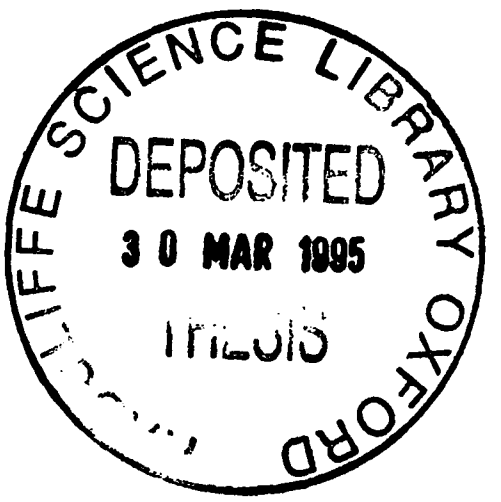
Surface Changes to Human Erythrocytes on Infection by *Plasmodium falciparum* Malaria

Jason Gardner

Christ Church



A thesis submitted to the University of Oxford in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Nuffield Department of Clinical Medicine

January, 1995

Abstract

Surface Changes to Human Erythrocytes on Infection by *Plasmodium falciparum* Malaria

Jason Paul Gardner

Christ Church

Thesis submitted for the degree of Doctor of Philosophy, University of Oxford, Hilary Term, 1995

Of the four *Plasmodium* species which cause malaria in humans, *P. falciparum* is responsible for the majority of the morbidity and mortality associated with this disease. The surface expression of parasite-derived proteins in the middle of the asexual cycle coincides with two important modifications of the host erythrocyte. First, a protective immune response is directed against a family of variant antigens, known as *P. falciparum* Erythrocyte Membrane Protein-1 (PfEMP1). Second, ligands are detected at the surface which mediate the specific cytoadherence of infected erythrocytes to vascular endothelium, such that infected cells are sequestered away from the peripheral circulation in deep vascular beds. The potentially fatal syndrome known as cerebral malaria can ensue when infected cells sequester at high density in the brain. Indirect studies have shown that the antigenic and adhesive phenotypes at the surface are linked to the expression of PfEMP1. However, there is a paucity of biochemical data which relate to PfEMP1, and this problem is addressed in this thesis.

This study has confirmed, at the biochemical level, inferences from serology that clonal antigenic variation occurred rapidly. Variation produced a number of novel antigenic and adhesive phenotypes which were associated with unique forms of PfEMP1.

Further insights into the mechanism of sequestration were possible because of the finding that single infected erythrocytes had the capacity to bind to at least three putative endothelial cell receptors; CD36, Intercellular Adhesion Molecule-1 (ICAM1), and Thrombospondin (TSP). It was demonstrated for the first time that PfEMP1 was responsible for cytoadherence to CD36 and ICAM1, but was probably not involved in adhesion to TSP. Extensive analysis with sequence-specific proteases proved that adhesive interactions with each receptor were separable properties of the surface, and facilitated the proposal of a domain model for PfEMP1.

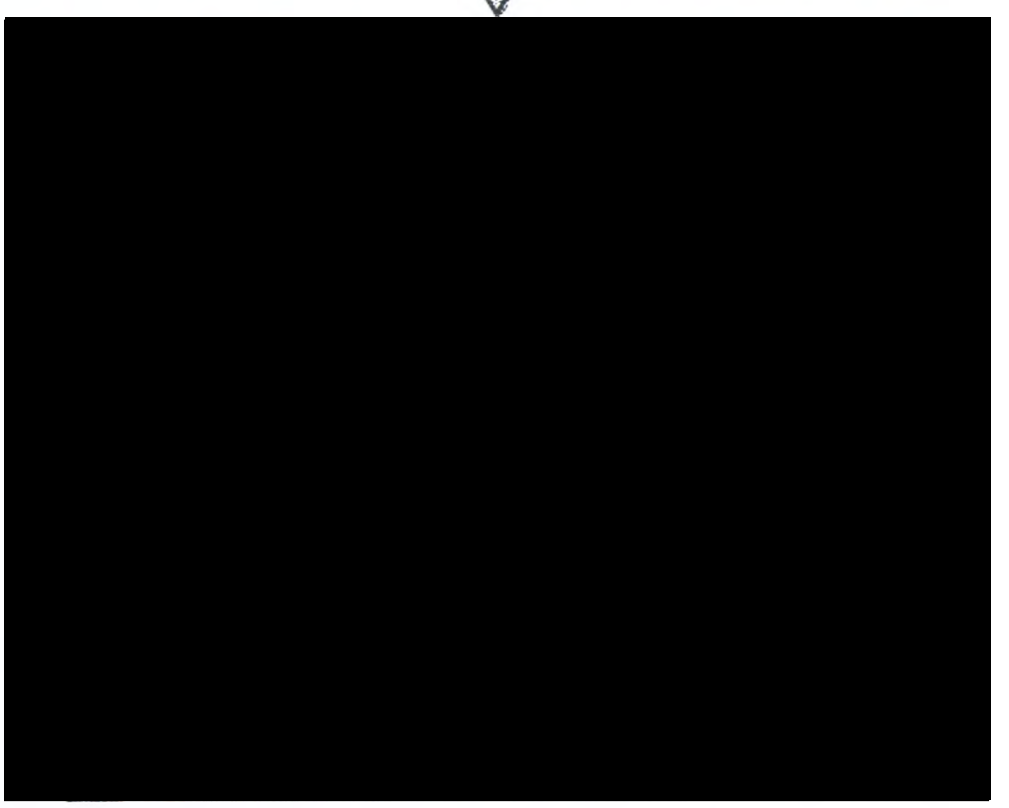
Detailed analysis of the antigenic and adhesive phenotypes of a series of clonally-derived parasites demonstrated that infected cells expressing all variant antigenic types could adhere to CD36 whereas adhesion to ICAM1 was seen in a restricted subset. This may be clinically relevant if, as current data suggests, adhesion of infected cells to ICAM1 is important in the development of cerebral malaria. Identification of all ICAM1 binding phenotypes could lead to the design of novel therapeutic strategies for this life-threatening condition.

The Challenge of Falciparum Malaria



A feeding female *Anopheles* mosquito.

How can we improve our understanding of this process?



Patient in coma with severe malaria due to *P. falciparum* infection.
Intensive care failed to save this Thai child.
(Photographs reproduced with permission from Knell, 1991)

To Mum and Dad,
for their love and tremendous support

and

To Bob Pinches,
for his time and patience

Table of Contents

Frontispiece i

Abstract ii

The Challenge of Malaria iii

Dedication iv

Table of Contents v - ix

Abbreviations x

Declaration xi

Chapter 1: INTRODUCTION 1

1.1. History of Malaria 2

1.1.1 Ancient 2
1.1.2 Modern 5

1.2. Life Cycle 8

1.3. The Vector 13

1.3.1 Mosquito Biology 13
1.3.2 Implications for Malaria Transmission 14

1.4. Epidemiology 17

1.5. The Clinical Picture 19

1.5.1.1 Symptoms and Diagnosis 19
1.5.1.2 Treatment 21

1.6. The Parasite 23

1.6.1 Aetiology 23
1.6.2 Phylogeny 23
1.6.3 Genetics 24
1.6.4 Antigenic Diversity 27
1.6.4.1 S Antigens 28
1.6.4.2 Circumsporozoite Protein 29
1.6.4.3 Merozoite Surface Proteins 30
1.6.4.4 Erythrocyte Associated Proteins 31
1.6.5 Metabolism 32
1.6.6 Transport 34

1.7. Immunity to Malaria 38

1.7.1 Innate Resistance 38
1.7.1.1 Thalassaemias 38
1.7.1.2 Sickle Cell Anaemia 39
1.7.1.3 Ovalocytosis 39
I.7.1.4 Duffy-antigen Negativity 40
1.7.2 Specific Immunity 41
1.7.2.1 Immunity in Endemic Areas 41
I.7.2.2 Asexual Blood Stage: Immune Responses and their Targets 43

1.8. Specific Background 51

1.8.1 Pathogenesis of Cerebral Malaria 52
1.8.2 Morphological Changes at the Surface of Infected Erythrocytes 57
1.8.3 Molecular Mechanisms of Sequestration 61
1.8.3.1 Endothelial cell receptors 61
1.8.3.2 Infected erythrocyte receptor(s) 69
1.8.4 Antigens at the Infected Red Cell Surface and Cytoadherence 71
1.8.5 Cytoadherence, Agglutination and PfEMP1 75
1.8.6 Host Membrane Protein Modifications 81
1.8.7 Other Membrane Alterations 82
1.8.8 Rationale and Aims 84

Chapter 2: MATERIALS AND METHODS 85

2.1. Materials 85

2.2. Methods 90

2.2.1 Parasites 90
2.2.2 Growth of Parasites 92
2.2.2.1 Routine Culture 92
2.2.2.2 Determination of Parasitaemia 96
2.2.2.3 Determination of Haematocrit 96
2.2.3 Electron Microscopy 97
2.2.4 Protease Digestion and Inhibition 97
2.2.5 Purification of Proteins 98
2.2.5.1 CD36 98
2.2.5.2 ICAM-1 98
2.2.5.3 TSP 99
2.2.6 Determination of Cytoadherent Phenotypes: Binding Assays 99
2.2.7 Selection of Parasites for Cytoadherence Phenotypes: Panning 101
2.2.8 Fluorescence Microscopy 101
2.2.9 Antibody-Mediated Agglutination 102
2.2.10 Determination of Antigenic Phenotypes: Mixed Agglutination Assay 103
2.2.11 Purification of antibody from polyclonal serum 104
2.2.11.1 Preparation of triple layers 104
2.2.11.2 Elution of antibody 104
2.2.12 Rosetting 105
2.2.13 Transport Experiments 105
2.2.14 ^{125}I Surface Labelling 106
2.2.15 ^{35}S Metabolic Labelling 107
2.2.15.1 Labelling of trophozoites 107
2.2.15.2 Dual labelling of rings 107
2.2.16 Detergent Extraction 108
2.2.17 Immunoprecipitation 108

2.2.18 SDS PAGE 109
2.2.18.1 Preparation of Gels 109
2.2.18.2 Preparation of Samples 111
2.2.18.3 Running Gels 111
2.2.18.4 Visualisation of Proteins 111
2.2.18.5 Sizing of Proteins 113
2.2.19 Autoradiography 113
2.2.20 Peptide Labelling 114
2.2.21 Chromatography 114
2.2.21.1 Dialysis 114
2.2.21.2 Ion Exchange Chromatography 115
2.2.21.3 Affinity Chromatography 115
2.2.22 Data Handling 116
2.2.23 Photography 116

Chapter 3: RELATIONSHIP OF ADHESION TO CD36 AND ANTIGENIC PHENOTYPE 117

3.1. Optimisation of the Agglutination Assay 120

3.2. Effect of proteases on Agglutination 122

3.2.1 $A4_{i+}$ 122
3.2.2 Other clones 126

3.3. Cytoadherence: Standardisation of the Spot Binding Assay 129

3.3.1 Manual 129
3.3.2 Belly Dancer™ 129
3.3.3 Hybridisation Machine 130

3.4. Effect of Proteases on Binding to CD36 132

3.4.1 $A4_{i+}$ 132
3.4.2 Other clones 135

3.5. Selection of A4-V8-CD36 137

3.6. Measurement of Antigenic Phenotypes 138

3.6.1 A4V8CD36 138
3.6.2 Effect of V8 Proteolysis 140
3.6.2.1 Normalisation of agglutination rates 140
3.6.2.2 Analysis of antigenic phenotypes post-digestion 141

3.7. Conclusions 143

Chapter 4: RELATIONSHIP OF ADHESION TO ICAM1 AND ANTIGENIC PHENOTYPE 149

4.1. Methods 151

4.1.1 COS cell expression 151
4.1.2 Purification of ICAM1-F_c 152

4.2. Selection of PRBC on purified ICAM1 154

4.2.1 Cytoadherent phenotypes of ICAM1 binding PRBC 155
4.2.2 Antigenic phenotypes of ICAM1 binding PRBC 156

4.3. Effect of proteases on binding to ICAM1 160

4.4. Selection for trypsin-resistant binding to ICAM1 162

4.5. Elution of VAT-specific antibodies 165

4.6. Pathway of phenotypic switching 166

4.7. Surface morphology of ICAM1 binding PRBC 169

4.8. Conclusions 173

Chapter 5: ADHESION TO TSP 180

5.1. Binding of different PRBC to TSP 182

5.2. Effect of Proteases 184

5.3. Selection of PRBC on TSP 186

5.4. Conclusions 187

Chapter 6: FUNCTIONAL MOLECULES AT THE PRBC SURFACE 190

6.1. Identification of parasite-derived surface molecules 192

6.2. Identification of variant antigens 196

6.3. Adhesion to CD36 201

6.4. Adhesion to ICAM1 207

6.5. Adhesion to TSP 213

6.6. Conclusions 215

Chapter 7: LINKAGE OF SURFACE PHENOTYPES 222

7.1. Effect of proteases on surface phenotypes 224

7.2. Effect of polyclonal serum on PRBC cytoadherence to purified receptors 226

7.2.1 Methodology 226
7.2.2 Reversal of cytoadherence to CD36, ICAM1 and TSP 228

7.3. Staging of surface phenotypes 233

7.4. Other PRBC surface functions 239

7.4.1 Rosetting 239
7.4.2 Transport 240

7.5. Conclusions 242

Chapter 8: DISCUSSION 245

8.1.1 Major Conclusions 245
8.1.2 Models of PRBC Surface Molecules 255

References **259 - 286**

Acknowledgements **287**

Appendix **288**

APS	ammonium persulphate
BSA	bovine serum albumin
CM	carboxymethyl
DAPI	4,6-diamidino-2-phenylindole dihydrochloride
DBD	Duffy binding domain
ddH_2O	deionised, distilled water
DEAE	diethylaminoethyl
DFP	di-isopropylfluorophosphate
DMSO	dimethyl sulphoxide
EtBr	ethidium bromide
Eur	European serum
g	gravity
g,mg,μg,ng	gram, milligram, microgram, nanogram
HEPES	N-2-hydroxy-ethyl-piperazine-N-2-ethanesulphonic acid
HUVEC	human umbilical cord endothelial cells
ICAM1	intercellular adhesion molecule-1
Ig	immunoglobulin
kDa	kilodaltons
ml, μl	millilitre, microlitre
M, mM	molar, millimolar
mAb	monoclonal antibody
mC_i, μC_i	milli-Curie, micro-Curie
MEM	minimal essential medium
MES	2-[N-morpholino] ethanesulphonic acid
M_r	molecular weight
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PFGE	pulsed field gel electrophoresis
PRBC	parasitised red blood cell(s)
RBC	red blood cell(s)
rpm	revolutions per minute
RPMI-1640	Roswell Park Memorial Institute medium 1640
R_M	rainbow markers
SDS	sodium dodecyl sulphate
SEM	standard error of the means
Sib	Sibanor serum
TBS	Tris buffered saline
TEM	transmission electron microscopy
TEMED	N, N, N', N' tetraethyl-ethylenediamine
TNF	tumour necrosis factor
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
TSP	thrombospondin
V	volts
VCAM1	vascular cell adhesion molecule-1
v/v	volume for volume
w/v	weight for volume

Declaration

Specific help with experimental work was provided by Bob Pinches, who assisted during the radio-labelling and staging of cells; Heather Horner, who helped with the transport experiment; and Alister Craig, who transfected the COS cells during the purification of ICAM1.

With these exceptions, I declare the work described in this thesis is the result of my own unaided efforts and has not been submitted to any other university.



Jason Gardner

January, 1995

Chapter 1

Introduction

Malaria has probably been the cause of more morbidity and mortality than any other pathogenic agent, including warfare, in the history of mankind (Desowitz, 1991). Infection by the parasite, *Plasmodium falciparum*, is responsible for the bulk of the 1-2 million estimated annual malaria fatalities (World Health Organisation, 1992). This figure shows no signs of declining and with the increased resistance to a diminishing armoury of antimalarial drugs, the situation for the tropical inhabitant is arguably worse than 20 years ago (Greenwood, 1985). As such, malaria represents a formidable challenge to the scientific and medical communities and even though the outlook is promising, there is no guaranteed solution on the horizon.

The work in this thesis is focused on the intraerythrocytic stage of the parasite's complex life cycle, where all the life-threatening symptoms of the disease are manifested. After infection by *P. falciparum*, the host erythrocyte undergoes dramatic surface modifications which are central to pathogenesis and are implicated in protective immunity.

This chapter is a summary of the literature relating to the history and biology of malaria, with emphasis on the biochemistry of the surface of the infected erythrocyte. The rationale and aims that underlie the scope of this work are described at the end of this first chapter and the specific background to each results section is detailed in the introductions to subsequent chapters.

1.1. History of Malaria

Malaria research is a fascinating cocktail of science, politics, suffering, misadventure and serendipity that make it an attractive topic for films, books, and conversation. It is characterised by significant observations of brilliant insight often made within scientifically flawed paradigms. Any historical account of malaria is usually long and intertwined with the development of civilisation because *Plasmodium* genus is probably older than mankind. For simplification and space, this chronicle is divided into **ancient** and **modern** periods of history and the cynical reader will notice that wars, not philanthropy, have been the impetus for the major discoveries in this field.

1.1.1 Ancient

Plasmodium that infect humans probably evolved in Africa and the parasite would have spread into Europe and Asia during the Neolithic period. *Plasmodium falciparum* is the only species which causes mortality in man. Therefore, this species requires a susceptible population that remains geographically stable and large enough to maintain transmission through dry seasons. It has been predicted that *P. falciparum* evolved later than the other three human malarias at a time, approximately 5000 years ago, that coincided with the advent of agriculture (Waters *et al.*, 1993). This hypothesis assumes that the mortality inflicted by *P. falciparum* has been constant.

There are numerous theories of how malaria affected early civilisation through its burden on armies and economies, and it is unquestionable that the disease had a dramatic influence on the settlement of new regions. An interesting account from the classical Greek historian Thucydides in 413 BC tells how the Athenian army was heavily defeated during its siege of Syracuse. The Athenians had been camped in marshland near the town in hot weather (optimal conditions for mosquito breeding) and had suffered a lethal fever epidemic that weakened their forces. It has been suggested that the epidemic was falciparum malaria and that Athens never recovered from this defeat. As a result, the city was subsequently destroyed by Sparta which signaled the downfall of Classical Greece and permitted Rome to dominate Europe (Bruce-Chwatt and de Zuleta, 1980).

Accounts of malaria before the end of the 19th century are understandably vague because neither the parasite nor its life cycle had been elucidated. Consequently, there is a variety of medically astute references in the ancient literature to symptoms which are similar to those of malaria, but their interpretation is open to conjecture (reviewed by Knell, 1991).

There are descriptions of acute fevers and of the mosquitoes that caused them in ancient Sanskrit manuscripts from Susruta, Asia in the 5th Century BC. One of the earliest 'doctors', Hippocrates, described tertian fevers (high temperature on days 1 and 3), that were less malign than quartan fevers (high temperature on days 1 and 4), and had probably observed the difference between *P. vivax* and *P. malariae* infections. He also reported the semi-tertian fevers associated with *P. falciparum* infection which had the worst prognosis and noted that people living near marshes were prone to having enlarged spleens, a common symptom of malaria.

Malaria was prevalent in most parts of the Old World that could sustain compatible mosquito vectors, which cannot survive long at extremes of temperature and high altitudes. Only the Arctic, some Pacific Islands, pockets of South America and areas above 6-7000 feet have never been affected. The disease probably spread into the New World via importation following the continent's discovery by Columbus. Old names, such as marsh fever or paludism (from the Latin word *palus*, meaning a marsh), reflect its occurrence near wetlands. Shakespeare, Chaucer and Pepys refer to the "agues" that seasonally blighted the marshy areas of Southern England (Major, 1932). The name, malaria is a corruption of the Italian *mala aria*, meaning bad air, that was coined by Horace Walpole in the 18th Century, when the disease was believed to be caused by the unwholesome air in swampy districts: the 'miasmic theory'.

The history of malaria is inexorably linked to the methods that have been used in its treatment. An ancient Chinese herbal remedy for fevers was to imbibe the crushed leaves of the plant *Artemisia annua* which contain the antimalarial, artemisinin. The Chinese kept this drug secret until 20 years ago, consequently, few of the problems associated with resistance have occurred, and now artemesinin is one of the most exciting current prospects for therapy and prophylaxis. For the rest of the malaria world before the Renaissance, divine intervention was the only treatment available until the discovery

of *Cinchona succirubra*, or 'fever-bark' tree in the early 17th century by Jesuit missionaries in South America (Knell, 1991).

The bark of this tree was found to be effective in curing tertian fever and was soon used widely all over Europe, where it was known as Jesuit's powder (Jaramillo-Arango, 1950). The preparations had variable efficacy, but Robert Tabor, an unqualified 'feverologist', earned his fortune and a knighthood when he used it to cure Charles II of the 'agues' (Major, 1932). By 1820, the active alkaloids, quinine and cinchonine had been isolated from the 'fever-bark' *cinchona* tree by Pelletier and Caventou (Gilles, 1993b) which allowed prescription of an accurate dosage. However, demand rapidly exceeded supply for this expensive drug when its value as a prophylactic was noticed. Eventually, a British entrepreneur, Charles Ledger, sold some seeds from an exceptional forest of *Cinchona* trees to the Dutch in 1861, which germinated and produced bark that yielded good quantities of quinine. The Dutch cultivated large plantations in Indonesia which gave them a profitable monopoly and the new species was termed *C. ledgeriana* in honour of its founder (Knell, 1991).

The spread of quinine kept pace with the colonisation of the world, and research into malaria was often a priority for governments who were committed to global domination via colonialism. However, ignorance of both the disease and of prohylaxis meant that malaria still had a devastating effect on mankind. For instance, the first attempt to construct the Panama Canal by the French between 1881 and 1888 failed due to the mortality and morbidity inflicted on a non-immune workforce by malaria and yellow fever.

The two most significant advances in the field were made at the end of the 19th Century and their importance was recognised by the reward of Nobel prizes. The first was the discovery of the aetiological agent of malaria in 1880 by Alphonse Laveran, a French army doctor in Algeria (Laveran, 1880). Using a crude microscope, he noticed black pigment (digested haemoglobin, haemazoin, produced by the parasite) inside blood cells and organs of patients suffering from malaria. He proposed that the pigment was a parasite and not a side effect of fever as was believed at the time. In common with many new theories, this idea was greeted with scepticism and most investigators maintained that malaria was a germ that lived in marshes. In 1885, Laveran demonstrated exflagellation of male gametocytes to convince Pasteur of his theory. Italian scientists, lead by Grassi,

distinguished the malaria parasites that were responsible for different fever patterns and they were classified in the *Plasmodium* genus (Knell, 1991).

In England, a leading specialist in tropical diseases, Sir Patrick Manson, had encouraged a colleague, Surgeon-Major Ronald Ross to "pursue the mosquito" in his work on malaria in the Indian army. The insect theory of malaria transmission had been proposed without evidence as early as 1717 by Lancisi and suggested by several other investigators (Gilles, 1993b). In 1897, Ross was serendipitously supplied with mosquito larvae from *Anopheles* species (he had previously been using *Culex* species, which is not a vector for human *Plasmodium*) and the insects were fed on malaria patients. After dissection of the mosquitoes, he saw the same black pigment that Laveran had previously described and went on to demonstrate conclusively that the mosquito was the vector for parasite transmission in avian malaria (Ross, 1897; reviewed in Ross, 1923). This was the second major discovery in the field and the first conclusive demonstration of a host-mosquito-host cycle for any infectious agent. With better knowledge and facilities, Bignami and Bastianelli were quickly able to follow up this finding with a near-complete description of the life cycle (Bastianelli, 1898).

1.1.2 Modern

These late 19th Century discoveries were a triumph for the rational approach to science and heralded the first attempts of malaria eradication. Unfortunately, larvicides (eg. Paris Green) and other methods (eg. oiling of breeding sites) which were used to destroy mosquito habitats were never comprehensively applied to any areas, and notable successes were based instead on knowledge acquired about mosquito biology. The Panama canal was completed by the USA only after mosquito control measures were employed which diminished the transmission of both malaria and yellow fever. Similarly, the Campagna marshes were drained in Mussolini's Italy to establish a fertile agricultural region near Rome that was free from malaria, a programme that won '*Il duce*' popular and political acclaim.

Screening programmes for efficient insecticides and antiparasite drugs were embarked upon soon after the turn of the Century. Ehrlich's concept of selective dyeing and his first chemotherapy trial of a drug (methylene blue) in a malaria patient formed the paradigm that was followed by the

pharmaceutical industry (Guttman and Ehrlich, 1891). German investigation was stimulated after its forces had been cut off from quinine supplies in East Africa during the First World War and began screening thousands of potential drugs. 8-aminoquinolines (eg. Atebrin, Plasmochin) and 4-aminoquinolines (Resochin, Sonotchin) were synthesised, and Sonotchin was ready for clinical trials by 1940 (Gilles, 1993b). Ironically, Resochin was discarded because of high toxicity, but was re-discovered 25 years later as chloroquine.

Over 250 000 drugs were screened for antimalarial activity during the Second World War when quinine became unavailable following the Japanese occupation of Java. However, it was only the capture of German soldiers in Algeria that allowed Sonotchin to fall into the hands (and mouths) of the British. Sonotchin was analysed and chloroquine was independently synthesised as a non-toxic and more effective antimalarial drug which then had a considerable effect on the course of the War in the Tropics. By the end of 1945, the Allies had also discovered that proguanil, pyrimethamine and sulphonamides were effective antimalarials and had embarked on crucial insecticide research. Dichloro-diphenyl-trichloroethane (DDT), which was synthesised in Switzerland as an anti-louse agent in 1941, was discovered to have anti-mosquito activity and was used successfully to interrupt the transmission of malaria (Pampona and Russell, 1955).

As a result of the Second World War, new tools were in place to meet the challenge of malaria and the newly-established World Health Organisation (WHO) declared that control of malaria was of paramount importance. A further 250 000 compounds were tested for antimalarial activity and great progress was made in Europe and the Indian-subcontinent with the combination of DDT and chloroquine. In 1955 the WHO announced that its policy was to eradicate malaria completely and this was achieved in many temperate regions of the world, despite the persistence of the vector. In spite of this, many warning signs of resistance were ignored as more funding was raised for this programme. Failure occurred dramatically in Africa, and by 1969, the WHO directive had switched back to control in those countries where eradication was impossible (Desowitz, 1991).

The collapse of these programmes had a long-lasting effect on the malaria field. Huge sums of money had been spent on eradication at the expense of basic research. Two decades of potential investigation were lost because malaria had been viewed by the scientific community in a similar manner to the way

smallpox is considered today. The decisions made by the WHO are shrouded in controversy due to the questionable motives of the bureaucrats in Geneva. It was another war that was to provide recognition of the importance of the disease, when the United States Army suffered severe setbacks from malaria infections in Vietnam during the early 1970s. Political motivation then stimulated an injection of funding into new research and the American Army is still active in the malaria field (Desowitz, 1991).

Significant advances have been made in the basic sciences that underpin malaria, and clinical studies have mirrored this revival during the last 25 years. Animal models, *in vitro* cultivation, molecular biology, immunology, vaccine and drug studies, genetics, epidemiology, vector biology, treatment regimes, and clinical management have been employed to investigate *Plasmodium* and malaria. Some of the techniques and ideas are at the forefront of research and the prospect of a vaccine is now realistic (and also not without controversy).

However, a perspective of this 'progress' is markedly different when viewed from the perspective of a tropical inhabitant. Alarming statistics illustrate that malaria today is still responsible for as much suffering and death as 20 years ago when there was no drug resistance (World Health Organisation, 1992). Modern science has not yet found a cure, but has provided the insecticide-soaked bed-net which limits transmission and protects against severe disease (Darriet *et al.*, 1984; Bradley *et al*, 1986; Snow *et al.*, 1988; Alonso *et al.*, 1991). Other current therapies include the antimalarial drugs that are based on quinine and artemesinin, which are far from novel! However, hi-tech procedures for clinical management of severe disease have been introduced as the pathogenesis and long-term effects of malaria are established. Complementary to these approaches, major emphasis is now placed on education and sanitation in the Third World which should influence all aspects of health. With nearly half of the world's population at risk, malaria remains a problem of global proportions and continues to ask the same questions of science and medicine that it has done since the beginning of mankind.

1.2. Life Cycle

An understanding of the complex life cycle of the parasite is central to a discussion of malaria. The time scales of different stages define constraints which determine the epidemiology and pathology of the disease. Ross' discovery of the mode of transmission marked the beginning of a new investigative era in infectious diseases and each of the stages within the human host has now surrendered to *in vitro* cultivation (Trager and Jenson, 1976; Mazier *et al.*, 1984, 1985; Sinden, 1985).

The cycle can be divided into four distinct stages, and is summarised in figure 1.1. **Fertilisation** is the sexual stage in the cycle and occurs in the mosquito gut, followed by three asexual stages whose purpose is multiplication: **sporogeny** occurs after fertilisation in the mosquito gut, followed by **hepatic schizogony** in the human liver, and **erythrocytic schizogony** in the human bloodstream. Transmission is by the bite of a female *Anopheles* mosquito (Bray and Garnham, 1982).

This description focuses on *P. falciparum* in man and concentrates on the intraerythrocytic stage, the subject of this thesis. Standard texts (Crewe and Haddock, 1985; Garnham, 1988) provide more detailed accounts with relevant electron micrographs to illustrate the cellular morphology. There are biological similarities between the asexual stages because they are preceded by an invasive form that penetrates the host cell. The intracellular parasite then grows and divides by internal segmentation to produce new invasive forms which are subsequently released. The parasite can remain for long periods inside its host cell without exposure (eg. many years in the case of *P. vivax* infected hepatocytes, known as hypnozoites). However, the invasive forms have shorter life-spans (in the order of minutes) when many novel proteins are exposed to the immune system. These are important considerations for the following discussions of acquired immunity and surface modifications.

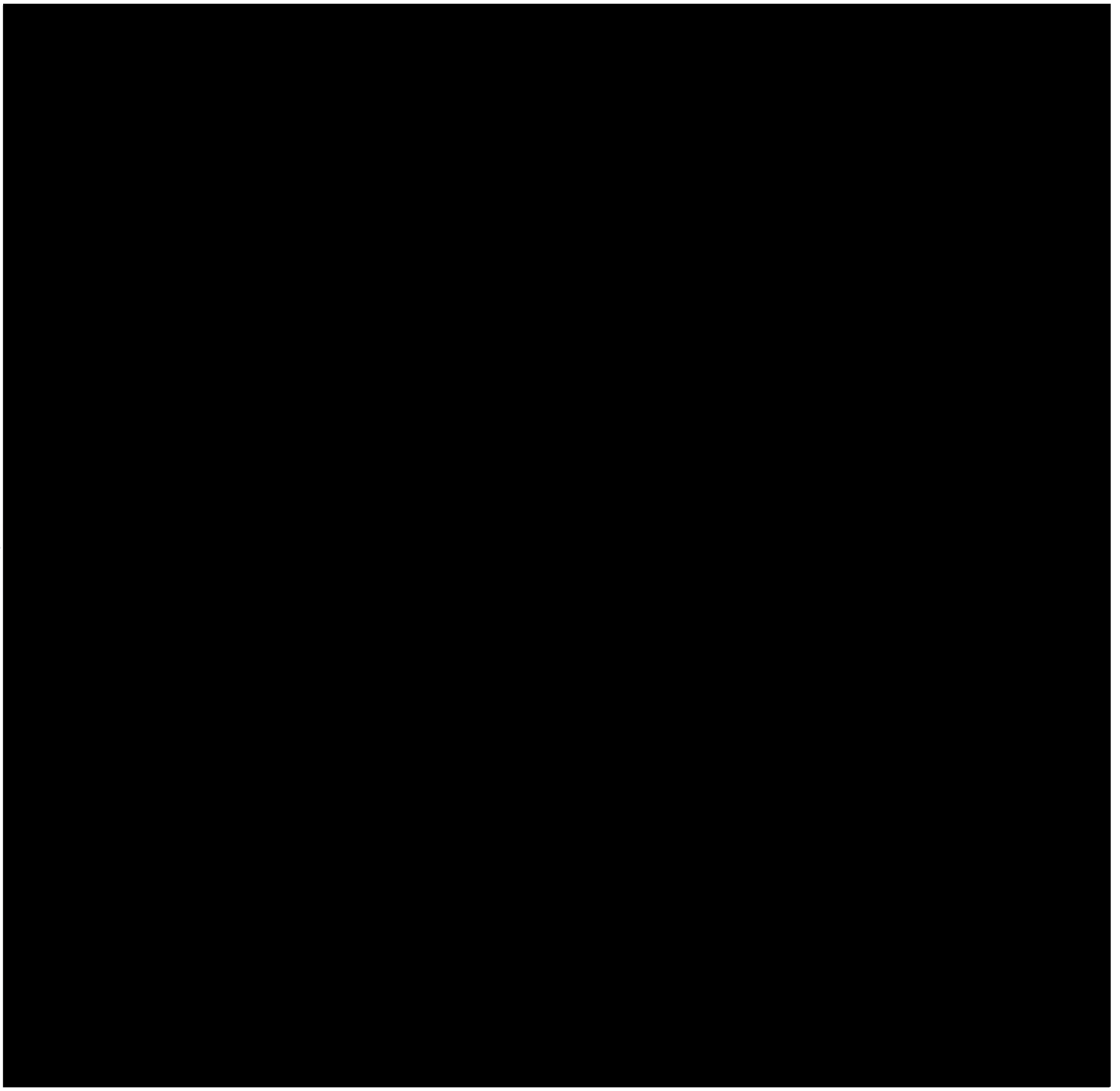


Figure 1.1. Summary of Plasmodium *life cycle. Numbers of different stages are referenced in the text (modified from A. J. Knell, 1991, and reproduced with permission).*

1. Fertilisation. After a blood meal containing mature gametocytes, the sex-cells (gametes) are released in the mosquito gut. Male gametocytes undergo exflagellation [*1*] and 6-8 gametes (containing one male nucleus each) are released from each cell and have a life span of 2 minutes inside the mosquito. Following fertilisation [*2*] of a female gamete by a male gamete, a zygote [*3*] is formed which differentiates over 12-24 hours into an invasive ookinete [*4*]. This period is when meiosis is assumed to occur. The ookinete adheres to the midgut wall and develops into an oocyst on the surface which is encased by the haemocoel.

2. Sporogeny. The oocyst grows by feeding on haemoglobin ingested during the blood meal and the black pigment that Ross noticed is produced as a by-product [*1*]. After about one week, but sooner at higher temperatures, the parasite will have divided to produce ~10 000 invasive sporozoites [*2*] which are released when the oocyst bursts [*3*] and migrate to the lumen of the salivary glands, up to 2 weeks post-infection.

3. Hepatic Schizogony. Sporozoites are injected into the bloodstream by the mosquito during a blood meal and invade liver hepatocytes within minutes. The number of sporozoites inoculated is remarkably constant (<20) and may influence the time course of the appearance of symptoms (Rosenberg *et al.*, 1990; Marsh, 1992). The intracellular parasite forms a trophozoite [*1*] which grows and differentiates into a schizont [*2,3*]. The incubation period for *P. falciparum* lasts for 9-12 days before there are any clinical symptoms. When each hepatocyte ruptures, 30000-40000 invasive merozoites are released into the bloodstream [*4*] (Shortt and Garnham, 1948).

4. Erythrocytic Schizogony (reviewed by Bannister and Dluzewski, 1990). Merozoites attach to the surface of erythrocytes by specific receptors only minutes after release from the hepatocyte [*1*]. *P. falciparum* merozoites will infect erythrocytes of all ages, but the other human malaria species have a predilection for either reticulocytes (*P. vivax* and *P. ovale*) or possibly for senescent erythrocytes (*P. malariae*). Invasion takes 20-30 seconds and is accomplished by a specific recognition event followed by an endocytic propulsion of the merozoite into the cytoplasm of the host cell. The merozoite has an apical complex which is adapted for this process and its outer coat (pellicle) is shed during invasion. The intraerythrocytic parasite is surrounded by the parasite membrane (PM), a parasitophorous

vacuolar membrane (PVM) and the red cell plasma membrane during the 48 hours it requires for growth and differentiation. Important parasite-induced functional and morphological changes occur in both membranes and these are detailed in a later section. During this stage, three cyclical intraerythrocytic forms of the parasite are usually visible when viewed under light-microscopy. Young forms (<15 hours post-invasion) of the parasite resemble **rings** [*2*] due to reorganisation of the nuclear material and their correct identification is a crucial part of malaria diagnosis. The prepatent period from inoculation to the appearance of a blood parasitaemia is 7-9 days for a *P. falciparum* infection. Rings grow by ingesting haemoglobin and host cell cytoplasm via specialised regions of the parasitophorous vacuole membrane called cytosomes and pigmented haemazoin is produced as a by-product in the food vacuole. The ring-form develops into a **trophozoite** after ~20 hours post invasion [*3,4*]. In a *P. falciparum* infection, mature erythrocytic forms are sequestered in deep vascular beds via specific adhesion to receptors on the surface of endothelial cells (section 1.8.3). Therefore, they are not normally visible in the peripheral blood and complete the rest of this stage in the vasculature of different organs. The endoplasmic reticulum proliferates and nuclear division takes place in the **schizont** form [*5*], and individual merozoites are formed by intracellular segmentation. When the schizont ruptures, 10-30 merozoites are released along with broken membranes, intracellular proteins and other pyrogens [*6,7*]. New erythrocytes are invaded and this cycle can be repeated many times in the bloodstream which contributes to the high parasitaemias associated with *P. falciparum* infection.

Some merozoites do not develop into schizonts after invasion and instead differentiate into crescent-shaped **gametocytes** inside the erythrocyte [*8*]. Commitment to gametocytogenesis occurs in the previous erythrocytic cycle, but the stimulus and mechanism of this switch is unknown. Metabolic stress or altered immune status of the host have been proposed (Bruce *et al.*, 1990), and some laboratory-adapted isolates lose the ability to form gametocytes which may be an irreversible genetic trait (Graves *et al.*, 1984). Gametocyte maturation requires 10-11 days but there is little metabolism or DNA replication and hence they are insensitive to most antimalarial drugs. Mature gametocytes [*9*] appear in the circulation and can be ingested during a mosquito blood meal to complete the cycle.

A biological consequence of parasitic life in two hosts is that the parasite is exposed to vastly different environments which present continuous challenges. For example, dramatic temperature fluctuations

(up to 17°C) occur on transmission from the mosquito to the human bloodstream. These changes are accompanied by simultaneous alterations in nutrient supply and host immune status. The ability of the parasite to survive in these hostile conditions is a testimony to its adaptability which is discussed later.

There are major differences between the life cycles of the four human *Plasmodium* species which are summarised in table 1.1. The length of the intraerythrocytic cycle mirrors the timings of fever which in turn correlate with the synchronicity of infection and pyrogen release on schizogony. Thus, quartan fevers are a hallmark of *P. malariae* infection, but irregular semi-tertian fevers indicate a *P. falciparum* infection. *P. vivax* is well-adapted for long-term survival in man because it can live undetected as silent hypnozoite forms in the liver for many months. Similarly, a single *P. malariae* infection can persist for several years, but even though its location in such cases has not been established, recrudescence of persistent erythrocytic forms can occur (Boyd, 1940). In contrast, the increased multiplicative capacity, shortened duration of specific stages and broad erythrocytic specificity are virulence factors unique to *P. falciparum*.

	P. falciparum	*P. vivax*	*P. ovale*	*P. malariae*
Sporogeny (days, at 28°C)	9-10	8-10	12-14	14-16
Hepatic schizogony (days)	5.5-7	6-8	9	14-15
Average number of merozoites per hepatocyte	30 000 - 40 000	~10 000	~15 000	~15 000
Erythrocytic schizogony (hours)	48	48	50	72
Average number of merozoites per erythrocyte	16	8	8	16

Table 1.1. Selected life cycle characteristics of human malaria species (modified from Gilles, 1993c).

1.3. The Vector

The mosquito has been a focus for malaria research since its discovery as the vector for the disease nearly 100 years ago. Since then, investigations have been directed at a greater understanding of its biology (reviewed by Service, 1993) to provide rational tools for control and eradication of malaria (reviewed by Onori *et al.*, 1993). This section is divided to reflect this background.

1.3.1 Mosquito Biology

Only 40 of the 360 mosquito species from the genus *Anopheles* can transmit the human malaria parasite and this limitation is one of the major factors that influences the disease epidemiology (Russell *et al.*, 1993). The posture of an *Anopheles* mosquito on resting and biting is distinctive because its body adopts a single-axis form on resting and feeds with its hind-legs raised. Also, adults have dark-spotted wings and long palps at the side of their proboscis which facilitate classification. However, differentiation between *Anopheles* species is more difficult due to their identical appearance and relies instead on karyotyping and chromosome analysis. The geographical distribution of species is variable and *A. gambiae* is the most important vector for malaria in Africa while *A. culcicifacies* is its equivalent in South Asia.

The *Anopheles* mosquito is a poikilotherm whose life cycle is 7 - 21 days within an optimum temperature range of 20-31°C. Males and females feed on nectar and fruit juices and copulation has to occur only once because the female can store sperm for subsequent egg production. However, she must take a blood-meal to complete all but her first egg cycle and each batch is laid at 2 - 3 day intervals under the surface of still water. The larvae and pupae develop under water, feeding on algae and bacteria and each batch produces 200 - 300 mosquitoes.

Adult *Anopheles* mosquitoes have species-specific behavioral patterns which are selective and determine their breeding and resting sites. Of particular importance is where the female mosquito will rest after digestion of her blood meal because she often remains stationary for over one day. *Anopheles* mosquitoes will often rest indoors on a wall or under the roof for long periods.

1.3.2 Implications for Malaria Transmission

Mammalian malaria is transmitted by the bite of an infectious adult female *Anopheles* mosquito but no vertical parasite transmission occurs in the vector (Garnham, 1966). To be capable of transmission, the mosquito must fulfill two criteria:

a) At least one male and one female gametocyte must be ingested by the mosquito during a blood meal.

b) For *P. falciparum*, at least 3 - 4 blood meals must be taken during the minimum 9 days that the parasite requires for fertilisation and sporogeny in the vector. This is arguably the most vulnerable period in the parasite's life cycle and some successful malaria control measures have been targeted against it (Onori *et al.*, 1993).

The frequency and choice of host for a blood meal influences transmission, thus, efficient vectors for malaria are usually **anthropophilic**. Transmission is proportional to the square of the man-biting rate because parasites are transferred via mosquito to man or vice versa (MacDonald, 1957). To this end, mosquitoes that are **endophagic** (feed indoors) transmit malaria well, and mosquito **density** is proportional to transmission, thus species that breed prolifically are effective. The mosquitoes must be situated near human habitation if density is to affect transmission. Introduction of large numbers of irradiated, sterile males can reduce breeding and thus transmission in these situations. Mosquito **longevity** has the most important influence on malaria because the female must survive long enough for the parasite to develop and be transmitted.

This factor was noted by Ross, who was the first person to produce mathematical models of transmission (Megroz, 1931). A convenient measurement of malaria transmission dynamics is the basic-case reproduction rate (BCCR), which assesses the average number of new cases of malaria that occur from a single case in a susceptible population (reviewed by Dietz, 1988). The main aim of malaria eradication is to reduce and maintain the BCCR to below 1. This variable is proportional to the probability of survival of the mosquito for one day to the power of the duration of the cycle in the vector. Thus, when the probability of daily survival for vectors is less than 65% , then *P. falciparum* transmission will be established. Below this figure, less than 1% of the females will live for the 10-12

day duration of sporogeny of *P. falciparum*. Lifespan is an intrinsic property of each mosquito species, and for *A. gambiae*, the major African vector, ~10% of mosquitoes can survive for the ~11 days required for *P. falciparum* development, based on estimates of a 85% chance of survival for one day (Dye, 1986). High humidity lengthens mosquito survival, but other factors that affect longevity are unknown. The vulnerability of the parasite at this stage has made the mosquito the target of several anti-malaria programmes (MacDonald, 1957).

Transmission control measures

Of all anti-malaria measures, mosquito control has probably given the greatest benefit to the most people (Kitron and Spielman, 1989). Insecticides have been used to spray the interiors of houses with organochlorides (e.g. DDT), organophosphates (e.g. Malathion), carbamates (e.g. Propoxur) or synthetic pyrethroids (e.g. Permethrin) (reviewed by Onori *et al.*, 1993). This has reduced the life expectancy of females by reducing numbers as they rest during digestion of a blood-meal (Pampona and Russell, 1955). However, resistance has spread (11 of the 57 resistant *Anopheles* species are malaria vectors) and insecticides have selected for mosquitoes with altered behavioural characteristics. Furthermore, the toxicity and environmental effects of insecticides make their use equivocal.

Several larval control measures have been used successfully to reduce malaria transmission after breeding sites have been located. This process of species sanitation was pioneered by Ross and is based on the selective removal of the vector habitat. The simplest and most effective method is drainage, but this is expensive and requires the cooperation of agricultural and public health communities. Alternative approaches include the introduction of either fish (eg. guppies) that feed on mosquito larva or bacteria (eg. *Bacillus thuringiensis israelensis*) which produce toxins and kill larval stages. The widespread use of these biological control measures has been prevented by fears for their adverse effects on the environment and ecology (Onori *et al.*, 1993).

Barrier-protection techniques are underestimated and highly effective methods that reduce transmission (Harries, 1993). Simple barriers such as mosquito screens on windows and insecticide-impregnated bed nets have produced dramatic decreases in malaria incidence and mortality in endemic areas (Darriet *et al.*, 1984; Bradley *et al*, 1986; Snow *et al.*, 1988; Alonso *et al.*, 1991). Also,

use of insect repellents and wearing clothes with long sleeves and trousers at evening time is likely to reduce the number of mosquito bites which, in turn, could reduce the likelihood of disease.

Genetic control of transmission has become a feasible alternative that has resulted from the recent explosion in molecular biology. A linkage map of the X chromosome of *A. gambiae* has been published (Zheng *et al.*, 1993) which investigators believe will lead to the identification of genes that make mosquitoes refractory to infection by *Plasmodium* species (Curtis, 1994). This is not unreasonable because there is a strain of *A. gambiae* which exhibits an enhanced encapsulation trait whereby it disables ookinetes by imprisoning them in the mid-gut wall and thus sporozoites are unable to proceed to the salivary glands (Collins *et al.*, 1986a; Paskewitz *et al.*, 1989). Gene(s) for this trait have been located to 10% of the mosquito genome, but the mode of insertion of this gene, once identified, into insects has no practical solution yet. Transfection of such genes, or those that reverse insecticide resistance, may rely on identification of a mosquito transposable element or use of a retroviral system, but both approaches are a long way in the future (Crampton *et al.*, 1990; Spielman, 1994).

An exciting 'altruistic' vaccine is under development that mimics proteins expressed by gametes and the ookinete which are not under human immune pressure. Monoclonal antibodies to such stage-specific proteins have been developed that interfere with the transmission of *P. yoelli* (Harte *et al.*, 1985) and block the development of *P. falciparum* oocysts (Vermuelen *et al.*, 1985). Although such antibodies would not confer individual protection, they may interrupt transmission and the recognised epitopes could become a component of a vaccine cocktail.

1.4. Epidemiology

Malaria is prevalent in 103 countries where over 50% of the world's population are at risk (Walsh, 1984; Greenwood *et al.*, 1991; World Health Organisation, 1992). Epidemiological features of the human disease are complex because transmission dynamics are determined by co-localisation of compatible species of parasite with vector, availability of a susceptible host and environmental factors (reviewed in Wernsdorfer, 1988). *P. falciparum* is the predominant species in endemic areas of Africa, and over 90% of the malaria-associated mortality occurs in sub-Saharan Africa. The microepidemiology of several south-central Pacific islands has been studied because harmonious mosquitoes and parasites are present but severe clinical disease is notably absent. This may be due to altered parasite virulence or innate host resistance (section 1.7.1). In West Africa, *P. ovale* has replaced *P. vivax* because the indigenous residents have evolved genetic resistance to erythrocyte invasion by the latter parasite (section 1.7.1.4), and other mutations that confer resistance to malaria have become established in malarious regions by selection pressure of the disease on the human genome (reviewed by Weatherall *et al.*, 1988).

The entomological factors that influence malaria epidemiology are relatively well understood and have been described in section 1.3. The distribution of compatible vectors is fundamental to successful malaria transmission, but only one or two will be of importance in each area of the world. Environmental factors such as altitude, temperature, humidity and rainfall during the seasons can affect epidemiology via their influence on mosquito behaviour.

The contrast between malaria epidemiology in different areas can be dramatic. At one extreme, in unstable malaria the transmission varies and can result in a sudden, devastating epidemic that may affect everyone in a community and then disappear. Under these conditions it is virtually impossible to acquire clinical immunity (section 1.7.2) and the disease can return with similar destructive results. This has been the case for Sri Lanka where the vector is *A. culciffacies*: in 1934-35 there were 3 million cases and 82 000 deaths, followed by a second epidemic in 1967, and another in 1986. By contrast, malaria can be present as an endemic part of the fabric of life and become an innocuous, yet potentially fatal condition that varies only with seasonally-associated changes in the climate. The

transition from high to low transmission (as assessed by spleen rates of indigenous children) is classified as holo-, hyper-, meso-, to hypo-endemicity. All young children in holoendemic regions West Africa have had an attack of clinical malaria, but immunity is generally well-established by the age of 10 when symptoms are rare despite persistent parasitaemia.

Attempts to mathematically model the disease and predict the effect of control or intervention measures have used epidemiological data as fundamental tenets. This has lead to the introduction of useful terms to describe the transmission and infectivity of the parasite in different communities. The basic case reproduction rate (BCCR) assumes that the susceptible population is initially uninfected and non-immune, and if the BCCR is <1, then malaria will disappear. Low BCCRs (between 1 - 10) are characteristic of unstable areas where epidemics are common and during which the BCCR can rise to over 1000. On the other hand, in holoendemic areas of high transmission such as Tanzania, the BCCR is stable at over 1000. However, recent mathematical modeling by Gupta and colleagues has shown that the BCCR was not as high as previously estimated in Papua New Guinea (Gupta *et al.*, 1994). They assumed that malaria in this endemic area was caused by a number of antigenically diverse strains, and the high risk of infection (as measured by the short time, typically < 1 year, before children became infected) was due to the slow build up of natural immunity to predominant infecting strains. The strain-specific nature of immunity is discussed later, but this shows the multifactoral approach necessary for an accurate explanation of malaria epidemiology.

An application of qualitative epidemiology has been in the accurate definition of clinical malaria cases in highly endemic areas. In these communities, it is difficult to determine how many people have febrile episodes that are caused by malaria because parasitaemia is universal. The attributable fraction (AF) is the proportion of fevers that are due to malaria parasitaemia alone and is estimated from blood smears and temperature readings. By taking factors such as age and semi-immunity into account and by using curve fitting regression analysis, more accurate estimates of the AF are possible and fewer false-positives are recorded. Hence, the effect of malaria on communities can be monitored accurately with a combination of epidemiological indicators and statistics. These techniques are a powerful tool that have allowed investigators to identify communities that suffer a high malaria burden, and to select regions for clinical trials of vaccines (Alonso *et al.*, 1994a, b).

1.5. The Clinical Picture

1.5.1.1 Symptoms and Diagnosis

The clinical symptoms of malaria depend on the species of infecting parasite and the immune status of the patient (reviewed by Warrell, 1993a). There is no single diagnostic feature of the disease, although the normal course of mild malaria is the development of episodes of chills, fevers, sweating interspersed with periods of well-being. The periodicity of the febrile stages is characteristic of the *Plasmodium* species and is determined by the duration of the intraerythrocytic cycle, if the infection is left untreated. The interval between febrile episodes is 72 hours in *P. malariae* and 48 hours in *P. falciparum*, *P. vivax*, and *P. ovale*, although synchronised infections are rare for *P. falciparum* malaria. These alternating paroxysmal attacks last for 8 - 12 hours and are usually accompanied by the presence of a blood-stage parasitaemia. The symptoms of subsequent episodes become less marked after several weeks, and further infections result in milder symptoms in spite of a persistent parasitaemia.

However, there is no direct relationship between parasitaemia and disease severity, because immune individuals are universally parasitised in endemic areas, but are asymptomatic. Parasitaemia is an unreliable indicator of disease or prognosis because recrudescences of *P. falciparum* and *P. malariae* may occur without any detectable parasite reservoir and relapses of *P. vivax* and *P. ovale* are caused by activation of hypnozoites in the liver. To avoid problems with asymptomatic parasitaemias, field studies have defined a clinical episode of malaria as an axillary temperature of >37.5 °C accompanied by a parasite density of between 5000 - 20000 parasites μl^{-1}, depending on the level of endemicity (Greenwood *et al.*, 1987).

In most cases, complete recovery is achieved, and eventually semi- or complete immunity will be acquired to the disease and symptoms will not occur in subsequent infections. However, a small proportion of patients, usually children or non-immune travellers, will develop severe life-threatening illness when infected with *P. falciparum* malaria. Factors which influence the occurrence of severe disease are poorly understood but probably include sporozoite dose, parasite virulence, socioeconomic variables (eg. nutrition status) and initial host immune status. Symptoms of severe malaria are

variable and can differ between age groups and geographical locations. Common complications are jaundice, shock, hypoglycemia, severe anaemia (haemoglobin <7.1 g dl^{-1}), and acute renal failure accompanied by multi-organ failure (Warrell *et al.*, 1982).

Cerebral malaria is the most notorious form of severe malaria where fever precedes convulsions and impairment of consciousness, which can be followed by death, particularly in young children. The importance of early diagnosis and prompt treatment is emphasised by the brief time (mean 2.8 days in The Gambia) between the onset of symptoms of cerebral malaria and death (Greenwood *et al.*, 1987).

The clinical definition of cerebral malaria is an acute diffuse, reversible encephalopathy accompanied by neurological complications where coma is essential. Other causes of coma must be confidently omitted, and a decreased level of consciousness may be an earlier sign. Although many fatalities occur at home and go unrecorded, population studies in Africa have estimated that the annual death rate from malaria in the under 5 age range is 1 - 10 per 1000 (Greenberg *et al.*, 1989). Severe malaria anaemia and cerebral malaria are the principal causes of death, and an unpublished study from a paediatric ward in The Gambia found that cerebral malaria was responsible for 17% of all childhood deaths between 1988 - 1990, and 8% were caused by severe malaria anaemia. In a separate study, neurological sequelae, for instance hemiplegia and cortical blindness, occurred in 10 - 15% of survivors of cerebral malaria, although it is more difficult to estimate long-term effects of malaria (Marsh, 1992). A case control study involving continuous follow-up of 200 patients in Kilifi, Kenya attempts to answer this question conclusively (C. I. Newbold, personal communication).

1.5.1.2 Treatment

The initial objective of treatment is the improvement of the patient's symptoms and often a radical cure is not attempted when there are multiple complications. In the simplest clinical scenario, mild malaria would be treated by the oral administration of one or a combination of antimalarial drugs. Treatment, carried out by a non-physician field worker, is usually oral chloroquine, mefloquine, proguanol or quinine and is normally effective within one week (Warrell, 1993b).

However, in many parts of the Tropics the range of drug treatments is restricted by cost, and the sporadic use of the limited prophylactic antimalarial drugs has enabled resistance to spread at an alarming rate. This means, in turn, that when a patient presents with malaria symptoms, there is a risk that a toxic drug overload will occur on treatment if there are already high levels of drug present. Consequently, there is now a recommended list of antimalarials, based on their pharmacokinetic properties, which are restricted for use in prophylaxis (eg. chloroquine), and others, such as quinine, are used for therapeutic purposes only (Warrell, 1993b).

Chloroquine had been considered the answer to the malaria 'problem' because of its high efficacy, low toxicity and low price. However, resistance has emerged gradually from one or two foci in South America and Southeast Asia over the past 40 years, with chloroquine-resistant *P. falciparum* reported throughout the malaria world (reviewed by Schapira *et al.*, 1990). Resistant parasites can rapidly export chloroquine from infected erythrocytes, but the molecular mechanism of this process remains unclear despite intense scrutiny. Genetic mapping has localised the chloroquine resistant locus, which should permit the isolation of the gene(s) responsible and suggest new therapies (reviewed by Wellems, 1991).

The spread of antimalarial resistance has been rapid and has led to the revival of the oldest antimalarial, quinine. Quinine use had been restricted because of its side-effects, termed cinchonism, which included deafness and nausea. Insulin secretion is stimulated by quinine which can also exacerbate the hypoglycaemia common in patients with high parasitaemias (reviewed by White and Pukrittayakamee, 1993). Consequently, selective use of quinine has resulted in limited resistance and the drug has found widespread application as the first-line of treatment for patients with severe

malaria. However, the delivery is complicated by disturbances to drug pharmacokinetics in severe disease conditions (White, 1987), and vomiting can mean that quinine must be delivered by intravenous infusion which limits its use to hospitals.

The clinical management of severe falciparum malaria has received much attention over recent years as improvement of medical facilities has attempted to keep pace with ever expanding knowledge of the pathogenesis of the disease (section 1.8.1). Strict definitions of which complications define the condition of severe disease have now been implemented which will lead to more accurate diagnoses (and problems in interpretation of antiquated literature). Rapid recognition of symptoms in patients at high risk, such as children and pregnant women, have led to fewer delayed diagnoses which was associated with higher mortality. A range of ancillary treatments for the broad complications of severe disease have been developed and tested in tropical hospitals. Severe anaemia can be treated with a screened blood transfusion, hypoglycaemic complications reduced with a single intravenous injection of 50% dextrose, and lactic acidosis alleviated by dichloroacetate (which stimulates pyruvate dehydrogenase activity). Focus on improved clinical attention to patients at the earliest signs of malarious symptoms has seen the implementation of hospital treatments founded on basic research. Continued development of appropriate research programmes will be vital to increase our understanding of malaria, especially if the spread of antimalarial resistance continues.

1.6. The Parasite

Plasmodium falciparum must be able to adapt to a multitude of hosts and vectors and this capacity has allowed its successful continuation as a species. This section outlines its evolutionary origins, the genetics which underlie its diversity, and the metabolic and transport processes that it uses for survival.

1.6.1 Aetiology

All malaria parasites belong to the phylum *Apicomplexa* (by virtue of the apicomplexes present inside the invasive forms), the class *Sporozoa* (because spores are formed in the life-cycle), the zoological family *Plasmodiidae* and are classified in the genus *Plasmodium* (reviewed by Gilles, 1993c). There are about 125 *Plasmodium* species, of which 20 are parasites of sub-human primates and 4 are parasites of man. Some of the 20 primate *Plasmodium* species are zoonotic and can produce the disease in man under natural and experimental conditions, but their significance is minor (Chin *et al.*, 1965).

The human *Plasmodium* species, *P. malariae*, *P. vivax*, *P. falciparum*, and *P. ovale* can interact with each other because superinfections of heterologous species are common. Finally, each human *Plasmodium* species can exist as morphologically identical strains that occur naturally in single and multiple infections, but can be distinguished by genetic analysis (reviewed by Gilles, 1993a).

1.6.2 Phylogeny

The origins of human malaria are obscure and have been examined by a range of phenomenological methods and recently, by recombinant DNA technology. Analysis provides inference pertaining to the evolutionary relationships between species and can thus direct investigations to alternative model systems for functional studies.

The DNA sequence of the asexually expressed small subunit ribosomal RNA (SSU rRNA) has been used to infer that *P. falciparum*, *P. vivax* and *P. malariae* have distinct lineages. Molecular analysis

has shown that *P. falciparum* was closely related to two avian species (*P. gallinaceum* and *P. lophurae*), whereas *P. vivax* was related to three simian malarias (*P. cynomolgi*, *P. knowlesi*, and *P. fragile*) and *P. malariae* was related to the rodent parasite, *P. berghei*. The sequence of the SSU rRNA genes *from P. ovale* and *P. reichenowi* (infection of higher apes) were not analysed.

These findings from a single molecule imply that lateral transfer of *P. falciparum* occurred millions of years ago from an avian to a human host (Brooks and McLennan, 1992), but there are still many contentious issues in this debate (reviewed by Waters *et al.*, 1993). Phylogenic trees are useful to identify functional homologues in parasites that share similar features and for homologues in other species that would aid the discovery of potential targets for antimalarial drugs.

1.6.3 Genetics

The explosion in molecular biology has permitted rapid advances to be made into the genetic structure of *Plasmodium* species. However, there are several unique features of *Plasmodium* which have restricted the application of classical and modern genetic techniques. Fundamental difficulties arise from the parasite's complex life cycle and the problems of maintaining each stage *in vitro*. A diploid stage exists briefly in the zygote in the mosquito gut wall, but the ploidy is 1N for the rest of the cycle when propagation is by mitosis (Walliker *et al.*, 1987). The sexual stages are difficult to study and only two genetic crosses have been performed by feeding mosquitoes with genetically-distinct *P. falciparum* gametocyte clones (Walliker *et al.*, 1987; Wellems *et al.*, 1990). PCR based analysis of asexual stages arising when sporozoites are transmitted to new vertebrate hosts has shown that genes are inherited in a Mendelian fashion, and that recombination occurs during zygote development (Walliker *et al.*, 1987; 1989).

The genome of each haploid *P. falciparum* cell is 25-30 Mb and contains 82% adenine-thymine (A-T) base pairs (Goman *et al.*, 1981; Pollack *et al.*, 1982). This unusual percentage is the highest recorded for any organism and means that the genetic code is more restricted in *P. falciparum*. Although the coding regions are not as A-T-rich, the upstream regulatory regions consist of up to 95% A-T base pairs which makes them inherently unstable and explains some of the problems encountered in cloning these regions into other organisms. Values for the guanine-cytosine (G-C) ratios and

interactions of DNA with probes for individual genes have indicated that *P. falciparum* is more closely related to *P. berghei* and *P. lophurae* than to other primate malaria species (Waters *et al.*, 1993).

Pulsed field gel electrophoresis (PFGE) has shown that all *Plasmodium* species comprise 14 chromosomes in the size range 0.5-3.5 Mb (Kemp *et al.*, 1987; Wellems *et al.*, 1987). Even though 14 kinetochores are visible inside intraerythrocytic cells (Prensier and Slomianny, 1986), no condensation occurs prior to mitosis which has prevented any *in situ* hybridisation studies. A large multi-centre project is underway to provide low and medium resolution maps of the *P. falciparum* genome from YAC libraries (Craig and Langsley, 1993). This will provide an integrated resource for the eventual sequencing of the entire genome and form a reference library of insert clones which together should facilitate the elucidation of the genetic basis of *Plasmodium* biology (Foster and Thompson, 1995).

In addition, there are two extra-chromosomal DNA elements in *P. falciparum* cells that resemble organelle-type genomes (reviewed by Palmer, 1992). The pseudo-mitochondrial genome is a multi-copy linear DNA that consists of 6kb tandem repeats and encodes three characteristic oxidative phosphorylation proteins and small fragments of large and small subunit rRNAs (Feagin *et al.*, 1992; Feagin, 1992). The other element is a 35kb circular DNA that has plastid characteristics and encodes genes for rRNAs, ribosomal proteins and functional tRNAs (Gardner *et al.*, 1991).

Malaria parasites exhibit a remarkable plasticity in the size of their chromosomes and consequently, intra-species variation of homologous chromosomes is commonplace. Size polymorphisms of up to 0.5Mb can occur by a variety of mechanisms including deletions (Corcoran *et al.*, 1988), recombination during meiosis (Vernick *et al.*, 1988), chromosomal breakage and healing (Corcoran *et al.*, 1986; Pologe and Ravetch, 1986, 1988) or amplification (Foote *et al.*, 1989; Wilson *et al.*, 1989).

Analysis of chromosome organisation has revealed that they are divided into distinct conserved and polymorphic domains. Regions of conservation occur in the centre and are transcribed, whereas polymorphic regions are located at the chromosome ends and tend to be transcriptionally inactive. The

polymorphic domains consist of multiple repeats that are implicated in recombination events during mitosis and meiosis (reviewed by Janse, 1993; Lanzer *et al.*, 1994a).

Processes occur during mitosis that lead to polymorphism and have been associated with functional changes such as the generation of antigenic diversity (Janse, 1993). It is not known whether these mitotic processes are spontaneous or programmed, but many are associated with DNA rearrangements at the chromosome ends. Deletions and insertions at subtelomeric regions can occur after chromosome breakage and repair (Lanzer *et al.*, 1994a), and chromosomes are always healed by insertion of telomeric repeats by telomerases (Pologe and Ravetch, 1986, 1988). Several chromosome breakage sites have been mapped to antigen-encoding genes, such as RESA, HRP1, HRP2 and Pf11.1 (Pologe and Ravetch, 1986, 1988; Pologe *et al.*, 1990; Scherf and Mattei, 1992; Scherf *et al.*, 1992). Recent reports that breakage events occur in nucleosome linker regions in KAHRP implicate a role for chromatin structure in availability of hot-spots (Lanzer *et al.*, 1994b) but this does not appear to be the case for Pf11.1 (A. G. Craig, personal communication). Loss of expression of antigens *in vitro* is associated with deletion events and the location of genes proximal to sub-telomeric ends provides an ideal site for generation of antigenic diversity by crossing-over mechanisms (Foote and Kemp, 1989; Kemp *et al.*, 1990). In support of this hypothesis, many genes for conserved housekeeping proteins (eg. actin, tubulin) have been mapped to central chromosome regions where they would not be prone to polymorphism (Wesseling, *et al.*, 1989; Delves *et al.*, 1990). Recent data supports 4 to 7 fold amplification a 30kb subtelomeric region on chromosome 4 of *P. falciparum*, but no function has been associated with this modification (J. Roubio, personal communication).

Early recombinant DNA technology focused on the production of cDNA and genomic DNA *Plasmodium* libraries in *E. coli*. DNA was cloned into λGT11 and λAmp3 vectors to construct expression libraries which were screened with polyclonal immune serum to identify antigens that could be vaccine candidates (Kemp *et al.*, 1983). These techniques led to the discovery of large polymorphisms at the protein level which correlated with high levels of diversity in parasite populations (reviewed by Day, 1992). Thus, the generation of increased diversity can be explained by examination of the structure and expression of the antigen encoding genes. The first example of parasite recombination has recently been reported for the merozoite surface protein genes, MSP1 and

MSP2 *of P. falciparum*. This mechanism generates new alleles at meiosis and may contribute to the enormous genetic diversity found in natural populations (section 1.6.4).

The possible role of these mechanisms in developmentally-regulated functions awaits characterisation of regulatory sequences and DNA binding proteins which are not likely to be polymorphic if they have important functions. Regulatory regions have been examined by cloning into prokaryotic vectors, but the inserts are inherently unstable due to their high A-T content. Experimental evidence from a number of genes suggests that regulation is predominantly at the level of transcription initiation, but upstream non-coding regions contain up to 95% A-T and this precludes the identification of TATA box homologues (reviewed in Lanzer *et al.*, 1993). Co-linear RNA is produced by the transcription machinery and processing occurs to trans-splice the infrequent introns and polyadenylate the 3'-end. Establishment of a transfection system for *Plasmodium* has been a long-term goal for the overall investigation of the control of gene expression. Transient transfection of *P. gallinaceum* female gametes by electroporation with the coding region of the pgs28 gene (gamete / zygote specific marker) linked to a firefly luciferase reporter gene was successful (Goonewardene *et al.*, 1993). This is the first step in the direct analysis of control regions and mechanisms, and may assist in the elucidation of a method for stable transfection.

1.6.4 Antigenic Diversity

Antigenic diversity occurs because of polymorphisms in allelic genes that result in the expression of structurally and antigenically distinct forms of a particular protein in different parasites and strains (Anders *et al.*, 1993). The genetic mechanisms described above underlie the polymorphisms that are characteristic of *Plasmodium* proteins (Anders and Smythe, 1989; McConkey *et al*, 1990). Several antigens which are recognised by immune sera from patients living in endemic areas have now been cloned. Sequence analysis has revealed that they are remarkably diverse and composed of multiple repeated segments. These repeats are thought to have a role in immune evasion and explain how *P. falciparum* can maintain long-term blood-stage infections, although the underlying mechanisms have remained elusive despite intense investigation.

To reflect these efforts, the most studied polymorphic antigens are those of the asexual blood stage haploid cells where the sequence of the allelic genes defines the expressed protein, and two specific examples are discussed below. A summary of the diversity observed in the proteins expressed at the surface of the infected erythrocyte is given to complement the later section on antigenic variation, the process whereby a clonal population of parasites can change its phenotype.

1.6.4.1 S Antigens

The S antigens (Sags) are the most polymorphic of the asexual blood stage antigens that have been characterised by sequencing studies, and protein size variations of 50-250 kDa can occur between Sags of field isolates (reviewed by Anders *et al.*, 1993). Sags are soluble, heat stable proteins synthesised by the schizont, exported to the parasitophorous vacuole and released into the circulation when the erythrocyte bursts. However, no function has been ascribed, although the presence of anti-Sag antibodies in a small proportion of individuals from endemic areas suggests that they may play a role in immune evasion. In support of this hypothesis was the finding that the same Sag did not occur in separate malarial episodes in the same individual (Wilson *et al.*, 1975). However, the study was not large enough to draw significant conclusions and the functional importance of these diverse antigens has remained enigmatic.

Although not universally accepted, the long-standing 'immune smokescreen' hypothesis proposes that the Sag is part of the parasite's mechanism to exhaust the host immune system. The Sag may achieve this by eliciting a low-affinity antibody response to the collective cross-reactive epitopes encoded by the central portion of the gene. These immune decoys could simultaneously divert any potentially harmful immune responses from other important epitopes.

The Sag gene exists as a single copy without introns and is ideally suited as a polymorphic marker in population biology studies because it varies according to strain type, but is stable *in vivo* and *in vitro* (reviewed by Anders and Smythe, 1989). There are four allele types that are defined on the basis of the 5' conserved regions and 3' semiconserved regions which flank a highly polymorphic region of tandem repeats. The repeat structure is the source of the antigenic diversity because the number (up to 100), length (21 - 45bp), sequence and reading frame of individual repeats can all vary. Natural

antibodies are found mainly in adults and are directed to repeat regions (Wilson *et al.*, 1975).These antibodies and monoclonal reagents have proved to be difficult to use in a typing system (Forsyth *et al.*, 1989a), but recently field and laboratory isolates have been examined by PCR amplification of the repeat regions (Kyes *et al.*, 1993). This technique is a sensitive diagnostic tool which can detect a single parasite (in theory) and unpublished results from a large African study imply the existence of a at least one new allele type (Dr. S. Kyes, personal communication). Comparative population studies of Sags and other polymorphic markers with such techniques will provide vital information on the spatial and temporal diversity of such proteins. One aim of such studies is to determine whether parasites exist as a single interbreeding population, or whether they occur as distinct inbreeding populations. Results may affect the planning of control measures for combating the spread of drug resistance or implementing new chemotherapy trials.

1.6.4.2 Circumsporozoite Protein

The plasma membrane of infective sporozoites is covered uniformly by a dense coat of circumsporozoite (CS) protein that is anchored to the pellicle and interacts with the surface the hepatocyte in invasion (reviewed by Nussenzweig and Nussenzweig, 1989). Immature sporozoites, which lack CS protein, were not infective and monoclonal antibodies to CS protein in mature sporozoites blocked hepatocyte attachment and invasion.

The CS gene was first cloned in *P. knowlesi* and later in *P. falciparum,* and extensive studies have shown that diversity occurs between species and between strains. It codes for an acidic polypeptide of 40-60 kDa, and the centre section consists of multiple short tandem repeats (NANP in *P. falciparum*) flanked by semi-conserved N- and C-termini. In common with Sags, the number, sequence and length of repeats varies remarkable between parasites even though they can invade the same host. For instance, the CS protein of *P. falciparum* contains 37 repeats of NANP interspersed with 4 copies of NVDP to create more diversity (reviewed by Arnot *et al.*, 1994).

The repeats are antigenic and early studies of CS protein concentrated on the central region and its role in immunity and hepatocyte invasion. When host antibodies bind to CS protein, the antigen-antibody complexes are sloughed off the surface and collect as a precipitate at the rear of the

sporozoite. This is known as the circumsporozoite precipitation (CSP) reaction and may be a form of defense against host immunity: the sporozoite remains infective because new CS protein is synthesised to replace the lost protein. Conserved cysteine residues at the C-terminus of CS protein have been implicated for invasion because they are essential for binding to sulphated sugars that are important components of the heparan sulphate proteoglycan counter-receptor on the hepatocyte surface.

The purpose of the antigenic diversity observed in CS protein is uncertain, but the presence of multiple immunodominant repeats may be a mechanism to neutralise harmful antibodies involved in the CSP reaction. These repeats may simultaneously divert the immune response from the functionally important regions of the molecule which are involved in invasion, but conclusive evidence has not been obtained in spite of this protein's importance as a potential vaccine candidate. A high proportion of non-synonymous mutations coincide with two immunodominant T-cell epitopes in the non-repetitive coding sequence of CS. These variations may be due to selection pressure exerted by the immune system, but this awaits conclusive demonstration (reviewed by Lockyer *et al.*, 1989).

1.6.4.3 Merozoite Surface Proteins

Early radiolabelling studies of merozoites indicated that there were more antigens on the merozoite surface than on the surface of the sporozoite. It now appears that homologues appear on the mature merozoite of all *Plasmodium* species as a result of proteolytic processing of a large (~200kDa) precursor polypeptide which is anchored to the membrane via a GPI-linkage at the C-terminus. referred to here as merozoite surface antigen 1 (MSA1), and has been otherwise known as the 195K protein, P190, the 200kDa protein, the polymorphic schizont antigen, gp185 and MSP1. Numerous alleles of MSA1 have been sequenced and the gene is extremely polymorphic between species and strains, for example 7 different allelic forms were identified using monoclonal antibodies in only 37 isolates of *P. falciparum*. Sequence alignment reveals 17 blocks which are either variable, conserved or semi-conserved (Miller *et al.*, 1993). The conserved and semi-conserved regions exhibit dimorphism which suggests that the major alleles of MSA-1 have been generated by intragenic recombination between two parental alleles during meiosis. The notable exception to this dimorphic generalisation occurs in block 2 which is constructed from repeats of 9bp and 27bp. Monoclonal

antibodies to MSA-1 that inhibit invasion and growth bind to the processed C-terminal 19kDa fragment which is conserved between parasites and remains associated with the erythrocyte membrane after invasion. When the same fragment was expressed in a baculovirus-insect cell *in vitro* system, antibodies were elicited that blocked growth. This has caused optimism that it may be possible to develop a vaccine that will be effective against diverse strains of *P. falciparum*.

MSA-2 is the product of a separate gene and is expressed on the merozoite surface as a ~28kDa GPI-anchored protein. Unlike MSA1, it is not proteolytically processed or carried through into ring stages, but it does exhibit polymorphism. Gene sequences from different isolates show that there is a large variable central region consisting of a tandem repeats which is flanked by non-repetitive 5' and 3' domains. Both types of region exhibit dimorphism and further antigenic diversity is created by intragenic recombination within the repeat regions (Marshall *et al.*, 1991). Repeats of MSA2 are analogous to those found in Sags because they are variable in sequence, length and number and are weakly immunogenic. Most indigenous residents from holoendemic malaria areas have antibodies to MSA2 in their sera and some monoclonal antibodies to this antigen can inhibit merozoite invasion, but functional characterisation is incomplete.

1.6.4.4 Erythrocyte Associated Proteins

During maturation of intraerythrocytic stages, over 10 polymorphic antigens have been identified that are synthesised and transported to the host cell surface where they are either inserted or secreted. In the case of *P. falciparum*, these antigens include PfEMP1, PfEMP2 (MESA), PfEMP3, Pf11.1, Pf332, HRP1, HRP2, HRP3, RESA (Pf155), FIRA, rosettins and SHARP. As a group, they are not as polymorphic as Sags (PfEMP1 may be an exception), even though they contain tandem repeats which are subject to single amino acid substitutions. Further antigenic diversity is generated by variations in repeat number, but the limited sequence data has restricted any detailed analysis. These proteins are extensively reviewed later in relationship to immunity (section 1.7.2) and biochemical and functional membrane changes of the erythrocyte (section 1.8.4). Their importance in antigenic diversity is emphasised by the large number of natural antibodies that recognise antigens produced by the infected erythrocyte.

1.6.5 Metabolism

Metabolism has been studied predominantly at the intraerythrocytic stage because infected erythrocytes can be purified in large numbers (reviewed in Sherman, 1979; Ginsburg, 1990). Consequently, little is known about metabolism in the other developmental stages, and current research has been driven by the need to identify novel pathways that may be targets for antimalarial drugs.

The mature erythrocyte is a biosynthetically inert cell and this presents a number of problems for the parasite because it requires nutrients for growth and development and must secrete toxic waste products. To grow efficiently, the parasite synthesises some enzymes for new metabolic pathways and transports substrates and other metabolites into its vicinity. For *P. falciparum* infected erythrocytes, few metabolic changes can be detected during the first half of the cycle. However, growth at trophozoite stage is accompanied by rapid changes in metabolism which are summarised below.

Energy Supply. Parasites do not store carbohydrates and must synthesise their own ATP necessary for growth. Thus, large quantities of glucose are acquired from the blood plasma and used in glycolysis which in turn produces ATP and lactate. NADPH for glycolysis is provided by the parasite- and host-encoded glucose-6-phosphate dehydrogenases (reviewed by Trigg, 1985), and because this process is anaerobic, it is unaffected by the oxygen tension of the erythrocyte. The rate of glycolysis is one to two orders of magnitude higher than the rate in uninfected erythrocytes. This contributes to hypoglycaemia (Molyneux *et al.*, 1989) and lactic acidosis (White, 1987), which are common conditions in malaria.

Protein Biosynthesis. Parasites can synthesise only glutamate, aspartate, alanine and leucine (Blum and Ginsburg, 1984) and must acquire the other amino acids from their surroundings (Siddiqui and Trager, 1967). Pinocytosis of cytoplasmic proteins occurs via internalisation of cytosomes located in the PVM which form food vacuoles inside the parasite. Haemoglobin is the major red cell cytoplasmic protein and is digested into haem groups and its constituent amino acids are used to synthesise new parasite proteins via a eukaryotic translation apparatus (Sherman and Tanigosh, 1970). The haem

molecules are oxidised and converted to haematin by haem polymerase. The haematin precipitates as pigment in the food vacuoles (reviewed by Goldberg and Slater, 1992).

Nucleic Acid Biosynthesis. Parasites must undergo rapid changes in nucleic acid synthesis to provide the characteristic AT-rich DNA for new merozoites at the late trophozoite stage. The parasite is incapable of *de novo* synthesis of purines (adenine and guanine) and salvage mechanisms are used to obtain adenosine and hypoxanthine precursors from blood plasma (Reyes *et al.*, 1982). Pyrimidines (cytidine, thymine, and uracil) are synthesised by parasite-encoded enzymes even though there are large excesses in the host cytoplasm and plasma (O'Sullivan and Ketley, 1980). The parasite encoded dihydrofolate reductase (DHFR) and thymidilate synthase (TS) enzymes exist as a single bifunctional protein which is involved in the conversion of uracil to thymine. This activity is sensitive to antifolate drugs (pyrimethamine and cycloguanil) at concentrations that do not affect human DHFR, and these drugs are specific to *Plasmodium* which implies that either the DHFR-TS enzyme or the pathway is unique. DHFR-TS requires folic acid as a cofactor and parasites do not grow efficiently in hosts who are deficient in PABA (para-amino benzoic acid; an essential precursor for folic acid).

Lipid Biosynthesis. Large numbers of diverse lipids are required for the construction of new merozoite membranes. The parasite synthesises some itself (e.g. phosphatidyl choline from serine and palmitate) (Vial and Philippot, 1982a, b) and the rest are imported from the plasma in the form of high density lipoproteins (Grellier *et al.*, 1991). The glycerol backbone of triglycerides and phospholipids is parasite-derived and lipid precursors are phosphorylated, resulting in a 5-fold increase in the total amount of phospholipid (Sherman, 1985). However, the parasite cannot synthesise new cholesterol or fatty acids, and is restricted in the number of modifications it can make to the latter. Thus, infected host membranes and parasite membranes have a lower cholesterol : phospholipid ratio than normal membranes (Sherman, 1985; Hsiao *et al.*, 1991). These factors may be protective against antioxidant stress (Kumaratilake *et al.*, 1992).

1.6.6 Transport

Efficient transport systems must exist to satisfy the extensive metabolic requirements of, and mediate protein secretion from the intraerythrocytic parasite (reviewed by Ginsburg, 1990; Cabantchik, 1990). However, there is no agreed paradigm for any proposed transport processes, because the compartmentalisation of the parasite and its access to the bloodstream are equivocal (reviewed by Haldar, 1994; Gero and Kirk, 1994). Three proposed models and their salient features are summarised in the annotated diagram (Figure 1.2). There is controversy over the existence of a parasitophorous duct that forms after invasion and allows the parasite plasma membrane (PM) direct access to the extracellular environment (reviewed by Taraschi and Nicolas, 1994). In addition to this structure, there is evidence for a tubovesicular membrane network (TVM) that mediates vesicular transport and has properties which are characteristic of a Golgi apparatus (reviewed by Elmendorf and Haldar, 1993).

In view of the inconsistencies relating to overall transport processes, it is simplest to consider the individual membranes and ducts with respect to their transport properties:

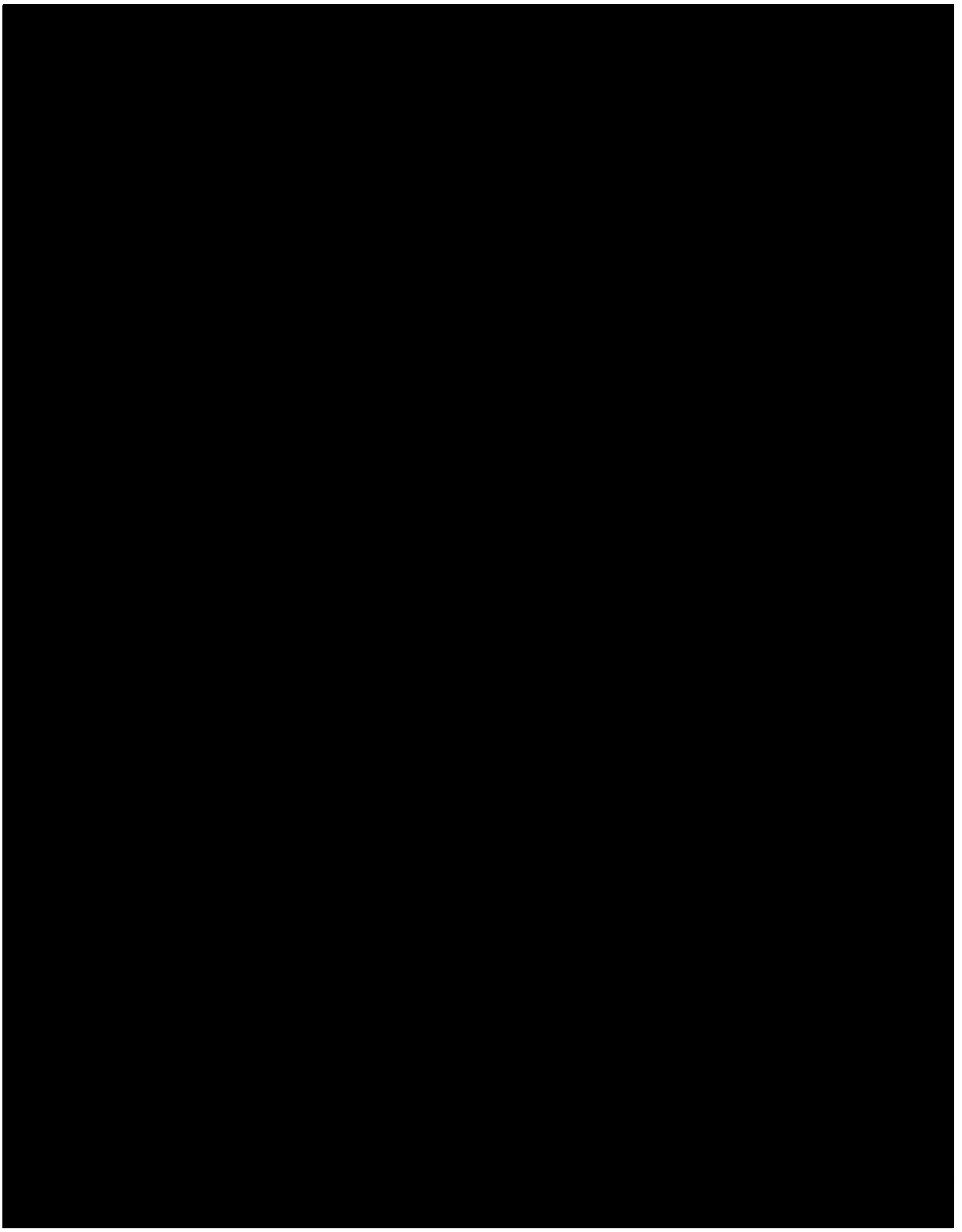


Figure 1.2. Proposed models for nutrient transport in infected erythrocytes (from Gero and Kirk, 1994).

a) Conventional model or b) direct access models where the intracellular parasite has access to the extracellular environment either (1) in the accolé position via a window opened by the fusion of PM and PVM or (2) by the formation of a duct.

Erythrocyte membrane (EM). Transport of solutes into and out of *P. falciparum* infected cells is by endogenous red cell transporters (eg. Band 3, glucose transporter, lactate transporter, Na^+-K^+ ATPase pump) in addition to parasite-induced modifications in the membrane. It has been established that changes occur in the erythrocyte membrane (EM) during maturation of the intracellular parasite which increase the permeability of the host cell to a variety of low molecular weight compounds. This occurs ~12 hours after merozoite invasion because iso-osmolar sorbitol causes selective lysis of trophozoites after this time (Lambros and Van der Berg, 1979). Sugars (Kutner *et al.*, 1985), nucleosides (reviewed by Gero and Wood, 1991), amino acids (Elford *et al.*, 1985) and choline (Elford *et al.*, 1990) are transported into infected cells by parasite-induced, non-saturable pathways with broad specificity and represent novel pathways (Kirk *et al.*, 1994). The molecular identity and biochemical properties of these pathways are unknown, and consequently it has not been conclusively established whether there are several different induced transport systems or whether a single species with broad specificity could be responsible for this induced transport. Recent evidence from studies using pharmacological inhibitors of anion transport demonstrated that induced transport of monovalent anions, cations, amino acids, sugars and nucleosides was blocked in the same order of potency for each substrate (Kirk *et al.*, 1994). This, and other data from the same laboratory strongly implied that there was a single type of transport pathway which behaved as an anion-selective pore or channel. There is not known to be any similar system present in normal human erythrocytes, but anion-selective 'chloride channels' with comparable pharmacological characteristics exist in other vertebrate cells including hippocampal neurones (Franciolini and Nonner, 1987), *Xenopus* oocytes (White and Aylwin, 1990) and human platelets (Manning and Williams, 1989).

Parasitophorous vacuolar membrane (PVM). Techniques to study transport across the PVM use methods that either permeabilise the EM to such an extent that it is no longer rate limiting, or involve the use of free parasites. Such studies have concluded that the PVM is not important for selectivity, but patch-clamping of infected cells following treatment with either digitonin or electric shock has detected a so-called 'nutrient channel', which has no specificity for either cations or anions (Desai *et al.*, 1993). An analogous channel has been identified in another parasite vacuolar membrane, *Toxoplasma gondii* (Schwab *et al.*, 1994). Therefore, the PVM could function as a molecular sieve.

Parasite plasma membrane (PM). There is evidence for a range of transport activities in the PM including an ATP-ADP exchange mechanism (Kanaani and Ginsburg, 1989), Na^+-H^+ antiport, glucose-H^+ cotransport, adenosine, Ca^{2+}, and lactate transport (Bosia *et al.*, 1993). However, due to the techniques used for studying the PM, these results could be attributed to transport across residual PVM. Thus, conclusive evidence for transport across the PM can only be obtained from studying trophozoites cultivated from free merozoites or other unambiguous methods (Trager *et al.*, 1992).

Metabolic window (PVM-PM fusion). The existence of a non-specific nutrient channel in the PVM is difficult to reconcile with the existence of a duct (see next) because low molecular weight solutes in the erythrocyte cytosol would be lost to the extracellular medium via the duct. The channel may be homologous to gap junctions and span the PVM and the PM to form a metabolic window (Gero and Kirk, 1994). Fusions of this kind are sometimes visible under electron microscopy when the intracellular parasite is in the accolé position, but there is no definitive evidence for the existence of a transport mechanism in this scenario.

Parasitophorous duct. There is persuasive evidence from biochemical studies that the intracellular parasite has direct access to the extracellular milieu via a duct structure that is continuous with the EM and the PVM (Pouvelle *et al.*, 1991). Investigations have shown that macromolecules (>10kDa) such as dextrans (Haldar and Uyetake, 1992; Fujioka and Aikawa, 1993), protein A and antibodies present in external medium directly enter the parasitophorous vacuole (Haldar, 1994). This theory is supported by evidence which suggested that iron chelators have direct access to parasite without passing through the host cell cytoplasm (Scott *et al.*, 1990). However, other investigators have been unable to repeat some of these findings and the existence of a duct remains highly controversial.

Tubovesicular membrane network (TVM). This is a dispersed network of membranes in the erythrocyte cytosol that was identified by examination of optical sections of infected cells. It appears to be continuous with the PVM, but does not seem to connect to the EM and accordingly there is no evidence that it participates in uptake of exogenous solutes (Elmendorf and Haldar, 1994). The localisation of typical golgi enzymatic activity (eg. sphingomyelin biosynthesis) suggests that it could participate in protein secretion (Elmendorf and Haldar, 1994).

1.7. Immunity to Malaria

When a person is first bitten by an infected *Anopheles* mosquito, there are a variety of outcomes ranging from death to complete well-being. In the latter scenario, resistance is non-specific and is usually innate: host genetic factors protect certain individuals against malaria disease without prior exposure or presence of maternal antibodies (section 1.7.1). This is different from immunity to malaria which is normally acquired after continuous exposure and protects only against disease but not infection (section 1.7.2).

1.7.1 Innate Resistance

Epidemiological data has lead to the identification of several genetic polymorphisms that are often deleterious, but confer protection against malaria. This "Malaria Hypothesis" was proposed by Haldane in 1949 after he noticed that the prevalence of thalassaemia in malaria endemic areas may have represented a state of balanced polymorphism (Haldane, 1949). Four common red cell disorders that have reached polymorphic frequencies in areas in which malaria has been endemic or is still endemic are summarised below. The 'malaria hypothesis' has stood the test of time, and it appears that the mutations responsible for these disorders have arisen independently and have reached their high frequencies by selection by malaria. Since then, the intraerythrocytic stage of the parasite's life cycle has received intense scrutiny because it is the period where all the symptoms of the disease are manifested. The rapid advances in red blood cell genetics during the same period have allowed the genetic basis for several mechanisms of innate resistance to be proposed. However, no unified mechanistic theory is apparent because the protective effects are likely to be manifested in concert with each other and may individually influence acquired immunity (reviewed by Weatherall *et al.*, 1988; Nagel and Roth, 1989).

1.7.1.1 Thalassaemias

These syndromes (α-thal and β-thal) result from an imbalance of globin chain synthesis and are geographically restricted to present or former *P. falciparum* endemic areas (Weatherall and Clegg, 1981; Livingstone, 1985). A single case control study has shown a 50% reduction of clinical malaria

cases in β-thal heterozygotes (Willcox, 1983) and a protective effect for both α-thal and β-thal against malaria has been demonstrated in a similar study in East Africa (Dr. A. V. S. Hill, personal communication). The protective mechanisms, however, are obscure but the increased neo-antigen expression on infected thalassaemic red cells may lead to enhanced splenic clearance, or elevated phagocytosis by macrophages which could reduce parasitaemia and clinical episodes (Luzzi *et al.*, 1991a, b).

1.7.1.2 Sickle Cell Anaemia

This disease is caused by a point mutation in the β-globin gene which results in an abnormal haemoglobin tetramer and confers a sickled appearance on the red cells of homozygotes (SS). This state is usually fatal in childhood due to secondary infections or sickle cell crises (usually vaso-occlusive). Hence, the maintenance of the heterozygotic genotype (AS) at high frequencies (over 20% of the population) in past and present malarious areas is believed to suggest a role in innate resistance (Hill *et al.*, 1991; Marsh, 1992). Again, the exact mechanism of protection is unresolved, but evidence that AS carriers are protected from disease and not infection in childhood implies that there may be favourable immune clearance of infected AS red blood cells. In addition, there is conflicting evidence pertaining to the influence of the intracellular environment on parasite growth in AS and SS red blood cells; an issue which awaits further investigation.

1.7.1.3 Ovalocytosis

This is a rare autosomal dominant cytoskeletal abnormality of red blood cells which results in reduced membrane deformability, reduced anion transport and an oval shape. High polymorphic gene frequencies (up to 50% in areas of Papua New Guinea) are achieved in some geographical regions which are consistent with a role in resistance to malaria. Two linked mutations in the major red cell membrane anion transporter, Band 3, appear to be the only genetic defects and occur in all cases of SE Asian ovalocytosis (Jarolim *et al.*, 1991, Schofield, 1992). However, there are no known disadvantages associated with these changes and the polymorphism may not be balanced against a protective effect against malaria, even though several mechanisms have been proposed. A more likely explanation is that ovalocytotic mutations will become fixed in such populations and it is those

individuals whose red cells will be less susceptible to invasion by *P. vivax* and *P. falciparum* (Kidson *et al.*, 1981).

1.7.1.4 Duffy-antigen Negativity

Merozoite invasion of erythrocytes is influenced by specific blood group antigens expressed on the erythrocyte plasma membrane (reviewed by Miller, 1988). The Duffy system is encoded by two alleles, Fy^aFy^b, and the Duffy antigen functions as the erythrocyte IL-8 cytokine receptor (Sim *et al.*, 1994). Homozygotic deficient red cells are resistant to infection by *P. knowlesi in vitro* (Miller *et al.*, 1975) and by *P. vivax in vivo* and *in vitro* (Miller *et al.*, 1976). Thus, a clear mechanism is available to explain epidemiological studies which have shown that *P. vivax* is not endemic in West Africa where the majority of indigenous residents are Duffy negative, $Fy^{a-}Fy^{b-}$. Selection pressure by malaria may have caused the fixation of this blood group mutation which implies that there must have been increased mortality due to *P. vivax* in the past (Pasvol *et al.*, 1982). Alternatively, the protective effect could be indirect and its influence on malaria may have been secondary to another disease (Livingstone, 1985).

1.7.2 Specific Immunity

Life in the human body provides a series of diverse challenges to the malaria parasite. These include 'simple' physical barriers, such as the membranes of the hepatocyte and erythrocyte which the invasive parasite must penetrate. Simultaneously, the host mounts a complex repertoire of immune responses to each stage of the life cycle with the ultimate goal of parasite destruction. The mechanisms of naturally-acquired immunity to malaria determine the outcome of an infection, but little is known about their origins or targets (reviewed by Day and Marsh, 1991). Consequently, a review of the field is beyond the scope of this chapter and instead I will outline findings from epidemiological studies that relate to immunity in endemic areas and emphasise the importance of antigens expressed during the intraerythrocytic stage as targets for immune responses.

1.7.2.1 Immunity in Endemic Areas

There are three important conclusions that can be drawn from immunoepidemiological studies in areas where malaria is holo- or hyper-endemic:

- immunity to malaria protects against disease and not infection: it is non-sterilising.
- protective immunity requires time and continuous exposure to develop.
- once developed, protective immunity is stage-, species- and strain-specific.

During the first 3-6 months after birth, neonates born to immune mothers are protected from disease even though parasites are frequently transferred transplacentally and persist in the child. Maternal antibodies are probably passed to the foetus and confer protection against disease. After this period, all infants become parasitised and some are susceptible to the clinical complications associated with severe *P. falciparum* malaria. The two most common life-threatening conditions are severe malaria anaemia and cerebral malaria, and have been discussed earlier in the clinical picture (section 1.5). The incidence of these conditions has been determined in a large case control study of children of different ages in West Africa where malaria is endemic. The mean age of peak incidence of severe malaria anaemia (28 months) was substantially lower than that of cerebral malaria (45 months)

(Brewster *et al.*, 1990). The implication of such results is that cerebral malaria develops after prior sensitisation, but this has not been tested and differences in parasite virulence and host genetic factors probably contribute (Greenwood *et al.*, 1991).

After the fourth year, children experience fewer disease events and only mild malaria symptoms are observed for the rest of life in that area (McGregor, 1963). The parasite rate (proportion of people with peripheral blood parasitaemias) in such areas is 20 - 40% (Greenwood *et al.*, 1987; Marsh *et al.*, 1988) but immunity is probably never complete, because people become susceptible to disease on exposure to antigenically different parasites by moving to other endemic areas. Furthermore, when people return to endemic areas, they become susceptible to disease on re-exposure, which suggests that the maintenance immunity requires continuous exposure to the same strains, or at least the same antigenic determinants (Targett, 1985).

These epidemiological findings are supported by data which were obtained when malariotherapy was used as a treatment for advanced neurosyphilis. Subjects were challenged with parasites to alleviate neurological symptoms and examination of data shows that immunity to *P. vivax* and *P. falciparum* was species-, strain- and stage- specific (Yorke and Macfie, 1924; Ciuca *et al.*, 1930, 1934). Acquired specific immunity in patients was achieved after one or two exposures, but potential confounding factors from the syphilis cannot be ruled out. These studies also demonstrated that geographical isolates differed in the severity of illness that ensued, which may indicate the differential expression of parasite virulence factors (Covell, 1951, Greenwood *et al.*, 1991).

The most obvious explanation for the epidemiological findings, neurosyphilis data and animal models is that immunity to malaria is strain-specific, at least in the early years in endemic areas (reviewed by Marsh, 1993). Individuals have to be infected by a large number of strains in their area and mount a successful immune response to each strain. When a child dies of malaria with parasitaemia, it is because he is infected with a novel virulent parasite that his immune system does not recognise. Lines of parasites differ in virulence in experimental models and virulent strains could exist in natural populations of *P. falciparum* (Cox, 1988). The frequency of attacks decreases as experience of more strains occurs and immunity to each one develops individually. In adults there could be a cross-reactive protective response that over-rides this mechanism to reduce the severity of of attacks.

However, it could be that the strain-specific epitopes remain variant and the efficiency of the responses to conserved T-helper epitopes increases instead. A summary of potential mechanisms of immune attack and their stage-specificity is shown in Figure 1.3.

1.7.2.2 Asexual Blood Stage: Immune Responses and their Targets

The asexual blood stage is the only symptomatic period of the life cycle and stage-specific immunity was demonstrated by the ability of rodents to clear injected blood stage parasites. Protective immunity occurs at this stage of the life cycle *in vivo*, but dissection of the key mechanisms and their targets has been arduous. The fundamental problems are that all the possible antibody-mediated and cell-mediated responses can occur at this stage, and the parasite simultaneously expresses a multitude of antigenically diverse proteins. One of the reasons for the slow development of immunity may be that these antigens are poorly immunogenic with regard to protective responses (reviewed by Mendis *et al.*, 1991). Hypotheses have suggested that immunodominant antigens function as immune decoys to create a smokescreen (Anders *et al.*, 1986) or that common repeated structures are T cell-independent antigens which protect critical antigens by epitope suppression (Schofield, 1991). In the past, it has been difficult to reconcile specific protective responses in *in vitro* models with *in vivo* and clinical data. The key molecules for protective immunity against *P. falciparum* in humans appear to be a family of parasite-derived neoantigens expressed at the red cell surface, and their biochemical characterisation will be central to an accurate description of immunity to malaria (section 1.8.5).

In any discussion of immunity to asexual stages, an understanding of the location and precise stage-specificity of the target antigens is essential (Figure 1.4).

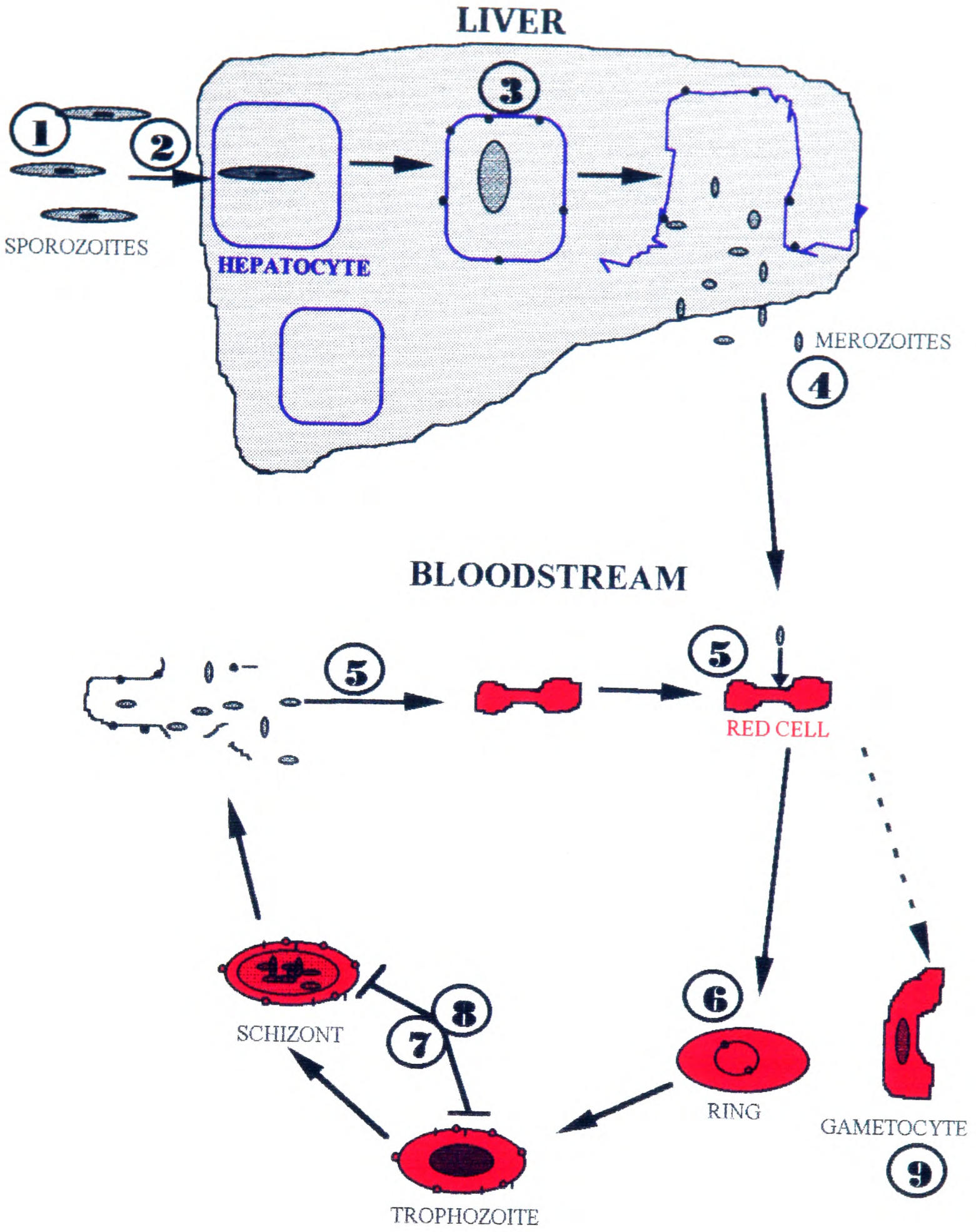


Figure 1.3. The parasite is exposed to several complex immune attacks after sporozoites are inoculated. These involve antibodies, cytokines and cell-mediated responses and the sites of potential attack of a selection of mechanisms in the life cycle are depicted (modified from Marsh, 1993).

