HOST GENETIC FACTORS IN SUSCEPTIBILITY TO MALARIA AND TUBERCULOSIS

by

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A thesis submitted for the degree of Doctor of Philosophy

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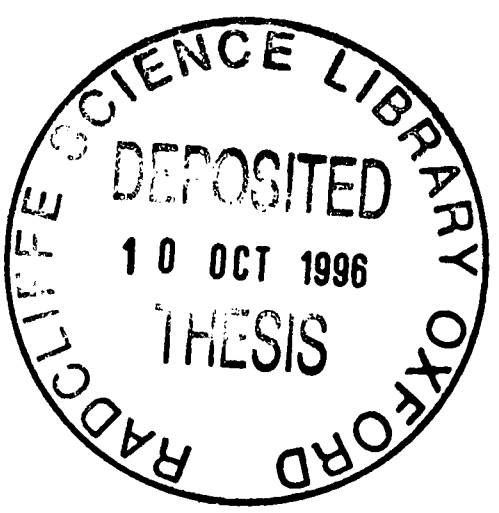

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Abstract

Plasmodium falciparum and *Mycobacterium tuberculosis* infections collectively cause as many as five million deaths world-wide each year. In the most afflicted populations, currently available drugs and vaccines appear inadequate. By offering insight into the pathophysiology of diseases, genetic studies provide options for new therapeutic approaches to major health problems. The results of case-control studies of genetic factors associated with disease outcomes in malaria and tuberculosis in an African setting are presented in this thesis.

Glucose-6-Phosphate dehydrogenase (G6PD) deficiency, the commonest enzymopathy of humans, affects over 400 million people. The geographical correlation of its distribution with the historical endemicity of malaria suggests that this disorder has risen in frequency through natural selection by malaria. However, attempts to confirm that G6PD deficiency is protective in case-control studies of malaria have yielded conflicting results. Hence, for this X-linked disorder, it is unclear whether both male hemizygotes and female heterozygotes are protected or, as frequently suggested, only females. Furthermore, how much protection may be afforded is unknown. In two large case-control studies of over 2000 African children, I found that the common African form of G6PD deficiency (G6PD A-) is associated with a 46-58% reduction in risk of severe malaria for both female heterozygotes and male hemizygotes. A mathematical model incorporating the measured selective advantage against malaria suggests that a counterbalancing selective disadvantage, associated with this enzyme deficiency, has retarded its rise in frequency in malaria-endemic regions.

There is some evidence that two T helper cell subsets, Th1 and Th2, regulate the immune response and thus influence the course of infections in mammalian hosts. These T cell subsets are reciprocal and associated with distinct cytokine profiles. Th2 T cell differentiation is promoted mainly by interleukin-4. Analysis of an IL-4 promoter polymorphism indicates that homozygosity for a putatively upregulatory IL-4 promoter variant is associated with a signficantly increased risk for severe malaria whilst heterozygotes are protected against this condition.

Epidemiological evidence implicates host genetic factors as major determinants of variable susceptibility to tuberculosis. Most attempts to define the genetic factor(s) have focused on the HLA genes but only one result, an association of HLA-DR2 with increased susceptibility to disease in Asian populations, has been reported with any consistency. The genetic component in tuberculosis is likely to be determined by multiple genes and, therefore, in this study, the role of both HLA and non-HLA candidate genes was investigated. No association was found with variants of the macrophage gene, *NRAMP1*, the homologue of which has been implicated in the regulation of genetic resistance in the mouse model. Examination of certain class I and class II HLA alleles as well as the -590 interleukin-4 promoter polymorphism also did not show any association with disease. However, heterozygotes for a promoter polymorphism at position -238 of the tumour necrosis factor gene and homozygotes for dysfunctional variants of the gene encoding the collectin, mannose binding protein, were both at increased risk of developing pulmonary tuberculosis.

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Attribution

*S. C. Khoo: The preliminary analysis of G6PD deficiency in Gambian male severe malaria cases and controls.

*Adrian Hill and Catherine Allsopp: The HLA typing on the Gambian adult controls in the tuberculosis study.

*Sunetra Gupta: The mathematical modelling on the G6PD data.

*William McGuire: The typing of Gambian adult controls for the TNF -308 promoter polymorphism

Publications

C Ruwende *et al.* (1995). Natural selection of hemi- and heterozygotes for G6PD deficiency in Africa by resistance to severe malaria. *Nature*. **376**, 246-249

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Figures and Tables

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Abbreviations

α	alpha
AIDS	Acquired immune deficiency syndrome
ANE	buffer solution containing sodium chloride, sodium acetate, EDTA & SDS
APC	antigen presenting cell
ATP	adenosine triphosphate
β	beta
bp	base pair
BCG	Bacillus Calmette Guerin
B35	HLA-B*35
B53	HLA-B*53
°C	degrees centigrade
Ca	calcium
CD (as CD4)	clustering determinant
CMI	cell mediated immunity
CRD	carbohydrate recognition domain
DIG	digoxigenin
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dNTP	deoxynucleotide triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleotide
DR	HLA-DRB
DR1	HLA-DRB1*01
DQ	HLA-DQB1
EDTA	ethylenediamine tetra-acetic acid disodium salt
γ	gamma
g	gram
Gam	Gambia
G6PD	glucose-6-phosphate dehydrogenase
Hb	haemoglobin
HbA	normal haemoglobin
HBV	hepatitis B virus
HIV	human immunodeficiency virus
HbS	sickle haemoglobin
HLA	human leucocyte antigen
HPFH	hereditary persistence of foetal haemoglobin
hr	hour
ICAM	intercellular adhesion molecule-1
IFN-γ	interferon-gamma
Ig	immunoglobulin
kb	kilobase
kD	kilodalton
kg	kilogram
Km	kilometre

Ken Kenya
IL-2,-4 interleukin-4
LMP low molecular weight protein
LPS lipopolysaccharide
μg microgram
μl microlitre
μM micromolar
Mb megabase
ml millilitre
mM millimolar
mal. malaria
MBP mannose binding protein
MHC major histocompatibility complex
min. minute
MRC Medical Research Council
mRNA messenger RNA
M. tb *Mycobacterium tuberculosis*
NADPH nicotinamide adenine dinucleotide phosphate, reduced form
ng nanogram
nm nanometre
Nramp1 natural resistance associated macrophage protein1 (mouse)
NRAMP1 natural resistance asociated macropahgeprotein1 (human)
OR odds ratio
^{32}P phosphorous radiosotope
PCR polymerase chain reaction
P.falciparum Plasmodium falciparum
RES reticuloendothelial system
RFLP restriction fragment length polymorphism
RNA ribonucleic acid
rpm revolutions per minute
SSC standard saline citrate
SDS sodium dodecyl sulphate
sec. second
sev severe
spp. species
TAP transporter associated with antigen processing (-1 and -2)
TB tuberculosis
TBE buffer containing tris, boric acid and EDTA
TCR T cell receptor
TE tris and EDTA buffer solution
Th T helper lymphocyte
Th1/2 T1/2 helper cell subset
TNF tumour necrosis factor
Tris tris(hydroxymethyl) aminithane
UNICEF United Nations Children's Education Fund
V volt
vs versus
WHO World Health Organisation

CHAPTER 1 - INTRODUCTION

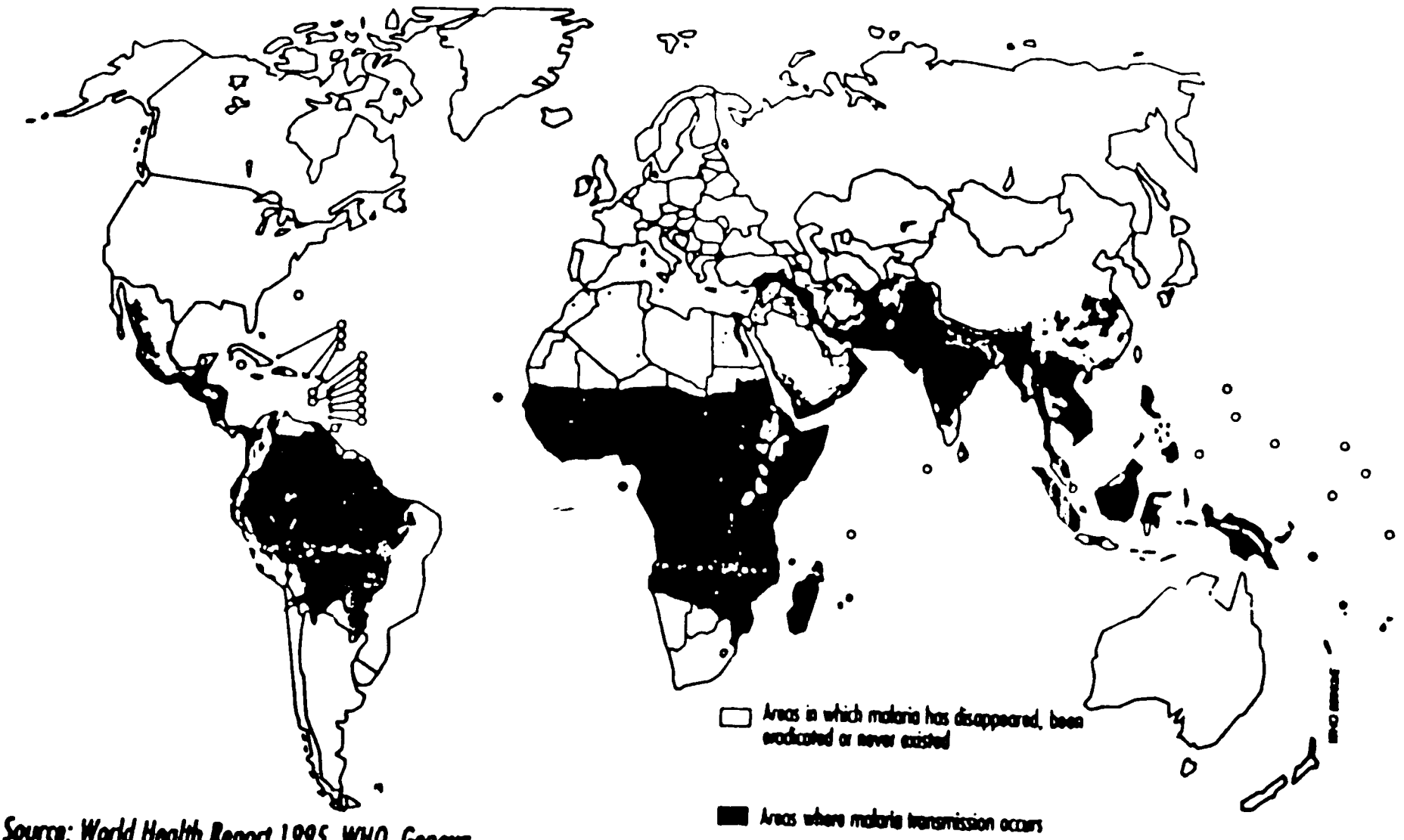
1.1 MALARIA

1.1.1 EPIDEMIOLOGY

Malaria is the world's most important tropical parasitic disease. The geographical area affected by malaria has shrunk considerably over the past 50 years as a result of sustained eradication and control programmes consisting essentially of chemotherapy against the parasite, vector reduction through insecticides and drainage of potential vector breeding sites. Nevertheless, control is becoming increasingly difficult and previous gains are quickly being eroded (WHO, World Health Report 1995). Today malaria is a public health problem in more than 90 countries (Figure 1A), inhabited by a total of some 2 400 million people - 40% of the world's population (World Health Report 1995, WHO). The worldwide incidence of the disease is estimated to be in the order of 300 - 500 million clinical cases each year with 90% of these cases occurring in sub-Saharan Africa. The majority of the remainder are concentrated in six countries - India, Sri lanka, Vietnam, Afghanistan, Columbia and Brazil.

The worsening of the malaria problem in developing countries today is partly linked to an increase in activities such as irrigation projects, deforestation, road building, and mining but it is also due to the disintegration of health services, armed conflicts and mass movement of displaced persons in these regions. In most of the affected countries, malaria exacts an enormous toll in lives, medical costs, and lost labour days (WHO, 1995). Annual mortality due to malaria is estimated at 1.5 - 2.7 million deaths

Figure 1. A Global distribution of malaria



Source: World Health Report 1995, WHO, Geneva.

each year (WHO 1995). The vast majority of deaths are in young children in the rural parts of Africa who on average experience 1-5 clinical acute malaria episodes per year (Greenwood *et al.* 1991.)

The global distribution of malaria is limited by the lower temperature limits, 15-20 °C, for the sporogony phase of the parasite's life cycle. However, even within areas bounded by these isotherms, transmission does not occur at altitudes above 1500 metres, in arid conditions or, as in most of the Central and South Pacific, where there are no suitable vectors. In affected countries, the seasonal pattern of clinical malaria is determined by the variations in vector density. Generally the vector density, and therefore malaria transmission, increase during the wet seasons.

After repeated infection as occurs in endemic areas, hosts usually develop a non-sterile immunity which is reflected less in reduced rates of infection but more in infection episodes of shorter duration and less severity (Molyneaux & Gramiccia, 1980). In endemic areas, the intensity of malaria transmission determines the clinical distribution of clinical symptoms in different age groups. The greater the intensity of transmission, the younger are the children that are most afflicted by severe disease (Greenwood *et al.* 1991).

1.1.2 THE CLINICAL PROBLEM

It is children that are particularly at risk from malaria but other high risk groups include primagravida pregnant women, non-immune travellers, refugees, displaced persons and labourers entering endemic areas. Most of deaths due to malaria in

children are attributable to cerebral malaria in the acute phase, and severe anaemia in chronic malaria. Results from several studies attempting to determine malaria mortality rates in African children suggest that each clinical attack of malaria has a mortality rate of approximately 0.5% (Greenwood *et al.* 1991), with this figure varying from country to country depending upon prevalent parasite drug sensitivity and levels of health resources (Sudre *et al.* 1990). The death rate in children under the age of 5 years is 1-10 per 1 000 per year (Greenwood *et al.* 1991). Estimates from studies conducted in The Gambia suggest that in Africa, 2% of children get severe malaria and that among those in this latter group that are referred to hospital, mortality is as high as 10-30% (Greenwood *et al.* 1991; Greenwood *et al.* 1987). The main complications of malaria, in addition to cerebral malaria and anaemia, include hypoglycaemia, lactic acidosis, acute renal failure, pulmonary oedema, nephrotic syndrome, thrombocytopaenia and, rarely, disseminated intravascular coagulation (DIC). In pregnant women, malaria is associated with higher rates of miscarriage, low birth weights and neonatal deaths (Watkinson & Rushton 1983).

A problem facing most countries in which malaria is endemic, is the emergence of parasite drug resistance. Chloroquine resistance is now common throughout Africa whilst widespread resistance to sulphadoxine-pyrimethamine is found in South East Asia and South America (WHO, 1995). Mefloquine resistance is less widely reported but it appears to be a problem in border areas of Thailand, Cambodia and Myanmar (WHO 1995,). Fortunately, there does not appear to be much resistance to quinine and it remains effective against severe malaria in most parts of Africa. Nevertheless, escalating drug resistance is definitely limiting the choices of anti-malarial drugs

choices available to physicians in endemic countries. New, more effective and affordable drugs are clearly needed but sadly there is little stimulus for the pharmaceutical industry to develop these because of the general poverty of those who suffer most from the disease.

1.1.3 LIFE-CYCLE

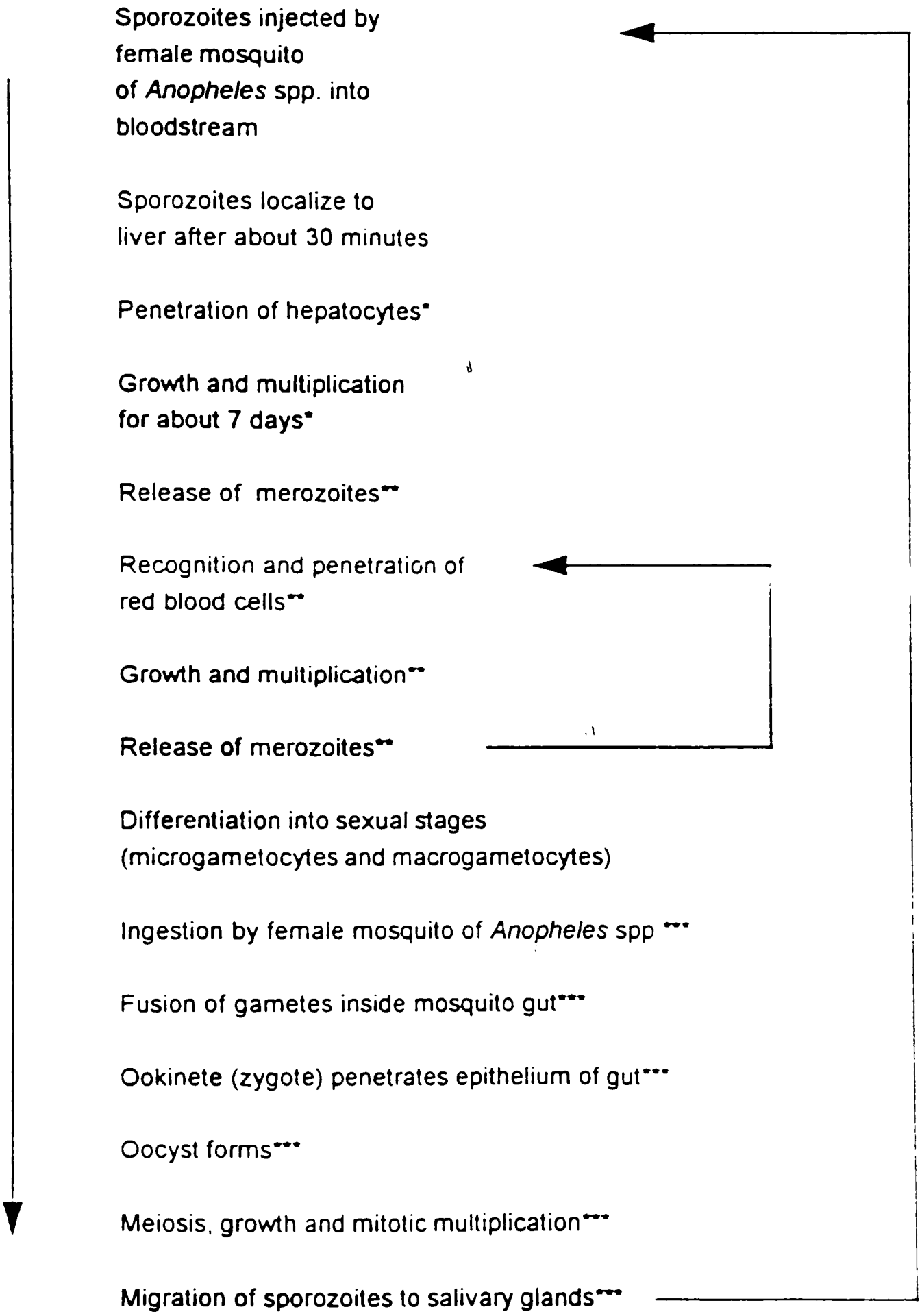
There are four species of *Plasmodium* that cause malaria in humans: *P. falciparum, P. vivax, P. ovale* and *P. malariae. P falciparum*, the most virulent, is responsible for most of the deaths caused by malaria. Malaria is transmitted by the *Anopheles* mosquito although only 40 out of a total of 360 Anopheles species can actually transmit malaria (Ramsdale & Coluzzi, 1975).

In life cycle of *P. falciparum* (Figure 1.B), sporozoite forms are inoculated into the bloodstream from the salivary glands of a female Anopheles mosquito. In this, the pre-erythrocytic stage of the parasite's life cycle, the sporozoites, which have a half life in the peripheral blood sytem of approximately 30 minutes, circulate briefly before binding to and entering hepatocytes. Here, rapid multiplication takes over 5-8 days to produce liver schizonts that give rise to an estimated 20, 000 merozoites per infected hepatocyte. These merozoites are released into the blood stream and the erythrocytic stage of the parasite's cycle begins. The merozoites bind to and then invade red blood cells and by the end of this process the red blood cell's plasma membrane invaginates to form the parasitophorous vacuole. For the first 10 hours, the developing parasites appear as 'ring forms'. After 30 hours, nuclear division begins and schizonts are formed which contain up to 32 merozoites. At 48 hours, the red blood cell ruptures

Figure 1.B

Plasmodium falciparum life cycle

The life cycle is divided in to three "phases", *=exoerythrocytic cycle, **=erythrocytic cycle, ***=mosquito cycle. The parasite is diploid only for a short time, predominantly as the ookinete. The parasite is haploid for the remainder of the life cycle.



and merozoites are released into the circulation to continue further cycles of asexual multiplication. This erythrocytic schizogony may achieve up to a ten fold increase in parasitaemia which is detectable, microscopically, 6 days after the liver stage is completed. After 2 cycles, infection becomes clinically apparent in the host manifesting as paroxysms of fever (accompanying release of merozoites), headache, general malaise, myalgia, arthralgia, chills, nausea and vomiting.

During the erythrocyte cycle some merozoites do not multiply but become committed to form male or female gametocytes (Bruce *et al.* 1990). It is still not really clear what the stimulus is for differentiation into the sexual forms but it may involve metabolic stress. The sexual stage of the parasite's life cycle starts after a male and female gametocyte are ingested by a feeding female *Anopheles* mosquito. Once it is in the midgut of the mosquito, the female gametocyte forms a single macrogamete whilst the male gametocyte undergoes several rounds of nuclear division to produce flagellated microgametes. These microgametes are motile and migrate to fertilise the macrogametes. The resulting zygote enlarges to form a mobile ookinete that migrates through the epithelial wall of of the mosquito midgut eventually resting on the external surface. Here, given the right temperature, the oocyst divides repeatedly to form up to 10 000 sporozoites. 5 days after they have escaped from the oocyst, the sporozoites travel up through the haematocoelomic fluid to enter the acinar glands of the mosquito's salivary glands. At this stage, the sporozoites are infectious when injected into the host.

There are a few major differences between the life-cycle of *P. falciparum* and the life-cycles of the other malaria-causing *Plasmodium* species. With *P. vivax* and *P. ovale*, some of the sporozoites entering the liver form dormant hypnozoites which subsequently only divide after a variable period of weeks to months to cause further blood-stage infections or relapses. In addition, *P. malariae* differs from the other species by having a longer erythrocytic schizogony cycle (72 hours) and blood stage parasites that can persist for up to 40 years. Unlike *P. falciparum* malaria, with *P. vivax*, *P. ovale* and *P. malariae* infections high parasitaemias are rare as erythrocyte invasion is more effectively restricted to reticulocytes than with the former. Moreover, the non-falciparum Plasmodial species do not undergo sequestration and hence infrequently cause organ-specific disease syndromes. As a result, they rarely cause mortality although death from splenic rupture or intercurrent illness may occur in previously debilitated individuals who get infected. *P. malariae* can also cause nephrotic syndrome particularly in children.

1.1.4 MALARIA RESISTANCE GENES

Malaria has been a common killer in human populations since ancient times (Bruce-Chwatt 1988) and, as a result, it has been a powerful selective agent in humans as both parasite and host have evolved to increase biological fitness through natural selection. Indeed, the genotypic changes that have arisen in the human genome over several generations to yield the so called 'malaria resistance genes' are not only of scientific interest as arguably the most outstanding examples of natural selection, but they are also of considerable medical importance too because of their impact on our understanding of the pathophysiology and control of this disease.

Malaria resistance genes (summarised in Table 1.a) can be broadly classified into those of innate host defense and those of the acquired immune system.

1.1.4.1 HOST INNATE IMMUNITY GENES

These resistance genes (Table 1.1) appear to act primarily on the erythrocytic stage of the parasite life cycle.

1.1.4.1.i Haemoglobin S (HbS)

The HbS variant which arises from a single base change in codon 6 of the β–globin gene that leads to a glutamate to valine substitution in the protein, is probably the best example of gene selection by disease and certainly the best known malaria resistance gene. It was Alison, noting the striking correlation between geographical distribution of HbS with areas with a history of endemic malaria, who first presented various types of evidence that *P. falciparum* was responsible for maintaining HbS in populations (Alison 1954a; Alison 1954b; Alison 1954c). He observed that while homozygous individuals for the sickle gene usually developed sickle cell anaemia rarely surviving beyond the age of 2 years, in malarious areas those with the sickle cell trait (HbS/HbA) appeared to have a survival advantage over homozygous HbA individuals. Individuals heterozygous for this β globin mutation were less likely to develop patent parasitaemia (Alison 1954) or die from severe malaria (Alison,1964) than normal controls. More recent studies have demonstrated that the sickle cell trait is associated with 90% protection against severe malaria (Hill *et al.* (1991).

Several hypotheses for the mechanism of HbS protection against malaria have been suggested. Under conditions of low oxygen tension HbS erythrocytes undergo sickling particularly when they are infected by a parasite and therefore one of the early hypotheses proposed that this phenomenon itself would adversely affect parasite survival (Luzzatto *et al.* 1970). The discovery that erythrocytic parasites require a high K+ environment for growth has prompted others to suggest that the protection enjoyed by Hb S carriers is based on increased potassium leakage from their erythrocytes in hypoxic environments (Friedman 1979). However, it is also possible that protection may also occur as a result of sickling-independent inhibition of parasite invasion and growth within the red cell under conditions of low oxygen tension (Pasvol *et al.* 1978). More recent data by Carlson and colleagues indicates that HbS erythrocytes may be less likely to form rosettes than normal erythrocytes and thus provide protection against cerebral malaria associated with this latter phenomenon (Carlson *et al.* 1994).

1.1.4.1.ii The Thalassaemias

The thalassaemias are the commonest genetic disorders in humans (Hill 1992). There are two forms of thalassaemia, α-thalassaemia and β-thalassaemia. α-thalassaemia is characterised by defective or absent α-globin gene expression usually caused by deletions within the α-globin gene complex located near the tip of the short arm of chromosome 16 (Buckle et al 1988). Large genetic deletions are associated with the more severe forms of the disorder denoted as α^{o}-thalassaemia, whilst two smaller deletions, by far the commonest in this group, are associated with milder forms of the disease, α^{+}-thalassaemia. Similarly in β-thalassaemia there is deficient or absent β-

Table 1.1 Genes implicated in variable resistance to malaria

β globin	HbS	**Duffy Blood Group**
	β thalassaemia	**Glycophorin A**
	HbE	**Glycophorin B**
	HbC	**ABO Blood group**
α globin	α thalassaemia	**TNF–α**
γ globin	HbF	**Band III** ovalocytosis
HLA	-B53	**Spectrin** elliptocyptosis
	-DRB1*13.02	**G6PD Deficiency**
	-DRB1*01.01	**Pyridoxal Kinase**

globin gene expression which occurs as a result of one or more point mutations within the β-globin gene complex on chromosome 11. As with HbS, the highest frequencies of the thalassaemias are in malarious areas although there are only a few reports in the literature of case-control studies or detailed geographical correlations. One of the few by Flint *et al.* (1986) provided strong evidence that α^+-thalassaemia was protective against malaria and this finding is corroborated by data generated in our own laboratory since then (SNR Yates D.Phil. thesis 1995) from large case-control studies in The Gambia and Kenya.

The mechanism of protection by the thalassaemias remains speculative although *in vitro* work suggests that this may be due to reduced growth of parasites when infected erythrocytes are subjected to oxidant stress (Friedman 1979). Furthermore, it has been demonstrated that thalassaemic heterozygotes express higher than normal levels of erythrocyte neoantigens (Luzzi *et al.* 1991) and this may be a protective mechanism whereby malaria-infected erythrocytes with variant haemoglobin undergo increased phagocytosis by macrophages (Bunvaratvej *et al.* 1986; Yuthavong *et al.* 1990).

1.1.4.1.iii Haemoglobin C (HbC)

Haemoglobin C is caused by a single base change in codon 6 of the β-globin gene. HbC is found mainly in sub-Saharan Africa, especially in the Sahel region of West Africa where the gene frequency reaches polymorphic frequencies (Mourant *et al.* 1976). Although no microepidemiological or clinical evidence has been advanced for a protective effect in malaria, *in vitro* erythrocytes in homozygous but not heterozygous HbC individuals show resistance to *P. falciparum* growth (Friedman 1979). It has been

suggested that this may be due to failure of merozoites to successfully break free from schizont infected HbC red blood cells (Nagel 1990).

1.1.4.1.iv Haemoglobin E (HbE)

Haemoglobin E is found mainly in malarious parts of Asia and is a β-globin gene variant that behaves like a mild β thalassaemia allele. As with HbC, homozygosity for HbE causes a mild anaemia (Kar *et al.* 1992). Data on the role of HbE in malaria resistance are conflicting. While results from some early studies suggested resistance to infection following *P vivax* innoculations (Ray *et al.* 1964) other studies failed to produce the same results (Kruatrachue *et al.* 1961). A more recent study in Ao Nagas in North India reported protection against both *P. vivax* and *P falciparum* malaria for both HbE heterozygotes and homozygotes (Kar *et al.* 1992). Resistance was greater against *P. vivax* than against *P falciparum*, and the heterozygous HbE genotype appeared more protected than homozygotes HbE state. With *in vitro* studies, resistance to *P. falciparum* infection in HbE heterozygous red blood cells has been reported in some studies (Nagel *et al.* 1981; Vernes *et al.* 1986) but disputed by others (Santiyanont *et al* 1981; Pasvol *et al.* 1982).

1.1.4.1.v Haemoglobin F (HbF)

Under 'normal' circumstances' production of HbF only occurs in the foetus and in early infancy but, in some individuals, particularly in populations from South East Asia, HbF production can persist into adult life and it has been proposed that this may be a host adaptation to malaria. This hypothesis is supported by several *in vitro* studies that

have demonstrated inhibition of *P falciparum* growth in HbF crythrocytes (Pasvol *et al.* 1976; Pasvol *et al.* 1977).

1.1.4.1.vi The Duffy Blood System

It has been known for a long time that, in sub-Saharan Africa, there is a high frequency of individuals who lack the Duffy blood group determinants Fy^a and Fy^b i.e Duffy negative individuals (Boyd *et al.* 1933; Bray 1958). In West Africa and to a lesser extent in East Africa, the majority of the population are Duffy blood group negative (Mourant *et al.* 1976). Proof of the relationship of this phenotype with malaria came from Miller *et al.* (1976) who showed that Duffy negative individuals were resistant to infection from *P. vivax*. Since then this observation has been replicated (Miller *et al.* 1978; Spencer *et al.* 1978).

These field studies considered in conjunction with extrapolations from *in vitro* work with *P knowlesi*, indicate that resistance to *P vivax* in Duffy blood group negative individuals probably occurs as a result of impaired parasite invasion of erythrocytes lacking both the Fy^a and Fy^b determinants on their surface (Miller 1988). Of interest are a few observations that provide indirect evidence that the different Plasmodial species may use different receptors for binding to and invasion of erythrocytes. Unlike *P. vivax*, *P. falciparum* appears to invade Duffy negative erythrocytes normally, and in populations with high frequencies of Duffy blood group negative individuals and which, therefore, are refractory to *P. vivax*, the latter has been replaced by *P. ovale* (Miller 1988).

1.1.4.1.vii The Glycophorins

Red blood cells with deficiencies or modifications in their glycophorins show increased resistance to *P. falciparum* invasion. Several studies have demontrated that En(a$^-$) erythrocyte that complete lack glycophorin A, a major erythrocyte sialoglycoprotein, have reduced invasion by *P. falciparum* (Miller *et al.* 1977; Pasvol *et al.* 1982; Perkins 1981). In addition M^kM^k erythrocytes deficient in both glycophorin A and B (Hadley *et al.* 1987), Tn erythrocytes that lack terminal sialyl and galactosyl residues of the O-linked oligosaccharide structures of glycophorin A (Dvorak *et al.* 1975; Cartron *et al.* 1983; Mitchell *et al.* 1986), Cad erythrocytes that have an additional N-acetylglucosamine residue on the O-linked oligosaccharide structures (Cartron *et al.* 1983), and Wrb$^-$ erythrocytes a variant form of glycophorin A (Pasvol *et al* 1982), have all been shown to exhibit reduced invasion by *P. falciparum*. These variant forms of the glycophorins are only rarely found at significant frequencies in human populations and are as such they are of comparatively minor importance in host resistance against malaria in endemic areas.

1.1.4.1.viii Elliptocytosis (Ovalocytosis)

Hereditary elliptocytosis (HE) refers to a heterogenous group of disorders which are found mainly in two malaria-endemic regions, South East Asia and Melanesia (Baer *et al.* 1976). The variants include aymptomatic or symptomatic HE, severe haemolytic HE (Hereditary pyropoikilocytosis, HPP), spherocytic HE and Melanesian ovalocytosis (stomatocytic elliptocytosis). This group of disorders, caused principally by a dysfunctional erythrocyte cytoskeleton, are characterised by the abnormal shape, oval or elliptical, of the red blood cells and are associated with a broad spectrum of

clinical manifestations that involve varying degrees of anaemia. The dysfunctional cytoskeleton is associated with a variety of molecular defects caused by mutations or codon deletions within the spectrin gene (Parquet *et al.* 1994) and band 3 (codons 400-408) (Jarolim *et al.* 1991; Palek & Lambert, 1990) of the erythrocyte. These changes interfere with the spectrin's ability to fully wind and unwind (Schofield *et al.* 1992; Mohandas *et al.* 1992) and since spectrin forms the elastic links of the cytoskeleton protein on which the mechanical stability of the erythrocyte depends, the resultant elliptocytes are more rigid or less distortable than normal erythrocytes. Unlike all the other variants that are caused by spectrin defects, in ovalocytosis the defect is in band 3 and probably disrupts the way in which the latter interacts with spectrin.

In addition to altered deformability, ovalocytes differ from normal erythrocytes in their lower expression of blood group antigens (Booth *et al.* 1977) and possibly increased sodium and potassium permeability (Honig *et al.* 1971; Schofield *et al.* 1992a). The net effect of these latter properties appears to make these ovalocytes resistant to *P falciparum* growth in both *in vitro* (Kidson et al 1981; Hadley et al 1983) and *in vivo* (Cattani et al 1987; Foo et al 1992) experiments. There is some evidence that the level of parasitaemia in *P. vivax* and *P. falciparum* infections is lower in individuals with ovalocytosis (Serjeantson *et al.* 1977) compared to controls and findings from a more recent field study in Papua New Guinea indicate that ovalocytosis is protective against cerebral malaria (Genton *et al.* 1995)

1.1.4.1.ix The ABO Blood Groups

There is not much known about the possible role of the ABO blood groups as markers for susceptibility to malaria but results from a large case-control study in The Gambia found a small but significant association of blood group O with resistance to malaria (Hill 1992). This may be due the reduced propensity of group O erythrocytes to rosette (Carlson *et al.* 1992), a phenomenon known to be associated with severe malaria (Carlson *et al.* 1990; Treutiger *et al.* 1992).

1.1.4.1.x Glucose-6-Phosphate Dehydrogenase (G6PD) Deficiency

There is considerable epidemiological and *in vitro* evidence that G6PD deficiency is protective against *P. falciparum* malaria (Alison 1961; Roth *et al.* 1983; Roth *et al.* 1983a; Friedman *et al.* 1979). Hitherto field studies have yielded conflicting results (Alison 1961; Gilles *et al.* 1967; Bienzle *et al.* 1972: Martin *et al.* 1979; Kar *et al.* 1992). Findings in the most widely referenced study and from which the dogma for many years has been derived suggested that protection was the sole prerogative of female heterozygotes (Bienzle *et al.* 1972). Chapter 3 gives greater details on the background to this question and describes the results of our own findings in two large case-control studies in West and East Africa.

1.1.4.1.xi Pyridoxal kinase

The observation in several studies of low erythrocyte pyridoxal kinase activity in African-Americans (Chern & Beutler 1975) and Africans (Solomon & Hillman 1978) led to the the hypothesis that reduced levels of this enzyme might confer some protection against malaria. Further support for this proposal came from Martin *et al.*

(1978) who reported that the pyridoxal kinase activity of malaria patients with higher *P. falciparum* parasitaemia was significantly higher than in the control group. Pyridoxal kinase and pyridoxine oxidase are required for the production from pyridoxine of pyridoxal phosphate which appears to be necessary for parasite development (Miller 1988). Interestingly, Anderson *et al.* (1979) found low levels of pyridoxine oxidase activity in thalassaemic individuals and speculated that these two disorders may have a similar mechanism of resistance to malaria.

1.4.2 HOST ACQUIRED IMMUNITY GENES

1.1.4.2i HLA Class I and Class II

The central role that these antigens have in the regulation of immune responses makes HLA genes important candidates as host genes that determine susceptibility to infections and this issue is discussed at length in Chapter 5 of this thesis. Associations with two HLA types have been reported from a large case-control study in The Gambia (Hill *et al.* 1991). HLA-B53 was asssociated with 40% protection from both cerebral malaria and severe malarial anaemia while an HLA class II antigen, DRB1*13.02 was associated with protection against severe malarial anaemia. It is likely that the class I association is mediated by cytotoxic T lymphocytes acting against the parasite during the pre-erythrocyte liver stage. The class II association may reflect more rapid clearance of the parasite during the erythrocytic stage, possibly because HLA-DRB1*13.02 carriers can generate a particularly effective antibody response against an important malarial antigen (Hill *et al.* 1992; Hill *et al.* 1992c). Results from another malaria case-control indicate that in Kenya, HLA-DRB1*0101 is associated protection from severe malaria (Yates SNR D. Phil .1995).

