"The molecular basis of tick-host interactions"

THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY AT THE UNIVERSITY OF OXFORD

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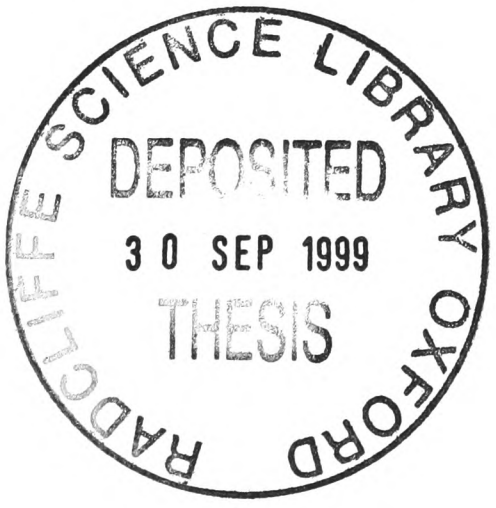
TRINITY TERM 1999



ABSTRACT

The Molecular Basis of Tick-Host Interactions

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Submitted for the degree of D.Phil. Trinity Term 1999

Ticks are obligate haematophagous arthropods that represent a major economic drain upon the world's livestock as well being a significant medical and veterinary risk through the transmission of tick-borne pathogens such as *Borrelia burgdorferi*, the causative agent of Lyme disease.

The tick-host relationship is a function of both ecological and physiological factors. Successful feeding requires the effective acquisition and digestion of a bloodmeal by the tick. Acquisition relies upon the ability of the tick to counteract host immune responses induced by the extended feeding periods of ixodid ticks (up to 2 weeks). The host response to tick infestation and the consequent countermeasures employed by the tick, constitute the tick-host interface.

The immune response of hosts to *Ixodes ricinus* infestations was examined through antigenic profiling. The antigens exposed to the host were shown to vary throughout the feeding period and differed between the different development stages of *I. ricinus*. It was also shown that different host species infested with *I. ricinus* recognised different antigens. This was true of both natural and non-natural hosts, and even closely related species.

Anti-complement activity was investigated in the salivary glands of *Ixodes* ticks. This activity was shown to inhibit some host species but not others. The pattern of inhibitory activity varied between the tick species tested in a way that was consistent with known tick host-preferences. The mechanisms of anti-complement activity in *I. ricinus* salivary glands were explored. The alternative but not the classical pathway of complement was inhibited. Activity was present in unfed ticks and throughout the feeding period. Three targets of the complement system were identified as being modulated by the tick.

Digestion of the bloodmeal was explored and a haemolytic activity was associated with the salivary glands of *I. ricinus* ticks. The activity was demonstrated to be Mg^{2+}-dependent.

In addition, a subtractive cDNA library enriched for saliva-associated transcripts was successfully produced. Random sampling identified putative differentially expressed genes.

The results of this thesis illustrate the complexity of tick-host interactions at the molecular level. It is apparent that the research described poses many more questions than answers.

ACKNOWLEDGEMENTS

This work was funded by NERC CASE studentship reference no. GT4/96/246/T. Special thanks to my supervisors Prof. Patricia A. Nuttall (IVEM) and Dr. Sarah E. Randolph (Zoology) for their support financially and technically. Thanks to all at IVEM, especially the 'tick-group', past and present for making this work a pleasurable experience. In particular I would like to thank Guido Paessen, Miles Nunn, Somchai Sangmanadech and Klaus Kurtenbach for their assistance and technical advice.

I am indebted to Dr. Robert Sim and all at his lab, as well as to Dr. Alister Dodds at the MRC Immunochemistry Unit for their assistance and for the supply of complement material. Special thanks to Dr. Hua-Tang Zhang (Institute of Molecular Medicine, John Radcliffe Hospital, Oxford) for his technical assistance in the production of the subtractive cDNA library.

Thanks to Dr. Sarah Wanless (Institute of Terrestrial Ecology, Banchory, UK.) for collecting *I. uriae* from seabird colonies. Thanks to Chris Hatton (IVEM) for photography, Tony Willis (MRC Immunochemistry) for N-terminal sequencing and to Val Cooper (Dyson Perrins) for primer synthesis.

Abbreviations

µg	microgram
µl	microlitre
µM	micromolar
^{0}C	degrees centigrade
AH_{50}	alternative pathway of complement haemolytic assay
amp	ampicillin
amp^r	ampicillin resistance
APS	ammonium persulphate
ATP	adenosine triphosphate
A_x	absorbance at *x* nanometres
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bis	N-N' methylenebisacrylamide
bp	basepair
BSA	bovine serum albumin
Ca^{2+}	calcium
cat. no.	catalogue number
cDNA	complementary DNA
CH_{50}	classical pathway of complement haemolytic assay
CR	complement receptor
CVF	cobra venom factor
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulphoxide

DNA	deoxyribonucleic acid
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphates
dsDNA(RNA)	double stranded DNA (RNA)
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
EGTA	ethyleneglycoltetra-acetic acid
EtBr	ethidium bromide
EtOH	ethanol
fc	flow cell (Biacore)
g	gram
GVB	gelatin, veronal buffered saline
h	hour
HRPO	horse radish peroxidase
I. hexagonus	*Ixodes hexagonus*
I. ricinus	*Ixodes ricinus*
I. scapularis	*Ixodes scapularis* (≡ *dammini*)
I. uriae	*Ixodes uriae*
Ig	immunoglobulin
IL-	interleukin
IPTG	isopropylthio-β-D-thiogalactopyranosidase
IVEM	Institute of Virology and Environmental Microbiology
kb	kilo base (pairs)

kDa	kilo Dalton
Krpm	kilo rpm
LB	Luria-Bertani medium
M	mole
mAb	monoclonal antibody
mg	milligram
Mg^{2+}	magnesium
min	minutes
ml	millilitre
mM	millimolar
mRNA	messenger RNA
M_w	relative molecular weight
ng	nanogram
nM	nanomolar
oligos	oligonucleotide primers
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline pH 7.4
PCR	polymerase chain reaction
pfu	plaque forming units
RdRp	RNA-dependent RNA polymerase
RNA	ribonucleic acid
RNase	ribonuclease

rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
RU	resonance units
SAT	saliva-activated transmission
SDS	sodium dodecyl sulphate
SE	standard error
SGE	salivary gland extract
ssDNA(RNA)	single stranded DNA (RNA)
SSH-PCR	suppression subtractive hybridisation polymerase chain reaction
STE	Salt, Tris, EDTA buffer.
TAE	Tris, acetate, EDTA buffer
TBE	Tris, borate, EDTA buffer
TE	Tris, EDTA buffer
TEMED	N-N-N'-N' tetramethylethylenediamine
Tm	melting temperature
TNFα	tumour necrosis factor alpha
UV	ultraviolet radiation
vol	volume
X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactosidase

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Ticks represent a major economic drain upon the world's agri-industry. This is the result of both the parasitic burden on hosts, including livestock, and the role of ticks as a major vector of pathogens. A wide range of pathogens are transmitted by ticks, including protozoa, bacteria and viruses, to many host species including reptiles, birds and mammals (including humans). Tick-vectored pathogens such as *Borrelia burgdorferi*, tick-borne encephalitis virus and *Babesia* are of great medical and veterinary importance. The successful transmission of these micro-parasites is dependent upon interactions that occur between the tick with its host at the feeding site. Compared with other parasite classes, there is relatively little research on ectoparasites in general and about ticks in particular. The aim of this study is to increase that knowledge base by investigating the molecular basis of the tick-host relationship.

1.2 Tick life cycles

Ticks are obligate haematophagous ectoparasites that have been found to infest every class of terrestrial vertebrates (Sonenshine, 1991). Ticks form part of the taxonomic order *Acarina* (ticks, mites), comprising three families (*Argasidae, Nuttalliellidae,*

Ixodidae) which are sub-divided into 19 genera and constitute over 850 species (Keirans, 1991).

The same basic life cycle is followed by all tick species. A hexapod larva hatches from the egg and develops into an octapod nymph before moulting into an adult, male or female. Each developmental stage requires a blood meal; if the female adult does not get a blood meal, vitellogenesis and consequently egg-laying are not possible, although some exceptions do occur (Aeschlimann, 1993). Argasidae (soft) ticks feed upon a host for a short time (from minutes to 1 hour, although larvae may feed for days) and are polyphasic with many (three to seven) nymphal instars. In contrast, Ixodidae (hard) ticks are long feeders (up to two weeks for some *Amblyomma* species) and can have a mono- di- or triphasic life cycle. *Ixodes* species are triphasic, and leave the host after each blood meal (one per developmental stage). Some *Ixodes* species such as *Ixodes ricinus* are tri-trophic, each developmental stage differentially parasitising a different, but sometimes overlapping range of host species (e.g. red deer is parasitised by all three stages of *I. ricinus*) (Fig 1.1).

1.3 *Ixodes ricinus* – European Lyme disease vector

Ixodidae represent the most economically important tick family, and the most extensively studied (Brossard *et al.*, 1993). Of the 637 species in this family, the Prostriata, genus *Ixodes*, contains about 240 species (Black IV and Piesman, 1994).

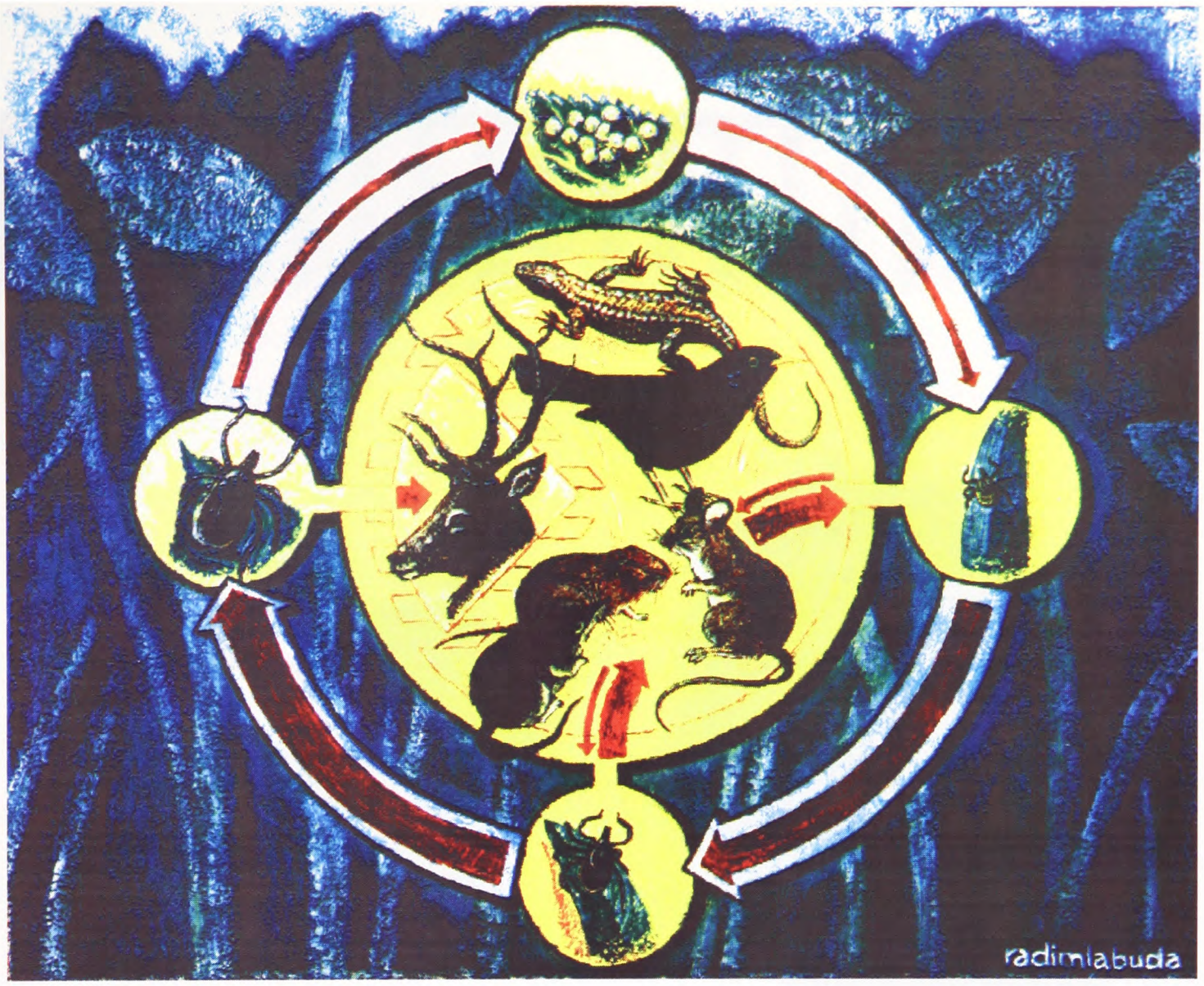

Fig 1.1 Schematic diagram of the life-cycle of *I. ricinus* illustrating the different life stages and typical hosts of those stages. Reproduced with kind permission from Pat Nuttall.

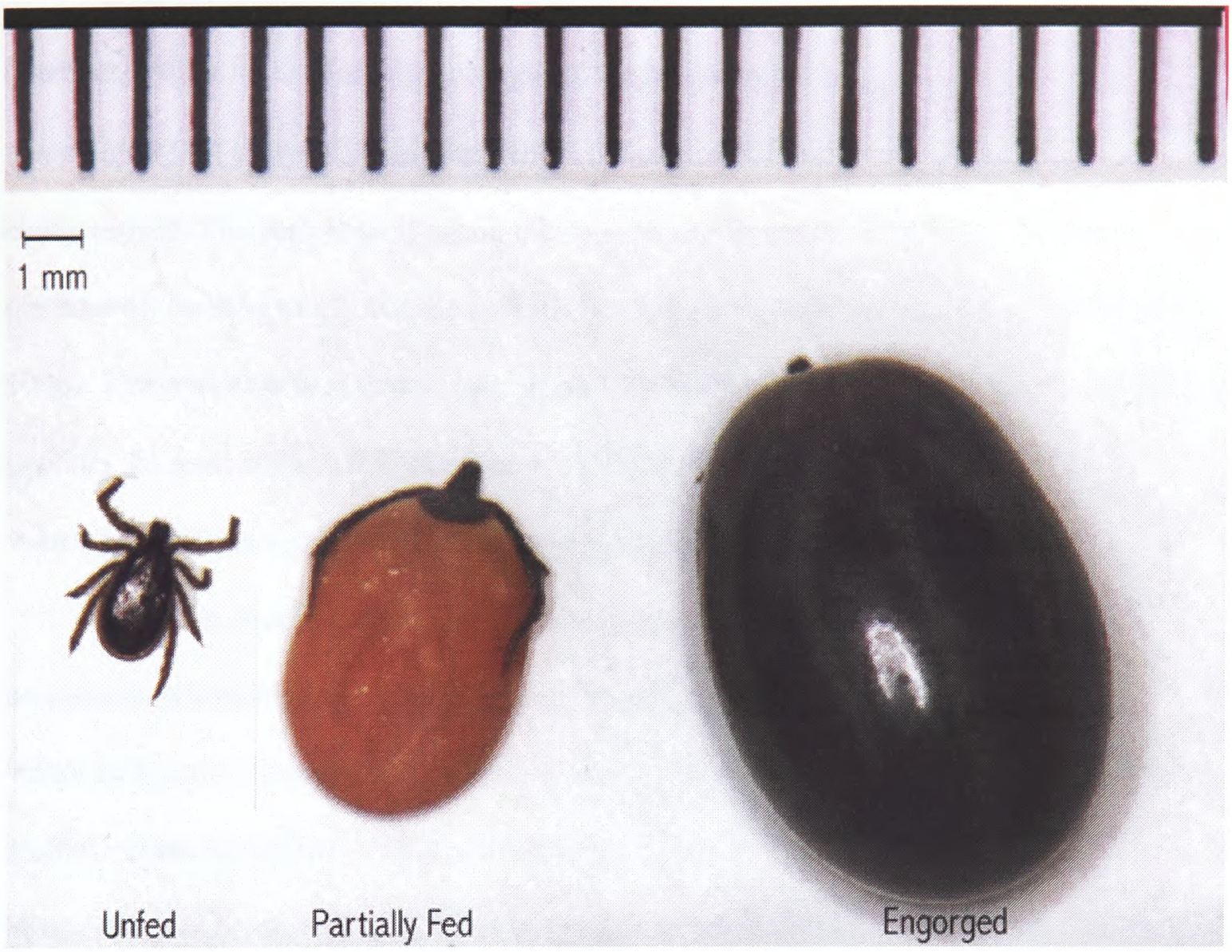

Fig 1.2 Photograph illustrating *I. ricinus* female adult tick development as a consequence of feeding. The unfed tick is an adult male.

Lyme borreliosis is probably the commonest tick-borne infection in the world (Bennett, 1995), being endemic throughout the Northern hemisphere. The disease was named after Old Lyme, Connecticut where the nature of the infection was first characterised. The aetiological agent, the spirochaete *Borrelia burgdorferi,* was first linked with incidences of juvenile arthritis in 1982 (Burgdorfer *et al.*, 1982). In the early 1990s, Lyme disease was ranked second only to AIDS in the degree of public concern towards diseases in the USA (Forschner, 1992) and in 1992 accounted for 90% of all vector-borne illnesses reported in the USA (Bennett, 1995).

In North America the major vector of Lyme disease is the deer tick, *Ixodes scapularis*, whilst from Eastern Europe to Japan it is *Ixodes persulcatus*. The major vector of Lyme disease in Europe and the major tick species of interest to this research is *Ixodes ricinus* (Krampitz, 1986) (shown in Fig 1.2). The distantly related *Ixodes hexagonus* and *Ixodes uriae*, which can vector *Borrelia*, are also investigated. *I. ricinus* is commonly found throughout northern Europe in areas below 1500m altitude. It has been found from 65° north to as far south as North Africa (Anderson and Magnarelli , 1993) and from Great Britain and Ireland into eastern Europe (Anderson, 1989).

The epidemiology of Lyme disease and other tick-borne pathogens is the result of the dynamic relationship between host, pathogen and vector, illustrated in Fig 1.3. A greater understanding of the tick-host interface may provide an insight into the mechanisms of transmission by ticks of such pathogens.

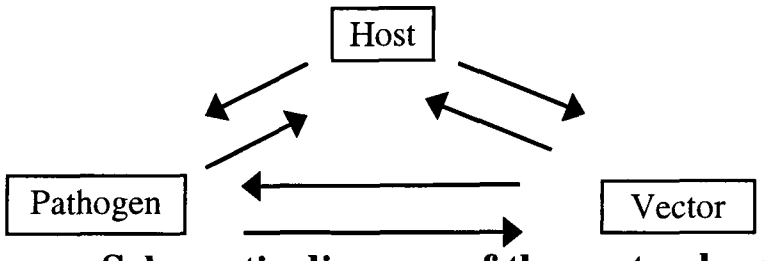

Fig 1.3 Schematic diagram of the vector-host-pathogen relationship.

1.4 The tick feeding site

During attachment to a host, the tick rostrum (hypostome and chelicerae) penetrates deeply into the dermis of the host, sometimes piercing cartilage to form a feeding lesion (Brossard and Fivaz, 1982). In contrast, other haematophagous arthropods such as mosquitoes and *Rhodnius prolixus* feed directly upon a dermal venule or arteriole (Lavoipierre *et al.*, 1959; Ribeiro, 1987b). Rabbit skin biopsies infested with female adult *Ixodes dammini*[1] ticks showed that at the beginning of a primary infestation, the rostrum is surrounded by a collagenous area (Wheeler *et al.*, 1989). A feeding lesion forms beneath the rostrum that is believed to be the result of host inflammatory reactions (Brossard *et al.*, 1993). Skin biopsies of dogs infested with *Rhipicephalus sanguineus* show a massive influx of neutrophils and release of hydrolytic granules, which is thought to form the feeding lesion (Brown and Knapp, 1980; Tatchell and Moorhouse, 1970). Cytolysins found in tick saliva may also contribute to the generation of the lesion (Kemp *et al.*, 1982). The tick feeds on primarily lysed tissue and leukocytes (at least in the case of *Dermacentor reticulatus*) as well as red blood cells during the slow phase of feeding, followed by the ingestion of blood contained in this lesion during the rapid phase of feeding (12-24 hours prior to drop off) (Brossard *et al.*, 1993). Ticks concentrate imbibed blood through osmoregulatory mechanisms, increasing the amount of protein available to the tick for digestion (Sauer *et al.*, 1992). In this fashion, ixodid ticks may increase their engorged body weight to over 100 times their unfed body weight (Bergman, 1996).

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[1] The conspecificity of *Ixodes dammini* and *Ixodes scapularis* has been established (Oliver *et al.*, 1993), and where mentioned in the text, the species descriptor used will be that used in the cited reference.

1.5 The tick-host interface

The tick-host relationship is the result of co-evolution between parasite and vertebrate host over 245 million years (Hoogstraal and Aeschlimann, 1982). Like other host-parasite relationships, co-evolution may be manifest at the cellular, molecular, and genetic levels (Hafner *et al.*, 1994). The tick-host relationship is a function of both ecological (probability of encountering a particular host) and physiological factors (i.e. the ability of the tick to feed successfully on a particular host). The feeding success of a tick upon a given host depends on its ability both to attach and to feed to repletion, which in turn relies upon the ability of the tick to antagonise host specific inflammatory responses (Ribeiro, 1987a). Literature describing the tick-host interface tends to fall into two categories; the demonstration of specific host responses to tick infestation, and the description of how tick salivary gland activities modulate these responses. These apparently contradictory areas of research illustrate the complex nature of the host-tick interface. The focus of this research is to examine host responses and tick counter-measures at the molecular level.

1.6 **Host immune systems**

The immune system of vertebrates has evolved in order to protect against pathogens, which include viruses, bacteria, protozoa and multicellular parasites. How the immune system of particular species responds is dependent upon the site of infection and the type of pathogen involved (Roitt *et al.*, 1996). Any immune response involves, firstly, recognition of the pathogen or foreign material, and secondly, mounting a reaction in order to eliminate it. In broad terms, immune responses can be classed as innate (non-specific) and adaptive (pathogen specific). Although the innate response does not alter on repeated exposure to a pathogen, the adaptive response improves with each successive exposure.

Immune responses involve both cellular and humoral factors that circulate constantly throughout the body. When an infection or tissue damage occurs, it is necessary to concentrate these factors and their products at the affected site. This process manifests itself as inflammation. An inflammatory response is characterised by three major events:

- Increase in blood supply to the area.
- Endothelial cells retract causing increased capillary permeability and allowing immune mediators to reach the site.
- Leukocytes migrate to the site. In the earliest stages of inflammation, mainly neutrophils are recruited; in later stages monocytes and lymphocytes migrate to the site.

The immunological interactions that occur at the tick-host interface involve both innate and adaptive responses by the host and the immunomodulatory countermeasures employed by the tick (Wikel and Bergman, 1997). The way in which immunity is expressed in responses to tick infestation varies greatly, depending upon the host species and tick species (Willadsen, 1980).

The tick-host interface occurs at the epidermal and dermal layer of the host; in consequence, these sites are the major initiators of both the innate and the adaptive response in hosts. Relatively recent studies have revealed the complexity and importance of these tissues in immune responses at both the cellular and humoral level. Distinct from systemic immunity, cutaneous immunity has been termed the skin immune system (Bos *et al.*, 1997). Immunocompetent cells are present in the cutaneous layer as resident, recruited and recirculating populations representing both adaptive (e.g. dermal dendritic cells, Langerhan cells and skin-associated T cells) as well as innate response cell types (Bos *et al.*, 1997). The keratinocytes, which comprise 95% of the mass of human epidermis, produce a multitude of cytokines including interleukins, chemokines, interferons, growth factors and angiogenic factors (Barker, 1997).

Multitudes of factors are involved in host responses to tick infestation. As a consequence of the extended feeding time of ixodid ticks, there is ample time for tick antigens to be presented by antigen-presenting cells to cells which are traditionally classified as being associated with the adaptive response such as lymphocytes. Hence the distinction between immune factors as being associated either with the adaptive or innate responses, masks the complexity of the immune system of vertebrates, particularly in respect to ixodid tick infestations.

1.6.1 **Acquired immunity to ticks**

Repeated tick infestations of some host species lead to the development of acquired resistance against the tick. This is manifested as reduced engorgement weight, longer feeding periods, decreased ova production, inhibited moulting and increased egg and tick mortality (Wikel, 1996a). Comparison of susceptible hosts with animals expressing acquired resistance to ticks shows that host cutaneous reactions differ grossly (and microscopically) at the attachment site (Wikel and Bergman, 1997). Tick salivary proteins have been shown to modulate these responses in a number of ways including the modulation of complement, antibody production, T-lymphocyte proliferative responses, and cytokine elaboration by antigen presenting cells and T lymphocytes (Wikel *et al.*, 1996b).

Studies of *Clethrionomys glareolus* (bank vole) and *Apodemus flavicollis* (woodmouse), both natural hosts of *I. ricinus*, showed that the former developed acquired resistance while the latter species did not. Inhibition of resistance through cyclosporin A treatment suggests the involvement of T-helper cells (Dizij and Kurtenbach, 1995). The reason for this difference is unknown, although the absence of acquired resistance in *C. glareolus* populations from North Sweden, where *I. ricinus* was introduced during the last 50 years, lead to the suggestion that acquired resistance is under selective pressure. The subject of acquired immunity to ticks has been extensively reviewed and will not be covered further here (Brossard *et al.*, 1993; Brown, 1985; Wikel, 1996a).

The adaptive response is characterised by the production of tick-specific antibodies, directed against tick material exposed during the feeding process, of which the salivary glands are the most important tissue involved (Uhlir *et al.*, 1994). How

particular hosts immunologically perceive ticks is explored in this thesis via antigenic profiling.

1.6.2 **Innate immunity to ticks**

When considering the innate (natural) immunity of a host to a tick, it is important to clarify what is meant by this term. In the context of this research, innate immunity describes the response of a host to the tick in the absence of acquired immune responses, i.e. host responses that are not tick-specific (Roitt *et al.*, 1996). This is not to be confused with 'innately resistant' hosts, which are described as a single animal of a species that is normally a host, being resistant to the parasite prior to previous exposure to that parasite (Willadsen, 1980). In other words, acquired resistance is a tick specific event, which can be thought of as being in addition to, rather than instead of, the host's innate resistance to infestation by ticks.

The innate response of a host to tick infestation, in common with acquired resistance, is mediated by both cellular and humoral factors. 'Recognition' of foreign material by the innate system relies mainly upon cellular phagocytes such as monocytes, macrophages and polymorphonuclear neutrophils, and by humoral factors, of which the alternative pathway of complement is arguably the most important. ixodid ticks have extended mouth-parts that pierce both epidermal and dermal layers. Tissue damage resulting from the initial incision by the tick, induces epidermal keratinocytes and endothelial cells to secrete the pro-inflammatory cytokines IL-1 and TNFα. These cytokines diffuse into the dermis where the dermal vasculature is activated to produce adhesion molecules necessary for the recruitment of circulating leukocytes. TNFα and

IL-1 produce autocrine and juxtacrine effects including induction of chemokines and other pro-inflammatory cytokines. Lymphocytes become aligned with antigen presenting cells, particularly Langerhan cells, and produce effector cytokines including IFN-γ (Barker, 1997). Cooperative interactions between Langerhan cells and dermal lymphocytes result in the coordinated shuttling of protein antigens to draining lymph nodes where sensitisation may occur most efficiently. Mast cells are effector cells present in the dermal layer that are stimulated by IgE, resulting in degranulation of these cells and the subsequent release of inflammatory mediators including histamine.

As a consequence the tick must deploy counter measures against such components of the immune system in order to feed successfully. Salivary gland extract (SGE) from female *Dermacentor reticulatus* reduces the ability of natural killer cells to kill tumour cells *in vitro* (Kubes, 1994). *Dermacentor andersoni* SGE suppresses macrophage elaboration *in vitro* (Ramachandra and Wikel, 1992). Histamine-binding proteins have been isolated from the salivary gland extract of *R. appendiculatus* and *D. andersoni* ((Paesen *et al.*, 1999) and Somchai Sangemandech- unpublished data). Tick anti-complement activity will be covered in detail later in the text.

It is the purpose of this research to obtain clues as to how a tick counteracts the innate response of a particular host through the investigation of salivary gland associated inhibition of the alternative pathway of complement.

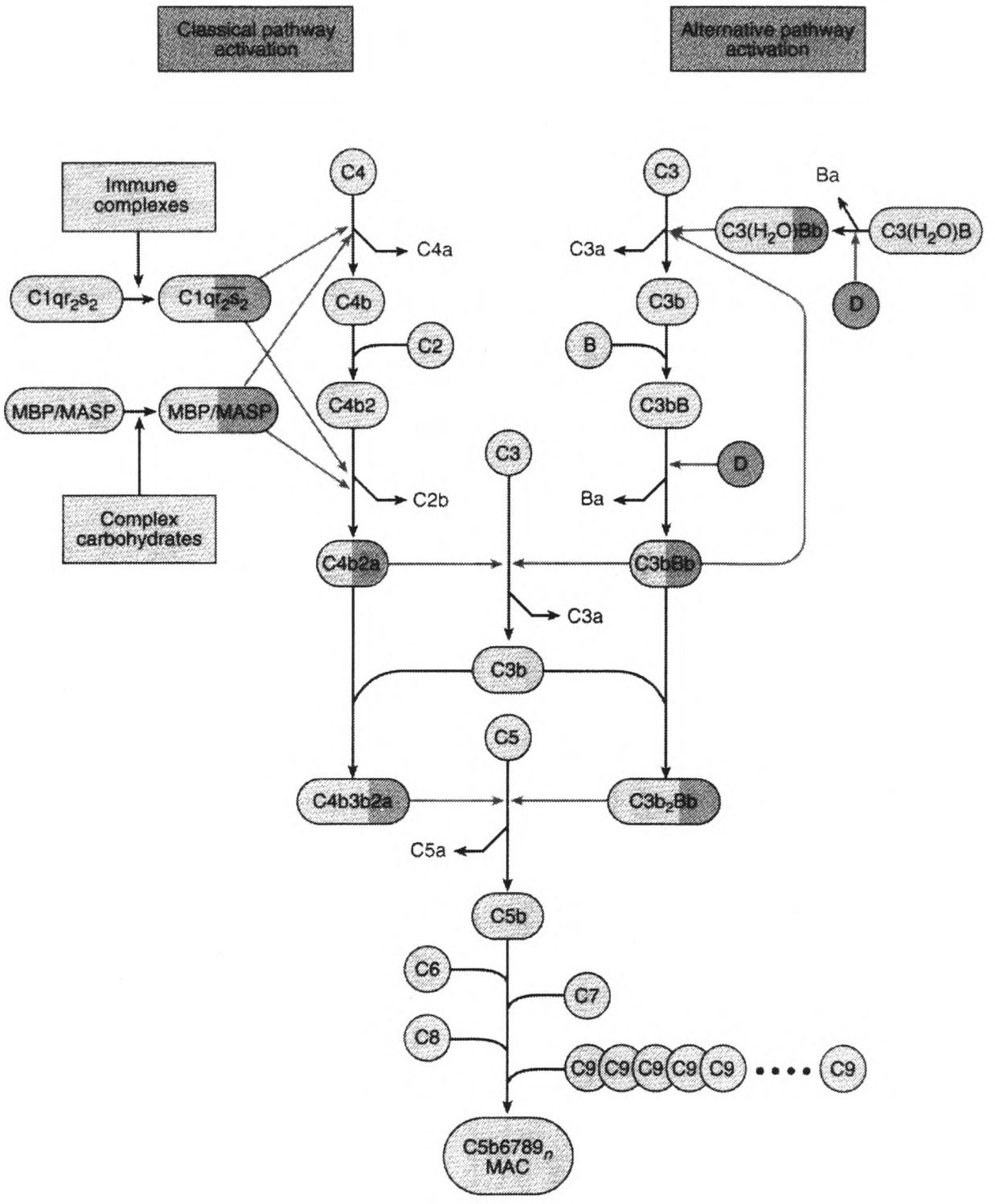


Fig 1.4 Schematic diagram of complement pathway. Reproduced from Law and Reid 1995.

1.7 The Complement System

Complement was first described in the 1890s as a heat-labile serum factor required along with heat stable antibody for bactericidal activity (Law and Reid , 1995). An alternative pathway, acting independently of antibodies, was proposed in the 1950s, but it was not until the 1970s that evidence for this pathway was accepted (Lepow, 1980). Today, more than 30 proteins in serum and on cell surfaces, have been shown to be closely involved in the complement system.

Complement forms the principal effector arm of the humoral immune system. In addition to its role in innate immunity, complement also interacts with the adaptive immune system, through recognition and activation via antigen-antibody complexes, regulation of B lymphocyte activity, and participation in antigen localisation to antigen-presenting cells (Sim and Dodds, 1997).

Complement can be activated via the classical pathway (which includes the antibody-independent lectin pathway) or via the alternative pathway (Fig 1.4). Activation of either pathway triggers a proteolytic cascade, ultimately resulting in the proteolytic conversion of C3 to C3b and C3a by either the classical pathway convertase C4b2a or the alternative pathway convertase C3bBb. C3a is an anaphylatoxin and consists of the first 77 amino acids of the α-chain of C3. Cleavage of C3a induces a conformational change in C3b resulting in the exposure of an internal thioester bond (Law and Dodds, 1997). This highly reactive thioester bond can bind covalently to any available nucleophiles including hydroxyl or amino sites of suitable surfaces.

Activated C3b can bind to an activating surface; the binding acts as a focus for phagocytosis (via complement receptor containing phagocytes) and/or for the formation of the lytic membrane attack complex (MAC). The short half-life of the thioester bond (10 μsec) acts to localise the response, negatively regulating systemic activation (Sepp *et al.*, 1993). This is largely due to the presence of the highly effective nucleophile, H_2O that is present at 55M. Thus the majority (~90%) of activated C3b forms inactive fluid phase C3b, which is cleaved by factor I and cofactor H (Law and Reid , 1995). The fate of C3 is considered further in Chapter6.

There is a constant, albeit slow, spontaneous turnover of C3 in which the thioester bond is hydrolysed without the loss of C3a. This molecule, designated $C3(H_2O)$, has a C3b like conformation which can act as a C3 convertase when bound to factor B and in the presence of factor D (Isenman *et al.*, 1981). It generates C3b constantly at a low level, which in turn can bind to host and 'foreign' surfaces. Consequently, there is a constant 'testing' of all serum exposed surfaces. The complement system also plays a major role in immune complex removal (Law and Reid , 1995).

Although the likelihood of activated C3b binding to an activating surface is low, such a binding event results in a positive amplification loop involving both classical and alternative pathway activation, causing rapid and effective opsonization of the activating surface.

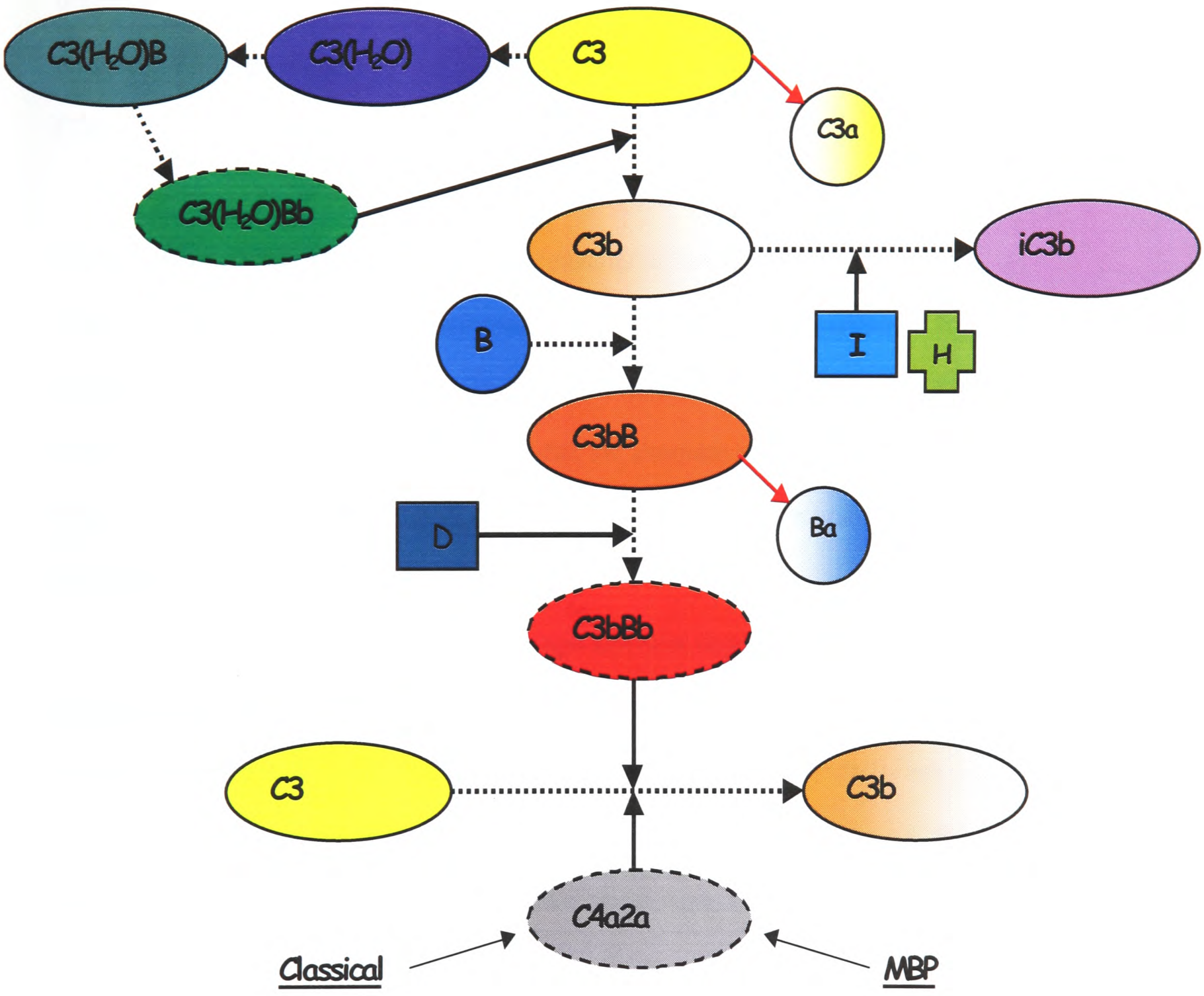


Fig 1.5 Schematic diagram of the alternative pathway of complement. Convertases are indicated as dotted lines.

1.7.1 **The alternative pathway of complement**

The alternative pathway of complement can be activated in an antibody-dependent manner via IgG and IgA and in an antibody-independent manner in the presence of foreign bodies containing suitable activating surfaces for the deposition of C3b.

Factor B can bind to membrane-bound C3b (fluid phase C3b is rapidly cleaved by factor I and co-factor H) in a Mg-dependent manner to form C3bB (Fig 1.5). Factor D binds to C3bB, cleaving factor B to form the alternative pathway convertase C3bBb. This results in positive amplification as more C3b is formed. The C3b portion of C3bBb can bind a second C3b molecule to form $(C3b)_2Bb$, the alternative pathway C5 convertase (Fig 1.4). Proteolytic cleavage of C5 results in initiation of the membrane attack complex formation.

Whether a surface is an activator or non-activator depends upon how long the deposited C3b survives. Non-activating surfaces have a high affinity for the regulatory protein factor H which binds the C3b allowing rapid degradation to iC3b by the protease factor I. Host cells contain surface receptors such as complement receptor 1 (CR1) and decay accelerating factor (DAF) which act in a similar way to factor H, positively regulating factor I mediated cleavage.

Factor I cleaves C3b at two positions in the α' chain to yield iC3b and C3f. iC3b is further cleaved by serum proteases to form C3c and C3dg which is further cleaved producing C3d and C3g (Harrison and Lachmann, 1980; Sim *et al.*, 1993) (Fig 1.6). Although these fragments have no role in the activation of complement, they are important ligands of CR2 and CR3 receptors. These receptors play an important role in

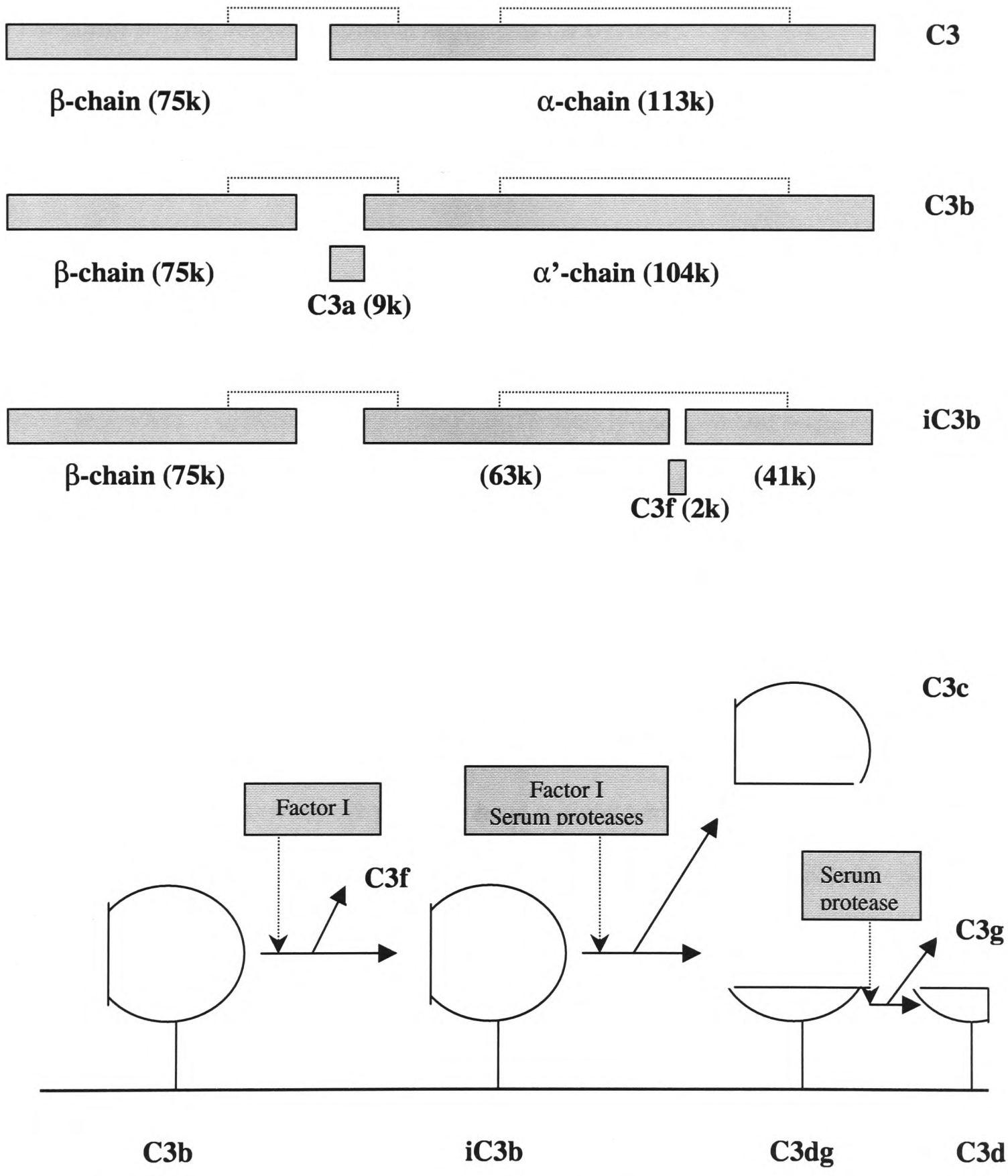


Fig 1.6 (Top) schematic diagram of C3 and cleavage products showing approximate molecular weights, disulphide bonds indicated by dotted lines. (Bottom) schematic diagram of surface bound C3b breakdown by factor I and serum proteases.

immune complex formation and antigen presentation, and are found on many immune cells including B-lymphocytes and neutrophils (Sim and Dodds, 1997). Another regulatory protein, properdin, the first alternative pathway component to be identified, positively controls the convertases C3bBb or $(C3b)_2Bb$ by stabilising the complexes.

1.7.2 **The classical pathway of complement**

Like the alternative pathway, the classical pathway can be activated in both an antibody-dependent (via Fc receptor binding) and antibody-independent manner. Activation has been most thoroughly investigated using IgG or IgM. Many other substances can activate the classical pathway independent of antibody, including chromatin, nucleic acids, mitochondrial membranes, viruses such as murine leukaemia virus as well as Gram-positive and Gram-negative bacteria (Taylor, 1993).

The initial step in classical pathway activation is the binding of C1q to an activator (Fig 1.4). C1q contains six globular head regions which can bind to immunoglobulins or other targets in a calcium-dependent manner. Activation requires multiple binding events and activators tend to contain repetitive structures (e.g. bacterial cell wall). When two or more C1q molecules bind to an activator, C1q undergoes a conformational change which results in autoactivation of the C1q-bound C1r. C1r in turn activates C1s which cleaves C4 to form C4b. C4b, like C3b, is activated proteolytically to expose a short-lived thioester bond which can react non-specifically with nucleophiles. C2 is a homologue of factor B that binds to C4b and is cleaved by C1s to form the classical pathway C3 convertase, C4b2a. The C3 convertase can cleave C3 resulting in activation of the alternative pathway. C3b can bind covalently to the C4b portion of the

complex to form the classical pathway C5 convertase, C4b3b2a. C5 cleavage results in initiation of the membrane attack complex.

Activation of the classical pathway can also occur via the lectin-binding pathway. Mannose-binding lectin (MBL) can substitute for C1q and purified MBL activated by mannose-rich structures can activate C1s and C1r. It is not known whether MBL activates C1r and C1s *in vivo* or is associated with MBL-associated proteases (MASPs) (Thiel *et al.*, 1997).

1.7.3 **Membrane attack complex formation**

Formation of either the alternative or classical pathway C3 convertase results in the further covalent bonding of C3b to these complexes, to form the C5 convertases, $(C3b)_2Bb$ and C4b3b2a respectively. The C5 convertases cleave C5 to form C5a and C5b. C5 cleavage initiates membrane attack complex (MAC) formation when the activating surface contains a lipid bilayer. C5a is the most potent of the anaphylatoxins and hence plays a major role in inflammation as well as being a powerful initiator of leukocyte migration.

The activated C5b binds to C6 forming a C5b-C6 complex and then to C7 to form a C5b-C7 complex that can bind to membranes, resulting in dissociation of the complex from bound C3b. This in turn allows C8 to bind to form a membrane-bound C5b-C8 complex. C5b-C8 acts as a receptor for C9. On binding, C9 undergoes a conformational change causing the hydrophobic regions to become exposed and hence become inserted into the membrane. Bound C9 shows a high affinity for C9-C9 binding resulting in multiple binding of C9 to form a 'pore' structure.

MAC formation allows the equilibration of small solute molecules across the membrane. The influx results in an osmotic imbalance as macromolecules contained in the cell cannot get out. The rapid influx of water causes the cell to swell and eventually to lyse.

1.7.4 **Activation of complement by tick infestation**

Pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1) are produced in response to tick-bite injury (Mbow *et al.*, 1994). TNF-α and IL-1 up-regulate the alternative pathway of complement and stimulate the acute phase response (Katz and Strunk, 1989; Perlmutter *et al.*, 1986). In addition, acute phase proteins, such as the mannan-binding lectin or the C-reactive protein, can activate and up-regulate the complement system (Suankratay *et al.*, 1998). Tissue damage caused during the initial incision of the skin by the tick would be expected to result in the rapid activation of host complement via the acute phase response. It has been observed that skin-derived fibroblasts, when exposed to IL-1 and TNF, increase production of factor B by over one hundred times and C3 by fifteen times (Katz and Strunk, 1989). Conversely, activation of the complement system up-regulates the inflammatory response as a result of the release of the anaphylatoxins C3a and C5a. Anaphylatoxin release induces many responses including mast cell de-granulation (e.g. histamine release), phagocyte migration, IL-1 release by monocytes and vasoactive activities (Marceau *et al.*, 1987).

Several researchers have looked at the interaction of ticks and the host complement system. *I. ricinus* adults that have fed on resistant rabbits show the presence of C3 within the midgut of the tick (Papatheodorou and Brossard, 1987). Treatment of

guinea pigs with cobra venom factor (resulting in alternative pathway decomplementation) significantly reduces resistance against *Dermacentor andersoni* larvae (Wikel and Allen, 1977). However, use of C4 deficient (i.e. classical pathway deficient) guinea pigs showed no decrease in resistance against *D. andersoni* larvae (Wikel, 1979). This evidence suggests that the alternative pathway of complement is an important mediator in the development of host resistance to tick infestation.

1.7.5 **Parasites, pathogens and complement - Response and counter-response**

Animal parasites can be species of protozoa, trematoda, cestoda, nematoda or arthropoda. Both adaptive and innate immunity are involved in anti-parasitic responses by hosts. The complement system is involved in many of these responses. If a parasite is to be successful it must overcome the barrage of host responses directed against it including activation of the complement system. How complement 'responds' to different parasites depends upon the nature of the parasite involved. For example, whether it is multi-cellular or uni-cellular and whether the parasite is an ectoparasite or an endoparasite. The majority of complement research has been carried out on protozoan parasites, and comparatively little research has been done on arthropods (Leid, 1988).

Parasites are continually co-evolving with their hosts and consequently there is a constant 'battle' between the development of vertebrate immune systems and the counter measures employed by the parasite. Many strategies are used by parasites to overcome complement detection/activation. For example the protozoan *Trypanosoma brucei gambiense* allows deposition of C3 convertase on the parasite surface, but the convertase is rapidly inactivated, possibly mediated by factor I (Devine *et al.*, 1986). The trematode,

Schistosoma mansoni, undergoes conversion from a complement sensitive form to a complement resistant form. The secretion of proteases accompanies the conversion; a 28 kDa serine protease has been shown to increase resistance against the alternative pathway of complement (Marikovsky *et al.*, 1988). The mechanism of these activities is unknown.

Similarly many strategies are used by pathogens to overcome complement detection/activation. *Echinococcus granulosus* has been demonstrated to inhibit complement activiation through sequestration of host factor H in the hydatid cyst wall (Diaz *et al.*, 1997). *Neisseria gonorrhoeae* conversion from serum sensitive to serum resistant strains was demonstrated to be the result of sialylation of the organism. Sialic acid is found on many eukaryotic cells and inactivates bound C3b by interacting with factor H (Ram *et al.*, 1998). The vaccina virus complement control protein has been shown to share homology with complement control proteins that contain short consensus repeat sequences (SCR) and to inactivate alternative complement activity by acting as a factor I cofactor (Sahu *et al.*, 1998). Similarly the Epstein-Barr virus has been shown to have factor I cofactor activity (Mold *et al.*, 1988).

This thesis will explore the mechanistic basis of anti-complement activity in tick species.

1.7.6 **Tick anti-complement activity**

Several researchers have investigated anti-complement activity in various ixodid ticks. The studies carried out so far suggest that different tick species utilise a number of different anti-complement strategies. *Dermacentor andersoni* salivary antigens specifically activate C5 cleavage to generate the anaphylatoxin C5a (Gordon and Allen,

1991b). In addition, C5 cleavage activity has been demonstrated in the SGE of *Dermacentor variabilis* (Berenberg *et al.*, 1972). It is suggested that this activity could act to decomplement the host sera through over-stimulation. In contrast, however, an anaphylatoxin inactivating activity has been described in *Ixodes scapularis* SGE, which is suggested to be a carboxypeptidase (Ribeiro and Spielman, 1986).

A protease inhibitor isolated from *Boophilus microplus* larvae has been shown to inhibit complement (Willadsen and Riding, 1980). Alternative pathway inhibitory activity has been found in the SGE of *Ornithodoros erraticus, O. moubata* (Astigarraga *et al.*, 1997) and *Ixodes scapularis* (Ribeiro, 1987a). The mechanisms of these anti-complement activities are unknown. The ubiquity of alternative pathway complement components in species including evolutionarily 'basic' creatures such as lampreys has lead to the suggestion that it is the ancestral immune system. It is believed that the MAC and classical pathway have arisen from gene duplications and/or translocations (Lachman and Hobart , 1979). For tick hosts such as reptiles that apparently lack adaptive immunity, the alternative complement pathway effectively is *the* immune system and consequently may be a crucial target for tick-mediated modulation.

This research will attempt to characterise the factor(s) involved in *Ixodes* species anti-complement activity, the mechanisms of that activity, and explore the relevance of activity to tick-host relationships.

1.8 Tick salivary gland products modulate host responses

The duration of feeding varies greatly amongst haematophagous arthropods (Ribeiro, 1987b). For example, *Rhodnius prolixus* engorges for a period of a few minutes to an hour (Lavoipierre *et al.*, 1959), whereas mosquito probing and blood ingestion occur in less than ten minutes (Ribeiro, 1987b). In sharp contrast, ixodid ticks take few bloodmeals (one per life-stage) of a relatively long duration (up to two weeks). The extended period of feeding is more than ample time for the host to mount immune responses. As a consequence the tick faces a barrage of host responses throughout the feeding period, including adaptive and innate immunity as well as inflammatory and haemostatic responses. The tick overcomes this armoury by utilising a pharmacopoeia of bioactive compounds which are produced by the salivary glands and secreted into the host.

A large number of components and activities of tick saliva have been described (Ribeiro, 1987b). These include anti-coagulants that target components of the haemolytic cascade, including factor Xa (Waxman *et al.*, 1990), V and VII (Gordon and Allen, 1991a) and thrombin (Hoffmann *et al.*, 1991). Vasodilators such as prostaglandin E_2 that increase the blood flow at the feeding site have also been described (Bowman *et al.*, 1996).

In addition there is a wide range of immunosuppressive activities associated with the salivary glands (Wikel *et al.*, 1994), whose targets include the complement system

(Ribeiro, 1987a), histamine release (Chinery and Ayitey-Smith, 1977) and IgG binding (Wang and Nuttall, 1994).

As well as being crucial to the tick's feeding success, the saliva of ticks can enhance the transmission success of pathogens as they enter an immuno-compromised site of the host, so called saliva-activated transmission (SAT) (Jones *et al.*, 1989; Jones *et al.*, 1992; Labuda *et al.*, 1993; Randolph and Nuttall, 1994).

All of the activities so far characterised relate to the effect of salivary gland proteins on *in vitro* systems (with the exception of SAT). There is little or no information available about the performance of these activities *in vivo*, such as the working concentration of active factors, nor about the localisation of such activities within the host.

1.9 Tick salivary gland immunomodulation: systemic or localised?

There is a 'deep conviction' within the literature to describe the 'good' parasite-host relationship as one where the parasite is essentially benign, and that only an 'unwise' parasite would cause harm or kill its host (Baker, 1974; Toft and Aeschlimann, 1993). This idea is consistent with the observation that ticks feeding on laboratory animals that are not 'natural hosts' often develop more intense immune responses than do natural host species (Ribeiro, 1989). However, unlike other haematophagous arthropods, ixodid ticks must elicit immune responses in order to form a feeding lesion. Indeed some authors suggest that salivary gland products may act to increase the immune responses of a host (Berenberg *et al.*, 1972). There is evidence that red blood cells are not essential for the development of the immature stages of *I. ricinus* (Fogie, 1959). It is also suggested that pathogens such as *B. burgdorferi* exploit immune responses, by disseminating from the feeding site via macrophage phagocytosis (Montgomery and Malawista, 1994). In other words, the tick must perturb the 'benign' balance of the parasite-host relationship in order to feed successfully upon a natural host. Consequently the tick faces a dilemma; it must utilise host responses in order to feed but without triggering a gross response by the host which would cause removal and/or destruction of the tick. For the tick to feed successfully, a balance must be struck between immunomodulatory measures employed by the tick, and the development of that response in the host, which facilitates the feeding process, but at the same time is potentially lethal to the tick. Based upon this reasoning it would be pertinent to assume that it is the localisation of immune responses in the host to the area of the feeding lesion, rather than the suppression of immunity (alluded to in most

of the literature), that is the true *modus operandi* of the immunomodulatory activities by tick salivary gland proteins.

The phenomenon of co-feeding, whereby ticks such as *I. ricinus* tend to cluster around a common feeding site on the host (Randolph *et al.*, 1996), is consistent with the idea that tick-induced modulation is a localised event in hosts *in vivo.* Ticks such as *Amblyomma hebraeum* produce specific assembly pheromones that promote assembly and co-feeding of ticks on hosts (Rechav, 1976). Indeed, the co-feeding behaviour of ticks has been 'exploited' by pathogens such as TBE virus and *B. burgdorferi* as a route of non-systemic transmission between co-feeding ticks (Gern and Rais, 1996; Labuda, 1993).

1.10 **Aims**

The tick-host relationship is a function of both ecology and of biological interactions at the tick-host interface. This thesis concerns the tick-host interface primarily at the molecular level. The following aims will be explored in this work.

- Haemolysis is critical for the survival of ticks as an obligate haematophagous arthropod. What are the mechanisms and possible roles of haemolysis in the salivary glands of *I. ricinus* ticks?

Although the tick-host interface occurs at the cutaneous level, the immunological interactions are as complex as any involving protozoan or metazoan endoparasites, viruses or bacteria (Wikel and Bergman, 1997). In order that the immunological basis of the tick-host interface is fully understood, both the host responses to tick infestation and the counter-measures employed by the tick must be considered. Any host immune response depends firstly upon perception and secondly upon the effector responses. The major mediators of perception by the adaptive immune system are tick-specific antibodies. Antigenic profiling will be used to characterise this response.

- How do different host species immunologically perceive tick infestations and does this change as a result of the feeding process and with the developmental stage feeding?

The complement system is the primary mediator of humoral perception of the innate immune system. The alternative pathway of complement along with the acute phase response are the first lines of defence in response to a tick infestation, and

consequently may be an important target for modulation by tick salivary gland bio-active compounds.

- This thesis will investigate anti-complement activity in the salivary glands of ixodid ticks and explore the mechanisms at the molecular level. In addition, the effect of this activity on different natural host species complement systems will be examined.

The isolation and characterization of tick saliva immuno-modulatory compounds will provide valuable insights into tick-host relationships and improve our far from complete knowledge of vertebrate immunology. The practical constraints of obtaining enough saliva material from ticks for traditional biochemical analysis are prohibitive. A genetic approach therefore appears to be a more promising option.

- A subtractive cDNA library will be created that is enriched for saliva-associated differentially expressed gene products from the salivary glands of unfed and feeding female adult *I. ricinus* ticks. Random sampling of this library will attempt to identify putative saliva-associated proteins.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Standard Reagents

All chemicals used were analytical reagent grade unless otherwise stated. Most reagents were obtained from Sigma Chemical Company Limited, Poole, UK or Merck Limited, Poole, UK. Reagents obtained from other sources are referred to in the text.

2.1.2 Water

Unless otherwise stated, water was obtained by purification through a 'Milli-Q' reagent system (Millipore). Water used for enzyme reactions and sterile work was purified and sterilised through autoclaving (ddH_2O).

2.1.3 General buffers and solutions

1 M Tris-HCl pH 6.8 (stacking)	12.1 g Tris, pH to pH 6.8 with HCl, made up to 100ml with ddH_2O.
1.5 M Tris-HCl pH 8.8 (resolving)	91 g Tris, pH to pH 8.8 with HCl, made up to 500ml with ddH_2O.
10% APS	1 g ammonium persulphate in 10ml ddH_2O.
Coomassie de-stain	10% acetic acid, 40% methanol

Coomassie stain	0.5g R-250 Coomassie brilliant blue dissolved in 10% acetic acid, 40% methanol.
DEPC treated H_2O	0.1% v/v diethyl pyrocarbonate, shaken vigorously and autoclaved
DNA loading buffer (6x)	0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol
EDTA 0.5M stock	adjusted to pH 8 with NaOH, autoclaved
Ethidium Bromide	10 mg/ml stock in ddH_2O, working concentration 0.5 µg/ml
GVB buffer (5x)	83 gNaCl, 10.19 g barbital, 10 g gelatin, made up to 2 l with ddH_2O. pH 7.35 ± 0.005 with 1 M HCl.
GVB/Mg buffer (1x)	40 ml of 5x GVB, 16 ml 0.1 M EGTA, 1 ml 2M $MgCl_2$, made up to 200 ml with ddH_2O.
IPTG	100mM stock in ddH_2O, filtered
$MgCl_2$ 2M stock	40.66g $MgCl_2$ in 100 ml ddH_2O
Native protein loading buffer (5x)	8 ml 1M Tris pH 6.8, 10 ml glycerol, 2.5 mg bromophenol blue made up to 20 ml with ddH_2O
SDS protein loading buffer (5x)	1.5g DTT, 2g SDS, 8 ml 1M Tris pH 6.8, 10 ml glycerol, 2.5 mg bromophenol blue made up to 20 ml with ddH_2O.
SSC (20x)	175.3g NaCl, 88.2g sodium citrate in 1 l ddH_2O.
TAE (50x)	242g tris base, 57.1 ml acetic acid, 100ml 0.5M EDTA (pH 8) in 1 l ddH_2O.