***1**. Antibody-mediated opsonisation of sporozoites. **2**. Antibody-inhibition of hepatocyte invasion. **3**. T-cell mediated responses to processed antigens at infected hepatocyte surface. **4**. Antibody-mediated opsonisation of merozoites. **5**. Antibody-inhibition of red cell invasion. **6**. Intracellular killing of parasites by soluble mediators. **7**. Complement activation by antibody binding to neoantigens on surface of infected red cells, leading to lysis. **8**. Opsonisation of infected red cells by macrophages. **9**. Antibody to gametocyte surface (transmitted to mosquito where it can interfere with normal fertilisation or development).*

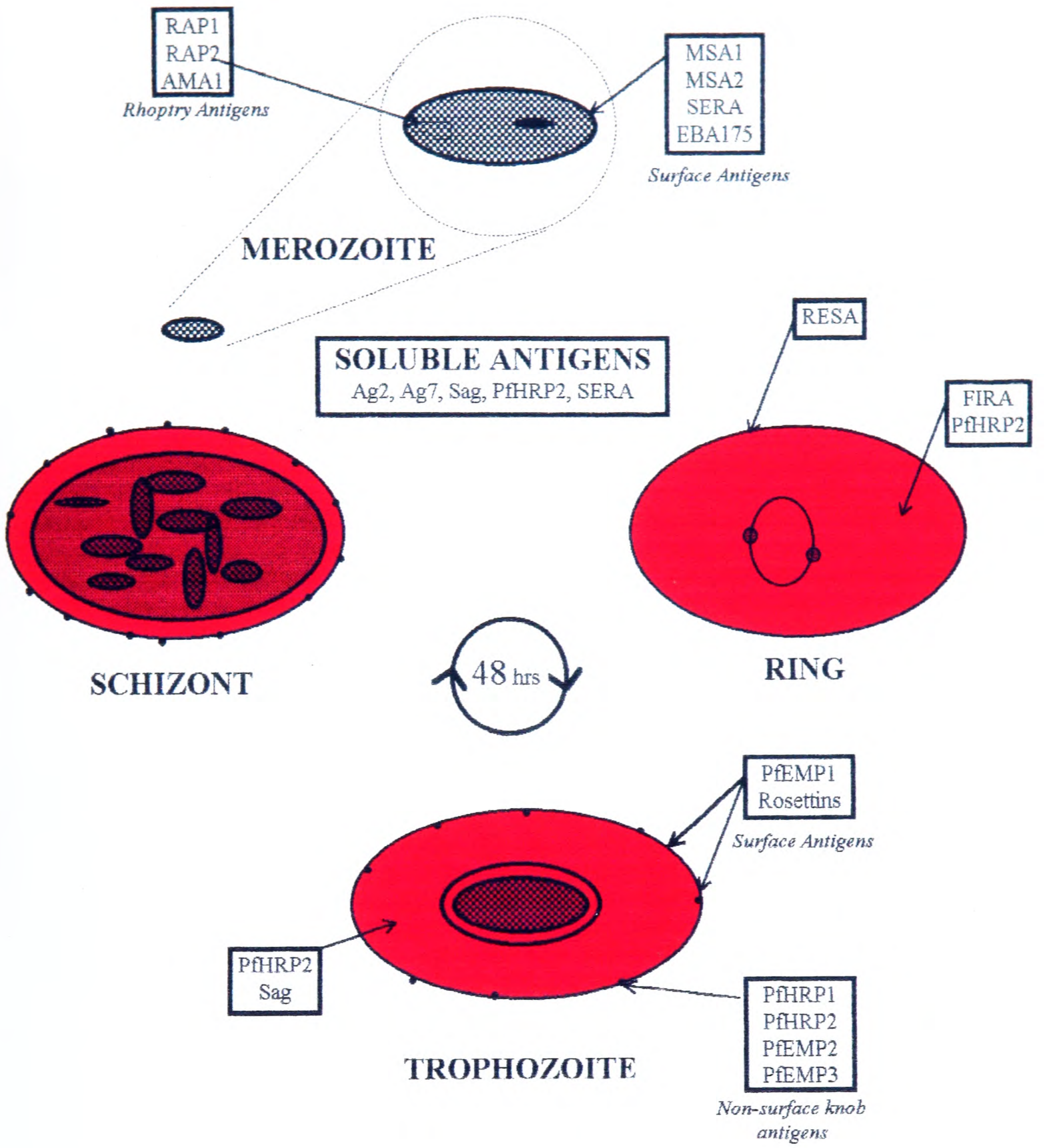


Figure 1.4. Schematic diagram showing location of a selection of asexual P. falciparum *antigens (Note that antigens expressed at trophozoite stage are maintained in the schizont stage).*

There is considerable evidence that merozoites express numerous proteins that are recognised by antibody mediated responses which concomitantly interfere with important parasite functions. Antibodies can inhibit invasion of erythrocytes by binding to the merozoite surface and inhibiting the interaction with the receptor on the red cell surface (Deans *et al.*, 1984; Sim *et al.*, 1994). The same effect can be achieved by antibodies that cause agglutination of free merozoites or those that prevent the release of merozoites from the ruptured schizont. Alternatively, antibodies may interfere with merozoite function after invasion by interacting with constituents of the micronemes or apical rhoptries which are only accessible after entry has commenced (Schofield *et al.*, 1986; Ridley *et al.*, 1990). In all cases, antibody binding can render the merozoite susceptible to secondary damage via complement mediated lysis or phagocytosis / opsonisation, but no protective mechanisms have been elucidated that recognise the *P. falciparum* merozoite in humans.

MSA1 and **MSA2** have been discussed with respect to their polymorphic natures in section 1.6.4.3. In animal models, protection against asexual challenge was observed after immunisation with purified *P. falciparum* antigens in *Aotus* and *Saimiri* monkeys for MSA1 (Siddiqui *et al.*, 1987; Etlinger *et al.*, 1991), and in mice for conserved regions *of P. falciparum* MSA2 (Saul *et al.*, 1992). Consequently, these antigens have been mooted as vaccine candidates (Howard and Pasloske, 1993).

RAP1 and **RAP2** are rhoptry associated proteins that form a complex known as QF3 (reviewed by Perkins, 1991). Monoclonal antibodies against *P. falciparum* QF3 inhibited *in vitro* growth (Schofield *et al.*, 1986) and *Saimiri* monkeys immunised with RAP1/RAP2 were protected (Ridley *et al.*, 1990).

Recombinant **RESA** (Pf155) affords weak protection in *Aotus* monkeys challenged with asexual stages (Collins *et al.*, 1986b), and anti-RESA antibodies inhibit *P. falciparum* growth *in vitro* (Berzins *et al.*, 1986; Ruangjiraporn *et al.*, 1988). This protein does not seem to be involved in naturally-acquired immunity because a cross-sectional and longitudinal study from The Gambia that measured anti-RESA showed no correlation with protection (Marsh *et al.*, 1989).

EBA-175 is implicated in erythrocyte binding because antibodies to a C-terminus sequence block invasion of erythrocytes by *P. falciparum in vitro* (Sim *et al.*, 1990; Sim *et al.*, 1992).

AMA-1 (Thomas *et al.*, 1990) monoclonal antibodies from *P. knowlesi* can block merozoite invasion of erythrocytes *in vitro* (Deans *et al.*, 1984). Immunisation of monkeys with AMA-1 from *P. knowlesi* or *P. fragile* results in protection against challenge with asexual parasites from the homologous species (Deans *et al.*, 1984, 1988).

Soluble antigens which are released into the plasma on schizont rupture are potential targets for anti-disease immune and coincide with the febrile episodes that characterise malaria infections. This material could include parasite-derived proteins, lipids, membranes, organelles and individual components which elicit the characteristic inflammatory response cascade. Antibodies to such targets may be expected to alleviate fever and could have protective effects if they interfere with a crucial parasite function (reviewed by Kwiaktowski, 1992). A crude mixture of soluble proteins from asexual *P. falciparum* culture supernatant gave complete protection in *Aotus* monkeys challenged with the same parasite (James *et al.*, 1985). However, the identification of protective targets and responses was not carried out. There are now several antigens that are known to be released by the parasite, but no functions have been ascribed.

S Antigens are the best-characterised polymorphic antigens of *P. falciparum* which are discharged after rupture of the infected erythrocyte and are summarised in section 1.6.4.1. S antigens are not believed to confer protection and may form part of an immune smokescreen mechanism, even though one monoclonal antibody can inhibit *in vitro* growth of parasites.

SERA is a serine rich protein that is released with Ag2 and Ag7 following schizont rupture (Jakobsen *et al.*, 1991). Recombinant yeast forms of SERA provided partial protection of *Aotus* monkeys against challenge *with P. falciparum* (Inselberg *et al.*, 1991). Purified *P. falciparum* SERA protected *Saimiri* monkeys against *P. falciparum* challenge (Perrin *et al.*, 1984).

PfHRP2 is synthesised by the intracellular parasite and is either secreted into the plasma or targeted to the cytoplasmic face of the erythrocyte membrane (Howard *et al.*, 1986; Rock *et*

al., 1988). The soluble form could be a target for immune attack if it has an essential function (see section 1.8.4).

At first sight, it would appear that the intraerythrocytic location of the parasite would be a perfect location to avoid recognition and attack by the host immune system. The parasite is protected inside a robust host plasma membrane inside a host cytoplasm in which there are few HLA molecules and no machinery to synthesise such proteins *de novo*. Thus, *a priori*, the parasite should be able to avoid both B cell mediated and T cell mediated responses.

However, this situation does not occur because the parasite synthesises antigens that are expressed at the surface of the erythrocyte plasma membrane. These parasite-derived neoantigens (PDNs) may mediate important functions in disease pathogenesis and are discussed in detail in section 1.8.5. Surface-exposed PDNs are ideal targets for immune attack because they bear epitopes which are displayed to the immune system for at least half of the intraerythrocytic cycle. One family of variable PDNs, known as PfEMP1, is believed to be the target of opsonising antibodies that confer protection in models *in vitro* and against clinical falciparum malaria *in vivo* (reviewed by Howard, 1988). Homologues to these important antigens exist in other *Plasmodium* species and initial evidence for protection and subsequent proof that they undergo antigenic variation was acquired in monkey and murine models of malaria (section 1.8.5).

Possible effector mechanisms that could be promoted by antibodies recognising the infected red cell surface include opsonisation. This mechanism occurs in *P. knowlesi* infections of monkeys and protection against challenge correlated with presence of opsonising antibody. In *P. chabaudi*, the protective murine response can distinguish between antigenic variants of the asexual blood stage. Parasitised erythrocytes are agglutinated by antibodies which recognise surface variants (Marsh and Howard, 1986). The repertoire of such antibodies is restricted in children but broad in adults who have immunity to *P. falciparum* disease because they have been exposed to different variants. A longitudinal study in The Gambia correlated antibody response to PDNs with protective immunity to clinical disease (Marsh *et al.*, 1989). Expression of PDNs may also be related to virulence by their proposed function in sequestration which may be blocked by protective immune responses. Evidence for this phenomenon and its consequences are discussed in section 1.8.

The size of the variant antigenic repertoire is likely to be large because PDNs of *P. falciparum* clones have recently been shown to undergo antigenic variation *in vitro* at 2% per generation in the absence of immune pressure (Roberts *et al.*, 1992). This would explain the phenotypic diversity at the erythrocyte surface observed in field isolates, and the agglutinating activity of serum from immune adults living in a hyperendemic area for malaria was shown to be variant-specific and not cross-reactive (Newbold *et al.*, 1992). Thus, the accumulation of variant-specific antibodies following repeated exposure to many strains would appear to be a possible mechanism for protective immunity (reviewed by Roberts *et al.*, 1993).

Variant-specific antibodies to *P. knowlesi* infected red cells resulted in phagocytosis of the infected cells by activated macrophages (Brown, 1971) . This mechanism may be important *in vivo* because accumulation of activated macrophages occurs in the liver and spleen during natural *P. falciparum* infections (Lee *et al.*, 1986), and infected erythrocytes which had been phagocytosed by macrophages were observed in *post mortem* specimens (Taliferro and Muligan, 1937; MacPherson *et al.*, 1985). Neutrophils may also play a role in immunity, however, a protective role for neutrophil phagocytosis was not observed using serum from a longitudinal study in The Gambia (Marsh *et al.*, 1989).

Macrophages are activated by opsonised parasites or parasitic debris and parasite antigens can stimulate cytokine release from T_h1 cells *in vitro*. Cytokine damage may explain the appearance of dead intracellular parasites, 'crisis forms', in the bloodstream of animals during recovery from infection (Quinn and Wyler, 1979). These soluble molecules may interact with reactive oxygen derivatives or nitric oxide derivatives which could have a role in the pathogenesis of severe disease because they were parasiticidal *in vitro* (Rockett, *et al.*, 1991). However, their importance remains to be established *in vivo*.

Splenic macrophages have an oxidative burst mechanism which is maximal at the time of recovery from *P. chabaudi* in mice (Dockrell *et al.*, 1986). Released oxygen derivatives can reduce parasite growth *in vitro* and *in vivo*, and is one of several important roles of the spleen in the initiation and execution of immunity (Clark *et al.*, 1983; Clark and Hunt, 1983). Splenic filtration of red cells with inclusions occurs by a phagocytic mechanism in the reticuloendothelial system, and thus clearance of infected erythrocytes is an important defence mechanism (Weatherall and Abdulla, 1982).

In addition, the spleen exerts selection pressure at the surface of the infected cell which can modulate the surface properties of infected erythrocytes and alter parasite virulence in monkeys (Barnwell *et al.*, 1983; Handunnetti *et al.*, 1987) and rodents (Gilks *et al.*, 1990). Little is known about the mechanisms involved, and the relevant literature is summarised in section 1.8.5.

Other antigens are expressed on the surface of the erythrocyte (Figure 1.4). However, in spite of a high degree of diversity, none have been correlated with protection in humans *in vivo*. The limited knowledge relating to their expression, structure and proposed function is discussed in section 1.8.4. Little attention has been focused on the parasite antigens expressed on the PM or PVM which may be accessible to antibodies via the proposed parasitophorous duct structure (Pouvelle *et al.*, 1991). Responses to such antigens may be parasiticidal if the proteins have essential functions, and may explain the otherwise paradoxical findings that growth-inhibiting IgG antibodies can act *before* schizont rupture and prevent merozoite release (Perrin *et al.*, 1981; Vande-Waa *et al.*, 1984; Li and Li, 1987). The existence of direct access mechanisms for intracellular parasites is controversial and may influence transport (section 1.6.6).

1.8. Specific Background

When this project was conceived three years ago, it was believed that the surface of the infected erythrocyte played a pivotal role in the pathogenesis of, and immunity to, *P. falciparum* malaria. The importance of this complex interface has been strengthened by subsequent investigations into the molecular and cellular basis of the host-parasite interaction. Such studies have provided significant insights into the mechanism of sequestration, which is involved in disease pathogenesis, and have facilitated the identification of targets for protective immunity. Both properties are believed to be attributable to a single family of parasite-derived neoantigens (PDNs) which are expressed at the parasitised red blood cell (PRBC) surface.

This section begins with a summary of the clinical studies on cerebral malaria and some of the theories concerning its pathogenesis. The parasite-induced modifications at the surface of the erythrocyte are described and the known functions of PDNs are discussed. The molecular basis of sequestration is expounded, followed by a synopsis of the relevant literature concerning the antigenic variation that occurs at the infected cell surface. We may now be on the verge of an exciting new phase of research in this area because the genes encoding the elusive parasite antigens responsible for the important functional changes have recently been cloned and sequenced (but are yet to be published).

1.8.1 Pathogenesis of Cerebral Malaria

The pathogenesis of severe malaria is complex and not well understood (White and Ho, 1992). Severe malaria anaemia and cerebral malaria are rare occurrences when compared to the total number of *P. falciparum* infections (Greenwood *et al.*, 1991). Outcome is variable and relates to the interaction of host and parasite factors (reviewed by Grau and de Kossoda, 1994; Clark and Rockett, 1994; Berendt *et al.*, 1994b; Miller *et al.*, 1994). Severe malaria anaemia is a fatal complication after infection by many *Plasmodium* species and will not be discussed here. Cerebral malaria is a life-threatening illness, which has an associated mortality of up to 30% even under good clinical management (Warrell *et al.*, 1982). High risk groups are children living in endemic areas and non-immune people in all malarious areas. There are several theories concerning its pathogenesis, but lack of basic knowledge about this process prevents the development of improved strategies for its treatment. Unlike severe anaemia, cerebral malaria is confined to *P. falciparum* infection and this restriction has guided researchers in the search for unique features of this *Plasmodium* species.

Cerebral malaria can have a heterogeneous presentation of symptoms (section 1.5.1.1), and is often difficult to diagnose. Modern clinical definitions of cerebral malaria are based on detailed pioneering research in South East Asia during the past 10 years. Clinical studies have utilised a modified version of the standard Glasgow scale that assesses the severity of coma and the associated neurological complications in malaria (Molyneux *et al.*, 1989; Warrell, 1989). Thus, diagnoses of cerebral malaria before this time were made using less strict criteria and conclusions from the same patients may be different today because inclusion criteria for patients are different and comparisons are difficult (reviewed by Berendt *et al.*, 1994b).

Early theories of pathogenesis were centred on the apparently paradoxical observation that fatal cases of 'cerebral malaria' had low peripheral blood parasitaemias which consisted of predominantly young, ring-stages (Bastianelli *et al.*, 1898). Parasitised erythrocytes of all stages of the other human malarias remain in the peripheral circulation (Table 1.2). This observation is a hallmark of *P. falciparum* infection and is due to the sequestration of mature infected erythrocytes in the deep vascular beds of several organs (Spitz, 1946; MacPherson *et al.*, 1985). Sequestered parasites can only be observed

after microscopic examination of *post-mortem* tissues and occurs at a significantly higher degree in the brains of patients who died of cerebral malaria (MacPherson *et al.*, 1985; Oo *et al.*, 1987; Pongponratn *et al.*, 1991). Sequestration results from the specific cytoadherence of mature parasitised red blood cells (PRBC) to the endothelial cells that line the microvasculature (Luse and Miller, 1971). Most investigators are agreed that sequestration via cytoadherence is the initiating event in cerebral malaria, and probably represents a unique virulence factor of *P. falciparum* (Warrell, 1987). Sequestration simultaneously confers a dual biological advantage on the parasite. Firstly, it allows parasitised erythrocytes to avoid destruction by splenic filtration (Barnwell, 1989) and secondly, the hypoxic conditions at sites of sequestration are known to facilitate growth *in vitro* of *P. falciparum* (Scheibel *et al.*, 1979).

The severity of *P. falciparum* malaria appears to be correlated with the degree of sequestration in the microvasculature of various organs, particularly the brain (Macpherson *et al.*, 1985; Pongponratn *et al.*, 1991). Several pathological studies of *post-mortem* tissue samples have shown that the brains of patients with cerebral malaria contained significantly more sequestered PRBC than other organs and brains of patients who died of other causes. In a study of fatal cases from Thailand, 94% of cerebral microvessels had sequestered parasites from patients who died from cerebral malaria with coma (Macpherson *et al.*, 1985). Only 13% of cerebral microvessels were sites of sequestration in patients who died of other causes with non-cerebral malaria. Ring haemorrhages were also observed at a statistically higher frequency in brains from the cerebral malaria group than in the control group which indicated a possible pathological consequence of local obstruction.

Therefore, brain-specific sequestration correlates with pathology observed in cerebral malaria, and sequestration has been the cornerstone of modern hypotheses of the disease pathogenesis (Berendt *et al.*, 1994b). Initially, it was assumed that PRBC were sequestered because they had difficulty in passing through the capillary bed due to agglutination or decreased deformability of infected cells (Marchiafava and Bignami, 1900). Consequently, blood flow to affected organs would be impeded, and in turn would be manifested as ring haemorrhages and lead to hypoxia and coma. Current evidence suggests sequestration occurs specifically in the post-capillary venules via cytoadherence of PRBC to endothelial cells, and this can result in local occlusion of blood flow and an increase in intra-

cranial pressure (Newton *et al.*, 1991). This mechanical obstruction theory may coexist with immunological theories that are based on studies which have detected immune complexes, complement components and inflammatory cells in the brains of cerebral malaria cases and are discussed later (Aikawa, 1988).

Downstream events of these pathological observations are not easy to discern because it is impossible to perform biopsy studies on the brains of living patients. The rapid reversibility of neurological complications and coma in most patients with cerebral malaria would suggest the involvement of transient metabolic disturbances (Clark *et al.*, 1994). Such a scenario could result from local microvascular obstruction by sequestered parasites which leads to hypoxaemia, acidosis and hypoglycaemia in the brain. Simultaneously, the immobile PRBC may release large quantities of toxins that cause the host to produce cytokines. Animal models (e.g. *P. berghei* ANKA strain in mice (Grau *et al.*, 1987)) of these states suggest that TNF may activate nitric oxide synthase causing elevated levels of nitric oxide that could lead to intracranial hypertension and aberrant neurotransmission (Clark *et al.*, 1991; Clark *et al.*, 1992). Elevated levels of IL-1 would result in lactic acidosis and hypoxia but experimental evidence is required to dissect these complex physiological events (Clark *et al.*, 1991).

Indeed, unidentified malaria toxins released on schizogony may stimulate macrophages to release TNF-α, which has been associated with disease severity in African children (Grau *et al.*, 1989) and particular TNF-α promoter polymorphisms confer susceptibility to severe disease (McGuire *et al.*, 1994). The ability to stimulate TNF-α production may be a parasite virulence factor because different clones produce varying levels of TNF-α *in vitro* (Bate *et al.*, 1992; Allan *et al.*, 1993). This cytokine is important because it causes the upregulation of expression of ICAM1, VCAM1 and E-selectin (section 1.8.3.1) which have been proven to mediate sequestration of infected erythrocytes *in vitro* (Berendt *et al.*, 1989; Ockenhouse *et al.*, 1992b). Recent studies suggest that this mechanism may occur preferentially in cerebral malaria cases, set against a background of widespread endothelial activation (Turner *et al.*, 1994).

Rosetting is an additional cytoadherent phenotype exclusive to mature infected erythrocytes (David *et al.*, 1988; Wahlgren *et al.*, 1990). Rosettes of normal erythrocytes around the infected cell are observed during *in vitro* culture and some studies have shown that they occur more frequently in patients with cerebral malaria (Carlson *et al.*, 1990; Dr. A. Rowe, personal communication). Rosetting may be important for margination of infected cells from flow which could precede sequestering events, and could simultaneously improve reinvasion rates by reducing merozoite diffusion distances. The molecular basis of this phenomenon awaits characterisation.

Unfortunately, there is no accurate animal model for studying pathogenesis of the human disease and many of the results of such animal-based research are often irrelevant, confusing and even misleading. The most relevant features are exhibited in the new *P. fragile* / Rhesus monkey combination that exhibits cerebral sequestration via putative cytoadherent receptors implicated in the human disease (Fujioka *et al.*, 1994). Some of the popular models are compared to the human sequestration patterns observed with *P. falciparum* in Table 1.2.

PARASITE	HOST	Pattern of Sequestration	Other Features
P. falciparum (K^+)	Humans	**cerebral malaria**: brain > heart, lung, liver, spleen **Non-cerebral malaria**: heart > lung, spleen, liver	Can lead to coma and death, and rosetting observed No death (Macpherson *et al.*, 1985)
	Saimiri monkey	brain vessels	Unpredictable symptoms (Fremount and Rossan, 1990; Gysin *et al.*, 1992)
	Aotus monkey	heart, intestine > adipose tissue, spleen	(Miller, 1969; Luse and Miller, 1971; Aikawa *et al.*, 1990a)
P. malariae (K^+), *P. vivax*(K^-), *P. ovale*(K^-)	Humans	NONE	
P. fragile(K^+)	Toque monkey	heart and small intestine	(Fremount and Miller, 1975)
	Rhesus monkey	brain, heart and adipose	cerebral rosetting and sequestration via knobs with neurological symptoms (Fujioka *et al.*, 1994)
P. coatneyi(K^+)	Rhesus monkey	heart, adipose tissue	(Aikawa *et al.*, 1992)
P. berghei(K^-)	Mice and Rats	bone marrow, liver, spleen	Vascular obstruction is by mononuclear cells and PRBC(Alger, 1963; Franz *et al.*, 1987)
P. berghei (ANKA)	CBA mice	brain, bone marrow, liver, spleen	Vascular obstruction is by mononuclear cells only (Mackey *et al.*, 1980; Grau *et al.*, 1987; Senaldi *et al.*, 1994)
P. chabaudi(K^-)	BALB/c Mice	liver, spleen, heart	(Cox *et al.*, 1987)

Table 1.2. Sequestration patterns in a range of parasite-host combinations. K^+, K^- refers to the presence or absence of knobs on the PRBC surface.

1.8.2 Morphological Changes at the Surface of Infected Erythrocytes

The parasite-induced surface changes which occur in the second half of the asexual intraerythrocytic cycle are essential for sequestration because uninfected cells and young ring forms do not participate. Thus, the host-parasite interface at the erythrocyte surface determines the molecular basis of sequestration, which in turn influences the outcome of infection by *P. falciparum* (Hommel, 1993).

Several gross morphological changes occur in the second half of the intraerythrocytic stage (Figures 1.5 and 1.6) (Aikawa, 1988, Sherman *et al.*, 1992). The erythrocyte loses its biconcave disc shape which is mirrored by a decrease in deformability and its ultrastructure is significantly altered. The most noticeable change occurs with the appearance of conoid protrusions (~100nm diameter, 30-40nm high) on the surface together with juxtaposed submembraneous electron dense material visible by transmission electron microscopy (Aikawa, 1988). These structures are called knobs and are synthesised by a dynamic process in the mature stages, reaching maximum density in the schizont where they can occupy up to 5% of the plasma membrane surface (Gruenberg *et al.*, 1983). Knobs appear as an ordered array and their detergent solubility properties suggest that they interact with the erythrocyte cytoskeleton. Their functional characterisation was an exciting period of malaria research and is reviewed in detail here because it illustrates many of the properties that a parasite-derived receptor must possess to be responsible for sequestration.

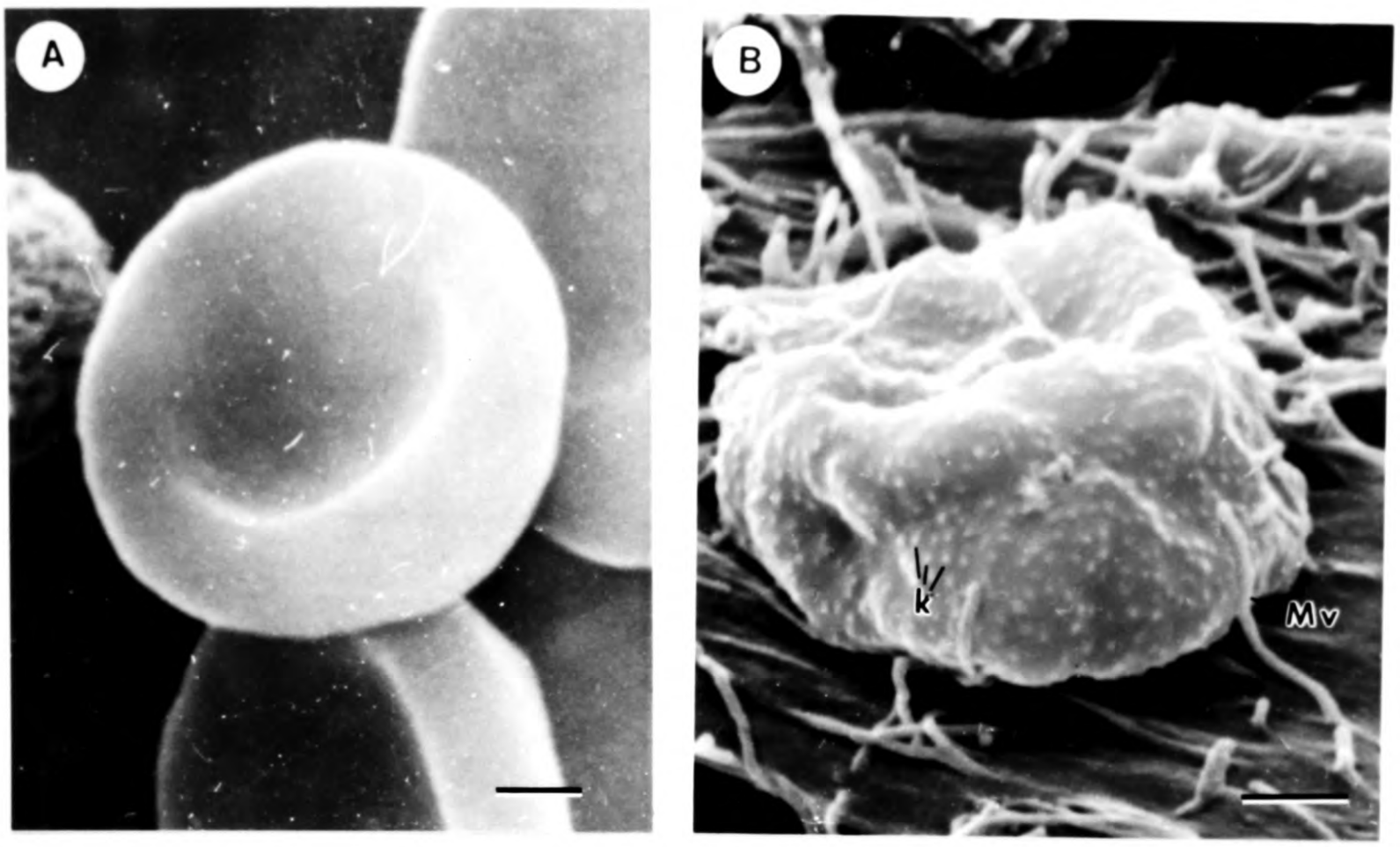


Figure 1.5. Scanning electron micrographs of A) normal erythrocytes and B) a parasitised erythrocyte adhering to a C32 melanoma cell. Numerous knobs (k) are present at the surface of the infected cell which is anchored by the microvilli (mv) that extend from the melanoma cell. Bar is 1 μm.

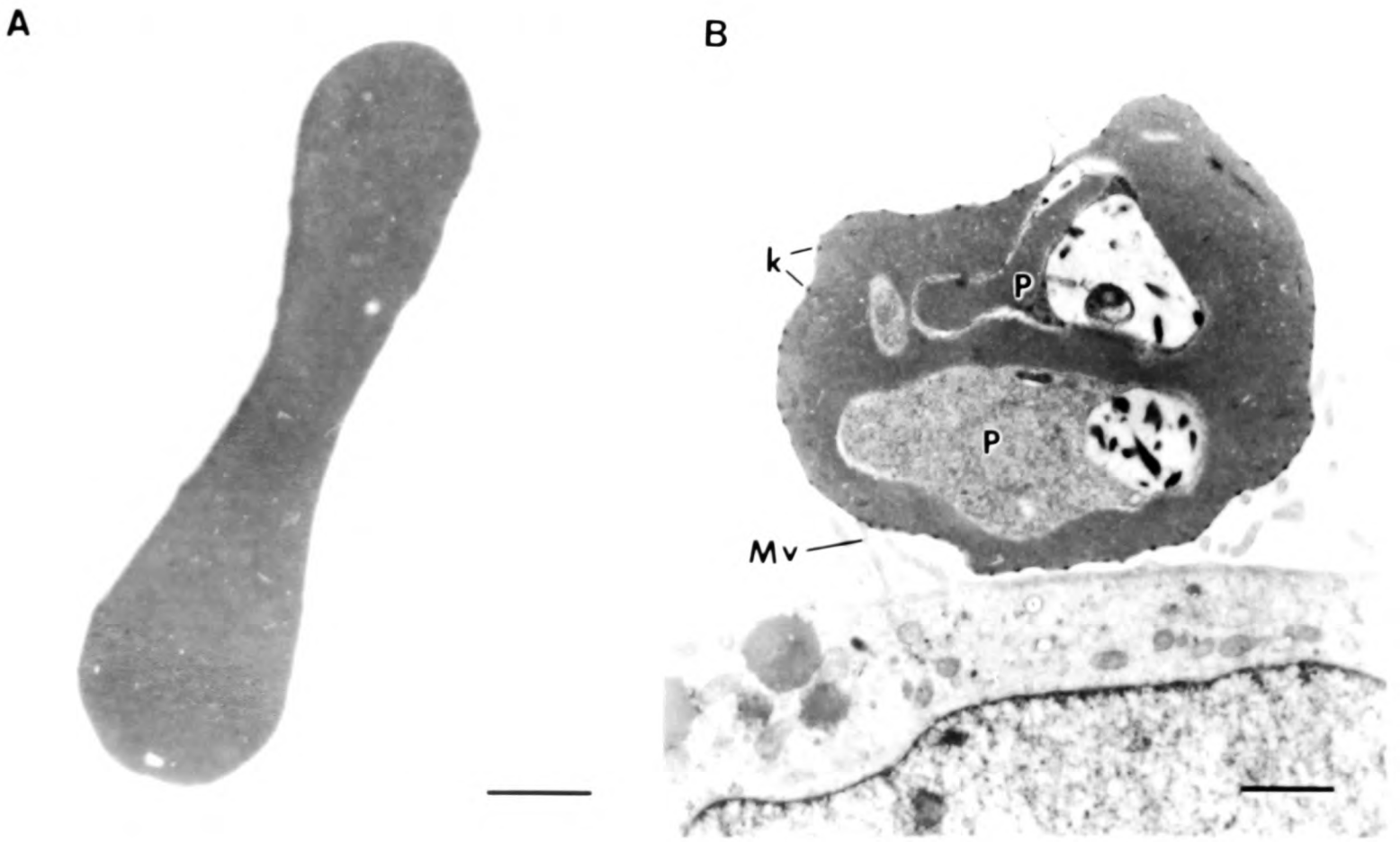


Figure 1.6. Transverse electron micrograph showing cross-section through A) a normal erythrocyte and B) a parasitised erythrocyte attached a C32 melanoma cell. Note the close apposition of the adherent membranes and that the erythrocyte appears to contain two intracellular parasites (P). Bar is 1 μm.

Initially, knobs were believed to be essential for sequestration because early studies showed that their stage-specificity correlated with this functional change and electron micrographs clearly demonstrated that knobs were sites of interaction of the parasitised erythrocyte surface with the surface of cultured endothelial cells (Luse and Miller, 1971). In animal models, *P. coatneyi* is closely related to *P. falciparum* and also expresses knobs at the trophozoite stage which are sequestered *in vivo* in rhesus monkeys (Desowitz *et al.*, 1969). Also, all field isolates of *P. falciparum* had knobby surfaces, i.e. they were knob-positive (designated K^+), and always showed sequestering phenotypes in *in vitro* and *ex vivo* models. Furthermore, the K^+ appearance was lost during laboratory culture without selection by Plasmagel™ flotation (Langreth *et al.*, 1978; Sherman and Valdez, 1989) and the knobless (K^-) parasites were non-sequestering *in vitro*. Hence, the ability of a parasite species to sequester was correlated with knob expression and this motivated investigations into the molecular and genetic basis of knobs.

Knob-negativity of *P. falciparum* asexual stages was shown to be an irreversible phenotype whose genetic basis was the spontaneous deletion of a subtelomeric region of chromosome 2 (Pologe and Ravetch, 1986; Kemp *et al.*, 1990). This resulted in the complete or partial loss of expression of a single protein that was preferentially labelled by radioactive histidine and designated histidine rich protein, PfHRP1 (also known as knob-associated histidine rich protein, KAHRP). PfHRP1 was detected only in K^+ parasites as a band of 80-120 kDa after SDS-PAGE of Triton X-100 insoluble extracts followed by Coomassie staining (Kilejian, 1979). This was the only biochemical correlate of knobs and there were no differences in expression of any other known malarial proteins in K^+ and K^- parasites. Immunolocalisation studies using a variety of antibodies detected PfHRP1 in the host cell cytoplasm and at the surface plasma membrane (Taylor *et al.*, 1987). However, immunoelectron microscopy has conclusively demonstrated that PfHRP1 is a constituent of the electron-dense spheres which have a submembraneous deposition at knobs (Taylor *et al.*, 1987).

There is now overwhelming evidence that PfHRP1 is not surface-exposed and knobs are neither necessary nor sufficient for sequestration. Table 1.2 shows that K^+ *P. falciparum* erythrocytes cultured in squirrel monkeys do not sequester and some laboratory adapted K^+ *P. falciparum* lines do not adhere to purified receptors *in vitro*. Other *Plasmodium* species have K^+ erythrocytes, but do not

sequester *in vivo*. These include, *P brasilianum* in New world monkeys and *P. malariae* in humans. Knob-negative sequestration can occur *in vivo*, eg. *P. berghei* in rodents, *P. chabaudi* in mice, and small numbers of K^- *P. falciparum* isolates are adherent in in *vitro* models (reviewed by Howard and Gilladoga, 1989).

Furthermore, knob-negative adhesion has been selected from three *P. falciparum* strains *in vitro*; Palo Alto (Udomsangpetch *et al.*, 1989) Ituxi (Biggs *et al.*, 1989) and Malayan Camp (Ockenhouse *et al.*, 1991b) and non-sequestering and sequestering forms of *P. falciparum* in splenectomised and intact *Saimiri* monkeys were both K^+ (the influence of the spleen on sequestration is discussed in section 1.8.3.2). Young *P. falciparum* gametocytes are K^- but adhere *in vivo*. Interestingly, mature gametocytes are also K^-, but do not sequester and this represents an important developmental switch that could facilitate transmission. Biochemical studies have used mild trypsin treatment to abolish adhesion *in vitro*, but knobs are still visible by electron microscopy. Finally, no antibodies to PfHRP1 block *in vitro* adherence, and PfHRP1 is expressed in non-cytoadherent lines (Taylor *et al.*, 1987).

The function of knobs is enigmatic, but an immunoelectron microscopic study has demonstrated that soluble labelled endothelial cell ligands, CD36 and TSP, bind to the surface of infected erythrocytes only at knobs (Nakamura *et al.*, 1992). This implied that knobs may be involved indirectly in cytoadherence by clustering of parasite adhesins.

1.8.3 Molecular Mechanisms of Sequestration

Sequestration is a specific event involving the adhesion (cytoadherence) of infected erythrocytes to venular endothelial cells via surface-exposed receptor molecules. *In vitro* models of sequestration were established to elucidate the nature of the interactions and molecules that mediate cytoadherence and this section reviews the current state of knowledge (reviewed by Berendt *et al.*, 1994a).

1.8.3.1 Endothelial Cell Receptors

In 1981, Udeinya and colleagues developed an *in vitro* model for cytoadherence using cultured human umbilical vein endothelial cells (HUVECs) which showed specific adhesion of erythrocytes infected with mature forms of *P. falciparum* maintained in *Aotus* monkeys. Many cell types were screened for adhesiveness and Schmidt discovered that C32 amelanotic melanoma cells (C32 melanoma cells), human amnion cells, and human aortic cells could mediate cytoadherence (Schmidt *et al.*, 1982). Bovine endothelial cells and other human cells, including lung fibroblasts and vascular smooth muscle cells, were non-adherent in the same study. Several other *in vitro* models of cytoadherence are routinely used, including human brain endothelial cells (HBECs), human dermal microvascular endothelial cells (HDMECs), peripheral human monocytes and platelets (Nash *et al.*, 1992; Swerlick *et al.*, 1992; Wick and Louis, 1991; Smith *et al.*, 1992; Johnson *et al.*, 1993).

An *ex vivo* model for studying cytoadherence under flow was developed using artificially perfused microvasculature from rat mesoappendix (Rock *et al.*, 1988). Although this never achieved popular status because of questions pertaining to the relevance of a rodent host, it did provide some insights into the effect of physiological shear forces on cytoadherence.

The identification of endothelial molecules that mediated *in vitro* and *ex vivo* cytoadherence was made possible by these whole cell models. There are currently five putative molecules, thrombospondin (TSP), CD36, intercellular adhesion molecule-1 (ICAM1), vascular cell adhesion molecule-1 (VCAM1) and E-selectin, which have been demonstrated to act as receptors for infected erythrocytes. Their characteristics are summarised in table 1.3.

Molecule	Structure	Site of Expression	Physiological Functions
TSP[1]	420kDa glycoprotein composed of 3 identical subunits	Secreted matrix protein (stored in platelet granules but synthesised by endothelial cells, macrophages and melanoma cells)	General cell-cell and cell-matrix adhesion protein. Binds to CD36, heparin, fibrinogen, fibronectin, and collagen.
CD36[2] (GPIV, GPIIIb, GP88, OKM5 antigen)	86 - 89kDa sialylated membrane glycoprotein monomer, and member of distinct gene family	Integral membrane monomer on surface of platelets, monocytes, macrophages, C32 melanoma cells, endothelial cells, some epidermal cells and foetal erythrocytes	Platelet aggregation, signal transduction. Binds to TSP, type I collagen, and oxidised low density lipoprotein.
ICAM1[3] (CD54)	95 - 122kDa membrane glycosylated monomeric member of immunoglobulin superfamily containing 5 Ig-like domains	Surface of fibroblasts, epithelial cells, leukocytes and endothelial cells. Cytokine-upregulated expression	T-cell co-stimulatory signal via its role as a receptor for LFA-1. One of mucosal invasion receptors for rhinovirus, and an endothelial adhesion molecule.
VCAM1[4]	6-7 Ig domains	Surface of B-cells, Bowman's capsule and activated HUVECs.	Receptor for VLA4 on lymphocytes, monocytes and some melanoma cell lines
E-Selectin[5] (ELAM1)	Typical selectin architecture, consisting of lectin domain, EGF-like domain and complement regulatory-like domain	Surface of activated endothelial cells	Receptor for neutrophils and monocytes via sialyl Lewis groups. Involved in metastasis and angiogenesis. Binds to carbohydrate structures (lectins).

Table 1.3. Summary of characteristics of known host receptors for P. falciparum *infected erythrocytes. General reviews are referred to in the footnotes.*

[1] Lawler, 1986; Frazier, 1987; Lahav, 1993

[2] Greenwalt *et al.*, 1992

[3] Hogg *et al.*, 1991

[4] Springer, 1990

[5] Bevilacqua and Nelson, 1993; Varki, 1994

A variety of methods have been used to establish conclusively that a molecule is a cytoadherence receptor. These include monoclonal antibody (mAb) blocking of cytoadherence, adhesion of parasitised erythrocytes to purified proteins or to transfected simian COS cells (which do not normally express the receptor) with genes that code for cytoadherent proteins. A summary is given in table 1.4 and extra information for each molecule is detailed below (reviewed by Berendt, 1994).

EVIDENCE FOR ROLE AS A RECEPTOR	**TSP**	**CD36**	**ICAM1**	**E-Selectin**	**VCAM1**
PRBC bind to purified protein on plastic					
Cytoadherent PRBC:					
Knobby cultured lines	+	+	+	+	+
Aotus-adapted lines	+	+	+	ND	ND
Patient isolates	+	+	+	+	+
Non-cytoadherent PRBC:					
Ring-infected erythrocytes	-	-	-	ND	ND
Knobless mature cultured lines	-	-	-	ND	ND
Trypsin-treated mature lines	-	-	ND	ND	ND
Soluble purified protein inhibit adhesion					
To homologous protein on plastic	+	+	+/-	ND	ND
To melanoma cells *in vitro*	+	+	ND	ND	ND
To endothelial cells *in vitro*	ND	-	ND	ND	ND
To rat endothelium *ex vivo*	+	ND	ND	ND	ND
Antibodies to protein inhibit adhesion					
To homologous protein on plastic	+	+	+	+	+
To melanoma cells *in vitro*	+	+	ND	ND	ND
To endothelial cells *in vitro*	+/-	-	+	ND	ND
To rat endothelium *ex vivo*	+	ND	ND	ND	ND
Induced binding by gene transfection	ND	+	+	ND	ND
Correlation of cytoadherence and expression of receptor on melanoma cells	-	+	+	ND	ND

Table 1.4. Summary of evidence for involvement of putative receptors in cytoadherence of P. falciparum *infected erythrocytes. This table is updated version from review by Chulay and Ockenhouse (1990) and specific references are in the text below (+/- denotes variable results).*

A diagrammatic representation of these diverse cytoadherence receptors is shown in figure 1.7 Thrombospondin was the first receptor identified that mediated the stage-specific adhesion of infected cells to purified protein absorbed onto plastic (Roberts *et al.*, 1985b). Most field isolates and laboratory adapted strains of *P. falciparum* bind to purified protein (Sherwood *et al.*, 1987), and adhesion was inhibited by soluble TSP, monoclonal and polyclonal antibodies to TSP (Roberts *et al.*, 1985b). The rat *ex vivo* mesoappendix model of cytoadherence also provided support for a role for specific TSP - PRBC interactions under flow (Rock *et al.*, 1988). The binding domain has not been mapped, but the C-terminal, Ca^{2+} dependent domain is essential for cytoadherence, whereas the N-terminal domain mediates binding to heparin (Haverstick *et al.*, 1984; Sherwood *et al.*, 1990). Its involvement during sequestration is unclear because it is a soluble, secreted molecule that can interact with a variety of receptors expressed on endothelium, including CD36 (reviewed by Pasloske and Howard, 1994), and is not constitutively expressed on endothelium in immunohistochemical studies (G. D. H. Turner, personal communication).

CD36 is a well-characterised cytoadherence receptor, and nearly all field isolates and laboratory adapted strains of *P. falciparum* will bind to purified protein on plastic (Barnwell *et al.*, 1989; Ockenhouse *et al.*, 1989; Oquendo *et al.*, 1989; Hasler *et al.*, 1990). *In vitro* cytoadherence is Ca^{2+}-independent and is inhibited by soluble CD36 and OKM5 anti-CD36 mAb (Barnwell *et al.*, 1985; Ockenhouse *et al.*, 1989). CD36 is expressed on the surface of C32 melanoma cells (Barnwell *et al.*, 1985; Ockenhouse and Chulay, 1988) and inhibition of glycoprotein synthesis in C32 melanoma cells prevented CD36 expression and cytoadherence (Wright *et al.*, 1991). COS cells transfected with a cDNA encoding CD36 are transformed into cytoadherent-competent cells, but the infected erythrocyte binding site has not been mapped (Oquendo *et al.*, 1989; Hasler *et al.*, 1993).

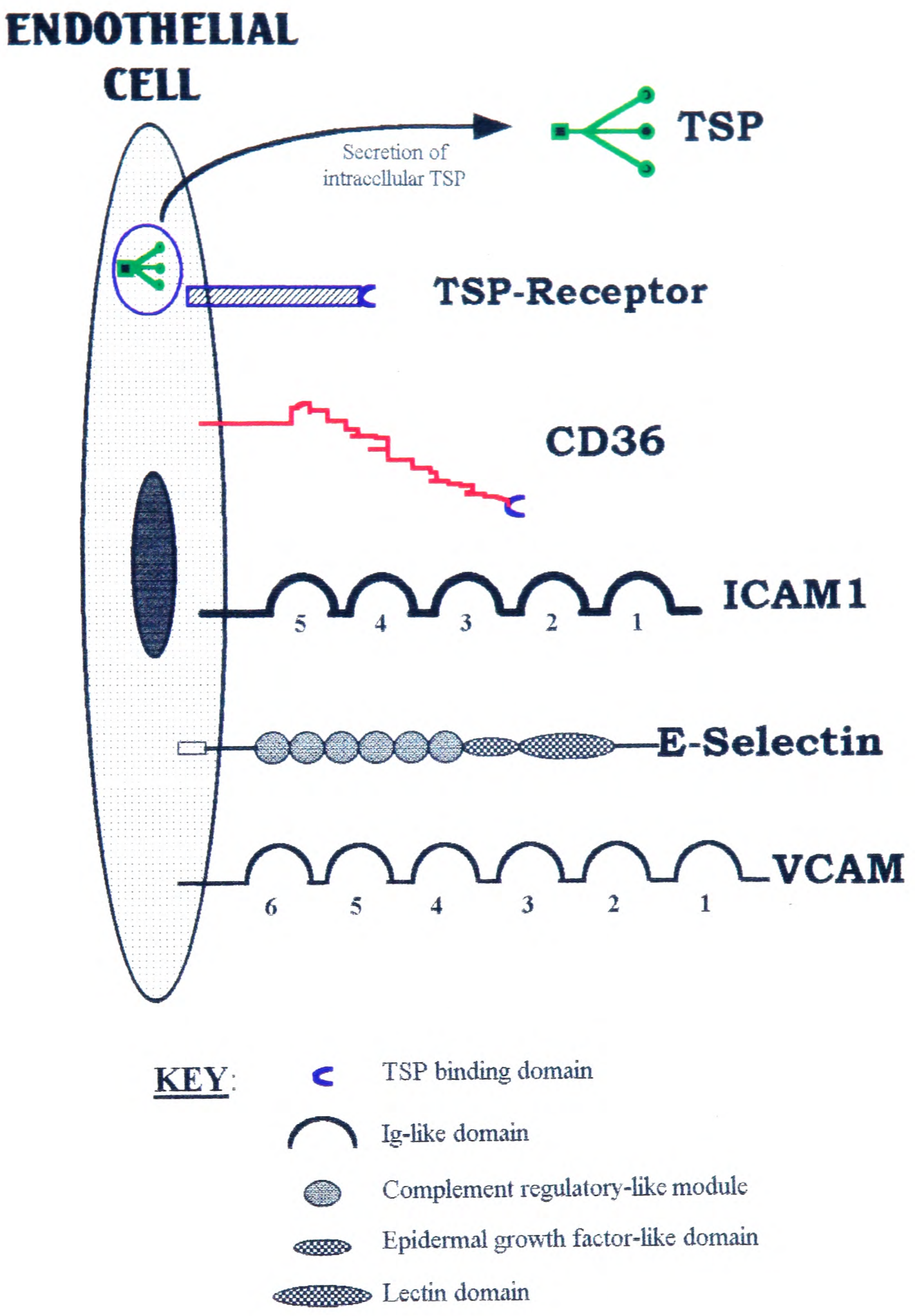


Figure 1.7. Diagrammatic summary of known receptors for cytoadherence of P. falciparum *infected erythrocytes to endothelium* in vivo *(note that CD36 is not expressed on HUVECs* in vitro*).*

This is in contrast to the Ig superfamily member, ICAM1. Domain deletion, mAb epitope mapping and chimaera construction techniques demonstrated that the binding site for infected erythrocytes is on domain 1 and is distinct from LFA1 and rhinovirus binding sites (Ockenhouse *et al.*, 1992a; Berendt *et al.*, 1992). Anti-ICAM1 mAbs block cytoadherence to HUVECs and to purified protein on plastic, but soluble ICAM1 does not readily block cytoadherence (A. G. Craig, personal communication). ICAM1 expression is upregulated by TNF-α on HUVECs (Rothlein *et al.*, 1986) and mAbs to ICAM1 can inhibit the cytokine-induced increase in cytoadherence. COS cells transfected with a cDNA encoding ICAM1 are transformed into cytoadherent-competent cells for some parasite lines (Berendt *et al.*, 1989; Hasler *et al.*, 1993). Not all field and laboratory adapted *P. falciparum* parasites bind to purified protein on plastic or to HUVEC (Reeder *et al.*, 1994) and there are dramatic differences in the levels of adhesion to purified proteins (Ockenhouse *et al.*, 1991a). The basis for this selectivity is discussed in the next section.

Table 1.4 demonstrates that the evidence for E-selectin and VCAM1 as cytoadherence receptors is not as strong as for TSP, CD36 and ICAM1. A single *P. falciparum* field isolate (CY36) from Thailand was shown to bind weakly to E-Selectin and VCAM1 adsorbed onto plastic and the same cells also bound to purified CD36 and ICAM1 (Ockenhouse *et al.*, 1992b). The avidity was increased by sequential selection of adherent parasites on the purified proteins, and a parasite clone (CY36.1B4) was isolated that bound well to CD36, ICAM1, VCAM1 and E-selectin. Anti-VCAM1 mAb partially inhibited binding of CY36.1B4 cells to purified VCAM1 on plastic, and had no effect on binding of the same clone to E-selectin. Polyclonal anti-E-selectin rabbit sera completely inhibited binding of CY36.1B4 cells to purified E-selectin on plastic, and had no effect on binding of the same clone to VCAM1 (Ockenhouse *et al.*, 1992b). A small minority of field isolates from Africa bound with low avidity to a purified recombinant form of VCAM1 but not to E-selectin on plastic (C. I. Newbold, personal communication).

Relationship to disease severity

Sequestration, as represented by a disappearance of mature infected erythrocytes from the peripheral circulation, is a ubiquitous feature of clinical falciparum malaria. One would predict *a priori* that the severity of infection would correlate with the degree of adhesion to host endothelium. Infected

erythrocytes have been sampled from patients with mild or severe malaria and adherence assays performed using the described model cell systems and purified proteins *in vitro* to test this hypothesis. Published studies to date using isolates from Malawi (Goldring *et al.*, 1992), Thailand (Ho *et al.*, 1991), The Gambia (Marsh *et al.*, 1988) and Papua New Guinea (Reeder *et al.*, 1994) have shown no strong correlations between clinical outcome and cytoadherence to any target *in vitro,* although there was a weak association of cerebral malaria with adhesion to C32 melanoma cells (Ho *et al.*, 1991). These studies have used small numbers of patients, which were insufficient to detect small differences and preliminary data from a large study in Kilifi, Kenya suggests that adhesion to purified ICAM1 is significantly greater in severe malaria than mild disease (C. I. Newbold, personal communication).

Reproducibility of results using different cytoadherence assays and in the same assays on different days is extremely difficult. Problems of interpretation are dramatically illustrated by a recent study from Papua New Guinea which did not find any correlation between binding of cells from patients with mild malaria to purified CD36 and C32 melanoma cells even though immuno-staining has demonstrated that CD36 was the main surface receptor on melanoma cells (Reeder *et al.*, 1994). In the same study, there was no correlation between isolate binding to HUVECs and purified ICAM1, the major receptor on these cells. These differences may have been due to the quality of infected cells and target cells or methodological problems because cells were not allowed to adhere under flow, which can affect adhesion (Wick and Louis, 1991). This is an important physiological consideration because it has been recently demonstrated that interactions of ICAM1, CD36 and TSP with parasitised erythrocytes are influenced by shear stress (Cooke *et al.*, 1994). Elegant rheological studies with purified receptors and HUVECs under conditions that mimic circulatory wall forces suggest that initial interactions made at high wall sheer stress may be of a rolling type and mediated exclusively by ICAM1. The subsequent immobilisation and static adhesion of infected erythrocytes occur via interaction with CD36 and / or TSP. Studies in progress imply that these three receptors may act synergistically during *in vitro* cytoadherence assays (C. J. McCormick, personal communication) and if this occurs *in vivo* then the relative spatial and temporal distribution of different receptors in tissues could influence which pathways of sequestration are employed.

There are two fundamental drawbacks in all *in vitro* studies. Firstly, the parasites tested are sampled from the peripheral blood and allowed to mature into trophozoites *in vitro*. Therefore, they are not the same parasites that are causing the disease and they are exposed to non-physiological factors during subsequent *in vitro* growth. Secondly, the parasites are not tested for adhesion against the endothelium of the host from which they were taken. The influence of host endothelial cell surface polymorphisms has not been assessed and may be important for susceptibility to cerebral malaria. Ideal studies would measure the adhesive phenotypes of sequestered cells using endothelial vasculature from *post-mortem* tissues under conditions that mimicked the pathophysiological states at time intervals leading up to death.

A novel circumvention of this problem has been to assay peripheral monocytes isolated from patients with malaria for their ability to bind to parasites from the same patients (Goldring and Hommel, 1992). The reasoning was that such cells represented physiological targets because they share 25 surface markers with endothelial cells, including ICAM1 and CD36, and respond to cytokines in a similar fashion. Hence, monocytes may represent realistic host targets and initial results from a small number of patients were promising. In general, isolates bound more avidly to monocytes isolated in the acute phase of cerebral malaria than those isolated during the convalescent phase. There were no differences between binding to monocytes isolated during either phase from patients with mild malaria (Goldring and Hommel, 1992). However, parasites were sampled only during the acute phase and these findings could be explained by parasite surface differences between acute and convalescent phases.

Immunohistochemistry has been used to assess the quantitative and qualitative expression of receptors in human studies. Studies with *post-mortem* specimens from Thailand have demonstrated variable expression of TSP, CD36, ICAM1, VCAM1 and E-selectin on the cerebral vasculature of patients who died from cerebral malaria (Ockenhouse *et al.*, 1992b). The same proteins were all absent from the brains of patients who died from other causes. The authors concluded that the co-expression of multiple adhesion molecules associated with cerebral malaria may direct sequestration to sites where adhesion is strongest. However, it is difficult to measure local distribution of receptors within organs although it is clear that there are multiple pathways for sequestration. In a recent quantitative

immunohistochemical study in Thailand, it was shown that there were significantly elevated levels of surface ICAM1, VCAM1 and E-selectin expressed on cerebral endothelium of patients who died from cerebral malaria (Turner *et al.*, 1994). This was not the case for CD36 and TSP, which were sparsely distributed. There was also a significant co-localisation of infected erythrocytes with ICAM1 that was associated with a greater risk of cerebral malaria and mortality was associated with a background of widespread endothelial activation (Turner *et al.*, 1994).

1.8.3.2 Infected Erythrocyte Receptor(s)

The existence of five unrelated host surface proteins which mediate sequestration implies that attachment of parasitised erythrocytes to endothelial cells is accomplished by multiple mechanisms, and one or more may be of critical importance to parasite survival. However, knowledge of the erythrocyte side of the interaction has not kept pace with advances in the understanding of the endothelial receptors. Consequently, the identity of a parasite-encoded counter-receptor for any of the host ligands remains elusive. It is not known whether there is one or more parasite molecules that mediate cytoadherence, or if one parasite molecule predominates in patients with cerebral malaria.

Criteria for infected erythrocyte receptor

Earlier disappointing experiments with knob-associated proteins have ensured that strict criteria below are fulfilled by the candidate molecules discussed in section 1.8.4. From the biological properties of sequestration outlined earlier, the cytoadherence receptor on the erythrocyte surface should be:

- present on the surface of sequestered parasites *in vivo* (although this is currently impossible to test).
- expressed on the surface at the same time as the adherence phenotype is conferred on the erythrocyte. This may be tested by northern blotting of mRNA (if suitable probes were available) or surface / metabolic labelling of infected erythrocytes to detect the protein.
- able to account for the difference between adherent and non-adherent parasites. This phenotype does not have to be linked to the knobby phenotype, but one would expect the expression of

conserved structures to account for the adherence of geographically diverse parasites to a small number of host receptors.

- able to mediate binding of infected cells to cultured endothelial cells *in vitro* or to purified proteins on plastic. Furthermore, the receptor must fail to bind to cultured cells or proteins that do not mediate *in vitro* cytoadherence.

- either a parasite-encoded ligand or a modified host protein that is only surface-exposed in mature forms. Perturbation of the erythrocyte membrane by insertion of parasite proteins may expose cryptic host structures or malarial enzymes may modify host proteins to create new domains that mediate cytoadherence.

- similar in those *Plasmodium* species that have comparable sequestration patterns *in vivo*. Comparative studies between those species which do not undergo sequestration should reveal some differences in expression of crucial parasite proteins, although host factors may contribute.

Mechanisms of parasite virulence are enigmatic, but it appears that the sequestration molecule of *P. falciparum* may be similar to the variant antigen of *P. knowlesi*. Antigenic variation may explain, in part, why this molecule has been difficult to clone and why no monospecific reagents exist. Before the development of long-term *in vitro* cultivation of asexual blood stages in 1976, it was impossible to obtain sufficient numbers of *P. falciparum* infected erythrocytes to perform vital experiments on their adhesive phenotypes. Consequently, sequestration was investigated by *in vivo* animal models and data have variable relevance to *P. falciparum* infection of humans (Table 1.2). Early studies focused on the mechanisms of antigenic changes at the surface of *P. knowlesi* infected erythrocytes and had a lasting effect on today's paradigms.

There is evidence for the expression of eleven parasite-induced proteins at the erythrocyte membrane, and the current state of knowledge is reviewed followed by a summary of the candidate molecules (reviewed by Howard, 1988).

1.8.4 Antigens at the Infected Red Cell Surface and Cytoadherence

The characteristics of HRP1 have been outlined above and the following proteins are discussed because most have been considered as cytoadherent moieties at one time. A diagrammatic representation of their distribution in relationship to knobs at the infected red cell surface is depicted in figure 1.8.

There are several large *P. falciparum* encoded antigens expressed in the erythrocyte membrane which do not fulfill the criteria of the cytoadherence receptor. The functions of these proteins have not been elucidated, and evidence suggests that they may play vital roles in membrane architecture because they interact with numerous erythrocyte proteins (reviewed by Howard, 1988).

Large malaria antigens are difficult to characterise because they are constructed from multiple linear repeats and may often share sequences that are immunogenically cross-reactive (reviewed by Anders and Smythe, 1989; Hommel and Semoff, 1988). For instance, the repetitive motif EEXXEE is the recognition epitope for the human IgM mAb 33G2, which cross reacts with D260, Ag332, Pf11.1 and RESA (Ahlborg *et al.*, 1991). The mAb 33G2 inhibited the adherence of a knob-negative isolate to C32 melanoma cells (Udomsanpetch *et al.*, 1989) and this has implicated all four antigens in cytoadherence, although the epitope may be surface expressed by PfEMP1 (see later).

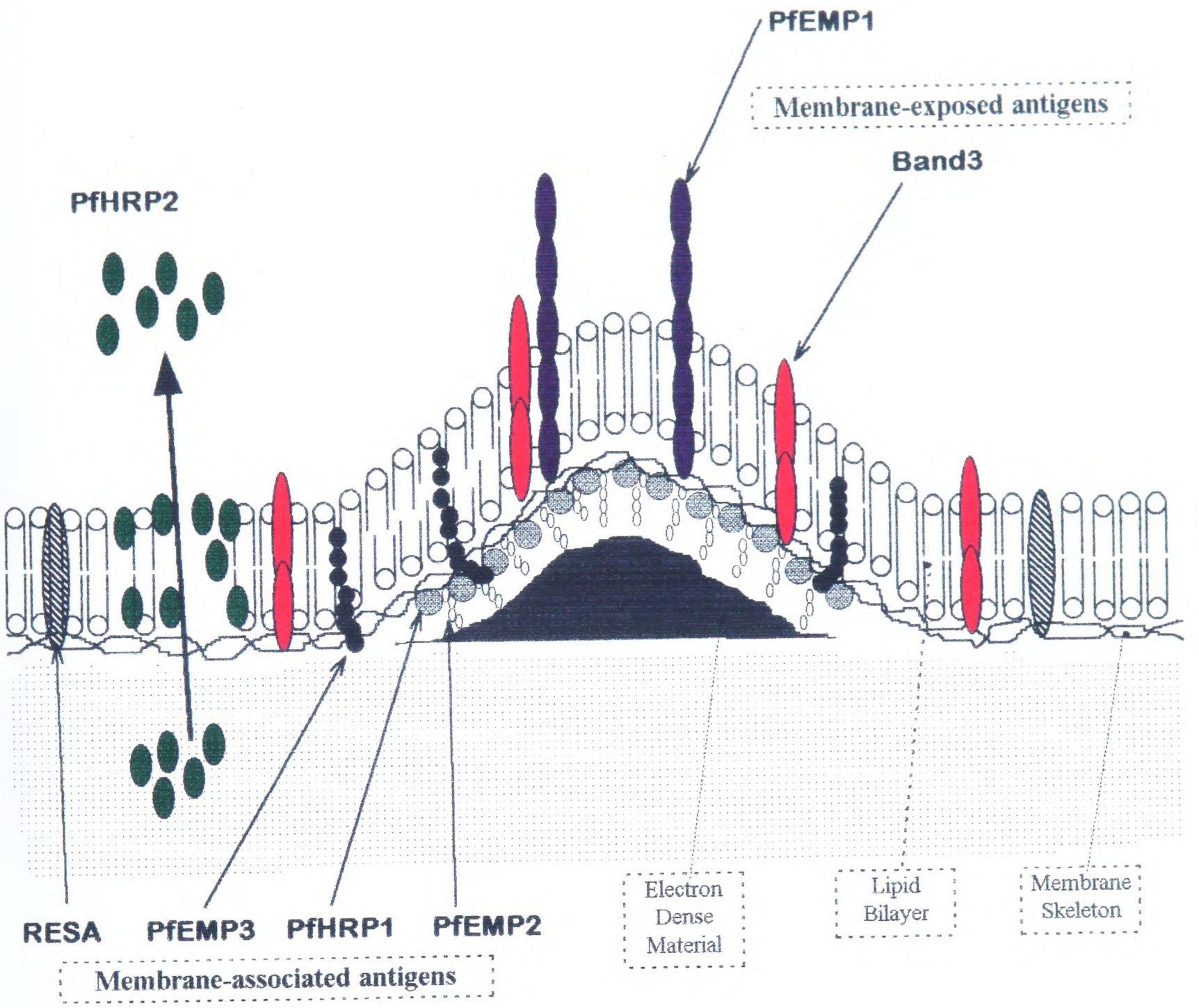


Figure 1.8. Diagrammatic representation of parasite-encoded antigens expressed in the membrane of P. falciparum *infected erythrocytes (Modified from Howard, 1988).*

PfEMP2 (a.k.a.: mature erythrocyte surface antigen (MESA), PP300):

This is a polymorphic phosphoprotein of 250-300kDa synthesised by mature stages of the intracellular parasite and can be readily labelled with radioactive amino acids (Howard *et al.*, 1988). It has been immunolocalised to the parasitophorous vacuolar membrane (PVM) and the inner face of the erythrocyte membrane (EM) in knobby regions. It is not surface-labelled or accessible to tryptic digestion, nor is it essential for knob formation or cytoadherence (Howard *et al.*, 1990). PfEMP2 is submembraneous, and it associates with the erythrocyte membrane skeletal protein, band 4.1 (Lustigman *et al.*, 1990). Hence, it could play a role in membrane stability or may be a precursor to PfEMP1.

PfEMP3:

PfEMP3 was identified in two (K^+) laboratory adapted isolates by monoclonal antibodies as a 310kDa invariant submembraneous antigen at the erythrocyte membrane of mature PRBC (Handunnetti *et al.*, 1992; Pasloske *et al.*, 1993; Van Schravendijk *et al.*, 1993). It was not surface-labelled, but could be metabolically labelled and extracted by SDS but not Triton X-100. Its function and relationship to knobs has not been determined, but it is antigenically distinct from PfEMP1.

RESA (a. k. a.: ring-infected erythrocyte surface antigen, Pf155):

RESA is located in the dense granules of merozoites and released into the parasitophorous vacuole after invasion (Culvenor *et al.*, 1991). RESA becomes associated with the erythrocyte membrane of ring stage parasites where it forms a ternary complex with spectrin, actin and band 4.1 (Foley *et al.*, 1991; Ruangjirachuporn *et al.*, 1991). Antibodies to RESA, which were affinity purified from immune serum, immunoprecipitated a biosynthetically labelled protein of 155kDa but only reacted with fixed PRBC. It is unlikely that RESA is involved in cytoadherence because prior treatment with trypsin did not affect the immunostaining pattern and ring-stage parasites were non-adherent. Furthermore, when it was expressed in non-cytoadherent lines, its level decreased as cytoadherence capacity increased.

PfHRP2:

This is a 70kDa histidine rich protein that is secreted from rings, trophozoite and schizont infected erythrocytes, although the mechanism of export is unknown (Parra *et al.*, 1991). It has been located in several intracellular compartments and appears as 'packets' within the cytosol and beneath the erythrocyte membrane. Staining with murine mAbs to PfHRP2 showed its deposition on the surface of cerebral microvessels by sequestered parasites (Aikawa *et al.*, 1990b). The same reagents had no effect on cytoadherence and the function of PfHRP2 is enigmatic because it is expressed independently of the knobby phenotype.

Pf11.1:

This was initially described as a 260kDa asexual protein (Koenan *et al.*, 1984), but subsequent studies established that its gDNA encoded a 1000kDa giant polypeptide that was surface iodinatable and insoluble in Triton X-100 (Petersen *et al.*, 1990). However, it was not correlated with cytoadherence and it has recently been associated with another malaria gene sequence called 332. Current evidence suggests that Pf11.1 is a sexual stage antigen and that the original 260kDa asexual protein is D260 which has not been characterised.

Rosettins:

A single study has identified two strain-specific ^{125}I labelled antigens of 22kDa and 28kDa at the surface of mature PRBC (Helmby *et al.*, 1993). Antibodies to these proteins inhibit rosetting but have no effect on cytoadherence. The relationship of rosettins to PfEMP1 is unknown.

1.8.5 Cytoadherence, Agglutination and PfEMP1

In the 1930s, Eaton demonstrated that parasite-induced antigenic modifications occurred at the surface and early investigations of the infected erythrocyte surface were motivated by questions relating to the mechanisms of acquired specific immunity (Eaton, 1938). Uncloned *P. knowlesi* parasitised erythrocytes were used to infect rhesus monkeys (*Macaca mulatta*), even though the natural host for this species is the kra monkey (*M. fascicularis*) where the parasite is avirulent (Brown and Brown, 1965; Brown *et al.*, 1968). Isolates were sampled during relapses from drug-cured animals and only schizonts (trophozoites and schizonts by today's classification) were agglutinated by sera from the same animals. This 'Schizont Infected Cell Agglutination (SICA) assay' was the first demonstration that parasites could modify the surface of a host cell and the recognised antigens were known as SICA.

This phenomenon was systematically investigated and a series of seminal papers published which established that SICA underwent antigenic variation and implicated a role for them in protective immunity (Brown *et al.*, 1970 a, b). Brown and colleagues injected cryopreserved *P. knowlesi* infected erythrocyte stabilates into monkeys and found that when the primary parasitaemia was drug-cured, there was a specific antibody response to the surface of the PRBC and a chronic infection was established. On re-challenge with parasites from the same original stabilates, a different antibody response followed drug-treatment. Thus, antibodies that were already present did not recognise the parasite serotype in the second wave because the isolates expressed antigenically distinct determinants. The same laboratory later demonstrated that variant-specific antibodies were present in the immunised animals that induced antigenic variation at the erythrocyte surface (Brown, 1973).

However, non-clonal starting populations of parasites were used so that the results could be attributed to minor parasite subpopulations of independent genetic origin which escaped the host immune responses against the major phenotype. This was proved not to be the case, when the original findings were extended later with cloned parasites in Rhesus monkeys to show conclusively that *P. knowlesi* underwent antigenic variation (Barnwell *et al.*, 1983). This process is distinct from antigenic diversity which represents the expression of polymorphic alleles at a single genetic locus (e.g. S Antigens and MSP1), and cells derived from a clone express the same antigen despite its diversity.

Erythrocyte surface antigens of many other species of *Plasmodium* have been demonstrated to undergo true antigenic variation. In 1979, Wrey and Timperman demonstrated that *P. berghei* underwent antigenic variation as deduced from homologous and heterologous challenges of white mice. Inbred mice were used in a series of studies by McLean and colleagues using transfer of variant-specific immune sera and analysis of recrudescent populations for the existence of *P chabaudi* infected red cell variants (1982 a, b). This group demonstrated that antigenic variation of *P. chabaudi* infected erythrocytes occurred and antigenic phenotype was altered on mosquito transmission (1986; 1987; 1990).

During the same period, significant advances into SICA expression were made by investigations into the role of the spleen in immunity to infection. Loss of SICA expression occurred when cloned *P. knowlesi* parasites were passaged in splenectomised rhesus monkeys (Barnwell *et al.*, 1983), and these $SICA^-$ parasites were capable of re-expressing the $SICA^+$ phenotype after several cycles following transfer to intact animals. It was concluded that the spleen could modulate antigen expression, but the basis is still unknown and is attributed to unidentified 'splenic factors'.

Consequently, the importance of the spleen in modulating protective immunity was studied in other animal models of malaria that closely resembled the pathogenesis of *P. falciparum* infections of humans. Using cloned *P. fragile* parasites to infect its natural host, the toque monkey (*Macaca sinicai*), parasite lines were isolated from splenectomised monkeys that failed to express surface neoantigens and did not undergo sequestration. The spleen was shown to modulate the antigenic phenotype of *P. fragile* infected erythrocytes when a single clone was used for infection of *M. sinicai*, and a defined order of variants appeared in successive recrudescent waves of parasitaemia (Handunnetti *et al.*, 1987). Splenic modulation of surface antigens and sequestration phenotype also occurred in the murine malaria *P. chabaudi* (Gilks *et al.*, 1990), and similar observations have been made in uncloned *P. falciparum* infections of the squirrel monkey (*Saimiri sciureus*). In the *P. falciparum* model, passage in splenectomised monkeys was accompanied by a change, not a loss, of surface antigenic phenotype, and mature forms were observed in the peripheral circulation. The loss of sequestration following splenectomy was described in a natural *P. falciparum* infection of man

(Israeli *et al.*, 1987). Thus, indirect evidence suggested that antigenic and adherent phenotypes were linked and switching could be influenced by the spleen.

The molecular basis for this phenomenon was pursued using cloned *P. knowlesi* infections of monkeys and labelling techniques to study the PRBC proteins. When surface labelled and metabolically labelled PRBC were immunoprecipitated by specific agglutinating sera, a high molecular weight antigen was recognised that varied in size between two clones (Howard *et al.*, 1983). The agglutinating properties of the antisera correlated with their capacity to immunoprecipitate the variant antigen, which was the same size using surface labelling or metabolic labelling techniques. This was the first molecular evidence for antigenic variation of a *Plasmodium* protein, and the indirect evidence available from earlier studies suggested that it was also functionally linked to the expression of the sequestration phenotype.

This research provided the impetus for the search for the biochemical correlate of SICA in *P. falciparum*, which was identified soon after the advent of a continuous *in vitro* culture system. PfEMP1 (*Plasmodium falciparum* Erythrocyte Membrane Protein-1) was first identified as the strain-specific molecule using monkey derived parasites that were labelled with the ^{125}I lactoperoxidase technique under conditions which did not label internal proteins (Leech *et al.*, 1984). The biochemical characteristics of PfEMP1 were similar to SICA and have since been shown to be a family of high molecular weight (200-300kDa) antigens that are operationally defined by their insolubility in Triton X-100 and sensitivity to low concentrations of trypsin. Strains and isolates differ in surface antigenicity and this is accompanied by alterations in the elecrophoretic mobility of ^{125}I labelled PfEMP1 (Howard, *et al.*, 1988; Van Schravendijk *et al.*, 1991). However, it may only be a quantitatively minor constituent because it is not easily detected by metabolic labelling despite enrichment of the Triton-insoluble extracts and immunoprecipitation (Howard *et al.*, 1988).

Several lines of evidence suggest that PfEMP1 undergoes antigenic variation and may mediate sequestration. Strain-specific sera can be elicited from monkeys infected with *P. falciparum* clones which immunoprecipitate ^{125}I labelled PfEMP1 and mediate agglutination of the homologous strain (Udeinya *et al.*, 1983; David *et al.*, 1983; Howard *et al.*, 1988). These sera inhibited the ability of the homologous strain and not heterologous strains to bind to C32 melanoma cells (Udeinya *et al.*, 1983;

David *et al.*, 1983). Therefore, the strain-specific antigenic determinants expressed on PfEMP1 must be linked to the expression of the adherence domains for C32 melanoma cells. Field isolates frequently lose their adherence characteristics when cultured *in vitro*, and this was always accompanied by a loss of PfEMP1 expression (reviewed by Howard *et al.*, 1990). The basis of this change is unknown, but it could be due to the absence of splenic pressure.

Selection of PRBC for adherence to C32 melanoma cells was always accompanied by a change in the molecular weight of ^{125}I labelled PfEMP1, and no other antigenic modifications were detected (Magowan *et al.*, 1988). Many studies have demonstrated that the trypsin sensitivity profiles of ^{125}I labelled PfEMP1, adhesion to C32 melanoma cells and agglutination are identical (reviewed by Howard, 1988).

There are no published monoclonal antibodies against PfEMP1 and indirect evidence that it mediates binding to individual endothelial host ligands has come from studies *in vitro* relating to the antigenic determinants at the PRBC surface. Selection of cloned parasites that only bind well to C32 melanoma cells (which express CD36 and TSP, and a little ICAM1), or for adhesion to HUVECs (express ICAM1 and no CD36), resulted in a 40-fold increase in adhesion to HUVECs, but only a 2-fold increase for adhesion to C32 melanoma cells (Biggs *et al.*, 1992). This selection also led to a different surface antigenicity as judged by the inability of antisera to the selected line to cross-react with the parental line and vice versa. The selected HUVEC binding line expressed a single PfEMP1 with higher molecular weight than PfEMP1 of the C32 melanoma cell binding line and these antigens were immunoprecipitated in a cross-reactive fashion by different strain-specific antisera.

Using erythrocytes that had been infected by a single *P. falciparum* parasite, it has been recently demonstrated that antigenic and adhesive phenotypes were linked (Roberts *et al.*, 1992). Antigenic variation occurred at 2% per generation from a single *P. falciparum* clone which could bind to purified CD36 and ICAM1 on plastic. The parental clone could switch to multiple variant antigenic subclones as determined by the mixed agglutination assay, and this rapid stochastic switching was always accompanied by a loss of ICAM1 binding. Selection for adhesion to purified ICAM1 restores the parental adherent and antigenic phenotype, and demonstrated clonal antigenic variation at the phenotypic level. A reduction in the size of PfEMP1 has been correlated with the loss of HUVEC

binding ability (Biggs *et al.*, 1992). Therefore, indirect evidence suggests that the binding domains for CD36 and ICAM1 are distinct, and their expression is linked to the antigenic phenotype of the parasite. There is no corresponding data for binding to TSP, VCAM1 and E-selectin.

One group has used anti-idiotype antibodies to the anti-CD36 monoclonal antibody, OKM5, to immunoprecipitate a molecule termed 'sequestrin' (Ockenhouse *et al.*, 1991b). This was a parasite-derived high molecular weight protein that was surface-labelled and immunoprecipitated from a single *P. falciparum* line which bound to CD36. The anti-idiotype antibodies gave partial inhibition of binding of the same line to C32 melanoma cells, but no details of their effect on binding to HUVECs were described. Sequestrin was sensitive to trypsin and soluble in Triton X-100 because it was derived from a knob-negative parasite, and thus has most of the features of a member of the PfEMP1 family.

The genetic basis for the antigenic and adhesive changes is unclear. There is evidence that the gene family for PfEMP1, or an essential regulatory element, may be located on the right arm of chromosome 9 (Kemp *et al.*, 1992; Day *et al.*, 1993). Deletions of a 0.3Mb subtelomeric region of this chromosome were observed in field isolates that had lost the ability to cytoadhere to C32 melanoma cells *in vitro* and no PfEMP1 was detectable by surface labelling. This was a reversible loss, and selection for binding to the same cells resulted in the appearance of a full length chromosome 9 and PfEMP1 molecule. Recent data using a clone from a field isolate with this deletion show that adherence can occur to HUVECs but not to purified CD36, ICAM1, VCAM1 and E-selectin (Chaiyaroj *et al.*, 1994a). This indicates that the 0.3Mb region on chromosome 9 may only be required for binding to an unidentified receptor(s) on C32 melanoma cells. The same study implicated other distinct loci which were associated with the ability to cytoadhere and also the existence of an additional receptor on HUVECs which may mediate trypsin-resistance adherence.

All of the available data suggest that PfEMP1 has a dual role that is central to the pathogenesis of malaria. Its definition as the variant antigen means that it is the strongest candidate target for the protective immune responses that are directed at antigens expressed at the erythrocyte surface. The subsequent demonstration that antigenic switching is linked to the adhesive properties of the infected erythrocyte has important implications for both the development of clinical immunity and for the sequestration phenotype of the infected cell (reviewed by Roberts *et al.*, 1993).

The high rate of phenotypic switching of *P. falciparum in vitro* is comparable to frequencies calculated in *Giardia* (Nash, 1992), *Candida* (Slutsky *et al.*, 1985), and *Trypanosoma* (Turner and Barry, 1989). This results in extensive phenotypic heterogeneity and even though the repertoire of antigenic variants has not been determined, it is likely to be large. Protective immunity develops against disease and not infection, and it is established when certain 'disease-causing' antigenic variants are recognised and destroyed. The numbers of a particular variant present during infection will be dependent on the rate of switching to that variant, the pre-existing level of specific antibody to that variant, and the rate at which the new antibody is synthesised. Mathematical models of this scenario predict that the variant repertoire would be rapidly exhausted *in vivo* due to the high switch rate (C. I. Newbold, personal communication). Thus, a spectrum of switching rates must operate to make up the overall rate of 2%, and the elucidation of serodemes within natural infections is an important goal. There is evidence for serodeme usage from the *P. fragile / M. sinicai* system where particular variants were associated with successive recrudescing waves of parasitaemias (Handunnetti *et al.*, 1987). Thus, antigenic variation may prevent the rapid recognition of erythrocytic stages and contribute to the chronicity of infection by *P. falciparum*.

The linkage of antigenic and adhesive phenotypes at the infected erythrocyte surface introduces further complexity because the levels of receptor expression in vascular beds will also affect the numbers of particular variants during infection via sequestration to host endothelium (reviewed by Roberts *et al.*, 1993). Expression of endothelial receptors will, in turn, be influenced by host polymorphisms and responses to circulating cytokines (reviewed by Berendt, 1994). The interaction of surface phenotypes implies a role for the spleen in the selective removal via filtration of particular adherent phenotypes (and hence antigenic variants) which have not sequestered. This is a different mechanism from earlier interpretations that this organ had a direct effect on parasite gene expression. Instead, the high rate of switching explains the low number of cycles that are required for the phenotypic changes observed when parasitised cells are transferred from spenectomised to intact monkeys.

This theory may explain why severe disease is a rare event, in comparison to the total number of infections. Severe disease only occurs when a particular variant infects an individual and gives rise to

a parasite which that does not elicit a protective immune response due to its antigenic phenotype. Severe disease would occur if this population also sequestered preferentially in the cerebral microvasculature due to its adhesive phenotype. Indirect evidence suggests that antigenic and adhesive phenotypes are mediated by PfEMP1 and no other surface antigens are involved. Thus, to elucidate its functional role in more detail, it is crucial to expand the biochemical data relating to PfEMP1.

At the time of writing, no sequence has been published for any members of the PfEMP1 family, but several laboratories may be on the verge of cloning this molecule. A description of the mechanism for antigenic variation in *Plasmodium* relies on sequence information and the genetic basis of variant surface glycoprotein switching in trypanosomes may provide a paradigm (Borst, 1991). The current status of this field is assessed in the discussion chapter in the light of findings in this thesis.

1.8.6 Host Membrane Protein Modifications

One laboratory has reported a series of experiments which indicated that there were parasite induced modifications to the host anion transporter, Band 3. This is the most abundant protein in the erythrocyte membrane (10^6 copies per cell) and exists as monomers, dimers and tetramers of a ~95kDa glycoprotein, which spans the bilayer 14 times. Anti-Band 3 IgG autoantibodies occur in normal human plasma as a result of red cell senescence, and react with a >240 kDa protein at the knobs of mature PRBC (Winograd *et al.*, 1987; Winograd and Sherman 1989b). This protein was surface-labelled by radioiodination, sensitive to trypsin and insoluble in Triton X-100. It was not biosynthetically labelled and was believed to be a tetrameric form of Band 3 induced on parasite maturation because it was absent from ring-stages (Winograd and Sherman, 1989b).

Several murine mAbs that give variable degrees of reactivity against the mature PRBC surface, are believed to recognise modified forms of Band 3. Two mAbs, 4A3 and IC4, immunoprecipitated ^{125}I-labelled 85kDa and 65kDa antigens respectively from a single adherent line that was absent from a non-adherent parasite (Winograd and Sherman, 1989a). Both antigens were Triton X100 insoluble and not biosynthetically labelled. Similar 2D gel electrophoretic patterns were observed on comparison of these antigens to the >240kDa senescent antigen and Band 3. It has been proposed that the 85kDa and 65kDa antigens are parasite-induced truncated forms of Band 3.

The mAbs 4A3 and IC4 blocked cytoadherence of one parasite line to C32 melanoma cells by varying degrees, and a soluble cryptic epitope (termed Pfalhesin) of Band 3 inhibited binding to purified CD36, but not to ICAM1 in the same adhesion assay (Crandall *et al.*, 1994). However, this system uses non-physiological conditions (eg. 50mM calcium lactate in adhesion buffer) and none of these results have been repeated in other laboratories. Thus, the hypothesis that modified forms of Band 3 mediate cytoadherence via CD36 is a controversial issue that will only be resolved when these findings are repeated on a range of laboratory and field isolates.

1.8.7 Other Membrane Alterations

Lipids play a crucial role in maintaining the membrane stability and they are a fundamental component of the fluid-mosaic model of plasma membranes which is based on a lipid bilayer. The precise lipid constitution can influence the membrane's fluidity and spatial differences in lipid distribution can affect localised membrane proteins. Lipid symmetry can affect red cell ghost adhesion to endothelial cells *in vitro* and a more ordered arrangement is favoured for this interaction. The importance of lipids to the surface membrane of PRBC has not been established, but indirect evidence suggests that they may affect its function.

Cholesterol levels are lowered in mature PRBC which makes the membranes more fluid and could facilitate the typical clustering of parasite antigens or adhesion molecules (Maguire and Sherman, 1990). There is an increase in exposed phosphatidylserine molecules in *P. falciparum* infected cells and this may increase adhesiveness by affecting the membrane lipid symmetry (Maguire *et al.*, 1991). The same effect is observed in reversibly sickled red cells in vitro and they show increased adhesion to endothelial cells that can be inhibited by endogenous phosphatidylserine, but not other phospholipids.

Glycophorin-bound sialic acid residues represent the major carbohydrate on the surface of the erythrocyte, and are responsible for the overall negative charge of the cell (Fukuda *et al.*, 1987). This is often reduced in aged erythrocytes and in *P. berghei*-infected cells (Howard and Day, 1981), and results in the exposure of β-galactosyl residues linked to glycophorin. Mature PRBC preferentially bind to lectins (concanavalin A, ricin, agglutinins) which implies that additional galactosyl residues are synthesised by the parasite during development (David *et al.*, 1981). Alternatively, endogenous

carbohydrate residues may be exposed or have increased lateral mobility induced by parasite maturation. In any case, it appears that the carbohydrates are clustered at knobs, but no studies have been performed on K^- parasites to date.

The presence of certain amino sugars in the medium reduced the *in vitro* cytoadherence of *P. falciparum* infected erythrocytes (Singh *et al.*, 1987). However, this result was probably due to the presence of high concentrations (50 - 100mM) of free amino groups and not due to specific competition by individual sugar residues. The same result was not observed when sugars were used in osmotically balanced media (C. I. Newbold, personal communication). Treatment of PRBC with neuraminidases or galactosidases did not abolish cytodherence to C32 melanoma cells, and instead, an increased level of binding of some parasite lines were observed (Sherman and Valdez, 1989). These findings may be due to modifications of the electrostatic surface properties, but the involvement of carbohydrate structures on antigenicity and adherent phenotypes is unclear.

1.8.8 Rationale and Aims

Studies by Brown and colleagues over 25 years ago demonstrated that *P. knowlesi* infected erythrocytes could undergo antigenic variation in monkeys (reviewed by Howard, 1984). Their work was a catalyst for the subsequent identification of the variant antigen in this parasite, and since then, the importance of the host-parasite relationship at the surface of the parasitised red blood cell (PRBC) has been recognised in *P. falciparum* malaria.

Parasite-derived neoantigens (PDNs) are expressed on the surface of erythrocytes infected by mature forms of *P. falciparum* which undergo sequestration in the cerebral microvasculature, a process which accounts for much of the pathology of cerebral malaria. The same PDNs have been identified as targets of the protective immune responses at this stage of the life cycle and the molecular and biochemical nature of PDNs has received intense scrutiny because they represent potential candidates for anti-adhesion therapy and could be components of a vaccine.

Indirect evidence from research over the past 10 years has shown that a single family of *P. falciparum* antigens, PfEMP1, satisfies the criteria required to fulfill a dual function as the cytoadherence receptor and the variant antigen. However, the cloning of this molecule has not been reported and there are no published monoclonal antibodies available. In the absence of sequence information, the aim of this thesis was to use biochemical techniques to investigate the variant antigenic properties of PfEMP1 and their relationship to cytoadherence.

Chapter 2

Materials and Methods

All chemicals and equipment that were used are tabulated in the first section of this chapter. General, well-established methods are described in the second section. Some procedures were modified for specific purposes in this study and these alterations are detailed in the appropriate chapters.

2.1. Materials

Table 2.1 shows the chemicals and equipment and their suppliers that were used in this study. Chemicals were analytical grade, except where stated.

SUPPLIER	CHEMICALS
Amersham plc.,	^{14}C-choline chloride
Buckinghamshire, UK.	Rainbow markers (^{14}C-labelled protein markers)
	Sodium ^{125}iodide
	L-[^{35}S]- *in vitro* cell labelling promix
BDH Biochemicals Ltd.,	Acetic acid
Warwickshire, UK.	Ammonium persulphate (APS; Electrophoresis grade)
	Bromophenol blue
	Calcium chloride
	D-glucose
	Di-sodium hydrogen orthophosphate
	Di-methyl sulphoxide (DMSO)
	Ethylene diaminotetra-acetic acid (EDTA)
	Giemsa's staining solution
	Glycerol
	Hydrochloric acid
	β-mercaptoethanol
	Methanol
	Potassium chloride
	Propan-2-ol

	Sorbitol Sodium azide Sodium chloride Sodium di-hydrogen orthophosphate Sodium hydroxide Triton (X-100 and X-114)
Bio-Rad Ltd., **Hertfordshire, UK.**	Acrylamide (electrophoresis grade) CM-2 cation exchange gel (sodium form) N, N'-methylenebisacrylamide (Bis, electrophoresis grade) N, N, N',N'-tetraethyl-ethylenediamine (TEMED) (electrophoresis grade) Prestained protein size markers (high and low M_r ranges)
Boerhinger Mannheim GmbH, **Mannheim, Germany.**	Chymotrypsin A_4 Dispase I (neutral protease, from *Bacillus polymyxa*) Tris (2-amino-2-(hydroxymethyl)-1,3-propandiol) V8 protease (endoproteinase Glu-C)
Coulter Electronics Ltd., **Luton, Bedfordshire, UK.**	Isoton II™
Flowindex plc., **Wigan, Lancashire, UK.**	Industrial methylated spirits (IMS, low grade)
Gibco Europe Ltd., **Uxbridge, Middlesex, UK.**	Phosphate buffered saline (PBS; with calcium and magnesium) Foetal calf serum (Myoclone™) L-glutamine N-2-hydroxyethylpiperazine, N-2-ethanesolphonic acid (HEPES) 2-[N-Morpholineo] ethanesulphonic acid (MES) Minimal Essential Media (MEM; with Earle's Salts, without L-arginine, L-cystine, glucose, L-glutamine, i-inositol, L-leucine, L-methionine) Penicillin RPMI 1640 medium Streptomycin
Nycomed Pharma AS, **Oslo, Norway.**	Lymphoprep™

Oxford Glycosystems Ltd., Oxford, UK.	Neuraminidase (from *Clostridium perfringens*, contamination-free) Neuraminidase (from *Vibrio cholerae*, contamination-free)
Pierce Luton, UK.	Iodogen™ iodination reagent (1, 3, 4, 6 - Tetrachloro - 3α - 6α - diphenylglycoluril)
Regional Transfusion Unit, John Radcliffe Hospital, Oxford, UK.	Acid citrate dextrose Group O red blood cells Human platelets Human serum
Rhone-Poulenc, Neully-sur-Seine, France.	Plasmagel™ Sodium chloride
Sigma Chemical Co., Poole, Dorset, UK.	Antipain Aprotinin Amberlite MB-3 beads Bovine serum albumin (BSA; sterile 30% solution, A-1662) Chymostatin Coomasssie brilliant blue R-250 4'-6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) DEAE-sephacel suspension Di-isopropyl-fluorophosphate (DFP) Ethidium bromide (EtBr) Gentamicin sulphate Glutaraldehyde (grade II) G-25 sephadex beads Lactoperoxidase Leupeptin Percoll Phenylmethylsulphonylfluoride (PMSF) Potassium iodide Protein A-sepharose beads (CL-4B) Sodium dodecyl sulphate (SDS, electrophoresis grade) N-tosyl-l-lysine-chloromethylketone (TLCK) N-tosly-l-phenylalanine-chloromethylketone (TPCK) Trypsin (TPCK treated, Type XIII) Trypsin inhibitor (purified chicken egg white, Type III-O)

Table 2.1 (a). Chemicals and suppliers used in this study.

SUPPLIER	EQUIPMENT
Alltech Assocs., **Carnforth, Lancashire, UK.**	Teflon wool
American National Can Co., **Greenwich, Connecticut, USA.**	Clingfilm™
Amicon Ltd., **Gloucestershire, UK.**	Ultrafiltration membranes Centricon tubes
Anachem Ltd., **Luton, Bedfordshire, UK.**	Gilson pipettes (P20, P200, P1000, P5000) and tips
Anderman and Co., **Kingston-on-Thames, Surrey, UK.**	Eppendorf™ microfuge tubes Eppendorf™ 1.5ml, 0.5ml tubes
Becton Dickenson Ltd., **Oxford, UK.**	Falcon 25cm^2, 75cm^2 tissue culture flasks Petri dishes (bacteriological grade) Polycarbonate and polypropylene tubes Sterile needles and syringes
Bio-Rad Ltd, **Hertfordshire, UK.**	Mini-Protean II SDS-PAGE kits Gel drier (model 583)
British Oxygen Plc., **Guildford, Surrey, UK.**	Gas cylinders (96%N_2, 1%O_2, 3%CO_2 and 78%N_2, 17%O_2, 5%CO_2)
Coulter Electronics Ltd., **Luton, Bedfordshire, UK.**	Coulter counter™ (Model D Industrial)
Dow Chemical Co., **New Jersey, USA.**	Saranwrap™
DuPont plc., **Stevenage, Hertfordshire, UK.**	Sorvall RT 6000B centrifuge
Fuji, **Tokyo, Japan.**	X-ray film
Genetic Research Instrumentation Ltd., Essex, UK.	Hybridisation machine
Gelman Sciences **Berks, UK.**	Syringe filters
C. A. Hendley Ltd., **Essex, UK.**	Multi-spot microscope slides

Hoeffer **Staffs, UK.**	Large PAGE kits
Kodak, **Hemel Hempstead, Hertfordshire, UK.**	Film cassettes and screens
Jencons Ltd., **Leighton Buzzard, Bedfordshire, UK.**	Liquid nitrogen storage tanks and accessories
Medicell International Ltd., **London, UK.**	Dialysis tubing (visking size 2-18/32")
Micro Instruments Ltd., **Oxford, UK.**	Nikon TMS phase contrast microscope Zeiss universal microscope Zeiss epifluorescence microscope
Mini Instruments, **Burnham-on-Crouch, Essex, UK.**	Geiger counter
Nelson Scientific Ltd., **Hertfordshire, UK.**	Corning 150cm^2 centrifuge tubes
Nunc **Kamstrup, Denmark**	Freezing vials
Philip Harris Ltd., **London, UK.**	Glass cover slips Microscope slides Pasteur pipettes
Sigma Chemical Co., **Poole, Dorset, UK.**	Protein gel-loading tips
Swann-Morton Ltd., **Sheffield, UK.**	Surgical blades
Whatman International, **Maidstone, Kent, UK.**	Whatman 3MM filter paper

Table 2.1.(b) Equipment and suppliers used in this study.

2.2. Methods

2.2.1 Parasites

Parasites are referred to as lines or clones depending on their mode of derivation. A parasite line is a named and defined population of cells (a culture) that has been taken during a natural infection and subsequently maintained *in vitro* or by passage in animals. A clone is a culture which has been derived from a single cell. The Ituxi parasite line had been selected for adhesion to C32 melanoma cells and endothelial cells (Berendt *et al.*, 1989). The resulting culture was termed ITO4 and was cloned by micromanipulation in our laboratory. Clones which were used in this study had been tested for their ability to bind to purified CD36 and ICAM1 and these results are summarised in table 2.2 (Roberts *et al.*, 1992). The parasite nomenclature has been modified by the addition of a subscript ($_{i+}$ or $_{i-}$) to denote the ability of the culture to bind to ICAM1. An outline of the derivations and known antigenic relationships between these clones is illustrated in figure 2.1.

PARASITE CLONE	CD36 Binding	ICAM1 Binding	MODE OF DERIVATION
$A4_{i+}$	+	+	Cloned from ITO4 (Roberts *et al.*, 1992). Gift of Dr. D. J. Roberts, Oxford, UK
$C9_{i+}$	+	+	Subcloned from $A4_{i+}$. (Roberts *et al* ., 1992). Gift of Dr. D. J. Roberts, Oxford, UK
$C18_{i-}$, $C24_{i-}$, $C28_{i-}$	+	-	Subcloned from $A4_{i+}$. (Roberts *et al.*, 1992) Gift of Dr. D. J. Roberts, Oxford, UK
C28-I_{i+}	+	+	Selected from $C28_{i-}$ clone by panning parasites for one round on purified ICAM1 (Roberts *et al*, 1992). Gift of Dr. A. R. Berendt, Oxford, UK
$IT6_{i+}$	+	+	Selected from the Ituxi line by six rounds of sequential panning on purified ICAM1 (Ockenhouse *et al.*, 1991a). Gift of Dr. C. F. Ockenhouse, Department of Immunology, Walter Reed Army Institute of Research, Washington, USA.
D4	-	-	Cloned from an artificial mixture of Palo Alto line and Wellcome line (Fandeur *et al.*, 1991). Gift of Dr. I. Wilson, Division of Parasitology, National Institute for Medical Research, The Ridgeway, Mill Hill, London.

Table 2.2. Summary of the parasite lines and clones used (± denotes the ability of a culture to bind to purified receptor proteins in vitro*).*

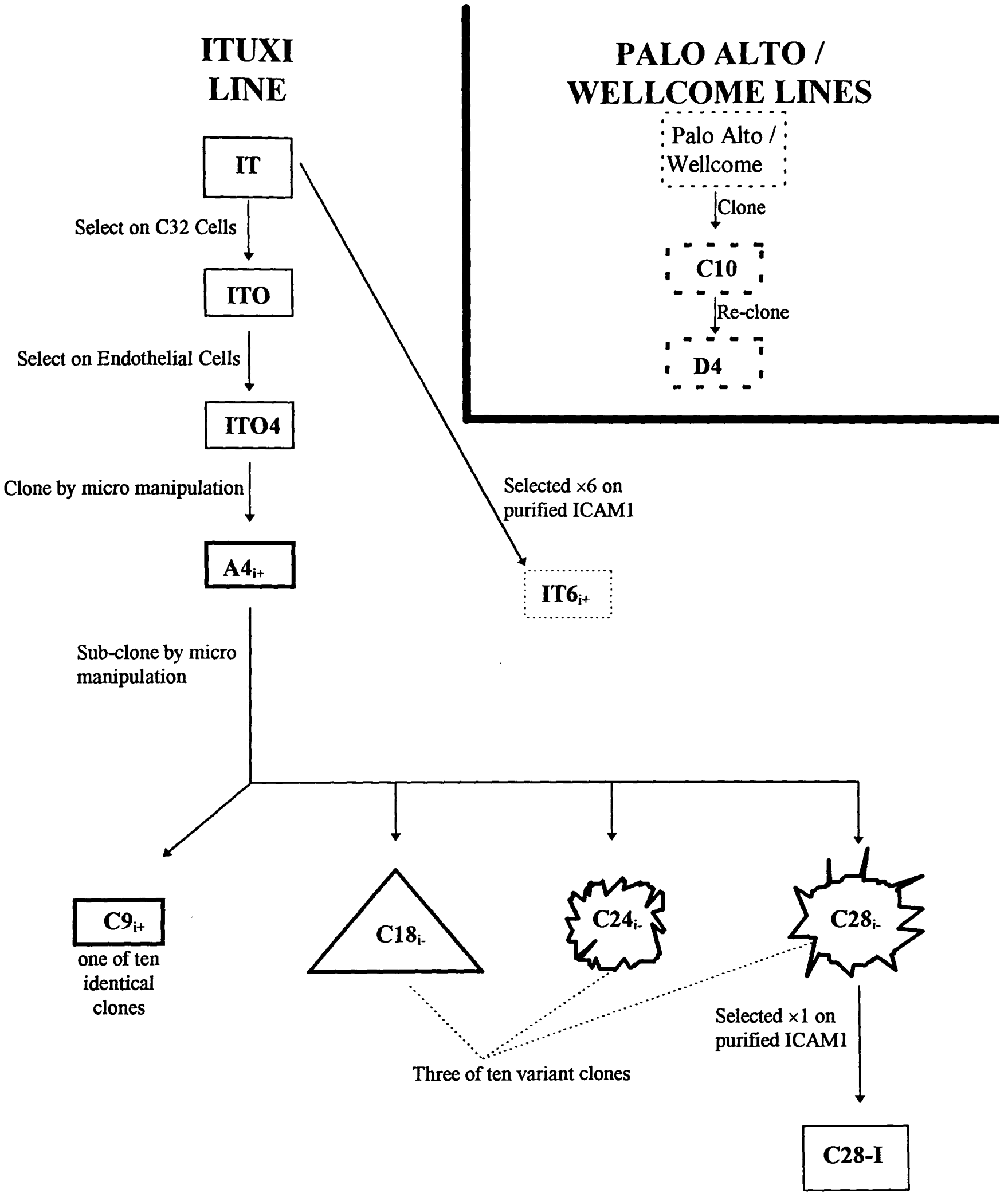


Figure 2.1 Tree diagram showing the derivation and antigenic relationships between parasites. The Ituxi line and Palo Alto / Wellcome lines are separate clone families and no cross-contamination had occurred (Dr. I. Wilson, personal communication). Cloned parasites are shown in heavy boxes and the shape of the box indicates the antigenic phenotype of the cultures that are relevant to this study. A dashed box indicates that the antigenic phenotype was unknown.

2.2.2 Growth of Parasites

2.2.2.1 Routine Culture

Parasites were grown in sterile conditions that mimicked a physiological post-capillary venular environment. The following techniques represent methods that have been established in our tissue culture laboratory (safety category II) for over ten years and are modifications of published methods (Trager and Jenson, 1976; Haynes *et al.*, 1976).

Cultures were maintained at low oxygen tension in a mixture of 96% N_2, 3% CO_2, 1% O_2 in sealed, flat-bottomed flasks in an incubator at 37°C. All manipulations were performed using aseptic techniques in a laminar flow hood that had been cleaned regularly by wiping the surfaces with 70% industrial methylated spirits (IMS) in water. Clones were handled individually in the hoods to eliminate cross-contamination by aerosols and solutions.

