PROTEINS RELEASED BY LARVAE OF SCHISTOSOMA MANSONI: THEIR CHARACTERISATION AND CLONING.

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DEDICATION

This thesis is dedicated to my parents

ABSTRACT

Despite the body of information which has accumulated on the mechanisms of immunity to *Schistosoma mansoni* operating in the irradiated cercaria vaccine model, little is known about the antigens involved. Biosynthetic labelling of parasites with ^{35}S-methionine, either prior to, or post cercarial transformation facilitated analysis of the kinetics of protein synthesis and release by schistosomula during *in vitro* culture. The proteins labelled during cercarial development in the snail were lost most rapidly within the first 3h after transformation to schistosomula, and at a much diminished rate thereafter. Additionally, the proteins released by schistosomula during *in vitro* culture were characterised. Two released proteins of M_r 61 and 20 kDa were dominant on autoradiographs and corresponded to areas of proteolytic activity detected on gelatin substrate gels. The *de novo* synthesis of proteins after transformation followed a complex pattern, rising to a peak at day 8 before declining sharply. The highest rate of protein synthesis corresponded to the period *in vivo* when schistosomula are undergoing the adaptations necessary for migration through capillary beds. During development to the lung-stage, schistosomula labelled post-transformation released at least 15 proteins of diverse molecular weights. Among them, three of 61, 45 and 20 kDa were especially prominent and appeared to be produced over the whole period. I believe that one or more of these proteins released in the skin-draining lymph nodes by irradiated parasites must serve as the inducers of the primary immune response, and released subsequently from challenge larvae in the lungs, as triggers of the effector response.

The ability of various antigenic fractions of *Schistosoma mansoni*, and in particular schistosomula-released proteins, to induce proliferation of lymph node cells, recovered from vaccinated mice, was tested in blastogenesis assays. Results from such experiments indicated that parasite-released proteins provided a good source of T cell immunogens, and reinforced their potential as vaccine candidates.

The production of antisera against early- and later-stage released proteins facilitated investigation of the ability of such molecules to induce antibody production, a further indicator of their immunogenicity. In this respect, antigens of 45, 20 and 12 kDa were recognised strongly by both sera on Western blots of protein preparations of *Schistosoma mansoni*. The ability of these sera to transfer resistance against a cercarial challenge infection in mice was tested. The serum raised against later-stage released proteins induced a moderate degree of immunity ranging from 19% to 29%, whereas the serum raised against early-stage released proteins conferred up to 51% resistance to mice when administered on days 4 and 7 post-challenge.

Subsequently, a unique cDNA library was generated with mRNA derived from 4 to 8 day old schistosomula. The library was screened with serum raised against proteins released by *in vitro*-cultured schistosomula between day 4 and day 8 post-

transformation. Bacterial colonies expressing recombinant proteins, identified by such sera, were isolated and initial steps towards their characterisation taken.

Finally, during the course of studying proteins released by schistosomula during *in vitro* culture, significant differences in the surface morphology of normal and irradiated larvae were observed. It is possible that such abnormalities could account for the ability of optimally-attenuated parasites to induce high levels of protective immunity against a cercarial challenge infection.

CONTENTS

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All of the work contained in this thesis is my own, with the following exceptions:

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ABBREVIATIONS

APC	Antigen presenting cell
BAL	Bronchoalveolar lavage
BCG	*Mycobacterium bovis* strain Bacillus Calmette-Guerin
cDNA	Complementary DNA
CFA	Complete Freund's adjuvant
c.p.m.	Counts per minute
D4-D8	Day 4 to day 8
D4-D8S	Day 4 to day 8 serum
DTH	Delayed-type hypersensitivity
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
Fig.	Figure
GST	Glutathione S-transferase
h	Hours
i.d.	Intradermal
IFNg	Interferon gamma
IL	Interleukin
i.v.	intravenous
kDa	Kilodaltons
krad.	Kilorads
M	Molar
M169	Medium 169
MHC	Major histocompatibility complex
min	Minutes
M_r	Relative molecular weight
NGS	Normal goat serum
NMS	Normal mouse serum
NO	Nitrogen oxide
NRS	Normal rabbit serum
0-3hS	0-3h serum
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
s	Seconds
^{35}S	35-Sulphur

s.c.	Subcutaneous
SCAP	Soluble cercarial antigen preparation
SDS	Sodium dodecyl sulphate
S.E.	Standard error
SLAP	Soluble larval antigen preparation
SOD	Superoxide dismutase
SWAP	Soluble worm antigen preparation
TCA	Trichloroacetic acid
Th	Helper T cell
TNF	Tumour necrosis factor
TPI	Triose-phosphate isomerase
VMS	Vaccinated mouse serum
W.H.O.	World health organisation

CHAPTER ONE

Introduction

INTRODUCTION

1.1 *Background*

Schistosomiasis is a disease caused by infection with dioecious parasites belonging to the family Schistosomatidae. Of the genera within this family, 7 are confined to birds and 5 to mammals, but only the genus *Schistosoma* is associated with humans. Of the mammalian blood flukes, this genus has achieved the greatest geographical distribution and diversity of the definitive hosts parasitised. Species of schistosome infective to man are found in 76 of the world's tropical and sub-tropical countries (W.H.O., 1985) and in some, the disease is extremely prevalent; for example, approximately 72% of the Ghanaian population is estimated to be infected (Doumenge *et al.* 1987). Seven species of *Schistosoma* parasitise humans; the three most common, *S. haematobium*, *S. japonicum* and *S. mansoni* account for 78, 69 and 57 million cases of schistosomiasis respectively, whilst the less common human parasites *S. intercalatum*, *S. mattheei*, *S. mekongi* and *S. malayensis* infect thousands of people (Peters & Gilles, 1991). However, these may represent conservative estimates, since it has been reported for *Schistosoma mansoni* that faecal egg screening, the standard method for assessing infection, usually underestimates the number of people carrying the disease (De Vlas & Gryseels, 1992).

Due to the relative ease in which the life-cycle of *Schistosoma mansoni* is maintained in the laboratory compared to other human schistosomes, this species has received the most attention. In York, the vast majority of research has centred upon this species using the murine host. Thus, the remainder of this thesis will refer, almost exclusively, to *Schistosoma mansoni*.

1.2 *Life-cycle of Schistosoma mansoni*

Schistosoma mansoni is transmitted via freshwater snails (including *Biomphalaria glabrata*) which are the intermediate hosts in the life-cycle, and in which asexual reproduction occurs. Owing to the losses of parasite numbers incurred during host location, a substantial amplification step occurs in the snail; penetration and development of only one miracidium leads to the production of several thousand cercariae (Theron, 1986). The cercaria, consisting of a head and a bifurcated tail, develops in approximately 1 week from the daughter sporocyst which is located in the digestive gland of the snail (Jourdane & Theron, 1987). Once mature, the cercaria leaves the snail in response to sunlight and then has between 5h and 8h, under optimal conditions, in which to find an appropriate host before its infectivity is impaired (Lawson & Wilson, 1983). The cercaria is covered by a carbohydrate-rich glycocalyx, which is thought to be anchored to the

underlying tegument via polypeptide chains (Marikovsky, Fishelson & Arnon, 1988), and functions to waterproof the cercaria whilst in search of a susceptible host. Host location by the cercaria is incompletely understood, but its probability of success is thought to be greatly enhanced by changes in motility in response to water turbulence, shadows and skin substances, the stimuli usually presented by a passing human. When a host is located, skin penetration is thought to have 3 distinct phases; attachment, surface exploration and penetration of the epidermis. The first 2 phases can be triggered by thermal and chemical stimuli, the third by chemical stimuli alone (Haas & Schmidt, 1982). Skin surface lipid has been shown to stimulate penetration (McKerrow *et al.* 1983), and also to induce eicosanoid production in cercariae; these substances are immunosuppressants and may therefore play a role in immune evasion by the parasite (Salafsky & Fusco, 1987). The cercarial head contains 3 functionally and structurally discrete secretory glands, the head gland and the pre- and post-acetabular glands. Secretion from the post-acetabular gland precedes but overlaps that of the pre-acetabular gland; however, both release proteases which facilitate skin penetration and possibly shedding of the glycocalyx (Marikovsky *et al.* 1988). Secretions from the head gland are thought to facilitate penetration of blood vessel walls in the dermis by parasites (Crabtree & Wilson, 1985).

Various protease activities have been reported from different laboratories. Landsperger, Stirewalt & Dresden (1982) identified the major protease species from a cercarial homogenate as a 25 kDa serine protease with an isoelectric point (pI) of 6 and with properties similar to chymotrypsin. Arnon and colleagues characterised two serine proteases secreted by transforming schistosomula. The major proteolytic activity was associated with a 28 kDa glycoprotein which had a pI of 11 and pH optimum of 9, whilst the minor activity was associated with a 60 kDa glycoprotein which had a pI of 9.2 (Marikovsky *et al.* 1988). The 28 kDa protease was later localised to both the pre- and post-acetabular glands of cercariae (Marikovsky, Arnon & Fishelson, 1990). McKerrow and colleagues have cloned a stage-specific proteinase which also facilitates infection of the host by cercariae (Newport *et al.* 1988). This serine proteinase of molecular weight (M_r) 30 kDa has a remarkably broad substrate specificity toward host tissue macromolecules, including keratin, fibronectin, laminin, type IV collagen and elastin (McKerrow *et al.* 1985). Despite the identification of different proteolytic species, which may be involved in skin penetration, recent structural analysis of the enzymes has shown that at least some of them are in fact post-translational derivatives of the same gene product (McKerrow, Newport & Fishelson, 1991). However, although evidence to date suggests that there is only a single serine protease gene in schistosomes, the origin of certain biochemical differences between the enzyme species remains obscure.

Strong muscular contractions around the acetabular glands, initiated by skin lipids, results in the release of proteases which are packaged in vesicles so as to protect the

parasite from its own degradative enzymes. Ruptured vesicles were noted adjacent to degraded epidermal cells and along the surface of the penetrating larvae themselves (Fishelson *et al.* 1992). It was suggested that these observations were consistent with the dual role for the enzymes in tissue invasion and in release of the glycocalyx. In concert with the action of proteases, the cercaria utilises its head spines to burrow through the channel that is created ahead of it. Shortly after shedding of the glycocalyx, schistosomula express a serine protease on their surface which appears to be identical, or very similar, to the secreted enzyme (McKerrow *et al.* 1991). This enzyme could degrade the complement components, C3, C3b, iC3b and C9 (Parizide *et al.* 1990) suggesting that it may also function to protect the larva against immune damage by cleaving off any complement proteins that become attached to the parasite surface. Skin penetration is accompanied by transformation of cercariae into schistosomula, which are adapted to life in the mammalian host.

For obvious reasons, the vast majority of information concerning the migratory pathway of *Schistosoma mansoni* and accompanying morphological and biochemical changes undergone by the parasite in the mammalian host comes from studies of infected rodents. Such adaptations will be described in more detail in section 1.5.1. The majority of schistosomula locate and penetrate a blood vessel and migrate via the lungs to the liver; this involves periods of active migration by schistosomula through capillary beds interspersed with passive carriage in the direction of blood flow. When the worms have paired, they begin to migrate up the hepatic vessels to the mesenteric veins, where the female commences egg-laying. Of the three most common schistosomes of humans, adult *S. mansoni* and *S. japonicum* inhabit the mesenteric vein and its tributaries. The eggs, after passing through the vessel walls to the lumen of the intestine are eventually voided in the faeces. In contrast, female adult worms of *S. haematobium* oviposit in the vesical plexus of the bladder, and eggs are excreted in the urine. The eggs hatch in freshwater releasing free-swimming miracidia which infect susceptible snails, and the life-cycle is perpetuated.

In humans, schistosomiasis is a chronic disease which presents a broad spectrum of morbidity ranging from sub-clinical infection to severe splenomegaly and hepatomegaly, which can be fatal. Worm pairs can persist within their host for many years causing only trivial direct pathology. The vast majority of damage is caused by the host's immune response to the eggs, of which approximately 300 are produced per day by an adult *Schistosoma mansoni* female. Unfortunately, roughly half of the eggs are washed into, and lodge in, the liver. The immune response to the trapped eggs results in granuloma formation around each egg, disruption of hepatic tissue organisation, portal hypertension and the development of porto-systemic anastomoses, which shunt a fraction of hepatic portal blood to the vena cava. Despite a low mortality rate from schistosomiasis of approximately 1%, an annual death toll of 2 million people has been recorded (Bergquist,

1987). As such, the disease is an important target for immune intervention.

1.3 *Control measures*

The requirement of a molluscan intermediate host for transmission of schistosomiasis to humans, means that the disease is centred around areas of freshwater, which can range from natural rivers and lakes to man–made dams and irrigation canals. Unfortunately, the latter two make a substantial contribution to the spread of schistosomiasis, for example in the Sudan, soon after 1 million acres of land was irrigated in 1963, the prevalence of *Schistosoma mansoni* infection reached 75% (Fenwick, 1987).

Many measures have been used to try to prevent, or limit, the spread of schistosomiasis, including the provision of public health education in tandem with latrines and clean water for washing and drinking. Latrines could reduce faecal and urinary contamination of water, thus preventing contact of eggs, and therefore miracidia, with the intermediate snail host and so reduce transmission. However, *Schistosoma* spp. have reservoir hosts which contribute to transmission of the disease to man. In endemic areas, water exposure time has been shown to correlate strongly with presence of infection in humans. Therefore, the existence of a clean water source could have a great impact on the level of parasite infection and transmission, simply by reducing the level of contact with 'contaminated' water. However, such measures require large amounts of money and a suitable water supply throughout the dry–season, both of which are difficult to obtain. Furthermore, a significant impact on transmission cannot be expected unless improvements in sanitation are also accompanied by changes in human behaviour. Unfortunately, it has been noted that even when better sanitation is available it is often not used (Cairncross, 1987).

Molluscicides, such as niclosamide have been used with limited success to reduce the population of susceptible snails. Such measures are not only expensive and potentially damaging to the local environment but with time could lead to the evolution of resistant snails. Furthermore, the snail populations have an impressive potential for regeneration and reinvasion, being able to re–establish in a period of months. However, there have been some successes, for example in an area of Egypt, the prevalence of infection was reduced from 46% in 1968 to 10% in 1973 by molluscicide treatment combined with chemotherapy (Fenwick, 1987). Thus, it has been suggested that there is still an important role to be played, by focal, rather than area–wide molluscicide treatment to reduce the transmission of schistosomiasis, alongside other control measures (Klumpp & Chu, 1987).

The mass treatment of infected human populations with chemotherapeutic drugs has, on the whole been very effective in reducing the prevalence and incidence of the disease (Mahmoud *et al.* 1983). This approach was advanced further by the availability of the

drug praziquantel, which has few side-effects, is given as a single dose, is highly effective against all species of schistosome and is relatively cheap (W.H.O., 1985). Unfortunately, such treatment does not prevent the pathological damage caused to the host as a result of the presence of eggs, nor reinfection. Thus, many patients require treatment more than once, which increases the overall cost of chemotherapy and necessitates the presence of an organised screening programme. Additionally, there is the possibility, as with molluscicide treatment, that resistance to the chemical could evolve. A recent outbreak of schistosomiasis has occurred in Northern Senegal, five years after two dams were completed on the Senegal river (W.H.O., 1992). Stool examinations of over 400 people showed >90% to be infected, of whom 41% had over 1000 eggs per gram of faeces, indicating intense infection. This previously "schistosome-naive" population showed a very low cure rate of 18% after praziquantel treatment compared to the 70-85% normally expected. Additionally, the drug caused severe although transient side-effects. Despite doubts that the results were due to praziquantel resistance, there is cause for concern.

For these and additional reasons, a single-shot vaccine would be a major step forward to relieve the suffering of many people who, without such treatment, will inevitably become infected with schistosomes. A vaccine, probably composed of recombinant protein(s) or synthetic peptides, presented in the appropriate manner should provide long-lived immunity, be cheap to manufacture and easy to transport and administer to people in endemic areas. Additionally, if given before natural exposure to the parasite, vaccination would have the added advantage over chemotherapy of preventing egg-induced pathology and reinfection, thus negating the need for further treatment. To date, no live or dead vaccine has been able to induce 100% immunity in laboratory rodent or primate hosts. Although sterile immunity is a goal to aim for, unlike malarial infections, schistosomes do not multiply within the definitive host and so a vaccine which induced >50% resistance is likely to be of benefit. Since only the most heavily infected individuals develop hepatosplenomegaly, which can be fatal, a substantial decrease in the level of infection would limit the number of people in this category and dramatically reduce the mortality rate, as well as decreasing the level of transmission.

Two other avenues of research are being pursued to try to prevent infection, or limit the pathology associated with the disease. One approach is to design compounds which will inhibit the action of the cercarial penetration enzyme, which is essential for skin invasion and therefore initiation of infection. Hotez *et al.* (1985) showed that dogs which were refractory to reinfection with another skin-invasive parasite, the hookworm, had high circulating titres of anti-elastase antibody and suggested the use of the enzyme as a protective vaccine. In schistosomiasis research, McKerrow and colleagues (Pino-Heiss *et al.* 1986) showed that monoclonal antibodies directed against the cercarial elastase could

kill transforming cercariae *in vitro*. Additionally, an inhibitor of the enzyme has been developed which, when applied to the surface of human skin inhibited cercarial invasion by 85% (Cohen *et al.* 1991). A different approach is to design an anti-fecundity vaccine which would act by reducing the number of eggs produced by adult female worms in an infected patient, and therefore decrease the level of pathology and transmission of the disease. The promising vaccine candidate glutathione S-transferase (Sm28GST; see section 1.9.4.1) not only protects various rodent and primate hosts through a reduction in worm burden but has also been shown to reduce female worm fecundity (Boulanger *et al.* 1991; Xu *et al.* 1993).

Additional research aimed at deciphering the complex interplay between the parasite and the host's immune system will undoubtedly yield more important information. Once understood, it may be possible to influence the outcome of infection, or at least limit the degree of pathology, by manipulating the immunological environment within the host. One way to achieve this could be through the addition or neutralisation of particular cytokines.

Along with the large body of evidence for acquired human resistance to schistosomiasis (Butterworth & Hagan, 1987; Hagan *et al.* 1991; Hagan, 1993) and the major advances in recombinant DNA technology, differentiation of T cells into functionally discrete sets and sub-sets, identification of cytokines and their functions and advances in the presentation and delivery of antigens, a vaccine against schistosomiasis is looking more promising.

1.4 *Experimental models of schistosomiasis*

Various animal models are available for the study of schistosomiasis in the laboratory. The most widely used are rats and mice and, to a lesser extent Guinea-pigs and non-human primates. These animals differ in their ability to support or, perhaps more correctly, inability to reject, a primary infection. Each has features which are analogous to human schistosomiasis. The chimpanzee develops disease most analogous to man and is the best model of pipestem fibrosis (Phillips *et al.* 1977). Moreover, resistance is minimal and develops very slowly; additionally spontaneous cure does not occur. Rhesus monkeys develop more dramatic resistance than chimpanzees and also demonstrate spontaneous cure. Obvious logistic and ethical problems exist in studying these animals. The mouse is the most highly studied susceptible rodent host and displays many similarities to human schistosomiasis in that liver pathology occurs, spontaneous cure does not, and resistance develops slowly. Furthermore, the existence of a wide range of inbred strains of mice has allowed dissection of the immunological interplay between the parasite and the host. Recent technological breakthroughs have permitted the development of transgenic (or knockout) mice in which a specific gene, for example one

which codes for a cytokine, can be made non-functional. This should allow further elucidation of the immune response to the parasite.

Unlike mice, rats are semi-permissive hosts which, although susceptible to infection, manifest a spontaneous and dramatic reduction in worm burden approximately 28 days after initial exposure (Stirewalt, Kuntz & Evans, 1951; Smithers & Terry, 1964). The elucidation of this mechanism of parasite elimination is of interest and is being actively researched; however, the rat model of schistosomiasis is perhaps the least analogous to that in humans (Phillips *et al.* 1977). Despite these facts, extrapolations of results achieved in a single animal model to the human disease are made with care. It is likely that all model systems will provide important information which will help in the development of a protective vaccine.

1.5 *The normal infection in laboratory mice*

1.5.1 *Migration and development*

Upon penetration of mouse skin, the cercarial tail is lost and the parasite commences its transformation into the next larval stage, the schistosomulum. The adaptations occurring at this stage include a change from cyanide-sensitive to cyanide-insensitive metabolism and intolerance to water. Parasites successfully penetrating the skin, reach the base of the epidermis within 30 min but then seem to be impeded by the epidermal basement membrane (Wilson & Lawson, 1980). On average, the skin phase of migration in the mouse takes approximately 88h (Miller & Wilson, 1978). During the first 3h post-transformation, membraneous vesicles pass from subtegumental cells to the tegument via cytoplasmic connections where they participate in the formation of the characteristic heptalamminate membrane of the schistosomulum (Hockley & McLaren, 1973). Additionally, the mid-body spines are progressively lost and have completely disappeared by day 3 post-transformation; this is thought to facilitate migration through the vascular system (Crabtree & Wilson, 1980). The loss of mid-body spines is accompanied by an increase in tegumental pits which are responsible for adding membrane to the surface of the schistosomulum (Hockley & McLaren, 1973; Wilson & Barnes, 1974) and result in an increase in surface area. The schistosomulum continues skin migration until it locates and penetrates a post-capillary venule in the dermis, a process which takes approximately 8h and may be facilitated by lytic secretions from the head gland (Crabtree & Wilson, 1985). Exit from the skin is not exclusively via the blood vessels, approximately 10-20% penetrate a lymphatic vessel (Miller & Wilson, 1978; Wheater & Wilson, 1979); this may be a chance event, simply reflecting the relative proportion of blood vessels to lymphatic vessels present in the dermis. This small percentage of parasites may have an important role to play in the induction of immunity in the irradiated cercaria vaccine model (see section 1.7.6).

The majority of parasites migrate in the direction of blood flow via the pulmonary artery to the lungs, the peak accumulation occurring at 6–7 days post–infection (Miller & Wilson, 1980; Mountford, Coulson & Wilson, 1988). There is a growth period of at least 72h in the lungs, and an increase in maximum length to 4 times that of skin worms (Wilson *et al.* 1978) implying synthesis of tegument membranes, and a concomitant decrease in diameter. A new inclusion body is seen at the lung–stage, the homogeneous body which may provide a lubricant for the surface of the schistosomulum (Crabtree & Wilson, 1986a). These changes are believed to facilitate the migration of larvae along the narrow pulmonary capillaries. Parasites which successfully migrate through the lungs, leave via the pulmonary vein and pass via the left side of the heart to be distributed, in the same proportion as cardiac output, to the systemic organs of the body. Wilson & Coulson (1986) speculated that 3 circuits of parasites around the pulmonary–systemic vasculature was required to recruit the entire hepatic portal population, which reaches a plateau by day 21. The high nutrient content of the hepatic portal system, low blood pressure or oxygen tension may be the triggers for parasite shortening, which probably terminates any further migration (Wilson *et al.* 1978). However, trapping of schistosomula in the liver is not completely efficient, and in a previously uninfected mouse, an estimated 14–30% may return to the lungs (Wilson, Coulson & Dixon, 1986).

Between days 28 and 35 after penetration, the parasites begin to pair and migrate up the hepatic vessels to the mesenteric veins where egg production commences approximately 5–6 weeks after infection (Warren & Peters, 1967). Some of the intravascularly released eggs fail to move on through tissues of the gut lumen. These ova are then carried by portal blood flow and can become trapped in the liver. Soluble egg antigens (SEA), secreted by the miracidium within the egg, induces a T cell–mediated granulomatous inflammatory response eventually resulting in tissue fibrosis and disruption of the hepatic architecture. The intensity of the granulomatous response is maximal during the acute stage (8–10 weeks post–exposure) of infection, and is accompanied by a strong T–helper (Th) cell activity (Doughty & Phillips, 1982a; 1982b) manifested by proliferation and inflammatory cytokine production (Chensue, Boros & David, 1980; Ragheb & Boros, 1989). As the infection progresses into the chronic stage (16–20 weeks post–exposure), the granulomatous response is spontaneously down–modulated resulting in diminished proliferation and cytokine production by lymphocytes (Chensue *et al.* 1980; Ragheb & Boros, 1989).

1.5.2 *Maturation of parasites*

Of the parasites which penetrate the skin of mice, between 50% and 70% fail to mature. Initial experiments, using techniques of tissue mincing and incubation to extract schistosomula suggested that the bulk of non–maturing parasites died in the skin soon

after penetration (Clegg & Smithers, 1968; Smithers & Gammage, 1980). Indeed, in some studies it was observed that an average of 30% of the schistosomula that could be recovered from the skin within 1h of penetration failed to exclude a dye, indicating that the parasites were not viable (Clegg & Smithers, 1968; Ghandour & Webbe, 1973). However, histological methods failed to detect significant numbers of dead or damaged schistosomula in this site (Stirewalt, 1959; Von Lichtenberg *et al.* 1976; Von Lichtenberg, Correa-Oliveira & Sher, 1985; Wheater & Wilson, 1979; Mastin, Bickle & Wilson, 1983). Additionally, 63% of parasites which penetrated the skin were detected in the lungs by quantitative histology 6-7 days after infection (Mastin *et al.* 1983). However, to determine the fate of non-maturing parasites, the techniques of mincing and incubation and quantitative histology have their disadvantages. The former suffers from the unknown efficiency of extraction (Miller & Wilson, 1978; 1980) whilst the latter is difficult to quantify, tedious and only a small number of samples can be processed (Wheater & Wilson, 1979; Mastin *et al.* 1983; Von Lichtenberg *et al.* 1985). These problems were overcome by the advent of the technique of compressed organ autoradiography (Georgi, 1982) in which isotopically labelled parasites can be tracked throughout the body of the host. This technique allowed essentially all of the organs of the mouse to be analysed and therefore did not suffer from the problem of lack of detection because of parasite migration to an unsampled organ.

Autoradiographic tracking studies of labelled parasites detected up to 78% of skin penetrants in the lungs of mice on day 7 (Mangold & Dean, 1983) and 92% in the lungs of rats on day 5 (Knopf *et al.* 1986). Additionally, Wilson *et al.* (1986) demonstrated that by day 14 post-infection, greater than 90% of parasites, detected in the skin on day 0, were present in other sites, although a small number lingered in the infection site as late as 35 days post-infection. Thus, it appears that only a minority of parasites are eliminated at the skin-stage of migration. The high degree of accuracy achieved by Wilson *et al.* (1986), which allowed the detection of parasites with only a low level of associated radioactivity, enabled them to predict that a reduction of parasite number did not occur until day 14 post-infection.

That the parasites experience difficulties in migrating through the pulmonary vasculature is suggested by the transit time through the lungs, which is considerably longer than through other organs (Wilson & Coulson, 1986). This inference was supported by ultrastructural studies (Crabtree & Wilson, 1986a) in which lung-stage parasites located in pulmonary capillaries were often seen to cause considerable distension of the vessels. From day 11 post-infection, it was found that an increasing proportion of schistosomula present in the lungs had been diverted into alveoli (Crabtree & Wilson, 1986b). Indeed by day 20, 80% of parasites present in the lungs were detected in alveoli. Whilst no cellular reactions were observed around intravascular parasites, large foci consisting initially of 50% polymorphonuclear cells and 50% mononuclear cells

were observed around alveolar parasites. However, if removed from their position in the lungs by mincing and incubation on days 7, 12 or 17 after infection and transferred to the hepatic portal system via the superior mesenteric vein, schistosomula were equally capable of maturing, irrespective of age (Coulson & Wilson, 1988). This suggests that parasites were not damaged in a manner which affected their maturation. However, 7 day old schistosomula delivered via the trachea to the alveoli had a limited capacity to reenter tissues and mature, since only 15% could be recovered by hepatic portal perfusion (Coulson & Wilson, 1988). Thus despite adaptive changes, it would appear that during parasite migration through the pulmonary capillaries, a large proportion of schistosomula rupture the narrow blood–air barrier and find themselves trapped in alveoli. From such a position, the majority of schistosomula, are unable to reenter tissues and therefore will ultimately starve to death. Thus, these results do not support a direct cytotoxic mechanism of parasite elimination.

1.6 *The concomitant immunity model*

Chronically infected mice are extremely resistant (up to 100%) to secondary cercarial challenge infection (reviewed by Dean, 1983). The elimination mechanism in such mice appears to be directed against the migrating larvae of the challenge infection, whilst the adult worms from the primary exposure remain unharmed. The term 'concomitant immunity' was coined to describe this situation (Smithers & Terry, 1969). The immunological nature of this mechanism was supported by the fact that T cell–deficient mice failed to develop concomitant immunity (Doenhoff & Long, 1979). Additionally, a role for macrophages in the resistance of infected mice to a subsequent infection was postulated (James *et al.* 1983). Furthermore, a proportion of immunity could be passively transferred to naive animals with chronic mouse sera, although the degree of resistance achieved rarely exceeded 50% of the resistance attained by the serum donor animals (Sher, Smithers & Mackenzie, 1975). However, mice that had been partially μ–suppressed developed a degree of resistance to challenge infection comparable to that of intact mice (Maddison *et al.* 1981) suggesting no role for antibody–mediated effector mechanisms.

Despite these results, the absolute role of the immune system in precipitating parasite attrition was called into question when it was found that the level of host resistance to a secondary challenge infection, correlated with the animal's pre–existing egg burden (Wilson, Coulson & McHugh, 1983 and reviewed by Wilson, 1990). Further evidence for the role of eggs in concomitant immunity was suggested by the failure of single–sex and mixed–sex infections, which were drug–terminated before oviposition, to induce immunity. It was found that when 7 day old schistosomula were injected into the hepatic portal vein of chronically infected mice, a significantly higher proportion could be

recovered from the lungs compared to uninfected control mice, suggesting a reduced trapping, or increased 'leakiness', in the liver. The chronically infected mice possessed well developed porto-caval anastomoses whereas the control mice did not. The greatly elevated leakiness of the liver in chronically infected mice caused many of the worms from the secondary infection to be redirected back to the circulation from which they could eventually reach the lungs. Since lung-stage schistosomula find this organ difficult to traverse, it is likely that the much larger liver worms would have a greater problem. Additionally, liver worms regain some mid-body spines which could cause further damage to the pulmonary capillaries. Taken together, these factors are likely to cause diverted liver worms to burst into alveoli, from which they are unlikely to be able to escape. Immune responses may enhance the observed resistance in chronically infected mice, but it is difficult to dissect their importance compared to the non-specific effector mechanisms caused by the presence of eggs. Further evidence of this non-specificity comes from observations that mice with chronic infections are protected against a heterologous challenge infection (Dean, 1983).

1.7 *The irradiated cercaria vaccine model*

One of the most promising breakthroughs towards the development of a vaccine against schistosomiasis occurred when Villella, Gomberg & Gould (1961) discovered that cercariae attenuated by gamma-irradiation, did not mature and hence produced no liver pathology, but were capable of inducing resistance in mice. Following this discovery, a series of experiments were performed to optimise the conditions required for the expression of resistance (Minard *et al.* 1978; Bickle *et al.* 1979a). Several other methods have been used to attenuate parasite migration and induce resistance in laboratory hosts. These include the use of chemicals (Bickle & Andrews, 1985) ultraviolet light (Dean, 1983) and X-rays (Hsu, Hsu & Burmeister, 1981). However, the use of gamma radiation is most widespread.

1.7.1 *Identification of the optimal conditions for the induction of protective immunity*

It was found that both the extent of migration and, the level of protection induced by attenuated larvae, were strongly influenced by the dose of radiation. Bickle, Dobinson & James (1979b) noted that while attenuation of cercariae with 2.3 krad. of radiation allowed a small number of intra-muscularly injected schistosomula to reach the liver, the majority of female worms were sterile. A dose of 2.3-10 krad. resulted in decreased worm recoveries from the lungs while 20 krad. caused a much reduced and 40 krad. a virtual absence of parasites in the lungs. Concomitantly, Bickle *et al.* (1979a) demonstrated that parasites irradiated with 20 krad. induced consistently greater levels of

protection (approximately 60%) than higher or lower doses of radiation. Conversely, in the U.S., a radiation dose of 50 krad. was found to induce the highest levels of resistance (Minard *et al.* 1978; Mangold & Dean, 1984). This discrepancy was thought to reflect differences in parasite maintenance and/or irradiation conditions (James & Dobinson, 1985). However, a recent report by Reynolds & Harn (1992) compared 15- and 50-krad. doses of gamma radiation and found that the former induced higher levels of protective immunity.

At York, the optimal irradiation dose was found to be 20 krad. (Coulson, unpublished observations); this consistently induces between 60-70% resistance to a cercarial challenge infection. Mastin *et al.* (1983) found that such optimally-attenuated parasites migrated to the lungs, persisted for at least 2 weeks and considerable numbers of larvae died there. Thus, in the majority of cases, the highest level of resistance induced by a vaccinating infection correlates with the radiation dose which causes premature termination of parasite migration in the lungs.

Optimal levels of protective immunity can be achieved with as few as 50 attenuated cercariae (Dean, 1983) and, there is no indication that resistance can be enhanced by increasing the numbers of vaccinating parasites above this dose (Minard *et al.* 1978; Bickle *et al.* 1979b). In the majority of cases, vaccine-induced resistance in mice appears to have a ceiling of approximately 70%. It is not known why the level of protection cannot be raised above this. It is possible that challenge parasites which arrive in the lungs and elongate more rapidly than other parasites, are able to negotiate the lungs before the effector mechanism has time to act.

1.7.2 *Effects of radiation on schistosomula and their ability to migrate*

Few studies which have analysed either directly or indirectly the effect irradiation has on the parasite, have noted any biochemical or morphological changes which could account for the ability to induce high levels of immunity. However, attenuated parasites were shown to migrate more slowly through the murine host. Mountford *et al.* (1988) demonstrated that by day 5 only 13% of applied parasites remained in the skin of mice infected with normal parasites while 43.4% were present in the skin of vaccinated mice. Normal parasites also migrated through the skin-draining lymph nodes more rapidly; less than 3% of applied parasites remained by day 7 compared with 10.4% of irradiated schistosomula. As mentioned previously, Mastin *et al.* (1983) showed that the migration of optimally-attenuated parasites was terminated in the lungs of vaccinated mice. Subsequently, an ultrastructural study on 20 krad. radiation-attenuated parasites fixed *in situ* in the lungs was performed (Mastin, Bickle & Wilson, 1985). It appeared that such parasites had undergone the normal developmental changes associated with the lung-stage of migration, including elongation of the body and loss of mid-body spines. No

evidence was found to suggest why migration was terminated in the lungs.

Biochemical observations (Simpson *et al.* 1985) have demonstrated that the same set of antigens, identified by serum from mice vaccinated with irradiated cercariae, were present on the surface of both normal and attenuated 3h schistosomula. Thus, at this early stage of development, irradiation seemed to have no effect on the expression of antigenic parasite surface proteins. Additionally, Vieira *et al.* (1987) showed that there was no difference in the ability of newly-transformed or 24h old, normal or irradiated, live or fixed schistosomula to induce proliferation of peripheral blood mononuclear cells from patients with chronic intestinal schistosomiasis. Thus, optimal levels of irradiation appears to have a subtle effect on the parasite, but one which can profoundly influence the induction of resistance. More recent studies have demonstrated some differences between normal and irradiated parasites. Wales *et al.* (1993) found that U.V. irradiation caused a pronounced modification of carbohydrate antigens expressed at the surface of cercariae and newly-transformed schistosomula. This could result in enhanced exposure of parasite surface proteins to the host's immune system. Additionally, it was demonstrated that protein synthesis was inhibited relative to normal larvae in the first 24h after irradiation but nevertheless recovered by 72h (Wales, Kusel & Jones, 1992). Whether the same results would be obtained with gamma-irradiated parasites is not known. Furthermore, recent morphological observations of *in vivo* and *in vitro*-cultured schistosomula (Harrop & Wilson, 1993; see appendix one) provide an alternative explanation for the premature termination of migration by gamma-irradiated parasites.

1.7.3 *The immunological nature of resistance*

Parasite attrition in the concomitant immunity model correlates with the host's pre-existing egg burden and crosses the species barrier. Conversely, there is much evidence to suggest that resistance in the irradiated cercaria vaccine model, which in most cases is only effective against homologous challenge (Cheever *et al.* 1983; Bickle *et al.* 1985), is the result of a specific immune response. For example, the immunity generated in vaccinated mice, but not those with chronic infections, was transferred to naive animals across a parabiotic union (Dean, Bukowski & Clark, 1981a) which suggested a role for circulating factors. Additionally, vaccination failed to protect congenitally athymic (nu/nu; nude) mice (Sher *et al.* 1982) whilst nude mice receiving thymus grafts became resistant. Although resistance was also abrogated by depletion of B cells with anti-μ chain serum (Sher *et al.* 1982) this result was subsequently invalidated by the discovery that the ablation also suppressed T helper cell function (Kim *et al.* 1984). Further support for the importance of T cells in the expression of resistance came from the discovery that the P/N strain of mouse developed poor resistance to a challenge infection, despite normal antibody levels and isotypes (James & Sher, 1983). Such mice were found to be

abnormal in their ability to activate macrophages due to a defect in T cell function which prevented the production of interferon gamma (IFNg; James *et al.* 1986). Subsequently, a pivotal role for IFNg, and therefore the Th1 cells that produce it, was demonstrated by Smythies, Coulson & Wilson (1992). Administration of a monoclonal antibody to IFNg on days 4, 8, 12 and 16 post-challenge gave an average 89.5% abrogation of protective immunity. This occurred in the presence of greatly elevated eosinophil levels suggesting no major role for these cells in this vaccine model. Indeed, it has been demonstrated previously that the ablation of eosinophils or strong depression of the IgE response, by treatment with monoclonal antibodies against IL-5 and IL-4 respectively, failed to influence the level of resistance attained (Sher *et al.* 1990). The evidence suggests that the ability to reject a challenge infection correlates with the induction of a delayed-type hypersensitivity (DTH) response.

1.7.4 *Immunological events accompanying vaccination*

1.7.4.1 *The skin and draining lymph nodes*

Following vaccination, 10-15% of attenuated larvae enter and persist in the skin-draining lymph nodes (inguinal and axillary) where they release substantial quantities of antigen (Mountford *et al.* 1988). The presence of antigenic material caused an increase in cell number which reached a peak on day 14 post-vaccination in the axillary lymph nodes, 13.5-fold higher than on day 0 (Constant, Mountford & Wilson, 1990). Additionally, the proportion of proliferating cells, determined by the incorporation of the thymidine analogue 5-bromo-2-deoxyuridine (BrdUrd) into axillary lymph node cells and detection by flow cytometry, increased reaching a peak on day 14, 3.4-fold higher than on day 0 (Constant & Wilson, 1992). Taking into account the elevated cell number, a 22.2-fold increase in $BrdUrd^+$ cells was detected on day 14 post-vaccination. The increase in cell number was accounted for by a significant change in both T- and B-cells, the former usually being the most common but the latter showing a greater proportional increase (Constant *et al.* 1990). However, the proportion of B cells which proliferated in the axillary lymph nodes was much smaller compared to the proportion of T cells (Constant & Wilson, 1992). It was suggested by these authors that after vaccination, T cells leave the skin-draining lymph nodes to enter the circulation, whilst the majority of B cells are retained. The reason for this retention has not been explained. However, it was postulated that B cells may have an important function in their role as antigen-presenting cells (APC) in the lymph nodes. In fact, Janeway, Ron & Katz (1987) postulated that B lymphocytes are the main type of APC in peripheral lymph nodes. Furthermore, it was suggested that B cells preferentially present antigen to DTH-inducing cells (Bottomly & Janeway, 1989).

T-helper ($CD4^+$) cells have been divided into two subsets which appear to be

phenotypically identical but differ in the profile of cytokines they secrete (Mosmann & Coffman, 1989). Th1 cells are characterised by their production of the cytokines IL-2 and IFNg whereas Th2 cells produce IL-4 and IL-5. The former mediates DTH, while only Th2 cells can act as helper cells for IgE production. Analysis of the cytokines produced by lymph node cells (Pemberton *et al.* 1991; Mountford *et al.* 1992) or spleen cells (Pearce *et al.* 1991; Caulada-Benedetti *et al.* 1991) of mice following vaccination with attenuated cercariae, was consistent with the prevalence of a Th1-type response. In comparison to a normal infection, vaccination caused a small increase in the number of cells in the skin- and lung-draining lymph nodes, yet the level of proliferation by both sets of lymph node cells was similar in both regimes (Pemberton *et al.* 1991). However, there was a substantial difference in the ability of lymph node cells from vaccinated mice to produce IFNg. It was calculated that on day 22 post-exposure, 24-fold more IFNg was produced per pair of axillary lymph nodes from mice receiving attenuated rather than normal parasites (Pemberton *et al*. 1991). This provides further evidence that the ability of Th1 cells from mice to produce IFNg after parasite antigen stimulation may correlate with the subsequent expression of protective immunity.

Following vaccination, schistosome-specific T cells traffic from the lymph nodes to the circulation where they can be detected by their ability to induce DTH as measured by the footpad assay (Ratcliffe & Wilson, 1991). The maximum degree of swelling occurred on day 10 post-vaccination in response to live lung-stage schistosomula and on day 17 to a soluble adult worm antigen preparation. This response could be completely abrogated by treatment of mice with an anti-CD4 antibody.

1.7.4.2 *Events in the lungs and their draining lymph node*

In an ultrastructural examination of the migration of attenuated parasites, Mastin *et al.* (1985) observed no host inflammatory reaction around schistosomula on day 7. By day 13, inflammation was noticeable but it was suggested that this was as a result of pulmonary tissue damage rather than the presence of parasites. The composition of cells, recruited to the lungs in response to the presence of vaccinating parasites, has been sampled by bronchoalveolar lavage (BAL). This provides cellular populations which reside in the respiratory airways but are free of contaminating leukocytes from the blood or the interstitium, and thus allows characterisation of the cells present in the lungs during the induction and effector phases of immunity. In mice exposed to 500 normal or irradiated (20 krad.) cercariae, no difference in the number of cells recovered by BAL was detectable at 2 weeks post-exposure (Aitken, Coulson & Wilson, 1988). However, by week 5, the numbers of leukocytes washed from the lungs of vaccinated mice was substantially greater than that from infected mice, and remained elevated beyond 10 weeks post-exposure. The sustained response may correlate with the long-term nature of immunity in this model, which can persist undiminished beyond 15 weeks (Correa-

Oliveira, Sher & James, 1984; Smith & Clegg, 1984).

While infected mice showed a small but significant increase in the number of monocytes recovered by BAL at week 5, vaccinated mice showed a 5–fold increase in monocytes and polymorphonuclear cells and a 15–fold increase in lymphocytes. Within this pool of lymphocytes, the percentage of $CD4^+$ (Th) cells did not differ significantly from the equivalent cell population of mice infected with normal parasites, however the absolute number did. Furthermore, it has been shown that a greater proportion of Th cells recovered from the airways responded more rapidly to mitogen stimulation by up–regulating the p55 subunit of the IL–2 receptor than did splenocytes from the same animal (Coulson & Wilson, 1993). This is indicative that many more previously–activated cells were present in the airways than the spleen. That this elevated proportion of sensitised cells was due to schistosome–specific lymphocytes was demonstrated by their 4–fold higher response to schistosome antigen. The majority of Th cells expressed high levels of the surface markers CD44 and low levels of the B isoform of CD45, compared to Th cells from the lymph nodes or the circulation, such phenotypes being characteristic of short–term effector/memory cells (Coulson & Wilson, 1993).