TBE (10x)	54g Tris base, 27.5g boric acid, 20 ml 0.5 M EDTA (pH 8) in 1 l ddH_2O.
Tris-glycine buffer	30 g Tris base, 144 g glycine, make up to 1 l with ddH_2O.
X-gal	2% v/v in demethylformamide

2.1.4 **Liquid Media**

The media technician at IVEM prepared LB and LB-agar containing media as indicated in the text. All other media were prepared as follows.

LB media was made by dissolving 50g yeast extract (Difco Laboratories), 100g bacto-peptone (Difco Laboratories) and 50g NaCl in 10 l of ddH_2O, the pH adjusted to 7.5 with 2M NaOH, and then autoclaved.

NZY broth was made by dissolving 5g NaCl, 2 g $MgSO_4.7(H_2O)$, 5g yeast extract and 10g of NZ amine (casein hydrolysate) in 1 l of ddH_2O, the pH adjusted to 7.5 with 2M NaOH and then autoclaved.

Agarose containing media contained 0.7% (w/v) agarose and was autoclaved.

2.1.5 **Equipment**

Atto Horizblot electroblotter (AE 6675)

Atto polyacrylamide gel electrophoresis tank (AE 6400)

Beckman ϕ10 pH meter

Beckman J2-HC centrifuge

Bio-Rad mini-sub DNA cell gel tank

Bio-Rad model 583 gel dryer

CECIL 6000 series UV spectrophotometer (CE 6600)

Fisons MSE Chillspin 2 centrifuge

Fotodyne UV transilluminator

Hybaid touchdown thermal cycler (HB-TD-MAN)

Juan A14 micro-centrifuge

Mini-instruments mini-assay type 6-20 (I^{125}- counter)

Mini-instruments scintillation meter type 540

Mitsubushi video copy processor

Novex X-cell II electrophoresis tank

Pharmacia electrophoresis constant power supply (ECPS 3000/150)

X-ograph compact X2 automatic film developer

2.1.6 **Suppliers addresses**

Amersham International	Amersham Place, Little Chalfont, Bucks. HP7 9NA
Biacore AB	2 Meadway Court, Meadway Technology Park, Stevenage, Herts. SG1 2EF
Bio-Rad laboratories Ltd.	Bio-Rad House, Maylands Ave., Hemel Hempstead, Herts. HP2 7TD
Boehringer Mannheim	Sandhofer Strasse 116, D-68305, Mannheim, Germany.

Calbiochem (UK) Ltd.	Boulevard Industrial Park, Padge Rd., Beeston, Nottingham. NG9 2JR
Clontech Laboratories	Unit 2, Intec 2, Wade Rd., Basingstoke, Hants. RG24 8NE
Invitrogen	P. O. Box 2312, 9704 CH Groningen, The Netherlands.
NBL	South Nelson Rd., Cramlington, Northumberland. NE23 9WF
New England Biolabs	Knowl Place, Wilbury Way, Hitchin, Herts. SG4 0TY
Novex	Brueningstrasse 50, Buildg. C584, D-65929, Frankfurt, Germany.
Pharmacia Biotech	23 Grosnenor Road, St. Albans, Herts. AL1 3AW
Promega UK	Delta House, Chilworth research centre, Southampton SO16 7NS
QUIAGEN Ltd.	Boundary Court, Gatwick Rd., Crawley, West Sussex. RH10 2AX
Sigma-aldrich Ltd.	Fancy Road, Poole, Dorset. BH12 4QH
Stratagene Ltd.	Cambridge Innovation centre, Cambridge Science Park, Milton Road, Cambridge. CB4 4GF.

2.2 **Enzymes**

All enzymes were purchased from biotechnology companies as indicated. All enzymes were stored at –20 ^{0}C. Enzymes used as parts of kits are indicated in the text.

proteinase K	Boerhinger Mannheim

Taq polymerase	Sigma
PNGase F	New England Biolabs

2.3 Miscellaneous methods

2.3.1 Sterile technique and sterilisation of buffers and solutions

All procedures requiring sterile conditions were performed in a class II micobiological safety cabinet (Envair Limited) or on the bench next to a Bunsen burner. Liquids were sterilised by autoclaving at 15lb/square inch on liquid cycle for 1h, where appropriate, or by filtration through 0.45 or 0.22μm filters (Millipore) which fit 'luer lock' syringes.

2.3.2 Spectrophotometry

A CECIL 6000 Series (CE 6600) UV spectrophotometer was used. Disposable plastic cuvettes were used to assay haemolysis, the density of bacteria and protein concentrations. Quartz cuvettes were used to determine nucleic acid concentrations.

2.3.3 Determination of protein concentration

Protein concentrations were determined using BIO-RAD protein assay solution (based on the Bradford dye binding procedure (Bradford, 1976)). Optical density was measured at 595nm on a spectrophotometer. The optical density of the sample was compared with a standard curve constructed by measuring the optical density of known amounts of BSA.

2.3.4 **Estimating DNA and RNA concentration**

DNA concentration was estimated by absorbance at 260nm, or by comparison to DNA of known concentration on a gel. λ–Hind III/EcoR I marker (NBL) is 0.375μg/μl and has a size of 45kb. An estimate of the concentration of a particular piece of DNA can be made by assessing the band of the marker that has similar luminosity to the DNA of interest, and calculating: (size of equivalent fragment/size of marker) x concentration marker x volume marker loaded. The answer to this sum, divided by the amount (in μl) of sample DNA of interest loaded gives the approximate concentration in μg/μl.

To estimate RNA concentration, 1μl RNA was added to 1ml ddH_2O and the absorbance measured at 260nm. $1A_{260}$ unit of ssRNA is equivalent to about 40μg/ml and $1A_{260}$ unit of dsRNA is equivalent to about 60μg/ml.

2.3.5 **Special precautions when working with RNA**

Except for buffers containing Tris, all solutions used for work with RNA (i.e. mRNA preparation, cDNA construction and RT-PCR) were treated with DEPC (0.1% v/v DEPC for 4h at 37°C, with shaking, autoclaved). Glassware used to make solutions for work with RNA was baked at 180°C for 12h. Chemicals for work with RNA were set aside, and when weighed were dispensed using flamed metal spatulas. Sterile disposable plasticware was used wherever possible. Disposable powder free latex gloves were worn.

2.3.6 **Special procedures used for I^{125} work**

All work with I^{125} was carried out in the MRC Immunochemistry Unit, Oxford under the supervision of the radiological safety officer. Waste disposal was in line with

University safety procedures. When handling samples, two pairs of powder free latex gloves were worn. Whenever possible, work was carried out behind lead-Perspex screening. Contamination was monitored using a scintillation meter.

2.3.8 **Centrifugation**

A benchtop Jouan A14 micro-centrifuge (top speed 14 Krpm) was used for the centrifugation of all samples smaller than 1.5 ml. Centrifugation of larger samples at low speeds (2K-5.5K), and at low temperature (4°C), was carried out in a Fisons MSE Chilspin 2. Higher speed centrifugation (e.g. Centricon concentration of proteins) was carried out in a Beckman J2-HC centrifuge.

2.4 **Standard molecular biology techniques**

2.4.1 **Phenol/chloroform extraction of nucleic acids**

Phenol/chloroform is a standard way in which to remove proteins such as enzymes from a nucleic acid reaction mixture (Sambrook *et al.*, 1989). An equal volume of buffered phenol was added to the sample, vortexed and then centrifuged for 5 minutes at 13 Krpm. The aqueous top phase was decanted into a fresh tube. Then an equal volume of chloroform was added to the aqueous phase, the sample vortexed and centrifuged for 5 minutes at 13 Krpm. The top phase was removed and the nucleic acids recovered by ethanol precipitation. Phenol/isoamyl alcohol/chloroform (1:24:1) extraction where indicated in the text was carried out as above, but with an additional extraction prior to phenol/chloroform extraction with an equal volume of phenol/isoamyl alcohol/chloroform.

2.4.2 **Ethanol precipitation of nucleic acids**

Ethanol precipitation is the standard technique of concentrating DNA by salt precipitation (Sambrook *et al.*, 1989). Adjusting solutions to 0.3M NaOAc using 1/10 volume 3M NaOAc pH 5.2 precipitated the DNA. Two volumes of 100% ethanol were added and the mixture incubated for 30 minutes at -20°C before centrifugation at 13 Krpm for 15 minutes at 4^{O}C. The pellet was washed with 1ml 70% ethanol, before centrifuging again at 13 Krpm for 5 minutes at 4^{O}C. After aspiration the pellet was dried for 30 minutes under vacuum before resuspending in a suitable volume of ddH_20 or TE buffer.

2.4.3 **Standard PCR procedure.**

PCR was performed in a total volume of 100µl using 1µl (2U) *Taq* polymerase and ≤1ng DNA as template. A 5 minute denaturation step, 30 cycles of denaturation/annealing/ extension, and a final 10 minutes extension step were typical programmes used. Denaturation was at 95°C for 30 seconds. Annealing was dependent upon the estimated annealing temperature of the primers, and extension was at 72°C for 30s/500bp of product. The annealing temperature of primers was estimated by the formula: 4°C per G/C + 2°C per A/T. Primer concentrations were 500µg/ml and 1µl of each primer was used in most reactions (i.e. about 0.5µM primer/100µl reaction). 10x PCR buffer was composed of 100mM TrisHCl pH 8.4, 500mM KCl, 1% gelatin, 0.5% NP-40, 0.5% Tween 20, 5-50mM $MgCl_2$ (normally 15mM $MgCl_2$). 2.5mM dNTP stocks were made in ddH_2O and stored at -20°C; dNTPs were added to final concentration

250μM. For each PCR experiment performed, single reaction mixtures were made which contained ddH_2O, 10x PCR buffer, dNTPs and primers. The reaction mixture was added to each tube followed by the template DNA; each reaction was overlayed with 75μl mineral oil. *Taq* polymerase was added during the initial 5 minute denaturation step. PCR reactions were performed using a Hybaid touchdown thermal cycler.

2.5 Gel electrophoresis of nucleic acids

2.5.1 Standard TAE agarose gels

Agarose gels (30-50ml) were prepared and run in 1 x TAE buffer. Gel concentrations were typically 0.7-2% (w/v) ultra pure agarose (Sigma). 1% agarose gels resolve linear dsDNA 0.5-7kb long. Higher percentage gels (up to 2%) were cast to separate smaller DNA fragments. Gels contained ethidium bromide at a final concentration of 10μg/ml. DNA samples were loaded in 1x sample buffer. Gels were run in a Bio-Rad minisub DNA electrophoresis tank at a constant voltage of 120V. The DNA was visualised using a Fotodyne UV transilluminator. The image was recorded on thermal paper via a camera and Mitsubishi video copy device. Unless otherwise indicated, the molecular weight marker used was EcoR1/Hind III digest of λ-phage DNA (NBL).

Formaldehyde containing gels (for RNA visualisation) were set up according to standard techniques (Sambrook *et al.*, 1989).

2.6 Polyacrylamide gel electrophoresis and analyses of proteins

2.6.1 Denaturing SDS-PAGE (reducing)

Unless otherwise stated a 10% polyacrylamide gel was set up and run as described by Laemmli using a discontinuous buffer system (King and Laemmli, 1971). Proteins were analysed by SDS-PAGE in 1mm thick, 8cm long upright slab gels using Atto PAGE model AE 6400 tanks.

The resolving gel (10%) was made from 10.3 ml acrylamide/bis (37.5:1- National Diagnostics), 7.5 ml 1.5 M Tris-HCl pH 8.8, 3 ml glycerol (87%), 0.3 ml 10 % SDS and 8.8 ml of ddH_2O. 200 µl of 10% TEMED was used as the catalyst and 200 µl of 10% APS as a source of free-radicals. The stacking gel (3%) was prepared from 2.4 ml arcrylamide/bis, 2.5 ml of 1 M Tris-HCl pH 6.8, 0.2 ml of 10% SDS and 14.8 ml ddH_2O. The gels were allowed to set at $4^{O}C$ for 1 hour. Protein samples were mixed with 5x buffer (containing DTT) and were denatured by boiling for 3 minutes before loading. Gels were run in tris-glycine buffer containing 1% SDS. Gels were run at a constant current of 20 mA per gel. Unless otherwise indicated, the molecular weight marker used was purchased from Sigma (cat. no. M-0671).

2.6.2 Native PAGE (non-reducing)

Native PAGE gels were prepared and run as described for reducing PAGE, except without the addition of SDS in the buffers. Samples were mixed in 5x native loading buffer (no DTT or SDS) and loaded as above (except not boiled).

2.6.3 **NuPAGE gel electrophoresis– reducing and non-reducing**

Where indicated pre-cast 4-12% bis-tris NuPAGE gels purchased from Novex technologies (Novex, cat. no. NP-0321) were used for electrophoresis. Samples were prepared as described above either under reducing or non-reducing conditions. Gels were run using supplied MES buffer (0.5 ml of anti-oxidant was added to anodal chamber under reducing conditions) according to manufacturers instructions at a constant voltage of 200 V. The Xcell II mini-cell vertical electrophoresis apparatus was used (Novex, cat. no. E19001). Unless otherwise indicated the MultiMark pre-stained molecular weight marker was used (Novex, cat. no. LC5725).

2.6.4 **Western Blotting**

Electroblotting of the gels onto nitrocellulose membranes were carried out as described (Sambrook *et al.*, 1989) either overnight at 20mA or at 90mA for 2 hours using a semi-dry blotting apparatus (Atto AE-6675 Horizblot). Where necessary protein was reversibly stained using Ponceau S. The membranes were cut and destained in ddH_2O. The membrane was blocked overnight in blocking solution (5% non-fat milk, 0.1% TWEEN-20, PBS). Incubations with primary and secondary antibodies were carried out in blocking buffer for 1 hour. Primary sera dilutions were calculated from dot-blot experiments (not shown). Secondary peroxidase conjugates were used at a 1 in 3000 dilution. Washing of membranes after incubations was carried out in washing buffer (0.1% TWEEN-20, PBS) in the following sequence: 2 x 1min, 1 x 15min and 2 x 5 min. Blots were developed using chemiluminesence ECL+ reagents (Amersham, cat. no. RPN

2132) as described in the manufacturer's instructions. Bands were visualised by exposing membranes to X-ray film.

2.6.5 **Autoradiogram preparation**

Gels that contained I^{125} samples were dried onto Whatman 3mm filter paper, using a Bio-Rad model 543-gel drier. The dried gels were tested for activity using a scintillation counter. Based on this information, the gel was exposed to X-ray film for varying quantities of time (approximately 1 day for 50000 cpm samples) before being developed. Film cassettes contained intensifying screens and were stored at $-70^{O}C$ during the exposure period.

2.6.6 **Coomassie blue staining**

Gels were fixed and stained in 40ml 42.5% methanol (v/v), 7% acetic acid (v/v) and 0.5% Coomassie Brilliant Blue R250 for 1 hour. Gels were destained in a large volume of 40% methanol and 10% acetic acid. The gels were then washed in distilled water and dried using a Bio-Rad gel air dryer.

2.7 Oligonucleotide primers

2.7.1 Primer design

Primers were designed based upon cDNA sequences as indicated in the text. Non-cDNA specific primers (e.g. vector, adapters etc.) as indicated in the text were designed and usually supplied by the relevant manufacturer (sequences not shown). cDNA specific primers were designed to be 20- 22 bases long, so as to give a T_m (section 2.4.3) of >60^{O}C. Primers were tested for possible primer-dimers and secondary structure using the PRIMER programme of the GCG suite.

2.7.2 Primer synthesis

Unless supplied, primer- DNA synthesis was carried out on an Applied Biosystems DNA synthesiser using the 0.2μM scale synthesis cycle (Val Cooper, Dyson-Perrins Laboratory, Oxford). Oligonucleotides were deprotected at 56°C overnight. The deprotected oligonucleotides (100μl) were purified and precipitated by the addition of 1ml butan-1-ol and centrifugation at 13 000rpm for 3 minutes. The butanol was removed and the pellet vacuum dried. The pellet was resuspended in 100 μl of TE buffer. Ten μl of the resuspended pellet was added to 2ml dH_2O and optical density at 260nm measured. The μg/μl of oligonucleotide at this dilution is 10x the optical density value at 260nm. The oligonucleotide was adjusted to a concentration of 500μg/μl with ddH_2O and stored in the -20°C freezer.

2.7.2 **Primer sequences**

The positions of the primer sequences on the relevant cDNA sequences are indicated in Chapter 4.

Primer	**Sequence**
Cyt c- 5'	GAAATTCCCCTATTATAGAACAAAT
Cyt c- 3'	GGAATTATAAATGAATCATA
Vaso- 5'	TTTCACACATGCTTTTAA
Vaso- 3'	CTCTTTTCAGATTTACTTCA

2.8 **Commercial kits**

The following commercially produced kits were used in this research. All procedures were carried out in accordance with supplied instructions unless otherwise indicated in the text.

ZAP Express cDNA synthesis kit	Stratagene (cat. no. 200403)
Advantage cDNA PCR kit	Clontech (cat. no. K1905-1)
PCR-Select cDNA Subtraction kit	Clontech (cat. no. PT1117-1)
ECL+ chemiluminesence kit	Amersham(cat. no.RPN2132)
pBAD-TOPO T/A cloning kit	Invitrogen (cat. no.180124)
Titan One-Tube RT-PCR	Boehringer Mannheim (cat. no. 1888382)

Packagene λ DNA packaging	Promega (cat. no. K3154)
QIAprep spin mini-prep kit	Quiagen (cat. no. 27106)
Micro-Fasttrack mRNA purification	Invitrogen (cat. no. 160228)
QIAquick PCR purification kit	Quiagen (cat. no. 28142)
DIG- High Prime kit	Boehringer Mannheim (cat. no. 1585606)

CHAPTER 3

THE CHANGING PROTEIN AND ANTIGENIC PROFILE OF FEEDING *I. ricinus* TICKS

3.1 Introduction

It has been widely observed that the gross morphology and histology of tick salivary glands changes throughout the feeding period (Binnington, 1978). An increase in the salivary gland soluble protein concentration in excess of ten times has been reported in feeding *Rhipicephalus appendiculatus* ticks (Wang and Nuttall , 1994). The increase in protein production throughout the feeding period is accompanied by differential expression (Oaks *et al.*, 1991). As a consequence, a changing protein profile of the salivary glands has also been reported for many tick species (Limo *et al.*, 1993; McSwain *et al.*, 1982; Sanders *et al.*, 1996; Shapiro *et al.*, 1986; Wang and Nuttall , 1994). Presumably these feeding associated proteins are produced temporally as a reflection of the changing tick-host interface throughout the extended feeding period. Whether such changes in salivary gland protein profile are a direct result of host responses (i.e. host modulated) or whether they represent a pre-programmed cycle is unknown. The protein profile and antigenic profile of the saliva of *Amblyomma hebraeum* was observed to change in response to the feeding process (Dharampaul *et al.*, 1993). The antigenic profile of the salivary glands also changes throughout the feeding period (Jaworski *et al.*, 1990; Limo *et al.*, 1993; Sanders *et al.*, 1996; Shapiro *et al.*, 1986; Wang and Nuttall , 1994). Some pathogens for example, *Trypanosma brucei*, use a changing antigenic profile in order to evade host immunity (Turner and Michael, 1997). Whether the

changing antigenic profiles of tick salivary glands are produced in order to compromise the host's immunity, or whether the effect is merely fortuitous, is not known.

Repeated infestations of ticks on some hosts results in the development of acquired resistance. Tick resistance is an immunological response that is mediated by antibody formation. The way in which immunity is expressed varies greatly, depending upon the host species and tick species that are investigated (Willadsen, 1980). There has been little if any, investigation of how different hosts immunologically perceive ticks. The majority of research to date has used 'laboratory' animals as opposed to the 'natural' hosts in order to study the antigenic profile of infestations.

Research reported in this chapter investigates how different hosts perceive *I. ricinus* infestations immunologically, as well as investigating how this perception changes with the different developmental stages of the tick and as a consequence of the feeding process.

3.2 Materials and methods

3.2.1 Serum preparation

All fresh sera were prepared from fresh blood clotted on ice for 1 hour. Serum was separated from the blood by centrifuging at 14 Krpm for 10 minutes. The serum was aliquoted and stored at –70° C. Human serum was obtained from two healthy male individuals. Rabbit serum was prepared by infesting three groups of three New Zealand White rabbits three times at 14 day intervals with *I. ricinus* larvae (about 500), nymphs (50) or adults (20). Serum from animals infested with the respective developmental stages was pooled. Hamsters (duplicates) were infested three times at 14 day intervals with either larvae (about 50), nymphs (20) or adults (5). Serum was pooled from the duplicate animals. Two individuals of laboratory reared *Clethrionomys glareolus* (bank vole), *Apodemus flavicollis* (woodmouse) or BALB/c mice were infested three times at 14-day intervals with *I. ricinus* larvae (50). Two animals of each species were used as sources of naïve serum. The duplicate sera were pooled. Serum dilutions used for western blotting as indicated were calculated using dot blots (not shown).

3.2.2 Salivary gland extract preparation

Salivary gland dissection was carried out immediately after removal of ticks from the host. Female adult *I. ricinus* were fed on hamsters for varying periods (days 1 to 6 and replete (i.e. day 7)), although unless otherwise indicated ticks were fed for 5 days. The salivary glands were excised and washed in PBS (500 μl) by vortexing and pelleting the salivary glands at 10 Krpm for 5 minutes. The salivary glands were homogenised in a

glass homogeniser using 10 µl per tick of phosphate buffered saline (PBS) on ice. Particulate matter was removed by centrifugation at 14000 rpm for 10 minutes. The soluble fraction (i.e. SGE) was aliquoted and stored at -70° C.

3.2.3 **Preparation of whole tick homogenates**

Ixodes ricinus larvae and nymphs were fed separately on hamsters until repletion. Female adult ticks were fed for five days on hamsters; mating males were also collected. Whole ticks were homogenised in liquid nitrogen using a mortar and pestle. The nitrogen was allowed to evaporate off and the homogenate resuspended in phosphate buffered saline. The homogenates were further homogenised using a glass homogeniser. Particulate matter was removed by centrifugation at 14 Krpm for 10 minutes. The homogenates were diluted to a protein concentration of 4 µg μl^{-1}. The soluble fraction was aliquoted and stored at -70° C.

3.2.4 **SDS-PAGE and western analysis**

NuPAGE electrophoresis under reducing conditions, Coomassie blue staining and western blotting were carried out as described in section 2.6.

3.2.5 **PNGase F and Proteinase K treatment of adult male homogenates**

PNGase F was purchased from New England Biolabs (cat. no. 701 S). Twenty µg of homogenate protein was denatured for 10 minutes in the supplied reducing buffer. The denatured protein was deglycosylated in 1% NP-40 and 1x reaction buffer, with 1 µl (10 units) of PNGase F. The reaction mixture was incubated at $37^{O}C$ for 1 hour.

Alternatively, 20 μg of homogenate was digested using 1 μg of proteinase K (Boehringer Mannheim). The reaction mixture was incubated at 37^{O}C for 1 hour.

All reactions were stopped by adding loading buffer and placing samples onto ice before loading onto a PAGE gel. PNGase F efficiency was visualised by the changing band pattern compared with the control, as visualised on blotted nitrocellulose membranes using Ponceau S total protein stain (data not shown).

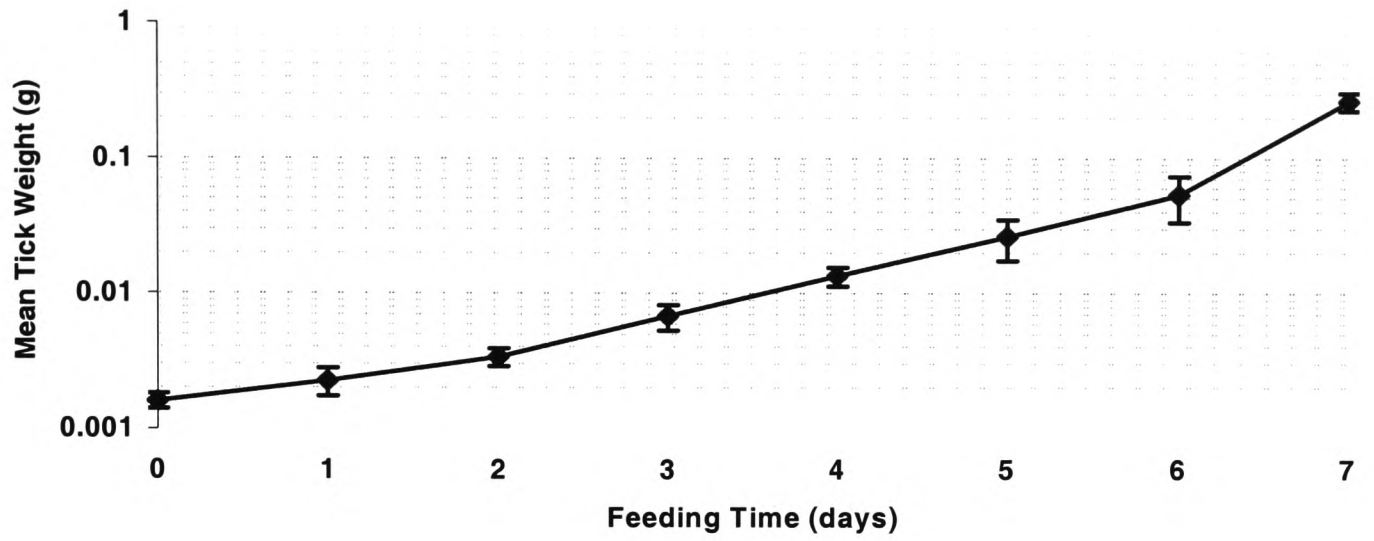


Fig 3.1- top Graph of *I. ricinus* female adult mean weight (g) against time of feeding (days) on rabbits (n = 10 pairs); plotted on a logarithmic scale . ± standard errors shown as bars.

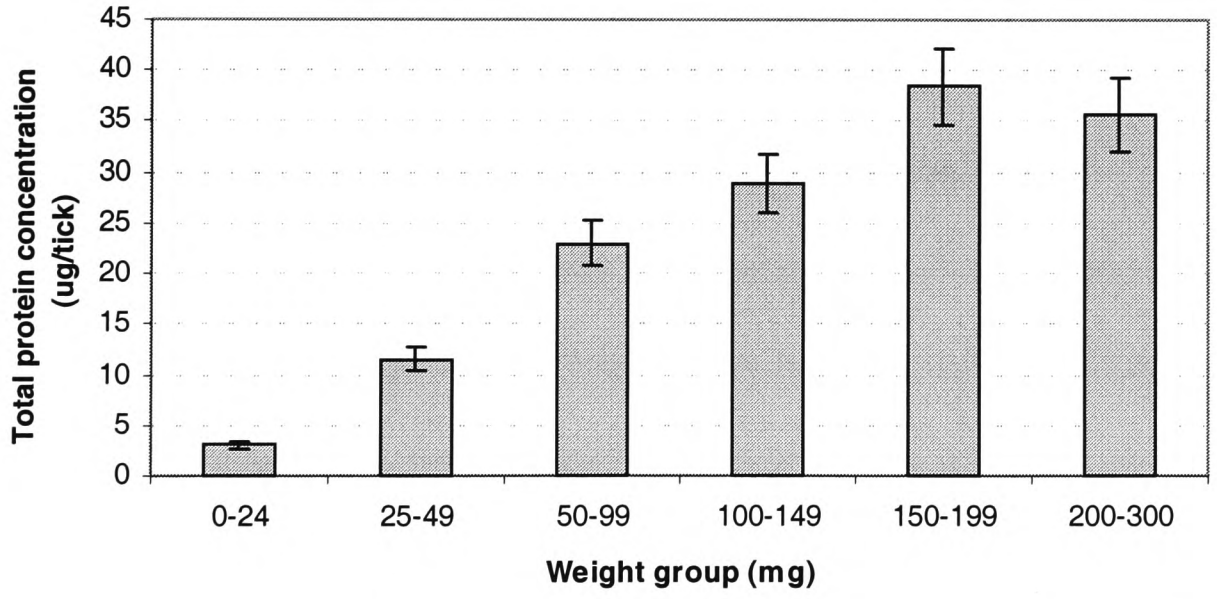


Fig 3.1- bottom Histogram showing the change in total SGE protein concentration as a function of increasing body weight. Concentrations were determined from pooled SGE from ticks collected at daily intervals and sorted into weight groups as shown (n = 5 pairs).

3.3 Results

3.3.1 Salivary gland soluble protein content change and mean weight change in *I. ricinus* adult females throughout the feeding period

One hundred and forty *I. ricinus* male + female adult ticks (70 pairs) were fed for varying amounts of time on three New Zealand white rabbits. The ticks were removed and weighed. Fig 3.1-top showed that the mean tick weight increases exponentially with number of days of feeding from unfed to day 5. The rate of increase in weight of ticks was markedly greater in ticks feeding for more than 6 days.

Thirty-five pairs (male + female) of adult *I. ricinus* ticks were fed on New Zealand white rabbits for various periods (day 1 (5 pairs), day 2 (5 pairs), day 3 (5 pairs), day 4 (5 pairs), day 5 (5 pairs), day 6 (5 pairs) and day 7 (i.e. replete- 5 pairs)). The ticks were removed from the hosts, weighed and sorted into weight groups (for brevity data not shown). The salivary glands were dissected and SGE prepared (section 3.2.2) in 100 µl PBS. A Bradford Protein assay was used to calculate the soluble protein concentration of SGE. Fig 3.1-bottom showed a linear relationship between the mean tick weight and the soluble protein content of salivary glands. The total soluble protein concentration increased from 3 µg/tick in females less than 25 mg to 38.4 µg/tick for ticks of 150-199 mg. There was a slight decrease in the soluble protein content of the salivary glands of ticks weighing 200-300 mg (ticks in this weight group had become replete- data not shown).

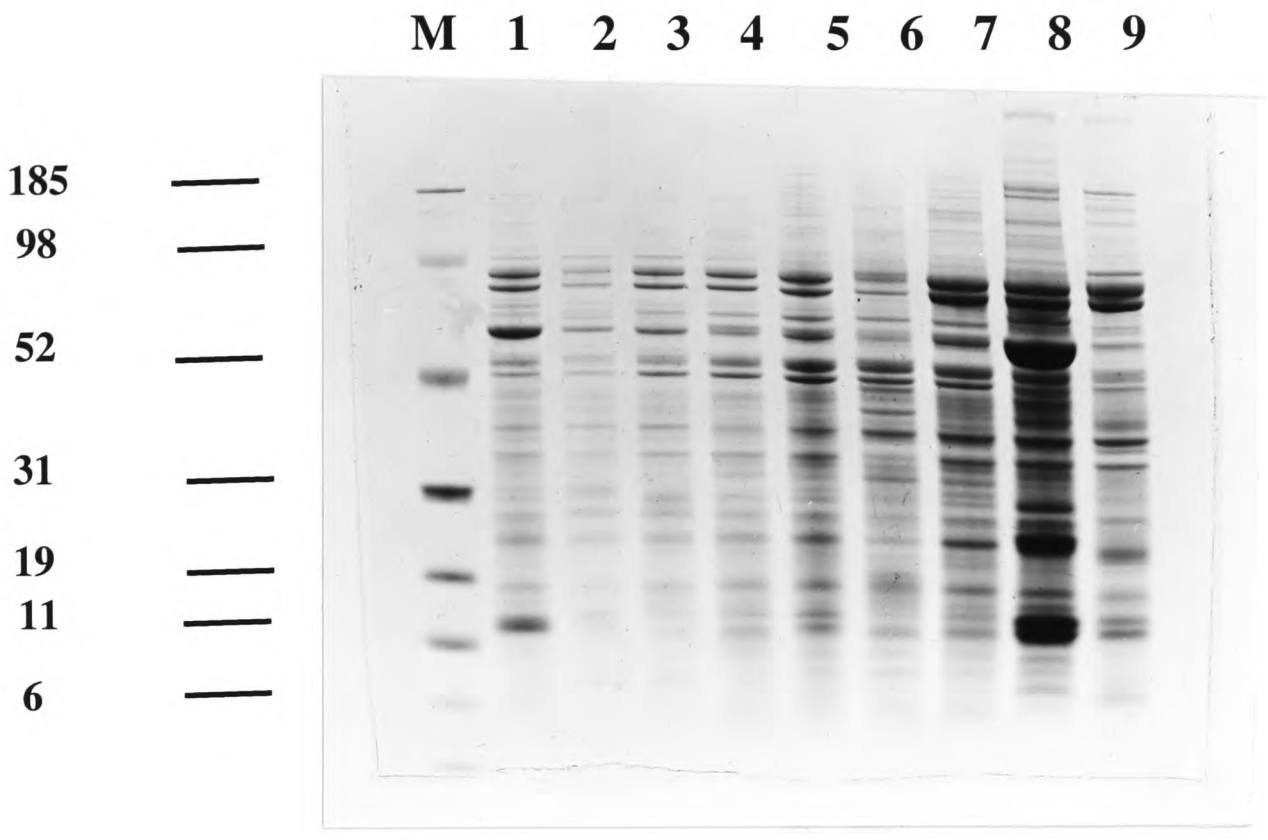
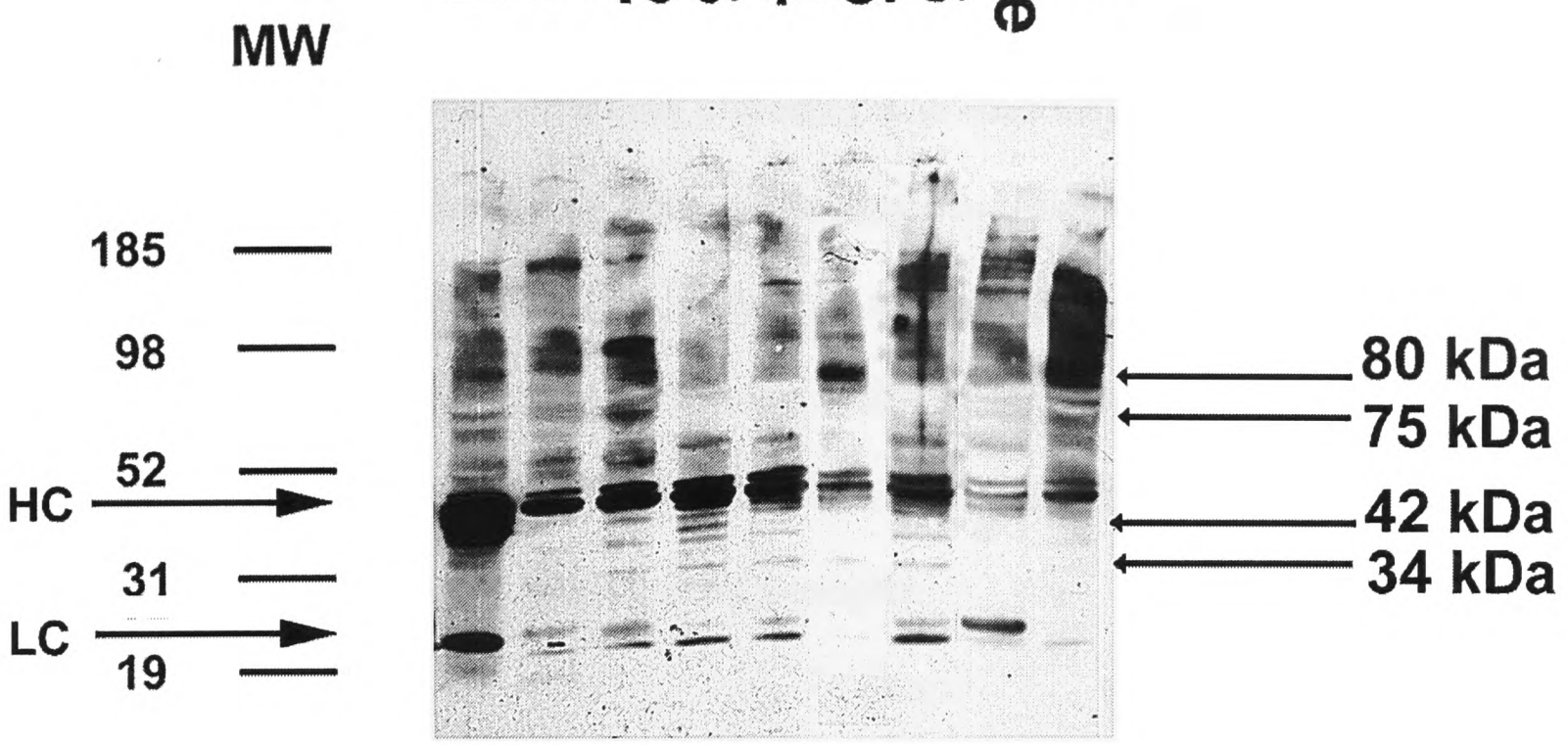


Fig 3.2 Changing protein profile of *I. ricinus* adult ticks throughout the feeding period. (M), molecular weight marker, (1) unfed SGE, (2) SGE (day 1), (3) SGE (day2), (4) SGE (day 3), (5) SGE (day 4), (6) SGE (day 5), (7) SGE (day 6), (8) whole tick female homogenate (day 5), (9) whole tick male homogenate. 20 μg of protein used. Visualised by Coomassie blue staining. (Nu-PAGE 4-12% MES gel run under reducing conditions).



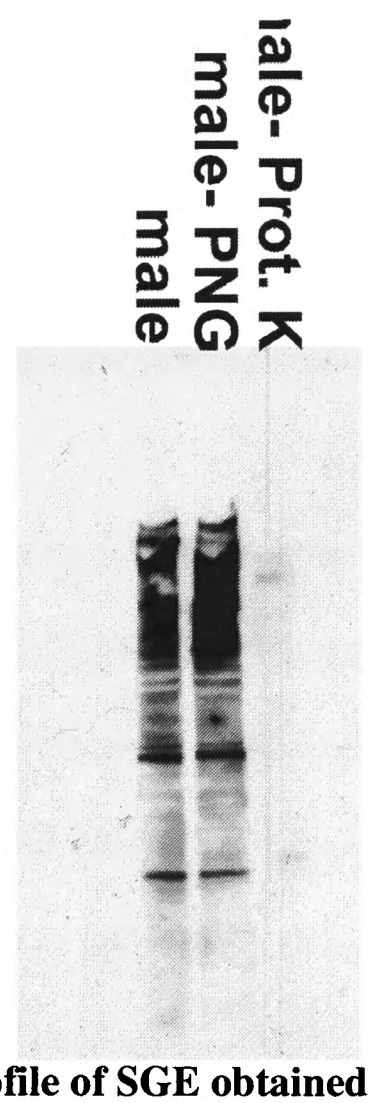


Fig 3.3 **(Top) Antigenic profile of SGE obtained from unfed and feeding female adults (days as indicated), female homogenate (day 5) and male homogenate. (Bottom) male homogenate treated with PNGase F or proteinase K as indicated. 20 μg of protein in each lane. Membranes were probed with 1 in 500 dilution of rabbit anti-adult tick serum. HC, LC- heavy chain and light chain of IgG respectively.**

3.3.2 **Changing protein profile throughout the feeding period**

SGE (20 μg) derived from unfed ticks or feeding ticks (day 1-6) and the whole homogenates of female (day 5) and male ticks were loaded onto a NuPAGE 4-12% bis-tris gel and run according to manufacturer's instructions under reducing conditions. Fig 3.2 shows that the protein profile of *I. ricinus* female adults changes throughout the feeding period. The protein profile of both female and male homogenates shared similar sized bands with some of the salivary gland extract samples.

3.3.3 **Changing antigenic profile throughout the feeding period**

A duplicate gel to that described in section 3.3.2 was set up and run in parallel with this gel. Twenty μg of SGE or homogenate was used per lane. Western blotting was carried out as described in section 2.6 using NuPAGE gels under denaturing conditions. The electro-blotted membrane was immuno-probed with a 1 in 500 dilution of rabbit anti-adult tick serum (section 3.2.1).

Several antigens (e.g. band above HC-IgG (about 52 kDa- not indicated) were observed throughout the feeding period in the salivary gland extract samples (Fig 3.3-top). A 42 kDa antigen is specific to SGE obtained from ticks fed for 3 days; similarly a 80 kDa band is present only in SGE obtained from ticks fed for 5 days. A 34 kDa antigen is present from day 2 onwards but not in unfed ticks or ticks fed for 1 day. A 75 kDa band is present in unfed ticks and in ticks fed for 1 or 2 days. These data suggest that salivary gland antigens of *I. ricinus* female adult ticks are differentially expressed in response to the feeding process. Similarly, with the results of protein profiling, the female

and male homogenates contain antigens that are the same sizes with bands observed in the salivary gland extract samples. IgG was observed in the SGE samples of unfed female and the whole tick homogenate of male ticks, this suggests that the IgG was residual from a previous bloodmeal (i.e. as a nymph).

3.3.4 Antigenic profile of adult male whole tick homogenate treated with PNGase F or proteinase K

The presence of antigens in the male whole tick homogenate was somewhat surprising, as the adult males of *I. ricinus* are widely believed not to feed. To investigate whether the cross-reactivity of antigens in the male homogenate reflect shared carbohydrate moieties or shared protein motifs, 20 μg of the homogenate was treated with PNGase F or proteinase K (section 3.2.5). The antigenic profile of the male homogenate treated with PNGase F did not differ significantly from that of the control, although some bands were absent (Fig 3.3- bottom). In contrast, proteinase K digestion removed almost all of the antigens. This suggests that the male adult antigens recognised by anti-adult tick serum are not due to shared carbohydrates, but are primarily protein specific epitopes.

3.3.5 Antigenic profile of different developmental stages of *I. ricinus*

Gut material was dissected from female adult ticks (fed for 5 days) and the homogenate prepared as described for SGE. Twenty μg of SGE or gut, larvae or nymph homogenate was used for western blotting as described in section 3.2.4. Blots were immuno-probed with a 1 in 500 dilution of the relevant rabbit anti-serum.

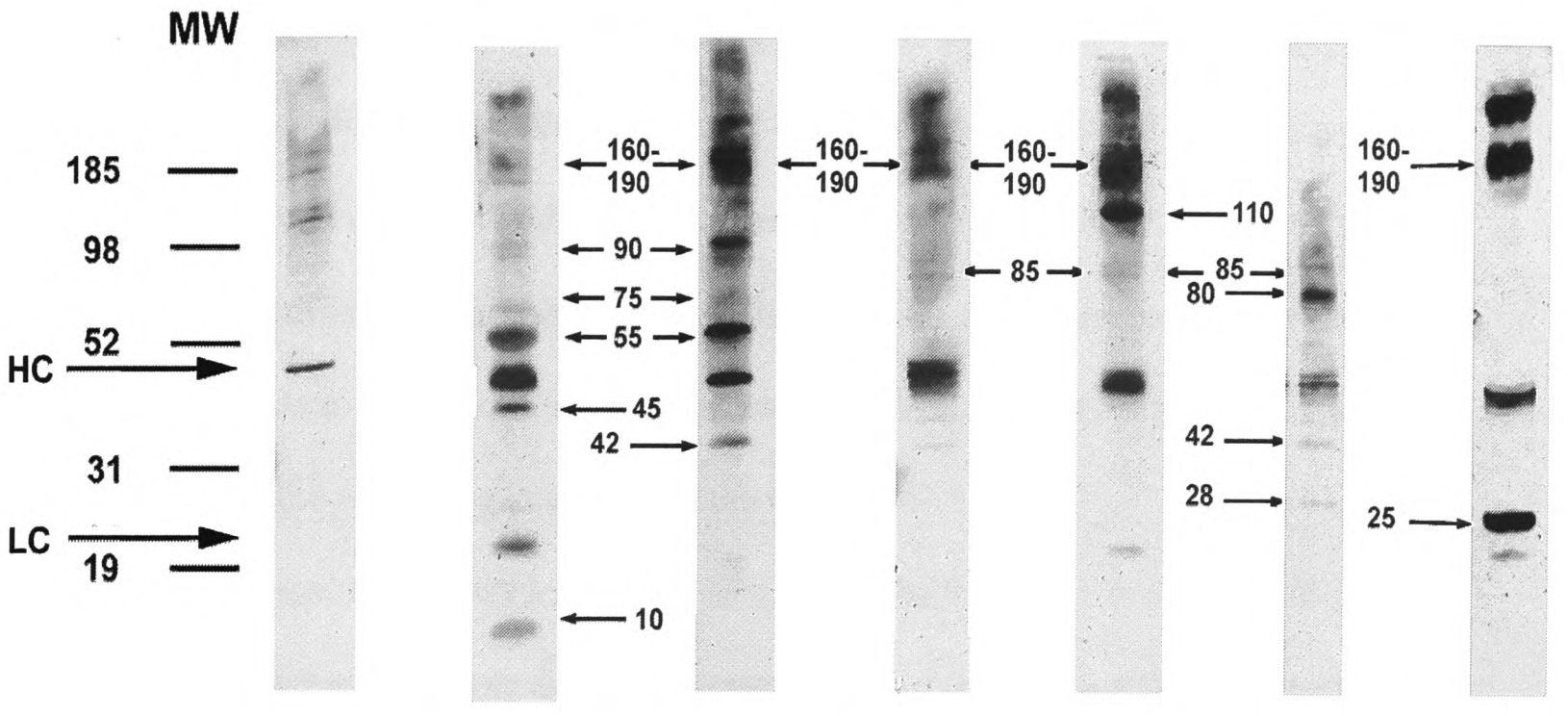


Fig 3.4 Antigenic profile of various developmental stages of *I. ricinus* and salivary glands and gut material from female adults (day 5). Immunoprobed with a 1 in 500 dilution of the relevant rabbit serum (as indicated). 20 μg of protein were used. HC, LC- heavy chain and light chain of IgG respectively.

The results shown in Fig 3.4 showed that the antigenic profile of SGE differs with the rabbit serum raised against infestations of the different developmental stages. Indeed, there are no major antigens recognised by all of the developmental stage rabbit serum. A 55 kDa antigen present in larval homogenate and SGE, is recognised by anti-larvae but not anti-nymph or anti-adult serum. Presuming that such antigens are also present in the SGE, this suggests that some proteins produced by female adult salivary glands are also present in the salivary glands of the different developmental stages, but are only exposed to the host at the different life stages, presumably through salivation. In other words, control of the protein profile of the saliva probably occurs at the cellular and/or the level of expression in the salivary glands of *I. ricinus* ticks.

Comparison of the relevant SGE profiles with the homogenate samples showed that extra bands were present in the homogenate samples (e.g. 45 and 10 kDa bands in larvae homogenate). Such antigens presumably represent non-salivary gland proteins exposed to the host during an infestation. Antigens such as the 42 kDa band present in the SGE but not the larval homogenate probably represent non-specific cross-reactivity. The majority of the developmental stage specific antigens were shared in both the relevant SGE and homogenate samples, suggesting that the salivary glands are the major source of antigens exposed to the host during feeding. The gut homogenate showed cross-reactivity with anti-adult antibodies, the major antigen was a 25 kDa species. The higher weight antigens shown in Fig 3.4 (e.g. 160-190 kDa) probably represent non-specific cross-reactivity. Antibodies recognise only a small segment of a protein (about 4-6 amino acids), the larger a protein, the greater the number of potential epitopes exposed, and hence the greater the chance of non-specific cross-reactivity.

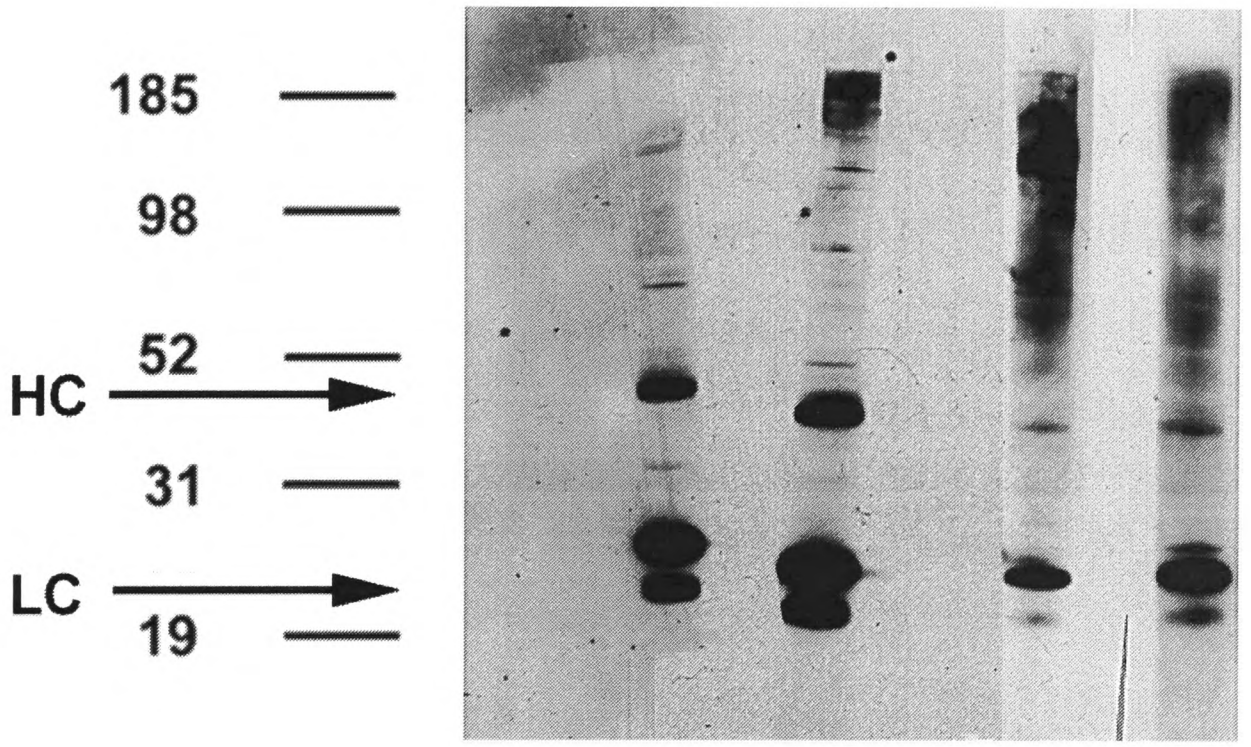


Fig 3.5 Antigenic profile of SGE (day 5) probed with a 1 in 500 dilution of serum from hamsters infested with the respective life stages of *I. ricinus* (as shown). 20 μg of SGE protein was used per lane. HC, LC- heavy chain and light chain of IgG respectively.

Serum obtained from hamsters infested with the various developmental stages of *I. ricinus* were used to immunoprobe SGE samples at a 1 in 500 dilution. Comparison of these results (Fig 3.5) with SGE samples probed with the relevant rabbit serum, showed that the pattern of antigens recognised by hamsters differed from that of rabbits. For example, 55 and 90 kDa antigens are strongly recognised by rabbit but not hamster anti-larval serum (Fig 3.7). Conversely, 50 and 80 kDa antigens react with the hamster but not the rabbit serum. Presuming that the same proteins are exposed to the different host species by the tick during feeding, this suggested that the two host species differ in their immunological perception of *I. ricinus* infestations.

3.3.6 Immunoprobing of SGE with serum from *Clethrionomys glareolus* (bank vole), *Apodemus flavicollis* (woodmouse) or BALB/c mice

The results of 3.3.5 suggested that different host species might recognise different antigens in response to tick infestations. However, the hosts used (i.e. rabbit and hamster) were not 'natural' hosts of *I. ricinus* ticks. In order to compare these results with natural rodent hosts of *I. ricinus* larvae, the antigenic profile of *Clethrionomys glareolus* (bank vole) and *Apodemus flavicollis* (woodmouse) were investigated. The laboratory strain BALB/c mouse was also tested in order to compare with the closely related woodmouse.

Western blotting was carried out as described in section 2.6 using NuPAGE gels under denaturing conditions (20µg of SGE was used per lane). The blots were immunoprobed using a 1 in 500 dilution of *C. glareolus, A. flavicollis* or BALB/c mice serum prepared as described in section 3.2.1, from either naïve or larvae infested animals.

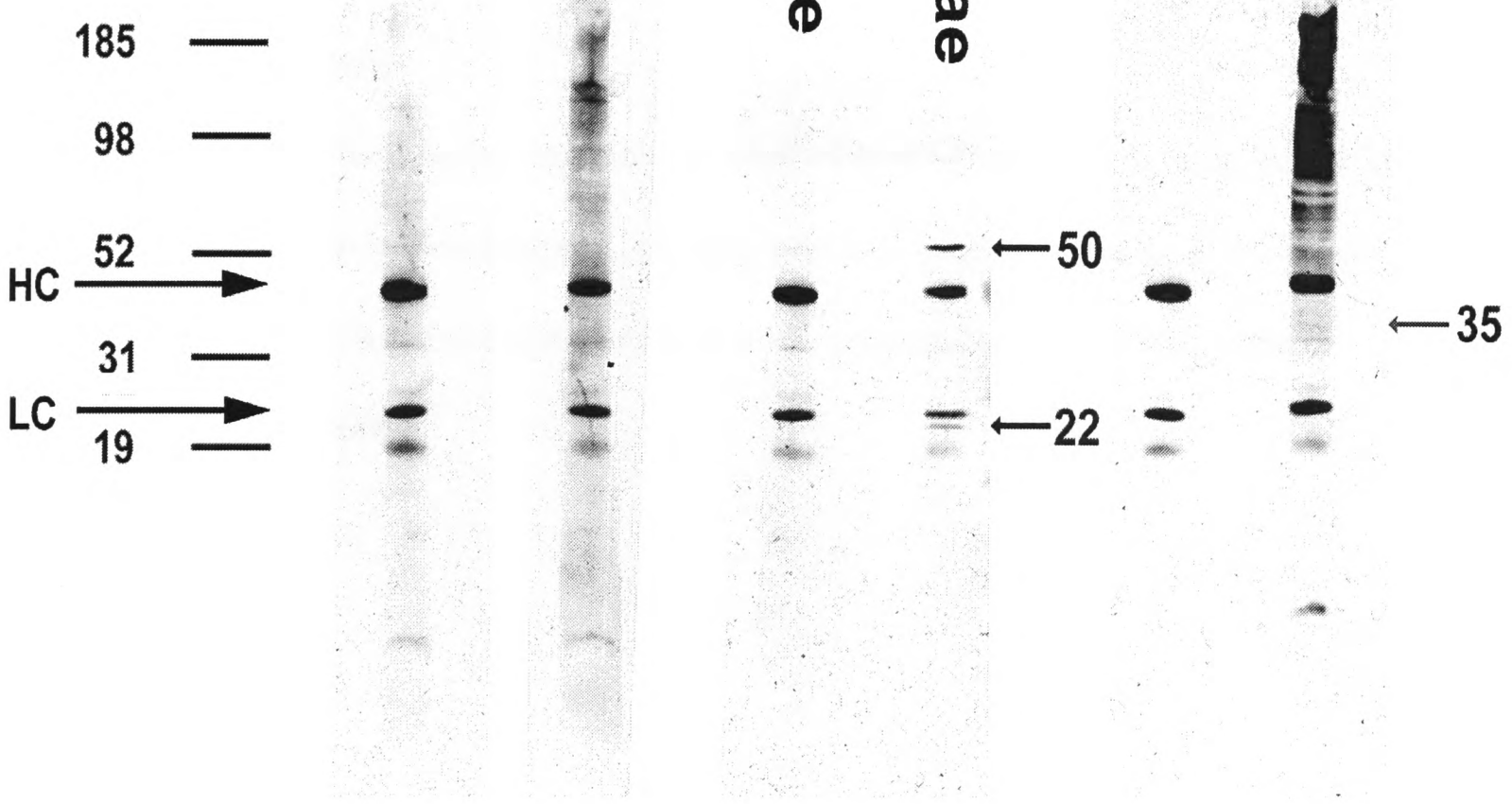


Fig 3.6 Antigenic profile of female adult *I. ricinus* SGE immunoprobed with a 1 in 500 dilution of BALB/c mouse, *C. glareolus* (vole) or *A. flavicollis* (woodmouse) serum as indicated. Twenty μg of SGE protein was used per lane. HC, LC- heavy chain and light chain of IgG respectively.

The results of Fig 3.6 showed that the pattern of SGE antigens recognised by the different host species differed significantly, and that the vole serum by far recognised the greatest number of antigens. Comparison of the woodmouse and BALB/c mouse antigenic profiles showed that the major antigens recognised by the BALB/c mouse are about 110 kDa, whereas in the woodmouse serum antigens of 50 and 22 kDa are the major species recognised. A duplicate reaction was set up except using mouse anti-IgM secondary antibody. The IgM profile was not significantly different from that of the IgG profile (data not shown).

The different anti-larval antigenic profiles of SGE of the various hosts tested are shown in Fig 3.7. These data suggests that the immunological perception of *I. ricinus* larvae differ between natural and non-natural hosts and between the closely related BALB/c and woodmouse.

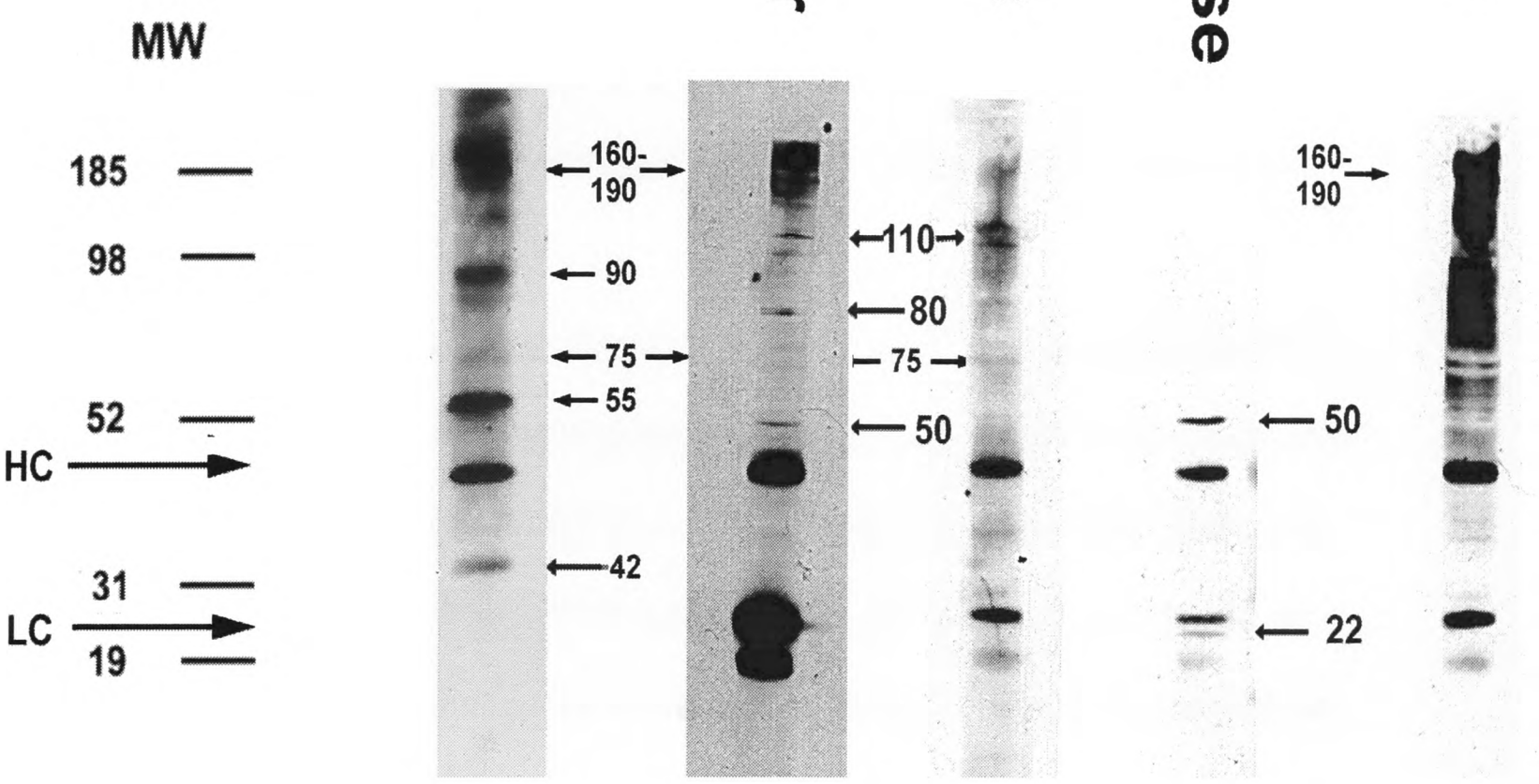


Fig 3.7 Antigenic profile of *I. ricinus* SGE immunoprobed with various species serum from larvae infested animals at a 1 in 500 dilution.

