

**MURINE AND HUMAN ANTIBODY RESPONSES TO *PLASMODIUM*
FALCIPARUM MEROZOITE SURFACE PROTEIN-1**

Richard Maurice Jennings

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This work is dedicated
to the memory of

Jon Chappel
1968 – 1999

An excellent scientist
and an irreplaceable friend

ABBREVIATIONS

ABC	Ammonium bicarbonate
ACN	Acetonitrile
AMP	Ampicillin
BSA	Bovine serum albumin
CDR	Complementarity-determining region
DEPC	Diethylpyrocarbonate
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetracetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunoadsorbant assay
g	Acceleration due to gravity
GPI	Glycosyl phosphatidylinositol
GST	Glutathione S-transferase
HRP	Horseradish peroxidase
IFA	Indirect immunofluorescence assay
IgG	Immunoglobulin G
IPTG	Isopropyl-B-D-thiogalactopyranoside
kDa	Kilodalton
mAb	Monoclonal antibody
MSP-1	Merozoite surface protein-1
MSP-1 ₄₂	42 kDa primary processing fragment of MSP-1
MSP-1 ₁₉	19 kDa secondary processing fragment of MSP-1 ₄₂
MSP-1 ₃₃	33 kDa secondary processing fragment of MSP-1 ₄₂

NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PBML	Peripheral blood mononuclear lymphocyte
PBS	Phosphate buffered saline
<i>P.</i>	<i>Plasmodium</i>
PCR	Polymerase chain reaction
PMSF	Phenyl methyl sulphonyl fluoride
rpm	Revolutions per minute
scFv	Single-chain variable fragment
SDS	Sodium dodecyl sulphate
TLCK	N- α -p-tosyl-L-lysine chloromethyl ketone
V _H	Variable region of the immunoglobulin heavy chain
V _L	Variable region of the immunoglobulin light chain
V _{κ}	Variable region of the immunoglobulin (light) kappa chain
V _{λ}	Variable region of the immunoglobulin (light) lambda chain
v/v	Volume per unit volume
w/v	Weight per unit volume

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ABSTRACT

A major surface protein complex on the *Plasmodium falciparum* merozoite, Merozoite Surface Protein-1 (MSP-1) undergoes a proteolytic cleavage at the time of erythrocyte invasion by the parasite. Two murine monoclonal antibodies, mAb 12.8 and mAb 12.10, are specific for a 19 kDa subunit of MSP-1 (MSP-1₁₉) and can prevent both this proteolytic cleavage and erythrocyte invasion. Such invasion-inhibitory mAbs may be generated by natural *P. falciparum* infection in man and may contribute to protection against malaria.

To isolate naturally occurring human anti-MSP-1₁₉ monoclonal antibodies, two phage display libraries were constructed, consisting of single chain Fv antibody fragments (scFvs) derived from malaria-exposed donors in The Gambia, West Africa. The libraries were panned with recombinant MSP-1₁₉ and a number of anti-MSP-1₁₉ scFvs were identified. Their ability to bind recombinant MSP-1₁₉ and native parasite antigen was demonstrated and their sequences were found to be diverse. The anti-MSP-1₁₉ scFv were shown to partially block the binding of mAbs 12.8 and 12.10 to MSP-1₁₉ but none of the scFvs isolated could be shown to inhibit erythrocyte invasion *in vitro*.

In order to further characterise the invasion-inhibitory murine hybridoma mAbs 12.8 and 12.10, the DNA sequences encoding the variable, antigen-binding, regions of these mAbs were obtained and confirmed by mass spectrometry of the antibodies themselves. The mAbs 12.8 and 12.10 were reconfigured into scFv format to serve as a future model in which to test ways of restoring invasion-inhibitory activity to scFvs. The scFv 12.10 was shown to bind its target antigen but could not be shown to inhibit erythrocyte invasion *in vitro*. Preliminary experiments were performed to examine ways in

which any invasion-inhibitory properties of the parent mAbs might be restored to the human and murine scFvs.

Chimeric murine/human versions of mAbs 12.8 and 12.10, engineered from the mAb sequences obtained, were shown to bind recombinant and native antigen, and were also shown to inhibit erythrocyte invasion *in vitro*.

In the future, the chimeric mAbs will be suitable reagents to test the ability of invasion-inhibitory mAbs to modify *P. falciparum* infection in a primate model, and the phage display libraries should provide a resource from which naturally occurring human invasion-inhibitory mAbs can be isolated.

CHAPTER 1

INTRODUCTION

1.1 Malaria

Malaria is a parasitic disease of man and other animals caused by apicomplexan protozoa of the genus *Plasmodium*. Of the four species of *Plasmodium* to infect man, *Plasmodium falciparum* is the cause of the great majority of morbidity and mortality due to malaria. The worldwide public health burden due to *P. falciparum* is very great (Breman, Egan et al. 2001). In many parts of the world that burden is increasing, both because of spread of the parasite into populations not previously exposed to malaria, and because of increasing drug resistance. There is an urgent need for new and affordable treatments and an effective vaccine. The fact that exposed populations develop protective, if not sterilising, immunity to malaria encourages the belief that an effective malaria vaccine will eventually be an achievable goal.

The malaria life cycle consists of a sexual stage in the anopheline mosquito and an asexual stage in man, with transmission occurring when the mosquito feeds on man. Clinical illness in man is caused by parasites in the blood stage of the life cycle. This stage is characterised by the invasion of host red blood cells by the *P. falciparum* merozoite, followed by intra-erythrocytic parasite growth and asexual replication to form a schizont, whose rupture releases many more merozoites into the plasma to continue the cycle. Most antimalarial drugs act exclusively on parasites in this blood stage of the life cycle. Vaccines have been proposed against other stages of the life cycle, but the blood stage is

widely considered to offer a promising target for a vaccine. This is particularly true for vaccines which aim to produce protection mediated by antibody, since the parasite is more vulnerable to antibody attack during the blood stage of the life cycle, when parasite antigens are exposed for considerable periods in the bloodstream.

Various parasite antigens expressed during the blood stage have been proposed as vaccine candidates, but an antigen which is considered particularly promising is the merozoite antigen, Merozoite Surface Protein 1 (MSP-1).

1.2 Merozoite Surface Protein 1 (MSP-1) and erythrocyte invasion

1.2.1 Merozoite Surface Protein 1 and secondary processing

Merozoite Surface Protein 1 (MSP-1) is present on the surface of the merozoite as a complex of polypeptides formed by the proteolytic cleavage, or primary processing, of the precursor protein. The complex is anchored to the merozoite by a 42 kDa fragment attached to the membrane by a GPI anchor. At the time of erythrocyte invasion, the 42 kDa fragment undergoes proteolytic cleavage, leaving a 19 kDa fragment (MSP-1₁₉) attached to the merozoite, which is carried into the erythrocyte as the parasite invades (Blackman, Heidrich et al. 1990). This proteolytic cleavage of MSP-1 is mediated by a parasite-derived protease, and is known as secondary processing. Secondary processing appears to be essential for successful erythrocyte invasion and is vulnerable to interruption by MSP-1₁₉-specific antibodies. Of a group of mAbs specific for epitopes within MSP-1₁₉, some have been shown to inhibit erythrocyte invasion, and these mAbs also

prevent secondary processing of MSP-1 (Blackman, Scott-Finnigan et al. 1994). The primary and secondary processing of MSP-1 are shown schematically in Figure 1.1, and the relationship between secondary processing and erythrocyte invasion is shown in Figure 1.3.

The solution structure of *P. falciparum* MSP-1₁₉ has been determined by nuclear magnetic resonance (Morgan, Birdsall et al. 1999) and the crystal structure of the homologous molecule from *P. cynomolgi* has also been determined (Chitarra, Holm et al. 1999). Both studies confirm that the protein structure has two domains with epidermal growth factor (EGF)-like folds. The protein has a U-shaped overall structure with the N-terminal proteolytic processing site close to the C-terminal GPI membrane anchor site, consistent with the involvement of a membrane bound protease in secondary processing. The structure of MSP-1₁₉ is illustrated in Figure 1.2

Many *P. falciparum* antigens display a high degree of sequence diversity between different parasite strains. The primary sequence of the MSP-1 precursor can be divided into 17 blocks. Blocks 15 and 16 comprise the N-terminus of MSP-1₄₂, and there are two major sequence types with about 47% identity. Block 17 corresponds to MSP-1₁₉, and the sequence is highly conserved (Miller, Roberts et al. 1993) (Jongwutiwes, Tanabe et al. 1993) with less than 5% variation in different alleles (Kang and Long 1995). Because of its role in erythrocyte invasion and its high degree of sequence conservation, MSP-1₁₉ is regarded as a leading candidate in a malaria vaccine.

1.2.2 The invasion-inhibitory anti-MSP-1₁₉ mAbs 12.8 and 12.10

In 1985 the results were published of a series of fusion experiments carried out to generate mAb-expressing hybridomas from the spleen cells of mice which had been immunised with crude *P. falciparum* schizont extract (McBride, Newbold et al. 1985). Two of the hybridomas, identified as 12.8 and 12.10, were found to secrete mAbs which recognise the 42 kDa component of MSP-1 (MSP-1₄₂) present on the surface of merozoites before erythrocyte invasion. The same mAbs also recognise the 19 kDa fragment of the MSP-1₄₂ which is produced by the secondary proteolytic processing of MSP-1 and which remains on the merozoite surface and is carried into the newly infected erythrocyte (Blackman, Heidrich et al. 1990). The mAbs 12.8 and 12.10 were shown to inhibit merozoite invasion of erythrocytes *in vitro*. This inhibitory action required high a concentration of mAb. At a concentration of 500 µg/ml, mAb 12.10 inhibited invasion by the T9/94 strain of *P. falciparum* by 72% and inhibited invasion by the T9/96 strain by 50% (Blackman, Heidrich et al. 1990). It was subsequently shown that of a group of mAbs specific for MSP-1₁₉, the two which inhibit erythrocyte invasion, mAb 12.8 and mAb 12.10, also inhibit the secondary processing of MSP-1 in a Western blot-based *in vitro* assay (Blackman, Scott-Finnigan et al. 1994). In the same study, other MSP-1₁₉-binding mAbs were identified which do not inhibit secondary processing or erythrocyte invasion, but which block this inhibition by mAbs 12.8 and 12.10.

These results suggest that the proteolytic cleavage or secondary processing of MSP-1 is necessary for erythrocyte invasion to proceed. The results also indicate that binding of different antibodies to adjacent but different epitopes on the EGF-like domains

of the MSP-1₁₉ molecule can have very distinct effects on the ability of the molecule to undergo secondary processing.

mAb 12.8 is an IgG2b isotype antibody which recognises an epitope in the first EGF-like domain of MSP-1₁₉ whereas mAb 12.10 is of the IgG1 isotype and recognises an epitope formed by the two EGF-like domains in combination (Chappel and Holder 1993). Both these epitopes are conserved between the two major allelic variants of MSP-1₁₉.

1.2.3 Blocking antibodies : antibodies which block inhibitory antibodies

The antibodies which inhibit erythrocyte invasion, and which would benefit the host, are conventionally referred to as inhibitory antibodies. The antibodies which block this inhibitory effect, and which would therefore be detrimental to the host, are referred to as blocking antibodies. Antibodies which recognise MSP-1₁₉ but neither inhibit invasion nor block the action of inhibitory antibodies are referred to as neutral antibodies.

Blocking antibodies may be directed against MSP-1₁₉ but they may also be directed to other parts of the MSP-1 complex. A series of polyclonal rabbit antibodies purified from immune sera raised against recombinant proteins corresponding to regions covering all of MSP-1 and expressed in *E. coli* (Holder, Sandhu et al. 1987) were examined for the presence of blocking antibodies (Guevara Patino, Holder et al. 1997). The ability of these antibodies to block the binding of mAb 12.8 and mAb 12.10 was measured using a competitive radio-immunoassay. Blocking activity was found in antibodies raised against recombinant proteins in which the EGF-like domains of MSP-1₁₉ were included, as

expected. However blocking activity was also found in antibodies raised against constructs corresponding to domains outside the COOH-terminal from which MSP-1₁₉ is derived. Sera raised against two constructs, pME3 and pME14, which include the NH2-terminal of MSP-1₄₂, and sera raised against pME6, a construct corresponding to a region from MSP-1₈₃, also showed potent blocking activity. Naturally acquired human antibodies against pME6 were then isolated from pooled Gambian adult immune serum by affinity chromatography on immobilised pME6 protein, and these affinity-purified antibodies were shown to block the processing-inhibitory effects of mAbs 12.8 and 12.10 in a processing assay (Guevara Patino, Holder et al. 1997).

These results show that blocking antibodies can be directed against epitopes both within MSP-1₁₉ and within other quite distinct regions of the molecule. They also show that such blocking antibodies are induced by natural infection in humans. MSP-1 epitopes inducing these blocking antibodies probably represent an immune evasion mechanism which has evolved in *P. falciparum* to circumvent the potentially neutralising effect of invasion-inhibitory antibodies (Holder, Guevara Patino et al. 1999). Naturally occurring polyclonal serum in malaria-exposed people probably therefore consists of a mixture of potentially protective inhibitory antibodies and potentially deleterious blocking antibodies. Assessment of the protective effect of naturally occurring polyclonal antibodies against MSP-1₁₉ therefore becomes difficult to interpret. Only by isolating monoclonal antibodies to MSP-1₁₉ generated by natural infection can the role of such antibodies in protection from malaria be reliably assessed.

1.2.4 Is the protective effect of invasion-inhibitory antibodies mediated by antibody alone ?

There has been debate about whether anti-MSP-1₁₉ antibodies alone confer protection or whether they recruit other arms of the immune system to mediate the protection which is observed. In the *in vitro* erythrocyte invasion assays used to identify the invasion-inhibitory activity of mAbs 12.8 and 12.10, the assay is carried out in cell-free medium in which there is no possibility of participation by monocytes (Blackman, Heidrich et al. 1990). While the medium is normally supplemented by 10% (v/v) decomplexed non-immune serum, the invasion-inhibitory properties can still be detected when bovine serum albumin, in the form of 0.5% (w/v) Albumax (GIBCO), is used instead of serum (Blackman 1994). Similarly, the *in vitro* assay of MSP-1 secondary processing used to demonstrate that mAbs 12.8 and 12.10 prevent the proteolytic cleavage of MSP-1 is also carried out in a cell-free and serum-free medium (Blackman, Scott-Finnigan et al. 1994). Nevertheless, there are studies suggesting that other arms of the immune system may be required for the protective effects of antibody to be exerted.

In a study involving passive transfer of polyclonal IgG from malaria-exposed African adults to eight malaria-infected non-immune Thai patients, clinical and parasitological improvement was reproducibly obtained in each case. *In vitro* before transfer, however, the IgG had no inhibitory effect on either erythrocyte invasion or intraerythrocytic development of the parasite, and even sometimes increased parasite growth. In contrast, IgG collected from the Thai patients 4 days after the passive transfer exerted an antibody-dependent cellular inhibitory (ADCI) effect in co-operation with blood

monocytes. This was interpreted by the authors as indicating that the protective effect seen with antibodies is not due to antibodies alone, but involves monocytes also (Bouharoun-Tayoun, Attanath et al. 1990). It must be noted, however, that the antibodies transferred were polyclonal, and the relative contribution of antibodies specific for MSP-1₁₉ to the protective effect cannot be determined. Another study examined the question specifically in relation to anti-MSP-1₁₉ antibodies, in mice infected with *P. yoelii*. The authors observed that while passive transfer of immune sera from mice immunised with *P. yoelii* MSP-1₁₉ protects naïve mice from very high parasitaemia and death, it does not provide as much protection from high parasitaemia as that conferred by active immunisation. This was felt to suggest that other arms of the immune system beyond antibody alone are involved in protection. The authors transferred immune serum into malaria naïve SCID, nude, CD4+ T-cell-depleted and B-cell knockout mice; all the mice died when challenged with *P. yoelii* (Hirunpetcharat, Vukovic et al. 1999). This study indicates that more than antibody alone is required to protect mice from fatal *P. yoelii* challenge. Whether the other arms of the immune system are contributing specifically to the protective effect mediated by anti-MSP-1₁₉ antibodies however, or whether they are rather involved in protected effects targeted to other stages or targets is not clear from this study. Anti-MSP-1₁₉ antibodies may exert their protective effect independently of any other arm of the immune system and yet not be sufficient on their own to protect against progressive infection.

Two studies suggest that protective antibodies can indeed exert their protective effect without invoking any other arm of the immune system. To determine whether Fc receptor-bearing cells are required to mediate the protective effect of antibodies in mice, immune sera was passively transferred into FcR gamma-chain knockout mice, in which macrophages were therefore unable to mediate phagocytosis or antibody-dependent

cell-mediated cytotoxicity (ADCC) through Fc γ receptors. Passive transfer of either *P. yoelii* hyperimmune sera or anti-GST-PyC2 sera (directed against a recombinant 15 kDa protein representing the C-terminus of *P. yoelii* MSP-1) protected the FcR knockout mice against lethal *P. yoelii* challenge. In this experiment, with polyclonal serum, the protective effect is therefore mediated by antibody alone, and does not require ADCC or the participation of Fc receptors (Rotman, Daly et al. 1998). A similar experiment was carried out using a monoclonal IgG3 specific for *P. yoelii* MSP-1₁₉, which passively transferred protection to mice deficient in the alpha chain of Fc γ RI, whose macrophages could not therefore bind IgG3 (Vukovic, Hogarth et al. 2000). This shows that, in the *P. yoelii* mouse model at least, the protective effect of an anti-MSP-1₁₉ mAb is due to the antibody alone.

1.2.5 Evidence of protective role of anti-MSP-1₁₉ antibodies in humans

The prevalence of antibodies against MSP-1₁₉ in areas of high transmission of *P. falciparum* appears to be high, although some studies have found a greater prevalence than others. In estimating the prevalence of anti-MSP-1₁₉ antibodies in a given population it is important that the conformational epitopes should be correctly folded in the antigen used for testing (Blackman and Holder 1993). In a study of adult Senegalese 74% of individuals were found to have antibodies to MSP-1₁₉ (Diallo, Nguer et al. 1999). In a study examining adult sera from The Gambia, 50% of donors possessed antibodies to MSP-1₁₉ although almost all possessed antibodies to the MSP-1₃₃ fragment which is shed at secondary processing of MSP-1₄₂ (Egan, Waterfall et al. 1997). In a population of 266 3-15-year old Ghanaian children, prevalence of antibodies recognising a recombinant

construct with both EGF-like domains of MSP-1₁₉ corresponding to the Wellcome allelic variant was about 30% (Dodoo, Theander et al. 1999). In another study of 111 plasma samples in western Kenya, using recombinant constructs representing the different allelic forms of MSP-1₁₉, 90% of samples reacted with the Ugandan-PA allelic form, 77% reacted with the MAD20 allelic form and 80% reacted with the Wellcome allelic form of MSP-1₁₉ (Udhayakumar, Anyona et al. 1995).

Both prevalence and level of antibody to MSP-1₁₉ increase with age after infancy. A study of 198 infants from a Tanzanian village highly endemic for *P. falciparum* found that anti-MSP-1₁₉ antibodies decreased for the first two months of life and then showed only a slight tendency to increase with age (Kitua, Urassa et al. 1999). A study of older children, aged between 6 months and 15 years, in Papua New Guinea found that both the prevalence and the concentration of antibodies to MSP-1₄₂ increased with age (al-Yaman, Genton et al. 1996). The same increase in prevalence and concentration with age was found in a study of people aged from 2 to 74 years in western Kenya (Shi, Sayed et al. 1996). A cross-sectional survey of 387 3-8 year old children from a malaria endemic area of The Gambia also showed a significant positive correlation between anti-MSP-1 antibody levels and age (Blackman and Holder 1993). In another study of children aged from 3 to 15 years in Ghana, however, no increase in prevalence or level of anti-MSP-1₁₉ antibodies with age was found (Dodoo, Theander et al. 1999).

It is not clear how long antibodies to MSP-1₁₉ persist for, but it may be that they are quite short-lived. A longitudinal study, in which monthly serum samples were collected from 24 infants in western Kenya from birth to one year of age, revealed several short-lived anti-MSP-1₁₉ IgG and IgM peaks throughout the infants' first year which correlated with

detection of parasitaemia (Branch, Udhayakumar et al. 1998). Another study assessed the antibody responses of 52 children and adults seasonally exposed to malaria in eastern Sudan over a period of 4 years. Antibodies to a recombinant protein corresponding to MSP-1₁₉ were found in 66% of acutely infected individuals with clinical malaria but in only 12% of individuals without clinical malaria. These antibody responses declined within a few months of drug treatment and parasite clearance (Cavanagh, Elhassan et al. 1998). A longitudinal study of serological responses to MSP-1 in adults in the village of Brefet in The Gambia (another area where malaria transmission is seasonally endemic), however, showed that anti-MSP-1 antibody levels were much more stable over time than cellular responses (Riley, Morris-Jones et al. 1993).

It may be, therefore, that cross-sectional studies which detect a relatively low antibody prevalence in fact reflect a situation in which many people produce anti-MSP-1₁₉ antibodies but those antibodies persist for only a short period of time after each infection (Holder and Riley 1996).

It seems that the majority of antibodies to MSP-1₁₉ recognise epitopes formed by a combination of both EGF domains rather than by one domain alone. In a study of serum antibodies from malaria-exposed people from The Gambia using recombinant proteins representing the two EGF domains both separately and in combination, 10-20% of individuals were found to have antibodies which recognised one or other of the EGF domains alone, but more than 40% of individuals had antibodies which could recognise the two EGF domains in combination (Egan, Chappel et al. 1995). Using the same recombinant proteins, a study of 266 3 to 15-year-old Ghanaian children found that the prevalence of antibodies recognising both EGF domains in combination was about 30%

while only about 15% of individuals had antibodies which recognised the first domain alone and only about 4% had antibodies which recognised the second domain alone (Dodoo, Theander et al. 1999).

The immunoglobulin isotype may be an important factor in protection, and the IgG1 subclass appears to predominate in anti-MSP-1₁₉ responses. Anti-MSP-1₁₉ immunoglobulins were found to be mainly of the subclass IgG1 in a study of Africans aged 6 months to 76 years from Sudan and Kenya (Cavanagh, Dobano et al. 2001) and the same was found in antibodies from malaria exposed people in The Gambia (Egan, Chappel et al. 1995). In another study anti-MSP-1₁₉ immunoglobulins were predominantly IgG1 and IgG3 although only IgG1 antibody was associated with protection (Shi, Sayed et al. 1996).

The majority of published studies to address the issue have suggested that anti-MSP-1₁₉ antibodies are associated with protection from clinical malaria. A case/control study was performed in western Kenya on 75 case/control pairs of infants who were either febrile (cases) or afebrile (controls) at the time of the first detected infection with *P. falciparum*. The presence and level of anti-MSP-1 antibodies was significantly higher in the afebrile group, both at the time of the first infection and at one month prior to the first infection. Anti-MSP-1₁₉ IgG was also associated with lower parasite densities and less infection-related haemoglobin loss. Mothers with anti-MSP-1₁₉ antibodies were protected against placental infection and infection in their infants (Branch, Udhayakumar et al. 1998). In a study of individuals aged 2 to 74 years old in western Kenya, higher levels of anti-MSP-1₁₉ IgG1 (but not IgG3) correlated with lower parasite densities among young age groups (Shi, Sayed et al. 1996). In a prospective study of children in Papua New Guinea antibodies against a baculovirus-expressed recombinant protein representing

P. falciparum MSP-1₄₂ were significantly associated with protection from clinical malaria and severe parasitaemia, while antibodies against the N-terminal part of MSP-1 were not (al-Yaman, Genton et al. 1996). Antibodies to MSP-1₁₉ were found to provide 40% protection against clinical malaria in children in Sierra Leone, and antibodies to one of the EGF-like motifs of MSP-1₁₉ were strongly associated with resistance to both clinical malaria and high parasitaemia in Gambian children (Egan, Morris et al. 1996). When individuals with symptomatic malaria infection in Guadalcanal were compared with people with asymptomatic infections, the asymptomatic group showed significantly higher levels of antibody to recombinant MSP-1₁₉ (Fu, Hato et al. 2000).

A few studies, however, have failed to detect any association between anti-MSP-1₁₉ antibodies and protection from malaria. When 266 Ghanaian children were tested for antibodies to recombinant proteins corresponding to the Wellcome and MAD20 allelic variants of MSP-1₁₉ and monitored clinically and parasitologically for 18 months, no protection associated with the antibodies was found (Dodoo, Theander et al. 1999). In another prospective study involving 198 Tanzanian infants whose antibodies to recombinant MSP-1₁₉ were measured, again no protective effect against clinical malaria was found (Kitua, Urassa et al. 1999).

1.2.6 Is the protective effect of antibody due to inhibition of invasion ?

It is difficult to demonstrate that a protective effect associated with anti-MSP-1₁₉ antibodies is directly caused by those antibodies, or that the mechanism of protection is analogous to the invasion-inhibitory activities of the murine mAbs 12.8 and 12.10. The

most direct way of demonstrating that antibodies are the cause of an associated protective effect is to demonstrate that the antibodies confer protection when passively transferred to a naïve host. Some of these passive transfer experiments have already been discussed in section 1.2.4 above. Three of these studies dealt specifically with anti-MSP-1₁₉ antibodies, but they used *P. yoelii* infection in mice as a model system (Hirunpetcharat, Vukovic et al. 1999) (Rotman, Daly et al. 1998) (Vukovic, Hogarth et al. 2000). The studies demonstrating protection against *P. falciparum* in human subjects examined the effect of passive transfer of purified total antibody rather than anti-MSP-1₁₉ antibodies specifically (Cohen, McGregor et al. 1961) (Bouharoun-Tayoun, Attanath et al. 1990). With the advent of the AIDS pandemic it is becoming less appropriate to conduct further experiments involving the passive transfer of immune globulin between human subjects, and a more practicable alternative is to study *P. falciparum* in a primate model. In one such study, purified rabbit immunoglobulin raised against yeast-expressed *P. falciparum* MSP-1₁₉ was passively transferred into malaria-naïve *Aotus nancymai* monkeys and failed to protect against homologous or heterologous challenge. However, the antibody under test was polyclonal, and rabbit immune serum has been shown to include blocking antibodies (Guevara Patino, Holder et al. 1997), whose presence may have complicated the result. Furthermore, while the purified rabbit immunoglobulin was shown to inhibit parasite growth *in vitro*, it did so only at concentrations of at least 4.8 mg/ml, and the antibody concentration achieved *in vivo* (1 mg/ml) was considerably less than this (Gozalo, Lucas et al. 1998).

To demonstrate that anti-MSP-1₁₉ antibodies associated with protection exert an effect through the inhibition of secondary processing and invasion, it is necessary to purify polyclonal anti-MSP-1₁₉ antibodies from immune serum in sufficient quantity to

demonstrate that they exert a protective effect in the relevant *in vitro* assays. Two such studies have been conducted. In one study, antibodies specific for the first EGF-like domain of MSP-1₁₉ were prepared by affinity chromatography from the total immunoglobulin G fraction of adult West African donors using a recombinant protein corresponding to this domain. These affinity-purified antibodies recognised their target and blocked the binding of an inhibitory mAb but did not inhibit parasite invasion in the *in vitro* invasion assay (Chappel, Egan et al. 1994). In a further study, IgG from malaria-exposed adults from the Ivory Coast was purified, and antibodies specific for the first EGF-like domain of MSP-1₁₉ were removed by affinity chromatography as above. Antibodies specific for the second EGF-like domain were then purified in the same way. The purified IgG was tested in the *in vitro* invasion assay. At a concentration of 100 µg/ml antibodies to the first EGF-like domain did not inhibit invasion but antibodies to the second EGF-like domain did show some invasion-inhibitory effect (Egan, Burghaus et al. 1999).

1.2.7 MSP-1₁₉ as a vaccine candidate; tests in animal models

MSP-1₁₉ –based vaccines have been tested in a number of experimental animal systems, with promising results. Studies have focussed mainly on *Plasmodium yoelii* infections in mice or *P. falciparum* infections in *Aotus* monkeys. The 11 kDa carboxyl-terminal of *P. yoelii yoelii* MSP-1, containing only the two EGF-like domains, protected mice against homologous strain blood stage (Daly and Long 1995) (De Souza, Ling et al. 1996) and sporozoite (Ling, Ogun et al. 1997) challenge. Furthermore, this resistance could be transferred passively by purified Ig, establishing the role of antibody in this protection (Daly and Long 1995). Some formulations of *P. falciparum* MSP-1₁₉ have

been successfully employed to protect *Aotus* monkeys against parasite challenge. MSP-1₁₉ expressed in *Saccharomyces cerevisiae* with Freund's adjuvant protected *Aotus nancymai* against lethal *P. falciparum* challenge (Kumar, Collins et al. 2000). *P. falciparum* MSP-1₁₉ fused with tetanus toxoid epitopes protected *Aotus vociferans* monkeys against *P. falciparum* challenge, and antibodies from some of the protected monkeys inhibited secondary processing of MSP-1 *in vitro* (Egan, Blackman et al. 2000). A recombinant form of MSP-1₄₂, which comprises the 42 kDa C-terminal portion of MSP-1 and includes MSP-1₁₉, expressed in baculovirus-infected insect cells, protected *Aotus nancymai* against *P. falciparum* challenge. This protection was better than that afforded by recombinant MSP-1₁₉ expressed in *Saccharomyces cerevisiae*, which was tested in the same series of experiments. With both antigens, however, protection was only seen when high antibody levels were obtained by formulation of the vaccines in Freund's adjuvant (Stowers, Cioce et al. 2001). In a primate model of *P. vivax* infection, recombinant baculovirus-expressed analogues of *P. falciparum* MSP-1₄₂ and MSP-1₁₉ from *P. cynomolgi*, a primate malaria parasite highly analogous to the human parasite *P. vivax*, provided lasting protection against *P. cynomolgi* challenge in the toque monkey, *Macaca sinica* (Perera, Handunnetti et al. 1998). As in the previous study, the vaccines were formulated in complete and incomplete Freund's adjuvant. Not all such studies have shown protection, however. In contrast to the protection afforded to *Aotus nancymai* in the study described above, a study in the same animal model using recombinant MSP-1₁₉ expressed in *E. coli*, incorporated into liposomes and adsorbed to alum as an adjuvant, failed to confer any protection (Burghaus, Welde et al. 1996). These contrasting results may well reflect the importance of the choice of adjuvant in inducing protective immune responses.

1.2.8 MSP-1₁₉ as a vaccine candidate; trials in humans

To date, only one trial of MSP-1₁₉ vaccines in humans has been published. A Phase I (safety and immunogenicity) trial was conducted of two yeast-derived MSP-1₁₉ vaccines, from the 3D7 or FVO strains of *P. falciparum*, fused to tetanus toxoid T-helper epitopes and adsorbed on alum. Serum antibody responses occurred in 5/16 and 9/16 healthy adults given a lower and a higher dose of vaccine respectively. Hypersensitivity reactions occurred in 3 subjects, one developing a generalised rash and another developing hypotension. The authors concluded that changes in formulation would be required to improve the safety and immunogenicity profiles of the vaccines (Keitel, Kester et al. 1999). One novel approach which could be used in the future design of MSP-1₁₉-based vaccines is to modify the protein by removing the epitopes recognised by blocking antibodies. Amino acid substitutions have been made in MSP-1₁₉ which prevent the binding of known blocking mAbs without affecting the binding of invasion-inhibitory mAbs (Uthairibull, Aufiero et al. 2001). Such modified molecules may provide more effective protection in a vaccine than molecules based on native MSP-1₁₉ (Holder, Guevara Patino et al. 1999).

1.2.9 Further studies of the role of anti-MSP-1₁₉ antibodies in protection against malaria

To evaluate the likelihood of an MSP-1₁₉-based vaccine being successful, two important questions need to be clarified. Does malaria infection in man induce

invasion-inhibitory antibodies to MSP-1₁₉, and if so, do such antibodies make an important contribution to naturally acquired immunity ?

As discussed above, it is difficult to assess the invasion-inhibitory capacity of polyclonal anti-MSP-1₁₉ antibodies because the natural immune response probably generates a mixture of protective invasion-inhibitory antibodies and deleterious blocking antibodies. To determine whether invasion-inhibitory antibodies to MSP-1₁₉ comprise a significant component of the natural immune response to malaria, it will be necessary to isolate and examine individual monoclonal antibodies to MSP-1₁₉. Only by examining a range of naturally occurring monoclonal antibodies to MSP-1₁₉ for invasion-inhibitory, blocking or neutral activity will it be possible to dissect the immune response in sufficient detail to answer this question clearly.

It is for this reason that the project described in this thesis was undertaken, namely to attempt to isolate and characterise monoclonal antibodies to MSP-1₁₉ from a library of human antibody fragments derived from malaria-immune donors and displayed on the surface of phage.

1.3 Obtaining monoclonal antibodies by phage display technology

1.3.1 The principles of phage display

Phage display technology offers a means of isolating high-affinity monoclonal antibodies of desired specificities without resort to hybridoma techniques. The power of the phage display technique rests upon the linkage of sequence information to selectable

function. In this technology, combinatorial libraries of recombinant antibody fragments are displayed on the surface of phage particles. The phage provides a vehicle by which each of the library members carries its own DNA coding sequence linked to the displayed protein product. This also enables library members to replicate themselves. Thus library members can be selected according to the binding characteristics of the displayed protein and then amplified by replication of the coding DNA within the phage. Repeated rounds of selection result in increasingly specific antibodies of increasing affinity. In this way it therefore becomes possible to select, out of tens or hundreds of millions of clones, those few phages which display a peptide that binds the target molecule (Cesareni 1992). This technique is therefore potentially very powerful, and has been likened to “finding a much-needed needle in a vast molecular haystack” (Rodi and Makowski 1999).

The successive steps of the phage display technique thus mimic the natural processes of immune selection (Winter, Griffiths et al. 1994). The two processes of natural and phage-display based antibody selection are compared schematically in Figure 1.4.

In the natural immune system, a finite number of V-genes encode sections of the variable regions of the heavy and light chains of antibodies. As stem cells mature to B cells, these V-genes are rearranged in many different permutations, yielding an immense repertoire of different antibody antigen-binding sites. Each B cell displays just one antibody of one antigen specificity. Interaction with the target antigen leads to selection of the relevant B cell clone, which may either proliferate to make short-lived plasma cells secreting the antibody or long-lived memory cells, capable of making antibodies of improved binding affinity through hypermutation on repeated stimulus with the antigen.

In the process of phage display, rearranged variable region sequences are amplified by PCR from B cells. Heavy and light chain variable region sequences are then randomly joined together in a further PCR to produce a repertoire of DNA molecules encoding antibody fragments analogous to the original antibody repertoire. The repertoire of antibody fragment-encoding DNA is inserted into a filamentous bacteriophage or phagemid vector to produce a fusion of the antibody fragment with the phage coat protein. In this way, each antibody fragment is displayed on the surface of a filamentous phage in a manner analogous to the display of antibody on the surface of a B cell. The antibody fragments displayed on phage are selected by binding to the relevant antigen, and can proliferate by replication in *E. coli*. Just as B cells may secrete antibody as short-lived plasma cells, so the *E. coli* can be induced to produce soluble antibody fragments. Likewise, just as B cells may become memory cells, their antibodies undergoing affinity maturation upon repeated exposure to antigen, so antibody fragments displayed on phage may undergo affinity maturation by repeated rounds of selection on antigen, or by the deliberate introduction of random mutations followed by re-selection (Winter, Griffiths et al. 1994).

Antibodies can be displayed on phage either as single polypeptide chains or as non-covalently bound heterodimers. To be displayed as single chains the heavy and light chain variable domains are linked together by a polypeptide spacer, to produce a single chain variable fragment (scFv) which preserves the binding characteristics of the original antibody binding site (McCafferty, Griffiths et al. 1990). To be displayed as non-covalently associated heavy and light chains, which are essentially Fab fragments, one chain is exported into the bacterial periplasm linked to the pIII phage protein and the other chain is co-expressed into the periplasm. There the two chains pair as they would during natural antibody expression (Hoogenboom, Griffiths et al. 1991). In general the scFv

format is favoured over the Fab format for antibody libraries because Fabs do not express as reliably, which results in reduced selection efficiency (Vaughan, Williams et al. 1996). Figure 1.5 illustrates how the structure of scFv relates to that of whole antibody.

1.3.2 Filamentous phage and the display of antibody fragments

Different types of bacteriophage have been employed to construct phage libraries, but the first and the most common type to be used is filamentous phage. Both fd filamentous phage and M13 filamentous phage have been used. Both are members of the Ff filamentous single stranded DNA phages that infect *E. coli* cells. The phage binds to the sex pili of *E. coli* and this binding is mediated by the gene 3-encoded minor coat protein pIII; also known as g3p (Hoogenboom, Griffiths et al. 1991).

Filamentous phage is unique in that of its five virion proteins, three can tolerate the insertion of foreign peptides. The manner in which the inserted peptide is displayed on the phage surface varies according to the phage protein with which it is fused. An intact filamentous phage is 930 nm long and contains a single stranded DNA genome of 6400 base pairs. The filamentous phage is covered throughout its length with 2800 copies of the major coat protein, pVIII. Peptides fused with this protein are therefore displayed in hundreds of copies over the surface of the phage virion. There are five copies of pIII and five copies of pVI on each phage, and these proteins are located at one end of the virion. Peptides fused to pVI are displayed with the carboxy terminus oriented outward. Peptides fused to pIII may be displayed in one to five copies per virion (Cabilly 1999). The crystal structure of the N-terminal domains of pIII have been determined (Lubkowski, Hennecke et

al. 1998). It appears that proteins displayed fused to pIII do not interfere with the infection of *E. coli* because they are fused far from the central part of the molecule which probably constitutes the binding site for the *E. coli* pilus. The pIII protein is the protein most commonly chosen to display peptides and proteins such as antibody fragments because it allows just one peptide or protein to be displayed per phage particle. If more than one antibody fragment is displayed, selection will be dominated by avidity rather than affinity, and high affinity antibodies cannot be so easily isolated (Marks, Hoogenboom et al. 1991).

The pIII fusion with scFv can be encoded with the other phage proteins within the same phage replicon (McCafferty, Griffiths et al. 1990) or it can be encoded separately on a phagemid, a plasmid containing a phage origin of replication (Hoogenboom, Griffiths et al. 1991). As phagemids do not carry the genes for the other phage proteins, they can only be packaged into phage particles if the other phage proteins are provided from another source. This is achieved by “rescue” through the addition of a helper phage such as M13K07. This helper phage contains the genes for all the phage proteins, including pIII, but due to a defective origin of replication it is poorly packaged in competition with the phagemids (Vieira and Messing 1987).

In selecting displayed antibody fragments for high affinity binding to antigen it is optimal for just one antibody fragment to be displayed per phage particle. If every pIII incorporated were fused with an antibody fragment each phage would display five such fragments. However, the average valency is reduced by the use of helper phage, as the wild-type helper pIII competes with the pIII fusion proteins for incorporation into the phage particle. The average valency is further reduced because the pIII fusion is often proteolysed, removing the antibody fragment before the phagemid pIII is incorporated.

The result of these two processes is that the phage are estimated on average to display less than a single fusion protein per phage particle, creating “monovalent” phage suitable for high affinity antibody selection (Winter, Griffiths et al. 1994). The use of phagemid vectors has an added advantage over the use of whole phage vectors, in that the phagemid vector has a transfection efficiency two or three orders of magnitude higher than the phage vector, allowing larger phage display libraries to be constructed (Hoogenboom, Griffiths et al. 1991).

1.3.3 Phagemid pHEN1 for the display of antibody fragments

The phagemid vector pHEN1 was constructed specifically for the purpose of displaying antibody fragments on phage (Hoogenboom, Griffiths et al. 1991). The pHEN1 phagemid, modified by the addition of a hexahistidine tag to give pHEN1H6, is illustrated in Figure 2.3 in Materials & Methods.

pHEN1 is a derivative of pUC119 (Vieira and Messing 1987), adapted by the inclusion of the coding region of pIII from the fd filamentous phage. The pIII signal sequence has been replaced by a pelB signal sequence. Between the signal sequence and the gene for pIII is an internal *Sfi*I site and a *Not*I site, allowing antibody fragments to be cloned as *Sfi*I/*Not*I fragments. Directly after the *Not*I site is a c-myc tag, enabling expressed antibody fragments to be detected with the anti-c-myc mAb 9E10. An amber stop codon (TAG) has been introduced between the tag and the region encoding the N-terminus of pIII. This amber codon enables the antibody fragment to be expressed either as a fusion with pIII to be displayed on phage, or as a soluble fragment without the pIII fusion. Growth of this phagemid in suppresser (*supE*) strains of *E. coli* such as TG1 *E*

coli, in which the amber codon is read as glutamine, results in the ribosome translating through the amber codon on to the pIII gene. This results in the antibody fragment being expressed as a fusion with the pIII protein. Growth of the same phagemid in a non-suppressor strain of *E. coli* such as HB2151, which reads the amber codon as a stop codon, results in the translation ending at the end of the antibody fragment, which is therefore expressed as a soluble fragment without the pIII protein (Hoogenboom, Griffiths et al. 1991).

Transcription of antibody-pIII fusions in pHEN1 is driven from the inducible lacZ promoter and the expressed protein is targeted to the bacterial periplasm by means of the pelB leader. The phagemid can be rescued with helper phage in medium without IPTG because the natural leakiness of the promoter provides sufficient expression of antibody-pIII. Increased expression by IPTG induction may lead to cell death due to the toxicity of the fusion protein (Hoogenboom, Griffiths et al. 1991). One further refinement to the phagemid is the addition of a hexahistidine tag to allow rapid purification of antibody fragments by nickel chelation (Vaughan, Williams et al. 1996).

1.3.4 Selection of specific high affinity antibodies

The ability to select high affinity antibodies of a desired specificity from a phage display library depends on a number of factors. Phage must be subjected to repeated rounds of selection by binding to antigen before rare and specific high affinity antibodies can be isolated. This process is known as panning the library. It is estimated after one round of panning rare binders are enriched by one in 10^3 and after two rounds they are

enriched by one in 10^6 (McCafferty, Griffiths et al. 1990). The representation of rare antibodies in the library should be enhanced by amplifying variable region sequences initially using PCR primers based on each of the heavy and light chain gene families to maximise the diversity of library members (Marks, Hoogenboom et al. 1991). The size of the library is also important. The larger and more diverse the library, the greater the chance of isolating high affinity antibodies (Perelson 1989). A medium size library of 10^7 members is similar in size to the B-cell repertoire of a mouse at any one moment (Marks, Hoogenboom et al. 1991). A larger library containing 1.4×10^{10} scFv fragments permitted the isolation of antibodies with sub-nanomolar affinities to a range of antigens (Vaughan, Williams et al. 1996).

Diversity is also increased by the fact that the original heavy and light chain pairings are scrambled in the process of creating the library, resulting in the association of a given heavy chain with any number of different light chains and vice versa (Marks, Hoogenboom et al. 1991).

With sufficiently large and diverse libraries, even the need for immunisation of the donor is bypassed. Such libraries are sufficiently diverse that high affinity antibodies can be isolated to any given antigen, even if the library is derived from naïve donors (Vaughan, Williams et al. 1996).

1.3.5 Applications of antibody phage display to clinical problems

Phage display technology has now yielded a considerable number of scFvs or mAbs with biological activity of clinical and potentially therapeutic relevance. A number of phage library-derived antibodies have been isolated which have been shown to neutralise viruses of clinical importance. These include rabies virus (Ray, Embleton et al. 2001), hepatitis E virus (Schofield, Glamann et al. 2000), respiratory syncytial virus (Nguyen, Hay et al. 2000), Ebola virus (Maruyama, Rodriguez et al. 1999), herpes simplex viruses 1 and 2 (Burioni, Williamson et al. 1994), human cytomegalovirus (Takekoshi, Maeda et al. 1998), and HIV-1 (Burton, Barbas et al. 1991). Antibodies with therapeutic potential have also been isolated against other, non-viral, infections. Phage-derived antibodies against bacterial toxins, such as the tetanus toxin (Lang, Vogel et al. 1995) and the pertussis toxin (Williamson and Matthews 1999) have the potential to replace plasma-derived immunoglobulins currently used for passive immunisation. Other infectious targets to which antibodies have been derived include the protozoan *Cryptosporidium parvum* (Baecher-Allan, Santora et al. 1999) and the pathogenic isoform of the prion protein (Williamson, Peretz et al. 1998). Antibodies have also been isolated with a potential role in autoimmune diseases, such as myasthenia gravis (Graus, de Baets et al. 1997) and haemolytic disease of the newborn (Miescher, Zahn-Zabal et al. 2000), and in specific malignancies such as Hodgkin's Disease (Klimka, Barth et al. 1999), or in tumour angiogenesis (Vitaliti, Wittmer et al. 2000).

Virus-neutralising or other biological activity in some instances is present in the scFv molecule alone. In other instances, the desired biological activity is obtained only

when the scFv is turned back into a whole antibody or otherwise engineered. Respiratory syncytial virus can be neutralised by scFv alone, and Fab fragments derived from libraries of Fab can neutralise Ebola virus (Maruyama, Rodriguez et al. 1999) and herpes simplex viruses 1 and 2 (Burioni, Williamson et al. 1994). To neutralise rabies virus in a standard *in vivo* assay, however, it was necessary to clone anti-rabies scFv into a mammalian expression vector carrying the human IgG1 Fc region (Ray, Embleton et al. 2001).

1.3.6 Applications of antibody phage display to malaria

Since the advent of phage display, the technology has been applied to studies of malaria on a number of occasions. The technology has been used in three main ways. Firstly, it has been used to display malaria proteins as immunogens. Secondly, it has been used to display random peptides to identify epitopes recognised by anti-malarial antibodies. Thirdly, it has been used to display antibody-fragments to obtain mAbs to given malaria antigens.

The first studies to apply phage display techniques to malaria examined ways in which malaria-derived peptides (rather than antibody fragments) displayed on phage could be used as immunogens, either to provide anti-malarial mAbs as reagents, or to serve as potential malaria vaccines. In the earliest study to apply phage display techniques to malaria, repeat regions of the circumsporozoite protein (CSP) of *P. falciparum* were cloned into the pIII coat protein gene and displayed on filamentous phage and shown to be antigenic and immunogenic in rabbits (de la Cruz, Lal et al. 1988). Regions of CSP were also displayed on the gpVIII major coat protein of bacteriophage Fd and shown to be

immunogenic in mice without the use of external adjuvants (Willis, Perham et al. 1993) (Greenwood, Willis et al. 1991). A putatively protective epitope of *P. falciparum* liver stage antigen-1 (LSA-1) was displayed on the coat protein of RNA bacteriophage MS2 and shown to be immunogenic in mice (Heal, Hill et al. 1999).

In later studies, libraries of random peptides displayed on phage were used to identify important malaria antigen B cell and T cell epitopes recognised by anti-malarial mAbs. Random peptide libraries were used to identify T cell epitopes recognised by malaria-protective MHC molecules (Davenport, Quinn et al. 1996), to identify mimotopes recognised by antibodies to *P. vivax* MSP-1₄₂ (Demangel, Rouyre et al. 1998), to identify mimotopes recognised by a mAb against *P. falciparum* ring-infected erythrocyte surface antigen (RESA) (Adda, Tilley et al. 1999) and to map epitopes of the glutamate-rich protein (GLURP) of *P. falciparum* using anti-GLURP IgG (Theisen, Soe et al. 2000).

Three studies have been published in which monoclonal antibodies recognising malaria antigens were isolated from libraries of antibody fragments (scFvs) displayed on phage. The first of these studies described the construction of a library of murine antibody fragments derived from mice protected from malaria challenge by immunisation with recombinant *P. chabaudi* DS apical membrane antigen (AMA-1). The library was panned with AMA-1 and yielded a population of scFvs which recognised AMA-1 from the *P. chabaudi* DS strain but not from the 556KA strain, although a subset of these scFv also recognised AMA-1 from *P. falciparum* (Fu, Shearing et al. 1997). Recently, two studies have been published describing the isolation of scFv from antibody libraries derived from malaria-exposed human donors. A monoclonal scFv recognising the block 2 region of *Plasmodium falciparum* was isolated from a library of scFv derived from ten patients with

clinical malaria from Cameroon (Sowa, Cavanagh et al. 2001). Another library derived from malaria-exposed individuals from Cameroon was used to select scFv against Pfs48/45, a *P. falciparum* gamete surface protein (Roeffen, Raats et al. 2001).

1.4 Chimeric and humanised antibodies

As discussed in section 1.2.6 above, it is important to determine whether invasion-inhibitory antibodies are generated in response to *P. falciparum* infection, but it is also important to determine whether such antibodies, if generated, can make an important contribution to protection. This question could be answered in part by determining whether the invasion-inhibitory mAbs 12.8 and 12.10 can protect against *P. falciparum* *in vivo*, in a primate model. Murine antibodies produced from hybridoma cell lines are not ideal reagents for such an experiment because they are difficult to produce in a form which is standardised and sufficiently pure, and because they will be recognised as foreign by the primate immune system. Recombinant versions of these mAbs would be easier to produce to the necessary quality, and could be engineered to be less immunogenic in primates. Chimeric versions of these mAbs, engineered to contain human instead of murine Fc regions, would be more suitable reagents for this purpose provided they retain their invasion-inhibitory activity. If recombinant chimeric versions of mAb 12.8 or mAb 12.10 can modify or control *P. falciparum* infections in primates, it is possible that they may have a potential therapeutic application in humans.

Murine monoclonal antibodies have been made to a wide range of therapeutic targets since the advent of hybridoma technology in the 1970s. Making human hybridoma

mAbs has proved much more difficult. Hybrids of human cells with mouse myeloma cells are unstable, with preferential loss of human chromosomes. Humans can rarely be hyperimmunised to order, and human cells from spleen or lymph nodes are not readily obtained. The alternative to hybridoma fusions, immortalisation of human B-cells with Epstein-Barr virus, leads to cell lines which are unstable and poor producers of antibody (Winter and Milstein 1991). Because of these difficulties, mAbs to targets of therapeutic importance have predominantly been derived from murine hybridomas.

As therapeutic agents in humans, however, murine mAbs have three serious disadvantages. Firstly they are recognised as foreign and induce an immune response in human patients. This human anti-mouse antibody (HAMA) response leads to rapid clearance of the murine mAb from the plasma. Secondly murine mAbs may sensitise the patient so that repeated administration may lead to allergic and even anaphylactic reactions. Thirdly, murine mAbs cannot efficiently recruit the human immune effector mechanisms that involve recognition of the Fc part of the molecule and lead to complement-dependent cytotoxicity or antibody-dependent cell-mediated cytotoxicity (Bendig 1995). Techniques have therefore been developed to attempt to overcome these difficulties by engineering murine antibodies to become more human.

