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DEPARTMENT OF MEDICAL PARASITOLOGY
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# MOLECULAR AND IMMUNOLOGICAL CHARACTERISATION OF TWO VACCINE DOMINANT ANTIGENS OF *SCHISTOSOMA MANSONI*

by
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## ABSTRACT

Exposure to radiation attenuated cercariae of *Schistosoma mansoni* induces immunologically mediated protection against a challenge infection. One approach towards the selection of putative vaccine candidate molecules has therefore been the identification of 'vaccine dominant' antigens. Such molecules can be defined as those which are recognised by sera from animals vaccinated with irradiated parasites but not by sera raised in non-immune animals harbouring a single sex infection. This thesis describes the immunological and molecular characterisation of a 16 kDa vaccine dominant schistosomula surface antigen of *S.mansoni* and a 15 kDa antigen found on all stages of the parasite which also meets this criteria.

The 16 kDa antigen extracted from mechanically transformed schistosomula and subsequently purified by immunoaffinity chromatography was shown to be a glycoprotein which incorporates both protein and carbohydrate epitopes. The latter, which include the target of a passively protective monoclonal antibody (Bickle *et al.*, 1986), bind the lectins peanut and ricin agglutinin and are believed to incorporate the monosaccharide N-acetylgalactosamine O-linked to the peptide core. Attempts to determine the amino acid sequence of the antigen by gas phase $NH_2$-terminal amino acid sequencing have also suggested that the protein moiety of the 16 kDa antigen is N-terminally blocked.

The immunoaffinity purified 16 kDa antigen was subsequently used to immunise groups of mice in conjunction with a number of different adjuvants. Significant, albeit low, levels of resistance were achieved following immunisation with the 16 kDa molecule plus the novasome adjuvant formulation.

Clones encoding the 15 kDa antigen were identified during the screening of a cDNA sporocyst library with sera specific for low molecular weight antigens. Sequence obtained for the antigen showed that it had some homology to members of a calcium binding protein superfamily, although the 15 kDa antigen itself was unable to bind calcium. The cDNA encoding this antigen was subsequently subcloned into a vector suitable for expression and the resulting fusion protein was used to immunise mice. Sera raised in these mice recognised the native 15 kDa antigen by Western blotting. However, the mice were not protected against a challenge infection.

**This thesis is dedicated to my Dad**

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## ABBREVIATIONS USED

| | |
|---|---|
| ADCC | antibody dependent cytotoxicity |
| BAL | broncho-alveolar lavage |
| bp | base pairs |
| CaBP | calcium binding protein |
| cDNA | complementary DNA |
| CIS | mouse chronic infection sera |
| ConA | *Canavalia ensiformis* (jackbean) agglutinin |
| DBA | *Dolichos biflorus* (horse gram) agglutinin |
| DTH | delayed type hypersensitivity |
| DTT | dithiothreitol |
| ECA | *Erythrina cristagalli* (coral tree) agglutinin |
| EDTA | ethylenediaminetetra-acetic acid |
| ER | endoplasmic reticulum |
| FCA | Freunds complete adjuvant |
| FIA | Freunds incomplete adjuvant |
| fuc | fucose |
| gal | galactose |
| galNAc | N-acetylgalactosamine |
| glc | glucose |
| glcNAc | N-acetylglucosamine |
| GS I | *Griffonia simplicifolia* I agglutinin |
| GS II | *Griffonia simplicifolia* II agglutinin |
| HRP | horseradish peroxidase |
| IHS | infected human sera |
| INFγ | interferon γ |
| IPTG | isopropyl-β-D thiogalactopyranoside |
| IRatS | infected rat sera |
| ISCOMs | immunostiumulating complexes |
| kb | kilobase |
| kDa | kilodalton |
| LcH | *Lens culinaris* (lentil) agglutinin |

| | |
|---|---|
| Lotus | *Lotus tetragonolobus* agglutinin |
| MAA | *Maackia amurensis* agglutinin |
| man | mannose |
| McAb | monoclonal antibody |
| MEGA 10 | decanoyl-N-methyl glucamide |
| MS | mechanically transformed schistosomula |
| NRatS | normal rat sera |
| Neu5Ac | sialic acid |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PEG | polyethylene glycol |
| pfu | plaque forming units |
| PIPLC | phosphatidylinositol-specific phospholipase C |
| PMN | polymorphonuclear leukocytes |
| PNA | *Arachis hypogaea* (peanut) agglutinin |
| PSB | phage storage buffer |
| RCA | *Ricinus communis* (ricin) agglutinin |
| SBA | *Glycine max* (soybean) agglutinin |
| SD | standard deviation |
| SDS | sodium dodecyl sulphate |
| SDW | sterile distilled water |
| SLN | skin draining lymph nodes |
| SNA | *Sambucus nigra* (elderberry) agglutinin |
| SSS | mouse single sex infection sera |
| TBS | Tris buffered saline |
| TNF$\alpha$ | tumour necrosis factor $\alpha$ |
| UEA I | *Ulex europaeus* I agglutinin |
| VMS | vaccinated mouse serum |
| VRabS | vaccinated rabbit serum |
| VRatS | vaccinated rat serum |
| WGA | *Triticum vulgare* (wheatgerm) agglutinin |

# TABLE OF CONTENTS


| | | Page |
|---|---|---|
| List of figures | | 8 |
| List of tables | | 11 |
| Chapter 1 | Introduction | 12 |
| Chapter 2 | Materials and Methods | 67 |
| Chapter 3 | Extraction and purification of the 16 kDa antigen | 108 |
| Chapter 4 | Characterisation of the 16 kDa antigen | 131 |
| Chapter 5 | Vaccination of mice with the immunoaffinity purified 16 kDa antigen | 154 |
| Chapter 6 | Screening of a sporocyst cDNA expression library with antibody probes | 189 |
| Chapter 7 | Characterisation of clones encoding a 15 kDa vaccine dominant antigen | 217 |
| Chapter 8 | General discussion | 246 |
| References | | 256 |
| Appendix | | 305 |

## LIST OF FIGURES


| | | Page |
|---|---|---|
| **Chapter 3** | | |
| Figure 3.1 | Extraction of the 16 kDa antigen with the detergent TX-114 | 111 |
| Figure 3.2 | Sequential extraction of MS with the detergents TX-114 and OTG | 113 |
| Figure 3.3 | Extraction of MS with OTG alone | 115 |
| Figure 3.4 | Immunoaffinity purification of the 16 kDa antigen | 117 |
| Figure 3.5 | Purification of the 16 kDa antigen using ion exchange chromatography | 120 |
| **Chapter 4** | | |
| Figure 4.1 | Two dimensional electrophoresis | 134 |
| Figure 4.2 | The effect of protease treatment upon the 16 kDa antigen | 136 |
| Figure 4.3 | The effect of periodate treatment upon the 16 kDa antigen | 140 |
| Figure 4.4 | Binding of lectins to the 16 kDa antigen | 143 |
| Figure 4.5 | Treatment of the 16 kDa antigen with the enzyme O-glycanase | 146 |
| **Chapter 5** | | |
| Figure 5.1 | The subclass of the anti-16 kDa antibodies within VMS | 157 |
| Figure 5.2 | The recognition of periodate treated 16 kDa antigen by VMS | 159 |
| Figure 5.3 | The subclass of the anti-16 kDa antibodies with Ribi(S) | 162 |
| Figure 5.4 | The recognition of periodate treated 16 kDa antigen by Ribi(S) | 164 |
| Figure 5.5 | Recognition of the schistosomula surface by Ribi(S) | 165 |

| | | Page |
|---|---|---|
| Figure 5.6 | Immunoprecipitation of radiolabelled schistosomula surface antigens | 167 |
| Figure 5.7 | ISCOMs incorporating the 16 kDa antigen | 170 |
| Figure 5.8 | The subclass of the anti-16 kDa antibodies within NOV(S) and ISC(S) | 176 |
| Figure 5.9 | The recognition of periodate treated 16 kDa antigen by ISC(S) and NOV(S) | 178 |

**Chapter 6**

| | | |
|---|---|---|
| Figure 6.1 | The recognition of the 16 kDa antigen by El$\alpha$16 | 193 |
| Figure 6.2 | Antibody select | 195 |
| Figure 6.3 | The recombinant protein expressed by clone 18.5 | 197 |
| Figure 6.4 | The vaccine dominant nature of FP 18.5/ß-gal | 198 |
| Figure 6.5 | The sequence of the peptide encoded by clone 18.5 | 201 |
| Figure 6.6 | Cloning irregularities | 202 |
| Figure 6.7 | Recovery of FP 18.5/ß-gal from a recombinant lysogen | 206 |
| Figure 6.8 | Recognition of a 17 kDa antigen by sera raised in mice immunised with FP 18.5/ß-gal | 207 |

**Chapter 7**

| | | |
|---|---|---|
| Figure 7.1 | The vaccine dominant nature of FP 2.1/ß-gal | 220 |
| Figure 7.2 | The sequence of the peptide encoded by clone 15V | 222 |
| Figure 7.3 | Comparison of the proposed calcium binding sites of the 15 kDa antigen with the EF hand test sequence | 226 |
| Figure 7.4 | The expression of FP 2.1/GST | 228 |

Page


| | | |
|---|---|---|
| Figure 7.5 | Investigating the calcium binding capacity of FP 2.1/GST | 229 |
| Figure 7.6 | The antigenicity of FP 2.1/GST | 231 |
| Figure 7.7 | Cleavage of FP 2.1/GST with thrombin | 232 |
| Figure 7.8 | The recognition of the 15 kDa antigen by mice immunised with FP 2.1/GST | 234 |
| Figure 7.9 | The recognition of the 15 kDa antigen amongst adult and egg antigens | 236 |

# LIST OF TABLES


Page

**Chapter 1**

Table 1.1 Monoclonal antibodies reacting with the surface of *S. mansoni* schistosomula 42

**Chapter 4**

Table 4.1 Data from sequence analysis of the 16 kDa antigen 137

Table 4.2 Biotinylated lectins used for the probing of Western blots 141

**Chapter 5**

Table 5.1 The immunisation of mice with the immunoaffinity purified 16 kDa antigen plus Ribi adjuvant 160

Table 5.2 Reduction in worm burden of mice immunised with ISCOMs incorporating the 16 kDa antigen 172

Table 5.3 Reduction in worm burden of mice immunised with the immunoaffinity purified 16 kDa antigen plus novasomes 173

**Chapter 7**

Table 7.1 Immunisation of mice with FP 2.1/GST 235

# CHAPTER 1
# INTRODUCTION

## 1.1 GENERAL INTRODUCTION

Schistosomiasis is a major health problem in the developing world. It is responsible for an estimated 200,000 human deaths per year, whilst a further 200 million people suffer from the disease and 600 million live in areas where they are at risk from infection (WHO, 1993). The disease, which is endemic in 74 countries worldwide, is caused by trematode parasites of the species *Schistosoma*. Of the three major species which infect humans, *S. mansoni* and *S. haematobium* are endemic in many parts of Africa and the eastern Mediterranean, *S, mansoni* is also prevalent in South America and the Caribbean, *S. japonicum* is found mainly in south east Asia and the western Pacific. Of lesser importance are the African parasite *S. intercalatum*, *S. mekongii* which is found mainly in the western Pacific regions and a Malayan relative of *S. mekongii*, *S. malayensis*.

The life cycle of the schistosome parasite is complex and involves passage through an intermediate snail host (see below). *S. mansoni* and *S. haematobium* are transmitted by aquatic snails of the genus *Biomphalaria* and *Bulinus* respectively, whilst amphibious snails of the genus *Oncomelania* transmit *S. japonicum*. The natural habitats of these snails range from small streams to the great lakes, the only requirement being that the water source is static or reasonably slow moving. Generally these habitats are widely dispersed, however under ideal conditions localised foci of high transmission can occur and on a number of occasions the development of new water bodies or the damming of existing ones has been responsible for the onset or spread of schistosomiasis. The risk of disease in these areas is also compounded by uncontrolled immigration and the movement of refugees. Thus, despite the success of a number of schistosomiasis control programmes, the global estimate of the number of people infected with schistosomes has not decreased within the last decade.

## 1.2 THE LIFE CYCLE OF *SCHISTOSOMA MANSONI*

The life cycle of *S. mansoni* is initiated when the free living cercarial stages of the parasite are shed from the intermediate snail host. These locate a mammalian host, attach themselves to the skin and penetrate the epidermis by the cytolytic action of enzymes secreted from the cephalic glands. On passing through the skin the cercariae shed their tails to become the larval or schistosomula stages of the parasite which quickly lose their glycocalyx covered tegument and develop a heptalaminate, double plasma membrane. The schistosomula pass into the peripheral lymphatic vessels or the blood venules and are

carried via the lungs to the liver. Here the worms couple before migrating to the mesenteric veins. Egg production begins approximately six weeks post infection and the worms, which may live for a number of years, remain *in copulo* with the female laying approximately 300 eggs per day in the fine venules around the gut (in Whitfield, 1982). About 50% of these eggs pass through the tissues of the gut wall and into the gut lumen from where they are shed from the body in the faeces. The eggs hatch in a light, freshwater environment to produce the miracidia larvae which infect the snail intermediate host. Within the snail each miracidia forms a mother sporocyst which then divides to produce daughter sporocysts. These migrate to the digestive glands of the snail and divide again to produce thousands of cercariae. The latter finally move to the pseudobranch from where they are shed periodically to continue the life cycle (in Smyth, 1976). The remainder of the eggs are not shed by the mammalian host and become trapped upon passage through the gut wall or are swept back by the blood flow into the liver, the lungs and other organs, where they become lodged. It is the immune response of the host towards these tissue eggs which is largely responsible for the pathology observed during schistosomiasis.

## 1.3 IMMUNOPATHOLOGY

Antigens secreted by the miracidia developing inside tissue eggs are released through microscopic pores within the rigid egg shell and induce the formation of granulomas consisting of lymphocytes, macrophages, eosinophils and fibroblasts. These granulomas together with subsequent fibrosis are the major cause of schistosome related pathology. During an infection with *S. mansoni* the formation within the liver and intestine of granulomas considerably larger than the egg itself, can impede hepatic blood flow and lead to the development of hypertension and hepatosplenomegaly. This may be further complicated by the development of irreversible Symmers clay pipe - stem fibrosis around branches of the portal veins and in severe cases by the formation of 'shunts' and bleeding oesophageal varices. The latter enable blood to by-pass the fibrotic liver and hence facilitate the carriage of eggs to other organs such as the lungs, kidneys or CNS where granulomas may also form.

The formation of granulomas during chronic schistosomiasis has been shown to be dependent upon T cell mediated immunity (Phillips *et al.*, 1977, Cheever *et al.*, 1985a, Hassounah and Doenhoff, 1993). Moreover, the studies of Mathew and Boros (1986)

demonstrated that depletion of murine CD4+ T cells abolished the formation of granulomas, whereas depletion of CD8+ cells had no effect. In mice the involvement of CD4+ cells in pathology has been further defined as a requirement for the production of cytokines by cells which are mainly of the T helper 2 (Th2) subclass. A number of studies have demonstrated that during a chronic infection a down-regulation in the production of T helper 1 (Th1) type cytokines such as IFNγ and IL-2, and an up-regulation in Th2 cytokines such as IL-4, IL-5 and IL-10, occurs at the onset of egg production (Pearce *et al.*, 1991a, Sher *et al.*, 1991, Grzych *et al.*, 1991). This contrasts with the situation observed in mice vaccinated once with irradiated cercariae or those supporting a single sex infection, in which a Th1 type response is maintained throughout. That this switch to a Th2 type response is vital to the induction of egg induced pathology in mice has been demonstrated via abolishment of hepatic collagen deposition with an anti-IL-4 monoclonal antibody (McAb) (Cheever, *et al.*, 1994, Eltoum, *et al.*, 1995). As yet the existence of two subclasses of CD4+ cells has not been demonstrated in humans. However, elevated IgE and eosinophilia are hallmarks of a patent human schistosome infection thus indicating the production of IL-4 and IL-5 which may be of importance to the development of pathology as well as to the onset of antibody mediated immunity (section 1.6).

An increase in the levels of tumour necrosis factor α (TNFα), a cytokine released by T cells and macrophages, has also been observed during schistosome infection (Chensue *et al.*, 1989). This cytokine is known to induce an inflammatory response and has been shown to have a role in immunopathology as severe combined immunodeficient (SCID) mice which lack T and B cells, failed to develop granulomas unless reconstituted with TNFα producing spleen cells or recombinant TNFα (Amiri *et al.*, 1992). It has therefore been suggested that during a patent infection, antigens released by schistosome eggs may prime endothelial cells to express leucocyte adhesion molecules which induce antigen sensitised T cells to release TNFα and stimulate the production of other granuloma mediators such as chemotactic and colony stimulating factors. Amplification of this response could then occur through the release of more TNFα by macrophages recruited to the granuloma (Amiri *et al.*, 1992). It is also of interest to note that TNFα stimulated an increase in the number of eggs produced by the female worm (Amiri *et al.*, 1992).

The role of B cell dependent immunity in immunopathology has not yet been clarified. B cell depleted mice have been shown to develop normal granulomas (Cheever *et al.*, 1985b) and although the transfer of immune lymphocytes to *S. mansoni* infected

thymectomised mice is able to reconstitute granuloma formation, the transfer of immune serum has little effect (Hassounah and Doenhoff, 1993). However, immune complexes have been located within the granuloma (Abdul-Aal and Attalah, 1987) and a role for immune sera in reducing circulating transaminase levels and hence preventing hepatocyte damage has been demonstrated (Hassounah and Doenhoff, 1993).

Finally, it has been shown that the immune response towards egg antigens is also responsible for the down-regulation of granuloma formation which is observed in mice and which may ameliorate pathology in humans. Cytokines secreted by Th1 cells are believed to have a role in this down-regulation and various studies in mice (Oswald *et al.*, 1994, Wynn *et al.*, 1994) have demonstrated that the abolition of interferon gamma (IFNγ) and IL-12, in addition to the depletion of natural killer (NK) cells, increases the size of the egg induced granulomas observed. Further studies have suggested that egg antigen stimulates an IL-12 independent release of IFNγ from NK cells which results in the expression of the IL-12 gene and subsequent release of this cytokine from macrophages. This in turn may stimulate the production of more IFNγ by Th1 type cells and both of these cytokines may then be involved in decreasing the production of IL-4 by Th2 cells and hence granuloma development. This hypothesis is supported in part by the observation that mice sensitised with eggs in combination with recombinant IL-12, developed only minimal granulomas upon subsequent egg challenge. It has also been demonstrated that suppressor T cells (Ts) and serum from chronically infected mice (CIS) are able to reduce granuloma formation upon passive transfer. Moreover, factors released by the parasite itself may be involved in the down-regulation of the immune response (reviewed by Phillips and Lammie, 1986).

## 1.4 THE CONTROL OF SCHISTOSOMIASIS

Health education, the provision of safe water and sanitation, snail control and most importantly chemotherapy all play a part in the control of schistosomiasis. However, in many developing countries the use of these methods and their integration within suitable control programmes is limited by economic constraints.

With regard to snail control, the application of one molluscicide, namely niclosamide, is the only method used to any extent. This can be applied either to small water bodies by spraying, or to large stretches of water via drip feeds upstream of the area to be treated. In some cases natural water sources have also been drained or eliminated to

decrease possible snail habitats and more consideration is now given to the possible spread of schistosomiasis during the design of new irrigation projects. However, in many cases the bodies of water which provide schistosomiasis transmission sites are immense and in spite of efforts to control the snail population, transmission in many areas remains high. Chemotherapy therefore remains the mainstay of schistosomiasis control.

Although oxamniquine is still used in some areas for the treatment of *S. mansoni* and metrifonate for the treatment of *S. haematobium*, Praziquantel (PZQ) is the preferred drug and is able to eliminate all species of schistosomes. It is a heterocyclic pyrazine-isoquinoline which acts by causing contraction of the adult worms, which lose their grip on the blood vessels, die and eventually disintegrate. It is generally effective when given as a single dose and has a cure rate of 60 - 90%, with egg reductions of 90 - 95% occurring in those who are not fully cured. The main problem encountered within control programmes which rely upon chemotherapy is therefore the cost. Although PZQ is a comparatively cheap drug, in areas of high transmission reinfection can occur very quickly and re-treatment is often required at least once a year (Cherfas, 1991). This can be targeted to particularly susceptible groups of individuals such as school children or those in a high risk occupation, (Butterworth, 1990) however, the cost of the drug and regular administration are still prohibitive in many areas where intervention is most required. Added to this is the growing concern regarding the possible development of drug resistance. Strains of schistosomes which are resistant to oxamniquine have already been reported in South America (Coles *et al.*, 1986) and although these infections were successfully treated with PZQ, laboratory evidence to suggest that *S. mansoni* in experimental mice can develop resistance to the latter has recently been published (Fallon and Doenhoff, 1994). One of the strains shown in this study to be less sensitive to PZQ treatment was from Senegal, a country in which a massive epidemic of human schistosomiasis has proved difficult to treat (Gryseels, 1994).

Thus in summary, schistosomiasis is at present controlled by a combination of snail elimination and more importantly chemotherapy. However, due to the requirement for constant re-application control programmes which rely upon the use of these strategies are both difficult and expensive to maintain. Hence it is widely accepted that new methods are required to control the onset and spread of schistosomiasis and the development of a suitable vaccine is now the ultimate goal of much of the work carried out within the field of schistosome research. In general vaccines are highly cost effective as compared to other

intervention procedures (World Bank, 1993). Moreover, newly developed vaccines could be incorporated within the operational WHO extended programme for childhood diseases, which already reaches 70 - 80% of the world's children. That effective vaccination against schistosomiasis infection is possible is supported by the demonstration of immunity in experimental animals (see below) and an increasing body of evidence which indicates that immunity to schistosomiasis develops, albeit slowly, in naturally infected humans (section 1.6).

## 1.5 IMMUNITY IN EXPERIMENTAL ANIMALS

Experimental hosts of *S. mansoni* include rodents, such as mice and rats and to a lesser extent primates, such as the rhesus monkey and the baboon. The baboon and the mouse are, like humans, permissive hosts which tolerate the development of the adult worm and the onset of egg production 4 - 5 weeks post infection (Smithers and Doenhoff, 1982). In contrast, the rat is a non-permissive host in which the majority of the worms are expelled between days 28 and 37 post infection whilst the rest remain stunted and unable to produce viable eggs (Cioli *et al.*, 1978). In the rhesus monkey, the situation is variable and has been shown to be dependent upon the size of the infection.

In all of these hosts resistance to reinfection can be induced by a normal unattenuated infection (concomitant immunity); by exposure to parasites attenuated with radiation or schistosomicides (vaccine immunity); or by immunisation with crude, purified or recombinant schistosome antigen preparations.

### 1.5.1 CONCOMITANT IMMUNITY

(a) The rhesus monkey

In the rhesus monkey resistance to reinfection develops within 12 weeks of a single exposure to unattenuated parasites (Smithers and Terry, 1965a). This resistance is assumed to be immunologically mediated and effective against the larvae of an incoming infection whilst the adult worms of the primary infection remain unaffected, hence the term concomitant immunity. The adults worms are known to be immunogenic as resistance to reinfection is observed following the direct transfer of mature worms into naive recipients (Smithers and Terry, 1967). However, they are able to protect themselves against the immune response stimulated by a variety of methods. These include rapid membrane turnover (Perez and Terry, 1973, Wilson and Barnes, 1974); the loss of

antigens which are expressed on the surface of the schistosomula (Samuelson *et al.*, 1980, Simpson *et al.*, 1984); the surface acquisition of host molecules i.e. blood group components (Clegg *et al.*, 1971) and histocompatibility antigens (Sher *et al.*, 1978), the expression of host like antigens and by increasing the intrinsic resistance of the parasite surface to host effector mechanisms (Tavares *et al.*, 1980, Moser *et al.*, 1980, Bickle and Ford, 1982). Furthermore, the adult worms secrete immunosuppressive factors and soluble antigens which form immune complexes and induce both suppression and tolerance in the host (Capron *et al.*, 1980a & b).

(b) Rats

As a non-permissive host, the infected rat expels a large proportion of the primary worm burden. Hence the resistance which develops approximately 4 weeks post-infection, persists in the presence of a low number of sterile, stunted worms (Smithers and Terry, 1965b). The resistance seen is believed to be immunologically mediated as the transfer of cells and serum (IRatS) from infected rats is able to confer immunity to naive recipients (Phillips *et al.*, 1975, Phillips *et al.*, 1978, Ford *et al.*, 1984a). Furthermore, the transfer of live worms into the liver has again demonstrated that the adult stages of the parasite possess the antigens required for the stimulation of concomitant immunity (Knopf and Cioli, 1980).

(c) Baboons

Rather less is known about the development of resistance following an unattenuated infection in the baboon. Early experiments involving the immunisation of baboons with *S. mansoni* cercariae (Sturrock *et al.*, 1978) indicated that the overall levels of resistance obtained were low although a small number of animals did demonstrate levels of resistance which were greater than 50%. Similarly, intrahepatic transfer studies have had varying degrees of success. Damian *et al.*, (1972) reported that infection with *S. mansoni* adult worms did not stimulate resistance to reinfection, however Webbe *et al.*, (1976) demonstrated the development of a low but significant level of resistance following the intra-hepatic transfer of *S. haematobium* adult worms.

(d) Mice

In mice resistance to a percutaneous challenge arises within 8 weeks of a single

unattenuated infection (Dean, 1983). However, it has become apparent that the resistance seen in this model is not immunologically specific but is associated with the pathological response of the host to parasite eggs deposited within the tissues (Wilson *et al.*, 1980, 1983, McHugh *et al.*, 1987). This claim is substantiated by the observations that B cell depletion has no effect on the resistance seen in infected mice (Maddison *et al.*, 1981) and that parabiosis, the transfer of sera and the transfer of T cells from the spleen or the lymph nodes of infected mice fails to protect naive animals (Dean *et al.*, 1981a, Harrison *et al.*, 1982). Moreover, resistance to a challenge infection does not develop until the primary infection reaches patency (Long *et al.*, 1978, 1980) nor is it seen during a single sex infection (Dean *et al.*, 1978, Bickle *et al.*, 1979a, Harrison *et al.*, 1982, Bickle *et al.*, 1983) or in mice in which the primary infection has been cured by chemotherapy (Doenhoff *et al.*, 1980). An inverse relationship has also been observed between the length of time that mice survive following a primary infection and their capacity to resist reinfection (Bickle *et al.*, 1980). Furthermore, resistance can not be induced by intra-hepatic transplantation of worms of a single sex (Boyer and Kalfayan, 1978) whereas the transfer of worms of both sexes confers resistance which develops following the onset of egg production (Peresan and Cioli, 1980). More specifically, the studies of Harrison *et al.* (1982) have demonstrated a positive correlation between the tissue egg count and resistance, whereas no correlation was seen between the latter and the adult worm burden.

The correlation between resistance and the presence of eggs within the tissues of the murine host is believed to a be a consequence of a pathology induced alteration in the ability of the liver to sequester challenge parasites. Studies by Wilson *et al.*, (1983) have demonstrated that almost immediately following the onset of egg production there is an increase in the number of challenge parasites which escape from the liver and die in other tissues. As these schistosomula do not contribute to the estimated adult worm burden, the host could be considered to be resistant even though the missing larvae have not been killed via immune mechanisms. This situation is exacerbated at a later time point by the development of major porta-cava anastomoses which allow the migrating larvae to travel from the liver back to the lung.

In spite of the obvious importance of egg related pathology to the resistance seen in infected mice, the injection of eggs alone has largely been unable to generate resistance (Moore *et al.*, 1963, von Lichtenberg, *et al.*, 1963, Doenhoff *et al.*, 1980, Harrison *et*

*al.*, 1982). It has been suggested that this is due to the failure of externally administered eggs to reach, and hence cause pathology, within areas of tissue which form part of the migratory route of the challenge parasites (Harrison *et al.*, 1982).

The aforementioned studies have demonstrated that both the rat and the mouse develop resistance to a challenge infection following exposure to unattenuated parasites. However, neither animal provides an ideal model for studies aimed at the development of a vaccine suitable for use in humans as the rat, unlike the human employs spontaneous expulsion of the primary worm burden and in the permissive mouse host the resistance observed is dependent upon egg related immunopathology. As a result, the work of several groups, including that at the London School of Hygiene and Tropical Medicine (LSHTM), has focused instead upon studying the development of immunity following a radiation attenuated infection. Here spontaneous expulsion, the release of adult and egg antigens and most importantly egg induced pathology are avoided by the death of the parasite in the larval stages.

### 1.5.2 VACCINE INDUCED IMMUNITY

The successful use of radiation attenuated parasites as a vaccine against parasitic bronchitis in cattle stimulated an interest in the use of such "live" vaccines for other parasitic diseases. Thus, following early studies with *S. japonicum* in primates (Hsü *et al*, 1962), Taylor *et al.*, (1976) demonstrated that sheep could be protected against infection with *S. mattheei* by immunisation with homologous irradiated schistosomula and that both sheep and cattle could be protected against *S. bovis* under laboratory conditions (Taylor *et al.*, 1979). These studies were subsequently extended into a field trial which demonstrated that immunisation with irradiated *S. bovis* schistosomula reduced worm and egg burdens as well as egg associated pathology (Majid *et al.*, 1980). Irradiated cercariae of *S. japonicum* have also been used successfully to immunise cattle in the laboratory (Hsü *et al.*, 1983) and the field (Hsü *et al.*, 1984). Thus the irradiated vaccine has been shown to stimulate protection against a schistosome infection occurring in a natural host animal. However, in order to characterise more specifically the nature of the immune response to radiation attenuated parasites it was necessary to focus instead upon the more easily studied mouse and rat laboratory models.

#### 1.5.2.1 The induction of immunity in laboratory hosts

(a) Mice

Vaccination of mice with γ-irradiated schistosome larvae has been shown consistently to induce species specific and long lasting (> 24 weeks) protection against a challenge infection (Minard *et al.*, 1978, Bickle *et al.*, 1979b, Bickle *et al.*, 1985). The studies of Bickle *et al.*, (1979b) clearly demonstrated that cercariae induced better resistance than schistosomula and that a single vaccination with 500 parasites was sufficient to induce significant levels of resistance by 3 weeks post vaccination. However, there has been some debate as to the dose of radiation which is required for the induction of optimal immunity. Initially, it was reported that maximum immunity was observed following the immunisation of mice with cercariae exposed to a low dose of irradiation. This prevented the production of eggs but allowed the development of some sterile worms within the liver (Villella *et al.*, 1961, Radke and Sadun, 1963). However, subsequent studies (Minard *et al.*, 1978, Bickle *et al.*, 1979b, Reynolds and Harn, 1992) demonstrated that better levels of immunity were stimulated by immunisation with parasites irradiated with a more moderate dose (i.e. 58, 20 or 15 krad respectively) although highly irradiated cercariae were poorly protective. Experiments employing parasite recovery techniques (Bickle *et al.*, 1979c) revealed that, unlike highly irradiated parasites which die in the skin, the majority of parasites attenuated with a moderate dose leave the skin and show a delayed migration to the lung, peaking on day 7 post infection i.e. one to two days later than the more pronounced peak observed following infection with unattenuated parasites. Only a very small fraction (1%) of these then migrate to the liver, thus suggesting that optimal immunity is induced by the death of moderately irradiated larvae in the lungs, or on route to the liver. Histological studies (Mastin *et al.*, 1983) and autoradiographic tracking techniques (Mangold and Dean, 1984, Elsaghier and McLaren, 1989), have since confirmed that the majority of moderately krad radiation attenuated parasites do die in the lungs. Moreover, although discrepancies have arisen regarding the specific dose which is required for the stimulation of optimal immunity, i.e. 50 krad (Minard *et al.*, 1978), 20 krad (Bickle *et al.*, 1979b), it has been shown that the optimal dose of radiation in each study produced immunising infections which behaved similarly with regard to their survival, migration and death in the lung. Six day old irradiated lung worms transplanted directly into the pulmonary vasculature have also been shown to confer levels of resistance almost equal to those seen following vaccination with irradiated cercariae (Dean

*et al.*, 1981b).

The induction of optimal immunity following the death of the 20 krad irradiated larvae in the lung could indicate that the stimulation of maximum resistance requires the release / expression of specific antigens which occurs upon the death of this particular stage of the parasite. Alternatively, the death of the parasite in this particular organ may be necessary or parasites may simply be required to survive for a particular length of time in the vaccinated host. In order to investigate the importance of these different parameters in the induction of optimal immunity, studies employing the schistosomicide Ro 11-3128 to attenuate infections were performed (Bickle and Andrews, 1985). In contrast to the situation seen with irradiated parasites whereby optimal immunity was stimulated by drug termination of the larvae on day 6 post infection, optimal immunity was stimulated by drug termination of an unirradiated infection on days 2-3 post infection. The former confirmed the importance of the death of the lung stage irradiated parasite in the generation of optimal immunity. However, the attainment of optimal immunity upon killing of the unirradiated parasites at a time when they would still be in the skin indicated that neither the site of death nor the persistence of the infection for a substantial length of time were important. It was therefore suggested that stimulation of optimal vaccine immunity is dependent upon the release / exposure of particular antigens on the death of the early larval stages and that the requirement for the killing of the irradiated lung stage as opposed to the normal skin stage is a consequence of a radiation induced decrease in protein synthesis which results in the delayed expression of such antigens by the irradiated larvae. Furthermore, the attenuation of parasites with high radiation doses could further delay or inhibit the expression of protective antigens and hence account for their poor ability to induce resistance. In the light of these results Bickle *et al.*, (1990) compared the pattern of larval surface antigen recognition by sera generated upon termination of an unirradiated infection with Ro 11-3128 at various intervals post infection (Ro 11S), with that of mice vaccinated with 20 krad irradiated parasites (VMS) or exposed to a chronic infection (CIS). This demonstrated that Ro 11S from 2 - 3 day terminations and VMS, preferentially recognised antigens of 32, 23, 16 and 15 kDa and that Ro 11S had the highest overall titre. Why termination of an infection with Ro 11-3128 stimulates such high levels of antibody is uncertain. However, skin stage schistosomula treated with Ro 11-3128 *in vitro* have been shown to produce an exudate and to develop vesicles termed "blebs" upon their surface (Bickle *et al.*, 1990). The latter represent released fractions of

particulate antigen and are believed to originate from the inner membrane leaflet of the lipid bilayer. More recent studies (Smith *et al.*, 1994) have demonstrated that following incubation with drug treated MS antigen presenting cells (APC) were better able to present antigen and hence stimulate the proliferation of sensitised T cells. Fractionation of the soluble antigens and isolated "blebs" demonstrated that this enhanced proliferation occurred largely in response to the latter, which also contained the majority of the proteinaceous material. Thus, it has been suggested (Bickle *et al.*, 1990) that if such "blebs" were formed following drug treatment *in vivo* the presentation of antigen to APC in the form of membranous vesicles may well be responsible for the development of the enhanced antibody response.

An alternative explanation for the enhanced immunogenicity of both the 20 krad irradiated parasites and Ro 11-3128 early terminated infections has been proposed by Mountford *et al.*, (1988, 1989). They suggested that the development of optimal protection in these two systems was a direct consequence of the persistence of antigenic material within the skin draining lymph nodes (SLN). It has been demonstrated by radiolabelling that 20 krad irradiated parasites on route to the lungs persist for much longer in the SLN than do unattenuated parasites. In addition, parasites attenuated by drug treatment on days 2 - 3 post infection showed a delayed migration through these nodes. The latter contrasted with the situation observed following drug termination of an irradiated infection at a similar point in time, as these parasites failed to reach the SLN and stimulated poor immunity. In addition, Coulson and Mountford, 1989 obtained poor levels of protection following the direct transfer of irradiated lung schistosomula into the pulmonary vasculature and it has been suggested (Wilson and Coulson 1989) that this may be a consequence of the absence of sensitisation by prior migration through the SLN. It is of course possible that both of the features described above are of importance, thus the generation of optimal immunity may require a protracted and enhanced release of specific antigens to occur within the SLN.

(b) Rats

Two vaccinations with 1000 $\gamma$ irradiated cercariae have been shown to stimulate high levels of protective immunity in rats (Smithers and Terry, 1965b, Phillips *et al.*, 1978, Ford *et al.*, 1984b). Although optimal resistance was seen following immunisation with parasites treated with a radiation dose of 0 - 20 krad (Ford *et al.*, 1984b), a proportion

of the unattenuated larvae or those irradiated at a very low dose did develop into adults and hence immunity associated with spontaneous elimination or the remaining stunted worms could contribute to the resistance seen. In contrast, 20 krad irradiated parasites stimulated optimal resistance in the absence of adult worms as they showed a delayed migration to the lung where they died 2 - 3 weeks post infection (Ford *et al.*, 1984b). Direct transfer of the irradiated lung stage into the pulmonary vasculature of naive rats also stimulated resistance equal to that observed following administration of irradiated cercariae (Ford *et al.*, 1984b).

#### 1.5.2.2 The site of challenge parasite elimination

(a) Mice

It is widely accepted that in the vaccinated mouse some of the challenge parasites are killed in the skin and some in the lungs. However, the question as to where the majority of elimination occurs has provoked much debate. Similar parasite recovery techniques, histopathological studies and autoradiographic tracking of $^{75}$Se labelled parasites have all been used in studies which concluded that the majority of parasites died either in the skin during the first 2 or 3 days post infection (Hsü *et al.*, 1979, Miller and Smithers, 1980, 1982, Hsü *et al.*, 1983, Ward and McLaren, 1988, Elsaghier and McLaren, 1989, Kamiya *et al.*, 1987) or in the lungs during the second or third week post infection (Stek *et al.*, 1981, Mastin *et al.*, 1983, von Lichtenberg *et al.*, 1984, Dean *et al.*, 1984, Wilson *et al.*, 1986). These two contradictory theories have also been supported by the transplantation of lung worms directly into the pulmonary vasculature of vaccinated mice. Thus, a reduction in the challenge worm burden of only 15% was seen following the direct transfer of 5 day old larvae into the lungs of mice in which the skin was considered to be the major site of attrition (Miller *et al.*, 1981, Smithers, 1982, McLaren *et al.*, 1985). In contrast, resistance almost equal to that seen following a percutaneous challenge (32 - 44%) was observed following the transfer of larvae into the lungs of a model system in which the majority of killing had been shown to occur in the lung (Dean *et al.*, 1981b). The passive transfer of sera from multiply vaccinated to naive mice (see below) has also been reported to confer optimal protection if carried out in accordance with the model used, i.e. at the time of challenge, when the parasites are killed in the skin (in Kamiya *et al.*, 1987) or on day 5 post infection if the larvae are killed in the lung (Mangold and Dean, 1986). The reasons for the differences between these two vaccinated

mouse models are as yet largely unresolved. The site of challenge elimination is not dependent upon the strain of mouse vaccinated nor is it affected by the site of vaccination or challenge administration (Elsaghier and McLaren, 1989). It has been suggested (Elsaghier and McLaren, 1989) that subtle variations in the parasite isolate used for vaccination may have a profound effect upon the site at which the challenge parasites are killed. However, on examining the elimination of a strain of parasite which had been shown by others to be killed in the skin, Dean *et al.*, (1995) demonstrated that in their hands the majority of the schistosomula were killed at some point after migration to the lungs.

(b) Rats

In the vaccinated rat, parasite recovery and histology techniques have clearly demonstrated that the majority of challenge parasites are killed in the lungs. Moreover, high levels of protection are consistently achieved upon transplantation of lung stage larvae into the pulmonary vasculature (Ford *et al.*, 1984b) and serum transfer at the time of challenge parasite migration through the lungs is able to passively protect naive animals (Ford *et al.*, 1984a, Ford *et al.*, 1987a, McLaren and Smithers, 1985).

### 1.5.3 MECHANISMS OF IMMUNITY

In contrast to the immunopathology related resistance seen in mice harbouring a chronic infection, the protection seen following a normal infection in the rat or vaccination of rats or mice with radiation attenuated cercariae is believed to be due to the generation of a specific immune response against the schistosome larvae. This proposal is supported by the species specific nature of the resistance induced (Bickle *et al.*, 1985) and also by the longevity of protection which demonstrates that the killing of the challenge parasite is independent of the inflammatory response observed in the lung following the death of the immunising infection (von Lichtenberg *et al.*, 1984). Studies using athymic and $\mu$-chain depleted rats and mice (Ford *et al.*, 1987b, Sher *et al.*, 1982) have indicated the involvement of both T and B cells in the development of protective immunity. However, the role of these lymphocytes is believed to differ in these two rodent species. Thus, studies with infected or vaccinated rats have indicated that T cells are required only to provide help for the production of protective antibody (Ford *et al.*, 1987a, section 1.5.3.2). In contrast, experiments using immunodeficient P strain mice

which, despite the production of antibodies capable of mediating macrophage killing *in vitro* develop poor resistance following vaccination with irradiated cercariae, suggested that T cell functions other than those required for B cell help are involved in the immunity seen in once vaccinated mice (James *et al*, 1984). The known deficiency of P strain mice in cell mediated immunity also provided the first evidence regarding the critical role of delayed type hypersensitivity (DTH) mechanisms in singly vaccinated mice. This is discussed in the following section.

#### 1.5.3.1 T cell mediated effector mechanisms

Studies by Aitken *et al.*, (1988) demonstrated that following a single vaccination of mice with irradiated cercariae there was a sustained and significant increase in the number of leucocytes present within the broncho-alveolar lavage (BAL) cell population. This infiltration began on day 7 post vaccination and peaked on day 21. As compared to the cell populations seen in naive mice it represented a five fold increase in the numbers of monocytes and polymorphonuclear leucocytes (PMN) present and more importantly a fifteen fold increase in the number of lymphocytes, all of which persisted for at least 10 weeks post vaccination. The extent of this lymphocyte influx was also shown to correlate with the degree of protection subsequently obtained. Thus, the maximum numbers of cells were seen in the lavage following the vaccination of mice with 20 krad attenuated parasites. As T cell proliferation has been shown to be stimulated to the same degree by unirradiated and irradiated larvae (James, 1985) it was unclear as to how this enhanced pulmonary response was mediated. However, studies by Mountford *et al.*, (1992) have demonstrated a positive correlation between the size and T cell content of the lymph nodes draining the skin (SLN) and the resistance generated following vaccination with parasites attenuated with varying doses of radiation. Moreover, removal of SLN prior to or immediately following vaccination results in a substantial decrease in the immunity generated. It has therefore been proposed that the radiation induced delay in parasite migration through SLN (section 1.5.2.1, Mountford *et al.*, 1988, 1989) elicits a population of sensitised T cells which appear in the circulation following vaccination, infiltrate the lung parenchyma and airways upon arrival of the irradiated parasite in the pulmonary arterioles and "arm" the lung against a challenge infection. Examination of the expanded lymphocyte populations of the pulmonary lavage revealed that both CD4+ and CD8+ cells were represented. This suggested a possible role for both helper (Th) and

cytotoxic (Tc) cells in vaccine induced immunity. However, the studies of Vignali *et al.*, (1989a) demonstrated that the depletion of Tc cells with an anti-CD8+ McAb resulted in a slight increase in the resistance observed, thus indicating that CD8+ cells do not play a role in the killing of the challenge parasite. The latter is consistent with *in vitro* observations which demonstrated that although CD8+ cells were able to bind to both skin and lung stage schistosomula via the recognition of absorbed host antigens, they were unable to kill (Butterworth *et al.*, 1979). In contrast, the depletion of Th cells by administration of an anti-CD4+ McAb resulted in the complete abrogation of both vaccine and drug attenuated immunity (Vignali *et al.*, 1989a). This suggested a vital role for CD4+ cells in the development of resistance and thus attempts were made to further characterise the subset of Th cells involved. Examination of cytokine profiles (reviewed by Smythies *et al.*, 1992) revealed that the majority of T cells removed from the lungs of vaccinated mice secreted IFNγ and IL-3 in response to antigenic stimulation. This indicated that Th1 cells were the main constituent of the enhanced pulmonary infiltrate. How the balance of T cells in favour of Th1 is attained is not fully understood. However, it has been proposed (Mountford *et al.*, 1994, Wynn *et al.*, 1995) that IL-12 secreted by NK cells, macrophages and B cells may play a role. This cytokine is known to be a potent stimulator of IFNγ production by CD4+, CD8+ and NK cells. Moreover, IL-12 stimulates the expansion of Th1 cells but suppresses the differentiation of Th2. It has therefore been suggested (Wynn *et al.*, 1995) that IL-12 released following vaccination with irradiated cercariae promotes a Th1 type response and hence the development of protective immunity. This hypothesis is supported by the demonstration that a small but significant increase in the levels of mRNA encoding a subunit of IL-12 occurred following vaccination with irradiated parasites (Wynn *et al.*, 1995). Furthermore, the administration of rIL-12 during vaccination with radiation attenuated parasites (Wynn *et al.*, 1995) or soluble lung stage antigens (Mountford *et al.*, 1994) increased the levels of resistance obtained beyond those normally seen following vaccination with these preparations.

It is largely accepted that the death of the irradiated parasite in the lungs is a prerequisite for the stimulation of optimal vaccine induced immunity. This is irrespective of whether the elimination of the challenge parasites occurs in the skin or in the lungs. Thus, the above describes the mechanisms which may be involved in the induction of

resistance in both of these models. However, it is believed that the mechanisms and cell population involved in the killing of the challenge parasites in these two sites differ considerably.

Thus, using vaccinated mice in which the major phase of challenge elimination occurs in the lungs, Aitken *et al.*, (1988) demonstrated that on the arrival of the challenge parasite in the lungs a second influx of T lymphocytes was observed. This was accompanied by an increase in the levels of IFNγ and IL-3, which peaked more rapidly after challenge than after vaccination and suggested that an anamnestic Th1 type response had occurred (reviewed by, Wilson *et al.*, 1992, Smythies *et al.*, 1993). The timing of IFNγ production was also coincident with phases of pulmonary macrophage activation. The latter which indicated the importance of both IFNγ and macrophage activation in the immunity induced in vaccinated mice is in accordance with the results of the original studies of James *et al.*, (1984). These demonstrated that P/N or P/J (P) strain mice, which failed to respond to the irradiated vaccine, gave a poor delayed type hypersensitivity (DTH) response to skin challenge with soluble worm antigens (SWAP). Further analysis revealed this to be due to an inability of T cells isolated from P strain mice to produce significant levels of INFγ, coupled with a deficiency of P strain macrophages to respond to this cytokine (James *et al.*, 1984). The importance of macrophage activation in the mouse model of vaccine immunity has also been indicated by the experiments of Lewis *et al.*, (1987) which demonstrated that the ability of macrophages taken from mice immunised with live irradiated and cryopreserved larvae to kill schistosomula *in vitro* correlated with the ability of these mice to resist a challenge infection. Moreover, the experiments of Vignali *et al.*, (1989a) showed that macrophages taken from mice immunised with 20 krad attenuated larvae had an increased ability to kill skin stage schistosomula *in vitro*. The upregulation of surface molecules (e.g. MHC Class II) on alveolar macrophages also demonstrated that activation of these cells occurred upon administration of the irradiated vaccine and increased following a challenge infection (Menson and Wilson, 1990). That the development of resistance is dependent upon the release of IFNγ has also been confirmed by the use of a McAb against this cytokine which abolished the development of vaccine immunity (Smythies *et al.*, 1992). However, subsequent studies (Williams *et al.*, 1995) have also indicated that a low level of TGFß, a cytokine which inhibits macrophage activation, is also required.

Despite this wide body of evidence the importance of T cell mediated macrophage

activation in vaccine immunity was questioned by the studies of Aitken *et al.*, (1987). These demonstrated that despite the abolition of DTH to a model antigen (bcg) the killing of the parasites in the lung persisted following whole body irradiation of the mouse host. However, further studies (Aitken *et al.*, 1987) revealed that although radiation reduced the numbers of peripheral leucocytes, the recirculation of T cells and hence DTH, vaccine sensitised T cells, resident in the pulmonary airways, were unaffected and were therefore able to activate macrophages in response to the arrival of the challenge parasite in the lungs. The radioresistant nature of the mechanisms involved in the killing of the parasite in the lungs also suggests that radiosensitive effector cells such as eosinophils, neutrophils and mast cells are not involved (Aitken *et al.*, 1987, Vignali *et al.*, 1988a). This was substantiated by the observation that mast cell deficient mice develop good levels of resistance following vaccination (Sher *et al.*, 1983) and that abolition of the eosinophil response by treatment with an anti-IL-5 McAb similarly has no effect upon the killing of parasites in the lungs (Sher *et al.*, 1990). Moreover, upon administration of an anti-IFNγ McAb the number of eosinophils and PMN in the lungs actually increased whilst immunity was abolished.

Rather less is known regarding the killing of challenge parasites in the skin. However, whole body irradiation has been shown to abolish skin phase immunity in mice (Delgado and McLaren, 1989) and the migration of neutrophils and at a later time point eosinophils, into the site of challenge has been observed (Ward and McLaren, 1988). In addition, agents which block macrophage function have no effect upon the development of skin phase immunity (Delgado and McLaren, 1989). This evidence therefore suggests that the mechanisms involved in the elimination of the challenge parasites from the skin differ from those employed in the lungs.

How the challenge schistosomula are killed following the generation of an immune response is as yet unclear. The secretion of IFNγ by memory T cells which follows the arrival of the challenge parasite in the lungs is believed to activate macrophages and induce an inflammatory response which results in the development of tight foci around the schistosomula by day 17 post challenge (Wilson, 1992). Similar foci are seen during the killing of the parasite in the skin although these contain radiosensitive leucocytes in addition to monocytes and T cells (Piper and McLaren, 1993, McLaren and Smithers,

1988). TNFα which is released by sensitised T cells and is known to upregulate the expression of vascular endothelial cells may be involved in the development of these foci, although ablation of this cytokine by anti-TNFα failed to affect the development of immunity (Smythies *et al.*, 1993). A similar role has also been suggested for IFNγ which has been seen to upregulate the expression of ligand receptor pairs (e.g. LFA / ICAM) and hence may determine the density of the inflammatory foci generated. The trapping of parasites in these inflammatory foci may be aided by eosinophil cationic proteins which have been seen to paralyse lung worms *in vitro* (McLaren *et al.*, 1984) and by macrophages which can traumatize the musculature of cultured larvae (McLaren and James 1985). Alternatively, the slow movement of the migrating parasites through the narrow vessels of the lung capillary beds may be sufficient to enable them to become targets of an anamnestic inflammatory response. Once trapped in inflammatory foci larvae may then be killed by toxic cell products or by the effects of antibody (McLaren, 1989). Nitric oxide (NO) is released by activated macrophages and has been shown to kill freshly transformed schistosomula *in vitro*. However, lung stage larvae are refractory to this type of killing (Pearce and James, 1986) and the role of NO *in vivo* is doubted as inhibition of NO production by N-monomethyl-L-arginine (L-NMMA) fails to deplete vaccine immunity (Smythies, *et al.*, 1993). Alternatively, the larvae may die simply as a result of their failure to migrate. This hypothesis is substantiated by the observation that schistosomula trapped within foci in the skin subsequently transform into lung stage parasites (Ward and McLaren, 1989). Moreover, parasites removed from either skin or lung foci are able to continue their migration if transplanted into a naive host (Coulson and Wilson, 1988). Finally it has been proposed that the development of foci around each parasite may result in a blockage of the migratory route of the challenge infection and result in a greater number of these parasites being shunted into the alveoli from where they cannot return (Coulson and Wilson, 1988).

#### 1.5.3.2 Antibody mediated effector mechanisms

(a) *In vitro*

Antibodies generated in response to a schistosome infection have been shown to kill newly transformed schistosomula *in vitro*. Initial experiments carried out using sera from infected rhesus monkeys, humans, rabbits and rats demonstrated that this cytotoxicity could be achieved via the action of antibodies known as lethal antibody in conjunction

with complement alone (reviewed in Capron *et al.*, 1982). However, subsequent studies (Butterworth *et al.*, 1974) which showed that phagocytic leucocytes were able to adhere to and kill schistosomula in the presence of heat inactivated sera suggested that antibody dependent cytotoxicity (ADCC) may be of greater importance.

Macrophages were the first leucocyte population to be shown to be involved in this process (Capron *et al.*, 1975). Cells incubated with IRatS and freshly transformed schistosomula were able to kill schistosome larvae within 16 hours (Capron *et al.*, 1977). Moreover, heating of the sera, treatment with anti-IgE and prior incubation of the macrophages with unrelated IgE were shown to prevent killing, as did the absorption of infection sera with schistosomula antigens. This indicated that the binding of macrophages to the larval surface occurred via interactions between low affinity Fc$\epsilon$RIIb receptors and IgE bound to schistosomula surface. Further studies then revealed that monomeric IgE was unable to promote the adherence of macrophages to schistosomula and led to the suggestion that immune complexes of circulating schistosome antigens and an excess of IgE were required for opsonisation (Capron *et al.*, 1977). How schistosomula are then killed is not clear. However, it has been shown that human alveolar macrophages specifically release lysosomal ß-glucoronidase, neutral proteases and superoxide anion following the cross linking of their IgE receptors (Joseph *et al.*, 1980).

The killing of schistosomula by eosinophils from uninfected rats has also been achieved following the incubation of larvae with infected human sera (IHS) or rat sera (Capron *et al.*, 1978, Butterworth *et al.*, 1979). Initial experiments using sera taken from rats 4 weeks post infection demonstrated that the depletion of IgE had little effect upon eosinophil cytotoxicity whereas absorption of the sera with anti-IgG2a, or prior incubation of eosinophils with aggregated antibodies of this subclass abolished the killing of the schistosomula (Capron *et al.*, 1978). Further studies then demonstrated that although sera removed from rats infected 4 weeks previously was cytotoxic despite IgE depletion, the cytotoxicity of sera taken 8 weeks following infection was depleted by removal of IgE but not IgG2a (Capron *et al.*, 1981). This suggested a requirement for IgG2a in early immunity which was superseded by a requirement for IgE.

Complement alone has also been shown to mediate the killing of schistosomula by eosinophils. Schistosomula are known to activate complement via the alternative pathway and to acquire C3 upon their surface. Hence, the demonstration that normal rat sera (NRatS) stimulates the killing of schistosomula by eosinophils from uninfected animals

indicates that eosinophils are able to bind via complement receptors to the C3 on the surface of the newly transformed larvae (Ramalho-Pinto *et al.*, 1978). Moreover, NRatS was better able to stimulate the adherence of eosinophils to schistosomula than heat inactivated IRatS and it has been suggested that complement coated larvae form better targets for eosinophils then those opsonised with antibody as a result of the chemotactic properties of other components generated upon activation of the complement cascade (i.e. C3b, C5, C567).

Once adhered to the surface of the schistosomula, eosinophils have been seen by electron microscopy to degranulate and release their toxic products in close proximity to the larval surface (McLaren *et al.*, 1981). Eosinophil major basic protein (MBP) makes up approximately 50% of these granular products and it is this together with eosinophil peroxidase which are believed to be the mediators of the killing observed (Butterworth *et al.*, 1978, Jong *et al.*, 1981).

In addition to eosinophils and macrophages, mast cells have been shown to play a role in the antibody dependent killing of schistosomula *in vitro*. Although unable to kill the parasite themselves, the depletion of mast cells greatly reduces the level of eosinophil mediated killing observed (Capron *et al.*, 1980b). Cytotoxicity is restored if mast cells are replaced with mast cell granular products, in particular eosinophil chemotactic factor A (ECF-A), and it has thus been suggested that during infection IgG2a and / or IgE activated mast cells release ECF-A, leukotriene B, PAF, IL-5, TNF, integrins and selectins, all of which are known to increase the migration of eosinophils and the expression of Fc and C3 receptors upon their surface (in Hagan *et al.*, 1991, Capron and Capron, 1994).

Neutrophils have also been seen to adhere to the schistosomula surface following incubation with sera from infected animals. Some studies have reported that complement is then required to bring about subsequent killing (Incani and McLaren, 1981). However, others have suggested that neutrophils are as cytotoxic as eosinophils in the presence of antibody alone (Anwar *et al.*, 1979).

Finally, platelets have been shown to adhere to and kill schistosomula in the presence of normal sera, although improved cytotoxicity is seen in the presence of sera from infected animals (Joseph *et al.*, 1983). This indicates that platelets are able to bind to the surface of the schistosomula via both complement and antibody, which has been shown to be of the IgE isotype in rats.

Although a number of antibody dependent immune mechanisms effectively mediate the killing of newly transformed schistosomula *in vitro*, it has been demonstrated that larvae become refractory to ADCC 24 hours after transformation (Ramalho-Pinto *et al.*, 1978, Bickle and Ford, 1982, Incani and McLaren, 1989). This can be attributed to the immune evasion tactics of the developing parasite (see section 1.5.1) and has led to questions regarding the role of antibody mediated mechanisms *in vivo* where the lung stage parasite is believed to be the target of the immune response.

(b) The role of antibody mediated mechanisms *in vivo*

Mice and rats which are B cell deficient are unable to develop resistance to reinfection (Mangold and Knopf, 1981, Sher *et al.*, 1982). Moreover, experiments involving the passive transfer of sera and McAbs have suggested that antibody has a role to play in the generation of protective immunity. The importance of antibody mediated mechanisms is however, believed to differ between the species of animal involved. Thus, the levels of immunity attained following the passive transfer of antibody from vaccinated mice are inconsistent, often poor, and rely upon the donor having received multiple vaccinations (Bickle *et al.*, 1985, Mangold and Dean, 1986). These observations are consistent with the demonstration that other T cell mediated mechanisms are of primary importance in once vaccinated mice (section 1.5.3). However, they also suggest that although there is no apparent increase in the immunity of the donor mouse following multiple vaccinations, an increase in the effectiveness of antibody mediated mechanisms does occur. This is substantiated by the demonstration that the depletion of CD4+ cells at the time of challenge abolishes the resistance observed in once immunised mice, yet has no effect on the resistance generated following multiple vaccinations (Kelly and Colley, 1988). Furthermore, the studies of Caulada-Benedetti *et al.*, (1991) have indicated that this change may be initiated by an alteration in the production of T cell cytokines. Thus the initial, predominantly Th1 type response, which results in the release of macrophage activating factors such as IFNγ and IL-2 in response to schistosomula antigens, was seen to switch upon multiple vaccination to a Th2 type response with the production of IL-4, IL-5 and hence IgG1. Some experiments using fractionated sera have indicated that the IgG fraction (Mangold and Dean, 1986) or more specifically IgG1, (Delgado and McLaren, 1990) is wholly responsible for the protective capacity of VMS. However, the studies of Jwo and LoVerde (1989) suggested that the non-IgG fraction

which contains mainly IgM and IgA, is also able to confer some protection. IgE has not been identified within VMS, and studies employing IgE deficient mice have demonstrated that this antibody isotype is not involved in the generation of immunity in multiply vaccinated mice (Sher *et al.*, 1983).

The levels of resistance seen upon the transfer of IRatS or sera from twice vaccinated rats (VRatS) to homologous recipients (25 - 60% and 60 - 70% respectively) are consistently higher than those observed following the passive transfer of VMS (Ford *et al.*, 1984a). Moreover, the importance of antibodies to the effector arm of rat immunity has been demonstrated by the observation that athymic recipients were protected by the transfer of VRatS (Ford *et al.*, 1987b). The isotype of antibodies which are required for the passive transfer of immunity differs according to the method used for the immunisation of the donors. Thus, the transfer of optimal immunity from infected rats relied upon the transfer of both IgG2a and IgE and hence confirmed the *in vivo* role of these two isotypes which is suggested by studies *in vitro*. In contrast, the transfer of vaccine immunity has been shown to rely totally upon the transfer of IgG2a. Indeed no specific IgE has been demonstrated in sera taken from rats immunised with radiation attenuated parasites (Ford *et al.*, 1987a).

How antibody kills schistosomes *in vivo* is not as yet fully understood. The lung stage schistosomula which form the targets of immunity *in vivo*, have been shown to be refractory to the antibody dependent mechanisms which kill freshly transformed schistosomula *in vitro*. However, complement depletion has been shown to reduce the immunity seen in both vaccinated and infected rats although in the vaccinated mouse the depletion of C3 had no effect (Vignali *et al.*, 1988b, Sher *et al.*, 1982). Moreover, the demonstration of immunity following the passive transfer of macrophages, platelets or eosinophils from infected rats (reviewed by Capron and Capron, 1994) has suggested that these cells armed with antibody / antigen complexes do have a role in the killing of the schistosomula *in vivo*. Following the challenge of passively immunised animals cellular reactions have been seen surrounding the dying parasites. These are similar in site and in composition to the foci seen following challenge of the donor animals. Thus, in rats passively immunised by the transfer of VRatS, the challenge parasites died in the lungs surrounded by inflammatory foci consisting mainly of radioresistant T cells and

macrophages (Vignali *et al.*, 1989b). In contrast, mice protected by the passive transfer of VMS from donors which eliminate the challenge infection in the skin, trapped the challenge larvae in the skin within radiosensitive foci of T cells, macrophages and eosinophils (Delgado and McLaren, 1989). Several suggestions have therefore been put forward regarding the role of antibody in the killing of parasites *in vivo*. Firstly, immune complexes which incorporate IgG1, IgG2a or IgE and circulating antigens could activate macrophages to release lysosomal proteins or trigger complement. The anaphylotoxins produced in this way would then attract more macrophages and eosinophils in addition to enhancing the expression of surface receptors on the latter. Products of both eosinophils and macrophages are known to damage the musculature of schistosomula, hence parasite migration could be delayed and an inflammatory response induced which traps the parasite and prevents further migration (McLaren and Smithers, 1988). Alternatively, antibodies may bind to the lung stage larvae hence hindering their migration through capillary beds and enabling them to become good targets for an antibody mediated inflammatory response (Dean *et al.*, 1987). Studies have demonstrated that, although the lung stage parasite is refractory to antibody mediated killing *in vitro*, VMS (Bickle and Ford, 1982), VRatS (Ford *et al.*, 1984a) and IRatS (Mangold, 1980) are able to bind to the surface of the lung stage schistosomula. The induction of killing by the prevention of migration could obviously not be reproduced *in vitro* and could therefore account for the antibody dependent elimination of the lung stage parasite despite its observed insusceptibility to ADCC mechanisms *in vitro*. Finally, it is feasible that the lung stage schistosomula is susceptible to *in vivo* antibody dependent immune mechanisms which are non-functional *in vitro*.

## 1.6 IMMUNITY IN HUMANS

### 1.6.1 *IN VITRO* KILLING BY HUMAN LEUCOCYTES

Studies performed *in vitro* have demonstrated that human effector cells are able to kill freshly transformed schistosomula in the presence of complement and / or antibody (Butterworth *et al.*, 1974, Anwar *et al.*, 1979, Hagan *et al.*, 1985). With regard to the latter, both the IgG (Anwar *et al.*, 1979) and IgE (Capron *et al.*, 1984) fractions of human infection sera have been shown to be capable of opsonising the schistosomula for killing. Moreover, although eosinophils are believed to be the major cytotoxic cells, a role for neutrophils and macrophages has also been proposed (Anwar *et al.*, 1979).

### 1.6.2 EVIDENCE FOR THE DEVELOPMENT OF IMMUNITY *IN VIVO*

In endemic areas the prevalence and intensity of schistosomiasis is seen to rise to a peak during the second decade of life and then decline slowly during adulthood (reviewed by Butterworth *et al.*, 1982). It was initially proposed that this decline was a consequence of the spontaneous death of the resident worms coupled with an age related decrease in water contact which prevents reinfection (Warren, 1973). However, studies in which the rates of reinfection following chemotherapy have been examined, have enabled the overall transmission rate and the water contact of individuals to be monitored during the period of reinfection and thus incorporated into the analysis of the data used to determine resistance. Such studies on *S. mansoni* in Kenya (Butterworth *et al.*, 1984, 1985), identified a group of children deemed to be resistant by virtue of their high water contact and low rates of reinfection and a group of susceptible children who became heavily reinfected following treatment. Comparison of the data for these two cohorts demonstrated that the average age of the resistant group was 13 and that of the susceptible group 11 and hence suggested the existence of age dependent factors that prevent reinfection despite high water contact. Longitudinal studies on *S. haematobium* in the Gambia (Wilkins *et al.*, 1984), have also demonstrated that individuals in a younger age group are more susceptible to reinfection after cure despite a level of water contact similar to that seen in older children.