1.1.5 MALARIA SUSCEPTIBILITY GENES

1.1.5.1 Tumour Necrosis Factor (TNF)

TNF is an important immunoregulatory cytokine produced predominantly by macrophages and T lymphocytes. There is now evidence that two TNF promoter polymorphisms at the -308 and the -238, may be associated with cerebral malaria (McGuire *et al* 1994) and severe malarial anaemia (McGuire *et al.* unpublished data) respectively. These two polymorphisms and their relationship with malaria and other infectious diseases are discussed in depth in Chapter 5. While there is only indirect evidence on the functional significance of the -238 polymorphism, Chloramphenicol acetyl transferase (CAT) reporter gene studies in B cell constructs indicate that the variant allele at -308, upregulates TNF-α expression (Wilson *et al.* 1994). Therefore the association of the -308 homozygosities with increased risk of cerebral malaria is consistent with other strong evidence linking elevated serum TNF-α with this syndrome (Kwiatkowski *et al.* 1990). Furthermore, TNF-α is known to upregulate the expression of host adhesion molecules that mediate parasite binding to vascular endothelium (Berendt *et al.* 1989). It has also been suggested that TNF-α may stimulate cerebral endothelium to produce excess nitric oxide and thus cause coma by interfering with neurotransmission (Clark *et al.* 1992)

1.2. TUBERCULOSIS

1.2.1 EPIDEMIOLOGY

In 1993, WHO declared tuberculosis a global public health emergency (WHO 1993 overview). It is estimated that 1.7 billion people, approximately one third of the

world's population, are infected by the causative organism *Mycobacterium tuberculosis* (Kochi 1991). There are as many as 60 million people suffering from tuberculosis today with an annual incidence of approximately 20 million active cases each year (Kochi 1993; Chan & Kaufmann 1994) (Figure 1.C). Today tuberculosis kills more people than any other communicable disease being responsible for an estimated 3.3 million deaths each year. Developing countries are the worst affected with tuberculosis accounting for 25% of all preventable adult deaths (Murray *et al.* 1990). As much as 95 % of the cases of tuberculosis are in the developing countries, with Asia alone having two thirds of all the world's infected individuals (Kochi 1993). While the annual risk of infection is probably below 0.1% in industrialised countries, it is probably between 1-2.5% in the developing countries (Cauthen & Ten Dam 1988; Smith & Moss 1994). If current trends are not reversed it is projected that 30 million people will die from tuberculosis in the 1990s with the annual death rate tripling from the current level to 10 million deaths per year by the year 2 000 (Kochi 1993).

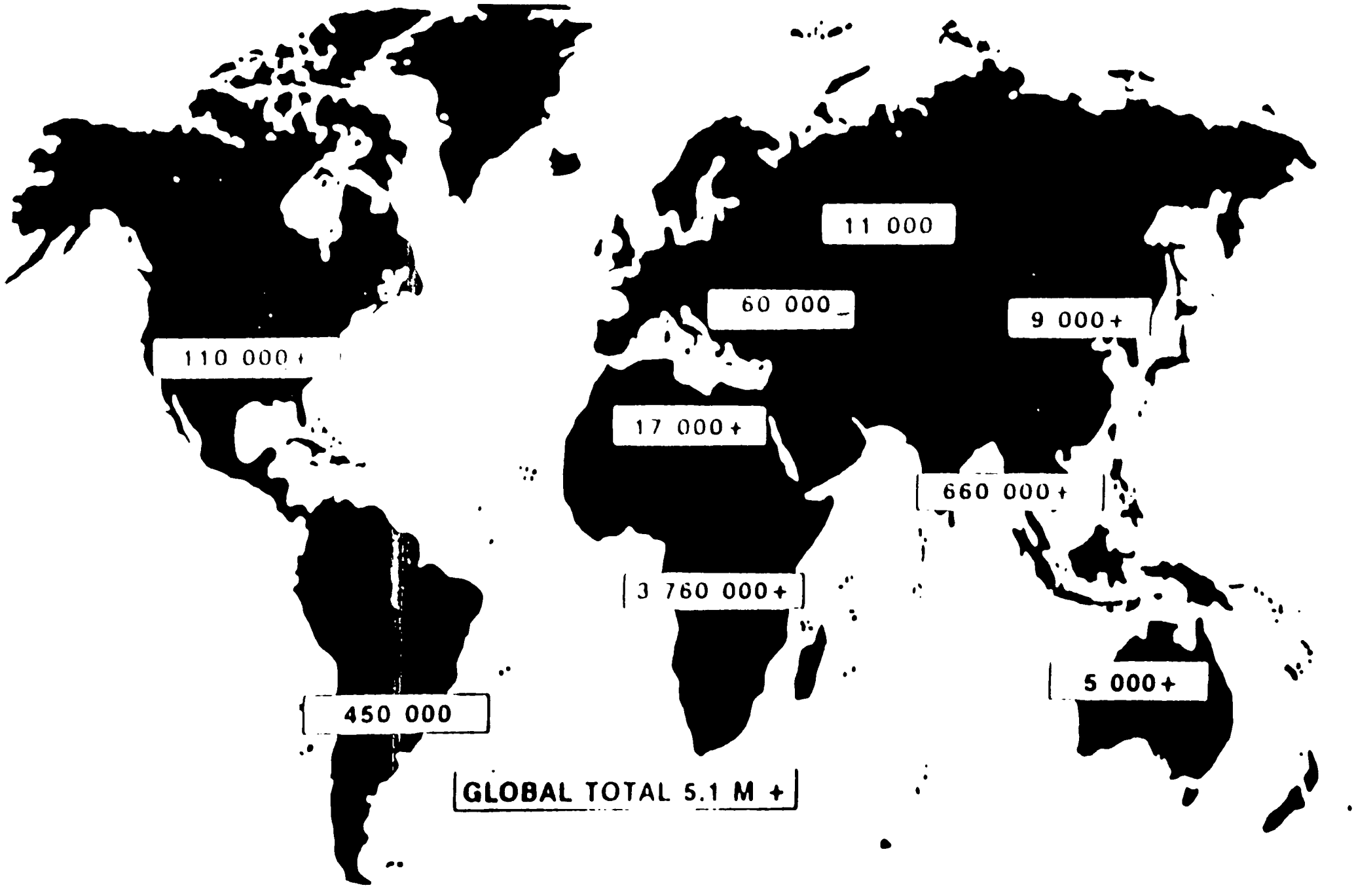
In the 19th century, tuberculosis was the most important cause of deaths in what are now the industrialised regions of the world (Williams 1908). During this period disease was caused by both *M. tuberculosis* and *M. bovis*. Improved socioeconomic conditions, in particular, were responsible for the decline of tuberculosis incidence seen in these countries in the early part of the century so that by the time tuberculin testing of dairy herds, pasteurisation of milk, sanatoria and specific chemotherapy were introduced in the early decades of this century, the decline was already well established. However, the introduction of chemotherapy did make a dramatic impact

on case mortality rate, more than halving the rate from the then alarming levels of 50-60% (Murray 1990)

In contrast, while tuberculosis was wreaking havoc in the industrialised countries in 18th and 19th centuries, there appears to have been relatively little tuberculosis in most developing countries and certainly in sub-Saharan Africa, the disease was probably only introduced with colonialisation at the turn of the 20th century (Diamond 1992; Livingstone 1857). Nevertheless, the population explosion coupled with less effective application of chemotherapy and other control programmes in these developing countries, has meant that throughout this century, tuberculosis has been a growing public health menace with annual mortality rates in these regions rising from less than 1 million at the start of the century to the current level of almost 3 million each year (Kochi 1993)

The global resurgence of tuberculosis since the late 1980's is attributable to three main causes. The first and, arguably, the most important cause for this crisis is the HIV epidemic particularly in sub-Saharan Africa where as much as 75% of HIV and *M. tuberculosis* dually infected individuals are found (Kochi 1993). Another major cause of the global TB epidemic is increasing drug resistance largely due to both the HIV epidemic and poorly managed, or inappropriately focused, TB control programmes. The rise in TB cases in the industrialised nations has been particularly marked in marginalised minority groups, the homeless, drug addicts and in immigrants from high prevalence countries (Kochi 1993). In the poorer countries of the world, wars, famines, and other natural disasters have created large populations of displaced

Figure 1. C Numbers of individuals worldwide infected with *M. tuberculosis* between 1990 -1993.



malnourished people who live in crowded conditions ideal for TB transmission. In some countries, the situation has been further compounded by worsening economic conditions that have led to the general collapse of health services. The tragedy in the developing countries is also that 80% of the cases of TB are within the economically most productive 15-59 year age group (WHO 1995) such that the overall economic impact of the disease in terms of lost labour and income, in addition to the costs of screening, diagnosing and treating cases, is considerable.

1.2.2 THE CLINICAL PROBLEM

Tuberculosis is remarkable in that it can affect every single organ of the human body and therefore present clinically in many unusual manifestations that are easily mis-diagnosed by unsuspecting physicians. Our current understanding of pathogenesis and protective immunity in tuberculosis is still limited. The tuberculin skin test as a marker of protective immunity is clearly inadequate especially in developing countries (Smith & Moss 1994) as there appears to be, at best, a weak correlation between skin-test conversion after BCG vaccination or exposure and protection. New vaccines and drugs are still needed as the current BCG has variable efficacy (0-80%) (Fine 1995) and, overall, probably only gives 50% protection against subsequent disease (Colditz *et al.* 1994).

It is still not totally understood why BCG vaccine has such variable efficacy against tuberculosis. Several reasons have been proposed which include differing methodologies in the various efficacy studies, differences in BCG administration, host genetic variation in different populations, BCG strain differences (Lagranderie *et al.*

1996) and the different modulating effects on BCG vaccination of the various environmental mycobacteria found in different geographical areas (Fine 1995). However, BCG does lower overall mortality, as well as reduce the incidence of disseminated disease and tuberculous meningitis in those vaccinated individuals who may later go on to develop disease (Colditz *et al.* 1994; Bloom & Fine 1994). In addition, the BCG vaccine does appear to confer significant protection against leprosy (Fine & Rodrigues 1990).

In the worst affected countries even when there is adequate availability of chemotherapeutic drugs, compliance is still a major problem today with less than 50% of detected cases being cured or completing chemotherapy (Snider 1993). Such cases remain infectious (Grzybowski 1991), and it is likely that death is the way a considerable proportion of these stop being so! The strengthening of tuberculosis (TB) control programmes and the introduction of WHO-recommended Directly Observed Treatment Short (DOTS) course in the first two months of the 6 month therapy will hopefully improve compliance and cure rates.

As indicated above, HIV infection has increased the burden of tuberculous disease in many parts of the world. In sub-Saharan Africa, HIV seroprevalence rates of more than 40% are common among patients with TB (Snider 1994). HIV appears to increase primary disease and secondary disease both by exogenous reinfection and reactivation (Lucas & Nelson 1994). In about 50% of cases, tuberculosis appears to precede other AIDS defining illnesses by up to 2 years (Chaisson *et al* 1987). Although sputum positivity rates are similar, the clinical patterns of TB in HIV positive patients

do differ from those in HIV negative individuals in several ways. HIV positive patients have a higher proportion of cases with extrapulmonary or disseminated disease, a higher frequency of false-negative tuberculin skin tests, atypical features of chest radiographs, fewer cavitating lesions, a higher rate of adverse drug reactions, the presence of other AIDS-associated manifestations, and a higher death rate (Elliot 1990). In the USA prior to the HIV epidemic, 85% of all tuberculosis cases were due solely to pulmonary disease (Farer *et al.* 1979), but now with the HIV epidemic, the latter only accounts for only 35% of cases with the remainder having either extrapulmonary tuberculosis alone or a mixture of both pulmonary and extrapulmonary tuberculosis (Small *et al.* 1991). The HIV epidemic has also generated difficulties in vaccination guidelines particularly for the newborn babies of infected mothers, as BCG vaccination can lead to disseminated disease in immunocompromised individuals.

The emergence of drug-resistant strains of *M. tuberculosis* has resulted in further treatment problems all over the world. As discussed this is related in part to the HIV epidemic but largely it is due to inadequate treatment practices. Fortunately, many countries are running WHO's DOTS strategy to ensure adequate treatment in the initial early phase of therapy. In the USA, the enormous clinical impact of multiple drug resistance (MDR) has been observed in tuberculosis case-fatality rates; the rates in these MDR cases are as high as 40% in immunocompetent individuals and over 80% in HIV infected individuals (Bloom 1994). The apparent reluctance for commercial reasons, of pharmaceutical companies to develop new antituberculosis drugs has meant that there have been no new anti-tuberculosis drugs for 30 years (Reichmann 1996). Antimicrobials with possible indications in tuberculosis have been reserved for what

have been perceived to be more prevalent conditions such as pneumonia and chronic bronchitis.

1.2.3 BACTERIOLOGY AND TRANSMISSION

Bacterial cell wall lipids can act as phage receptors and it is on this basis that 4 phage types of *M. tuberculosis* have been identified: A, B, C and I. Phage type A, by far the commonest strain in sub-Saharan Africa, is the the most prevalent phage type worldwide (Citron & Girling 1987). In Europe and Northern America both phage types A and B are predominant whilst strain I is found mainly in Asia where it accounts for as much as one third of all phage types. 50% of all adult TB cases are sputum positive (Styblo 1984) and individuals get infected principally by inhaling air droplets from such infectious source cases (Shaw & Wynn-Williams 1954; Bloom & Murray 1992). An untreated source case can infect between 10-14 people although even higher figures have been reported in the literature (Styblo 1994). It is estimated that in developed countries, two or three cases may be infected by a sputum positive case before the latter is detected whereas in the developing countries where there are higher numbers of close contacts, the number may be four or five (Murray *et al.* 1990)

The organism is not only found in aerosol particles but can also be carried in dust particles. These infection-laden particles can remain suspended in the air for long periods of time. Only 6% of inhaled particles actually reach the alveoli following inhalation; the rest settle in the upper respiratory mucosa and are expelled by the 'ciliated respiratory escalator' (Smith & Moss 1994). Effective transmission is determined by several factors: the number and concentration of bacilli present in a

source case, the duration of exposure and the aerodynamics of the infection-laden particles. It is estimated that the number of exhaled bacilli in a source case with solid or lung lesions is between 10^2 and 10^4 bacilli whilst in those with cavitary lesions, it is anything between 10^7 -10^8 infectious particles (Smith & Moss 1994). Ingestion of the particles is a 10, 000 fold less effective mode of infection probably duc to the bactericidal action of gastric acid (Gaudier & Gernez-Rieux 1962).

Only 5-15% of infected individual actually go on to develop disease or primary tuberculosis (Citron & Girling). In high prevalence regions it is usually children that develop primary disease. Primary tuberculosis cases are usually sputum negative and are therefore not infectious. The majority of infected individuals do not develop disease and are assumed to mount an effective immune response against the infection. Control of the infection with or without chemotherapy does not necesarily imply complete killing of *M tuberculosis* and the organism can remain dormant inside tissue macrophages for years. Infection of immunocompetent individuals is associated with a 10% lifetime risk of developing clinical disease (Comstock 1982) whereas, in HIV positive individuals, this risk is increased to 8% per year (Selwyn *et al.* 1989). Years after the initial infection, individuals may develop secondary tuberculosis as a result of either exogenous reinfection or reactivation of latent infection often due to a failure of immune surveillance.

Epidemiological models suggest that in countries with high prevalence, exogenous reinfection is thought to be responsible for the majority of secondary tuberculosis cases whilst in countries of lower prevalence it is reactivation of disease that is more

common (Styblo 1984). Most (70-90%) of all TB cases are pulmonary TB with the remainder (10-30%) being extrapulmonary (Citron & Girling 1987).

1.2.4 GENETIC FACTORS IN VARIABLE SUSCEPTIBILITY TO TUBERCULOSIS

1.2.4.1 Background

For more than a century it has been recognised that host genetic factors are important in susceptibility to tuberculosis (Biggs 1888-89; Hirsch 1886). And while the familial clustering of the disease and observations of individual and racial differences in resistance during epidemics or in disease-endemic areas (Stead 1992), have all strengthened this belief, for a long time formal proof has been difficult to establish. About forty years ago, Lurie and others reported the findings of extensive work with inbred rabbits which provided convincing experimental evidence of a genetic factor in resistance to tuberculosis (Lurie *et al.* 1955; Lurie 1964). During the same period, Motulsky was also making a strong case for a genetic basis to variation in resistance to infection in humans (Motulsky 1960) He described how when tuberculosis was first introduced into the Qu'Appelle Indian Reservation in North America, the annual tuberculosis death rate was 10% of the population yet 40 years later, when presumably most of the susceptible individuals had died, the annual death rate fell to 0.2% suggesting that genetic factors have a major influence on tuberculosis susceptibility in humans.

There is other evidence that indicates that, although environmental factors such as nutrition, overcrowding and bacterial strain are important, genetic factors are also

There is some evidence that there may be racial differences in tuberculosis susceptibility. In the USA, the incidence and prevalence of tuberculosis is as about as twice as high in blacks as in whites living in the same institution (Stead *et al.* 1989) and although these differences may be ascribed to social factors, Stead has observed that among groups of comparably housed and fed nursing home residents, blacks are more readily infected by M. tuberculosis than whites (Stead *et al.* 1990). Interestingly, these observations are supported by *in vitro* studies that suggest that there may indeed be some inherent racial differences in macrophage function (Crowle & Elkins 1990; McPeek *et al.* 1992).

Work from animal models of mycobacterial diseases has also indicated a role for host genetic factors in clinical outcomes. In mice, it has been demonstrated that innate resistance to intracellular infections with *Mycobacteria, Salmonella* and *Leishmania* is under the control of an autosomal dominant gene, Natural Resistance-Associated Macrophage Protein 1 (*Nramp1*) (Skamene *et al.* 1982; Vidal *et al.* 1993). The gene is expressed in macrophage populations from the reticuloendothelial organs and is believed to encode a membrane transporter protein. *Nramp1* has a variety of pleiotropic effects on macrophage activation (Buschman *et al.* 1989; Roach *et al.* 1994). Macrophages from innately resistant strains respond more vigorously to infection than susceptible mice whose *Nramp1* protein has been shown to contain a glycine to aspartate amino acid substitution within the second predicted transmembrane domain. The homologous gene in humans has been cloned and sequenced (Vidal S *et al.* 1993) and in Chapter 6, I describe my investigation of the

strongly implicated in the selective morbidity and mortality of *M. tuberculosis* infection. In a tragic incident in Lubeck in 1927, infants, inadvertently immunised with a single viable virulent *M. tuberculosis* strain, displayed marked differences in susceptibility ranging from death to recovery (Anonymous 1935) once again pointing to a genetic basis for variable TB resistance. In addition, studies conducted 20 years ago on the natural history of tuberculosis have also provided some indirect evidence showing that in the absence of chemotherapy, 50% of sputum positive TB cases died within 5 years of diagnosis, 30% developed disease and 'self cured' with the other 20% remaining disease-free (Springett 1971; National Tuberculosis Institute 1974). However, these differences could have been attributable to differences in the bacterium (strain or infectious load) or environmental effects such as socioeconomic status, nutrition, and smoking.

The proposal that genetic factors contribute to variable resistance to tuberculosis is probably best supported by several twin studies (summarized in Table 1.2) which have shown substantially greater concordance for tuberculosis among monozygotic compared to dizygotic twins (Kallmann & Reisner 1942; Comstock 1978). Therefore if one of a twin pair developed tuberculosis then the chance that the other would also contract the disease was significantly greater if the twins were monozygotic than if they were dizygotic. Since monozygotic twins are identical genetically whereas dizygotic twins share only half of their genetic material, these results were strongly suggestive of a role for genetic factors in the disease.

Table 1.2 Summary of major twin studies of tuberculosis showing greater concordance rates for disease incidence in monozygotic twins.

Authors & year	Total twin pairs	Monozygotic concordance rates	Dizygotic concordance rates
Diehl & von Verschuer, 1936	205	65%	25%
Dehlinger & Kunsch, 1938	46	58%	6%
Kallman & Reisner, 1943	308	62%	18%
Harvald & Hauge, 1956	143	38%	19%
Simmonds & Comstock 1978	205	32%	14%

role of the human homologue of this gene, *NRAMP1*, in pulmonary tuberculosis in The Gambia.

1.2.4.2 Genetic associations with tuberculosis

1.2.4.2.i HLA genes

In the past most investigators examining the role of genetic factors in tuberculosis have understandably paid most of their attention to the HLA genes. Although several associations have been reported in the literature, the only one that has been reported with any consistency is an association in predominantly Asian populations, of HLA-DR2 with increased susceptibility to tuberculosis (Brahmajothi *et al.* 1991; Bothamley *et al.*1989; Khomenko *et al.* 1990). Skewed transmission of this allele from parents with tuberculosis to affected offspring has also been demonstrated in segregation analysis of affected pedigrees (Singh *et al.* 1983). The role of HLA in tuberculosis is discussed more extensively in chapter 5.

1.2.4.2.ii Non-HLA genes

Although studies quoted above implicate a role for HLA in tuberculosis suceptibility, this is insufficient to account for the large genetic component to tuberculosis susceptibility identified by twin studies. Over the years there have been several published studies that have suggested roles for the ABO groups in tuberculosis (Mourant 1978; Saha & Banerjee 1968; Jain 1970; Overfield & Klauber 1980). Other polymorphic loci that have been investigated with which equivocal associations were found include Rhesus and MN blood groups, PTC tasting, secretor, haptoglobin, group specific component, the phosphoglucomutase system and acetylation status

(Kooptzoff & Walsh 1957; Milunicova & Dominec 1966; Hever 1969; Sidhu 1974; Papiha *et al.* 1987; Karim *et al* 1981). Hence the non-HLA genetic component in disease susceptibility has remained largely undefined and in chapters 6 and 7 of this thesis, I discuss the results that I obtained from studies on the role of 3 non-HLA candidate gene loci.

1.3. APPROACHES TO ANALYSIS OF HOST GENETIC FACTORS IN HUMAN INFECTIOUS DISEASES

1.3.1 Twin Studies

The ability to compare the concordance rate for a disease phenotype between monozygotic twins and dizygotic twins, makes twin studies extremely useful for identifying the contribution of any heritable component for a given disease phenotype such as an infectious disease. This approach is based on the the fact that monozygotic twins are genetically identical whilst dizygotic twins, like any other full sibling pair, share only half of their genetic material. In the presence of a genetic component in governing susceptibility to disease, a greater degree of similarity with respect to the presence of disease (i.e concordance) is observed between monozyotic twins compared to dizygotic twins. The advantage of twin studies is that, generally, they allow assessment of individuals who share similar environmental conditions. Twin studies also have the special advantage of allowing a direct measure of the penetrance of the genetic component in a given disease. Twin studies have provided considerable evidence for the presence of a genetic contribution to the clinical response to both

tuberculosis (discussed above in section 1.2.4) and malaria infections (Jepson *et al.* 1995).

There are, however, limitations to twin studies. Twin studies only give an indication of the genetic contribution to increased disease risk and still leave the challenging problem of identification of not only the genes involved with a given disease trait but also the latter's relative contributions to the disease phenotype. In addition, inaccurate assessment and classification of twin pairs can give rise to misleading results. Furthermore, it has been suggested that there are two fundamental assumptions in twin studies that may not be wholly accurate and which, therefore, may give rise to misleading results. The first is that monozygotic twins are genetically identical. It is argued that genomic imprinting, X-chromosome inactivation and somatic rearrangements particularly of immunoglobulin and T cell receptor loci in the latter case, may cause genetic and immunological differences between monozygotic twins (Gregersen 1993). However, none of these differences have ever been shown to directly lead to phenotypic differences in B or T cell function in monozygotic twins.

The second purportedly erroneous assumption in twin studies is that there are no differences in the environmental experiences between the two types of twin pairs. It is argued that there may subtle differences in shared environment between monozygotic and dizyotic twins with the former tending to have greater environmental sharing (Phillips 1993). This increased environmental sharing in monozygotic twins may actually begin in utero. Prenatal differences in environment between monozygous and dizygous twins may result in differences in fetal growth and survival (Bulmer 1970). It

is known that the majority (66%) of monozygotic twins are monochorionic whereas dizygotic twins are always dichorionic. It is argued that there are significant differences in the shared environment between these two types because for monochorionic twins it is likely that there is some competition for a limited supply of nutrients and, in addition, there is a higher likelihood that vascular anastomoses may be formed between the two circulations of the two twins (Phillips 1993).

1.3.2 Adoptee Studies

Adoptee studies are particularly useful for assessing the separate contributions of genetic and environmental factors to adult morbidity or mortality. This type of study assesses the alteration in risk of an event in adoptees when their biological or adoptive parent has suffered the same event. The greater the relative risk for a disease in the adoptee for a given outcome in his or her biologic parent, the greater the genetic contribution to the given outcome. However, a significantly increased relative risk for a given outcome in an adoptee given the same outcome in his or her adoptive parent, implies a role for environmental factors in the measured outcome. If the relative risk for a designated outcome is stronger between a biological parent and an adoptee than it is between an adoptive parent and an adoptee, this implies that genetic factors are more important than environmental factors and vice versa.

The adoptee study which best illustrates the merits of this approach was conducted in Denmark in the 1980's (Sorenson *et al.* 1988). In this study, the authors followed up adult subjects adopted early in life by parents unrelated to them and analysed the adoptees' general and cause-specific mortality rates up until the sixth decade in relation

to ages at death and causes of death of both their biological and adoptive parents. The study demonstrated that death from natural causes of a biological parent before the age of 50 year doubled the mortality rate for adoptees. In contrast, such early death in an adoptive parent had no effect on the mortality rate of adoptees. Of particular interest, was the observation that premature death from infection in biological parents was associated with a statistically increased relative risk of 5.81 for premature death in adoptees whereas there was no such effect in adoptive parents (relative risk 0.73) suggesting that with infectious diseases in general, the genetic contribution to disease mortality is significantly more important than that of environmental factors. In accordance with current understanding of the underlying pathophysiological processes, the results showed that for early death from cardio- and cerebrovascular diseases, genetic and environmental factors are both important with relative risks of 4.52 and 3.02 respectively, whilst for cancers, the environmental factors appeared to be more important than the genetic factors, relative risks 5.16 and 1.19 respectively.

Adoptee studies do have several drawbacks. They may take a long time to complete and invariably large numbers of study individuals are needed. In addition, they can only be conducted effectively where medical diagnoses, documentation, and record-keeping is uniform and of high standard and, unfortunately, this is not always the case in manpower- and economically-deprived countries.

1.3.3 Linkage studies

The identification of thousands of highly informative microsatellite markers consisting of dinucleotide repeats in the human genome has revolutionised genetic linkage

analysis and it is now possible to screen the entire human genome for susceptibility genes in multifactorial diseases (Davies *et al.* 1994; Hashimoto *et al.* 1994). Sets of these dinucleotide repeats have now been assembled that are fairly evenly spaced, typically at 10 megabase intervals, across the human genome and they can be used to screen families for linkage to any phenotype or disease. Although these microsatellite markers can be used in small numbers of large pedigrees as in traditional studies of single gene disorders, for mapping of susceptibility genes in complex diseases influenced by both genetic and environmental factors, analysis of many nuclear families is preferred (Hill 1996). Traditional likelihood odds ratio (Lod) analysis of large infectious disease pedigrees may be confounded by non-diseased individuals who carry susceptibility genes but are not affected because of limited or non-exposure to the infectious pathogen. In contrast, in affected sib pair studies of infectious diseases only affected and, therefore, exposed sibs are considered and the segregation of markers from parents to these siblings is determined.

1.3.3.1 The affected sib pair method

The affected sib pair method is based on a comparison of the observed and expected distributions of the number of alleles shared identical by descent (ibd). Two alleles are said to be ibd when they both originate from the same parental allele. The number of alleles shared ibd by two affected sibs is thus 0, 1, or 2, these numbers occurring in expected proportions of 1:2:1 in the absence of linkage. Significantly increased sharing of a genetic marker by affected siblings indicates that the marker is linked to a susceptibility gene (Risch 1990a). The ability to detect linkage to a disease susceptibility locus may be measured in terms of the risk ratio, λ_s, of the risk to a sib of

an affected proband versus the population prevalence (Risch 1990a). This λ_s is an overall risk ratio that summarizes the collective effect of all disease loci; the higher the λ_s is, the stronger the genetic effect. The power of an affected sib pair study to detect linkage is function of λ_s, and also of how many genes there are and how they interact with one another. The sib pair approach has already been used successfully by Davies *et al.* (1994) and Hashimoto *et al.* (1994) to screen the entire human genome for type I diabetes susceptibility loci using fluorescence based PCR analysis of microsatellite markers.

The advantage of the affected sib pair method is that it is easy to use and relatively robust to factors such as phenotype ascertainment and mode of inheritance. However, the method relies on unambiguous determination of the number of alleles that are shared ibd by affected siblings. This is not always possible if a particular marker is not polymorphic enough. Possible solutions for dealing with this problem are identity by state (ibs) analyses which focus on the number of alleles of the same type shared by siblings regardless of whether these have originated from the same parental allele, or likelihood-ratio methods which take into account both parental markers and the study population marker allele frequences (Risch 1990b)

Linkage results may localise a gene to a relatively broad region and to delineate identified regions more closely one needs to do association analyses. This can be done with suitable polymorphic gene candidates in standard case-control studies (discussed below) or using transmission disequilibrium testing (tdt) (Spielman *et al.* 1993). Instead of using a control population, in the tdt test one measures the segregation of an

allele at a locus from a heterozygous parent to an affected child. The expected rate of transmission is 50% and a significant increase above this is evidence of both linkage and association (Copeman *et al.* 1995).

Linkage studies provide one with an opportunity to identify all the major genes in the human genome that may be involved in susceptibility to a given disease. Their disadvantages include the large numbers of families that need to be recruited and the considerable expense associated with these studies especially as they only identify broad chromosomal loci, further work being needed to identify the actual disease-causing genes.

1.3.4 Complex Segregation Analysis studies

Another approach that can be used be used to analyse genetic susceptibility to infectious disease is complex segregation analysis of large pedigrees. It is a method which involves proposing a model to explain the inheritance pattern of phenotypes (e.g disease) observed in a pedigree. The model that is chosen for testing on the data will usually specify information such as the allele frequency at the marker locus, the location of the trait-causing locus, the penetrance function and the transmission frequencies from parent to child. This analysis is ideal for simple Mendelian traits because the permissible models are few and easily tested (Lander & Schork 1994). Unfortunately, a large number of different models will usually fit infectious disease pedigrees since if given a free choice of allele frequency parameters, it will occasionally be possible to propose a plausible a major gene model. Naturally, a plausible major gene model thus derived is not any more likely than a complex multi-gene model which

in reality, for infectious diseases, is probably the more appropriate model. Complex segregation studies only provide information on the type of genetic component for a disease still leaving the task of locating and identifying the gene(s).

1.3.5 Candidate Gene Association studies

1.3.5(i) Cohort studies

Analytically the simplest way to ascertain whether a specific allele or genotype is associated with risk for an infectious disease is the cohort study. Initially, cohorts of comparable individuals are identified who have and do not have specific alleles or genotypes. These individuals are then followed prospectively for a given time to determine whether their exposure affects their risk of disease. The difference in the incidence of disease between those with or without a given risk factor is used to calculate the relative risk. There are several disadvantages to cohort studies. Because the follow-up period is usually determined by the study itself rather than by some inherent biological process, the relative risk derived may be a somewhat arbitrary measure of risk. Secondly, in the study of infectious diseases it would be unethical not to use prophylaxis or prompt treatment for individuals known to be at high risk of infection and disease. However such measures reduce the numbers available for the study and preclude the more useful analysis of non-diseased individuals with those at the severe end of the diseased spectrum. The major drawback of cohort studies is the huge numbers of study individuals that need to be followed up. This together with other logistic problems make these type of studies difficult to conduct especially in those very poor countries whose populations suffer most from infectious diseases.

1.3.5(ii) Case-control studies

Whereas a cohort study identifies and classifies individuals intially by their exposure status for a putative risk factor and then tests for subsequent disease, a case-control study identifies subjects by disease status and tests retrospectively for exposure. More specifically, case-control studies in genetic analyses compare the frequency of particular alleles or genotypes in cases and control populations. Significant differences between the two suggest that the alleles either cause the trait being tested or are in linkage disequilibrium with a causative allele. These tests do not necessarily indicate a causative relationship between an allele and a given disease but only demonstrate an association between the two. Since linkage disequilibrium is only detectable over small regions, usually less than 1 centimorgan (cM), an association indicates close proximity of the measured allele to the disease-causing gene.

Most of the malaria resistance genes have been identified using case-control studies. With infectious diseases it has been found that the evolutionarily more appropriate use of comparisons of controls with severe cases of diseases enhances the chances of observing effects of candidate gene alleles on susceptibility to disease (Hill *et al.* 1991; McGuire W *et al.* 1994; Ruwende *et al.* 1995). Case-control studies have the advantage that they are less laborious and expensive than large family studies. Furthermore, they are much more sensitive than linkage studies which may miss disease loci if the disease allele frequency is high or if there is considerable genetic heterogeneity present in the disease.

Identification of suitable candidate genes for association studies requires an understanding of the pathophysiology of the disease. Table 1.3 lists some of the genes which given our current knowledge of host defense mechanisms are good candidates for these type of studies of infectious diseases (Hill 1996). In this regard, data from inbred mouse models on host resistance loci for a given pathogen may be useful in the choice of candidate gene homologues in humans (Wakelin & Blackwell 1988; Vidal *et al.* 1993) although differences between animal models of disease with human disease may limit the usefulness of this approach. For case-control studies, polymorphisms need to be identified within candidate genes and so the use of this approach may be restricted to only those genes that have been cloned and sequenced. The major drawback with case-control sudies relates to the recruitment of suitable matched controls. Inadequately matched controls or population admixture within the study individuals can all give rise to false positive results. Problems of this nature can be avoided by using ethnically homogenous populations, using internal family members instead of unrelated individuals used as controls and replicating tentative associations with family-based controls (Lander & Schork 1994; Thomson 1994). Unlike the genome screen linkage approaches, candidate gene approaches may miss important unknown genes that may contribute to increased disease risk. Furthermore, the more candidate genes that one tests for the greater the chance of spurious false positive results unless corrections are made for mutiple comparisons.

Table 1.3 Some candidate genes in infectious diseases

HLA	Interferon-γ
TNF-α and -β	Interferon-γ receptor
TNF receptors	*NRAMP1*
Inducible nitric oxide synthase	T cell receptor variants
Interleukin-1 α and β	Fcγ RII (CD32)
Interleukin-1 receptor	Fcγ RIII (CD16)
Interleukin-1 receptor antagonist	IgH allotypes
Interleukin-4	K_m allotypes
Interleukin -10	Mannose binding protein
Interleukin -12	Duffy chemokine receptor
Fucosyl transferase-2 (Secretor)	ICAM-1
vitamin D receptor	

1.4 THIS PROJECT

As our understanding of disease processes expands, it is becoming increasingly apparent that in infectious disease, infection and disease are not always synonomous. Infection is now recognised as being only the first part in a complex interaction between between pathogen and host. We are only just beginning to grasp how important host factors are in determining not just the establishment of infection but, more pertinently, the clinical outcome of these infections in the host. In this project, I set out to examine the role of some host factors in two important infectious diseases, malaria and tuberculosis. Firstly, I investigated the role of G6PD deficiency in clinical malaria in children from East and West Africa. Secondly, using primarily a candidate gene approach, I examined the role of certain host genetic factors in susceptibility to tuberculosis in The Gambia.

CHAPTER 2 - MATERIALS AND METHODS

2.1 AMPLIFICATION OF DNA USING THE POLYMERASE CHAIN REACTION (PCR)

Genomic DNA was amplified in 96 well plates on a thermal cycler. The reaction buffer and amplification primers (Table 2.1) used varied according to the sequence to be amplified. Reactions were carried out in volumes of between 15 and 50 µl with template concentrations of 1µg/100µl and primer concentration of 50pM. Samples were overlaid with approximately 40 µl of mineral oil to prevent evaporation. Cycling conditions (Table 2.2) also varied with the primers used and the target sequence to be amplified. Generally, reactions had an initial denaturation step of 95-100°C for 3- 10 minutes. After denaturation, the thermal cycler was paused and 1-2 units of Taq polymerase enzyme added and the programme then allowed to proceed until completion.

2.2 AGAROSE GEL ELECTROPHORESIS

DNA samples were analysed on 1-2% agarose gels made up in 1 x TBE electrophoresis buffer. Agarose powder was dissolved by microwave oven boiling and the gel cooled to 60°C before it was poured into the gel trays. Running buffer (0.25% bromophenol blue) was added to approximately 5µl of each DNA sample and electrophoresed in TBE running buffer at 50-100 V for 30 mminutes to 1 hour. DNA fragments were visualised by staining the gels with ethidium bromide (5µl of 20µg/µl stock per 100ml) and UV light transillumination using an Eagle Eye (Stratagene). The size of the DNA fragments was estimated by running the DNA samples with a

molecular weight marker usually ϕX174 fragments: fragment sizes 72, 118, 194, 234, 271, 310, 603, 872, 1078, 1353 base pairs.

2.3 POLYACRYLAMIDE GEL ELECTROPHORESIS

Of all the genes examined, only the two NRAMP1 microsatellites D2S1471 and the NRAMP1 5' promoter dinucleotide repeat were investigated using this method.