There are two basic methods by which a murine antibody may be rendered more human. One is to create a chimeric antibody and the other is to engineer a “humanised” antibody. The relationship between a murine, chimeric and humanised (CDR-grafted) antibody is shown in Figure 1.6.

The simpler of these two methods is that of creating a chimeric antibody. This involves taking only the variable domains from the murine antibody and joining these to the constant regions of a human antibody. This technique has the advantage of being relatively easy technically, and murine antibodies thus engineered may have considerably reduced immunogenicity in man (LoBuglio, Wheeler et al. 1989). However, approximately 35% of the final chimeric antibody sequence remains derived from the mouse antibody (Bendig 1995). Thus while single doses of such mAbs may not generate a major immune response in man, repeated dosing may cause a HAMA response in over 50% of patients (Elliott, Maini et al. 1994).

A more elaborate method of rendering murine mAbs more human is to take only the antigen-binding loops or complementarity determining regions (CDRs) from the murine hybridoma mAb and to graft these into the framework regions of human variable domains. These reshaped variable regions are then joined to human constant domains to yield an antibody which is fully “humanised” (Jones, Dear et al. 1986). Humanised antibodies are less immunogenic because only about 9% of the final antibody sequence is derived from the murine mAb (Bendig 1995). However, the process of humanisation is much more time consuming than that of creating a chimeric antibody. The transfer of murine CDRs alone onto human framework regions may often lead to a loss of affinity, which may only be restored by reintroducing some foreign framework residues. The number and identity of these residues has to be determined empirically, working with homologous human variable antibody domains and computer models of the variable domain structure (Bendig 1995). A recent approach to humanisation bypasses much of the empirical work described above by making use of phage display techniques. In this approach, only the third CDRs of the original murine heavy and light chains are preserved, and the rest of the variable domains

are replaced with human sequences selected from phage-displayed antibody libraries (Rader, Cheresch et al. 1998).

More than a decade elapsed from the development of hybridoma techniques to the first use of monoclonal antibodies as therapeutic agents in man. Until recently very few monoclonal antibodies were in routine clinical use, but the number of therapeutic mAbs available on the market has increased sharply in the past few years. The first murine mAb used therapeutically was OKTC, a murine hybridoma IgG2a mAb which targets CD3 on the surface of T lymphocytes. OKTC is a potent immunosuppressant, and has proved useful in the prevention of rejection of kidney and liver transplants (Wilde and Goa 1996). Half the patients who receive this murine mAb however, generate OKTC-neutralising anti-idiotypic antibodies, which limit re-use of this therapy, and first administration can produce a dangerous acute clinical syndrome due to massive cytokine release (Chatenoud 1993). The murine/human chimeric mAbs in clinical use appear to be less immunogenic. Rituximab, an anti-CD20 chimeric antibody, is useful in the treatment of non-Hodgkin's lymphoma, and causes relatively mild immune response-mediated side effects (Grillo-Lopez, White et al. 1999). Infliximab, an anti-tumour necrosis factor (TNF α) chimeric mAb, is effective against the inflammatory bowel condition Crohn's disease (Hyams, Markowitz et al. 2000) and rheumatoid arthritis, and causes few side effects although about 10% of treated patients develop a HAMA response (Markham and Lamb 2000). Another chimeric mAb has been developed as a therapeutic agent in the form of a Fab fragment. Abciximab, which is directed against the glycoprotein IIb/IIIa receptor on platelets and inhibits platelet aggregation, is now a mainstream therapy in widespread use for unstable angina (van den Brand, Laarman et al. 1999). Antimurine antibodies have been

demonstrated in patients receiving abciximab, but these HAMA responses do not seem to limit its use.

Most of the therapeutic mAbs currently on the market are fully humanised. CAMPATH-1H is a humanised anti-CD52 IgG1 that binds to the lymphocyte surface antigen CD52 and is an effective treatment for various lymphomas and leukaemias (Flynn and Byrd 2000). Daclizumab (Zenapax) is a humanised mAb against the interleukin-2 receptor on T lymphocytes and is effective in preventing acute rejection of kidney transplants (Eckhoff, McGuire et al. 2000) and trastuzumab (Herceptin) is a humanised mAb against the human epidermal growth factor receptor-2 and has proven useful in treating women with metastatic breast cancer (Baselga 2001). Accumulating evidence from clinical use appears to confirm the expectation that humanised antibodies will prove less immunogenic than murine or chimeric mAbs. In a phase II study of 46 patients receiving weekly intravenous trastuzumab, no antibodies against trastuzumab could be detected in any of the subjects (Baselga, Tripathy et al. 1999).

While monoclonal antibodies alone can be therapeutically useful, their therapeutic potential may be greatly enhanced if they are used to selectively target drugs or toxins to specific target cells. Two such immunoconjugates are already available clinically. Rituximab has been linked to yttrium 90 to create a tumour-targeted radioimmunoconjugate, which appears more effective against non-Hodgkin's lymphoma than rituximab alone (Witzig 2000). Mylotarg is a humanised anti-CD33 mAb linked to a cytotoxic antibiotic, and can induce complete remission of acute myeloid leukaemia (Sievers, Larson et al. 2001).

Only one therapeutic mAb is available so far against an infectious disease. Palivizumab (Synagis) is a humanised IgG1 directed against respiratory syncytial virus (RSV), a major pathogen of children and premature infants. Palivizumab is very effective; used prophylactically in 1502 high-risk children, palivizumab reduced hospitalisation due to RSV by 55% (1998).

No mAbs derived from phage display libraries are yet on the market, although a number are undergoing trials. The most advanced of these, D2E7, is a fully human anti-tumour necrosis factor mAb which has been shown to be effective in the control of rheumatoid arthritis (Taylor 2001).

In the last few years the number of monoclonal antibodies in use or in development as therapeutic agents has increased dramatically. Much of this increase so far is due to improved techniques for engineering safe, effective and non-immunogenic agents from murine mAbs. Agents derived from human phage display libraries are almost certain to come into similarly widespread use in the near future. Against this background, it is possible that mAbs against the malaria parasite, as well as having a role in basic research, may potentially have a therapeutic role in the future.

1.5 The aims of this project

This project has two main aims. One aim is to isolate and characterise naturally occurring human monoclonal antibodies to MSP-1₁₉ in the form of single chain variable fragments (scFvs) obtained from phage display libraries derived from malaria-exposed

donors. By examining the sequence diversity and binding characteristics of a variety of MSP-1₁₉-specific antibody fragments derived in this way, it should be possible to dissect the natural human immune response at the molecular level. To detect any invasion-inhibitory activity in these antibody fragments it will probably be necessary to engineer them back to a format approaching that of a whole antibody. If this can be done, it should then be possible to use the techniques and materials developed in this project to isolate natural human mAbs with invasion-inhibitory activity. This would provide a very useful insight into the mechanism of natural immunity to malaria.

The other aim of this project is to clone and sequence the antigen-binding variable regions of two murine hybridoma monoclonal antibodies which are known to inhibit erythrocyte invasion by *P. falciparum*, and to use those sequences to create and characterise scFv and murine/human chimeric versions of those mAbs. The scFv versions of these mAbs should serve as a useful model with which to develop techniques for restoring invasion-inhibitory activity to scFvs, which may then be applied to human scFvs derived from the phage libraries. The recombinant chimeric mAbs can, by being shown to retain invasion-inhibitory activity, confirm the identity of the sequences obtained from the parent murine hybridomas. The chimeric versions of these mAbs can be produced in a standardised high quality form which should be less immunogenic in primates than murine hybridoma mAbs would be. The chimeric mAbs will therefore also be appropriate agents to test in a primate model of *P. falciparum* in order to determine whether these mAbs can modify or prevent clinical malaria. This would provide useful insights into the biological relevance of invasion-inhibitory mAbs to naturally acquired malaria immunity. It is also possible that these invasion-inhibitory recombinant mAbs may be developed as therapeutic agents for clinical malaria in the future.

Figure 1.1

The primary and secondary processing of *Plasmodium falciparum*

Merozoite Surface Protein-1

- After primary processing, MSP-1 exists as a complex attached to the merozoite surface by a GPI anchor. Secondary processing occurs at the time of erythrocyte invasion. During secondary processing, a parasite-encoded protease cleaves MSP-1₄₂ to produce MSP-1₁₉ and MSP-1₃₃. MSP-1₃₃ is shed along with the rest of the protein complex. MSP-1₁₉ remains on the surface of the merozoite and is carried into the erythrocyte.

Figure 1.1

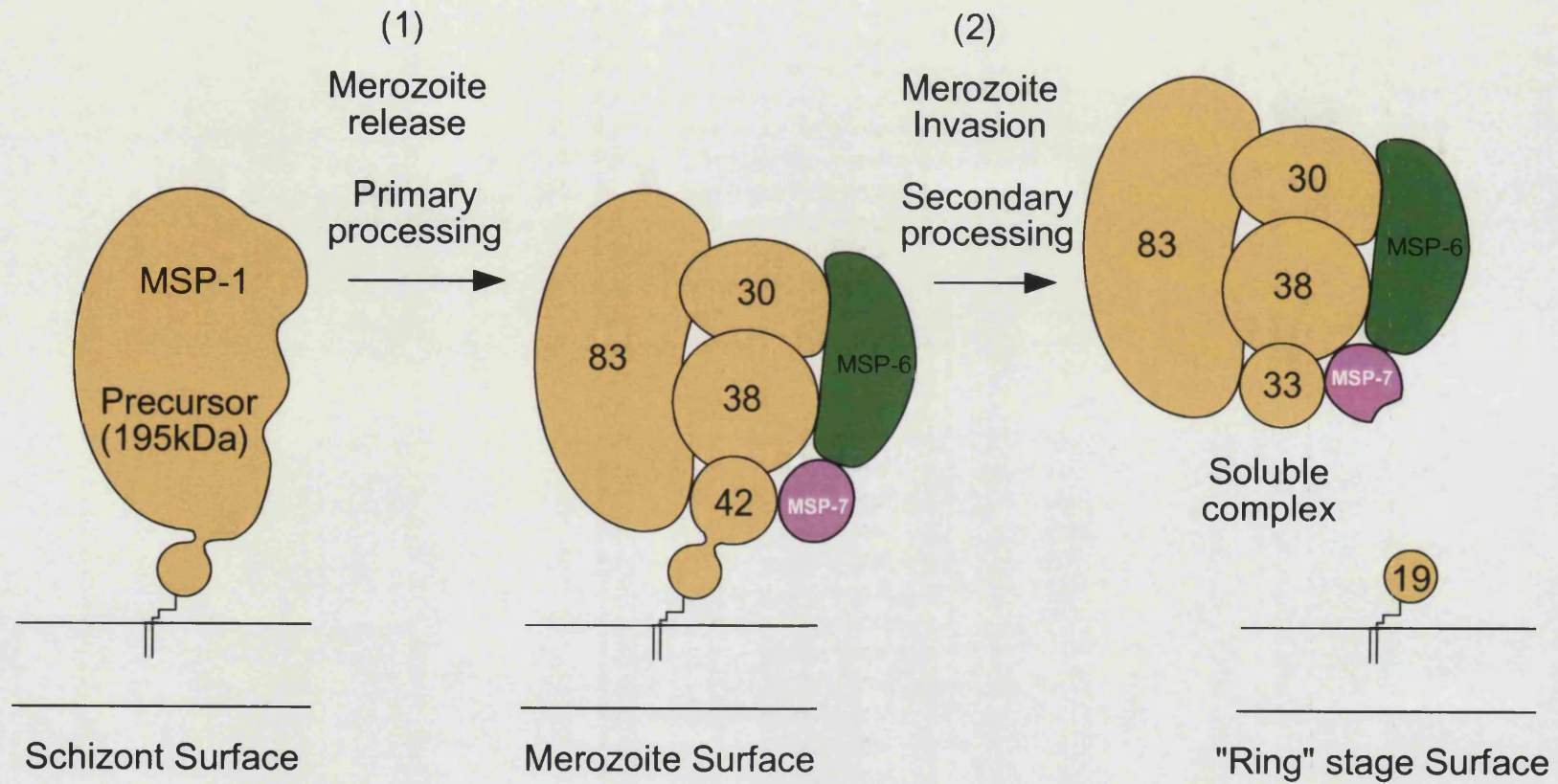


Figure 1.2

The structure of MSP-1₁₉

The solution structure of *P. falciparum* MSP-1₁₉ has been determined by nuclear magnetic resonance (Morgan, Birdsall et al. 1999) and the crystal structure of the homologous molecule from *P. cynomolgi* has also been determined (Chitarra, Holm et al. 1999). Both studies confirm that the protein structure has two domains with epidermal growth factor (EGF)-like folds. The protein has a U-shaped overall structure with the N-terminal proteolytic processing site close to the C-terminal GPI membrane anchor site, consistent with the involvement of a membrane bound protease in secondary processing.

Here MSP-1₁₉ is shown in four different orientations. The N-terminus (pink) and the C-terminus (dark grey) are identified, and can be seen to be close to each other. The first EGF-like fold is shown in lighter grey and the second EGF-like fold is shown in darker grey.

Figure 1.2

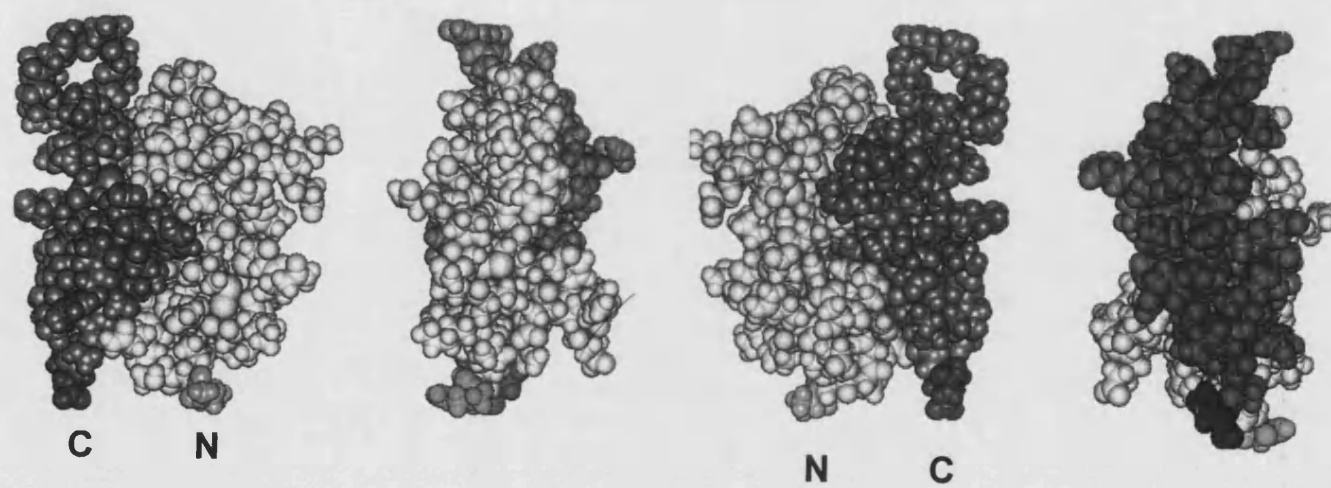


Figure 1.3

MSP-1 secondary processing and erythrocyte invasion

Merozoite Surface Antigen-1 (MSP-1) covers the surface of the merozoite. MSP-1 exists as a protein complex, and at the time of erythrocyte invasion, the MSP-1₄₂ component of the complex is cleaved by a parasite-encoded protease. Most of the MSP-1 complex is shed, including a 33 kDa fragment of MSP-1₄₂. A 19 kDa fragment, MSP-1₁₉, remains on the surface of the invading merozoite and is carried into the erythrocyte. Antibodies which prevent this cleavage also prevent erythrocyte invasion.

Figure 1.3

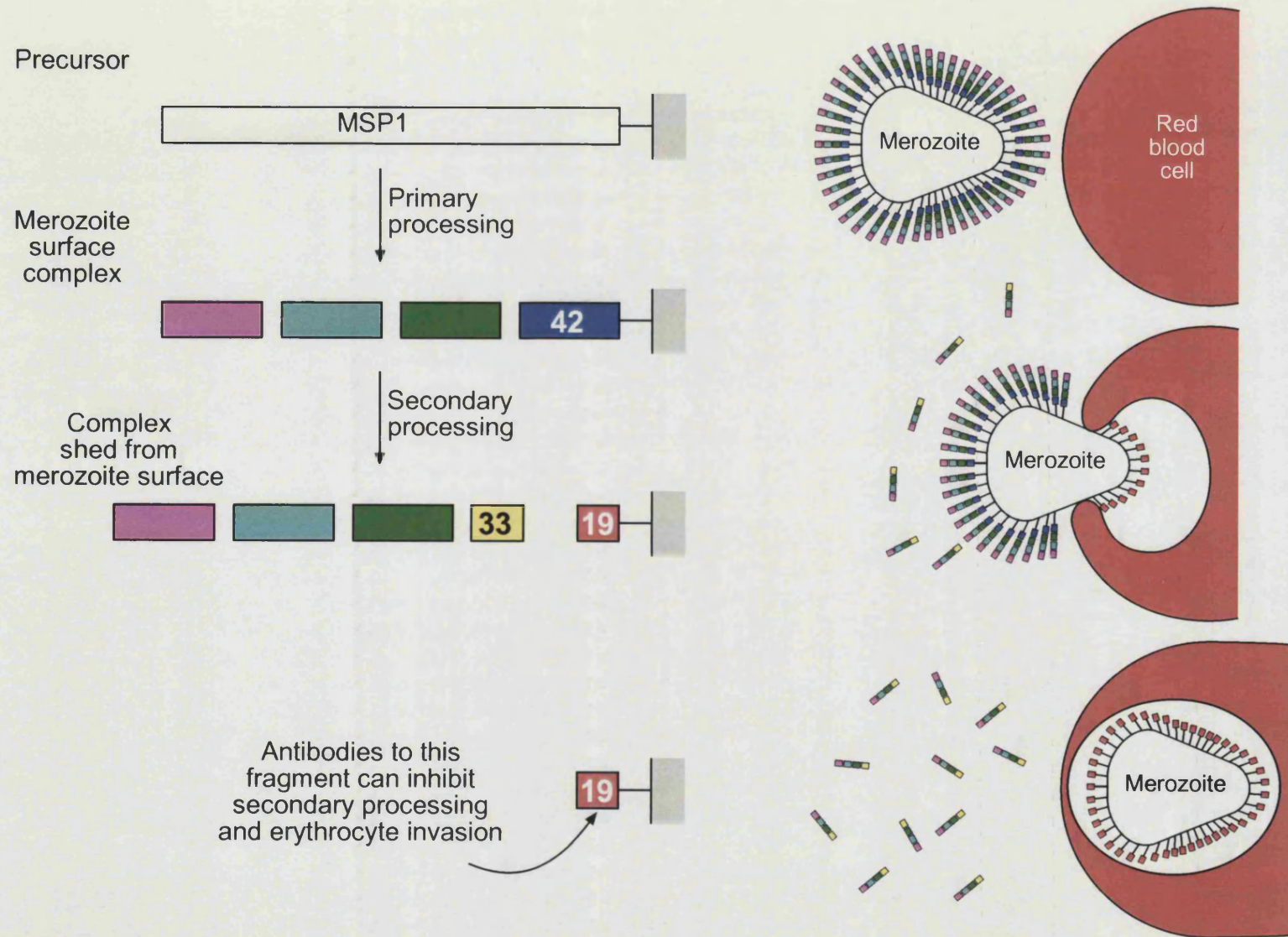


Figure 1.4

Natural and phage display-based antibody selection

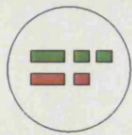
The successive steps of the phage display technique mimic the natural processes of immune selection (Winter, Griffiths et al. 1994). The two processes of natural and phage-display based antibody selection are compared schematically here.

In the natural immune system, a finite number of V-genes encode sections of the variable regions of the heavy and light chains of antibodies. As stem cells mature to B cells, these V-genes are rearranged in many different permutations, yielding an immense repertoire of different antibody antigen-binding sites. Each B cell displays just one antibody of one antigen specificity. Interaction with the target antigen leads to selection of the relevant B cell clone, which may either proliferate to make short-lived plasma cells secreting the antibody or long-lived memory cells, capable of making antibodies of improved binding affinity through hypermutation on repeated stimulus with the antigen.

In the process of phage display, rearranged V-genes are amplified by PCR from B cells. Heavy and light chain V-genes are then randomly joined together in a further PCR to produce a repertoire of DNA encoding antibody fragments analogous to the original antibody repertoire. Each antibody fragment is displayed on the surface of a filamentous phage in a manner analogous to the display of antibody on the surface of a B cell. The antibody fragments displayed on phage are selected by binding to the relevant antigen, and can proliferate by replication in *E. coli*. Just as B cells may become plasma cells and secrete antibody or become memory cells developing affinity matured antibody, so antibody fragments displayed on phage may be secreted or undergo affinity maturation by repeated rounds of selection on antigen or by the introduction of random mutations.

Figure 1.4

Natural Immune Selection



Stem Cell:
Unrearranged V-genes



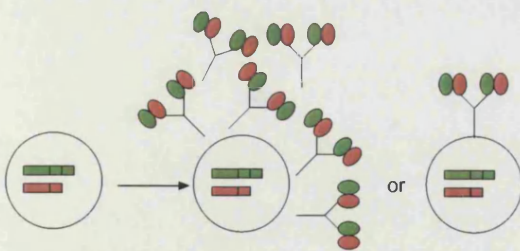
B Cell:
Rearranged V-genes:
random combinations
of V_H and V_L



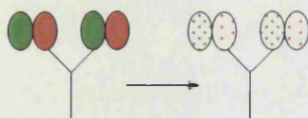
B Cell displays antibody on surface



Specific B Cell selected by
antigen and proliferates



B cell differentiates into
a) Plasma cell secreting antibody
b) Memory cell displaying antibody

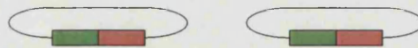


Antibody undergoes affinity maturation

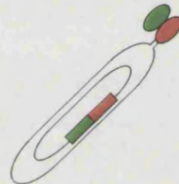
Phage Display



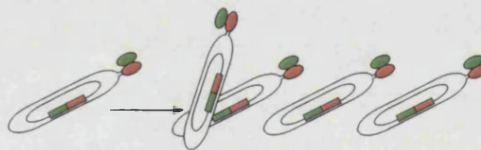
Rearranged V-genes
(amplified by PCR)



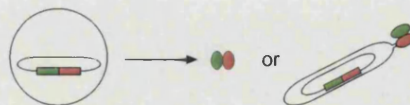
V-genes joined as scFv in phagemid:
random combinations of V_H and V_L



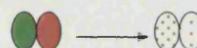
Phage displays scFv on surface



Specific phage selected by antigen,
eluted and amplified



E. coli recombinants induced to
a) secrete soluble scFv
b) produce phage displaying antibody



Affinity improved by
a) repeated rounds of selection
b) introduction of random mutations

Figure 1.5

Single chain variable fragment (scFv) and antibody

The whole IgG molecule is made up of two heavy chains and two light chains, providing two antigen-binding sites. Antigen specificity is determined by the variable regions of both the heavy chain (V_H , shown in red) and the light chain (V_L , shown in green). The remainder of the IgG molecule consists of constant regions (C_L and $C_{H1}-C_{H3}$, shown in pink) whose sequences are conserved.

The antigen-binding site of an IgG molecule can be reconfigured as a single chain molecule (scFv) which retains the antigen specificity of the parent antibody. The scFv consists of the V_H and V_L regions, joined together by a flexible linker (shown in blue).

The scFv molecule differs from the whole IgG molecule in avidity, having only one antigen-binding site, and in size, having a molecular mass of approximately 30 kDa, compared with a mass of approximately 150 kDa for the whole IgG.

Figure 1.5

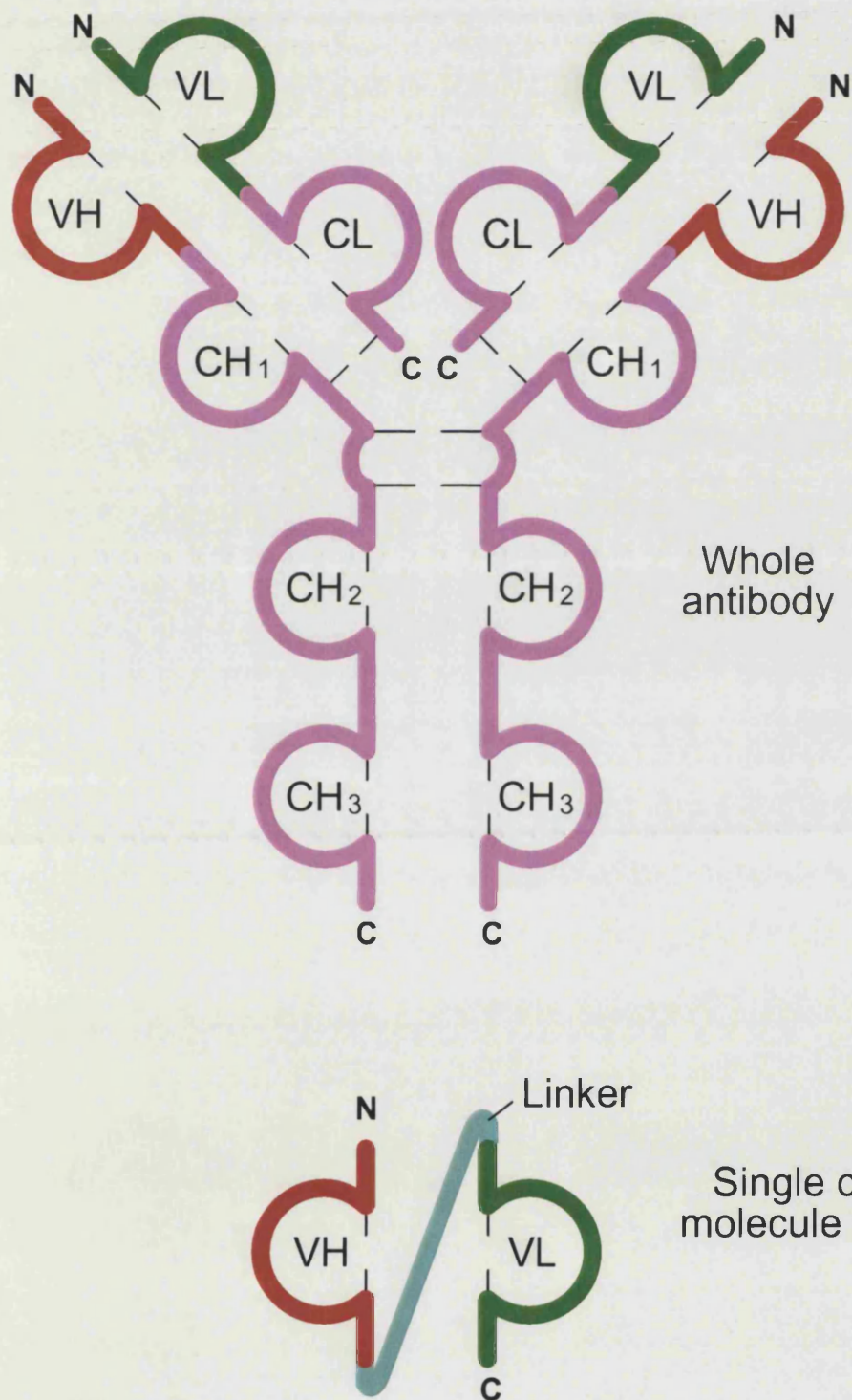


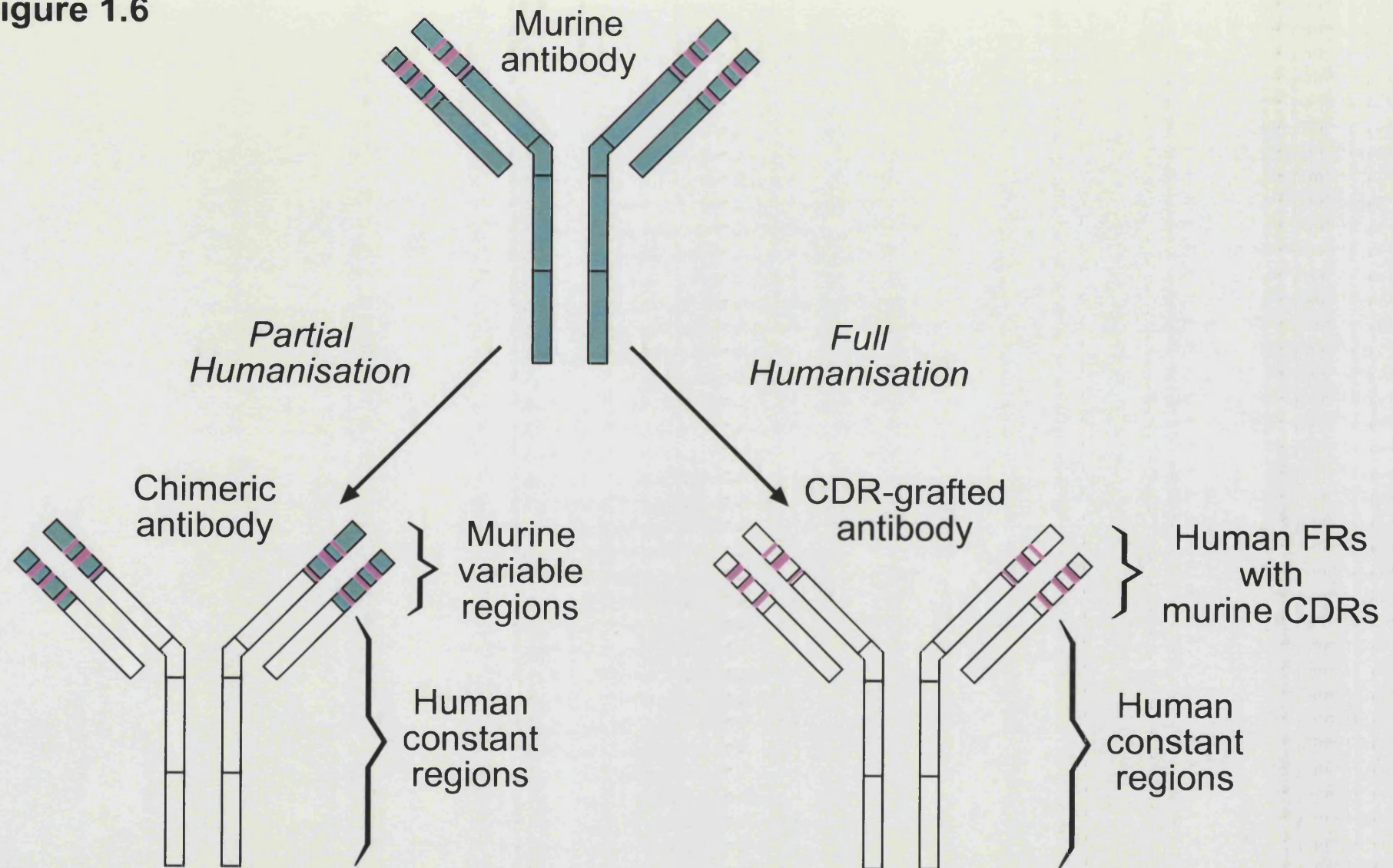
Figure 1.6

Murine, chimeric and humanised antibodies

A rodent antibody may be adapted to reduce potential immunogenicity prior to any therapeutic application in man. An antibody may be “partially humanised” by grafting the rodent variable regions of the antibody onto human constant regions to make a chimeric antibody. To reduce potential immunogenicity still further, an antibody may be “fully humanised” by grafting only the rodent complementarity-determining regions (CDRs) into an otherwise human antibody. This is achieved by changing the framework residues (FRs) between the CDRs to residues commonly found in human antibody sequences. The humanised variable regions are then grafted onto human constant regions as before.

In this figure, the rodent CDRs are shown in pink, rodent framework and constant regions are shown in blue, and human framework and constant regions are shown in white.

Figure 1.6



CHAPTER 2

MATERIALS AND METHODS

2.1 mAb 12.8 and mAb 12.10 variable region cloning and sequencing

2.1.1 Preparation of whole RNA and cDNA from 12.8 and 12.10 murine hybridoma cells

Murine hybridoma cell lines 12.8 and 12.10 (McBride, Newbold et al. 1985) (kindly supplied by Dr Jana McBride of the University of Edinburgh) were grown up, and total RNA extracted and purified on a caesium chloride gradient as follows. 10^7 cells were pelleted and resuspended in 7.7 ml filter sterilised GIT buffer (4M guanidine thiocyanate, 4 mM sodium acetate pH 6.0, in 100 ml RNase-free H₂O, to which 64 μ l mercaptoethanol was added), and lysed by vortex. The lysate was stored on ice for 5 min, layered onto 4 ml of 5.7 M caesium chloride in diethylpyrocarbonate (DEPC)-treated H₂O and centrifuged at 174,000 x g in a swing out rotor overnight at 20°C. The RNA pellet was resuspended in 300 μ l H₂O, visualised by electrophoresis on a 1% agarose gel with ethidium bromide staining, and the RNA concentration was measured by absorbance at 260 nm. The RNA was stored in 75% ethanol at -70°C.

Alternatively total RNA was extracted using the RNA Stat-60 kit (Biogenesis Ltd).

First strand cDNA was produced for amplification using the First-Strand cDNA Synthesis Kit (Pharmacia Biotech) with the *Not1*-d(T)₁₈ primer according to the manufacturer's instructions.

2.1.2 PCR amplification of mAb 12.8 and mAb 12.10 heavy and light chain variable region sequences from cDNA

The DNA encoding the variable regions of the antibody heavy and light chains was amplified from hybridoma cDNA using 12 different sets of degenerate primers designed to anneal to the different possible leader sequences of the variable region (MHV and MKV primers for mouse IgG heavy and light (kappa) chains respectively), and a single primer or primer mix designed to anneal to the 5' end of the constant region (MHC and MKC primer for mouse IgG heavy and light chains respectively). The MHC primer was an equimolar mix of four primers, MHCG1, MHCG2a, MHCG2b and MHCG3, designed to anneal to the constant regions of mouse IgG1, IgG2a, IgG2b and IgG3 respectively. The primer sequences are shown in Table 2.1.

The PCR reaction was carried out in a total volume of 50 μ l, constituted as follows;

cDNA/mRNA hybrid	1 μ l
10x PCR Buffer II	5 μ l
25 mM MgCl ₂	3 μ l
10 mM dNTPs	4 μ l
10 μ M MHV or MKV primer	1.25 μ l
10 μ M MHC or MKC primer	1.25 μ l
H ₂ O	32 μ l
AmpliTaq polymerase 1 unit	2.5 μ l

PCR was carried out using a hot start, with 25 cycles melting at 94°C for 1 minute, annealing at 55°C for 1 minute, extending at 72°C for 2 minutes, followed by extension at 72°C for 10 minutes. PCR products were run on a 1% low melting point agarose electrophoresis gel, and extracted using the Wizard Prep kit (Promega).

2.1.3 Cloning of mAb 12.8 and mAb 12.10 variable region DNA sequences

PCR products were ligated into the pCRTM2.1 vector (Invitrogen) and then used to transform *E. coli* XL-1 Blue cells by heat shock according to the Invitrogen protocol. Transformants with inserts were identified by PCR screen using the same MHV/MKV and MHC/MKC primers as were used for the initial amplification of the IgG variable region DNA. The same PCR conditions were used. Cycling was carried out for 20 cycles, melting at 94°C for 1 minute, annealing at 60°C for 1 minute, extending at 72°C for 2 minutes, finally extending at 72°C for 10 minutes. The products were analysed by gel electrophoresis. Positive clones identified by PCR screening were stored as glycerol stocks at -80°C. The mAb 12.8 and mAb 12.10 variable region sequence inserts in the pCRTM2.1 vector were sequenced using TA Forward and TA Reverse primers, cycling with an annealing temperature of 40°C.

TA Forward Primer: CGAGCTCGTACGTAGA

TA Reverse Primer: TACCGAGCTCGGATCCACTA

2.1.4 Attempted N-terminal amino acid sequencing of mAb 12.8 and mAb 12.10 heavy and light chains

Using 2 µg of each mAb, 12.8 and 12.10, the heavy and light chains were resolved by SDS-PAGE using CAPS buffer (100 mM 3-[cyclohexylamino]-1-propanesulphonic acid [CAPS] pH 11.0). The resolved proteins were transferred onto an immobilon membrane. Electroblotting buffer was 10 mM CAPS and 10% (v/v) methanol in H₂O. After transfer the membrane was saturated with 100% methanol for 5 seconds then stained with 0.1% Coomassie Blue in 40% (v/v) methanol, 10% (v/v) acetic acid in H₂O for 1 min. The membrane was destained with 50% methanol (v/v) in H₂O. The transferred stained protein was sent to Dr Arthur Moir of the Department of Molecular Biology and Biotechnology at the University of Sheffield for N-terminal sequencing based on the Edman degradation reaction.

To overcome N-terminal blocking of the proteins, the mAbs were incubated with pyroglutamate aminopeptidase to remove putative N-terminal pyrrolidone carboxylic acid residues prior to SDS-PAGE. Incubation with pyroglutamate aminopeptidase was carried out using 10 µg of mAb in 0.1 M sodium phosphate pH 8.0 containing 5 mM dithiothreitol (DTT) and 10 mM EDTA. Approximately 0.2 µg of pyroglutamate aminopeptidase was added and the reaction was allowed to proceed at room temperature overnight.

2.1.5 Amino acid sequencing of hybridoma antibodies by nanospray tandem mass spectrometry

The mAb 12.8 and mAb 12.10 IgG heavy and light chains were fractionated by electrophoresis using a 12% polyacrylamide gel under reducing conditions. The fractionated proteins were stained for 5 minutes with 50% (v/v) MeOH, 10% (v/v) acetic acid, 0.1% (w/v) Coomassie Blue, and destained with 12% (v/v) MeOH, 7% (v/v) acetic acid. The stained bands were excised and SDS extracted by incubating with 200 mM ammonium bicarbonate (ABC)/50% acetonitrile (ACN) buffer for 30 minutes at 30°C. The protein was then reduced with excess 20 mM dithiothreitol in 200 mM ABC for 1 hr at 30°C, and then washed with 200 mM ABC buffer. Cysteines were alkylated with freshly prepared 50 mM iodoacetamide in 200 mM ABC in the dark for 20 min at room temperature, and the bands were then washed extensively with 50 mM ABC to remove excess iodoacetamide. The gel containing the protein was then cut in small pieces and covered in 100% ACN for 15 min, and dried in a vacuum centrifuge.

The protein was digested with trypsin. A sufficient volume of 2 ng/μl trypsin (modified sequencing grade Promega, Madison, WI) in 5 mM NH_4HCO_3 was added to cover the gel pieces and digestion performed overnight at 32°C in an incubator. The digests were then acidified by the addition of a 1/10 volume of 2% trifluoroacetic acid prior to analysis.

Digestion supernatant was loaded onto a 2mm x 0.8mm C18 microcolumn (LC Packings, Amsterdam, Netherlands), washed and step eluted with 60% methanol, 0.1% formic acid directly into an Econo12 nanospray needle (New Objective Inc, Cambridge, MA). Nanospray (Wilm and Mann 1996) tandem mass spectra were acquired on an LCQ “classic” quadrupole ion trap mass spectrometer (ThermoQuest Corporation, Austin, TX)

equipped with a nanospray source (Protana, Odense, Denmark) operated at a spray voltage of 800V and a capillary temperature of 150°C. Tandem (MS²) mass spectra were acquired at a collision energy of 30% and a parent ion isolation width of 3 Da. Masses of all tryptic fragments, charged and uncharged, were computer-predicted from candidate sequences derived from DNA sequencing. Fragments corresponding to predicted masses were isolated, and the daughter ion spectrum determined to confirm amino acid sequences.

2.2 Murine/Human chimeric mAbs 12.8 and 12.10

2.2.1 Transient expression of chimeric mAbs 12.8 and 12.10 in *cos* cells

Cos-7 cells (American Type Culture Collection CRL 1651) were grown until confluent in Dulbecco's Modified Eagles Medium (DMEM) (Life Technologies 41966-029) supplemented with 10% (v/v) foetal calf serum (Hyclone A 1101-L), 580 µg/ml L-glutamine, 50 units/ml penicillin and 50 µg/ml streptomycin in a 150 cm² flask. The cells were then trypsinised, centrifuged at 250g for 5 min then resuspended in 6 ml of medium as above. They were then divided equally between three 150 cm² flasks each containing 25 ml of fresh pre-warmed medium as above. The cells were incubated overnight at 37°C in 5% CO₂ in air and then harvested while growing exponentially. The cells were trypsinised, centrifuged again as above, and then washed in 20 ml PBS and resuspended in PBS to a concentration of 1x10⁷ cells/ml. Seven hundred microlitres of these cells were then pipetted into a Gene Pulser cuvette (Bio-Rad 165-2088). Ten micrograms of both the heavy chain and light chain expression vector DNA for either chimeric mAb 12.8 or 12.10 was added and electroporated with a 1900 Volt 25 µFarad capacitance pulse using the Bio-Rad Gene Pulser (165-2078). The electroporated *cos* cells

were left at room temperature for 10 minutes then gently added to a 10 cm diameter tissue culture dish containing 8 ml of pre-warmed DMEM supplemented with 5% (v/v) γ -globulin free foetal calf serum plus L-glutamine, penicillin and streptomycin as above. The cells were incubated in 5% CO₂ at 37°C for 72 hours and then the supernatant containing expressed chimeric antibody was harvested for analysis.

2.2.2 Purification of chimeric mAbs 12.8 and 12.10 using Protein A

Expressed chimeric mAbs 12.8 and 12.10 were purified from *cos* cell supernatants using the ImmunoPure IgG (Protein A) Purification Kit (Pierce, 44667). The Protein A column was equilibrated with 5 ml of PBS pH 8.0 or the Immunopure IgG Binding Buffer. The *cos* cell supernatant was diluted with an equal volume of PBS and added to the column. The column was then washed with 15 ml PBS. The bound IgG was eluted with 5 ml ImmunoPure IgG Elution Buffer.

2.2.3 Quantification of expressed chimeric mAbs 12.8 and 12.10 by ELISA

A 96-well Nunc-Immuno MaxiSorp plate was coated with 100 μ l/well of 0.4 μ g/ml goat anti-human IgG antibody, Fc γ fragment-specific (Jackson ImmunoResearch Labs, 109-005-098) in PBS. This was left to incubate at 4°C overnight. The excess coating solution was removed and the plate was washed 3 times with 200 μ l/well washing buffer (1x PBS with 0.1% [v/v] Tween). 100 μ l SEC buffer (1x PBS with 0.02% [v/v] Tween 20, 0.2% (w/v) BSA) was added to each well. 200 μ l of a solution of the chimeric mAb at a known concentration of 1 μ g/ml in SEC buffer was added to two of the wells to serve as a standard. 200 μ l of experimental *cos* cell supernatants under test were added to the

appropriate wells in serial 2-fold dilutions in SEC buffer. The plate was incubated at 37°C for 1 hour. The wells were rinsed 6 times with 200 µl wash buffer. 100 µl peroxidase-conjugated goat anti-human kappa light chain (Sigma, A-7164), diluted 1:5000 in SEC buffer, was added to each well. The incubation and wash were repeated as above. Colour was developed by adding 150 µl K-BLUE substrate (Sky Bio, KB176) to each well and leaving in darkness for 10 minutes then adding 50 µl RED STOP (Sky Bio, RS20) solution to each well. The absorbance was read at 655 nm.

2.3 Construction and expression of scFv fragments from mAb 12.8 and mAb 12.10 DNA

2.3.1 Construction of single-chain variable fragments (scFvs) from mAb 12.8 and mAb 12.10

The amplified heavy chain and light chain variable (V_H and V_L) DNA sequences were assembled into a construct encoding a single-chain Fv by means of a three-step PCR reaction, illustrated in Figure 2.1. The oligonucleotide primers used are shown in Table 2.2.

In the first step, the V_H DNA was amplified with a 5' primer, 12.8 V_H .BackSfi or 12.10 V_H .BackSfi, (designed to anneal to the 5' end of the gene just after the leader sequence) which introduced an *Sfi*I and *Nco*I site, and a 3' primer, 12.8 V_H .For or 12.10 V_H .For. The V_L DNA was amplified with a 5' primer, 12.8 V_L .Back or 12.10 V_L .Back, and a 3' primer, 12.8 V_L .ForNotI or 12.10 V_L .ForNotI, which added a *Not*I site. DNA encoding an 18 amino acid flexible linker was amplified with a 5' primer, 12.8Linker218.Back or 12.10Linker218.Back, designed to overlap with the 3' end of the V_H , and a 3' primer,

12.8Linker218.For or 12.10Linker218.For, designed to overlap with the 5' end of the V_L.

The sequence of the flexible linker is shown in Table 2.2.

In the second step, the V_H PCR product from step 1 was combined with the linker DNA using 12.8V_H.BackSfi or 12.10V_H.BackSfi and 12.8Linker218.For or 12.10Linker218.For.

The V_L PCR product from step 1 was also combined with the linker DNA, using 12.8Linker218.Back or 12.10Linker218.Back and 12.8V_L.ForNotI or 12.10V_L.ForNotI.

In the third and final step, the PCR products from step 2 were united into a single chain Fv using 12.8V_H.BackSfi or 12.10V_H.BackSfi and 12.8V_L.ForNotI or 12.10V_L.ForNotI.

PCR reactions were carried out as before, but in a volume of 100 µl, using 20 nanograms of template DNA, and amplifying with Deep Vent polymerase. An annealing temperature of 65°C was used for steps 1 and 2, and an annealing temperature of 55°C was used for step 3.

The scFvs were cloned in *E. coli* XL1-Blue and sequenced as before.

2.3.2 Repair of scFv 12.10 DNA sequence by site-directed mutagenesis

On sequencing the DNA encoding scFv 12.10, an unwanted single base pair mutation was found encoding the substitution of proline for serine at position 17 of the first framework region of the heavy chain. A primer, N1210V_HFr1.rev, was designed to enable correction of the sequence using PCR to produce a site-directed mutagenesis. The method is illustrated diagrammatically in Figure 2.2. N1210V_HFr1.rev was designed to anneal to the framework 1 region of the heavy chain, and this primer incorporated the correct DNA sequence without the unwanted mutation. The sequence of N1210V_HFr1.rev is shown below with the nucleotide correction underlined.

N1210V_HFr1.rev : GACAGCTTCACTGAAGCCCCAGGCTTC.

Using the mutated scFv DNA as a template, this primer was used with 1210V_H.BackSfi to amplify a 5' portion of scFv 12.10 approximately 75 base pairs long, which incorporated the corrected sequence rather than the unwanted mutation. The 75 base pair product from this PCR was then used as a primer in conjunction with the primer 1210.V_LForNotI, to amplify the whole of scFv 12.10 (again using the DNA with the unwanted mutation as a template). This second PCR was intended to yield a full-length scFv product in which the mutation had reverted back to the desired sequence.

The first step PCR in the site-directed mutagenesis was set up as follows:

DNA template* 5 ng/μl	10 μl
10x Pfu buffer	5 μl
25 mM MgCl ₂	2 μl
10 mM dNTP	4 μl
10 μM N1210scFv.V _H Fr1.rev	1.25 μl
10 μM 1210V _H .BackSfi	1.25 μl
H ₂ O	25.5 μl
Pfu	1 μl

*The DNA template was the DNA encoding the scFv 12.10 with the unwanted mutation

The reaction was cycled at 94°C 5 min, then 94°C 1 min, 55°C 1 min, 72°C 2 min for 25 cycles, then 72°C 10 min.

Pfu was used in preference to AmpliTaq polymerase because AmpliTaq would add A overhangs which would introduce further mutations. The product of the first PCR above was gel-purified using the Wizard Prep kit (Promega) to a volume of 50 μl and 1 μl of this was used as Primer 2 in the second PCR reaction below. The DNA template was the same as that used in the first PCR, above. The second PCR was set up as follows:

DNA template 5 ng/μl	10 μl
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10x Pfu buffer	5 µl
25 mM MgCl ₂	2 µl
10 mM dNTP	4 µl
10 µM N1210scFv.V _L ForNotI	1.25 µl
Primer 2 (3 ng/µl)	2 µl
H ₂ O	24.75 µl
Pfu	1 µl

The reaction was cycled as before.

2.3.3 Subcloning of scFv 12.8 and scFv 12.10 sequences into expression plasmid and phagemid vectors

Plasmids with the scFv 12.8 and scFv 12.10 inserts were digested with *SfiI* and *NotI*, and the DNA was extracted with phenol/chloroform. The scFv sequences were ligated as *SfiI/NotI* fragments into *SfiI/NotI*-cut expression vectors. Two vectors were used. Initially the scFvs were ligated into pUC/c-*myc*/H6, a pUC-derived expression vector with C-terminal c-*myc* and hexahistidine tag sequences (obtained from Dr Olivier Leger, MRC Collaborative Centre) and, subsequently, the scFvs were ligated into pHEN1H6. pHEN1 is a phagemid vector designed for the expression of scFvs either on the surface of M13 filamentous phage (as a fusion with the phage gene III structural protein), or as a soluble free protein with a C-terminal c-*myc* tag (Hoogenboom, Griffiths et al. 1991). pHEN1H6 is adapted from pHEN1 by the addition of a C-terminal hexahistidine tag in front of the C-terminal c-*myc* tag. Ligations were performed in a volume of 10 µl using approximately 50 ng vector DNA, 100 ng insert DNA, 1 µl (4 Weiss units) T4 DNA Ligase (Invitrogen) and 1 µl 10x Ligation Buffer (Invitrogen), overnight at 16°C. Forty microlitres of *E. coli*

TG1 or HB21/51 competent cells were transformed using 1 μ l of the ligation reactions (approximately 10 ng of DNA) by electroporation (1.7kV), resuspended in 1 ml SOC buffer (2% [w/v] bacto-tryptone, 0.5% [w/v] bacto-yeast extract, 8.5 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 20 mM glucose pH 7.0) at 37°C, shaken in an Innova 4000 incubator shaker (New Brunswick Scientific) at 30 rpm for 1 hour at 37°C and then plated onto agar.