Although it has largely been accepted that an age related increase in resistance to schistosome infection does occur, there is still much debate as to the factors involved in its development. It has been suggested that resistance takes the form of a slowly acquired immunity which is dependent upon previous exposure. Alternatively, the onset of resistance may be dependent upon age related physiological or immunological alterations. In most endemic areas age and exposure are intricately related and it is impossible to separate history of exposure from other age related effects. However, this is possible in areas where, due to immigration or recent establishment of transmission, entire communities have been exposed for a relatively short and identical period of time. Thus, the early studies of Kloetzel and Da Silva (1967), which examined the prevalence and intensity of infections in adult immigrants to an endemic area, demonstrated that the general pattern of intensity was similar to that seen in a stable endemic population. A peak in intensity occurred approximately 20 years following primary exposure, hence suggesting that age related resistance was dependent upon a long history of prior

infection. In contrast, more recent and perhaps more thorough studies (reviewed by Gryseels, 1994) have suggested that the age dependent increase in resistance is in fact independent of the extent of previous exposure. For example, studies amongst populations of recent immigrants into endemic regions in Burundi, have demonstrated that both prevalence and intensity show a peak in children of 10 - 15 years, being lower in similarly exposed adults. Studies in an area of Senegal, in which transmission was recently established following the building of an irrigation system, also gave similar results (Gryseels, 1994). Thus, these studies suggested that age related changes which occur around the time of sexual maturation, stimulate a strengthening of physical barriers or a shifting in the immune response, which protect against infection with schistosomes.

Although the extent to which the development of resistance is dependent upon age or upon previous exposure is not clear, alterations in the immune response of the host are likely to be involved. The following section details some of the studies carried out with the aim of examining the relationship between the expression of resistance and the presence of potentially protective immune responses.

### 1.6.3. THE DEVELOPMENT OF HUMAN IMMUNITY

#### 1.6.3.1 The balance of antibody isotypes

Using blood samples taken from children deemed either resistant or susceptible to reinfection (as above), Butterworth *et al.*, (1985) examined the antibody response to the schistosomula, adult and egg stages of the parasite and measured the levels of eosinophilia. The results obtained demonstrated that, although a difference in the activity of the eosinophils taken from the two groups could not be discounted, no positive correlation between resistance and the levels of antibody or number of eosinophils was detected. In contrast, negative correlations were observed between the levels of antibody directed against the egg stages and resistance to reinfection (Butterworth *et al.*, 1987). Thus, it is now believed that the slow development of human immunity may be a consequence of the need to down regulate the production of anti-egg antibodies (i.e. blocking antibodies) which are produced early in infection and prevent the cytotoxic action of other antibodies.

Blocking antibodies were initially described by Grzych *et al.*, (1982, 1984) who showed that the *in vitro* cytotoxicity and the ability to passively transfer immunity with a rat IgG2a McAb (IPLSm1), was blocked by a second McAb of the IgG2c subclass

(IPLSm3). Both McAbs recognised a 38 kDa schistosomula surface antigen (section 1.9.2.1(a)), although the latter also bound to a 20 kDa molecule suggesting that the two antibodies recognised overlapping rather than identical epitopes. McAbs of the IgM isotype which are able to block *in vitro* killing of schistosomula by IHS and eosinophils have also been described (Dunne *et al.*, 1987). These were shown to recognise the major egg polysaccharide antigen K3, in addition to periodate sensitive epitopes of the 38 and 20 kDa schistosomular antigens (Bickle and Andrews, 1988). It has therefore been suggested that antibodies raised against polysaccharide antigens secreted in large quantities by the schistosome egg block the recognition of cross reactive epitopes present within glycoproteins or other glycoconjugates exposed upon the surface of the schistosomula. Evidence that blocking antibodies are raised during a natural infection in humans has been provided by Khalife *et al.*, (1986) who demonstrated that the *in vitro* killing of schistosomula by the IgG fraction of sera taken from infected individuals was abolished by prior incubation of the larvae with the IgM fraction. Further analysis of the binding patterns of the sera revealed that both fractions recognised the previously described 38 kDa schistosomula surface antigen. Moreover, the levels of IgM against the 38 kDa antigen were shown to be greater in sera taken from children susceptible to reinfection than in those which were resistant.

The levels of IgG2 in human sera have also been shown to correlate negatively with resistance to reinfection (Dunne *et al.*, 1987, Demeure *et al.*, 1993). As IgG2, like IgM, is raised in response to carbohydrate epitopes, it has been suggested that antibodies of this subclass stimulated by carbohydrate epitopes on the egg also contribute to the lack of immunity seen in the young by preventing the binding of effector antibodies of a different IgG subclass to cross reactive epitopes upon the surface of the schistosomula.

In contrast to the killing mediated by IgG, that mediated by eosinophils and IgE was not affected by prior incubation of schistosomula with IgM (Khalife *et al.*, 1986). This suggests that IgE antibodies recognise different epitopes to those recognised by blocking antibodies of the IgM isotype. Moreover, it has been shown in studies with *S. haematobium* (Hagan *et al.*, 1991) and *S. mansoni* (Rihet *et al.*, 1991, Dunne *et al.*, 1992, Demeure *et al.*, 1993) that the levels of IgE directed against larval (Rihet *et al.*, 1991, Demeure *et al.*, 1993) and adult (Hagan *et al.*, 1991, Dunne *et al.*, 1992) schistosome antigens are higher in older, i.e. resistant individuals than in those which remain susceptible to reinfection. However, in addition to the elevated levels of IgE seen

in schistosomiasis, levels of IgG4 are also raised (Bocter and Peter, 1990). These are higher in younger susceptible children than in the older resistant age group. Thus, it has been suggested that for immunity to develop a high IgE response must be coupled with a lower IgG4. How this balance is derived is not obvious as the production of both IgE and IgG4 are controlled by the release of IL-4. Nevertheless it has been shown that the levels of IFNγ which are necessary to inhibit the IL-4 dependent production of IgE are lower than those required to inhibit the generation of IgG4. It has therefore been proposed that the onset of immunity may represent an immunoglobulin class switch which is a consequence of varying levels of different T cell cytokines (Hagan *et al.*, 1991). Like the IgM and IgG2 isotypes which block the IgG cytotoxic killing of the schistosomula, IgG4 is believed to be directed against egg antigens which cross react with larval antigens and hence prevents the binding of IgE effector antibodies. However, unlike the former isotypes, IgG4 is raised predominantly in response to periodate insensitive epitopes of a peptide nature (Dunne *et al.*, 1988).

#### 1.6.3.2 Differential recognition of schistosome antigens

In addition to examining the nature of the overall antibody response to schistosome infection, attempts have been made to identify responses to individual antigens which correlate with the development of immunity.

The negative correlation between resistance to reinfection and the level of blocking antibodies raised against the 38 kDa schistosomula surface antigen (described above) suggests that a switch to an effective antibody response against this molecule may be involved in the onset of immunity and is consistent with the ability of McAbs against this antigen to passively transfer resistance (Grzych *et al.*, 1982, Kelly *et al.*, 1986).

A schistosomula surface antigen which is recognised preferentially by IgG antibodies within the sera of subjects with a high level of resistance to reinfection has also been described. This 37 kDa antigen is recognised by sera from 80 - 90% of resistant individuals and only 14 - 33% of sera from patients susceptible to reinfection (Dessein, *et al.*, 1988). The molecule is thus considered to be a of interest as a vaccine candidate antigen, and the corresponding gene has been cloned, sequenced and shown to encode the homologue of human and mouse glyceraldehyde-3P-dehydrogenase (Goudot-Crozel *et al.*, 1989).

Schistosome paramyosin (James *et al.*, 1985) has also been reported to be recognised

preferentially by sera from individuals living in an endemic area who fail to secrete eggs (Correa-Oliveira *et al.*, 1989). Moreover, following chemotherapy, the levels of antibody against this molecule remain high only in those individuals which are cured and remain stool negative. It has therefore been suggested that an active infection may suppress the levels of anti-paramyosin antibodies and that a response to paramyosin may play a role in the onset of age dependent immunity.

An increase in the IgA response to the 28 kDa schistosome equivalent of mammalian glutathione-S-transferase (Sm28 GST) has also been demonstrated to correlate positively with age and hence with an increase in resistance to schistosome infection (Grzych *et al.*, 1993). Furthermore, the IgA fraction of sera raised against this antigen has been shown to have a direct negative effect upon the fecundity of the female worm (section 1.9.2.2 (a)).

Finally, age dependent resistance to reinfection has been positively correlated with an increased IgE response to adult worm antigens of 22 and 15 kDa (Dunne *et al.*, 1992). Both of these antigens have been purified and a clone encoding the former (Sm22) identified. Sequencing of this clone revealed the antigen to be identical to that previously described by Stein and David (1986) which is present within the tegument of the 5 day old schistosomula and the adult worm but is not exposed upon the surface. It is not yet known whether Sm22 represents a true target of immune attack or whether it is simply a marker of immunity (reviewed by Butterworth, 1994).

## 1.7 MONOCLONAL ANTIBODIES

In an attempt to identify molecules of particular interest as vaccine candidate antigens, a large number of McAbs have been produced. As it is believed that the larval stages of the schistosome are important targets of immunity, a significant proportion of those selected for further studies recognise antigens upon the surface of the schistosomula (see Table 1.1). On some occasions, the production of such McAbs was enhanced by the use of restricted antigen preparations e.g. detergent extracts of schistosomula (Harn *et al.*, 1985, Bickle *et al.*, 1986), to immunise the donors of the spleen cells which were subsequently used for fusions. On other occasions, whole unattenuated parasites (Grzych *et al.*, 1982, Zodda and Phillips, 1982, Hazdai, *et al.*, 1985), irradiated cercariae (Taylor and Butterworth, 1982, Gregoire *et al.*, 1987) or even egg homogenates have been employed (Harn *et al.*, 1984). Many of the McAbs which recognise the surface of the

**TABLE 1.1** **Monoclonal antibodies reacting with the surface of *S. mansoni* schistosomula**

| McAb | Target antigens (kDa) | Isotype | Passive protection | Immunising preparation* | Reference |
|---|---|---|---|---|---|
| IPL Sm1[r] | 38[s,c], >150[c] | IgG2a | 53 - 62% | Unattenuated cercariae | Grzych *et al.*, 1982<br>Dissous *et al.*, 1982 |
| 3AF12 - D6 | >200[s], 38[s], 20[s], 17[s]<br>160[c], 130[c] | IgG1 | 40 - 50% | Unattenuated cercariae | Zodda & Phillips, 1982<br>Kelly *et al.*, 1986 |
| 2CH12-HI | >200[s] | IgG3 | 38% | Unattenuated cercariae | Kelly *et al.*, 1986 |
| 129A3/1/3 | 200[cm], 160[cm], 155[cm], 43[cm] | - | N.A. | Purified cercarial glycoproteins | Strand *et al.*, 1982 |
| MSM1.37 | 24[s] | IgG1 | N.A. | Unattenuated cercariae | Taylor & Butterworth, 1982 |
| MSM1.29 | 24[s] | IgM | N.A. | Unattenuated cercariae | Taylor & Butterworth, 1982 |
| E1 | 160[s], 130[s], >200[c], 200[c], 111[e] | IgG2b | 21 - 41% | Egg homogenate | Ham *et al.*, 1984 |
| IPL Sm3 | 38[s], 20[s] | IgG2c | Blocks killing by IPL Sm1 | Unattenuated cercariae | Grzych *et al.*, 1984 |
| WP66.4 | 155[s,cm] | IgM | 32 - 43% | Unattenuated cercariae | Smith *et al.*, 1982<br>Smith & Clegg, 1985 |
| 9B | 200[s,m] | IgG1 | 35% | Unattenuated cercariae | Hazdai *et al.*, 1985 |

**TABLE 1.1 cont:-**

| 1C | $200^{*,nr}$ | IgG1 | 38% | Unattenuated cercariae | Hazdai *et al.*, 1985 |
|---|---|---|---|---|---|
| M1 | $28^{s,aw}$ | IgG1 | 41 - 50% | Detergent extract of schistosomula | Ham *et al.*, 1985a<br>Ham *et al.*, 1992 |
| M2 | $23^{c,s,aw}$ | IgG1 | 35% | Detergent extract of schistosomula | Ham *et al.*, 1985b<br>Ham *et al.*, 1987b |
| M7B3A | $16^{s,wb}$ | IgG3 | 33 - 70% | Detergent extract of schistosomula | Bickle *et al.*, 1986 |
| M22H12C | $32^{s,aw}$ | IgG2a | 27 - 58% | Irradiated cercariae | Bickle *et al.*, 1986 |
| 2CH12-H1 | $>200^{s}$ | IgG3 | 38% | Unattenuated cercariae | Kelly *et al.*, 1986 |
| 1.G1 | $85\text{-}130^{s}, 97^{aw}$ | IgM | 38% | Irradiated cercariae | Gregoire *et al.*, 1987 |
| 4.4B | $85\text{-}130^{s}, 97^{aw}$ | IgM | 51% | Irradiated cercariae | Gregoire *et al.*, 1987 |
| 204-3E4 | $68^{s,aw}$ | IgG2a | N.A. | Freeze / thaw schistosomula | Weist *et al.*, 1991 |

(*) Antigen preparation used for the immunisation of mice from which spleen cells were taken for fusion.

(r) Rat McAb.

(s) Mr of schistosomular antigen recognised by McAb

(c) Mr of cercarial antigen recognised by McAb

(aw) Mr of adult worm antigen recognised by McAb

(e) Mr of egg antigen recognised by McAb

(nr) Mr on antigen under non reducing conditions, antigens of 30 and 45 kDa seen under reducing conditions

(wb) Seen on Western blots only

larvae as judged by immunofluorescence, have been further tested for their protective potential via *in vitro* cytotoxicity assays and *in vivo* passive transfer experiments. The antigens recognised by those which were protective could then be identified and in some cases have been purified by immunoaffinity chromatography.

The ability of McAbs to protect mice and rats from a challenge infection confirmed a role for antibody mediated mechanisms in both of these species. Furthermore, despite the observed importance of particular antibody isotypes in infected rats (IgE/IgG2a), vaccinated rats (IgG2a) and in vaccinated mice (IgG1), studies using McAbs have demonstrated that antibodies of various isotypes, which recognise carbohydrate or peptide epitopes, can confer protection. Obtaining high levels of protection following the administration of antibody against a single antigenic moiety also indicates that vaccination with defined antigen preparations could be successful.

Table 1.1 describes some of the more prominent McAbs produced to date. Further details regarding those which target vaccine candidate molecules are also given in the following sections.

## 1.8 CHARACTERISATION OF ANTIGENS AT THE SCHISTOSOME SURFACE

### 1.8.1 SCHISTOSOMULA MEMBRANE ANTIGENS

The larval stages of the schistosome parasite are considered to be good targets for a vaccine against schistosomiasis. Unlike older parasites, the schistosomula are susceptible to killing *in vitro* by a variety of antibody dependent mechanisms. Moreover, studies using radiation attenuated schistosomula have demonstrated that the death of the larval stage alone stimulates good, immunologically specific protection. Amongst the antigens expressed by the schistosomula, those present within the surface membrane are of particular interest as vaccine candidate antigens. The membrane represents the barrier between the parasite and the host and as such may contain molecules involved in ion and molecular transport, signal transduction and other physiological or metabolic functions which are vital to parasite survival. Furthermore, it seems probable that changes in the expression of surface membrane antigens are involved in the increased resistance of the later stages of the parasite to immune mechanisms (Pearce *et al.*, 1986). Attempts have therefore been made to characterise the molecules present within the schistosomula membrane and to examine their recognition by a variety of sera.

Two dimensional (2D) electrophoresis of $^{125}$I (IODOGEN) labelled schistosomula

surface components revealed about twenty heavily labelled molecules of which a significant proportion were judged to be antigenic following immunoprecipitation experiments (Kelly *et al.*, 1985). Thus, for parasites residing in an immunologically hostile environment, schistosomula express a surprisingly large repertoire of surface antigens. This may reflect the importance of the surface membrane in the uptake of nutrients etc. from the host. The schistosomula surface antigens precipitated by various immune sera covered a broad range of molecular weights, although the majority of the heavily labelled molecules ranged from 10 - 20 kDa with the exception of an acidic 32 - 38 kDa antigen complex. The latter, which was originally described by Dissous *et al.*, (1981), has been labelled by the IODOGEN method (Simpson *et al.*, 1984, Payares *et al.*, 1985a, Omer Ali *et al.*, 1986) and by lactoperoxidase catalysed iodination (Dissous *et al.*, 1981, Simpson *et al.*, 1983a) and is recognised by CIS, VMS, IHS and a rabbit sera raised against purified membranes of the adult worm (Rabαm). Further analysis of this antigenic complex revealed that it consisted of a number of antigens which include the 38 kDa antigen described by Grzych *et al.*, (1982, 1984) as the target of both a protective and a blocking McAb (Dissous *et al.*, 1982, section 1.6.3.1) and a 32 kDa antigen recognised by a passively protective McAb described by Bickle and Andrews (1986). The precipitation of the 38 and the 32 kDa molecules by different McAbs demonstrates that these are two distinct antigens. However, the 38, the 32 and a 20 kDa antigen which is also recognised amongst surface membrane molecules, have been shown to be differently glycosylated versions of the same peptide backbone (Payares *et al.*, 1985b).

In addition to the 32 - 38 kDa complex, antigens of 200, 94, 68, 45, and 17 kDa have been precipitated from labelled surface antigens with CIS. Similar antigens were also recognised by IHS and by VMS although the latter failed to precipitate the 17 kDa antigen and recognised an additional antigen of 15 kDa. With the exception of the 68 and the 45 kDa molecules, all of these antigens failed to precipitate following prior incubation of CIS / VMS with living schistosomula (Simpson *et al.*, 1983a) thus indicating that they are exposed upon the surface of the membrane. With regard to the 68 and 45 kDa antigens, it is possible that these are present within the surface membrane although their immunogenic moieties remain unexposed. Alternatively, some permeation of the $^{125}$I label into the parasite surface could have occurred (Simpson *et al.*, 1983a). An 18 kDa molecule has also been recognised amongst surface membrane molecules by sera from

rabbits vaccinated with irradiated cercariae (VRabS) (Liberti *et al.*, 1986, Cioli *et al.*, 1987). This molecule is not recognised by CIS, IRatS nor IHS and as such has similar properties to the 16 kDa antigen identified by Bickle *et al.*, (1986), which is the focus of much of the work carried out here. The 16 kDa antigen is not however, readily radiolabelled, although it is recognised amongst a detergent extract of schistosomula following Western blotting and is present upon the surface of the schistosomula as demonstrated by McAb immunofluorescence.

During parasite maturation alterations occur in the expression of larval surface antigens. Thus, after culturing of young schistosomula for 24 hours *in vitro* the 17 and 200 kDa antigens are lost from the surface, whilst following incubation for 48 hours, the 38 kDa antigen is lost and the 32 kDa molecule becomes the major antigen precipitated by CIS, VMS and Rabαm (Simpson *et al.*, 1984, Payares *et al.*, 1985a). The 32 - 38 kDa complex can be radiolabelled in the cercarial stages and precipitated with VMS, CIS (Payares *et al.*, 1985a) or a McAb raised against the 38 kDa antigen (Dissous *et al.*, 1985). It has therefore been suggested that the 38 kDa antigen and other antigens seen upon the surface of the freshly transformed schistosomula may represent the remnants of the cercarial glycocalyx which upon the completion of transformation are lost and replaced by a dominant 32 kDa glycoprotein present within the new heptalaminate membrane. Alternatively, the loss of some of these antigens could be a consequence of their release from the newly formed heptalaminate membrane. As the 38 and 200 kDa antigens have been shown to be held within the membrane via a glycosylphosphatidylinositol (GPI) anchor rather than a transmembrane domain, it has been suggested that their release could be facilitated by the cleavage of this anchor with endogenous or host phosphatidylinositol-specific phospholipase C (PIPLC), or by the absence of a cytoplasmic domain (Pearce and Sher, 1989, Sauma and Strand, 1990). However, Simpson *et al.*, (1984) have demonstrated that the 17 and 38 kDa antigens are not present within the culture fluid, thus the incorporation of molecules into the lipid bilayer offers an alternative explanation for the disappearance of some antigens from the parasite surface. Newly exposed antigens of 8 and 15 kDa are also recognised by Rabαm upon the surface of the larvae following 24 hour *in vitro* culture (Simpson *et al.*, 1984). This 15 kDa molecule is believed to be a different antigen to that of 15 kDa labelled with $^{125}I$ upon the surface of the freshly transformed schistosomula (see above) as the latter is

not recognised by Rabαm. Moreover, the precipitation of the former is ablated following prior incubation of VMS with whole worm homogenate whilst the latter is still observed.

Lung stage worms removed from the host continue to express the newly exposed 8 and 15 kDa antigens and the 32, 20 and 15 kDa antigens originally observed upon the surface of younger larvae. In addition, major antigens of 25 and 97 kDa are seen by Rabαm and a 65 kDa antigen by antisera raised against the 32 kDa molecule partially purified from adult worm membranes (Payares *et al.*, 1985a). The 25 kDa antigen is of interest as a vaccine candidate molecule (section 1.9.2.1 (c)) and the 65 kDa molecule has been shown to represent schistosome alkaline phosphatase. The latter may be expressed particularly upon the surface of older parasites to meet the increased nutritional and / or physiological requirements of the developing parasite. Further membrane alterations then occur as the parasite matures from the lung stage larvae into the adult worm.

### 1.8.2 ADULT MEMBRANE ANTIGENS

Using the IODOGEN method, Payares *et al.*, (1985a, 1985c) were able to radiolabel a wide range of molecules on the surface of 3 week old juvenile worms. These included the 32 and 20 kDa antigens originally recognised on the young schistosomula by VMS, CIS and Rabαm, in addition to the major antigens of 25, 97 and 65 kDa which were initially precipitated by Rabαm from the molecules of the lung stage larvae. In contrast, the labelling of 6 week old worms has proved problematic (Hayunga *et al.*, 1979). Some authors have observed the labelling of a small number of proteins only after a particularly long exposure (Snary *et al.*, 1980) whilst others have been unable to label any proteins at all (Payares *et al.*, 1985a). It is believed that this inability to label proteins upon the surface of the adults is a consequence of the sequestration of parasite antigens (Payares *et al.*, 1985a) and / or the masking of proteins by other surface molecules. With regard to the latter, the surface of the adult worm has been shown to be rich in lipid molecules which are able to bind the radiolabel, and glycolipids and glycoproteins of host origin are also absorbed (Clegg *et al.*, 1971, Sher *et al.*, 1978). The removal of lipid with organic solvents (Hayunga *et al.*, 1979) or the stimulation of membrane turnover *in vitro* (Roberts *et al.*, 1983) has also been shown to increase the binding of radiolabel to the surface of the adult worm.

As an alternative to labelling the surface of the intact adult parasites, the tegument

of the mature worm has been isolated by a variety of methods and subjected to radioiodination (Payares *et al.*, 1985a, Simpson *et al.*, 1989, 1990). This has demonstrated that the previously described antigens of 32 and 20 kDa are still present with the membrane, as are those of 8, 15, 25 and 97 kDa, which were initially recognised upon the older schistosomula. A 13 kDa antigen and another antigen of 15 kDa which failed to radiolabel have also been shown to be major antigens recognised by Rabαm on Western blots of isolated adult membranes (Smithers *et al.*, 1990). The absence of a signal following prior absorption of Rabαm with living schistosomula demonstrated that the majority of these antigens shared epitopes with antigens present upon the surface of the newly transformed larvae. Thus, it has been concluded that the adult worm tegument contains many proteins which are identical to, or cross reactive with, those of the schistosomula. However, these are not accessible to IODOGEN catalysed iodination and are unavailable for antibody binding *in vivo*, thus enabling the adult worms to evade the immune mechanisms of the host.

## 1.9 VACCINATION

### 1.9.1. CRUDE ANTIGEN PREPARATIONS

As described in previous sections immunisation with live or radiation attenuated parasites has stimulated good levels of protective immunity in a number of animal models. Immunisation with dead material has however, proved less effective and despite stimulating the production of significant levels of cytotoxic antibody, initial attempts to immunise animals with various worm, cercarial and egg preparations in conjunction with a number of different adjuvants, failed to provide good levels of protection (Murrell *et al.*, 1975, reviewed by Clegg and Smith, 1978 and by Dean, 1983). Thus the immunisation of mice with ground worms in conjunction with *Corynebacterium parvum* (Maddison *et al.*, 1978) or with whole worm homogenate plus a saponin adjuvant (Smithers *et al.*, 1989) has been reported to stimulate significant levels of resistance. However, the observed reductions in the worm burdens were poor (29% and 19 - 37%) and in the experiments of Maddison *et al.*, (1978) particularly large doses of bacterial adjuvant were required. Similarly, isolated adult worm teguments have been used with FCA and *C. parvum* to immunise rabbits (Tendler *et al.*, 1986). Here, 81% and 61% reductions in worm burdens were observed. However, immunisation with FCA and *C. parvum*, gave 52% and 32% protection when administered alone.

More promising results have been obtained following immunisation with the larval stages of the parasite or with their released products. Thus, frozen and thawed schistosomula have been seen to stimulate significant levels of immunity when administered to mice in conjunction with BCG (35% - 54%) (James, 1985, Keisari, *et al.*, 1993). The studies of James (1985) demonstrated that this was dependent upon intradermal administration of the vaccine and in both studies the development of protection appeared to correlate with T cell proliferation and macrophage activation. In contrast, the studies of Horowitz *et al.*, (1982) and Auriault *et al.*, (1984) suggested a link between the development of protection and the titre of specific IgE raised following vaccination with cercarial antigens. With regard to the former studies, sonicated cercarial antigens were used to immunise mice together with alum, an adjuvant known to stimulate particularly high levels of IgE. Analysis of the data obtained demonstrated that the highest levels of protection (34% - 91%) were observed in those mice with the highest titre of IgE. The studies of Auriault *et al.*, (1984, 1985) used the material released into the media following 16 hour *in vitro* culture of schistosomula (SRP-A) to immunise rats in the absence of adjuvant. Again high titre IgE was seen and it was demonstrated that this was capable of killing schistosomula in concert with macrophages, eosinophils and platelets from rats, monkeys and humans. Moreover, protective immunity was generated in rats following immunisation with SRP-A (46 - 83%) or passive transfer of anti-SRP-A antibodies (32 - 83%) (Auriault *et al.*, 1985, Damonneville *et al.*, 1986). In view of the observations made regarding the role of IgE and ADCC in both infected and vaccinated rats (section 1.5.3.2) the association of this antibody isotype with the high levels of immunity observed in the studies of Auriault *et al.*, (1985) is not surprising. However, the aforementioned findings of Horowitz *et al.* (1982) are somewhat unexpected as a role for IgE has not been proposed in the development of immunity in the mouse. In addition, it has been demonstrated that mice depleted of IL-4, IL-5 and IgE are able to develop good levels of immunity in response to the irradiated vaccine (Sher *et al.*, 1983, 1990).

Finally, it has recently been demonstrated that immunisation of mice with the antigens released during *in vitro* culture of lung stage schistosomula plus recombinant IL-12, stimulates significant levels of protection (53%). This is consistent with the role of IL-12 in the development of murine vaccine immunity (section 1.5.3.1) and further studies regarding the use of this cytokine as an adjuvant are being performed (Mountford *et al.*, 1994).

## 1.9.2 DEFINED ANTIGEN VACCINES

Although the immunisation of laboratory animals with radiation attenuated parasites has been shown to produce good, immunologically mediated resistance, the use of the irradiated vaccine in humans is not ethically acceptable nor is it practically viable due to the large amounts of parasite material which would be required. The latter point also applies to the use of the few crude antigen preparations which have been shown to stimulate significant levels of immunity and thus recent work has concentrated on attempts to define the protective antigens which are responsible for the immunity generated in resistant laboratory animals. Many different strategies have been employed with the aim of defining such antigens. These include the identification of immunogenic molecules within crude parasite extracts, the isolation of the target antigens of passively protective McAbs and the selection of antigens or recombinant clones which are recognised predominantly by protective sera. It is hoped that protective antigens or epitopes identified may ultimately form the basis of a defined antigen vaccine which can be produced by recombinant technology or chemical synthesis. In addition to abolishing the requirement for large amounts of parasite material the production of such a vaccine would have the added advantage of enabling components which are toxic, autoimmunogenic or unnecessary for the development of resistance, to be omitted.

Thus far a number of vaccine candidate molecules have been defined. The most prominent of these are discussed in further detail below.

### 1.9.2.1 Surface membrane antigens

(a) The 38 kDa antigen

Following the demonstration that rat antibodies of the subclass IgG2a were involved in the killing of schistosomula *in vitro* (section 1.5.3.2), Grzych *et al.*, (1982) produced hybridomas secreting antibodies of this isotype which were able to induce eosinophil mediated ADCC. One of these antibodies, IPLSm1, which was seen to kill up to 85% of young schistosomula was also able to transfer passive protection to naive rats (27% - 58%). Subsequent studies (Dissous *et al.*, 1982) demonstrated that IPLSm1 recognised a 38 kDa molecule which formed part of the 32 - 38 kDa antigenic complex initially described by Dissous *et al.*, (1981) as the major antigens precipitated following probing of radiolabelled schistosomula surface antigens with VMS, CIS or IHS. The 38 kDa antigen itself is present only upon the cercariae and the young schistosomula (section

1.8.1). However, IPLSm1 together with a host of other anti-38 kDa protective McAbs have been shown to recognise an additional antigen of >200kDa on the surface of the schistosomula, a range of other differently sized antigens upon the miracidia, cercariae, adult and egg stages of the parasite (see Table 1.1) and perhaps, most surprisingly, an antigen present in the tissues of the intermediate snail host (Zodda & Phillips, 1982, Harn *et al*, 1984, 1987a, Kelly *et al.*, 1986, Dissous *et al.*, 1986). The latter was serendipitously shown to be a consequence of cross-reaction between the 38 kDa antigen and keyhole limpet haemocyanin (KLH) (Dissous *et al.*, 1986). It has therefore been concluded that the 38 kDa antigen is a glycoprotein containing immunodominant carbohydrate epitopes which are also present upon other molecule of parasite and snail origin.

In addition to being the target of a range of protective McAbs, the carbohydrate epitopes present on the 38 kDa and / or other cross reactive antigens stimulate the production of blocking antibodies experimentally (e.g. IPLSm3) and *in vivo* (see section 1.6.3.1). It is this, coupled with an inability to produce carbohydrate epitopes using recombinant technology, which makes the use of the 38 kDa vaccine candidate glycoprotein particularly problematic. Grzych *et al.*, (1985) attempted to overcome these problems by raising rat anti-idiotype McAbs to IPLSm1. One of these McAbs (JM8-36) which was able to inhibit the binding of IPLSm1 to the 38 kDa antigen, was then used to immunise rats. Analysis of the results obtained demonstrated that anti-anti-idiotype antibodies were raised in response to JM8-36. These resembled the original McAb IPLSm1 with regard to their ability to passively transfer immunity *in vivo* and to mediate eosinophil cytotoxicity *in vitro*. Moreover, a significantly reduced worm burden (50% - 80%) was observed following a challenge infection of JM8-36 immunised rats.

Finally, as a consequence of the difficulties faced in obtaining substantial amounts of the 38 kDa glycoprotein for immunisation, attempts have been made to immunise animals with KLH. This molecule, which is often used as a carrier protein in immunisation protocols, can be obtained in large amounts commercially and has been shown to stimulate significant levels of immunity in rats (Grzych *et al.*, 1987). KLH is at present being used to vaccinate cattle and sheep against natural infections of both *S. bovis* and *S. japonicum* (Taylor *et al.*, 1994). Initial studies have demonstrated that following the immunisation of cattle with KLH, a decrease in the fecundity of the female *S. bovis* worm is observed although the overall worm burden is not affected (Bushara *et*

*al.*, 1993).

(b) <u>The 23 kDa antigen (Sm23)</u>

Sm23 is an integral membrane protein which was first described by Harn *et al.*, (1985b) as the target of a McAb (M2) raised from animals immunised with a membrane enriched schistosomula preparation. The McAb was initially selected on account of its ability to bind to the surface of both cercariae and freshly transformed schistosomula. However, subsequent immunofluorescence studies revealed that Sm23 was also retained upon the surface of the schistosomula following 96 hours in culture, was seen upon the surface of the lung stage larvae following the removal of host antigen and was precipitated from a radiolabelled extract of adult worms. As the lung stage larvae are believed to be the target of the immune response *in vivo*, the binding of M2 to lung worms suggested that Sm23 was of particular interest as a possible vaccine candidate antigen. This was substantiated by the demonstration that Sm23 was one of the major schistosomula antigens precipitated by VMS (Oligno *et al.*, 1988, Bickle *et al.*, 1990) and by sera raised in mice optimally immunised by infection plus treatment with Ro 11-3128 whilst the parasites were still in the skin (section 1.5.2.1, Bickle *et al.*, 1990). The passive transfer of M2 has also been shown to stimulate a 35% decrease in the observed worm burden of naive recipients (Harn *et al.*, 1987b). Furthermore, active immunisation with Sm23 purified by McAb immunoaffinity chromatography and subsequent electroelution, induced significant albeit low levels of protection (11 - 27%) (Harn *et al.*, 1987b).

Further information regarding the structure of Sm23 has been obtained following the cloning experiments of Wright *et al.*, (1990) and Dalton *et al.*, (1987a). With regard to the former, sera eluted from the 23 - 25 kDa region of a Western blot of integral membrane molecules was used to screen an adult cDNA library. The isolated clones were shown to encode a 23 kDa antigen which was subsequently realised to be identical to the molecule described by Harn *et al.*, (1985b) (i.e. Sm23). Analysis of the sequence obtained for this antigen revealed the presence of three N-terminal transmembrane regions, a large hydroph ic domain which contained putative sites for N-glycosylation and a fourth C-terminal hydrophobic domain. However, despite the obvious presence of domains suitable for the anchoring of Sm23 within the parasite membrane, recent studies have demonstrated that during post-translational processing the C terminal hydrophobic domain is replaced by a GPI anchor. Further experiments subsequently revealed that

cleavage of this anchor with phosphatidylinositol-specific phospholipase C (PIPLC) failed to release Sm23 from the membrane. Hence this molecule is unusual in that it appears to use both transmembrane domains and a GPI group for its anchorage within the membrane (Köster and Strand, 1994). Comparison of the sequence for Sm23 with others in the database has also revealed that this antigen is homologous (84%) to a 23 kDa molecule of *S. japonicum* (Sj23) and to various members of a superfamily of molecules expressed upon the surface of mammalian cells. The latter include the tumour associated antigen ME491 (see below).

With regard to the studies of Dalton *et al.*, (1987a), an 18 kDa antigen was selected from radiolabelled schistosomula surface antigens using a McAb produced in mice vaccinated with irradiated cercariae. This antigen which was also recognised by CIS and more predominantly by VMS, was precipitated from the metabolically labelled products of both schistosomula and adult worms. Sera raised against this molecule were used to screen a cDNA library and a clone isolated. Subsequent sequencing of this clone and comparison with the sequence obtained by Wright *et al*, (1990) revealed that this clone also encoded Sm23 (Wright *et al.*, 1991a).

More recently studies aimed at identifying the immunogenic regions of Sm23 have been performed. Thus, synthetic peptides representing particular regions of the molecule, have been probed with a variety of sera and used to stimulate T cells (Reynolds *et al.*, 1992). Not surprisingly both B and T cell epitopes were located within the hydrophilic region of the molecule which is presumed to be extracellular and is also the region which shows the least homology to other Sm23 related surface molecules. Treatment of the synthetic peptides and the whole antigen expressed as a recombinant protein revealed that the majority of these epitopes were also susceptible to denaturing with reducing agents and hence conformational. Despite the latter a multiple antigenic peptide (MAP) incorporating linearly synthesised B and T cell epitopes, has been shown to stimulate approximately 70% protection in mice when administered with alum (Harn and Reynolds, 1993).

Finally, several suggestions regarding the function of Sm23 have been put forward. Its homology to the tumour associated antigen ME491 and other molecules which may represent receptors for growth cell factors (e.g. CD37, TAPA-1), has suggested a role for Sm23 in cellular proliferation and parasite growth (Wright *et al.*, 1991a). Alternatively, members of this family of related proteins and hence Sm23 could have a

role in adhesion or as vehicles of cell motility (in Reynolds *et al.*, 1992). Furthermore, as many of these mammalian antigens have been seen to be exposed upon the surface of haemapoietic cells, it has been suggested that the expression of Sm23 by the blood dwelling schistosome parasite may act as a decoy and / or cause a down-regulation of the immune response. In this regard, it has been reported that the response to a peptide incorporating both B and T cell epitopes of Sm23 is decreased following multiple exposures to cercariae (Reynolds *et al.*, 1992).

(c) The 25 kDa antigen (Sm25)

The development of immunity following the intrahepatic transfer of adult worms has demonstrated that antigens present within this stage are able to stimulate an immune response which protects against reinfection. Thus, Knight *et al.*, (1989) attempted to identify the antigens responsible for this protective immunity by screening an adult cDNA library with sera raised in rabbits immunised with purified adult worm teguments. A recombinant clone was isolated and shown to encode the 25 kDa antigen (Sm25) previously described by Payares *et al.*, (1985a) as the dominant antigen precipitated by Rabαm.

Sm25 is believed to be of importance in the development of protective immunity as following the immunisation of mice with adult tegumental antigens the levels of protection obtained were observed to correlate with the titre of the antibody raised against this antigen (Smithers *et al.*, 1989). Furthermore, Sm25 is recognised by protective sera raised in Fischer rats but not by non-protective sera raised in rats of the Wistar-Furth strain (El-Sherbieni *et al.*, 1990). Recognition of Sm25 also correlated with the inheritance of resistance in crosses between resistant WEHI 129/J mice and mice of the susceptible Balb/c strain (Wright *et al.*, 1988).

Sm25 is not seen upon the surface of the cercariae or schistosomula stages. It is, however, present within the lung stage larvae (Payares *et al.*, 1985a) and it has been suggested that the antigen is associated with the cytoplasmic face of the adult surface membrane (Payares *et al.*, 1985c). Extraction of Sm25 with the detergent TX-114 has indicated that the antigen has an integral membrane nature (Karcz *et al.*, 1988, Omer Ali *et al.*, 1991) and the probing of adult sections with sera raised against a recombinant protein expressed by the partial length clone isolated by Knight *et al.*, (1989) has shown that the antigen is restricted to the tegument and the cytons underlying the muscle layer.

The partial length clone isolated by Knight *et al.*, (1989) was also used to obtain a full length clone encoding Sm25 (Omer Ali *et al.*, 1991). Analysis of the sequence data obtained from this clone revealed a stretch of hydrophobic amino acids at the C-terminus of the predicated sequence (Omer Ali *et al.*, 1991). This domain appeared to be too short to span the membrane and also contained a number of polar residues. However, it has subsequently been demonstrated (Pearce *et al.*, 1991b) that the attachment of a palmitic acid to a cysteine residue within this domain is involved in the stabilising of this region and the anchoring of Sm25 within the surface membrane.

**1.9.2.2 Soluble antigens**

Description of the surface molecules of schistosomes has largely focused upon those which are intrinsic to the surface membrane. However, a number of vaccine candidate molecules have been described which are soluble proteins only peripherally and often transiently associated with the schistosome surface. Of these a number appear to be parasite enzymes whilst one is a muscle component.

(a) 28 kDa schistosome glutathione-S-transferase (Sm28 GST)

The schistosome GSTs are a group of molecules which are homologous to mammalian glutathione-S-transferases. These enzymes are involved in the maintenance of reduced glutathione (GSH) and in catalysing the detoxification of a variety of xenobiotics by conjugation with GSH. Two major GST isoenzymes have been characterised in both *S. mansoni* and *S. japonicum*. These have molecular weights of 28 kDa (Sm28 GST and Sj28 GST) and 26 kDa (Sm26 GST and Sj26 GST). A third, little characterised 28 kDa isoenzyme has also been described in *S. mansoni* (Tiu *et al.*, 1988, Wright *et al.*, 1991b).

With regard to *S. mansoni*, work has largely centred upon the characterisation of Sm28 GST. Interest in this molecule was stimulated when Balloul *et al.*, (1985) demonstrated that rat sera, raised against a 28 kDa band of electrophoretically separated adult worm molecules precipitated an antigen expressed upon the surface of the susceptible schistosomula stage of the parasite. Subsequently, the 28 kDa molecule was purified from SDS PAGE gels and used to immunise rats and mice. The sera obtained from immunised rats was able to passively transfer protection to naive recipients and to act in concert with eosinophils to kill schistosomula *in vitro*. Moreover, both rats and

mice immunised with the electroeluted antigen demonstrated a significantly reduced worm burden (50% - 70% and 39% - 43% respectively) (Balloul *et al.*, 1987a). Sera raised in animals vaccinated with the eluted protein were then used to screen an adult worm cDNA library and clones encoding the 28 kDa antigen were isolated (Balloul *et al.*, 1987b). Sequencing of these clones revealed that the 28 kDa molecule represented a schistosome equivalent of mammalian GST. A recombinant form of Sm28 GST (rSm28 GST) was then produced and used to extend the protection experiments to primates (Balloul *et al.*, 1987c, Boulanger *et al.*, 1991). In baboons the protection afforded by immunisation with rSm28 GST in terms of worm reduction was somewhat erratic (0% - 80%) and was dependent upon the amount of protein used, the number of doses given and the adjuvant employed. However, in addition to causing a reduction in worm burden, Sm28 GST was also shown to have an anti-fecundity effect. Thus, Boulanger *et al.*, (1991) demonstrated that in some baboons vaccinated twice with rSm28 GST, the number of eggs produced per female worm was significantly reduced. Sera from these animals was also shown to inhibit egg production *in vitro* and the viability of the eggs laid was also decreased. More recently the native forms of both 28 and 26 kDa *S. bovis* GST have been purified from *S. bovis* adult worms and used to immunise cattle against a natural homologous challenge. The results obtained were similar to those observed following immunisation with rSm28 GST in baboons as immunisation with *S. bovis* GST gave variable reductions in worm burdens but a consistent and significant decrease in worm fecundity.

The way in which GST exerts its protective effect is not as yet fully understood. The antigen has been shown to be present within the tegument of the schistosomula and the tegument, parenchyma and excretory cells of the adult worm (Taylor *et al.*, 1988). However, it does not have the characteristics of an integral membrane or membrane associated protein and its presence at the larval surface is thought to be as an excretory / secretory product which is expressed transiently and released as part of a group of highly immunogenic molecules (in Capron *et al.*, 1987). This transient expression may however be sufficient to mediate immune attack, as demonstrated by the ability of antibodies raised *in vivo* following immunisation with Sm28 GST to kill schistosomula in an ADCC type reaction *in vitro* (Balloul *et al*, 1987a). Heating of the sera raised in these rats was shown to deplete this cytotoxicity ability (Balloul *et al.*, 1987a) thus suggesting a role for IgE in the development of protective immunity in this model. In addition, a role for anti-GST IgA has been indicated. It has been demonstrated using sera from infected

humans that the titre of this isotype increased following drug treatment and showed a positive correlation with age and hence resistance to reinfection (Grzych *et al.*, 1993). Moreover, the IgA fraction of human sera was shown to block the enzymatic activity of Sm28 GST *in vitro* and to decrease the fecundity of the female worm (Grzych *et al.*, 1993). A causal relationship between decreased fecundity and inhibition of GST activity has also been suggested by the experiments of Xu *et al.*, (1991) as the passive transfer of an anti-GST McAb (S13) which blocks the enzyme binding site, was shown to result in a decrease in both worm fecundity and egg viability. In contrast, passive transfer of a second McAb (H12) which does not affect enzymatic activity, failed to reduce either. Transfer of this McAb did however mediate a significant reduction in the worm burden of both rats and mice, thus suggesting that the reduction of the worm burden and the reduction in fecundity are mediated by the binding of antibodies to different epitopes upon Sm28 GST.

Immunisation with both native and recombinant Sm28 GST has also been shown to stimulate a protective T cell response. T cells removed from the spleen of GST immunised rats and mice have been shown to proliferate in response to Sm28 GST and various crude antigen preparations (Wolowczuk *et al.*, 1989). Moreover, when transferred to naive animals these lymphocytes were able to confer protective immunity (Auriault *et al.*, 1987, Wolowczuk *et al.*, 1989). It has been suggested that the latter may be a consequence of the production of IFNγ and other cytokines (e.g. TNFα) by Th1 and / or Tc cells as the supernatants removed from stimulated anti-Sm28 GST T cell cultures were able to activate macrophages and platelets to kill schistosomula *in vitro* (Wolowczuk *et al.*, 1989). Mice immunised with Sm28 GST and the recipients of T cells from such animals, have also been shown to have reduced egg related pathology. Thus, a reduction in the number of granulomas and a decrease in the collagen content of the liver were observed. Both CD4+ and CD8+ cells have a role in this mechanism and it is believed that IFNγ, which is known to down-regulate collagen production, is again involved. The latter is substantiated by the observation that prior treatment of mice with anti-IFNγ antibodies abolishes the anti-pathology effects of Sm28 GST immunisation (Pancre *et al.*, 1994).

Finally it should be noted that synthetic constructs of particular regions of the Sm28 GST molecule have been produced and shown to stimulate immunity. Perhaps the most successful of these is an octameric construct which has been shown to incorporate both

B and T cell epitopes. Immunisation of rats, mice and baboons with this peptide has been seen to stimulate a good antibody and T cell response. Moreover, immunisation of rats resulted in a 40% - 50% reduction in worm burden (Wolowczuk *et al.*, 1991).

(b) Schistosome triose-phosphate isomerase (TPI)

In an attempt to characterise larval surface antigens Harn *et al.*, (1985a) produced a number of McAbs. One of these (M1) bound uniformly to the surface of the cercarial and schistosomula stages and was shown to precipitate an antigen of 28 kDa. Further analysis of the binding pattern of M1 demonstrated that this antigen was lost from the surface of the larvae following 24 hours *in vitro* culture, however it was present amongst the metabolic products of miracidia, lung worms, adults and eggs. It has therefore been suggested that the 28 kDa antigen represents a molecule expressed upon the surface of the early larval stages which is covered upon the completion of the new heptalaminate membrane. Immunofluorescence has shown that the target of M1 is expressed by the majority of the cells within the adult worm. These include the lining of the gut, muscles and the tegument (Harn *et al.*, 1992).

M1 was used in passive transfer experiments and shown to mediate a 41% - 49% reduction in the worm burden of naive mice (Harn *et al.*, 1987b, 1992). Moreover, immunisation of mice with the 28 kDa antigen purified by McAb immunoaffinity chromatography, was shown to stimulate a significant level of protection (39%) (Harn *et al.*, 1987b). This suggested that the 28 kDa antigen was indeed a good vaccine candidate and further characterisation studies were therefore carried out. Attempts to obtain sequence data via N-terminal amino acid sequencing of the whole molecule demonstrated that it was N-terminally blocked. However, following tryptic digestion sequence data for three distinct peptides was obtained and used to scan the database for homology to other molecules. This revealed that the 28 kDa antigen was homologous to the mammalian glycolytic and gluconeogenic enzyme triose phosphate isomerase (TPI) (Harn *et al.*, 1992). The antigen was then shown to be enzymatically active by catalysing the production of glyceraldehyde 3-phosphate. This activity was blocked by prior incubation of the antigen with M1. The latter result gives rise to the possibility that the McAb antibody may kill the parasite via inhibition of the catalytic activity of TPI. Furthermore, compounds which mimic the antigen binding site of M1 could be considered for specific pharmacological control of the schistosome parasite.

Following the demonstration that the 28 kDa antigen represented schistosome TPI (sTPI), a human TPI cDNA was used to screen a cercarial cDNA library and isolate a full length clone. The insert from this clone was then expressed in a suitable vector and the recombinant protein obtained was demonstrated to be enzymatically active and to bind the McAb, M1. Preliminary studies with this recombinant antigen have suggested that sTPI is capable of inducing levels of protection similar to those obtained upon immunisation with the native molecule (Shoemaker *et al.*, 1992).

The status of sTPI as a vaccine candidate has also generated interest in the possibility of producing MAPs which incorporate B and T cell epitopes of this molecule. The use of MAPs is of particular interest with regard to this antigen, as it should enable those regions which show the most homology to human TPI to be omitted from the construct and hence decrease the likelihood of stimulating an auto-immune response. The production of a MAP incorporating B and T cell epitopes from a non-conserved region of sTPI has been described (Reynolds *et al.*, 1994) and immunisation with this construct has been shown to stimulate Th1 cells which proliferate in response to both the MAP and sTPI itself. The stimulation of a predominantly Th1 type response appears to be a particular feature of sTPI (Richter *et al.*, 1993) and suggests that this antigen may be a particularly good vaccine candidate as Th1 cells are believed to be responsible for the development of irradiated vaccine induced immunity (Smythies *et al.*, 1992) and the down-regulation of immunopathology in the mouse model. This is substantiated by the observation that immunisation of mice with the sTPI MAP results in levels of protection which range form 38% to 82% (Harn and Reynolds, 1993).

(c) Schistosome paramyosin (Sm97)

The results of various experimental studies in mice vaccinated with irradiated cercariae led James *et al.*, (1985) to conclude that the development of immunity in this model was dependent upon the stimulation of T cell mediated mechanisms. Thus, they immunised mice intradermally (i.d.) with the soluble components of schistosomula (SCHLAP) or adult worms (SWAP), in conjunction with BCG, an adjuvant known to preferentially stimulate cell mediated immunity. The immunised mice had a significantly reduced worm burden (48% (SCHLAP), 70% (SWAP)) and although it was believed that the resistance observed was a consequence of T cell mediated mechanisms, Pearce *et al.*, (1986) examined the antibody response generated in an attempt to identify the major

immunogens. Surprisingly, on probing Western blots of SWAP with sera raised in SWAP / SCHLAP immunised mice only one major schistosome protein, an antigen of 97 kDa (Sm97) was recognised. Moreover, if various size separated fractions of SWAP were used for immunisation, only that fraction containing molecules of a high molecular weight induced protection (Sher *et al.*, 1986). These results therefore indicated that high molecular weight antigens and in particular Sm97, may be responsible for the protection observed in mice immunised i.d. with SWAP plus BCG. Thus McAbs were generated following i.d. immunisation of mice with this high Mr fraction (Pearce *et al.*, 1986) and used to purify the 97 kDa antigen from SWAP. Mice given two 20 $\mu$g injections of purified Sm97 developed protection equal to that observed following two 1 mg injections of SWAP (39%) (Pearce *et al.*, 1988). The purified 97 kDa antigen was then used to immunise rabbits and the sera raised utilised as a probe for screening an adult cDNA library. Sequencing of one of the partial length clones isolated suggested that Sm97 represented schistosome paramyosin, a previously uncloned $\alpha$ helical coiled protein which forms the core of the myosin filaments in invertebrate muscle (Lanar *et al.*, 1986). This molecule is important in the "catch mechanism" of invertebrates and it has been suggested that Sm97 may play a role in the attachment of the schistosomes to host blood vessels, thereby enabling the parasite to avoid dislodgement by the blood flow. Subsequent examination of the recombinant protein (rSm97) expressed by the isolated clone demonstrated that a 52 kDa portion of Sm97 was encoded. However, as this portion incorporated the epitope recognised by one of the McAb raised against Sm97, immunoaffinity purification was carried out and the purified recombinant antigen used in further protection studies (Pearce *et al.*, 1988). Rats immunised with rSm97 had a significantly reduced worm burden (26%) although the reduction was not as large as that observed following immunisation with native Sm97. This suggested that the 52 kDa fragment of Sm97 which was represented by rSm97 lacked a number of antigenic moieties. This was substantiated by the failure of Sm97 to bind to all of the anti-Sm97 McAbs.

Despite the development of a monospecific antibody response in animals protected by immunisation with Sm97, it has been demonstrated that the mechanisms involved in the generation of resistance are T rather than B cell mediated. Thus, although i.d. immunisation with SWAP can protect B cell deficient mice, nude mice are not protected by vaccination in this manner. Further experiments (in James *et al.*, 1987) have also

demonstrated that immediate hypersensitivity, NK activity and complement activation are not involved in the development of immunity in this model. However, the failure of P strain mice to respond to vaccination with Sm97 or SWAP (James *et al.*, 1988) has demonstrated the importance of a DTH type response (see section 1.5.3.1). This hypothesis is substantiated by the observation that *in vitro* cultures of T lymphocytes from SWAP or Sm97 immunised mice proliferated in response to Sm97 (Pearce *et al.*, 1986) and released IFN$\gamma$ which activated macrophages to kill newly transformed schistosomula *in vitro* (James *et al.*, 1986). Moreover, mice immunised with SWAP gave a positive skin response when sensitised with Sm97 (Pearce *et al.*, 1986).

Sm97 is present in both the adult and schistosomula stages of the parasite (James *et al.*, 1985). However, as would be expected of a protein associated with muscle filament, it is not expressed upon the parasite surface. Thus, it is difficult to envisage how immunisation with Sm97 stimulates an immune response which is capable of killing the intact parasites of a challenge infection. Two hypotheses have been suggested. Firstly, it is possible that Sm97 is released as an excretory / secretory product of intact parasites which is then able to stimulate T cells sensitised by vaccination and so generate a DTH response directed specifically at the living parasite. Sm97 has indeed been detected in the media surrounding adult worms cultured *in vitro* and the antigen has been shown to exist predominantly within the tegument just below the parasite surface (Pearce *et al.*, 1986, Matsumoto *et al.*, 1988). Alternatively, it has been proposed that sensitised T cells, stimulated by the release of Sm97 from spontaneously dying parasites, induce a non-specific inflammatory reaction which traps this and other "bystander" parasites and thus halts migration and further development.

Finally, attempts have been made to characterise the antibody response to Sm97 in humans living in an area endemic for schistosomiasis (Correa-Oliveira *et al.*, 1989). Anti-Sm97 antibody levels were shown to be significantly higher in resistant individuals than in those who are susceptible to reinfection (section 1.6.3.2).

#### 1.9.2.3 Vaccine dominant antigens

As described above, animals vaccinated with radiation attenuated cercariae develop good immunologically specific resistance which protects against a challenge infection. In contrast, mice harbouring a single sex infection are not protected and in chronically infected mice the resistance observed is believed to be dependent upon egg induced

pathology. Thus, a number of studies have been performed with the aim of identifying "vaccine dominant" antigens, i.e. those molecules preferentially or uniquely recognised by VMS. A number of antigens meeting this criteria have been identified and are considered to be vaccine candidate molecules. Two of these, which are also schistosome surface antigens, are described below.

(a) The 200 kDa antigen (schistosome myosin)

The vaccine dominant nature of the 200 kDa antigen was initially described by Dalton and Strand (1987) following the probing of Western blots of Con A binding schistosome glycoproteins with CIS and sera from mice vaccinated once or twice with radiation attenuated parasites. The 200 kDa molecule together with a number of other antigens were recognised only by VMS within both the adult and schistosomula stages of the parasite. Immunoaffinity columns of VMS or CIS bound to Protein A Sepharose were then produced and sera specific for vaccine dominant glycoproteins (anti-Irradiated Vaccine, anti-IrV) was raised by immunisation of a rabbit with those molecules which bound to the former column but not the latter. This sera was shown to precipitate all those glycoproteins initially described as vaccine dominant from adult and larval metabolic products. Moreover, anti-IrV bound to the surface of both freshly transformed and lung stage schistosomula. Thus, this antisera recognised antigens seen upon the surface of the stages known to form the target of immune attack *in vivo* (Tom *et al.*, 1987).

An adult cDNA library was screened with anti-IrV and a strongly positive clone (IrV-5) was isolated. Serum raised against the ß-gal recombinant protein expressed by this clone was seen to immunoprecipitate the 200 kDa and a 38 kDa antigen from radiolabelled schistosomula surface molecules (Tom *et al.*, 1987). These antigens were seen to correspond to those previously reported to be the targets of numerous passively protective McAbs (section 1.9.2.1 (a)). Moreover, Kelly *et al.*, (1986) has suggested that the recognition of the 200 kDa antigen by these McAbs correlated with their ability to transfer resistance. In addition the cDNA insert of clone IrV-5 was sequenced and shown to have 50% homology with the human ß myosin chain. Thus, the 200 kDa antigen expressed upon the surface of the schistosomula is presumed to be schistosome myosin.

The ß-gal recombinant protein expressed by clone IrV-5 was isolated by electroelution and used to immunise mice (Soisson *et al.*, 1992). The levels of protection obtained (31%) were promising but not statistically significant, hence further immunisation

experiments were carried out using the schistosome part (62 kDa) of the recombinant protein expressed in pGEX, from which GST had been cleaved with thrombin (rIrV-5). The removal of this vector component was seen to enhance the antigenicity of the expressed protein and protection levels of up to 75% were observed in mice immunised three times with 10 $\mu$g of rIrV-5 incorporated into proteosomes containing the outer membrane protein of meningococcus (OMP). As proteosomes which represent large (700 kDa) conglomerates of the antigen to be used for immunisation, are believed to enhance immunogenicity via enabling the antigen to be folded in such a way as to expose the hydrophilic regions of the molecule, immunisation experiments were then attempted using IrV-5 proteosomes lacking the OMP adjuvant. A statistically significant reduction in worm burden of 62% was seen. Subsequent experiments using rIrV-5 have also demonstrated that the antigen is immunogenic in rats and in outbred baboons. With regard to the former, a reduction in worm burden of 94% was seen following three immunisations with 25 $\mu$g rIrV-5 in the form of micelles (Soisson and Strand, 1993). With regard to the latter, five immunisations with 50 $\mu$g of rIrV-5 in micelles or OMP containing proteosomes stimulated a significant anti-rIrV-5 antibody titre. However, the baboons showed variable (0% - 53%) levels of protection. Further analysis of the sera obtained from the immunised baboons demonstrated that the titre of antibodies raised against IrV-5 correlated positively with the level of resistance observed. Moreover, there was a similar striking correlation between the titre of anti-rIrV-5 antibodies raised and the protection observed in baboons vaccinated with irradiated cercariae. Animals immunised with rIrV-5 also had a reduced number of egg related granulomas although neither the severity of these lesions nor the fecundity of the worm were affected. At present, different immunisation protocols are being explored in the hope of devising one which stimulates the production of antibodies in a large majority of the immunised animals (Soisson *et al.*, 1993).

(b) The 16 kDa antigen

With regard to the theory that it is the surface of the schistosomula which forms the target of immune attack *in vivo*, Bickle *et al.*, (1986) derived a number of McAbs from fusions of spleen cells of mice immunised with a detergent extract of mechanically transformed schistosomula (MS). One of these McAbs, M7B3A (B3A) which was selected upon the basis of its ability to bind to the surface of MS, was subsequently shown to bind

with increasing intensity to skin stage schistosomula recovered up to 48 hours post infection. However, when tested against larvae removed from the host 72 hours post infection, the fluorescence observed was notably weaker. The McAb also failed to bind to the surface of 5 day old lung schistosomula, 10 day old liver worms and the cercarial stages of the parasite. The probing of frozen sections of the mature worm has also indicated that the target of this antibody is not present within the internal organs of the adult parasite (Dr.Q. Bickle, personal communication). The passive transfer of B3A to naive mice was shown to stimulate levels of resistance (30% - 70%) which compared favourably to those observed following the transfer of other McAbs (Table 1.1). Moreover, significant levels of immunity were only seen following the administration of B3A at the time of challenge (Andrews, 1986). This was consistent with the restricted binding of the McAb to the early larval stages of the parasite.

Despite the binding of B3A to the surface of the schistosomula, attempts to identify the target of this protective McAb amongst IODOGEN labelled larval surface antigens failed to produce results. The target antigen also failed to label with the Bolton and Hunter reagent which suggests a paucity of tyrosine residues. Western blotting of a detergent extract of MS was, however, more successful and the target of the McAb was identified as a low molecular weight antigen of 16 kDa. Subsequent studies demonstrated that this molecule was also vaccine dominant. Moreover, the failure of the antigen to bind sera from mice vaccinated with irradiated cercariae of *S. japonicum* demonstrated that the 16 kDa antigen was species specific, as is the resistance induced by the irradiated vaccine (Bickle *et al.*, 1985). The 16 kDa antigen is also recognised preferentially by sera from mice protected by exposure to Ro 11-3128 abbreviated infections (Bickle *et al.*, 1990).

Finally, B3A has been shown to stimulate previously inactivated macrophages to kill schistosomula *in vitro* (54%). The level of killing observed with this IgG3 McAb was significantly higher than that induced by a similar titre of CIS or VMS. Other surface binding McAbs of the same isotype also failed to stimulate significant levels of killing. Further analysis revealed that the killing of schistosomula by B3A was blocked by McAbs against the $\alpha$ chain of the macrophage adhesion molecule, Mac-1. Thus, it was suggested that Mac-1 may share a functional relationship with the molecule responsible for the binding of IgG3 antibodies to the macrophage. Alternatively, Mac-1 itself may bind this particular antibody subclass and hence play a critical role in the B3A mediated killing of the schistosomula, at least *in vitro* (Vignali *et al.*, 1990).

## 1.10 PROJECT AIMS

The 16 kDa antigen described above has a number of features which suggest that it may be of interest as a putative vaccine candidate antigen. The molecule is expressed upon the surface of the schistosomula which is believed to be both an inducer and a target of immune attack. It is the target of a passively protective McAb (B3A), and it is recognised preferentially by immune sera raised in mice vaccinated with radiation attenuated parasites. Thus, the main aim of the research described here was to characterise the biochemical and molecular nature of this antigen and to explore its potential for vaccination.

During the course of these studies a second low molecular weight antigen was identified. This molecule was shown to be vaccine dominant and attempts were therefore made to characterise this molecule using molecular biological techniques in particular.

The specific aims of the work detailed in each chapter were as follows:-

Chapter 3 — Experiments were performed with the aim of optimising the strategies used for the extraction of the 16 kDa antigen from the larval stages of *S. mansoni* and for its purification in preparation for immunisation studies.

Chapter 4 — Studies aimed at investigating the biochemical and molecular nature of the 16 kDa antigen were performed. Particular emphasis was placed upon the characterisation of the epitopes recognised by the McAb and polyclonal sera raised in mice immunised with irradiated parasites.

Chapter 5 — Antigen purified as described in Chapter 3 was used to immunise mice in conjunction with a variety of adjuvants. This was done primarily to assess the protective ability of the 16 kDa antigen although the nature of the immune response generated was also of interest.

Chapter 6 — In an attempt to identify clones encoding the 16 kDa antigen a sporocyst cDNA library was screened with affinity purified antibodies and the isolated clones characterised by a variety of techniques. This work was carried out in collaboration with my supervisor Dr. Q.D. Bickle.

Chapter 7 During the screening of the cDNA library with affinity purified antibodies, clones encoding a 15 kDa antigen were isolated. The recombinant protein expressed by these clones was shown to be vaccine dominant and thus further studies were carried out with the aim of characterising this second vaccine dominant molecule.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 STRAIN OF PARASITE AND HOSTS

### 2.1.1 Maintenance of the life cycle

Throughout this research the parasite used was a Puerto Rican strain of *S. mansoni* maintained in *Biomphalaria glabrata* and TO outbred mice (A. Tuck & Son Ltd., Battesbridge, Essex) as described by Andrews (1987).

### 2.1.2 Experimental hosts

Male C57Bl/10 mice were obtained from The National Institute for Medical Research (Mill Hill, London), female CBA and C57/Bl10 mice from Charles River UK Ltd., (Margate, Kent) and female Balb/c mice from A. Tuck & Son Ltd., (Battesbridge, Essex). Fischer rats were also obtained from Charles River UK Ltd (Margate, Kent) and New Zealand white rabbits and a half lop rabbit from Rosemead Rabbits (Waltham Abbey, Essex).

## 2.2 IRRADIATION OF CERCARIAE

MS were irradiated as described by Bickle *et al.*, (1979(b)). Briefly, cercariae were concentrated to approximately 500/ml using a Millipore apparatus with a filter of 8 $\mu$m pore size. These were then irradiated in a 'Gammabeam 60' cobalt source at a dose rate of 12 - 13 krad/minute. The cercariae were then concentrated and transformed as below.

## 2.3 MECHANICAL TRANSFORMATION OF SCHISTOSOMULA

Cercariae were mechanically transformed as described by James and Taylor (1976). Briefly cercariae were concentrated using a Millipore concentration apparatus and 8 $\mu$m filters to a volume of approximately 10 ml in water. The water was then replaced by ELAC media (Earls media plus lactalbumin hydrolysate supplemented with 100 U/ml penicillin, streptomycin and genomycin, (Gibco)) and re-concentrated to a volume of 5 ml. To disrupt the cercarial heads from tails the parasites were then passed through a 21 G needle (Beckton Dickinson) attached to a Luer Lok syringe. Mechanically transformed schistosomula (MS) were then checked under a light microscope to ensure that the separation of heads from tails was complete.

