THE SECRETORY ANTIGENS OF SCHISTOSOMA MANSONI AND THEIR INVOLVEMENT IN THE IMMUNE ELIMINATION OF WORMS FROM RATS.

Lucy Cutts

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DEDICATION

This thesis is dedicated to my parents

ABSTRACT

Laboratory rats are able to expel schistosomes from the hepatic portal system around 28 days post–infection. Parasite–specific IgE titers rise and mast cells are recruited to the liver in large numbers, coincident with parasite expulsion. Since the worms appear viable prior to day 28, it is assumed that IgE production and mast cell degranulation are stimulated by antigens released by healthy parasites. The present study identified molecules released by adult worms, which could participate in stimulating IgE production and mast cell degranulation.

By surgically transferring adult schistosomes from donor C57BL/6 mice to naive recipients of the same strain, it was possible to sustain viable parasite function for a period of 8 weeks. Larval development was bypassed, and since the recipient animals were exposed to healthy adult worms they mounted an antibody response directed against those antigens released by mature parasites only. Serum collected from the recipient mice (WTS) was used in western blotting studies to probe fractionated parasite protein. Twelve immunodominant moieties were identified ranging in molecular weight from 14 to 208 kDa. Eight of these antigens were immunolocalised to the tegument membrane or gastrodermis of adult worms.

To investigate the nature of the IgE response during rat schistosomiasis, a longitudinal study was performed in which Fischer rats were exposed to male and female (mixed–sex), or male–only cercariae. Worms were recovered at days 28 and 42 post–infection to assess whether worm elimination had taken place. The levels of serum IgE and systemic mast cell protease (RMCP II; a mediator released following mast cell degranulation) were evaluated by ELISA. The results showed that total IgE production proceeded from day 21 and peaked at day 42 post–infection, whereas RMCP II release was high at days 28 and 35, but had diminished by day 42. Furthermore, it appeared that IgE levels were enhanced by products released from adult female worms, since mixed–sex infections induced greater IgE titers in comparison to male–only infections; this elevated IgE production was not attributed to egg secretions. Two dominant allergens Mw of 67 and 36–38 kDa were detected by western blotting using immunopurified IgE from infection serum.

Parasite–specific IgE levels in the rat infection serum were quantified using a functional rat basophilic leukemia–serotonin release assay (RBL–SRA). Results from this assay not only corroborated evidence for enhanced IgE production following mixed–sex infections, but also modelled the complex interactions between

IgE, mast cells and allergen. From the RBL–SRA it was concluded that female schistosomes were more allergenic than their male counterparts, and that this reactivity appeared to be directed against carbohydrate determinants. As very low levels of IgE are able to sensitise mast cells, and degranulation can be stimulated with microgram quantities of schistosome antigen, the results presented in this study support a role for mast cells and IgE in mediating worm elimination in rats.

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ABBREVIATIONS

ADCC	Antibody-dependent cell cytotoxicity
APC	Antigen presenting cell
ATP	Adenosine tri-phosphate
AMP	Adenosine mono-phosphate
a.u.	Abitrary units
BMMC	Bone marrow-derived mast cells
BSA	Bovine serum albumin
CAA	Circulating anodic antigen
CB	Coupling buffer
CCA	Circulating cathodic antigen
CMS	Chronic mouse serum
CTMC	Connective tissue-type mast cells
DAB	Diaminobenzidine
DB	Discoid bodies
DTH	Delayed-type hypersensitivity
EA	Egg albumin
EAF	Eosinophil activating factor
ECF	Eosinophil chemotactic factor
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
epg	Eggs per gram
E/S	Excretory/secretory
Fc∈R	Fc-epsilon-receptor
FcγR	Fc-gamma-receptor
FCS	Foetal calf serum
Fig.	Figure
FPLC	Fast protein liquid chromatography
GPI	Glycophosphatidylinositol
GST	Glutathione S-transferase
h	Hours
^{3}H	Tritiated thymidine
HBSS	Hank's balanced salt solution
HI	Heat-inactivated
HR	High responder

HRP	Horseradish peroxidase
IFN-γ	Interferon gamma
IH	Immediate-type hypersensitivity
IL	Interleukin
IP	Immunopurified
kDa	Kilodaltons
M	Molar
mAb	Monoclonal antibody
MARE	Mouse anti-rat IgE
min	Minutes
MEM	Minimal eagle's medium
MES	Morpholinoethane sulfonic acid
MLB	Multilamellar bodies
MMCP	Mouse mast cell protease
MMC	Mucosal mast cells
Mw	Molecular weight
NCF	Neutrophil chemotactic factor
NGtS	Normal goat serum
NMS	Normal mouse serum
NRS	Normal rat serum
NRbS	Normal rabbit serum
OD	Optical density
Ole e I	Olive tree pollen
PAF	Platelet activating factor
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCA	Passive cutaneous anaphylaxis assay
PK	Prausnitz Kustner test
PI	Protease inhibitors
RAST	Radioallergosorbent assay
RBL	Rat basophilic leukemia
RIA	Radioimmunoassay
RMCP	Rat mast cell protease
s	Seconds
^{35}-S	35-sulphur
SCF	Stem cell factor

SRP	Schistosomula-released products
SDS	Sodium dodecyl sulphate
S.E.	Standard error
SEA	Soluble egg antigen
SRBC	Sheep red blood cells
SWAP	Soluble worm antigen preparation
TBS	Tris buffered saline
TCA	Trichloroacetic acid
Th	Helper T cell
TNF	Tumour necrosis factor
VCAM	Vascular cell adhesion molecule
VLA	Very late antigen
VRS	Vaccinated rat serum
W.H.O.	World Health Organisation
WTS	Worm transfer serum

CHAPTER ONE

Introduction

PART ONE: SCHISTOSOMIASIS-INFECTION AND IMMUNITY

1.1 *Background*

Schistosomiasis is a chronic and debilitating parasitic disease caused by infection with a blood-dwelling fluke belonging to the genus *Schistosoma* (Family, *Trematoda*; Sub-family, *Schistosomatidae*). Eighteen species of *Schistosoma* are recognised; of these 5 species are considered important parasites of man. *S. mansoni*, *S. haematobium* and *S. japonicum* are the most prevalent, while *S. mekongi* and *S. intercalatum* have a more localised distribution. Other species occasionally known to infect man such as *S. mattheei* and *S. incognitum* have been reported as zoonoses. Schistosomiasis is endemic in 76 countries from the African continent to S.E. Asia, isolated regions of China and S. America (WHO Report, 1985); the extensive geographical spread and diversification in terms of numbers of recorded species and variety of hosts parasitised make this infection one of the most important and widespread parasitic diseases of man. Current estimates suggest that 200 million people are affected, with approximately 1 million deaths occurring per annum (Abdel-Wahab *et al.* 1993). More recently, it has been proposed that official figures may underestimate the prevalence of morbidity, since the conventional method of screening, by faecal egg count, does not account for a certain proportion of the infected patients (DeVlas & Gryseels, 1992).

S. mansoni has received the most attention due to the relative ease with which this parasite is maintained in the laboratory (Coles, 1984). This thesis deals with the nature of *S. mansoni* infection in rats, and hence it is to this species of schistosome that the remainder of the study will refer.

1.2 *The life-cycle of Schistosoma mansoni*

S. mansoni is dependent upon specific freshwater snails (of the genus *Biomphalaria*) for its asexual phase of reproduction and subsequent transmission to a definitive mammalian host. Under the influence of a variety of stimuli, physical and chemical, ciliated miracidia locate a snail host and penetrate successfully within 24 hours. Immediately after entering the haemocoel of the vector snail, the parasites migrate to the hepatopancreas and metamorphose into mother sporocysts, undergoing asexual reproduction to produce numerous daughter sporocysts. Further amplification of parasite numbers occurs following the differentiation of daughter

sporocysts into cercariae (Cheng & Bier, 1972), thereby replenishing the substantial losses of larvae incurred during snail location. A single miracidium gives rise to many thousands of cercariae following a pre-patent period of four weeks. However, their release is sporadic and rarely exceeds more than 1500 cercariae per snail, per day (Webbe, 1965). In response to sunlight and warmth the cercariae, comprised of a head and a typical bifurcate tail (Theron, 1986), migrate through the haemocoel of the molluscan host to the mantle cavity where they burrow through the tissue and enter the freshwater environment.

Each cercaria is protected against its new aqueous surroundings by a carbohydrate-rich glycocalyx, which is thought to be anchored to the underlying tegument via polypeptide chains (Marikovsky, Fishelson and Arnon, 1988). Owing to limited glycogen stores the cercariae have a restricted period of 5-8h during which they must successfully locate a susceptible mammalian host. Although poorly understood, the location of an appropriate host is thought to be triggered by changes in movement of the water column, shadows and host skin secretions; by decreasing motility in response to these stimuli the parasites enhance the probability of making contact with a host. Mucus secretions released from glands posterior to the ventral sucker enable the cercariae to adhere to the skin's surface. Attachment precedes skin penetration which involves 2 discrete phases: surface exploration, triggered by thermal and chemical stimuli, and epidermal penetration initiated by chemical stimuli alone (Haas & Schmidt, 1982). In addition to stimulating skin invasion, the volatile mammalian skin lipids induce production of fatty acid metabolites (eicosanoids) from the parasite. Eicosanoids are immunosuppressive substances thought to play a role in the immune evasion strategy of schistosomes (Furlong, 1991). Furthermore, lytic enzymes released by 3 functionally and structurally distinct secretory organs, the head glands, and the pre- and post-acetabular glands, are directly involved in the penetration of host skin and the concurrent loss of the surface glycocalyx (Marikovsky *et al.* 1988). Once this carbohydrate-rich envelope has been shed the larvae are only viable in physiological media. However, there is still a detectable residue (40%) of glycocalyx material on the schistosomulum surface, the loss of which continues over the ensuing hours (Wilson, 1987). Once in the skin, the released proteolytic enzymes degrade the extracellular matrix of the epidermis facilitating passage of the spine-covered cercarial heads (McKerrow & Doenhoff, 1988). The cercarial tails are now lost and the parasites transform into schistosomula, a stage adapted for life in the mammalian host.