The phagemid vector pHEN1H6 is illustrated in Figure 2.3.

scFv 12.10 was also subcloned into the phagemid vector pHEN1H6ABD2. The vector pHEN1H6ABD2 was constructed by Dr Jonathan Chappel and is adapted from pHEN1H6 by the addition of two albumin-binding domains (ABDs) positioned immediately after the *NotI* site and immediately before the hexahistidine tag. Expression in this vector results in a scFv expressed as a fusion with the two albumin-binding domains and the hexahistidine and *c-myc* tags. The purpose of creating such a fusion is to express scFv which, in the presence of albumin, will bind two albumin molecules (molecular weight 68 kDa) and thereby acquire a total mass approximating to that of a whole antibody.

The albumin-binding domains are flanked with 3 additional glycine residues in order to facilitate flexibility in the fusion protein and to preserve the binding of the scFv, ABDs and hexahistidine and *c-myc* tags. The albumin-binding domain sequence was obtained from the plasmid pASK90-abd-D1.3 supplied by Arne Skerra (Technische Universität München).

The vector pHEN1H6ABD2, with the sequence of the albumin-binding domain, is shown in Figure 2.4.

2.4 Sequencing, expressing, purifying and detecting scFvs

2.4.1 DNA sequencing

To prepare DNA for sequencing, glycerol stocks were inoculated into either 15 ml or 50 ml of 2TY medium (16 g tryptone, 10 g yeast extract, 5 g NaCl per litre) + 100 µg/ml ampicillin, and grown with shaking in an Innova 4000 incubator shaker at 300 rpm overnight at 37°C. Plasmid DNA was then purified using the Qiagen Mini/Midiprep Plasmid Purification Protocol. DNA to be sequenced was prepared using the ABI PRISM dRhodamine terminator cycle sequencing ready reaction kit (Applied Biosystems). For each sequencing reaction, 200-500 ng of the template DNA to be sequenced and 3.2 pmol of primer DNA were added to 8.0 µl of Terminator Ready Reaction Mix and made up with H₂O to a volume of 20 µl. Reactions were cycled at 96°C for 45 seconds, 50°C for 30 seconds, 60°C for 4 minutes, for 25 cycles, with rapid thermal ramping. The resulting extension products were purified by adding them to 2.0 µl 3 M sodium acetate pH 4.6 with 50 µl 95% (v/v) ethanol. The tubes were vortexed and placed on ice for 10 minutes to precipitate the extension products. The tubes were then spun in an Eppendorf microcentrifuge 5415 C at 13,000 rpm (11,340 g) for 30 minutes. The supernatant was aspirated and discarded and the pellet was rinsed with 250 µl 70% (v/v) ethanol. The tubes were centrifuged again as above for 5 minutes and the supernatant was discarded and the pellets were air-dried. Pellets were resuspended in loading buffer (deionised formamide in a 5:1 ratio with 25 mM EDTA [pH 8.0] with blue dextran [50 mg/ml]). The samples were vortexed and centrifuged and heated at 95°C for 2 minutes to denature and then loaded onto the sequencing gel. Sequencing was carried out using the Perkin Elmer ABI Prism 377 DNA Sequencer.

2.4.2 Expression of scFvs in *E. coli*

An agar plate was streaked from a glycerol stock of the scFv clone, and incubated at 37°C overnight. A fresh colony was inoculated into 25 ml 2TYAG (16g tryptone, 10g yeast extract, 5g NaCl per litre + 100µg/ml ampicillin, supplemented with 2% w/v glucose), and grown overnight at 30°C in an Innova 4000 incubator shaker at 300 rpm. The culture was then added to 1 litre of pre-warmed 2TYAG and grown in an Innova 4000 incubator shaker at 300 rpm for 4 hours at 30°C in a baffled flask. The cells were then pelleted by centrifugation at 3000 rpm (1240 g) for 15 minutes in a pre-cooled Beckman centrifuge with a JA-17 rotor, and resuspended in 1 litre 2TY containing 100 µg/ml ampicillin and 1 mM isopropylthiogalactopyranoside (IPTG), pre-warmed to 30°C, and grown in an Innova 4000 incubator shaker at 300 rpm in a baffled flask for 4 hours. The cells were then pelleted as before and frozen at -80°C for later periplasmic extraction.

Periplasmic extracts were made by thawing the cell pellet into 20 ml periplasmic buffer (25 mM Tris-HCl pH 7.5, 20% sucrose, 200 mM NaCl, 1 mM EDTA) and shaking at 4°C for 1 hour. If proteolytic breakdown was known to occur, phenyl methyl sulphonyl fluoride (PMSF) was added to the periplasmic buffer to 0.1 mM before use. The suspension was then centrifuged at 14,000 rpm (27,000 g) in a Beckman centrifuge with a JA-17 rotor at 4°C for 15 min to pellet the cell debris, and the supernatant containing the periplasmic fraction was retained.

2.4.3 Purification of expressed scFvs using hexahistidine tag

MgCl₂ was added to the periplasmic fraction to a concentration of 2 mM, and then 100 µg DNaseI was added. One millilitre of 50% Ni-NTA agarose slurry (Qiagen), pre-washed 3 times in wash buffer (50 mM Na phosphate, 300 mM NaCl, 10 mM imidazole, pH 8.0), was added to the periplasmic fraction and incubated on a rotating platform at 4°C for 1 hour. The resin was then pelleted in an Eppendorf microcentrifuge 5415C at 1000 rpm (70 g) for 1 minute and, after removal of 90% of the supernatant, the slurry was added to a Qiagen 1 ml column (#34024) which was allowed to drain under gravity. Two x 4 ml wash buffer was applied, and then the bound scFv was eluted with 4x 0.5 ml elution buffer (50 mM Na phosphate, 300 mM NaCl, 250 mM imidazole, pH 8.0). Eluted scFv was dialysed in 3 changes of PBS at 4°C over 24 hr using Pierce Slide-A-Lyzer 10 kDa cut-off dialysis cassettes, and stored at 4°C.

2.4.4 Detection of expressed scFvs by Western blot using *c-myc* or hexahistidine tag

The scFv was resolved by SDS-PAGE on a 12% polyacrylamide gel under non-reducing conditions, and transferred to an Immobilon nitrocellulose membrane. The scFv was detected using Qiagen Anti-PentaHis antibody according to the Qiagen protocol, using horseradish peroxidase conjugated goat anti-mouse IgG (H+L) (Sigma) and ECL chemiluminescent detection (Amersham Life Science). Alternatively, anti-*c-myc* 9E10 hybridoma supernatant was used in place of the Anti-PentaHis antibody.

2.5 Construction of the phage display libraries

2.5.1 Selection of malaria-exposed donors

Blood donors were selected from among adult men and women resident in the village of Brefet and its environs, in The Gambia, West Africa. Of 20 donors selected, 10 were selected on the basis of previously noted high levels of anti-MSP-1 IgG as detected by ELISA in a previous study (Egan, Waterfall et al. 1997). The remainder was chosen at random from among volunteers in the village. Equal numbers of male and female donors were selected, and the majority of female donors had had one or more pregnancies. Details of donors are shown in Table 2.3.

Blood was collected for one library in March 1997 (dry season; low prevalence of clinical malaria) and for the other library in October 1997 (end of wet season; high prevalence of clinical malaria).

2.5.2 Isolation of donor peripheral blood mononuclear leucocytes (PBMLs)

Twenty millilitres of venous blood was taken from each donor and added to a 25 ml universal container containing 5 ml RPMI 1640 with 50 units/ml penicillin, 50 µg/ml streptomycin and 250 units of heparin. The universal containers were filled to the top with medium, and samples were transported from the field to the laboratory (approximately 2 hours) at 4°C and then allowed to return to room temperature in the laboratory. The blood was then further diluted 1:1 with RPMI 1640 plus penicillin and streptomycin as above and 20 ml of diluted blood was layered carefully onto 15 ml Ficoll-Paque PLUS (Pharmacia Biotech 17-1440-02) and centrifuged in a Sigma 4-10 centrifuge at 3500 rpm (2340 g) for

25 minutes at room temperature with the brake off. The plasma was then removed, aliquoted and frozen at -20°C . The layer containing PBMLs was diluted in 10 ml RPMI 1640 plus 5% foetal calf serum and centrifuged at 3500 rpm (2340 g) for 15 minutes at room temperature. The pellet was then resuspended in 6 ml RPMI 1640 plus 5% foetal calf serum. This wash was repeated twice, spinning once at 3500 rpm (2340 g) and then at 1700 rpm (510 g) and the cells were then resuspended in 3 ml RPMI 1640 plus 5% foetal calf serum, and stored briefly at 37°C . Cell counts and viability tests were performed with a haemocytometer using 25 μl of cell suspension mixed with 25 μl of trypan blue.

2.5.3 Extraction of total RNA from donor PBMLs

RNA extractions were performed using the Qiagen RNeasy Midiprep kit. Cells from 5-6 separate blood donations were pooled for RNA extraction. One millilitre of cell suspension in RPMI 1640 was taken from each processed donation. The cells were spun at 300 g for 5 minutes and the supernatant aspirated. The pellet was loosened and 3.8 ml Qiagen Buffer RLT plus β -mercaptoethanol was added to lyse the cells. The cell lysate was vortexed for 10 seconds and then passed 10 times through a 20-gauge needle. An equal volume of 70% ethanol was added and the cell lysate was vortexed again. The lysate was then added to a Qiagen RNeasy midi spin column and centrifuged at 4000 g for 5 minutes. The column was washed with 3.8 ml Qiagen buffer RW1 and spun at 4000 g for 5 minutes. The column was then washed again with 2.5 ml Qiagen buffer RPE diluted with 100% ethanol 1:4 v/v and centrifuged at 4000 g for 2 minutes. Finally 2.5 ml Qiagen buffer RPE was added and the column was spun at 4000 g to dry the spin column membrane, and the total RNA was eluted from the column by adding 150 μl RNase-free H_2O for 1 minute, then centrifuging at 4000 g for 3 minutes. This step was then repeated

with a further 150 µl RNase-free H₂O. RNA was quantified by measuring the absorbance at 260 nm.

2.5.4 Separation of MSP-1₁₉-specific PBMLs using Dynabeads

An attempt was made to separate out PBMLs expressing MSP-1₁₉-specific antibody on the cell surface for subsequent RNA extraction. 8×10^8 tosyl-activated Dynabeads 450 (Dyna) were coated with MSP-1₁₉-GST according to the Dynal protocol. PBMLs were pooled (from 5-6 separate donations) using 1 ml of cells suspended in RPMI 1640 from each donation, in a 15 ml falcon tube. The coated Dynabeads were added to a concentration of 2×10^7 beads/ml and the cells and beads were shaken together on a shaker at 30 rpm for 1 hour at 4°C. The cell/bead mix was then placed on a Dynal magnet for 60 seconds, the medium was removed, and the cells and beads were resuspended in a further 5-6 ml RPMI 1640 plus 5% foetal calf serum. This wash was repeated three times. The cell/bead mix was then resuspended in 1 ml RPMI 1640 plus 5% foetal calf serum. Cell count and cell viability was assessed as above, counting only those cells visibly attached to a bead. RNA extraction was then attempted as described above.

2.5.5 Synthesis of cDNA from total RNA

The steps involved in constructing the scFv libraries, from cDNA synthesis to the construction of *SfiI/NotI*-digested scFv inserts ready for cloning, are shown diagrammatically in Figure 2.5. cDNA was made from total RNA using the First-Strand cDNA Synthesis Kit (Pharmacia Biotech XY-028-00-15). This step is shown in Figure 2.5 Step 1. Reactions were set up as follows:

Bulk First-Strand Reaction Mix	77 µl
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Oligonucleotide Primer (20 pmol/μl)	7 μl
DTT Solution	7 μl
RNA	140 μl

Four reactions were carried out using four different oligonucleotide primers. The primers used were:

3 HuIg1-4 (20 pmol/μl) to amplify the variable regions of the immunoglobulin heavy chains

3 HuIgκ (20 pmol/μl) to amplify the variable regions of the immunoglobulin light κ chains

3 HuIgλ (20 pmol/μl) to amplify the variable regions of the immunoglobulin light λ chains

NotI-d(T)₁₈ (Pharmacia Biotech) (0.2 μg/μl) to non-specifically amplify mRNA

3 HuIg1-4 GTCCACCTTGGTGTGCTGGGCTT

3 HuIgκ ACACTCTCCCCTGTTGAAGCTCTT

3 HuIgλ TGAAGATTCTGTAGGGGCCACTGTCTT

RNA was heated to 65°C for 10 minutes then chilled on ice prior to the reactions. The reactions were carried out at 37°C for 1 hour. The cDNA was then flown back from The Gambia to the UK at 4°C and then stored at -20°C.

2.5.6 Amplification of antibody variable region sequences from human donor PBML cDNA

The DNA sequences encoding the variable regions of the human heavy chains, kappa light chains and lambda light chains were amplified separately from the cDNA. This step is shown diagrammatically in Figure 2.5 Step 2. The multiple primers used were based on each of the human heavy and light chain gene families in order to maximise the diversity of the libraries (Marks, Tristem et al. 1991). The primers were designed to anneal to the 5' and 3' ends of the DNA encoding the rearranged variable region sequences. Back primers (denoted by "BACK" in the primer name) anneal to the 5' end and forward primers (denoted by "FOR" in the primer name) anneal to the 3' end of the variable region sequence. Most of the primers have already been described (Marks, Hoogenboom et al. 1991) but some were newly designed by Dr Jonathan Chappel at the MRC Collaborative Centre, Mill Hill, London. Primers for the different gene families were only used if a functionally rearranged gene from that family was listed in the V-BASE GOLD database (which lists only functionally rearranged sequences) on 27 September 1996. The primer sequences are shown in Table 2.4. The PCR reactions carried out to amplify variable region sequences from the different heavy and light chain gene families are shown in Table 2.5.

PCR reactions were set up in a final volume of 50 μ l as follows:

Perkin Elmer buffer II	5 μ l
25 mM MgCl ₂	3 μ l
10 mM dNTPs	4 μ l
H ₂ O	32 μ l
Primer 1 (10 pmol/ μ l)	2 μ l
Primer 2 (10 pmol/ μ l)	2 μ l

cDNA	1 μ l
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AmpliTaq (5u/ μ l)	1 μ l
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Reactions were carried out under oil with a hot start at 94°C for 5 minutes and then 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute, for 30 cycles.

Five microlitres of PCR product was visualised after electrophoresis on a 2.5% agarose gel with ethidium bromide. PCRs giving only a weak band were repeated using Deep Vent DNA polymerase (NEB). These reactions were set up as follows:

10x Deep Vent Buffer	5 μ l
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dNTPs (10 mM)	4 μ l
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H ₂ O	35 μ l
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Primer 1(10 pmol/ μ l)	2 μ l
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Primer 2(10 pmol/ μ l)	2 μ l
----------------------------	-----------

cDNA	1 μ l
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Deep Vent (2 u/ μ l)	1 μ l
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Reactions were cycled as above with a hot start and oil overlay, and products were visualised as above. PCRs still giving only weak bands were repeated using 5 times more cDNA and PCR was carried out under a hot lid rather than under oil overlay.

PCR products were resolved on a 2.5% low melting point agarose gel containing ethidium bromide, and 1 mM guanosine was added to the gel and buffer as an ultraviolet protectant (Grundemann and Schomig 1996) when constructing the second (October 97) library. Forty-five microlitres of PCR reaction was added to each well.

Bands containing amplified variable region sequences were excised from the gel and purified using the phenol/lithium chloride melt procedure (Falson 1992). DNA pellets were resuspended in 20 μ l H₂O and 2 μ l of each purification was used to quantify the recovery.

Purified variable region sequence mixes were prepared containing 10 ng of each variable region PCR product. Three mixes were made, V_H mix, containing the PCR products from the 10 V_H PCR reactions, V_K mix, containing the PCR products from the 9 V_K PCR reactions, and V_λ mix, containing the PCR products from the 13 V_λ PCR reactions. Each mix was diluted to a concentration of 5 ng/ μ l to prepare for the first stage of scFv assembly.

2.5.7 Preparation of linker DNA

To assemble V_H DNA with V_K or V_λ DNA to make scFv, it is necessary to join them together with DNA encoding a flexible 15 amino acid linker. The linker has the amino acid sequence (Gly-Gly-Gly-Gly-Ser)₃, and the DNA sequence GGTGGCGGTGGCTCGGGCGGTGGTGGGTCTGGGTGGCGGCGGATCT.

In order to be able to join the linker DNA to the V_H DNA and the V_K or V_λ DNA, the linker was amplified using primers designed to add extensions to the linker DNA which would overlap with the V_H DNA and the V_K or V_λ DNA. This step is shown diagrammatically in Figure 2.5 Step 2. The primers were designed so that the 5' end of the linker would overlap with the 3' end of the V_H DNA, and the 3' end of the linker would overlap with the 5' end of the V_K or V_λ DNA. This arrangement enables the creation of scFv of the form V_H -linker- V_K or V_H -linker- V_λ .

Four different primers were used to produce overlaps between the 5' end of the linker and the 3' end of the different families of V_H genes. Nine different primers were used to produce overlaps between the 3' end of the linker and the 5' end of the different families of V_K genes. Thirteen different primers were used to produce overlaps between the 3' end of the linker and the 5' end of the different families of V_λ genes. Each of the 4

V_H primers were used with each of the 9 V_K or 13 V_λ primers in 88 different PCR reactions.

The primer sequences are shown in Table 2.6. The PCR reactions are shown in Table 2.7.

PCR reactions were set up as follows:

10x PCR Buffer II	5 µl
MgCl ₂ (25 mM)	3 µl
dNTPs (10 mM)	4 µl
Primer 1 (10 pmol/µl)	2 µl
Primer 2 (10 pmol/µl)	2 µl
Linker template (10 ng/µl)	1 µl
H ₂ O	32.5 µl
AmpliTaq	0.5 µl

The reactions were set up in a 96 well plate format and cycled on a TRIO-Thermoblock (Biometra) thermal cycler at 94°C for 5 minutes, then at 94°C for 1 minute, 45°C for 1 minute, 72°C for 1 minute for 30 cycles. Each reaction was set up both with template linker DNA and, as a negative control, without template linker DNA.

The PCR products were resolved on a 2.5% agarose gel, and bands containing the amplified linker were excised and gel purified using a phenol/gel melt procedure previously described (Falson 1992). The final washed pellets were resuspended in 20 µl H₂O and frozen at -20°C until use in the scFv assembly PCR.

2.5.8 scFv assembly stage 1: attaching V_H heavy chain sequences and V_κ and V_λ light chain sequences to scFv linkers

The amplified V_H, V_κ and V_λ sequences were attached to the scFv linker by PCR. This step is shown diagrammatically in Figure 2.5 Step 3. Reactions were set up as shown in Table 2.8. Ten different PCR reactions were set up.

Two PCR reactions attached the V_H sequences to either the linker with a 3' end designed to overlap with the 5' end of the V_κ sequences, or to the linker with a 3' end designed to overlap with the 5' end of the V_λ sequences. These are reactions 1 and 2 in Table 2.8. In these reactions, two template DNA mixes were used. One template was a mix of all the amplified V_H sequences as described in section 2.5.6. The other template was a mix of all the linkers amplified with regions overlapping both the 3' end of the V_H sequences and the 5' end of the V_κ sequences or the V_λ sequences, as described in section 2.5.7. An equimolar mix of all 10 of the HuVHBACK primers (see Table 2.4 a) was used to anneal to the 5' end of the V_H sequences and a single primer, HuLINKFOR, was used to anneal to the 3' end of the linker.

HuLINKFOR AGATCCGCCGCCACCCGACCC

Five PCR reactions attached the linker to the V_κ sequences. These are reactions 3-7 in Table 2.8. Again two template DNA mixes were used. One template was a mix of all the amplified V_κ sequences as described in section 2.5.6. The other template was a mix of all the linkers amplified with regions overlapping both the 3' end of the V_H sequences and the 5' end of the V_κ sequences, as described in section 2.5.7. In all 5 reactions, a single primer, HuLINKBACK, was used to anneal to the 5' end of the linker. Five different primers were used in the 5 reactions to anneal to the 3' end of the V_κ sequences. These primers were the 5 HuJκFOR primers used in section 2.5.6 and shown in Table 2.4 b).

HuLINKBACK GGTGGCGGTGGCTCGGGCGGT

Three PCR reactions attached the linker to the V_λ sequences. These are reactions 8-10 in Table 2.8. Again two template DNA mixes were used. One template was a mix of all the amplified V_λ sequences as described in section 2.5.6. The other template was a mix of all the linkers amplified with regions overlapping both the 3' end of the V_H sequences and the 5' end of the V_λ sequences, as described in section 2.5.7. As before, in all 3 reactions a single primer, HuLINKBACK, was used to anneal to the 5' end of the linker. Three different primers were used in the 3 reactions to anneal to the 3' end of the V_λ sequences. These primers were the 3 HuJλFOR primers used in section 2.5.6 and shown in Table 2.4 b). Reactions were set up in a volume of 100 µl as follows:

10x PE Buffer II	10 µl
25 mM MgCl ₂	6 µl
10 mM dNTPs	8 µl
Template 1: V_H (5 ng/µl) or V_κ or V_λ (2 ng/µl) PCR mix	1 µl
Template 2: Linkers with κ or λ overlaps (2 ng/µl)	1 µl
Primer 1 (10 pmol/µl)	4 µl
Primer 2 (10 pmol/µl)	4 µl
H ₂ O	65 µl
AmpliTaq	1 µl

PCR reactions were performed with a hot start at 94°C for 3 minutes and cycled at 94°C for 90 seconds, 55°C for 1 minute, 72°C for 2 minutes, for 25 cycles, then 72°C for 10 minutes.

To provide sufficient product, multiple PCRs were performed. Reactions using V_H or V_κ sequences (reactions 1-7 in Table 2.8) were set up in duplicate and reactions using V_λ sequences (reactions 8-10 in Table 2.8) were each set up in 4 tubes.

PCR products were precipitated with ethanol and resolved on a 1.4% agarose gel with 1 mM guanosine. The appropriate bands were excised from the gel and purified using the Wizard Prep Kit (Promega) and eluted with 50 μ l H₂O. The DNA was quantified by comparison to standard DNA concentrations. The purified DNA eluates were diluted to 2.5 ng/ μ l in H₂O for the second and final stage scFv assembly PCR. The purified DNA from PCR reactions 1 and 2 above (V_H joined to the linker with κ overlaps and V_H joined to the linker with λ overlaps) were pooled for the second stage assembly PCR.

2.5.9 scFv assembly stage 2: attaching V_H-linker to linker-V _{κ} or linker-V _{λ} to make complete scFvs with restriction sites

Reactions were set up to combine V_H genes with either V _{κ} genes or V _{λ} genes, via the linkers attached to them in the previous PCR, to make full-length scFvs. *Sfi*I and *Not*I restriction sites were also incorporated at either end of the scFvs in this PCR step. This stage in the scFv construction is shown diagrammatically in Figure 2.5 Step 4. The primers used in these reactions are shown in Table 2.9.

Three reactions were performed.

One reaction was designed to join the V_H-linker sequences to the linker-V _{κ} sequences to make V_H-V _{κ} scFv. In this reaction, one DNA template was the mix of V_H-linker sequences described in section 2.5.8 and the other DNA template was the mix of linker-V _{κ} sequences described in the same section. Ten primers (designated HuVH...BACKSfi) were designed to anneal to the 5' ends of the 10 families of V_H sequences and to incorporate a *Sfi*I restriction site, and these primers were used in an equimolar mix. Five primers were designed to anneal to the 3' end of the V _{κ} sequences

and to incorporate a *NotI* restriction site. Again, these 5 primers were used in an equimolar mix.

Another reaction was designed to join the V_H-linker sequences to the linker-V_λ sequences to make V_H-V_λ scFv. In this reaction, one DNA template was the mix of V_H-linker sequences described in section 2.5.8 and the other DNA template was the mix of linker-V_λ sequences described in the same section. Again, the 10 HuVH...BACKSfi primers were used in equimolar mix as described above. Three primers were designed to anneal to the 3' end of the V_κ sequences and to incorporate a *NotI* restriction site. Two of these primers, HuJλ1FORNot and HuJλ2-3FORNot, were used in this reaction to anneal to the 3' ends of the different families of V_λ sequences. Again, these 2 primers were used in an equimolar mix.

One extra reaction was set up using the HuVH...BACKSfi primer mix as above and using Huλ7xFORNot as the other primer. This reaction was done separately because it was found that the Huλ7xFORNot primer worked better in the final assembly PCR if an annealing temperature of 72°C was used instead of 69°C.

Reactions were set up in a volume of 100 µl as follows:

Perkin Elmer 10x Buffer	10 µl
25 mM MgCl ₂	6 µl
10 mM dNTPs	8 µl
H ₂ O	69 µl
Template 1: V _H -linker (2.5 ng/µL)	1 µl
Template 2: linker-V _κ or linker-V _λ (2.5 ng/ µl)	1 µl
Primer 1: HuV _H BACKSfi mix	2 µl

Primer 2: HuJκFORNot mix or HuJλFORNot mix or λ7x alone) 2 μl

AmpliTaq Gold 1 μl

All reactions except those using primer λ7x were cycled as follows:

94°C for 90 seconds, 69°C for 1 minute, 72°C for 2 minutes, for 30 cycles, then 72°C for 10 minutes.

Reactions using primer λ7x were cycled as follows:

94°C for 90 seconds, 72°C for 2 minutes, for 30 cycles, then 72°C for 10 minutes.

Reactions were carried out under oil. To provide sufficient DNA, each reaction using V_κ-linker was set up in duplicate and each reaction using V_λ-linker was set up in 4 tubes.

The PCR products were precipitated and resuspended in TE buffer (40 μl for V_κ and 80 μl for V_λ PCRs). The PCR products were then resolved on a 1.4% agarose gel (40 μl per well) and the appropriate bands were excised and purified using the Wizard Prep kit (Promega) and eluted with 3x 50 μl H₂O. The purified PCR product was quantified and stored at -20°C.

2.5.10 Reamplification of assembled scFvs with long primers

The library scFvs were reamplified with primers designed to give an extension of 100 bases beyond the restriction sites in order to improve the efficiency of the restriction digestion of the PCR products prior to ligation. This is illustrated diagrammatically in Figure 2.5 Step 5.

BACKSfi was designed to anneal to the 5' (V_H) end of the scFv and FORNot was designed to anneal to the 3' (V_κ or V_λ) end of the scFv. The sequences of BACKSfi and FORNot are shown in Table 2.10.

The PCR was set up as follows:

Perkin Elmer 10x Buffer II	10 μ l
MgCl ₂ (25 mM)	6 μ l
dNTPs (10 mM)	8 μ l
BACKSfi (10 μ M)	2 μ l
FORNot (10 μ M)	2 μ l
V _H V _{κ} scFv or V _H V _{λ} scFv (5 ng/ μ l)	2 μ l
H ₂ O	69 μ l
AmpliTaq	1 μ l

Each of the two PCRs (amplifying either V_HV _{κ} scFv or V_HV _{λ} scFv) was set up in 10 separate identical reactions. Reactions were carried out with a hot start at 94°C for 4 minutes and then cycled at 94°C for 1 minute, 72°C for 2 minutes, for 15 cycles, followed by an extension at 72°C for 10 minutes.

2.5.11 *Sfi*I/*Not*I restriction digestion of scFvs

This step is illustrated diagrammatically in Figure 2.5 Step 6. After reamplification of the library scFvs with long primers, the 10 PCR products from each reaction were combined and extracted once with phenol/chloroform, then again with chloroform, then ethanol precipitated and resuspended in 100 μ l H₂O. The V_HV _{κ} scFv and V_HV _{λ} scFv DNA were each digested with 250 u *Sfi*I (Boehringer). Restriction digests were set up in a volume of 500 μ l as follows:

10x Buffer M	50 μ l
V _H V _{κ} scFv or V _H V _{λ} scFv DNA	100 μ l
H ₂ O	325 μ l

<i>Sfi</i> I (10 u/μl)	25 μl
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The digest was carried out at 50°C overnight.

The DNA was extracted and precipitated as above and the *Not*I digestion was set up as follows:

10x Buffer M	50 μl
V _H V _κ scFv or V _H V _λ scFv DNA	100 μl
H ₂ O	325 μl
<i>Not</i> I (10 u/μl)	25 μl

This digest was carried out at 37°C for 4 hours. The DNA was extracted and precipitated as above (March library) or gel-purified from 1.4% gels as above (October library) in preparation for ligation.

2.5.12 Ligation of scFvs into pHEN1H6 vector

The V_HV_κ scFv or V_HV_λ scFv DNA inserts were ligated into the phagemid vector pHEN1H6.

Prior to the ligation, 30 μg of caesium chloride-purified pHEN1H6 vector was digested with *Sfi*I/*Not*I/*Pst*I (Boehringer). Successive digestions were each carried out in a volume of 500 μl with 50 μl 10x Buffer M. *Sfi*I digestion was carried out at 50°C for 4 hours, *Not*I digestion at 37°C for 4 hours and *Pst*I digestion at 37°C for 2 hours. The DNA was phenol/chloroform extracted and ethanol precipitated after each digest. The digested vector was resuspended in 100 μl H₂O, passed over a chromaspin 1000 column and quantified.

The ligations were carried out in a volume of 500 μ l using 400 ng of vector, 100 ng of V_HV_K scFv or V_HV_λ scFv, 10x ligation buffer and T4 ligase (New England Biolabs).

The ligations were set up as follows:

pHEN1H6	8 μ l
V_HV_K scFv or V_HV_λ scFv	10 μ l
10x ligation buffer	50 μ l
H ₂ O	382 μ l
T4 Ligase (400 u/ml)	50 μ l

The ligations were each split into 5 aliquots of 100 μ l and purified using the QIAEX II kit (Qiagen) and eluted at 50°C with 20 μ l 10 mM Tris pH 8.5.

2.5.13 Transformation of *E. coli* with scFv in pHEN1H6

Fresh electrocompetent *E. coli* TG1-TR cells were prepared. Fifty cuvettes were prepared on ice and 2 μ l aliquots of ligated DNA were placed in each cuvette. Fifty microlitres of *E. coli* cells were added to the cuvettes, using 10 at a time, and the cells were electroporated with 1.7 kV. After electroporation, 1 ml 2TYG was added to the cuvette and removed, and the cuvette was rinsed with a further 1 ml 2TYG and the transformed cells were pooled and shaken at 220 rpm for 1 hour. An aliquot of cells was removed for titration and the remainder was centrifuged at 2340 g for 10 minutes then resuspended in 3 ml 2TYG. Five lots of 600 μ l of these cells were plated on each of 5 large plates of 2TY agar with 100 μ g/ml ampicillin and 2% glucose, and incubated overnight at 30°C. The colonies were then scraped and resuspended in 10 ml 2TY plus 15% (v/v) glycerol and stored as 48 x 1ml aliquots of V_HV_K scFv and 48 x 1ml of V_HV_λ scFv at -70°C.

2.6 Panning the libraries

2.6.1 Preparation of phage from library glycerol stocks

An aliquot of 1 ml of each library glycerol stock (i.e. March library V_HV_K , March library V_HV_λ , October library V_HV_K and October library V_HV_λ) was added to 500 ml 2TYAG and grown at 37°C in a baffled flask in an Innova 4000 incubator shaker at 300 rpm until the A_{600} was 0.5 (80 minutes). M13KO7 helper phage (Gibco BRL) 0.5 ml (= 2.5×10^{11} phage) was added to give a final concentration of 5×10^8 phage/ml. The cells were incubated at 37°C for 30 minutes without shaking and then for 30 minutes shaking at 200 rpm to allow infection. The cells were then centrifuged at in a Beckman J-6B centrifuge at 5000 rpm (2380 g) for 10 minutes and resuspended in 500 ml 2TYA (without glucose) plus kanamycin 50 µg/ml and grown at 30°C for 16 hours shaking at 300 rpm.

The phage was concentrated by precipitation with polyethylene glycol. The culture was centrifuged in a Beckman JA-17 centrifuge at 8000 rpm (8820 g) in 10 x 50 ml Oakridge tubes for 15 minutes. The phage-containing supernatant was transferred to a sterile flask and 150 ml of 20% polyethylene glycol/2.5 M NaCl was added and mixed. The mix was left on ice for 90 minutes to precipitate the phage, and then centrifuged at 8820 g for 15 minutes at 4°C to pellet the phage. The supernatant was removed, and the remaining phage was centrifuged again as above and the residual supernatant removed with a pipette. Each pellet of precipitated phage was then resuspended in 1 ml of TE buffer (10 mM Tris pH 8.0, 0.1 mM EDTA) and the ten 1 ml phage suspensions were pooled. Residual bacterial debris was removed from the 10 ml suspension of phage in TE buffer by centrifuging at 8820 g for 5 minutes, and the phage-containing supernatant was transferred to a fresh tube.

Further purification of the phage was carried out as follows. Five grams of caesium chloride (CsCl) were added to the 10 ml of phage stock in TE buffer to give a final CsCl concentration of 0.5 g/ml and mixed to dissolve. The phage was then placed in a 13.5 ml 16 x 76 mm polyallomer tube (Beckman, Cat #342413) and ultracentrifuged at 42,000 rpm in a Beckman XL-90 Ultracentrifuge with a 70.1 TI rotor for 24 hours. The ultracentrifuge was stopped with the brake off. Three distinct bands were seen; a clear band at the top of the tube, an opaque band below it and another clear band below that. Each band was carefully removed separately with a 19 G needle and dialysed against 1L TE buffer in a Slide-A-Lyzer 10K dialysis cassette at 4°C overnight to remove the CsCl.

To determine which band contained the greatest quantity of phage, the quantity of phage was titrated from each band as follows. The dialysed phage-containing material from each Slide-A-Lyzer cassette was made up to 2 ml with TE buffer to create purified library phage stocks. Aliquots from each of these stocks were diluted into successive tenfold dilutions in 2TY on a 96 well plate. TG1-TR *E. coli* cells were grown in 50 ml of medium at 37°C for approximately 4 hours until they reached A_{600} 0.5-1.0. Duplicates of 10 µl of the phage stock dilutions were added to 1 ml of the TG1-TR cells and allowed to infect for 30 minutes at 37°C without shaking. One hundred microlitres from each of these dilutions of phage culture were then plated in duplicate onto TYAG plates and left overnight at 30°C. The colonies were counted to estimate the original concentration of phage.

Library phage stocks were stored as above in TE buffer at 4°C until used for panning.

2.6.2 Panning for MSP-1₁₉-specific scFvs

A plastic Nunc Immuno tube (Maxisorp 75 x 12 mm, Cat. No. 4-44202) was coated with recombinant MSP-1₁₉-GST (Burghaus and Holder 1994) as follows. One millilitre of MSP-1₁₉-GST (10 µg/ml in PBS) was added to the Immuno tube and kept at 4°C for 16 hours. The coated Immuno tube was then rinsed three times with PBS, and then blocked by filling to the brim with 3% (w/v) skimmed milk powder in PBS for 2 hours at room temperature, then rinsed 3 times with PBS. Concentrated library phage was pre-blocked as follows. Approximately 1×10^{14} phage from each of the two libraries (03/97 and 10/97) were pooled together in a volume of 1 ml in a 1.5 ml Eppendorf tube. Eighteen percent (w/v) skimmed milk powder in 200 µl of 6x PBS was added and left at room temperature for 1 hour. GST was then added to the phage at a concentration of 100 µg/ml for 1 hour to prevent selection of anti-GST scFvs during panning. 1 millilitre of pre-blocked phage was then added to the MSP-1₁₉-GST-coated Immuno tube and incubated (stationary) at 37°C for 1 hour. The Immuno tube was then rinsed with PBS/0.1% Tween 20 (PBS/Tween) 5 times (10 times for panning rounds 2, 3 and 4) and then rinsed 20 times with PBS. Bound phage was then eluted by adding 1 ml freshly made 100 mM triethylamine (pH 11.0) to the Immuno-tube and incubating at room temperature for 10 minutes. Eluted phage was then transferred to a fresh 1.5 ml Eppendorf tube, neutralised by adding 500 µl 1M Tris-HCl pH 7.4 and stored on ice.

2.6.3 Re-infection of *E. coli* TG1 cells with eluted phage

Exponentially growing *E. coli* TG1 cells were prepared as follows. A 50 ml 2TY broth (16g tryptone, 10g yeast extract, 5g NaCl, in 1L) was inoculated with a single colony of *E. coli* TG1 taken from a streaked minimal agar plate, and incubated with shaking (Innova 4000) at 37°C for 4 hours (until A_{600} was 0.5-1.0). Five millilitres of the TG1 culture was added to a 50 ml universal tube, and 1.4 ml of the eluted phage was added (the remaining 100 μ l of eluted phage was stored at 4°C). The eluted phage was left to infect the *E. coli* at 37°C for 30 minutes stationary and then at 37°C for 30 minutes shaking at 100 rpm. Two hundred microlitres of culture was removed to make serial dilutions for plating and counting as follows. A 2TYAG agar plate was plated with 100 μ l of phage-infected *E. coli* and 3 more 2TYAG agar plates were plated with 100 μ l of three serial ten-fold dilutions (made on ice) of infected *E. coli*, and incubated overnight. Colonies on these plates were then counted to estimate the number of phage retrieved by panning. The remaining 6.2 ml of phage-infected *E. coli* culture was centrifuged (Beckman JA-17) at 3500 rpm (690 g) for 10 minutes and the pellet was resuspended in 0.6 ml 2TY broth and spread on a large (243 x 243 mm) 2TYAG agar plate and incubated at 30°C for 16 hours.

2.6.4 Storage and rescue of phagemid scFvs after selection

The colonies from each round of panning were scraped from the large 2TYAG agar plate into 10 ml of 2TY broth in a 50 ml universal tube. Fifty microlitres of this broth was then used to inoculate 25 ml of 2TYAG and shaken at 37°C for 90 minutes (until A_{600} was 0.5-1.0). The remainder of the broth was mixed with 5 ml 50% v/v glycerol, rotated for 10 minutes at room temperature and then stored in 1 ml aliquots at -70°C. The inoculated 25 ml 2TYAG culture was infected with helper phage as follows. M13K07

helper phage was added to a final concentration of 5×10^8 pfu/ml. The cells were left stationary at 37°C for 30 minutes, shaken in an Innova 4000 incubator shaker at 100 rpm at 37°C for 30 minutes, then transferred to a 50 ml universal tube and centrifuged (Beckman JA-17) at 3500 rpm (1690 g) for 10 minutes. The bacterial pellet was then resuspended in 25 ml prewarmed 2TY with 50 µg/ml kanamycin and 100 µg/ml ampicillin. The cell suspension was then transferred to a 250 ml flask and shaken at 300 rpm at 30°C for 16 hours to produce phage particles. One millilitre of culture was then transferred to a 1.5 ml Eppendorf tube and centrifuged at 13000 rpm (11,340 g) for 5 minutes. The phage-containing supernatant (containing about 1×10^{12} /ml) was transferred to a fresh tube and used to perform the next round of panning as described above in section 2.6.2.

2.6.5 Assessment of diversity of MSP-1₁₉-GST-binding scFvs by *Bst*NI digest

To give an indication of the sequence diversity of MSP-1₁₉-GST-binding scFv clones, *Bst*NI restriction digest patterns were obtained as follows. scFvs were amplified by PCR in a volume of 20 µl containing:

Perkin Elmer II 10x buffer	2 µl
25 mM MgCl ₂	1.2 µl
10 mM dNTPs	1.6 µl
RSP 48 primer (10 pmol/µl)	1 µl
fdTET primer (10 pmol/µl)	1 µl

Sterile H₂O 12.7 µl

AmpliTaq DNA polymerase 0.5 µl

A small amount of an ELISA-positive MSP-1₁₉-GST-binding clone was transferred from the glycerol stock of the master plate into the PCR tube using a sterile pipette tip, and the PCR mix was overlaid with mineral oil. The PCR product was amplified using a Biometra thermal cycler using 30 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes followed by extension at 72°C for 10 minutes. Three microlitres of PCR product was analysed by electrophoresis on a 1.5% agarose gel, to confirm the presence of a band of approximately 1 kb. The remaining PCR products were made up to 1x NEB2 Buffer with 100 mg/ml BSA, and 2 units of *Bst*NI were added. After mixing, digests were incubated at 60°C for 2 hours under oil. Digests were resolved by electrophoresis on a 4% NuSeive agarose gel with ethidium bromide.

2.6.6 DNA sequencing of selected MSP-1₁₉-binding scFv clones

Sequencing was carried out using the Perkin Elmer ABI Prism 377 DNA Sequencer, using Perkin Elmer Terminator Ready Reaction Mix.

scFv inserts in the phagemid vector pHEN1H6 were amplified for DNA sequencing using the following primers:

FdTET 5' GTCGTCTTTCCAGACGTTAGT 3'

RSP 48 5' AGCGGATAACAATTTACACAGGA 3'

2.7 Assessment of binding of mAbs and scFvs to target antigen

2.7.1 Binding of scFvs and mAbs to acetone-fixed *P. falciparum* by indirect immunofluorescence (IFA)

Binding of scFvs and chimeric mAbs to parasites was assessed by IFA on acetone-fixed blood stage *P. falciparum* (FCB-1 and T9/96 lines). Multiwell glass slides of acetone-fixed *P. falciparum* smears were dried, then 5 µl of Ni-NTA-purified scFv was added to each well in concentrations of 30 µg/ml, 3 µg/ml, 0.3 µg/ml, and 0.03 µg/ml in PBS, and incubated in a humid chamber at 37°C for 30 minutes. Polyclonal scFv was tested at 20 µg/ml. The slides were then washed in PBS, and 15 µl of 9E10 (anti-c-myc) mouse hybridoma supernatant was added to each well, and incubated as above. Slides were then washed again as described and a 1:100 dilution of stock FITC-labelled anti-mouse antibody in PBS/1% bovine serum albumin (BSA) was added to each well, incubating again as above. The slides were then washed again and covered, and then examined under the fluorescence microscope.

Chimeric mAbs, made by transient expression in *cos* cells and protein A-purified, were tested at the same concentrations as above, and revealed with a 1:100 dilution of stock FITC-labelled anti-human antibody in PBS/1% BSA.

Murine hybridoma mAbs 12.8 and 12.10 at 30 µg/ml were used as positive controls and revealed with FITC-labelled anti-mouse antibody. An anti-hen's egg lysozyme scFv, scFv D1.3, was used at 30 µg/ml as a negative control for IFA with scFv, and mAb BX version 2 (an unrelated non-*P. falciparum*-specific humanised mAb, directed against a cancer-specific antigen), donated by Susan Potts, MRC Collaborative Centre, was used at 30 µg/ml as a negative control for IFA with chimeric mAbs.

2.7.2 Binding of scFvs and chimeric mAbs to recombinant MSP-1₁₉ by ELISA

A Nunc Maxisorb 96-well ELISA plate was coated with 100 μ l 1 μ g/ml recombinant MSP-1₁₉-GST fusion protein in PBS and left at 4°C overnight to coat. The plate was rinsed 3 times with PBS and then blocked with 280 μ l per well of 3% Marvel/PBS for 1 hour at 37°C. The plate was then washed again 3 times with PBS and 50 μ l scFv was added at 100 μ g/ml, and allowed to bind for 1 hour at 37°C. mAb 12.8 and mAb 12.10 were used as positive controls, and an anti-hen's egg lysozyme scFv, scFv D1.3, was used as a negative control. The plate was then washed 3 times with PBS/Tween and then PBS, and to each well containing scFv was added 100 μ l of 9E10 anti-c-*myc* mouse hybridoma cell culture supernatant, and the plate was incubated at 37°C for 1 hour. The plate was washed 3 times with PBS/Tween and then PBS, and then to each well was added 100 μ l of a 1/5000 dilution in 3% marvel/PBS of peroxidase-conjugated anti-mouse kappa antibody (Harlan Sera-Lab). The plate was incubated again at 37°C for 1 hour, and then washed 3 times in PBS/Tween then PBS. The colour was developed by adding 150 μ l Neogen K-BLUE substrate (Sky Bio) to each well and leaving in darkness for 10 minutes then adding 50 μ l Neogen RED STOP solution (Sky Bio). The plate was read on a BIO-RAD 3550 Microplate reader at 655 nm.

For ELISA using chimeric mAbs 12.8 and 12.10, negative control wells were coated with hen's egg lysozyme (1 μ g/ml in PBS). One hundred microlitres of mAb was added to each well, in serial 1:1 dilutions, starting with a concentration of 50 μ g/ml, and murine hybridoma mAbs 12.8 and 12.10 were used as positive controls and humanised mAb BX version 2 (see above) was used as a negative control mAb. Bound chimeric

antibodies were detected with 100 μ l of a 1/5000 dilution in 3% marvel/PBS of peroxidase-conjugated goat anti-human kappa light chain antibody (Sigma).

2.7.3 Identification of MSP-1₁₉-binding scFvs by ELISA

In order to isolate MSP-1₁₉-binding scFvs from the polyclonal scFv obtained in successive rounds of library panning, a soluble ELISA of 80 clones was carried out. Individual colonies were picked at random from the third and fourth rounds of library panning using a sterile pipette tip and transferred into 100 μ l 2TYAG medium in a 96 well master plate and grown with shaking in an Innova 4000 incubator shaker at 100 rpm for 16 hours at 30°C. A replica 96 well microtitre plate was prepared and inoculated from the master plate. The master plate was then stored at -70°C after adding 50 μ l 50% glycerol to each well. The replica plate was shaken at 100 rpm for 6 hours at 37°C. The plate was then centrifuged in a Centaur 2 MSE centrifuge at 2000 rpm for 10 min and the supernatant was removed. The pellets were resuspended in 100 μ l 2TY with 50 μ g/ml kanamycin, 100 μ g/ml ampicillin, and 1 mM IPTG and shaken at 100 rpm at 30°C for 16 hours to produce scFv for ELISA.

A 96-well Nunc Maxisorb microtitre plate was coated with MSP-1₁₉-GST using 100 μ l per well of MSP-1₁₉-GST 1 μ g/ml in PBS. Positive control wells were coated with hen's egg lysozyme 100 μ g/ml in 100 mM sodium bicarbonate pH 9.6. Negative control wells were left uncoated. The plate was left to coat at 4°C for 16 hours. The coating solution was then flicked out, the plates were rinsed three times with PBS, and then blocked with 280 μ l/well of 3% dried milk powder in PBS for 2 hr at 37°C. The coated and blocked plates were then washed 3 times with PBS.

The scFvs were blocked as follows. The scFv-containing culture plate was centrifuged at 2000 rpm for 10 min. One hundred microlitres of each scFv-containing supernatant was transferred to the corresponding well of a microtitre plate containing 20 μ l of 6x PBS with 18% dried milk powder and mixed. The plate was left for 1 hour at room temperature to block the scFv. Fifty microlitres of pre-blocked scFv was then transferred from each well of the blocking plate into the corresponding well of the antigen-coated plate. Blocked anti-hen's egg lysozyme scFv D1.3 was added to the positive control wells. The scFv was allowed to bind for 1 hour at 37°C. The plate was then washed 3 times with PBS/Tween and 3 times with PBS using a Sanofi LP35 plate-washer (Pasteur Diagnostics). One hundred microlitres of 9E10 anti-c-*myc* hybridoma supernatant was added to each well and the plate was incubated for 1 hour at 37°C. The plate was washed again as above and 100 μ l of a 1/5000 dilution peroxidase-conjugated goat anti-mouse antibody (Jackson) in 3% Marvel/PBS was added and the plate was incubated for 1 hour at 37°C. The plate was washed again as above and then washed once with saline. The colour was developed by adding 150 μ l Neogen K-BLUE substrate (Sky Bio) to each well and leaving in darkness for 10 minutes then adding 50 μ l Neogen RED STOP solution (Sky Bio) and read at 655 nm on a BIO-RAD 3550 Microplate reader.

2.7.4 Competition ELISA with MSP-1₁₉-binding scFvs and mAbs 12.8 and 12.10

A competition ELISA was designed to determine whether the phage library scFvs could compete with mAbs 12.8 and 12.10 for binding sites on the MSP-1₁₉ molecule. Competition ELISA plates were coated with MSP-1₁₉-GST at 0.5 µg/ml for competition with mAb 12.8 and at 0.05 µg/ml for competition with mAb 12.10. The scFvs were expressed in a 96-well replica plate inoculated from the master plate, as above, and the pre-blocked scFv-containing supernatants were transferred to the ELISA plate and allowed to bind. The plates were washed and then mAb 12.8 (at 0.5 µg/ml) or mAb 12.10 (at 0.05 µg/ml) were allowed to bind also. Bound mAb was then revealed as described previously.

2.7.5 Binding of polyclonal scFv to *P. falciparum* by Western blot

Ten microlitres each of T9/96 and FCB-1 *P. falciparum* merozoites were incubated for 1 hour at 37°C with 1 mM MgCl₂, 1 mM CaCl₂, Leupeptin, Antipain, Aprotinin & TLCK (Tos-Lys-chloromethylketone.HCl) in 200 µl PBS. The merozoites were then boiled for 10 minutes with 200 µl of non-reducing buffer, and 5 µl was loaded into each well of a 15% SDS polyacrylamide gel, and the gel was run and transferred onto a nitrocellulose membrane. The membrane was blocked with PBS/3%Marvel on a rotating platform at room temp for 2 hours, then the polyclonal scFv was added to the blocking solution at a final concentration of 1 µg/ml for 1 hour. The membrane was washed with PBS/Tween 3 times for 5 minutes and then incubated with 9E10 supernatant for 1 hour at room temperature. Peroxidase-conjugated goat anti-mouse IgG/1gM was added (diluted 1 in 25,000 in PBS/3%Marvel) for 1 hour at room temp. The membrane was then washed

three times as above and developed using the ECL chemiluminescent detection system (Amersham Life Science).

2.8 Assessing invasion-inhibitory activity of mAbs and scFvs

2.8.1 *In vitro* culture of *P. falciparum*

P. falciparum parasites were cultured in RPMI-1640 supplemented with 25 mM HEPES, 25 mM NaHCO₃, 0.2% (w/v) glucose, 25 µg/ml gentamicin, 20 µg/ml hypoxanthine, plus either 10% (v/v) ABO-compatible human serum or 0.5% AlbumaxTM (Gibco BRL) and 2 mM glutamine with fresh washed human erythrocytes at a haematocrit of 1-2%. Cultures were maintained at 37°C in 1L NunclonTM flasks (NUNC) gassed to contain an atmosphere of 7% CO₂, 5% O₂, 88% N₂. The medium was changed every 48 hours. To replace the medium, cultures were centrifuged in a Beckman J-6B centrifuge at 2200 rpm (460 g) for 5 minutes and resuspended in fresh medium and recultured as above. Fresh human erythrocytes were added approximately twice weekly to maintain a parasitaemia of approximately 10-20%. Parasitaemias were measured every 48 hours using blood smears. Blood smears were air-dried, fixed in 100% methanol and stained for 20 minutes with 10% (v/v) Giemsa stain in phosphate buffer pH 7.2.