3.4 **Discussion**

Much of the tick antigenic research to date relies upon immunising hosts with crude SGE extract and may result in normally 'hidden' antigens being exposed. Therefore for the experiments described here, the purpose of which was to investigate immunological perception by hosts, female adult *I. ricinus* SGE and whole tick homogenates were tested against sera obtained from hosts infested with the relevant developmental stages.

Throughout the feeding period, the mean weight of *I. ricinus* female adult ticks increased from a mean weight of 6 mg for unfed ticks to 264 mg for ticks fed for six days or more (Fig 3.1-top). This is an increase of over forty times in body weight. There was an exponential increase in tick weight from unfed to ticks fed for five days; the rate of this increase was greater after six days of feeding. This probably reflects the onset of the fast phase of feeding which occurs 12-24 hours prior to drop off and after the extended slow phase of feeding (Brossard *et al.*, 1993). It is generally believed that it is during the slow phase of feeding that the tick must adapt to the host responses, forming a feeding lesion, and that the fast phase can only occur once a 'stable' host-tick interface has been established. It is plausable to assume that the majority of saliva-mediated modulation at the host site occurs during the slow phase of feeding. The saliva of *A. habraeum* was shown to be antigenically more complex in partially fed small ticks (<100 mg) when compared with larger feeding ticks (Dharampaul *et al.*, 1993). The results of this chapter suggest that the fast phase of feeding occurs after six or more days in feeding *I. ricinus*

female adult ticks. For this reason, ticks used for this research (unless otherwise stated) were fed for five days. Consistent with the findings of other researchers, the soluble protein concentration of the salivary glands increases in a linear fashion in unfed and throughout the feeding period (represented by different weight groups) (Fig 3.1-bottom). There was however, a slight decrease in the protein content of ticks weighing 200-300 mg. The ticks in weight group 200-300 mg represent ticks that were replete (data not shown). The observed loss of SGE protein content in this group could be due to degradation of the salivary glands which takes place after completion of feeding (Harris and Kaufman, 1981). Salivary gland degeneration was observed to occur within 24 hours post-engorgement in the ixodid tick *A. habraeum* (Lomas *et al.*, 1998). Alternatively, the excess washing required to remove large quantity of haemoglobin in these salivary glands may have lead to the observed reduction in protein content. The observed range of protein concentrations in the salivary glands of *I. ricinus* adult female ticks are consistent with those reported for *Rhipicephalus appendiculatus* (Wang and Nuttall , 1994) and *Amblyomma americanum* (McSwain *et al.*, 1982).

The results in this chapter demonstrated that both the protein profile and the antigenic profile of female adult *I. ricinus* salivary glands changes throughout the feeding period, and from that of unfed female ticks. These data are consistent with the idea that attachment and feeding are the major modulators of salivary gland protein synthesis in ticks (Binnington, 1978). The range of antigens exposed to hosts by ticks differs throughout the feeding cycle (Jaworski *et al.*, 1990). Fig 3.3- top showed that antigens recognised by rabbit anti-adult serum differ in the SGE derived from *I. ricinus* female adult ticks unfed and throughout the feeding period. The changing pattern is presumably

a direct result of the changing protein expression of the salivary glands. It is not known whether, like other parasites such as *Trypanosoma brucei*, the changing antigenic profile of feeding adult female *I. ricinus* salivary glands has an immunoevasive role (Turner and Michael, 1997).

The male and female whole tick homogenate protein and antigenic profiles were surprisingly similar to those observed with the salivary gland extract samples. This might suggest that many protein components of salivary glands are common to other tissue types in the tick. It has been observed that haemolymph proteins pass into the salivary glands, and subsequently the saliva of feeding *Rhipicephalus appendiculatus* ticks (Wang and Nuttall , 1994). It is also likely that many unrelated protein species share identical mobilities to salivary gland proteins, or contain cross-reactive epitopes. SGE derived antigens are believed to be mainly glycoproteins, many of which contain cross-species reactivity (Wheeler *et al.*, 1991). PNGase F treatment of the male adult whole tick homogenate did not significantly reduce the number of epitopes recognised by rabbit anti-adult serum whereas proteinase K treatment removed all but a few antigens (Fig 3.3-bottom). This suggests that the cross-reactive antigens of male adult *I. ricinus* ticks are primarily based on common protein motifs and not upon shared carbohydrate moieties. Despite the fact that male *I. ricinus* are generally believed not to feed, salivary gland structures have been described in these ticks, their function is unknown (Balashov, 1965). The results of Fig 3.3 suggest that the male adult *I. ricinus* salivary glands contain many shared proteins with the salivary glands of female adult ticks. Whether bioactive compounds are present in the salivary glands of male adult *I. ricinus* ticks is unknown.

Probing of whole homogenates and SGE proteins with sera raised in rabbit and hamster against the different developmental stages of *I. ricinus* showed that different antigens are exposed to the host at the different life stages as a consequence of feeding (Fig 3.4 and 3.5). Similar results were obtained in previous *I. ricinus* antigenic profile studies (Rutti and Brossard , 1989; Uhlir *et al.*, 1994). The majority of antigens observed in the whole tick homogenate samples were also present in the SGE samples probed with the respective sera. This suggests that these proteins are probably salivary gland derived proteins in the crude homogenates. This suggests that the salivary glands are the major source of antigens exposed during feeding. The differing antigenic profile of SGE samples with respect to the different developmental stage anti-serum used, suggests that proteins are present in adult female salivary glands that are differentially exposed to the host by the salivary glands only at the specific developmental stages (Fig 3.4). Presuming that such antigens do not represent non-specific cross-reactivity, this suggests that the different developmental stages of *I. ricinus* expose differing antigens to the host. However, larvae and nymph specific antigens are present in the SGE protein of female adults that are not recognised by the anti-adult serum (e.g. 55 kDa band- larvae specific). If adult females do not expose these antigens to the host during the feeding process (presumably as saliva), this suggests that these expressed proteins are sequestered intracellularly within the salivary glands. Consequently, it is probable that regulation of the protein content of saliva occurs at the cellular level as well as at the level of expression in the salivary glands. Secretary proteins can be stored intracellularly in specialised secretary granules, for release upon response to a specific stimulus ('regulated secretion') (Miller and Moore, 1990). Agranular (Type I) and granular (Type II, III and

IV) accini are found in the salivary glands of ixodid ticks (Kemp *et al.*, 1982). *Ixodes ricinus* salivary glands have been observed to contain type II and III accini (Balashov, 1965). In ixodids, attachment to the host appears to trigger the production of granules in some cells and, at the same time, stimulate the secretion of granules from other cells (Kemp *et al.*, 1982).

The presence of antigens in the larvae and nymph whole tick homogenates, but not in the respective SGE, probably represent proteins exposed to the host from a part of the tick other than the salivary glands. A 25 kDa integumental antigen has been described in *I. ricinus* (Rutti and Brossard , 1989). It is suggested that mid-gut derived antigens of *Amblyomma americanum*, are probably exposed to the host as a result of regurgitation of mid-gut material during feeding (Brown, 1988). The presence of gut-specific antigens in *I. ricinus* was consistent with this hypothesis, a 25 kDa antigen is present in the gut but not SGE samples (Fig 3.4). This antigen may be the integumental antigen described by Rutti *et al.*

Serum from hamsters infested with the various developmental stages of *I. ricinus* was used to investigate the antigenic profile of SGE in Fig 3.5. Consistent with the observations of Fig 3.4, these results showed that different antigens in the SGE samples were recognised by serum against the respective life stages of *I. ricinus*. However, these profiles were different from those obtained with the respective rabbit serum. These data suggest that the antigens recognised by a host in response to *I. ricinus* infestation varies between these host species.

The majority of research detailing the immune responses of hosts to tick infestations is based upon 'laboratory' animals such as rabbits and mice being used as

host models. The antigenic profile of SGE immunoprobed with serum raised in various species against *I. ricinus* larvae infestation are shown in Fig 3.7. Fig 3.6 and 3.7 showed that the pattern of reactive antibodies produced in response to infestation varies with the species used as a host. Furthermore, the 'laboratory' models used (i.e. rabbit, hamster and BALB/c mouse) differed markedly from serum obtained from infested 'natural' hosts (i.e. woodmouse and bank-vole). In other words, the immunological 'perception' and presumably the effector response of a host differs between the particularly species infested by a tick.

Clethrionomys glareolus, but not *Apodemus flavicollis*, acquires resistance to repeated *Ixodes ricinus* larval infestations (Dizij and Kurtenbach, 1995). The results of Fig 3.6 showed that specific antibodies are produced by *A. flavicollis* in response to infestations of *I. ricinus* larvae. This suggests that *A. flavicollis* perceives the parasite immunologically, but does not mount a sufficient response to affect the tick-feeding process. Perhaps the immunmodulatory properties of the tick saliva (Ribeiro *et al.*, 1985) are sufficient to suppress the effector responses of *A. flavicollis,* but not *C. glareolus*. It should be noted that there was no quantitative measurement of the antibody response in these hosts. Alternatively, antigens that are present in blots probed with *C. glareolus* but not *A. flavicollis,* serum such as the 35 kDa band, may confer resistance to larval *I. ricinus* (Fig 3.6). Further work is being carried out to test this hypothesis.

Any immune response involves, firstly recognition of the pathogen or foreign material, such as is presented to the host during tick feeding, and secondly, mounting a reaction to eliminate it (i.e. the effector response). The development of acquired immunity against ticks is mediated largely by antibody formation (Willadsen, 1980).

Serum obtained from rabbits repeatedly infested with *I. ricinus* was shown to transfer resistance to naïve animals (Brossard and Girardin, 1979). This chapter provides evidence that the immunological perception of tick infestation by hosts, as shown through antigenic profiling, is dependent upon the feeding process, the particular life-stage that is feeding, and the host species on which the tick is feeding.

These findings suggest that the tick-host interface is more complex than was previously recognised. In view of the development of immunological based anti-tick vaccines, this study further reinforces the importance of 'natural' vs. 'laboratory' research in the study of tick-host interactions (Randolph and Nuttall, 1994).

CHAPTER 4

PRODUCTION OF A SUBTRACTIVE cDNA LIBRARY FROM UNFED AND PARTIALLY FED *Ixodes ricinus* FEMALE ADULT TICK SALIVARY GLANDS

4.1 Introduction

Unlike other haematophagous arthropods, ixodid ticks take few blood meals of a relatively long duration. During the feeding process, ticks secrete a wide range of bioactive compounds into the host as saliva (Ribeiro *et al.*, 1985). Saliva contains proteins that facilitate feeding of the tick by counteracting host inflammatory responses. A number of saliva activities have been characterised including immunomodulation, anti-coagulation and vasodilation (Wikel, 1996b). Elucidation of how tick saliva modulates host immunity can provide a valuable insight on vertebrate immunology. However, the practical constraints of obtaining enough tick material for traditional biochemical analysis are prohibitive. This is especially true for the smaller *Ixodes* species ticks, such as *Ixodes ricinus.* Therefore a genetic approach, utilising recombinant cDNA library technology appears to be a more promising way in which to study tick proteins in theoretically unlimited quantities.

mRNA typically represents only 1% of the total RNA population of a tissue. In addition, transcripts expressed in response to a specific event or state (i.e differentially expressed genes), represent only a tiny fraction of this mRNA population. Consequently,

the identification of differentially expressed genes has been the attention of considerable research.

Rare transcripts may account for only 1 in 10^6 clones of a standard cDNA library. Consequently, the practical number of clones required to produce an effective cDNA library is huge; hence the random sampling of clones as a way to isolate a differentially expressed gene, is a punitive task. A more productive approach is to look at ways of enriching mRNA populations for differentially expressed genes. By enriching salivary gland mRNA populations in such a way, it should be possible to identify feeding-associated expression, and saliva-associated expression in particular.

The first aim of the research described in this chapter was to produce a λ-phage library from 'full-length' cDNA constructs derived from the total mRNA populations of the salivary glands of feeding *I. ricinus* adult females. This library was used primarily for screening purposes. The second aim was to use a subtractive approach to produce a cDNA population enriched for differentially expressed saliva-associated sequences. Using this method in combination with differential screening of the subtracted cDNA clones, should increase the likelihood of obtaining random clones that are saliva-associated. Recombinant expression of such cDNA should in theory allow for the production of '*in vitro*' saliva proteins from female adult *I. ricinus* ticks.

4.1.2 λ-phage cDNA library construction

The main objective in generating a useful recombinant DNA library from a cDNA population is the creation of the huge population of clones necessary to ensure that the library contains at least one version of every sequence of interest.

In general terms, λ cDNA library construction involves first conversion of a mRNA population into the more stable cDNA form, and secondly the cloning of the resulting cDNA population into a λ-phage derived vector. A brief description of the technique using Startagene's ZAP Express kit is given as follows. Reverse transcriptase (Moloney murine leukaemia virus) and a poly (T) primer containing a XhoI site are used for first strand synthesis. 5'-methyl dCTP is incorporated in first strand synthesis, in order that the first strand cDNA produced is not cleaved by the restriction enzymes used in subsequent cloning steps. The RNA portion of the cDNA-RNA hybrid is cut using RNase H, which produces multiple fragments that can be used by DNA polymerase I as primers for 'nick-translation'. The uneven termini of the cDNA are 'nibbled' back using *pfu* DNA polymerase, and the blunt ends ligated to EcoR I adapter sequences. Xho I digestion releases the adapter from the 3' end, forming directional 5'-EcoR I-cDNA-Xho I-3' cDNAs. The cleaved adapters are separated from the cDNA on a Sephacryl containing column. The size-fractionated cDNA is then precipitated, and directionally ligated into λ-phage arms containing complementary EcoRI and Xho I cloning sites. The λ-library is then packaged into XL1-Blue MRF', *E. coli* cells.

The λ-ZAP Express (Stratagene) vectors used in this research allow for both eukaryotic and prokaryotic expression of inserts via CMV and *lac* promoters, respectively. Based on λgt11/10 vectors, λ-ZAP clones can be screened either with DNA or antibody probes. Excision of the pBK-CMV phagemid allows for the characterisation of clones within plasmid systems.

4.1.2 A subtractive approach to differential mRNA expression

A subtracted cDNA library consists of cDNA clones that correspond to mRNA present in one state (tester) but not another (driver). During the feeding period both the protein and mRNA profile of tick salivary glands changes as a consequence of saliva protein production (Oaks *et al.*, 1991; Wang and Nuttall , 1994). By 'subtracting' the mRNA population of salivary glands derived from unfed ticks (representing a quiescent state) from mRNA produced during feeding (quiescent plus saliva-associated), the resulting mRNA population should be enriched for differentially expressed genes associated with the feeding process.

Many techniques have been used to identify differentially expressed genes. In general terms, cDNA subtraction techniques rely upon the hybridisation of an excess of driver mRNA (or cDNA) to tester cDNA, and then analysing the unhybridized tester ssDNA. The separation of ssDNA from the dsDNA (or DNA/RNA hybrid) can be performed by several techniques including hydroxylapatite chromatography (Hendrick *et al.*, 1984), avidin-biotin binding (Sargent and Dawid, 1983), and oligo (dT) latex beads (Hara *et al.*, 1991). Despite the successful identification of numerous important genes using these methods (e.g. T-cell receptors (Hendrick *et al.*, 1984)), such techniques are often inefficient in obtaining low abundance transcripts. In addition, more than 20 μg of driver mRNA is routinely required for such techniques. This is more than could be practically collected from the salivary glands of unfed *I. ricinus* ticks.

The technique of choice for this research was suppression subtractive hybridisation-PCR (SSH-PCR). The SSH-PCR technique has been successfully used to produce subtractive cDNA libraries from a range of sources from as little as 500ng of

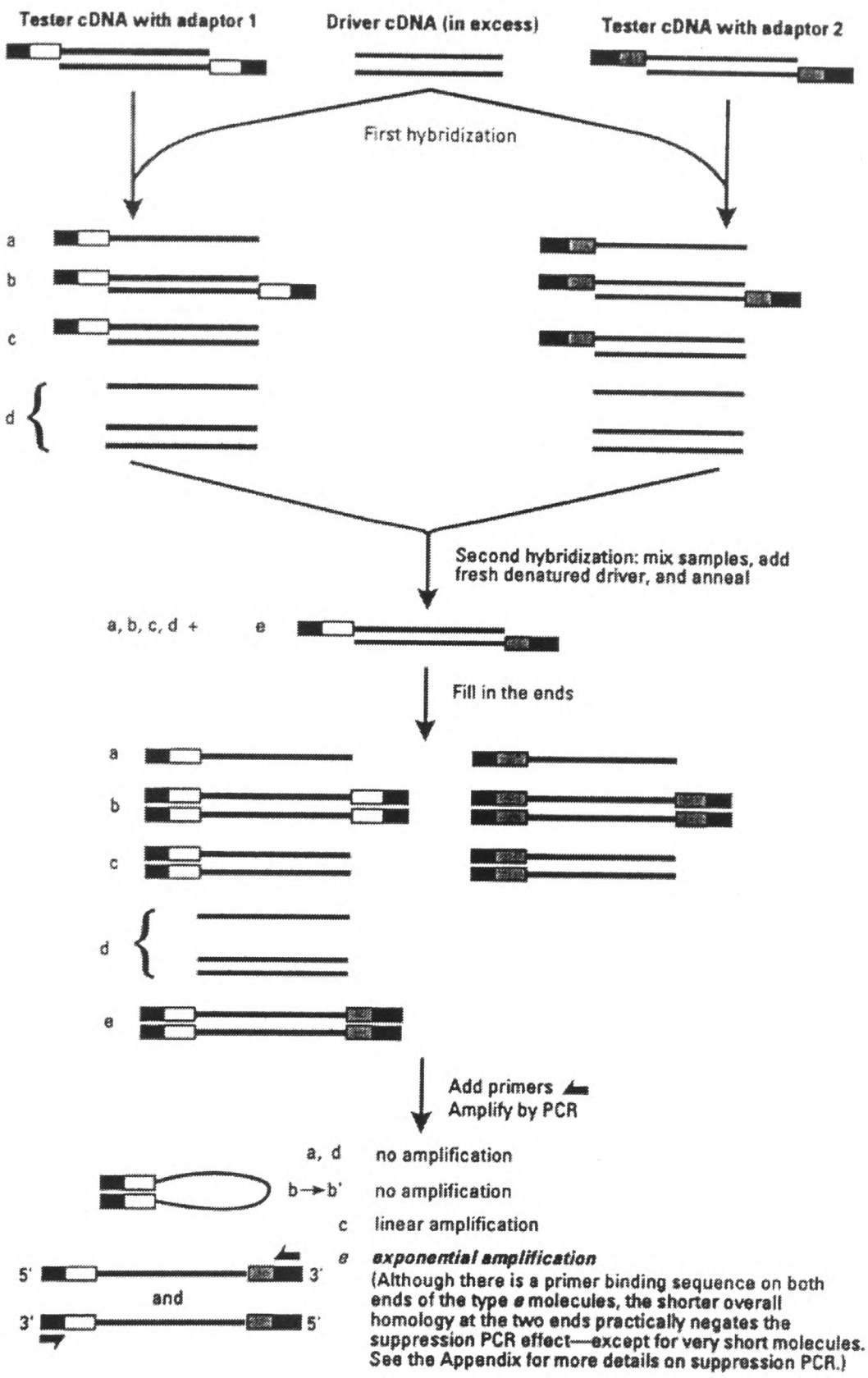


Fig 4.1 Schematic diagram of SSH-PCR technique. Reproduced from Clontech PCR-Select manual.

mRNA (Chan *et al.*, 1997; Groenink and Leegwater, 1996). SSH-PCR selectively amplifies differentially expressed cDNA by PCR, whilst suppressing the PCR of non-target cDNAs. Equalisation of rare and abundant transcripts occurs during the first hybridisation step (Diatchenko *et al.*, 1996). The PCR-Select kit used in this research (Clonetech, cat. no. K1804-1) is based upon the SSH-PCR technique, and has been optimised for use with small quantities of mRNA.

4.1.3 **Suppression subtractive hybridisation- PCR**

Fig 4.1 shows an outline of the SSH-PCR technique. Driver and tester mRNA (i.e. unfed and fed salivary glands respectively) are reverse transcribed using a $dT_{(30)}$ primer containing a Hind III and Rsa I site to form first strand cDNA. Second strand synthesis is carried out as described in section 4.1.2. Both cDNA populations are separately digested with the blunt-ending four-base restriction enzyme, Rsa I. This allows the larger cDNA to be cut to a size more suitable for cloning as well as providing a common sequence for cloning. The tester cDNA population is split in two; one half is ligated to adapter 1 and the other half to adapter 2. These adapters lack phosphate groups, and so ligate only to 5' ends of cDNA. Both adapters contain a common sequence at the 5' end (PCR primer 1(PCR1)) but individual 3' sequences (corresponding to PCR nested primers 1 (PN1) and 2 (PN2) respectively).

During the first round of hybridisation, an excess of driver cDNA is added to each adapter-ligated tester sample. The mixtures are then heat-denatured and allowed to re-anneal. The possible combinations that form during re-annealing (i.e. type a, b, c, d and e molecules) are shown in Fig 4.1. Type a molecules (representing adapter ligated tester

only cDNA) are normalised during this step due to the second order kinetics of hybridisation (i.e. the more abundant molecules re-anneal faster to form type b molecules). Non-differentially expressed molecules or molecules present only in driver population form type c or d molecules, respectively. In the second round of hybridisation, the two-cDNA populations are mixed together without denaturing and then an excess of freshly denatured driver cDNA is added to the mixture. This acts to further enrich the target population for differentially expressed cDNA as well as only allowing hybridisation of type a-adapter1 molecules with type a-adapter2 molecules to form the hybrid type e molecules (i.e. tester specific cDNA).

Filling in the ends with DNA polymerase generates type e molecules containing both adapters at the 5' and 3' ends. The entire population is then subjected to PCR using PCR primer 1; type a and d molecules lack the adapter specific primer sites and consequently are not amplified. Type c molecules contain only one primer site, resulting in the linear amplification of this population. The adapters contain long inverted repeat sequences; the result is that type b molecules form pan-handle like structures which inhibit PCR, so called suppression PCR (Siebert *et al.*, 1995). Only type e molecules containing both primer sites can undergo exponential amplification. Secondary PCR using the nested primers further enriches the population for differentially expressed cDNAs. The resulting cDNAs can then be cloned directly into T/A vectors. The construction of both forwards (i.e. tester-driver (+)) and backwards (i.e. driver-tester (-)) cDNA populations allows for the differential screening of 'forward' clones with probes derived from both cDNA populations, therefore reducing the number of false positive clones obtained.

4.2 Materials and methods - λ library construction

4.2.1 Preparation of mRNA

The freshly dissected salivary glands of 50 + 46 female adult ticks fed for four and six days respectively, were pooled and placed on dry ice in a DEPC treated tube. Homogenisation was carried out using glass homogenisers in the supplied protein/RNAase degrading solution. mRNA preparation was carried out using a poly-d(T) cellulose spin column (Micro-FastTrack kit (Invitrogen, cat. no. K1520-02)) according to the supplied protocol. The resulting mRNA was ethanol precipitated and re-suspended in 24μl 10mM Tris-EDTA pH 7.5. The concentration of mRNA was measured spectrophotometrically at 260nm. The RNA concentration was 0.128 $\mu g\ \mu l^{-1}$, and the total mRNA obtained was 2.56 μg. All of this was used for cDNA synthesis.

4.2.2 cDNA Library Construction

cDNA library construction was carried out according to manufacturers instructions using the ZAP Express cDNA Synthesis kit (Stratagene, cat. no. 200403). All reactions were carried out under sterile conditions using DEPC treated / sterile consumables. cDNA construction was carried out in accordance with manufacturers instructions. No control [α-^{32}P]dNTP containing reaction was used to follow first strand synthesis (no isotopes were available at the time). EcoR1 adapters were ligated to the cDNA at 8^{O}C for two days. The adapters were separated from the cDNA using a supplied spin column containing Sepharyl S-500; 4x 60 μl fractions were collected in STE buffer. Of the four fractions collected, fractions 1 and 2 (1+2), and 3 and 4 (3+4) were pooled.

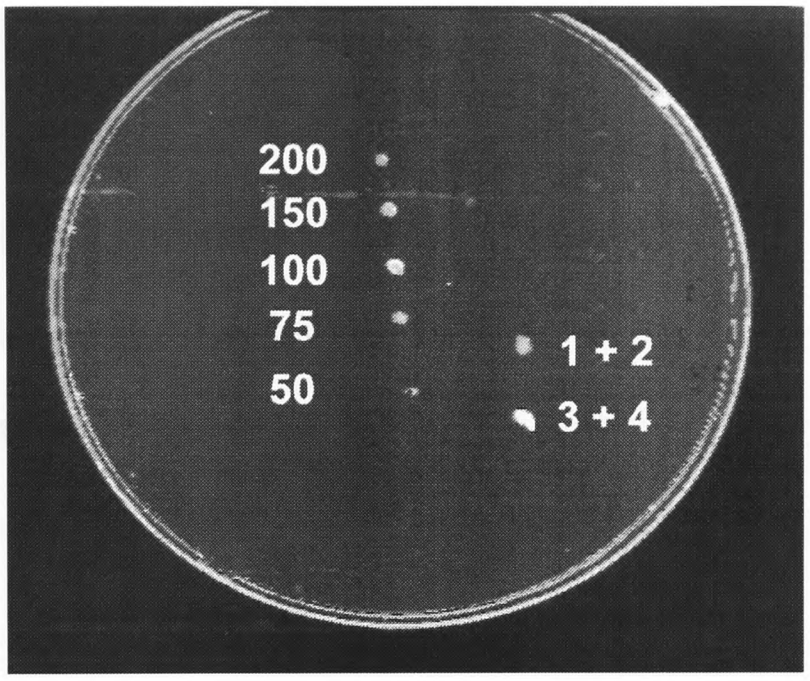


Fig. 4.2 EtBr containing agarose plate. Standard DNA concentrations as shown (ng μl^{-1}). 0.5 µl of cDNA containing fractions were spotted on plate as shown. Visualized by UV light.

0.5 µl (total 3 µl) of the size-fractionated cDNA were visualised using EtBr agarose plates. This indicated a concentration of ~150 ng μl^{-1} (Fig 4.2). 1 µl of each cDNA population was used to ligate λ vector arms at 4°C for two days according to supplied protocol (Packagene Lambda DNA Packaging System (Promega, cat. no. K3152)). To check the titre of the primary libraries, 1 µl (total 500 µl) of fraction 1+2 was used to transfect XL1-MRF'cells as described in the manufacturer's protocol. A titre of 2465 pfu μl^{-1} equivalent to a total of 1.23 x 10^6 pfu was obtained. Plaques were tested for the presence of insert using X-gal / IPTG containing plates. The ratio of white/blue colonies was ~30.

Because of the instability of primary libraries it is suggested that the library should be amplified before storage. The equivalent of 5 x 10^4 pfu (i.e. 20 • l) per plate (25 plates) was used to amplify the primary library in accordance with manufacturer's instructions. Titration of the amplified library (in 120ml SM buffer + 0.3% CH_3Cl) gave a total of 2.49 x 10^9 pfu.

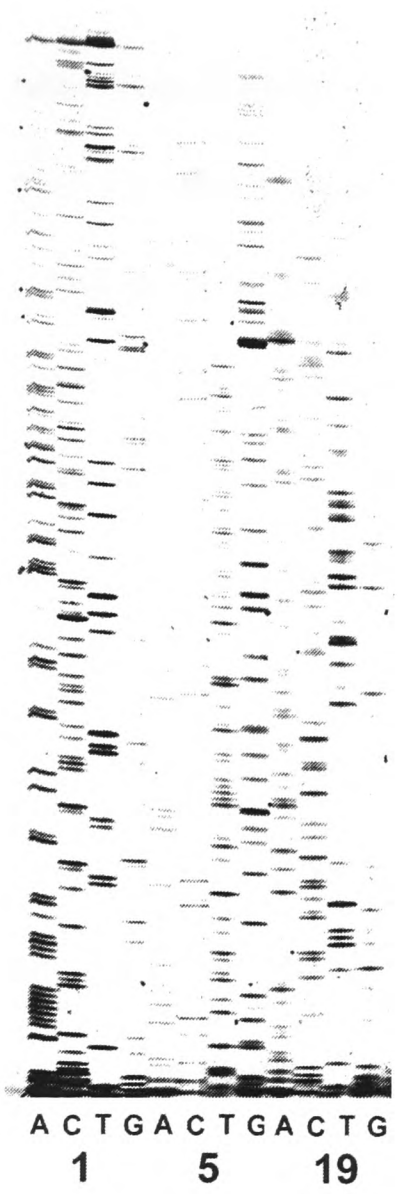


Fig 4.3 Example of sequencing ^{32}P-labelled autoradiogram sequenced using Sequenase dideoxy method. Sequences shown are λ-phage cDNA library derived (sequences in Fig 4.5).

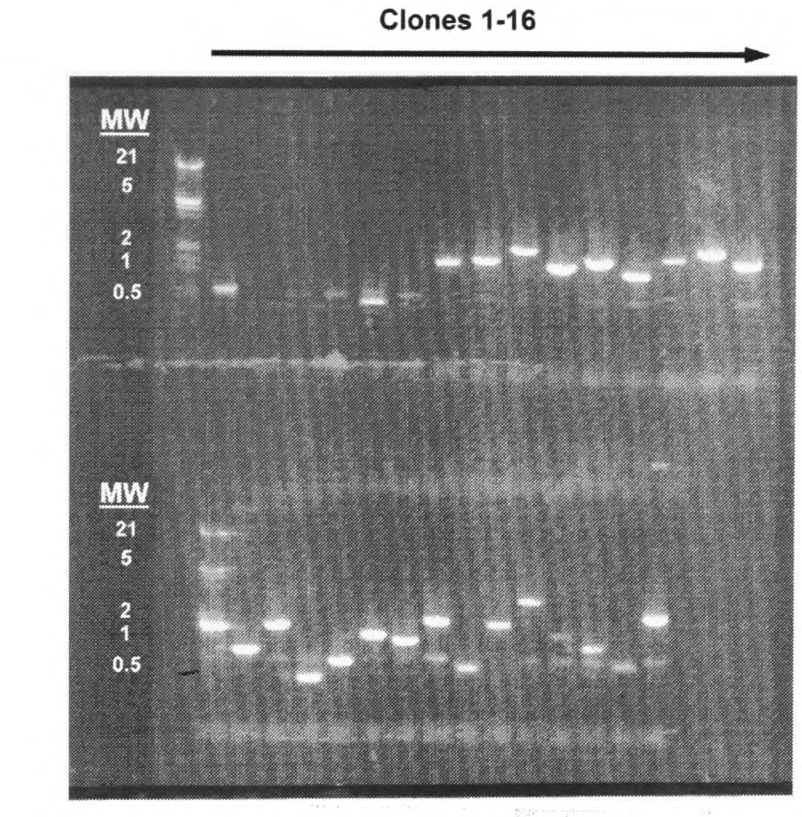
4.3 Results - λ library construction

4.3.1 Random sampling of clones from λ library

In order to test the validity of the cDNA library constructed, random clones were picked and the inserts sequenced. Plaques were grown up as described in the supplied protocol on X-gal/IPTG containing NZY plates. Thirty white colonies were 'cored' from the plate and diffused overnight in 500 µl SM buffer + 20 µl CH_3Cl at 4°C. Two µl of the phage-containing solution was used as template DNA for PCR reactions. PCR reactions were set up as described in section 2.4.3, using vector specific sequences, T3 and T7 as primers (Fig 4.4). Six of the lowest molecular weight clones (i.e. clones no. 1, 5, 19, 20, 24 and 29) were chosen for sequencing (Fig 4.3). For the purposes of sequencing, the clones were phagemid excised into XL1-Blue cells according to the manufacturer's instructions. Colonies were plated out onto kanomycin containing plates (50 µg ml^{-1}). Single colonies were picked and used for inoculating overnight cultures in LB containing 50 µg ml^{-1} of kanomycin. Phagemid DNA was prepared from cultures using the QIAprep spin mini-prep kit (Quiagen- cat. no. 27106). Five µg of the DNA was denatured and enzymatically sequenced using a Sequenase 2.0 kit (United States Biochemical, cat.no. 70775) in accordance with the supplied protocol.

4.3.2 Sequencing of clones

Sequences were read from gels as shown (Fig 4.3). Vector-derived sequence was removed and the truncated sequences used for homology searching (Fig 4.5). Searches were carried out using the GENEMBL and swissprot databases, and the BLAST



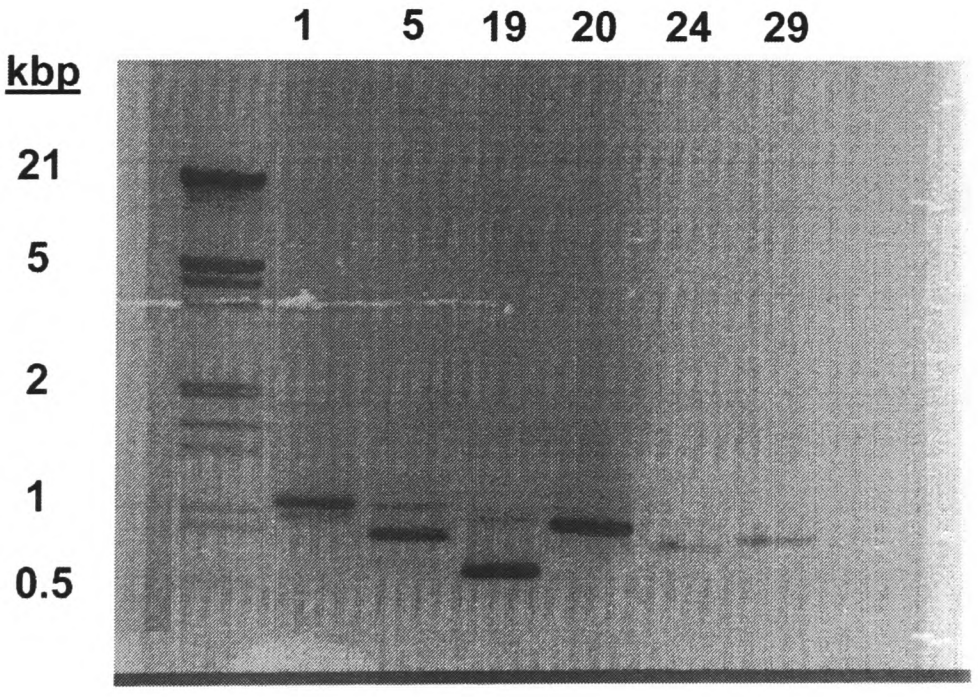


Fig 4.4 PCR products of randomly picked clones from λ cDNA library. 2% TAE agarose gel. Top- 30 random clones, bottom- clones chosen for sequencing. Clone numbers as indicated.

algorithm. Clone # 20 shows little homology with any published sequences. Clone #1 has homology (smallest sum probability P(N) = 2.0 x 10^{-3}) to several regulatory proteins from *Saccharomyces cerevisiae* and no obvious signal sequence. This suggests the clone maybe a nuclear protein, although the clone may not represent the complete transcript. Clones #5 and #19 possess a putative signal sequence (i.e. a run of hydrophobic residues downstream of the start codon), and although they have no significant homology to any published sequences, they may encode secretion products. Clone #29 is a homologue of cytochrome c oxidase polypeptide II (P(N) = 3.1 x 10^{-100}), a protein that is widely used in phylogenetic studies. Clone #29 shows the highest degree of homology to *Tegeticula maculata* (a type of moth). Clone #24 is a homologue of elongation factor-2 (P(N) = 2.8 x 10^{-12}).

These results showed that the construction of the λ-phage cDNA library was successful and that the sequence information obtained from the random sampling of this library shows the cDNA is tick-derived.

The λ-phage cDNA library provides a resource for the screening of 'full-length' clones using either antibody or DNA probes. The library was expressed and screened using serum obtained from rabbits infested with the various developmental stages of *I. ricinus* (section 3.2.1). Several positive clones were obtained using the DIG detection system (Boheringer Mannheim- data not shown), but further rounds of screening showed no positive clones. The library was also screened with DIG labelled DNA probes obtained from a *Rhipicephalus appendiculatus* cDNA library (obtained from Guido Paesen- IVEM), however no positive colonies were obtained (data not shown).

(1)AAAGAATTCGGCACGAGCAGCCCGTAGCAAAAAAACCCAAAAGCA

GAACTTCCGGAATTCCAACCGCTTGCTCAAGCCCCAACAGCGTCTCTCCAAAT

CAACACTCCAAATGCCGATCAACGGAAGGAAGCAAACCACTAACTGGATGG

AATCTGGCAACTCCGACATACGTCAAGTCAACTCAAAAAA(215)

Clone #1

(1)GTTACACGTGCAAGCTGATGAATTATTCTGTACCTACGATATGTAGAAGTT

TTTGATTGATGGTCTACCTTGACAGTTCTAAGTATGTAATTAGCATTTTGCAA

TACGTTTGTTT(115)

Clone #5

(1)AAGAATTCGGCACGAGCAACAAAGGAAAGCGAGACGAACTGCCTTTCCGT

TTGTCACCGAGTGCACCAACAGACCAACCAGATCGATAATCCTTCATCCACT

CTACCC(108)

Clone # 19

Fig 4.5 Sequences of λ-library derived cDNA clones. Vector sequences have been removed.

(1)AAGTCTACCAGTCATGAAGACTGCGCTGACCTGTGCGCTTTTCGGGATTTT

GTTTTTCGGAAGCCTGTGTTCTTCTAACGAAGAAATCCAGGAACAAGAGCCG

AGCCCAACCCTCAGGACGAACTCTACAACGAA(135)

Clone # 20

(1)CACGAGGCCGTGGGAGGCATCATCGGGGTGCTCAACAGGCGCAGGGGAC

AGCTCTTCGAGGAGTCTCAAGTGACCGGCACGCCCATGTTCGTGGTCAAGGA

CTCTACCT(108)

Clone # 24

(1)CNNNCTTTCNTTNAGCCATTTGGGTACACTTACCTGNTACCCCNCCCGGGG

AAATTCCCCTATTATAGAACAAATTGGAAAATCGATGGGCCCGCGGCCGCTC

TAGAAGTACTCTCGAGTTTTTTTTTTTTTTTTTAAAAAGATTTTAGTCATTT

AATAAAATTATTTGAGGATGTNATTTCAAGAGAAATAGGTATAAATCTATGA

TTTGCTCCACAAATTTCAGAACATTGCCCAAAAAATAAACCTGGGCGTGAAG

CAAAGGAAAAAGACTGATTAAGGCGACCAGGAACGGCATCTATTTTTATTCC

TAATGATGGAACTGTTCATGAATGAATTACATCTGCTGATGAAATTAAAAATT

TAATGTGAGAATTAAAAGGAATTACTATACGGTTGTCTACGTCAAGAATCGG

AATGAGTTTTTTATTATTTCATTACNAGGAATTATAAATGAATCATATTCNAT

ATTAAAATCTGAGAATTCATAAGATCAATATCATTGATGTCCAATAATTTTTA

CAGTAATTGAAGGTGAGTAAGATTCCCNNGTATNTAAAGAAGACTTTTGAN

GGTTTGCCATAAANATTNATGTTTTGCTGGGATANT (586)

Clone # 29 (cyt 5' and cyt 3' primer sequences are underlined)

4.4 Materials and methods – Subtractive library construction

4.4.1 mRNA preparation.

Two hundred unfed female adult *I. ricinus* ticks were dissected, and their salivary glands removed and placed on dry ice. One hundred and fifty female + male adult *I. ricinus* ticks (75 day 4 and 75 day 6) were fed on rabbits; the salivary glands of these ticks were dissected out and placed on dry ice. The two fed populations were pooled in order to give a representation of the changing mRNA profile throughout feeding. mRNA was prepared as described in section 4.2.1. The concentration of mRNA was measured spectrophotometrically at 260nm.

Unfed mRNA = 0.056 μg μl^{-1} in 41 μl (total = 2.296 μg)

Fed mRNA = 0.488 μg μl^{-1} in 41 μl (total = 20.0 μg)

4.4.2 First strand cDNA synthesis

To assess the efficiency of the PCR-Select procedure supplied human skeletal muscle poly (A) mRNA was used as a control. Unfed (driver) and fed (tester) tick mRNA (2 μg) was used for first strand synthesis. The mRNA samples were denatured at 70°C for 2 minutes. First strand cDNA synthesis was carried out using MMLV reverse transcriptase (200 units), at 42°C for 1.5 hours, incorporating 1 μl of [α^{32}P]dCTP (1 mCi/ml) as described in the supplied protocol.

4.4.3 **Second strand cDNA synthesis**

The first strand mixtures were mixed with the supplied second-strand enzyme cocktail (DNA polymerase I, RNase H and *E. coli* DNA ligase) according to supplied protocol, incubation was carried out for 2 hours at 16°C. The cDNA was blunt-ended using 6 units of T4 DNA polymerase by incubating for 30 minutes at 16°C. Synthesis was stopped using EDTA/glycogen mix (10mM EDTA/ 50 μg ml^{-1}). The samples (volume 86 μl) were phenol: chloroform: isoamyl alcohol and then chloroform: isoamyl alcohol extracted according to supplied protocol (in order to remove enzymes etc.). The cDNA was then precipitated using 0.5 volumes of 4M NH_4OAc and 2.5 volumes of 95% ethanol. The pellets were detected using a Geiger-Muller tube. The cDNA was resuspeneded in 50 μl of H_2O.

4.4.4 **Rsa I digestion of cDNA**

The ds cDNA was digested using supplied Rsa I (15 units) as described in the protocol; incubation was carried out at 37°C for 1.5 hours. Reactions were stopped by adding 2.5 μl of EDTA/glycogen mixture. Five μl (total 50 μl) of the reaction mixtures was used to assess the efficiency of the synthesis and digestion by running the products on a 2% TAE-agarose gel (section 4.5.2). The digested cDNA was then phenol: chloroform: isoamyl alcohol extracted and ethanol precipitated as described in section 2.4 and the pellet resuspended in 5.5 μl of H_2O.

4.4.5 **Adapter ligation**

Rsa I digested cDNA (1.5 µl) was diluted in 7.5 µl of H_2O. The control skeletal muscle tester cDNA was prepared as described in the supplied protocol. Each tester sample was split in two; one half was ligated to adapter 1 and the other half to adapter 2 (designated tester (+)-1, (+)-2 etc.). 2 µl each of (+)tester cDNA (i.e. fed), (-)tester cDNA (i.e. unfed) and tester control cDNA were used for each ligation reaction (total volume 10 µl). A control ligation containing both adapters (i.e. unsubtracted tester control (+)-c etc.) was also prepared. The ligation reactions were incubated overnight at 16°C and stopped by adding EDTA/glycogen mix. Heating the samples at 72 OC for 5 minutes inactivated the ligase.

4.4.6 **First hybridisation**

The adapter 1 and adapter 2 ligated tester cDNA samples were mixed separately with an excess of the respective digested driver cDNA as described in the supplied protocol. The cDNA mixtures were denatured at 98°C for 1.5 minutes. Hybridisation was carried out at 68°C for 8 hours in supplied hybridisation buffer.

4.4.7 **Second hybridisation**

The primary hybridisation samples (i.e (+)-1 and (+)-2 etc.) were pooled. An excess of the respective driver cDNA, freshly denatured at 98°C for 1.5 minutes was added to each of the pooled tester samples. Hybridisation was carried out overnight at 68°C in accordance with the supplied protocol. The samples were diluted in 200 µl of the supplied dilution buffer and stored at –20°C.

4.4.8 **Primary PCR**

One μl of each of the diluted subtracted and unsubtracted (i.e. 1 μl of (+)-c etc.) cDNA samples was used as template DNA for primary PCR reactions using the PCR1 primer. PCR was carried out (total volume 25 μl), using the Advantage cDNA PCR kit (4.4.11) according to the supplied protocol. The efficiency of the subtraction was measured by comparison of PCR products obtained with the control skeletal muscle cDNA subtraction with that of a supplied control subtraction. 8 μl of each reaction was loaded on a 2% TAE-agarose gel. The remainder was stored at $-20^{O}C$.

Because the quantity of cDNA used as a template in primary PCR was so low, rare transcripts may not be represented in individual PCR reactions. In order to increase the representation of such sequences in the subtracted library, four identical primary PCR reactions were set up for each sample used.

4.4.9 **Secondary PCR**

For each primary PCR sample, 3 μl of the product was diluted in 27 μl of H_2O. The four replica reactions were pooled at this stage. 1 μl of the diluted-pooled PCR mixture was used as template DNA for secondary PCR. PCR was carried out (total volume 25 μl) using the Advantage cDNA PCR kit and the nested primers PN1 and PN2 as described in the supplied protocol. 8 μl of each secondary PCR sample was loaded on a 2% TAE-agarose gel. The remainder was stored at $-20^{O}C$.

4.4.10 **Cloning of cDNA into T/A vector**

Three μl of the forward subtracted cDNA (i.e. fed-unfed) secondary PCR reaction was used for ligation into the pBAD-TOPO cloning vector (Invitrogen) in accordance with the manufacturer's instructions. The resulting plasmids were transformed into supplied TOP10 competent cells as described in the supplied protocol. The resulting cells were grown up overnight at 37^{O}C on LB plates containing 50 μg ml^{-1} ampicillin. Random clones were picked from these plates and used for sequencing as described in section 4.5.7. The plates were stored at 4^{O}C.

4.4.11 **Advantage cDNA PCR**

The amplification procedures for the PCR-Select kit were optimised using the Advantage cDNA PCR kit (Clontech, cat. no. PT1580-1). No products were obtained when using Bioline or Sigma *Taq* polymerase (data not shown). PCR reactions were carried out in accordance with the supplier's instructions using a Hybaid Touchdown thermal cycler.

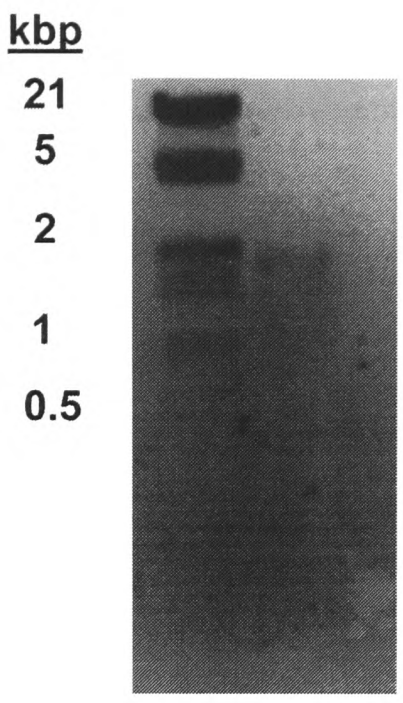


Fig 4.6 Visualisation of partially fed *I. ricinus* salivary gland mRNA. 1% agarose-formaldehyde containing gel. DNA molecular weight marker as shown. 2.5 μg of fed mRNA was loaded.

4.5 Results – Subtractive library construction

4.5.1 mRNA preparation

In order to check the size and presence of the prepared tick mRNA, a 1% agarose formaldehyde containing gel was set up as described (Sambrook *et al.*, 1989). Because only a small quantity of unfed mRNA was available (see section 4.4.1), only fed mRNA (5 µl = 2.5 µg) was loaded onto the gel. The gel was run at 50V.

The results (Fig 4.6) showed that the RNA species were mainly less than 2 kbp in size. These sizes are consistent with previous results obtained with non-mammalian mRNA (Clontech PCR-Select manual).

4.5.2 Rsa I digestion

Uncut (2.5 µl) and of Rsa I digested cDNA (5 µl) of each sample was loaded onto 2% TAE-agarose gel. Although some cDNA could be seen on the gel after UV exposure, no clear banding could be seen (not shown). Therefore the gel was dried and exposed to X-ray film overnight. The autoradiogram (Fig 4.7) shows that cDNA synthesis was successful for the unfed, fed and control mRNA populations. The changing banding patterns and size of the labelled cDNA after digestion suggest that the digestion with Rsa I was successful.

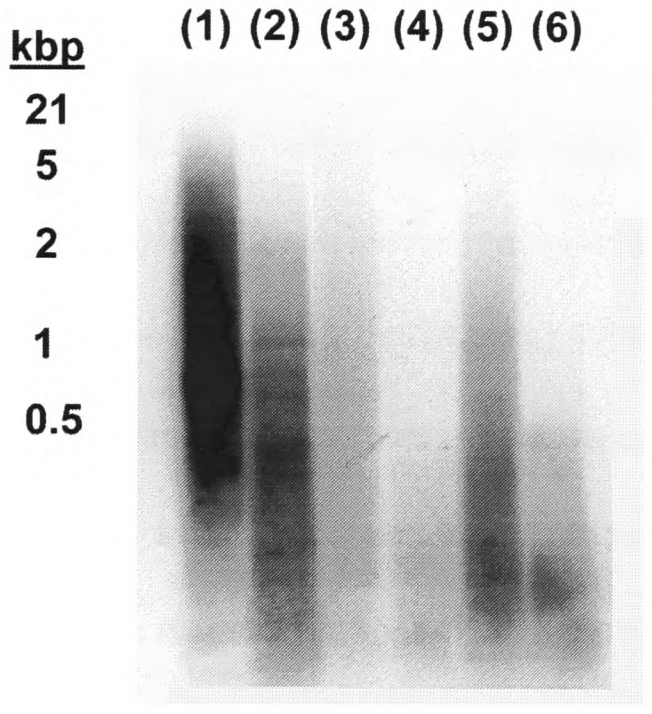


Fig 4.7 Rsa I digestion of cDNA. Autoradiogram of 2% TAE agarose gel containing ^{32}P-labelled cDNA. (1) Control cDNA – uncut, (2) control cDNA – Rsa I digested, (3) unfed cDNA – uncut, (4) unfed cDNA – Rsa I digested, (5) fed cDNA – uncut, (6) fed cDNA – Rsa I digested.

4.5.3 **Adapter ligation**

One µl of each ligated cDNA sample was diluted in 200 µl of H_2O. The diluted cDNA (1 µl) was used as template DNA for PCR as described in the supplied protocol. To assess the efficiency of the ligation, the relative quantity of PCR product obtained from 5' and 3' 'housekeeping gene' specific primers (representing non-ligated cDNA) was compared with the PCR products obtained using the adapter specific 5' primer (PCR1) and the 3' housekeeper primer. The supplied primers, human G3DPH, are suitable for the human derived control cDNA, however no PCR products were obtained with the tick samples. Primers were designed from the sequence of *Rhipicephalus appendiculatus* ubiquitin (not shown). The sequence was obtained from Guido Paesen. No PCR products were observed. A further set of primers were designed based on the cytochrome oxidase polypeptide II sequence obtained from *I. ricinus* λ cDNA library clone # 29 (Fig 4.5). No PCR products were obtained even after attempts to optimise the PCR e.g. using a range of annealing temperatures, $MgCl_2$ concentration etc.

As a consequence the result obtained with the muscle control cDNA was used as an indication of overall ligation efficiency (Fig 4.8). These results indicate that a significant proportion of the cDNA was ligated successfully to the adapters 1 and 2.

4.5.4 **Analysis of primary PCR**

To test the efficiency of the subtraction procedure, the supplied subtracted control skeletal muscle cDNA PCR products were compared with that of the control cDNA prepared during this research.

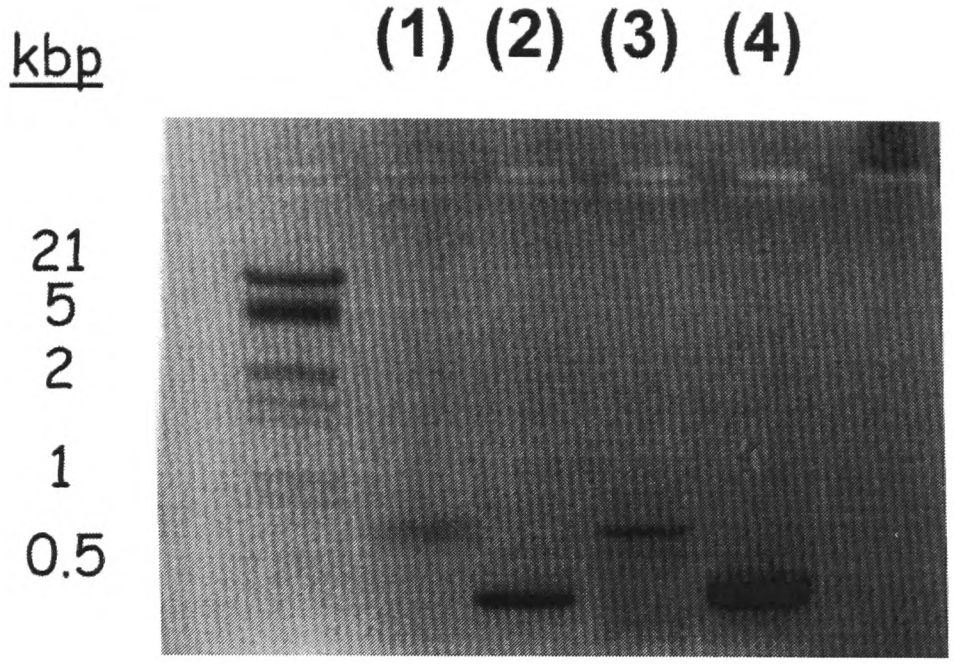


Fig 4.8 Control cDNA adapter ligation. 2% TAE agarose gel containing (1) PCR using G3DPH 3' and PCR1 primers – adapter1 ligated cDNA, (2) PCR using G3DPH 3' and 5' primers – adapter1 ligated cDNA, (3) PCR using G3DPH 3' and PCR1 primers – adapter2 ligated cDNA, (4) PCR using G3DPH 3' and 5' primers – adapter2 ligated cDNA. Visualized using UV.

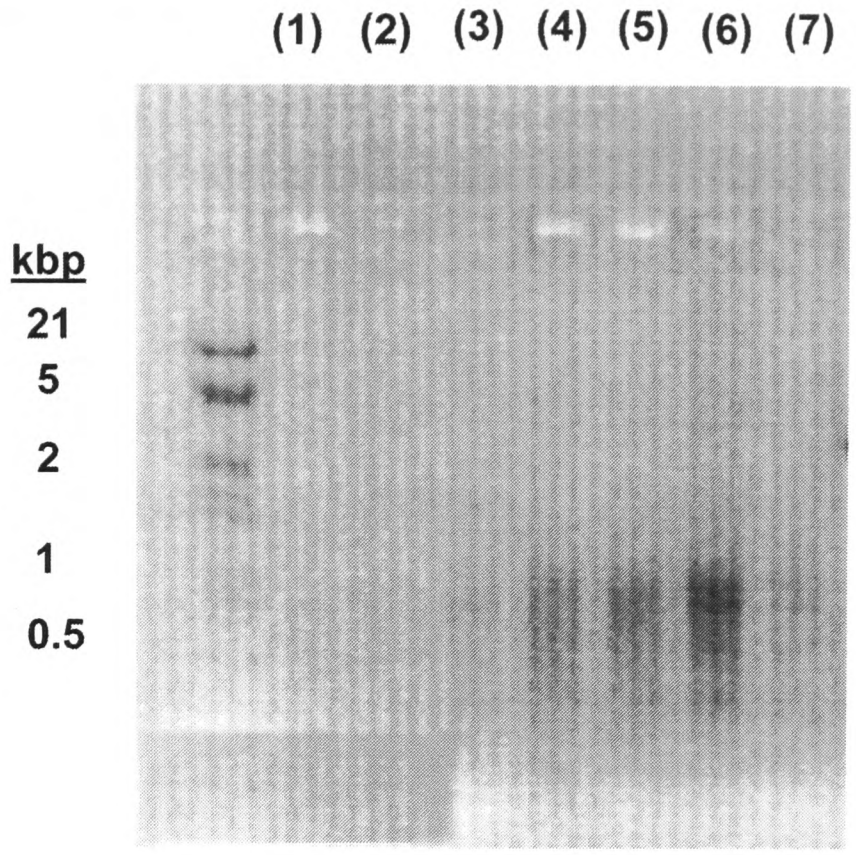


Fig 4.9 Primary PCR products of subtracted and non-subtracted cDNA using PCR1 primers. 2% TAE agarose gel containing (1) subtracted fed cDNA, (2) subtracted unfed cDNA, (3) control subtracted cDNA, (4) non-subtracted fed cDNA, (5) non-subtracted unfed cDNA, (6) non-subtracted control cDNA, (7) supplied subtraction control cDNA. Visualized by UV.

A 2% TAE-agarose gel was loaded with 8 µl of a total volume of 25 µl from each primary PCR reaction (section 4.4.8) (Fig 4.9). The forward (i.e. fed-unfed (+)) and reverse (i.e. unfed-fed (-)) subtracted cDNA were designated fed subtracted and unfed subtracted respectively.

Very little DNA can be seen in either the tick-derived samples or in the control subtracted samples (Fig 4.9). This is not unusual as the quantity of DNA produced by primary PCR is often too little to detect at this stage (Clontech manual). However, cDNA was observed in all of the unsubtracted PCR reactions. This suggests that the PCR reactions were successful.

4.5.5 **Analysis of nested PCR**

PCR was carried out as described in the supplied protocol using diluted primary PCR products as template DNA and the nested primers PN1 and PN2. Each sample reaction was visualised on a 2% TAE-agarose gel (8 µl- total volume 25 µl) .

Discrete bands were seen in most of the cDNA samples (Fig 4.10). Three discrete bands were seen in the supplied subtraction control. At least two of these bands were also present in the experimental control reaction suggesting that subtraction was successful. Both control-subtracted samples show a different banding pattern to that of the unsubtracted control sample. Bands present in the subtracted tick-derived cDNA are significantly different to those seen in the unsubtracted control samples. These results suggest that subtraction of these samples was also successful.

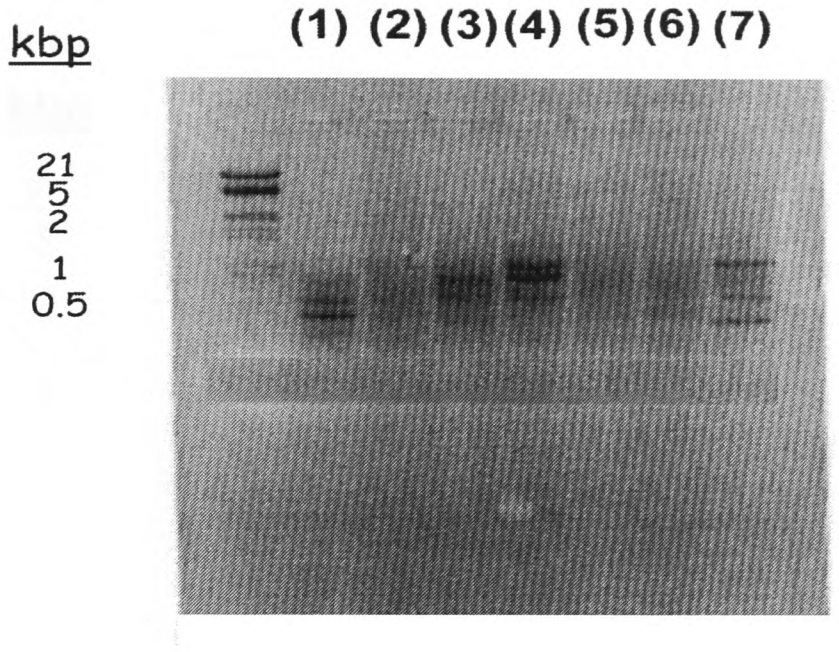


Fig 4.10 Secondary PCR of subtracted and non-subtracted cDNA using nested primers, PN1 and PN2. 2% TAE agarose gel containing (1) subtracted unfed cDNA, (2) subtracted fed cDNA, (3) control subtracted cDNA, (4) supplied subtraction control cDNA, (5) non-subtracted unfed cDNA, (6) non-subtracted fed cDNA, (7) non-subtracted control cDNA. Visualized by UV.