Additionally, flow cytometric analysis of BAL cells recovered from vaccinated mice was used to quantify the proportion of macrophages displaying specific surface markers (Menson & Wilson, 1990). The proportion of Ia^+ macrophages increased 5–fold over days 14–28 post–vaccination whilst there was a sharp decrease in F4/80 expression between days 14–21. These phenotypic changes are characteristic of activated macrophages and coincide with the production of IFNg, which can activate macrophages, by Th cells in the mediastinal lymph node, which drains the lungs.

Following the arrival of schistosomula in the lungs, small quantities of parasite–released material could be detected in the mediastinal lymph node, as judged by the presence of radio–labelled proteins after either infection or vaccination of mice with ^{75}Se–labelled parasites (Mountford *et al.* 1988). However, the method used in this study, only allowed detection of material that was synthesised and therefore labelled prior to cercarial transformation. By the lung–stage of development much of this labelled material has been lost. Therefore, this result is likely to provide an underestimate of the amount of parasite–derived protein present in the mediastinal lymph node. In response to this parasite material, changes in the cellular composition of the mediastinal lymph node could be observed (Constant *et al.* 1990). The peak percentage of $BrdUrd^+$ cells detected in the mediastinal lymph node occurred on day 14, with the greatest absolute number of proliferating cells occurring on day 21, a value 10.3–fold higher than from naive mice (Constant & Wilson, 1992). This enhanced level of proliferation persisted beyond day 35 post–vaccination.

Restimulation of mediastinal lymph node cells, recovered from vaccinated mice, *in vitro* with a soluble schistosomula preparation resulted in a peak of proliferation by

approximately 18 days post-exposure, while T-cell growth factor production peaked on day 18 and IFNg was first detected on day 15 and was still increasing by day 22 (Pemberton *et al.* 1991). Mediastinal lymph node cells recovered from vaccinated mice showed a 3- to 6-fold increase in their ability to produce IFNg after *in vitro* restimulation compared to equivalent cells from infected mice.

Both the increases in lymph node cell number (Constant *et al.* 1990) and degree of proliferation after restimulation *in vitro* (Lewis & Wilson, 1982a; Pemberton *et al.* 1991) reflected the kinetics of parasite migration. Thus, axillary lymph node cells, recovered from vaccinated mice, showed a peak responsiveness to a soluble schistosome antigen preparation on day 5 whereas schistosome-specific proliferation by cells from the mediastinal lymph node was not detected until at least day 10 post-vaccination.

A significant increase in cell number was detected in the spleen only at days 14 and 21 post-vaccination, the maximum at day 14 representing a 1.7-fold increase over day 0, thus indicating that vaccination with attenuated parasites causes marked localised rather than systemic responses. The limited splenic response is not surprising as no parasites and little of their released antigens were found in this site after vaccination (Mountford *et al.* 1988).

1.7.5 *Source of antigens and exposure time essential for the induction of immunity*

Since irradiated cercariae are capable of inducing consistently high levels of resistance to a challenge infection, it is pertinent to ask what is the nature of the immunogens responsible for immunity? It has proved extremely difficult to pinpoint both the stage of parasite and the relevant antigen(s), derived from such larvae, that are responsible for the induction of protective immunity.

Antigens unique to the cercaria can be excluded, since it has been demonstrated that mice receiving an intra-dermal immunisation of 8 day old lung-stage schistosomula, derived from irradiated cercariae, were as resistant to a challenge infection as mice vaccinated percutaneously with irradiated cercariae (Coulson & Mountford, 1989). Conversely, irradiated 3-4 week old schistosomula were much less effective inducers of resistance than skin- or lung-stage larvae (Dean, Cioli & Bukowski, 1981b; Sher & Benno, 1982). This would seem to indicate that larval antigens expressed within the first 1-2 weeks after penetration are crucial for the induction of protection. However, the latter experiments used 3-4 week old schistosomula irradiated at the time of recovery, rather than as cercariae. Since this may have altered their migratory potential and possibly their antigenic profile, interpretation of the results is complicated.

Certain schistosomulicidal drugs, which rapidly kill parasites *in vivo*, have been used to determine the period of contact between host and attenuated parasites required before resistance is induced against a subsequent challenge infection. Utilising this approach,

Bickle (1982) concluded that at least 1–2 weeks contact was required to elicit a protective response. Subsequently, a refined study (Bickle & Andrews 1985) using the drug RO11–3128 showed that only when the drug was administered 8 days after vaccination, was the level of resistance relatively unaffected compared with vaccinated control animals. Additionally, by varying the time between vaccination and challenge, Ratcliffe & Wilson (1992) showed a gap of not more than 10 days was required to induce near optimal levels of resistance. However, it must be remembered that challenge elimination does not occur immediately and therefore the induction phase could be prolonged beyond day 10 post–vaccination. Ratcliffe & Wilson (1992) suggested that the protective mechanism operating in the lungs of vaccinated mice developed primarily between days 11 and 20.

The surgical removal of the site of immunisation at times post–vaccination has also been used to assess the timing of the induction mechanism. Excision of the exposure site at 24h prevented the development of resistance, yet if delayed until day 7 or 14 partial immunity developed (Bickle, 1982). In a similar study, Mangold & Dean (1984) showed that removal of the tail exposure site on days 2 and 4 blocked the induction of resistance whereas excision on days 5 and 6 had less effect, and by day 8 resistance levels were comparable to vaccinated control mice. Between days 5 and 8, an increasing proportion of applied parasites would have migrated to post–skin sites, especially the lungs, suggesting that antigen presentation at, and/or cell recruitment to, such sites could be important to the induction process Alternatively, it was possible that the induction process was complete by day 8.

1.7.6 *Site requirements for the induction of immunity*

To investigate any site requirements for the induction of immunity, the vaccinating parasites can be delivered via various routes so as to by–pass an organ, or the organ (specifically lymph nodes) can simply be removed by surgery. These methods were used by various researchers to investigate any such requirements. Dean *et al.* (1981b) found little difference in the ability of day 6 schistosomula, irradiated at the time of recovery, to induce resistance when delivered intravenously, intra–muscularly or intra–peritoneally. Nevertheless, percutaneous vaccination with irradiated cercariae, a proportion of which migrate through the skin–draining lymph nodes, was found to induce similar or higher levels of resistance. Coulson & Mountford (1989) found that intra–dermal administration of attenuated 3h or 8 day old schistosomula, a proportion of which migrate to the draining lymph nodes and lungs, induced high levels of protection comparable to percutaneous vaccination. Intermediate levels were elicited by delivery of parasites via intra–peritoneal or intra–tracheal routes, whilst intravenous injection yielded little or no resistance. The latter result was corroborated by a recent paper from the same group (Mountford *et al.* 1992). Thus, the induction of optimal levels of resistance appears to require that some

attenuated parasites migrate to, and are sequestered in, lymph nodes draining the vaccination site. Since lymph nodes are potent sites for antigen processing and presentation, their importance is perhaps not surprising.

Removal of the skin–draining lymph nodes provided more direct evidence of their role in the expression of resistance (Mountford & Wilson, 1990). Surgical excision of the axillary and inguinal lymph nodes, which drain the skin exposure site, five days prior to vaccination, reduced the level of resistance by two–thirds following challenge infection, compared to sham–operated controls. Since abrogation was not complete suggests that priming of schistosome–specific lymphocytes may also occur at other sites, for example in the mediastinal lymph node. Additionally, removal of the skin–draining lymph nodes on days 5, 10, 15 or 20 post–vaccination resulted in progressively smaller reductions in immunity. Presumably this is as a result of the removal of primed lymphocytes resident in the skin–draining lymph nodes. The later the excision time, the more cells will have left the node and entered the circulation from which they may be recruited to sites of antigenic challenge, for example the lungs. Removal of lymph nodes not draining the site of vaccination had no effect on the induction of resistance, indicating that a major fraction of the proliferative responses relevant to protection occurs in the lymph nodes draining the skin–exposure site.

Nevertheless, the sensitisation of schistosome–specific T cells in the skin–draining lymph nodes may not be the only requirement for the induction of immunity, since parasites attenuated with 80 krad. radiation which fail to migrate beyond the skin but promote changes in lymph node cell number, fail to induce resistance (Constant *et al.* 1990; Mountford *et al.* 1992). However, vaccination of mice with 80 krad. irradiated cercariae followed 8 days later by intravenous injection of attenuated lung–stage schistosomula resulted in the recruitment of IFNg–secreting cells to the lungs and significant levels of resistance following challenge infection (Mountford *et al.* 1992).

1.8 *Challenge infection of vaccinated mice*

1.8.1 *Immunological events accompanying challenge infection*

Exposure of vaccinated mice to a cercarial challenge provokes a classical anamnestic immune response in the skin–draining lymph nodes, which is both more rapid in appearance and of greater magnitude than following primary exposure (Correa–Oliveira *et al.* 1984). Similarly, an anamnestic response was noted in the lungs following challenge infection, as judged by increase in cell number (Aitken *et al.* 1988). Changes in macrophage phenotype were also observed, though these were slight compared to the responses detected after vaccination (Menson & Wilson, 1990). Aitken *et al.* (1988) observed a strong association between pulmonary leukocytic responses and the

elimination of challenge infections by vaccinated mice. However, in the same study, mice vaccinated with irradiated cercariae of *Schistosoma mansoni* and challenged with *Schistosoma margrebowiei* failed to show a pulmonary anamnestic response, and were not protected. Thus, it appeared that the population of cells sensitised by the vaccinating parasites failed to react to the antigens presented by a heterologous challenge.

Despite the observed correlation between the occurrence of an anamnestic response and resulting protection, it has been shown that the effector mechanism operative against challenge schistosomula is radiation-resistant (Aitken *et al.* 1987; Vignali, Bickle & Taylor, 1988). Whole-body irradiation of vaccinated mice prior to challenge infection causes a dramatic decrease in the number of blood leukocytes but has no effect on the level of resistance induced, or the number of cells recoverable by BAL. Thus, it appears that the cells which are recruited to the lungs after vaccination are responsible for challenge parasite elimination, suggesting that further expansion of the cellular population as a result of the challenge infection is not required.

1.8.2 *Target and site of challenge elimination*

The parasite stage which is the target of immune elimination and the site at which it occurs, are in many respects intimately interlinked. However, experimental analysis of either has proved to be a controversial subject for many years. Investigations by some groups suggested early parasite death in the skin (Miller & Smithers, 1980; Hsu *et al.* 1983; Kamiya, Smithers & McLaren, 1987; Li Hsu *et al.* 1990), whereas, Mastin *et al.* (1983), using histologic techniques concluded that challenge schistosomula were eliminated during, or after their migration through the lungs. Since 1983, this finding has been corroborated by several workers (Dean *et al.* 1984; Von Lichtenberg *et al.* 1985) despite the detection of intense eosinophil-enriched inflammatory responses in the skin of vaccinated and challenged mice. Thus, although immune responses do occur in the skin, their relevance to immunity in this vaccine model is questionable. Autoradiographic tracking of challenge parasites has confirmed that the bulk of elimination occurs in the lungs of mice vaccinated against the York isolate of *Schistosoma mansoni* (Wilson *et al.* 1986). There is now overwhelming evidence to suggest that the "lung-stage" parasite is the major target of immune elimination. However, the site of lung-stage parasite attrition remains unresolved, being reported to occur either in the lungs or the skin (see Wilson & Coulson, 1989 or McLaren & Smithers, 1988; McLaren, 1989 respectively).

Further support for the importance of post-skin sites in the elimination of parasites has come from passive transfer studies with various protective sera. Several groups have found that the highest level of protection was achieved when the serum was transferred at a time coincidental with parasite residence in the lungs of mice (Mangold & Dean 1986; and see section 4.3.6) or rats (Ford *et al.* 1984). Furthermore, the ablation of IFNg,

described in section 1.7.3 provided additional evidence for the importance of post-skin sites. In this study, treatment was not started until day 4, by which time most parasites are *en route* to the lungs, yet resistance to a cercarial challenge was reduced by 89.5%.

As far as parasite attrition is concerned, the lungs may represent a unique site for various reasons. Firstly, parasites obviously find this a difficult organ to migrate through, since a large percentage of schistosomula from a primary infection fail to migrate beyond the lungs (see section 1.5.2). Also, the adaptive changes required to facilitate migration through this site necessitates that the parasite is a stationary target for several days, which may allow the immune response time to act. Secondly, following vaccination, the lungs contain a reservoir of schistosome-specific cells, which have been said to arm the lungs against a subsequent challenge infection. Finally, if parasite killing is the result of a non-specific blocking mechanism (see section 1.8.3) rather than direct cytotoxic action, it is more likely to be effective in the lungs than for example the skin. Parasite migration through the lungs is essentially unidirectional, any obstacle which manifests itself in front of the migrating larvae can retard or prevent onward migration. Rupturing of the narrow blood vessel walls may result in diversion into alveoli which trap the parasite. Conversely in the skin, parasites can migrate in any direction and there is no trap such as that provided by the alveoli in the lungs.

1.8.3 *Role of cell-mediated mechanisms in challenge elimination of parasites*

Two main mechanisms of cell-mediated elimination of challenge parasites have been proposed, which differ only in their specific mode of killing. Both rely on the recruitment of schistosome-specific Th cells to the lungs of vaccinated mice and the formation of cellular foci rich in mononuclear cells, including Th cells, around challenge parasites. These responses are characteristic of a DTH mechanism. Wilson and colleagues believe that the vast majority of parasite attrition is the result of a non-specific blocking effect caused by the compact cellular foci which form around the challenge parasite. In contradiction to this hypothesis, the ablation of IFNg during challenge infection caused larger pulmonary foci around parasites compared to untreated mice, yet immunity was almost totally abrogated. However, the foci were less compact and of different cellular composition, containing increased numbers of eosinophils and multinucleated giant cells (Smythies *et al.* 1992). It was postulated that the looser foci were not capable of preventing parasite migration through the pulmonary vasculature, accounting for the observed abrogation of immunity. Furthermore, despite the presence of extensive cellular infiltrates in the lungs of vaccinated mice, ultrastructural studies detected no dead or obviously damaged schistosomula (Crabtree & Wilson, 1986b). Indeed, the recovery of parasites from the lungs on days 7, 12 or 17 post-challenge, and their injection into the superior mesenteric vein resulted in an equal maturation rate irrespective of age. Many of

these parasites would not have matured if left *in situ*. This suggests that the parasites were not suffering any cytotoxic damage that would affect their ability to mature.

Conversely, James and colleagues believe that macrophages recruited to the lungs following vaccination have a direct cytotoxic effect on the challenge larvae. Macrophages obtained from normal humans and activated with recombinant human IFNg (Cottrel, Pye & Butterworth, 1989), or recovered from the peritoneal cavity of mice infected with *Schistosoma mansoni* (James *et al.* 1983), are very efficient effector cells in *in vitro* killing assays against newly-transformed schistosomula. The process can occur independently of antibody but is very slow; by 48h targetted parasites displayed massive internal disruption but the tegument remained intact. Ultrastructural studies of the interaction between macrophage and parasite *in vitro* showed contact to occur only transiently yet changes in the sub-tegumental mitochondria and muscle cells were observed (McLaren & James, 1985). Thus, unlike granulocyte-mediated killing, macrophage-mediated cytotoxicity did not appear to be directed against the surface tissues of the parasite. The mechanism of macrophage killing is not clear. However, the macrophage cell line, IC-21 can kill schistosomula *in vitro* despite its inability to produce oxygen products associated with the respiratory burst (McLaren & James, 1985). Thus it may be assumed that toxic oxygen radicals and/or H_2O_2 are not responsible for the manifestations of damage observed. Instead, the role of nitric oxides has been postulated, since treatment of macrophages with arginase, which inhibits nitrogen oxide (NO) synthesis, decreased *in vitro* killing of schistosomula. NO is thought to diffuse through the membrane and act on enzymes, probably in the mitochondria.

By using a nonliving vaccine to induce substantial levels of resistance to reinfection (see section 1.9.1), James (1986) demonstrated that sensitisation for cell-mediated immune responses, including lymphokine production and macrophage activation, correlated with induction of resistance to *Schistosoma mansoni* in this model. However, despite this correlation, using *in vitro* assays Pearce & James (1986) showed that 3h schistosomula and 2 and 3 week old worms were susceptible to macrophage-mediated killing yet 7-10 day old lung-stage larvae, the postulated targets of the effector mechanism, were not. It has been suggested that schistosomula which persist in the lungs for 2 weeks or more could become susceptible to direct cytotoxic killing. A situation can be envisaged in which parasites which are impeded by the cellular foci in the lungs and have not died from starvation in alveoli, become targets of direct cytotoxic mechanisms.

1.8.4 *Role of humoral mechanisms in challenge elimination of parasites*

Many studies have described the ability of various sources of antibodies to kill schistosomula *in vitro* in combination with eosinophils, macrophages or platelets (reviewed by Capron *et al.* 1982); these have been termed antibody-dependent cellular

cytotoxicity (ADCC) mechanisms. It appears that only young schistosomula are susceptible to this killing mechanism since later-stage parasites fail to bind antibodies to their surface. Various mechanisms were proposed to explain this, including the action of parasite proteases against bound immunoglobulins, rapid surface membrane turnover, the inert nature of the secreted outer bilayer of the tegument and the acquisition of host molecules to mask parasite proteins. The latter explanation is unlikely since parasites cultured *in vitro* in a defined medium also became refractory to the effector response (Dean, 1977; Samuelson, Sher & Caulfield, 1980; Dessein *et al.* 1981).

An *in vivo* role for antibodies in parasite elimination was suggested by the ability of some sera to confer protective immunity upon passive transfer to naive recipients. However, despite singly-vaccinated mice being highly protected against a challenge infection, and serum (vaccinated mouse serum; VMS) from such mice being able to recognise parasite surface proteins (Simpson *et al.* 1985), the passive transfer of VMS to naive mice has failed to confer any protection (Bickle *et al.* 1985; Mangold & Dean, 1986). Conversely, serum from multiply-vaccinated mice was capable of transferring a degree of resistance, although this was never as great as that shown by the donors (Mangold & Dean, 1986; Jwo & LoVerde, 1989). Multiple vaccination has been shown to predispose towards a Th2-type response compared with single vaccination which induces a Th1-type response (Caulada-Benedetti *et al.* 1991). Thus, multiple vaccination is likely to result in both quantitative and qualitative differences in antibody titre. Indeed, Dalton & Strand (1987) showed that while antibodies from VMS were directed against glycoproteins ranging in M_r from >300 to < 10 kDa, antibodies from mice vaccinated twice were directed predominantly towards high M_r glycoproteins. Such differences may account for the ability of multiply-vaccinated sera to transfer resistance.

The failure of serum from singly-vaccinated mice to transfer resistance could be viewed as circumstantial evidence for the importance of cell-mediated immunity in this model. Unfortunately, adoptive transfers of lymphoid cells from vaccinated donors (Bickle *et al.* 1985) or of Th1 cell clones (Pemberton *et al.* 1993) have failed to transfer resistance. There are various possible explanations for these failures. However, after prolonged culture *in vitro*, a Sm28GST-specific T cell line containing primarily Th cells secreting IFNg and IL-2, conferred approximately 50% protection to recipient mice (Wolowczuk *et al.* 1989).

It is possible that two different effector mechanisms occur in singly- and in multiply- vaccinated mice, a DTH response predominantly in the former and an antibody-mediated response in the latter. Supportive evidence for such ideas has been provided by $CD4^+$ T cell depletion experiments. As mentioned previously, depletion of this T cell sub-set can reduce immunity in singly-vaccinated mice by up to 100%. However, in multiply- vaccinated mice one study demonstrated that resistance was unaffected following $CD4^+$ T cell depletion (Kelly & Colley, 1988) whilst another

(Vignali *et al.* 1989), reported up to 70% abrogation of resistance.

1.8.5 *A summary of the induction and effector phases of immunity, and implications for the nature of the relevant antigens*

Optimally–attenuated parasites perform two key roles which facilitates their ability to induce protective immunity. Firstly, through their persistence in the skin–draining lymph nodes, a site of active antigen processing and presentation, they release proportionally greater amounts of material than normal parasites and stimulate a preferential proliferation of T lymphocytes. Secondly, their migration to, and eventual death in, the lungs recruits a population of schistosome–specific T cells which arms this organ against a subsequent challenge infection.

When normal schistosomula of a challenge infection reach the lungs of vaccinated mice, they provoke focal inflammatory responses rich in mononuclear cells, including $CD4^+$ T cells characteristic of DTH. The inflammation appears to function by blocking the migration of challenge parasites and, IFNg plays a key role via its capacity to act as an inflammatory cytokine.

The antigens involved in the induction phase of immunity which promote expansion of the T cell population are likely to be released from the viable vaccinating parasite, although it is possible that dead or dying schistosomula also prime immune cells. However, since the pulmonary effector mechanism in singly–vaccinated mice is T cell–mediated, it must be triggered by antigen(s) released from intact challenge schistosomula during the course of their migration. The released antigen(s) must then be processed by accessory cells and presented in association with MHC class II to the pulmonary T cells to initiate formation of an effector focus.

1.9 *Identification of candidate vaccine antigens*

Although live attenuated parasites consistently induce high levels of resistance against a cercarial challenge infection in mice, rats and primates, their use as a vaccine in humans is considered to be too risky for several reasons. Firstly, it would be essential that each vaccinating parasite was irradiated to such a degree as to prevent egg production. Failure to achieve this with essentially 100% of the parasites could lead to infection rather than immunisation. Secondly, there are recurring reports of an association of spinal cord complications associated with acute schistosomiasis (Neves *et al.* 1973). For these and additional reasons, attempts have been made to induce resistance by using non–living parasite preparations.

In the search for a vaccine against schistosomiasis, a vast number of antigens have been described and to list them all would be beyond the scope of this thesis. Therefore, I

shall try to concentrate on antigens which have received most attention. Initially, crude mixtures of parasite antigens from various stages of the life-cycle were tested for their ability to induce resistance, with moderate success. More recently, as research has progressed, defined antigens which have been cloned and sequenced have been used; these will be described later in section 1.9.4.

1.9.1 *Induction of resistance by complex non-living parasite preparations*

One of the highest protection results achieved with a crude antigen preparation utilised a cercarial sonicate (Horowitz, Smolarsky & Arnon, 1982). Small quantities of this complex antigen preparation (0.2μg – 2.0μg) adsorbed on alum were able to induce between 34% and 91% protection in mice against a subsequent challenge infection. The choice of mouse strain, adjuvant and immunisation protocol (two boosts on days 28 and 70 post-primary immunisation) were chosen to yield high specific IgE levels, a hallmark of infection by helminth parasites. IgE has been postulated to play a role in resistance to schistosomiasis, although depletion of IgE appeared to have no effect on the level of immunity in the irradiated cercaria vaccine model (Sher *et al.* 1990). The use of complete Freund's adjuvant instead of alum produced a higher titre of total anti-cercarial antibodies but low levels of anti-parasite IgE and provided no significant protection to the mice. In contrast to this encouraging result, immunisation of mice with a cercarial glycocalyx preparation actually caused a significant increase in the adult worm burden recovered after challenge infection (Harn, Cianci & Caulfield, 1989).

James and colleagues developed a vaccination regime specifically aimed at stimulating the cellular arm of the immune response. Either freeze-thawed larvae or soluble parasite preparations in combination with the adjuvant BCG injected intra-dermally into mice resulted in significant levels of protection, with a mean of 51% after a single immunisation (James, 1985). This has probably become the most consistent method for inducing significant levels of resistance using a non-living vaccine. Further characterisation of the immune response following this vaccination regime led to the identification of the protein paramyosin as an important inducer of the effector response (see section 1.9.4).

1.9.2 *Parasite surface proteins*

Since the tegument is in direct contact with the potentially hostile environment of the host, antigens expressed on the surface of the parasite have been extensively studied. Furthermore, many immune responses act at the surface of pathogens via antibodies. These include ADCC mechanisms which have been implicated in parasite killing. Thus, much time has been invested in the characterisation of tegumental proteins. Two major

approaches have been taken to analyse such proteins. One method involves the labelling of surface molecules using extrinsic non-permeant probes, followed by membrane isolation, gel electrophoresis and autoradiography. The immunogenicity of such labelled surface proteins can be assessed by immunoprecipitation with various sera from vaccinated or infected hosts. In the second approach, the target antigens of monoclonal or polyclonal antibodies which bind to the parasite surface have been identified by immunoprecipitation, affinity chromatography or Western blotting.

The two main surface labelling techniques which have been used by researchers are the lactoperoxidase (Dissous, Dissous & Capron, 1981) and the Iodogen methods (Knight *et al.* 1984). Both techniques demonstrated that 6 major polypeptide surface antigens of *Schistosoma mansoni* could be precipitated by human or animal infection sera. These were of M_r 92, 38, 32, 20, 17 and 15 kDa (Simpson & Smithers, 1985; Payares *et al.* 1985). However, many more surface proteins have been described. Wilson (1987) compiled a table of 17 proteins which are expressed on the surface of 3h schistosomula and have been reported more than once in the literature. Such proteins ranged in M_r from >150 to <20 kDa. Taylor & Wells (1984) detected at least 35 labelled surface polypeptides of schistosomula after 2-D SDS-PAGE. Of these, not all were precipitated by a pool of human infection serum, and there was a marked heterogeneity in the antibody response of infected individuals to the tegument preparation. Additionally, 2-D SDS-PAGE of ^{125}I-labelled surface proteins resolved 23 polypeptides of which 14 could be immunoprecipitated by either chronic mouse serum or vaccinated mouse serum. Proteins of M_r 32-38 kDa and 20 kDa could be precipitated by either sera (Kelly *et al.* 1985). Thus, the schistosomulum appears to display a diverse range of surface proteins, which is unusual for a parasite which is in direct contact with an immunologically hostile environment.

Evidence that schistosomula surface antigens play a role in immunity is provided by a number of studies which have shown that monoclonal antibodies which recognise such antigens can often be used to transfer passive immunity. Grzych *et al.* (1982) produced a rat monoclonal IgG2a antibody which transferred significant levels of immunity to rats (53-61%) and which immunoprecipitated surface antigens of M_r 200 and 38 kDa. Zodda & Phillips (1982) described a mouse monoclonal antibody which conferred 48% resistance to mice and recognised two proteins of M_r 160 and 130 kDa. More recently, the antibody was shown to bind to schistosomulum surface antigens of 200, 38, 17 kDa and weakly to the 20 kDa antigen (Kelly *et al.* 1986). This poly-specific recognition was due to the presence of a common carbohydrate epitope. Harn *et al.* (1985) produced a protective monoclonal antibody following immunisation of mice with membrane-enriched extracts of mechanically-transformed schistosomula, which later lead to the identification of the vaccine candidate triose-phosphate isomerase (see section 1.9.4). Bickle, Andrews & Taylor (1986) identified two monoclonal antibodies against surface

antigens present on schistosomula which reacted with proteins of 32 kDa and 16 kDa and which conferred significant protection to mice of between 28–70% and 14–58%, respectively. Furthermore, Smith & Clegg (1985) used two surface antigens of M_r 155 and 53 kDa, isolated from young or adult schistosomes, to vaccinate mice. Following challenge infection small yet statistically significant reductions in mean worm burdens were observed compared to challenge control mice. Such results would seem to suggest that this line of research is valid. Indeed, most of the promising vaccine candidates described in section 1.9.4 have been shown to be expressed, at least transiently, on the surface of the parasite.

1.9.3 *Parasite released proteins*

Released (i.e. secreted, excreted or shed) proteins of *Schistosoma mansoni* have received most attention for their potential as immunodiagnostic markers of infection, rather than vaccine candidates. In this respect, the majority of work has focused on proteins released by adult worms, since antigens of the adult worm digestive tract are recognised by the immune system of the host early after infection. Five proteins associated with the worm gut have been identified, some or all are thought to be components of the mucin–like layer shielding the gastrodermal syncitium from low pH and immune attack (Chappel *et al.* 1990). Of these antigens, two have received most attention, circulating cathodic antigen (CCA) and circulating anodic antigen (CAA). Both show a large molecular weight range of approximately 50 kDa to >300 kDa and partition in the TCA soluble fraction of adult worms (Deelder *et al.* 1980). Their presence could be demonstrated in the vomitus and excretory/secretory antigens of adult worms. Using an IgG1 monoclonal antibody against CAA in a sandwich ELISA, the level of antigen in serum of infected patients was found to correlate with faecal egg output (Deelder *et al.* 1989). Additionally, the lower detection level corresponded to approximately 10 eggs per gram of faeces. Thus, such antigens are proving valuable in immunodiagnosis of schistosomiasis.

Much less attention has focused on released proteins, of surface or other origin, as potential vaccine candidates. Of the few studies which have taken this approach, vaccination of mice with proteins released by parasites during *in vitro* culture has met with limited success. Murrel & Clay (1972) used a complex multiple vaccination regime to deliver proteins released by adult worms, and achieved 40% resistance. However, the culture conditions are unlikely to have been satisfactory for normal parasite maintenance and therefore the released proteins may have been contaminated with a large proportion of somatic proteins. Conversely, no protection was achieved by immunising mice with secretions derived from cercariae (Minard, Murrel & Stirewalt, 1977).

However, studies from Capron's group on proteins released from newly–transformed

schistosomula during 16h of *in vitro* culture were more promising. Rat IgE directed against such schistosomula-released proteins (SRP) were cytotoxic for larvae *in vitro* in the presence of macrophages, eosinophils or platelets (Auriault *et al.* 1984a) and recognised two surface proteins of M_r 26 and 22 kDa. Additionally, a large number of SRP with a wide M_r range was recognised by infected rat and human sera (Verwaerde *et al.* 1985). IgG antibodies obtained by immunisation of rats with SRP reacted with 3 major schistosomula surface proteins of M_r 38, 32 and 21 kDa. These antibodies facilitated killing of schistosomula *in vitro* in the presence of complement or eosinophils. Active immunisation of rats with SRP achieved between 46% and 83% resistance, whilst passive transfer of anti-SRP antisera conferred up to 83% protection (Damonneville *et al.* 1986). An important role for IgE in parasite attrition was suggested by the significant reduction in immunity following IgE depletion of the antiserum. Subsequently, the 26 kDa antigen was found to be the main target of the cytotoxic IgE response. Additionally, T cell lines specific for SRP passively transferred to naive rats induced an increase in the IgE response and conferred 46-66% resistance to a challenge infection (Damonneville *et al.* 1987).

1.9.4 *Vaccine candidates*

In 1991, a World Health Organisation report stated that a limited number of vaccine candidates had reached the stage at which development, with the objective of trials in humans, might be considered. These included glutathione S-transferase, paramyosin and triose-phosphate isomerase. It was noted that many additional proteins of potential utility had been described but were not as well defined as the aforementioned antigens. Table 1.1 lists some of the protection data achieved with these and other promising vaccine candidates.

1.9.4.1 *Glutathione S-transferase (Sm28GST)*

In terms of vaccine production, glutathione S-transferase (Sm28GST) is the most advanced, since it has been cloned, sequenced, crystallised and tested for protective immunity in various animal models. This antigen was first described by Balloul *et al.* (1985). Constituents of adult worms were fractionated by SDS-PAGE, and proteins electroeluted from the gel used to immunise Fischer rats. Three antisera were obtained, one of which recognised a 28 kDa protein. This antigen could be detected among the *in vitro* translation products from adult worm mRNA and among ^{125}I surface antigens of schistosomula. Furthermore, the antiserum could mediate antibody-dependent cellular cytotoxicity (ADCC) of schistosomula *in vitro* in the presence of eosinophils. By 1987, active immunisation of Fischer rats or BALB/c mice with the purified antigen resulted in significant levels of resistance (Balloul *et al.* 1987a). Additionally, antiserum raised

against this antigen conferred passive protection upon recipient rats. In the same year the anti–28 kDa antiserum was used to screen an adult worm cDNA library. A positive clone was purified to homogeneity and found to code for a 25 kDa recombinant protein (Balloul *et al.* 1987b). Serum from rats immunised with this fusion protein was capable of inducing up to 80% cytotoxicity of schistosomula in an *in vitro* ADCC assay in the presence of normal rat eosinophils. This cytotoxicity could be reduced to 35% by heat–inactivation or IgE depletion.

Subsequently, the recombinant protein was found to possess glutathione S–transferase activity (Taylor *et al.* 1988). This enzyme is ubiquitous amongst eukaryotic organisms and performs a series of reactions which are essential in protecting cell constituents from oxidative attack by oxygen and oxygen–free radicals. The enzyme was found to be present in the tegument, protonephridial and subtegumental cells. Additionally, no significant immunological cross–reactivity between parasite and human and rat GST was observed, an important consideration for vaccine development.

T cells from mice immunised with recombinant Sm28GST (rSm28GST) proliferated in response to schistosome antigens from different developmental parasite stages, and to 3 rSm28GST–derived synthetic peptides, of which the most significant response occurred to amino acid residues 24–43 (Wolowczuk *et al.* 1989). Sm28GST specific Th cell lines maintained in culture for two months and containing predominantly IL–2 and IFNg secreting cells (Th1), passively transferred 50% protection to mice. Subsequently, a peptide (115–131) derived from Sm28GST was shown to have both T and B cell epitopes and, when delivered as an octameric construction in conjunction with CFA, resulted in 40–50% protection in rats (Wolowczuk *et al.* 1991). Immunisation of baboons with rSm28GST in alum resulted in a significant but very variable level of protection (Boulanger *et al.* 1991). In addition to the decrease of worm burden, immunisation could reduce female worm fecundity by 33% and faecal egg output by 66%.

A monoclonal antibody raised against Sm28GST and which inhibited its enzymatic activity was shown to recognise the carboxyl terminal amino acids 190–211 and to a lesser extent the amino terminus residual 10–43. Active immunisation of mice with amino acid residues 190–211 decreased the worm burden and also tissue egg deposition and egg hatching. It was postulated that by using both the amino acid residues 115–131 and the N– and C–terminal peptides, a peptidic construction could be produced which decreased both worm burden and female worm fecundity (Xu *et al.* 1993). Recently, vaccination of Zebu cattle with *Schistosoma bovis* GST was shown to have a small effect on the worm burden following challenge infection, but decreased faecal egg counts by 56–82% (Bushara *et al.* 1993).

1.9.4.2 *Paramyosin (Sm97)*

In 1986, Pearce *et al.* reported that mice protected against a challenge infection by

intra-dermal vaccination with a nonliving antigen preparation in conjunction with BCG, produced antibodies against a single antigen of 97 kDa present in soluble adult worm preparations. Hybridomas, prepared from such mice, produced antibodies which recognised the 97 kDa protein on Western blots. Furthermore, the antibodies bound to regions just below the tegumental and gut syncitia on transverse sections of adult worms. The purified antigen could elicit DTH in intra-dermally vaccinated mice indicating that it was also capable of evoking cell-mediated responses. Subsequently, antibodies were used to isolate a clone from an adult worm lambda gt11 cDNA library. Following purification and sequence analysis, the protein was found to have homology to paramyosin, a myofibrillar muscle protein (Lanar *et al.* 1986).

The internal location of this protein made it distinct from other vaccine candidates which are expressed, at least transiently, on the surface of the parasite. As such, this protein would not be available for direct attack by antibodies. It was postulated that the cell-mediated response could be stimulated by Sm97 molecules released from parasites as a consequence of normal protein metabolism. Subsequently, when administered at 4-40μg per mouse, both the native molecule and a recombinant expression product conferred resistance against a challenge infection (Pearce *et al.* 1988). Additionally, lymphocytes recovered from mice vaccinated with Sm97 produced IFNg in response to living schistosomula suggesting that this protein is released from live parasites.

1.9.4.3 *Triose-phosphate isomerase*

This protein was defined following the preparation of monoclonal antibodies from mice vaccinated with detergent extracts of living mechanically-transformed schistosomula (Harn *et al.* 1985). The monoclonal antibody M.1 was found to recognise a 28 kDa antigen in all stages of the parasite, and transiently bound to the surface of newly-transformed schistosomula, therefore it was tested for its ability to transfer resistance to mice. When administered intra-peritoneally 3-4h prior to challenge infection, the monoclonal antibody conferred 41-49% protection to mice (Harn *et al.* 1992). By immunofluorescence on transverse sections of adult worms, the antigen was found in all cells. Amino terminus sequences of tryptic peptides of the 28 kDa antigen had high (79-87%) sequence homology with the mammalian glycolytic/gluconeogenic enzyme triose-phosphate isomerase. Subsequently, the complete coding DNA for *Schistosoma mansoni* TPI was isolated (Shoemaker *et al.* 1992) and confirmed that this cDNA encoded the 28 kDa antigen recognised by the monoclonal antibody M.1. The purified native enzyme was shown to function enzymatically in a manner similar to yeast and mammalian TPI. Addition of the monoclonal antibody to the reaction altered the catalytic activity of the schistosome TPI. The monoclonal antibody M.1 was found to be immunologically specific for the schistosome enzyme.

1.9.4.4 *Glyceraldehyde 3-phosphate dehydrogenase* (G3PDH)

In a hyper-endemic area for *Schistosoma mansoni* in Brazil, the ability of adolescents to resist reinfection, following parasitologic cure with oxamniquine, was assessed. Subsequently, the subjects were grouped into those with either a low or high susceptibility to reinfection. The ability of the most resistant group to specifically detect any parasite antigen was determined (Dessein *et al.* 1988). It was found that IgG antibodies from those subjects with a low susceptibility to reinfection preferentially reacted with a 37 kDa larval surface antigen. Subsequently, this 37 kDa antigen was cloned and the encoded polypeptide found to have a high amino acid sequence homology with chicken and human G3PDH (Goudot-Crozel *et al.* 1989). This protein is highly conserved, therefore regions of low conservation need to be identified for use as a vaccine in humans.

1.9.4.5 *Other vaccine candidates*

Strand and colleagues identified potentially protective antigens by comparing the recognition of proteins by sera from protectively vaccinated mice to that of patently-infected mice (Strand, Dalton & Tom, 1987). Sera from mice vaccinated with irradiated cercariae recognised antigens present in schistosomula and adult worms, among them, an antigen of 200 kDa. A cDNA clone encoding a 62 kDa protein of this antigen was sequenced and found to have homology with myosins of other species (Amory Soisson *et al.* 1992). The recombinant protein presented to mice in the form of proteosome complexes, with or without the outer membrane protein of meningococcus, resulted in impressive levels of protection.

A further protective glycoprotein antigen of 68 kDa was isolated from adult worms by immunoaffinity chromatography using a protective monoclonal antibody. Immunisation of mice with this antigen induced 30-66% resistance against a challenge infection without the use of adjuvant (King *et al.* 1987). Additionally, in a group of chronically infected Egyptian patients, the antibody response to this antigen showed a significant negative correlation with intensity of infection (King *et al.* 1989). Subsequently, a monoclonal antibody against this protein conferred partial protection to mice (Blanton *et al.* 1991) and, by immunoelectron microscopy, was found to bind within the head and preacetabular glands of cercariae and schistosomula. In adult worms the protein was found to be widely distributed, being most visible in the gut and tegument.

Mr (kDa)	FORM	ANIMAL MODEL	ADJUVANT	IMMUNISATION ACTIVE	IMMUNISATION PASSIVE	REFERENCE
GLUTATHIONE S-TRANSFERASE (GST)						
28	NATIVE	RAT (FISCHER)	CFA or $AL(OH)_3$	52-72%		BALLOUL ET AL. 1987a
		MOUSE (BALB/c)	CFA	40-43%		
		RAT (FISCHER)			60-65%	
	RECOMBINANT	RAT (FISCHER)	$AL(OH)_3$	67%		BALLOUL ET AL. 1987b
		HAMSTER	$AL(OH)_3$	52%		
	RECOMBINANT	BABOON	$AL(OH)_3$	0-80%		BOULANGER ET AL. 1991
	PEPTIDE	RAT (FISCHER)	CFA	40-50%		WOLOWCZUK ET AL. 1991
	RECOMBINANT	RAT (FISCHER)	$AL(OH)_3$ or BCG	32-60%		GREZEL ET AL. 1993
	RECOMBINANT	MOUSE (BALB/c)	CFA	28-52%		XU ET AL. 1993
	PEPTIDE	MOUSE (BALB/c)	CFA	37-40%		
	PEPTIDE	MOUSE (BALB/c)	CFA	9-20%		

Table 1.1. Candidate vaccine antigens against schistosomiasis

Mr (kDa)	FORM	ANIMAL MODEL	ADJUVANT	IMMUNISATION ACTIVE	IMMUNISATION PASSIVE	REFERENCE
PARAMYOSIN (Sm97)						
97	NATIVE	MOUSE (C57Bl/6j)	BCG	31–39%		PEARCE ET AL. 1988
	RECOMBINANT	MOUSE (C57Bl/6j)	BCG	26%		
	NATIVE	MOUSE (CFI)	NONE	24–53%		FLANIGAN ET AL. 1989
TRIOSE-PHOSPHATE ISOMERASE (TPI)						
28		MOUSE (C57Bl/6j)			41–49%	HARN ET AL. 1992
MYOSIN						
62	RECOMBINANT	MOUSE (C57Bl/6j)	+ ADJUVANT – ADJUVANT	UP TO 82% UP TO 62%		AMORY SOISSON ET AL. 1992
SmW68						
68	NATIVE	MOUSE (C57Bl/6j)	NONE	30–66%		KING ET AL. 1987

Table 1.1 (cont.). Candidate vaccine antigens against schistosomiasis

1.10 *Aims of this study*

From the preceding introduction, it should be clear that T cells play a pivotal role in the expression of resistance in mice vaccinated with irradiated cercariae of *Schistosoma mansoni*. Since the pulmonary effector mechanism is T cell–mediated, it must be triggered by antigen(s) released from intact challenge schistosomula during the course of their normal biological activities in order to initiate an effector focus. The released antigen(s) must be processed by accessory cells before presentation, in association with MHC class II, to the pulmonary T cells. Thus, parasite–released proteins are a source of potential vaccine candidates.

A large body of information is available about tegumental proteins of schistosomula and adult worms. Conversely, much less is known about proteins released by *Schistosoma mansoni* during maturation in the mammalian host. Although proteins released by newly–transformed schistosomula have been described previously, most work examined the secreted proteases, which facilitate skin penetration by cercariae, to the exclusion of other proteins. No study has analysed the proteins released by schistosomula up to the lung–stage of development. It is during this period that the induction and effector mechanisms of immunity occur in the irradiated cercaria vaccine model. Therefore, the main aim of this study was to characterise such released proteins.

Initial work, prior to that described in chapter two, led to the development of a suitable culture system which supported parasite development to the lung–stage and, maintained a high level of viability. Once this was achieved, several biochemical parameters of parasite metabolism were measured. The biosynthetic labelling of parasites either prior to or post–cercarial transformation, facilitated analysis of the kinetics of protein synthesis and loss during *in vitro* culture. Additionally, the proteins released into the culture medium over a period of 7 days were characterised following SDS–PAGE plus autoradiography.

Chapter three describes an *in vitro* T cell blastogenesis assay which was used to assess the ability of such schistosomula–released proteins to induce a proliferative response by lymph node cells recovered from vaccinated mice. A comparative analysis was carried out in which the proliferative responses induced by soluble, particulate and released proteins of *Schistosoma mansoni* and by live schistosomula were tested. It was hoped that such a study would provide an indication of the potency of released proteins as T cell immunogens, and hence their potential as vaccine candidates.

Chapter 4 describes the production of two sera, 0–3hS and D4–D8S, raised in rabbits against proteins released by schistosomula during the first 3h and between day 4 and day 8 post–transformation respectively. The D4–D8S was produced primarily to screen a cDNA library. However, both sera were tested for their ability to transfer passive protection to mice and were also used to identify antigenic moieties in various protein

fractions of *Schistosoma mansoni*.