2.8.2 Synchronisation of *P. falciparum* cultures and purification of schizonts

The *P. falciparum* culture was centrifuged for 5 minutes in a Beckman J-6B centrifuge at 2400 rpm (550 g). The pellet was resuspended in 30 ml RPMI-1640 supplemented as above plus 10% (v/v) ABO-compatible human serum, and centrifuged again in a Sigma 4-10 centrifuge at 1800 rpm (570 g) for 5 minutes. The supernatant was removed and the volume of the pellet was estimated. RPMI-1640 plus 10% serum, pre-warmed to 37°C, was added at a volume of 2.4 times the pellet volume. An equal volume of Plasmagel (Laboratoire Roger Bellon), pre-warmed to 37°C, was added, and the pellet was gently resuspended and left undisturbed for 30 minutes in an incubator at 37°C. The top layer, containing the schizonts, was carefully removed to a fresh tube, and an equal volume of pre-warmed RPMI-1640 was added. The cells were centrifuged in a Sigma 4-10 centrifuge at 1700 rpm (510 g) and resuspended in fresh pre-warmed RPMI-1640 plus 10% serum. To provide purified schizonts for invasion assays, schizonts from this stage of the procedure were used. To synchronise ongoing *P. falciparum* cultures, 6 ml of fresh ABO-compatible red cells, washed in RPMI-1640, were added to the schizonts and resuspended to a final haematocrit of 10%. The culture was then left in a gassed flask at 37°C for 2 hours.

The culture was then centrifuged in a Sigma 4-10 centrifuge at 2000 rpm (770 g) for 5 minutes. The supernatant was removed and the pellet was resuspended in 10 volumes of 5% (w/v) sorbitol in water, to neutralise all but the ring forms of *P. falciparum*. The culture was left in sorbitol at room temperature for 10 minutes, then centrifuged as above and washed once in RPMI-1640. The *P. falciparum* ring-forms thus isolated were then recultured in fresh pre-warmed RPMI-1640 plus 10% serum plus 2 mM glutamine at a

haematocrit of 1-2% in a gassed flask. This process was repeated weekly to maintain synchrony.

2.8.3 Quantification of antibody/scFv mediated inhibition of erythrocyte invasion

Invasion assays were carried out essentially as previously described (Blackman, Heidrich et al. 1990). Specifically, purified antibodies/scFv were prepared by dialysis in RPMI 1640 (without Albumax) (Sigma) overnight at 4°C using a Slide-a-lyser dialysis cassette (50 kDa cut-off). Test antibodies/scFvs in RPMI 1640 (without Albumax) were placed in a flat-bottomed Costar 3596 96-well polystyrene plate. Each well was supplemented with 5 µl ABO-compatible human serum (i.e. 10% by final volume) or bovine serum albumin in the form of 0.5% (w/v) AlbumaxTM (Gibco BRL) (again at 10% by final volume) and with glutamine (final concentration 2 mM). 3D7 or FCB-1 *P. falciparum* schizonts, collected and purified at 40 hours post invasion, were added with fresh erythrocytes (blood group O Rhesus Negative) to give a final parasitaemia of 2% and a final haematocrit of 3-5% in a total volume of 50 µl in each well. Final concentration of test antibody in each well was 0.5-1 mg/ml. scFvs were tested at a final concentration of approximately 200 µg/ml.

Positive control assays (“no invasion”) were carried out using mouse mAbs 12.8 and 12.10 at 0.5-1 mg/ml, and using EGTA (0.4 mM final concentration). Negative control assays (“full invasion”) were carried out using 0.5-1 mg/ml mAb BC1, a murine mAb not directed against any *P. falciparum* antigen, or the anti-hen’s egg lysozyme scFv D1.3, and also using medium alone.

scFv 12.10-ABD2 was tested in the presence of 0.05% (w/v) human albumin. Human scFvs were also tested in the presence of 25-50 µg/ml anti-PentaHIS antibody (Qiagen) or a 1:8 dilution of hybridoma 9E10 supernatant.

The plates were placed in a gassed box containing an atmosphere of 7% CO₂, 5% O₂, 88% N₂, and incubated at 37°C for 20 hours.

Each antibody was tested in two identical wells. The percentage of erythrocytes invaded after 20 hours was determined separately from two Giemsa-stained thin-film slides from each well. One thousand erythrocytes were counted on each slide, and slides were examined blind. The mean of these four independent counts was taken to be the final parasitaemia.

Figure 2.1

PCR construction of a single chain variable fragment (scFv) from mAb

12.10

This figure shows the construction of scFv 12.10 from DNA encoding the variable regions of the mAb 12.10 heavy chain (V_H , shown in blue) and light chain (V_L , shown in red) and a flexible 18 amino acid linker, linker 218 (shown in green). The V_H and V_L sequences were assembled into a construct encoding a scFv by means of a three-step PCR reaction.

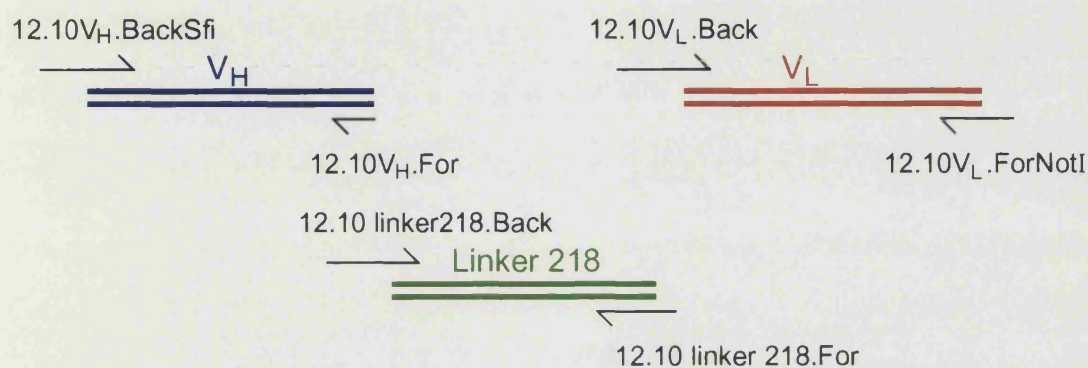
In Step 1, the V_H DNA was amplified with a 5' primer, 12.10 V_H .BackSfi, (designed to anneal to the 5' end of the gene just after the leader sequence) which introduced an *Sfi*I and *Nco*I site, and a 3' primer, 12.10 V_H .For. The V_L DNA was amplified with a 5' primer, 12.10 V_L .Back, and a 3' primer, 12.10 V_L .ForNotI, which added a *Not*I site. DNA encoding an 18 amino acid flexible linker was amplified with a 5' primer, 12.10linker218.Back, designed to overlap with the 3' end of the V_H , and a 3' primer, 12.10linker218.For, designed to overlap with the 5' end of the V_L .

In Step 2, the V_H PCR product from step 1 was combined with the linker using 12.10 V_H .BackSfi and 12.10linker218.For. The V_L PCR product from step 1 was also combined with the linker, using 12.10linker218.Back and 12.10 V_L .ForNotI.

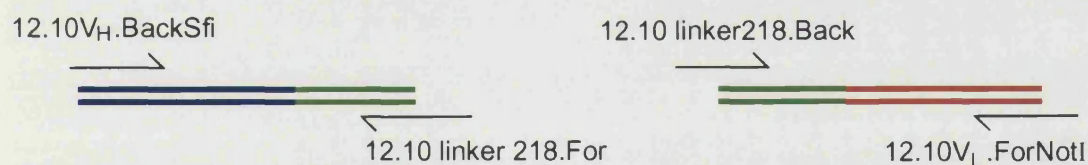
In Step 3, the PCR products from step 2 were united into a single chain variable fragment (scFv) using 12.10 V_H .BackSfi and 12.10 V_L .ForNotI.

Figure 2.1

PCR Step 1 (3 reactions)



PCR Step 2 (2 reactions)



PCR Step 3 (1 reaction)

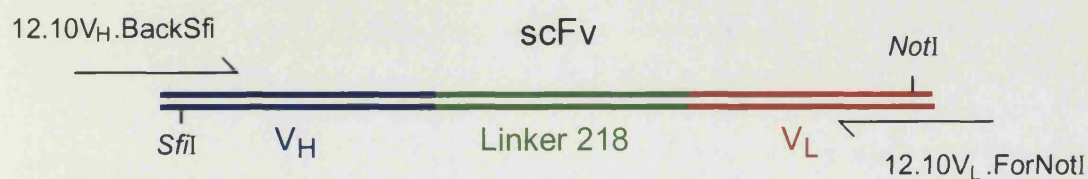


Figure 2.2

PCR site-directed mutagenesis to correct the DNA sequence of scFv 12.10

On sequencing the DNA encoding scFv 12.10, an unwanted single base pair mutation was found encoding the substitution of proline for serine at position 17 of the first framework region of the heavy chain (marked here in blue). A primer, N1210V_HFr1.rev, was designed to enable correction of the sequence using PCR to produce a site-directed mutagenesis. The method is illustrated here. N1210V_HFr1.rev was designed to anneal to the framework 1 region of the heavy chain, and this primer incorporated the correct DNA sequence (marked here in black) without the unwanted mutation.

Using the mutated scFv DNA as a template, this primer was used with 1210V_H.BackSfi to amplify a 5' portion of scFv 12.10 approximately 75 base pairs long (shown here in pink), which incorporated the corrected sequence rather than the unwanted mutation. The 75 base pair product from this PCR was then used as a primer in conjunction with the primer 1210.V_LForNotI, to amplify the whole of scFv 12.10 (again using the DNA with the unwanted mutation as a template). This second PCR was designed to yield a full-length scFv product in which the mutation had reverted back to the desired sequence.

Figure 2.2

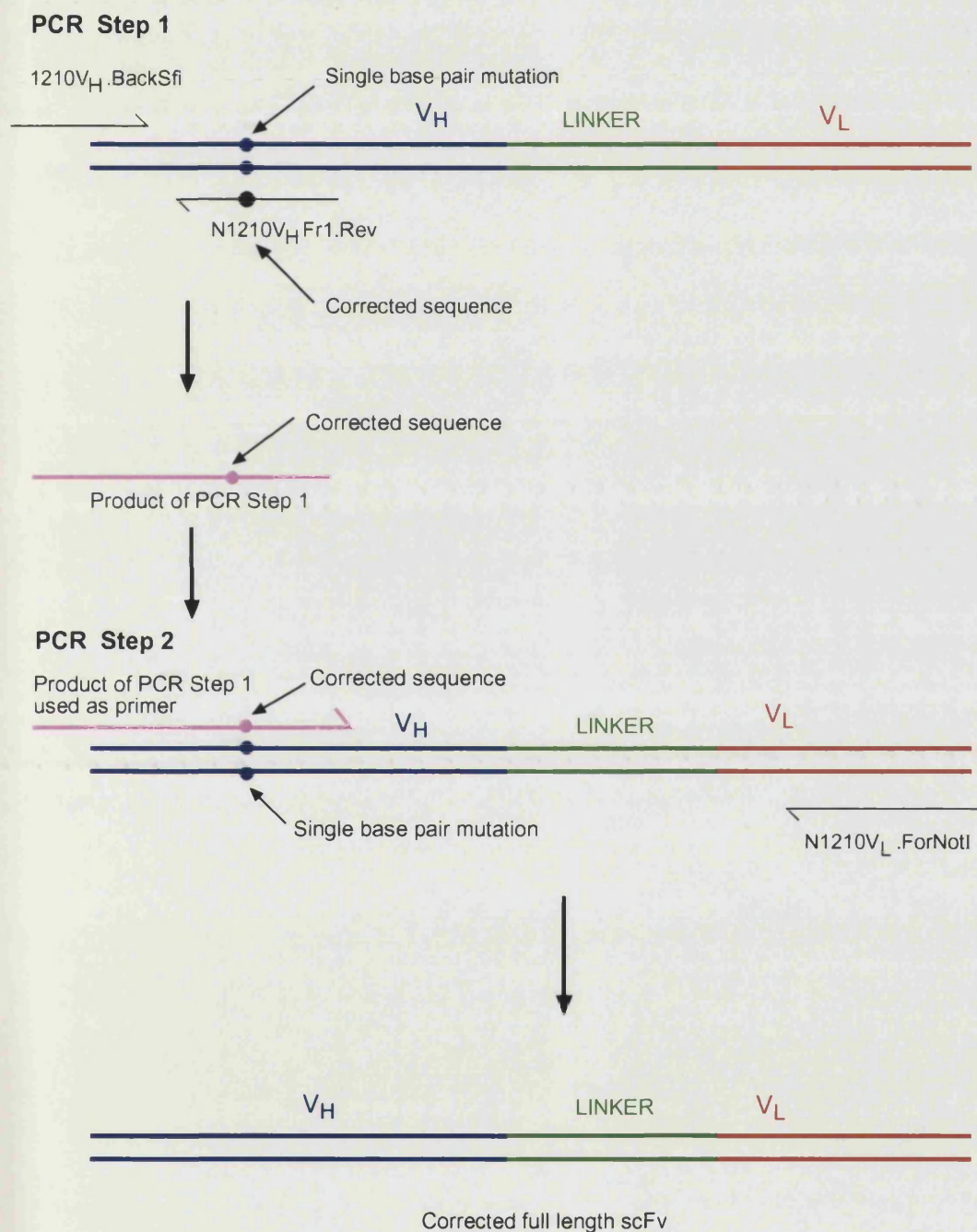


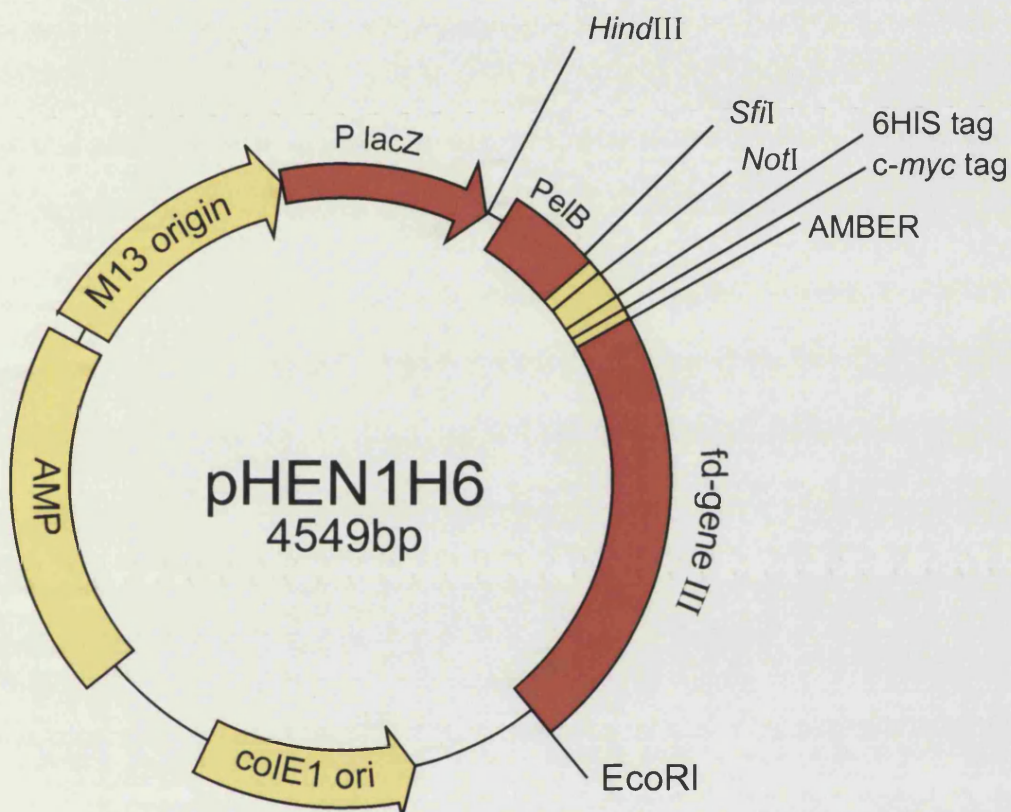
Figure 2.3

Phagemid vector pHEN1H6

The phagemid vector pHEN1 was constructed specifically for the purpose of displaying antibody fragments on phage (Hoogenboom, Griffiths et al. 1991). The pHEN1 phagemid, modified by the addition of a hexahistidine tag to give pHEN1H6, is illustrated here.

pHEN1H6 is a derivative, via pHEN1, of pUC119 (Vieira and Messing 1987), adapted by the inclusion of the coding region of pIII from the fd filamentous phage. The pIII signal sequence has been replaced by a *pelB* signal sequence which targets the expressed scFv to the bacterial periplasm. Between the signal sequence and the gene for pIII are an internal *SfiI* site and a *NotI* site, allowing antibody fragments to be cloned as *SfiI/NotI* fragments. Directly after the *NotI* site is a hexahistidine tag followed by a *c-myc* tag, both for immunodetection of antibody fragments. An amber stop codon (TAG) has been introduced between the tag and the region encoding the N-terminus of pIII. Growth of this phagemid in suppresser (*supE*) strains of *E. coli* such as TG1 *E. coli*, in which the amber codon is read as glutamine, results in the ribosome translating through the amber codon on to the pIII gene. This results in the antibody fragment being expressed as a fusion with the pIII protein. Growth of the same phagemid in a non-suppresser strain of *E. coli* such as HB2151, which reads the amber codon as a stop codon, results in the translation ending at the end of the antibody fragment, which is therefore expressed as a soluble fragment without the pIII protein (Hoogenboom, Griffiths et al. 1991). Transcription is driven from the inducible *lacZ* promoter.

Figure 2.3



PHEN1H6

```

PelB LEADER                                -1  +1 POLYLINKER
L  L  A  A  Q  P  A  M  A  Q  V  Q  L  Q  V  D  L  E  I  K  R
TTA CTC GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTG CAG GTC GAC CTC GAG ATC AAA CGG
                SfiI                                PstI                                XhoI

[=== Poly H TAG =====]                  [===== C-Myc TAG =====]
A  A  A  H  H  H  H  H  H  G  A  A  E  Q  K  L  I  S  E  E  D
GCG GCC GCA CAT CAC CAT CAC CAT CAC GGG GCC GCA GAA CAA AAA CTC ATC TCA GAA GAG GAT
NotI

==]                                +2 gIII
L  N  G  A  A  (E)  T  V  E
CTG AAT GGG GCC GCA TAG ACT GTT GAA
                amber

```

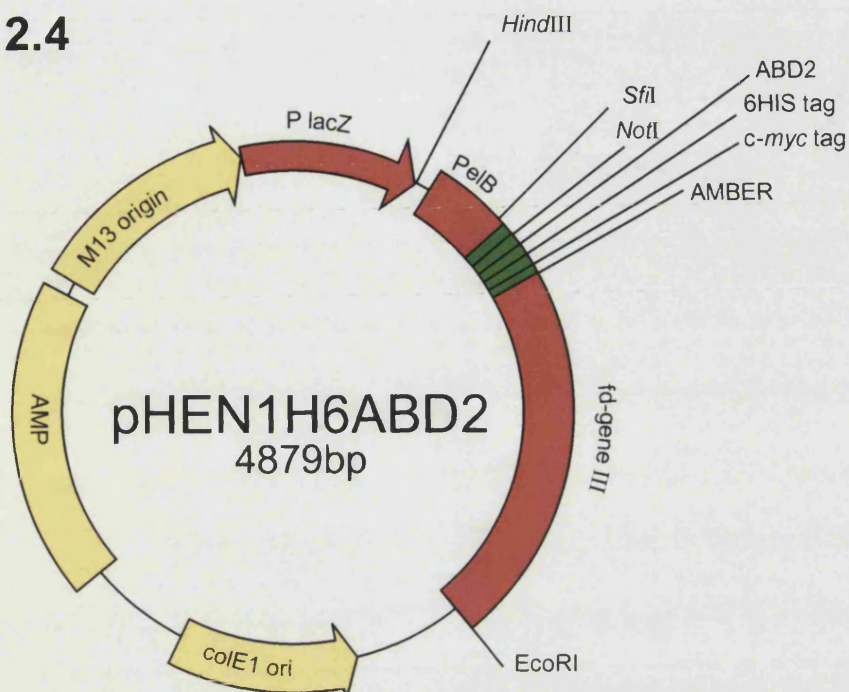
Figure 2.4

Phagemid vector pHEN1H6ABD2

pHEN1H6ABD2 is modified from pHEN1H6 by the insertion of two albumin-binding domains (ABDs) positioned immediately after the *NotI* site and immediately before the hexahistidine tag. Expression in this vector results in a scFv expressed as a fusion with the two albumin-binding domains followed by the hexahistidine and *c-myc* tags. The purpose of creating such a fusion is to express scFv which, in the presence of albumin, will bind two albumin molecules (molecular weight 68 kDa) and thereby acquire a total mass approximating to that of a whole antibody.

The albumin-binding domains are flanked with 3 additional glycine residues in order to facilitate flexibility in the fusion protein and to preserve the binding of the scFv, ABDs and hexahistidine and *c-myc* tags.

Figure 2.4



pHEN1H6ABD2

PelB LEADER

```
.L...L...A...A...Q...P...A...M...A...Q...V...Q...L...Q...V...D...L...E...I...K...R
TTA.CTC.GCG.GCC.CAG.CCG.GCC.ATG.GCC.CAG.GTG.CAG.CTG.CAG.GTC.GAC.CTG.GAG.ATC.AAA.CGG.
          SfiI                                PstI.                                XhoI
```

```
[=====
.A...A...A...G G G L A E A K V L A N R E L D K Y
GCG.GCC.GCA.GGC.GGT.GGA.CTG.GCT.GAA.GCT.AAA.GTT.CTG.GCT.AAC.CGT.GAA.CTG.GAC.AAA.TAC.
NotI
```

```
=====FIRST ALBUMIN BINDING DOMAIN=====
G V S D Y Y K N L I N N A K T V E G V K A
GGT.GTT.TCC.GAC.TAC.TAC.AAA.AAC.CTC.ATC.AAC.AAC.GCT.AAA.ACC.GTT.GAA.GGT.GTT.AAA.GCT.
```

```
=====] . [XXXXXXXXXXXXXXXXXXXXX
L I D E I L A A L P G G G G A A G G G L A
CTG.ATC.GAC.GAA.ATT.CTC.GCA.GCA.CTG.CCG.GGT.GGC.GGA.GGG.GCC.GCA.GGC.GGT.GGA.CTG.GCT.
```

```
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
E A K V L A N R E L D K Y G V S D Y Y K N
GAA.GCT.AAA.GTT.CTG.GCT.AAC.CGT.GAA.CTG.GAC.AAA.TAC.GGT.GTT.TCC.GAC.TAC.TAC.AAA.AAC.
```

```
XXXXXXXXXXXXSECOND ALBUMIN BINDING DOMAINXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
L I N N A K T V E G V K A L I D E I L A A
CTC.ATC.AAC.AAC.GCT.AAA.ACC.GTT.GAA.GGT.GTT.AAA.GCT.CTG.ATC.GAC.GAA.ATT.CTC.GCA.GCA.
```

```
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX[====POLY H TAG=====] [==C-MYC TAG==
L P G G G G A A...H...H...H...H...G...A...A...E Q K L
CTG.CCG.GGT.GGC.GGA.GGG.GCC.GCA.CAT CAC CAT CAC CAT CAC GGG GCC GCA GAA CAA AAA CTC
```

```
=====]
I S E E D L N G A A (E) T V E
ATC TCA GAA GAG GAT.CTG AAT GGG GCC GCA TAG ACT GTT GAA
Amber
```

Figure 2.5

Construction of the phage display libraries

This figure illustrates the steps involved in constructing the phage display libraries.

Step 1

Total RNA, extracted from the PBMLs of malaria-exposed donors, is used as a template for first-strand cDNA synthesis using specific primers.

Step 2

The variable regions of the antibody heavy chains and κ and λ light chains are amplified from cDNA using PCR primers specific for each of the different gene families. DNA encoding the linker is amplified with primers designed to create overlaps with the sequences of the heavy and light chains.

Step 3

The linker sequences are joined to either the heavy chain sequence or the κ or λ light chain sequences.

Step 4

The products from step 3 are joined to make full-length scFv sequences, and the primers used to do this also insert *SfiI* and *NotI* restriction sites for cloning.

Step 5

Long PCR primers are used to introduce long extensions to the scFv sequences to facilitate efficient *SfiI/NotI* restriction digestion.

Step 6

The products from step 5 are digested with *SfiI/NotI* to yield scFv inserts ready for cloning into pHEN1H6.

Figure 2.5

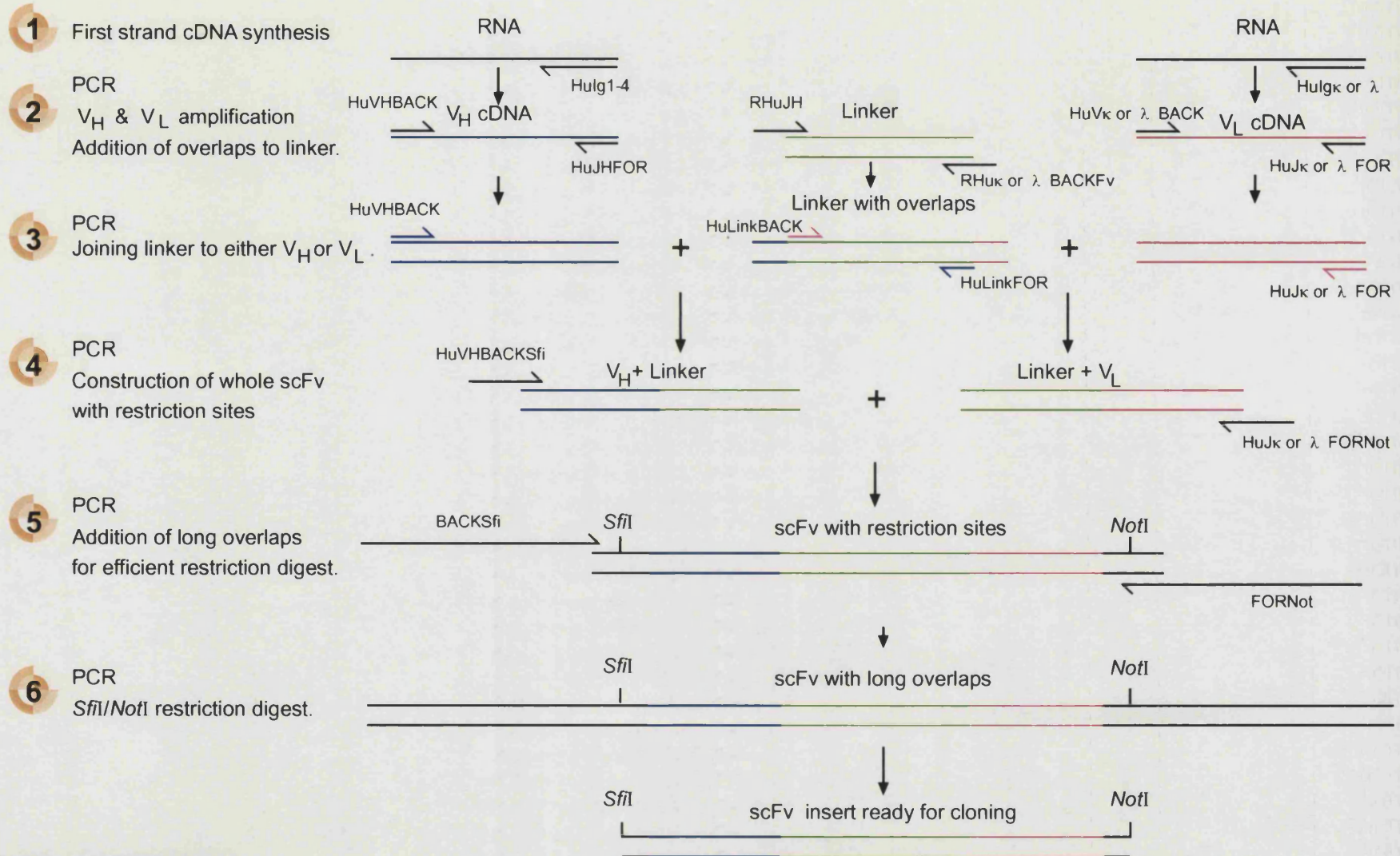


Table 2.1 a)**PCR primers for cloning mouse heavy chain variable regions**

Name	Sequence
MHV1 ^a (27mer)	ATGAAATGCAGCTGGGGCATCTTCTTC G
MHV2 (26mer)	ATGGGATGGAGCTGTATCATGTTCTT A CC
MHV3 (27mer)	ATGAAGTTGTGGTTAAACTGGGTTTTT A
MHV4 (25mer)	ATGAACTTTGGGCTCAGCTTGATTT G T G
MHV5 (30mer)	ATGGACTCCAGGCTCAATTTAGTTTTCTT
MHV6 (27mer)	ATGGCTGTCCTAGGGCTACTCTTCTGC T G C G
MHV7 (26mer)	ATGGGATGGAGCGGGATCTTTCTCTT A T G A
MHV8 (23mer)	ATGAGAGTGCTGATTCTTTTGTG
MHV9 (30mer)	ATGGATTGGGTGTGGACCTTGCTATTCCTG C A
MHV10 (27mer)	ATGGGCAGACTTACATTCTCATTCCTG
MHV11 (28mer)	ATGGATTTTGGGCTGATTTTTTTTATTG
MHV12 (27mer)	ATGATGGTGTAAAGTCTTCTGTACCTG
MHCG1 (21mer)	CAGTGGATAGACAGATGGGGG
MHCG2a (21mer)	CAGTGGATAGACCGATGGGGG
MHCG2b (21mer)	CAGTGGATGAGCTGATGGGGG
MHCG3 (21mer)	CAAGGGATAGACAGATGGGGC

^a MHV indicates primers that hybridise to leader sequences of mouse heavy chain variable region genes.

Table 2.1 b)
PCR primers for cloning mouse kappa light chain variable regions

Name	Sequence
MKV1 ^a (30mer)	ATGAAGTTGCCTGTTAGGCTGTTGGTGCTG
MKV2 (30mer)	ATGGAGACAGACACACTCCTGCTATGGGTG T T
MKV3 (30mer)	ATGAGTGTGCTCACTCAGGTCCTGGCGTTG G
MKV4 (33mer)	ATGAGGGCCCCTGCTCAGTTTTTTGGCTTCTTG A A C AA
MKV5 (30mer)	ATGGATTTTTCAGGTGCAGATTATCAGCTTC A T
MKV6 (27mer)	ATGAGGTGCCCTGTTTCAGTTCCTGGGG T TT C G C T A
MKV7 (31mer)	ATGGGCATCAAGATGGAGTCACAGACCCAG T TTTT T G
MKV8 (31mer)	ATGTGGGGACCTTTTTTCCCTTTTTCAATTG T G C AA
MKV9 (25mer)	ATGGTATCCACACCTCAGTTCCTTG G T G
MKV10 (27mer)	ATGTATATATGTTTGTTGTCTATTTCT
MKV11 (28mer)	ATGGAAGCCCCAGCTCAGCTTCTCTTCC
MKV13 (26mer)	ATGGATTACAGGCCCAAGTTCTTAT
MKC (20mer)	ACTGGATGGTGGGAAGATGG

^a MKV indicates primers that hybridise to leader sequences of mouse kappa light chain variable region genes.

Table 2.2 PCR primers and linker for construction of scFvs 12.8 and

12.10

12.8V_H.BackSfi

GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG ATC CAC TTG GTG CAG TCT
GG

12.8V_H.For

TGA GGA GAC TGT GAG AGT G

12.8V_L.Back

CAA ATT GTT CTC ACC CAG TCT CC

12.8V_L.ForNotI

GAG TCA TTC TCG ACT TGC GGC CGC TCG TTT TAT TTC CAA CTT TGT CCC

12.8Linker218.For

GGA GAC TGG GTG AGA ACA ATT TGA CCT TTA GTG GAA CCT TCA CCG G

12.8Linker218.Back

CAC TCT CAC AGT CTC CTC AGG CTC TAC CTC TGG

12.10V_H.BackSfi

GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTC CAA CTG CAG CA

12.10 V_H.For

TGA GGA GAC GGT GAC CGT G

12.10V_L.Back

GAC ATT GTG ATG ACC CAG TCT CA

12.10V_L.ForNotI

GAG TCA TTC TCG ACT TGC GGC CGC TCG TTT CAG CTC CAG CTT GGT CCC

12.10Linker218.Back

CAG GGT CAC CGT CTC CTC AGG CTC TAC CTC TGG

12.10Linker218.For

GAG ACT GGG TCA TCA CAA TGT CAC CTT TAG TGG AAC CTT CAC CGG

Linker218

GGC TCT ACC TCT GGC TCT GGT AAA CCG GGT TCC GGT GAA GGT CCA CTA A

Table 2.3

Adult Gambian blood donors from whom the libraries were derived

ID number	Age	Sex	Location	Donated to 1 st library	Donated to 2 nd library
Z999	68	M	Bundung	Y	N
Z157	24	F	Brefet	Y	Y
Z165	25	F	Brefet	Y	N
Z152	40	F	Brefet	Y	N
Z149	44	M	Brefet	Y	Y
Z151	30	M	Brefet	Y	Y
Z142	30	M	Brefet	Y	Y
Z144	23	M	Bundung	Y	N
Z49	35	M	Brefet	Y	Y
Z168	31	F	Brefet	Y	Y
Z88	44	F	Brefet	Y	Y
Z71	32	F	Sukuta	Y	Y
Z141	54	F	Brefet	Y	Y
Z158	24	F	Somita	Y	Y
Z176	44	F	Somita	Y	Y
Z16	44	F	Tubakuta	Y	Y
Z15	52	F	Brefet	Y	Y
Z5	71	M	Brefet	Y	N
Z21	34	M	Mandinaring	Y	Y
Z143	25	M	Bundung	Y	N

Table 2.4 a)

PCR primers to amplify human V_H heavy chain sequences

HuVHBACK primers anneal to the 5' end of the V_H sequences.

HuJHFOR primers anneal to the 3' end of the V_H sequences.

Sources of linkers are noted as follows:

Marks	As previously described (Marks, Hoogenboom et al. 1991)
JAC	Dr Jonathan Chappel, MRC Collaborative Centre
VB	V BASE GOLD database

<u>Primer</u>	<u>Source(s)</u>	<u>Sequence</u>
HuVH1aBACK	Marks	CAGGTGCAGCTGGTGCAGTCTGG
HuVH2xBACK	V BASE/JAC	CAGGTCACCTTGAAGGAGTCTGG
HuVH3aBACK	Marks	GAGGTGCAGCTGGTGGAGTCTGG
HuVH3xBACK	V BASE/JAC	GAGGTGCAGCTGGTGGAG
HuVH4aBACK	Marks	CAGGTGCAGCTGCAGGAGTCGGG
HuVH4xBACK	V BASE/JAC	CAGCTGCAGCTGCAGGAGTC
HuVH4yBACK	V BASE/JAC	CAGGTGCAGCTACAGCAGTG
HuVH5xBACK	V BASE/JAC	GAGGTGCAGCTGGTGCAGTCTGG
HuVH6aBACK	Marks	CAGGTACAGCTGCAGCAGTCAGG
HuVH7xBACK	V BASE/JAC	CAGGTGCAGCTGGTGCATCTGG
HuJH1-2FOR	Marks	TGAGGAGACGGTGACCAGGGTGCC
HuJH3FOR	Marks	TGAAGAGACGGTGACCATTTGTCCC
HuJH4-5FOR	Marks	TGAGGAGACGGTGACCAGGGTTCC
HuJH6FOR	Marks	TGAGGAGACGGTGACCGTGGTCCC

Table 2.4 b)**PCR primers to amplify human V_κ and V_λ light chain sequences**

HuV_κBACK and HuλBACK primers anneal to the 5' end of the V_κ and V_λ sequences respectively.

HuJ_κFOR and HuJλFOR primers anneal to the 3' end of the V_κ and V_λ sequences respectively.

HuV _κ 1aBACK	Marks	GACATCCAGATGACCCAGTCTCC
HuV _κ 2aBACK	Marks	GATGTTGTGATGACTCAGTCTCC
HuV _κ 2xBACK	V BASE/JAC	GATATTGTGATGACCCAGACTCC
HuV _κ 3aBACK	Marks	GAAATTGTGTTGACGCAGTCTCC
HuV _κ 3xBACK	V BASE/JAC	GAAATTGTAATGACACAGTCTCC
HuV _κ 4aBACK	Marks	GACATCGTGATGACCCAGTCTCC
HuV _κ 5aBACK	Marks	GAAACGACACTCACGCAGTCTCC
HuV _κ 6aBACK	Marks	GAAATTGTGCTGACTCAGTCTCC
HuV _κ 6xBACK	V BASE/JAC	GATGTTGTGATGACACAGTCTCC
HuJ _κ 1FOR	Marks	ACGTTTGATTTCCACCTTGGTCCC
HuJ _κ 2FOR	Marks	ACGTTTGATCTCCAGCTTGGTCCC
HuJ _κ 3FOR	Marks	ACGTTTGATATCCACTTTGGTCCC
HuJ _κ 4FOR	Marks	ACGTTTGATCTCCACCTTGGTCCC
HuJ _κ 5FOR	Marks	ACGTTTAATCTCCAGTCGTGTCCC
Huλ1BACK	Marks	CAGTCTGTGTTGACGCAGCCGCC
Huλ1xBACK	V BASE/JAC	CAGTCTGTGCTGACTCAGCCACC
Huλ2xBACK	V BASE/JAC	CAGTCTGCCCTGACTCAGCCT
Huλ3aBACK	Marks	TCCTATGTGCTGACTCAGCCACC
Huλ3bBACK	Marks	TCTTCTGAGCTGACTCAGGACCC
Huλ3xBACK	V BASE/JAC	TCCTATGAGCTGACTCAGCCAC
Huλ4xBACK	V BASE/JAC	CTGCCTGTGCTGACTCAGCCCCC
Huλ4yBACK	V BASE/JAC	CAGCCTGTGCTGACTCAATC
Huλ5xBACK	V BASE/JAC	CAGCCTGTGCTGACTCAGCC
Huλ6BACK	Marks	AATTTTATGCTGACTCAGCCCCA
Huλ7xBACK	V BASE/JAC	CAGACTGTGGTGACTCAGGAGCC
Huλ8xBACK	V BASE/JAC	CAGACTGTGGTGACCCAGGAGCC
Huλ10xBACK	V BASE/JAC	CAGGCAGGGCTGACTCAGCCACC
HuJλ1FOR	Marks	ACCTAGGACGGTGACCTTGGTCCC
HuJλ2-3FOR	Marks	ACCTAGGACGGTCAGCTTGGTCCC
HuJλ7xFOR	V BASE/JAC	ACCGAGGACGGTCAGCTGGGTGCC

Table 2.5 PCR reactions to amplify human V_H , V_K and V_λ sequences

PCRs TO AMPLIFY HEAVY CHAIN VARIABLE REGION (V_H)				
5' PRIMER	3' PRIMER	cDNA	Polymerase 03/97	Polymerase 10/97
HuVH1aBACK	JHFOR mix	V_H	Deep Vent	Deep Vent
HuVH2xBACK	JHFOR mix	V_H	Deep Vent	AmpliTaq
HuVH3aBACK	JHFOR mix	V_H	AmpliTaq	Deep Vent
HuVH3xBACK	JHFOR mix	V_H	Deep Vent	Deep Vent
HuVH4aBACK	JHFOR mix	V_H	AmpliTaq	AmpliTaq
HuVH4xBACK	JHFOR mix	V_H	AmpliTaq	Deep Vent
HuVH4yBACK	JHFOR mix	V_H	Deep Vent	Deep Vent
HuVH5xBACK	JHFOR mix	V_H	Deep Vent	AmpliTaq
HuVH6aBACK	JHFOR mix	V_H	Deep Vent	Deep Vent
HuVH7xBACK	JHFOR mix	V_H	AmpliTaq	Deep Vent
PCRs TO AMPLIFY KAPPA LIGHT CHAIN VARIABLE REGION (V_K)				
5' PRIMER	3' PRIMER	cDNA	Polymerase 03/97	Polymerase 10/97
HuVK1aBACK	JkFOR mix	V_K	Deep Vent	AmpliTaq
HuVK2aBACK	JkFOR mix	V_K	AmpliTaq	AmpliTaq
HuVK2xBACK	JkFOR mix	V_K	Deep Vent	AmpliTaq
HuVK3aBACK	JkFOR mix	V_K	AmpliTaq	AmpliTaq
HuVK3xBACK	JkFOR mix	V_K	AmpliTaq	AmpliTaq
HuVK4aBACK	JkFOR mix	V_K	AmpliTaq	AmpliTaq
HuVK5aBACK	JkFOR mix	V_K	AmpliTaq	AmpliTaq
HuVK6aBACK	JkFOR mix	V_K	AmpliTaq	AmpliTaq
HuVK6xBACK	JkFOR mix	V_K	AmpliTaq	AmpliTaq
PCRs TO AMPLIFY LAMBDA LIGHT CHAIN VARIABLE REGION (V_λ)				
5' PRIMER	3' PRIMER	cDNA	Polymerase 03/97	Polymerase 10/97
Hu λ 1BACK	J λ FOR mix	V_λ	AmpliTaq	AmpliTaq
Hu λ 1xBACK	J λ FOR mix	V_λ	AmpliTaq	AmpliTaq
Hu λ 2xBACK	J λ FOR mix	V_λ	AmpliTaq	Deep Vent
Hu λ 3aBACK	J λ FOR mix	V_λ	AmpliTaq	Deep Vent
Hu λ 3bBACK	J λ FOR mix	V_λ	AmpliTaq	Deep Vent
Hu λ 3xBACK	J λ FOR mix	V_λ	AmpliTaq	AmpliTaq
Hu λ 4xBACK	J λ FOR mix	V_λ	AmpliTaq	AmpliTaq
Hu λ 4yBACK	J λ FOR mix	V_λ	AmpliTaq	AmpliTaq
Hu λ 5xBACK	J λ FOR mix	V_λ	AmpliTaq	AmpliTaq
Hu λ 6BACK	J λ FOR mix	V_λ	Deep Vent	Deep Vent
Hu λ 7xBACK	J λ FOR mix	V_λ	Deep Vent	AmpliTaq
Hu λ 8xBACK	J λ FOR mix	V_λ	AmpliTaq	AmpliTaq
Hu λ 10xBACK	J λ FOR mix	V_λ	AmpliTaq	AmpliTaq

Table 2.6

PCR primers to amplify the library (GGGGS)₃ scFv linker

Regions which should hybridise to the linker are shown in bold type.

Sources of linkers are noted as follows:

Marks	As previously described (Marks, Hoogenboom et al. 1991)
JAC	Dr Jonathan Chappel, MRC Collaborative Centre
KK	Dr Katy Kettleborough, MRC Collaborative Centre
VB	V BASE GOLD database

<u>Primer</u>	<u>Source(s)</u>	<u>Sequence</u>
RHuJH1-2	Marks	GCACCCCTGGTCACCGTCTCCTCAGGTGG
RHuJH3	Marks	GGACAATGGTCACCGTCTCTTCAGGTGG
RHuJH4-5	Marks	GAACCCCTGGTCACCGTCTCCTCAGGTGG
RHuJH6	Marks	GGACCACGGTCACCGTCTCCTCAGGTGG
RHuVκ1aBACKFv	Marks/KK	GGAGACTGGGTCATCTGGATGTCAGATCCGCC
RHuVκ2aBACKFv	Marks/KK	GGAGACTGAGTCATCACAACATCAGATCCGCC
RHuVκ2xBACKFv	V B/JAC/KK	GGAGTCTGGGTCATCACAATATCAGATCCGCC
RHuVκ3aBACKFv	Marks/KK	GGAGACTGCGTCAACACAATTTTCAGATCCGCC
RHuVκ3xBACKFv	V B/JAC/KK	GGAGACTGTGTCTATTACAATTTTCAGATCCGCC
RHuVκ4aBACKFv	Marks/KK	GGAGACTGGGTCATCACGATGTCAGATCCGCC
RHuVκ5aBACKFv	Marks/KK	GGAGACTGCGTGAGTGTCTGTTTCAGATCCGCC
RHuVκ6aBACKFv	Marks/KK	GGAGACTGAGTCAGCACAATTTTCAGATCCGCC
RHuVκ6xBACKFv	V B/JAC/KK	GGAGACTGTGTCTATCACAACATCAGATCCGCC
RHuλ1BACKFv	Marks/KK	GGCGGCTGCGTCAACACAGACTGAGATCCGCCGCCACCCGAC
RHuλ1xBACKFv	V B/JAC/KK	GGTGGCTGAGTCAGCACAGACTGAGATCCGCCGCCACCCGAC
RHuλ2xBACKFv	V B/JAC/KK	AGGCTGAGTCAGGGCAGACTGAGATCCGCCGCCACCCGAC
RHuλ3aBACKFv	Marks/KK	GGTGGCTGAGTCAGCACATAGGAAGATCCGCCGCCACCCGAC
RHuλ3bBACKFv	Marks/KK	GGGTCCTGAGTCAGCTCAGAAGAAGATCCGCCGCCACCCGAC
RHuλ3xBACKFv	V B/JAC/KK	GTGGCTGAGTCAGCTCATAGGAAGATCCGCCGCCACCCGAC
RHuλ4xBACKFv	V B/JAC/KK	GGGGGCTGAGTCAGCACAGGCAGAGATCCGCCGCCACCCGAC
RHuλ4yBACKFv	V B/JAC/KK	GATTGAGTCAGCACAGGCTGAGATCCGCCGCCACCCGAC
RHuλ5xBACKFv	V B/JAC/KK	GGCTGAGTCAGCACAGGCTGAGATCCGCCGCCACCCGAC
RHuλ6BACKFv	Marks/KK	TGGGGCTGAGTCAGCATAAAATTAGATCCGCCGCCACCCGAC
RHuλ7xBACKFv	V B/JAC/KK	GGCTCCTGAGTCACCACAGTCTGAGATCCGCCGCCACCCGAC
RHuλ8xBACKFv	V B/JAC/KK	GGCTCCTGGGTCACCACAGTCTGAGATCCGCCGCCACCCGAC
RHuλ10xBACKFv	V B/JAC/KK	GGTGGCTGAGTCAGCCCTGCCTGAGATCCGCCGCCACCCGAC

Table 2.7 a)

PCR reactions to amplify the linker for incorporation in V_H-V_K scFv

PRIMER 1		PRIMER 2
RHuVκ1aBACKFv	+	RHuJH1-2
RHuVκ2aBACKFv	+	RHuJH1-2
RHuVκ2xBACKFv	+	RHuJH1-2
RHuVκ3aBACKFv	+	RHuJH1-2
RHuVκ3xBACKFv	+	RHuJH1-2
RHuVκ4aBACKFv	+	RHuJH1-2
RHuVκ5aBACKFv	+	RHuJH1-2
RHuVκ6aBACKFv	+	RHuJH1-2
RHuVκ6xBACKFv	+	RHuJH1-2
RHuVκ1aBACKFv	+	RHuJH3
RHuVκ2aBACKFv	+	RHuJH3
RHuVκ2xBACKFv	+	RHuJH3
RHuVκ3aBACKFv	+	RHuJH3
RHuVκ3xBACKFv	+	RHuJH3
RHuVκ4aBACKFv	+	RHuJH3
RHuVκ5aBACKFv	+	RHuJH3
RHuVκ6aBACKFv	+	RHuJH3
RHuVκ6xBACKFv	+	RHuJH3
RHuVκ1aBACKFv	+	RHuJH4-5
RHuVκ2aBACKFv	+	RHuJH4-5
RHuVκ2xBACKFv	+	RHuJH4-5
RHuVκ3aBACKFv	+	RHuJH4-5
RHuVκ3xBACKFv	+	RHuJH4-5
RHuVκ4aBACKFv	+	RHuJH4-5
RHuVκ5aBACKFv	+	RHuJH4-5
RHuVκ6aBACKFv	+	RHuJH4-5
RHuVκ6xBACKFv	+	RHuJH4-5
RHuVκ1aBACKFv	+	RHuJH6
RHuVκ2aBACKFv	+	RHuJH6
RHuVκ2xBACKFv	+	RHuJH6
RHuVκ3aBACKFv	+	RHuJH6
RHuVκ3xBACKFv	+	RHuJH6
RHuVκ4aBACKFv	+	RHuJH6
RHuVκ5aBACKFv	+	RHuJH6
RHuVκ6aBACKFv	+	RHuJH6
RHuVκ6xBACKFv	+	RHuJH6

Table 2.7 b)

PCR reactions to amplify the linker for incorporation in V_H-V_λ scFv

PRIMER 1		PRIMER 2
RHuλ1BACKFv	+	RHuJH1-2
RHuλ1xBACKFv	+	RHuJH1-2
RHuλ2xBACKFv	+	RHuJH1-2
RHuλ3aBACKFv	+	RHuJH1-2
RHuλ3bBACKFv	+	RHuJH1-2
RHuλ3xBACKFv	+	RHuJH1-2
RHuλ4xBACKFv	+	RHuJH1-2
RHuλ4yBACKFv	+	RHuJH1-2
RHuλ5xBACKFv	+	RHuJH1-2
RHuλ6BACKFv	+	RHuJH1-2
RHuλ7xBACKFv	+	RHuJH1-2
RHuλ8xBACKFv	+	RHuJH1-2
RHuλ10xBACKFv	+	RHuJH1-2
RHuλ1BACKFv	+	RHuJH3
RHuλ1xBACKFv	+	RHuJH3
RHuλ2xBACKFv	+	RHuJH3
RHuλ3aBACKFv	+	RHuJH3
RHuλ3bBACKFv	+	RHuJH3
RHuλ3xBACKFv	+	RHuJH3
RHuλ4xBACKFv	+	RHuJH3
RHuλ4yBACKFv	+	RHuJH3
RHuλ5xBACKFv	+	RHuJH3
RHuλ6BACKFv	+	RHuJH3
RHuλ7xBACKFv	+	RHuJH3
RHuλ8xBACKFv	+	RHuJH3
RHuλ10xBACKFv	+	RHuJH3
RHuλ1BACKFv	+	RHuJH4-5
RHuλ1xBACKFv	+	RHuJH4-5
RHuλ2xBACKFv	+	RHuJH4-5
RHuλ3aBACKFv	+	RHuJH4-5
RHuλ3bBACKFv	+	RHuJH4-5
RHuλ3xBACKFv	+	RHuJH4-5
RHuλ4xBACKFv	+	RHuJH4-5
RHuλ4yBACKFv	+	RHuJH4-5
RHuλ5xBACKFv	+	RHuJH4-5
RHuλ6BACKFv	+	RHuJH4-5
RHuλ7xBACKFv	+	RHuJH4-5
RHuλ8xBACKFv	+	RHuJH4-5
RHuλ10xBACKFv	+	RHuJH4-5
RHuλ1BACKFv	+	RHuJH6
RHuλ1xBACKFv	+	RHuJH6
RHuλ2xBACKFv	+	RHuJH6
RHuλ3aBACKFv	+	RHuJH6
RHuλ3bBACKFv	+	RHuJH6
RHuλ3xBACKFv	+	RHuJH6
RHuλ4xBACKFv	+	RHuJH6
RHuλ4yBACKFv	+	RHuJH6
RHuλ5xBACKFv	+	RHuJH6
RHuλ6BACKFv	+	RHuJH6
RHuλ7xBACKFv	+	RHuJH6
RHuλ8xBACKFv	+	RHuJH6
RHuλ10xBACKFv	+	RHuJH6

Table 2.8**PCR reactions to attach V_H and V_K and V_λ sequences to linker DNA**

PCR Reaction	1	2	3	4	5	6	7	8	9	10
10xPEBufferII/MgCl ₂ /dNTPs	24	24	24	24	24	24	24	24	24	24
DNA template: V_H PCR product mix (5 ng/ μ l)	1	1	0	0	0	0	0	0	0	0
DNA template: K linkers 2 ng/ μ l	1	0	1	1	1	1	1	0	0	0
DNA template: Λ linkers 2 ng/ μ l	0	1	0	0	0	0	0	1	1	1
Primer VHback mix	4	4	0	0	0	0	0	0	0	0
Primer HulinkFOR	4	4	0	0	0	0	0	0	0	0
Primer HulinkBACK	0	0	4	4	4	4	4	4	4	4
DNA template: V_K PCR product mix (5 ng/ μ l)	0	0	1	1	1	1	1	0	0	0
DNA template: V_λ PCR product mix (5 ng/ μ l)	0	0	0	0	0	0	0	1	1	1
Primer HuJkFOR	0	0	4 K1	4 K2	4 K3	4 K4	4 K5	0	0	0
Primer HuJ λ FOR	0	0	0	0	0	0	0	4 Λ 1	4 Λ 2-3	4 Λ 7x
H ₂ O	65	65	65	65	65	65	65	65	65	65
AmpliTaq	1	1	1	1	1	1	1	1	1	1

Table 2.9 a)

5' Primers used to assemble whole scFv and introduce *Sfi*I restriction enzyme sites

HuBACKSfi primers anneal to the 5' end of the V_H sequence and introduce a *Sfi*I restriction site to the 5' end of the scFv

(*Sfi*I, bold type; *Nco*I, underlined)

<u>Primer</u>	<u>Source(s)</u>	<u>Sequence</u>
HuVH1aBACKSfi	Marks	GTCCTCGCAACTGCGGCCCAGCCGGCCATGGCCCAGGTGCAGCTGGTGCAGTCTGG
HuVH2xBACKSfi	V BASE/JAC	GTCCTCGCAACTGCGGCCCAGCCGGCCATGGCCCAGGTGCACCTGAAGGAGTCTGG
HuVH3aBACKSfi	Marks	GTCCTCGCAACTGCGGCCCAGCCGGCCATGGCCGAGGTGCAGCTGGTGGAGTCTGG
HuVH3xBACKSfi	V BASE/JAC	GTCCTCGCAACTGCGGCCCAGCCGGCCATGGCCGAGGTGCAGCTGGTGGAG
HuVH4aBACKSfi	Marks	GTCCTCGCAACTGCGGCCCAGCCGGCCATGGCCCAGGTGCAGCTGCAGGAGTCGGG
HuVH4xBACKSfi	V BASE/JAC	GTCCTCGCAACTGCGGCCCAGCCGGCCATGGCCCAGGTGCAGCTGCAGGAGTC
HuVH4yBACKSfi	V BASE/JAC	GTCCTCGCAACTGCGGCCCAGCCGGCCATGGCCCAGGTGCAGCTACAGCAGTG
HuVH5xBACKSfi	V BASE/JAC	GTCCTCGCAACTGCGGCCCAGCCGGCCATGGCCGAGGTGCAGCTGGTGCAGTCTGG
HuVH6aBACKSfi	Marks	GTCCTCGCAACTGCGGCCCAGCCGGCCATGGCCCAGGTACAGCTGCAGCAGTCAGG
HuVH7xBACKSfi	V BASE/JAC	GTCCTCGCAACTGCGGCCCAGCCGGCCATGGCCCAGGTGCAGCTGGTGCAATCTGG

Table 2.9 b)

3' Primers used to assemble whole scFv and introduce *NotI* restriction enzyme sites

HuFORNot primers anneal to the 3' end of the V_{κ} or V_{λ} sequence and introduce a *SfiI* restriction site to the 5' end of the scFv

(*NotI*, bold type)

<u>Primer</u>	<u>Source(s)</u>	<u>Sequence</u>
HuJ κ 1FORNot	Marks	GAGTCATTCTCGACTT GCGGCCGC ACGTTTGATTTCACCTTGGTCCC
HuJ κ 2FORNot	Marks	GAGTCATTCTCGACTT GCGGCCGC ACGTTTGATCTCCAGCTTGGTCCC
HuJ κ 3FORNot	Marks	GAGTCATTCTCGACTT GCGGCCGC ACGTTTGATATCCACTTGGTCCC
HuJ κ 4FORNot	Marks	GAGTCATTCTCGACTT GCGGCCGC ACGTTTGATCTCCACCTTGGTCCC
HuJ κ 5FORNot	Marks	GAGTCATTCTCGACTT GCGGCCGC ACGTTTAATCTCCAGTCGTGTCCC
HuJ λ 1FORNot	Marks	GAGTCATTCTCGACTT GCGGCCGC ACCTAGGACGGTGACCTTGGTCCC
HuJ λ 2-3FORNot	Marks	GAGTCATTCTCGACTT GCGGCCGC ACCTAGGACGGTCAGCTTGGTCCC
HuJ λ 7xFORNot	V BASE/JAC	GAGTCATTCTCGACTT GCGGCCGC ACCGAGGACGGTCAGCTGGGTGCC

Table 2.10

PCR primers designed to anneal to the 5' and 3' ends of the library scFv to produce long extensions

These primers were designed to hybridise to either the HuVHBACKSfi primer series, or the HuJk/λFORNot primer series, giving an extension of 100 bases outside the restriction sites. The extended sequences of these primers match the sequences of the corresponding flanking sequences of the pHEN1H6 phagemid, and are added to the scFv to improve the cutting efficiency of the restriction enzymes *Sfi*I and *Not*I. Restriction sites are shown in bold type.