A synchronised *P. falciparum* culture requires fresh media and red cells every 48 hours or when it has passed through schizogony. To ensure optimal merozoite reinvasion and growth of cultures, the volume of medium was adjusted so that the depth did not exceed 1cm in the flask. The approximate volumes of media and red blood cells required during each cycle are based on the empirical observation that asexual growth was impaired at parasite concentrations $>2\times10^8$ cells ml^{-1}. Thus:

medium required (ml) = 5 × parasitaemia (%) × packed cell volume (ml)

A five-fold multiplication rate was assumed for *in vitro* growth of *P. falciparum* and cultures were routinely maintained at 5 - 10% parasitaemia. Thus, if a culture of trophozoites had been diluted with fresh red blood cells to 2% parasitaemia, 1ml of such a culture could be maintained for 48 hours in 100ml of medium in a 150cm^2 flask and be expected to produce a final parasitaemia of approximately 10% trophozoites in the following cycle.

Media and Red Cells

Media were prepared from stock solutions that had been sterilised by autoclaving or by passing through a 0.22μm filter. All solutions were stored at 4°C and warmed to 37°C in a water bath before use.

- **RPMI-S**

  RPMI 1640 medium was supplemented with 37.5mM HEPES, 20mM D-glucose, 2mM L-glutamine, 25μg ml^{-1} gentamicin sulphate and adjusted to pH 7.2 with 1M NaOH.

- **RPMI+S**

  RPMI-S supplemented with 8-10% (v/v) pooled human serum. Serum for use in cultures was derived from pools of undefined blood groups from at least six healthy individuals and stored at -20°C in aliquots. RPMI+S was used within one month of preparation. Separate solutions were used to avoid cross-contamination when different parasites were cultured simultaneously.

- **Red Cells**

  Group O blood was collected into Acid-Citrate-Dextrose (15% v/v) and centrifuged through an equivolume of Lymphprep™ at 1500*g* for 15 minutes. Erythrocytes separated to the bottom of the preparation and white cells and plasma were aspirated from the top. The red cell pellet was washed twice in RPMI-S after sequential centrifugation at 1500*g* for 5 minutes and resuspended to 25% haematocrit in RPMI-S. Red cells were stored at 4°C for up to one month before white cells and plasma were removed or used within two weeks after purification.

Cryopreservation

Long-term storage of viable parasites was achieved by cryopreservation in liquid nitrogen at -196°C following the methods of Haynes and colleagues (1976). Mature parasites were lysed by the thawing procedure and ring-stage parasites were observed in smears of cultures immediately post-thaw.

- **Freezing Parasites**

  The culture was centrifuged at 1000g for 5 minutes and the supernatant removed. The pellet was loosened and glycerolyte (57.0g of glycerol, 1.60g of Na lactate, 0.03g of KCl, 1.38g of $Na_2H_2PO_4$ in 100ml ddH_2O, pH 6.8 with 1M NaOH) added dropwise through a 25 gauge needle. A total of 5 volumes of glycerolyte was added to 3 volumes of packed cells, having allowed the cells to equilibrate for 5 minutes after addition of the first volume. Stabilates were made by pipetting 1ml aliquots into screw-top freezing vials which were quickly stored at -80°C in a polystyrene holder for 24 hours to minimise the potentially deleterious temperature gradient. Vials were transferred to indexed boxes in cylinders containing liquid nitrogen.

- **Thawing Stabilates**

  Stabilates were thawed rapidly at 37°C and transferred to 50ml centrifuge tubes. Cells were washed by the sequential addition of saline solutions with decreasing osmolarity: 0.2ml of 12% (w/v) NaCl was added dropwise through a 25 gauge needle while agitating the suspension. The cells were allowed to equilibrate for 5 minutes before the dropwise addition of 10ml of 1.8% (w/v) NaCl through a 25 gauge needle. The mixture was continually agitated during this process. A further 10ml of 0.9% (w/v) NaCl / 0.2% (w/v) glucose was added dropwise through a 25 gauge needle. After centrifugation at 900*g* for 5 minutes, the supernatant was removed and the cells were washed in 20ml of RPMI-S. The final supernatant was removed and the cells cultured as described.

Synchronisation

Cultures were synchronised to facilitate the measurement of stage-dependent properties and prevent the development of unwanted phenotypes (eg. K^-). When comparative experiments between different parasites were performed, cultures were grown in the same red cells and serum pool. Synchronous ring-stage parasites were produced by sorbitol lysis (Lambros and Van der Berg, 1979), and K^+ trophozoites were enriched by gelatin flotation (Pasvol *et al.*, 1978).

- **Sorbitol Lysis**

  Older parasites can be destroyed by osmotic lysis because the infected red cell membrane becomes permeable to a range of low molecular weight compounds (Sherman, 1985). Young, ring stage parasites and uninfected cells remain after resuspension of a cell pellet in 5% (w/v) sorbitol at 20% haematocrit for 15 minutes at 37°C. Trophozoite debris was removed by two washes in RPMI-S and the cells returned to standard culture conditions.

- **Gelatin Flotation**

  Changes in the red cell membrane during parasite growth result in differential sedimentation coefficients of infected cells. Trophozoites float in a gelatin solution but young parasites and uninfected cells form rouleaux and settle into a pellet under gravity. To effect this, a culture at 50% haematocrit in RPMI-S was mixed with an equivolume of gelatin (Plasmagel™) and the mixture incubated at 37°C under 1g until a visible pellet had formed. The purified trophozoite suspension was removed and washed once in RPMI-S. A ten-fold enrichment of trophozoites was normally achieved, resulting in a parasitaemia of 85-98%.

- **Percoll Flotation**

  Mature knob-negative infected erythrocytes (from D4 or D4-I) sediment with uninfected red cells when treated with Plasmagel™. Therefore, these parasites were enriched by layering cells at 15% haematocrit in RPMI-S over a prewarmed 65% (v/v) percoll (colloidal PVP coated silica) solution in RPMI-S. The tube was centrifuged (1500*g*, 10 minutes) at 37°C and the enriched trophozoites washed twice in RPMI-S before further manipulation.

2.2.2.2 Determination of Parasitaemia

Estimates of the parasitaemia (% of infected cells) and stage distribution of cultures were done by counting Giemsa-stained parasites in a thin-film blood smear examined with a light microscope.

Approximately 5µl of packed cells at 50% haematocrit were removed from the culture and smeared in a thin film on a glass microscope slide. The sample was air-dried, fixed with methanol for 5 seconds and stained with Giemsa's staining solution (Giemsa's stain (10% v/v) in 21mM Na_2HPO_4, 4.4mM KH_2PO_4, at pH 7.35 filtered through a 0.22µm filter) for >20 minutes. The stain was washed off with tap water, the slide dried and a light microscope used to examine the smear. A high-power objective (×100 magnification) and oil immersion were essential to distinguish between young ring-forms, pigmented trophozoites and dividing schizonts. During routine culture, the parasitaemia was determined from counts of at least 500 cells and >50 infected cells were counted in duplicate smears for statistical purposes.

Thick films were occasionally prepared during the initial cycles of growth immediately post-selection in order to detect low (<0.1%) parasitaemias. A circular, translucent smear was made by pipetting 10µl of packed cells onto a glass slide. The cells were not fixed, but air-dried and stained directly with Giemsa's staining solution as above. This method was more sensitive than thin films because the density of applied cells was increased. They were visible because plasma membranes were destroyed and only 'naked' parasites remained. An estimation of parasitaemia was not attempted and thick films were only employed to determine if there were any parasites in the culture (>1 parasite in 50 high-power fields). Parallel thin films were examined to ensure that the culture was not contaminated.

2.2.2.3 Determination of Haematocrit

A Coulter counter™ was used to determine the haematocrit (combined concentration of uninfected and infected red blood cells) of a culture. This instrument detected particles in a suspension by the alteration of electrical resistance as a stream of particles passed through a circular electrode. 10µl of a well-resuspended sample of the culture was diluted in 990µl of Isoton II™ diluent and mixed vigorously. 100µl of this solution was added to 10ml of Isoton II™ in a Coulter vial™ and mixed 5 times by inversion. The number of cells in 0.5ml of the diluted suspension was counted by the machine and automatically corrected for the coincidental passage of red cells. Triplicate

measurements of two test and two control samples (Isoton II™ alone) were made. The counter was calibrated regularly with a standard solution of fixed chicken erythrocytes supplied by the manufacturer and the cell concentration calculated by the equation:

cell concentration (cells ml^{-1}) = (test mean - control mean) × (1/0.01) × (10.1/0.1) × 2

For the purposes of this study, it was assumed that 1µl of packed erythrocytes contained 10^7 cells.

2.2.3 Electron Microscopy

Transmission electron microsopy was used to investigate the morphology of fixed parasites. This method gave superior resolution to light microscopy and could reveal intracellular structures and parasite-induced membrane modifications. 10-50µl of cells in an Eppendorf™ tube were washed twice in RPMI-S and 1ml of osmotically balanced fixative (2.5% (v/v) glutaraldehyde (EM Grade) in 0.1% cacodylate buffer) was added. Cells were allowed to settle under 1*g* at 4°C for >24 hours and prepared for examination in the Electron Microscopy Laboratory, John Radcliffe Hospital, Oxford. All subsequent manipulations and photography were carried out by Dr. D. J. Ferguson.

2.2.4 Protease Digestion and Inhibition

A range of proteases and neuraminidases was used in this study, and protocols were based on published methods (Leech *et al.*, 1984). Table 2.3 summarises the conditions required for proteolysis and inhibition. All digestions were carried out at approximately 10% haematocrit in Eppendorf™ tubes. For agglutination and binding assays (sections 2.2.6 and 2.2.9), 1.0 - 5.0 × 10^9 cells at 2 - 10% parasitaemia were used, and 2.0 - 4.0 × 10^8 cells at 85-98% parasitaemia were employed after ^{125}I surface labelling (section 2.2.14). Cells were washed twice in RPMI-S and proteases were used at equivalent activities. 1mg trypsin was equal to 10U of other enzymes (1 U = 1 chromozyme unit†, which is equivalent to 140 BAEE units‡). Cell pellets were washed three times in RPMI-S (1000*g*, 5 seconds) before further use, and post-digestion supernatants from the first wash were transferred to clean tubes which were centrifuged at 10000*g* for 10 minutes to remove membrane debris.

---

† 1 chromozyme unit (U) is the enzyme activity that liberates amino acids from casein in 1 minute at 37°C and pH 7.5.

‡ 1 BAEE unit is enzyme activity with benzoyl-L-arginine ethylester (BAEE) as a substrate and is used in the case of trypsin.

ENZYME	Digestion Conditions			Inhibition Conditions	
	Time (min)	pH	Temp (°C)	Inhibitor	Time (min)
Chymotrypsin[1]	5	7.2	25-28	0.1mM TPCK in 1ml of RPMI-S	5
Dispase	5	7.2	25-28	800µl 1mM EDTA in 1ml of PBS	5
Neuraminidase	60[2]	6.0[3]	37	equivolume RPMI-S, 2 washes	5
Trypsin	5	7.2	25-28	equivolume 2mg ml^{-1} trypsin inhibitor in RPMI-S	5
V8 protease	5	7.8	25-28	5µl of 500mM DFP in isopropanol	5

Table 2.3. Summary of digestion and inhibition conditions used in this study.

2.2.5 Purification of Proteins

2.2.5.1 CD36

CD36 was isolated from outdated human platelets after solubilisation of the membrane proteins in Triton X-100, followed by phase-partition in Triton X-114, ion exchange chromatography and affinity chromatography with a wheat germ agglutinin-sepharose column (Tandon *et al.*, 1989; gift of Dr. C. I. Newbold). Purified CD36 at 4µg ml^{-1} was stored at 4°C in PBS (Phosphate buffered saline) / 0.1%(v/v) Triton X-100 / 0.02%(w/v) sodium azide, and contained no contaminating ICAM1 or TSP activity (Dr. G. D. H. Turner, personal communication).

2.2.5.2 ICAM1

An ICAM1 F_c chimaera composed of five, three, or two domains of ICAM1 F_c linked to three domains of the IgG F_c fragment was prepared by transient expression in COS cells (Berendt *et al.*, 1992). The ICAM1 F_c was absorbed onto a protein A-sepharose column and purified by a two-stage, pH dependent elution step. Detailed results of isolation procedures are given in chapter 4.

---

[1] Chymotrypsin was pretreated with 0.2mM TLCK to inhibit any contaminating trypsin activity.

[2] Cells were resuspended every 15 minutes.

[3] 0.25mM MES replaced HEPES to maintain the buffering capacity of RPMI-S.

2.2.5.3 TSP

Calcium-replete thrombospondin (TSP) at 0.85mg ml^{-1} was purified from fresh human platelets by the method of Haverstick *et al.*, 1984 (gift of Dr. J. McGregor, Lyons, France). TSP was stored at 4°C in 50mM TBS / 5mM $CaCl_2$ (TBS: Tris-buffered saline; 137mM NaCl, 2.7mM KCl, 0.5mM $MgCl_2$, pH 7.8). There was a single 180kDa band visible on 10% SDS-PAGE gels after Coomassie blue staining, and no 88kDa band was observed. There was no contaminating CD36 or ICAM1 activity as assayed by western blotting (Dr. G. D. H. Turner, personal communication).

2.2.6 Determination of Cytoadherent Phenotypes: Binding Assays

The cytoadherent phenotype of a culture was assessed by incubating infected cells in Petri dishes with radially arranged spots of purified receptor proteins which had been adsorbed onto the plastic surface (Roberts *et al.*, 1992).

Solutions of CD36 (12.5ng ml^{-1}), ICAM1-F_C (25μg ml^{-1}) or TSP (40μg ml^{-1}) were adsorbed as individual 3μl spots arranged radially on to 6cm diameter plastic dishes (Falcon no. 1007) in a moist atmosphere at 37°C for 2 hours. Triplicate spots of each protein were used in duplicate dishes in at least two separate experiments for each determination. The spots were aspirated and the dishes blocked with PBS / 0.02%(w/v) sodium azide / 2% BSA for 2 hours at 37°C to prevent non-specific adherence. The blocking solution was replaced with 5mM $CaCl_2$ / 50mM TBS / 0.02%(w/v) sodium azide / 2% BSA, pH 7.8 when TSP was used. This solution was aspirated and the dishes were washed four times in sterile binding medium (RPMI 1640 without added $NaHCO_3$, supplemented with 16mM D-glucose, 37.5mM HEPES at pH 7.0 with NaOH). The parasitaemia(s) of the test culture(s) were determined by counting 1000 erythrocytes in a Giemsa-stained thin film. The cell concentration of the culture and of a suspension of washed, uninfected erythrocytes was measured using a Coulter counter™. Sufficient cells were removed from the suspensions of infected and uninfected cells to produce 1.25ml suspension of erythrocytes at 1 - 2% haematocrit and 2 - 10% parasitaemia per binding assay dish (cytoadherence to the three receptors was linear in these ranges, see chapter 3).

The final parasitaemia of the suspension was enumerated from a count of >50 infected cells in a thin film and the final haematocrit determined as in section 2.2.2.3.

The dishes were incubated at 37°C for 30 minutes with a constant gentle rocking motion in a hybridisation machine (Boekel Industries, model number 136500). Unbound cells were removed by carefully washing at least five times with binding medium. The same number of washes was performed for each dish and repeated until cells had been removed from areas of plastic where no receptor had been added. The adherent PRBC were fixed with 1% (v/v) glutaraldehyde (grade II) in binding medium for 2 hours at room temperature and stained for 30 minutes with filtered 10%(v/v) Giemsa's staining solution. The stain was rinsed by dip-washing in a beaker of tap water and the dishes were dried in air. The number of parasites bound in 10 low power (×10 objective) grids was counted using a light microscope. This number was converted to cells mm^{-2} using a scaling factor (area of one low power grid = $0.4mm^{-2}$) and the ×100 objective with oil immersion was used to confirm that uninfected cells had not bound to purified proteins under any condition. Background binding was assessed by counting one low power field from an area of each dish without spotted protein. The same microscope was used for all counting, and the cytoadherent phenotype of a culture was measured on at least two different days.

Reproducible binding was achieved after ensuring that the protein spots did not become dry during the washing steps and that resuspensions and washes were performed gently. Inter-dish variation was minimised by adding and removing wash suspensions from the same point on each dish and by counting parasites bound to the same area of each spot. Intra-dish variation was reduced by using the hybridisation machine which was more efficient for resuspensions than the belly dancer™ or manual resuspensions, and full results are presented in chapter 3.

2.2.7 Selection of Parasites for Cytoadherence Phenotypes: Panning

Parasites that could bind to CD36 or to ICAM1 were selected from cultures by panning on Petri dishes coated with the purified receptor in sterile conditions using a modification of the method of Roberts *et al.* (1992).

6cm diameter bacteriological plastic dishes (Falcon no. 1007) were coated with receptor protein by pipetting 2.4ml of CD36 (12.5 ng ml^{-1} in PBS / 2.5μg ml^{-1} gentamicin sulphate) or 3.0ml of ICAM1 F_c (10μg ml^{-1} in PBS / 2.5μg ml^{-1} gentamicin sulphate) onto the dishes and incubating at 37°C in a moist atmosphere for 2 hours. The protein solution was removed by aspiration and the dishes blocked by the addition of 3.0ml of PBS / 2%(v/v) BSA / 2.5μg ml^{-1} gentamicin sulphate / 0.02%(w/v) sodium azide for 2 hours at 37°C. The blocking solution was removed and the dishes washed four times with binding medium. 1.25ml of a suspension of trophozoites at 85-95% parasitaemia and 2% haematocrit in binding medium was added to each dish which was incubated at 37°C for 30 minutes with constant rocking in the hybridisation machine. The unbound parasites were gently washed off 5 times in binding medium and the efficiency of each washing step was monitored by viewing the surface of the dishes with a inverted phase-contrast microscope. The adherent parasites were removed by rinsing the dishes with binding medium under strong pressure from a pasteur pipette and cultured as described above in RPMI-S / 10%(v/v) complement-inactivated human serum. Infected cells were cryopreserved when the parasitaemia of the culture was >1%.

2.2.8 Fluorescence Microscopy

DNA-chelating dyes were used to selectively stain mature parasites which were observed at ×40 magnification in a wet film with an immunofluorescence microscope.

PRBC at trophozoite stage between 2 - 10% parasitaemia were washed twice in RPMI-S and resuspended in Eppendorf™ tubes at approximately 10% haematocrit in RPMI-S. EtBr (10μg ml^{-1} final concentration from 10mg ml^{-1} stock solution in ddH_2O) or DAPI (20μg ml^{-1} final concentration from 1mg ml^{-1} stock solution in DMSO) was added and the suspension mixed 5 times by inversion. After incubation at 37°C for 10 minutes, the cells were washed three times in RPMI-S.

For examination by indirect immunofluorescence, 2µl of a stained suspension was pipetted onto a single spot on a multi-spot slide which was covered by a greased coverslip to form a wet film. A Leitz epifluorescence microscope with a G 365 excitation filter, an FT 395 beam splitter and an LP 420 barrier filter was able to detect the emission spectra of DAPI and EtBr, permitting the analysis of single- or double-stained test cultures.

2.2.9 Antibody-Mediated Agglutination

Antigenic 'agglutinins' expressed at the surface of mature infected erythrocytes can be recognised by antibodies in sera from immune patients from malaria-endemic areas (Hommel *et al.*, 1982). Variant-specific antibodies cross-react with the surface of parasites and parasites that express identical agglutinins form clumps of infected cells known as agglutinates. The agglutination assay was based on the published protocol of Newbold and colleagues (1992) and provided a quantitative measurement of antigenicity. Pooled adult endemic area serum was obtained from ten 'immune' Gambian adults living in Sibanor, and termed 'Sib serum'. The pool was diluted 1:1 with RPMI 1640, complement inactivated by heating at 56°C for 30 minutes and stored in aliquots at -20°C. Human non-immune control serum was obtained from a pool of serum donations from random, healthy adults, not exposed to malaria and living in Europe. The control serum pool was referred to as 'Eur serum' and was stored in aliquots at -20°C.

PRBC at 5 - 10% parasitaemia were labelled with EtBr as described in section 2.2.8, and resuspended in the appropriate serum dilution in Eppendorf™ tubes. Mixing was carried out either on a rotator for 30 - 90 minutes where a minimum volume of 150µl at 10% haematocrit was necessary or on a shaker for 15 - 60 minutes where a final volume of 50µl at 20% haematocrit was used. 2µl of the suspension was pipetted onto duplicate spots on a multi-spot slide which was covered with a greased coverslip and examined as described in section 2.2.8.

1000 stained cells were counted in each spot from two separate experiments, and the percentage of infected cells in agglutinates was calculated. The number of agglutinates in defined size categories was also scored to give an indication of the size distribution of agglutinates in the sample. Agglutinate size categories were 3-5, 6-10, 11-20 and >20 infected cells per agglutinate. It was difficult to

enumerate accurately the number of parasites in giant agglutinates (>50 cells) due to their three dimensional nature, and an approximation was made in these cases.

2.2.10 Determination of Antigenic Phenotypes: Mixed Agglutination Assay

The antigenic similarity of intact parasitised cells was compared using the Mixed Agglutination Assay (Newbold *et al.*, 1992). This method allowed a semi-quantitative assessment of the relative antigenic phenotype of a culture. Two cultures of parasites were labelled independently with either EtBr or DAPI as described in section 2.2.8 and mixed in Sib serum at 1:4 in RPMI-S. Cells were mixed, sampled and examined as described in section 2.2.9 and agglutinates were scored for whether they contained all red cells (stained with EtBr), all blue cells (stained with DAPI) or mixture of red cells and blue cells. The criterion for mixed-colour agglutinates was that an intact parasitised cell of the second colour was clearly visible and emeshed within the primary agglutinate. 100 agglutinates of 5-10 cells were counted in at least two separate experiments for each pair of samples tested.

The proportion of mixed colour agglutinates was an indication of the degree of similarity of the antigenic phenotype expressed at the cell surface. Parasites with identical antigenic phenotypes have >90% mixed colour agglutinates as predicted by a binomial distribution (Roberts *et al.*, 1992). If the probability of homologous agglutination is p for an agglutinate of size n, then the probability of an agglutinate being a single colour is p^{n-1} and the proportion of mixed agglutinates is $1-p^{n-1}$.

Equal numbers of parasites from the test cultures were used and the degree of similarity between them is the ratio of chance of heterologous agglutination to the chance of homologous agglutination, or (1-p) / p. Assuming that all agglutinates consisted of 5 cells, and cultures were antigenically indistinguishable (p=0.5), the proportion of mixed agglutinates would be 0.94 and the degree of similarity would be 1. The degree of similarity of the test cultures was categorised into a range based on the percentage of mixed agglutinates formed in the test. Table 2.4 shows the ranges used in this study, which were identical to those used in previous investigations (Roberts *et al.*, 1992).

% Mixed Agglutinates	% Antigenic Similarity
0-20	0-5
21-40	6-14
41-90	15-78
91-100	>79

Table 2.4. Ranges used for estimating the antigenic similarity of two cultures from results of the mixed agglutination assay.

2.2.11 Purification of antibody from polyclonal serum

Antibodies from polyclonal sera recognise multiple antigenic phenotypes expressed at the surface of infected erythrocytes (Newbold *et al.*, 1992). Bound antibodies were eluted from the surface of cloned parasites to enrich variant-specific antibodies from adult endemic Sib serum (Marsh and Howard, 1986).

2.2.11.1 Preparation of triple layers

200µl of 40%(v/v) percoll in RPMI-S was pipetted into multiple Eppendorf™ tubes followed by 500µl of dibutyl phthalate. 500µl of 0.1M glycine (pH 3.0 with HCl) / 0.1M NaCl was carefully added to form the top layer. The tubes were centrifuged at 10000*g* for 30 seconds to ensure that the layers had separated and then equilibrated on ice for >2 hours. Occasionally, some preparations were discarded due to the uneven formation of the triple layers.

2.2.11.2 Elution of antibody

Plasmagel™ purified trophozoites were washed twice in RPMI-S and resuspended in Eppendorf™ tubes in an equivolume of Sib serum for 20 minutes at 37°C. The tubes were centrifuged gently (1000*g*, 1 minute), and the pellets were washed four times in RPMI-S. 30 - 40µl of the pellet was quickly added to the top layer of each triple-layered elution tube on ice and mixed thoroughly with the glycine by resuspension through the pipette tip. The tubes were centrifuged at 10000*g* for 30 seconds and 10µl of 1M NaOH added to each top layer and mixed carefully on ice. The top layers were removed into a clean vial and dialysed overnight in 500ml of sterile PBS / 0.05%(w/v) sodium azide with continuous stirring at 4°C. The dialysed antibody was transferred into the top compartment of a Centricon tube with a 30kDa molecular filter which was centrifuged at 1500*g* for >90 minutes at 4°C.

The concentrated antibody solution was collected from above the filter and stored at 4°C in a clean vial.

2.2.12 Rosetting

An infected cell was considered to form a rosette if it attached to two or more uninfected red cells immediately after removal from the culture (Rowe *et al.*, 1994). Trophozoites were stained with EtBr and examined in a wet film by immunofluorescent microscopy (section 2.2.8). The percentage of rosetting parasites was measured by counting 500 parasites in triplicate samples.

2.2.13 Transport Experiments

Unidirectional influx across the infected erythrocyte plasma membrane was studied by following the uptake of ^{14}C-labelled choline chloride into *P. falciparum* infected erythrocytes (Kirk *et al.*, 1991). Fluxes were measured at 37°C both in the presence and absence of furosemide, an effective inhibitor of parasite-induced choline transport (Kirk *et al.*, 1994).

Mature trophozoites at >90% parasitaemia were washed three times in 'malaria saline' (MS; 125mM NaCl / 5mM KCl / 25mM HEPES / 5mM glucose, pH 7.4) by centrifugation (5 min × 1000*g*) and resuspended in 1ml of MS in a single microcentrifuge tube at 37°C and allowed to equilibrate for >10 minutes. Cells were aliquotted into duplicate tubes with either furosemide (in DMSO) or DMSO controls. The flux commenced with the addition of ^{14}C choline chloride with unlabelled choline chloride such that the final volume was 0.5ml, the final choline concentration was 1mM, and the activity was ~2 μC_i ml^{-1}. After thorough mixing the tubes were left for 12 minutes. To terminate the flux, 110µl aliquots of the suspension were removed and transferred to triplicate microcentrifuge tubes containing 0.75ml of ice-cold "stopping solution" (MS supplemented with 100µM furosemide) layered over 0.5ml dibutylphthalate. These tubes were immediately centrifuged (30 sec × 10000*g*) to sediment the cells below the oil.

The aqueous supernatant solution was removed by aspiration, and the radioactivity remaining on the walls of the tubes was removed by rinsing three times in ddH_2O. Any residual water droplets were removed from the tube and the lid, then the dibutylphthalate was aspirated. The cell pellet was lysed by mixing vigorously with 0.5ml of 0.1% (v/v) Triton X-100 and deproteinised by the addition of

0.5ml of 5.0% (w/v) trichloacetic acid followed by centrifugation (10 min × 10000*g*). Radioactivity was measured using a β-scintillation counter. The amount of radiolabel trapped in the extracellular space between the cell pellets was estimated from "zero-time" samples which were taken within a few seconds of combining the cells and radiolabelled substrate.

2.2.14 ^{125}I Surface Labelling

Intact erythrocytes infected with mature forms of *P. falciparum* were surface labelled by the lactoperoxidse-catalysed addition of radiolabelled iodide (Leech *et al.*, 1984). This method relies on the production of an electrophilic iodine species ($^{125}I^{+}$) from sodium iodide using oxidative conditions. The radioactive iodine can react with any aromatic moiety present on the surface of cells. The protocol used in this study tagged surface proteins on accessible tyrosine residues and was a modification of the published technique (Howard *et al.*, 1982), although little is known about the extent of labelling at histidine and tryptophan.

Cultures of young infected erythrocytes were synchronised by sorbitol lysis and immediately subjected to Plasmagel™ flotation to remove trophozoites, broken parasites and membrane debris. Trophozoites were enriched 24 hours later by a second treatment with Plasmagel™. Uninfected erythrocytes from the same batch of red cells were cultured for 48 hours in the same RPMI+S medium and incubated with Plasmagel™ before labelling. Purified trophozoites (85-98% parasitemia) were resuspended in RPMI+S at approximately 0.1% haematocrit and maintained at low oxygen tension in tissue culture flasks to allow recovery. A thin film was made prior to labelling to ensure the absence of dividing schizonts and ruptured cells.

$2.0 - 4.0 \times 10^{8}$ cells were washed twice in RPMI-S and twice in PBS. The cells were resuspended in 0.5ml PBS in Eppendorf™ tubes and 5μl of 10μM KI added. 1.2 Units of lactoperoxidase (EC 1.11.1.17) and 5μl of 100 $mC_i\,ml^{-1}$ sodium ^{125}iodide were added to each tube. 6μl of 0.03%(v/v) hydrogen peroxide (prepared daily) was added at time zero and the contents of the Eppendorf™ tube mixed vigorously by inversion to initiate the reaction. 3μl additions of 0.03% hydrogen peroxide were made at one minute intervals for five minutes, and the suspension mixed thoroughly each time. The reaction was quenched by the addition of a reducing agent (0.5ml of PBS / 50μM $Na_2S_2O_3$) after 6

minutes and the suspension transferred to clean Eppendorf™ tubes and pelleted in a microfuge (1000*g*, 5 seconds). The cells were washed twice in 1ml of PBS / 10mM KI and either digested with protease or extracted by detergent.

2.2.15 ^{35}S Metabolic Labelling

Parasites are metabolically active and use ^{35}S labelled amino acids for the biosynthesis of new proteins (Howard, 1988). Two protocols were employed that were modifications of the method of Howard *et al.*, 1983 using a cocktail of ^{35}S-methionine and ^{35}S-cysteine to maximise the number of labelled parasite proteins.

2.2.15.1 Labelling of trophozoites

20µl of Plasmagel™-enriched trophozoites at >90% parasitaemia were resuspended in 20ml of methionine-free and cysteine-free culture media without serum (MEM-S: Minimal Essential Medium supplemented with 2.0 g l^{-1} D-glucose, 292 mg l^{-1} glutamine, 2 mg l^{-1} i-inositol, 126.4 mg l^{-1} arginine, 52.5 mg l^{-1} leucine) for 20 minutes. The cells were transferred to 10ml of MEM-S supplemented with 2%(v/v) human serum (MEM+S) and 0.5 mC_i (25μC_i ml^{-1}) of L-[^{35}S]-*in vitro* cell labelling mix (>65% methionine, >25% cysteine, <10% impurities (leucine, isoleucine)) (Amersham International). The mixture was cultured at low oxygen tension for 2 hours. The cells were washed twice in MEM-S and resuspended in MEM-S at low oxygen tension for 10 minutes. After two further washes in MEM-S, infected cells were harvested and labelled proteins were extracted with detergents as described in section 2.2.16.

2.2.15.2 Dual labelling of rings

The exact time of onset of protein synthesis of *P. falciparum* infected erythrocytes is unknown. In order to increase the number of labelled proteins, cultures were incubated with ^{35}S labelled amino acids for two periods during the erythrocytic cycle.

100µl of synchronous young ring stage infected cells between 10-20% parasitaemia were resuspended in 20ml of MEM-S for 20 minutes. The cells were transferred to 10ml MEM+S / 0.5mC_i (25μC_i ml^{-1}) of L-[^{35}S]-*in vitro* cell labelling mix and the mixture cultured at low oxygen tension for 2 hours. The radioactive cell supernatant was removed after centrifugation of the culture at 1000*g* for 5 minutes

and stored at 37°C. The cells were resuspended in RPMI+S for 5 hours to allow the cells to recover. After two washes in MEM-S, the culture was incubated at low oxygen tension for 20 minutes in the same media to starve. The cells were resuspended in the recovered radioactive cell supernatant for 2 hours at low oxygen tension for a second-labelling pulse. The cells were transferred to RPMI+S and allowed to mature into trophozoites and purified as described earlier.

2.2.16 Detergent Extraction

Infected red cell membrane proteins are differentially soluble in low concentrations of Triton X-100 probably by virtue of their mode of attachment in the lipid bilayer. Triton soluble (TS) proteins were purified from membranes by an initial treatment with Triton X-100 and the Triton X-100 insoluble (TI) proteins were extracted by an extra treatment with SDS.

2.0 - 4.0×10^8 of ^{125}I-labelled cells were extracted with 0.5ml NET (150mM sodium chloride / 5mM EDTA / 50mM Tris, pH 8.0) with 1%(w/v) TX-100 in the presence of protease inhibitors (0.2mM N-tosyl-L-lysine-chloromethyl-ketone (TLCK), 0.1mM N-tosyl-L-phenylalanine-chloromethyl-ketone (TPCK), 1mM phenyl-methyl-sulphonyl-fluoride (PMSF), 10μg ml^{-1} each of antipain, aprotinin, leupeptin and chymostatin) on ice, followed by centrifugation (10000*g* for 5 minutes) to prepare a TS fraction.

The Triton-insoluble pellet was disrupted by drawing up and down through a yellow pipette tip in 20mM Tris / 150mM NaCl / 0.02%(w/v) sodium azide, pH 8.0 / 2%(w/v) sodium dodecylsulphate (SDS) and the suspension was solubilised and parasite DNA sheared by drawing up and down several times through a 25 gauge needle with a 1ml syringe until a dark, homogeneous suspension resulted. The samples were centrifuged at 10000*g* for 5 minutes to prepare the Triton-insoluble supernatant.

Aliquots of the TS and TI proteins were stored at -20°C and used for SDS-PAGE or immunoprecipitation experiments.

2.2.17 Immunoprecipitation

Radiolabelled proteins were precipitated by antibodies bound to protein A-sepharose beads after prior removal of non-specific protein A-binding proteins. The methods used in this study were modified from the published protocols of Van Schravendijk *et al.* (1993).

25 - 50µl of ^{125}I- or ^{35}S- labelled detergent extracts of cells or supernatants was precleared by incubation in 0.5ml of NET / 1% (v/v) TX-100 with 10µl of protein-A sepharose beads (pre-swollen overnight at 4°C in NET / 0.1%(w/v) sodium azide) for 2 hours on a rotator (10 rpm) at 4°C. The percentage of TX-100 was reduced to 0.1% (v/v) in all manipulations where TS samples or membrane-free digestion supernatants were immunoprecipated. The precleared lysate was divided into two Eppendorf™ tubes, and each tube was incubated with 25µl of serum or 5µl of monoclonal antibody for 2 hours on a rotator at 4°C. 20-30µl of pre-swollen protein-A sepharose was added in 0.5ml NET / TX-100 and the tubes placed on the rotator overnight at 4°C. The beads were washed sequentially at 4°C in 1ml volumes of NET / TX-100, NET / 1%(v/v) BSA / TX-100, NET / TX-100, NET / 350mM sodium chloride / TX-100, 20mM Tris / 150mM sodium chloride, pH 8.0. The beads were transferred to new tubes, and the precipitated immunocomplexes were dissociated by boiling for 5 minutes in 50µl electrophoresis sample buffer and analysed by SDS-PAGE. The monoclonal antibodies to internal regions of Band 3 (Wainwright *et al.*, 1989; Wainwright *et al.*, 1990) were gifts from Dr. M. Tanner, Biochemistry Department, Bristol University, Bristol.

2.2.18 SDS PAGE

Proteins and peptides were separated by discontinuous SDS - polyacrylamide gel electrophoresis (SDS-PAGE) based on a modification of the method of Laemmli (1970). There were four separate stages to SDS-PAGE: preparation of gels, preparation of samples, separation of proteins, and visualisation of proteins:

2.2.18.1 Preparation of Gels

Gel solutions listed in table 2.5 were routinely used to prepare separating and stacking gels. Solutions A, B and C were stored in brown glass bottles at 4°C for up to 1 month. The volumes required for different percentage acrylamide gels are shown in table 2.6. Gels were assembled using commercial equipment in large (Hoeffer maxi gel apparatus) or mini (Bio-Rad mini-Protean II kits) sizes. Glass plates and teflon separating strips were cleaned thoroughly by wiping with IMS and assembled in a sealed stand to prevent leakage. Gels were cast with freshly prepared mixtures using clean, plastic syringes. After the separating gel had been poured, water-saturated butan-1-ol was carefully added to

cover the meniscus to prevent oxidation of the gel surface during polymerisation. When the gel had polymerised , the overlay was decanted and the upper portion washed 5 times with ddH_2O. The glass plates were dried with a piece of filter paper and the stacking solution added. A plastic comb was carefully placed into the un-polymerised stacking gel to form a series of parallel lanes on polymeristion.

STOCK SOLUTION	RECIPE
Solution A	1M HCl (48ml), Tris (36.3g), TEMED (0.23ml) Made up to 100ml with ddH_2O
Solution B	1M HCl (48ml), Tris (5.98g), TEMED (0.46ml) Made up to 100ml with ddH_2O
Solution C	Acrylamide (30g), Bis (0.4g) Made up to 100ml with ddH_2O and equilibrated over Amberlite MB-3 beads
10% SDS	10% SDS (w/v) in ddH_2O
10% APS	10% APS (w/v) in ddH_2O, prepared daily

Table 2.5. Stock solutions used to prepare acrylamide gels.

Type of Gel (%acrylamide (w/v))	Solution	Volume per large gel (15 × 15 × 0.15 cm)	Volume per mini-gel (7.5 × 7.5 × 0.075 cm)
Separating (5%)	Solution A	5.0ml	1.25ml
	Solution C	3.2ml	0.8ml
	ddH_2O	11.4ml	2.85ml
	10% SDS	0.2ml	0.05ml
	10% APS	0.2ml	0.05ml
Separating (10%)	Solution A	5.0ml	1.25ml
	Solution C	6.65ml	1.66ml
	ddH_2O	8.0ml	2.0ml
	10% SDS	0.2ml	0.05ml
	10% APS	0.2ml	0.05ml
Stacking (3%)	Solution B	1.9ml	0.48ml
	Solution C	2.5ml	0.63ml
	ddH_2O	10.0ml	2.5ml
	10% SDS	0.15ml	0.038ml
	10% APS	0.15ml	0.038ml

Table 2.6. Volumes of solutions used to prepare acrylamide gels.

2.2.18.2 Preparation of Samples

Protein samples were denatured before electrophoresis by the addition of an equivolume of freshly-prepared stock sample buffer (200µl of 10% SDS, 100µl of 1M Tris, 100µl of β-mercaptoethanol, 100µl of glycerol, 442µl ddH_2O and a few crystals of bromophenol blue). The closed, but ventilated tubes were placed in a boiling water bath for 5 minutes and precipitates removed by centrifugation at 10000*g* for 5 minutes.

2.2.18.3 Running Gels

The plastic comb was removed from the polymerised stacking gel and the wells filled with running buffer (3.0g of Tris, 14.4g of glycine, 1.0g of SDS in 1000ml of ddH_20 at pH 8.3) to remove air bubbles. Samples were loaded into the wells with fine-pointed flat pipettes to avoid cross-contamination by overflow. The complete gel was transferred to an electrophoresis tank and running buffer added to the top (cathode) and bottom (anode) reservoirs. Large gels were run at 60mA gel^{-1} and mini gels at 200V until the blue dye-front had reached the base of the gel.

2.2.18.4 Visualisation of Proteins

Two types of staining procedure were used to visualise separated proteins after the apparatus had been removed and the glass plates separated. After staining, all gels were transferred to Whatman 3MM filter paper, covered by Saranwrap™ and dried under a vacuum at 80°C for 2 hours.

2.2.18.4.1 Coomassie Stain

Gels were simultaneously fixed and stained by incubation for >1 hour on a belly dancer with at least 5 gel volumes of Coomassie staining solution (0.125%(w/v) Coomassie brilliant R-250 dye in 25%(v/v) propan-2-ol, 10%(v/v) glacial acetic acid). Background stain was removed by two or more 1 hour washes with destain solution (20%(v/v) methanol, 10%(v/v) glacial acetic acid). Occasionally the solutions were warmed to 37°C to reduce the wash times to 20 minutes. This method could detect protein at >10 ng mm^{-2}.

2.2.18.4.2 Silver Stain

When increased sensitivity was required, gels were stained using a modification of the method of Blum *et al.*, 1988 which is summarised in table 2.7. This method takes advantage of the affinity of

silver ions for nucleophilic groups in macromolecules in gels. Following the chemical reduction of the ions, an image of the electrophoretic band is produced. The sensitivity varies with type of protein, but 0.1ng nm^{-2} protein are routinely detected (Blum *et al*, 1988). All manipulations were carried out at room temperature in clean glassware using a belly dancer to ensure efficient mixing. Care was taken to ensure that gels were completely immersed in the solution and to avoid fingerprints and distortion of gels by folding.

Procedure (notes)	**Solution**	**Length × number of incubations**
Fixing (prevented diffusion of proteins)	50%(v/v) methanol, 12%(v/v) acetic acid, 0.5ml 37% paraformaldehyde in 1l ddH_2O	≥1 hour ×1
Wash (removed acetic acid which can interfere with next step)	50 %(v/v) ethanol	20 minutes ×3
Pretreatment (thiosulphate increased contrast)	0.02% (w/v) $Na_2S_2O_3.5H_2O$	exactly 1 minute ×1
Rinse (removed excess thiosulphate which would cause surface precipitation by Ag_2S)	ddH_2O	20 seconds ×3
Impregnate	0.2%(w/v) $AgNO_3$, 0.75ml 37% paraformaldehyde in 1l ddH_2O	20 minutes ×1
Rinse (removed excess silver nitrate which would saturate thiosulphate in next step)	ddH_2O	20 seconds ×3
Develop (appearance of protein bands)	fresh 6%(w/v) Na_2CO_3, 0.5ml 37% paraformaldehyde , $Na_2S_2O_3.5H_2O$ (4mg) in 1l ddH_2O	10 minutes, and replaced if solution turned brown
Wash (removed excess thiosulphate which would increase background)	ddH_2O	2 minutes ×2
Stop	50%(v/v) methanol, 12%(v/v) acetic acid	10 minutes ×1
Wash (removed acetic acid before drying)	50%(v/v) methanol	≥20 minutes ×1

Table 2.7. Summary of stages in visualising gels by silver staining

2.2.18.5 Sizing of Proteins

The molecular weight (M_r) of proteins was determined by comparison of relative mobilities against protein standards (table 2.8) which had been electrophoresed on the same gel. A calibration curve of R_f value (distance migrated for each protein / total distance of gel front) against log M_r was plotted on semi-log paper, and the R_f values for each of the unknown proteins was measured. The log (M_r) was determined from the curve and converted to M_r. The spectrin doublet (220kDa and 240kDa) was used as an additional internal standard for the accurate determination of high Mr proteins (>200kDa) from erythrocyte membrane preparations, when the SDS PAGE gels had been stained with Coomassie blue.

Rainbow Markers (^{14}C labelled; from Amersham) Broad Range		**Prestained Standards** (Unlabelled; from Bio-Rad) High Range		Low Range	
Myosin	200 000	Myosin	221 000	Phosphorylase B	107 000
Phosphorylase b	97 400	β-galactosidase	115 000	BSA	76 000
BSA	69 000	BSA	63 000	Ovalbumin	52 000
Ovalbumin	46 000	Ovalbumin	46 000	Carbonic anhydrase	36 800
Carbonic anhydrase	30 000			Trypsin inhibitor	27 200
Trypsin inhibitor	21 500			Lysosyme	19 000
Lysosyme	14 300				

Table 2.8. Protein standards used. The individual proteins and their M_r values are shown (units are Da), as quoted by the manufacturers on individual vials.

2.2.19 Autoradiography

Radiolabelled proteins on SDS-PAGE acrylamide gels were visualised by autoradiography. The dried gel was placed next to a sheet of X-ray film in a light-proof cassette. The sensitivity of the film to emitted radiation was increased by pre-flashing the film (Swanstrom and Shank, 1978) and placing between intensifying screens (Laskey and Mills, 1975). Exposure times ranged from 12 hours at -80°C for well-labelled gels to 3 months at room temperature for low activity gels. Films were developed with a X-O-graph varia-speed X-150 machine.

2.2.20 Peptide Labelling

The sparingly-soluble catalyst Iodogen™ (Pierce), which had been adsorbed onto the surface of 1.5 ml Eppendorf™ tubes, was used to label peptides with sodium ^{125}iodide. This was a quick and efficient (>60% incorporation) method for the incorporation of ^{125}I onto the aromatic ring of tyrosine residues of soluble peptides (Fraker and Speck, 1978). No reducing agent was required and the reaction was terminated by decantation of the solution.

Desiccated Iodogen™ was dissolved at 1mg ml^{-1} in chloroform in clean, dry glassware. 2μg of Iodogen™ was added to each Eppendorf tube in 50μl of chloroform after dilution of the stock solution to 40μg ml^{-1}. The solvent was evaporated carefully for approximately 5 minutes using a flow of gas so that the minimum surface area of the tube was "plated" with catalyst. 50μl of peptide in PBS was added to each coated tube with 0.2mC$_i$ of sodium ^{125}iodide for 10 minutes with constant agitation to aid diffusion. The tubes were centrifuged at 10000*g* for 10 seconds and the sample applied to pre-spun G-25 Sephadex columns (1ml plastic syringe without plunger and end-sealed with Teflon wool, filled with G-25 Sephadex and equilibrated with PBS / 10mM KI by centrifugation three times at 1000*g* for 2 minutes). Columns were centrifuged at 1500*g* for 2.5 minutes to separate the labelled peptide from the free ^{125}iodide which was incorporated into the G-25 Sephadex. Labelled samples were stored in lead containers at -20°C and the columns were washed extensively with 1M KI before disposal.

2.2.21 Chromatography

Aliquots of proteins and peptides were analysed with a range of chromatographic techniques to enrich and purify components.

2.2.21.1 Dialysis

Dialysis was used to re-equilibrate samples without significant reduction of the sample concentration. Efficient dialysis requires diffusion across a semi-permeable barrier that separates a small volume of the original sample from a large volume of the new solution.

Dialysis tubing was boiled for 10 minutes in 1mM EDTA in ddH_2O to ensure that uniform pore size, and then stored in sterile ddH_2O at 4°C for up to 1 month. A suitable length of tubing was removed for use, washed twice with ddH_2O and one end was sealed with a tight double-knot. The sample (0.5 -

5.0ml) was added with a plastic pasteur pipette and the open end was sealed with a plastic clip. The tubing was completely immersed in the new solution (500 - 1000ml) which was stirred continually for >12 hours at 4°C for equilibration.

Microdialysis of small volumes (<0.5ml) was carried out using Eppendorf™ tubes that had the centre of the plastic lid previously removed with a hot wire. The sample was added to the bottom of the modified tube and a small piece of washed dialysis tubing was used to cover the top of the tube before sealing with the lid and a extra clip. The tube was inverted and placed in a polystyrene holder on the surface of a large volume (100 - 200ml) of the new solution in a beaker, such that air bubbles were absent from the interface of the solutions and dialysis membrane. The second solution was stirred continually for >12 hours at 4°C to assist diffusion.

2.2.21.2 Ion Exchange Chromatography

Separation by ion exchange chromatography is dependent on the adsorption of charged molecules to an immobilised ion exchange group of the opposite charge. DEAE-sephacel (cross-linked cellulose matrix) and CM-sepharose (agarose matrix) were used to exchange anions and cations respectively. Separations were carried out in a batch procedure using 50µl of equilibrated ion exchange matrix in Eppendorf™ tubes. The matrix was washed ten times without centrifugation with the required buffer at 4°C and 200µl aliquots of sample were added for 2 hours with resuspension every 30 minutes at 4°C. Supernatants were aspirated carefully via a yellow pipette tip and bound substances removed by boiling the matrix in sample buffer. Separated extracts were analysed by SDS-PAGE using mini-protean II™ kits.

2.2.21.3 Affinity Chromatography

Affinity chromatography is a form of separation by which the molecules to be purified are specifically and reversibly adsorbed by a complementary binding substance (ligand) that has been immobilised on an insoluble support (matrix). Unlike ion exchange, affinity chromatography has a concentrating effect based on the natural specificities of the interacting molecules and was used to process large and small volumes.

Immobilised protein A on sepharose beads in affinity columns was used to purify ICAM1 F_c chimaeras synthesised by transfected COS cells. This protocol is discussed in detail in chapter 4.

2.2.22 Data Handling

Data from agglutination and binding assays were analysed using an IBM-based spreadsheet application (Excel 5, Microsoft). Mean values ± standard error of the means (SEM) were calculated and values expressed as percentage of controls. A two-tailed paired t-test was performed to check statistical significance, and correlation coefficients calculated.

2.2.23 Photography

Photographs of cells illuminated by light or immunofluorescence were taken with a microscope-mounted camera and a high-speed colour film. 400ASA Kodak film was used for fixed, Giemsa-stained infected cells and 1600ASA Kodak film for wet films of immuofluorescent cells. Autoradiographs and stained SDS-PAGE gels were photographed by staff at Medical Illustration Department, John Radcliffe Hospital. Electron microscope images were photographed by Dr. D. J. Ferguson using monochrome film.

Chapter 3

Relationship of Adhesion to CD36 and Antigenic Phenotype

The first demonstration that the malaria parasite could modify the host erythrocyte surface came in the 1930s when Eaton observed that serum from Rhesus monkeys infected with *P. knowelsi* could agglutinate schizont-infected erythrocytes (Eaton, 1938). Since then, it has been shown that field- and laboratory-derived isolates of *P. falciparum* will form agglutinates in sera from immune adults living in endemic areas (Hommel *et al.*, 1982; Marsh and Howard, 1986; Van Schravendijk *et al.*, 1991; Aguiar *et al.*, 1992; Iqbal *et al.*, 1993). Variant-specific antibodies present in polyclonal serum mediate agglutination of cells that bear the same surface epitope due to the bivalent nature of antibody-antigen binding (Newbold *et al.*, 1992). The *P. falciparum* "agglutinins" that are recognised in this assay define the antigenic phenotype of the culture. Agglutinins are a family of surface proteins known as PfEMP1 (*Plasmodium falciparum* Erythrocyte Membrane Protein 1) that is diverse in field isolates (Marsh and Howard, 1986; Forsyth *et al.*, 1989b) and undergoes clonal variation at a high rate *in vitro* (Biggs *et al.*, 1991; Roberts *et al.*, 1992). Consequently, a cloned parasite will rapidly become heterogeneous during cultivation, and multiple antigenic phenotypes and PfEMP1s will be present.

At the same time as antigenic determinants are detected, the parasite also expresses receptors for cytoadherence to host endothelial cells (Udeinya *et al.*, 1981; David *et al.*, 1983). The mature parasitised red blood cells (PRBC) sequester in the vascular beds of specific organs and cerebral malaria can ensue if this occurs at high levels in the brain (MacPherson *et al.*, 1985). Several animal models and *in vitro* cell binding models have been developed to imitate the pathogenesis of human cerebral malaria and investigate the basis of sequestration (reviewed by Chulay and Ockenhouse, 1990; Berendt *et al.*, 1994b). Early studies found that PRBC from most patients and laboratory isolates bound well to C32 amelanotic melanoma cells (Udeinya *et al.*, 1985; Marsh *et al.*, 1988), and the major surface receptor on this cell line was the surface glycoprotein, CD36 (Barnwell *et al.*, 1985). Adhesion was inhibited by anti-CD36 antibodies and by soluble CD36 which indicated that the interaction between the infected cell surface and CD36 was specific (Ockenhouse *et al.*, 1988). It was

subsequently demonstrated that purified CD36 and COS cells transfected with cDNA encoding CD36 also mediated cytoadherence (Barnwell *et al.*, 1989; Oquendo *et al.*, 1989).

CD36 is one of 5 putative cytoadherence receptors, and a small study showed that it was present on the cerebral endotheliuni from 6 out of 13 patients who died with cerebral malaria, but absent from similar *post mortem* tissue in 5 patients who died from other causes (Ockenhouse *et al.*, 1992b). However, several lines of evidence suggest that adhesion of PRBC to CD36 is not involved directly in cerebral malaria. A larger study in Thailand demonstrated that CD36 was located on the surface of cerebral endothelium of patients who died with cerebral malaria and non-uninfected controls at low levels (Turner *et al.*, 1994). A detailed analysis of expression of endothelial receptors and sequestered PRBC in individual vessels showed that expression of CD36 was not associated with a significantly increased risk of cerebral malaria (Turner *et al.*, 1994).

Analysis of adhesion characteristics of PRBC sampled from peripheral blood of patients has not detected a correlation of binding to CD36 with disease severity. A study of 18 Thai patients (9 with severe malaria, 9 with uncomplicated malaria) showed no correlation of adhesion to C32 melanoma cells by PRBC from patients with disease severity (Ockenhouse *et al.*, 1991a). A similar conclusion was reached by a larger study from Thailand which analysed the adhesion of 59 patient isolates to the same target cells (Ho *et al.*, 1991). A larger study with 200 patients in Africa has shown that PRBC from all patients can adhere to CD36, and this phenomenon was not associated with cerebral malaria (C. I. Newbold, personal communication).

The adherent phenotype and antigenic phenotype at the PRBC surface appear to be linked, and their expression is comodulated by PfEMP1 (reviewed by Berendt *et al.*, 1994a). Changes in the capacity of a parasite line to bind to C32 melanoma cells was accompanied by a change in the molecular weight of PfEMP1 (Magowan *et al.*, 1988), and infected monkey sera inhibited adherence to C32 melanoma cells in a variant-specific fashion (Udeinya *et al.*, 1983). In a clonally-derived set of parasites, antigenic switching was always accompanied by a change in ICAM1 adhesion (Roberts *et al.*, 1992). *In vivo*, the adhesive phenotype may be crucial for determining the organ-specific pattern of sequestration that in turn could influence the severity of disease (Roberts *et al.*, 1993). Subtle differences in adherent and antigenic phenotypes between parasite isolates may be responsible for the

variability of clinical presentations. The clone tree of PRBC which expressed known antigenic and adherent phenotypes was able to show a linkage between antigenic phenotype and ICAM1 binding. (Roberts *et al.*, 1992). However, the high rate of switching can lead to significant problems for the dissection of the relationship of antigenic phenotype and other adhesive phenotypes. Agglutination assays are more likely to detect major populations of PRBC that will be recognised by the polyclonal serum used, whereas binding assays will identify major and minor populations that can be enriched by binding to purified receptors.

Since the balance of evidence suggested that the antigenic and adhesive functions were mediated by variable surface proteins, it was reasoned that further analysis of the relationship between agglutination and adhesive phenotypes could be accomplished with a range of sequence specific proteases. While earlier studies have shown that adherence to C32 melanoma cells, purified CD36, and agglutination of PRBC were sensitive to low concentrations of trypsin, which was specific for basic amino acids (David *et al.*, 1983; Leech *et al.*, 1984; Magowan *et al.*, 1988). The effect of this and other proteases on a range of adherent clones with defined antigenic phenotypes has not been examined.

Therefore, it was decided to extend the number of proteases used and digest cloned PRBC *in vitro*. The effects of the proteases on characterised antigenic and adherent phenotypes of clones were measured with optimised agglutination and cytoadherence assays. This would permit the contribution of minor phenotypes to the overall agglutination and adhesion of a culture to be determined against the background of rapid variation.

3.1. Optimisation of the Agglutination Assay

A series of preliminary experiments was performed to determine the optimum conditions for the agglutination assay. The general protocol followed is detailed in Chapter 2 (Section 2.2.9) and was based on a published method (Marsh and Howard, 1986).

The published agglutination assays differ primarily in their mode of mixing the test cells and serum. There were two approaches that had been used in our laboratory. The first was to mount the tubes in a rotator, which slowly mixed the contents by inversion at a constant speed (10 rpm). The other method involved the use of an Eppendorf™ shaker which mixed the cells by high-speed agitation of the tubes, but became noticeably hot after extended use. Before undertaking experiments using this assay, qualitative comparisons were made in order to establish the best methodology and conditions.

The kinetics of agglutination of each method were investigated over time using PRBC from $A4_{i+}$ stained with ethidium bromide (EtBr) at 4-6% parasitaemia in the presence of serum from immune adults (Sib serum) at 1:3 dilution in RPMI-S (Figure 3.1).The percentage of cells in agglutinates increased with time (t) on the rotator until the reaction had saturated ($t > 40$ minutes). When cells were mixed on the shaker, agglutinates formed rapidly, but dissociated at longer reaction times. The temperature of the shaker increased to > 38°C after 25 minutes and the decrease in agglutination was associated with cell lysis as judged by the appearance of haemoglobin in sample supernatants. Therefore, the rotator was used to mix agglutination samples in this study.

A serial dilution of Sib serum was prepared to determine the optimum concentration to use for future assays (Figure 3.2). The same pools of Sib and Eur sera were used throughout this study and were stored in aliquots at -20°C. The Sib serum was effective down to dilutions of 1:32 in RPMI-S (equivalent to 1:64 because stock serum had been diluted 1:1 in RPMI-S during collection), but the agglutination reaction proceeded slowly at these serum levels. European serum was titrated and no infected cell agglutinates were observed, but stacking of uninfected cells (rouleaux) occurred at high Eur serum concentrations (1:2 and 1:4 dilutions in RPMI-S). Sib serum was used at 1:4 (effectively 1:8) for all measurements and gave high levels of agglutination after 30 minutes of mixing on the rotator. Eur serum was used at 1:8 in RPMI-S for all control experiments in this study.

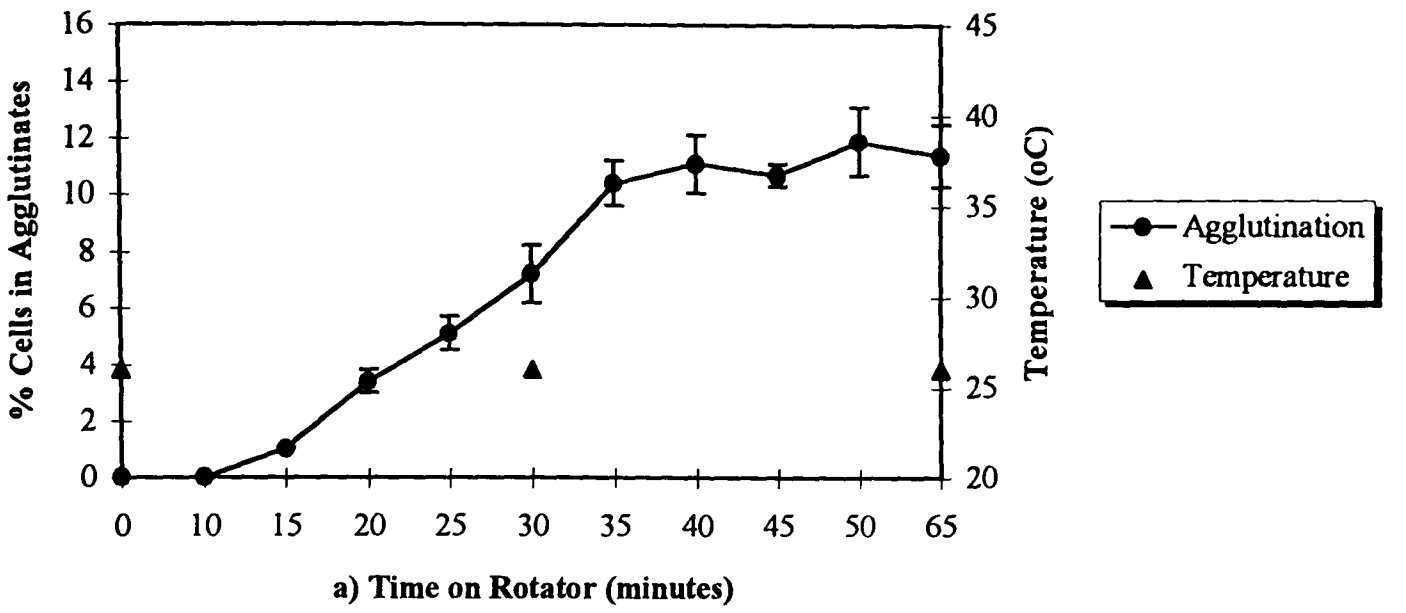


Figure 3.1. Comparison of mixing methods for agglutination assays. a) Rotator, and b) Shaker, temperature of tubes is also shown. PRBC from $A4_{i+}$ were stained with ethidium bromide (EtBr) at 4-6% parasitaemia in the presence of Sib serum at 1:4 dilution in RPMI-S. Data are mean ± SEM of duplicate experiments.

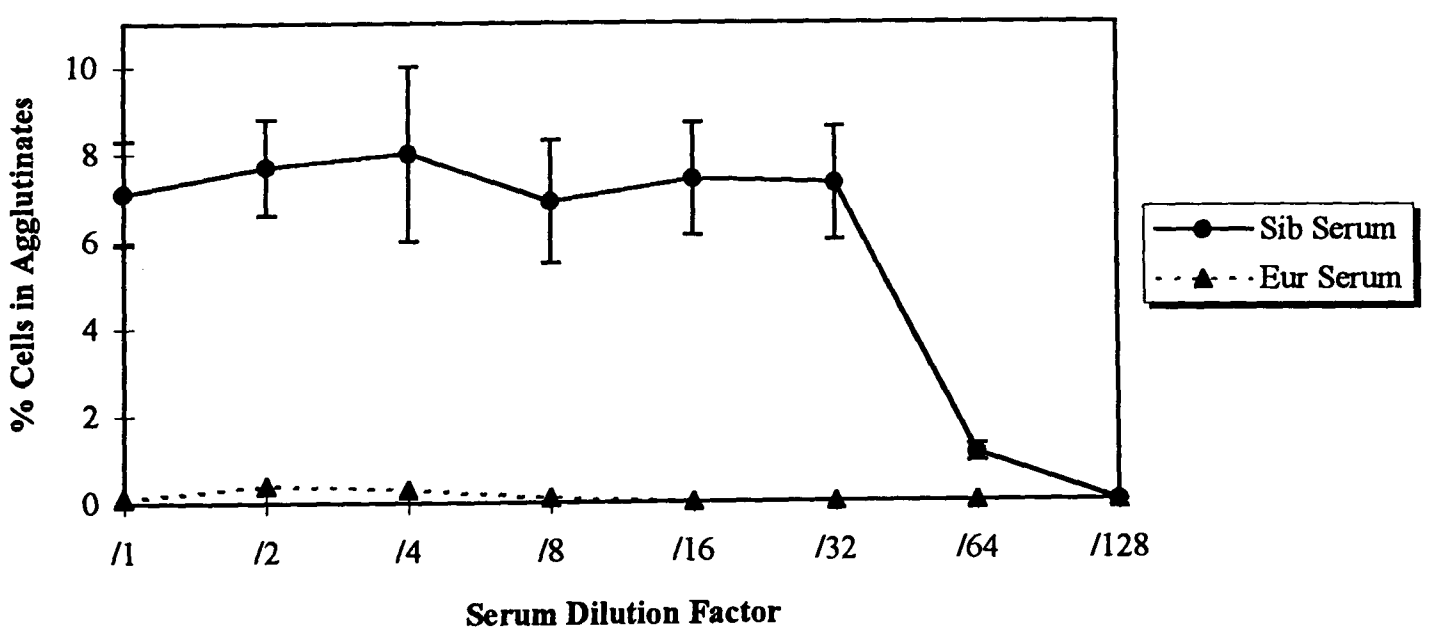


Figure 3.2. Effect of serum concentration on agglutination on rotator. PRBC from $A4_{i+}$ at 5% parasitaemia were stained with ethidium bromide and mixed with either Sib serum or Eur serum on the rotator for 30 minutes.

3.2. Effect of proteases on Agglutination

Having established optimum parameters for the assay, a number of proteases were tested at equal activities† for their effect on the agglutination of parasites from $A4_{i+}$ in Sib and Eur sera. The same proteases were screened for their effect on subclones of $A4_{i+}$ using the same assay.

3.2.1 $A4_{i+}$

This parasite was chosen first because it had been used in earlier subcloning experiments to produce a range of clones with different antigenic phenotypes that had been characterised by the mixed agglutination assay (Roberts *et al.* 1992; section 2.2.1). The results from a single experiment are summarised in table 3.1 and show that chymotrypsin, dispase and trypsin caused a decrease in the percentage of PRBC in agglutinates. In the case of V8 protease however, agglutination was resistant and multiple giant (>20 cells) agglutinates were observed after digestion (Figure 3.3). Agglutination was not affected by treatment of PRBC with neuraminidase from *C. perfringens* but was enhanced by treatment with neuraminidase from *V. cholerae*. However, unlike the case with V8 protease, the resistant agglutinates were small. The observed effects were enzyme-specific because addition of inhibitors without protease did not affect agglutination. No significant agglutination occurred in Eur serum before or after digestion.

These experiments were repeated several times and it was established that agglutination of $A4_{i+}$ parasites was differentially sensitive to a range of sequence-specific proteases (Figure 3.4). $A4_{i+}$ had $25.0 \pm 3.2\%$ ($n = 4$) control agglutination after trypsin treatment at $1 mg\ ml^{-1}$, $102.0 \pm 6.1\ \%$ ($n = 5$) of control agglutination after V8 protease digestion at $10U\ ml^{-1}$, but was completely sensitive to chymotrypsin and dispase.

† protease activity is defined by its ability to cleave a standard substrate under defined conditions, and is influenced by its source and concentration (section 2.2.4).

CONDITION	% Parasites in Agglutinates		Number of Agglutinates in size category (Sib serum)			
	Sib Serum	Eur Serum	3-5	6-10	11-20	>20
RPMI-S *(CONTROL)*	10.0 ± 3.8	0.1 ± 0.2	20	6	0	0
Chymotrypsin	0	0	0	0	0	0
Dispase	0	0	0	0	0	0
Neuraminidase (from *C. perfringens*)	11.1 ± 1.2	0	17	7	0	0
Neuraminidase (from *V. cholerae*)	18.2 ± 2.2	0	35	10	0	0
Trypsin	1.9 ± 1.1	0	6	0	0	0
V8 Protease	11.5 ± 2.1	0	6	3	2	2
TPCK *(inhibitor of chymotrypsin)*	9.1 ± 2.1	0.2 ± 0.1	25	3	0	0
EDTA *(inhibitor of dispase)*	10.4 ± 1.2	0	21	4	0	0
Trypsin inhibitor	11.1 ± 2.4	0.2 ± 0.1	21	3	1	0
DFP *(inhibitor of V8 protease)*	11.1 ± 2.1	0	26	3	0	0

Table 3.1. Effect of a range of proteases and inhibitors on agglutination of $A4_{i+}$ cells at 4% parasitaemia. Results from a single representative experiment are shown and data are mean values of percentage of PRBC in agglutinates ± SEM from duplicate determinations made in a single experiment on the same cells (section 2.2.9).

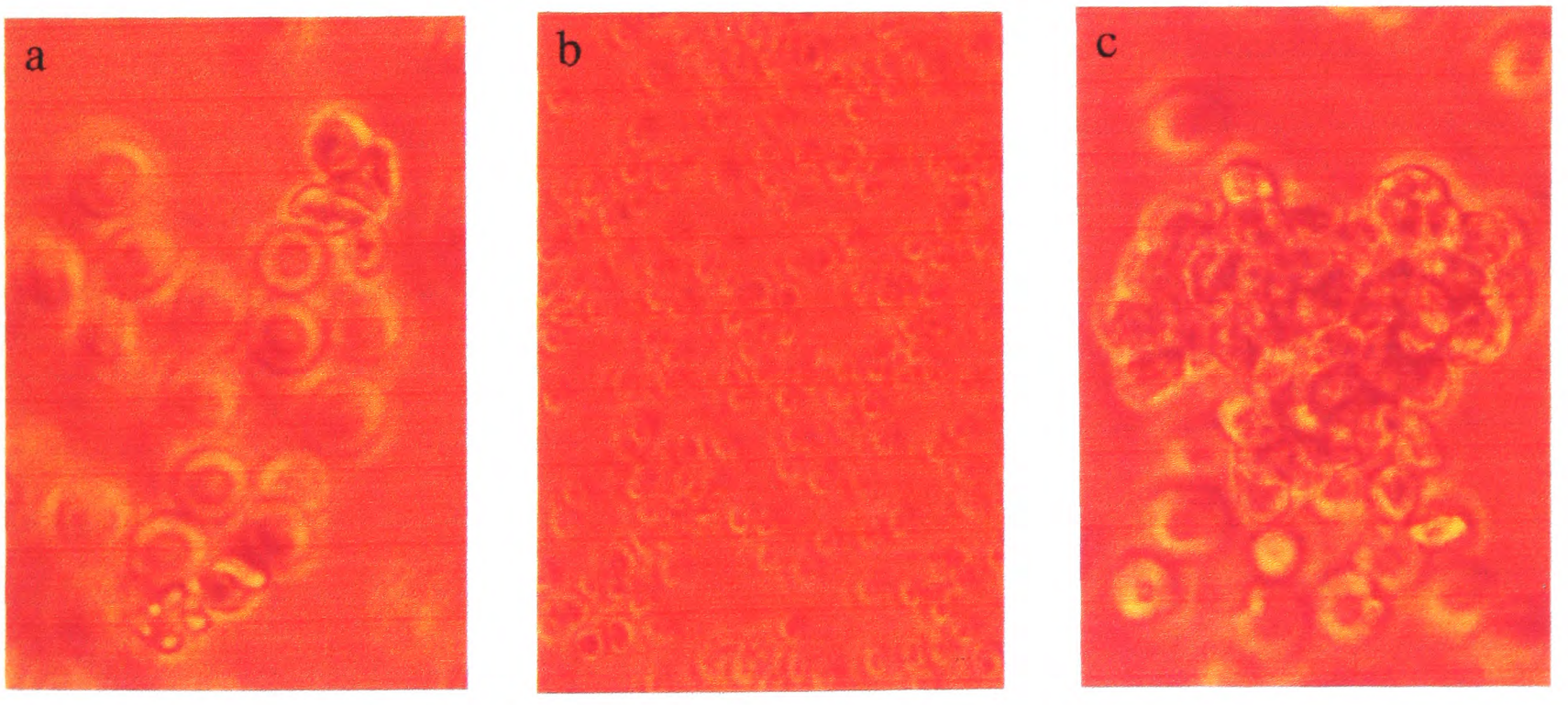


Figure 3.3. Effect of proteases on agglutination of PRBC from $A4_{i+}$ in Sib serum. Light field views of a) control PRBC (×40 objective), b) PRBC digested with 1mg ml^{-1} dispase before mixing (×16 objective), and c) PRBC digested with 10U ml^{-1} V8 protease before mixing (×40 objective). Note that the size of agglutinates formed after V8 protease was significantly increased.

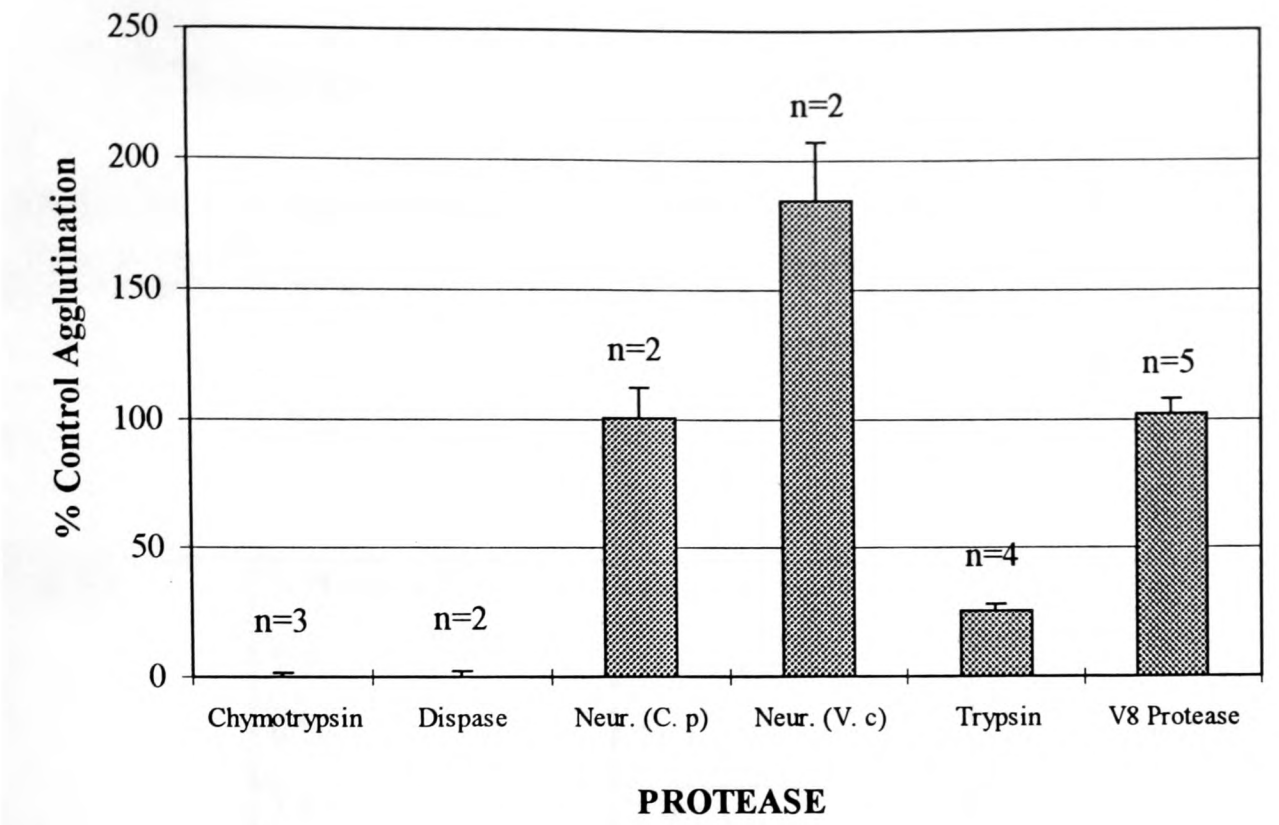


Figure 3.4. Bar chart to summarise the effect of a range of proteases on agglutination of $A4_{i+}$ cells in Sib serum after a number of experiments (n). Data are from mean values from six determinations in each. Key: Neur. (C. p) - neuraminidase from C. perfringens, *Neur. (V. c) - neuraminidase from* V. cholerae

To investigate further the differential effect of proteolysis on agglutination of $A4_{i+}$ cells in more detail, serial dilutions of chymotrypsin, trypsin and V8 protease were used (Table 3.2). Agglutination was completely sensitive to low concentrations (0.1mg ml^{-1}) of chymotrypsin, but showed a significant and transient increase in both percentage and size of agglutinates at 0.1mg ml^{-1} of trypsin ($p < 0.025$). 1U ml^{-1} of V8 protease caused an increase in the size of agglutinates formed by $A4_{i+}$ cells and had no effect on the overall percentage of cells in agglutinates.

CONDITION		% PRBC in Agglutinates ± SEM	Number of Agglutinates in size category			
PROTEASE	CONCENTRATION		3-5	6-10	11-20	>20
Chymotrypsin	0.00(mg ml^{-1})	16.0 ± 1.7	24	6	0	0
	0.01	3.2 ± 0.4	7	0	0	0
	0.10	0.0	0	0	0	0
	1.00	0.0	0	0	0	0
Trypsin	0.00 (mg ml^{-1})	18.3 ± 1.2	29	8	0	0
	0.01	15.3 ± 2.1	16	4	3	0
	0.10	21.3 ± 3.1	15	3	2	1
	1.0	2.0 ± 1.1	6	0	0	0
V8 Protease	0.00 (U ml^{-1})	14.3 ± 2.1	22	7	0	0
	0.10	12.2 ± 2.1	21	6	1	0
	1.00	15.1 ± 1.4	17	8	3	1
	10.0	14.1 ± 3.1	10	4	4	2

Table 3.2. Effect of serial dilutions of chymotrypsin, trypsin and V8 protease on agglutination of parasites from $A4_{i+}$ in Sib serum. Experiments were performed on different days on the same culture at 4-6% parasitaemia.

Elevated concentrations of V8 protease were used for extended times to discover if a limit digestion had occurred. Agglutination of $A4_{i+}$ was resistant to 40U ml^{-1} V8 protease (equivalent to 4mg ml^{-1} trypsin) and large (>40 cells) agglutinates occurred, even after 15 minutes of digestion (data not shown). When tubes were mixed for longer times on the rotator, the percentage of cells in agglutinates increased and massive (>100 cells) agglutinates were observed. This result confirmed that the digestion had proceeded to a limit under the concentrations of protease used.

Thus, it appeared that agglutination of $A4_{i+}$ was resistant to high concentrations of V8 protease, but was sensitive to other proteases. Protease-resistant agglutination of infected erythrocytes had not been reported before, and in order to determine whether the effect of V8 protease on $A4_{i+}$ was a variant specific result or was a general effect, a range of subclones of $A4_{i+}$ with different antigenic phenotypes were tested.

3.2.2 Other clones

Five other parasites were used to investigate the effect of V8 protease on agglutination. The relative antigenic phenotypes of the four subclones from $A4_{i+}$ had been previously characterised by the mixed agglutination assay (Roberts *et al.*, 1992). Because the stabilates used in the experiments were not precisely the same as those used in earlier studies, their relative antigenic types were first assessed by the mixed agglutination assay (Table 3.3 and Figure 3.5).

EtBr (red) stained test culture	**DAPI (blue) stained test culture**											
	$A4_{i+}$			**$C18_{i-}$**			**$C24_{i-}$**			**$IT6_{i+}$**		
	Red	Mix	Blue	Red	Mix	Blue	Red	Mix	Blue	Red	Mix	Blue
$A4_{i+}$	1	96	3	41	19	40	41	15	44	40	15	45
$C18_{i-}$	42	15	43	2	97	1	49	1	50	43	16	39
$C24_{i-}$	43	13	44	49	5	46	0	100	0	45	11	44
$IT6_{i+}$	44	8	48	43	12	45	48	4	48	1	97	2

Table 3.3. Representative mixed agglutination data from a single experiment. Cultures were stained with either EtBr or DAPI and mixed with Sib serum at 1:4 in RPMI-S on the rotator. 100 agglutinates of 5 - 10 cells each were counted. Red - EtBr only stained agglutinates, Blue - DAPI only stained and Mix - both EtBr and DAPI stained cells in agglutinates (section 2.2.10).

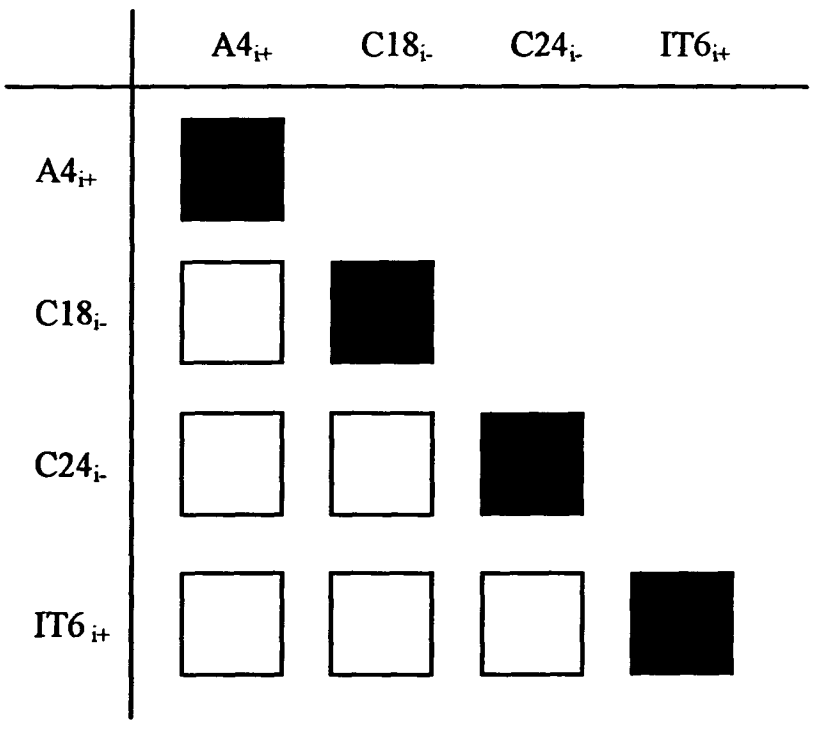


Figure 3.5. Mixed agglutination analysis of $A4_{i+}$, $C18_{i-}$, $C24_{i-}$ and $IT6_{i+}$.

Key: ■ 100 - 80%, □ 5 - 0% similarity between test cultures.

The antigenic phenotype of $IT6_{i+}$ was characterised by the mixed agglutination assay and shown to be different from $A4_{i+}$, $C18_{i-}$ and $C24_{i-}$ because few mixed colour agglutinates were observed in heterologous reactions (for basis of assay and analysis of results, see section 2.2.10). Other chosen clones had already been characterised: $C9_{i+}$ expressed the same variant antigenic type (VAT) as $A4_{i+}$, but $C18_{i-}$, $C24_{i-}$ and $C28_{i-}$ were unique antigenic variants (Roberts *et al.*, 1992).

- **V8 Protease**

When PRBC from $C9_{i+}$ (A4VAT) were digested with V8 protease at 10U ml^{-1}, agglutination was resistant and giant agglutinates were observed (Figure 3.6). Agglutination of cells from $IT6_{i+}$, $C24_{i-}$ and $C28_{i-}$ was completely sensitive, but giant agglutinates were formed after $C18_{i-}$ was digested and the percentage of PRBC in agglutinates was enhanced (171 ± 18.8% control, n = 4). Therefore, agglutination of PRBC that expressed the A4VAT was resistant to V8 protease, and agglutination of parasites that expressed the C18VAT was enhanced by the same protease.

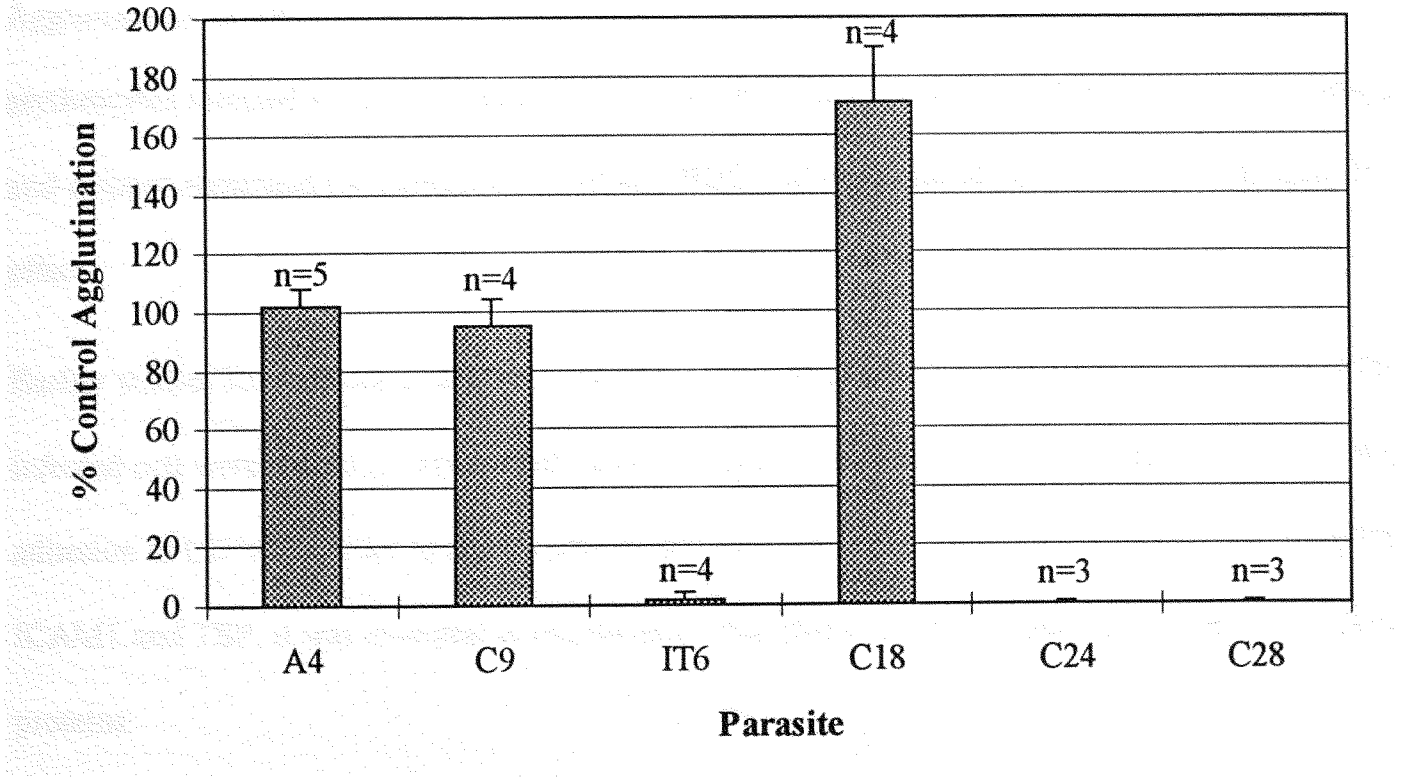


Figure 3.6 Effect of V8 protease on agglutination of a range of PRBC. Results are mean values ± SEM from a number of separate experiments (n).

- **Trypsin**

Low concentrations of trypsin abolished the agglutination of all parasites except $C9_{i+}$, which was partially resistant. This finding was investigated in more detail in chapter 4.

- **Neuraminidases**

Agglutination of cells from $C18_{i-}$ was unaffected by treatment with neuraminidase from *C. perfringens* (95.3 ± 5.3% control, n = 2), and enhanced by neuraminidase from *V. cholerae* (145.3 ± 23.7% control, n = 2). These results were similar to those determined using $A4_{i+}$ cells and implied that the sensitive carbohydrate moieties were not related to the VAT of the culture. The increase of agglutination following neuraminidase digestion was probably due to an alteration of surface charge.

Thus, these data indicated that the sensitivity to proteolysis of antigenic epitopes expressed exclusively on the surface of *P. falciparum* infected erythrocytes was in some cases at least variant-specific. Agglutination of PRBC from $A4_{i+}$, $C9_{i+}$ and $C18_{i-}$ was resistant to V8 protease, while agglutination of erythrocytes infected with other clones was sensitive to the same treatment. Conversely, chymotypsin and dispase abolished the agglutination of all PRBC and neuraminidases had no variant-specific effect.

Earlier studies have demonstrated that the antigenic and adherent phenotypes at the surface of the infected cell were linked (Roberts *et al.*, 1992), thus it was decided to test the effect of proteases on adhesion to CD36. In order to able to investigate the effect of proteases on other putative receptors, ICAM1 and TSP, it was essential to use an assay that had been standardised for adhesion to all three proteins.

3.3. Cytoadherence: Standardisation of the Spot Binding Assay

The adhesion of PRBC to purified receptor protein was assessed quantitatively by measuring the number of cells bound to immobilised protein on plastic. For these studies, individual spots of the putative receptors, CD36, ICAM1 and TSP were employed throughout. The benefit of this approach over *in vitro* whole cell models (eg. human umbilical vein endothelial cells (HUVECs) and C32 melanoma cells) of cytoadherence is that specific adhesion to individual proteins can be quantified without other confounding factors. Further justification for the use of spot binding assay is given by experiments that showed specific inhibition of parasite binding to purified receptors on plastic by monoclonal antibodies (Roberts *et al*, 1985b; Barnwell *et al.*, 1989; Berendt *et al.*, 1992). The general protocol followed is detailed in section 2.3.6.

Initial experiments indicated that the reproducibility of data from these assays was a significant problem. Before embarking on an extensive study of several clones, the assay was standardised by comparing three reported mixing / washing methods and by using concentrations of CD36, ICAM1 and TSP that were known to mediate efficient binding of PRBC from $A4_{i+}$ (Roberts *et al.*, 1985b; Roberts *et al.*, 1992).

3.3.1 Manual

Cells were resuspended in binding medium in the dishes for 30 minutes at 37°C and were mixed every 5 minutes by gentle manual rocking to resuspend the cells (Roberts *et al.*, 1992). The dishes were washed 5 times by manual rocking to remove unbound cells, but dish-to dish and spot-to-spot variation was large (Table 3.4), and results were not reproducible on different days. The kinetics of the assay under these conditions were not ideal, because cells quickly settled out of suspension and temperature fluctuations were introduced when dishes were removed from the incubator during resuspensions.

3.3.2 Belly Dancer™

A Belly Dancer™ was routinely used in the laboratory for mixing solutions during SDS-PAGE gel staining or western blotting. The machine consisted of a platform that rotated in the horizontal plane at a constant speed determined by a motor. Dishes were carefully mounted on the rotating stand during binding and washing steps. However, cells rapidly pooled in the centre of the dish due to centripetal acceleration resulting from the circular motion of the platform. This resulted in large variation (Table 3.4), and because the assay could only be performed at room temperature, this method was not used.

3.3.3 Hybridisation Machine

This machine was used for mixing probes with blots or filters during molecular biology experiments. It was found to be an efficient method for mixing cells during binding assays and provided reproducible results with low variation. Dishes were placed on trays which rocked gently in the vertical plane at constant speed in a chamber where the temperature was set to 37°C. Cells were kept in suspension for 30 minutes and wash conditions were standardised (5 × 1 minute washes of 2ml binding medium). Spot-to-spot, dish-to-dish and day-to-day variation were lowest when results for both receptors were considered, and this method was superior to the previous two because it permitted adhesion at constant temperature and conditions were easier to control (Table 3.4).

RESUSPENSION METHOD	DAY	Binding to CD36 ± SEM	Binding to ICAM1 ± SEM
MANUAL	1	165.1 ± 78.8	32.1 ± 24.2
	2	234 ± 107.4	74.1 ± 43.2
BELLY DANCER™	1	187.3 ± 125.1	59.2 ± 45.2
	2	274.1 ± 149.3	43.1 ± 24.1
HYBRIDISATION MACHINE	1	211.5 ± 18.5	53.3 ± 8.6
	2	245.1 ± 11.3	75.1 ± 9.2

Table 3.4. Comparison of alternative mixing / washing methods used in binding assays. $A4_{i+}$ cells at 5 - 10% parasitaemia and 2 - 3% haematocit were used in duplicate dishes on two different days. Data are the mean binding mm^{-2} ± SEM of triplicate spots and are corrected to 1% parasitaemia and 1% haematocrit.

Having decided on the optimum methodology, adhesion of PRBC from $A4_{i+}$ to purified CD36, ICAM1 and TSP was determined using the hybridisation machine for resuspensions over a range of haematocrits and parasitaemias to optimise the conditions for binding to each receptor (Figure 3.7). Binding to all receptors was linearly correlated with haematocrit over the range 0.4 - 5.2% ($r > 0.98$) at a constant parasitaemia (6.0%). Binding to all receptors were linearly correlated with parasitaemia between 1.8 - 10.5% ($r > 0.94$) at a constant haematocrit (2.0%). The relative binding of PRBC was assessed in subsequent experiments using cell suspensions diluted to between 2 - 10% parasitaemia and 1 - 5% haematocrit. The absolute number of cells bound was corrected to 1% haematocrit and 1% parasitaemia because adhesion was proportional to cell number in these conditions.

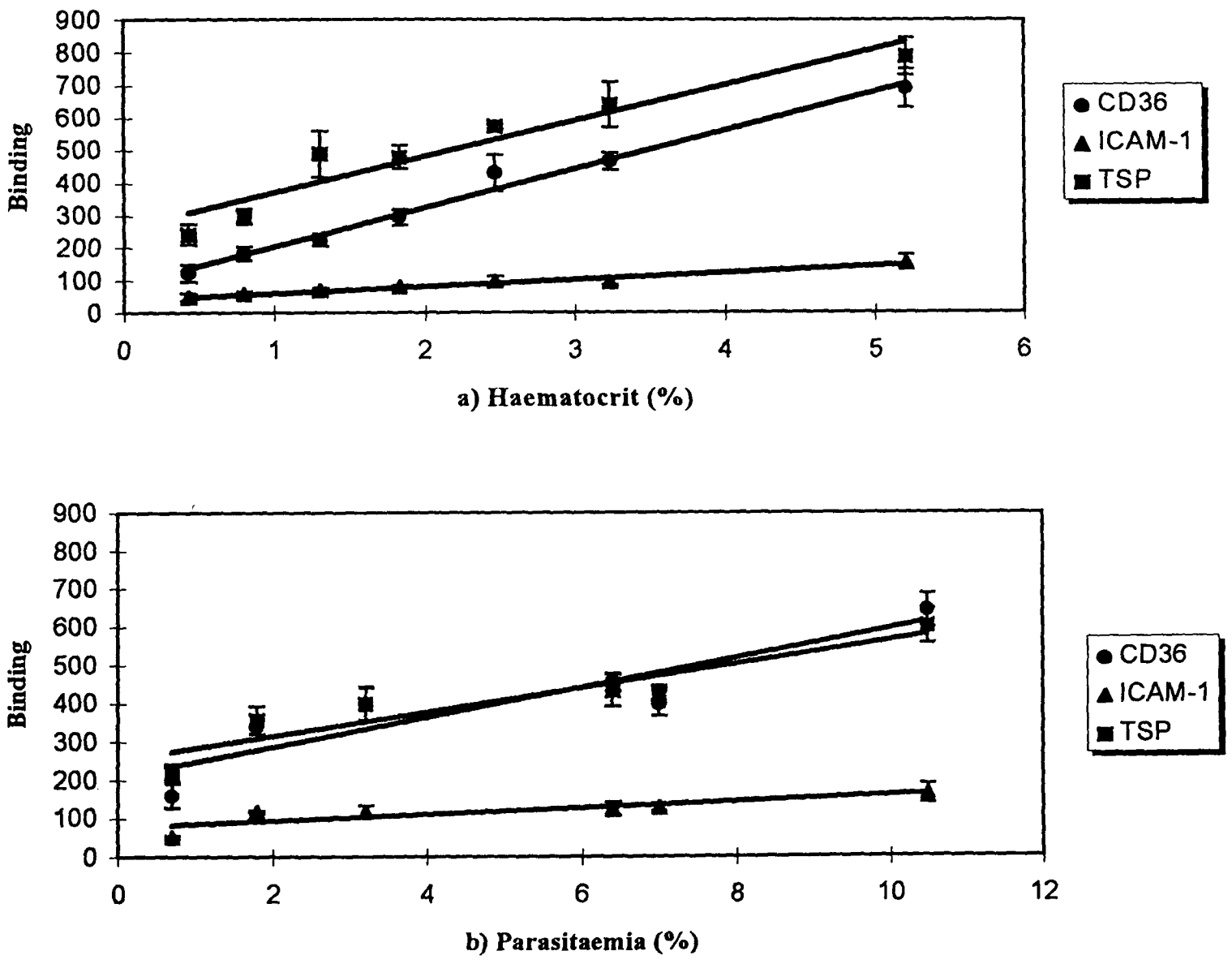


Figure 3.7. Effect of varying parasite numbers on binding using the hybridisation machine. a) Haematocrit and b) Parasitaemia of $A4_{i+}$ cells was varied in binding assays. Data are mean cells bound $mm^{-2} \pm SEM$ from triplicate determinations in a single experiment.

3.4. Effect of Proteases on Binding to CD36

Having optimised the conditions for the spot binding assay so that results were reproducible and reliable, two strategies were employed to determine whether or not there was an association between antigenic phenotype and binding to CD36:

a) Cells from $A4_{i+}$ were digested with the same proteases that had been characterised previously for their effect on agglutination, and digested cells were tested for their ability to bind to CD36.

b) Cells from other clones were examined to discover whether or not the effect of V8 protease on binding to CD36 correlated with its effect on agglutination.

3.4.1 $A4_{i+}$

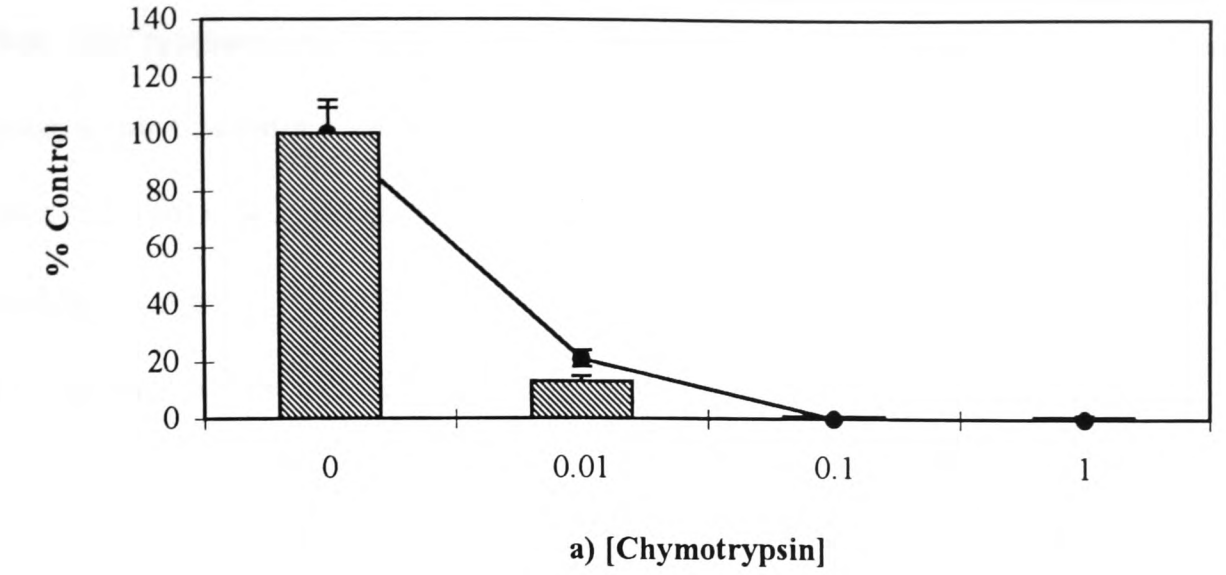
PRBC from $A4_{i+}$ did not bind to CD36 after digestion with chymotrypsin, dispase or trypsin (table 3.2) which correlated with the reduction in agglutination observed with the same proteases. Treatment with neuraminidases or protease inhibitors alone had no effect on binding and supported previous conclusions that surface proteins were important for mediating adherence to CD36 (Ockenhouse and Chulay, 1990). In the case of V8 protease, 22.5 ± 6.3% (n = 5) of control binding remained after digestion.

CONDITION	**Binding to CD36 (% control)**	**n**
RPMI-S	100.0 ± 8.7	5
Chymotrypsin	2.0 ± 1.2	3
Dispase	0.0 ± 0.3	3
Neuraminidase (from *C. perfringens*)	77.7 ± 11.2	2
Neuraminidase (from *V. cholerae*)	95.5 ± 9.7	2
Trypsin	0.0 ± 0.5	5
V8 protease	22.5 ± 6.3	5
TPCK *(inhibitor of chymotrypsin)*	103.1 ± 4.7	2
EDTA *(inhibitor of dispase)*	99.7 ± 3.4	2
Trypsin inhibitor	104.5 ± 6.7	2
DFP*(inhibitor of V8 protease)*	97.7 ± 8.1	2

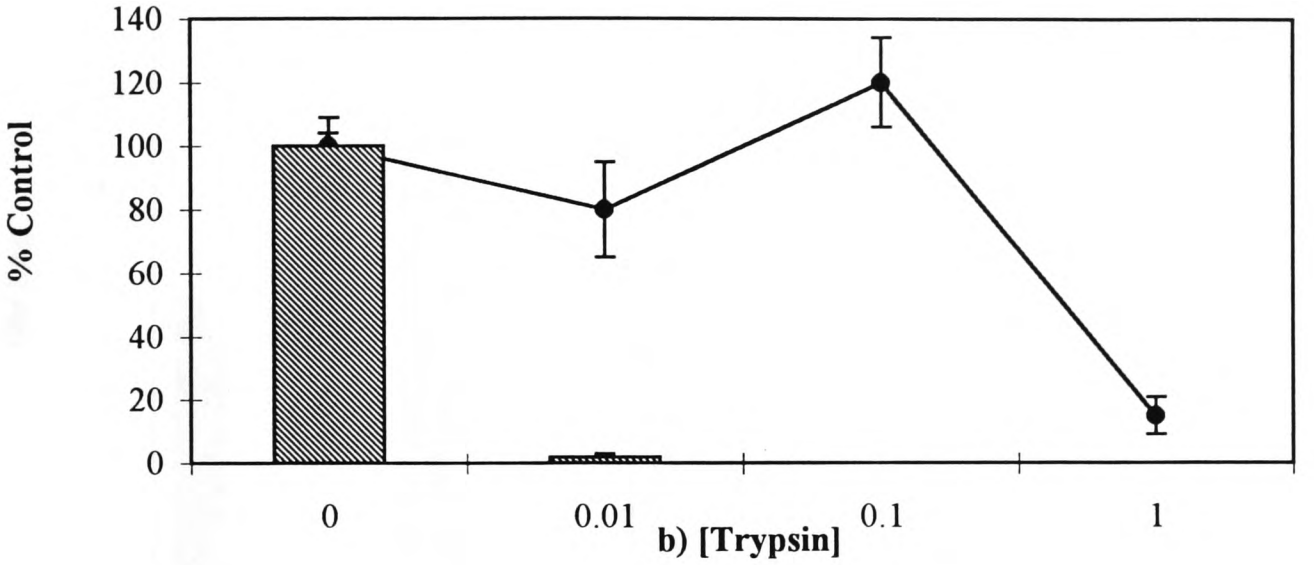
Table 3.2. Summary of the effect of a range of proteases on binding of $A4_{i+}$ cells to CD36. Results are % control (undigested PRBC) binding ± SEM from a number of experiments (n) are shown.

The effect of three proteases, chymotrypsin, trypsin and V8 protease, on adhesion to CD36 and agglutination was then compared on the same batch of PRBC from $A4_{i+}$ (Figure 3.8). Binding to CD36 was completely sensitive to low concentrations of chymotrypsin and trypsin and the effect of chymotrypsin was identical to earlier findings in agglutination experiments. However, two observations were unusual. Firstly, low concentrations of trypsin that had caused a transient increase in the % cells in agglutinates ablated binding to CD36. This indicated that these surface phenotypes may be differentially sensitive to trypsin, and was investigated in more detail in chapter 4.

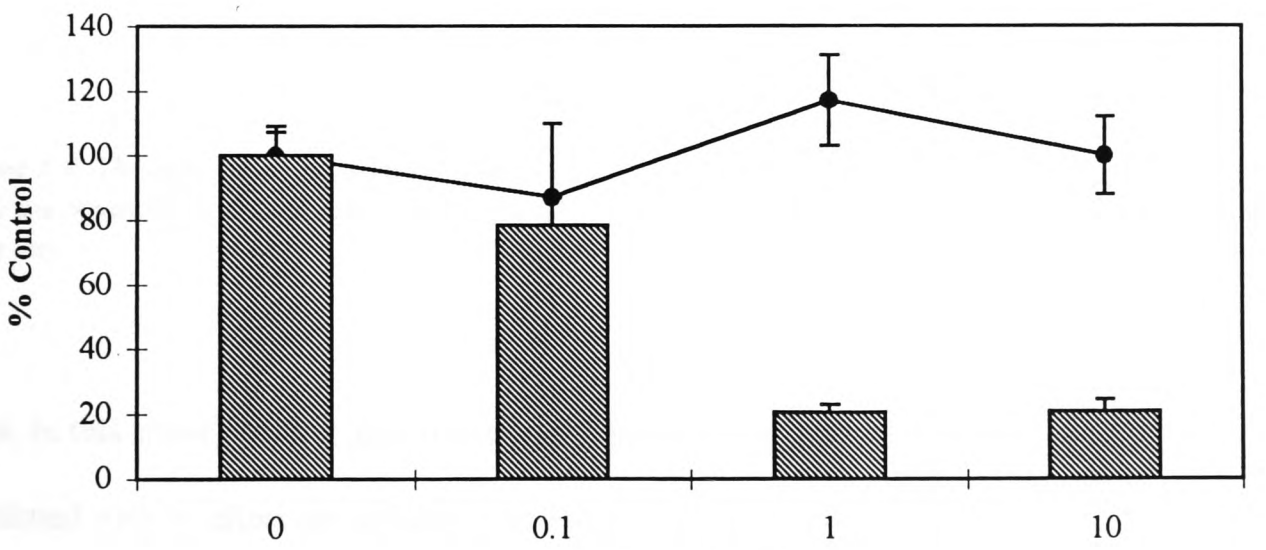
More intriguing however, was the observation that V8 protease did not completely abolish adhesion of $A4_{i+}$ cells to CD36, even at elevated concentrations of protease (40U ml^{-1}) (data not shown). Thus, there appeared to be a similarity in the effect of a sequence-specific protease on agglutination and cytoadherence: partially resistant binding of $A4_{i+}$ cells to CD36 was observed after treatment with V8 protease and the same protease had no effect on percentage of cells in agglutination. The effect of V8 protease on other cloned parasites with different antigenic phenotypes was therefore investigated to determine if this relationship was also variant specific.



a) [Chymotrypsin]



b) [Trypsin]



c) [V8 Protease]

Figure 3.8. Effect of different concentrations of proteases on agglutination and binding to CD36 of $A4_{i+}$ cells. a) Chymotrypsin (mg ml^{-1}), b) Tryspin (mg ml^{-1}), c) V8 protease (U ml^{-1}). Results are the mean values ± SEM from duplicate determinations during a single experiment. Key: line graph - agglutination in Sib serum, bar chart - binding to CD36.