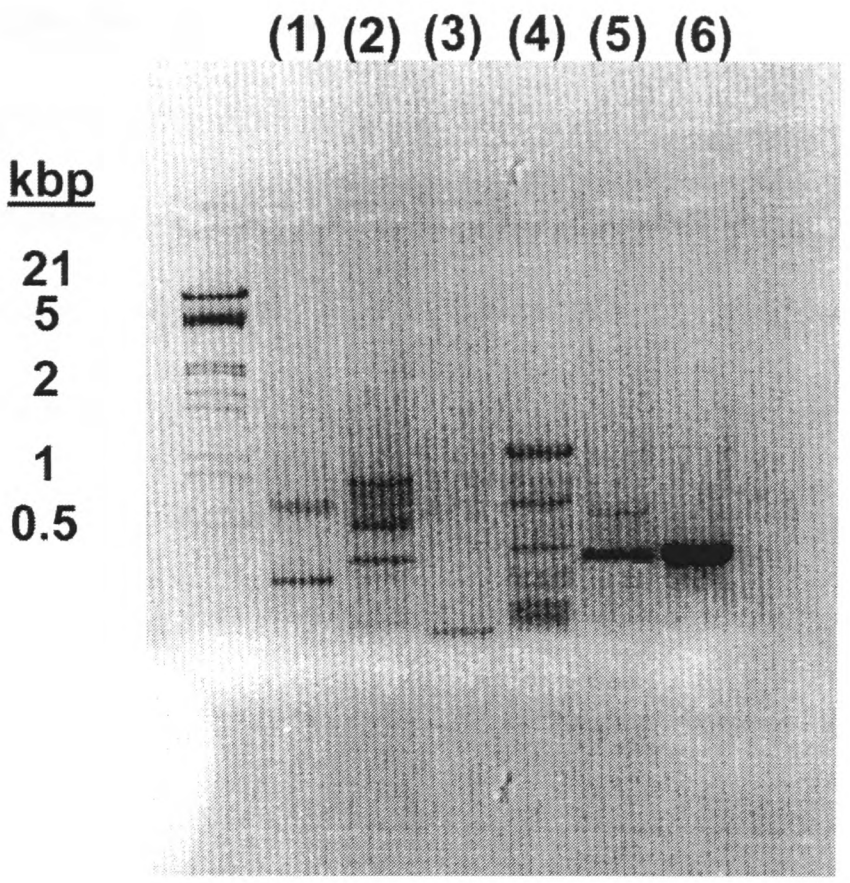


Fig 4.11 Efficiency of subtraction using 'house-keeping' genes as primers for PCR. Cytochrome oxidase primers (see section 4.5.3) were used for tick samples; G3DPH primers were used for the control samples . 2% TAE agarose gel containing (1) subtracted fed cDNA, (2) non-subtracted fed cDNA, (3) subtracted unfed cDNA, (4) non-subtracted unfed cDNA, (5) subtracted control cDNA, (6) non-subtracted control cDNA. Visualised using UV.

4.5.6 **Subtraction efficiency**

In order to access the efficiency of the subtraction procedure, PCR was carried out using housekeeper specific gene derived primers (5' and 3') using both subtracted and unsubtracted cDNA samples as template DNA according to the supplied protocol. The supplied G3DPH primers were used with the control cDNA samples. The tick cDNA samples were amplified using primers based on the *I. ricinus* cytochrome oxidase polypeptide II transcript (section 4.3.2). A 2% TAE agarose gel was prepared and 8 μl of the PCR products (total 25 μl) were loaded and run on the gel.

Fig 4.11 showed that G3DPH specific bands were present in both the unsubtracted and subtracted control cDNA. However, much greater quantities of DNA were observed in the unsubtracted control. This suggests that subtraction was successful. The results of the tick samples using the cytochrome oxidase primers showed that several PCR products were present in the unsubtracted samples, but fewer species were seen in the corresponding subtracted samples. This suggests that subtraction was also successful with these samples.

4.5.7 **T/A cloning of forward subtracted cDNA**

Taq polymerase has a non template-dependent terminal transferase activity, which adds a single, overhanging 3' thymidine (T). T/A vectors exploit this fact and contain cloning sites containing a single overhanging adenine (A). The technique used for this research (i.e. pBAD-TOPO cloning kit (Invitrogen)) exploits the ligation activity of topo-isomerase to allow ligation to occur at room temperature in 5 minutes. This results in improved ligation efficiency (Shuman, 1994). The pBAD-TOPO vector contains an

arabinose-based promoter (pBAD) which allows tight control over expression levels. The promoter region is both negatively and positively regulated by the product of the *ara*C gene (Ogden *et al.*, 1980). Basal expression levels can be repressed by growing in the presence of glucose. Glucose lowers cAMP levels which decreases binding of the CAP protein that subsequently inhibits transcription (Miyada *et al.*, 1984). The vector also contains the V5 epitope to allow for detection of recombinant product and a C-terminal polyhistidine tag to allow for ease of purification.

Three μl of the fed subtracted secondary PCR product was used to ligate into the pBAD/TOPO vector. The plasmids were transformed into the supplied TOP10 *E. coli* competent cells as described in the manufacturer's protocol. Cells were plated out on LB agar plates containing 50 μg ml^{-1} ampicillin. About 100 colonies were obtained. The transformation was repeated several times on the dates indicated in Table 4.1.

4.5.8 **Random sampling of cDNA clones**

Eighty colonies (obtained from multiple transformations) were picked and used to inoculate overnight 5ml cultures (LB broth + 50 μg ml^{-1} ampicillin). Plasmid DNA was prepared from 47 of these cultures using the QIAprep spin mini-prep kit (Quiagen- cat. no. 27106) in accordance with the manufacturer's instructions. DNA concentrations were measured spectrophotometrically at 260/280 nm (data not shown). The inserts were sequenced using chemiluminescense cycle sequencing by Steve Howard or Bob Possee using the IVEM ABI automatic sequencer. Two μg of the plasmid DNA was used as template DNA with the adapter specific PN1 primer for PCR cycle sequencing according to the manufacturer's instructions.

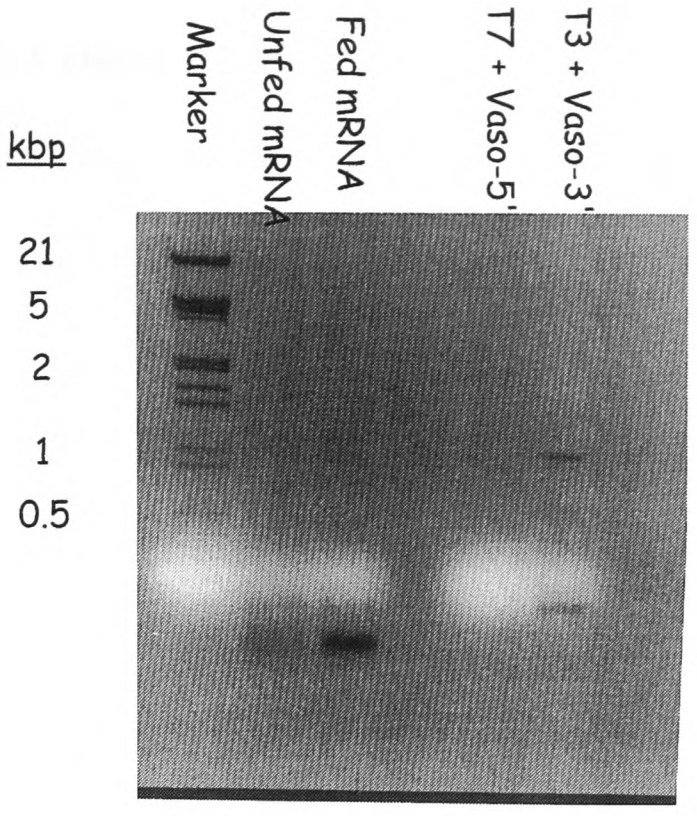


Fig 4.12 RT-PCR and λ-PCR products obtained from mRNA and λ cDNA library respectively. RT-PCR was carried using clone 38 specific primers (i.e. Vaso 5' and Vaso 3'), λ-PCR using clone specific and vector specific primers as indicated. 2% TAE agarose gel. Visualised by UV.

4.5.9 **Sequencing of cDNA clones**

Steve Howard or Bob Possee supplied the sequence data. Vector-derived sequences were removed using the SEQED program and the truncated sequences used for homology searching (Table 4.1). Searches were carried out using the NBLAST algorithm against both the GENEMBL (DNA) and the SWISSPROT (amino acid) databases.

17 of the 47 sequences were mitochondrially associated; 13 of these corresponding to known *Ixodes ricinus* 16s rRNA sequences and 1 clone each of cytochrome oxidase polypeptide chain I, II and III (clones 23, 22 and 3 respectively); NADH-ubiquinone oxireductase (clone 9). Clone 1 and 31 are homologous to the *Drosophila* and *Ixodes hexagonus* mitochondrial genomic sequences respectively. Clone 5 shows a high degree of homology ($P(N)= 2 \times 10^{-9}$) to the sik1 protein of *Saccharomyces cerivisiae*. Sik 1 is a 504 amino acid protein that possibly acts as a nuclear microtubule binding protein (Morin *et al.*, 1995). The tick derived sequence shares 57% identity at the amino acid level, suggesting that this clone may be a nuclear protein. Clone 14 shares 59% identity to the nucleotide sequence of Chimpanzee *Alu* type DNA ($P(N)= 8 \times 10^{-7}$)(Fig 4.13). It is likely that the clone represents *Alu*-like sequences in the genome of the tick presumably present as an impurity in the mRNA preparation.

Clone 6 shares 86% identity with the nucleotide sequence of *Gallus gallus* mRNA for ABC transporter protein ($P(N)= 8.5 \times 10^{-5}$) over a 174 bases. The ABC (ATP-binding cassette) transporter is a cell membrane associated protein involved in the control of steroid export (Edelmann *et al.*, 1999). The tick sequence shares homology against this sequence from the initiator codon onwards (i.e. ATG- Fig 4.13), suggesting that the clone could be a secretary product. This suggests that the tick sequence (in part) may contain a

putative signal sequence involved in the secretary pathway of salivary gland cells. Further work is necessary to test this hypothesis.

Fifteen of the sequences show no significant homology to any sequences in the database searched. Whether these sequences represent differentially expressed genes or not is unknown. Further work is needed in order to identify putative secretary products etc.

One of the earlier derived clones (number 38 on Table 4.1) shares homology with the pre-peptide sequence of vasotocin from *Buffus japonicus* (Japanese toad). Vasotocin and its mammalian analogue arginine vasopressin, are nonapeptide hormones with many physiological functions including the stimulation of vasodilation (Moore and Lowry, 1998). Despite the low level of homology (P(N)= 0.29)), this clone was chosen for further investigation as vasodilatory compounds and their possible functions, have been reported in the saliva of *A. americanum* (Bowman *et al.*, 1996). To look for differential expression of this clone, the semi-quantitative reverse transcriptase PCR (RT-PCR) method was used. Primers were designed based upon this sequence; the primers separated by about 170 nucleotides (Fig 4.13- designated Vaso 5' and 3'). These primers were used along with 0.2 μg of mRNA from unfed and fed ticks (section 4.4.1) to prepare RT-PCR reactions. RT-PCR was carried out using the Titan One-tube kit (Boehringer Mannheim- cat. no. 1888382) in accordance with manufacturer's instructions. Ten μl of the PCR reaction (total 25 μl) was run on a 2% TAE agarose gel (Fig 4.12). The results of Fig 4.12 showed a RT-PCR product of about 170 bp to be present in significantly higher quantities in the fed compared with unfed mRNA samples. These results suggest that this gene may

be differentially expressed in the salivary glands of *I. ricinus* female adults during the feeding process.

In order to see whether this clone was represented as full-length cDNA in the λ-phage library (because the subtracted library cDNA was restriction digested), combinations of vector specific (i.e. T7 or T3) and clone specific (i.e. Vaso 5' and Vaso 3' respectively) primers were used for PCR. The λ-phage cDNA library (section 4.2.2) was used as template DNA (2 μl). PCR was carried out as described in section 2.4.3. The same agarose gel (i.e Fig 4.12) was used to visualise 10 μl of the PCR products (total 100 μl). Two products of about 1000 and 400 bp were seen in the PCR sample containing the primers T3 and Vaso 3'. These results suggest that two clones are present in the λ library corresponding to the Vaso sequence obtained from the subtractive library. The remaining PCR products were purified using QIAquick PCR purification kit (Qiagen cat no. 28142). The DNA was DIG labelled using the DIG High Prime kit (Boehringer Mannheim cat no. 1585606) according to manufacturer's instructions. The DNA was used as a probe to screen the λ library (1×10^5 pfu were used) in accordance with the supplied protocol (Stratagene- ZAP Express manual). Despite using various hybridisation temperatures, positive clones obtained in the first round of screening could not be detected in further rounds of screening (data not shown). Future work will include the direct cloning of the PCR products into a T/A vector.

Clone 37 showed a high degree of homology with the replicase polyprotein sequence of *Drosophila* C virus ($P(N) = 1.8 \times 10^{-16}$), swissprot accession no. TRVRL:O36966. The translated sequence of clone 37 has been designated virus_tick. The homologous region (49% positive homology over a 180 amino acid stretch as

measured using NBLAST program) relates to the RNA-dependent RNA polymerase (RdRp) region of the polyprotein amino acid sequence (Johnson and Christian, 1998). Homology searching against sequences obtained from Guido Paesen (IVEM), revealed homology to a sequence from clone gp-205 (virus_guido), derived from the random sampling of a *Boophilus microplus* salivary gland cDNA library. Amino acid alignment of the RdRp regions (truncated at the C-terminus for illustrative reasons) of the picornaviruses, *Drosophila* C virus (virus_dcv; TRVRL:O36966), cow-pea mosaic virus (virus_cpmv; SW:VGNB_CPMV), red clover mottle virus (virus_rcmv; SW:VGNB_RCMV), tomato black ring virus (virus_tbrv; SW:POL1_TBRVS) and rice tungro spherical virus (virus_rtsv; TRVRL:Q98645), with the tick sequences are shown in Fig 4.14. The alignment was created using GCG pileup command (gap creation penalty = 3.0, gap extension penalty = 0.1) and plotted using PRETTYPLOT. Regions of homology, which include the virus_tick sequence, are shaded. The motifs designated by Koonin (1993) are labelled I-VI. Homology is observed between the tick sequences and all of the viral conserved motifs with the exception of motif I (5 amino acid). The virus_tick sequence is homologous with 7 of the 28 amino acids in motif II, of which 2 amino acids are conserved in both tick sequences. 8 of the 12 amino acid of motif III are conserved in virus_tick of which only 1 is shared by both tick sequences. 5 of the 11 amino acid sequences of motif IV are conserved in virus_tick of which 3 are shared by both tick sequences. 16 of the 27 amino acid sequences of motif V are conserved in virus_tick, 6 of these are conserved in both tick sequences.

These results perhaps suggest the presence of viruses associated with the salivary glands of these ticks. Future work will attempt to sequence upstream and downstream

regions of the sequence in order to clarify whether the sequence represents viral cDNA or a tick cDNA homologue.

It is apparent that, although the research described so far, has provided several putative clones of interest, full exploitation of this resource still remains a Herculean task. Future work will include further random sampling of the library and differential screening of the library in order to reduce the number of false positives obtained. RT-PCR is only a semi-quantitative technique and northern blotting should be carried out in order to clarify differential expression of cDNA. Due to the difficulty in obtaining sufficient mRNA from the salivary glands of *I. ricinus* ticks for this technique, a 'virtual' northern blot would be a more realistic method (i.e. mRNA populations are PCR amplified as cDNA). Expression of the library will also be carried out and the subsequent protein products investigated.

Clone #	Length (bases)	Blast (DNA/ AA)	Database	Homology	P (N)	Database Accession No.	Date	Alt. Name
1	667	DNA	genembl	*Drosophila* mitochondrial genome	1.2×10^{-21}	EMORG:MIDMMU375	4/10	clpbadfu1
2	675	DNA	genembl	None	-	-	4/10	clpbadfu2
3	675	DNA	genembl	*Ixodes pacificus* cytochrome oxidase III	1.2×10^{-43}	GBINV_N:AF082986	4/10	clpbadfu3
4	672	DNA	genembl	*Ixodes ricinus* 16s rRNA	2.6×10^{-44}	EMORG:MIIRRGDG1	4/10	clpbadfu4
5	670	AA	swissprot	*Saccharomyces cerevisiae* sik1 protein	2.0×10^{-9}	SW:SIK1_YEAST Q12460	4/10	clpbadfu5
6	618	DNA	genembl	*Gallus gallus* mRNA for ABC transporter protein	8.5×10^{-5}	EMVRT:GGA9799	4/10	clpbadfu6
7	611	DNA	genembl	None	-	-	4/10	clpbadfu7
8	616	DNA	genembl	None	-	-	4/10	clpbadfu8
9	605	AA	swissprot	*Albinaria coerulea* NADH-ubiquinone oxidoreductase	5.1×10^{-13}	SW:NU4M_ALBCO P48914	4/10	clpbadfu9
10	613	DNA	genembl	*Ixodes ricinus* rRNA	2.5×10^{-31}	EMORG:MIIRRGDG1	4/10	clpbadfu10
11	607	DNA	genembl	None	-	-	4/10	clpbadfu11
12	599	DNA	genembl	None	-	-	4/10	clpbadfu12
13	614	DNA	genembl	None	-	-	4/10	clpbadfu13
14	628	DNA	genembl	Chimpanzee Alu type DNA	8.0×10^{-7}	EMMAM:MORSA	4/10	clpbadfu14

15	608	DNA	genembl	*Ixodes ricinus* 16s rRNA	2.5×10^{-26}	EMORG:MIIRRGDG1	4/10	clpbadfu15
16	602	DNA	genembl	*Homo sapiens* spinal atrophy like gene	0.15	EMHUM1:HSW55c20	4/10	clpbadfu16
17	594	DNA	genembl	None	-	-	4/10	clpbadfu17
18	604	DNA	genembl	None	-	-	4/10	clpbadfu18
19	604	AA	swissprot	extensin	9.2×10^{-3}	RPLN:Q39600	4/10	clpbadfu19
20	605	DNA	genembl	*Ixodes ricinus* 16s rRNA	1.2×10^{-60}	EMORG:MIIRRGDG1	4/10	clpbadfu20
21	603	DNA	genembl	*Ixodes ricinus* 16s rRNA	1.5×10^{-50}	EMORG:MIIRRGDG1	4/10	clpbadfu21
22	605	DNA	genembl	*Choristoneura rosaaceana* cytochrome oxidase II	2.3×10^{-41}	EMORG:MICROIIU	4/10	clpbadfu22
23	613	DNA	genembl	*Phaenicia sericat* cytochrome oxidase I	2.9×10^{-39}	EMORG:MIPSPIEA	4/10	clpbadfu23
24	605	DNA	genembl	*Ixodes ricinus* 16s rRNA	1.9×10^{-30}	EMORG:MIIRRGDG1	4/10	clpbadfu24
25	604	DNA	genembl	None	-	-	4/10	clpbadfu25
26	622	DNA	genembl	*Ixodes ricinus* 16s rRNA	1.2×10^{-17}	EMORG:MIIRRGDG1	11/11	clpbadfu26
27	632	DNA	genembl	None	-	-	11/11	clpbadfu27
28	603	AA	swissprot	extensin	0.33	TRPLN:Q01943	11/11	clpbadfu28
29	606	DNA	genembl	None	-	-	11/11	clpbadfu29
30	665	DNA	genembl	None	-	-	11/11	clpbadfu30
31	661	DNA	genembl	*Ixodes hexagonus* mitochondrial DNA genome	7.7×10^{-15}	EMORG:AF081828	11/11	clpbadfu31

32	624	DNA	genembl	*Ixodes ricinus* 16s rRNA	2.1×10^{-15}	EMORG:MIIRRGDG1	11/11	clpbadfu32
33	628	DNA	genembl	*Ixodes ricinus* 16s rRNA	6.9×10^{-24}	EMORG:MIIRRGDG1	11/11	clpbadfu33
34	656	DNA	genembl	*Ixodes ricinus* 16s rRNA	7.5×10^{-15}	EMORG:MIIRRGDG1	11/11	clpbadfu36
35	908	DNA	genembl	None	-	-	15/5	CLUF1
36	714	DNA	genembl	None	-	-	15/5	CLUF2
37	742	AA	swissprot	*Drosophila* C virus	1.8×10^{-16}	TRVRL:O36966	15/5	CLUF3
38	367	AA	swissprot	vasotocin	0.29	-	15/5	CLFU5
39	588	DNA	genembl	None	-	-	23/5	cl1
40	612	DNA	genembl	*Ixodes ricinus* 16s rRNA	2.3×10^{-80}	EMORG:MIIRRGDG1	23/5	cl2
41	690	DNA	genembl	*Ixodes ricinus* 16s rRNA	9.0×10^{-68}	EMORG:MIIRRGDG1	23/5	cl3
42	695	DNA	genembl	*Ixodes ricinus* 16s rRNA	7.0×10^{-44}	EMORG:MIIRRGDG1	23/5	cl4
43	615	AA	swissprot	*Caenorhabditis elegans*. collagen alpha 2(iv) chain precursor.	0.87	SW:CA24_CAEEL	23/5	cl5
44	615	AA	swissprot	*Homo sapien*s beta-defensin 1 precursor	0.96	SW:BD01_HUMAN	23/5	cl6
45	605	AA	swissprot	*Homo sapiens* basic proline-rich peptide	5.1×10^{-5}	SW:PRPE_HUMAN	23/5	cl7
46	618	AA	swissprot	human adenovirus type	0.85	SW:HEX3_ADE12	23/5	cl8

47	605	AA	swissprot	12.peripentonal hexon-associated protein *Homo sapiens* neurofilament triplet h protein	1.7×10^{-3}	SW:NFH_HUMAN	23/5	cl9

Table 4.1 Results of homology searches with sequences of SSH-PCR obtained clones using the BLAST algorithm of the GCG suite of programs. Both swissprot and GENEMBL databases were used for searches. Homologous sequences shown and the relevant database and accession code correspond to the smallest sum of probability scores (P(N)). The dates given refer to the date of transformation.

(1)GGCNTCTTTC TNAGCCTNTA GCGGATCCTA CCTGACGCTT TTTATCGCAA

CTCTCTACTG TTTCTCCATA CCCGTTTTTT TGGGCTAGAA ATAATTTTGT

TTAACTTTAA GAAGGAGATA TACATACCCA TGGGCTCTGG ATCCGGTGAT

GACGATGACA AGCTCGCCCT TCTAATACGA CTCACTATAG GGCTCGAGCG

GCCGCCCGGG CAGGTACCGA CAGACACTG(A TG)ACGACACT CCTGCGCCCA

CTAAGAAGCC GAAGCNAAAA AAGAAGAAAC AGAAGAAGAA TAAGAAACCT

AAGGGCAACT ANAAGAAGAA TTAGGAGGGA TATTTCTAAT GAAAAGCCCT

GAAACTAAAC ATGGCTAAAT AAAGTTGAGA CACTGACTGT TCGTAAAACA

NACNANCTTT NNANANTCCN NTACCTTNTC NCTTCTCACC CTNTCANTCT

NTTTNTNNNT CCTTCCTCNT NTNTNNCCNT NTNTACNTCN NTTTCCNTTT

CTTTCNTNNT NNCTCTNTCT TNTCNNNTTC ATANTCNANN NNTAAANAAN

TCCNNGGGGG GANCCCCCCC NCTTTTTTTT TTTTTTAANN GGGNGNTTGA

NNGGTCTCNC NCCCCCCC(618)

Clone 6

(1)GCTCGCCCTT NTTCGCGGCC TNTAGCGGGA TCCGNACCTG ACGCTTTTTA

TCGCAACTCT CTACTGTTTC TCCATACCCG TTTTTTTGGG CTAGAAATAA

TTTTGTTTAA CTTTAANAAG GAGATATACA TACCCATGGG CTCTGGATCC

GGTGATGACN ATNACAAGCT CGCCCTTCTA ATACGACTCA CNATAGGGCA

GCGTGGTCGT GGCCGAGGTC TTGTGGGTAT ATAAGACATT GAGGAAATAA

ACNCAGTANC TGAGTTTGAG AATNNCNNNN TATATCATCN CNAAANTTNN

AACCACNNTN NACNTAACAA NCTAATNAAT TNNATCCCTT ANTCNNTTAC

TNTNTCTCTT CCCTTTCCCT CTCNCCCTNC NCTTTTCCCN TNNTCACCTN

CNCCCTCACA TCTTCTTTTC TTTCTCNNCC NCCTCNTTTC TTCCTCTNAT

CNGTTNNTTT CCTCTTTCTT TTTTNCCNGC GCGGGGGGGG CNNTCTCTCN

CCTTCTTTTT TNTTTTCTAA NGGGGCAAAC ANCGGTTTCT TCACCTCCAT

CCCTCNCCNN GNTCATANNC CTTTANGGNT NTCCCCCCCC TTCACTNTAT

NATNCTCNTC CNCTCCNTCN GTCTTTNT(628)

Clone 14

(1)TTNNCCCNNC TTTTAAANNC ATNTNCCAAC AANATCNCAT ATATAACTNA

TTAACTANNT CATTAATCTC AACANTTAAT NCAAANCCAN AANNAATTCC

ACAAAACATT TTATACATNA CATTANTTNCT AAATNANTNA CATTAAACA

ATTCCTCCAT TAAAACTTTT CTATANTCAT TTNCTACACT NTCAAAATAA

TNTTCATACC ATATAAAAAT ANCANAAAAA NCACCTTCCA TCACATCACN

TTNCAAANTA TCACCAAAAN CCTTATANTC ACCAACNACA AAATTATTAC

CTTTANCCAA TAATNAACNA NCAANATNAN TCCATTCTAA ACTATTAACA

TTAATACCTA TTNCCNTTCC ATTATCTATT CTANCATTCT NATATNCANC

TTNAAANACA CCAAAATACT TNTNAATCNC CCAANTAAAT TCTACTNNNC

TANTANANAA CATTCNANTC TTTCCCATTT TTAATTTCTC CNNTAATACC

NCNAATCTTA ANANTTCCAC AAAAATANTC NCTNNAATTC NCCNTTTAAT

CNTAATTTAT NATNNTNATT NTAANANTTT NNNANTTCAN NANNAATCTC

TCTNAAACCA CTCCTTCTCN NNNNTNCTCN NCNNNACACC TANNNNNACT

NAANTANCCT ACCTAACCCT CCNNCCNATC ACCNTCNNNC ATTCCNNCCA

TNATTTNT(708)

Clone 37

(1)TTTCACACAT GCTTTTAANN ANCCNNNNAA CANAAAAANA NCTTTCNATN

NNATCATTTT CCCTNNCTCA ANAAANCATC CTNNNTTNCA NTNTTAATAT

ATCTNTCTTN AAANCACCNT TNNTCNTAAC TTTTCTNTNT ATCNTANCAN

TTNNTANTNC CTCTTTTCAG ATTTACTTCA CTCNTNNTTN NAAATAAANN

CNTCTATTNT NNTTNANANN AAAAAAAAAA ATAAANAAAN AAANAAAAAA

CCCNCNNCCN CAACCACNCT AANNNCAANC TTNAANNTNA NCCTATCCCT

AACCCTCTCC NCNNTCTCAA TTCTACNCTT ACCNNTCNTC ATCACCATCA

CCNTTNATTT TAAACNN(367)

Clone 38 (Vaso 5' and 3' primers sequences are underlined)

Fig 4.13 Sequence information of clones of interest obtained by random sampling of subtractive library clones. Primer sequence positions underlined as indicated. N's represent nucleotide of indeterminate assignment.

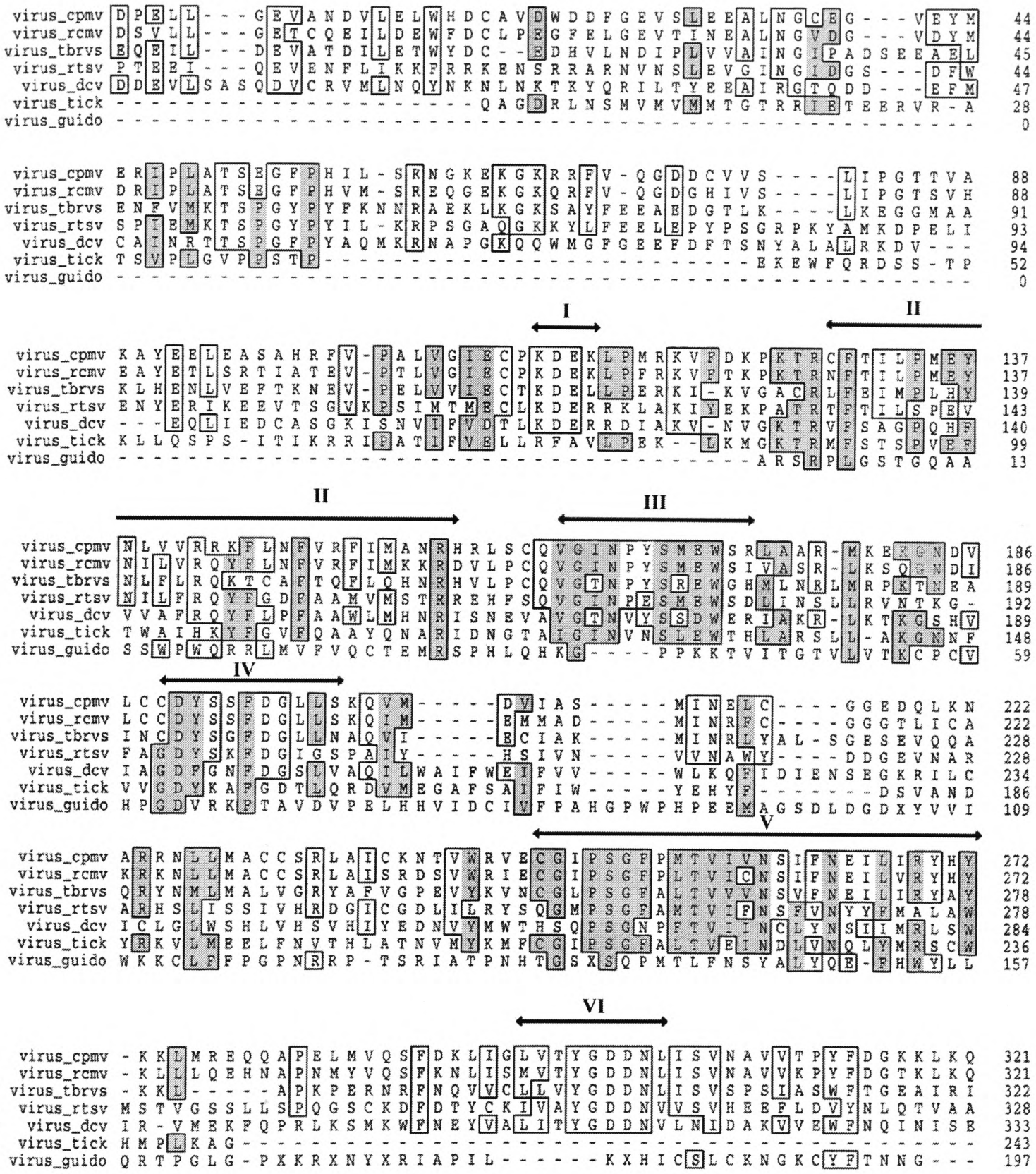


Fig 4.14 **Alignment of *I. ricinus* clone 37 (virus_tick) and *B. microplus* clone gp205 (virus_guido) with RNA-dependent RNA-polymerase regions of polypeptide sequences from *Drosophila* C virus (virus_dcv), cow-pea mosiac virus (virus_cpmv), red clover mottle virus (virus_rcmv), tomato black ring virus (virus_tbrv) and rice tungro spherical virus (virus_rtsv). The alignment was created using GCG pileup command (gap creation penalty = 3.0, gap extension penalty = 0.1) and plotted using PRETTYPLOT. Regions of homology which include the virus_tick sequence are shaded. The motifs designated by Koonin (1993) are labelled I-VI.**

4.4 Discussion

The results of this chapter demonstrate the successful production of a λ-phage cDNA library from the mRNA of partially fed (day 4 + 6) female adult *I. ricinus* salivary glands. The random sampling of clones from this library showed that some sequence inserts (e.g. clone #29- Fig 4.5) are *I. ricinus* derived on the basis of homology searches against published database sequences. Screening of the library using both antibody (e.g. resistant serum) and DNA probes (e.g. probes prepared by Guido Paesen) identified colonies which appeared to be positive. However, secondary and tertiary screening of the clones failed to confirm these results (data not shown). This was probably due to the presence of false positive clones and my failure to optimise conditions for screening using the DIG system of detection (Boehringer-Manheim).

Several researchers have reported the use of recombinant technology in order to produce gene products derived from tick salivary glands. The random sampling of clones from a λ-phage cDNA library constructed from feeding *Rhipicephalus appendiculatus* salivary glands identified a tick homologue of the human DNA helicase II sequence (Paesen *et al.*, 1996). λ-phage cDNA libraries were constructed from the salivary glands of unfed and feeding *Amblyomma americanum* (Jaworski *et al.*, 1989; Needham *et al.*, 1989). These libraries were screened with resistant rabbit serum raised against injected crude SGE. No data have been published as to the success of this work in isolating putative clones. mRNA obtained from the salivary glands of feeding *Amblyomma americanum* was *in vitro* translated using a rabbit reticulocyte translation system and incorporating [^{35}S]methionine (Oaks *et al.*, 1991). To date, there are no published data relating to the study of differential expression in ticks.

Using the SSH-PCR technique, a subtractive cDNA library was produced successfully from the salivary glands of female *I. ricinus* ticks, as judged from results obtained with the supplied skeletal muscle control. Both forward (i.e. fed – unfed) and backwards (i.e. unfed – fed) tick-derived libraries were created. Results as judged by *I. ricinus* specific house-keeping gene PCR (i.e. cytochrome oxidase) suggest that the forward cDNA population has been deletion enriched transcripts expressed differentially in association with feeding. The subtracted cDNA was cloned into the T/A vector pBAD-TOPO. Random sampling and sequencing of clones from this library (Table 4.1) showed that several of the sequences are clearly *I. ricinus* derived on the basis of homology searches against published database sequences (e.g. *I. ricinus* 16s rRNA). The presence of clones that are clearly not differentially expressed in the subtracted library is not a surprise. This subtractive technique, like all others to date is not absolute, but enriches the cDNA population for differentially expressed sequences. The success of the subtraction varies from 5% to 50% depending upon the starting material used and levels of differential expression between the two tissues (Clontech manual). There was however, no quantitative measurement of the success of this subtraction. In addition, the kit has been optimised for use with mammalian material and thus far no data existed on the efficiency of the system using invertebrate mRNA. The presence of apparently genomic *Alu* sequences (clone 14- Table 4.1, Fig 4.13) suggests nucleic acid impurities were present in the initial mRNA purification and presumably a proportion of the clones corresponding to 16s rRNA are the result of such impurities.

Repetitive DNA accounts for at least 20% of the human genome and has been classified into four major families. *Alu* sequences are approximately 280 bp long and

account for 6-13% of human genomic DNA. *Alu* sequences are postulated to be retrotransposons that have inserted into the genome via a ssRNA intermediate (Mighell *et al.*, 1997). Repetitive DNA has been widely reported in the genome of invertebrates, primarily in *Drosophila* and *Bombyx mori* (silkmoth), the latter containing mainly *Alu*-like sequences (Sternberg *et al.*, 1992). Some retrotransposons of *Drosophila* are proposed to originate from the *gypsy* retrovirus (the only invertebrate retrovirus identified so far) (Pelisson *et al.*, 1997). Whether the sequence of clone 14 originated from a past tick retroviral infection is unknown.

Targeted proteins such as secretary products and membrane bound proteins (e.g. ABC transporter), often contain translated N-terminal signal peptides that act to direct protein translocation from the cytosol to intracellular organelles, cell membrane, or for export (Martoglio and Dobberstein, 1998). Clone 6 contained a high degree of homology to the N-terminal sequence of the targeted protein, *Gallus gallus* ABC transporter protein. Although no obvious signal sequence (i.e. a run of hydrophobic residues) is found in this protein or the tick sequence (Fig 4.13), the secretion of proteins lacking hydrophobic residues is well established (Kuchler and Thorner, 1992). Such sequence information could provide a useful tool in isolating saliva-associated proteins in *I. ricinus* ticks. Further work is needed to test this hypothesis.

Clone 38 shares homology to the secreted vasodilatory nonapeptide hormone vasotocin. Despite low levels of homology, this sequence was investigated for differential expression using RT-PCR and sequence specific primers. The results of Fig 4.12 suggest that feeding tick salivary gland mRNA contains a significantly greater number of transcripts than mRNA prepared from the salivary glands of unfed ticks. This suggests

that this gene is differentially expressed in response to a feeding process and subsequently may represent a saliva-associated protein of female adult *I. ricinus* ticks. PCR of the λ-phage cDNA library using sequence and vector specific primers suggests that products of 1000 and 400 bp are present in the library. Screening of the library using the DIG-labelled PCR products failed to isolate any positive clones (data not shown). Future work will clarify the differential expression and sequence of this protein.

Clone 37 shows a high degree of homology with the RdRp region of the *Drosophila* C virus ($P(N)=1.8 \times 10^{-16}$) as well as to many other positive-strand RNA viruses (not shown). *Drosophila* C virus is an ungrouped member of the *Picornaviridae* virus family (Murphy *et al.*, 1995). The RNA-dependent RNA polymerase (RdRp) region of this family has been shown to contain eight conserved protein motifs (Koonin and Dolja, 1993). Overall, 71 of the 243 amino acid stretch in question (corresponding to the virus_tick sequence) shown in Fig 4.14 are present in these conserved motifs amongst the picorna-virus family. Of these, 37 amino acids (i.e. 52%) are conserved in the virus_tick sequence, compared with 20% homology outside this region (i.e. 35 of 172 amino acids).

Comparative analysis of positive-strand RNA viral amino acid sequences showed that RdRp was by far the best-conserved region amongst this group (Koonin and Dolja, 1993). Only three of the eight designated motifs, IV, V and VI were unequivocally conserved throughout the class. The importance of these 'core' RdRp motifs for polymerase activity has been shown by site-directed mutagenesis of the EMCV polymerase (Sankar and Porter, 1992). As well as showing a higher level of homology with the assigned motifs, the *I. ricinus* sequence is more highly conserved in these 'core' motifs (52.4% in motifs IV and V compared to 30.3% homology against motifs I, II and

III). Unfortunately, the virus_tick sequence was too short to compare homology with motif VI.

This evidence suggests that the tick cDNA may code for an active RNA-dependent RNA polymerase. RdRp's have only been described as occurring in positive-strand RNA viruses. In addition, the fact that the tick sequence contains highest homology with an insect picornavirus, *Drosophila* C virus, suggests that the salivary glands of female *I. ricinus* ticks may contain a related virus. The presence of a homologous sequence in *Boophilus microplus* salivary gland cDNA lends weight to this hypothesis. Alternatively, the sequences could represent tick expressed genomic DNA of an unknown function; no analogous activity with RdRp has been described in eukaryotes. No recombination has been demonstrated between positive-strand RNA viruses and host cells.

Presuming that the sequence is part of a previously undescribed viral RNA genome, the fact that both tick colonies were maintained at IVEM, suggests that such a virus is benign to the ticks and to the hosts used for feeding. A lot of work is necessary to confirm this hypothesis. Although viruses have been previously described in ticks, for example tick-borne encephalitis (*Flaviviridae*) and Thogoto virus (*Orthomyxoviridae*), no picornaviruses have been described that infect members of the order Acari. Due to the benign nature of the virus and its presence in the salivary glands, such a virus could prove a valuable vector for the future genetic engineering of ticks.

The results of this chapter showed the production of a successful subtractive library from mRNA enriched for feeding-associated transcripts from the salivary glands of feeding female adult *I. ricinus* ticks. Several of the clones obtained by random

sampling and sequencing represent putative differentially expressed and secreted proteins. Considering that the probability of obtaining such clones by chance is almost negligible and based on the evidence of RT-PCR and subtraction efficiency (section 4.5.6), this suggests that the enrichment procedure was successful. Until differential screening is carried out, a quantitative estimate of the enrichment is not possible. This work provides a valuable resource for the future characterisation of saliva-associated activities as yet undescribed. These results of this chapter provide the first steps towards the production of 'in vitro' *I. ricinus* saliva.

CHAPTER 5

HAEMOLYTIC ACTIVITY IN THE SALIVARY GLANDS OF FEEDING *I. ricinus* TICKS

5.1 Introduction

Ticks are obligate haematophagous arthropods. Protein represents the most abundant constituent of blood, however the majority of this protein is locked away in erythrocytes (36.8 g 100 ml^{-1} compared with 7.41 g 100 ml^{-1} in the plasma of humans). These proportions are largely uniform throughout host species (Altman and Dittmer, 1971; Lehane, 1996). Consequently, one of the most important events in digestion for haematophagous arthropods is haemolysis. Despite the central importance of haemolysis very little is known of the mechanisms involved (Lehane, 1996).

In some fleas and mosquitoes, haemolysis is partially achieved through physical means for example, erythrocytes are punctured by cuticular spines and armatures present in the foregut (Lehane, 1996). A haemolysin of unknown chemical nature has been detected in the salivary glands of the bedbug *Climex* (Sangiorgi and Frosini, 1940). The haemolysin of *Rhodnius prolixus* has been shown to consist of a basic peptide (de Azambuga *et al.*, 1983). In the mosquito *Aedes aegypti* proteases and possibly phospholipases are reportedly involved in haemolysis (Geering, 1975). Free fatty acids

are thought to disrupt the erythrocyte membrane through detergent action in *Stomoxys calcitrans* (Spates *et al.*, 1982).

Blood consists primarily of water (80% in humans), and consequently like many other haematophagous arthropods, ticks concentrate the bloodmeal through osmoregulatory mechanisms, hence increasing the concentration of protein available for digestion (Sauer *et al.*, 1996).

Digestion of erythrocytes in ticks was thought to be an exclusively intracellular event, haemolysis occurring before nutrient absorption (Coons *et al.*, 1986). Tick digestion of host-erythrocytes has been observed to occur intracellularly in the midgut of *I. scapularis* (Ribeiro, 1988). This is in contrast to haematophagous insects, where haemolysis is primarily extracellular (Akov, 1982). The apparent lack of intact erythrocytes in the tick gut (Walker and Fletcher , 1987) and the demonstration of haemolytic activity in the saliva of *Amblyomma americanum* have led to the suggestion that initial haemolysis probably occurs extracellularly, perhaps acting to facilitate the digestive process (Zhu *et al.*, 1997). A secreted haemolysin has been described in *Argas persicus* (Tatchell, 1964).

A successful bloodmeal is a pre-requisite for any tick to enter the next developmental stage (Aeschlimann, 1993). Consequently, tick haemolysins (in the midgut and/or the salivary glands) are essential for survival of the tick. The use of immunogens as effective anti-tick vaccines has been reported (Tellam *et al.*, 1992). The formation of antibodies directed against tick haemolysins could provide a valuable tool in the development of anti-tick vaccines.

This chapter reports haemolytic activity associated with the salivary glands of female adult *I. ricinus* ticks. The aim was to investigate this activity with a view towards functional characterisation and future isolation of the factor(s) involved.

5.2 Materials and Methods

5.2.1 Haemolytic Assays

Five ml of fresh rabbit blood in Alsevers solution (1:1 vol/vol) were washed three times in 50ml GVB/Mg (2mM) EGTA buffer by centrifuging at 1500g for 10mins between washes. The unsensitized red blood cells were diluted to a concentration of 2 x 10^8 cells ml^{-1}. The assay volume was made up to 150 µl with 50 µl of red blood cells and GVB/Mg buffer. SGE (unless otherwise stated 5 µl (20 µg)) or PBS (control) was added last, and then the reactions were incubated at 37^{O}C for 1 hour. Adding 1.2 ml of cold 0.15 M NaCl stopped haemolysis. Whole cells were spun down and haemolysis measured spectrophotometrically at 412nm (Coligan, 1994). All assays were carried out in duplicate.

Assays related to in section 5.3.2 were set up as above except using guinea-pig or sheep blood as a source of erythrocytes. For the assays described in section 5.3.3, rabbit erythrocytes were prepared as described above using GVB instead of GVB/Mg buffer. EDTA, $MgCl_2$ or $CaCl_2$ was added to the GVB buffered erythrocytes and incubated at 37^{O}C for 30 minutes prior to adding the SGE.

5.2.2 Kinetic analysis of SGE-mediated haemolysis

The optimal wavelength for scattering was calculated by creating a differential spectrum from lysed and non-lysed cells (Figure 5.1). The differential spectrum was created automatically using the DIV algorithm of the Cecil 6000 series (CE 6600)

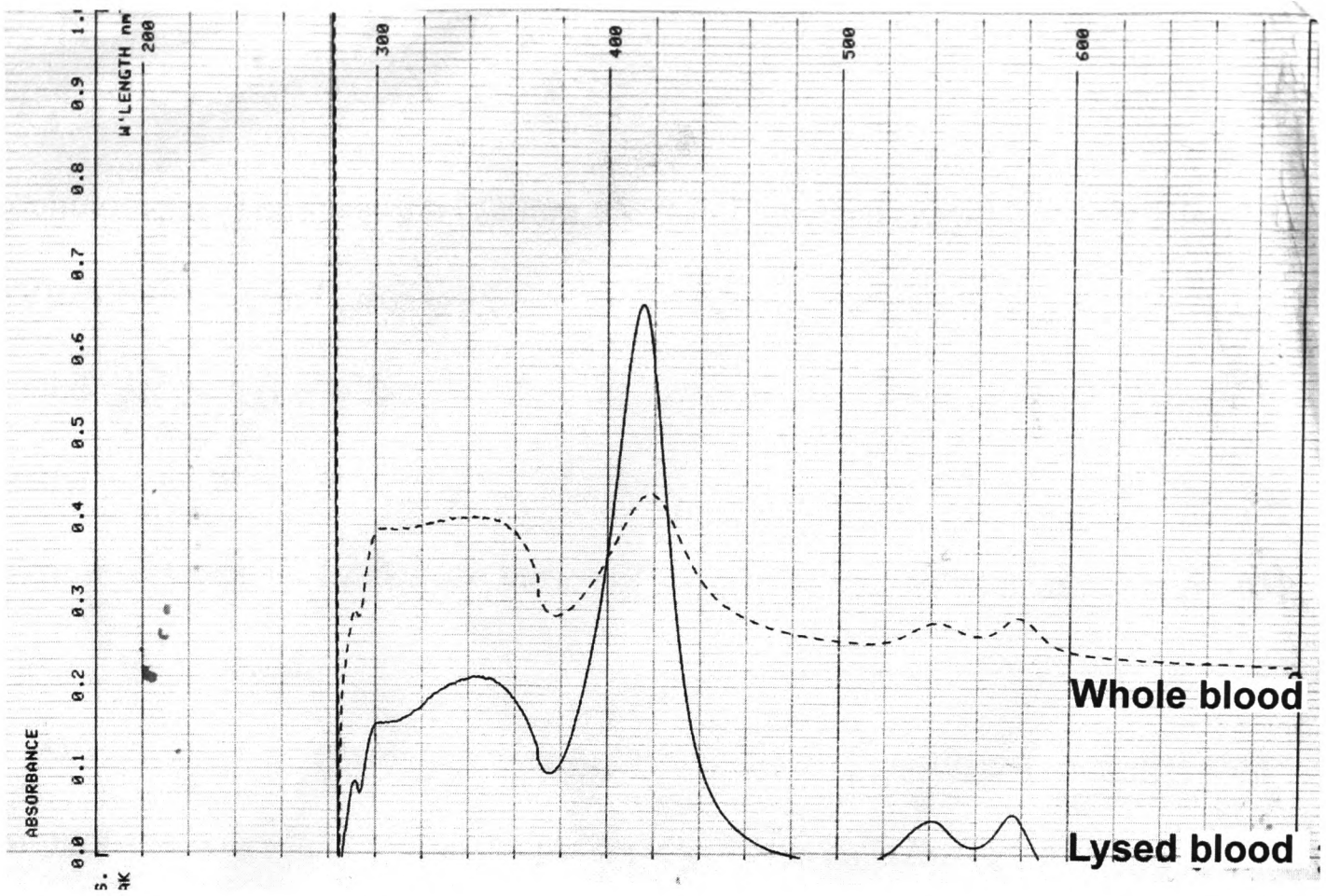


Fig 5.1 Absorbance spectrum of whole and lysed erythrocytes measured using a Cecil CE6600 spectrophotometer (range 200- 700 nm). (Top) individual spectra, (bottom) differential spectrum (whole – lysed spectra). The maximum is 595.9 nm (as indicated).

spectrophotometer. The spectrum (280nm – 700nm) obtained from whole erythrocytes (i.e. GVB/Mg buffer) and lysed erythrocytes (i.e. H_2O) are shown in Fig 5.1 (top). The differential spectrum (i.e. whole spectrum – lysed spectrum) showed that the largest differential occurs at 595.9nm (Fig 5.1 – bottom). Wavelengths that are greater than the size of the interacting molecules (erythrocytes are greater than 600 nm in size) give rise to scattering phenomena.

A haemolytic assay was set up as described above. The quantity of erythrocytes used in the assay was calculated semi-empirically to give an O.D. (595.9 nm) of about 0.7 (2.8×10^7 erythrocytes) in a total volume of 250 µl (including 5 µl SGE). Incubations were carried out at 37^{O}C. Spectrophotometric measurements were taken at various time intervals as indicated (Fig 5.3). Before each measurement, the mixture was gently shaken to re-distribute the erythrocytes. Control samples (i.e. no SGE) were also set up and measured throughout the incubation time; no lysis was observed (data not shown).

5.3 Results

5.3.1 Detection of haemolysis in the salivary gland extract of feeding female adult *I. ricinus* ticks

In order to maintain the integrity of the erythrocytes in haemolytic assays, buffers used and experimental conditions are identical to those described for AH_{50} assays (section 6.2.2), except for using 2 mM $MgCl_2$ (c.f. 10 mM) and no serum source. Varying quantities of *I. ricinus* SGE were tested for activity (4 $\mu g\ \mu l^{-1}$ as measured by the Bradford assay).

Treatment	Mean Absorbance (412nm)	% Haemolysis
0% lysis (i.e. no SGE)	0.068	**0**
100% lysis(i.e. H_2O)	0.839	**100**
0.5 µl SGE	0.078	**1.2**
1 µl SGE	0.152	**10.9**
2 µl SGE	0.198	**16.9**
5 µl SGE	0.681	**79.5**

Table 5.1: Haemolysis of rabbit erythrocytes using various volumes of *I. ricinus* SGE protein (section 5.3.1).

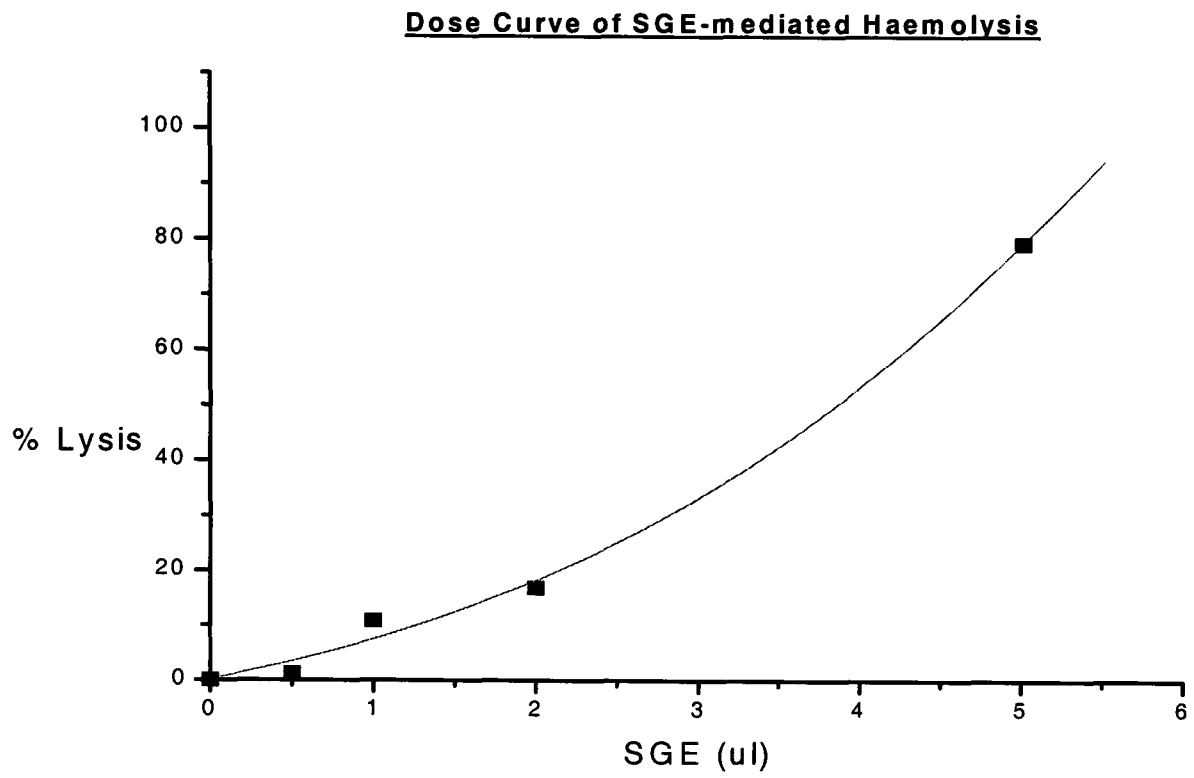


Fig 5.2 Graph of % haemolysis of rabbit erythrocytes against volume of *I. ricinus* SGE (section 5.3.1).

Haemolysis of rabbit erythrocytes in the haemolytic assays used increased in a dose-dependent manner with the volume of *I. ricinus* SGE used (Fig 5.2).

5.3.2 Kinetic analysis of SGE-mediated haemolysis

To investigate the kinetics of SGE-mediated haemolysis, a haemolytic assay was set up using a fixed quantity of SGE (20 μg) and an excess of substrate (i.e. erythrocytes). Haemolysis was measured at various time points by spectrophotometrically measuring scattering due to the presence of whole erythrocytes. The optimum wavelength was calculated as 595.9 nm (section 5.2.2). The results shown in Fig 5.3 showed that SGE-

mediated haemolysis displays saturation kinetics. No significant increase in the amount of haemolysis was observed after 30 minutes incubation time.

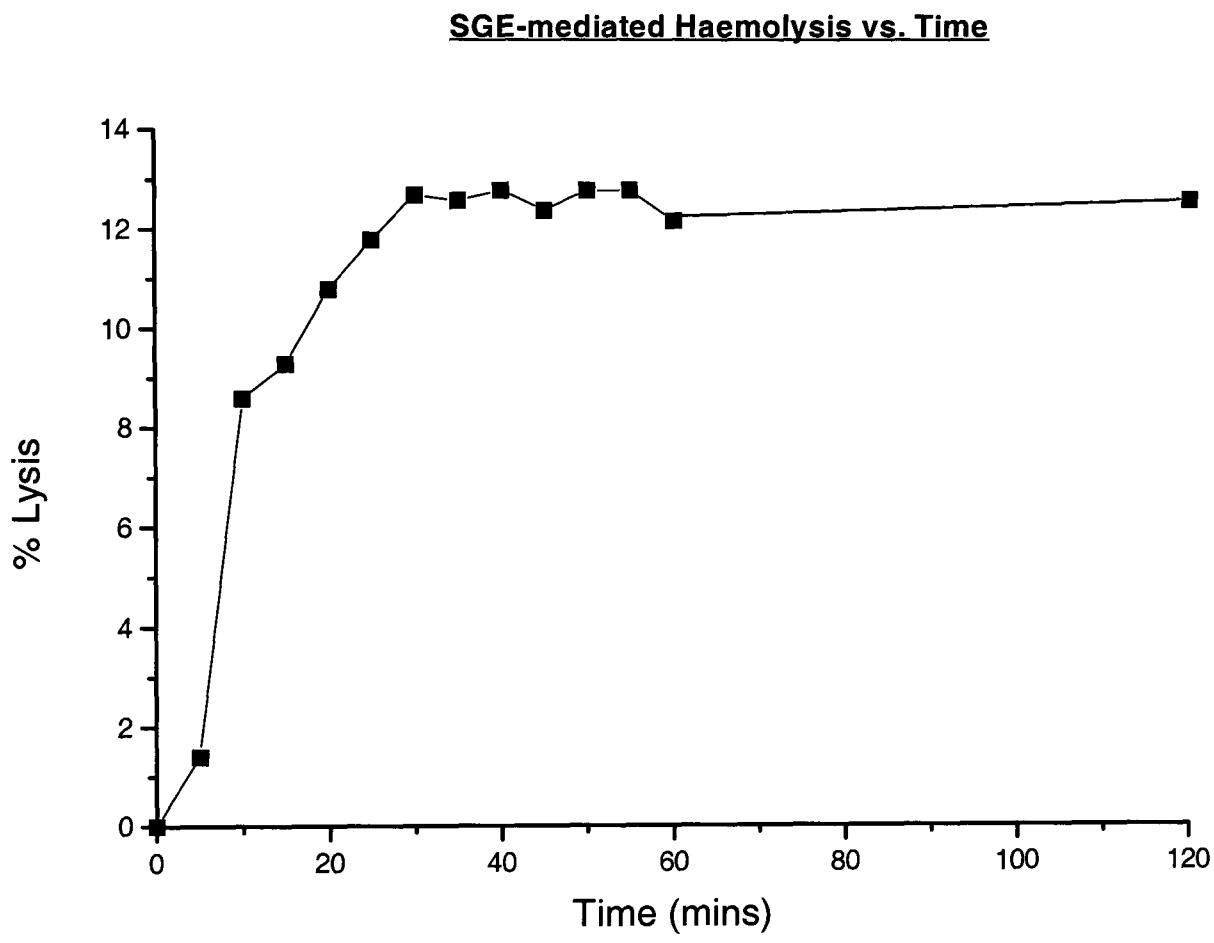


Fig 5.3 Kinetic analysis of SGE-mediated haemolysis as measured through whole erythrocyte scattering at 595.9 nm (section 5.3.2).

5.3.3 Characterisation of haemolytic activity

In order to test whether haemolysis was host species specific, haemolytic assays were set up as described above using rabbit, guinea-pig or sheep erythrocytes.

Treatment	Mean Absorbance (412nm)	% Haemolysis
0% (rabbit)	0.068	**0**
100% (rabbit)	0.839	**100**
SGE (rabbit)	0.681	**81**
0% (sheep)	0.192	**0**
100% (sheep)	1.810	**100**
SGE (sheep)	1.507	**83**
0% (guinea-pig)	0.122	**0**
100% (guinea-pig)	0.829	**100**
SGE (guinea-pig)	0.799	**95**

Table 5.2 *I. ricinus* SGE-mediated haemolysis using various species as sources of erythrocytes. 20 μg of SGE protein was used (section 5.3.3).

The results of table 5.2 showed that SGE-mediated haemolysis occurred when rabbit, sheep or guinea-pig erythrocytes were used in the haemolytic assays.

5.3.4 Effect of ions upon SGE-mediated haemolysis

The buffer used in the haemolytic assays described thus far (i.e. GVB/Mg buffer), contained Mg^{2+} (2 mM) and EGTA (10 mM). To investigate whether either of these factors was directly effecting SGE-mediated haemolysis, haemolytic assays were set up using EDTA, Ca^{2+} and Mg^{2+}. Rabbit erythrocytes were prepared as above in GVB buffer

(i.e. no EGTA or Mg). EDTA, $MgCl_2$ or $CaCl_2$ were added to erythrocyte suspension and incubated at $37^{O}C$ for 30 minutes prior to the addition of SGE.

Treatment	Mean Absorbance (412nm)	% Haemolysis
0% (0 mM)	0.059	**0**
0% (2 mM Mg)	0.017	**0**
0% (10 mM Mg)	0.020	**0**
0% (2mM Ca)	0.233	**0**
0% (10mM Ca)	0.173	**0**
0% (10mM EDTA)	0.102	**0**
100%	0.848	**100**
SGE (0 mM)	0.284	**28.5**
SGE (2mM Mg)	0.587	**68.5**
SGE (10mM Mg)	0.032	**1.4**
SGE(2mM Ca)	0.244	**1.8**
SGE (10mM Ca)	0.187	**2.1**
SGE (10mM EDTA)	0.122	**2.8**

Table 5.3 Testing the effects of divalent cations on *I. ricinus* SGE-mediated haemolysis. 20 μg of SGE protein was used (section 5.3.4).

The results of Table 5.3 showed that EDTA treatment inhibited haemolysis. Limited haemolysis occured in the absence of excess ions; in contrast, haemolytic activity appeared to be inhibited by the presence of extra Ca^{2+}. Haemolysis was greatest in the presence of 2 mM Mg^{2+}, but was inhibited in excess Mg^{2+} (10 mM).

5.4 Discussion

Haemolytic activity is essential to the survival of ticks. Despite this fact, little is known of the processes involved. The results of this chapter provide evidence for the presence of haemolytic activity associated with the salivary glands of feeding adult female *I. ricinus* ticks. It should be noted however, due to practical constraints, the saliva was not tested for haemolytic activity. Consequently, there is no unequivocal evidence that the haemolytic activity of *I. ricinus* SGE described in this chapter occurs exclusively in the saliva of the ticks. The ixodid tick, *Amblyomma americanum*, provides a premise for the existence of extracellular saliva-associated haemolysis in ticks (Zhu *et al.*, 1997). The role of saliva-associated haemolysis is unclear. Tick salivary secretions are thought to be responsible for tissue destruction and haemorrhage in the development of the feeding lesion (Kemp *et al.*, 1982). It is possible that haemolytic activity in the saliva of ixodid ticks may aid formation of the feeding lesion.