## 2.4 PRODUCTION OF ANTISERA

### 2.4.1 Vaccinated rabbit sera

Vaccinated rabbit sera (VRabS) were raised in New Zealand white rabbits given five, monthly exposures to 5,000 *S. mansoni* cercariae irradiated with 20 krad γ radiation.

### 2.4.2 Vaccinated mouse sera

Vaccinated mouse sera (VMS) were raised in C57/Bl10 or CBA mice given four, monthly exposures to 600 *S. mansoni* cercariae irradiated with 20 krad γ radiation.

### 2.4.3 Vaccinated rat sera

Vaccinated rat sera (VRatS) were raised in Fischer rats given three, monthly exposures to 1000 *S. mansoni* cercariae irradiated with 20 krad γ radiation.

### 2.4.4 Chronic infection sera

Chronic infection sera (CIS) were obtained from CBA mice infected with 25 unattenuated cercariae and bled between 15 and 30 weeks following infection.

### 2.4.5 Single sex infection sera

Single sex infection sera (SSS) were raised in mice infected with 100 male cercariae obtained from individual snails infected with a single miracidia. The mice were bled between 15 and 20 weeks following infection.

### 2.4.6 The monoclonal antibody, M7B3A

Production as ascites fluid of the anti-16 kDa McAb, M7B3A (B3A) was as described by Bickle *et al.*, (1986).

## 2.5 ETHANOL PRECIPITATION OF PROTEINS

The sample to be precipitated was diluted with 9 volumes of ice cold ethanol and incubated overnight at -20°C. The precipitated protein was pelleted by centrifugation at 6,000 x g for 10 minutes at 4°C, the ethanol removed and the pellet dried under vacuum.

## 2.6 SDS POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS PAGE)

Unless otherwise stated SDS PAGE was carried out according to the method of

Laemmli *et al.*, (1970) using a discontinuous gel system and Biorad mini gel apparatus. A 30% acrylamide : N N', methylene bisacrylamide (ratio = 39.5 : 1) stock solution was made and used at an appropriate dilution to make both resolving and stacking gels. In all cases a 15% or an 8% resolving gel (15%/8% acrylamide : bisacrylamide solution, 375 mM Tris/HCl (pH 8.8), 0.1% SDS, 0.05% ammonium persulphate, 0.05% N N N' N' tetramethylenediamine (TEMED)) was used with a 5% stacking gel (5% acrylamide : bisacrylamide solution, 125 mM Tris/HCl (pH 6.8), 0.1% SDS, 0.1% ammonium persulphate, 0.125% TEMED). Prior to setting, the resolving gel was overlaid with water saturated butanol which was removed prior to the pouring of the stacker. Samples were prepared for electrophoresis by boiling for 3 minutes in an appropriate volume of either 2 or 4 times concentrated SDS PAGE sample buffer (final concentration:- 625 mM Tris/HCl (pH 6.8), 2.3% SDS, 10% glycerol, 5% (v/v) ß mercaptoethanol, 0.001% bromophenol blue) and were electrophoresed alongside 5 - 10 $\mu$l of low or high molecular weight markers (Remazol). The size of the markers were as follows:-

| Low molecular weight (Da) | High molecular weight (Da) |
|---|---|
| 94,000 | 205,000 |
| 67,000 | 116,000 |
| 43,000 | 97,000 |
| 30,000 | 66,000 |
| 20,000 | 45,000 |
| 14,000 | 29,000 |

Mini gels were run for 1 hour at a constant voltage of 180 V in SDS PAGE running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS).

## 2.7 STAINING OF POLYACRYLAMIDE GELS

### 2.7.1 Staining with Coomassie blue

Following electrophoresis gels to be stained were normally placed in Coomassie blue stain (0.1% Coomassie blue, 45% methanol, 10% acetic acid) for 20 minutes and then in destain (5% methanol, 7% acetic acid) for as long as was necessary to remove excess colour. However, if the antigen was to be excised and used for immunisation it was

necessary to minimise the occurrence of acid hydrolysis and staining and destaining were done in the minimum time required for visualisation of the antigen band.

### 2.7.2 Staining with silver nitrate

Following electrophoresis, gels were fixed for 30 minutes (30% ethanol, 10% acetic acid), incubated for 30 minutes in 30% ethanol including 0.5 M sodium acetate, 0.5% gluteraldehyde and 0.2% sodium thiosulphate, and rinsed three times for ten minutes in sterile distilled water (SDW). Staining (0.1% silver nitrate, 0.02% formaldehyde) was then carried out for 45 minutes in the dark at room temperature. Following staining the gel was rinsed briefly in SDW and the developing solution (2.5% sodium carbonate, 0.01% formaldehyde) was added. The substrate solution was changed every 30 seconds until the gel was fully developed. The reaction was then stopped by the addition of EDTA to a final concentration of 50 mM.

## 2.8 WESTERN BLOTTING

Western blotting was carried out according to the method of Towbin *et al.*, (1979) using the Biorad Mini Trans-Blot Electrophoretic Transfer Cell. Unless otherwise stated 0.45 $\mu$m nitrocellulose paper (Hybond C, Amersham) was used and both the nitrocellulose paper and the gel were soaked briefly in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) prior to blotting. The transfer was carried out for 1 hour at a constant 350 mA in transfer buffer.

## 2.9 VISUALISATION OF SIGNALS ON WESTERN BLOTS

### 2.9.1 Probing of Western blots with antisera

(a) Following transfer Western blots were incubated for 20 minutes at room temperature in 5% dried milk powder (Marvel) in PBS (see appendix) in order to block the remaining protein binding sites. The blots were then incubated overnight at 4°C in antisera diluted in 5% milk solution. Dilutions were made according to the sera used and are given in the appropriate section. Following incubation in antisera the blots were washed 3 times for 10 minutes in washing buffer (PBS, 0.05% Tween 20) prior to addition of the horseradish peroxidase (HRP) labelled conjugate. Conjugates were used at a dilution of 1 in 3,000 (Biorad goat anti-mouse / rabbit / rat IgG) or 1 in 1,000 (Serotec rabbit anti- mouse IgG subclass specific conjugates) in washing buffer and the blots were incubated for at least

30 minutes. Blots were then washed as above and developed with substrate solution (0.625 mg/ml diaminobenzadine, 0.004% cobalt chloride, 0.01% hydrogen peroxide in PBS). The reaction was stopped by rinsing the blot in water.

(b) Western blots were probed with primary and secondary antibodies as described above. The blots were then developed using radioiodinated Protein A as described by Burnett *et al.*, (1981). Briefly, Western blots were incubated for 1 hour in radioiodinated Protein A (a gift from Dr. Q.Bickle), washed 3 times for 10 minutes in washing buffer, attached to appropriately sized pieces of filter paper (Whatman) and covered with Saranwrap. The blots were then used to expose scientific imaging film (Kodak XAR-5).

### 2.9.2 Probing of Western blots with biotinylated lectins

Following transfer Western blots were blocked by incubation in 2.5% bovine serum albumin (BSA) for 1 hour at 37°C, or overnight at 4°C. The blots were then washed twice for 10 minutes in TBS (50 mM Tris/HCl (pH 7.5), 0. 15 M NaCl) and once for 10 minutes in TBS including metal ions (1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$). The blots were incubated for one hour at room temperature in a variety of biotinylated lectins (EY Laboratories) at a concentration of 10 $\mu$g/ml in TBS, including metal ions as above. In experiments carried out to examine the specificity of lectin binding a competing sugar was added at a final concentration of 0.2 M. Following incubation with lectin the blots were washed 3 times for 10 minutes in TBS and incubated for 1 hour with a 1 in 50 dilution of avidin complexed with HRP (ABComplex, Dako). The blots were finally washed 3 times for 10 minutes in TBS plus 0.1% Tween 20 and developed with diaminobenzadine as above.

### 2.9.3 Staining of Western blots with amido black

Following transfer Western blots were incubated in amido black stain (0.1% amido black, 45% methanol, 10% acetic acid) for 2 minutes and destained (5% methanol, 7% acetic acid) until all excess colour was removed.

## 2.10 DETERGENT EXTRACTION OF MS

### 2.10.1 Extraction of MS with the detergent Triton X-114 (TX-114)

Pellets of approximately 230,000 MS or cercariae were resuspended by vortexing in

1 ml of 0.5% pre-condensed TX-114 in PBS and the suspension incubated on ice for 30 minutes. The preparation was then centrifuged at 6,000 x g for 5 minutes at 4°C and the supernatant (supernatant 1) removed and placed on ice. The remaining pellet was resuspended in 1 ml PBS by sonicating three times for 30 seconds at a frequency of 14 microns peak to peak and 50 $\mu$l of pre-condensed TX-114 were added (final concentration approximately 0.5%). Incubation and centrifugation were carried out as above and the supernatant (supernatant 2) removed. Both supernatants were then separated into detergent and aqueous phases using a procedure adapted from that of Bordier (1981):- A 5 ml cushion of 6% sucrose, 0.06% TX-114 in PBS was placed at the bottom of a centrifuge tube and the supernatants overlaid. The tube was then incubated for 5 minutes at 37°C and centrifuged at 600 x g for 5 minutes at room temperature. Following centrifugation the aqueous phase was removed from above the sucrose cushion whilst the detergent phase remained as an oily droplet at the bottom of the tube. In order to ensure a complete separation of the two phases a further 50 $\mu$l TX-114 were added to the aqueous phase which was then overlaid on a second sucrose cushion and warmed and centrifuged as above. The aqueous phases and detergent phases following each separation were then combined to give a single aqueous and detergent phase for each supernatant. The detergent phases were made up to a volume of 1 ml by the addition of PBS and the final insoluble pellet resuspended in 500 $\mu$l PBS. 100 $\mu$l of each aqueous and detergent phase were ethanol precipitated overnight at -20°C and the resulting pellets resuspended in 50 $\mu$l of PBS. The detergent and aqueous phase antigen plus 50 $\mu$l of the insoluble pellet were then diluted with an equal volume of 2 x SDS PAGE sample buffer and 10 $\mu$l of each sample were run on a gel which was stained with Coomassie blue. Samples containing equivalent amounts of protein were then separated by SDS PAGE and analysed by Western blotting.

### 2.10.2 Sequential extraction of MS with TX-114 and a variety of other detergents

A pellet of approximately 400,000 MS was disrupted in 1 ml of 50 mM Tris/HCl (pH 8.0) by sonicating 3 times for 30 seconds at a frequency of 14 microns peak to peak. Pre-condensed TX-114 (11.4%) was added to give a final concentration of 1% and the tube incubated on ice for 30 minutes. The preparation was then centrifuged at 6,000 x g for 5 minutes at 4°C and the supernatant removed. The supernatant was warmed at 37°C for 5 minutes and the aqueous and detergent phases separated as described. The detergent

phase was then made up to 250 μl with Tris/HCl (50 mM, pH 8.0) and pellets insoluble in TX-114 were resuspended in 100 μl of Tris/HCl (50 mM, pH 8.0) using a sonicating water bath. An equal amount of either Triton X-100 (TX-100), sodium deoxycholate (DOC),3-[3-cholamidopropyl)-dimethylammonia]-1-propane-sulfonate(CHAPS),octyl-ß-D thio glucopyranoside (OTG) or deconyl-n-methylglucamide (MEGA 10) were added to a final concentration of 1.5%. The tubes were incubated at room temperature for one hour with occasional mixing then centrifuged at 6,000 x g for 10 minutes at 4°C and the supernatant collected. The remaining pellets were resuspended in 125 μl of Tris/HCl (50 mM, pH 8.0).

### 2.10.3 Extraction of MS with OTG alone

MS at a concentration of 400,000/ml in 50 mM Tris/HCl (pH 7.4), were disrupted by sonication at 14 microns peak to peak (3 x 30 seconds), centrifuged for 1 hour at 100,000g, 4°C and the aqueous phase removed. The remaining pellet was resuspended by sonication in 500 μl/400,000 MS of 50 mM Tris/HCl (pH 7.4) and an equal volume of OTG at a concentration of 3% in 50 mM Tris/HCl (pH 7.4) was added. The preparation was incubated at room temperature for 1 hour with occasional mixing, centrifuged as above, and the detergent fraction removed. The remaining pellets were resuspended in 125 μl of Tris/HCl (50 mM, pH 7.4).

## 2.11 PREPARATION OF ADULT WORM ANTIGEN

10 mg of freeze dried *S. mansoni* adult worms were homogenised in 2 ml of PBS and the detergent Nonidet P-40 (NP-40) added to a final concentration of 0.1%. The preparation was then centrifuged at 6,000 x g for 20 minutes at 4°C and the supernatant removed and stored at -20°C.

## 2.12 PURIFICATION OF THE 16 KDA ANTIGEN FROM MS

### 2.12.1 Immunoaffinity chromatography

(a) Preparation of the immunoaffinity column

The McAb, B3A was purified by passing clarified ascites fluid slowly through a 5 ml column of Protein A Sepharose beads, washing the column extensively with 100 ml TBS (50 mM Tris/HCl (pH 8.6), 0.15 M NaCl) and eluting the bound antibody with a buffer of pH 4.3 (50 mM sodium acetate, 0.15 M NaCl). 20 fractions (1 ml) were

collected and assessed for antibody content by SDS PAGE and Coomassie blue staining. Fractions containing the purified antibody were then dialysed extensively against TBS and rotated overnight with Protein A Sepharose beads at a concentration of approximately 18 mg of immunoglobulin (as assessed by Coomassie blue staining) per 250 mg of beads. The unbound material was then removed and the Protein A Sepharose beads washed 3 times for 10 minutes with borate buffer (pH 8.0, see appendix). Cross-linking of the McAb to the beads was then achieved by incubation for 1 hour at room temperature in 10 ml borate buffer (pH 8.0) plus 50 mg of freshly prepared dimethylpimelimidate (Sigma). The beads were washed once with borate buffer (pH 9.0) and then incubated for 10 minutes in borate buffer (pH 9.0) plus 20 mM ethanolamine. The beads were transferred to a 10 ml Poly Prep chromatography column (Biorad) and stored in borate buffer (pH 9.0) plus 0.02% sodium azide at 4°C.

(b) Purification of the 16 kDa antigen using the immunoaffinity column

The immunoaffinity column prepared as above was pre-eluted by the addition of 5 ml diethylamine (DEA) (pH 11.5) and the pH restored with Tris/HCl (10 mM pH 7.4) prior to use. OTG extracted material was then prepared as above and circulated through the immunoaffinity column a minimum of three times at a flow rate of approximately 1 ml/9 minutes. The column was washed extensively with 100 ml of 50 mM Tris/HCl (pH 7.4), 0.15 M NaCl, 1 mM EDTA, 0.1% OTG followed by 10 ml of 10 mM Tris/HCl (pH 7.4), 0.1% OTG. Bound material was eluted with 10 ml of 50 mM DEA (pH 11.5) including 0.1 % OTG. 500 $\mu$l fractions were collected and neutralised by the addition of 50 $\mu$l 0.5 M $NaH_2PO_4$.

### 2.12.2 Ion exchange chromatography

4 ml columns of the anion exchange gel, Diethyl-aminoethyl (DEAE) Bio-Gel A and the cation exchange gel, carboxymethyl (CM) Bio-Gel A were used. Extraction of antigens from MS was as above with the exception that sonication and OTG extraction were done in a buffer suitable for use with each column i.e. for the DEAE column 25 mM Tris/HCl (pH 8.0), 1 mM EDTA, 1.5% OTG was used and for the CM column a pH 6.0 phosphate buffer (89% 25 mM $NaH_2PO_4$, 11% 25 mM $Na_2HPO_4$ 1 mM EDTA, 1.5% OTG). Following equilibration of the column with 4 bed volumes of suitable buffer the detergent extracts were passed through the columns which were then washed with 10 ml of the appropriate buffer. Bound material was eluted either by the addition of a step

or continuously increasing salt gradient (0 to 0.5 M KCl) using a Biorad Model 385 Gradient Former for the latter. In all cases 1.5 ml eluates were collected and ethanol precipitated to remove excess salt prior to SDS PAGE.

### 2.12.3 Hydrophobic interaction chromatography

An OTG extract prepared as above was diluted to a final concentration of 25% ammonium sulphate ($(NH_4)_2SO_4$) and applied to a 2 ml column of Phenyl Sepharose CL-4B (Pharmacia) previously equilibrated with 25% ammonium sulphate. The column was then washed with 25% ammonium sulphate and eluted with a decreasing salt gradient (25% ammonium sulphate (3 ml), 10% ammonium sulphate (3 ml) and Tris/HCl (50 mM, pH 7.5,) (3 ml)). 1 ml of ethylene glycol was then applied as a final elution step. Prior to running on a gel all the fractions and the starting material were dialysed and ethanol precipitated to remove excess salt.

### 2.12.4 Lectin affinity chromatography

OTG extracted material or immunoaffinity purified antigen were rotated for at least one hour with agarose beads coated with peanut agglutinin (PNA) (Sigma). The beads were allowed to settle, the unbound fraction was removed and the beads were washed 3 times for 30 minutes with 50 mM Tris/HCl (pH 7.4), 0.15 M NaCl, 1 mM EDTA, 0.1% OTG. The beads were then eluted with increasing concentrations (0.1 M - 0.5 M) of galactose and / or lactose. Borate buffer (0.45 M, pH 6.0) was used as a final elution step.

### 2.12.5 The Biorad 491 Prep cell

Immunoaffinity column eluates were centrifuged through a Centricon 10 microconcentrator (Amicon, molecular weight cut off of 10 kDa) and the antigen which collected on the membrane resuspended in 250 $\mu$l 0.2 M ammonium acetate buffer. The volatile buffer was then removed by freeze drying under vacuum overnight and the precipitate was resuspended in 20 $\mu$l of sterile PBS in preparation for electrophoresis. The Biorad 491 Prep cell apparatus was assembled and the gel poured in accordance with the protocol supplied. As with SDS PAGE a 15% acrylamide resolving gel and a 5% stacking gel were used. Prior to loading the concentrated sample was denatured by boiling in SDS PAGE sample buffer. The gel was run at a constant 40 mA and 20, 2.5 ml fractions were

collected immediately following the elution of the dye front.

### 2.12.6 Staining of purified antigen on ProBlott membrane

Several eluates containing partially purified 16 kDa antigen eluted from the McAb affinity column were concentrated into a volume of 250 $\mu$l 0.2 M ammonium acetate buffer (pH 7.5) using a Centricon 10 microconcentrator and freeze dried overnight. The precipitate was then resuspended in 20 $\mu$l of sterile PBS, separated by SDS PAGE and transferred onto ProBlott membrane (Perkin-Elmar Ltd) using the Biorad Mini Trans-Blot Electrophoretic Transfer Cell. Prior to transfer the ProBlott membrane was soaked for a few seconds in methanol and then both the membrane and the gel were soaked for 5 minutes in electroblotting buffer (10 mM cyclohexylamino-1-propanesulphonic acid (CAPS), pH to 11.0 with 0.2 M NaOH, 10% methanol). Transfer was carried out at a constant 50 V at room temperature for 30 minutes in the above buffer. Following transfer the ProBlott was saturated in 100% methanol for a few seconds and then stained for 1 minute in Coomassie blue R-250 (40% methanol, 1% acetic acid, 0.1% Coomassie blue R-250). The ProBlott was destained to remove excess colour (50% methanol) and the region of the blot corresponding to the 16 kDa antigen excised.

## 2.13 GAS PHASE $NH_2$ TERMINAL AMINO ACID SEQUENCING

Gas phase $NH_2$-terminal amino acid sequencing was very kindly performed by Dr. Alan Harris in the Laboratory of Protein Structure at the National Institute for Medical Research, Mill Hill, London.

## 2.14 TWO DIMENSIONAL ELECTROPHORESIS

500 $\mu$l of OTG extracted MS antigen was ethanol precipitated and resuspended in 125 $\mu$l of 2D sample buffer plus 125 $\mu$l of SDW. 25 $\mu$l of sample was then loaded per tube gel and 2D electrophoresis was carried out for 3.5 hours at 750 V according to the protocol supplied with the Biorad Mini Protean II Tube Cell apparatus, with the following exceptions:-

a) Sample overlay buffer made up as specified in the protocol proved to be too dense and passed through the loaded sample. It was thus diluted 1 part buffer to 2 parts SDW prior to use.

b) The acid (bottom) end of the tubes were marked by spotting with Coomassie blue powder before placing them in SDS sample buffer for storage at -20°C. This was done so that the basic and acidic ends of the first dimension gels could be readily differentiated.

## 2.15 TREATMENT OF THE 16 KDA ANTIGEN WITH A VARIETY OF PROTEASES

A pellet of MS were boiled in SDS PAGE sample buffer without mercaptoethanol and centrifuged for 15 minutes at 14,000 g at room temperature. The preparation was then diluted to 0.1% SDS with 50 mM Tris/HCl (pH 8.0), 5 mM $CaCl_2$ and treated for 1 hour with either protease K (Boehringer Mannheim), chymotrypsin, trypsin, or protease XIV (Sigma). Pronase E (Sigma) was predigested for 2 hours at 37°C in 10 mM Tris/HCl (pH 7.8), 10 mM EDTA, 0.5% SDS and the antigen digested in the same buffer. Papain digestion was carried out in 100 mM Tris/HCl (pH 7.4). All proteases were used at final concentrations of 20 and 200 $\mu$g/ml.

## 2.16 TREATMENT OF WESTERN BLOTS WITH SODIUM META PERIODATE

Vicinal hydroxyl groups were oxidized using sodium meta periodate according to the method of Woodward *et al.*, (1985):- Following transfer Western blots were incubated for 20 minutes in 5% milk solution or for 1 hour in 2.5% BSA, washed for 10 minutes in washing buffer (PBS, 0.05% Tween 20) and cut into strips. Strips were then treated with 20 mM sodium periodate in 50 mM sodium acetate buffer (pH 4.5) for either one or four hours in the dark at room temperature. Control strips were incubated for four hours in the acidic buffer alone. Following treatment strips were washed for 30 minutes in 50 mM sodium borohydride and rinsed for 10 minutes in washing buffer. The blots were then probed with antibody or lectin as described above (section 2.9).

## 2.17 NEURAMINIDASE TREATMENT OF WESTERN BLOTS

Following transfer Western blots were blocked for 1 hour in 2.5% BSA at 37°C and washed twice for 10 minutes in TBS (50 mM Tris/HCl (pH 7.5), 0.15 M NaCl). The blots were then cut into strips and each strip treated for 1 hour at room temperature with 500 $\mu$l of neuraminidase (Sigma) at a concentration of 6 mU/ml in 0.2 M sodium acetate buffer (pH 5.5). Negative control strips were incubated for 1 hour in the acetate buffer alone. Following treatment the blots were then washed for 10 minutes in TBS plus metal

ions (1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$) and probed with lectin as above (section 2.9.2). As a positive control 200 $\mu$g of fetuin were treated with neuraminidase and probed with PNA.

## 2.18 <u>CLEAVAGE OF O-LINKED CARBOHYDRATES WITH THE ENZYME O-GLYCANASE</u>

### 2.18.1 Treatment of the 16 kDa antigen with O-glycanase

30 $\mu$l of an OTG extract of MS (400,000 MS/ml) was added to each of four microcentrifuge tubes. The antigen was then ethanol precipitated overnight at -20°C. This step was included in order to remove parasite material and detergent which may affect the activity of the O-glycanase enzyme (Adrian Grey, Oxford Glycosystems, personal communication). Following precipitation the antigen in one tube was resuspended in 5 $\mu$l incubation buffer (0.1 M sodium citrate (pH 6.0), BSA (100 $\mu$g/ml), 0.02% sodium azide) and as a positive control 25 $\mu$l of asialofetuin (10 mg/ml, Sigma) was added to the resuspended antigen along with 5 $\mu$l (1.5 mU) of the enzyme Endo-$\alpha$-N-acetylgalactosaminidase (O-glycanase, Oxford Glycosystems). The amount of enzyme used was that recommended for cleavage of 250 $\mu$g of asialofetuin (Adrian Grey, Oxford Glycosystems). The antigen in a further two tubes was then resuspended in 1 $\mu$l or 6 $\mu$l of incubation buffer and an equal volume (0.3 mU / 1.8 mU) of O-glycanase enzyme added. The samples were then made up to a total of 7 $\mu$l or 42 $\mu$l respectively with SDW. This was done to ensure that the concentration of the enzyme and the components of the incubation buffer in the two experimental samples were equal to those used in the positive control. The antigen in the final tube was resuspended in 6 $\mu$l of incubation buffer and 36 $\mu$l of SDW added. This tube was used as a no enzyme negative control. All the tubes were then incubated for 20 hours at 37°C. Following incubation appropriate amounts of 2 x SDS PAGE sample buffer were added to the experimental reactions and negative control. This stopped the reactions and the samples were then run on SDS PAGE gels, Western blotted and probed with antibodies or lectin. The positive control reaction was stopped by the addition of 10 $\mu$l of boric acid (0.8 M, pH 9.1) and the activity of the enzyme assessed using the Elson-Morgan assay (see below).

### 2.18.2 Assessment of O-glycanase activity

O-glycanase activity was assessed by measuring the disaccharide, Gal-ß (1-3)

GalNAc, liberated from the enzyme treated asialofetuin using an adapted version of the Elson-Morgan assay (Reissig *et al.*, 1955):- Following the addition of the boric acid the preparation was boiled for exactly 3 minutes and then incubated on ice for 5 minutes. 225 $\mu$l of p-dimethylaminobezaldehyde (DMAB) solution was added (0 .15 M DMAB, 98.75% acetic acid, 1.1% HCl) and the preparation incubated for a further 20 minutes at 37°C. The OD of the solution was then read at 585 nm.

## 2.19 IMMUNOSTIMULATING COMPLEXES (ISCOMs)

### 2.19.1 Preparation of ISCOMs incorporating the 16 kDa antigen

ISCOMs were made according to the method of Lövgren *et al.*, (1987):- An OTG extract of approximately 8 million MS was passed down an immunoaffinity column which was washed and eluted as above (section 2.12.1). Comparison of the silver stained eluates with ovalbumin standards demonstrated that the best two eluates combined contained approximately 0.5 - 1 mg/ml of partially purified antigen. Thus, these fractions were used for the formation of ISCOMs incorporating the 16 kDa molecule whilst 1 ml of the immunoaffinity column elution buffer (50 mM DEA, 50 mM $NaH_2PO_4$, 0. 1% OTG) was used for the formation of protein free ISCOMs. 19 mg of MEGA 10 was added to each sample to give a final detergent concentration of 2% and the solutions were warmed at 37°C for 3 minutes to enable the MEGA 10 to dissolve. 1 mg of the adjuvant Spikoside (Iscotec, AB) was then added to each tube followed by 50 $\mu$l of lipid mix (50 mg phosphatidylcholine plus 50 mg cholesterol dissolved in 1 ml chloroform and added to 10 ml 20% MEGA 10). The preparations were sonicated at room temperature for 15 minutes to disrupt any protein aggregates which may have formed, left to stand for 1 hour and then transferred to pre-boiled dialysis tubing with a molecular weight cut off of 14 kDa. Dialysis was carried out against 5 L of 50 mM Tris/HCl (pH 8.5), 0.001% sodium azide at room temperature for 8 hours or until the dialysates became slightly cloudy, an indication of the formation of ISCOMs. The buffer was then exchanged for 5 L PBS and dialysis continued for a further 20 hours at 4°C. On completion of dialysis the samples were removed from the dialysis tubing and the ISCOMs concentrated by centrifugation through a sucrose gradient (see below).

### 2.19.2 Purification of ISCOMs by density gradient centrifugation

2.25 ml of 40% sucrose in 50 mM Tris/HCl (pH 8.5) were placed in the bottom of

an ultraclear centrifuge tube (Beckman) with care being taken to ensure that droplets of sucrose did not adhere to the sides of the tube. Using a 1 ml syringe 2.25 ml of 10% sucrose in 50 mM Tris/HCl (pH 8.5) were overlaid onto the 40% sucrose. The control and antigen containing dialysates were then overlaid onto the sucrose gradients (500 $\mu$l / gradient) and centrifuged for 9 hours at 60,000 x g (27,000 rpm in a Beckman SW 55 swinging bucket rotor) at 20°C. The rotor was allowed to coast to a stop with the brake off. 250 $\mu$l fractions were then removed from the top of the tube using a 1 ml syringe. The fractions containing the ISCOMs, which form a visible band at the interface between the two sucrose concentrations, were noted. 22.5 $\mu$l of all the fractions taken from the gradients used to purify the ISCOMs containing the 16 kDa antigen were then separated by SDS PAGE, transferred by Western blotting and the blots probed with antibody in order to identify those fractions containing antigen. If the fraction corresponding to the position of the ISCOMs within the gradient was shown to contain a substantial amount of antigen, this fraction together with those removed from immediately above and below were dialysed against 5 L PBS plus 0.001% Na azide at 45°C for 12 hours to remove sucrose. Corresponding fractions removed from the sucrose gradients on which the control samples were separated were treated in a similar manner. The presence of ISCOMs within all these fractions was then verified by electron microscopy (see below).

### 2.19.3 Electron microscopy

The dialysed samples were checked for the formation of ISCOMs using negative staining:- 50 $\mu$l of each fraction was applied to a carbon coated grid and stained using 2% ammonium molybdate as the contrasting agent. The grids were then examined using a Jeol 1200 EX transmission electron microscope at an accelerating voltage of 60 / 70 kV.

## 2.20 IMMUNISATION STUDIES IN MICE USING THE PURIFIED 16 KDA ANTIGEN

By comparing the levels of Coomassie blue or silver staining observed following the electrophoresis of particular column eluates and samples of a known protein concentration, it was possible to demonstrate that approximately 500 $\mu$g of 16 kDa antigen were present in the first two fractions (total 1 ml) eluted from the immunoaffinity column following purification of the antigen from 8 million parasites. This figure has subsequently been used to estimate the total amounts of 16 kDa antigen used in

conjunction with Ribi or novasome adjuvant to immunise mice (see below). However, it should be noted that these are very approximate figures as the glycanic nature of the 16 kDa molecule effects the efficiency with which it is stained and hence ensures that it is difficult to estimate accurately the amounts of antigen present in the column eluates. Attempts to estimate the amount of 16 kDa antigen incorporated into ISCOMs have not been made as the 16 kDa antigen was not visible in the ISCOM preparation following Coomassie blue or silver staining (see Chapter 5).

### 2.20.1 Immunisation of mice with the immunoaffinity purified 16 kDa antigen plus Ribi adjuvant

Fractions eluted from the immunoaffinity column (as above) were prepared for immunisation by dialysing to remove DEA using Spectra/Por dialysis membrane (3.5 kDa cut off). Dialysis was carried out against 3 x 1 L PBS for 90 minutes at room temperature and then overnight at 4°C. Ribi adjuvant (Ribi Immunochem Research Incorporation) was then incubated at 42°C for 5 to 10 minutes and reconstituted by the addition of 2 ml sterile saline. A group of eight male C57Bl/10 mice were immunised with approximately 150 μg of 16 kDa antigen per mouse plus Ribi adjuvant. The antigen was given over the course of 12 weeks with immunisations on days 0, 14, 49 and 84. In all cases the total volume of the immunising preparation was 100 μl per mouse. For the first three immunisations this incorporated 56 μl of antigen preparation plus 44 μl of Ribi and was given in each of two sub-cutaneous (s.c) sites. For the final immunisation 78 μl of antigen and 22 μl of Ribi were used and administered both s.c (50 μl) and intraperitoneally (i.p) (50 μl). A second group of 8 male C57Bl/10 mice were used as control animals and immunised in conjunction with the experimental group, the 16 kDa antigen being replaced with an equivalent amount of sterile saline. In all cases the immunisation preparation was vortexed for 2 to 3 minutes prior to injection in order to form an emulsion. Both groups of mice were challenged with 200 cercariae on day 112, killed and perfused on day 142 and the worm burden counted.

### 2.20.2 Immunisation of mice with the 16 kDa antigen plus novasomes

7 female CBA mice were immunised with approximately 165 μg of partially purified 16 kDa antigen per mouse plus novasomes which were a kind gift from Dr. C. Wright (Novavax Inc.). The antigen was given over the course of three immunisations with

injections on days 0, 15 and 33. On each occasion 90 $\mu$l of antigen preparation was combined with 10 $\mu$l of novasomes and administered in each of two ip sites. A group of five female CBA mice were included as control animals and immunised in conjunction with the experimental animals, the 16 kDa antigen being replaced with a solution of 50 mM DEA (pH 11.5), 50 mM $NaH_2PO_4$, 0.1 % OTG. In all cases the antigen / DEA / adjuvant preparation was vortexed briefly prior to injection. It was not necessary to form an emulsion. All of the mice were challenged with 200 cercariae on day 99 and killed and perfused 35 days later.

### 2.20.3 Immunisation of mice with the 16 kDa antigen incorporated into ISCOMs

7 female CBA mice were immunised with the 16 kDa antigen incorporated into ISCOMs as described (section 2.19). Prior to immunisation ISCOMs were dialysed against 5 L of PBS overnight at 4°C using dialysis tubing with a molecular weight cut off of 14 kDa (Medicell International Ltd). This was done in order to remove sodium azide which might otherwise have been toxic on immunisation. For each immunisation 70 $\mu$l of ISCOMs per mouse was used from a total of 3 ml prepared using 16 kDa antigen purified from 18 million MS (approximately 1.5 mg). A control group of 5 female CBA mice were also immunised, the 16 kDa ISCOMs being replaced by 35 $\mu$l of ISCOMs formed without the incorporation of protein plus 35 $\mu$l sterile saline. As Spikoside, the adjuvant used in the formation of ISCOMs, is toxic if given in large amounts it was necessary to check the safety of the preparation prior to each of the immunisations. Thus, a single mouse from each group was immunised 48 hours earlier than the rest. The mice were immunised subcutaneously in each of two sites on days 0, 15, 33, 64, 85 and challenged with 200 cercariae on day 99. The mice were then perfused on day 134 and the worm burden estimated.

## 2.21 INDIRECT IMMUNOFLUORESCENCE

This was performed in 6 x 50 mm cytology tubes (Sterilin Ltd) as described by Bickle *et al.*, 1986. Briefly, following a 3 hour incubation in medium, approximately 50 MS in a volume of 10 $\mu$l were placed in each tube. The sera to be tested was then diluted in cold PBS to give a total volume of 200 $\mu$l and the MS were incubated in the sera for 20 - 30 minutes at 4°C. Following incubation the MS were washed 3 times in cold PBS, the final wash leaving the MS resuspended in 50 $\mu$l PBS. The parasites were then

incubated in fluorescein-conjugated rabbit anti-mouse antibody (Nordic) diluted 1 in 5 in PBS for 20 - 30 minutes at 4°C. Washing was repeated as before and the MS were then examined using a Leutz Diaplan ultraviolet microscope and the degree of fluorescence rated from negative (-) to very bright (+ + +). Normal mouse sera and a McAb known to bind to the surface of the schistosomula were used in all cases as negative and positive controls respectively.

## 2.22 $^{125}$I RADIOLABELLING OF SCHISTOSOMULAR SURFACE ANTIGENS

Newly transformed schistosomula were radiolabelled according to the method of Fracker and Speck (1979) as described by Andrews (1987). 200 $\mu$g of Iodogen (1,3,4,6-tetrachloro-3-$\alpha$-6-$\alpha$ diphenylglycoluril, Pierce) was dissolved in chloroform and pipetted into a 5 ml scintillation tube. The chloroform was allowed to evaporate and 100,000 MS in 195 $\mu$l of media were added. The radiolabelling reaction was then performed by the addition of 5 $\mu$l of Na$^{125}$I (0.5 mCi) and incubating at room temperature for 8 minutes with shaking every minute. Following labelling the MS were transferred to a clean microcentrifuge tube and washed 6 times with PBS to remove unbound radioactive label. 300 $\mu$l of 1% TX-100 in PBS was then added to the tube and the intact MS incubated for 45 minutes at 4°C. The MS were homogenised, incubated for a further 45 minutes at 4°C and the TX-100 extract removed. The parasites were then resuspended in 300 $\mu$l of 1.5% OTG, incubated for 45 minutes at 4°C and the OTG extract removed. The final parasite pellet was incubated overnight at 4°C in a further 300 $\mu$l of 1.5% OTG.

## 2.23 IMMUNOPRECIPITATION OF RADIOLABELLED SCHISTOSOMULA SURFACE ANTIGENS

10 $\mu$l (1 - 2 x $10^6$ counts/minute) aliquots of the TX-100 and the initial OTG extracted material were transferred to microcentrifuge tubes and 50 $\mu$l of immunoprecipitation buffer (50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 10 mM EDTA, 0.05% TX 100) added. Each sera to be tested (5 $\mu$l neat or 10 $\mu$l in 50% glycerol) was added to one each of the TX-100 and OTG extracted aliquots and the preparations incubated overnight at 4°C. Protein A sepharose (Sigma) was then prepared by swelling in immunoprecipitation buffer (250 mg for 1 ml of beads) and 40 $\mu$l of a 50% suspension was added to each of the reactions which were incubated for 1 hour at room temperature with occasional mixing. Following incubation the beads were sedimented by a pulse spin,

washed three times with 1 ml of immunoprecipitation buffer and resuspended in 25 $\mu$l 2 x SDS PAGE sample buffer. The samples were electrophoresed and the gels stained with Coomassie blue. This enables the proteins to be fixed prior to drying of the gel and also the presence of antibody in each reaction to be observed. The gels were dried using a Biorad Model 583 gel drier for 1 hour at 80°C and then placed in a cassette next to scientific imaging film (Kodak XAR-5) and incubated at -70°C for at least 24 hours.

## 2.24 THE cDNA EXPRESSION LIBRARY

The library used was made by Phillipa Francis using RNA extracted from the hepatopancreas of *S. mansoni* infected *B. glabrata* snails, an Amersham cDNA synthesis kit and λgt11 arms (Francis, 1989). The unamplified library consisted of approximately $10^6$ recombinants. However, a proportion of this had been amplified after construction to a titre of 3 x $10^9$ plaque forming units (pfu)/ml and this was used in the following studies.

## 2.25 SCREENING OF THE cDNA EXPRESSION LIBRARY USING ANTIBODY PROBES

### 2.25.1 Preparation of sera for screening

(a) Immunisation of a rabbit with the purified 16 kDa antigen

A 500 $\mu$l fraction of immunoaffinity purified 16 kDa antigen was run on an SDS PAGE gel. The gel was stained with fresh Coomassie blue for 2 minutes, destained quickly and the band corresponding to the 16 kDa antigen excised. The gel slice was then snap frozen on dry ice and ground into a fine powder using a mortar and pestle. This was stored at -70°C until required. Immediately prior to injection Ribi adjuvant (Ribi Immunochem Research Incorporation) was incubated at 42°C for 5 to 10 minutes then reconstituted by the addition of 2 ml sterile saline. The powdered antigen was resuspended in 500 $\mu$l of sterile saline, added to 250 $\mu$l of the adjuvant and the preparation vortexed for 2 to 3 minutes. The resulting emulsion was then injected directly into one of the popliteal lymph nodes of a half lop rabbit at Imperial College of Science and Technology. The rabbit was bled approximately every ten days and given boost injections 2 and 4 weeks after the first. The first boost was given in multiple sites and the second directly into the other popliteal lymph node.

(b) Elution of antibodies from the 16 kDa region of Western blots

Elution of antibodies from Western blots was based on the method of Beall and Mitchell (1986). MS were extracted with TX-114 (as described in section 2.10.1). The insoluble pellets were then boiled in SDS PAGE sample buffer and the antigen separated by SDS PAGE using 6 large gels (40,000 MS/gel). The antigen was transferred by Western blotting and the blots blocked by incubation in 5% milk solution. Vertical strips cut from both ends of each blot were probed with VRabS and B3A to indicate the position of the 16 kDa antigen and the area of nitrocellulose to which the 16 kDa antigen was bound was then excised from each blot. The strips were re-blocked, treated with 20 mM sodium periodate in 50 mM sodium acetate buffer (pH 4.5) for 1 hour in the dark and incubated overnight at 4°C with 20 ml VRabS at a dilution of 1 in 20. Following removal of the antibody, the strips were washed 5 times for 30 minutes in 50 mM Tris/HCl (pH 8.0), 0.15 M NaCl, 0.02% Tween 20, once for 30 minutes in 0.1 M borate buffer (pH 8.0), 0.15 M NaCl, once for 30 minutes in PBS and finally for 30 minutes in 10 mM Tris/HCl (pH 8.0). This final wash was carried out in low molarity Tris to ensure that buffer remaining prior to elution of antibody from the strips had a low buffering capacity and was therefore unable to cause an increase in the pH of the elution buffer. Antibody was then eluted by addition of 9 ml 0.1 M glycine/HCl (pH 2.5), to the strips in a petri dish previously blocked with 1% BSA in the acidic buffer to prevent absorption of eluted antibody onto the plastic surface. The eluted antibodies were removed after 5 minutes, neutralised by the addition of 700 $\mu$l 1 M Tris/HCl (pH 8.0) and BSA added to a final concentration of 1%. The strips were then washed for 10 minutes in PBS followed by Tris/HCl (10 mM, pH 8.0) and the elution steps repeated. Both aliquots of eluted antibody were stored at -20°C prior to use. It was subsequently demonstrated that the strips of antigen could be used repeatedly for the elution of antibodies.

### 2.25.2 Preparation of competent *E. coli* Y1090 for screening

A glycerol stock of Y1090 was used to streak a fresh YT agar plate (1.5%, see appendix) supplemented with ampicillin (50 $\mu$g/ml) (YT amp50) which was then incubated overnight at 37°C. A single colony of Y1090 was used to inoculate 10 ml of YT medium supplemented with 10 mM $MgSO_4$, 0.2% maltose and 50 $\mu$g/ml ampicillin. The culture was grown overnight at 37°C in a shaking incubator. Bacterial cells were then pelleted by centrifugation at 500 x g for 10 minutes at room temperature and resuspended

in 5 ml of sterile 10 mM $MgSO_4$.

### 2.25.3 Primary screening of the cDNA library

For each agar plate used in screening, approximately 1.5 x $10^4$ λgt11 pfu were used to infect 600 μl of competent Y1090 (prepared as above) by incubation for 15 minutes at 37°C. The cells were then added to 9 ml of soft agar (0.8%) containing 10 mM $MgSO_4$ at 50°C and plated onto fresh 140 mm YT amp 50 plates and grown at 42°C until plaques were visible (3 - 4 hours). Nitrocellulose filters (130 mm, 45 μm, Millipore) were soaked in 10 mM isopropyl-ß-D thiogalactopyranoside (IPTG) for 5 minutes and excess solution was blotted away such that the filters remained damp. The IPTG impregnated filters were then overlaid onto the plates and the expression of recombinant proteins induced by incubating the plates for a further 2 hours at 37°C. The filters were then orientated using a needle to puncture both the filter and the underlying agar and carefully removed. Following removal the filters were washed for 5 minutes in TBST (20 mM Tris/HCl (pH 8.0), 0.15 M NaCl, 0.1% Tween 20) to remove bacterial debris and blocked (see below). The filters were then probed overnight with undiluted sera eluted from the 16 kDa region of Western blots (as above). The plates were stored at 4°C.

### 2.25.4 Detection of positive recombinants

Two systems employing different conjugates and substrates were used as follows:-

(a) Alkaline phosphatase labelled conjugate

Filters to be developed using this system were blocked in 0.1% Tween 20, 1% BSA in TBS for 45 minutes at room temperature and incubated overnight in sera diluted in TBS. Following removal of the antisera the filters were washed 4 times for 15 minutes in TBST and incubated for 1½ hours in alkaline phosphatase labelled anti-rabbit conjugate (Biorad) diluted 1 in 7,500 in 0.1 M Tris/HCl (pH 9.5), 0.1 M NaCl, 5 mM $MgCl_2$. The washing step was then repeated and the filters developed by the addition of substrate solution (375 μg/ml naphthol phosphate (NBT, taken from a stock of 75 mg/ml in 70% dimethylformamide), 188 μg/ml 5-bromo-4-chloro-3 indoly phosphate (BCIP, taken from a stock of 50 mg/ml in 100% dimethylformamide), 0.1 M Tris/HCl (pH 9.5), 0.1 M NaCl, 50 mM $MgCl_2$). The reaction was stopped by the addition of 20 mM Tris/HCl (pH 8.0), 5 mM EDTA.

(b) HRP-labelled conjugate

Filters to be developed using this system were blocked in 5% milk solution and incubated overnight in sera diluted in the same. Following removal of the antisera the filters were washed five times for 5 minutes with PBS / 0.1% Tween 20 and incubated in biotinylated donkey anti-rabbit species specific conjugate diluted 1 in 200 in PBS. The washing step was repeated and the blots then incubated in streptavidin horseradish peroxidase complex (Amersham) diluted 1 in 400 in PBS. Following a final repeat of the washing step the bound antibody was then visualised by the addition of the substrate (0.625 mg/ml diaminobenzadine, 0.004% cobalt chloride, 0.01% hydrogen peroxide in PBS). The reaction was stopped by rinsing the filters with water.

### 2.25.5 Secondary screening

Clones that were positive with the sera eluted from the 16 kDa region of Western blots were picked using the sharp end of a pasteur pipette into 100 $\mu$l of SM buffer (0.1 M NaCl, 50 mM Tris/HCl (pH 7.5), 8 mM $MgSO_4$, 2% gelatine) containing 3 $\mu$l of chloroform. The tubes were incubated for at least 15 minutes at room temperature to enable the phage to elute from the agar plug. The phage stock was then diluted appropriately and plated at a density of $10^2$ - $10^3$ pfu per 90 mm plate. The phage were then grown and screened as described above using a 1 in 200 dilution of VRabS.

### 2.25.6 Purification of positive recombinants

Plaques positive with VRabS were picked using the sharp end of a Pasteur pipette into 100 $\mu$l SM buffer. The phage were allowed to elute from the agar plug and then used in a tertiary array. 150 $\mu$l of Y1090 were plated out in 3 ml soft agar onto 90 mm YT amp50 plates and 50 - 100 pfu from each phage stock were spotted onto the surface in 2 - 5 $\mu$l of SM buffer. The plates were incubated and recombinant proteins expressed and screened using a 1 in 200 dilution of VRabS. This procedure either confirmed the purity of the secondary phage stock or enabled a plaque to be selected for a tertiary stock.

### 2.25.7 Differential screening of tertiary arrays

Clones positive with VRabS on secondary screening were used in a tertiary array as described above (section 2.25.6) with the exception that following the removal of the first nitrocellulose filter a second IPTG impregnated filter was overlaid onto the plate. The

plate was then incubated for a further 2 hours at 37°C and the second filter removed. Both filters were blocked and the first set incubated overnight in CIS diluted 1 in 50 in 5% milk solution and the second in VRabS at a dilution of 1 in 200.

### 2.25.8 Amplification of positive clones

Clones of interest were amplified and plate stocks made as follows:- Competent Y1088 were prepared as described for Y1090 (section 2.25.2) and infected with a high density of phage (5,000 pfus). The bacteria were then plated on fresh YT amp50 plates and grown overnight at 42°C in order to achieve confluent bacterial cell lysis. The plates were then overlaid with 5 ml of SM buffer and the phage eluted by gentle shaking for 2 hours at 40°C. The SM buffer was removed and the bacterial cell debris pelleted by centrifugation at 2,000 x g for 10 minutes at room temperature. The supernatant was then aliquoted into 1 ml fractions and 15 µl of chloroform added per 1 ml. Phage stocks were stored at 4°C.

## 2.26 ANTIBODY SELECT

Plaque specific antibody purification (antibody select) was carried out using a modified version of the procedure described by Ozaki *et al.*, (1986):- Clones of interest were plated in duplicate at a density of 3 x $10^5$ pfu per 90 mm YT amp50 plate. The plates were incubated at 42°C until plaques were just visible (3 - 4 hours) then overlaid with filters impregnated with 10 mM IPTG and incubated for 2 hours at 37°C. The filters were then turned over and the plates incubated for a further 2 hours to enable recombinant protein to bind to both sides of the filter. The filters were then removed, blocked in 5% milk solution for 2 hours and incubated overnight in a 1 in 40 dilution of VRabS at 4°C. Following removal of the antibody solution the filters were washed 5 times for 30 minutes (50 mM Tris/HCl (pH 8.0), 0.15 M NaCl, 0.02% Tween 20), once for 30 minutes (0.1 M borate buffer (pH 8.0), 0.15 M NaCl), once for 30 minutes in PBS and finally for 30 minutes in 10 mM Tris/HCl (pH 8.0). A petri dish was then blocked with 1% BSA in 0.1 M glycine/HCl (pH 2.7), and the filters incubated in the dish for 5 minutes in 4 ml of 0.1 M glycine/HCl (pH 2.7) at room temperature. The eluted antibodies were removed and neutralised by the addition of 75 µl 2 M Tris/HCl (pH 8.0) and 400 µl 10% BSA were added. The antibodies were then used to probe Western blots as necessary.

## 2.27 EXAMINATION OF THE RECOMBINANT PROTEINS EXPRESSED BY λgt11 in Y1090

Competent Y1090 were infected with 3 x $10^5$ pfu and plated in 3 ml of soft agar supplemented with 2 mM IPTG onto fresh 90 mm YT amp50 plates. The plates were incubated at 37°C for 6 hours. The soft agar was then scraped away into 2 microcentrifuge tubes and 1 ml of each scrape was boiled for 3 minutes with 333 μl of 4 x SDS PAGE sample buffer. 2 - 10 μl of each sample was then loaded whilst molten onto an 8% polyacrylamide gel. Following electrophoresis, gels were initially stained with Coomassie blue to enable the amount of recombinant protein produced by individual clones to be assessed. Comparable amounts of recombinant protein were then loaded across a 7 cm slot, electrophoresed and transferred by Western blotting. The blot was then cut into strips and probed with various sera pre-absorbed by rolling overnight with 200 μl of non-recombinant λgt11 lysate (see section 2.38.1).

## 2.28 EXTRACTION OF DNA WITH ORGANIC SOLVENTS

### 2.28.1 Phenol extraction

A volume of TE (see appendix) equilibrated phenol equal to half the volume of the sample was added and the tube was vortexed and incubated at room temperature for 5 - 15 minutes. The sample was then centrifuged at 6,000 x g for 10 minutes at room temperature and the aqueous layer containing the DNA removed. To ensure that the maximum amount of DNA was obtained the remaining phenol was then overlaid with a suitable volume of SDW and vortexing, centrifugation and removal of the aqueous phase were repeated as above.

### 2.28.2 Chloroform extraction

Chloroform diluted 24 parts to 1 with isoamyl alcohol ($CHCl_3$/IAA) was used to extract DNA using a procedure identical to that described for phenol extraction.

### 2.28.3 Phenol / chloroform extraction

A 1 to 1 mixture of phenol and $CHCl_3$/IAA was also used for purification of DNA. The procedure used was identical to that described above for phenol extraction.

## 2.29 ETHANOL PRECIPITATION OF DNA

DNA was precipitated by the addition of 3 M Na acetate to a final concentration of 300 mM plus 2 volumes of -20°C absolute ethanol (Aristar grade, BDH) and incubation for 30 minutes at -70°C or overnight at -20°C. Following incubation the precipitated DNA was pelleted by centrifugation at 6,000 x g for 10 minutes at 4°C and the ethanol removed. The pellet was then washed with 70% -20°C ethanol, dried under vacuum and resuspended in an appropriate amount of SDW or TE.

## 2.30 LARGE SCALE PREPARATION OF λgt11 DNA

A single colony of Y1088 was used to inoculate 10 ml of YT media supplemented with ampicillin (50 μg/ml) and 0.2% maltose. The culture was grown overnight in a shaking incubator at 37°C. The bacteria were then pelleted by centrifugation at 4,000 x g for 10 minutes at 4°C and resuspended in 10 ml 10 mM $MgSO_4$, 3 ml of which was used along with 3 x $10^5$ recombinant λgt11 phage to inoculate 75 ml JLB media (see appendix) supplemented with 2 mM $MgSO_4$. The culture was again grown overnight in a shaking incubator at 37°C, pelleted by centrifugation as above and the supernatant removed. Ribonuclease (RNAase, Sigma) at a final concentration of 40 μg/ml and deoxyribonuclease (DNAase, Sigma) at a final concentration of 16 μg/ml were then added to the supernatant which was incubated for 1 hour at 37°C. The phage were then precipitated by the addition of polyethylene glycol (PEG) 1000 and 2.5 M NaCl to a final concentration of 2.7% and 0.3 M respectively, followed by incubation at 4°C for 2 hours. The tubes were then centrifuged for 40 minutes at 14,000 x g and the pellets resuspended in 1 ml of TE plus 4 mM EDTA and incubated for 10 minutes at room temperature. The resuspended phage were then extracted once with phenol alone and repeatedly with phenol / chloroform until precipitated protein was no longer seen at the interface between the aqueous and organic phases. The contents of the three tubes were then pooled and ethanol precipitated to concentrate the DNA. Following precipitation the remaining pellets were resuspended in 1 ml of TE, divided into two and phenol / chloroform extraction was repeated a further 5 times. The samples were then combined once more, ethanol precipitated as above and the pellet resuspended in an appropriate volume of TE.

## 2.31 POLYMERASE CHAIN REACTION (PCR)

### 2.31.1 Amplification of DNA from λgt11 phage stocks

2 μl of phage stock was added to 18 μl of SDW and boiled for 3 minutes. 4 μl of 10 x PCR buffer (Bioline), 4 μl dNTP's (2 mM, Boehringer Mannheim), 0.6 μl of each primer (20 μM), a further 10.3 μl of SDW and 0.5 μl of Taq polymerase (5U/μl), Bioline) were then added to each sample. The sample was covered with a layer of sterile paraffin to prevent evaporation and the PCR reaction was carried out using 35 cycles as follows:-

| | |
|---|---|
| Denaturation | 94°C for 60 seconds |
| Annealing | 55°C for 60 seconds |
| Extension | 72°C for 100 seconds |

### 2.31.2 Amplification of DNA from cDNA libraries

PCR reactions were carried out using DNA from both adult and sporocyst cDNA libraries constructed in λgt11 bacteriophage. 200 μl of 2 M NaCl / 20% PEG were added to a 1 ml aliquot of the amplified library, the sample vortexed briefly and left at room temperature for 15 minutes. The preparation was then centrifuged at 6,000 x g for 5 minutes at room temperature and the supernatant removed with care being taken to ensure that no PEG remained. The precipitated DNA was resuspended in 60 μl of SDW and 7 μl of 10 x PCR buffer (Bioline), 7 μl dNTP's (2 mM, Boehringer Mannheim), 1 μl of each primer (20 μM) and 0.5 μl Taq polymerase (5U/μl, Bioline) were added to 53.5 μl of the DNA sample. The preparation was then overlaid with a layer of sterile paraffin and the PCR reaction carried out as follows:-

| | | |
|---|---|---|
| Cycle 1 | Denaturation | 94°C for 5 minutes |
| | Annealing | 55°C for 60 seconds |
| | Extension | 72°C for 60 seconds |
| Cycles 2 - 31 | Denaturation | 94°C for 60 seconds |
| | Annealing | 55°C for 60 seconds |
| | Extension | 72°C for 60 seconds |

### 2.31.3 PCR primers

The following primers were used for PCR reactions.

λgt11 forward primer:- 5'd(GGTGGCGACGACTCCTGGAGCCCG)3'

λgt11 reverse primer:- 5'd(TTGACACCAGACCAACTGGTAATG)3'

2.1 primer:- 5'd(GCGCGAATTCGAAAGTTTCTTATTTGT)3'

## 2.32 DNA ELECTROPHORESIS

### 2.32.1 Agarose gel electrophoresis

An appropriate amount of 6 x concentrated agarose gel loading buffer (30% glycerol, 0.25% xylene cyanol FF, 0.25% bromophenol blue) was added to the DNA to be separated by electrophoresis. The sample was then loaded onto a 1% agarose gel (Biorad) made in TAE (see appendix) and electrophoresed alongside high and / or low molecular weight DNA markers. The molecular weights of the markers used were as follows:-

| λ Hind III (bp) | φX Hae III (bp) |
|---|---|
| 23,130 | 1,353 |
| 9,416 | 1,078 |
| 6,557 | 872 |
| 4,361 | 603 |
| 2,322 | 310 |
| 2,027 | 281 |
| 564 | 271 |
| 125 | 234 |
| | 194 |
| | 118 |
| | 72 |

1 μl of λ Hind III (USB) and / or 0.5 μl of φX Hae III (Cambridge Bioscience) were run per agarose gel. The gel was electrophoresed at 80 V for 30 - 45 minutes using the Biorad mini sub DNA cell electrophoresis system.

### 2.32.2 Staining of agarose gels

On all occasions DNA agarose gels were stained with ethidium bromide (Sigma) taken from a 10 mg/ml stock. If the DNA was not to be isolated from the agarose gel and

used in subsequent procedures then 1 $\mu$l of ethidium bromide was added to the molten agarose and incorporated into the gel prior to electrophoresis. If the DNA was to be used in subsequent procedures then the gel was stained following electrophoresis by incubation in a solution of ethidium bromide in TAE for as long as was necessary to visualise the DNA. DNA was visualised by examination of the gel on a UV light transilluminator and gels photographed under UV light using an orange filter.

## 2.33 PURIFICATION OF DNA FROM LOW MELTING POINT AGAROSE

DNA electrophoresis was performed as above with the exception that 1% low melting point agarose (Biorad) was used and the gel was electrophoresed at 40 V for 1 to 1½ hours. The DNA was then visualised by staining as briefly as possible with ethidium bromide and the region of the gel containing the DNA was excised using a sterile scalpel blade and transferred to a microcentrifuge tube. A volume of SDW approximately equal to that of the gel was added together with 5 M NaCl to give a final concentration of 0.25 M and the sample was placed at 65°C for 10 minutes or until the agarose was molten. DNA was extracted from the molten solution with phenol and then chloroform and ethanol precipitated overnight at -20°C.

## 2.34 DIGESTION OF DNA WITH RESTRICTION ENZYMES

Digestions were carried out in 10 $\mu$l of the appropriate buffer at 37°C for 90 minutes. The reaction was then stopped by freezing the sample or by phenol / chloroform extraction. The following restriction enzymes were used at a concentration of 5 - 10 units per 1 $\mu$g of DNA to be digested.

| Enzyme | Buffer |
|---|---|
| *Eco*R I (NBL) | High |
| *Pvu* II (NBL) | Medium (USB) |
| *Kpn* I / *Sac* I (NBL) | Low |

| Buffers (10X) | NaCl | Tris/HCl (pH 7.4) | $MgCl_2$ | DTT |
|---|---|---|---|---|
| High | 1 M | 0.5 M | 0.1 M | 10 mM |
| Medium | 0.5 M | 0.1 M | 0.1 M | 10 mM |
| Low | - | 0.1 M | 0.1 M | 10 mM |

## 2.35 SUBCLONING

### 2.35.1 Plasmid vectors

(a) M13 mp18/mp19

M13 vectors are often used to subclone DNA prior to sequencing as they enable single stranded DNA to be obtained. A further attraction of these vectors is that insertion of DNA into the cloning site inactivates the $\alpha$ peptide thus preventing the synthesis of ß-galactosidase and allowing bacteria harbouring recombinant phage to be selected on X-gal / IPTG plates. Here the *Kpn* / *Sac* fragment of λgt11 clone 18.5 was subcloned into *Kpn* / *Sac* cut M13 mp19 using 10 ng of de-phosphorylated vector DNA and a 1 : 3 vector to insert ratio. *Eco*R I cut PCR products from clones 18.5 and 2.1 were similarly subcloned into *Eco*R I cut M13 mp18.

(b) pGEM-T vector

The pGEM-T vector (Promega) is specially designed for the cloning of PCR products. It is prepared by digestion of the pGEM-5Zf vector (Promega) with the enzyme *Eco*R V followed by the addition of a single 3′ thymidine residue to both strands. This creates T overhangs which increase the efficiency with which PCR products are cloned into the pGEM-T vector by forming base pairs with the single deoxyadenosine residue added to the 3′ end of PCR products by many Taq polymerases. The pGEM-T vector also employs insertional inactivation of the $\alpha$ peptide allowing colour selection of recombinants. A PCR product of clone 2.1 was subcloned into the pGEM-T vector using 25 ng of vector DNA and a 1 : 5 vector to insert ratio.

(c) TA vector

TA vector was kindly prepared and the ligation performed by Dr. Phillipa Francis at the Middlesex Hospital. The TA vector is prepared by digestion of the Bluescript SK +/- vector with the enzyme *Eco*R V and the addition of 3′ terminal thymidine to both strands. As with the pGEM-T vector the efficiency of ligation of PCR products into the TA vector is improved by the formation of T overhangs. Recombinant TA plasmids can also be identified by colour selection. A PCR product of clone 2.1 was subcloned into the TA vector using 100 ng of vector DNA and a 1 : 2 vector to insert ratio.

(d) pGEX vector

The pGEX vectors are versions of a plasmid (pSj5) (Smith and Johnson, 1988) which is able to direct the synthesis of the 26 kDa *S. japonicum* GST isoenzyme (Sj28 GST) in *E. coli* under the control of an IPTG inducible promotor. The vectors are modified in such a way as to allow the expression of foreign peptides as a fusion with the C-terminal of Sj28 GST. Such recombinant proteins are easily purified using glutathione bound to agarose beads and the protease thrombin can then be used to remove Sj28 GST. One of the pGEX vectors, pGEX-2T, has a unique *Eco*R I site within its cloning region which is in frame with the *Eco*R I site of λgt11. Thus, following subcloning of the PCR product of clone 2.1 into the TA vector, the insert was excised by cutting with *Eco*R I and subcloned into pGEX-2T. For each ligation 25 ng of vector DNA was used and a 1 : 2 vector to insert ratio.

### 2.35.2 DNA ligations

DNA ligations were carried out overnight at 16°C using 1 μl of T4 ligase and 1 μl 10 X ligase buffer (NBL) in a 10 μl reaction.

### 2.35.3 Preparation of competent TG2s

All of the plasmids were used to transform the bacterial strain TG2 (*sup*E *hsd*Δ5 *thi* Δ(*lac-pro*AB) Δ(*srl-rec*A)306::Tn*10*(*tet*$^r$) F' [*tra*D36 *pro*AB' *lac*I$^q$ *lac*ZΔM15]):- A glycerol stock of TG2 was used to streak a minimal plate (see appendix) which was grown overnight at 37°C. A single colony of TG2 was then used to inoculate 10 ml of YT which was grown overnight at 37°C with shaking. 2 ml of the overnight culture was then used to inoculate a further 40 ml of YT and the culture grown until an $OD_{600}$ of 0.4 - 0.6 was reached (approximately 2 hours). The bacteria were then pelleted in sterile tubes by centrifugation at 400 x g for 10 minutes at 4°C and the pellet resuspended in 20 ml ice cold 50 mM $CaCl_2$. The cells were incubated on ice for 20 minutes and then centrifuged as above. The final pellet was resuspended in 4 ml of 50 mM $CaCl_2$ and the bacteria stored at 4°C until needed. Competent cells were most efficient when used immediately although transformation of the cells was possible for up to 5 days following treatment.

### 2.35.4 Transformation of competent bacteria with plasmid DNA

The procedure used for the transformation of bacteria with the single stranded

plasmids M13 mp18/mp19 is given in section 2.37.1. Bacterial cells were transformed with pGEM-T, TA or pGEX plasmids as below.

10 μl of the ligation reaction containing recombinant plasmid DNA were added to 200 μl of fresh competent cells. The preparation was incubated on ice for at least 30 minutes to enable the DNA to bind to the surface of the bacteria and movement of the DNA into the cell was then induced by heat shocking in a water bath at 42°C for 90 seconds. The cells were transferred onto ice and the volume of the preparation made up to 5 ml by the addition of YT. The culture was then incubated at 37°C for 1 hour with gentle shaking. This enables the enzymes encoded by the plasmid which detoxify antibiotic to be produced within the bacteria prior to plating out on ampicillin plates. Following incubation the cells were pelleted by centrifugation at 600 x g for 10 minutes and the majority of the supernatant removed. The cells were plated out in the remaining media (approximately 100 μl) on 90 mm YT plates supplemented as appropriate for the selection of recombinants and grown overnight at 37°C.

### 2.35.5 Selection of recombinants

The procedure used for the selection of recombinants was determined by the phenotype of the plasmid.

(a) Selection on ampicillin plates

All the plasmids employed carry the ampicillin resistance gene. Thus, in all cases transformed cells were plated on ampicillin plates which allow only those bacteria harbouring plasmids to grow and form colonies. Cells transformed with the pGEM-T plasmid were grown on YT plates supplemented with ampicillin at a concentration of 100 μg/ml. For cells transformed with other plasmids ampicillin at a concentration of 50 μg/ml was used.