Gels were made from 6% acrylamide/7 M urea (Severn Biotech). Polymerisation of acrylamide was initiated by addition of 400 µl of 10% w/v ammonium persulphate and 12 µl of TEMED. In all cases 24 cm 36 well gels were used. Gels were left to set for at least 1 hour. The surfaces of the glass plates were cleaned with water and ethanol before being placed into 373A Sequencers (AppliedBiosystems). 1 x TBE buffer was used in the anode and cathode buffer tanks. DNA samples were loaded and gels run at 900 V limiting power for 7.5 hours.

DNA samples were first PCR amplified with one of primer pair pre-labelled with a fluorescent dyes either FAM, HEX or TAMRA. PCR products were prepared for electrophoresis by pooling those samples that were to be run simultaneously. Quantities for pooling were adjusted according to the intensity of the PCR product observed but generally 1 in 40 dilutions of the PCR product was loaded into the gel. To each DNA sample was added 2.5 µl formamide (containing a trace of dextran) and 0.5 µl of GENESCAN2500-ROX.. Samples were denatured at 94°C for 2 minutes and placed on ice during loading.

2.4 DATA COLLECTION FROM POLYACRYLAMIDE GELS

Gene analysis was performed using the GENESCAN^{TM}672 (Ver 1.2) software (Applied Biosystems) according to the manufacturer's manual. Gel lane tracking was checked manually. GENESCAN 2500 Split Peak and Local Southern Method sizing options were used to calculate fragment sizes. Size standard User Defined, and sizes given in mobility units (MU) which are roughly equivalent to size in base pairs.

Genotyper^{TM} was used to determine and record the alleles at each marker locus, as described in the manufacturer's manual (Genotyper^{TM} Ver. 1.0). The Filter Labels option with default parameters was used to discriminate shadow peaks. Alleles were determined as the highest two peaks within the stated allele range, and were labelled by size. Homozygotes were identified when, shadow peaks having been excluded, no other peak was present.

2.5 DOT BLOT TRANSFER OF AMPLIFIED DNA TO NITROCELLULOSE OR NYLON MEMBRANES

Amplifcation product was rendered single stranded before transfer to a membrane as follows. 10 µl of PCR product was transferred to a clean 96 well microtitre plate and mixed with 100ml of denaturation solution (86µl of TE, 6µl of 0.5mM EDTA pH 7.5, 8µl of 6M NaOH) and incubated on ice for 10 minutes. To neutralise the reaction, 110 µl 2M ammonium acetate was added and the samples then transferred to a pre-wetted nylon membrane using a BRL Hybridot manifold. After transfer, each well was washed out with a further 200 µl of the 1M ammonium acetate used before for pre-wetting the

precipitated with two and half volumes of ethanol and centrifuged at 10 000 rpm for 15 minutes. The pellet was then dried and resuspended in distilled water as above. The concentration of the product was determined by spectrophotometry at 260 nM UV.

2.8 RADIOACTIVE END LABELLING OF OLIGONUCLEOTIDE PROBES

400ng of oligonucleotide was placed in a 1.5 ml microcentrifuge tube with 46.5 µl of kinase buffer (0.66 M Tris-HCl pH 7.5, 5mM $MgCl_2$, 5mM DDT, 1mM ATP), 1.5 µl of T4 Polynucleotide Kinase enzyme, 2µl γ ^{32}P-ATP (Amersham) to make up a final volume of 50 µl. The sample was incubated behind a screen for 1 hour at 37°C. The reaction was terminated by the addition of 450 ml TE buffer. Unincorporated nucleotides were removed by running the probe through a NAP-5 tm column (Pharmacia) according to the manufacturer's instructions. The probe was eluted off the column with 1ml TE buffer and 5 µl counted in a scintillation counter to determine its specific activity.

2.9 RADIOACTIVE HYBRIDISATION, WASHING AND AUTORADIO-GRAPHY FOR AMPLIFIED DNA

The membranes were placed in hybridisation bottles and incubated at 32 °C for 30 minutes with 10mls of prehybridisation buffer (6x SSC, 1x denhardt's solution, 2.5 mg/ml salmon Sperm DNA [denatured and sonicated], 2% SDS) and incubated at 32°C for 30 minutes. The prehybridisation solution was discarded and the labelled oligonucleotide (20 million counts/minute) added to 10ml of hybridisation solution (6x SSC, 0,01% SDS). The filters were hybridised overnight at 37°C. The hybridisation solution was washed twice, first in 6xSSC at room temperature for 10 minutes and

filter. The membrane was placed on damp Whatmann paper and the DNA fixed either by baking at 80°C for 2 hours or using UV fixation.

2.6 SOUTHERN TRANSFER OF DNA TO NITROCELLULOSE MEMBRANES

A tray was filled with 20x SSC and a glass plate balanced above with a sheet of 3mm Whatmann paper acting as a wick. The neutralised agarose gel slab was placed onto the paper wick with a wet nitrocellulose membrane laid over the gel ensuring that no bubbles formed between the two. The tray was covered with saran wrap and cut so that only the gel and membrane were free for transfer of DNA. Lastly, the gel and membranes were covered with three sheets of 3mm Whatmann paper soaked in 20x SSC, several layers of paper towels and a heavy weight. The gel was blotted overnight and then nitrocellulose membrane was removed and baked at 80°C for two hours to fix the DNA.

2.7 DEPROTECTION OF NEWLY SYNTHESISED OLIGONUCLEOTIDES

The oligonucleotide amplification primers and detection probes for the G6PD deficiency study were synthesised on a DNA Synthesizer, 381A (Applied Biosystems) and were supplied covalently attached to an inert substrate on a column. The oligonucleotide was removed by extracting the column slowly over a period of 30 minutes to 1 hour with 2mls of concentrated ammonium hydroxide (Andover Chemicals). The eluate was then transferred to 2 ml sealable tubes and deprotected overnight at 55°C. Once deprotected the oligonucleotides were either dried down using a Speedivac centrifuge and then resuspended in 500µl - 1 ml distilled water or

finally in 6x SSC at the melting temperature of the probe (calculated using the formula TM(°C) = 4[G+C] + 2[T + A]) for 10 minutes. The membranes were then dried and wrapped in cling film and exposed to X-ray film (Kodak X-Omat) for 4 hours at -80°C. Subsequent exposures were determined according to the intensity of the dots.

2.10 STRIPPING OF RADIOACTIVELY HYBRIDISED FILTERS

The filters were incubated in Strip buffer (6x SSC, 50mM EDTA) at 30°C for 30 minutes and then rinsed in 2x SSC solution at room temperature.

2.11 DIGOXIGENIN (DIG) LABELLING OF OLIGONUCLEOTIDE PROBES

(Boehring Mannheim)

To 1 µl of the oligonucleotide probe was added 4µl of tailing buffer, 4µl of 25 mM cobalt chloride, 1µl of DIG-dUTP and 9µl of distilled deionised water. 1µl of terminal transferase enzyme was the added and the 20µl reaction incubated at 37°C for 15 minutes. The labelling reaction was terminated by the tube being placed on ice and adding 2µl of glycogen solution (1 µl glycogen stock in 200µl 0.2M EDTA). 78µl of water was then added to the tube to make a total volume of 100µl. For each hybridisation bottle 25µl of the labelled probe was added.

2.12 DIG HYBRIDISATION AND STRINGENCY WASHING

All the reaction were carried out in Hybaid bottles. The nylon membranes, on which the amplified PCR product had been fixed, were placed in Hybaid bottles and initially blocked for 30 minutes at room temperature. The blocking solution (4x SSPE, 1% blocking reagent, 0.1% laurylsarcosine) was discarded and the membranes

prehybridised in a TMAC-based hybridisation solution (3M TMAC, 50mM Tris-HCl pH8.0, 0.1% SDS, 2mM EDTA) at 46-53° C depending on the amplified gene. 25µl of the labelled probe was then added and hybridisation allowed to take place for at least one hour at the same temperature as prehybridisation. After the hybridisation was completed the membranes were washed first for 20 minutes at room temperature in Wash Buffer 1 (2x SSPE, 0.1% SDS) and then for 15 minutes at the stringency temperature 47-59°C.

2.13 DIG DETECTION

These steps in the DIG detection process were all done at room temperature. The filters were first rinsed in Buffer 1 (NaCl, Maleate pH 7.5) for 5 minutes and then blocked with 20 ml of Buffer 2 (Buffer 1 and 1.0% Blocking reagent) for 30 minutes. 2 µl of anti-DIG antibody was then added to the Buffer 2 used for blocking and the reaction solution incubated for a further 30 minutes. Excess antibody was then washed off in two 15 minute washes with 25 ml of Tween wash buffer (Buffer 1 and 0.3% Tween). The filters were then equilibrated with 10 ml of Buffer 3 for 5 minutes. After the bottles had been properly drained the filters were incubated for 5-10 minutes in 5ml of CSPD solution (1:100) CSPD dilution in Buffer 3. The filters were then removed from the Hybaid bottles, wrapped in Saran cling film and then incubated at 37°C for 15 minutes to activate the CSPD. The filters were then exposed to X-ray film for approximately 10-20 minutes.

2.14 STRIPPING OF DIG-HYBRIDISED FILTERS

Initially, the filters were washed at 65°C for 30 minutes in 20ml Stripping Buffer 1 (20mM EDTA, 2xSSC), then at room temperature for 10 minutes in 20ml of Stripping Buffer 2 (2xSSC, 0.1% SDS) before a final third wash at 37°C for 30 minutes in 20ml of Stripping Buffer 3 (0.2M NaOH, 0.1%SDS). The filters were then rinsed with 2xSSC wrapped in cling film and exposed to X-ray film to ensure that the stripping process had been successful with all previous probes actually removed. The membranes were stored at -4°C .

2.15 DNA EXTRACTION FROM WHOLE BLOOD

All DNA extraction performed by myself were done using the Nucleon kit (Scotlab). 10ml of whole blood were mixed with 40ml of Reagent A (10mM Tris-HCl, 320mM Sucrose, 5mM MgCl2, 1% Triton, pH 8.0) in a 50 ml polypropylene centrifuge tube and the tube shaken for 4 minutes before it was centrifuged at 3 500 rpm for 5 minutes. The supernatant was discarded and 2ml of Reagent B (400 mM Tris-HCl pH 8.0, 60mM EDTA, 150mM NaCl, 1% SDS) lysis solution added and the tube vortexed briefly to resuspend the pellet. The suspension was transferred to a 15ml screw-capped polypropylene centrifuge tube, 500µl of sodium perchlorate added and the mixture shaken for 15 minutes at room temperature before being incubated in a water bath at 37°C for 30 minutes for deproteinisation. For the DNA extraction step, 2 ml of Chloroform (stored at -20°C) was added and the tube shaken for 15 minutes at room temperature then incubated in a shaking water bath at 65°C for 25 minutes. 300µl of the nucleon silica suspension was then added and the tube centrifuged at 2 500 rpm for 3 minutes. The tubes were then held vertically and the aqueous DNA-containing phase

above the silica suspension removed and transferred to a fresh tube. To this DNA phase, two volumes of cold ethanol (4°C) were added and the tube inverted gently to precipitate the DNA. The DNA was the resuspended in 500µl of distilled deionised water and the DNA concentration measured using a fluorometer.

2.16 HIV testing

This was done using the Wellcozyme HIV 1 and HIV 2 enzyme immunoassay for antibodies to immunodominant antigens: the envelope glycoprotein of both virus types and the cross reacting viral core protein. Only samples that were positive on 2 separate tests were considered HIV positive.

2.17 Statistical analysis

Comparisons between clinical groups in both the malaria and tuberculosis studies were made using the χ^2 test with continuity corrections. Odds ratios were calculated using the Statclac programme and are shown with 95% confidence intervals. The possible confounding effects of ethnic group heterogeneity in The Gambia on the odds ratio was assessed using a Mantel-Haenszel test. For the G6PD study, χ^2 tests for heterogeneity showed no significant differences between the odds ratios in the Gambian and Kenyan studies. So, combined odds ratios were calculated to provide pooled estimates, which are shown with 95% confidence intervals. Comparison of parasite density between groups for the G6PD study was performed using t-tests on log transformed data.

Table 2.1 Oligonucleotide sequences of primers used to amplify specific gene fragments using Polymerase chain reaction. Of primer pairs, 5' primer given first.

Genes amplified	Primer Sequences
G6PD (202)	5'-GTG GCT GTT CCG GGA TGG CCT TCT G-3'
	5'-CTT GAA GAA GGG CTC ACT CTG TTT G-3'
G6PD (376)	5'-CCC CAG AGG AGA AGC TCA -3'
	5'-CCA GGT AGA AGA GGC GGT-3'
HLA-DRB1	5'-GCC GCT GCA CTG TGA AGC TCT C-3
	5'-TTC TTC AAT GGG ACG GAG CG-3'
HLA-DR13.02	5'-TCC TGG ACA GAT ACT TCC-3
	5'-GCA CTG TGA AGC TCT CAC-3'
HLA-Class I	5'-CAC TCC ATG AGG TAT TTC-3'
α1 & 2 domain	5'- GTC TCC TTC CCG TTC TC-3'
HLA-B53, -35	5'- CCG GAA CAC ACA GAT CTT-3'
	5'-GTC GTA GGC GGA CTG GTC-3'
TNF-α	5'-CAA ACA CAG GCC TCA GGA CTC-3'
	5'-AGG GAG CGT CTG CTG GCT G-3'

Table 2.1 continued

NRAMP1 promoter	5'-GAG GGG TCT TGG AAC TCC A-3'
microsatellite	5'-CAC CTT CTC CTC CGG CAG CCC-3'
internal reverse primer:	TAC CCC ATG ACC ACA CC
D2S1471	5'-TAC CAT TCA CAC ATC GAA GAA C-3'
	5'-GAT CTC TCC CCC ACA ATA AAC-3'
MBP	5'-GCA CCC AGA TTG TAG GAC AGA G-3'
	5'-CAG GCA GTT TCC TCT GGA AGG-3'
IL-4	5'-ACT AGG CCT CAC CTG ATA CG-3'
	5'-GTT GTA ATG CAG TCC TCC TG-3'

Table 2.2 Cycling programmes used for various PCR amplifications

Gene amplified	Fragment size (bp)	Cycling conditions	$MgCl_2$	Cycles
G6PD (202)	109	hot start of 94 °C x 240 sec. then 94 °C x 45 sec. 60 °C x 60 sec 72 °C x 120 sec.	2.0mM	30
G6PD (376)	150	hot start of 94 °C x 240 sec. then 94 °C x 45 sec. 57 °C x 60 sec 72 °C x 120 sec.	2.0mM	35
HLA-DRB1	270	hot start of 95 °C x 300sec. then 94 °C x 90 sec. 64 °C x 120 sec 72 °C x 120 sec.	2.0mM	35

Table 2.2 continued

		hot start of 95 °C x 300sec. then		
HLA-DR13.02		94 °C x 60 sec. 58 °C x 60 sec 72 °C x 60 sec.	2.0mM	35
		hot start of 95 °C x 360sec. then		
HLA-Class I α1 & 2 domain	790	94 °C x 90 sec. 65 °C x 90 sec 72 °C x 120 sec.	1.5mM	35
		hot start of 95 °C x 300sec. then		
HLA-B53, -35	603	94 °C x 90 sec. 58 °C x 120 sec 72 °C x 120 sec.	1.5mM	35
		hot start of 95 °C x 600sec. then		
TNF-α	519	94 °C x 60 sec. 58 °C x 90 sec 72 °C x 120 sec.	2.0mM	35

Table 2.2 continued

NRAMP1 Promoter microsatellite, *1 PCR*	800	94 °C x 60 sec. 58 °C x 60 sec 72 °C x 180 sec.	2.5mM	15
NRAMP1 Promoter microsatellite, *2° PCR*	108-124	94 °C x 60 sec. 58 °C x 60 sec 72 °C x 45 sec.	2.5mM	30
D2S1471	78-122	94 °C x 45 sec. 53 °C x 60 sec 72 °C x 60 sec.	2.5mM	28
MBP	328	95 °C x 240sec. then 94 °C x 90 sec. 58 °C x 90 sec 72 °C x 120 sec.	3.5mM	40
IL-4	253	hot start of 95 °C x 300sec. then 94 °C x 60 sec. 58 °C x 45 sec 72 °C x 600 sec.	4.5mM	38

Table 2.3 Sequence-specific oligonucleotide probes used to determine the allele present in amplified product and their washing temperatures

Allele	Oligonucleotide Sequence	Washing temperature
G6PD A-	5'-TTC ATC ATG GGC TA-3'	38 °C
G6PD non A-	5'-TTC ATC GTG GGC TA-3'	36 °C
G6PD A	5'-GCC ACA TGG ATG CCC TC-3'	54 °C
G6PD B	5'-GCC ACA TGA ATG CCC TC-3'	52 °C
HLA-DRALL	5'-AGC TGG GGC GGC CT-3'	51°C
HLA-DR1	5'-GGA AAG ATG CAT CTA TA-3'	52 °C
HLA-DR2	5'-GCG GTT CCT GCA CAG AG-3'	53 °C
HLA-DR3	5'-GGG TGG ACA ACT ACT GC-3'	53 °C
HLA-DR4	5'-ACT TCT ATC ACC AAG AG-3'	54 °C
HLA-DR7	5'-GGA AAG ACT CTT CTA TA-3'	52 °C
HLA-DR8	5'-GGC GGG CCC TGG TGG AC-3'	53 °C
HLA-DR9	5'-TGC GGT ATC TGC ACA GA-3'	53 °C
HLA-DR10	5'-GGA AAG ACG CGT CCA TA-3'	52 °C
HLA-DR11	5'-GGC CTG ATG AGG AGT AC-3'	53 °C
HLA-DR13.02	5'-ACA TCC TGG AAG ACG AGC G-3'	53 °C
HLA-DR13.04	5'-GGC GGC CTA GCG CCG AG-3'	54 °C
PS28	5'-ACA TCC TGG AAG ACG AGC-3'	52°C

Table 2.3 continued

HLA-B35 (Bw6)	5'-GGC ACC TGC GCG GC-3'	38 °C
HLA-B53 (Bw4)	5'-CGG ATC GCG CTC CGC TAC-3'	38 °C
HLA-B18	5'-TAG AGC AAG AGG GGC-3'	38 °C
-308 TNF '1'	5'-AGG GGC ATG GGG ACG GG-3'	59 °C
-308 TNF '2'	5'-AGG GGC ATG AGG ACG GG-3'	56 °C
-238 TNF 'G'	5'-CCC TGC TCC GAT TCC GAG-3'	56 °C
-238 TNF 'A'	5'-CCT CGG AAT CAG AGC AGG G-3'	58 °C
MBP-57 w	5'-CAC CAA GGG AGA AAA GGG-3'	54 °C
MBP-57 m or 'C'	5'-CAC CAA GGA AGA AAA GGG-3'	57 °C
MBP-52 w	5'-AAG ATG GGC GTG ATG GCA-3'	54 °C
MBP-52 m or 'D'	5'-AAG ATG GGT GTG ATG GCA-3'	56 °C
MBP-54 w	5'-CGT GAT GGC ACC AAG GGA-3'	54 °C
MBP-54 m or 'B'	5'-CGT GAT GAC ACC AAG GGA-3'	56 °C
IL-4 '1'	5'-GAA CAT TGT CCC CCA GTG-3'	47 °C
IL-4 '2'	5'-GAA CAT TGT TCC CCA GTG-3'	49.5 °C

2.18 STOCK SOLUTIONS

500mM EDTA - 18.6g EDTA, 10g NaOH/100mlS dH_2O

10% SDS - 10G Sodium lauryl sulphate/100mls dH2O

100 x DENHARDT'S SOLUTION - 2% w/v Ficoll, 2% w/v polyvinylpyrrolidene, 2% w/v Bovine serum albumin

10mg/ml SALMON SPERM DNA - Sonicate DNA for 4x 15secs. at 4°C. Boil for 5 minutes, chill on ice and then freeze.

TBE BUFFER - 89mM Tris-HCl, 89mM Boric Acid, 2.5mM EDTA

40% POLYACRYLAMIDE STOCK - 38% w/v Polyacrylamide, 2% w/v Bis-Acrylamide

RADIOACTIVE PREHYBRIDISATION SOLUTION - 1.8x SSC, 10xDenhardt's, 0.4mg/ml Salmon Sperm DNA, 0.5% w/v SDS

20x SSPE - 175.3g NaCl, 31.2g $NaH_2PO4.2H2O$

RADIOACTIVE HYBRIDISATION SOLUTION - 1.8x SSC, 0.001% w/vSDS

DIG BLOCKING SOLUTION - 4x SSPE, 1% Blocking solution, 0.1% lauryl sarcosine

DIG HYBRIDISATION SOLUTION - 3M TMAC,50mM Tris pH 8.0, 0.1% SDS, 2mM EDTA

DIG 10x BUFFER 1 - 87,66g NaCl, 116.1g Maleate, pH 8.0

DIG WASH BUFFER 1 - 2x SSPE, 0.1% SDS

DIG BUFFER 2 - DIG Buffer 1, 1.0% Blocking reagent

DIG TWEEN WASH BUFFER - Buffer 1, 0.3% Tween

DIG BUFFER 3 - 100mM Tris (pH 9.5), 100mM NaCl, 20mM $MgCl_2$

CSPD SOLUTION - 1%CSPD, Buffer 3

DIG STRIPPING BUFFER 1 - 20mM EDTA, 2x SSC

DIG STRIPPING BUFFER 2 - 2x SSC, 0.1% SDS

DIG STRIPPING BUFFER 3 - 0.2M NaOH, 0.1% SDS

NUCLEON REAGENT A - 10mM Tris-HCl, 320mM Sucrose, 5mM Sucrose, 1% Triton, pH 8.0

NUCLEON REAGENT B - 400mM Tris-HCl pH 8.0, 60mM EDTA, 150 mM NaCl, 1% SDS

CHAPTER 3 GLUCOSE-6-PHOSPHATE DEHYDROGE-NASE (G6PD) DEFICIENCY AND P. FALCIPARUM MALARIA IN THE GAMBIA AND KENYA

3.1 G6PD

G6PD is a cytoplasmic so called 'house-keeping' enzyme found in all cells of the body. It catalyses the first and rate-limiting step of the hexose monophosphate pathway which facilitates pentose phosphate synthesis and is an important source of NADPH. NADPH is required for several biosynthetic reactions and is essential for maintaining adequate intracellular levels of reduced forms of glutathione and other forms of sulphydryl groups. Hence by preserving and regenerating reduced forms of glutathione as well as promoting the stability of catalase, an enzyme that breaks down toxic oxygen compounds such as hydrogen peroxide, NADPH plays a major role in a cell's ability to withstand oxidant stress. The hexose monophosphate shunt catalysed by G6PD is also an important source ribose which is essential for production of nucleotide coenzymes, the replication of nucleic acids and therefore cell division (Sodeinde 1992). The biological importance of this enzyme is underlined by the fact G6PD has been detected in virtually every cell type of every contemporary organism so far tested (Vulliamy *et al.* 1992).

Along with the loci encoding colour blindness and factor VIII, the G6PD gene is located in the telomeric region on the long arm (Xq28) of the X chromosome (Pai *et al.* 1980; Keats 1983). The G6PD gene consists of 13 exons and spans approximately 18 kb (Chen *et al.* 1991). The G6PD protein has a molecular weight of 59 kd and the

active enzyme is composed variably of two or four identical 515 amino acid subunits (Persico *et al.* 1986).

3.2 Genetic polymorphism of G6PD

The G6PD locus is probably the most polymorphic locus in humans with over 300 allelic variants known, at least 87 of which have reached polymorphic (i.e >1%) frequencies. (Luzzatto & Mehta 1989; Luzzatto & Battistuzzi 1985). These variants have been characterised biochemically based primarily on their differing residual enzyme activities and electrophoretic mobility patterns but also on their physicochemical (thermostability, chromatographic behaviour) and kinetic properties (K_m for Glucose-6 phosphate or NADPH, pH dependence and utilisation of substrate analogues). Interestingly, most of these polymorphic alleles are associated with variable forms of G6PD enzyme deficiency. The enzyme deficiency appears to result from a combination of decreased catalytic activity and increased degradation of the encoded mutant protein.

The G6PD variants are grouped into different classes depending on the degree of enzyme deficiency and consequent clinical symptoms (Table 3.1). To date, at least 34 different mutations have been identified through comparisons of genes encoding variant enzymes with the sequence of the normal G6PD B gene. These mutations are widely spread throughout the gene being found in all exons except exon 3 and exon 13. All but one of these are point mutations which cause amino acid substitutions (Vulliamy *et al.* 1992). The exception is G6PD Sunderland which is due to a 3bp

Table 3.1 Classes of G6PD variants

	enzyme activity
Class I	sevcrely deficient associated with chronic non-spherocytic anaemia
Class II	severely deficient, <10% residual enzyme activity
Class III	moderately deficient, 10 - 60% enzyme activity
Class IV	near normal or normal enzyme activity, 60 - 150% enzyme activity
Class V	increased enzyme activity, >150%

deletion and results in the loss of an isoleucine residue (MacDonald *et al.* 1991). The absence in the G6PD gene of larger deletions, or other mutations such as nonsense mutations or frameshift mutations that would completely abolish the function of the protein, suggests that complete absence of the G6PD enzyme is lethal (Vulliamy *et al.* 1992).

One of the most common variants associated with deficiency and the variant that is investigated in this study, is the class III variant, G6PD A-. G6PD A-, which is the predominant deficiency variant in Africa, is unique in that it contains two mutations. The first at nucleotide 376, which on its own gives rise to G6PD A (Vulliamy *et al.* 1988), is an adenine to guanine subsitution that results in an asparagine to aspartate amino acid substitution whilst the second mutation, usually a guanine to adenine substitution at nucleotide 202, leads to a valine to methionine substitution (Hirono & Beutler 1988). In a few people two other alternative second mutation sites have been identified at nucleotides 680 and 968, but the nucleotide 202 substitution accounts for at least 95% of the G6PD A- molecular variants in Africa (Hirono & Beutler 1988; Beutler *et al.* 1989).

In Africa, G6PD is essentially a tri-allelic polymorphism (Table 3.2). G6PD B, the nomal variant associated with normal or 100% enzyme activity, is the commonest allele with frequencies of 60-80%. G6PD A which has 90% of the activity of G6PD B is the next commonest allele with frequency between 15-40%. The third allele which is the common deficiency allele in Africa is G6PD A-. It is a class III variant with 12% enzyme activity and it varies in frequency from 0 to 25% (Luzzatto & Mehta 1989).

Table 3.2 G6PD alleles in Africa and their enzyme activities

alleles	classs	enzyme activity	frequency
G6PD B	**IV**	**100%**	**0.60 - 0.80**
G6PD A	**IV**	**80%**	**0.15 - 0.40**
G6PD A-	**III**	**12%**	**0.00 - 0.25**

3.3 G6PD deficiency

3.3.1 Frequency and distribution

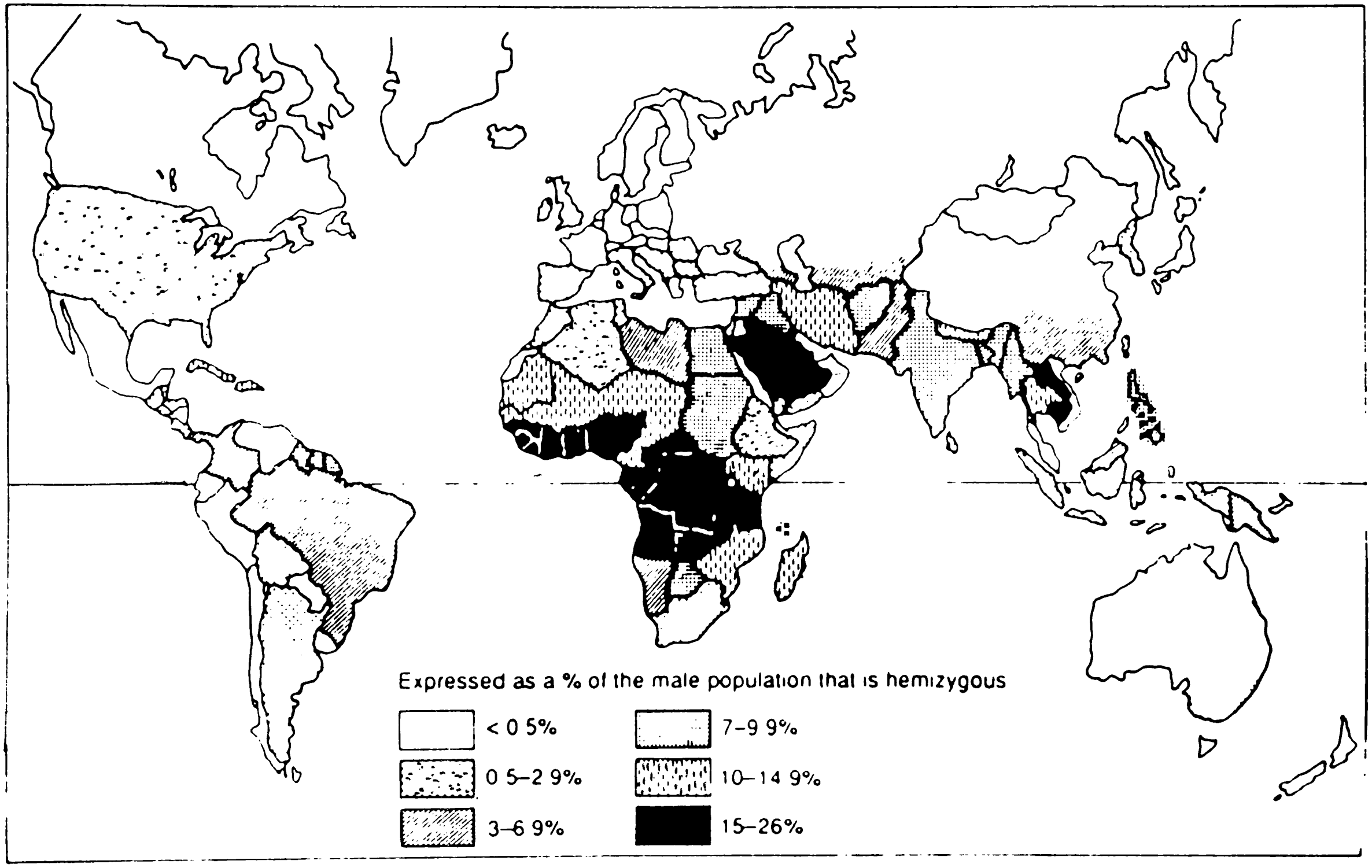
G6PD deficiency is the commonest enzymopathy in man affecting over 400 million people worldwide (Beutler 1991). This disorder which is caused by a multitude of the different structural allelic mutants of the G6PD gene referred to above, is found mainly in the tropical and sub-tropical regions of the world with the highest rates, usually 5-30%, being found in Africa, Asia, the Middle East, the Mediterranean and Papua New Guinea (Figure 3.A; Luzzatto & Mehta 1989). Worldwide the frequency figures range from 62% in Kurdish Jews to 0.1% in Japan and Northern Europe (WHO Working Group, 1989b).

3.3.2 Clinical features

Clinical expression of G6PD deficiency is probably dependant on an interaction of the molecular properties of a given deficiency variant, exogenous factors and, possibly, additional genetic factors (Luzzatto & Mehta 1989). In unstressed normal cells, G6PD activity is only 2% of total capacity (WHO working group 1989) and therefore it is hardly surprising that most individuals with the more common class II and III G6PD deficiency variants are usually asymptomatic. Although this has not been proved categorically there does appear to be a correlation between the degree of enzyme deficiency and the propensity to develop clinical symptoms.

The most striking clinical syndrome associated with G6PD deficiency, acute haemolytic anaemia, occurs as a manifestation of this disorder on the mature red blood

Figure 3.A showing worldwide distribution of G6PD deficiency



cell. On account of its long non-nucleated life-span, the mature erythrocyte has a diminished reductive capacity to respond to oxidant stress because of its impaired ability to generate adequate levels of NADPH and therefore reduced forms of glutathione. Uncompensated oxidant stress in the erythrocyte leads to oxidation of haemoglobin to methaemoglobin, heinz body formation and membrane damage (Beutler 1983). In the extreme, this leads to haemolysis while a less severe stress increases the deformability of the erythrocyte and probably increases the likelihood that the stressed cell will be removed from circulation by the reticuloendothelial system (Arese *et al.* 1986; Turrini *et al.* 1993).

Acute haemolytic anaemia is therefore the most frequent clinical manifestation of G6PD deficiency. The haemolysis is precipitated most commonly by infections but also can occur after the ingestion of drugs or foodstuffs that contain oxidant components and in certain metabolic conditions such as diabetic ketoacidosis (Greene 1993). Quantitatively, infections are likely to be the most important precipitants. With drugs, definite associations have been identified with certain antimalarial agents such as primaquine, some sulphonamides, nitrofurantoin and several anti-inflammatory agents (Greene 1993). Fava beans (Vicia faba) commonly ingested in the Mediterranean are the most well documented causative dietary agent and are associated with a well characterised condition, favism. Favism is associated with the severely deficient class II G6PD Mediterranean form and not the moderately deficient G6PD A- form that is common in Africa. Although all victims of favism are G6PD deficient, not all (only 25%) G6PD deficient individuals develop favism after consumption of the fava beans

(Luzzatto & Mehta1989; Sodeinde 1992), suggesting that they may be other genetic or environmental factors involved.

For Class I G6PD deficiency variants, the formed enzyme is functionally so poor that the red cell life-span is shortened even in the absence of stress and hence Class I variants are associated with a chronic non-spherocytic hemolytic anaemia (Beutler 1991). Affected individuals typically have mild to moderate anaemia and splenomegaly. The disadvantage of the chronic anaemia probably outweighs any survival advantage afforded by these class I mutations and not surprisingly most of these mutations arise sporadically as new mutations but are not usually propogated in a population (Luzzatto & Mehta 1989). Interestingly, these class I variants are clustered near the carboxyl terminus of the G6PD protein (Beutler 1991).

Another serious clinical effect of G6PD deficiency, is icterus neonatorum or neonatal jaundice which in severe cases can lead to permanent neurological damage or death. Increased red blood cell destruction accounts for some of the hyperbilirubinaemia observed in this syndrome but it is likely that a severe enzyme deficiency in the hepatocyte may impair the catabolism of bilirubin and thus also contribute to the development of jaundice (Beutler 1991).

3.3.3 Diagnosis and management of G6PD deficiency-related conditons

Diagnosis of G6PD deficiency is easy and there are a variety of quantitative and semi-quantitative tests available that do not measure G6PD protein levels but rather enzyme activity as reflected in NADPH production. As mentioned earlier, the vast majority of

G6PD deficient individuals are asymptomatic and therefore do not need treatment. In acute haemolytic anaemia, management is directed at the underlying cause i.e treatment of infection and any co-existent metabolic derangements, withdrawal of the offending drug or foodstuff. In severe cases blood transfusion (exchange transfusion in neonatal jaundice) with blood of normal G6PD activity may be necessary. Screening of all newborns for deficiency has been proposed in populations with high prevalence rates as this would facilitate appropriate dietary management and prophylactic avoidance of potentially toxic drugs in identified G6PD deficient individuals.

3.3.4 X-chromosome activation and G6PD deficiency

The G6PD gene is on the X chromosome and therefore in females one of the two G6PD alleles is subject to inactivation. Consequently expression of G6PD deficiency varies markedly among female heterozygotes as their red blood cell populations are a mosaic of deficient and normal cells due to variable X-chromosome inactivation (Beutler *et al.* 1962). In fact, this phenomenon affects all somatic cells in the body such that G6PD phenotypes have been successfully used in the past to determine the clonal origins of certain tumours and embryonal tissues in such females heterozygotes (Linder D & Gartler1965; Beutler 1967; Fialkow 1976).

3.4 The G6PD deficiency and malaria hypothesis

3.4.1 Epidemiological evidence

It has been proposed that G6PD deficiency has been selected for in different populations because it may aid fecundity (Lisa *et al.* 1994) and enhance metabolism of a common environmental pollutant, lead (Sodiende 1992). However, the striking

geographical correlation between the distribution of these polymorphic alleles with areas with historical endemicity of *P.falciparum* malaria suggests that disorder has risen in frequency through natural selection by malaria. Furthermore, micromapping within relatively restricted geographical areas such as Kenya (Alison 1960), Papua New Guinea (Yenchitsomanus *et al.* 1986), Greece (Stamatoyannopoulos *et al.* 1966) and Sardinia (Siniscalco *et al.* 1966) has also shown a similarly remarkable geographical correlation beteen altitude and the distribution of this disorder with the lower altitude (<1 000 metres) areas, which are known to have more intense malaria transmission, being clearly associated with higher frequencies for G6PD deficiency. It is unlikely that the distribution of G6PD deficiency is attributable to gene flow and the presence of many diverse G6PD variants that have arisen independently and reached polymorphic frequencies in geographically disparate areas (Luzzatto & Mehta 1989) further supports the occurrence of natural selection for this disorder.

3.4.2 *In vitro* evidence

Reports from early field studies that *P. falciparum* and *P. vivax* parasites preferentially invade younger red blood cells that have relatively higher G6PD activity (Alison & Clyde 1961; Kruatrachue *et al.* 1962), as well as the observation that in the presence of normal and deficient erythrocytes, malaria parasites preferentially develop in the normal cells (Luzzatto *et al.* 1969), led investigators to propose that G6PD deficient erythrocytes might confer protection against malaria by inhibiting erythrocyte invasion or intracellular development of the malaria parasite (Luzzatto 1979; Friedman *et al.* 1979). Since then there have been several independent studies in the literature reporting impaired growth of *P falciparum* in G6PD deficient erythrocytes (Roth *et al.*

1983; Miller *et al.* 1984), although in some studies this was only observed when cultures were subjected to oxidative stress (Friedman *et al.* 1979). Furthermore, there are data that indicate that in heterozygous females who have different proportions of normal and deficient cells owing to variable X-chromosome inactivation, the degree of parasite growth inhibition is proportional to the percentage of deficient cells present (Roth *et al.* 1983a).