In this chapter, haemolytic activity was demonstrated to be associated with the salivary glands of female adult *I. ricinus* ticks in a dose-dependent manner (Fig 3.2). Kinetic analysis of the haemolytic activity showed that haemolysis displayed saturation kinetics under the experimental conditions used (Fig 3.3). This suggests either that the haemolytic activity is non-enzymatic (such as basic peptides present in *R. prolixus*) or that a co-factor required for enzymatic haemolysis becomes limiting. Haemolysis occurred irrespective of the host erythrocyte species tested in the haemolytic assays.

The haemolytic activity of *A. americanum* saliva has been attributed to a Ca^{2+}-dependent activity (Zhu *et al.*, 1997). In contrast the haemolytic activity of *I. dammini* ticks appears to be Ca^{2+}-independent (Ribeiro, 1988). The results of the investigation reported here show that haemolysis was inhibited by the divalent ion chelator, EDTA. The addition of extraneous Ca^{2+} indicates that haemolysis is not Ca^{2+}-dependent, indeed the presence of extra Ca^{2+} inhibited activity. This may be the result of the extra Ca^{2+} out-competing intrinsically membrane-associated Mg^{2+}. SGE-mediated haemolysis in *I. ricinus* ticks appears to be Mg^{2+} dependent. Interestingly, optimal haemolysis occurs in the presence of 2 mM Mg^{2+}, but was almost completely inhibited by 10 mM Mg^{2+}. Haemolysis in the absence of extraneous Mg^{2+}, presumably reflects the presence of intrinsically erythrocyte associated Mg^{2+}. Mg^{2+} has been observed to be bound to lipid molecules of cell membranes, stabilising these structures (Cowan, 1995). The fact that EDTA (Ca^{2+} and Mg^{2+} chelator) but not EGTA (Ca^{2+} chelator) inhibited haemolysis is consistent with this presumption. Do these data suggest that Mg^{2+} concentration acts as a regulator for *I. ricinus* haemolytic digestion? It should be noted that there is no evidence to suggest that such high levels of Mg^{2+} are ever present in the tick. Consequently, the physiological relevance of such inhibition is unclear.

Magnesium is a relatively abundant element in biological systems typically present at a concentration of ~1 mM extracellularly and intracellularly (the 'real' concentration in a cell is 30 mM, most of which is bound to proteins and nucleic acids). Magnesium has been shown to be a major regulator of activity in many enzymes including key enzymes of the glycolytic pathway (Cowan, 1995).

The results of this investigation may provide clues about the mechanisms of haemolytic activity in *I. ricinus* ticks and forms the basis for the future characterisation and isolation of haemolysins.

CHAPTER 6

ANTI-COMPLEMENT ACTIVITY IN THE SALIVARY GLANDS OF *I. ricinus* TICKS

6.1 Introduction

Complement forms the principal effector arm of the humoral immune system. The complement system is involved in both innate and adaptive immunity. Along with the acute phase proteins, the complement system, particularly the alternative pathway, are the first lines of defence employed by a host in response to tissue disruption caused by tick feeding (Ribeiro, 1987a). Increased levels of C3 have been reported in the serum of rabbits infested with *Ixodes ricinus* female adults, along with the presence of C3 in the midgut extracts of these ticks (Papatheodorou and Brossard, 1987). C3 deposition has been demonstrated at both the dermo-epidermal junction and within epidermal vesicles of guinea-pigs infested with *Dermacentor andersoni* larvae (Allen *et al.*, 1979).

Many strategies are employed by pathogens and parasites in order to avoid detection/destruction by the complement system (section 1.7.5). Several researchers have reported tick salivary gland associated anti-complement activity. Alternative pathway inhibitory activity has been associated with the salivary glands of *Ornithodoros erraticus, Ornithodoros moubata* (Astigarraga *et al.* 1997) and *Ixodes dammini* (Ribeiro 1987). This chapter investigated anti-complement activity in *Ixodes ricinus* ticks.

Inhibition of the alternative pathway of complement by *I. dammini* was shown to inhibit C3b deposition on activating surfaces as shown by ELISA. C3a generation was observed to be inhibited in subsequent experiments. Size-exclusion column chromatography showed that the activity eluted off the column as a single peak corresponding to a 49 kDa protein. These observations suggest that C3 cleavage is being inhibited by the SGE. However, as a consequence of the central and complex role played by C3 in both pathways (Fig 1.4), inhibition of cleavage could be caused at numerous points in the complement pathways.

" The mechanism of action of *I. dammini* salivary anti-complement activity remains to be explored." (Ribeiro 1987).

It is the aim of this chapter to elucidate the molecular mechanism of the anti-complement activity in female adult *Ixodes ricinus* salivary glands.

6.1 Materials and Methods

6.2.1 Gel electrophoresis

Both standard and NuPAGE gels under reducing and non-reducing conditions were prepared and run as described in section 2.6. Western blotting and Coommasie staining was carried out as described in section 2.6. Dilutions of the relevant anti-serum used for immunoprobing are as indicated in the text. I^{125} labelled samples were prepared, run on NuPAGE gels and autoradiograms prepared as described as described in section 2.6.5.

6.2.2 Alternative pathway of complement haemolytic assay (AH_{50})

Five ml of fresh rabbit blood in Alsevers solution (1:1 vol/vol) were washed three times in 50ml GVB/Mg (10mM) EGTA buffer by centrifuging at 1500g for 10mins between washes. EGTA inhibits the classical pathway by chelating Ca^{2+} that is essential for activation of this pathway. Mg^{2+} is required for both pathways. The unsensitized rabbit red blood cells were diluted to a concentration of 2×10^8 cells ml^{-1}. Serum was diluted in GVB/Mg EGTA buffer. The assay volume was made up to 150 µl with 50 µl of prepared red blood cells. SGE or PBS (5 µl) was added last, and then the reactions were incubated at $37^{O}C$ for 1 hour. Adding 1.2 ml of 0.15 M NaCl stopped haemolysis. Whole cells were spun down and haemolysis measured spectrophotometrically at 412nm (Coligan, 1994). All assays were carried out in duplicate. AH_{50} assays used for investigating complement component activation or proteolysis over time (i.e. PAGE analysis) were set up as above and unless otherwise stated contained 5 µl of human

serum. The tubes were shaken before each time sample was removed. Reactions were stopped by mixing samples with the appropriate loading buffers and placing on ice.

6.2.3 Classical pathway of complement haemolytic assay (CH_{50})

Five ml of fresh sheep blood in Alsevers solution (1:1 vol/vol) was washed once in GVB-EDTA (50ml) and three times in 50ml GVB^{++} buffer (GVB buffer with Mg^{2+} and Ca^{2+}). The red blood cells were diluted to a concentration of 1 x 10^9 cells ml^{-1}. The erythrocytes were sensitised using rabbit hemolysin (Sigma), titrated as described (Coligan, 1994). Assays were carried out using a 1/40 dilution of guinea pig sera (Sigma; CH_{50} titre= 147 units/ml) as a source of complement in accordance with standard protocols (Mayer, 1961). Adding 1.2 ml of 0.15 M NaCl stopped haemolysis. Whole cells were spun down and haemolysis measured spectrophotometrically at 412nm (Coligan, 1994). All assays were carried out in duplicate.

6.2.4 Preparation of factor D from human serum

Fresh human sera were prepared from blood as described (Coligan, 1994). One ml of human serum was loaded onto a G75 Sephadex column (1 cm (diameter) x 30 cm (length)) equilibrated with 1 x GVB buffer (Pharmacia, cat. no. 17-0050-01). Flow rate was 0.2 ml min^{-1} and 1 ml fractions were collected. Protein concentration was measured spectrophotometrically using absorbance at 280 nm. Fractions 13-21 were collected and pooled as factor D depleted serum (Reid *et al.*, 1988) (Fig 6.1). Fractions 26-33 were pooled as a source of enriched factor D. The pooled fractions were concentrated down to physiological strength using centricon-3 columns (Amicon, cat. no. 4202) in accordance

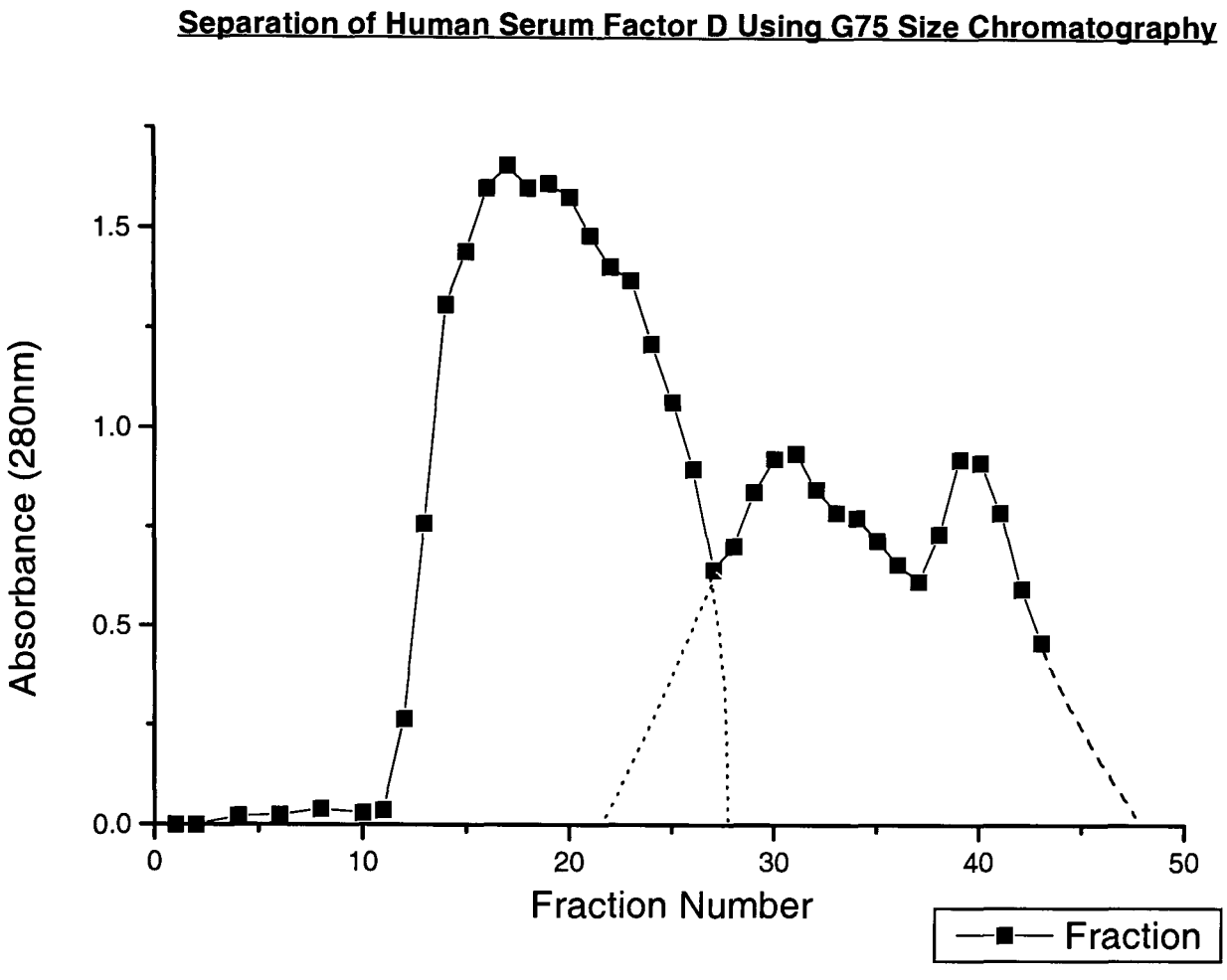


Fig 6.1 Factor D preparation from serum (section 6.2.4). Graph of protein concentration (280 nm) against number of serum fractions collected.

with the manufacturer's instructions. Deficient serum and factor D containing serum were tested for functional activity using AH_{50} assays (data not shown).

6.2.5 Iodination of C3 and C3(H_2O) with I^{125}

C3(H_2O) or C3 (200 μg) purified from fresh serum by Alister Dodds (MRC Immunochemistry Unit), were used for the iodination reactions. The C3 or C3(H_2O) in PBS containing 5 mM EDTA was added to an Iodogen coated tube (20 μg in CH_3Cl which was evaporated off), 1 mCi of I^{125} was added and the mixture incubated for 5 minutes at room temperature. A PD-10 (Pharmacia) size exclusion column, pre-equilibrated with BSA (10 mg), was used to separate the free I^{125} from the C3-bound I^{125} (in PBS). Fractions (6 x 1 ml) were collected and tested for radioactivity using a scintillation tube held at fixed distance. Fractions 3 and 4 were collected as containing the I^{125} labelled C3 and C3(H_2O). The unbound I^{125} came off at fraction 6-10. The fractions were pooled and 2 μl of each C3 and C3(H_2O) counted for activity.

Activity was counted over 10 seconds using a I^{125} counter (mini-assay type 6-20) with a counting efficiency of 70%. Three replicates were measured.

Tube	Mean background	Mean count (10s)	cpm μl^{-1}
C3	44	74365	**222963**
C3(H_2O)	48	22783	**68205**

Table 6.1 Activity in counts per minute of I^{125} labelled C3 and C3(H_2O).

6.2.6 **Effect of SGE on C3 cleavage and deposition on rabbit erythrocytes in an AH_{50} assay**

An AH_{50} assay was set up as described in section 6.2.5 using 2 x 10^8 rabbit erythrocytes (measured spectrophotometrically). Human serum (10 µl), 10 µl SGE/PBS and 40 µl of I^{125}-C3 were added to the reaction mixture. The total reaction volume was 150 µl. Samples (30 µl) were taken at various time intervals as shown (Fig 6.10). Samples were spun down at 14 Krpm for 10 minutes, to pellet whole erythrocytes and ghost membranes. Ten µl of the supernatants were removed and designated supernatant samples (s/n). The pellets were washed two times in GVB/Mg buffer, before re-suspending in 10 µl of GVB/Mg buffer. All sample reactions were stopped by mixing with 5x loading buffer (containing DTT) and placing on ice until loading. Samples were run on a NuPAGE 4-12% bis-tris gel under reducing conditions, and an autoradiogram prepared.

6.2.7 **Effect of SGE on turnover of C3(H_2O) to iC3b visualised by autoradiography**

Master mixes containing 20 µl of C3(H_2O), 1 µl of human serum and 25 µl of SGE (100 µg)/PBS were made up to a total volume of 60 µl using GVB/Mg buffer. The control (PBS) and extract samples (SGE) were incubated at 37^{O}C. Samples (5x 10 µl) were removed at various time periods as indicated (Fig 6.11). All sample reactions were stopped by mixing with 5x loading buffer (containing DTT) and placing on ice until

loading. Samples were run on a NuPAGE 4-12% bis-tris gel under reducing conditions, and an autoradiogram prepared.

6.2.8 **Effect of SGE on alternative pathway C3-convertase kinetics visualised through estimation of factor B cleavage by Coomassie-blue staining**

In order to optimise experimental conditions for convertase formation, several combinations of factor D, C3b and factor B were tested (data not shown). 200 ng of purified C3b (from Alister Dodds- MRC Immunochemistry, Oxford), 1 ng of purified factor D (section 6.2.4) and 2 μg of factor B (Sigma) were made up to 20 μl using GVB/Mg buffer. Incubations were carried out at 37^{O}C and samples incubated for various periods of time as indicated (Fig 6.12). All sample reactions were stopped by mixing with 5x loading buffer (containing DTT) and placing on ice until loading. Samples were run on a NuPAGE 4-12% bis-tris gel under reducing conditions, and Coomassie blue stained as described (section 2.6). SGE containing samples contained 2 μg of protein (Fig 6.13).

6.2.9 **Effect of SGE on convertase stability visualised by turnover of I^{125} labelled C3**

Reactions were set up as described above (section 6.2.13), except using 5 μl (20 μg) of SGE for each sample. Convertase formation was carried out as above using an incubation time of 20 minutes in the presence or absence of SGE. Further convertase formation was inhibited by adding 10 mM EDTA. At this point, 2 μl of I^{125} labelled C3 (about 50000 cpm) in the presence or absence of SGE were added to the reaction mixtures. Incubations were carried out at 37^{O}C for various time periods as shown (Fig

6.14). All sample reactions were stopped by mixing with 5x loading buffer (containing DTT) and placing on ice until loading. Samples were run on a NuPAGE 4-12% bis-tris gel under reducing conditions, and an autoradiogram prepared .

6.2.10 Effect of SGE on I^{125} labelled C3 and C3(H_2O) as visualised by reducing PAGE autoradiography

Five μl (20 μg) of SGE were added to 50000 cpm of C3 or C3(H_2O) made up to 10 μl volume with $MgCl_2$ (final 10mM) and PBS. Incubations were carried out at 37^{O}C for various time periods as indicated (Fig 6.15). Reactions were stopped by mixing with 5x loading buffer (containing DTT) and placing on ice until loading. Samples were run on a NuPAGE 4-12% bis-tris gel, and an autoradiogram prepared.

6.2.11 Iodination of SGE with I^{125}

Fifty μg of *I. ricinus* SGE in PBS (200 μl) was used for the iodination reaction. The SGE was added to an Iodogen coated tube (20 μg in CH_3Cl which was evaporated off), 2 mCi of I^{125} was added and the mixture incubated for 5 minutes at room temperature. A PD-10 (Pharmacia) size exclusion column, pre-equilibrated with BSA (10 mg), was used to separate the free I^{125} from the SGE-bound I^{125}. Fractions (6 x 1 ml) were collected and tested for radioactivity using a scintillation tube held at fixed distance. Fractions 3 and 4 were collected as containing the I^{125} labelled SGE. The unbound I^{125} came off at fraction 6-10. The fractions were pooled and 2 μl of I^{125}-SGE counted for activity. Approximately 50000 cpm of the I^{125} labelled SGE was run on a NuPAGE gel under reducing and non-reducing conditions. The autoradiogram is shown in Fig 6.25.

Tube	mean background	mean count (10s)	cpm μl^{-1}
SGE	37	28815	**86445**

Table 6.2 Activity in counts per minute of I^{125} labelled SGE.

Approximately 50000 cpm of I^{125} labelled SGE was run on a NuPAGE gel under reducing and native (i.e. different loading buffers used etc.) and an autoradiogram prepared (Fig 6.24).

6.2.12 Affinity chromatography of I^{125} labelled SGE

To look for possible interactions between C3 or factor D and SGE, affinity columns containing bound C3 or factor D were set up and I^{125} labelled SGE run through the columns. In order to eliminate possible steric hindrance caused by binding these proteins directly to the column, monoclonal antibodies were bound to protein G-Sepharose and the proteins bound in turn to the respective antibodies. 0.3 ml of protein G-Sepharose fast flow (Sigma (binding capacity 2 mg ml^{-1})) were loaded into two 2 ml columns (i.e. 0.15 ml per column- dimensions 0.5 cm x 4 cm). Columns were washed through with 5 x 1 ml PBS. JA4-2, an anti-factor D monoclonal from John Volanakis, University of Alabama, Birmingham (0.25 ml of 0.6 mg ml^{-1}), diluted in 1 ml PBS was loaded onto one column. The other column was loaded with 0.6 ml (0.23 mg ml^{-1}) of WM-1, an anti-C3c monoclonal from Steve Whitehead (ICRF), diluted in 1 ml PBS. The monoclonals were loaded onto the columns 3 times. The columns were then washed with

5 x 2 ml of PBS. Five ml of human serum (from Bob Sim (MRC Immunochemistry Unit, Oxford), pre-absorbed on IgG Sepharose) and purified C3 from Bob Sim (MRC Immunochemistry Unit, Oxford) (200 µl, 0.77 mg ml^{-1}) diluted in 5 ml PBS and were loaded onto the respective columns 3 times. The columns were washed with 5 x 2 ml of PBS. In addition a column was set up containing 0.5 ml of Sepharose bound antibody against factor B (Bob Sim (MRC Immunochemistry Unit, Oxford)- 200 µg ml^{-1} binding capacity). The column was loaded with 4 ml of pre-absorbed serum, 3 times. The column was washed with 5 x 2 ml PBS and in all other respects treated like the other columns.

Two ml of the I^{125} labelled SGE (i.e. 1.6 x 10^9 cpm) was loaded onto the respective columns three times. The columns were washed with 5 x 2 ml of GVB buffer. In order to quantitate binding of the radiolabelled SGE macromolecules to the columns, the washed contents of the columns (i.e. resin and bound species) were removed (in 500 µl GVB buffer) transferred to a fresh tube, and counted.

	mean background (10s)	**Bound SGE mean count (10s)**	**Bound SGE (cpm)**
factor D	32	45766	**274596**
C3	30	22815	**136890**
factor B	31	23433	**140598**

Table 6.3 Activity in counts per minute of affinity chromatography bound I^{125} labelled SGE.

Elution of the bound SGE materials was carried out using 1 M NaCl (on the assumption that binding was likely to have a major ionic component). Fractions (6 x 50 µl) were collected in tubes pre-treated with 10 % Triton-100, in order to prevent the small quantities of protein becoming absorbed onto the tubes. The collected fractions were counted for activity.

Column	Fraction	Mean background (10s)	Mean count (10s)	Eluted count (cpm)
factor D	GVB wash	25	301	**1656**
	1	22	1379	**8142**
	2	30	1135	**6630**
	3	30	1169	**6834**
	4	18	1033	**6090**
	5	34	1282	**7488**
	6	30	996	**5796**
C3	GVB wash	23	389	**2196**
	1	19	245	**1356**
	2	23	1596	**9438**
	3	21	2125	**12624**
	4	23	1696	**10038**
	5	19	1475	**8736**
	6	26	1149	**6738**
factor B	GVB wash	26	253	**1362**
	1	27	355	**1968**
	2	27	273	**1476**
	3	28	259	**1386**
	4	16	321	**1830**
	5	38	257	**1314**
	6	26	293	**1602**

Table 6.4 Mean activity in counts per minute eluted from C3, factor D or factor B column. GVB washes (50 µl) were collected before elution. Fractions collected were 50 µl 1 M NaCl.

Several of the fractions (5 µl volume) were run on a gel and Coomassie blue stained. No protein was observed in these fractions (data not shown). Based upon the above information 20 µl each of C3-3, C3-4, D-1 and D-3 were run on a NuPAGE gel as described in section 2.6. Because of the high salt concentration of the samples, the gel was run at a constant voltage of 50 V in the cold room. An autoradiogram was prepared as described in Chapter2 (Fig 6.25).

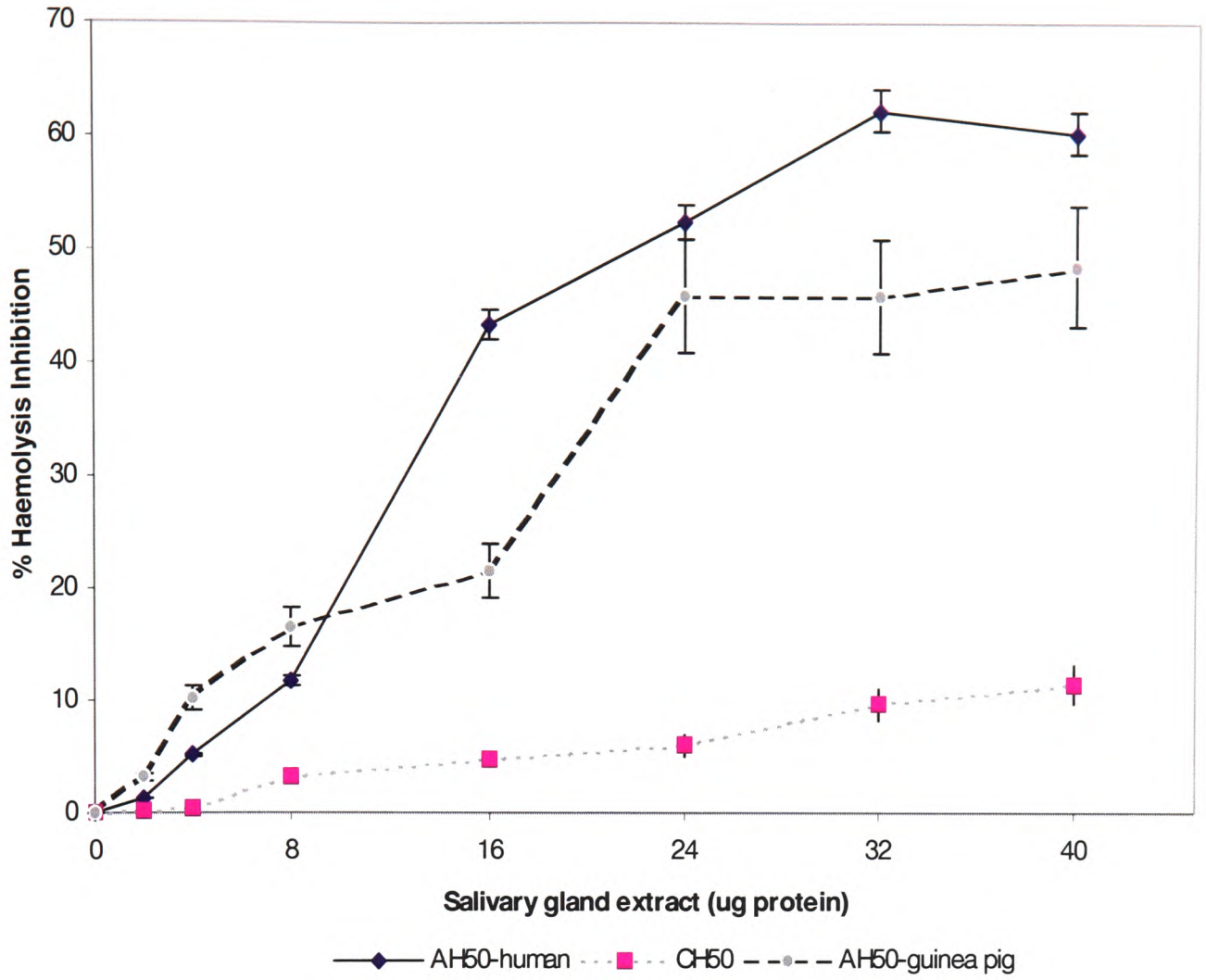


Fig 6.2 Mean percentage inhibition of complement-mediated hemolysis relative to the dose of SGE taken from adult female *I. ricinus* that had fed for 5 days. 10 μl of human serum (equivalent to 83% hemolysis in absence of SGE) and 12 μl of guinea-pig serum (equivalent to 67% hemolysis in the absence of SGE) were used for the AH_{50} assays. 2.5 μl of guinea pig serum (equivalent to 92% hemolysis in absence of SGE) was used for the CH_{50} assays. Assays were carried out in triplicate, ± standard errors are shown as bars. Continuous line, AH_{50} human serum; dashed line, AH_{50} guinea-pig serum; dotted line, CH_{50}.

6.3 Results

ANTI-COMPLEMENT ACTIVITY

6.3.1 Anti-complement activity of *I. ricinus* SGE

I. ricinus SGE inhibited the alternative pathway (AH_{50}) of complement (using either guinea-pig or human serum) but not the classical pathway (CH_{50}) (Figure 6.2). Twenty μg of SGE protein (one salivary gland equivalent) inhibited about 50% of the haemolytic activity of 10 μl of human serum (83% haemolysis in the absence of SGE).

6.3.2 Anti-complement activity of *I. ricinus* SGE throughout the feeding period

SGE (20 μg) prepared from ticks either unfed or fed on hamsters for 1, 2, 3, 4, 5, or 6 days were tested in AH_{50} assays using 8.3 μl of human serum (72% haemolysis in the absence of SGE) (Table 6.5). Anti-alternative pathway activity was present in the salivary glands of adult female *I. ricinus* ticks when unfed and throughout the feeding period, but mean activity per μg of SGE protein was greatest at approximately five days of feeding.

Treatment	Absorbency Range (412nm)	Mean Haemolysis Inhibition (% of PBS control)
PBS (5 µl) (i.e. no SGE)	0.336-0.350	**(0)**
Day 0 SGE	0.151-0.157	**55.2**
Day 1 SGE	0.129-0.139	**60.9**
Day 2 SGE	0.141-0.145	**58.3**
Day 3 SGE	0.030-0.040	**89.8**
Day 4 SGE	0.091-0.099	**72.3**
Day 5 SGE	0.027-0.029	**91.9**
Day 6 SGE	0.051-0.059	**83.9**

Table 6.5 Assay to determine alternative pathway activity (AH_{50} assay) in the presence or absence of *Ixodes ricinus* SGE (20 µg) prepared from unfed (day 0) or feeding (Day 1-6) ticks. 8.3 µl of human serum was used (equivalent to 72% haemolysis in absence of SGE).

6.3.3 Testing *Rhipicephalus appendiculatus*, *Ornithodoros moubata*, *Anopheles gambesi*, and *Dermacentor andersoni* salivary glands for inhibition of the alternative pathway of complement

SGE derived from *Anopheles gambesi* and *Rhipicephalus appendiculatus* (male day 3) were obtained from Miles Nunn (IVEM). *Dermacentor andersoni* (female day 4) SGE was obtained from Somchai Sangemanadach (IVEM). *Ornithodoros moubata* ticks (females fed two times) were dissected and the salivary glands prepared as described

above. The various salivary gland extracts were tested in AH_{50} assays. Anti-complement activity was present in *O. moubata* SGE although inhibition per μg of protein was slightly less than that of *I. ricinus* SGE. No inhibition of the alternative pathway of complement activity was observed with *R. appendiculatus, D. andersoni* or *A. gambesi* SGE (Table 6.6).

Treatment	Absorbency Range (412nm)	Mean Haemolysis Inhibition (% of PBS control)
PBS (5 μl)	0.742-0.750	**(0)**
I. ricinus	0.080-0.092	**88.5**
R. appendiculatus	0.735-0.741	**1.1**
D. andersoni	0.637-0.641	**14.4**
O. moubata	0.121-0.143	**82.3**
A. gambesi	0.734-0.738	**1.4**

Table 6.6 Assay to determine alternative pathway activity (AH_{50} assay) in the presence of *I. ricinus, R. appendiculatus, D. andersoni, O. moubata* and *A. gambesi* SGE. 20 μg of protein and, 5 μl of human serum were used (equivalent to 96.3 % haemolysis in the absence of SGE).

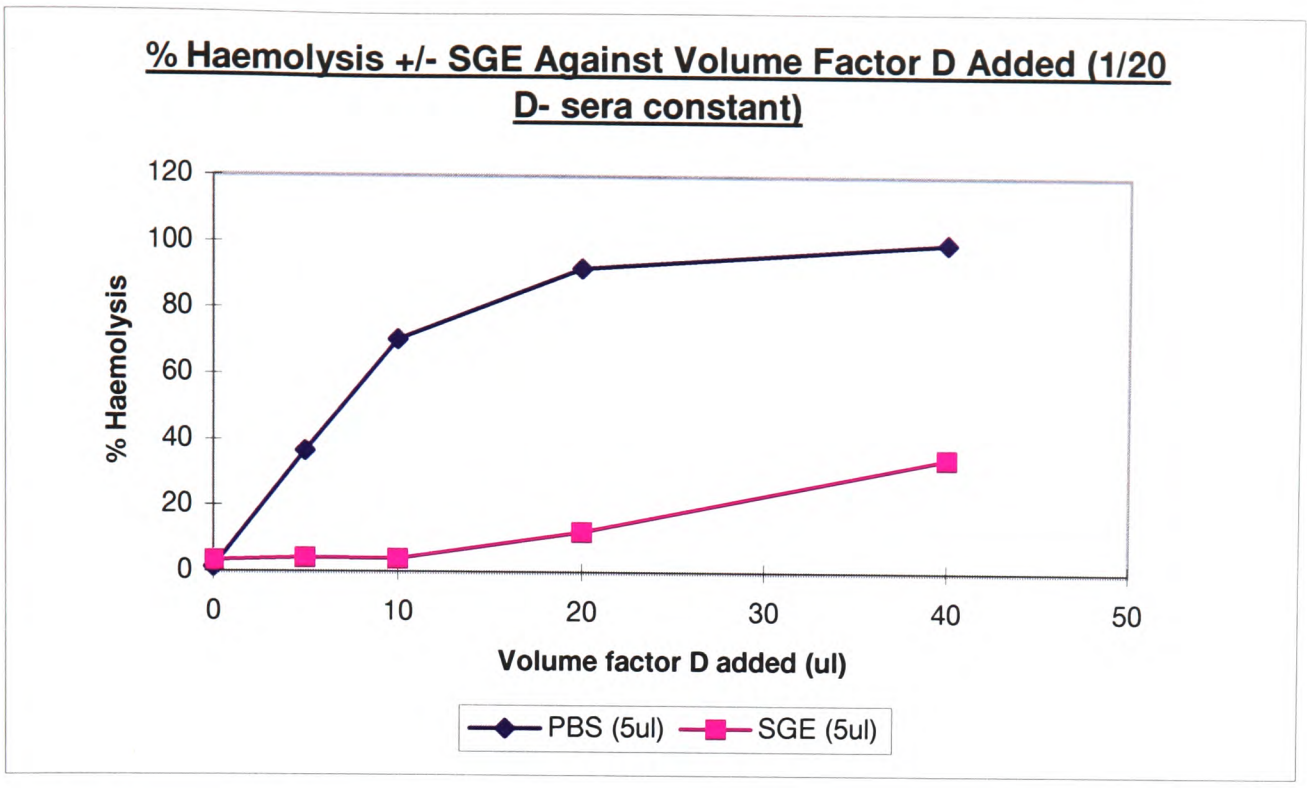
6.3.4 Testing *I. ricinus* adult male, female and larvae and nymphal whole tick homogenates for haemolysis in the presence and absence of serum.

Thirty nymphs and fifty larvae (*I. ricinus*) were fed separately on hamsters for 2 days before being removed. Fifteen female and male adult ticks were fed on hamsters for five days before being removed. The ticks were ground in liquid nitrogen using a mortar and pestle. Samples were further homogenised using a glass homogeniser in 100 µl of PBS. Particulate material was removed by centrifugation at 14,000 rpm for 10 minutes. Twenty µg protein of each extract was used in AH_{50} assays (as measured by the Bradford assay). In order to test non-complement dependent haemolysis, control reactions in the absence of serum were set up for each of the homogenate samples tested.

The adult female, nymphal and larval whole tick homogenates showed significant haemolytic activity in the absence of serum (Table 6.7). Haemolytic activity was probably due to the presence of haemolysins in the gut material of the whole tick homogenates. In contrast, the male homogenate had little haemolytic activity in the absence of serum. Conversely, adult female, nymphal and larval whole tick homogenates showed reduced haemolysis in the presence of serum, whereas male adult homogenates showed little difference from the haemolytic activity of the PBS control.

Treatment	Absorbency Range (412nm)	Mean Haemolysis (% of PBS control)
PBS (5 µl)	0.659-0.661	**(100)**
Larvae homog.- no serum	0.831-0.789	**123**
Larvae hoinog.- serum	0.509-0.512	**77.3**
Nymph homog.- no serum	0.934-0.884	**137**
Nymph homog.- serum	0.596-0.601	**90.6**
Female SGE- no serum	0.024-0.028	**3.9**
Female SGE- serum	0.038-0.041	**6.0**
Female homog.- no serum	1.114-0.998	**160**
Female homog.- serum	0.782-0.852	**123**
Male homog.-no seruin	0.167-0.176	**25.9**
Male homog.-serum	0.692-0.724	**107**

Table 6.7 Assay to determine alternative pathway activity (AH_{50} assay) in the presence of *I. ricinus* adult SGE, male, female, nymphal and larval extracts. 5 µl of human serum was used (equivalent to 56% haemolysis in the absence of SGE).



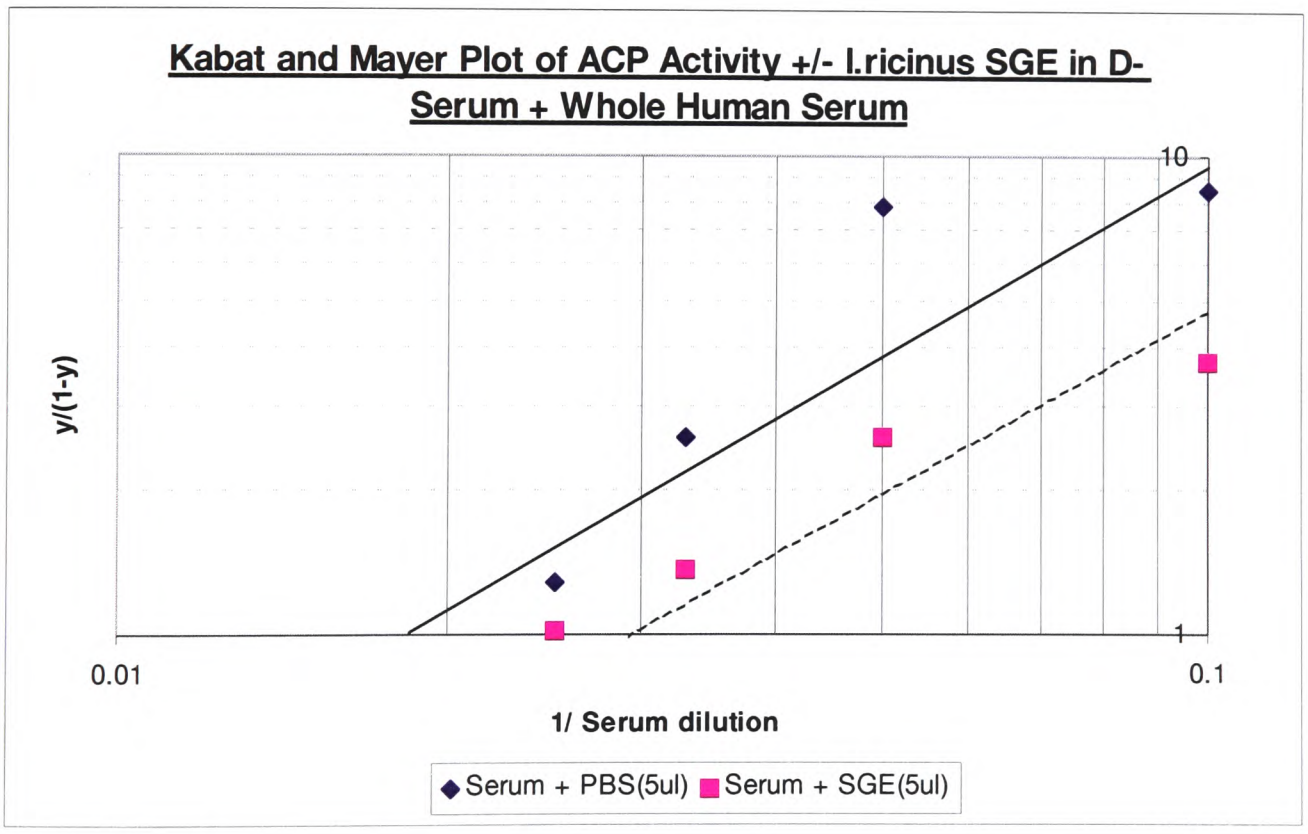
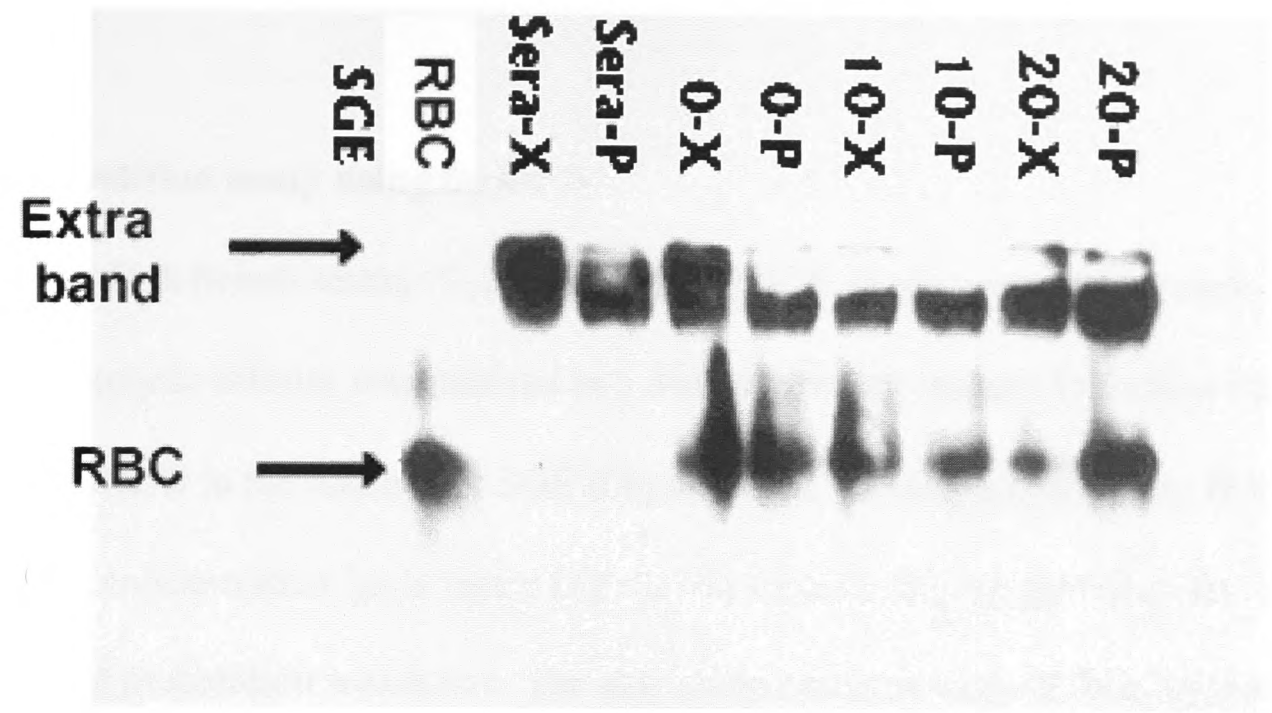


Fig 6.3 **Competition assays using an excess of factor D. (Top) 20 µl of factor D deficient serum, volume of purified factor D as shown. (Bottom) 20 µl of factor D deficient serum, whole serum dilution as shown. 20 µg of SGE protein was used.**



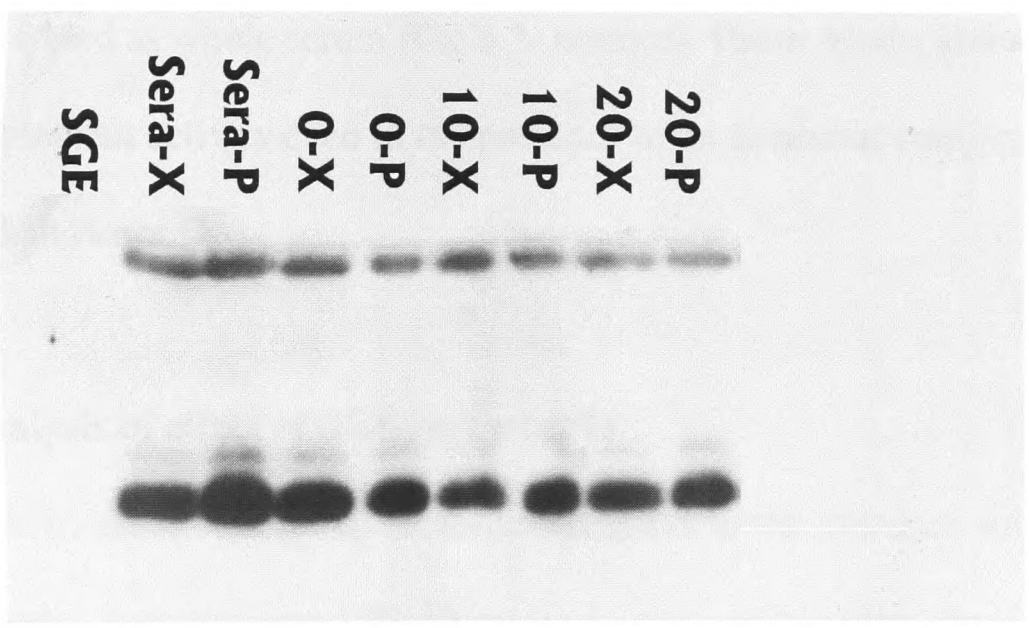


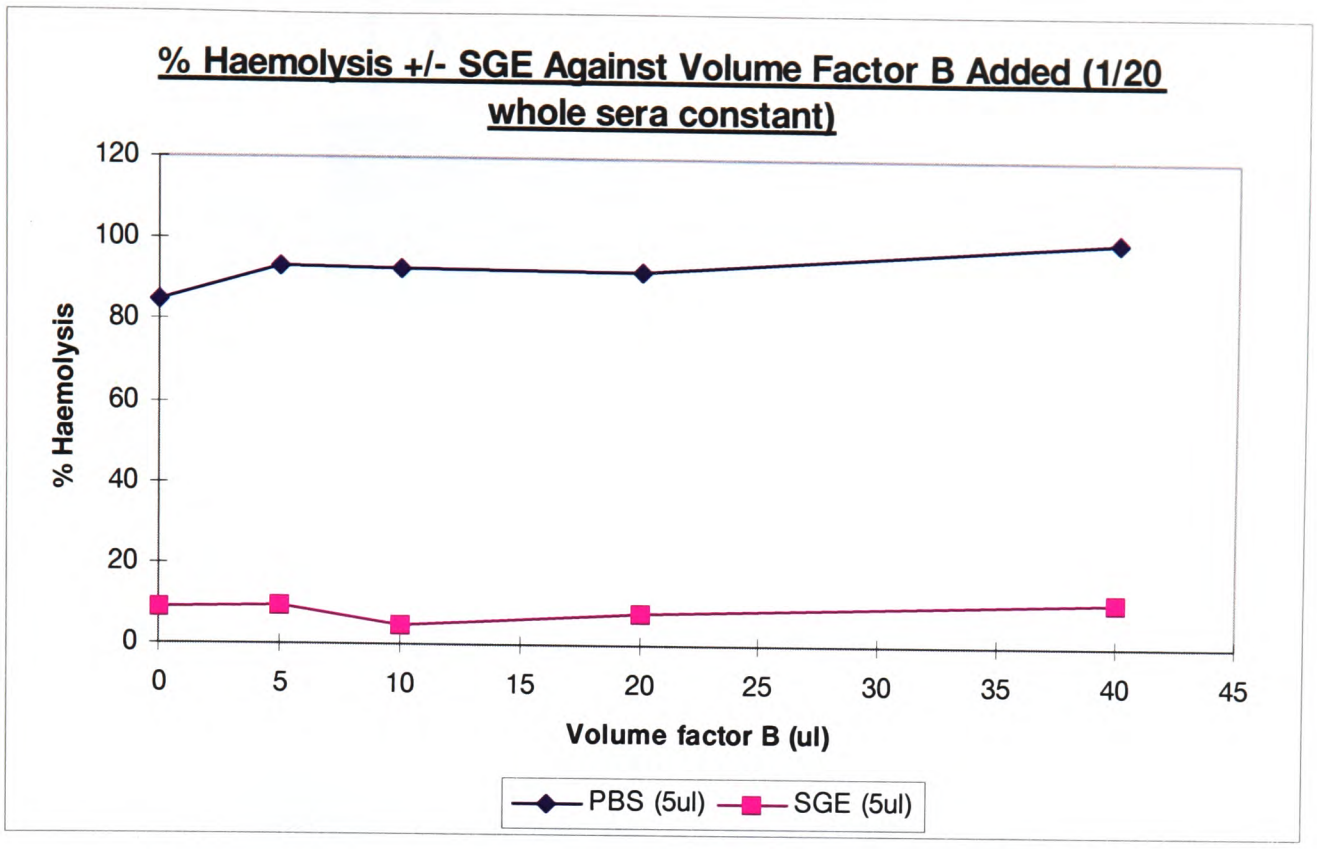
Fig 6.4 Effect of SGE on factor D in AH_{50} assay over time. Western blot probed with 1 in 300 dilution of JA-4-2 mAb against factor D. Time points as indicated, X- extract containing, P- PBS containing, RBC- red blood cells only, Sera- serum only (i.e. no red blood cells). (Top) native PAGE, (Bottom) SDS PAGE.

6.3.5 Competition assay using factor D

Factor D deficient serum (5 μl) and 20 μg of SGE (5 μl) was used for each sample. Haemolytic activity was restored in a dose dependent manner becoming saturated at 20 μl of factor D in the absence of SGE (Fig 6.3- top). As the purified factor D is at physiological concentration (as is factor D deficient serum), this suggests that about 25% of the factor D preparation was active. The anti-complement activity of SGE appears to be out-competed in a dose dependent manner by the addition of factor D (Fig. 6.3- top). An excess of factor D deficient serum (20 μl) was used in standard AH_{50} assays, and active factor D was added as whole serum (Fig 6.3- bottom). These results showed that SGE inhibited complement activity even in the presence of an excess of complement components other than factor D.

6.3.6 Western analysis of effect of SGE on factor D

A standard AH_{50} assay was set up as described above in the presence and absence of SGE. Five μl samples (total volume 150 μl) were taken at various time intervals as indicated (Fig 6.4). Western blotting was carried out as described (section 2.6); the membranes were probed with JA4-2 monoclonal anti-factor D serum at a 1 in 600 dilution. The results of SDS-PAGE analysis are inconclusive (Fig 6.4-bottom). Sensitivity was greatly reduced compared with that of native PAGE analysis. This is probably due to the epitope specificity of the monoclonal antibody, which was raised against factor D in its native form.



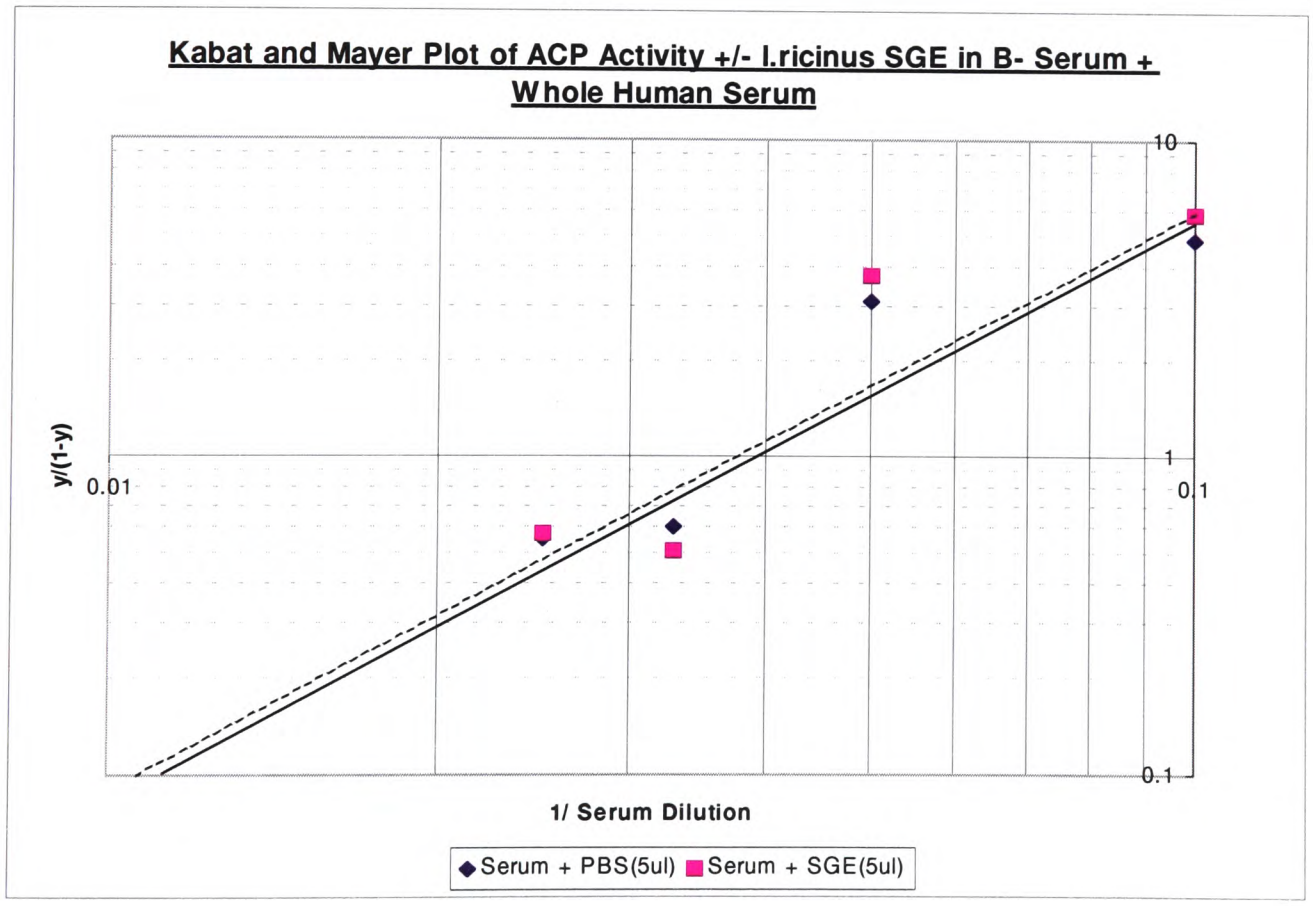


Fig 6.5 **Competition assays using an excess of factor B. (Top) 5 μl of whole serum, volume of purified factor B as shown. (Bottom) 20 μl of factor B deficient serum, whole serum dilutions as shown. 20 μg of SGE protein was used.**

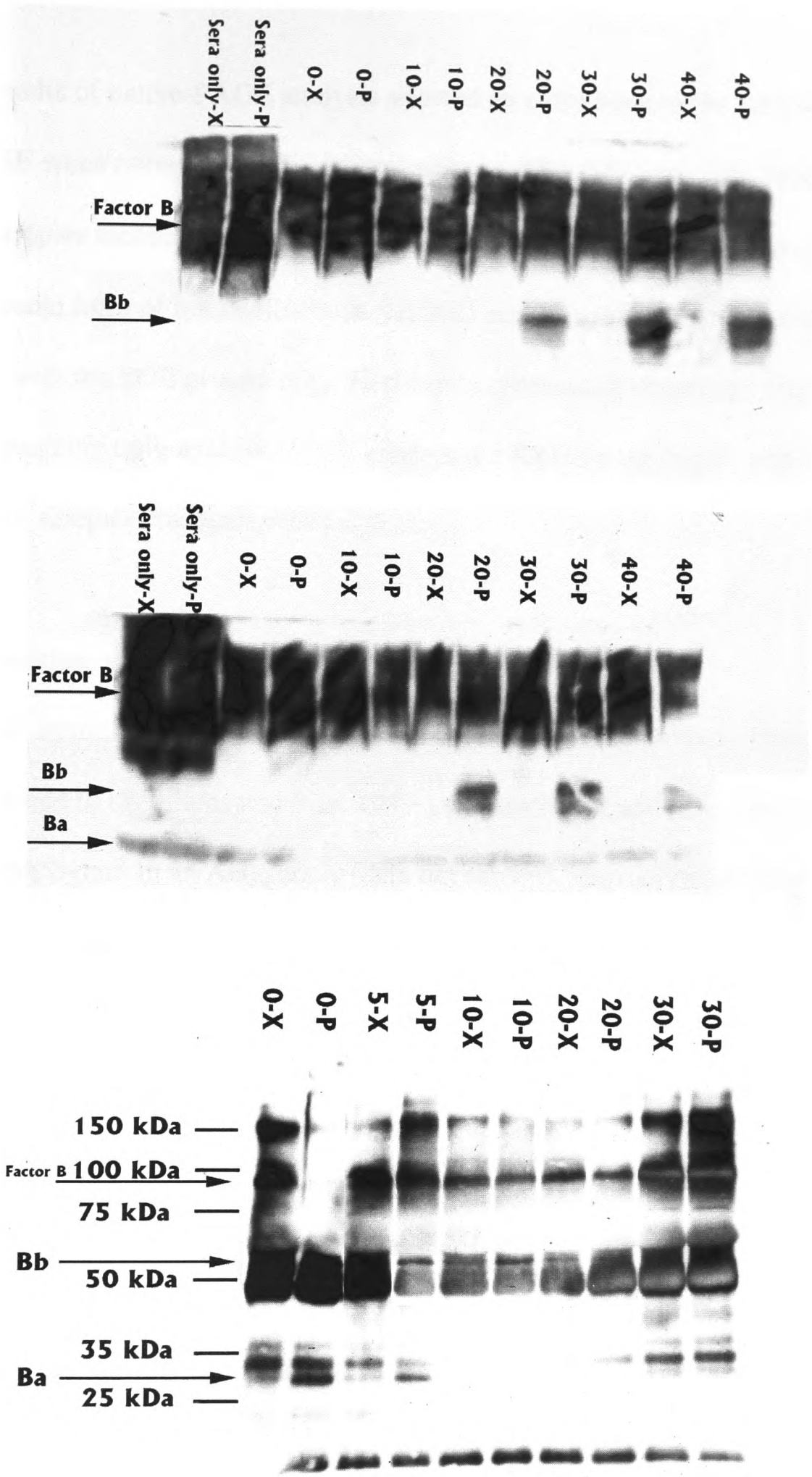


Fig 6.6 **Effect of SGE on factor B in AH_{50} assay over time. Western blot probed with 1 in 600 dilution of polyclonal against factor B. Time points as indicated, X-extract containing, P-PBS containing, Sera- without erythrocytes. (Top) native PAGE (*I. ricinus)*, (middle) native PAGE (*I. hexagonus)*, (Bottom) SDS PAGE (*I. ricinus).***

The results of native-PAGE analysis showed an extra band in the samples containing SGE when compared to the control samples (Fig 6.4- top). This band is not observed in samples incubated for 10 or 20 minutes. Such a change in mobility is indicative of some form of interaction between SGE and factor D. No cross-reactivity was observed with the SGE protein only. However a cross-reactive antigen was present in the sample containing only red blood cells (designated RBC on the figure) and AH_{50} samples but not samples containing only serum.

6.3.7 **Competition assay using factor B**

Purified factor B obtained from Bob Sim (MRC Immunochemistry Unit, Oxford) (purified according to (Williams and Sim, 1993)) was tested for activity using factor B deficient serum (Sigma) in an AH_{50} assay (data not shown). Haemolytic activity was not restored suggesting that the factor B preparation was not functionally active (unlike the factor B deficient serum, the purified factor B had undergone multiple freeze/thaw cycles). As a consequence, 5 µl of whole serum (instead of factor B deficient serum) (± 20 µg SGE) was used in each AH_{50} assay. The anti-complement activity of SGE was not significantly changed by the addition of the purified factor B (Fig. 6.5- top). An excess of factor B deficient serum (20 µl) was used in AH_{50} assays; active factor B was provided using whole serum (Fig. 6.5- bottom). These results suggest that an excess of complement components, with the exception of factor B, inhibited the anti-complement activity of the SGE. In other words, factor B is unlikely to be directly involved in anti-complement activity.

6.3.8 **Western analysis of effect of SGE on factor B**

AH_{50} assays were set up as described above using 20 μg of SGE from *I. ricinus* or *I. hexagonus* (total volume 150 μl). Samples (5 μl) were taken at time intervals as indicated (Fig 6.6). Mixing samples with loading buffer and placing on ice stopped reactions. The samples were loaded onto a polyacrylamide gel, run and western blotted as described in section 2.6. Polyclonal rabbit anti-factor B serum obtained from Bob Sim (MRC Immunochemistry Unit, Oxford) was used to probe membranes at a 1 in 600 dilution.

The results shown in Fig 6.6- bottom demonstrated that less Bb (62 kDa) and Ba (30 kDa) were generated in samples containing SGE compared with the control samples. No extra bands which could be indicative of factor B binding are seen in samples containing SGE in native PAGE analysis (Fig. 6.6- top, middle), it is clear however that factor B cleavage is inhibited in these samples. The native PAGE results (Fig 6.6- middle) using *I. hexagonus* SGE show the same pattern as that of *I. ricinus*. This suggests that both adult female *I. ricinus* and *I. hexagonus* salivary glands contains a factor that inhibits cleavage of factor B (but not necessarily by a direct effect on factor B).