(b) Selection on IPTG / X-gal plates

Bacterial cells transformed with the TA or pGEM-T plasmids were grown on YT plates supplemented with appropriate amounts of ampicillin and overlaid with IPTG and X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside):- 4 μl of IPTG (1 M) and 4 μl of X-gal (10%) in 100 μl YT were used per 90 mm plate and the plates were spread at least half an hour prior to plating of the bacteria. The plates were then dried and the

bacteria plated. White colonies of cells harbouring recombinant plasmids could be distinguished from colonies of cells harbouring self ligated vector which are blue in colour.

## 2.36 PREPARATION OF DOUBLE STRANDED PLASMID DNA

The procedure used for the preparation of single stranded DNA from the M13 vector is given in section 2.37.1. The following methods were used for the preparation of TA and pGEM-T double stranded plasmid DNA.

### 2.36.1 Mini preparations

(a) Quick boiling method

This method was used for identifying recombinant clones by size differentiation. Putative recombinant clones were picked using a sterile bacterial loop and used to inoculate 3 ml of YT plus ampicillin (50 - 100 $\mu$g/ml). The cultures were incubated overnight at 37°C with shaking. Bacteria from 1.5 ml of each overnight culture were then pelleted by centrifugation at 6,000 x g for 10 minutes at room temperature and the supernatant removed. The pellet was resuspended by vortexing in 100 $\mu$l STET (0.1 M NaCl, 10 mM Tris/HCl (pH 8.0), 1 mM EDTA, 5% TX-100) and 8 $\mu$l of fresh lysozyme solution (a few crystals in 1 ml 10 mM Tris (pH 8.0)) was added. The tubes were incubated on ice for 5 minutes prior to boiling for 40 seconds. Chromosomal DNA was then sedimented by centrifugation for 10 minutes at 4°C and removed using a sterile pipette tip. 20 $\mu$l of the remaining supernatant was electrophoresed on a 1% agarose gel alongside identically prepared DNA from a non-recombinant vector. An increase in the size of the plasmid was used as an indicator of the insertion of foreign DNA.

(b) Alkaline lysis method

This method was used to prepare small amounts of recombinant DNA to a purity suitable for cutting with restriction enzymes and subcloning. A single colony of recombinant bacteria was used to inoculate 3 ml of YT plus ampicillin (50 - 100 $\mu$g/ml) and the culture grown overnight at 37°C with shaking. 1.5 ml of the culture was then was centrifuged at 6,000 x g for 10 minutes at room temperature, the supernatant discarded and the pelleted bacteria resuspended in 100 $\mu$l of 15% sucrose, 10 mM EDTA, 25 mM Tris/HCl (pH 8.0) plus a few crystals of lysozyme. The solution was incubated on ice for

5 minutes and chromosomal DNA then denatured by the addition of 200 μl 0.2 M NaOH / 1% SDS and incubation on ice for a further 5 minutes. The preparation was neutralised by the addition of 150 μl 3 M potassium acetate, 12% acetic acid and the denatured chromosomal DNA sedimented by centrifugation at 6000 x g for 15 minutes at 4°C. The supernatant was removed, extracted with phenol / chloroform and the DNA precipitated with ethanol at -70°C for 30 minutes. Following precipitation the pellet was resuspended in 50 μl TE and treated with RNAase (final concentration of 50 μg/ml) for 90 minutes at 37°C. The preparation was re-extracted with phenol and phenol / chloroform until the DNA was free from contaminating protein and then ethanol precipitated overnight. The final pellet was resuspended in 10 μl TE.

**2.36.2 Large scale preparation of DNA from recombinant plasmids**

A single colony was used to inoculate 10 ml of YT plus ampicillin (50 μg/ml) and the culture grown overnight in a shaking incubator at 37°C. 200 μl of this overnight culture was then used to inoculate a further 100 ml of YT which was grown overnight as above. Following incubation bacteria were pelleted by centrifugation at 400 x g and the supernatant removed. The pelleted bacteria were resuspended in 5 ml 15% sucrose, 10 mM EDTA, 25 mM Tris/HCl (pH 8.0) plus a few crystals of lysozyme and the preparation incubated on ice for 5 minutes. 10 ml 0.2 M NaOH / 1% SDS were then added and the tube incubated for a further 5 minutes. The solution was neutralised by the addition of 7.5 ml 3 M potassium acetate, 12% acetic acid and incubated on ice for 30 - 60 minutes. Denatured chromosomal DNA was then sedimented by centrifugation at 4,000 x g for 20 minutes at 4 °C. The supernatant was collected and extracted once with phenol / chloroform. The plasmid DNA was then precipitated by the addition of 1 volume of isopropanol and incubation on ice for at least 10 minutes. The DNA was pelleted by centrifugation at 400 x g for 10 minutes at 4°C, the isopropanol removed and the pelleted rinsed with 70% ethanol and dried under vacuum. Once free of ethanol the pellet was resuspended in 4 ml of TE and incubated with 50 μg RNAase for 1 hour at 37°C. The reaction was stopped by phenol extraction followed by extraction with phenol / chloroform and the plasmid DNA was then concentrated by ethanol precipitation. Following precipitation the pellet was resuspended in 606 μl of SDW and incubated with PEG / NaCl at a final concentration of 10% and 0.4 M respectively for 20 minutes on ice. The DNA was then pelleted by centrifugation at 6,000 x g for 15 minutes at 4°C,

rinsed with 70% ethanol, dried under vacuum and resuspended in 50 μl of TE.

## 2.37 DNA SEQUENCING

### 2.37.1 Preparation of M13 DNA for sequencing

A plaque containing recombinant M13 phage was resuspended in 1.5 ml of a 1 in 100 dilution of an overnight culture of TG2. This was then grown for 5½ hours at 37°C in a shaking incubator. The bacteria were pelleted by centrifugation at 6,000 x g for 10 minutes at room temperature and a 20 μl aliquot of the supernatant was incubated at 65°C for 5 minutes with 1 μl 2% SDS. 5 μl of DNA sample buffer were then added and the sample was electrophoresed on a 1% agarose gel alongside non-recombinant plasmid. An increase in size was used as conformation of the presence of an insert and if observed 200 μl of the remainder of the supernatant was precipitated with 20% PEG / 2M NaCl by incubation for 15 minutes at room temperature. The DNA was then pelleted by centrifugation at 6,000 x g for 5 minutes at room temperature and the supernatant removed. This procedure was repeated to ensure complete removal of PEG. The pellet was then resuspended in 100 μl TE, subjected to repeated phenol / chloroform extraction until the DNA was free from contaminating protein and ethanol precipitated overnight. 1 μl of the preparation was electrophoresed alongside a sample of M13 of a known concentration to estimate the yield. 1 μg of recombinant DNA was used per sequencing reaction.

### 2.37.2 Preparation of double stranded DNA for sequencing

Double stranded plasmid DNA was isolated from bacterial cells as described (section 2.36.2). For each sequencing reaction to be performed 2.5 μg of DNA was then denatured as follows:- The volume of the sample containing the DNA to be denatured was made up to 16 μl by the addition of SDW if necessary. 4 μl of 2 M NaOH, 1 mM EDTA were then added and the preparation was incubated for 5 minutes at room temperature. The solution was placed on ice, neutralised by the addition of 2 μl of 2 M ammonium acetate (pH 5.6) and the DNA precipitated by the immediate addition of 55 μl of ice cold 95% ethanol. The sample was then incubated at -80°C for at least 10 minutes, the ethanol removed and the pellet washed twice with 1 ml of 70% -20°C ethanol. The final pellet was freeze dried under vacuum and resuspended in 7 μl of SDW.

### 2.37.3 Sequencing reactions

DNA sequencing was performed using a USB Sequenase kit according to the manufacturers instructions:-

(a) Annealing the sequencing primers

The primers to be employed were diluted to a concentration of 0.5 $\mu$M and 1 $\mu$l (0.5 pM) was added to 7 $\mu$l of resuspended DNA plus 2 $\mu$l of sequenase buffer (0.2 M Tris/HCl (pH 7.5), 0.25 M NaCl, 0.1 M $MgCl_2$). The preparations were then warmed to 65°C for 2 minutes and cooled slowly until the temperature reached 30°C. The tubes were then placed on ice. The primers used for sequencing were as follows:-

| | |
|---|---|
| λgt11 forward primer:- | 5′d(GGTGGCGACGACTCCTGGAGCCCG)3′ |
| λgt11 reverse primer:- | 5′d(TTGACACCAGACCAACTGGTAATG)3′ |
| M13 (-40) primer:- | 5′d(GTTTTCCCAGTCACGAC)3′ |
| T3 forward primer:- | 5′d(TAATACGACTCACTATAGGGCGA)3′ |
| 2.1 primer:- | 5′d(GCGCGAATTCGAAAGTTTCTTATTTGT)3′ |

(b) Labelling reactions

For standard reactions (reading up to 500 base pairs from the primer) the labelling mix (7.5 $\mu$M dGTP, 7.5 $\mu$M dCTP, 7.5 $\mu$M dTTP) was diluted 1 in 5 with SDW and 2 $\mu$l of diluted labelling mix, 1 $\mu$l of 0.1 M DTT, 0.5 $\mu$l [$\alpha$-$^{35}$S]dATP (10 $\mu$Ci/$\mu$l, 600 Ci/mM) and 2 $\mu$l of sequenase diluted 1 in 8 in enzyme dilution buffer (10 mM Tris/HCl (pH 7.5), 5 mM DTT, 0.5 mg/ml BSA) were added. The reaction was then incubated at room temperature for 5 minutes.

(c) Termination reactions

Once the labelling reaction was complete 3.5 $\mu$l of the reaction was transferred to each of 4 pre-warmed (37°C for 1 minute) tubes containing 2.5 $\mu$l of a termination mix (each termination mix contains 80 $\mu$M dGTP, 80 $\mu$M dATP, 80 $\mu$M dCTP, 80 $\mu$l dTTP, 50 mM NaCl plus 8 $\mu$M ddGTP (ddGTP termination mix) or 8 $\mu$M ddATP (ddATP) termination mix, 8 $\mu$M ddCTP (ddCTP termination mix) or 8 $\mu$M ddTTP (ddTTP termination mix)). The tubes were then incubated for 5 minutes at 37°C. Termination reactions were stopped by the addition of 4 $\mu$l stop solution (95% formamide, 20 mM

EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). The samples were then stored overnight at -20°C.

(d) Alteration of reaction conditions

(a) Sequencing close to the primer:- For sequencing close to the primer (less than 200 nucleotides). The labelling mix was diluted 1 in 20 in SDW and both the labelling and termination reactions were carried out for only 3 minutes.

(b) Extending sequences away from the primer:- To sequence up to 600 nucleotides away from the primer the termination reaction was altered by the replacement of 2.5 $\mu$l of termination mix with a mixture of 1.5 $\mu$l Sequence Extending Mix plus 1 $\mu$l of the appropriate termination mix.

### 2.37.4 Denaturing gel electrophoresis

40 cm by 33 cm glass plates were used with 0.4 mm spacers. Prior to pouring the gel the plates were thoroughly cleaned with NaOH / ethanol and treated with Repelsilane (small plate) (Hopkins & Williams) or 15 $\mu$l silane (large plate) (BDH), 165 $\mu$l 10% acetic acid in 5 ml methanol (large plate). A 40% acrylamide : N N', methylene bisacrylamide (ratio 19 : 1) stock solution was then used to make a 6% polyacrylamide sequencing gel (6% acrylamide : bisacrylamide solution, 50 mM Tris, 50 mM orthoboric acid, 1 mM EDTA, 7.6 M urea, 0.04% ammonium persulphate, 0.0005% TEMED). The gel was poured and allowed to set overnight at room temperature. Immediately prior to electrophoresis the samples to be loaded were heated to 75°C for 2 minutes and the gel was pre-run for 20 minutes at a constant 40 W in running buffer (0.1 M Tris, 0.1 M orthoboric acid, 2 mM EDTA). The wells were then cleared of urea released from the gel and the samples were loaded and electrophoresed at a constant 40 W. If sequence close to the primer alone was required potassium acetate was added to the lower buffer chamber to a final concentration of 1 M and the gel was electrophoresed until the highest dye line was approximately 22 cm from the bottom of the gel. If sequencing further from the primer was also required then potassium acetate was not used, electrophoresis was continued until the lower dye line reached 3 cm from the bottom of the gel and a second set of reactions were loaded and electrophoresed until the higher dye line was 22 cm from the bottom of the gel. Following electrophoresis the gel was soaked in 10% acetic acid for 10 minutes at room temperature, rinsed with water for 20 minutes and then dried for

1 hour at 80°C. Dry gels were then put down on Kodak XAR-5 scientific imaging film.

## 2.38 PREPARATION OF λgt11 RECOMBINANT PROTEINS FOR IMMUNISATION

### 2.38.1 Preparation of λgt11 lysogens

A glycerol stock of Y1089 was used to streak a fresh YT amp50 plate and the plate incubated at 37°C overnight. A single colony of Y1089 was then used to inoculate 10 ml of YT supplemented with 0.4% maltose and ampicillin (50 μg/ml) and the culture grown overnight at 37°C in a shaking incubator. 1 ml of this overnight culture was used to inoculate 50 ml of pre-warmed YT supplemented as above and the culture incubated with shaking at 37°C until an $OD_{600}$ of 0.5 was reached (approximately 3 hours). $MgCl_2$ was then added to the cell culture to give a final concentration of 10 mM and the cells aliquoted into volumes of 100 μl. Each 100 μl aliquot was then infected with approximately 1.25 x $10^8$ pfu of the λgt11 18.5 phage stock and incubated for 20 minutes at 32°C. A 1 x $10^{-6}$ dilution of infected cells were plated out onto 90 mm YT amp50 plates to give approximately 250 pfu per plate and grown overnight at 32°C. Following overnight incubation 12 colonies were picked using a sterile pipette tip and each colony streaked onto two separate YT amp50 plates. One plate was then incubated overnight at 32°C and the other at 43°C. Clones which contained lysogenic phage were able to grow at 32°C but not at 43°C. Such clones were picked and used to produce recombinant proteins.

### 2.38.2 Expression of the recombinant protein by lysogenic phage

A single colony of Y1089 harbouring lysogenic phage was used to inoculate 5 ml of YT plus ampicillin (50 μg/ml) and the culture was grown overnight at 32°C with shaking. This overnight culture was then used to inoculate a further 100 ml of YT supplemented with ampicillin (50 μg/ml) and the culture grown until an $OD_{600}$ of 0.5 - 0.6 was reached (1.5 - 2 hours). The lytic cycle of the phage was then induced by incubating the culture at 42°C for 20 minutes with occasional mixing and IPTG was added to a final concentration of 10 mM to induce protein expression. The culture was then grown for a further 1.5 hours at 37°C. The bacteria were pelleted by spinning at 400 x g for 10 minutes at room temperature, resuspended in 2 ml of PBS and stored at -20°C.

### 2.38.3 Purification of the recombinant protein on a sucrose cushion

A few crystals of lysozyme were added to lysogens prepared as above and the samples incubated on ice for 20 minutes. The detergent TX-100 was then added to a final concentration of 1% and the preparation sonicated 3 times for 30 seconds. Sucrose cushions were prepared by pipetting 3.2 ml of 25% sucrose in PBS plus 0.05% TX-100 into ultracentrifuge tubes (Ultraclear centrifuge tubes, Beckman). 1.8 ml of sonicated control or 18.5 lysogen was then overlaid onto the sucrose gradients and the tubes centrifuged at 60,000 x g (27,000 rpm in a Beckman SW 55 swinging bucket rotor) for 30 minutes at 4°C. Following centrifugation the supernatant was removed followed by the sucrose itself. Both phases were placed on ice while the remaining pellet was washed with PBS plus 0.05% TX-100. The pellet was then resuspended in 1 ml PBS. 20 μl of the control and 18.5 lysogen supernatant and pellet phases were separated by electrophoresis through 8% polyacrylamide gels. The gels were stained with Coomassie blue or transferred by Western blotting and probed with VRabS in order to determine the phase in which the recombinant protein (FP 18.5/ß-gal) was present.

## 2.39 IMMUNISATION OF MICE WITH FP 18.5/ß-gal

2 female Balb/c mice and 2 female CBA mice were immunised with the supernatant phase obtained by separation on sucrose gradients of a lysate of bacteria harbouring the 18.5 recombinant phage (section 2.38). 2 control animals were immunised with the supernatant phase prepared from bacteria harbouring the wild type phage. The amount of FP 18.5/ß-gal or ß-gal alone present in this preparations was estimated to be 10 μg / 50 μl by comparison with protein standards following SDS PAGE and staining with Coomassie blue. Each mouse was immunised with a total of 150 μl (30 μg of protein) given over the course of three immunisations (days 0, 14 and 47). For the first of these immunisations the protein was given s.c in conjunction with FCA, whilst FIA was used for the latter two. All of the mice were challenged with 200 cercariae on day 59 and killed and perfused 55 days later.

## 2.40 PREPARATION OF GST RECOMBINANT PROTEINS FOR IMMUNISATION

### 2.40.1 Expression of recombinant proteins in pGEX

Following the subcloning of the 2.1 insert into the pGEX expression vector a single

colony of bacteria harbouring the recombinant plasmid was used to inoculate 5 ml of YT plus ampicillin (50 $\mu$g/ml). The culture was incubated overnight at 37°C with shaking. 500 $\mu$l of this overnight culture was then used to inoculate 4.5 ml of YT plus ampicillin (50 $\mu$g/ml) and the culture grown for 2 hours as above. To induce the expression of the recombinant protein IPTG was added (1 mM final concentration) and the culture was grown for a further 2.5 hours. Bacteria were then pelleted by centrifugation at 500 x g for 10 minutes at room temperature, the supernatant removed and the cells disrupted by resuspension in 500 $\mu$l of 1% TX-100 in PBS and 3 freeze / thaw cycles. Centrifugation was then repeated as above and the supernatant containing the released recombinant protein removed. 40 $\mu$l of a 50% suspension of glutathione agarose beads (Sigma) in PBS were added to the supernatant which was rotated overnight at room temperature. The beads were then pelleted by centrifugation, the unbound fraction removed and the beads washed three times with 1 ml of 1% TX-100 in PBS. The beads were boiled for 5 minutes in an equal volume of SDS PAGE sample buffer, sedimented by pulse centrifugation and the supernatant electrophoresed alongside proteins expressed by non-recombinant pGEX prepared in an identical manner.

### 2.40.2 Large scale preparation and purification of FP 2.1/GST

The above method was scaled up to provide a 1 L culture of bacteria harbouring the recombinant pGEX plasmid. The bacteria were then pelleted and antigen released from the pellet by resuspension in 20 ml of 1% TX-100 in PBS and sonication three times for 30 seconds. The bacterial debris was then pelleted by centrifugation as above and the supernatant passed through a 2 ml column of glutathione agarose beads (pre-eluted with 5 mM reduced glutathione, 50 mM Tris/HCl (pH 8.0)). The column was washed with 25 ml 1% TX-100 in PBS followed by 25 ml PBS alone. Bound recombinant proteins were then eluted with 10 ml 5 mM reduced glutathione, 50 mM Tris/HCl (pH 8.0) and 1 ml fractions collected. The fractions containing recombinant protein were identified using SDS PAGE and Coomassie blue staining and stored at -20°C until required for immunisation.

### 2.40.3 Thrombin cleavage of FP 2.1/GST

10 $\mu$g of purified recombinant protein in 33 $\mu$l of 5 mM glutathione, 50 mM Tris/HCl (pH 8.0), 100 mM NaCl, 1.5 mM $CaCl_2$ was initially digested overnight at

25°C with 8 μl (2.7 U) of thrombin (Sigma). This amount of thrombin was latter used to cleave 50 μg of recombinant protein.

## 2.41 PREPARATION OF GST FOR IMMUNISATION

GST alone was prepared from non-recombinant pGEX using a protocol identical to that used for the preparation of recombinant proteins.

## 2.42 IMMUNISATION OF MICE WITH FP 2.1/GST

### 2.42.1 Investigating the antigenicity of the recombinant protein

In a preliminary experiment a single female Balb/c and a single female CBA mouse were immunised with 10 μg of purified FP 2.1/GST. A single female CBA mouse was immunised with thrombin cleaved material from 30 μg of FP 2.1/GST and a third female CBA mouse was immunised with 10 μg of GST alone. The FP 2.1/GST and GST used were prepared as described (section 2.40, 2.41) and the protein concentration of these samples estimated by comparison with protein standards following SDS PAGE and Coomassie blue staining. The volume of the sample was then adjusted by the addition of sterile saline to 50 - 75μl prior to immunisation. In all cases the antigen was injected with FCA and divided between two s.c. sites. A boost immunisation was given after 14 days using the adjuvant FIA.

### 2.42.2 Investigating the immunising potential of FP 2.1/GST

Following the above preliminary experiment a group of 8 female Balb/c mice were immunised with 50 μl (10 μg) of FP 2.1/GST plus 50μl of FCA by s.c. injections in each of two sites (day 0). Two groups of 8 control animals were also immunised. In one group FP 2.1/GST was replaced by 10 μg of GST and in another by sterile saline. Repeated immunisations in which FCA was replaced by FIA were then given on days 23, 59, 162 and 197. The mice were challenged with 200 cercariae per mouse on day 232 and perfused 7 weeks latter.

## 2.43 INVESTIGATING THE BINDING OF RADIOACTIVE CALCIUM TO THE 15 KDA ANTIGEN

Equivalent amounts of FP 2.1/GST protein and a known calcium binding protein (recombinant *S. japonicum* calpain, a gift from Dr. M. Huggins of L.S.H.T.M.) were

separated by SDS PAGE and transferred by Western blotting. Half of the blot was then stained with amido black. The other half was washed for a total of 1 hour in three changes of calcium binding buffer (60 mM KCl, 5 mM $CaCl_2$, 10 mM imidazole) and incubated in 10 ml $^{45}CaCl_2$ (1 mCi/L, Amersham) for 10 minutes at room temperature. Following incubation the latter blot was washed in SDW for 5 minutes at room temperature, rinsed briefly in 50% ethanol and air dried overnight. When completely dry the blot was used to expose scientific imaging film (Kodak XAR-5) for at least 24 hours.

## 2.44 CALCULATION OF RESISTANCE

Percentage resistance was calculated as follows:-

$$\%R = 100x \left[ 1 - \frac{\text{mean worm recovery from experimental group}}{\text{mean worm recovery from control group}} \right]$$

Statistical significance was assessed using Student's 't' test.

# CHAPTER 3

# EXTRACTION AND PURIFICATION OF THE 16 kDa ANTIGEN

## 3.1 INTRODUCTION

As described in Chapter 1, a 16 kDa antigen present on the surface of *S. mansoni* schistosomula has been shown to be of particular interest as a vaccine candidate molecule. As well as having a surface location, the antigen is the target of a passively protective McAb and is recognised preferentially by sera taken from demonstrably immune animals vaccinated with irradiated parasites. Two of the main aims of this research have therefore been to characterise the biochemical nature of this molecule and to examine the immune response generated on immunisation with the 16 kDa antigen alone. In order to be able to carry out such studies it was first necessary to devise suitable strategies for the extraction and purification of the antigen from the parasite. It is with this that the experiments detailed in this chapter are concerned.

Previous studies have demonstrated that the 16 kDa antigen separates into the detergent, rather than the aqueous phase, following extraction of MS with Triton X-114 (TX-114) (Bickle *et al.*, 1990). This indicates that the antigen is an integral membrane molecule and hence that the use of a detergent is essential for its extraction. The first section of this chapter describes studies aimed at assessing the efficiency of a number of detergent extraction protocols with regard to their ability to extract the 16 kDa antigen from the schistosomula and cercarial stages of the parasite.

Having achieved efficient extraction of the 16 kDa antigen, attempts were made to purify the molecule in preparation for immunisation and further characterisation studies. The use of immunoaffinity chromatography employing the McAb (B3A), a variety of other chromatography techniques, and the isolation of the 16 kDa antigen following SDS PAGE were investigated. In each case the purity of the antigen obtained was assessed by silver and Coomassie blue staining and by the probing of Western blots with both B3A and VRabS.

## 3.2 RESULTS

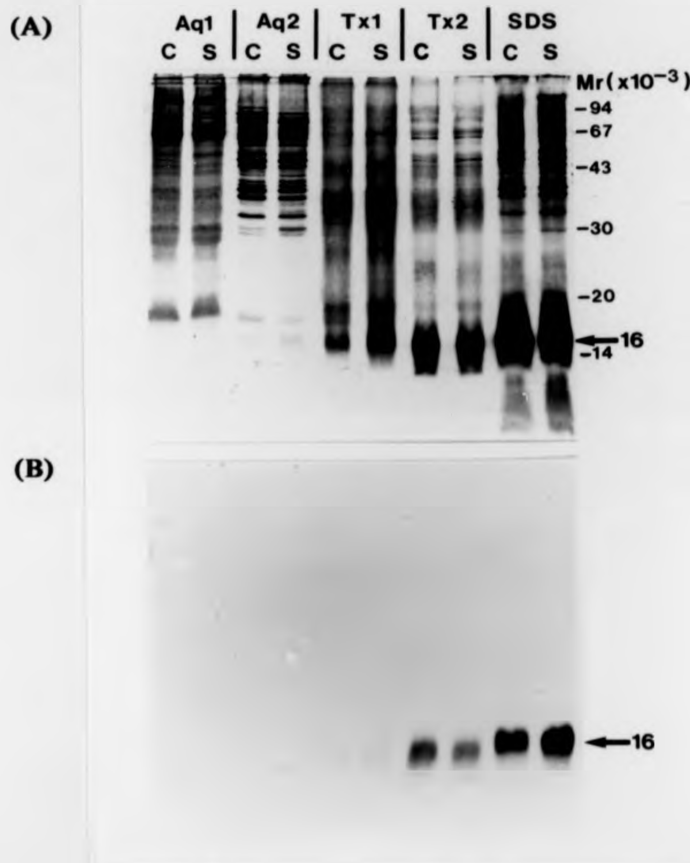
### 3.2.1 DETERGENT EXTRACTION OF THE 16 kDa ANTIGEN

#### 3.2.1.1 Extraction of the 16 kDa antigen with the non-ionic detergent TX-114

Following extraction with TX-114, soluble and integral membrane molecules can be separated into the aqueous and detergent phases of the preparation respectively (Bordier, 1981). One advantage of using this detergent for the large scale extraction of the 16 kDa integral membrane antigen may therefore be the attainment of an accompanying degree

of purification via the removal of soluble molecules which partition into the aqueous phase. However, TX-114 is also a mild non-ionic detergent which is known to be relatively inefficient at solubilising membranes (Helenius, 1979). Thus, although it has been demonstrated that some 16 kDa antigen does separate into the detergent phase following extraction of MS with TX-114 (Bickle *et al.*, 1990) it is possible that this represents only a small proportion of that antigen which is available. Before TX-114 could be used in large scale extraction procedures it was therefore deemed necessary to determine what proportion of the antigen was efficiently extracted by this detergent. Both cercariae and MS were compared as possible sources of the molecule:- Equivalent numbers of intact cercariae and MS were incubated in 0.5% TX-114 for 30 minutes at 4°C. The supernatant was then removed and separated into aqueous and detergent phases (Aq1 and Tx1) whilst the remaining parasites were disrupted by sonication in PBS and the extraction and separation procedures repeated (Aq2 and Tx2). Comparable amounts of protein from both aqueous and detergent phases and the final insoluble pellet were then separated by SDS PAGE and transferred by Western blotting. Figure 3.1 demonstrates that a number of aqueous (Aq1) and detergent (Tx1) phase antigens were extracted by incubation of intact parasites in TX-114. These included the 16 kDa antigen which was clearly observed following the probing of Western blots with VRabS (Figure 3.1(A)). However, only a very poor 16 kDa signal was seen following the probing of similar blots with B3A (Figure 3.1(B)) thus indicating that TX-114 fails to extract much 16 kDa antigen from the intact parasite. In contrast, TX-114 extraction of parasites disrupted by sonication was successful in removing significant amounts of 16 kDa antigen from both MS (S) and cercariae (C). As expected, the antigen partitioned into the detergent phase (Tx2) of the preparation where it was a major antigen recognised by VRabS and by B3A. Again numerous soluble antigens separated into the aqueous phase (Aq2). Thus, some 16 kDa antigen was extracted from sonicated parasites using the detergent TX-114 and as hoped a degree of purification was obtained by the separation of this integral membrane molecule from the aqueous phase antigens. However, extraction of the remaining TX-114 insoluble pellet by boiling in SDS PAGE sample buffer (SDS) then revealed that a far greater proportion of the 16 kDa antigen clearly remained insoluble. It was therefore concluded that extraction with TX-114 failed to remove a significant proportion of the available antigen and, on consideration of the difficulties involved in the production of large quantities of larval stage starting material, it was decided that despite the partial

**Figure 3.1** Extraction of the 16 kDa antigen with the detergent TX-114




230,000 intact cercariae (C) and MS (S) were incubated in 0.5% TX-114 in PBS at 4°C for 30 minutes. The preparation was then centrifuged and the supernatant removed, incubated at 37°C for 5 minutes and separated into aqueous (Aq1) and detergent (Tx1) phases by centrifugation through sucrose. Parasite bodies in the remaining pellet were then disrupted by high frequency sonication and the incubation and separation procedures repeated to give rise to a second aqueous (Aq2) and detergent (Tx2) phase. The final insoluble pellet (SDS) was resuspended in PBS. Samples containing comparable amounts of protein as judged by SDS PAGE and Coomassie blue staining were then boiled in SDS PAGE sample buffer, electrophoresed on 15% gels, transferred by Western blotting and probed with VRabS (A) and B3A (B) at dilutions of 1 in 200 and 1 in 20 respectively.

purification achieved, TX-114 was not a suitable detergent for routine extraction of the 16 kDa molecule.

The Western blotting studies described here also indicated that slightly larger amounts of the 16 kDa antigen were recovered from MS following incubation of intact parasites in TX-114 (Tx1). However, this most probably represented an increase in the susceptibility of the antigen to detergent extraction following the removal of the glycocalyx upon mechanical transformation. This is substantiated by the observation that following removal of the glycocalyx from cercariae during the sonication procedure, slightly larger amounts of 16 kDa antigen are extracted from cercariae as compared to those recovered from MS (Tx2). Both these stages of the parasite therefore appear to contain similar quantities of the 16 kDa antigen, however MS were chosen for use in subsequent extraction procedures as they are comparatively easy to prepare.

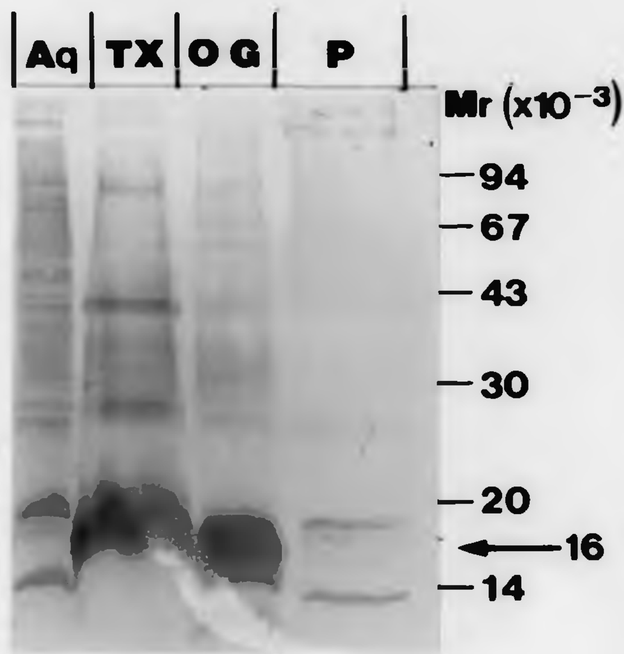
#### 3.2.1.2 Sequential extraction of MS with TX-114 and a variety of other detergents

As described above, incubation with TX-114 failed to remove a large proportion of the 16 kDa antigen from MS. Thus, in an attempt to optimise the extraction procedure, the insoluble pellet remaining after TX-114 extraction was resuspended and extracted with a range of other detergents. The detergents Triton X-100, sodium deoxycholate (DOC) and CHAPS proved inefficient at removal of the remaining 16 kDa antigen (data not shown). However, incubation of the TX-114 insoluble pellet for 1 hour at room temperature with either 1.5% octyl-ß-D thio glucopyranoside (OTG) or 1.5% deconyl-n-methylglucamide (MEGA-10) resulted in the extraction of all of the remaining 16 kDa antigen. Figure 3.2 shows a Western blot of material removed from 400,000 sonicated MS by sequential extraction with TX-114 (Aq, TX), OTG (OG) and SDS PAGE sample buffer (P). Similar results were obtained when using MEGA-10 as a second detergent (data not shown). However, as difficulties were encountered on solubilising MEGA-10 at room temperature, OTG was chosen for use in subsequent studies.

#### 3.2.1.3 Extraction of the 16 kDa antigen with OTG alone

The non-ionic detergent OTG efficiently extracts the remaining 16 kDa antigen from MS previously treated with TX-114. However, if the TX-114 extraction step could be omitted without a reduction in the overall recovery of the 16 kDa antigen, this would result in a more simple procedure in which all of the antigen was obtained in a single

Figure 3.2 Sequential extraction of MS with the detergents TX-114 and OTG




400,000 sonicated MS were incubated in 1% TX-114 for 30 minutes at 4°C, centrifuged and the supernatant removed, warmed and separated into aqueous (Aq) and detergent (TX) phases. The TX-114 insoluble pellet was then resuspended by sonication and extracted for 1 hour at room temperature with 1.5% OTG, (OG). The final insoluble pellet (P) was resuspended in 50 mM Tris/HCl (pH 8.0). Aliquots of each fraction containing antigen equivalent to that extracted from approximately 5,000 MS was boiled in SDS PAGE buffer, electrophoresed and transferred by Western blotting. The blot was probed with VRabS at a dilution of 1 in 200.

detergent phase. The presence of only OTG in the preparation containing all of the 16 kDa antigen would enable the detergent to retain its predictable characteristics such as a high CMC, a small micelle size and hence the ability to be removed by dialysis. OTG was therefore used in an attempt to extract the 16 kDa antigen from MS without the preceding TX-114 incubation. In the hope of retaining the degree of purification obtained by removal of the TX-114 aqueous phase antigens in the initial protocol, MS were first sonicated in 50 mM Tris/HCl (pH 7.4), spun at 100,000 x g and the supernatant removed. Figure 3.3 (S) demonstrates that a number of soluble antigens were removed in this supernatant whilst the 16 kDa antigen integral membrane molecule remained in the Tris insoluble pellet. This pellet was then extracted with 1.5% OTG for 1 hour at room temperature. Figure 3.3 (OG, P) shows that such treatment resulted in the efficient extraction of almost all of the 16 kDa antigen in a single detergent phase. This procedure was therefore used to provide detergent extracts containing the 16 kDa antigen for all subsequent experiments. It should however be noted that despite the high efficiency of OTG extraction, no obvious silver or Coomassie blue staining band was observed in the 16 kDa region following electrophoresis of the material extracted from as many as 10,000 MS (data not shown). This indicates that although the 16 kDa molecule is highly antigenic, it is only present in very small amounts.

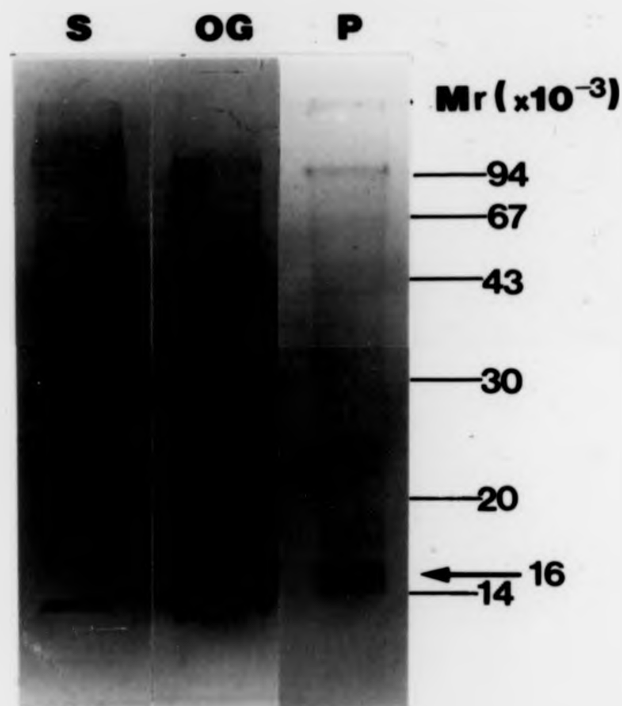
As the 16 kDa antigen is an integral membrane molecule, the presence of a detergent is a constant requirement if the molecule is to remain in solution. Thus, by dialysing against different concentrations of OTG it was also observed (data not shown) that following extraction, a detergent concentration as low as 0.1% was sufficient for this purpose. This concentration of OTG was therefore used in all solutions to contain the 16 kDa antigen e.g. column elution buffers.

### 3.2.2 PURIFICATION OF THE 16 kDa ANTIGEN

#### 3.2.2.1 Immunoaffinity chromatography

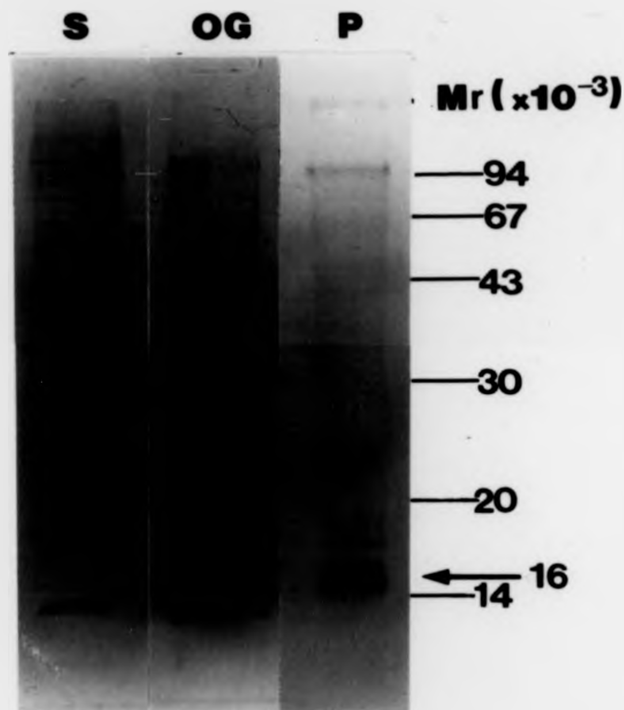
In order to obtain purified 16 kDa antigen for characterisation and immunisation studies, an OTG extract prepared as above was circulated through a pre-eluted column of B3A bound to Protein A Sepharose beads. The column was then washed extensively and the bound antigen eluted with diethylamine (pH 11.5). Figure 3.4(A) shows a Western blot of the column eluates and unbound fraction probed with VRabS and Figure 3.4(B) an equivalent blot probed with B3A. As can be seen, the 16 kDa molecule was the major

**Figure 3.3** **Extraction of MS with OTG alone**




A pellet of approximately 400,000 MS was disrupted by sonication in Tris/HCl (50 mM, pH 7.4), centrifuged and the supernatant removed (S). The remaining pellet was then resuspended by sonication and incubated in 1.5% OTG for one hour at room temperature. The second supernatant was removed (OG) and the pellet resuspended in 50 mM Tris/HCl (pH 7.4) (P). Aliquots of each fraction containing the equivalent of antigen extracted from 4,000 MS was boiled in SDS PAGE buffer, electrophoresed and transferred by Western blotting. The blot was probed with VRabS at a dilution of 1 in 200.

**Figure 3.3** Extraction of MS with OTG alone

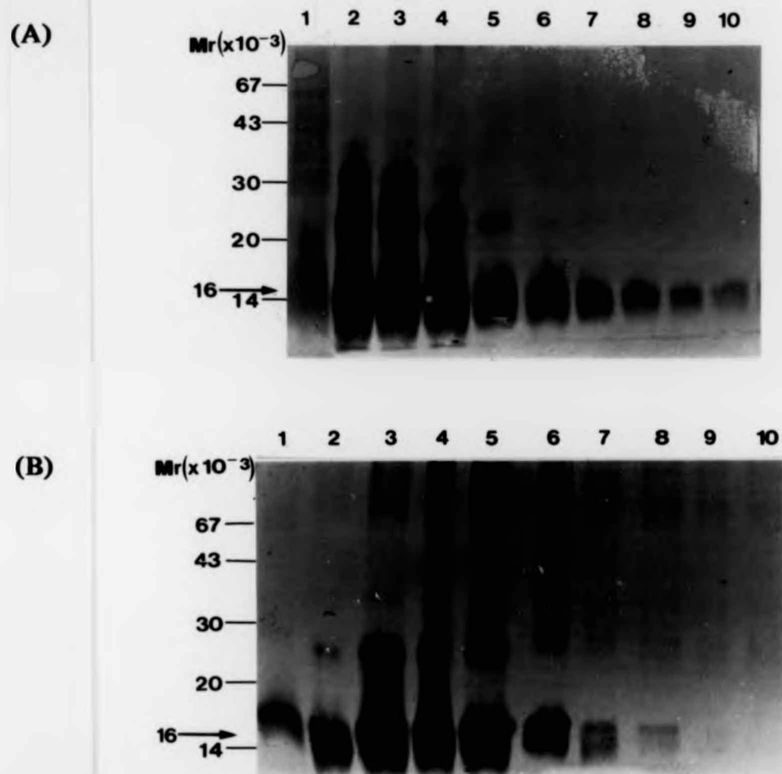



A pellet of approximately 400,000 MS was disrupted by sonication in Tris/HCl (50 mM, pH 7.4), centrifuged and the supernatant removed (S). The remaining pellet was then resuspended by sonication and incubated in 1.5% OTG for one hour at room temperature. The second supernatant was removed (OG) and the pellet resuspended in 50 mM Tris/HCl (pH 7.4) (P). Aliquots of each fraction containing the equivalent of antigen extracted from 4,000 MS was boiled in SDS PAGE buffer, electrophoresed and transferred by Western blotting. The blot was probed with VRabS at a dilution of 1 in 200.

antigen present in the column eluates, although a signal of approximately 25 kDa was recognised on probing with VRabS or with B3A, whilst a 32 kDa molecule was seen only on probing with VRabS. Probing of Western blots with antibody also revealed that some of the 16 kDa antigen remained within the unbound fraction, thus indicating that the saturation point of the immunoaffinity column had been reached. Further aliquots of the first four fractions eluted from the column were then separated by SDS PAGE and stained with Coomassie blue (Figure 3.4(D)) or with silver nitrate (Figure 3.4(C)). On staining with Coomassie blue a band corresponding to the 16 kDa molecule was clearly seen in a single eluted fraction. With the exception of a doublet of approximately 58 kDa no other contaminating molecules were visible in this eluate. As this 58 kDa doublet has also been seen in numerous unrelated samples following separation by SDS PAGE, it is believed to be a contaminant introduced in the SDS PAGE sample buffer. Thus, the 16 kDa antigen is the only eluted molecule visible by staining with Coomassie blue. However, on staining with silver nitrate, which is a much more sensitive system, the 16 kDa molecule was visible in three fractions (a total volume of approximately 1.5 ml) and a small number of contaminating molecules were also visible in these eluates. Of these, the band of approximately 32 kDa was seen in all three eluates containing the 16 kDa antigen whilst weaker signals of approximately 18 - 20 and 25 kDa were visible only in the fraction containing the majority of the 16 kDa molecule.

Although a reasonable degree of purification was achieved using immunoaffinity chromatography, a small number of contaminating molecules were present in those fractions containing the 16 kDa antigen and attempts were therefore made to improve the immunoaffinity purification technique. These included the incorporation of an additional wash using a buffer containing a low concentration of SDS (0.1%) and elution of the column with the low pH buffers 0.2 M ammonium acetate (pH 3.0) or 0.1 M sodium citrate (pH 2.5). In all cases a significantly lower recovery of the 16 kDa antigen was obtained and the purity of the eluted antigen was not improved. Passing the crude detergent extract down a column of Protein A Sepharose prior to passage through the immunoaffinity column also failed to remove the contaminating material. As a result the original immunoaffinity protocol was reinstated and other methods of purification were investigated with regard to the possibility of using another technique in conjunction with, or as an alternative to, immunoaffinity chromatography.

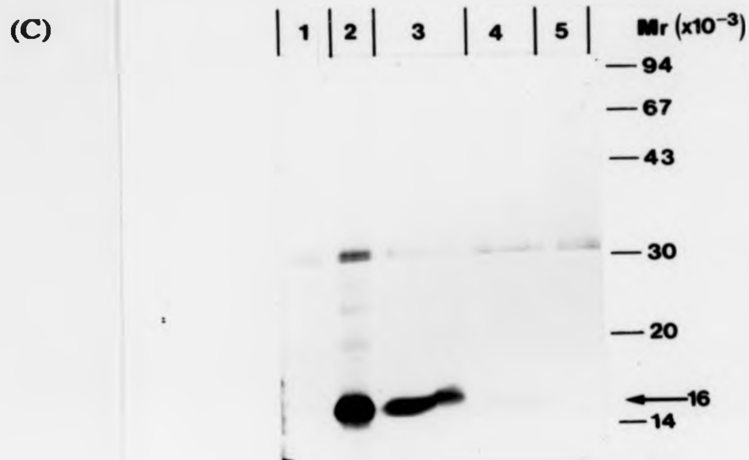
**Figure 3.4 Immunoaffinity purification of the 16 kDa antigen**



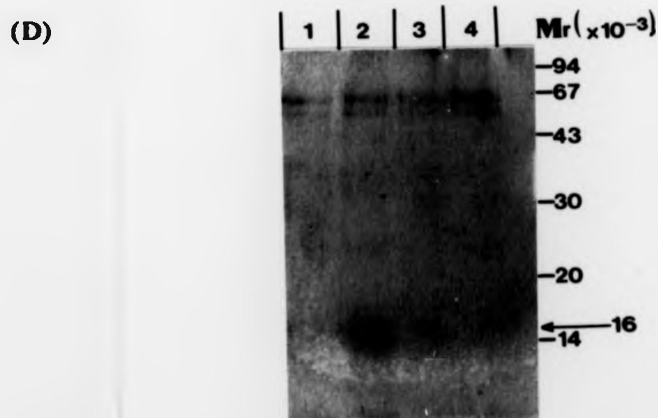

The 16 kDa antigen was purified by passage of material extracted from approximately 15 million MS through the McAb immunoaffinity column. 10 $\mu$l of fractions 1 to 9 (lanes 2 - 10) eluted by the application of 50 mM diethylamine (pH 11.5) and 10 $\mu$l of unbound material (lane 1) were then separated by SDS PAGE and transferred by Western blotting. The blots were probed with VRabS (A) or B3A (B) at a dilution of 1 in 200 and 1 in 20 respectively.

**Figure 3.4 continued**

(C)




(D)

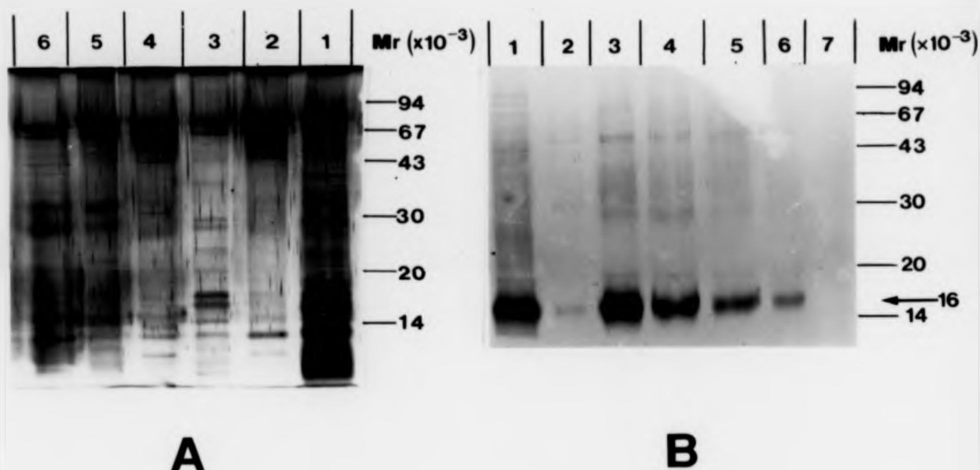



10 $\mu$l aliquots of fractions 1 - 4 (C) or 1 - 3 (D) (lanes 2 - 4/5) eluted by the application of 50 mM diethylamine (pH 11.5) were electrophoresed alongside 10 $\mu$l of column unbound material (lane 1) and the gel stained with silver nitrate (C) or with Coomassie blue (D).

### 3.2.2.2 Ion exchange chromatography

At a particular pH a molecule which includes a protein moiety would be expected to be either positively or negatively charged. Ion exchange chromatography exploits this phenomenon by using columns of oppositely charged beads to bind the molecule. Initially both the anion exchange matrix, diethyl aminoethyl (DEAE) Bio-Gel A, and the cation exchange matrix carboxymethyl (CM) Bio-Gel A were used in an attempt to purify the 16 kDa antigen. The DEAE column was equilibrated with a Tris buffer (25 mM Tris/HCl (pH 8.0), 1 mM EDTA, 1.5% OTG) and the CM column with a pH 6.0 phosphate buffer (89% 25 mM $NaH_2PO_4$, 11% 25 mM $Na_2HPO_4$ 1 mM EDTA, 1.5% OTG) and OTG extracts of MS were then prepared as above with the exception that extraction was carried out in the buffer appropriate for use with each column. This was done to ensure that the pH of the pre-equilibrated column was not altered by the addition of the starting material. Following application of the sample, each column was washed extensively with the appropriate buffer and eluted with a continuously increasing potassium salt gradient. Starting material, unbound molecules and the eluted fractions following ethanol precipitation were then run on SDS PAGE gels and transferred by Western blotting. Probing of these blots with VRabS and B3A demonstrated that the CM column failed to bind any 16 kDa antigen (data not shown) and attempts to use this method for the purification of the 16 kDa antigen were therefore abandoned. In contrast, a small proportion of the 16 kDa antigen was seen to bind and subsequently elute from the DEAE column (data not shown). Reducing the conductivity of the starting material by diluting 1 in 4 with 25 mM Tris/HCl (pH 8.0) prior to application, was then shown to result in the proportion of the 16 kDa antigen binding and eluting from the column being raised to almost 100% (Figure 3.5). However, other molecules were present in the eluates containing the 16 kDa antigen and silver staining of an SDS PAGE gel demonstrated that although there were some qualitative differences between the starting, unbound and eluted fractions, a distinct band at 16 kDa was not visible within the column eluates (Figure 3.5(A)). Moreover, the probing of Western blots of the column eluates also demonstrated that the 16 kDa antigen eluted in fractions corresponding to 0.2 - 0.3 M KCl which had a total volume of 6 ml (Figure 3.5(B)). If ion exchange chromatography were to be used as an initial purification step, buffer exchange would be required before fractions eluted from the DEAE column could be applied to the immunoaffinity column. This would inevitably involve some antigen loss. Thus it was decided that the benefits of attaining the

**Figure 3.5** **Purification of the 16 kDa antigen using ion exchange chromatography**




An OTG extract of 200,000 MS was diluted 1 in 4 in Tris/HCl (25 mM, pH 8.0) and passed down a column of DEAE Biogel A. The fractions generated by elution with a continuously increasing salt gradient were then ethanol precipitated and the equivalent of 125 μl of the fractions eluted with approximately 0.25M were electrophoresed (lanes 3 - 7) alongside 25 μl of starting material and 25 μl of unbound material (lane 2). The gel was then stained with silver nitrate (panel A) or transferred by Western blotting and probed with VRabS at a dilution of 1 in 200 (panel B).

low level of purification observed on carrying out ion exchange chromatography did not outweigh the disadvantages of including buffer exchange. Furthermore, the large volume of high salt buffer in which the 16 kDa antigen was eluted from the DEAE column prohibits the use of ion exchange chromatography as a final purification procedure.

#### 3.2.2.3 Hydrophobic interaction chromatography

Hydrophobic interaction chromatography employs a hydrophobic stationary phase to bind those molecules which have a hydrophobic moiety. As an integral membrane molecule the 16 kDa antigen is presumed to have a hydrophobic domain which should therefore enable it to interact with such a stationary phase. To test this an OTG extract of MS was circulated through a Phenyl Sepharose column. The column was then washed with 25% ammonium sulphate and eluted with a stepwise decreasing salt gradient of 25% and 10% ammonium sulphate, followed by ethylene glycol. Probing of Western blots of the starting material, unbound fraction and the column eluates demonstrated that the binding of the 16 kDa antigen to phenyl sepharose was irreversible. Matrices with weaker binding properties (amino-ethyl, propyl, butyl or pentyl agarose) were also tried, however these did not bind the 16 kDa antigen (data not shown).

#### 3.2.2.4 Lectin affinity chromatography

It has been demonstrated (see Chapter 4) that the plant lectin *Arachis hypogaea* (peanut agglutinin, PNA) binds to the 16 kDa antigen on Western blots. Thus, lectin affinity chromatography was investigated as an additional method of purifying this molecule. An OTG extract containing the 16 kDa antigen was rotated for at least one hour with agarose beads coated with PNA. The beads were then washed and eluted with increasing concentrations of galactose, increasing concentrations of lactose, with borate buffer (0.45 M, pH 6.0) and finally by boiling in SDS PAGE sample buffer. Equivalent amounts of the unbound and eluted fractions were separated by SDS PAGE and transferred by Western blotting. Probing of the Western blot with VRabS (data not shown) demonstrated that a large proportion of the 16 kDa antigen failed to bind to the PNA agarose and it was speculated that this may well be due to the presence of inhibitory substances within the crude detergent extract which was applied to the lectin column. Thus, lectin affinity chromatography would not be suitable for use as an initial step in the purification of the 16 kDa antigen from OTG extracts. As an alternative, partially purified

antigen eluted from the immunoaffinity column was rotated with PNA agarose beads. The beads were washed and eluted as above and the unbound and eluted fractions again separated by SDS PAGE and Western blotted. The results obtained demonstrated that a large proportion of the partially purified 16 kDa antigen now bound to the PNA agarose beads. However, although this antigen could be eluted by boiling of the beads in SDS PAGE sample buffer, the binding was irreversible using techniques which would enable viable antigen to be obtained (data not shown). Thus, the use of lectin affinity chromatography as a final purification step is also infeasible.

For a variety of reasons, none of the purification techniques detailed above were suitable for use in conjunction with immunoaffinity chromatography. Thus, attempts were made to further purify the eluted 16 kDa antigen by recovery of the molecule following SDS PAGE.

#### 3.2.2.5 The Biorad 491 Prep cell

A dozen eluates from the immunoaffinity column which contained enough 16 kDa antigen to give a signal on Western blots probed with B3A but not on gels stained with silver nitrate nor Coomassie blue, were combined and concentrated into a volume suitable for loading onto the Biorad 491 Prep cell (500 $\mu$l) using a Centricon 10 microconcentrator. The preparation was then electrophoresed and 2.5 ml eluates collected. Probing of Western blots of 25 $\mu$l of each of these elutes with VRabS and B3A demonstrated that the 16 kDa antigen obtained appeared to be pure (data not shown), however the molecule eluted in a volume of 1% SDS which totalled more than 20 ml. This procedure is therefore inappropriate for use as a final purification step as concentration of the 16 kDa antigen into a volume of buffer suitable for use in further characterisation studies would by infeasible. Furthermore, the antigen losses which would be involved in the transfer of material eluted from the Prep Cell into a buffer suitable for passage through the immunoaffinity column make the use of this technique as a preliminary purification step impractical.

#### 3.2.2.6 Transfer to ProBlott membrane

Molecules separated by SDS PAGE and transferred by Western blotting to ProBlott membrane can be visualised by staining with Coomassie blue. Antigens of interest can then be isolated by excision of the appropriate region of the blot. As a consequence of the

fixing and staining procedures, antigen obtained in this way is not suitable for use in immunisation experiments. However, attempts were made here to use this method to obtain pure 16 kDa antigen suitable for gas phase N terminal amino acid sequencing. To ensure that sufficient amounts of antigen were used for this purpose, six fractions eluted from the immunoaffinity column following purification of the 16 kDa antigen from 15 million MS were selected and their antigen content investigated. Following electrophoresis of 25 $\mu$l aliquots of each 0.5 ml fraction, the 16 kDa molecule could be visualised by Coomassie blue staining in four of the six fractions and by silver staining in all. These six antigen rich fractions were therefore combined, concentrated using a Centricon 10 microconcentrator and loaded into a single well of a 15% polyacrylamide gel. Following SDS PAGE the antigen was transferred to ProBlott membrane which was stained with Coomassie blue. A strong but rather smeared signal was seen in the 16 kDa region of the blot. This was excised and sent to the National Institute for Medical Research at Mill Hill for N terminal amino acid sequencing (see Chapter 4).

## 3.3 DISCUSSION

A 16 kDa antigen of *S. mansoni* has been shown to be of interest as a putative vaccine candidate molecule. The aim of this chapter was therefore to extract and purify this antigen for use in subsequent immunisation and characterisation studies.

Indirect immunofluorescence using the protective McAb B3A has previously demonstrated that the 16 kDa antigen is present upon the surface of living schistosomula (Bickle *et al.*, 1986). Further experiments involving the separation of the antigen into the detergent phase following TX-114 extraction of MS (Bickle *et al.*, 1990 and this chapter) have since demonstrated that, as defined by Bordier (1981), the 16 kDa antigen is an integral membrane molecule as opposed to one which is only peripherally associated with the surface. Integral membrane molecules are firmly attached to plasma membranes via hydrophobic interactions between their own hydrophobic domain(s) and the core of the lipid bilayer. These domains often consist of a stretch of hydrophobic amino acid residues which occur within the peptide moiety of a molecule. However, acylation via the addition of either a complex GPI linkage, or a fatty acid linked directly to the peptide backbone can also be used as a means of anchoring a molecule within a membrane. Regardless of the nature of this hydrophobic domain(s), the strength of the interactions between an integral membrane antigen and the lipid bilayer are such that a detergent (or an organic

solvent) will always be required for antigen extraction. During the course of such an extraction, detergent molecules replace most of the lipid moieties which contact the integral membrane molecule. This results in the formation of protein-detergent micelles which are held together by interactions between the aforementioned hydrophobic entities of the integral membrane molecule and those of the amphipathic detergent. Water soluble or hydrophilic molecules do not interact with detergent and hence do not form micelles. If the detergent used for extraction is one of the Triton X series, the integral membrane molecules can then be separated from soluble molecules, as on warming of the preparation to a particular temperature, the detergent-protein micelles form large aggregates which dissociate from the monomeric forms of the soluble molecules. This results in the formation of two distinct phases which can be easily separated. The temperature at which the formation of micellar aggregates occurs is known as the cloud point and varies according to the type of Triton used. For TX-114 the cloud point is about 20°C and this enables this detergent to be used for extraction and separation of soluble and integral membrane molecules at a temperature which does not induce protein degradation (Bordier, 1981).

The possibility of obtaining a degree of purification by separation of the 16 kDa integral membrane antigen from soluble molecules, ensured that the detergent TX-114 was the first to be assessed with regard to its ability to extract the 16 kDa molecule from MS. However, it was demonstrated here that extraction of MS with TX-114 failed to remove a large proportion of the 16 kDa molecule. This coincides with the observation (Helenius, 1979) that TX-114 is relatively inefficient at the solubilisation of membranes and hence the extraction of integral membrane molecules. The 16 kDa antigen remaining following extraction of MS with TX-114 could be solubilised by boiling in SDS PAGE sample buffer. However, at concentrations suitable for the solubilisation of membranes, ionic detergents such as SDS, often cause drastic conformational changes and hence a decrease in the biological activity and / or antigenicity of the extracted molecule (Helenius, 1979). This is because in addition to replacing lipid moieties within the bilayer, ionic detergent molecules bind to both the hydrophilic and the hydrophobic regions of the integral membrane molecule itself. Although antibody recognition of the 16 kDa antigen extracted by boiling in SDS PAGE sample buffer indicates that at least some epitopes are resistant to the denaturing effects of this detergent, it is possible that other structural features of the 16 kDa antigen are destroyed by such harsh treatment. Furthermore, problems can

arise on attempting to purify molecules from ionic detergent extracts, as immunoaffinity columns may be destabilised by the deleterious effect of such detergents on antibody structure and the use of ion exchange chromatography is not possible as the charge of the native antigens is masked by the charge of bound SDS. In general, non-ionic detergents are less harsh than ionic detergents. They bind only to the hydrophobic region of the integral membrane molecule and this rarely leads to conformational changes or loss of activity. Moreover, non-ionic detergents are suitable for the preparation of extracts to be used in subsequent purification procedures. Here, the non-ionic detergents OTG and MEGA-10 were shown to extract almost all of the 16 kDa antigen from MS. In addition, a degree of purification similar to that seen when using TX-114 was obtained by removal of molecules soluble in 50 mM Tris prior to detergent extraction. The more readily soluble OTG was therefore selected for subsequent extraction of the 16 kDa antigen from MS.

The existence of a large amount of 16 kDa antigen which is resistant to extraction with TX-114 (and TX-100, section 3.2.1.2) but sensitive to extraction with the high CMC detergents OTG and MEGA-10 is also of some interest in the light of studies carried out by Hooper and Turner (1988) and Hooper and Bashir (1991). These demonstrated that the detergent extraction profile of a number of mammalian integral membrane ectoenzymes reflected the manner in which the molecules were associated with the plasma membrane. Thus, a whole range of detergents were equally efficient at extracting antigens anchored within the membrane via a hydrophobic stretch of amino acids. In contrast, molecules possessing a GPI anchor were extracted efficiently by detergents with a high CMC (OTG, CHAPS and DOC) but were partially resistant to those with a low CMC (TX-100, TX-114 and Nonidet P-40). Thus, some aspects of the detergent extraction profile of the 16 kDa antigen suggest the possibility of it being attached to the membrane via a GPI anchor. In contrast, other observations have been made which contradict this suggestion. Firstly, a large proportion of the 16 kDa antigen remaining following TX-114 extraction is not removed by the detergents CHAPS and DOC which have a CMC equal to that of MEGA-10 (section 3.2.1.2). Secondly, it has been demonstrated (Dr.Q. Bickle, personal communication) that the 16 kDa antigen is not released from the plasma membrane by treatment with PIPLC, an enzyme which specifically cleaves the GPI linkage. However, some GPI anchors are resistance to treatment with this enzyme and should further work be carried out with regard to the nature of the attachment of the 16 kDa antigen to the

membrane it may be useful to attempt to release the 16 kDa molecule from the membrane with a second GPI specific enzyme, PIPLD.

Following detergent extraction of the 16 kDa antigen from MS it was necessary to purify the molecule in preparation for immunisation experiments and further characterisation studies. As one of the major problems when working with the 16 kDa antigen is its low abundance, it was hoped that a single step purification procedure could be devised. This would avoid encountering the antigen losses which invariably occur during the buffer exchange and / or concentration steps normally required when more than one purification technique is used. Immunoaffinity chromatography using a McAb cross linked to a matrix, is a highly specific procedure which has been used successfully to purify a single molecule from a crude extract of numerous organisms including schistosomes (Hazdai *et al.*, 1985, Oligno *et al.*, 1988, Harn *et al.*, 1992). This method was therefore the first choice when looking for a technique which could enable the 16 kDa antigen to be purified in a single step. Here, the passively protective McAb, B3A was cross linked to Protein A Sepharose via its Fc receptor, a procedure designed to leave both Fab regions available for antigen binding. The column was then used to purify the 16 kDa antigen from an OTG extract of MS. The results obtained demonstrated that the 16 kDa antigen was the major molecule observed following Coomassie blue or silver staining of the column eluates. It was also the major antigen recognised on probing Western blots with VRabS and B3A. However, small amounts of a number of other molecules were also present in the fractions containing the majority of the 16 kDa antigen. Of these, a 58 kDa doublet which is most probably introduced during SDS PAGE and therefore not present in the original column eluates, is the major contaminant, although smaller molecules of 32, 25 and 20 - 18 kDa are also seen. It seems most likely that the 25 kDa signal corresponds to antibody light chains which have leached from the immunoaffinity column upon application of the high pH elution buffer. This is suggested by the similarity in the estimated size of this molecule to that of light chains (24 kDa) and also by the observation that a signal of approximately 25 kDa is seen on Western blots of the column eluates probed with anti-mouse conjugate without exposure to a primary antibody (data not shown). The recognition of the 25 kDa antigen on blots probed with VRabS suggests that mouse McAb light chains are also recognised by the anti-rabbit conjugate used. The leaching of antibody from immunoaffinity columns has been described previously. For example, on using a McAb immunoaffinity column to purify

a 22 kDa antigen from *S. mansoni* schistosomula membrane extracts, Oligno *et al.*, (1988) reported the presence of what was presumed to be light and heavy antibody chains in the column eluates. With regard to the molecule which runs at 32 kDa, its size suggests the possibility of it representing dimers of the 16 kDa antigen. However, the recognition of this molecule following electrophoresis under reducing conditions deems this unlikely. Moreover, although recognised by VRabS, the 32 kDa molecule is not seen on probing Western blots of the column eluates with B3A. This latter point also indicates that the 32 kDa molecule does not bind to the column via cross reactive epitopes which are recognised by the McAb and suggests that in addition to the silver staining molecule(s) of 18 - 20 kDa, the 32 kDa molecule binds non-specifically to the immunoaffinity column. It is possible that such molecules have a particular affinity for antibody or perhaps for protein in general. Despite the high specificity of antibody - antigen interactions, the non-specific binding of molecules to immunoaffinity columns is commonly encountered. Although, as stated above, several investigators have used affinity chromatography alone to obtain completely pure antigen, others have reported that the use of a second purification technique, either prior to or following immunoaffinity chromatography, was required to remove contaminating material (Carlsson 1993, Oligno *et al.*, 1988). Various attempts were made here to devise useful additional purification procedures.