Although there is growth inhibition in G6PD deficient erythrocytes, it is now clear that after a few growth cycles, the parasite can overcome the inhibition and it had been suggested that the parasite achieved this by producing its own G6PD enzyme (Hempelmann & Wilson 1981; Usanga & Luzzatto 1985). On the premise that expression of parasite G6PD enzyme was affected by host erythrocyte deficiency, an ingenious mechanism was put forward (Usanga & Luzzatto 1985) to explain the then current dogma that only female heterozygotes were protected against malaria (Bienzle *et al.* 1972). Hence in uniformly deficient red blood cells such as those found in deficient hemizygous males or deficient hemizygous females, the parasite's own induced G6PD enzyme would compensate for the lack of the host's enzyme. However, in female heterozygotes, who necessarily have mixed populations of deficient and non-deficient erythrocytes, parasite adaptation would be compromised and thus parasite growth impaired by the the need to repeatedly switch on its enzyme as a parasite moved from a non-deficient to a deficient host red blood cell. Unfortunately, while confirming the phenomenon of adaptation, subsequent studies (Roth & Schulman 1988; Ling & Wilson 1988) including one by Luzzatto's group

(Kurdi-Haidar & Luzzatto 1990) have found that the parasite G6PD levels do not appear to be affected by the host red cell genotype.

3.4.3 Field studies

Researchers have long recognised that definitive answers on the malaria /G6PD hypothesis had to come from field studies. Such studies would answer several important questions. Firstly, is G6PD deficiency protective against uncomplicated mild malaria, severe malaria or both and, if so, what is the extent of protection conferred? Secondly, if the disorder is protective against malaria, do all various deficiency genotypes in males and females afford protection and, if so, are there any differences in the amount of protection associated with the various genotypes? The literature on previous G6PD deficiency/malaria field studies is full of conflicting reports (Alison & Clyde 1961; Gilles *et al.* 1967; Bienzle *et al* 1972; Butler 1973; Kar *et al.* 1992; Martin *et al.* 1979; Kruatrachue *et al.* 1962; summarised in Table 3.3).

The most widely quoted field study from which came the dogma that only female heterozygotes were protected against malaria, was carried out by Lucio Luzzatto's group in Nigeria in the early 1970s (Bienzle *et al.* 1972). In their study they recruited 702 children aged between 9 months to 6 years, and 189 young adults between 14 and 20 years of age who had presented to a hospital with an acute febrile illness. 66% of the children had *P. falciparum* parasitaemia.The frequencies of the G6PD A males and G6PD A-/B females were lower in those children with malaria suggesting that these genotypes were protective against the disease. Subsequent reanalysis of the same study looking at parasitisation rates in the children also concurred with the initial findings

demonstrating that the G6PD A-/B females had the lowest rates whilst, in marked contrast, hemizygous and homozygous deficient subjects had the highest infection rates (Bienzle *et al.* 1979). Their observation that only mildly deficient G6PD A males, and not the more markedly deficient hemizygous males and homozygous females, were protected against malaria does not concur with the view that the degree of enzyme deficiency is central to the mechanism of protection against in G6PD deficiency. Furthermore, their assertion, based on their results, that only G6PD A-/B heterozygotes and not G6PDA-/A heterozygotes who probably have comparable G6PD activities, are protected appeared puzzling with regard to the possible mechanism of protection of this disorder against malaria. A subsequent field sudy on a similar population in Nigeria disputed the findings of Luzzatto's group (Martin *et al.* 1979).

3.4.4 G6PD deficiency and non-falciparum malaria

The malaria G6PD hypothesis primarily concerns the more common *P. falciparum* malaria. The comparatively small numbers of studies with non-falciparum malaria has made this isssue difficult to assess although there are a few reports in the literature. A study in Nigeria reported a lower than expected frequency of female heterozygotes amongst 33 girls with *P. malariae* - associated nephrotic syndrome (Sodeinde 1992). In addition, a large study by Kar *et al.* (1992) in North India reported protection in female heterozygotes and male hemizygotes against both *P. falciparum* and *P. vivax* malaria.

Table 3.3 Summary of some field studies on G6PD deficiency and malaria

i. studies supporting protective role of G6PD deficiency

investigator	study numbers	comment	population
Alison & Clyde, 1961	532	lower parasite rates and densities in deficient male children; reduced levels similar to children with sickle trait for both indices	East Africa
Gilles et al. 1967	100	reduced frequency of both deficient males and females in cases with very high parasite counts (>100, 000 mm^3) compared to controls	Nigeria
Luzzatto et al 1969	-	greater rates of parasitisation of non-deficient erythrocytes in mixed erythrocyte populations of female heterozygotes with acute malaria	Nigeria
Bienzle et al 1972	702	reduced frequency of female heterozygotes only in paediatric cases; reduced parasite densities in same group	Nigeria
Butler 1973	277	lower incidence of malaria in nonimmune deficient adult African-American males compared to their non-deficient counterparts	Vietnam
Kar et al 1992	708	significantly reduced parasitisation rates in deficient males and females	India

Table 3.3 continued

ii. studies indicating no protective role of G6PD deficiency

investigator	**study numbers**	**comment**	**population**
Kruatrachue et al 1962	203	increased malaria in deficient males (1-3 years old) compared to non-deficient males in same age group	Thailand
Powell & Brewer 1965	16	no difference in parasitisation and parasitaemia levels in experimentally infected deficient and non-deficient nonimmune adult African-American male prison inmates	U.S.A
Martin et al 1979	68	no protection against cerebral malaria for any of the deficiency genotypes	Nigeria

3.4.5 Why the difficulty in verification of the Malaria/G6PD deficiency hypothesis?

Unlike the sickle cell trait whose protective effect in malaria has been relatively straightforward to prove in *in vitro* and field studies, G6PD deficiency's suspected protective role in malaria has proved difficult to to verify. There are several reasons that may explain why this might be so. In contrast to HbS, G6PD follows a sex-linked rather than an autosomal inheritance pattern and so males have distinctly different genotypes from females. In addition, there is considerable genetic heterogeneity associated with G6PD deficiency and in some populations more than one deficiency variant is present. More pertinently, it is known that fitness of the deficiency phenotype is decreased significantly only under a limited number of specific circumstances and therefore on average very little. Lastly, G6PD deficiency may interact with other genetic factors and population specific non-malaria enviromental factors, such as diet, to modify the net fitness of the carrier (Martin 1994).

In retrospect, perhaps it would have been surprising if a significant effect of G6PD deficiency had been clearly demonstrated in some of the major field studies done in the past. Invariably, these clinical studies attempted to define the various genotypes from phenotypic measurements of enzyme levels, electrophoretic mobility and cytochemical staining patterns (Gilles *et al.* Bienzle *et al.* 1972; Martin *et al.* 1979). This is difficult because overlapping levels of enzyme activity are seen among genotypes, partly related to variable inactivation of X chromosomes in female heterozygotes, and partly to altered rates of erythrocyte turnover in acute malaria (Luzzatto & Mehta 1989). Furthermore, several of these studies were studying parasite densities in cases of the

more common clinical condition, mainly mild or uncomplicated malaria (Bienzle *et al.* 1972; Martin *et al.* 1979). Recently, it has been found that comparison of genotype frequencies in children with the relatively rare condition of complicated or severe malaria (Greenwood *et al.* 1991; Marsh 1992) with those of matched controls seems to offer a more sensitive measure of the effect of malaria resistance alleles (Hill *et al.* 1991; McGuire *et al.* 1994).

3.5 Aims of this study

In this study we set out to measure the frequencies of the G6PD A-, G6PD A and G6PD B alleles and genotypes in over 2000 DNA samples that already been collected as part of large case-control studies of severe malaria in West and East Africa. This was going to be one of the the first and certainly the largest field study to use precise molecular techniques to unequivocally define G6PD genotypes in an investigation of the proposed protective effect of G6PD deficiency alleles, such as G6PD A-, against malaria. Our main aim was to establish definitively whether females, males or both with G6PD deficiency are protected primarily against malaria, particularly severe malaria, and to provide the first estimate of the amount of protection afforded. We hoped that this information would assist in understanding the mechanism of protection against malaria associated with this disorder as well as clarifying the evolutionary mechanisms responsible for the high prevalence of this genetic disorder in most tropical and sub-tropical populations.

3.6 Study designs for the two case-control studies

Children up to 10 years of age were studied in two regions of endemic malaria, The Gambia in West Africa and Kenya in East Africa (Figure 3B).

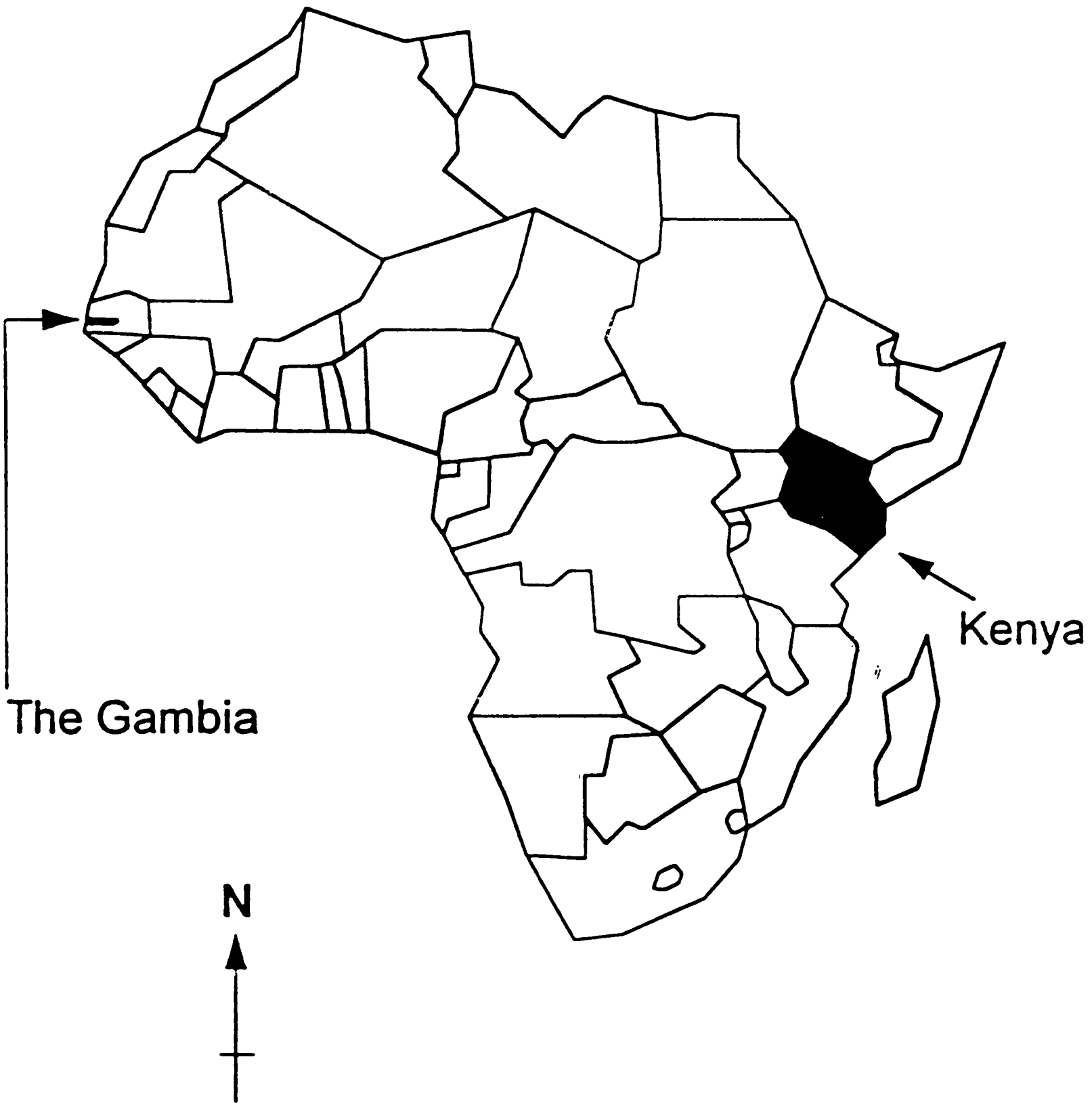
3.6.1 The Gambian study

Children were recruited over 3 years during the peak malaria transmission season from the central hospital in the capital Banjul. A diagnosis of acute malaria was made if a child with an appropriate clinical picture had a parasitaemia >2, 500 μl^{-1}, a level likely to be associated with illness in this setting, and relevant clinical and laboratory investigations excluded other diagnoses. Malaria cases were assigned to the severe group if any of the following features were present: (1) cerebral malaria, unrousable coma of score <3 by standard criteria (Molyneux *et al.* 1989), or repeated prolonged (>30 minutes) seizures; (2) severe malarial anaemia, haemoglobin level <5g dl^{-1} 1 on admission; (3) hypoglycaemia, blood glucose < 2.2mmol. The control children consisted of inpatients and outpatients with a variety of non-malarial illnesses and without *P. falciparum* parasitaemia who were matched for age and location as a group to the severe malaria cases. Mild malaria cases were those that fulfilled the above-mentioned criteria for diagnosis of malaria infection but without any of the specific features ascribed to severe cases as described above.

3.6.2 The Kenyan study

The Kenyan cases were recruited at Kilifi District Hospital on the Kenyan coast and matched for age and area of residence to healthy community controls, as part of a larger case-control study (Marsh K et al., in preparation). Cases of severe malaria were

Figure 3.B Map of Africa showing The Gambia and Kenya



classified as either cerebral malaria or severe malarial anaemia; a small minority of children had both conditions. The criteria for inclusion in these clinical categories were as described for the Gambian study, except that in Kilifi the threshold parasite density deemed appropriate for inclusion in the severe malarial anaemia group was 10,000 μl^{-1} with a haemoglobin level < 5g dl^{-1} (but 2,500μl^{-1} in The Gambia), reflecting the higher transmission of malaria in the Kilifi area than in the Gambian study area. The severe malaria cases were slightly younger in Kenya (mean 27 months) than in The Gambia (mean 36 months).The Kenyan control group consisted of healthy community children, recruited irrespective of the presence or absence of asymptomatic parasitaemia, who were individually matched for age and location to the cases of severe and mild malaria.

3.7 RESULTS

G6PD genotypes were typed by PCR amplification and oligonucleotide-specific hybridisation. Table 3.4 shows the frequencies of individuals with different genotypes in the various clinical groups. There was no significant heterogeneity in odds ratios between the two populations studied, so combined odds ratios and significance levels were calculated The frequency of female heterozygotes was lower in the children with severe malaria than in controls in both studies. Analysis of the combined data sets indicated that this genotype was associated with about 46% protection against severe malaria (odds ratio = 0.54 [95% confidence intervals 0.34-0.84] $p < 0.006$) and also with significant protection against mild malaria (O.R = 0.59 [95% c.i. 0.36-0.94] $p < 0.03$). Male hemizygotes were also less frequent amongst the children with severe malaria, with the combined odds ratio indicating that this genotype was associated with

about 58% protection (O.R.= 0.42 [95% c.i. 0.22-0.77] $p < 0.005$). The decrease in frequency of this genotype amongst the cases of mild malaria was not statistically significant (O.R. 0.63 [0.36-1.11] $p = 0.12$). In some instances, individuals had both cerebral malaria and severe anaemia. Female G6PD A- homozygotes were too rare to allow measurement of the susceptibility of this genotype to severe malaria.

Clinical evidence of haemolysis during severe malaria is rare in both populations and no difference in haemoglobin levels (g/dl, mean ± S.E.) was found between G6PD genotypes (e.g. for Kenya: in normals, males and females without the A- allele, 7.94 ± 0.11; in heterozygotes 8.07 ± 0.32; in hemi- or homozygotes 7.78 ± 0.29). Heterozygosity for haemoglobin S was strongly protective against both severe and mild malaria in both areas but no clear evidence of interaction betweeen G6PD and haemoglobin S genotypes was observed, although the power of the study to detect this was low. G6PD enzyme levels measured at recruitment, in Kenya only, showed a correlation of enzyme level (in units per 10^{12} RBC, mean ± S.E.) with G6PD genotype (normals 292 ± 7; heterozygotes 238 ± 14; hemi- or homozygotes 141 ± 16) (Table 3.5(i)), but no significant difference in mean enyzme activity was observed between clinical groups (controls 277 ± 9; mild malaria 249 ± 10; severe malaria 289 ± 12).

Parasite densities (geometric mean with 95% confidence interval x 10^3 μl^{-1}) amongst malaria cases (studied within 2 hours of arrival at hospital) in The Gambia did not differ significantly between genotypes (normal 75.8, 67.1-85.6; heterozygotes 116.8, 65.9-207.0; hemizygotes 42.1, 6.6-266.8) (Table 3.5(ii)). A larger proportion of the children with severe malaria had received some form of anti-malarial prior to

Table 3.4 Frequencies (%) of G6PD A- female heterozygotes and male hemizygotes in The Gambia and Kenya

		CONTROLS	MILD MALARIA	*Severe Mal. Anaemia*	*Cerebral Malaria*	All SEVERE MALARIA	ODDS RATIOS Mild v Controls	ODDS RATIOS Severe v Controls
FEMALE HETERO-ZYGOTES	Gambia	**13.7%** n=182	**9.1%** n=165	5.3% n=94	6.8% n=174	**6.7%** n=255	0.62 [0.30-1.29] *P*=0.23	0.45 [0.22-0.89] *P*=0.02
	Kenya	**27.3%** n=143	**17.2%** n=116	20.6% n=68	16.9% n=65	**18.8%** n=133	0.52 [0.27-0.99] *P*=0.047	0.60 [0.33-1.11] *P*=0.11
	Combined odds ratio	**19.7%** n=325	**12.3%** n=285			**10.8%** n=388	0.59 [0.35-0.90] *P*=0.011	0.54 [0.34-0.84] *P*=0.006
MALE HEMI-ZYGOTES	Gambia	**5.9%** n=239	**2.7%** n=182	1.3% n=80	1.9% n=208	**1.4%** n=279	0.45 [0.14-1.38] *P*=0.20	0.23 [0.06-0.77] *P*=0.012
	Kenya	**18.8%** n=149	**14.3%** n=133	14.9% n=47	8.6% n=70	**11.1%** n=117	0.72 [0.36-1.42] *P*=0.40	0.54 [0.25-1.15] *P*=0.12
	Combined odds ratio	**10.8%** n=388	**7.6%** 315			**4.3%** n=396	0.63 [0.36-1.11] *P*=0.12	0.42 [0.22-0.77] *P*=0.004

Table 3.5 G6PD enzyme activities and parasite densities for the various G6PD genotypes and clinical groups. G6PD genotypes analysed in three groups: 'normal' = normal females and males; heterozygote = heterozygote females; deficient = G6PD A- hemizygous males and homozygous deficient females

(i) G6PD enzyme levels (Units per 10^{12} red blood cells) in Kenyan study showing correlation with G6PD A- genotypes but not in the clinical groups).

genotype	enzyme activity	**clinical status**	enzyme activity
normal	292 ± 7	controls	277 ± 9
heterozygote	238 ± 14	mild malaria	249 ± 10
deficient	41 ± 16	severe malaria	289 ± 12.

(ii) Parasite densities ($x10^3$ μl^{-1}): geometric mean with 95% confidence intervals. There were no significant differences in the G6PD genotypes in The Gambian but the Kenyan data showed lower parasite densities in deficient individuals.

G6PD genotypes	**The Gambia**	**Kenya**
normal	75.8 [67.1-85.6]	79.7 [70.3-90.4]
heterozygote	116.8 [65.9-207.0]	98.0 [67.4-142.5]
deficient	42.1 [6.6-266.8]	45.4 [31.8-64.7]

Table 3.6 G6PD genotypes in males in the control and severe malaria groups. A possible protective effect of the G6PD A genotype, with 85% of normal enzyme activity, was sought by comparing the frequencies of this genotype between the clinical groups in which an effect was perhaps most likely to be detectable. i.e males with severe malaria to control males. But this genotype did not differ in frequency between the severe malaria cases (22.2% in The Gambia, 24.4% in Kenya) and the control group (21%, 22.5% respectively) in either country.

(i) The Gambia

genotype	controls (%)	severes (%)
	n=224	n=212
A-	5.9	1.4
A	21.0	22.2
B	73.1	76.4

(ii) Kenya

genotype	controls (%)	severes (%)
	n=155	n=114
A-	18.8	11.1
A	22.5	24.4
B	58.7	64.5

presentation and admission to the hospital from where they were recruited and bled for the study. In Kenya, where this was less of a problem, the parasite densities amongst hemizygotes (45.4, 31.8-64.7) was lower than those of heterozygotes (98.0, 67.4-142.5; $P<0.01$) and normals (79.7, 70.3-90.4; $P<0.01$).

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3.8 DISCUSSION

Clearly, the G6PD A- allele is associated with substantial resistance to severe malaria in hemizygous males as well as in heterozygous females. Protection of hemizygotes as well as heterozygotes assists in assessing the proposed mechanisms underlying protection in this disorder and is consistent with *in vitro* studies showing impaired growth of *P. falciparum* in enzyme deficient erythrocytes (Roth 1983). Our results support the notion that the degree of enzyme deficiency is central to the protective mechanism of G6PD deficiency against malaria. There are several mechanisms which might explain this phenomenon at the molecular level. The most likely attributes protection to reduced multiplication in deficient erythrocytes probably as a result of the intracellular accumulation of toxic oxidized substances such as disulphide glutathione (GSSG), oxidised membrane thiols (Miller *et al.* 1984) and or haemozoin (Turrini *et*

al. 1993). However, there is some evidence that infected deficient erythrocytes may either be more susceptible to haemolysis as a result of increased haemoglobin and release of ferriheme a known cytolytic agent (Janney *et al.* 1986) or they may be more readily phagocytosed by cells of the reticuloendothelial system as a result of the erythrocyte changes (i.e methaemoglobin and heinz body formation, membrane damage) that are associated with the attendant oxidant stress imposed by parasite infection (Turrini *et al.* 1993).

The degree of protection associated with G6PD deficiency reported here is less than that afforded to carriers of sickle haemoglobin but equal to or greater than that associated with some HLA variants and thalassaemias (Hill *et al.* 1991; Hill *et al* 1992c). Compared to the most prevalent Mediterranean and some Asian types of G6PD deficiency, the African A- variant has a higher level of enzyme activity and the associated disorder is milder (Luzzatto & Mehta 1989), so that we would predict that protection against malaria conferred by G6PD deficiency may be at least as great in many non-African populations.

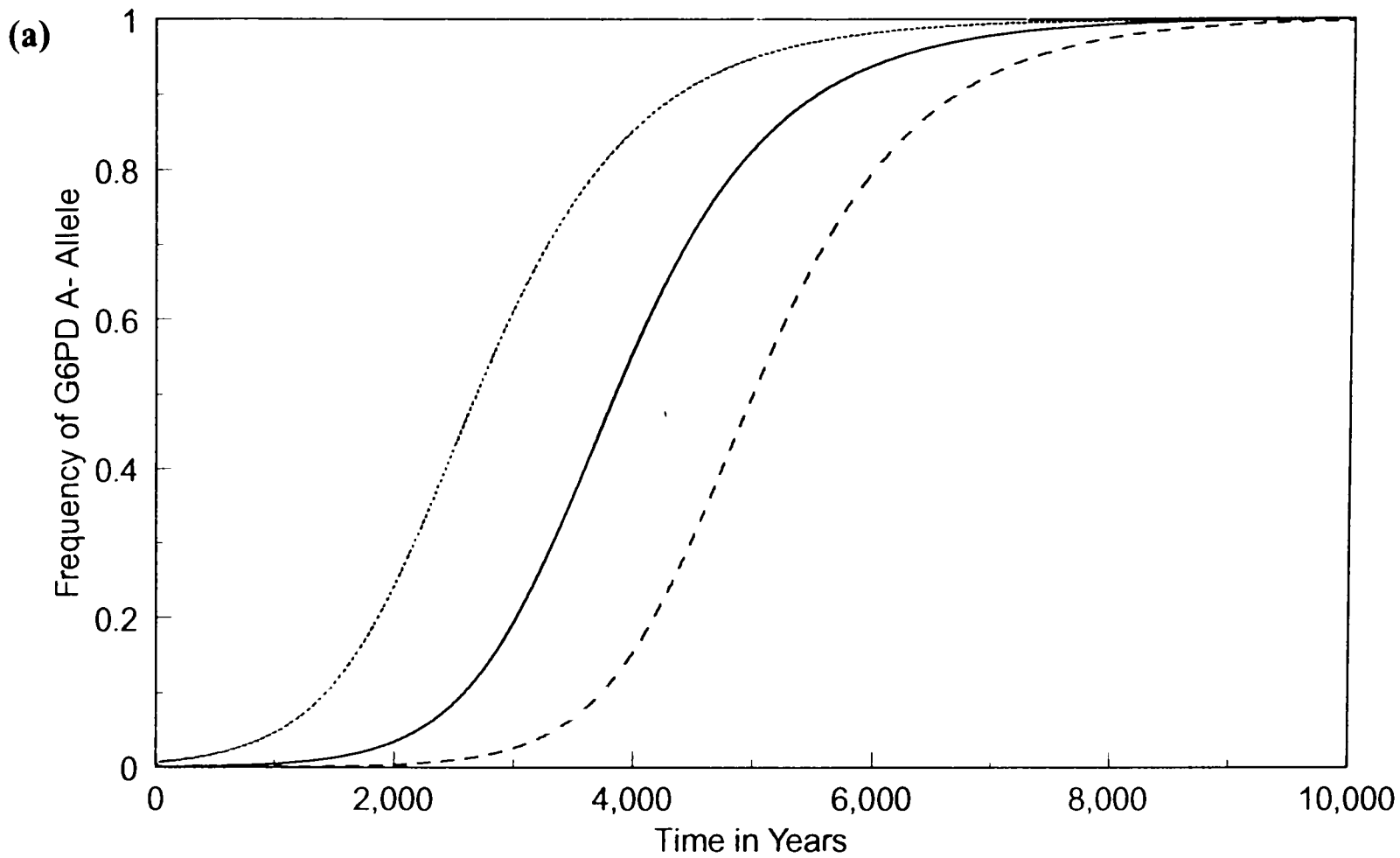
An important consequence of protection of male hemizygotes as well as female heterozygotes is that this allows a deficiency allele to eventually reach fixation (i.e. a frequency of 100%), whereas with heterozygote protection alone the deficiency allele frequency will stabilize at 50%. The availability of data on malaria mortality rates from sub-Saharan Africa (Greenwood *et al.* 1987) allows estimation of the time it would take for the G6PD A- allele to rise in frequency under this degree of positive selection by malaria. Interestingly, the time required is short, approximately 2000 to 3000 years

(figure 3.C). Estimates of the time depth of malarial selection in Africa based on archaeology, population history and human genetics are greater: at least 5000 to 7000 years and possibly much longer (Bruce-Chwatt 1988). Furthermore, outside of Africa in the many populations where G6PD deficiency is prevalent, deficiency alleles have generally failed to reach frequencies greater than 5-40% (Livingstone 1985). Hence, the magnitude of protection against malaria observed here suggests that a counterbalancing selective disadvantage associated with the deficiency genotypes (see figure 3.C legend) may have prevented G6PD deficiency from reaching higher frequencies in so many countries.

The main clinical complication associated with G6PD deficiency today is acute haemolysis following ingestion of oxidant drugs, after the consumption of fava beans and occasionally as a consequence of various infections (Luzzatto & Mehta 1989). In the past infections may have been more frequent precipitants of severe haemolytic reactions. Additionally, G6PD deficiency is associated with an increased risk of neonatal jaundice and environmental factors may influence the rate of this complication (Luzzatto & Mehta 1989). Individuals with more severe enzyme deficiency appear more susceptible to all of these manifestations, but the estimate of the degree of malaria resistance afforded to female heterozygotes (see Table 3.4) is little different from that of male hemizygotes. Hence, the overall fitness of female heterozygotes is likely to be greater than that of hemizygotes and homozygotes. This situation constitutes a balanced polymorphism (Motulsky 1960) and would explain the observed rarity of populations in which frequencies of G6PD deficiency are in excess of 50% (Luzzatto & Mehta 1985). Thus, the magnitude of the protection afforded against

malaria by the African G6PD A- variant suggests that the associated enzyme deficiency disorder, which is relatively benign in a modern medical setting (Luzzatto & Mehta 1989) may have been selectively much more disadvantageous in the past.

Figure 3.C Mathematical modelling of changes in the frequencies of the G6PD A- alleles in males and females over time (see legend overleaf).



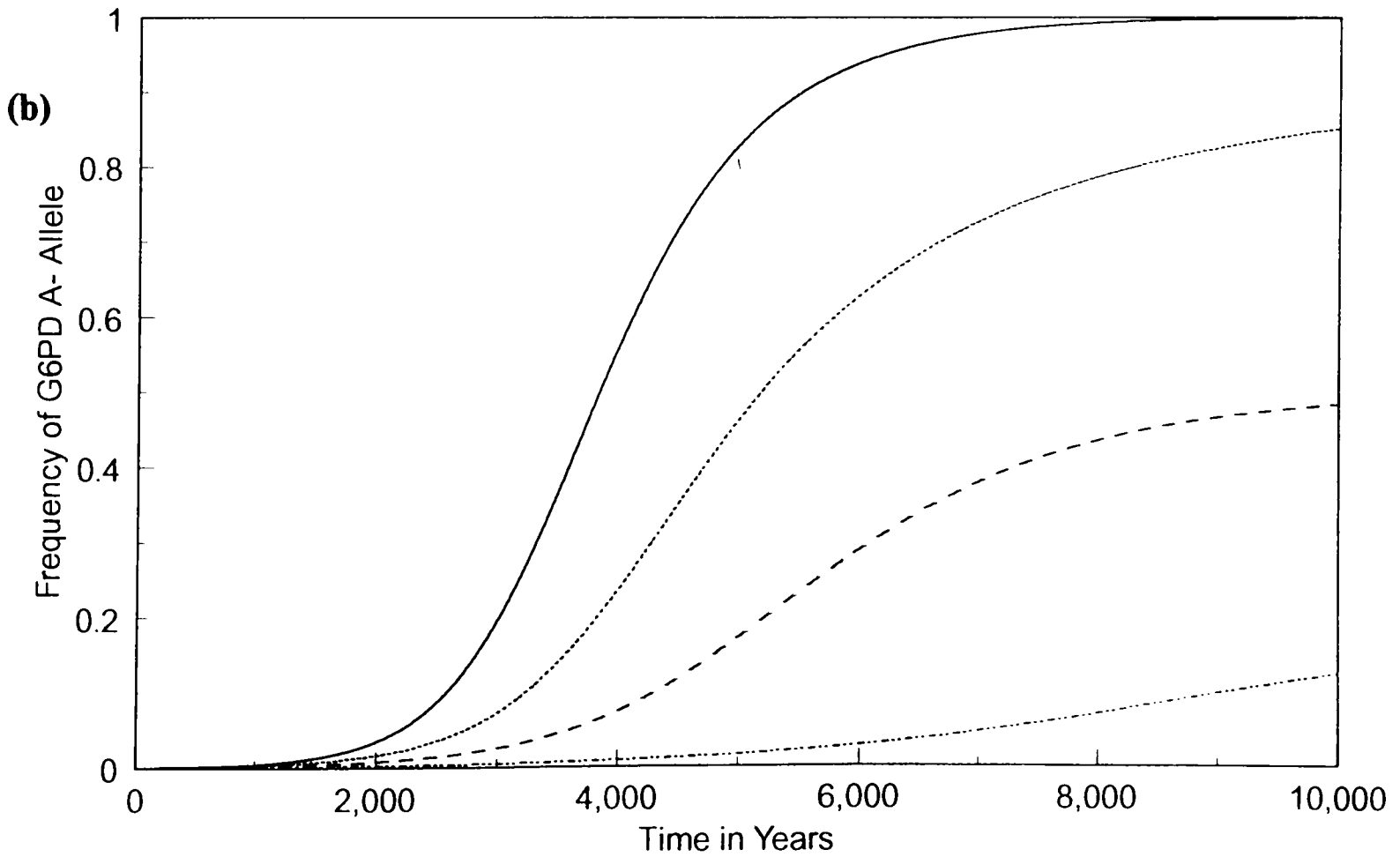


Figure 3.C legend

Figure 3.C (a) Changes in the frequencies of the G6PD A- allele in males and females over time under a single selection pressure with varying initial frequencies of the A- allele of 1% (dotted line), 0.1% (continuous) and 0.01% (dashed). The male and female frequencies are effectively superimposed and cannot be distinguished. It can be seen that using a starting frequency of 0.1% (continuous line) the allele reaches the current frequency in The Gambia (of 5.9%, see table 3.d) in 2280 years and nears fixation after 7000 years. To reach the frequency found in Kilifi, Kenya requires 2970 years. Here, the protection against death from malaria afforded to G6PD heterozygotes and hemizygotes is equivalent to the reduction in risk of severe malaria associated with these genotypes in the combined analysis shown in the Table. It is assumed that the protection afforded to female homozygotes against malaria is equivalent to that of male hemizygotes. The malaria mortality rate here is 8%, estimated from contemporary surveys in The Gambia (Greenwood *et al.* 1987) and from the local haemoglobin S heterozygote frequency of 15% (Hill *et al.* 1991) which is assumed to be at equilibrium. The other parameter used in the model is the overall population mortality rate which here corresponds to a mean lifespan of 20 years. A longer mean lifespan would slow the rate of rise of the A- allele. A higher mortality rate from malaria, an expanding population size, a shorter mean lifespan and a higher initial frequency of the A- allele would all shorten the time required to reach present day frequencies.

Figure 3.C(b) As malaria appears to have been a major selective force in Africa for at least 5000 to 7000 years (Bruce-Chwatt 1988) we assessed which alterations in genotype fitness would reduce the rate of increase in frequency of the G6PD A- allele. If the selective advantage to the hemi- and homozygote that is produced by malaria resistance is exactly counterbalanced by their increased mortality from other causes, so that their overall fitness is equal to that of individuals with a normal G6PD genotype, the rate of rise of allele frequency is reduced and the allele eventually fixes at 50%

frequency (dotted line). The continuous line is as in figure 3.C(a). The dotted line represents a counterbalancing selection pressure corresponding to half the size of the fitness advantage conferred malaria resistance, and the lowest line shows an overall reduction in fitness of hemizygotes and heterozygotes equivalent to the increase in their fitness conferred by malaria resistance, i.e. the selective disadvantage is double the size of the selective advantage. If the G6PD A- frequencies observed in Kenya and The Gambia are now at equilibrium, the selective disadvantage to hemizygotes and homozygotes may be calculated to be 1.75 and 2.34 times, respectively, greater than the selective advantage against malaria.

Model dynamics. The dynamics of this system may be modelled as a set of coupled differential equations

$$\{dx_i/dt = \gamma f(i) - \mu(i) \sim x_i$$

where the subscript i (=1,.....5) structures the population according to sex and G6PD genotype (1: normal female; 2: heterozygous female; 3: homozygous female; 4: normal male; 5: hemizygous male). x_i represents the proportion of the total population of genotype i. $\mu(i)$ is the mortality rate associated with genotype i, f(i) is the proportion of the total reproductive output, $\gamma(=\mu(i)x_i)$, assigned to genotype i. If p_f $(= (x_3+0.5x_2)/(x_1+x_2+x_3))$ and p_m $(= x_5/(x_4+x_5))$ are the G6PD A- allele frequencies in the females and males respectively, then: $f(1)=(1-p_f)(1-p_m)/2$; $f(2)=(p_f(1-p_m)+p_m(1-p_f))/2$; $f(3)=p_fp_m/2$; $f(4)=(1-p_m)/2$; $f(5)=p_m/2$.