After transformation the parasites, covered by a membraneous outer bilayer,

maintain their proteolytic activities, thereby continuing the process of skin invasion (Keene *et al.* 1983). Furthermore, metalloproteinase release has been associated with the cleaving of schistosomicidal antibody from the surface of worms, again minimising immune-mediated damage incurred during skin invasion (Auriault *et al.* 1981). The schistosomula may remain in the skin from 48h to more than 4 days post-transformation (Crabtree & Wilson, 1985; Miller & Wilson, 1978). Upon location and entry into a venule, larvae are carried by the blood-flow to the lungs (Miller & Wilson, 1980). Alternatively, some of the parasites enter lymphatic vessels from where they are transported to the skin-draining lymph nodes (Mountford, Coulson and Wilson, 1988; Wheater & Wilson, 1979). The details of normal parasite migration and development in mice and rats will be reviewed in section 1.5. Schistosomula lodged in the lungs are indistinguishable initially in size and morphology from skin worms of the same age (Wilson *et al.* 1978). However, spatial constraints experienced within the pulmonary capillary beds appear to induce significant morphological changes in the parasites. In the ensuing days the volume of the lung worms does not increase. However their length is more than doubled and body spines are lost, thereby increasing the surface area by an estimated 52% (Crabtree & Wilson, 1980). Morphological changes are accompanied by behavioural adaptations; the worms undergo rhythmic cycles of extension and contraction of 6-9 seconds duration to facilitate their migration along the lumina of blood vessels (Wilson *et al.* 1978). Those parasites that traverse the narrow alveolar capillaries successfully are carried passively in the direction of the blood flow to the systemic organs of the host. The final destination for the schistosomes is the hepatic portal system, however this is dependent upon the trapping of parasites in the mesenteric veins of the gut, and the effective recirculation of worms lodged in other tissues. The wholly intravascular route permits relocation from unfavourable environments to the migratory goal. However it is worth emphasising that the mechanism of trapping is not 100% efficient. Why the parasites should take up residence in the hepatic portal system is not completely understood. It is thought that two major factors operate to trap incoming parasites, the lower blood pressure encountered in the wide bore distributaries (Miller & Wilson, 1980) and/or the nutrient-rich composition of the portal blood (Wheater & Wilson, 1979).

Once the larvae have arrived in the hepatic portal system they transform into blood-feeding adults, losing the ability to undertake intravascular migration by reducing their length (Miller & Wilson, 1980). As a consequence of shortening, the tegument becomes highly convoluted to accommodate the surplus membrane, with

numerous pits distinguishable at the surface (McLaren, 1980). The pitted nature of the schistosome tegument imparts an enormous increase in surface area, indicative of an absorptive function. The dorsal surface of the male worm forms into large bulbous tubercules, enabling the parasite to gain purchase along the mesenteric veins, while the female is longer and more slender. Respective modifications are unnecessary for the female since it lies protected within the male's gynaeophoric canal; only the head and tail of the female are thus exposed. It appears that the male worm communicates with its partner by nutritional and hormonal means; the muscular action of the clasping male appears to help the immature female to pump blood into her intestine (Gupta & Basch, 1987). The female schistosome ingests a larger number of red blood cells, 13 times the number and 9 times the rate of male worms (Lawrence, 1973). The reason for the difference is probably due to a larger utilisation of amino acids by females in connection with oogenesis. In view of this, haemoglobinases have been described with higher activities in female *S. mansoni* than males (Timms & Beuding, 1959).

By pairing, the female matures and begins to oviposit. The eggs become lodged in the mesenteric veins and capillaries of the host. The terminally spined ova use mechanical and enzymatic processes to break through the tissue barriers, thereby permitting entry into the intestinal lumen where they are voided with the faeces. These deposited eggs hatch upon contact with fresh water and release free-living miracidia which infect the appropriate snail host and continue the cycle of parasitism.

The life-span of adult worms in humans has been estimated to average 3.5 to 12 years, with some worms living for up to 30 years. Consequently, schistosomiasis is a chronic disease which presents a broad spectrum of morbidity ranging from sub-clinical infection to severe splenomegaly and hepatomegaly. The most important pathology is caused by the trapping of eggs in the liver of the host. An inappropriate inflammatory cell response to antigen secreted from the eggs results in the formation of granulomas and the destruction of the hepatic tissue (Boros & Warren, 1970). In advanced cases, fibrosis occurs, with further disruption of hepatic tissue organisation and the development of "Symmers' pipestem fibrosis" leading to the occlusion of the portal system. The ensuing portal hypertension causes ascites, the development of porto-systemic anastamoses, which shunt a fraction of the hepatic blood to the vena cava; the latter may cause fatal haemorrhaging. The hepatocellular function remains in principle preserved.

The severity of disease depends upon the intensity and duration of the infection,

and also of still largely unknown immunological and genetic factors. Since schistosomaisis constitutes a major public health problem many control strategies have been proposed and implemented (Liese, 1986).

1.3 *Control Measures*

Complete eradication of this parasite is unlikely without profound modification of the ecological and sanitary factors which determine the transmission level of schistosomes. Owing to the lack of significant socio-economic progress in many endemic countries a more realistic objective has been pursued by reducing transmission following chemotherapy of infected individuals and/or by controlling snail populations with chemical molluscicides. These approaches should result in the reduction of prevalences and intensities of infection to levels at which morbidity becomes tolerable.

1.3.1 *Chemotherapy*

Three basic strategies have been used to combat increasing morbidity in human populations. In selective treatment, members of the community are screened by faecal egg counts or by serum sampling, and the positive individuals chosen for chemotherapy. In targeted therapy, the screening is followed by treatment of individuals with high egg counts only. Mass treatment refers to the administration of drugs to the entire population without prior individual diagnosis. The decision to implement each of these schemes or a combined phase approach must take into consideration the epidemiological data for the community of interest, the effectiveness of the drug used, cost of delivery and potential logistical problems.

A programme of selective treatment has been used recently in preference to indiscriminate mass-treatment of communities. In Maniema (Zaire) the treatment of heavily-infected individuals (>600 epg) has almost eliminated the occurrence of severe hepatosplenic morbidity, although the intensity and prevalence of infection has hardly been affected as rapid reinfection is the rule rather than the exception in areas of intense transmission. A similar national research and control programme was initiated in Burundi in 1982. In contrast to the Manieman community the prevalence, intensity and morbidity were moderate, and transmission focal and erratic in time and space. However, despite effective screening strategies the application of selective treatment did not affect the levels of transmission although it did result in the rapid control of morbidity (Gryseels, Polderman and Engels, 1992).

In the absence of transmission control, treatment must be repeated at intervals of a few years or less for an undetermined period. The cost of drugs and, even more, of screening and drug delivery is high, therefore large scale chemotherapy is out of the reach of most national budgets.

Dependency upon chemotherapy alone is not a viable solution for schistosomiasis control, even with the advent of the curative drug Praziquantel which has few side-effects, is administered as a single dose, is highly effective against all species of schistosome and is relatively cheap (WHO, 1985). The widely reported drug resistance in schistosomes, confined essentially to compounds of the hycanthone/oxamniquine family (Cioli, Pica-Mattoccia & Archer, 1993), limits any long-term reliance upon this chemical. If community-based chemotherapy is envisaged, integration with other methods of control is crucial.

1.3.2 *Snail Control*

The demise of molluscicidng as the primary means of controlling schistosome transmission in endemic areas has largely been as a consequence of cheap, safe and effective oral drugs. Further, the cost of the molluscicide of choice, Niclosamide (18US$/kg), is a factor in the decline of chemical snail control. A third reason has been the failure of blanket or area-wide molluscicidng to control transmission in major projects. Focal molluscicidng targets those small water bodies, canals or points/sites along a river that are "hot-spots" for transmission (Klumpp & Chu, 1987). However, difficulties arise in the identification of microfoci and periods of peak transmission. In addition, snail populations exhibit an impressive potential for regeneration and reinvasion of an area, re-establishing in a period of months.

1.3.3 *Primary Healthcare*

The most singularly effective approach in schistosomiasis control is sanitation and health education. By increasing awareness of the problem within the affected community significant improvements can be implemented almost immediately. Faecal or urinary contamination can be reduced by the construction of latrines. This facility however must be consistently used by a large majority of the population in order to lower transmission rates. With adequate designs adapted to the needs and attitudes of the population the establishment of safe water supplies, foot bridges, showers etc, can reduce human exposure directly. Any modification must be supported by a health education program using realistic messages, targeted approaches and considerations of cultural attitudes – all are key factors to success.