The final aim of the project was to characterise further the proteins released by lung-stage schistosomula. Since only tiny quantities of such molecules are available, chapter 5 describes the generation and screening of a cDNA library with the D4-D8S. The identification of recombinant proteins and, initial steps taken towards their characterisation are described.

CHAPTER TWO

Protein synthesis and release by cultured schistosomula of *Schistosoma mansoni*

Protein synthesis and release by cultured schistosomula of *Schistosoma mansoni*

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SUMMARY

The lung schistosomulum of *Schistosoma mansoni* is the target of protective immunity in mice singly vaccinated with irradiated cercariae. Since the effector responses are T cell-mediated, their initiation requires the release of antigens from the intact parasite. We have used the technique of biosynthetic labelling with [^{35}S]methionine, before and after transformation of the cercariae, to analyse the kinetics of protein synthesis and release by the schistosomulum. In addition, the proteins present in the soluble fraction of the parasite and those released during *in vitro* culture have been characterized. During a 7-day culture period schistosomula derived from labelled cercariae lost proteins most rapidly within the first 3 h after transformation. Two proteins of molecular weight 61 and 20 kDa were dominant and may correspond to areas of proteolytic activity. Analysis of the rate of protein synthesis of schistosomula labelled after transformation revealed four different phases, which may relate to the developmental processes occurring *in vivo*. During the first 24 h, synthesis was very low, increasing to a plateau and then rising to a peak at day 8; thereafter the rate declined rapidly. Whilst some stage-specific synthesis of proteins was detected in the soluble fractions of the parasite bodies, the pattern of proteins released by cultured larvae was remarkably uniform. At least 15 proteins were detected by autoradiography with bands at 61, 45 and 20 kDa being particularly prominent. These proteins merit further study as potential mediators of the protective immune response.

Key words: *Schistosoma mansoni*, protein synthesis, schistosomulum, protective immune response.

INTRODUCTION

Vaccination of C57Bl/6 mice with optimally irradiated cercariae of *Schistosoma mansoni* remains the most consistent method of inducing a high level of protective immunity. However, it is still not completely understood why this vaccination regime is so successful. Irradiated larvae persist in the skin and draining lymph nodes for longer than their normal counterparts, and migrate no further than the lungs where they die (Mangold & Dean, 1984; Mountford, Coulson & Wilson, 1988). Priming of schistosome-specific lymphocytes with Th1 characteristics takes place in the skin-draining lymph nodes (Pemberton *et al.* 1991; Mountford *et al.* 1992). The recruitment of these cells to the lungs, in response to the arrival and sequestration of vaccinating parasites, is thought to be an essential feature of immunity in this model (Wilson & Coulson, 1989; Mountford *et al.* 1992).

It has proved difficult to pinpoint the precise developmental stage of the parasite which induces protective immunity. When irradiated day 8 lung schistosomula are administered intradermally, they prove to be at least as immunogenic as irradiated cercariae (Coulson & Mountford, 1989), implying that secretions unique to the latter are not the relevant immunogens. Conversely, irradiated 3 to 4-week-old schistosomula are much less effective than skin- or lung-stage larvae (Dean, Cioli & Bukowski, 1981; Sher & Benno, 1982). These observations indicate that larval antigens expressed within the first 1–2 weeks after penetration are crucial for the induction of protection. This conclusion is supported by experiments in which the time of excision of skin-exposure sites or draining lymph nodes was varied (Mangold & Dean, 1984; Mountford & Wilson, 1990), or the period between vaccination and challenge altered (Ratcliffe & Wilson, 1992). Tracking studies have revealed that the majority of challenge parasites are eliminated in the lungs of once-vaccinated mice (Wilson, Coulson & Dixon, 1986), following stimulation of a focal delayed-type hypersensitivity (DTH) response which blocks onward migration (Crabtree & Wilson, 1986; Smythies, Coulson & Wilson, 1992). Each focal response to a challenge larva appears to be an independent event. Since immunity in these mice is cell-mediated, the antigens must be processed by accessory cells and presented in the context of MHC class II, to be recognized by the antigen receptors on reactive $CD4^+$ T cells. Triggering of an effector response in the lungs will therefore require the release of antigens from intact challenge schistosomula. It is also implicit that the vaccinating parasites must release an identical set of antigens to prime T cells in the lymph nodes.

The loss of significant quantities of material from labelled schistosomula *in vivo* has been noted

(Wilson & Coulson, 1986; Mountford *et al.* 1988), yet no study has characterized the proteins released from schistosomula during the period corresponding to migration from the skin to the lungs. Proteolytic enzymes released from cercariae upon transformation have been described previously (see review by McKerrow & Doenhoff, 1988), and the proteins released by adult worms during *in vitro* culture have recently been studied by Lewis & Strand (1991). The formulation of an efficient medium for the *in vitro* culture of schistosomula (Basch, 1981) has allowed the analysis of many biochemical parameters which would be impossible to study *in vivo*. This culture system is capable of supporting worm growth and development up to the stage of pairing, and electron microscope studies have detected no obvious morphological differences between *in vivo* and *in vitro* cultured worms (Basch & Basch, 1982).

Using this system, we have investigated the kinetics of protein synthesis and loss during *in vitro* culture of schistosomula up to the lung-stage of development. For this purpose, two different pools of proteins were labelled, those synthesized by the developing cercariae in the snail, and those synthesized by the schistosomula after transformation. The pattern of proteins labelled in the soluble fraction of parasites prior to and post-transformation has been determined. In addition, the subset of proteins released into the culture medium between transformation and day 7 has been characterized. The dominant released proteins which may mediate lung-phase immunity are highlighted.

MATERIALS AND METHODS

Preparation of parasite material

A Puerto Rican isolate of *Schistosoma mansoni* was maintained by routine passage through albino *Biomphalaria glabrata* and LACA mice. Snails harbouring a patent infection were induced to shed cercariae by exposure to bright light. The cercariae were pooled, concentrated on ice by sedimentation and mechanically transformed as described by Ramalho-Pinto *et al.* (1974). Cercarial heads were isolated from tails by centrifugation on a discontinuous Percoll gradient (Lazdins *et al.* 1982).

Parasite culture system

Medium 169 (M169) as defined by Basch (1981) was used throughout for the culture of schistosomula, without the addition of human serum or red blood cells; the absence of the latter prevents transformation of parasites to the liver stage of development. Medium 169 containing 300 U/ml penicillin, 300 μg/ml streptomycin and 160 μg/ml gentamicin (Sigma) was used as a wash medium (M169W). This was supplemented with 1% foetal calf serum (M169S; Globepharm) or 20 μg/ml bovine serum albumin (M169A; Sigma), depending on the experimental protocol followed. M169S was used when culture supernatants were not to be collected, as the excess of proteins contributed by the serum supplement caused distortions on gels. Isolated cercarial heads were washed 4 times in 10 ml of M169W, and cultured in either M169S or M169A (2000–3000 parasites/ml) in 24-well plates (Corning) at 37 °C in 5% CO_2.

Assessment of parasite viability and maturation

The viability of schistosomula throughout the culture period was assessed by scoring the percentage which were motile, and by means of a Trypan blue dye exclusion test. Viability on day 3 and day 7 was also tested by culturing 3 different aliquots of schistosomula for 4·5, 9 or 20 h in 10 ml of M169A containing 100 μCi [^{35}S]methionine. Subsequently, the TCA-precipitable cpm/parasite were determined. A linear increase in the incorporation of isotope into proteins with time indicated that there was no downturn in metabolism. The ability of *in vitro*-cultured schistosomula to mature *in vivo* was determined by their surgical transfer to the superior mesenteric vein of naive mice (Coulson & Wilson, 1988). The experiment was performed with hand-counted day 7 and day 12 schistosomula and aliquotted samples of day 7 larvae. Five weeks later, adult worms were recovered by perfusion of the hepatic-portal system and counted to determine the percentage maturation.

Radio-isotope labelling of parasites

In order to identify proteins synthesized by the developing cercariae prior to transformation, snails previously screened for their ability to yield a large number of parasites were labelled with [^{35}S]methionine (20 μCi/snail; NEN; specific activity 1186 μCi/mmol) in 1 ml of pond water/snail for 5 h, according to the method of Wilson & Coulson (1986). Labelled cercariae were obtained 5 and 7 days later (shed I and II respectively), transformed, and cultured as described in the experimental design. Following centrifugation at 150 *g* for 60 s, parasite-free culture supernatants were collected at predetermined times.

To label those proteins synthesized after transformation, schistosomula were first cultured in M169S for defined times, washed 4 times to remove any dead parasites and serum proteins, and cultured in approximately 10 ml of M169A containing 200 μCi [^{35}S]methionine for a defined period, as described in the experimental design. Schistosomula were then washed and chase-cultured in M169A, or pelleted and used to obtain a soluble protein fraction. Culture supernatants were collected at various times

and then passed through a 10DG desalting column (Bio-Rad) to remove any unincorporated [^{35}S]methionine.

Preparation of culture supernatants and schistosomula for analysis by SDS–PAGE and autoradiography

Culture supernatants were concentrated to 500 μl in a stirred ultrafiltration cell containing a 3 kDa cut-off Diaflo membrane (Amicon), under N_2 at a pressure of 40 psi, and stored at −80 °C. Soluble proteins were released from larval bodies by sonication (21 kHz at 6·5 μm amplitude) for 90 s. Soluble and particulate material was partitioned by centrifugation for 1 h at 105000 *g* and 4 °C. The soluble sonicate was recovered and stored at −80 °C. Most of the secretory proteins should be a subset of the soluble fraction, although proteins of membrane origin may be absent. Conversely, many soluble proteins, for example cytoplasmic enzymes will not be secreted and their appearance in the culture medium would be an indication of leakage from dead or dying parasites.

Proteins in the culture supernatants and soluble fractions were separated under reducing conditions by SDS–PAGE on 6–16% density gradient gels. Gels were fixed, stained with Coomassie R-250, impregnated with the fluorographic reagent Amplify (Amersham) and then dried. Labelled proteins were visualized by autoradiography following exposure of gels to Hyperfilm-β max (Amersham) for 4–6 weeks.

Analysis of samples by substrate gel electrophoresis

Substrate gel electrophoresis was carried out essentially as described by Lockwood *et al.* (1987). Briefly, culture supernatants and soluble protein fractions were diluted in sample buffer without dithiothreitol and loaded, without prior boiling, onto 9% acrylamide gels impregnated with gelatin to a final concentration of 0·2%. After electrophoretic separation, the gels were washed briefly in distilled water and then for 2 × 30 min in 2·5% Triton X-100. Gels were incubated for 48 h at 37 °C in 0·1 M glycine–NaOH, 5 mM $CaCl_2$, pH 9, stained in Coomassie blue and then destained to allow visualization of areas of proteolytic activity.

Experimental design

Pre-transformation labelling. The loss of [^{35}S]methionine labelled material from schistosomula was monitored throughout the 7-day culture period. Replicates of 10 cercariae, cercarial tails, or schistosomula (0 h, 3 h, 24 h and day 7) and 5 μl aliquots of the corresponding culture supernatants, were applied to glass microfibre filters (Whatman). These were dried and, after addition of scintillant, counted in a liquid scintillation analyser (Packard).

In order to obtain proteins for analysis by SDS–PAGE and autoradiography, schistosomula derived from shed I of the labelled snails were cultured for 7 days in M169A. At 3 h and 24 h, parasite-free culture supernatants were removed, the schistosomula resuspended and cultured in fresh medium. On day 7, the culture supernatant was removed and then the 0–3 h, 3–24 h and 24 h–day 7 supernatants, concentrated as described previously. The schistosomula were used to yield a day 7 soluble fraction. Cultures of parasites from shed II were terminated at 24 h to yield a soluble protein fraction. *Post-transformation labelling.* (i) *Kinetics of protein synthesis during development.* Separate aliquots of newly-transformed schistosomula, derived from a single pool of cercariae, were pulsed with [^{35}S]methionine for 24 h on consecutive days (up to day 10). At the end of the 24 h pulse-period, schistosomula were washed 6 times in 10 ml of M169W to remove unincorporated isotope. The incorporation of [^{35}S]methionine into proteins of schistosomula was assessed by measuring the TCA-precipitable cpm. Replicate samples of 50 washed schistosomula in 200 μl of M169W were lysed by sonication, and protein precipitated by the addition of 600 μl of 15% TCA. After 15 min on ice, the lysates were applied to individual glass microfibre filters (Whatman), washed with 15% TCA, dried and counted. The background radioactivity from 200 μl of the final wash medium was determined and subtracted from the value of the parasite lysates. (ii) *Analysis of released and soluble proteins.* Schistosomula derived from a single pool were divided into 7 or 8 equal aliquots and cultured in M169S for periods ranging from 0 to 7 days, after which they were washed 4 times and pulsed with [^{35}S]methionine in M169A for 24 h. The culture supernatant was removed after centrifugation, and the number of parasites in each culture determined. Soluble proteins were prepared from the larval bodies, whilst culture supernatants were treated to remove unincorporated isotope (as described above), and then concentrated. All samples were analysed by SDS–PAGE and autoradiography. (iii) *Continuous 7-day labelling.* A single pool of schistosomula was cultured in approximately 10 ml of M169A containing [^{35}S]methionine for 7 days. At the end of this period, the culture supernatant was removed and treated as described previously. The number of parasites in the culture was determined and the larval bodies used to yield a soluble protein fraction for analysis by SDS–PAGE and autoradiography, in comparison with the culture supernatant. (iv) *Pulse-chase labelling.* In order to investigate the kinetics of labelled protein loss, schistosomula derived from a single pool of cercariae were divided into 3 equal aliquots, and cultured in M169S prior to labelling. Each culture was pulsed with [^{35}S]methionine in M169A for 24 h on days 3, 6 or 10. The pulse supernatant was then recovered, the

parasites washed extensively to remove any unincorporated label, and chase-cultured for 4 days in M169A. An aliquot of approximately 500 schistosomula was taken immediately after the 24 h pulse period and 2 and 4 days later and the TCA-precipitable cpm/parasite determined.

RESULTS

Viability and maturation of schistosomula throughout the culture period

To ensure that the proteins detected in the culture medium were products released from live schistosomula and not material leaked by dead or dying parasites, viability was monitored by several methods. Following a 7-day continuous culture period in M169S (without any washes), approximately 95% of parasites were motile and excluded Trypan blue. Viability was slightly lower in M169A, and therefore M169S was used in preference, except where the presence of serum would interfere with analysis of proteins by SDS–PAGE. Most deaths occurred during the first 2–4 days when the parasites were much less motile, less elongate and more fragile upon centrifugation than at later times. From day 5 onwards, the schistosomula began to elongate and became much more motile. When schistosomula were cultured for 7 days and then pulsed for 4·5, 9 and 20 h, they incorporated 2·8±0·15, 5·89±0·26 and 13·62±0·66 cpm/parasite respectively, i.e. a linear increase with time (data not shown). An identical experiment using day 3 schistosomula produced a similar linear increase but with a lower level of incorporation.

When hand-counted schistosomula were transferred to naive mice after 7 or 12 days of *in vitro* culture, 70·3 and 86·6% respectively were recovered as adult worms by perfusion 5 weeks later. When day 7 larvae were aliquotted for transfer, to avoid a biased selection, 74·2% matured *in vivo*.

Pre-transformation labelling

Kinetics of protein release during development. The rate of labelled protein loss, determined over a 7-day period, is illustrated in Fig. 1. Upon transformation, 32% of the total cercarial cpm were lost with the tail, whilst a further 16% was released into the medium over the first 3 h of culture. During this period, the mean rate of labelled protein loss was 5·4% of the total cercarial cpm/h. This rate decreased markedly to 0·2%/h between 3 and 24 h, and to 0·077%/h between 24 h and day 7. Only 29% of the total cercarial counts remained associated with the schistosomula by day 7. This compares to a figure of 16% for day 7 *ex vivo* schistosomula (data not shown). At each sampling time, the sum of the

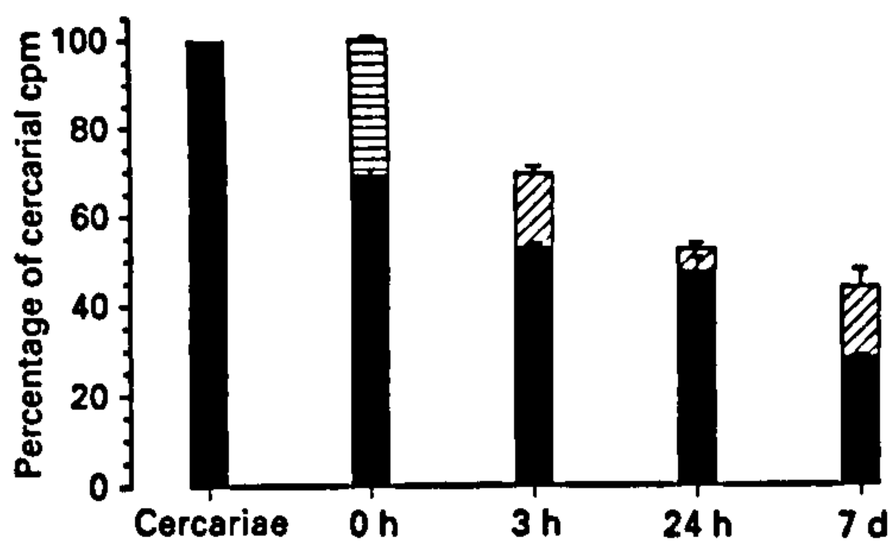

Fig. 1. Analysis of the distribution of labelled proteins, in the cercaria, larval body (■), tail (▤) or culture supernatant (▨) derived from schistosomula during *in vitro* culture. The results are expressed as a mean percentage of the cercarial cpm±S.E. The values are from 10 experiments for cercariae, 0 h body, tail, 3 h body and 0–3 h supernatant, 5 experiments for 24 h body and 3–24 h supernatant, and 3 experiments for the 7-d body and 24 h–day 7 supernatant.

supernatant and larval body counts is approximately equal to the body counts of the previous sampling time indicating the accuracy of the balance sheet.

Analysis of soluble and released proteins. The pattern of labelled proteins in the soluble and released fractions was analysed by electrophoresis followed by autoradiography; a representative example is shown in Fig. 2. The proteins released between 0–3 h and 3–24 h showed qualitatively similar banding patterns. Two proteins of M_r 61 and 20 kDa were the most dominant, and could be detected within 2 min of transformation (data not shown). Unique to the first 24 h, were 2 proteins of M_r 47 and 43 kDa, while minor proteins of M_r 90, 88, 70, 38 and 28 kDa (arrowed) were released only after 24 h. Many proteins with M_r similar to those in the culture supernatants were also detectable in the 24 h or day 7 soluble fractions. However, the 61 and 20 kDa proteins were enriched in all 3 culture supernatants; indeed, by day 7 the 20 kDa protein was barely detectable in the soluble fraction. A number of proteins in both the 24 h and day 7 soluble fractions, most of which were < 20 kDa, were not released during the 7-day culture period.

Proteolytic activity in the soluble and released protein fractions was analysed on gelatin substrate gels (data not shown). In both 0–3 h and 3–24 h culture supernatants areas of proteolytic activity in the region of 61 and 20 kDa were visible, yet only a low level of activity was present in the 24 h soluble fraction. No proteolytic activity could be detected when 24 h–day 7 supernatants were analysed, despite two proteins of M_r 61 and 20 kDa remaining dominant on SDS–PAGE gels throughout this period.

Post-transformation labelling

Kinetics of protein synthesis during development. The rate of [^{35}S]methionine incorporation into TCA-

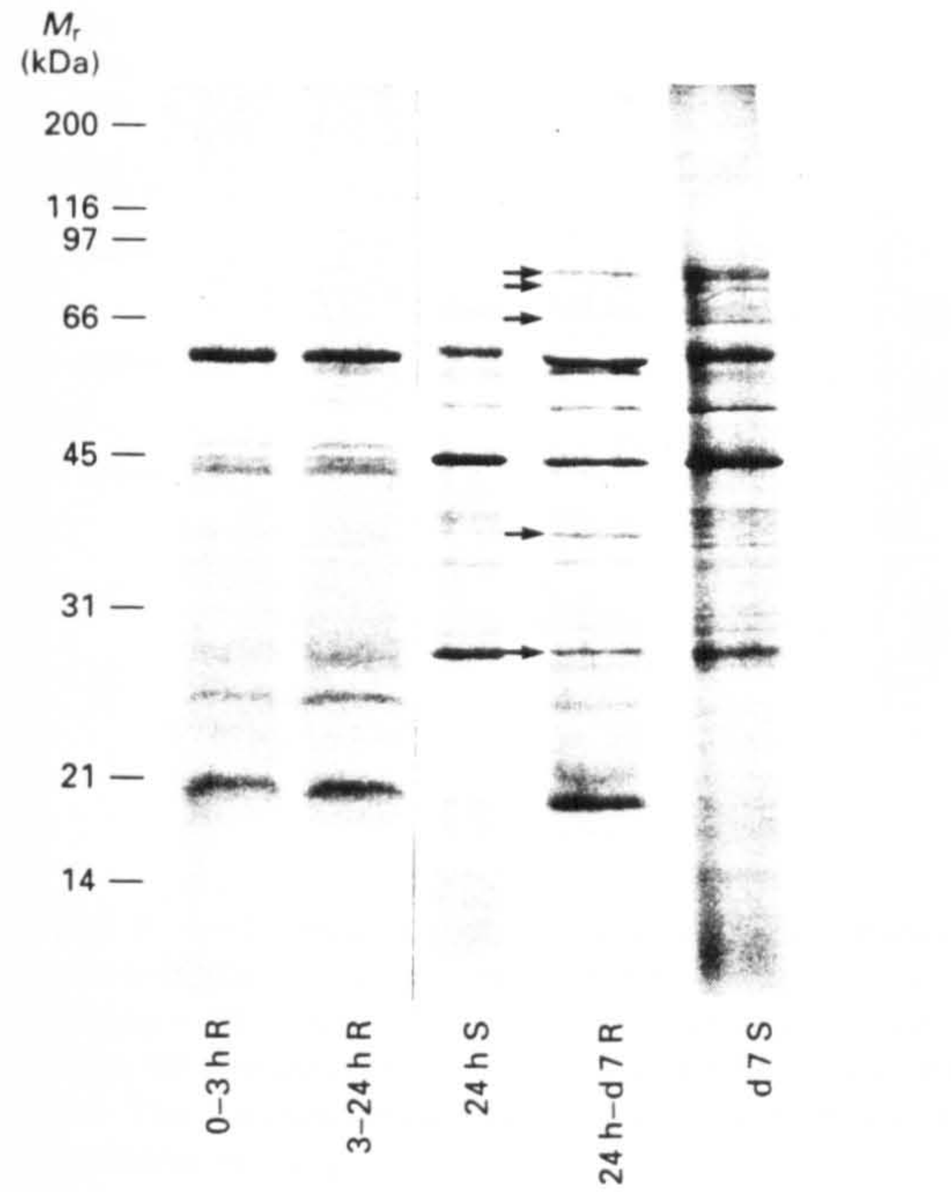

Fig. 2. Autoradiograph of electrophoretically separated soluble (S) and released (R) proteins, derived from schistosomula labelled prior to transformation. The equivalent of 10000 cpm was run down each lane of the gel. The autoradiograph was developed after 4 weeks exposure to the gel.

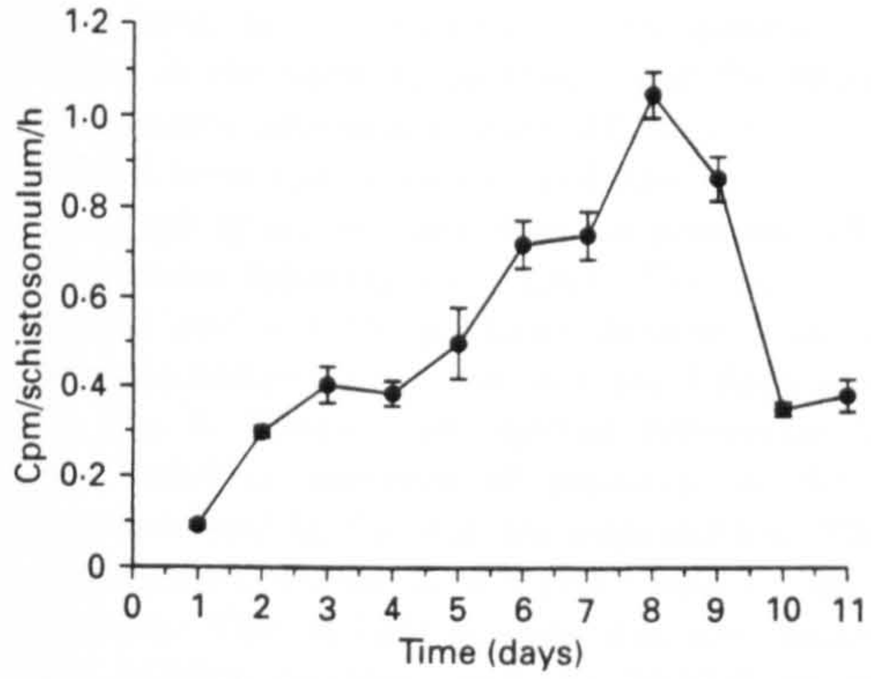

Fig. 3. Rate of protein synthesis by schistosomula pulsed for 24 h with [^{35}S]methionine at 24 h intervals. Values represent the mean cpm/schistosomulum/h ± S.E.

precipitable material of schistosomula, following a 24 h pulse on consecutive days, was determined over an 11-day period (Fig. 3). An initial lag phase of approximately 24 h occurred during which there was only a low level of protein synthesis. There was then a rise to a plateau phase which lasted for 2–3 days, followed by a further increase in the rate of synthesis which reached a peak on day 8. The rate then declined rapidly so that by day 10, the level was similar to that seen between days 2 and 4. The peak rate of protein synthesis was 11·5 times greater than within the first 24 h after transformation. A similar profile of the rate of protein synthesis was seen in repeat experiments ($n = 3$). However, the timing of the peak and subsequent rapid decline of synthesis varied by 24 h either side of the result shown here.

Analysis of soluble proteins. A one-dimensional separation of labelled proteins present in the soluble fraction of schistosomula pulsed for 24 h on successive days, is shown in Fig. 4. The intensity of the banding patterns of this subset of parasite proteins to a degree mirrored the observed rate of protein synthesis (cf. Fig. 3). Few proteins were synthesized during the first 24 h, yet the two most dominant, of M_r 28 and 20 kDa, were synthesized at a higher rate during this period than at subsequent times. Several minor proteins were only synthesized within the first 24 h, while other proteins of M_r 130, 110, 97, 70, 43, 42, 38 and 32 kDa (arrowed) showed stage-specific synthesis at later times. The most prominent of these were of M_r 97 and 38 kDa, the former of which was synthesized predominantly on days 6 and 7, and the latter only between days 3 and 6.

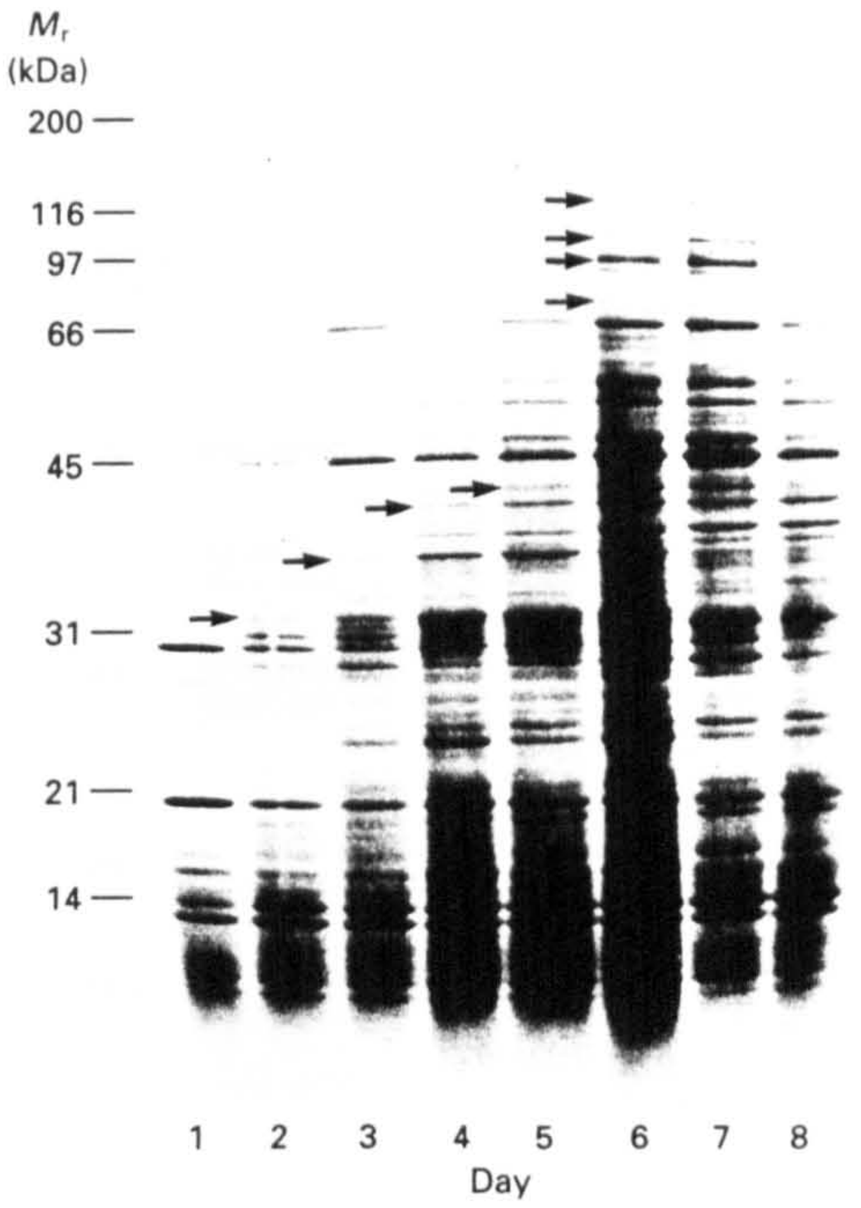

Fig. 4. Autoradiograph of electrophoretically separated proteins of the soluble fraction of *in vitro*-cultured schistosomula following a 24 h pulse on successive days. The equivalent soluble protein from 4000 schistosomula was run down each lane of the gel. The autoradiograph was developed after 4 weeks exposure to the gel.

Analysis of released proteins. Schistosomula of different ages released a complex mixture of proteins into the culture medium during a 24 h pulse (Fig. 5). As would be predicted from the rate of isotope

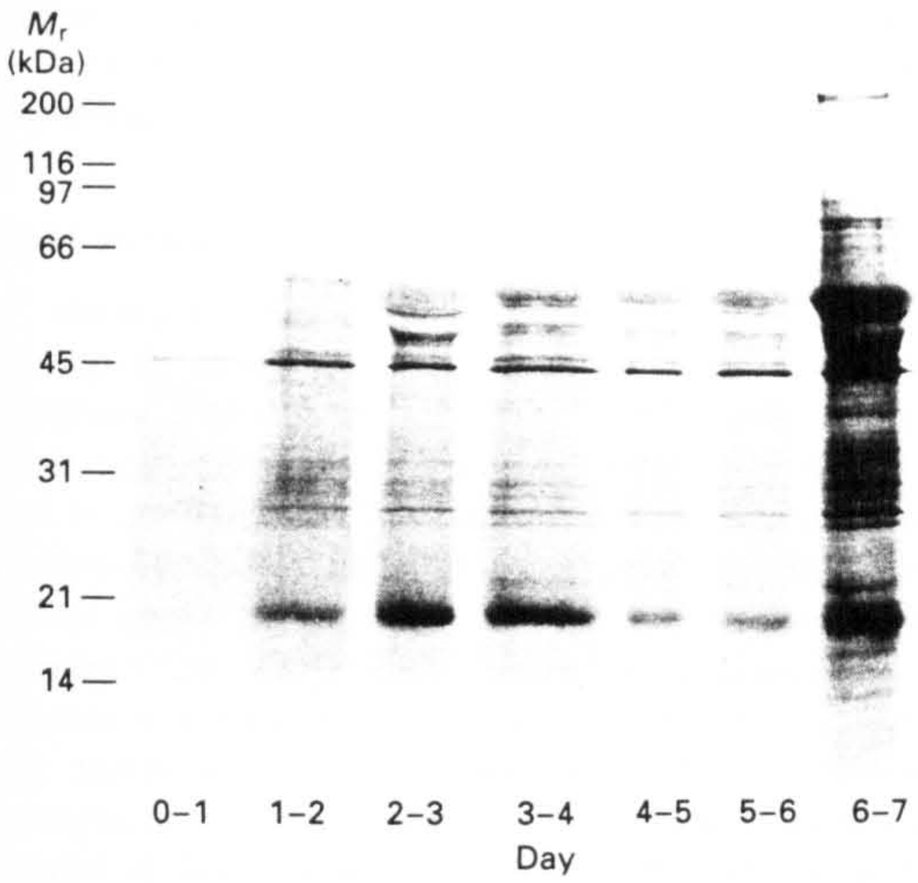

Fig. 5. Autoradiograph of electrophoretically separated proteins released by *in vitro*-cultured schistosomula during a 24 h pulse. The equivalent protein released from 6000 schistosomula was run down each lane of the gel. The autoradiograph was developed after 4 weeks exposure to the gel.

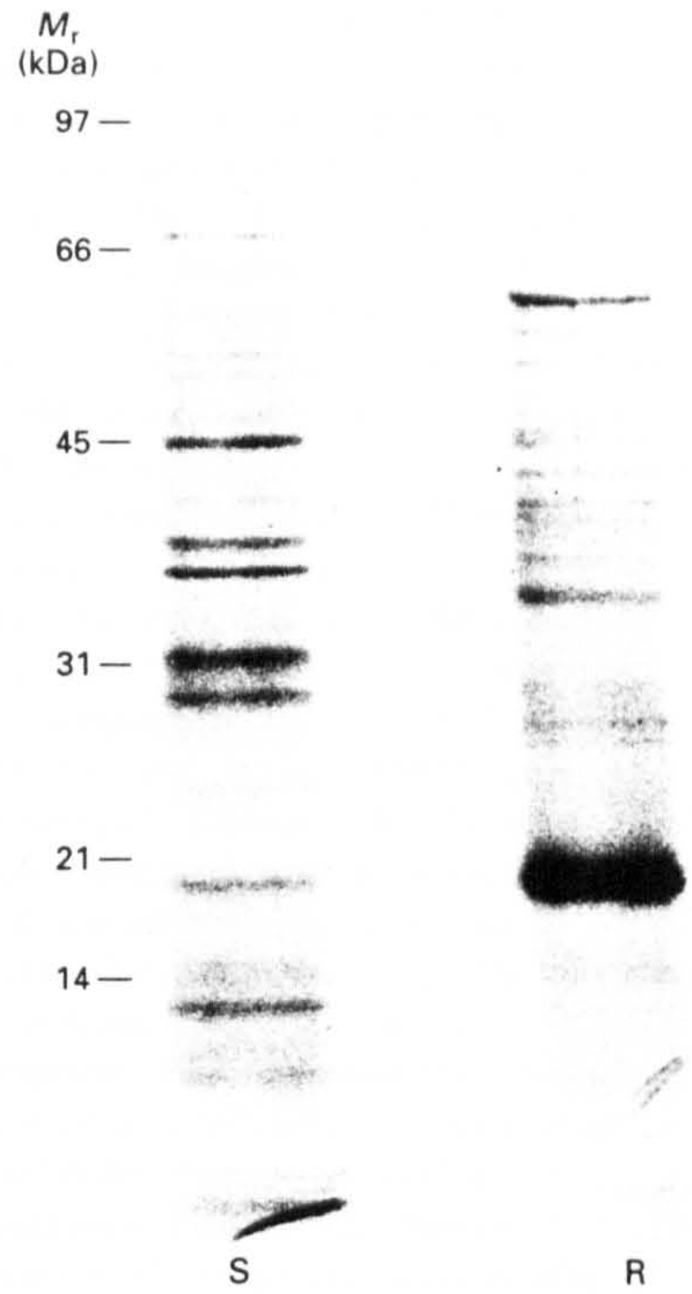

Fig. 6. Autoradiograph of electrophoretically separated soluble (S) and released (R) proteins of schistosomula following a continuous 7-d labelling. The equivalent protein from 10000 schistosomula was loaded onto gels and separated by SDS–PAGE. The autoradiograph was developed after 4 weeks exposure to the gel.

incorporation (Fig. 3), few newly synthesized proteins were detectable in the culture supernatant within the first 24 h. At later times, more than 15 proteins were released into the culture medium, ranging in M_r from < 20 to > 100 kDa, the two most dominant being 45 kDa and 20 kDa. During this latter period, there were few qualitative differences in the banding patterns, with the exception of two minor proteins of high M_r and one of 23 kDa, which were not released until day 3.

Analysis of soluble and released proteins following a continuous labelling for 7 days. The cumulative released and soluble proteins derived from schistosomula continuously labelled for 7 days are shown in Fig. 6. There were marked differences between the banding patterns of proteins in the soluble fraction and in the culture supernatant. The most dominant proteins in the latter had M_r of 61 and 20 kDa. The 61 kDa protein was not detectable in the soluble fraction, and the 20 kDa protein was highly enriched in the culture supernatant, compared to the soluble fraction.

Pulse-chase labelling of parasites. Schistosomula were pulse-labelled on days chosen to correspond to different phases of protein synthesis. The loss of parasite-associated labelled protein was then followed over a 4-day chase period (Fig. 7). As predicted from the results in Fig. 3, there was differential incorporation of isotope into TCA-precipitable material of parasites at each time point (i.e. the parasites pulsed on day 6 incorporated more isotope than those pulsed on days 3 and 10). However, the percentage loss of cpm showed little difference. In each case, more than 50% of the cpm, associated

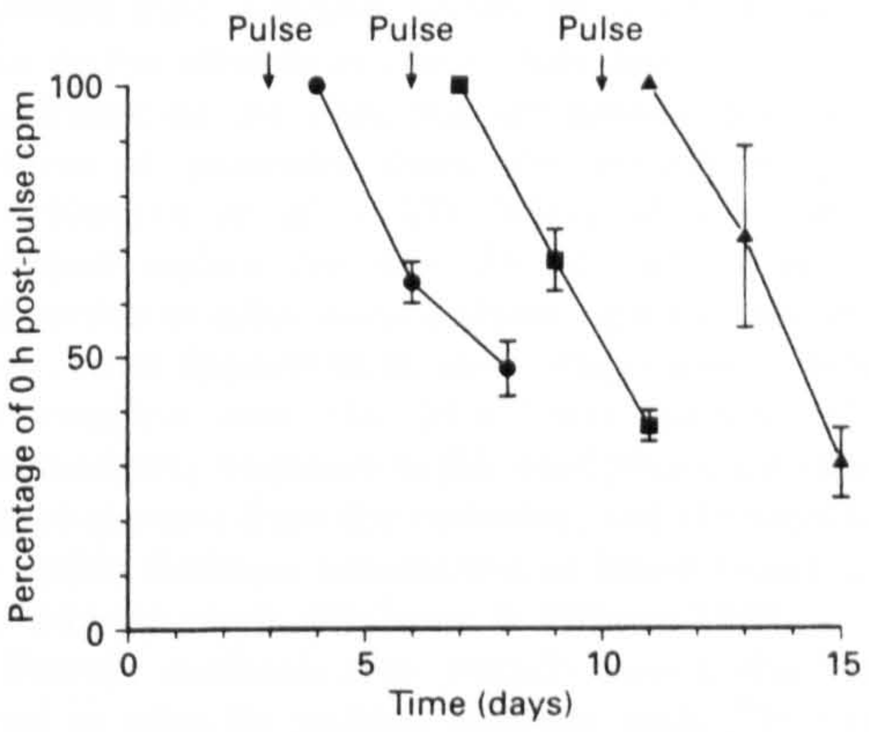

Fig. 7. Rate of loss of parasite-associated labelled protein following a 24 h pulse on day 3, 6, or 10 and a subsequent 4-d chase. Results are expressed as a mean percentage ($n = 3$) of the cpm/parasite immediately after the 24 h pulse ± S.E.

with the parasite immediately after the pulse, was lost during the chase period.

When the pulse and chase supernatants were compared by SDS–PAGE and autoradiography (data not shown), essentially the same pattern of

proteins was seen in each. The most dominant proteins were of M_r 61, 45 and 20 kDa, the latter becoming less prominent with time.

DISCUSSION

In order to characterize proteins released by developing schistosomula *in vitro*, it is obviously important that essentially 100% of parasites are viable and develop normally. Several criteria were used to confirm that the culture system was capable of supporting a high level of parasite viability and did not impair maturation. The linear increase in the incorporation of [^{35}S]methionine into schistosomula proteins was a good indication that there was little, if any, parasite death or loss of anabolic activity throughout the pulse period. Additionally, surgical transfer of day 7 or day 12 *in vitro*-cultured worms to naive mice resulted in maturation rates comparable to, or better than, those reported by Coulson & Wilson (1988) for *ex vivo* worms of identical ages. Randomly chosen (aliquotted) day 7 worms were equally as capable of maturing as hand-picked schistosomula indicating that our results were not biased by the selection procedure. Thus, we conclude, that 12 days of *in vitro* culture have no effect on the ability of larvae to mature *in vivo*.

Cercariae, labelled during development in the snail, lost 32% of their total cpm with the tail upon transformation, a value identical to that reported by Wilson & Coulson (1986). These workers also found that during development of labelled parasites *in vivo*, 89% of the total cercarial cpm had been lost by day 7, compared to the value we report of 71% after 7 days of *in vitro* culture. There are a number of possible explanations for this disparity. Firstly, development is known to occur slightly faster *in vivo* than *in vitro* (Basch, 1981). Secondly, the environment *in vivo* is probably more hostile than that *in vitro*, and a higher rate of protein turnover is likely, due to repair processes. Thirdly, secretion of some proteins may require a stimulus such as a change in O_2 tension or nutrient levels, or the physical pressure of squeezing along a blood vessel; these stimuli may not be present *in vitro*. Finally, this study used [^{35}S]methionine to label parasites whereas Wilson & Coulson (1986) used [^{75}Se]methionine. However, after 7 days of *in vivo* culture [^{35}S]methionine-labelled parasites lost 84% of the total cercarial cpm compared to the 89% reported by Wilson & Coulson (1986). This suggests that the use of different isotopes does not account for the disparity of labelled protein losses *in vitro* and *in vivo*.