BACKSfi Marks/JAC
ACCATGATTACGCCAAGCTTGCATGCAAATTCTATTTCAAGGAGACAGTCATAATGAAATACCTATTGCCTAC
GGCAGCCGCTGGAGTCCTCGCAACTGCGGGCCAGCCGGCCATGGCC

FORNot Marks/JAC
GACGTTAGTAAATGAATTTTCTGTATGAGGTTTTGCTAAACAACTTTCAACAGTCTATGCGGCCCCATTCAGA
TCCTCTTCTGAGAGTCATTCTCGACTTGCGGCCGCAC

CHAPTER 3

CLONING AND SEQUENCING OF MURINE INVASION-

INHIBITORY MONOCLONAL ANTIBODIES

3.1 Introduction

Around the time of erythrocyte invasion, the MSP-1 polypeptide complex on the surface of the invading merozoite undergoes a proteolytic cleavage, leaving only a small fragment, MSP-1₁₉, remaining on the merozoite surface. Two murine hybridoma mAbs specific for MSP-1₁₉, mAb 12.8 and mAb 12.10, inhibit erythrocyte invasion (Blackman, Heidrich et al. 1990) by blocking the protease-mediated cleavage of MSP-1 (Blackman, Scott-Finnigan et al. 1994).

Two questions of major interest arise from these observations. The first question is whether such invasion-inhibitory antibodies are made as part of the natural human immune response to *P. falciparum* infection. This project aimed to investigate this question by isolating and characterising natural human monoclonal antibodies to MSP-1₁₉ from a phage display library made from malaria exposed human donors. A second question of particular interest is whether antibodies which are invasion-inhibitory *in vitro* have the capacity to modify or prevent *P. falciparum* infection *in vivo*. This project aimed to take a step closer to answering this question by characterising recombinant human/murine chimeric versions of these mAbs which are likely to be suitable for testing in a primate model of *P. falciparum* infection.

An essential prelude to these studies was the identification, cloning and sequencing of the variable regions of the genes encoding the antigen binding sites of mAbs 12.8 and 12.10, and this work is described in this chapter.

It proved to be difficult to identify all the DNA sequences encoding the variable portions of the light and heavy chains of these antibodies, because it was difficult to distinguish the mAb 12.10 light chain sequence from a non-functional light chain pseudogene also present in the hybridoma. Hybridoma heavy and light chain pseudogenes are transcribed and may be partially translated, but are not generally secreted, and so do not contribute to the secreted hybridoma antibody. One light chain pseudogene, the MOPC-21 kappa light chain gene, has been identified in the myeloma cell line from which the 12.8 and 12.10 hybridomas were derived (Cowan, Secher et al. 1974) and it has been shown to be non-functional due to a deletion of 4 nucleotides leading to a frameshift (Walfield, Selsing et al. 1981). Its sequence has been described (Carroll, Mendel et al. 1988). However, another pseudogene was identified in the mAb 12.10 hybridoma, which had not been previously described. Misleadingly, this pseudogene in the 12.10 hybridoma was almost identical to the functional sequence encoding the mAb 12.8 light chain, and consequently the 12.10 pseudogene was initially thought to be the functional gene for the mAb 12.10 light chain. To resolve the uncertainty it became necessary to obtain the amino acid sequences of the mAb 12.8 and mAb 12.10 heavy and light chains. These sequences could not be obtained by N-terminal sequencing, presumably due to N-terminal blocking of the proteins, and were eventually obtained by mass spectrometry. In this way the identity and sequences of the antigen-specific variable regions of these invasion-inhibitory mAbs were finally confirmed.

3.2 Results

3.2.1 Amplification of DNA encoding the variable regions of mAb 12.8 and mAb 12.10

DNA encoding the variable regions of the heavy and light chains of mAbs 12.8 and 12.10 was amplified using a single primer (MHC or MKC for heavy and light chains respectively) designed to anneal to the constant region of the heavy or light chain, and 12 different primers (MHV or MKV for heavy and light chains respectively) designed to anneal to the different possible leader sequences of the gene. 12 PCR reactions were therefore carried out to amplify each heavy and each light chain variable sequence. In each case more than one of the 12 reactions gave a product of the expected size. This is because the primers amplify not only the functional genes but also the two known non-functional pseudogenes (the light chain sequence described above and a heavy chain sequence) which are transcribed but not translated, and are derived from the parent myeloma cell line (Cowan, Secher et al. 1974). From previous experience, it is known that the MHV5 primer anneals to the known heavy chain pseudogene and MKV2 anneals to the known light chain pseudogene. After attempting PCR amplification with all the primers, the variable region sequences were amplified along with a number of pseudogenes. After further work to investigate these sequences (described below) it was possible to determine that the following variable region sequences had been amplified with the following primers:

mAb 12.8 heavy chain	MHV7
mAb 12.10 heavy chain	MHV9
Parent myeloma heavy chain pseudogene	MHV5
mAb 12.8 light chain	MKV5

mAb 12.10 light chain	MKV7
Parent myeloma light chain pseudogene	MKV2 & MKV7
Previously unknown 12.10 light chain pseudogene	MKV5

Figure 3.1 shows the products of the 12 PCR reactions carried out to amplify the variable region of the heavy chain of mAb 12.10, in which both the functional gene DNA and the parent myeloma pseudogene DNA have been amplified.

3.2.2 Cloning and sequencing of DNA encoding variable regions of mAbs 12.8 and 12.10

The mAb 12.8 and mAb 12.10 heavy and light chain variable region DNA products were cloned into the pCRTM2.1 vector (Invitrogen) and sequenced using the Perkin Elmer ABI Prism 377 DNA Sequencer. The variable regions of both the heavy and the light chains differ significantly in DNA sequence between the two mAbs, which is consistent with the finding that they bind non-identical epitopes on the MSP-1₁₉ molecule (Chappel and Holder 1993).

Figure 3.2 shows the DNA and amino acid sequences of the variable regions of the heavy chains and light chains of mAb 12.8 and mAb 12.10, with the two mAbs compared.

3.2.3 A new non-functional light chain pseudogene found in the hybridoma 12.10

The myeloma cell line from which the hybridomas were made, P3/NS-1/1-Ag4-1 (NS-1), is derived from the MOPC-21 plasmacytoma (McBride, Newbold et al. 1985), which is known to contain a light chain pseudogene, the MOPC-21 pseudogene, whose

sequence is known (Carroll, Mendel et al. 1988). This pseudogene is transcribed, but is non-functional, having a frameshift in the third complementarity-determining region (CDR3). The MOPC-21 pseudogene is not secreted and so does not contribute to the secreted hybridoma antibody. The primers MKV2 and MKV7 are known from previous experience to anneal to the MOPC-21 pseudogene. However, the mAb 12.10 hybridoma was found to contain a further light chain pseudogene, which amplified with primer MKV5. This pseudogene also has a frameshift in CDR3. However, it bears a great similarity in sequence to the functional light chain of the mAb 12.8 hybridoma, which was derived from the same series of fusion experiments. Because of this similarity, it was initially assumed that this frameshifted gene was the functional gene, and that the frameshift represented either an artefact of the PCR amplification or of the DNA sequencing. Repeated amplifications and sequencing, however, showed a consistent frameshift in this gene. Sequencing of the PCR product obtained with primer MKV7 (and previously assumed to be the MOPC-21 pseudogene) showed it to be another completely different light chain gene in the 12.10 hybridoma, with an open reading frame throughout. With two candidate light chain DNA sequences to choose between, the functional DNA sequence was then identified by mass spectrometry of mAb 12.10 itself and mAb 12.10 Fab. There was no evidence on mass spectrometry of any expression of the mAb 12.10 pseudogene which amplified with primer MKV5, whereas the expressed product of the light chain region amplified with primer MKV7 was identified, confirming this as the variable region sequence of the functional gene.

Figure 3.3a) shows a comparison between the variable region DNA sequences of the mAb 12.10 functional light chain gene and light chain pseudo gene, which are very different from each other. Figure 3.3a) also shows the close similarity between the functional mAb 12.8 light chain variable region and the mAb 12.10 non-functional

pseudogene variable region. Figure 3.3b) shows the same comparisons, translated into amino acid sequences.

3.2.4 Attempted confirmation of mAb 12.8 and mAb 12.10 heavy and light chain amino acid sequences by N-terminal sequencing

Primarily because of the difficulty in distinguishing the mAb 12.10 light chain functional gene from the pseudogene, an attempt was made to determine the amino acid sequences of the variable regions of mAbs 12.8 and 12.10 by N-terminal sequencing. Approximately 2 µg of each of mAb 12.8 and mAb 12.10 was used and the heavy and light chains were resolved by SDS-PAGE using CAPS buffer as described in section 2.1.4, and then blotted onto an immobilon membrane and stained with Coomassie blue. The transferred protein was sent to Dr Arthur Moir of the Department of Molecular Biology and Biotechnology at the University of Sheffield for N-terminal sequencing based on the Edman degradation reaction. It did not prove possible to obtain any amino acid sequence information, and it was presumed that this was because of N-terminal blocking of the proteins. In case this blocking was due to the presence of N-terminal pyrrolidone carboxylic acid residues, an attempt was made to remove any such groups by incubating the proteins with pyroglutamate aminopeptidase. The treated proteins were then processed as before. Despite this measure, again no sequence information was obtained.

3.2.5 Confirmation of mAb 12.8 and mAb 12.10 heavy and light chain amino acid sequences by mass spectrometry

Since protein sequence information could not be obtained by N-terminal sequencing, it was decided to determine the amino acid sequences of the mAbs 12.8 and 12.10 by mass spectrometry. The proteins were processed as described in section 2.1.5, and mass spectrometry was carried out with Dr Steve Howell of the Division of Protein Structure at the National Institute for Medical Research. From candidate DNA sequences, predictions of expected tryptic fragments and their masses (as determined uncharged and charged) were made. Figure 3.4 shows such a prediction of the tryptic fragments anticipated from the mAb 12.10 light chain sequence amplified with the primer MKV7, which proved to be the functional gene. The figure also shows the actual mass spectrogram of the tryptic fragments obtained by digesting the light chain band of the mAb 12.10 Fab fragment. As an example, a peak is seen at mass 543.8 Da, corresponding to the predicted tryptic fragment T5, doubly charged ($[M+2H]$ in Figure 3.4). This fragment was accordingly isolated in the mass spectrometer, and its constituent ions analysed to determine the amino acid sequence. Figure 3.5 shows the predicted masses of the fragment ions, or “daughter ions”, of tryptic fragment T5, and the same figure shows the actual “daughter ion” spectrum, whose peaks correspond almost precisely to the predicted sizes, thus confirming the amino acid sequence.

Other tryptic fragments from both mAbs were analysed in the same way. By this method it became possible to identify the functional gene for the 12.10 light chain, and also to confirm the other DNA sequences.

Figure 3.6 shows the amino acid sequences as deduced from the DNA sequences, with underlining of those fragments which have been additionally confirmed at the protein level by mass spectrometry.

3.3 Discussion

The variable regions of the IgG heavy and light chains of the murine hybridoma mAbs 12.8 and 12.10 have now been cloned and sequenced. The amino acid sequences of the antigen-binding sites of these invasion-inhibitory IgGs are therefore now known. Although a non-functional light chain pseudogene has been identified in mAb 12.10 which is almost identical to the functional gene in mAb 12.8, the functional gene DNA sequences have been validated by mass spectrometry of the mAbs themselves. Much of the amino acid sequence of the mAb variable regions has been independently determined and confirmed in this way. The two invasion-inhibitory mAbs show marked differences in their amino acid sequences, both in the heavy and the light chains. This is consistent with previous observations that the two mAbs bind to non-identical epitopes in MSP-1₁₉, and demonstrates that two very different antibody variable region sequences can both have invasion-inhibitory properties.

It is assumed that the hybridoma 12.8 functional light chain gene and the hybridoma 12.10 light chain pseudogene are derived from the same original light chain gene, as the two hybridomas were made in the same series of fusion experiments (McBride, Newbold et al. 1985). Presumably the gene remained functional in hybridoma 12.8, but through a frameshift became non-functional in hybridoma 12.10, in which there was another functional light chain gene contributing to the secreted antibody instead. It is possible, however, that this pseudogene was already present in some precursor cell lines from

which these hybridomas were derived. If this is the case, this study's identification of this pseudogene may prove useful in the future. Knowledge of the pseudogenes present in a hybridoma is very useful when trying to identify and amplify antibody variable region sequences for the purposes of engineering antibodies into chimeric or humanised form. Various methods have been described for neutralising any pseudogenes which may interfere with this process (Nicholls, Johnson et al. 1993) (Cochet, Martin et al. 1999).

This work also underlines the potential usefulness and efficiency of sequencing proteins by mass spectrometry when DNA sequencing and N-terminal protein sequencing fail to resolve ambiguities.

Figure 3.1

PCR amplification of mAb 12.10 heavy chain variable region sequence

One PCR primer was used to anneal to the constant region of the heavy chain (primer MHC) and 12 different primers were used to anneal to the different possible leader sequences (primers MHV1-MHV12). PCR reactions were run on a 1% agarose gel.

Lanes 1 to 12 contain PCR reactions carried out with primers MHV1 to MHV12 respectively. Bands of the expected size (approximately 450 base pairs) are seen in lanes 5 and 9. From previous experience, primer MHV5 is known to amplify the parent myeloma heavy chain pseudogene, which is transcribed but not translated. The product in lane 5 was therefore disregarded, and the product in lane 9 was purified, cloned and sequenced, and found to be the variable region of the mAb 12.10 heavy chain as expected.

Figure 3.1

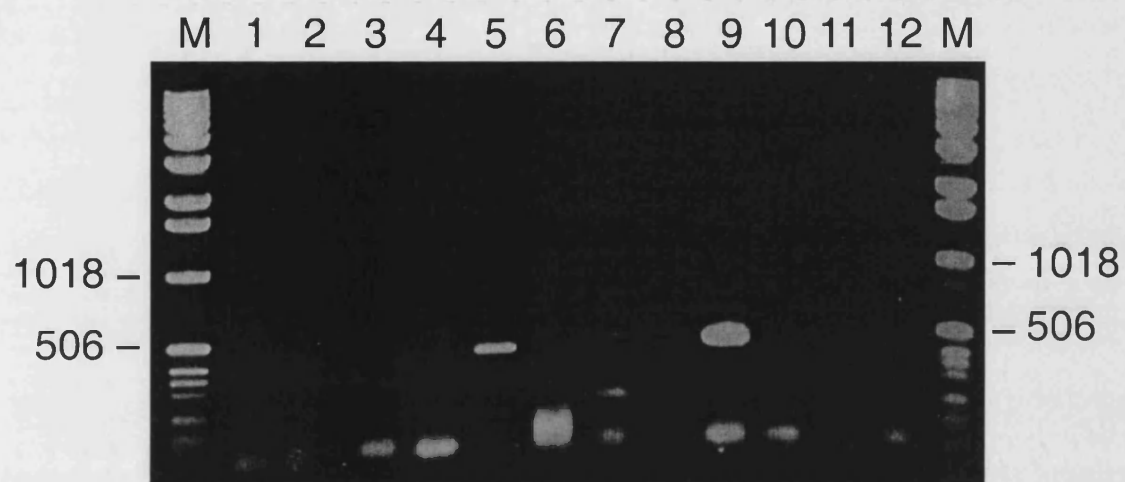


Figure 3.2a)

DNA sequences of mAb 12.8 and mAb 12.10 heavy and light chain variable region sequences

Above is shown a comparison between the heavy chain variable region DNA sequences of mAb 12.8 (12.8VH) and mAb 12.10 (12.10VH).

Below is shown a comparison between the light chain variable region DNA sequences of mAb 12.8 (12.8Vκ) and mAb 12.10 (12.10Vκ).

Identical nucleotides in the two heavy chains are highlighted in blue. Identical nucleotides in the two light chains are highlighted in green. There are significant differences between the two mAbs in both the heavy and light chain sequences.

Figure 3.2a)

mAb 12.8 and mAb 12.10 heavy chain variable region DNA sequences

	10	20	30	40	50	60
12.8VH	CAGATCCACT	TGGTGCAGTC	TGGACCTGAG	CTGAGGAAGC	CTGGAGAGAC	AGTCAAGATC
12.10VH	CAGGTCCAAC	TGCAGCAGCC	TGGGGCTGAA	CTEGTGAAGC	CTGGGGCTTC	AGTGAAGCTG
	70	80	90	100	110	120
12.8VH	TCCTGCAAGG	CTTCTGGGTA	TACCTTCACA	AACTATGGAA	TGAACTGGGT	GAAGCAGGAT
12.10VH	TCCTGCAAGG	CTTCTGGCTA	CACCTTCACC	AGCTACTGGA	TAACTGGGT	GAAGCAGAGG
	130	140	150	160	170	180
12.8VH	ACAGGAAAGG	GTTTAAAGTG	GATGGGCTGG	ATCAACACCT	TCTCTGGAGA	GCCAAACATAT
12.10VH	CCTGCACAAG	GCCTTGAGTG	GATTGGAGAG	ATTATTCCCTA	ACAACGGTCG	TAGTAATTAT
	190	200	210	220	230	240
12.8VH	GCTGATGACT	TCAAGGCACG	GTTTGCCTTC	TCTTTGGAAA	CCTCTGCCAC	CACTGCCTAT
12.10VH	AATGAGAAAT	TCAAGAGCAA	GGCCACACTG	ACTGTAGACA	AATCCTCCAG	CACAGCCTAC
	250	260	270	280	290	300
12.8VH	TTGCAGATCA	ACAACCTCAA	AAATGAGGAC	ATGGCTACAT	ATTTCTGT--	--G----TA-
12.10VH	ATGCAACTCA	GCAGCCTGAC	ATCTGAGGAC	TCTGCGGTCT	ATTTCTGTGC	AAGAAACTGG
	310	320	330	340	350	
12.8VH	CAGTCGG----	TAATAGACAA	CTGGGGCCAA	GGCACCACTC	TCACAGTCTC	CTCA
12.10VH	GCCTACTGGT	TCTTCGATGT	CTGGGGCGCA	GGGACCAGGG	TCACCGTCTC	CTCA

mAb 12.8 and mAb 12.10 light chain variable region DNA sequences

	10	20	30	40	50	60
12.8Vk	CAAATTGTTT	TCACCCAGTG	TCACAGCAATC	ATGTCTGCAT	CTCCAGGGGA	GAAGGTCACC
12.10Vk	GACATTGTTG	TGACCCAGTG	TCACAAATTC	ATGTCCACAT	CAATAGGAGA	CAGGGTCAGC
	70	80	90	100	110	120
12.8Vk	ATGACCTGCA	GTGCCAGCTC	AAG-TAT-AG	ATTACATGCA	--CTGGTACC	AGCAGAGAGG
12.10Vk	ATCACCTGCA	AGGCCAG-TC	AGGATGTGAG	TTCTGCTGTA	GCTGGGTATC	AACAGAGACC
	130	140	150	160	170	180
12.8Vk	AGGCACCTTC	CCGAAAAGAT	GGATTTATGA	CACATCCAAA	CTGGCTTCTG	GAGTCCCTGC
12.10Vk	AGGACAAATC	CCTAAACTAC	TGATTTACTC	GGCATCCTAC	CGGTACACTG	GAGTCCCTGA
	190	200	210	220	230	240
12.8Vk	TCGCTTCAGT	GGCAGTGGGT	CTGGGACCTC	TTATTCTCTC	ACAATCAGCA	GCATGGAGGC
12.10Vk	TCGCTTCGCT	GGCAGTGGAT	CTGGGACGGA	TTTCACTTTC	ACCATCAGCA	GTGTGCAGGC
	250	260	270	280	290	300
12.8Vk	TGAAGATGCT	GCCA-CTTAT	TACTGCCATC	AGCGGAGTAG	TTACCATTCG	ACGTTCCGCT
12.10Vk	TGAAGA-CCT	GGCAGTTTAT	TACTGTCAGC	AACATTCTAG	TACTCCGCTC	ACGTTCCGCTG
	310	320				
12.8Vk	CTGGGACAAA	GTGGGAAATA	AAA			
12.10Vk	CTGGGACAAA	GCTGGAGCTG	AAA			

Figure 3.2b)

Amino acid sequences of mAb 12.8 and mAb 12.10 heavy and light chain variable region sequences

Above is shown a comparison between the heavy chain variable region amino acid sequences of mAb 12.8 (12.8VH) and mAb 12.10 (12.10VH). Identical residues within the framework regions of the two heavy chains are highlighted in pink. Identical residues within the complementarity-determining regions (CDRs) of the two heavy chains are highlighted in blue.

Below is shown a comparison between the light chain variable region amino acid sequences of mAb 12.8 (12.8Vk) and mAb 12.10 (12.10Vk). Identical residues within the framework regions of the two light chains are highlighted in green. Identical residues within the complementarity-determining regions (CDRs) of the two light chains are highlighted in yellow.

Marked differences are seen in all three CDRs of both the heavy and the light chains, which is consistent with the observation that they bind to different epitopes within MSP-1₁₉.

Figure 3.2b)

mAb 12.8 and mAb 12.10 heavy chain variable regions

	10	20	30	40	50	60
12.8VH	QIHLVCSGPE	LRKEGETVKI	SCKASGYTFT	NYGMNWKQD	TCKGLKWMGW	INTFSGEPTM
12.10VH	CVQLQCPGAE	IVKPGASVKL	SCKASGYTFT	SYWIHWVKQR	PGQGLEWIGE	IIPNNGRSNY

	70	80	90	100	110	
12.8VH	ADDFKGRFAF	SLETSATTAY	LCINNLKNEE	MATYFCVQ--	S--VIDNWQ	GTTTLTVSS
12.10VH	NEKFKSKATL	TVDKSSSTAY	MCLSSLTSED	SAVYFCARNW	AYWFFDVWGA	GTTTVTVSS

mAb 12.8 and mAb 12.10 light chain variable regions

	10	20	30	40	50	60
12.8VK	QIVLTQSPAI	MSASPGEKNT	MTCSASSSI-	DYMHNYOQKE	GTSPKRWIYD	TSKLASGVIA
12.10VK	DIYMTQSHKF	MTTSIGDRVS	ITCKASQDVS	SAVANYOQRE	QSPKLLIYS	ASYRYTGVPD

	70	80	90	100	
12.8VK	RFSGSGSGTS	YSLTISSEEA	EDAATYYCHQ	RSSYPFTFGS	GTKLEIR
12.10VK	RFAGSGSETD	FTFTISSEVQA	EDLAVYYCQ	HSSTPLTFGA	GTKLELR

Figure 3.3a)

DNA sequences of hybridoma 12.10 light chain pseudogene and mAb 12.8 and mAb 12.10 functional light chain variable region sequences

Above is shown a comparison between the DNA sequence of the hybridoma 12.10 light chain pseudogene (12.10V κ Ψ) and the mAb 12.10 light chain variable region (12.10V κ). Identical nucleotides are highlighted in blue. The hybridoma 12.10 light chain pseudogene was found when the PCR product amplified with primer MKV5 was cloned and sequenced. This pseudogene is distinct from the known MOPC-21 kappa light chain pseudogene, also present in the hybridoma and derived from the parent plasmacytoma, which has already been described. The 12.10V κ pseudogene is transcribed, but there is a frameshift at base pair 282 (residue 95), where there is a single base pair deletion. This pseudogene is apparently not secreted, as mass spectrometry of mAb 12.10 showed no evidence of an expressed product. It is quite different in sequence from the functional mAb 12.10 light chain variable region, 12.10V κ .

Below is shown a comparison between the DNA sequence of the hybridoma 12.10 light chain pseudogene variable region (12.10 κ Ψ) and the DNA sequence of the mAb 12.8 light chain functional variable region (12.8V κ). Identical nucleotides are highlighted in green. The two sequences are almost identical. The 12.8 and 12.10 hybridomas were made in the same series of fusion experiments, and presumably the two sequences derive ultimately from the same parent light chain gene.

Figure 3.3a)

Hybridoma 12.10 Vk pseudogene and mAb 12.10 Vk variable region

	10	20	30	40	50	60
12.10Vk Ψ	CAAATTGTTTC	TCACCCAGTC	TCCAGCAATC	ATGTCTGCAT	CTCCAGGGGA	CAAGGTCACC
12.10Vk	GACATTGTGA	TGACCCAGTC	TCACAAATTC	ATGTCCACAT	CAATAGGAGA	CAGGGTCAGC
	70	80	90	100	110	120
12.10Vk Ψ	ATGACCTGCA	GTGCCACCTC	AAG-TATAAG	TT-ACATGCA	--CTGGTACC	AGCAGAAGCC
12.10Vk2	ATCACCTGCA	AGGCCA-GTC	AGGATGTGAG	TTCTGCTGTA	GCCTGGTATC	AACAGAGACC
	130	140	150	160	170	180
12.10Vk Ψ	AGGCACCTCC	CCCAAAAGCT	GGATTTATGA	CACATCCAAA	CTGGCTTCTG	GAGTCCCTGT
12.10Vk	AGGACAATCT	CCTAAACTAC	TGATTTACTC	GGCATCCATC	CGGTACACTG	GAGTCCCTGA
	190	200	210	220	230	240
12.10Vk Ψ	TCGCTTCAGT	GGCAGTGGGT	CTGGGACCTC	TTATTCTCTC	ACAATCAGCA	GCATGGAGGC
12.10Vk	TCGCTTCGCT	GGCAGTGGAT	CTGGGACGGA	TTTCACTTTC	ACCATCAGCA	GTGTGCAGGC
	250	260	270	280	290	300
12.10Vk Ψ	TGAAGATGCT	GCCA-CTTAT	TACTGCCATC	AGCGGAGTAG	GTACCCACGT	GGACGTTCCG
12.10Vk	TGAAGA-CCT	GGCAGTTTAT	TACTGTCAGC	AACATTCTA-	GTACTC-CGC	TCACGTTCCG
	310	320				
12.10Vk Ψ	TGGAGGCATC	AAGCTGGAAA	TCAAA			
12.10Vk	TGCTGGGACC	AAGCTGGAGC	TGAAA			

Hybridoma 12.10 Vk pseudogene and mAb 12.8 Vk variable region

	10	20	30	40	50	60
12.10Vk Ψ	CAAATTGTTTC	TCACCCAGTC	TCCAGCAATC	ATGTCTGCAT	CTCCAGGGGA	CAAGGTCACC
12.8Vk	CAAATTGTTTC	TCACCCAGTC	TCCAGCAATC	ATGTCTGCAT	CTCCAGGGGA	GAAGGTCACC
	70	80	90	100	110	120
12.10Vk Ψ	ATGACCTGCA	GTGCCACCTC	AAGTATAAGT	TACATGCACT	GGTACCAGCA	GAAGCCAGGC
12.8Vk	ATGACCTGCA	GTGCCAGCTC	AAGTATAGAT	TACATGCACT	GGTACCAGCA	GAAGCCAGGC
	130	140	150	160	170	180
12.10Vk Ψ	ACCTCCCCCA	AAAGCTGGAT	TTATGACACA	TCCAAACTGG	CTTCTGGAGT	CCCTGTTTCG
12.8Vk	ACCTCCCCCA	AAAGATGGAT	TTATGACACA	TCCAAACTGG	CTTCTGGAGT	CCCTGCTTCG
	190	200	210	220	230	240
12.10Vk Ψ	TTCACTGGCA	GTGGGTCTGG	GACCTCTTAT	TCTCTCACAA	TCAGCAGCAT	GGAGGCTGAA
12.8Vk	TTCACTGGCA	GTGGGTCTGG	GACCTCTTAT	TCTCTCACAA	TCAGCAGCAT	GGAGGCTGAA
	250	260	270	280	290	300
12.10Vk Ψ	GATGCTGCCA	CTTATTACTG	CCATCAGCGG	AGTAGGTACC	CACGTGGACG	TTCCGTGGAG
12.8Vk	GATGCTGCCA	CTTATTACTG	CCATCAGCGG	AGTAGTTACC	CA--TTCACG	TTCCGCTCCG
	310	320				
12.10Vk Ψ	GCATCAAGCT	GGAAATCAAA				
12.8Vk	GGACAAGTT	GGAAATAAAA				

Figure 3.3b)

Amino acid sequences of the hybridoma 12.10 light chain pseudogene and mAb 12.8 and mAb 12.10 functional light chain variable regions

Above is shown a comparison between the amino acid sequences of the hybridoma 12.10 light chain pseudogene (12.10V κ Ψ) and the mAb 12.10 light chain variable region (12.10V κ). Identical residues are highlighted in blue.

Below is shown a comparison between the amino acid sequence of the hybridoma 12.10 light chain pseudogene variable region (12.10 κ Ψ) and the amino acid sequence of the mAb 12.8 light chain functional variable region (12.8V κ). Identical residues are highlighted in blue. The two sequences are almost identical. The frameshift at base pair 282 (residue 95) in the hybridoma 12.10 pseudogene is represented in this translation by the letter X.

Figure 3.3b)

Hybridoma 12.10 pseudogene translation and mAb 12.10 Vκ variable region

	10	20	30	40	50	60
12.10VκΨ	QIVLTQSPAI	MSASPGDKVT	MTCSATSSI-	SYMHWYQKPF	GTSPKSWIYD	TSKLASGVFV
12.10Vκ	DIVMTQSHKF	MSTSIGDRVS	ITCKASQDVS	SAVAWYQORP	GQSPKLLIYS	ASYRYTGVPD
	70	80	90	100		
12.10VκΨ	RFSGSGSGTS	YSLTISSMEA	EDAATYYCHQ	RSRYPXWTFG	GGIKLEIK	
12.10Vκ	RFAGSGSGTD	FTFTISSVOA	EDLAVYYCQ	HSSTPL-TFG	AGTKLELK	

Hybridoma 12.10 pseudogene translation and mAb 12.8 Vκ variable region

	10	20	30	40	50	60
12.10VκΨ	QIVLTQSPAI	MSASPGDKVT	MTCSATSSIS	YMHWYQKPG	TSPKSWIYDT	SKLASGVFVR
12.8Vκ	QIVLTQSPAI	MSASPGDKVT	MTCSASSSID	YMHWYQKPG	TSPKRWIYDT	SKLASGVFAR
	70	80	90	100		
12.10VκΨ	FSGSGSGTSY	SLTISSMEAE	DAATYYCHQR	SRYPXWTFG	GIKLEI K	
12.8Vκ	FSGSGSGTSY	SLTISSMEAE	DAATYYCHQR	SSYPF-TFGS	GTKLEI K	

Figure 3.4

Mass spectrometry of mAbs: Predicted tryptic fragments and mass spectrogram of 12.10 Fab light chain

Above are shown the predicted fragments from a tryptic digestion of the mAb 12.10 light chain variable region. Fragments are numbered T1 to T8 according to their position in the light chain, T1 being at the N-terminus. The predicted apparent masses for each fragment are given below as they would appear by mass spectrometry if the fragments had one, two or three positive charges ($[M+H]$, $[M+2H]$ & $[M+3H]$ respectively)

Below is shown the mass spectrogram of the Fab 12.10 light chain. As an example, a peak with apparent mass 543.8 Da is highlighted with an arrow. This peak corresponds to the predicted tryptic fragment T5 (LLIYSASYR) with two positive charges (T5 $[M+2H]$, predicted mass 543.3).

Figure 3.4

Fragment	Residues	Sequence
T8	104 - 107	LELK
T3	19 - 24	VSITB K
T6	55 - 61	YTGVP DR
T2	10 - 18	FMSTS IGDR
T1	1 - 9	DIVMT QSHK
T5	46 - 54	LLIYS ASYR
T4	25 - 45	ASQDV SSAVA WYQQR PGQSP K
T7	62 - 103	FAGSG SGTDF TFTIS SVQAE DLAVY YBQQH SSTPL TFGAG TK

Fragment	[M+H]	[M+2H]	[M+3H]
T8	502.32	251.67	168.11
T3	547.35	274.18	183.12
T6	807.40	404.20	269.81
T2	1013.47	507.24	338.50
T1	1058.53	529.77	353.52
T5	1085.60	543.30	362.54
T4	2290.12	1145.56	764.05
T7	4274.02	2137.51	1425.35

S# 1-40 RT: 0.01-1.19 AV: 40 SM: 7B NL: 3.59E6
T: + p ms

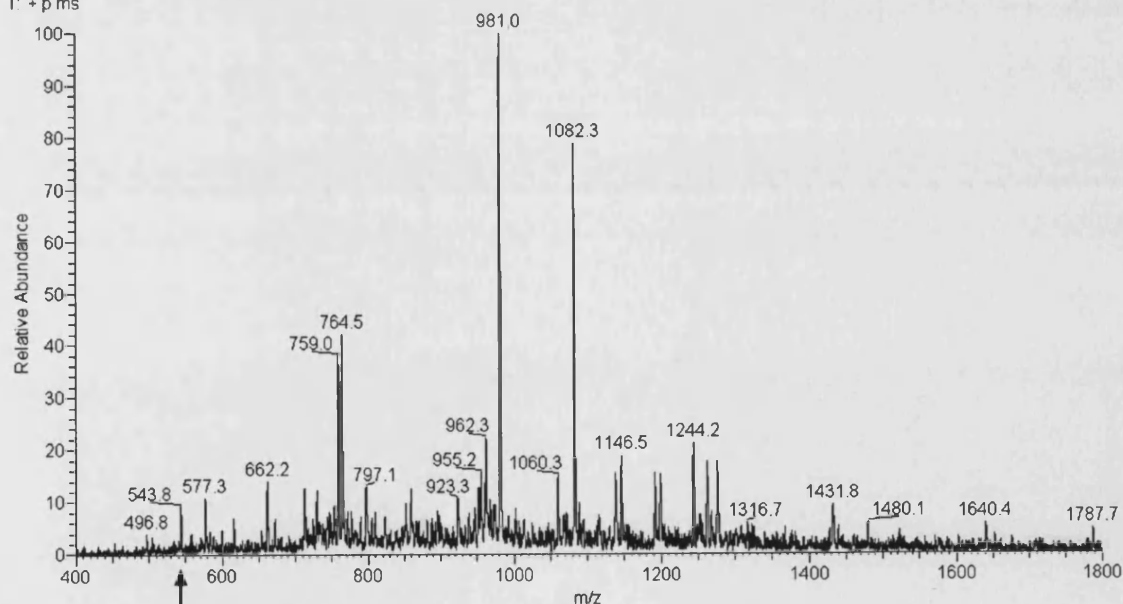


Figure 3.5

Mass spectrometry of mAbs: predicted “daughter ions” from tryptic fragment 5 of 12.10 Fab light chain, and mass spectrogram of fragment 5

Above are shown the predicted masses of every possible “daughter ion”, or fragment, of the tryptic peptide T5 as those “daughter ions” would appear if they had a single positive charge.

Below is the mass spectrogram of the tryptic peptide T5 after isolation of T5 from the other tryptic fragments, followed by breakdown of T5 into all possible constituent “daughter ions”. Peaks are seen with masses corresponding almost precisely to the predicted “daughter ion” masses, thus confirming the amino acid sequence of the peptide as predicted by the candidate DNA sequence.

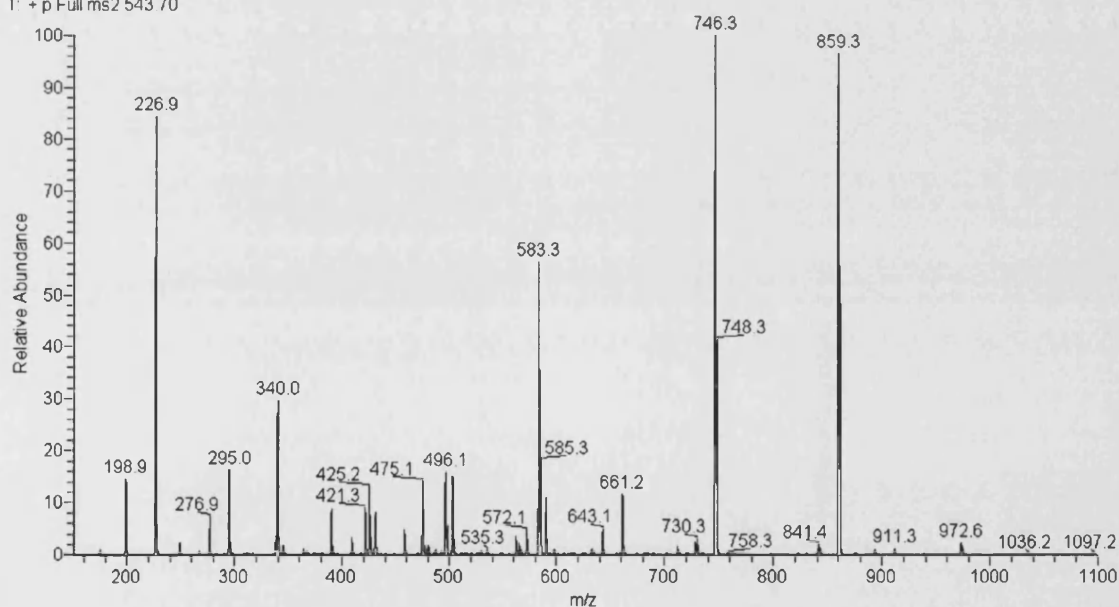
Figure 3.5

Tryptic fragment 5 Monoisotopic Mass = 1084.59 Residues 1 - 9

Fragment ions: Monoisotopic m/z ratios with 1 positive charge

b	114.1	227.2	340.3	503.3	590.4	661.4	748.4	911.5	-
	1	2	3	4	5	6	7	8	9
	Leu	Leu	Ile	Tyr	Ser	Ala	Ser	Tyr	Arg
	9	8	7	6	5	4	3	2	1
y	-	972.5	859.4	746.3	583.3	496.3	425.2	338.2	175.1

S# 1-37 RT: 0.02-1.19 AV: 37 SM: 7B NL: 8.73E3
T: + p Full ms2 543 70



N.I.M.R. London, NW7 1AA.

Jennings 1 3/9/01 NC

Figure 3.6

Amino acid sequences of 12.8 & 12.10 heavy and light chains as determined by mass spectrometry

The amino acid sequences of the variable regions of the mAb 12.8 and mAb 12.10 heavy and light chains, and the predicted amino acid sequence of the hybridoma 12.10 light chain pseudogene, are shown. Underlining denotes tryptic fragment peptide sequences confirmed by mass spectrometry. Tryptic fragments identified and sequenced by mass spectrometry are listed after each sequence. Red (heavy chains) or green (light chains) residues in the sequences denote residues in the complementarity-determining regions (CDRs). Asterisks (*) denote ends of tryptic fragments. J denotes pyroglutamic acid, with a mass 17 Da less than glutamine). B denotes cysteine residues which have been altered to carboxyamidomethyl-cysteine through treatment of the protein with iodoacetamide.

It can be seen that a substantial proportion of the DNA sequences of the variable regions of both mAbs 12.8 and 12.10 heavy and light chains have been independently confirmed by amino acid sequencing. In the case of the variable region of the light chain of mAb 12.10, mass spectrometry has identified all but one of the peptides predicted from the open reading frame DNA sequence obtained with the PCR primer MKV7. None of the peptides predicted from the frameshifted DNA sequence obtained with PCR primer MKV5 are identified, thus clearly establishing that the sequence obtained with the PCR primer MKV7 is derived from the functional gene, whereas the frameshifted sequence obtained with PCR primer MKV5 is derived from a non-functional pseudogene which does not contribute the mAb 12.10.

Figure 3.6

mAb 128 heavy chain variable region

JIHLV QSGPE LR*KPG ETVK*I SBK*AS GYTFT NYGMN WVK*QD TGK*GL K*WMCW INTFS
GEPTY ADDFK* GR*FAF SLETS ATTAY LQINN LK*NED MATYF BVQSV IDNWG QGTTL TVSS

Tryptic fragments identified are T1 (mass 1359 [M+H]) T4 (mass 1739 [M+H]) T7 (mass 2265 [M+H]) and T9 (mass 2232 [M+H])

mAb 12.8 light chain variable region

JIVLT QSPAI MSASP GEK*VT MTBSA SSSID YMHWY QOKPG TSPK*R* WIYDT SK*LAS
GVPAR* FSGSG SGTSY SLTIS SMEAE DAATY YBHQR* SSYPF TFGSG TK*LEI K

Tryptic fragments identified are T1 (mass 1840 [M+H]), T2 (mass 2991 [M+H]) T4 (mass 912 [M+H]) T5 (mass 770 [M+H]) T6 (mass 3264 [M+H]) T7 (mass 1279 [M+H]) and T8 (mass 502 [M+H])

mAb 1210 heavy chain variable region

JVQLQ QPGAE LVKPG ASVK*L SBK*AS GYTFT SYWIH WVK*QR PGQGL EWIGE IIPNN
GR*SNY NEK*FK* SK*ATL TVDK*S SSTAY MQLSS LTSED SAVYF BAR*NW AYWFF DVWGA
GTTVT VSS

Tryptic fragments identified are T1 (mass 1960 [M+H]) and T3 (mass 1846 [M+H])

mAb 1210 light chain variable region (encoded by functional gene)

DIVMT QSHK*F MSTSI GDR*VS ITBK*A SQDVS SAVAW YQORP GQSPK* LLIYS ASYR*Y
TGVPD R*FAGS GSGTD FTFTI SSVQA EDLAV YYBQQ HSSTP LTFGA GTK*LE LK

Tryptic fragments identified are T4 (mass 2290 [M+H], 1146 [M+2H]) T5 (mass 1086 [M+H], 543 [M+2H]) and T7 (mass 4274 [M+H])

Hybridoma 12.10 light chain variable region (encoded by pseudogene)

QIVLT QSPAI MSASP GDK*VT MTBSA TYMHSSIS WY QOKPG TSPK*S WIYDT SK*LAS
GVPVR* FSGSG SGTSY SLTIS SMEAE DAATY YBHQR* SR*YPX XXFGG GIK*LE IK*

No predicted tryptic fragments found

CHAPTER 4

SINGLE CHAIN VARIABLE FRAGMENTS AND CHIMERIC ANTIBODIES DERIVED FROM INVASION-INHIBITORY MURINE MONOCLONAL ANTIBODIES

4.1 Introduction

Once the DNA sequences of the variable regions of mAb 12.8 and mAb 12.10 were determined, a decision was made to construct single chain variable fragments (scFv) of these antibodies. This was done for a number of reasons.

Firstly, it was important to determine whether scFv versions of these invasion-inhibitory mAbs would retain the ability to inhibit erythrocyte invasion. One long-term aim of this project was to isolate novel human invasion-inhibitory anti-MSP-1₁₉ antibodies from the phage display libraries. Any such antibodies obtained from the phage display libraries would be obtained initially as scFv fragments. It was therefore important to know whether such scFv fragments could be expected to retain any invasion-inhibitory activity present in the parent antibodies. A previous study (Guevara Patino 1997) showed that Fab and F(ab')₂ fragments of mAb 12.10 do not retain the ability of the parent antibody to inhibit both MSP-1 secondary processing and erythrocyte invasion. It was anticipated therefore that scFv 12.10 would be unlikely to retain invasion-inhibitory activity, but this was not certain. Nor was it possible to anticipate the likely activity of a scFv version of mAb 12.8, since at this time no Fab fragments of mAb 12.8 had ever been made or studied.

Secondly, if antibodies in scFv format will not retain the invasion-inhibitory activity of the parent antibodies, then techniques will be required to restore invasion-inhibitory activity to scFvs. Such techniques will be needed if scFvs obtained by panning the libraries are to be screened for potential invasion-inhibitory activity. Any technique for restoring invasion-inhibitory activity would need to be tested on a scFv version of a known invasion-inhibitory antibody such as mAb 12.8 or mAb 12.10.