In the long-term this rationale for control will reinforce chemotherapeutic and mollusciciding measures and should be the priority for governments and funding agencies in view of the broadness of its impact.

1.3.4 *An alternative approach*

Although the treatment of individual, uncomplicated infections is now much improved, control of transmission on the population level continues to be a problem. In the present socio-economic climate of most endemic countries such control programmes can be difficult to sustain. Thus, the development of alternative, durable control measures is a necessity to relieve the suffering of many millions of people, and in particular the susceptible younger generation. Principal among these long-term solutions would be the advent of a cheap and effective vaccine. "Single-shot" immunisation would be ideal, with a vaccine composed of recombinant protein(s) or peptide(s) capable of generating a potent anamnestic response. Furthermore, this solution would prevent the egg-induced hepatic pathology associated with acute and chronic schistosomiasis and as a consequence lower the prevalence, morbidity and possibly the rate of infection/reinfection and transmission.

The efficacy of such a vaccine would have to be 100%, although to date no live or dead vaccine has been shown to induce this level of immunity in laboratory rodent or primate hosts. A more tangible goal might be the development of sterile immunity, since the schistosomes do not replicate within the mammalian host. Hence, the induction of >50% resistance might limit the number of people afflicted with hepatosplenomegaly, the severe form of the disease. Lively debate on this issue argues that any vaccine which is not 100% effective would result in some egg deposition. Over a prolonged period of exposure the deposition of parasite eggs would skew the immune response and promote chronic hepatic pathology, with the ensuing clinical complications outlined in section 1.2 (Ridi, 1995).

Despite the lack of a successful vaccine, it is appropriate to discuss alternative approaches and rationales. Since it is evident that the deposition of eggs provokes the damaging cellular reactivity associated with disease, some studies have focused upon the amelioration of egg-induced pathology as a vaccine strategy. The design of an anti-fecundity vaccine would, in the short-term, eliminate/reduce egg production from female worms thereby preventing the induction of harmful immunopathology. In the long-term, the transmission rates could be lowered within the treated community. With respect to this approach, glutathione S-transferase (Sm28GST) has been proposed as a vaccine candidate since it not only protects

various rodent and primate hosts through a reduction in worm burden (Balloul *et al.* 1987), but has also been shown to reduce female worm fecundity (Xu *et al.* 1993). Similarly, the prevention of the immunopathological consequences of infection has also formed the basis of a vaccine rationale by Wynn and colleagues (1995a). Earlier work had demonstrated that administration of the cytokine IL–12, increased the protective immunity afforded by an attenuated larval schistosome vaccine (Wynn *et al.* 1995b). When Wynn introduced eggs and IL–12 into the peritoneal cavities of C57BL/6 mice, mice were sensitised to a percutaneous challenge with *S. mansoni* cercariae. Although the inoculation did not significantly reduce the number of worms or eggs recovered at either 8 or 12 weeks post–challenge, the granulomas were significantly smaller and contained fewer eosinophils than those observed in infected, unsensitised mice. Further, the amelioration of fibrotic lesions reflected the down–modulation of the detrimental inflammatory response to ova. This immunisation protocol demonstrates how the host's immune response may be manipulated to fight infection.

A multifactorial vaccine designed to polarise a beneficial immune response would be composed of protective antigenic molecules or determinants, an appropriate adjuvant reactivity and would neutralise/enhance specific cytokines, thereby optimising the method of presentation to the immune system and augmenting the vaccine's protective capacity. However, further progress is contingent upon the elucidation of the complex induction and effector mechanisms manifest in the protection of hosts against schistosomiasis. The fact that workers have succeeded in harnessing and manipulating the immune system to their advantage, justifies the optimism felt by many researchers involved in the development of an anti–schistosome vaccine.

1.4 *Experimental models of Schistosomiasis*

Recent advances in our knowledge of immunity to schistosomes have been facilitated by studies on experimental animal models since, for obvious reasons, there are inherent difficulties in describing immunological events directed against parasites in human hosts. The most widely used animals are rats and mice, and to a lesser extent guinea pigs and non–human primates. Each model has its own disadvantages and advantages dependent upon the nature of the investigation. For example the rat host does not support the full development of adult worms (Ritchie, Garson & Knight, 1963) and is therefore an inappropriate model in the study of egg–

induced pathology or, the generation of pathology and resistance. Nevertheless, the elimination of primary and challenge infections in the rat make this an ideal candidate for the study of immune mechanisms directed at the larval and adult worm stages, and their respective antigens. On the other hand, the mouse is considered by some to be most analogous to the situation in man, as it displays the same immunopathological consequences of infection. However, the severity of disease in this model indicates that at best, it can only represent an extreme of the human host–parasite relationship. Despite the various analogies and disparities drawn between infected laboratory hosts and the clinical disease, each model is likely to provide insights into the immunobiology of the parasite as well as information regarding the induction and development of effective anti–schistosome immune responses. However, caution must be exercised in extrapolating between the nature of immunity observed in laboratory hosts and the expression of resistance in man.

A brief resume will be given later, on the nature of the normal infection in mice (section 1.7), followed by an extensive review of the maturation of schistosomes in rats (section 1.9.1), focusing upon the immunity directed against parasites in this non–permissive host.

1.5 *Migration and development of schistosomes in laboratory rodents*

Owing to the bulk of information available on the migration and development of schistosomes in mice and the similarity with the migratory pattern observed in rats much of this topic will be discussed with reference to the mouse model. However, where it is deemed important, details of similar studies carried out in rats will be included to aid clarity and prevent repetition in later sections.

In order to understand the immunobiology of infection it is necessary to investigate the developmental strategy of parasites in the definitive mammalian host. The cercarial tail is lost as the larvae penetrate the mouse/rat skin (Stirewalt, 1974) and begin their transformation into the next life–stage, the schistosomulum. Structural and biochemical changes occur as the larvae migrate through the epidermis of the host, successfully reaching the epidermal basement membrane within 30min of attachment (Wilson & Lawson, 1980; Wheater & Wilson, 1979). Estimates regarding the duration of stay in the skin of mice and Wistar rats give the half–life of exit as 88h and 70h respectively (Miller & Wilson, 1978). Within the first 3h of transformation greater numbers of membraneous vesicles are detected within the tegument where they participate in the formation of the heptalaminate

membrane of the schistosomulum (Hockley & McLaren, 1973), a modification for life in the mammalian host.

Histological examination of lymph nodes draining the infection site has revealed evidence for the exit of parasites from the skin via the lymphatics. Migration through the lymphatics is a chance event and results in approximately 10–20% of applied parasites entering lymph vessels in mice (Mountford, Coulson & Wilson, 1988; Wheater & Wilson, 1979; Miller & Wilson, 1978). Additionally, autoradiographic tracking of larvae present in the axillary and inguinal lymph nodes from mice and rats indicated that this figure was greater than suggested, 23 and 2.4% respectively (Coulson & Wilson, unpublished data). Finally, the transit time of schistosomula through the skin was more than 90% complete in rats by 96h and in mice by 120h (Coulson & Wilson, unpublished data).

By days 6 and 7 post–infection, histological analysis revealed that a large proportion of the parasites (48–63%) had accumulated in the pulmonary vasculature of mice (Mastin, Bickle & Wilson, 1983) compared to 92% of skin penetrants in rats on day 5 (Ward & McLaren, 1989; Knopf *et al.* 1986; Coulson & Wilson, unpublished data). Parasites which successfully traverse the pulmonary vasculature enter the venous compartment of the lungs and are carried to the left side of the heart to be distributed to systemic organs in proportion to the cardiac output (Wilson, Coulson & Dixon, 1986; Coulson & Wilson, unpublished data). Those schistosomula lodged in non–splanchnic organs migrate to the hepatic portal system with the majority becoming trapped within the pre–sinusoidal venules of the liver. Parasites dispersed to other organs have to cross the capillary beds and return to the lungs to repeat the process of migration (Wilson & Coulson, 1986). In mice and rats schistosomula were initially detected in the liver on days 6 and 7 respectively (Miller & Wilson, 1978). However, since the technique of portal perfusion may not account for small larvae, the profile of incoming parasites is probably an underestimate (Miller & Wilson, 1980). Results obtained from autoradiographic tracking studies showed that the recruitment of parasites to the mouse liver was complete by day 21 (Wilson *et al.* 1986), whilst in rats accumulation appeared to plateau as early as day 12 post–infection (Ward & McLaren, 1989; Knopf *et al.* 1986; Coulson & Wilson, unpublished data).

The hepatic environment of the mouse host encourages the maturing schistosomes to pair up and migrate along the lumen of the hepatic vessel to the mesenteric veins where egg deposition commences from day 28 onwards after infection. Conversely, the majority of those schistosomula that reach the liver in laboratory inbred rats do

not migrate to the mesenteric veins but remain lodged in the hepatic venules. These parasites are cleared from the rat from day 28 post–infection onwards. Thus, the course of infection is incomplete, as those worms not eliminated remain stunted, producing few mature eggs, with output consisting of predominately degenerate forms and empty shells (Cioli, Knopf & Senft, 1977; Phillips *et al.* 1975; Ritchie, Garson & Knight, 1963).