The most rapid release of proteins, labelled in the developing cercariae, occurred during the first 3 h of culture. The cercarial glycocalyx, shed at this time, is composed of glycoprotein (M_r 5×10^3 kDa; Caulfield *et al.* 1987) too large to enter electrophoretic gels. However, it is possible that some of the macromolecules detected in the 0–3 h culture supernatants are derived from it. During the 0–3 h period, the most dominant polypeptides on SDS-PAGE reduced gels were of M_r 61 and 20 kDa. Two bands of proteolytic activity against gelatin, had similar molecular weights but it is not possible to confirm if they are identical because of the different electrophoretic conditions. Marikovsky, Fishelson & Arnon (1988) described two serine proteases of M_r 60 and 28 kDa which were secreted by transforming cercariae, predominantly within the first 30 min. They showed that these enzymes play an important role in skin penetration and shedding of the cercarial glycocalyx. Other secreted proteases of M_r 25 kDa (Landsperger, Stirewalt & Dresden, 1982), 30 kDa (McKerrow *et al.* 1985) and 47 kDa (Chavez-Olortegui, Resende & Tavares, 1992) have been described, and may be involved in skin penetration and transformation. Proteins of M_r 61 and 20 kDa were also prominent in the 24 h–day 7 culture supernatant, yet no proteolytic activity could be detected. This suggests that there is more than one protein migrating to the same position in the gel or, less likely, that some developmental regulation of the proteases is occurring. Keene *et al.* (1983) noted the absence of proteolytic activity after 24 h and showed that secretion of enzymes from the preacetabular glands was complete by this time. Furthermore, Cousin, Stirewalt & Dorsey (1981) described empty and shrunken acetabular gland cells and ducts of schistosomula within 1 h of skin penetration, although artificially transformed cercariae were found to release their contents more slowly. It is possible that secretion occurs more slowly *in vitro* due to the absence of short-chain fatty acids which are found on the skin, and are known to stimulate release of proteases from the acetabular glands (McKerrow *et al.* 1983). Most of the proteins released within the first 3 h of culture are also detectable in subsequent culture supernatants. However, there appears to be some stage-specific release of proteins over the 24 h–7-day period. These proteins may originate in the head gland, a structure which persists from the cercariae, and the secretions of which facilitate penetration of blood vessel walls by schistosomula (Crabtree & Wilson, 1985).

Protein synthesis after transformation was measured *in vitro* by pulsing cultures with [^{35}S]methionine for 24 h periods. This pulse duration was chosen as a compromise between incorporating enough label into proteins to allow their detection by autoradiography, whilst minimizing the potential catabolism of the proteins within parasite tissues. The procedure under-estimates the rate of synthesis because some proteins are released from the body during the pulse period. However, our methods permitted these secreted proteins to be characterized. The rate of protein synthesis can be divided into four different phases over an 11-day period. During the

first 24 h after cercarial transformation, a very low rate of protein synthesis was measured; a result which has been reported previously (Nagai *et al.* 1977; Yuckenburg, Poupin & Mansour, 1987; Blanton & Licate, 1992), and presumably occurs because the parasite utilizes proteins pre-synthesized in the cercariae for the transformation process (Hockley & McLaren, 1973). Blanton & Licate (1992) noted that this lag phase was due, not to a paucity of mRNA, but to a post-transcriptional block on translation. This block was reversed over time and allowed the expression of available mRNA species. Heat-shock proteins are produced during the first 6–8 h of transformation (Yuckenburg *et al.* 1987; Blanton & Licate, 1992) and could prevent protein synthesis; as their expression decreases the block on synthesis would be reversed. The increasing rate of synthesis up to day 8 corresponds to the period over which parasites reach the lungs and undergo the adaptive changes necessary for intravascular migration (Wilson *et al.* 1978). The subsequent decline in synthesis to the level seen between days 2 and 4 may represent a quiescent phase in development. Such a phase was postulated by Lawson & Wilson (1980) on the basis of biochemical analysis, and may indicate that the parasite has acquired all the adaptations necessary for migration to the portal system.

Autoradiographic analysis of labelled proteins present in the soluble fraction of the larval body revealed some stage-specific synthesis, which may correspond to the physiological changes occurring during schistosomulum development. One such change is the appearance of homogeneous bodies, which are specific to the lung-stage (McLaren *et al.* 1978); their secretions may help to minimize friction between parasite tegument and pulmonary endothelium (Crabtree & Wilson, 1986). Other workers have shown stage-specific synthesis of proteins, employing a very similar labelling protocol to our own but fractionating an homogenate of the whole worm body using 2-D SDS–PAGE (Yuckenburg *et al.* 1987).

During development to the lung-stage, schistosomula, labelled after transformation, released at least 15 proteins of diverse M_r. The distinct banding pattern of the soluble fraction compared with the culture supernatant, derived from schistosomula continuously labelled for 7 days, provides strong evidence that the proteins in the latter are true secretions, not molecules leaked from dying parasites. Among the proteins released within most culture periods, three of M_r 61, 45 and 20 kDa are dominant (the 61 kDa protein is often poorly visualized due to the ballooning effect caused by the BSA additive in the culture medium). Since all three bands are present in the sequential 24 h pulse supernatants from day 1 to day 7, we conclude that these proteins are synthesized and released over most of the developmental period. The bands at 61 and 20 kDa coincide with major proteins released from the cercariae during transformation. However, unlike the latter they do not correspond to regions of proteolytic activity and may therefore represent completely different proteins. We are currently attempting to address this question using 2-D SDS–PAGE, but are limited by the amount of labelled secretion available for analysis.

This is the first report to characterize proteins synthesized and released by schistosomula during development to the lung-stage. Many proteins of diverse molecular weight were identified in the culture supernatants. These molecules released within the lymph nodes draining the vaccination site must serve as inducers of the primary protective response, and in the lungs of vaccinated mice as initiators of the cell-mediated effector mechanism elicited by challenge larvae. The ability of these released proteins to stimulate proliferation by lymph node cells recovered from protectively vaccinated mice, will be reported in a later publication. In order to make further progress in understanding their biological role for the parasite, and their interaction with the host immune system, an abundant supply of purified proteins is required. With this in mind, we are currently screening a larval cDNA library, constructed in the lambda ZAP vector to identify recombinants coding for released proteins.

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CHAPTER THREE

The proliferative responses of lymph node cells to antigenic fractions of larval *Schistosoma mansoni*, and to live schistosomula.

3.1 Introduction

The importance of skin-draining lymph nodes, in the induction of resistance against *Schistosoma mansoni*, has been demonstrated previously by their excision prior to vaccination or by administering irradiated parasites intravenously, thus by-passing this site; in both cases immunity to a challenge infection was greatly reduced (Mountford & Wilson, 1990; Coulson & Mountford, 1989 respectively). It is likely that the skin-draining lymph nodes are the main site for the priming of schistosome-specific T cells, an event which is essential to the induction of protective immunity (Constant *et al.* 1990).

Since lymphocytes are important for the development of resistance in the irradiated cercaria vaccine model, it is pertinent to ask whether their degree of proliferation in response to parasite antigen(s) correlates with the immune status of the host. Proliferation of lymphocytes *in vitro* in response to the presence of protein confirms two related facts, firstly that the macromolecule is immunogenic and secondly that lymphocytes have been sensitised to it previously. Several studies have used mouse strains which develop differential levels of resistance after vaccination, and tested the ability of lymphocytes recovered from lymph nodes or spleens of such animals to proliferate in response to crude antigen preparations (James, Labine & Sher, 1981; Lewis & Wilson, 1982b). No correlation was found between the immune status of the animal and its ability to respond to the antigen preparation. Similarly, Colley *et al.* (1977) found no statistical correlation between the intensity of schistosome infection in human patients (determined by eggs per gram of faeces) and the degree of cell-mediated reactivity, assessed by lymphocyte proliferation, to soluble preparations of either eggs, cercariae or adult worms. However, interpretation of such studies can be hindered by the complex nature of the antigen preparation. For example, it has been reported that schistosomes contain material which can non-specifically inhibit (Dessaint *et al.* 1977; Camus *et al.* 1981) or promote (Auriault *et al.* 1984b) lymphocyte proliferation.

Furthermore, certain experimental parameters can complicate results. James (1981) used lymph node cells recovered from mice with chronic infections. Such mice would have been exposed to three different life-cycle stages of the parasite, therefore lymphocytes derived from them would display a broad cross-reacting spectrum of antigen recognition. Vieira *et al.* (1987) analysed the proliferative response of peripheral blood mononuclear cells, from patients with chronic intestinal schistosomiasis, to live schistosomula. Unfortunately, by the end of the culture period most of the parasites had died, therefore the proliferative response could not be assigned exclusively to parasite-released proteins. The use of an isolated protein, or at least a defined preparation with a restricted number of molecules would allow easier interpretation of results from such

experiments.

There is a large body of information available on the antibody responses of vaccinated mice to specific parasite proteins fractionated from complex antigenic mixtures, utilising SDS–PAGE and immunoprecipitation or Western blotting (Simpson, James & Sher, 1983; Simpson *et al.* 1984; Simpson *et al.* 1985; Richter & Harn, 1993). Similar techniques have also been applied using serum from human patients (Taylor & Wells, 1984; Roberts *et al.* 1987; Dessein *et al.* 1988). However, there are relatively few reports which describe T cell responses to fractionated parasite proteins. The specificity of an animal's T–cell repertoire can be investigated by electrophoretic separation of complex antigenic mixtures and subsequent transfer to nitrocellulose. This circumvents the need to purify each polypeptide species (Lamb, Ohehir & Young, 1988). Despite the usefulness of this technique, technical problems such as the difficulty of transferring sufficient quantities of antigen to the solid support can result in failure to identify potentially important proteins (Rothbard & Lamb, 1990). Alternatively, Mountford & Wilson (1993) separated antigenic mixtures by high performance electrophoretic chromatography (HPEC) prior to analysis in T cell proliferation assays. Utilising this approach, they were able to fractionate relatively crude parasite protein mixtures into samples of limited antigenic composition. Each sample was then analysed for its ability to induce proliferation of, and cytokine production by, lymph node cells recovered from vaccinated mice. This allows both the immunogenic potential of the small number of proteins present in each fraction to be assessed, and their ability to induce a Th1 or Th2 type response, important considerations for vaccine design against schistosomiasis.

As detailed in section 1.7.5, comparable levels of immunity can be achieved following either percutaneous (p.c.) vaccination of C57Bl/6 mice with irradiated cercariae, or by intra–dermal (i.d.) vaccination with day 8 schistosomula derived from irradiated cercariae (Coulson & Mountford, 1989). By using p.c. or i.d. vaccination protocols and lymphocytes extracted from the main skin–draining lymph nodes (axillary and cervical respectively) on day 5 post–vaccination, an attempt has been made to simplify the experimental design to facilitate easier interpretation of the results. In the previous chapter, the proteins present in the soluble fraction of, and those released by, schistosomula up to the lung–stage of development were analysed. Since we believe released proteins, whatever their origin, to be important in both induction and effector phases of immunity, the aim of this work was to examine the ability of this sub–set of parasite proteins to induce proliferative responses in lymphocytes recovered from protectively vaccinated mice. In addition, the responses to soluble and particulate parasite protein fractions and live schistosomula were analysed. The soluble preparation was used because many secretory molecules are likely to constitute a sub–set of this fraction, whereas shed proteins, such as membrane fragments, are more likely to be present in the particulate fraction.

3.2 Materials and methods

3.2.1 *Parasite culture medium*

Two media were used consecutively for the cultivation of parasites. Medium 169 defined by Basch (1981) and adapted by Harrop & Wilson (1993) was used because of its ability to support a high level of parasite viability. This medium was supplemented with antibiotics alone (M169W) or with foetal calf serum (1%; M169S) to serve as a wash medium or parasite culture medium respectively. For the final 24h of culture, parasites were transferred to RPMI (RPMI–1640; Flow laboratories) containing 1% foetal calf serum (FCS; Globepharm Ltd, Esher, Surrey, U.K.), penicillin (200U/ml), streptomycin (100μg/ml) and 2mM L–glutamine (designated RPMI/1). Over a 24h period, this medium maintained a high level of parasite viability (>95%).

3.2.2 *Preparation of parasite material*

A Puerto–Rican isolate of *Schistosoma mansoni* was maintained by routine passage through LACA mice and albino *Biomphalaria glabrata*. Snails, harbouring a patent infection, were induced to shed cercariae, the parasites concentrated by sedimentation at 4°C and then mechanically transformed as described by Ramalho–Pinto *et al.* (1974). Resulting schistosomula were isolated by centrifugation on a discontinuous 40–70% Percoll gradient (Lazdins *et al.* 1982; SIGMA) and washed 4 times in 10ml M169W. They were then resuspended in M169S and cultured in 24–well plates (Corning) for defined periods of time at 37°C, 6% CO_2. Such parasites were used either directly in lymphocyte blastogenesis assays or to yield culture supernatants, soluble and particulate fractions. In order to obtain these samples, schistosomula, derived from a single pool of cercariae, were divided into 7 equal aliquots and cultured in M169S for periods ranging from 0 to 6 days (see Fig. 3.1), after which they were then washed 6 times and cultured for 24h in RPMI/1. At the end of this period, the parasite suspension was centrifuged (150*g* for 60s), the culture supernatant removed and the number of parasites in each culture determined. Culture supernatants were concentrated to 1ml in a stirred ultrafiltration cell, containing a 3 kDa cut–off Diaflo membrane (Amicon), under N_2 at a pressure of 40 psi. Culture medium which had not been exposed to parasites was treated in the same way and served as a negative control (medium blank) in proliferation experiments using released proteins. Soluble proteins were released from larval bodies by sonication (21 kHz at 6.5μm amplitude) for 90s, and partitioned from particulate material by centrifugation for 1h at 105,000*g*, 4°C. The particulate material was then resuspended in RPMI/10 (RPMI containing 10% FCS, penicillin (200U/ml), streptomycin (100μg/ml),

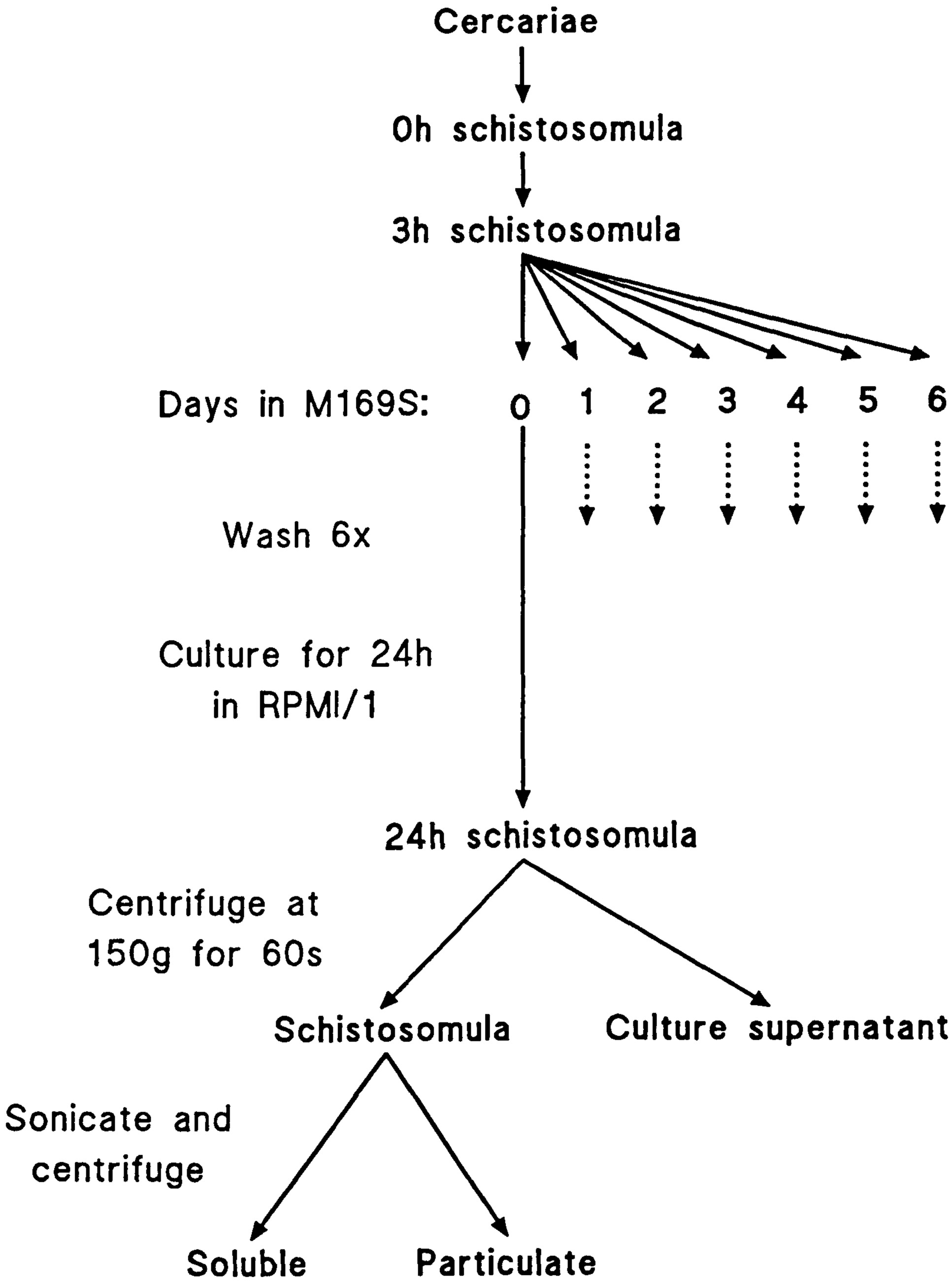


Fig. 3.1. Generation of culture supernatants, soluble and particulate parasite fractions.

2mM L–glutamine and $5x10^{-5}$M 2–mercaptoethanol). Soluble and particulate protein fractions and those released by schistosomula during *in vitro* culture were filter–sterilised and the protein content of the soluble fractions determined by the Bradford assay. Both soluble and particulate fractions were reconstituted to 1ml with RPMI/10. All fractions were stored at –20°C until required. Schistosomula to be used directly in proliferation assays, were cultured in M169S for defined times, washed 6 times in RPMI/10 and aliquoted into flat–bottomed 96–well microtitre plates in 100μl RPMI/10. In experiments using live larvae, soluble or particulate fractions, unconcentrated RPMI/10 was used as a medium blank (no antigen control).

3.2.3 *Vaccination regimes*

Two vaccination regimes were used, percutaneous (p.c.) and intra–dermal (i.d.). The former involved the exposure of anaesthetised mice (Wilson & Coulson, 1986) to 500 cercariae, attenuated with 20 krad. of gamma radiation from a ^{60}Co source (Department of Radiobiology, Cookridge Hospital, Leeds), via the shaved abdomen. For i.d. vaccination, attenuated day 8 parasites were recovered from the lungs of vaccinated mice by mincing and incubation (Wilson and Coulson, 1986). Resulting lung–stage schistosomula were washed in M169S and samples of 300 were injected in 15μl M169S into the dermis of the mouse pinna (Coulson & Mountford, 1989).

3.2.4 *Preparation of responder lymphocytes and antigen presenting cells (APC)*

Responder lymphocytes were obtained by the surgical removal of the axillary lymph nodes from p.c. vaccinated mice and the cervical lymph nodes from i.d. vaccinated mice, 5 days after exposure to parasites. Single cell suspensions were obtained by teasing lymph nodes apart with sterile forceps. Cells were washed and resuspended in RPMI/10. Irradiated splenocytes were used as a source of APC. These were prepared by removing spleens from naive mice and exposing them, whilst on ice, to a ^{137}Cs source for 30 min. The irradiated spleens were pressed through sterile stainless steel mesh and then washed in RPMI/10. The number of lymph node cells and splenocytes was determined, using a haemocytometer, and adjusted to 2 x 10^6/ml for the former and 4 x 10^6/ml for the latter.

3.2.5 *Assay of lymphocyte proliferation*

Responder cells (10^5/well) recovered from either p.c. or i.d. vaccinated mice and APC (2 x 10^5/well) were cultured for 5 days (37°C, 6% CO_2) in either 200μl RPMI/10 alone (or the relevant medium blank), or in the presence of live parasites, released proteins isolated from live larvae, soluble or particulate parasite proteins. Since the parasite–

released proteins were collected in medium containing FCS, a protein estimate could not be made. Therefore, in experiments which used released proteins, proliferation was expressed per number of parasites from which the material was derived. Additionally, it was difficult to obtain an accurate protein estimate for particulate protein and so proliferation in response to these samples was also expressed in the same way.

Each well received 18.5 kBq ^{3}H–thymidine (specific activity 185 GBq/mmol, 37MBq/ml; Amersham) for the final 18h of culture, and the incorporation of isotope into cellular DNA was measured, after harvesting (cell harvester, ILACON), by liquid scintillation counting. Results are expressed as mean c.p.m. (±S.E.M.) for triplicate samples. In all experiments, wells containing APC but no responder cells were included to control for ^{3}H–thymidine incorporation due to the irradiated splenocytes. Additionally, in assays using live schistosomula, cultures which contained live larvae but no responder cells or APC were included. In such experiments, values were plotted as total ^{3}H–thymidine incorporation by cells and schistosomula minus the value incorporated by the parasites alone. It has been demonstrated previously (James, 1981) that the presence of cells does not stimulate uptake of ^{3}H–thymidine by live larvae.

Statistical significance of the results was determined using the Student's *t*–test.

3.3 Results

3.3.1 *Inhibition of lymphocyte proliferation by a component of M169*

Initially, parasite–released proteins were collected in M169. Unfortunately, a constituent of this medium was found to inhibit cell proliferation. Figure 3.2 illustrates this fact using a soluble worm antigen preparation (SWAP) as the test antigen. SWAP was diluted either in unconcentrated RPMI/10, concentrated RPMI/1 or concentrated M169. Cellular proliferation to the antigen preparation was severely inhibited by the presence of concentrated M169, but unaffected by RPMI/10 or concentrated RPMI/1. Subsequently, all parasite–released proteins were collected in RPMI/1.

3.3.2 *Analysis of proliferative responses of axillary lymph node cells recovered from percutaneously vaccinated mice*

The proliferative response of axillary lymph node cells, recovered from p.c. vaccinated mice, to soluble protein fractions of schistosomula (1μg/well) collected at 24h intervals over a 7 day period is shown in figure 3.3. All samples induced significantly higher responses than background proliferation to medium alone (RPMI/10). The lowest level of proliferation was induced by proteins present in the day 1 soluble fraction, this

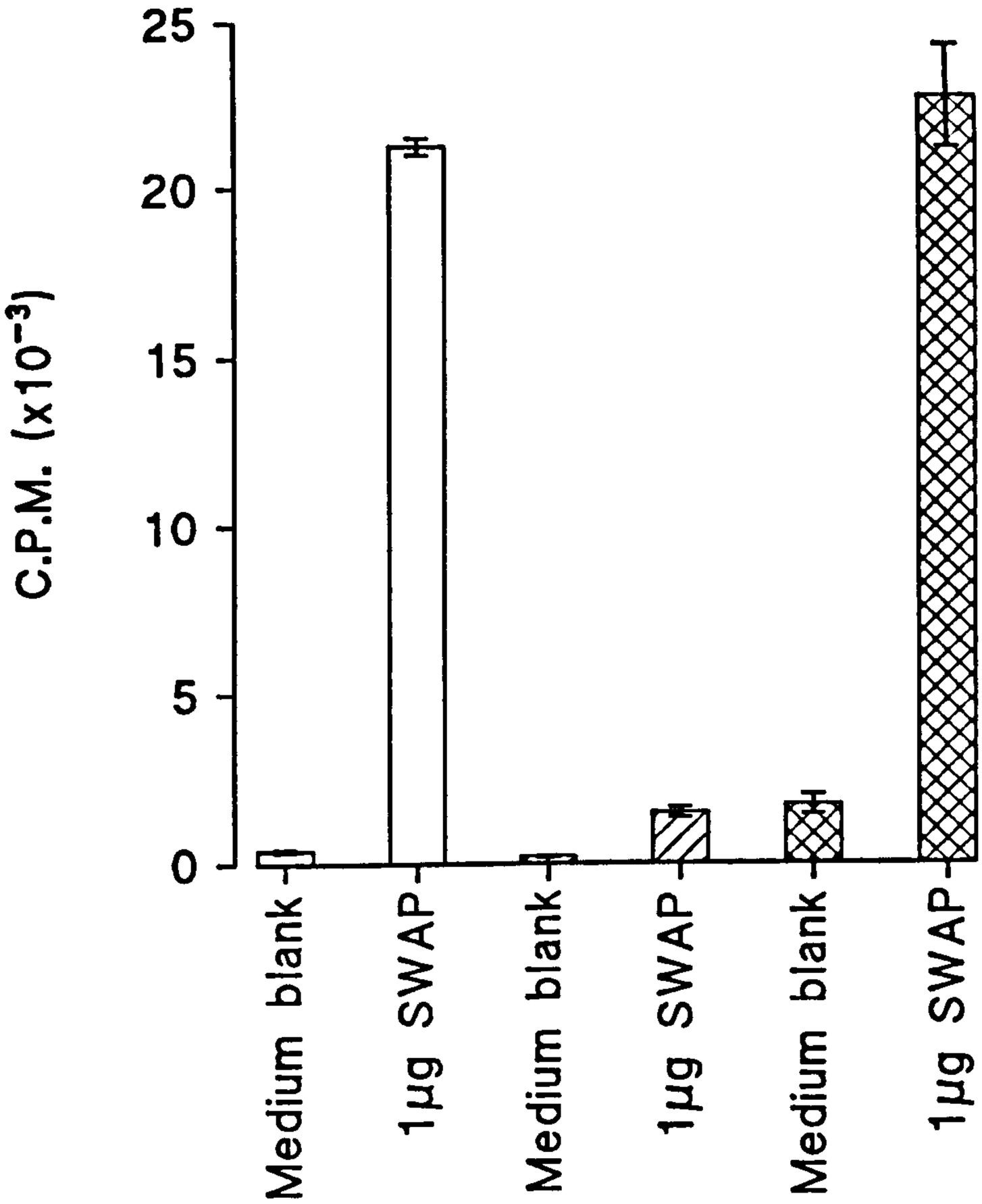


Fig. 3.2. Inhibition of lymphocyte proliferation by a factor present in M169. Values are expressed as the mean c.p.m. ± S.E.M. from triplicate wells and represent the proliferative response of axillary lymph node cells, recovered from percutaneously vaccinated mice, to 1µg SWAP diluted in: RPMI/10 (□), concentrated M169 (▨) or concentrated RPMI/1 (▩). Relevant medium blanks are shown alongside.

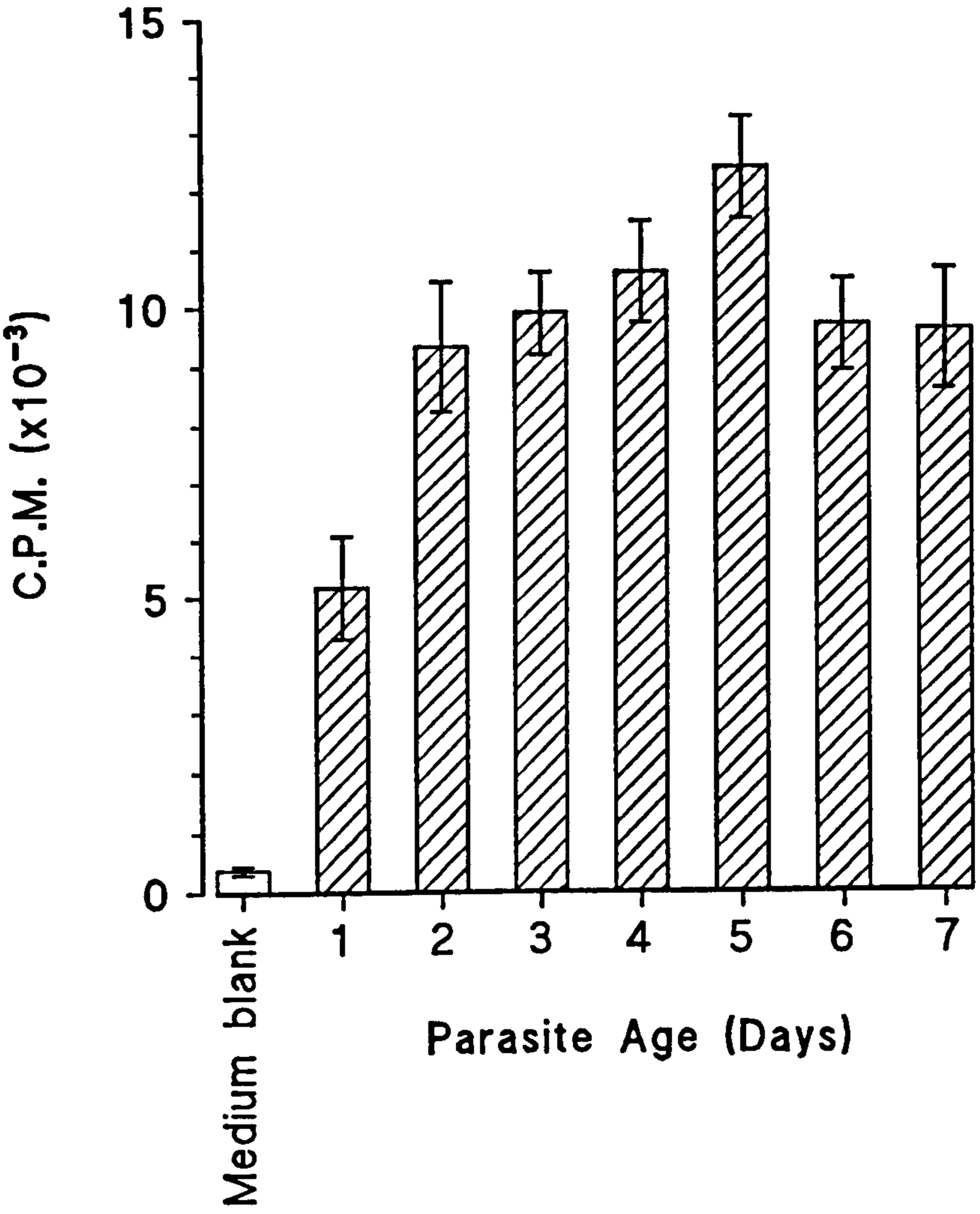


Fig. 3.3. Proliferative response of axillary lymph node cells, recovered from percutaneously vaccinated mice, to 1μg soluble protein derived from schistosomula and collected at 24h intervals over a 7 day period. Values are expressed as the mean c.p.m. ± S.E.M. for triplicate wells from 3 experiments.

value being significantly lower than all others ($P < 0.05$), but still 13 times greater than the medium blank. All other samples induced approximately 2 times greater levels of proliferation than the day 1 soluble fraction and up to 30 times greater levels than background.

The pattern of proliferation to the equivalent amount of protein released from 1000 schistosomula during a 24h period was very different (Fig. 3.4). The highest level of proliferation was induced by material released within the first 48h, these values being almost 30 times greater than background proliferation to the medium blank (concentrated RPMI/1). Thereafter, the proliferative response decreased reaching its lowest level to proteins released between day 5 and day 6, a value only 3 times greater than the background.

Axillary lymph node cells responded well to live 5 day old schistosomula (Fig. 3.5), all values being higher than background proliferation. When exposed to 400 schistosomula, the level of proliferation was 11 times greater than background and, the response appeared to plateau at this point. However, the degree of proliferation induced by sonicated compared to live parasites was greatly enhanced, the highest level occurring to 200–400 sonicated parasites, a value 55 times greater than background. When 400 live schistosomula of different ages were used directly in lymphocyte blastogenesis assays (Fig. 3.6), a pattern of proliferation similar to that in figure 3.4 was observed. The highest level of proliferation was induced by 1 day old, and the lowest by 11 day old schistosomula.

3.3.3 *Analysis of proliferative responses of cervical lymph node cells recovered from intra-dermally vaccinated mice*

The proliferative responses of cervical lymph node cells recovered from i.d. vaccinated mice to titrated soluble parasite proteins are shown in figure 3.7. In all cases, despite a high background, elevated levels of proliferation occurred to all protein samples. The proliferative response appeared to plateau at 10μg per well (Fig. 3.7 inset), and the magnitude of the response at this protein concentration was very similar whichever fraction was used. However, 1μg protein/well was used subsequently to conserve scarce samples. Cellular responses to 1μg soluble parasite proteins collected every 24h over a 7 day period are shown in figure 3.8. The lowest level of proliferation occurred to proteins present in the day 1 soluble fraction, this value being significantly lower than all other samples ($P < 0.05$). After 24h, the magnitude of the response to soluble parasite fractions was similar, being almost 3 times greater than the background level.

The pattern of proliferation to the equivalent amount of protein released from 1000 schistosomula (Fig. 3.9) showed a similar profile to that observed in figure 3.8. A

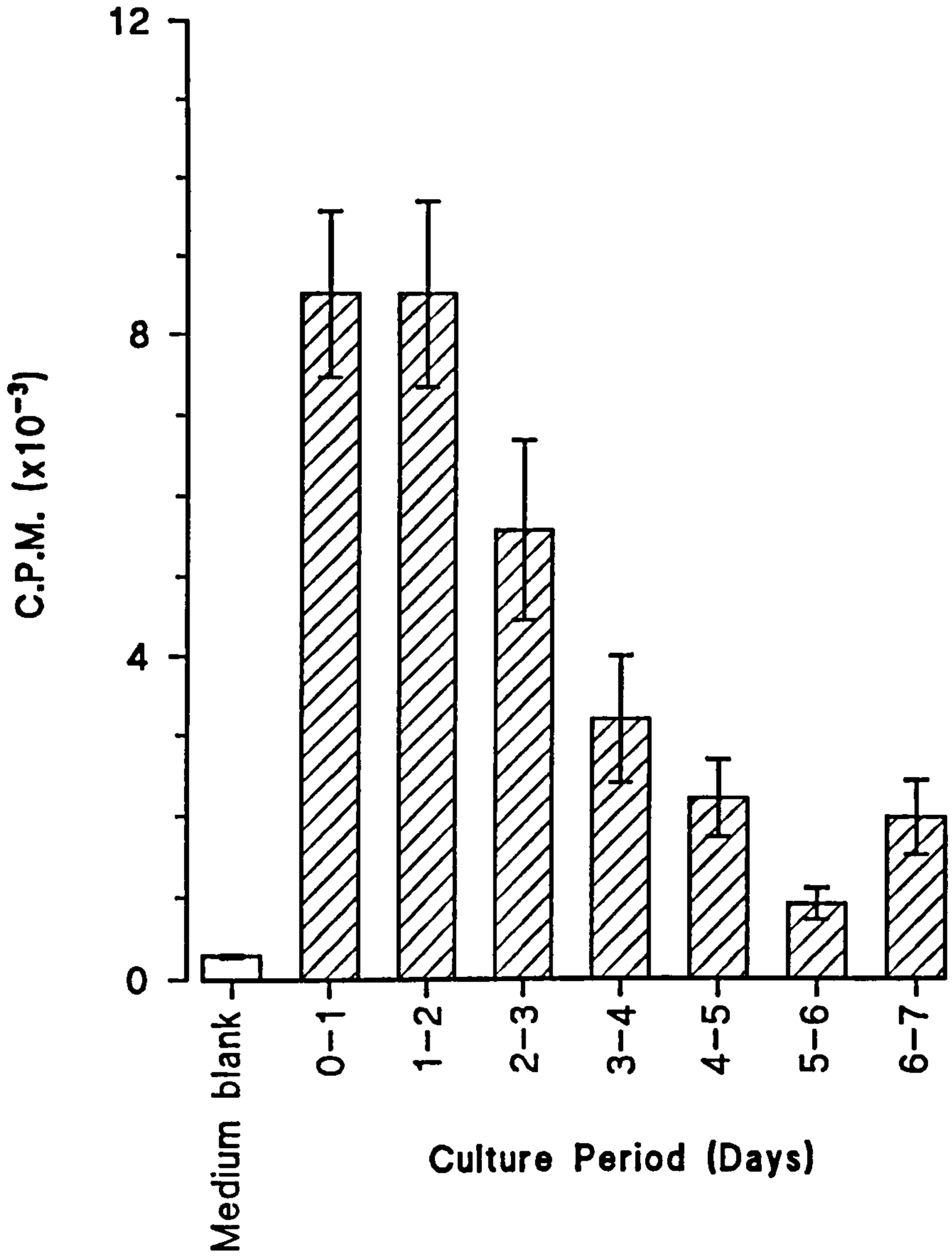


Fig. 3.4. Proliferative response of axillary lymph node cells, recovered from percutaneously vaccinated mice, to schistosomula–released proteins collected at 24h intervals over a 7 day period. Values are expressed as the mean c.p.m. ± S.E.M. for triplicate wells from 3 experiments. The equivalent amount of protein released from 1000 schistosomula was added to each well.

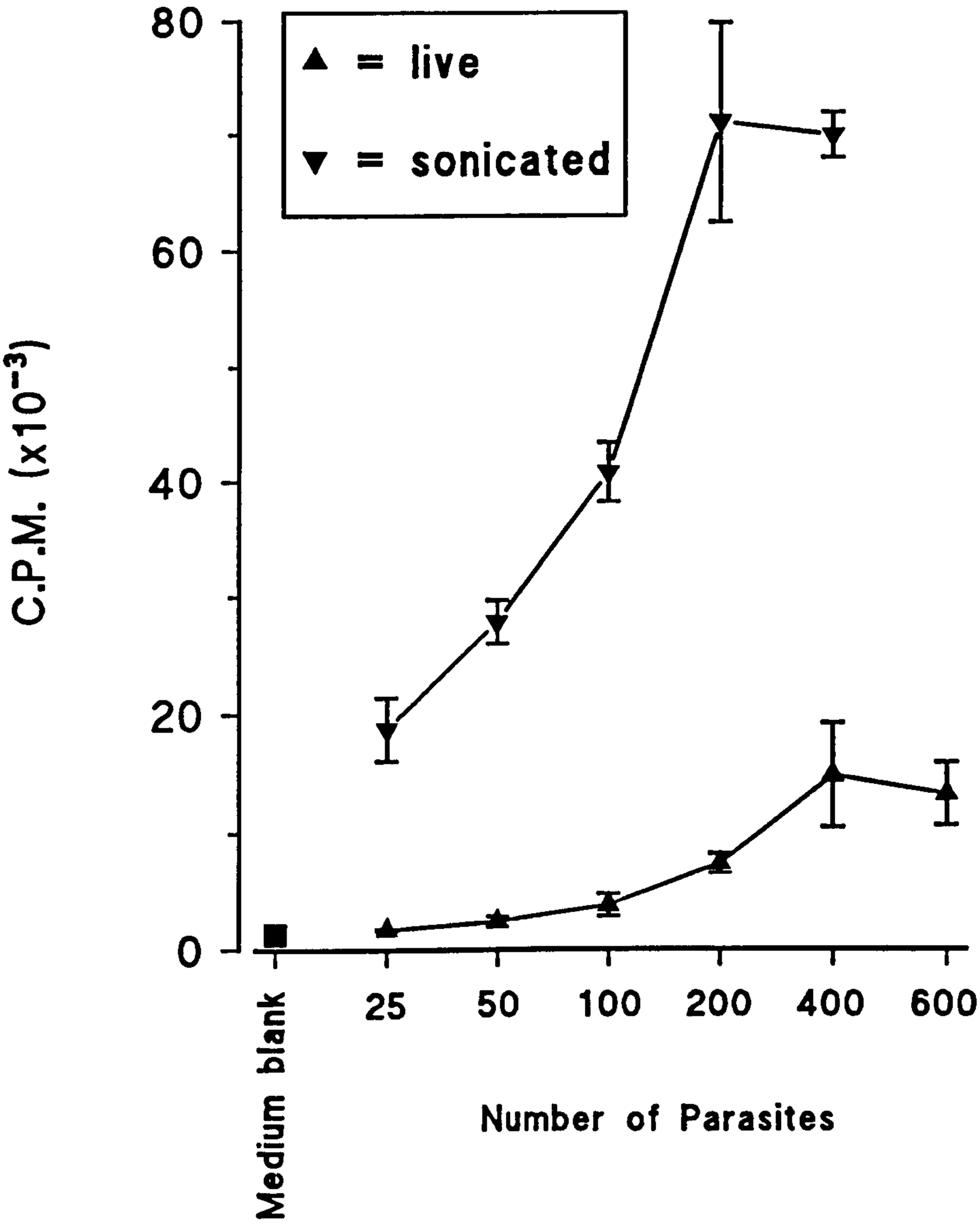


Fig. 3.5. Proliferative response of axillary lymph node cells, recovered from percutaneously vaccinated mice, to live or sonicated 5 day old schistosomula.
Values are expressed as the mean c.p.m. ± S.E.M. of triplicate wells.

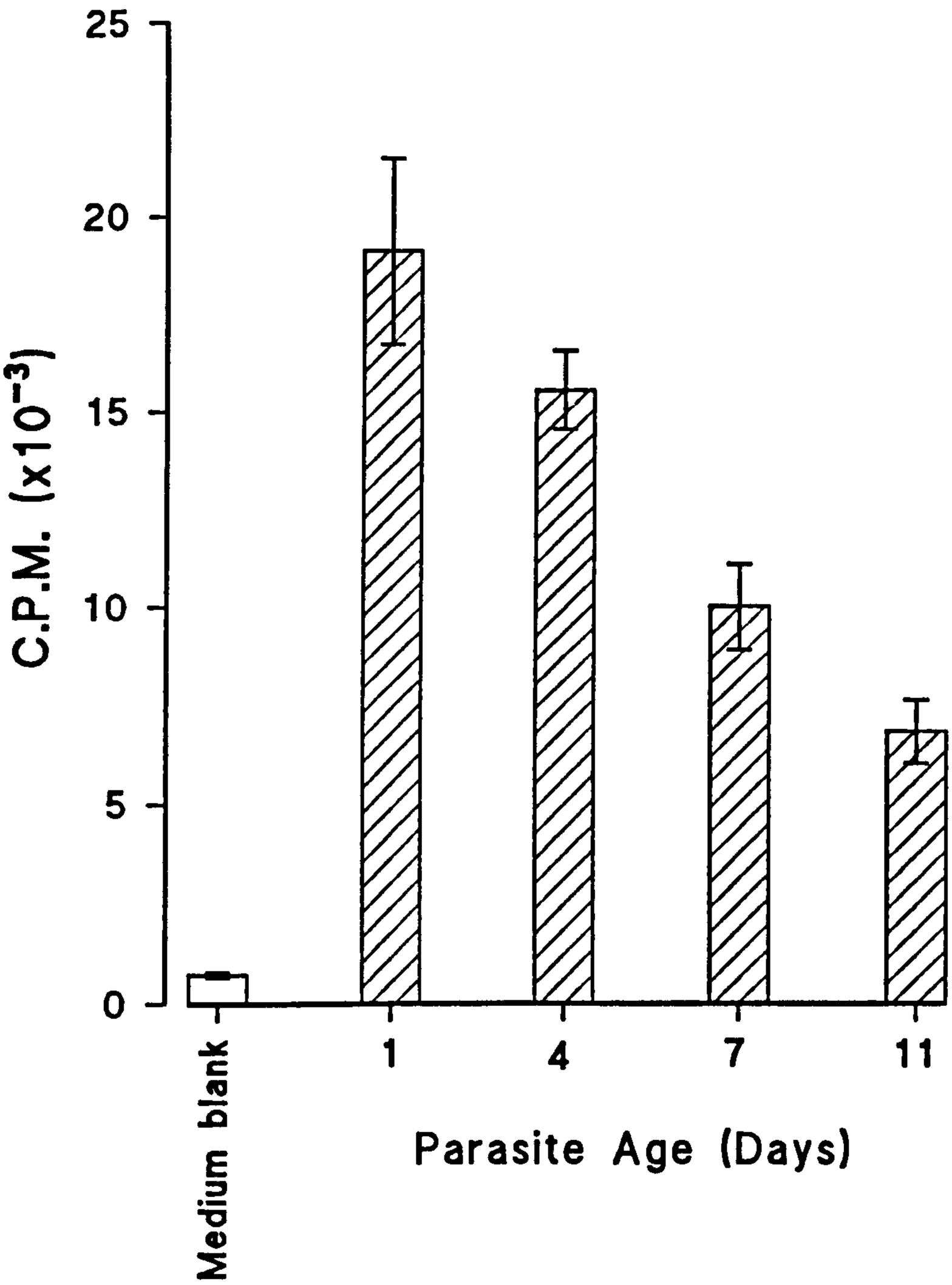
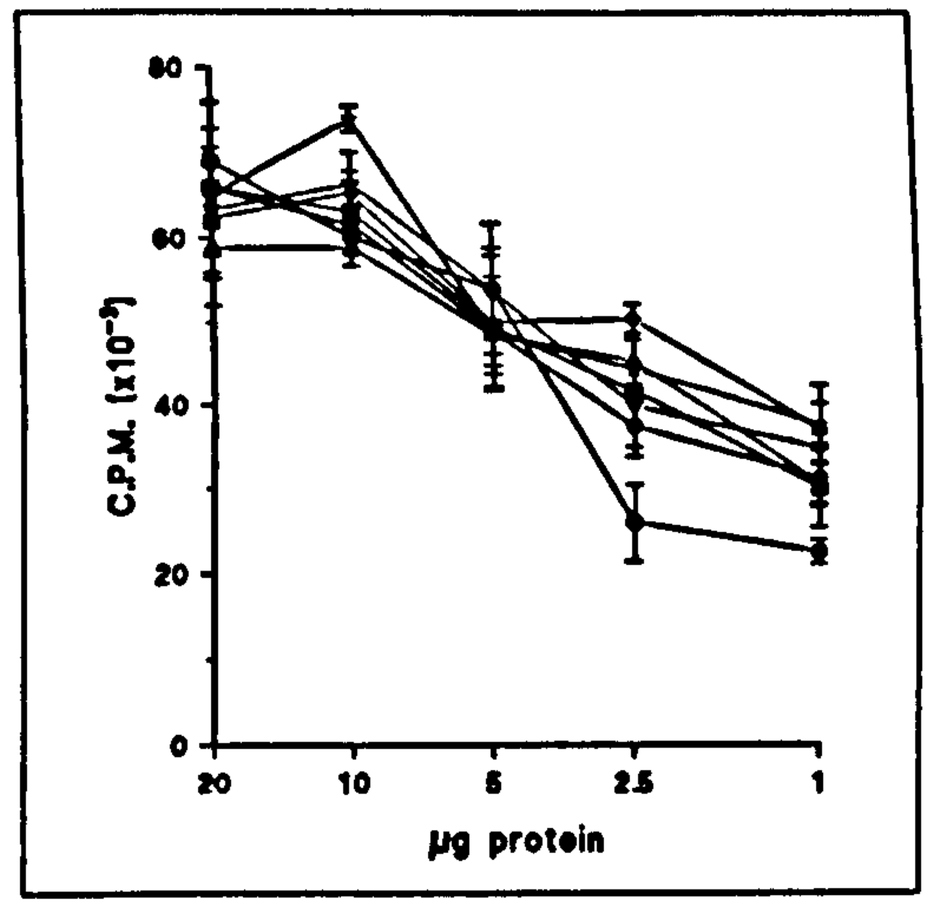


Fig. 3.6. Proliferative response of axillary lymph node cells, recovered from percutaneously vaccinated mice, to 400 live 1, 4, 7 or 11 day old schistosomula.