Preliminary experiments were done to test a possible method of restoring invasion-inhibitory activity to scFv 12.10. This method rests on the assumption that the Fc portion of the antibody, whose loss leads to loss of biological activity in the case of mAb 12.10, is essential because its size is required to contribute some steric effect required for the inhibition of secondary processing. Possible techniques to restore invasion-inhibitory activity might therefore depend on increasing the mass of the scFv molecule until it approximates to the mass of a whole antibody. Engineering scFv back into whole antibodies is time consuming and labour intensive, and so is not suitable for screening large numbers of scFv quickly. An alternative way of increasing the size of scFv 12.10 was attempted. The scFv was adapted to express as a fusion with two albumin-binding domains so that two albumin molecules (molecular weight 68 kDa) could be bound to the scFv to increase its mass to approximate to the mass of a whole antibody. The albumin-binding domain was originally identified in the streptococcal protein G (Nygren, Ljungquist et al. 1990) and has been used to indirectly immobilise proteins to solid phase via albumin for the panning of a phage display library (Grob, Baumann et al. 1998). After attempting to modify scFv 12.10 in this way, an invasion assay was performed with the modified scFv to see if invasion-inhibitory activity was restored.

Finally, the DNA sequences obtained for the variable regions of mAb 12.8 and mAb 12.10 were used to construct recombinant mouse/human whole antibodies. The chimeric antibodies were made by Eilish Cullen of the Antibody Engineering Group, MRC Collaborative Centre, Mill Hill, London. As part of this project, these antibodies were then tested for their ability to bind to recombinant and native antigen and inhibit erythrocyte invasion. This work was done for three reasons. Firstly, and most importantly, demonstrating that whole antibodies constructed from these sequences can inhibit erythrocyte invasion offers unequivocal proof that the variable region sequences described in the previous chapter do indeed define the invasion-inhibitory antigen-binding sites of the antibodies. Secondly, recombinant versions of mAbs 12.8 and 12.10, with known sequences, enable production of antibodies of guaranteed standardised specificity. This should be helpful in standardising the degree of biological activity present in different batches of mAbs 12.8 and 12.10. Such standardisation should be useful, since mAbs 12.8 and 12.10 are routinely used as the positive controls in erythrocyte invasion assays, and the level of biological activity in different batches of antibody derived from hybridoma cell cultures has appeared to vary in the past. Thirdly, to demonstrate the potential biological relevance of invasion-inhibitory antibodies, they should be tested for their ability to modify or prevent *P. falciparum* infection in an animal model such as the *Aotus* monkey. Recombinant antibodies would be suitable for this purpose. They are antibodies of known sequence and specificity, and can be readily produced to the high quality needed for use in animals. Chimeric mouse/human antibodies should be particularly suitable for this purpose, since they contain human rather than murine constant regions, and human sequences would be anticipated to be less potentially immunogenic than mouse sequences when injected into primates.

4.2 Results

4.2.1 Construction of scFv DNA from the variable region sequences of mAbs 12.8 and 12.10

The variable region sequences obtained from mAbs 12.8 and 12.10 described in the previous chapter were used to construct DNA encoding single chain variable fragments (scFv) of these antibodies. The scFvs were constructed by a three-stage process involving three successive PCR reactions, as described in section 2.3.1. The process of construction of the scFvs is illustrated diagrammatically in Figure 2.1. The PCR product of the PCR creating the full-length scFv 12.8 DNA sequence is shown in Figure 4.1.

4.2.2 Repair of scFv 12.10 DNA sequence by site-directed mutagenesis

On sequencing the DNA encoding scFv 12.10, an unwanted single base pair mutation was found encoding the substitution of proline for serine at position 17 of the first framework region of the heavy chain. This was corrected using PCR to produce a site directed mutagenesis back to the correct sequence. The method is described section 2.3.2 and illustrated in Figure 2.2. DNA sequencing confirmed that the mutation had indeed been removed.

4.2.3 DNA sequences of scFv 12.8 and scFv 12.10

The scFv 12.8 and scFv 12.10 sequences were cloned into the vector pCRTM2.1 (Invitrogen) for DNA sequencing. Sequencing was carried out using a Perkin Elmer ABI Prism 377 DNA Sequencer as described. The DNA sequences and translations of scFv 12.8 and scFv 12.10 are shown in Figure 4.2.

4.2.4 Expression of scFv 12.8 and scFv 12.10

scFv 12.8 and scFv 12.10 were expressed and purified as described in sections 2.4.2 – 2.4.3. Expressed scFv was detected by Western blot using anti-PentaHis antibody (Qiagen) as described. The scFv purity was assessed by SDS-PAGE gel staining with silver stain and Coomassie blue and the protein concentration was measured by absorbance at 280 nm, assuming that $A_{280} = 1$ for 520 $\mu\text{g/ml}$ scFv 12.10.

scFv 12.10 expressed well, with 0.8 μg protein expressed per ml of bacterial culture.

Expression levels for scFv 12.8, however, were very poor. Expressed scFv 12.8 could be clearly seen by Western blot using Anti-PentaHis antibody, but could not be seen with Coomassie stain and was not clearly seen with SDS-PAGE gel silver staining. Expression levels of scFv 12.8 were too low to be accurately quantified by absorbance at 280 nm. It was not possible to improve the expression level of scFv 12.8 by varying either the temperature or the duration of expression. This low expression level meant that it was not possible to obtain sufficient quantities of scFv 12.8 to assess its binding to recombinant or native MSP-1₁₉ by ELISA or IFA. Nor was it possible to test scFv 12.8 in the invasion assay, for which much more material is required than could be obtained.

The expression levels of scFv 12.8 and scFv 12.10 did not vary significantly between expression temperatures of 25°C for 8-12hr, 30°C for 4-12 hr and 37°C for 4-12 hr.

Both scFvs were found in the bacterial culture supernatant as well as in the periplasmic fraction.

Western Blots of both scFv 12.8 and scFv 12.10 revealed some breakdown of the full-length protein. In both cases, detecting with Anti-PentaHis antibody, the full-length scFv was seen as a band at 30 kDa, and a breakdown product was seen at 18 kDa. Breakdown of scFv was reduced by expression at 30°C rather than 37°C and minimised, although not abolished, by expression over 8 hours at 25°C and by the addition of PMSF to the periplasmic fraction.

Figure 4.3 shows scFv 12.8 separated by SDS-PAGE using a 12% polyacrylamide gel and detected with Anti-PentaHis antibody (Qiagen). Both the 30 kDa full-length scFv and the 18 kDa breakdown product can be seen.

4.2.5 Binding of 12.10 scFv to MSP-1₁₉ by ELISA

A Nunc maxisorb 96-well ELISA plate was coated with 1 µg/ml recombinant MSP-1₁₉-GST fusion protein in PBS as described in section 2.7.2. One hundred microlitres of scFv 12.10 was added to the wells in serial double dilutions, starting at a concentration of 100 µg/ml and going down to 3.13 µg/ml. The mouse hybridoma mAbs 12.8 and 12.10 were used as positive controls and the non-*Plasmodium*-binding humanised mAb BX (version 2) (supplied by Sue Potts, MRC Collaborative Centre) was used as a negative control. Positive and negative control antibodies were also added in serial double dilutions, starting at a concentration of 50 µg/ml. The plate was then incubated and washed as described, and 100 µl of 9E10 (murine hybridoma anti-c-*myc* mAb) culture supernatant was added to the wells containing scFv, and the plate was incubated at 37°C for 1 hour. The plate was then washed again as described and the bound antibodies were

revealed with 100 µl of a 1 in 5000 (v/v) dilution of either HRP-conjugated anti-mouse kappa (Harlan Sera-Lab) (to reveal bound mAbs 12.8, 12.10 and 9E10) or HRP-conjugated anti-human kappa light chain, bound and free (Sigma) (to reveal bound humanised mAb BX (version 2)). The plate was incubated and washed again as described and the colour was developed by adding 150 µl Neogen K-BLUE substrate (Sky Bio) to each well and leaving in darkness for 10 minutes then adding 50 µl Neogen RED STOP solution (Sky Bio). The plate was read on a BIO-RAD 3550 Microplate reader at 655 nm.

scFv 12.10 bound to MSP-1₁₉-GST by ELISA and these results are illustrated in Figure 4.4.

4.2.6 Binding of 12.10 scFv to acetone-fixed *P. falciparum* by IFA

Binding of scFv to parasites was assessed by indirect immunofluorescence (IFA) on acetone-fixed *P. falciparum* (FCB-1 and T9/96 strains) as described in section 2.7.1. Ni-NTA-purified scFv 12.10 was tested at concentrations of 30 µg/ml, 3 µg/ml and 0.3 µg/ml. The slides were incubated and washed as described, and 15 µl of 9E10 (anti-c-myc) mouse hybridoma supernatant was added to each well to bind to the c-myc tag of the scFv. Bound antibody was revealed with FITC anti-mouse antibody as described.

scFv 12.10 was seen to bind to *P. falciparum* and yielded a very similar immunofluorescence pattern to that given by mAb 12.8 and mAb 12.10 (the immunofluorescence results are not shown).

4.2.7 scFv 12.10 in the erythrocyte invasion assay, alone and bound to two albumin molecules

To determine whether scFv 12.10 retained the invasion-inhibitory property of the parent mAb 12.10, scFv 12.10 was tested in the erythrocyte invasion assay at a concentration of approximately 200 µg/ml. scFv 12.10 did not inhibit erythrocyte invasion in this experiment (data not shown).

In order to try to restore invasion-inhibitory activity to the scFv version of mAb 12.10, it was decided to incorporate either one or two albumin-binding domains to the C-terminus of the scFv. This was done in the hope that the addition of one or two bound albumin molecules to the scFv might restore invasion-inhibitory activity by increasing the bulk of the scFv so that its mass approximated to that of a whole antibody.

A new expression vector, pHEN1H6ABD2, was created by Dr Jonathan Chappel of the Antibody Engineering Group, MRC Collaborative Centre, Mill Hill, London. This vector is identical to pHEN1H6 except for the addition of two albumin-binding domains between the *NotI* site and the hexahistidine tag. The vector pHEN1H6ABD2 is shown in Figure 2.4. The scFv 12.10 insert was ligated into this vector and expressed, as scFv 12.10-ABD2, with two albumin-binding domains fused to the C-terminus of the scFv. Expression levels were similar to those obtained with scFv 12.10 although, as with scFv 12.10, some breakdown of the proteins was observed on Western blot, despite the addition of PMSF at the time of elution of the protein from the Ni-NTA column. The binding of scFv 12.10-ABD2 to human albumin was confirmed by ELISA, and scFv 12.10-ABD2 was tested in the invasion assay at a concentration of 241 µg/ml. Albumin was present at a final concentration of 0.05% (w/v). scFv X509-ABD2 (made by Dr Jonathan Chappel)

was used as a negative control (mAb X509 recognises MSP-1₃₃). In this experiment scFv 12.10-ABD2 did not inhibit erythrocyte invasion (data not shown).

4.2.8 Binding of chimeric 12.8 & 12.10 to MSP-1₁₉-GST by ELISA

Using the mAb 12.8 and mAb 12.10 variable region sequences, chimeric mouse/human versions of mAbs 12.8 and 12.10 were made by Eilish Cullen of the Antibody Engineering Group (now AERES Biomedical) of the MRC Collaborative Centre, Mill Hill, London. An ELISA was carried out to confirm that the chimeric mAbs retained the ability to bind to MSP-1₁₉. An ELISA plate was coated with 1 µg/ml recombinant MSP-1₁₉-GST fusion protein as described above. Chimeric mAbs 12.8 and 12.10 were added to the wells in serial double dilutions, starting at a concentration of 50 µg/ml and going down to 0.024 µg/ml. The mouse hybridoma mAbs 12.8 and 12.10 were used as positive controls and humanised mAb BXv2 was used as a negative control, in the same concentrations. The plate was then incubated and washed as described, and the bound antibodies were revealed with HRP-conjugated anti-mouse or anti-human antibodies and read as described above.

The chimeric versions of mAb 12.8 and mAb 12.10 bound to MSP-1₁₉-GST by ELISA. Binding of the chimeric mAbs to MSP-1₁₉-GST compared favourably with binding by the parent hybridoma mAbs 12.8 and 12.10, and these results are illustrated in Figure 4.5.

4.2.9 Binding of chimeric 12.8 & 12.10 to parasites by IFA

The chimeric versions of mAb 12.8 and 12.10 were tested for their ability to bind acetone-fixed *Plasmodium falciparum* by IFA. The antibodies were tested against two strains of *P. falciparum*, T9/96 and FCB-1. The immunofluorescence experiments were carried out as described in section 2.7.1. The mouse hybridoma mAbs 12.8 and 12.10 were used as positive controls and the non-*Plasmodium*-binding humanised mAb BX (version 2) (supplied by Sue Potts, MRC Collaborative Centre) was used as a negative control. All antibodies were tested at 30 µg/ml, 3 µg/ml and 0.3 µg/ml.

The chimeric mAbs 12.8 and 12.10 both showed strong binding to acetone-fixed *P. falciparum* by immunofluorescence, showing an immunofluorescence pattern identical to that of the parent hybridoma mAbs. These results are shown in Table 4.1.

4.2.10 Inhibition of erythrocyte invasion by chimeric mAbs 12.8 and 12.10

The chimeric versions of mAb 12.8 and mAb 12.10 were tested for their ability to inhibit *P. falciparum* erythrocyte invasion. The invasion assays were carried out as described in section 2.8.3. The assays were done in a flat-bottomed Costar 3596 96-well polystyrene plate. All antibodies were dialysed overnight into RPMI (without albumax). Murine hybridoma mAbs 12.8 and 12.10 and EDTA were used as positive controls and a non-*Plasmodium*-specific murine mAb, BC1 (obtained from Alex Brown of the Antibody Engineering Group, MRC Collaborative Centre, and directed against an anti-cancer target antigen) and RPMI alone were used as negative controls. Fresh washed O+ve human red blood cells were used and 3D7 *P. falciparum* schizonts were purified as described and supplied by Muni Grainger, Division of Parasitology, NIMR. Volumes were chosen to

provide a final haematocrit in each well of 5%, and a parasitaemia at the start of the experiment of approximately 1%.

Each well contained a total volume of 50 μ l made up as follows:

Antibody in RPMI	40 μ l
AB+ve human serum	5 μ l (10% v/v)
Glutamine 200 mM	0.5 μ l (final concentration 2 mM)
RBCs and schizonts in RPMI	4.5 μ l (haematocrit 5%, parasitaemia at time zero 1%)

Final concentrations of antibodies were as follows:

Chimeric mAb 12.10	560 μ g/ml
Hybridoma mAb 12.8	1280 μ g/ml
Hybridoma mAb 12.10	4130 μ g/ml
mAb BC1	800 μ g/ml

The 96-well plate was incubated under an atmosphere of 7% CO₂, 5% O₂, 88% N₂ at 37°C for 24 hours and then thin blood films were made from each well and stained with Giemsa stain. The percentage of erythrocytes invaded was calculated by counting one thousand erythrocytes on each film and noting the number parasitised.

Medium alone was tested in three separate wells, mAb BC1 was tested in four separate wells, chimeric mAb 12.10 was tested in five separate wells, and hybridoma mAbs 12.8 and 12.10, and EDTA, were tested in one well each.

The percentage of red cells invaded in each well after 24 hours are shown in Table 4.2a).

The chimeric mAb 12.10, at a concentration of 560 µg/ml, reduced the percentage of red cells invaded by *P. falciparum* by 57%.

This result is illustrated as a bar chart showing the percentage inhibition of erythrocyte invasion, in Figure 4.6.

In a second invasion assay, set up in an identical way to that described above, chimeric mAb 12.8 was tested at a concentration of 1200 µg/ml, with mAb BC1 as a negative control at 1280 µg/ml as before. In this experiment the parasitaemia at the start of the experiment was 0.1%. Each antibody was tested in three separate wells.

The percentage of red cells invaded in each well after 24 hours is shown in Table 4.2b).

The chimeric mAb 12.8, at a concentration of 1200 µg/ml, reduced the percentage of red cells invaded by *P. falciparum* by 54%.

4.3 Discussion

The work described in this chapter demonstrates that the antibody variable region sequences whose isolation was described in Chapter 3 are indeed the sequences encoding the invasion-inhibitory antigen-binding sites of mAbs 12.8 and 12.10. It has also been shown that these variable regions can retain their binding to their target antigen when engineered into a single chain variable fragment (scFv) format.

In additional experiments, not described here, both scFv 12.8 and scFv 12.10 were shown to bind to recombinant MSP-1₁₉-GST by Biacore surface plasmon resonance.

Biacore studies carried out by Dr Jonathan Chappel showed that scFv 12.10 binds recombinant MSP-1₁₉-GST with a high affinity. The scFv 12.10 K_d was shown to be $6 \times 10^{-10}M$, which lies between the affinities previously determined for the Fab 12.10 (K_d $1.3 \times 10^{-10}M$) and the mAb 12.10 (K_d $1 \times 10^{-11}M$) (Guevara Patino 1997).

It appears from this work that scFv 12.10 loses the invasion-inhibitory activity of the parent antibody, as anticipated from the lack of invasion-inhibitory activity in Fab and F(ab')₂ fragments of the same antibody. It has been shown here, however, that this invasion-inhibitory activity is restored when the variable region sequences are re-engineered into a chimeric whole antibody format with human constant regions.

An initial attempt was made to restore invasion-inhibitory activity to scFv 12.10 by increasing the bulk of the molecule through the addition of albumin-binding sites binding two albumin molecules per scFv. There was not time in the course of this study to pursue these attempts to completion. There was evidence of some breakdown of both scFv 12.10 and scFv-12.10-ABD2, and the intact scFvs were not purified away from the breakdown products prior to their use in these invasion assays. Only limited quantities of scFv-12.10-ABD2 were available and it may be that higher concentrations would be needed to inhibit invasion. Also the actual binding of albumin to the albumin binding domain(s) was not demonstrated in the invasion assay described, although scFv 12.10-ABD2 has been shown to bind to human albumin by ELISA. It should not, therefore, be concluded that this strategy cannot restore invasion-inhibitory activity to scFvs simply from the negative results obtained in this limited study. Once the problems outlined above have been addressed, the use of albumin bound to albumin-binding domains to bulk up scFvs will merit further investigation. Other possible methods of restoring invasion-inhibitory

activity to scFvs, based upon increasing the bulk of the molecule through the binding of antibodies to the scFv *c-myc* or hexahistidine tags, are discussed in Chapter 6. It might also be helpful if the intact scFv could be purified away from the breakdown products prior to its use in these invasion assays. It could also be advantageous if an expression system could be found which minimises the breakdown of scFv and yields higher quantities of material to work with in the assays.

It was not possible to determine the properties of scFv 12.8 because of its poor expression levels in *E. coli*. Expression levels of this molecule might be improved if a different expression system, such as a baculovirus system, were used. Expression levels of scFv have also been achieved by altering amino acids in the framework regions (Saldanha, Martin et al. 1999) and such alterations might improve the expression of scFv 12.8. It would be of some interest to produce scFv 12.8 in sufficient quantity to discover whether scFv 12.8 also fails to retain the invasion-inhibitory property of the parent antibody. This experiment could however also be done using Fab 12.8, which has just been made (Dr Matthew Lock, Division of Parasitology, NIMR, unpublished data).

The chimeric antibodies express well and inhibit invasion. It would be very interesting to know whether invasion inhibitory antibodies can modify *P. falciparum* infection in a primate model. The human/murine chimeric versions of mAbs 12.8 and 12.10 should be an ideal source of material for such an experiment, since they are of a known sequence, they can be produced in sufficient quantity and to a high quality, and they should be less immunogenic to primates than the original murine antibodies. This experiment is planned and quantities of chimeric mAbs 12.8 and 12.10 are currently being

expressed at AERES Biomedical (formerly the Antibody Engineering Group, MRC Collaborative Centre, Mill Hill, London) for this purpose.

Figure 4.1

DNA encoding scFv 12.8, assembled by three-step PCR

Lanes 1,2,3 & 4 show the PCR product from four identical PCR reactions corresponding to PCR 3 in the three-step PCR construction of scFv 12.8, as described in section 2.3.1, and shown schematically (for scFv 12.10) in Figure 2.1. PCR products were separated on a 1% agarose gel with 10 ng/ml ethidium bromide, and photographed under ultraviolet light. A band can be seen in each lane corresponding to the predicted size of 717 base pairs for scFv 12.8.

Figure 4.1

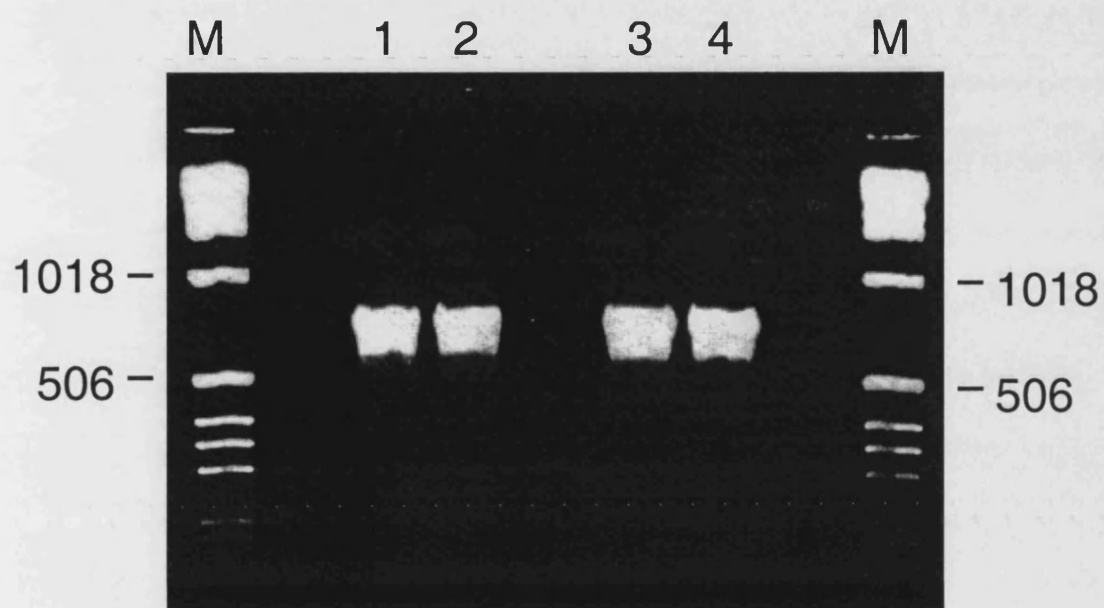


Figure 4.2

scFv 12.8 and scFv 12.10 DNA and amino acid sequences

Above are shown the DNA sequences of scFv 12.8 and scFv 12.10. The scFvs are formed by the joining of the heavy chain variable region sequence (shown in blue) to the light chain variable region sequence (shown in red) via DNA encoding a flexible 18 amino acid linker (shown in green).

Below are shown the amino acid sequences of the scFvs. Heavy chain framework residues are shown in blue, heavy chain complementarity-determining region (CDR) residues are shown in violet, light chain framework residues are shown in red, light chain CDR residues are shown in pink, and linker residues are shown in green. A terminal arginine residue (R) introduced for cloning purposes, is shown in brown.

Figure 4.2

scFv 12.8 DNA sequence

CAGATCCACTTGGTGCAGTCTGGACCTGAGCTGAGGAAGCCTGGAGAGACAGTCAAGATCTCCTGCAAGGCTT
CTGGGTATACCTTCACAACTATGGAATGAACTGGGTGAAGCAGGATACAGGAAAGGGTTTAAAGTGGATGGG
CTGGATCAACACCTTCTCTGGAGAGCCAACATATGCTGATGACTTCAAGGGACGGTTTGCCTTCTCTTTGGAA
ACCTCTGCCACCACTGCCTATTTGCAGATCAACAACCTCAAAAATGAGGACATGGCTACATATTTCTGTGTAC
AGTCGGTAATAGACAACCTGGGGCCAAGGCACCACTCTCACAGTCTCCTCAGGCTCTACCTCTGGCTCTGGTAA
ACCGGGTTCCGGTGAAGGTTCCACTAAAGGTCAAATTGTTCTCACCCAGTCTCCAGCAATCATGTCTGCATCT
CCAGGGGAGAAGGTCACCATGACCTGCAGTGCCAGCTCAAGTATAGATTACATGCACTGGTACCAGCAGAAGC
CAGGCACCTCCCCAAAAGATGGATTTATGACACATCCAACTGGCTTCTGGAGTCCCTGCTCGCTTCAGTGG
CAGTGGGTCTGGGACCTCTTATTCTCTCACAATCAGCAGCATGGAGGCTGAAGATGCTGCCACTTATTACTGC
CATCAGCGGAGTAGTTACCCATTACGTTTCGGCTCGGGGACAAAGTTGGAAATAAAACGA

scFv 12.10 DNA sequence

CAGGTCCAACCTGCAGCAGCCTGGGGCTGAACTGGTGAAGCCTGGGGCTTCAGTGAAGCTGTCCTGCAAGGCTT
CTGGCTACACCTTCACCAGCTACTGGATACACTGGGTGAAGCAGAGGCCCTGGACAAGGCCTTGAGTGGATTGG
AGAGATTATTCTTAACAACGGTCGTAGTAATTATAATGAGAAATTCAGAGCAAGGCCACACTGACTGTAGAC
AAATCCTCCAGCACAGCCTACATGCAACTCAGCAGCCTGACATCTGAGGACTCTGCGGTCTATTTCTGTGCAA
GAACTGGGCCTACTGGTTCTTCGATGTCTGGGGCGCAGGGACCACGGTCACCGTCTCCTCAGGCTCTACCTC
TGGCTCTGGTAAACCGGGTTCCGGTGAAGGTTCCACTAAAGGTGACATTGTGATGACCCAGTCTCACAAATTC
ATGTCCACATCAATAGGAGACAGGGTCAGCATCACCTGCAAGGCCAGTCAGGATGTGAGTTCTGCTGTAGCCT
GGTATCAACAGAGACCAGGACAATCTCCTAAACTACTGATTTACTCGGCATCCTACCGGTACACTGGAGTCCC
TGATCGCTTCGCTGGCAGTGGATCTGGGACGGATTTCACTTTCACCATCAGCAGTGTGCAGGCTGAAGACCTG
GCAGTTTATTACTGTGAGCAACATTCTAGTACTCCGCTCACGTTTCGGTGTGTTGGGACCAAGCTGGAGCTGAAAC
GA

scFv 12.8 amino acid sequence

10	20	30	40	50	60
QIHLVQSGPE	LRKPGETVKI	SCKASGYTFT	NYGMNWKQD	TGKGLKWMGW	INTFSGEPTY
70	80	90	100	110	120
ADDFKGRFAF	SLETSATTAY	LQINNLIKNE	MATYFCVQSV	IDNWQGTTL	TVSSGSTSGS
130	140	150	160	170	180
GKPGSGEGST	KGQIVLTQSP	AIMSASPGEK	VTMTCSASS	IDYMHWYQOK	PGTSPKRWIY
190	200	210	220	230	
DTSKLASGVP	ARFSGSGSGT	SYSLTISME	AEDAATYYCH	QRSSYPFTFG	SGTKLEIKR

scFv 12.10 amino acid sequence

10	20	30	40	50	60
QVQLQPGAE	LVKPGASVKL	SCKASGYTFT	SYWIHWVKQR	PGQGLEWIGE	IIPNNGRSNY
70	80	90	100	110	120
NEKFKSKATL	TVDKSSSTAY	MQLSSLTSED	SAVYFCARNW	AYWFFDVWGA	GTTVTVSSGS
130	140	150	160	170	180
TSGSGKPGSG	EGSTKGDIVM	TQSHKFMSTS	IGDRVSITCK	ASQDVSSAVA	WYQQRPGQSP
190	200	210	220	230	240
KLLIYSASYR	YTGVPDRFAG	SGSGTDFTFT	ISSVQAEDLA	VYYCQQHSST	PLTFGAGTKL

ELKR

Figure 4.3

Western blot of scFv 12.8

As described in sections 2.4.2 and 2.4.3, scFv 12.8 was expressed in a pUC-derived expression vector in a 1 litre culture of *E.coli* TG1 cells for 4 hrs at 30°C, and a periplasmic fraction was prepared from the cells. The expressed scFv was purified using the hexahistidine tag using 1ml of 50% Ni-NTA agarose slurry (Qiagen) and a 1ml nickel column (Qiagen) according to the manufacturer's guidelines. Proteins were separated by SDS-PAGE on a 12% polyacrylamide gel under non-reducing conditions, transferred to an Immobilon nitrocellulose membrane and detected with anti-PentaHis antibody (Qiagen), using peroxidase-conjugated goat anti-mouse antibody and the ECL chemiluminescent detection system (Amersham Life Science).

Lane 1 was loaded with 40 µl of culture supernatant (without purification). Lane 2 was loaded with 40 µl fresh periplasmic extract prior to Ni-NTA purification. Lane 3 was loaded with 20 µl of the first 200 µl volume of eluate after Ni-NTA purification. In each case a band is seen corresponding to the predicted size of 30 kDa for the intact scFv, which is found secreted into the supernatant as well as in the periplasm. A band of 18 kDa is also seen in lanes 2 and 3, which is presumed to be a breakdown product of the scFv. While some breakdown has occurred in the fresh periplasmic extract, more breakdown has occurred during Ni-NTA purification. Breakdown was minimised, although not altogether prevented, by expressing the scFv for 8 hrs at 25°C, and by the addition of PMSF to the Qiagen nickel column.

Figure 4.3

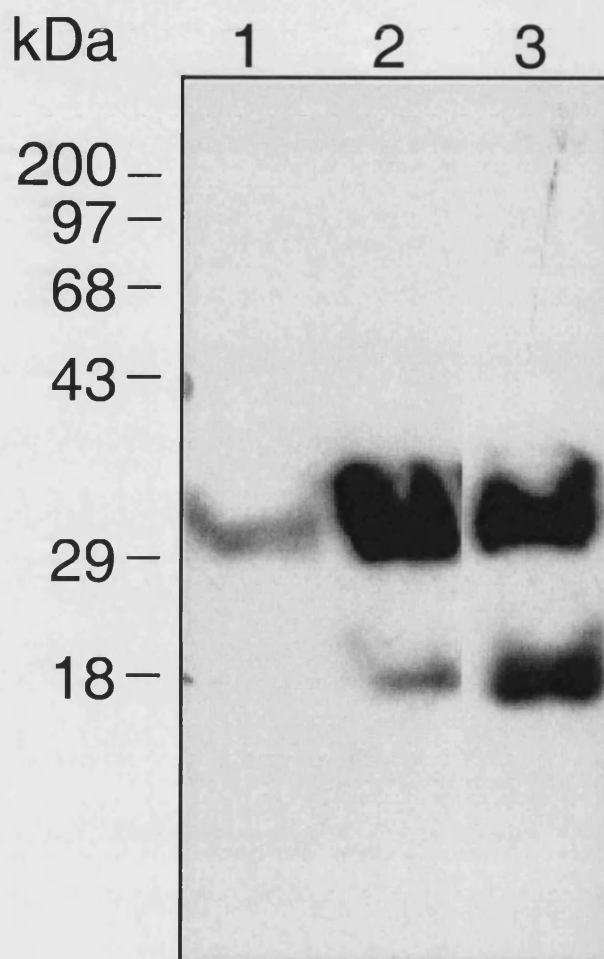


Figure 4.4

Binding of scFv 12.10 to MSP-1₁₉-GST by ELISA

The binding of scFv 12.10 to recombinant MSP-1₁₉-GST was examined by ELISA as described in section 2.7.2. The ELISA plate was coated with 100 µl 1 µg/ml recombinant MSP-1₁₉-GST fusion protein in PBS and blocked with 3% Marvel/PBS. Fifty microlitres of scFv was added at 100 µg/ml, and allowed to bind for 1 hr at 37°C. mAb 12.10 was used as a positive control, and mAb BX version 2 (BXv2) was used as a negative control. One hundred microlitres of 9E10 anti-*c-myc* mouse hybridoma cell culture supernatant was added to each well containing scFv, and bound antibody was detected with 100 µl of a 1/5000 dilution in 3% marvel/PBS of peroxidase-conjugated anti-mouse kappa antibody (Harlan Sera-Lab). The colour was developed with Neogen K-BLUE substrate and Neogen RED STOP solution (Sky Bio). The plate was read on a BIO-RAD 3550 Microplate reader at 655 nm.

It can be seen that this ELISA confirms that scFv 12.10 binds to recombinant MSP-1₁₉. The scFv 12.10 gives absorbances similar to those obtained with mAb 12.10, although the lower concentrations of scFv 12.10 were not tested. No binding of the negative control mAb to MSP-1₁₉ has occurred.

Figure 4.4

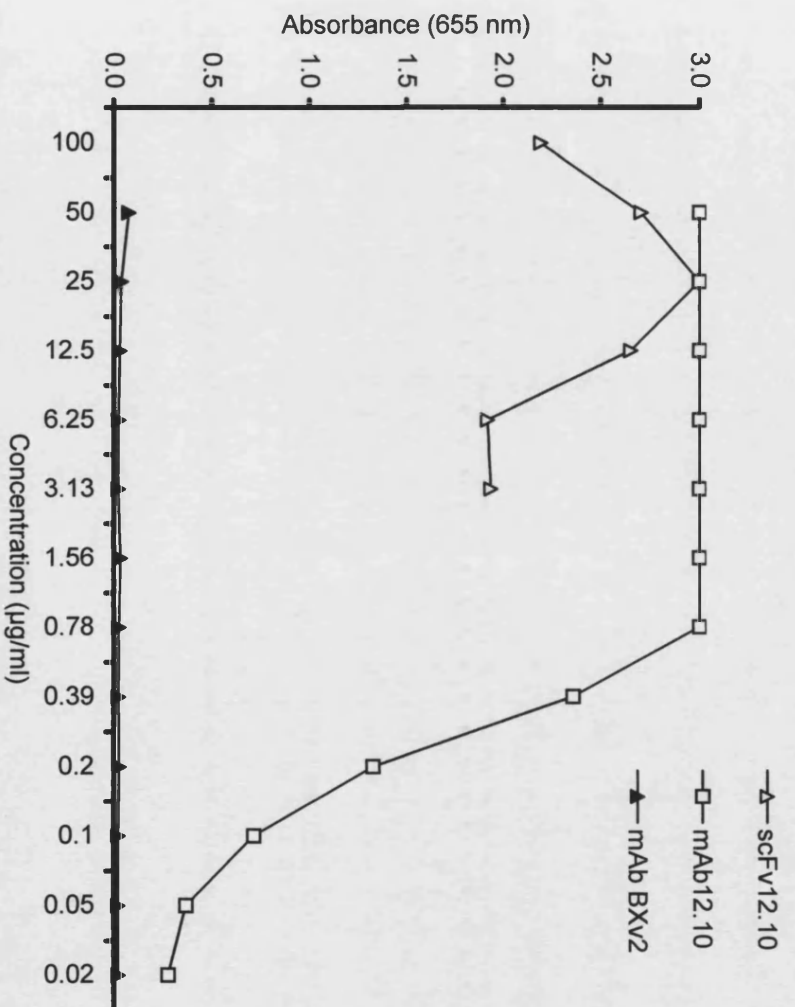


Figure 4.5

Binding of chimeric mAbs 12.8 and 12.10 to MSP-1₁₉-GST by ELISA

The binding of the mouse/human chimeric versions of mAbs 12.8 and 12.10 to recombinant MSP-1₁₉-GST was examined by ELISA as described in section 2.7.2. One hundred microlitres of mAb was added to each well, in serial 1:1 dilutions, starting with a concentration of 50 µg/ml, and murine hybridoma mAbs 12.8 and 12.10 were used as positive controls and humanised mAb BX version 2 was used as a negative control mAb. Bound chimeric antibodies were detected with 100 µl of a 1/5000 dilution in 3% marvel/PBS of peroxidase-conjugated goat anti-human kappa light chain antibody (Sigma).

The chimeric versions of mAb 12.8 and 12.10 and the parent murine mAbs 12.8 and 12.10 bind equally well to MSP-1₁₉. No binding of the negative control mAb is seen.

Figure 4.5

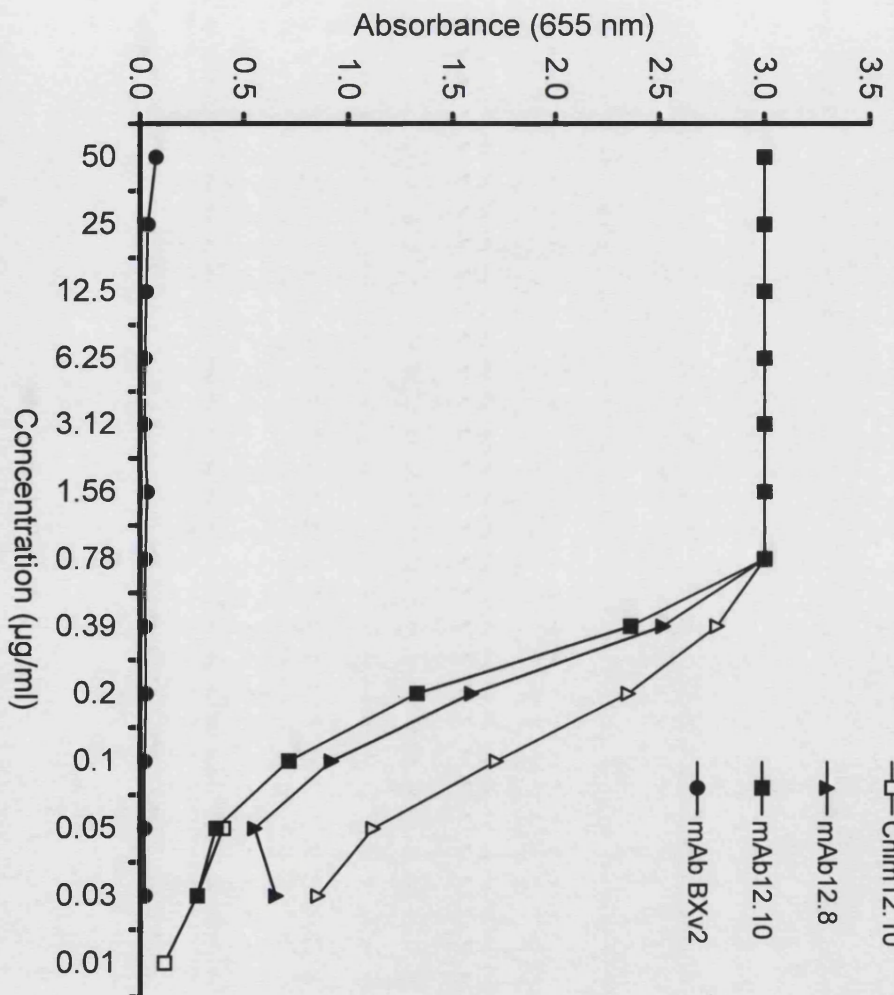


Figure 4.6

Inhibition of erythrocyte invasion by chimeric mAb 12.10

The ability of the chimeric versions of mAbs 12.8 and 12.10 to inhibit invasion of erythrocytes by *Plasmodium falciparum* was tested in an *in vitro* assay as described in section 2.8.3. The results of the invasion assay with chimeric mAb 12.10 are shown here. The chimeric mAb 12.10 was tested at 560 µg/ml. The parent murine mAb 12.10 and the murine mAb 12.8 and EDTA were used as positive controls and the non-*Plasmodium*-specific murine mAb BC1 was used as a negative control. 3D7 parasites were used and the starting parasitaemia at time zero was 1%. The *P. falciparum* cultures were incubated for 24 hours, and parasitaemias were calculated by counting one thousand erythrocytes on each thin blood film.

Using medium alone, ("full invasion") the parasitaemia after 24 hours was 3.1%. Other parasitaemias are shown here as percentages of the full invasion parasitaemia. The parasitaemia after 24 hours with chimeric mAb 12.10 was only 43% of the full invasion parasitaemia, demonstrating that the chimeric version of mAb 12.10 does indeed inhibit erythrocyte invasion. Interestingly, more inhibition of invasion is shown with chimeric mAb 12.10 than with the parent murine mAb 12.10, which was used at 4130 µg/ml. This reflects our experience that the degree to which mAb 12.10 inhibits invasion appears to vary between different batches of antibody prepared from different culture flasks of 12.10 hybridoma cells. The error bars represent the maximum and minimum parasitaemias obtained from counting multiple thin film slides.

Figure 4.6

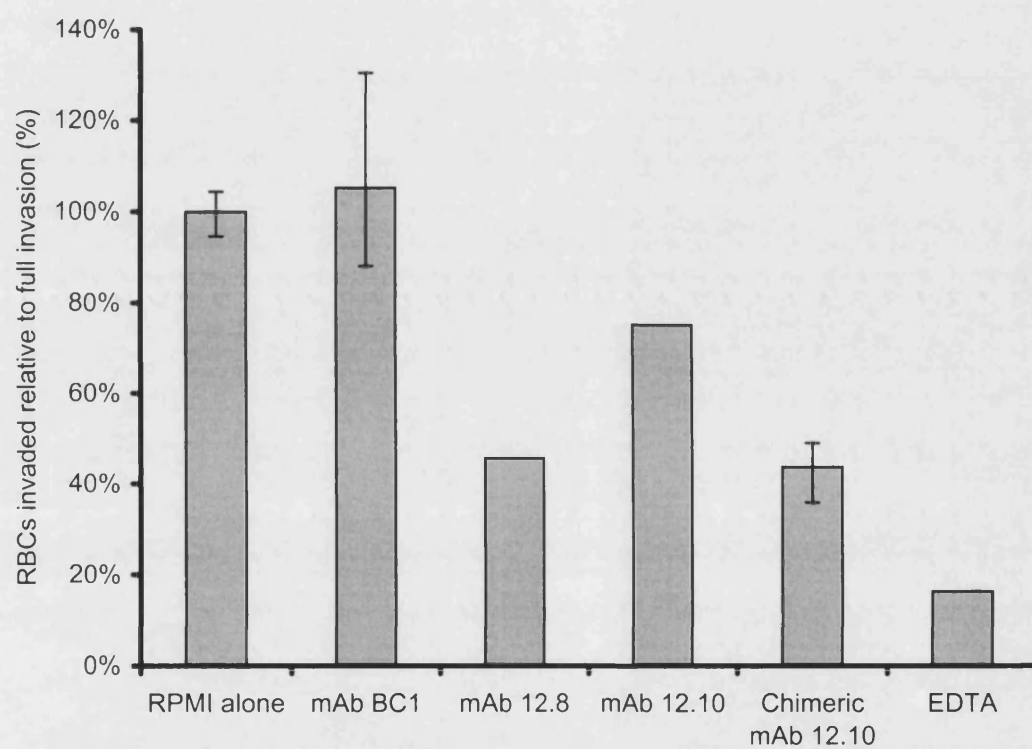


Table 4.1**Binding of chimeric 12.8 & 12.10 to parasites by IFA**

<i>Plasmodium falciparum</i> T9/96 strain			
	Concentration of antibody		
Antibody	30 µg/ml	3 µg/ml	0.3 µg/ml
Mouse mAb 12.8	Strongly positive	Strongly positive	Positive
Mouse mAb 12.10	Strongly positive	Positive	Negative
Chimeric mAb 12.8	Strongly positive	Positive	Negative
Chimeric mAb 12.10	Strongly positive	Positive	Negative
mAb BAT	Negative	Negative	Negative

<i>Plasmodium falciparum</i> FCB-1 strain			
	Concentration of antibody		
Antibody	30 µg/ml	3 µg/ml	0.3 µg/ml
Mouse mAb 12.8	Strongly positive	Positive	Negative
Mouse mAb 12.10	Strongly positive	Positive	Negative
Chimeric mAb 12.8	Positive	Negative	Negative
Chimeric mAb 12.10	Strongly positive	Positive	Negative
mAb BAT	Negative	Negative	Negative

Table 4.2

Inhibition of erythrocyte invasion by chimeric mAbs 12.8 and 12.10

Table 4.2a)

Inhibition of invasion by chimeric mAb 12.10

Reagent tested	% parasitaemias on multiple counts	Mean % parasitaemia
Medium alone	3.2%, 3.1%, 2.9%	3.1%
mAb BC1	2.7%, 3.2%, 4.0%, 3.0%	3.2%
Chimeric mAb 12.10	1.5%, 1.3%, 1.4%, 1.4%, 1.1%	1.3%
mAb 12.10	2.3%	2.3%
EDTA	0.5%	0.5%

Table 4.2b)

Inhibition of invasion by chimeric mAb 12.8

Reagent tested	% parasitaemias on multiple counts	Mean % parasitaemia
mAb BC1	3.1%, 2.0%, 2.2%	2.4%
Chimeric mAb 12.8	1.2%, 0.8%, 1.3%	1.1%

CHAPTER 5

CONSTRUCTION AND PANNING OF HUMAN ANTIBODY PHAGE

DISPLAY LIBRARIES

5.1 Introduction

This chapter describes the construction of two human phage display libraries of antibody fragments derived from malaria-exposed donors, and the results of the panning of those libraries with recombinant MSP-1₁₉-GST in order to isolate MSP-1₁₉-specific scFvs.

The decision was made to construct the libraries from peripheral blood mononuclear lymphocytes donated by malaria-exposed individuals. It is possible to isolate high affinity antibodies to a wide range of antigens using libraries constructed from donors who have not been immunised beforehand. For this to be done, however, it is necessary to use a very large library containing in the order of 10^{10} members (Vaughan, Williams et al. 1996). Furthermore, the aim of this work was not simply to isolate human antibody fragments specific for MSP-1₁₉, but to isolate them from individuals previously exposed to *P. falciparum* in order to gain insights into the natural human antibody response to malaria infection.

The village of Brefet on the south bank of the River Gambia in West Africa was chosen as the location for the collection of blood donations. Brefet was selected because it is within two hours journey from the Medical Research Council Laboratories at Fajara in The Gambia where the initial stages of the library construction could be carried out. Brefet was also chosen because malaria is prevalent in this area (Greenwood, Bradley et al. 1987),

and because the people of Brefet have been the subject of a previous study showing that 50% of adults possess antibodies to MSP-1₁₉ (Egan, Waterfall et al. 1997).

Clinical malaria in this area is highly seasonal, and occurs during a limited period at the end of the rainy season (Greenwood, Bradley et al. 1987). The best time to collect blood donations for a phage display library would be when the maximum number of MSP-1₁₉-specific PBMLs are circulating in the peripheral blood. The antigen stimulation associated with clinical malaria should cause MSP-1₁₉-specific lymphocytes to proliferate, but it is not known whether such lymphocytes would then remain in the peripheral blood or migrate to the reticuloendothelial tissue and be depleted peripherally. The decision was therefore made to construct two phage display libraries. One of these libraries was derived from blood donations collected in March (the middle of the dry season) when clinical malaria is very infrequent, and the other was derived from blood collected in October (the end of the wet season) when clinical malaria is more common.

Because a previous study found that only 50% of adults in this area had evidence of antibodies to MSP-1₁₉ (Egan, Waterfall et al. 1997), the libraries were constructed from blood donations from a fairly large number of individuals. An approximately equal number of male and female donors were chosen and most of the female donors chosen were multigravidae, so that the libraries might also contain representatives of any antibody responses made specifically during pregnancy.

The steps involved in constructing the library are summarised in Figure 2.5. PBMLs were separated out from blood samples and the total RNA was extracted. Using both primers specific for human variable region sequences and a NotI-d(T)₁₈ primer

(Pharmacia Biotech), first-strand cDNA was synthesised. These steps were carried out in The Gambia, as it was thought that cDNA would be more likely than RNA to remain stable in transit from West Africa to London. The variable sequences of the human antibody heavy and light chains were then amplified. To maximise the library diversity, the PCR primers used were designed to hybridise with each of those gene families which have previously been shown to generate functionally rearranged antibody variable region sequences. In a further PCR step, the heavy or light chain variable sequences were joined to the DNA encoding the flexible scFv linker. In a final PCR step the heavy and light chain variable sequences were joined via the linker DNA to create DNA encoding full-length scFvs with appropriate restriction sites for cloning. The scFvs were ligated into the phagemid vector pHEN1H6 (shown in Figure 2.3) and electroporated into TG1 *E. coli* to create the libraries. Using the cDNA obtained in The Gambia, the libraries were constructed at the MRC Collaborative Centre at Mill Hill by Dr Jonathan Chappel, and sections 5.2.3 – 5.2.9 and Figures 5.1 – 5.5 in this chapter describe and illustrate his work.

In an alternative approach to the library construction, MSP-1₁₉-specific PBMLs were pre-selected and separated out from part of each blood donation using MSP-1₁₉-coated dynabeads (Dynal). Whole RNA was prepared from these PBMLs and an attempt was made to amplify antibody variable region sequences from cDNA derived from these cells. This was done to try to maximise the likelihood of MSP-1₁₉-specific variable region sequences being represented in one of the libraries eventually constructed. It was not known at the outset how many members the libraries were going to have, but the size of a library is important since it is known that high affinity antibodies can usually be isolated only from fairly large libraries (Griffiths, Williams et al. 1994). The pre-selection strategy

was therefore attempted as a backup measure in case the other libraries should prove not to be large enough to contain the antibody fragments of interest.

The libraries were panned with MSP-1₁₉. A recombinant version of MSP-1₁₉ was used, expressed in *E. coli* as a fusion with glutathione-S-transferase (GST) as previously described (Burghaus and Holder 1994). In a separate series of experiments, carried out by Dr Jonathan Chappel and not described here, the libraries were also panned against whole *P. falciparum* merozoites. Both merozoites in which secondary processing had proceeded, and merozoites in which secondary processing had been prevented, were used. The strain of *P. falciparum* used (T9/96 or FCB-1) to pan the libraries was alternated between panning rounds, in an attempt to isolate only phage binding to antigens conserved between strains. The results of these experiments are not shown in this thesis, although they are referred to in the course of the discussion of the results obtained with recombinant MSP-1₁₉.

Both the panning of the library and the production of specific scFv for further analysis (described in Chapter 6) were carried out using the TG1 strain of *E. coli*. As discussed in section 1.3.2, an amber stop codon in pHEN1H6 separates the scFv sequence from the gene encoding the phage pIII structural protein. TG1 *E. coli* suppresses the amber stop codon, reading it as glutamine, so that the scFv is expressed as a single fusion protein with the phage pIII protein. If the suppression of the amber stop codon were complete in TG1 *E. coli*, it would not be possible to produce soluble scFv alone, as it would always be expressed fused with the phage pIII protein. In fact the suppression of the amber stop codon in TG1 *E. coli* is far from complete. This has the advantage that both scFv/pIII fusion protein (for phage display) and soluble scFv (for analysis) can be produced in the same cell, and consequently most manipulations are performed in the TG1 strain.

5.2 Results

5.2.1 Number and viability of PBMLs obtained from malaria-exposed donors

Peripheral blood mononuclear lymphocytes (PBMLs) were obtained from twenty malaria-exposed blood donors in the village of Brefet, The Gambia, in March 1997, and these were used to construct the first phage display library (the March library). PBMLs were again obtained from fifteen of the same donors in October 1997 and used to construct the second phage display library (the October library).