1.6 *The murine model*

1.6.1 *The nature of the normal infection in laboratory mice*

The mouse is considered as a permissive host, since parasites develop into healthy adult worms and the infection becomes patent 4–6 weeks post–exposure to cercariae of *S. mansoni*. The passage of normal parasites in mice is similar to that in rats, albeit a little slower. Infection of naive mice with *S. mansoni* results in the complete maturation of between 30 and 50% of the cercariae applied. The failure of the remaining skin penetrants to reach the hepatic portal system has been the focus of much debate and speculation. Preliminary studies employed a tissue mincing and incubation technique to extract migratory larvae from the tissues and organs of interest. It was concluded from these investigations that the majority of schistosomes were retained in the skin; unable to migrate further they eventually die (Smithers & Gammage, 1980; Clegg & Smithers, 1968). Conversely, other workers used histological examination of the skin to reveal that a significant proportion of invading schistosomula *did* traverse the dermal barrier successfully (Crabtree & Wilson, 1985; von Lichtenberg *et al.* 1985; Mastin *et al.* 1983; Wheater & Wilson, 1979). However, both of these experimental approaches have their disadvantages and advantages; the efficiency of parasite extraction by mincing and incubation is dubious (Miller & Wilson, 1980), whilst histology is difficult to quantify, tedious to perform and only a small number of tissue samples can be processed (von Lichtenberg *et al.* 1985; Mastin *et al.* 1983; Wheater & Wilson, 1979). This issue of skin phase migration was greatly clarified with the autoradiographic tracking of isotopically labelled schistosomula in the compressed organs of infected mice (Georgi, 1982). It was found that the vast majority of parasites (78%) migrated as far as the lungs of mice by day 7 after infection (Mangold & Dean, 1983). Following the introduction of an intensifying screen to detect parasites with only a low level of associated radioactivity 100% efficiency was achieved (Wilson *et al.* 1986). This adaptation led to the demonstration that >90%

of skin schistosomula survived passage to the lungs by day 14 post-infection. Thus, it is apparent that only a minority of worms are eliminated at the skin-stage of migration.

Subsequently, parasites migrate to the hepatic portal system of the mouse, with the remaining larvae found distributed in unfavourable locations such as the lungs, systemic organs and skin. It was noted that up to 30% of parasites detected between days 14 and 21 did not reach the liver from the lungs (Wilson *et al.* 1986). Evidently, difficulties were encountered by the parasites in traversing the pulmonary capillary beds, this observation corroborated by the prolonged pulmonary transit time (30-35h) of schistosomula compared to their more rapid migration (16h) through non-splanchnic organs (Wilson & Coulson, 1986).

Additionally, histological and ultrastructural examination of schistosomula in the lungs of naive mice showed that at day 7 post-infection all schistosomula were intravascular. However, from day 11 a number of parasites were located in the alveoli of the lungs, until finally at day 20, 80% of the elongate schistosomula were detected in the air spaces (Crabtree & Wilson, 1986). Diversion of worms into the alveoli presumably occurs as a result of the pressure exerted by the parasites against the delicate capillary walls. Ultimately it is the limited capacity of the impacted larvae to reenter the circulation that accounts for a large proportion of worm attrition in the lungs of mice following a primary infection (Coulson & Wilson, 1988). It is assumed therefore that these lung worms die from starvation. With the notable absence of inflammatory cell reactivity directed against the intravascular parasite it is evident that worm attrition in the naive mouse model is a consequence of physical constraints rather than immunological mechanisms.

1.6.2 *Concomitant immunity*

Chronically infected mice appear to acquire resistance to the larvae of challenge infections whilst adult worms from the intial exposure remain unharmed. The term 'concomitant immunity' was used to describe this phenomenon of resistance to challenge (Smithers & Terry, 1969). The substantial levels of immunity recorded (70-100%) in NMRI mice (Dean, Bukowski & Cheever, 1981) were positively correlated with the presence of eggs in the pre-sinusoidal venules of the liver (Harrison, Bickle & Doenhoff, 1982; Dean *et al.* 1978). Evidence for the importance of oviposition in the development of protection was supported by investigations using single-sex parasites, which produced no overt pathology and generally failed to induce resistance (Bickle *et al.* 1979). However, immunisation with isolated eggs

or egg extracts did not generate any schistosomicidal immune response (Bickle, Ford & Andrews, 1983). Initially, worm death was attributed to a protective humoral response, since partial transfer of immunity to naive animals was achieved with chronic mouse serum; although the degree of resistance attained rarely exceeded 50% (Sher, Smithers & MacKenzie, 1975). However, the preliminary evidence for protective blood-borne factors was challenged later following the unsuccessful transfer of resistance to naive animals by parabiotic union with previously infected mice (Dean, Bukowski & Clark, 1981). This latter study was substantiated by passive transfer experiments, using serum or cells from highly resistant donors to naive recipients, which demonstrated that negligible levels of immunity were transferred to naive mice (Harrison *et al.* 1982; Doenhoff & Long, 1979). Thus, it appeared that antibody-mediated effector mechanisms were unimportant in the expression of immunity against schistosomes in mice.

An alternative explanation of concomitant immunity was provided by Wilson (1990) who suggested that the loss of integrity in the hepatic vasculature accompanying a chronic infection might enhance the recirculation of challenge larvae to unfavourable locations, thereby promoting their demise. Indeed Wilson and coworkers found that microspheres, injected via the superior mesenteric vein, escaped from the hepatic system in proportion to the extent of egg deposition, and hence the severity of the pathological changes in the vasculature (Wilson, Coulson & McHugh, 1983). The development of minor connections within the liver linking the portal and hepatic veins permits the passage of elongate schistosomula through the liver to the lungs. Additionally, with the progression of infection, major extrahepatic anastomoses formed between the portal and systemic vascular beds allow the carriage of adult worms with the portal blood to the lungs, thus precipitating their death (Wilson, 1990).

1.7 *Maturation and elimination of a primary infection in the rat*

In order to address the complex mechanism of immunity operating in the non-permissive rat host it is first necessary to identify the site(s) where parasite elimination occurs. In contrast to the mouse model comparatively few studies have defined the dynamics of migration of schistosomula in the rat. Although larvae have been recovered from the skin, draining lymph nodes, lungs and livers of the normal rat using a variety of techniques (Ward & McLaren, 1989; Knopf *et al.* 1986; Knopf *et al.* 1983; Miller & Wilson, 1978) only one study has enumerated parasites from

other systemic and non-splanchnic organs (Coulson & Wilson, unpublished data). In laboratory rats only a small percentage of the cercariae from a primary infection are recoverable as worms beyond 6 weeks, and these parasites are stunted, remain within the hepatic portal veins and produce few, non-viable eggs (Cioli, Knopf & Senft, 1977; Phillips *et al.* 1975; Ritchie, Garson & Knight, 1963). It was initially thought that attrition of schistosomula occurred in the skin, as only half the number of penetrants could be recovered when this tissue was minced and incubated in culture medium for several hours (Clegg & Smithers, 1968). However, as mentioned in section 1.7 this crude extraction technique led to gross misinterpretation of the migratory capacity of worms in mice (Miller & Wilson, 1980). Using another approach, Knopf *et al.* (1983) recovered parasites following amputation of the tail infection site at various times post-exposure with *S. mansoni*. These authors found that 32–34% of the infecting cercariae progressively migrated from the skin to the lungs from day 3 after infection. However, autoradiographic tracking of isotopically labelled parasites revealed that >90% were present in the skin at day 3 and that most had disappeared from this tissue by day 6 (Ward & McLaren, 1989; Knopf *et al.* 1986; Coulson & Wilson, unpublished data). It was therefore concluded that no significant elimination of schistosomula occurred in the skin of infected rats.

As anticipated from the skin exit data, accumulation of parasites in the rat lung was rapid, with a peak value of 85% of penetrants detected on day 6 post-infection (Ward & McLaren, 1989; Knopf *et al.* 1986; Coulson & Wilson, unpublished data). The subsequent enumeration of foci in the hepatic portal system of rats suggests that recruitment of parasites to the liver is complete by day 12 (Ward & McLaren, 1989; Knopf *et al.* 1986; Coulson & Wilson, unpublished data), with 79% of penetrants detected in non-splanchnic locations (Coulson & Wilson, unpublished data). The profile of parasite numbers entering the liver was much lower than the peak number detected in the lungs by autoradiography, hence Coulson and Wilson concluded that a large proportion of the elimination had taken place when penetrants were present in non-splanchnic sites; predominately the lungs (Ward & McLaren, 1989; Knopf *et al.* 1986; Knopf *et al.* 1983; Coulson & Wilson, unpublished data). In addition, when lung schistosomula from donor rats were injected by an intravenous route to the hepatic portal system of naive recipients the level of maturation did not differ significantly from that which followed percutaneous infection (Knopf *et al.* 1986). Thus, there appeared to be no major rejection of intravascular parasites from the liver, until day 21 (Coulson & Wilson, unpublished data).

However, the exact timing of elimination can only be defined by establishing

whether a decrease in worm numbers in a tissue constitutes death and clearance or migration to another organ on the migration route. Several studies have postulated the lungs as an important site of parasite attrition in rats, as a decline in worm numbers was observed from day 5 post-infection, a period coincident with the residence of schistosomula in the pulmonary vasculature (Ward & McLaren, 1989; Knopf *et al.* 1986; Knopf *et al.* 1983). Irrespective of these putative conclusions, a complete balance sheet of parasite migration in all organs and tissues must be established. Coulson and Wilson corroborated evidence for lung phase killing by showing that after day 12 the number of parasite foci fell in the lungs at a time when recruitment of parasites to the liver appeared complete (Coulson & Wilson, unpublished data).