Values are expressed as the mean c.p.m. ± S.E.M. from triplicate wells.



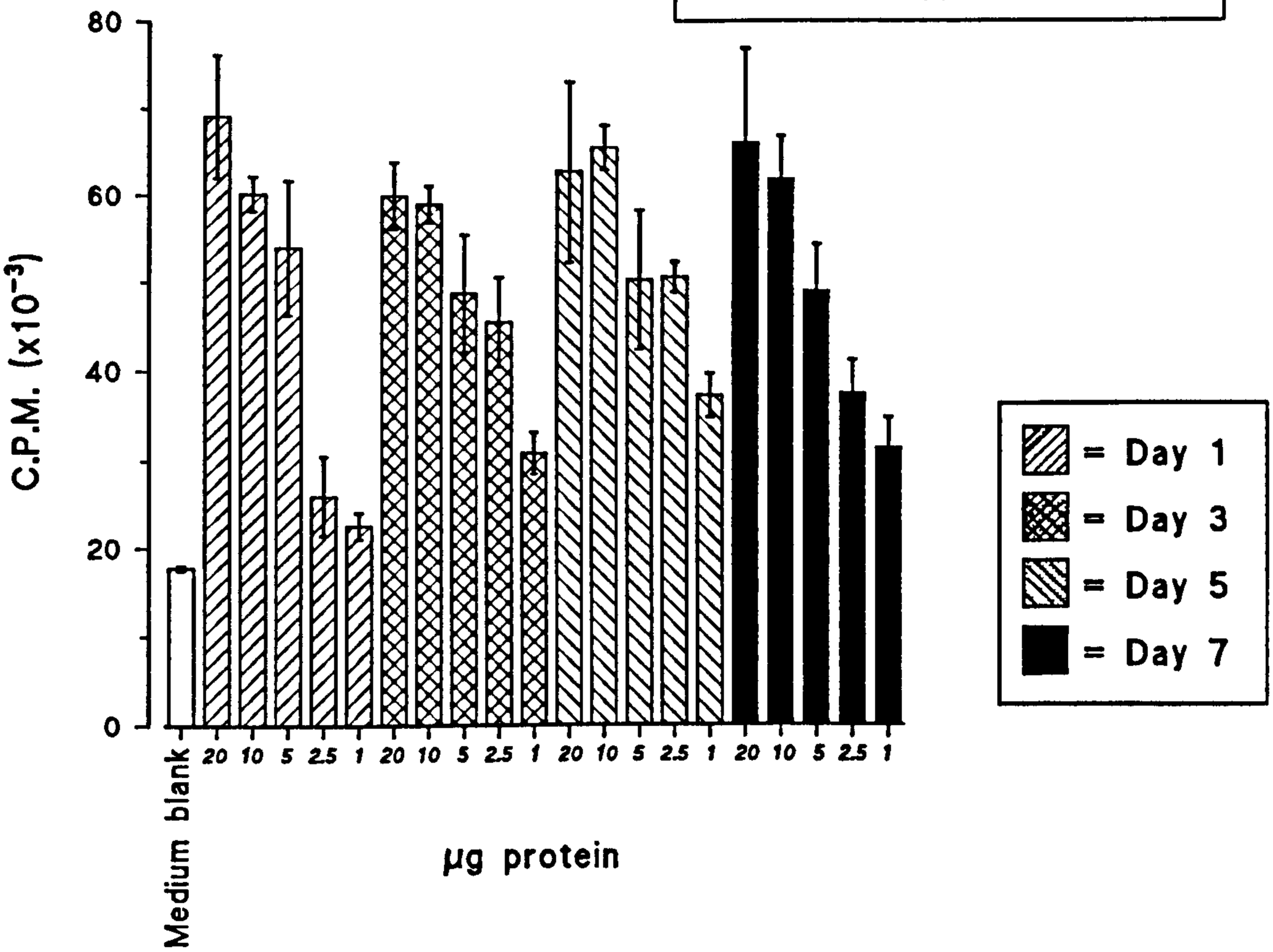


Fig. 3.7. Proliferative response of cervical lymph node cells, recovered from intra–dermally vaccinated mice, to titrated soluble protein from 1, 3, 5 and 7 day old schistosomula. Values are expressed as the mean c.p.m. ± S.E.M. from triplicate wells. The inset figure shows the response to all 7 soluble fractions collected at 24h intervals over a 7 day period, which clearly shows the plateau of proliferation at 10µg protein/well.

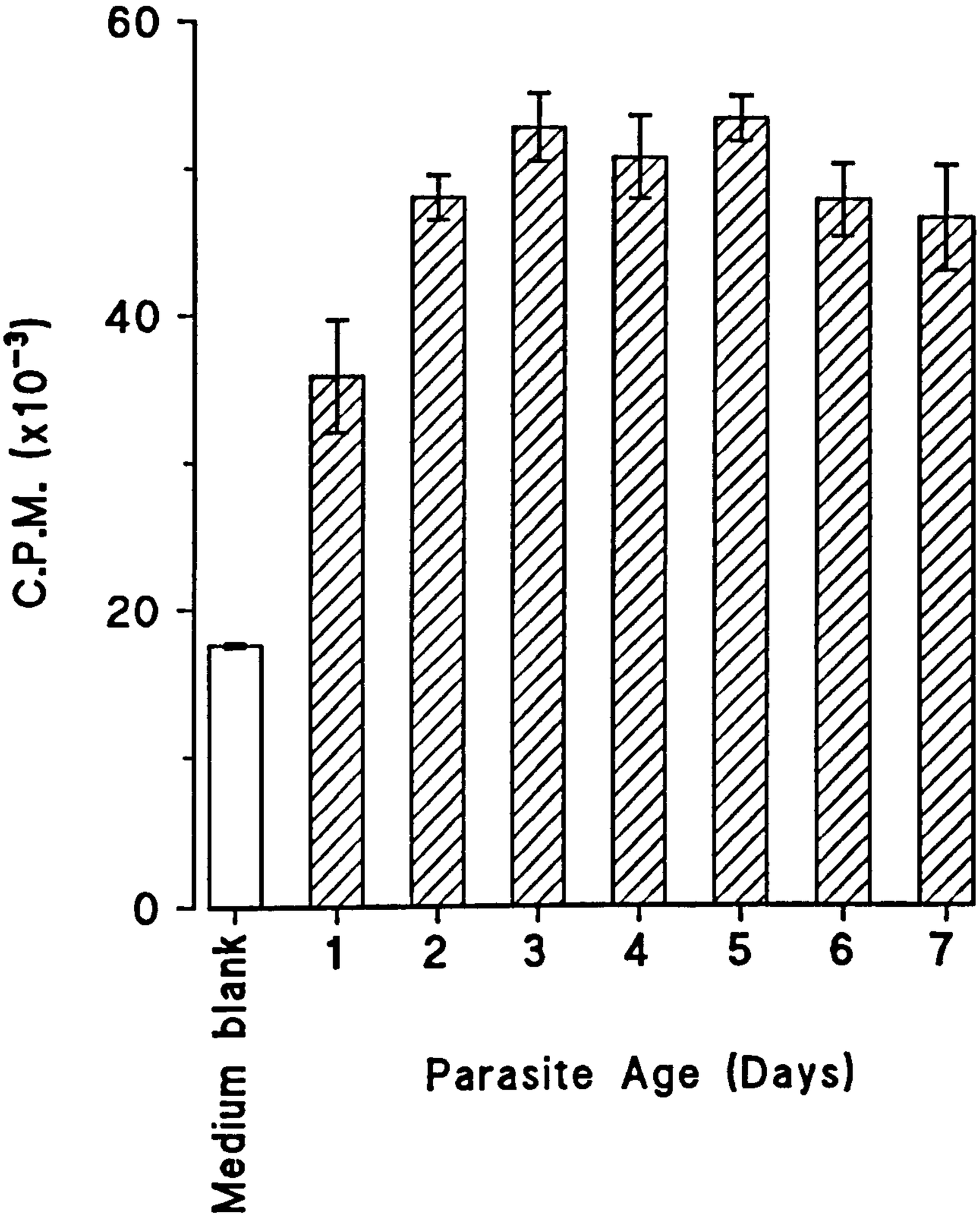


Fig. 3.8. Proliferative response of cervical lymph node cells, recovered from intra-dermally vaccinated mice, to 1μg soluble protein derived from schistosomula and collected at 24h intervals over a 7 day period. Values are expressed as the mean c.p.m. ± S.E.M. for triplicate wells from 3 experiments.

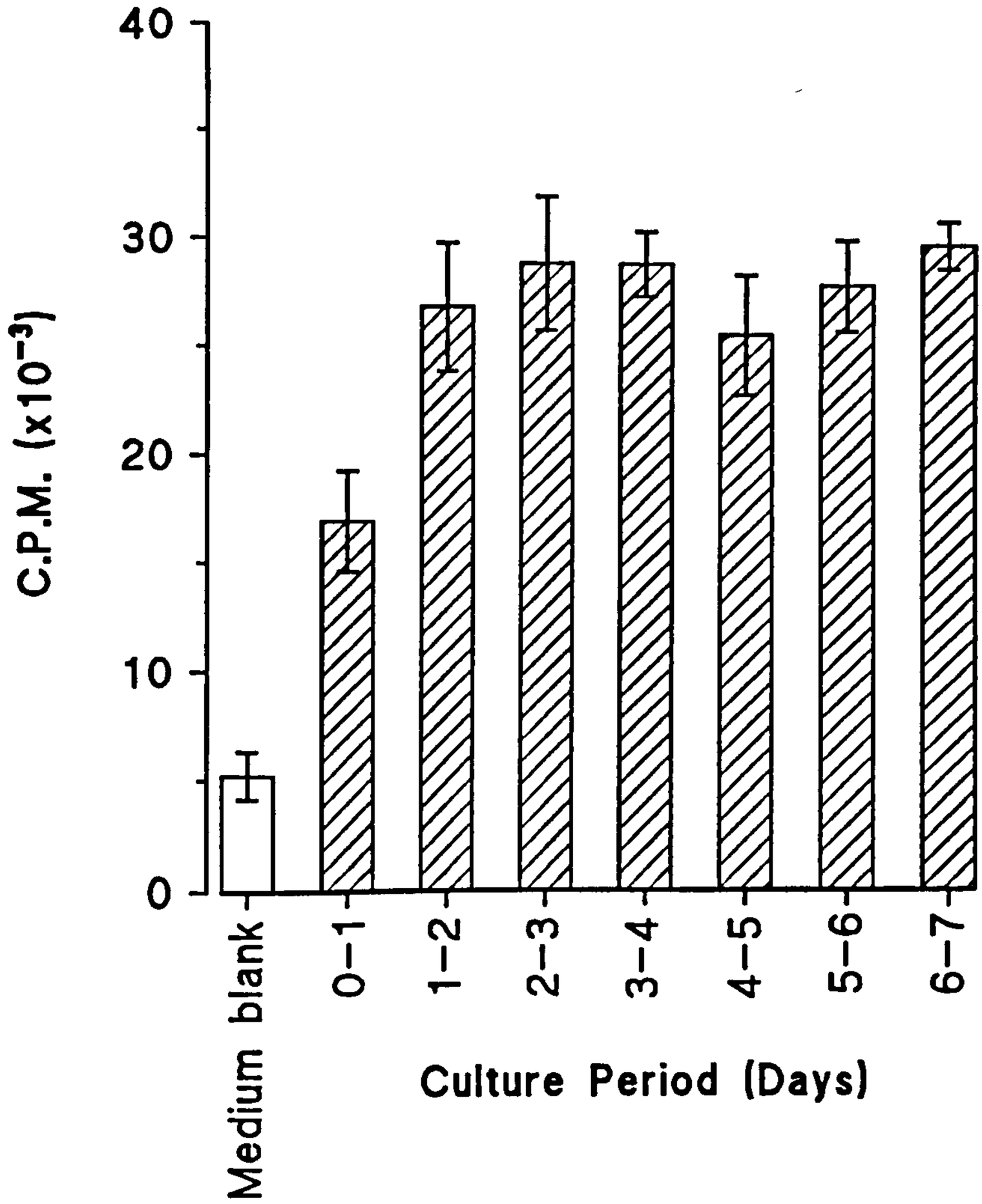


Fig. 3.9. Proliferative response of cervical lymph node cells, recovered from intra-dermally vaccinated mice, to schistosomula-released proteins collected at 24h intervals over a 7 day period. Values are expressed as the mean c.p.m. ± S.E.M. for triplicate wells from 3 experiments. The equivalent amount of protein released from 1000 schistosomula was added to each well.

significantly lower response ($P < 0.05$) was induced by proteins released during the first 24h of culture than at subsequent times, this value being 3 times greater than background levels. Supernatants collected at later time points, induced a higher level of proliferation, approximately 5 times greater than background levels. Figure 3.10 illustrates the proliferative response of cervical lymph node cells to particulate parasite fractions collected every 24h over a 7 day period. All fractions gave similarly high responses, each being at least 4 times greater than background proliferation.

3.4 Discussion

3.4.1 *Blastogenesis assay conditions*

Since M169 is a complex medium, it is impossible, without much time-consuming work, to identify the component(s) responsible for the inhibition of cellular proliferation. However, despite the superior nature of M169 as a parasite culture medium, RPMI/1 was capable of supporting a high level of parasite viability, and thus could be used as a substitute.

In all blastogenesis experiments, negative controls were included to account for ^{3}H-thymidine incorporation by APC, live larvae, or responder cells in the presence of medium alone, whilst SWAP was used as a positive control. In some experiments, lymph node cells recovered from naive mice were included to control for any non-specific stimulation of proliferation. Ideally, each assay would have included such lymph node cell controls; however, test material was often limiting. Also, a concanavalin A control would have been useful to allow comparison of cell viability from experiment to experiment.

Blastogenesis experiments used the axillary or cervical lymph nodes excised from mice five days after either percutaneous vaccination with optimally-attenuated cercariae, or intra-dermal vaccination with day 8 schistosomula respectively. During the period between exposure to parasites and excision of lymph nodes, little parasite death occurs (Wilson *et al.* 1986), therefore most T cell responses should be directed towards material released by transforming cercariae or by migrating schistosomula. Supportive evidence for this inference comes from experiments in which a DTH footpad assay was used to probe *in vivo* cellular responses of mice, 10 or 17 days after vaccination with irradiated cercariae (Ratcliffe & Wilson, 1992). A much lower response was observed to fixed (dead) compared to live lung-stage schistosomula, suggesting that up to 17 days post-vaccination the majority of T cell specificities are towards secretory or released proteins, not somatic proteins derived from dead parasites. It has been determined that lymph node cells recovered on day 5 yield the highest level of proliferation after *in vitro* restimulation with parasite antigen (Pemberton *et al.* 1991). Presumably, the 5 day exposure period

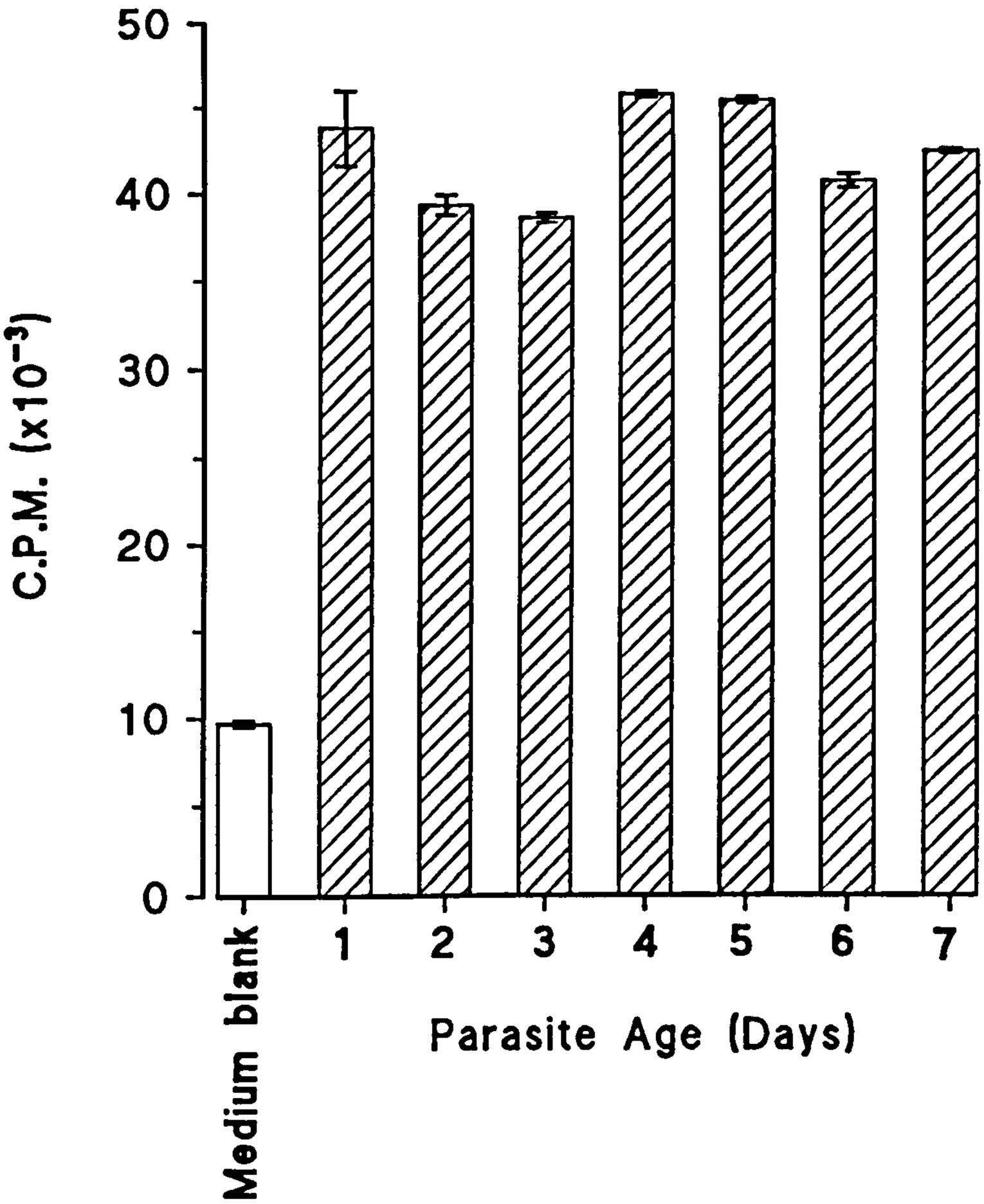


Fig. 3.10. Proliferative response of cervical lymph node cells, recovered from intra-dermally vaccinated mice, to particulate parasite fractions collected at 24h intervals over a 7 day period. Values are expressed as the mean c.p.m. ± S.E.M. for triplicate wells from 3 experiments. The equivalent amount of particulate protein derived from 1000 schistosomula was added to each well.

allowed enough time for cells to be primed to parasite material but for few to have left the lymph nodes as part of their normal trafficking.

3.4.2 *Proliferative responses of axillary lymph node cells recovered from p.c. vaccinated mice*

Since the immune system has a 'lag phase' before a response to antigen is detectable, it was likely that cells recovered on day 5 would have a specificity which was biased towards proteins released by larvae within the first one to two days after vaccination. It may seem surprising therefore that the lowest proliferative response of axillary lymph node cells to soluble parasite proteins was to those present in the 24h schistosomula. However, two results from chapter 2 may help to explain this. During the first 24h after transformation, the schistosomulum synthesises very little protein. However, within this time there is a massive release of proteins pre-synthesised in the cercaria. Thus, in terms of protein composition, the 24h parasite can be regarded as a cercaria minus the proteins released within this period. After 24h, the rate of protein synthesis increases, therefore the proportion of total released proteins derived from material synthesised post-transformation is also likely to increase. It was possible then, that after the first 24h, more proteins to which the mouse had been sensitised were present in the soluble fraction of parasites and hence caused the higher degree of proliferation.

The highest levels of proliferation induced by proteins released *in vitro* by 1000 schistosomula occurred to those lost within the first 24h to 48h of culture. It was likely that this material constituted the major set of proteins to which the vaccinated mouse had been primed. Additionally, during this period, the rate of protein loss is high. Therefore, the enhanced degree of proliferation may also reflect the quantity of antigen used in the assay as much as its quality. However this is a reflection of the events which occur during a natural infection i.e. a host is exposed to a particular number of parasites not to a standard quantity of antigen at all stages.

The proliferative response of axillary lymph node cells to 400 live schistosomula was marginally greater than that induced by isolated released proteins (cf. Fig. 3.4). However, it must be remembered that the live parasites were in culture with responder cells for 5 days. Thus, 400 schistosomula released antigenic material over a period of 120h compared with the isolated released proteins which were collected after culture of 1000 schistosomula for 24h. Although not an accurate comparison, it appears that the presence of a live metabolising parasite does not provide a dramatically enhanced antigenic stimulus compared to isolated released proteins. This result was in contrast to that obtained by James (1981) who demonstrated a highly elevated proliferative response of cells to live schistosomula compared to their isolated released proteins. Several factors

may account for this discrepancy, firstly James pooled cells from disparate lymph nodes of chronically infected mice and as such the results are not directly comparable. Secondly, proteins released over 24h or 48h were collected, whereas live schistosomula were present in the assay for 62h and therefore had a longer period in which to release protein. However, the proliferative response to titrated numbers of live schistosomula agreed with those of James (1981), who found that the level of proliferation reached a plateau at 400 larvae per well. That sonicated 5 day old schistosomula induced a much greater response than intact parasites suggests either the quantity of antigen released from viable schistosomula or the number of specific T cells to such released proteins was limiting. The latter hypothesis would seem to be correct, since increasing the number of live parasites from 400 to 600 did not increase the level of proliferation. The pattern of proliferation induced by schistosomula up to 11 days old supported the result obtained using isolated released proteins, the lowest level of proliferation occurring to older parasites. This indicates that either quantitatively less protein to which the mouse had been sensitised was released by later-stage parasites, or that there were qualitative differences in the secreted material.

3.4.3 *Proliferative responses of cervical lymph node cells recovered from i.d. vaccinated mice*

Initially, i.d. injection of day 8 schistosomula was carried out in FCS-containing medium. However, high background levels of proliferation (approximately 20,000 c.p.m.) were obtained, since cervical lymph node cells would proliferate in response to the FCS present in RPMI/10. Subsequently, schistosomula were washed and injected into the dermis of the pinna in M169 containing normal mouse serum. This reduced the background proliferation to approximately 5,000 c.p.m..

The proliferative response of lymph node cells from i.d. vaccinated mice to titrated soluble protein derived from 1 to 7 day old schistosomula reached a plateau at approximately 10μg/well. The magnitude of the response at this antigen concentration was similar for all soluble fractions analysed, suggesting either that the isotope or the number of antigen-specific T cells was limiting (i.e. antigen was in excess). If the latter explanation is correct then the use of limiting quantities of antigen allows smaller differences in the number of specific T cells to be detected, in terms of magnitude of proliferation. When 1μg soluble protein was used per well, the lowest proliferative response by lymphocytes was to proteins derived from 1 day old schistosomula. As mentioned previously, a parasite of this age can be regarded, in terms of protein composition, as a cercaria minus the early-stage secretions. Following i.d. vaccination of mice with 8 day old schistosomula, it was likely that the majority of T cell specificities would be to proteins synthesised after transformation, since most proteins synthesised

prior to transformation have been lost from the schistosomula by day 8 (chapter 2). Therefore, it was not surprising that the soluble protein derived from 1 day old schistosomula induced the lowest level of proliferation. Subsequent protein fractions caused similar levels of proliferation, indicative of the relative homogeneity of this sub-set of parasite proteins throughout the first week of development (chapter 2).

The relatively low response of cervical lymph node cells to proteins released during the first 24h after transformation compared with later fractions may be explained by qualitative rather than quantitative factors. Although it was likely that quantitatively more protein was present in this fraction it would be comprised predominantly of proteins that were synthesised prior to transformation. Since 8 day old schistosomula were the youngest parasites to which mice in this vaccination regime had been exposed, lymphocytes from such animals would not have been primed to stage-specific proteins released during the first 24h. After this time, an increasing proportion of total released proteins would be comprised of those synthesised post-transformation. This is likely to explain the enhanced proliferative response to proteins released after 24h.

Particulate parasite fractions were capable of inducing a good, yet fairly uniform proliferative response. This is probably because the fraction contains many structural proteins which are more likely to be conserved throughout the 7 day period. Any fluctuations in proliferation were probably due to inaccuracies in resuspending the pellet, resulting in slightly different protein concentrations, rather than real qualitative differences.

3.4.4 *Conclusion*

As would be expected, all parasite fractions tested in these assays induced substantial levels of proliferation above that caused by medium alone. In terms of fold increase in incorporation of ^{3}H-thymidine above background levels, proteins released from the equivalent of 1000 schistosomula were as potent as 1μg soluble parasite protein in most cases. However, to achieve an accurate comparison it would be essential to compare 1μg released protein with 1μg soluble protein. This could be achieved by collecting released proteins in serum-free medium, in which only schistosomula older than 5 days can survive sufficiently well. Thus, this investigation would be limited to proteins released by older larvae. Such experiments are currently underway in this laboratory. Having established that released proteins can stimulate proliferation of T cells recovered from protectively vaccinated mice (and were therefore released *in vivo* in order to prime lymphocytes), it would be interesting to determine the type and magnitude of response induced i.e. Th1 or Th2. A good indication of this would be provided by cytokine assays performed on culture supernatants taken from T cell proliferation assays.

For an effective immune response to occur against pathogens, foreign proteins must

induce proliferation and therefore clonal expansion of the specific arm of the body's defence mechanism. If a high degree of proliferation by lymphocytes is a good indicator of the immunogenic and therefore vaccine potential of a group of proteins, then the results presented here are encouraging. However, for the purpose of vaccine design against schistosomiasis, it is important to define which cellular sub-set is being induced to proliferate most by the antigenic fraction. Induction of the 'wrong' sub-set could lead to increased pathology rather than cure. Although schistosomula-released proteins represent a relatively defined sub-set of total parasite proteins, their composition is still complex. One of the problems of working with crude antigen preparations and trying to identify differences in the ability of each to induce proliferation, is the homogeneity which exists between fractions. It has been reported previously that lymph node cells recovered from mice, 1 week after vaccination, could respond equally well to soluble cercarial, adult worm and egg antigen preparations, despite the host never having been exposed to the latter two life-cycle stages (Mak & Sanderson, 1985). However, at the clonal level many differences in reactivity to the various antigenic preparations were detected. Thus, one explanation for the failure of experiments discussed in section 3.1 to show a correlation between immune status of the host and ability of lymphocytes to proliferate in response to specific antigen maybe the complex nature of the antigen mixture used. If more restricted protein mixtures were used, correlations would possibly be more obvious. Collection of enough schistosomula-released proteins for separation by HPEC could potentially help to select candidate vaccine antigens which were capable of eliciting strong immune responses of a Th1 nature. Alternatively, parasite-released proteins could be identified from a gene library and the recombinant proteins then tested for their ability to induce proliferation of, and cytokine production by, lymph node cells recovered from protectively vaccinated mice. Initial steps towards this goal are described in chapter 5.

CHAPTER FOUR

Detection of antigenic fractions of *Schistosoma mansoni* using monospecific sera, and sera raised against schistosomula-released proteins.

4.1 Introduction

In chapter 1, it was argued that challenge parasites of mice vaccinated once with attenuated cercariae were eliminated via a T cell rather than antibody–mediated mechanism. Conversely, in multiply–vaccinated mice, the process of elimination is either enhanced by, or totally dependent upon antibody–mediated mechanisms. Antibodies usually bind to pathogens via accessible epitopes, such as those present on surface–exposed proteins. Therefore, polypeptides released by schistosomula, unless enriched with surface membrane–derived macromolecules, are unlikely to be a good source of target antigens of protective antibodies. They may nevertheless contain important T cell antigens.

This chapter will describe the characteristics of two antisera, 0–3hS and D4–D8S, raised in rabbits against proteins released by schistosomula within the first 3h, and between day 4 and day 8 post–transformation, respectively. These sera were produced for several reasons. Firstly, analysis of their specificities facilitates investigation of the ability of constituents of either 0–3h or D4–D8 released proteins to induce antibody production, an indicator of their immunogenicity. However, for the purpose of this work, the most important reason was that they could be used to identify proteins expressed from a cDNA library; the serum raised against D4–D8 released proteins was produced primarily for this purpose. Screening of a larval cDNA library with this serum forms the subject matter of the following chapter.

The 0–3hS was produced for several reasons, but primarily to allow a comparative analysis of both 0–3hS and D4–D8S by ELISA and Western blotting experiments against their respective homologous antigen preparations or heterologous protein fractions. Additionally, 0–3h released proteins were readily available and derived from parasites, essentially 100% of which were viable. Furthermore, secretions from newly–transformed schistosomula contain stage–specific proteins, including enzymes involved in skin penetration, and as such have a different composition from later–stage released molecules. In an attempt to compare the relative abundance of a particular protein in an antigen preparation and its ability to induce an antibody response in rabbits, molecules detected by Western blotting and following Coomassie staining or autoradiography, will be described.

Additionally, monospecific sera and monoclonal antibodies directed against defined proteins were used to characterise the soluble and released protein fractions, by Western blotting. Finally, sera raised against both early– and later–stage released proteins were tested for their ability to confer passive protection to mice, against a *Schistosoma mansoni* challenge infection.

4.2 Materials and methods

4.2.1 *Preparation of parasite material*

Schistosomula were obtained as described in section 3.2.2. For the first 3h, parasites were cultured in M169W, the suspension centrifuged at 150*g* for 60s and the parasite–free culture supernatant (0–3h) removed. Schistosomula were resuspended and cultured in M169S for a further 4 days after which they were washed 6 times in 10ml M169W, to remove serum proteins and dead parasites, then resuspended in M169W containing 1% normal rabbit serum (NRS; GIBCO) and cultured for 4 more days. At the end of this period, the parasite suspension was centrifuged and the supernatant (day 4 – day 8 culture supernatant; D4–D8) removed. Several 0–3h or D4–D8 supernatants, collected from different parasite cultures, were pooled, concentrated using a stirred ultrafiltration cell (Amicon) as described previously (section 3.2.2), and stored at –20°C. Such samples were used to raise antiserum (see section 4.2.2).

For enzyme linked immunosorbent assays (ELISA) and Western blotting experiments, soluble antigen preparations of cercariae (soluble cercarial antigen preparation; SCAP), *in vitro*–cultured lung–stage larvae (soluble larval antigen preparation; SLAP) and adult worms (soluble worm antigen preparation; SWAP), recovered by portal perfusion of infected mice (Smithers & Terry, 1965), were prepared, as detailed in chapter 2. Additionally, 0–3h released proteins were produced as described above. However, because protein estimates were required, prior to use of samples in ELISA or Western blotting experiments, lung–stage larval secretions had to be collected in serum–free medium. It was noted previously (chapter 2) that within the first 5 to 6 days after transformation, schistosomula were fragile upon centrifugation and 'sensitive' to the absence of serum. If D4–D8 culture supernatants had been collected in serum–free medium, an unacceptable level of parasite death would have occurred. Previous experimental results indicated a strong degree of homology in the proteins released between days 4 to 6 and 6 to 8. Since a high level of parasite viability could be maintained during *in vitro* culture from day 6 to day 8 in M169W (which contains no serum supplement), proteins released during this time were collected instead of between day 4 and day 8. On day 6, schistosomula were washed 6 times in 10ml M169W and then cultured for an additional 2 days in M169W. At the end of this period, the culture supernatant was recovered and concentrated, whilst the schistosomula were used to yield a soluble antigen preparation (SLAP). The protein content of the soluble and released fractions was estimated by the Bradford assay (Bio–Rad).

4.2.2 *Production of antisera*

Antisera to proteins released within the first 3h and between day 4 and day 8 post-transformation were raised in New Zealand white rabbits. Prior to the first immunisation, a pre-bleed was taken from each animal to serve as a normal rabbit serum (NRS) control in subsequent experiments. For the primary inoculation, 500μl of the appropriate concentrated culture supernatant was mixed, by vortexing, with an equal volume of Freund's complete adjuvant (SIGMA) and injected sub-cutaneously (s.c.) at 4 or 5 sites on the back of the rabbit. Subsequent immunisations were given in Freund's incomplete adjuvant (SIGMA) at approximately 4 week intervals. Small test bleeds were taken from each rabbit at approximately 2 week intervals to allow changes in the antibody titres to be monitored by ELISA. Each blood sample was allowed to clot for 1h at room temperature, ringed with a mounted needle and allowed to stand for several hours at 4°C. The serum was removed, pipetted into eppendorfs and spun at 9000*g* for 90s to remove any contaminating red blood cells. All sera were stored in small aliquots at -70°C.

4.2.3 *Monoclonal antibodies and monospecific sera*

In an attempt to identify constituents of the soluble and, in particular, released parasite proteins by Western blotting, monoclonal antibodies and monospecific sera were obtained from research groups around the world. The following samples were kindly donated:

Target antigen	M_r (kDa)	Specificity	Raised in	Group
Superoxide dismutase (SOD)	16	polyclonal	rabbits	Pierce
Superoxide dismutase (SOD)	20	monoclonal	mice	LoVerde
Glutathione S-transferase (Sm26)	26	polyclonal	rabbits	Pierce
Glutathione S-transferase (Sm28)	28	polyclonal	rabbits	Pierce
Triose-phosphate isomerase (TPI)	28	monoclonal	mice	Harn
Haemoglobinase (Sm32)	32	monoclonal	rabbits	Rotmans
Calreticulin (Sm58)	58	polyclonal	rabbits	Pierce
Paramyosin (Sm97)	97	monoclonal	mice	Pearce

4.2.4 *Measurement of antigen-specific antibody titre*

The antibody titres of the 0-3h serum (0-3hS) and the D4-D8 serum (D4-D8S) to their essentially homologous antigen preparations (0-3h and D6-D8 released proteins respectively) or to various other antigenic fractions of *Schistosoma mansoni* was

determined by ELISA. Microtitre plates (Nunc) were coated with antigen, diluted to 100μl with carbonate coating buffer pH 9.6 (15mM Na_2CO_3, 35mM $NaHCO_3$), by incubation at 4°C overnight in a humid chamber. Subsequently, plates were washed with PBS Tween (137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4 containing 0.3% Tween 20) and the wells blocked, to inhibit non-specific binding of antibodies, by incubation with blocking buffer (carbonate coating buffer containing 3% normal goat serum (NGS)) for 2h at 37°C in a humid chamber. The sera were diluted in blocking buffer as required, 100μl of the appropriate sample added to each well and the plates incubated in a humid chamber for 2h at room temperature. Plates were washed 3 times with PBS Tween and, after the addition of 100μl goat anti-rabbit peroxidase conjugate (GAR PO conjugate; SIGMA), diluted to 1:5000 with blocking buffer, incubated in a humid chamber for 2h at room temperature. Plates were washed 3 times with PBS Tween and then 100μl enzyme substrate (10mM $Na_2HPO_4.12H_2O$, 4.9mM citric acid, containing 0.05% Tween 20 and, immediately before use 0.2% O-phenylenediamine and 0.02% H_2O_2) added to each well. When sufficient colour had developed, the reaction was stopped by the addition of 30μl 8M sulphuric acid and, spectrophometric readings taken at 490nm in a Dynatech MR5000 plate reader.

4.2.5 *SDS Polyacrylamide gel electrophoresis (SDS-PAGE)*

Separation of various protein fractions of *Schistosoma mansoni* was performed by 1-D electrophoresis under reducing conditions according to the method of Laemmli (1970; see also chapter 2). Known quantities of protein were boiled for 2 min in sample buffer (4 volumes sample: 1 volume sample buffer; 10% SDS, 25% glycerol, 0.3125M Tris-HCl pH 6.8, 0.025% bromophenol blue, 50mM dithiothreitol) and loaded on to gels consisting of a running gel (6-16% acrylamide, 0.16-0.43% bis-acrylamide in 0.375M Tris-HCl pH 8.8 containing 0.1% SDS) overlaid by a stacking gel (4.5% acrylamide, 0.12% bis-acrylamide in 0.125M Tris-HCl pH 6.8 containing 0.1% SDS). Molecular weight standards (Bio-Rad) were treated in the same way as test antigen fractions, and loaded on to the gel. A constant current of 20mA per gel was applied until the dye front reached the running gel, when the current was increased to 30mA per gel. Electrophoresis continued until the dye front was approximately 20mm from the base of the gel. Where required, gels were fixed in 15% TCA, stained with Coomassie brilliant blue (1.25% Coomassie brilliant blue in 40% methanol, 10% acetic acid), destained in a 40% methanol, 10% acetic acid mixture and dried onto filter paper.

4.2.6 *Western blotting*

After electrophoretic separation of proteins, gels were sandwiched between an

immobilon–P membrane (MILLIPORE), which had been pre–soaked in methanol for 5s and then deionised water for 2 min, and filter paper pre–soaked in 0.5% SDS. Blotting of proteins took place at 40V for 16h in an LKB "Transphor" apparatus containing transfer buffer (25mM Tris, 192mM glycine in 20% methanol) cooled to 4°C by a heat exchanger. Upon completion of transfer, the segment of the membrane containing the molecular weight markers was sliced off and stained for 5 min with Amido Black (0.1% Amido black, 50% methanol, 10% acetic acid), then destained as required in 50% methanol, 10% acetic acid. In order to reduce non–specific binding of antibodies, the remainder of the membrane was incubated in blocking buffer (PBS containing 0.3% Tween 20 and 5% NGS) for 2h at room temperature. If required, the membrane was cut into strips and then incubated with the test sera, diluted as appropriate in blocking buffer, for 2h at room temperature. For each antigen preparation tested, one strip was always probed with NRS to serve as a negative control. Strips were washed in 5 changes of PBS Tween over 1h and were then incubated in GAR PO conjugate diluted to 1:20000 in blocking buffer or, in experiments where anti–Sm97, anti–TPI or anti–SOD (20 kDa) were used, diluted (1:15000) goat anti–mouse peroxidase conjugate (SIGMA), for 2h at room temperature. Strips were washed again for 1h in PBS Tween with 5 changes of buffer. The sensitive technique of enhanced chemiluminescence (ECL; Amersham) was used to detect antigen/antibody complexes as described by the manufacturer. The novel step utilised by this method is the oxidation of luminol, catalysed by peroxidase conjugated antibodies, which results in the emission of light. Such emissions are detected on film (Hyper–film ECL; Amersham) and visualised after incubation in Kodak D19 developer for approximately 1 min and fixing in Unifix (Kodak; 1 volume Unifix: 2 volumes water) for 5 min.

4.2.7 *Passive transfer of serum*

Prior to transfer, the various sera (NRS, 0–3hS and D4–D8S) were heat inactivated at 56°C for 30 min to destroy complement activity and then incubated with 2ml packed mouse red blood cells for 2h at 37°C to adsorb potentially damaging antibodies directed against mouse antigens. Mouse red blood cells were obtained by cardiac puncture using a heparinised syringe and needle. Blood was collected into a tube, on ice, containing 1ml heparinised saline. The cells were centrifuged at 200*g* for 10 min at 4°C, the supernatant removed and the cells resuspended in heparinised saline. The centrifugation and wash steps were repeated, the cells left in suspension for 10 min, centrifuged and the supernatant then removed. The cells were then ready to be used for adsorption.

For all passive transfer experiments, C57Bl/6 mice were used, either as recipients of NRS, test serum or as challenge controls. Each experimental group contained 5 mice. The treatment received by groups of mice in each experiment is displayed in table 4.1.

Experiment number	Bleed number	Group	Treatment
1	4	1 2 3 4 5	Challenge controls NRS on D0 0-3hS on D0 NRS on D4 and D7 D4-D8S on D4 and D7
2	7	1 2 3	Challenge controls NRS on D4 and D7 D4-D8S on D4 and D7
3	7	1 2 3 4 5	Challenge controls NRS on D0 0-3hS on D0 0-3hS on D1 0-3hS on D4 and D7
4	9	1 2 3 4 5	Challenge controls NRS on D4 and D7 D4-D8S on D0 D4-D8S on D1 D4-D8S on D4 and D7

Table 4.1. Experimental design for the passive transfer of sera to mice. The experiment number, the bleed from which the test serum was derived, and the treatment groups are tabulated. Additional experimental details are given in the text.