CHAPTER 4 - THE GAMBIAN TUBERCULOSIS STUDY - CASE AND FAMILY COLLECTION

4.1 THE GAMBIA: GENERAL INFORMATION

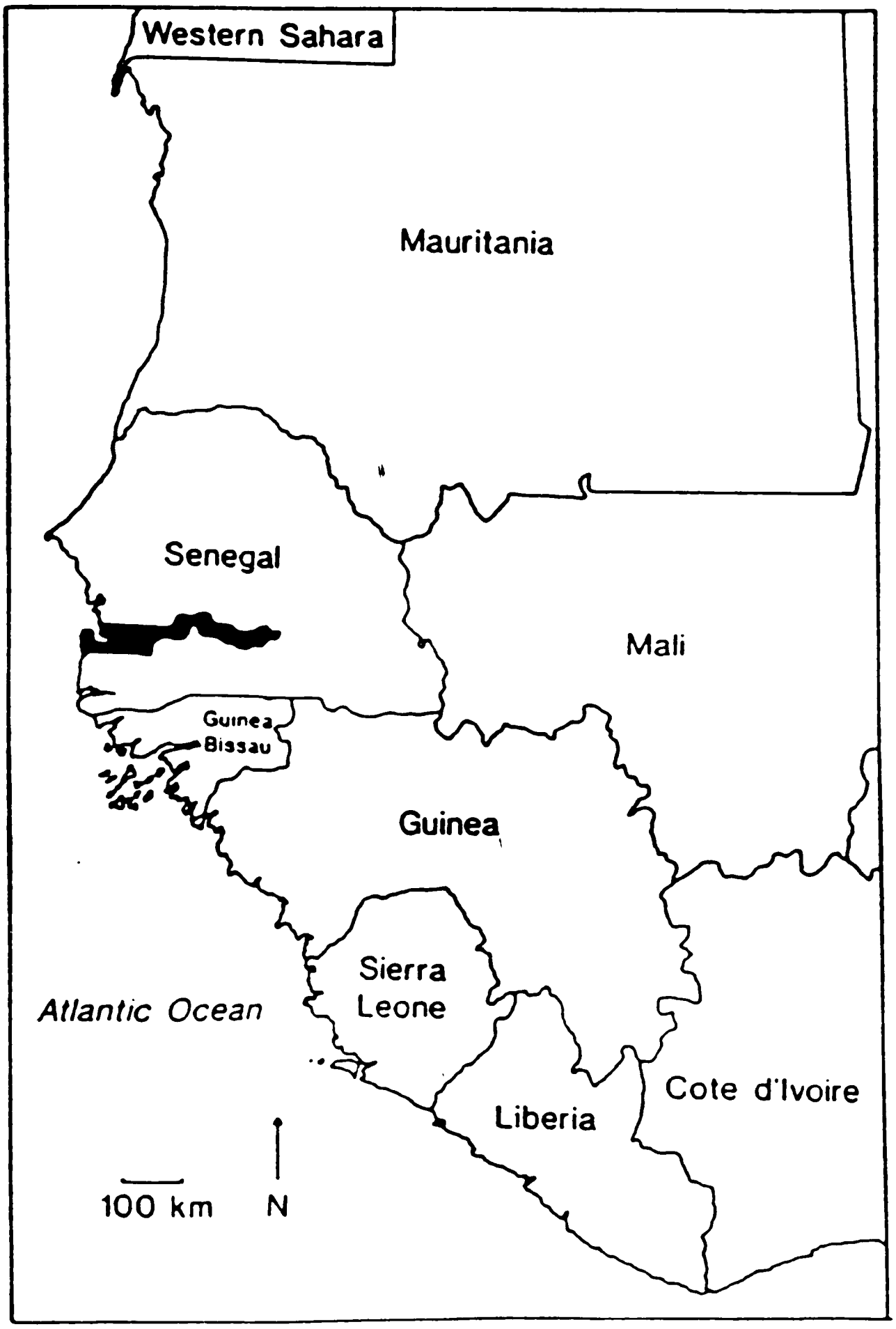
4.1.1 Geography

The Gambia is a small country that lies on the West African coast. The country is surrounded by francophone Senegal and occupies a land area of 10, 690 km^2. The Gambia lies on either side of the the River Gambia and stretches for 330 km from east to west. Most of the country has savannah-type vegetation and secondary forests with dense mangrove swamps along the river banks.

4.1.2 Demography

The last census to be carried out in The Gambia took place in 1983 and and current figures are derived from extrapolations made from data obtained from this census. It is estimated that at present The Gambia has a population of 800 thousand to 1 million inhabitants. The country has a high population density of 64 km^{-2} with 75% of the population living in the rural areas and most of the remaining 25% clustered in the urban and periurban areas of Banjul, the capital, Brikama, Basse and Farafenni. Approximately two fifths of the entire population is located in the coastal region of the country in and around the capital Banjul. Based on figures from the last census, it is estimated that 44% of the population was below the age of 15 years. In 1983 the country had an infant mortality rate of 125 deaths per 1, 000 live births and life

Figure 4.A Map of the West African Coast. The Gambia is shaded black



expectancy at approximately 42 years. There are four major ethnic groups with the Mandinkas (36.6%), Fulas (17%), Wollofs (13.2%) and Jolas (9.4%) accounting for most of the population. Other smaller ethnic groups include Serahulis, Sereres, Manjagos, Akus, Lebanese and Mauritanians. Although all the ethnic groups, particularly the most populous Mandinkas, are represented in all parts of the country, the Wollofs and the various minority groups are clustered mainly in the Western coastal provinces while the bulk of the Fulas and Serahulis are principally in the Eastern 'up-country' region. Polygamy is quite common in the country particularly in the rural areas. The four major ethnic groups co-exist in relative harmony but there appears to be very little intermarriage between the groups. 95% of the population is Muslim.

4.1.3 Economy

The Gambia is a rural economy with agriculture contributing significantly to the gross domestic product. The majority of the population depend on subsistence farming of rice, sorghum, millet and a variety of fruits and vegetables. Tourism and groundnuts are the country's main foreign currency earners. The country has a gross national product per capita of US$250 although this appears to be falling in recent times due to a combination of declining groundnut prices on the international market, droughts and political instability (T Corrah Ph.D thesis 1995).

4.2 TUBERCULOSIS IN THE GAMBIA

4.2.1 Historical perspective

There are no records on the incidence of the disease in thc pre-20th century era. Although there was intermittent contact with slave-trading Europeans from then TB-endemic industrialised countries for much of the 19th century, it is still likely that, as in other sub-Saharan regions, there may not have been much tuberculosis on the West African coast. However, the records do indicate that tuberculosis has been in The Gambia for most of this century. Until recent times it is likely that the pervasive stigma attached to the disease resulted in gross under-reporting of cases. The construction by the Goverment in the late 1940s, of a 40 bed sanatorium right next to the cemetery in Banjul (T Corrah Ph.D thesis 1995) suggests that the authorities then clearly recognised tuberculosis as a highly communicable disease with high mortality. Unfortunately, it is likely that these circumstances only reinforced pervasive negative attitudes towards the disease and probably resulted in fewer cases coming forward for treatment at a time when, paradoxically, effective chemotherapeutic agents were becoming increasingly available.

Unfortunately, until the late 1970s no proper records were kept and, in fact, most of these old records are now lost such that no accurate morbidity or mortality data are available from this time. Prior to the initiation of the Extended Programme of Immunisation (EPI) programme in May 1979, BCG immunisation appears to have been sporadic with communities served by missionaries in the rural areas and, in the later years, those near the capital being the main beneficiaries of this vaccination (Annual Health Statistics Report 1980, Gambian Medical & Health Department) .

Since its institution in 1979, the EPI programme which targets Tuberculosis, Diptheria, Pertussis, Tetanus, Poliomyelitis, Measles and Yellow fever, has made remarkable progress and by the late 1980's the programme was achieving 80% coverage of the entire Gambian population (The Children and Women of The Gambia. UNICEF 1992).

4.2.2 Tuberculosis in The Gambia Today

4.2.2.i EPIDEMIOLOGY

Recent annual records indicate that The Gambia has an annual incidence of 800 - 1000 cases of tuberculosis (Leprosy/Tb Annual Services Report, The Gambia 1990). In adults 90% of the cases have pulmonary tuberculosis and most of these (80%) are confirmed by sputum microscopy or culture. Although it is increasingly becoming a problem, hitherto, drug-resistance has not been a major problem. 8 - 10 % of patients with pulmonary tuberculosis have HIV infection and 25-30% of HIV patients with the AIDS Related Complex (ARC) or AIDS have some form of tuberculosis (V Bouchier personal communication).

4.2.2.ii THE NATIONAL TUBERCULOSIS CONTROL PROGRAMME

Tuberculosis is a notifiable disease in The Gambia. The Tuberculosis Control Programme in The Gambia is run by the Leprosy/Tuberculosis Unit in the Medical and Health Department of the Gambian Government. Since 1986, the National Leprosy/Tuberculosis Control Programme (LTCP) has been administered under an agreement between the Gambian Government and the Dutch agency Nederlandse Stichting voor Leprobestrijding (NSL). When it was established the project's short

term objectives were to establish and maintain adequate case finding and clinical and bacteriological diagnostic services through out the country. Its long term goals were firstly, to reduce the incidence and prevalence of tuberculosis (particularly sputum positive cases) and leprosy such that these two diseases would cease to be public health problems, and secondly, to incorporate the programme's activities within the country's Primary Health Care system.

4.2.2.iii ORGANISATION OF THE TUBERCULOSIS CONTROL PROGRAMME

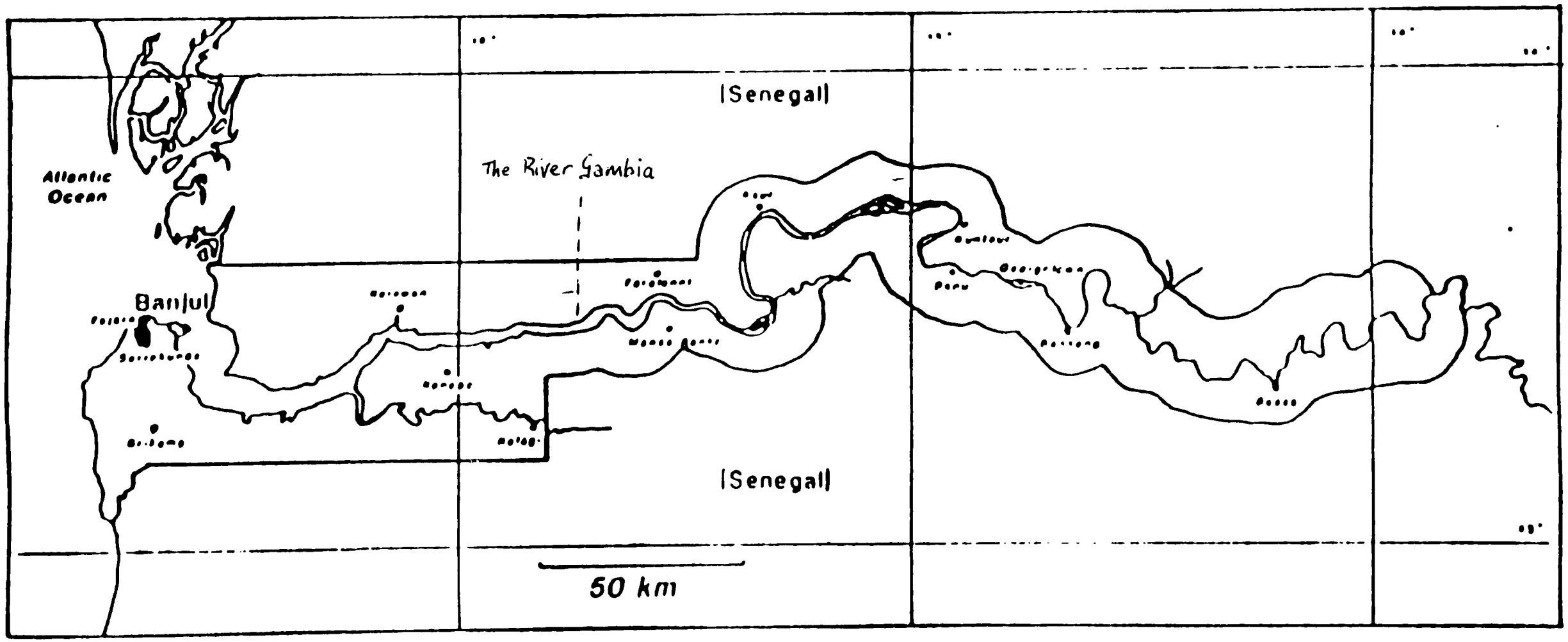
Centrally, the LTCP unit is headed by Senior Medical Officer and the Senior Leprosy Tuberculosis Control Officer who together are responsible for managing and coordinating the LTCP programme nationally, and for continually monitoring and evaluating the programme in addition to supervising and training the programme's Leprosy /Tuberculosis (TB) officers. Each region has a Leprosy/Tuberculosis Inspector as part of the Regional Health Team that oversees the activities of the Primary Health Care programme (Table 4.1). Each region has polyclinics or regional health centres which serve as secondary level health institutions and each polyclinic has a Leprosy/TB officer who is in charge of the TB/Leprosy service for the area served by the Polyclinic. All the health centres within the polyclinics catchment area refer cases to the Leprosy/TB Officer based at the polyclinic.

The TB/Leprosy officer is usually a civilian with at least a minimal level of 'O' level grade formal education who has been trained specifically to carry the duties of a leprosy/TB officer. The responsibilities of a Leprosy/TB officer include clinical and microscopic diagnoses of tuberculosis cases, the prescription of standard treatment for

Table 4.1 Organisation of the Leprosy/Tuberculosis Control Program in The Gambia

Level 1	**Central Leprosy/Tuberculosis Unit**
	Medical and Health Department, Banjul
	Senior Medical Leprosy/Tuberculosis Officer
	Leprosy/Tuberculosis Control Officer
Level 2	**4 Regional Health Headquarters**
	(Banjul, Mansakonko, Bansang and Kaure)
	Leprosy/Tuberculosis Control Inspectors
Level 3	**10 Polyclinics/Regional Health Centres**
	Leprosy/Tuberculosis Officers
	Dispensaries
	Patients
Level 4	**Peripheral Health Centres**
	Village Health Workers
	Community Health Workers
	Patients

Figure 4.B Map of The Gambia



proven cases, the maintenance of good records and contact tracing of newly diagnosed cases. Diagnostically or therapeutically challenging cases are seen by the programme's Senior Medical Officers who visit each Polyclinic LPCP unit approximately once a month. Village health workers (VHW) and Community Health Workers (CHW) at the various health centres liase with the Polyclinic TB/Leprosy officer for diagnosis of suspected cases, supervision of prescribed treatment for diagnosed cases, and tracing of case contacts. Leprosy/TB Officers at the various Polyclinics forward monthly statistics to the central LTCP Unit in the Medical and Health Department.

4.2.2.iv DIAGNOSIS AND MANGEMENT OF TUBERCULOSIS

The Leprosy/TB officers in all the polyclinics are trained to take histories, perform physical examinations, measure patients' weights and keep good records of their findings. In addition, these officers are expected to carry out microscopic sputum examination (and split skin smears in suspected leprosy cases) and prescribe standard chemotherapy in proven cases. Leprosy/TB officers in and around the capital Banjul have the facility of sputum culture available at the Medical Reseach Council laboratories in Fajara. Since 1985, the short course chemotherapy (SCOC) regimen has been used. This course consists of an intensive 8 weeks of rifampicin, isoniazid, pyrazinamide, ethambutol ('TB4') three times a week. This part of the course is supervised by the Leprosy/TB Oficer and hence patients have to travel to the Polyclinic thrice weekly, usually Monday, Wednesday and Friday, during this 2 month period.

The initial intensive phase of treatment is then followed by a further 16 weeks of rifampicin and isoniazid ('TB2') three times a week. During this latter phase patients

only report to the Leprosy/TB officer once a month. Microscopic sputum examination is repeated after the first intensive phase of treament. Cases that are either still sputum positive at this stage or that are generally not improving clinically are referrred to the senior Medical Officer. Non-compliant cases and suspected or proven cases of drug-resistant tuberculosis are admitted at the sanatorium and treated as inpatients for the entire 6 months of therapy.

4.2.3 Overall assessment of the Gambian Tuberculosis Control Programme

The Tuberculosis Control Programme appears to be running well but nevertheless, there are several areas which are in need of attention. Awareness and understanding of the disease in the general population could be improved and, to this end, health education campaigns would most certainly be useful. The busiest Leprosy/TB unit in the country is located in Serrekunda, Banjul. The Officers in this one unit alone probably see 50-70% of all tuberculosis cases in the country and their small numbers and meagre facilities mean that they are unable to carry some of their duties fully. There is no doubting their committment and their diagnostic capability both clinically and bacteriologically, but their sparse number in the face of the overwhelming number of cases whose treatment they supervise, means that they can only spend a limited amount of time doing tracing and work-up of patient contacts. The other great drawback faced by TB/Leprosy Officers nationally with regard to the tracing and investigation of patient contacts, is the limited availability of motor-cycles and the inadequate weekly fuel allowance. Past record keeping has been satisfactory but can clearly be improved, particularly, in the documentation of patient addresses.

4.3 COLLECTION OF AFFECTED SIB-PAIR FAMILIES AND INDIVIDUALS CASES OF TUBERCULOSIS

The Gambia was chosen for our study of tuberculosis host genetics in sub-Saharan Africa for two main reasons. Firstly, the relatively low prevalence of HIV infection in The Gambia would allow us to study the role of tuberculosis host genetics independently of the confounding effect of HIV infection on susceptibility to tuberculous disease. HIV infection increases the risk of tuberculosis by weakening the immune response and therefore in study populations with high HIV prevalence rates, detection of the effects of genetic variation on immune responses to tuberculosis in these populations is likely to be more difficult than in populations with low HIV prevalence rates. Whilst in The Gambia the HIV prevalence is 0.5-1% in the general population and 8-10% in tuberculosis cases (V Bouchier personal communication), in several other sub-Saharan countries such as Zaire, Uganda, Zambia and Zimbabwe the equivalent HIV prevalence rates are significantly higher, 8-25% and 25-67% respectively (Lucas & Nelson 1994). Secondly, our research group already had strong links from previous collaborations, with the British Medical Research Council (MRC) Laboratories that has been present in The Gambia for over 30 years.

4.3.1 Strategy

This study was approved by the joint Gambian Government/Medical Research Council Ethical Committee. Study individuals and families were to be identified in collaboration with the Medical Research Council and the Gambian National TB/ Leprosy Units. While the affected sib-pair families would be recruited from all over the country, the individual cases would only be recruited from the Kombos coastal

province from where the blood donor controls came. The project's goals at the outset were to recruit a total of 200 sib-pair families and at least 250 individual cases of tuberculosis over a two year period. In the first year, 1995, the aim was to collect at least 100 affected sib-pair families for the linkage study and 100 individual cases for the concurrent association study.

4.3.2 Operation

The identification, tracking and locating, interviewing and bleeding of study families and individuals was done primarily by myself and Yerro Sowe from the MRC in liason with TB/Leprosy officers from the various Polyclinics countrywide. Recruitment took place over two trips. The first was a 9 week visit from January to March 1995, and the second trip a 7 week visit from July to August 1995. Informed consent was obtained from all the individuals recruited as cases for the study. During both these trips I relied on the records at the Polyclinics for 'passive' detection of potential sib-pair families.

The loose use, by both patients and the TB/Leprosy officers, of the terms 'brother' and 'sister' in the context of the extended African family meant that the majority of potential sib-pair cases that we followed up turned out not to be true biological siblings. The term brother or sister was used often to refer variously to cousins, long term friends or unrelated individuals from the same village or place of abode. The widespread prevalence of polygamy amongst many Gambian households meant that a not inconsiderable fraction of sib-pair families that we collected were half siblings. The other major problem experienced during the recruitment was inadequate address

details in the records. The absence of road names and house numbers coupled to the rare availability of telephones in households made the locating of individuals laborious and in many cases unsuccessful. The great mobility of many Gambians both within The Gambia and also in neighbouring Senegal and Guinea Bissau also proved to be an important negative factor in locating members of potential sib-pair families identified from the records. The collection of families using 'active' detection i.e via contact tracing of siblings of diagnosed cases currently on treatment was initiated by Richard Bellamy and is still ongoing.

Recruitment of individuals for our case-control study was infinitely easier than that of families described above. Whereas any sib-pairs with tuberculosis were recruited for the family linkage study, only individuals with sputum positive clinical pulmonary tuberculosis whose diagnoses had been confirmed either by sputum positive Ziehl Neelson staining or sputum culture were admitted for the association study. Individual cases were typically on the initial intensive phase of anti-TB treatment when recruited and as such they were identified on any one of the thrice weekly visits that they made to the coastal Polyclinics for their drug therapy. Individuals who were known to be HIV positive were not recruited into the study. Testing for HIV 1 and 2 was done on all samples after informed consent was obtained and appropriate pre-counselling given. 10 ml of whole blood was obtained from all individuals recruited into the study and the DNA extracted at the MRC laboratories in The Gambia before transportation to Oxford.

4.3.3 Summary of field trip

A total of 216 cases of bacteriologically proven clinical pulmonary tuberculosis were recruited into our study (Table 4.2). 69% were males and 31% females and this is consistent with recent records in The Gambia that show a preponderance of males in tuberculosis cases. 306 DNA specimens from healthy adult male blood donors in the coastal region were available for the association study. Table 4.2 shows the average age and ethnic group composition of the cases and controls. There were 8 individuals who were HIV positive amongst cases and none in the control group. Exclusion of the 8 HIV positive individuals made no significant difference to the odds ratios observed in all our analyses.

As shown in Table 4.3(i), 69 families were collected. 27 were ideal sib-pair families suitable for identity by descent linkage analysis whilst 42 were either incomplete families due to non-availability or death, or families where affected individuals were half siblings or close cousins. This latter group were to be used in identity by state linkage analysis.

4.4 LINKAGE STUDY

We were hoping to have collected 100 families by the end of the second visit to The Gambia. Given that the statistical power of detecting any linkages using the affected sib-pair approach with the small number of Gambian families (27) families is relatively low, it was decided that The Gambian families would be pooled with families collected by Matthew Collin from Natal, South Africa. In the end a total of 32 families were

Table 4.2 Summary of individuals used in case-control study

	Controls	**Cases**
total number:	306	216
sex:	all male	males: 69%
	blood donors	females: 31%
average age:	29 years	33 years
% HIV positive:	0	3.7
ethnic group (%)		
Mandinka:	31	35
Wollof:	27	20
Fula:	12	20
Jola:	13	15
Serrahule	6	3
Manjiago:	5	3
Other:	6	4

available for analysis. Unfortunately, preliminary genotype data for selected chromosome 5 and 6 markers showed a high degree of non-paternity in our families (30%) and in addition a considerable number of the meioses were uninformative assome of the markers turned out not to be polymorphic enough.

The case-control arm of this study discussed had shown that a TNF-α promoter variant to be significantly associated with increased risk of pulmonary tuberculosis (described in chapter 5). Not surprisingly given the small sample size and the modest relative risk, 2.57, observed in the association study, analysis of the TNF microsatellite marker close to the TNF-α gene did not demonstrate significant linkage (Table 4.3 ii) and confirmed our view that the first full phase of our linkage study be delayed until more families had been collected. Fortunately, the association studies on individual pulmonary tuberculosis cases have proved to be very useful and in Chapters 5,6 and 7 I describe results that I have obtained from this arm of the project.

Table 4.3. (i) Summary of families collected in The Gambia

affected sib pair families suitable for identical by descent (ibd) analysis: 27

incomplete affected families suitable for identical by state (ibs) analysis: 42

Table 4.3 (ii) Ibd analysis of TNF microsatellite data for pooled Gambian and South African families

potential informative meioses: 64

actual informative meioses: 31

ibd allele sharing:	**2**	**1**	**0**
observed :	9	16	6
expected:	7.5	15.5	7.5

CHAPTER 5 -THE MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) AND TUBERCULOSIS

5.1 THE MHC

Evidence in animals and man indicates that the immune response is controlled to a considerable degree by genes linked to the MHC (Benacerraf & McDevitt 1972; Klein 1986). In many species including man, the MHC is the most polymorphic expressed gene family (Klein 1986) and in human population genetics, it has proved extremely useful as a marker of population diversity and in tracing population migration patterns (Serjeantson 1982). The remarkable polymorphism of the MHC has sparked considerable debate about the fundamental mechanims that have generated and maintained this extreme diversity and it is now widely accepted that the evolution of this remarkable polymorphism has been largely pathogen-driven (Hill 1992).

In humans, there are three classes of MHC genes: human leucocyte antigen (HLA) Class I, II genes and MHC Class III region genes. The MHC genes span 4 Mb accounting for 0.1% of the human genome (Trowsdale 1993). The Class I and II loci, the principal gene products of the MHC complex, encode transmembrane proteins expressed on the surface of specific subset of cells. These Class I and Class II transmembrane proteins are similar in structure overall and have extracellular amino terminal domains that bind antigen for presentation to T cells. Class I molecules are composed of two polypeptide chains; the polymorphic chromosome-6 encoded α or heavy chain (44 kd) and the non-polymorphic $\beta 2$ microglobulin or light chain (1.2 kd) encoded by a separate single locus on chromosome 15. For Class II molecules both the

α chain (33 kd) and the β chain (28 kd) that make up the molecule are coded on chromosome 6.

The sequence diversity between different HLA alleles is found in the α and β chains that make up the peptide binding site (Bjorkman *et al.* 1987) for the Class I gene family HLA A, B, and C. The HLA-B locus is the most polymorphic in this group and functionally may be the most important. There are approximately 40 HLA-A alleles, 60 HLA-B alleles and 18 HLA-C alleles. HLA-C molecules are the most weakly expressed in Class I. The Class II gene family DR, DQ and DP are also very polymorphic. There are now over 60 DRB1, 14 DQA, 19 DQB, 8 DPA and 38 DPB alleles that have been identified (WHO committee for HLA nomenclature, 1992). Several other genes that map to the Class II region have been described and these include the TAP 1 and 2 genes believed to have important roles in peptide transport across internal membranes, and the LMP 2 and 7 genes involved in proteolysis in several cytoplasmic compartments (Parham 1990). The HLA class III genes lying between the Class I and II code for a large number of different proteins with diverse functions; included in this group are tumour necrosis factor-α (TNF-α) and -β, a heat shock protein, Factor B, and several members of the complement system such as C2 and C4.

HLA Class I and II molecules are principal components of acquired or specific immunity but have different functions. Via their cell surface TCR receptors, T cells recognise peptide ligands bound to MHC molecules on the surface of cells. The processing pathways of MHC Class I and Class II molecules are different and

determine which peptide antigens are exposed to the T cells. The two classes capture and present peptides from intracellular locations. Class I molecules present peptides from the endoplasmic reticulum derived from proteins synthesised in the cell or peptides from the cytosol. In contrast, Class II molecules present peptides from internalised exogenous proteins and cell surface peptides. The different MHC molecules exhibit preferences for specific structural requirements or motifs for peptide binding. The striking diversity of the MHC in humans ensures a wide array of different peptide binding specificities and, in evolutionary terms, this is probably associated with a selective advantage.

Generally, peptides presented by Class I HLA molecules are 8-10 amino acids in length (Hunt *et al.* 1992) whilst peptides presented by Class II HLA molecules are more variable ranging from 12-20 amino acids in length (Rudensky *et al.* 1992; Chicz *et al.* 1993). Class I molecules are expressed on virtually all nucleated cells and in intracellular infections, infected host cells present foreign antigen to CD8+ mainly cytotoxic T cells. Class II molecules whose expression is restricted to specialised cells such as B cells, macrophages and other antigen presenting cells, present processed foreign antigens to CD4+ T cells. When stimulated, CD4+ cells secrete various regulatory cytokines that activate macrophages and help antibody formation as well as cytotoxic CD8+ T cells.

5.2 HLA ASSOCIATIONS WITH DISEASES

As alluded to earlier, numerous associations of HLA with diseases have been reported in the literature (Table 5.1). The most striking association is that of HLA B27 with

ankylosing spondylitis where 90 % of patients with the disease have HLA B27 compared to 5-10% in controls (Brewerton *et al.* 1973; Schlosstein *et al.* 1977). Although several other associations of autoimmune diseases with specific HLA alleles, particularly with Class II, have also been reported the results that have arguably been even more exciting have been those associations of HLA types with fatal infectious diseases such as malaria and HIV. Table 5.1 summarizes some of the important associations described to date. Some of these include an association in The Gambia between HLA B53 and DR13.02 and protection from severe malaria (Hill *et al.* 1991); in HIV infection, an association between the HLA haplotype A1-B8-DR3 with more rapid disease progression (Steel *et al.* 1988; Kaslow *et al.* 1990); and in *M. leprae* infection, an association of HLA DR2 with increased susceptibility to leprosy (Todd *et al.* 1990). These results provide compelling evidence for the hypothesis that the remarkable diversity of the MHC has been driven primarily by parasite selection where, by virtue of a diverse array of MHC molecules and hence large variety of pathogen antigens that the latter therefore can recognise, hosts are able to generate and maintain antigen-specific immunity to rapidly-evolving pathogens.

5.3 HLA IN TUBERCULOSIS

5.3.1 Background

Most attempts at defining the genes governing variable susceptibility to tuberculosis have concentrated on MHC polymorphisms and numerous HLA antigen frequency comparisons have been made between tuberculous patients and both purified protein derivative (PPD) positive and negative healthy individuals in several different places.

Table 5.1 Some reported HLA associations with Infectious Diseases (excluding tuberculosis) in humans

disease	**antigen**	***effect***	**population**	**reference**
leprosy	DR2	*susceptibility*	Asian	Rani *et al.* 1993
				Roy *et al.* unpublished
			Korean	Kim *et al.* 1987
			several	Todd *et al.* 1990
severe				
malaria	B53, DR1302	*protection*	Gambian	Hill *et al.* 1991
HIV/AIDS	A1-B8-DR3	*susceptibility*	British	Steel *et al.* 1988
			American	Kaslow *et al.* 1990
	B35	*susceptibility*	Italian	Scorza Smeraldi *et al.* 1986
Persistent Hepatitis				
B carriage	DR6	*protection*	North Europeans	van Hattum *et al.* 1987
	DR1302	*protection*	Gambian	Thurz *et al.* 1995
	DR2	*protection*		Almarri *et al.* 1994
	DR7	*susceptibility*		Almarri *et al.* 1994
Human Papilloma				
Virus associated				
cervical cancer	DQ3	*susceptibility*	Europeans	Wank *et al.* 1991
				Mehal 1994
			African-American	Gregoire *et al.* 1994
			Japanese	Nawa *et al.* 1995

Table 5.2 gives a summary of the numerous associations reported between various HLA types and tuberculosis. Although there has been a great deal of inconsistency in the reports, one result, an association of HLA DR2 with susceptibility to tuberculosis, has been obtained repeatedly (Bothamley *et al.* 1989; Khomenko *et al.* 1990; Brahmajothi *et al.* 1991; Singh *et al.* 1983).

There are several reasons that may explain the variation and inconsistencies in results of most of the other HLA studies. For some studies, the claimed associations are very likely to be false positive results as no statistical corrections were made for the multiple comparisons of different HLA alleles. Other less mundane reasons that may explain the inconsistencies in these HLA studies include: differences in the population frequencies and distribution of HLA alleles, geographical variations in the bacterial strains, ethnic group differences in host susceptibility allele frequencies, the HLA genes being closely linked to but not allelic with the actual susceptibility locus in tuberculosis, and variable interactions in different populations between any HLA susceptibility or resistance allele(s) with other non-HLA tuberculosis susceptibility or resistance alleles in the human genome. Other more fundamental reasons would include inadequate or inaccurate case definition, recruitment of inappropriate controls and the use in the past of relatively crude HLA typing and subtyping techniques. Over the last decade there has been a re-focusing of attention on HLA class II in contrast to HLA class I alleles and increased use of specific oligonucleotide probing over less specific serotyping and RFLP typing methods. These changes will probably serve to improve the investigation and detection of any real HLA associations with disease in *M.tuberculosis* infection.

Table 5.2 Studies reporting HLA associations with tuberculosis in humans

antigen	effect	population	reference
DR2	*susceptibility*	Asian	Singh *et al.* 1983
			Bothamley *et al.* 1989
			Khomenko *et al.* 1990
			Brahmajothi *et al.* 1991
A10, B8	*susceptibility*	Asian	Brahmajothi *et al.* 1991
A2, B5	*susceptibility*	Egyptians	Hafez *et al.* 1985
B8	*susceptibility*	Newfoundland Canadians	Selby *et al.* 1978
Bw15	*susceptibility*	African American	Al-Arif *et al.* 1979
Bw35	*susceptibility*	Chinese	Jiang *et al.* 1983

Despite the abundance of literature on HLA and tuberculosis, very few such studies have been conducted in African populations. This is surprising given that Africa, particularly sub-Saharan Africa with its distinctive genetic profile and and unmatched HLA diversity has the potential of revealing other HLA associations with tuberculosis that have not yet been described in the literature. It is with this in mind that we typed our cases primarily for common HLA Class II alleles to observe whether in our population, a population known to have twice as many different HLA alleles as Europeans (Olerup *et al.* 1991), there were any HLA associations with variable susceptiblity or resistance to clinical pulmonary tuberculosis.

5.3.2 RESULTS

All the typing was done using PCR amplification of relevant regions of the HLA genes and hybridisation with specific ologinucleotides. In order to limit the burden of multiple comparisons in the statistical analysis of our results and also to increase the statistical power of our subsequent analyses, only a limited number of common DRB1 alleles were considered in our analysis. Therefore, we measured the frequency of 10 DR alleles, DR-1, DR-3, DR-4, DR-7, DR-8, DR-9, DR-10, DR-11, DR13.02 and DR-1304 (Table 5.3), that we knew to be common from previous studies by our group in the same Gambian population Hill *et al.* 1991. In addition, we also measured the frequency of HLA-DR2 allele as several different studies had shown that in Asian populations, this allele was associated with susceptibility to tuberculosis. For class I, only three alleles were typed HLA-B53, -B35 and -B18 (Table 5.4). HLA-B53 and -B18 had been shown to be in linkage disequilibrium with a TNF promoter variant that this study had demonstrated to be significantly associated with increased susceptibility

to pulmonary tuberculosis (Table 5.8). We therefore sought to esablish whether these HLA molecules were in fact the actual susceptibility alleles. HLA data from our control population was already available from previous work done by Adrian Hill and Catherine Allsopp.

There were no significant differences in the frequencies between our cases and controls for either the various class II DR alleles or the HLA Class I alleles tested. The effect of the TNF promoter variant on suceptibility discussed later in this chapter was found to be independent of the Class I alleles with which it was demonstrated be in linkage disequilibrium.

5.3.3 DISCUSSION

Lymphocytes are important in the development of immunity against pathogens. T cell responsiveness, in particular, is believed to be under genetic control in large part by the HLA system. As with all other intracellular infections, Class I and Class II HLA molecules probably affect the development of specific immunity to *M. tuberculosis* infection (Collins 1983; Cox *et al.* 1988). Although bacteriostasis or bactericidal activities against tubercle bacilli occur within the macrophage, it is the nature of the T-cell mediated immune response that most influences the outcome of infection with the MHC class II restricted CD4 T cells, in particular, playing a pivotal role in the development of specific immunity.

In mice, T cells appear to be important for anti-mycobaterial resistance as nude (nu/nu) and SCID mice suffer more severely from *M. tuberculosis (M.tb)* and BCG infections

Table 5.3 HLA Class II DRB1 antigen frequencies in cases and controls

antigen	**Controls (%)**	**Cases (%)**	***P* value (corrected)**
	n=306	**n=215**	
DR1	4.9	5.6	not significant (ns)
DR2	4.9	6.0	ns
DR3	11.1	7.4	ns
DR4	10.8	11.2	ns
DR7	8.5	6.0	ns
DR8	9.2	13.0	ns
DR9	12.7	9.8	ns
DR10	14.1	12.1	ns
DR11	19.6	23.3	ns
DR1302	24.2	16.3	ns uncorrected P=0.038
DR1304	23.9	21.4	ns

Table 5.4 Selected HLA Class I antigen frequencies in cases and controls

antigen	**Controls (%)**	**Cases (%)**	***P* value (corrected)**
	n=306	n=215	
B53	21.2	18.6	ns
B35	10.5	16.7	ns
B18	5.9	3.3	ns

(Sher 1975; Izzo & North 1992). In addition, inhibition of *in vivo* growth of *M. tuberculosis* in mice correlates with the persistence of MHC Class II expression by peritoneal macrophages (McPeek *et al*). In humans, *in vivo* phagocytosis of either live or killed Mtb results in reduced Class II expression on monocyte surfaces as well as decreased processing and presentation of exogenous protein (Gercken *et al.* 1994).

Association studies and family segregation studies based on HLA polymorphisms in humans provide convincing evidence for involvement of HLA associated genes in variable susceptibility to tuberculosis and leprosy. Several multi-case family studies have reported that affected progeny have a significantly increased probability of inheriting the HLA haplotype of parent who also has tuberculosis (Singh *et al.* 1983: Khomenko *et al* 1990). In addition, unpublished data from Jepson *et al.* suggest that the MHC accounts for 20-25% of the genetic component determining the cellular immune response to PPD.

Several possible mechanisms could be invoked to explain the occurrence of associations between resistance or susceptibility to tuberculosis (or any other infectious disease) with specific HLA alleles. One explanation would be that the various HLA alleles differ in their abilities to regulate an effective immune response against important antigens of any given pathogen. Evidence from *in vitro* studies are suggestive of differences in the cellular and humoral responses of different HLA alleles. Data from Bothamley *et al.* (1989) have shown that sera from the susceptible DR2 individuals appear to have significantly increased levels of antibodies against

specific peptides within a 38kd *M.tb* antigen. Khomenko and colleagues (1990) have also demonstrated clear differences in antibody production and PPD skin responses between tuberculosis susceptible (DR2, B15) and resistant (DR3, B27) individuals.

Cytokine profiles are likely to be important in the development of appropriate immunity to combat *M.tb* infection and it is conceivable that different HLA molecules may variably influence the nature (Th1//Th2) of the cytokine profile that occurs in response to an infection and thereby cause altered susceptibility to any given infectious disease. It is likely that the numerous different *M.tb* antigens probably have varying pathogenic significance and as such the the use of reverse immunogenetics to characterise the peptides that bind HLA alleles which may be involved with increased or decreased risk to any given infectious disease such as tuberculosis and and the subsequent identification of antigens from which these peptides are derived, would be useful information for purposes of designing vaccines and other therapeutic interventions.