At a cellular level, histological examination of the rat lung following a primary infection with *S. mansoni*, revealed that larvae were located in the pulmonary vasculature (Ward & McLaren, 1989; Oshman *et al.* 1986; von Lichtenberg & Byram, 1980), with negligible numbers of parasites observed within alveoli (Ward & McLaren, 1989). Furthermore, the rat was able to mount significant cellular reactions in the lungs 5 to 7 days after primary infection (von Lichtenberg & Byram, 1980). At day 15 post-infection, extravascular inflammatory foci had developed around the pulmonary worms, which were dominated by macrophages, rather than by granulocytic cells, with some foci having the appearance of granulomas (Oshman *et al.* 1986). Neither Oshman *et al.* (1986) nor von Lichtenberg and Byram (1980) found evidence for cell-inflicted damage at the lung level that would account for the reduction in parasite numbers at the portal level. However, Ward and McLaren (1989) did note an increase in worm density with time post-infection that could indicate degradation of parasite tissue. Therefore, it appears that blocking of parasite migration, and as a consequence their retention within the pulmonary vasculature, could contrive to prevent the accumulation of worms within hepatic portal system, in a manner similar to that observed following the normal infection of mice (Wilson, 1990; Wilson *et al.* 1986). Furthermore, the rapidity of immune recognition of the migrating schistosomula, and establishment of trapping foci seemed to be of greater importance than the size and the composition of the enveloping granulomata (von Lichtenberg & Byram, 1980). Ultimately, as in the mouse model, the lung schistosomula die due to starvation.

It is emphasised that only 10% of skin penetrants successfully negotiate the pulmonary vasculature to become resident in the hepatic portal system of the rat host (Wilson & Coulson, unpublished data). Thereafter a second phase of parasite

attrition proceeds in the liver. Beginning at about day 28 post–infection, worm recoveries from the portal system show a rather sharp drop up until day 42, with a subsequent slower phase of worm elimination (Smithers & Terry, 1965). As the infection progresses the number of worms seems to approach a baseline asymptotically, but an average of about 5 worms could be recovered one year after infection with 1000 cercariae. In addition, the number of worms seems to reach a somewhat constant level late post–exposure (e.g. 16 weeks) irrespective of the number of cercariae used for infection (Phillips *et al.* 1978). The occurrence of spontaneous elimination of the remaining parasite burden approximately 28 days after initial infection (Cioli, Knopf & Senft, 1977; Phillips *et al* 1975; Smithers & Terry, 1965; Ritchie, Garson & Knight, 1963) means that the rat represents a unique host for *S. mansoni*. These characteristics of intrinsic resistance have been of interest to many workers who have attempted to identify the mechanisms involved in elimination. Intense inflammatory foci surrounding the parasites in the lungs appear to be responsible for the limited migration of schistosomula from the lungs to the liver of the naive rat. However, the processes involved in the termination and impairment of worm maturation in the liver from day 28 seem less clear. The mechanisms contributing to natural immunity in rats are complex, comprised of a series of cellular and humoral interactions often working in concert or in apparent contradictary roles. In addition, it is evident that non–immunological factors influence adult worm survival.

1.8 *Adult worm elimination: Non–immunological mechanisms*

In proposing possible mechanisms for the phenomenon of adult worm expulsion from laboratory rats Smithers and Terry (1965) were cautious in assuming a role for acquired immunity. The nature of elimination was initially studied with immunosuppressed rats exposed to cercariae of *S. mansoni*. High levels of immunosuppression were achieved with thymectomised inbred rats that had been irradiated and then reconstituted with T cell–free bone marrow cells (B rats). Although an involvement of the immune system in the self–cure phenomenon was strongly suggested by the significantly higher number of worms recovered from the immunosuppressed rats, compared to intact controls, it was found that elimination eventually occurred in thymectomised animals also (Cioli & Dennert, 1975). Upon close examination it appeared that worms recovered from B rats were as stunted as those from normal animals. Females did not achieve maturation and hence egg

deposition remained abortive and worm pairs failed to migrate from liver to mesenteric veins. Thus, despite the important role of the immune system in worm elimination it was proposed that immunological phenomena were restricted to the final phases of killing and clearance of worms which were impaired initially by some undefined non–immune mechanism.

To test this hypothesis a series of experiments were designed by Cioli, Blum & Ruppel (1978) to assess whether the commencement of rejection (from the 4th week onwards) was dependent upon the time required by rats to mount an immune response sufficient to kill worms, or whether this timing was dictated by extraneous factors vital to worm development. Schistosomes obtained from rats which had been infected 2, 3, or 4 weeks previously were transferred into the mesenteric veins of normal rats and the onset of elimination determined in recipients by portal perfusion at weekly intervals thereafter (Cioli *et al.* 1978). It was found that 2 week old worms were rejected 2 weeks after transfer, 3 week old worms 1 week after transfer, and 4 week old worms immediately after transfer. Schistosomes obtained from donor mice or hamsters showed the same pattern of elimination once transferred into rats. Thus, it was concluded that the onset of *S. mansoni* elimination in laboratory rats was dependent upon the total age of the parasites and independent of the length of contact with the host in which rejection occurred (Cioli *et al.* 1978). This kind of evidence can be taken as an additional argument against the hypothesis that schistosome expulsion is primarily an immune phenomenon.

Using a different approach to gain additional information on the nature of the "inhibition" exerted by the rat upon *S. mansoni*, 4 and 8 week old schistosomes were transferred from non–permissive rats to permissive hamster recipients (Cioli, Knopf & Senft, 1977). It appeared that worms were subjected to reversible constraints, since schistosomes obtained from rats at any time post–infection and subsequently introduced into permissive hosts developed very rapidly into normal adult worms; they migrated to mesenteric veins and started depositing the usual high number of viable eggs. Conversely, when fertile adult worms (8 weeks of age) grown in a permissive mouse host were transferred into the portal veins of rats, their numbers were progressively reduced, and any survivors failed to migrate to mesenteric veins. Furthermore, the remaining worms became stunted and were dramatically inhibited in their rate of oviposition. These experiments indicate that the non–permissive host not only blocks worm development, but also imposes restrictions upon the continuous maintenance of the adult state. In a separate study Knopf and Soliman (1980) infected thyroidectomised rats, or rats whose hypothalamus had been

surgically removed, with cercariae of *S. mansoni*, and found that the delayed onset of worm elimination was correlated with raised levels of parasite ecdysteroid hormone. Thus, it seemed apparent that specific host hormones could influence the permissive or non–permissive status of rats to schistosome infection.

1.9 *Adult worm elimination: T cell–mediated immunity*

The participation of immune effector mechanisms in the specific rejection of schistosomes was first suggested by the delayed onset of worm elimination in thymectomised (Cioli & Dennert, 1975), or athymic rats (Phillips *et al.* 1983). It was evident that clearance of worm populations between 3 and 5 weeks after infection was partly contingent upon T cell function. By 9 weeks there was a significant decline (>40%) of parasite numbers recovered from infected athymic animals. However, this value was far less than that in heterozygous control rats (>90%) (Phillips *et al.* 1983). T cell competence also appeared to have a qualitative effect upon parasite development, as histopathological examination revealed that worms obtained from immunosuppressed rats were superior to those recovered from control animals, in their respective abilities to oviposit fertile eggs (Phillips *et al.* 1983; Cioli & Dennert, 1975). The enhanced maturation of adult worms is thought to be dependent upon the location of schistosomes within the mesenteric veins of the athymic/thymectomised rat, relative to more hostile environment of the pre–sinusoidal venules in the intact animal (Phillips *et al.* 1983). More specifically, the lodging of parasites in portal vessels necessitates close association with circulating cells, host endothelium and schistosomulum tegument, thereby attracting granulomatous lesions around the adult worms in control rats (Phillips *et al.* 1983). The composition and cytotoxicity of these cellular aggregates will be described in the later section 1.9.3c.

Since it may be inferred, from the reports above, that T cell responsiveness is essential in mediating the initial immune response to schistosomula, it is relevant to question the timing of this event with regard to protection. Cells obtained from infected donor rats at various times post–infection have been injected into syngeneic recipients followed by the exposure of these recipients to cercariae of *S. mansoni*. Passively transferred protection was observed in naive rats which had received cells collected from donor animals infected 3–4 weeks earlier with cercariae; the expression of resistance was obviated following the depletion of the thymocyte population by anti–thymocyte antibody (Phillips *et al.* 1975).

Additional support for a cell–mediated mechanism of immunity operating against adult worms was provided by a T cell ablation and proliferation study. Thymocyte and splenocyte responsiveness to an *in vitro* homologous antigenic challenge showed that the peak proliferative response in rats occurred 4 to 5 weeks after infection (Mendlovic *et al.* 1987). Furthermore, it appeared that the rat thymocyte population exhibited very high proliferative responses (x3) to *S mansoni* antigens, compared to rat splenoctyes. Comparative studies in mice revealed a different pattern of proliferation, as thymocyte responses were muted compared to the growth of the splenocyte population stimulated with the same antigens. These data not only implicate a role for T cells in natural infection and permissiveness to infection but also indicates that the adult worm is the inducer of T cell reactivity. More importantly, the demonstration that thymocyte responses predominate in rats, compared to the lower responsiveness of mouse thymocytes, suggests that a challenge infection of rats may trigger a more rapid, stronger anamnestic mechanism of immunity. However, the high thymocyte responsiveness in rats, and the suggestion that challenge infections may trigger an anamnestic response, assumes the existence of two–way traffic between the rat thymus and the periphery.