In each case, mice were exposed to a mean of either 200 (experiment 1) or 120 (experiments 2, 3 and 4) hand-counted cercariae via the tail on day 0. All groups of mice which received serum on day 0 were injected several hours prior to challenge infection. For each inoculation, 0.5ml serum was administered intravenously. Portal perfusion of the mice was carried out 5 weeks after challenge infection and the adult worm burden determined. Resistance was expressed as the percentage reduction in the number of adult worms recovered from mice receiving the test serum compared to that of challenge control mice or mice receiving NRS, and was calculated from the formula

$$\% R = (C - T)/C \times 100$$

where T is the mean number of worms recovered from the test group, C is the mean number of worms recovered from the control group and R is the level of resistance. Differences in worm burdens were tested for statistical significance using the Student's *t* test.

4.3 Results

4.3.1 *SDS-PAGE analysis of soluble and released proteins of Schistosoma mansoni*

A Coomassie-stained gel of the protein fractions used in subsequent ELISA or Western blot experiments is displayed in figure 4.1. Each sample was loaded at 25μg/lane. The soluble cercarial antigen preparation contained two dominant proteins of molecular weight (M_r) 31 and 28 kDa, the latter being a major component of both 0-3h and D6-D8 released proteins. Two polypeptides of M_r 61 and 20 kDa present in SCAP were barely visible in SLAP and could not be detected in SWAP. Constituents of the lung-stage soluble preparation more closely resembled SWAP than SCAP but contained a dominant, and possibly unique, protein at approximately 45 kDa. The dominant doublet in SCAP at 31 and 28 kDa became a triplet in SLAP and SWAP, the additional protein occurring at 32 kDa. The latter two preparations contained several minor polypeptides at approximately 90 kDa which could not be detected in SCAP or either of the released protein fractions.

Approximately 12 polypeptides could be detected in the 0-3h released material; of the two most dominant, the moiety at 61 kDa could not be detected in D6-D8 released proteins whilst the 28 kDa antigen remained prominent. In the D6-D8 released fraction approximately 15-20 proteins were detectable, ranging in M_r from 70 kDa to <10 kDa. The most dominant protein occurred at 28 kDa whilst a polypeptide at approximately 20 kDa was also prominent. Thus, the early- and later-stage released proteins were not

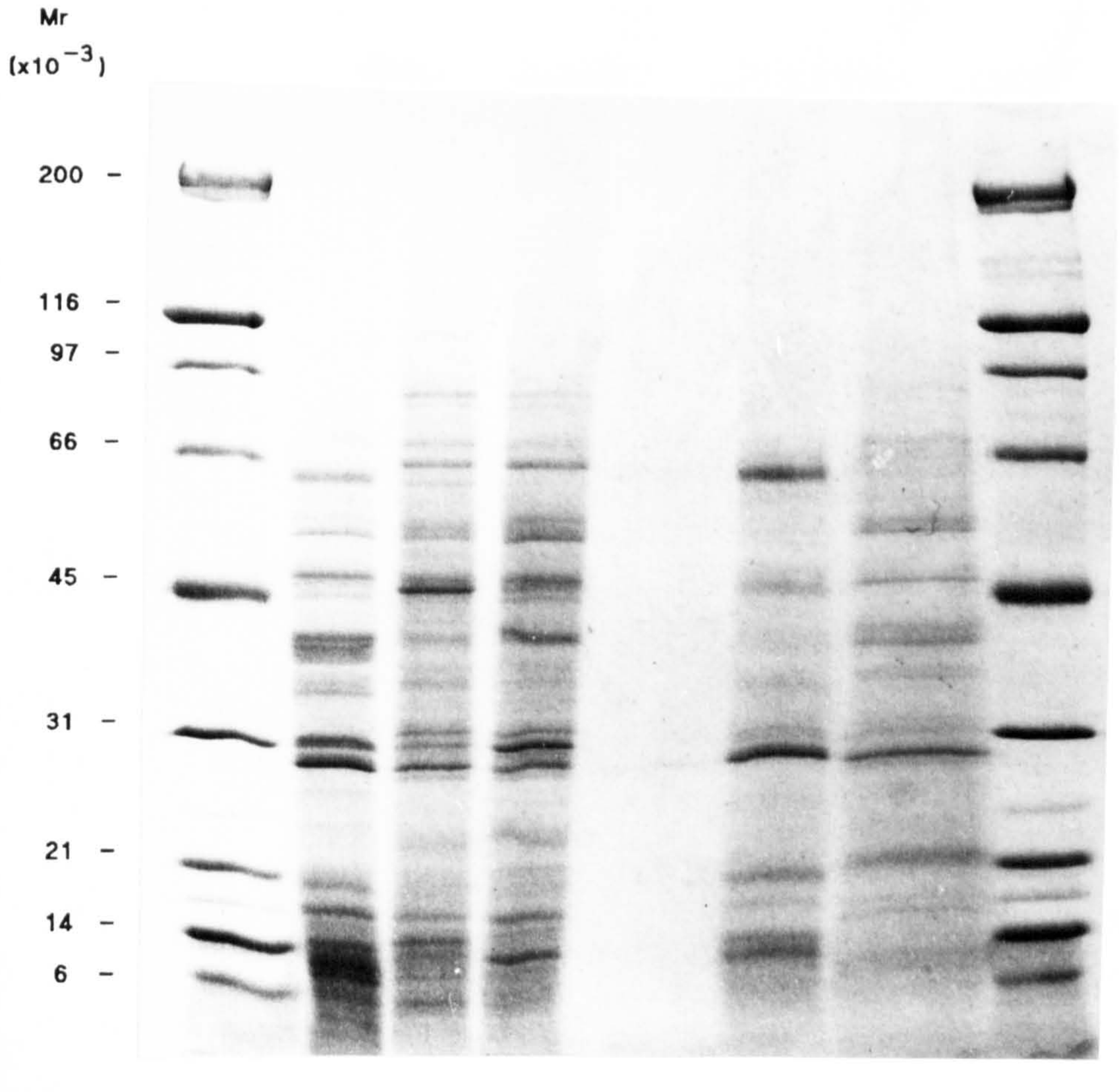


Fig. 4.1. Coomassie–stained gel of soluble preparations of cercariae (SCAP), lung–stage larvae (SLAP) and adult worms (SWAP) and early–stage (0–3h) and later–stage (D6–D8) released proteins of *Schistosoma mansoni.* The molecular weights (kDa) of the standards are illustrated.

identical in composition. Additionally, in comparison to either SCAP or SLAP, the 0–3h and D6–D8 released proteins, which are likely to be sub–sets of these cruder soluble parasite preparations, showed both quantitative and qualitative differences in their composition. Some constituents of the soluble preparations appeared to be released in relatively large quantities whilst others did not seem to be released at all. However, using 1–D SDS–PAGE, it was not possible to state unequivocally that proteins of identical M_r, present in both soluble and released protein fractions, were indeed the same molecule.

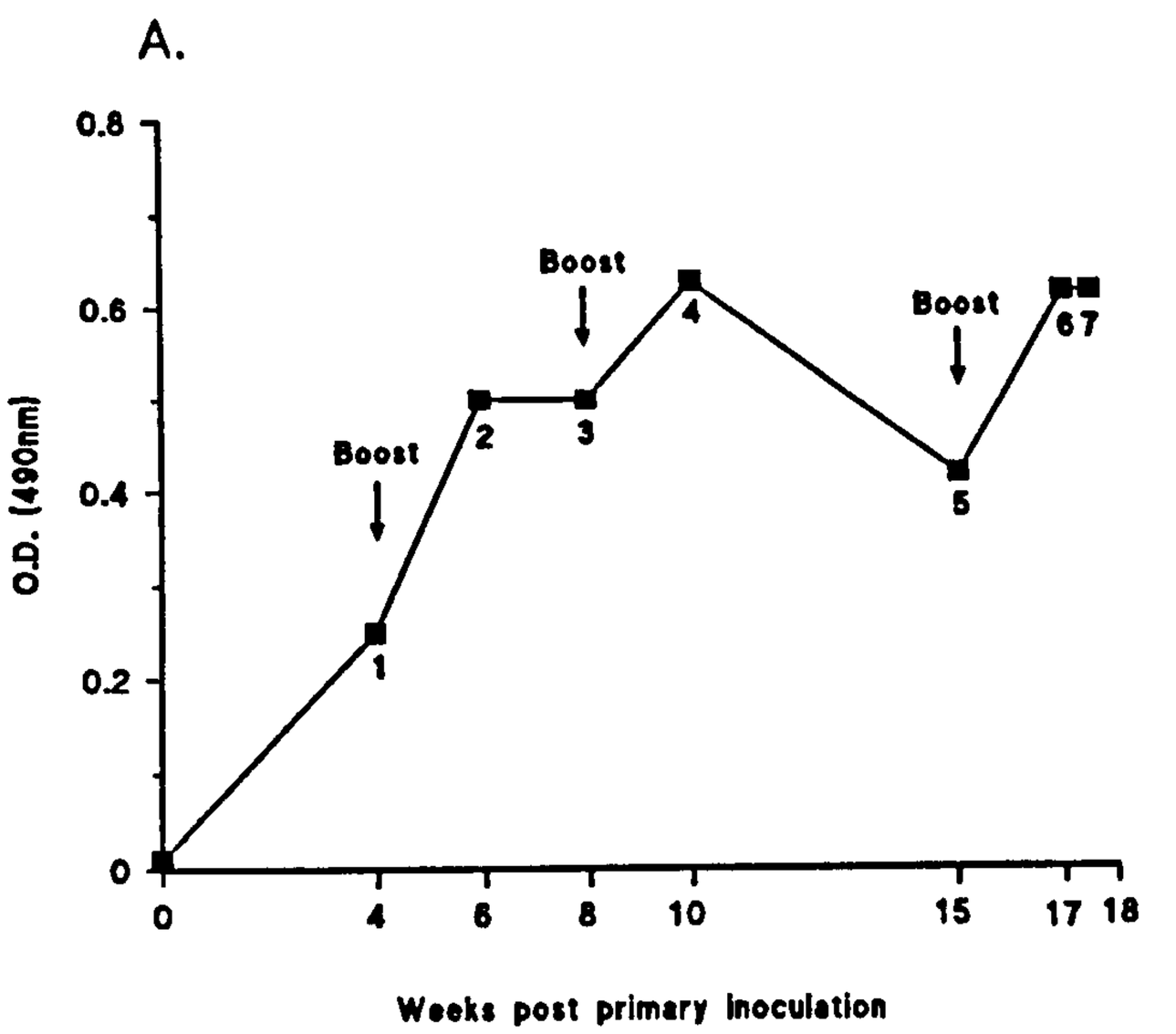
4.3.2 *Production of antisera and vaccination schedule*

The vaccination schedule for each rabbit, inoculated with either 0–3h or D4–D8 released proteins, and the resulting antibody titre determined by ELISA against their homologous antigen preparation (0.025μg/well), is shown in figure 4.2. In these experiments, the 0–3hS was used at a dilution of 1:12800 and the D4–D8S at 1:800. The antigen–specific antibody titre of the rabbit immunised with 0–3h released proteins reached a plateau after 2 boosts (Fig. 4.2A). Approximately 2 weeks after the third boost, whilst the antibody titre was high, this rabbit was terminally bled (bleed 7). In contrast, the antibody titre of the rabbit immunised with proteins released by schistosomula between days 4 and 8, increased dramatically after the second boost (Fig. 4.2B) and was still rising after the third boost. Between weeks 17 and 37 after the primary inoculation, the titre fell very little. The rabbit received one more boost at week 37 and was then terminally bled 2 weeks later (bleed 9).

4.3.3 *Analysis of serum reactivity by ELISA*

In order to identify the appropriate antibody and antigen dilutions to be used in subsequent ELISA experiments, a matrix was set up in which the sera (bleed 7) were diluted down the plate and the antigen concentration across the plate. The D4–D8S gave a good optical density reading, which fell on the linear part of the dilution curve (data not shown), at a serum dilution of 1:800 and using D6–D8 released proteins at a concentration of 0.025μg/well. The 0–3hS was many times more potent than the D4–D8S and could be used at a dilution of 1:12800 when plates were coated with 0–3h released proteins at 0.025μg/well. However, most experiments incorporated an antibody dilution curve which commenced at 1:800 and an antigen concentration of 0.025μg/well.

The reactivity of each serum (bleed 7) to its homologous and heterologous antigen fraction determined by ELISA is illustrated in figure 4.3. The 0–3hS reacted very strongly to its homologous antigen preparation but much less so to the heterologous D6–D8 released protein fraction. In contrast, the D4–D8S reacted more strongly to proteins released within the first 3h post–transformation than it did to its essentially



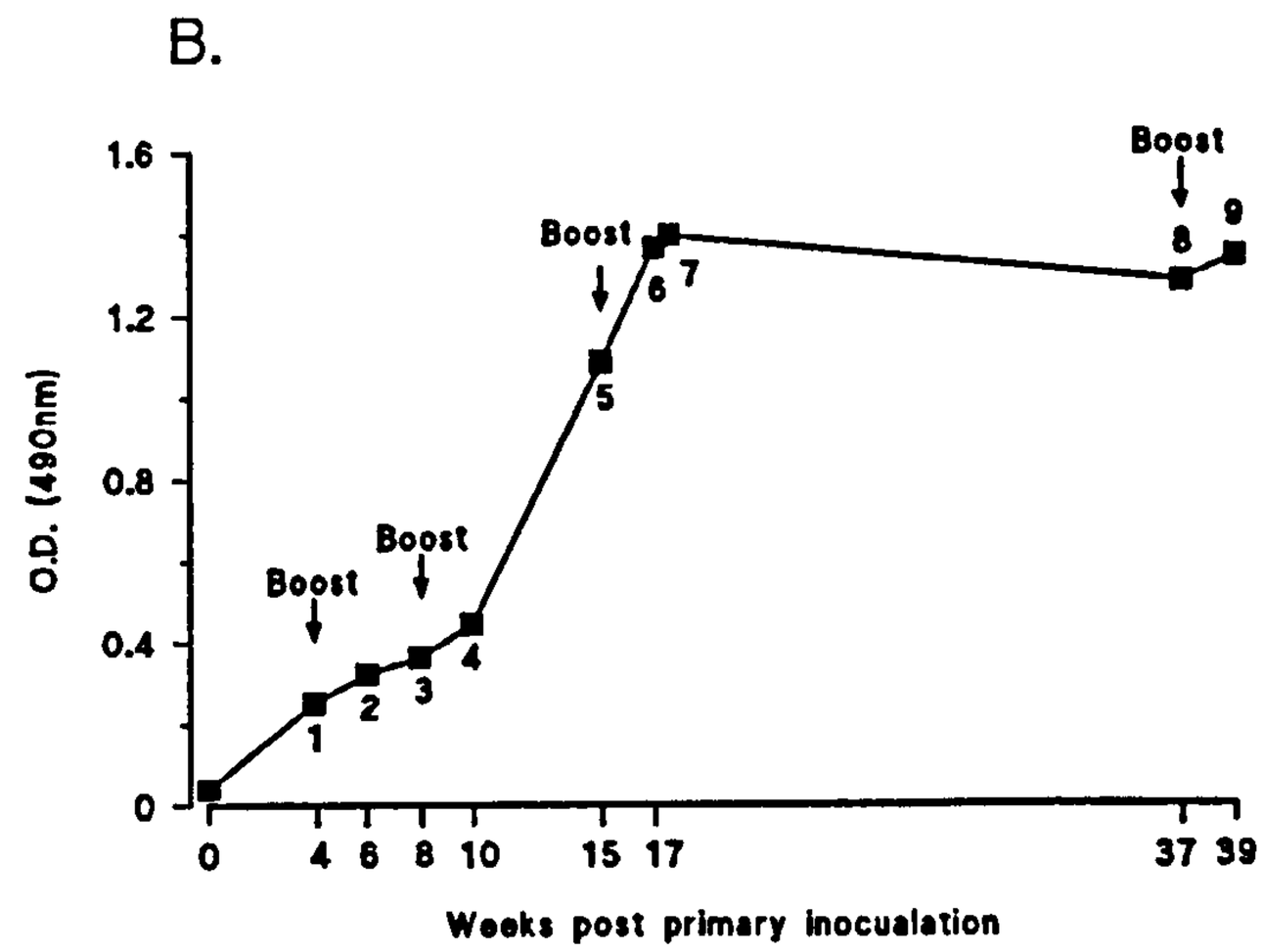


Fig. 4.2. Vaccination schedule and antibody titre, determined by ELISA, of serum recovered from rabbits immunised with proteins released by schistosomula during the first 3h (A) or between day 4 and day 8 (B) post-transformation.

Plates were coated with the homologous antigen (0–3h or D6–D8 released proteins) at 0.025μg/well. Numbers indicate times at which blood samples were taken.

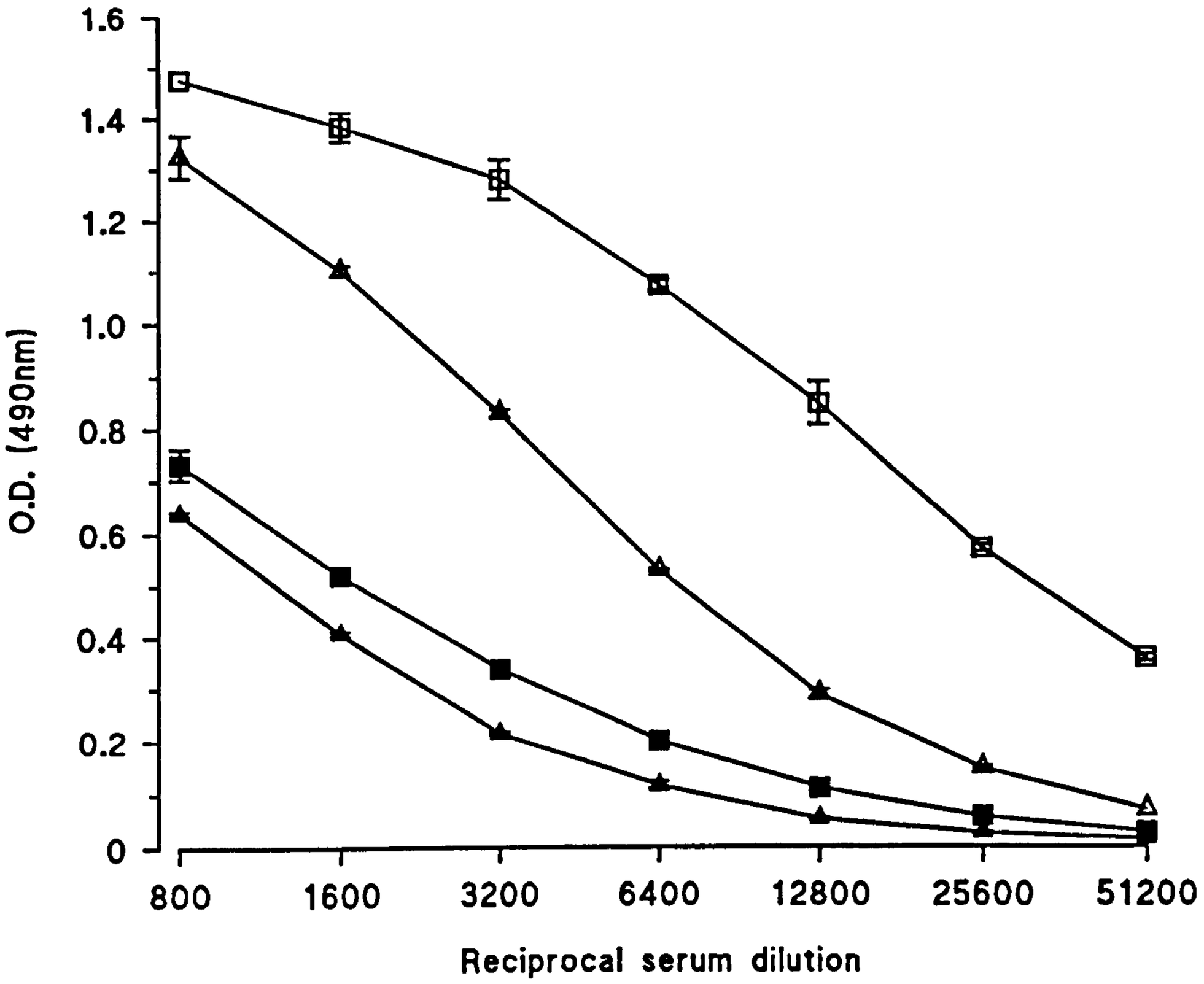


Fig. 4.3. Titration of 0–3hS and D4–D8S in an ELISA against their homologous and heterologous antigen preparations.

The graph represents the reactivity of the 0–3hS (squares) against 0–3h (□) or D6–D8 (■) released proteins and D4–D8S (triangles) against 0–3h (△) or D6–D8 (▲) released proteins. Both antigen preparations were used at 0.025µg/well. All values are expressed as the mean O.D. ± S.E..

homologous fraction (D6–D8 released proteins). The reactivity of the two sera against their homologous antigen fraction (0–3h or D6–D8 released proteins) and soluble cercarial or lung–stage larval preparations (data not shown) was compared by ELISA. This experiment demonstrated that the sera reacted more strongly to their homologous antigens than to the soluble preparations.

The reactivity of the sera in an ELISA, using plates coated with soluble preparations of either cercariae, lung–stage schistosomula or adult worms is shown in figure 4.4. The 0–3hS reacted strongly to SCAP yet relatively poorly to both SLAP and SWAP. Similarly, the D4–D8S gave a high O.D. against SCAP but much lower levels to SLAP or SWAP. The pattern of reactivity of the two sera is depicted more clearly in the inset of figure 4.4. The 0–3hS reacted more strongly to SCAP than did the D4–D8S. However, antibodies from the two sera showed an equal affinity for proteins present in SLAP whilst the D4–D8S reacted more strongly than the 0–3hS to proteins present in SWAP. In a further experiment which used different cercarial, lung–stage larval and adult worm antigen preparations, the pattern of reactivity was similar (data not shown). However, antibodies from the 0–3hS showed a slightly higher affinity for SLAP than the D4–D8S, whilst the reactivities to SWAP were almost identical.

4.3.4 *Analysis of serum specificity by Western blotting*

The specificity of both sera to antigens present in 0–3h or D6–D8 released protein fractions (loaded at 2.5μg/cm in a 3cm trough lane) detected by Western blotting is shown in figure 4.5. Since the 0–3hS and D4–D8S reacted less strongly to D6–D8 than to 0–3h released proteins, the sera had to be used at higher concentrations (commencing at 1:250 instead of 1:1000) to allow their antibody specificities against this preparation to be defined. The 0–3hS recognised at least 7 distinct moieties in its homologous antigen preparation, with four of M_r 70, 45, 20 and 12 kDa being particularly prominent. At the serum concentrations used, the D4–D8S recognised a smaller number of antigens but, as for the 0–3hS, proteins of 20 and 12 kDa were dominant. Both 0–3hS and D4–D8S detected a similar set of antigens in the D6–D8 released proteins. However, a minor protein of <20 kDa (arrowed) was only recognised by the 0–3hS whilst a 47 kDa antigen (arrowed) was only identified by the D4–D8S. Unlike the pattern of reactivity against the 0–3h released proteins, neither serum recognised any antigen particularly strongly, perhaps the most prominent being of M_r 28 and 20 kDa.

When the two sera were used to probe Western blots of three soluble parasite fractions (SCAP, SLAP and SWAP), the intensity of staining mirrored the result obtained by ELISA (data not shown). However, by using the same range of serum dilutions for all three antigen preparations, few proteins could be detected in either SLAP or SWAP. Therefore, subsequent experiments used higher serum concentrations for SLAP

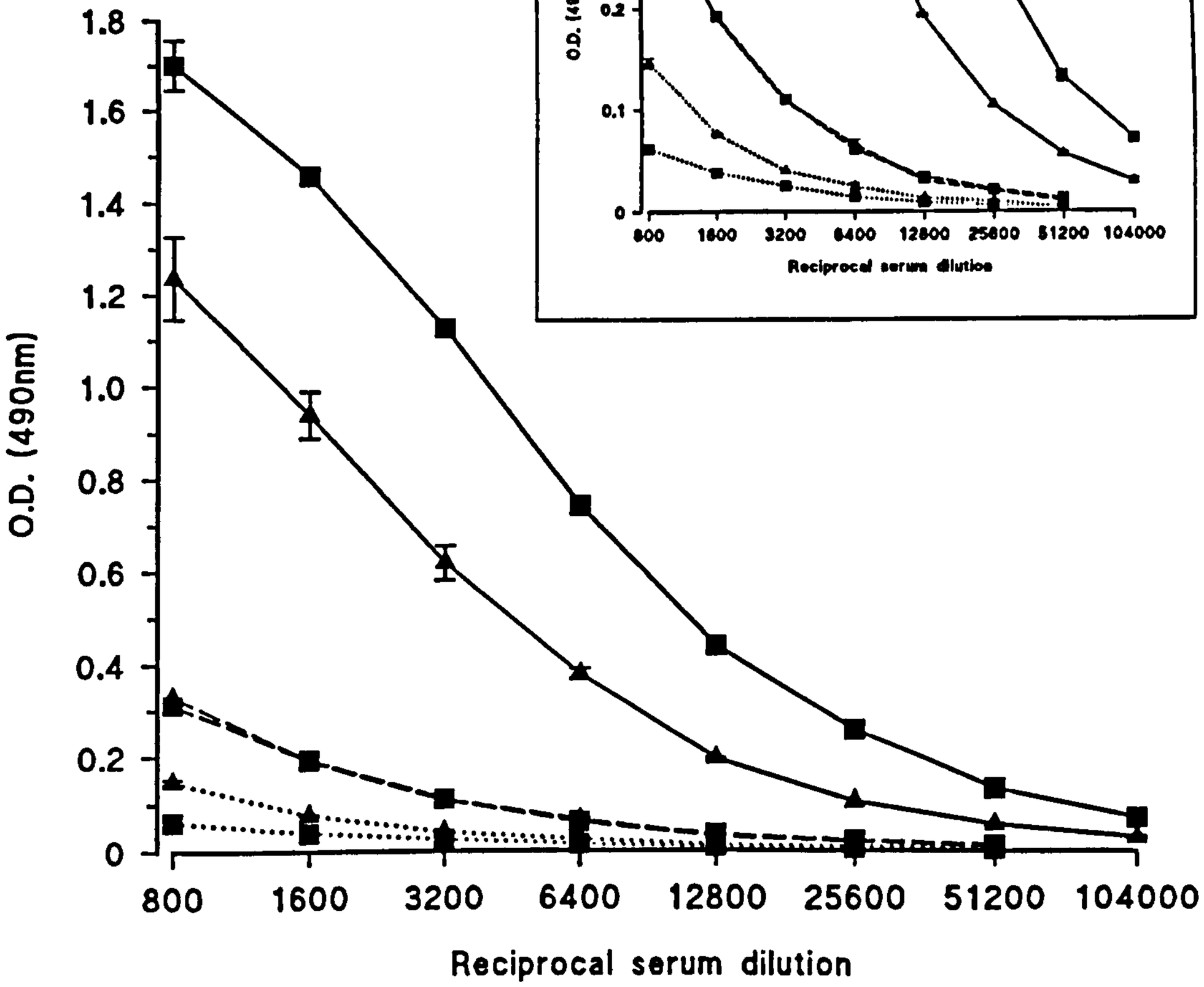


Fig. 4.4. Titration of O–3hS and D4–D8S in an ELISA against soluble preparations of cercariae (solid lines), lung–stage schistosomula (dashed lines) or adult worms (dotted lines).

The reactivities of the O–3h serum (■) and D4–D8 serum (▲) against each preparation is expressed as the mean O.D. ± S.E.. The inset figure shows an enlarged segment of the graph (see text for details).

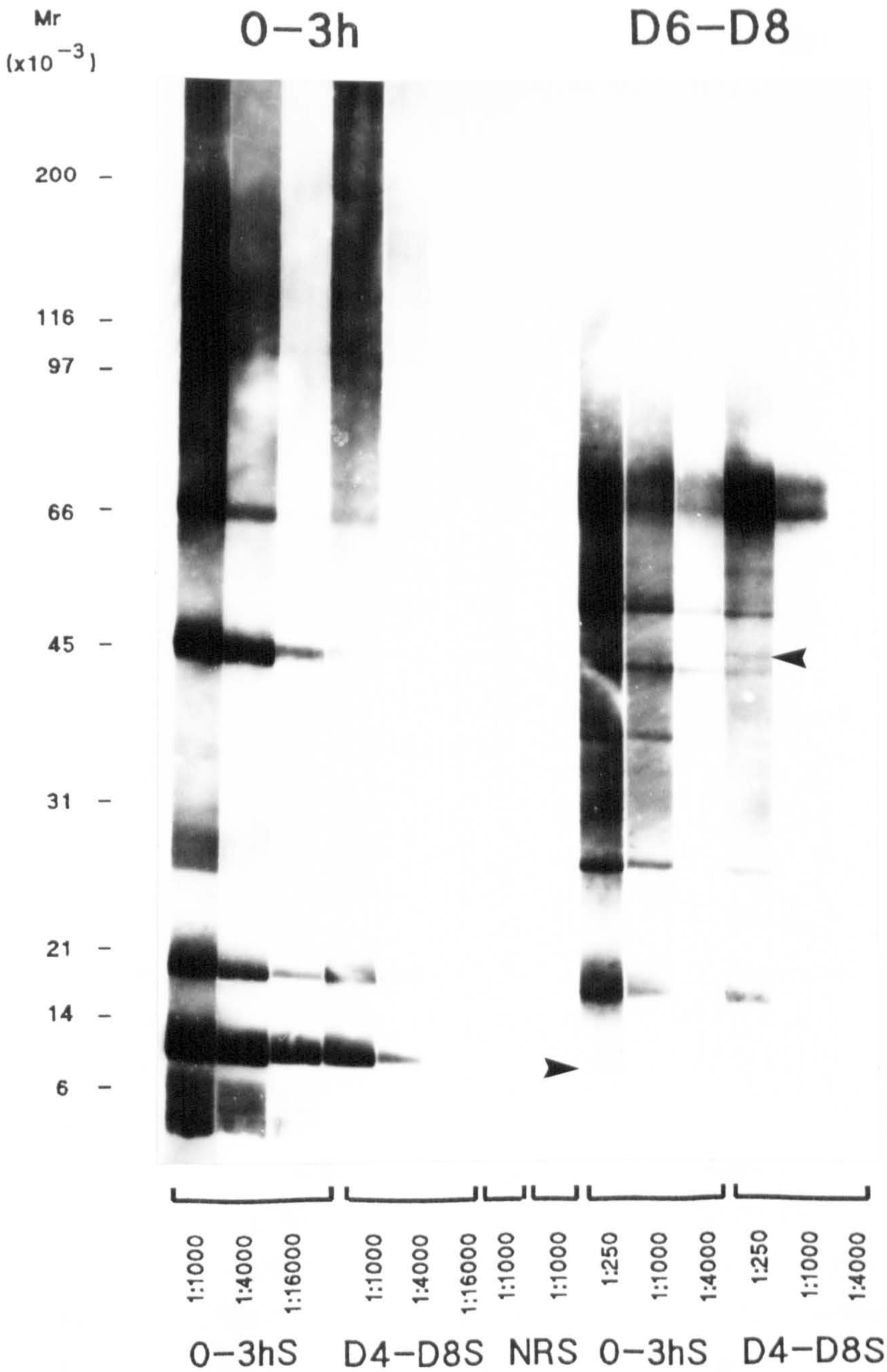


Fig. 4.5. Western blot analysis of 0–3h and D6–D8 released proteins. Antigen preparations (loaded at 4μg/cm per 3cm trough lane) were probed with 0–3hS, D4–D8S and NRS at the serum dilutions indicated underneath the brackets.

and SWAP to allow the specificity of the sera to be determined (Fig. 4.6). A broad array of proteins was identified by both 0–3hS and D4–D8S in SCAP, ranging in M_r from >200 kDa to <10 kDa. When the 0–3hS was used to probe blots of SCAP, antigens of 45, 20 and 12 kDa were particularly prominent, whilst only the latter two were recognised strongly by the D4–D8S. Both sera recognised a diverse range, but more restricted number of proteins in SLAP and SWAP. The D4–D8S detected several high M_r antigens which were unique to SLAP and SWAP, especially one at approximately 97 kDa. This protein was recognised by the 0–3hS in SLAP but not in SWAP. Additionally, the dominant protein at 12 kDa in SCAP which was identified by both sera, could not be detected in either SLAP or SWAP. Furthermore, the 45 kDa protein was only identified by the 0–3hS in SCAP but not in SLAP or SWAP, whilst the dominant 20 kDa protein which was detected by both sera could only be visualised at the highest serum concentration in SLAP and SWAP. No proteins were identified by antibodies from NRS in any of the soluble parasite preparations tested.

4.3.5 *Identification of parasite proteins with monospecific sera or monoclonal antibodies*

Monospecific sera or monoclonal antibodies were used in an attempt to identify constituents of soluble and released protein fractions derived from schistosomula. The dilutions used for each monospecific sera varied. Suitable dilutions were arrived at by using the suggested working concentration and testing it on Western blots of SWAP. If the staining was either too strong or too weak, the dilution was altered accordingly. Figure 4.7 shows the pattern of antigen/antibody staining using the defined sera, against SCAP, SLAP and SWAP loaded at identical antigen concentrations (10μg/cm of a 3cm trough lane). It was possible to detect superoxide dismutase (Sm16), both glutathione S–transferases (Sm26 and Sm28), haemoglobinase (Sm32), calreticulin (Sm58) and paramyosin (Sm97) in most antigen preparations, but not superoxide dismutase (20 kDa) or triose–phosphate isomerase. Of the proteins detected, superoxide dismutase (16 kDa), calreticulin and paramyosin appeared to constitute a higher percentage of the total soluble protein in later– compared to earlier–stage parasites. In contrast, both GSTs showed a similar staining intensity throughout the three life–cycle stages. Haemoglobinase could be detected in both SLAP and SWAP but not in SCAP.

The 0–3h and D6–D8 released proteins were analysed in a similar manner (Fig. 4.8). However, due to the scarcity of the latter, both preparations were used at a lower antigen concentration (2.5μg/cm of a 3cm trough lane). In such experiments, the serum concentrations were increased, which allowed the detection of Sm26 and Sm28 in both the 0–3h and D6–D8 released proteins. Additionally, SOD (16 kDa) and calreticulin could be detected in proteins released between day 6 and day 8.

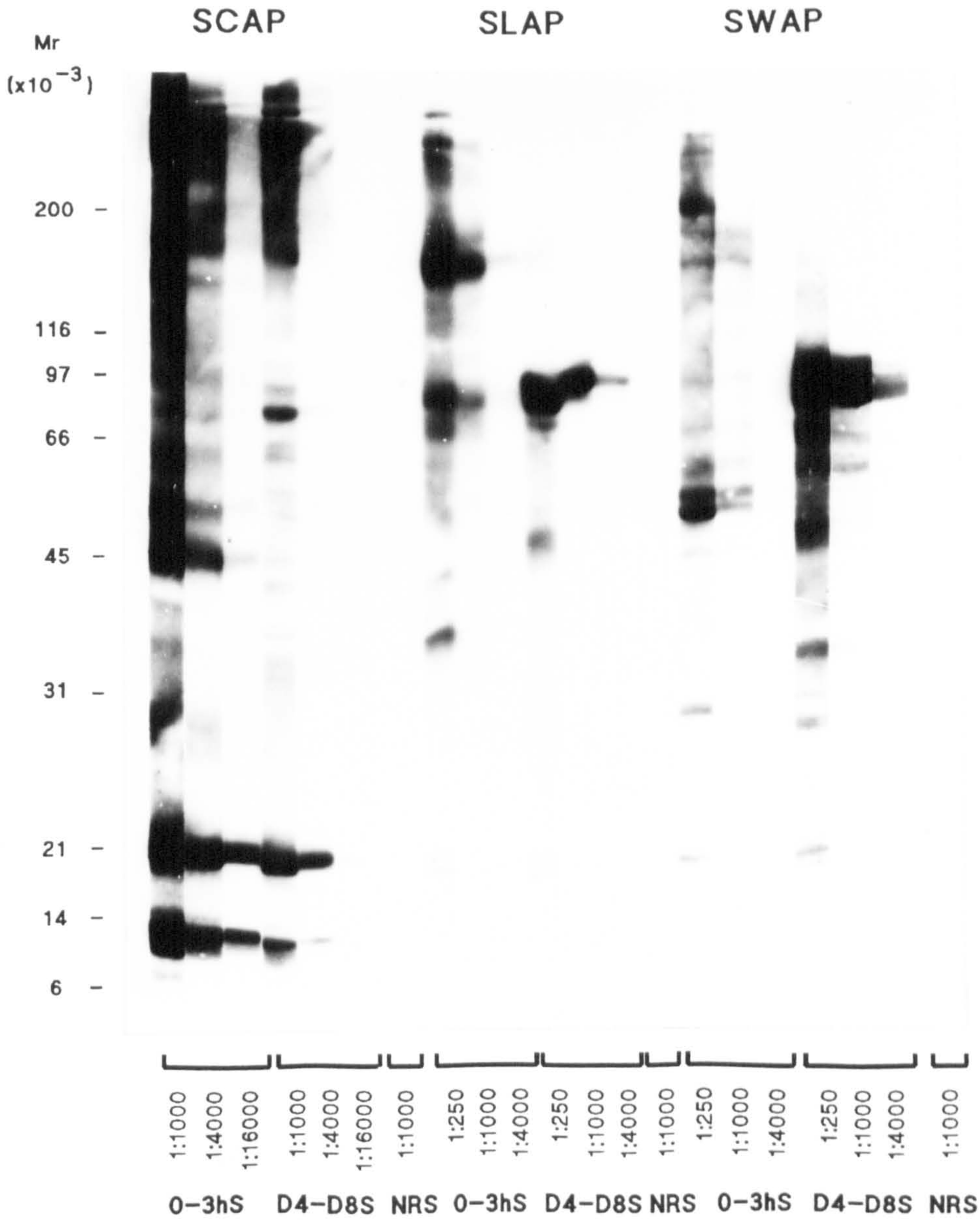


Fig. 4.6. Western blot analysis of soluble preparations of cercariae (SCAP), lung–stage larvae (SLAP) and adult worms (SWAP) of *Schistosoma mansoni.*

Antigen preparations (loaded at 10μg/cm per 3cm trough lane) were probed with 0–3hS, D4–D8S or NRS at the serum dilutions indicated underneath the brackets.

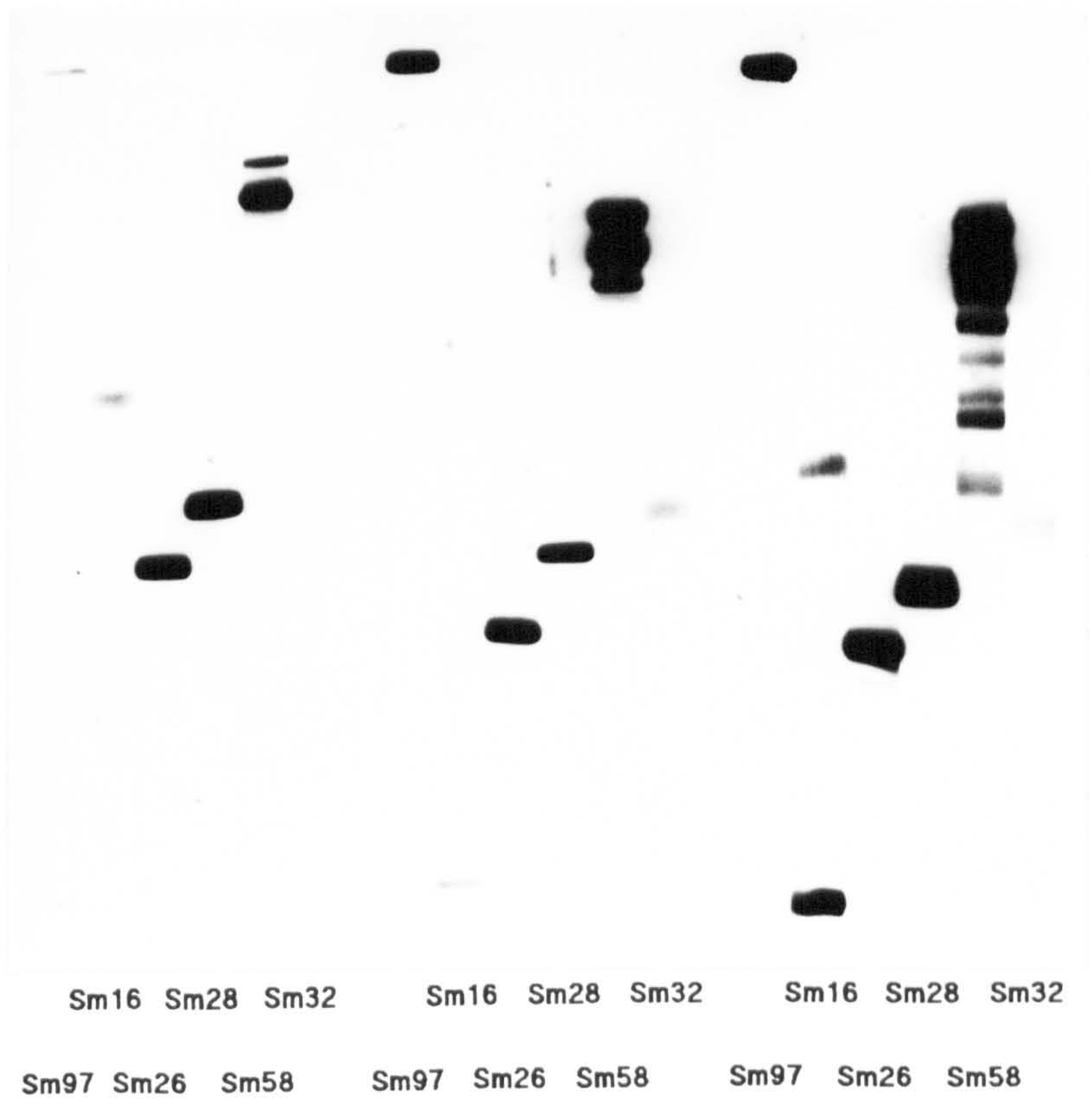


Fig. 4.7. Western blot analysis of soluble preparations of cercariae (SCAP), lung–stage larvae (SLAP) and adult worms (SWAP) of *Schistosoma mansoni.* Parasite proteins were detected with monospecific sera or monoclonal antibodies against paramyosin (Sm97), superoxide dismutase (Sm16), glutathione S–transferases (Sm26 and Sm28), calreticulin (Sm58) or haemoglobinase (Sm32). All antigens were loaded at 10μg/cm.