3.4.2 Other clones

When other parasites were examined (Figure 3.9), binding to CD36 was universally sensitive to trypsin at low concentrations, and was >90% sensitive to chymotrypsin. On the other hand, only a subset of parasites were sensitive to V8 protease: $IT6_{i+}$, $C24_{i-}$, and $C28_{i-}$ could not bind to CD36 after V8 proteolysis, but parasites from $C9_{i+}$ bound at 23.1 ± 15.4% of control levels after digestion (n = 3). CD36 binding of $C18_{i-}$ cells was completely resistant to V8 protease (99.4 ± 4.0% control, n = 3).

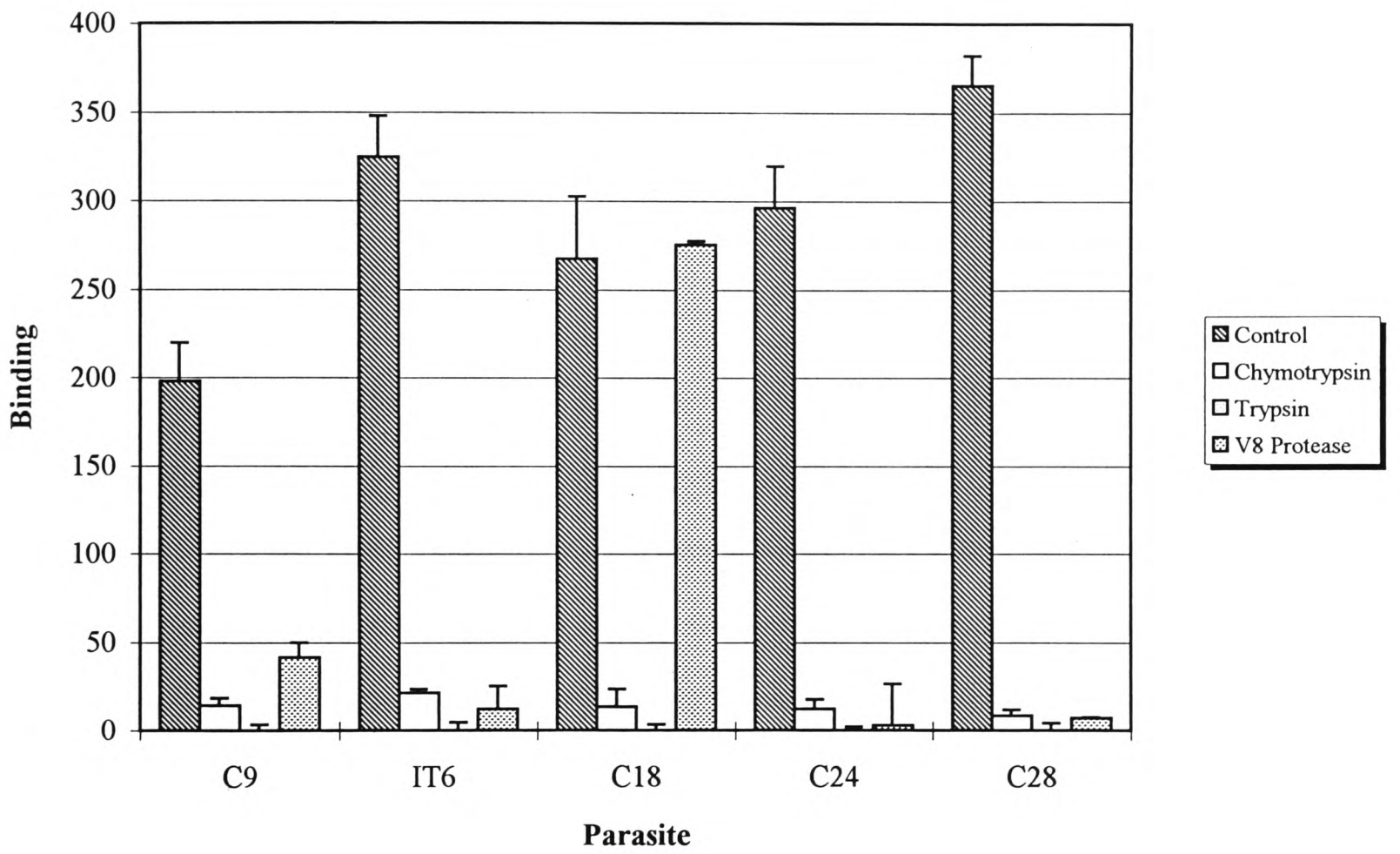


Figure 3.9. The effect of chymotrypsin, trypsin and V8 protease on binding to CD36 of a range of parasites. Data are mean PRBC bound mm^{-2} ± SEM of cells bound to triplicate spots of receptor in duplicate dishes on the same day.

Thus, in this clone family it appeared that the effect of V8 protease on binding to CD36 was closely correlated with its effect on agglutination. Parasites could be grouped by the sensitivity of their surface properties to V8 protease (Table 3.4).

GROUP	Parasite	Agglutination	Binding to CD36
I	$IT6_{i+}$, $C24_{i-}$, $C28_{i-}$	Completely Sensitive	Completely Sensitive
II	$A4_{i+}$, $C9_{i+}$	Completely Resistant	Partially Resistant (~20%)
III	$C18_{i-}$	Enhanced	Completely Resistant

Table 3.4. Summary of the effect of V8 protease on agglutination and binding to CD36 of a range of parasites.

The V8 protease-resistant CD36 binding phenotype was associated with the expression of two antigenic phenotypes, the A4VAT (i.e. $A4_{i+}$ and $C9_{i+}$) or C18 VAT. Parasites that expressed the A4VAT had partially resistant CD36 binding after digestion, but the C18 VAT showed completely resistant binding.

Studies in the laboratory had previously demonstrated that *P. falciparum* undergoes antigenic variation at a high rate *in vitro* leading to the rapid generation of multiple VATs within a clone. Clones are thus not phenotypically homogeneous, and the most obvious explanation for these data was that the high rate of clonal variation resulted in the expression of a V8 protease-resistant VAT within $A4_{i+}$ and $C9_{i+}$. Hence, V8 protease-resistant PRBC could be present as a subpopulation of $A4_{i+}$ and $C9_{i+}$, and could mediate both the resistant binding to CD36 and the resistant agglutination observed in these parasites.

Two experimental approaches were available to test this hypothesis:

- Panning of $A4_{i+}$ parasites on CD36 after V8 proteolysis. This would allow enrichment of the resistant subpopulation within the culture.
- Analysis of the V8 protease-digested cells from $A4_{i+}$ and $C18_{i-}$ by the mixed agglutination assay. This would provide an indication of the antigenic phenotypes of the V8 protease resistant PRBC.

3.5. Selection of A4V8CD36

PRBC from $A4_{i+}$ were treated with 10U ml^{-1} of V8 protease and selected on purified CD36. The adherent cells were grown on for three cycles and termed A4V8CD36. This selected population bound strongly to CD36 but not to ICAM1 and formed agglutinates in Sib serum but not in Eur serum (Table 3.5). These properties were sensitive to trypsin at 1mg ml^{-1} but unchanged after digestion with 10U ml^{-1} V8 protease. Agglutinates formed after V8 proteolysis were significantly larger than those in controls, and giant (>50 cells) agglutinates were common.

A4V8CD36	**AGGLUTINATION** (% Cells in Agglutinates)		**BINDING** (Cells mm^{-2})	
	Sib serum	**Eur serum**	**CD36**	**ICAM1**
CONTROL	12.4 ± 4.2	0.2 ± 0.2	364.6 ± 55.6	10.0 ± 2.4
TRYPSIN (1mg ml^{-1})	0	0	0	0
V8 PROTEASE (10U ml^{-1})	16.7 ± 3.6	0	361.7 ± 25.8	0.5 ± 0.2

Table 3.5. Effect of trypsin and V8 protease on agglutination and cytoadherence of A4V8CD36. Results are mean values ± SEM from two experiments.

3.6. Measurement of Antigenic Phenotypes

3.6.1 A4V8CD36

Since the phenotype of A4V8CD36 was similar to $C18_{i-}$, the mixed agglutination assay was used to characterise its antigenic relationship to both $C18_{i-}$ and $A4_{i+}$. These data are presented in table 3.6 and summarised in figure 3.10 and illustrate that a significant proportion of A4V8CD36 was antigenically indistinguishable from $C18_{i-}$ (39.4% overall identity[¶]) and was only 3.8%[¶] similar to $A4_{i+}$, within the limits of the assay ($A4_{i+}$ and $C18_{i-}$ were 4.6% identical). A characteristic mixed colour agglutinate formed when PRBC from A4V8CD36 and $C18_{i-}$ were tested is shown in figure 3.11.

	DAPI (blue) stained test culture								
	$A4_{i+}$			**$C18_{i-}$**			**A4V8CD36**		
EtBr (red) stained test culture	Red	Mix	Blue	Red	Mix	Blue	Red	Mix	Blue
$A4_{i+}$	1	97	2	42	17	41	36	12	40
$C18_{i-}$	43	16	44	2	97	1	15	72	13
A4V8CD36	43	16	35	11	75	14	1	99	1

Table 3.6 Mixed agglutination data using $A4_{i+}$, $C18_{i-}$ and A4V8CD36 cells in the same experiment. Red - EtBr only stained agglutinates, Blue - DAPI only stained and Mix - both EtBr and DAPI stained cells in agglutinates.

[¶] Estimates of the degree of antigenic similarity were calculated from the proportion of mixed colour agglutinates (x) formed in the mixed agglutination assay, and applying the formula:

$$x = 1-p^{(n-1)}$$

where p is the chance of homologous agglutination and n is the number of cells per agglutinate (5).

The value of p is related to the degree of antigenic similarity of the test cultures by the formula:

$$\text{antigenic similarity (\%)} = 100 \times (1-p) / p$$

Thus, in the case of A4V8CD36 tested against $C18_{i-}$, the mean of two determinations was 73.5% mixed agglutinates, then x = 0.735 and p = 0.717, and the antigenic similarity was 39.4% (see section 2.2.10 for basis of assay).

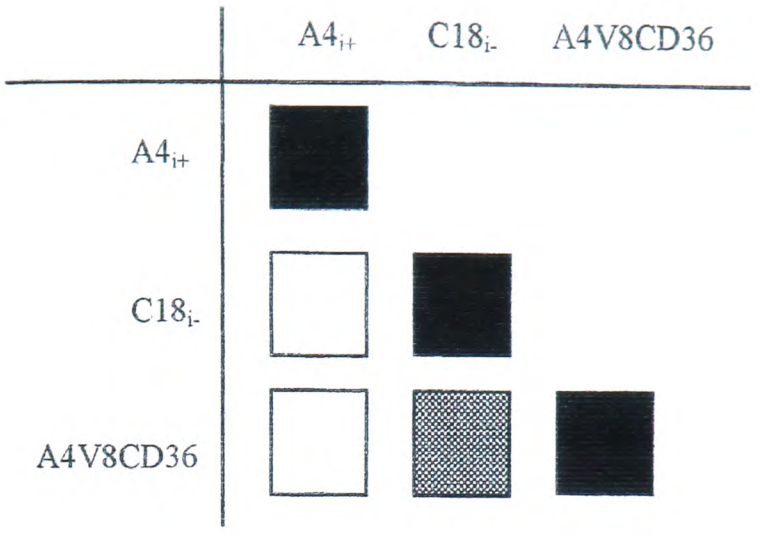


Figure 3.10. Mixed agglutination analysis of cells from $A4_{i+}$, $C18_{i-}$, and A4V8CD36.

Key: ■ 100 - 80%, ▩ 39.4%, □ 5 -0% similarity.

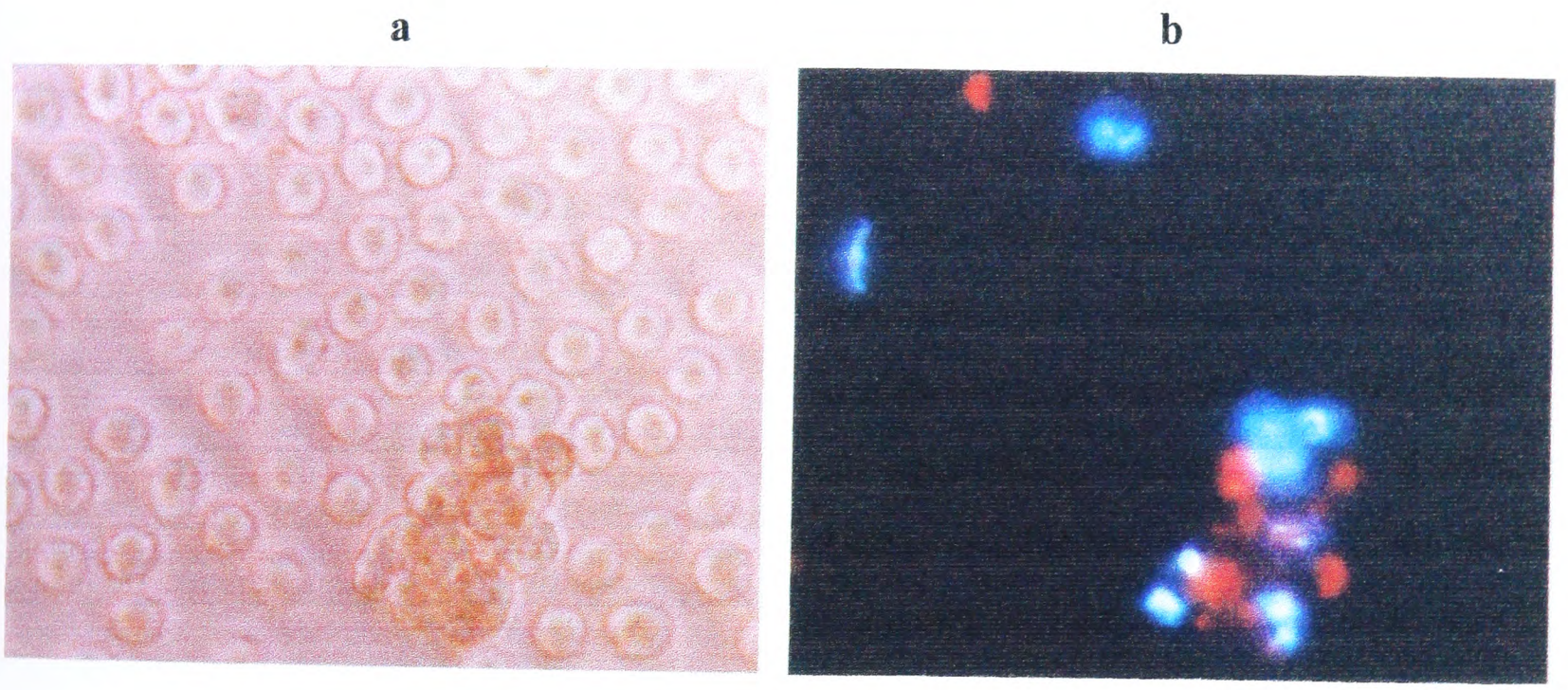


Figure 3.11 a) light field view (×40 objective) of an agglutinate formed when PRBC from A4V8CD36 and $C18_{i-}$ were mixed in Sib serum. b) same field by immunofluorescence: PRBC from A4V8CD36 were stained with EtBR (red) and $C18_{i-}$ with DAPI (blue).

Thus, it appeared that A4V8CD36 showed a high degree of phenotypic similarity to $C18_{i-}$ and could represent the V8 protease-resistant subpopulation within $A4_{i+}$. To confirm that this population had not arisen by chance during the three cycles after selection, parasites were used in the mixed agglutination assay immediately after digestion.

3.6.2 Effect of V8 Proteolysis

3.6.2.1 Normalisation of agglutination rates

For the mixed agglutination assay to be informative, the rates of agglutination of the test cultures must be identical. In previous experiments, it was assumed that parasites agglutinated at the same rate, which was independent of their antigenic phenotype, provided that the numbers of each test culture were comparable. However, cells from $C18_{i-}$ and A4V8CD36 have enhanced agglutination after V8 protease digestion, and $A4_{i+}$ cells will agglutinate at high levels after digestion if left on the rotator for long periods. Thus, for comparisons of antigenic phenotypes of digested and undigested cells to be valid, rates of agglutination of test cells were normalised in preliminary experiments.

10μl of cells from $C18_{i-}$ and A4V8CD36 at 5% parasitemia post-digestion were stained with EtBr and resuspended in different volumes of fresh, washed red blood cells at 25.0% haematocrit. 10μl of each aliquot was mixed with 10μl of the test culture stained with DAPI at 5% parasitaemia. The samples were mixed in Sib serum (Section 2.2.9) on the rotator and the results from a single experiment are summarised in table 3.7. The rates of agglutination of the test cells were equal when similar numbers of single-coloured agglutinates were observed (shown by shading), i.e. the proportion of red agglutinates was the same as the proportion of blue agglutinates. V8 protease-treated cells from $C18_{i-}$ and A4V8CD36 were diluted 1:2 in red blood cells when mixed with undigested cells from $C18_{i-}$ and A4V8CD36 for comparable rates of agglutination in further assays. However, the same digested cells gave similar rates of agglutination to undigested $A4_{i+}$ cells when diluted 1:5 in red blood cells, and identical results were obtained after the experiment was repeated twice. PRBC from $A4_{i+}$, $C18_{i-}$, A4V8CD36 and V8 protease treated $A4_{i+}$ agglutinated at similar rates and were used at the same concentrations (data not shown).

Test Cultures	$C18_{i-}$	vs	*$C18_{i-}$*	A4V8 CD36	vs	*A4V8 CD36*	$A4_{i+}$	vs	*$C18_{i-}$*
Agglutinates / Ratio of test cultures	Red	Mixed	Blue	Red	Mixed	Blue	Red	Mixed	Blue
1:1	3	5	92	12	6	82	10	22	68
1:2	14	68	18	17	66	17	15	31	54
1:5	73	15	12	65	18	17	13	71	16
1:10	88	6	6	75	11	14	65	31	4

Table 3.7. Normalisation of the rates of agglutination of test cultures used in the mixed agglutination assay after V8 protease digestion. Data are the number of agglutinates in each category from a single experiment. Italics and underlining denotes V8 protease digested cells. Shading denotes the ratio when the rate of agglutination of each culture are comparable.

3.6.2.2 Analysis of antigenic phenotypes post-digestion

Mixed agglutination assays were repeated on cells from $A4_{i+}$, $C18_{i-}$ and A4V8CD36 before and after digestion by V8 protease using the optimum ratios of cells determined above. The percentage of mixed colour agglutinates only is shown in Table 3.8, and these data are summarised in Figure 3.12. These results demonstrated that the V8 protease-treated cells from $A4_{i+}$ which formed agglutinates were antigenically similar to undigested $C18_{i-}$ and A4V8CD36. Also, the V8 protease treated parasites from $A4_{i+}$, $C18_{i-}$ and A4V8CD36 were antigenically related to each other and were similar to the undigested C18VAT. Thus, in the clones examined it appeared that V8 protease resistant agglutination was restricted to a single VAT which was preserved after digestion.

	DAPI (blue) stained test culture					
EtBr culture	**$A4_{i+}$**	***$A4_{i+}$***	**$C18_{i-}$**	***$C18_{i-}$***	**A4V8CD36**	***A4V8CD36***
$A4_{i+}$	99					
$A4_{i+}$	14.5	100				
$C18_{i-}$	18	59	100			
$C18_{i-}$	15.5	67	64	98		
A4V8CD36	14	68.5	63	64	98	
A4V8CD36	15.5	74	68.5	70	77	97

Table 3.8. Mixed agglutination data from $A4_{i+}$, $C18_{i-}$, and A4V8CD36. Mean number of agglutinates stained with both EtBr and DAPI are shown in a single representative experiment. Underlining and italics denotes V8 protease-digested cultures.

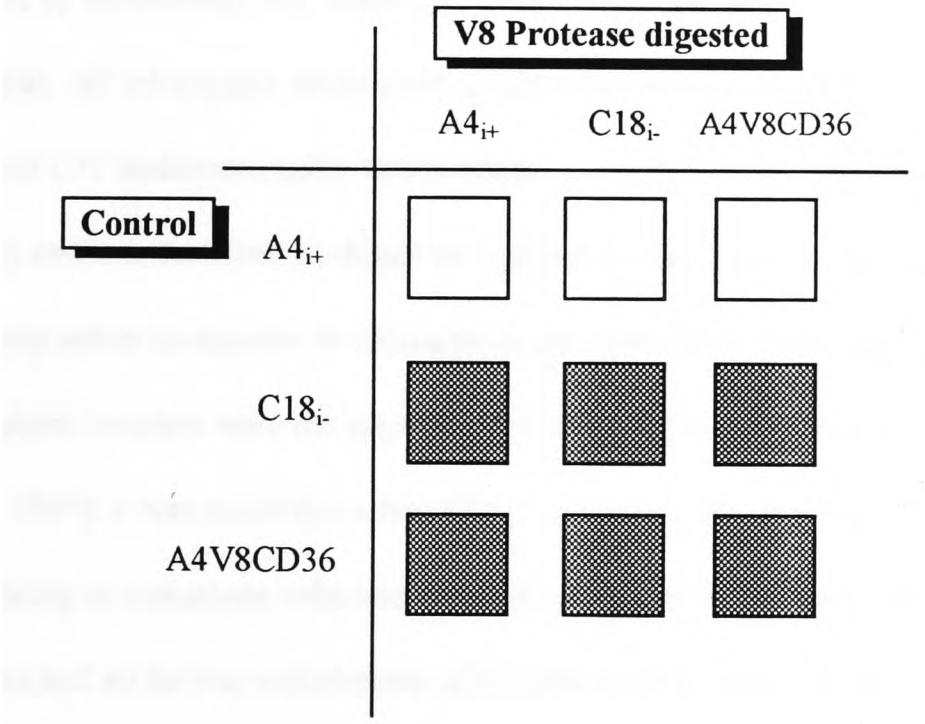


Figure 3.12. Mixed agglutination analysis of cells from $A4_{i+}$, $C18_{i-}$ and A4V8CD36, before and after V8 protease digestion at 10 Uml^{-1}. Key: ▩ 31-19%, □ 5-0% similarity, calculated as described in earlier footnote and section 2.2.10. Data are from duplicate determinations from at least two separate experiments

3.7. Conclusions

It was rationalised that surface phenotypes were mediated by variable molecules and should be differentially sensitive to sequence-specific proteases. The initial objectives of this chapter were to investigate the variant-specificity of defined proteases as a means of dissecting phenotypic heterogeneity that was a consequence of the high rate of clonal antigenic variation (Roberts *et al.*, 1992). This was successful, and this study has therefore repeated and extended previous observations made by others that had suffered by the use of relatively undefined parasites (Table 3.9).

It was first noted in 1983 by David and colleagues that low concentrations of trypsin and chymotrypsin abolished binding of *P. falciparum* infected squirrel monkey red blood cells to C32 melanoma cells *in vitro*. Conversely, high concentrations of neuraminidase resulted in enhanced binding in the same study. Since then, it has been established that CD36 is the major surface ligand expressed by melanoma cells, although ICAM1 was sometimes detected at low levels (Ockenhouse *et al.*, 1991a). All subsequent investigations have concluded that parasite binding to CD36 (using fixed or unfixed C32 melanoma cells) was sensitive to trypsin, chymotrypsin and pronase (non-specific protease) and was therefore mediated by exposed surface proteins. Binding of parasites to the same targets was either insensitive or enhanced by neuraminidase treatment which implied that carbohydrate moieties were not important for this interaction. However, in one study (Sherman and Valdez, 1989), it was noted that when FCR-3 cells were digested with TPCK-treated trypsin at 0.1mg ml^{-1}, binding to melanoma cells was reduced by only 60%. No comment was made on this apparent resistance and no further experiments were carried out to dissect this finding.

Reference	Parasite(s)	Target	Protease	Effect
David *et al.*, 1983	Palo Alto PLF-3, clone PLF-3 / B11	C32 melanoma cells	chymotrypsin trypsin neuraminidase	abolished binding abolished binding enhanced binding
Hommel *et al.*, 1983	FCR-3, Indochina-1, Palo Alto PLF-3	IFAT[1]	chymotrypsin trypsin neuraminidase	abolished antigenicity abolished antigenicity no effect
Leech *et al.*, 1984	Camp, St Lucia	C32 melanoma cells	trypsin	abolished binding
Marsh and Howard, 1986[2]	Field isolates	agglutination	tryspin neuraminidase	abolished agglutination no effect
Magowan *et al.*, 1988	ITG2F6 and derived clones	fixed C32 melanoma cells	tryspin	abolished binding
Sherwood *et al.*, 1989	Camp	fixed C32 melanoma cells	trypsin	1% resistant binding
Ockenhouse *et al.*, 1989	Camp	purified CD36	chymotrypsin trypsin (0.1mg ml^{-1}) neuraminidase	abolished binding abolished binding no effect
Sherman and Valdez, 1989	FCR-3	C32 melanoma cells	chymotrypsin trypsin pronase neuraminidase	abolished binding 40% resistant binding abolished binding enhanced binding by 50%
Biggs *et al.*, 1990	FAC8 and B8C(K^-)	C32 melanoma cells	trypsin	abolished binding
Chaiyaroj *et al.*, 1994a	FAF6 and B8C(K^-)	C32 melanoma cells and agglutination	trypsin	abolished binding and agglutination

Table 3.9. Summary of published data related to protease sensitivity and binding to CD36.

[1] IFAT - Indirect Fluorescent Antibody Test: measures immunofluorescence and provides an indication of antigenicity

[2] unpublished reference in paper

There were numerous faults and drawbacks in the published studies which are summarised below:

a) It was unclear as to whether contaminating protease activities had been inhibited or not. Many low-grade proteases contain contaminating activities and this is of particular importance for chymotrypsin and trypsin which are extracted from the same source (bovine pancreas). Thus, chymotrypsin should have been pretreated with TLCK and trypsin pretreated with TPCK to avoid any misleading results. This was not always stated.

b) Neuraminidases cleave sialic acid residues off carbohydrate chains, but have different specficities which are dependent on their source. The specificity and source of neuraminidases were rarely stated.

c) Different proteases were not compared at identical activities. Protease activities are measured by assessing rate constants for digestion of standard substrates. Often, these substrates vary and units are not consistent between manufacturers. Activities were not stated and similar concentrations were used instead.

d) A limited number of parasites were used with undefined antigenic phenotypes. Thus, no attempt could be made to investigate the influence of antigenic phenotype on the measured properties, and only binding to CD36 was studied.

e) The intrinsic variation of the infected cell surface phenotypes makes accurate analysis of agglutination and binding more complicated. Several methods for studying cytodherence were non-physiological (eg. allowing parasites to settle for 20 minutes on melanoma cells (Sherman and Valdez, 1989)), or characterised by inconsistent results (radiolabelled parasite cell binding assay (David *et al.*, 1983)).

In this chapter, a number of parasites with well-characterised surface phenotypes were digested with 6 different proteases. The effects of proteolysis on agglutination, antigenic phenotype and binding to purified CD36 of a culture were examined using improved, standardised assays that gave reproducible results. In accordance with reports from other laboratories, it was found that agglutination in pooled adult endemic area serum and binding to CD36 were both reduced by trypsin and chymotrypsin. However, low concentrations of trypsin caused an increase in agglutination but destroyed binding to

CD36 in one clone ($A4_{i+}$). This phenomenon was studied in relationship to adhesion to ICAM1 in more detail in chapter 4.

Two recent studies by Chaiyaroj and colleagues have found that some parasites exhibited trypsin resistant agglutination when selected independently for adhesion to HUVECs (1994a) or C32 melanoma cells (1994b). Trypsin resistant binding occurred only to the cultured cell that each parasite had been selected on, but the receptors that mediated the resistant binding have yet to be identified because neither parasite bound to CD36, ICAM1 or TSP after digestion. These data are discussed in more detail in chapter 8, but it is clear that there are additional complexities introduced when whole cell models are used to dissect cytoadherence.

Involvement of surface carbohydrate structures in binding to CD36 and agglutination was less clear. Normal erythrocytes are negatively charged due to the presence of sialic acids on surface glycophorin which contains 15 O-linked sialotetrasaccharides and a single N-linked oligosaccharide per molecule (reviewed by Anstee, 1990). High concentrations of neuraminidases with different specificities did not affect binding to CD36 of PRBC from two antigenically distinct clones. Neuraminidase from *C. perfringens* did not affect agglutination and has a broad specificity with no preferences in practice between the linkage of the sialic acids to monosaccharides, because the K_m and V_{max} values are high (Corfield *et al.*, 1981). Neuraminidase from *V. cholerae* enhanced the agglutination of PRBC from both clones and has a preference for α2-3 linkages over α2-6 or α2-8 linkages (Greenwalt *et al.*, 1973; Schauer, 1973). This is likely to be due to the decrease in negative charge by preferential cleavage of the α2-3 linked sialic acid residues from glycophorin (Rogers *et al.*, 1992). This linkage is more abundant on glycophorin than α2-6 or α2-8 linkages (Fukuda *et al.*, 1987), and cleavage resulted in a decrease in the repulsion of digested PRBC which can in turn, form agglutinates more readily, but did not affect binding to CD36 (Sherman *et al.*, 1992). One report has indicated that carbohydrate chains are important for the overall antigenicity of the parasite (Ramasamy and Reese, 1985), but these results make it unlikely that carbohydrate chains contribute to the surface antigenicity of variant clones because similar results were obtained from two different VATs.

The most illuminating results were obtained with the acidic amino acid specific enzyme, V8 protease. Agglutination of cells from $A4_{i+}$ was completely resistant to high concentrations of this protease and

there was a dramatic increase in the mean size of the agglutinates. This was observed at 40U ml^{-1} V8 protease (equivalent to 4mg ml^{-1} trypsin) and after extended lengths of digestion, indicating that a limit digestion had occurred.

The same pattern of resistance was observed in $C9_{i+}$, a subclone of $A4_{i+}$, which was antigenically indistinguishable from its parent. Agglutination of other clones was completely sensitive, except $C18_{i-}$ which formed larger agglutinates after V8 proteolysis and at significantly higher levels than controls. When binding to CD36 was examined, parasites that expressed the A4VAT were approximately 20% resistant to V8 protease and cells from $C18_{i-}$ were completely resistant. All other clones exhibited V8 protease sensitive adhesion to CD36.

The apparent correlation between resistant agglutination and resistant binding to CD36 was tested by selection of the resistant cells within $A4_{i+}$ after V8 proteolysis on CD36 (summarised in Figure 3.13). The selected culture (A4V8CD36) was antigenically similar to $C18_{i-}$ and showed identical protease sensitivities to $C18_{i-}$ (enhanced agglutination and resistant CD36 binding after V8 proteolysis). Further analysis of V8 protease-digested cultures by the mixed agglutination assay demonstrated that V8 protease resistant agglutination within $A4_{i+}$ was mediated by cells that expressed the C18VAT. Taken together, these findings strongly implied that A4V8CD36 was the C18VAT expressing population of cells within $A4_{i+}$. The C18VAT was preserved after V8 digestion of $C18_{i-}$ and A4V8CD36 cells demonstrating that the resistant phenotype was restricted to a single VAT. The increase in mean size of agglutinates after digestion by V8 protease could be due to a change in the electrostatic surface charge of the infected red cells which could decrease their repulsion of each other. Alternatively, the proteins that carry the epitopes crucial to agglutination could be more accessible or increased numbers could be exposed to antibodies after digestion. This may explain the apparent paradox that complete resistance of $A4_{i+}$ agglutination occurs after V8 protease digestion, but only a subpopulation of PRBC within the culture can form agglutinates. This hypothesis is supported by the dilution factors necessary to normalise the rates of agglutination of digested and undigested PRBC during mixed agglutination analysis.

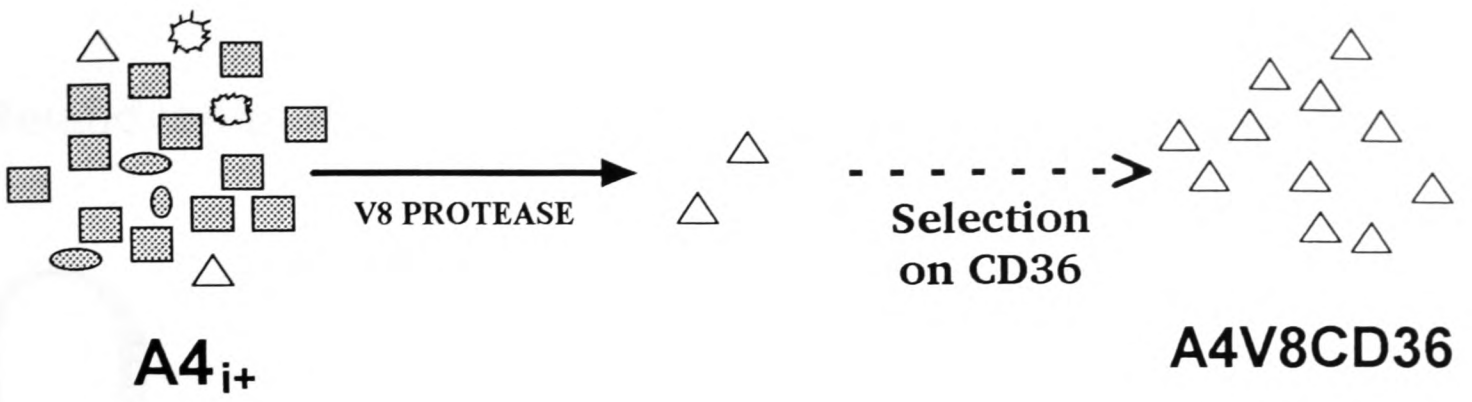


Figure 3.13 Diagrammatic summary of derivation of A4V8CD36 from $A4_{i+}$. Shape of surrounding denotes the antigenic phenotype of the PRBC: square = A4VAT, triangle = C18VAT. Shading represents ability to bind to ICAM1: dark shading = positive binding, no shading = no binding.

Thus, at the simplest level, these results provide biochemical confirmation of the heterogeneity of cloned parasites, since this study has demonstrated unequivocally that the C18VAT was present as a subpopulation within $A4_{i+}$. Field studies have shown that most parasites can adhere to purified CD36. Thus a conserved functional domain would have to be coexpressed with variable antigenic determinants to explain the apparent linkage between these properties.

In a recent study by Reeder and colleagues (1994), parasites from 20 patients with non-severe malaria in Papua New Guinea were tested for their ability to bind to C32 melanoma cells, HUVECs, purified ICAM1, and purified CD36. One isolate was found to bind with much greater avidity to purified CD36 than the other isolates, although no laboratory strain was included as an internal control. The same isolate did not bind to ICAM1 and was unique in its ability to form massive (> 100 cells) agglutinates in the available immune sera. The protease sensitivities of these antigenic phenotypes were not characterised, but this isolate could represent a wild-type parasite with similar characteristics to the V8-protease treated $C18_{i-}$ clone.

Another important finding was that these data represent the first demonstration of protease resistant agglutination and binding to CD36. The restriction of this phenotype to a single variant antigenic phenotype implies that these functions were mediated by a particular variant antigen. The variability of such a molecule would explain the differential availability of V8 protease cleavage sites between antigenic variants. This hypothesis was studied further by examining the effect of trypsin and V8 protease on surface-labelled PRBC to identify the molecules that were responsible for these phenotypes.

Chapter 4

Relationship of Adhesion to ICAM1 and Antigenic Phenotype

Observations that sequestration in cerebral vessels may be related to the pathogenesis of cerebral malaria have focused attention on the molecular mechanisms of cytoadherence. Parasitised red blood cells (PRBC) from field isolates and laboratory lines can adhere at different levels to thrombospondin (TSP), CD36, ICAM1, VCAM1 and E-selectin (reviewed by Pasloske *et al.*, 1994). However, the contribution of each receptor to disease severity and the relative importance of adhesive phenotypes at the PRBC surface are unclear. Immunohistochemical studies of *post mortem* tissues have detected ICAM1 on the surface on cerebral microvessels in patients with cerebral malaria (Ockenhouse *et al*,. 1992b; Turner *et al.*, 1994). Furthermore, expression of ICAM1 by endothelial cells is upregulated by TNF during an inflammatory response (Rothlein *et al.*, 1986), and circulating levels of this cytokine are increased in patients with severe disease compared to patients with mild disease (Grau *et al.*, 1989; Kern *et al.*, 1989; Kwiatkowski *et al.*, 1990; Ringwald *et al.*, 1991). Thus, cytoadherence to ICAM1 could represent a preferential pathway of sequestration in severe disease. Also, *in vitro* cell models have shown that ICAM1 mediates the TNF-induced cytoadherence to human umbilical vein endothelial cells (HUVECs) (Berendt *et al.*, 1989; Udeinya *et al.*, 1993).

These studies have suggested that adherence to ICAM1 may be a parasite virulence factor. The relatively low occurrence of cerebral malaria cases compared to the total number of infections could then be explained by the low frequency with which ICAM1 binding parasites occur (Roberts *et al.*, 1993; Hommel, 1993). Conclusive evidence for this hypothesis has not been obtained. However, *in vitro* studies have established that only a subset of field isolates and laboratory-derived lines can adhere to purified ICAM1 (Reeder *et al.*, 1994; C. I. Newbold, personal communication).

Indirect data has linked the ICAM1 binding phenotype at the PRBC surface to the antigenic phenotype of the culture. Using cloned parasites, Roberts and colleagues (1992) proved that antigenic phenotypes could switch at a high rate and switching was always accompanied by a loss of ICAM1 binding. This could be reversed by selection on purified ICAM1, and the original antigenic phenotype

was expressed. Biggs and colleagues (1992) found that adherence of PRBC to HUVECs selected for the same antigenic variant in three separate experiments and concluded that adherence to ICAM1 (the main receptor on HUVECs) was linked to the PRBC antigenic phenotype.

A second important conclusion from both studies was that the antigenic phenotype associated with ICAM1 adherence was relatively restricted. Even though different typing methods were utilised in each experiment, the expression of a single variant antigenic phenotype was correlated with the ability of PRBC to bind to ICAM1. Thus, it was proposed that ICAM1 binding variants may represent a subset of the total antigenic repertoire. If ICAM1 binding parasites are involved in the pathogenesis of severe disease then virulent parasites may represent a subset of the total variant repertoire.

Elucidation of the relationship of ICAM1 adherence to antigenic phenotype is thus central to an understanding of parasite virulence. The extent of the antigenic repertoire and switching pathways involved in the production of the apparent antigenic restriction in ICAM1 binding parasites have not been examined. Also, factors, including surface morphology, which may influence the cytoadherent and antigenic properties of ICAM1 binding parasite lines have not been investigated in a comparative fashion. All of these issues are addressed in this chapter.

4.1. Methods

ICAM1 is a member of the immunoglobulin (Ig) superfamily with 5 tandemly linked Ig-like domains (see table 1.3 for summary). A series of PCR-generated Ig domain expression constructs of ICAM1 had been synthesised in COS cells to determine the site of interaction for adherence to the infected erythrocyte (Berendt *et al.*, 1992). Antibody blocking experiments using a range of constructs located the PRBC binding region to domain 1 on ICAM1 and also showed that domain 2 was necessary for maximal binding. This suggested that domain 2 was involved in maintaining an accessible conformation of domain 1 and all constructs containing both domains would mediate efficient PRBC adhesion.

It was anticipated that a large amount of ICAM1 would be required for the cytoadherence assays discussed in this chapter and for the immunoprecipitation experiments in chapter 6. An unpublished protocol had been developed in the laboratory by Drs. A. R. Berendt and A. G. Craig which used transfected COS cells to synthesise ICAM1-F_c chimaeras which could be subsequently purified by affinity chromatography. This protocol was used repeatedly to generate several ICAM1-F_c constructs that mediated the adhesion of infected erythrocytes. An outline and the principles of the methodology are presented here and a more detailed manuscript is in preparation (A. G. Craig, personal communication).

4.1.1 COS cell expression

A number of ICAM1-human IgG1-F_c chimaera plasmid constructs were constructed by Dr. A. G. Craig that encoded 2 domain ICAM1 (2d; domains 1 and 2), 3 domain ICAM1 (3d; domains 1, 2 and 3), and 5 domain ICAM1 (5d; domains 1, 2, 3, 4 and 5). These DNA molecules were transiently transfected into confluent COS cells in petri dishes by DEAE dextran followed by DMSO shock, or by electroporation. After 2-5 days of expression, cell supernatants that contained ICAM1-F_c were harvested.

4.1.2 Purification of ICAM1-F_c

ICAM1-F_c chimaeras were purified by virtue of the affinity of the human F_c portion for protein A. The filtered COS cell supernatants were loaded onto a column consisting of protein A which had been coupled to sepharose beads. The column was pre-equilibrated with sodium phosphate buffer, pH 7, flow rate of 1ml min^{-1}, to permit efficient binding of the F_c portion to the protein A. Unbound material was washed away with 5 column volumes of buffer at pH 7 and the bovine Ig molecules from the culture medium were eluted by first washing in sodium citrate buffer at pH 5. A characteristic peak occurred on the chart recorder which measured the absorbance of the column eluates and translated the value into a deflection on the trace (Figure 4.1). When the reading had returned to the base-line, all of the contaminating bovine Ig molecules had been eluted from the column, and the buffer was replaced with a sodium citrate buffer at pH 3 to elute the bound ICAM1-F_c. A second peak occurred on the chart trace when the ICAM1-F_c protein was detected by the recorder and the eluate was collected into bijou tubes that contained a small volume of 1M Tris at pH 8.8 for neutralisation. The trace returned to base-line when all of the protein had been eluted, and the column was washed thoroughly in sodium phosphate buffer at pH 7 before storage at 4°C.

The area under the peak gave an indication of the quantity of protein eluted, and the gradient of the peak was influenced by the purity of the protein and the physical characteristics of the column. A sharp, high peak was frequently observed which indicated that a good yield of pure ICAM1-F_c had been obtained. The concentration of eluted protein was determined accurately after dialysis against PBS / 0.1%(w/v) sodium azide either by measuring the optical density of the solution or by comparison with known standards on a Coomassie-stained SDS PAGE gel (Figure 4.2). The efficiency of each preparation of ICAM1 F_c for mediating PRBC cytoadherence was determined by titration of the solution in the spot binding assay. The 2d, 3d, and 5d ICAM1-F_c constructs mediated adhesion at equivalent levels (A. G. Craig, personal communication), and were used throughout this study in standardised batches of known concentration and avidity. Aliquots that contained large amounts of contaminating bovine immunoglobulin or those that did not bind infected cells were re-purified by a second cycle of affinity chromatography.

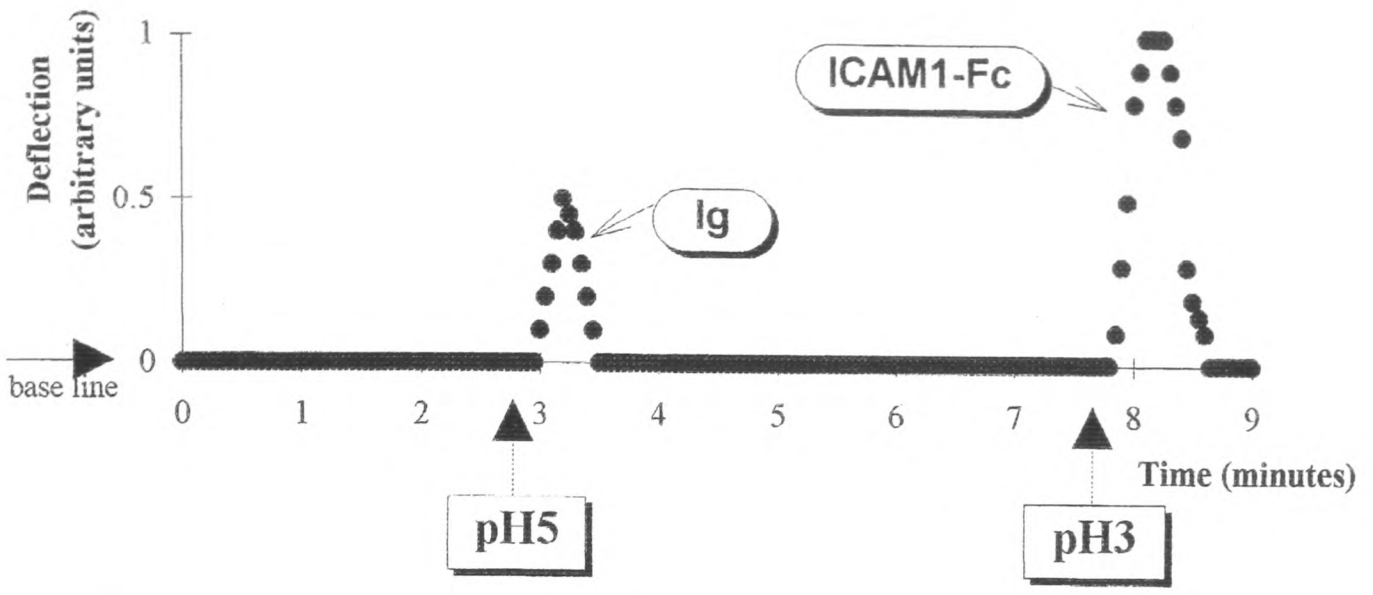


Figure 4.1. Representative chart recorder trace during the affinity chromatography purification of an ICAM1-F_c construct after expression in COS cells. Deflection is a caused by an increase in light absorbance in the column flow-through solution measured by a light sensor in the chart recorder. The arrows denote the time when the pH5 and pH3 buffers were applied, and the peaks correspond to the bovine Ig molecules or the ICAM1-F_c chimaera eluants. The timings are not to scale.

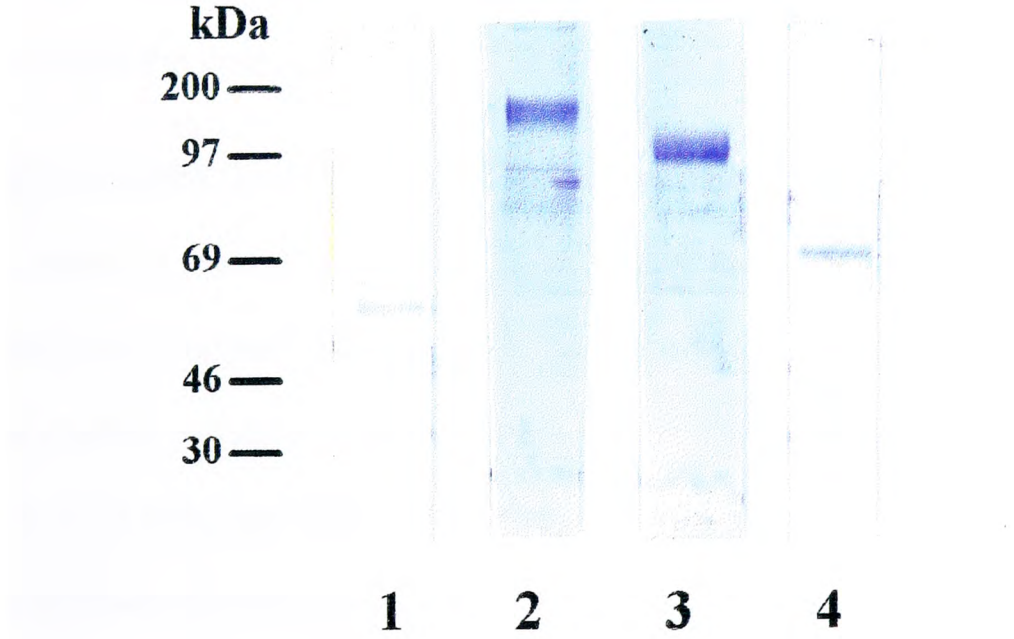


Figure 4.2. SDS PAGE gel of eluted proteins stained with Coomassie blue. KEY: lane 1: pH5 eluate containing bovine Ig heavy and light chains, lane 2: 5d ICAM1F_c (M_r = 115kDa), lane 3: 3d ICAM1F_c (M_r = 85kDa), lane 4: 2d ICAM1F_c (M_r = 65 kDa). Protein standards (kDa) are shown on left.

4.2. Selection of PRBC on purified ICAM1

Phenotypic antigenic switching had been observed by selecting a single parasite for adherence to purified ICAM1 (Roberts *et al.*, 1992). In this study, one round of panning of a non-ICAM1 binding clone, $C28_{i-}$, selected an ICAM1 binding line, C28-I. The antigenic phenotype of C28-I was different from $C28_{i-}$ but was similar to $A4_{i+}$, the ICAM1 binding parent from which $C28_{i-}$ had been subcloned (Figure 2.1). This was a clear demonstration of clonal antigenic variation in *P. falciparum*, and implied that the genes conferring the ability to bind to ICAM1 were conserved during switching. In the same study, all ICAM1 binding subclones of $A4_{i+}$ expressed the same antigenic phenotype, referred to as A4VAT, as assessed by the mixed agglutination assay using Sib serum.

Thus, there was only one antigenic phenotype associated with ICAM1 binding PRBC in this clone family. In view of the results in the previous chapter in which it had been possible to dissect the variant-specific effect of a number of proteases, it was decided to select several other clones on ICAM1 in attempt to widen the range of variants adhering to this ligand.

Two other non-ICAM1 binding clones, $C18_{i-}$ and $C24_{i-}$, were used to select ICAM1 binding lines using two rounds of panning (section 2.2.7). In addition, the ICAM1 binding population within the parent clone, $A4_{i+}$, was studied by two selections of this parasite on purified protein, and C28-I was reselected a further two times using the same technique. The adherent and antigenic phenotypes of the resultant ICAM1 binding parasites, C18-I(2), C24-I, A4-I and C28-I* were extensively characterised using the optimised techniques described in chapter 3. In addition, the $IT6_{i+}$ parasite was studied because it had been derived in another laboratory from IT by 6 rounds of selection on ICAM1 (Figure 2.1), but had not been further characterised in our laboratory (Ockenhouse *et al.*, 1991a).

4.2.1 Cytoadherent phenotypes of ICAM1 binding PRBC

A direct comparison of levels of adherence to purified ICAM1 and CD36 in the optimised spot binding assay (section 2.2.6) was carried out before and after selection of clones on ICAM1 (Figure 4.3).

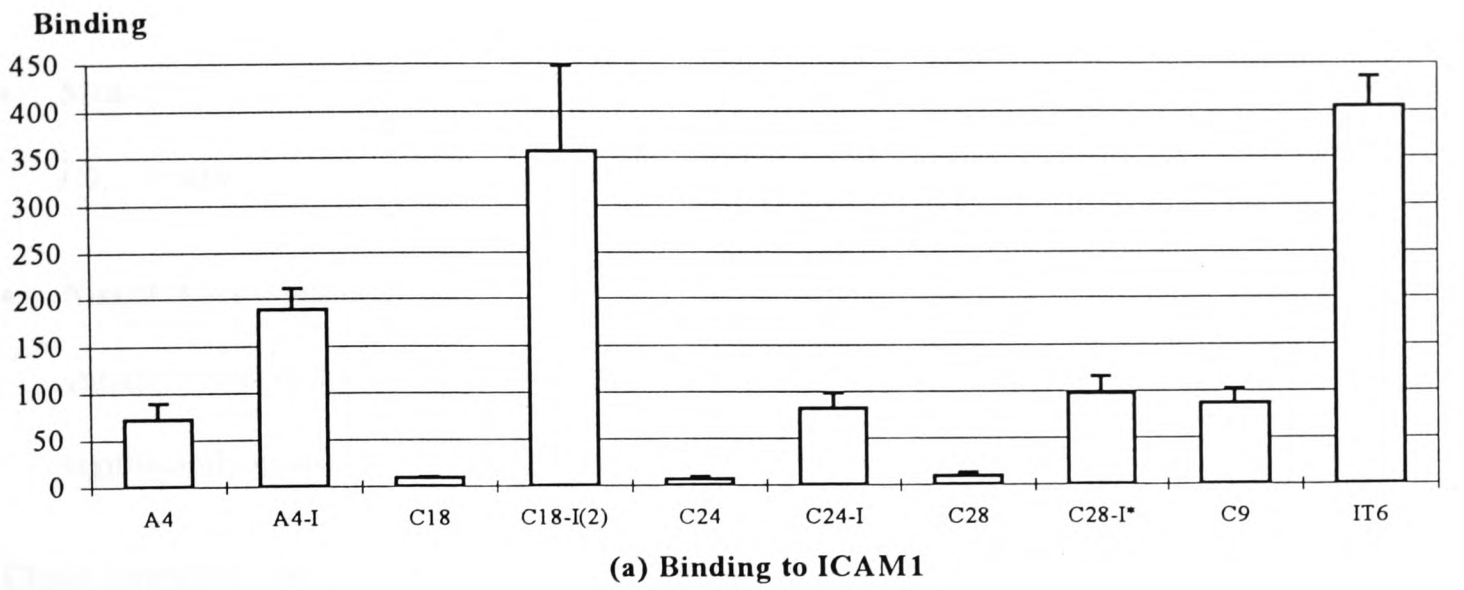


Figure 4.3. Comparison of absolute binding of a series of lines and clones to (a) ICAM1 and (b) CD36. Results are mean cells bound mm^{-2} ± SEM from at least four experiments. Data were corrected to 1% haematocrit and parasitaemia within ranges that allowed for comparison between parasites (section 3.3 and Roberts et al., 1992).

All of the selection procedures increased the overall levels of binding to ICAM1 without a concomitant increase in CD36 binding. Examination of the levels of binding to ICAM1 reveals three categories of parasites with differing levels of avidity.

- **High**-ICAM1 binding parasites (>350 cells bound mm^{-2}) include C18-I(2) and $IT6_{i+}$. Binding to CD36 was in a similar range.

- **Mid**-ICAM1 binding parasites (70-190 cells bound mm^{-2}) include $A4_{i+}$, A4-I, C24-I, C28-I* and $C9_{i+}$. Binding to CD36 was in similar range.

- **Non**-ICAM1 binding parasites (<10 cells bound mm^{-2}) include $C18_{i-}$, $C24_{i-}$, $C28_{i-}$ and other variant clones of $A4_{i+}$ as described before (Roberts *et al.*, 1992). Binding to CD36 was significantly greater than binding to ICAM1 and was in the range 267-365 cells bound mm^{-2}.

Closer inspection revealed that selection for binding to ICAM1 can increase the levels of binding to this ligand and either have no effect on levels on CD36 binding (i.e. A4-I and C18-I(2)), or can produce a large reduction (~50%) in the levels of CD36 binding (i.e. C24-I and C28-I*). This apparent reciprocal linkage in these two lines may reflect an enrichment of the ICAM1 binding population over the CD36 binding population and is discussed later.

4.2.2 Antigenic phenotypes of ICAM1 binding PRBC

The mixed agglutination assay was used to characterise the antigenic phenotypes of the ICAM1 binding parasites (section 2.2.10). These experiments were performed in duplicate on two separate days in conjunction with Dr. D. J. Roberts and R. A. Pinches (both Oxford). The results generated by myself are shown in table 4.1(a) and the data obtained by my collaborators in table 4.1(b). All methods and analysis of data were identical throughout this study and were based on those used by Roberts *et al.*, 1992 and Newbold *et al.*, 1992.

EtBr (red) stained test culture	DAPI (blue) stained test culture				
	$A4_{i+}$	**A4-I**	**$C18_{i-}$**	**C18-I(2)**	**$IT6_{i+}$**
$A4_{i+}$	99				
A4-I	72	100			
$C18_{i-}$	17.5	ND	96		
C18-I(2)	13	25	15.5	97	
$IT6_{i+}$	11.5	16	14	92	99

Table 4.1a. Mixed agglutination data using PRBC stained with EtBr or DAPI. Test cultures were mixed with Sib *serum on the rotator as described in section 2.2.10, and 100 agglutinates of 5 - 10 cells each were counted. The number of mixed agglutinates only is recorded, and is the mean of 4 determinations in 2 separate experiments. ND - not done.*

EtBr (red) stained test culture	DAPI (blue) stained test culture				
	$A4_{i+}$	**$C9_{i+}$**	**C24-I**	**C28-I***	**$IT6_{i+}$**
$A4_{i+}$	98				
$C9_{i+}$	95	98			
C24-I	22	ND	98		
C28-I*	61.5	ND	51.5	97	
$IT6_{i+}$	10	ND	54	65	97

Table 4.1b. Mixed agglutination data obtained by Dr. D. J. Roberts and R. A. Pinches. Key as above, and mean values of 4 determinations from 2 experiments are quoted. Cultures were stained with either EtBr or DAPI and mixed with Sib *serum on the rotator. 100 agglutinates of 5 - 10 cells each were counted, and the number of mixed agglutinates only is recorded. ND - not done.*

The antigenic similarity of the test cultures was calculated from the % of mixed colour agglutinates formed in the above assays (section 2.2.10). A summary of the results is shown in figure 4.4, using ranges proposed by Roberts *et al.*, 1992 (section 2.2.10).

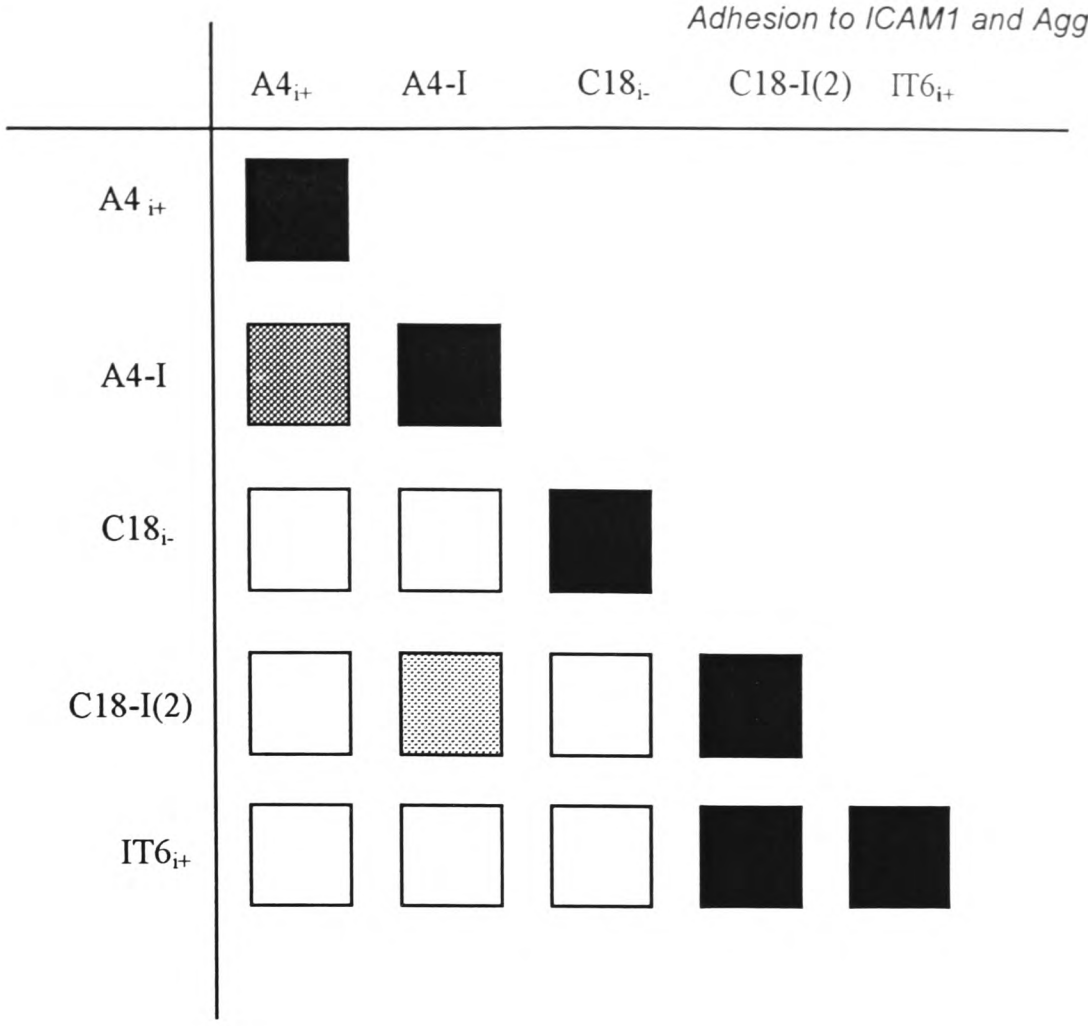


Figure 4.4a). Mixed agglutination analysis of cells from $A4_{i+}$, $C18_{i-}$, C18-I(2), and $IT6_{i+}$.

Key: ■ 100 - 79%, ▩ 78 - 15%, ▨ 14 - 6%, □ 5 -0% similarity.

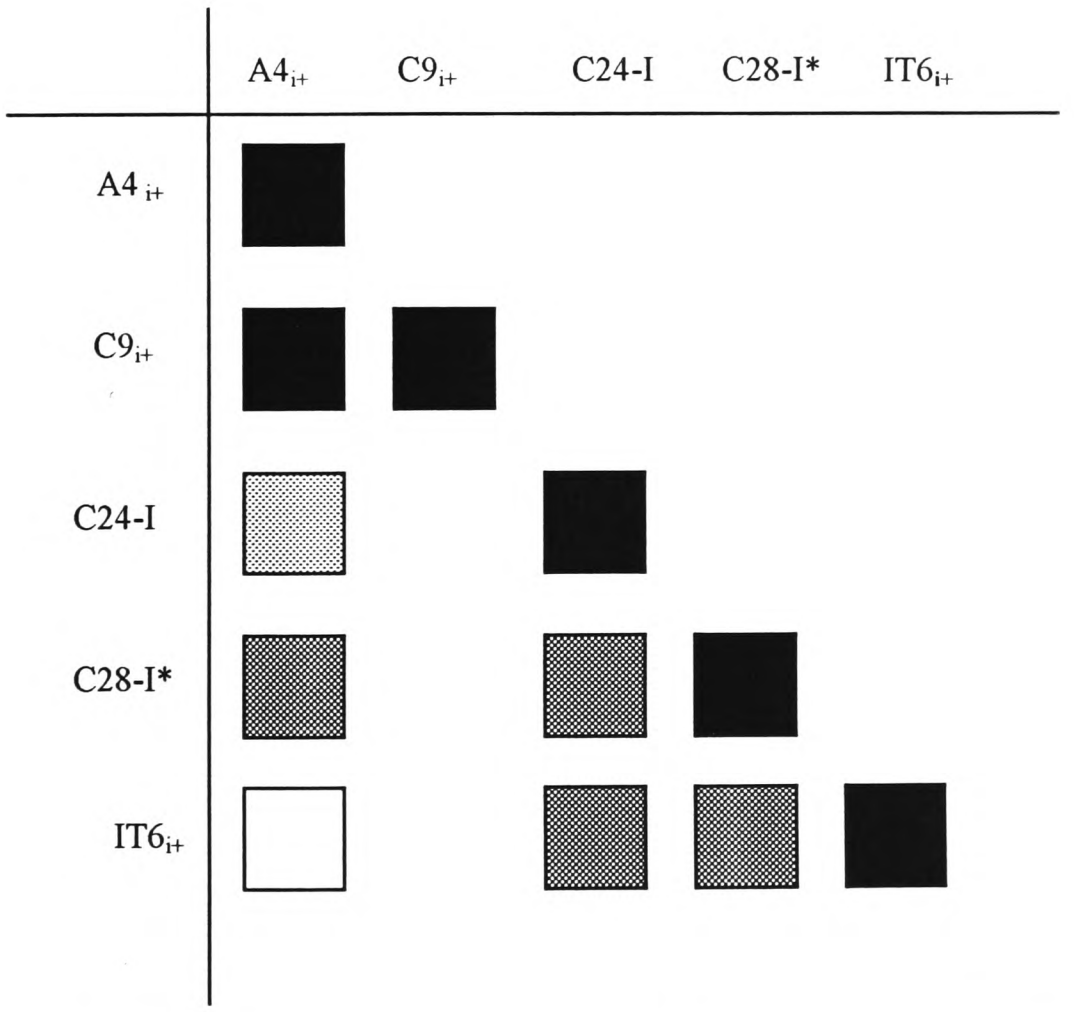


Figure 4.4b). Mixed agglutination analysis of cells from $A4_{i+}$, $C9_{i+}$, C24-I, C28-I and $IT6_{i+}$.*

Key: ■ 100 - 79%, ▩ 78 - 15%, ▨ 14 - 6%, □ 5 -0% similarity.

Examination of figure 4.4 shows that the A4VAT was the predominant antigenic type expressed by ICAM1 binding PRBC and was known to be the major VAT associated with all 10 ICAM1 binding C clones (eg. $C9_{i+}$) derived in previous subcloning experiments (Roberts *et al.*, 1992). Other non-ICAM1 binding C clones can be selected for adherence to ICAM1 and always undergo a concomitant switch in antigenic phenotype. $C24_{i-}$ behaved in a similar fashion to $C28_{i-}$ in an earlier study and switched back to the parental A4VAT phenotype after selection on ICAM1. Separate selections for adherence starting with PRBC from $A4_{i+}$ and C28-I had no effect on the antigenic phenotypes. $C18_{i-}$ was unique, because even though it had been subcloned from $A4_{i+}$, selection on ICAM1 produced a novel antigenic phenotype which was indistinguishable from that expressed by $IT6_{i+}$.

Thus, it appeared that there were at least two major antigenic phenotypes expressed by the ICAM1 binding PRBC derived from the IT line. The A4VAT was detected in a large proportion of A4-I, C24-I, C28-I*, and these lines had similar antigenic phenotypes to each other and to $C9_{i+}$. The antigenic phenotype of these PRBC was markedly different from the major antigenic phenotype expressed by C18-I(2) and $IT6_{i+}$.

Hence there was a correlation between antigenic phenotype and cytoadherent phenotype of these ICAM1 binding parasites: **mid** ICAM1 binders expressed the A4VAT and **high** ICAM1 binders expressed a novel VAT, referred to as the IT6VAT. The association of the antigenic and cytoadherent phenotypes was dissected with sequence specific proteases.

4.3. Effect of proteases on binding to ICAM1

Sequence-specific proteases had already proved to be a useful tool in the biochemical analysis of surface phenotypes of *P. falciparum* infected erythrocytes (chapter 3). Results with V8 protease from a range of cloned parasites demonstrated that protease-resistant binding to CD36 was correlated with the expression of a unique antigenic phenotype, C18VAT. This protease-resistant VAT could be expressed by minor subpopulations in other 'clones' because of the high rate of clonal antigenic variation and resulted in partially resistant phenotypes. The protease sensitivities of the antigenic and cytoadherent phenotypes of the **mid-** and **high-** ICAM1 binding PRBC were extensively examined with V8 protease and trypsin (Table 4.2). By analogy with the results in chapter 3, low levels of resistance were attributed to the presence of minor subpopulations.

Parasite	Binding to ICAM1	Binding to CD36	Agglutination
A4	2.2 ± 1.2	22.5 ± 6.3	102.0 ± 6.1
A4-I	1.7 ± 0.5	1.8 ± 1.2	0
C9	2.0 ± 0.3	23.1 ± 15.4	96.3 ± 10.2
C24-I	1.8 ± 0.5	0.7 ± 0.2	0
C28-I*	1.1 ± 0.8	2.3 ± 1.1	0
C18-I(2)	5.6 ± 3.5	6.2 ± 3.2	1.1 ± 0.3
IT6	6.3 ± 2.2	1.1 ± 0.7	2.2 ± 2.1

*Table 4.2a) Summary effect of **V8 PROTEASE** on ICAM1 binding parasites. Values are the resistance as expressed as percentage of untreated control ± SEM for at least three experiments. Parasites are grouped by antigenic phenotypes: no shading = A4VAT, light shading = IT6VAT.*

Parasite	Binding to ICAM1	Binding to CD36	Agglutination
A4	70.3 ± 8.2	0	25.0 ± 3.2
A4-I	8.7 ± 0.3	0	4.2 ± 1.3
C9	87.3 ± 16.4	0	10.2 ± 1.3
C24-I	12.1 ± 6.2	0	1.1 ± 1.5
C28-I*	4.8 ± 2.3	0	1.5 ± 1.7
C18-I(2)	5.6 ± 2.5	0	1.1 ± 1.2
IT6	2.3 ± 1.1	0	0.9 ± 1.3

*Table 4.2b) Summary effect of **TRYPSIN** on ICAM1 binding parasites. Values are the resistance as expressed as percentage of untreated control ± SEM for at least three experiments. Parasites are grouped by antigenic phenotypes: no shading = A4VAT, light shading = IT6VAT.*

The salient features of these tables and other data with neuraminidases can be summarised:

- ICAM1 binding was sensitive to **V8 protease** in all parasites, and resistant binding to CD36 was restricted to those PRBC that probably contained a low level of C18VAT within the culture. Agglutination of ICAM1 binding PRBC was also sensitive to V8 protease, except when the C18VAT was expressed as a minor subpopulation (i.e. within $A4_{i+}$ and $C9_{i+}$). The subpopulation of cells within $A4_{i+}$ that was known to express the C18VAT was diminished in A4-I, as demonstrated by a decrease in the % of mixed agglutinates formed when PRBC from A4-I and $C18_{i-}$ were tested (data not shown). Thus the C18VAT was refractory to selection on ICAM1 as expected, because $C18_{i-}$ did not bind to ICAM1.

- **Trypsin** has a variable effect on PRBC binding to ICAM1: cells from $A4_{i+}$ and $C9_{i+}$ exhibited high levels of resistant binding to ICAM1, but cytoadherence of other lines was sensitive to the same protease. PRBC from $A4_{i+}$ and $C9_{i+}$ also had significantly higher levels of resistant agglutination than other ICAM1 binders which was transiently increased at low trypsin concentrations (figure 3.8b, chapter 3). PRBC binding to CD36 was universally sensitive to trypsin.

- Digestion with **Neuraminidases** (from *V. cholerae* and *C. perfringens*), had no effect on ICAM1 binding of parasites from $A4_{i+}$ (126.9 ± 12.7 % control, n = 2) and C18-I(2) (105.2 ± 6.7 % control, n = 2). This implied that sialic acid residues on the PRBC surface were not essential for binding to ICAM1 by these cells.

A novel finding from this study was that trypsin-resistant binding to ICAM1 occurred in $A4_{i+}$, $C9_{i+}$ and in a small proportion of other ICAM1 binding lines. The protease sensitivities of parasite lines (C18-I(2), C24-I and C28-I*) which had been derived by selection of variant C clones on ICAM1 were identical, even though they expressed different antigenic phenotypes.

The available evidence suggested that the trypsin resistant ICAM1 binding phenomenon was not linked to expression of the A4VAT *per se*, but due instead to the expression of a novel, minor antigenic phenotype. Resistant binding to ICAM1 and agglutination were not observed in PRBC from A4-I, C24-I and C28-I*, which expressed the A4VAT. Using paradigms developed in chapter 3, it was rationalised that the observed trypsin-resistant ICAM1 binding and agglutination was due to an

antigenically distinct subpopulation within $A4_{i+}$ and $C9_{i+}$. The novel resistant phenotype would be expected to be distinct from the A4VAT and IT6VAT which confer protease sensitive surface properties.

4.4. Selection for trypsin-resistant binding to ICAM1

To investigate the trypsin resistant cytoadherence phenomenon, mature PRBC from $A4_{i+}$ were selected on purified ICAM1 immediately following trypsin digestion at 1mg ml^{-1}. The adherent cells were cultured for 5 cycles, and then re-selected on ICAM1 after trypsinisation to increase the homogeneity of the resistant population. A4TRY-I was cultured after the second selection and its antigenic and cytoadherent phenotypes were examined together with the protease sensitivities of these phenotypes (Table 4.3).

PRBC from A4TRY-I formed agglutinates in Sib serum but not Eur serum, and agglutination was resistant to trypsin, but sensitive to V8 protease. Undigested PRBC from A4TRY-I bound to both ICAM1 and CD36 which was interesting because it had been selected from PRBC (*trypsinised*-A4) that could only bind to ICAM1 (see conclusions). Digestion of A4TRY-I with trypsin at 1mg ml^{-1} resulted in enhanced binding to ICAM1 by approximately 400%, but binding to CD36 was ablated. This was the first demonstration of complete trypsin-resistant agglutination, and the only example of enhanced cytoadherence following trypsinisation. Agglutination and cytoadherence of A4TRY-I were sensitive to V8 protease, indicating that the domains that mediated these interactions had different sensitivities to each protease.

A4TRY-I	AGGLUTINATION (% Cells in Agglutinates)		CYTOADHERENCE (Cells mm^{-2})	
	Sib Serum	**Eur Serum**	**ICAM1**	**CD36**
Control	16.7 ± 5.3	1.2 ± 1.5	70.4 ± 18.5	145.2 ± 23.6
Trypsin (1mg ml^{-1})	15.8 ± 6.5	0	349.9 ± 55.2	0.1 ± 0.9
V8 Protease (10U ml^{-1})	1.2 ± 0.8	0.2 ± 1.5	0.4 ± 1.7	0.3 ± 1.3

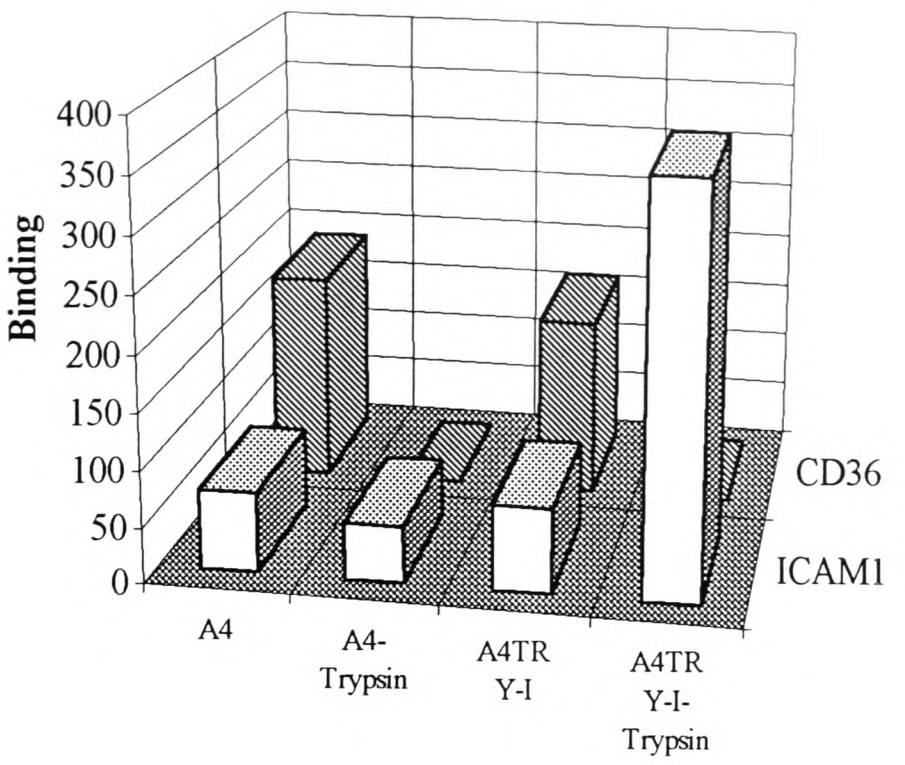
Table 4.3. Data from a representative experiment showing the effects of trypsin and V8 protease on the agglutination and cytoadherence of the selected culture, A4TRY-I. Results are the mean ± SEM of triplicate determinations from two experiments.

The relationship of the antigenic phenotypes of undigested and trypsinised $A4_{i+}$ and A4TRY-I were investigated. Table 4.4 shows that A4TRY-I was antigenically distinct from $A4_{i+}$, and that trypsinisation produced a novel phenotype that was not present in the control cultures. These phenotypes were distinct from the other identified ICAM1 binding phenotype, IT6VAT (data not shown).

There was a low degree of antigenic similarity between trypsinised cells from $A4_{i+}$ and A4TRY-I, which suggested that this novel phenotype was only revealed after trypsinisation and was associated with the dramatic increase in binding to ICAM1 observed after digestion of A4TRY-I. Thus, the apparent trypsin resistant ICAM1 binding observed in $A4_{i+}$ (70.3 ± 8.2% control, n = 5) was probably due to the trypsin-enhanced binding to ICAM1 of the A4TRY-I subpopulation (406.2 ± 120.7% control, n = 6). Using these values, A4TRY-I was estimated to constitute ~17% (i.e. 70 × 100/406) of the ICAM1 binding cells within $A4_{i+}$, assuming that all of the resistant binding to ICAM1 was due to A4TRY-I. However, this subpopulation was not detected by the mixed agglutination assay when $A4_{i+}$ and A4TRY-I were tested. A summary of the effect of trypsin on $A4_{i+}$ and A4TRY-I is shown in figure 4.5.

EtBr (red) stained test culture	**DAPI (blue) stained test culture**			
	$A4_{i+}$	**A4-*Trypsin***	**A4TRY-I**	**A4TRY-I-*Trypsin***
$A4_{i+}$	98			
A4-*Trypsin*	3.5	95		
A4TRY-I	7	8.5	99	
A4TRY-I-*Trypsin*	7.5	24	7	97

Table 4.4. Mixed agglutination data using cultures stained with either EtBr or DAPI and mixed with Sib *serum on the rotator. 100 agglutinates of 5 - 10 cells each were counted, and the number of mixed agglutinates from 4 determinations in 2 separate experiments is recorded.* Trypsin-*denotes that the cells had been digested with trypsin at 1mg* ml^{-1}*. The parasitaemia of A4-*Trypsin *was 4 times that of the other test cultures to ensure that agglutinates were formed at comparable rates (data not shown).*



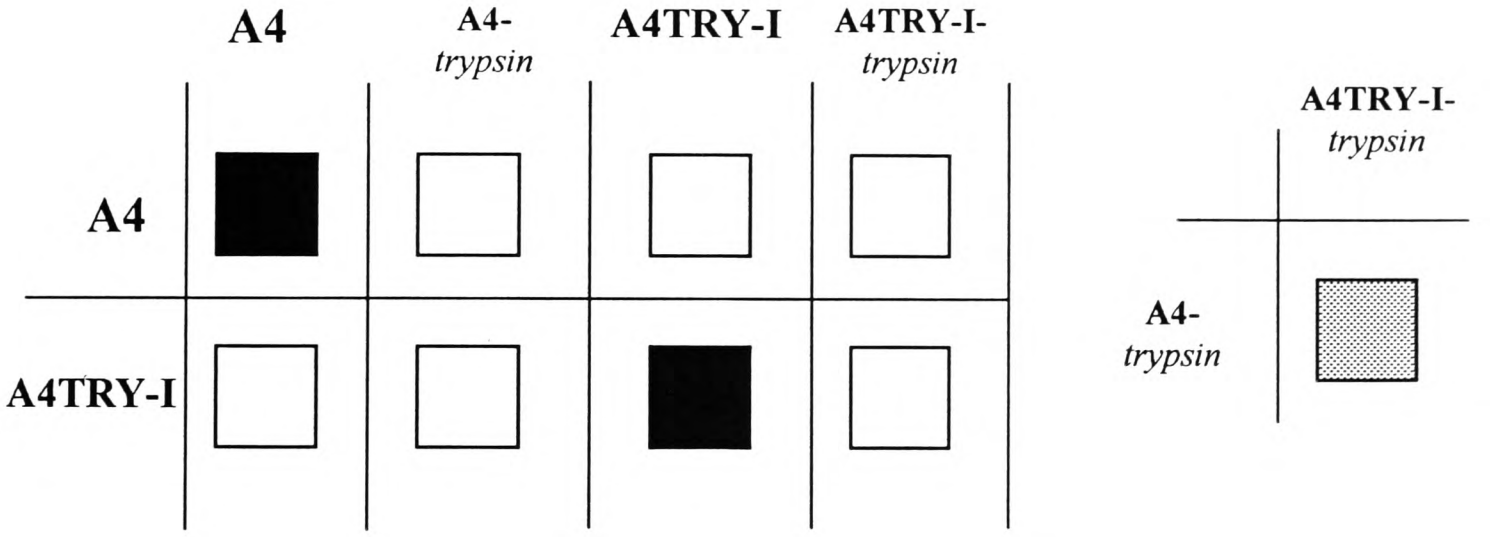


Figure 4.5. Summary of the effect of trypsin on $A4_{i+}$ and its trypsin-resistant subpopulation, A4TRY-I. 3D bar chart shows effect of trypsin on binding (PRBC mm^{-2} ; SEM were less than <10%) to CD36 and ICAM1 in three separate experiments, and boxes denote the antigenic similarities of the cultures to undigested populations as determined by the mixed agglutination assay (two experiments). Key:

■ *100-80%*, ▩ *7.1%*, □ *2.2-0% similarity.*

4.5. Elution of VAT-specific antibodies

An alternative approach to studying the antigenic characteristics of PRBC is to elute antibodies that bind to the antigenic determinants expressed at the surface (Marsh and Howard, 1986). Previous studies have shown that sera from immune adults are mixtures of variant specific antibodies which bind to the PRBC surface and mediate agglutination (Newbold *et al.*, 1992). It was reasoned that purification of VAT-specific antibodies from Sib serum would provide a useful reagent that could facilitate the purification of the variant antigen(s). Several earlier attempts at eluting antibodies from two C clones had failed (data not shown), possibly because variant-specific antibodies were present at low titres in the original polyclonal serum, or were not concentrated following elution.

The mixed agglutination data demonstrated an apparent correlation between adherence to ICAM1 and antigenic phenotype after typsinisation of A4TRY-I. To aid the molecular charaterisation of the ICAM1 binding ligand, it was decided to attempt to elute antibodies from untreated and trypsinised A4TRY-I and test their efficacy following a 10-fold concentration (section 2.2.11). A Coomassie-stained 10% polyacrylamide gel under reducing conditions showed the light and heavy antibody chains in the preparations (Figure 4.6). Results from agglutination assays with the same antibodies are shown in table 4.5.

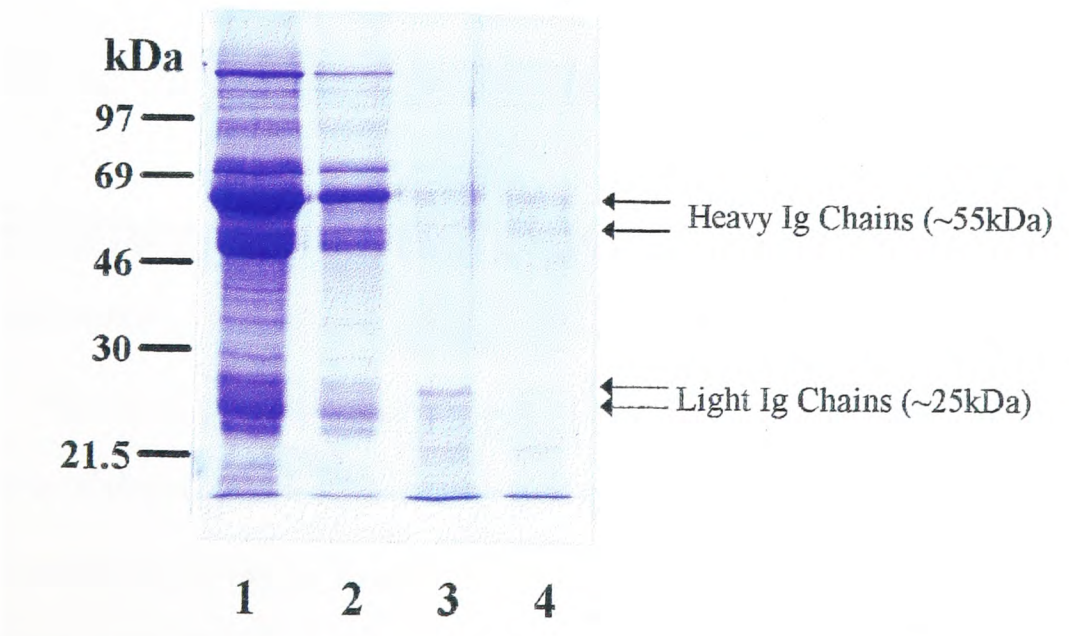


Figure 4.6. Coomassie-stained 10% acrylamide SDS PAGE gel of eluted antibody preparations from Sib *serum. Key: lane 1:* Sib *serum (1:10 dilution in RPMI-S), lane 2:* Sib *serum (1:50 dilution in RPMI-S), lane 3: antibody eluted from non-trypsinised PRBC, lane 4: antibody eluted from trypsinised PRBC. Protein standards in kDa are shown on left.*

PARASITE	Agglutination (% cells in agglutinates)					
	Controls		Antibody from control A4TRY-I PRBC		Antibody from A4TRY-I-*trypsin* PRBC	
	Sib Serum	Eur Serum	Eluted	Concentrated (×10)	Eluted	Concentrated (×10)
A4	16.2 ± 5.2	0.5 ± 0.6	0	0	0	0
A4-*trypsin*	2.3 ± 2.0	0	0	0	0	2.5 ± 1.7
A4TRY-I	15.2± 3.8	0.5 ± 1.1	0	0	0	0
A4TRY-I-*trypsin*	13.7 ± 3.9	0	0	0	0	5.3 ± 1.3
RBC	0	0	0	0	0	0
RBC-*trypsin*	0	0	0	0	0	0

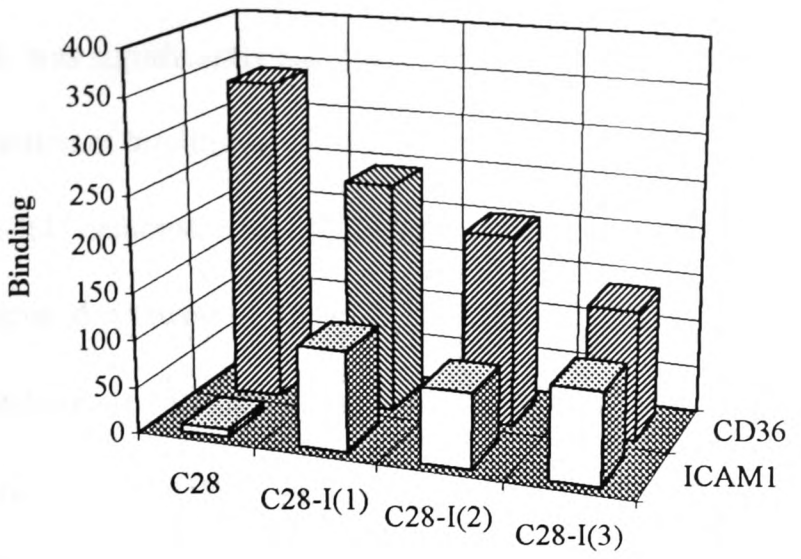
*Table 4.5. Effect of eluted antibodies from A4TRY-I and A4TRY-I-*trypsinised *on agglutination of PRBC and red blood cells (RBC). $A4_{i+}$ and A4TRY-I cells at 7% parasitaemia were digested with 1mg ml^{-1} of trypsin and standard agglutination assays performed (section 2.2.9 and chapter 3).* Sib *serum was used as a positive control at an actual dilution of 1:4 in RPMI-S, and Eur serum was used as a negative control at a dilution of 1:8 in RPMI-S. Results are the mean % of PRBC in agglutinates from duplicate determinations in 2 separate experiments.*

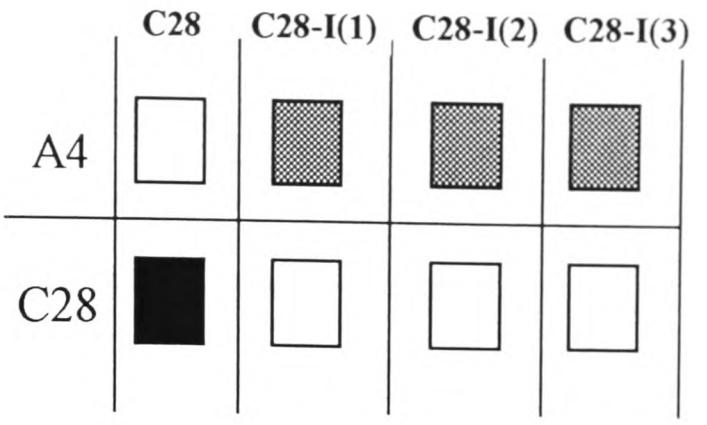
Table 4.5 shows that concentrated antibody from trypsinised A4TRY-I cells selectively agglutinated trypsinised PRBC from $A4_{i+}$ and A4TRY-I, but had no effect on untreated PRBC or uninfected erythrocytes. Antibodies eluted from untreated A4TRY-I did not mediate agglutination of any cells.

This was the first successful elution of a variant-specific antibody from the infected erythrocyte surface and indicates that there were common antigenic determinants revealed on trypsinisation of $A4_{i+}$ and A4TRY-I which are absent from untreated cultures. This reagent was used in later experiments in attempts to identify the surface ligand that mediated this interaction (chapter 6).

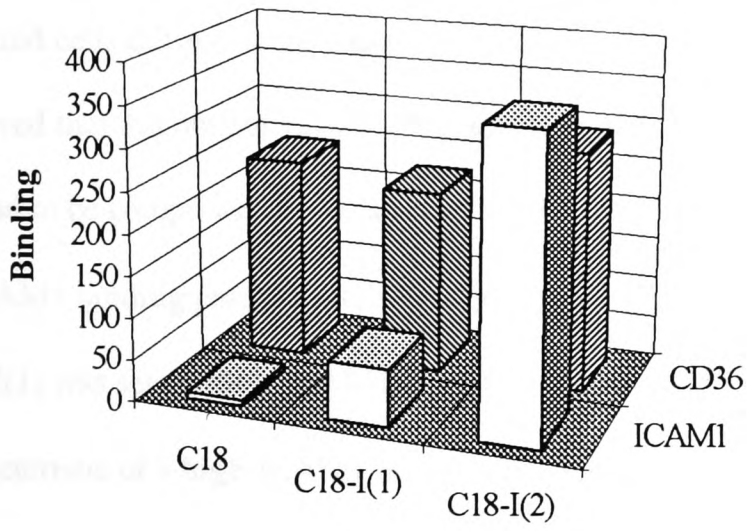
4.6. Pathway of phenotypic switching

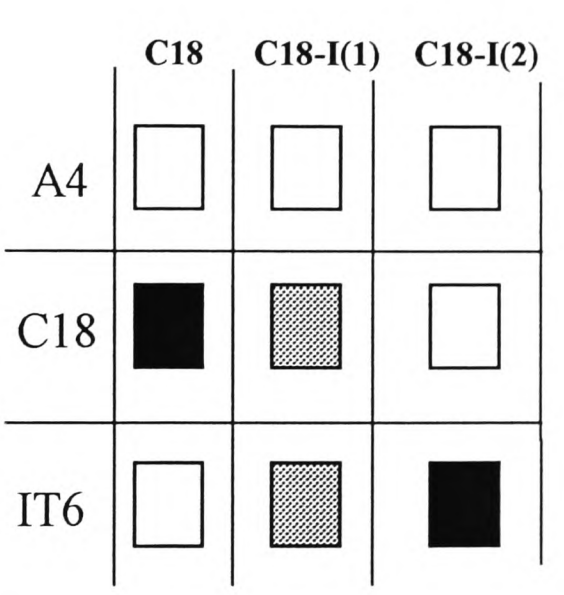
In earlier experiments, the phenotypes of PRBC were tested at the end of multiple rounds of selection on ICAM1. Aliquots of intermediates after each panning were cryopreserved for future investigations of the pathway of phenotypic switching. Such stabilates from $C28_{i-}$ and $C18_{i-}$ were tested to provide an insight into possible switching pathways of two antigenically distinct C clones. Mixed agglutination analysis was performed using PRBC from $A4_{i+}$ and the starting clone ($C28_{i-}$ or $C18_{i-}$) as references for relative antigenic phenotypes, and adhesion of the intermediate cultures to purified ICAM1 and CD36 was tested as before (Figure 4.7).





a) Analysis of the cytoadherent and antigenic phenotypes of intermediates derived during the selection of $C28_i$- on ICAM1.





a) Analysis of the cytoadherent and antigenic phenotypes of intermediates derived during the selection of $C18_i$- on ICAM1.

Figure 4.7. Summary of cytoadherent and antigenic phenotypes of intermediates from the selection of (a) $C28_i$- and (b) $C18_i$- on ICAM1. The number in parenthesis denotes the number of rounds of selection. i.e. $C28_i$- was selected on ICAM1 to produce C28-I(1), which was grown up and tested then selected on ICAM1 to produce C28-I(2). Bars represent the mean binding from two experiments and SEMs were <10% in all cases. The key to mixed agglutination assay data is: ■ 100-80% ▩ 79-15% ▨ 14-6% □ 5-0% antigenic similarity.

The original study by Roberts and colleagues (1992) reported that binding to ICAM1 of PRBC from $C28_{i-}$ was significantly elevated after one round of panning on ICAM1 and was accompanied by a reduction of binding to CD36. On re-testing, it was confirmed that this primary intermediate line C28-I(1) demonstrated a high degree of antigenic similarity to $A4_{i+}$, but none to $C28_{i-}$. However, the associated decrease in the level of binding to CD36 was not as marked as had been reported (5-fold reduction). In this series of selections, the binding to CD36 decreased gradually after each round of panning, but the levels of binding to ICAM1 and the antigenic phenotype of the culture remained unchanged after two further selections.

On the other hand, $C18_{i-}$ showed a significant increase in ICAM1 binding ability after a single selection on ICAM1, but C18-I(1) did not show any antigenic similarity to $A4_{i+}$. C18-I(1) had a low similarity to IT6 ($3.5 \pm 1.3\%$, as determined accurately by the mixed agglutination assay[†]) and to $C18_{i-}$ ($6.7 \pm 5.1\%$[†]). This latter phenotype could represent a subpopulation of the starting culture that was linked to the ICAM1 binding phenotype or could be due to the failure to remove all of the unbound cells during the panning. In either case, a unique **mid** ICAM1 binding phenotype was observed that did not correlate with expression of the A4VAT as a major phenotype and seemed instead to be comprised of a mixture of the C18VAT and IT6VAT. A more homogeneous population of ICAM1 binding parasites that showed near identity ($88.0 \pm 6.4\%$[†]) to IT6 was produced when C18-I(1) was selected on ICAM1. C18-I(2) bound to ICAM1 on plastic at levels that were characteristic of a **high** ICAM1 binder, and no change in overall levels of PRBC binding to CD36 were observed after either panning. This pathway was distinct from that observed in $C28_{i-}$ (and probably $C24_{i-}$) because selection revealed a novel intermediate culture, C18-I(1), that consisted of at least two antigenic subpopulations.

[†] Estimates of the degree of antigenic similarity were calculated from the proportion of mixed colour agglutinates (x) formed in the mixed agglutination assay, and applying the formula:

$$x = 1\text{-}p^{(n-1)}$$

where p is the chance of homologous agglutination and n is the number of cells per agglutinate (5).

The p value is related to the degree of antigenic similarity of the test cultures by the formula:

$$\text{antigenic similarity (\%)} = 100 \times (1\text{-}p) / p$$

Thus, if 13% of the agglutinates formed in the test are mixed colour, then $x = 0.13$ and $p = 0.966$, and the antigenic similarity is 3.5% (see section 2.2.10 for basis of assay).

These conclusions were confirmed by protease digestions. C28-I(1) and C28-I(2) had identical sensitivities to V8 protease and trypsin as C28-I* (data not shown), whereas C18-I(1) had overlapping sensitivities (Table 4.6). Agglutination and binding to CD36 of PRBC from C18-I(1) were resistant to V8 protease which was attributed to the low levels of C18VAT present in the culture. Adhesion to ICAM1 was sensitive to V8 protease and trypsin, which ablated agglutination and binding to CD36. Thus, the presence of the antigenically-restricted V8 protease-resistant binding to CD36 in C18-I(1), but not in C18-I(2) correlated with the shift away from the C18VAT by further selections on ICAM1.

C18-I(1)	Binding to ICAM1 (% control)	Binding to CD36 (% control)	% Cells in Agglutinates
V8 PROTEASE	3.7 ± 1.4	32.7 ± 9.0	93.2 ± 15.9
TRYPSIN	5.7 ± 2.7	0	4.5 ± 1.2

Table 4.6. Effect of V8 protease (10U ml^{-1}) and trypsin (1mg ml^{-1}) on cytoadherence and agglutination of PRBC from C18-I(1) intermediate. Values are means of % control levels ± SEM from two separate experiments. Note that C18-I(1) contains at least two populations of antigenically distinct PRBC that express either the C18VAT or the IT6VAT.

4.7. Surface morphology of ICAM1 binding PRBC

Transmission electron microscopy (TEM) was used to produce high resolution images of the surface of ICAM1 binding PRBC. Figure 4.8 shows that knobs (**k**) were associated with the surface of four ICAM1 binding lines examined, and there was no observable difference in their distribution or density between antigenically distinct parasites (D. J. Ferguson, personal communication). Typical parasite-induced internal erythrocytic modifications were observed including Maurer's clefts (**M**) in the host cell cytoplasm, and the appearance of a food vacuole (**fv**), nucleus (**N**), and rhoptries (**R**) inside the parasite (P).

Trypsinisation did not remove surface knobs and had no visible effect on the surface of the parasitised erythrocytes (Figure 4.9), in common with V8 protease (data not shown). Thus, it was evident that the knobs were not essential for adhesion to ICAM1, because knobs were observed on the surface of trypsinised PRBC that did not bind to ICAM1 and non-ICAM1 binding C clones (Dr. D. J. Roberts, personal communication).

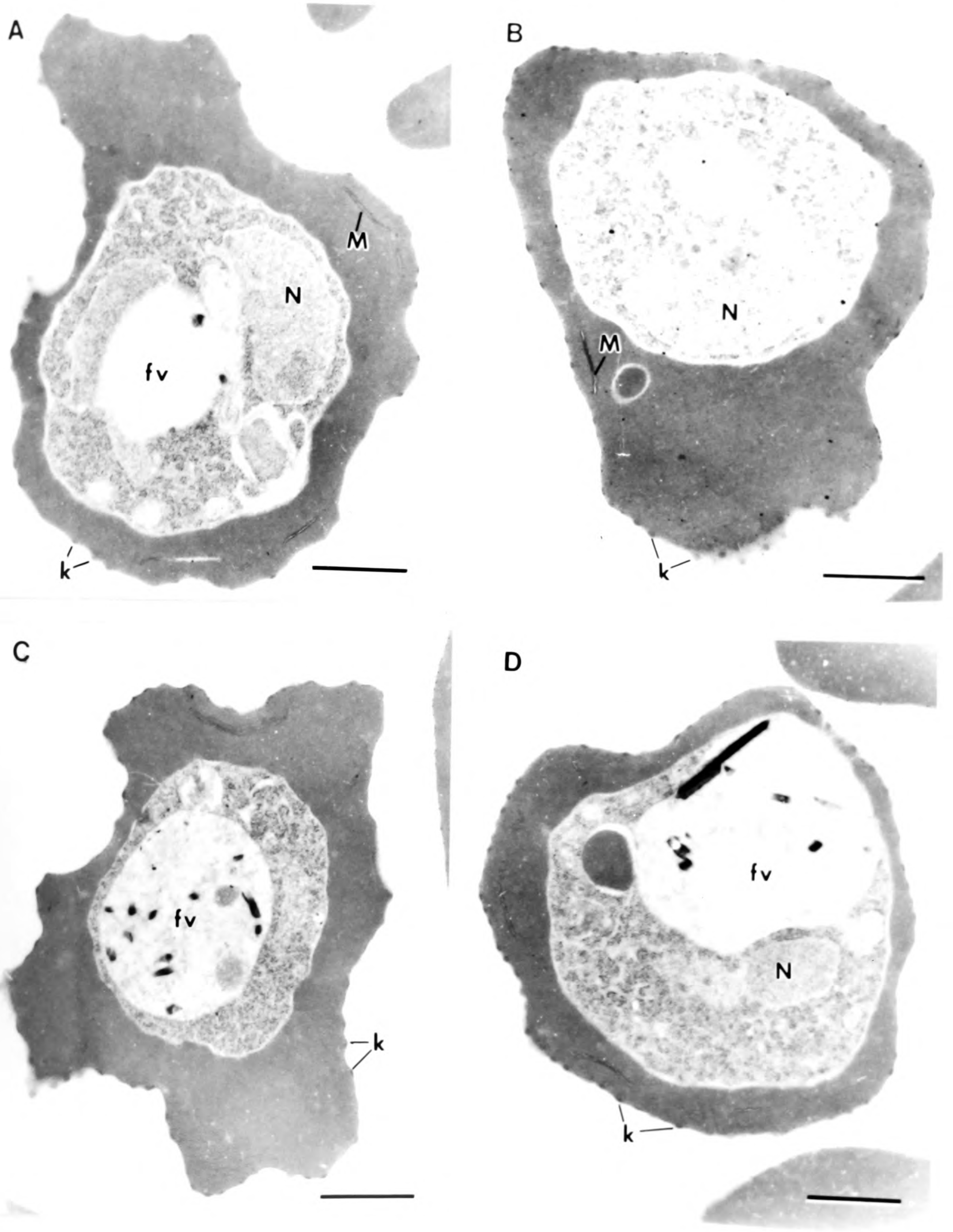


Figure 4.8. Electron micrographs of PRBC from four ICAM1 binding lines. A) A4, B) A4RI-I, C) I16, D) C1S-I(2). All have a similar density of knobs (k) at the PRBC surface (D. J. Ferguson, personal communication). Other parasite- induced modifications are indicated, including the parasite nucleus (N), food vacuole (fv), and Maurer's clefts (M). Bar is 1 μm.