6.3.9 **Effect of CVF treatment on anti-complement activity as visualised by western analysis of factor B turnover**

CVF (cobra venom factor) is an analogue of C3b that is insensitive to factor I-mediated cleavage. The result is that the serum is decomplemented as all the C3 is converted to C3b by the fluid phase CVF convertase (i.e. CVFBb). To investigate

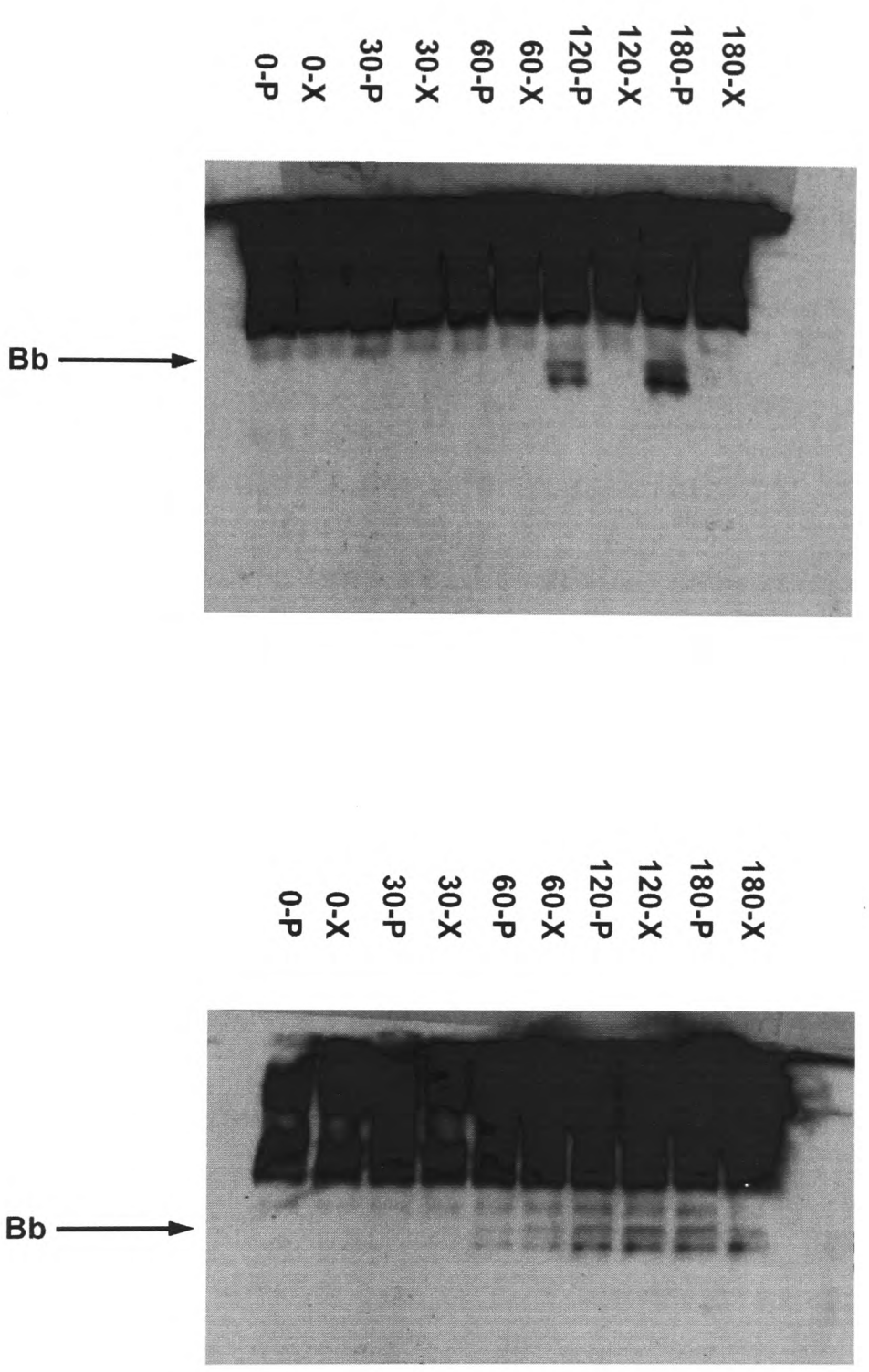
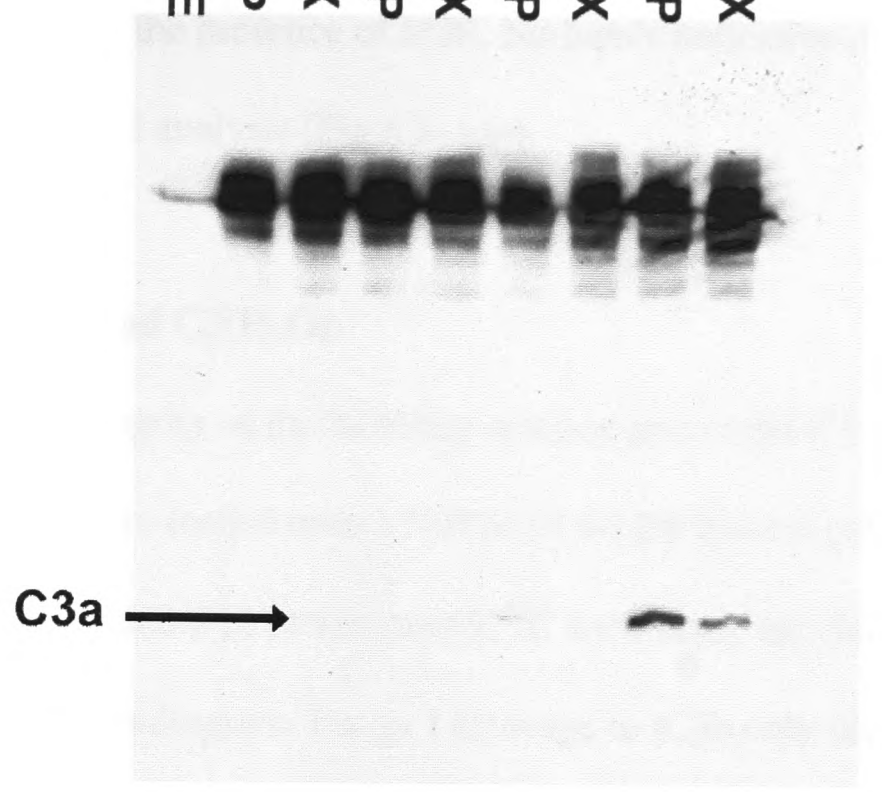


Fig 6.7 **Effect of SGE on factor B in AH_{50} assay over time in the presence (bottom) or absence (top) of CVF (native PAGE). Western blot probed with 1 in 300 dilution of factor B polyclonal serum. Time points as indicated (in minutes). X-extract containing, P-PBS containing.**

whether the anti-complement activity of SGE is dependent upon factor I mediated cleavage of C3 to iC3b, AH_{50} assays were set up as above in the presence or absence of an excess of CVF (5 μg- Sigma). 5 μl samples (150 μl total volume) were taken at various time periods and loaded onto a native PAGE as described in section 2.6. Complement activation in the presence or absence of SGE was measured by factor B cleavage; visualised as described in section 6.3.8 using native-PAGE western blotting. The times of sample collection in the presence of CVF were calculated empirically. The results of Fig 6.7 showed that in the absence of CVF, SGE-containing samples inhibited the turnover of factor B to Bb. In contrast, in the presence of CVF no such inhibition was observed. These results suggest that CVFBb but not C3bBb is able to form in the presence of SGE. Because CVFBb is insensitive to factor I cleavage, it is possible that anti-complement activity has factor I like activity converting fluid phase C3b to iC3b. Alternatively, because CVF is outcompeting the C3 in these assays, it is possible that the anti-complement activity of the SGE directly involves C3 or a derivative of C3.

6.3.10 **Western analysis of the effect of SGE on C3a formation**

A standard AH_{50} assay was set up as described above in the presence or absence of SGE (20 μg). Samples (7.5 μl- total volume 150 μl) were taken at various time intervals as indicated (Fig 6.8). Samples were loaded onto NuPAGE gels under both reducing and non-reducing conditions and western blotted as described (section 2.6). The membranes were probed with rabbit monospecific polyclonal anti-C3a serum at a 1 in 750 dilution (Calbiochem, cat no. 204859). The results of Fig 6.8 clearly showed that C3a cleavage was reduced in the SGE containing samples. This indicates that C3 cleavage by



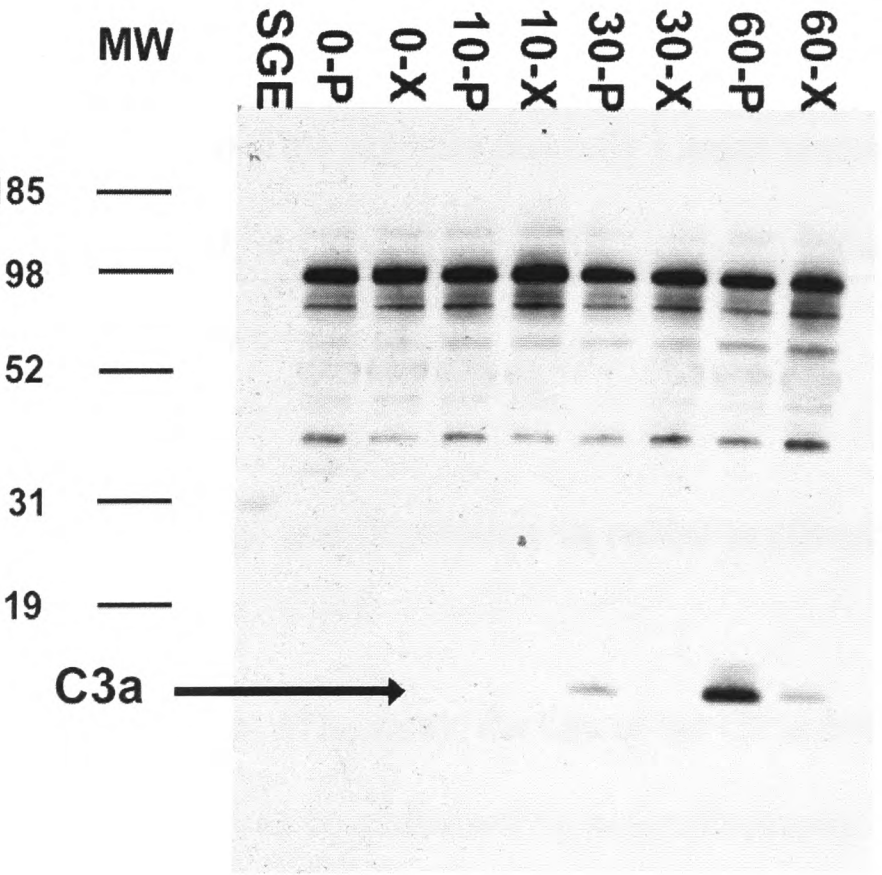


Fig 6.8 Effect of SGE on C3a formation in AH_{50} assays as measured by western analysis. Polyclonal monospecific anti-C3a serum was used at a 1 in 750 dilution. Incubation periods (in min) as indicated, P- PBS (control), X- extract (SGE), SGE- SGE only. (Top) native PAGE, (bottom) denaturing PAGE.

the convertases is inhibited in the presence of SGE. No bands indicative of SGE binding were observed in native PAGE analysis (Fig 6.8- top).

6.3.11 I^{125}-labelling of C3 and C3(H_2O)

In order to test the integrity of the labelling reaction and of the C3 and C3(H_2O), approximately 50000 cpm were loaded onto a NuPAGE 4-12% bis-tris gel. Both the C3 and C3(H_2O) were treated with 0.5 µl of serum at 37^{O}C for 1 hour; the cleavage products were visualised using an autoradiogram. Factor I cleavage to iC3b only occurs for C3(H_2O) and fluid-phase C3b; C3 is not a substrate. Both the alpha chain (113 kDa) and the beta chain (75 kDa) of C3 and C3(H_2O) were labelled successfully (Fig 6.9). The results showed that only a small proportion of C3 compared with C3(H_2O) was cleaved by factor I into iC3b (63 and 41kDa). This suggests that only a small percentage of the C3 preparation was dead (i.e. hydrolysed to C3(H_2O)). The cleavage products of C3 by factor I and serum proteases are shown in Fig 1.6.

6.3.12 Effect of SGE on C3 cleavage and deposition on rabbit erythrocytes in an AH_{50} assay

By using I^{125}-labelled C3 in an AH_{50} assay, the fate of the C3 in both the fluid and solid (i.e. erythrocyte bound) phases can be visualised by autoradiography. Samples were prepared as described in section 6.2.11.Before loading onto the gel, the solid phase samples were counted for activity. Activity was counted over 10 sec using a I^{125} counter (Table 6.8). The relatively low counts obtained in sample 80-P were probably due to insufficient pelleting of the membranes in this sample.

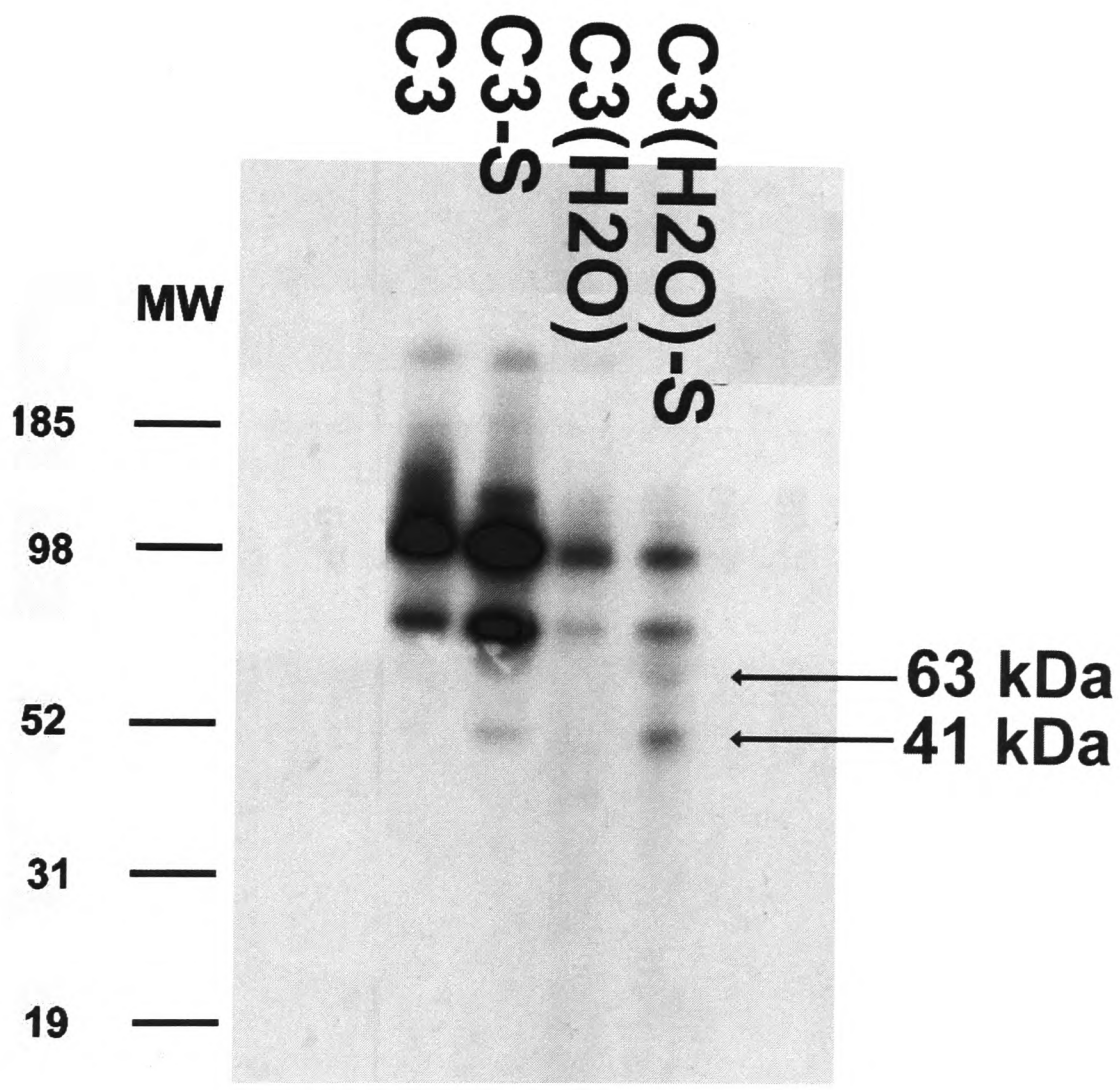


Fig 6.9 Autoradiogram of I^{125} labeled C3 and C3(H_2O). C3-S and C3(H_2O)-S samples were treated with 0.25 μl of human serum for 1 hour at 37^{O}C.

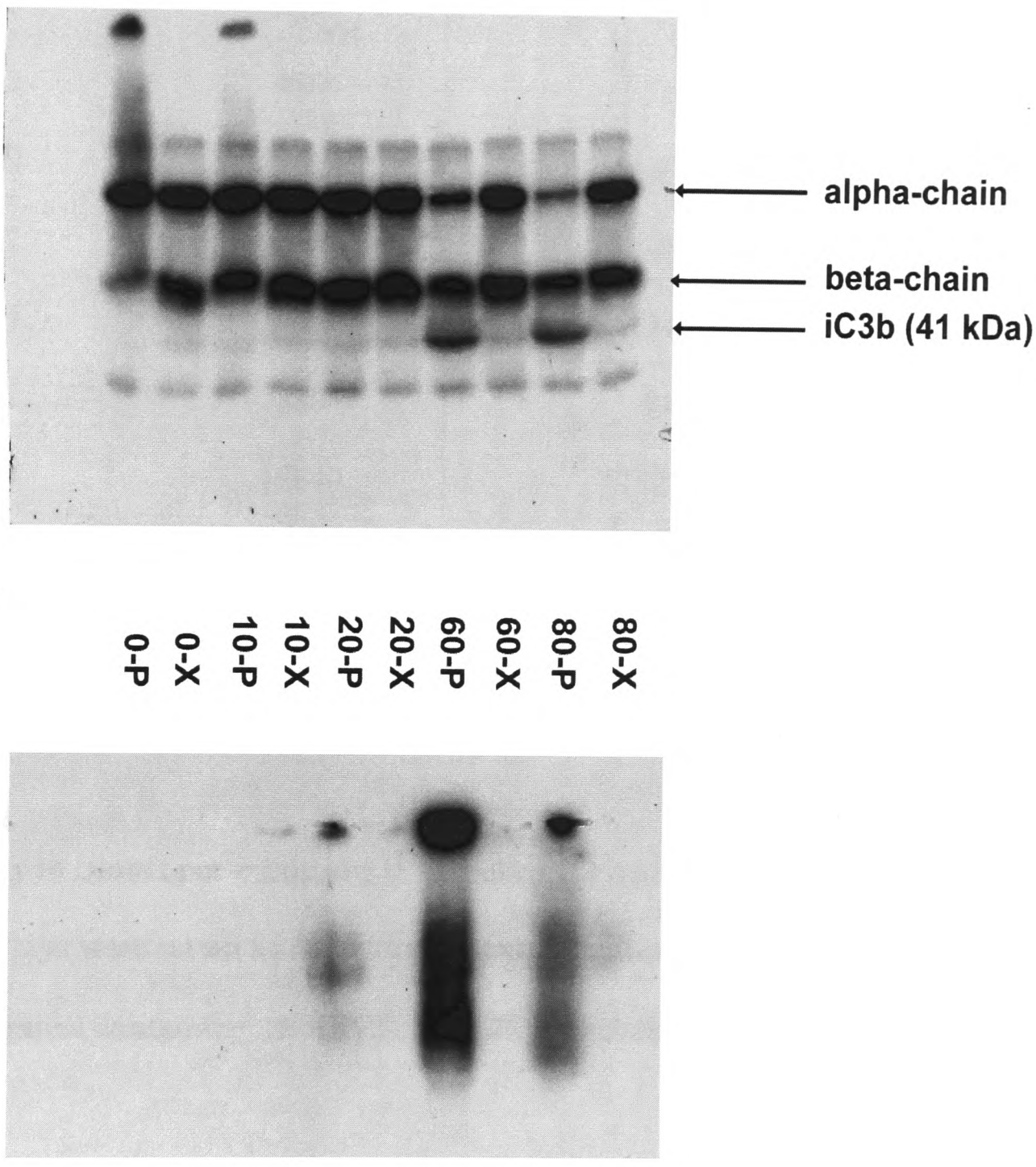


Fig 6.10 Effect of SGE on fate of I^{125} labeled C3 in AH_{50} assay over time. Visualised by autoradiography. Time points as indicated, X-extract containing, P-PBS containing. (Top) supernatant (s/n) samples, (bottom) erythrocyte and ghost membrane samples.

Sample	mean background	mean count (10s)	cpm
0-P	48	1050	**6012**
0-X	52	997	**5670**
10-P	59	998	**5634**
10-X	47	694	**3882**
20-P	54	2248	**13164**
20-X	62	880	**4908**
60-P	44	18840	**112776**
60-X	58	1383	**7950**
80-P	56	8525	**49014**
80-X	44	1455	**8466**

Table 6.8 Activity in counts per minute of I^{125} labelled C3 bound to erythrocyte membranes. AH_{50} assays were set up as described in text. Samples taken at various times as shown; P, control containing 10 µl PBS, X, SGE containing (10 µl ≡ 40 µg).

PAGE autoradiograms demonstrated that in the fluid phase (i.e. supernatant) C3 in the control samples is cleaved to iC3b with increasing incubation time (Fig 6.10- top). In contrast, SGE containing samples showed no significant cleavage. The erythrocyte membrane-containing samples show that in the control samples, I^{125}-labelled C3 is being deposited on the cell membranes, but not in the SGE containing samples (Fig 6.10- bottom). These results suggest that in the fluid phase SGE is preventing C3 from being

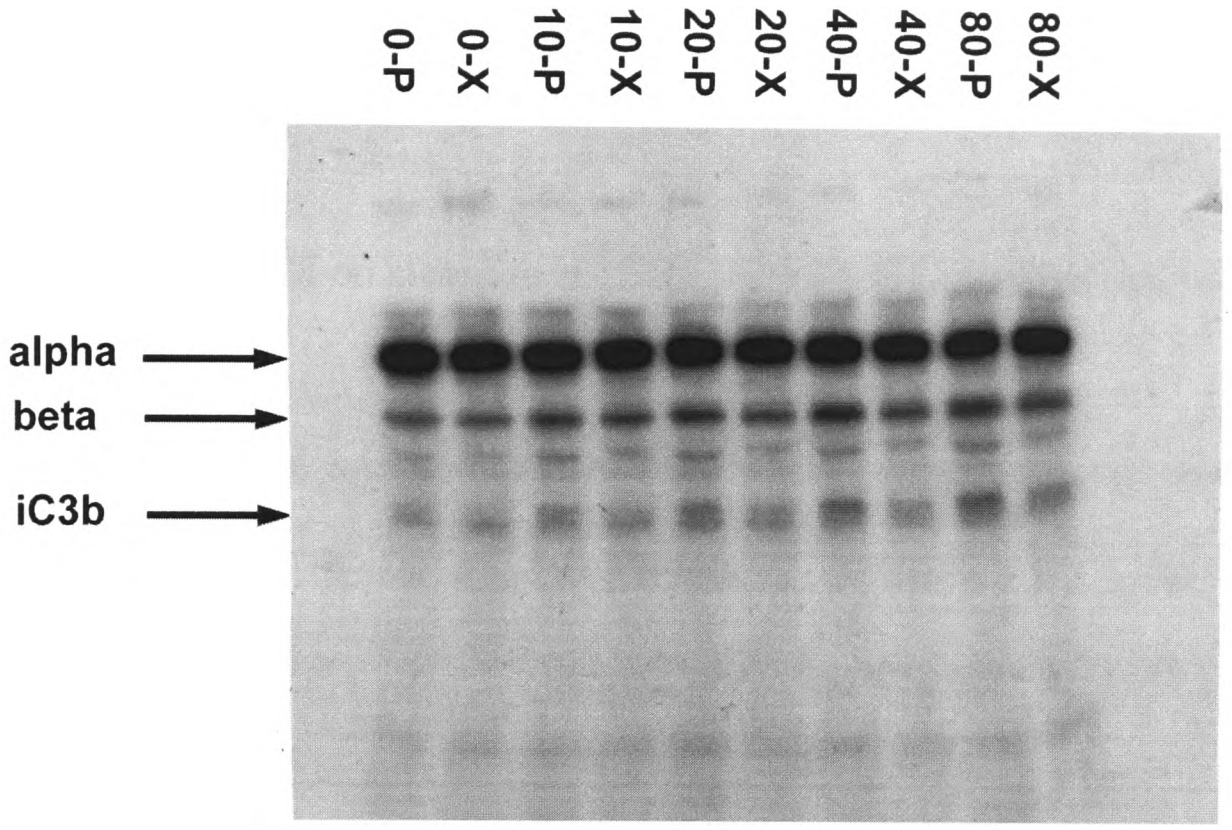


Fig 6.11 Effect of SGE upon cleavage of C3(H_2O) to iC3(H_2O) by serum proteases. Visualised by autoradiography. Time points as indicated, X-extract containing, P-PBS containing.

cleaved to C3b (which would be subsequently degraded to iC3b). In view of the fact that C3b is not being deposited upon erythrocyte membranes in the presence of SGE, it is likely that SGE is inhibiting the formation of C3b and subsequently iC3b.

6.3.13 **Effect of SGE on cleavage of C3(H_2O) to iC3(H_2O) visualised by autoradiography**

To investigate whether SGE anti-complement activity effects the turnover of C3(H_2O) to iC3(H_2O) by factor I and cofactors, I^{125} labelled C3(H_2O) was incubated with serum at $37^{O}C$ in the presence or absence of SGE. Samples (about 50000 cpm) were incubated for various periods of time as indicated in Fig 6.11. These results showed a reduced level of iC3(H_2O) formation in the presence of SGE. This suggests that SGE directly effects C3(H_2O) possibly through binding/modification in such a way that it is no longer a substrate for factor I cleavage.

6.3.14 **Effect of SGE on alternative pathway C3-convertase: kinetics of factor B cleavage as visualised by SDS-PAGE Coomassie-blue staining**

It is possible that the SGE acts by destabilising the C3 convertase (i.e. C3bBb) by not allowing the convertase to form or through destabilisation of the complex, once formed. The rate of formation of the convertase can be measured by observing the conversion of factor B (92 kDa) to Bb (62 kDa) and Ba (30 kDa) via factor D cleavage. Factor D, in contrast to other serine proteases, is highly specific for its substrate (i.e. C3bB) (Kim *et al.*, 1995). As a consequence, factor B can only be cleaved when associated with C3b. By using small quantities of factor D and C3b (i.e. not

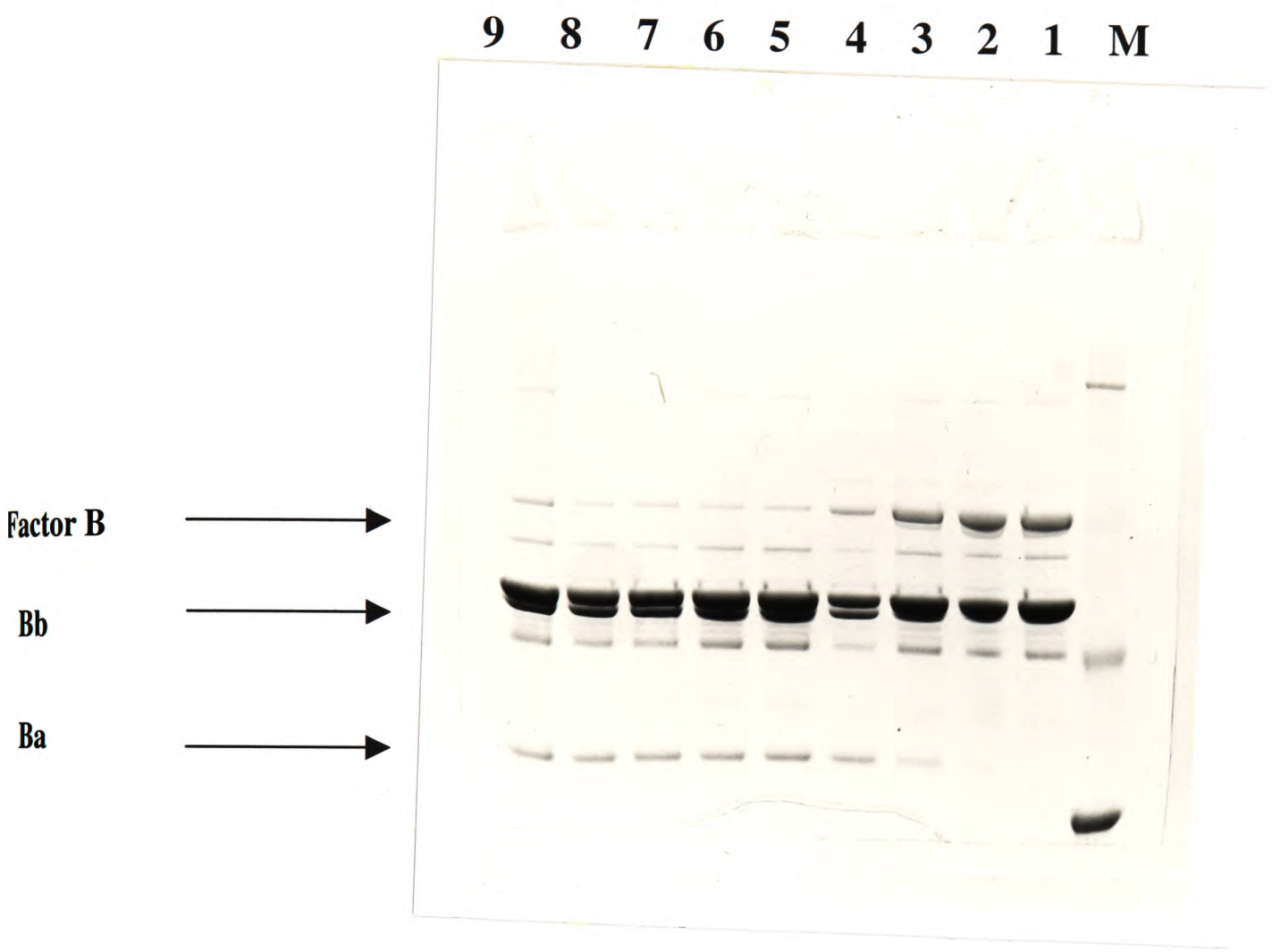


Fig 6.12 Kinetics of convertase formation as measured by factor B turnover and visualised though Coomassie blue staining. Incubations were carried out for various periods as indicated. (M), molecular weight marker, (1) 0 min, (2) 1 min, (3) 10 min, (4) 30 min, (5) 60 min, (6) 120 min, (7) 180 min, (8) 240 min, (9) overnight. The band above Bb (present in all samples) is albumin present in the factor D preparation.

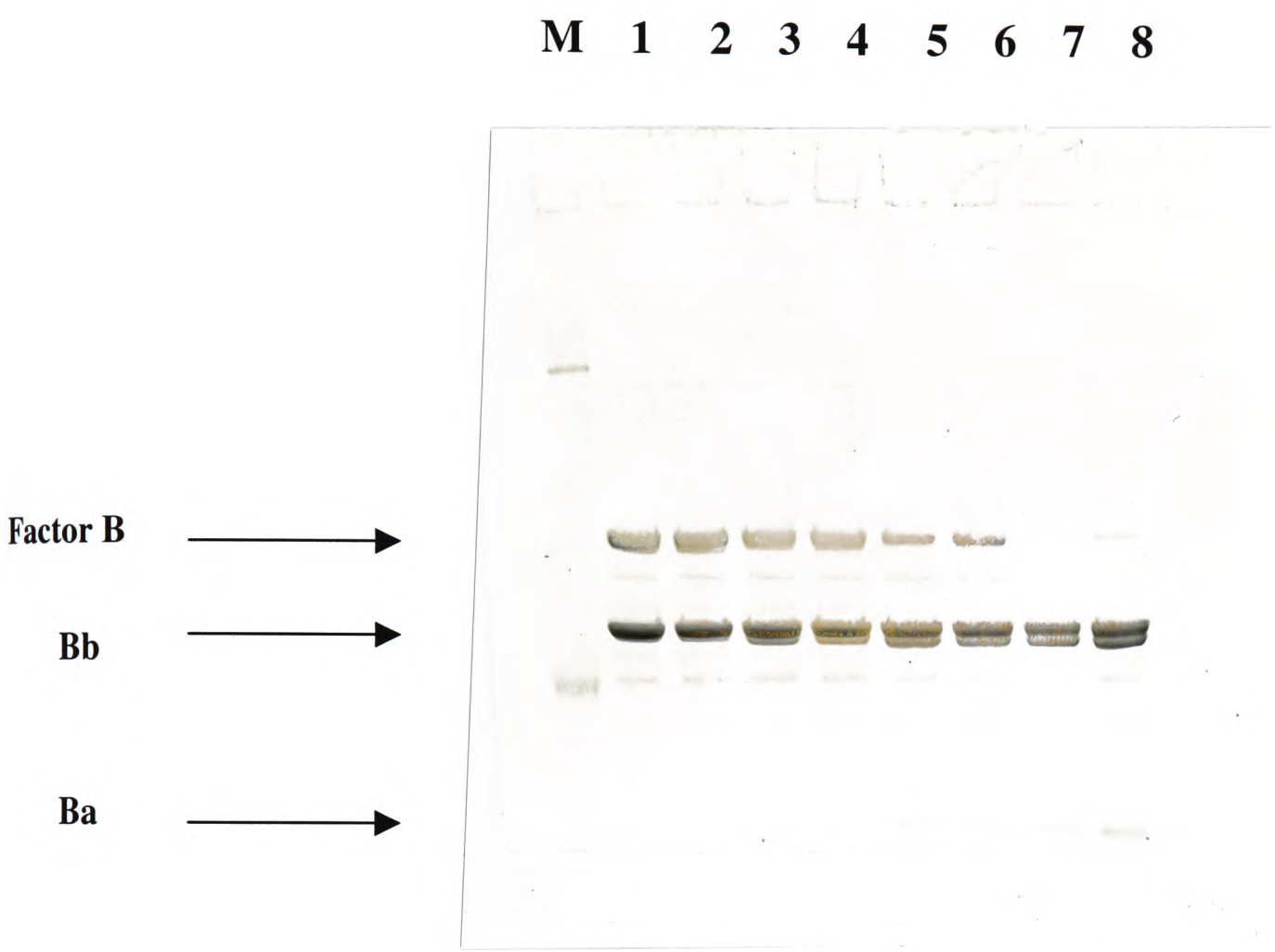
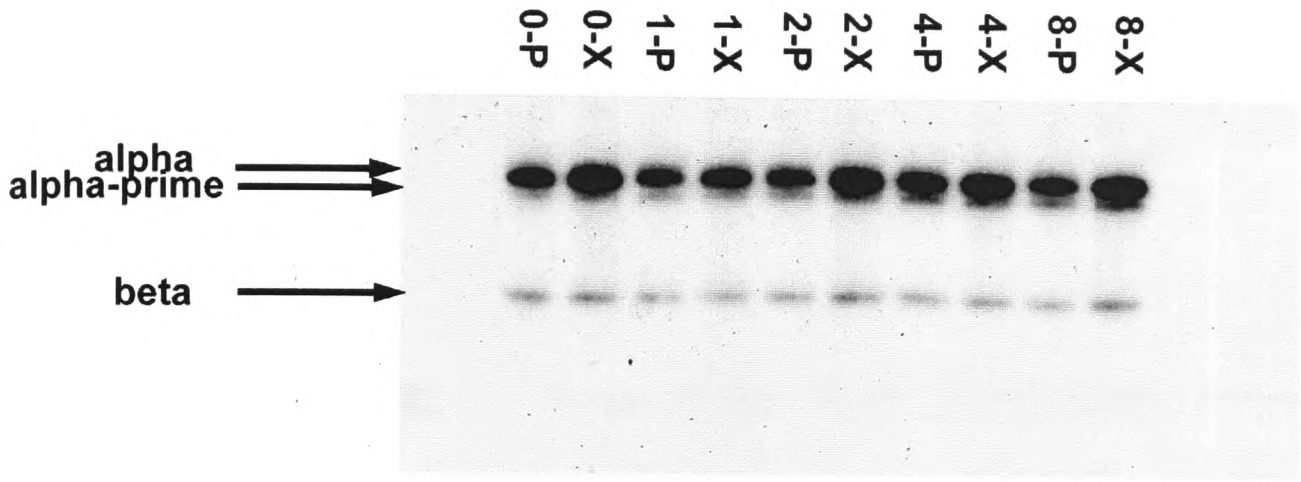


Fig 6.13 **Kinetics of convertase formation as measured by factor B turnover and visualised though Coomassie blue staining in the presence and absence of SGE. Incubations were carried out for various periods as indicated. (M), molecular weight marker, (1) 0 min (PBS), (2) 0 min (SGE), (3) 10 min (PBS), (4) 10 min (SGE), (5) 20 min (PBS), (6) 20 min (SGE), (7) 40 min (PBS), (8) 40 min (SGE). The band above Bb (present in all samples) is albumin present in the factor D preparation. 400 ng of SGE protein was used.**

enough to be observed on a Coomassie stained gel) and an excess of factor B, convertase formation and decay (i.e. formation and subsequent dissociation of C3bB complexes) can be measured by observing the turnover of factor B in the presence and absence of SGE via Coomassie staining. In order to investigate both the kinetics and stability of convertase in the system used, it was necessary to calculate the time period when the convertase was actually active in the control samples (i.e. at equilibrium). The results of Fig 6.12 demonstrate that factor B was cleaved between 10 and 30 minutes of incubation time under the experimental conditions used. No significant differences in the kinetics of convertase formation are seen in the presence of SGE (Fig 6.13). This did not change when more SGE (12 μg) was used in this experiment (data not shown). This suggests that SGE does not affect the formation/decay of the convertase. These results are in contrast to those obtained in section 6.3.8 when SGE was observed to inhibit the cleavage of factor B. Because C3 is provided as C3b in this assay (c.f. serum as source of C3 in section 6.3.8), this suggests that the anti-complement activity of the SGE is preventing the cleavage of C3 to C3b.

6.3.15 **Effect of SGE on convertase stability visualised by turnover of I^{125} labelled C3**

Under the above experimental conditions, convertase formation was shown to occur after 20 minutes of incubation time. By stopping further convertase formation by the addition of EDTA (Mg^{2+} required for convertase formation) at this time point, convertase activity can be measured over time by the conversion of I^{125} labelled C3 to C3b (i.e. α to α' cleavage). By adding the SGE (20 μg) to the reaction mixture before



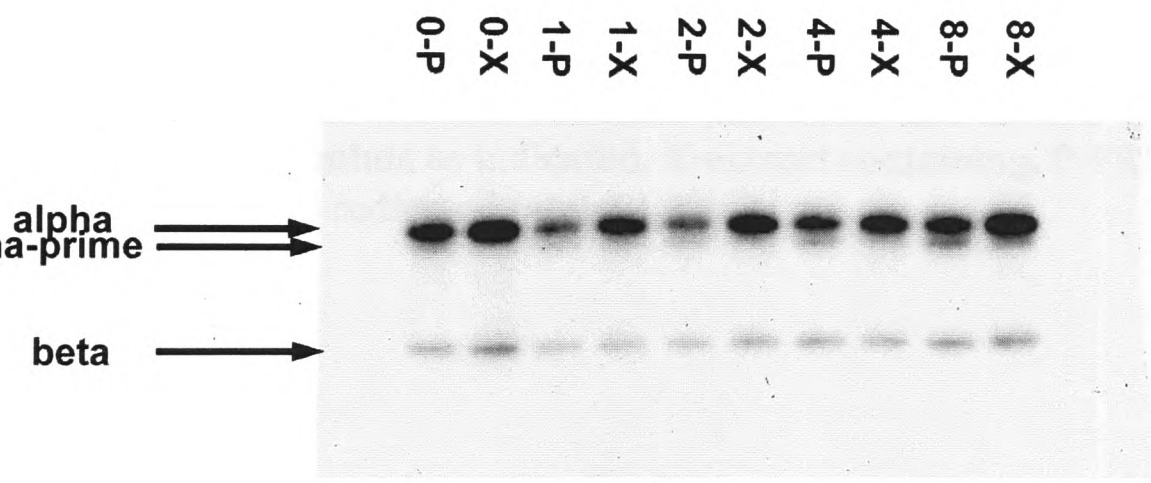


Fig 6.14 Effect of SGE on convertase integrity, visualised through I^{125} labeled C3 cleavage. Visualised by autoradiography. Time points as indicated, X-extract containing, P-PBS containing. (Top) SGE addition prior to EDTA, (bottom) SGE addition concurrent with EDTA addition.

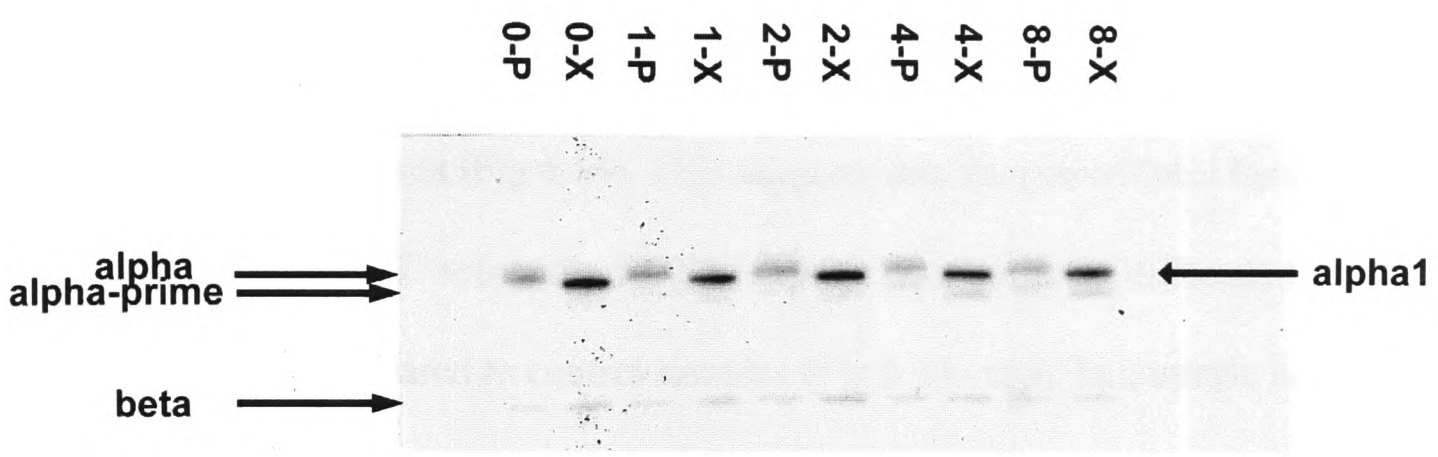


Fig 6.15 Effect of SGE on convertase integrity, visualised through I^{125} labeled C3 cleavage. The SGE was added at the same time as C3 and EDTA. Visualised by autoradiography. Time points as indicated, X-extract containing, P-PBS containing. SGE cleaved α1-chain as indicated (alpha 1).

adding the EDTA, the effect of SGE on convertase formation can be visualised using autoradiography. By adding SGE at the same time as the EDTA and C3, the effect of SGE on the stability or activity of the formed convertase can be visualised through autoradiography. Cleavage of C3 to C3b (i.e. α to α') was observed under the experimental conditions used (Fig 6.14). This suggests that the convertases formed were active. Addition of the SGE before the EDTA, showed no significant differences in α-band cleavage when compared to control samples (Fig 6.14– top). In contrast, addition of SGE at the same time as the EDTA, caused reduced cleavage of α to α' when compared to the control samples (Fig 6.14- middle).

The experiment was repeated (i.e. SGE added with EDTA and C3), except that the gel was run for a longer period of time (2.5 hours c.f. 1.5 hours) in order to improve resolution of the α and α' bands (Fig 6.15). Unfortunately, it is apparent that more I^{125} labelled C3 was present in the SGE containing samples than in the control samples. Although the absolute quantities of α' formed does not significantly differ between the SGE and the control samples, it is a clear that a greater proportion of the total α-chain in the control samples was cleaved compared with the SGE-containing samples. Fig 6.15 also demonstrated that the α-band of C3 in the SGE containing samples differed in mobility (hereby designated α1) from that of the control samples, such a shift is indicative of a cleavage event (the β-chain size does not differ from the control samples). The fact that the C3, SGE and EDTA were pre-mixed before adding to the convertase reactions, and that the shift in mobility is seen at time 0, suggests that the SGE is directly cleaving the α-band of C3. The appearance of α'-type bands in the SGE containing samples suggests that the α1 band is still a substrate for the convertase, albeit to a lesser

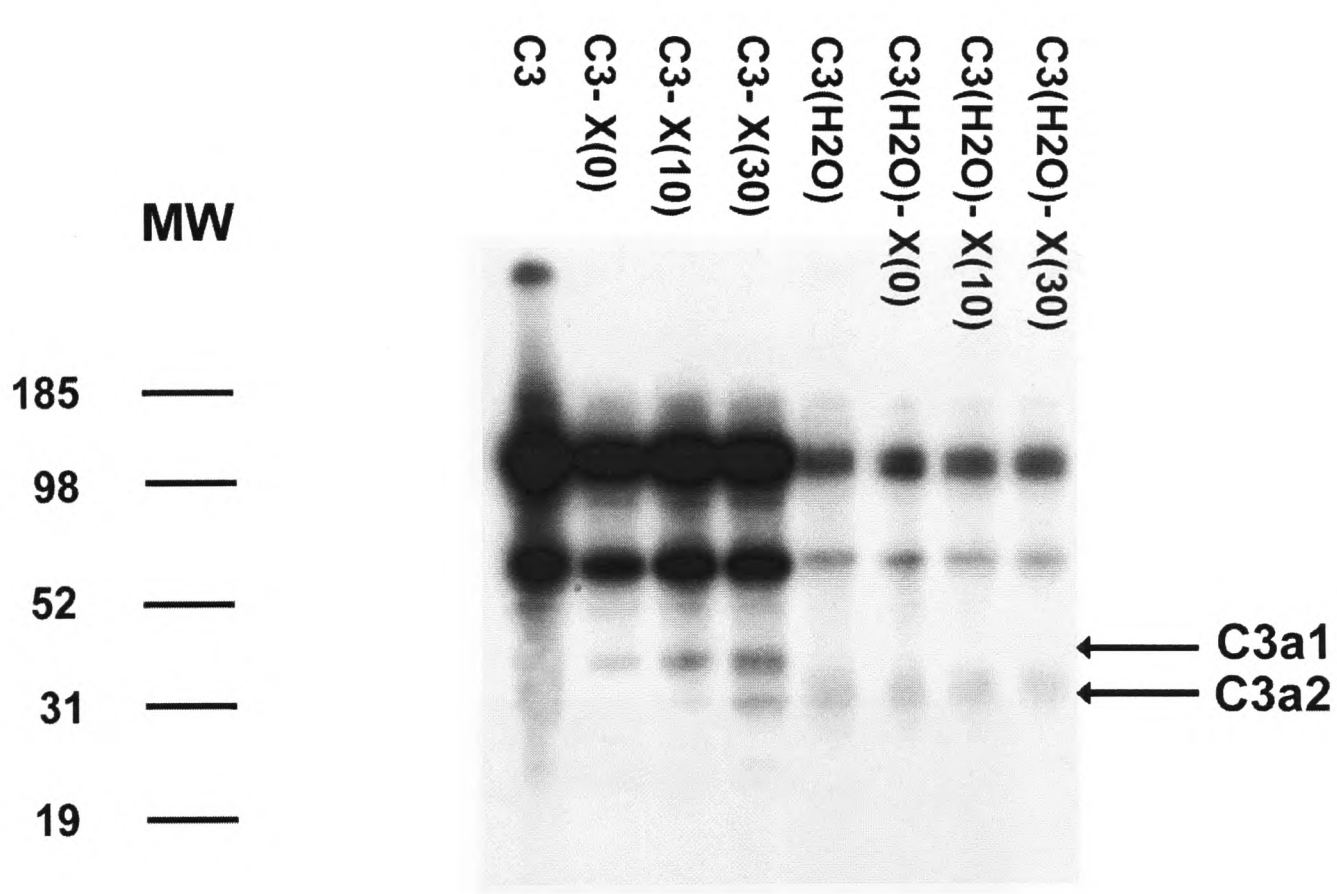


Fig 6.16 Effect of SGE on labelled I^{125} C3 and C3(H_2O) as visualised by autoradiography. Various incubation times (in min) as indicated, (-X) SGE extract containing. 20 μg of SGE protein was used.

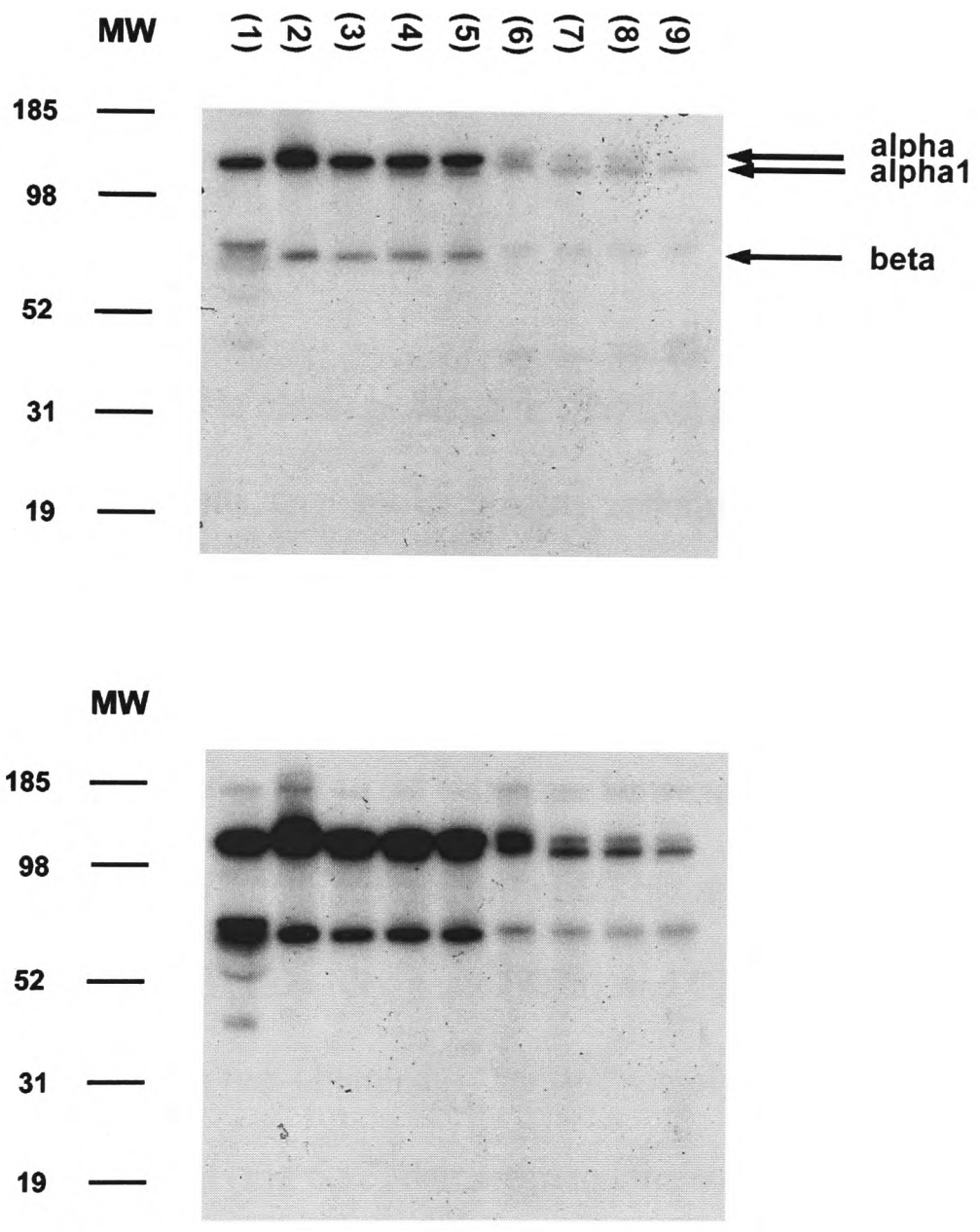


Fig 6.17 Effect of SGE on I^{125} labeled C3 and C3(H_2O). Visualised by autoradiography. (1), C3 + serum, (2) C3 only, (3) C3 + SGE (time 0), (4) C3 + SGE (time 30), (5) C3 + SGE (time 60), (6) C3 (H_2O) only, (7) C3(H_2O) + SGE (time 0), (8) C3(H_2O) + SGE (time 30),(9) C3(H_2O) + SGE (time 60). (Top) short exposure, (bottom) long exposure.

extent than the 'native' C3 α-chain. This also suggests that the N-terminal of the α1-chain is still intact, as the convertase product appears have an identical change in mobility to the change observed from α to α'-chain in the control samples (i.e. C3a is produced by the convertase).

6.3.16 Effect of SGE on I^{125} labelled C3 and C3(H_2O) as visualised by reducing PAGE autoradiograms

To investigate possible cleavage of C3 or C3(H_2O) by SGE in the absence of other complement components, changes in mobility under reducing (i.e. denaturing) conditions were visualised by autoradiography (Fig 6.16). Assays were set up as described in section 6.2.10. Unfortunately the resolution in this experiment was insufficient to say anything about α to α1 cleavage. However, the results of Fig 6.16 suggest that the α-chain (presumably α1-chain) of C3 but not C3(H_2O) is further cleaved into fragments of about 36, 31 kDa (designated C3a1 and C3a2 respectively). These fragments differ in size from that obtained by serum proteolysis (Fig 6.17 and Fig 6.9). Repetition of this experiment (Fig 6.17) demonstrated the α-band of both C3 and C3(H_2O) were cleaved by the SGE. Furthermore, it is clear that cleavage increases with increasing incubation time in the C3 samples. The cleavage products C3a1 and C3a2 are not seen in this or subsequent I^{125}-labelled experiments, the results of Fig 6.16 were obtained after a prolonged exposure time. It is suggested that these fragments are not sufficiently labelled to be observed in subsequent radiographic data. These data suggest that the SGE cleaves the α-chain of both C3 and C3(H_2O) to generate a C3b like form,

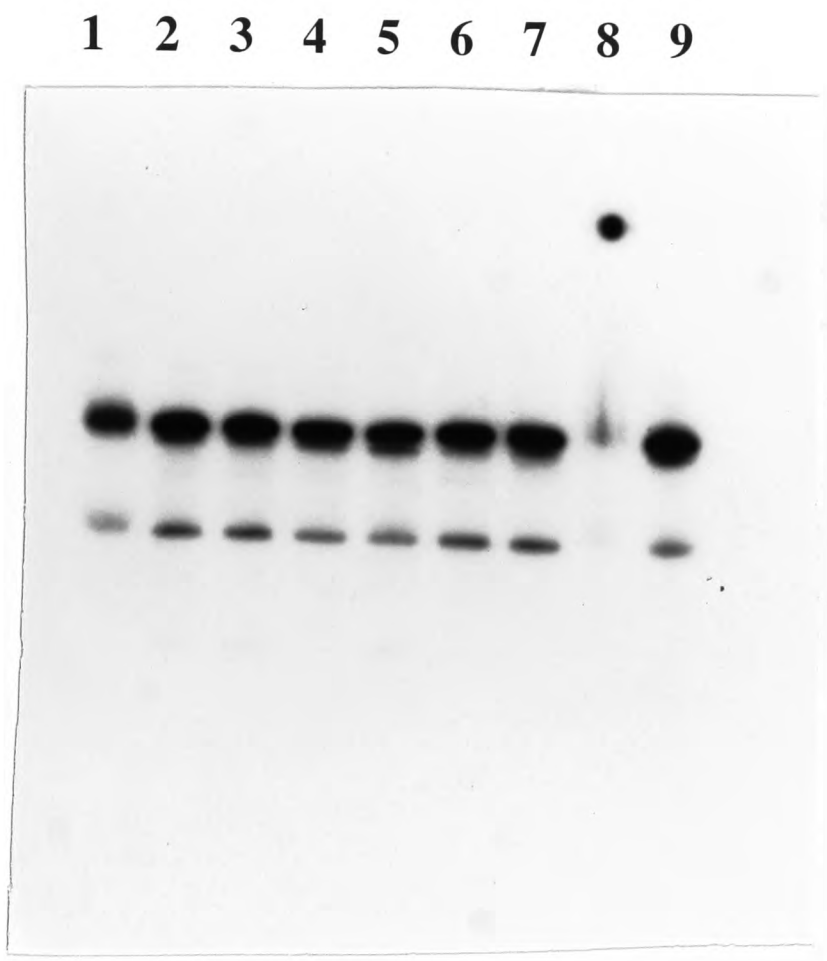


Fig 6.18 Autoradiogram of I^{125} labeled C3 in the presence or absence of varying quantities of SGE. (1), C3 only, (2) C3 + SGE (20 μg) (0 min), (3) C3 + SGE (4 μg), (4) C3 + SGE (20 μg), (5) C3 + SGE (40 μg), (6) boiled for 10 minutes, (7) treated with DTT, (8) treated with β-mercaptoethanol, (9) overnight digestion. Unless otherwise indicated incubations were carried out for 30 minutes at $37^{O}C$, and 20 μg of SGE was used.

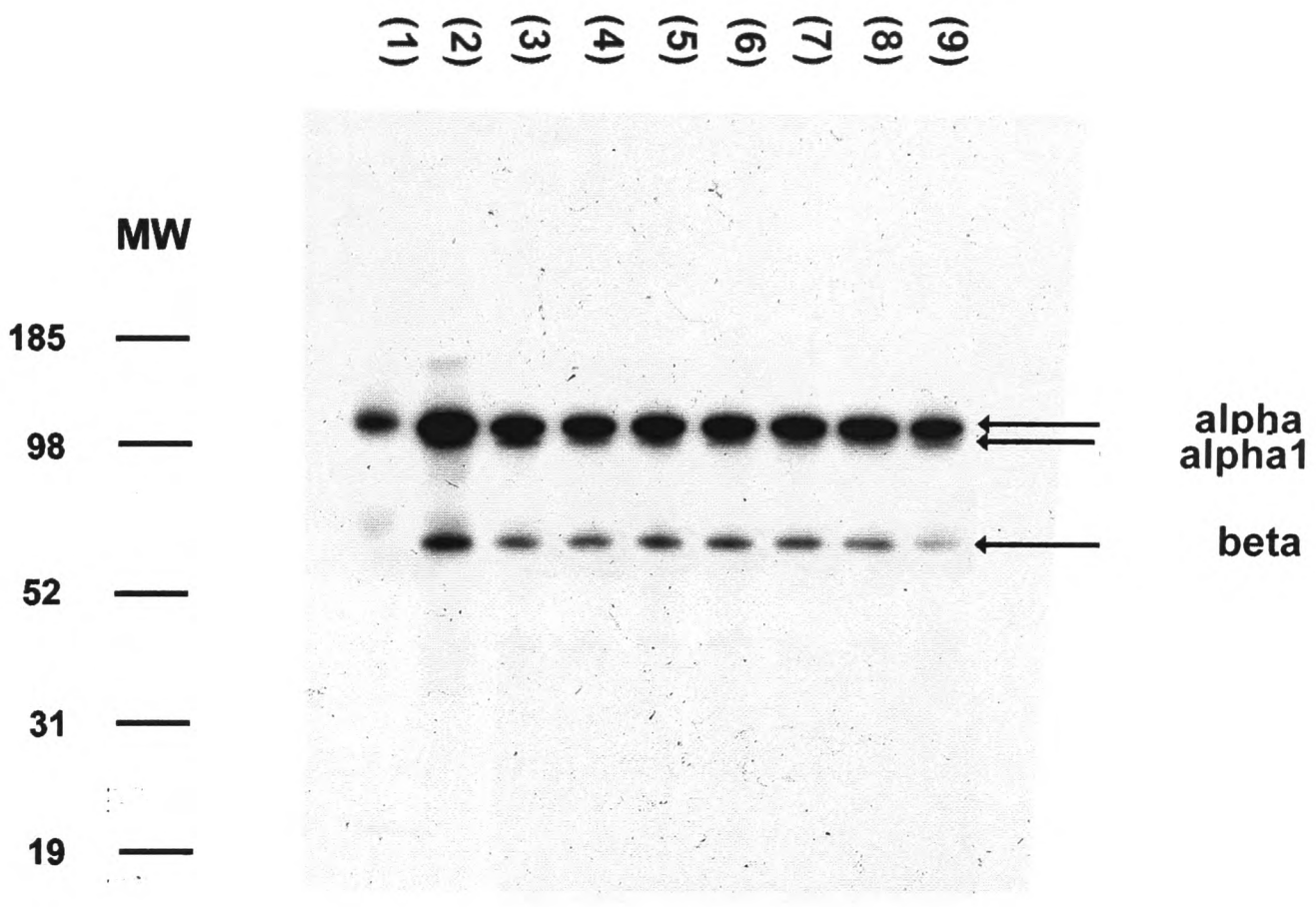


Fig 6.19 The effect of protease inhibitors on SGE-mediated cleavage as visualised by autoradiography. (1), C3 only, (2) C3 + SGE (0 minutes), (3) C3 + SGE (60 minutes), (4) soya bean trypsin inhibitor, (5) E-64, (6) pepstatin A, (7) inhibitor cocktail, (8) 10 mM EDTA, (9) Mg^{2+} (10 mM). 20 μg of SGE was used for each sample. Samples were in PBS (i.e. neutral conditions).