1.9.1 *T cell subsets*

To resolve, in a more direct way, which subset of T cells is involved in the immune response of the rat, and whether T helper (Th) cells play a role in permissiveness to infection, the effect of specific monoclonal antibody (mAb) against rat T helper cells (W3/25) was investigated both on the *in vitro* proliferation of the cells and on the susceptibility of the rats to infection *in vivo* (Mendlovic *et al.* 1987). In the presence of mAb the proliferative reactivity of rat thymocytes towards the antigens of either larvae or adult worms was drastically reduced. Furthermore, *in vivo* administration of the W3/25 mAb to rats, prior to infection, led to the depletion of Th cells and a significant increase in adult worm survival compared to untreated controls. This was manifested not only by an increase in worm burden at weeks 4 and 8 post–infection, but also by a more severe infection as observed both macroscopically and histologically (Mendlovic *et al.* 1987). Taken together, the priming of the T helper subset appears paramount for the induction of schistosomicidal activity (Mendlovic *et al.* 1987) and the subsequent recruitment of effector cells (Phillips *et al.* 1983).

The existence of functionally distinct Th subsets in rats has been proven recently. Rat recipients of cardiac allografts differentially express the cytokines interleukin 2

(IL–2) (T–helper 1 subset; Th1) or IL–4 (Th2), as determined by mRNA expression *in vivo* (Papp *et al.* 1992). The Th1 subset produces IL–2 and interferon (IFN) γ which participate in delayed type hypersensitivity (DTH) responses, in addition to stimulating IgG2a and IgG2b production (Mosmann & Coffman, 1989). Whereas the Th2 subset produces IL–4 and IL–5, which stimulate the production of IgA, IgG1 and IgE isotypes in immediate hypersensitivity responses (IH). However, the supposed cell marker for rat Th subsets, OX22 (Spickett *et al.* 1983), is gradually lost from the surface of T cells following their activation (Wang *et al.* 1990). Thus, it may be inferred that Th subsets do exist in rats and that they may be distinguished by the release of IL–2 and IL–4 *in vivo*, but not by the differential expression of the OX22 marker.

Experiments by Phillips *et al.* (1991) were designed to characterise the T cell reactivity involved in the mechanism of protective immunity to schistosomes in rats. Monoclonal antibodies to IL–2 receptor (IL–2R) bearing T cells (ART18 mAb), soluble IL–4 (11B11 mAb) and the Th cell marker (W3/25 mAb) were used to deplete the respective T cell populations and cytokines *in vivo* and *in vitro*. Administration of 11B11 completely abrogated IgE production in rats, whilst ART18 reduced all cellular and humoral responsiveness against T cell dependent antigens, such as purified protein derivative (PPD). The abrogation of IL–4 and IgE production did not affect worm elimination. However, in contrast, the ablation of IL–2R–bearing T cells not only blocked the development of the W3/25 population, previously demonstrated to be protective in passive transfer experiments (Mendlovic *et al.* 1987; Phillips *et al.* 1987), but also significantly reduced worm elimination compared to untreated animals (Phillips *et al.* 1991). The population of rat OX8$^+$ cells remained unaffected by the administration of ART18 mAb. Moreover, these OX8$^+$ cells were able to suppress the ability of Th cells to adoptively transfer resistance (Phillips *et al.* 1991). However, this finding is not consistent with the observation made by Noble and co–workers (1993) who showed that OX8$^+$ cells reduced IL–4, IL–5 and IL–10 secretion by rat Th splenocytes cultured *in vitro*. Thus, it appears possible that in Phillip's study IL–4 release from Th cells *in vivo* was reduced by the presence of OX8$^+$ cells, which delay worm elimination, yet paradoxically the same study shows that IL–4 and IgE production are not correlated with immunity to schistosomes. Obviously this contention demands further clarification. Nevertheless, Phillips *et al.* (1991) argue that resistance in rats to initial and challenge infection is contingent upon the development of IL–2 dependent T lymphocyte subpopulations and not IL–4 mediated reactivity.

1.10 *Adult worm elimination: Humoral immunity*

The role of humoral immunity, including the effect of both antibodies and complement in the progression of schistosomiasis is rather complex (reviewed by Capron *et al.* 1992; Capron & Capron, 1986; Phillips & Colley, 1978). The injection of anti-μ antibody into neonatal rats, prior to exposure with cercariae of *S. mansoni*, resulted in the active suppression of immunoglobulin synthesis, but did not significantly alter the worm burden, compared to irrelevant Ig controls (Bazin *et al.* 1980). However, in this study the primary worm burden was recovered by portal perfusion at day 28 post-infection, at a time when schistosome elimination has been initiated rather than completed (Cioli, Knopf & Senft, 1977; Phillips *et al* 1975; Smithers & Terry, 1965; Ritchie, Garson & Knight, 1963). Thus, it proved difficult to negate a role for antibody in the early immune response following an initial infection (Bazin *et al.* 1980).

1.10.1 *Passive transfer of immune serum*

As an alternative approach, serum from previously exposed animals (immune serum) was passively transferred to unexposed syngeneic recipients, in order to determine the nature of humoral immunity during infection. It was shown that immune serum, obtained from donor rats infected for 3.5 weeks, conferred no protection when transferred to recipients. In fact, large 3ml doses of serum appeared to promote worm development in these animals. Moreover, when cells, which transferred protection to recipient rats, and serum were injected simultaneously into naive animals, the serum abrogated the anticipated protective effect (Phillips *et al.* 1975). Conversely, serum from an animal infected for 7 weeks transferred high levels of resistance (up to 51%). In explaining this apparent paradox Santoro *et al.* (1978) found that sera obtained from schistosome-infected rats contained antigen in the form of antigen/antibody complexes, and hence it was inferred that the appearance of protective antibody in the serum would only be observed once the bulk of antigen had been neutralised. Furthermore, since serum collected from donor rats infected for less than 3.5 weeks did not passively transfer resistance (Phillips *et al.* 1975), the adult worm was implicated as the target of protective humoral responses. Additionally, by using the techniques of ion exchange chromatography and solid phase immunoabsorption with goat anti-rat IgG, adoptive resistance was attributed to the presence of IgG in the anti-serum (Phillips *et al.* 1975). Phillips *et al.* (1975) concluded that the protective humoral response was directed against

mature parasites and that antibody, particularly IgG, was able to influence the course of infection, albeit in an extremely complex manner.

A second series of passive transfer experiments proved that T cell competence was crucial to the development of an effective humoral response. Athymic rats infected with *S. mansoni* cercariae were bled at weekly intervals after exposure and the serum collected was passively transferred to thymus-intact rats (Capron *et al.* 1983; Phillips *et al.* 1983). The results showed that serum collected from athymic rats did not transfer the same degree of resistance to naive intact animals when compared to the successful transfer of immune serum from T cell competent donors. Therefore, it appears that T cells are required in the development of a protective antibody response. Additionally, upon performing analogous studies using serum from intact rats passively transferred into athymic individuals, it was found that the adult worm burden was significantly diminished compared to control animals which received normal rat serum alone (NRS; Capron *et al.* 1983; Phillips *et al.* 1983). Thus, the athymic animals possess an intact effector mechanism(s), but require the transfer of schistosome-specific antibody from heterozygous animals to prime the protective effector cell population(s).

1.10.2 *The IgE isotype*

The participation of anaphylactic antibodies in the immune response to schistosomes was suggested by Sadun and Gore (1970) who found an inverse relationship between the level of homocytotropic antibody and susceptibility to infection in various animal species. Later, additional support for the involvement of reaginic antibody was provided from the study of Bazin *et al.* (1980) who demonstrated that from day 28 post-infection rats which were injected with NRS, and not anti-μ immunoglobulin, produced elevated titers of IgE and IgG isotypes compared to other antibody classes.

The role of IgE in anaphylaxis has been well documented, and hence several studies evaluated IgE antibody production in rats during an infection with *S. mansoni* (recent review by Capron *et al.* 1992; Reiner & Zahner, 1986; Rousseaux-Prevost *et al.* 1978; Rousseaux-Prevost, Bazin & Capron, 1977). Workers found that total IgE levels were raised after about 3 weeks post-infection, and reached a plateau after 5 weeks, whereas the antigen-specific IgE increased steadily until week 15 (Reiner & Zahner, 1986; Rousseaux-Prevost *et al.* 1978) following a low infection dose of 500 cercariae per animal (Reiner & Zahner, 1986). Approximately 10-20% of total IgE was found to be parasite-specific in *S. mansoni*-infected rats (Rousseaux-Prevost *et*

al. 1978). The difference between total and specific IgE levels is ascribed to the ability of schistosome infections to potentiate IgE responses to non-parasite antigens, as seen in other parasite infections, such as *Nippostrongylus brasiliensis* (Jarrett & Miller, 1982; Orr & Blair, 1969). Furthermore, the longevity of the reagin response has been attributed to the low level persistence of live parasites in the host (Phillips *et al.* 1978; Rousseaux-Prevost *et al.* 1977).