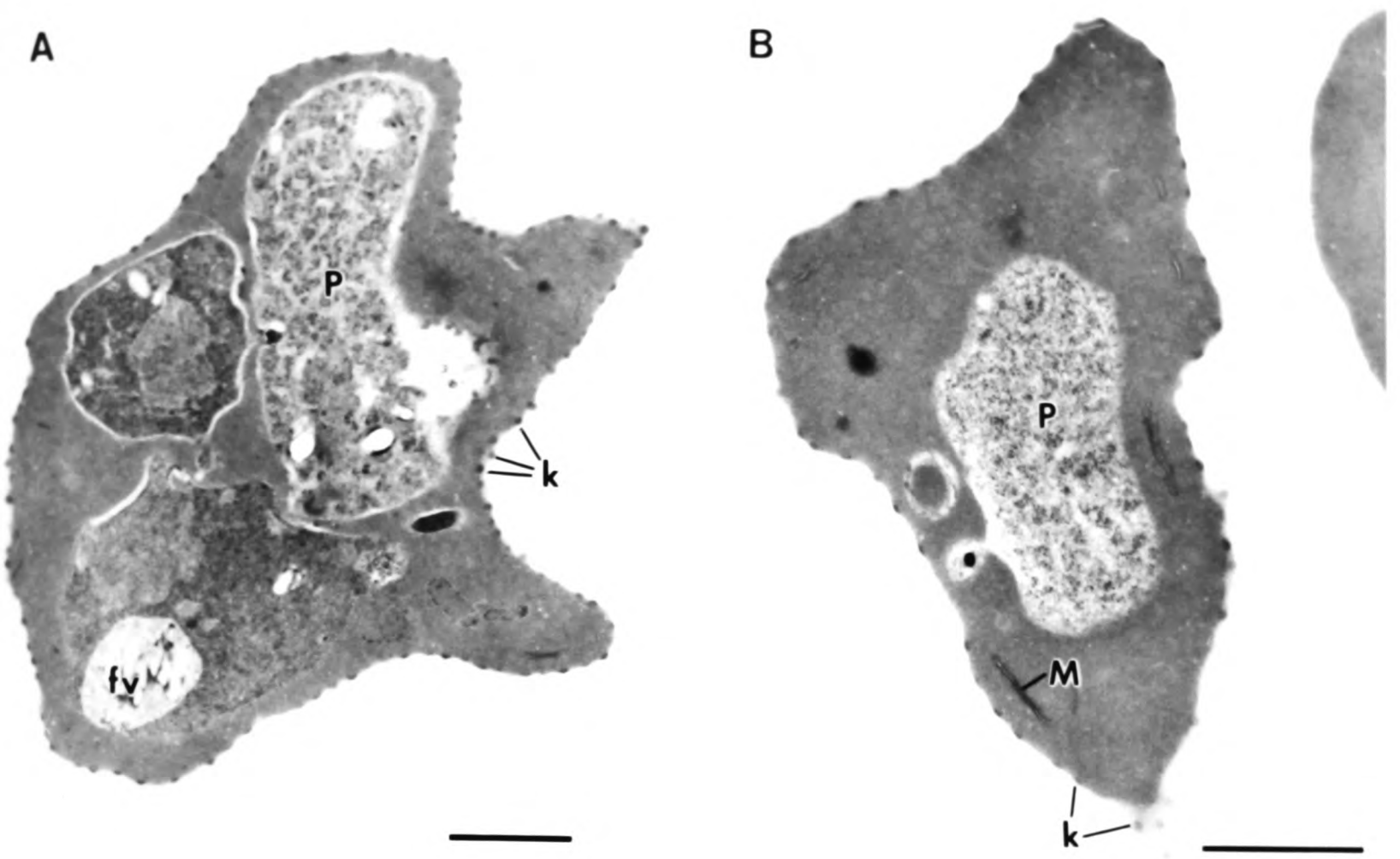


Figure 4.9. Electron micrographs of PRBC from A) IT6 and B) A4TRY-I digested with 1mg ml^{-1}. Knobs (k) are present at the PRBC surface and key is same as figure 4.8, and bar is 1μm.

It was rationalised that knob-negative parasites would bind to ICAM1, and these infected cells would be suitable for the isolation of the ICAM1 binding ligand, which would not be associated with the red cell cytoskeleton and could be extracted by mild detergents. A similar strategy had been employed for the characterisation of sequestrin, a potential CD36 binding ligand, which had been extracted by Triton X-100 from knob-negative parasites (Ockenhouse *et al.*, 1991). Therefore, a knob-negative clone, D4, from the Palo Alto line, that did not cytoadhere well to any purified receptor *in vitro* was selected for adherence to ICAM1 (in conjunction with Dr. A. R Berendt). After 4 rounds of panning, D4-I bound to ICAM1 at a low level while retaining a knob-negative phenotype (Figure 4.10). Unfortunately, neither D4 nor D4-I formed agglutinates in Sib serum, thus the antigenic phenotypes of these cells could not be assessed by the mixed agglutination assay. Cytoadherence of D4-I was partially resistant to trypsin and V8 protease implying that it was a heterogeneous population (data not shown).

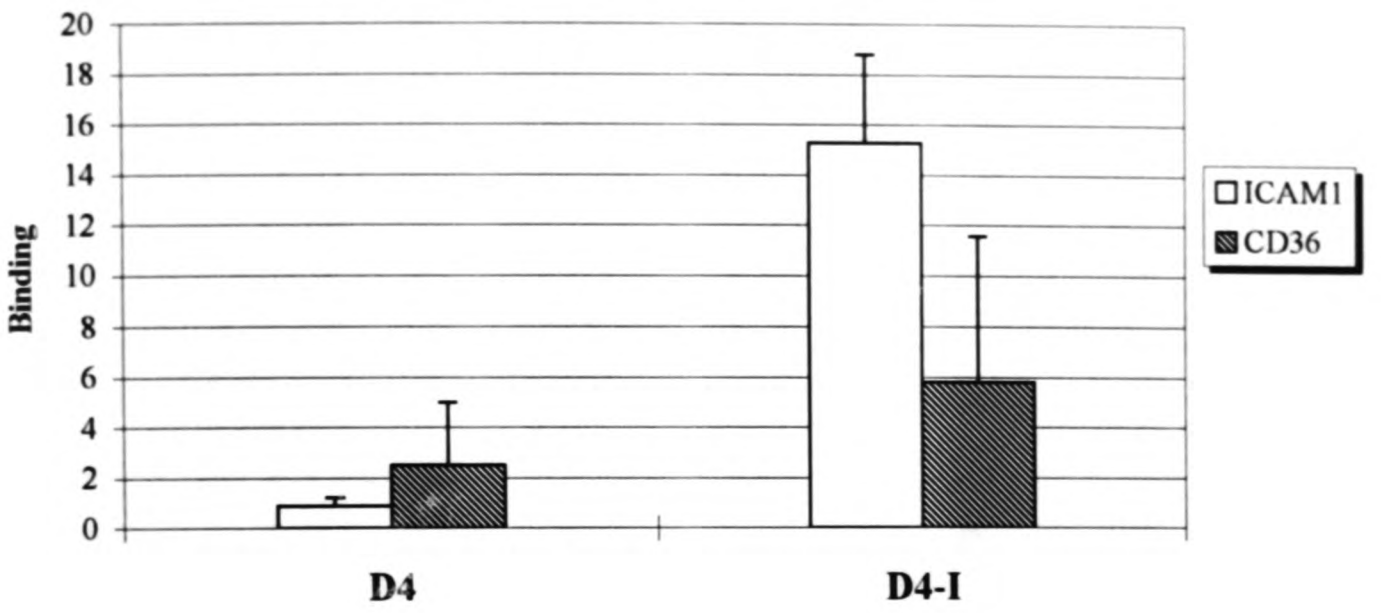


Figure 4.10.a) Adhesion of PRBC from D4 and D4-I to purified CD36 and ICAM1. Data are mean values (PRBC mm^{-2}) ± SEM from two separate experiments.

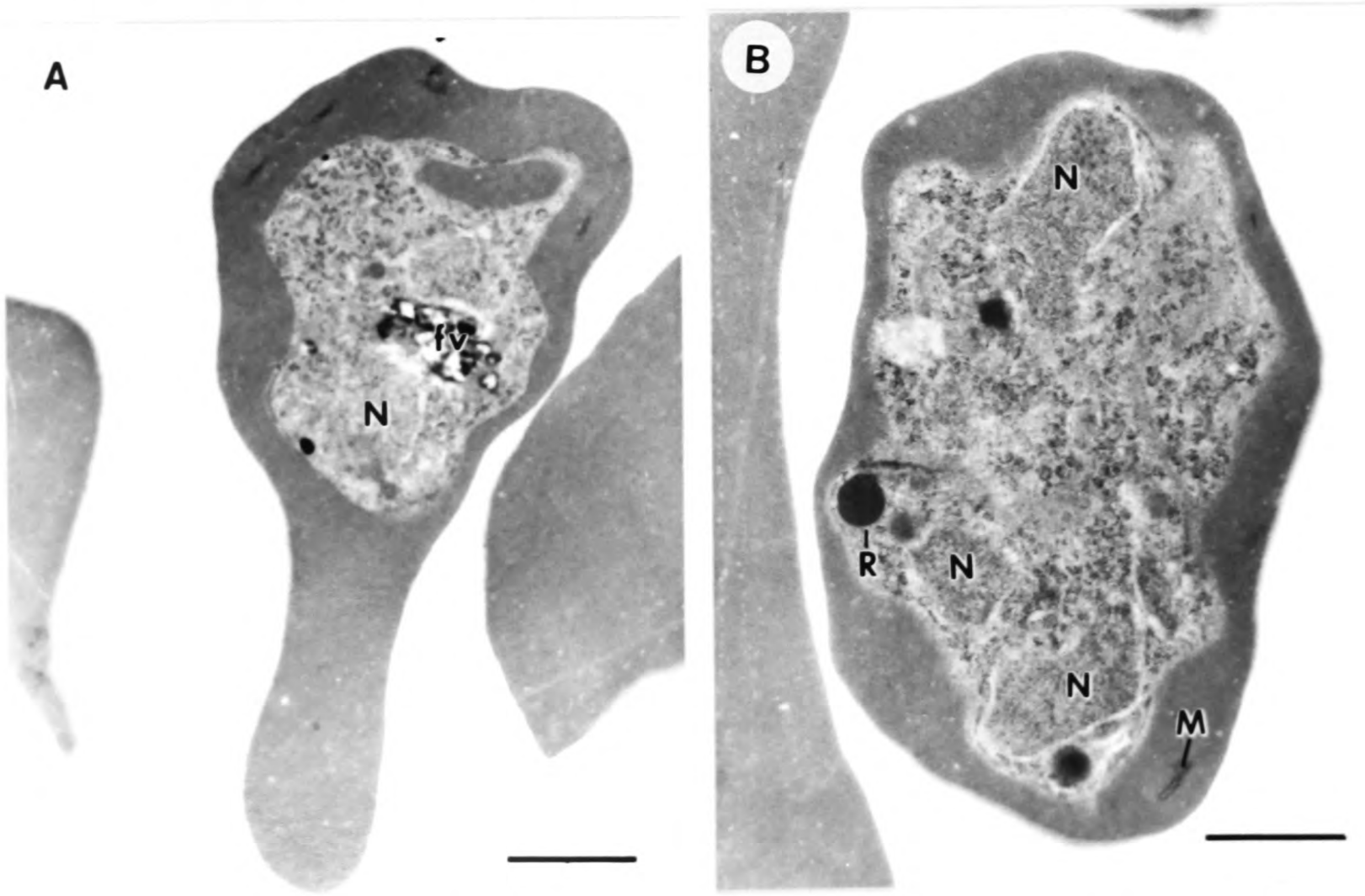


Figure 4.10 b) Electron micrograph of PRBC from A) D4 and B) D4-I showing that knobs are not present at the PRBC surface, but erythrocytes retain other typical parasite-induced modifications, including parasite nucleus (N), food vacuole (fv), Maurer's clefts (M) and rhoptries (R). Bar is 1 μm.

4.8. Conclusions

ICAM1 is one of five putative adhesion ligands that mediates sequestration of parasitised red blood cells (PRBC) in the post-capillary venules. PRBC undergo sequestration in all organs during infection by *P. falciparum*, but when PRBC accumulate at high frequency in the brain, a life-threatening clinical syndrome known as cerebral malaria can result (MacPherson *et al.*, 1985). Cerebral malaria occurs in a small minority of *P. falciparum* infections and identification of the parasite virulence factors has been difficult because it is impossible to directly characterise the sequestered PRBC which are causing the disease. Indirect evidence suggests that PRBC adherence to ICAM1 is important in disease pathology, because immunohistochemical studies from patients who died from cerebral malaria have shown that ICAM1 expression in cerebral vessels was associated with PRBC sequestration in the same vessels (Turner *et al.*, 1994). Also, a large case-control study in Kenya has shown that adhesion of PRBC to ICAM1 is greater in PRBC from patients with clinical disease than from asymptomatic controls (C. I. Newbold, personal communication).

Recent studies have shown that the antigenic phenotype expressed by mature PRBC was variable *in vitro* and was linked to the ICAM1 adherent phenotype (Biggs *et al.*, 1992; Roberts *et al.*, 1992). The ability to bind to ICAM1 has been associated with a single antigenic phenotype in two studies, and this relative restriction in PRBC surface phenotypes could represent an important virulence factor *in vivo*. In contrast, all PRBC bound to CD36 and this function was associated with multiple antigenic phenotypes.

To address this issue, two non-ICAM1 binding clones ($C18_{i-}$ and $C24_{i-}$) were selected for adherence to ICAM1 and the antigenic and cytoadherent phenotypes of the adherent lines (C18-I and C24-I) were tested. Other ICAM1 binding parasites ($A4_{i+}$ and C28-I) were reselected on ICAM1 and examined with IT6 which had been produced by 6 rounds of panning of IT parasites on ICAM1. A summary of the derivation and phenotypic relationships of these ICAM1 binding parasites is shown in figure 4.11.

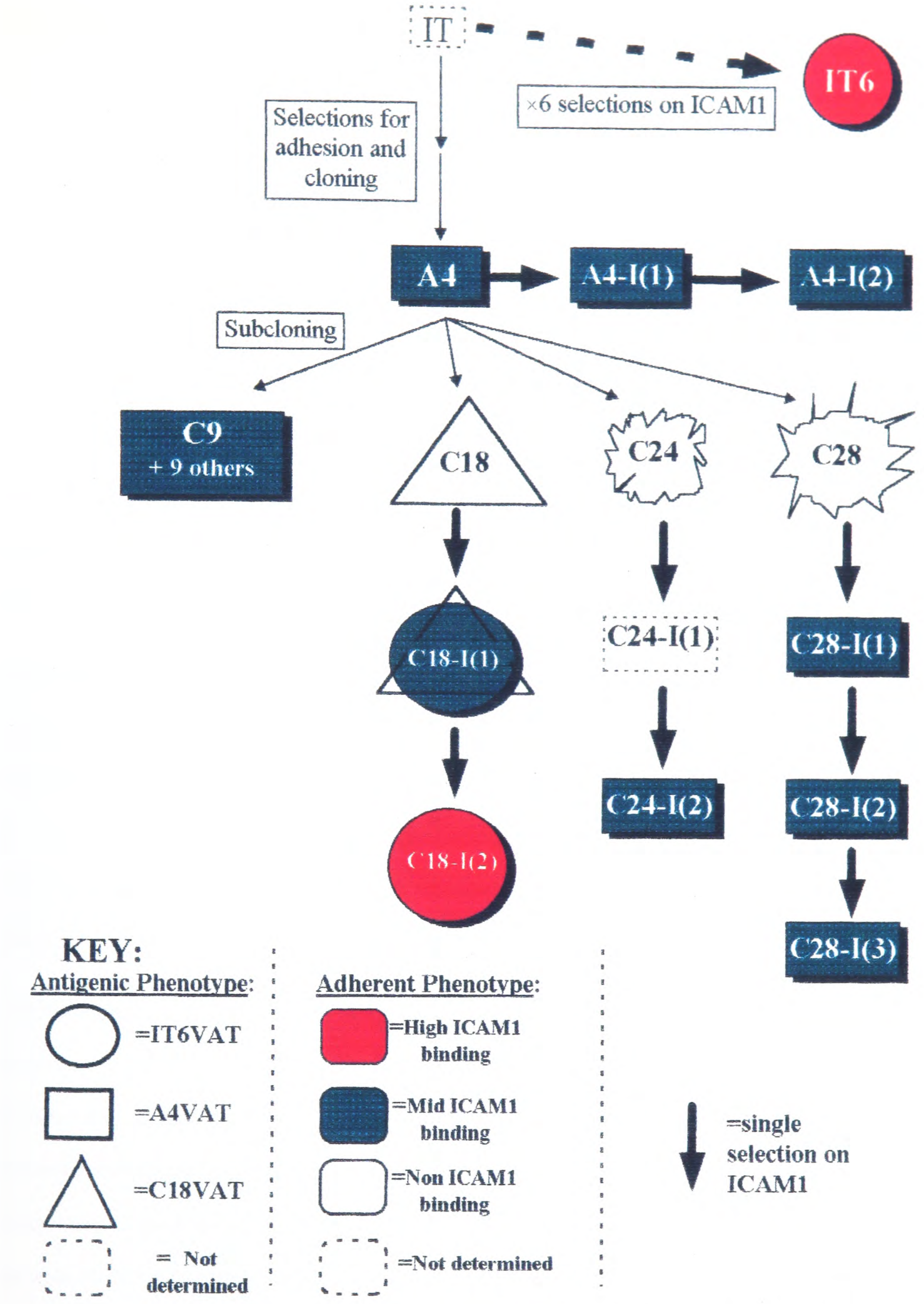


Figure 4.11. Summary of the derivation and phenotypic relationships of the ICAM1 binding PRBC used in this study. The shape of the surrounding box denotes the antigenic phenotype of the parasite, and the fill colour represents the level of binding to ICAM1.

There were only two antigenic phenotypes associated with ICAM1 binding phenotypes in this clone tree. The A4VAT was the predominant phenotype which was expressed by 10 C subclones derived from $A4_{i+}$ and 6 lines selected on ICAM1. PRBC that expressed the A4VAT bound to ICAM1 at **mid**-levels. The IT6VAT was associated with IT6 and C18-I(2) and these selected lines exhibited significantly higher levels of binding to ICAM1. The selected populations maintained the ability to bind to CD36 which suggested that a single PRBC was capable of binding to both receptors.

One consequence of the high rate of clonal antigenic variation was the rapid appearance of variant subpopulations within a parasite line *in vitro*. Proteases have been used in chapter 3 to demonstrate that complete V8 protease resistant binding to CD36 was intrinsically linked to the expression of a variant antigenic phenotype, C18VAT. This phenotype was present as a minor subpopulation detected by mixed agglutination assays, within other parasites and explained the partially resistant phenotypes observed in such lines. Proteases were used to analyse the extent of phenotypic heterogeneity within this extended range of ICAM1 binding parasites.

While adhesion to ICAM1 was universally sensitive to V8 protease, partially resistant binding to CD36 occurred in parasites that expressed the C18VAT as a subpopulation ($A4_{i+}$, $C9_{i+}$ and C18-I(1)). The same parasites also showed completely resistant agglutination after V8 proteolysis which had been previously demonstrated in chapter 3 to be mediated by the C18VAT subpopulation. Sialic acids were not essential for PRBC to adhere to ICAM1 because digestion with neuraminidases had no effect.

Trypsin digestion of ICAM1 binding parasites provided more interesting and complex results. Two parasites, $A4_{i+}$ and $C9_{i+}$ showed trypsin-resistant ICAM1 binding, and agglutination was partially resistant after the same treatment. Binding to CD36 was universally sensitive to trypsin and this was the first demonstration that binding to ICAM1 could be dissociated from binding to CD36. The relationship of this phenomenon to antigenic phenotype was complicated because ICAM1 binding of all other PRBC was abolished by trypsin treatment, including the selected lines (C24-I, C28-I* and A4-I) which expressed the A4VAT.

To determine if there was a linkage between trypsin-resistant binding and antigenic phenotype, PRBC from $A4_{i+}$ were selected on ICAM1 after trypsinisation and the selected line was examined. This strategy had proved successful in explaining the basis for V8 protease resistant binding to CD36. Analysis of the trypsin-resistant ICAM1 binding population showed that a novel antigenic phenotype (A4TRY-I) existed within $A4_{i+}$ which was modified to a second novel antigenic phenotype after trypsinisation. The mixed agglutination assay demonstrated that the trypsin resistant populations of $A4_{i+}$ and A4TRY-I were antigenically similar. The undigested cells were antigenically distinct which implied that A4TRY-I was present at a very low frequency within $A4_{i+}$ (summarised in Figure 4.12). The antigenic similarity of the trypsinised populations was confirmed by the specific agglutination of digested cells by antibodies eluted from trypsinised A4TRY-I. These antibodies did not agglutinate undigested cells or trypsinised red cells implying that a cryptic parasite-derived epitope had been revealed on trypsinisation and mediated the resistant agglutination. It was unlikely that A4TRY-I had arisen by chance during selection on ICAM1 because two rounds of panning of undigested $A4_{i+}$ cells failed to show any similarity between A4-I(2) and A4TRY-I.

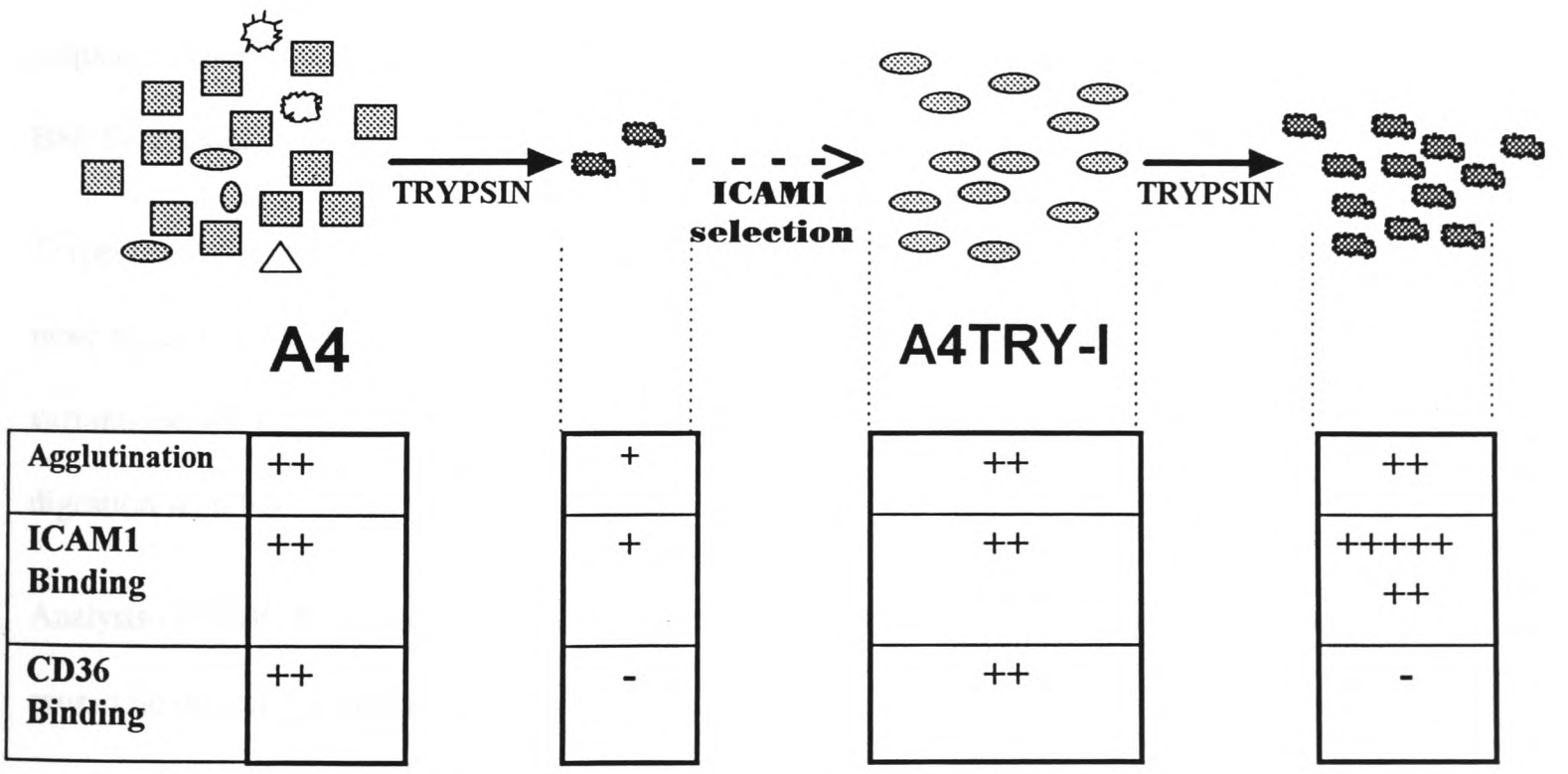


Figure 4.12. Summary of derivation and phenotypic relationships of A4 and A4TRY-I, and the effect of trypsinisation. Surrounding of cells denotes antigenic phenotype and degree of shading represents ability to bind to ICAM1. Note that a novel antigenic phenotype is produced on trypsinisation that is absent from undigested cells.

A4TRY-I could bind to both ICAM1 and CD36, even though it had been selected from trypsinised A4 PRBC which could only bind to ICAM1. This implied that the ligand(s) for these host receptors were expressed on the same cell, otherwise CD36 binding would not be recovered. Trypsinisation of A4TRY-I completely ablated binding to CD36, had no effect on agglutination levels, but enhanced binding to ICAM1 by approximately 4-fold. Thus the trypsin-resistant binding to ICAM1 of PRBC from $A4_{i+}$ was due to this expansion of adherence by the A4TRY-I.

Trypsin-resistant adhesion to HUVECs has been recently demonstrated in a single knob-positive clone (BE-8) that had been subcloned from clone B3C (from a Papua New Guinean isolate) which had been selected 8 times on unstimulated HUVECs (Chaiyaroj *et al.*, 1994a). BE-8 was antigenically distinct from its parent population as determined by cross reactivity with defined antisera, and did not adhere to any known purified cytoadherence receptor (including ICAM1) or C32 melanoma cells. PRBC agglutination and adherence to the unstimulated HUVECs was resistant to trypsin digestion at 0.1 mg ml^{-1}, but the digested cells were not tested for binding to purified ICAM1 and their antigenic phenotype was not characterised. It remains unclear if BE-8 is the equivalent A4TRY-I in this clone family, although binding of BE-8 cells to HUVEC was not enhanced by trypsinisation. Unpublished data suggests that the novel receptor expressed on unstimulated HUVEC may be a chondroitin-4-sulphate moiety, and this would constitute a novel mechanism for cytoadherence (S. C. Chaiyaroj, BSP Symposium, Liverpool, 1994).

Trypsin is known to cleave many PRBC surface proteins and was likely to make the resistant proteins more accessible for cytoadherence and antibody binding. This may explain the successful elution of variant-specific antibody from such cells and the transient increase in agglutination of $A4_{i+}$ following digestion with low concentrations of trypsin.

Analysis of PRBC by transmission electron microscopy (TEM) demonstrated that trypsinisation caused no discernible effects on the surface morphology of ICAM1 binding parasites, and knobs were not destroyed. Furthermore, all ICAM1 binding parasites expressed surface knobs and unpublished studies had found that non-ICAM1 binding variants also expressed knobby protrusions. Thus, it was concluded that knobs were not essential for adherence to ICAM1 and that a knob-negative ICAM1 binding parasite line would provide an ideal target for isolation of the parasite ligand which binds to

ICAM1. In the absence of knobs, parasite-derived erythrocyte membrane proteins are extractable by non-ionic detergents because they are not associated with the erythrocyte cytoskeleton (Ockenhouse *et al.*, 1991b). D4 was a non-cytadherent knob-negative clone from a different parasite line that was successfully selected on ICAM1. The selected parasite, D4-I, bound at low levels to ICAM1 and TEM confirmed that it continued to express a knob-negative phenotype. This parasite was not examined in detail because it did not form agglutinates in Sib serum but was analysed in chapter 6 during investigation of the surface molecules involved in PRBC adhesion to ICAM1.

Intermediates during the switching pathway of non-ICAM1 binding clones were studied after individual rounds of selection on ICAM1. Mixed agglutination analysis revealed that the A4VAT was the preferred antigenic phenotype for two variant C clones ($C24_{i-}$ and $C28_{i-}$) selected on ICAM1 (C24-I and C28-I. This phenotype was maintained with no change in the levels of binding to ICAM1 after further selections of C28-I. However, this was not the only switching pathway to ICAM1 binding parasites utilised by variant C clones, because the IT6VAT was produced after two rounds of panning of $C18_{i-}$ on ICAM1. This was the antigenic phenotype expressed by $IT6_{i+}$, a line selected for adherence from IT. The IT6VAT appeared gradually during the selection of $C18_{i-}$, unlike the A4VAT which appeared after a single round of selection of $C28_{i-}$ on ICAM1 and was refractory to further selections. A large proportion of $C18_{i-}$ was still detectable in the primary intermediate, C18-I(1), together with a population cells that expressed the IT6VAT. A second selection of C18-I(1) dramatically enriched the IT6VAT to the extent that C18-I(2) and IT6 were antigenically indistinguishable. These data provide further evidence that the capacity to express all VATs is retained after antigenic switching, because $IT6_{i+}$ and C18-I(2) are derived from a common parasite line, IT and the gene(s) encoding the IT6VAT must have been conserved through the cloning of $A4_{i+}$ and its subsequent subcloning stages. However, the IT6VAT was probably not expressed within $A4_{i+}$ (or was present only at extremely low levels), because A4-I still expresses that A4VAT after two selections on ICAM1, and the high ICAM1 binding IT6VAT was not observed.

There are two lines of evidence which suggest that switches to the IT6VAT are rare. Firstly, subcloning of A4 produced 10 clones which bound to ICAM1 at **mid**-levels and all expressed the A4VAT. Hence, the IT6VAT was probably not switched to during the derivation of $A4_{i+}$. Secondly, if

the IT6VAT was present as a subpopulation within a culture, it would be selected more rapidly than the A4VAT by virtue of its greater binding to ICAM1. This was not observed, and only the $C18_{i-}$ clone switched to the IT6VAT after selection. The basis of the alternative mechanism in this clone was unclear, but it was not surprising that the IT6VAT has arisen after selection of the uncloned IT line because the highest affinity ICAM1 binding parasites would predominate after 6 selections.

The molecular interaction of the antigenic epitopes and the ICAM1 binding domain was unclear, but their expression must be comodulated at the surface of the PRBC to explain the linkage of antigenic and adhesive phenotypes. Variation could occur in the ICAM1 binding domain to modulate the specificity of interaction between **high-**, **mid-** and **non-** ICAM1 binding phenotypes. Host cell specificity is determined in this manner by antigenic variation of the *influenza* haemagglutinin protein (Schild *et al.*, 1983; Rogers *et al.*, 1983).

The restricted antigenic phenotypes associated with ICAM1 binding *in vitro* and the failure to observe antigenic switching without selection in one ICAM1 binding parasite implied that constant selection pressure would be required *in vivo* to maintain this surface property. Adhesion to CD36 appears to be a conserved property of the majority of antigenic variants and would explain why more parasites from patients bind to this receptor than to ICAM1. However a significant decrease in CD36 binding was not consistently observed by selecting for ICAM1 adherence, which suggested that these properties were co-expressed on the same cell. This conclusion was supported by the absence of non-CD36 binding PRBC. The gradual reduction of adhesion to CD36 during the selection of $C24_{i-}$ and $C28_{i-}$ was probably due to the enrichment of PRBC that bound with greater avidity to ICAM1 than to CD36.

This study has confirmed that the antigenic phenotypes associated with ICAM1 adherence are restricted, which may be important *in vivo* if the onset of severe disease is determined by a limited number of antigenic phenotypes or adherent specificities. Expression of a **high** ICAM1 binding phenotype would be a prerequisite for sequestration of PRBC in microvessels that expressed low levels of ICAM1. If switching to this phenotype was rare *in vivo*, this may be a parasite virulence factor that contributes to the low frequency of severe disease.

Chapter 5

Adhesion to Thrombospondin

Thrombospondin (TSP) is a large, multi-functional protein that is synthesised by several cell types and secreted as a water-soluble extracellular molecule (Lahav, 1993). TSP was the first host cytoadherent ligand to be identified by the fact that mature, knob-positive PRBC bound to purified protein immobilised on plastic (Roberts *et al.*, 1985b). This interaction was inhibited by high concentrations of fluid-phase TSP and anti-TSP monoclonal antibodies (mAbs). The binding site for PRBC on TSP is unknown, but biochemical studies have localised the epitope of a mAb which inhibits cytoadherence to the C-terminus (Sherwood *et al.*, 1990). This globular region of TSP has a Ca^{2+}-dependent structure which correlates with the absolute requirement for this cation for PRBC adhesion *in vitro* (Sherwood *et al.*, 1990).

The physiological function of TSP is to act as an adhesive 'bridge' molecule that links membrane proteins during platelet aggregation (Tuszynski *et al.*, 1985). Its function in sequestration is intriguing because CD36 is a known receptor for TSP and *in vitro* cell models of cytoadherence have suggested that TSP may function as an ancillary molecule during PRBC binding to CD36 (Asch *et al.*, 1987; McGregor *et al.*, 1989). Inhibition of PRBC attachment to C32 melanoma cells by soluble TSP (80% inhibition), and anti-TSP mAbs (50-70% inhibition) suggest that TSP contributes to adhesion in this model (Sherwood *et al.*, 1989).

However, the role of TSP in PRBC sequestration is equivocal for two reasons. Firstly, sensitive radiolabelling techniques have demonstrated that levels of surface-associated TSP on C32 melanoma cells and non-adherent melanoma cell lines (eg. G361 and RPMI-8252) were comparable (Sherwood *et al.*, 1989). There were no differences in the overall levels of TSP secreted by these cell lines, and evidence to date suggests that there are other receptor(s) which bind PRBC in these models. Secondly, *in vivo* results from immunohistochemical analysis of *post mortem* tissues from Thai patients, detected TSP at low levels on endothelium from all organs and these levels were unchanged between mild and severe malaria cases (Turner *et al*, 1994).

Local concentrations of surface TSP have not been measured on the melanoma cell surface or *in vivo*, and could be relevant in malaria pathology. Several cell types, including platelets, monocytes and endothelial cells, secrete TSP *in vitro* and can contribute to the circulating pool of TSP *in vivo* (Gartner *et al.*, 1981; Mosher *et al.* 1982; Raugi *et al.*, 1982). The concentration of TSP in normal plasma is low (20ng ml^{-1}), but local concentrations following release by activated platelets can reach 2μg ml^{-1} in serum (Saglio *et al.* 1982). This phenomenon may be relevant to sequestration because other surface proteins, eg. sulphated glycolipids (Roberts *et al.*, 1985a) and proteoglycans (Kaesberg *et al.*, 1989; Sun *et al.*, 1989), are expressed by endothelial cells that can bind to TSP *in vitro*. However, the expression of potential TSP receptors (excluding CD36) in different vascular beds during alternative disease states has not been examined by extensive immunochemical studies.

Uninfected cells, ring-form PRBC and knob-negative mature PRBC do not adhere to TSP, which implies that its counter-receptor is a parasite-derived knob surface protein (Roberts *et al.*, 1985b). Immunogold staining techniques have shown that TSP binds to mature PRBC only at knobs, and the same study identified these regions as the sites of attachment for CD36 (Nakamura *et al.*, 1992). All field isolates tested can bind to purified TSP at different levels which are proportional to the parasitaemia and percentage of mature PRBC present in the culture (Sherwood *et al.*, 1987, 1989). It is not known if PRBC adhesion to TSP is a parasite virulence factor because only small numbers of patients have been analysed to date.

When TSP was discovered to function as a receptor for cytodherence in 1985, early research into the basis of adhesion focused primarily on the role of knobs at the PRBC surface. Since the demonstration in 1989 that knobs were neither necessary nor sufficient for cytoadherence (Udomsangpetch *et al.*, 1989; Biggs *et al.*, 1989), there have been few reports which relate to the biochemical basis of PRBC adhesion to TSP. Consequently, the functional relationship of adhesion to TSP and to other subsequently discovered cytoadherence receptors (eg. CD36 and ICAM1) has not been studied in detail. Differential receptor usage may play an important role in malaria pathology and studies in preceding chapters have found that adhesion to CD36 and ICAM1 is related to the antigenic phenotype expressed at the PRBC surface. Since binding to TSP appears to be a constant property of the PRBC, it is important to determine its relationship to other surface phenotypes.

5.1. Binding of different PRBC to TSP

The original study by Roberts and colleagues (1985b) demonstrated that Ca^{2+} replete, purified TSP at concentrations of 20 - 200 $\mu g\ ml^{-1}$ immobilised on plastic could mediate adherence of *Aotus* erythrocytes infected with two knob-positive strains of *P. falciparum* (Malayian Camp and St. Lucia). The same report also found that five other knobby *P. falciparum* strains from Brazil (ItG2F6), Vietnam/Cambodia (VI), Thailand (T2), Kenya (K3) and Liberia (L), when cultured in human erythrocytes also bound to purified TSP. Levels of binding to TSP were significantly higher for *Aotus* derived PRBC (7100 ± 240 and 4100 ± 600 PRBC mm^{-2}) than human erythrocyte derived PRBC (mean binding of five isolates 332 ± 219 PRBC mm^{-2}) (Roberts *et al.*, 1985b). The dichotomy in adhesion to TSP could be due to parasite strain variation or differences between host erythrocytes. There was a small correlation with binding to TSP and binding to C32 melanoma cells, but the results were not normalised for parasitaemia, which can have a profound influence on adhesion (Sherwood *et al.*, 1989). The antigenic phenotypes of the PRBC were not examined, but they would be expected to be different due to their wide geographical distribution.

PRBC with well-characterised surface phenotypes were tested for their ability to bind to TSP at 40$\mu g\ ml^{-1}$, to investigate the relationship of binding to TSP to other known receptors (CD36 and ICAM1) (Figure 5.1). Seven knob-positive PRBC were chosen that expressed 6 variant antigenic types (VATs) to assess simultaneously the effect of antigenic determinants on adherence. Four of these lines ($A4_{i+}$, $C9_{i+}$, IT6, A4TRY-I) bound to both CD36 and ICAM1, and three clones ($C18_{i-}$, $C24_{i-}$, $C28_{i-}$) bound to CD36 only. A knob-negative line, D4-I, of different genotype derived by selection on ICAM1 was also tested, and was known to bind weakly to CD36 and ICAM1. The phenotypic properties of these PRBC is summaried by the boxes in figure 5.1; the shape denotes the antigenic phenotype and the degree of shading represents the level of adhesion to ICAM1 determined in section 4.2.1.

All knob-positive PRBC derived from IT bound to TSP at high levels (166 - 217 cells mm^{-2}). These data were within the reported ranges for other laboratory-derived PRBC and field isolates of different origins after correction for parasitaemias. The knob-negative line D4-I, derived from the Palo Alto / Wellcome strain, bound at significantly lower levels (27.3 ± 5.9 PRBC mm^{-2}), and uninfected

erythrocytes were found to adhere at 4.5 ± 3.3 cells mm^{-2} to this concentration of TSP. This is the first report of knob-negative adhesion to TSP, and it is interesting that D4-I had been derived by selection on ICAM1 from a parasite, D4, which did not bind to TSP (data not shown). However, adhesion to TSP does not appear to be related to the ability of the PRBC to bind to ICAM1, because **high-**, **mid-** and non-ICAM1 binding PRBC all bound at similar levels.

The overall levels of adhesion to TSP of IT-derived PRBC with distinct antigenic phenotypes were comparable, and the antigenic phenotype of D4-I was unknown because PRBC did not form agglutinates in the available endemic area serum. Thus, it was impossible to conclude that a particular antigenic phenotype was associated with low levels of adhesion to TSP. It was rationalised that sequence specific proteases would be a more sensitive tool to test this relationship, because adhesion to TSP would be differentially sensitive to digestion if it was mediated by variable surface molecules.

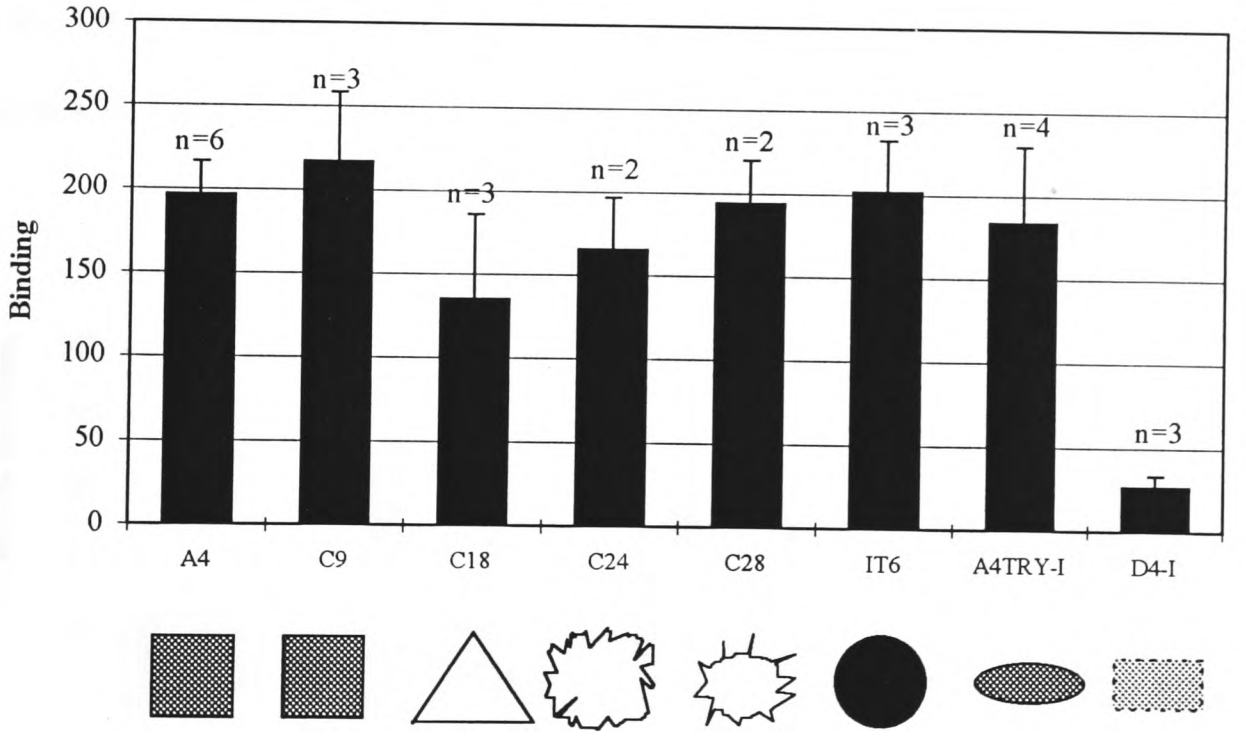


Figure 5.1. Bar chart summary of adhesion to purified thrombospondin of PRBC with different surface phenotypes. Data are mean binding mm^{-2} ±SEM from several (n) experiments. Shapes below chart represent antigenic phenotypes of PRBC, i.e. square is A4VAT, triangle is C18VAT, circle is IT6VAT etc. (D4-I was uncharacterised and is denoted by dashed rectangle). Shading denotes degree of PRBC binding to ICAM1 as determined in chapter 4: black = high binding to ICAM1, dark grey = mid binding, light grey = low binding, unshaded = no binding. Note that all PRBC can bind to CD36 (chapter 3).

5.2. Effect of Proteases

A single study by Sherwood and colleagues (1989) has shown that low concentrations of trypsin between 0.1 - 10μg ml^{-1} can reduce the binding of *Aotus* erythrocytes infected with the Malayian Camp strain of *P. falciparum*. PRBC binding to TSP (at 5μg ml^{-1} and 10μg ml^{-1}) was completely abolished by the highest concentration of trypsin (10μg ml^{-1}), but the same concentration of protease reduced binding to TSP (at 30μg ml^{-1}) by only 81%. The authors concluded that this 'large excess' of TSP may permit binding to a small number of PRBC receptors that had not been cleaved, but offered no explanation as to why these receptors were resistant (Sherwood *et al.*, 1989).

Sequence specific proteases have been used in this study to investigate the relationship of surface phenotypes by testing their effects on PRBC agglutination and binding to CD36 and to ICAM1. Protease resistant binding has been associated with the expression of particular antigenic phenotypes: V8 protease resistant PRBC adherence to CD36 was restricted to the C18VAT and trypsin-resistant PRBC binding to ICAM1 was linked to the A4TRYIVAT. Thus V8 protease and trypsin were tested for their effect on binding to TSP, at concentrations that were known to affect binding to other ligands (Figure 5.2).

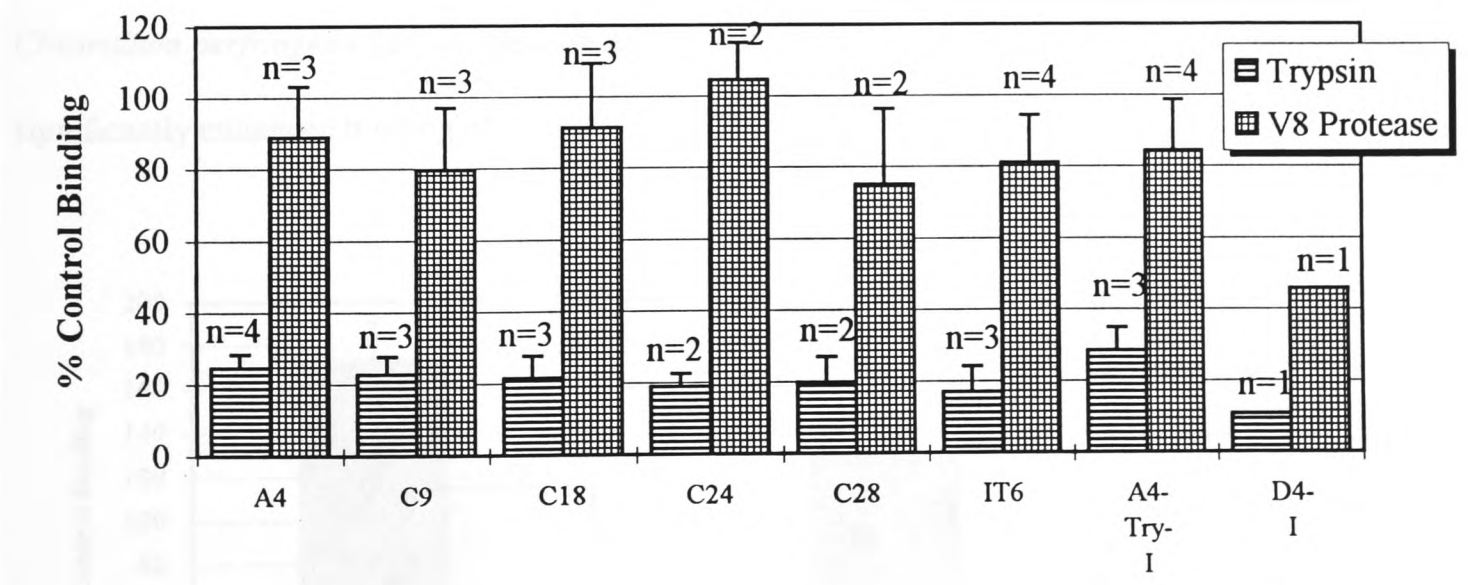


Figure 5.2. Effect of proteases on PRBC adherence to TSP. PRBC were digested with 1mg ml^{-1} trypsin or 10U ml^{-1} V8 protease and spot binding assays were performed using purified TSP at 40μg ml^{-1}. The data are from several (n) experiments and are mean percentage values ± SEM of undigested control PRBC treated with RPMI-S and inhibitor only.

Figure 5.2 shows that the two different proteases had a surprisingly uniform effect on the adhesion of IT-derived PRBC to TSP. Binding to TSP was partially resistant to trypsin: 16.8 - 29.4% of control binding remained after digestion. On the other hand, binding to TSP was generally insensitive to V8 protease: 75 - 104.6% control binding remained after digestion. A single experiment showed that the same trend of resistant binding to TSP was observed when PRBC from D4-I were digested with trypsin and V8 protease.

These findings are in marked contrast to the variant-specific effects of the same proteases on adherence to CD36 and ICAM1, and suggest that binding to TSP was not related to the antigenic phenotype of the PRBC. However, it is possible that the resistant binding could be mediated by a variant antigenic phenotype that is present at a constant level within each culture. Using paradigms established in the two preceding chapters, it would be anticipated that this subpopulation would be completely protease resistant or even exhibit protease-enhanced binding to TSP. This hypothesis seems improbable, because the high rate of clonal variation means that 6 cultures with different major antigenic phenotypes were unlikely to express similar levels of resistant subpopulations.

The influence of surface sialic acid residues on PRBC binding to TSP was investigated by treating PRBC from $A4_{i+}$ and $C18_{i-}$ with different neuraminidases. Figure 5.3 shows that neuraminidase from *Clostridium perfringens* had no effect on adhesion to TSP, but neuraminidase from *Vibrio cholerae* significantly enhanced binding of both sets of PRBC to TSP.

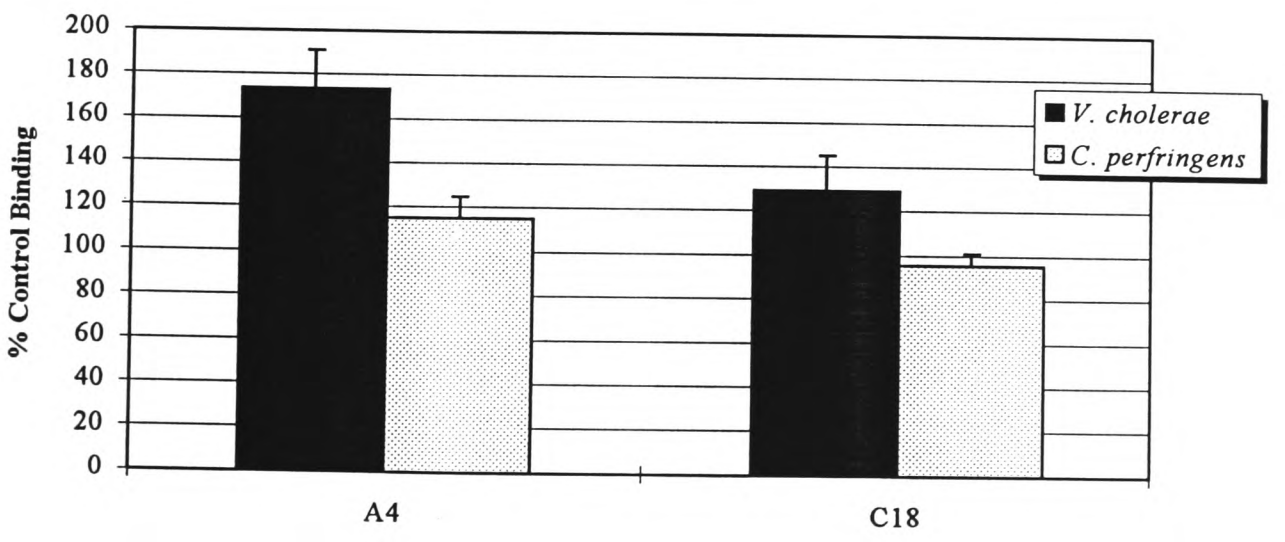


Figure 5.3. Effect of neuraminidases on PRBC binding to TSP at 40μg ml^{-1}. Data are mean cells bound mm^{-2} ± SEM from two experiments after PRBC were digested with neuraminidases from either V. cholerae *or* C. perfringens.

5.3. Selection of PRBC on TSP

In view of the separate nature of adhesion to TSP and antigenic phenotype, it was decided to select PRBC on purified TSP. This would enrich the PRBC within the culture that bound to TSP and analysis of the selected population would determine if these phenotypes were linked. $C24_{i-}$ was chosen because it had been selected in the previous chapter on ICAM1 to produce a parasite line, C24-I, that had switched to the A4VAT.

C24-TSP was produced by a single round of selection of mature PRBC from $C24_{i-}$ on Ca^{2+}-replete TSP (at 40 $\mu g\ ml^{-1}$) and was analysed immediately after removal from the TSP-coated dish (section 2.2.7). Mixed agglutination analysis showed that the relative antigenic phenotype of the cells that bound to TSP were identical to the C24VAT and no 'back-switching' had occurred to the A4VAT (Figure 5.4).

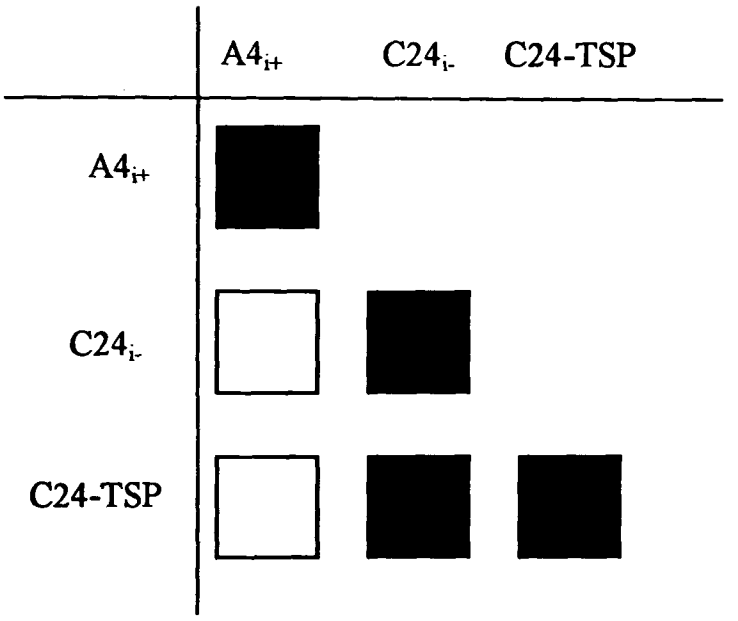


Figure 5.4. Mixed agglutination analysis of PRBC from $A4_{i+}$, $C24_{i-}$ and C24-TSP immediately following selection on TSP. Data are duplicate determinations from a single experiment. Key: dark shading = 100 - 80% antigenic similarity, clear box = 5 -0% antigenic similarity between test cultures.

A single spot binding assay demonstrated that PRBC could bind to CD36 immediately after selection of TSP (Table 5.1). PRBC from $C24_{i-}$ (mock-selected on a dish without TSP) and C24-TSP bound at similar levels to CD36 and TSP, and this strongly suggested that ligand(s) for binding to TSP and CD36 were expressed on the same cell. PRBC from C24-TSP were cultured for 2 further cycles and cryopreserved without further analysis.

PARASITE	Binding	
	TSP	CD36
C24	173.2 ± 25.5	267.3 ± 35.2
C24-TSP	188.6 ± 36.9	261.3 ± 22.4

Table 5.1. Binding of PRBC from $C24_{i-}$ (mock-selected on a dish without TSP) and C24-TSP immediately after selection on TSP. Binding (PRBC mm^{-2} ± SEM) was normalised for parasitaemia and haematocrit and values quoted are means from triplicate spots in duplicate dishes on the same day.

5.4. Conclusions

TSP has been known for a decade to mediate cytoadherence of PRBC. However, its involvement during sequestration is complex because it is a soluble protein that is difficult to measure accurately. The situation is complicated further because TSP can interact with several different surface proteins expressed by endothelial cells, including CD36, a known receptor for PRBC, which can function independently in PRBC cytoadherence. The relative importance of CD36 and TSP *in vivo* has not been elucidated due to practical difficulties in estimating circulating TSP in different malaria disease states.

Even less is known about the PRBC surface and its interaction with TSP, but results in this chapter have shown that adhesion to TSP was a constant property of PRBC that was partially resistant to trypsin and completely resistant to V8 protease. The main advantage of this study over previous investigations was that the surface phenotypes of PRBC employed were already characterised and the effects of proteases on agglutination, binding to CD36 and binding to ICAM1 were known. Consequently, several conclusions can be made that relate adhesion to TSP to other PRBC surface properties:

- **Adhesion to TSP was independent from antigenic phenotype**. PRBC which expressed different antigenic phenotypes bound to TSP at similar levels, and sequence-specific proteases did not have a variant specific effect on adhesion to TSP. Adhesion to TSP was maintained after digestion of PRBC with proteases that abolished agglutination. Thus, PRBC epitopes essential for agglutination were separable from binding domains for TSP. This conclusion was supported by the finding that PRBC which had been selected for adhesion to TSP were antigenically indistinguishable from the major antigenic phenotype of the mock-selected control PRBC. This implied that binding to TSP was a constant property of the PRBC surface that was not related to the antigenic phenotype of the PRBC.

- **Adhesion to TSP was separable from adhesion to CD36 and ICAM1**. PRBC bound to TSP after digestion with concentrations of trypsin and V8 protease that abolished adhesion to CD36 and ICAM1. PRBC that had been selected on TSP could bind to CD36 at the same levels as mock-selected PRBC, which implied that receptors for these ligands were on the same cell. Specific carbohydrate

structures are not essential for PRBC adhesion to TSP because neuraminidases did not abolish cytoadherence to TSP. In fact, digestion of PRBC with neuraminidase from *V. cholerae* caused an increase in binding to TSP which may be due to an alteration in the electrostatic charge of the PRBC, as a consequence of the relative contribution of sensitive sialic acid residues to the overall charge at the surface.

- **Adhesion to TSP was independent of the knobby phenotype**. PRBC from a knob-negative line, D4-I, adhered at low levels to TSP. This was the first demonstration of knob-independent *P. falciparum* infected erythrocyte adhesion to purified TSP which mirrored earlier findings with *Babesia*-infected erythrocytes. *B. bovis* is a parasite which infects cattle erythrocytes without any concomitant surface changes, but causes PRBC sequestration during infection (reviewed by Wright *et al.*, 1988). Infection of cattle with virulent strains of this parasite leads to cerebral symptoms, and PRBC can adhere to purified TSP *in vitro* (Parrodi *et al.*, 1989). Avirulent strains of *B. bovis* and other species, including *B. bigemina* and *B. rodhaini*, do not express knobs or cytoadhere *in vivo* (Parrodi *et al.*, 1990). However, PRBC from these strains bound to purified TSP at similar levels which suggests that adhesion to TSP is a constant property of PRBC infected with *Babesia* or *P. falciparum* and is independent of knob expression.

To interpret these results, a model of the interaction of PRBC with TSP and CD36 is proposed in figure 5.4. This differs from that proposed by Barnwell and colleagues (1989), because there are separate ligands on the PRBC surface which interact independently with TSP and CD36. This arrangement agrees with unpublished data from Chulay and Ockenhouse who have shown that fluid phase TSP and CD36 inhibit PRBC adherence to the homologous, but not the heterologous receptor (Chulay and Ockenhouse, 1990). The same authors have also referred to unpublished results using 'certain enzymes' to reduce PRBC adhesion to one, but not the other receptor (Chulay and Ockenhouse, 1990).

If the PRBC adhesion domains were on the same molecule, the domain for TSP adhesion would have to be closer to the membrane than the CD36 adhesion domain to explain the protease resistant PRBC binding to the former receptor. Alternatively, the adhesion domains could be on separate molecules. These possibilities were probed further in chapter 6, and are discussed in detail in chapter 8.

In addition to parasite-derived proteins, cryptic host epitopes may be exposed on infection by *P. falciparum* that can mediate cytoadherence (reviewed by Sherman and Winograd, 1990). This is pertinent for PRBC adhesion to TSP for two reasons. Firstly, TSP has lectin-like activity (Haverstick *et al.*, 1984) and secondly, modified erythrocytes can interact with TSP as shown by the ability of soluble protein to mediate agglutination of trypsinised, glutaraldehye-fixed human erythrocytes (Jaffe *et al.*, 1982). If similar proteins are exposed during intraerythrocytic parasite maturation, they may contribute in PRBC adhesion to TSP, and explain why this is a constant function of PRBC and is not linked to the expression of variable surface phenotypes. Other laboratories have found that trypsin does not completely abolish PRBC binding to TSP (Sherwood *et al.* 1989, Dr. D. Baruch, personal. communication). Thus, cryptic domains of trypsin-insensitive host proteins (eg. Band 3) may become accessible during parasite maturation as has been proposed to mediate adhesion to CD36 (Crandall *et al.*, 1994). Therefore, the elevated levels of binding to TSP of PRBC from *Aotus* monkeys compared to the human erythrocytes in the original study (Roberts *et al.*, 1985b) may have been due to differential expression of important erythrocyte molecules in these species.

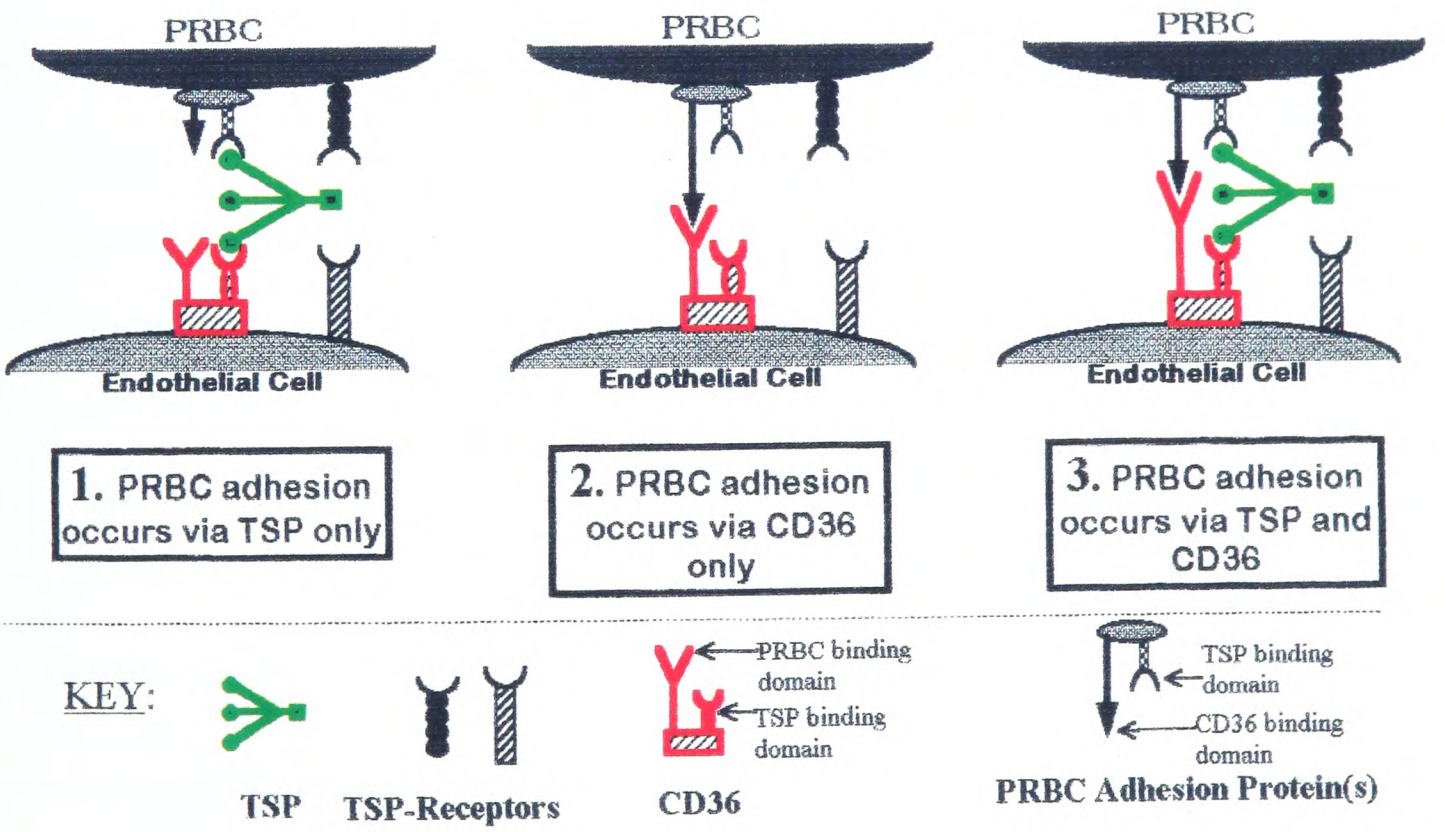


Figure 5.4. Three theoretical models of the interaction of PRBC with TSP and CD36. In model 1, adhesion is via TSP interaction with the PRBC surface, whereas in model 2, only CD36 interacts with the surface. Model 3 shows the mechanism when both TSP and CD36 interact independently with the PRBC surface, as has been demonstrated by soluble receptor blockade experiments (Chulay and Ockenhouse, 1990). Note that TSP could contribute to cytoadherence by forming bridges between the PRBC and the endothelial cell using either parasite derived proteins or other invariant TSP receptors on the PRBC surface. There are other combinations possible because there may be multiple potential TSP-receptors on the surface of PRBC, which may be of host origin and may share homology with other TSP-receptors expressed by endothelial cells.

Chapter 6

Functional Molecules at the PRBC Surface

Indirect evidence has linked the cytoadherent and antigenic phenotypes at the surface of the *P. falciparum* infected erythrocyte (reviewed by Roberts *et al.*, 1993). Three candidate molecules have been proposed which mediate PRBC cytoadherence and agglutination.

The most persuasive results support a role for the parasite-derived family of proteins, PfEMP1, in carrying the antigenically-variant determinants and in mediating PRBC cytoadherence to C32 melanoma cells (Howard *et al.*, 1988), HUVECs (Biggs *et al.*, 1992). PfEMP1 is defined in operational terms: it is a surface-labelled high, variable molecular weight (200 - 300kDa) protein that is insoluble in Triton X-100, but soluble in SDS and sensitive to low concentrations of trypsin (Leech *et al.*, 1984; Howard *et al.*, 1988). The variant-specific antibodies which mediate agglutination of PRBC in immune serum also precipitate PfEMP1 in a variant-specific fashion (Howard *et al.*, 1988). Identical serum can also reverse adhesion of *Aotus*-derived *P. falciparum* infected erythrocytes to C32 melanoma cells in a strain-specific manner, suggesting that the antigenic epitopes and adherent domains are in close proximity or are on the same surface molecule (David *et al.*, 1983). Roberts and colleagues (1992) proved that clonal antigenic variation was associated with modifications in the adhesion of PRBC clones to purified ICAM1. A similar study demonstrated that changes in PRBC adhesion to HUVECs was always accompanied by a switch in the PRBC antigenic phenotype and loss of adhesion was associated with a concomitant reduction in the size of the PfEMP1 expressed (Biggs *et al.*, 1992). Biochemical investigations showed that adhesion of PRBC to C32 melanoma cells (David *et al.*, 1983), HUVECs (Biggs *et al.* 1992), purified CD36 (Barnwell *et al.*, 1989) and purified TSP (Sherwood *et al.*, 1989) was universally sensitive to trypsin and PfEMP1 had an identical pattern of sensitivity to the same protease (Leech *et al.*, 1984).

A second candidate molecule has been identified with the use of anti-idiotype (Anti-Id) antibodies against OKM8, a monoclonal antibody to CD36 that inhibits PRBC adhesion (Ockenhouse *et al*, 1991b). Anti-Id antibodies molecularly mimicked CD36 and immunoprecipitated a high molecular weight protein, termed sequestrin, from surface- and metabolically-labelled knob-negative PRBC derived from a single parasite line. While sequestrin had similar biochemical characteristics to

PfEMP1 expressed by other parasites, no further information has appeared and it is not clear how the two proteins are related.

This situation is in contrast to the host-encoded adhesin, Pfalhesin, that has been proposed to mediate the interaction between the PRBC and CD36 (Crandall *et al.*, 1994). Pfalhesin is reported to be a cryptic region of the host anion transport protein, Band 3 (residues 546 - 553), which is only exposed on the surface of erythrocytes infected with mature forms of *P. falciparum*. Peptides from this region inhibited adhesion of one PRBC line to C32 melanoma cells and to CHO cells that were stably transfected with a cDNA encoding CD36 (Crandall *et al.*, 1994). There was no effect of soluble Pfalhesin on PRBC adhesion to ICAM1-transfected CHO cells and the authors concluded that a modified form of Band 3 was important for PRBC adhesion to CD36. This was the latest report from the same laboratory in a series of studies over the last 6 years which have suggested that parasite-induced alterations to Band 3 can mediate specific adhesive interactions (Winograd and Sherman, 1989; Crandall and Sherman, 1991, 1993; Crandall *et al.*, 1993; Land *et al.*, 1995).

Results from this study presented in earlier chapters have provided an insight into the phenotypic relationships of cytoadherence and agglutination of a range of cloned PRBC *in vitro*. The use of sequence-specific proteases to digest PRBC demonstrated that adhesion to CD36 and ICAM1 were variant-specific functions which were related to the antigenic phenotype of the PRBC. The findings suggested that adhesion to CD36 and ICAM1 would be mediated by variable molecules. However, adhesion to TSP was a constant property of all PRBC which was not affected by proteases in a variant-specific fashion and was likely to be mediated by invariant proteins. These hypotheses were examined by methods that could distinguish firstly between host and parasite-derived proteins and secondly between molecules which are expressed on the surface or inside the infected erythrocyte.

6.1. Identification of parasite-derived surface molecules

Several techniques were employed to differentiate between host and parasite-derived surface molecules. Coomassie staining of SDS PAGE gels gave an indication of the relative mobilities of internal and external proteins (section 2.2.18), and metabolic labelling (section 2.2.15) was used to establish that a protein was parasite-derived. Radioiodination was routinely employed throughout this study with conditions that labelled only surface proteins at available tyrosine residues (2.2.14). Surface-exposed PRBC proteins have been defined historically by their sensitivity to trypsin (Leech *et al.*, 1984), and thus labelled cells were digested with this protease at 1mg ml^{-1} before subsequent analysis. Figures in this chapter have been annotated with **0** to denote PRBC that were treated with RPMI-S as controls or with **T** to denote digestion with trypsin at 1mg ml^{-1}. The detergent solubility characteristics of radioiodinated proteins were exploited to separate labelled material into a Triton soluble (TS) extract and a Triton insoluble (TI) pellet, which was subsequently solubilised in SDS.

- **Coomassie staining**

Proteins were separated by SDS PAGE, and typical Coomassie-stained gels of uninfected erythrocytes and parasitised erythrocytes from the $A4_{i+}$ clone are shown in Figure 6.1(a). Protein standards (Rainbow markers; R_M in kDa) were separated on the same gels and their mobilities were used to estimate the size of bands. These gels provided a quantitative analysis of the relative proportions of proteins present and indicate that the majority of protein in PRBC was host-derived. Major membrane proteins included the α and β chains of spectrin (240kDa and 220kDa respectively) (Anderson, 1979) and Band 3. Trypsin-sensitive proteins were absent, which implied that either they constituted a minor proportion of the total protein or that they were not stained by Coomassie dye (e.g. glycophorin).

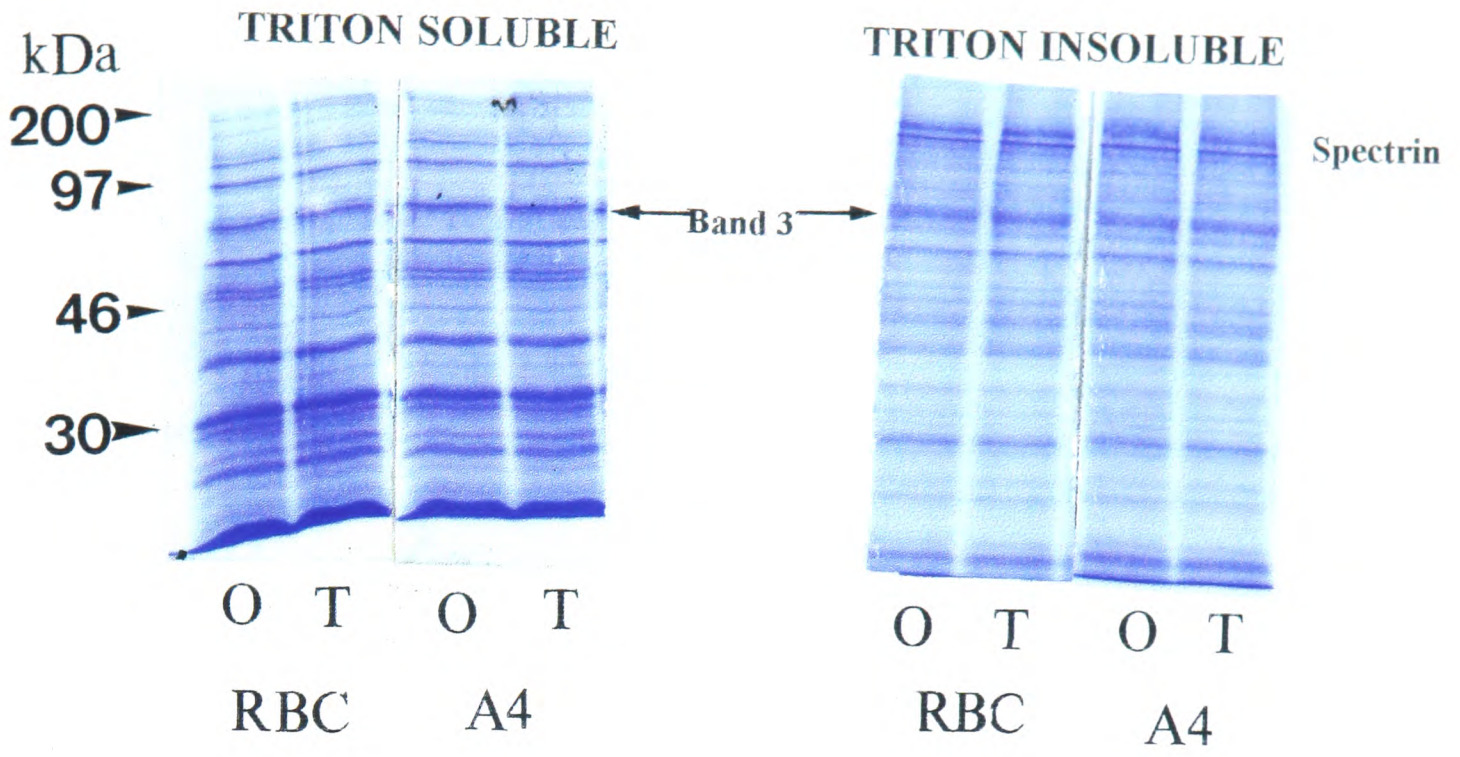


Figure 6.1a. 10% Coomassie stained SDS PAGE gels of Triton-soluble (TS) and Triton-insoluble (TI) extracts from uninfected cells and PRBC infected with mature forms of $A4_{i+}$. Cells had been treated with RRMI-S or trypsin at 1mg ml^{-1} before detergent extraction.

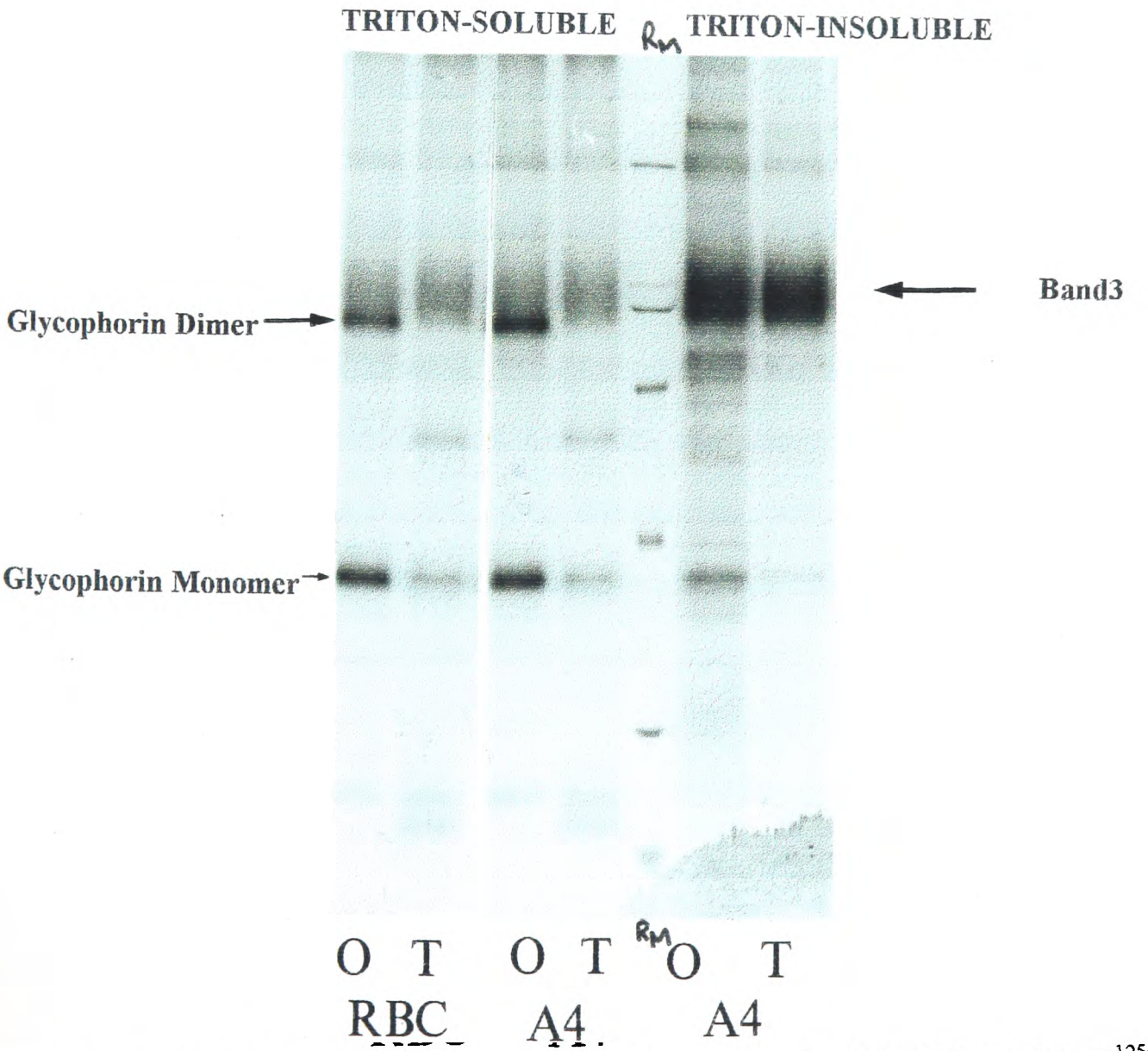


Figure 6.1b. Autoradiograph of Triton-soluble (TS) and Triton-insoluble (TI) extracts of ^{125}I surface labelled PRBC ($A4_{i+}$) and uninfected erythrocytes separated on 10% SDS PAGE gels.

- **Surface labelling**

Analysis of detergent-extracted ^{125}I-labelled PRBC by SDS PAGE gels and autoradiography showed that there was a range of surface labelled proteins only present in parasitised cells. Multiple proteins were trypsin-sensitive (Figure 6.1b), and labelled fragments were observed after digestion. Glycophorin was identified in the Triton soluble extracts as a trypsin sensitive monomer and dimer. High molecular weight parasite-derived TI proteins were sensitive to trypsin and could only be resolved in detail on 5% gels (Figure 6.1c). These proteins fulfilled the operational criteria for PfEMP1, and migrated with M_r 300, 290, 280, 260, 240kDa. There were no equivalent proteins in the TS extracts. The anion-exchange protein, Band 3 was the most abundant erythrocyte membrane protein present at 1×10^6 copies per cell (Tanner *et al*, 1988), and migrated as a characteristic smear of 85-110kDa (Fairbanks *et al.*, 1971). Band 3 had a Triton-soluble component and a Triton-insoluble component and despite its exposure on the surface, it was trypsin-resistant in both uninfected erythrocytes and PRBC, as previously reported (Steck *et al.*, 1971; Dzandu *et al.*, 1985; Leech *et al.*, 1984).

- **Metabolic Labelling**

The host erythrocyte does not synthesise new proteins, thus parasite-derived proteins can be identified by their ability to incorporate radiolabelled amino acid precursors (Howard *et al.*, 1988). PRBC were cultured in medium with a cocktail of ^{35}S-labelled methionine and cysteine, and proteins analysed following detergent extraction. All the proteins in Figure 6.1d were of parasite origin, and several TI proteins were trypsin-sensitive. Molecular weights were assigned to these bands by measurement of their R_f mobility values and are discussed later. Multiple trypsin-sensitive proteins were detected in the high molecular weight range of the TI extract which satisfied the criteria for PfEMP1, and are labelled with arrow heads.

Therefore, it was possible to identify parasite-derived surface proteins with a combination of these techniques. Coomassie staining, radioiodination, metabolic labelling, and autoradiography were applied to different PRBC clones to identify molecules that may mediate functional changes at the infected erythrocyte membrane.

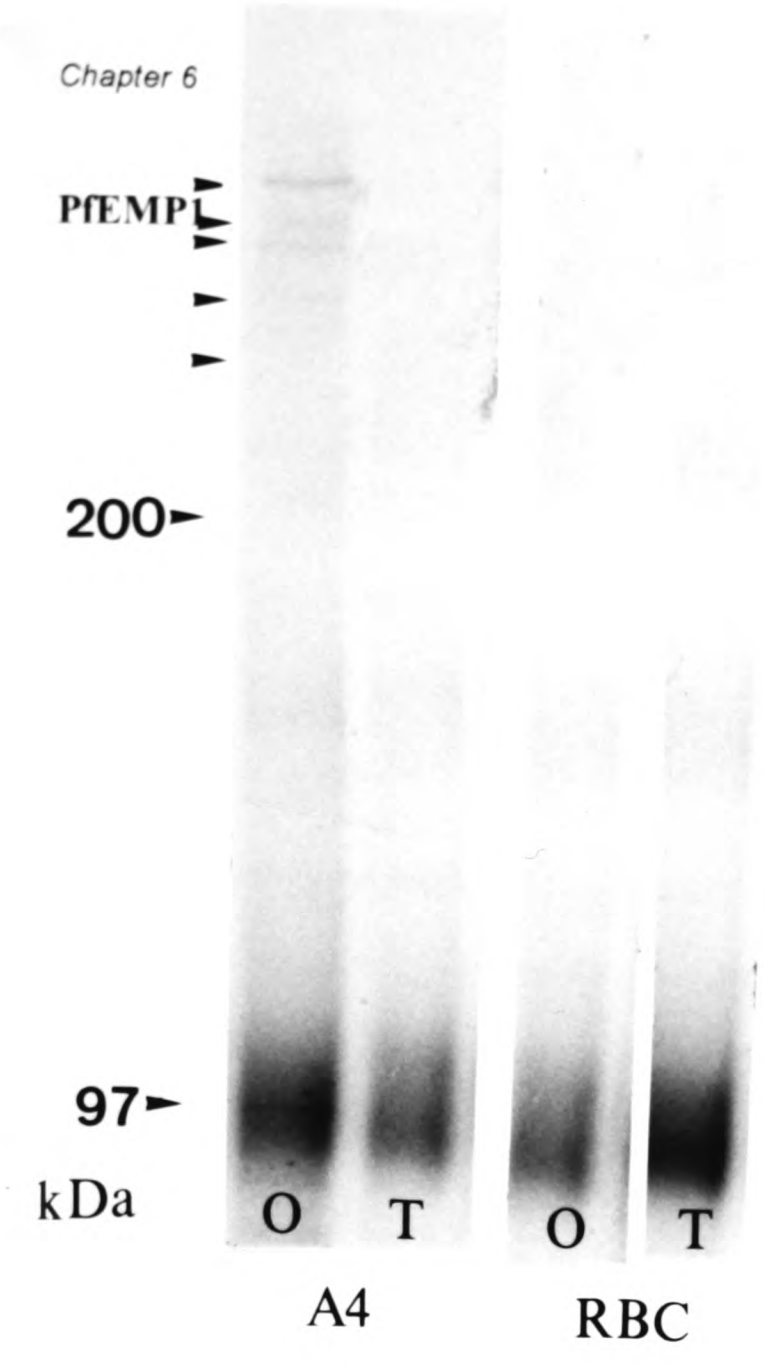


Figure 6.1c Autoradiograph of Triton-insoluble (TI) extracts from ^{125}I-surface labelled PRBC infected with A4$_{1.1}$ or erythrocyte controls (RBC), separated on a 5% SDS PAGE gel to resolve proteins with the characteristics of PfEMP1

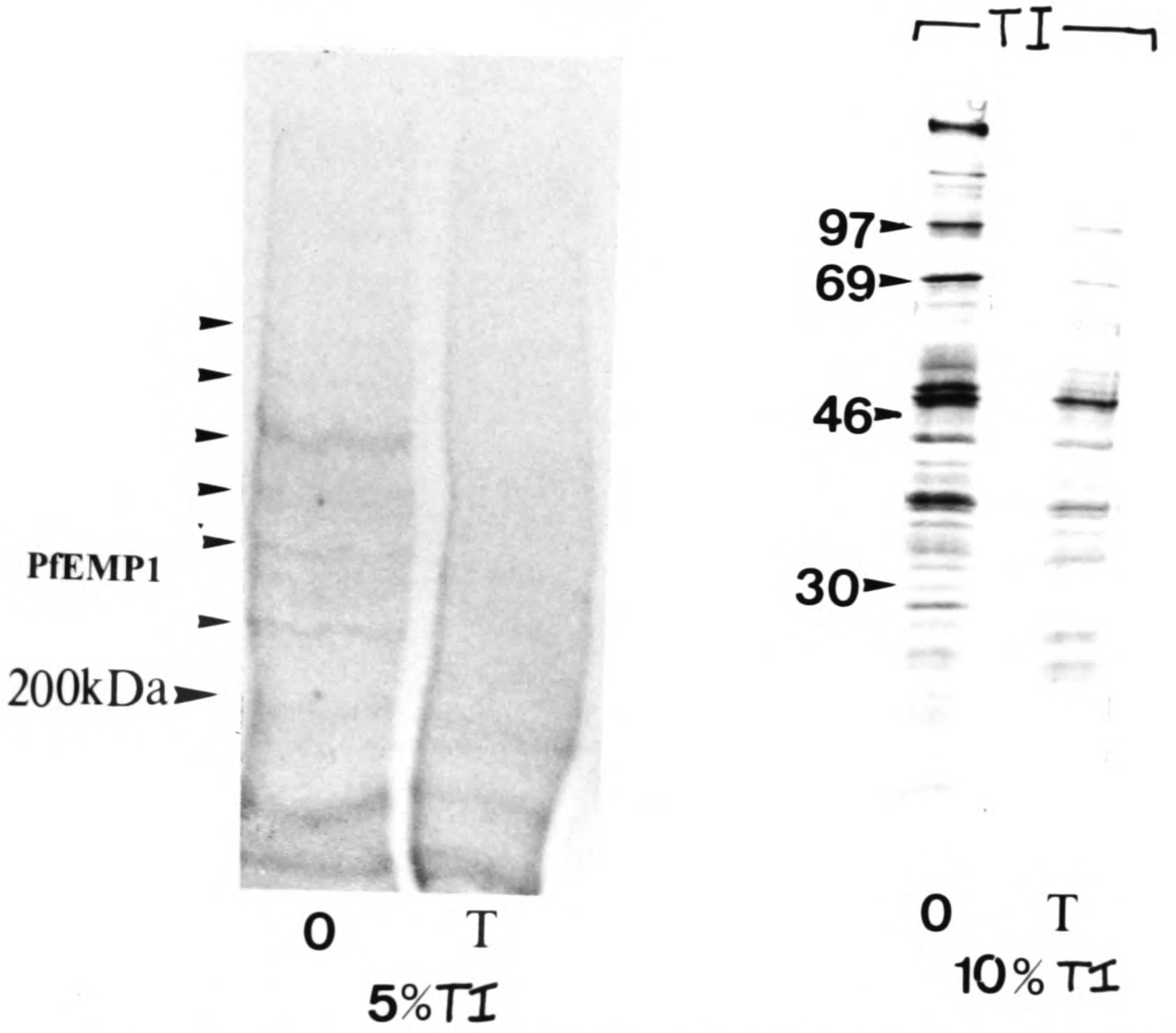


Figure 6.1d. ^{35}S-metabolic labelled extracts of PRBC from A4$_{1.1}$ separated on 10% and 5% SDS PAGE gels. PRBC were treated with either RPMI-S (O) or trypsin at 1mg ml^{-1} (T).

6.2. Identification of variant antigens

Variant-specific epitopes determine the antigenic phenotype of the PRBC and clones derived from $A4_{i+}$ expressed either the parental, A4VAT, antigenic type (e.g. $C9_{i+}$) or a variant antigenic phenotype (eg. C18VAT, C24VAT, C28VAT etc.). The relative antigenic phenotypes had been determined using the mixed agglutination assay which required antibodies in Sib serum to cross react with the surface of PRBC. Techniques described in the preceding section were used to investigate the variant molecule(s) on the mature PRBC surface that mediated agglutination in this clone family.

PRBC from a range of subclones of $A4_{i+}$ were surface-labelled with ^{125}I to investigate the properties of molecules that fulfilled the operational characteristics of PfEMP1. 5% gels of surface labelled Triton-insoluble proteins revealed a family of trypsin-sensitive molecules that had different elecrophoretic mobility between clones (Figure 6.2a, b). These molecules were specific for the antigenic phenotype of each clone (Table 6.1) and were the only proteins to satisfy the criteria for PfEMP1. Characteristic variant molecules of M_r 290, 280, 260 and 225kDa were observed in PRBC from $A4_{i+}$ (Figure 6.2a). The major variant molecule of $C18_{i-}$ was 225kDa (Figure 6.2a), in $C9_{i+}$ it was 280kDa, and in $C24_{i-}$ it was 260kDa (Figure 6.2b).

Parasite	Size of PfEMP1(s)	n
$A4_{i+}$	290, 280, 260, 240, 225	4
$C9_{i+}$	280, 240, 225	2
$C18_{i-}$	225	4
$C24_{i-}$	260	2
$C28_{i-}$	240	2

Table 6.1. Summary of the size of PfEMP1s expressed by different clones as measured in several surface labelling experiments (n). PfEMP1 was defined as a trypsin-sensitive high molecular weight(M_r) triton insoluble ^{125}I labelled variant protein. The size of PfEMP1 was determined by plotting the log(M_r) vs the R_f value (distance migrated / total migration of gel front) for commercially-available radiolabelled 'Rainbow' standards. To improve the accuracy of estimates at the high molecular weight range, the Coomassie-stained erythrocyte spectrin doublet (240kDa and 220kDa) from the same gel was used in addition to standards.

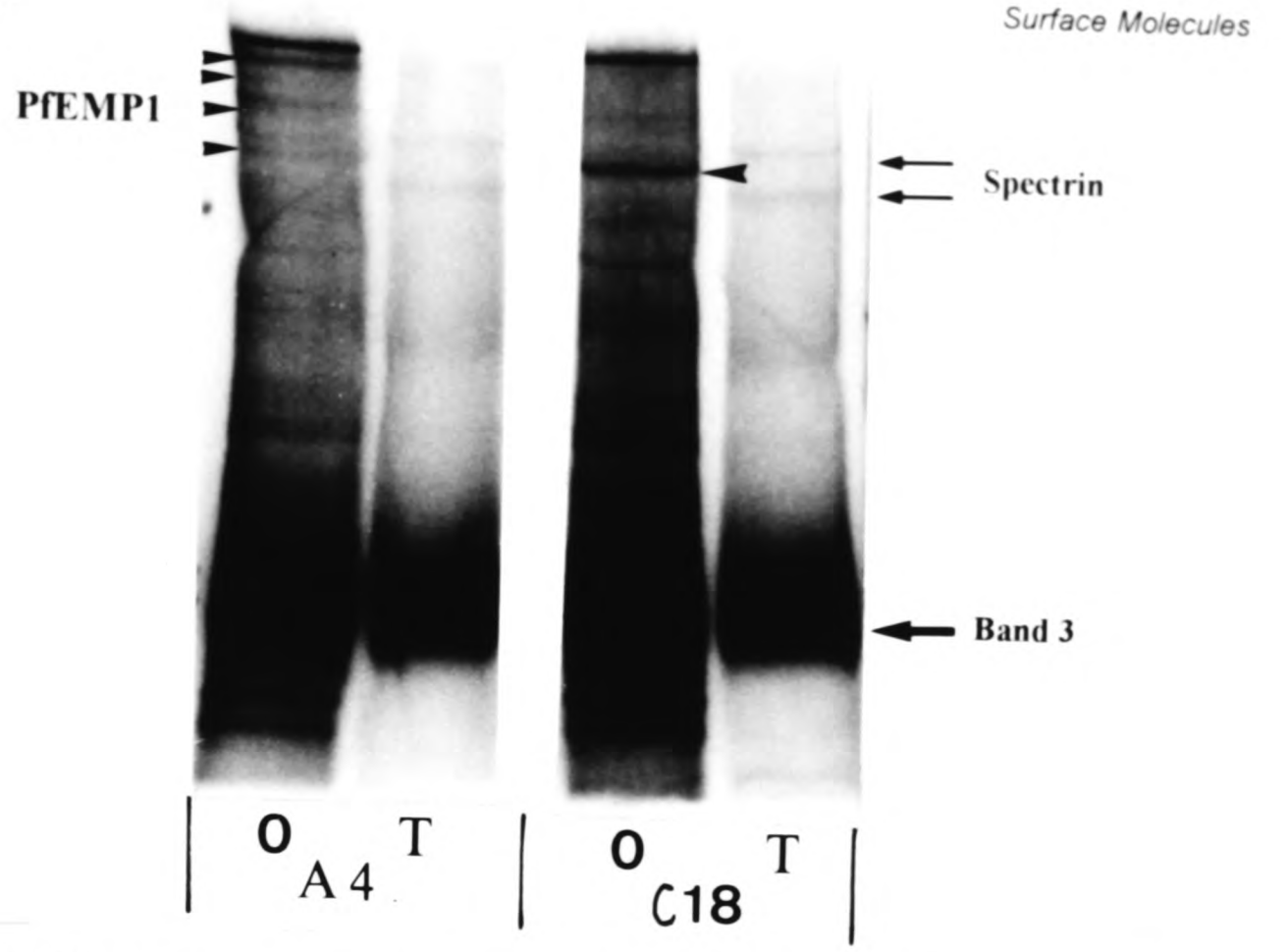


Figure 6.2a ^{125}I-surface labelled Triton-insoluble (TI) extracts from PRBC infected with $A4_{t+}$ or $C18_{t-}$ separated on 5% SDS PAGE gels to resolve proteins with the characteristics of PfEMP1. The major PfEMP1 of $C18_{t-}$ is 225kDa, and Band3 is resistant to trypsin. Note that spectrin has been labelled in a small number of lysed cells.

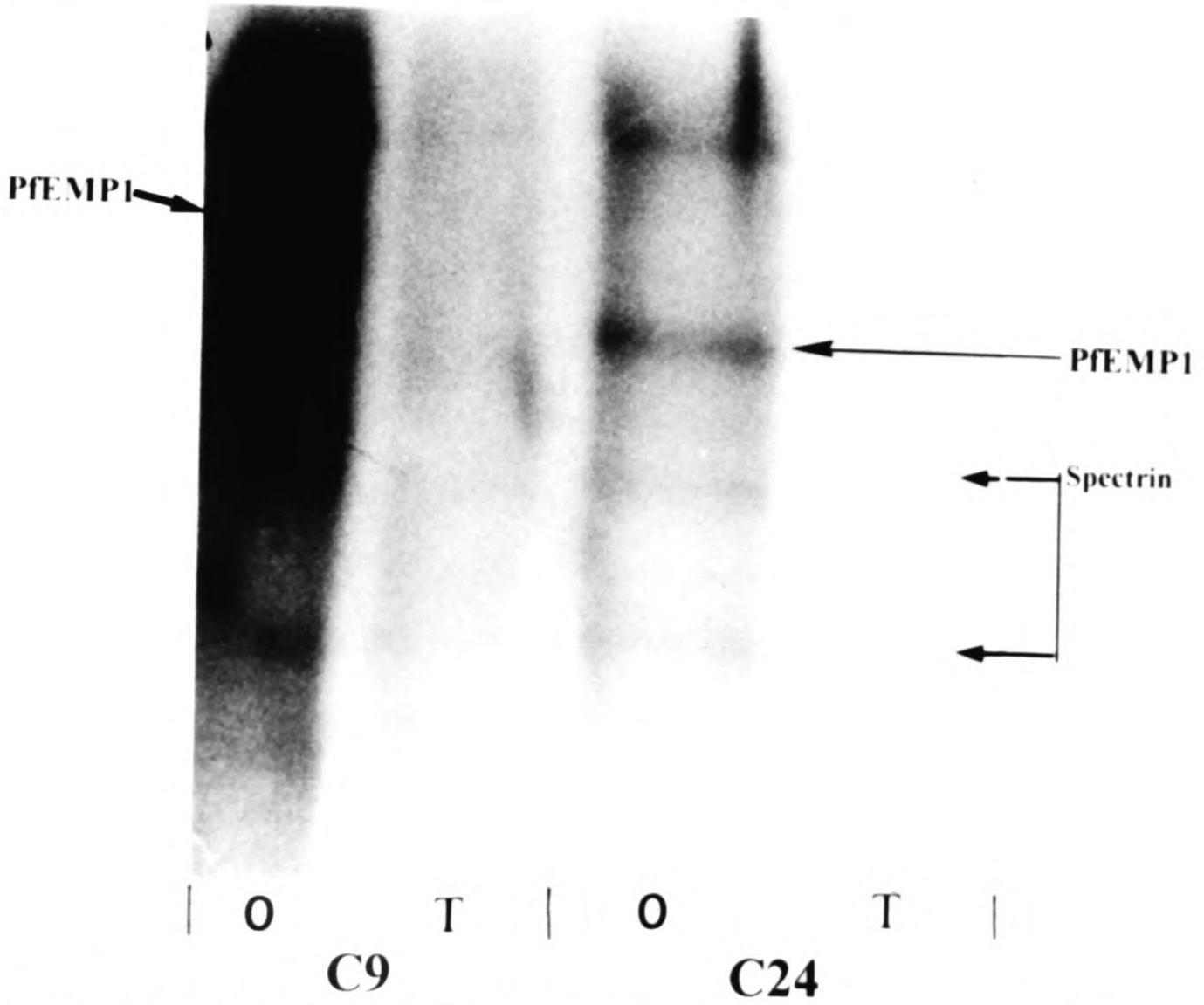


Figure 6.2b ^{125}I-surface labelled Triton-insoluble (TI) extracts from PRBC infected with $C9_{t-}$ and $C24_{t-}$ separated on 5% SDS PAGE gels to resolve proteins with the characteristics of PfEMP1. Major PfEMP1 in $C9_{t-}$ is 280kDa and in $C24_{t-}$ is 260kDa. Note that spectrin has been labelled in a small number of lysed cells.

A surface protein of constant molecular mass (~300 kDa) was occasionally labelled on PRBC and was sensitive to trypsin. The 300kDa protein was consistently detected when PRBC were grown in the same erythrocytes and labelled at the same time. However, it was absent from identical clones in different erythrocytes, therefore this protein could be a red cell senescent antigen. A 300kDa trypsin sensitive protein was labelled in uninfected cells, and erythrocytes infected with young ring stages which agreed with this hypothesis (Figure 6.2c).

The variant PfEMP1-like proteins were not detected by Coomassie staining and were probably present at low copy number (Figure 6.2d).This was confirmed by low intensity bands with the characteristics of PfEMP1 observed after identical gels were silver stained (data not shown). A constant 290kDa (n=6) parasite-derived internal protein with the characteristics of PfEMP2 (Howard *et al.*, 1988), was observed in Coomassie blue stained 10% SDS PAGE gels of TI samples. Extensive analysis of TI and TS proteins by 5% and 10% SDS PAGE gels did not reveal additional variable surface proteins and typical invariant Triton soluble proteins are shown for PRBC from $C18_{i-}$ and $C28_{i-}$, compared to uninfected cells (figure 6.2e).

Immunoprecipitation of TS and TI extracts from surface labelled and metabolically labelled PRBC was carried out to identify proteins recognised by antibodies from immune serum. The Sib serum was obtained from the same pool as the serum used in all agglutination assays and control European (Eur) serum was from a pool obtained from individuals that had not been exposed to malaria. Results with surface labelled PRBC from $A4_{i+}$ and $C18_{i-}$ are shown in Figure 6.3 and illustrate that multiple molecules with the characteristics of PfEMP1 were specifically precipitated by antibodies in Sib serum. These molecules had a M_r of 290, 280, 240 and 225kDa in $A4_{i+}$ and a single molecule of M_r 225 kDa was identified in $C18_{i-}$. The putative 300kDa senescent red cell antigen was not immunoprecipated by Sib or Eur sera in any clone. No PfEMP1-like molecules were immunoprecipitated after PRBC had been digested with trypsin or V8 protease. An important exception was a 175kDa molecule with the characteristics of PfEMP1 which was immunoprecipitated only from $C18_{i-}$ after V8 protease treatment (Figure 6.3; discussed in next section). Unfortunately, none of the surface labelling results could be repeated using metabolically labelled extracts implying that either the variant proteins were present at low copy number or were of host origin.

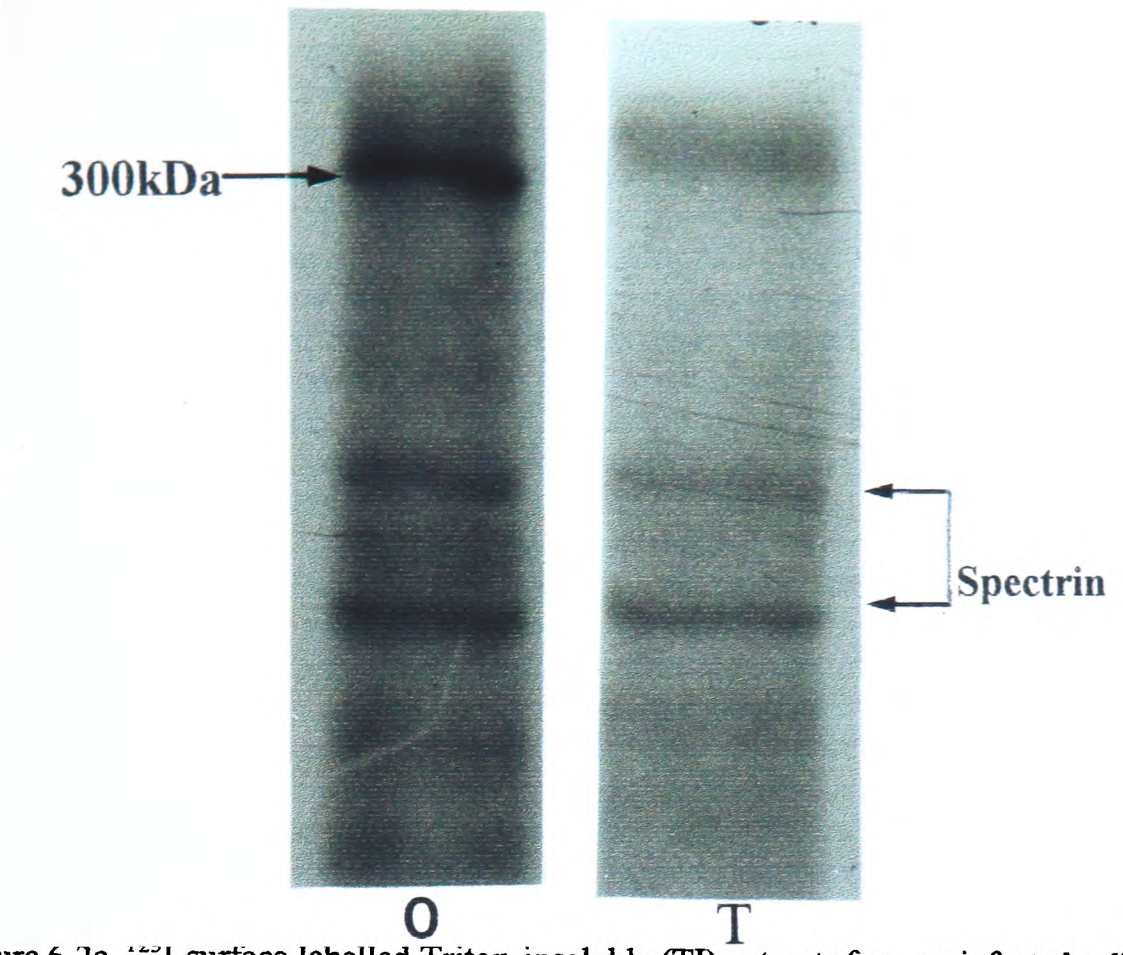


Figure 6.2c. ^{125}I-surface labelled Triton-insoluble (TI) extracts from uninfected cells and ring stage PRBC at the bottom of a Plasmagel™ preparation of $A4_{i+}$ demonstrating that putative 300kDa senescent antigen is trypsin sensitive. Note that spectrin has been labelled in a small number of lysed cells.