In accordance with its pI of 6.1 (see chapter 4) the 16 kDa antigen bound to the anion exchanger DEAE Bio Gel A at a pH of 8.0. The initially low levels of binding were increased by dilution of the OTG extract prior to application. This has the effect of decreasing the likelihood of a strongly charged antigen being surrounded by salt molecules of parasite origin which prevent its binding to the beads. Unfortunately, many other molecules also bound and eluted from the DEAE matrix and it was decided that the advantages gained by using ion exchange chromatography in conjunction with immunoaffinity chromatography, would be outweighed by the disadvantages of carrying out the processes required for buffer exchange.

Lectin affinity chromatography is a widely used technique which employs the specific binding of a lectin immobilised on a solid matrix to a carbohydrate entity of a glycoprotein. In some cases this technique is used simply to identify the presence of particular sugar moieties. For example, Nyame *et al.*, (1987, 1988a, 1988b, 1989) exploited the specific binding ability of a variety of lectins to demonstrate the presence

of particular N- and O-linked sugars in adult schistosomes and in schistosomula. Alternatively, lectin affinity chromatography has been used to isolate groups of molecules which are of interest. Thus, Strand *et al.*, (1982) used Con A chromatography to isolate schistosomula surface glycoproteins which are believed to be of importance in the development of the human immune response. Dalton and Strand (1987) have used a similar procedure as the first step in a protocol aimed at isolating glycoproteins recognised preferentially by sera from mice vaccinated with radiation attenuated cercariae. However, of more relevance to the studies carried out here, is the demonstration that lectin affinity chromatography can be used to purify individual glycoproteins. In general, this technique is used in the early stages of a purification scheme and is followed by other more highly specific purification techniques. For example, Kelly *et al.*, (1987) isolated a subset of antigens from a preparation of schistosomula membrane molecules by affinity chromatography on lentil lectin Sepharose. The antigens of specific interest, a 32 and a 20 kDa glycoprotein, were further purified by electroelution from 2D gels. However, the level of purification which can be obtained using lectin affinity chromatography obviously depends upon the sugar composition of the molecule to be isolated. Although lectins such as lentil lectin and Con A recognise the common sugars mannose and glucose and hence bind to a large number of glycoproteins, other lectins such as soybean agglutinin (SBA), which recognises the more unusual N-acetylgalactosamine (GalNAc), have been shown to bind very few schistosome glycoproteins and have therefore been used in single step procedures to purify antigens to a level suitable for further characterisation (Linder *et al.*, 1991). Previous studies using lectins to probe radiolabelled antigens from adult schistosomes separated on SDS PAGE gels have demonstrated that PNA like SBA recognises very few schistosome antigens (McGregor *et al.*, 1985). In addition, the studies described in Chapter 4 of this thesis demonstrate that biotinylated PNA binds to very few schistosomula antigens on Western blots. The 16 kDa antigen did, however, produce a strong signal with this biotinylated lectin and it was hoped that PNA lectin affinity chromatography would provide a useful additional step towards the purification of this molecule. It was therefore surprising that the majority of the 16 kDa antigen did not bind to PNA coated agarose beads on application of a detergent extract of MS. One explanation for this discrepancy, is the presence of inhibitors of binding such as the high concentration of detergent or material of parasite origin e.g. salts or enzymes, in the crude MS extract. Alternatively, it is possible that lectin binding entities are only exposed

following boiling of the 16 kDa antigen in SDS PAGE sample buffer prior to Western blotting. Although crude detergent extracts have been used successfully in lectin affinity chromatography procedures (Hayunga and Sumner, 1986(a), 1986(b), Strand *et al.*, 1982) the binding of the immunoaffinity purified 16 kDa antigen to PNA agarose demonstrates that prior denaturation of the molecule is not required and suggests that inhibitory substances do indeed contribute to the initial lack of binding observed. This inhibition could be a characteristic of OTG in particular or perhaps the configuration of the 16 kDa antigen itself in the presence of a high concentration of detergent. Although the 16 kDa antigen present within an immunoaffinity column eluate bound to the PNA agarose beads, the molecule failed to elute on application of a saturated solution of galactose. Such a solution is often sufficient to elute PNA affinity columns, however galactose is known to be a comparatively inefficient inhibitor of PNA binding as in solution it undergoes mutarotation. This is a process whereby galactose continually inter-converts from the $\alpha$ to the $\beta$ anomer through an open ring structure. As this open ring is unable to bind to PNA, at any particular point in time, a proportion of the molecules in a solution of galactose are unable to act as inhibitors. In contrast, lactose ($\beta$-D gal, 1,4, $\beta$-D glc) is an efficient inhibitor of PNA binding. Its disaccharide nature ensures that it is unable to mutarotate and thus lactose molecules are always present in the pyranose form which is capable of inhibiting lectin binding. Despite this, incubation of PNA agarose beads with a saturated solution of lactose also failed to elute the 16 kDa antigen. Finally a solution of 0.45 M borate was used. This is often successful at removing glycoproteins which bind to lectin with a particularly high affinity, as borate forms complexes with the 4- and 6-hydroxyl groups of non-reducing pyranosides (Hayunga and Sumner, 1986a). Again the 16 kDa antigen remained bound to the beads and the binding was thus deemed irreversible. The irreversible binding of glycoproteins to lectin agaroses is not unusual. For example, Hayunga and Sumner (1986a) using a variety of lectin coated beads to isolate radiolabelled adult schistosome glycoproteins, reported that between 20% and 40% of labelled material bound irreversibly. The high affinity with which some glycoproteins bind to lectins is believed to be due to the formation of multivalent interactions between the sugar residues of the glycoprotein and the lectin molecules. The development of such interactions relies heavily upon the close proximity of the sugar residues to one another, a feature which is provided by the clustering of carbohydrate chains seen on many glycoproteins. Displacement of a glycoprotein from lectin agarose by the addition of an

eluting sugar with a single lectin binding site is hence often inefficient (Goldstein and Hayes, 1978).

As none of the additional chromatography techniques investigated were considered suitable for enhancing the purity of the 16 kDa antigen obtained following immunoaffinity procedures, the use of SDS PAGE as a second purification step was examined. Use of the Biorad 491 Prep Cell was shown to be a potentially powerful purification technique, however, the elution of a small amount of antigen in an extremely large volume of SDS containing buffer posed insurmountable practical problems. The possibility of electroeluting the 16 kDa antigen following standard SDS PAGE of immunoaffinity purified antigen was also considered. However, this method was never attempted in view of the probability of encountering substantial losses of this small molecular weight antigen either via its binding to the electroelution membrane or during subsequent concentration procedures. As an alternative, attempts to purify the 16 kDa molecule using SDS PAGE, Western blotting and Coomassie blue staining of ProBlott membrane were made. This was done with the particular aim of obtaining pure antigen in a form suitable for N terminal amino acid sequencing. Significant Coomassie blue staining was observed in the 16 kDa area of the blot and this enabled the appropriate region of the ProBlott membrane to be excised. More details regarding the purity of the 16 kDa molecule isolated in this way were obtained on sequencing and are given in Chapter 4.

Following staining with Coomassie blue, the 16 kDa molecule excised from ProBlott membrane is unsuitable for use in immunisation studies. Thus, in view of the failure of other more suitable techniques to enhance the purity of the 16 kDa molecule obtained following immunoaffinity chromatography, it was decided to use the immunoaffinity column eluates without further purification in experiments aimed at establishing whether the 16 kDa molecule has an immunising potential. Although some contaminating molecules are present within these eluates, the 16 kDa antigen is the only molecule visualised following Coomassie blue staining and corresponds to the most predominant signal seen with silver stain. Moreover, probing of Western blots of the column eluates with McAb and VRabS demonstrated that the 16 kDa molecule retains its strong antigenicity and is hence the major antigenic species present.

# CHAPTER 4

# CHARACTERISATION OF THE 16 kDa ANTIGEN

## 4.1 INTRODUCTION

This chapter describes experiments performed with the aim of investigating the physical and biochemical characteristics of the 16 kDa vaccine candidate antigen.

The diffuse nature of the 16 kDa signal seen following single dimension SDS PAGE and Western blotting suggests that the 16 kDa antigen may be a glycoprotein. Thus, whilst initial attempts were made to improve the resolution of the 16 kDa signal using two dimensional (2D) electrophoresis, experiments were also carried out with the aim of confirming the presence of both carbohydrate and peptide moieties.

With regard to confirming the presence of the latter, the 16 kDa antigen was treated with a variety of proteases and the effect of such treatment upon the size and the antigenicity of the molecule was investigated. Attempts were then made to obtain sequence data for the peptide moiety of the antigen in the hope of acquiring further information regarding the structure, and perhaps, the function of the molecule. Here experiments aimed at obtaining sequence data by gas phase N-terminal amino acid sequencing are described. Additional attempts to obtain this data via the cloning of the gene encoding the 16 kDa antigen are described in Chapter 6.

There are available several techniques which allow the presence of carbohydrate entities to be determined. One of the more frequently used of these is treatment with sodium meta periodate, a substance known to cleave between the vicinal hydroxyl groups of sugar rings. Oxidation with periodate results in the cleavage rather than the removal of sugar molecules and hence a change in the mobility of an oxidised antigen following SDS PAGE is not always apparent. The conformational changes associated with periodate cleavage do however often lead to a decrease in the antigenicity of a glycoprotein and hence the glycosylated nature of an antigen often becomes apparent on probing with antibodies. The use of this technique can also provide additional information regarding the biochemical nature of the epitopes recognised by a variety of sera. To investigate the possibility of the 16 kDa molecule being a glycoprotein, the antigen bound to Western blots was treated with sodium meta periodate and the effects of this treatment were examined by probing of the blot with both VRabS and the McAb specific for the 16 kDa antigen, B3A.

The carbohydrate entities of glycoproteins in general are important in the maintenance of conformational stability, protease resistance, charge and other important physiological properties of the molecule. With regard to antigenic glycoproteins in particular,

carbohydrates often contribute towards epitopes and in many cases McAbs are directed towards the terminal sugars of carbohydrate chains (Woodward *et al.*, 1985). Thus, having established that a molecule is glycosylated a variety of techniques can be used to obtain further details regarding the precise structure of the carbohydrate moiety. Plant lectins provide specific probes for a variety of sugar residues, and a range of glycosidase enzymes are available to investigate the way in which these sugars are linked to the peptide backbone. In the latter part of this chapter efforts to characterise the carbohydrate moiety of the 16 kDa antigen using a wide range of biotinylated lectins and an endoglycosidase specific for an O-linkage are described.

## 4.2 RESULTS

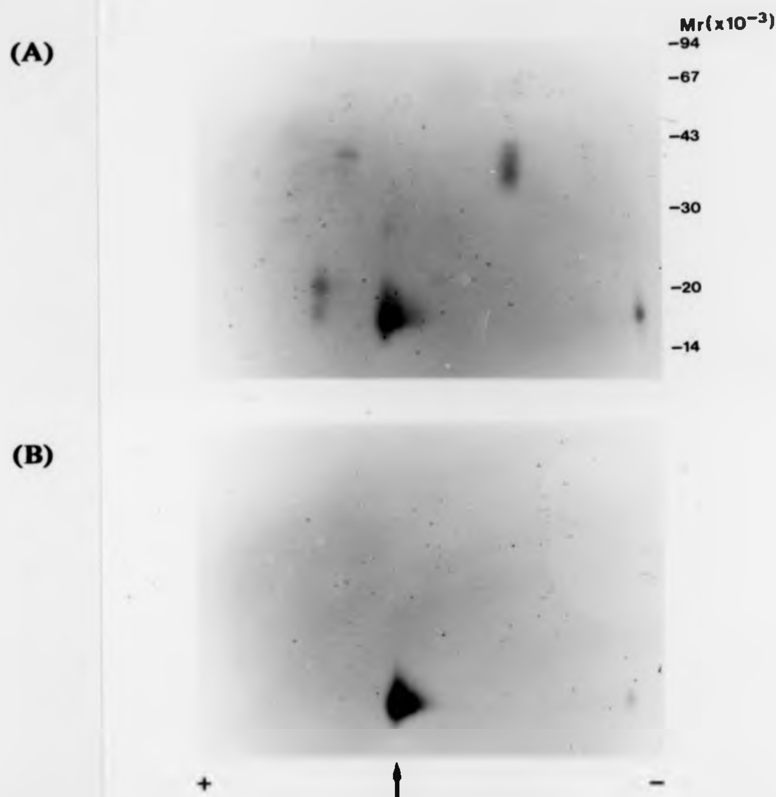
### 4.2.1. TWO DIMENSIONAL ELECTROPHORESIS

An OTG extract of 18,000 MS was separated by 2D electrophoresis, transferred by Western blotting and probed with VRabS or B3A. Figure 4.1(A) demonstrates that probing with VRabS produced a number of signals of varying intensities. Of these, two signals of approximately 16 kDa were also visible on probing with B3A (Figure 4.1(B)). The dominant of these two 16 kDa signals shows a spread which covers a narrow range of pIs and indicates that a number of 16 kDa isomorphs exist. These may represent differently glycosylated versions of the same peptide. Comparison of these blots with two dimensional blots of carbonic anhydrase standards (not shown) demonstrated that the centre of this signal had a pI of 6.1. The position of the minor 16 kDa signal at the extreme edge of the second dimension gel suggested that it could represent antigen left behind at the origin of the first dimension gel. However, although many antigens are recognised on probing with VRabS, signals which align vertically with this 16 kDa signal were not present. This would be expected if a proportion of the molecules present within the OTG detergent extract had been left at the origin of the first dimension gel and then separated in the second. These results therefore suggest that the minor 16 kDa signal may represent a very basic isomorph of the 16 kDa antigen. The markers used did not cover a broad enough range to enable the pI of this minor signal to be determined.

**Figure 4.1** Two dimensional electrophoresis

(A)

(B)




An OTG extract of 18,000 MS was separated by 2D electrophoresis and transferred by Western blotting. The blots were then probed with VRabS (A) or B3A (B) at a dilution of 1 in 200 and 1 in 20 respectively. + and - correspond to the acidic and basic ends of the gel. The major 16 kDa signal is indicated by an arrow.

### 4.2.2 CHARACTERISATION OF THE PEPTIDE MOIETY OF THE 16 kDa ANTIGEN

#### 4.2.2.1 Treatment of the 16 kDa antigen with a broad range of proteases

An SDS extract of MS was treated with a broad range of non-specific proteases. The effects of this treatment upon the 16 kDa antigen were then monitored by separation of the extract using SDS PAGE, Western blotting and probing of the blots with VRabS. - Figure 4.2 demonstrates that the 16 kDa signal was completely lost following incubation of the extract for 1 hour with 20 $\mu$g/ml of pronase, protease Type XIV (Sigma), papain or proteinase K. Treatment with 20 $\mu$g/ml of chymotrypsin or trypsin resulted in a decrease in the intensity of the 16 kDa signal which was completely ablated by increasing the concentration of the enzymes to 200 $\mu$g/ml. These results demonstrated that the 16 kDa antigen has a peptide component.

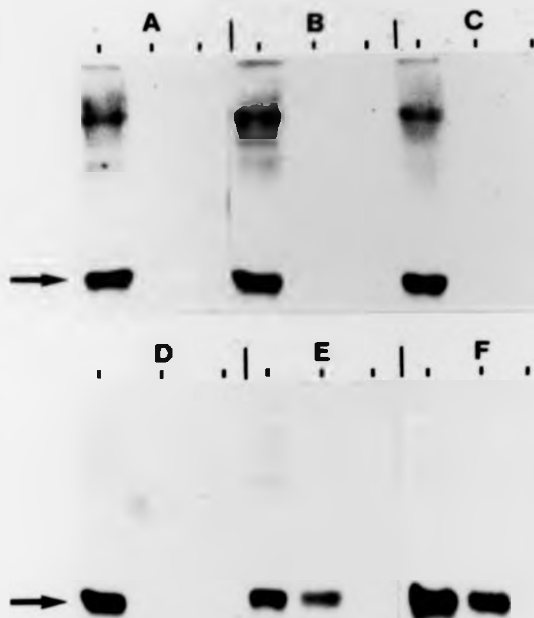
#### 4.2.2.2 Gas phase N-terminal amino acid sequencing

To provide as much antigen as possible for sequencing, McAb immunoaffinity purified material extracted from 15 million MS was concentrated, subjected to SDS PAGE and transferred to ProBlott membrane as described in Chapter 3. The final amount of antigen transferred was equivalent to approximately 150 times as much as is required to see a 16 kDa signal following Coomassie blue staining of a polyacrylamide gel (Figure 3.4(D)). Thus, on staining of the ProBlott membrane with Coomassie blue a strong, but smeared, signal was observed in the 16 kDa region of the blot. The most intensely stained areas of this signal were excised and sent to The Laboratory of Protein Structure at Mill Hill where N-terminal amino acid sequencing was performed. The data obtained plus additional data from previous attempts to sequence the 16 kDa antigen using immunoaffinity purified antigen (Dr Q. Bickle) is shown in Table 4.1. Although a best consensus sequence of:-

H/S/V - H/S/V - P - ? - P - R - A - L - P - N.

was suggested it was impossible to deduce, with any confidence, a sequence for the 16 kDa antigen from these results. The level of staining of the excised ProBlott membrane indicated that micromoles of protein were available for sequencing. However, in each cycle only picomoles of amino acids were obtained. This together with the occurrence of numerous different amino acids in each cycle, suggested that the 16 kDa antigen, presumed to contribute the major Coomassie blue stained signal seen on the ProBlott, may

**Figure 4.2** The effect of protease treatment upon the 16 kDa antigen




A Western blot of a detergent extract of MS was treated for 1 hour at 37°C with a range of proteases. Panel A = pronase, panel B = protease Type XIV (Sigma), panel C = papain, panel D = proteinase K, panel E = chymotrypsin and panel F = trypsin. In each panel, lane 1 = untreated antigen, lane 2 = antigen treated with 20 μg/ml of protease and lane 3 = antigen treated with protease at a concentration of 200 μg/ml. The blot was developed with VRabS at a dilution of 1 in 200. The position of the 16 kDa antigen is indicated by an arrow.

**TABLE 4.1 Data from sequence analysis of the 16 kDa antigen**

| CYCLE | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| BAND 1<br>AMINO ACID (pg) | G<br>S | E(2.0) | P(2.0) | P(2.0) | | K(1.0) | L(1.0) | G(1.0) | N(1.0) | R(0.5) |
| BAND 2<br>AMINO ACID (pg) | G<br>A(4.0)<br>K(1.0) | S(2.0)<br>T(2.0) | E(1.0)<br>P(1.0) | F(1.0)<br>Y(I.O)<br>P(1.0) | A(0.5)<br>S(0.5) | | | | | |
| BAND 3<br>AMINO ACID (pg) | D(2.7)<br>A(2.5)<br>S(2.0)<br>H(1.8)<br>K(1.6) | I(1.8)<br>E(1.3)<br>V(1.0)<br>P(1.0)<br>Q(1.0) | P(5.0) | P(7.0)<br>R(2.0) | P(7.0) | A(0.4) | L(0.4) | | | |
| BAND 4<br>AMINO ACID (pg) | D(1.2)<br>G(1.0) | K(1.0)<br>L(0.8)<br>V(1.0)<br>Q(0.8)<br>N(0.6)<br>E(0.4) | P(1.2)<br>L(0.6)<br>K(0.6) | D(0.6) | P(1.0)<br>Q(0.4)<br>E(0.3) | | A(0.6)<br>E(0.3) | L(1.0)<br>D(0.6)<br>G(0.2) | P(0.5)<br>V(0.1) | |

**TABLE 4.1:- cont.**

| BAND 5 | V(1.0) | H(2.0) | P(2.0) | Y(0.5) | P(1.8) | R(1.5) | A(0.6) | S(0.3) | N(0.4) | S(0.2) |
|---|---|---|---|---|---|---|---|---|---|---|
| AMINO ACID (pg) | S(1.5) | V(1.5) | F(0.2) | Q(0.3) | K(0.50 | K(1.2) | F(0.2) | Q(0.3) | Y(0.2) | F(0.1) |
| | A(0.5) | S(1.5) | | | | Q(0.5) | | | | |
| | | D(1.5) | | | | E(0.5) | | | | |
| | | Q(0.5) | | | | | | | | |
| | | L(0.5) | | | | | | | | |

Antigen rich immunoaffinity column eluates were concentrated, electrophoresed, transferred to ProBlott membrane and stained with Coomassie blue. The 16 kDa region was then excised and used for N-terminal amino acid sequencing. The data obtained for three separate regions of the 16 kDa signal (bands 3, 4 and 5) is shown above. Also shown is data obtained previously by Dr. Q. Bickle, following N-terminal sequencing of the immunoaffinity purified 16 kDa antigen (bands 1 and 2).

itself be N-terminally blocked and that the amino acids seen are derived from trace contamination products. If the 16 kDa antigen is N-terminally blocked, sequencing from the N-terminus of the whole peptide cannot be performed.

### 4.2.3 CHARACTERISATION OF THE CARBOHYDRATE MOIETY OF THE 16 kDa ANTIGEN

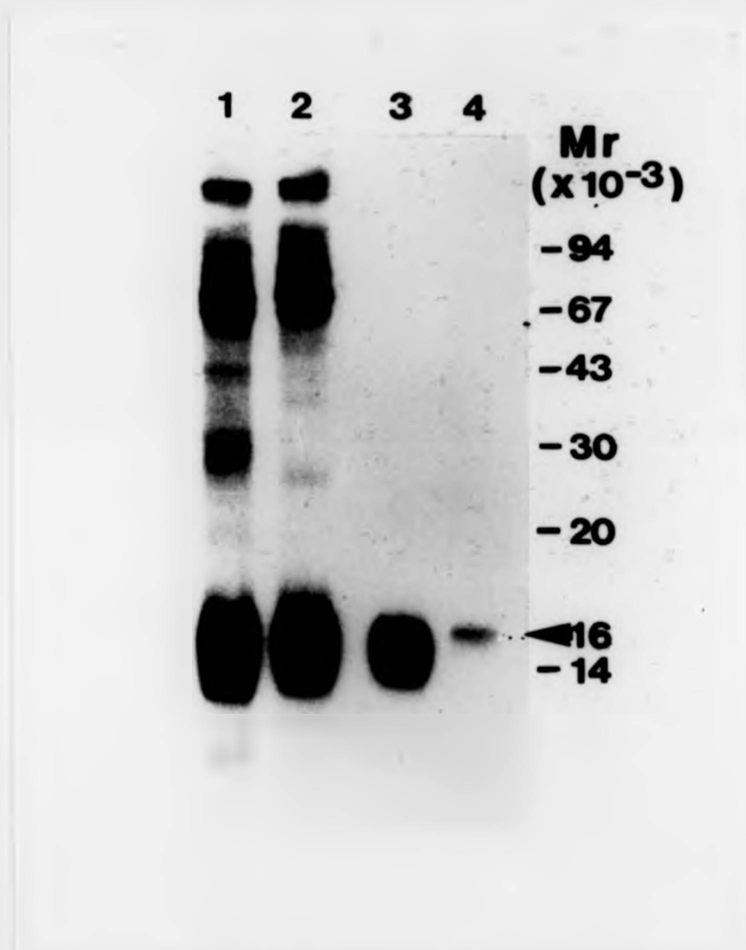
#### 4.2.3.1 Treatment of the 16 kDa antigen with sodium meta periodate

The diffuse 16 kDa signal observed following one or two dimensional electrophoresis suggests that the 16 kDa antigen may be a glycoprotein. Thus, attempts were made to determine whether the antigen incorporates carbohydrate by treating with sodium meta periodate. Western blots of a detergent extract of MS were treated with 20 mM sodium meta periodate in 50 mM sodium acetate buffer (pH 4.5) for 1 hour or for four hours in the dark at room temperature. Control samples were similarly treated with acetate buffer alone. The binding of B3A to the 16 kDa antigen was significantly depleted after treatment for 1 hour (Figure 4.3) whilst treatment for 4 hours completely ablated the signal (data not shown). This indicated that the 16 kDa antigen is indeed a glycoprotein and moreover that a carbohydrate epitope is the target of the passively protective McAb. In contrast, the binding of VRabS to the 16 kDa antigen was not noticeably affected by periodate treatment thus suggesting that the 16 kDa antigen also has some peptide epitopes.

#### 4.2.3.2 The binding of lectins to the 16 kDa antigen

Having demonstrated that the 16 kDa antigen is glycosylated, analysis of the nature of the carbohydrate portion of the molecule was carried out by examining its lectin binding specificity. For this purpose, Western blots of a detergent extract of MS were incubated overnight with a wide range of biotinylated lectins. These covered all the major carbohydrate binding groups (see Table 4.2), yet only *Arachis hypogaea* (peanut agglutinin (PNA)) and *Ricinus communis* (ricin agglutinin (RCA)) were seen to bind to the 16 kDa molecule (Figure 4.4). Although unaffected by the presence of galactose this binding was shown to be abolished by inclusion of the stronger inhibitor 0.2 M lactose (data not shown). This indicates that the binding of the lectins to the antigen is specific and of high affinity.

**Figure 4.3** The effect of periodate treatment upon the 16 kDa antigen




A Western blot of an OTG extract of MS was cut into strips and incubated for one hour with 20 mM sodium meta periodate in 50 mM sodium acetate buffer (pH 4.5) (lanes 2 and 4) or with acidic buffer alone (lanes 1 and 3). Lanes 1 and 2 were probed with VRabS at a dilution of 1 in 200 and lanes 3 and 4 with a 1 in 20 dilution of B3A.

**TABLE 4.2 Biotinylated lectins used for the probing of Western blots**

**Glucose / Mannose group**

| LECTIN | SPECIFICITY | BINDING |
|---|---|---|
| *Canavalia ensiformis* (Con A) | αMan > αGlc > GlcNAc | - |
| *Lens culinaris* (LcH) | αMan > αGlc > GlcNAc | - |

**N-Acetylglucosamine group**

| LECTIN | SPECIFICITY | BINDING |
|---|---|---|
| *Triticum vulgare* (WGA)[¥] | GlcNAc(β1,4GlcNAc)$_{1-2}$ > βGlcNAc > Neu5Ac | - |
| *Ulex europaeus* II (UEA II)[*] | L-Fucα1,2Galβ1,4GlcNAc > GlcNAc(β1,4,GlcNAc)$_{1-3}$ | - |
| *Griffonia simplicifolia* II (GS II) | α and βGlcNAc | - |

**L-Fucose group**

| LECTIN | SPECIFICITY | BINDING |
|---|---|---|
| *Lotus tetragonolobus* (Lotus) | αL-Fuc > L-Fucα1,2Galβ1,4GlcNAc >> L-Fucα1,2Galβ1,3,GlcNAc | - |

**TABLE 4.2 cont.**

N-Acetylgalactosamine / galactose group

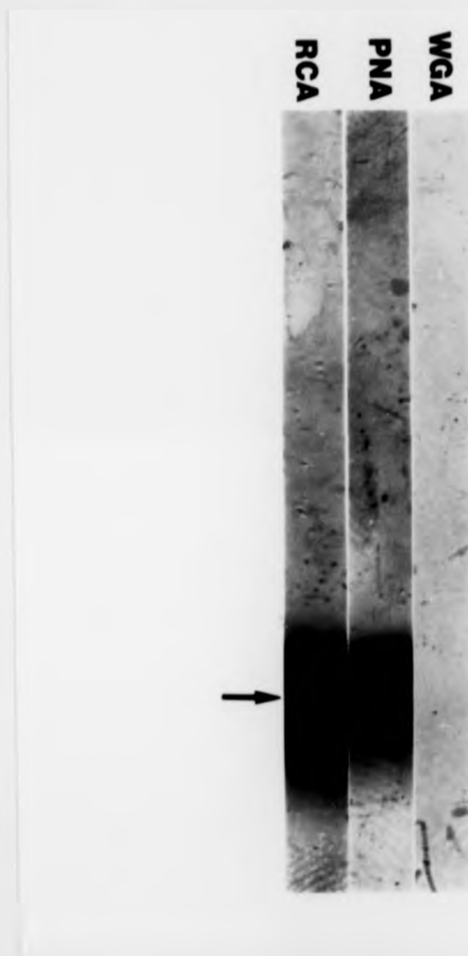
| LECTIN | SPECIFICITY | BINDING |
|---|---|---|
| *Dolichos biflorus* (DBA)[*] | GalNAcα1,3GalNAc >> αGalNAc | - |
| *Glycine max* (SBA)[*] | αGalNAc > ßGalNAc > αGal > ßGal | - |
| *Griffonia simplicifolia* I (GS I)[*] | αGalNAc and αGal | - |
| *Arachis hypogaea* (PNA)[*] | Galß1,3GalNAc > α and ßGal and α and ßGalNAc | + |
| *Erythrina cristagalli* (ECA)[*] | Galß1,4GlcNAc > αGalNAc | - |
| *Ricinus communis* (RCA)[*] | ß and αGal > GalNAc | + |
| *Sambucus nigra* (SNA) | Neu5Acα2,6,Gal and Neu5Acα2,6,GalNAc > GalNAc | - |

Sialic acid group

| LECTIN | SPECIFICITY | BINDING |
|---|---|---|
| *Maackia amurensis* (MAA)[¥] | Neu5Acα2,3Gal | - |

* These lectins only bind to terminal structures.
φ These lectins have a preference for terminal sugars.
*/φ The binding of lectins in either of these groups may be increased by treatment of the target glycoprotein with neuraminidase.
¥ The binding of these lectins may be decreased by treatment with neuraminidase.

**Figure 4.4** Binding of lectins to the 16 kDa antigen




A Western blot of an OTG extract of MS was cut into strips and probed with biotinylated ricin agglutinin (RCA), peanut agglutinin (PNA) and wheatgerm agglutinin (WGA). The position of the 16 kDa antigen is marked with an arrow.

#### 4.2.3.3 The effect of neuraminidase treatment on the binding of lectins to the 16 kDa antigen

The binding ability of a number of the lectins used in the above experiment is decreased or abolished by the masking of their target carbohydrates with sialic acid residues. The binding of these lectins can therefore be induced or increased by treatment of glycoproteins with neuraminidase, an enzyme which removes sialic acid. As PNA does not bind to sugars masked by sialic acid it is already known that at least some sugars which do not incorporate this molecule are present within the 16 kDa antigen. However, if on treatment with neuraminidase, the binding of PNA or RCA were to be increased, or the binding of additional lectins (*Glycine max* (SBA), *Griffonia simplicifolia* I (GS I), *Ulex europaeus* I, (UEA I) or *Erythrina cristagalli* (ECA)) induced, this would demonstrate the presence of sialic acid within the 16 kDa antigen and enable previously masked sugars to be identified. Strips of a Western blot of immunoaffinity purified 16 kDa antigen were therefore treated with 10 mU of neuraminidase in 200 mM sodium acetate (pH 5.5) for 1 hour. As a positive control, 100 $\mu$g of fetuin bound to nitrocellulose was similarly treated and, as a negative control, strips were incubated in acidic buffer alone. The blots were then probed with biotinylated lectins as described above. Although treatment of fetuin with neuraminidase increased the binding of PNA, thus demonstrating the activity of the enzyme, treatment of Western blots of the 16 kDa antigen had no effect on the binding of any of the lectins used (data not shown). This indicates that sugars incorporating sialic acid are not present within the 16 kDa molecule, although, it is possible that sialated oligosaccharides are present which are not recognised by the lectins used either before or after treatment with neuraminidase. In view of the large number and wide range of lectins employed this seems unlikely.

#### 4.2.3.4 Attempts to characterise the carbohydrate-peptide linkage using O-glycanase

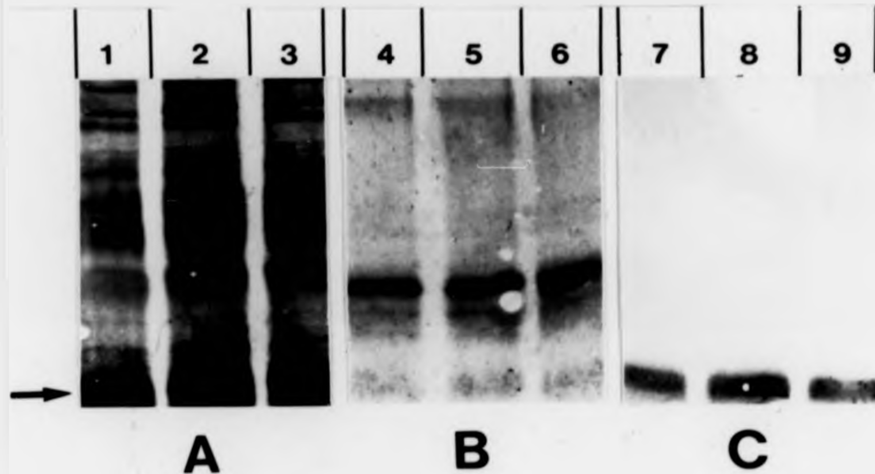
The carbohydrate chains of a glycoprotein can be linked to the peptide backbone via an N-link to an asparagine residue or via an O-link to a serine or threonine. Previous experiments (Francis, 1989) using the enzyme Glycopeptidase F, which specifically cleaves N-linked oligosaccharides from the peptide moiety of a molecule, have indicated that the carbohydrate epitope recognised by B3A is not an N-linked sugar and provide no evidence to suggest that the 16 kDa antigen possesses sugars of this kind. Thus, attempts

were made here to determine whether the epitope recognised by B3A is an O-linked sugar using the enzyme O-glycanase (Oxford Glycosystems). This enzyme cleaves specifically between the disaccharide Gal ß (1-3) GalNAc and a serine or threonine residue within the peptide core. Thus, although cleavage of a carbohydrate entity with this enzyme demonstrates unequivocally the presence of this particular O-linkage, other types of O-linked sugars are not detected by this method. As enzymes with a more broad specificity are not available and the binding of PNA and RCA to the 16 kDa antigen indicates the presence of Gal and / or GalNAc, a detergent extract of MS was treated with O-glycanase in sodium citrate phosphate (pH 6.0) for 20 hours at 37°C. A no enzyme negative control reaction was also used, and as a positive control for the activity of the enzyme, 250 $\mu$g of asialofetuin was treated with O-glycanase in a reaction also containing OTG extracted MS antigens. Following treatment the activity of the enzyme was confirmed by using the positive control in the Elson Morgan assay (Reissig *et al.*, 1955). This assay enables the liberation of the Gal ß (1-3) GalNAc disaccharide from asialofetuin to be measured following the generation of a colour reaction with the DMAB substrate. The possible liberation of the disaccharide from the 16 kDa antigen could not however be assessed in this manner as the assay is not particularly sensitive and therefore even if significant release of the disaccharide occurred it would be necessary to treat unavailable amounts of the 16 kDa antigen for it to be detected in this way. The enzyme treated 16 kDa antigen and the negative control were therefore run on SDS PAGE gels, transferred by Western blotting and the blots probed with B3A, VRabS or PNA. Figure 4.5 shows that treatment of the 16 kDa antigen with O-glycanase did not affect the binding of the antibodies or the lectin, nor did it alter the size of the signal observed. This demonstrates that the epitope recognised by the McAb and / or the sugar recognised by PNA is not O-linked Gal ß (1-3) GalNAc.

## 4.3 DISCUSSION

Experiments detailed in this chapter showed that, as judged by a loss in antigenicity, the 16 kDa molecule was susceptible to degradation by a wide range of non-specific proteases. This confirmed the presence of a peptide moiety. An alteration in the antibody binding pattern of the 16 kDa antigen was also used to demonstrate its susceptibility to treatment with sodium meta periodate. Although on rare occasions prolonged treatment with periodate has resulted in the degradation of protein molecules, the short oxidation

**Figure 4.5 Treatment of the 16 kDa antigen with the enzyme O-glycanase**




30 $\mu$l aliquots of an OTG extract of MS (400,000 MS/ml) were treated with O-glycanase for 20 hours at 37°C. The enzyme was used at a concentration of 0.3 mU (lanes 2, 5 and 8) or 1.8 mU (lanes 3, 6 and 9). Aliquots incubated in the absence of enzyme were also included as negative controls (lanes 1, 4 and 7). Following incubation antigens were separated by SDS PAGE and transferred by Western blotting. The blots were probed with VRabS at a dilution of 1 in 200 (panel A), biotinylated PNA (panel B) or B3A at a dilution of 1 in 20 (panel C). The position of the 16 kDa antigen is marked with an arrow.

time and acidic conditions employed here should have allowed the denaturation of carbohydrates via the cleaving of vicinal hydroxyl groups to occur, without affecting the structure of the peptide entity (Clamp and Hough, 1965). Thus, the failure of B3A to bind to periodate treated 16 kDa antigen indicated that the molecule is indeed a glycoprotein and that a carbohydrate epitope is the target of the McAb. The continued binding of VRabS to the 16 kDa on periodate treated Western blots substantiated the claim that the peptide moiety is unaffected by the conditions used for periodate oxidation and indicated that the 16 kDa molecule also incorporates some peptide epitopes. Having identified the 16 kDa antigen as a glycoprotein, studies were carried out with the aim of characterising both the peptide and carbohydrate moieties.

Attempts to obtain sequence data for the peptide moiety of the 16 kDa antigen by gas phase N-terminal amino acid sequencing were unsuccessful. Whereas the level of Coomassie blue staining of the ProBlott membrane suggested that micromoles of protein were available for sequencing only picomoles of a variety of amino acids were present in each sequencing cycle. The most plausible explanation for this discrepancy is that the 16 kDa antigen which contributes the major Coomassie blue stained signal is N-terminally blocked and that the amino acid sequence data obtained represents that of minor contaminating molecules. The presence of the latter indicates that the 16 kDa antigen excised from the ProBlott membrane was not completely pure. However, had the 16 kDa antigen not been N-terminally blocked, the amino acids derived from the micromolar concentrations of antigen present would have far outweighed those derived from trace contamination products and the sequence of the 16 kDa molecule would have been discernible. With proteins not amenable to sequencing from the N-terminus, it is a common procedure to sequence internal peptides generated by cleavage of the molecule with enzymes (e.g. trypsin) or with cyanogen bromide and isolated by HPLC. However, Coomassie blue stained antigen bound to ProBlott membrane is unsuitable for use in this protocol and in view of the small quantities and partial purity of the antigen present, it was considered impractical to attempt digestion of the 16 kDa molecule within the immunoaffinity column eluates. Further efforts to obtain sequence data for the 16 kDa antigen were therefore focused on the screening of recombinant cDNA libraries (see chapter 6).

With regard to the characterisation of the carbohydrate entity of the 16 kDa antigen, the binding of B3A to the molecule following incubation with the enzyme Glycopeptidase

F has suggested that an N-linked sugar is not the target of the McAb (Francis, 1989). Moreover, as no alteration in the size of the molecule was observed following this treatment, it seems unlikely that the 16 kDa antigen possesses any sugars of this kind. However, although Glycopeptidase F cleaves between the asparagine residue of the peptide component and the GlcNAc-GlcNAc-Man trisaccharide which forms the core of all N-linked oligosaccharides, chains in which a fucose is $\alpha$ 1-3 linked to the primary GlcNAc (Tretter *et al.*, 1991) and chains which are N-linked to a terminal asparagine residue (Tarentino *et al.*, 1985) are resistant to cleavage. The former have been identified in schistosomes (Nyame *et al.*, 1988a) and suggest the possibility of Glycopeptidase F resistant N-linked sugars existing within the 16 kDa antigen. However, the lectin experiments carried out here also support the suggestion that the 16 kDa does not include N-linked oligosaccharides. *Triticum vulgaris* (wheatgerm agglutinin, WGA) which recognises the trisaccharide core of all N-linked sugars did not bind to the 16 kDa antigen, nor did *Canavalia enisformis*, (ConA), *Lens culinaris*, (lentil) nor *Griffonia simplicifolia* II (GS II) which recognise GlcNAc. Although the 16 kDa antigen does not contain such sugars, other researchers have demonstrated that molecules containing high mannose and / or complex type N-linked oligosaccharides are present in both schistosomula (Nyame *et al.*, 1988a) and the adult stage of the parasite (Nyame *et al.*, 1988b, 1989).

The assertion that the 16 kDa antigen does not contain any N-linked oligosaccharides leads to the suggestion that the carbohydrate(s) which bind the McAb and the lectins PNA and RCA, are attached to the peptide core via an O-linkage. Although O-glycosylation is one of the most common post translational modifications to occur, its function is still not fully understood. However, as a consequence of steric interactions between the carbohydrate and peptide moieties of a glycoprotein and the overpowering of the hydrophobic interactions normally involved in protein folding by the hydrophilic nature of the carbohydrate chains, secondary and tertiary structures which are typical of non-glycosylated peptides are completely altered by the addition of O-linked sugars. It has therefore been suggested that O-glycosylation is of paramount importance to the establishment of the overall structure of a glycoprotein (Jentoft, 1990). One of the predominant effects of glycosylation on the structure of a molecule is the extension of the glycosylated region to form a semi-flexible rod. This can lead to an increase in the length covered by a small number of amino acids and it has been proposed (Jentoft, 1990) that

this may enable the extracellular portion of some glycoproteins to stretch far above the plasma membrane (e.g. leucosialin, epiglycanin). A large number of such glycoproteins could then be used in the formation of a protective layer over the cell surface. Alternatively, the extension resulting from glycosylation of a short peptide sequence between a membrane bound domain and a functional domain, may enable the latter to appear above the cell surface and the glycoprotein to act as a signalling molecule. O-linked sugars are also known to provide glycoproteins with charge and water binding properties and to protect regions of the peptide backbone from degradation by proteases (Paulson, 1989).

For most secreted and membrane glycoproteins, O-glycosylation initially involves the linkage of a GalNAc residue to the hydroxyl group of a serine or threonine residue within the peptide moiety (Piller and Piller, 1993). Several core structures can then be constructed around this basic linkage, although one of the most commonly occurring is formed by the addition of a galactose residue to give the structure Gal ß (1-3) GalNAc - Ser / Thr. As both PNA which has a high affinity for this disaccharide and RCA, which targets terminal galactose, bind to the 16 kDa antigen on Western blots, the enzyme O-glycanase was used to determine whether this core structure was present within the 16 kDa glycoprotein. O-glycanase is specific for this particular linkage and cleaves the disaccharide from the peptide core only if it is unsubstituted (ie. no other sugar groups are attached). The continued binding of B3A to the 16 kDa antigen following treatment with O-glycanase demonstrates that Gal ß (1-3) GalNAc does not represent the target of the McAb. Moreover, the inability of this treatment to decrease the binding of PNA to the antigen suggests that this simple disaccharide is not present. The latter is somewhat surprising when considering the high affinity with which PNA binds to the 16 kDa antigen and the preference of PNA for this disaccharide. However, although PNA binds with greatest affinity to the Gal ß (1-3) GalNAc structure, it will also recognise sugars which incorporate terminal $\alpha$ or ß galactose and / or terminal $\alpha$ or ß GalNAc. Similarly, although RCA has a preference for terminal ß galactose, it will bind to terminal $\alpha$ galactose or terminal $\alpha$ or ß GalNAc. Thus, other possibilities for the structure of the lectin binding O-linked carbohydrate(s) present within the 16 kDa antigen, include complex oligosaccharides terminating in galactose or GalNAc and more simple structures involving these residues linked directly to the peptide core. Although unusual, the latter have already been shown to be present in schistosomes. Nyame *et al.*, (1987, 1988a)

demonstrated that whilst the major O-linked sugar in both schistosomula and adult worms was found to be GlcNAc linked directly to the peptide backbone, a small amount of directly linked unsubstituted GalNac was also seen. Furthermore, complex sugars including the disaccharide Gal ß (1-3) GalNAc were not seen in the schistosomula stage. The results of Nyame *et al.*, are therefore consistent with the suggestion made above that Gal ß (1-3) GalNAc is not present within the 16 kDa antigen and also indicate that complex oligosaccharides terminating in suitable residues do not represent the target of PNA, RCA or B3A. Thus, taking into account the conclusions of the studies of Nyame *et al.*, (1988) in addition to those carried out here, the probable target of the lectins binding to the 16 kDa antigen is the monosaccharide GalNAc attached directly to the peptide core. The inability of *Glycine max* (SBA), which has a high affinity for $\alpha$ GalNAc, to bind to the 16 kDa antigen suggest that these residues are in the ß configuration. In addition, the inability of the 16 kDa antigen to bind lectins which are specific for GlcNAc indicates that this residue is not present and hence suggests that the McAb, like the lectins, also targets ß GalNAc residues attached directly to the peptide core. This is consistent with the observed periodate sensitivity of the epitope recognised by the McAb as GalNAc residues have vicinal hydroxyl groups and would therefore be destroyed by periodate. Furthermore, the binding of PNA to the 16 kDa antigen is abolished by periodate treatment (data not shown). The indication that the target of the McAb is a single monosaccharide attached to the peptide core is somewhat surprising, as the majority of previously described carbohydrate epitopes have a more complex structure (Hakomori and Kannagi, 1983). However, a number of McAbs have been described which recognise epitopes associated with O-linked monosaccharides found on glycoproteins of rat renal tissue (Holt *et al.*, 1987, Park *et al.*, 1987). The binding of these McAbs was shown to depend not only on the presence of unsubstituted GlcNAc residues but also on the integrity of the peptide chain to which the sugars are attached. Similarly, the demonstration that the binding of B3A to the 16 kDa antigen is not blocked by the inclusion of lactose also suggests that this McAb does not bind in a lectin type fashion to a monosaccharide alone. Other possibilities for the nature of the epitopes to which these McAbs do bind include an epitope formed by the linkage between the carbohydrate and peptide entities which is reliant on the conformation of both. Alternatively, the antibodies may recognise peptide epitopes which are dependent on the attachment of sugar residues for their conformation. As suggested by Jentoft (1990) (see

above) glycosylation can drastically alter the conformation of a peptide structure. Thus, it seems possible that removal or cleavage of sugar residues may lead to a loss in the integrity of some peptide epitopes.

The study of lectin binding to antigens upon the schistosome surface is also of interest as several studies have indicated that major alterations in the binding pattern of lectins to the surface of the schistosome occur as the parasite becomes refractory to immune attack i.e. as the newly transformed schistosomula develop into lung stage larvae. It has therefore been proposed that the developing resistance of older schistosomula may be due, in part, to alterations in the nature of the glycoproteins present upon the surface membrane. Thus, further studies were carried out using lectins specific for a number of sugar groups to look in more detail at these changes. Studies using fluorescein labelled lectins have demonstrated that the surface of the newly transformed schistosomula has a large number of binding sites for Con A, WGA and *Lotus tetragonolobus* (Lotus) and a smaller number of binding sites for PNA and RCA (Murrell *et al.*, 1978, Simpson *et al.*, 1983b). In contrast, five day old lung schistosomula have 70% - 75% less binding sites for Con A, RCA, WGA and PNA whilst the binding of Lotus lectin is absent (Simpson *et al.*, 1983b). This observed decrease in the binding of PNA may be of particular relevance to the studies described here as it has been demonstrated that the 16 kDa antigen is the major molecule recognised by PNA on probing of Western blots of a detergent extract of MS. Thus, it is proposed that a change in the expression of the 16 kDa antigen could play a substantial part in the decreased level of PNA binding to the surface of the developing schistosomula and hence in the general reduction in the number of glycoproteins available upon the surface, a process which is thought to be involved in the evasion of host immunity. In this context it is also of interest to note that the pattern of binding of fluorescently labelled PNA reported by Simpson *et al.*, (1983b) corresponds to that of B3A which also binds to the surface of newly transformed but not lung stage schistosomula (Bickle *et al.*, 1986).

How the loss of surface glycoproteins available for lectin binding occurs is not fully understood. One possibility is that the addition of sialic acid residues to existing sugars masks the target sugars of some lectins. This is supported by the studies of Simpson *et al.*, (1983b), which demonstrated that the binding of PNA to freshly transformed schistosomula was not increased by treatment with neuraminidase, whereas similar treatment of lung stage parasites increased the binding of PNA and also induced the

binding of SBA. The former observation is in agreement with the results obtained here which show that the binding of PNA to Western blots of the 16 kDa schistosomula surface antigen was not increased by neuraminidase treatment. Moreover, although it is possible, that previously unexposed glycoproteins which incorporate PNA binding groups masked by sialic acid are newly exposed on the surface of the lung stage parasite, these results may also suggest that the 16 kDa antigen present in MS is modified by the addition of sialic acid in the lung stage schistosomula. This would account for the decrease in the binding of PNA and possibly the inability of B3A to bind to the lung stage larvae. Whether the sialic acid which masks glycoproteins in the lung stage larvae is of host or parasite origin is unknown. However, Nyame *et al.*, (1987) failed to demonstrate the synthesis of sialic acid by schistosomula or by adult worms and it is well documented that schistosomes are capable of absorbing host antigens onto their surface. Once incorporated into the surface, sialic acid may play an important role in enabling the lung stage schistosomula to resist the host's immune response. In addition to masking the sugar groups of some glycoproteins, sialic acid has previously been shown to be capable of preventing complement activation (Fearon, 1978, Pangburn and Muller-Eberhard, 1978) and reducing the immunogenicity of certain cells (Currie and Bagshawe, 1969). Alternative explanations for the decrease in binding of lectins to the lung stage schistosomula surface are the occurrence of membrane turnover with the loss of major glycoproteins or, as previously mentioned, an alteration in the structure of the surface membrane which results in the exposure of different membrane molecules which perhaps include some asialated glycoproteins (Hayunga and Sumner, 1986a).

Also of interest in the context of the work described in this chapter are studies which have employed labelled lectins to examine the effect of irradiation on the glycoproteins present upon the surface of the parasite. Wales *et al.*, (1993) demonstrated that following UV irradiation the binding of Con A, WGA and PNA to cercariae was greatly increased as compared to that seen with normal parasites. Moreover, the binding of lectins to the surface of irradiated parasites was maximal when parasites were irradiated with doses shown to stimulate the greatest level of immunity in the UV irradiated vaccine model. This increased binding was lost once transformation of the cercariae had been completed. Thus, it has been proposed (Wales *et al.*, 1993) that irradiation causes a disruption of the glycocalyx which leads to an increase in the lectin binding ability and more importantly the immunogenicity of the parasites, by exposure of cryptic epitopes upon glycoproteins

within the glycocalyx or within the membrane beneath. If the 16 kDa glycoprotein which is not normally recognised on the surface of cercariae were to be exposed in such a way, this may explain the increased binding of PNA seen following irradiation and the increased immunogenicity of the antigen which is seen in the irradiated vaccine model.

In addition to the 16 kDa molecule, a number of antigens expressed upon the surface of the schistosomula have been shown to be glycoproteins (Simpson *et al.*, 1984, Oligno *et al.*, 1988, Soisson *et al.*, 1992). Of these, a proportion express surface exposed epitopes of a carbohydrate nature and in the case of the 38 kDa vaccine candidate antigen originally described by Dissous *et al.*, (1985) (section 1.9.2.1) such epitopes have been seen to represent the targets of passively protective McAbs (Grzych *et al.*, 1982, Kelly *et al.*, 1986). In accordance with the results obtained here which demonstrate that the target of the passively protective McAb, B3A is a carbohydrate epitope of the 16 kDa antigen, these observations indicate that the binding of antibodies to carbohydrate epitopes upon the surface of the schistosomula is able to mediate protection. However, although some glycoproteins may represent good vaccine candidates the glycanic nature of their epitopes limits their production by DNA recombinant technology. With regard to the 38 kDa antigen this problem has been circumvented by the use of an anti-idiotype vaccine which was shown to confer significant protection in rats (Grzych *et al.*, 1985, section 1.9.2.1). It is possible that similar techniques, or the use of mimeotopes could be employed to immunise against the carbohydrate epitope recognised by B3A upon the 16 kDa antigen. However, it should be noted that the binding of VRabS to the 16 kDa antigen remains unaffected by periodate treatment, as this indicates that potentially clonable peptide epitopes are also present upon this molecule.

Thus, in summary the 16 kDa antigen has been characterised as a glycoprotein consisting of an N-terminally blocked peptide component which is most probably O-glycosylated by the attachment of a number of GalNAc residues directly to the peptide core. The latter either form the target of the passively protective McAb or are critical to its formation.

# CHAPTER 5

# VACCINATION OF MICE WITH THE IMMUNOAFFINITY PURIFIED 16 kDa ANTIGEN

## 5.1 INTRODUCTION

The purpose of the studies detailed in this chapter was to immunise mice with the partially purified 16 kDa antigen. This was done in order to raise a good immune response and thus investigate the ability of the molecule to protect against a challenge infection. However, immunising with the purified antigen might also result in the generation of monospecific sera which could be used as a probe with which to screen a cDNA expression library (see Chapter 6).

It is known that the development of a protective antibody response relies not only upon the titre of the antibody raised against the immunogen but also upon the major antibody isotype, the antibody affinity and the specific nature of the antigenic determinants recognised. Prior to immunisation with the partially purified 16 kDa antigen, it was therefore deemed useful to carry out experiments aimed at characterising the antibody response generated against the 16 kDa antigen in immune mice multiply vaccinated with irradiated cercariae. As it is proposed that the anti-16 kDa response may be of importance to the development of the protection seen in this model, it was hoped that such studies would indicate the features of this response which might be necessary for the development of protective immunity and thus those which the injections with the purified antigen were intended to reproduce. The first section of this chapter describes the characterisation of VMS with regard to the titre and subclass of the antibody raised against the 16 kDa antigen and the nature of the epitopes recognised.

One of the major problems encountered when immunising with purified antigens is their poor antigenicity. This is believed to be due to the simple monomeric form in which molecules isolated from the parasite exist, and the problem is traditionally overcome by the use of immunopotentiating chemicals known as adjuvants (Alving *et al.*, 1992). In addition to enhancing the immune response in general, adjuvants also have the ability to drive the response in a particular direction (Audibert and Lise, 1993). This is largely due to two factors. Firstly, adjuvanticity is linked to an ability to selectively stimulate one of the T cell subsets that control the immune response (Grun and Maurer, 1989). This dictates the type of cytokines produced and ultimately the isotype of the antibody response generated. Secondly, the type of adjuvant used may affect the way in which an antigen is presented. For example, micelle and vesicle type structures are particularly suited to the presentation of membrane antigens. Thus, in order to optimise the likelihood of achieving protective immunity and to examine the effect of using different adjuvants upon

the antibody response raised against the 16 kDa antigen, the immunoaffinity purified antigen was used in conjunction with the oil in water suspension Ribi, the novel adjuvant novasomes (Novavax Inc.) and the Quil A derivative, Spikoside, in the form of immunostimulating complexes (ISCOMs). Following immunisation, the features of the antibody response generated were investigated and compared to those of VMS. Protection data was also obtained.

## 5.2 RESULTS

### 5.2.1 CHARACTERISATION OF THE ANTIBODIES RAISED AGAINST THE 16 kDa ANTIGEN IN MICE VACCINATED WITH RADIATION ATTENUATED CERCARIAE

The 16 kDa molecule is a major antigen recognised by sera raised in mice multiply vaccinated with irradiated cercariae (VMS) (Bickle *et al.*, 1986). VMS taken from four times immunised female CBA mice has been used in the following characterisation studies.

#### 5.2.1.1 Antibody titre

Strips of a Western blot of an OTG extract of MS were incubated in increasing dilutions of VMS. Antibodies recognising the 16 kDa antigen had a titre of greater than 1 in 5,000 by this method (data not shown).

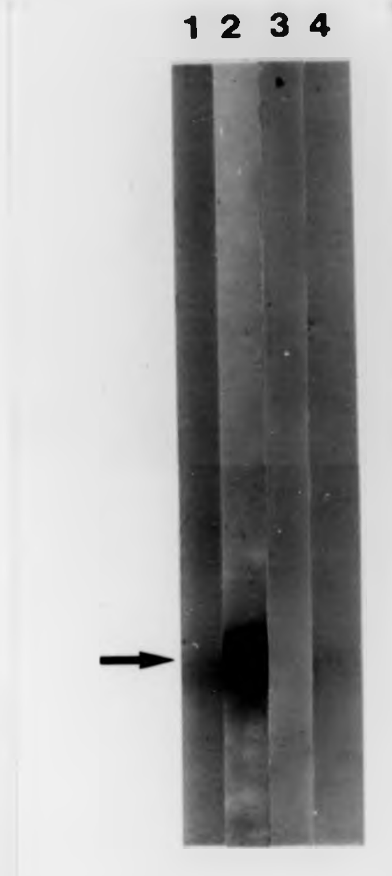
#### 5.2.1.2 Antibody subclass

Strips of a Western blot of immunoaffinity purified antigen were incubated overnight in a 1 in 200 dilution of VMS and developed with IgG subclass specific, peroxidase labelled, rabbit anti-mouse conjugates. Figure 5.1 demonstrates that the predominant subclass of the IgG antibodies recognising the 16 kDa antigen was IgG2a. No signal was seen with any of the other conjugates.

#### 5.2.1.3 The biochemical nature of the epitopes recognised

The recognition of periodate sensitive and insensitive moieties upon the 16 kDa molecule by B3A and VRabS respectively (see Chapter 4) has indicated that the antigen has both carbohydrate and peptide epitopes. In order to determine the nature of the epitopes recognised by VMS, strips of a Western blot of immunoaffinity purified antigen

**Figure 5.1** **The subclass of the anti-16 kDa antibodies within VMS**




A Western blot of immunoaffinity purified 16 kDa antigen was cut into strips and incubated overnight with a 1 in 200 dilution of VMS. The strips were then probed using subclass specific conjugates. Lane 1 = IgG1, lane 2 = IgG2a, lane 3 = IgG2b and lane 4 = IgG3. The position of the 16 kDa antigen is marked with an arrow.

were treated with sodium meta periodate for 1 hour and for 4 hours and probed with a 1 in 50 dilution of sera. Figure 5.2 demonstrates that the 16 kDa signal was depleted by treatment with 20 mM sodium meta periodate for one hour. These results indicate that antibodies within VMS recognised predominantly carbohydrate epitopes.

### 5.2.2 IMMUNISATION OF MICE WITH IMMUNOAFFINITY PURIFIED 16 kDa ANTIGEN PLUS RIBI ADJUVANT

The experiments detailed in Chapter 3 demonstrated that the material eluted from the immunoaffinity column was very much enriched with regard to the 16 kDa antigen. Although these eluates also contain a small number of contaminating molecules, the 16 kDa antigen was the only molecule seen by Coomassie blue staining, the most predominant molecule visible on silver staining and the major antigen recognised by VRabS following SDS PAGE and Western blotting. Thus, in view of the failure of other techniques to enhance the purity of the immunoaffinity purified 16 kDa molecule, the most antigen rich eluates obtained following purification of the antigen from a total of 23 million MS were dialysed to remove excess salt and diethylamine (DEA) and used for immunisation (section 2.20.1). A group of 8 male C57Bl/10 mice were immunised subcutaneously with the partially purified antigen plus Ribi adjuvant. A similar control group were immunised with the adjuvant alone. Following a total of four injections both groups of mice were challenged, perfused after 6 weeks and the worm burdens counted. Table 5.1 demonstrates that no significant protection was obtained.

### 5.2.3 CHARACTERISATION OF THE ANTIBODY RESPONSE IN MICE IMMUNISED WITH THE 16 kDa ANTIGEN PLUS RIBI ADJUVANT

#### 5.2.3.1. Recognition of the 16 kDa antigen by Western blotting

A pool of the sera raised in mice immunised twice with the purified 16 kDa antigen plus Ribi, was shown to give a weak signal with the immunoaffinity purified molecule on Western blots (data not shown). The strength of this response was increased considerably following two further injections and as can be seen in Figure 5.3 the 16 kDa molecule was the major antigen recognised on probing of Western blots of an OTG extract of MS antigens. The only other signal, which was only occasionally observed, was the doublet of approximately 58 kDa which has been described previously (see Chapter 3) and is believed to represent a contaminant which is introduced during SDS PAGE.

**Figure 5.2** **The recognition of periodate treated 16 kDa antigen by VMS**

A Western blot of immunoaffinity purified 16 kDa antigen was cut into strips and treated with 20 mM sodium meta periodate in 50 mM sodium acetate (pH 4.5) for 1 hour (lane 2) or for 4 hours (lane 3). A control strip was incubated for 4 hours in the acidic buffer alone (lane 1). The strips were then probed with a 1 in 50 dilution of VMS. The position of the 16 kDa antigen is marked with an arrow.

**TABLE 5.1** **The immunisation of mice with the immunoaffinity purified 16 kDa antigen plus Ribi adjuvant**

| Number of mice per group | Immunisation protocol | Worm burden (+/- S.D.) | Reduction (%) | Significance |
|---|---|---|---|---|
| 7 | Saline plus Ribi | 74.3 +/- 9.8 | - | - |
| 7 | Purified 16 kDa plus Ribi | 69.7 +/- 10.6 | 6.2 | N.S. |

C57Bl/10 mice were immunised s.c with immunoaffinity purified 16 kDa antigen plus Ribi adjuvant or with Ribi plus sterile saline. The mice were then challenged with 200 cercariae 4 weeks after the last immunisation and killed and perfused 30 days later. The average worm burden was obtained for both groups and % resistance calculated as described in Chapter 2. S.D. = standard deviation.

Thus, it appeared that, despite the lack of protection observed, a strong monospecific response had been generated against the 16 kDa antigen.

#### 5.2.3.2 Recognition of the 16 kDa antigen by sera raised in individual mice

The worm burdens seen in Table 5.1 represent the average values for the immunised and the control groups. However, on observation of the worm burdens of individual mice it became apparent that within the immunised group a number of animals had worm burdens which differed substantially from the average. Sera taken from individual mice immunised four times with the immunoaffinity purified antigen was therefore used to probe Western blots of an OTG extract of MS (data not shown). The results obtained demonstrated that, although sera from all of the mice recognised the 16 kDa antigen to some extent, sera from some individuals gave a signal which was substantially stronger than that given by others. However, comparison of the blots with the protection data demonstrated that those mice which responded strongly to immunisation with the 16 kDa were not those with lower than average worm burdens. A pool of sera from mice immunised with the 16 kDa antigen plus Ribi adjuvant (Ribi(S)) was used for the following studies.

#### 5.2.3.3 Antibody titre

The probing of Western blots of OTG extracted MS antigens with increasing dilutions of Ribi(S) demonstrated that the anti-16 kDa antibodies within this sera had a titre of at least 1 in 5,000 (data not shown). This was comparable to that of VMS.

#### 5.2.3.4 Antibody subclass

Western blots of an OTG extract of MS were probed with a 1 in 500 dilution of Ribi(S) and developed with IgG subclass specific conjugates. As can be seen in Figure 5.3 the major subclass of the antibodies recognising the 16 kDa antigen was IgG2a. A much weaker signal was also visible following probing with the anti-IgG2b conjugate. However, no signal was seen with the IgG1 or IgG3 specific conjugates.

#### 5.2.3.5 The nature of the epitopes recognised

(a) Treatment with sodium meta periodate

To determine the biochemical nature of the epitopes recognised by Ribi(S) on the 16

**Figure 5.3 The subclass of the anti-16 kDa antibodies within Ribi(S)**

A Western blot of an OTG extract of MS was cut into strips and incubated with a 1 in 500 dilution of Ribi(S). The strips were then developed using isotype specific conjugates. Lane 1 = IgG1, lane 2 = IgG2a, lane 3 = IgG2b and lane 4 = IgG3. The position of the 16 kDa antigen is marked with an arrow.