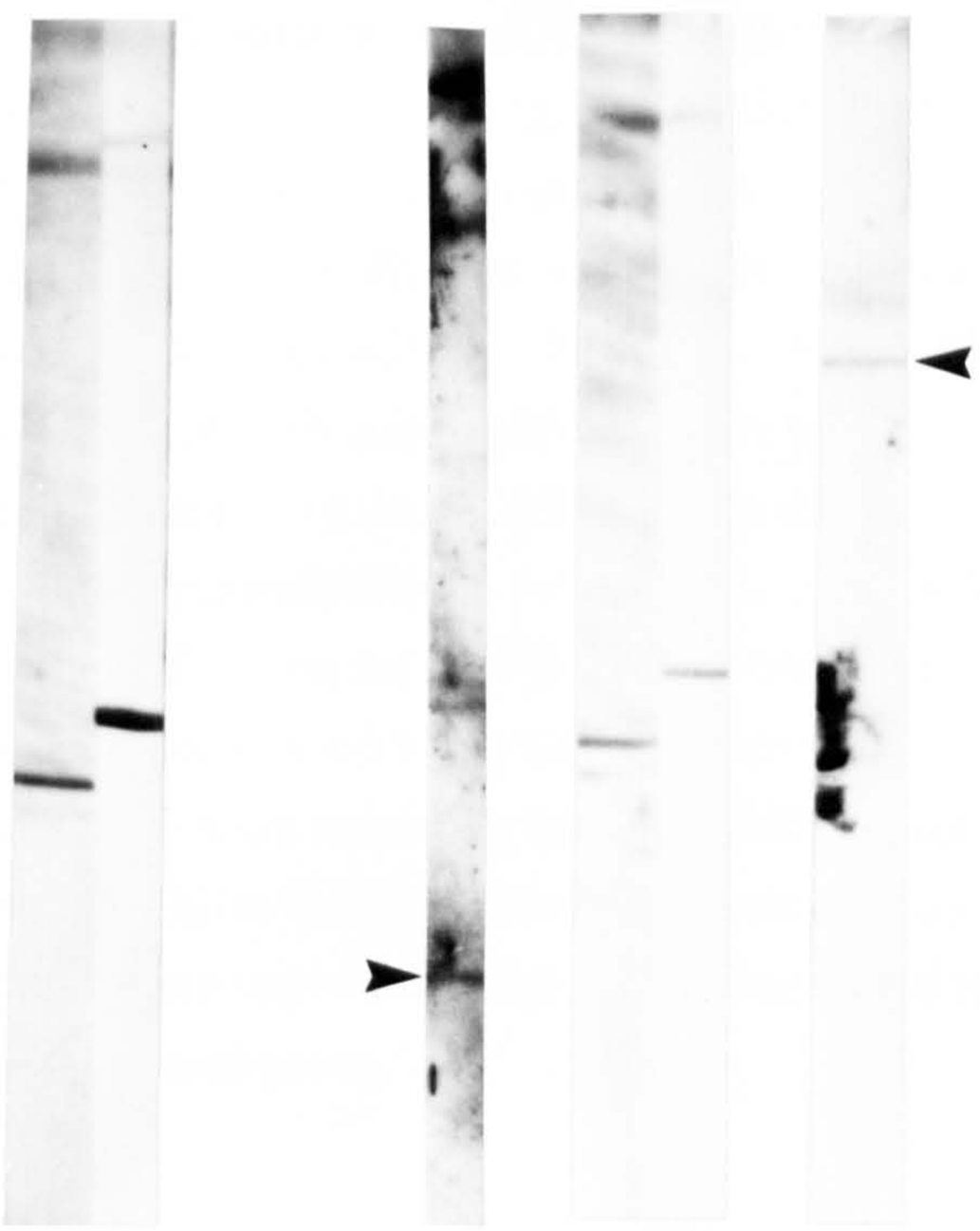


Fig. 4.8. Western blot analysis of 0–3h and D6–D8 released proteins of *Schistosoma mansoni.*

Parasite proteins were detected with monospecific sera or monoclonal antibodies against superoxide dismutase (Sm16), glutathione S–transferases (Sm26 and Sm28) and calreticulin (Sm58). All antigens were loaded at 10μg/cm per 3cm trough lane.

4.3.6 *Passive transfer of 0–3hS and D4–D8S to mice*

Both 0–3hS and D4–D8S were tested for their ability to provide passive protection to mice challenged with cercariae of *Schistosoma mansoni*. The sera were transferred on various days to assess the importance of skin– and lung–stage parasites as targets of immune elimination by each serum. The mean worm burdens, and calculated resistance values compared to the NRS control group, from 4 individual experiments are displayed in table 4.2. In experiment 1, mice receiving 0–3hS (bleed 4) intravenously (i.v.) on day 0 or D4–D8S (bleed 4) i.v. on days 4 and 7 showed reductions in worm burdens of 25% and 25.7% respectively. However, the results were not statistically significant. Likewise, experiment 2 in which mice received either NRS or D4–D8S (bleed 7) on days 4 and 7, a reduction in worm burden of 22% was achieved which was not statistically significant. In experiment 3 where mice received 0–3hS (bleed 7) on day 0, day 1 or days 4 and 7, statistically significant reductions in adult worm burdens of 29.3%, 47% and 50.5% respectively were achieved compared with the single control group which received NRS on day 0. However, when compared to the challenge control group, reductions of –0.05%, 24.7% and 29.6% were recorded, none of which attained statistical significance. In experiment 4, in which mice received D4–D8S on day 0, day 1 or days 4 and 7, none of the resistance values were statistically significant compared to the mean worm burden of mice from the NRS control group.

4.4 Discussion

4.4.1 *A comparative analysis of soluble and released proteins of Schistosoma mansoni, detected by Coomassie staining and autoradiography*

Soluble and released proteins derived from radio–labelled larvae of *Schistosoma mansoni* have been identified previously by autoradiography (chapter 2). At the time, limitations on parasite material, imposed by the size of the snail colony, prevented the detection of released proteins by conventional staining methods. Therefore, only proteins released from parasites radio–labelled with ^{35}S–methionine either prior to, or post–transformation could be detected. Expansion of the snail colony allowed more parasites to be obtained and therefore enough released proteins to be collected for detection by Coomassie staining, following electrophoretic separation, irrespective of time of synthesis. The gel displayed in figure 4.1 showed many similarities to the proteins detected by autoradiography, described in chapter 2.

As expected, there was a degree of antigenic conservation in the soluble protein fractions from cercariae to schistosomula through to adult worms; however there were

Experiment number	Treatment	Mean worm burden ± S.E.	Resistance cf. NRS control group
1 (Bleed 4)	Challenge controls	66.8 ± 4.5	—
	NRS on D0	69.6 ± 7.7	—
	0-3hS on D0	52.2 ± 5.6	25% (P>0.05)
	NRS on D4 + D7	69.5 ± 14.0	—
	D4-D8S on D4 + D7	51.6 ± 9.1	26% (P>0.05)
2 (Bleed 7)	Challenge controls	28.2 ± 1.1	—
	NRS on D4 + D7	35.4 ± 2.2	—
	D4-D8S on D4 + D7	27.6 ± 2.8	22% (P>0.05)
3 (Bleed 7)	Challenge controls	36.4 ± 4.9	—
	NRS on D0	51.7 ± 4.1	—
	0-3hS on D0	36.6 ± 2.7	29% (P<0.05)
	0-3hS on D1	27.4 ± 2.1	47% (P<0.01)
	0-3hS on D4 + D7	25.6 ± 3.7	51% (P<0.01)
4 (Bleed 9)	Challenge controls	39.2 ± 2.1	—
	NRS on D4 + D7	36.2 ± 3.7	—
	D4-D8S on D0	25.8 ± 3.4	29% (P>0.05)
	D4-D8S on D1	26.5 ± 2.3	27% (P>0.05)
	D4-D8S on D4 + D7	29.5 ± 3.7	19% (P>0.05)

Table 4.2. Protection data achieved with 0-3hS and D4-D8S upon passive transfer to mice on either day 0, 1, or days 4 and 7. The mean worm burden ± S.E. from each group is shown. All resistance values were calculated as detailed in section 4.2.7 using the mean worm burden of mice receiving NRS alone as the control group.

also both qualitative and quantitative differences. By separating sonicated extracts of cercariae, schistosomula and adult worms using 2-D SDS-PAGE, Miller, Rekosh & LoVerde (1989) calculated that adult worms shared 60% of the polypeptides of schistosomula. However, they found no differences between proteins of the cercarial extract and those of 1, 3, 5 and 6h schistosomula. Presumably, this was because only a low level of protein synthesis occurs within the first 6h post-transformation (Nagai *et al.* 1977; Yuckenburg, Poupin & Mansour, 1987; Blanton & Licate, 1992) and stage-specific proteins, associated with skin invasion and the transformation process, may still persist. It was also interesting to note, from the same study, that a protein of 20 kDa was identified in extracts of schistosomula but not adult worms. In this study, a protein (or proteins) at approximately 20 kDa could be detected in SCAP but not SWAP. The dominant protein present in SLAP was of 45 kDa. A protein of the same M_r was detected after autoradiography of labelled soluble parasite proteins (chapter 2). This macromolecule was barely detectable in the 24h soluble schistosomula preparation, but became one of the prominent proteins in 7 day old schistosomula.

The two most dominant released proteins detected by radio-labelling of parasites, were of M_r 61 and 20 kDa. Following Coomassie staining, the former was prominent in the 0-3h but not the D6-D8 released proteins. The latter could be detected in the 0-3h released proteins but did not stain strongly, whereas, in the D6-D8 released fraction, only a protein of marginally higher M_r could be seen. Thus, it appeared that two macromolecules of slightly differing M_r, occurring at approximately 20 kDa, were present in parasite-released proteins, an hypothesis which was also suggested in chapter two on the basis of the presence or absence of protease activity. The strong intensity of this protein (or proteins) on autoradiographs compared to Coomassie-stained gels suggests that it either has a relatively high proportion of methionine residues or is synthesised and released at a high rate during the labelling period. Likewise, a protein at 45 kDa, released by labelled schistosomula during *in vitro* culture, was prominent on autoradiographs yet was barely detectable by Coomassie staining. Conversely, the dominant moiety at 28 kDa in both 0-3h and D6-D8 released proteins on Coomassie-stained gels was not prominent on autoradiographs of similar fractions. This suggests that the protein either has a low methionine content or a low rate of synthesis and release during the labelling period. A comparison of the proteins released by schistosomula, and those present in soluble preparations of cercariae, schistosomula and adult worms, showed that there were both quantitative and qualitative differences in their composition. This provided further evidence that the released proteins were derived from viable parasites and not artefacts from dead or dying schistosomula.

4.4.2 *Analysis of serum reactivity by ELISA*

Since much more protein is released by schistosomula during the first 3h after transformation than at later stages (Harrop, unpublished observations), the higher titre of the 0–3hS compared with the D4–D8S may simply reflect the quantity of proteins used to inoculate the rabbits, rather than their antigenicity. A recommended vaccination dose for rabbits ranges from 50μg to 1000μg antigen per inoculation (Harlow & Lane, 1988). The rabbit immunised with 0–3h released proteins received at least 50μg for each dose whereas the recipient of D4–D8 released molecules was inoculated with approximately 10μg. This may provide one explanation for the higher titre of the 0–3hS. However, antibodies present in the D4–D8S bound more strongly to material released during the first 3h post–transformation than to D6–D8 released proteins. This suggests that moieties common to both 0–3h and D4–D8 released proteins are more immunogenic, in terms of antibody production, than those unique to the latter. A further potential explanation which would affect interpretation of the titre of the D4–D8S, was that an inaccurate estimate of the quantity of parasite protein used in ELISA experiments was made. The D6–D8 released proteins were obtained by culturing schistosomula in medium containing FCS for the first 6 days, washing thoroughly and then culturing in serum–free medium for the final 2 days. It was possible that the washing step was not sufficient to prevent some contamination of parasite–derived material with FCS, which would yield an incorrect estimate of the amount of parasite–derived protein. Hence, if less parasite–released proteins were used to coat wells of plates used in ELISA experiments than calculated, an underestimate of the antigen–specific antibody titre would be made. Subsequently, this potential problem has been overcome by applying the 6 day old schistosomula to a Percoll gradient, centrifuging and then washing thoroughly.

As expected, the 0–3hS bound more strongly to its homologous antigen preparation, than to a heterologous fraction. However, this serum was more reactive against D6–D8 released proteins in ELISA experiments than the D4–D8S. The higher titre of the 0–3hS compared to the D4–D8S may provide a simple explanation for this result. Ideally, the D4–D8S would be tested in ELISA experiments against D4–D8 released proteins. However, for the reason stated in section 4.2.1, this was not possible.

Both sera reacted much more strongly to SCAP than to SLAP or SWAP in ELISA experiments indicating that the released proteins to which they were raised constituted a greater proportion of the soluble preparation of cercariae than later–stage parasites. Since the biosynthetic labelling of parasites gave no quantitative feel for the proportion of released proteins derived from either cercariae or schistosomula, this was an interesting observation. Both sera reacted equally well to SLAP, and there was an indication that antibodies present in the D4–D8S bound more strongly, than those from 0–3hS to SWAP. The very low reactivity of both sera to SWAP could suggest that the 0–3h and

D4–D8 released proteins represent a much smaller percentage of SWAP compared to SCAP. Alternatively, it is possible that some of the released proteins may not be present at all in SWAP, indicative of stage–specific expression of such released molecules at pre–adult phases of the life–cycle.

4.4.3 *Detection of antigenic fractions of Schistosoma mansoni with antibodies present in 0–3hS and D4–D8S*

In all Western blots of released proteins, using either conventional diaminobenzidine staining (data not shown) or ECL, a high background reactivity was observed, even with the strong blocking step used to prevent non–specific binding of antibodies to the membrane. A possible explanation for this, was provided by observations of Coomassie–stained gels. Soluble preparations of *Schistosoma mansoni* yielded sharp bands with a low background staining, whereas many gels of released proteins showed more diffuse bands and a higher background throughout much of the lane. This background appeared to be amplified in Western blotting experiments, especially when 0–3h released proteins were used, where the staining extended into the stacking gel. Within the first 3h post–transformation, much of the cercarial glycocalyx is lost; indeed during *in vitro* culture of transforming radiolabelled cercariae, Caulfield *et al.* (1987) detected 70% of the labelled glycocalyx in the culture medium. The glycocalyx is highly antigenic and is composed predominantly of carbohydrate residues of high M_r (Caulfield *et al.* 1987). Glycoproteins and carbohydrates are known to separate less readily into discrete bands by SDS–PAGE than proteins. Complexes may form between carbohydrates and proteins which may prevent entry into the gel or poor resolution (Hames & Rickwood, 1990). Thus, the high background staining may be caused by antibodies binding to immunogenic carbohydrate residues or glycoproteins derived from the glycocalyx which, due to their poor resolution by SDS–PAGE, result in a broad smear.

Of the discrete proteins detected by antibodies present in the 0–3hS on blots of 0–3h released proteins, an antigen at approximately 20 kDa was dominant. A protein of identical M_r has been described previously (chapter 2) and was the most intense band detected following autoradiography, but not Coomassie staining, in both 0–3h and later–stage released proteins. The two most prominent proteins on Coomassie–stained gels of 0–3h released proteins, at 31 and 28 kDa, were not recognised strongly by the 0–3hS, suggesting that they were not very immunogenic in terms of antibody production. A comparative analysis of the antibody specificities of 0–3hS and D4–D8S was difficult to determine because of the differences in their titre. However, the D4–D8S recognised the 4 most dominant immunogens (M_r 70, 45, 20 and 12 kDa) detected by the 0–3hS. Both sera recognised additional antigens in the D6–D8 released proteins which, in most cases, were of identical M_r. A protein of 28 kDa was recognised more strongly by both sera in

this fraction than in 0–3h released proteins. Again, a protein of 20 kDa was identified by both sera.

Why the 0–3hS recognised proteins in the D6–D8, but not the 0–3h released proteins to which it was raised was puzzling. For example, proteins of approximate M_r 55 and 38 kDa were recognised by the 0–3hS in D6–D8, but not in 0–3h, released proteins. There are two possible explanations for this result. Firstly, variations in antigen preparations may have affected the result. Secondly, it was possible that antibodies present in the serum were binding to glycosylated regions of proteins shared by several molecules. Indeed, Zodda & Phillips (1982) described a monoclonal antibody reactive against two proteins of 160 and 130 kDa, whereas Kelly *et al.* (1986) showed it to bind to antigens of 200, 38, 20 and 17 kDa. This poly–specific recognition was due to a common carbohydrate epitope. Similarly, the 0–3hS recognised a few antigens (38 kDa and <14 kDa) in the D6–D8 released proteins that the D4–D8S did not. This could be explained by the fact that antibody responses vary significantly from rabbit to rabbit (Hammerl, Hartl, & Thalhamer, 1993). Therefore, one antigen may produce a good antibody response in one rabbit but a poor response in another. This potential problem will be remedied in future by raising antisera in more than one rabbit and/or by using different species e.g. mice and rats.

The range of antibody specificities of 0–3hS and D4–D8S against soluble preparations of *Schistosoma mansoni* was also complex. Both sera recognised a protein of 20 kDa very strongly in SCAP, but only weakly at the highest serum concentrations in SLAP or SWAP. Thus, this immunologically dominant protein appears to be highly enriched in cercariae compared with schistosomula or adult worms. A broad smear at approximately 97 kDa was present on blots of SLAP and SWAP probed with D4–D8S. A protein of this M_r has been characterised previously and found to be paramyosin (Lanar *et al.* 1986), which will be referred to later (section 4.4.4). In general, there was a moderate level of homology between the banding patterns seen on Coomassie stained gels of soluble and released proteins and those detected by antibodies, from either serum, on Western blots of the same samples. Thus, it would appear that even if a protein is well represented in a preparation, it does not guarantee that it will induce a good antibody response in an animal. However, the validity of using only one animal to raise each serum, and variations which may occur in the preparation of antigens, are factors which must be considered when drawing such conclusions.

4.4.4. *Identification of parasite proteins with monospecific sera or monoclonal antibodies*

In an attempt to identify some of the proteins present in the complex antigenic mixtures used in this study, monospecific sera or monoclonal antibodies were used to

probe Western blots. As would be expected, most of the target antigens of the antibodies were detected in soluble preparations of *Schistosoma mansoni*. However, neither SOD (20 kDa) nor TPI could be detected. There are several possible explanations for this failure. Either such proteins were absent, or only present in minute quantities, in the fractions tested or that the antibodies were denatured upon transport. The latter explanation was more likely for the monoclonal antibody directed against TPI, since this enzyme is universal in cells performing glycolysis and has previously been immunoprecipitated from all stages of the parasite life-cycle (Harn *et al.* 1985).

The proteins detectable in this study included the antioxidant enzymes SOD (16 kDa) and GST which are important in protecting parasites from potentially lethal molecules generated by the exposure of leukocytes to appropriate stimuli. The sensitivity of *Schistosoma mansoni* from different life-cycle stages to H_2O_2 and oxygen-free radicals has been described previously (Nare, Smith & Prichard, 1990). It was found that newly-transformed schistosomula, lung-stage and 2 week old larvae were much more susceptible to the presence of H_2O_2 or oxygen-free radicals than were 4 and 8 week old worms. Concurrently, the level of activity of some antioxidant enzymes was elevated in the older parasites. In particular, the GST activity increased dramatically from newly-transformed schistosomula through to 8 week old worms. SOD showed a small but not statistically significant increase in activity. Nare *et al.* (1990), postulated that the lower level of antioxidant activity in juvenile worms could account for their greater susceptibility to immune elimination compared to more mature parasites. Obviously, this hypothesis does not hold true in the rat model of schistosomiasis, in which the majority of parasites are eliminated 4-6 weeks after primary infection (Stirewalt *et al.* 1951; Smithers & Terry, 1964). In the work described in this chapter, no attempt was made to measure enzyme activity. However, in terms of the quantity of protein, SOD (16 kDa) showed a significant increase in the soluble sub-set of proteins from cercariae through to the adult worm stage. Haemoglobinase, the acid-thiol proteinase responsible for the breakdown of haemoglobin from which the parasite obtains much of its source of amino acids, could be detected in SLAP and SWAP but not SCAP. This supports the findings of Dresden, Payne & Basch (1982) who could not detect haemoglobinase activity in cercariae but could in 12 day old worms. Since schistosomula do not begin feeding avidly on red blood cells until after arrival in the liver, such stage-specific expression of the enzyme would seem logical. However, recently Gotz & Klinkert (1993) cast doubt on the identity of Sm32 as the 'haemoglobinase' of *Schistosoma mansoni*. Instead, evidence was presented which suggested that Sm31 was the enzyme responsible for the breakdown of haemoglobin. If this result proves to be correct, then the work described here would need to be repeated with a monospecific serum or monoclonal antibodies directed against Sm31.

Although it has been surmised that Sm28 was an excretory/secretory metabolite of

the parasite (Capron *et al.* 1987), as far as I am aware the identification of either Sm28, Sm26, SOD or calreticulin as proteins released from cultured schistosomula has not been described previously. However, two forms of SOD were found to be actively secreted by adult stages of the human parasite *Onchocerca volvulus* (Henkle *et al.* 1991). In view of the protective function of superoxide dismutases in inhibiting killing mechanisms based on the production of oxygen-free radicals, it was suggested by Cordeiro da Silva *et al.* (1992) that schistosome SODs may be involved in parasite defence against the host immune response. Thus, the observation that the 16 kDa SOD could be detected in proteins released by schistosomula between day 6 and day 8 lends weight to this suggestion. It was interesting to note that the promising vaccine candidate Sm28 was detected in proteins released both immediately after transformation and 6-8 days later. Whether the protein is lost during the turnover of the outer bilayer of the tegument, or is actively secreted, is not known. The detection of calreticulin in proteins released by schistosomula during *in vitro* culture was not expected. However, little is known about either the function or location of this molecule in *Schistosoma mansoni* (Khalife *et al.* 1993).

4.4.5 *Passive transfer of sera*

The protection provided to mice against a cercarial challenge by transfer of 0-3hS or D4-D8S was not very successful. The D4-D8S showed little difference in ability to confer protection when transferred on day 0, 1 or days 4 and 7. None of the results attained statistical significance at the 95% level when compared to the mean worm burdens of either NRS or challenge control groups. The most encouraging results were obtained with the 0-3hS when transferred on day 0, day 1 or days 4 and 7. These results were comparable to those obtained by Bickle *et al.* (1985) and Mangold & Dean (1992). Bickle *et al.* (1985) used serum from rabbits vaccinated up to 16 times with optimally-attenuated cercariae and achieved between 34% and 69% protection (mean=43%) when serum was transferred to mice on the day of challenge and 31% to 56% (mean=41%) when given 5-6 days post-challenge. Mangold & Dean (1992) used purified IgG, from serum raised in rabbits which had been vaccinated 3 times with 50 krad. irradiated cercariae, in passive transfer experiments. When transferred to mice on days 4 and 7 post-challenge, 43%-61% reductions in the adult worm burdens were achieved. It was found that the level of resistance induced was very much dependent on the quantity of IgG administered. Resistance was calculated compared to the worm burdens of mice receiving NRS alone. In an attempt to use fewer mice, only one NRS control group was included in the experiments described in this chapter. This group received NRS on day 0, therefore there were no NRS controls for 0-3hS transferred on day 1 or days 4 and 7, and as such the statistical significance of these results cannot be corroborated. Furthermore,

NRS increased the mean worm burden of this group compared to the challenge controls. It is possible that by increasing the volume of fluid in the vascular system, the diameter of capillaries could be widened slightly, allowing parasites to pass through sites, such as the pulmonary capillaries more easily. However, if this were true, an even greater difference in the worm burdens should have been detected between the group of mice which received 0.5ml NRS on two days and the challenge control group. None of the results from this experiment attained statistical significance when compared to the mean worm burden of the challenge control group. Therefore, it depended upon which control group was used for comparison, whether or not the results were statistically significant.

The mechanism by which the serum reduced the worm burden is not known. The elimination of pathogens by antibodies is usually brought about by binding to epitopes exposed on the surface of the foreign body. Several methods of elimination can occur thereafter, which include: receptor–mediated phagocytosis (an unlikely effector mechanism against a large multicellular pathogen), antibody–dependent complement activation, ADCC and possibly even inhibition of important enzymes which are expressed, at least transiently, on the parasite surface, for example GST or TPI. Mangold & Dean (1992) found that removal of the Fc fragment of IgG antibodies by pepsin treatment prior to passive transfer to mice, completely abrogated immunity. This suggested that an effector mechanism which involved host components bearing Fc receptors was required for immune elimination.

Thus, antibody–mediated elimination mechanisms usually act directly on the surface of the target. Since the sera used in these experiments were raised against parasite–released proteins it is perhaps not surprising that they did not confer high levels of protection. There are several possible explanations for the relative success of the 0–3hS compared to the D4–D8S at transferring resistance. Firstly, the 0–3hS had a much greater antibody titre than the D4–D8S. Since the injection of serum i.v. results in its widespread distribution throughout the tissues of the recipient, it is important that along the route of parasite migration there is a high antibody concentration. If the concentration were too low, an insufficient number of antibodies would bind to the target to facilitate the induction of effector mechanisms. Secondly, during the first 3h the glycocalyx is shed, and may be accompanied by underlying material derived from the tegument. Thus, the 0–3hS could contain more antibodies capable of binding to proteins exposed on the parasite surface, than the D4–D8S. Although the rate of turnover of the outer bilayer of the tegument is not known for schistosomula, in adult worms the process occurs relatively slowly, with a t½ of approximately 5 days (Saunders, Wilson & Coulson, 1987). If a similar turnover rate occurs in schistosomula, the D4–D8S is unlikely to contain many antibodies with specificities towards proteins of tegumental origin. However, Miller *et al.* (1989) showed that a monospecific serum against a 12.5 kDa antigen was capable of mediating a high degree of parasite killing in an *in vitro* assay using eosinophils or

complement despite the proteins internal location in flame cells of the parasite excretory system.

Although the most successful serum at conferring passive protection to mice was raised against early–stage released proteins, there was an indication that it acted most efficiently when administered on days 4 and 7 post–challenge. This corresponds to the time during which challenge schistosomula would be resident in the lungs. However, it must be borne in mind that this group of mice received twice as much serum as other experimental groups and therefore effectively had double the antibody titre. Mangold & Dean (1986; 1992) found that the most consistent level of resistance achieved with serum from multiply vaccinated mice was obtained when transferred at a time coincident with parasite migration to the lungs. Additionally, Gregoire *et al.* (1987) found that their protective IgM monoclonal antibody transferred significant resistance to mice when administered on the day of challenge or 8 days, but not 15 days, later. Ford *et al.* (1984) showed that serum from infected or vaccinated rats was equally as protective when transferred to recipient rats on the day of challenge or 5–7 days later. Thus, it has been shown that either sera or monoclonal antibodies can effectively cause a reduction in worm burden of mice or rats, when administered at a time coincident with challenge parasite residence in the lungs. Since the 0–3hS was effective against schistosomula older than 4 days, suggests that there is a degree of antigenic conservation between cercariae and lung–stage parasites, an hypothesis that was confirmed by the results described previously (chapter 2 and section 4.3.4) and by others (Atkinson & Atkinson, 1982; Miller *et al.* 1989).

In conclusion, sera raised against early (0–3h) and later–stage (D4–D8) schistosomula–released proteins recognised a complex pattern of antigens in both soluble and released protein fractions. It is likely that some of these molecules correspond to proteins detected by autoradiography (chapter 2) or by Coomassie staining (this chapter). Proteins of 45, 20 and 12 kDa were detected by antibodies on blots of various antigen fractions. In particular, the 20 kDa antigen(s), although not particularly prominent on Coomassie–stained gels, was dominant on autoradiographs (chapter 2) and could be detected by both sera on Western blots of all parasite protein fractions used in this study. As such, this protein (or proteins) warrants further investigation.

CHAPTER FIVE

Identification of recombinant proteins expressed from a larval *Schistosoma mansoni* cDNA library.

5.1 Introduction

In the previous chapters, the proteins released by schistosomula during *in vitro* culture (chapter 2), their ability to stimulate proliferation of lymph node cells recovered from vaccinated mice (chapter 3) and the properties of sera raised against sub-sets of these proteins (chapter 4) were described. Further work was impeded by the small quantities of schistosomula-released proteins which could be obtained from parasite cultures (approximately 15μg per culture). While this amount was sufficient to analyse T cell responsiveness (chapter 4) and possibly allow small-scale vaccination experiments with mice, it could not provide a source for purification of individual constituents.

Antibody screening of schistosome cDNA libraries, the majority of which have been generated from adult worm mRNA, has identified and facilitated further characterisation of several potential vaccine candidates including GST, TPI and paramyosin. It is important that candidate vaccine antigen(s) are available in recombinant form for several reasons. Firstly, the cDNA coding for the recombinant protein can be sequenced and its homology to any other cloned protein determined. The existence of homologies could provide an insight to the function of the molecule in the host. Secondly, by using high level expression systems, large amounts of the protein can be obtained. Since it is unlikely that sufficient quantities of the purified protein(s) could be obtained from parasites to vaccinate the population at risk from the disease, this is an important factor. Additionally, a plentiful supply of the protein enables more experiments to be undertaken to investigate the nature of the molecule. For example, the amino acid sequence of the protein can be determined. Furthermore, if the protein is suspected to be an enzyme, it can be used in functional assays e.g. GST.

From the amino acid sequence, it is possible to predict potential B and T cell epitopes. This can be achieved by using computer programmes or, by using synthetic peptides in assays of T cell proliferation or in ELISA experiments as described by Reynolds, Shoemaker & Harn (1992). Synthetic peptides containing the appropriate B and/or T cell epitopes, derived from candidate antigens have several advantages as potential vaccines, which were detailed by Taylor, Cordingley & Butterworth (1984). Such moieties would be cheap to manufacture, an important consideration for a vaccine destined for use in developing countries. Furthermore, quality control would be easy to maintain and the possibility of undesirable side-effects should be greatly reduced compared to use of complex antigenic mixtures. As described in section 1.9.4, Capron and colleagues have identified peptides, derived from GST, which when used to vaccinate animals, not only caused a reduction in the worm burden but also decreased female worm fecundity.

With these considerations in mind, it was decided that a cDNA library would be constructed and screened in order to identify, and subsequently obtain sufficient

quantities of, schistosomula-released proteins. It is not known whether the protective antigen(s) which mediate lung phase immunity are stage-specific or common to other life-cycle stages. Therefore, to maximise the chances that mRNA species coding for the relevant released proteins were present, a cDNA library in the lambda Zap II expression vector was constructed using mRNA extracted from 4 to 8 day old *in vitro*-cultured schistosomula. Larvae in this age range have a high rate of protein synthesis (chapter 2) and therefore should contain greater quantities of mRNA compared to earlier stages. The elevated level of synthesis during this period was due to the expression of a broad array of proteins rather than a restricted few. Thus, a diverse range of mRNA species should also be present. Furthermore, by using such parasite material, mRNA coding for cercaria-specific proteins, which are not important to immunity against schistosomiasis (section 1.7.5), would be absent. As described in the previous chapter, an antiserum raised against proteins released by *in vitro*-cultured schistosomula between day 4 and day 8 has been characterised. Antibodies present in the serum bound to parasite proteins on Western blots indicating that it would be suitable for screening a gene library, since recombinant antigens immobilised on nitrocellulose filters are also likely to be in a denatured form. This chapter describes initial results aimed at identifying parasite-released proteins expressed from a larval cDNA library using serum raised against such molecules.

5.2 Materials and Methods

5.2.1 *Collection of parasite material*

Schistosomula were obtained from snails with patent *S. mansoni* infections, as described previously in section 3.2.2, and cultured in M169 + FCS (1%). Cultures were terminated on either day 4, 5, 6, 7 or 8, the schistosomula pelleted by centrifugation and then stored at −80°C until required.

5.2.2 *Isolation of total RNA from schistosomula*

In order to prevent degradation by contaminating RNase activity, all procedures used in the isolation of RNA were carried out using Diethyl pyrocarbonate (DEPC) treated reagents, autoclaved glassware, sterile plasticware and RNase-free reagents.

RNA was recovered from schistosomula using the method described by Sambrook, Fritsch & Maniatis (1989). Briefly, frozen 4 to 8 day old schistosomula were resuspended and homogenised in guanidine isothiocyanate homogenisation buffer (4M guanidine isothiocyanate (GIBCO), 0.1M Tris-HCl pH 7.5, 1% 2-mercaptoethanol) using an Ystral shearer (Fryma Ltd., Hemel Hempstead, U.K.). Sodium lauryl sarcosinate (BDH) was

then added to a final concentration of 0.5% and the mixture centrifuged at 5000*g* for 10 min at room temperature. The resulting supernatant was layered onto a cushion of 5.7M caesium chloride (CsCl), 0.01M EDTA pH 7.5 and centrifuged in an SW41 swing-out rotor at 32,000 rpm for 24h, 20°C. The majority of the supernatant above the resulting RNA pellet was removed with a pasteur pipette and the bottom of the centrifuge tube, containing the RNA, detached using a red-hot razor blade. The RNA pellet was washed once in 70% ethanol and then left to dissolve in TES (10mM Tris-HCl pH 7.5, 1mM EDTA pH 8.0, 0.1% SDS) overnight at 4°C on a shaking platform. Subsequently, the RNA solution was extracted once with phenol:chloroform:isoamylalcohol (25:24:1) and twice with chloroform:isoamylalcohol (24:1), followed by overnight sodium acetate:ethanol (1/10th volume 3M sodium acetate pH 5.2 : 3 volumes ice cold 100% ethanol) precipitation at 0°C. This solution was then centrifuged for 20 min at 9000*g*, 4°C, the supernatant removed carefully and discarded. The pellet was washed in ice-cold 70% ethanol and centrifuged again for 15 min after which the supernatant was removed. The pellet was resuspended in 100μl DEPC-treated water and the RNA yield determined spectrophometrically at 260nm. The integrity and quality of the preparation was assessed by agarose gel electrophoresis (1% agarose in 1x TBE; from a 5x stock of 0.45M Tris, 0.44M boric acid, 0.01M EDTA). To an appropriate volume of the sample, 1μl loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 15% Ficoll 400) was added and the mixture made up to 8μl with 1x TBE. The gel tank was filled with 1x TBE and the sample loaded into wells of the gel. Electrophoresis took place at 100V. At the end of the run, gels were soaked in a solution of 0.005% ethidium bromide for approximately 10 min, washed briefly in distilled water and the RNA visualised on a U.V. transilluminator.

5.2.3 *Generation and screening of a larval cDNA library*

A larval cDNA library in the lambda Zap II expression vector was constructed by Clontech laboratories (Palo Alto, California, U.S.A) using total RNA isolated from *in vitro*-cultured schistosomula. The procedure used to manufacture the library included the purification of mRNA from total RNA, denaturation of any secondary structures by methylmercuric hydroxide treatment and, following second strand synthesis by reverse transcriptase, the quantity of DNA amplified by PCR. Low molecular weight (<400 base pairs) cDNAs were removed, whilst those remaining were cloned into a unique *Eco*RI site in the lambda Zap II vector using *Eco*RI linkers. The library was amplified once.

Clontech reported that the unamplified library had a complexity of 2×10^6 plaque forming units/ml (pfu/ml) and that 96% of the amplified library, which had a titre of approximately 10^{10} pfu/ml, contained cDNA inserts which ranged in size from 1.0 kb to 5.0 kb.

Prior to screening of the library, plating cells were produced by inoculating 10ml

Luria–Bertani media (LB media; 1% Tryptone, 0.5% yeast extract, 0.17M NaCl, pH 7.0) containing 0.2% maltose, with a bacterial colony (strain BB4; see appendix 2), and incubated overnight at 37°C on a shaking platform (250 rpm). The cells were pelleted by centrifugation at 3000*g* for 10 min and then resuspended in 4ml 10mM $MgSO_4$. The library was diluted in SM buffer (100mM NaCl, 50mM Tris pH7.5, 5mM $MgSO_4$, 0.01% gelatin) to yield approximately 70,000 pfu per 400 cm^2 plate. An appropriate volume of plating cells was added to the diluted bacteriophage and the mixture incubated in a water bath for 20 min at 37°C. Molten top agar (LB media containing 0.7% bacto agar) was added to the mixture which was then carefully poured on to the top of 1–2 day old LB Agar plates (LB media containing 1.5% bacto agar). Once solidified, the plates were incubated in an inverted position at 42°C.

An estimate of the proportion of bacteriophage which contained no cDNA insert was made by utilising the ability of lambda Zap, containing an intact β–galactosidase gene, to convert X–gal (5–bromo–4–chloro–3–indolyl–β–D–galactopyranoside), a lactose analogue, to a product that is coloured deep blue. Bacteriophage which contain a cDNA insert have a disrupted lac Z' gene and are unable to synthesise a functional β–galactosidase enzyme. Therefore, resultant plaques are clear. The addition of 40μl X–gal (20mg/ml in dimethylformamide) plus an inducer of the lac Z' gene, such as isopropyl β–D–thiogalactopyranoside (IPTG; 4μl from a 1M stock) to the LB agar on which the library was plated out, following incubation at 42°C for approximately 4h, allowed non–recombinant colonies to be distinguished from those containing cDNA inserts. Several of these 'blue' plaques were picked and purified to homogeneity.

The library was screened essentially as described by Huynh, Young & Davis (1986) with antiserum, raised against proteins released by schistosomula during *in vitro* culture between days 4 and 8 post–transformation (D4–D8S). Briefly, LB plates were seeded with bacteria infected with bacteriophage as described previously, and incubated at 42°C until small plaques were visible. Nitrocellulose filters (Millipore) which had been pre–soaked in 10mM IPTG and allowed to air dry, were carefully placed onto the plates and incubated at 37°C for a further 4h. Before removal of the filters, small pin pricks were made through to the agar support, to allow correct orientation at a later time. Filters were washed briefly in blot wash buffer (see section 4.2.6) and incubated in blocking buffer (blot wash buffer containing 5% NGS) overnight at 4°C. Dilution of the primary serum to 1:1000 yielded a good positive signal, using diaminobenzidine staining, against released proteins spotted onto nitrocellulose filters and a low background against bacterial lysates (data not shown), and was therefore used in all screening procedures. Filters were incubated for 2h in D4–D8S, diluted to 1:1000 in blocking buffer, washed for 3 x 20 min with blot wash buffer and then incubated for 2h with goat anti–rabbit peroxidase conjugate diluted to 1:1000 in blocking buffer. The filters were washed again and then incubated with peroxidase substrate solution (0.17M ammonium acetate, 2.9 mM citric

acid warmed to 40°C plus 0.01% diaminobenzidine and 0.0003% H_2O_2 added immediately prior to use). The reaction was terminated by washing filters in water. Any clone expressing a protein which was detected by the serum stained more strongly than remaining clones. Such 'positive' clones were isolated from the test plate and purified to homogeneity by several rounds of re–screening.

5.2.4 *Excision of plasmids from Lambda Zap*

From the bacteriophage DNA of isolated plaques, pBluescript containing the inserted cDNA was excised through the use of a helper phage. To perform this, 100μl plating cells (strain XL1–blue; see appendix 2), 200μl of the isolated clone (approximately 10^{10} pfu/ml) and 20μl R408 helper phage (10^6–10^7 pfu/ml) were added to an eppendorf tube and incubated for 15–20 min at 37°C. This mixture was added to 5ml LB media in a small conical flask, incubated at 37°C for 2–6h on a shaking platform and then heated at 70°C for 20 min to kill bacteria and inactivate the parent phage. Cell debris was removed by centrifugation (5000*g*) for 5 min and the supernatant, containing packaged filamentous phage particles (phagemid), decanted into sterile eppendorf tubes and stored at 4°C until required.

To obtain single colonies, 200μl of the phagemid and 100μl plating cells were added to an eppendorf tube, the mixture incubated at 37°C for 15 min and then the suspension streaked out on ampicillin–containing (50μg/ml) LB agar plates and incubated at 37°C overnight. XL1–blue host cells infected with phagemid gave ampicillin–resistant colonies, whereas cells infected with helper phage did not form colonies. The plates were stored at 4°C.

5.2.5 *Plasmid mini preparations*

Plasmid preparations were prepared essentially as described by Birnboim & Doly (1979). Single colonies, obtained from the procedure described above (section 5.2.4), were picked and used to inoculate 5ml LB medium containing ampicillin (50μg/ml) which was then incubated overnight at 37°C on a shaking platform. The culture was centrifuged (10000*g* for 10 min), the supernatant discarded and the pellet resuspended in 100μl solution I (50mM glucose, 25mM Tris–HCl pH 8.0, 10 mM EDTA). To this, 200μl ice cold, freshly prepared solution II (0.2M NaOH, 1% SDS) was added, the suspension mixed gently by inverting and then left on ice for 5 min. Subsequently, 150μl ice cold solution III (60% 5M potassium acetate, 11.5% glacial acetic acid) was added, the mixture vortexed, left on ice for 5 min and then centrifuged at 9000*g* for 5 min. The supernatant was transferred to a new tube and then extracted once with phenol and once with chloroform:isoamylalcohol. To the recovered aqueous phase, 250μl isopropanol was

added, left for 5 min at room temperature and then centrifuged at 9000*g* for 10 min. The supernatant was removed and the pellet washed carefully with 70% ethanol. Once dry, the pellet was resuspended in 50μl TE (10mM Tris–HCl pH 7.5, 1mM EDTA pH 8.0).

5.2.6 *Restriction digestion of plasmids and estimation of the cDNA insert size*

Since cDNA was cloned into the unique *Eco*RI site of lambda Zap, the insert could be excised by *Eco*RI digestion. Plasmid preparations were digested to completion with *Eco*RI (Pharmacia) in *Eco*RI buffer following incubation at 37°C as described by the manufacturer. Subsequently, 1μl RNase A (10mg/ml) was added and the mixture incubated for a further hour at 37°C. At the end of this period, the reaction was terminated by incubation of the mixture for 10 min at 65°C. The sample was stored at –20°C until required for analysis by gel electrophoresis. The insert size was calculated by running the restriction digest, on a 1% agarose gel alongside an *Eco*RI/*Hind*III digest of lambda DNA. The gel was stained with ethidium bromide and the DNA visualised on a U.V. transilluminator.

5.2.7 *DNA isolation*

Adult worms of *Schistosoma mansoni* were recovered by portal perfusion of infected mice (Smithers & Terry, 1965), and then DNA isolated from the parasites as described by Jeffs & Simpson (1993). Briefly, adult worms were ground to a powder in the presence of a small amount of liquid nitrogen in a mortar and pestle. The powder was thawed in an equal volume of extraction buffer (50mM Tris–HCl pH 8.0, 50mM EDTA pH 8.0, 100mM NaCl) at 37°C. Subsequently, an equal volume of extraction buffer containing 1% SDS and 100μg/ml proteinase K was added, the mixture incubated for approximately 2h at 37°C and then spun at 3000*g* for 3 min to remove any cell debris. The supernatant was transferred to a sterile eppendorf tube and extracted twice with phenol:chloroform:isoamylalcohol (25:24:1) and twice with chloroform:isoamylalcohol. The resulting aqueous layer was removed to a fresh tube, RNase A (100μg/ml) added, and the solution incubated for 1h at 37°C. The mixture was then extracted again as described above. To the aqueous layer, 1/10th volume 3M sodium acetate pH 5.2 and 2.5 volumes ice cold 100% ethanol was added, mixed gently, placed on ice for approximately 30 min to precipitate and then centrifuged at 9000*g* for 15 min at 4°C. The supernatant was removed carefully, discarded and the pellet washed with ice cold 70% ethanol, the centrifugation step repeated and the supernatant discarded. The pellet was gently resuspended in TE buffer, pH 8.0 and then stored at –20°C.

5.2.8 *Random primed labelling*

Owing to the initial difficulties in obtaining DNA labelled to a high specific activity, the DNA was cleaned using a GENECLEAN kit (BIO 101 Inc. La Jolla, California, U.S.A.), prior to the labelling step. To prevent possible steric inhibition during subsequent labelling, the DNA (50–100ng) was digested for 3h at 37°C with an *Eco*RI, *Pst*, *Hind*III mixture in one phor–all buffer (Pharmacia). Digestion was terminated by heating to 65°C for 10 min and was followed by a single extraction with phenol and then chloroform:isoamylalcohol. The DNA was precipitated by adding 4μl 3M sodium acetate and 80μl 100% ethanol and leaving on ice for 15 min followed by centrifugation for 15 min at 9000*g*, 4°C. The supernatant was removed and the pellet washed with ice cold 70% ethanol. Once dry, the pellet was resuspended in 10μl TE.