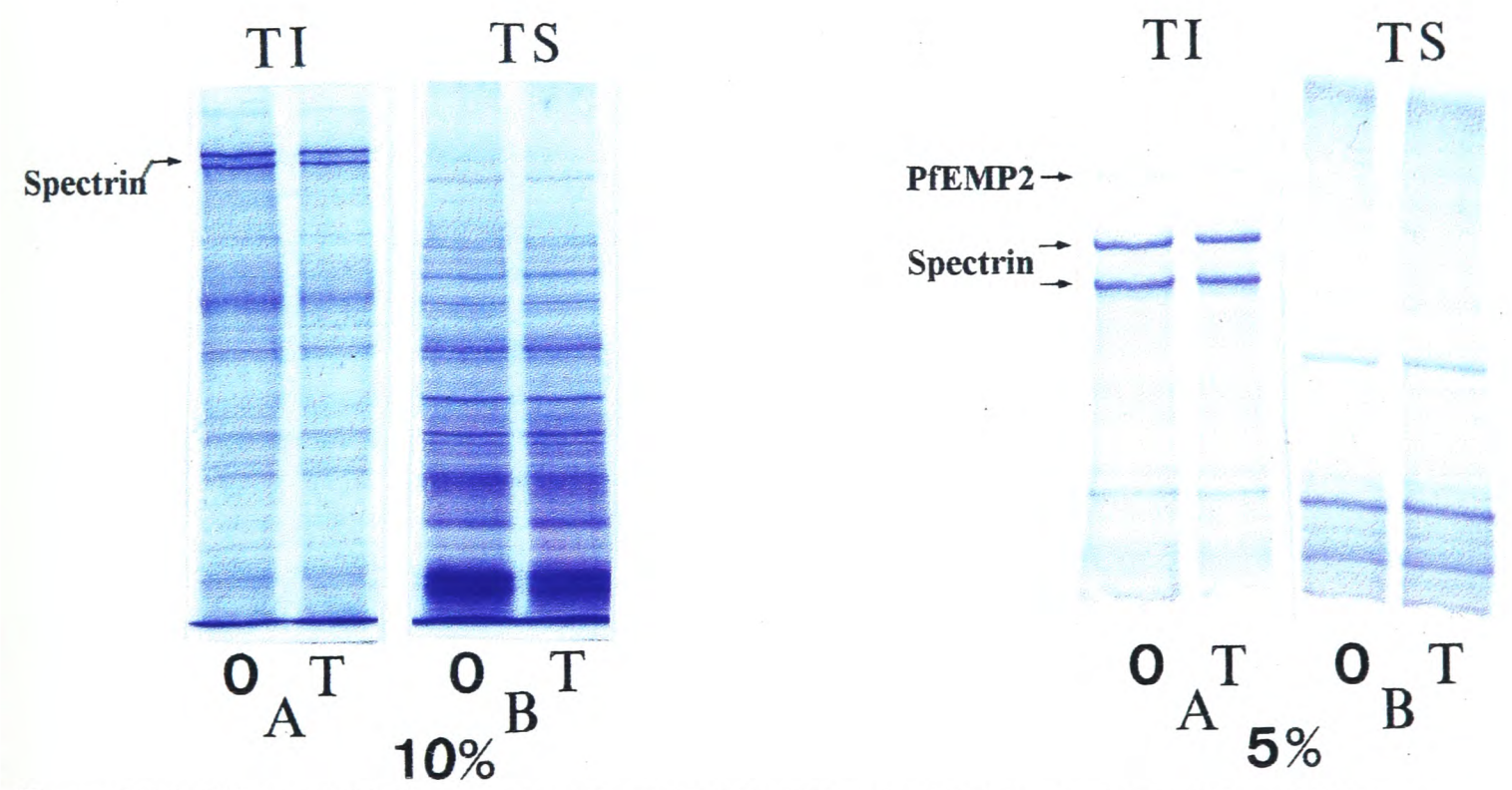


Figure 6.2d. 5% and 10% Coomassie stained SDS PAGE gels of Triton-insoluble (TI) and Triton-soluble (TS) extracts from PRBC infected with mature forms of $C18_{i-}$ (A) and $C28_{i-}$ (B).

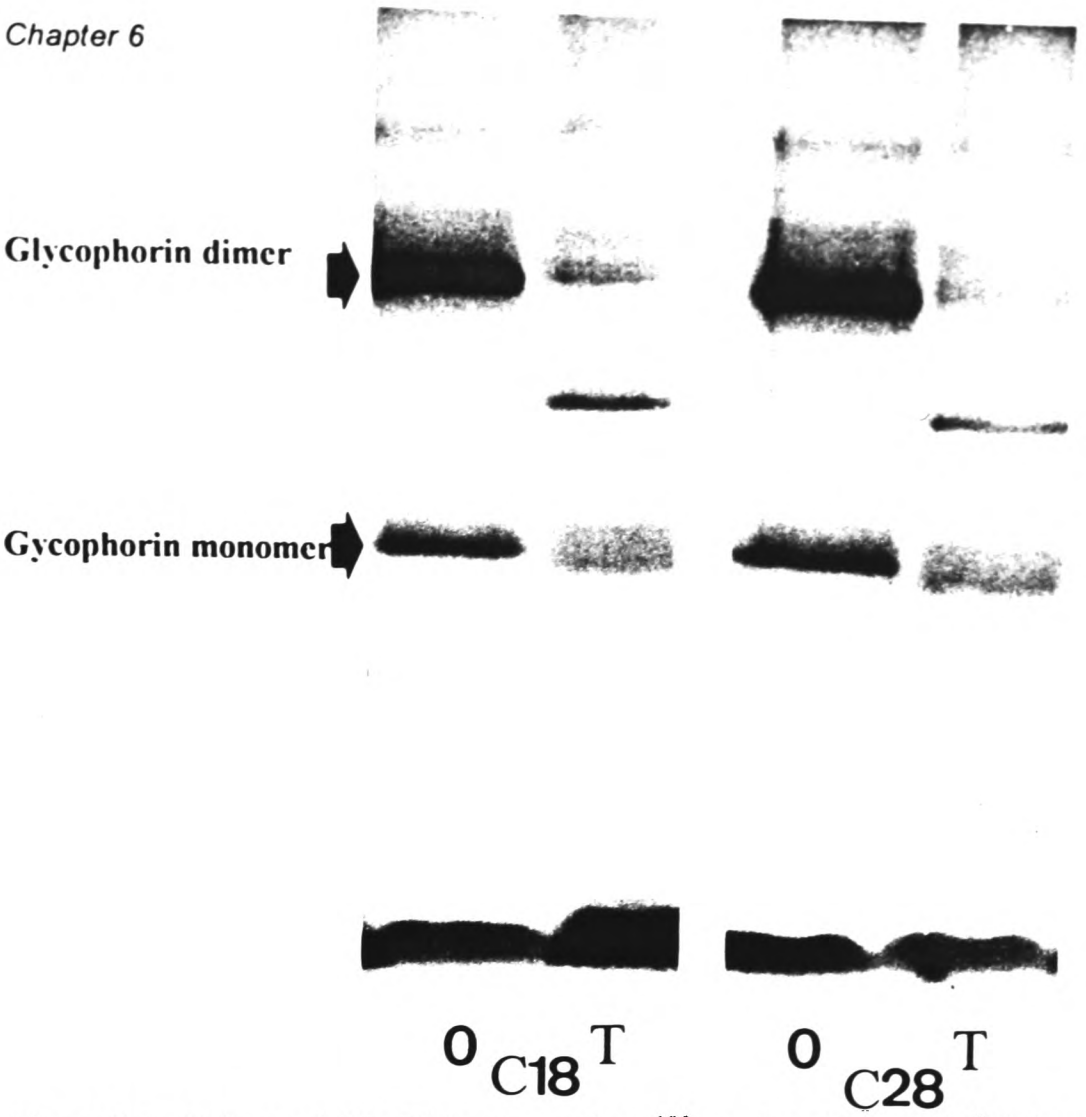


Figure 6.2e. Triton-soluble (TS) extracts from ^{125}I-surface labelled PRBC from $C18_t$. and $C28_t$. treated with RPMI-S or trypsin.

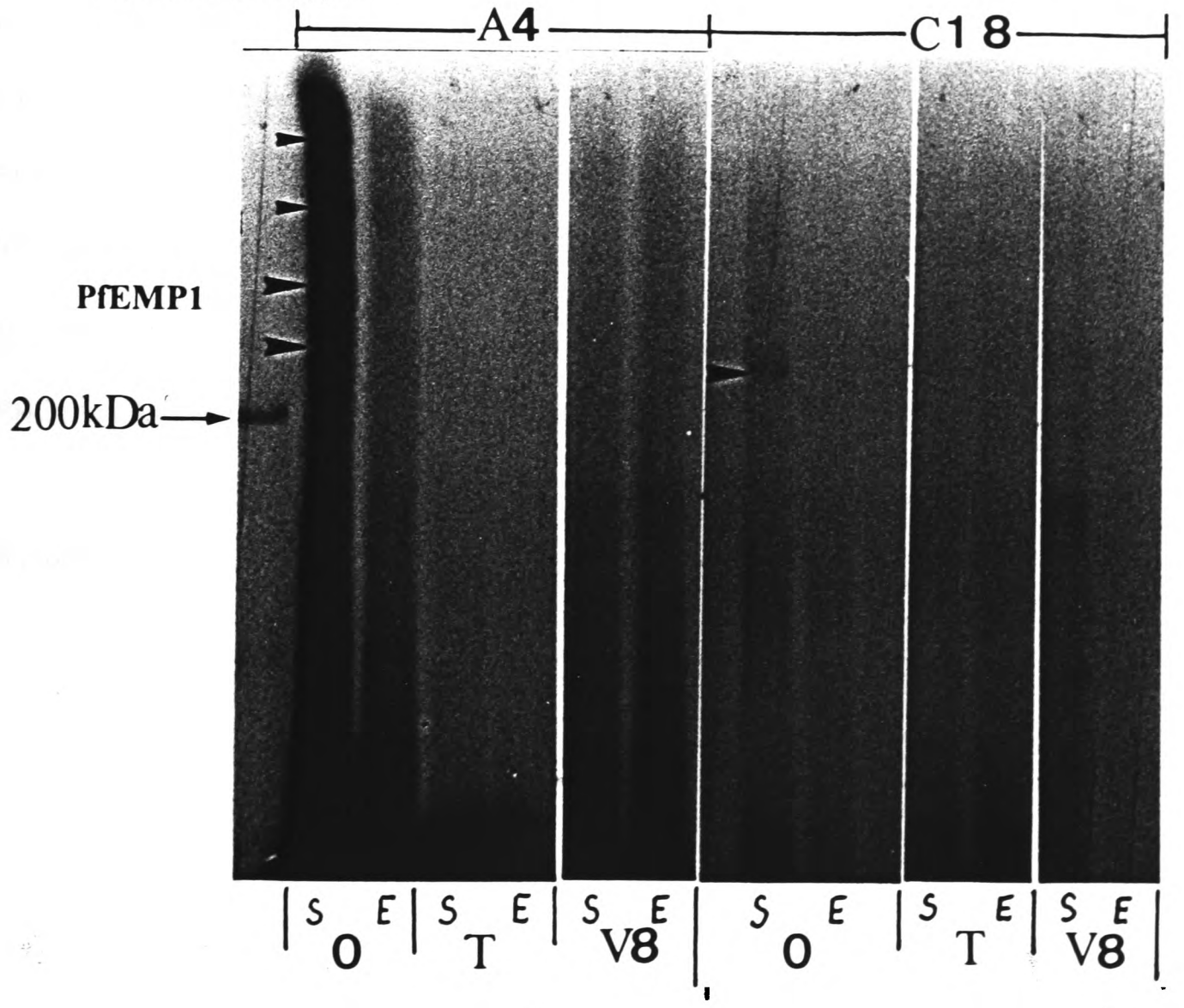


Figure 6.3. Immunoprecipitation of ^{125}I Triton-insoluble (TI) antigens from $A4_t$. and $C18_t$ separated on a 5% SDS PAGE gel. PRBC were treated with RPMI-S (0), 1mg ml^{-1} trypsin (T) or 10U ml^{-1} V8 protease and immunoprecipitated on protein A sepharose beads linked to antibodies from either European serum (E) or Sib serum (S).

6.3. Adhesion to CD36

Data presented in chapter 3 have demonstrated that all PRBC tested can bind to CD36, but only clones that express a unique antigenic phenotype (C18VAT) can bind to the same receptor after digestion by V8 protease. Due to the high rate of clonal antigenic variation (Roberts *et al.*, 1992), the C18VAT was detectable as a minor subpopulation within other clones and mediated the V8 protease-resistant binding to CD36 detected in these cultures. When a partially resistant culture ($A4_{i+}$) was digested with V8 protease and selected on purified CD36, a parasite line (A4V8CD36) was produced which had identical antigenic and cytoadherent phenotypes to the C18VAT. A single PfEMP1 with identical mobility (225kDa) to the PfEMP1 expressed by $C18_{i-}$ was observed when PRBC from A4V8CD36 were surface labelled (Figure 6.4a), and there were no other differences in the TI and TS profiles between this selected line and other parasites. A large (175kDa) Triton insoluble protein remained after V8 protease digestion of A4V8CD36 and $C18_{i-}$, and a band with identical M_r and detergent solubility characteristics was present at low levels within $C9_{i+}$ (Figure 6.4b). The 175kDa protein was absent from other C clones and was therefore a variant-specific membrane-associated fragment produced by V8 proteolysis of surface labelled PRBC and its presence correlated quantitatively with the resistant binding to CD36 by the same PRBC.

A band of 175kDa with the same characteristics was immunoprecipitated by antibodies in Sib serum after PRBC from $A4_{i+}$ and $C18_{i-}$ had been treated with V8 protease (Figure 6.3). Several other labelled bands were immunoprecipitated by Sib and Eur sera, but no variant-specific proteins were detected after V8 proteolysis of labelled PRBC from other clones.

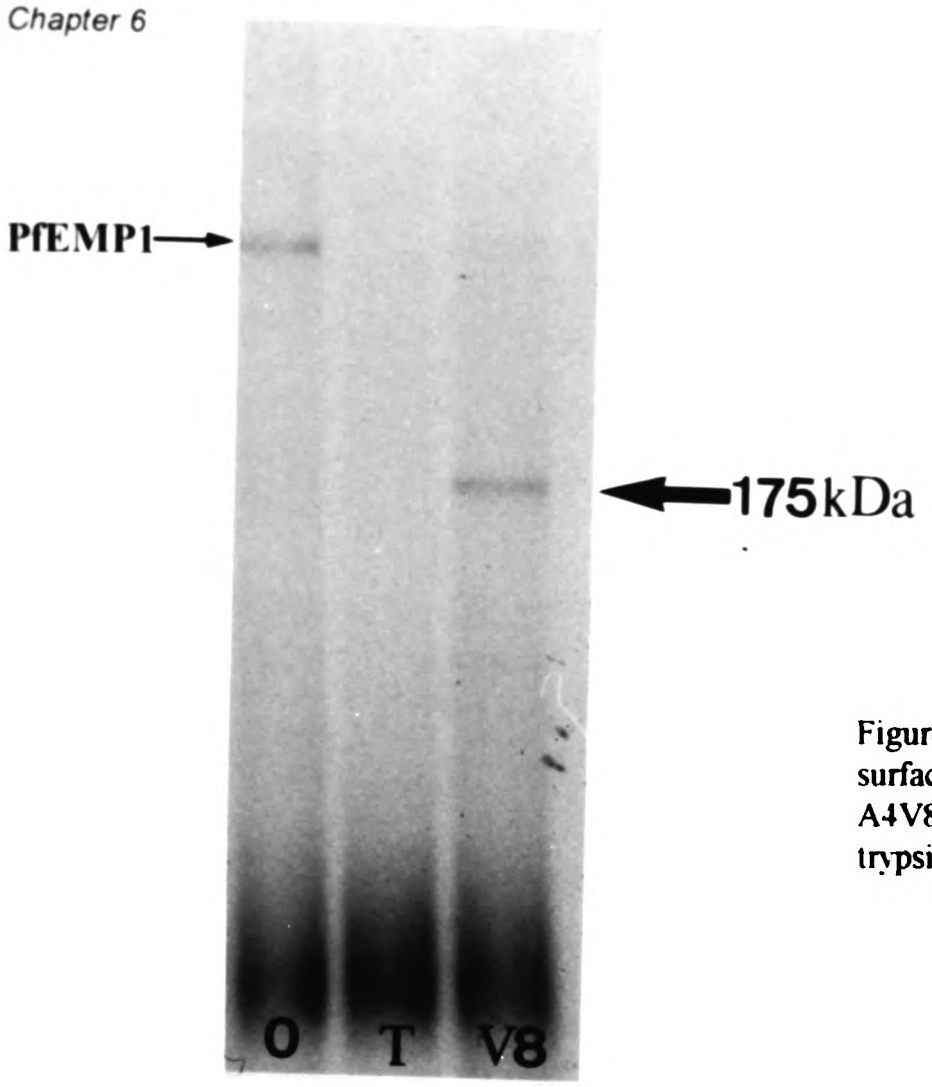


Figure 6.4a. Triton-insoluble extracts from ^{125}I surface labelled PRBC infected with A4V8CD36 after treatment with RPMI-S (0), trypsin (T) or V8 protease (V8).

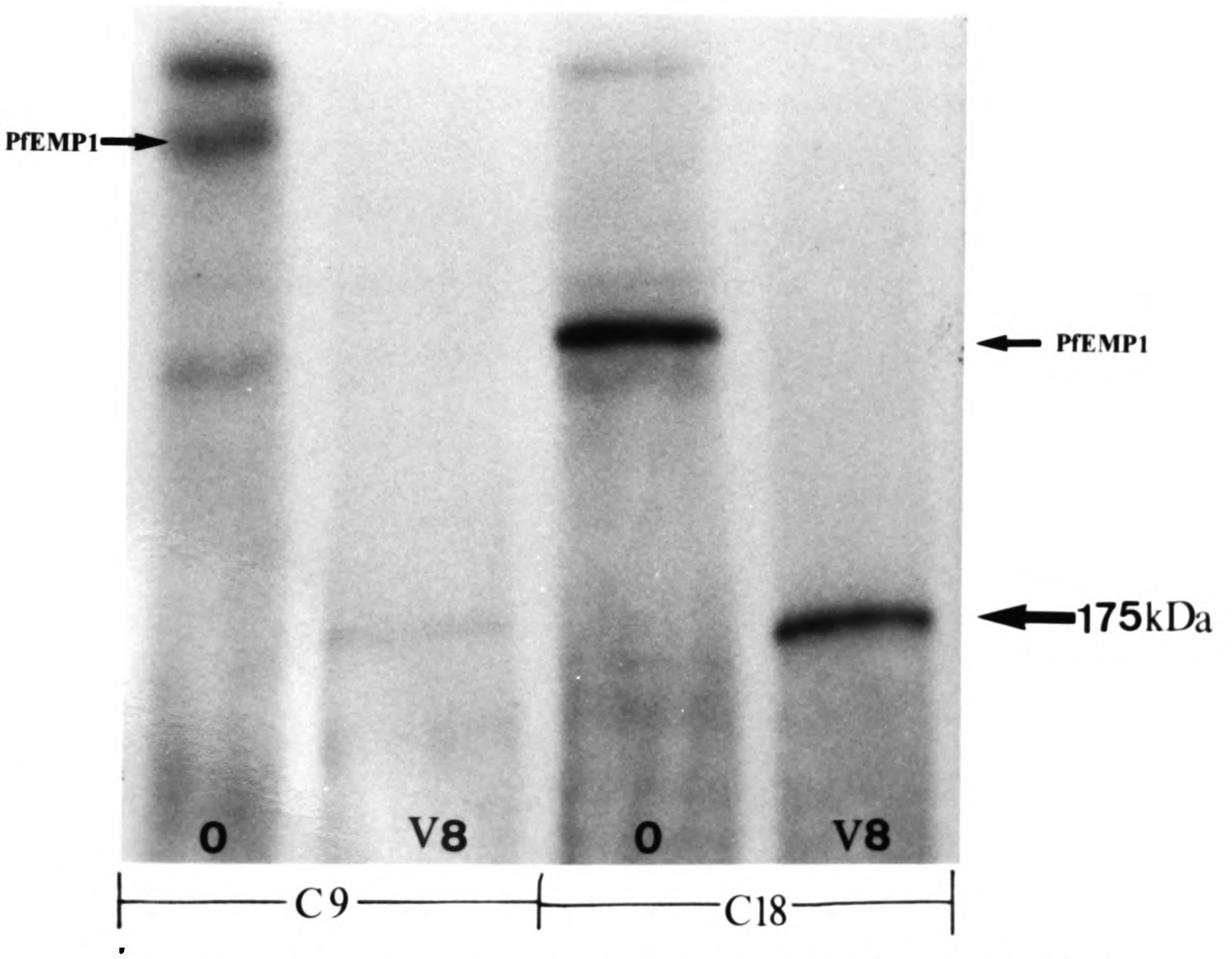


Figure 6.4b. Triton-insoluble extracts from ^{125}I-surface labelled PRBC infected with C9, or C18, after treatment with RPMI-S (0) or V8 protease (V8)

A corresponding 175kDa protein was not observed when metabolically labelled PRBC from C18 were digested with V8 protease. Immunoprecipitation experiments to enrich ^{35}S-labelled variant-specific bands using Sib serum were also unsuccessful. It was therefore questionable whether or not this molecule was of parasite or modified-host origin. A chance observation turned out to be useful in resolving this matter.

When DFP was unintentionally omitted during one surface labelling experiment, it was noticed that the 175kDa band was still present in $A4_{i+}$ and $C18_{i-}$, but absent from other clones (Figure 6.4c). Hence, this protein was completely resistant to V8 protease, even when PRBC membranes had been disrupted by detergents. This fortuitous discovery was exploited to show that the 175kDa protein was metabolically-labelled and present at low level after V8 digestion of PRBC from $C18_{i-}$ when the protease was not inhibited by DFP (Figure 6.4d). Other proteins were cleaved by the prolonged V8 protease activity following removal of membranes, and the low copy number of this protein was emphasised by the long exposure times (>10weeks) of autoradiographs necessary for detection. Hence the 175kDa protein was a parasite-derived surface antigen that was produced after V8 protease digestion and correlated with resistant binding to CD36. It was absent from all clones that did not bind to CD36 after treatment with V8 protease. Its variant-specific nature suggested that it was derived from the C18VAT associated 225kDa member of the PfEMP1 family.

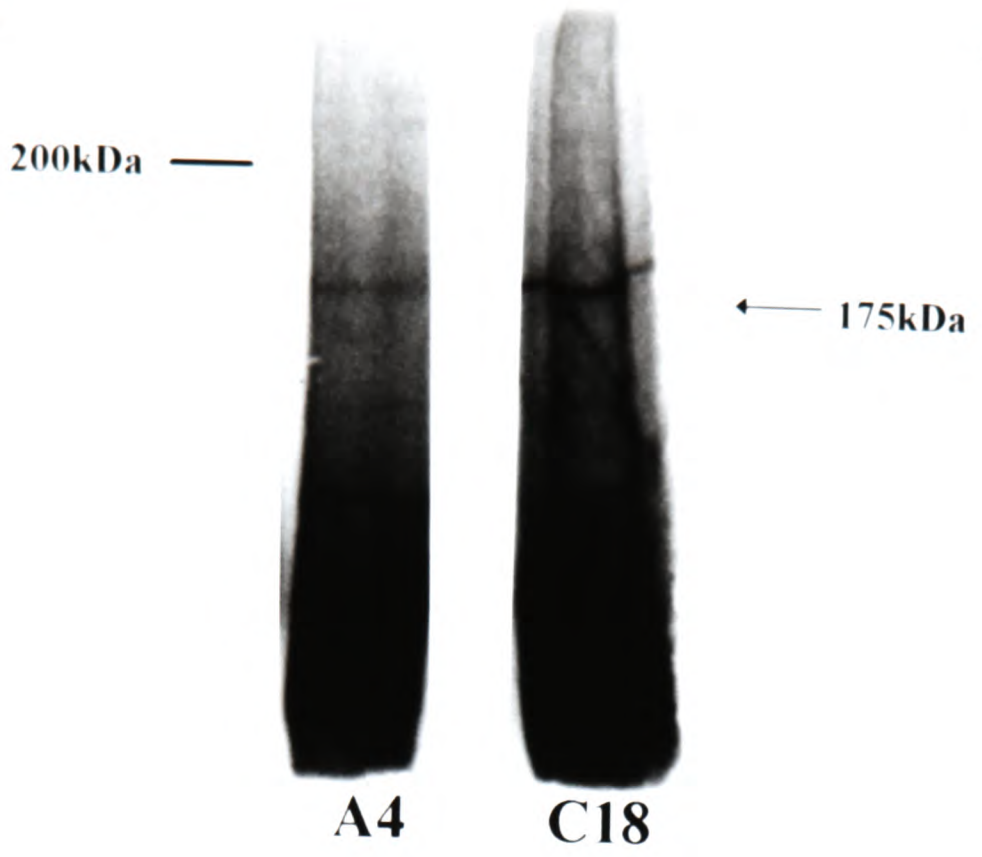


Figure 6.4c. Triton-insoluble (TI) extracts from ^{125}I-surface labelled PRBC infected with $A4_1$ or $C18_1$ after digestion with V8 protease without inhibition by DFP.

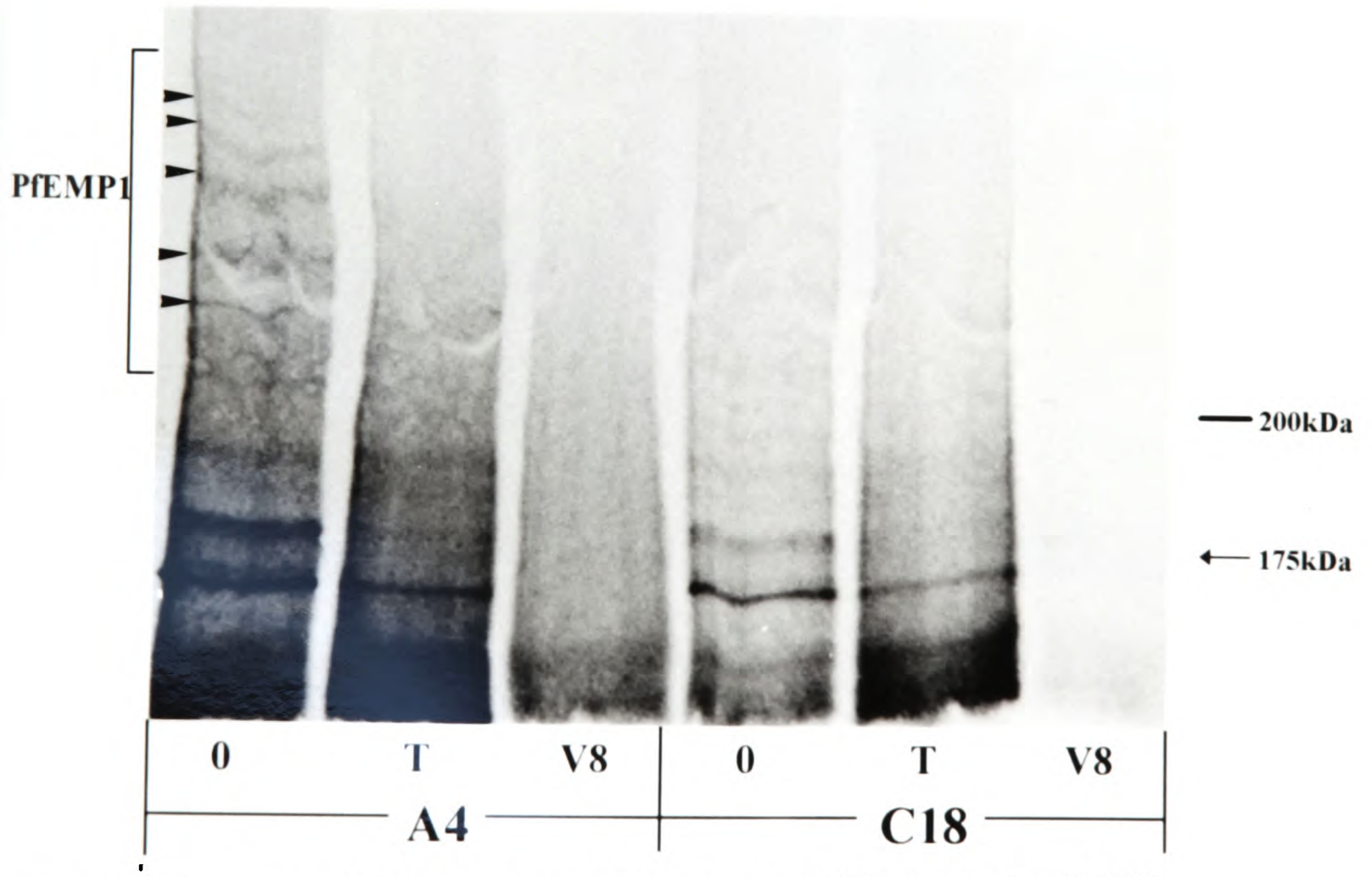


Figure 6.4d. ^{35}S-metabolic labelled extracts of PRBC from $A4_1$ and $C18_1$ separated on 5% SDS PAGE gels. Cells were treated with RPMI-S (O), trypsin (T), or V8 protease (V8) at trophozoite stage. Note that V8 protease was not inhibited with DFP, and a 175kDa fragment was present at a low level in PRBC from C18.

The supernatants following digestion of labelled PRBC were analysed by SDS PAGE to discover if variant-specific fragments had been released. Figure 6.5 shows that a 50kDa fragment was produced by V8 proteolysis of ^{125}I labelled PRBC from $C18_{i-}$, and was present at low levels in supernatants from PRBC from $A4_{i+}$ and $C9_{i+}$ (data not shown). This fragment is likely to be derived from the 225kDa PfEMP1 expressed by these PRBC because it is absent from all other clones tested and uninfected red cells. Furthermore, the size of the fragment (50kDa) represents the difference between the 225kDa PfEMP1 of $C18_{i-}$, and the 175kDa surface associated variant-specific protein remaining after digestion.

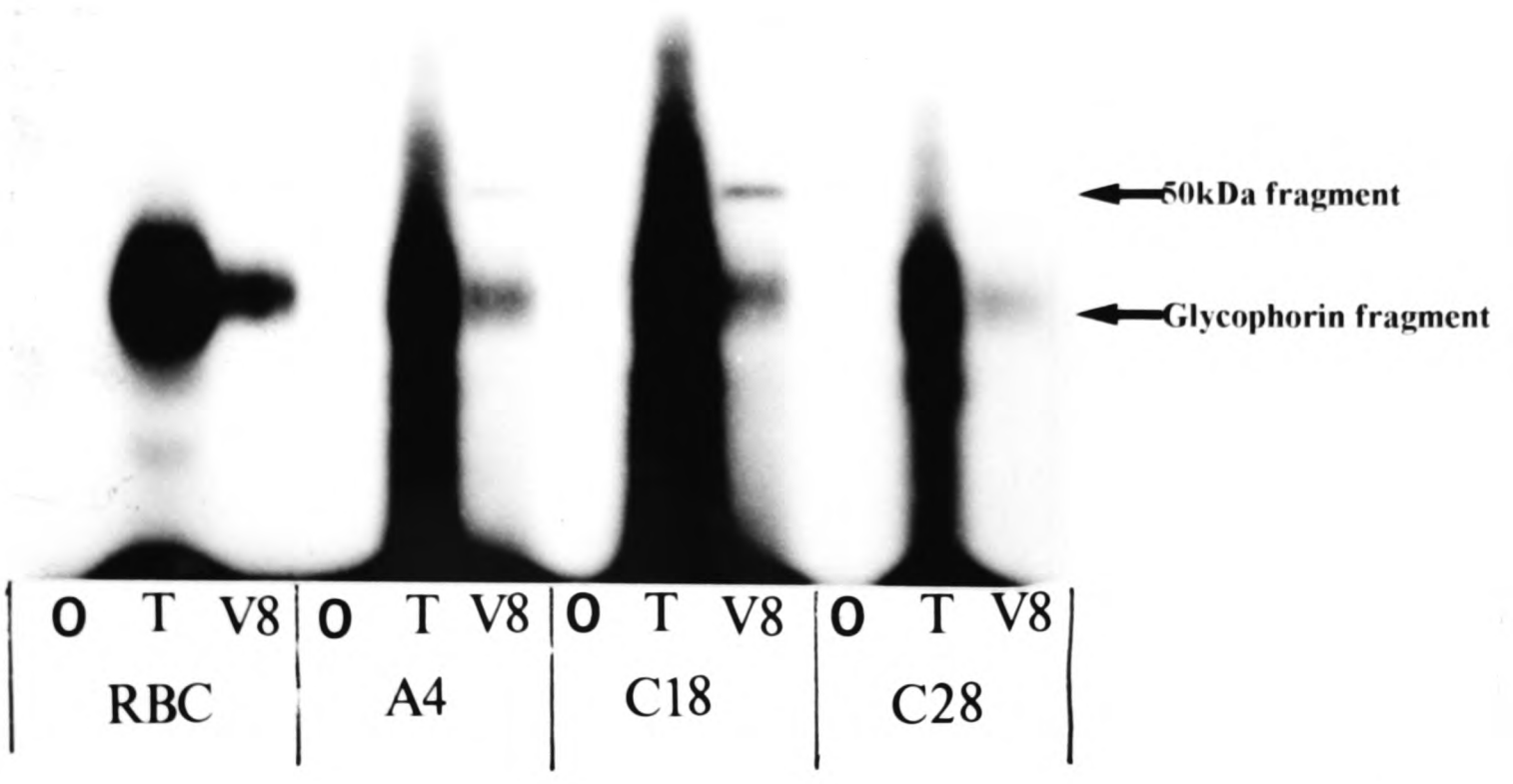


Figure 6.5. 10% SDSPAGE gels of supernatants released on digestion of ^{125}I-surface labelled PRBC or uninfected cells (RBC) with trypsin (T) or V8 protease (V8). Autoradiograph is low molecular weight range only.

To investigate the possible origins of the 50kDa fragment, surface labelled PRBC from $C18_{i-}$ were digested with a serial dilution of V8 protease concentrations, and the TI samples and supernatants were examined (Figure 6.6a, b). The 225kDa PfEMP1 of $C18_{i-}$ was sensitive to low concentrations (1U ml^{-1}) of V8 protease and a 50kDa fragment was detected in the supernatants of PRBC from $C18_{i-}$ cells only. The relative intensities of appearing bands in the supernatants and disappearing bands and the TI extracts during V8 proteolysis was compared. The appearance of the 50kDa fragment followed exactly the removal, as assessed by intensity of bands and timing of release, of the 225kDa PfEMP1 expressed by $C18_{i-}$. This implied that the 50kDa fragment was derived from the variant antigen in this clone. Longer exposure of supernatants from digested cells showed that many peptides were cleaved off PRBC by V8 protease, but only the 50kDa was variant-specific (Figure 6.6c, d)

Extensive immunoprecipiation experiments were performed with Sib serum and Eur serum to examine the immunogenic nature of the released fragments. The 50kDa C18VAT specific protein was not recognised by either serum when three labelled supernatant samples derived from independent PRBC surface labellings were analysed (n = 4), even when the variant-specific (225kDa) PfEMP1s were labelled. The simplest explanations for the failure of antibodies to recognise the 50kDa peptide was that the peptide did not contain any immunogenic epitopes or that Sib serum did not contain any specific antibodies to this region. Another reason proposed was that high quantities of residual V8 protease (inhibited by DFP) in the cell supernatants blocked the crucial antibody-antigen interaction via a non-specific mechanism. To remove this possible confounding factor, the V8 protease was extracted from labelled supernatants by ion exchange chromatography prior to immunoprecipitation. The physical characteristics of the enzyme were analysed batch-wise using DEAE dextran and a serial dilution of KCl buffer to determine the optimum conditions for removal, as based on published methods (Drapeau *et al.*, 1972). Coomassie stained SDS PAGE gels confirmed that V8 protease had been removed from supernatants by the digestion of PRBC from $C18_{i-}$. However, further immunoprecipitation experiments using supernatants from which the V8 protease had been removed were still unsuccessful, and it was impossible to detect the 50kDa fragment after DEAE treatment. This suggested that the fragment was removed in conjunction with V8 protease and further purification attempts with carboxymethyl sepharose and lectins proved unsuccessful (data not shown)

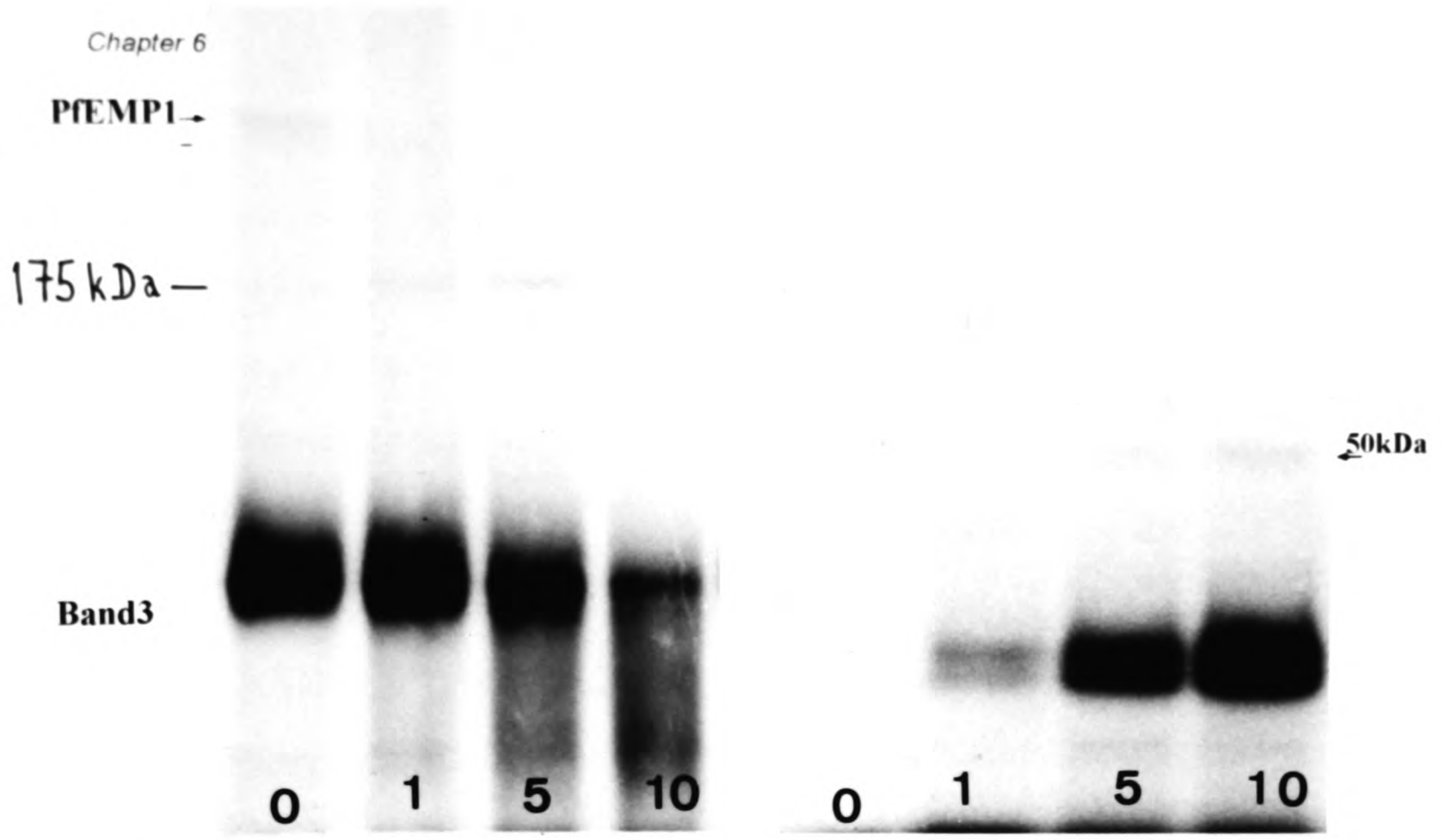
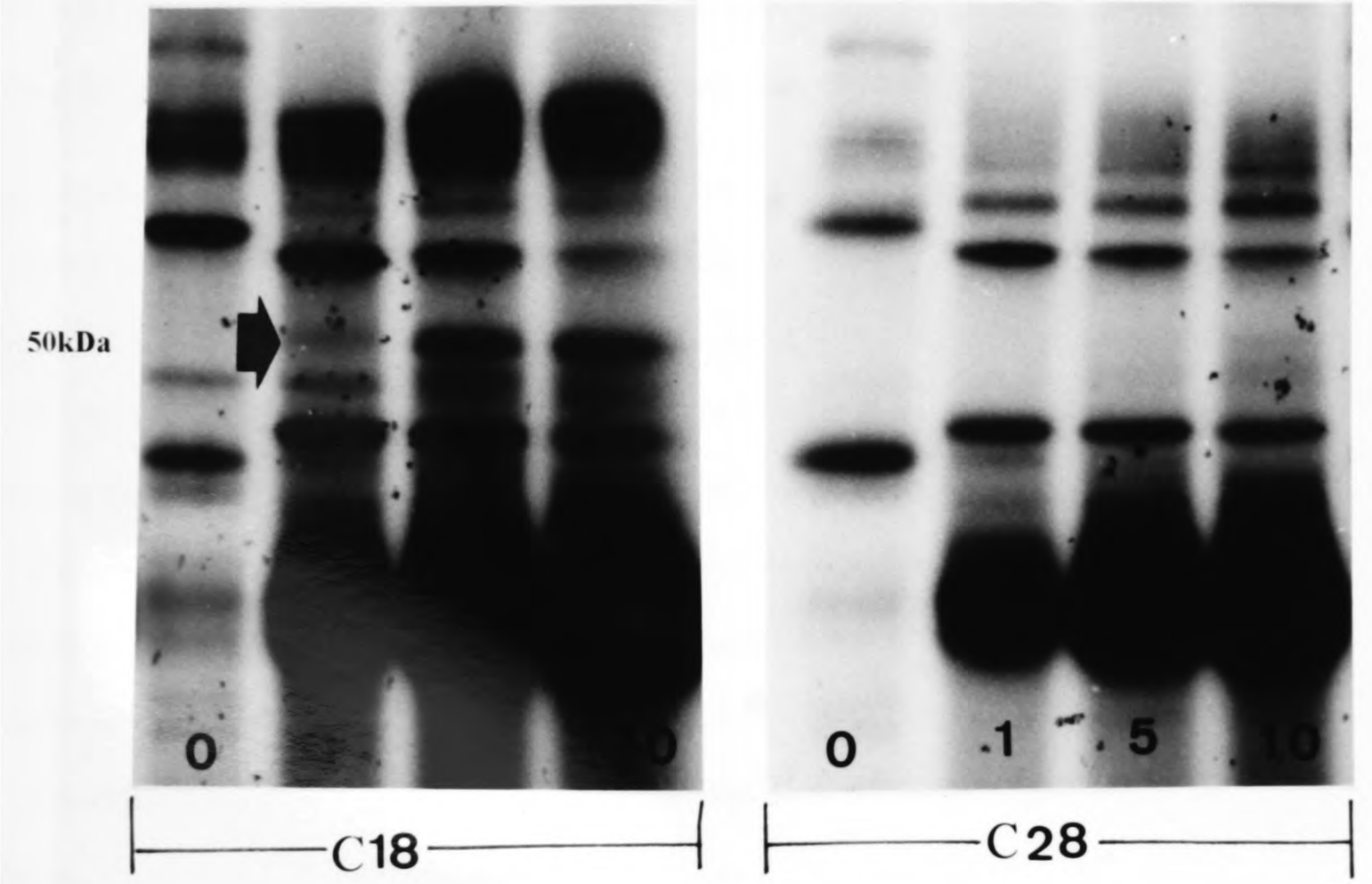


Figure 6.6a. Effect of V8 protease dilution on ^{125}I-surface labelled PRBC from C18. . Triton-insoluble extracts are shown, and 225kDa PfEMP1 is partially cleaved at 1U ml^{-1} , and completely removed at 5U ml^{-1} V8 protease.

b. Supernatants from PRBC from C18 following digestion with a dilution of V8 protease. A 50kDa fragment appears in samples after digestion at 5U ml^{-1} V8 protease. Glycophorin fragment is visible at 1U ml^{-1}

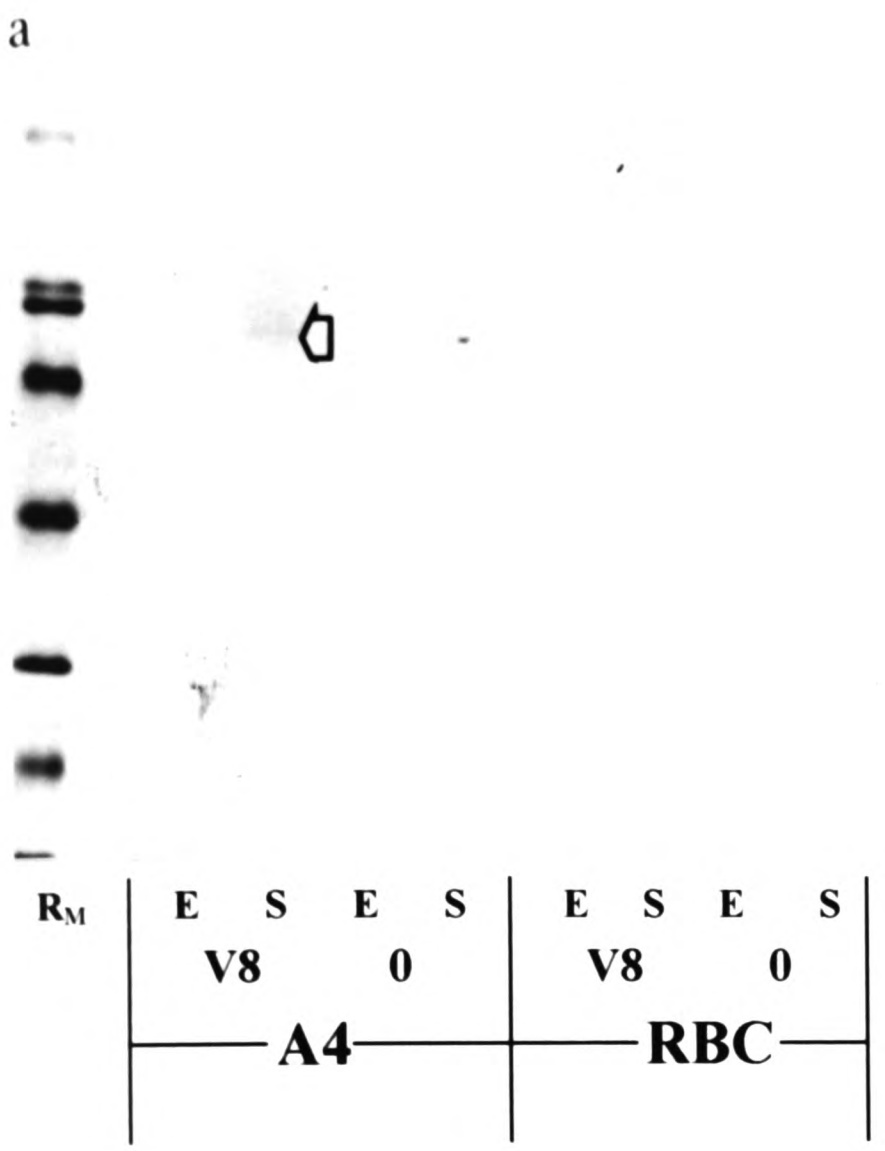


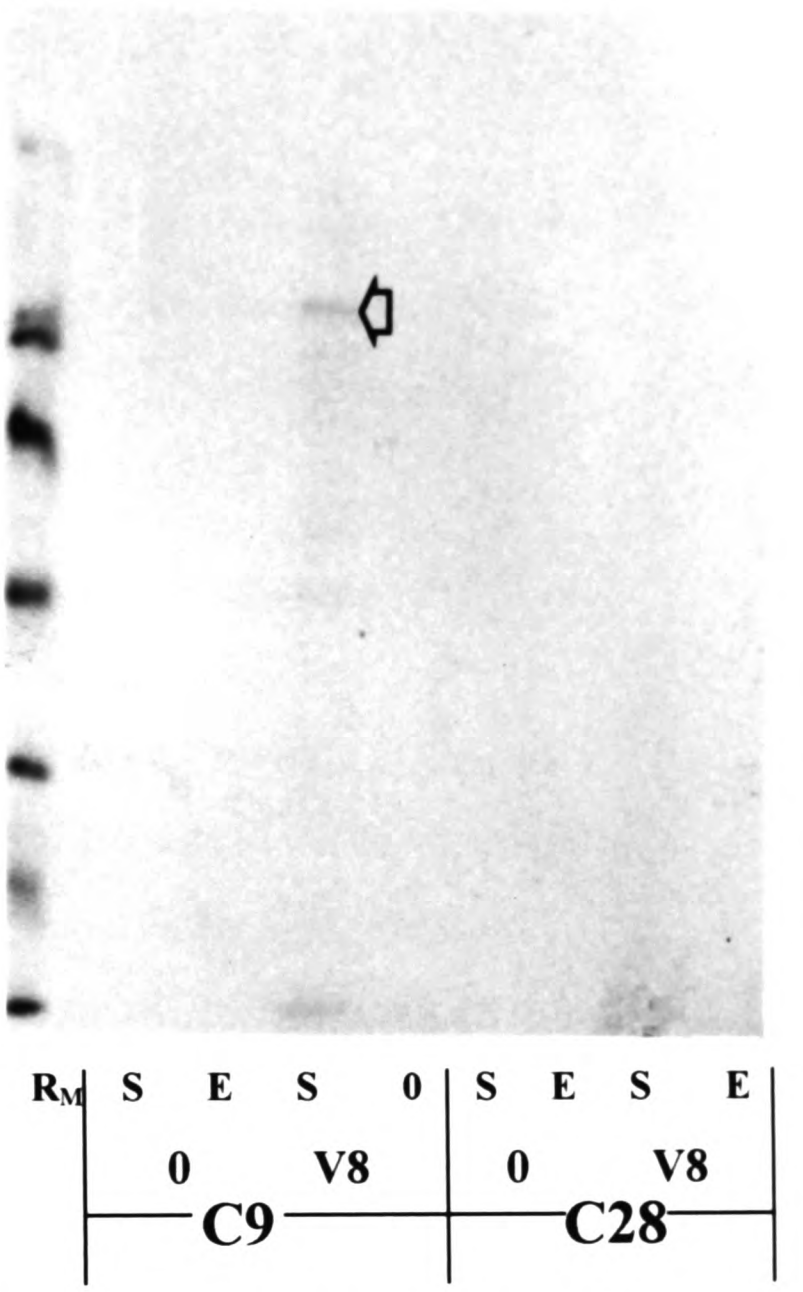
c Longer exposure of autoradiographs of supernatants after ^{125}I-surface labelled PRBC from C18 and C28 had been digested with V8 protease. The 50kDa fragment is specific for C18 and first present at low intensity in samples after treatment with V8 protease at 1U ml^{-1}.

An alternative explanation for the failure of these experiments was based on the low copy number of the fragment. This factor made potential positive results difficult to detect by autoradiography. To increase the specific activity of recognised proteins, immunoprecipitation of non-labelled fragments was carried out, and the precipitated complexes were iodogen™ labelled by ^{125}I on the proteinA sepharose beads. These experiments failed to identify a 50kDa fragment or any other variant-specific fragments, regardless of whether V8 protease was present or not in the sample (data not shown).

More successful results were obtained with the A4VAT specific 90-110kDa doublet, which was selectively recognised by Sib serum in independent experiments when surface labelled PRBC from $A4_{i+}$ were tested (Figure 6.7a), and $C9_{i+}$ (Figure 6.7b). An antibody eluted from $A4_{i+}$ cells specifically recognised the doublet when supernatants from surface labelled $A4_{i+}$ PRBC were tested (Figure 6.7c), and did not recognise similar proteins when PRBC from $C18_{i-}$, $C24_{i-}$, $C28_{i-}$ were examined (data not shown). The same eluted antibody specifically agglutinated PRBC that expressed the A4VAT (i.e. $A4_{i+}$ and $C9_{i+}$), and this suggested that the doublet contained variant-specific epitopes from PfEMP1. The variant-specific doublet was not detected by immunoprecipitation of supernatants following digestion of metabolically-labelled PRBC, confirming the low copy number of these molecules.

In summary, PfEMP1 is likely to mediate PRBC adhesion to CD36 because its sensitivity to V8 protease correlates exactly with the effect of the same protease on adhesive phenotypes in this clone family. A 225kDa member of the PfEMP1 family is the only protein associated with the antigenically restricted V8 protease-resistant binding observed in the C18VAT. A non-immunogenic variant-specific fragment of 50kDa is cleaved by V8 protease and released into the cell supernatant and a 175kDa protein remains associated with the PRBC membrane. The failure to immunoprecipitate the 50kDa fragment is not surprising if it does not bear antigenic epitopes. This explanation is in agreement with phenotypic data that demonstrated that the antigenic phenotype of PRBC from $C18_{i-}$ is not modified by V8 proteolysis and digested cells from this clone still formed agglutinates in Sib serum.





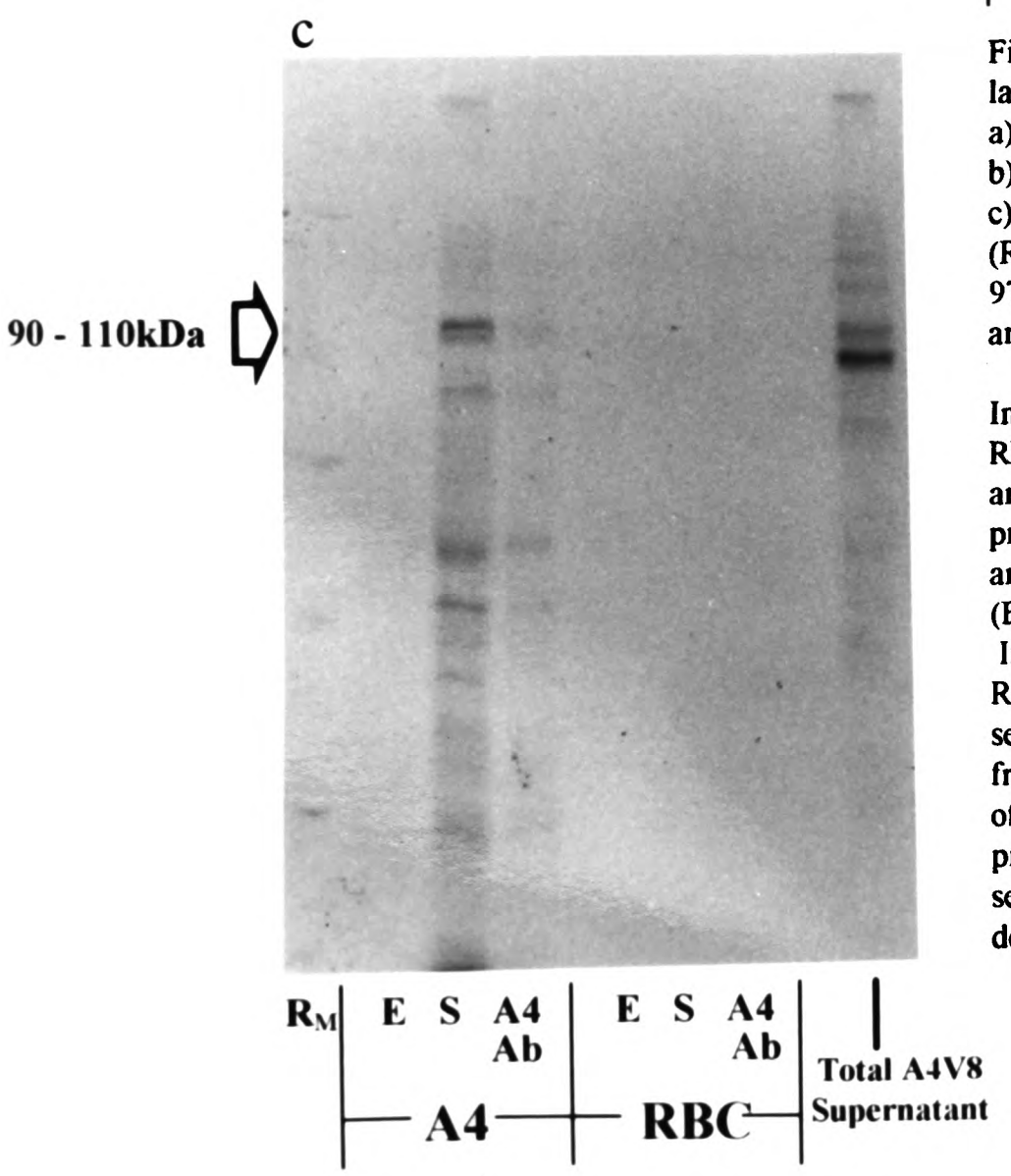


Figure 6.7. Immunoprecipitation of ^{125}I labelled supernatant samples from
a) $A4_{i+}$ and uninfected red cells (RBC)
b) $C9_{i+}$ and $C28_{i-}$
c) $A4_{i+}$ and RBC
(R_M = Rainbow markers; 200kDa, 97kDa, 69kDa, 46kDa, 30kDa, 21.5kDa), arrow denotes 89-110kDa doublet).

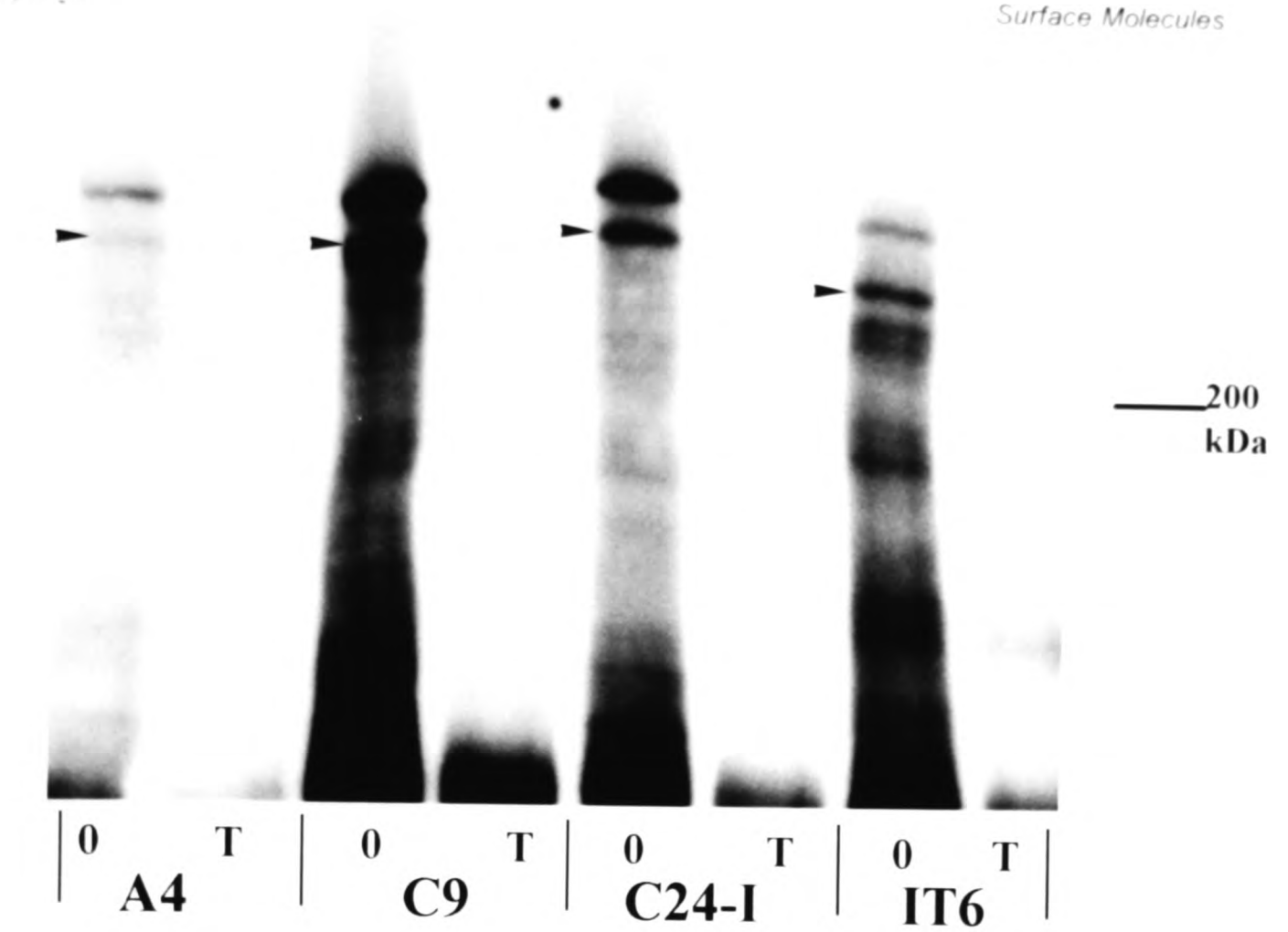
In (a) and (b), cells were treated with RPMI-S(0), or 10U ml^{-1} V8 protease (V8) and supernatants immunoprecipitated on protein A sepharose beads linked to antibodies from either European serum (Eur) or immune serum (Sib).
In (c), identical samples from $A4_{i+}$ and RBC were immunoprecipitated with Eur serum, Sib serum and antibody eluted from $A4_{i+}$ PRBC (A4Ab). A small volume of total supernatant sample from V8 protease digested $A4_{i+}$ PRBC were separated on the same gel to indicate the degree of enrichment.

6.4. Adhesion to ICAM1

Adhesion to ICAM1 is a variant-specific property of PRBC that is linked to the expression of unique antigenic phenotypes (Roberts *et al.*, 1992; Biggs *et al.*, 1992). PRBC that express the IT6VAT (eg. IT6 and C18-I(2)) bind to ICAM at high levels, whereas mid-ICAM1 binding PRBC express the A4VAT and include the ICAM1 binding lines C24-I and C28-I* (Chapter 4).

Surface labelling of PRBC from ICAM1 binding lines generated in chapter 4 showed that PRBC express trypsin-sensitive Triton insoluble PfEMP1s which were associated with the antigenic phenotype of the culture (Figure 6.8a, b, c). PRBC from IT6 and C18-I(2) expressed a 270kDa member of the PfEMP1 family, in addition to a constant 300kDa protein. C24-I and C28-I* expressed multiple high molecular weight PfEMP1s which had similar mobility to those associated with the A4VAT (Figure 6.8c). There was no difference in the intensity of labelling of variant bands and metabolic labelling confirmed this result (data not shown). Therefore, high levels of binding to ICAM1 were probably not attributable to increased levels of PfEMP1. No differences were observed in the TS proteins expressed by ICAM1 binding parasites and non-binding parasites. The high M_r TI proteins were immunoprecipated by Sib serum and not by European serum, confirming their immunogenicity (Figure 6.8d).

Molecules with the characteristics of PfEMP1 were not detected when knob-negative PRBC from D4 and D4-I were labeled with ^{125}I following enrichment on a percol gradient. No high M_r trypsin-sensitive proteins were labelled in the TI or TS extracts and the patterns of labelling were identical for PRBC from D4 and D4-I (data not shown). This correlated with the failure of PRBC from these lines to form agglutinates and their low levels of cytoadherence to all three purified receptors. Unfortunately, this prevented purification attempts of Triton-soluble PfEMP1 that are known to exist in other knob-negative parasite lines (Ockenhouse *et al.*, 1991b).



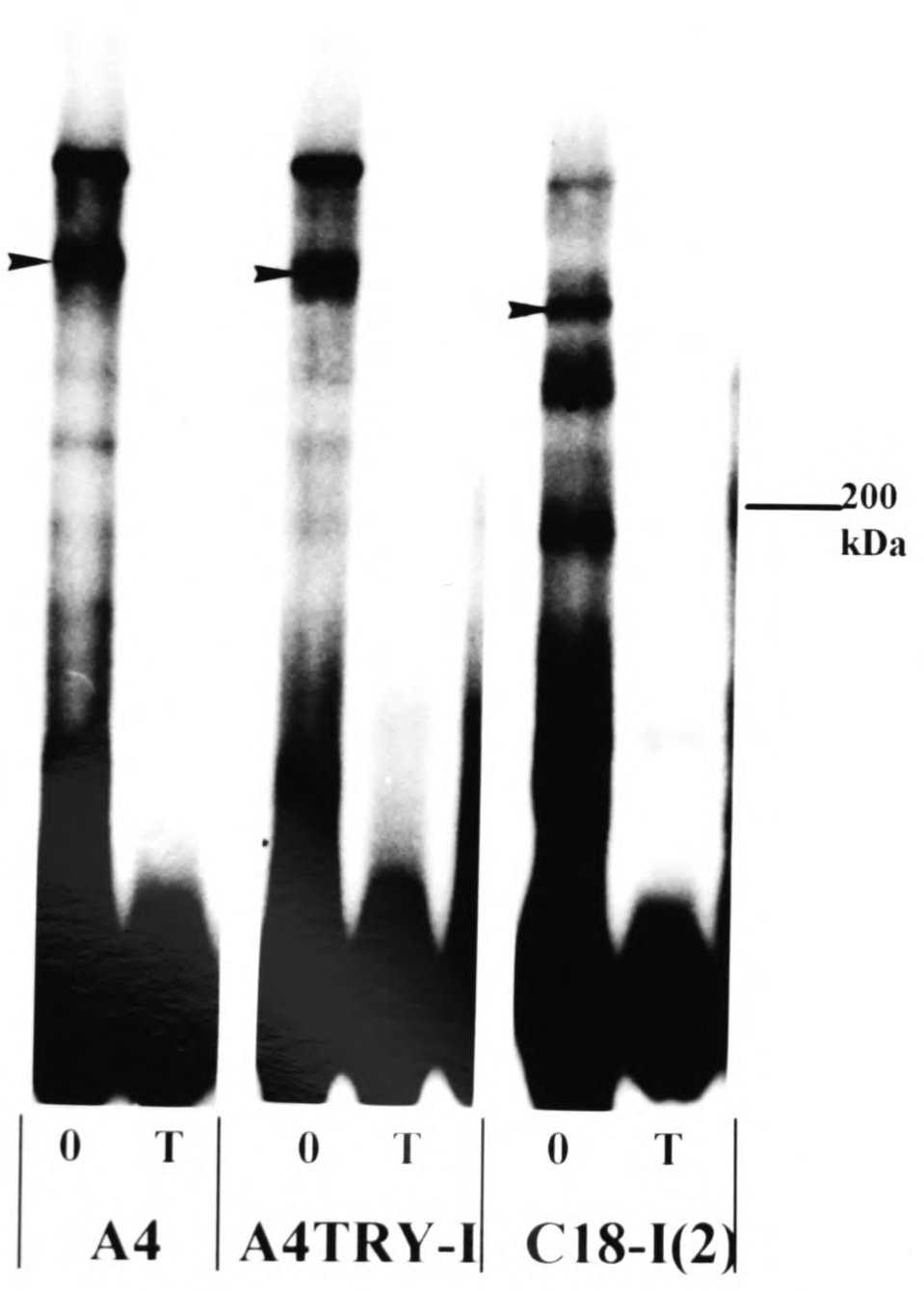


Figure 6.8. Triton-insoluble extracts from ^{125}I surface labelled PRBC infected with ICAM1 binding clones labelled on the same day.
a) A4, C9, C24-I, IT6 and
b) A4, A4TRY-I and C18-I(2).
PRBC were treated with RPMI-S (0) or trypsin (T), and samples were separated on 5% SDS PAGE gels to resolve proteins with the characteristics of PfEMP1 (denoted by an arrow head). Note that spectrin has been labelled in a small number of lysed cells.

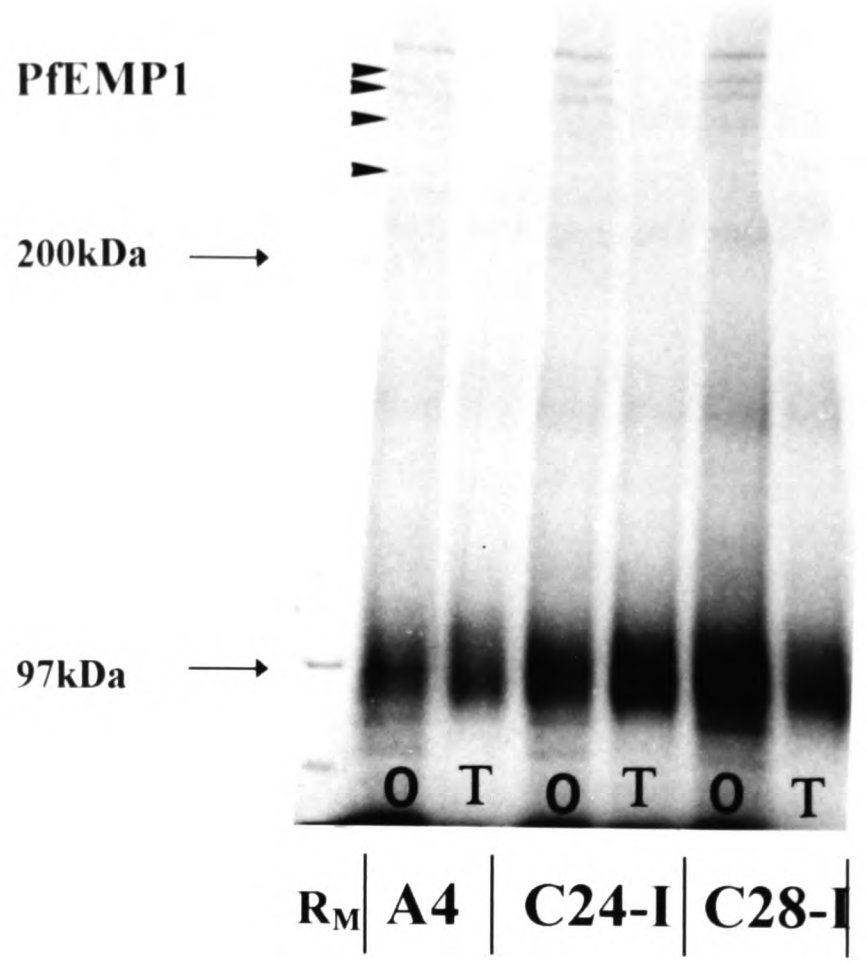


Figure 6.8c Triton-insoluble extracts from I-surface labelled PRBC infected with ICAM1 binding clones A4, C24-I and C28-I separated on 5% SDS PAGE gels to resolve proteins with the characteristics of PfEMP1 (denoted by an arrow head) Rainbow markers R..

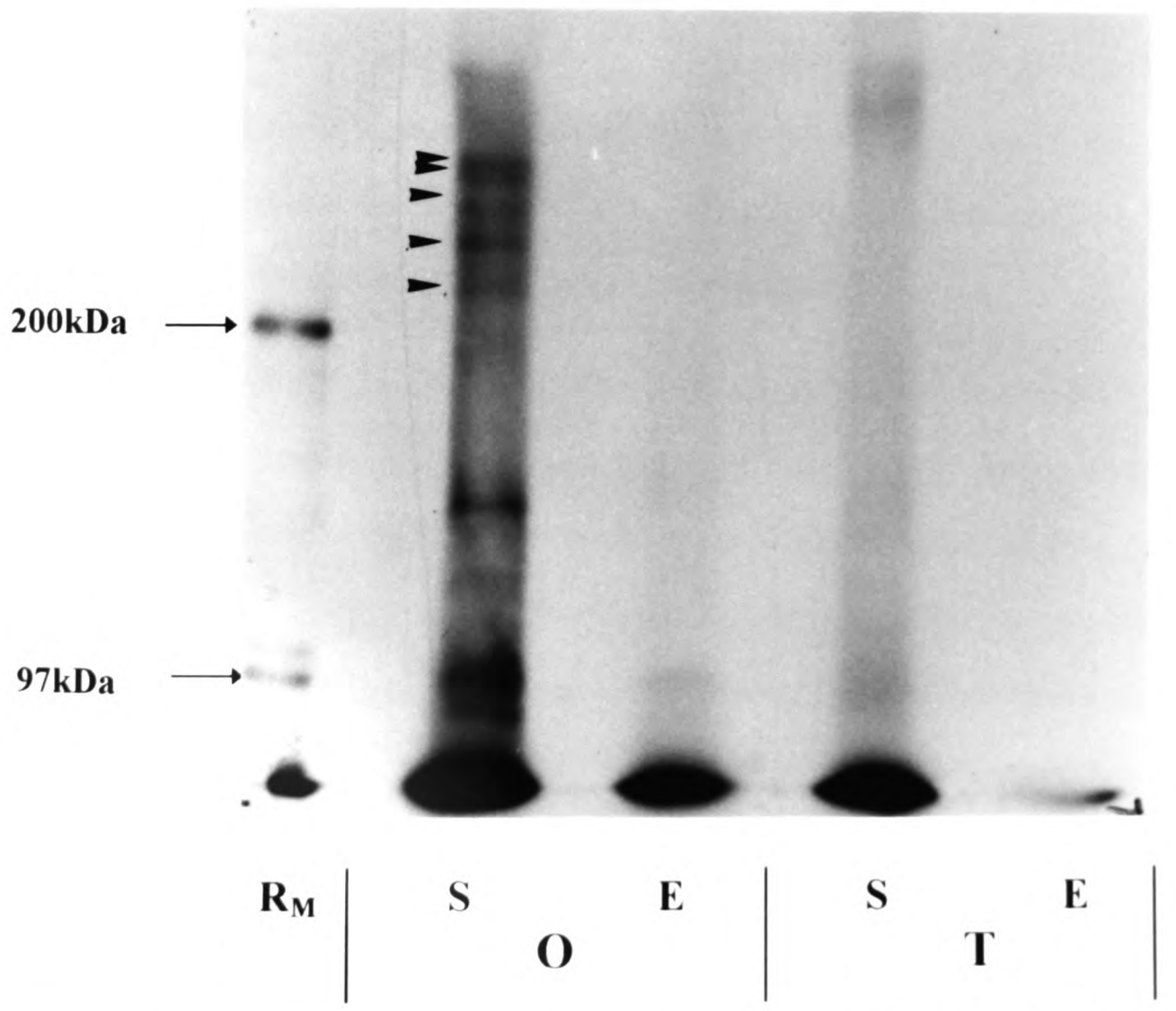
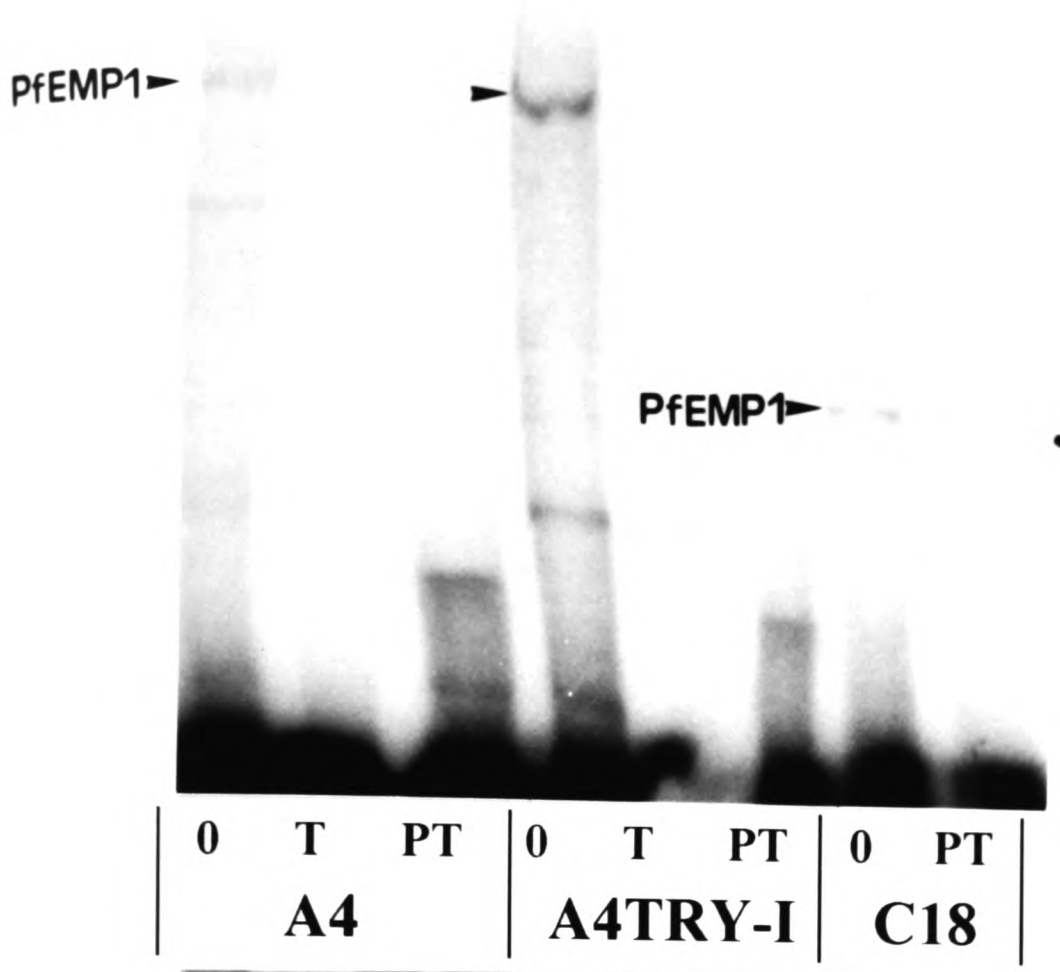


Figure 6.8d Immunoprecipitation of ^{125}I Triton-insoluble antigens from C28-I separated on a 5% SDS PAGE gel. PRBC were treated with RPMI-S (O) or 1mg ml^{-1} trypsin (T) and immunoprecipitated on protein A sepharose beads linked to antibodies from either European serum (E) or Sib serum (S). Proteins with the characteristics of PfEMP1 are indicated. R_M Rainbow markers

In summary, switches from a non-ICAM1 binding phenotype were associated only with the expression of higher M_r PfEMP1s which suggested that ICAM1 binding was mediated by PfEMP1. The **high**-ICAM1 binding phenotype associated with the IT6VAT was linked to the expression of a unique PfEMP1 molecule, which was not expressed by other PRBC. These data do not exclude the possibility that the PRBC may express non-PfEMP1 proteins which mediate binding to ICAM1 and are associated with the antigenic determinants of the PRBC.

To investigate this possibility, batches of surface-labelled and metabolically-labelled PRBC from ICAM1 binding lines were digested with proteases and the profiles analysed. Previous results had shown that trypsin-enhanced binding to ICAM1 and resistant agglutination were associated with PRBC that expressed a particular antigenic phenotype, A4TRY-I. The pattern of surface labelled proteins in PRBC from A4-TRY-I was identical to those observed in PRBC from $A4_{i+}$, except that a major 280kDa PfEMP1 was expressed (Figure 6.8a). This PfEMP1 was trypsin-sensitive, and no labelled variant-specific TS or TI proteins or soluble fragments were observed after digestion (data not shown). It was rationalised that trypsin had cleaved the labelled portion of the putative ICAM1 binding ligand into small fragments which were not detectable in the cell supernatants (data not shown). If this hypothesis was correct, then a variant-specific fragment would remain on the surface of PRBC from A4-TRY-I that could mediate binding to ICAM1.

This idea was tested by surface labelling PRBC from A4-TRY-I and two different clones following trypsin treatment (Figure 6.9a, b). The only differences between the PRBC labelled after trypsinisation occurred in the TI extracts which had been solubilised in SDS as before. At least 5 proteins (Bands #1-5) were expressed by PRBC from A4-TRY-I and $A4_{i+}$ that were absent from $C18_{i-}$. Close examination of 5% gels of TI proteins revealed a protein of 170-180kDa labelled after trypsinisation of the two ICAM1 binding cultures. None of these proteins were observed in uninfected red cells. There were several bands of low intensity that were only expressed by $A4_{i+}$ and A4-TRY-I following trypsinisation and were candidates for the trypsin-resistant domain of the ICAM1 binding ligand (bands #1-5). The most likely protein was band #5 (25kDa), because this was present at a greater intensity in A4-TRY-I than $A4_{i+}$, and was absent from other parasites (i. e. $C18_{i-}$ and $IT6_{i+}$ (data not shown)) which did not bind to ICAM1 after trypsinisation.



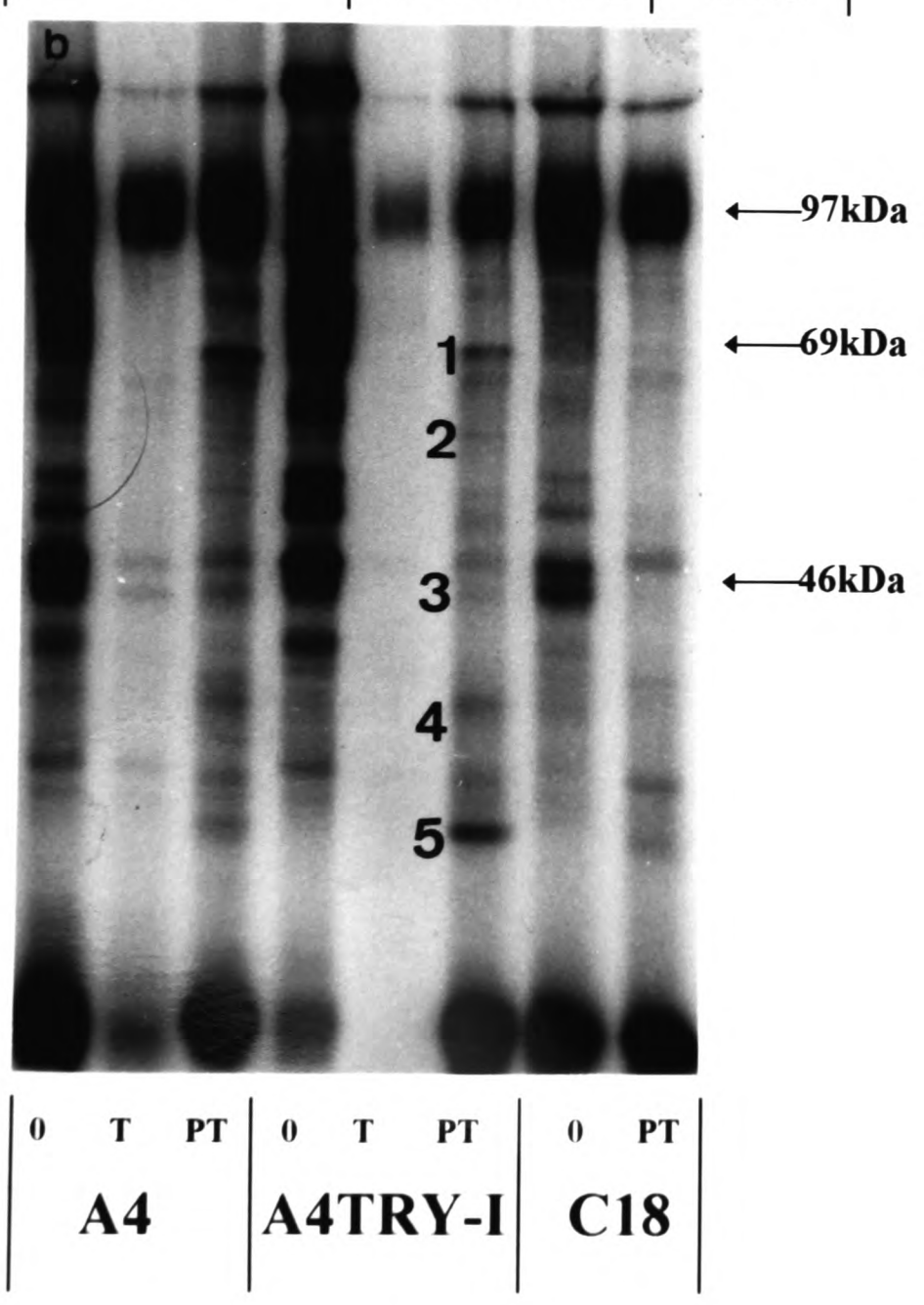


Figure 6.9.(a) 5% and (b) 10% autoradiographs of ^{125}I surface labelled PRBC from A4, A4TRY-I and C18. PRBC were labelled and treated with RPMI-S (O) or trypsin (T). Identical PRBC were digested with trypsin before labelling on the same day (PT) to identical proteins that were candidates for the trypsin-resistant ICAM1 binding ligand (Bands 1-5). PfEMP1s are indicated by the arrow heads.

Metabolic labelling of PRBC was used to provide an alternative profile of ICAM1 binding lines. In accordance with earlier labellings, low quantities of trypsin-sensitive molecules with the operational characteristics of PfEMP1 were labelled. Unfortunately, no variant specific proteins were detected due to the low intensity of bands (data not shown). A 10% SDS PAGE gel of TI proteins from metabolically labelled PRBC showed the existence of at least 5 trypsin-sensitive proteins in all clones (Figure 6.10). No variant-specific bands were observed after trypsinisation, thus it was impossible to identify a parasite derived protein that fulfilled the criteria for the ICAM1 binding receptor.

Immunoprecipitation of labelled molecules with ICAM1-F_c was attempted to prove that candidate molecules contained ICAM1 binding domains. ICAM1-F_c chimaeras that were known to mediate efficient cytoadherence of PRBC were used and CD8-F_c chimaeras were used as controls. Unfortunately, no proteins were selectively recognised by the ICAM1-F_c that were not also immunoprecipitated by the CD8 F_c. A wide range of labelled PRBC proteins was tested, including TI extracts, TS extracts and soluble supernatants. Samples before and after trypsinisation were examined without success, and immunoprecipitation of unlabelled extracts followed by iodination with Iodogen™ was carried out. This latter technique proved that ICAM1-F_c was indeed binding to protein A sepharose beads under the conditions used (Figure 6.11), but there were no successful experiments. Variant proteins from the same samples were recognised by Sib serum and not by European serum and thus three explanations were proposed for the apparent failure of ICAM1 to interact with PfEMP1-like molecules. Firstly, the interaction of ICAM1 with the infected erythrocyte receptor could be of low avidity and the concentration of ICAM1-F_c used in the immunoprecipitations (10-50$\mu g\ ml^{-1}$) was too low to mediate efficient binding. Alternatively, the detergent-extracted parasite receptor does not interact with ICAM1 in a solubilised form and only binds when associated with the PRBC membrane. Cross-linking experiments could be performed to test this possibility. Thirdly, the PRBC receptor for ICAM1 may bind to the CD8F_c or the protein A used in the assay thus no specificity will be achieved by using ICAM1-F_c.

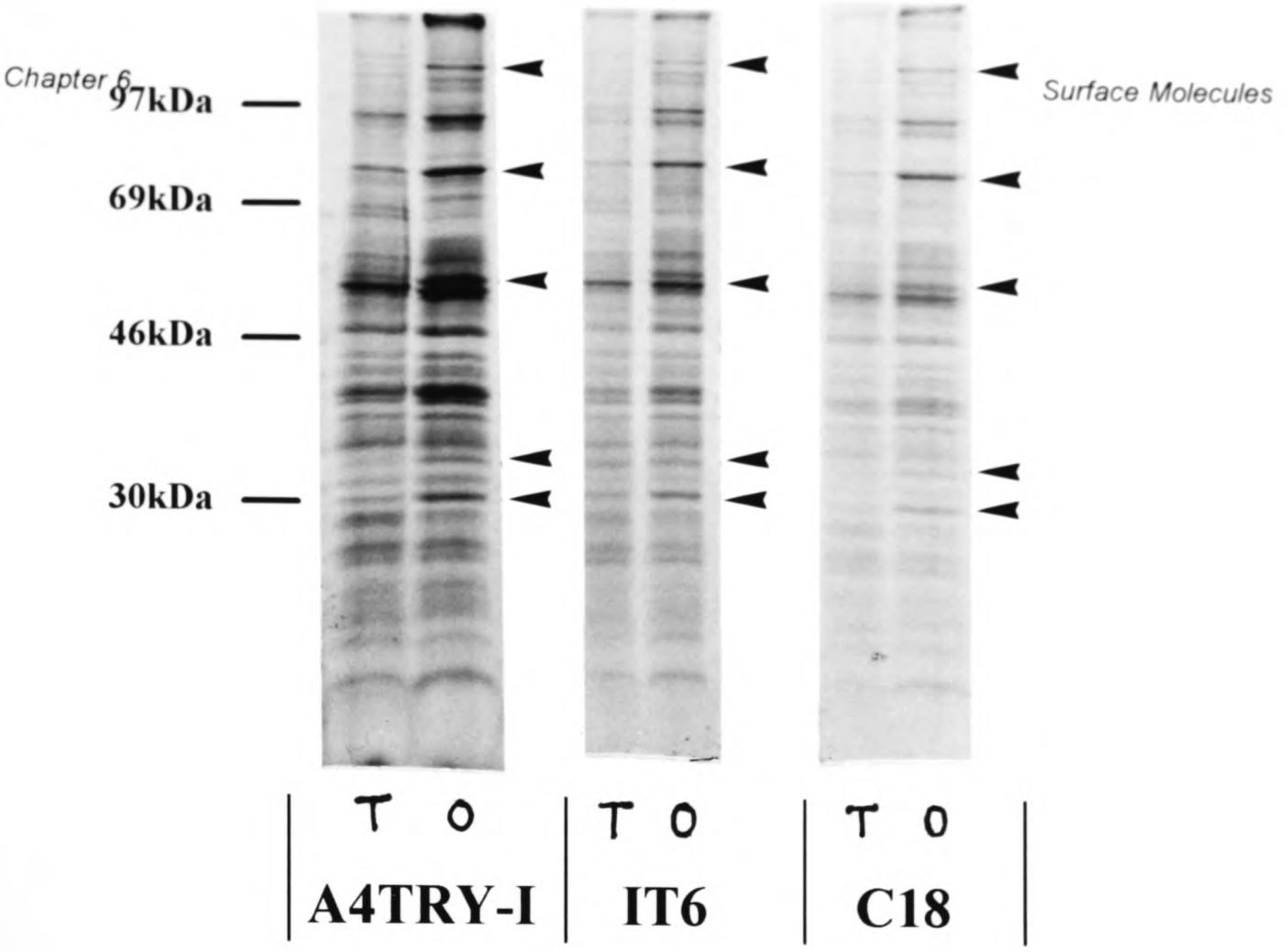


Figure 6.10. Triton insoluble extracts from ^{35}S-metabolic labelled PRBC from A4TRY-I, IT6 and $C18_i$. separated on a 10% SDS PAGE gel. PRBC were treated with either RPMI-S (0) or trypsin at 1mg ml^{-1} (T). Arrows denote trypsin-sensitive parasite derived proteins. Protein standards are shown on left and trypsin sensitive molecules are depicted by arrows.

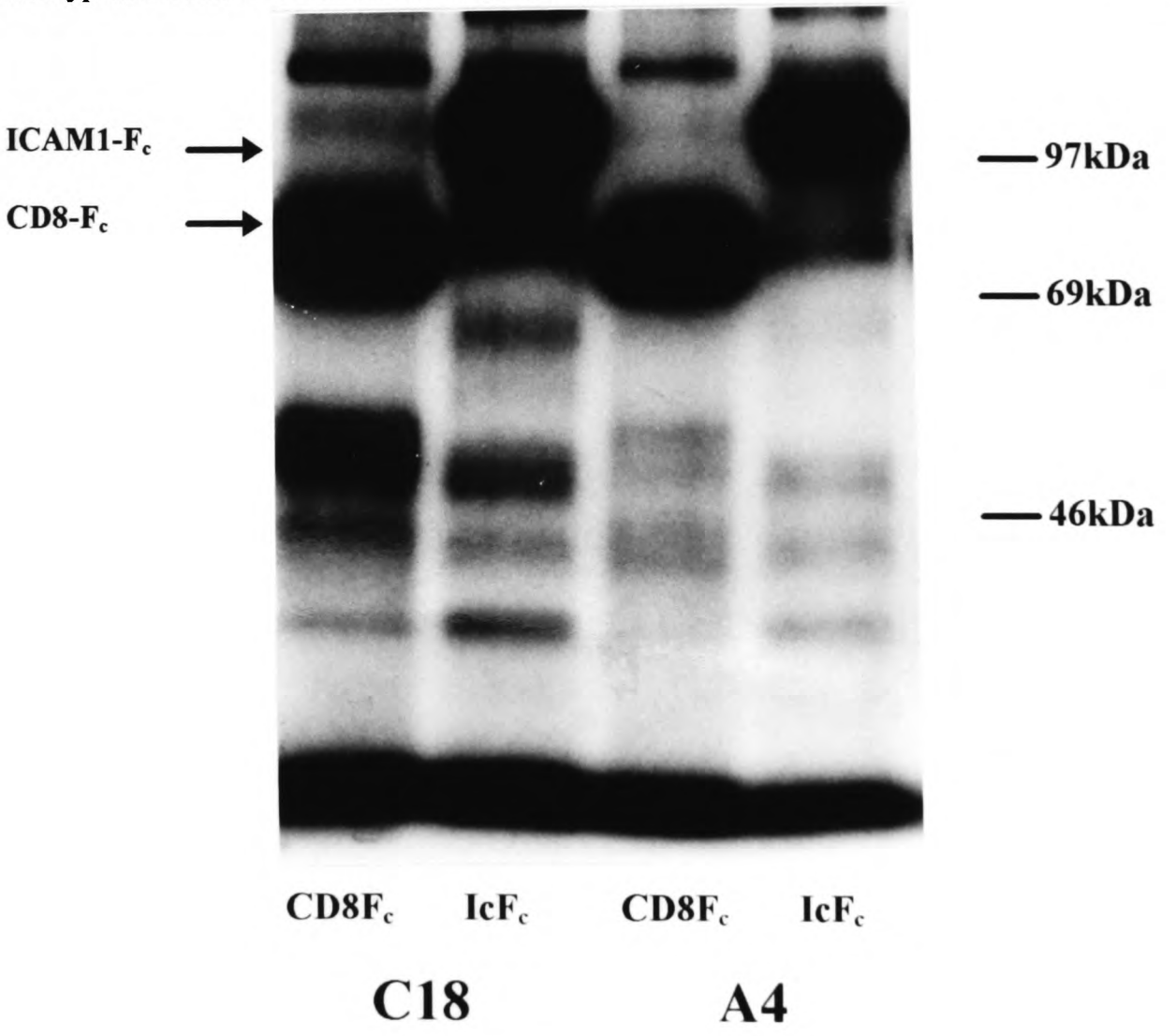


Figure 6.11. Iodogen™ labelled protein A sepharose beads after immunoprecipitation of TI extracts of PRBC from $C18_i$ and $A4_i$, with Control F_c (CD8-F_c) or ICAM1-F_c (IcF_c; 5 domain) constructs

The antibodies eluted off trypsinised PRBC A4TRY-I which showed variant-specific agglutination were used in immunoprecipitation experiments with metabolic labelled and surface labelled extracts from ICAM1 binding lines. No variant-specific proteins were observed in any experiments, although the antibodies did cross react with a 175kDa Triton-insoluble protein specifically from A4-TRY-I cells which had been labelled following trypsinisation. Unfortunately, this experiment could not be repeated due to availability of eluted antibodies.

6.5. Adhesion to TSP

The infected erythrocyte receptor that binds to TSP has not been identified, even though this multifunctional protein was discovered as a cytoadherence ligand in 1985 (Roberts *et al*, 1985b). One study demonstrated that knob-positive PRBC adhesion to low concentrations of TSP was sensitive to trypsin (Sherwood *et al.*, 1989), and the PfEMP1s expressed by the same PRBC (Malayan Camp) were also sensitive to trypsin (Leech *et al.*, 1984). Further information relating to the molecular nature of this interaction has not been published.

Results presented in chapter 5 demonstrated that adhesion to TSP was independent of the antigenic phenotype of the PRBC, partially resistant to trypsin and completely resistant to V8 protease. Hence, the infected cell receptor for TSP was either a constant molecule that was resistant to tryspin and resistant to V8 protease, or was a domain on a variable molecule with similar characteristics.

Analysis of the effects of the two proteases on labelled PRBC from different clones identified a number of proteins which fulfilled the criteria for the TSP receptor at the PRBC surface (eg. low molecular weight parasite derived surface labelled triton soluble proteins). In addition, there were multiple trypsin- and V8 protease-resistant host surface proteins expressed in all PRBC clones. For a host protein to act as a receptor for TSP, it must undergo parasite-induced modifications, because uninfected erythrocytes do not bind.

A candidate host protein which fulfilled these criteria was Band 3 because published reports suggest that it is modified in mature *P. falciparum* infected erythrocytes (Winograd and Sherman, 1989). Band 3 was trypsin-resistant but sensitive to V8 protease in all PRBC tested in this thesis, and surface-labelled fragments of this molecule remain associated with the membrane after V8 proteolysis. This was confirmed using monoclonal antibodies (gifts of Dr. M. Tanner) to internal regions of Band3 in immunoprecipiation assays with surface labelled TI extracts from two different clones. Figure 6.12 shows a representative autoradiograph when either of the monoclonal antibodies was used with PRBC from $C18_{i-}$ and $C28_{i-}$. Hence a modified form of Band 3 satisfied the criteria of the PRBC receptor for TSP, as based on labelling and phenotypic data in this clone family.

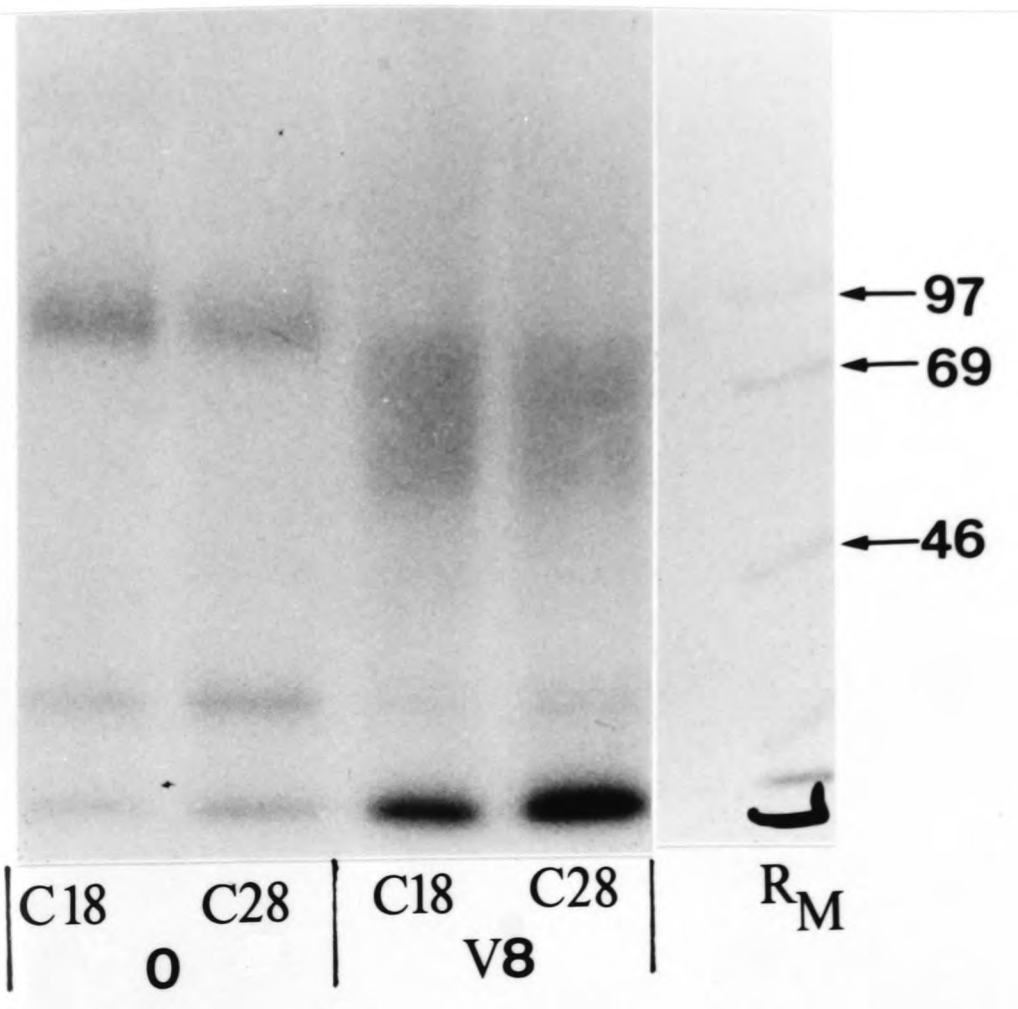


Figure 6.12. Immunoprecipitation of ^{125}I Triton-insoluble antigens from C18 and C28 with monoclonal antibodies to internal regions of Band3 (gift of Dr. M. Tanner). PRBC were treated with RPMI-S (0) or V8 protease at 10U ml^{-1} (V8) before immunoprecipitation. Note that Band3 migrates as a characteristic ~80kDa smear in controls and a smear of ~65kDa after protease treatment.

6.6. Conclusions

Previous studies and evidence presented in this thesis suggest that the parasite-derived antigens which are recognised by immune sera also mediate sequestration of mature PRBC. The molecular basis of these two important surface functions was investigated in this chapter using a range of PRBC that had been phenotypically characterised in earlier chapters.

Variant-specific V8 protease-resistant binding to CD36 was associated with expression of a single parasite-derived 225kDa member of the PfEMP1 at the surface of the infected erythrocyte. Due to the high rate of antigenic variation of PfEMP1, multiple forms were detected in cloned PRBC, and the levels of V8 protease resistant binding of cloned PRBC to CD36 correlated with the amount of the 225kDa PfEMP1 present in a culture. This was confirmed by selection of the resistant subpopulation of PRBC on CD36 from a clone that expressed an alternative major antigenic phenotype (A4VAT). The selected line (A4V8CD36) expressed the C18VAT and a single PfEMP1 of 225kDa.

A 175kDa molecule of parasite origin with the operational characteristics of PfEMP1 was produced by V8 proteolysis of PRBC which expressed the C18VAT. Therefore, this membrane associated protein was likely to contain the binding domain for CD36. A band of 175kDa was immunoprecipitated by antibodies in Sib serum which confirmed that the resistant protein also contained antigenic epitopes which could mediate PRBC agglutination after V8 protease treatment. A non-immunogenic soluble fragment of 50kDa, derived from the 225kDa PfEMP1, was released on V8 proteolysis of PRBC which expressed the C18VAT. Additional immunogenic variant-specific fragments were cleaved by V8 protease digestion of other clones: a 90-110 kDa doublet was immunoprecipitated by Sib serum from digestion supernatants of A4VAT-expressing PRBC. A doublet with identical mobility was immunoprecipitated from the same samples by A4VAT-specific antibodies, but not by control sera. This doublet was probably derived form the 280kDa PfEMP1 associated with this VAT.