**Figure 5.3** The subclass of the anti-16 kDa antibodies within Ribi(S)

A Western blot of an OTG extract of MS was cut into strips and incubated with a 1 in 500 dilution of Ribi(S). The strips were then developed using isotype specific conjugates. Lane 1 = IgG1, lane 2 = IgG2a, lane 3 = IgG2b and lane 4 = IgG3. The position of the 16 kDa antigen is marked with an arrow.

kDa antigen, strips of a Western blot of immunoaffinity purified antigen were treated with sodium meta periodate for 1 or for 4 hours and then probed with a 1 in 50 dilution of sera. Figure 5.4 demonstrates that the recognition of the 16 kDa antigen by Ribi(S) was significantly diminished following treatment with periodate for 1 hour, although a residual signal was still observed following four hour treatment. These results indciate that the majority of antibodies within Ribi(S) recognise epitopes of a carbohydrate nature.

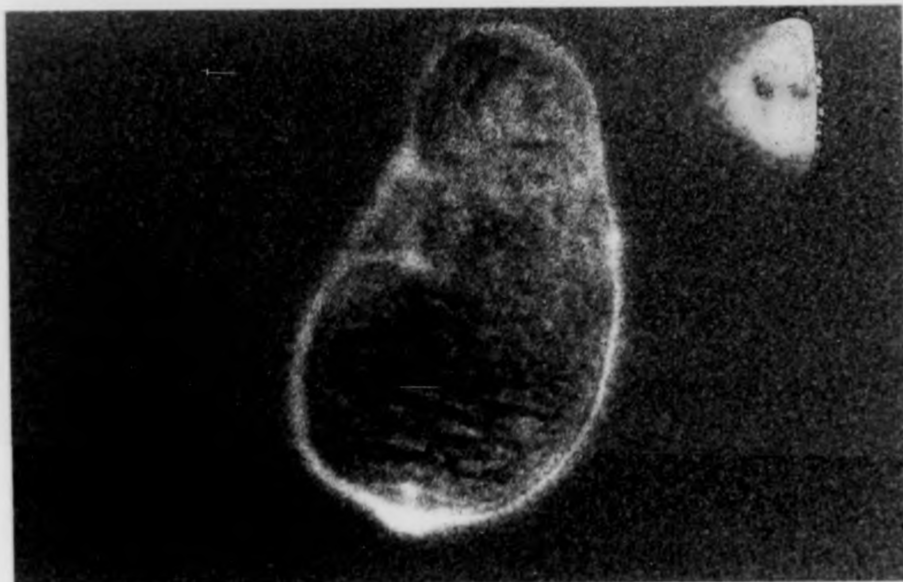
(b) The recognition of surface exposed epitopes

Despite the recognition of carbohydrate epitopes on the 16 kDa antigen by antibodies of an equal titre and a similar IgG subclass to the anti-16 kDa antibodies within VMS, mice immunised with the 16 kDa molecule plus Ribi adjuvant were not protected against a challenge infection. One of many possible explanations for this observation is that Ribi(S) failed to recognise carbohydrate epitopes which were appropriate as targets of a protective immune response. For example, it could well be important that surface exposed epitopes are recognised if the intact parasite of the challenge infection are to be killed effectively. To investigate whether surface exposed epitopes are recognised by antibodies within Ribi(S) living schistosomula were probed with this sera and a fluorescein labelled anti-mouse conjugate. Schistosomula similarly probed with B3A were used as a positive control. Figure 5.5 demonstrates that on probing with Ribi(S), fluorescence which was judged to be equal to that seen on probing with the McAb was observed. This suggested that Ribi(S) does indeed bind to epitopes exposed upon the surface of the schistosomula. However, it was noted that in addition to binding to the schistosomula heads, Ribi(S) bound to the cercarial tails still present in the MS preparation. This contrasted with the binding pattern observed for B3A which bound only to the former. The 16 kDa antigen is known to be present in cercarial tails and has previously been extracted in small amounts using OTG (data not shown). It is therefore possible that Ribi(S) recognises epitopes of the 16 kDa antigen which are not targets of the McAb and are exposed on the surface of cercarial tails as well as on schistosomula. Alternatively, it is possible that the binding of Ribi(S) to the surface of the parasite could be due to the recognition of epitopes of antigens other than the 16 kDa molecule which are exposed on the surface of the cercarial tails as well as the schistosomula. In order to investigate whether Ribi(S) recognised molecules other than the *S. mansoni* specific 16 kDa antigen, *S. japonicum* schistosomula were probed with this sera and the fluorescein labelled conjugate. Despite

**Figure 5.4** **The recognition of periodate treated 16 kDa antigen by Ribi(S)**

Strips of a Western blot of immunoaffinity purified 16 kDa antigen were incubated for 1 hour (lane 2) or for 4 hours (lane 3) with 20 mM sodium periodate in 50 mM sodium acetate buffer (pH 4.5). A control strip (lane 1) was incubated for 4 hours in the acidic buffer alone. The strips were then probed with Ribi(S) at a dilution of 1 in 50. The position of the 16 kDa antigen is marked with an arrow.

**Figure 5.5** **Recognition of the schistosomula surface by Ribi(S)**



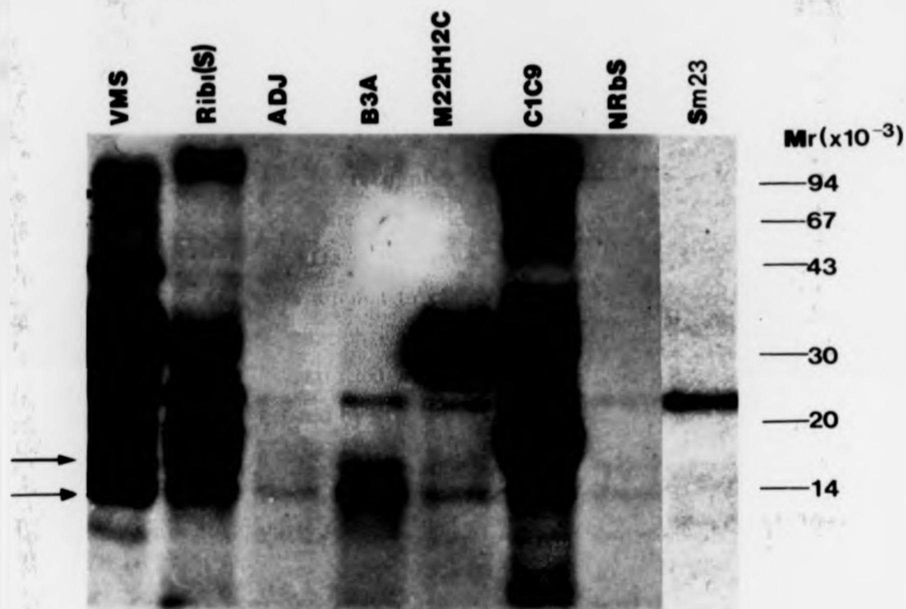
Live schistosomula were incubated with Ribi(S) diluted 1 in 50 and then probed with a fluorescein conjugated secondary antibody.

the species specificity of the 16 kDa antigen strong fluorescence was observed (data not shown). This demonstrated that, although the 16 kDa antigen was the only signal observed following the probing of Western blots with Ribi(S), this sera does recognise other surface exposed antigens which probably represent contaminants present in the affinity purified preparation used for immunisation. It was therefore not possible to conclude from these results whether or not Ribi(S) recognised surface exposed epitopes of the 16 kDa antigen.

#### 5.2.3.6. The surface antigens recognised by Ribi(S)

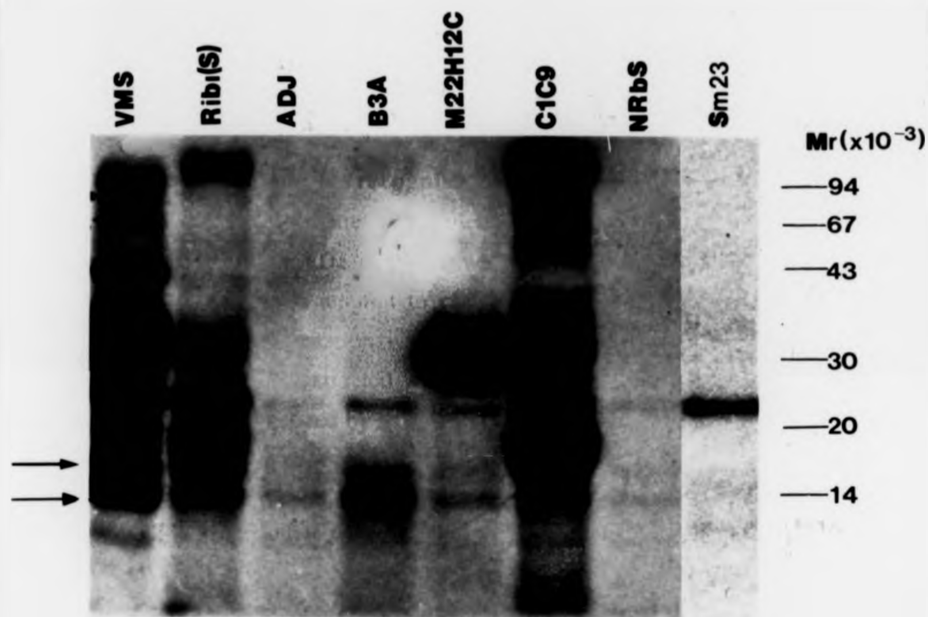
The immunofluorescence studies detailed above suggested that Ribi(S) recognised antigens other than the 16 kDa molecule which were not visible upon the probing of Western blots. Thus, in an attempt to characterise these antigens the more sensitive technique of immunoprecipitation was used. Newly transformed schistosomula were radiolabelled with $^{125}I$ and extracted with TX-100 followed by OTG. Labelled surface antigens were then precipitated by a range of sera including a number of McAbs shown previously to recognise surface antigens. All of the sera gave stronger signals with the antigens extracted with OTG and Figure 5.6 demonstrates that Ribi(S) precipitated a number of OTG extracted antigens of varying molecular weights. Comparison of the pattern of molecules precipitated by Ribi(S) to those precipitated by B3A suggested that the 16 kDa antigen may be amongst those antigens recognised. However, the low molecular weight signals obtained with both Ribi(S) and with B3A were somewhat difficult to distinguish with certainty from the background response. This was not unexpected as previous attempts to radiolabel the 16 kDa antigen have either failed completely or provided similarly confusing data (Dr. Q. Bickle, personal communication). Other antigens precipitated by Ribi(S) included a signal of 18 to 20 kDa and antigens of approximately 23, 32 and 97 kDa. Comparison of the precipitation pattern of Ribi(S) with that of the McAb, M22H12C suggested that the 32 kDa antigen corresponded to that described as the target of this McAb by Bickle *et al.*, (1986). Furthermore, comparison of the antigens precipitated by Ribi(S) with those precipitated by sera raised in rabbits against a recombinant form of the vaccine candidate antigen, Sm23 (a gift from Dr. Q. Bickle) suggested that Ribi(S) also precipitated this surface antigen (Harn *et al.*, 1985b). However, a 23 kDa antigen was also weakly precipitated by a number of the McAbs and by NRabS. This suggested that Sm23 may be a particularly "sticky" molecule with a

**Figure 5.6 Immunoprecipitation of radiolabelled schistosomula surface antigens**




Living schistosomula were surface labelled with $I^{125}$ and solubilised by incubation in 1.5% OTG. The extracted antigens were then precipitated by incubation with a variety of sera followed by Protein A Sepharose and the immunoprecipitates were separated by SDS PAGE. Lane 1 = 1 in 100 dilution of VMS, lane 2 = 1 in 100 dilution of Ribi(S), lane 3 = 1 in 100 dilution of sera taken from mice immunised with the Ribi adjuvant alone, lane 4 = 1 in 20 dilution of B3A, lanes 5 and 6 = 1 in 20 dilutions of the McAbs, M22H12C and C1C9 respectively, lanes 7 = 1 in 200 dilution of NRabS and lane 9 = 1 in 200 dilution of sera raised against a recombinant form of Sm23. Possible positions of the 16 kDa antigen are arrowed.

**Figure 5.6 Immunoprecipitation of radiolabelled schistosomula surface antigens**




Living schistosomula were surface labelled with I[125] and solubilised by incubation in 1.5% OTG. The extracted antigens were then precipitated by incubation with a variety of sera followed by Protein A Sepharose and the immunoprecipitates were separated by SDS PAGE. Lane 1 = 1 in 100 dilution of VMS, lane 2 = 1 in 100 dilution of Ribi(S), lane 3 = 1 in 100 dilution of sera taken from mice immunised with the Ribi adjuvant alone, lane 4 = 1 in 20 dilution of B3A, lanes 5 and 6 = 1 in 20 dilutions of the McAbs, M22H12C and C1C9 respectively, lanes 7 = 1 in 200 dilution of NRabS and lane 9 = 1 in 200 dilution of sera raised against a recombinant form of Sm23. Possible positions of the 16 kDa antigen are arrowed.

degree of affinity for immunoglobulin in general. It is also of interest to note that an antigen of 32 kDa and a signal of 18 - 20 kDa were observed on silver staining of the immunoaffinity column eluates which were used to immunise these mice (section 3.2.2.1). These results therefore demonstrate that these contaminating molecules, although minor signals, were present in sufficient amounts to stimulate an antibody response.

### 5.2.4 IMMUNISATION OF MICE WITH THE 16 kDa ANTIGEN PLUS ALTERNATIVE ADJUVANTS

In addition to enhancing the specific response to immunising antigens, a particular adjuvant may selectively boost certain components of the immune system and hence have a profound effect on the type of immune response generated and the subsequent development of resistance. In a second attempt to generate a protective response by immunising with the partially purified 16 kDa antigen two different adjuvants were therefore used. As immunisation with the antigen plus Ribi failed to provide protection despite stimulating an antibody response of a titre and IgG subclass equal to that seen in mice vaccinated with irradiated cercariae, particular attention was paid to the possibility of presenting the antigen in a way which would stimulate a response against different epitopes. The adjuvant Spikoside which is a derivative of Quil A was therefore used to form ISCOMs into which the antigen could be incorporated. As incorporation into ISCOMs requires interactions between the hydrophobic region of the antigen and the lipid moieties of the ISCOM which are similar to those required for anchoring a surface molecule within the bilayer, it was hoped that this may lead to the exposure of epitopes of the 16 kDa antigen which are normally exposed upon the surface of the intact schistosomula. In addition, the novel adjuvant novasomes, a kind gift from Dr. C. Wright, Novavax, U.S.A. was used. This adjuvant is currently undergoing trials and as yet little is known about the way in which it stimulates an immune response. However, the preparation consists of a detergent (Brij 52) in a squalene emulsion and is designed to produce multimeric micelles incorporating the immunising antigen.

#### 5.2.4.1 Immunisation of mice with the 16 kDa antigen incorporated into ISCOMs

(a) Incorporation of the 16 kDa antigen into ISCOMs

Appropriate amounts of the detergent MEGA 10, Spikoside and lipids were added to the most antigen rich fractions eluted from the immunoaffinity column following

purification of the 16 kDa antigen from a total of 18 million MS. The preparation was then dialysed against PBS to enable the formation of the cage-like ISCOMs to occur. After 48 hours the dialysate became cloudy indicating that ISCOMs had been formed.

(b) Purification of ISCOMs

The ISCOMs were purified by centrifugation of the dialysate through a sucrose gradient. This enabled an opaque band of ISCOMs to be seen at the interface between the two sucrose concentrations. Twenty 200 $\mu$l fractions were then removed from the sucrose gradient and the fraction containing the band and the fractions directly above and below this region were examined for the presence of ISCOMs by electron microscopy. Figure 5.7 demonstrates that the formation of typical cage like ISCOMs had occurred. These were observed in all three of the fractions examined although the greater majority were found in the fraction containing the visible band.

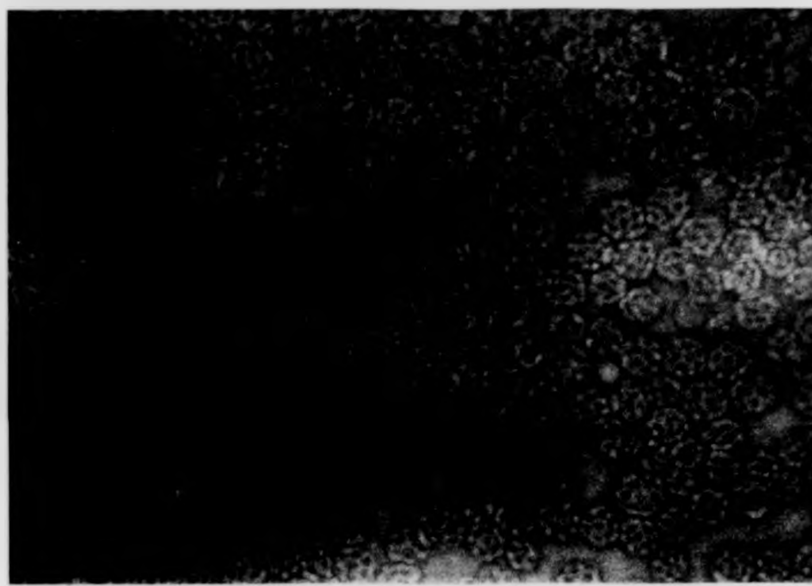
(c) Localisation of the 16 kDa antigen

The 20 fractions removed from the sucrose gradient following centrifugation were separated by SDS PAGE, transferred by Western blotting and probed with VRabS. Although small amounts of the 16 kDa molecule were seen in numerous fractions, the majority of the antigen was concentrated in those fractions containing ISCOMs (data not shown). This suggested that the 16 kDa antigen had been successfully incorporated into the ISCOM structure. However, although the 16 kDa antigen was easily detectable in the fractions containing ISCOMs by probing of Western blots with VRabS, a signal was no longer visible on silver staining of similar gels. Thus, a large proportion of the 16 kDa antigen present in the immunoaffinity column eluates appeared to have been lost during the process of forming ISCOMs. No other antigens were visible in the fractions containing ISCOMs following silver staining or probing of Western blots with VRabS.

(d) Immunisation

In an attempt to compensate for the small amount of antigen present within the ISCOMs a mouse was immunised with the largest feasible volume of the ISCOM containing preparation. However, as a consequence of the toxicity of the Spikoside present in ISCOMs, this resulted in the death of the animal within 24 hours. A group of 7 CBA mice were therefore immunised with 70 $\mu$l of ISCOMs incorporating the 16 kDa

**Figure 5.7** **ISCOMs incorporating the 16 kDa antigen**




Immunoaffinity purified 16 kDa antigen was incubated with detergent, lipid and the Quil A derivative, Spikoside and dialysed for 48 hours against PBS. The ISCOMs formed were then purified by centrifugation through a sucrose gradient and examined using electron microscopy. The above shows ISCOMs containing the 16 kDa antigen following negative staining with ammonium molybdate.

antigen. This dose was tolerated without obvious side effects, moreover, only 5 $\mu$l of an identical preparation gave a strong signal on dot blots probed with VRabS. A group of 5 control mice were immunised with ISCOMs formed without the incorporation of protein. Following a total of five injections, sera raised in mice immunised with ISCOMs containing the 16 kDa antigen were shown to recognise the 16 kDa molecule on Western blots of immunoaffinity purified material. No signal was observed on probing with sera raised in the control animals. All the mice were then challenged, perfused 5 weeks later and individual worm burdens obtained. Table 5.2 demonstrates that the mice immunised with ISCOMs containing the 16 kDa antigen showed a reduction in worm burden of 16.4% on comparison with mice immunised with the empty structure. This difference was not however statistically significant. In view of the smaller size of the control group used, comparisons were then made between the average worm burden obtained for mice immunised with the 16 kDa antigen incorporated into ISCOMs and that of a larger control group which included the worm counts for mice immunised with the novasome adjuvant alone (see below) in addition to those for mice immunised with the empty ISCOM structure. Such comparisons were feasible as these studies were done in conjunction and there was no significant difference between the worm burdens obtained for these two groups of control animals. When compared with the larger control group, mice immunised with the 16 kDa antigen incorporated into ISCOMs had a reduction in worm burden of 18.7% which was statistically significant ($p < 0.025$).

#### 5.2.4.2 Immunisation of mice with the immunoaffinity purified 16 kDa antigen plus novasomes

The most antigen rich eluates obtained following immunoaffinity purification of the 16 kDa antigen from a total of 18 million MS were used in conjunction with novasomes to immunise a group of 7 CBA mice (section 2.20.2). A group of 5 control mice were immunised with the adjuvant alone. Following a total of four immunisations the sera taken from the immunised group was shown to recognise the immunoaffinity purified 16 kDa antigen on Western blots. No signal was seen with sera taken from the control group. The mice were then challenged, perfused and the number of worms counted. The results obtained (Table 5.3) demonstrated that the worm burden in the immunised mice was reduced by 25.3% as compared to that of the novasomes alone control group. This difference was shown to be statistically significant ($p < 0.025$). Further comparisons

**TABLE 5.2** **Reduction in worm burden of mice immunised with ISCOMs incorporating the 16 kDa antigen**

| Number of mice per group | Immunisation protocol | Worm burden (+/- S.D.) | Reduction (%) | Significance |
|---|---|---|---|---|
| 5 | ISCOMs alone | 94.8 +/- 14.3 | - | |
| 7 | ISCOMs incorporating the 16 kDa antigen | 79.2 +/- 17.5 | 16.4 | $p < 0.1$ |

A group of 7 female CBA mice were immunised s.c with ISCOMs incorporating the immunoaffinity purified 16 kDa antigen or with ISCOMs formed without the incorporation of protein. The mice were then challenged with 200 cercariae 2 weeks after the final immunisation and killed and perfused 35 days later. The average worm burden for each group was obtained and resistance calculated as described in Chapter 2.

**TABLE 5.3 Reduction in the worm burden of mice immunised with the immunoaffinity purified 16 kDa antigen plus novasomes**

| Number of mice per group | Immunisation protocol | Worm burden (+/- S D ) | Reduction (%) | Significance |
|---|---|---|---|---|
| 5 | Novasomes alone | 99 4 +/- 12.2 | - | |
| 7 | 16 kDa antigen plus novasomes | 74 3 +/- 20 8 | 25.3 | $p < 0.025$ |

7 female CBA mice were immunised i p with the immunoaffinity purified 16 kDa antigen plus novasomes or with novasomes alone The mice were then challenged with 200 cercariae 9 weeks after the final immunisation and killed and perfused 35 days later Following perfusion the average worm burden was obtained and used to calculate resistance as described in Chapter 2.

were then made between the average worm burden of mice immunised with the 16 kDa antigen plus novasomes and a larger control group which included animals immunised with ISCOMs formed without the incorporation of antigen (as above) in addition to those immunised with novasomes alone. Such comparisons resulted in a smaller reduction in worm burden (23.7%), which was however more statistically significant ie. $p < 0.01$.

### 5.2.5 CHARACTERISATION OF THE ANTIBODY RESPONSE IN MICE IMMUNISED WITH THE 16 kDa ANTIGEN PLUS ALTERNATIVE ADJUVANTS

#### 5.2.5.1 Recognition of the 16 kDa antigen

A pool of sera taken immediately prior to challenge from animals immunised with the 16 kDa antigen plus novasomes or the molecule incorporated into ISCOMs was shown to recognise the antigen on Western blots of immunoaffinity purified material (see Figure 5.9). The doublet of approximately 58 kDa, which is a non-specific signal introduced during SDS PAGE, was also seen on blots probed with sera from immunised and control groups. However, difficulty was experienced in obtaining further information regarding the specificity of the sera obtained from immunised mice, as their titres were such that antigens including that of 16 kDa were not visible on probing Western blots of a crude detergent extract.

#### 5.2.5.2 Recognition of the 16 kDa antigen by sera taken from individual mice

Tables 5.2 and 5.3 give the average worm burdens for the groups of animals immunised with the 16 kDa antigen incorporated into ISCOMs or the antigen plus novasomes respectively. However, closer examination of the results obtained revealed that a number of individuals within each group had worm burdens which differed substantially from the average. Thus, sera taken from individual mice immunised with the 16 kDa antigen incorporated into ISCOMs or with the antigen in conjunction with novasomes, were used to probe Western blots of the immunoaffinity purified molecule. Only four of the mice immunised with ISCOMs gave a detectable response against the 16 kDa molecule. In all cases this was not as strong as that observed in mice immunised with the antigen plus novasomes. Furthermore, comparison of the blots with the protection data demonstrated that the four responders had three of the highest worm burdens. Thus, the development of an anti-16 kDa antibody response following immunisation with ISCOMs

did not correlate with a low worm burden. In contrast, sera taken from all of the mice immunised with the 16 kDa antigen plus novasomes recognised the immunoaffinity purified molecule. However, differences in the strength of the signals obtained were observed and comparison of the blots with the protection data demonstrated that in general mice with the stronger responses were amongst those with the lower worm burdens. Thus, the two highest worm burdens were seen in two of the three mice showing a minimal response. However, the third of these mice did have the lowest worm burden.

Pooled sera from mice immunised with the 16 kDa antigen incorporated into ISCOMs (ISC(S)) or the antigen in conjunction with novasomes (NOV(S)) were then used in further characterisation studies.

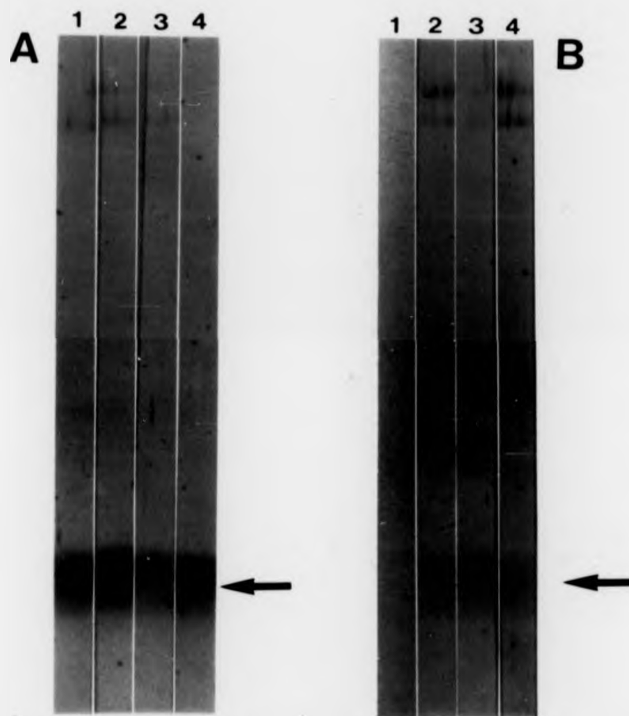
#### 5.2.5.3 Antibody titre

Western blots of immunoaffinity purified 16 kDa antigen were probed with increasing dilutions of NOV(S) or ISC(S). The results obtained demonstrated that a signal could not be observed with ISC(S) beyond a dilution of only 1 in 10 whereas NOV(S) had a titre of greater than 1 in 500 (data not shown).

#### 5.2.5.4 Antibody subclass

Western blots of immunoaffinity purified 16 kDa antigen were probed with a 1 in 10 dilution of ISC(S) or a 1 in 50 dilution of NOV(S) and developed with IgG subclass specific conjugates. The results shown in Figure 5.8 demonstrate that the subclasses present in both sera covered a broader range than those within VMS or Ribi(S). With regard to NOV(S) (Figure 5.8(A)), the major subclass appeared to be IgG2a, although the signal with this conjugate was only marginally stronger than that obtained on probing with anti-IgG1 antibodies. A reasonable signal was also observed with the anti IgG3 conjugate and a weaker one with anti-Ig2b. The poor titre of ISC(S) resulted in very weak signals being obtained on probing with the IgG subclass specific conjugates (Figure 5.8(B)). However, signals easily discernible on the original blot were present following probing with the IgG2a, IgG2b and IgG3 specific conjugates, that obtained with the anti-IgG2a conjugate being marginally the strongest. No signal was visible on probing with anti-IgG1.

**Figure 5.8** **The subclass of anti-16 kDa antibodies within NOV(S) and ISC(S)**




Strips of a Western blot of immunoaffinity purified 16 kDa antigen were incubated overnight with a 1 in 50 dilution of sera raised in mice immunised with the 16 kDa antigen plus novasomes (panel A) or a 1 in 10 dilution of sera raised in mice immunised with ISCOMs incorporating the 16 kDa antigen (panel B). The strips were then probed with IgG subclass specific conjugates. Lane 1 = IgG1, lane 2 = IgG2a, lane 3 = IgG2b and lane 4 = IgG3. The position of the 16 kDa antigen is marked with an arrow.

#### 5.2.5.5 The nature of the epitopes recognised

(a) Treatment with sodium meta periodate

Western blots of immunoaffinity purified material were treated with sodium meta periodate for 1 hour or for 4 hours and probed with a 1 in 10 dilution of ISC(S) or a 1 in 50 dilution of NOV(S). Figure 5.9(A) demonstrates that the recognition of the 16 kDa antigen by ISC(S) was abolished following treatment with sodium meta periodate for 1 hour. This suggests that only carbohydrate epitopes are recognised by this sera. In contrast, the recognition of the 16 kDa antigen by NOV(S) was not noticeably affected by periodate treatment for 1 hour (Figure 5.9(B)) nor for 4 hours (data not shown). This suggests that peptide epitopes are preferentially recognised following immunisation with the 16 kDa antigen plus the novasome adjuvant.

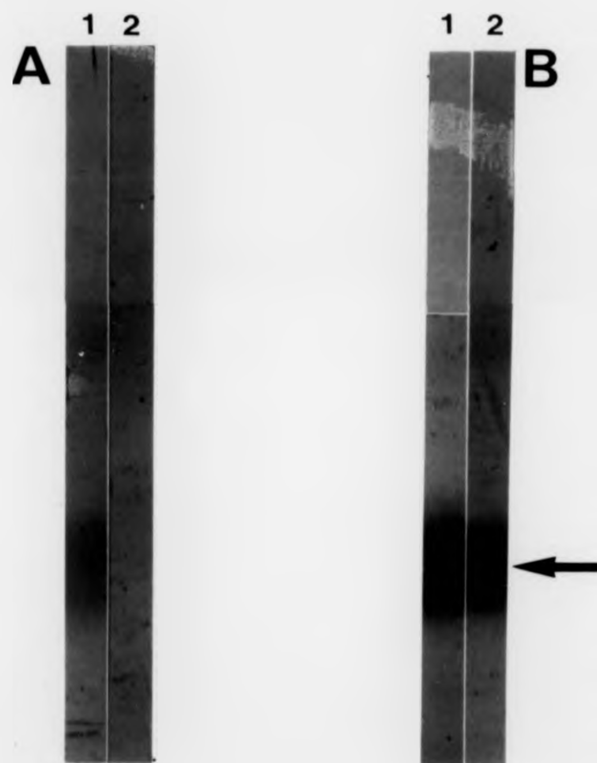
(b) Recognition of surface epitopes

Freshly transformed schistosomula were incubated in a 1 in 10 dilution of ISC(S) or a 1 in 50 dilution of NOV(S) and probed with a fluorescein labelled anti-mouse conjugate. No fluorescence was seen following probing with ISC(S). However, probing with sera from mice immunised with the 16 kDa antigen plus novasomes gave fluorescence which was of an equal intensity to that observed following probing with B3A (data not shown). Unlike B3A, NOV(S) bound to both the heads and tails of MS thus raising the possibility that this sera recognises molecules in addition to the 16 kDa antigen, which are not detected following the probing of Western blots (see section 5.2.3.5). It was therefore impossible to deduce whether NOV(S) recognises epitopes of the 16 kDa antigen which are exposed upon the surface of the schistosomula. However, following immunofluorescence the heads of the parasites were more intensely labelled than the tails. This suggested that some antigens exposed upon the surface of the former but not the latter were recognised.

## 5.3 DISCUSSION

It has frequently been demonstrated that an adjuvant has the ability not only to augment an immune response but also to influence its protective capacity by enhancing particular features (Audibert and Lise, 1993). Thus, to increase the likelihood of obtaining a protective response following immunisation of mice with the partially purified 16 kDa antigen, a variety of adjuvants were used. Initially, the use of FCA, a water in mineral

**Figure 5.9** **The recognition of periodate treated 16 kDa antigen by ISC(S) and NOV(S)**




Strips were cut from a Western blot of immunoaffinity purified 16 kDa antigen and treated for 1 hour with 20 mM sodium meta periodate in 50 mM sodium acetate buffer (pH 4.5) (lane 2), or with the acidic buffer alone (lane 1). The strips were then probed with ISC(S) (panel A) or NOV(S) (panel B) at a dilution of 1 in 10 and 1 in 50 respectively. The position of the 16 kDa antigen is marked with an arrow.

oil emulsion which incorporates heat killed mycobacteria in the mineral oil phase and the antigen for immunisation in aqueous phase droplets, was considered. However, although FCA is one of the most powerful adjuvants known, it is also highly toxic and is not licensed for use in either human or veterinary vaccines. Moreover, although it is licensed for use in experimental animals, chronic inflammation, ulceration at the site of injection and more long term auto-immune complications frequently occur. It was therefore decided that FCA was not suitable for use in experiments to determine the protective potential of the immunoaffinity purified 16 kDa antigen, as the small amounts of material available made it infeasible to immunise large groups of mice and hence essential that animals were not lost due to the ill effects of adjuvant during the course of the experiment. Furthermore, immunisation with FCA results in the production of a large amount of non-specific antibody which may have proved a complication if the sera raised was subsequently used for screening libraries.

As an alternative to FCA, Ribi adjuvant was chosen. Ribi incorporates two immunogenic components, monophosphoryl lipid A (MPL) and trehalose dicorynomycolate (TDM), which are suspended in an oil in water emulsion of 2% squalene plus 0.2% Tween 80. MPL is a non toxic form of lipid A, the biologically active, immunogenic moiety of the endotoxin lipopolysaccharide (LPS), which is a component of mycobacterial cell walls. MPL is formed by acid hydrolysis of diphosphoryl lipid A (DPL) and although it is 1000 times less potent than DPL in eliciting toxic and pyrogenic responses, it retains the adjuvanticity of DPL and of LPS as a whole (Ribi *et al.*, 1986). MPL has been shown to stimulate good cell mediated immunity and a specific antibody response equal to that obtained on immunisation with FCA, even when used with as little as 25 ng of antigen (Rudbach *et al.*, 1988, Hui *et al.*, 1994). Its immunogenicity is believed to be largely derived from its ability to incorporate rapidly into macrophages in the spleen and liver and to induce a state of activation which augments the ability of these cells to phagocytose and enhances their properties as antigen presenting cells (APCs) by increasing the expression of MHC class II (Alving, 1993). Cytokines released by these activated macrophages also stimulate the influx and subsequent activation of more macrophages and other effector cells. MPL also has the ability to enhance antibody production via the direct stimulation of Th cells, (Audibert and Lise, 1993) and to act as a T cell independent polyclonal B cell mitogen. Moreover, there is some evidence that MPL is able to inhibit the activity of suppressor T cells (Gupta *et al.*, 1993).

TDM was originally purified from the waxy "cord factor" of the tubercle bacillus by Asselineau and Lederer (1949). The form used in Ribi is however, a synthetic product which retains the adjuvanticity of the original TDM whilst lacking its toxicity. The incorporation of TDM in addition to MPL is believed to increase the adjuvanticity of the Ribi formulation as the two substances are thought to act synergistically. TDM binds the immunising antigen to the surface of the squalene oil droplets which effects the way in which the antigen is presented by APCs. This together with the cytokine profile stimulated by MPL is believed to influence the activation of different T cell subsets and hence the production of particular types of antibody (see below). As yet the complete Ribi formulation has not been licensed for human use although both major components of this adjuvant do have minimal toxicity and trials with MPL in humans have demonstrated that although some side effects were observed these were tolerable (Takada and Kotani, 1992).

Despite the generation of an anti-16 kDa antibody response which had a titre and characteristics similar to those of VMS (see below) no reduction in the worm burden of mice immunised with the immunoaffinity purified antigen plus Ribi adjuvant was seen. One explanation for this is that the antigen was presented in such a way as to stimulate a response against epitopes which were inappropriate as targets of a protective immune response. It was therefore decided to test other adjuvants likely to present the 16 kDa antigen in a different and perhaps more appropriate way. ISCOMs were chosen for use with the 16 kDa antigen as they are particularly suited to the presentation of membrane molecules. The Spikoside adjuvant used in their formation is a mixture of naturally occurring saponins, which are surface active glycosides extracted from the bark of the South American soaptree *Quillaja saponaria* (Morein *et al.*, 1984). Spikoside has similar composition to the adjuvant Quil A although it has been specially selected for its ability to form ISCOMs. At its CMC (0.03%) Spikoside combines via hydrophobic interactions with cholesterol to form mixed micelles into which molecules with a hydrophobic domain can be incorporated. This results in the presentation of the molecule in an accessible, multimeric form. Immunisation with various viral glycoproteins incorporated into ISCOMs has been shown to stimulate the production of antibodies against epitopes not seen following immunisation with the antigen and other adjuvants (Morein *et al.*, 1984, De Vries *et al.*, 1988). Furthermore, immunisation with ISCOMs incorporating one of over 20 different membrane proteins have been shown to generate both humoral and cell mediated immunity which is long lasting and in many cases protective (Lovgren *et al.*,

1990, Ertuck *et al.*, 1991, Mumford *et al.*, 1994, Kazanji *et al.*, 1994).

The enhanced immunogenicity of the ISCOM structure is believed to be based upon the ability of saponin to bind to the cholesterol of leucocyte membranes and hence enable the ISCOMs to interact with a number of different cell populations (Bomford, 1980). Examination of the fate of radiolabelled glycoproteins incorporated into ISCOMs (reviewed by Morein *et al.*, 1990a) has demonstrated that they are rapidly and efficiently taken up by macrophages and transported from the site of injection into the lymphoid organs, in particular the spleen (Claassen and Osterhaus, 1992). Here the adjuvant activated macrophages phagocytose and serve as antigen presenting cells (APCs) for T cells. The latter results in the proliferation of T cells, the provision of B cell help and the generation of a DTH type response via the production of cytokines, in particular IL-2 and IFNγ (Heath *et al.*, 1991). Neutrophils may also have an important role to play in the adjuvanticity of saponins. The number of PMNs in the peritoneal lavage has been shown to increase by up to 80% following immunisation with an antigen incorporated into ISCOMs (Watson *et al.*, 1989) and the production of neutrophil proteases capable of stimulating B cells has also been described. ISCOMs are also taken up directly by B cells which then form particularly efficient APCs for the presentation of antigen to specific T cells (Morein *et al.*, 1990a). Finally, and of particular importance with regard to the success of anti-viral ISCOM vaccines, antigens incorporated into ISCOMs have been shown to enter the intracellular pathway of antigen processing and hence are able to stimulate Tc cells when presented in conjunction with MHC Class I (Morein *et al.*, 1987, Takahashi *et al.*, 1990).

ISCOMs were prepared here by extensive dialysis of the immunoaffinity purified 16 kDa antigen, MEGA-10, Spikoside, cholesterol and phosphatidlycholine. They were purified on a sucrose gradient and shown by EM to be of the correct size (approximately 35 nm in diameter) and to possess the characteristic loose translucent cage-like ISCOM structure which consists of ring-like subunits each with a diameter of approximately 12 nm. Western blotting of the sucrose gradient fractions then demonstrated that the 16 kDa antigen was present in those fractions containing the ISCOMs. This was taken as indicative of the incorporation of the 16 kDa antigen into the ISCOM itself, as a molecule of 16 kDa would not have been present at this position in the sucrose gradient had it retained its monomeric form. Moreover, large non-ISCOM micellar aggregates of the 16 kDa antigen would have been discernible on carrying out electron microscopy. However,

although some 16 kDa antigen was successfully incorporated into ISCOMs, silver staining of the fractions containing the purified structures demonstrated that a significant proportion of the molecule had not been incorporated. Such a phenomenon is not an unusual occurrence and although the incorporation of as much as 80% of a purified viral glycoprotein into ISCOMs has been described (Akerblom *et al.*, 1989), the incorporation of only 33 to 64% of a palmitified sporozoite protein (Kazanji *et al.*, 1994) and only 15% of a purified HIV envelope glycoprotein (Pyle *et al.*, 1989) has also been reported. Furthermore, it has been shown that the amount of antigen which incorporates into ISCOMs depends not only upon the successful application of the procedure used in the formation of the ISCOMs but also upon more specific characteristics of the antigen itself. For example, the studies of Mougin *et al.*, (1988) have demonstrated that ISCOMs formed by the dialysis of Quil A and a mixture of hydrophobic surface glycoproteins incorporated a substantially greater percentage of one antigen than another. Moreover, the work of Kazanji *et al.*, (1994) demonstrated that the proportion of a molecule which incorporated into ISCOMs correlated positively with the amount available in the starting material. Thus, the incorporation of a small percentage of the 16 kDa antigen into ISCOMs may be a consequence of the small amounts of antigen available within the immunoaffinity column eluates coupled with particular but as yet unspecified features of the molecule itself. Fortunately, one of the main advantages of the ISCOM adjuvant system is that very small amounts of antigen (0.1 $\mu$g) incorporated into ISCOMs have been shown to generate a good immune response (Morein *et al.*, 1984, 1990b). Here, the glycosylated nature of the 16 kDa molecule made it impossible to use conventional protein estimation techniques to determine precisely the amount of antigen present in the ISCOM preparation. However, the probing of dot blots with VRabS did demonstrate that a strong signal was obtained with as little as 5 $\mu$l of the preparation and it was therefore decided to continue with the immunisations as planned. Initial immunisations demonstrated that a dose per mouse of 275 $\mu$l proved fatal, the mouse dying within 24 hours of inoculation. This was presumed to be due to the toxicity of the Spikoside adjuvant which although partially purified still contains a mixture of saponins, some of which are known to be haemolytic (Kensil *et al.*, 1991). When incorporated into ISCOMs the toxicity of saponin is reduced to a tenth of that of free molecules and this has led to ISCOMs being used regularly for the immunisation of animals, in addition to allowing their consideration for human use. However, toxicity in mice has been seen by other workers (Dr. R. Jennings,

University of Sheffield, personal communication) and it seems feasible that the large dose of ISCOMs used here contained enough saponin to cause toxicity even when incorporated into ISCOMs. Moreover, when considering the large number of ISCOMs and the small amounts of antigen which were present in the immunising preparation it is likely that a significant proportion of the ISCOMs present did not contain the 16 kDa molecule. Such "empty" ISCOMs have been shown to incorporate more saponin than those incorporating an antigen (Morein *et al.*, 1990a) and hence may have increased toxicity. This suggestion is substantiated by the observation made here that although a 70 $\mu$l dose of the ISCOM preparation containing the 16 kDa antigen was shown to be non-toxic, a similar dose of "empty" ISCOMs was fatal to the control animals and half of this amount was subsequently used. Following five immunisations, sera raised in the antigen immunised mice was shown to recognise the 16 kDa molecule by Western blotting and all of mice were then challenged. A 16.4% reduction in the worm burden of the immunised mice was observed, although this was not statistically significant.

Novasomes are a novel adjuvant which consist of the detergent Brij 52 in a squalene emulsion. As yet little is known about the properties of this adjuvant, however it was shown here that immunisation with the 16 kDa antigen plus novasomes induced an antibody response against the 16 kDa antigen and the best, albeit still low, level of protection (25.3%) which was statistically significant ($p < 0.025$).

In addition to examining the protection data obtained following immunisation with the 16 kDa antigen plus different adjuvant systems, the sera raised in each case were characterised according to titre, subclass and the nature of the epitopes recognised. These characteristics were then compared with those of VMS and attempts are made here to relate the different features of the antibody responses generated to the varying levels of protection obtained. Comparisons of this kind are obviously somewhat limited in that the T cell response which has been shown to be of importance in the mouse model is not examined. Furthermore, the experiments carried out here have explored only the nature of the IgG response to the 16 kDa antigen. However, passive transfer studies employing VMS (Mangold and Dean, 1986, section 1.4.3.2) and protective McAbs (section 1.7) have demonstrated the ability of an appropriate antibody response to confer immunity and indicate the importance of the IgG antibody isotype.

With regard to the titre of the IgG antibody response obtained; both VMS and Ribi(S) had a titre of 1 in 5,000 as judged by the recognition of the 16 kDa antigen on Western

blots of an OTG extract of MS. In contrast, the recognition of a 16 kDa signal with NOV(S) or ISC(S) required Western blots of the immunoaffinity purified antigen to be probed. The titre of NOV(S) was then reasonable (1 in 500), however ISC(S) was still very weak with a titre of only 1 in 10. Although the generation of an antibody response has been observed following immunisation with as little as 0.1 $\mu$g of BSA incorporated into ISCOMs (Morein *et al.*, 1990b), a single dose of 5 - 10 $\mu$g of antigen in ISCOMs is more usually quoted as suitable for the generation of cell mediated immunity and the induction of an antibody response may require a larger dose in addition to more frequent immunisations. It therefore seems possible that the small amounts of 16 kDa antigen which were incorporated into ISCOMs could account for the poor titre of ISC(S). Despite inducing lower titres of anti-16 kDa antibodies, the immunisation of mice with the 16 kDa molecule incorporated into ISCOMs or with the antigen plus novasomes stimulated better levels of protection than those obtained following immunisation with the antigen in conjunction with Ribi. These results therefore demonstrate that as expected, the development of resistance was not dependent upon antibody titre alone.

Further analysis of the characteristics of VMS revealed that the antibodies recognising the 16 kDa antigen were restricted to the IgG2a subclass. This indicated that the nature of the antibody response generated against the 16 kDa antigen was not typical of the overall character of VMS which has been shown by ELISA and immunofluorescence to consist largely of IgG1 with smaller amounts of IgG2a, IgG2b and IgG3 (Caulada-Benedetti *et al.*, 1991, Delgado and McLaren, 1990). The studies of Delgado and Mclaren (1990) which demonstrated that the IgG1 fraction of VMS was the only isotype capable of transferring resistance to naive animals also suggest that the antibody response against the 16 kDa antigen may be unimportant to the development of vaccine immunity. However, the studies of Richter *et al.*, (1993) have demonstrated that the isotype response of mice vaccinated with radiation attenuated parasites to a variety of candidate vaccine antigens does differ and can depend upon the number of immunisations given, the strain of mouse vaccinated and the dose of radiation used for attenuation. For example, although HSP70 is recognised predominantly by IgG1, GST is seen predominantly by IgM and the vaccine candidate molecule TPI by IgG2a. Thus, it is possible that the 16 kDa molecule represents an antigen which tends to stimulate a Th1 type response and hence the production of specific IgG2a, within the context of the predominantly Th2 / IgG1 type response which is characteristic of mice multiply vaccinated with irradiated cercariae.

Immunisation with the purified 16 kDa antigen plus Ribi adjuvant also generated a restricted antibody profile. IgG2a was the only subclass produced in significant amounts although a very much weaker signal was seen with the IgG2b specific conjugate. Again this observation contrasted with published reports which describe the production of the three major IgG subclasses of mice, namely IgG1, IgG2a and IgG2b in response to immunisation with a number of antigens plus Ribi adjuvant, MPL and TDM, or MPL alone (Takayama *et al.*, 1991, Kenney *et al.*, 1989). However, the predominant subclass seen in these latter studies was also seen to differ. Thus, Takayama *et al.*, (1991) demonstrated that IgG2a was the major subclass produced on immunisation, whereas the studies of Kenney *et al.*, (1989) reported that the IgG1 response was predominant following the immunisation of mice with human serum albumin. It has been suggested that the mouse strain used for vaccination may affect the results obtained and naive C57Bl/10 mice, the strain used for immunisation with the 16 kDa antigen plus Ribi, do produce measurably more IgG2b and IgG2a than IgG1 (Satsuume-Sakai, *et al.*, 1977). However, it appears unlikely that the complete lack of IgG1 production seen in the experiments carried out here could be accounted for in this way.

Despite the low titre of the sera raised in mice immunised with the 16 kDa antigen incorporated into ISCOMs, signals were observed with the anti-IgG2a, IgG2b and IgG3 specific conjugates, the signal with the former being slightly stronger than that with the latter two. The broad range of subclasses was as expected following vaccination with an antigen incorporated into ISCOMs as immunisation with influenza ISCOMs has been shown to stimulate the production of significant amounts of IgG1, marginally less IgG2a and small amounts of IgG2b and IgG3 (Ben Ahmeida *et al.*, 1992). Other experiments employing the purified saponin QS-21 have provided similar results although a reversal in the proportions of IgG2a and IgG1 was seen and the suggestion made that the variation in the results observed was a consequence of the presence of a variety of saponins with slightly different immunogenic qualities within different preparations of Quil A (Campbell and Peerbaye, 1992). However, although ISC(S) contained IgG2b and IgG3 in addition to IgG2a, the results obtained here demonstrated that, again, no IgG1 was raised in response to the 16 kDa antigen.

As yet very little information regarding the nature of the antibody response generated against other antigens used in conjunction with novasomes is available. However, the results obtained here which demonstrates the production of IgG1 on immunisation with

the 16 kDa antigen plus novasomes, suggests that the novasome adjuvant is capable of stimulating either a different type of response to those epitopes recognised by VMS, Ribi(S) and ISC(S), or that different epitopes are recognised following the presentation of the antigen in this way.

The nature of the epitopes recognised by VMS and by sera generated in animals immunised with the 16 kDa molecule plus different adjuvants was also examined. The results with VMS were somewhat surprising in that the binding of the sera to the 16 kDa antigen was completely abolished by treatment with sodium meta periodate. This suggests that predominantly carbohydrate epitopes were recognised and contrasts with the recognition of the 16 kDa antigen by VRabS and with the previously reported properties of VMS as a whole. With regard to the latter, the studies of Omer Ali *et al.*, (1986) demonstrated that although the binding of CIS to the surface of the schistosomula was reduced by 90% upon treatment of the parasite with sodium meta periodate, the binding of VMS was reduced by only 10%. Further studies (Omer Ali *et al.*, 1988) then indicated that this was a consequence of the binding of CIS to abundant non-species specific carbohydrate epitopes which cross react with those upon the egg whereas the recognition of the larvae by VMS appeared to be due predominantly to the recognition of species specific peptide epitopes some of which cross react with the adult worm. However, in addition to the work carried out here, more recent studies (Richter *et al.*, 1993) have shown that antibodies against carbohydrate epitopes are present within VMS. Moreover, the 16 kDa antigen has been shown to be a membrane glycoprotein with carbohydrate epitopes (see Chapter 4) and as the carbohydrate portion of glycoproteins are often exposed at the surface of an organism it seems feasible that antibodies against such epitopes may dominate the response to this particular molecule. The periodate sensitive nature of the signal recognised by Ribi(S) and ISC(S) confirms the dominance of immunogenic carbohydrate moieties within the 16 kDa antigen and as the molecule was incorporated into ISCOMs with the express purpose of presenting the molecule in a manner similar to that seen in the intact parasite, the latter also substantiates the above suggestion that carbohydrate epitopes of the 16 kDa antigen may normally be exposed upon the surface of the parasite. However, immunisation with the 16 kDa antigen plus novasomes again produced unique results in that there was no apparent reduction in the binding of NOV(S) to the 16 kDa antigen upon Western blots following periodate treatment of the blot for 4 hours. This indicates that NOV(S) recognises predominantly

peptide epitopes and coincides with the presence of IgG1 within this sera as this subclass is known to be raised predominantly against T cell dependent peptide epitopes (in Mazza *et al.*, 1990). In addition, the recognition of peptide epitopes by NOV(S) suggests that the administration of the 16 kDa antigen in conjunction with novasomes results in the presentation of different epitopes rather than the generation of an IgG1 response to those epitopes also seen by VMS, Ribi(S) and ISC(S).

Immunofluorescence studies employing the McAb, B3A have demonstrated that the 16 kDa antigen is available for antibody binding upon the surface of living schistosomula. However, none of the sera raised in response to immunisation with the immunoaffinity purified molecule could be demonstrated unequivocally to bind to surface exposed epitopes of the 16 kDa molecule. With regard to Ribi(S) and NOV (S), fluorescence equal to that seen with B3A was observed on probing live schistosomula. However, with Ribi(S) the situation was shown to be complicated by the recognition of several surface antigens in addition to that of 16 kDa. The possibility that NOV(S) also recognises contaminating antigens is substantiated by the binding of this sera to cercarial tails in addition to heads. In contrast, ISC(S) failed to bind to the living schistosomula, a feature which could be explained by the particularly low titre of this sera.

Thus, sera raised in mice immunised with the 16 kDa antigen plus different adjuvants show some variation with regard to titre, major IgG subclass and the nature of the epitopes recognised. How do these differences relate to the levels of resistance seen? The lack of a relationship between resistance and the levels of antibodies raised against the 16 kDa antigen in individual mice immunised with ISCOMs incorporating the 16 kDa molecule suggests that factors other than the anti-16 kDa IgG response were responsible for the reduced worm burdens seen in this model. However, similar comparisons made between the antibody titres and worm burdens of mice immunised with the 16 kDa antigen plus novasomes, do tentatively suggest that the antibody response stimulated in these animals may be of importance to the levels of protection observed. Hence these results are of particular interest, as in addition to providing the highest levels of protection, immunisation with the 16 kDa molecule plus novasomes stimulated an antibody response which was unique with regard to the recognition of peptide epitopes and the presence of IgG1. This could suggest that either or both of these two factors are necessary for the development of protective antibody mediated immunity. However, this is not substantiated by the results obtained upon characterisation of VMS nor by the

recognition of a periodate sensitive epitope by the passively protective IgG3 McAb, B3A.

Thus, in summary, immunisation of mice with the 16 kDa molecule plus novasomes or with the antigen incorporated into ISCOMs, produced low levels of protection which in the case of the former were statistically significant and related to the development of a suitable antibody response. The latter indicates that higher levels of resistance may be achieved as a consequence of higher antibody titres and suggests that varying the vaccination protocol and / or increasing the immunising dose may stimulate better levels of protection. However, in view of the difficulties involved in producing significant amounts of purified antigen from MS, an alternative source of 16 kDa antigen would be required if further immunisation studies were to be performed. In this regard, attempts have been made to identify a recombinant clone encoding the 16 kDa antigen (see Chapter 6).

# CHAPTER 6

# SCREENING OF A SPOROCYST cDNA EXPRESSION LIBRARY WITH ANTIBODY PROBES

## 6.1 INTRODUCTION

Experiments detailed in Chapter 4 have demonstrated that obtaining sequence data for the 16 kDa antigen by gas phase N-terminal amino acid sequencing is infeasible. The peptide moiety of the antigen appears to be N-terminally blocked and the small amount of antigen available makes trypsin digestion and subsequent sequencing of any resulting peptides impractical. This chapter therefore describes attempts to obtain sequence data for this antigen using the alternative approach of identifying a recombinant clone encoding the molecule within a cDNA expression library. Identification of such a clone could also enable substantial amounts of recombinant protein to be produced for immunisation, thus abolishing the need for large amounts of parasite material and the use of time consuming purification techniques.

Clones encoding many schistosome proteins have previously been identified by screening adult cDNA expression libraries. These include some of the most promising vaccine candidate molecules such as GST (Balloul *et al.*, 1987b), paramyosin (Lanar *et al.*, 1986), TPI (Shoemaker *et al.*, 1992) and schistosome myosin (Soisson *et al.*, 1992). However, much evidence suggests that the 16 kDa antigen is a larval specific molecule which is not expressed in the later stages of the parasite, thus here, a sporocyst cDNA expression library constructed in λgt11 (Francis and Bickle, 1992) was screened. That the 16 kDa antigen is present within the sporocyst stage has already been demonstrated by the probing of Western blots of an SDS extract of sporocysts with the McAb, B3A (Dr. Q. Bickle, personal communication).

If a clone is to be isolated from a prokaryotic library using immunoscreening, it is necessary that the molecule has some peptide epitopes which are recognised by the antibody probe to be used. Furthermore, the presence of antigenic regions of a peptide nature are vital if a corresponding recombinant protein, produced in *E. coli* and hence not glycosylated, is to be of value for immunisation. The recognition of periodate sensitive epitopes by a variety of sera raised against the 16 kDa antigen has indicated the immunodominance of carbohydrate epitopes present within this molecule. However, the continued recognition of the periodate treated molecule by VRabS suggests that potentially clonable peptide epitopes are also present and identifiable by immunoscreening (see Chapter 4).

The following chapter describes attempts to produce sera specific for peptide epitopes of the 16 kDa antigen and details its use in screening the sporocyst cDNA expression

library. Also described are experiments aimed at characterising the native antigens encoded by clones isolated using "antibody select" methods, sequencing and expression of the encoded DNA as recombinant proteins.

## 6.2 RESULTS

### 6.2.1 PRODUCTION OF ANTISERA FOR SCREENING OF THE cDNA LIBRARY

In order to identify clones encoding the 16 kDa antigen within the sporocyst cDNA expression library it was necessary to have a specific antibody probe. The McAb, B3A is unsuitable for this purpose as it recognises a carbohydrate epitope upon the 16 kDa antigen. Similarly, periodate sensitive epitopes are recognised by sera raised in mice immunised with the immunoaffinity purified 16 kDa antigen plus Ribi adjuvant or with the antigen incorporated into ISCOMs (see Chapter 5). In contrast, sera raised in mice immunised with the 16 kDa antigen plus novasomes did recognise some periodate insensitive epitopes. However, the use of these sera for screening libraries is impractical due to its low titre and the small amounts available. Furthermore, the recognition of antigens in addition to that of 16 kDa by sera taken from mice immunised with the immunoaffinity purified antigen plus Ribi suggests that any sera raised against this partially purified preparation would not be monospecific. Alternative methods were therefore used in an attempt to produce a monospecific antibody reagent which recognises peptide epitopes of the 16 kDa antigen.

#### 6.2.1.1 Immunisation of a rabbit with purified 16 kDa antigen

A detergent extract of 10 million MS was passed down an immunoaffinity column and the column washed and eluted as described previously. In an attempt to increase the purity of the 16 kDa molecule prior to immunisation the whole of the most antigen rich fraction was separated by SDS PAGE and the band corresponding to the 16 kDa antigen identified by Coomassie blue staining. The region of the gel containing this band was then excised, snap frozen and ground into a fine powder. The powdered gel was prepared for immunisation by resuspension in saline and the addition of Ribi adjuvant.

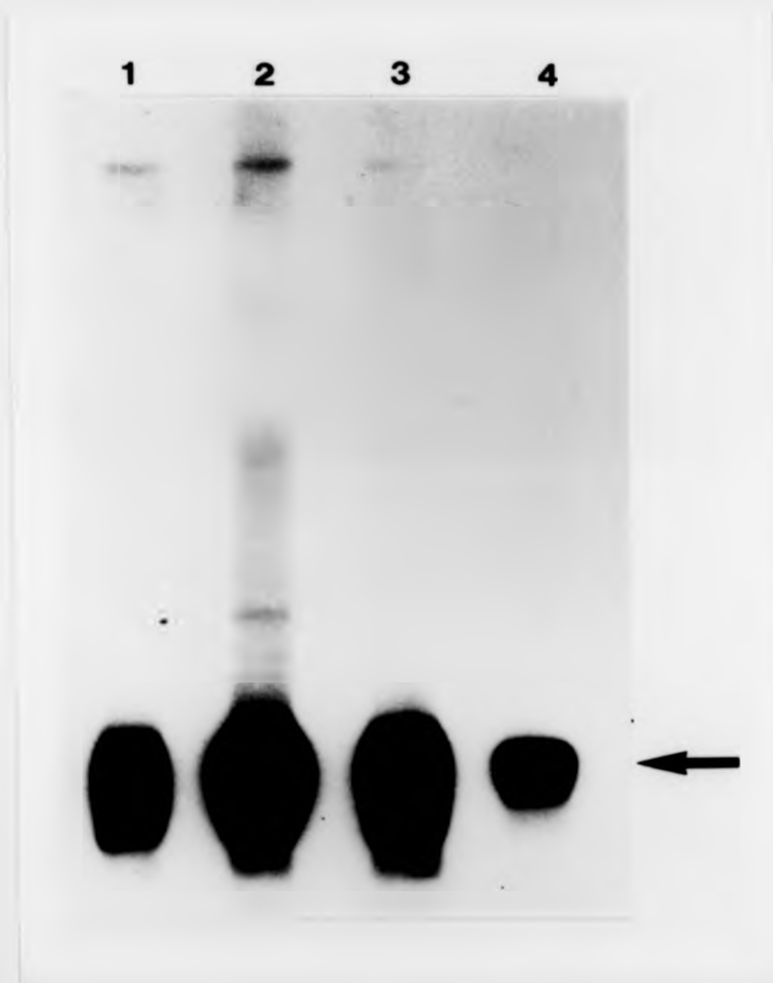
Previous experiments involving subcutaneous immunisation of rabbits with immunoaffinity purified 16 kDa antigen plus CFA have resulted in the generation of sera with a low anti-16 kDa antibody titre (Dr. Q. Bickle, personal communication). Thus,

in an attempt to obtain sera with a titre suitable for use in screening, the 16 kDa antigen prepared as above was injected directly into the popliteal lymph node of a rabbit. This route of immunisation has been shown to be particularly efficient at generating high titre antibody responses with small amounts of antigen (Dr. T Bianco, personal communication). A total of three immunisations were given, one in each of the popliteal lymph nodes and one in multiple sites. Following the third immunisation the animal had to be destroyed due to paralysis of its hind legs. Serum taken at or before the onset of paralysis did not recognise the 16 kDa antigen on Western blots and was therefore not of use for the screening of the cDNA library.

#### 6.2.1.2 Elution of antibodies from the 16 kDa region of sodium meta periodate treated Western blots

A second approach to obtaining antibody suitable for screening the cDNA expression library was based on the elution of antibody from Western blots as described by Beall and Mitchell (1986). A TX-114 insoluble pellet of MS was boiled in SDS and the extracted molecules subjected to SDS PAGE and Western blotting. Regions of the blots corresponding to the 16 kDa antigen were then identified by probing strips cut from either end with B3A and the appropriate region of the nitrocellulose paper was excised. In order to reduce the binding of antibodies specific for carbohydrate epitopes, the strips were then treated with sodium meta periodate for 4 hours at room temperature in the dark. This procedure has already been shown to destroy the binding of the McAb to the 16 kDa antigen (section 4.2.3.1). Following periodate treatment the blots were incubated overnight with VRabS, washed extensively and the antibody binding to the strips eluted by the application of a low pH buffer. The eluted antibodies (El$\alpha$16) were then used to probe Western blots of SDS extracted antigens. Figure 6.1 demonstrates that the 16 kDa antigen was the only major antigen recognised by El$\alpha$16, although weak signals were also seen at approximately 20 kDa and $<$ 100 kDa. El$\alpha$16 was then shown to have a titre of greater than 1 in 100 by Western blotting. As periodate treatment of the Western blot prior to incubation with VRabS would have prevented the binding of antibodies specific for periodate sensitive epitopes, the eluted antibodies should be enriched for those which recognise peptide epitopes of the 16 kDa antigen and thus suitable for screening the sporocyst cDNA library.

**Figure 6.1** **The recognition of the 16 kDa antigen by El$\alpha$16**




Strips of nitrocellulose cut from the 16 kDa region of Western blots of SDS extracted MS antigens were treated with sodium meta periodate and incubated overnight with a 1 in 200 dilution of VRabS. Three successive elutions with low pH buffer yielded antibody solutions which were tested at a 1 in 10 dilution on a Western blot of SDS extracted MS antigens (lanes 2, 3 and 4). The blot was also probed with a 1 in 20 dilution of B3A (lane 1). The position of the 16 kDa antigen is marked with an arrow.