The changes in serum IgE levels are correlated with the parasite population in laboratory rats. It appears that worm rejection, starting on day 28, occurs rapidly until day 35, then continues less quickly until day 70 (Cioli, Knopf & Senft, 1977; Phillips *et al.* 1977). Rousseaux-Prevost *et al.* (1978) recovered adult worms from rats over a longitudinal time course and found that the highest IgE level was obtained on day 42 approximately 2 weeks after the start of worm rejection. Furthermore, the decline in total IgE levels between days 42 and 50, followed a period of rapid parasite clearance (Rousseaux-Prevost *et al.* 1978; Rousseaux-Prevost *et al.* 1977). This correlation suggests a relationship between worm burden in the infected rat and IgE production.

Additional support for the importance of IgE in worm elimination was given following a series of passive transfer experiments using immune serum selectively depleted of IgE by immunoabsorptive procedures. The removal of this antibody class resulted in a highly significant, though partial decrease (50%) in immunity to schistosomes, as determined by the recovery of a primary infection from recipient rats (Capron *et al.* 1980). This finding supported the involvement of IgE in protection. Furthermore, a more recent study used an IgE mAb (Verwaerde *et al.* 1987), against an adult worm secretory product, to immunise naive rats, prior to a schistosome infection. The passive transfer of this mAb achieved high levels of resistance (40–60%), as assessed from worm recoveries on day 21 post-infection.

Although an elevation of total IgE is a consistent feature of infection with schistosomes, the level attained depends upon the genetically determined IgE-producing capacity of the host. Thus, while peak levels of >50μgIgE/ml are the norm for *S.mansoni*-infected Hooded Lister rats, Fischer rats only produce IgE in the region of 11μg/ml after infection (Rousseaux-Prevost *et al.* 1977). For the most part, studies of *S. mansoni* in rats have used the inbred Fischer strain and hence most of the work described in this introduction will refer to this strain, unless stated otherwise.

Results obtained from specific radioimmunoassays and immunoabsorptive procedures have established the presence of circulating immune complexes in

infected rat hosts. Almost 70% of the total circulating antigens and 13% of the IgE antibody were found within the immune complex fraction (Santoro *et al.* 1978). Thus, it was proposed that following infection a significant proportion of the specific–IgE in the serum becomes associated with antigen, again demonstrating the importance of IgE in schistosomiasis. However, since most of the IgE in the body is bound to the surfaces of cells expressing receptors for the Fc portion of IgE (Fc∈RI/II), the value obtained for parasite–specific IgE is probably an underestimate (Chen & Enerback, 1992). Hence, the amount of IgE actually bound to antigen in the host bloodstream most likely represents a small fraction of total IgE (serum and cell–bound immunoglobulin).

Other factors limit the accurate determination of total IgE and in particular parasite–specific IgE levels. Firstly, only a minute amount of IgE is present in the bloodstream compared to other isotypes. For example the amount of IgE in humans is 50–300ng/ml compared with 10mg/ml of IgG (Sutton & Gould, 1993). Secondly, evidence exists for the blocking of IgE reactivity by IgG4 antibody in humans infected with schistosomes (Demeure *et al.* 1993; Rihet *et al.* 1992; Hagan *et al.* 1991; Boctor & Peter, 1990; Butterworth *et al.* 1987). This inhibitory IgG activity is also observed in rats, thereby making the detection of parasite–specific IgE difficult. Different experimental approaches have been designed to counteract the problem of low IgE titers, such as the passive cutaneous anaphylaxis assay (PCA) and the radioallergosorbent test (RAST). However, PCA requires the use of large numbers of animals, and both methods are still affected by IgG blocking reactivity. With more recent technological advances it is now possible to purify IgE, thereby effectively removing IgG antibodies from infection serum (Rihet *et al.* 1992; Ahmad *et al.* 1991).

The T–cell derived cytokine IL–4 appears to be a crucial factor in the selective switching of antibody responses to IgE and the subsequent production of IgE from B cells (Mosmann & Coffman, 1989). Moreover, this effect appears to be exerted at the level of the germline, since it has been shown that IL–4 directs recombination to the constant region of the ∈–heavy chain (Xu & Rothman, 1994). In man, two other signals are also involved in the regulation of IgE responses namely, the CD40 ligand (CD40L) and the Fc∈RII (Gordon *et al.* 1991; Noelle, Ledbetter & Aruffo, 1992). The finding that Th cells (Mendlovic *et al.* 1987; Phillips *et al.* 1987; Phillips *et al.* 1983) and IgE responses (Capron *et al.* 1980) are involved in the expression of immunity to schistosomes is consistent with the observation that Th cells are the primary source of IL–4 in the early activation phase of the immune response.

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Additionally, it is now clear that mast cells are also able to enhance the production of IgE from B cells by the release of IL–4 upon IgE–dependent or IgE–independent stimulation (Gauchat *et al.* 1993; Williams *et al.* 1993; Plaut *et al.* 1989).

1.10.3 *The IgG2a isotype*

The IgG2a antibody isotype is also associated with anaphylactic reactivity. In addition, the production of IgG2a has been shown to increase during schistosome infections. By using anti–immunoglobulin–coated sheep red blood cells (SRBC) to bind eosinophils from infected rats, the sequential expression of various antibody isotypes (IgG, IgG2a and IgE) was demonstrated during infection (Capron *et al.* 1984). It appeared that at the early stage of worm elimination (4 weeks post–infection) eosinophils bound to IgG2a–coated SRBC, whereas eosinophils obtained from rats infected for 5 to 8 weeks, preferentially bound SRBC coated with IgE (Capron *et al.* 1984). These results are in agreement with earlier *in vitro* findings, which required the same sequence of antibody isotypes for rat eosinophil–mediated killing of schistosomula; IgG2a from immune serum collected at 4 weeks (Capron *et al.* 1978a) and IgE antibody from immune serum collected at 6 to 8 weeks (Capron *et al.* 1980). However, it must be emphasised that the artificial *in vitro* system does not reflect the situation *in vivo*, as eosinophils are rarely found in direct contact with schistosomula (Bentley, Carlisle & Phillips, 1981). Furthermore, the older parasite stages are refractory to eosinophil–mediated cytotoxicity (Auriault *et al.* 1981).

A more direct proof of IgG2a participation in immunity was given by experiments which used serum depleted of IgG2a, or untreated serum, to passively transfer resistance to recipient animals (Capron *et al.* 1980). As was the case for IgE, removal of the IgG2a fraction partially abrogated (50%) protection to schistosomes in rats. In a second series of experiments Grzych *et al.* (1982) found that a rat IgG2a mAb injected into normal rats 4h after infection with *S. mansoni* conferred highly significant levels of protection (between 53 and 62%). In comparison, no measurable protective effect was achieved in rats who received ascitic fluids, either induced by myeloma cells alone, or ascitic fluid obtained from clones producing monoclonal IgG2a directed against an unrelated antigen (Grzych *et al.* 1982). Thus, it appears that the induction of immunity to schistosomes in rats is dependent, in part, upon the early production of antigen–specific IgG2a following infection. Furthermore, the priming of rat eosinophils *in vitro* requires the presence of this antibody isotype.

From this resume on humoral immunity to schistosomes in rats it is apparent that IgE

and IgG2a could be important components in the immunological mechanism of worm elimination. However, their unequivocal role in the protective immune response *in vivo* remains to be clarified. It is envisaged that with the advent of genetically manipulated animals, and the identification of Th subsets in rats, definitive investigations into the multifactorial antibody response directed against schistosomes will become possible.

1.11 *Adult worm elimination: The effector cell response*

Although T cells and specific antibody production have been associated with the elimination of adult schistosomes, it is debatable whether these factors induce the non-specific attrition of worms. The protective effects of cytotoxic T cells and complement-mediated immunity have been reported in other infections, but equivalent roles for these components in schistosomiasis have not been proven. The following section will focus upon histological evidence for the involvement of specific effector cells in parasite elimination, the proposed antibody-dependent cell cytotoxicity (ADCC) mechanism, and finally proof of the protective role of immune effector cells *in vivo* by the passive transfer of such cells to naive recipients.

1.11.1 *Evidence for the involvement of specific effector mechanisms*

Since it appeared that a specific mechanism was involved in worm elimination immunoparasitologists turned their attention to the role of eosinophils, macrophages and mast cells in rats. Histopathological examination had demonstrated that these effector cells were found in large numbers within parasitised tissue. Worms in the hepatic portal system 17 to 19 days post-infection, were consistently observed near the outer surface of the liver, in vessels only slightly larger than the parasites themselves. A number of cell types, eosinophils, mononuclear cells and mast cells were recruited to the hepatic tissues surrounding the portal venules, thereby forming extravascular inflammatory foci immediately adjacent to the endothelium and to the parasites (Bentley, Carlisle & Phillips, 1981; Phillips *et al.* 1983). The eosinophils were fully mature but were not degranulating, in contrast to the few mast cells present which were in the process of degranulation (Miller *et al.* 1994; Bentley, Carlisle & Phillips, 1981; Phillips *et al.* 1983). Additionally, cells were not found in direct contact with schistosomes, which appeared intact despite the intense inflammatory reactivity in surrounding tissues. Conversely, electron microscopy studies of infected rat tissues by Phillips *et al.* (1983) showed that, in a few cases,

eosinophils were found bound to the surface of liver parasites. Often their characteristic cytoplasmic granules were seen to be orientated toward the parasite membrane, which appeared very dense, highly vacuolated and irregular. Further evidence for the tegument as the target of immune attrition came from earlier studies of 35 day old worms obtained from permissive mice/hamsters and non-permissive rat hosts (Senft, Gibler & Knopf, 1978). In rats, the maturation of the schistosome tegument was both delayed and incomplete, and thus may partly explain the rapid degeneration of parasites in non-permissive hosts.