PBML numbers and cell viability were determined by examining cells in a haemocytometer after staining with trypan blue. The numbers of PBMLs recovered from the first six donors in March 1997, and the numbers of these cells which appeared viable with trypan blue, are shown in Table 5.1.

One third of each donated blood sample from March 1997 was used to perform a total RNA extraction from the PBMLs immediately, and it was from this one third of donated blood that the March library was made. (Another third of each sample was used to attempt EBV transformation of PBMLs, and the remaining third was used to attempt RNA extraction from pre-selected MSP-1₁₉-specific PBMLs.)

The total number of PBMLs from which the March Library was constructed was 3.26×10^8 .

Either one third or one half of each donated blood sample obtained in October 1997 was used to extract total RNA immediately, and it was from these RNA extractions that the October library was eventually made. (The other two thirds or one half of each donated sample were again used to attempt RNA extraction from pre-selected MSP-1₁₉-specific

PBMLs.) The percentages of PBMLs which appeared viable on trypan blue staining were similar to those in March 1997, i.e. over 95%. The total number of PBMLs from which the October library was constructed was 1.5×10^8 . The number of cells used to construct the libraries represents the theoretical maximum for the number of different heavy and light chains contained in the final libraries. The total numbers of PBMLs obtained from the fifteen donors bled in October 1997, and the numbers used in the eventual construction of the library, are shown in Table 5.2.

5.2.2 RNA extraction from PBMLs

PBMLs were isolated and then pooled and total RNA was extracted from the pool of PBMLs on the same days as the blood was donated. RNA was extracted as described in section 2.5.3, and eluted into $2 \times 150 \mu\text{l}$ DEPC H_2O . Fifty microlitres of this RNA solution was diluted 1:20 into 1 ml DEPC H_2O and the A_{260} measured in a quartz cuvette (previously immersed in 1:1 concentrated HCl/methanol to destroy RNAses). The RNA concentration was calculated by assuming that an RNA solution of $40 \mu\text{g/ml}$ gives an A_{260} of 1. An average of $13.9 \mu\text{g}$ total RNA was obtained per 20 ml blood donation. The results of the RNA quantification are shown in Table 5.3.

5.2.3 Amplification of antibody variable region sequences from human donor PBML cDNA

The variable regions of the heavy and light chain genes were amplified by PCR from the PBML cDNA, using a range of primers, as described in section 2.5.6. Thirty-two reactions, with 32 different combinations of primers, were performed using AmpliTaq. All the PCR reactions yielded products of approximately 350-400 bp as expected. 11 of the reactions yielded faint bands with AmpliTaq and were repeated with Deep Vent DNA polymerase, yielding stronger bands. The reaction products are shown in Figure 5.1. The amplified variable region sequences were excised from the gel and gel-purified. The quantity of DNA recovered from each reaction ranged from 180 – 1000 ng.

5.2.4 scFv assembly stage 1: Attaching heavy chain sequences and light chain sequences to scFv linkers

The amplified variable region sequences were joined together to construct scFv fragments by PCR in a two stage process, illustrated schematically in Figure 2.5. In the first stage, DNA encoding a flexible 15 amino acid linker was attached either to the 3' end of the V_H sequence or the 5' end of the V_λ or V_κ sequences. To attach the appropriate linkers to the appropriate heavy and light chain sequences, 10 different PCR reactions were carried out as shown in Table 2.8. All 10 reactions yielded products of approximately 400-450bp as expected, and these reaction products are shown in Figure 5.2.

The bands were excised from the gel and gel-purified, yielding between 500 and 2500 ng of DNA per reaction.

5.2.5 scFv assembly stage 2: Attaching V_H-linker to linker-V_κ or linker-V_λ to make complete scFvs with restriction sites

The PCR products from the above reactions, consisting of either V_H, or V_λ or V_κ sequences, attached to linker DNA, were then used as templates in a second PCR reaction. This was designed to join the V_H sequences to the V_λ or V_κ sequences via the linker to make DNA encoding complete scFv fragments. This assembly step is illustrated schematically in step 4 of Figure 2.5. The primers were designed to also incorporate *Sfi*I and *Not*I restriction sites for subsequent cloning. Eight reactions were performed, linking the V_H sequences to the V_{κ1}, V_{κ2}, V_{κ3}, V_{κ4}, V_{κ5} and V_{λ1}, V_{λ2-3}, V_{λ7κ} sequences respectively, in order to make all the desired combinations of heavy and light chain gene families. These PCR reactions were carried out as described in section 2.5.9 using the PCR primers shown in Table 2.9. All 8 reactions yielded products of approximately 800 bp as expected, and these reaction products are shown in 5.3.

5.2.6 Addition of long extensions beyond the *Sfi*I & *Not*I restriction sites by PCR

In order to be able to digest the scFv DNA with *Sfi*I and *Not*I for insertion into the phagemid vector, a further PCR was carried out with long primers, using the products from the above PCR as templates. This PCR was designed to add long extensions, overlapping the flanking sequences of pHEN1H6, beyond the *Sfi*I restriction site at the 5' end and the *Not*I restriction site at the 3' end of the scFv DNA. These long extensions were added to improve the efficiency of *Sfi*I/*Not*I digestion. This PCR is illustrated schematically in step 5 of Figure 2.5. The primers are shown in Table 2.10 and the reactions were carried out as described in section 2.5.10. Two reactions were carried out, one using V_H-V_κ scFv DNA

as a template and one using V_H-V_λ scFv DNA as a template. Both reactions were carried out in 10 separate reaction tubes. The PCR reactions both yielded products of the expected size.

Prior to setting up this PCR, a *Sfi*I & *Not*I & *Nco*I restriction digest was carried out with the template scFv DNA to ensure that none of the scFv contained any internal *Sfi*I or *Not*I or *Nco*I restriction sites. There was no evidence of any of the scFv DNA being digested by any of these enzymes.

5.2.7 *Sfi*I/*Not*I restriction digest of V_H-V_κ and V_H-V_λ scFv DNA

The V_H-V_κ and V_H-V_λ scFv DNA from the above PCR was chloroform-extracted, ethanol precipitated and resuspended and then *Sfi*I/*Not*I digested. Digestion occurred appropriately and the digested products were of the anticipated sizes and are shown in Figure 5.4.

The *Sfi*I/*Not*I digested V_H-V_κ and V_H-V_λ scFv DNA products were gel purified and quantified. 7.5 µg of V_H-V_κ and 7.5 µg of V_H-V_λ DNA was recovered.

5.2.8 Ligation of scFv inserts into phagemid vector and transformation of

***E. coli* TG1-TR cells**

The *Sfi*I/*Not*I digested scFv inserts were ligated into the phagemid vector pHEN1H6 and the ligation products were purified and electroporated into *E. coli* TG1-TR competent cells as described in sections 2.5.12 – 2.5.13. Using 50 µl of TG1-TR cells each, 50 electroporations were carried out using V_H-V_κ scFv DNA and 50 electroporations were carried out using V_H-V_λ scFv DNA. Each lot of 50 electroporated cells was pooled and (after removal of a 2.5 ml aliquot of cells for titration) plated. The next day the colonies

were scraped from the plates and resuspended and stored as two lots of 48 1ml aliquots (either V_H - V_K scFv or V_H - V_λ scFv) as glycerol stocks at -70°C .

The aliquots removed for titration were plated and the number of colonies were counted to estimate the number of scFv members in the whole library. The results are shown in Table 5.4.

5.2.9 Presence and diversity of scFv inserts

After transformation and plating, 50 colonies of the V_H - V_K transformed cells and 50 colonies of the V_H - V_λ transformed cells were randomly picked and PCR screened for the presence of inserts. All 100 of these colonies yielded a PCR product of the expected insert size. Part of each of these products was digested with the frequent-cutting restriction enzyme *Bst*NI to make an initial assessment of the diversity of the inserts. Diverse *Bst*NI restriction patterns were obtained and are shown in Figure 5.5.

5.2.10 PBML numbers pre-selected with MSP-1₁₉-GST-coated dynabeads

A proportion of PBMLs were set aside to attempt to separate out those PBMLs expressing MSP-1₁₉-specific antibody on the cell surface, for subsequent RNA extraction. 8×10^8 tosyl-activated dynabeads 450 (Dyna) were coated with recombinant MSP-1₁₉-GST. The PBMLs were pooled and the coated dynabeads were added to a concentration of 2×10^7 beads/ml and the cells and beads were shaken together and those cells sticking to the beads were separated out on a Dynal magnet. Cell count and cell viability were assessed as above, counting only those cells visibly attached to a bead. RNA extraction was then attempted as described above.

From 8.1×10^7 PBMLs (comprising one third of the total PBMLs obtained from the first six 20 ml blood donations in March 1997), 3×10^5 cells (i.e. approximately 0.5% of the starting number) were separated out by recombinant MSP-1₁₉-GST-coated magnetic beads.

5.2.11 Attempted antibody variable region amplification from cDNA of preselected PBMLs

RNA extraction was carried out on these preselected cells as described above. The quantity of RNA obtained was too low to be accurately estimated by spectroscopy. cDNA synthesis was carried out as above, but attempts at antibody variable region amplification from this cDNA were not successful.

5.2.12 Numbers of phage retrieved by panning against recombinant MSP-1₁₉-GST

Aliquots from each of the March and October phage display libraries were pooled for panning against antigen. The initial amount of phage used in the panning experiments was 1×10^{14} colony-forming units (cfu). The pooled library phage was panned against recombinant MSP-1₁₉-GST as described in sections 2.6.2 – 2.6.4. The number of phage recovered on successive rounds of panning is expected to fall initially, as non-binding phage is eliminated, and then to rise sharply, as high affinity antigen-binding phage is selected and amplified. This expected pattern was seen when panning against recombinant MSP-1₁₉-GST. After the first round of panning, 1.5×10^6 cfu were recovered. After the second round 6.4×10^4 cfu were recovered. After the third round 1.4×10^7 cfu were

recovered, and after the fourth round 1.0×10^8 cfu were recovered. These results are illustrated in Figure 5.6.

5.3 Discussion

The results in this chapter describe the successful construction of two phage display libraries derived from blood donations from malaria-exposed donors. The phage numbers obtained by successive rounds of panning of the combined libraries with recombinant MSP-1₁₉ are consistent with the successive enrichment of MSP-1₁₉-binding phage.

The combined size of the two libraries (approximately 1.3×10^9 members) is sufficiently large to anticipate that they will yield high affinity antibody fragments (Griffiths, Williams et al. 1994). The results of the *Bst*NI restriction digest of 100 randomly selected clones suggest that the library is indeed diverse. In an additional experiment to estimate diversity, a number of clones were randomly selected from the unpanned libraries and sequenced by Jonathan Chappel and Alicia Sedo of the MRC Collaborative Centre. These clones were found to be diverse in sequence and were derived from a wide range of different variable gene families (data not shown).

In retrospect, there was a degree of redundancy in the number of PCRs used to amplify the linker DNA prior to its incorporation with either the heavy chain or light chain variable sequences. Linker DNA was prepared in 88 separate PCRs as described in section 2.5.7. These PCRS were designed to produce overlapping regions at both ends of the linker DNA, designed to anneal to the 3' end of the heavy chain sequences and the 5' end of the κ or λ light chain sequences in all possible combinations. Using the linkers thus

amplified, it is theoretically possible to create DNA encoding full-length scFv in a single PCR, using heavy chain, light chain and linker sequences as templates in the same reaction. This would involve essentially omitting step 3 as illustrated in Figure 2.5. Phage display libraries have been made in this way (Marks, Hoogenboom et al. 1991) but attempts to assemble the scFv in these libraries by such a single step PCR were not successful. An intermediate step was therefore introduced (step 3 in Figure 2.5) to join the linker DNA to either the heavy chain or the light chain sequences, but not both at once. The linker PCR primers involved in this step, HuLinkBACK and HuLinkFor, do not preserve both of the overlapping linker ends created in the 88 PCRs described above, but with this intermediate step, those overlapping ends are no longer both required. This is because in the final assembly PCR (step 4 in Figure 2.5) the V_H-linker and linker-V_L anneal to each other via the two complementary strands of the linker itself.

It is not clear why the attempt to amplify antibody variable region sequences from cDNA derived from pre-selected PBMLs was unsuccessful. The most likely explanation is that the quantity of total RNA which was prepared from these cells was insufficient for the synthesis of adequate quality first strand cDNA. Although the proportion of PBMLs binding MSP-1₁₉-coated dynabeads was quite high (about 0.5% of the total) the actual numbers of pre-selected PBMLs used for each RNA preparation was smaller than the minimum number recommended by the Qiagen RNeasy Midiprep kit. The yield of RNA might be improved with the addition of carrier RNA to the cells prior to the RNA extraction, were the experiment to be repeated. In retrospect, however, the success of this pre-selection approach was not essential to the project to create libraries from which MSP-1₁₉-specific scFv can be isolated, as the libraries made from unselected PBMLs were successfully constructed and proved able to yield scFvs of interest.

Figure 5.1

Amplification of antibody variable region sequences from human donor PBML cDNA

Variable region sequences were amplified using 32 different primer combinations in 32 separate reactions, using AmpliTaq, as described in section 2.5.6. The PCR primers and reactions used are shown in Table 2.4 and Table 2.5 respectively. This figure shows 5 µl of each 50 µl reaction resolved on a 2.5% agarose gel.

Lanes 1-10 show amplified V_H sequences, i.e. the variable part of the rearranged gene encoding the heavy chain of the antibody. These are:

V_H1a (lane 1), V_H2x (lane 2), V_H3a (lane 3), V_H3x (lane 4), V_H4a (lane 5), V_H4x (lane 6), V_H4y (lane 7), V_H5x (lane 8), V_H6a (lane 9), V_H7x (lane 10).

Lanes 11-19 show amplified V_κ sequences, i.e. the variable part of the rearranged gene encoding the κ light chain of the antibody. These are:

V_κ1a (lane 11), V_κ2a (lane 12), V_κ2x (lane 13), V_κ3a (lane 14), V_κ3x (lane 15), V_κ4a (lane 16), V_κ5a (lane 17), V_κ6a (lane 18), V_κ6x (lane 19).

Lanes 20-32 show amplified V_λ sequences, i.e. the variable part of the rearranged gene encoding the λ light chain of the antibody. These are:

V_λ1 (lane 20), V_λ1x (lane 21), V_λ2x (lane 22), V_λ3a (lane 23), V_λ3b (lane 24), V_λ3x (lane 25), V_λ4x (lane 26), V_λ4y (lane 27), V_λ5x (lane 28), V_λ6 (lane 29), V_λ7x (lane 30), V_λ8x (lane 31), V_λ10x (lane 32).

Standard DNA marker sizes are indicated.

Figure 5.1

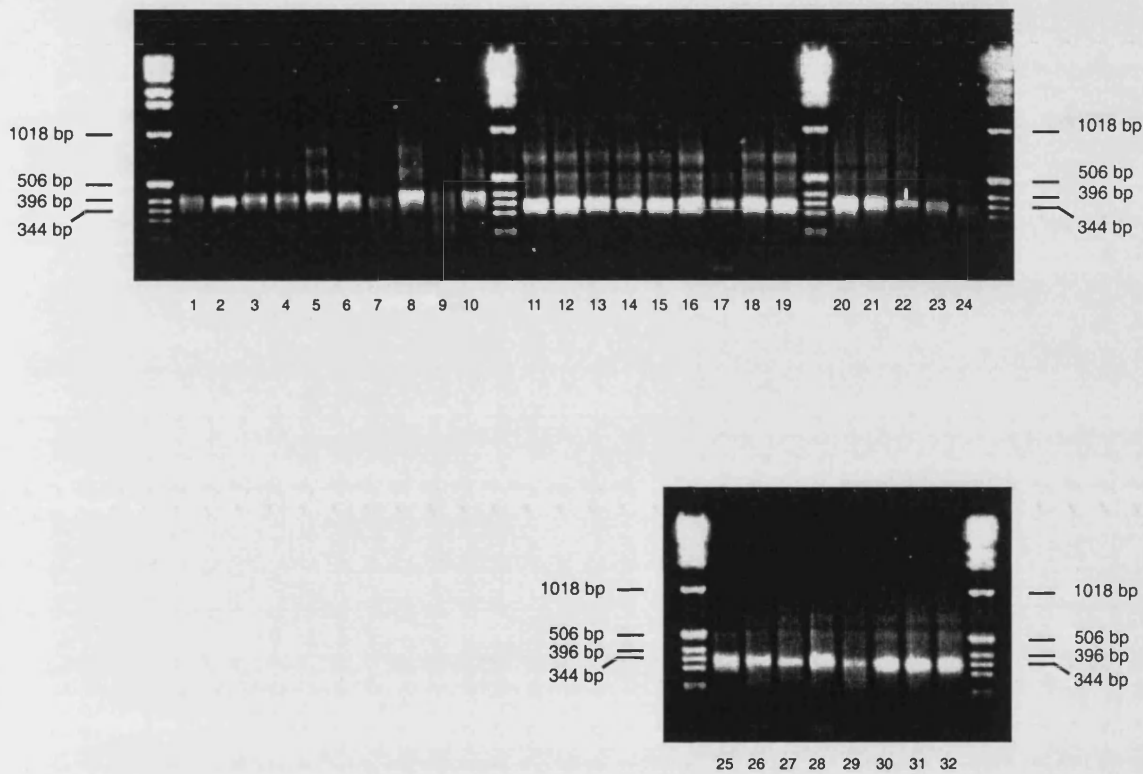


Figure 5.2

scFv assembly stage 1:

Attaching heavy chain sequences and light chain sequences to scFv linkers

This figure illustrates the first stage of a two-stage process by which the amplified variable region sequences were joined together to construct scFv fragments by PCR, as described in section 2.5.8. In the first stage, DNA encoding a flexible 15 amino acid linker was attached either to the 3' end of the V_H sequence or the 5' end of the V_λ or V_κ sequences. To attach the appropriate linkers to the appropriate heavy and light chain sequences, 10 different PCR reactions were carried out as shown in Table 2.8. Ten microlitres of each 100 μ l reaction were resolved on a 2.5% agarose gel, shown here.

Lanes 1-10 show the products of PCR reactions 1-10, performed as described in Table 2.8

DNA size markers were used as previously described.

Figure 5.2

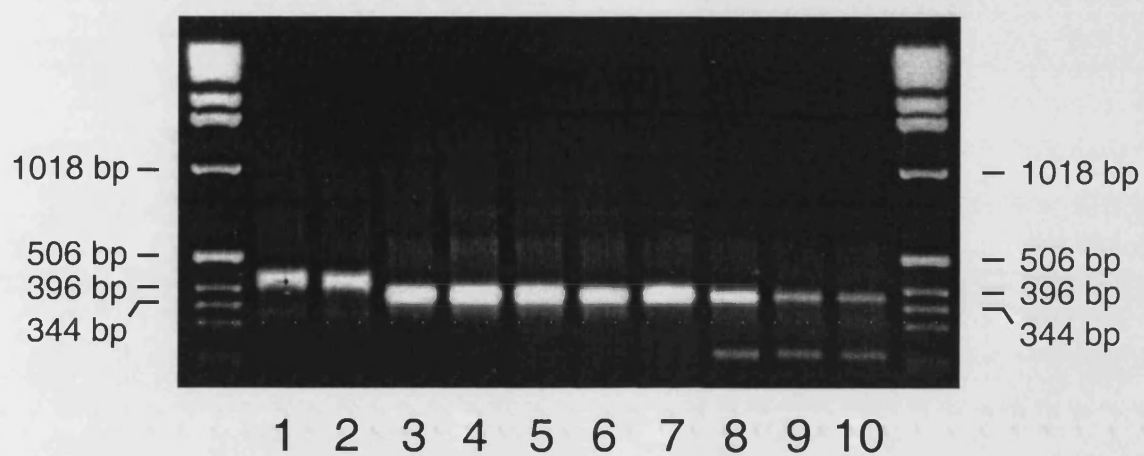


Figure 5.3

scFv assembly stage 2:

Attaching V_H -linker to linker- V_K or linker- V_λ to make complete scFvs with *SfiI*/*NotI* restriction sites.

This figure illustrates the second stage of the PCR assembly of scFv from V_H and V_λ or V_K sequences, as described in section 2.5.9.

The PCR products shown in Figure 5.2, consisting of either V_H or V_λ or V_K sequences attached to linker DNA, were then used as templates in a second PCR reaction. This was designed to join the V_H sequences to the V_λ or V_K sequences via the linker to make DNA encoding complete scFv fragments, and the primers were designed to incorporate *SfiI* and *NotI* restriction sites for subsequent cloning. Eight reactions were performed, linking the V_H sequences to the V_{K1} , V_{K2} , V_{K3} , V_{K4} , V_{K5} and $V_{\lambda1}$ $V_{\lambda2-3}$ $V_{\lambda7x}$ sequences respectively, in order to make all the possible combinations of heavy and light chain gene families. These PCR reactions were carried out using the PCR primers shown in Table 2.9. Ten microlitres of each 100 μ l reaction were resolved on a 2.5% agarose gel, shown here.

The bands represent DNA encoding whole scFv. The lanes are labelled according to the gene family of light chain which has been joined to the heavy chain.

Figure 5.3

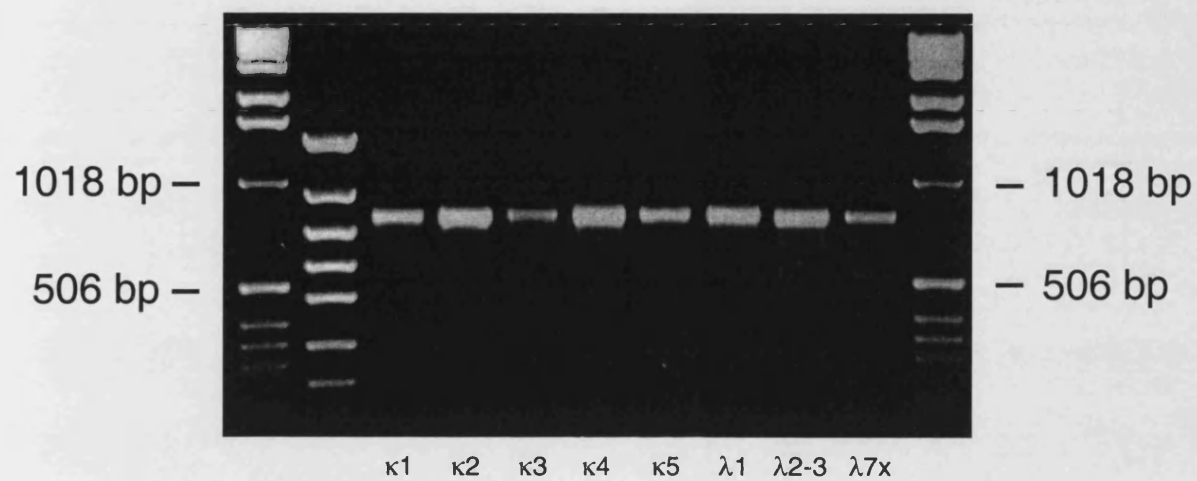


Figure 5.4

***SfiI/NotI* restriction digest of V_H - V_K and V_H - V_L scFv DNA**

This figure shows uncut scFv DNA, *SfiI*-digested scFv DNA and *SfiI/NotI*-digested scFv DNA, produced by digestion of the scFv DNA shown in Figure 5.3, as described in section 2.5.11. Restriction digests were performed after the addition of long DNA overlaps to facilitate digestion, as described in section 2.5.10.

The digestion products are resolved on a 2.5% agarose gel.

Lanes 1-3 show V_H - V_K scFv DNA uncut (lane 1), cut with *SfiI* (lane 2) and cut with *SfiI/NotI* (lane 3).

Lanes 4-6 show V_H - V_L scFv DNA uncut (lane 4), cut with *SfiI* (lane 5) and cut with *SfiI/NotI* (lane 6).

The DNA size markers are the same as in Figure 5.3.

Figure 5.4

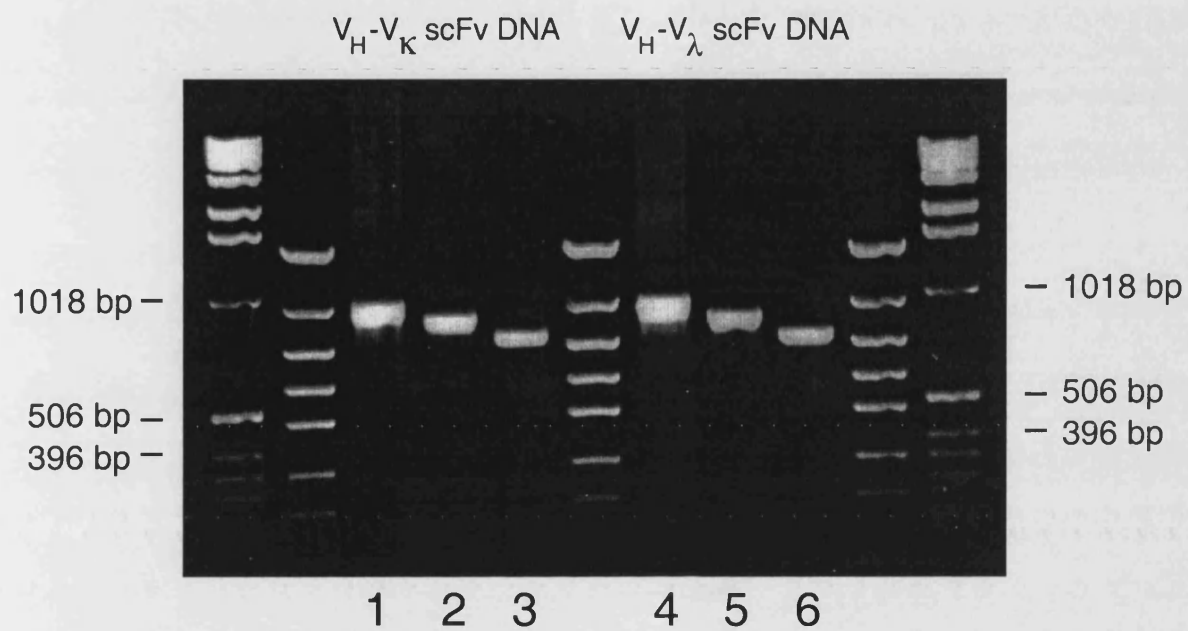


Figure 5.5

Estimation of diversity of library scFv inserts by *Bst*NI digestion

This figure shows the different restriction-digest patterns of 50 randomly selected scFvs from the October library.

Fifty colonies of the V_H - V_K transformed cells and 50 colonies of the V_H - V_λ transformed cells were randomly picked and PCR screened for the presence of inserts. All 100 of these colonies yielded a PCR product of the expected insert size. Part of each of these products was digested with the frequent-cutting restriction enzyme *Bst*NI to make an initial assessment of the diversity of the inserts. The products of this digestion were resolved on a 4% NuSieve agarose gel.

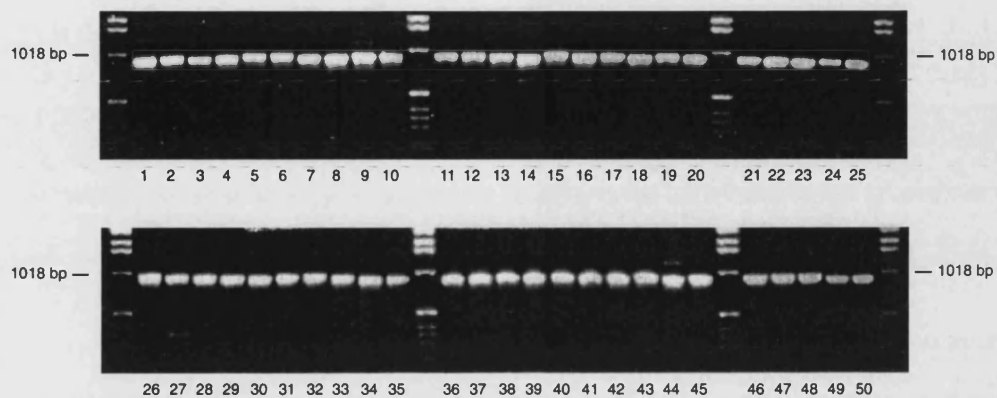
Figure 5.5 a) shows the V_H - V_K scFv inserts amplified by PCR screen from 50 randomly selected colonies.

Figure 5.5b) shows the same V_H - V_K scFv inserts after digestion with *Bst*NI.

A large number of different digest patterns are seen, suggesting a high degree of DNA sequence diversity among the library scFv inserts.

Figure 5.5

a)



b)

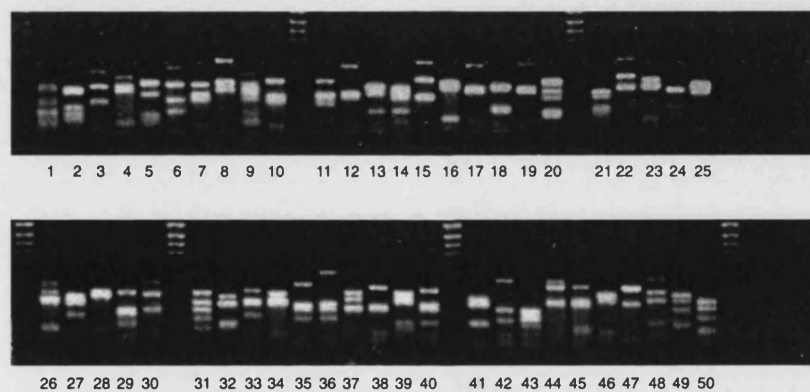


Figure 5.6

Quantity of phage recovered from successive rounds of panning

The phage display libraries were subjected to four rounds of panning with recombinant MSP-1₁₉-GST in order to isolate MSP-1₁₉-specific scFvs, as described in sections 2.6.2 – 2.6.4. The initial amount of phage used in the panning was 1×10^{14} colony-forming units (cfu). The number of phage recovered on successive rounds of panning was expected to fall initially, as non-binding phage is eliminated, and then to rise sharply, as high affinity antigen-binding phage is selected and amplified. This expected pattern was seen on panning with MSP-1₁₉ and is shown in this figure. These results suggested that MSP-1₁₉-specific scFv had indeed been selected for and amplified. Note that the scale on the y-axis is logarithmic.

Figure 5.6

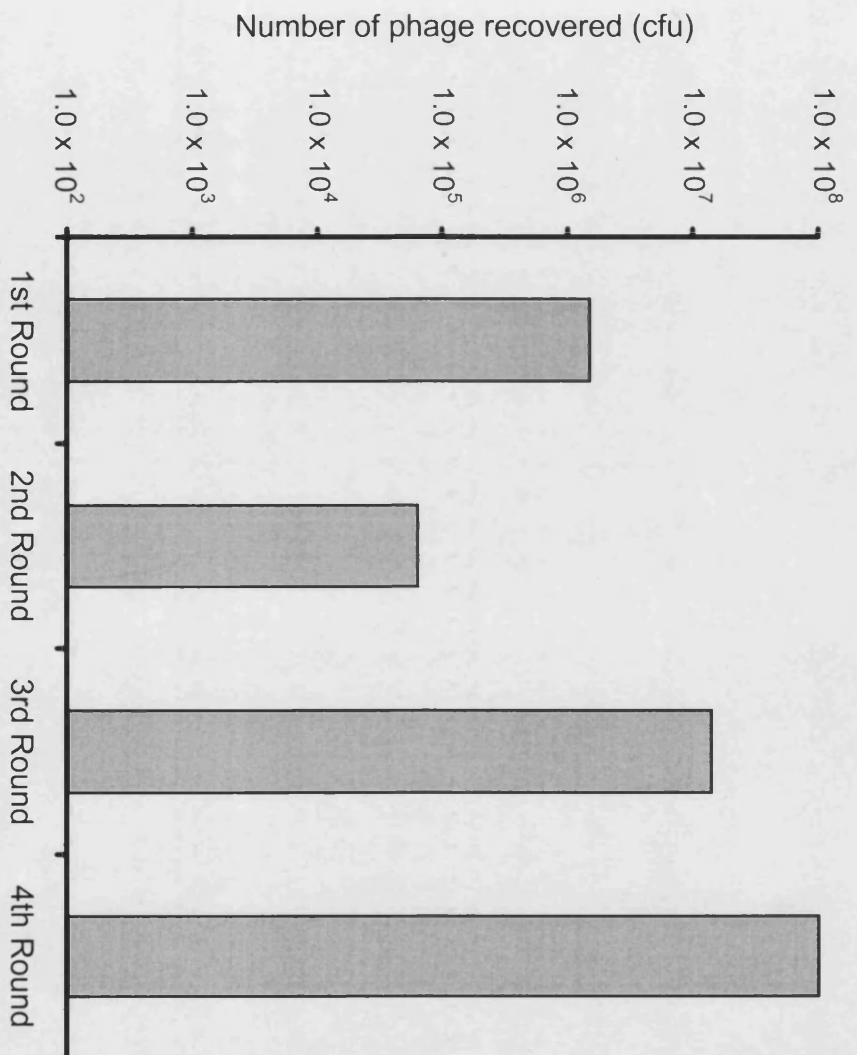


Table 5.1**Recovery and viability of PBMLs from individual donors, March 1997**

Donor	Volume of blood donated	No. of PBMLs recovered	No. of viable cells (%)
Z5	20ml	5.0×10^7	> 95%
Z15	20ml	1.2×10^7	> 95%
Z21	20ml	5.2×10^7	> 95%
Z71	20ml	3.2×10^7	> 95%
Z88	20ml	6.0×10^7	> 95%
Z168	20ml	2.2×10^7	> 95%

Table 5.2

Number of PBMLs recovered and used to construct the October library

Donor	Volume of blood donated	No. of PBMLs recovered	No. of PBMLs used to make library
Z157	20ml	2.63×10^7	0.88×10^7
Z152	20ml	3.36×10^7	1.12×10^7
Z149	20ml	2.23×10^7	0.74×10^7
Z151	20ml	4.16×10^7	1.39×10^7
Z142	20ml	1.73×10^7	0.58×10^7
Z49	20ml	0.49×10^7	0.16×10^7
Z168	20ml	2.36×10^7	0.79×10^7
Z88	20ml	1.67×10^7	0.56×10^7
Z71	20ml	1.69×10^7	0.56×10^7
Z141	20ml	3.48×10^7	1.16×10^7
Z158	20ml	1.56×10^7	0.78×10^7
Z176	20ml	1.36×10^7	0.68×10^7
Z16	20ml	5.24×10^7	2.62×10^7
Z15	20ml	3.38×10^7	1.69×10^7
Z21	20ml	2.13×10^7	1.07×10^7

Table 5.3**Quantity of total RNA obtained from blood donations**

PBML pool used in library construction	No. of donors	A ₂₆₀	RNA concentration	Total RNA obtained
March 1	6	0.295	236 µg/ml	70.8 µg
March 2	6	0.398	318 µg/ml	95.5 µg
March 3	8	0.373	298 µg/ml	89.4 µg
October 1	8	0.300	240 µg/ml	72.0 µg
October 2	2	0.104	31.2 µg/ml	09.4 µg
October 3	5	0.624	499 µg/ml	149.7 µg

Table 5.4

Estimation of the size of the March and October phage display libraries

	The March library	The October library
No of blood donors used	20	15
No of PBMLs used	3.3×10^8	1.5×10^8
No. of V_H - V_K members	3.9×10^8	1.0×10^8
No. of V_H - V_L members	4.5×10^8	3.5×10^8
Total No. of members	8.4×10^8	4.5×10^8

CHAPTER 6

CHARACTERISATION OF HUMAN LIBRARY-DERIVED ANTI-

MSP-1₁₉ SCFVS

6.1 Introduction

The panning of the phage display libraries with recombinant MSP-1₁₉ led to the isolation of increasing numbers of phage with successive rounds of panning, as described in the previous chapter. This chapter describes the work done to demonstrate that there are MSP-1₁₉-binding clones among the amplified phage, to isolate as many different MSP-1₁₉-binding clones as possible, and to characterise the properties of these clones.

Firstly the polyclonal phage from successive panning rounds was assessed for the presence of MSP-1₁₉-binding scFv. To do this, the total polyclonal scFv obtained from each successive round of panning was examined for binding to acetone-fixed *P. falciparum* by IFA. The IFA signal from the unpanned library and the first two rounds of panning was negative as expected, but the signal from the third and fourth rounds of panning was similar to that obtained with mAbs 12.8 and 12.10, indicating successful isolation and amplification of MSP-1₁₉-binding scFvs from the library. This was confirmed by demonstrating that the polyclonal scFv also bound to *P. falciparum* merozoite lysate on a Western blot.

Individual MSP-1₁₉-binding clones were then isolated from the polyclonal phage by ELISA of scFvs from 160 randomly selected clones. Of these clones, 114 (71%) bound to MSP-1₁₉. In order to assess the DNA sequence diversity of these clones, the

diversity of the restriction fragment patterns produced by the frequent cutting enzyme *Bst*NI was examined, and 11 different restriction patterns were identified.

To determine which of these scFvs might be most likely to have invasion-inhibitory potential, the ability of individual scFvs to block the binding of the invasion-inhibitory mAbs 12.8 and 12.10 to MSP-1₁₉ was examined by competition ELISA. The majority of MSP-1₁₉-binding scFvs appeared to partially block the binding of these mAbs, suggesting that the scFv epitopes at least overlap the invasion-inhibitory epitopes recognised by mAbs 12.8 and 12.10. Of these scFvs, 20 clones were selected, on the basis of their *Bst*NI restriction digest patterns, for DNA sequencing. Of these 20 clones, some were found to contain identical inserts but a total of 6 different scFv sequences were found. There was more sequence diversity in the light chains than in the heavy chains. All 6 sequences were similar to each other. Representative clones for each of the different sequences were expressed and the expression levels of the different scFvs determined. The binding of each of these scFvs to *P. falciparum* was confirmed by IFA.

Those scFvs which could be expressed in sufficient quantity were tested for the ability to inhibit erythrocyte invasion *in vitro*. No invasion-inhibitory scFvs were identified, although this is not surprising given that Fab fragments of mAb 12.10 do not retain the invasion-inhibitory activity of the parent mAb (Guevara Patino 1997). An initial attempt was made to examine ways in which invasion-inhibitory activity might be restored to the scFvs in the future. An attempt was made to increase the size of the scFvs used in the invasion assay to the size of a whole antibody by the addition either mAb 9E10 or anti-PentaHIS antibody (Qiagen) to the scFv so that these antibodies could bind to the scFv *c-myc* or hexahistidine tag respectively. This was done on the

assumption that increasing the size of the scFv back to a size approximating to that of a whole antibody might restore any invasion-inhibitory activity present in the parent antibody.

6.2 Results

6.2.1 Binding of polyclonal scFv from panning rounds 1-4 to *P. falciparum* by IFA

In order to ascertain whether successive rounds of panning against recombinant MSP-1₁₉-GST had indeed isolated *P. falciparum* merozoite-binding scFvs, polyclonal scFv from each round of panning was tested for its ability to bind *P. falciparum* parasites by immunofluorescence. Soluble antibody was purified from phage recovered from each round of panning by periplasmic extraction followed by purification on a Qiagen mini-His column. IFA was carried out using acetone-fixed parasites, and the polyclonal scFv was tested against both T9-96 and FCB-1 strains of *P. falciparum*. The polyclonal scFv was tested at a concentration of 20 µg/ml. The monoclonal antibodies 12.8 and 12.10 were used as positive controls, and polyclonal scFv prepared from the unpanned March and October libraries was used as a negative control. The polyclonal scFv from the unpanned libraries and from the first and second rounds of panning against recombinant MSP-1₁₉-GST showed no appreciable binding, but the polyclonal scFv from the third and fourth rounds produced an immunofluorescence pattern similar to that of the positive control mAbs. The immunofluorescence patterns obtained with polyclonal scFv from successive rounds of panning against recombinant MSP-1₁₉-GST are shown in Figure 6.1.

6.2.2 Binding of polyclonal scFv from panning rounds 1-4 to *P. falciparum* by Western blot

To confirm that the polyclonal scFv from later rounds of panning did bind native merozoite protein, binding was also demonstrated by Western blot, as described in section 2.7.5. T9/96 and FCB-1 *P. falciparum* merozoites were incubated for 1 hour at 37°C with 1 mM MgCl₂, 1 mM CaCl₂, and the protease inhibitors Leupeptin, Antipain, Aprotinin & TLCK in 200 µl PBS. The merozoites were then boiled for 10 minutes with 200 µl non-reducing buffer and the proteins were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. The polyclonal scFv was added to the membrane at a final concentration of 1 µg/ml for 1 hour. The membrane was incubated with 9E10 supernatant and peroxidase-conjugated goat anti-mouse IgG/IgM was added and detected using the ECL chemiluminescent detection system (Amersham Life Science).

The results are shown in Figure 6.2

Polyclonal scFv from the fourth round of panning against recombinant MSP-1₁₉-GST recognised both MSP-1₁₉ and MSP-1₄₂ (from which MSP-1₁₉ is derived at secondary processing). Interestingly, polyclonal scFv obtained by Dr Jonathan Chappel, by panning against whole merozoites in which processing had been allowed to proceed, gave almost identical patterns, recognising MSP-1₁₉ and MSP-1₄₂.

6.2.3 Identification of individual MSP-1₁₉-GST-binding scFv clones by ELISA

Individual MSP-1₁₉-binding scFv clones were identified by ELISA on a 96-well plate. Individual colonies from the third and fourth rounds of panning against MSP-1₁₉-GST were picked and transferred to a 96-well master plate and grown overnight. A replica plate was inoculated from the master plate and grown to induce scFv expression. Fifty microlitres of 50% glycerol was added to each well of the master plate, which was then stored frozen at -70°C. Pre-blocked culture supernatants containing scFv were transferred to an ELISA plate coated with MSP-1₁₉-GST.

Eighty clones were picked from the third round of panning and 80 clones were picked from the fourth round of panning.

Ninety-two percent of the scFvs from the third round of panning and 53% of the scFvs from the fourth round of panning bound to MSP-1₁₉.

6.2.4 Assessment of clone diversity by *Bst*NI digest

An initial assessment of the sequence diversity of the MSP-1₁₉-binding scFvs was made. This was done by examining restriction fragments produced by the frequent cutting restriction enzyme *Bst*NI. Selected scFv DNA sequences were amplified by PCR using the primers RSP 48 and fdTET. The completed PCR reactions were then digested with *Bst*NI at 60°C for 2 hours and the digestion products were resolved on a 3% agarose gel.

From the scFv obtained by panning against MSP-1₁₉-GST, 78 clones were examined, of which 68 were MSP-1₁₉-binders and 10 were non-binders (as previously demonstrated by ELISA).

The amplified undigested scFv and the corresponding restriction digest products are shown in Figure 6.3.

On this random sample of 64 MSP-1₁₉-binding clones, 11 different *Bst*NI restriction digest patterns were identified, suggesting therefore that some degree of diversity existed among the MSP-1₁₉-binding scFvs obtained from the libraries.

6.2.5 Investigation of scFv-binding MSP-1₁₉ epitopes by competition

ELISA

In order to see whether the MSP-1₁₉-binding scFvs bind to epitopes overlapping those of the invasion inhibitory mAbs 12.8 and 12.10, or whether they bind to some other region of the molecule, a competition ELISA was carried out. This was designed to determine whether the scFvs could compete with mAbs 12.8 and 12.10 for binding sites on the MSP-1₁₉ molecule.

ELISA plates were coated with MSP-1₁₉-GST at 0.5 µg/ml for competition with mAb 12.8 and at 0.05 µg/ml for competition with mAb 12.10. The scFvs were expressed in a 96-well replica plate inoculated from the master plate, as above, and the pre-blocked scFv-containing supernatants were transferred to the ELISA plate and allowed to bind. The plates were washed and then mAb 12.8 (at 0.5 µg/ml) or mAb 12.10 (at 0.05 µg/ml) were allowed to bind also.

The results are shown in Figure 6.4.

Most of the scFv which bound to MSP-1₁₉-GST by ELISA gave a reduction in signal from mAb 12.8 of more than 40%, and a reduction in signal from mAb 12.10 of more than 25%. This suggests that the epitopes to which the scFvs bind may overlap the epitopes of mAb 12.8 and mAb 12.10.

6.2.6 DNA and amino acid sequences of MSP-1₁₉-binding scFvs

From those scFvs obtained from the third and fourth rounds of panning against recombinant MSP-1₁₉-GST, 20 clones were selected for sequencing. The clones were chosen on the basis of their binding to MSP-1₁₉-GST by ELISA, their variety of restriction fragment patterns on *Bst*NI digest and their ability to inhibit the binding of mAbs 12.8 and 12.10 in the competition ELISA. Each clone was given a name consisting of two letters and two numbers, which identify the antigen used for panning, the round of panning from which the clone was derived, and the position of the clone on the original 96-well plate of glycerol stocks described in section 6.2.3. Thus the scFv T3D6 refers to the scFv clone obtained by panning against MSP-1₁₉-GST (denoted by the letter T), from the third round of panning, and located in position D6 on the 96-well master plate.

A number of the scFv clones were found on sequencing to be identical, and from among the 20 clones sequenced, six different scFv sequences were found. These scFv clones are scFvs T3B4, T3C2, T3C8, T3D3, T3D6 and T4D4. There is more diversity among the light chain variable regions of these clones than among the heavy chain variable regions.

Two significantly different heavy chain sequences were identified. The commoner heavy chain is present in scFvs T3B4, T3C2, T3C8, T3D6 and T4D4, although the CDR 1 of scFv T4D4 differs from the others in this group by one residue. The other heavy chain sequence is found in scFv T3D3, and differs from the commoner heavy chain in both CDR 2 and CDR 3.

Five different light chain sequences were identified among these 6 scFvs. Variation is seen in all three CDRs. Full sequence information was not obtained on two of the scFv, T3B4 and T3C8, which were not sequenced through the whole of the third CDR and the fourth framework region.

The amino acid sequences of the scFvs are shown in Figure 6.5.

6.2.7 Expression levels of selected MSP-1₁₉-binding scFvs

Selected scFvs were made by periplasmic extraction from a culture volume of 10 ml and purified on mini-His columns (Qiagen). Purified scFv was eluted in a volume of 200 µl. The quantity of purified scFv obtained from 10 ml of culture was estimated by measuring the absorbance at 280 nm, assuming that 600 µg/ml of scFv gives an A₂₈₀ of 1.0.

The results are shown in Table 6.1.

6.2.8 Binding of selected scFvs to *P. falciparum* parasites by IFA

Selected purified scFv clones were tested for their ability to bind to acetone-fixed T9/96 & FCB-1 *P. falciparum* by IFA. mAb 12.10 was used as a positive control and scFv D1.3 (anti-hen's egg lysozyme) was used as a negative control.

Immunofluorescence patterns were graded as negative, poorly positive, positive or very positive depending on the strength of the signal compared with that of mAb 12.10. All the library scFvs tested gave a positive immunofluorescence pattern similar to that obtained with mAb 12.10.

The results are shown in Table 6.2.

6.2.9 Examination of selected scFvs for invasion-inhibitory activity

Selected scFvs were tested for the ability to inhibit erythrocyte invasion *in vitro*. Invasion assays were carried out as described in section 2.8.3. The scFvs T3B4, T3C2, T3D3, T3D6, and T4D4 were tested. The scFvs were dialysed in RPMI and tested at a final concentration of approximately 200 µg/ml. None of the scFvs tested inhibited erythrocyte invasion. The same scFvs were also tested in the presence of 9E10 hybridoma supernatant (final concentration 10% [v/v]) or anti-PentaHIS antibody (Qiagen) (final concentration 25 µg/ml or 50 µg/ml). This was done because it was hoped that these mAbs might increase the size of the scFv by binding to the *c-myc* and hexahistidine tags respectively, and thereby restore putative invasion-inhibitory activity. None of the scFvs tested inhibited erythrocyte invasion in these experiments (data not shown).

6.3 Discussion

The work described in this chapter demonstrates that it is possible to isolate human antibody fragments that recognise MSP-1₁₉ from phage display libraries derived from malaria-exposed human donors. It has been shown here that the scFvs isolated by panning the libraries with recombinant MSP-1₁₉ do indeed recognise both recombinant MSP-1₁₉ and native merozoite MSP-1₁₉ and whole *P. falciparum* parasites. Binding to recombinant MSP-1₁₉ has been shown by ELISA, binding to native MSP-1₁₉ (in both strains FCB-1 and T9/96) has been shown by Western blot, and binding to whole acetone-fixed *P. falciparum* parasites (again in both strains FCB-1 and T9/96) has been shown by IFA.

It has also been shown that a number of different anti-MSP-1₁₉ antibody fragments can be isolated in one series of panning rounds with a single antigen. This was indicated by the *Bst*NI restriction digest patterns and confirmed by DNA sequencing of the scFvs obtained.

Furthermore this work suggests that the scFvs that bind to MSP-1₁₉ compete with the invasion-inhibitory mAbs 12.8 and 12.10 for binding to antigen. This has been demonstrated by competition ELISA, in which the binding of the invasion-inhibitory mAbs to recombinant MSP-1₁₉ was reduced by MSP-1₁₉-specific scFvs but not by those scFvs which do not themselves bind to MSP-1₁₉ by ELISA.

None of the scFvs selected for testing in this study showed any inhibition of erythrocyte invasion in the *in vitro* assay. Preliminary experiments were done to attempt to restore putative invasion-inhibitory activity to these scFvs by increasing their

size, but no invasion-inhibitory effect could be detected. Further experiments are needed to optimise such strategies and determine whether they can restore invasion-inhibitory activity to scFvs in the future.

It is estimated that successive rounds of panning enrich the population of phage specific for the antigen by one in 10^3 at each round (McCafferty, Griffiths et al. 1990). It can be seen however from the results of the ELISA described in section 6.2.3, that in a test of 80 randomly chosen clones from the third and fourth panning rounds, the proportion of isolated scFv fragments specific for MSP-1₁₉ actually decreased between the third and fourth rounds of panning. This probably occurs because by the fourth round of panning, scFvs on phage are being selected for some property other than binding to the antigen of interest. It is possible that plastic-binding phage are being selected, and this has been described previously (Adey, Mataragnon et al. 1995). It is also possible that mutated scFvs with unwanted stop codons in the DNA sequence are being selected because a truncated scFv will relieve the cells of a metabolic burden, conferring a selection advantage on such mutants. Given these considerations, and also considering that further rounds of panning can only enrich specific phage already isolated in previous panning rounds, it seems very unlikely that a fifth round of panning would lead to the isolation of any further scFvs of interest.