There were no observable differences in the effect of V8 protease on surface labelled Band 3 in different clones and the evidence presented was not consistent with the conclusions from one laboratory that modified Band 3 was important for PRBC adhesion to CD36 (Crandall *et al.*, 1994).

Furthermore, it is difficult to reconcile the well-established trypsin-resistant properties of Band 3 monomer with the widespread trypsin sensitivity of adhesion to CD36.

Comparison of labelled proteins from a range of non-ICAM1 binding PRBC before and after selection on purified ICAM revealed that the only surface modifications occurred in the mobility of PfEMP1 molecules. Selection for adhesion to ICAM1 produced PRBC with multiple high molecular weight PfEMP1s that were associated with the antigenic phenotype of the culture. Further evidence that PfEMP1 mediated binding to this receptor was obtained by analysing the effect of trypsin on surface labelled proteins. Trypsin-resistant binding to ICAM1 was associated with the expression of a single 280kDa PfEMP1 in a line that was selected for this phenotype (A4TRY-I). A protein with identical characteristics was present at low copy number in other PRBC due to the high rate of switching. However, trypsin digestion of labelled PRBC cleaved all surface proteins and there were no trypsin-resistant variant-specific proteins associated with binding to ICAM1. Surface labelling after digestion revealed several proteins (bands #1-5) that could mediate this function and it was concluded that trypsin had cleaved the ^{125}I labelled tyrosine (s) from the 280kDa PfEMP1. A 25kDa protein (band #5) was detectable at a higher level on PRBC from A4-TRY-I than $A4_{i+}$, and was absent from other clones that could not bind to ICAM1 after trypsinisation. This was the most-likely candidate identified for the ligand which mediated the trypsin-enhanced adhesion to ICAM1 observed in this parasite. The relative surface labelling of the 25kDa band suggested that it was a membrane associated fragment of PfEMP1, although firm conclusions require further experiments. Metabolic labelling of PRBC confirmed that trypsin sensitive surface parasite-derived proteins were present on clones, but were independent of the ability to bind to ICAM1.

The data in this chapter are thus consistent with the interpretation that adhesive domains for CD36 and ICAM1 are expressed on the same PRBC. Surface labelling profiles suggest that only one form of PfEMP1 is expressed at one time, and the biochemical separation of these functions by proteases emphasises the domain architecture of PfEMP1. If a single PfEMP1 mediated PRBC adhesion to CD36 and to ICAM1 it must consist of distinct domains for adhesion to each receptor, and also carry epitopes which were recognised by antibodies. Modification of ICAM1 binding domains by antigenic variation would explain the low molecular weight PfEMP1s that are associated with the non-ICAM1

binding phenotype. Switching of the cytoadherent phenotype was accompanied by antigenic switching, and novel epitopes on PfEMP1 could be produced by the same process.

Variant-specific protease-resistant cytoadherence is consistent with the modification of protease cleavage site availability in variable epitopes of PfEMP1. Trypsin destroyed the ability of all PRBC to bind to CD36, but ICAM1 binding remained in parasites (A4, A4TRY-I) that expressed a particular PfEMP1 (280kDa). Thus the CD36 binding domain must lie in a region of this PfEMP1 that is further from the membrane than the ICAM1 binding domain and is removed by trypsin. The data do not rule out the possibility that binding domains for ICAM1 and CD36 are on separate PfEMP1s expressed on the same cell.

The nature of the molecule that mediates PRBC adhesion to TSP is equivocal. The simplest explanation that was consistent with the surface labelling patterns and phenotypic data was that binding to TSP was mediated by a single invariant molecule which was resistant to V8 protease and trypsin. This constant molecule could be parasite derived or a host derived. In either case, the trypsin resistant adhesion would be attributed to modification of electrostatic charge of the PRBC following digestion of other surface proteins. This would allow the PRBC to adhere to TSP, which is a heterogeneous molecule in terms of charge, at a lower level (Clemetson *et al.*, 1980). These models are discussed in more detail in Chapter 8.

Chapter 7

Linkage of Surface Functions

In spite of the progress that has been made into elucidating the mechanisms of cytoadherence and antigenicity, there are many simple issues relating to the interdependence of the new surface phenotypes that remain unclear. For example, the temporal relationship between the expression of antigenic and cytoadherent phenotypes during the life cycle has not been studied in detail.

Results in previous chapters have provided the first direct evidence that adhesion to the putative cytoadherence receptors, CD36 and ICAM1, was mediated by a family of variable antigenic proteins (PfEMP1) expressed at the PRBC surface. These observations implied that the domains for agglutination and cytoadherence to CD36 and ICAM1 were on a single molecule. In this case, high concentrations of antibodies in immune serum would be expected to interfere with cytoadherence if the adhesive domains were in close proximity to the antigenic epitopes. This was first suggested by studies with immune serum from *Aotus* monkeys which inhibited and reversed the binding of PRBC to C32 melanoma cells in a strain-specific manner (Udeinya *et al.*, 1983). Subsequent studies have used the same model of cytoadherence *in vitro* to address this issue, and thus the effect of immune serum on PRBC adhesion to individual receptors has not been tested. Preliminary studies with *in vitro* models of cytoadherence using isolates and sera obtained from patients with mild *P. falciparum* malaria in Thailand (Singh *et al.*, 1988), West Africa (Van Schravendijk *et al.*, 1991) and Pakistan (Iqbal *et al.*, 1993) have been encouraging. Antimalarial immunoglobulin (IgG) inhibited or reversed PRBC adhesion to C32 melanoma cells to different degrees in an isolate-specific fashion.

These investigations have been motivated by the possibility of using immunotherapy to treat patients with cerebral malaria, especially in areas where drug resistance is a major problem. Seminal studies by McGregor and colleagues investigated the effect of gammaglobulin, pooled from healthy individuals living in an area where malaria was endemic, in local children with uncomplicated falciparum malaria (Cohen *et al.*, 1961; McGregor *et al.*, 1963). In these studies, treatment reduced parasitaemia and led to complete recovery. Recent trials of a pool of intravenous immunoglobulin

from adults living in malaria endemic areas only to treat patients with mild *P. falciparum* malaria have shown a non-sterilising protective effect against symptoms in 8 Thai patients with mild malaria with rapid clearance of circulating forms (Sabchareon *et al.*, 1991).

The therapeutic value of an immunoglobulin pool for severe malaria was tested in a small study in 16 patients with cerebral symptoms from Malawi (Taylor *et al.*, 1992). Unfortunately no differences were observed between the treated and untreated groups even though the immunoglobulin inhibited adhesion of isolates from the same patients to C32 melanoma cells. Therefore, reduction of cerebral malaria symptoms may depend on its ability to inhibit the initial pathological process of sequestration in the cerebral microvasculature, which is probably different from the *in vitro* models used (reviewed by Berendt, 1994).

Additional problems of interpretation stem from the paucity of data relating to endothelial cell receptor usage in different pathological states of malaria. *In vitro* adhesion assays and immunohistochemical evidence have shown that no single receptor appears to mediate sequestration of PRBC in cerebral microvasculature. Available data suggest that preferential adhesion of PRBC to ICAM1 may be associated with severe disease (Turner *et al.*, 1994; C. I. Newbold, personal communication). PfEMP1 mediates binding to ICAM1 and is the target of antibody-mediated immune responses directed to the PRBC surface (Marsh *et al.*, 1989).

If further progress is to made in this area, it is clearly important to understand how the binding to each receptor inter-relates, and to determine if the effect of immune serum is receptor-specific. In this chapter, careful examination of highly-synchronised parasites was used to investigate the former, while variant-specific adhesion to ICAM1, CD36 and TSP in the presence or absence of immune serum was used to study the latter.

7.1. Effect of proteases on surface phenotypes

PRBCs with different antigenic phenotypes had been derived in an earlier study and used to show that clonal antigenic variation was accompanied by switches in the cytoadherent phenotype expressed at the PRBC surface (Roberts *et al.*, 1992). Two proteases, trypsin and V8 protease, with different specificities were used in earlier chapters to dissect the surface phenotypes of an extended range of PRBCs. A summary of the effect of these proteases on agglutination and cytoadherence of PRBC is shown in Table 7.1.

When the results were examined as a whole, it was clear that the parasite-induced surface functions were differentially sensitive to trypsin and V8 protease. Adhesion to CD36, ICAM1 and TSP was biochemically separable at the PRBC surface, and the molecular implications of this finding were studied in chapter 6. An important observation from table 7.1 was that protease resistant adhesion to either CD36 or ICAM1 was always accompanied by detectable PRBC agglutination. This correlated with other data which suggested that adhesion to these two ligands was linked to the expression of a variant antigen (PfEMP1) which mediated agglutination.

On the other hand, adhesion to TSP was completely separable from other known surface functions by trypsin and V8 protease. The protease sensitivity of PRBC binding to TSP was independent of the major antigenic phenotype of the culture and all PRBC derived from IT had similar levels of resistant binding after digestion with trypsin or V8 protease. Significantly, protease-resistant PRBC binding to TSP occurred in the absence of detectable agglutination and the balance of phenotypic evidence suggested that this function was not linked to the antigenic determinants at the infected erythrocyte surface. To probe the linkage of agglutination and cytoadherence in more detail, the effect of Sib serum on PRBC adhesion to the individual receptors was examined.

PARASITE	Major Antigenic Phenotype	ICAM1 Binding Phenotype	PROTEASE RESISTANCE							
			Agglutination		CD36 Binding		ICAM1 Binding		TSP Binding	
			TRY	*V8*	*TRY*	*V8*	*TRY*	*V8*	*TRY*	*V8*
A4	A4VAT	MID	25	102	0	23	70	2	25	89
C9	A4VAT	MID	10	96	0	23	87	2	23	80
C18	C18VAT	NON	0	171	0	99			21	91
C24	C24VAT	NON	0	0	0	1			19	105
C28	C28VAT	NON	0	0	0	2			26	75
A4V8CD36	C18VAT	NON	0	135	0	95				
IT6	IT6VAT	HIGH	1	2	0	1	2	6	17	81
C18-I(2)	IT6VAT	HIGH	1	1	0	6	6	6		
C24-I(2)	A4VAT	MID	1	0	0	1	12	2		
C28-I*	A4VAT	MID	2	0	0	2	5	1		
A4-I(2)	A4VAT	MID	4	0	0	2	6	2		
A4TRY-I	A4TRYIVAT	MID	95	7	0	0	406	1		
D4-I		LOW			0	10	27	18	6	46

Table 7.1. Summary of the surface phenotypes of PRBCs examined in this study. The major antigenic phenotype was determined by the relative degree of mixed colour agglutinates formed in the mixed agglutination assay, and the ICAM1 binding phenotype was determined by the spot binding assay. Values for protease resistance are the percentage of control (undigested) agglutination or binding after PRBC had been digested with Trypsin (TRY) at 1 mg ml^{-1} or V8 protease (V8) at 10 U ml^{-1}. Dark squares denote that the experiment was not done. Note that all PRBC had surface knobs and were from the IT genotype, except D4-I which was knob-negative and derived from Palo Alto / Wellcome lines (section 2.2.1).

7.2. Effect of polyclonal serum on PRBC cytoadherence to purified receptors

7.2.1 Methodology

The effect of antibodies on cytoadherence has been previously investigated by two different experimental approaches. The first method was to incubate PRBC with polyclonal serum for a fixed period of time and then test these cells in adhesion assays. This approach is unreliable because many PRBC formed agglutinates in immune serum and thus the observed inhibition of adhesion could be attributed to non-specific mechanisms, i.e. PRBC would not cytoadhere to purified receptors if they were in the centre of a large agglutinate. The second methodology was pioneered by Udeinya and colleagues (1983) and was based on the principle of antibody-reversal of cytoadherence. This group used the technique to demonstrate that PRBC adhesion to C32 melanoma cells was reversed by strain-specific immune sera from *Aotus* monkeys, and the methodology described below is a modification of their protocol.

The effect of antibodies in Sib serum on PRBC cytoadherence to purified CD36 (12.5 ng ml^{-1}), ICAM1 (25 μg ml^{-1}) and TSP (40 μg ml^{-1}) was examined by the standard spot binding assay (section 2.2.6). The hybridisation oven was used to resuspend cells, and PRBC were allowed to adhere for 15 minutes at 37°C. Unbound cells were removed by gently washing the dishes four times with binding medium without serum. 1.25ml of Sib serum from the same pool that was used for agglutination assays was pipetted onto the dishes at different dilutions in binding medium at pH 7.0. Binding medium and serum pooled from patients with no prior exposure to malaria (European serum) were used as controls. The dishes were rocked on the hybridisation oven for a further 15 minutes, and unbound cells were removed by five gentle washes in binding medium without serum. Bound cells were fixed and stained as before and the results from two independent experiments are shown in Figure 7.1.

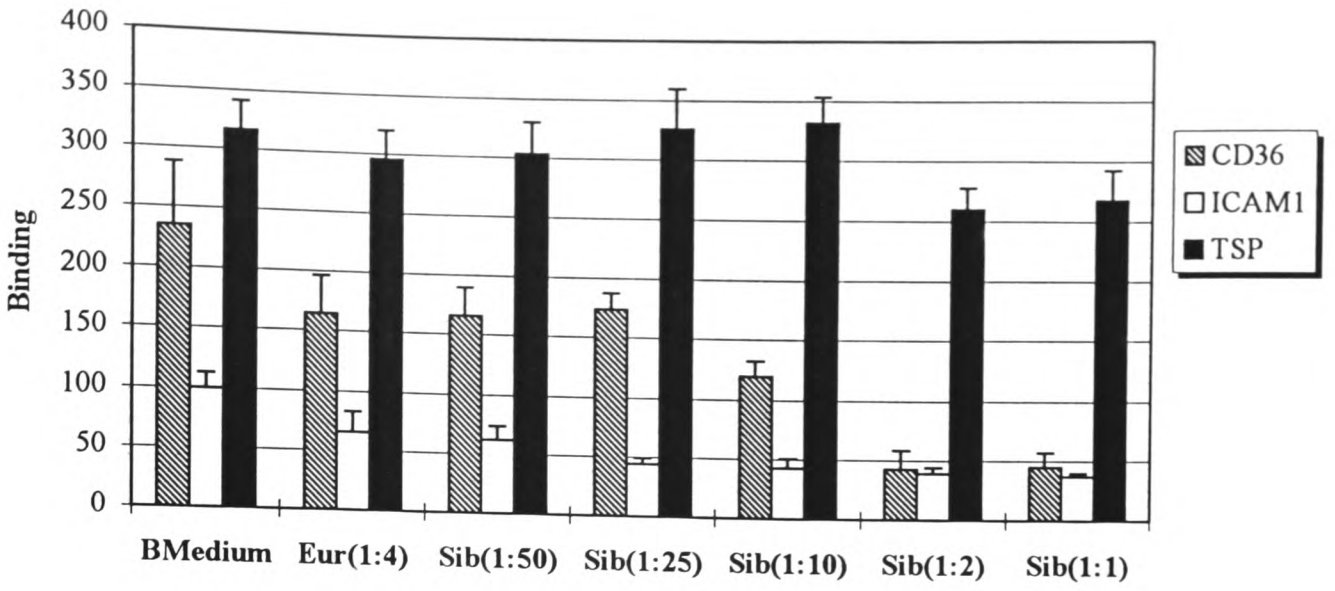


Figure 7.1. Effect of sera on PRBC bound to CD36, ICAM1 and TSP in the spot binding assay. PRBC from $A4_{i+}$ at 6% parasitaemia and 2% haematocrit were allowed to adhere to purified receptors for 15 minutes. Unbound cells were washed off and binding medium (BMedium), European serum (Eur), or Sib serum was added at different concentrations for a further 15 minutes. The number of cells bound was counted and results are mean values ± SEM for two experiments. Different dilutions of European serum were tested, but the degree of reversal was always significantly lower than the values obtained when equivalent concentrations of Sib serum were used.

These data demonstrated that antibodies in Sib serum specifically reversed PRBC adhesion to purified CD36 and ICAM1, but had little effect on adhesion to TSP at the concentrations of receptor used. The reversal effect reached a plateau at a serum dilution of 1:2 in binding medium, but pooled non-immune serum (Eur) at 1:4 dilution in binding medium also reversed adhesion to CD36 and ICAM1 to a lesser degree. The low levels of reversal by Eur serum implied that there was non-specific dissociation of bound PRBC, which was probably caused by the high quantities of protein present in the sera. European serum was twice the protein concentration of Sib serum due to the different mode of collection and this was confirmed by SDS PAGE (data not shown).

7.2.2 Reversal of cytoadherence to CD36, ICAM1 and TSP

To discover if the reversal of cytoadherence to CD36 and ICAM1 was specific to PRBC from $A4_{i+}$, or was a general phenomenon, the effect of Sib serum at 1:2 dilution in binding medium was compared to Eur serum at 1:4 dilution for three other antigenically distinct parasite lines (Figure 7.2).

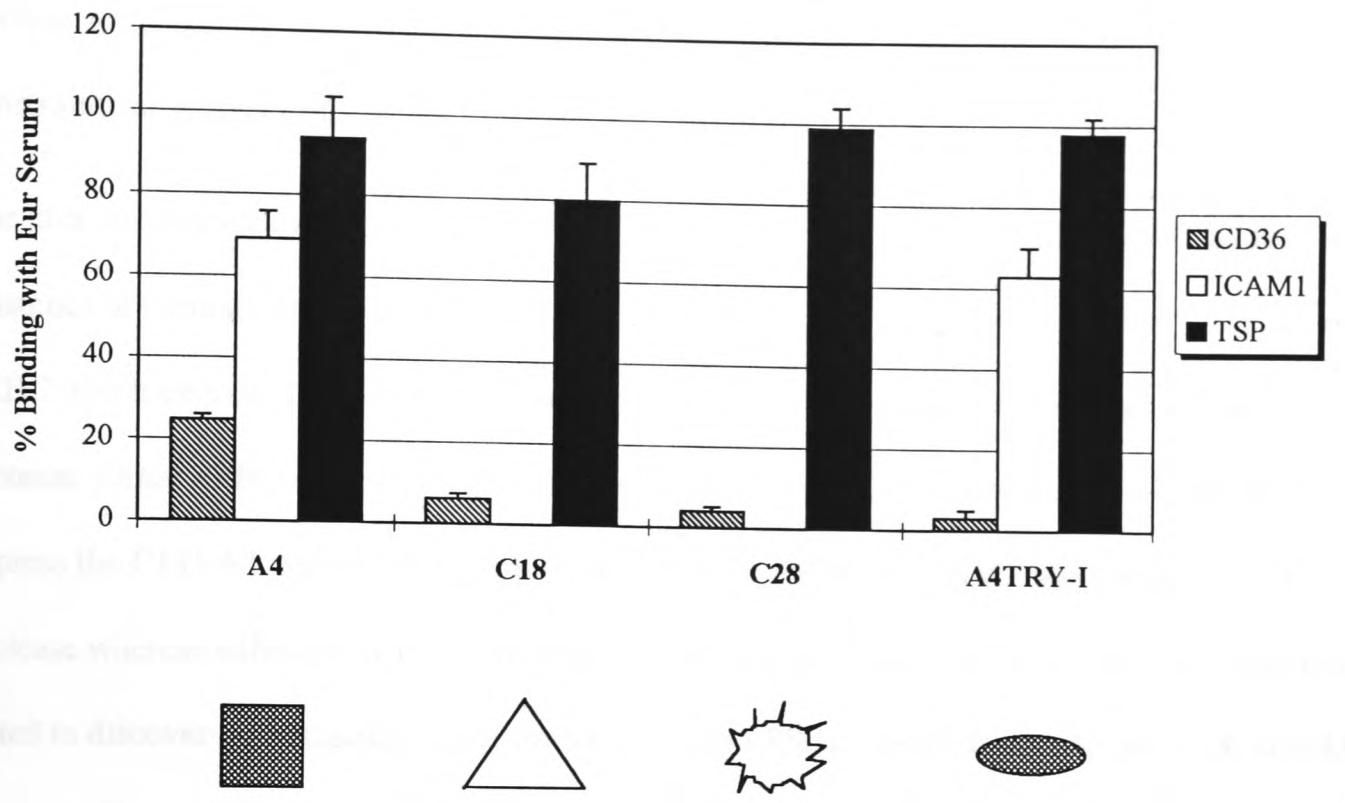
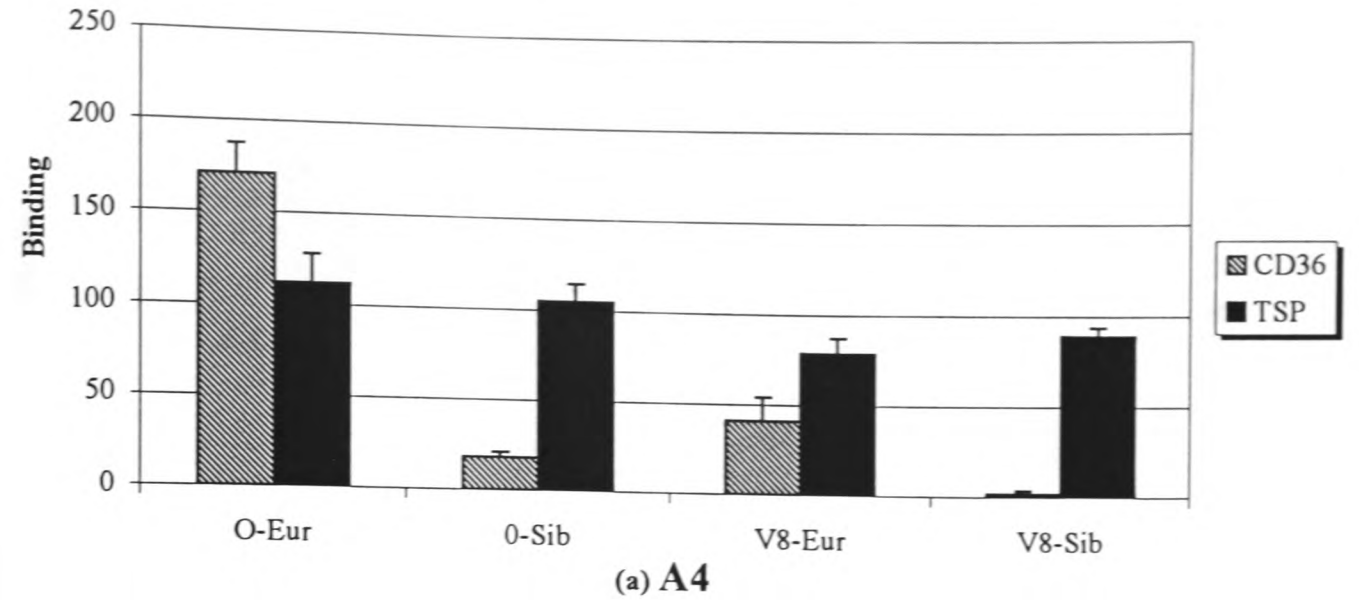


Figure 7.2. The effect of Sib serum on reversal of adherence of PRBC to CD36, ICAM1 and TSP. The results are the mean PRBC bound mm^{-2} ± SEM for duplicate dishes from at least three experiments for each parasite line. The control dishes were incubated with Eur serum at 1:4 dilution in binding medium. The surrounding shape denotes the antigenic phenotype of the PRBC and the shading signifies that the PRBC can bind to ICAM1.

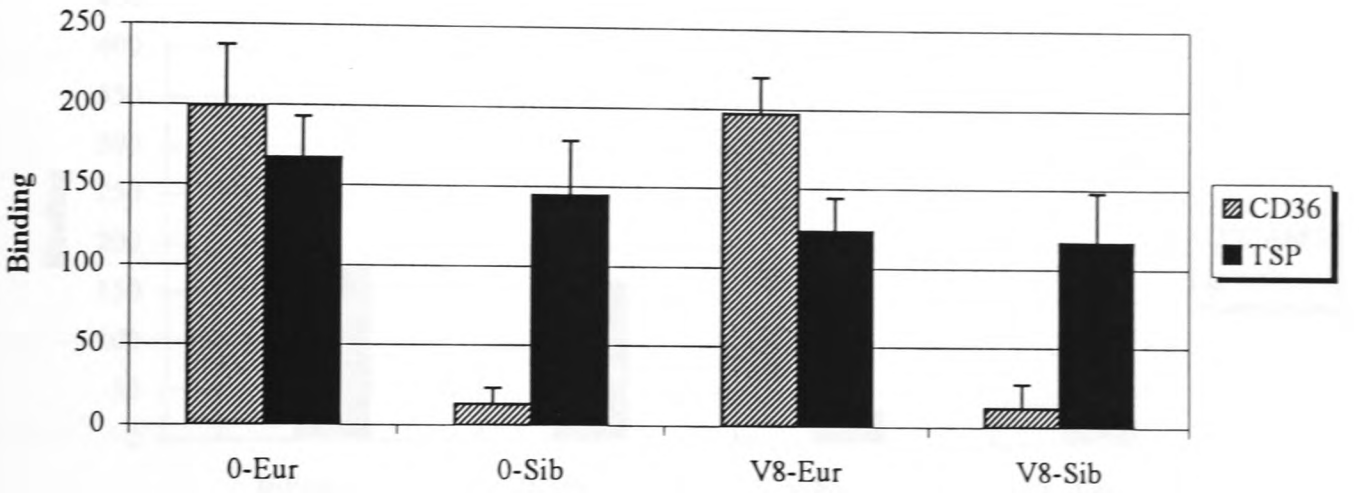
The effects of serum on cytoadherence differed quantitatively between parasite line and receptor tested. Sib serum reversed adhesion of PRBC to CD36 and ICAM1, but had little effect on adhesion to TSP (mean reversal = 7.2 ± 3.1% control, n = 12). Adhesion to CD36 was reversed by 75.3 ± 4.4% (n = 5) for PRBC from $A4_{i+}$, and by between 93.8 - 97.0% for PRBC from the three other parasite lines. The reason for these significantly different ($p<0.0001$) levels of reversal of adhesion to CD36 was unclear, and is discussed in the conclusions. Only two parasites lines ($A4_{i+}$ and A4TRY-I) were tested for the effect of serum on binding to ICAM1, but the results were comparable (mean reversal = 37.9 ± 5.1% control, n = 8) and were intermediate between levels of reversal for CD36 and TSP.

Thus, it appeared that for all PRBC tested, adhesion to purified CD36 and ICAM1 was reversed by specific antibodies in Sib serum, whereas adhesion to TSP was unaffected. This implied that domains for adhesion to the former receptors were in close apposition to antigenic epitopes at the PRBC surface. The apparent insensitivity of PRBC binding to TSP could be attributed to the lack of specific antibodies in Sib serum which interfere with binding to this receptor. Alternatively, the TSP binding domain at the surface was not recognised by antibodies because it was inaccessible due to spatial constraints or was part of a non-immunogenic molecule.

The protease-resistant adhesion to CD36 and to ICAM1 has been attributed in earlier chapters to the existence of variant antigenic phenotypes which were insensitive to proteases. Table 7.1 shows that PRBC which express the C18VAT have completely resistant binding to CD36 after digestion with V8 protease. Other PRBC show levels of partial resistance that correlate with the number PRBC which express the C18VAT within the culture. Adhesion of all PRBC to ICAM1 was sensitive to V8 protease whereas adhesion to TSP is resistant to the same protease. Sib serum and Eur serum were tested to discover if the resistant adhesion to CD36 and TSP of PRBC from $A4_{i+}$ and $C18_{i-}$ could be reversed (Figure 7.3).



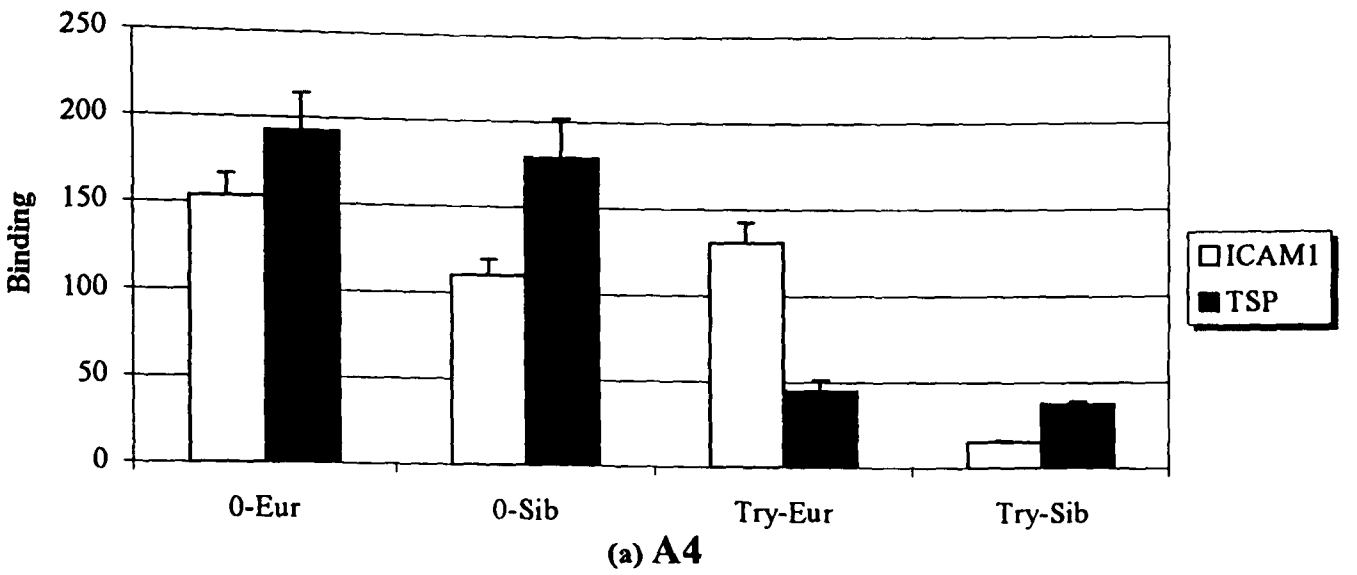
(a) **A4**



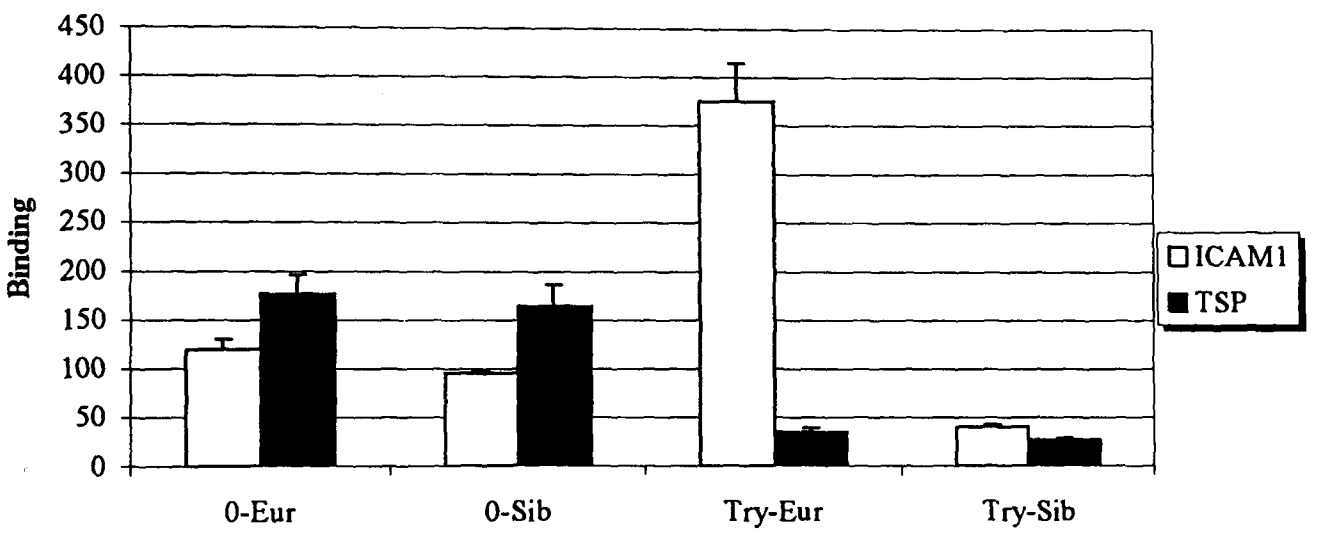
(b) **C18**

Figure 7.3. Effect of sera on adhesion of PRBC from (a) $A4_{i+}$ and (b) $C18_{i-}$ to CD36 and TSP. Undigested PRBC (0) or V8 protease (10U ml^{-1}) digested PRBC (V8) were allowed to adhere to CD36 and TSP for 15 minutes. After free cells were washed off, Eur serum (1:4) or Sib serum (1:2) in binding medium were added for a further 15 minutes. Unbound cells were washed off and cells were fixed and stained before counting. Data are mean values of PRBC binding mm^{-2} ± SEM from triplicate dishes in two experiments.

The V8 protease-resistant binding to CD36 was reversed in PRBC from $A4_{i+}$ and $C18_{i-}$, but the resistant binding to TSP was unaffected by Sib serum in both parasites. This finding implied that the binding domain for CD36 was close to an antigenic epitope recognised by antibodies in Sib serum and provided more support for the hypothesis that resistant binding to CD36 was linked to the expression of particular antigenic phenotypes.



(a) **A4**



(b) **A4TRY-I**

Figure 7.4. Effect of sera on adhesion of PRBC from (a) $A4_{i+}$ and (b) A4TRY-I to ICAM1 and TSP. Undigested PRBC (0) or Trypsin (1mg ml^{-1}) digested PRBC (TRY) were allowed to adhere to ICAM1 and TSP for 15 minutes. After free cells were washed off, Eur serum (1:4) or Sib serum (1:2) were added for 15 minutes. Unbound cells were washed off and cells were fixed and stained before counting. Data are mean cells bound mm^{-2} ± SEM from duplicate dishes in two experiments. Note that the scales on the y-axes are different to display the trypsin-enhanced adhesion of PRBC from A4TRY-I to ICAM1.

The trypsin-resistant binding to ICAM1 of PRBC from $A4_{i+}$ and A4TRY-I was more sensitive to antibody reversal than undigested PRBC binding to the same ligand (Figure 7.4). The resistant binding has been demonstrated to be associated with the expression of a novel antigenic phenotype that is not detectable in the control cultures. Thus, the increased antibody reversal of cytoadherence could be due to the increased spatial access of antibody to epitopes at the PRBC surface made possible by the removal of bulky proteins by trypsin. In addition, there may be a higher titre of antibodies in Sib serum that recognise this novel phenotype which are responsible for the observed effect. This was suggested by the successful elution of variant-specific antibodies to the trypsin-resistant phenotype which agglutinated trypsinised PRBC from $A4_{i+}$ and A4TRY-I. There were insufficient quantities of variant-specific antibody to test its effect on cytoadherence, but these results confirmed earlier findings which suggested that binding to ICAM1 was closely linked biochemically to the antigenic epitopes at the PRBC surface.

The trypsin-resistant PRBC binding to TSP observed in $A4_{i+}$ and A4TRY-I was refractory to antibodies in Sib serum. This was in accordance with all results obtained and strongly implied that the binding domain for TSP was separable from the antigenic epitopes which were recognised by agglutinating antibodies in Sib serum.

Thus, it is clear from these results that antibodies in Sib serum can reverse PRBC cytoadherence to purified CD36 and ICAM1, but have little effect on adhesion to TSP. Identical serum mediated PRBC agglutination, which suggested that cytoadherence to CD36, ICAM1 and agglutination share a common mechanism that may be distinct from adhesion to TSP. Another approach taken to probe this relationship was to determine the time of onset of these functions during the intraerythrocytic life-cycle.

7.3. Staging of surface phenotypes

A highly-synchronised culture is a prerequisite for the accurate measurement of the time when a PRBC will agglutinate or cytoadhere. To synchronise cultures using sorbitol lysis of mature forms would not be the method of choice, because large numbers of PRBC would be lost due to lysis induced by sorbitol, which would have to be applied 1 hour after reinvasion to accomplish tight synchrony. Schizonts from the previous cycle would be destroyed, and reinvasion rates would be low. Hence a non-standard method for synchronisation was tested which employed low concentrations of aphidicolin, a toxic inhibitor of eukaryotic DNA synthesis.

A single study demonstrated that the addition of 1.5 $\mu g\ ml^{-1}$ of aphidicolin to an asynchronous culture for 15 hours could synchronise PRBC at the mature trophozoite stage (Inselberg and Banyal, 1984). Ring-infected erythrocytes were observed in thin films 9 hours after the inhibitor had been removed from the culture. In this study, the reinvasion rates were approximately 90% of control cultures, and the synchrony was 1 hour with this concentration of aphidicolin (Inselberg and Banyal, 1984).

Aphidicolin was used under identical conditions to those quoted in the report by Inselberg and Banyal (1984) with an asynchronous culture of PRBC from $A4_{i+}$ (Figure 7.5a). However, no reinvasion occurred and close inspection of thin films of cultures revealed that aphidicolin at 1.5 $\mu g\ ml^{-1}$ was toxic to infected erythrocytes and trophozoites were morphologically abnormal (Figure 7.5b). Similar results were observed when different concentrations of aphidicolin were tested for different lengths of time in the culture. Control rates of reinvasion were achieved only when a lower concentration ($<0.5\ \mu g\ ml^{-1}$) of aphidicolin was used, but there was no improvement in the degree of synchrony. Thus, aphidicolin was impractical for synchronising PRBC from $A4_{i+}$, and the sorbitol method was used, despite its limitations.

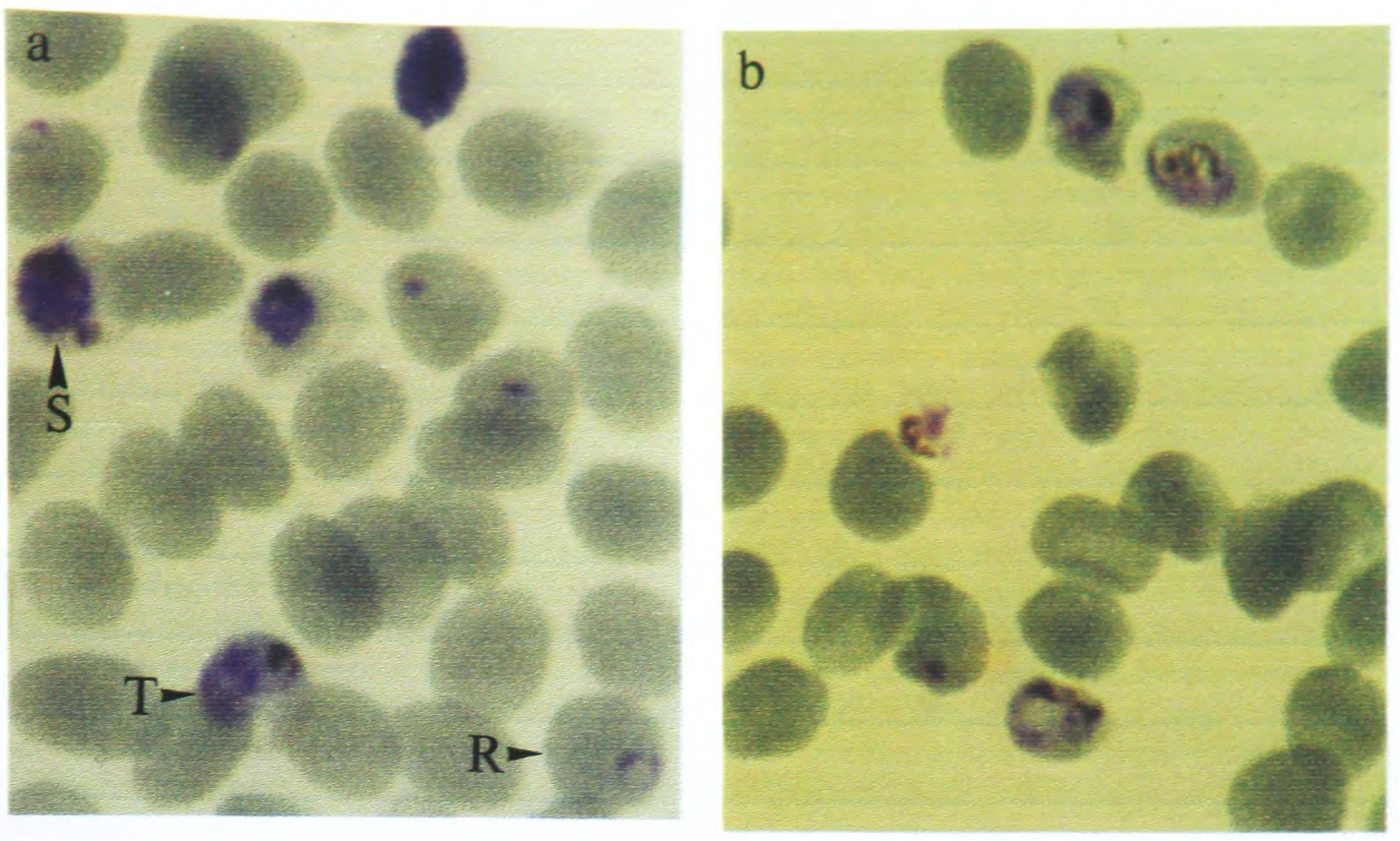


Figure 7.5. (a)Thin film of an asynchronous culture of PRBC from $A4_{1+}$ stained with Giemsa. Rings (R), trophozoites (T), and Schizonts (S) are shown. (b) Thin film of the same culture following incubation with aphidicolin at 1.5μg ml^{-1} for 15 hours.

Large numbers of infected erythrocytes (1×10^{10}) from $A4_{1+}$ were cultured and synchronised by sequential applications of sorbitol in four successive cycles of growth. The last two additions of sorbitol were applied exactly 1 hour after bursting schizonts were observed on thin films, because this was empirically decided to be the age of the intracellular parasite that could be determined most accurately by Giemsa staining. The sorbitol procedure considerably reduced the number of PRBC and the tightly synchronised (1 hour) young ring forms produced were only readily visible on Giemsa-stained thin films approximately 5 hours following reinvasion.

Approximately 24 hours after each of the four reinvasions, mature trophozoites were enriched by Plasmagel™ flotation and resuspended in fresh, washed erythrocytes. The number of PRBC after the final addition of sorbitol was typically 1×10^{8}. Several different working definitions were used to describe the morphology of the synchronous PRBC in the subsequent cycle (Figure 7.6 and Table 7.2), to improve the accuracy and reproducibility of results (recommended by Marsh *et al.*, 1988).

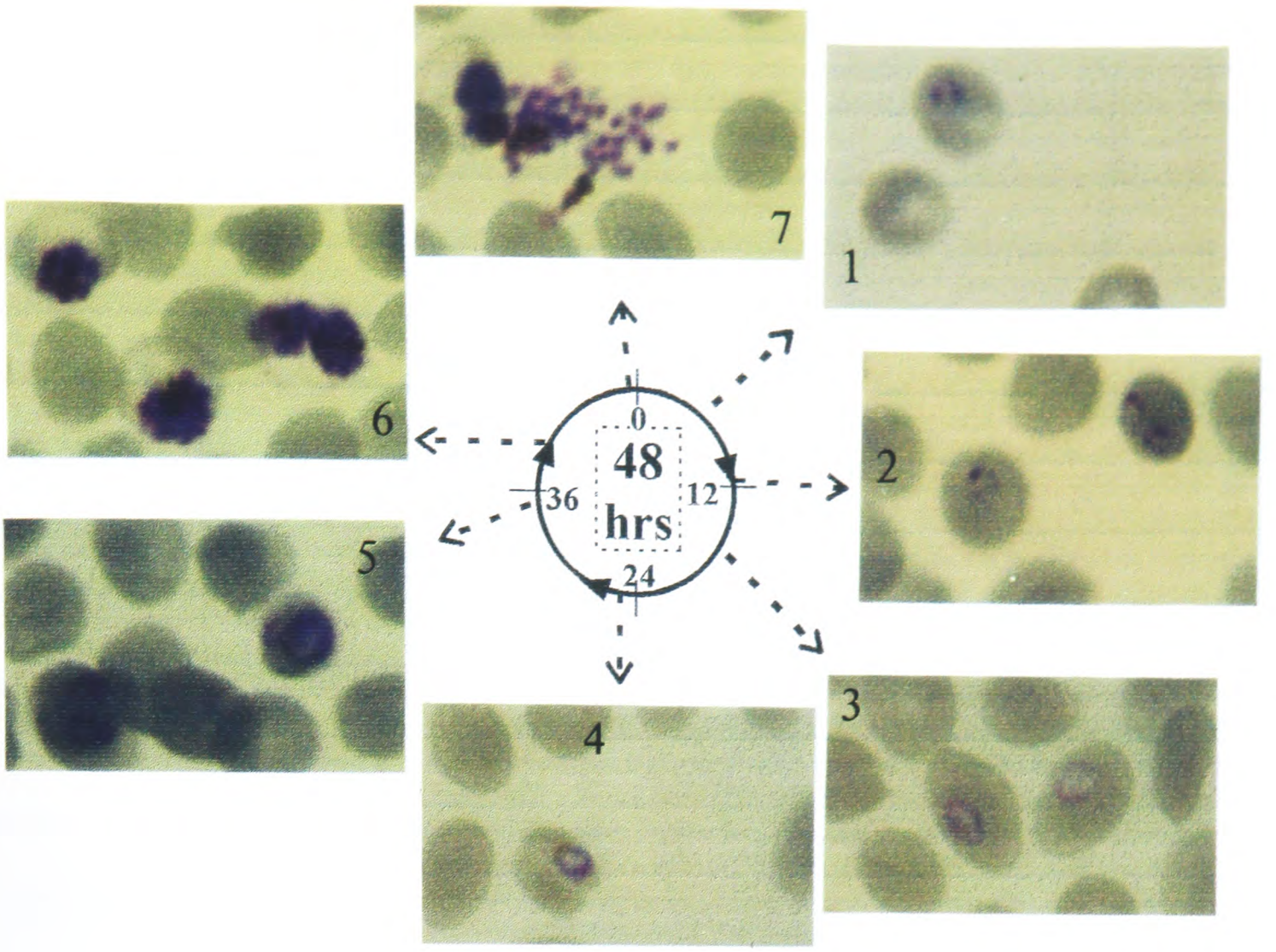


Figure 7.6. Photographs of thin film smears of PRBC from $A4_{i+}$ at different stages following sorbitol synchronisation.

Photo. N° (Age)	Age Range (hours)	Working Definition	Morphological Features of Parasite
1 (8)	6 - 8	Young Ring	Annular appearance, chromatin granules at one or both poles of small ring form. Parasite is approximately 1μm diameter.
2 (12)	9 - 16	Mature Ring	Vacuole visible. Oldest form observed post sorbitol synchronisation.
3 (18)	17 - 21	Young Trophozoite	Pigment (haemazoin) deposited. Youngest form observed post-Plasmagel™ flotation.
4 (26)	19 - 32	Mid Trophozoite	Globular appearance. Nuclear division commences.
5 (36)	33 - 39	Mature Trophozoites	Heavily pigmented, and nuclear division has produced smaller nuclei surrounded by cytoplasm. Occupies ~2/3 of erythrocyte.
6 (44)	40 - 46	Segmenter (Mature Schizont)	Segmentation produces 8 - 32 merozoites per cell.
7 (47)	47 - 48	Bursting Schizont	Pigment and merozoites released after infected erythrocyte has burst.

Table 7.2. Summary of features of P. falciparum *infected erythrocytes observed on thin films at different stages during the 48 hour life cycle. Photograph numbers relate to Figure 7.6, and Age is time after reinvasion (hours).*

The synchronous culture was divided into separate tissue culture flasks which were assayed independently at different time points following reinvasion. This was done to avoid disturbances to the culture conditions that would have occurred by continually removing samples from a single flask and would consequently have altered the rate of PRBC growth. At each time point, a thin film of each sample was made and shorter agglutination and spot binding assays were performed so that cells were fixed as soon as possible after the smear was made. Sib serum at 1:4 dilution in RPMI-S was used in the agglutination assay, PRBC were resuspended in duplicate tubes on the rotator for 15 minutes and two slides from each sample were counted immediately. Duplicate binding assay dishes were rocked for 15 minutes in the hybridisation oven and washed and fixed as described in the general methods chapter. An agglutination reaction was scored as negative (-), if there was less than two agglutinates of >3 PRBC observed when 200 PRBC were counted from duplicate samples. The binding assays were counted without knowledge of the time of sampling (i.e. blinded) and the number of cells bound mm^{-2} for each receptor was calculated.

A preliminary experiment established that the time of onset of agglutination and cytoadherence after reinvasion occurred at the same time as the appearance of old rings and young trophozoites in the culture. A more accurate experiment was performed by sampling at 2 hour intervals during the period 10 - 20 hours post reinvasion.

The results from one experiment are shown in figure 7.7, and show that erythrocytes infected with ring forms (up to 12 hours post-invasion) of $A4_{i+}$ do not form agglutinates or cytoadhere. Mature PRBC bind to all receptors at comparable levels to previous experiments and form agglutinates in Sib serum. The timing of these phenotypes is different, because binding to TSP was observed in cultures at 14 hours post reinvasion and the same cells did not bind to other ligands or form agglutinates. PRBC adhesion to CD36 and ICAM1 and agglutination occurred simultaneously at 16 hours post invasion and identical times of onset were observed in a second experiment from a different stabilate of $A4_{i+}$ at the same number of cycles post-thaw.

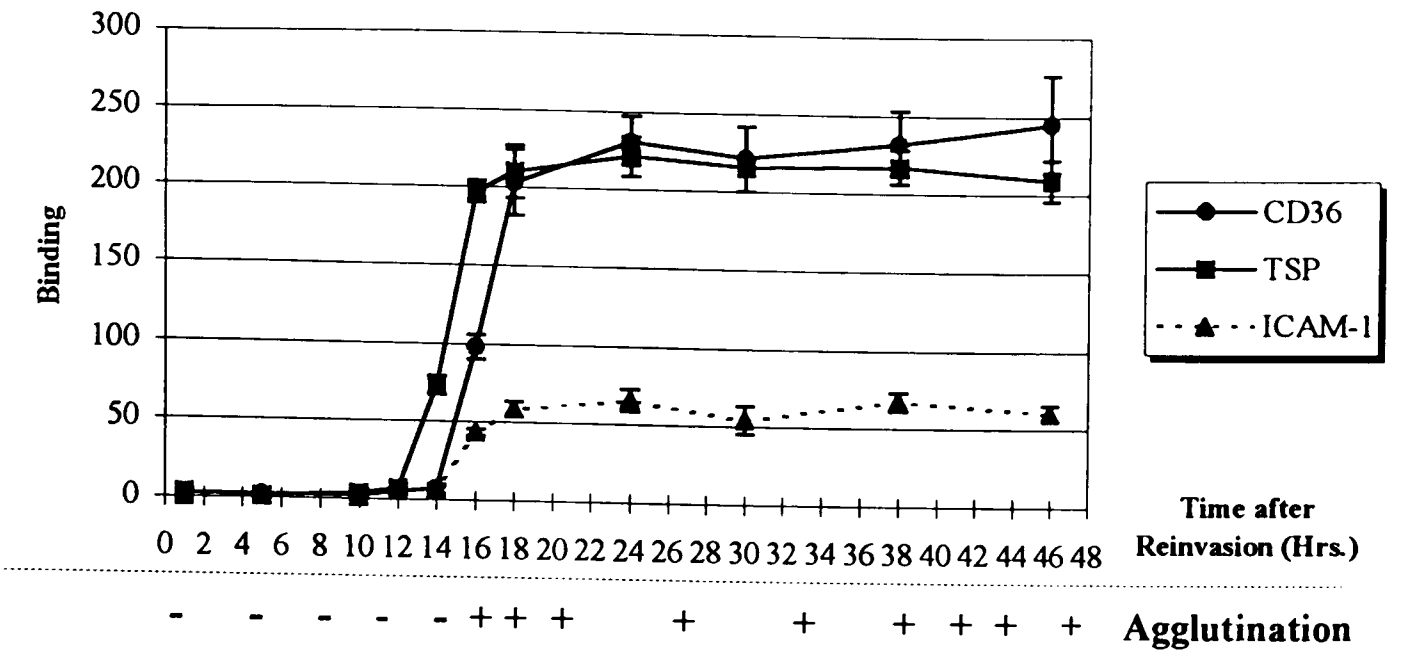
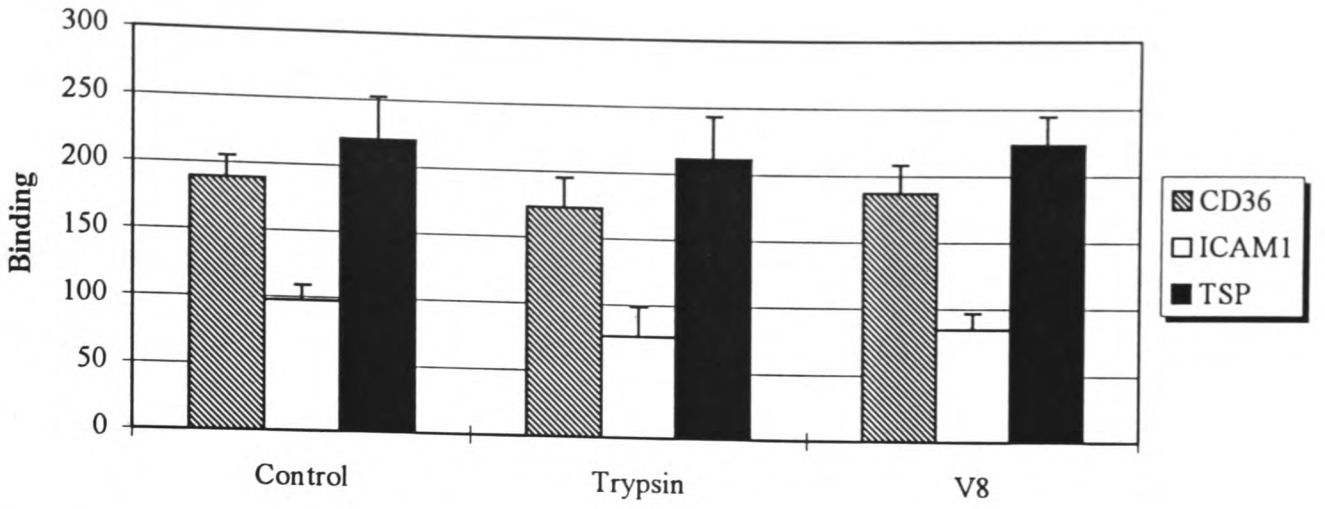


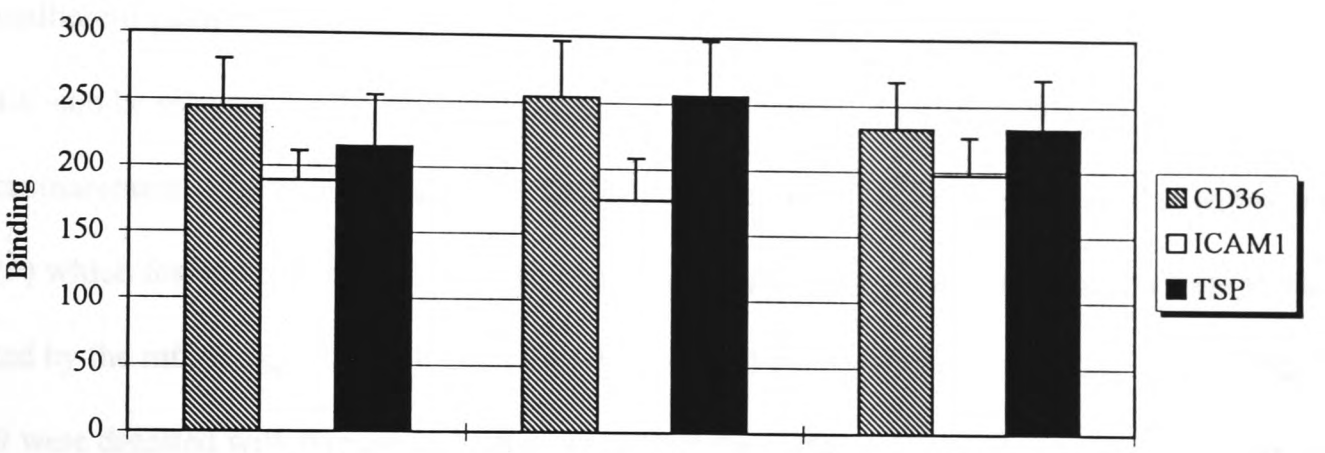
Figure 7.7. The time of onset of cytoadherence and agglutination of PRBC from $A4_{i+}$. Shortened versions of the spot binding assay and the agglutination assay were used to measure the surface functions of a culture of PRBC that had been synchronised to 1 hour. Values for binding are mean cells bound mm^{-2} ± SEM from triplicate determinations made in single experiment.

Thus, it was concluded that the time of onset of binding to TSP was earlier than the other surface phenotypes, which was consistent with its phenotypic and biochemical separation. Similarly, the association of PRBC adhesion to CD36 and ICAM1 with agglutination, and hence antigenic phenotype, was strengthened by the observation that these functions were activated simultaneously.

A complementary approach employed to examine the importance of surface changes which occurred during the first half of the intraerythrocytic stage was to digest ring-form PRBC with proteases, and assess their agglutination and cytoadherence properties at trophozoite stage. Sorbitol-synchronised PRBC from $A4_{i+}$ and IT6 were washed twice in RPMI-S and digested with trypsin (1mg ml^{-1}) or V8 protease (10U ml^{-1}). After the proteases had been inhibited, PRBC were washed twice in RPMI-S and cultured for 24 hours in standard conditions. Levels of adhesion to CD36, ICAM1 and TSP of PRBC digested at ring-stage and tested at trophozoite stage were comparable to undigested controls in two independent experiments (Figure 7.8). Agglutination and parasitaemia were unchanged (data not shown).



(a) **A4**



(b) **IT6**

Figure 7.8. Effect of proteases on cytoadherence of PRBC from either (a) $A4_{i+}$ or (b) IT6 after cells had been digested at ring stage and tested 24 hours later. Data are mean binding in cells mm^{-2} ± SEM from two separate experiments.

These results were different from those obtained when trophozoites were digested with the same proteases and implied that the modifications to the PRBC surface which were important for agglutination and cytoadherence occurred after ring stage. Furthermore, uninfected erythrocytes which had been digested did not bind to any of the receptors tested and did not form agglutinates which confirmed that the functional changes to the erythrocyte were parasite-derived.

7.4. Other PRBC surface functions

7.4.1 Rosetting

Mature infected erythrocytes can cytoadhere to uninfected erythrocytes and form rosettes (David *et al.*, 1988). This is a variable characteristic of PRBC, but its occurrence in the cerebral capillaries of patients infected with *P. falciparum* may be associated with severe disease (Carlson *et al.*, 1990; Treutiger *et al.*, 1992; A. Rowe, personal communication). The molecules on the uninfected cell and the PRBC surface that mediate rosetting have not been elucidated, but low molecular weight parasite-derived rosettins have been associated with this function in one study (Helmby *et al.*, 1993).

Recent evidence has suggested that the rosetting phenotype may be related to the antigenic and cytoadherent phenotypes at the erythrocyte surface. Roberts and colleagues (1992) have shown that PRBC can be selected for the rosetting phenotype, and cloning of selected PRBC by micromanipulation was accompanied by antigenic switching. This method was used to isolate a clone (R29) which formed rosettes at high frequency. R29 expressed a unique antigenic phenotype when tested by the mixed agglutination assay and did not bind to ICAM1 (Roberts *et al.*, 1992). PRBC from R29 were digested with trypsin and V8 protease and the surface phenotypes examined (Figure 7.9). Rosetting, agglutination in Sib serum, and binding to CD36 were abolished by both proteases, and it was concluded that the molecules on the PRBC surface that mediated these functions in R29 were protease-sensitive.

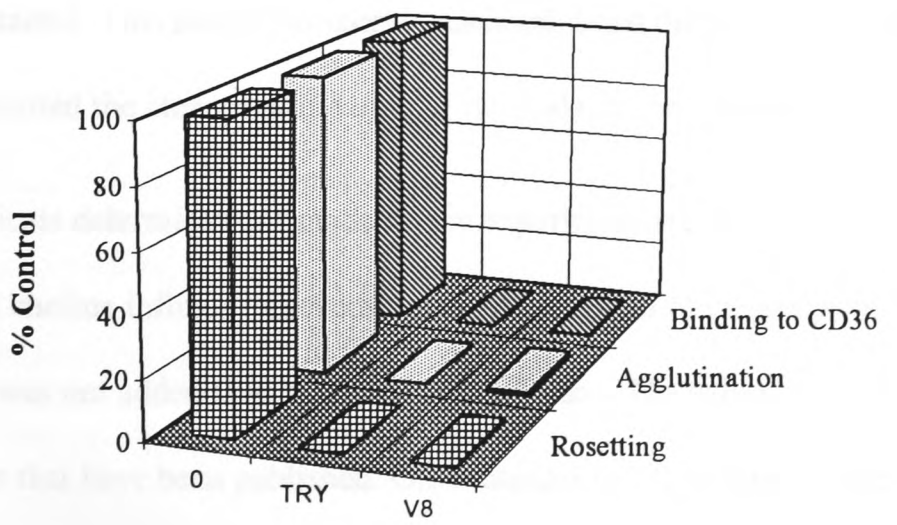


Figure 7.9. Effect of trypsin (TRY; 1mg ml^{-1}) and V8 protease (V8; 10U ml^{-1}) on rosetting, agglutination and binding to CD36 of PRBC from R29 clone.

7.4.2 Transport

The PRBC membrane shows increased permeability to a wide range of solutes during the intraerythrocytic development of *P. falciparum* (Elford *et al.*, 1985). These transport pathways are thought to be functionally distinct from endogenous host mechanisms and are responsible for the import of biosynthetic substrates and export of the potentially harmful products of parasite metabolism. Recent work by Kirk and colleagues (1991, 1994) has demonstrated that the majority of the non-saturable transport of low M_r solutes (e.g. monovalent cations (choline, K^+) and anions (lactate, Cl^-), amino acids, sugars and nucleosides) across the PRBC membrane *in vitro* is via broad specificity pathways of a single type. Pharmacological evidence and substrate-specificity experiments have shown that the parasite-induced pathways have the characteristics of a chloride channel. To discover if malaria-induced solute transport was linked to other surface functions in the infected cell, the protease sensitivity of this channel was tested in a single experiment performed in Dr. Kirk's laboratory in Oxford.

The protocol followed has been described in the general methods chapter (section 2.2.13) and is based on the published method (Kirk *et al.*, 1994). PRBC from $C18_i$ and uninfected erythrocytes (RBC) were resuspended in 1mM choline chloride (and a trace of radiolabel), which was sufficient to saturate the host cell choline transporter and thereby minimise the flux via this system. The uptake of radioactive substrate by cells treated with RPMI-S, trypsin (1mg ml^{-1}) or chymotrypsin (1mg ml^{-1}) was measured over a fixed time interval. Furosemide (0.1mM) was added to half of the samples before the time course was started. This anion-transport blocker inhibited the parasite-induced uptake of choline and therefore permitted the clear identification of the malaria-induced transport component.

Results from triplicate determinations made in one experiment are shown in Table 7.3. These data demonstrated that choline influx was several times higher in all three groups of PRBC than RBC when furosemide was not added (PRBC / RBC choline flux). The values were in agreement with the >20-fold increases that have been published. Other studies have estimated values of PRBC choline influx to range from 60 - 218 μmol $(10^{12}$ cells·hr$)^{-1}$ compared to 1.3 - 7.4 μmol $(10^{12}$ cells·hr$)^{-1}$ in RBC. The influx of choline that was induced by the parasite was furosemide-sensitive, but this component was not significantly altered by pretreatment of the cells with proteases (Figure 7.10).

CONDITION	Choline flux in **PRBC**		Choline flux in **RBC**		PRBC / RBC choline flux	Parasite induced choline flux
	-Fur	+Fur	-Fur	+Fur		
Undigested	111.8	31.2	3.7	10.0	×30	108.1
Trypsin	148.7	50.5	5.7	9.3	×26	143.0
Chymotrypsin	108.9	33.5	4.0	4.8	×27	104.9

Table 7.3. Influx of choline into infected and uninfected erythrocytes in the presence and absence of furosemide. Trophozoite infected cells from $C18_{i-}$ were purified to >90% by Plasmagel™ flotation and RBC were co-cultured from the same batch from The Blood Transfusion Service. Flux values are means from triplicate determinations made during a single experiment, and units are μmol $(10^{12}$ cells·hr$)^{-1}$.

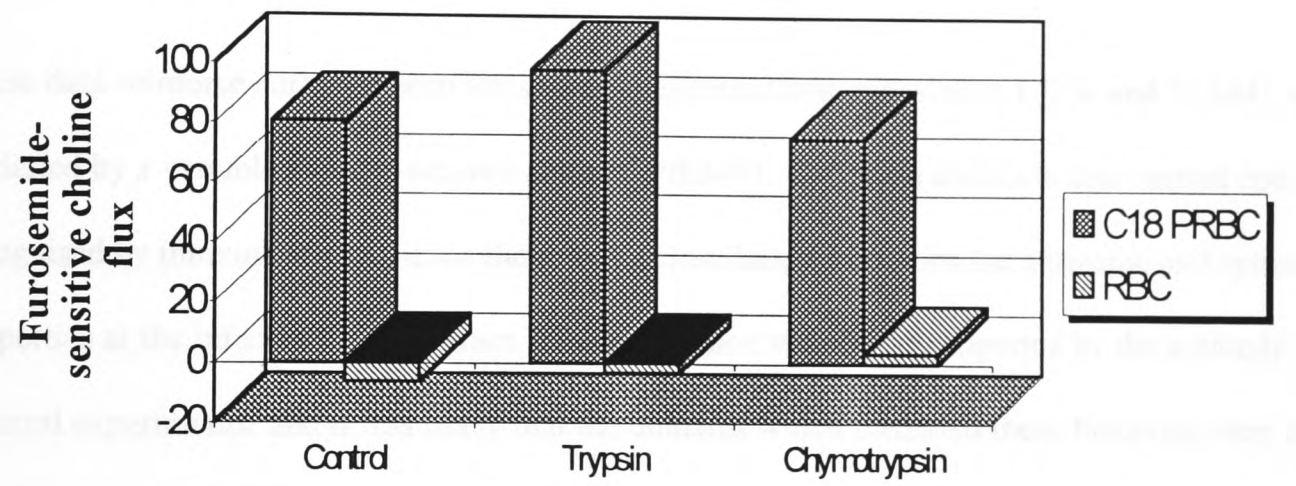


Figure 7.10. The effect of trypsin and chymotrypsin on the furosemide-sensitive component of choline transport of choline into PRBC from $C18_{i-}$ or uninfected erythrocytes (RBC). This flux represents transport via the parasite-induced pathway. Data are mean values in μmol choline $(10^{12}$ cells·hr$)^{-1}$ from triplicate determinations from a single experiment.

Thus, the same proteases which abolished the agglutination and adhesion of PRBC from $C18_{i-}$ to CD36 had no effect on the parasite-induced transport of choline. Trypsin and chymotrypsin were chosen in these experiments because the major membrane protein in RBCs, Band3, is an anion exchanger that is resistant to trypsin, but sensitive to chymotrypsin (Dzandu *et al.*, 1985). Therefore, there was no evidence for linkage of the altered membrane transport and altered cytoadherence properties of *P. falciparum* infected erythrocytes.

7.5. Conclusions

Results in this chapter have demonstrated that antibodies from immune serum reversed cytoadherence to CD36 and ICAM1, but have no effect on adhesion to TSP. The mechanism of this interaction could either be via competitive inhibition of PRBC adhesion at the receptor binding site, or through an indirect mechanism of antibody-mediated inactivation via blockade due to steric hindrance or conformational change at another epitope. The degree of reversal was greatest for adhesion to CD36 and was comparable to levels (>77%) found by Udeinya and colleagues (1983) using C32 melanoma cells. Binding to ICAM1 was reversed by ~40%, but trypsin resistant adhesion to the same receptor was completely reversed. The difference was probably due to the greater accessibility of antibody to surface epitopes following cleavage of trypsin sensitive proteins. On the other hand, the degree of antibody-reversal of binding to TSP, either before or after digestion, was negligible.

These data reinforce earlier experiments which suggested that adhesion to CD36 and ICAM1 was mediated by a variable parasite-derived antigen, PfEMP1. The same molecule also carried epitopes recognised by immune serum, hence there was a close linkage between the antigenic and cytoadherent properties at the infected PRBC surface. This conclusion which was supported by the antibody reversal experiments, and it was likely that the domains which mediated these functions were in close proximity at the PRBC surface. The adhesive domains for TSP were separable from the other surface phenotypes by biochemical methods (e.g. proteolysis) and this was mirrored by antibody-reversal results.

The stage specificity of these phenotypes and their precise timing of onset during the erythrocytic cycle provided further support for the hypothesis that adhesion to TSP was distinct from other surface properties. Binding to CD36, ICAM1 and agglutination were activated simultaneously 16 hours post-invasion at young trophozoite stage, whereas adhesion to TSP occurred two hours earlier. These estimates were sooner than the reported time (20 - 25 hours; young trophozoite) for field isolate binding to C32 melanoma cells (Marsh *et al.*, 1988). The authors noted that their cultures were asynchronous and suggested that morphological criteria be used instead to assess the time of onset of cytoadherence. Therefore, the findings in this chapter were comparable to the published study and changes to the erythrocyte membrane occur during the second third of the intraerythrocytic cycle

which result in cytoadherence (to purified receptors and C32 melanoma cells) and agglutination. Hence, proteases were only effective at ablating these phenotypes when applied at the trophozoite stage. This indicated that the parasite molecules responsible were not exposed at young ring stage, or host molecules were not modified and exposed by the parasite until young trophozoite forms were observed.

These results have important bearing on the possibility of immunotherapy to treat cerebral malaria. Passive transfer of immune serum into intact *Aotus* monkeys was followed by a rapid increase of circulating trophozoites and schizonts (David *et al.*, 1983). Thus, reversal of binding can occur *in vivo*, and the crisis forms of infected erythrocytes observed in humans preceding a successful immune response could represent cells which had been dislodged from sequestered sites by antibodies. Furthermore, released mature stage infected erythrocytes will be destroyed by splenic filtration in the reticuloendothelial system, leading to a reduction in parasitaemia.

However, this was not implied by observations from Cohen *et al.*, 1961, McGregor *et al.*, 1963 and Sabchareon *et al.*, 1991, who found that alleviation of symptoms of mild malaria were associated with the absence of mature forms in the peripheral circulation. An alternative explanation for their findings was that variant-specific antibody reversed the sequestration of PRBC, which were quickly removed by opsonisation before detection in the peripheral circulation. The theory that reversal of cytoadherence may alleviate clinical severity in cerebral malaria was tested in Malawi by Taylor and colleagues (1992), but no beneficial effect was observed even though the immunoglobulin pool inhibited adhesion of the isolates from the same patients to C32 melanoma cells.

Thus, the potential of immunotherapy as treatment for cerebral malaria is equivocal, and deserves more attention. Results in this chapter are pertinent because CD36 and ICAM1 are both expressed in the cerebral microvasculature of patients with cerebral malaria (Turner *et al.*, 1994), and in these cases, the interaction of PRBC in certain disease states may be reversed by immune serum. Unfortunately, two observations predict that this approach may be problematic. Firstly, reversal of binding of PRBC adhesion to CD36 was only ~80%, even under optimised *in vitro* conditions. This figure is likely to be lower *in vivo*, because the concentration of administered antibody will be considerably reduced. Secondly, reversal of ICAM1 binding was even less successful, which was

disappointing because other studies have suggested that PRBC adhesion to this receptor is likely to be important for the development of severe malaria (Turner *et al.*, 1994; C. I. Newbold, personal communication).

Therefore, highly specific antibodies to conserved binding domains would be essential to reverse adhesion of different isolates *in vivo*. Serum pooled from immune adults living in endemic areas would not be an efficient reagent in this respect, because it consists of variant-specific antibodies to the PRBC surface (Newbold *et al.*, 1992). If the reversal of sequestration is pursued as a means of therapy for cerebral malaria, specific reagents that recognise the receptor binding domains at the PRBC surface will need to be developed. The success of this novel type of approach will be determined by a detailed knowledge of the host and parasite molecules that mediate sequestration.

Chapter 8

Discussion

The past three years have seen a rapid expansion of information relating to the infected erythrocyte surface. Recent developments have aroused optimism that the molecular basis and functional consequences of the parasite-induced modifications at this crucial host-parasite interface will soon be established. This study has confirmed at the biochemical level that clonal antigenic variation of *P. falciparum* infected erythrocytes occurs, and has provided strong evidence that the variant-surface antigen, PfEMP1, mediates PRBC cytoadherence to CD36 and ICAM1. Thus, in addition to its implicated role in immune evasion by antigenic variation, PfEMP1 has a clear functional role that is central to the pathogenesis of cerebral malaria.

These findings coincide with an exciting period in this field, because the culmination of a world-wide collaborative effort has led to the (as yet unpublished) cloning of PfEMP1. To reflect this situation, this chapter is divided into two sections; the first summarises the major conclusions of this work, which have been discussed individually at the end of each results chapter, and their relationship to the published literature. In addition, the influence of these conclusions on potential novel therapeutic interventions for cerebral malaria are discussed. Two molecular models of the PRBC surface, which are consistent with the data, are proposed in the second section, in conjunction with a selection of experiments that could be performed to test their validity.

8.1.1 Major Conclusions

- **Phenotypic heterogeneity**

At the simplest level, the findings in this thesis provide support for the process of clonal antigenic variation of *P. falciparum* at the biochemical level. This conclusion has been derived from two important results. Firstly, multiple molecules with the properties of PfEMP1 were observed at the surface following clonal expansion of *P. falciparum* infected erythrocytes. These proteins had identical molecular weight in clones that expressed similar relative antigenic phenotypes determined by the mixed agglutination assay. All the evidence obtained to date suggests that PfEMP1 is the only

antigen recognised in the agglutination assay and is associated uniquely with the antigenic phenotype of a culture. The high rate of antigenic switching (2.4% per generation) (Roberts *et al*, 1992) means that a clone rapidly becomes heterogeneous and PRBC with variant antigenic phenotypes (VATs) can be subcloned after a limited number of growth cycles (summaried in Figure 8.1). Each VAT was associated with the expression of a unique major PfEMP1 that was always of lower molecular weight than the major parental equivalent in this clone family (chapter 6). Previous studies in this laboratory had shown that antigenic switching was accompanied by a loss of ICAM1 binding (Roberts *et al.*, 1992). Therefore, the reduction in the size of PfEMP1 reported in this thesis, which was correlated with a switch to a non-ICAM1 binding phenotype, could be due the deletion of domains necessary for adhesion to this ligand. A similar result was found by Biggs and colleagues (1992) for PRBC that had switched to a non-HUVEC binding phenotype, and interaction of adhesive domains and antigenic epitopes has been suggested before (Roberts *et al.*, 1993).

The second important biochemical finding was that protease-resistant agglutination occurred in a variant specific fashion. Agglutination by immune serum of PRBC that expressed particular antigenic phenotypes was completely resistant to digestion with either trypsin or V8 protease. The low levels of resistance that were observed in other clones were a consequence of the high rate of switching, and the resistant subpopulations were selected from the parental clone, $A4_{i+}$, to prove this hypothesis. A4TRYI and A4V8CD36 expressed different antigenic phenotypes from the major phenotype of the starting population, $A4_{i+}$, and this was taken as further evidence of the heterogeneity of *P. falciparum* infected erythrocytes. The lines selected for protease-resistant adhesion each expressed a single major PfEMP1 that was present in the parental clone at a lower level and hence provided a biochemical correlate of the earlier phenotypic observations.

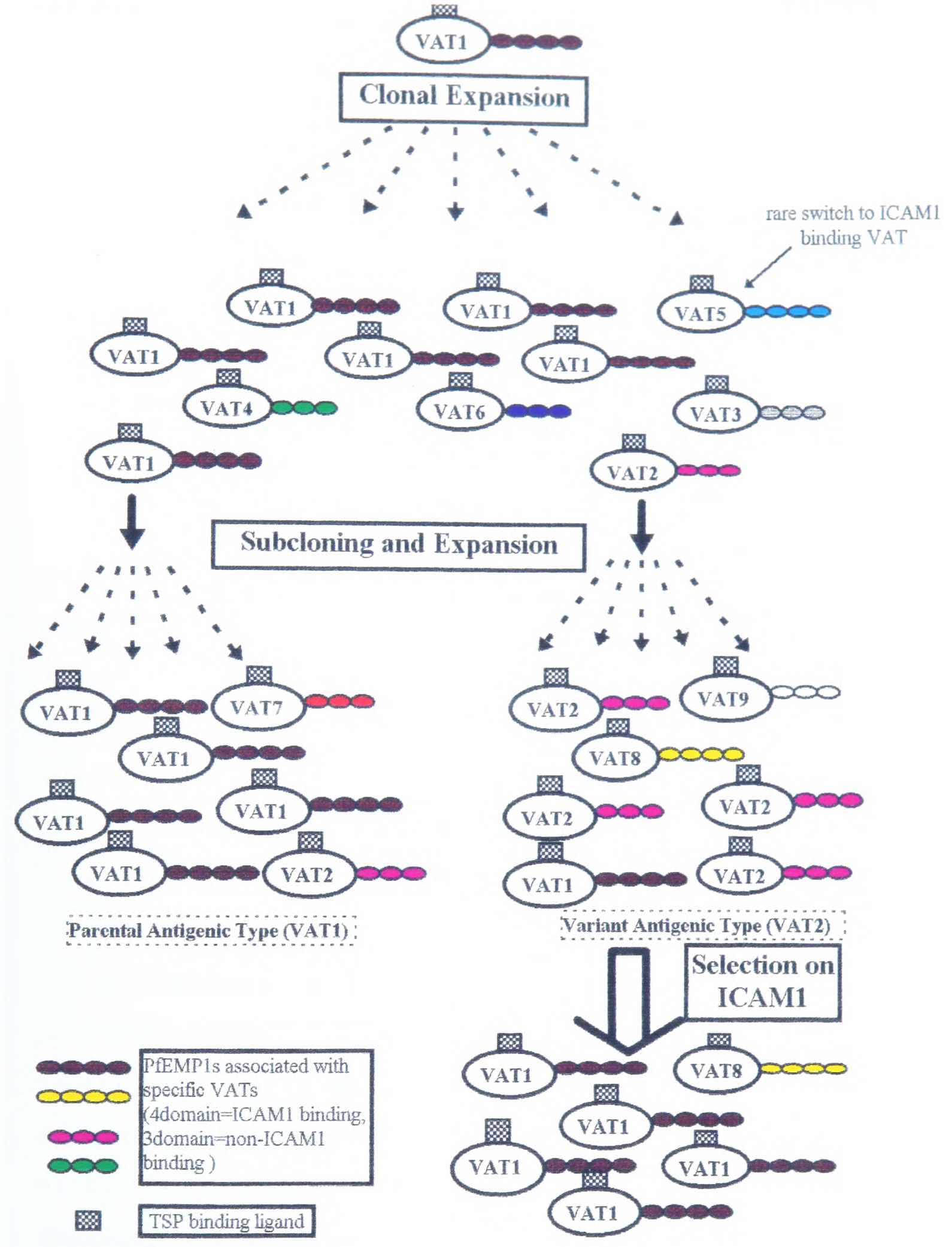


Figure 8.1. Model of effect of switching of PfEMP1 in clonal populations of PRBC. Each PfEMP1 is uniquely associated with a particular antigenic phenotype. VAT1 is the starting, parental phenotype which rapidly becomes heterogeneous on expansion. Subcloning this population can result in the appearance of a different VAT (right), or the re-appearance of the parental VAT (left). Switches to VATs associated with ICAM1 binding phenotypes are rare, therefore the parental VAT is usually enriched when a variant subclone is selected on ICAM1. Only one PfEMP1 per cell is shown for simplicity. The TSP receptor is separate from PfEMP1 at the PRBC surface.

- **A single PRBC can bind to CD36 and ICAM1**

An important conclusion from this work was that adhesion to CD36 and ICAM1 were properties of the same cell, which had not been demonstrated conclusively before. The observation that PRBC derived by selection on ICAM1 after trypsinisation, which ablated binding to CD36, and subsequently bound to both receptors after expansion, could only be explained by the presence of adhesive domains for each receptor on a single cell. This confirmed unpublished results in the laboratory which showed that PRBC which had been selected on CD36 could bind directly to ICAM1, and *vice versa*, without further cultivation (A. G. Craig and R. A. Pinches, personal communication).

A recent report by Chaiyaroj and colleagues (1994b) has demonstrated that multiple ligands for adhesion to C32 melanoma cells and HUVECs can be present on a single PRBC. They have shown that PRBC which were selected on C32 melanoma cells or HUVECs could bind to both cell lines and to purified CD36 and ICAM1. An interesting result was that selected cells could bind to C32 melanoma cells after trypsinisation, whereas adhesion to HUVECs, CD36 and ICAM1 was completely sensitive to the same protease. Multiple rounds of selection for trypsin-resistant binding to C32 melanoma cells produced a PRBC line which expressed a trypsin-resistant member of the PfEMP1 family and exhibited resistant or enhanced agglutination in the available immune sera. However, this parasite was not an equivalent of A4TRY-I because it did not bind to ICAM1 following trypsinisation. The balance of evidence suggests that the receptor for trypsin-resistant binding to C32 melanoma cells is an N-linked oligosaccharide (Chaiyaroj *et al.*, 1994b). This report emphasises the diversity of phenotypes at the PRBC surface, but the use of whole cell models adds a further level of complexity to the interpretation of results. The major drawback of this approach is that no firm conclusions can be made regarding the ability of single PRBC to bind to known individual cytoadherent receptors. Therefore, the authors are incorrect to assume that a single cell could bind to CD36 and ICAM1, although their evidence supports earlier observations that other receptors for cytoadherence must exist (Udeinya and Akogyeram, 1993; Chaiyaroj *et al.*, 1994a).

- **A single molecule (PfEMP1) mediates PRBC agglutination and cytoadherence to CD36 and ICAM1**

All of the results in this study were consistent with the reported linkage of particular antigenic and adhesive phenotypes expressed at the PRBC surface (Biggs *et al.*, 1992; Roberts *et al.*, 1992). The general observation that protease-resistant binding to either receptor was universally observed with resistant agglutination was strong evidence that they were closely-related functions. The fact that protease treatment could not separate agglutination from binding to either CD36 or ICAM1, and resistant phenotypes were variant-specific, implied that these functions were mediated by PfEMP1.

Furthermore, the onset of agglutination coincided exactly with the ability of PRBC to bind to CD36 and ICAM1, which was further evidence that these functions were comodulated. The timing of expression of PfEMP1 by surface labelling was not attempted, but could be measured more readily with monoclonal antibodies or other monospecific reagents.

Binding to CD36 was a constant property of all clones that was independent of antigenic phenotype and reversed by approximately 83% (n = 11) by antibodies in polyclonal immune serum. This indicated that the binding domain for CD36 was in close apposition to, but not identical to, antibody binding sites and could be mediated by a conserved domain expressed by all PfEMP1s irrespective of molecular weight.

The most persuasive evidence that adhesion to CD36 was mediated by PfEMP1 was obtained by surface labelling PRBC selected for V8 protease-resistant binding to CD36. This line (A4V8CD36) expressed a PfEMP1 with identical characteristics to PRBC from a variant clone, $C18_{i-}$, which exhibited complete V8 protease-resistant agglutination and adhesion to CD36. A4V8CD36 and $C18_{i-}$ expressed a 175kDa protein with the characteristics of PfEMP1 after V8 protease digestion that was a proteolytic fragment of PfEMP1. An identical 175kDa protein was also present at low levels, due to the high rate of antigenic switching, in other clones that showed partial V8 protease-resistant binding to CD36.

These results contradict findings by Crandall and colleagues (1994) who have maintained for the past 6 years that a modified form of Band 3 was responsible for adhesion to CD36 (reviewed by Sherman,

1990). This now seems unlikely for three reasons. Firstly, V8 protease-resistant adhesion to CD36 was variant specific and associated uniquely with changes to PfEMP1. There were no observable differences in the properties of Band 3, which was sensitive to V8 protease in all clones tested. Secondly, adhesion to CD36 was sensitive to trypsin in all PRBC clones, but Band 3 was resistant to the same protease. Finally, results using identical reagents based on modified forms of Band 3 produced by Dr. Sherman's laboratory (Crandall and Sherman, 1994) could not be repeated using PRBC under standardised conditions in Oxford (Dr. D. J. Roberts and P. Warn, personal communications). These reagents included putative monoclonal antibodies to modified Band 3, and cryptic regions of Band 3 that were reported to inhibit PRBC adhesion to CD36 (reviewed by Land *et al.*, 1995). Taken together, the above observations are strong evidence that modified forms of Band 3 are not involved in mediating PRBC adhesion to CD36 and further controversial issues in this debate are summarised by Newbold and Marsh, 1990.

On the other hand, PfEMP1 correlated exactly with the variant-specific protease-resistant adhesion to CD36, and it was concluded that the epitopes for agglutination and adhesive domains for CD36 were on the same molecule. A similar argument can be applied to the trypsin-resistant adhesion to ICAM1 which was associated with a particular VAT (A4TRY-I), and strongly implied that this function was mediated by PfEMP1.

Two indirect observations suggested that there was only one form of PfEMP1 expressed at the PRBC surface. Firstly, earlier observations that cloned PRBC expressed particular VATs which were different from each other implies that a single PRBC is capable of expressing only one form of antigenic epitope at one time. This was confirmed by the very low numbers of mixed agglutinates when the same variant clones were tested against other variant clones (eg. $C18_{i-}$ *vs* $C24_{i-}$) (Roberts *et al.*, 1992). The second indirect observation was that variant clones expressed a single PfEMP1 which was correlated with the expression of a particular VAT. If there were multiple forms of PfEMP1 on the PRBC surface, it would be likely that several molecules with the characteristics of PfEMP1 would be labelled in all clones. This was not the case, and only ICAM1 binding PRBC expressed multiple PfEMP1-like molecules which was attributed to the high rate of clonal variation. Therefore, the

simplest explanation was that agglutination, and adhesion to CD36 and ICAM1 were mediated by PfEMP1 and only one form of this molecule was present on the PRBC surface in each cycle.

This paradigm assists the interpretation of the findings from the extensive phenotypic analysis of the range of clones investigated in this thesis. The inability of variant clones to bind to ICAM1 is due to the loss of an adhesive domain, and this correlated with the low molecular weight forms of PfEMP1 associated with this phenotype (Figure 8.1). Selection of variant PRBC on purified ICAM1 restores the ICAM1 binding function and simultaneously selects for PRBC which express high molecular weight forms of PfEMP1. A single 270kDa member of the PfEMP1 family was uniquely associated with the high-ICAM1 binding phenotype associated with the IT6VAT, which was interpreted as further evidence that this molecule was central to the antigenic and adherent phenotypes at the PRBC surface.

Results from protease digestion suggested that the domain for adhesion to ICAM1 on PfEMP1 was proximal to the domain for CD36 binding in all clones. Paradoxically, trypsin-resistant binding to ICAM1 was not associated with a PfEMP1-like molecule with similar protease sensitivity on surface labelled PRBC. An explanation for this phenomenon is that in PRBC which express these VATs, iodinated tyrosine residues on PfEMP1 are cleaved by trypsin. Subsequent surface labelling post-trypsinisation identified a 25kDa variant specific surface protein which was a candidate for the trypsin-resistant domain(s) for adhesion to ICAM1.

The single molecule hypothesis can be used to explain the different levels of binding to CD36 and ICAM1. These phenotypes could be attributed to **(1)** sequence variation in binding sites for these receptors on PfEMP1, **(2)** copy number of PfEMP1, or **(3)** heterogeneity of PfEMP1s present in PRBC populations measured due to the high switch rate. A combination of all three possibilities probably operates, and can be used to explain the different levels of adhesion to putative cytoadherent receptors. Selected PRBC, e.g. A4V8CD36 and A4TRY-I, from the same PRBC clone ($A4_{i+}$) bound to CD36 at different levels, which implied that different PfEMP1 molecules vary in their capacity to bind to this receptor. Non-CD36 binding PRBC were absent from the IT clone tree, and selection for adhesion to ICAM1 sometimes reduced the levels of binding to CD36, as shown by C28-I*, but not C18-I(2). This

was probably due to the relative enrichment of a population of PRBC in C28-I* which could bind to both receptors rather than only to CD36.

The majority of PRBC bind to ICAM1 at lower levels than to CD36 which may be due to the proposed domain architecture of PfEMP1. The location of ICAM1 adhesive domains on PfEMP1 close to the membrane may not afford an optimal receptor-ligand environment whereas adhesive domains for CD36 are predicted to be distal to these regions and may be more accessible. The levels of reversal of adhesion to ICAM1 by antibodies in immune serum support this hypothesis. The intermediate levels (~40%, n = 8) of reversal of adhesion to ICAM1 in $A4_{i+}$ and A4TRY-I can be attributed to the spatial constraints conferred by this domain architecture. Trypsin digestion cleaves many proteins from the PRBC surface, including the CD36 binding domains from PfEMP1, and therefore allows greater access of antibody to the ICAM1 adhesive domains. This explains the high levels (~80 - 90%) of reversal of trypsin-resistant adhesion to ICAM1 in the same PRBC.

- **PRBC adhesion to TSP is a separate surface function at the PRBC surface**

The argument for binding to TSP being mediated by PfEMP1 is not strong. Certain PRBC which did not form agglutinates after protease digestion could still bind to TSP at control levels. Also, untreated PRBC with variable antigenic phenotypes bound at similar levels to TSP, and two sequence specific proteases had a constant effect on adhesion. Taken together, these results suggested that adhesion to TSP was unrelated to the antigenic phenotype of the culture and this hypothesis was supported by the inability of antibodies in polyclonal immune serum to reverse adhesion to this receptor. This could not be attributed to the reduced access of antibody to the receptor binding site, because immune serum had no effect on adhesion of protease-digested PRBC to TSP.

There were several PRBC surface molecules that fulfilled the biochemical criteria for the TSP receptor, which must express constant domains to account for the conserved function. Three alternative explanations are possible: (1) a modified host protein is involved, (2) a novel conserved parasite-derived protein could be responsible, or (3) a conserved domain on PfEMP1 may mediate adhesion to TSP. Modified Band 3 was a strong candidate if situation (1) was true and unpublished immunoprecipitation results with labelled PRBC extracts indicated clearly that Band 3 was one of

several surface proteins (including PfEMP1) that bound to immobilised TSP (Dr. D. Baruch, personal communication). A novel, unidentified parasite-derived protein would fulfill the criteria for (2), but none were apparent from this study, and few parasite derived proteins are believed to be surface-exposed (Howard, 1988).

In order to explain the effect of proteases, if (3) was correct, the TSP adhesive domain on PfEMP1 would have to be closer to the membrane than domains for adhesion to ICAM1 and CD36. However, assuming that a single molecule mediates adhesion to the three receptors, it is difficult to explain how trypsin reduces adhesion to TSP, but has no effect on binding to ICAM1 in PRBC from A4TRY-I. In addition, a complex mechanism would be required to account for the early appearance of the TSP binding phenotype before the PRBC can bind to CD36, ICAM1 and form agglutinates.

Therefore, the balance of evidence suggests that an invariant protein mediated PRBC adhesion to TSP. This was confirmed by selection of a single PRBC clone ($C24_{i-}$) on TSP; there was no change to the major antigenic phenotype expressed by the cells which bound to this receptor. This result was in contrast to the observed switch in antigenic phenotype expressed when the same PRBC clone had been selected on ICAM1 (C24-I). Therefore, adhesion to TSP was a constant function of the infected cell surface and was probably unrelated to the antigenic phenotype of the cell. PRBC removed immediately from TSP-coated dishes could bind directly to CD36 which proved that the receptor(s) for TSP and CD36 (and, by inference, ICAM1) were expressed by a single cell.

- **Adhesion to ICAM1 is restricted to a limited number of VATs**

Multiple pathways exist to allow the PRBC to adhere to different receptors which may be expressed at variable levels in different organs in different patients. Sequestration has three advantages for the parasite. Firstly, it protects the infected erythrocyte from destruction by splenic filtration mechanisms. Secondly, sequestration facilitates growth under optimal hypoxic conditions and thirdly the obstruction caused by adherent parasites in vessels reduces the blood flow to specific organs and facilitates reinvasion by minimising diffusion times for merozoites. Therefore, the capacity of PRBC to adhere to at least five different receptors signifies the importance of sequestration to the parasite.

Several studies have implicated adhesion to ICAM1 as a central event in the development of severe disease (Turner *et al.*, 1994; Dr. C. I. Newbold, personal communication). The cytokine-induced upregulation of expression of this receptor in cerebral microvessels suggests that ICAM1 binding may be a parasite virulence factor (reviewed by Berendt, 1994). The finding that the antigenic phenotypes associated with ICAM1 binding PRBC in this clone family was relatively restricted may be relevant in clinical disease. If ICAM1 binding parasites represent a virulent subpopulation in natural infections, and switches to ICAM1 binding phenotypes are rare (as implied by this study), then this may in part explain the rarity of severe disease in comparison to the total number of infections. The apparent restriction of ICAM1 adhesive phenotypes to a limited number of VATs raises the intriguing possibility of designing a set of reagents that can inhibit binding to all ICAM1 binding PRBC *in vivo*.

In this respect, characterisation of ICAM1 binding domains on PfEMP1 is essential, because it could lead to the design of anti-adhesion peptides. This is a realistic possibility that could lead to the inhibition or reversal of adhesion to this ligand and consequently reduce the numbers of sequestered PRBC in vital organs. Approaches of this kind may provide much-needed therapeutic interventions for the treatment of severe malaria in those individuals who are most at risk (e.g. young children in endemic areas). By reversing sequestration, successful anti-adhesion therapy would alleviate the symptoms of coma associated with cerebral malaria, and allow treatment with available anti-malarial drugs.

8.1.2 Models of PRBC Surface Molecules

Figure 8.1 shows the consequence of the high rate of antigenic variation of PfEMP1 on a clonal PRBC population. After few cycles *in vitro*, multiple forms of PfEMP1 will be present on different cells, and subcloning and expansion of individual PRBC at this stage will result in the enrichment of particular variant antigenic types (VATs). The constant molecule for TSP adhesion is unaffected by switching of PfEMP1 and this explains the conserved features of this phenotype in PRBC with different VATs.

The above conclusions allow a model of the PRBC surface to be proposed that predict a domain architecture for the molecules which mediate agglutination and binding to putative receptors. In the absence of sequence data, the following hypothesis is based on three assumptions that were obtained from indirect functional experiments in this study: first, PfEMP1 mediates PRBC agglutination via epitopes which are recognised by antibodies in immune serum. Second, PfEMP1 contains separate domains which mediate adhesion to both CD36 and ICAM1. Third, adhesion to TSP is mediated by a separate invariant surface molecule that is either a parasite-derived protein or a modified host protein.

Figure 8.2 shows the predicted domain architecture of PfEMP1 within a number of PRBC with different VATs. Expression of a particular VAT is associated with the presence of variant-specific epitopes along the molecule, which are recognised by antibodies in immune serum that mediate agglutination and reverse adherence to CD36 and ICAM1. The minimum number of cleavage sites for trypsin and V8 protease are indicated, and the possible sites of accessible tyrosine residues labelled by the lactoperoxidase addition of ^{125}I are shown. The models are consistent with the protease sensitivity results (summarised in table 7.1), antibody reversal experiments, and labelling profiles of PRBC before and after digestion. It should be noted that the order of domains and epitopes has been determined only by protease analysis and antibody reversal experiments. Therefore, these predictions do not account for patterns of protein folding which partially determine the secondary and tertiary structure of proteins. Consequently, many other conformations exist other than a single-spanning membrane protein depicted in the models, and it is unlikely that this simplified architecture occurs *in vivo*.

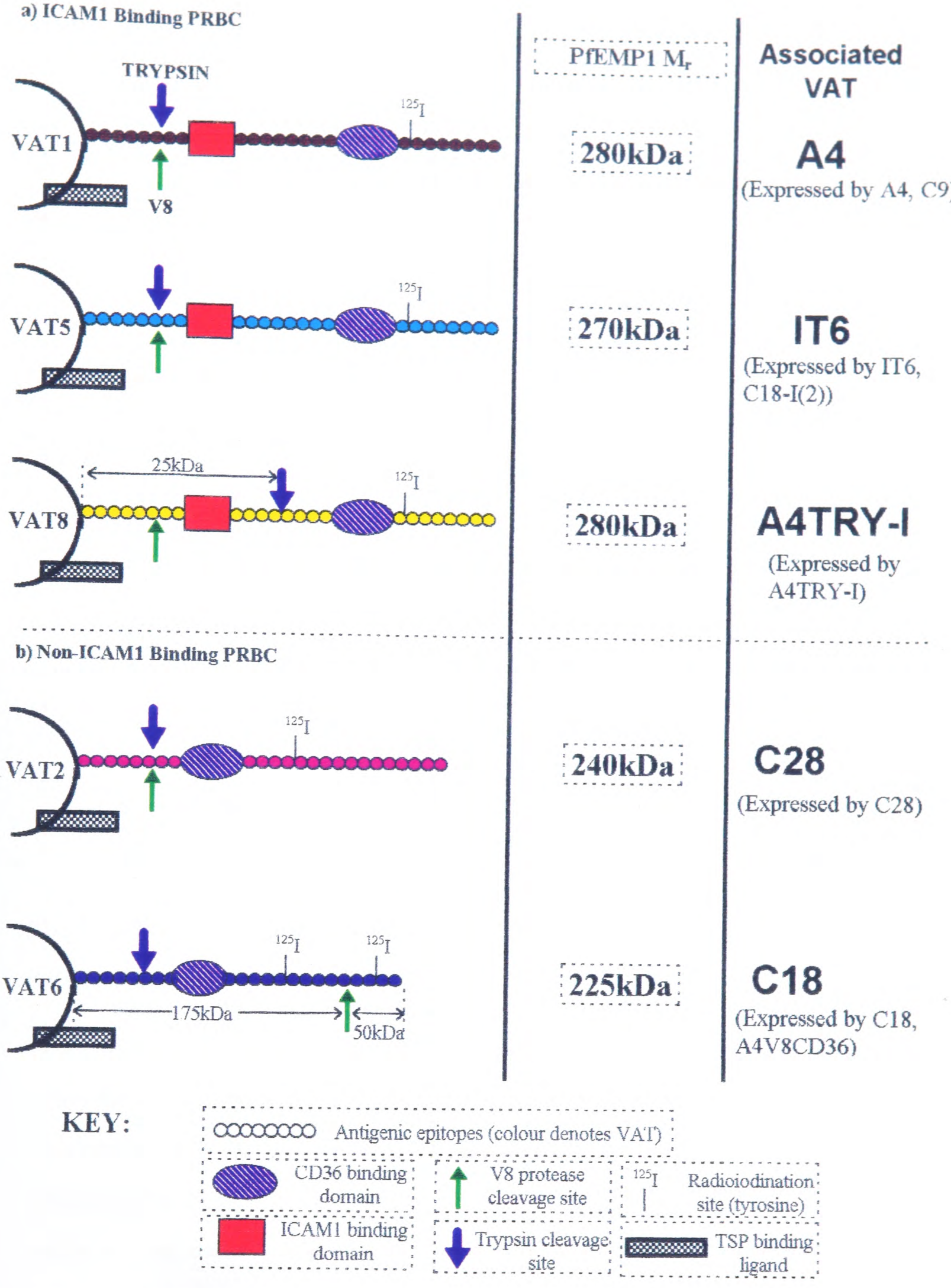


Figure 8.2. Domain model of PfEMP1 in particular variant antigen types within the IT clone tree (not to scale). Antigenic epitopes are recognised by antibodies in immune serum in agglutination assays and cytoadherence reversal experiments. A single site for each protease is depicted for simplicity and summarised in Table 7.1.

The genetic basis of these phenotypes is one of the most rapidly advancing areas of current malaria research. For some time, it was believed that the gene(s) encoding PfEMP1, or a region essential for the expression of PfEMP1, were located on the right arm of chromosome 9, and the majority of this region has now been cloned (Day *et al.*, 1993; Barnes *et al.*, 1994). Recently, it has been reported that a cloned parasite with a deletion of this region of chromosome 9 expressed a trypsin-resistant cytoadherence phenotype (Chaiyaroj *et al.*, 1994a), and this study implied the existence of at least two distinct genetic loci for cytoadherence.

Unpublished data presented at recent meetings have clarified this situation and have identified a family of genes encoding PfEMP1 (T. E. Wellems, BSP Symposium, Liverpool, 1994; T. E. Wellems and D. Baruch; Festschrift for Dr. Neil Brown, The Wellcome Trust, London; 1994). In brief, it appears that there are members of a multi-gene family (termed *var*) located on, and expressed from, several chromosomes of *P. falciparum* which encode diverse proteins with predicted homologies to the Duffy binding domains (DBD) of *P. knowlesi* (Miller *et al.*, 1975). Reverse transcriptase PCR with degenerate primers to semi-conserved regions of *var* genes has been used to demonstrate that variant clones derived from $A4_{i+}$ showed a range of different RTPCR products, whereas PRBC which expressed the parental A4VAT had the same RTPCR product as $A4_{i+}$. The level of variant-specific transcripts in this clone family agreed with the antigenic similarity measured by the mixed agglutination assay. Genomic sequencing shows that each *var* gene has between 1 - 4 copies of the DBD sequence, and if these regions correspond to CD36 binding domains then this fits exactly with the protease data presented in this thesis.

To date, no functional studies have been performed on the cloned members of the *var* gene family. Indirect fluorescence antigen tests (IFAT) have demonstrated that *var* gene products are transported to the infected erythrocyte surface, and the genes are predicted to encode proteins of 250 - 300kDa (T. E. Wellems, BSP Symposium, Liverpool, 1994). Functional studies of cloned sequences of PfEMP1 will soon be underway to identify regions responsible for the implicated cytoadherent phenotypes. One approach would be to analyse the ability of recombinant PfEMP1 regions expressed in the baculovirus-insect system to mediate cytoadherence to specific receptors. Experiments of this kind will provide a stringent test for the domain models proposed in figure 8.2, and will directly determine the

functional role of different regions of PfEMP1. One potentially confounding factor in these studies is that PfEMP1 is a membrane protein and is likely to have regions of hydrophobic amino acids. These regions will interact non-specifically with receptor proteins when exposed in solution, and will also be difficult to crystallise. If these approaches are successful, such knowledge will be applied to a crystal structure of PfEMP1 which will facilitate the design of vaccine components and novel reagents for anti-adhesion therapy.

References

Aguiar, J. C., G. R. Albrecht, P. Cegielski, B. M. Greenwood, J. B. Jensen, G. Lallinger, A. Martinez, I. A. McGregor, J. N. Minjas, and J. Neequaye. "Agglutination of *Plasmodium falciparum*-infected erythrocytes from East and West African isolates by human sera from distant geographic regions." Am J Trop Med Hyg 47 (1992): 621-32.

Ahlborg, N., K. Berzins, and P. Perlmann. "Definition of the epitope recognized by the *Plasmodium falciparum*-reactive human monoclonal antibody 33G2." Mol Biochem Parasitol 46 (1991): 89-95.