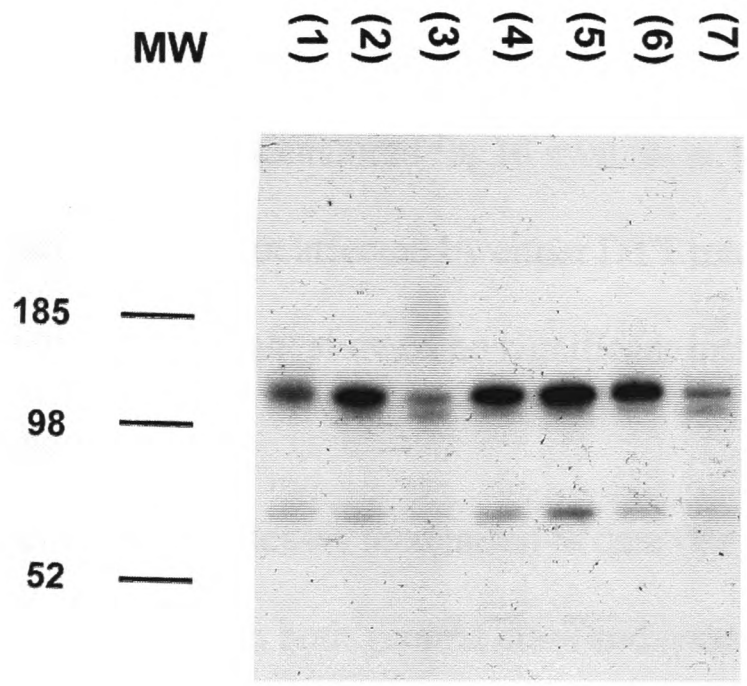


Fig 6.20 Effect of pH, ATP and various treatments on SGE-mediated I^{125} labeled C3 cleavage. Visualised by autoradiography. (1), C3 only, (2) C3 + SGE, (3) C3 + SGE (pH 4), (4) C3 + SGE (pH 9), (5) C3 + SGE (boiled), (6) C3 + SGE (proteinase K treated), (7) C3 + SGE (10mM ATP). 20 μg of SGE protein was used.

designated C3b' and C3(H_2O)' respectively, but that the α1-chain of C3b' is specifically cleaved into further fragments.

6.3.17 **Characterisation of SGE C3 protease activity**

Various volumes of SGE were tested to see if proteolysis was dose-dependent. Fig 6.18 showed that cleavage was dose dependent. To investigate the possibility that the activity is due to a thiol containing protease (i.e. activated upon reduction), the SGE was pre-incubated with 2 mM DTT or β-mercaptoethanol (2 mM) for 2 hours at 37^{O}C before incubating with C3. SGE was also denatured for 10 minutes at 100^{O}C before incubating with C3. Proteolytic activity was not affected by either DTT treatment or by boiling. Incubation of SGE with C3 overnight showed no significant increase in the proportion of C3 cleaved by the SGE-protease.

There are six classes of proteases recognised to date (Beyon and Bond, 1989). These are assigned mechanistically; both general class and specific inhibitors have been identified for most of the proteases described so far. By testing these inhibitors with the SGE C3 protease activity, a mechanistic class can be assigned to the protease. Several of these class-specific inhibitors as well as an inhibitor cocktail were tested on the proteolytic activity. The results of Fig 6.19 showed that cleavage activity was not significantly affected by any of the inhibitors tested under neutral conditions.

The effect of pH and of ATP on the protease activity was investigated, the results are shown in Fig 6.20. Treatment with proteinase K, boiling (at neutral pH) or pH 9 treatment (10 mM Na_2HPO_4) did not significantly affect the protease activity. However,

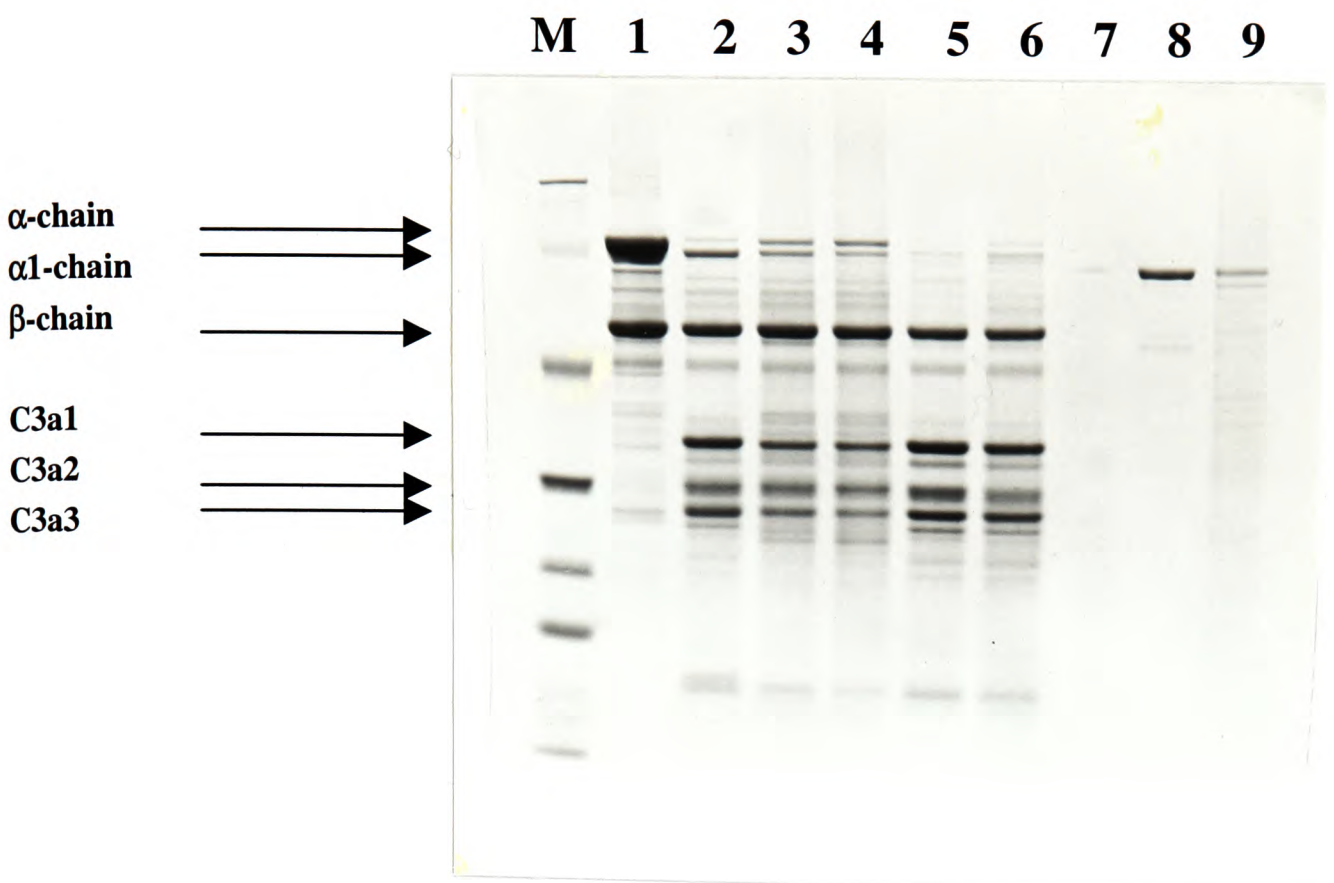


Fig 6.21 **Effect of extended incubation time on SGE-mediated C3 proteolytic activity. (M), molecular weight marker, (1) C3 control, (2) C3+SGE (2 days), (3) C3+ SGE (3 days), (4) C3+SGE (4 days), (5) C3+SGE- E64, (6) C3+SGE- pepstatin A, (7)SGE only, (8) factor B control, (9) factor B+SGE (4 days). Visualised by Coommasie blue staining. 400 ng of SGE protein was used.**

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  1  MGPTSGPSLL LLLLTHLPLA LGSPMYSIIT PNILRLESEE TMVLEAHDAQ
 51  GDVPVTVTVH DFPGKKLVLS SEKTVLTPAT NHMGNVTFTI PANREFKSEK
101  GRNKFVTVQA TFGTQVVEKV VLVSLQSGYL FIQTDKTIYT PGSTVLYRIF
151  TVNHKLLPVG RTVMVNIENP EGIPVKQDSL SSQNQLGVLP LSWDIPELVN
201  MGQWKIRAYY ENSPQQVFST EFEVKEYVLP SFEVIVEPTE KFYYIYNEKG
251  LEVTITARFL YGKKVEGTAF VIFGIQDGEQ RISLPESLKR IPIEDGSGEV
301  VLSRKVLLDG VQNLRAEDLV GKSLYVSATV ILHSGSDMVQ AERSGIPIVT
351  SPYQIHFTKT PKYFKPGMPF DLMVFVTNPD GSPAYRVPVA VQGEDTVQSL
401  TQGDGVAKLS INTHPSQKPL SITVRTKKQE LSEAEQATRT MQALPYSTVG
451  NSNNYLHLSV LRTELRPGET LNVNFLLRMD RAHEAKIRYY TYLIMNKGRL
501  LKAGRQVREP GQDLVVLPLS ITTDFIPSFR LVAYYTLIGA SGQREVVADS
551  VWVDVKDSCV GSLVVKSGQS EDRQPVPGQQ MTLKIEGDHG ARVVLVAVDK
601  GVFVLNKKNK LTQSKIWDVV EKADIGCTPG SGKDYAGVFS DAGLTFTSSS
651  GQQTAQRAEL QCPQPAARRR RSVQLTEKRM DKVGKYPKEL RKCCEDGMRE
701  NPMRFSCQRR TRFISLGEAC KKVFLDCCNY ITELRRQHAR ASHLGLARSN
751  LDEDIIAEEN IVSRSEFPES WLWNVEDLKE PPKNGISTKL MNIFLKDSIT
801  TWEILAVSMS DKKGICVADP FEVTVMQDFF IDLRLPYSVV RNEQVEIRAV
851  LYNYRQNQEL KVRVELLHNP AFCSLATTKR RHQQTVTIPP KSSLSVPYVI
901  VPLKTGLQEV EVKAAVYHHF ISDGVRKSLK VVPEGIRMNK TVAVRTLDPE
951  RLGREGVQKE DIPPADLSDQ VPDTESETRI LLQGTPVAQM TEDAVDAERL
1001 KHLIVTPSGC GEQNMIGMTP TVIAVHYLDE TEQWEKFGLE KRQGALELIK
1051 KGYTQQLAFR QPSSAFAAFV KRAPSTWLTA YVVKVFSLAV NLIAIDSQVL
1101 CGAVKWLILE KQKPDGVFQE DAPVIHQEMI GGLRNNNEKD MALTAFVLIS
1151 LQEAKDICEE QVNSLPGSIT KAGDFLEANY MNLQRSYTVA IAGYALAQMG
1201 RLKGPLLNKF LTTAKDKNRW EDPGKQLYNV EATSYALLAL LQLKDFDFVP
1251 PVVRWLNEQR YYGGGYGSTQ ATFMVFQALA QYQKDAPDHQ ELNLDVSLQL
1301 PSRSSKITHR IHWESASLLR SEETKENEGF TVTAEGKGQG TLSVVTMYHA
1351 KAKDQLTCNK FDLKVTIKPA PETEKRPQDA KNTMILEICT RYRGDQDATM
1401 SILDISMMTG FAPDTDDLKQ LANGVDRYIS KYELDKAFSD RNTLIIYLDK
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1451  VSHSEDDCLA FKVHQYFNVE LIQPGAVKVY AYYNLEESCT RFYHPEKEDG