Among the scFvs obtained by panning the libraries with recombinant MSP-1₁₉-GST and selected for sequencing, the sequences are all relatively similar to each other. More diversity is found among the light chains than among the heavy chains. There are essentially two different heavy chains among the scFvs, with a third minor variant. The two heavy chains differ from each other both in the second and the third complementarity determining regions (CDRs). There are five different light chain

sequences, and variations are seen in all three light chain CDRs. The greater diversity seen among the light chain sequences is consistent with previous observations of light chain promiscuity and probably reflects the lesser contribution made by the light chain than the heavy chain to the antigen specificity of antibodies and scFvs. Interestingly, Dr Jonathan Chappel panned these libraries with whole merozoites which had undergone secondary processing of MSP-1, and obtained very similar scFv sequences to those shown here. One scFv obtained from merozoite panning, N4E4E9, has an amino acid sequence identical to that of scFv T3D6 except for some differences in the fourth framework region of the light chain. The close similarity between the scFvs obtained by panning with either processed merozoites or recombinant MSP-1₁₉ suggest that natural antibody responses to the merozoite after secondary processing are directed more towards MSP-1₁₉ than any other antigen remaining on the merozoite surface.

The affinity of the scFvs described here for MSP-1₁₉ was not determined. However, Dr Jonathan Chappel used Biacore to analyse the affinity of the scFv N4E4E9 for recombinant MSP-1₁₉-GST immobilised on a carboxymethyl dextran hydrogen sensor chip (Pharmacia Biacore). scFv N4E4E9 was found to have a K_D of 3.5×10^{-9} . As the amino acid sequence of scFv N4E4E9 is very similar to that of the scFvs described here, and almost identical to one, it is likely that the scFvs described in this chapter will also have affinities of the order of 10^{-9} .

In order to determine which scFvs may have the potential to inhibit erythrocyte invasion it is important to determine as clearly as possible where their epitopes are located on the MSP-1₁₉ molecule. As has been discussed, antibodies to MSP-1₁₉ may be classified as inhibitory (those which block invasion), blocking (those which block the neutralising effect of the inhibitory antibodies) or neutral (those which neither inhibit

invasion nor block the invasion-inhibitory antibodies). It is known that malaria-exposed people make all three types of antibody and so the scFvs obtained by panning with MSP-1₁₉ may be derived from parent antibodies from any of these three groups. Those scFvs whose binding sites do not overlap with the invasion-inhibitory mAbs 12.8 and 12.10 are probably unlikely to have invasion-inhibitory potential themselves. Those scFvs whose binding sites do overlap with those of mAbs 12.8 and 12.10 may be derived from parent antibodies that are either invasion-inhibitory or blocking. In selecting scFvs for further study therefore, those that can block the binding of mAb 12.8 or mAb 12.10 to MSP-1₁₉ are likely to be of more interest than those which cannot.

The results of the competition ELISA described in this chapter suggest that the MSP-1₁₉-binding scFvs obtained recognise epitopes on the MSP-1₁₉ molecule which are either identical to, or overlap with, those epitopes recognised by mAbs 12.8 and 12.10. The epitopes of mAbs 12.8 and 12.10 overlap each other, so it is not surprising that scFvs may be found whose epitopes overlap with both. The competition ELISA described here was carried out in two 96 well plates in order to screen the same 160 library clones (80 from the third round and 80 from the fourth round of panning) as were screened for binding to MSP-1₁₉ in the ELISA described in section 6.2.3. In fact almost all the clones which showed binding to MSP-1₁₉ in the ELISA also showed some inhibition of binding of mAbs 12.8 and 12.10 in the competition ELISA. This suggests that all the MSP-1₁₉-binding scFvs tested bind to similar or identical epitopes, and that if engineered back to full antibodies they would either be invasion-inhibitory or blocking but not neutral. There are, however, ways in which this experiment could be repeated in a different way to give clearer results. Because the competition ELISA described here was done to screen 160 clones, the amount of scFv present in each well

was not controlled and will have varied with the level of expression of the different scFvs. Also, the library scFvs were tested for their ability to block whole antibodies rather than scFv forms of mAb 12.8 and mAb 12.10. This is not ideal because the avidity of whole IgG will be greater than that of monovalent scFv. At the time this experiment was done scFv 12.10 was not yet available in a form which could be distinguished from the library scFvs on detection. The experiment could be repeated using the scFv 12.10 fused with two albumin binding domain tags as previously described. The ELISA plate could then be coated with albumin, and then the scFv 12.10-ABD2 could be bound to the albumin and then recombinant MSP-1₁₉ could be bound to scFv 12.10-ABD2. The library scFv could then be tested for their ability to bind to the MSP-1₁₉. The scFv version of mAb 12.8 did not express well enough to be used in such a way. However, Fab fragments of mAb 12.8, which would be distinguishable from the human library scFv because they are murine, have recently been made using papain (Dr Matthew Lock, Division of Parasitology, NIMR; personal communication) and could be used instead of scFv. It is likely that the inhibition of binding of the invasion-inhibitory mAbs by the library scFvs would be greater if monovalent versions of the mAbs were tested in this way.

Another way of determining which epitopes are recognised by these scFv would be to use Biacore surface plasmon resonance to perform epitope mapping. The mAbs 12.8 or 12.10 could be bound to the Biacore chip, and MSP-1₁₉ could be then captured by the bound antibody. If the scFv obtained could still bind to the MSP-1₁₉, this would imply that they recognise an epitope on MSP-1₁₉ other than that recognised by mAb 12.8 or mAb 12.10.

The experiments carried out to attempt to restore putative invasion-inhibitory activity to the library scFvs could be refined in a number of ways. The scFvs under test should be quantified after they have been purified away from any breakdown products to ensure that the concentration of intact scFv is accurately determined. The scFv should be tested at high concentrations. In the case of mAbs 12.10, a concentration of around 500 µg/ml is required to produce at least 50% inhibition of erythrocyte invasion by T9-96 *P. falciparum* parasites. A scFv expressed with a *c-myc* and hexahistidine tag has a mass of approximately 30% that of a whole antibody. It might therefore be anticipated that the concentration required for an invasion-inhibitory scFv to inhibit would only be 30% that of the concentration required for a whole antibody, and that by analogy with mAb 12.10 an scFv might inhibit invasion at around 170 µl/ml. An antibody has two antigen-binding domains however, whereas a scFv has only one, and so twice the above concentration of scFv may be required to be equivalent to the invasion-inhibitory concentrations of mAbs 12.8 and 12.10. This is not known, however, and so concentrations considerably higher than this should be tested. Furthermore, antibodies directed against the scFv tags (mAb 9E10 or anti-PentaHIS antibody) should be used in a purified form. They should also be used at a concentration sufficient to bind all of the scFv present, and again by analogy with mAb 12.10 it would be appropriate to use mAb 9E10 or anti-PentaHIS antibody at a concentration of at least 500 µg/ml. The binding of these antibodies to the scFv under test could be confirmed using an ELISA in which scFv is first bound to MSP-1₁₉ and then mAb 9E10 or anti-PentaHIS are tested for their ability to bind the scFv. Other potential strategies for restoring invasion-inhibitory activity to scFvs are discussed further in Chapter 7.

With a combined size of 1.3×10^9 members, these phage display libraries derived from malaria-exposed donors are large. The results described in this chapter clearly demonstrate that the libraries are also diverse and of a high quality, and that antibody fragments to antigens of interest can be isolated from them. The libraries are therefore a valuable resource from which antibody fragments to a wide variety of malaria antigens may be isolated in other studies in the future.

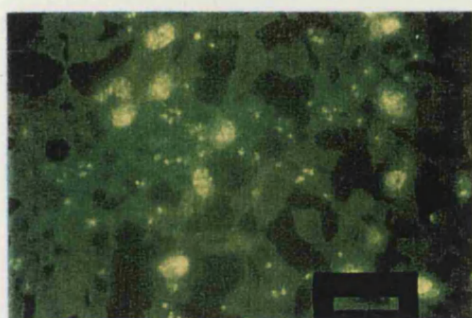
Figure 6.1

Binding of polyclonal scFv from successive rounds of library panning to *P. falciparum* by immunofluorescence

Polyclonal scFv from each successive round of panning against MSP-1₁₉ was examined for the ability to bind acetone-fixed *P. falciparum* parasites by immunofluorescence as described in section 2.7.1. Shown here are the immunofluorescence patterns obtained from polyclonal scFv obtained from the March phage display library before panning, and from the combined March and October phage display libraries after one, two, three and four rounds of panning against recombinant MSP-1₁₉-GST. T9/96 parasites have been used. mAb 12.8 has been used as a positive control.

It can be seen that mAb 12.8 yields a characteristic immunofluorescence pattern revealing *P. falciparum* schizonts. No immunofluorescence is seen with the polyclonal scFv from panning round 1, and very little immunofluorescence is seen from panning round 2. By panning round 3, however, the polyclonal scFv yields an immunofluorescence pattern very similar to that obtained with mAb 12.8, and a similar positive signal is seen, although less brightly, with polyclonal scFv from panning round 4. These results prove that MSP-1₁₉-specific scFvs have been successfully isolated and amplified from the libraries after three rounds of panning. The slight reduction in the immunofluorescent signal in round 4 may reflect the fall in the proportion of MSP-1₁₉-binding scFvs present after the fourth round of panning, which was demonstrated by the examination of 80 randomly selected clones from each round by ELISA, as described in section 6.2.3.

Figure 6.1



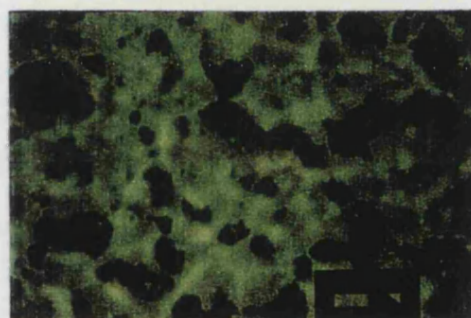
mAb 12.8



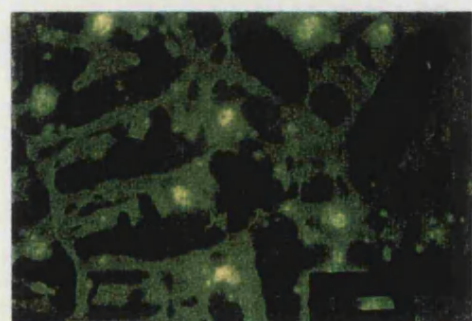
Before panning



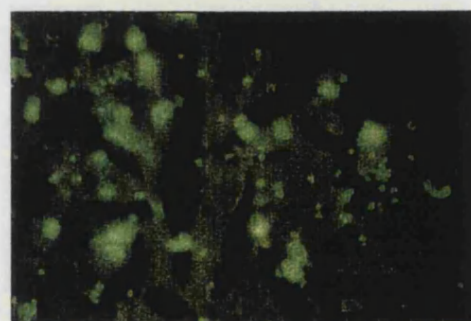
Panning round 1



Panning round 2



Panning round 3



Panning round 4

Figure 6.2

Binding of polyclonal scFv from the fourth round of library panning to *P. falciparum* by Western blot

Binding of polyclonal scFv obtained from the fourth round of panning to *P. falciparum* merozoite proteins was examined by Western blot as described in section 2.7.5. T9/96 or FCB-1 merozoites were prepared as described, resolved by SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was then cut into strips and incubated with various polyclonal scFv, and bound scFv was revealed with anti-c-myc 9E10 antibody as described. Above is the result using T9/96 merozoites, and below using FCB-1 merozoites. Different lanes were loaded with polyclonal scFv obtained from a number of different panning experiments using different antigens. Only the panning with recombinant MSP-1₁₉ is described in this thesis, and scFv obtained with MSP-1₁₉ is used in Lane 9. The lanes were probed as follows:

Lane 1	Polyclonal scFv from March library, unpanned
Lane 2	Polyclonal scFv from October library, unpanned
Lane 3	Polyclonal scFv from 4 th round of panning with unprocessed merozoites (rounds 1, 2 & 4 with FCB-1, round 3 with T9/96, March library only)
Lane 4	Polyclonal scFv from 4 th round of panning with unprocessed merozoites (rounds 1, 2 & 4 with T9/96, round 3 with FCB-1, March library only)
Lane 5	Polyclonal scFv from 4 th round of panning with unprocessed merozoites (rounds 1, 2 & 4 with FCB-1, round 3 with T9/96, October library only)
Lane 6	Polyclonal scFv from 4 th round of panning with unprocessed merozoites (rounds 1, 2 & 4 with T9/96, round 3 with FCB-1, October library only)
Lane 7	Polyclonal scFv from 4 th round of panning with processed merozoites (rounds 1, 2 & 4 with FCB-1, round 3 with T9/96)
Lane 8	Polyclonal scFv from 4 th round of panning with processed merozoites (rounds 1, 2 & 4 with T9/96, round 3 with FCB-1)
Lane 9	Polyclonal scFv from 4 th round of panning with MSP-1 ₁₉ -GST
Lane 10	scFv D1.3 (anti-hen's egg lysozyme)
Lane 11	scFv X509 (anti-MSP-1 ₃₃)
Lane 12	scFv 89.1 (anti-MSP-1 ₈₃)
Lane 13	mAb 12.8 (anti MSP-1 ₄₂ and MSP-1 ₁₉)

Figure 6.2

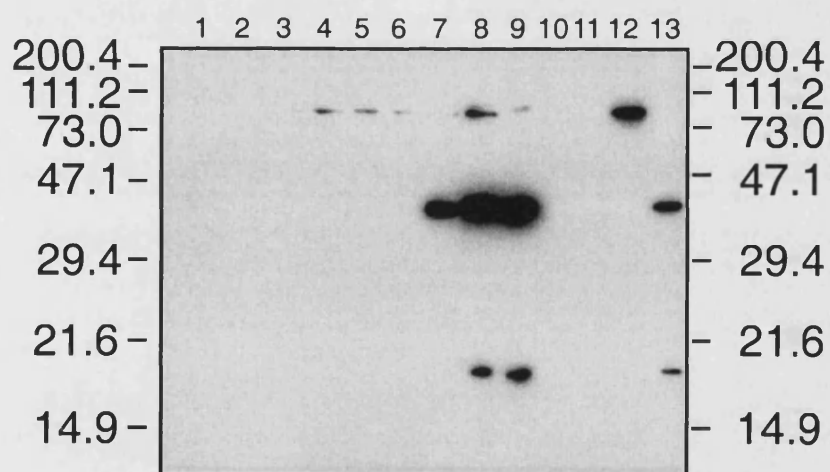
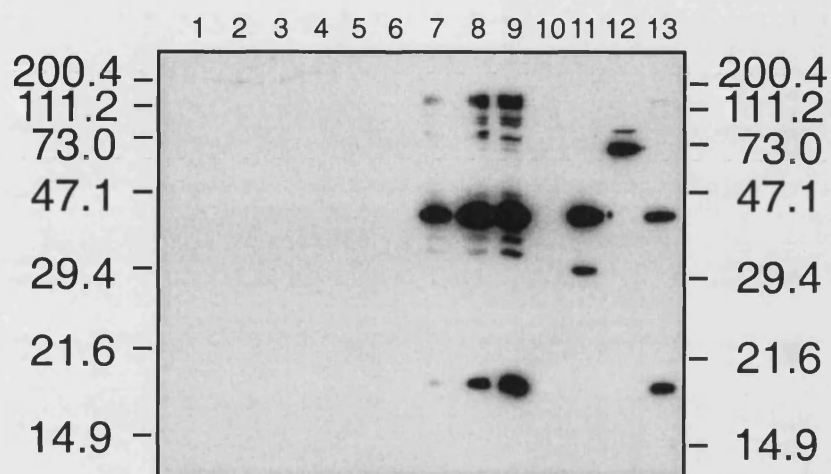


Figure 6.3

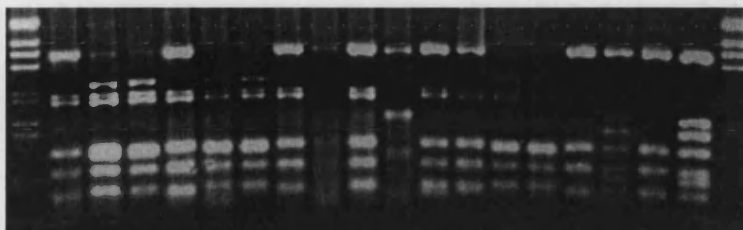
Assessment of DNA sequence diversity of MSP-1₁₉-binding scFvs by *Bst*NI restriction digest

This figure shows the different restriction-digest patterns of 78 randomly selected scFvs, 36 selected from the third round of panning against MSP-1₁₉ and 36 selected from the fourth round of panning against MSP-1₁₉.

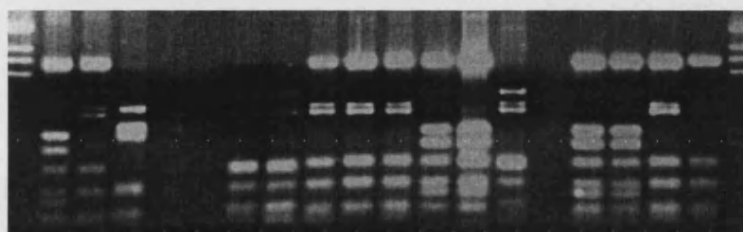
The 78 colonies were randomly picked and PCR screened for the presence of inserts. All 100 of these colonies yielded a PCR product of the expected insert size. Part of each of these products was digested with the frequent-cutting restriction enzyme *Bst*NI as described in section 2.6.5 to make an initial assessment of the diversity of the inserts. The products of this digestion were resolved on a 4% NuSieve agarose gel.

A large number of different digest patterns are seen, suggesting a high degree of DNA sequence diversity among the MSP-1₁₉-specific scFv inserts.

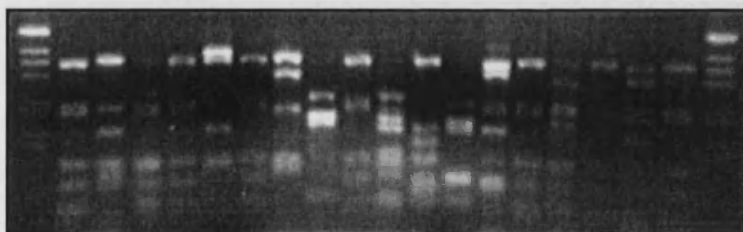
Figure 6.3



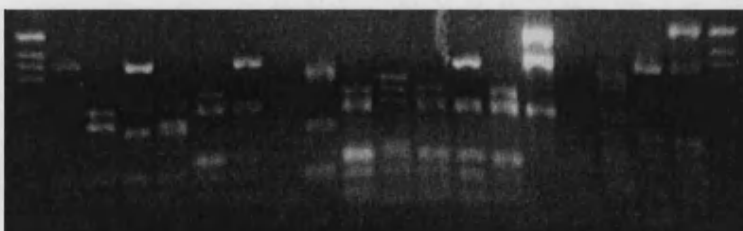
Panning round 3: clones 1-18



Panning round 3: clones 19-36



Panning round 4: clones 41-59



Panning round 4: clones 60-78

Figure 6.4a)

Inhibition of binding of mAbs 12.8 and 12.10 to MSP-1₁₉ by scFvs by competition ELISA: Effect of non-MSP-1₁₉-binding scFvs

A competition ELISA was designed to determine whether the scFvs obtained by panning against MSP-1₁₉ could compete with mAbs 12.8 and 12.10 for binding sites on the MSP-1₁₉ molecule, as described in section 2.7.4. Competition ELISA plates were coated with recombinant MSP-1₁₉-GST at 0.5 µg/ml for competition with mAb 12.8 and at 0.05 µg/ml for competition with mAb 12.10. The scFv were expressed in a 96-well plate as described and the pre-blocked scFv-containing supernatants were transferred to the ELISA plate and allowed to bind. The plates were washed and then mAb 12.8 (at 0.5 µg/ml) or mAb 12.10 (at 0.05 µg/ml) were allowed to bind also. Bound mAb was then revealed as described previously.

This figure shows the results of the negative control experiment. The bar chart shows the effect which scFvs from the fourth round of panning, which had been shown by ELISA *not* to bind to MSP-1₁₉, have on the binding of mAbs 12.8 and 12.10. The binding of mAb 12.8 and mAb 12.10 is shown as a percentage of the binding (as measured by absorbance at 655 nm) of mAb 12.8 and mAb 12.10 in the absence of any scFv. As expected, scFvs from the fourth round of panning which do not bind MSP-1₁₉ do not inhibit the binding of mAbs 12.8 and 12.10 to MSP-1₁₉.

Figure 6.4 a)

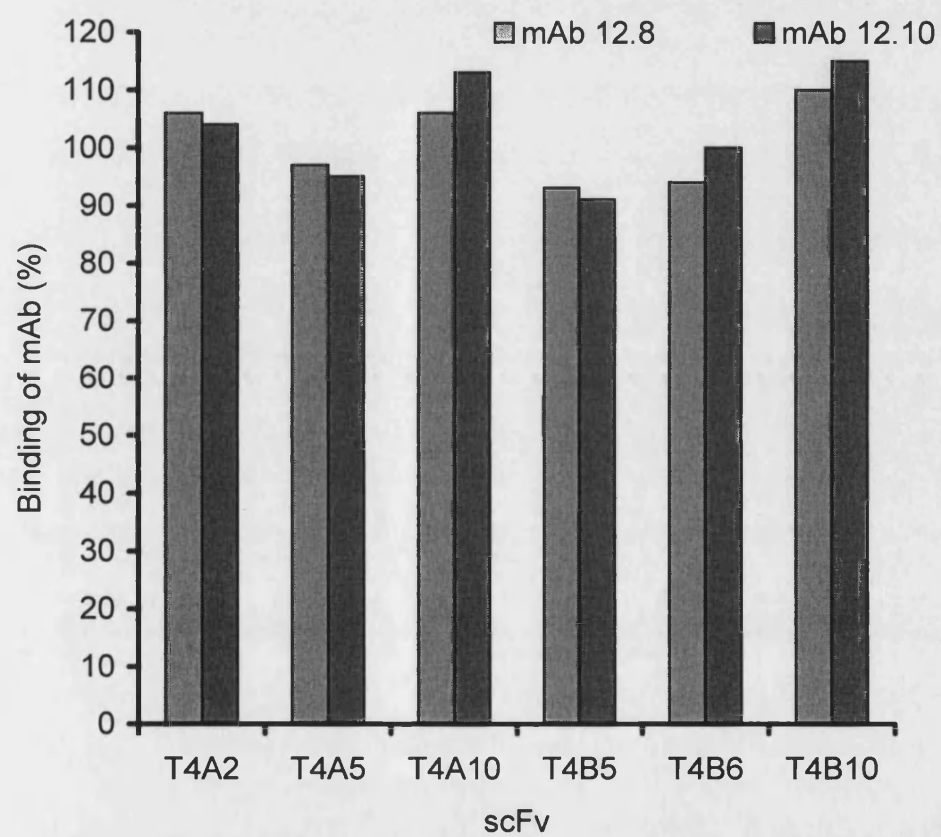


Figure 6.4b)

Inhibition of binding of mAbs 12.8 and 12.10 to MSP-1₁₉ by scFvs by competition ELISA: Effect of MSP-1₁₉-binding scFvs

This figure shows the effect of MSP-1₁₉-binding scFvs from the third and fourth rounds of panning on the binding of mAbs 12.8 and 12.10 to MSP-1₁₉.

In some cases the binding of mAb 12.8 (as measured by absorbance at 655 nm) is reduced by over 60% and the binding of mAb 12.10 is reduced by over 30%. This result suggests that at least some of the anti-MSP-1₁₉ scFvs compete with mAbs 12.8 and 12.10 for a similar, or identical, epitope.

Figure 6.4 b)

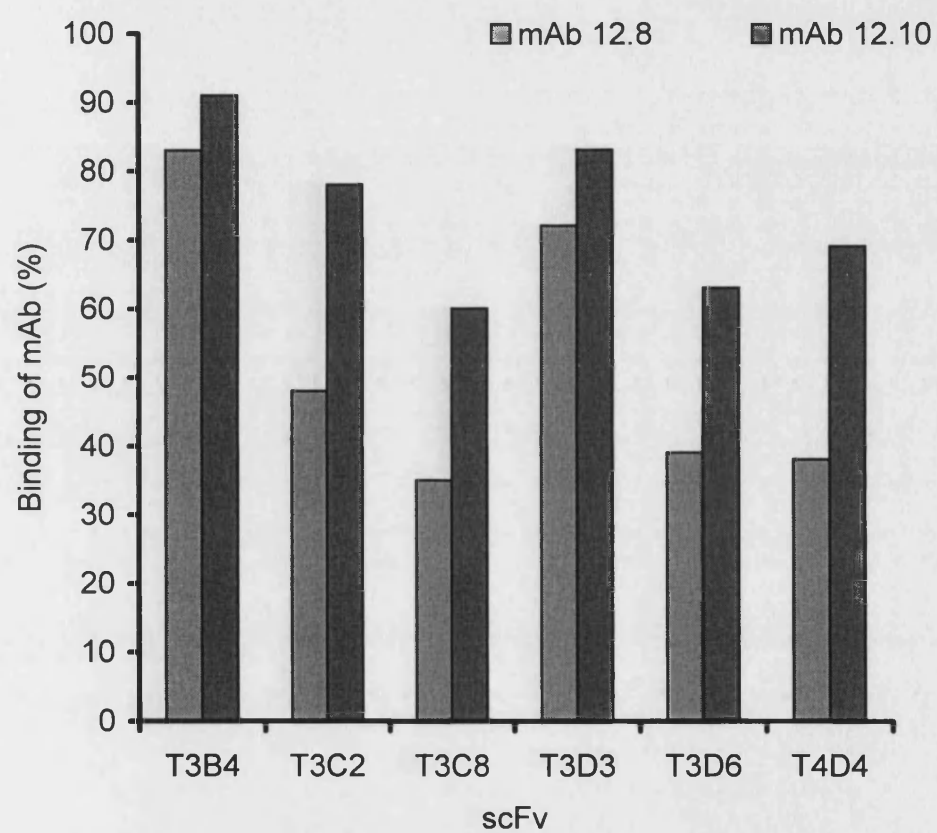


Figure 6.5

Amino acid sequences of MSP-1₁₉-binding scFvs

This figure shows an alignment of six selected scFvs obtained by panning the phage display libraries with recombinant MSP-1₁₉, and one scFv (N4E4E9) obtained (by Dr Jonathan Chappel) by panning the libraries with *P. falciparum* merozoites in which secondary processing had been allowed to proceed. Residues which differ from the consensus sequence are highlighted in pink. Complementarity-determining regions (CDRs) are outlined in green.

There is more diversity among the light chain variable regions of these clones than among the heavy chain variable regions. Two significantly different heavy chain sequences are seen. The commoner heavy chain is present in scFvs T3B4, T3C2, T3C8, T3D6 and T4D4, although the CDR 1 of scFv T4D4 differs from the others in this group by one residue. The other heavy chain sequence is found in scFv T3D3, and differs from the commoner heavy chain in both CDR 2 and CDR 3.

Six different light chain sequences are seen among these 6 scFvs. Variation is seen in all three CDRs. Full sequence information was not obtained on two of the scFv, T3B4 and T3C8, which were not sequenced through the whole of the third CDR and the fourth framework region.

It is interesting that a scFv obtained by panning with processed merozoites (N4E4E9) is all but identical to a scFv (T3D6) obtained by panning with recombinant MSP-1₁₉.

Figure 6.5

Heavy chain sequences

		H1	H2	H3
T3D3	QVQLVQSG ¹ EVKKPGASVKVSCK ² SGY ³ FT ⁴ SYDIN ⁵ WVRQAPGQGLEW ⁶ LG ⁷ [RISPYN ⁸ GT ⁹ LY ¹⁰ QT ¹¹ LOG ¹²]RVTMT ¹³ TDTS ¹⁴ NT ¹⁵ TY ¹⁶ ELRSL ¹⁷ SDDTAVYYCAR ¹⁸ EGVWF ¹⁹ EL ²⁰ LS ²¹ WGQGT ²² LVT ²³ VSS ²⁴			
T4D4	QVQLVQSGAEVKKPGASVKVSCKASGYTFT ⁴ SYDIN ⁵ WVRQAPGQGLEWLG ⁷ [RISPYNSNTNYAQKFHD ¹²]RVTMT ¹³ TD ¹⁴ TSTSTAYMELRSLRSDDTAVYYCAR ¹⁸ EGVWFGELSY ¹⁹ WGQGT ²² LVT ²³ VSS ²⁴			
T3C2	EVQLVQSGAEVKKPGASVKVSCKASGYTFT ⁴ SYDIN ⁵ WVRQAPGQGLEWLG ⁷ [RISPYNSNTNYAQKFHD ¹²]RVTMT ¹³ TD ¹⁴ TSTSTAYMELRSLRSDDTAVYYCAR ¹⁸ EGVWFGELSY ¹⁹ WGQGT ²² LVT ²³ VSS ²⁴			
T3B4	EVQLVQSG ¹ EVKKPGASVKVSCKASG ² TFT ⁴ SYDIN ⁵ SVRQAPGQGLEWLG ⁷ [RISPYNSNTNYAQKFHD ¹²]RVTMT ¹³ TD ¹⁴ TST ¹⁵ TAYMELRSLRSDDTAVYYCAR ¹⁸ EGVWFGELSY ¹⁹ WGQGT ²² LVT ²³ VSS ²⁴			
T3D6	EVQLVQSGAEVKKPGASVKVSCKASGYTFT ⁴ SYDIN ⁵ WVRQAPGQGLEWLG ⁷ [RISPYNSNTNYAQKFHD ¹²]RVTMT ¹³ TD ¹⁴ TSTSTAYMELRSLRSDDTAVYYCAR ¹⁸ EGVWFGELSY ¹⁹ WGQGT ²² LVT ²³ VSS ²⁴			
T3C8	EVQLVQSGAEVKKPGASVKVSCKASGYTFT ⁴ SYDIN ⁵ WVRQAPGQGLEWLG ⁷ [RISPYNSNTNYAQKFHD ¹²]RVTMT ¹³ TD ¹⁴ TSTSTAYMELRSLRSDDTAVYYCAR ¹⁸ EGVWFGELSY ¹⁹ WGQGT ²² LVT ²³ VSS ²⁴			
N4E4E9	EVQLVQSGAEVKKPGASVKVSCKASGYTFT ⁴ SYDIN ⁵ WVRQAPGQGLEWLG ⁷ [RISPYNSNTNYAQKFHD ¹²]RVTMT ¹³ TD ¹⁴ TSTSTAYMELRSLRSDDTAVYYCAR ¹⁸ EGVWFGELSY ¹⁹ WGQGT ²² LVT ²³ VSS ²⁴			

Light chain sequences

		L1	L2	L3
T3D3	EV ¹ VTQSPATLSLSPGERATLSC ² [RASQSVSS ³ -YLA ⁴]WYQQKPGQAPRLLIY ⁵ [DAS ⁶ NRAT ⁷]GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC ⁸ QQYGS ⁹ SS ¹⁰ -IT ¹¹ FGQGTKVEIK ¹²			
T4D4	EV ¹ VTQSP ² TL ³ SLSPGERATLSC ⁴ [RASQSVSS ⁵ SYLA ⁶]WYQQKPGQAPRLLIY ⁷ [GASSRAT ⁸]GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC ⁹ QQYGS ¹⁰ SP ¹¹ -TI ¹² FGQGTKVEIK ¹³			
T3C2	DV ¹ VTQSP ² TL ³ SLSPGERATLSC ⁴ [RASQSVSS ⁵ -ALA ⁶]WYQQKPGQAPRLLIY ⁷ [KASFL ⁸ EN ⁹]GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC ¹⁰ QQYNT ¹¹ YP ¹² YS ¹³ FGQGTKVEIK ¹⁴			
T3B4	EV ¹ TT ² ITQSP ³ TL ⁴ SLSPGERATLSC ⁵ [RASQS ⁶ SS ⁷ SNLA ⁸]WYQQKPGQAPRLLIY ⁹ [DAS ¹⁰ NRAT ¹¹]GIPDRFS ¹² --TSGTDFTLTISRLEPEDFAVY ¹³			
T3D6	DV ¹ VTQSPATLS ² SLSPGERATLSC ³ [RASQSVSSK ⁴ -LA ⁵]WYQQKPGQAPRLLIY ⁶ [AASSRAT ⁷]GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC ⁸ QQYDT ⁹ SP ¹⁰ YN ¹¹ FG ¹² GGQGGIK ¹³			
T3C8	DV ¹ VTQSPATLS ² SLSPGERATLSC ³ [RASQSVSSK ⁴ -LA ⁵]WYQQKPGQAPRLLIY ⁶ [AASSRAT ⁷]GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC ⁸ QQYDT ⁹ SP ¹⁰ YN ¹¹ FG ¹² GGQGGIK ¹³			
N4E4E9	DV ¹ VTQSPATLS ² SLSPGERATLSC ³ [RASQSVSSK ⁴ -LA ⁵]WYQQKPGQAPRLLIY ⁶ [AASSRAT ⁷]GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC ⁸ QQYDT ⁹ SP ¹⁰ YN ¹¹ FG ¹² GGQGGIK ¹³			

Table 6.1**Expression levels of selected MSP-1₁₉-binding scFv clones**

scFv	Amount of protein from 1ml of culture
T3A2	2.2 µg/ml
T3A10	3.4 µg/ml
T3B4	3.2 µg/ml
T3C2	3.4 µg/ml
T3C8	4.0 µg/ml
T3D3	1.3 µg/ml
T3D6	4.4 µg/ml
T4A4	3.8 µg/ml
T4B3	0.4 µg/ml
T4B8	1.3 µg/ml
T4D4	3.8 µg/ml

Table 6.2**Binding of selected MSP-1₁₉-binding scFv clones to *P. falciparum* parasites by**

Immunofluorescence patterns were graded as negative, poorly positive, positive or very positive depending on the strength of the signal compared with that of mAb 12.10.

scFv	Result with FCB-1	Result with T9/96
T3A2	negative	negative
T3A10	very positive	ND
T3B4	negative	poorly positive
T3B6	ND	positive
T3C2	very positive	very positive
T3C8	very positive	very positive
T3D3	negative	poorly positive
T3D6	positive	positive
T4A4	very positive	very positive
T4B3	positive	very positive
T4B8	positive	positive
T4D4	positive	positive
D1.3	negative	negative
mAb 12.10	very positive	very positive

CHAPTER 7

GENERAL DISCUSSION

The work described in the first part of this thesis shows that it is possible to engineer murine hybridoma monoclonal antibodies which can inhibit erythrocyte invasion by *Plasmodium falciparum* into recombinant partially humanised antibodies which retain invasion-inhibitory activity. It has also been shown that one of these mAbs can be engineered into a single chain variable fragment (scFv) which expresses well and retains the ability to bind to the target antigen with good affinity.

The demonstration of antigen binding and inhibition of invasion by the recombinant mouse/human chimeric versions of the hybridoma mAbs 12.8 and 12.10 provides confirmation that the sequences of the variable regions of the parent hybridoma mAbs have been correctly identified. The chimeric mAbs will also be suitable reagents with which to determine whether invasion-inhibitory mAbs can modify or prevent clinical malaria in a primate model of malaria. These chimeric mAbs have a number of advantages over mAbs purified from hybridoma cell cultures. Firstly they should be less immunogenic in primates because they have been engineered with human antibody constant regions. Human antibody constant region sequences will be much closer to primate sequences than murine sequences would be, and these antibodies should evoke a correspondingly more muted immune response in primates than would be evoked by murine mAbs. Secondly, being recombinant constructs of known sequence, these mAbs provide a completely standardised form of the antibodies which can be produced to the rigorously high standard necessary for testing in primates. A previous study designed to test these invasion-inhibitory mAbs in *Aotus* monkeys had

to be abandoned because the purified hybridoma mAbs were contaminated with lipopolysaccharide (LPS) at quantities which precluded their safe use in primates. It has also been observed that different batches of mAb 12.10 prepared from different cultures of 12.10 hybridoma cells may vary in the degree to which they inhibit erythrocyte invasion. It is hoped that controlled expression of recombinant forms of these mAbs under rigorous conditions may prevent some of these variations in quality.

Because the concentrations of antibody required to inhibit invasion *in vitro* are quite high, at around 400 µg/ml, the quantity of antibody which is anticipated to be required in order to test these antibodies in primates is correspondingly large. The expression levels of both chimeric mAbs 12.8 and 12.10 are good, and chimeric mAb 12.10 is currently being produced in large quantities at AERES Biomedical (formerly the Antibody Engineering Group of the MRC Collaborative Centre at Mill Hill, London). This is being done with the intention that chimeric mAb 12.10 should be tested for the ability to modify or prevent *P. falciparum* infection in *Aotus* monkeys, in collaboration with the Walter Reed Army Institute for Research, Washington D.C.

If the chimeric antibodies do modify or control *P. falciparum* infection in a primate model, the question of their potential use as therapeutic agents in humans would naturally arise. The number of therapeutic antibodies in standard use in resource-rich countries is steadily increasing, and it may be anticipated that their cost will fall as this expansion continues. At present, cost is a central issue in the usefulness of any new malaria therapy, and the main burden of disease falls upon countries least able to afford expensive treatments. Furthermore, currently available drugs, used judiciously, can still normally control individual infections. This situation may change however, particularly if multidrug resistance in malaria worsens and spreads to cause major public

health problems in countries (for instance in South East Asia) with greater economic resources. In such a situation, it is conceivable that therapeutic antibodies for malaria may have a future.

The single chain variable fragment (scFv) engineered from the sequences obtained for the variable regions of the heavy and light chains of mAb 12.10 binds to MSP-1₁₉ with good affinity. Like Fab 12.10, the scFv version of 12.10 does not appear to retain invasion-inhibitory activity. The scFv version of mAb 12.10 will therefore be useful as a model with which to test different strategies for restoring putative invasion-inhibitory activity to human scFvs derived from phage display libraries. A number of possible strategies for achieving this are discussed below.

The work described in the second part of this thesis shows that it is possible to isolate high affinity human monoclonal antibody fragments to MSP-1₁₉ from a phage display library derived from malaria-exposed donors. A number of different single chain variable fragments (scFvs) were isolated by panning two medium-sized libraries (with a combined size of approximately 1×10^9 members) with recombinant MSP-1₁₉-GST. These scFvs were shown to bind to recombinant MSP-1₁₉-GST by ELISA and were shown to bind to native *Plasmodium falciparum* parasites by immunofluorescence and by Western blot. These scFvs also partially blocked the binding of the invasion-inhibitory murine mAbs 12.8 and 12.10, suggesting that the scFvs recognise epitopes which overlap with those epitopes recognised by the invasion-inhibitory mAbs. Selected scFvs were tested in an *in vitro* assay of erythrocyte invasion. None of the scFvs showed any inhibition of invasion, but further experiments are needed to examine possible ways in which putative invasion-inhibitory activity might be restored to antibody fragments presented in the scFv format.

It is likely that any scFv with the potential to inhibit erythrocyte invasion in a manner analogous to that of the murine hybridoma mAbs 12.8 and 12.10 would need to be engineered back into a format approaching that of a whole antibody before any invasion-inhibitory activity would be displayed. This seems likely because Fab and F(ab')₂ fragments of mAb 12.10 do not retain the invasion-inhibitory property of the parent antibody (Guevara Patino 1997). The most unequivocal way of engineering scFv to be more like the parent antibody is to actually use the sequence of the scFv to construct a whole recombinant antibody. Biological activity has been restored to scFvs in this way in a number of studies (Miescher, Zahn-Zabal et al. 2000), and techniques have been developed to facilitate the transformation of scFvs into whole antibodies (Ames, Tornetta et al. 1995) (Mahler, Marquis et al. 1997). In one study, biological activity has also been restored to an scFv against the glycoprotein antigen of the rabies virus by expressing the scFv as a fusion with the human IgG1 Fc region (Ray, Embleton et al. 2001). The problem with these approaches is that they are labour-intensive and time-consuming, and are therefore unsuitable for screening large numbers of scFvs efficiently. An ideal technique for rapid screening would readily increase the size of the scFv (approximately 30 kDa) to that of a whole antibody (approximately 150 kDa) without requiring each scFv to be engineered individually. For some biological properties of antibodies the Fc portion of the antibody has to be restored to the scFv to recruit other essential immune effector mechanisms (Ray, Embleton et al. 2001). In the case of protective antibodies against MSP-1₁₉ however, as discussed in section 1.2.4, there is evidence that these other effector functions, and the Fc portion of the antibody, are not required for protection (Rotman, Daly et al. 1998) (Vukovic, Hogarth et al. 2000).

A number of different strategies might be used to bulk up the scFvs to a size approaching that of a whole antibody. Three such strategies have already been discussed. Two involve the binding of a second antibody to a tag at the C-terminus of the scFv. Purified mAb 9E10 might be bound to the *c-myc* tag or anti-PentaHIS antibody might be bound to the hexahistidine tag. Anti-PentaHIS antibody is a very expensive reagent, whereas mAb 9E10 can be produced in quantity quite easily from 9E10 hybridoma cells. Furthermore 9E10 has already been used successfully to restore biological activity to a scFv with a *c-myc* tag. A scFv against influenza haemagglutinin regained the ability to inhibit haemagglutination (and by implication to neutralise influenza virus) when used in conjunction with mAb 9E10 (Keith Ansell, MRC Collaborative Centre, Mill Hill, London, unpublished data). Another approach already discussed in the context of scFv 12.10 is to express the scFvs as a fusion with two albumin-binding domains. In the presence of albumin, two albumin molecules (molecular weight 68 kDa) would bind to each scFv, increasing the mass of the scFv by around 136 kDa.

An alternative and possibly more robust strategy for increasing the mass of the scFv would be to express the scFv as a fusion with another protein which would itself increase the mass by the required amount. The additional mass would then be covalently bound, which might prove more reliable under a wider range of conditions than relying on the non-covalent binding of a second antibody or of albumin.

It is possible that other antibodies are generated by natural infection which inhibit erythrocyte invasion by a different mechanism to that of the known invasion-inhibitory mAbs 12.8 and 12.10. If this is the case, scFv fragments of such antibodies might retain invasion-inhibitory activity without the requirement for an

increase in size to that of the parent antibody. There is some evidence that such invasion-inhibitory antibodies may exist. A study of blood samples from malaria-exposed children aged up to 15 years from Nigeria has demonstrated the presence of antibodies which inhibit secondary processing of MSP-1 but which do not compete with mAbs 12.8 and 12.10 for binding to MSP-1₁₉ (Roseangela Nwuba, Division of Parasitology, NIMR, unpublished data). In another study, antibodies raised against a recombinant mutated form of MSP-1₁₉ incorporating three amino acid substitutions showed substantially more inhibition of erythrocyte invasion than that produced by mAbs 12.8 and 12.10. The same antibodies showed no more inhibition of secondary processing than that shown by mAbs 12.8 and 12.10, suggesting that some other mechanism is involved in their inhibition of erythrocyte invasion (Chairat Uthaipibull, Division of Parasitology, NIMR, unpublished data).

The scFvs isolated from these phage display libraries by panning with recombinant MSP-1₁₉ do not appear to be particularly diverse from the examples which were sequenced. Essentially only two different heavy chains were detected and they were quite similar to each other. There was more diversity among the light chains but they probably contribute less to antibody specificity than the heavy chains. Interestingly, those scFvs obtained by Dr Jonathan Chappel by panning the libraries with whole merozoites (in which secondary processing of MSP-1 had been allowed to proceed) showed very similar, or in some case near identical, sequences to those obtained by panning with recombinant MSP-1₁₉ and described in this work. Those scFvs derived from merozoite panning gave a near identical pattern on binding to merozoite proteins on a Western blot as did the scFvs derived from MSP-1₁₉ panning. This suggests that the antigen which most readily generates an antibody response on the surface of processed merozoites is MSP-1₁₉. Furthermore, virtually all the scFvs which

bound to MSP-1₁₉ in the ELISA also blocked the binding of mAbs 12.8 and 12.10. It seems possible therefore that all the MSP-1₁₉-binding scFvs isolated in the third and fourth rounds of panning in fact bind to the same or a very similar epitope. If that epitope does indeed overlap with the epitope recognised by mAbs 12.8 and 12.10, as suggested by the competition ELISA, then the scFvs, if restored to a whole antibody format, would presumably be either invasion-inhibitory or blocking, but not neutral. The scFv N4E4E9 was obtained by Dr Jonathan Chappel of the MRC Collaborative Centre by panning these libraries with whole processed *P. falciparum* merozoites. The sequence of N4E4E9 is very similar to the sequences of the scFvs described in this work, and almost identical to one of them, scFv T3D6. Dr Jonathan Chappel engineered scFv N4E4E9 back into a whole recombinant antibody and showed that as a whole antibody, N4E4E9 does not appear to have much inhibitory effect in the *in vitro* assay of secondary processing of MSP-1 (unpublished data). It seems quite possible therefore that the panning of these libraries with either recombinant MSP-1₁₉ or whole merozoites has resulted, at the third and fourth rounds of panning, in the isolation of a panel of closely related blocking antibodies rather than invasion-inhibitory antibodies. If this does prove to be the case, it will be necessary to consider other panning strategies which may be more likely to isolate scFv with invasion-inhibitory potential from these libraries.

There are a number of ways in which the strategy described in this work might be modified in order to isolate a wider variety of MSP-1₁₉-binding scFvs from these libraries. It is unlikely that a fifth round of panning against recombinant MSP-1₁₉ would yield any further scFvs of interest beyond those already obtained by four rounds of panning, for the reasons discussed in section 5.3. One approach which might lead to the identification of a more diverse population of MSP-1₁₉-specific scFvs could be to

examine the scFvs isolated from the second round of panning against MSP-1₁₉. So far, only scFvs from the third and fourth rounds of panning against MSP-1₁₉ have been examined. It is possible that MSP-1₁₉ binding scFvs will have been sufficiently enriched after two panning rounds that some may be detected if a sufficient number of randomly selected clones are examined by ELISA. The scFvs from the second round of panning were not examined individually in this study. Polyclonal scFv from the second round was examined for binding to *P. falciparum* by IFA (see Figure 6.1) and did not give a strong signal. There did appear to be some slight immunofluorescence signal, however, in contrast to the complete absence of signal from the polyclonal scFv retrieved after one round of panning. It may be therefore that some scFvs with invasion-inhibitory potential are present in the phage obtained from the second round of panning, but that these scFvs are out-competed by other scFvs in subsequent rounds of panning. This might occur if they have a lower affinity for MSP-1₁₉ than the scFvs which were isolated in this study, or if they are at some other selective disadvantage.

The disadvantage of selecting scFvs from the second round of panning is that they may have a relatively low affinity for MSP-1₁₉, since higher affinity scFvs tend to be selectively enriched only on repeated rounds of panning. The scFvs of most interest are those with affinities comparable to the affinities of antibodies generated by a secondary immune response. There are however a number of techniques for improving the affinities of antibody fragments isolated by phage display. The affinity of isolated heavy chain variable regions (V_H) has been improved by making random amino acid substitutions in the first and second complementarity determining regions (CDRs) (Davies and Riechmann 1996) and also by making substitutions in the second and third CDRs (Thompson, Pope et al. 1996). CDRs can be targeted for random mutagenesis followed by further affinity-based selection by phage display in a process termed CDR

walking (Barbas, Hu et al. 1994) and this process has been used to improve the affinity of a prototype anti-HIV-1 antibody 420-fold (Yang, Green et al. 1995). Affinity of scFvs has also been improved by introducing variations in the sequence of the linker that joins the heavy and light chain variable domains of the molecule (Turner, Ritter et al. 1997). Thus if MSP-1₁₉-specific scFvs were isolated from the second round of panning but were found to be of low affinities, there are a number of techniques by which their affinities might be improved.

Another recognised strategy for re-panning a library to get a different population of scFvs against a given antigen than those initially isolated is that of epitope masking. In this procedure, a highly immunogenic epitope on an antigen is blocked, or masked, with a scFv which has already been isolated against that epitope. This prevents the previously dominant epitope being exposed when panning is repeated, permitting the isolation of a broader range of scFvs, directed against other epitopes, which were not previously selected for. This technique has been used successfully to access neutralising human antibodies to a weakly immunogenic epitope of the HIV-1 gp120 antigen (Ditzel, Binley et al. 1995). The libraries described in this thesis could be panned again with MSP-1₁₉, having masked the previously dominant epitope with one of the scFvs already obtained, to enable the isolation of other scFvs with invasion-inhibitory potential analogous to that of mAbs 12.8 and 12.10. As discussed above, it is also possible that other epitopes exist on the MSP-1₁₉ molecule, capable of generating invasion-inhibitory antibodies which do not recognise the same epitopes as mAbs 12.8 or 12.10. To search for scFvs against such epitopes, the libraries could be panned using an epitope-masking strategy in which MSP-1₁₉ is blocked with Fab 12.8 or Fab 12.10.

It may be that the scFvs isolated so far would act as blocking antibodies rather than invasion-inhibitory antibodies if they were restored to a whole antibody format. To avoid the isolation of scFvs with the potential only for blocking activity, the libraries could be panned with mutant forms of MSP-1₁₉. A number of such mutants have been constructed, some of which are recognised by the invasion-inhibitory mAbs 12.8 and 12.10 but are not recognised by any of a panel of known blocking antibodies (Uthaipibull, Aufiero et al. 2001). The libraries could be panned again with a mutant form of MSP-1₁₉ that does not bind any of the known blocking antibodies. This may permit the isolation of different scFvs, recognising similar epitopes to those recognised by the scFvs obtained in this study, but with potential invasion-inhibitory properties rather than blocking properties.

In summary, therefore, there are a number of methods by which putative invasion-inhibitory activity might be restored to antibody fragments isolated in the scFv format. If such a technique can be developed and validated using the scFv form of mAb 12.10, it can then be applied to the scFvs described in this study. If the scFvs described in this study prove not to have invasion-inhibitory properties, there are a number of ways in which the libraries might be panned again in order to search for scFvs which do have the potential to inhibit invasion. Given the wealth of evidence that anti-MSP-1₁₉ antibodies do have an important role in naturally acquired human immunity to *P. falciparum*, it seems likely that such antibody fragments are represented in these libraries, and that they can be isolated and identified provided the search is conducted in the right way.

The work described in this thesis has shown that MSP-1₁₉-specific human monoclonal antibody fragments can be isolated from phage display libraries made from

malaria-exposed donors. It has also shown that murine MSP-1₁₉-specific mAbs which inhibit the invasion of erythrocytes by *P. falciparum* can be engineered into a partially humanised form and still retain their invasion-inhibitory activity. This work provides a basis for the further dissection of the human antibody response to MSP-1₁₉ at the molecular level. It also provides a basis from which the role of invasion-inhibitory antibodies in protection may be investigated *in vivo* and their potential as therapeutic agents may be further investigated.

CHAPTER 8

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