The HLA-DR2 allele, associated with susceptibility to tuberculosis in some Asian populations (Brahmajothi *et al.* 1991; Bothamley *et al.* 1989; Khomenko *et al.* 1990) was not significantly increased in these Gambian tuberculosis cases. The difference in the HLA-DR2 results for susceptibility to tuberculosis between these two populations might be explained by differences in the frequencies and distribution of the various DR2 subtypes. Clinical outcomes might differ for individuals of different subtypes perhaps as a result of the latter's varying capacities to bind and present the peptide

epitopes (Davenport & Hill 1996) of either protective or pathogenetic *M. tuberculosis* antigens. In Asia, it is the DR15 subtypes of HLA DR2 that appear to be associated with susceptibility to mycobacterial disease (Bothamley & Schreuder 1992; Rani *et al.* 1993; Bothamley et al 1995). 15*01 and 15*02 are the commonest HLA-DR2 subtypes in Asia (Rani *et al.* 1993). Preliminary analysis of our adult controls indicates in The Gambia, two HLA-DR2 subtypes, 15-2 (53.3%) and 16-1 (46.7%), are predominant. Sequence analysis of these identified HLA-DR2 RFLP subtypes in The Gambia and other African populations will certainly be enlightening.

As discussed above, the lack of extensive subtype data for the HLA Class II molecules investigated in this study may also be responsible for our failure to detect any HLA Class II association with disease. In addition, it is also possible that any HLA Class II DRB1 alterations on tuberculosis susceptibility that may be present in our study population may be relatively minor effects and as such a much bigger study than our this would be required to detect these. Moreover, this study did not analyse any HLA Class II DP and DQ alleles and although these genes are not as polymorphic as the DR gene, there may still be associations between individual alleles at these loci with tuberculosis .

Although *M. tuberculosis* enters the macrophage via phagocytosis and as such its degraded antigen peptides are ordinarily processed and presented on the cell surface via the MHC class II pathway, there is evidence that virulent bacilli may evade killing by escaping from phagocytic vacuoles into the cytoplasm, from which they can be

routed via the MHC Class I antigen processing pathway (McDonough *et al.* 1993)). This supports growing evidence that HLA Class I and CD8+ T cells in particular, are important in immunity to *M. tuberculosis* infection (Chan & Kaufmann 1994). In mice, it has been demonstrated that adoptive transfer of enriched CD8 cells from immune mice confers protection against disease (Orme & Collins 1994). Moreover, *in vivo* depletion of CD8 cells (Cox *et al.*1989) or disruption of β_2 microglobin (Flynn *et al.* 1992) in mice leads to diminished resistance to *M. tuberculosis* infection. Our limited analysis of HLA Class I alleles namely HLA-B53, -B35, and -B18 did not show any association between any of these alleles with disease. A more extensive study of other HLA Class I alleles in our Gambian tuberculosis cases and controls is clearly necessary.

Therefore while our data do not provide any evidence for an HLA association with altered susceptibility to tuberculosis, they do not rule out this possibility either. It is likely that for an HLA study, our study may not have been large enough in terms of the number of cases and controls. The on-going recruitment of more cases and controls and the increased use of molecular subtyping of HLA molecules, will no doubt facilitate a more robust appaisal of the role of HLA, and particularly of HLA Class I antigens, in pulmonary tuberculosis in the future.

5.4 TUMOUR NECROSIS FACTOR-α

5.4.1 Background

With the possible exception of interferon-γ, tumour necrosis factor-α (TNF- α) is probably the most extensively studied cytokine. It is encoded by a gene that lies within the MHC class III region on the short arm of chromosome 6. The MHC Class II HLA DR genes are 900 kb centromeric and the MHC Class I HLA B genes 250 kb telomeric to the TNF–α gene. The gene for TNF-β or lymphotoxin, with which TNF-α has 30 amino acid homology and similar biological actions, is approximately 1 kb 5' to TNF-α's transcription start site. Although it is secreted primarily by macrophages, T cells, B cells, NK cells, Kuppfer cells, glial cells and adipocytes can also produce TNF-α (Tracey 1993).

TNF- α is an important immunoregulatory cytokine with a wide range of effects particularly on the cellular elements of the immune system. These effects include: increased cell surface expression of MHC class I and class II molecules and the IL-2 receptor, enhanced phagocytic activity and cytotoxicity, degranulation and respiratory burst activity, upregulation of complement receptor expression, and enhanced IL-1 and IL-6 synthesis. Due to its extensive pleiotropic activity, TNF-α's biological role is not only important in inflammation but also in tissue remodelling and wound healing (Tracey 1993). In animal models, injection of TNF-α into solid tumours causes haemorrhagic necrosis, hence its name, and intra-arterial infusions of TNF–α are known to be effectively tumoricidal against some liver and brain tumours.

In its biologically active state, TNF-α exists as a trimer of 17 kd 157 amino acid subunits. A cell membrane associated form of TNF-α is also bioactive and, when proteolytically cleaved, releases the active 17 kd moiety that is secreted together with a smaller membrane-associated 14 kd fragment that is detectable in plasma by antibody (Jue *et al.* 1990; Kriegler *et al.* 1988). Two TNF–α receptors referred to as p55 and p75 have been identified. The two receptors share 25% homology in their cysteine rich extracellular domains but their cytoplasmic domains are totally different. Both receptors bind to TNF–α with equal affinity but most functions of TNF–α appear to be mediated through the p55 receptor. Surface expression of both types of receptor is upregulated by IFN-γ, IL-2, proteinase A activators and concanavalin A (Thoma *et al.* 1990; Tsujimoto *et al* 1986; Ungluab *et al.* 1987).

5.4.2 TNF and disease

Cytokines produced by immunologically competent cells are an essential component of the inflammatory host response to infection or injury but it is now recognised that over-expression of cytokines may result in tissue damage. TNF-α, in particular has been implicated as the major effector of immunopathological tissue damage in several disease conditions (Strieter 1993). The most well documented example of TNF–α mediated tissue damage is septic shock, a syndrome associated with grossly elevated serum TNF-α levels. Other examples, shown in Table 5.5, include cerebral malaria, activation of latent HIV infection, the anaemia and cachexia of cancers and possibly malaria, burns, graft versus host disease (GVHD), adult respiratory distress syndrome (ARDS), diabetes mellitis (via B cell and insulin resistance), rheumatoid arthritis (RA),

systemic lupus erythematosis (SLE) (Streiter 1993), the Jarisch-Herxheimer reaction (Negussie *et al.* 1992) and the reversal syndromes of leprosy (Khanokar-Young *et al.* 1995). Although it is possible that in some of these conditions, raised TNF–α levels may just be an epiphenomen, in others the evidence pointing to a causal role for elevated TNF–α in the associated tissue damaging processes is compelling. For example, animals subjected to anti-TNF pre-treatment are protected against endotoxaemic or bacteraemic shock (Mathison *et al.* 1988; Ogata *et al.* 1993; Jin *et al.* 1994). High levels of TNF-α are associated with rejection in graft versus host disease (GVHD) and, in rats, anti-TNF–α therapy improves cardiac allograft survival (Streiter 1993).

5.4.3 TUMOUR NECROSIS FACTOR-α IN TUBERCULOSIS

5.4.3.i Background

To the extent that tuberculous disease can be considered as an interplay between protective and tissue damaging immune responses following *M. tuberculosis* infection, the role of TNF-α in tuberculosis is similarly complex. The lipoarrabinomannan (LAM) and lipomannan components of the mycobacterial cell wall are recognised as potent TNF-α stimulators in macrophages (Bradbury & Moreno 1993; Adams *et al.* 1993; Chatterjee *et al.* 1992). Although this cytokine probably has an important protective role in tuberculosis, it is also strongly implicated in much of the immunopathology that characterises this disease (Rook & Hernadez-Pando 1994).

In mice, TNF–α clearly has a protective role against mycobacterial disease. It is required for proper granuloma formation and enhances the macrophage's bactericidal

Table 5.5 Diseases in which high TNF production is implicated in the process of tissue injury.

disease	**complications**
septic shock	hypotension and tissue injury
adult respiratory distress syndrome	endothelial injury and oedema
graft versus host disease	organ/tissue inflammation and infarction
diabetes mellitus	beta cell cytotoxicity and insulin resistance
systemic lupus erythematosis	tissue injury and renal failure
rheumatoid arthritis	inflammation and acute phase responses
cerebral malaria	cerebral inflammation and coma
AIDS	activation of latent infection
erythema nodosum leprosum	tissue injury
cancer	anaemia and cachexia
tuberculosis	cachexia, night sweats
relapsing fever	Jarisch-Herxheimer reaction

and bacteriostactic potential by triggering nitric oxide (NO) production in IFN-γ activated macrophages (Flynn *et al.* 1995). Mice pre-treated with anti-TNF–α antibody are unable to contain *M. tuberculosis* infection, developing high bacillary loads in their lung, liver and spleens and quickly succumbing to disseminated disease (Flynn *et al.* 1995). In addition, a virulent laboratory strain of *M tuberculosis*, H37v, appears to differ from the avirulent Mtb H37a strain in its propensity to stimulate TNF–α production in mouse macrophages *in vitro*. The virulent strain induces little or no TNF–α production whereas the avirulent H37a strain stimulates macrophages to release copious amounts of the cytokine (Falcone *et al.* 1994; Adams *et al.* 1993).

In humans too there is some evidence for a beneficial role for TNF–α in antituberculosis immunity. In TB pleuritis, which is considered a good immunological model of protective immunity in tuberculosis because the syndrome can resolve itself without the need for chemotherapy, both pleural TNF protein and mRNA levels are significantly higher than those in blood (Barnes *et al.* 1990). However, as appears to be typical of its role generally in tuberculous disease, TNF is probably also responsible for the fever, exudative effusion and tissue necrosis observed in this condition. Furthermore, lung granulomas in patients with *M. tuberculosis, S mansoni,* or sarcoidosis-induced lung lesions usually contain large amounts of TNF–α mRNA and seem to be actively producing this cytokine (Myatt *et al.* 1994). In another mycobacterial disease, leprosy, monocytes from the immunologically more competent patients with tuberculoid leprosy release more TNF–α when stimulated *in vitro* with LAM or *M.leprae* than monocytes from patients with the more severe lepromatous form of the disease (Barnes *et al.* 1992).

However, the evidence for a critical role for TNF–α in the pathophysiology of human tuberculosis is strong. TNF–α is believed to be the major mediator of the fever, weakness, night sweats, necrosis and progressive weight loss that characterise tuberculosis (Tramontana *et al.* 1995). Numerous studies have demonstrated that blood monocytes and alveolar macrophages from patients with active tuberculous disease secrete higher levels of TNF–α than those from healthy controls and patients with chronic refractory tuberculosis (Takashima *et al* 1990; Kim *et al* 1991; Cadranel *et al.* 1993). One study reported that alveolar macrophages isolated from sputum culture-positive TB patients produce significantly more TNF–α than those from sputum culture-negative patients (Kim *et al.* 1991). Findings from the same study suggest that there may also be a positive correlation between serum TNF–α levels and the severity of tuberculosis as determined by radiographic evidence of advanced lung disease (Kim *et al.* 1991). There is some evidence from a recently published study of male tuberculosis patients, that administration of a 14 day course of thalidomide, a drug known to inhibit TNF–α expression, in conjunction with standard anti-TB chemotherapy, is associated with with accelerated weight gain (Tramontana *et al.* 1995).

5.4.4 REGULATION OF TNF PRODUCTION

LPS is a widely studied potent inducer of TNF (Tracey 1993). Exposure of blood monocytes to LPS leads to a 3-fold increase in transcription, a 100-fold increase in TNF–α mRNA content, and a 10, 000-fold increase in TNF–α protein secretion (Beutler *et al.* 1986). The presence in the gene's 5' flanking region of several highly conserved LPS-responsive regulatory sites, which include NFkB elements, cyclic

AMP response elements, a cytokine-1-type κB enhancer variant homologue, an Ig chain κB enhancer homologue, a homologue of an MHC class II promoter element and GC and TATA boxes, provides strong evidence that TNF–α is an inducible gene (Spriggs *et al.* 1992; Tracey 1993).

There is some evidence from recent *in vitro* experiments that suggests that there are stable population differences in TNF production in response to various antigens (E Coleman *et al.* unpublished data). It is likely that these stable differences are due to genetic regulation of TNF responses. This genetic regulation may be influenced by polymorphisms in either the TNF gene itself, the HLA genes or other non-HLA genes. Nucleotide changes in critical regulatory regions can result in significant differences in the transcriptional activities of otherwise identical promoters (Emery *et al.* 1993) and therefore the presence of such polymorphisms in the numerous regulatory sites in the gene's 5' promoter region, makes these TNF polymorphisms strong candidates as important host regulators of TNF production.

5.4.5 ASSOCIATIONS OF TNF-α PROMOTER POLYMORPHISMS WITH DISEASE

Two di-allelic promoter polymorphisms have been described in the promoter region of the TNF-α gene. The first one identified is located at the -308 position of the promoter (Wilson *et al.* 1994). Chloramphenicol acetyl transferase (CAT) reporter gene studies using B cell transfectants demonstrated that the G-A single base substitution at this -308 position is associated with increased constitutive and inducible TNF-α production *in vitro* (Wilson *et al.* 1994). McGuire and colleagues (1994) have described an

association between homozygotes for this variant allele and cerebral malaria (Table 5.6). There is strong evidence linking elevated serum TNF-α with cerebral malaria (Kwiatkowski *et al.* (1990). In addition, TNF-α is known to upregulate expression of of host adhesion molecules that mediate parasite binding to vascular endothelium (Berendt *et al.* 1989). It has also been suggested that TNF-α may stimulate cerebral endothelium to overproduce nitric oxide, thus causing coma by interfering with neurotransmission (Clark *et al.* 1992). Individuals homozygous for the -308 variant allele, also appear to be at increased risk for lepromatous leprosy (M Roy unpublished data) and mucocutaneous Leishmaniasis (Cabrera *et al* 1995) and at higher risk of death from meningococcal disease (M Levine *et al.* unpublished data).

The second polymorphism at position -238, another G-A dimorphism, is located within a putative regulatory Y box within the promoter of the TNF-α gene (D'Alfonso *et al.* 1994). Although as yet no *in vitro* studies have been reported, inferences from studies of malaria in mice and humans suggest that this variant may be associated with increased constitutive TNF-α production. Firstly, McGuire *et al.* have reported an association of heterozygotes for this -238 polymorphism with severe malaria anaemia in children from both Kenya and The Gambia. Secondly, experimental models of murine malaria incriminate TNF–α as a cause of a dyserythopoeitic anaemia that is reversed by the administration of anti-TNF antibodies (Clark & Chaudri 1988; Miller *et al.* 1989). In addition, chronic exposure to TNF achieved by implanting nude mice with TNF secreting CHO cells causes inhibition of erythropoeisis (Johnson *et al.* 1989). Moreover, in vitro, TNF–α suppresses proliferation of erythroid progenitor cells in human bone marrow cultures (Goodman *et al.* 1987). There is also additional

Table 5.6 Infectious diseases associated with TNF-α promoter polymorphisms.

i. -308 promoter polymorphism

disease (population)	**genotype**	**effect**	**reference**
cerebral malaria (Gambians)	variant homozygotes	*increased susceptibility*	McGuire *et al.* 1994
lepromatous leprosy (Indians)	heterozygotes	*increased susceptibility*	Roy *et al.* unpublished
mucocutaneous leishmaniasis (Brazilians)	variant heterozygotes	*increased susceptibility*	Cabrera *et al.* 1995
meningococcal meningitis (Europeans)	heterozygotes	*increased mortality*	Levin *et al.* unpublished
trachoma (Gambians)	heterozygotes	*increased susceptibility*	Conway *et al.* unpublished

ii. -238 promoter polymorphism

severe malaria anaemia (Gambians)	heterozygotes	*increased susceptibility*	McGuire *et al.* unpublished
trachoma (Gambians)	heterozygotes	*increased susceptibility*	Conway *et al.* unpublished

evidence from transgenic mice in which TNF–α is constitutively overexpressed which suggests that TNF–α may also cause anaemia by enhancing erythrophagocytosis (Taverne *et al.* 1994).

5.4.6 RESULTS

We typed our cases and controls for these two TNF–α promoter polymorphisms using PCR amplification of the promoter and specific oligonucleotide hybridisation of the the amplified product. No association was found between the -308 promoter polymorphism and susceptibility to tuberculosis (Table 5.7i). However, heterozygotes for the -238 TNF-α promoter polymorphism were more frequent amongst the cases of pulmonary tuberculosis (odds ratio 2.49; P=0.00002, Table 5.7ii). Too few homozygotes were identified to allow definition of the risk associated with this genotype. Although the -308 variant was not more frequent among the tuberculosis cases, double heterozygotes for the -238 and -308 variants were at higher risk than those heterozygous for the -238 variant alone (Table 5.7iii). This is consistent with the hypothesis that the -238 variant, like the -308 variant, is associated with increased rather than decreased transcription of TNF–α, so that the presence of these alleles in trans may have an additive effect leading to an pathological excess of TNF–α production in response to *M. tuberculosis* infection.

TNF-α is located in the Class III region of the MHC and the -238 variant allele is in linkage disequilibrium with certain Class I and Class II HLA alleles, namely HLA-B18, -B53, -DR3, -DR9, and -DR13.02. However, appropriate HLA-stratified analysis of

Table 5.7 Frequencies of tumour necrosis factor genotypes in the study groups.

(a) For the -308 $_{G->A}$ promoter polymorphism the common allele is denoted 1 and the variant allele 2. (b) For the -238 $_{G->A}$ promoter polymorphism the common allele is denoted G and the variant allele A. The frequency of heterozygotes was increased in the cases compared to controls as shown. After stratification for ethnic group (Mantel-Haenzel odds ratio 2.54 $P < 0.0000085$) and multiple regression analysis allowing for ethnic group and the MBP genotype ($P=<10^{-4}$) this association remained highly significant. In the combined analysis shown in (c) the odds ratio is higher for -238 heterozygotes with the variant -308 allele (6.19) than for those without (2.05).

(i) **-308 $_{G->A}$ promoter polymorphism**

Genotype	Controls (%)		Cases	(%)
	n=263		n=203	
11	190	(72)	140	(69)
12	61	(23)	59	(29)
22	12	(5)	4	(2)

(ii) **-238 $_{G->A}$ promoter polymorphism**

Genotype	Controls (%)		Cases	(%)	Odds ratio	P value
	n=229		n=206			
GG	197	(86)	136	(66)		
AG	28	(12)	66	(32)	2.49 [1.60 - 3.9]	0.000023
AA	4	(2)	4	(2)		

Table 5.7 continued

(iii) **-238 $_{G->A}$ and -308 $_{G->A}$ promoter polymorphisms**

Genotype	Controls (%)		Cases (%)		Odds ratio	P value
	n=229		n=203			
AA & 11	4	(2)	4	(2)		
AA & 12	0		0			
AA & 22	0		0			
AG & 11	22	(10)	37	(18)	2.05 [1.12-3.75]	0.01
AG & 12	6	(3)	29	(14)	6.19 [2.38-17.03]	$< 10^{-4}$
AG & 22	0		0	(14)		

Table 5.8 List of HLA phenotypes shown in a previous study (McGuire *et al.* unpublished) to be significantly associated with TNF-α promoter -238 variant allele in Gambian population.

	antigen frequency (%)	**association (95% CI of odds ratio)**
DR3	6	2 - 7
DR9	14	5 - 10
DR1302	29	0.3 - 0.7
B18	5	8 - 44
B53	25	2 - 6

the -238 data showed that the observed association with susceptibility to tuberculosiswas independent of these HLA types.

5.4.7 DISCUSSION

These results provide further evidence for a role for cytokine gene polymorphisms in determining the clinical outcome of human infections. Specifically, these data concur with *in vitro* studies that implicate TNF-α in the clinical expression of pulmonary tuberculosis in humans. Clearly, *in vitro* expression experiments are needed to assess the functional significance of TNF-α -238 promoter polymorphism. The interaction of -238 promoter variant with a known TNF-α upregulating -308 promoter variant showing an even greater increase in the risk of disease compared to that attributable to the -238 variant alone, suggests along with other evidence from the literature that, functionally, the -238 promoter variant also upregulates TNF-α expression.

Several hypothesis have been put forward to explain the complex role of TNF–α in the host response to *M.tb* infection that appears to vary from protective immunity on one end of the spectrum to disease immunopathology on the other. It has been suggested that the local cytokine profile, the hormonal milieu, the tissue specificity and the pathogen strain are all factors that may govern the effects, benefical or deleterious, of TNF–α in tuberculous disease (Rook & Hernandez-Pando 1994). It has also been observed that *M tuberculosis* infection of cells, particularly those cells outside the reticulo-endothelial system, renders these cells exquisitely sensitive to the toxic effects of TNF-α (Filley & Rook 1991; Filley et al. 1992). If TNF expression studies do demonstrate that the -238 variant causes upregulation of TNF–α expression, then our

data, while not proving or disproving these other hypotheses, would indicate that the amount of TNF–α produced in itself is a major determinant of the overall effects of this cytokine in host response to *M. tuberculosis* infection.

There are potential clinical applications of these findings (discussed in greater detail in the concluding chapter of this thesis) but several questions have still to be addressed. There is *in vitro* evidence (Kim *et al.* 1991) that suggests that there may be differences in the contribution of altered TNF production to the pathophysiology of different tuberculous disease phenotypes. As in mice (Bradbury & Moreno 1993) there may be variation in TNF production by macrophages in different tissues. Therefore the role, if any, of these and other cytokine polymorphisms on different forms of the disease need to be investigated. Similarly, it will be important examine the role of TNF-α polymorphisms with TB cases from other populations as there may be different susceptibility genes in different populations.

Preliminary results from a small Indian case-control study suggest that, in India, the -238 and -308 polymorphisms may not be associated with altered susceptibility to tuberculosis (Ruwende *et al.* unpublished data). *M. tuberculosis* strain differences in the two populations may explain the observed difference in the two study populations. It is known that variation in the antigens (e.g *M.tb* cell wall lipoarabinomannan*)* of different strains may cause strain differences in a pathogen's capacity to stimulate TNF-a production by macrophages *in vitro* (Chatterjee et al 1992, Chatterjee at al 1992a, Falcone et al. 1994; Allan *et al.* 1993; Allan *et al.* 1995).

Susceptibility genes for a polygenic trait such as tuberculosis may vary between populations and this could explain the different results between Indians and Gambians for this TNF promoter polymorphism. In addition, this may be due to differences in disease phenotype between the clinical tuberculosis cases in the two study groups. *In vitro* data from Kim *et al.* (1991) indicate that monocytes from patients with sputum-positive tuberculosis produce more TNF than the monocytes from sputum negative cases. Interestingly, it appears that in an ethnically heterogenous population of cases in London, the same association has been demonstrated between the -238 TNF promoter variant and increased susceptibility to clinical tuberculosis (R Wilkinson, personal communication). This would suggest that, even in the presence of putative heterogeneity in disease pathophysiology, TNF–α is a common denominator in tuberculous disease processes.

Evidently, these TNF-α promoter variant alleles have deleterious effects in several infectious disease syndromes (i.e cerebral malaria, MCL, lepromatous leprosy and meningococcal disease [-308]; severe malaria anaemia and pulmonary tuberculosis [-238]), but to reach current frequencies (-238, 6-8%; -308, 14-16%) these variant alleles must confer some selective advantage against what historically was, and or presently is, a major disease in this population. In this regard it will be interesting to observe the effect of these polymorphisms on the outcomes to pneumococcus, hepatitis B infection, yellow fever, trypanosomiasis, onchocerciasis and other common non-infectious conditions such as hypertension, hepatomas, gynaecological cancers e.t.c. *In vitro*, TNF is known to have some anti-viral effects (Wong & Goeddel 1986; Mestan *et al.* 1986; van Strijp *et al.* 1991). Demonstration that these latter effects

accurately reflect *in vivo* events would be relevant with regard to the maintenance of these promoter polymorphisms in human populations.

CHAPTER 6 - *NRAMP1* AND MANNOSE BINDING PROTEIN (MBP) IN TUBERCULOSIS

6.1 *NRAMP1* IN TUBERCULOSIS

6.1.1 Background

There is considerable evidence for evolutionary conservation of chromosomal segments between mice and humans (Edwards 1994). Comparative mapping has been used successfully to identify several genes important in a large spectrum of diseases that affect humans (Nadeau *et al*; Liu *et al.* 1993; Searle *et al.* 1994). One of the most extensively studied genetic determinants of variable natural resistance to infectious diseases in mice is the *Bcg* locus (Skamene *et al.* 1982). Indeed, it appears that in inbred mouse strains, innate susceptibility to several mycobacterial species, *Mycobacterium bovis(BCG), Mycobacterium lepremarium, Mycobacterium intracellulare,* in addition to other intracellular infections such as *Salmonella typhimurium* and *Leishmania donovani,* is under the control of a single gene in the *Bcg/Ity/Lsh* locus located in the proximal region of mouse chromosome 1 locus (Schurr *et al.* 1990, Blackwell 1989).

In inbred mouse strains, infection with *Mycobacterium bovis* (BCG*)* is biphasic. Infection outcome in the early phase is influenced by non-specific immunity whilst control of the later phase of infection is dependent on specific immunity (Skamene 1994). It became apparent from early studies that, following the intravenous injection of low dose (10+CFU) dispersed BCG into inbred mice, variation at the *Bcg* locus determined which of two distinct outcomes, susceptible or resistant, occurred in the

early non-immune phase of infection (Skamene *et al.* 1982). In the susceptible Bcg^s mice, there was rapid uncontrolled multiplication of BCG in the reticulo-endothelial organs (spleen, liver, lungs) with absent bacterial growth in Bcg^r mice (Skamene *et al.* 1984). Mendelian segregation analysis (Gros *et.* 1981) concurred with other studies that suggested that the non-immune phase of BCG infection in these inbred mouse strains was probably under the control of a single autosomal recessive gene whilst the late phase (3-4 weeks) characterised either by complete clearance of the bacterial load or by persistent infection in the reticulo-endothelial (RE) organs of susceptible mice, was under the control of the H-2 linked immune response genes (Forget *et al.* 1981; Pelletier *et al.* 1982; Brett *et al.* 1992).

The identification of similarly distinct groups of resistant and susceptible mice for other antigenically and taxonomically unrelated intracellular pathogens such as *Salmonella. typhimurium* and *Leishmania donovani* (Bradley 1974; Plant *et al.* 1982) led investigators to propose that the macrophage was the most likely cellular compartment expressing this *Bcg* locus and it was suggested then that this gene may an important role as a regulator in the priming of macrophage activation (Buschman *et al.* 1989).

The candidate gene from the *Bcg* locus, originally named *Nramp* and now redesignated *Nramp1* in mice and *NRAMP1* in humans, was isolated by positional cloning and subsequently shown to encode a macrophage-specific membrane protein with 12 putative transmembrane domains (Malo *et al* 1991). Sequence analysis of the *Nramp1* gene demonstrated that susceptibility to infection was associated with a non-conservative substitution of glycine for aspartic acid at amino acid position 105 located

in the second putative transmembrane domain (Malo *et al* 1991). Recent evidence showing that natural resistance to intracellular infection in these inbred mice is abrogated by disruption of the *Nramp1* gene has provided unequivocal confirmation that *Ity/Lsh/Bcg* locus is indeed *Nramp1* (Vidal *et al.* 1995).

Gros *et al.* (1983) have provided compelling evidence which implicates the macrophage as the cell which determines Bcg phenotype expression. They were able to demonstrate firstly, that expression of the Bcg susceptibility or resistance phenotype was dependent on a radioresistant bone marrow-derived cell, and secondly, that administration of silica, a macrophage poison, to *Bcg* resistant or susceptible mice, abolished expression of these phenotypes (Gros *et al* 1983). In support of these findings have been *in vitro* studies with *Salmonella* (Lissner *et al.* 1983), *Leishmania* (Crocker *et al.* 1984), BCG and other mycobacterial species (Goto *et al.* 1989; Stach *et al* 1984; Stokes *et al.* 1986; Denis *et al.* 1989; Blackwell *et al.* 1989) which have clearly established that the major difference in the early infection phase resistance mechanisms between Bcg^r and Bcg^s mice, is attributable to greater T-, B- and NK cell independent anti-microbial activity in the macrophages of *Bcg* resistant animals.

Further evidence of the central role of macrophages in *Nramp1* gene expression came from several reports confirming constant differences in the phenotypic measurements of macrophage activation between resistant and susceptible mice (E Buschman *et al.* 1989). *Nramp1* resistant mice have consistently greater macrophage activation as measured by a variety of parameters such as responses to interferon-γ, respiratory burst activity (as measured by hydrogen peroxide production and hexose

monophosphate shunt activity), and expression of cell surface activation markers (Buschman *et al.* 1989, Blackwell *et al.* 1991; Zwilling *et al.* 1994; Dennis *et al.* 1988). However, recent evidence from mouse knock-outs for the *Nramp1* gene suggests that this gene may play a key role only in the early part of the macrophage-parasite interaction via a cytocidal or cytostatic mechanism that is distinct from that associated with activated macrophages (Vidal *et al.* 1995).

The human homologue of the mouse *Nramp1, NRAMP1,* lies in close proximity to the villin gene on chromosome 2q35 and has been recently cloned (Vidal *et al.* 1993 Blackwell *et al.* 1995). Although several sequence variants of the human *NRAMP1* gene have been described (Blackwell *et al.* 1995, Liu *et al.* 1995) the region encoding the mouse mutation in the second transmembrane domain is conserved in humans (Vidal *et al.* 1993; Blackwell *et al.* 1995). Two distinct genes *NRAMP1* and *NRAMP2* with 80% overall homology, have now been described in both mice and humans (Vidal *et al.* 1995; Gruenheid *et al.* 1994) and it appears there may still be yet another member in the the *NRAMP* family (Dosik *et al.* 1994). Whilst expression of *NRAMP1* gene on human chromosome 2 is restricted to phagocytic cells, *NRAMP2* gene found on chromosome 12q is constitutively expressed, albeit at low levels, in all tissues (Vidal *et al.* 1995).

The human *NRAMP1* gene which is 12 kb long with 15 exons, appears to encode an integral membrane protein containing both a conserved transport motif and a putative SH3 binding domain (Blackwell *et al.* 1995). Although there have been great advances in determining the nucleotide sequence of *NRAMP1*, its functional role remains elusive.

Strong sequence homology between the human *NRAMP1* and transporter proteins in other phylogenetically distinct organisms points to the gene encoding an important transporter molecule (Vidal *et al.* 1993). Between transmembrane domains 6 and 7 lies a 'binding protein dependent transport system inner membrane signature' which is a motif found in prokaryote and eukaryote nitrate transporters. 20 to 30% similarity with the nitrate transporter of *Aspergillus nidulans* led Vidal *et al.* (1993) to propose that perhaps *NRAMP1* was a nitrate transporter that facilitated delivery of nitrates into the phagolysosomes of infected macrophages. This proposal is certainly consistent with the observation that phenotypic expression of the Bcg resistance and susceptibility phenotypes is restricted to cells of the reticuloendothelial system particularly macrophages (Cellier *et al.* 1994). More tantalisingly, however, it also concurs with data that implicate a critical role for the production of reactive nitric oxide intermediates in the control of *Mycobacterial tuberculosis (M.tb)* infection.

Nevertheless, the proposed single function for *NRAMP1* as a nitrate transporter gene does not adequately explain the pleiotropic effects of this gene and it is likely that *NRAMP1* functions as a multi-substrate transporter (Blackwell *et al.* 1995). The tight linkage of the human *NRAMP1* gene to cytoskeleton genes (villin) in a conserved region of chromosome 2 has led some investigators to speculate that *NRAMP1* may be involved in signal transduction (Schurr *et al.* 1989; Blackwell 1989). *NRAMP1* is very similar to a nitrate transporter of *Aspergillus nidulans* (Vidal *et al.* 1993) but it appears there is an even greater similarity observed between *NRAMP1* and the yeast mitochondrial proteins SMF1/2 (Blackwell *et al.* 1995) which may indicate that *NRAMP 1* may not encode a nitrate transporter but rather a hydrophobic transporter

on the mitochondrial membrane. To cloud the picturc a little further, recent experiments with *Nramp1* null allele mouse transgenics do support a role for this protein in macrophage transport but suggest that, contrary to current thinking, *Nramp1* is not critical for elimination of intracellular parasites by activated murine marophages (Vidal *et al.* 1995). Evidently, the functional role of *Nramp1* is still unclear and more transgenic mouse experiments and reporter gene studies with macrophage constructs need to be done.

Several sequence variants in the 5' promoter region and within the human *NRAMP1* gene sequence itself have been described (Blackwell *et al.* 1995; Liu *et al.* 1995). This is particularly useful as these described sequence variants provide researchers with tools with which to study the possible role of *NRAMP1* in genetic susceptibility to tuberculosis and other parasitic and non-parasitic diseases that involve the macrophage in humans. Of nine variants described to date, only two cause amino acid substitutions, the other seven are silent substitutions or are located in introns (three) or UTRs (four) of the gene (Blackwell *et al.* 1995; Liu *et al.* 1995). Unfortunately, heterozygosity for these nine polymorphisms is low to moderate with the highest value being approximately 47 % and this reduces the power of studies to detect any possible associations or linkages between the various alleles with tuberculosis. Fortunately there are other highy polymorphic microsatellite markers that are linked to the *NRAMP1* gene which can be used instead to investigate the possible role of this gene in differential genetic susceptibility to tuberculous disease.

Interestingly, one of the *NRAMP1* sequence variants described lies within the 440 bp 5' promoter region which has several regulatory elements such interferon-γ, W elements, API and NFκB sites that are commonly associated with other known inducible genes such as iNOS and TNF-α (Blackwell *et al.* 1995). It is therefore possible that *NRAMP1* expression may be regulated by macrophage priming or activation stimuli. Also located within the 5' flanking region of *NRAMP1* is a putative Z-DNA-forming dinucleotide repeat (Blackwell *et al.* 1995). DNA in such Z-conformation is known to influence regulatory signalling (Rich *et al.* 1984) and, therefore, this dinucleotide repeat or microsatellite marker may influence *NRAMP1* gene expression (Blackwell *et al.* 1995). It is possible, therefore, that this promoter microsatellite may be useful as a functional marker of differential *NRAMP1* macrophage expression and therefore macropahge activation and subsequent disease phenotype in macrophage-related disease traits.

Recent unpublished work by Blackwell *et al.* indicates that the different alleles in the promoter microsatellite marker may be associated with functionally variable levels of *NRAMP1* expression and therefore macrophage activation. Of the 4 alleles described (Blackwell *et al.* 1995), allele 3 of this microsatellite is reported to be associated with relatively higher *NRAMP1* expression and in a case-control study of tuberculosis in Brazil, this allele appears to be protective against tuberculosis (Blackwell *et al.* unpublished data).

Given the relatively low heterozygosity observed in the published human *NRAMP1* polymorphisms, it is particularly useful that there are a number of highly polymorphic

microsatellite markers that are linked to the *NRAMP1* gene and may also serve as useful markers for the assessment of the role of *NRAMP1* in human diseases. In this study, I used two microsatellite markers to examine the possible role of *NRAMP1* in clinical pulmonary tuberculosis cases and controls. The first of these, D2S1471, was isolated from a YAC containing the villin gene VIL1 (White *et al.* 1994) and maps to within 155 kb of the *NRAMP1* gene. The second marker used is the microsatellite marker that lies within *NRAMP1* 's promoter region.

6.1.2 RESULTS

For both the D2S1471 and NRAMP1 5' promoter microsatellite, DNA samples were PCR amplified with fluorescent-labelled primers. PCR products were run on polyacrylamide gels in ABI sequencers and analysed with the Genotyper programme. The D2S1471 microsatellite was the first marker that was evaluated for a possible association with altered susceptibility to pulmonary tuberculosis in our cases and controls. No association was found between any of the alleles for this marker with disease outcome in our study (Table 6.1 & Figure 6.Aa). For the Nramp1 5' promoter region, the 4 alleles ascribed this marker locus in a previous study (Blackwell *et al.* 1995) were indeed all present our population. As for the D2S1471 microsatellite, no association was found between this promoter microsatellite marker with resistance or susceptibility to pulmonary tuberculosis (Table 6.2 & Figure 6.Ab).