Aikawa, M. "Human cerebral malaria." Am J Trop Med Hyg 39 (1988): 3-10.

Aikawa, M., A. Brown, C. D. Smith, T. Tegoshi, R. J. Howard, T. H. Hasler, Y. Ito, G. Perry, W. E. Collins, and K. Webster. "A primate model for human cerebral malaria: *Plasmodium coatneyi*-infected rhesus monkeys." Am J Trop Med Hyg 46 (1992): 391-7.

Aikawa, M., M. Iseki, J. W. Barnwell, D. Taylor, M. M. Oo, and R. J. Howard. "The pathology of human cerebral malaria." Am J Trop Med Hyg 43 (1990a): 30-7.

Aikawa, M., G. Jacobs, H. E. Whiteley, I. Igarashi, and M. Ristic. "Glomerulopathy in squirrel monkeys with acute *Plasmodium falciparum* infection." Am J Trop Med Hyg 38 (1988): 7-14.

Aikawa, M., M. Torii, A. Sjolander, K. Berzins, P. Perlmann, and L. H. Miller. "Pf155/RESA antigen is localized in dense granules of *Plasmodium falciparum* merozoites." Exp Parasitol 71 (1990b): 326-9.

Alger, N. E. "Distribution of schizonts of *Plasmodium berghei* in tissues in rats, mice and hamsters." J. Protozool. 10 (1963): 6-10.

Allan, R. J., A. Rowe, and D. Kwiatkowski. "*Plasmodium falciparum* varies in its ability to induce tumor necrosis factor." Infect Immun 61 (1993): 4772-6.

Alonso, P. L., S. W. Lindsay, J. R. Armstrong, M. Conteh, A. G. Hill, P. H. David, G. Fegan, A. de-Francisco, A. J. Hall, F. C. Shenton, *et al.* "The effect of insecticide-treated bed nets on mortality of Gambian children [see comments]." Lancet 337 (1991): 1499-502.

Alonso, P. L., M. Tanner, T. Smith, R. J. Hayes, J. A. Schellenberg, M. C. Lopez, I. Bastos-de-Azevedo, C. Menendez, E. Lyimo, and N. Weiss. "A trial of the synthetic malaria vaccine SPf66 in Tanzania: rationale and design." Vaccine 12 (1994a): 181-6.

Alonso, P. L., T. Teuscher, and M. Tanner. "SPf66: research for development or development of research?" Vaccine 12 (1994b): 99-101.

Anders, R, D. J. McColl, and R. L. Coppel. "Molecular variation in *Plasmodium falciparum*: polymorphic antigens of asexual erythrocytic stages." Acta Tropica 53 (1993): 239-253.

Anders, R. F., G. V. Brown, R. L. Coppel, and D. J. Kemp. "Induction of protective immunity to malaria." Lepr Rev 2 (1986): 245-53.

Anders, R. F. and J. A. Smythe. "Polymorphic antigens in *Plasmodium falciparum*." Blood 74 (1989): 1865-75.

Anderson, J.M. "Structural studies on human spectrin: comparison of subunits and fragmentation of native spectrin." Journal of Biological Chemistry 254 (1979): 939-944.

Anstee, D. "Blood group-active surface molecules of the human red blood cell." Vox Sang 58 (1990): 1-20.

Armstrong-Schellenberg, J.R.M., T. Smith, P.L. Alonso, and R.J. Hayes. "What is clinical malaria? Finding case definitions for field research in highly endemic areas." Parasitology Today 10 (1994): 439-442.

Arnot, D.E., C. Roper, and A.A. Sultan. "MVR-PCR analysis of hypervariable DNA sequence variation." Parasitology Today 10 (1994): 324-327.

Asch, A.S., J. Barnwell, R.L. Silerverstein, and R.L. Nachman. "Isolation of the thrombospondin membrane receptor." Journal of Clinical Investigation 79 (1987): 1054-1061.

Asch, A. S. and R. L. Nachman. "Thrombospondin: phenomenology to function." Prog Hemost Thromb 9 (1989): 157-76.

Bannister, L. H. and A. R. Dluzewski. "The ultrastructure of red cell invasion in malaria infections: a review." Blood Cells 16 (1990): 257-92.

Barnes, D.A., J. Thompson, T. Triglia, K. Day, and D.J. Kemp. "Mapping the genetic locus implicated in cytoadherence of *Plasmodium falciparum* to melanoma cells." Molecual and Biochemical Pharmacology 66 (1994): 21-29.

Barnwell, J. W. "Cytoadherence and sequestration in falciparum malaria." Exp Parasitol 69 (1989): 407-12.

Barnwell, J. W., A. S. Asch, R. L. Nachman, M. Yamaya, M. Aikawa, and P. Ingravallo. "A human 88-kD membrane glycoprotein (CD36) functions in vitro as a receptor for a cytoadherence ligand on *Plasmodium falciparum*-infected erythrocytes." J Clin Invest 84 (1989): 765-72.

Barnwell, J.W., R.J. Howard, H.G. Coon, and L.H. Miller. "Splenic requirement for antigenic variation and expression of the variant antigen on the erythrocyte membrane in cloned *Plasmodium knowlesi* malaria." Infect. Immun. 40 (1983): 985-994.

Barnwell, J. W., C. F. Ockenhouse, and D.M. Knowles. "Monoclonal antibody OKM5 inhibits the in vitro binding of *Plasmodium falciparum*-infected erythrocytes to monocytes, endothelial, and C32 melanoma cells." J Immunol 135 (1985): 3494-7.

Bastianelli, G., A. Bignami, and B. Grassi. "Coltivazione delle semilune malariche dell'uomo nell *Anopheles* claviger." Atti della Reale Accademia dei Lincei 55 (1898): 313-317.

Bate, C. A., J. Taverne, E. Roman, C. Moreno, and J. H. Playfair. "Tumour necrosis factor induction by malaria exoantigens depends upon phospholipid." Immunology 75 (1992): 129-35.

Berendt, A. R. "Sequestration and its discontents: infected erythrocyte-endothelial cell interactions in *Plasmodium falciparum* malaria." Res Immunol 144 (1993): 740-5.

Berendt, A. R., D. J. Ferguson, J. Gardner, G. Turner, A. Rowe, C. McCormick, D. Roberts, A. Craig, R. Pinches, B. C. Elford, and C. I. Newbold. "Molecular mechanisms of sequestration in malaria." Parasitology 108 (1994a): S19-28.

Berendt, A. R., A. McDowall, A. G. Craig, P. A. Bates, M. J. Sternberg, K. Marsh, C. I. Newbold, and N. Hogg. "The binding site on ICAM-1 for *Plasmodium falciparum*-infected erythrocytes overlaps, but is distinct from, the LFA-1-binding site." Cell 68 (1992): 71-81.

Berendt, A. R., D. L. Simmons, J. Tansey, C. I. Newbold, and K. Marsh. "Intercellular adhesion molecule-1 is an endothelial cell adhesion receptor for *Plasmodium falciparum*." Nature 341 (1989): 57-9.

Berendt, A. R., G. D. H. Turner, and C. I. Newbold. "Cerebral malaria: the sequestration hypothesis." Parasitology Today 10 (1994b): 412-414.

Berzins, K., H. Perlmann, B. Wahlin, J. Carlsson, M. Wahlgren, R. Udomsangpetch, A. Bjorkman, M. E. Patarroyo, and P. Perlmann. "Rabbit and human antibodies to a repeated amino acid sequence of a *Plasmodium falciparum* antigen, Pf 155, react with the native protein and inhibit merozoite invasion." Proc Natl Acad Sci U S A 83 (1986): 1065-9.

Bevilacqua, M.P. and R.M. Nelson. "Selectins." Journal of Clinical Investigation 91 (1993): 379-387.

Biggs, B.-A., R. F. Anders, H. E. Dillon, K. M. Davern, M. Martin, C. Petersen, and G. V. Brown. "Adherence of infected erythrocytes to venular endothelium selects for antigenic variants of *Plasmodium falciparum*." The Journal of Immunology 149 (1992): 2047-2054.

Biggs, B.-A., J. G. Culvenor, J. S. Ng, D. J. Kemp, and G. V. Brown. "*Plasmodium falciparum*: cytoadherence of a knobless clone." Exp Parasitol 69 (1989): 189-97.

Biggs, B.-A., L. Gooze, K. Wycherley, D. Wilkinson, A. W. Boyd, K. P. Forsyth, L. Edelman, G. V. Brown, and J. H. Leech. "Knob-independent cytoadherence of *Plasmodium falciparum* to the leukocyte differentiation antigen CD36." J Exp Med 171 (1990): 1883-92.

Biggs, B.-A., L. Gooze, K. Wycherley, W. Wollish, B. Southwell, J. H. Leech, and G. V. Brown. "Antigenic variation in *Plasmodium falciparum*." Proc Natl Acad Sci U S A 88 (1991): 9171-4.

Blum, H., H. Beier, and H.J. Gross. "Improved silver staining of plant proteins, RNA and DNA by polyacrylamide gels." Electrophoresis 8 (1987): 93-99.

Blum, J.J. and H. Ginsburg. "Absence of α-ketoglutarate dehydrogenase activity and presence of CO_2-fixing activity in *Plasmodium falciparum* grown in vitro in human erythrocytes." Journal of Protozoology 31 (1984): 167-169.

Borst, P. "Molecular genetics of antigenic variation." Immunoparasitology Today 12 (1991): A29-A33.

Bosia, A., D. Ghigo, F. Turrini, E. Nissani, G. P. Pescarmona, and H. Ginsburg. "Kinetic characterization of Na^+/H^+ antiport of *Plasmodium falciparum* membrane." J Cell Physiol 154 (1993): 527-34.

Boyd, M.F. "Observations on naturally and artifically induced quartan malaria." American Journal of Tropical Medicine 20 (1940): 749-798.

Bradley, A. K., B. M. Greenwood, A. M. Greenwood, K. Marsh, P. Byass, S. Tulloch, and R. Hayes. "Bed-nets (mosquito-nets) and morbidity from malaria." Lancet 2 (1986): 204-7.

Bray, R.S. and P.C. Garnham. "Life-cycle of primate malaria parasites." British Medical Bulletin 38 (1982): 117-123.

Brewster, D. R., D. Kwiatkowski, and N. J. White. "Neurological sequelae of cerebral malaria in children." Lancet 336 (1990): 1039-1043.

Brooks, D. R. and D. A. McLennan. "The evolutionary origin of *Plasmodium falciparum*." J Parasitol 78 (1992): 564-6.

Brown, I. N., A. C. Allison, and R. B. Taylor. "*Plasmodium berghei* infections in thymectomized rats." Nature 219 (1968): 292-293.

Brown, K. N. "Protective immunity to malaria provides a model for the survival of cells in an immunologically hostile environment." Nature 230 (1971): 163-167.

Brown, K. N. "Antibody induced variation in malaria parasites." Nature 242 (1973): 49-50.

Brown, K. N. and I. N. Brown. "Immunity to malaria : antigenic variation in chronic infections of *Plasmodium knowelsi*." Nature 208 (1965): 1286-1288.

Brown, K.N., I.N. Brown, and L.A. Hills. "Immunity to malaria: I. Protection against *Plasmodium knowlesi* shown by monkeys sensitized with drug-suppressed infections or by dead parasites in Freund's adjuvant." Experimental Parasitology 28 (1970a): 304-317.

Brown, K. N., I. N. Brown, P. I. Trigg, R. S. Phillips, and L. A. Hills. "Immunity to malaria II. Serological response of monkeys sensitised by drug-suppressed infected or parasitised cell in Freund's complete adjuvant." Exp. Parasitol. 28 (1970b): 318-338.

Bruce, M. C., P. Alano, S. Duthie, and R. Carter. "Commitment of the malaria pararsite *Plasmodium falciparum* to sexual and asexual development." Parasitology 100 (1990): 191-200.

Bruce-Chwatt, L.J., de Zuleta,J. The Rise and Fall of Malaria in Europe. Oxford: Oxford University Press, 1980.

Carlson, J., H. Helmby, A. V. Hill, D. Brewster, B. M. Greenwood, and M. Wahlgren. "Human cerebral malaria: association with erythrocyte rosetting and lack of anti-rosetting antibodies." Lancet 336 (1990): 1457-60.

Chaiyaroj, S.C., R.L. Coppel, C. Magowan, and G.V. Brown. "A *Plasmodium falciparum* isolate with a chromosome 9 deletion expresses a trypsin-resistant cytoadherence molecule." Molecular and Biochemical Parasitology 67 (1994a): 21-30.

Chaiyaroj, S.C., R.L. Coppel, S. Novakovic, and G.V. Brown. "Multiple ligands for cytoadherence can be present simultaneously on the surface of *Plasmodium falciparum*-infected erythrocytes." Proceedings of the National Academy of Sciences, USA 91 (1994b): 10805-10808.

Chin, W., P.G. Contacos, G.R. Coatney, and H.R. Kimball. "A naturally acquired quotidian-type malaria in man transferrable to monkeys." Science 149 (1965): 865-867.

Chulay, J. D. and C. F. Ockenhouse. "Host receptors for malaria-infected erythrocytes." Am J Trop Med Hyg 43 (1990): 6-14.

Ciuca, M., L. Ballif, and M. Chelarescu-Vieru. "Immunity in malaria." Transactions of the Royal Society of Tropical Medicine and Hygeine 27 (1934): 619-622.

Ciuca, M., L. Ballif, and M. Vieru. "Immunite dans le paludisme experimentale." Archive Roumaines de Pathologie Experimentale et de Microbiologie 3 (1930): 209-229.

Clark, I. A. "Suggested importance of monokines in pathophysiology of endotoxin shock and malaria." Klin Wochenschr 60 (1982): 756-8.

Clark, I. A., W. B. Cowden, and G. A. Butcher. "Free oxygen radical generators as antimalarial drugs." Lancet i (1983): 234.

Clark, I. A., W. B. Cowden, and K. A. Rockett. "The pathogenisis of human cerebral malaria." Parasitology Today 10 (1994): 417-418.

Clark, I. A. and N. H. Hunt. "Evidence for reactive oxygen intermediates causing hemolysis and parasite death in malaria." Infection and Immunity 39 (1983): 1-6.

Clark, I. A. and K. A. Rockett. "The cytokine theory of human cerebral malaria." Parasitology Today 10 (1994): 410-412.

Clark, I. A., K. A. Rockett, and W. B. Cowden. "Proposed link between cytokines, nitric oxide and human cerebral malaria." Parasitology Today 7 (1991): 205-207.

Clark, I. A., K. A. Rockett, and W. B. Cowden. "Possible central role of nitric oxide in conditions clinically similar to cerebral malaria." Lancet 340 (1992): 894-6.

Clemetson, K.J., A. Capitanio, F.L. Pareti, J.L. McGregor, and E.F. Luscher. "Additional platelet membrane glycoprotein abnormalities in Glanzmann's thrombasthenia: A comparison with normals by high resolution two-dimensional polyacrylamide gel electrophoresis." Thrombin Research 18 (1980): 797.

Cohen, S., I. A. McGregor, and S. Carrington. "Gamma-globulin and acquired immunity to human malaria." Nature 192 (1961): 733-737.

Collins, F. H., R. K. Sakai, K. D. Vernick, S. Paskewitz, D. C. Seeley, L. H. Miller, W. E. Collins, C. C. Campbell, and R. W. Gwadz. "Genetic selection of a Plasmodium-refractory strain of the malaria vector *Anopheles gambiae*." Science 234 (1986a): 607-10.

Collins, W. E., R. F. Anders, M. Pappaioanou, G. H. Campbell, G. V. Brown, D. J. Kemp, R. L. Coppel, J. C. Skinner, P. M. Andrysiak, and J. M. Favaloro. "Immunization of *Aotus* monkeys with recombinant proteins of an erythrocyte surface antigen of *Plasmodium falciparum*." Nature 323 (1986b): 259-62.

Cooke, B.M., A.R. Berendt, A.G. Craig, J. MacGregor, C.I. Newbold, and G.B. Nash. "Rolling and stationary cytoadhesion of red bloods cells parasitized by *Plasmodium falciparum:* separate roles for ICAM-1, CD36 and thrombospondin." British Journal of Haematology 87 (1994): 162-170.

Corcoran, L. M., K. P. Forsyth, A. E. Bianco, G. V. Brown, and D. J. Kemp. "Chromosome size polymorphisms in *Plasmodium falciparum* can involve deletions and are frequent in natural parasite populations." Cell 44 (1986): 87-95.

Corcoran, L. M., J. K. Thompson, D. Walliker, and D. J. Kemp. "Homologous recombination within subtelomeric repeat sequences generates chromosome size polymorphisms in *P. falciparum*." Cell 53 (1988): 807-13.

Corfield, P., R.W. Veh, M. Wember, J.-C. Michalski, and R. Schauer. "The release of N-acetyl- and N-glycolloyl-neuramic acid from soluble complex carbohydrates and erythrocytes by bacterial, viral and mammalian sialidases." Biochemical Journal 197 (1981): 293-299.

Covell, G. "Clinical, chemotherapeutic and immunological studies on induced malaria." British Medical Bulletin 8 (1951): 51-55.

Cox, F. E. G. "Major models in animal research: rodent." In Malaria, The Principles and Practice of Malariology, ed. W. J. H. Wernsdorfer and I. MacGregor. 1503-1544. 2. London: Churchill Livingstone, 1988.

Cox, J., S. Semoff, and M. Hommel. "*Plasmodium chabaudi*: a rodent malaria model for in-vivo and in-vitro cytoadherence of malaria parasites in the absence of knobs." Parasite Immunol 9 (1987): 543-61.

Craig, A. and G. Langsley. "A malaria genome project: how a good idea is being put into practice." Bulletin Institut Pasteur 91 (1993): 179-187.

Crampton, J., A Morris, G. Lycett, A. Warren, and P. Eggleston. "Transgenic mosquitos: A future vector control strategy." Parasitology Today 6 (1990): 31-36.

Crandall, I., W. E. Collins, J. Gysin, and I. W. Sherman. "Synthetic peptides based on motifs present in human band 3 protein inhibit cytoadherence/sequestration of the malaria parasite *Plasmodium falciparum*." Proc Natl Acad Sci U S A 90 (1993): 4703-7.

Crandall, I., K. M. Land, and I. W. Sherman. "*Plasmodium falciparum*: pfalhesin and CD36 form an adhesin/receptor pair that is responsible for the pH-dependent portion of cytoadherence/sequestration." Exp Parasitol 78 (1994): 203-9.

Crandall, I. and I. W. Sherman. "*Plasmodium falciparum* (human malaria)-induced modifications in human erythrocyte band 3 protein." Parasitology 3 (1991): 335-40.

Crewe, W. and D. R. W. Haddock. Parasites and Human Disease. London: Edward Arnold, 1985.

Culvenor, J. G., K. P. Day, and R. F. Anders. "*Plasmodium falciparum* ring-infected erythrocyte surface antigen is released from merozoite dense granules after erythrocyte invasion." Infect Immun 59 (1991): 1183-7.

Curtis, C.F. "The case for malaria control by genetic manipulation of its vectors." Parasitology Today 10 (1994): 371-374.

Darriet, F., V Robert, N. Thu Vien, and P. Carneule. Evalualtion of the efficacy of permetrin impregnated intact and perforated mosquito nets against the vectors of malaria. WHO, Geneva, 1984. UBC/84.879.

David, P. H., S. M. Handunnetti, J. H. Leech, P. Gamage, and K. N. Mendis. "Rosetting: a new cytoadherence property of malaria-infected erythrocytes." Am J Trop Med Hyg 38 (1988): 289-97.

David, P. H., M. Hommel, L. H. Miller, I. J. Udeinya, and L. D. Oligino. "Parasite sequestration in *Plasmodium falciparum* malaria: spleen and antibody modulation of cytoadherence of infected erythrocytes." Proc. Natl. Acad. Sci. USA. 80 (1983): 5075-5079.

David, P.H., M. Hommel, and L.D. Oligino. "Interactions of *Plasmodium falciparum* infected erythrocytes with ligand-coated agarose beads." Molecular and Biochemical Parasitology 4 (1981): 195-204.

Day, K. P., F. Karamalis, J. Thompson, D. A. Barnes, C. Peterson, H. Brown, G. V. Brown, and D. J. Kemp. "Genes necessary for expression of a virulence determinant and for transmission of *Plasmodium falciparum* are located on a 0.3-megabase region of chromosome 9." Proc Natl Acad Sci U S A 90 (1993): 8292-6.

Day, K. P., J. C. Koella, S. Nee, S. Gupta, and A. F. Read. "Population genetics and dynamics of *Plasmodium falciparum*: an ecological view." Parasitology 104 (1992): S35-52.

Day, K.P. and K. Marsh. "Naturally acquired immunity to *Plasmodium falciparum*." Immunoparasitology Today 12 (1991): A68-A71.

Deans, J. A. "Protective antigens of bloodstage *Plasmodium knowlesi* parasites." Philos Trans R Soc Lond Biol 307 (1984): 159-69.

Deans, J. A., A. M. Knight, W. C. Jean, A. P. Waters, S. Cohen, and G. H. Mitchell. "Vaccination trials in rhesus monkeys with a minor, invariant, *Plasmodium knowlesi* 66 kD merozoite antigen." Parasite Immunol 10 (1988): 535-52.

Delves, C. J., P. Alano, R. G. Ridley, M. Goman, S. P. Holloway, J. E. Hyde, and J. G. Scaife. "Expression of alpha and beta tubulin genes during the asexual and sexual blood stages of *Plasmodium falciparum*." Mol Biochem Parasitol 43 (1990): 271-8.

Desai, S. A., D. J. Krogstad, and E. W. McCleskey. "A nutrient-permeable channel on the intraerythrocytic malaria parasite." Nature 362 (1993): 643-6.

Desowitz, R.S. The Malaria Capers. London and New York: Norton, 1991.

Desowitz, R. S., L. H. Miller, R. D. Buchanan, and B. Perenpanick. "The sites of deep vascular schizogony in *Plasmodium coatneyi* malaria." Trans. R. Soc. Trop. Med. Hyg. 63 (1969): 198-202.

Dietz, K. "Mathematical Models for Transmission and Control of Malaria." In Malaria: Principles and practice of malariology, ed. W.H. Wernsdorfer and I. McGregor. 1091-1133. Edinburgh: Churchill Livingstone, 1988.

Dockrell, H. M., A. Alavi, and J. H. L. Playfair. "Changes in oxidative burst capacity during murine malarias and the effect of vaccination." Clinical Experimental Immunology 66 (1986): 37-43.

Drapeau, G.R., Y. Boily, and J. Houmard. "Purification and properties of an extracellular protease of *Staphylococcus aureus*." Journal of Biological Chemistry 247 (1972): 6720-6726.

Dye, C. and G. Hasibeder. "Population dynamics of mosquito-borne disease: effects of flies which bite some people more frequently than others." Trans R Soc Trop Med Hyg 80 (1986): 69-77.

Dzandu, J.K., M. Deh, and G. Wise. "A re-examination of the effects of chymotrypsin and trypsin on the erythrocyte membrane surface topology." Biochemical and Biophysical Research Communications 126 (1985): 50-58.

Eaton, M. D. "The agglutination of *Plasmodium knowlesi* by immune serum." J. Exp. Med. 67 (1938): 857-869.

Elford, B. C., J. D. Haynes, J. D. Chulay, and R. J. Wilson. "Selective stage-specific changes in the permeability to small hydrophilic solutes of human erythrocytes infected with *Plasmodium falciparum*." Mol Biochem Parasitol 16 (1985): 43-60.

Elford, B. C., R. A. Pinches, C. I. Newbold, and J. C. Ellory. "Heterogeneous and substrate-specific membrane transport pathways induced in malaria-infected erythrocytes [comment]." Blood Cells 16 (1990): 433-6.

Elmendorf, H. G. and K. Haldar. "*Plasmodium falciparum* exports the Golgi marker sphingomyelin synthase into a tubovesicular network in the cytoplasm of mature erythrocytes." J Cell Biol 124 (1994): 449-62.

Etlinger, H. M., P. Caspers, H. Matile, H. J. Schoenfeld, D. Stueber, and B. Takacs. "Ability of recombinant or native proteins to protect monkeys against heterologous challenge with *Plasmodium falciparum*." Infect Immun 59 (1991): 3498-503.

Fairbanks, G., T.L. Steck, and D.F.H. Wallach. "Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane." Biochemistry 10 (1971): 2606-2617.

Fandeur, T., S. Bonnefoy, and O. Mercereau-Puijalon. "In vivo and in vitro derived Palo Alto lines of *Plasmodium falciparum* are genetically unrelated." Mol Biochem Parasitol 47 (1991): 167-78.

Feagin, J.E. "The 6kb element of *Plasmodium falciparum* encodes mitochondrial cytochrome genes." Molecular and Biochemical Parasitology 52 (1992): 145-148.

Feagin, J. E., E. Werner, M. J. Gardner, D. H. Williamson, and R. J. Wilson. "Homologies between the contiguous and fragmented rRNAs of the two *Plasmodium falciparum* extrachromosomal DNAs are limited to core sequences." Nucleic Acids Res 20 (1992): 879-87.

Foley, M., L. Tilley, W. H. Sawyer, and R. F. Anders. "The ring-infected erythrocyte surface antigen of *Plasmodium falciparum* associates with spectrin in the erythrocyte membrane." Mol Biochem Parasitol 46 (1991): 137-47.

Foote, S. J. and D. J. Kemp. "Chromosomes of malaria parasites." Trends Genet 5 (1989): 337-42.

Foote, S. J., J. K. Thompson, A. F. Cowman, and D. J. Kemp. "Amplification of the multidrug resistance gene in some chloroquine-resistant isolates of *P. falciparum*." Cell 57 (1989): 921-30.

Forsyth, K. P., R. F. Anders, J. A. Cattani, and M. P. Alpers. "Small area variation in prevalence of an S-antigen serotype of *Plasmodium falciparum* in villages of Madang, Papua New Guinea." Am J Trop Med Hyg 40 (1989a): 344-50.

Forsyth, K. P., G. Philip, T. Smith, E. Kum, B. Southwell, and G. V. Brown. "Diversity of antigens expressed on the surface of erythrocytes infected with mature *Plasmodium falciparum* parasites in Papua New Guinea." Am J Trop Med Hyg 41 (1989b): 259-65.

Foster, J. and J. Thompson. "The *Plasmodium falciparum* genome project: a resource for researchers." Parasitology Today 11 (1995): 1-4.

Fraker, P.J. and J.C. Speck. "Protein and cell membrane iodinations with a sparingly soluble chloramide 1,3,4,6-tertachloro-3 alpha, 6 alpha-diphenylglycoluril." Biochemical and Biophysical Research Communications 80 (1978): 849-857.

Franciolini, F. and W. Nonner. "Anion and cation permeability of a chloride channel in rat hippocampal meurons." Journal of General Physiology 90 (1987): 453-478.

Franz, D. R., M. Lee, L. T. Seng, G. D. Young, W. B. Baze, and Ge Lewis Jr. "Peripheral vascular pathophysiology of *Plasmodium berghei* infection: a comparative study in the cheek pouch and brain of the golden hamster." Am J Trop Med Hyg 36 (1987): 474-80.

Frazier, W. A., V. M. Dixit, N. J. Galvin, and P. R. Rotwein. "Structure of human thrombospondin: complete amino acid sequence derived from cDNA." Semin Thromb Hemost 13 (1987): 255-60.

Fremount, H. N. and L. H. Miller. "Deep vascular schizogony in *Plasmodium fragile*: organ distribution and ultrastructure of erythrocytes adherent to vascular endothelium." Am. J. Trop. Med. Hyg. 24 (1975): 1-8.

Fremount, H. N. and R. N. Rossan. "Anatomical distribution of developing trophozoites and schizonts of *Plasmodium vivax* in *Aotus lemurinus lemurinus* and *Saimiri sciureus*." J Parasitol 76 (1990): 428-30.

Fujioka, H. and M. Aikawa. "Morphological changes of clefts in Plasmodium-infected erythrocytes under adverse conditions." Exp Parasitol 76 (1993): 302-7.

Fujioka, H., P. Millet, Y. Maeno, S. Nakazawa, Y. Ito, R. J. Howard, W. E. Collins, and M. Aikawa. "A nonhuman primate model for human cerebral malaria: rhesus monkeys experimentally infected with *Plasmodium fragile*." Exp Parasitol 78 (1994): 371-6.

Fukuda, M., M. Lauffenburger, and H Sasaki et al. "Structures of novel sialylated O-linked oligosaccharides isolated from human erythrocyte glycophorins." Journal of Biological Chemistry 262 (1987): 11952-11957.

Gardner, M. J., D. H. Williamson, and R. J. Wilson. "A circular DNA in malaria parasites encodes an RNA polymerase like that of prokaryotes and chloroplasts." Mol Biochem Parasitol 44 (1991): 115-23.

Garnham, P. C. C. Malaria Parasites and other Haemosporidia. Oxford: Blackwell Scientific Publications, 1966.

Garnham, P. C. C. "Malaria parasites of man: life cycles and morphology (excluding ultrastructure)." In Malaria, The Principles and Practice of Malariology, ed. W. J. H. Wernsdorfer and I. MacGregor. 61-96. 1. London: Churchill Livingstone, 1988.

Gartner, T.K., J.M. Gerrard, J.G. White, and D.C. Williams. "The endogenous lectin of human platelets is an alpha granule component." Blood 58 (1981): 153.

Gero, A.M. and K. Kirk. "Nutrient transport pathways in *Plasmodium*-infected erythrocytes: what and where are they?" Parasitology Today 10 (1994): 395-399.

Gero, A. M. and A. M. Wood. "New nucleoside transport pathways induced in the host erythrocyte membrane of malaria and *Babesia* infected cells." Adv Exp Med Biol (1991): 169-72.

Gilks, C. F., D. Walliker, and C. I. Newbold. "Relationships between sequestration, antigenic variation and chronic parasitism in *Plasmodium chabaudi chabaudi*--a rodent malaria model." Parasite Immunol 12 (1990): 45-64.

Gilles, H.M. "Epidemiology of malaria." In Bruce-Chwatt's Essential Malariology, ed. H.M. Gilles and D.A. Warrell. 124-164. third ed., Vol. Boston: Edward Arnold, 1993a.

Gilles, H.M. "Historical outline." In Bruce-Chwatt's Essential Malariology, ed. H.M. Gilles and D.A. Warrell. 1-12. third ed., Vol. Boston: Edward Arnold, 1993b.

Gilles, H.M. "The malaria parasites." In Bruce-Chwatt's Essential Malariology, ed. H.M. Gilles and D.A. Warrell. 12-35. third ed., Vol. Boston: Edward Arnold, 1993c.

Ginsburg, H. Malaria and the Blood Cell. Vol. 16 (2/3). New York: Springer-Verlag, 1990.

Goldberg, D. E. and A. F. G. Slater. "The pathway of hemoglobin degredation in malaria parasites." Parasitology Today 8 (1992): 280-283.

Goldring, J. D. and M. Hommel. "Variation in the cytoadherence characteristics of malaria parasites: Is this a true virulence factor?" Mem. Inst. Oswaldo Cruz 87 (1992): 313-322.

Goldring, J. D., M. E. Molyneux, T. Taylor, J. Wirima, and M. Hommel. "*Plasmodium falciparum*: diversity of isolates from Malawi in their cytoadherence to melanoma cells and monocytes in vitro." Br J Haematol 81 (1992): 413-8.

Goman, M., G. Langsley, J.E. Hyde, N.K. Yankovsky, J.W. Zolg, and J.G. Scaife. "The establishment of genomic DNA libraries for the human parasite *Plasmodium falciparum* and identification of individual clones by hybridization." Molecular and Biochemical Parasitology 5 (1982): 391-400.

Goonewardene, R., J. Daily, D. Kaslow, T. J. Sullivan, P. Duffy, R. Carter, K. Mendis, and D. Wirth. "Transfection of the malaria parasite and expression of firefly luciferase." Proc Natl Acad Sci U S A 90 (1993): 5234-6.

Grau, G. E. and S. de Kossodo. "Cerebral Malaria: Mediators, Mechanical Obstruction or More?" Parasitology Today 10 (1994): 408-409.

Grau, G. E., L. F. Fajardo, P. F. Piguet, B. Allet, P. H. Lambert, and P. Vassalli. "Tumor necrosis factor (cachectin) as an essential mediator in murine cerebral malaria." Science 237 (1987): 1210-2.

Grau, G. E., T. E. Taylor, M. E. Molyneux, J. J. Wirima, P. Vassalli, M. Hommel, and P. H. Lambert. "Tumor necrosis factor and disease severity in children with falciparum malaria." N Engl J Med 320 (1989): 1586-91.

Graves, P. M., R. Carter, and K. M. McNeill. "Gametocyte production in cloned lines of *Plasmodium falciparum*." Am J Trop Med Hyg 33 (1984): 1045-50.

Greenberg, A. E., M. Ntumbanzondo, N. Ntula, L. Mawa, J. Howell, and F. Davachi. "Hospital-based surveillance of malaria-related paediatric morbidity and mortality in Kinshasa, Zaire." Bull World Health Organ 67 (1989): 189-96.

Greenwalt, D.E., R.H. Lipsky, C.F. Ockenhouse, H. Ikeda, N.N. Tandon, and G.A. Jamieson. "Membrane glycoprotein CD36: A review of its roles in adherence, signal transduction, and transfusion medicine." Blood 80 (1992): 1105-1115.

Greenwalt, T.J. and E.A. Steane. "Quantitative haemagglutination. IV. Effect of neuraminidase treatment on agglutination by blood group antibodies." British Journal of Haematology 25 (1973): 207-215.

Greenwood, B., K. Marsh, and R. Snow. "Why do some African children develop severe malaria?" Parasitology Today 7 (1991): 277-281.

Greenwood, B. M. "The immunology of malaria--a view from the bush." Parasite Immunol 7 (1985): 191-9.

Greenwood, B. M., A. K. Bradley, A. M. Greenwood, P. Byass, K. Jammeh, K. Marsh, S. Tulloch, F. S. Oldfield, and R. Hayes. "Mortality and morbidity from malaria among children in a rural area of The Gambia, West Africa." Trans R Soc Trop Med Hyg 81 (1987): 478-86.

Grellier, P., D. Rigomier, V. Clavey, J. C. Fruchart, and J. Schrevel. "Lipid traffic between high density lipoproteins and *Plasmodium falciparum*-infected red blood cells." J Cell Biol 112 (1991): 267-77.

Gruenberg, J., P.R. Allred, and I.W. Sherman. "A scanning electron microscope analysis of the protrusions (knobs) present on the surface of *Plasmodium falciparum*-infected erythrocytes." Journal of Cell Biology 97 (1983): 795-802.

Gupta, S., K. Trenholme, R. M. Anderson, and K. P. Day. "Antigenic diversity and the transmission dynamics of *Plasmodium falciparum*." Science 263 (1994): 961-3.

Guttman, P. and P. Ehrlich. Berl. Klin. Wochenschr. 28 (1891): 953-956.

Gysin, J., M. Aikawa, N. Tourneur, and T. Tegoshi. "Experimental *Plasmodium falciparum* cerebral malaria in the squirrel monkey *Saimiri sciureus*." Exp Parasitol 75 (1992): 390-8.

Haldane, J. B. S. "Disease and evolution." La Ricerce Scientifica 19 (1949): 68-75.

Haldar, K. "Ducts, channels and transporters in *Plasmodium*-infected erythrocytes." Parasitology Today 10 (1994): 393-395.

Haldar, K. and L. Uyetake. "The movement of fluorescent endocytic tracers in *Plasmodium falciparum* infected erythrocytes." Mol Biochem Parasitol 50 (1992): 161-77.

Handunnetti, S. M., K. N. Mendis, and P. H. David. "Antigenic variation of cloned *Plasmodium fragile* in its natural host *Macaca sinica*. Sequential appearance of successive variant antigenic types." J Exp Med 165 (1987): 1269-83.

Handunnetti, S. M., B. L. Pasloske, M. R. van-Schravendijk, J. C. Aguiar, T. F. Taraschi, J. A. Gormley, and R. J. Howard. "The characterization of two monoclonal antibodies which react with high molecular weight antigens of asexual *Plasmodium falciparum*." Mol Biochem Parasitol 54 (1992): 231-46.

Harries, A. D. "Malaria: keeping the mosquitoes at bay." Lancet 342 (1993): 506-7.

Harte, P. G., N. Rogers, and G. A. Targett. "Vaccination with purified microgamete antigens prevents transmission of rodent malaria." Nature 316 (1985): 258-9.

Hasler, T., G. R. Albrecht, M. R. Van-Schravendijk, J. C. Aguiar, K. E. Morehead, B. L. Pasloske, C. Ma, J. W. Barnwell, B. Greenwood, and R. J. Howard. "An improved microassay for *Plasmodium falciparum* cytoadherence using stable transformants of Chinese hamster ovary cells expressing CD36 or intercellular adhesion molecule-1." Am J Trop Med Hyg 48 (1993): 332-47.

Haverstick, D.M., V.M. Dixit, G.A. Grant, W.A. Frazier, and S.A. Santoro. "Localization of the hemagglutinating activity of platelet thrombospondin to a 140,000-dalton thermolytic fragment." Biochemistry 23 (1984): 5597-5603.

Haynes, J. D., Diggs, C. L, Hines, F. A. and Desjardins, R. E. "Culture of human malaria parasites *Plasmodium falciparum*." Nature 263 (1976): 767-769.

Helmby, H., L. Cavelier, U. Pettersson, and M. Wahlgren. "Rosetting *Plasmodium falciparum*-infected erythrocytes express unique strain-specific antigens on their surface." Infect Immun 61 (1993): 284-8.

Hill, A. V., C. E. Allsopp, D. Kwiatkowski, N. M. Anstey, P. Twumasi, P. A. Rowe, S. Bennett, D. Brewster, A. J. McMichael, and B. M. Greenwood. "Common west African HLA antigens are associated with protection from severe malaria [see comments]." Nature 352 (1991): 595-600.

Ho, M., B. Singh, S. Looareesuwan, T. M. Davis, D. Bunnag, and N. J. White. "Clinical correlates of in vitro *Plasmodium falciparum* cytoadherence." Infect Immun 59 (1991): 873-8.

Hogg, N., P.A. Bates, and J. Harvey. "Structure and function of intercellular adhesion molecule-1." Chemical Immunology 50 (1991): 98-115.

Hommel, M. "Amplification of cytoadherence in cerebral malaria: towards a more rational explanation of disease pathophysiology." Ann Trop Med Parasitol 87 (1993): 627-35.

Hommel, M., P. H. David, and L. D. Oligino. "Surface alterations of erythrocytes in *Plasmodium falciparum* malaria. Antigenic variation, antigenic diversity, and the role of the spleen." J Exp Med 157 (1983): 1137-48.

Hommel, M., P. H. David, L. D. Oligino, and J. R. David. "Expression of strain-specific surface antigens on *Plasmodium falciparum*-infected erythrocytes." Parasite Immunol 4 (1982): 409-19.

Hommel, M. and S. Semoff. "Expression and function of erythrocyte-associated surface antigens in malaria." Biol Cell 64 (1988): 183-203.

Howard, R.J. "Antigenic variation of bloodstage malaria parasites." Philosophical Transactions of the Royal Society, London 307 (1984): 141-158.

Howard, R. J. "Malarial proteins at the membrane of *Plasmodium falciparum*-infected erythrocytes and their involvement in cytoadherence to endothelial cells." Prog Allergy 41 (1988): 98-147.

Howard, R.J., J.W. Barnwell, and V Kao. "Antigenic variation of *Plasmodium knowlesi* malaria: identification of the variant antigen on infected erythrocytes." Proc. Natl. Acad. Sci. USA 80 (1983): 4129-4133.

Howard, R.J., J.W. Barnwell, V. Kao, W.A. Daniel, and S.B. Aley. "Radioiodination of new protein antigens of the surface of *Plasmodium knowlesi* schizont-infected erythrocytes." Molecular and Biochemical Parasitology 6 (1982): 343-367.

Howard, R. J., J. W. Barnwell, E. P. Rock, J. Neequaye, Adjei D. Ofori, W. L. Maloy, J. A. Lyon, and A. Saul. "Two approximately 300 kilodalton *Plasmodium falciparum* proteins at the surface membrane of infected erythrocytes." Mol Biochem Parasitol 27 (1988): 207-23.

Howard, R.J. and K.P. Day. "*Plasmodium berghei:* Modification of sialic acid on red cells from infected mouse blood." Experimental Parasitology 51 (1981): 95-103.

Howard, R. J. and A. D. Gilladoga. "Molecular studies related to the pathogenesis of cerebral malaria." Blood 74 (1989): 2603-18.

Howard, R. J., S. M. Handunnetti, T. Hasler, A. Gilladoga, Aguiar Jc de, B. L. Pasloske, K. Morehead, G. R. Albrecht, and Schravendijk Mr van. "Surface molecules on *Plasmodium falciparum*-infected erythrocytes involved in adherence." Am J Trop Med Hyg (1990): 15-29.

Howard, R.J. and B.L. Pasloske. "Target antigens for asexual malaria vaccine development." Parasitology Today 9 (1993): 369-372.

Howard, R. J., S. Uni, M. Aikawa, S. B. Aley, J. H. Leech, A. M. Lew, T. E. Wellems, J. Rener, and D. W. Taylor. "Secretion of a malarial histidine-rich protein (Pf HRP II) from *Plasmodium falciparum*-infected erythrocytes." J Cell Biol 103 (1986): 1269-77.

Hsiao, L.L. Howard, R.J. Aikawa,M. Taraschi,T.F. "Modification of host cell membrane lipid composition by the intra-erythrocytic human malaria parasite *Plasmodium falciparum*." Biochemical Journal 274 (1991): 121-132.

Inselburg, J. and H. S. Banyal. "*Plasmodium falciparum*: synchronization of asexual development with aphidicolin, a DNA synthesis inhibitor." Exp Parasitol 57 (1984): 48-54.

Inselburg, J., D. J. Bzik, W. B. Li, K. M. Green, J. Kansopon, B. K. Hahm, I. C. Bathurst, P. J. Barr, and R. N. Rossan. "Protective immunity induced in *Aotus* monkeys by recombinant SERA proteins of *Plasmodium falciparum*." Infect Immun 59 (1991): 1247-50.

Iqbal, J., P. Perlmann, and K. Berzins. "Serological diversity of antigens expressed on the surface of erythrocytes infected with *Plasmodium falciparum*." Trans R Soc Trop Med Hyg 87 (1993): 583-8.

Israeli, A., M. Shapiro, and M. A. Ephros. "*Plasmodium falciparum* malaria in an asplenic man." Trans R Soc Trop Med Hyg 81 (1987): 233-4.

Jaffe, E.A., L.L.K. Leung, R.L. Nachman, R.I. Levin, and D.F. Mosher. "Thrombospondin is the endogenous lectin of human platelets." Nature 295 (1982): 246.

Jakobsen, P. H., P. Grellier, T. G. Theander, N. Behrendt, M. Torii, M. Aikawa, J. Schrevel, and S. Jepsen. "Identification and localization of a soluble antigen, Ag2, of 136 kDa from *Plasmodium falciparum* in vitro cultures." Apmis 99 (1991): 155-62.

James, M. A., I. Kakoma, M. Ristic, and M. Cagnard. "Induction of protective immunity to *Plasmodium falciparum* in *Saimiri sciureus* monkeys with partially purified exoantigens." Infect Immun 49 (1985): 476-80.

Janse, C. J. "Chromosome size polymorhism and DNA rearrangements in *Plasmodium*." Parasitology Today 9 (1993): 19-22.

Jaramillo-Arango, J. The Conquest of Malaria. London: Heinemann Medical Books, 1950.

Jarolim, P., J. Palek, D. Amato, K. Hassan, P. Sapak, G. T. Nurse, H. L. Rubin, S. Zhai, K. E. Sahr, and S. C. Liu. "Deletion in erythrocyte band 3 gene in malaria-resistant Southeast Asian ovalocytosis." Proc Natl Acad Sci U S A 88 (1991): 11022-6.

Johnson, J. K., R. A. Swerlick, K. K. Grady, P. Millet, and T. M. Wick. "Cytoadherence of *Plasmodium falciparum*-infected erythrocytes to microvascular endothelium is regulatable by cytokines and phorbol ester." J Infect Dis 167 (1993): 698-703.

Kaesberg, P.R., W.B. Ershler, J.D. Esko, and D.F. Mosher. "Chinese hamster ovary cell adhesion to human platelet thrombospondin is dependent on cell surface heparan sulfate proteoglycan." Journal of Clinical Investigation 83 (1989): 994.

Kanaani, J. and H. Ginsburg. "Metabolic interconnection between the human malarial parasite *Plasmodium falciparum* and its host erythrocyte. Regulation of ATP levels by means of an adenylate translocator and adenylate kinase." J Biol Chem 264 (1989): 3194-9.

Kemp, D.J., R.L. Coppel, A.F. Cowman, R.B. Saint, G.V. Brown, and R.F. Anders. "Expression of *Plasmodium falciparum* blood-stage antigens in *E. coli*: detection with antibodies from immune humans." Proceedings of National Academy of Sciences, USA 80 (1983): 3787-3791.

Kemp, D. J., A. F. Cowman, and D. J. Walliker. "Genetic diversity in *Plasmodium falciparum*." Adv. Parasitol. 29 (1990): 75-149.

Kemp, D. J., J. Thompson, D. A. Barnes, T. Triglia, F. Karamalis, C. Petersen, G. V. Brown, and K. P. Day. "A chromosome 9 deletion in *Plasmodium falciparum* results in loss of cytoadherence." Mem Inst Oswaldo Cruz 3 (1992): 85-9.

Kemp, D. J., J. K. Thompson, D. Walliker, and L. M. Corcoran. "Molecular karyotype of *Plasmodium falciparum*: conserved linkage groups and expendable histidine-rich protein genes." Proc Natl Acad Sci U S A 84 (1987): 7672-6.

Kern, P., C.J. Hemmer, and J.C. Van Damme. "Elevated tumor necrosis factor alpha for complicated *Plasmodium falciparum* malaria." American Journal of Medicine 87 (1989): 139-143.

Kidson, C., G. Lamont, A. Saul, and G. T. Nurse. "Ovalocytic erythrocytes from Melanesians are resistant to invasion by malaria parasites in culture." Proc. Natl. Acad. Sci. 78 (1981): 5829-5832.

Kilejian, A. "Characterization of a protein correlated with the production of knob-like protrusions on membranes of erythrocytes infected with *Plasmodium falciparum*." Proceedings of the National Academy of Sciences, USA 76 (1979): 4650-4653.

Kirk, K., H. A. Horner, B. C. Elford, J. C. Ellory, and C. I. Newbold. "Transport of diverse substrates into malaria-infected erythrocytes via a pathway showing functional characteristics of a chloride channel." J Biol Chem 269 (1994): 3339-47.

Kirk, K., H. Y. Wong, B. C. Elford, C. I. Newbold, and J. C. Ellory. "Enhanced choline and Rb^+ transport in human erythrocytes infected with the malaria parasite *Plasmodium falciparum*." Biochem J 278 (1991): 521-5.

Kitron, U. and A. Spielman. "Suppression of transmission of malaria through source reduction: antianopheline measures applied in Israel, the United States, and Italy." Rev Infect Dis 11 (1989): 391-406.

Knell, A.J. Malaria. first ed., Oxford: Oxford University Press, 1991.

Koenen, M., A. Scherf, O. Mercereau, G. Langsley, L. Sibilli, P. Dubois, L. Pereira-da-Silva, and B. Muller-Hill. "Human antisera detect a *Plasmodium falciparum* genomic clone encoding a nonapeptide repeat." Nature 311 (1984): 382-5.

Kumaratilake, L. M., B. S. Robinson, A. Ferrante, and A. Poulos. "Antimalarial properties of n-3 and n-6 polyunsaturated fatty acids: in vitro effects on *Plasmodium falciparum* and in vivo effects on P. berghei." J Clin Invest 89 (1992): 961-7.

Kutner, S., W. V. Breuer, H. Ginsburg, S. B. Aley, and Z. I. Cabantchik. "Characterization of permeation pathways in the plasma membrane of human erythrocytes infected with early stages of *Plasmodium falciparum*: association with parasite development." J Cell Physiol 125 (1985): 521-7.

Kwiatkowski, D. "Malaria: becoming more specific about non-specific immunity." Curr Opin Immunol 4 (1992): 425-31.

Kyes, S., A. G. Craig, K. Marsh, and C. I. Newbold. "*Plasmodium falciparum*: a method for the amplification of S antigens and its application to laboratory and field samples." Exp Parasitol 77 (1993): 473-83.

Laemmli, U. K. "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." Nature 227 (1970): 680-685.

Lahav, J. "The functions of thrombospondin and its involvement in physiology and pathophysiology." Biochim Biophys Acta 1182 (1993): 1-14.

Lambros, C. and J. P. Van der Berg. "Synchronisation of *Plasmodium falciparum* erythrocytic stages in culture." J. Parasitol. 65 (1979): 418-420.

Land, K.M., I.W. Sherman, J. Gysin, and I.E. Crandall. "Anti-adhesive antibodies and peptides as potential therapeutics for *Plasmodium falciparum* malaria." Parasitology Today 11 (1995): 19-23.

Langreth, S. G., J. B. Jensen, R. T. Reese, and W. Trager. "Fine structure of human malaria *in vitro*." J. Protozool. 25 (1978): 443-452.

Lanzer, M., D. de Bruin, S.P. Wertheimer, and J.V. Ravetch. "Organization of chromosomes in *Plasmodium falciparum:* a model for generating karyotypic diversity." Parasitology Today 10 (1994a): 114-117.

Lanzer, M., S.P. Wertheimer, D. De Bruin, and J.V. Ravetch. "Plasmodium: control of gene expression in malaria parasites." Experimental Parasitology 77 (1993): 121-128.

Lanzer, M., S. P. Wertheimer, D. de-Bruin, and J. V. Ravetch. "Chromatin structure determines the sites of chromosome breakages in *Plasmodium falciparum*." Nucleic Acids Res 22 (1994b): 3099-103.

Laskey, R. A. and A. D. Mills. "Quantitative detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography." Eur. J. Biochem. 56 (1975): 335-341.

Laveran, A. "Note sur un noveau parasite trove dans le sang de pluisiers malades attients de fievre palustre." Bulletin de l'Academie Medicine 9 (1880): 1235-136.

Lawler, J. and R. O. Hynes. "The structure of human thrombospondin, an adhesive glycoprotein with multiple calcium-binding sites and homologies with several different proteins." J Cell Biol 103 (1986): 1635-48.

Lee, S-H., P. Crocker, and S. Gordon. "Macrophage plasma membrane and secretory properties in murine malaria. Effects of *Plasmodium yoelii* blood-stage infection on macrophages in liver, spleen and blood." Journal of Experimental Medicine 163 (1986): 54-74.

Leech, J. H., J. W. Barnwell, L. H. Miller, and R. J. Howard. "Identification of a strain-specific malarial antigen exposed on the surface of *Plasmodium falciparum*-infected erythrocytes." J Exp Med 159 (1984): 1567-75.

Li, J. L. and Y. J. Li. "Inhibitory, opsonic and cytotoxic activities of monoclonal antibodies against asexual erythrocytic stages of *Plasmodium falciparum*." Parasitology 95 (1987): 229-40.

Livingstone, F.B. "The malaria hypothesis." In Distribution and Evolution of the Haemoglobin and Globin Loci, ed. J.E. Bowman. Elsevier Science Publishing Co., Inc., 1983.

Lockyer, M. J., K. Marsh, and C. I. Newbold. "Wild isolates of *Plasmodium falciparum* show extensive polymorphism in T cell epitopes of the circumsporozoite protein." Mol Biochem Parasitol 37 (1989): 275-80.

Luse, S. A. and L. H. Miller. "*Plasmodium falciparum* malaria. Ultrastructure of parasitized erythrocytes in cardiac vessels." Am J. Trop. Med. Hyg. 20 (1971): 655-660.

Lustigman, S., R. F. Anders, G. V. Brown, and R. L. Coppel. "The mature-parasite-infected erythrocyte surface antigen (MESA) of *Plasmodium falciparum* associates with the erythrocyte membrane skeletal protein, band 4.1." Mol Biochem Parasitol 38 (1990): 261-70.

Luzzi, G. A., A. H. Merry, C. I. Newbold, K. Marsh, and G. Pasvol. "Protection by alpha-thalassaemia against *Plasmodium falciparum* malaria: modified surface antigen expression rather than impaired growth or cytoadherence." Immunol Lett 30 (1991a): 233-40.

Luzzi, G. A., A. H. Merry, C. I. Newbold, K. Marsh, G. Pasvol, and D. J. Weatherall. "Surface antigen expression on *Plasmodium falciparum*-infected erythrocytes is modified in alpha- and beta-thalassemia." J Exp Med 173 (1991b): 785-91.

MacDonald, G. The Epidemiology and Control of Malaria. London: Oxford University Press, 1957.

Mackey, L. J., A. Hochmann, C. H. June, C. E. Contreras, and P. H. Lambert. "Immunopathological aspects of *Plasmodium berghei* infection in mice. II Immunopathology of cerebral and other tissues lesions during infections." Clin Exp Immunol 42 (1980): 412-420.

MacPherson, G. G., M. J. Warrell, N. J. White, S. Looareesuwan, and D. A. Warrell. "Human cerebral malaria. A quantitative ultrastructural analysis of parasitized erythrocyte sequestration." Am J Pathol 119 (1985): 385-401.

Magowan, C., W. Wollish, L. Anderson, and J. Leech. "Cytoadherence by *Plasmodium falciparum*-infected erythrocytes is correlated with the expression of a family of variable proteins on infected erythrocytes." J Exp Med 168 (1988): 1307-20.

Maguire, P. A., J. Prudhomme, and I. W. Sherman. "Alterations in erythrocyte membrane phospholipid organization due to the intracellular growth of the human malaria parasite, *Plasmodium falciparum*." Parasitology 2 (1991): 179-86.

Maguire, P. A. and I. W. Sherman. "Phospholipid composition, cholesterol content and cholesterol exchange in *Plasmodium falciparum*-infected red cells." Mol Biochem Parasitol 38 (1990): 105-12.

Major, R.H. Classic Description of Disease. Oxford: Blackwell, 1932.

Manning, S.D. and A.J. Williams. "Conduction and blocking properties of a predominantly anion-selective channel from human platelet surface membrane reconstituted into planar phospholipid bilayers." Journal of Membrane Biology 109 (1989): 113-122.

Marchiafava, E. and A. Bignami. "English translation in Steadman's twentieth century practice of medicine." 155. 19. London: 1900.

Marsh, K. "Malaria--a neglected disease?" Parasitology 104 (1992): S53-69.

Marsh, K. "Immunology of human malaria." In Bruce-Chwatt's Essential Malariology, ed. H.M. Gilles and D.A. Warrell. 60-78. third ed., Vol. Boston: Edward Arnold, 1993.

Marsh, K. and R. J. Howard. "Antigens induced on erythrocytes by *P. falciparum*: expression of diverse and conserved determinants." Science 231 (1986): 150-3.

Marsh, K., V.M. Marsh, J. Brown, H.C. Whittle, and B.M. Greenwood. "*Plasmodium falciparum*: The behaviour of clinical isolates in an in vitro model of infected red blood cell sequestration." Experimental Parasitology 65 (1988): 202-208.

Marsh, K., L. Otoo, R. J. Hayes, D. C. Carson, and B. M. Greenwood. "Antibodies to blood stage antigens of *Plasmodium falciparum* in rural Gambians and their relation to protection against infection." Trans R Soc Trop Med Hyg 83 (1989): 293-303.

Marshall, V. M., R. L. Coppel, R. K. Martin, A. M. Oduola, R. F. Anders, and D. J. Kemp. "A *Plasmodium falciparum* MSA-2 gene apparently generated by intragenic recombination between the two allelic families." Mol Biochem Parasitol 45 (1991): 349-51.

Mazier, D., I. Landau, P. Druilhe, F. Miltgen, C. Guguen-Guillouzo, D. Baccam, J. Baxter, J. P. Chigot, and M. Gentilini. "Cultivation of the liver forms of *Plasmodium vivax* in human hepatocytes." Nature 307 (1984): 367-9.

McConkey, G. A., A. P. Waters, and T. F. McCutchan. "The generation of genetic diversity in malaria parasites." Annu Rev Microbiol 44 (1990): 479-98.

McGregor, I. A., S. P. Carrington, and S. Cohen. "Treatment of East African *P. falciparum* malaria with West African human gammaglobulin." Trans. R. Soc. Trop. Med. Hyg. 57 (1963): 170-175.

McGregor, J.L., B. Catimel, S. Parmentier, P. Clezardin, M. Dechavanne, and L.L. Leung. "Rapid purification and partial characterization of human platelet glycoprotein IIIb. Interaction with thrombospondin and its role in platelet aggregation." Journal of Biological Chemistry 264 (1989): 501-506.

McGuire, W., A.V.S. Hill, C.E.M. Allsopp, B.M. Greenwood, and D. Kwiatkowski. "Variation in the TNF-alpha promoter region associated with susceptibility to cerebral malaria." Nature 371 (1994): 508-511.

McLean, S. A., L. M. MacDougall, and R. S. Phillips. "Early appearance of variant parasites in *Plasmodium chabaudi* infections." Parasite Immunol 12 (1990): 97-103.

McLean, S. A., C. D. Pearson, and R. S. Phillips. "*Plasmodium chabaudi*: antigenic variation during recrudescent parasitaemias in mice." Exp Parasitol 54 (1982a): 296-302.

McLean, S. A., C. D. Pearson, and R. S. Phillips. "*Plasmodium chabaudi*: relationship between the occurrence of recrudescent parasitaemias in mice and the effective levels of acquired immunity." Exp Parasitol 54 (1982b): 213-21.

McLean, S. A., C. D. Pearson, and R. S. Phillips. "Antigenic variation in *Plasmodium chabaudi*: analysis of parent and variant populations by cloning." Parasite Immunol 8 (1986): 415-24.

McLean, S. A., R. S. Phillips, C. D. Pearson, and D. Walliker. "The effect of mosquito transmission of antigenic variants of *Plasmodium chabaudi*." Parasitology (1987):

Megroz, R. L. Ronald Ross - Discoverer and Creator. London: Allen and Unwin, 1931.

Mendis, K.N., P.H. David, and R. Carter. "Antigenic polymorphism in malaria: is it an important mechanism for immune evasion?" Immunoparasitology Today 12 (1991): A34-A37.

Miller, L.H. "Distribution of mature trophozoites and schizonts of *Plasmodium falciparum* in the organs of *Aotus trivirgatus*, the night monkey." American Journal of Tropical Medicine and Hygiene 18 (1969): 860-865.

Miller, L. H. "Genetically determined human resistance factors." In Malaria, The Principles and Practice of Malariology, ed. W. J. H. Wernsdorfer and I. MacGregor. 487-500. 1. London: Churchill Livingstone, 1988.

Miller, L. H., M. F. Good, and G. Milon. "Malaria pathogenesis." Science 264 (1994): 1878-83.

Miller, L.H., S. J. Mason, D.F. Clyde, and M. H. McGinniss. "The resistance factor to *Plasmodium vivax* in blacks: The Duffy-blood-group genotype, FyFy." New Eng. J. Med. 295 (1976): 302-304.

Miller, L.H., S. J. Mason, J. A. Dvorak, M. H. McGinniss, and I. K. Rothman. "Erythrocyte receptors for *(Plasmodium knowlesi)* malaria: The Duffy blood group determinants." Science 189 (1975): 561-562.

Miller, L. H., T. Roberts, M. Shahabuddin, and T. F. McCutchan. "Analysis of sequence diversity in the *Plasmodium falciparum* merozoite surface protein-1 (MSP-1)." Mol Biochem Parasitol 59 (1993): 1-14.

Molyneux, M. E., T. E. Taylor, J. J. Wirima, and A. Borgstein. "Clinical features and prognostic indicators in paediatric cerebral malaria: a study of 131 comatose Malawian children [see comments]." Q J Med 71 (1989): 441-59.

Mosher, D.F., M.J. Doylw, and E.A. Jaffe. "Synthesis and secretion of thrombospondin by cultured human endothelial cells." Journal of Cell Biology 93 (1982): 343-348.

Nagel, R.L. and E.F. Roth Jr. "Malaria and red cell genetic defects." Blood 74 (1989): 1213-1221.

Nakamura, K., T. Hasler, K. Morehead, R. J. Howard, and M. Aikawa. "*Plasmodium falciparum*-infected erythrocyte receptor(s) for CD36 and thrombospondin are restricted to knobs on the erythrocyte surface." J Histochem Cytochem 40 (1992): 1419-22.

Nash, G. B., B. M. Cooke, K. Marsh, A. Berendt, C. Newbold, and J. Stuart. "Rheological analysis of the adhesive interactions of red blood cells parasitized by *Plasmodium falciparum*." Blood 79 (1992): 798-807.

Nash, T. "Surface antigen variability and variation in *Giardia lamblia*." Parasitology Today 8 (1992): 229-234.

Newbold, C. I. and K. Marsh. "Antigens on the *Plasmodium falciparum* infected erythrocyte surface are parasite derived: A reply." Parasitology Today 6 (1990): 320-324.

Newbold, C. I., R. Pinches, D. J. Roberts, and K. Marsh. "*Plasmodium falciparum*: the human agglutinating antibody response to the infected red cell surface is predominantly variant specific." Exp Parasitol 75 (1992): 281-92.

Newton, C. R., F. J. Kirkham, P. A. Winstanley, G. Pasvol, N. Peshu, D. A. Warrell, and K. Marsh. "Intracranial pressure in African children with cerebral malaria [see comments]." Lancet 337 (1991): 573-6.

Nussenzweig, V. and R. S. Nussenzweig. "Circumsporozoite proteins of malaria parasites." Bull Mem Acad R Med Belg 144 (1989): 493-504.

O'Sullivan, W.J. Ketley, K. "Biosynthesis of uridine monophosphate in *Plasmodium berghei*." Annals of Tropical Medicine and Parasitology 74 (1980): 109-114.

Ockenhouse, C. F., R. Betageri, T. A. Springer, and D. E. Staunton. "*Plasmodium falciparum*-infected erythrocytes bind ICAM-1 at a site distinct from LFA-1, Mac-1, and human rhinovirus." Cell 68 (1992a): 63-9.

Ockenhouse, C. F. and J. D. Chulay. "*Plasmodium falciparum* sequestration: OKM5 antigen (CD36) mediates cytoadherence of parasitized erythrocytes to a myelomonocytic cell line." J Infect Dis 157 (1988): 584-8.

Ockenhouse, C. F., M. Ho, N. N. Tandon, G. A. Van-Seventer, S. Shaw, N. J. White, G. A. Jamieson, J. D. Chulay, and H. K. Webster. "Molecular basis of sequestration in severe and uncomplicated *Plasmodium falciparum* malaria: differential adhesion of infected erythrocytes to CD36 and ICAM-1." J Infect Dis 164 (1991a): 163-9.

Ockenhouse, C. F., F. W. Klotz, N. N. Tandon, and G. A. Jamieson. "Sequestrin, a CD36 recognition protein on *Plasmodium falciparum* malaria-infected erythrocytes identified by anti-idiotype antibodies." Proc Natl Acad Sci U S A 88 (1991b): 3175-9.

Ockenhouse, C. F., N. N. Tandon, C. Magowan, G. A. Jamieson, and J. D. Chulay. "Identification of a platelet membrane glycoprotein as a falciparum malaria sequestration receptor [see comments]." Science 243 (1989): 1469-71.

Ockenhouse, C. F., T. Tegoshi, Y. Maeno, C. Benjamin, M. Ho, K. E. Kan, Y. Thway, K. Win, M. Aikawa, and R. R. Lobb. "Human vascular endothelial cell adhesion receptors for *Plasmodium falciparum*-infected erythrocytes: roles for endothelial leukocyte adhesion molecule 1 and vascular cell adhesion molecule 1." J Exp Med 176 (1992b): 1183-9.

Onori, E., P.F. Beales, and H.M. Gilles. "Rationale and technique of malaria control." In Bruce-Chwatt's Essential Malariology, ed. H.M. Gilles and D.A. Warrell. 196-267. third ed., Vol. Boston: Edward Arnold, 1993.

Oo, M. M., M. Aikawa, T. Than, T. M. Aye, P. T. Myint, I. Igarashi, and W. C. Schoene. "Human cerebral malaria: a pathological study." J Neuropathol Exp Neurol 46 (1987): 223-31.

Oquendo, P., E. Hundt, J. Lawler, and B. Seed. "CD36 directly mediates cytoadherence of *Plasmodium falciparum* parasitized erythrocytes." Cell 58 (1989): 95-101.

Palmer, J.D. "Green ancestry of malarial parasites." Current Biology 2 (1992): 318-319.

Pampona, E.J., Russel,P.F.,. "Malaria a world problem." World Health Organisation Chronicle 9 (1955): 31-96.

Parra, M. E., C. B. Evans, and D. W. Taylor. "Identification of *Plasmodium falciparum* histidine-rich protein 2 in the plasma of humans with malaria." J Clin Microbiol 29 (1991): 1629-34.

Parrodi, F., I.G. Wright, A.S.. Bourne, and C. Dobson. "In vitro adherence of bovine erythrocytes infected with *Babesia bovis* to thrombospondin and laminin." International Journal for Parasitology 19 (1989): 567-569.

Parrodi, F., I.G. Wright, J.D. Kerr, and C. Dobson. "In vitro adherence of erythrocytes infected with *Babesia bigemina* and *Babesia rodhaini* to thrombospondin." International Journal for Parasitology 20 (1990): 899-903.

Paskewitz, S. M., M. R. Brown, F. H. Collins, and A. O. Lea. "Ultrastructural localization of phenoloxidase in the midgut of refractory *Anopheles gambiae* and association of the enzyme with encapsulated *Plasmodium cynomolgi*." J Parasitol 75 (1989): 594-600.

Pasloske, B. L., D. I. Baruch, M. R. van-Schravendijk, S. M. Handunnetti, M. Aikawa, H. Fujioka, T. F. Taraschi, J. A. Gormley, and R. J. Howard. "Cloning and characterization of a *Plasmodium*

falciparum gene encoding a novel high-molecular weight host membrane-associated protein, PfEMP3." Mol Biochem Parasitol 59 (1993): 59-72.

Pasloske, B. L. and R. J. Howard. "Malaria, the red cell, and the endothelium." Annu. Rev. Med. 45 (1994): 283-295.

Pasvol, G., J. S. Wainscoat, and D. J. Weatherall. "Erythrocytes deficiency in glycophorin resist invasion by the malarial parasite *Plasmodium falciparum*." Nature 297 (1982): 64-6.

Pasvol, G., R.J.M. Wilson, M.E. Smalley, and J. Brown. "Separation of viable schizont-infected cells of *Plasmodium falciparum* from human blood." Annals of Tropical Medicine and Parasitology 65 (1978): 87-88.

Perkins, M. E. "Approaches to study merozoite invasion of erythrocytes." Res Immunol 142 (1991): 662-5.

Perrin, L. H., M. Loche, J. P. Dedet, C. Roussilhon, and T. Fandeur. "Immunization against *Plasmodium falciparum* asexual blood stages using soluble antigens." Clin Exp Immunol 56 (1984): 67-72.

Perrin, L. H., E. Ramirez, P. H. Lambert, and P. A. Miescher. "Inhibition of *P. falciparum* growth in human erythrocytes by monoclonal antibodies." Nature 289 (1981): 301-303.

Petersen, C., R. Nelson, J. Leech, J. Jensen, W. Wollish, and A. Scherf. "The gene product of the *Plasmodium falciparum* 11.1 locus is a protein larger than one megadalton." Mol Biochem Parasitol 42 (1990): 189-95.

Phillips, R.S., R.A. Wolstencroft, I.N. Brown, K.N. Brown, and D.C. Dumonde. "Immunity to Malaria: III. Possible occurrence of a cell-mediated immunity to *Plasmodium knowlesi* in chronically infected and Freund's complete adjuvant-sensitized monkeys." Experimental Parasitology 28 (1970): 304-317.

Pollack, Y., A.L. Katzen, D.T. Spira, and J. Golenser. "The genome of *Plasmodium falciparum*. I. DNA base composition." Nucleic Acids Research 10 (1982): 539-546.

Pologe, L. G., D. de-Bruin, and J. V. Ravetch. "A and T homopolymeric stretches mediate a DNA inversion in *Plasmodium falciparum* which results in loss of gene expression." Mol Cell Biol 10 (1990): 3243-6.

Pologe, L. G. and J. V. Ravetch. "A chromosomal rearrangement in a *P. falciparum* histidine-rich protein gene is associated with the knobless phenotype." Nature 322 (1986): 474-7.

Pologe, L. G. and J. V. Ravetch. "Large deletions result from breakage and healing of *P. falciparum* chromosomes." Cell 55 (1988): 869-74.

Pongponratn, E., M. Riganti, B. Punpoowong, and M. Aikawa. "Microvascular sequestration of parasitized erythrocytes in human falciparum malaria: a pathological study." Am J Trop Med Hyg 44 (1991): 168-75.

Pouvelle, B., R. Spiegel, L. Hsiao, R. J. Howard, R. L. Morris, A. P. Thomas, and T. F. Taraschi. "Direct access to serum macromolecules by intraerythrocytic malaria parasites." Nature 353 (1991): 73-5.

Prensier, G. and C. Slomianny. "The karyotype of *Plasmodium falciparum* determined by ultrastructural serial sectioning and 3D reconstruction." J Parasitol 72 (1986): 731-6.

Quinn, T. C. and D. J. Wyler. "Intravascular clearance of parasitized erythrocytes in rodent malaria." Journal of Clinical Investigation 63 (1979): 1187-1195.

Ramasamy, R. and R. T. Reese. "A role for carbohydrate moieties in the immune response to malaria." J Immunol 134 (1985): 1952-5.

Raugi, G.J., S.M. Mumby, D. Abbott Brown, and P. Bornstein. "Thrombospondin: synthesis and secretion by cells in culture." Journal of Cell Biology 95 (1982): 351-354.

Reeder, J. C., S. J. Rogerson, F. al-Yaman, R. F. Anders, R. L. Coppel, S. Novakovic, M. P. Alpers, and G. V. Brown. "Diversity of agglutinating phenotype, cytoadherence, and rosette-forming characteristics of *Plasmodium falciparum* isolates from Papua New Guinean children." Am J Trop Med Hyg 51 (1994): 45-55.

Reyes, P. Rathod, P.K. Sanchez,D.J. Mrema,J.E.K. Rieckmann,K.H. Heidrich,H.G. "Enzymes of purine and pyrimidine metabolism from the human malarial parasite, *Plasmodium falciparum*." Molecular and Biochemical Parasitology 5 (1982): 275-290.

Ridley, R. G., B. Takacs, H. W. Lahm, C. J. Delves, M. Goman, U. Certa, H. Matile, G. R. Woollett, and J. G. Scaife. "Characterisation and sequence of a protective rhoptry antigen from *Plasmodium falciparum*." Mol Biochem Parasitol 41 (1990): 125-34.

Ringwald, P., F. Peyron, J. P. Lepers, P. Rabarison, C. Rakotomalala, M. Razanamparany, M. Rabodonirina, J. Roux, and J. Le-Bras. "Parasite virulence factors during falciparum malaria: rosetting, cytoadherence, and modulation of cytoadherence by cytokines." Infect Immun 61 (1993): 5198-204.

Ringwald, P., F. Peyron, and J.P. Vuillez. "Levels of cytokines in plasma during *Plasmodium falciparum* malaria attacks." Journal of Clinical Microbiology 29 (1991): 2076-2078.

Roberts, D.D., D.M. Haverstick, V.M. Dixit, W.A. Frazier, S.A. Santoro, and V. Ginsburg. "The platelet glycoprotein thrombospondin binds specifically to sulfated glycolipids." Journal of Biological Chemistry 260 (1985a): 9405.

Roberts, D. D., J. A. Sherwood, S. L. Spitalnik, L. J. Panton, R. J. Howard, V. M. Dixit, W. A. Frazier, L. H. Miller, and V. Ginsburg. "Thrombospondin binds falciparum malaria parasitized erythrocytes and may mediate cytoadherence." Nature 318 (1985b): 64-6.

Roberts, D.J., B.-A. Biggs, G. Brown, and C.I. Newbold. "Protection, pathogenesis and phenotypic plasticity in *Plasmodium falciparum* malaria." Parasitology Today 9 (1993): 281-286.

Roberts, D. J., A. G. Craig, A. R. Berendt, R. Pinches, G. Nash, K. Marsh, and C. I. Newbold. "Rapid switching to multiple antigenic and adhesive phenotypes in malaria [see comments]." Nature 357 (1992): 689-92.

Rock, E. P., Ef Roth Jr., R. R. Rojas-Corona, J. A. Sherwood, R. L. Nagel, R. J. Howard, and D. K. Kaul. "Thrombospondin mediates the cytoadherence of *Plasmodium falciparum*-infected red cells to vascular endothelium in shear flow conditions." Blood 71 (1988a): 71-5.

Rock, E. P., A. J. Saul, D. W. Taylor, J. H. Leech, J. A. Sherwood, and R. J. Howard. "Expression of the histidine-rich protein PfHRP1 by knob-positive *Plasmodium falciparum* is not sufficient for cytoadherence of infected erythrocytes." Infect Immun 56 (1988b): 3301-4.

Rockett, K. A., M. M. Awburn, W. B. Cowden, and I. A. Clark. "Killing of *Plasmodium falciparum* in vitro by nitric oxide derivatives." Infect Immun 59 (1991): 3280-3.

Rogers, G. N., J. C. Paulson, R. S. Daniels, J. J. Skehel, I. A. Wilson, and D. C. Wiley. "Single amino acid substitutions in *Infulenza* haemagglutinin change receptor binding specificity." Nature 304 (1983): 76.

Rogers, M.E., D.T. Williams, R. Niththyananthan, M.W. Rampling, K.E. Heslop, and D.G. Johnston. "Decrease in erythrocyte glycophorin sialic acid content is associated with increased erythrocyte aggregation in human diabetes." Clinical Science 82 (1992): 309-313.

Rosenberg, R., R. A. Wirtz, I. Schneider, and R. Burge. "An estimation of the number of malaria sporozoites ejected by a feeding mosquito." Trans R Soc Trop Med Hyg 84 (1990): 209-12.

Ross, R. "On some peculiar pigmented cells found in two mosquitos fed on malarial blood." British Medical Journal 2 (1897): 1786-1788.

Ross, R. Memoirs, with a full account of the great malaria problem and its solution. London: John Murray, 1923.

Rothlein, R., M.L. Dustin, S.D. Marlin, and T.A. Springer. "A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1." Journal of Immunology 137 (1986): 1270-1274.

Rowe, A., A.R. Berendt, K. Marsh, and C.I. Newbold. "*Plasmodium falciparum:* A family of sulphated glycoconjugates disrupts erythrocyte rosettes." Experimental Parasitology 79 (1994): 506-516.

Ruangjirachuporn, W., R. Udomsangpetch, J. Carlsson, D. Drenckhahn, P. Perlmann, and K. Berzins. "*Plasmodium falciparum*: analysis of the interaction of antigen Pf155/RESA with the erythrocyte membrane." Exp Parasitol 73 (1991): 62-72.

Ruangjirachuporn, W., B. Wahlin, H. Perlmann, J. Carlsson, K. Berzins, M. Wahlgren, R. Udomsangpetch, H. Wigzell, and P. Perlmann. "Monoclonal antibodies to a synthetic peptide corresponding to a repeated sequence in the *Plasmodium falciparum* antigen Pf155." Mol Biochem Parasitol 29 (1988): 19-28.

Russell, R. C. "Vector-borne diseases and their control." Med J Aust 158 (1993): 684-90.

Sabchareon, A., T. Burnouf, D. Ouattara, P. Attanath, H. Bouharoun-Tayoun, P. Chantavanich, C. Foucault, T. Chongsuphajaisiddhi, and P. Druilhe. "Parasitologic and clinical human response to immunoglobulin administration in falciparum malaria." Am J Trop Med Hyg 45 (1991): 297-308.

Saglio, S.D. and H.S. Slayter. "Use of a radioimmunoassay to quantify thrombospondin." Blood 59 (1982): 162-166.

Saul, A., R. Lord, G. L. Jones, and L. Spencer. "Protective immunization with invariant peptides of the *Plasmodium falciparum* antigen MSA2." J Immunol 148 (1992): 208-11.

Schapira, A. "The resistance of falciparum malaria in Africa to 4-aminoquinolines and antifolates." Scand J Infect Dis Suppl 75 (1990): 1-64.

Schauer, R. "Chemistry and biology of the acylneuraminic acids." Angew. Chem. internat. edit 12 (1973): 127-138.

Scheibel, L.W., S.H. Ashton, and W. Trager. "*Plasmodium falciparum* microaerophilic requirements in human red blood cells." Experimental Parasitology 47 (1979): 410-418.

Scherf, A., R. Carter, C. Petersen, P. Alano, R. Nelson, M. Aikawa, D. Mattei, L. Pereira-da-Silva, and J. Leech. "Gene inactivation of Pf11-1 of *Plasmodium falciparum* by chromosome breakage and healing: identification of a gametocyte-specific protein with a potential role in gametogenesis." Embo J 11 (1992): 2293-301.

Scherf, A. and D. Mattei. "Cloning and characterization of chromosome breakpoints of *Plasmodium falciparum*: breakage and new telomere formation occurs frequently and randomly in subtelomeric genes." Nucleic Acids Res 20 (1992): 1491-6.

Schild, G. C. , J. S. Oxford, J. C. de Jong, and R. G. Webster. "Evidence for host-cell selection of *Influenza* virus antigenic variants." Nature 303 (1983): 706.

Schmidt, J. A., I. J. Udeinya, J. H. Leech, R. J. Hay, M. Aikawa, J. Barnwell, I. Green, and L. H. Miller. "*Plasmodium falciparum* malaria. An amelanotic melanoma cell line bears receptors for the knob ligand on infected erythrocytes." J. Clin. Invest. 70 (1982): 379-386.

Schofield, A. E., D. M. Reardon, and M. J. Tanner. "Defective anion transport activity of the abnormal band 3 in hereditary ovalocytic red blood cells." Nature 355 (1992): 836-8.

Schofield, L. "On the function of repetitive domains in protein antigens of *Plasmodium* and other eurkaryotic parasites." Parasitology Today 7 (1991): 99-105.

Schofield, L., G. R. Bushell, J. A. Cooper, A. J. Saul, J. A. Upcroft, and C. Kidson. "A rhoptry antigen of *Plasmodium falciparum* contains conserved and variable epitopes recognized by inhibitory monoclonal antibodies." Mol Biochem Parasitol 18 (1986): 183-95.

Schwab, J.C., C.J. Beckers, and K.A. Loiner. "The parasitophorous vacuole membrane surrounding intracellular *Toxoplasma gondii* functions as a molecular sieve." Proceedings of the National Academy of Sciences, USA 91 (1994): 509-513.

Scott, M. D., A. Ranz, F. A. Kuypers, B. H. Lubin, and S. R. Meshnick. "Parasite uptake of desferroxamine: a prerequisite for antimalarial activity." Br J Haematol 75 (1990): 598-602.

Senaldi, G., C. Vesin, R. Chang, G. E. Grau, and P. F. Piguet. "Role of polymorphonuclear neutrophil leukocytes and their integrin CD11a (LFA-1) in the pathogenesis of severe murine malaria." Infect Immun 62 (1994): 1144-9.

Service, M.V. "The *Anopheles* vector." In Bruce-Chwatt's Essential Malariology, ed. H.M. Gilles and D.A. Warrell. 96-124. third ed., Vol. Boston: Edward Arnold, 1993.

Sherman, I.W. "Biochemistry of Plasmodium (malarial parasites)." Microbiological Reviews 43 (1979): 453-495.

Sherman, I. W. "Membrane structure and function of malaria parasites and the infected erythrocyte." Parasitology 91 (1985): 609-45.

Sherman, I. W., I. Crandall, and H. Smith. "Membrane proteins involved in the adherence of *Plasmodium falciparum*-infected erythrocytes to the endothelium." Biol Cell 74 (1992): 161-78.

Sherman, I. W. and E. Valdez. "In vitro cytoadherence of *Plasmodium falciparum*-infected erythrocytes to melanoma cells: factors affecting adhesion." Parasitology 3 (1989): 359-69.

Sherman, I.W. and E. Winograd. "Antigens on the *Plasmodium falciparum* infected erythrocyte surface are not parasite derived." Parasitology Today 6 (1990): 317-320.

Sherman, I.W. Tanigoshi, L. "Incorporation of ^{14}C-amino-acids by malaria (*Plasmodium lophurae*). IV. In vivo utilization of host cell haemoglobin." International Journal of Biochemistry 1 (1970): 635-637.

Sherwood, J. A., D. D. Roberts, K. Marsh, E. B. Harvey, S. L. Spitalnik, L. H. Miller, and R. J. Howard. "Thrombospondin binding by parasitized erythrocyte isolates in falciparum malaria." Am J Trop Med Hyg 36 (1987): 228-33.

Sherwood, J. A., D. D. Roberts, S. L. Spitalnik, J. W. Lawler, L. H. Miller, and R. J. Howard. "Falciparum malaria parasitized erythrocytes bind to a carboxy-terminal thrombospondin fragment and not the amino-terminal heparin-binding region." Mol Biochem Parasitol 40 (1990): 173-81.

Sherwood, J. A., D. D. Roberts, S. L. Spitalnik, K. Marsh, E. B. Harvey, L. H. Miller, and R. J. Howard. "Studies of the receptors on melanoma cells for *Plasmodium falciparum* infected erythrocytes." Am J Trop Med Hyg 40 (1989): 119-27.

Shortt, H.E. Garnham.P.C.C. "Demonstration of a persisting exoerythrocytic cycle of *Plasmodium cynomolgi* and its bearing on the production of relapses." British Medical Journal 1 (1948): 1225-1228.

Siddiqui, W. A., L. Q. Tam, K. J. Kramer, G. S. Hui, S. E. Case, K. M. Yamaga, S. P. Chang, E. B. Chan, and S. C. Kan. "Merozoite surface coat precursor protein completely protects *Aotus* monkeys against *Plasmodium falciparum* malaria." Proc Natl Acad Sci U S A 84 (1987): 3014-8.

Siddiqui, W.A. and W. Trager. "Free amino-acids of blood plasma and erythrocytes of normal ducks and ducks infected with malarial parasite *Plasmodium lophurae*." Nature 214 (1967): 1046-1047.

Sim, B. K., C. E. Chitnis, K. Wasniowska, T. J. Hadley, and L. H. Miller. "Receptor and ligand domains for invasion of erythrocytes by *Plasmodium falciparum*." Science 264 (1994): 1941-4.

Sim, B. K., P. A. Orlandi, J. D. Haynes, F. W. Klotz, J. M. Carter, D. Camus, M. E. Zegans, and J. D. Chulay. "Primary structure of the 175K *Plasmodium falciparum* erythrocyte binding antigen and identification of a peptide which elicits antibodies that inhibit malaria merozoite invasion." J Cell Biol 111 (1990): 1877-84.

Sim, B. K., T. Toyoshima, J. D. Haynes, and M. Aikawa. "Localization of the 175-kilodalton erythrocyte binding antigen in micronemes of *Plasmodium falciparum* merozoites." Mol Biochem Parasitol 51 (1992): 157-9.

Sinden, R. E. "Gametocytogenesis in Plasmodium spp., and observations on the meiotic division." Ann Soc Belg Med Trop 2 (1985): 21-33.

Singh, B., M. Ho, S. Looareesuwan, E. Mathai, D. A. Warrell, and M. Hommel. "*Plasmodium falciparum*: inhibition/reversal of cytoadherence of Thai isolates to melanoma cells by local immune sera." Clin Exp Immunol 72 (1988): 145-50.

Singh, B., M. Monsigny, and M. Hommel. "Amino-sugars inhibit the in vitro cytoadherence of *Plasmodium falciparum*-infected erythrocytes to melanoma cells." Mol Biochem Parasitol 23 (1987): 47-53.

Slutsky, B., J. Buffo, and D. Soll. "High-Frequency Switching of Colony Morphology in *Candida Albicans*." Science 230 (1985): 666-669.

Smith, H., J. A. Nelson, C. G. Gahmberg, I. Crandall, and I. W. Sherman. "*Plasmodium falciparum*: cytoadherence of malaria-infected erythrocytes to human brain capillary and umbilical vein endothelial cells--a comparative study of adhesive ligands." Exp Parasitol 75 (1992): 269-80.

Snow, R. W., K. M. Rowan, S. W. Lindsay, and B. M. Greenwood. "A trial of bed nets (mosquito nets) as a malaria control strategy in a rural area of The Gambia, West Africa." Trans R Soc Trop Med Hyg 82 (1988): 212-5.

Spielman, A. "Why entomological antimalaria research should not focus on transgenic mosquitoes." Parasitology Today 10 (1994): 374-376.

Spitz, S. "The pathology of acute falciprum malaria." Milit. Surg. 99 (1946): 555-572.

Springer, T.A. "Adhesion receptors of the immune system." Nature 346 (1990): 425.

Steck, T.L., G. Fairbanks, and D.F.H. Wallach. "Disposition of the major proteins in the isolated erythrocyte membrane. Proteolytic dissection." Biochemistry 10 (1971): 2617-2624.

Sun, X., D.F. Mosher, and A. Rapraeger. "Heparan sulfate-mediated binding of epithelial cell surface proteoglycan to thrombospondin." Journal of Biological Chemistry 264 (1989): 2885.

Swanstrom, R. and P. R. Shank. "X-ray intensifying screens greatly enhance the detection of radioactive isotopes ^{32}P and ^{125}I." Anal. Biochem. 86 (1978): 184.

Swerlick, R. A., K. H. Lee, T. M. Wick, and T. J. Lawley. "Human dermal microvacular endothelial but not umbilical vein endothelial cells express CD36 in vivo and in vitro." J. Immunol. 148 (1992): 78-83.

Taliaferro, W. H. and H. W. Mulligan. "The histopathology of malaria with special reference to the function and origin of the macrophages in defense." Indian med. Res. Mem. 29 (1937): 1-138.

Tandon, N. N., R. H. Lipsky, W. H. Burgess, and G. A. Jamieson. "Isolation and characterization of platelet glycoprotein IV (CD36)." J. Biol. Chem. 264 (1989): 7570.

Tanner, M.J.A. "Erythrocyte membrane structure and function." In Malaria and the Red Cell (Ciba Foundation Symposium 94), ed. D. Evered and J. Whelan. 3-23. London: Pitman Press, 1983.

Taraschi, T.F. and E. Nicolas. "The parasitophorous duct pathway: new opportunities for antimalarial drug and vaccine development." Parasitology Today 10 (1994): 399-402.

Targett, G. A. "Chemotherapy and the immune response in parasitic infections." Parasitology 90 (1985): 661-73.

Taylor, D. W., M. Parra, G. B. Chapman, M. E. Stearns, J. Rener, M. Aikawa, S. Uni, S. B. Aley, L. J. Panton, and R. J. Howard. "Localization of *Plasmodium falciparum* histidine-rich protein 1 in the erythrocyte skeleton under knobs." Mol Biochem Parasitol 25 (1987): 165-74.

Taylor, T. E., M. E. Molyneux, J. J. Wirima, A. Borgstein, J. D. Goldring, and M. Hommel. "Intravenous immunoglobulin in the treatment of paediatric cerebral malaria." Clin Exp Immunol 90 (1992): 357-62.

Thomas, A. W., L. H. Bannister, and A. P. Waters. "Sixty-six kilodalton-related antigens of *Plasmodium knowlesi* are merozoite surface antigens associated with the apical prominence." Parasite Immunol 12 (1990): 105-13.

Trager, W. and J. B. Jensen. "Human malaria parasites in continuous culture." Science 193 (1976): 673-675.

Trager, W. and J. Williams. "Extracellular (axenic) development in vitro of the erythrocytic cycle of *Plasmodium falciparum*." Proc Natl Acad Sci U S A 89 (1992): 5351-5.

Treutiger, C. J., I. Hedlund, H. Helmby, J. Carlson, A. Jepson, P. Twumasi, D. Kwiatkowski, B. M. Greenwood, and M. Wahlgren. "Rosette formation in *Plasmodium falciparum* isolates and anti-rosette activity of sera from Gambians with cerebral or uncomplicated malaria." Am J Trop Med Hyg 46 (1992): 503-10.

Trigg, P.I. Plasmodiidae. Methods of cultivating parasites in vitro, ed. A.E.R. Taylor and J. R. Baker. New York: Academic Press, 1978.

Trigg, P. I. "Recent advances in malaria research: parasite biology, chemotherapy and host/parasite relationships." Ann Ist Super Sanita 21 (1985): 271-90.

Turner, C. M. R. and J. D. Barry. "High frequency of antigenic variation in *Trypanosoma brucei rhodesiense* infections." Parasitology 99 (1989): 67-75.

Turner, G.D.H., H. Morrison, M. Jones, T.M.E. Davis, S. Looareesuwan, I.D. Buley, K.C. Gatter, C.I. Newbold, S. Pukritayakamee, B. Nagachinnta, N.J. White, and A.R. Berendt. "An immunohistochemical study of the pathology of fatal malaria." American Journal of Pathology 145 (1994): 1057-1069.

Tuszynski, G.P, S. Srivastava, H.I. Switalska, J.C. Holt, C.S. Cierniewski, and S. Niewiarowski. "The interaction of human platelet thrombospondin with fibrinogen thrombospondin purification and specificity of interaction." Journal of Biological Chemistry 260 (1985): 12240-12245.

Udeinya, I. J. and C. O. Akogyeram. "Induction of adhesiveness in human endothelial cells by *Plasmodium falciparum*-infected erythrocytes." Am J Trop Med Hyg 48 (1993): 488-95.

Udeinya, I. J., J. Leech, M. Aikawa, and L. H. Miller. "An in vitro assay for sequestration: binding of *Plasmodium falciparum*-infected erythrocytes to formalin-fixed endothelial cells and amelanotic melanoma cells." J Protozool 32 (1985): 88-90.

Udeinya, I.J., L.H. Miller, I.A. McGregor, and J.B. Jensen. "*Plasmodium falciparum* strain-specific antibody blocks binding of infected erythrocytes to amelanotic melanoma cells." Nature 303 (1983): 429-431.

Udeinya, I. J., J. A. Schmidt, M. Aikawa, L. H. Miller, and I. Green. "Falciparum malaria infected erythrocytes specifically bind to cultured human endothelial cells." Science 213 (1981): 555-557.

Udomsangpetch, R., M. Aikawa, K. Berzins, M. Wahlgren, and P. Perlmann. "Cytoadherence of knobless *Plasmodium falciparum*-infected erythrocytes and its inhibition by a human monoclonal antibody [see comments]." Nature 338 (1989): 763-5.

Van-Schravendijk, M. R., B. L. Pasloske, D. I. Baruch, S. M. Handunnetti, and R. J. Howard. "Immunochemical characterization and differentiation of two approximately 300-kD erythrocyte membrane-associated proteins of *Plasmodium falciparum*, PfEMP1 and PfEMP3." Am J Trop Med Hyg 49 (1993): 552-65.

Van-Schravendijk, M. R., E. P. Rock, K. Marsh, Y. Ito, M. Aikawa, J. Neequaye, D. Ofori-Adjei, R. Rodriguez, M. E. Patarroyo, and R. J. Howard. "Characterization and localization of *Plasmodium falciparum* surface antigens on infected erythrocytes from west African patients." Blood 78 (1991): 226-36.

Vande-Waa, J. A., J. B. Jensen, M. A. Akood, and R. Bayoumi. "Longitudinal study on the in vitro immune response to *Plasmodium falciparum* in Sudan." Infect Immun 45 (1984): 505-10.

Varki, A. "Selectin ligands." Proceedings of the National Academy of Sciences 91 (1994): 7390-7397

Vermeulen, A. N., T. Ponnudurai, P. J. Beckers, J. P. Verhave, M. A. Smits, and J. H. Meuwissen. "Sequential expression of antigens on sexual stages of *Plasmodium falciparum* accessible to transmission-blocking antibodies in the mosquito." J Exp Med 162 (1985): 1460-76.

Vernick, K. D. and T. F. McCutchan. "Sequence and structure of a *Plasmodium falciparum* telomere." Mol Biochem Parasitol 28 (1988): 85-94.

Vial, H.J. , M.J. Thuet, and J.R. Philippot. "Phospholipid biosynthesis by *Plasmodium knowlesi*-infected erythrocytes. The incorporation of phospholipid precursors and the identification of previously undetected metabolic pathways." Journal of Parasitology 68 (1982a): 379-391.

Vial, H.J. , M.J. Thuet, and J.R. Philippot. "Phospholipid biosynthesis in synchronous *Plasmodium falciparum* cultures." Journal of Protozoology 29 (1982b): 258-263.

Wahlgren, M., J. Carlson, W. Ruangjirachuporn, D. Conway, H. Helmby, A. Martinez, M. E. Patarroyo, and E. Riley. "Geographical distribution of *Plasmodium falciparum* erythrocyte rosetting and frequency of rosetting antibodies in human sera." Am J Trop Med Hyg 43 (1990): 333-8.

Wainwright, S.D., W.J. Mawby, and M.J.A. Tanner. "The membrane domain of the human erythrocyte anion transport protein." Biochemical Journal 272 (1990): 265-268.

Wainwright, S.D., M.J.A. Tanner, G.E.M. Martin, J.E. Yendle, and C. Holmes. "Monoclonal antibodies to the membrane domain of the human erythrocyte anion transport protein." Biochemical Journal 258 (1989): 211-220.

Walliker, D. "Genetic recombination in malaria parasites." Exp Parasitol 69 (1989): 303-9.

Walliker, D., I. A. Quakyi, T. E. Wellems, T. F. McCutchan, A. Szarfman, W. T. London, L. M. Corcoran, T. R. Burkot, and R. Carter. "Genetic analysis of the human malaria parasite *Plasmodium falciparum*." Science 236 (1987): 1661-6.

Walsh, J. A. "Estimating the burden of illness in the tropics." In Tropical and Geographic Medicine, ed. K. S. Warren and A. A. F. Mahmoud. 1073. New York: MaGraw-Hill, 1984.

Warrell, D.A. "Pathophysiology of severe falciparum malaria in man." Parasitology 94 (1987): s53-76.

Warrell, D. A. "Treatment of severe malaria." J R Soc Med 17 (1989): 44-50.

Warrell, D. A. "Pathophysiology of severe falciparum malaria [editorial]." P N G Med J 35 (1992): 229-32.

Warrell, D.A. "Clinical features of malaria." In Bruce-Chwatt's Essential Malariology, ed. H.M. Gilles and D.A. Warrell. 35-50. third ed., Vol. Boston: Edward Arnold, 1993a.

Warrell, D.A. "Treatment and prevention of malaria." In Bruce-Chwatt's Essential Malariology, ed. H.M. Gilles and D.A. Warrell. 164-196. third ed., Vol. Boston: Edward Arnold, 1993b.

Warrell, D.A., S. Looareesuwan, and M.J. Warrell. "Dexamethasone proves deleterious in cerebral malaria: A double blind study in 100 comatose patiennts." New England Journal of Medicine 306 (1982): 313-319.

Warrell, D A., M. E. Molyneux, and P. F. Beales. "Severe and complicated malaria." Trans. R. Soc. Trop. Med. Hyg. 84 (1990): 1-65.

Waters, A.P., D.G. Higgins, and T.F. McCutchan. "The phylogeny of malaria: a useful study." Parasitology Today 9 (1993): 246-250.

Weatherall, D. J. and S. Abdalla. "The anaemia of *Plasmodium falciparum* malaria." Br Med Bull 38 (1982): 147-51.

Weatherall, D. J., J. I. Bell, J. B. Clegg, J. Flint, D. R. Higgs, A. V. Hill, G. Pasvol, and S. L. Thein. "Genetic factors as determinants of infectious disease transmission in human communities." Philos Trans R Soc Lond Biol 321 (1988): 327-48.

Weatherall, D. J. and J. B. Clegg. The Thalassaemia Syndromes. 3rd edn. ed., Oxford: Blackwell Scientific, 1981.

Wellems, T.E. "Molecular genetics of drug resistance in *Plasmodium falciparum* malaria." Parasitology Today 7 (1991): 110-112.

Wellems, T. E., L. J. Panton, I. Y. Gluzman, V. E. do-Rosario, R. W. Gwadz, A. Walker-Jonah, and D. J. Krogstad. "Chloroquine resistance not linked to mdr-like genes in a *Plasmodium falciparum* cross [see comments]." Nature 345 (1990): 253-5.

Wellems, T. E., D. Walliker, C. L. Smith, Rosario Ve do, W. L. Maloy, R. J. Howard, R. Carter, and T. F. McCutchan. "A histidine-rich protein gene marks a linkage group favored strongly in a genetic cross of *Plasmodium falciparum*." Cell 49 (1987): 633-42.

Wernsdorfer, W. J. H. and I. MacGregor. Malaria, The Principles and Practice of Malariology. London: Churchill Livingstone, 1988.

Wesseling, J. G., R. Dirks, M. A. Smits, and J. G. Schoenmakers. "Nucleotide sequence and expression of a beta-tubulin gene from *Plasmodium falciparum*, a malarial parasite of man." Gene 83 (1989): 301-9.

White, M.M. and M. Aylwin. "Niflumic and flufenamic acids are potent reversible blockers of $Ca(2^{+})$-activated Cl-channels in *Xenopus* oocytes." Molecular Pharmacology 37 (1990): 720-724.

White, N. J. "Clinical and pathological aspects of severe malaria." Acta Leiden 56 (1987): 27-46.

White, N. J. and M. Ho. "The pathophysiology of malaria." Adv Parasitol 31 (1992): 83-173.

White, N. J. and S. Pukrittayakamee. "Clinical malaria in the tropics." Med J Aust 159 (1993): 197-203.

Wick, T. M. and V. Louis. "Cytoadherence of *Plasmodium falciparum*-infected erythrocytes to human umbilical vein and human dermal microvascular endothelial cells under shear conditions." Am J Trop Med Hyg 45 (1991): 578-86.

Willcox, M., A. Bjorkman, and J. Brohult. "Falciparum malaria and beta-thalassaemia trait in northern Liberia." Ann Trop Med Parasitol 77 (1983): 335-47.

Wilson, C. M., A. E. Serrano, A. Wasley, M. P. Bogenschutz, A. H. Shankar, and D. F. Wirth. "Amplification of a gene related to mammalian mdr genes in drug-resistant *Plasmodium falciparum*." Science 244 (1989): 1184-6.

Wilson, R. J., I. A. McGregor, and P. J. Hall. "Persistence and recurrence of S antigens in *Plasmodium falciparum* infections in man." Trans. Roy. Soc. Med. Hyg. 69 (1975): 460-467.

Winograd, E., J. R. Greenan, and I. W. Sherman. "Expression of senescent antigen on erythrocytes infected with a knobby variant of the human malaria parasite *Plasmodium falciparum*." Proc Natl Acad Sci U S A 84 (1987): 1931-5.

Winograd, E. and I. W. Sherman. "Characterization of a modified red cell membrane protein expressed on erythrocytes infected with the human malaria parasite *Plasmodium falciparum*: possible role as a cytoadherent mediating protein." J Cell Biol 108 (1989a): 23-30.

Winograd, E. and I. W. Sherman. "Naturally occurring anti-band 3 autoantibodies recognize a high molecular weight protein on the surface of *Plasmodium falciparum* infected erythrocytes." Biochem Biophys Res Commun 160 (1989b): 1357-63.

WHO. World declaration of the control of malaria; Ministerial conference on Malaria. 1992.

Wrey, M. and G. Timperman. "Observations on the virulence and the antigenic characters of cloned and uncloned lines of the ANKA isolate of *Plasmodium berghei*." Ann. Soc. belge Med. trop. 59 (1979):

Wright, I.G., B.V. Goodger, and I.A. Clark. "Immunopathophysiology of *Babesia bovis* and *Plasmodium falciparum* infections." Parasitology Today 4 (1988): 214-218.

Wright, P. S., D. E. Cross-Doersen, K. K. Schroeder, T. L. Bowlin, P. P. McCann, and A. J. Bitonti. "Disruption of *Plasmodium falciparum*-infected erythrocyte cytoadherence to human melanoma cells with inhibitors of glycoprotein processing." Biochem Pharmacol 41 (1991): 1855-61.

Yourke, W. and J.W.S. Macfie. "Observations on malaria made during treatment of general paralysis." Transactions of the Royal Society of Tropical Medicine and Hygiene 18 (1924): 13-44.

Zheng, L., F. H. Collins, V. Kumar, and F. C. Kafatos. "A detailed genetic map for the X chromosome of the malaria vector, *Anopheles gambiae* [see comments]." Science 261 (1993): 605-8.

Acknowledgements

This thesis was funded by the award of a Wellcome Trust Prize Studentship, and I acknowledge the generous financial support I have received from The Wellcome Trust and my parents during the past three years.

I am extremely grateful to two people without whom this work would not have been possible. Dr Chris Newbold has been my supervisor for the past three years, and his guidance throughout the project and assistance with the writing of this thesis is much-appreciated. Secondly, I have been fortunate to share a bench in the laboratory with Bob Pinches, and it has been a pleasure to work with him. Bob was always helpful and patient towards me, and his guidance and tuition of techniques were invaluable in the early stages.

It has been a pleasure to work in the 'Malaria Lab', and I will miss the friends I have made at the IMM. I would especially like to thank Dr Sue Kyes who provided support in different guises: hotel services, cinema advice, reference typing, etc. I am grateful to Dr Dave Roberts for his critical advice and assistance with my post-doctoral fellowship applications. I thank Marie Meaden for her patient help with reference-hunting and 'stationery management'. I thank everyone who helped to ~~proov-reed~~ proof-read this thesis, and Rachael Ritchie for the lunches (and for agreeing to marry my brother!).

Away from science, I would like to thank Emma for her love and friendship which are always appreciated. I thank her for not giving up through the good and bad times in the past three years and her support during the last three months has been immeasurable.

And finally.... I thank my Mum for the million and one things (the list would be another thesis), especially during the past three months at home and for the twenty three years before that. Thanks to my Dad for use of his study and computer, for the encouragement, and for the best advice of the lot!

Appendix

Results obtained during the course of this thesis have been communicated in the following presentations:

Presentation	Type	Venue	Date
Oxford University Tropical Network Meeting	Oral	Oxford	Feb 1992
Oxford University Tropical Network Meeting	Oral	Oxford	Feb 1993
Wellcome Trust Red Cell Meeting	Poster	London	Mar 1993
British Society of Parasitology: Malaria Meeting	Oral	Oxford	Apr 1994
Oxford University Tropical Network Meeting	Oral	Oxford	May 1994
Wellcome Trust Prize Students' Meeting	Oral	London	Jul 1994
International Molecular Parasitology Meeting	Poster	Cape Cod, USA	Sep 1994
Invited Seminar, Stanford Medical School	Oral	San Francisco, USA	Sep 1994
Invited Seminar, Scripps Research Institute	Oral	San Diego, USA	Oct 1994
Invited Seminar, Festschrift for Dr. Neil Brown	Oral	London	Nov 1994

PUBLICATIONS

Two papers based on this work are in preparation, and part of this study has been reviewed in:

Molecular mechanisms of sequestration in malaria.
A. R. Berendt, D. J. P. Ferguson, J. Gardner, G. Turner, A. Rowe, C. McCormick, D. Roberts, A. Craig, R. Pinches, B. C. Elford and C. I. Newbold.
Parasitology (1994) **108**, S19-28