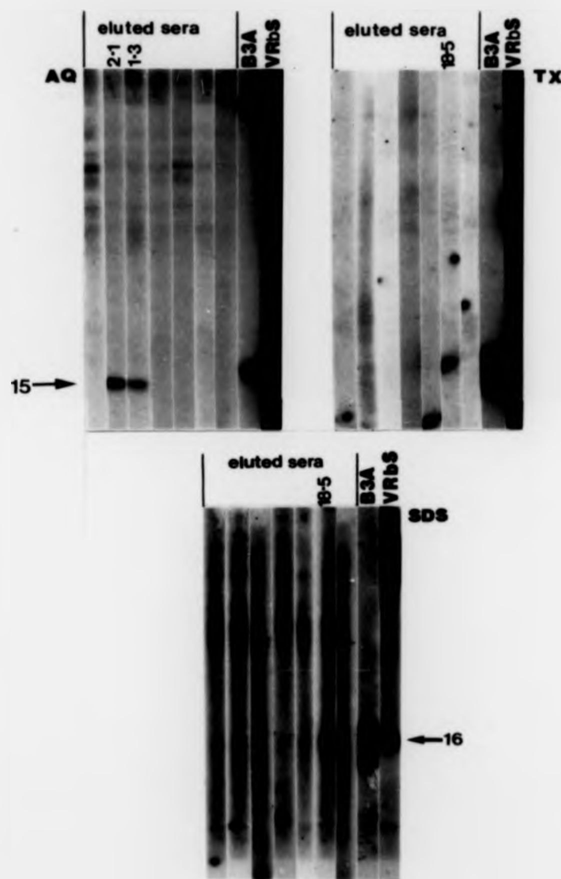
### 6.2.2 SCREENING OF THE SPOROCYST cDNA EXPRESSION LIBRARY

90,000 recombinant clones were screened with Elα16 prepared as above. 20 positive clones were identified of which 9 were positive on secondary screening with VRabS. A number of these clones were then plaque purified and seven (2.1, 1.3, 18.5, 5.1, 6.2, D.30 and 12.7) were taken forward for further study.

### 6.2.3 ANTIBODY SELECT

Using the method of Ozaki *et al.*, (1986) attempts were made to identify the native antigens encoded by the seven clones selected. The clones were plated at a high density, grown at 37°C and overlaid with filters impregnated with IPTG. This induces the expression of proteins which subsequently bind to the nitrocellulose filters. Antibodies recognising recombinant proteins were then selected from VRabS by overnight incubation of the filters with VRabS and elution of bound antibodies with a low pH buffer. The eluted antibodies were used to probe Western blots of aqueous and detergent extracts of MS. Figure 6.2 demonstrates that of the antibodies eluted from the clones, three, namely those eluted from clones 2.1, 1.3 and 18.5, recognised MS antigens. Antibodies eluted from clones 2.1 and 1.3 appeared to recognise the same molecule, an antigen of 15 kDa which unlike the 16 kDa antigen is seen predominantly in the aqueous phase of the TX-114 extraction (Figure 6.2 (Aq)). It is possible that this molecule corresponds to a 15 kDa molecule previously described by Francis (1989) as a soluble vaccine dominant antigen (see Chapter 7). In contrast, the antibodies eluted from clone 18.5 recognised an appropriately sized antigen in the detergent phase of the TX-114 extraction (Figure 6.2 (Tx)) and amongst those antigens extracted from the TX-114 insoluble pellet by boiling in SDS (Figure 6.2 (SDS)). It therefore appeared possible that this clone encoded the 16 kDa antigen, although the recognition of this low molecule weight molecule by affinity eluted antibodies was inconsistent. The signal was observed on one other occasion but failed to appear on two more. Furthermore, the recognition of this signal appeared to be somewhat dependent on the use of fresh antibodies eluted from filters overlaid on plates with a high titre of phage. Antibodies eluted from a second clone (clone 18.4) which was picked from the same plate as 18.5 following secondary screening with VRabS, also failed to produce a signal on an occasion on which a signal was seen using sera eluted from clone 18.5. Further studies were therefore necessary to determine whether a clone encoding the 16 kDa antigen had indeed been identified.

**Figure 6.2** **Antibody select**

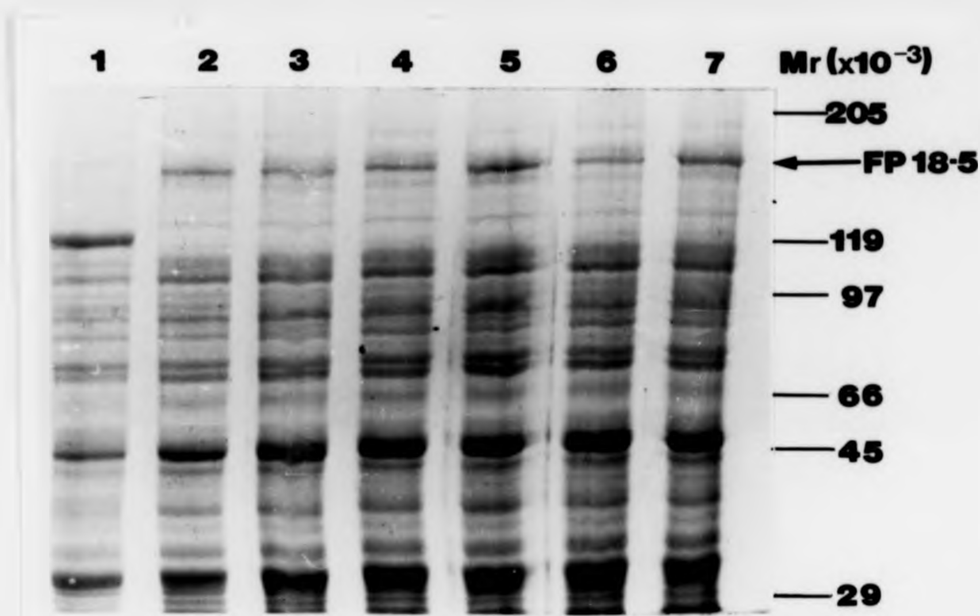



Antibodies were eluted from 7 different λgt11 clones selected using Elα16. These were then used to probe Western blots of aqueous (AQ), TX-114 (TX) and SDS extracted MS antigens. The blots were also probed with B3A and VRabS at a dilution of 1 in 20 and 1 in 200 respectively. Antigens of interest are denoted by arrows and the designated clone number indicated at the top of the relevant strips (i.e. 2.1, 1.3, 18.5).

### 6.2.4 INVESTIGATING THE PATTERN OF ANTIBODY BINDING TO THE PEPTIDE ENCODED BY CLONE 18.5

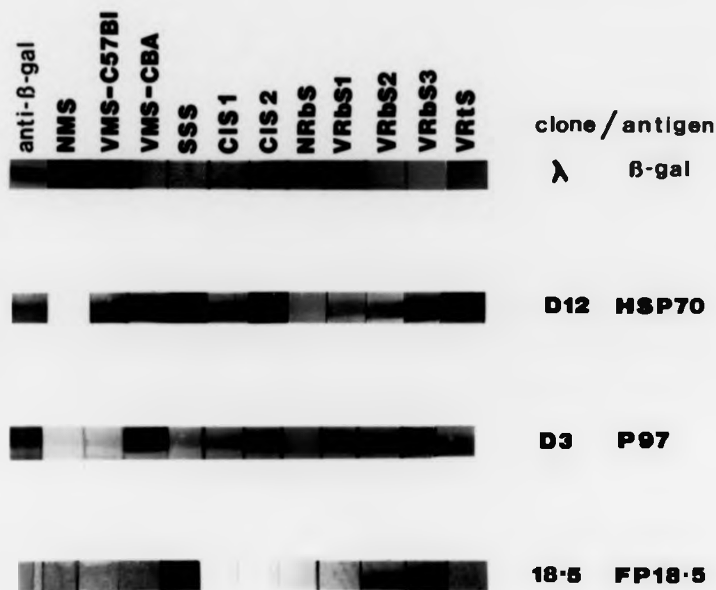
As detailed in previous chapters the 16 kDa molecule has an antibody binding pattern which is characteristic of a "vaccine dominant" antigen. Recombinant proteins expressed by clones encoding this molecule would therefore be expected to display the same or a similar pattern of antibody binding. Thus, in order to further characterise the peptide encoded by clone 18.5 (P 18.5) and to examine the possibility of this being the 16 kDa antigen, experiments were carried out to determine the antibody binding pattern of the ß galactose (ß-gal) recombinant protein expressed by this λgt11 clone. Clone 18.5 was used to infect *E. coli* Y1090 and plated at a high titre in soft agar supplemented with IPTG to induce the expression of proteins. The soft agar containing the expressed proteins was then removed, boiled in sample buffer and subjected to SDS PAGE on an 8% polyacrylamide gel. Staining of the gel with Coomassie blue revealed that clone 18.5 expresses a recombinant protein of approximately 138 kDa (FP 18.5/ß-gal) (fusion protein 18.5/ß-gal) which is not expressed by non-recombinant phage (Figure 6.3). This protein represents the 116 kDa ß-gal fused to a schistosome peptide of approximately 22 kDa. Comparable amounts of FP 18.5/ß-gal, recombinant proteins comprised of *S. mansoni* HSP70 and paramyosin similarly expressed in λgt11, and ß-gal expressed by non-recombinant phage were then electrophoresed and transferred by Western blotting. Strips of each blot were probed with a variety of sera which had been pre-absorbed with lysates of induced non-recombinant λgt11 grown in *E. coli*, to remove those antibodies which recognise bacterial antigens alone. As can be seen in Figure 6.4 FP 18.5/ß-gal is recognised by VRabS (2 and 3) but not by chronic infection sera (CIS) nor by sera raised in mice harbouring a single sex infection (SSS). This recognition pattern can be contrasted with those of HSP70 and paramyosin which are recognised by all but normal sera and with that of ß-gal alone which is not recognised by any of the pre-absorbed sera used. However, although similar to the 16 kDa antigen with regard to its recognition by VRAbS but not CIS nor SSS, FP 18.5/ß-gal differs in that it is not recognised by either VMS nor VRatS. Although the lack of recognition by VMS could be explained by the observation that this sera recognises only carbohydrate epitopes of the 16 kDa antigen (see Chapter 4), VRatS has been shown to recognise some periodate insensitive epitopes (data not shown). However, this lack of recognition by VRatS does not demonstrate unequivocally that FP 18.5/ß-gal does not represent the 16 kDa antigen, as there are several possible

**Figure 6.3** The recombinant protein expressed by clone 18.5




Increasing volumes of soft agar containing the proteins expressed by clone 18.5 (lanes 2 - 7) or those expressed by a non-recombinant control (lane 1) were boiled in SDS PAGE sample buffer and separated by electrophoresis. The gel was then stained with Coomassie blue.

**Figure 6.4 The vaccine dominant nature of FP 18.5/ß-gal**




The proteins expressed by clone 18.5, a number of other recombinant clones and non-recombinant λgt11, were separated by SDS PAGE and transferred by Western blotting. The blots were then probed with a variety of *E. coli* lysate absorbed sera. VMS, NMS, SSS, CIS and VRatS were used at a dilution of 1 in 100. VRabS, NRabS and anti-ß-gal (a gift from Dr. M. Huggins) were used at a dilution of 1 in 200.

explanations for differences between the antibody recognition pattern of a native antigen and its cloned counterpart. For example, it is possible that the DNA insert of clone 18.5 does not represent a full length gene and that the epitopes recognised by VRatS within the native 16 kDa antigen are not encoded by clone 18.5. Alternatively, the peptide epitopes recognised by VRatS on the 16 kDa antigen may be conformational epitopes which are not expressed by a ß-gal recombinant protein produced in a prokaryotic cloning system. It was therefore decided that the binding of VRabS but not CIS nor SSS to FP 18.5/ß-gal warranted still further investigations into the characteristics of P 18.5 and the possibility of it corresponding to the 16 kDa antigen.

### 6.2.5 SEQUENCING OF CLONE 18.5

Much information regarding the structure of a molecule can be acquired by obtaining nucleotide and amino acid sequence data. For example, if clone 18.5 does encode the 16 kDa antigen, sequence data obtained for the encoded peptide may reveal the presence of regions of amino acids suitable for the attachment of O-linked carbohydrates. Similarly, as the 16 kDa antigen is an integral membrane molecule either a membrane spanning domain or a region suitable for acylation may be observed. Even if clone 18.5 does not encode the 16 kDa antigen, any sequence data obtained would still be of value, as the scanning of databases for homologous sequences could reveal a more likely identity for the encoded protein. Thus, the insert encoded by clone 18.5 was amplified in a PCR reaction using the forward and reverse λgt11 primers and a product of approximately 500 bp was obtained. In view of the small size of this insert, clone 18.5 was then cut with the enzymes *Kpn* I and *Sac* I to obtain DNA for subcloning and sequencing. The resulting 2.5 kb fragment was purified on low melting point agarose and subcloned into the paired sequencing vectors M13 mp18 and mp19. Performing cloning and sequencing with both of these vectors should have enabled the DNA insert to be sequenced in both orientations, however recombinant clones were only obtained in M13 mp19. Thus, although some data was obtained by sequencing from M13 mp19 with the λgt11 forward primer, sequencing in the opposite direction was not possible and data for the DNA close to the binding site of the forward primer was not obtained. To confirm the existing data and to obtain the missing sequence, the PCR product obtained on amplification of the DNA encoded by clone 18.5, was cut with *Eco*R I to remove the PCR primers, purified using low melting point agarose and subcloned into *Eco*R I cut M13 mp18. Although only one of the M13

mp18 / 19 vector pair was used for subcloning, digestion of both the vector and the PCR product with *Eco*R I enabled the DNA to be cloned into M13 mp18 in both orientations. The complete sequence of the DNA encoded by clone 18.5 was obtained by sequencing several recombinant clones using the M13 (-40) forward primer. This data confirmed that obtained on sequencing of the *Kpn* I / *Sac* I fragment and is shown in Figure 6.5.

Initial analysis of the sequencing data obtained revealed that cloning irregularities had occurred during the construction of the cDNA expression library and during the subcloning of the PCR product into M13 mp18. Figure 6.6 shows that, as expected, an *Eco*R I site is present at the junction of the 5′ end of the cut PCR product with the M13 vector DNA. This demonstrates that the λgt11 forward PCR primer and the λgt11 DNA found immediately upstream of the cloning site have been removed from the 5′ end of the PCR product by cleavage of an *Eco*R I site at the junction of λgt11 and the 5′ end of the cloned insert. This has produced an appropriate 'sticky end' thus allowing conventional ligation of the *Eco*R I cut PCR product to similarly cut M13 mp18. In contrast, an *Eco*R I site is absent at the junction of the 3′ end of the PCR product and M13. Moreover, the DNA ligated directly to the M13 vector represents not the 3′ end of the cDNA encoded by clone 18.5 but a short section of λgt11 DNA which corresponds to that observed immediately downstream of the cloning site. Closer examination of the sequence data obtained revealed that the junction between λgt11 and the adenosine residues which represent the 3′ end of the cDNA encoded by clone 18.5 can be observed upstream of this point, again an *Eco*R I site is absent. Thus, the absence of an *Eco*R I site between the 3′ end of the cDNA insert and λgt11 of the clone 18.5 used as a template for the PCR reaction, has led to a similar absence in the PCR product. The λgt11 DNA observed immediately downstream of the cloning site and the reverse PCR primer have thus not been removed from the 3′ end of the PCR product on digestion with *Eco*R I. Despite this the *Eco*R I digested PCR product has been subcloned into M13 via an unconventional ligation between the remaining reverse primer and *Eco*R I cut M13.

A single open reading frame which is in frame with β-gal was observed on translation of the nucleotide sequence obtained for P 18.5 (Figure 6.5). This was as expected as the inserted cDNA had been shown to be expressed as a recombinant protein in λgt11. Examination of the amino acid sequence data then revealed that a methionine residue was present at position 27. Moreover, a stop codon is absent and an open reading frame maintained throughout the length of the clone. The absence of a stop codon and the

**Figure 6.5** **The sequence of the peptide encoded by clone 18.5**

10 20 30 40
ACAATAGTCCAGCAAGAGTATCACCTAACCTCGCTTAAAACTAGA
T I V Q Q E Y H L T S L K T R
10

50 60 70 80 90
GAGGTAGAAAATACTCGGGAGTCAGAAGTGTTCATGGCACAAAAA
E V E N T R E S E V F M A Q K
20 30

100 110 120 130
GAATCACCATTACCATATGTTTGCACTTTTTCAACAGAAACAGAG
E S P L P Y V C T F S T E T E
40

140 150 160 170 180
GCGGCGAAACCAGATATCCCGCGTGCCTCTATAGTAACAGATAAG
A A K P D I P R A S I V T D K
50 60

190 200 210 220
AATCTTAATGACAACACTAACTCTGCTTTAATGTCAATAGCAGGT
N L N D N T N S A L M S I A G
70

230 240 250 260
CAACAAGATGCCACAGTTTCTTCACCTTCTTCAGATCTGTGTCCT
Q Q D A T V S S P S S D L C P
80 90

270 280 290 300 310
ACAGAAGACACGCCAATTGTTGCAGAAATTTCCGAACAAGACTCG
T E D T P I V A E I S E Q D S

320 330 340 350
TTCAAAGCAGACGAAACAGTAAAATGTGAAGCTATTGCCAGCGAG
F K A D E T V K C E A I A S E
100

360 370 380 390 400
AACCCGACTAGTAAGGACAACACCATGACAAGAAGGAATGATGAA
N P T S K D N T M T R R N D E
110 120

410
AAAAAAAA
K K

The *Kpn* I / *Sac* I fragment from the lambda gtll clone 18 5 was subcloned into M13 mp19 and sequence data obtained This data was then confirmed and completed by sequencing of a PCR product obtained by amplification of the DNA encoded by clone 18 5 The nucleotide sequence obtained is shown with the corresponding amino acid residues beneath

**Figure 6.6** **Cloning irregularities**

A PCR product obtained following amplification of the DNA encoded by λgt11 clone 18.5 was subcloned into the vector M13 mp18 and sequenced. The above figure highlights the cloning irregularities which have occured during this subcloning and upon construction of the sporocyst cDNA library.

ambiguity surrounding the size of the native antigen encoded by clone 18.5 makes it impossible to determine whether the methionine at position 27 is the initiating methionine. However, this seems unlikely as the sequence 5′ to this residue is in the same open reading frame as that which is 3′. If this methionine were indeed the initiating residue prior sequence would represent the 5′ untranslated region of the gene which would be more likely to contain stop codons. In addition, the nucleotide sequence surrounding the ATG which encodes the methionine at position 27 does not conform to the Kozak consensus sequence (i.e. AXXATGG) for the initiation of translation (Kozak, 1984). The absence of a stop codon prior to the string of ten adenosine residues at the 3′ end of the clone also suggests that these encode lysine residues rather than representing the poly(A) tail. This is substantiated by the observation that the AATAA nucleotide sequence which often represents the signal for polyadenylation is not present upstream of this adenosine string. Thus, it appears that clone 18.5 is not a full length clone and that both 5′ and 3′ ends of the coding region are missing.

### 6.2.6 COMPUTER ANALYSIS OF THE SEQUENCE DATA OBTAINED FOR THE PEPTIDE ENCODED BY CLONE 18.5

The nucleotide and amino acid sequence data obtained for P 18.5 was used to search the Swissprot database. No homology with other molecules was found. Thus, the PC / Gene computer programme was used in an attempt to characterise the structural features of the encoded peptide. Firstly, the programme was used to employ the method of Kyte and Doolittle (1982) and a plot of the hydropathic index of the whole amino acid sequence was obtained. This demonstrated that there were no regions of particularly high hydrophobicity or of high hydrophilicity within the amino acid sequence. The hydropathic index generated by this method was then used as described by Klein *et al.*, (1985) to determine the likelihood of this amino acid sequence encoding an integral membrane protein. As was expected from observation of the initial hydropathic index no membrane spanning regions were predicted. Using this method P 18.5 was therefore classified as being peripheral to the membrane rather than integral. This suggested that it does not correspond to the 16 kDa antigen. However, it is possible that rather than incorporating a stretch of hydrophobic amino acids, the 16 kDa antigen uses acylation i.e. the addition of fatty acids to the peptide, for attachment to the lipid bilayer. Acylation can involve the incorporation of the complex fatty acid containing GPI anchor. Alternatively, a fatty acid

can be linked directly to the peptide backbone. As discussed in Chapter 3 the presence of a GPI anchor within the 16 kDa antigen is a possibility made plausible by the susceptibility of the antigen to extraction by a particular type of detergent. Furthermore, scanning of the amino acid data obtained using the Prosite computer programme revealed that following particular post-translational modifications the glycine at position 77 could form part of a site for the attachment of the fatty acid, myristate. The attachment of fatty acid to the N-terminus of proteins has also been observed to block N-terminal amino acid sequencing (Aitken *et al.*, 1982, Ozols *et al.*, 1984). Thus, despite the absence of hydrophobic regions within the amino acid sequence encoded by the DNA insert of clone 18.5 it is possible that the encoded molecule could become integral to the membrane. Further analysis of the amino acid sequence obtained for clone 18.5 also revealed the presence of potential sites of phosphorylation by both protein kinase C and casein kinase II. However, potential sites for O-glycosylation i.e. serine or threonine residues within a proline rich region were not observed.

### 6.2.7 IMMUNISATION OF MICE WITH THE PEPTIDE ENCODED BY CLONE 18.5

Experiments to determine whether clone 18.5 encodes the 16 kDa antigen by examination of its antibody binding pattern and by obtaining sequence data proved inconclusive. It was therefore decided to carry out immunisation experiments with the recombinant antigen, FP 18.5/ß-gal. This should enable sera raised against the peptide *in vivo* to be used for screening Western blots of MS antigens in order to determine whether the 16 kDa antigen is recognised.

#### 6.2.7.1 Preparation of recombinant antigen

To obtain protein for immunisation, clone 18.5 was used to produce a recombinant lysogen in *E. coli* Y1089. This procedure has been used successfully on numerous occasions for the production of ß-gal recombinant proteins in relatively high abundance (Young and Davis, 1983). Proteins expressed by the recombinant and a wild type lysogen were solubilised by boiling a sample of Y1089 in SDS PAGE sample buffer and separated by electrophoresis through a 8% polyacrylamide gel. Coomassie blue staining of the gel revealed the expression of a protein of approximately 138 kDa by the recombinant but not the control lysogen. The remaining bacterial cells were then lysed and the soluble fraction

was separated from the insoluble material by sucrose gradient centrifugation. Both fractions were subjected to SDS PAGE and Western blotting. Figure 6.7 demonstrates that the 138 kDa protein expressed by the recombinant lysogen was recognised by VRabS but not NRabS and that the protein separated into the supernatant following sedimentation through sucrose. Although numerous other bacterial proteins are also seen in this phase a degree of purification had been achieved. In particular, the partitioning into the insoluble pellet of an antigen of approximately 55 kDa which is recognised strongly by both VRabS and NRabS, ensured that the 138 kDa recombinant protein was the major antigen in the supernatant.

**6.2.7.2 Immunisation of mice with the FP 18.5/ß-gal**

The supernatant containing FP 18.5/ß-gal was used to immunise mice in conjunction with CFA / IFA. A total of three subcutaneous immunisations were given to each of two female Balb/c and two female CBA mice. Similar groups of control mice were immunised with the supernatant obtained by separation through sucrose of the proteins expressed by a non-recombinant lysogen. Western blots of the soluble and detergent fractions obtained by extraction of MS with OTG were probed with the sera raised. Figure 6.8 demonstrates that a low molecular weight antigen was recognised by sera from animals immunised with the recombinant antigen but not by sera taken from the controls. However, this antigen had a molecular weight of approximately 17 kDa and was present in the soluble (Aq) rather than the detergent (OTG) phase of the MS extract. These results indicate that P 18.5 does not represent a portion of the 16 kDa antigen.

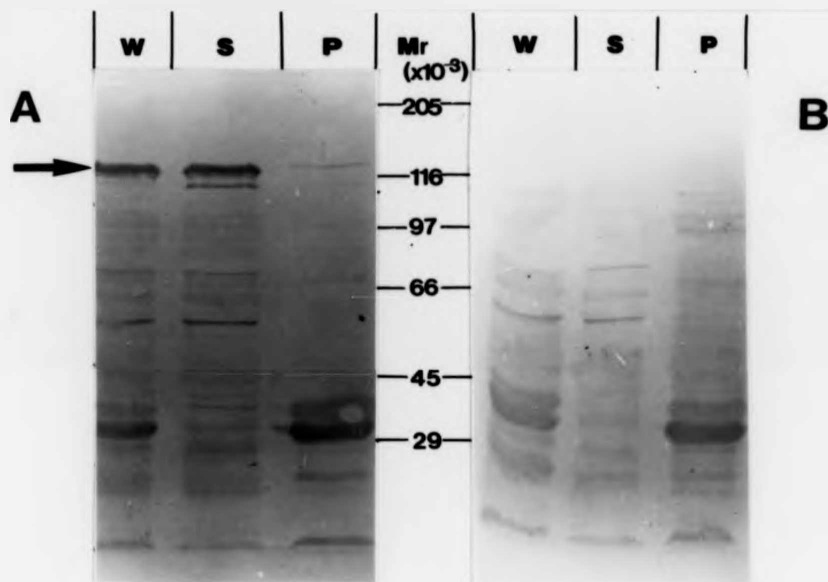
**6.2.7.3 The protective capacity of the peptide encoded by clone 18.5**

Although the above results suggested that P 18.5 does not represent the 16 kDa antigen, the immunised mice were challenged two weeks after the final immunisation and perfused eight weeks later. The worm burdens of the immunised mice were not significantly different from those of the controls. These results gave no indication that this antigen was worthy of further study.

**6.2.9 <u>RESCREENING OF THE cDNA LIBRARY</u>**

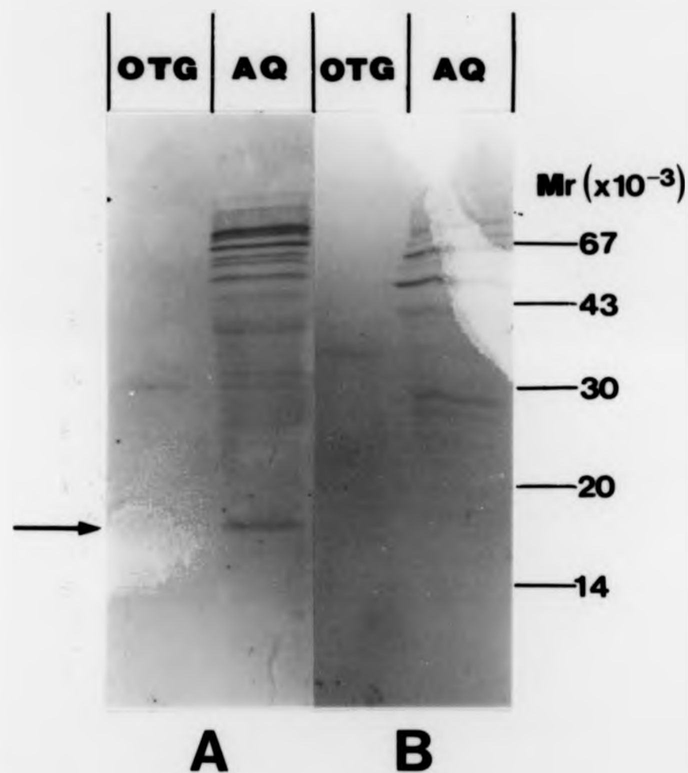
As it was demonstrated that the protein encoded by clone 18.5 does not represent the 16 kDa antigen, a further 70,000 recombinant cDNA clones were screened in the hope

**Figure 6.7** Recovery of FP 18.5/ß-gal from a recombinant lysogen




The λgt11 clone 18.5 was used to produce a recombinant lysogen in *E. coli* Y1089. An aliquot of the antigens expressed by this lysogen were then separated by centrifugation through a sucrose cushion and the soluble (S) and insoluble (P) proteins obtained were electrophoresed and Western blotted alongside an aliquot of unfractionated lysate (W). The blot was probed with VRabS (panel A) or NRabS (panel B) at a dilution of 1 in 200. The position of the 138 kDa recombinant protein is marked with an arrow.

**Figure 6.8** Recognition of a 17 kDa antigen by sera raised in mice immunised with FP 18.5/ß-gal




Sera raised in mice immunised with the soluble fraction of the 18.5 lysogen (panel A) or with the soluble fraction of the non-recombinant lysogen (panel B) were used at a dilution of 1 in 50 to probe aqueous (Aq) and detergent (OTG) extracts of MS. The position of the 17 kDa antigen recognised by sera raised in mice immunised with FP 18.5/ß-gal is marked with an arrow.

of identifying clones which do encode this molecule. Primary and secondary screening was carried out as described previously (section 6.2.2) using fresh antibodies eluted from the 16 kDa region of periodate treated Western blots. However, rather than subjecting the clones isolated to "antibody select" procedures, they were first plated on tertiary arrays and double lifts were taken and screened with either VRabS or CIS. Clones putatively encoding the 16 kDa antigen should be positive with the former but not the latter. Repeated screening of the tertiary arrays failed to identify clones which met this criteria.

## 6.3 DISCUSSION

The identification of a clone encoding a peptide of interest has many advantages. Firstly, it enables sequence data to be obtained which may be used to provide information about the structure and perhaps the function of the molecule. Secondly, incorporation of the DNA encoding the peptide of interest into a suitable expression vector may enable substantial amounts of recombinant protein to be produced in bacteria. This latter point is of particular importance in the field of schistosome research as the availability of parasite material relies heavily on the maintenance of a complex life cycle. Maintenance of such a life cycle is difficult and time consuming and the amount of parasite material which can be produced in this way is often limiting.

In order to identify clones encoding the 16 kDa antigen within a sporocyst cDNA expression library a monospecific serum which recognised at least some peptide epitopes was required. Neither the McAb nor sera obtained on immunisation of mice with the immunoaffinity purified antigen were suitable for this purpose, thus other methods were employed in an attempt to obtain a specific antibody probe. Initially, powdered polyacrylamide gel containing the 16 kDa antigen eluted from the immunoaffinity column and additionally purified by SDS PAGE was injected into the popliteal lymph nodes of a rabbit. It has been demonstrated (Miller *et al.*, 1989) that on subcutaneous injection, polymerised acrylamide acts as an adjuvant by slowly releasing the antigen from the gel matrix and thus allowing even weak immunogens to induce a good antibody response. However, injection of the polyacrylamide gel into the lymph nodes of a rabbit proved to be unsuccessful, as following the third injection the animal had to be destroyed due to paralysis of its hind legs. Moreover, sera taken prior to, or following the onset of paralysis did not produce a signal in the 16 kDa region of Western blots. Although injection directly into the lymph node has been carried out successfully on previous

occasions the antigens used were in solution rather than absorbed onto polyacrylamide (Dr. T. Bianco, personal communication). It therefore seems likely that the paralysis observed here was due to the injection into the lymph node of either the neurotoxic gel components, acrylamide and bis acrylamide, or a high level of SDS. Further studies involving electroelution of the 16 kDa antigen from the gel prior to immunisation were considered and this may have avoided the toxic side effects observed on immunising with the polyacrylamide gel. However, as described in Chapter 3, the amounts of 16 kDa antigen available for immunisation were very small and it was decided that the losses which invariably occur during electroelution would be unacceptable.

In a second approach to obtaining a specific probe for screening, antibodies were eluted from the 16 kDa region of periodate treated Western blots of SDS extracted MS antigens. Probing of Western blots of similarly extracted antigens with these eluted antibodies (El$\alpha$16) demonstrated that the major signal corresponded to the 16 kDa antigen and that El$\alpha$16 had an anti-16 kDa titre of a least 1 in 100. This sera was then used to screen approximately 90,000 recombinant clones of which seven were eventually selected for further studies. On identification of the antigens encoded by these clones using antibody select methods it became apparent that molecules other than the 16 kDa antigen were recognised by El$\alpha$16. For example, clones 2.1 and 1.3 were shown to encode a 15 kDa aqueous phase molecule thought to be the 15 kDa vaccine dominant antigen described by Francis (1989). The isolation of clones encoding an aqueous phase molecule was surprising in that the antibodies used to screen the cDNA library were selected on the basis of their ability to bind to MS antigens extracted by SDS from a pellet previously extracted with TX-114. Such a method was used with the specific intent of excluding those antibodies which recognise TX-114 soluble molecules and hence enriching El$\alpha$16 for antibodies against the 16 kDa antigen which remains largely insoluble. The presence within El$\alpha$16 of antibodies recognising a clone encoding an aqueous phase antigen suggests that a proportion of some soluble molecules remains associated with the MS pellet despite prior extraction with TX-114. Further studies have been carried out with this 15 kDa molecule and are detailed in Chapter 7.

Antibodies eluted from a third clone (clone 18.5) did appear to recognise an antigen of approximately 16 kDa which was present within both the TX-114 detergent phase and the SDS phase of the MS extract. However, the inconsistency of this recognition on repetition of the antibody select procedure and the requirement for antibodies eluted from

a high titre of phage, led to doubts as to the validity of this result. It seemed possible that the inconsistency of the results obtained could be explained by a requirement for the absorption of an aliquot of antibodies from Elα16 which when eluted have a sufficient titre for the recognition of the low molecular weight molecule on Western blots. Thus, a lower titre of phage may express too little recombinant protein for the absorption of a suitable aliquot of antibodies. Similarly, antibodies eluted from the sibling clone, clone 18.4, may have failed to recognise the low molecular weight antigen on Western blots if clone 18.4 expressed less recombinant antigen than clone 18.5. A strong signal with VRabS which was used initially as a criteria for its selection for further studies does indicate that clone 18.5 may be particularly efficient at the expression of the encoded protein. Alternatively it is possible that the inconsistency of the results obtained was due to the presence of a spurious signal on some occasions. If the low molecular weight molecule recognised by antibodies eluted from clone 18.5 has a tendency towards the non-specific binding of antibodies or protein molecules in general, then it is possible that varying results may be obtained which are dependent on the success of the washing protocol used within the antibody select procedure.

Although immunisation studies carried out at a later date indicated that the low molecular weight signal was a spurious signal (see section 6.2.7.2. and below), initial attempts to dispel the ambiguity surrounding the identity of P 18.5 involved examination of its antibody recognition profile and the obtaining of sequence data. Characterisation of the antibody binding properties of the peptide expressed by clone 18.5 as a fusion with ß-gal (FP 18.5/ß-gal) did not provide sufficient evidence to determine whether it represented the 16 kDa antigen or not. Thus, the cDNA insert from clone 18.5 was subcloned into M13 and sequenced. Examination of the nucleotide and amino acid sequence data obtained revealed that 413 bases representing 137 amino acids were encoded. Translation of this sequence would result in the production of a peptide of approximately 15 kDa. This contrasted significantly with the observation that the recombinant protein expressed by clone 18.5 had an estimated size of 138 kDa, as this corresponds to the fusion of a 22 kDa encoded peptide to the 116 kDa ß-gal. However, further examination of the sequence data obtained indicated that the partial length cDNA encoded by clone 18.5 incorporated neither the 5′ nor the 3′ end of the coding region of the gene. The absence of the latter and hence a stop signal, provided a possible explanation for the large size of the recombinant protein expressed, as translation could

continue beyond the 3′ end of the cDNA insert and through λgt11 DNA until a stop codon was encountered. That clone 18.5 encodes a 15 kDa peptide despite the absence of both the 5′ and the 3′ ends of the coding region of the gene also suggested that the complete gene may encode a peptide too big to correspond to the 16 kDa antigen. However, it was considered possible that the antigen could be represented by a larger precursor molecule which is post / co-translationally modified to produce a glycoprotein of an appropriate size. For example, a number of proteins destined for insertion into the surface membrane are processed by the cleavage, from a larger precursor protein, of a signal sequence similar to that seen on the precursors of secretory molecules (Kreil, 1981). This N-terminal signal consists of a core of at least nine hydrophobic amino acid residues and up to thirty residues (i.e. 3 kDa) in total. The sequence, which is normally present at the N-terminus of the protein, is required for the initiation of the binding of the ribosome on which the growing polypeptide chain is being synthesised to the lipid molecules within the membrane of the ER. This binding enables the polypeptide to pass co-translationally into the lumen of the ER through a channel formed in the membrane. Here, cleavage of the signal sequence and preliminary glycosylation take place prior to the transportation of the molecule to the cell surface (Kreil, 1981). The absence of data for the 5′ untranslated region of the gene partially encoded by clone 18.5 made it impossible to determine if a putative N-terminal amino acid signal sequence was encoded.

Computer analysis of the amino acid sequence data obtained for P 18.5 also revealed that the sequence lacked features which may be expected to be associated with that encoding the 16 kDa antigen. A hydrophobic region suitable for anchoring the molecule within the surface membrane was not observed nor were possible sites of O-glycosylation. With regard to the latter, it is known that in many glycoproteins the sugar chains are clustered around a single short region of the core peptide (Blochberger *et al.*, 1989). Thus, it was considered possible that ample glycosylation sites could be provided within a relatively short stretch of amino acids, which are encoded by the 5′ or 3′ regions absent from the partial length clone 18.5. With regard to the former, it has been suggested that molecules which are exposed at the surface of the schistosome membrane are more likely to be covalently linked to the membrane via acylation (i.e. the addition of fatty acids) than by virtue of their own transmembrane domain (Rogers and Tiu, 1991). Thus, clones can not be discounted from encoding a membrane antigen solely on the basis of their lacking a region of hydrophobic peptide sequence suitable for spanning the lipid bilayer.

One form of acylation commonly used to anchor a molecule within a membrane is the addition of the complex fatty acid containing GPI structure. The possibility of the 16 kDa antigen incorporating such an anchor has already been suggested by the susceptibility of this molecule to extraction by detergents with high CMCs. The likelihood of such an anchor being associated with a particular cloned molecule can also be assessed by analysis of cDNA sequence data. mRNA encoding proteins destined to be GPI anchored contains a short stretch of hydrophobic amino acids at its 3′ end. This region of hydrophobic residues is never seen on the mature protein and it is believed to represent a signal sequence which directs its own cleavage from a precursor molecule and the subsequent addition of the GPI anchor (Low, 1987, Ferguson and Williams, 1988, Pearce *et al.*, 1991b). How this region acts as a signal for cleavage is not clearly understood. However, as no consensus sequence has been found on comparison of numerous GPI anchored protein precursor molecules it is thought that the conformation rather than the precise sequence of this hydrophobic region is important. The absence of sequence data for the 3′ untranslated region of the gene partially encoded by clone 18.5 made it impossible to exclude the possibility of this clone encoding a GPI linked protein and hence the 16 kDa antigen. If such a structure, which has a molecular weight of approximately 1.2 - 1.5 kDa (in Hall *et al.*, 1995), were to be required for the anchoring of P 18.5 this would increase the size of the mature product beyond 16 kDa. However, it has been noted that due, most probably, to the inefficient binding of SDS to the GPI structure, removal of the GPI anchor from GPI anchored proteins leads to an increase in the estimated size of the protein on SDS PAGE (Littlewood *et al.*, 1989). Thus, if the 16 kDa antigen is GPI anchored, its size may be under estimated by SDS PAGE and the peptide moiety of the antigen could be represented by a sequence longer than that initially anticipated.

A second way of anchoring membrane proteins which do not possess a hydrophobic membrane spanning domain is using acylation via the addition of fatty acid directly to the peptide backbone. This increases the hydrophobicity of the region to which the fatty acid is added and enables it to interact with the lipid molecules within the bilayer. In general this type of acylation involves either the addition of myristic acid via an amide bond to the amino group of an N-terminal glycine residue or the addition of myristic, stearic, oleic or palmitic acid via a thioester or ester linkage to a cysteine or serine / threonine residue respectively (Simon and Aderem, 1992). The addition of myristic acid via linkage to internal lysine residues has also been described (Stevenson *et al.*, 1993). Acylation of

soluble forms of immunoglobulin has previously been shown to enable molecules to become membrane bound (Huang *et al.*, 1980). Similarly, deacylation of many proteins has been shown to abolish the ability of the molecule to be incorporated into vesicles (Petri *et al.*, 1981). However, of more relevance to the studies detailed here is the observation that direct acylation has been shown to be involved in the anchoring of some proteins to the schistosome bilayer. Thus, Pearce *et al.*, (1991b) demonstrated that the addition of palmitic acid to a residue within a short C terminal hydrophobic region of a 25 kDa vaccine candidate antigen (Sm25, see section 1.9.2.1), stabilised the otherwise unsuitable region for interaction with the lipid bilayer. Removal of the palmitate led to the transfer of Sm25 from the detergent to the aqueous phase following extraction with TX-114. On analysing the sequence data obtained for P 18.5 the Prosite computer program suggested the possibility of the peptide being acylated via the addition of myristic acid to the glycine residue at amino acid position 77. In order for this to occur the glycine must form part of a hexapeptide sequence which is recognised by the enzyme responsible for myristoylation, myristyl Coenzyme A : protein N-myristyl (NMT). This sequence consists of an N-terminal glycine at position one together with uncharged residues at positions two and five. Any residues are allowed at positions three, four and six (Gordon 1990). Although the glutamine and alanine residues at amino acids 78 and 81 respectively are uncharged and hence appropriate residues for positions two and five of this hexapeptide sequence, the glycine residue at position 77 is obviously not N-terminal. Despite this, amino acids 77 to 82 of P 18.5 were selected by the program as a possible site of myristoylation as the occurrence of post / co-translational modifications which result in the exposure of previously internal residues can not be dismissed. Whilst enabling the glycine at position 77 of P 18.5 to form an N-terminus, the post / co-translational removal of amino acids preceding this residue would obviously result in the production of a peptide of much reduced size. It was considered possible that a mature molecule of 16 kDa could then be produced from this peptide by the addition of myristic acid, the inclusion of the sequence encoded by the absent 3′ end of the coding region of the gene and subsequent glycosylation. However, although acylation following the cleavage of a large precursor molecule to expose a previously internal glycine residue has been described for picornaviral polyprotein precursors (Palmenberg, 1990), the removal of an initiating methionine to reveal an N-terminal glycine residue is the only modification described thus far in acylated eukaryotic proteins (Towler *et al.*, 1988). It is therefore

improbable that the antigen encoded by clone 18.5 is processed in such a way as to enable myristoylation to occur. Furthermore, it has been shown that although, in general, acylation serves to anchor many molecules within the surface membrane, some acylated proteins and in particular myristoylated proteins, are found within the aqueous phase following TX-114 extraction (Towler *et al.*, 1988). Thus the presence of myristic acid does not guarantee a membrane location. Other functions of myristoylation such as the targeting of aqueous phase molecules which are peripherally associated with the plasma membrane to appropriate locations have also been suggested (Resh, 1989).

Obtaining sequence data for the DNA encoded by clone 18.5 also demonstrated that an *Eco*R I restriction site was absent at the junction of the 3′ end of the cloned sporocyst cDNA with λgt11. Thus, *Eco*R I restriction of the PCR product amplified from this clone did not result in the cleavage of the λgt11 sequence downstream of the cloning site nor of the reverse PCR primer. Despite this it was observed that following subcloning of the improperly cut PCR product into *Eco*R I cut M13 only six of the twenty four nucleotides complementary to the λgt11 reverse primer are present at the junction of the 3′ end of the inserted PCR product and the M13 DNA. It seems probable that the original PCR product incorporated more of the sequence complementary to that of the reverse primer as amplification is unlikely to have occurred following ligation of the primer to a stretch of just six bases. These results therefore suggest that a number of bases have been removed from the 3′ end of the PCR product by exonuclease activity in the solutions used either for the attempted restriction with *Eco*R I or during the ligation reaction. The absence of four nucleotides (AATT) from the 5′ terminus of the *Eco*R I cut sticky end of M13 mp18 suggests that exonuclease activity has also removed bases from the vector DNA. The "nibbled" PCR product and vector have then been able to join via a blunt ended ligation and produce recombinant clones suitable for sequencing. During the course of this project the absence of an *Eco*R I restriction site at the 3′ end of the cDNA encoded by several clones within the sporocyst expression library has been demonstrated. The possible reasons for this are discussed in more detail in Chapter 7.

As sequencing of P 18.5 did not enable the possibility of it representing the 16 kDa antigen to be dismissed, attempts were made to raise antibody against this peptide and hence characterise the corresponding native molecule. Thus, clone 18.5 was used to produce a lysogen in Y1089 and the recombinant protein expressed (FP 18.5/ß-gal) injected in conjunction with CFA / IFA into a small group of mice. Sera raised in these

mice were shown to recognise an antigen of approximately 17 kDa present within a 50 mM Tris/HCl (pH 7.4) extract of MS. It is possible that mice immunised with FP 18.5/ß-gal do not produce antibodies against the schistosome part of the recombinant protein and that the 17 kDa signal represents recognition of a molecule of bacterial origin. However, this molecule is not seen by sera raised in mice immunised with the proteins expressed by a non recombinant lysogen, thus suggesting that recognition of the 17 kDa molecule by recombinant immunised mice does indeed represent the recognition of the schistosome peptide encoded by clone 18.5. That the peptide of approximately 15 kDa encoded by the partial length clone 18.5 represents a portion of a 17 kDa native antigen is also compatible with the sequencing data obtained and suggests that a further 2 kDa of molecule is synthesised by translation of the complete gene and inclusion of any subsequent post / co-translational modifications. However, the recognition of a 17 kDa aqueous phase antigen by mice immunised with FP 18.5/ß-gal is obviously not consistent with the suggestion that clone 18.5 encodes the 16 kDa integral membrane antigen. Thus, it became apparent that the original low molecular weight signal observed on probing detergent phase antigens with sera eluted from clone 18.5 by antibody select procedures, represented a spurious one. This may be due to inefficient washing and perhaps a degree of affinity of the low molecular weight molecule for Elα16. However, it is not clear why a 17 kDa aqueous phase antigen was not recognised by sera eluted from clone 18.5. Moreover, it is difficult to deduce why screening with antibodies selected on the basis of their ability to bind to the 16 kDa region of Western blots of detergent phase antigens (i.e.Elα16), isolated a clone encoding a molecule present within the aqueous phase of the extraction. However, experiments aimed at characterising the specificity of Elα16 by probing of a Western blot of SDS extracted antigens prior to screening, did demonstrate that a weak signal of approximately 17 kDa was recognised by this sera (section 6.2.1.2).

The selection of clones encoding a 15 kDa and a 17 kDa antigen demonstrates that Elα16 does recognise low molecular weight molecules. Despite this, further rounds of screening with fresh antibodies eluted from the low molecular weight region of the periodate treated Western blot failed to identify clones encoding the 16 kDa antigen. Thus, despite the screening of more than 160,000 recombinant clones, a clone encoding the 16 kDa antigen was not isolated. There are several possible reasons for this failure. Firstly, Elα16 could have recognised periodate insensitive carbohydrate epitopes rather than peptide epitopes as hoped. However, in view of the results discussed in Chapter 4

which suggest that the only sugar present within the 16 kDa antigen is the periodate sensitive monosaccharide ß GalNAc this seems unlikely. Secondly, it is possible that a large proportion of the antibodies present in Elα16 recognise conformational epitopes which although peptide in nature are not expressed in a prokaryotic system. The selection of antibodies from VRabS on the basis of their ability to bind to antigens following SDS PAGE should have enriched Elα16 for antibodies which recognise linearised epitopes. However, a proportion of the antibodies selected could still have recognised reformed conformational epitopes or any which are resistant to denaturation by SDS. Alternatively it is possible that clones encoding the 16 kDa antigen are poorly represented in the library screened. It has been demonstrated (Buell *et al.*, 1988) that the efficiency of reverse transcription varies between mRNA species. Moreover, the cDNA library used had been subjected to amplification. Although during amplification λgt11 phage were used to infect Y1088, a bacterial strain which carry the *lac* repressor to prevent the expression of recombinant proteins until IPTG is added, this system is "leaky" and small amounts of protein can be expressed prior to induction. If the protein expressed is toxic to the phage or the bacterial cell host this can lead to slow growth of the recombinant clone and thus under representation within the amplified library. Again this is an unlikely explanation for the failure to identify clones encoding the 16 kDa antigen within the cDNA library, as experiments detailed by Francis (1989) describe similar results on screening of the library prior to amplification. Finally although mature 16 kDa antigen has been identified in the sporocyst stage, mRNA encoding for this antigen may be in low abundance. The detection of clones encoding non abundant mRNA is particularly difficult when screening a cDNA expression library with antibody probes as only clones in which the cDNA is encoded in the correct orientation and reading frame can be identified. Screening of yet more recombinants may therefore be required to reveal a clone encoding the 16 kDa antigen.

# CHAPTER 7

# CHARACTERISATION OF CLONES ENCODING A 15 KDA VACCINE DOMINANT ANTIGEN

## 7.1 INTRODUCTION

As described in Chapter 6 several clones were isolated following the screening of a sporocyst cDNA expression library with affinity purified antibodies specific for low molecular weight antigens (Elα16). Using the method of Ozaki *et al.*, (1986) the native antigens encoded by these clones were then identified within a TX-114 extract of MS. Two of the clones, 2.1 and 1.3, were shown to encode a 15 kDa molecule present within the aqueous phase of the preparation. Several pieces of evidence suggest that this molecule may be the same as that encoded by a clone (15V) described by Francis (1989). Firstly, clones 2.1, 1.3 and 15V were all isolated from the same sporocyst cDNA library as a consequence of their ability to bind antibodies within VRabS. Secondly, the 15 kDa antigen encoded by clone 15V was described by Francis (1989) as being present amongst MS antigens extracted with 6 M guanidinium hydrochloride (G/HCl) but not those extracted with 1% sodium deoxycholate (DOC). The separation of different subsets of antigens into these extracts had already suggested that soluble antigens were removed by G/HCl whereas the detergent DOC was required to extract those molecules integral to the membrane. Thus, the presence of the 15 kDa antigen encoded by 15V in the G/HCl extract suggests that like the antigen encoded by clones 2.1 and 1.3 the molecule has the characteristics of a soluble protein.

The recombinant protein expressed by clone 15V has been shown to be recognised by VRabS which can confer passive protection and by VMS, but not by sera from non-immune mice harbouring a chronic (CIS) or single sex (SSS) infection (Francis and Bickle, 1992). Thus, it is known that the antigen encoded by clone 15V is to some extent vaccine specific. Studies described in the first section of this chapter were therefore aimed at characterising the antibody binding pattern of the peptide encoded by clone 2.1. Such studies should reveal whether the molecule encoded by this clone is also vaccine dominant and hence of interest as a potential vaccine candidate antigen.

The second part of this chapter describes experiments aimed at acquiring information regarding the structure and possible function of the 15 kDa molecule encoded by the isolated clones. This involved obtaining nucleotide and amino acid sequence data and making comparisons with those sequences already recorded in established databases.

Finally the protective potential of the 15 kDa antigen was examined following its expression as a recombinant protein.

## 7.2 RESULTS

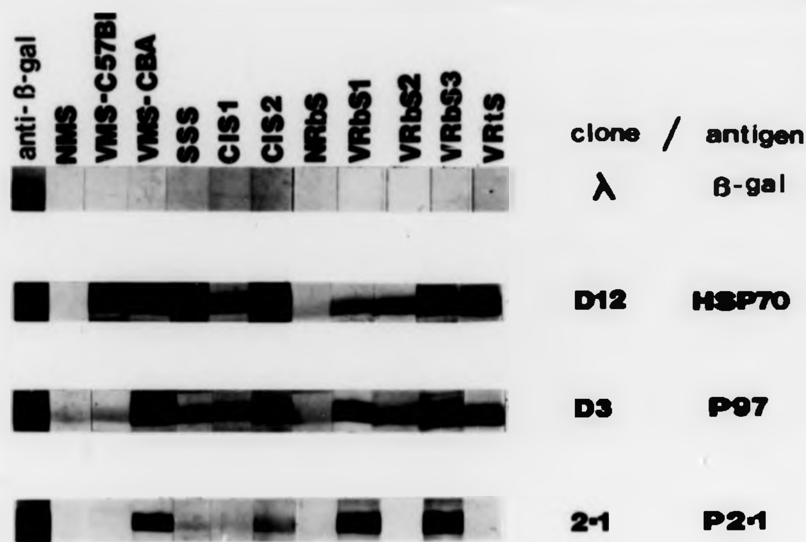
### 7.2.1 INVESTIGATING THE PATTERN OF ANTIBODY BINDING TO THE PEPTIDE ENCODED BY CLONE 2.1

To determine whether the peptide encoded by clone 2.1 is vaccine dominant the clone was plated at a high titre in soft agar supplemented with IPTG. The agar containing expressed proteins was then removed, boiled in SDS PAGE sample buffer and electrophoresed on an 8% polyacrylamide gel. Staining of the gel with Coomassie blue revealed that clone 2.1 expressed a protein of approximately 126 kDa (FP 2.1/ß-gal) which was not expressed by the non recombinant phage (data not shown). This represented the 116 kDa ß-gal plus a fused peptide of approximately 10 kDa (P 2.1). Comparable amounts of FP 2.1/ß-gal, a recombinant form of the heat shock protein HSP70, recombinant paramyosin (P 97) and ß-gal expressed by non recombinant phage were then separated by SDS PAGE and transferred by Western blotting. Strips of each blot were probed with a variety of sera pre-absorbed against the bacterial proteins expressed by a culture of non-recombinant λgt11 which include a significant amount of ß-gal. Figure 7.1 demonstrates that FP 2.1/ß-gal was recognised strongly by VRabS (1 and 3) and VMS(CBA). In contrast, a very weak signal was seen with SSS and the recognition of the antigen by CIS was either weak CIS(2) or absent CIS(1). This vaccine dominant pattern of recognition can be contrasted with that of HSP70 and P97 which were recognised by all except the normal sera and that of ß-gal which was recognised only by sera raised against itself. The latter point demonstrates the effectiveness of the pre-absorption of the sera used.

### 7.2.2 OBTAINING SEQUENCE DATA FOR THE DNA ENCODED BY CLONES 15V, 2.1 AND 1.3

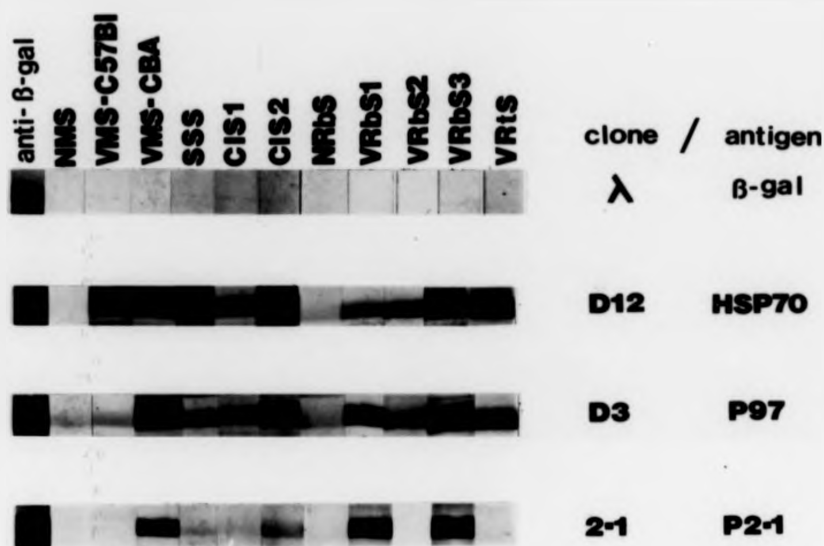
The recognition of FP 2.1/ß-gal by VMS(CBA) and VRabS but not by SSS nor CIS suggests that the protein encoded by clone 2.1 is of interest as a vaccine dominant antigen. In addition, the similarities observed between the antibody binding pattern of FP 2.1/ß-gal and the 15 kDa antigen encoded by clone 15V (Francis, 1989) suggests that these clones may indeed encode the same molecule. In order to test this hypothesis and also to obtain further information regarding the structure and possible function of the 15 kDa molecule(s) attempts were made to amplify and sequence the DNA encoded by clones 15V, 2.1 and also 1.3.

Figure 7.1 The vaccine dominant nature of FP 2.1/ß-gal




The recombinant proteins expressed by clone 2.1, a number of other recombinant clones and non-recombinant λgt11, were separated by SDS PAGE and transferred by Western blotting. The blots were then probed with a variety of *E. coli* lysate absorbed sera. VMS, NMS, SSS, CIS and VRatS were used at a dilution of 1 in 100. VRabS, NRabS and anti-ß-gal were used at a dilution of 1 in 200.

**Figure 7.1** **The vaccine dominant nature of FP 2.1/ß-gal**




The recombinant proteins expressed by clone 2.1, a number of other recombinant clones and non-recombinant λgt11, were separated by SDS PAGE and transferred by Western blotting. The blots were then probed with a variety of *E. coli* lysate absorbed sera. VMS, NMS, SSS, CIS and VRatS were used at a dilution of 1 in 100. VRabS, NRabS and anti-ß-gal were used at a dilution of 1 in 200.

#### 7.2.2.1 PCR reactions

The cDNA encoded by all three clones was amplified from plate stocks by PCR reactions using the forward and reverse λgt11 primers. The PCR products obtained were then electrophoresed on agarose gels and their sizes estimated (data not shown). Amplification of the DNA encoded by clones 15V and 2.1 produced a product of the same size i.e. approximately 400 bp. In contrast, the PCR product obtained from clone 1.3 had an estimated size of 1.7 kb. This data suggests that clones 2.1 and 15V may represent partial length clones of the gene encoding the 15 kDa antigen whereas clone 1.3 might conceivably contain the complete coding region together with additional untranslated flanking regions. The DNA inserts of all three clones were therefore sequenced.

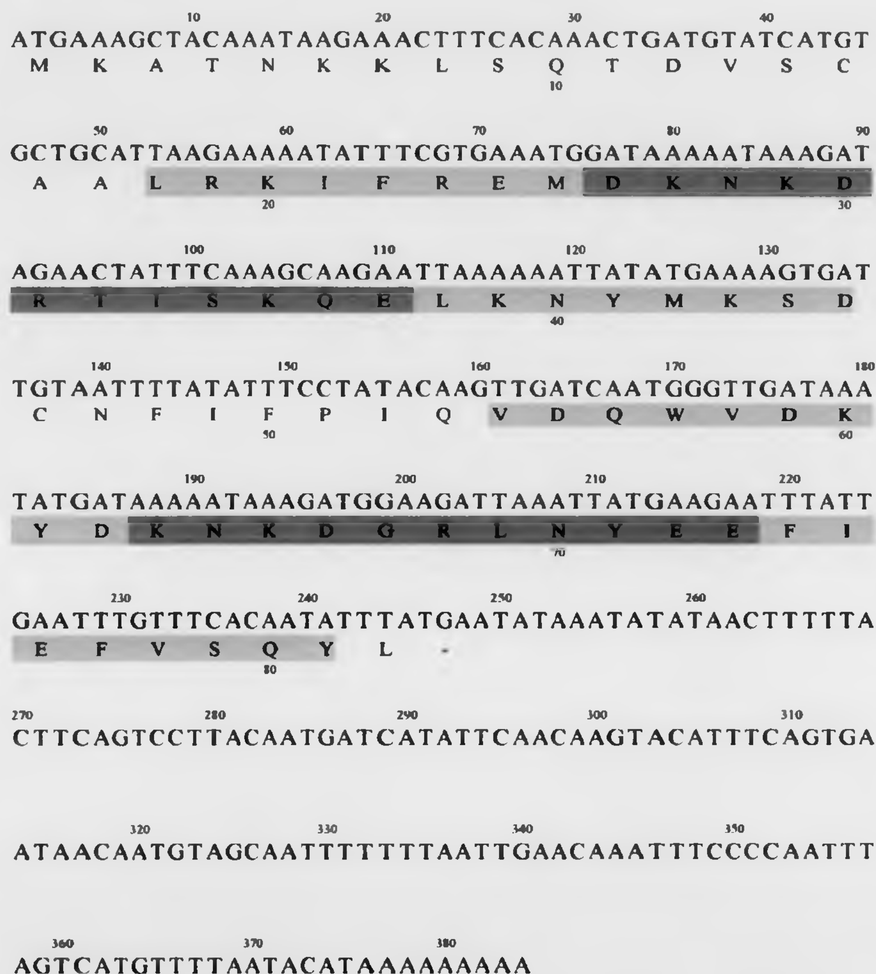
#### 7.2.2.2 Sequencing

(a) Sequencing of the DNA encoded by clone 15V

Purified λgt11 DNA from clone 15V was cut with the restriction enzymes *Kpn* I and *Sac* I. The resulting 2.5 kb product which contained the *Eco*R I cloning site and the cDNA insert, was then purified using low melting point agarose and subcloned into each of the *Kpn I* / *Sac* I cut M13 mp18 / mp19 paired vectors. The full length sequence of the cDNA encoded by clone 15V (393 bp) was obtained by sequencing from recombinant mp19 and mp18 with the forward and reverse λgt11 primers respectively (Figure 7.2). Analysis of the nucleotide sequence data obtained demonstrated that as with clone 18.5 (see Chapter 6) no *Eco*R I site was present at the junction of the 3′ end of the cDNA insert of clone 15V and the λgt11 DNA. Instead a string of nine adenosine residues lead directly into λgt11 sequence which is identical to that seen at the junction of λgt11 and the cDNA encoded by clone 18.5. This string of adenosine residues may represent the poly(A) tail, however, a polyadenylation signal (AATAA) was not present 10 to 30 residues downstream of the first of these adenosines, thus suggesting that these represent not the poly(A) tail but a string of adenosines within the 3′ untranslated region of the gene.

As the cDNA encoded by clone 15V had been expressed as a recombinant protein in λgt11, the reading frame of the subcloned *Kpn* I / *Sac* I fragment could be determined and the nucleotide sequence was translated (Figure 7.2). Analysis of the data obtained demonstrated that amino acid 1 of the translated sequence represented a methionine

**Figure 7.2** **The sequence of the peptide encoded by clone 15V**




The *Kpn* I / *Sac* I fragment from the lambda gt11 clone 15V was subcloned into the vectors M13 mp19 and mp18 and sequenced using the forward and reverse lambda gt11 primers respectively. The nucleotide sequence obtained is shown with the corresponding amino acid residues beneath. The shaded regions denote those residues which are involved in the formation of the putative calcium binding domain. The loop is represented by the darker shade and the helices by the lighter shade.

residue and that a TGA stop codon was present at nucleotide positions 246 - 248. Despite this it seems unlikely that clone 15V incorporates the whole coding region of the gene encoding a 15 kDa antigen, as translation from the methionine residue at amino acid position 1 through to the proposed stop codon would result in the production of a peptide with an approximate size of only 9.2 kDa. It therefore seems more likely that the residue at position 1 of the amino acid sequence represents an internal rather than the initiating methionine and that the 5′ end of the gene partially encoded by clone 15V is absent.

(b) Sequencing of the DNA encoded by clone 2.1

The PCR product obtained following amplification of the cDNA encoded by clone 2.1 was cut with *Eco*R I, purified on low melting point agarose and subcloned into similarly cut M13 mp18. The sequence obtained from just one clone demonstrated that the 5′ end of the cDNA insert encoded by clone 2.1 was identical to that of the cDNA encoded by clone 15V and hence confirms that the two clones do indeed encode part of the same gene. No additional sequence was obtained on sequencing of the insert from clone 2.1.

(c) Sequencing of the DNA encoded by clone 1.3

DNA obtained by PCR from the λgt11 clone 1.3 was purified on low melting point agarose and subcloned into the pGEM-T vector. Sequencing of one of the clones obtained, with the M13 (-40) reverse primer and the T3 forward primer gave non-overlapping sequence for the 5′ and 3′ ends of the DNA encoded. Comparison of this sequence with that already obtained demonstrate that the 5′ end of the cDNA encoded by clone 1.3 was identical to that of the cDNAs encoded by clones 15V and 2.1. Thus, these results demonstrated that clone 1.3 encodes part of the same gene as that encoded by clones 15V and 2.1 and that the larger size of the cDNA insert was not a consequence of the incorporation of additional 5′ sequence. Examination of the sequence obtained for the 3′ end of the cDNA insert of clone 1.3 demonstrated similarities to clone 15V and clone 18.5 (see Chapter 6) in that an *Eco*R I site was not present at the junction of the 3′ end of the cDNA insert with λgt11. Instead a stretch of seven adenosine residues leads directly into λgt11 sequence which is identical to that seen at the junction of the vector and the cDNAs encoded by clones 18.5 and 15V. However, as expected, the sequence immediately upstream of the seven 3′ adenosine residues encoded by clone 1.3 differed

from that seen immediately upstream of the string of nine adenosines at the 3′ end of clone 15V. These results therefore suggest that the large size of the insert encoded by clone 1.3 is due to the incorporation of a concatemer of different cDNA's or to the inclusion of an extremely long 3′ untranslated region. A polyadenylation signal was not seen towards the 3′ end of the cDNA encoded by clone 1.3 thus suggesting that the terminal string of seven adenosine residues may not represent a poly(A) tail.