6.1.3 DISCUSSION

In the light of the considerable attention given to the isolating and sequencing of the *NRAMP1* gene, there is a paucity of data in the literature on studies examining the role

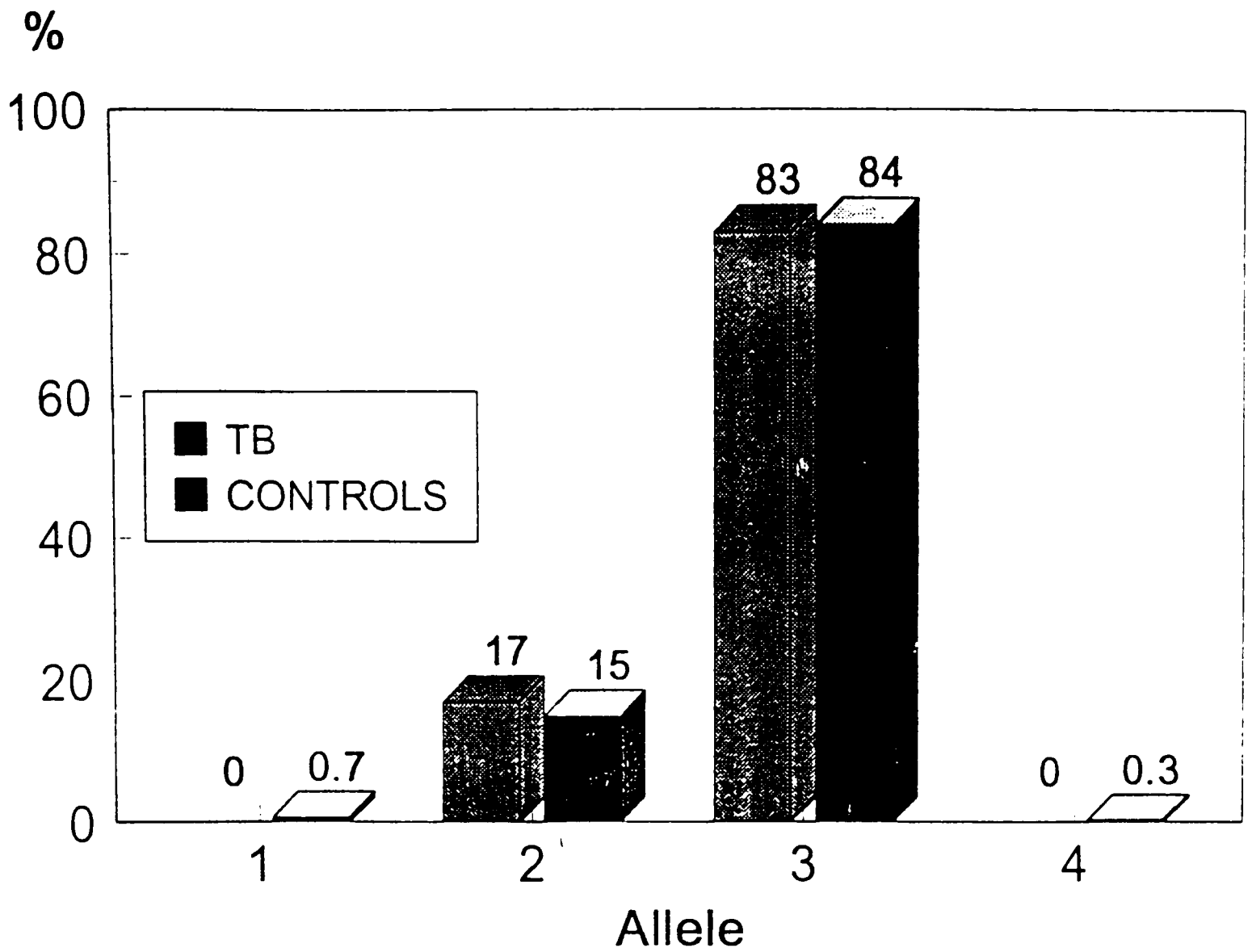
Table 6.1 Allele frequencies (%) for D2S1471 microsatellite marker that is 155kb from the NRAMP1 gene (White *et al.* 1994). None of the frequency differences were significant after correction for multiple comparisons.

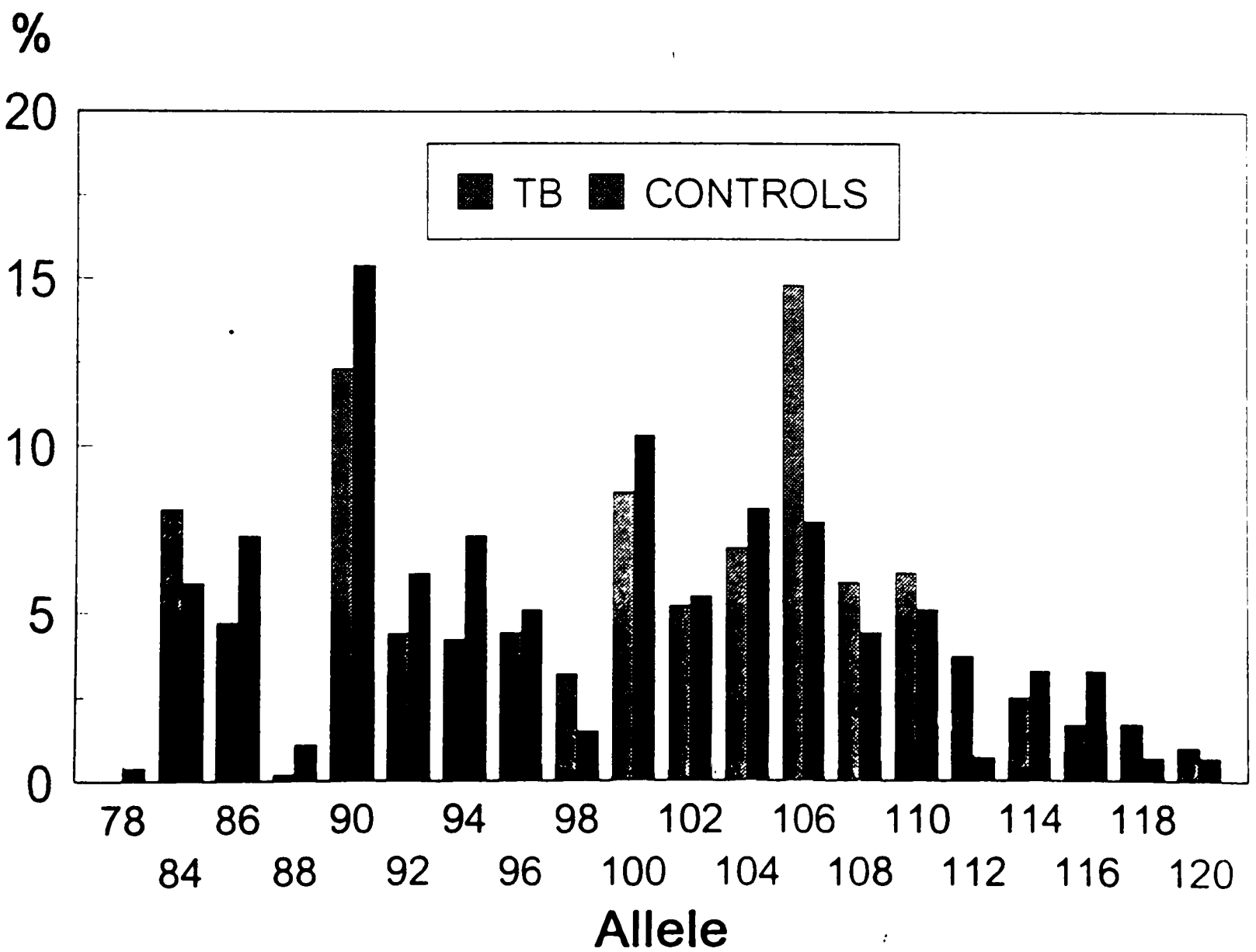
allele size (bp)	controls n=137		cases n=205		*P* value
	no.	frequency (%)	no.	frequency (%)	
78	1	0.4	0		ns
84	16	5.9	33	8.1	ns
86	21	7.7	19	4.7	ns
88	3	1.1	1	0.2	ns
90	42	15.4	50	12.3	ns
92	17	6.2	18	4.4	ns
94	20	7.3	17	4.2	ns
96	14	5.1	18	4.4	ns
98	4	1.5	13	3.2	ns
100	28	10.3	35	8.6	ns
102	15	5.5	21	5.2	ns
104	22	8.1	28	6.9	ns
106	21	7.7	60	14.8	ns (0.2)
108	12	4.4	24	5.9	ns
110	14	5.1	25	6.2	ns
112	2	0.7	15	3.7	ns (0.2)
114	9	3.3	10	2.5	ns
116	9	3.3	7	1.7	ns
118	2	0.7	7	1.7	ns
120	2	0.7	4	1.0	ns

Table 6.2 *NRAMP1* promoter microsatellite allele in cases and controls showing no significant frequencies differences. Allele '3' that may be associated with increased *NRAMP1* expression (J Blackwell unpublished data) is the most common allele and is not associated altered susceptibility to tuberculosis in these Gambian cases and controls

allele	**(bp)**	**controls**		**cases**	
		n=191		**n=184**	
'1'	122	2	(0.7%)	0	
'2'	120	58	(15%)	62	(17%)
'3'	118	321	(84%)	306	(83%)
'4'	108	1	(0.3%)	0	

Figure 6.A


NRAMP1 Promoter Alleles
%
100
80
60
40
20
0
TB
CONTROLS
0
0.7
17
15
83
84
0
0.3
1
2
3
4
Allele


NRAMP1 D2S1471 Alleles
%
20
15
10
5
0
TB
CONTROLS
78
84
86
88
90
92
94
96
98
100
102
104
106
108
110
112
114
116
118
120
Allele

of this candidate in the outcome of *Mycobacterial tuberculosis* infections in humans. It may be be the case that other investigators that have been and that, presumably, are still are looking for possible effects of the different *NRAMP1* gene sequence variants on *M.tb* disease outcomes have, as in our case, simply not yet found any significant associations or linkages. Indeed, the only published data that implicate *NRAMP1* in human tuberculosis come from a study on an extended multisib-case Canadian-Indian family from which came the somewhat tentative report of 'significant LOD score' linkage of chromosome 2 allele variants with tuberculosis (Skamene 1994).

The majority of the sputum-positive pulmonary tuberculosis cases in this study probably would have been adults with post-primary rather than primary tuberculosis. This may be an important consideration as, in mice, the effects of the *Nramp1* alleles are clearly most marked in the early non-immune phase of infection (Gros *et al.* 1981) that probably more closely resemble primary rather than post-primary tuberculosis. The observation in inbred mice of similar resistance/susceptibility *Nramp1* phenotypes with different intracellular organisms (ie BCG, *Leishmania*, *Salmonella*) also suggests that the non-immune phase of primary *Mtb* infection that may lead to primary tuberculosis in humans, that is more likely to be influenced, if at all, by the *NRAMP1* gene. For this reason, therefore, one might not expect this study to show any significant associations for tuberculosis with either of the two markers investigated.

However, there is some evidence that, in mice, the *Bcg/Lsh* resistance and susceptibility genotypes also influence the subsequent development of acquired immunity. Firstly, it is known that T cells are required for complete clearance of

Leishmania donovani infection and that development of memory is clearly better in resistant compared to susceptible strains (Kaye & Blackwell 1989). Furthermore, the observation that Ia (Class II) expression is more persistant in macrophages from resistant mice (Buschman *et al.* 1989, Blackwell *et al.* 1991; Zwilling *et al.*1994) indicates that the antimicrobial resistance mechanisms that govern the *Nramp1* resistance and susceptibility phenotypes also influence T cell function. These observations would therefore suggest that any effect that *NRAMP1* polymorphisms have on disease outcome in *M.tb* infection may be detectable in cases of post-primary tuberculosis such as those in this study. However, in view of the evidence from the mouse model, linkage and association studies of paediatric cases of primary tuberculosis are clearly necessary before a role of the *NRAMP1* gene in determining clinical outcomes of tuberculosis in humans can be ruled out.

There are still many aspects of the *NRAMP1* gene primarily related to its function that need to be clarified and this may help investigators to design studies to optimally assess the role of *NRAMP1* in human infectious diseases. Why the *Nramp1* Gly-105 variant in mice causes differential susceptibility for some intracellular infections and not others, remains unresolved. It is possible that any effects of *NRAMP1* on *M.tb* infection outcome in humans may be influenced by the level of exposure to *M.tb* and other mycobacteria. Therefore, the high prevalence of both *M.tb* and environmental mycobacteria in The Gambia (T Corrah Ph.D. thesis 1995) might be a factor that obscures any effects that *NRAMP1* may have on variable tuberculosis susceptibility.

Despite the undisputed relevance of this gene in the clinical outcome of mycobacterial and other intracellular infections in mice, it may be the case that, as this study suggests, *NRAMP1* does not play a significant role in host resistance to *M.tb* infection in humans. The absence in the homologous domain 2 of human *NRAMP1*, of the glycine 105 polymorphism which gives rise to the different susceptibility phenotypes in mice may be seen as evidence to support this view especially as no other sequence variants in the mouse *Nramp1* yield the same striking susceptibility and resistant phenotypes as the amino acid 105 aspartate-glycine dimorphism. Hence the putative deleterious effects of any *NRAMP1* deficiency alleles may have prevented their reaching significant frequencies in human populations.

6.2 MANNOSE BINDING PROTEIN IN TUBERCULOSIS

6.2.1 Background

Mannose binding protein (MBP) is a member of the collectin (collagenous lectins) family of glycoproteins that may play an important role in first line host defence against various infectious pathogens (Weis *et al.* 1991; Tabona *et al.* 1995). MBP is produced in the liver and exists structurally as a multimeric assembly of 32 kd trimer subunits each comprising of cysteine-rich, collagenous and carbohydrate-recognition domains (Super *et al.* 1992). Along with lung surfactant (SP-A & SP-D), conglutinin and collectin-43 (CL-43), MBP is a member of the non-membrane bound C-type group of lectins (Quesenberry & Drickamer 1992; Drickamer *et al.* 198). The presence of inducer elements in the 5' region of the MBP gene on chromosome 10 in addition to the high serum levels of this protein that are observed in inflammatory reactions, all

indicate that MBP is secreted by the liver in acute phase responses to invading pathogens (Ezekowitz *et al.* 1988; Weis *et al.* 1991; Sastry *et al.* 1989).

MBP functions as a pattern recognition molecule that enables the host to distinguish self from non-self via selective calcium-dependent binding of its carbohydrate recognition domain (CRD) to non-host N-acetylglucosamine, mannose or fucose carbohydrate moieties on the surfaces of invading organisms (Super *et al.* 1992). This sugar-specific recognition function of MBP is thought to be essential not only for host defence but, perhaps, also for host intercellular recognition and interaction (Ezekowitz & Stahl 1988b; Ezekowitz 1991). Functionally, MBP acts as both an antibody- and C1q-independent activator of the classical complement pathway as well as an opsonin through binding to the C1q receptor (Kuhlman *et al.* 1989; Ikeda *et al.* 1987). There is evidence that the MBP activation of the classical complement pathway occurs through a recently described MBP-associated serine protease (MASP) (Matsushita & Fujita 1992).

Low serum MBP levels are strikingly associated with the presence of variant MBP alleles (Sumiya *et al.* 1991; Lipscombe *et al.* 1992; Madsen *et al.* 1994). There are three structural variants of the polypeptide corresponding to single nucleotide substitutions in codons 57, 54 and 52 of the MBP gene (Table 6.3). Although, some promoter polymorphisms are also known to influence the serum MBP levels (Madsen *et al.* 1995) these three structural variants are clearly the most important deteminants of serum MBP levels. All three arise from single base changes that cause amino acid substitutions (Sumiya *et al.* 1991; Lipscombe *et al.* 1992; Madsen *et al.* 1994). For the

codon 54 (allele B) and codon 57 (allele C) substitutions the glycine is replaced by aspartic acid and glutamic acid respectively. The codon 52 variant (allele D) results from an exhange of a cysteine for an arginine residue.

Each of the codon mutations found in the variant alleles gives rise to reduced serum MBP levels because the associated amino acid (aa) substitutions cause disruptions of the Glyaa-Xaa-Yaa structure of the collagenous triple helix (Sumiya *et al.* 1991; Madsen *et al.* 1995). The precise mechanism by which the latter lead to low serum MBP levels is not totally clear. It may be that the presence of these disruptions of the collagenous tail hinder formation of the fundamental 32 kD MBP subunit. Alternatively, the MBP subunit may form naturally enough but there may either be reduced hepatocyte secretion of the subunit protein, defective polymerisation of the formed subunits or increased intra- or extracellular degradation of assembled polymers.

Homozygotes for the wild type allele, allele A, are associated with highest serum MBP levels while carriage of either of these variants (heterozygotes) gives rise to a reduction to intermediate range serum levels that are less than half of normal levels (Turner *et al.* 1992; Madsen *et al.* 1994). Homozygotes for each of the variant alleles give rise to very low or undetectable serum levels (Sumiya *et al.* 1991; Lipscombe *et al.* 1992; Madsen *et al.* 1994). The results of MBP sequencing studies indicate that two variant alleles are never in *cis* with one another and therefore individuals who possess two different variant alleles (ie B,C,or D) are recognised functionally as variant homozygotes as they too have very low or undetectable serum MBP levels (Madsen *et al.* 1995).

Table 6.3 Mannose binding protein (MBP) structural allele frequency ranges found in different populations in several studies (Sumiya *et al.* 1991; Turner *et al.* 1993; Garred *et al.* 1993; Madsen *et al.* 1994; Garred *et al.* 1995; Summerfield *et al.* 1995)

	allele	**population**	**allele frequency**
wild type allele	= A	(all populations)	> 0.6
codon 54 mutation	= B	(Caucasian/Eskimo)	0.1 - 0.20
codon 57 mutation	= C	(African populations)	0.2 - 0.3
codon 52 mutation	= D	(all populations)	0.05

There are clear population differences in the distribution and frequencies of the MBP variants (Madsen *et al.* 1994) (Table 6.3). The wild type allele, A, is the commonest in all populations. Allele B is found at relatively high allele frequencies (10-20%) in Caucasian, Chinese and Eskimo populations whilst allele C is mostly found in Africa where it has an allele frequency of 20-30%. Allele D has been described in all the populations studied to date albeit at a much lower allele frequency (5%) (Madsen *et al.* 1994; Garred *et al.* 1995).

6.2.2 MBP IN HOST IMMUNITY

There are several reports which all point to a role for MBP in host defence against invading pathogens. There is evidence that MBP is important in the control of influenza infection in the pre-immune phase (Anders *et al.* 1994; Hartshorn *et al.* 1993). In HIV infection, MBP and is capable of neutralising the virus by binding to viral envelope glycoproteins and activating complement mediated viral lysis (Spear *et al.* 1990). There are also some data that suggest that MBP may inhibit *in vitro* HIV infection of cells (Ezekowitz *et al.* 1989) Moreover, MBP has been shown to enhance serum bactericidal activity on mannose rich Salmonella isolates *in vitro* (Schweinle *et al.* 1989).

Interest in MBP was increased by a report that low serum MBP levels were associated with a common opsonic defect associated with recurrent infections in childhood (Super *et al.* 1989). It appeared that this immune deficiency syndrome manifested itself most strikingly in children between 6 to 24 months at a time when they are at their most vulnerable on account of their immature specific immune system and the disappearance

of maternal antibodies (Hammarstrom 1986). Furthermore, the observation that most of the recurrent infections were with encapsulated bacteria and enteric and respiratory viruses supported the assertion that MBP had an important role in innate immunity (Super *et al.* 1989; Ezekowitz 1991). Whereas early studies suggested that it was young children that were particularly immunocompromised by MBP deficiency (Super *et al.* 1989; Sumiya *et al.* 1991), more recent studies suggest that absent or extremely low levels of serum MBP may confer a life-long risk of infection (Summerfield *et al.* 1995; Garred *et al.* 1995).

Despite the evidence for a role of MBP in innate immunity, MBP deficiency has not been demonstrated to be clearly associated with any specific infectious disease. However, findings in a case-control study in Ethiopia did suggest that cases with leprosy had significantly elevated levels of serum MBP compared to controls (Garred *et al.* 1994). Because previous *in vitro* work by the same group had demonstrated that MBP binds to sonicates of *M. leprae* with strong affinity, it was proposed that perhaps the high frequency of variant MBP alleles in so many diverse populations may be due to a selective advantage conferred by the associated low serum MBP levels in protection against mycobacterial infections (Garred *et al.* 1994). Against the backdrop of this information, we set out to examine the frequencies of the various MBP genotypes in our cases and controls.

6.2.3 RESULTS

Typing of the three MBP codon variants was performed using PCR amplification and oligonucleotide-specific hybridisation on Gambian controls and sputum positive

pulmonary tuberculosis cases. All three MBP variants found at varying frequencies in other populations, were present in our study populations and, as expected, the codon 57 variant allele C was by far the commonest variant allelle (Table 6.4). Because only individuals who are functionally homozygous for MBP deficiency alleles appear to be somewhat immunodeficient, our primary hypothesis was that this group might show altered susceptibility to tuberculosis. The results (Table 6.4) show such individuals were significantly more frequent among cases than controls (odds ratio 2.59 [1.25-5.41], P=0.008). There was no significant difference in the frequency of heterozygotes between cases and controls. Thus individuals that are functionally homozygous for MBP deficiency were found to be at increased risk of pulmonary tuberculosis.

6.2.4 DISCUSSION

The remarkably high carriage of these MBP structural variants in so many diverse populations (Africans 50%, Caucasians 40%, and Eskimos 20% carriage) is something of a mystery especially in view of the fact that attendant low serum MBP levels carry such a striking disadvantage, being associated with opsonic deficiencies which lead to recurrent infections in childhood (Super *et al.* 1989; Sumiya *et al.* 1991) and in adults (Garred *et al.* 1995; Summerfield *et al.* 1995). Several reports in the literature suggest that, as with several other known resistance genes such as HbS, while the MBP variants may have certain selective disadvantages, these structural polymorphisms have been maintained as result of selection for resistance against other infectious diseases. Low MBP levels may be protective against intracellular infections such as HIV and *Herpes Simplex* (Fischer *et al.* 1994) that may use MBP and other antibody-independent opsonisation mechanisms to enhance their infectivity of host cells

Table 6.4 Frequencies of mannose-binding protein genotypes in the tuberculosis study groups. For the primary analysis subjects were classified into three genotype groups as shown in the left-hand column: those with a normal genotype, those that are heterozygous for MBP mutation, and those designated functional homozygotes. This latter group includes those homozygous for any mutation as well as double heterozygotes.

	Genotype	**Controls** (n=218)		**Cases** (n=211)		
Normal	AA	126	(58%)	114		(54%)
	AC	68		57		
Carriers	AD	13	79 (36%)	8	67	(32%)
	AB	1		2		
	BC	0		1		
	CD	3		5		
Functional	BD	0		1		
Homozygotes	CC	10	13 (6%)	22	30	(14%)*
	DD	0		1		
	BB	0		0		

*The frequency of functional homozygote genotypes is significantly higher in the cases than controls (odds ratio 2.59 [1.25-5.41], P=0.008). This difference remained significant following stratification for ethnic group differences in the study population (P=0.001) and logistic regression analysis allowing for the ethnic group and TNF -238 and -308 promoter genotypes (P=0.0077). The alleles are designated as follows: normal allele =A, codon 54 variant allele =B; codon 57 variant allele =C; codon 52 variant allele =D.

(Robinson *et al.* 1990; Reisinger *et al.* 1990). In addition, it is possible that another possible advantage associated with low serum MBP may be reduced susceptibility to complement-mediated tissue damage. The lower frequency of autoimmune diseases in Africa, where there is the highest carriage of these MBP structural variants, might also be considered to be indirect evidence that supports to this idea.

Our data do not support the hypothesis of Garred *et al* (1994), that the high frequencies of MBP deficiency variants observed in Africans have been selected for as a result of increased resistance to tuberculosis. In fact, these data show the opposite effect with functionally MBP-deficient homozygotes being at an increased risk for pulmonary tuberculosis. The finding of the highest frequency of the MBP structural variants amongst Africans who, if anything, appear to be at an increased risk for *M. tb* infection and disease (Stead *et al.* 1989; Stead *et al.* 1990; Stead *et al.* 1992; Crowle & Elkins 1990; McPeek *et al.* 1992), is at variance with Garred's proposal that resistance to tuberculosis is the major positive selective force that is maintaining the high frequency of the MBP deficiency variant (allele C) in Africa. Garred's proposal is derived from his finding of significantly elevated serum levels in leprosy patients (Garred *et al.* 1994). However, MBP is an acute phase protein and therefore other intercurrent inflammatory stresses may may be responsible for elevated serum levels at any given time point. Hence the results of any association studies such as Garred's that attempt to correlate a chronic disease such as leprosy with single time point raised serum MBP levels without any genotype data, need to be interpreted with caution.

Historical (Livingstone 1857; Diamond 1992) and epidemiological (Stead *et al.* 1992). information indicate that the introduction of *M. tb* infection to sub-Saharan Africa is probably a relatively recent event and probably occurred in tandem with colonialisation at the end of the last century. Therefore even if one ignores our data and assumes that Garred *et al.* are correct in their hypothesis that the low MBP levels are protective against tuberculosis, it is still unlikely that, as proposed, *M. tb* has had enough time to exert the major selective force which would account for the high allele C population carriage rate observed in Africa today. However, it is still possible that even if *M. tb* is not responsible for the selective force behind the high frequency of allele C, other fatal infections such as malaria and Yellow fever may be important in this regard. Case-control studies of malaria in the same study population, have not found any significant association between malaria disease and the allele C structural variant (J Summerfield unpublished data; R Bellamy *et al.* unpublished data).

The knowledge that MBP binds very strongly to mycobacterial lipoarabinomannan (LAM) and lipomannan (LM) cell wall components (Garred *et al.* 1994) allows us to speculate on possible cellular mechanisms by which very low or absent serum MBP levels might make individuals susceptible to tuberculous disease as suggested by our results. *In vivo, Mtb* is known to reside primarily in macrophages and entry of the bacterium into macrophages can occur via several receptors including CD14 (Peterson *et al.* 1995) , the complement receptors CR1, CR3 and CR4, the mannose receptor and the MBP or C1q receptor (Chan & Kaufmann 1994). Activation enhances the macrophage's bacteriostatic or bacteriocidal activity against resident *M. tb* bacteria. It is recognised that there are differences in the levels of macrophage activation that

occur following entry of an organism into the macrophage. Macrophage entry of *M.tb* via the CR1 and CR3 receptors does not always trigger respiratory burst activity with the attendant production of reactive oxygen and nitric oxide intermediates as is observed when other receptors are used (Wright & Silverstein 1983; Schlesinger *et al.* 1990)). Such quantitative differences in activation may therefore give rise to variation in the efficiency with which *M.tb* multiplication is contained within the macrophage. In such a scenario, in individuals with very low or absent MBP levels and diminished MBP opsonisation, *M.tb* macrophage entry would necessarily have to occur via other 'activation-inefficient' receptors such as the CR1 and CR3 receptors therefore making such individuals more prone to develop tuberculosis through increased survival of *M.tb* in macrophages.

Our patients mainly had post-primary tuberculosis some of which will be a result of reactivation and the rest of reinfection. It is important to establish whether the functionally MBP-deficient variant homozygotes are predisposed to one or both processes. MBP is probably important primarily in innate immunity (Ezekowitz 1991; Tabona *et al.* 1995) but it is, nevertheless, still possible that MBP plays a role in the host immune response not only in primary tuberculosis but also in post-primary reinfection and reactivation tuberculosis. Work on animal models indicates that in populations with high *M.tb* prevalence rates such as The Gambia, the majority of post-primary tuberculosis cases will be attributable to reinfection as opposed to reactivation of old disease. It is conceivable therefore, that events within the macrophage during primary infection may in part determine not only the likelihood of individuals controlling infection in the first instance but also their chances of subsequently

developing reactivation or reinfection post-primary tuberculosis in later years. Whilst distinguishing between reactivation and reinfection cases amongst post-primary tuberculosis patients is at the moment still difficult to ascertain, primary TB cases are easier to identify and it will be interesting to undertake a similar case-control study to this with such cases.

However, our identification of MBP genotypes that enhance *Mtb* disease susceptibility provides tantalising opportunities for the design of future anti-tuberculous strategies as it permits the identification of high risk individuals who might benefit from TB prophylaxis. However, there are still several issues that need to be addressed. Do these MBP deficiency alleles cause altered susceptibility to primary tuberculosis? Are they associated with increased susceptibility to pulmonary tuberculosis in different populations both within and without sub-Saharan Africa? What are the effects of the MBP structural variants in other important infectious diseases and in non-infectious diseases such as autoimmne diseases? Do the other C-type lectins, particularly pulmonary surfactant, also influence tuberculosis susceptibility either directly or through interaction with MBP? Most importantly, the exact molecular effects of the various MBP structural variants at the macrophage cell level need to be clarified before these findings can be employed in new strategies for combating tuberculous disease.

CHAPTER 7 - INTERLEUKIN-4 (IL-4) IN MALARIA AND TUBERCULOSIS

7.1 TH1/TH2 T HELPER CELL DIFFERENTIATION

The importance of cytokine networks in determining the host response to infection is becoming increasingly evident as more work is done in identifying and characterizing the functions of members of the cytokine family. Our understanding of how cytokine networks operate to control immune responses was greatly enhanced by the identification, first in mice (Mossman *et al.*1986; Cherwinski *et al.* 1987) and subsequently in humans (Barnes *et al.* 1993), of two subsets of regulatory CD4 + T helper (Th) cell clones associated with distinctive cytokine profiles. The two subsets termed Th1 and Th2 were defined primarily by distinctive patterns of cytokine secretion with Th1 cells secreting interleukin-2 (IL-2), gamma interferon (IFN-γ) and tumour necrosis factor while the Th2 cells produced IL-4, IL-5, IL-6, IL-10 and IL-13 (Mossman & Sad 1996).

The functional dichotomy between these two regulatory subsets has been confirmed (Romagnani 1991) with Th1 cells enhancing cellular immunity and Th2 are best adapted to bolstering humoral immunity. In addition, the cytokine profiles also influence isotype switching in antibody responses with immunoglobulin E (IgE) and IgG1 responses in mice promoted by Il-4(Th2) whilst IG2a production is promoted by IFN-γ (Th1)(Finkelman *et al.* 1990; Mossman & Sad 1996). The products of the two subsets regulate one another reciprocally. IFN-γ from the Th1 subset inhibits Th2 proliferation while IL-4 and IL-10 which are produced by TH2 cells, inhibit synthesis

of Th1 cytokines as well as blocking the development of Th1 cells (Fernandez-Botran *et al.* 1988; Fiorentio *et al.* 1989; Gajewski *et al.* 1991; Fitch *et al.* 1989).

Although the Th1 and Th2 patterns of cytokine production were first observed in mice, similar cytokine patterns were subsequently described among human CD4+ T-cell clones (Del Prete *et al.* 1991; Romagnani 1991) and although the profiles are very similar to those described in mice, it is clear that synthesis of several cytokines notably IL-2, IL-6, IL-10 and IL-13, is not as tightly restricted to a single subset as in mouse T cells. Other cytokine patterns have also been described such as Th0 cells that express both Th1 and Th2 like cyokine profiles and Th3 cells that produce high amounts of transforming growth factor beta (TGF-β) (Chen *et al.* 1994; Mossman & Sad 1996).

It has been argued that the cytokine patterns expressed by CD4+ T cells are not discrete subsets but rather a continuum of different random combinations of cytokine secretion (Kelso 1995) but there is considerable evidence that suggests otherwise. *In vitro* work with both mice and human CD4+ clones demonstrate the following: consistent co-expression of IL-4 and Il-5, striking inverse correlation in the synthesis of IL-4 and IFN-γ (Street *et al.* 1990), reproducible inhibition of Th1 cells by IL-10 and IL-4 and enhancement of IFNγ synthesis by IL-2 and IL-12 (Fiorentino *et al.* 1989). All this evidence together with those from other studies (Assenmacher *et al.* 1994; Openshaw *et al.* 1995) strongly indicates that the Th1/Th2 dichotomy is a real phenomenon and that the expression of the distinctive cytokine profiles associated with the latter is unlikely to be a random process.

Although it is generally accepted that Th1 cells are involved in cell mediated inflammatory reactions such as delayed-type hypersensitivity (DTH) reactions and that Th2 cells encourage antibody production, particularly IgE, in addition to enhancing eosinophil proliferation and function (Mossman *et al.* 1989; Romagnani 1991; Mossman & Sad 1996), however, there do remain several aspects of the characteristics, interactions and functional roles of the Th cell regulatory subsets that require more precise elucidation. These include the factors, be these host, parasitic or environmental, that determine Th1/Th2 differentiation, and the *in vivo* stability over time of the different cytokine profiles.

Th1 and Th2 cytokine patterns have now been identified in immune responses in infections, allergy and autoimmunity (Mossman & Sad 1996). Interestingly, it has been suggested that the Th cell polarisation that occurs in response to infection may be an evolutionary consequence of an adaptation of non-specific immunity to most appropriately counter different infections (Garside & Mowat 1995).

7.2 THE ROLE OF TH1/TH2 RESPONSES IN DISEASE

There is increasing evidence that Th1/Th2 differentiation has an important role to play in determining susceptibility and resistance to infectious pathogens in humans (Table 7.1). The most striking demonstration of the pathophysiological significance of distinct Th1-Th2 phenotypes has emerged from studies of protozoan parasitic infections, notably leishmaniasis, in which the outcome of *Leishmania major* infection in mice is critically dependent upon which Th subset is preferentially activated (Sher *et al.*1992). A Th1-mediated response leads to resistance and a Th2 response results in

susceptibility (Locksley *et al.* 1987). Significantly, various immunological manipulations most effective in the first week of infection, can alter the pattern of resistance and susceptibility. Treatment of susceptible BALB/c mice with anti-IL4 converts a Th2 pattern to a Th1 cytokine profile that confers resistance (Chatelain *et al.* 1992) whereas depletion of IFN-γ in resistant C3H/HeN mice leads to susceptibility (Belosevic *et al.* 1989). The importance of differential Th1/Th2 expansion has also been observed with bacterial (Haak-Frednscho *et al.* 1992), helminth (Urban *et al.* 1991) and fungal infections (Romani *et al.* 1994). Generally, the results from these studies show that Th1 mediated responses are protective against intracellular pathogens while Th2 responses are protective against infections by extracellular microbes.

In leprosy, the severe lepromatous type lesions are associated with elevation of Th2 cytokines such as IL-4, IL-5 and IL-10 while Th1 cytokines such as IFNγ and TNF predominate in the DTH type tuberculoid lesions (Modlin *et al.* 1984; Barnes *et al.* 1992; Yamamura *et al.*1991). In leishmaniasis, the lesions in the more severe human chronic mucocutaneous (MCL) disease type are associated with a Th2-type cytokine profile characterised by an abundance of Il-4 which is distinct from that found in the more benign localised cutaneous leishmaniasis type (LCL) (Pirmez *et al.* 1991). Even more topical than these reports on leprosy and leishmaniasis, have been the accounts in the literature on the effects of Th1/Th2 cytokine profiles on infection and disease progression in HIV infection. The data are controversial but suggest that Th2 response is associated with more rapid disease progression and development of full

Table 7.1 Murine (M) and human (H) infectious diseases in which Th1/Th2 responses are implicated in infection outcomes

pathogen	**host**	**effect**	**reference**
Leishmania major	M	Th1 responses in resistant mice; anti-IL4 protects, anti IFN-γ exacerbates	Scott *et al.* 1989
Leishmania donovani	H	IL-4 higher in generalised visceral disease; IFN-γ higher in localised cutaneous disease	Kurtzhals *et al.* 1994
Plasmodium chabaudi	M	Th1 provides early NO-dependent defense; Th2 provides late antibody-dependent defense	von der Weid *et al.* 1993
Trichuris muris	M	Th2 response in resistant mice; IL-4 protects, anti-IL4 exacerbates	Else *et al.* 1994
Schistosoma mansoni	M	Schistosome eggs induce Th2 response associated with granuloma formation; granuloma formation reduced by IL-12 or anti-IL4 treatment	Oswald *et al.* 1994
HIV	H	Possible Th1 to Th2 switch with progression to AIDS	Clerci *et al.* 1993 Mosmann 1994
murine AIDS	M	Th2 response associated with disease progression	Gazzinelli *et al.* 1992
Measles	H	Vaccination causes increased IL-4 production	Ward *et al.* 1993
BCG	H	Th1 response at site of tuberculin (DTH) reaction	Tsicopoulos *et al.* 1992
M. leprae	H	Th1 response expressed in tuberculoid leprosy lesions, Th2 response expressed in lepromatous lesions	Yamamura *et al.* 1991
M. tuberculosis	H	elevated IFN-γ in resistance-associated pleurisy response	Barnes *et al.* 1993

blown AIDS and, possibly, even greater initial infection rates (Clerici *et al.* 1993; Clerici & Shearer 1994; Clerici & Shearer 1994a). Other serological studies in humans indicate that the Th1/Th2 balance may also be relevant in hepatitis B virus (HBV) infection with a vigorous Th2-type antibody responses associated with chronic HBV while Th1-type cellular responses dominated in transient acute HBV (Maruyama *et al.* 1993; Milich *et al.* 1995).

In pregnancy, there is evidence that there are definite cytokine profile changes that that may be important for the interaction of the maternal immune system with the feto-placental unit. Successful pregnancy appears to favour a Th2 type pattern and while this enhances the development of the feto-placental unit, it also leads to increased susceptibility to certain intracellular infections and auto-immune diseases (Wegmann *et al.* 1993). Although the hormonal changes of pregnancy also cause a degree of immunosuppression, the induced Th2 cytokine state is believed to be responsible in part for observed exacerbations of intracellular infections such as malaria (Watkinson et al. 1983), HIV (Minkoff *et al.* 1987) and toxoplasmosis (Luft *et al.* 1982). The symptoms of rheumatoid arthritis, a cell mediated autoimmune disorder, are reduced in pregnancy (Da Silva & Spector 1992); and in systemic lupus erythematosis (SLE) in which excessive autoantibody production is responsible for the principal pathology, the condition worsens in pregnancy (Varner 1991).

Evidence from murine models suggest that the factors that influence Th1/Th2 differentiation may include the dose of the infective organism, the solubility or particulate nature of the pathogen's antigens, the predominant type of antigen

presenting cell (APC) for a given infection, recent or concomitant infection by other pathogens, the current hormonal milieu and other genetic factors particularly MHC haplotypes (Mossman & Sad 1996; Milich *et al.* 1995). In addition, it is also possible that even given the same genetic and environmental background, Th1/Th2 T cell differentiation may well vary temporally during the same infection and also in subsequent infections with the same pathogen (Orme 1993; Andersen 1995). Interestingly there are indications that there may be a non-pathogen specific association between chronic infections and Th2 patterns (Rook *et al.* 1993; Mossman & Sad 1996). It is not clear whether, as suspected in HBV (Maruyama *et al.* 1993; Milich *et al.* 1995), HIV infections (Mossman *et al.* 1994) and other such chronic infections, the Th2 cytokine profile is responsible for persistence of the infection or whether it is the chronicity of infection that is the stimulus for the observed Th2 bias.