1501  KLNKLCRDEL CRCAEENCFI QKSDDKVTLE ERLDKACEPG VDYVYKTRLV

1551  KVQLSNDFDE YIMAIEQTIK SGSDEVQVGQ QRTFISPIKC REALKLEEKK

1601  HYLMWGLSSD FWGEKPNLSY IIGKDTWVEH WPEEDECQDE ENQKQCQDLG

1651  AFTESMVVFG CPN
```

Fig 6.22 Amino acid sequence of human C3 precursor (accession: A01257). 1-22, signal sequence; 23-667, beta chain; 672-1663, alpha chain. 672-748, C3a peptide (shown in red); 749-1663, C3b (α'-chain). 955-1001, C3g fragment (shown in grey); 1002-1303, C3d fragment (shown in blue); 1304-1320 C3f fragment (shown in violet). 749-790, sequence implicated in factor B binding (highlighted in yellow)(Lambris *et al.* 1996). Thioester bond (grey box). α and α1 chain (C3 and C3b' respectively) N-terminal sequence underlined and bold (667-689). C3a2 N-terminal sequences underlined and bold (994-1001 and 1021-1030). C3a3 N-terminal sequence underlined and bold (760-768).

reactions at pH 4 (10 mM acetate) and containing10 mM ATP significantly increased cleavage. This suggests that proteolytic activity is probably due to an acid protease.

Experiments were set up using un-labelled C3 (3 μg) and 0.5 μl (2 μg) of SGE at pH 4 and incubated overnight, or for 2, 3 or 4 days. Bands were visualised by Coomassie-blue staining. Results of Fig 6.21 show that consistent with the results of autoradiography, SGE cleaves C3 α-chain into α1 which is further cleaved into C3a1 and C3a2, an additional band of 26 kDa was also observed (designated C3a3). Based on these results an identical reaction was set up.

The α-chain, α1-chain, C3a2 and C3a3 were N-terminally sequenced by Tony Willis (MRC Immunochemistry Unit). The results in relation to the sequence of human C3 precursor are shown in Fig 6.22 (de Bruijn and Fey, 1985). The N-terminal sequence of the α and α1 chain are identical. In other words, the proteolytic activity of SGE occurs at the C-terminus of C3. From the change in mobility observed between the α and α1-chain, this fragment is probably about 7 kDa (compared with α to α1- Fig 6.23). A band of 7 kDa is observed in Fig 6.21, it is possible that this band corresponds to the C-terminal fragment produced by SGE-proteolysis (designated C3x). N-terminal sequencing should be carried out in order to confirm this. C-terminal cleavage is consistent with the results of Fig 6.15 (using pre-formed convertase), presumably the α1-chain of C3b' can undergo cleavage by C3bBb to produce the C3a fragment (i.e. C3a sequence is present in α1-chain).

N-terminal sequencing of the C3a2 fragment revealed two sequences either side of the thioester bond in the C3d region of C3 (Fig 6.22). These sequences are only 29 amino acids apart and it is possible that the downstream sequence represents degradation

of C3a2. Presuming this is the case, cleavage occurs at amino acid 993 and 994 between an aspartic acid and alanine residue respectively. It is also possible that the two sites represent separate cleavage events. The proposed model of the cleavage of the α-chain of C3 by *I. ricinus* SGE-protease(s) is shown in Fig 6.32.

The N-terminal sequence of the C3a3 fragment (PAGE molecular weight approximately 26 kDa) would be expected to produce a C-terminal fragment of 233 amino acids from the C3a3 cleavage site to the C3a2 cleavage site (i.e. 760-993 (Fig 6.22)). This suggests that a 88 amino acid product should be present, perhaps co-migrating with C3x. Cleavage occurs between an asparagine and an isoleucine residue in the 42 amino acid stretch proposed to contain the binding site of the C3b molecule for factor B, CR1, factor H and membrane cofactor protein (Lambris *et al.*, 1996). Perhaps such cleavage may serve to interfere directly with factor B binding. The fact that sequencing results demonstrated that the α1-chain was not cleaved at the N-terminal suggests that cleavage from C3b' to C3a2 and C3a3 may occur only after the α-chain has been cleaved to the α1-chain (Fig 6.32).

Both cleavage sites (i.e. C3a3 and C3a2) occur between a charged residue (i.e. Asn and Asp) and a hydrophobic residue (i.e. Ile and Ala respectively). This might suggest that a single SGE-associated protease is generating these cleavage products, it is equally possible that more than one protease is implicated.

In order to investigate the specificity of the cleavage of C3 by the proteolytic activity, an extended incubation time was used. The results of Fig 6.21 showed that even after 4 days of incubation the pattern of the digested fragments did not significantly

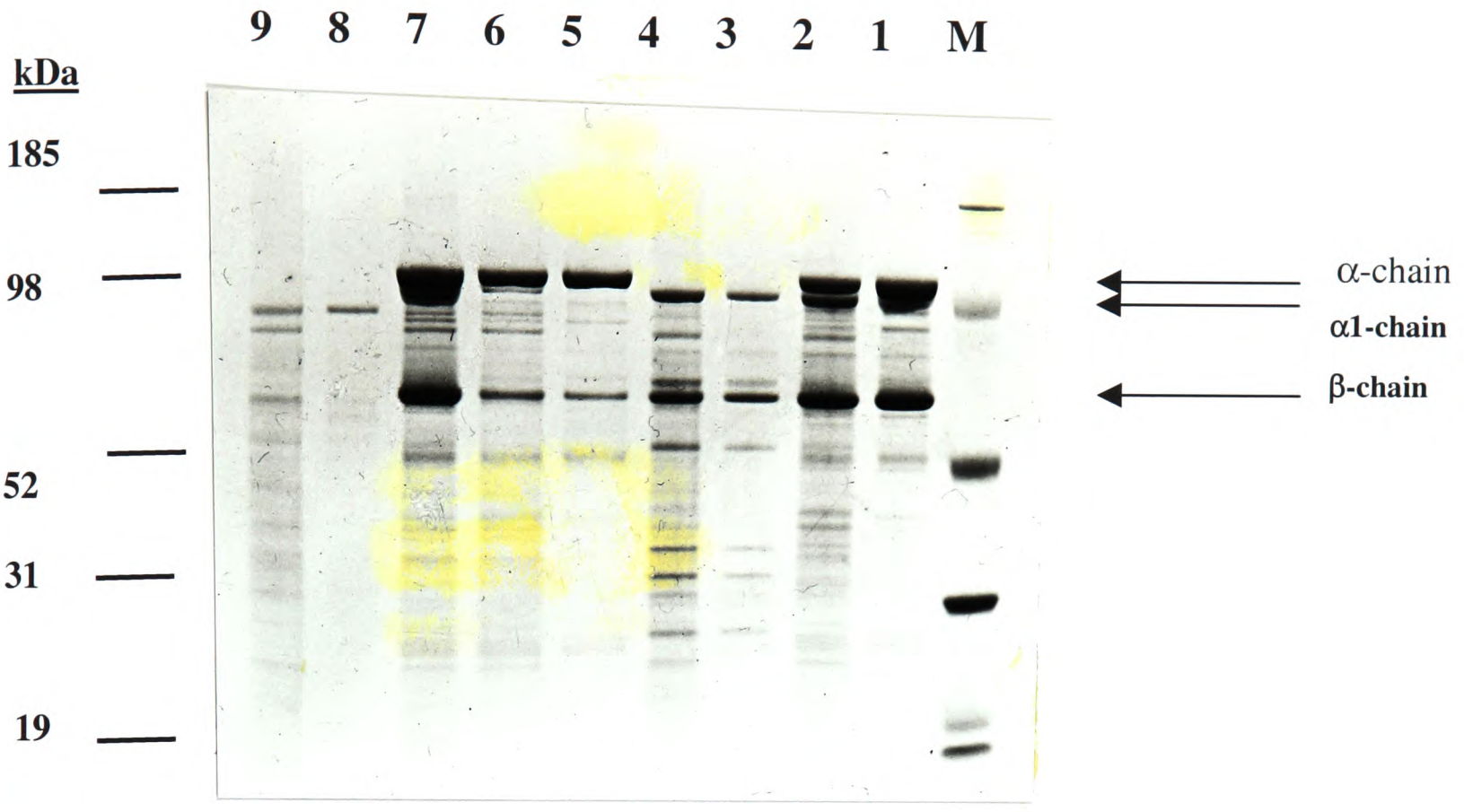


Fig 6.23 **Proteolytic activity of SGE on C3, C3(H_2O), C3b and factor B. (M), molecular weight marker, (1) C3 only, (2) C3+SGE, (3) C3b only, (4) C3b+SGE, (5) C3(H_2O) only, (6) C3(H_2O)+SGE, (7) C3+SGE+pepstatin A, (8) factor B only, (9) factor B+SGE. Visualised by Coomassie blue staining.**

change. Neither the β-chain of C3 or factor B was significantly cleaved over this period by the SGE, suggesting specificity for the α-chain of C3.

Specificity of cleavage was further investigated by setting up overnight incubations with C3, C3b or C3(H_2O). The results of Fig 6.23 show that proteolytic cleavage appears to be specific for the α-chain of C3, and that the α'-chain of C3b is not a substrate for cleavage by the SGE-protease. The α-chain of C3(H_2O) is cleaved but a much lesser degree to the α-chain of C3. This suggests that proteolytic cleavage of C3 by *I. ricinus* SGE is conformationally specific.

6.3.18 **Affinity chromatographic purification of I^{125} labelled SGE with factor D, factor B and C3 bound columns**

To investigate possible binding of factor D, factor B and C3 with *I. ricinus* SGE, the SGE was I^{125} labelled (section 6.2.11) and affinity purified by binding to a column containing bound factor D, factor B or C3. The fractions were eluted using 1 M NaCl. The results of Table 6.4 (section 6.2.12) showed that bound SGE material was eluted successfully from the factor D and C3 containing columns, as measured by the increase in radioactivity compared with GVB buffer wash through fractions. The pattern of radioactivity varied between these two columns. In contrast the factor B-containing column did not show elution of significant radioactivity above that obtained with the GVB buffer wash through fractions. Similar results were obtained from counts measured after elution with HCl-glycine (data not shown). This suggests that factor B is not a ligand for any factor in the I^{125} labelled SGE.

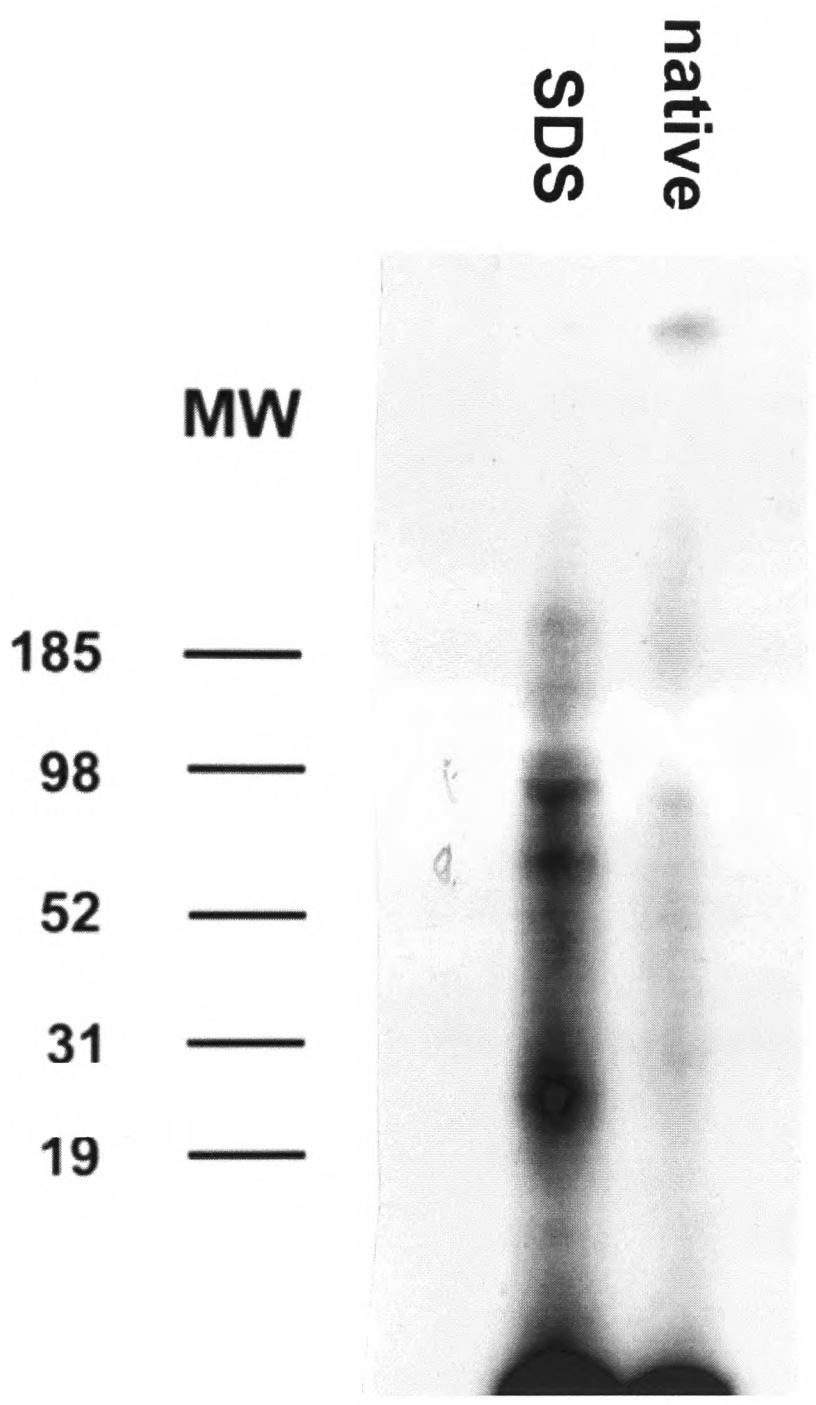


Fig 6.24 Autoradiogram of I^{125} labelled *I. ricinus* SGE, run under denaturing (SDS) and native conditions

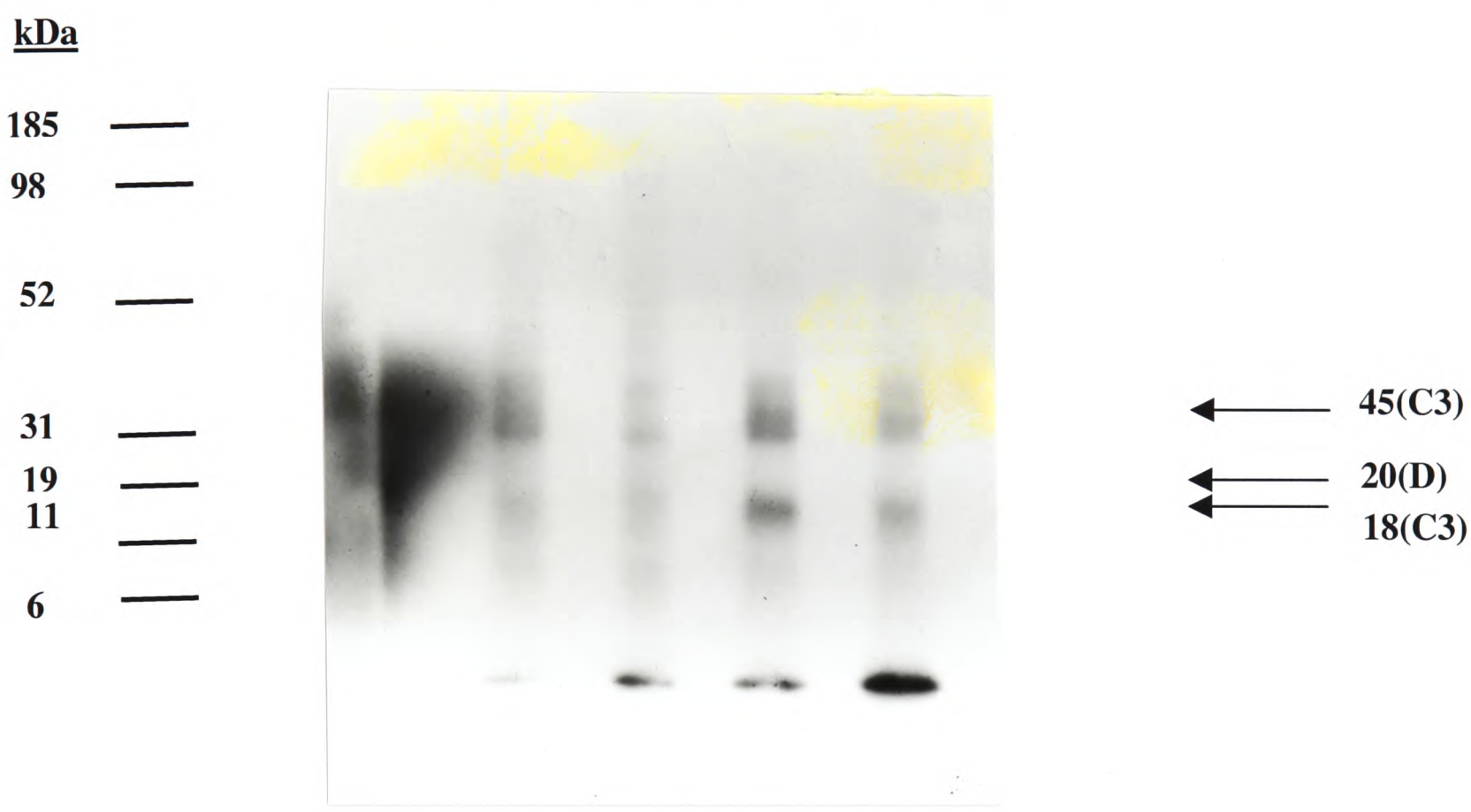


Fig 6.25 Autoradiogram of I^{125} labelled SGE affinity purified on columns containing bound C3 or factor D. Relevant column and eluted fraction number as indicated.

Based on these results, fractions C3-3, C3-4, D-1 and D-3 were used to prepare an autoradiogram. The pattern of these proteins differs significantly from the protein profile of the un-purified I^{125} labelled SGE (Fig 6.24). The results of Fig 6.25 showed several protein species common to both C3 and factor D column elutions. These bands probably represent non-specific interactions between the SGE and the matrix and/or IgG. Bands of 18 kDa and 45 kDa, and of 20 kDa are seen specifically for C3 and factor D bound columns respectively. Unfortunately, due to the high salt concentrations in the samples, resolution of the autoradiogram was poor. It is possible that these proteins represent specific interactions between *I. ricinus* SGE and the bound complement components. It must be noted however, that the complement reactive species of the SGE may not be radio-labelled.

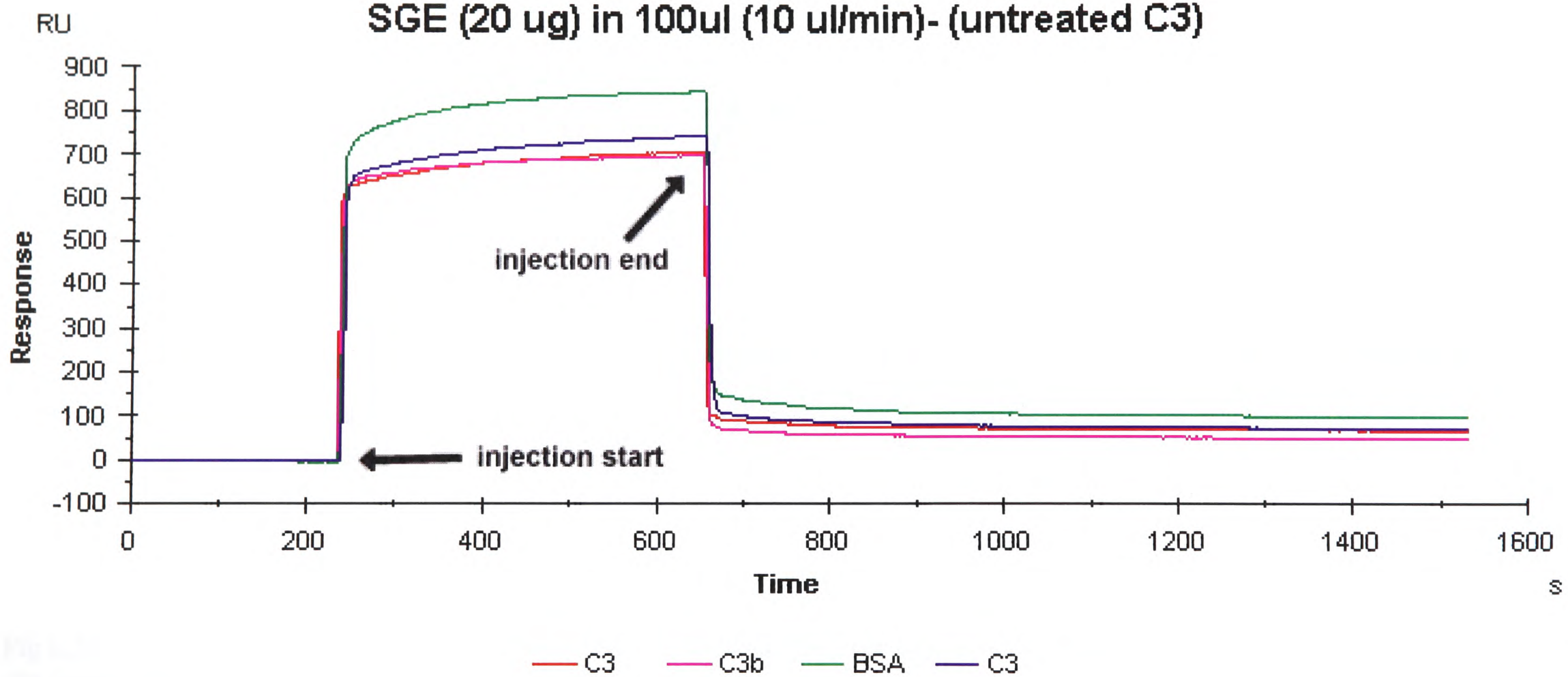


Fig 6.26 **Sensorgram of SGE (20 µg) in 100 µl (10 µl/min) injected over all four flow cells (untreated C3)**

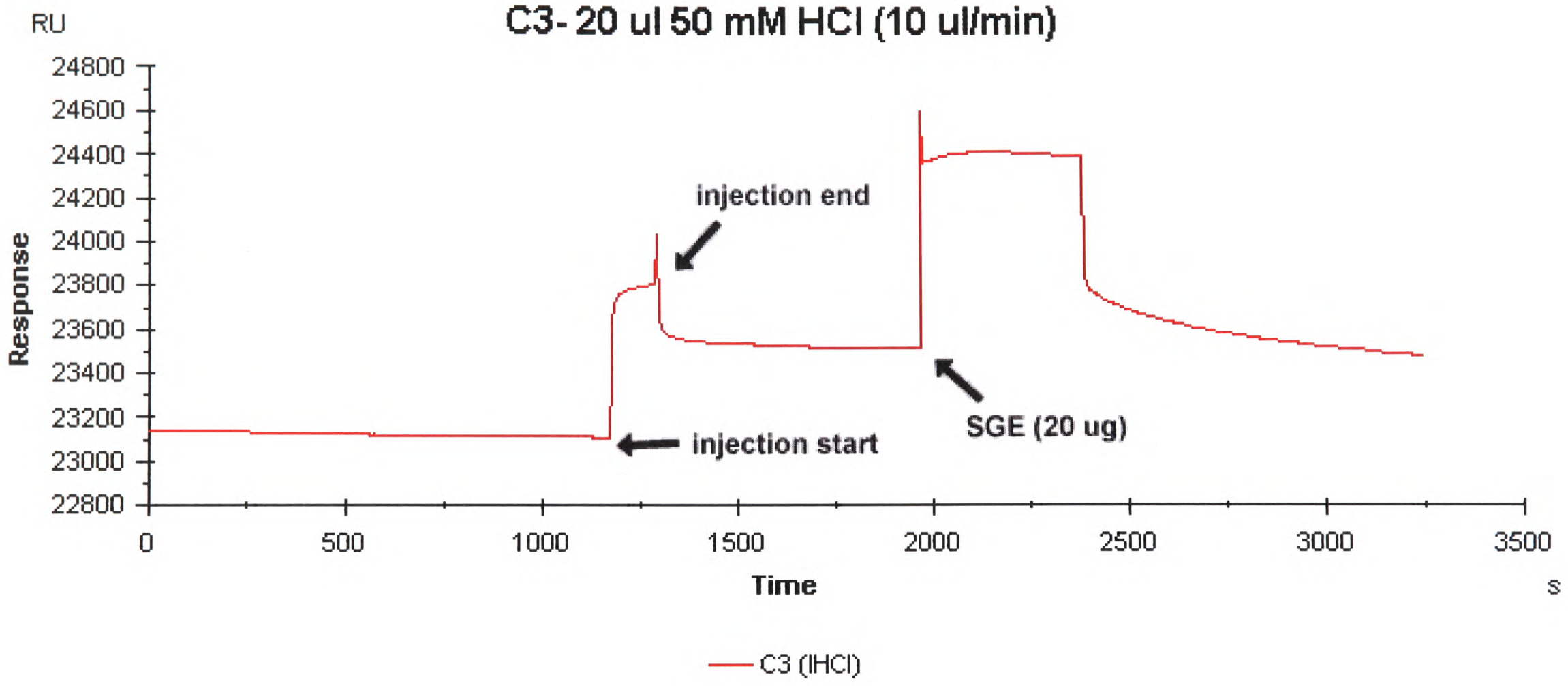


Fig 6.27 Sensorgram of 20 µl 50 mM HCl (10 µl/min) injected over untreated C3 flow cell followed by injection of SGE (20 µg) in 100 µl.

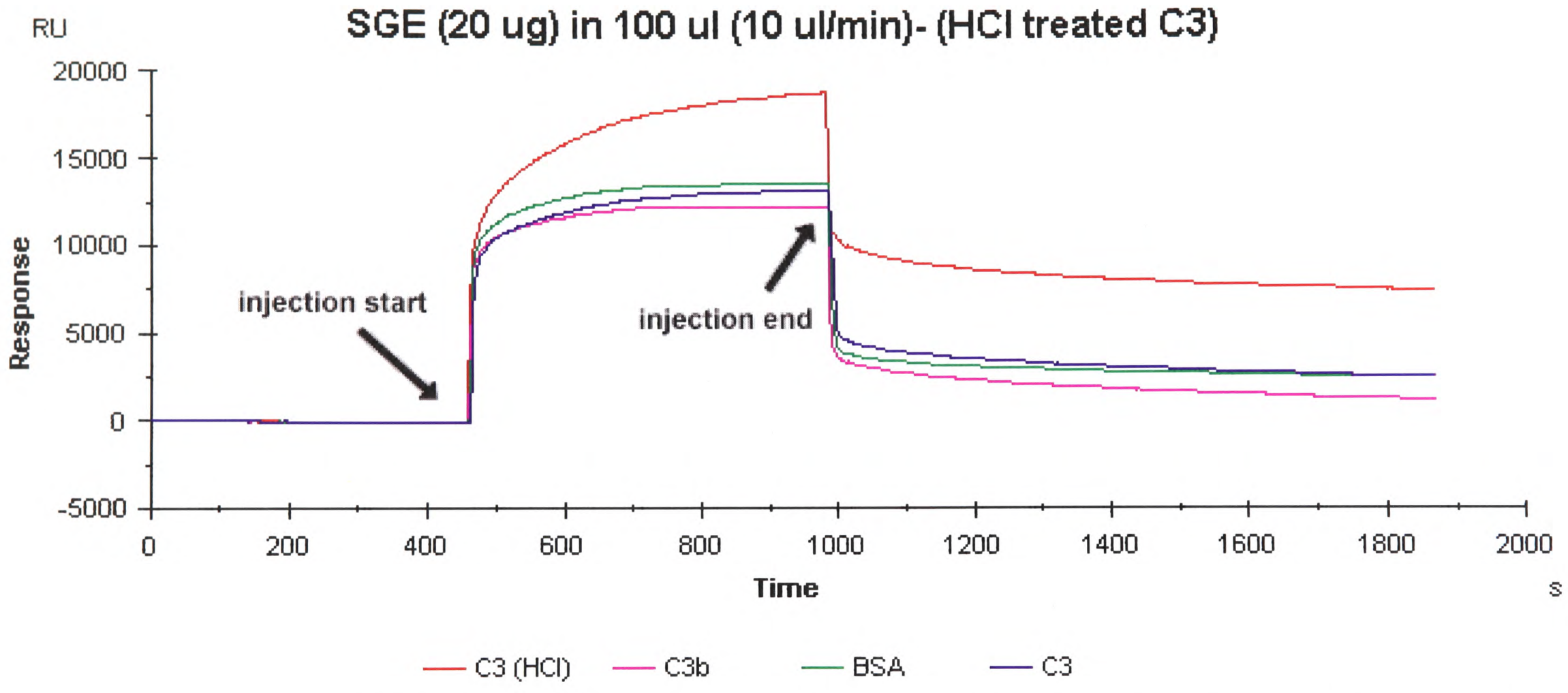


Fig 6.28 **Sensorgram of SGE (20 µg) in 100 µl (10 µl/min) injected over all four flow cells (HCl treated C3; untreated C3)**

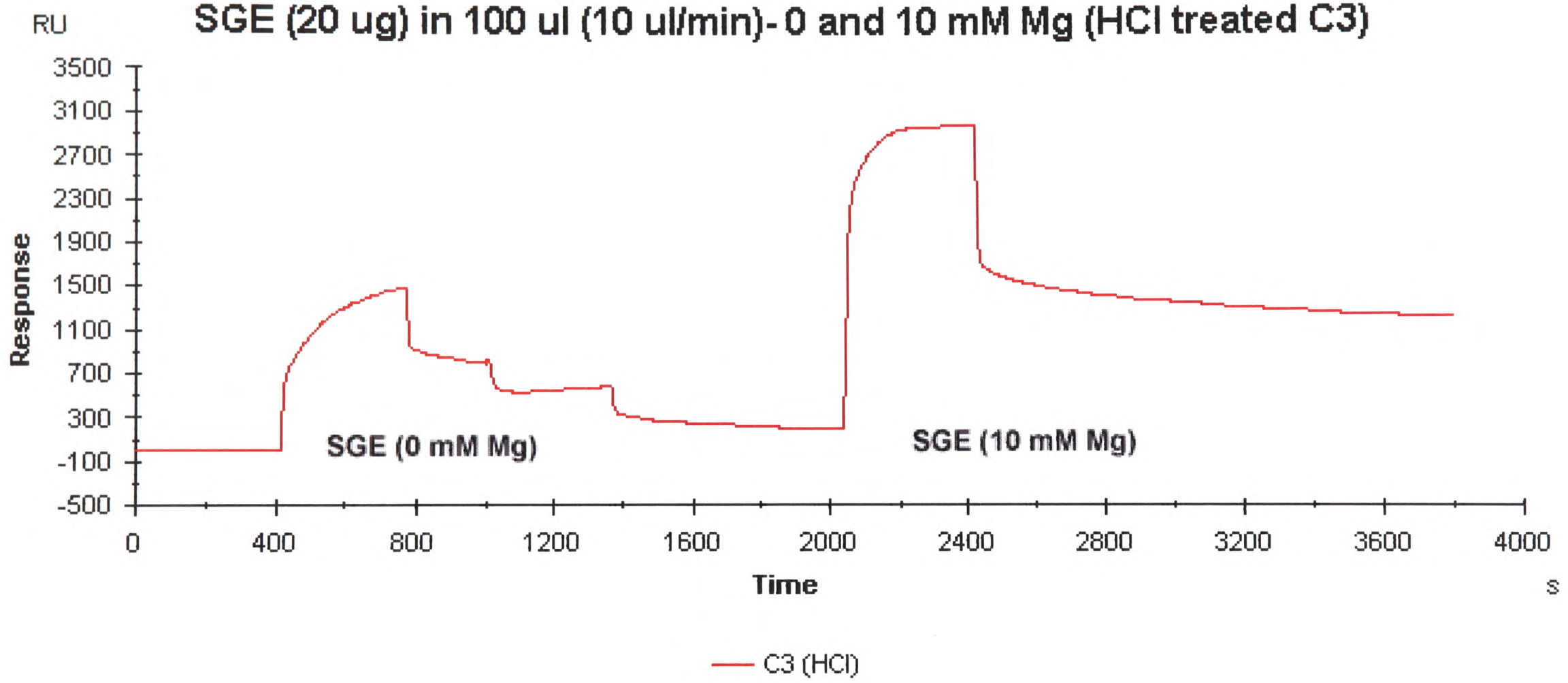


Fig 6.29 Sensorgram of SGE (20 µg) in 100 µl PBS (0 and 10 mM Mg^{2+}) injected over HCl treated C3 flow cell (10 µl/min).

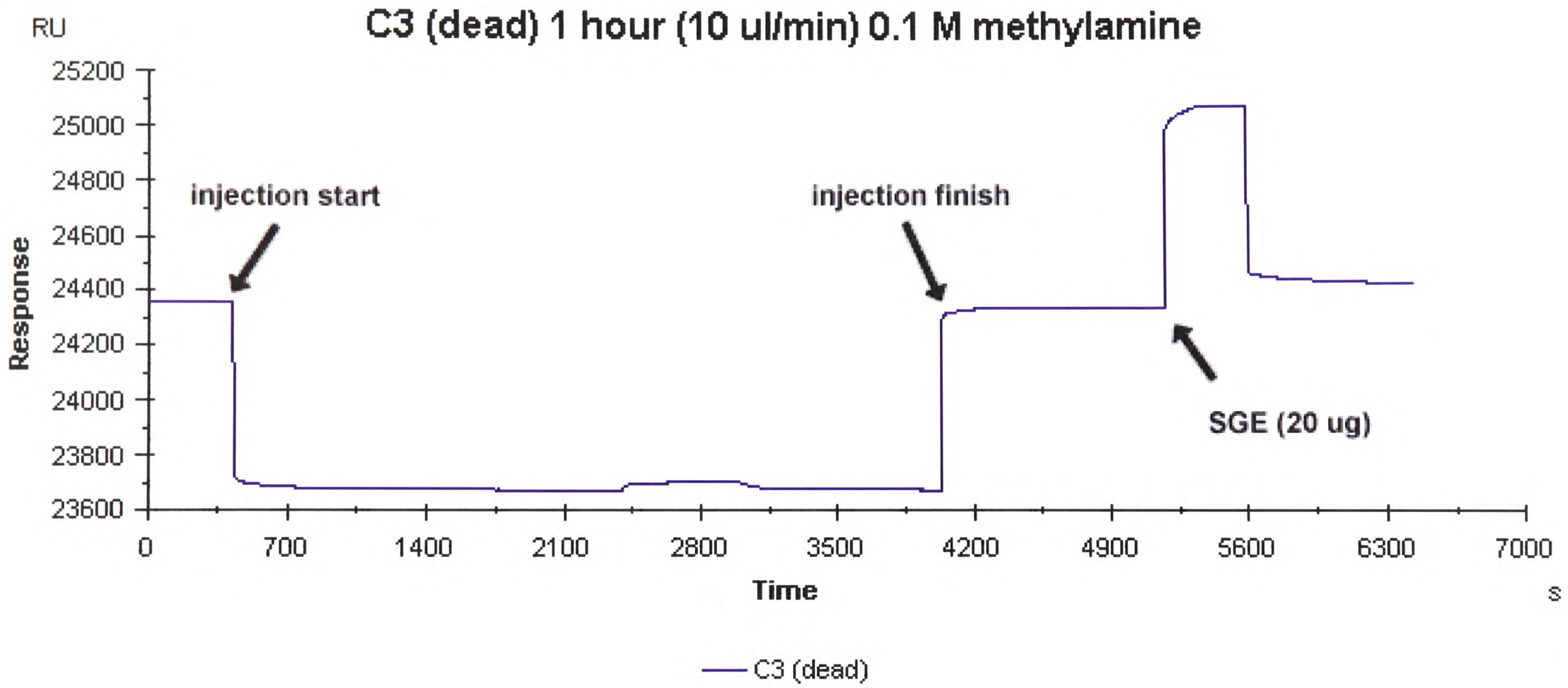


Fig 6.30 Sensorgram of C3 (untreated flow cell) injected with 0.1 M methylamine pH 8.5 for 1 hour (10 µl/min) followed by injection with SGE (20 µg).

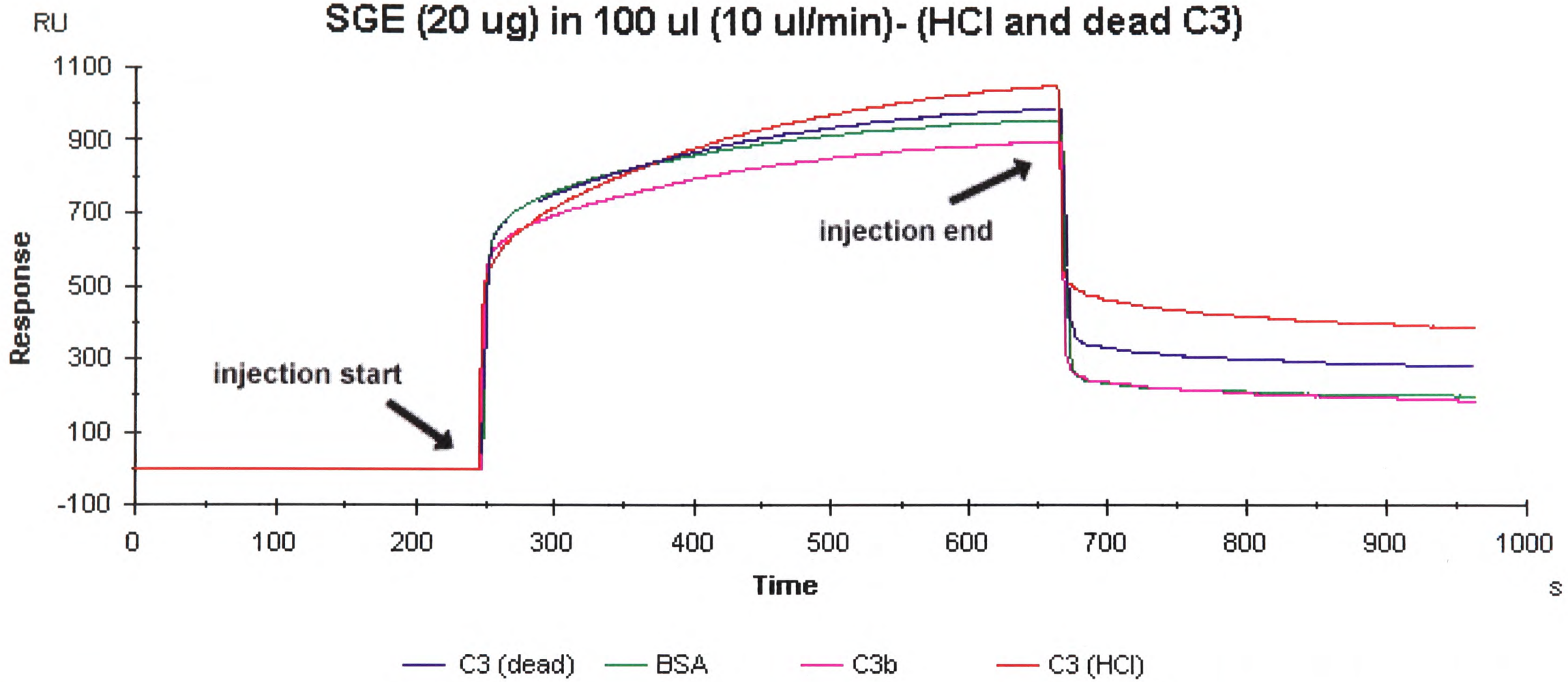


Fig 6.31 **Sensorgram of SGE (20 µg) in 100 µl (10 µl/min) injected over all four flow cells (HCl treated C3; dead C3)**

6.3.19 **Plasmon resonance studies of SGE binding to immobilised complement components**

At the interface between two transparent media of differing refractive indices, incident light at an angle greater than the critical angle is totally internally reflected. Although the incident light is totally reflected, an electromagnetic field component, the evanescent wave, penetrates a short distance (about one wavelength) into the medium of lower refractive index. If the interface is coated with a thin layer of metal, and the light is monochromatic and p-polarised (i.e. the electric vector is parallel to the plane of incidence), the intensity of the reflected light is markedly reduced at a specific incidence angle, producing a sharp shadow. This phenomenon is termed surface plasmon resonance (SPR) (Kretschmann and Raether, 1968). The SPR angle depends upon several factors, one of which is the refractive index of the medium into which the signal propagates, i.e. the non-illuminated side. The refractive index of the surface depends upon the concentration of solutes at the surface. By immobilising components onto the surface (i.e. chip) interactions between the ligand (i.e. immobilised phase) and analytes (i.e. fluid phase) can be measured through the changing refractive index of the surface as measured in real time. The sensorgrams shown in this section are plots of the changing SPR signal over time. Surface plasmon resonance studies were carried out using a Biacore 2000 system. By immobilising complement components, possible binding interactions with the fluid phase SGE can be measured in real time through changes caused in the refractive index.

Unless otherwise indicated ligands were bound to CM5 chips through amine coupling in accordance with supplied instructions at pH 5.0 (10000 RU). Results using purified factor D (section 6.2.4), C3b (1 μg ul^{-1} – from Alister Dodds- MRC Immunochemistry, Oxford) and C4b (1 μg ul^{-1}- Sigma) immobilised directly onto a CM5 chip, failed to show any significant binding activity with the SGE (data not shown). This was despite using various buffers (e.g. GVB/Mg, PBS) and flow rates (1-10 μl min^{-1}). Purified factor B was also immobilised onto a CM5 chip and no significant binding was observed (not shown). To test the binding of both C3 and factor B, the respective polyclonal rabbit antiserum (from Bob Sim (MRC Immunochemistry Unit, Oxford)) were tested for binding activity. Specific binding activity was observed (not shown). To investigate whether the binding of these components to the chips was interfering with possible SGE binding due to conformational changes or through steric hindrance (particularly the 25 kDa factor D), monoclonal antibodies against both C3c (WM1) and factor D (JA4-2) were bound onto CM5 chips (15000 RU; JA4-2 was put on at both 15000 RU and 5000 RU). No binding was observed when purified C3 (from Bob Sim (MRC Immunochemistry Unit, Oxford)) or factor D (both the purified and functionally active (section 6.2.4) were tested), respectively, were passed over the chip. Various buffers and flow rates were used, and in addition binding at both 25^{O}C and 37^{O}C were tested (not shown). A streptavidin chip was used to bind biotinylated mouse anti-IgG (Sigma) at pH 4.8 (15000 RU) onto two flow cells. Both WM1 and JA4-2 (10 μg) were passed over separate flow cells. Binding was observed (about 1500 RU) with both monoclonal antibodies (also confirmed by regeneration of the chip using 10mM HCl).

However, no binding was observed when either factor D or C3 was passed over the chips (data not shown).

Previous results (section 6.3.16) suggested that SGE might be interacting differentially with respect to C3 and C3(H_2O). To investigate this, C3, C3b, C3(H_2O) and BSA were bound to a CM5 chip at pH 5.0 (10000 RU). Initially C3(H_2O) was prepared by incubating C3 with 10 mM methylamine pH 8.5 for 1 hour at 37^{O}C. When SGE was passed over the chip at 10 µl min^{-1}, no significant binding was observed (Fig 6.26). However, binding of SGE was found to occur when tested on a chip prepared by Miles Nunn containing bound C3 (not shown). This chip was prepared in an identical way, but had undergone regeneration conditions (i.e. 20mM HCl treatment). By treatment of one of the flow cells (C3 containing) of my chip with 20 µl of 50 mM HCl (5 µl min^{-1}), a significant change in the base-line value of the C3 was observed (Fig 6.27). When this flow cell was tested with SGE, significant binding was shown to occur (Fig 6.27 and Fig 6.28). Binding was observed to increase in the presence of 10 mM Mg^{2+} (Fig 6.29). Treatment of the other flow cell (i.e. C3(H_2O) containing) with 100 mM methylamine pH 8.5 for 1 hour (10 µl min^{-1}) resulted in a change in the baseline value of this flow cell (Fig 6.30). In contrast to the previous results, SGE binding was shown to occur in this flow cell, albeit less than that of the HCl treated flow cell (Fig 6.31).

These data suggest that *I. ricinus* SGE contains a factor(s) that binds to C3. However, consistent with above results, the basis of this interaction appears to be conformationally specific. Bound 'live' C3 only showed significant binding of SGE only when chemically treated with either HCl or methylamine (0.1 M). Attempts were made to recover the bound analyte, but to no avail (not shown).

6.4 Discussion

6.4.1 Anti-complement activity

The complement system is involved in many host inflammatory responses, consequently modulation of complement activity is essential for the successful feeding of ticks. Inhibition of the alternative pathway of complement has been demonstrated in the female adults of *Ixodes dammini* (classical activity was not tested) (Ribeiro, 1987a). The results of this chapter extend this activity to the salivary glands of female adult *I. ricinus* and *O. moubata*. The fact that SGE of *I. ricinus* inhibited the alternative but not the classical pathway of complement (Fig 1.4), may reflect the evolution of vertebrate immunity. The ubiquity of alternative pathway components in all vertebrate classes, and possibly also invertebrates, has led to the proposal that the classical pathway of higher vertebrates evolved later by gene duplications and/or translocations (Dodds and Day, 1993). More recent data suggests that the MBL-pathway may be more ancient than the alternative pathway, however supporting evidence for this hypothesis is lacking at present.

Unlike the tick immunoglobulin-binding proteins that are expressed only during the latter half of the feeding period (Wang and Nuttall, 1995a; Wang and Nuttall, 1995b), the anti-complement activity of *I. ricinus* was detected in SGE of unfed ticks as well as throughout the feeding process (Table 6.5). Presumably the active factor(s) is stored in the salivary gland acini prior to secretion. Pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1) are produced in response to tick-bite injury (Mbow *et al.*, 1994). TNF-α and IL-1 up-regulate the alternative pathway of

complement and stimulate the acute phase response (Katz and Strunk, 1989; Perlmutter *et al.*, 1986). Acute phase proteins such as mannan-binding lectin and C-reactive protein can activate and up-regulate the complement system (Suankratay *et al.*, 1998). Tissue damage caused during the initial incision of the skin by the tick should result in rapid activation of host complement via the acute phase response. Consequently immediate inhibition of host alternative pathway activity by the tick may be necessary for successful feeding.

Testing the salivary glands of other haematophagous arthropods, i.e. *Anopheles gambesi* as well as the salivary glands of the metastriate ticks, *Dermacentor andersoni* and *Rhipicephalus appendiculatus* showed no significant inhibition of the alternative pathway of complement. In contrast, the SGE of the argasid tick *Ornithodoros moubata,* did inhibit the alternative pathway of complement. The fact that argasid ticks feed for a relatively short period of time (about 20 minutes is typical), again reinforces the presumption that the alternative pathway of complement activation occurs rapidly in the host in response to tick infestation. However, the salivary gland extract of *R. appendiculatus* has been demonstrated to inhibit the classical pathway of complement (Miles Nunn (IVEM)– unpublished data). This suggests that different anti-complement strategies may have developed between prostriate and metastriate ticks. Consistent with this hypothesis is the demonstration of alternative pathway of complement inhibition by the salivary gland extracts of the prostriate ticks, *I. dammini*, *I. uriae, I. hexagonus* and *I. ricinus* (Chapter 7). The differences in anti-complement strategy may be a reflection of tick evolution. Phlyogenetic studies based on morphology (Hoogstraal and Aeschlimann, 1982) and upon 16s rRNA sequences (Black IV and Piesman, 1994), suggest that

Argasidae are the ancestral tick family from which first prostriata and later the metastriata ticks evolved. This would suggest that in parallel with vertebrate evolution, anti-alternative activity evolved before the anti-classical activity of metastriate ticks. Further work is required to test this hypothesis. Presumably, anti-complement activity has evolved primarily in order to modulate host inflammatory responses, whether such activity also serves to protect the midgut from imbibed complement components is unknown.

Testing of whole and larval homogenates showed no significant inhibition of complement activity. However, control samples (without serum) showed that the whole tick homogenates of adult female, larvae and nymphs possessed haemolytic activity. The lytic activity is probably the result of haemolysins present in the gut material of the whole tick homogenates (salivary gland-associated haemolysis reported in Chapter 5 was shown to be inhibited at 10mM Mg^{2+} (section 5.3.4)). Male adult homogenates had no such haemolytic activity; this is consistent with the belief that these ticks do not feed on hosts. The lytic activity is not in any way associated with anti-complement activity, but is associated with digestion. The homogenates of adult females, nymphs and larvae showed reduced haemolysis in the presence of serum when compared with the control samples, in contrast with the results of the male homogenates. These data perhaps suggests that larvae and nymphs as well as adult female *I. ricinus* ticks can inhibit the alternative pathway of complement. Non-haemolytic complement assays should be carried out to confirm this.

Because *I. ricinus* SGE has been demonstrated to inhibit the alternative but not the classical pathway of complement, only components of this pathway were considered in the characterisation of the mechanisms involved in anti-complement activity.

6.4.2 **Factor D analysis**

Factor D is a 24kDa serine protease that cleaves factor B to Bb and Ba only when factor B is bound to C3b (i.e. C3bB) to form the alternative pathway convertase C3bBb (Fig. 1.5). Unlike most other serine proteases, factor D exists only in active form (i.e. has no pro-enzyme form). Control of activity appears to be at the level of substrate specificity. Factor D has very low activity against low molecular weight synthetic substrates. Crystallographic data suggest that factor D becomes active only upon binding of C3bB via binding-induced conformational changes (Kim *et al.*, 1995). Factor D is present in sera at relatively low levels (1 $\mu g\ ml^{-1}$ c.f. 210 $\mu g\ ml^{-1}$ Factor B) and as such may present a good target for tick-derived immunosuppression.

The results of Fig 6.4 showed the presence of an extra species in the SGE containing samples, as analysed by native-PAGE and immunoprobed with JA4-2, an anti-factor D monoclonal. Such a change in mobility suggests an interaction between the SGE and factor D. This could be due to a binding event or through cleavage of factor D. Functionally active purified factor D or factor D supplied as whole serum decreased the anti-complement activity of *I. ricinus* SGE (Fig 6.3). The addition of 40 µl of purified factor D increased haemolysis from 12.3% to 34.8% (c.f. 92.3% in control reaction). The results of Fig 6.25, showed that a specific species in the I^{125} labelled SGE of approximately 20 kDa eluted off the factor D containing column at 1M NaCl.

The inability to produce demonstrable binding using plasmon resonance studies, was possibly due to steric hindrance of the small factor D (25 kDa) bound to the chip. Consistent with this idea, no binding was observed when JA4-2 was passed over the immobilised factor D (data not shown). Taken together these data suggest that *I. ricinus* SGE interacts with factor D, presumably through binding of the active site or other site, acting to prevent conformational activation of factor D. A similar activity has not been described previously in the literature. It should be noted however that the SGE did not appear to inhibit turnover of factor B in the presence of C3b, factor B and factor D (section 6.3.13).

6.4.3 **Factor B analysis**

Factor B is a 92 kDa protein present in serum at 210 μg ml^{-1} plasma (Law and Reid , 1995). Factor B forms a Mg^{2+} dependent complex with C3b to form C3bB. Factor B is cleaved to Bb (62 kDa) and Ba (32 kDa) through the action of factor D. Factor B possesses the catalytic sites of both the C3 and C5 convertase (Jensen and Koch, 1991). Western analysis using polyclonal rabbit serum analysed by both native and SDS-PAGE, showed no bands indicative of binding or of cleavage of factor B in samples containing SGE (Fig. 6.6). Indeed, compared with the formation of Bb and Ba in the control samples, it is clear that factor B cleavage is inhibited in the presence of both *I. ricinus* and *I. hexagonus* SGE. This suggests that the anti-complement mechanism is probably similar in both species. In contrast, when turnover of factor B was tested in the presence of C3b (or CVF), SGE did not inhibit the turnover of factor B. This shows that C3bB (or

CVFB) can form in the presence of SGE, suggesting that the primary activity of the SGE anti-complement activity is to prevent the conversion of C3 to C3b.

Purified factor B or factor B supplied as whole serum did not decrease the anti-complement activity of *I. ricinus* SGE (Fig 6.5). Plasmon resonance studies with immobilised factor B showed no significant binding events (data not shown). Similarly, affinity chromatographic purification of I^{125} labelled SGE, showed little specific activity to be eluted from the factor B bound column. These data suggest that *I. ricinus* SGE does not interact directly with factor B, but that the cleavage of factor B is inhibited indirectly as a result of SGE activity.

6.4.4 **C3 analysis**

C3 is a 185 kDa protein composed of an α (110 kDa) and β (75 kDa) chain and that is present in plasma at a concentration of 2100 μg ml^{-1} (Law and Reid , 1995). C3 plays a key role in both the classical and the alternative pathways of complement, as discussed in detail in Chapter 1.

Western analysis of C3a formation in an AH_{50} assay showed that C3 cleavage to form C3a is inhibited by SGE activity (Fig 6.8). CVF treatment of serum complement activity apparently inhibits the anti-complement activity of SGE, as visualised by factor B cleavage (Fig 6.8). As CVF is insensitive to factor I cleavage, this suggests that either factor I is implicated in anti-complement activity, or that the excess of CVF used is out-competing the anti-complement activity, suggesting that C3 is a direct target for modulation by the tick.

By using I^{125}-labelled C3, the effect of SGE upon the fate of C3 can be measured with high sensitivity. The results of Fig 6.10 showed that SGE inhibits the deposition of C3 upon rabbit erythrocyte membranes (i.e. solid phase). The supernatant samples of the same experiment (Fig 6.10), showed that in the absence of SGE, control sample fluid phase C3b was cleaved to iC3b by factor I. In contrast, SGE containing samples showed no significant cleavage under the experimental conditions used, suggesting that fluid phase C3b is not being formed in these samples. These data suggest that the main effect of *I. ricinus* SGE anti-complement activity is the inhibition of turnover of C3 to C3a and C3b.

To investigate the effect of SGE upon the formation of iC3b, cleavage was visualised in the absence of erythrocytes by using I^{125}-labelled C3(H_2O) in the presence of serum. Fig 6.11 showed that iC3(H_2O) was being formed in the control sample by the serum proteases, but that cleavage was partially inhibited by the presence of SGE. These data suggest that C3 is modified in such a way that it is not recognised by factor I as a substrate for cleavage.

The effect of SGE on convertase formation was investigated in the presence of pre-formed C3b, by Coomassie blue staining of the turnover of factor B (Fig 6.12, 6.13). No significant differences in the kinetics of convertase formation were observed in the presence or absence of SGE. Similarly by using I^{125}-labelled C3 as a substrate for the convertase, and by adding the SGE before stopping further convertase formation with EDTA, initial convertase formation and the kinetics of formation were not significantly changed (Fig 6.14- top). In contrast, by adding the SGE concurrent with EDTA (i.e. when further convertase formation has been inhibited), the effect of SGE upon the

stability of the formed convertase was visualised. The results of Fig 6.14- bottom, showed that the cleavage of α to α' was reduced in the presence of SGE, when compared to the control samples. Repetition of this experiment (Fig 6.15), but running the gel for a longer period, was consistent with these observations. However from the increased resolution it was apparent that the mobilities of both α and α' chains are significantly different from those of the control samples. Such a change in mobility suggests that the α chain of C3 is being cleaved by a factor in SGE to a C3b-like form (designated C3b'). These data suggest that the C3 is modified in such a way that C3b' is still recognised as a substrate by the pre-formed convertase, albeit to a lesser degree (i.e. the N-terminal fragment C3a is produced).

The effect of SGE upon only C3 or C3(H_2O) was visualised by autoradiography (Fig 6.16). Consistent with the above data, these results suggested that *I. ricinus* SGE is cleaving the α chain of C3 to form a species of a similar size to the α'-chain of C3b (designated the α1-chain) in the absence of other complement components. Both C3 and C3(H_2O) are substrates for this cleavage (Fig 6.16). However the results of Fig 6.16 suggest that the α1-chain of C3b' specifically (i.e. not C3b'(H_2O)) is further cleaved by proteolytic activity to produce three major fragments designated C3a1, C3a2 and C3a3. The sizes of these fragments (i.e. 36, 31 and 28 kDa respectively) differ from those produced by serum protease cleavage. Cleavage of C3 to C3b' was demonstrated to be both dose and time dependent.

N-terminal sequencing of the α1-chain showed that the N-terminus was not cleaved by an SGE factor suggesting that cleavage occurs at the C-terminus rather than the N-terminus that is the site for convertase cleavage (i.e. C3a). This is consistent with

the observation that the α1-chain still appears to be a substrate for cleavage by the convertase (Fig 6.15). A 7 kDa fragment presumably representing the C-terminal fragment and designated C3x is observed in the SGE treated samples of Fig 6.21.

Little is known about the possible function of the C-terminus region of the α-chain of C3. Indeed little is known about the whole C3c region, although it is proposed to be a ligand for CR1 (Lambris, 1988). It is not known what effect the cleavage of a C-terminal fragment such as C3x would have upon the conformation of C3.

It is proposed that C3b' is conformationally different to C3b, acting to inhibit formation of the C3b'B complex and effectively stopping convertase formation. Consistent with this observation, C3b' appears to be a less successful substrate for factor I-mediated cleavage (Fig 6.11).

Characterisation of the proteolytic activity suggests that an acid protease is present in the SGE of female adult *I. ricinus* ticks. The lack of inhibitory action of all of the proteolytic inhibitors tested as well as the insensitivity to physical treatments when tested at neutral pH suggests that the active site may only become exposed as the result of acid treatment. Treatment with various class specific proteolytic inhibitors under acidic conditions failed to identify a mechanistic class (data not shown). Extended incubation times (over 4 days) and use of factor B as a substrate suggest that the proteolytic activity is specific for the α-chain of C3 (Fig 6.21). Furthermore, that the C3 proteolytic activity is conformationally specific, only the α-chain of C3 but not C3b or C3(H_2O) were cleaved (Fig 6.23).

Using acidic conditions C3 was shown to be cleaved into three fragments by SGE-proteolytic activity, that corresponded in molecular weight of the C3a1, C3a2 and

C3a3 fragments observed in Fig 6.16 under physiological pH conditions. Whether the acid activated proteolytic activity is the same as that observed under physiological conditions is not known. It should be noted that 20 μg of SGE protein (c.f. with 400 ng in Fig 6.21) was used in the experiment shown in Fig 6.16.

It is possible that acid treatment of the protease(s) causes a conformational change similar to that obtained upon binding to a yet un-identified cofactor present in serum. Alternatively, in common with complement proteases, the SGE-protease(s) may be activated by cleavage, perhaps mediated by the activated complement components themselves.

N-terminal sequencing of the C3a2 fragment showed that cleavage occurred between an arspartic acid (993) and alanine (994) residue of the α-chain of C3 (Fig 6.22). This corresponds to a fragment of 321 amino acids (approximately 31 kDa). Cleavage occurs in the C3dg region of the α-chain of C3. The serum proteases trypsin, elastase and plasmin have been shown cleave the C3dg region to generate C3d (Ross *et al.*, 1982). The SGE-mediated cleavage occurs 8 amino acids upstream of C3d cleavage. C3d has been demonstrated to contain the binding site for the CR2 receptor between amino acid residues 1227 and 1236 (Fig 6.22) (Ross *et al.*, 1982). CR2 receptors are primarily found on B lymphocytes (Nickells and Atkinson, 1997). Whether SGE cleavage in this region could interfere with this activity is unknown (possibly acting on bound C3b).

N-terminal sequencing of the C3a3 fragment showed it to correspond to a region 233 amino acids up-stream of the C3a2 cleavage site (consistent with apparent molecular weight of 26 kDa (Fig 6.21). Cleavage occurs within the 42 amino acid region identified as containing the binding sites for factor B, factor H, CR1 and membrane cofactor protein

(Fig 6.22) (Lambris *et al.*, 1996). Site-directed mutagenesis identified two amino acid residues in this region (Glu^{758}-Glu^{759}) as being implicated in factor B binding (Taniguchi-Sidle and Isenman, 1994). The C3a3 cleavage site occurs between the adjacent Asn^{760} and Ile^{761} residues. It is possible that cleavage at this site by an SGE-protease interferes with the binding of C3b' to factor B. Further work is required to test this hypothesis.

The fact that the major high molecular weight species of C3 present after SGE treatment (i.e. α1-chain) is not N-terminally cleaved suggests that the C3a3 cleavage occurs only after C3b' cleavage. The proposed model of cleavage is shown in Fig 6.32. It should be noted that N-terminal sequencing of the C3a1 fragment is necessary to confirm this model. Both C3a3 and C3a2 cleavage occurs between a charged residue (i.e. Asn^{760} and Asp^{993}) and a hydrophobic residue (i.e. Ile^{761} and Ala^{994}) suggesting that both cleavage events may be catalysed by a common proteolytic factor.

6.4.5 **Surface plasmon resonance studies**

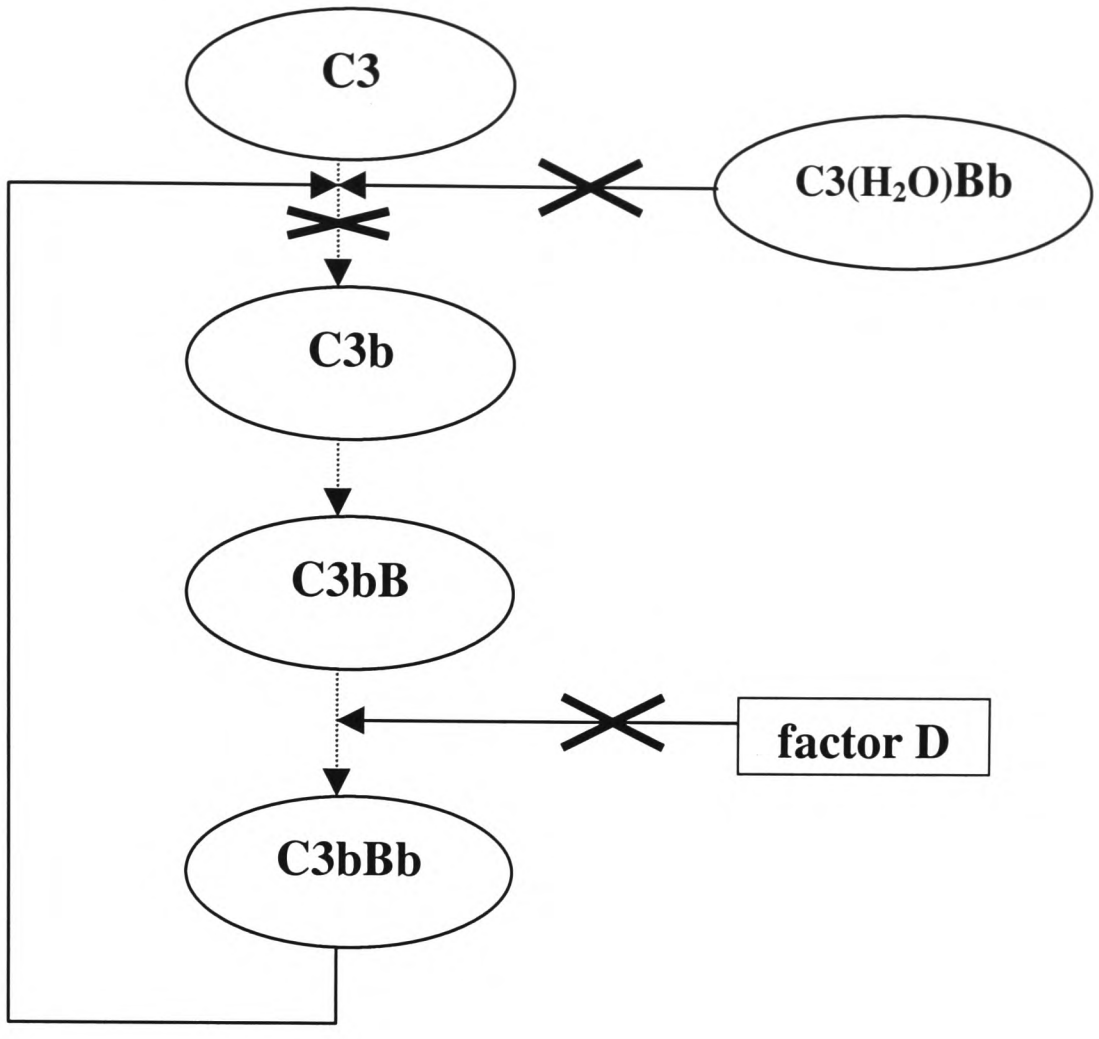
C3 was immobilised on a CM5 chip as described in section 6.3.17. Initial results using SGE as an analyte showed no significant binding activity of the C3 (Fig 6.26). However, there was binding to C3 bound to a chip by Miles Nunn (not shown) which had been subject to regeneration treatments (i.e. 50 mM HCl). Treatment of one of the flow cells (fc) with 50 mM HCl showed that the baseline of the C3 changed significantly (Fig 6.27); this is indicative of a conformational change in the bound C3. The treated flow cell showed significant binding to SGE when tested (Fig 6.28). This suggests that a factor in the SGE is binding to C3, but that the binding is conformationally specific. There is, however, no published data upon conformational changes of C3 by acid treatment (only

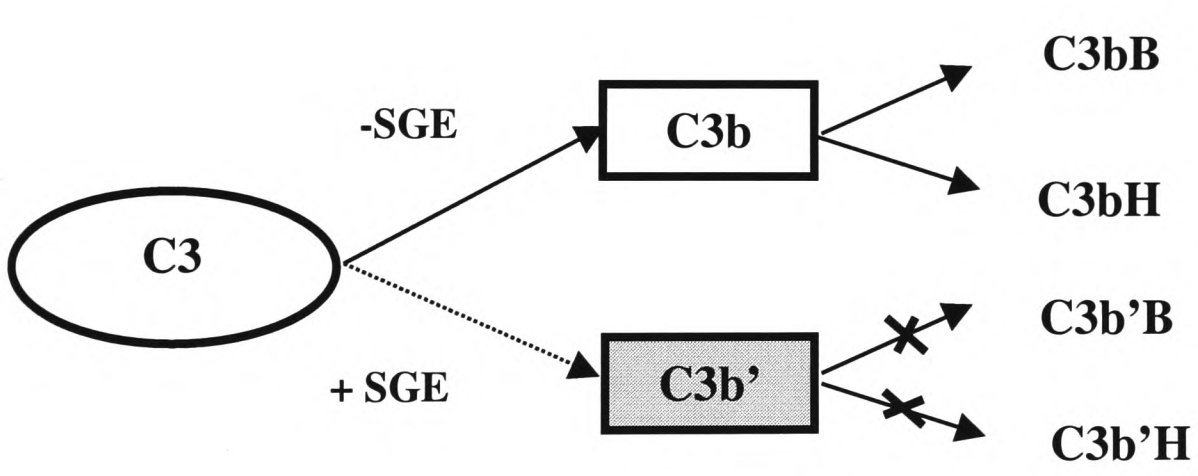
alkaline), in addition no information is available upon the conformational state of C3 immobilised upon a chip. Treatment of the remaining C3-containing flow cell with 0.1 M methylamine pH 8.5 for 1 hour (10 μl min^{-1}) again showed a significant change in the baseline suggesting a conformational change in the bound C3 (Fig 6.27). This treatment is the standard way in which to create C3(H_2O) (Tack *et al.*, 1980). Testing SGE with this treated chip showed that significant binding occurred in the treated flow cell. These data suggest that a factor(s) in SGE may be specifically binding C3(H_2O) but not C3.

6.4.6 **Conclusion**

The results of this chapter provide valuable insights into the mechanisms of anti-complement activity in *I. ricinus* ticks. Perhaps unsurprisingly, the anti-complement activity appears to be more complex than was at first envisaged; indeed the results of this study pose more questions than answers. It is apparent that both factor D, C3(H_2O) and C3 are targets of modulation by the saliva of the ticks (Fig 6.32). Although some form of interaction was demonstrated between SGE and factor D, it is not apparent whether such activity effects the function of factor D.

It is clear however, that the principal action of SGE anti-complement activity is upon C3. There appears to be no effect upon the formation of CVFB, CVFBb, C3bB or C3bBb. In other words, the major effect is upon the conversion of C3 to C3b via cleavage of the α-chain by the convertase. Cleavage of the α-chain was observed in the presence


C3
C3(H2O)Bb
C3b
C3bB
factor D
C3bBb


C3bB
-SGE
C3b
C3bH
C3
C3b'B
+ SGE
C3b'
C3b'H

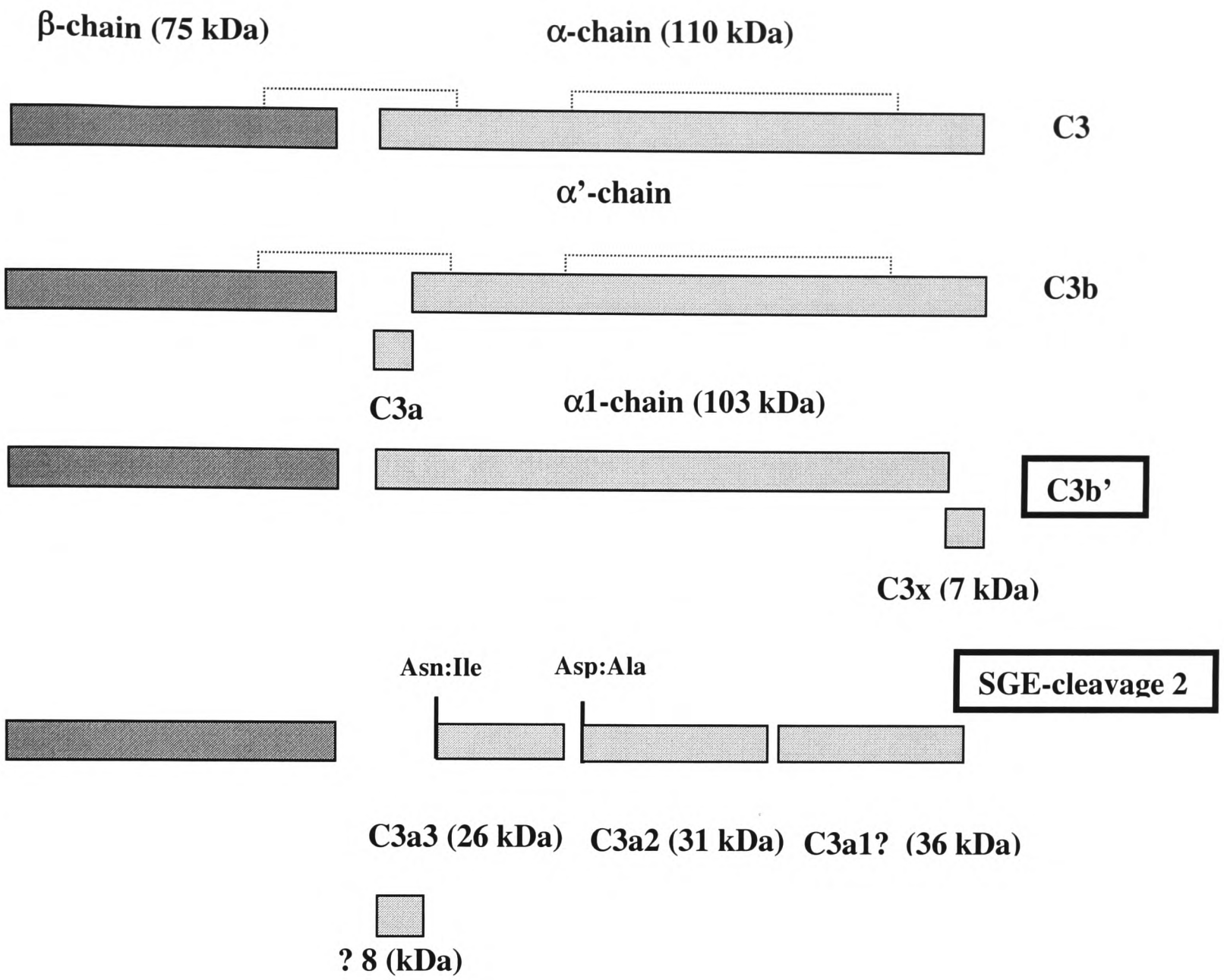


Fig 6.32 **(Top), schematic diagram of alternative pathway of complement showing proposed sites of inhibition by *I. ricinus* SGE (indicated as crosses). (Middle) proposed model of SGE modification on the fate of C3. (Bottom) schematic diagram of proposed proteolytic cleavage of C3 by SGE protease(s), showing approximate molecular weights and cleavage sites where known. Dotted lines indicate di-sulphide bonds, the state of bonds in the SGE cleaved products is unknown. Unidentified fragments are depicted by a question mark.**

of SGE, but to a different form, C3b', by an SGE-associated proteolytic activity. The cleavage event occurs at the C-terminus of the α-chain of C3 (to form α1-chain). It is suggested that C3b' does not form functional C3b'B or C3b'Bb complexes, but does appear to be a substrate for C3bBb cleavage (Fig 6.32). In addition it appears that C3b' appears not to form C3b'H complexes (similar to C3bH, in which C3b is a substrate for factor I cleavage).

A proteolytic activity specific for the α-chain of C3 (i.e. not C3b or C3(H_2O)), was identified in the SGE of *I. ricinus* ticks. This activity was shown to cleave the α1-chain of C3b' into three major products (C3a1, C3a2 and C3a3). It is suggested that such cleavage may enhance the proposed C3b' anti-complement activity and only occur after the α to α1-chain cleavage event. The activity was shown to be due to an acid protease and consequently, it is not clear whether proteolysis is a separate phenomenon from the anti-complement activity of *I. ricinus* SGE.

The results of this chapter suggest that SGE may be interacting differently with respect to the conformationally different C3 and C3(H_2O). Evidence was presented to suggest that C3(H_2O) specifically may be bound by an SGE factor; perhaps in such a way as not to let factor B bind, inhibiting formation of the 'seed' convertase, C3(H_2O)Bb. Quantities of this form in serum represent a minute proportion of the total C3. The formation of C3(H_2O)Bb is the focus of the constant testing of serum exposed surfaces for non-self by the alternative pathway of complement. As a consequence C3(H_2O) would be a good target for modulation by the tick.

In conclusion, it is clear that several factors, acting on several points in the alternative pathway, are involved in the inhibition of complement by the salivary glands

of female *I. ricinus* ticks (Fig 6.32). It is the aim of this researcher to further characterise and isolate the factors involved in anti-complement activity in future work.

CHAPTER 7

SPECIES SPECIFIC ANTI-COMPLEMENT ACTIVITY IN *Ixodes* SPECIES TICKS

7.1 Introduction

Most if not all tick species show some degree of host specificity. The European Lyme disease vector, *I. ricinus*, is a highly promiscuous feeder; it has been found on over 65 host species in the UK including birds, reptiles and mammals (Milne, 1949). At the other extreme, some species, such as *I. jellisoni* are associated with a single host species (Keirans *et al.*, 1996). The host range of a particular tick species is a function of ecological (probability of encountering a particular host) and physiological factors (Sonenshine and Mather, 1994). The feeding success of a tick of any species upon a given host depends on its ability both to attach and to feed to completion, which in turn relies upon the ability of the tick to antagonize host specific inflammatory responses (Ribeiro, 1987a).

In this chapter evidence is presented to suggest that the ability of any particular *Ixodes* species to feed successfully upon a given host may be influenced by the ability of the tick to inhibit activation of the alternative pathway of complement of that host.

7.2 Materials and Methods

7.2.1 Preparation of salivary gland extract

Ixodes ricinus and *Ixodes hexagonus* ticks were reared and maintained at the Institute of Virology and Environmental Microbiology, Oxford according to standard methods (Jones *et al.*, 1988). Adult male and female ticks were fed on hamsters for five days (unless otherwise stated) before the females were removed and their salivary glands dissected. Partially engorged female adult *Ixodes uriae* ticks were collected from guillemot (*Uria aalge)* and kittiwake (*Rissa tridactyla*) colonies on the Isle of May, Firth of Forth, Scotland. Salivary gland dissection was carried out immediately after their removal from the host. Salivary glands were excised, washed, and homogenized in 10 µl per tick of phosphate buffered saline (PBS) on ice. Particulate matter was removed by centrifugation at 14000 rpm for 10 minutes. The soluble fraction (i.e. SGE) was aliquoted and stored at -70° C. Protein concentration was determined using a modified Bradford assay (Bio-Rad protein assay) (Bradford, 1976). Standard curves were prepared using bovine serum albumin.

7.2.2 Serum preparation

All fresh sera were prepared from fresh blood clotted on ice for 1 hour. Serum was separated from the blood by centrifuging at 14000 rpm for 10 minutes. Serum was aliquoted and stored at –70° C; aliquots were only used once (Whaley and North, 1997). Pigeon and canine sera, both obtained from Sigma (pooled from a number of animals),

were reconstituted with H_2O according to the manufacturer's instructions, aliquoted and stored as above. Hedgehog (*Erinaceus europaeus)* serum was obtained from two male hedgehogs from Mrs. Tiggy-Winkle's Sanctuary, Somerset, UK. Human sera were obtained from two healthy male individuals, fresh canine sera from 3 individuals and pheasant (*Phasianus colchicus)* serum from 4 birds (2 male, 2 female). Two individual red deer (*Cervus elaphus scoticus)* were also used as serum donors.

7.2.3 Immunoaffinity chromatography of *I. ricinus* SGE with canine serum

To remove antigens that cross-reacted with canine serum, *I. ricinus* SGE was passed through a column that had been pre-absorbed with canine immunoglobulins. 0.5ml of protein A agarose (4%) (Sigma) was packed into a 2.5ml column suspended in PBS. The column was washed with 5x 1ml of PBS. 300 µl of canine serum (Sigma) in 3ml PBS was run through the column three times before washing the column with 5x 1ml PBS washes. 200 µl of *I. ricinus* SGE (equivalent to 40 tick salivary glands collected on day five of feeding) were loaded onto the column three times before collection. The column was then washed as above and the immunoglobulin bound fractions were eluted from the column using 200 µl pH4 (10mM acetate), pH3 (10mM glycine) and finally pH2 (10mM glycine) buffers.

7.3 Results

7.3.1 Serum species sensitivity of anti-complement activity of *Ixodes* ticks

To eliminate the possibility of serum mediated haemolysis being non-specific in the assays, every animal serum that was used, was tested in an AH_{50} assay containing EDTA (10mM) and by treating serum for 30 minutes at 56^0C; both these treatments abolish complement activity (Coligan, 1994). Non-complement haemolysis was not observed for any of the species serum tested (data not shown). Serum from different individuals of the same species and pooled serum were compared in AH_{50} assays with and without *I. ricinus* SGE; no significant intraspecific differences in AH_{50} values were observed (data not shown).

The AH_{50} value of a particular serum is a semi-empirical value calculated by haemolytic assay (section 6.2.2) and corresponds to the serum dilution required to lyse 50% of cells in the assay. Results are expressed as the reciprocal of this dilution in AH_{50} units per ml of serum (Coligan, 1994). Five dilutions were used to determine the AH_{50} values of serum; the dilution range used for each individual serum was calculated empirically. The results were plotted according to Kabat and Mayer (1/serum dilution vs. y/(1-y) where y= proportional lysis) (Mayer, 1961). Lines were plotted using linear regression and the AH_{50} value of the serum was calculated using interpolation where y/(1-y)=1 (i.e. 50% haemolysis). Standard errors given are a measure of deviation from mean AH_{50} value (three replicants). AH_{50} values per ml of each serum tested with or

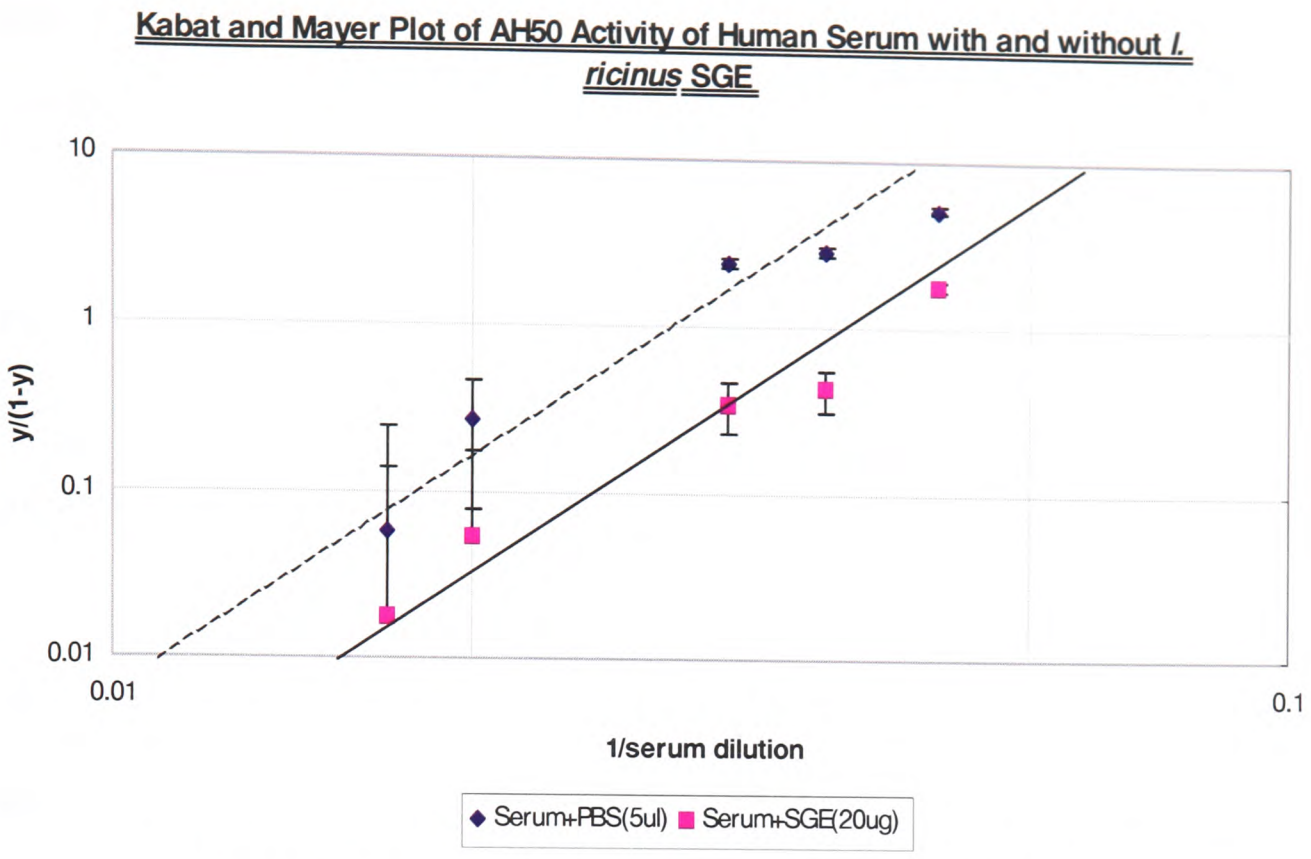


Fig 7.1 Examples of Kabat and Mayer plots (1/serum dilution vs. y/(1-y) where y= proportional lysis) used to calculate AH_{50} values of sera given in Table 7.1. AH_{50} assays were carried out over a range of 5 serum dilutions with and without *Ixodes ricinus* SGE (one salivary gland equivalent), using (top) human and (bottom) pheasant sera as a source of complement. Lines were fitted using linear regression (bars show ± standard error). The mean AH_{50} value of a serum, i.e. the reciprocal of the serum dilution where y/(1-y)=1 (i.e. 50% lysis), was calculated by interpolation. All values are expressed as AH_{50} units per ml of serum.

without SGE from the three species of tick (Table 7.1) were derived from Kabat-Mayer plots of the sort shown in Fig 7.1.

Ixodes ricinus SGE inhibited alternative pathway activity when human, red deer, hedgehog or pigeon serum were used as a complement source, but not when canine or pheasant serum were used (Table 7.1). Likewise, *I. hexagonus* SGE inhibited complement activity of human, red deer and hedgehog serum but, unlike *I. ricinus* SGE, it showed inhibition with canine but not pigeon serum. SGE from the seabird tick, *I. uriae*, inhibited complement when tested with pigeon serum, but not with human or pheasant serum.

SGE source	Serum	Treatment	[2]AH_{50}	[3]± S. E.	[4]Δ AH_{50}
I. ricinus	Human	PBS	315.4	7.946	**-104.0**
		SGE	211.4	10.104	
	Red Deer	PBS	377.4	13.457	**-78.0**
		SGE	299.4	8.866	
	Dog	PBS	178.6	3.676	**+19.7**
		SGE	198.3	2.940	
	Hedgehog	PBS	312.5	10.233	**-105.8**
		SGE	206.7	5.848	
	Pigeon	PBS	153.3	3.066	**-31.3**
		SGE	122.0	3.027	
	Pheasant	PBS	259.0	6.929	**+9.8**
		SGE	268.8	5.148	
I. hexagonus	Human	PBS	299.4	4.063	**-65.3**
		SGE	234.1	4.703	
	Red Deer	PBS	434.8	5.853	**-83.9**
		SGE	350.9	5.341	
	Dog	PBS	195.7	2.548	**-75.6**
		SGE	120.1	2.456	
	Hedgehog	PBS	312.5	5.802	**-205.8**
		SGE	106.7	5.051	
	Pigeon	PBS	120.5	4.692	**+2.6**
		SGE	123.1	1.986	
	Pheasant	PBS	193.8	4.932	**+4.2**
		SGE	198.0	2.227	
I. uriae	Human	PBS	147.1	2.629	**+12.4**
		SGE	159.5	4.362	
	Pigeon	PBS	170.3	5.663	**-32.1**
		SGE	138.2	3.940	
	Pheasant	PBS	174.1	5.464	**+6.0**
		SGE	185.2	2.603	

Table 7.1 AH_{50} values per ml of serum of various species in the presence or absence of SGE (20 μg) from *Ixodes* ticks

[2] AH_{50} units per ml of serum are calculated from Kabat-Mayer plots (i.e. volume of serum required for y/(1-y)=1 unit)

[3] ± standard error AH_{50} units per ml (three replicants)

[3]ΔAH_{50} value = AH_{50} (SGE) – AH_{50} (PBS) (inhibition shown in bold type)

7.3.2 **Immunoaffinity chromatography of *I. ricinus* SGE with canine serum**

Dot blot tests were carried out using *I. ricinus* and *I. hexagonus* SGE immobilised on nitrocellulose and immunoprobed with either human or canine serum and the relevant anti-IgG HRP-conjugate. These results showed SGE reactivity against both human and canine serum (data not shown). This might suggest that the lack of anti-complement activity of *I. ricinus* SGE against dog serum (Table 7.1) could be caused by the presence of antibodies directed against *I. ricinus* in the serum. To test this possibility, the SGE was immunoaffinity purified using a protein A agarose (IgG binding) column pre-absorbed with canine serum. Five µl of immunoabsorbed SGE and the eluted immunoglobulin bound fractions (pH 4, 3 and 2) were tested for activity in an AH_{50} assay using human serum (5 µl-equivalent to 81% haemolysis in the absence of SGE) (Table 7.3). The immunoabsorbed SGE inhibited 83.8% of the complement activity of the PBS control, whereas the antigen containing fractions showed little inhibition of haemolysis. This suggests that the lack of inhibitory activity of *I. ricinus* SGE against the alternative pathway of complement in canine serum is not due to the presence of specific antibodies against the activity, but reflects serum species specific sensitivity.

Treatment	Absorbency range (412nm)	Mean Haemolysis Inhibition (% of PBS control)
PBS	0.431-0.433	**(0)**
Untreated SGE	0.043-0.049	**89.4**
Immunoabsorbed SGE	0.068-0.072	**83.8**
Eluted fraction pH 4	0.358-0.365	**14.6**
Eluted fraction pH 3	0.383-0.391	**10.5**
Eluted fraction pH 2	0.425-0.428	**1.4**

Table 7.2 Anti-complement activity of *Ixodes ricinus* SGE immunoabsorbed with dog serum. AH_{50} assays were carried out with 5 µl of human serum (equivalent to 81% haemolysis in the absence of SGE) and 5 µl of each sample.

7.4 Discussion

The complement system is involved in many host inflammatory responses, consequently modulation of complement activity is essential for the successful feeding of ticks. Inhibition of the alternative pathway of complement has been shown in the female adults of *Ixodes dammini* (Ribeiro, 1987a). The results of this chapter this observation is extended to the European *Ixodes* ticks, *I. ricinus*, *I. hexagonus* and *I. uriae*. In addition, the results show a correlation between host preference and inhibitory activity.

The differential ability of salivary products from ticks of each *Ixodes* species to inhibit the activity of complement in sera from various vertebrate species (Table 7.1) is largely consistent with the ticks' recorded feeding habits. *I. ricinus* SGE inhibited alternative pathway activity in human, red deer, hedgehog and (weakly) pigeon sera, of which only pigeons are unrecorded hosts. Humans are incidental, but not uncommon hosts. Complement from pheasant or dog was not inhibited. Although many dog-owners report ticks feeding on their pets, they do not usually distinguish between tick species (*I. canisuga, I. hexagonus* and *I. ricinus* all feed on canids), nor record the success with which attached ticks feed to engorgement. In the UK, pheasants are now very significant hosts for nymphal *I. ricinus* (Hoodless *et al.*, 1999), despite being exotic to Europe (Tapper, 1992). However, there are no records of *I. ricinus* adult females feeding on pheasants in nature. In this study, SGE was used from adult female ticks. If the immature stages also inhibit the alternative pathway of complement (section 6.3.4), they may show a different pattern of activity that reflects the different host preferences compared with

adults. Although *I. ricinus* has been recorded on a wide range of hosts, its adults feed mainly on large mammals such as deer and sheep and not on the smaller mammals and birds that feed the immature stages (Hillyard, 1996).

Ixodes hexagonus SGE inhibited alternative complement activity in canine, hedgehog, human and red deer serum but not pheasant or pigeon. *I. hexagonus* is known as the hedgehog tick because it is nidiculous and commonly recorded in the easily accessible nests of hedgehogs, especially in suburbia. It also frequently parasitizes other "nest-dwelling" mammals such as foxes and mustelids, but not birds (Arthur, 1953). The complement activity of hedgehog serum is inhibited twice as strongly by SGE from *I. hexagonus* as from *I. ricinus* (Table 7.1). This is consistent with the much higher proportion of records of *I. hexagonus* (27%) compared with *I. ricinus* (0.25%) feeding on hedgehogs (Martyn, 1988). Canines and humans may pick up *I. hexagonus* in semi-natural settings such as parks and gardens. An unusually high number of these ticks were recorded feeding on humans in the summer of 1944 when people sought refuge in garden air-raid shelters (Arthur, 1953).

The natural hosts of *I. uriae* are seabirds, of which forty-eight species have been recorded as hosts (Wilson, 1970), but whose sera were not tested. *I. uriae* SGE inhibited the complement activity of pigeon serum but not pheasant or human. Unlike pheasants and other game birds, pigeons and doves *(Columbidae)* frequent sea cliffs.

All three of the *Ixodes* species tested inhibited the alternative pathway of complement in one or more of the species serum tested. This suggests the evolution of a common anti-complement mechanism in prostriate ticks. From the presented data it is apparent that anti-complement activity of the *Ixodes* species tested is host species-

specific, suggesting divergent targets of anti-complement activity between the different *Ixodes* species. There is little information about how the complement system differs between species (Nonaka, 1997). The species specificity of *Ixodes* tick anti-complement activity may provide new insights into these variations. Based upon the results of this chapter, it is clear that both ecological and physiological factors play a role in determining the host range of a particular tick species, but as yet it is not possible to distinguish cause and effect.

CHAPTER 8

CONCLUDING DISCUSSION

8.1 Introduction

Ixodid ticks are obligate haematophagous arthropods that feed for extended periods of time. A successful bloodmeal by the tick is a pre-requisite for the transition to the next developmental stage or for egg development in the female adult. As with any haematophagous arthropod, a successful bloodmeal by ticks depends firstly upon the acquisition and secondly upon digestion of the imbibed blood. The latter process is investigated in Chapter5. Haemolytic activity is described in the salivary glands of female *I. ricinus* ticks. This activity was shown to be Mg^{2+}-dependent, but was inhibited at higher concentrations of Mg^{2+} (i.e. 10 mM). The role of such regulation *in vivo* is unknown.

The acquisition of a bloodmeal by the tick is a function of both ecological (i.e. the ability to find a host) and physiological factors (i.e. the ability of the tick to feed successfully on a particular host). The feeding success of a tick upon a given host depends on its ability both to attach and to feed to repletion, which in turn relies upon the ability of the tick to antagonise host specific inflammatory responses (Ribeiro, 1987a). The manifestation of this relationship at the physiological level is described as the 'tick-host interface'. The extended feeding period of ixodid ticks allows ample time for the vertebrate host to mount both innate and adaptive immune responses. Chapter 3

investigated the adaptive immune response of host species to tick infestations as shown through antigenic profiling. Chapter 6 and 7 investigated the innate response of host species as exemplified by examination of the alternative pathway of complement activity and mechanisms of the countermeasures employed by *Ixodes* ticks.

The tick-host relationship is the result of co-evolution between parasite and vertebrate host over 245 million years (Hoogstraal and Aeschlimann, 1982). The development of a veritable pharmacopia of bioactive compounds in the salivary glands of ticks has resulted in the successful parasitism of all classes of terrestrial vertebrates and the evolution of over 850 species. Our current understanding of vertebrate immunology is at best fragmentary. Consequently, the immunomodulatory activities of tick saliva can provide a valuable insight into previously undescribed vertebrate immune responses. The problems associated with obtaining enough protein from tick salivary glands for traditional biochemical analysis are prohibitive. The production of a subtractive cDNA library from cDNA enriched for saliva-associated transcripts described in Chapter4 provides a valuable resource for the future investigation of salivary compounds in theoretically unlimited quantities.

8.2 Salivary gland changes during the feeding process

The results of Chapter3 showed that the mean weight of female adult *I. ricinus* ticks feeding on rabbits increased from a mean weight of 6 mg for unfed ticks to 264 mg for ticks fed six days or more. This is an increase of over forty times in body weight. There was an exponential increase in tick weight from unfed to ticks fed for five days; the

rate of this increase was much greater after six days of feeding. This probably reflects the onset of the fast phase of feeding. Concurrently, the soluble protein concentration of the salivary glands was shown to increase in a linear fashion throughout the feeding period. The increase in the protein content was accompanied by a changing protein profile in the salivary glands throughout the feeding period. Presumably, these feeding associated proteins are produced temporally as a reflection of the changing tick-host interface throughout the extended feeding period. Whether such changes in salivary gland protein profile are as a direct result of host responses (i.e. host modulated) or whether they represent a pre-programmed cycle is unknown. In addition, immunoprobing with anti-adult rabbit serum showed that the antigenic profile of SGE proteins changes throughout the feeding period. The changing pattern is presumably a direct result of the changing protein expression of the salivary glands. It is not known whether, like other parasites such as *Trypanosoma brucei*, the changing antigenic profile of feeding adult female *I. ricinus* salivary glands has an immunoevasive role (Turner and Michael, 1997).

8.3 The antigenic profile of the different developmental stages of *I. ricinus*

Any immune response involves, firstly, recognition of a pathogen or of foreign material, such as is presented to the host during tick feeding, and secondly, mounting a reaction to eliminate it (effector response). The development of acquired immunity against ticks is mediated largely by antibody formation (Willadsen, 1980). The antigenic profiling of tick proteins provides a way in which to measure the immunological

perception of tick infestation (i.e. immunogenic substances which are exposed to host resulting in the production of specific IgG antibodies).

In Chapter 3 evidence is presented to suggest that the salivary glands are the major source of antigens exposed to the host during tick infestation. Antigenic profiling of whole tick homogenates showed that the majority of antigens were common to the SGE samples. In contrast, immunoprobing of female adult gut homogenate with rabbit anti-adult serum showed only one major antigen, presumably exposed to the host as the result of regurgitation of gut material. Surprisingly, the male adult whole tick homogenate showed a similar antigenic profile to that of the female whole tick homogenate. This suggests that many of antigens are common to the salivary glands of male and female adult *I. ricinus* ticks, despite the fact that the former is widely believed not to feed.

Immunoprobing of the whole tick homogenates and SGE of the different developmental stages of *I. ricinus* with the respective resistant rabbit serum revealed that different antigens are exposed to the host during infestation by the various life stages. Results consistent with this hypothesis were observed with SGE samples immunprobed with serum obtained from hamsters infested with the various developmental stages of *I. ricinus*. Some of the life-stage specific antigens were present in the SGE samples probed with either anti-larvae or anti-nymph but not anti-adult rabbit serum. These proteins are presumably present in the salivary glands of adult females but are not exposed to the host during the bloodmeal. This suggests that the proteins may be sequestered intracellularly in the salivary glands, suggesting some form of secretary regulation in the salivary glands of *I. ricinus* ticks.

8.4 Host species specific immunological perception of *I. ricinus* infestation

Comparison of both 'natural' and 'laboratory' host antigenic responses to *I. ricinus* larvae are shown in Fig 3.7. These data show that the antigens that elicit an antigenic response during a feeding session vary between host species; this is true even in the closely related *A. flavicollis* and BALB/c mouse. Presumably, such different immunological perception of tick infestation by different host species is also reflected at the effector level. The way in which immunity is expressed varies greatly, depending upon the host species and tick species that are investigated (Willadsen, 1980). Comparison of the natural hosts of *I. ricinus, C. glareolus* and *A. flavicollis* showed that both hosts developed specific antibodies against infestation. However, the former host develops resistance after repeated infestations whereas the latter species does not. This suggests that resistance against tick infestations occurs primarily at the effector level. Consequently, it is likely that tick immunomodulatory activity is the major factor in the feeding success of the tick. The observation of host species specific anti-complement activity (Chapter7) is consistent with this hypothesis. This research is particularly relevant in light of the growing research towards the development of anti-tick vaccines based upon immunogens (Wikel *et al.*, 1996a).

8.5 Anti-complement activity in *Ixodes* ticks

Anti-complement activity was described in Chapter7 associated with the salivary glands of the *Ixodes* species ticks, *I. ricinus, I. hexagonus* and *I. uriae*. Chapter 6 investigated the mechanisms of the anti-complement activity of *I. ricinus*. SGE was shown to inhibit the alternative but not the classical pathway of complement. The salivary glands of the argasid tick *O. moubata*, but not the metastriate ticks *D. andersoni* and *R. appendiculatus*, were also shown to have anti-alternative pathway activity. The latter species was however shown to have anti-classical pathway activity (Miles Nunn- IVEM). It is possible that anti-complement activity in ticks co-evolved with the development of the vertebrate immune system. Inhibitory activity against the more ancient alternative pathway of complement was found in salivary glands of argasid and prostriate ticks, in contrast the relatively recent lineage, metastriate ticks, were shown to inhibit the classical pathway of complement which is restricted to higher vertebrates.

Anti-complement activity was observed in the salivary glands of unfed and feeding adult female *I. ricinus* ticks. It was proposed that this might be a reflection of the necessity to inhibit complement activity rapidly after the initial incision by the tick, which would quickly cause activation of complement during the acute phase response. The presence of activity in the salivary glands of unfed female adult ticks, suggests that the activity may be sequestered intracellularly in the salivary glands, possibly stored in the granules of type II or III acini. Whether the proposed sequestered antigens described in Chapter3, represent anti-complement factors is unknown. Anti-complement activity was tested in the whole tick homogenates of the various developmental stages of *I.*

ricinus, however the presence of non-complement specific haemolytic activity in these samples, presumably gut-associated, means that these results were unclear.

The molecular basis of the anti-complement activity of *I. ricinus* SGE was examined in Chapter6. These data suggested that the anti-complement activity probably act at several points on the alternative pathway of complement (Fig 1.5). Consequently, it is likely that several SGE factors are involved in inhibition. The proposed model of inhibitory activity is shown in Fig 6.32. The results of Chapter 6 demonstrated that SGE interacts in some way with factor D; this was indicated from the results of competition assays, native-PAGE western analysis and by affinity chromatography. It possible that an SGE factor(s) activity binds factor D in a way that prevents conformational activation of the protease by its substrate (i.e. C3bB). It should be noted however that the activity of factor D was apparently not inhibited when tested in assays containing pre-formed convertase (section 6.3.13).

In contrast, investigation of the role of factor B in anti-complement activity suggested that this component was not directly implicated. Factor B cleavage was inhibited by SGE in AH_{50} assays but not in assays containing pre-formed convertases, or CVF suggesting that the inhibitory activity was an indirect effect of the SGE anti-complement activity. Consistent with these data, competition assays using an excess of purified factor B did not effect SGE anti-complement activity and little or no specific binding was observed in affinity chromatography experiments.

The results of experiments described in Chapter 6 suggest that the main target of complement-modulation by *I. ricinus* SGE is C3. The evidence suggests that C3 is not cleaved to C3b and C3a by the convertase in the presence of SGE. This is not due to a

direct effect upon the convertase formation or its stability, but is probably due to the specific cleavage of the α-chain of C3 (to form α1-chain) by an SGE factor to form a C3b-like molecule, that was designated C3b'. Cleavage occurs at the C-terminus of C3 presumably resulting in a conformational change in the C3b'. It is proposed that the C3b' molecule does not bind factor B or factor H (i.e. C3b'B or C3b'H are not formed), effectively preventing the formation of the alternative convertase. A proteolytic activity was characterised in *I. ricinus* SGE as being specific for the α1-chain of C3b' (i.e. not C3b (α'-chain) or C3(H_2O)), that results in further cleavage of C3b' into three fragments of 36, 31 and 26 kDa, designated C3a1, C3a2 and C3a3 respectively. N-terminal sequencing of the C3a2 and C3a3 products suggest that this cleavage probably occurs after C3b' cleavage. The α1-chain is cleaved at three sites by SGE-proteolytic activity, one adjacent to residues implicated in the binding of factor B by C3b, the other in the C3dg region, representing the products C3a3 and C3a2 respectively. The other cleavage site representing C3a1 has yet to be confirmed. The proposed model of cleavage of C3 by *I. ricinus* salivary gland activity is shown in Fig 6.32. It is suggested that this activity further inhibits complement activity of the C3b' molecule. Similar cleavage sites in both molecules suggest that a single species of SGE-protease maybe involved. Proteolysis of the α1-chain was greatly enhanced by acid treatment of the SGE. In light of this information, it is not clear if the proteolytic activity reflects anti-complement activity in the SGE of the ticks or whether it is a separate phenomenon. Alternatively, it is possible that acid treatment may reflect a conformational change caused by a serum factor, perhaps through the proteolytic activated complement components themselves. Such an

exquisite regulatory mechanism, would provide a magnificent example of the dynamism of the tick-host interface at the molecular level.

Plasmon resonance studies, as well as the proteolytic studies demonstrated that the interactions of SGE with C3 are probably conformationally specific. Evidence was presented to suggest that an SGE factor may bind to C3(H_2O) but not C3.

It is clear that the anti-complement activity of *I. ricinus* SGE probably involves several factors acting at different points within the alternative pathway of complement. This is probably necessary due to the elaborate regulation and dynamics of this pathway and of the complement system in general (Chapter1). The observation that activity inhibits the alternative but not the classical pathway, although C3 is a central component of both pathways, suggests that SGE activity acts at another point specific to the alternative pathway. The mechanistic reason behind the serum species sensitivity of anti-complement activity in the different *Ixodes* species ticks tested in Chapter7 remains unknown. Very little research has been carried out upon the differences between complement components in different species. C3 is the most widely studied component of non-human complement and complete or partial cDNA sequences have been described for the mammals; rabbit, rat, mouse and guinea-pig. Comparison of these sequences with human C3 showed a high level of conservation (about 80%) at the nucleic and amino acid level (Nonaka, 1997). The snake C3b analogue, CVF successfully forms a fluid-phase convertase with factor B from many species; the almost universal toxicity of the venom to animal species serves as a testimony to its cross-species reactivity. No data exists upon factor D in non-humans, although factor D has been isolated from carp, the primary structure has yet to be determined (Yano and Nakao, 1994). This might suggest that the

serum species sensitivity of anti-complement activity is due to a factor other than C3, possibly factor D. Alternatively, it is possible that the C3 cleavage sites recognised by SGE-proteases are not conserved between the host species tested in Chapter 7. The relevance of the cleavage of iC3b to form C3dg and C3c by factor I has been called into question because the cleavage site is not conserved between human, mouse and rabbit C3 (Lambris, 1988).

Anti-complement activity in ixodid tick saliva may also play a role in pathogen transmissibility. The causative agent of Lyme disease, *Borrelia burgdorferi,* is vectored by *Ixodes* species, particularly those of the *I. ricinus* complex. Different genospecies of *B. burgdorferi s.l.* show differential host-specific transmissibility from vertebrates to ticks (Kurtenbach *et al.*, 1998a). The reservoir competence of a given host for a particular genospecies is consistent with the resistance of that genospecies to alternative complement activity of that host serum (Kurtenbach *et al.*, 1998b). Treatment of those genospecies that are sensitive to mammalian sera (e.g. *B. garinii*) with *I. ricinus* SGE reduces the borrelicidal activity of the serum (K. Kurtenbach, personal communication). Thus anti-complement activity in *Ixodes* ticks may help protect susceptible genospecies of *Borrelia* as they are transmitted in saliva from tick to a host, although not from host to tick when *Borrelia* are unprotected in the mid-gut of the tick.

Knowledge of how parasites such as ticks modulate host responses may provide insights into previously undescribed territories of vertebrate immunology. Characterisation of the mechanisms of tick anti-complement activity will clarify the roles and operation of the complement system in vertebrate immunity and its impact on host-vector-pathogen interactions.

8.6 'Natural' vs. 'laboratory' models of the tick-host interface

The aim of this thesis was to investigate the molecular basis of tick-host interactions. The research described in this thesis, far from being an exhaustive treatment of this subject, has served to illustrate the complexity of the tick-host interface. In addition, it has reinforced the necessity of investigating 'natural' as well as 'laboratory' models of tick-host interactions when describing the molecular interactions between ticks and their host species. There is a gap in the literature between the '*in vivo*' ecological observations of 'natural' tick-host models and the '*in vitro*' laboratory approach of study which is based primarily on 'laboratory' hosts. The results of this thesis provide evidence that the host immune response to a tick infestation varies with the host species used at both the innate (Chapter7) and the adaptive level (Chapter3). In addition, the effectiveness of tick counter-measures against host responses (i.e. anti-complement activity) was shown to be host species specific (Chapter7). The pattern of the serum sensitivity of anti-complement activity in the *Ixodes* species tested was consistent with the host range of the tick species tested.

These results not only suggest that the 'laboratory' models used differ from 'natural' models of the tick-host interface, but that the tick-host interface varies significantly between even closely related host species. This suggests that the tick-host interface is much more complex and dynamic than was previously envisaged. This realisation is consistent with the increasing complexity observed between tick-host-pathogen relationships as exemplified by the serum species complement sensitivity of

Borellia genospecies (Kurtenbach *et al.*, 1998b). These observations should be considered in the future development of potential pharmacological compounds from tick salivary glands and of anti-acarine research.

8.7 Future work

It is clear that the findings of this thesis provide some answers to the original aims described in Chapter1. However, it is also apparent that the results described pose many more questions and subsequently research.

In particular, the anti-complement activity of *I. ricinus* SGE will continue to be explored and isolation of the factors involved will be the focus of future work. In addition, the characterisation of clones generated by the subtractive library remains a gargantuan task, but one in which the potential results generated could prove particularly fruitful in the light of future disclosure of the processes involved in the vertebrate immune system.

8.8 Potential uses of complement inhibitory compounds

As a footnote, a few examples are given below where the development of anti-complement compounds could be of clinical use. Complement has been shown to be involved in many non-pathogen mediated immune responses, particularly those involving auto-immunity and inflammatory reactions.

Complement has been shown to play a major role in xenotransplant hyperacute rejection. The involvement of the alternative pathway has been demonstrated in the rejection of rabbit hearts by human blood (Forty *et al.*, 1992) and of rat hearts in foetal sheep (Rajasinghe *et al.*, 1996). Transgenic pig livers expressing human decay accelerating factor (hDAF) (Pascher *et al.*, 1997), and selective inhibition of the alternative pathway by soluble complement receptor1 (sCR1[desLHR-A]) (Gralinski, 1996) was to shown to protect tissue from complement-mediated damage and to reduce the severity of hyperacute rejection. sCR1 was also successfully used to inhibit complement activation during cardiopulmonary bypass (Gillinov *et al.*, 1993). Alternative pathway activation is a major mediator of ischemia/reperfusion injury (Rubin *et al.*, 1989) and the inhibition of complement using sCR1 reduces reperfusion injury in tissue (Shandelya, 1993).

Activation of the alternative complement activity is the major mediator of autoimmune diseases such as systemic lupus erythematosus (Shandelya, 1993). Activation has also been associated with the synovial tissue from patients with osteoarthritis and rheumatoid arthritis (Guc *et al.*, 1993) and the anti-bronchial asthma compound ibudilast acts through inhibition of the alternative pathway (Onodera *et al.*, 1991). Alternative complement activation is one of the most important mediators of renal injury (Levy and Habib, 1981; Stapleton *et al.*, 1981), for example glomerulnephritis (Linshaw *et al.*, 1987) and glomerular sclerosis. Activation has also been linked to burn injury in mice, while decomplementation by cobra venom factor (CVF) was reduces mortality due to this injury (Gelfand *et al.*, 1982).

Finally the alternative pathway may also enhance HIV-1 infection and mask HIV-1 neutralising antibody activity in conjunction with antibody (Robinson, 1988), as well as being necessary for the binding of HIV to complement receptor CR2 expressed abundantly on follicular dendritic cells and immature B cells (Montefiori *et al.*, 1993).

Any advances in our understanding of the ways in which the alternative pathway of complement maybe inhibited, therefore offers significant potential in many areas of medicine. Indeed, it is not without the realms of possibility that the anti-complement activity of tick saliva may one-day be utilised for more general clinical purposes.

CHAPTER 9

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