Prior to random primed labelling (described by Feinberg & Vogelstein, 1983), the DNA was denatured by heating for 10 min at 100°C and then rapidly cooled on ice. To an eppendorf tube, 25ng of denatured DNA, 3μl dNTP mixture (1μl dATP, dGTP and dTTP from 0.5mM stocks), 2μl 10x hexanucleotide mixture (Boehringer Mannheim) and 5μl [α^{32}P] dCTP (NEN DuPont, Stevenage, U.K.; 3000Ci/mmol) were added. This mixture was made up to 19μl with sterile water and 1μl (approximately 2U) Klenow enzyme (Boehringer Mannheim) then added. The mixture was incubated for 30 min at 37°C and the reaction terminated by adding 2μl 0.2M EDTA pH 8.0 and by heating to 65°C for 10 min. Unincorporated [α^{32}P] dCTP was removed by spun column chromatography. A Sephadex G50 column (Pharmacia) was equilibrated with STE (10mM Tris–HCl pH 8.0, 1mM EDTA pH 8.0, 100mM sodium chloride) and the labelling mixture applied to the top of the column which was then centrifuged at 1600*g* for 4 min. The solution which passed through the column was collected and the degree of labelling measured by liquid scintillation counting.

5.2.9 *Filter hybridisation*

Approximately equal quantities of previously denatured plasmid preparations derived from isolated clones, were spotted onto nitrocellulose filters in 1μl drops. As controls, denatured adult worm DNA served as the positive and a plasmid preparation isolated from a non–recombinant colony as the negative. In addition a filter which had been applied to LB agar containing the larval cDNA library plated at low density was used as another test. The DNA was baked onto the filters by incubation in a vacuum oven at 80°C for 30 min. The filters were placed into hybridisation bottles and 20ml pre–hybridisation buffer (4x STE, 10x Denhardt's solution (2% BSA, 2% Ficoll, 2% polyvinylpyrrolidone, 0.1% SDS, 0.1% sodium pyrophosphate)) containing denatured fragmented herring sperm DNA (100μg/ml) added and incubated for 4h at 68°C. This buffer was poured off

and replaced with pre-hybridisation buffer containing the labelled DNA (3.5 x 10^6 cpm/ml pre-hybridisation buffer) which had previously been denatured. The mixture was incubated overnight at 68°C. After the hybridisation step, the filters were washed to a high stringency with 2x SSC (from a 20x stock of 3M sodium chloride, 0.3M sodium citrate, pH 7.0), 0.1% SDS at room temperature for 5 min. This was followed by 2 x 20 min washes with 1x SSC, 0.1% SDS at 68°C and 2 x 20 min washes with 0.2x SSC, 0.1% SDS at 68°C. The filters were dried, wrapped in cellophane and exposed to Hyperfilm MP (Amersham) in the presence of intensifying screens and placed up for autoradiography at -70°C. After an exposure time of 24h, the film was developed as described in section 4.2.6.

5.3 Results

Collection of approximately 150,000 4 to 8 day old schistosomula, enabled the recovery of 10μg total RNA. This provided enough material for a cDNA library to be generated by Clontech laboratories. By plating out the library on the bacterial strain XL1-blue in the presence of IPTG and X-gal, approximately 15% of the resulting plaques were blue suggesting that 85% of bacteriophage contained a cDNA insert. This compares to 96% recombinants reported by Clontech. Twenty randomly-picked 'clear' plaques were purified to homogeneity and the bacteriophage DNA converted into the plasmid form. Restriction digests with *Eco*RI of the purified plasmids followed by electrophoretic separation on 1% agarose gels, enabled the average cDNA insert size of the library to be calculated. A value of 1.3 kb was obtained which compared to 1.6 kb reported by Clontech who used PCR amplification of the inserts from 6 randomly picked clones followed by agarose gel electrophoresis, to determine the average insert size.

After screening approximately 1 x 10^6 plaques with the D4-D8S, only 5 positive clones were identified on separate plates. Figure 5.1 shows these 5, alongside a non-recombinant clone, after they had been plaque purified. The positive clones were not recognised by NRS nor by sera raised against proteins released by newly-transformed schistosomula *in vitro* (0-3hS) or those released by adult worms *in vivo* (data not shown). Monospecific sera and monoclonal antibodies to proteins, which have been cloned in other laboratories, were obtained (see section 4.2.3). These were used to ascertain whether the positive clones identified in this study expressed proteins which were recognised by any of the antibodies. Two clones (18 and 39) expressed proteins which were recognised by an anti-Sm97 monoclonal antibody (Fig. 5.2). None of the positive clones expressed proteins which were detected by antibodies directed against calreticulin, 26 or 28 kDa glutathione S-transferases, triose phosphate-isomerase or superoxide dismutase (data not shown).

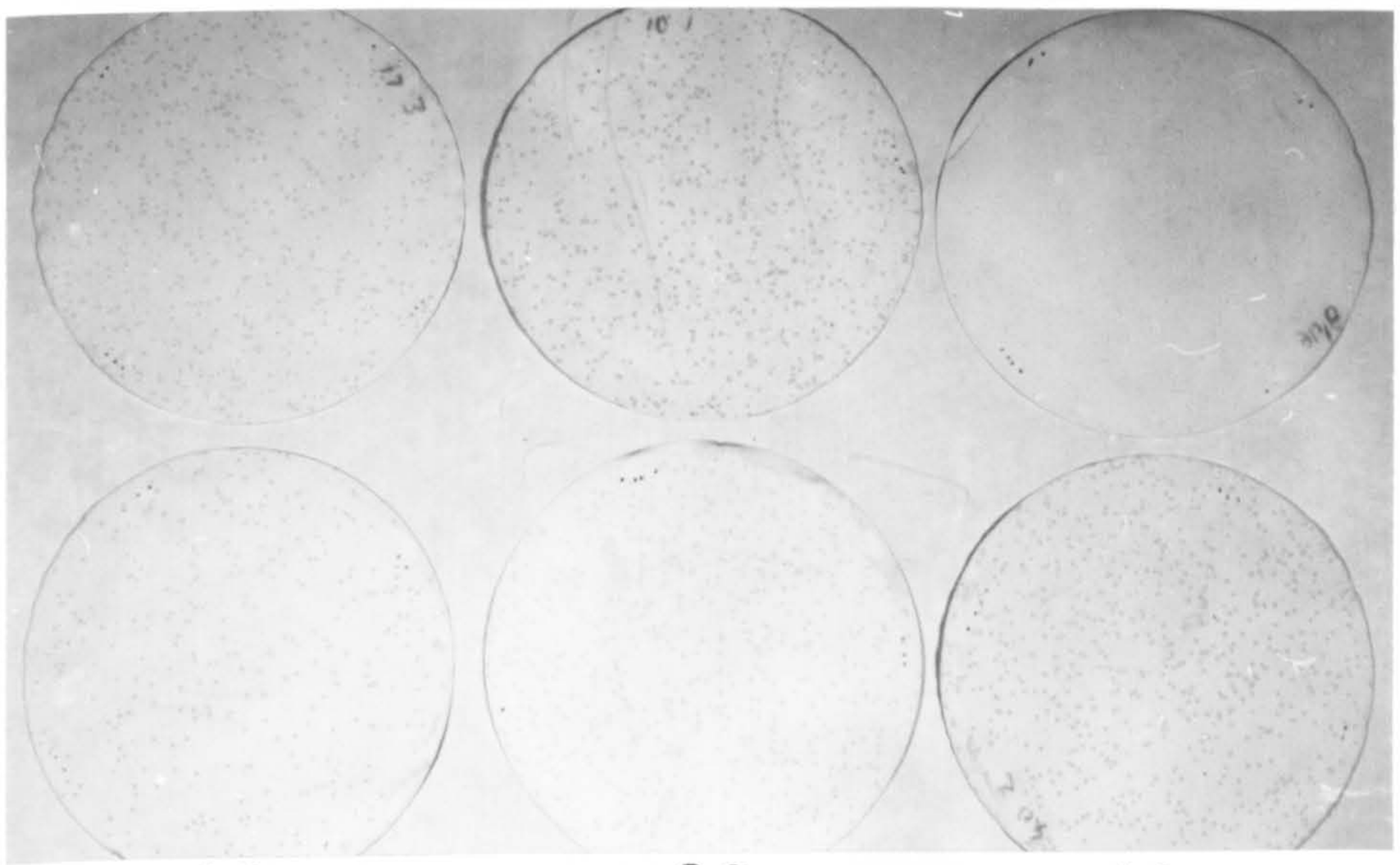


Fig. 5.1. Screening of filters of isolated positive clones (10, 17, 18, 39 and 40) and a non-recombinant clone (N.R.) with serum raised against parasite-released proteins (D4-D8S).

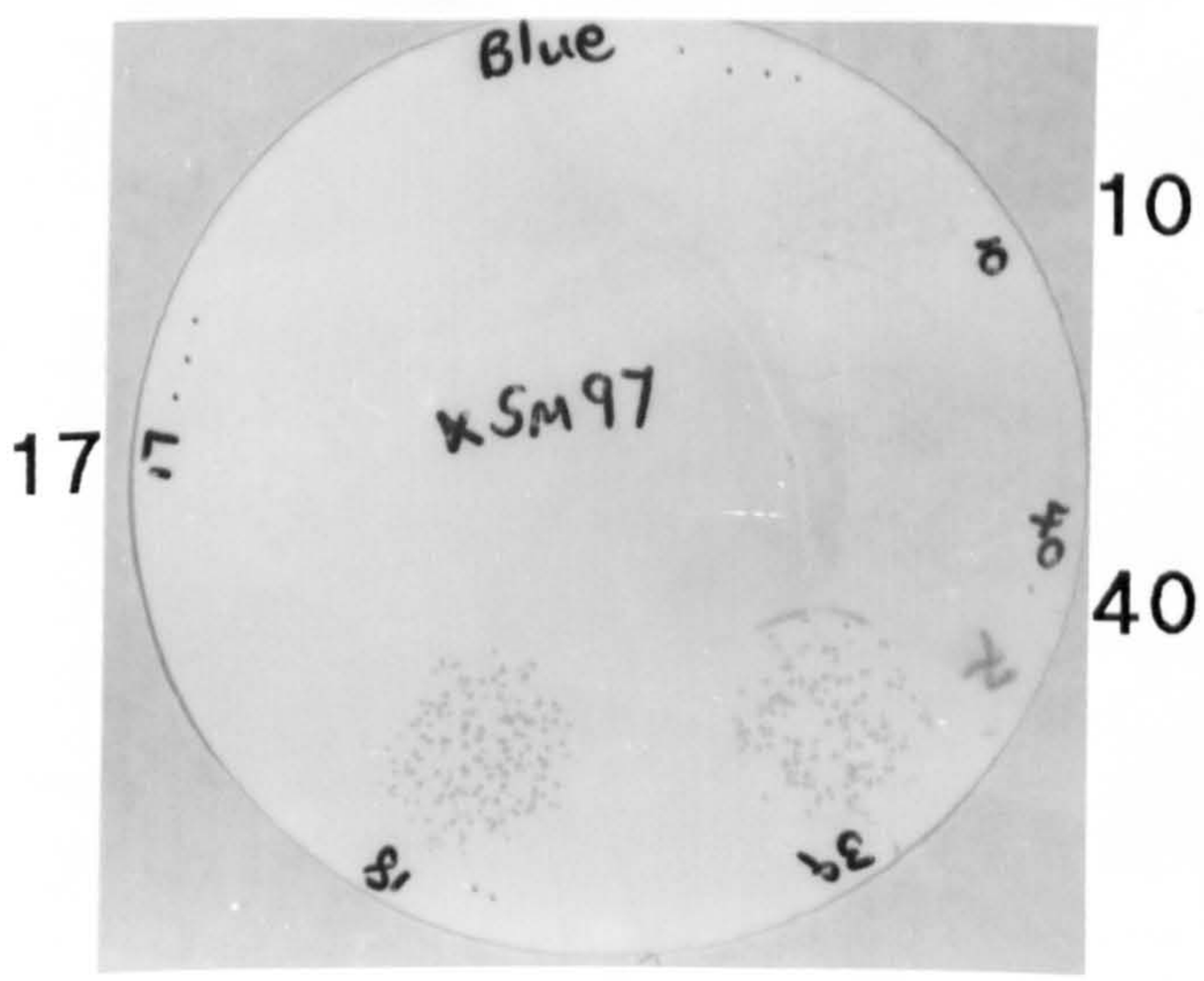


Fig. 5.2. Screening of positive clones with a monoclonal antibody against paramyosin (Sm97). Numbers represent the corresponding positive clone whereas N.R. is an isolated non-recombinant clone.

DNA from all of the positive clones was converted to the plasmid form and, after restriction digestion, their cDNA insert size determined on agarose gels (Fig. 5.3). The insert sizes were approximately 1.3 kb (clone 10), 1.2 kb (clone 17), 1.3 kb (clone 18), 0.75 kb (clone 39) and 1.2 kb (clone 40). Clones 10 and 18 gave two bands on gels suggesting that they had an internal *Eco*RI cleavage site in their cDNA insert.

The low frequency of identification of positive clones raised doubts about the quality the schistosome library. In order to ensure that the library was of schistosome origin and did not contain a high proportion of contaminating DNA, schistosome DNA was labelled with ^{32}P and used to probe filter-bound DNA derived from the lambda Zap library. High stringency washes were used to minimise non-specific binding of the complex labelled genomic DNA. A photograph of the resulting autoradiograph is shown in figure 5.4. Binding of the DNA to the bluescript vector containing no insert (negative control) was hardly visible whilst binding to the immobilised genomic DNA (positive control) was very strong. All of the plasmid preparations derived from positive clones (10, 17, 18, 39 and 40) or randomly-picked plaques (A-F) gave a good signal although the intensity varied from clone to clone. The nitrocellulose filter containing plaques derived from the library also bound the labelled DNA (data not shown) although not as strongly as the plasmid preparations as judged by the intensity of the signal.

5.4 Discussion

Both the number of recombinant bacteriophage and the average insert size of the library compared favourably to the results reported by Clontech.

Since the serum used for screening (D4-D8S) was capable of recognising parasite antigens by Western blotting (chapter 4), and the library appeared to be of good quality, the relatively low frequency of identification of positive clones was disappointing. The use of hybridisation to check that the bacteriophage contained cDNA inserts of *Schistosoma mansoni* origin confirmed that they did. Additional supportive evidence for the origin of the library is provided by the recognition of two of the positive clones by an anti-Sm97 (paramyosin) monoclonal antibody. Despite the fact that this protein is universal in invertebrates and is probably well conserved, the result is unlikely to be coincidental.

There are several possible explanations for the low yield of positive clones. Firstly, the mRNA which encodes released proteins may represent only a tiny proportion of other mRNA species and would thus be relatively rare in the library. Secondly, the antibodies may recognise predominantly glycosylated regions of proteins, which would not be available for binding during the screening procedure. Thirdly, it was shown (section 4.3.3) that the D4-D8S had a higher affinity for proteins present in soluble preparations

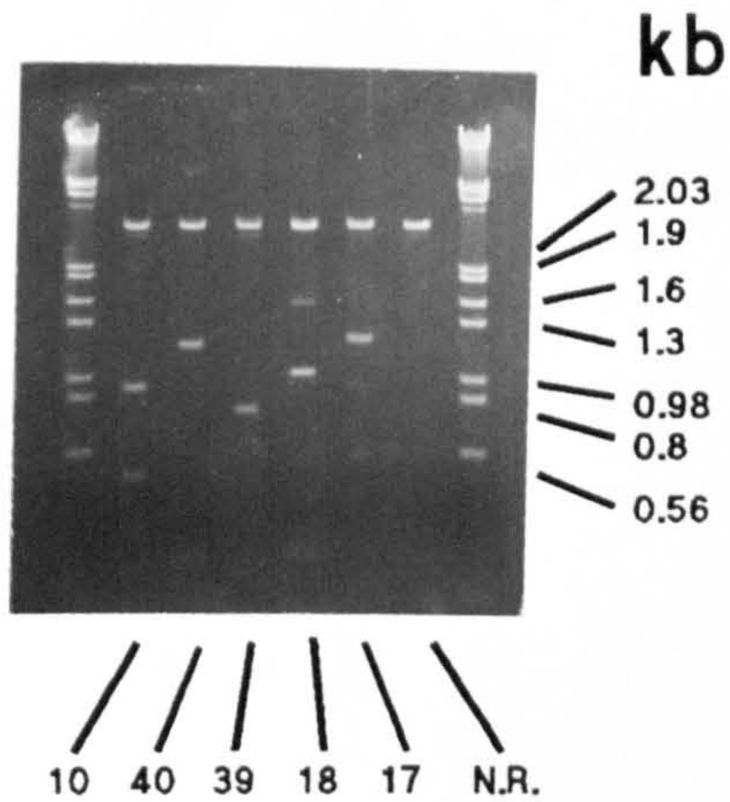


Fig. 5.3. Analysis of EcoRI restriction digests of purified plasmid preparations of non-recombinant and 'positive' clones (10, 17, 18, 39 and 40). Samples were separated on 1% agarose gels alongside an EcoRI/HindIII lambda DNA digest, the fragment sizes of which are indicated at the side of the photograph, in kilobases.

Fig. 5.4. Filter hybridisation of labelled total genomic DNA to either homologous genomic DNA (+) or purified plasmids of non−recombinant phage (−), positive clones (10, 17, 18, 39 and 40) and randomly picked clones (A, B, C, D, E and F).

of cercariae rather than schistosomula. This indicated that the immunogenic proteins which were released at the lung-stage of development were highly enriched in soluble preparations of cercariae compared to schistosomula. As such, a cDNA library generated from cercarial mRNA may be more appropriate for the detection of recombinants using the D4-D8S. A further consideration to bear in mind is the validity of raising serum in a single animal; such serum may not be representative since it might only contain antibodies directed against a limited number of potential antigens present in the released proteins. In the near future it is hoped that anti-serum can be raised in mice, rats and rabbits to schistosomula-released proteins.

Of the 5 positive clones identified, none expressed proteins which were recognised by serum raised against 0-3h released proteins or those released by adult worms. Therefore, it is possible that the clones code for molecules which are only released during a short period of parasite development. It was of interest to note that of the five positive clones identified, two were recognised by an anti-paramyosin monoclonal antibody. One of the clones recognised by this antibody appeared to have an internal *Eco*RI site. Although the D4-D8S detected a protein of 97 kDa in soluble preparations of schistosomula and adult worms (section 4.3.4), it did not do so in D6-D8 released protein preparations. Additionally, the anti-Sm97 monoclonal antibody did not react with any of the D6-D8 released proteins. Unless paramyosin was only released between days 4 and 6, it is difficult to explain why the D4-D8S contained antibodies directed against paramyosin. Since there is a large body of information available about this protein, it is unlikely that characterisation of the two 'paramyosin' clones will be taken much further. Although the anti-Sm97 monoclonal antibody did not bind to proteins expressed by the 3 remaining positive clones, the possibility remains that they may still code for paramyosin. In order to resolve this question, antibodies bound to proteins expressed by the 3 clones could be eluted from nitrocellulose filters and used to probe a blot of purified paramyosin.

Future work will be described in the concluding discussion.

CHAPTER SIX

Concluding discussion

Throughout the course of studying proteins released by schistosomula during *in vitro* culture, significant differences in the surface morphology of normal and attenuated larvae were consistently observed. After 8 days in culture, schistosomula derived from normal cercariae exhibited the characteristics of parasites recovered from the lungs (Crabtree & Wilson, 1986a). Although 20 krad.–irradiated schistosomula had lost their mid–body spines, an indication that development had proceeded normally, they exhibited random muscular constrictions along the length of their body. Additionally, the alternating extensions and contractions of the body, which are characteristic of lung–stage schistosomula, occurred more slowly and the maximum extension was less in irradiated, compared to normal parasites. It was suggested that these abnormalities could account for the persistence of attenuated larvae in the skin–draining lymph nodes and lungs, two events which are essential for the induction of protective immunity in this vaccine model.

Despite the large body of information which is available on many aspects of the irradiated cercaria vaccine model, there is still little known about the antigens which mediate immunity. In a recent review Sher *et al.* (1989) stated that four basic strategies have been employed in the identification of potential vaccine candidates against schistosomiasis. The first, and most widely used approach, has been the generation of monoclonal antibodies, followed by the identification and synthesis of the relevant target epitopes (the identification of TPI represents an example of this approach). The second, is to identify antigens seen uniquely by naturally resistant hosts. The third approach is to induce resistance in a host by immunisation with a crude antigen preparation, and then analyse the immune response induced subsequently. This procedure led to the identification of paramyosin. The final approach is to identify an "Achilles heel" in the life–cycle of the parasite, i.e. a component which is functionally essential to parasite survival (the cercarial proteases probably fall into this category). None of these approaches was followed directly to identify the antigens which mediate lung–phase immunity in the irradiated cercaria vaccine model. Instead, based on the detailed information available on the induction and effector phases of immunity in this model, it was hypothesised that proteins released from schistosomula would provide a source of potential vaccine candidates.

Biosynthetic labelling with ^{35}S–methionine either prior to, or post–cercarial transformation, facilitated analysis of the kinetics of protein synthesis and release by schistosomula during *in vitro* culture. Over an 11 day period, approximately four different phases of protein synthesis could be identified, which may relate to the developmental processes undergone by schistosomula *in vivo*. Furthermore, the proteins released by larvae during development to the lung–stage, were characterised. Schistosomula labelled prior to transformation released two dominant proteins of M_r 61 and 20 kDa whilst parasites labelled post–transformation released three dominant molecules of 61, 45 and 20 kDa.

To assign a function to any of these molecules would, at the moment, be speculation. Nevertheless, there are several potential sources from which such proteins could be derived. The acetabular glands are likely to make a large contribution to material released within the first few hours post-transformation. However, such structures have completely disappeared before the lung-stage is reached. Conversely, the head gland does persist to this stage; its secretions have been postulated to facilitate parasite entry into the blood vessels of the dermis (Crabtree & Wilson, 1985). The tegument of the lung schistosomulum also contains a novel secretory vesicle, the homogeneous body (Crabtree & Wilson, 1986a), the products of which may act as a lubricant to aid progress along a capillary. Additionally, the embryonic gut becomes active at the lung-stage, and the nephridial system enlarges; both could act as sources of secretions to trigger the pulmonary effector response. Whilst the possibility remains that *in vivo*, schistosomula release qualitatively or quantitatively different proteins than those cultured *in vitro*, the high level of parasite viability maintained during *in vitro* culture and their ability to mature when surgically transferred to naive mice suggests that the molecules detected are, at worst, a good representation of those released *in vivo*. Although much of the work in this thesis was based on the irradiated cercaria vaccine model, the analysis of parasite-released proteins is not constrained by this model. If the proteins released by parasites cultured *in vitro* or *in vivo* are indeed identical, then the characterisation of such molecules is equally as relevant to human schistosomiasis as it is to the mouse model.

Parasite-released proteins are unlikely to be a good source of vaccine candidates for immune elimination mechanisms such as ADCC, which are dependent upon opsonisation by antibodies. However, they may be a good source of T cell immunogens. James (1987) suggested that "a vaccine based on T cell-mediated immunity offers certain theoretical advantages over a vaccine dependent on antibody. For example, virtually any antigen, whether surface, internal, or secreted, against which the host is immunised in a way that specifically sensitizes the correct T cell subset and to which the host is reexposed upon challenge infection could, theoretically, be of protective value in inducing lymphokine production." "...Since cell-mediated immunity is antigen specific in the induction phase, but can operate non-specifically in the effector phase, the cytotoxic reactivity of lymphokine activated macrophage effector cells need not be directed toward specific surface epitopes on the target. This offers the distinct advantage of overcoming the parasite's immune evasion strategies of antigen masking or shedding and antibody cleavage. Finally, the epitopes triggering T cell responses are more likely to be protein than carbohydrate in nature and less likely to depend on conformation possibilities, that would expédite vaccine production." However, despite such statements, the relevance of T cell-mediated effector mechanisms in human schistosomiasis is not clear.

Antibody-mediated killing of *Schistosoma mansoni* has been well documented in experimental systems (Capron & Capron, 1986; Capron *et al.* 1987; Sher *et al.* 1989) and

also in sera of patients with schistosomiasis (Butterworth *et al.* 1985; Butterworth *et al.* 1987; Dessein *et al.* 1988). Although cellular immune responses have been studied in experimental systems (Sher *et al.* 1982; Constant *et al.* 1990; Pemberton *et al.* 1991; Ratcliffe & Wilson, 1991; Constant & Wilson, 1992; Richter, Reynolds & Harn, 1993) and infected subjects (Colley *et al.* 1977; Vieira *et al.* 1987), very little is known about the relationship between the specific cellular immune response to *Schistosoma mansoni* antigens and the degree of infection. Recent studies on infected patients in Brazil have addressed these issues. Gazzinelli *et al.* (1992) identified a group of individuals who lived in endemic areas and had continued contact with 'contaminated' water but were repeatedly stool negative; these people were termed endemic normals. It was found that peripheral blood mononuclear cells (PBMC) taken from this group of people were highly responsive to schistosome antigen preparations, particularly soluble egg antigens. Furthermore, upon stimulation, the cells produced significantly higher levels of IFNg than equivalent cells from infected patients. More recently, Ribeiro de Jesus *et al.* (1993) identified 50 subjects, living in an endemic area of schistosomiasis, who had the same degree of exposure to cercariae. Despite this, the degree of infection, assessed by eggs per gram of faeces (epg faeces) was variable (0–5604). It was found that the proliferative response of PBMC to schistosomula and adult worm antigens was higher in individuals with a low degree of infection (<400 epg faeces) than the response by heavily infected individuals (>400 epg faeces; $P<0.001$). An inverse correlation between the proliferative response to *Schistosoma mansoni* antigens and the degree of re-infection was also observed ($P=0.02$). It was suggested that cellular immune mechanisms could be involved in human resistance to *Schistosoma mansoni* infection.

The work described in this thesis used an assay of lymph node cell blastogenesis as an indicator of the potency of various antigen preparations as T cell immunogens. The results from such experiments demonstrated that schistosomula-released proteins were capable of inducing a strong proliferative response by lymph node cells recovered from protectively vaccinated mice. This proves that cells from such mice have been sensitised to some, or all, of these macromolecules previously, and that released proteins provide a good source of T cell immunogens. Additionally, live schistosomula induced a strong proliferative response. Fractionation of the parasite-released proteins into samples of limited antigenic composition, prior to use in blastogenesis assays would provide an indication as to which specific molecule(s) may serve as candidate vaccine antigens. Unfortunately, due to the limited quantity of material available, this was not a feasible option. Therefore, further characterisation of the proteins is required before any particular antigen can be regarded as a vaccine candidate. However, the released protein or proteins at approximately 20 kDa perhaps deserve special attention. As suggested in chapters two and four, it appears that more than one molecule is represented at this molecular weight. Such molecules were dominant on autoradiographs of released proteins derived from

schistosomula labelled either prior to, or post-transformation. In addition, antibodies from serum raised against released macromolecules bound to protein(s) at 20 kDa very strongly. Although neither of these facts guarantee that this protein would be a potent stimulator of Th cells, if such a result was obtained, it would be of great interest. A protein of similar molecular weight has been identified by Dunne *et al.* (1992). In this study, a group of infected Kenyan patients were treated with chemotherapeutic drugs and then the intensities of reinfection followed over the next two years. It was found that their IgE responses against an adult worm antigen preparation correlated negatively with intensities of reinfection. By Western blot analysis, a dominant 22 kDa antigen was recognised by IgE antibodies from most, but not all, high IgE responders. This antigen was found to be located in the tegument of the adult worm, and of lung and liver worms, but not in skin-stage schistosomula.

Since further characterisation of the released proteins described in this thesis was impeded by their low availability, recombinant DNA technology was utilised. Initial attempts made to screen a larval cDNA library with serum raised against schistosomula-released proteins, resulted in the identification of 5 positive clones. Of these, two expressed proteins which were recognised by a monoclonal antibody directed against paramyosin. It is hoped that further screening of the library will lead to the identification of the dominant 20 kDa antigen(s) described in this study, and other schistosomula-released proteins. Initially, it should be possible to obtain microgram quantities of the fusion proteins which could be purified by affinity chromatography or eluted from electrophoretic gels. After concentration, such molecules can be used in T cell blastogenesis assays, and their ability to induce proliferation of, and cytokine production by, such cells tested. Results obtained from such experiments should allow the selection of a small number of potential vaccine antigens. By using high level expression systems, large quantities of the selected molecules should eventually be obtained. This would facilitate further characterisation of such moieties.

In summary, the work described in this thesis has provided a firm foundation for the hypothesis that schistosomula-released proteins are an important source of candidate T cell vaccine antigens. It is hoped that the further characterisation of one, or more, of the released proteins described in this study, will eventually progress to the stage at which trial vaccination experiments in mice can be implemented.

APPENDIX ONE

Irradiation of *Schistosoma mansoni* cercariae impairs neuromuscular function in developing schistosomula.

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Irradiation of *Schistosoma mansoni* Cercariae Impairs Neuromuscular Function in Developing Schistosomula

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ABSTRACT: Optimally attenuated larvae of *Schistosoma mansoni* (20 krad) are incapable of migrating farther than the lungs, and they induce high levels of resistance in mice to a subsequent challenge infection. The effect of gamma radiation on the parasite is described and related to the migratory pattern of attenuated larvae. Scanning electron microscopy revealed marked morphological differences between normal and irradiated larvae at the lung stage of development. The latter exhibited random constrictions, probably resulting from contraction of circular muscle fibers, at intervals along the length of the body and showed subtle differences in motility. We suggest these abnormalities account for persistence of attenuated larvae in the skin-draining lymph nodes and lungs, 2 events that are instrumental to the induction of protective immunity in this vaccine model.

A single exposure of C57Bl/6 strain mice to 500 gamma-irradiated cercariae of *Schistosoma mansoni* induces up to 70% protection against a challenge with normal parasites (Dean, 1983). The optimal radiation dose has been defined as 20 krad in our experimental system, but other investigators use up to 56 krad. Recently, Reynolds and Harn (1992) compared 15- and 50-krad doses of gamma radiation and found the former induced higher levels of protective immunity. An exposure regime involving normal parasites but precluding pathological changes in the liver (e.g., single sex, or drug-terminated infections) elicits only low levels of protection. Gamma irradiation of cercariae appears to have no effect on the subsequent surface antigenicity of schistosomula (Simpson et al., 1985), their ultrastructure (Mastin et al., 1985), or their ability to stimulate proliferation of human mononuclear cells (Vieira et al., 1987). Recently, it has been demonstrated that protein synthesis is inhibited, relative to normal larvae, in the first 24 hr after irradiation but nevertheless recovers by 72 hr (Wales et al., 1992). However, the major difference between irradiated and normal larvae that has been highlighted is in the pattern of migration through the mammalian host. Optimally irradiated larvae show a retarded exit from the skin. Some enter and persist in lymph nodes draining the exposure site, whereas others reach the lungs but apparently travel no farther (Mountford et al., 1988). The migration of hyperirradiated parasites, which induce little or no resistance, is even more abbreviated, none actually reaching the lungs (Constant et al., 1990).

The altered pattern of migration shown by optimally irradiated larvae has been proposed to be the key factor responsible for the induction of protection (Mountford et al., 1988). There is a greater, more prolonged proliferation of T cells in lymph nodes draining the skin and lungs after exposure to irradiated than to normal parasites (Constant and Wilson, 1992). Additionally, the antigen-stimulated production of cytokines, particularly interferon gamma and interleukin 3, is more persistent (Pemberton et al., 1991). The attenuated schistosomula that reach the lungs are also an important component in vaccination success. They stimulate the recruitment of schisto-

some-specific CD4+ T cells to the pulmonary parenchyma and airways (Aitken et al., 1988). This persistent population of T lymphocytes may serve to arm the lungs against the arrival of challenge parasites, providing the organ with the capacity for a rapid memory response on secondary exposure to antigen. However, the question of how irradiation modifies parasites, causing them to stimulate the more intense immune response, remains unanswered. In the course of studying larval excretory–secretory antigens, we consistently observed significant differences in surface structure between attenuated and normal larvae that may provide an explanation.

Cercariae of a Puerto Rican strain of *S. mansoni* were shed from *Biomphalaria glabrata* snails and one-half was exposed to 20 krad of gamma radiation from a ^{60}Co source (Department of Radiobiology, Cookridge Hospital, Leeds, U.K.). Bodies were sheared from normal and irradiated cercariae in 5% glucose (Ramalho-Pinto et al., 1974) and recovered after separation from tails on a discontinuous Percoll gradient (Lazdins et al., 1982). The bodies were cultured in 24-well plates at 37 C in a 5% CO_2 : 95% air mixture, in Medium 169 (Basch, 1981) lacking erythrocytes and with 1% fetal calf serum replacing human serum. The absence of erythrocytes in the medium prevents transformation of schistosomula to the liver stage (Clegg and Smithers, 1972). After some days in culture, gross morphological differences between normal and irradiated larvae were detectable by light microscopy. To characterize these, schistosomula were fixed, prepared for scanning electron microscopy (Crabtree and Wilson, 1980) and examined in an ISI (International Scientific Instruments, Harpur Hill, Derbyshire, England. U.K.) 100A microscope.

After 8 days in culture, schistosomula derived from normal cercariae (Fig. 1A) exhibited the characteristics of schistosomula recovered from the lungs (Crabtree and Wilson, 1986). The body was elongate and lacked spines apart from the extreme anterior and posterior regions. The disappearance of midbody spines is an indication that development had proceeded normally in vitro. Schistosomula derived from irradiated cercariae (Fig. 1B) showed a similar pattern of spination, but differed in appearance in 1 marked respect. They showed a series of tight constrictions (between 1 and 10) distributed randomly along the length of the body. These were not shrinkage artifacts resulting from specimen preparation. The constrictions were first apparent by light microscopy on day 5 of culture and only prominent by day 6–7; they were a consistent feature of all cultures of attenuated larvae. Similar constrictions were present on attenuated larvae recovered from the lungs of vaccinated mice on day 8. Subtle differences in motility between normal and attenuated larvae could also be observed. The alternating extensions and contractions of the body, which are characteristic of lung schistosomula, occurred more slowly and the maximum extension was less in the irradiated parasites. Both sets of larvae remained indefinitely in an arrested state of development with more than 50% surviving for at least 6 wk, the irradiated larvae retaining constrictions throughout.

The musculature of the schistosomulum is an arrangement of inner longitudinal and outer circular fibers, lying immediately below the tegument and external to the parenchyma and other cells of the body. These internal tissues serve as a hydrostatic skeleton on which the muscles act

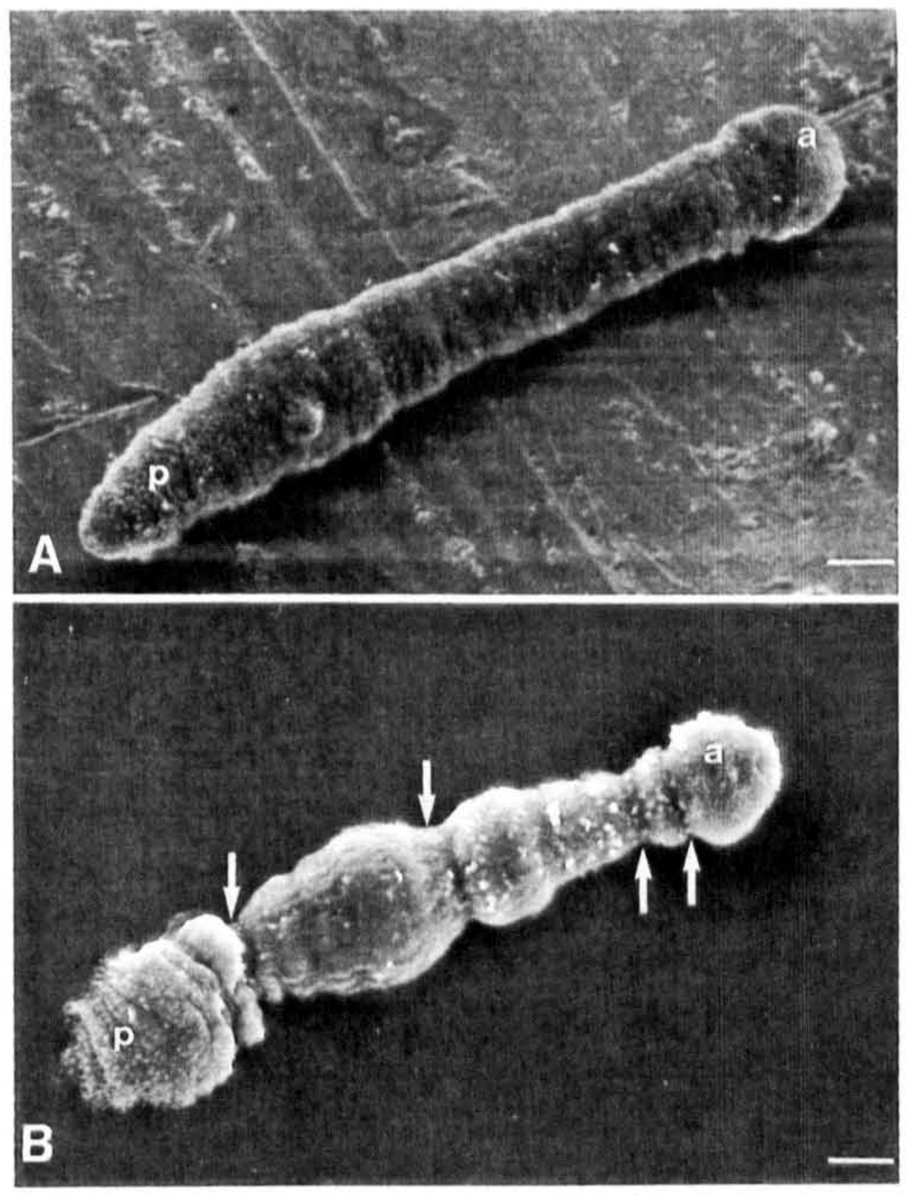

FIGURE 1. Scanning electron micrographs of schistosomula cultured for 8 days in vitro. Scale bar = 10 μm. **A.** Normal schistosomulum showing the smooth outline of the body and characteristic loss of midbody spines but presence of anterior (a) and posterior (p) spines. **B.** Schistosomulum derived from an irradiated cercaria showing constrictions of the body at random points along its length (arrows), and presence of anterior (a) and posterior (p) spines.

in a concerted manner to achieve the alterations in body form involved in the process of intravascular migration (Crabtree and Wilson, 1986). Thus, extension of the body occurs by the simultaneous contraction of the circular and relaxation of the longitudinal fibers, and vice versa for contraction. The function of the anterior and posterior spines is to provide purchase against the vessel wall and to facilitate movement along the lumen (Crabtree and Wilson, 1980).

We suggest that each random constriction in the body wall is produced by the overcontraction of 1 or 2 circular muscle fibers to their maximum extent, whereas other circular muscle fibers remain in balance with the extended longitudinal fibers. Thus, gamma radiation exerts a delayed effect on the neuromuscular coordination of the parasite without causing its death. This interpretation of the altered structure provides one possible explanation of the truncated migration pattern of attenuated schistosomula. The differences in surface structure and motility only become apparent after 5 days in culture, a fact consistent with the observation that larvae are able to commence their migration from the skin before becoming sequestered in a lymph node or the lungs (the $t_{1/2}$ for the migration of normal parasites out of mouse skin is approximately 4 days [Miller and Wilson, 1978]).

We conclude that the optimal dose of gamma radiation exerts an effect on the parasite at a crucial stage in its development 5–7 days after exposure. This effect is manifest as a progressive disruption of neuromuscular coordination that impairs parasite motility. The result is to trap attenuated larvae in the draining lymph nodes and lungs, where they are likely to release larger quantities of antigen due to the prolonged period of residence, thus stimulating the immune response and generating the protective state. A high radiation dose (80 krad) presumably acts earlier, preventing parasite migration from the skin, whereas a low dose (5 krad) permits a relatively normal migration; in both extreme situations the attenuated larvae fail to induce a high level of immunity (Constant et al., 1990; Coulson and Wilson, unpubl. data).

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Ultrastructural Determination of Cystogenesis by Various *Toxoplasma gondii* Isolates in Cell Culture

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ABSTRACT: The tissue cyst stage of *Toxoplasma gondii* is important in relapsing disease seen in toxoplasmic encephalitis and retinochoroiditis. An in vitro culture system to examine the developmental biology of the tissue cyst stage would greatly aid in our understanding of this stage of the parasite's life cycle. We used transmission electron microscopy (TEM) and acid–pepsin digestion of infected cell cultures to determine the capability of 21 isolates of *T. gondii* to produce tissue cysts in cell cultures. All 21 of the isolates had acid–pepsin-resistant stages present, and tissue cysts could be demonstrated in 19 using TEM. The present study demonstrates that tissue cyst formation in vitro is a common phenomenon for *T. gondii* isolates.

Toxoplasma gondii is a ubiquitous protozoan parasite that infects human beings and most other warm-blooded animals (Dubey and Beattie, 1988). Its facultatively heteroxenous life cycle consists of the following polymorphic stages: sporozoites within sporulated oocysts; tachyzoites, the rapidly dividing stages that occur early in infections and are the stages that are transplacentally transmitted; and bradyzoites, the slowly dividing stages that are present in tissue cysts. Biological and antigenic differences exist among the 3 stages of the parasite (Frenkel et al., 1970; Dubey and Frenkel, 1976; Freyre et al., 1989; Kasper, 1989).

Toxoplasma gondii infections in immunocompetent adult animals usually are asymptomatic, whereas serious disease can occur in transplacentally infected, young or immunocompromised animals. Toxoplasmic encephalitis now is recognized as a serious, often fatal, manifestation of the infection in many patients with the acquired immunodeficiency syndrome (Luft and Remington, 1988, 1992; Strittmatter et al., 1992). Toxoplasmic encephalitis and retinochoroiditis probably are caused by reactivation or rupture of latent tissue cyst stages (Frenkel and Escajadillo, 1987).

Cell culture systems for examining the developmental biology of bradyzoites and tissue cysts would aid in our knowledge of these important stages of the parasite's life cycle. Certain isolates of *T. gondii* produce tissue cysts in cell cultures (Hogan et al., 1960; Matsubayashi and Akao, 1963; Kambara et al., 1971; Shimada et al., 1974; Hoff et al., 1977; Jones et al., 1986; Lindsay et al., 1991). Two of these studies (Hogan et al., 1960; Shimada et al., 1974) examined the RH isolate of *T. gondii*, whereas different isolates were used in the other studies.

The present study was conducted to determine the ability of various isolates of *T. gondii* to produce tissue cysts in cell cultures.

Four commonly used laboratory isolates, RH, TS-4, ME-49, and GT-1, and 17 isolates from various wild mammals and birds were examined for the ability to produce tissue cysts in cell cultures (Table I). The GT-1 isolate was used as a positive control because it previously has been shown to produce tissue cysts in cell culture (Lindsay et al., 1991). All *T. gondii* isolates, except RH and TS-4, were maintained as chronic infections in female ICR mice prior to use in cell culture studies. Most isolates were used after only 1 passage in mice; none had been passaged in mice more than 3 times. The RH and TS-4 iso-

APPENDIX TWO

Genotype of bacterial strains

Strain	Genotype
BB4	*sup*F58 *sup*E44 *hsdR*514 *galK*2 *galT*22 *trpR*55 *metB*1 *tonA* ▲*lac*U169F [*proAB*$^{+}$*lacI*^{q}*lacZ*▲M15 Tn10(tet^{r})]
XL1-Blue	*supE*44 *hsdR*17 *rec*A1 *end*A1 *gyr*A46 *thi rel*A1 *lac*$^{-}$

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