### 7.2.3 ATTEMPTS TO OBTAIN THE 5′ END OF THE GENE ENCODING THE 15 KDA ANTIGEN

The above sequencing suggested that the 5′ end of the coding region of the gene encoding the 15 kDa antigen is absent from the cDNA inserts of all three of the clones isolated. Thus, in an attempt to obtain data for this region a PCR reaction was carried out using the λgt11 forward primer and a specially designed reverse primer which incorporates amino acids complementary to nucleotides 10 - 26 of the sequence already obtained for the 15 kDa antigen. The primers were used to amplify DNA from a high titre stock of λgt11 libraries constructed from adult or sporocyst cDNA. The products obtained were separated by electrophoresis on agarose gels (data not shown). Amplification of DNA within the adult cDNA library produced a number of diffuse bands which ranged in size from approximately 200 to 400 base pairs. However, separation and purification of these weak and diffuse bands using low melting point agarose proved problematic and was therefore abandoned. In contrast, a single, stronger and more distinct band of 120 bp was seen following amplification of DNA within the sporocyst cDNA library (data not shown). This appeared to be big enough to incorporate a portion of the gene encoding for the 15 kDa antigen in addition to the PCR primers and the λgt11 DNA present between the binding site of the forward primer and the *Eco*R I cloning site (total of 78 bp). Thus, the band was purified and subcloned by blunt ended ligation into the pGEM-T vector. Restriction with the enzyme *Pvu* II was then used to confirm the presence of the small insert within the pGEM clones isolated and sequencing of one of these clones was performed. Analysis of the data obtained demonstrated that the subcloned PCR product encoded only the two PCR primers and the λgt11 DNA found between the region to which the forward primer binds and the *Eco*R I cloning site. As no other bands were apparent following PCR from the sporocyst cDNA library these results therefore indicate that clones encoding the 5′ end of the gene partially encoded by clones 2.1, 1.3

and 15V are not present.

### 7.2.4 ANALYSIS OF THE AMINO ACID SEQUENCE OBTAINED FOR THE 15 KDA ANTIGEN

The partial nucleotide and amino acid sequence obtained for the 15 kDa antigen was used to search the SwissProt data base for homology to other molecules. No complete identity with molecules within the database was observed, however, homologies of up to 42% were seen with parvalbumin alpha (Zuehlke *et al.*, 1989), members of the troponin C superfamily (Carpenter *et al.*, 1984) and calcium binding proteins in general. Further examination of the data obtained revealed that this homology was limited to two particular regions of the sequence obtained for the 15 kDa antigen. These were similar to those encoding the calcium binding domains of the aforementioned molecules, thus suggesting a putative role for the 15 kDa antigen as a calcium binding protein. However, in order for a molecule to bind calcium it is essential that within the proposed calcium binding domain (CaBD) certain structurally and functionally important residues are conserved, as these allow the formation of an EF hand (see section 7.3). A test sequence detailing the residues required for the formation of such a motif has been published (Ram *et al.*, 1989) and a comparison between the test sequence and the appropriate regions of the 15 kDa antigen was made (Figure 7.3). Some variation was observed between the these two sequences, the most crucial of these being the absence of the central glycine in the loop structure of the first putative calcium binding domain (pCaBDI) of the 15 kDa antigen. A number of other residues within the helix region of both putative calcium binding domains were also not in accordance with the test sequence. However, as a number of functional $Ca^{2+}$ binding proteins have been described which lack perfect EF hands (Ram *et al.*, 1989, Havercroft *et al.*, 1990, Moser *et al.*, 1992) it was considered necessary to investigate experimentally the possibility of the 15 kDa antigen binding calcium.

### 7.2.5 SUBCLONING OF THE cDNA ENCODED BY CLONE 2.1 INTO A VECTOR SUITABLE FOR EXPRESSION

Recombinant antigen was required for use in experiments to assess the calcium binding ability of the 15 kDa antigen and for testing in immunisation studies. It was therefore considered necessary to subclone the DNA encoding P 2.1 into a suitable expression vector. Sequencing of all three clones isolated from the sporocyst cDNA

**Figure 7.3** Comparison of the proposed calcium binding sites of the 15 kDa antigen with the EF hand test sequence

| | ← | | | helix | | | | → | ← | | | | loop | | | | | | | → | ← | | | helix | | | | → |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Calcium ion binding positions | | | | | | | | | X | | Y | | Z | | -Y | | -X | | | -Z | | | | | | | | |
| EF hand test sequence | n | - | - | n | n | - | - | n | D | - | * | - | * | G | - | n | * | - | - | E | n | - | - | n | n | - | - | n |
| 15V (amino acids 18 - 45) | **L** | - | - | **I** | **F** | - | - | **M** | **D** | - | **N** | - | **D** | R | - | **I** | **S** | - | - | **E** | **L** | - | - | Y | **M** | - | - | **D** |
| 15V (amino acids 54 - 81) | **V** | - | - | **W** | **V** | - | - | Y | **D** | - | **N** | - | **D** | **G** | - | **L** | **N** | - | - | **E** | **F** | - | - | **F** | **V** | - | - | Y |

Amino acid residues 18 - 45 and 54 - 81 of the peptide encoded by clone 15V were aligned with the EF hand test sequence devised by Ram *et al.*, (1989). The loop structure comprises 6 $Ca^{2+}$ ion ligating positions (X, Y, Z, -X, -Y, -Z). * = any residue with side chain oxygen (D, E, N, Q, S, T). n = any non polar residue (A, D, E, F, I, K, L, M, P, R, V, W) and - = any amino acid. Amino acids shown in **bold** accord with the test sequence.

library demonstrated the absence of an *Eco*R I site at the junction of the 3′ end of the cDNA inserts and λgtII. Thus, excision of DNA from these clones and direct subcloning into an expression vector may have proved problematic. A PCR product obtained by amplification of the cDNA encoded by clone 2.1 was therefore subcloned by blunt ended ligation into the TA plasmid. This vector has a unique *Eco*R I site situated 8 bases downstream of the site into which PCR products are cloned. Five recombinant clones were produced which on restriction with *Eco*R I released inserts of approximately 400 bp. The insert from one of these clones was then subcloned into *Eco*R I digested pGEX.

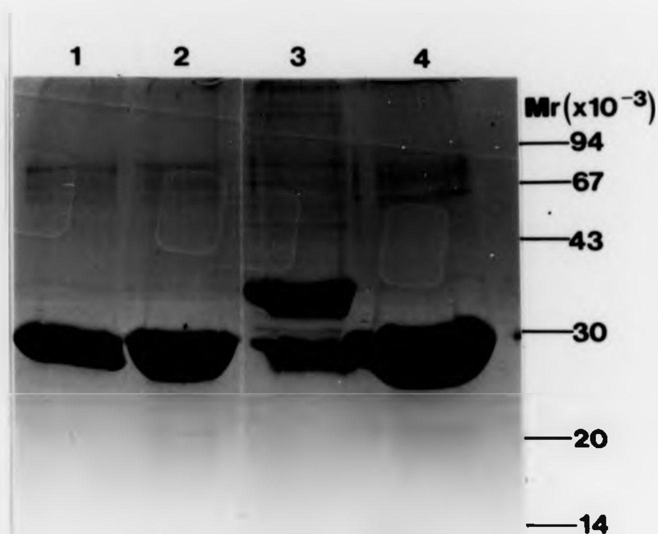
### 7.2.6 EXPRESSION OF THE RECOMBINANT ANTIGEN IN pGEX

The pGEX vector expresses foreign peptides as a fusion with the C-terminus of a 26 kDa *S. japonicum* GST. Recombinant proteins can therefore be separated from other bacterial products as a consequence of their ability to bind agarose beads coated with glutathione. Subcloning of the DNA insert excised from the TA vector into pGEX, produced five putative recombinant clones. The expression of proteins by these clones was then induced by the addition of IPTG to a high titre plasmid culture and the culture supernatant was rolled overnight with glutathione coated agarose beads. Bound proteins were eluted from the beads by boiling in SDS PAGE sample buffer and separated by SDS PAGE. Coomassie blue staining of the resulting gel demonstrated that a single recombinant clone expressed a fusion protein of approximately 36 kDa (FP 2.1/GST) (Figure 7.4). This represents *S. japonicum* 26 kDa GST fused to a 10 kDa (P 2.1) peptide of the 15 kDa antigen. A prominent protein band was also seen at 26 kDa and was presumed to represent GST derived from the degradation of FP 2.1/GST.

### 7.2.7 ANALYSIS OF THE CALCIUM BINDING ABILITY OF THE 15 KDA ANTIGEN

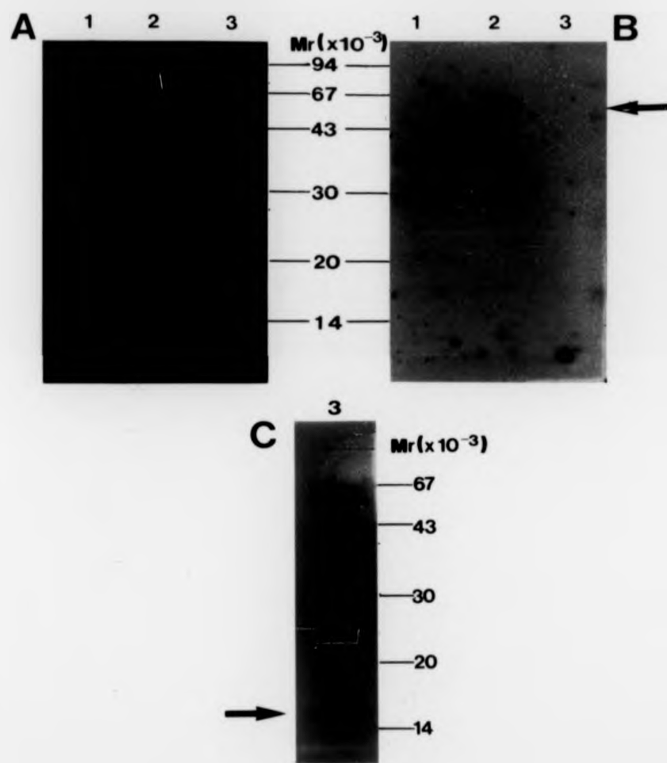
Multiple samples of the recombinant protein FP 2.1/GST, the native 15 kDa antigen within the aqueous phase following TX-114 extraction of MS and recombinant derived *S. japonicum* calpain, were subjected to SDS PAGE and Western blotting. Staining of part of the Western blot with amido black demonstrated that equivalent amounts of recombinant FP 2.1/GST and the calpain positive control were used (Figure 7.5 (A)). Moreover, probing of the aqueous phase antigens with sera raised against FP 2.1/GST (section 7.2.8.2) demonstrated that the native 15 kDa molecule was present (Figure 7.5

**Figure 7.4** The expression of FP 2.1/GST




The DNA insert of λgt11 clone 2.1 was amplified by PCR and subcloned into the pGEX expression vector. The expression of proteins by putative recombinants was then induced by the addition of IPTG to a high titre plasmid culture. Cleared bacterial lysate were rolled overnight with glutathione coated agarose beads and bound proteins were eluted from the beads by boiling in SDS PAGE sample buffer. The eluted proteins were then separated by SDS PAGE and the gel stained with Coomassie blue. A single recombinant clone expressed a fusion protein of approximately 36 kDa (FP 2.1/GST) (lane 3). The remaining clones expressed GST alone (lanes 1, 2 and 4).

**Figure 7.5 Investigating the calcium binding capacity of FP 2.1/GST**




Equivalent amounts of FP 2.1/GST (lane 1), recombinant derived *S. japonicum* calpain (lane 2) and aqueous phase antigens extracted from MS (lane 3) were electrophoresed in duplicate and transferred by Western blotting. One half of the blot was then stained with amido black (panel A) and the other probed with radiolabelled $^{45}$Ca (panel B). A single track of aqueous phase antigens was also probed with a 1 in 50 dilution of sera raised against FP 2.1/GST (panel C). The latter demonstrates the presence of the 15 kDa antigen amongst the aqueous phase antigens (arrowed in panel C). Only *S. japonicum* calpain showed binding of $^{45}$Ca (arrowed in panel B).

(C)). The remaining half of the blot was therefore probed with radioactive calcium ($^{45}Ca$) and overlaid on photographic film which was developed several weeks later. Figure 7.5(B) demonstrates that only *S. japonicum* calpain showed any ability to bind calcium.

### 7.2.8 IMMUNISATION OF MICE WITH FP 2.1/GST

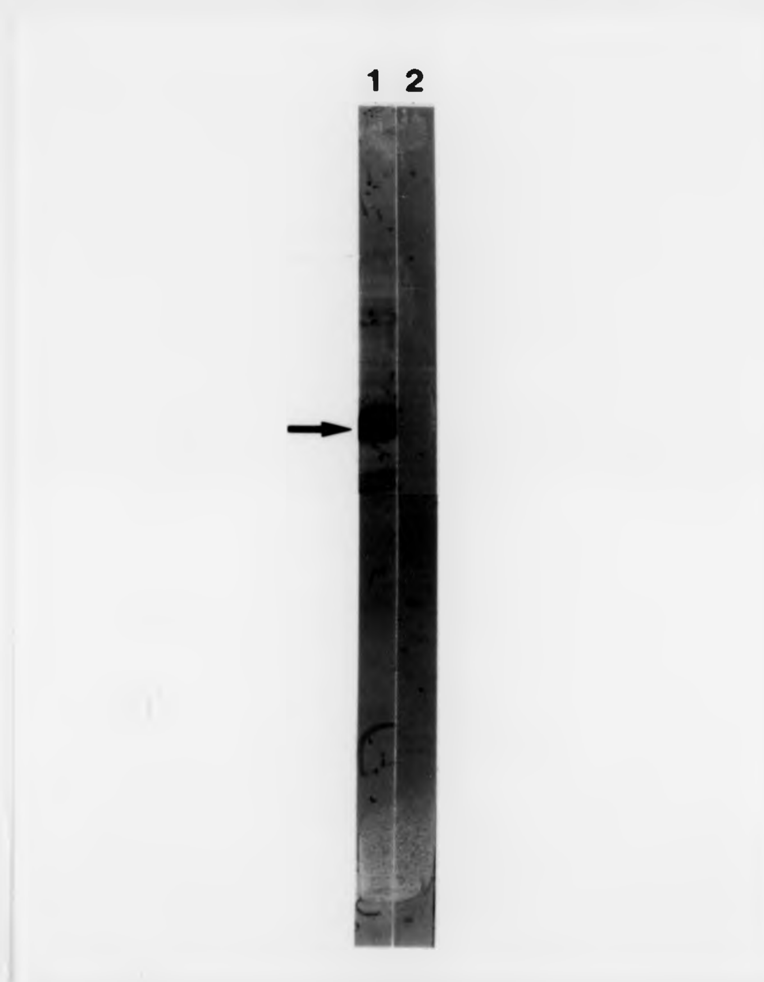
#### 7.2.8.1 Preparation of FP 2.1/GST for immunisation

Comparable amounts of FP 2.1/GST and GST expressed by non recombinant pGEX were separated by SDS PAGE, Western blotted and probed with rabbit sera. Figure 7.6 shows that unlike GST alone the recombinant protein was recognised by VRabS but not by NRabS. This demonstrated that the P 2.1 retains its antigenicity when expressed as a fusion with GST and thus FP 2.1/GST was suitable for use in immunisation studies. However, a further advantage of using the pGEX expression vector is that once a recombinant protein has been purified, the GST part of the fusion can be removed by cleavage with thrombin. FP 2.1/GST was therefore digested with this enzyme and the products examined by SDS PAGE. Figure 7.7 (A) demonstrates that on Coomassie blue staining a 26 kDa GST and a 10 kDa peptide (P 2.1) were observed as expected. However, as can be seen in Figure 7.7 (B) Western blotting of similarly cleaved material revealed that following removal of GST the 10 kDa peptide was only weakly recognised by VRabS. This indicated that the P 2.1 epitope(s) recognised by this sera rely upon the fusion with GST for their structural integrity, their antigenicity and possibly their immunogenicity. It was therefore decided to use the whole rather than the cleaved recombinant protein for immunisation.

#### 7.2.8.2 Immunisation of mice with FP 2.1/GST

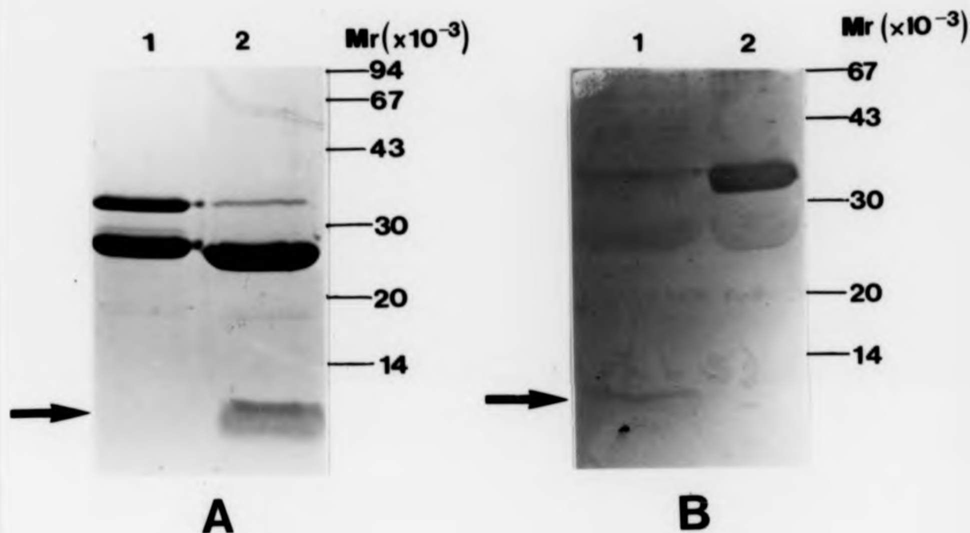
In a preliminary experiment to verify the immunogenicity of FP 2.1/GST and to identify the strain of mice which gives the most appropriate antibody response on immunisation, two female Balb/c and two female CBA mice were immunised with FP 2.1/GST plus CFA / IFA. Similar groups of control mice were immunised with either adjuvant alone or adjuvant plus GST expressed by non-recombinant pGEX. These preliminary experiments demonstrated that both strains of mice produced antibody which recognised FP 2.1/GST on Western blots. However, the sera raised in CBA mice immunised with FP 2.1/GST gave an equal signal when used to probe GST alone. In contrast, the response seen in FP 2.1/GST immunised Balb/c mice had a greater

**Figure 7.6** The antigenicity of FP 2.1/GST




A Western blot of FP 2.1/GST was cut into strips and probed with a 1 in 200 dilution of VRabS (lane 1) or NRabS (lane 2). The position of FP 2.1/GST is marked with an arrow.

**Figure 7.7** **Cleavage of FP 2.1/GST with thrombin**



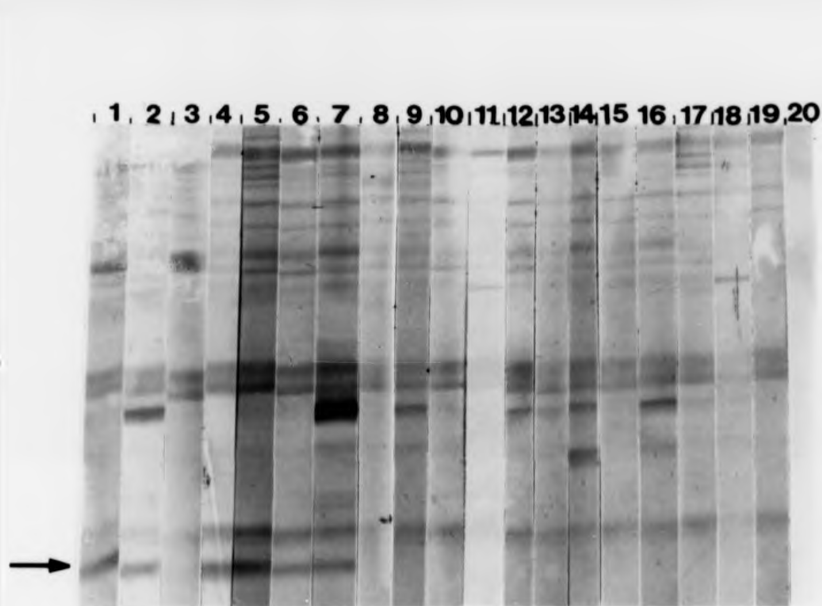

10 $\mu$g of FP 2.1/GST (lane 1) was electrophoresed alongside an equivalent amount of FP 2.1/GST digested with thrombin overnight at 25°C (lane 2). Gels were then stained with Coomassie blue (A) or Western blotted and probed with a 1 in 200 dilution of VRabS (B). The position of the thrombin released schistosome portion of the recombinant protein is marked with an arrow.

specificity for the fused peptide (data not shown). Thus, groups of 8 Balb/c mice were immunised with either FP 2.1/GST plus CFA / IFA, adjuvant alone or adjuvant plus non recombinant GST. Following the last of five injections mice immunised with FP 2.1/GST were shown to recognise a 15 kDa antigen on Western blots of the aqueous phase of a TX-114 extraction of MS (Figure 7.8). This molecule was not recognised by sera taken from mice in either of the control groups. The mice in all three groups were then challenged and perfused 5 weeks later. The average worm burden for each group is given in Table 7.1. As can be seen the group of mice immunised with FP 2.1/GST had a mean worm burden which was not significantly different to that of the control groups. However, a number of mice within the FP 2.1/GST immunised group did have worm burdens which were noticeably different to the group average. Sera taken from individual mice was therefore used to probe strips of a Western blot of TX-114 aqueous phase antigens. Figure 7.8 demonstrates that sera from one of the mice immunised with the recombinant protein did not recognise the 15 kDa native antigen (lane 3). Moreover, the signal seen with two other sera was slightly weaker than that obtained with the rest (lanes 6 and 7). However, comparison of the Western blots with the protection data revealed that these poor responders were amongst those mice with the lowest worm burdens. Thus, no correlation between recognition of the 15 kDa antigen and a low worm burden was observed. It should however be noted that the antibody response generated towards the 15 kDa antigen was in general poor. In all cases a 1 in 50 dilution of sera was required for recognition of the molecule on a Western blot.

### 7.2.9 IDENTIFICATION OF THE 15 KDA ANTIGEN IN DIFFERENT STAGES OF THE SCHISTOSOME LIFE CYCLE

The clones encoding the 15 kDa antigen characterised here were isolated from a sporocyst cDNA library and antibodies selected on these clones recognise the 15 kDa antigens amongst the aqueous phase MS antigens. Hence it has already been demonstrated that the 15 kDa molecule is present in the early stages of the schistosome life cycle. Here attempts were made to determine whether the molecule is also present amongst antigens extracted from adult worms or from eggs. Thus, a detergent extract of adult worms and a PBS extract of eggs (TDR, WHO) were separated by SDS PAGE, Western blotted and probed with the positive sera raised against FP 2.1/GST (section 7.2.8.2). Figure 7.9 demonstrates that unlike the stage specific 16 kDa antigen the 15 kDa antigen is present

Figure 7.8 **The recognition of the 15 kDa antigen by mice immunised with FP 2.1/GST**



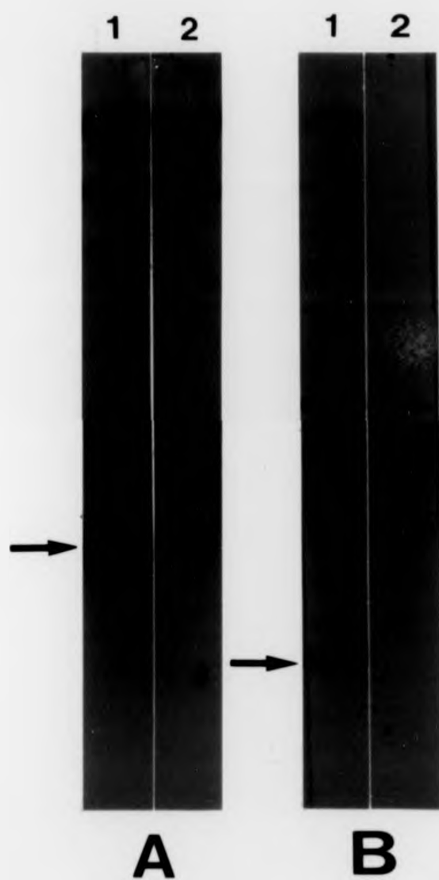

Mice immunised with FP 2.1/GST (lanes 1 - 7), non-recombinant GST (lanes 8 - 14) or CFA/IFA alone (lanes 15 - 20) were bled immediately prior to challenge and the sera obtained from individual animals used to probe strips of a Western blot of aqueous phase MS antigens. All of the sera were used at a dilution of 1 in 50 and the position of the 15 kDa antigen is marked with an arrow.

**TABLE 7.1** Immunisation of mice with FP 2.1/GST

| Number of mice per group | Immunisation protocol | Worm burden (+/- S.D.) | Reduction (%) | Significance |
|---|---|---|---|---|
| 6 | CFA / IFA adjuvant alone | 68.5 +/- 14.9 | - | |
| 7 | Non-recombinant GST plus CFA / IFA | 69.1 +/- 11.0 | - | |
| 7 | FP 2.1/GST plus CFA / IFA | 61.6 +/- 13.5 | 10.8% | $p < 0.25$ |

Female CBA mice were immunised s.c with FP 2.1/GST, non-recombinant GST or with adjuvant alone. The mice were then challenged with 200 cercariae 7 weeks after the last immunisation and killed and perfused 35 days later. The average worm burden for each of the groups was obtained and resistance calculated as described in Chapter 2.

Figure 7.9 **The recognition of the 15 kDa antigen amongst adult and egg antigens**




Strips of a Western blot of a detergent extract of adult worms (panel A) or soluble egg antigens (panel B) were probed with a 1 in 50 dilution of sera raised against FP 2.1/GST (lane 1) or non-recombinant GST (lane 2). The position of the 15 kDa antigen is marked with an arrow.

in both the adult worm (Figure 7.9 (A)) and the schistosome egg (Figure 7.9 (B)).

## 7.3 DISCUSSION

Two clones (clones 2.1 and 1.3) isolated from the sporocyst cDNA library were shown by antibody select methods to encode a 15 kDa antigen present within the aqueous phase of a TX-114 extract of MS. This antigen was confirmed as being the same as that encoded by a third clone (clone 15V) which was isolated by Francis (1989) upon screening of the same cDNA library.

The exclusion of the 15 kDa molecule from the detergent phase of a TX-114 extract of MS demonstrates that this antigen is not integral to the membrane and hence suggests either an intracellular location or an extrinsic / peripheral association with the parasite surface (Towler *et al.*, 1988). That the 15 kDa antigen shows some homology to a family of EF hand bearing intracellular calcium binding proteins (see below) indicates that perhaps the former is more likely. Despite their location a number of intracellular enzymes and muscle components are found amongst the most promising of the vaccine candidate antigens thus far described (e.g. GST, paramyosin, TPI). In some cases (e.g. TPI) the molecule is believed to be located adjacent to the surface membrane, for example, within the cells of the tegument and to become transiently exposed upon the surface of the parasite during the process of transformation (Harn *et al.*, 1992). In other cases (e.g. GST) the antigen has been shown to be amongst a highly immunogenic group of molecules released from the surface of the parasite (Capron *et al.*, 1987). EF hand intracellular calcium binding proteins such as the calmodulin homologue Sm20, have already been observed in cells within the tegument of the schistosomula (Havercroft *et al.*, 1990). Thus, a similar location and hence release or transient surface expression of the intracellular 15 kDa antigen described here seems possible.

The 15 kDa antigen is of particular interest as the recombinant peptide expressed by clone 2.1 (FP 2.1/ß-gal) was recognised predominantly by sera from animals vaccinated with irradiated cercariae. However, FP 2.1/ß-gal was not seen by VRatS which is passively protective nor by sera from vaccinated C57Bl/10 mice which develop high levels of protection. The absence of recognition by VMS(C57) could be attributed to a genetically restricted (H-2) inability of this particular strain of mouse to recognise the 15 kDa antigen. The studies of Richter and Harn (1993) and Richter *et al.*, (1993) have demonstrated that a different pattern of antigen recognition develops in CBA and

C57Bl/10 mice following vaccination with irradiated cercariae. For example, the recognition of a recombinant form of paramyosin was shown to be largely restricted to VMS raised in CBA mice, an observation which has been confirmed by the studies carried out here (see Figure 7.1). Alternatively, it is possible that VMS(C57) and VRatS recognise epitopes present on the native 15 kDa molecule which were not expressed by FP 2.1/ß-gal. Attempts to determine here whether VMS(C57) and VRatS recognise the native 15 kDa antigen on Western blots were unsuccessful as the large number of low molecular weight antigens seen by these sera ensured that it was impossible to determine definitively whether the 15 kDa molecule was amongst them (data not shown). The vaccine dominant nature of the 15 kDa antigen is somewhat surprising as the molecule is present in the egg and the adult as well as in the schistosomula stages of the parasite. This suggests that the 15 kDa antigen may be immunogenic only upon death of the irradiated schistosomula in the lungs, or that the restricted recognition of the molecule may arise as a direct result of structural changes which occur during the irradiation process itself. Radiation damage to the glycocalyx has been shown to allow normally unexposed molecules to be made available on the surface of the parasite (see Chapter 4). Furthermore, irradiation inhibits protein and glycoprotein synthesis and it has been observed that some secretory molecules may be considerably altered and more easily processed following such treatment (Kusel *et al.*, 1989).

Having identified the 15 kDa molecule as a vaccine dominant antigen, sequence data was obtained for the cDNA inserts of clones 15V, 2.1 and 1.3. The large size of the cDNA encoded by clone 1.3 fostered hopes that it may represent the full length gene encoding the 15 kDa antigen. However, analysis of the sequence data obtained indicated that all three clones lacked the 5′ end of the coding region. More surprisingly, all three clones were shown to have identical 5′ termini. It is possible that this could have arisen via the cleavage of an internal *Eco*R I site within the gene encoding the 15 kDa antigen which has enabled cDNAs to be ligated to λgt11 without the addition of *Eco*R I linkers. Such an internal site should have been protected by methylation and as none of the clones thus far isolated from this library have been definitively shown to possess an insert cleaved at an internal *Eco*R I site it is not possible to conclude that this process was inefficient. However, the *Eco*R I linkers used during the construction of the cDNA sporocyst library do possess a cysteine residue 3′ to the *Eco*R I cloning site (i.e. GAATTC<u>C</u>). Hence if ligation had occurred between the λgt11 vector and an *Eco*R I cut

linker attached to the 5′ end of the synthesised cDNA, a sequence of GAATTCC would be expected a the junction of λgt11 and the 5′ end of the cDNA inserts of clones 15V, 2.1 and 1.3. The GAATTC sequence which was seen at the junction of λgt11 and the 5′ end of the cDNAs encoded by these three clones therefore suggests that ligation has occurred between the vector DNA and a *Eco*R I cut internal restriction site situated towards the 5′ end of the gene encoding the 15 kDa antigen. It also became apparent on sequencing clones 15V and 1.3 that as with clone 18.5 (see Chapter 6) an *Eco*R I site was not present at the junction between the 3′ termini of the encoded cDNAs and λgt11. Other clones selected from this library have also been shown to lack such a site (Dr Q. Bickle, personal communication). The most likely explanation for this occurrence is that during the construction of the cDNA library *Eco*R I linkers were not ligated to the ends of a number of synthesised cDNAs. In view of the presence of an *Eco*R I site at the junction of the vector and the 5′ end of the cDNA inserts of a number of isolated clones, it was initially assumed that this applied only to the 3′ termini. However, if as suggested above the cleavage of an internal *Eco*R I site has resulted in the formation of 5′ *Eco*R I cut ends it is possible that linkers have not been ligated to either end of some cDNAs. This could be due to an inability of the T4 DNA polymerase used to fill in the ragged ends of the synthesised cDNA and thus produce blunt ends to which the linkers can ligate. Alternatively linkers may not have been added due to an inefficiency in the ligation reaction itself. Despite the absence of 3′ *Eco*R I cut linkers the cDNAs encoded by clones 1.3, 15V and 18.5 have ligated to the λgt11 vector. Analysis of the data obtained here revealed that in each case this ligation had taken place between 3′ cDNA sequence which was represented by a string of adenosine residues and λgt11 sequence represented by the *Eco*R I cut end from which the 5′ sequence AATT had been removed. The removal of these residues from the *Eco*R I cut end of the M13 vector had already been seen to enable the subcloning of the 18.5 PCR product (see Chapter 6) and may be a consequence of exonuclease activity in one of the solutions used either for *Eco*R I restriction or ligation. The results obtained here suggest that, similarly 'nibbled' λgt11 DNA has a particular ability to join via a blunt ended ligation to cDNAs ending in a 3′ string of adenosine residues.

Although all three of the clones encoding the 15 kDa antigen had identical 5′ termini the 3′ terminus of clone 1.3 was seen to differ from that of clone 15V. This was not unexpected in view of the large size of the 1.3 cDNA insert and suggested that this insert

may represent either a concatemer of cDNAs, which starts with that encoded by clones 2.1 and 15V, or the cDNA encoded by clone 15V plus 3′ untranslated cDNA which is absent from the shorter clones. The latter is not inconsistent with the observation that the 3′ end of the 15V sequence did not contain a polyadenylation signal upstream of the terminal adenosines which might therefore be upstream of the true Poly(A) tail. However, if the cDNA encoded by clone 1.3 does represent the product of a single 15V gene it would include an untranslated region of approximately 1.3 kb. Moreover, the absence of a polyadenylation signal at the 3′ end of the cDNA encoded by clone 1.3 also suggested that the string of adenosines at the 3′ end of this clone did not represent a Poly(A) tail. It should however be noted that whilst most characterised *S. mansoni* cDNAs have been reported to have the AATAA signal upstream of the Poly(A) tail, exceptions to the rule are common (Wright *et al.*, 1990, Laclette *et al.*, 1991). Alternatively, if the cDNA insert encoded by clone 1.3 represents a concatemer of cDNAs joined via the ligation of *Eco*R I cut ends, it should be susceptible to *Eco*R I digestion. The PCR product from clone 1.3 was not susceptible to such cleavage (data not shown). However, as a number of clones isolated from the cDNA sporocyst library have been shown to contain cDNA inserts which lack a 3′ *Eco*R I site it seems possible that some non-conventional ligation of cDNAs may have occurred and resulted in the formation of concatemers which are resistant to *Eco*R I digestion. Northern blotting could be used to establish the size of the mRNA representing the gene partially encoded by clone 15V and hence determine the length of the untranslated regions and the likelihood of clone 1.3 encoding the product of a single gene. However, this was not a priority in the present studies as attention was focused instead upon obtaining data for the 5′ end of the coding region of the gene. Attempts to use a 15V specific 5′ oligonucleotide and the λgt11 forward primer for amplifying DNA encoding this region from the cDNA sporocyst library were unsuccessful and resulted in the amplification and subcloning of a single band which proved to be the PCR primers. The absence of other PCR products suggested that the cDNA encoding the 5′ region of the 15V gene is not present within the cDNA sporocyst library. This could be a consequence of the cleavage of an internal *Eco*R I site following inefficient methylation of the cDNA used in its construction. A similar experiment using the above primers to amplify DNA within an adult cDNA library did however result in the production of several bands of various sizes. As a consequence of problems encountered during attempts to purify these weak, diffuse bands, further work was not carried out on

these PCR products here. However, were further attempts to obtain the complete sequence of the gene encoding the 15 kDa antigen to be made, purification, subcloning and sequencing of these PCR products may prove successful.

Despite the absence of data for the 5′ end of the coding region of the gene encoding the 15 kDa antigen, sequence representing 393 nucleotides and hence 82 amino acids was obtained. This was used to search the Swissprot computer database whereby it was revealed that the 15 kDa antigen shared some homology with members of a family of calcium binding proteins (CaBPs). CaBPs regulate the levels of systemic and intracellular $Ca^{2+}$ and thereby control processes as diverse as muscle contraction, enzyme activation and exocytosis. They can be broadly separated into two groups; the transmembrane calcium transporters and the intracellular calcium binding proteins (Stewart *et al.*, 1992). The latter includes those possessing a calcium binding domain known as an EF hand, with which the 15 kDa antigen has some homology. Within this particular group of CaBPs a further functional distinction can be made. The regulatory EF hand CaBPs such as troponin C and calmodulin, undergo a change in conformation on binding calcium which enables them to interact with particular molecules or enzymes often in specific cellular compartments. This leads to the generation of a specific action, for example, muscle contraction in the case of troponin C. In contrast, the second functional group of EF hand CaBPs act as physiological $Ca^{2+}$ buffers and thus play a role in modulating the level of intracellular $Ca^{2+}$ One such molecule is parvalbumin, a protein located primarily in fast twitch muscles. Following relaxation of the muscle parvalbumin functions by binding the $Ca^{2+}$ released by other CaBPs such as troponin C and thus preventing the immediate re-initiation of muscle contraction (Strynadka and James, 1989). It is with parvalbumin that the 15 kDa antigen shares the greatest degree of homology. A number of CaBPs possessing EF hands have already been described in *S. mansoni*. These include a 20 kDa antigen with homology to calmodulin which is located in the muscle cells of all stages of the parasite (Havercroft *et al.*, 1990, Stewart *et al.*, 1992); an antigen of approximately 60 kDa which has homology to calpain and is located in the sporocyst and adult stages (Andresen *et al.*, 1991); a 9 kDa antigen, also with homology to calmodulin which is transiently expressed in cercariae and early schistosomula (Ram *et al.*, 1989) and a 16 kDa antigen, again with homology to calmodulin, which is found only in the schistosome egg (Moser *et al.*, 1992). A number of CaBPs have also been identified in the envelope and apical membrane of the adult worm (Siddiqui *et al.*, 1991) however these have not

yet been characterised and it is not known if they possess an EF hand motif.

The motif known as the EF hand is in fact a structure which consists of a twelve amino acid loop surrounded by two, twelve residue $\alpha$ helices. In almost all CaBPs this motif occurs in pairs which are separated by only five to ten amino acid residues. Typically the loops of these paired domains interact via anti-parallel ß sheet hydrogen bonds to form the bottom of a "cup" like structure, the sides of which are formed by the four amphipathic $\alpha$ helices. A $Ca^{2+}$ ion binds to each loop via seven oxygen ligands, six of which are provided by the side chains of five amino acid residues within the loop (X, Y, -Y, Z, -Z, see Figure 7.3) and the seventh by an associated water molecule (-X). Other residues present within the loop stabilise its structure via the formation of hydrogen bonds (Strynadka and James, 1989). Thus, in order to form an EF hand, particular residues are required at specific positions within a putative $Ca^{2+}$ binding domain (CaBD) and a consensus sequence which indicates these residues has therefore been derived (Ram *et al.*, 1989). Comparison of this consensus sequence with the sequence for the putative calcium binding domains of the 15 kDa antigen demonstrated that some differences were apparent. However, as it has been shown previously that many CaBPs function despite incomplete identity with the consensus sequence, the ability of both the native 15 kDa antigen and the recombinant protein (F.P 2.1/GST) to bind radiolabelled calcium was examined experimentally. The results obtained demonstrated that the 15 kDa antigen was non-functional as a CaBP. It seems most probable that the failure of the first putative calcium binding domain (pCaBDI) (amino acids 18 - 45) of the 15 kDa molecule to bind calcium is a consequence of the substitution by arginine of the invariant glycine residue at position six within its loop structure. This glycine residue normally assists the peptide chain to make a $90^{\circ}$ turn and thus ensures that the remaining $Ca^{2+}$ ligands are in coordinating positions. Although replacement of this residue has been observed in the loop of the functional intestinal CaBP (Strynadka and James, 1989) two additional amino acids are also present in the early part of this loop which enable it to turn instead at an asparagine residue at position 8. pCaBDI of the 15 kDa antigen does not have these extra amino acids present in the early part of its loop nor the asparagine residue at position 8. It is therefore unlikely that this loop turns despite the absence of the central glycine residue. A substitution of this central glycine has also been described for a 21.7 kDa putative CaBP encoded by another clone isolated from the cDNA library screened here. Computer modelling of the structure of this antigen predicted that the presence of a side

chain on the non-glycine residue at position 6 of its putative calcium binding domain disrupted the structure of the loop and thus prevented the binding of calcium (Francis and Bickle, 1992). Although calcium binding domains are almost always paired, in many cases only one of the pair is required to be functional for the protein to bind some calcium (Andresen *et al.*, 1991, Stewart *et al.*, 1992). Thus, it might have been expected that binding of calcium to the 15 kDa antigen would have occurred via the second putative calcium domain (pCaBDII, amino acids 54 - 81) which, unlike the first, incorporates a loop which showed complete accordance with the consensus sequence. However, as stated above the 15 kDa antigen failed to bind any calcium at all thus suggesting that pCaBDII was also non-functional. The inability of pCaBDII to bind calcium may be a consequence of its close proximity to pCaBDI. As described earlier the deviations from the consensus sequence which are seen in the loop of pCaBDI are likely to result in an alteration in the overall structure of this region. Hence the conformation and the calcium binding ability of the closely associated pCaBDII may also be effected. Alternatively, the deviations from the consensus sequence within the helices of both putative calcium binding domains may play a role in their lack of $Ca^{2+}$ binding. Both helices of pCaBDII and the C-terminal helix of pCaBDI are imperfect in that polar tyrosines are present in some of the positions which ideally require a hydrophobic residue. Many CaBPs, including some of those cloned from *S. mansoni*, have been observed to function despite similar alterations. However, the use of the PC/Gene sequence analysis programme to predict the secondary structure of the 15 kDa antigen demonstrated that here such substitutions had resulted in a drastic shortening of the C-terminal helix of pCaBDI and the N-terminal helix of pCaBDII. Moreover, the C-terminal "helix" of pCaBDII was represented by a completely extended conformation which may well contribute to the inability of the 15 kDa antigen to bind calcium. Despite the failure of the 15 kDa antigen to bind calcium, it seems likely that the molecule evolved from an ancestral protein which contained functional EF hand binding domains. Mutation of a single nucleotide could have led to the replacement of the glycine residue usually seen at loop position 6 with the arginine residue observed. Similarly the tyrosine residues responsible for the absence of $\alpha$ helical conformation could have arisen from a single mutation of hydrophobic residues such as aspartate and phenylalanine. Thus, in addition to the 21.7 kDa antigen described by Francis and Bickle (1992), the 15 kDa antigen described here has been shown to represent a non-functional EF hand containing protein.

The reasons for the apparent loss of function of these putative CaBPs encoded by the sporocyst cDNA are unknown, as is the current function of these molecules within the parasite.

In order to obtain antigen for assessing the protective potential of the 15 kDa antigen, a blunt ended PCR product from clone 2.1 was subcloned into the TA vector, excised with *Eco*R I and subcloned into pGEX. Of the five recombinant clones produced, only one expressed a fusion protein of 36 kDa (FP 2.1/GST). This represented the 26 kDa *S. japonicum* GST fused to a 10 kDa peptide (P 2.1). As judged by the probing of Western blots with VRabS removal of GST from FP 2.1/GST via cleavage with thrombin resulted in a substantial decrease in the antigenicity of P 2.1. Thus, uncleaved FP 2.1/GST was used to immunise a group of 8 Balb/c mice and a group of mice immunised with non-recombinant GST were included in addition to the normal adjuvant alone control. Despite the recognition of Western blotted, parasite derived 15 kDa antigen by sera raised in mice immunised with FP 2.1/GST, no significant protection against a challenge infection was observed. Comparison of the average worm burden of the group immunised with non-recombinant GST to that of mice immunised with adjuvant alone also demonstrated that, as expected, immunisation with *S. japonicum* GST did not protect mice against a *S. mansoni* challenge infection. Analysis of individual sera taken from mice immunised with FP 2.1/GST did demonstrate that some of the mice did not produce a detectable antibody response against the 15 kDa antigen. Furthermore, sera taken from those mice which did recognise the 15 kDa molecule gave a signal which was weak even when a 1 : 50 dilution of sera was used. Thus, it is possible that a low antibody titre could contribute to the lack of protection seen in the FP 2.1/GST immunised mice. However, this explanation seems unlikely as comparison of the protection data with the blots probed with sera from individual mice demonstrated that those mice giving the strongest antibody response following immunisation were amongst those with the higher worm burdens. Alternatively, the lack of protection observed could be a consequence of the recognition of inappropriate epitopes on the native molecule or of the production of antibodies of an inappropriate isotype. It is therefore difficult to conclude definitively that a protective response can not be obtained on immunisation with the 15 kDa antigen and the use of a different route of immunisation or of a different adjuvant could be considered. However, the results obtained here do suggest that despite its vaccine dominant nature, the 15 kDa molecule has no potential as a vaccine candidate antigen.

Finally, it seems unlikely that the 15 kDa antigen described here (and by Francis (1989)) represents any previously described molecule. A 15 kDa vaccine dominant antigen was observed by Simpson *et al.*, (1983a) amongst a detergent extract of $^{125}I$ labelled schistosomular antigens. However, this molecule appeared to be expressed on the surface of the schistosomula and was larval specific (Omer Ali *et al.*, 1989). Similarly, a vaccine dominant 15 kDa antigen was described amongst the antigens present in radiolabelled tegumental membranes of the adult worm (Simpson *et al.*, 1989). This antigen was shown to separate into the detergent phase of a TX-114 extract of homogenised worms and is therefore unlikely to represent the 15 kDa antigen seen here to be extracted by aqueous buffers. Lastly, a 15 kDa antigen has been described amongst the molecules of the schistosome glycocalyx (Dalton *et al.*, 1987b). This is lost on transformation of the cercariae and hence was not present within latter stages of the life cycle.

Thus, it appears that the 15 kDa vaccine dominant antigen detailed here represents a previously undescribed antigen which although non functional is homologous to members of the EF hand calcium binding proteins.

# CHAPTER 8
# GENERAL DISCUSSION

## 8.1 GENERAL DISCUSSION

Despite the success of a number of nationally organised integrated control programmes, the overall global estimate of the number of people infected with schistosomes has not decreased within the past decade. It has therefore been accepted that new methods of controlling this important helminthic disease are required and much of recent research has focused upon attempts to develop a suitable vaccine. The feasibility of achieving this aim is supported by the demonstration that protective immunity can be stimulated in a range of animal models following immunisation with radiation attenuated parasites (reviewed by Taylor, 1994). Moreover, there is an ever increasing body of evidence to suggest that immunity develops, albeit slowly, in naturally infected humans (Butterworth *et al.*, 1984, 1985, Wilkins *et al.*, 1984).

A wide variety of strategies have been employed with the aim of identifying molecules suitable for incorporation within a vaccine against schistosomiasis. As a result a number of promising vaccine candidate antigens have been identified. These include both intracellular and surface molecules, cross reactive and species specific molecules and molecules which are located on particular, or on all stages of the parasite. Studies in rodents and in non-human primates have demonstrated that a number of these antigens are able to stimulate protective immunity either in their native form and / or as recombinant molecules (Harn *et al.*, 1987b, Balloul *et al.*, 1987a, 1987c, Pearce *et al.*, 1988, Soisson *et al.*, 1992). However, many fail to stimulate levels of resistance equal to those obtained following immunisation with irradiated parasites and as yet none have been considered suitable for use in human trials. Thus, the search for vaccine candidate antigens continues.

The higher levels of resistance observed following vaccination with irradiated parasites as opposed to a non-attenuated infection indicate that the identification of vaccine dominant molecules may be a valid approach towards the selection of putative vaccine candidate antigens. Such molecules can be defined as those recognised by sera raised in animals vaccinated with irradiated parasites but not by sera raised in animals harbouring a chronic (CIS) or a single sex infection (SSS). This strategy does have its limitations, as some defined antigens which are recognised by CIS and SSS as well as by sera from vaccinated animals (e.g. the 38 kDa surface glycoprotein ), have previously been shown to stimulate protective immunity when presented in the absence of the inappropriate responses which are generated against them in chronically infected mice. However,

schistosomes are complex organisms and when searching for vaccine candidate antigens it is necessary to employ some sort of criterion aimed at decreasing the number of molecules upon which further studies are performed. The isolation of vaccine dominant antigens has been used successfully to identify the 200 kDa schistosome myosin protein which is perhaps the most promising of the newer vaccine candidate molecules (Dalton and Strand, 1987, Soisson *et al.*, 1992, Soisson *et al.*, 1993). The 16 kDa and 15 kDa antigens which are the focus of the research described here are also vaccine dominant.

The 16 kDa antigen which was originally identified as the target antigen of the protective McAb, M7B3A (Bickle *et al.*, 1986) is present upon the surface of the early schistosomula and is the major antigen recognised following the probing of Western blots of cercarial and MS antigens with VRabS. It is not, however, found amongst soluble egg antigens nor is it labelled following the probing of cross-sections of adult worms with B3A (Dr. Q. Bickle, personal communication). Although highly immunogenic in the context of the irradiated vaccine, the 16 kDa molecule is of low abundance in both MS and cercariae (Bickle *et al.*, 1986, Chapter 3). This has proved a limiting factor throughout the course of this project although initial studies were aimed at counteracting this problem by optimising the techniques used for obtaining and purifying the 16 kDa antigen. Previous studies had demonstrated the 16 kDa antigen to be an integral membrane molecule requiring detergent for extraction. Here extraction of almost all of the available antigen was achieved by incubation of the Tris insoluble fraction of sonicated MS with the non-ionic detergent OTG. Removal of the soluble antigens prior to detergent extraction also provided a significant initial enrichment for the 16 kDa antigen and this protocol was therefore used routinely for large scale preparations. A resistance to extraction with low CMC detergents (e.g. Triton / NP40) coupled with a susceptibility to extraction by some detergents with a high CMC (e.g. MEGA-10, OTG) was also noted during the performance of experiments aimed at optimising the extraction protocol. This suggested that the 16 kDa antigen may be held within the surface membrane via a GPI linkage (Hooper and Turner, 1988, Hooper and Bashir, 1991), although the resistance of the molecule to extraction with other high CMC detergents (e.g. DOC, CHAPS) and to cleavage with the GPI specific enzyme PIPLC (Dr. Q. Bickle, personal communication) contradicts this suggestion. It therefore seems more likely that the requirement for strong detergents may be due to the 16 kDa antigen being somehow linked to internal elements of the parasite. However, clones should not be discounted from putatively encoding the

16 kDa antigen solely on the basis of their lacking a region of hydrophobic peptide sequence suitable for spanning the lipid bilayer.

The extent to which the 16 kDa antigen was able to be purified following McAb affinity purification was disappointing, as a number of contaminating molecules were observed within the fractions containing the majority of the 16 kDa molecule. It was also hoped that lectin affinity chromatography may have provided a useful additional purification step as the 16 kDa antigen was the only molecule recognised by PNA when biotinylated lectin was used to probe Western blots of MS antigens. The specificity of the binding of the biotinylated lectin to the 16 kDa molecule was demonstrated by the inclusion of lactose during probing of the Western blots. Moreover, the signal was not negated by the addition of the weaker inhibitor galactose, thus suggesting that the interaction between PNA and the 16 kDa antigen was of a fairly high affinity. The latter proved however to be problematic as following the passage of the immunoaffinity purified 16 kDa antigen down a column of PNA agarose coated beads, the multivalent interactions between the beads and the 16 kDa antigen were so strong as to be irreversible. The high affinity of this interaction is somewhat surprising as subsequent analysis of the results of Nyame *et al* (1987, 1988a, 1988b, 1989) coupled with the observations made here regarding the absence of the O-linked disaccharide Gal ß (1-3) GalNAc, suggested that PNA binds to the 16 kDa antigen via recognition of the monosaccharide ß-galactose. Although PNA binds to this simple sugar, it does so with a low affinity and hence it is surprising that the 16 kDa antigen was not able to be eluted from the PNA agarose column.

Although complete purification of the 16 kDa antigen was not obtained, substantial enrichment was achieved. Whereas the molecule had previously been observed only on probing Western blots with antisera, it was seen here by Coomassie blue and silver staining to be the major component of the best column eluates. It should also be noted that the 16 kDa antigen was shown to be a glycoprotein and as glycosylated molecules are often poorly stained with Coomassie blue and silver nitrate, the actual amount of antigen obtained may have been greater than estimated. Staining of gels with the carbohydrate specific Periodic acid Schiff reagent was considered. However, this method would not have provided any additional information regarding the amount of 16 kDa antigen obtained, as the levels of staining observed with this reagent cannot be quantified unless it is known exactly to what extent an antigen is glycosylated.

Although some contaminating molecules were observed, the 16 kDa antigen was the major component of several column eluates. On consideration of this, the failure of other techniques to enhance the purity of the immunoaffinity purified 16 kDa molecule, and the relative lack of larval material available to continue investigating the potential of other purification methods, it was decided to use the immunoaffinity column eluates for the immunisation of mice. Several different adjuvants were used in conjunction with the 16 kDa antigen and although none of the immunisation protocols induced high levels of protection, some resistance was seen following immunisation with the 16 kDa antigen incorporated into ISCOMs and with the antigen plus novasomes. In the case of the latter the reduction obtained was statistically significant, a feature which was of particular interest, as in addition to providing the highest level of protection, this protocol also induced an immune response which differed from that seen following immunisation with the antigen plus other adjuvants. Firstly, an IgG1 type response was observed in addition to the predominant IgG2a response which was seen following all immunisation protocols. Secondly, periodate insensitive epitopes of the 16 kDa antigen were recognised by NOV(S) as opposed to periodate sensitive carbohydrate entities. These results suggested that the recognition of peptide epitopes by antibodies of the subclass IgG1 may be important for the generation of immunity at least in this model.

It could be argued that the low but significant levels of immunity generated following immunisation with the 16 kDa antigen plus novasomes are also encouraging as they were observed despite the poor antibody titres obtained. A 1 in 500 dilution of NOV(S) was required for the recognition of affinity purified 16 kDa antigen on Western blots. The importance of antibody titre to specific larval surface antigens in immunity to *S. mansoni* has been strongly suggested by the extremely close correlation between immunity and antibody levels to the 200 kDa vaccine candidate antigen in baboons vaccinated with irradiated cercariae or the purified recombinant antigen (Soisson *et al.*, 1993, section 1.9.2.3(a)). Moreover, a tendency towards lower worm burdens was observed in mice which had higher anti-16 kDa antibody titres following immunisation with the purified antigen plus novasomes. It is therefore possible that increasing the antibody titres against the 16 kDa antigen may generate a higher level of resistance and were further immunisations to be carried out it would be important to examine various ways of achieving this aim. These should include increasing the amounts of antigen used for each immunisation and / or altering the timing and frequency of injections. It would also be

of interest to examine the protective potential of the 16 kDa antigen in other animals. Immunisation of rats with the molecule in conjunction with various adjuvants is perhaps the most obvious choice as rats generate high levels of protection following vaccination with radiation attenuated parasites. Moreover, protective immunity in the rat is clearly dependent upon antibody mediated mechanisms and is believed to be based upon the generation of ADCC type reactions which could involve IgG1. This animal may therefore be a particularly good model for the generation of protection following immunisation with the 16 kDa antigen plus novasomes.

The possibility of trying various immunisation protocols with the 16 kDa antigen is however limited by the small amounts of immunoaffinity purified 16 kDa antigen which are available. It therefore seems likely that any further immunisation studies would need to be deferred until an alternative immunogen could be developed. In this regard, attempts were made here to identify a clone encoding the 16 kDa antigen, a procedure which could enable the peptide component of the antigen to be sequenced and produced in substantial quantities either as a recombinant or synthetic molecule. That the 16 kDa molecule incorporates a peptide component was confirmed by its susceptibility to proteases, that this peptide component would be suitably immunogenic is suggested by the demonstration that NOV(S) and more importantly passively protective VRabS, recognised epitopes of a periodate insensitive nature. Here approximately 160,000 recombinant clones from a sporocyst cDNA library were screened using antibodies eluted from the 16 kDa region of Western blots incubated with VRabS. No clones encoding the 16 kDa antigen were isolated. Previous attempts to isolate clones encoding vaccine dominant antigens by screening both the amplified and unamplified cDNA library with VRabS have also failed to isolate clones encoding the 16 kDa antigen (Francis, 1989, Dr.Q. Bickle, personal communication). Thus, in total approximately 220,000 recombinant clones have been screened without success. There are a number of possible explanations as to why cDNA encoding a particular antigen may be under represented in a good cDNA library (see Chapter 7). However, a further possibility which came to light as the result of the experiments carried out here was that internal *Eco*R I sites may not have been methylated and hence protected from cleavage during the construction of the cDNA sporocyst library. Thus, cleavage of cDNAs encoding the 16 kDa antigen could have occurred and negated the expression of a significant proportion of the molecule and / or prevented the expression of epitopes capable of binding the antibodies used for screening. Were further

screening to be carried out with the aim of isolating clones encoding the 16 kDa antigen it may therefore be necessary to utilise a different cDNA library.

A second way of obtaining sequence data for a peptide molecule is to carry out gas phase N-terminal amino acid sequencing. This is a sensitive method which enables the sequence of up to 50 amino acids at the amino terminal of a peptide to be determined. By sequencing a number of different peptide fragments it is possible to determine the whole sequence of a protein using this method. Alternatively, the data obtained for the N-terminus of the protein can be used to design a probe which is then used to detect cloned DNA encoding the whole molecule. Attempts to sequence the 16 kDa antigen in this manner failed, most probably as a consequence of the molecule being N-terminally blocked. An N-terminal block can be induced by impure reagents used in purification procedures. However, as special care was taken with regard to the purity of the reagents used for the preparation of the 16 kDa antigen it seems more likely that the N-terminus of this molecule is blocked by a naturally occurring blocking group. Such groups include the fatty acids myristate and palmitate which may also be involved in anchoring molecules within the plasma membrane (Aitken *et al.*, 1982, Ozols *et al.*, 1984). If a molecule is N-terminally blocked amino acid sequencing can only be performed following cleavage of the protein component with enzymes such as trypsin, or with cyanogen bromide. The smaller peptides obtained are then isolated using high performance liquid chromatography (HPLC) and sequenced. This procedure was not attempted here as it requires very pure antigen. Thus, the immunoaffinity column eluates were unsuitable for this purpose and antigen additionally purified by SDS PAGE and transferred to ProBlott can not be cleaved by trypsin or cyanogen bromide.

The production of recombinant or synthetic peptides is only one of a number of methods which may be used to obtain antigen for immunisation. An alternative way is via the generation of anti-idiotype antibodies. The use of this method to provide an immunising preparation is particularly attractive with regard to the 16 kDa antigen, as it provides the possibility of raising an immune response against carbohydrate epitopes. Such epitopes can not be produced using conventional recombinant technology and at least one such epitope, namely that which is recognised by the passively protective McAb, B3A, appears to be a suitable target for antibody mediated protection. Alternatively the McAb could be used to screen peptide libraries in order to detect mimeotopes.

The possible function of the 16 kDa antigen at the larval surface can only be surmised

at present. In the absence of sequence data it is impossible to determine if there are any similarities between the 16 kDa antigen and other previously characterised molecules of known function. However, the appearance of this antigen at the larval surface following the loss of the glycocalyx and its presence exclusively at the surface of the skin stage schistosomula suggests that it may have a role to play in protecting the newly exposed surface of the early larval parasite against specific or non-specific immune attack. Alternatively, the 16 kDa antigen may have a function which is associated with the maturation of the heptalaminate surface membrane or the transport of metabolites and other molecules across the host / parasite interface. This would be aided by the O-glycosylation described here as occurring within the 16 kDa antigen, as O-glycosylation has frequently been shown to extend the length of a short peptide region and hence enable the extracellular domain of many glycoproteins to stretch above the cell surface.

Finally, it is important to note that the 16 kDa antigen initially described by Bickle *et al.* (1986) and further characterised as part of this thesis, is unlikely to represent any other previously described schistosomula antigen. This seems somewhat surprising in view of the large body of work carried out with the aim of identifying surface antigens recognised by sera raised in animals vaccinated with irradiated parasites. However, much of this work has involved the precipitation of radiolabelled surface antigens with a variety of sera and although there is a suggestion that the 16 kDa antigen may be labelled with IODOGEN (see Chapter 5) this labelling is not very efficient. The antigen has also failed to label with Bolton and Hunter reagent or lactoperoxidase. Furthermore, although the 16 kDa antigen is a vaccine dominant glycoprotein, the studies carried out here demonstrate that it does not bind to Con A and thus would not have been isolated following the experiments of Dalton and Strand (1987) which utilised Con A chromatography to isolate vaccine dominant glycoproteins.

Clones encoding the 15 kDa antigen described here, were identified during the screening of the sporocyst cDNA library with sera specific for low molecular weight antigens. Although this screening was initially performed in the hope of identifying clones encoding the 16 kDa antigen, work was continued upon the 15 kDa molecule, as the recombinant protein expressed by clones encoding this antigen was shown to be vaccine dominant.

The 15 kDa antigen differs significantly from the 16 kDa antigen in that it is a soluble

protein which is seen in all stages of the life cycle. Thus, it seems most likely that this antigen represents an intracellular protein which may be presented to the immune system following its release from the parasite. That the antigen is present in the latter stages of the parasite yet is not recognised by CIS nor by SSS indicates that the molecule is not presented to the immune system during the course of a normal infection whereas its release is enhanced following the death of the irradiated larvae in the lungs. Alternatively the antigenicity of this molecule may be altered by the irradiation process itself.

The 15 kDa antigen was shown by sequencing of the clones obtained to have some homology to a group of EF hand bearing intracellular calcium binding proteins. This homology was limited to two regions of the 15 kDa molecule which had similarities to the calcium binding domains (CaBD) of these proteins. Comparison of the partial sequence obtained for the 15 kDa antigen with an EF hand test motif did, however, indicate that the putative CaBDs of the 15 kDa molecule lacked residues essential for the formation of the loop and helical regions which make up this structure. This was also indicated by the demonstration that both the recombinant and native 15 kDa antigen failed to bind radiolabelled calcium. In this respect the 15 kDa antigen is similar to a 21.7 kDa vaccine dominant antigen which was identified by Francis and Bickle (1992) following the isolation of clones from the cDNA library used here. The question remains as to whether these antigens represent molecules which at some time functioned as calcium binding proteins and also as to what their function might now be.

Despite the generation of sera which recognised both the native and recombinant forms of the 15 kDa antigen, mice immunised with a portion of the 15 kDa antigen expressed as a fusion with *S. japonicum* GST, were not protected against an *S. mansoni* challenge infection. Although the antibody response observed by Western blotting was poor in all cases, it seems unlikely that attempts to increase this antibody titre would have any positive effects upon the development of resistance, as those mice which had the highest antibody titre were amongst those with the highest worm burdens. The 15 kDa molecule therefore failed to show any promise as a vaccine candidate antigen and no further studies were indicated.

Thus, in summary, although the 15 kDa antigen failed to show any promise as a vaccine candidate antigen, immunisation of mice with the 16 kDa plus novasomes stimulated low but significant levels of immunity. This immunity was observed despite

the low titre of the antibody generated in response to the 16 kDa antigen and it is therefore possible that further experiments employing different immunisation protocols, may stimulate higher antibody titres and hence better levels of protection. The paucity and partial purity of the immunoaffinity purified antigen available at the present time makes such experiments infeasible unless an alternative immunogen can be found. It may be possible to meet this aim via the production of a recombinant or synthetic peptide molecule or the development of an anti-idiotype vaccine.

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# APPENDIX

## SOLUTIONS

The following is a list of solutions used frequently. In all cases solutions were made up in distilled or double distilled water and sterilised where appropriate by autoclaving at 121°C for 20 minutes.

### Borate buffer

| | (per litre) |
|---|---|
| (a) Sodium tetraborate | 38.1g |
| (b) Orthoboric acid | 6.1g |

Add (a) to (b) to give a buffer of appropriate pH

### 20 x Phosphate buffered saline (PBS)

| | (per litre) |
|---|---|
| NaCl | 160g |
| KCl | 4g |
| $NaH_2PO_4$ (anhydrous) | 22g |
| $KH_2PO_4$ | 4g |

### 10 x TAE

| | (per litre) |
|---|---|
| Tris | 48.4g |
| Sodium acetate | 13.4g |
| EDTA | 7.4g |

pH to 8.1 with glacial acetic acid

### TE

| | (per litre) |
|---|---|
| Tris | 12.1g |
| EDTA | 3.7g |

pH to 8.0 with HCl

## MEDIA

### YT

| | (per litre) |
|---|---|
| Yeast extract | 5.0g |
| Tryptone | 10.0g |
| NaCl | 10.0g |

### JLB

| | (per litre) |
|---|---|
| Tryptone | 10.0g |
| Yeast extract | 5.0g |
| NaCl | 15.0g |
| Glucose | 1.0g |

### Minimal Agar

| | (per litre) |
|---|---|
| $Na_2HPO_4$ | 60.0g |
| $KH_2PO_4$ | 30.0g |
| $NH_4Cl$ | 10.0g |
| NaCl | 5.0g |
| Bacto agar | 15.0g |

The solution prepared as above was sterilised by autoclaving and the following added to 1 L.

| | |
|---|---|
| 1 M $MgCl_2$ | 1 ml |
| 0.1 M $CaCl_2$ | 1 ml |
| 1 M thiamine | 1 ml |
| 50% glucose | 4 ml |

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