7.3 INTERLEUKIN-4 (IL-4)

IL-4 is a cytokine produced primarily by activated T lymphocytes and its pleiotropic activities include increasing the size and viability of B lymphoblastoid cell lines, antibody isotype switching and the induction of MHC class II antigens, Fcε low affinity receptors, CD40, and the p75 chain of the IL-2 receptor (Song *et al.* 1996). Therefore, IL-4 does appear to have an important regulatory role in the immune system and it is certainly pivotal in the development of the Th2-type response that governs humoral immunity, eosinophilia, and inflammatory macrophage deactivation.

The bulk of the evidence on the function of IL-4 has come from animal models and indicates that early IL-4 secretion leads to polarisation towards Th2 differentiation via

IL-10 and autocrine IL-4 secretion by Th 2 cells and that these two cytokines in turn downregulate Th1 responses by suppressing IL-12 production (Garside & Mowat 1996). In several infections administration of IL-4 diverts the ensuing response away from Th1 cells towards Th2 subpopulations of T helper effectors (Sadick *et al.* 1990). Anti-IL4 antibodies block the development of Th2 responses and IgE production in a variety of immune responses in mice (Finkelman *et al.* 1990). In those mouse strains that expel the intestinal *Trichuris muris* within the context of a Th2-type immune response, in vivo depletion of IL-4 causes a conversion to the susceptible Th1-type phenotype (Garside & Mowat 1996). Moreover, mutant mice whose IL-4 gene has been disrupted are completely unable to produce IgE and have impaired IgG1 development (Kuhn *et al.* 1991).

In humans, the role of IL-4 in host immune and inflammatory responses is less clear but nonetheless several lines of evidence suggest that it has a similar role to that found in mice. IL-4 is probably involved in allergic disease since its effect on isotype switching implicates it in the generation of the high serum IgE levels observed in atopic individuals (Purkerson & Isakson 1992; Lebman & Coffman 1988). It has been known for quite some time that total IgE levels show evidence of heritability (Gerrard *et al.* 1978; Lee *et al.* 1974). In more recent times, linkage has been described between total serum IgE levels and markers on chromosome 5q31 in the approximate vicinity of the the IL-4 gene (Marsh *et al.* 1994). In addition to its role in allergic conditions, IL-4 is also likely to determine infection outcomes because of its effect on Th1/Th2 differentiation.

It is possible that one of the many factors that may affect Th1/Th2 differentiation and hence influence host susceptibility or resistance to malarial disease may be variable expression of IL-4. Regulatory elements within the IL-4 gene may result in varying levels of expression of the gene. There is now good evidence that the human IL-4 promoter exists in multiple allelic forms and that some of these may lead to overexpression of the IL-4 gene hence amplifying Th2 cell differentiation and Ig class switching to IgE (Song *et al.* 1996). A study on Amish kindreds in Pennsylvania has found linkage between total IgE levels and markers around the Th2 cytokine cluster where the IL-4 gene lies on chromosome 5 (Marsh *et al.* 1994). More recently, a single C to T single base substitution has been identified at the -590 bp of the IL-4 promoter region (Rosenwasser 1995; Borish *et al.* (1995), and luciferase reporter gene transfection experiments indicate that the C to T substitution results in increased Il-4 expression (Rosenwasser 1995) and there is a tentative report that this polymorphism may be linked to IgE levels (Borish *et al.* 1995).

7.4 Th1/Th2 PROFILES IN MALARIA

7.4.1 BACKGROUND

The long time taken for humans in endemic areas to develop immunity to malaria is probably related to the gradual acquisition over several seasons of specific antibody and effector T cell responses (Good 1995). T helper cells, therefore, have a major role in the development of effective cell mediated and humoral malaria immunity. In some rodent models, selection of the appropriate cytokine response appears to be crucial for control of malaria infections (von der Weid & Langhorne 1993). In *Plasmodium chabaudii* infection, Th1 cells are necessary for effective host defence in the initial pre-

erythrocytic stages of primary malaria infection, while Th2 cells are important for control of the later stages of infection and the development of long term immunity (von der Weid *et al.* 1993). Even though Th1 and Th2 effector functions both play a role in parasite clearance there are indications that, in some circumstances, these cytokine profiles may also be associated with malaria immunopathogenesis.

Evidence to support the central role of IL4 in the Th2 response, believed to be important in the development of humoral immunity to malaria, comes from mouse experiments in which T cell clones from T cells taken after clearance of secondary infection have been demonstrated to produce significant amounts of IL-4 (Von der Weid *et al.* 1993; Von der Weid *et al.* 1994). However, pregnant mice, that immunologically tend to be Th2-switched, appear to suffer more severe malaria infections (Wegmann *et al.* 1993).

Therefore with the possibility that Th1/Th2 pattern differentiation might critially affect the host immune response and therefore the clinical outcome of infection in humans, I examined the role in *P. falciparum* malaria cases and controls from The Gambia and Kenya, of the -590 promoter polymorphism that purportedly causes differential expression of the IL-4 gene.

7.4.2 RESULTS

I typed cases and controls collected from a large malaria case-control study conducted in The Gambia (described in chapter 4) for the bi-allelic polymorphism at the -590 position of the IL-4 gene. Typing was performed using standard PCR amplification and

hybridisation with specific olgonucleotides. The results (Table 7.2(i) show that allele 2, which is the less common variant allele in all the caucasian populations studied thus far with a frequency of approximately 15-35% (Rosenwasser *et al.* (1995) Dormy House meeting; A Walley personal communication) is significantly more frequent (60%) in the Gambian population. No difference in the frequency of the three genotypes was observed between mild controls and mild malaria cases. However, in the comparison between mild controls the group that best represents the general population in this case-control study, and severe malaria cases, there were significant differences in the frequencies of heterozygotes and allele 2 homozygotes. Whilst there was an excess of allele 2 homozygotes in severe malaria cases in contrast to the mild controls, there was significantly reduced frequency of heterozygotes in the severe malaria group compared to mild controls (Table 7.2(i) & (ii)). There were no significant differences in the frequencies of allele 1 homozygotes in the two groups.

As with several other association studies of other malaria susceptibility/resistance loci, the significant results were only observed in the comparison of controls with individuals with severe disease and not in individual cases of mild malaria (Hill *et al.* 1990; McGuire *et al.* 1994; Ruwende *et al.* 1995). Interestingly, the severe controls group which consisted of individuals in hospital with other acute non-malarial severe disease had similar genotype distribution as the severe malaria cases. The bulk of these severe controls had an infectious aetiology and included acute respiratory tract infections, bacterial meningitis, diarrhoeal disease and malnutrition.

Tables 7.2(i) Frequencies (%) of genotypes for -590 IL-4 promoter polymorphism in Gambian malaria cases and controls

genotype	mild controls	mild malaria	severe controls	severe malaria cases	cerebral malaria	severe anaemia
	n=324	n=298	n=222	n=361	n=228	n=150
'11'	13.9	10.1	10	8.6	7.5	10
'12'	53.1	56.0	45.9	40.7	39.9	40.7
'22'	33.0	33.9	44.1	50.7	52.6	49.3

Table 7.2 (ii) Statistical comparison of mild controls vs Gambian severe malaria cases

for the '22' genotype

odds ratio = 2.09 [1.51-2.8]

P = 0.00000432

Mantel Haenzel for ethnic group

odds ratio =2.16 [1.57-3.01]

P = 0.0000015

for the '12' genotype

odds ratio = 0.61 [0.44-0.83]

P = 0.0016

Mantel Haenzel for ethnic group

odds ratio = 0.63 [0.46-0.87]

P = 0.004

Table 7.2(iii) Frequencies (%) of genotypes for -590 IL-4 promoter polymorphism in Kenyan severe malaria cases and controls children

genotype	controls	severe cases	cerebral malaria	severe anaemia	controls vs severe malaria odds ratio	P value
	n=122	n=173	n=87	n=86		
'11'	4.1	10.4	12.6	8.1		0.3
'12'	46.7	40.5	41.4	39.6	0.77[0.47-1.27]	0.34
'22'	49.2	49.1	46.0	52.3	1.01 [0.62-1.65]	0.96

A limited analyses of severe malaria cases and controls from the Kenyan case-control study (also described in chapter 4) again showed that, as in The Gambia, allele 2 was the commonest allele. However, unlike the Gambian data, the Kenyan data did not demonstrate increased susceptibility to severe malaria in allele 2 homozygotes (Table 7.b(iii). There was also evidence of heterozygote protection against severe malaria was present in the Kenyan study but this effect did not reach statistical significance.

7.4.3 DISCUSSION

Our relatively crude understanding of the role of Th1/Th2 differentiation in infection clearance and control comes primarily from murine studies of *P. chabaudi* infection. Th1 derived cytokines enhancing cellular immunity are primarily produced in the acute phase of primary infection and as the infection progresses the pattern of cytokine production shifts towards a Th2 like response (Von der Weid *et al.* 1994; Von der Weid *et al.* 1993). However, other mouse malaria models show different Th1 and Th2 disease-risk effects (de Kossodo & Grau 1993; Perlmann *et al.* 1995) and therefore it is difficult to extrapolate with any certainty on the role of Th1/Th2 differentiation on *Plasmodium falciparum* infection clinical outcomes in humans.

TNF-α is produced in excess in severe malaria (Kwiatkowski *et al.* 1990) and there are genetic loci that have been implicated in this phenomenon (McGuire et 1994). However, TNFα is produced by both Th1 and Th2 CD4+ clones in humans (Mossman & Sad 1996) and so it is difficult to infer which particular cytokine profile, if any, may similarly be associated with the development of severe malarial disease. While acknowledging the importance of Th1 responses for cellular immune response in the

early stage of malaria, some researchers still feel that severe malaria occurs as a consequence of immunopathogenesis within the context of a Th1 cytokine profile (Von der Weid *et al.* 1994).

Viewed together with the transfection results by Rosenwasser's group showing higher IL-4 expression in allele 2, the data from our comparison of severe cases and mild controls for this IL-4 promoter polymorphism suggest that a Th2 type response in early infection may also lead to severe malaria. There are several lines of evidence that support this view. As mentioned earlier, the Th2 shift that occurs in pregnancy is known to exacerbate acute malaria (Wegmann *et al* 1993). Secondly, work by Perlmann and colleagues (1994) demonstrated an association of total serum IgE levels with cerebral malaria but not with uncomplicated mild malaria. Therefore, if Il-4 is the most important locus that determines total IgE levels and the allele 2 does indeed upregulate Il-4 expression (Rosenwasser 1995), then these results concur with the results of Perlmann in implicating Th2 responses in the pathogenesis of severe malaria cases.

Interestingly, preliminary results from another study of Gambian children did not demonstrate raised total IgE antibodies in cases of cerebral malaria (Faux *et al.* unpublished data). However, these cases were bled several months after the acute malaria episodes and this may be inappropriate if *in vivo* Th1/Th2 differentiation is not temporally stable for that extended a time period.

The observation from our Gambian results that heterozygotes for the promoter polymorphism which presumably have intermediate levels of IL-4 expression, are associated with protection from severe malaria raises the possibility that this may be a balanced polymorphism and indeed, data from the Kenyan case control study (Table 7.2iii) show a similar trend although they do not reach statistical significance.

Benefits conferred by elevated high Il-4 expression associated with allele 2 may include greater fecundity as the resultant Th2 bias which is known to enhance successful pregnancy, and which presumably would be a consequence of high Il-4 expression. In addition, Th2-switched individuals may have greater resistance towards those predominantly extra-cellular pathogens such as encapsulated bacterial (e.g *Salmonella*, *Haemophilus*, *Meningococcus*) and helminthic infections that are best cleared by a Th2 biased cytokine profile. The disadvantages of a Th2 bias that may occur as a result of high IL-4 expression, may be an increased susceptibility to more severe disease outcomes in intracellular infections such as malaria.

Given the high prevalence of extracellular pathogens in most parts of sub-Saharan Africa and the fact that long term immunity to what is unequivocally the largest selective force, malaria, may depend on Th2 dependent specific humoral immunity, it is not inconceivable that the higher frequency of allele 2 and the under-representation of heterozygotes in both severe malaria cases and in the severe disease group in general may reflect a balanced polymorphism. For an infection such as malaria with different phases (pre-erythrocytic and erythrocytic) it is likely that both Th1 and Th2 immune responses are required for protective immunity. Therefore, the heterozygote genotype

associated with intermediate IL-4 expression and therefore which, presumably, would not tilt the immune response to either of the Th1 or Th2 extremes, might be best suited to effectively deal with such a complex infection.

In recent months, other allelic nucleotides have been identified in the promoter, particularly at positions -81 and -285 positions, that cause overexpression of the IL-4 gene (Song *et al.* 1996). In view of the discordant results for the allele 2 homozygotes in the Gambian and Kenyan studies, it seeems likely that allele 2 of the -590 IL-4 promoter polymorphism may not be a major IL-4 transcription upregulatory locus. It is possible that, in The Gambia, allele 2 may be in linkage disequilibrium with another upregulatory polymorphism amongst these recently described promoter variants whereas in Kenya it is not in such linkage disequilibrium.

7.5 Th1/Th2 PROFILES IN HEPATITIS B VIRUS INFECTION

7.5.1 BACKGROUND

Several serological studies have suggested that the Th1/Th2 balance may be relevant in acute and chronic hepatitis B virus infection (Ferrari *et al.* 1990; Maruyama *et al.* 1993; Maruyama *et al.* 1994). Since hepatitis B serological test results were available from the children in the Gambian malaria case-control study that had already been typed for the -590 Il-4 promoter polymorphism as discussed above, an attempt was made to assess the role, if any, of this polymorphism on hepatitis B infection and carriage. This was also particularly relevant as it was known that chronic hepatitis B infection was associated with an increased risk of severe malaria in The Gambia (Thurz

et al. 1995) and this study had already demonstrated that allele 2 homozygotes for the -590 IL-4 promoter polymorphism were also associated with the same condition.

7.5.2 RESULTS

The results show that although there are no significant differences in the frequencies of the various -590 IL-4 genotypes for either clearance or persistent hepatitis B surface antigen carriage (Table 7.3(i), allele 2 homozygotes are at increased risk of getting HBV infected, (odds ratio 1.33 [1.00-1.74], P=0.038) (Table 7.3(ii.). Interestingly, as for severe malaria, heterozygotes are associated with a reduced risk of infection (odds ratio 0.72 [0.55-0.95] P=0.018). In the overall analysis, the average age of the non-infected children is lower than that of the infected children and therefore this group may not have been as exposed to hepatitis B infection as their older infected counterparts. However, even when only children above 2 years of age are considered, there is still significant over-representation of allele 2 homozygotes and under-representation of heterozygotes in the infected group (Table 7.3(iii). Stratification for malaria status showed that the effect of increased susceptibility to infection of allele 2 homozygotes in the severe malaria group (Table 7.3(iv) although this result did not reach statistical significance .

7.5.3 DISCUSSION

There are several observations from serological studies of hepatitis B virus (HBV) infection that indicate that the different Th subsets are important host factors that may play a role determining the outcome of hepatitis B virus infection. In one study, 50% of asymptomatic chronic HBV infected patients demonstrated antibody production in

Table 7.3 (i) Frequencies of genotypes for -590 IL-4 promoter polymorphism in Gambian children for persistent Hepatitis B virus carriage

genotype	cleared infection n=180	persistent carriers n=185	odds ratio	P value
'11'	11.1	11.4		ns
'12'	46.1	40.5		ns
'22'	42.8	48.1		ns

Table 7.3(ii) Frequencies of genotypes for -590 IL-4 promoter polymorphism in Gambian children for Hepatitis B virus infection

genotype	uninfected n=618	infected n=365	odds ratio	P value
'11'	10.2	11.2		
'12'	51.3	43.3	0.72 [0.55-0.95]	0.018
'22'	38.5	45.5	1.33 [1.02-1.75]	0.038
	Mantel Haenzel for ethnic group		1.32 [1.00-1.74]	0.05

Table 7.3(iii) Frequencies of genotypes for -590 IL-4 promoter polymorphism in Gambian children over 2 years for Hepatitis B virus infection

genotype	uninfected n=327	infected n=284	odds ratio	P value
'11'	9.5	10.9		
'12'	52.6	43.0	0.67 [0.48-0.94]	0.018
'22'	37.9	46.1	1.33 [1.02-1.75]	0.049

Table 7.3(iv) Frequencies (%) of genotypes for -590 IL-4 promoter polymorphism in Gambian children for Hepatitis B virus infection when stratified for malaria status. There were no significant differences between the HBV groups within either the malaria case or malaria control group but there was a clear over-representation of allele 2 homozygotes and and an under-representation of heterozygotes in the severe malaria group compared to the controls.

	SEVERE MALARIA			**MILD CONTROLS**		
genotype	**uninfected n=160**	**infected n=134**	***P value***	**uninfected n=59**	**infected n=158**	***P value***
'11'	7.5	9.0	0.9	20.3	12.7	0.23
'12'	44.4	34.3	0.15	50.9	55.1	0.69
'22'	48.1	56.7	0.18	28.8	32.2	0.75

the absence of immune-mediated liver disease (Maruyama *et al.*1993) suggesting that these individuals are Th2 switched immunologically. There is evidence that hepatitis B virus chronically infected individuals exhibit Th-2 associated greater antibody responses than those individuals with Th1-type cellular immune responses, who experience acute self-limited hepatitis B virus infection (Maruyama *et al.* 1994; Ferrari e*t al.*1990). Furthermore, it has been shown in mouse experiments that there is epitope-MHC dependent differential recognition of hepatitis B nucleocapsid antigens by Th1 and Th2 cells.

While our data do not reveal any associations between the -590 promoter genotypes with HBV carriage, they suggest, if the previously described expression data on this polymorphism by Rosenwasser are valid, that whilst Th2-type immune responses may cause increased susceptibility to infection, mixed Th1/Th2 responses are associated with resistance to infection. Most data on infectious diseases implicate a critical role for either the Th1 or Th2 subset in the clinical course of infections (reviewed Mossman & Sad 1996). This finding differs with these other studies in that it suggests that the cytokine profile may critically determine not just the clinical course of an infection but, perhaps more importantly, the initial establishment of infection.

The IL-4 results for HBV infected and non-infected individuals after stratification for malaria status suggest that children who have had HBV infection may be at a greater risk for severe malaria. However, although the results do not reach statistical significance, they might be providing yet another small indication that the immune response to, and hence the clinical outcome of, any given infection may be governed by

the effects of other interactions, past or concurrent, between the host and other pathogens.

7.6 Th1/Th2 PROFILES IN TUBERCULOSIS

7.6.1 BACKGROUND Th1/Th2 IN TUBERCULOSIS

The development of acquired to immunity to mycobacterial infections is thought to depend primarily on an interplay between macrophage and T cell function with macrophages as the principal effectors and T lymphocytes as the prime inducers of acquired resistance (Barnes *et al.* 1994). *In vitro* studies show that, as with mice, CD4+ Tcell clones stably differentiate into functional Th1 or Th2 patterns after stimulation with purified protein derivative (PPD) antigen from *M. tuberculosis* (Del-Prete *et al.* 1991).

Th1/Th2 differentiation in host immune responses to mycobacterial infection has been extensively studied particularly with regard to its potential role as a determinant of variable susceptibility or resistance to another mycobacterial disease leprosy. Leprosy has immunologically and clinically distinct disease phenotypes ranging from tuberculoid leprosy at one end of the spectrum to lepromatous leprosy at the other end. There are numerous conflicting reports in the literature on this issue but it is still possible to arrive at some convincing conclusions. Patients with tuberculoid leprosy mount a strong T cell response to *M. leprae* and are able to restrict growth of the organism while lepromatous patients manifest disseminated disease with their T cells responding weakly to *M. leprae* (Sieling *et al.* 1994). Quantitively, antibody responses are highest at the lepromatous end of the spectrum and decreased towards the

tuberculoid pole (Hussain & Kifayet 1995). In both mice and humans, CD4+ T cells isolated from tuberculoid lesions secrete predominantly Th1 type cytokines such as IFNγ, IL-2 and IL-12 whereas lepromatous lesions appear to have IL-4 producing T cells (Sieling *et al.* 1994). Lymphocyes from DTH lesions in both leprosy and tuberculosis secrete large quantities of Th1 type cytokines especially IFN-γ (Barnes *et al.* 1993; Barnes *et al.* 1994). Moreover, administration of the Th1 cytokine IL-12 appears to confer protection against *M.tb* infection in mice (Flynn & Bloom 1994).

The early phase of acquired immunity to *Mycobacterium tuberculosis* infection seems to be mediated by the emergence of protective IFNγ secreting CD4 T lymphocytes (Cooper *et al.* 1995). In mice, adoptive transfer of protection against *M.tb* and BCG infection depends on transfer of the same Th1-associated subset of selected CD4 cells (Orme 1987; Orme & Collins 1984). Indeed, in mice IFNγ, a Th1 cytokine, appears to be central to the development of resistance to tuberculosis probably by increasing the production of reactive nitric oxide metabolites (Flesch & Kaufmann 1990).

Tuberculous pleurisy is believed to provide a model of protective anti-tuberculous immunity since this condition usually resolves itself when left untreated (Barnes *et al* 1994). In pleurisy, there is a significant excess of the Th1 cytokines IFNγ and IL-12 in the pleural fluid compared to serum. Although IL-10 is also raised in pleural fluid the other Th2 cytokines such as IL-4 and IL-5 are undetectable (Barnes *et al.* 1993). In addition, natural killer (NK)cells, which probably have a role in cell-mediated host resistance to *Mycobacterial tuberculosis* infection, are known to secrete Th1 type cytokines (Barnes *et al.* 1994).

There certainly seems to be enough evidence to support the notion that in tubercle infection, a Th1 host response such in DTH is protective, a mixed Th1Th2 response leads to progression to pulmonary tuberculosis, whilst a Th2 response with poor Th1 DTH response, gives rise to disseminated disease such as miliary tuberculosis (Barnes *et al.* 1994). Surcel and colleagues (1994) have demonstrated significantly increased numbers of IL-4 secreting cells in patients with active tuberculosis compared with sensitised healthy controls. There are also several reports in the literature that report the finding of hypergammaglobinaemia and elevated levels of the Th2-associated IgE isotype in patients with active tuberculosis (Papiha *et al.* 1987 ; Gatner *et al.* 1982). In mice, the Th1/Th2 profile has important consequences in intestinal helminth infections and generally Th2 responses lead to clearance of the infection. Interestingly, a study by Grange *et al.* (1984) showed some evidence of a higher incidence of helminth infections in tuberculosis patients compared to controls.

The data may not not be conclusive but there certainly is enough evidence that the Th1/Th2 cytokine profiles may be relevant *M tuberculosis* infection outcomes.

7.6.2 RESULTS

Our results (Table 7.4) did not show any differences in the frequencies genotypes for the IL-4 promoter polymorphism between the pulmonary tuberculosis cases and controls.

7.6.3 DISCUSSION

In addition to the evidence described earlier on the role of Th1/Th2 cytokines in

Table 7.4 Frequencies of genotypes for -590 IL-4 promoter polymorphism in Gambian controls and pulmonary tuberculosis cases.

genotype	**controls n=239**	**cases n=216**	**odds ratio**	**P value**
'11'	10.0	11.6		ns
'12'	51.0	48.1		ns
'22'	39.0	40.3		ns

tuberculosis disease risk, there are also several studies that have reported raised levels of both total and specific serum IgE levels in tuberculosis (Young *et al.* 1989; Papiha *et al.* 1985; Greinert *et al.* 1990). It has been suggested that IgE levels may be important parameters for the prognosis and course of the disease as low serum levels are found in patients with minimal radiographic TB lesions while individuals with radiographic evidence of advanced lung pathology, miliary or extrapulmonary disease have higher IgE levels (Etz & Schmidt 1987). There is evidence that indicates IL-4 may be the major locus that controls IgE levels (Marsh *et al.* 1994; Borish *et al.* 1995; Song *et al.* 1996). Moeover, *in vitro* data from by Surcel *et al.* (1994) looking at the proliferation and cytokine production responses of blood mononuclear cells stimulated with mycobacterial antigens in TB patients and sensitised healthy controls also implicates raised IL-4 production in the pathogenesis of tuberculosis.

Our data do not show any association with any of the -590 IL-4 promoter genotypes.There are several possible interpretations of these data. The first is that our extrapolation that the differentially expressive alleles for this IL-4 promoter polymorphism do indeed affect the Th1/Th2 T cell differentiation may be incorrect. Clearly further work is needed to clarify this issue. Secondly, if we are correct in our assumption that allele 2 carrying individuals are relatively more Th2 switched compared to allele 1 carriers, it may well be the case that this differentiation does not have any effect on the development of pulmonary tuberculosis disease. Alternatively, differential IL-4 expression and therefore Th1/Th2 differentiation may indeed affect disease outcome following tubercle infection but that this IL-4 promoter locus may be just one of numerous factors that interactively affect the IL-4 production and therefore

the overall cytokine profile. Thus a relatively small contribution to the overall genetic component that governs disease risk may make the detection of the association with the given locus extremely difficult.

Another possible confounding factor in our analysis may be possible heterogeneity within these cases of pulmonary tuberculosis. Unlike leprosy whose distinctive signs lend themselves towards relatively easy phenotypic classification, pulmonary tuberculosis is different. It is possible that our group of sputum positive pulmonary tuberculosis cases may immunologically and pathophysiologically be a heterogenous group. If any effects of the IL-4 promoter polymorphism on Th cell differentiation and hence variable disease outcome, is specific to restricted disease processes, analysis of the group as a single entity would lower the chances of detecting associations.

Nevertheless, we can conclude that whatever its functional role in IL-4 transcription levels and Th cell differentiation, the -590 IL-4 promoter polymorphism does not appear to have any direct major effect on disease outcome in clinical pulmonary tuberculosis in The Gambia.

CHAPTER 8 - CONCLUDING REMARKS

The identification of genetic variants that contribute to susceptibility to multifactorial diseases such as infectious diseases has important biological and public health consequences. Firstly, it provides insights into the basic pathophysiological processes that underly such diseases thereby unravelling important information on the immunological aspects, the biochemical pathways and the structural biology of implicated protein variants in various diseases. In addition, genetic studies also enhance our understanding of protective immunity as well as providing us with targets for novel therapeutic interventions ranging from chemotherapy, adjunctive or supplementary therapy, vaccine design or even gene therapy. From a public health perspective, genetic studies provide us with an opportunity to identify high risk individuals who, once detected by population screening programmes, may benefit from a variety of prophylactic measures such as immunisation, prophylactic chemotherapy or behaviour modification. Knowledge of the role of genetic variants in diseased individuals may also aid medical management decisons in diseased individuals by clarifying prognoses and providing information on optimal treatment options. In a wider scientific context, genetic studies are of great interest because they contribute to our knowledge of the aetiology of complex diseases as well as enhancing our understanding of evolutionary processes. In the event that, rather than identify susceptibility or resistance genes, these studies concretely establish the absence of genetic contribution to disease risk, environmental factors affecting the development of the disease can then be pinpointed and individuals at risk thus given the opportunity to modify their exposure to these environmental risk factors.

The Gambian and Kenyan case-control studies on the relationship between G6PD deficiency and malaria are now the largest field studies that have examined this controversial question and the findings provide clear evidence of the protective role for this disorder against severe malaria. The results provide strong evidence that all G6PD deficiency genotypes are probably protected against severe disease and to a less extent against mild disease. This is of great significance for it provides a basis for establishing the likely mechanism of disease. Our results suggest that G6PD enzyme activity levels are central to the mechanism of protection and that, despite the presence of parasite G6PD, oxidative stress in deficient erythrocytes probably impairs growth of the parasite within the red blood cell. *In vitro* studies have demonstrated reduced growth of *P. falciparum* in G6PD-deficient red cells particularly at high oxygen tensions. In addition, there is evidence that indicates that there may be more efficient erythrophagocytosis of infected enzyme-deficient red cells by macrophages.

Our findings also have important evolutionary implications on our understanding of the relationship between G6PD deficiency and malaria, especially in the light of existing knowledge on evolutionary significance of other malaria resistance genes such as sickle haemoglobin and HLA-B53. Not only do the results establish the existence of protection for the various enzyme-deficient genotypes, they also provide the first estimate of the amount of protection conferred by this disorder. Protection against severe malaria in both male hemizygotes and female heterozygotes means that deficiency alleles can eventually reach fixation under natural selection. Given the amount of protection (46-58%) that we have identified in our study, one can make estimates of the time required to reach fixation. For the levels of protection we

observed for the G6PD A- allele that we investigated, it would take 2,000-3,000 years for the allele to reach fixation under this degree of positive selection. Since it is known from data on archaeology, population, history and human genetics that malaria has been a selection factor in Africa in sub-Saharan Africa for at least 5,000-7,000 years, one must conclude that although G6PD deficiency is now a relatively benign clinical disorder, in the past it may have been selectively much more disadvantageous.

Clearly, it is necessary for our results to be reproduced both for different populations in malaria-endemic areas and also for the numerous other known G6PD deficiency variants. Data on the the role of enzyme-deficient female homozygotes in severe malaria are still needed as this study had too few of this group of individuals for any meaningful analysis. It will also be interesting to follow up the work of Kar and colleagues and examine the role of G6PD deficiency in *P .vivax* infections. Furthermore, *in vitro* work examining the role of G6PD deficiency on important host-parasite phenomena such as sequestration, rosetting and macrophage release of key cytokines such as TNF, IL-4, IFN-γ in response to parasite infection is also needed. With knowledge derived from *in vitro* studies on the precise biochemical pathways with which oxidative stress in deficient erythrocytes interferes with parasite growth, novel immunotherapeutic and or chemotherapeutic agents might be designed which better target essential enzymes and cofactors in the parasite growth process within the infected erythrocyte.

This study also identifies non-HLA genes that influence the clinical outcome of *M. tuberculosis* infection. Logistic regression indicates that the disease risks associated

with either of the susceptibility genes remain significant after stratification for possible confounding variables. Individuals who possess susceptibility genotypes for both MBP and the TNF promoter are at an increased risk for pulmonary tuberculosis although, on account of the small numbers, there is no clear evidence that this is due to a multiplicative or additive process. It is clearly necessary to establish the effects of these genes on susceptibility in different populations. Interestingly, both the -238 TNF polymorphism and particularly the MBP deficiency alleles are relatively common in Africans compared to other population groups, so that these results may in part account for the apparent increased susceptibility of blacks to tuberculosis. It is believed that *M. tuberculosis* has been in Africa for only a century (Diamond, 1992) and therefore it is unlikely that this infection has been a major selective force responsible for current frequencies of these variants. There are likely to be other diseases, infectious or non-infectious or both, that are responsible for the observed high frequencies and it will be both interesting and essential to identify these diseases before we consider interventions that counter the effects that thousands of years of evolution have enforced on our genome as humans.

Furthermore, more work on the pathophysiological manifestations and interactions at the cellular level of the various disease-associated genotypes is essential. In this regard, *in vitro* studies on the function of macrophage constructs of these variants and specifically the cells' response to either *M. tuberculosis* infection or stimulation with the latter's antigens, will be enlightening as will be the results of on-going functional studies on the effect on TNF-α expression of the -238 promoter polymorphism.

These markers, the -238 TNF-α promoter variant and homozygotes for the MBP structural variants, may have future applications in the identification of high risk individuals either for specific prophylaxis against tuberculosis or for adjunctive therapy to supplement antituberculosis chemotherapy in an era of increasing mycobacterial drug resistance. The TNF association suggests that the use of anti-TNF agents may be valuable in the therapy of tuberculosis and several agents such as pentoxyifyline, thalidomide and a new generation of TNF converting enzyme inhibitors may be valuable (McGeehan et al., 1994; Mohler et al., 1994; Gearing et al., 1994; Zabel & Schade 1993). Of these thalidomide has already been shown to be of use in cases of tuberculosis (Tramontana et al., 1995). MBP replacement therapy has been contemplated for MBP-deficient individuals (Chang et al., 1994) and it is possible that MBP supplementation may become feasible for high-risk groups particularly in countries with high tuberculosis prevalence rates. Such high-risk groups would include immunocompromised or otherwise vulnerable individuals such as HIV infected cases, young infants, the elderly as well as those individuals that screening programmes would have been identified as genetically MBP deficient.

However, before all above-described interventions against tuberculosis are contemplated, it is imperative that researchers examine the relevance to other diseases of any gene variants such as TNF and MBP that may alter suceptibility to tuberculosis. It is possible that for some genes, the variants may be balanced polymorphisms maintained through differential selection by several diseases. There is some evidence that the high TNF levels are associated with the putatively upregulatory -238 and -308 TNF promoter variants, may be protective against viral infections (Wong & Goeddel

1986; Mestan *et al.* 1986; van Strijp *et al.,* 1991). Similarly, it is possible that MBP deficiency may lower susceptibility to other intracellular pathogens that use MBP opsonisation to gain cell entry. In addition, since MBP can facilitate activation of the classical complement pathway, MBP deficiency may also confer protection against the complement-mediated tissue damage that is observed in various autoimmune syndromes.

Interestingly, the HLA-DR2 association with susceptibility to tuberculosis has only been described in Asia. We did not find the same association in The Gambia and this may be due to a variety of reasons perhaps the simplest being that this study was not large enough. It is not inconceivable that this difference in HLA-DR2 susceptibility may be attributable to variation in the bacterial strain in the two populations. It is also possible that inter-population variation in HLA-DR2 susceptibility may reflect differences in the frequencies of the various HLA-DR2 subtypes. In Asia, it is the DR15 subtypes of HLA DR2 that are associated with susceptibility to tuberculosis and leprosy (Bothamley & Schreuder 1992; *Rani et al.* 1993; Bothamley *et al.* 1995). HLA-DR2 15*01 and 15*02 are the commonest HLA-DR2 subtype in Asia (Rani *et al.* 1993). Preliminary analysis of DR2 in our Gambian adult controls, indicates that there are two major HLA-DR2 RFLP subtypes, DR15 (53.3%) and DR16 (46.7%) and it will be useful to sequence these these to establish whether they represent similar or different subtypes to those found in India.

At present, there is still much about the various host, parasite and other environmental factors that influence invasion, infection and the various forms of disease in

tuberculosis that we do not know about. There is a great need for us to improve our understanding of pathogenesis and protective immunity in tuberculosis. More specifically, the *M. tuberculosis* antigens that evoke either tissue-damaging or tissue-protective processes in the host need to be identified. In addition, it is essential for us to pinpoint the host factors that govern the responses to specific *M. tuberculosis* antigens that may be implicated in protection or disease pathogenesis. An example, would be the effect of both HLA and non-HLA genes on Th1/Th2 differentiation in response to specific *M.tb* antigens. Data from Bothamley *et al.* have already demonstrated that sera from the susceptible DR2 individuals appear to have significantly increased levels of antibodies against specific peptides within a 38kd *M. tuberculosis* antigen (Bothamley *et al.* 1989). It may be that the 38kd *M. tuberculosis* antigen may selectively stimulate Th2 T cell subsets which in turn would promote a vigorous but ineffective antibody response and downregulate a potentially protective Th1 response.

This study has studied the role of some genetic factors in sputum positive clinical cases of predominantly post-primary pulmonary tuberculosis. With the use of ever-improving molecular epidemiological techniques, it may be possible in the future to separate cases of reactivation and reinfection post-primary tuberculosis and to thus establish whether or not the genetic factors that influence these two processes are the same. It is also clearly necessary to examine the role of identified genetic factors in other types of tuberculosis such as primary tuberculosis and the various forms of extra-pulmonary tuberculosis, and tuberculosis in HIV infected individuals. It may also be interesting to

examine the role of the identified genetic variants on skin-test and *in vitro* cellular responsiveness to various *M. tuberculosis* antigens.

As in the past, animal models continue to be extremely useful to geneticists seeking to elucidate the possible molecular mechanisms or pathways that underly various diseases. Unlike in humans, the opportunity with rodent models to alter specific genes and use experimental crosses to create genetically distinct inbred strains that differ in their susceptibility to specific pathogens permits genetic analysis of the complex manner in which multiple genes regulate resistance to various infectious diseases. However, it is always necessary to assess the appropriateness of a given animal model to the infectious disease of interest in humans. At present, there are no good animal models for cerebral malaria in humans. Furthermore, it is likely that while there may be reasonable animal models for primary tuberculosis in humans, there are no satisfactory models for post-primary tuberculosis in humans. It is therefore interesting to speculate as to whether use of the mouse model, for instance, would have identified the genes, TNF and MBP, that these studies have shown to influence susceptibility to predominantly post-primary tuberculosis. This is also relevant to this study's finding that suggests that, in humans, *NRAMP1* may not influence tuberculosis susceptibility as has been demonstrated in the mouse model.

Host resistance to infection is clearly a complex process governed by numerous genes. The more work that is done on the role of host genetic factors in infectious diseases, the more we realise how crude our current understanding is of the genetic heterogeneity and genetic interactions in the pathophysiology of diseases. For malaria,

several such genes have already been identified but it is possible that there may still be a few more. In contrast, for tuberculosis, we only just beginning to unravel susceptibility genes and it is likely that there are several more genes which alter tuberculosis resistance that are yet to be identified. Fortunately, the availability of a wide range of genetic approaches provides us with the tools to systematically dissect out these genes for these complex traits. These studies on both malaria and tuberculosis support the use of a candidate gene approach to investigate genetic susceptibility to infectious diseases in humans and indicate that, as for malaria, variable susceptibility to tuberculosis in humans may also involve a large number of host genes.

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