Alternatively, direct damage to the majority of parasites may require a longer period of time, as proposed by Knopf (1979). This author observed granulomatous liver foci in *S. mansoni*-infected rats at later stages of infection (5, 11 and 16 weeks). Whilst some schistosomes appeared intact others were infiltrated by inflammatory cells, mostly eosinophils. Hence, eosinophils were implicated in the killing of worms from week five post-infection (Knopf, 1979). However, it is not clear from Knopf's study whether the eosinophils mediated direct damage to the schistosomes *in vivo*, or whether they were recruited after parasite death.

Additional support for the *in vivo* schistosomicidal activity of eosinophils was given by another histological study that examined tissues from athymic rats, which had been exposed to *S.mansoni* cercariae (Phillips *et al.* 1983). Immunosuppressed animals exhibited an impaired peripheral and tissue eosinophilia, and increased worm survival, compared to intact animals. This observation corroborated earlier work by Knopf (1979) who reported a peak in peripheral eosinophilia corresponding to the period of worm elimination in the liver. Furthermore, the parasites in athymic rats inhabited the larger portal venules and the intestinal mesenteries. However, it must be noted that both these studies do not rule out the participation of other cells in the effector mechanism(s). Furthermore, it is not clear whether the delayed onset of worm elimination in T cell depleted rats is correlated with an impaired eosinophilia, or with the establishment of worms in more favourable environments, within the larger portal venules and the mesenteries. Nonetheless, it appears that the development and recruitment of eosinophils to parasitised sites is T cell dependent.

1.11.2 *Parasite killing in vitro – ADCC mechanisms*

Most of the studies which have analysed immunity to infection in rats have relied upon a plethora of *in vitro* investigations which most certainly fail to reflect the synergistic and orchestrated responses that occur *in vivo*. Furthermore, the cell cytotoxicity assays measure the extent of antibody-mediated killing against 3h skin-

stage schistosomula. Thus, it is difficult to extrapolate these observations to the immune mechanism operating against adult worms *in vivo*, particularly in light of the immunopathological data which indicate that these effector cells remain in extravascular sites. Nevertheless, information has been derived regarding the activation of eosinophils, macrophages and mast cells by anaphylactic antibody, and thus has proved valuable in understanding cell–cell co–operation, as well as cell–parasite interaction.

Hence, a large bulk of information has accumulated regarding ADCC mechanisms directed against schistosomes and their role in protective immunity to primary and secondary infections in rats. Bearing in mind that evidence for ADCC mediated killing of adult worms has not been demonstrated *in vivo* the following resumé of such effector mechanisms should be viewed with scepticism.

a. *Eosinophils*

It has been proposed that two stages of eosinophil activation are pivotal in the establishment of parasite killing *in vitro*. Initially, the production of significant levels of IgG2a during worm elimination, has been associated with the induction of eosinophil–mediated cytotoxicity in culture (Capron *et al.* 1978a). Indirect evidence supporting this hypothesis showed that eosinophils from infected rats could kill non–opsonised schistosomula directly, at the very period when IgG2a from immune serum activated killing by normal rat eosinophils. Additionally, a longitudinal study revealed that co–operation between IgG2a and eosinophils was required to mediate the attrition of schistosomula at about 4 weeks of infection (Capron & Dessaint, 1985; Capron *et al.* 1978a). However, by the ninth week after exposure to cercariae the protective effector function induced by IgG2a *in vitro* was inhibited (Capron, Torpier & Capron, 1979).

It was envisaged that IgE antibody would also promote eosinophil activation *in vitro*, since IgE levels increase significantly following schistosome infection (Reiner & Zahner, 1986; Rousseaux–Prevost *et al.* 1978; Rousseaux–Prevost *et al.* 1977). Indeed, it was found that after 6 weeks a heat–labile, complement independent antibody was responsible for the observed cytotoxicity against schistosomula *in vitro*, which was no longer attributable to IgG2a (Capron *et al.* 1980).

b. *Macrophages*

Macrophage cytotoxicity assays, directly analogous to those described for eosinophils above, have shown that the schistosomicidal activity of macrophages is effective *in vitro*. However, as in the case of eosinophils the proposed role for macrophage–mediated schistosome killing has not been demonstrated conclusively

in vivo.

Preliminary longitudinal studies demonstrated that normal rat peritoneal macrophages could be stimulated to kill schistosomula *in vitro* when preincubated with immune serum obtained from rats infected for 6 weeks (Capron *et al.* 1976). IgE was proposed as the heat-labile, complement-independent activation factor involved; this hypothesis was confirmed by the reduced ability of IgE-depleted immune serum to prime macrophages for schistosome killing (Capron *et al.* 1976). Moreover, marked alterations of the target parasite were found in ultrastructural studies of macrophage-induced schistosomula killing *in vitro*. After 18h of incubation, the antibody-primed mononuclear phagocytes were found adherent to the larval surface, with the macrophage microvilli associated with perforations of the schistosome tegument (Capron, Dessaint & Capron, 1977).

c. *Platelets*

In the context of ADCC mechanisms, it is relevant to mention the anti-schistosome activity of rat blood platelets. Platelets are common blood components, comprising nearly 34% of the total blood leucocyte volume. Using the same approaches of *in vitro* cytotoxicity assays and adoptive transfer of immune cells to naive rats, described for eosinophils and macrophages above, it was found that the killing properties of rat platelets was significant at day 54 (week 7 to 8) post-infection (Joseph *et al.* 1983). Moreover, the adoptive transfer of immune platelets, obtained from rats at day 42 after exposure, conferred a high degree of protection (63%) when injected into normal syngeneic recipients. Further, the antibody-dependent nature of activation has been confirmed, since it is known that human platelets express FcϵRII at their surfaces (Capron *et al.* 1986).

d. *Mast cells*

Preliminary *in vitro* experiments indicated that mast cell depletion from eosinophil-rich cell populations resulted in decreased eosinophil cytotoxicity (from 50% in the presence of mast cells to 20% without) to cultured schistosomula (Capron *et al.* 1978b). Furthermore, optimal levels of eosinophil-effected parasite killing could be restored with the addition of more than 1% purified mast cells to the assay. However, mast cells preincubated with immune rat serum were unable to mediate schistosomula killing directly *in vitro*. Thus, this data implicates mast cells as an important accessory cell for eosinophil activation.

Additionally, the requirement for intact mast cells was obviated following the demonstration that medium removed from degranulated rat mast cells, was able to trigger eosinophil activation in the presence of immune serum (Capron *et al.* 1978b).

These authors supposed that eosinophil chemotactic factor (ECF–A tetrapeptides) released from degranulating mast cells could be the soluble mediator involved in eosinophil priming. However, this does not mean that ECF–A is the only signal required, since it is known that many potent chemokines and cytokines are also secreted by these cells (Plaut *et al.* 1989).

1.11.3 *Effector cell responses that mediate parasite killing in vivo*

Although these experiments have suggested a direct role for anaphylactic antibody, eosinophils, macrophages, platelets and mast cells in schistosomula killing *in vitro*, it must be remembered that the biologic reactivity of these cells *in vivo* is probably more complex than presumed. Furthermore, all of the ADCC assays described have focused upon the killing of larvae and not adult worms. Thus, several different approaches have been adopted to investigate the effector mechanisms which mediate the killing of adult worms *in vivo*. The first approach involves the recovery of effector cells from infected rats at various intervals post–infection, and subsequent analysis of the immunoglobulin isotypes bound to their surfaces.

SRBC coated with various antibody isotypes permitted identification of the immunoglobulins which bound eosinophils from infected rats. At 4 weeks post–infection eosinophils adhered to IgG2a–coated SRBC, whereas by six to eight weeks eosinophils bound to IgE–coated erythrocytes (Capron *et al.* 1984). However, this technique only demonstrates the presence of antibody at effector cell surfaces, it does not provide an insight into the anti–schistosome function of eosinophils, macrophages, platelets and mast cells.

Thus, a second approach was designed to address the protective capacity of potential effector cells *in vivo*. Eosinophils and macrophages were transferred from infected donors, at different times post–infection, to naive recipients which were then exposed to schistosome cercariae. Attempts to sensitise normal rat eosinophils with IgG2a from immune serum, prior to injection into recipient animals, has proved largely unsuccessful. More recently, Pestel *et al.* (1988) found that Fc∈R, in association with IgE–rich serum, contributed to enhanced levels of worm elimination in rats which received activated macrophages. The level of immunity to schistosomes was determined following portal perfusion 21 days post–infection. Up to 64% resistance was afforded using immune macrophages injected simultaneously with immune serum (6 weeks), compared to 31% resistance acquired with the transfer of normal macrophages and immune serum (Pestel *et al.* 1988). Thus, the data support the antigen–specific nature of macrophage cytotoxicity and suggest an

active role for Fc∈R-bearing cells in the expression of protection to schistosomes. However, the protective nature of these activated cell populations was evaluated by recovering worms from recipients 21 days post-infection, and since the elimination of adult worms does not proceed before day 28 any resistance measured was as a consequence of larval killing. Furthermore, much of the protection achieved by the administration of immune serum could be attributed to the binding of anaphylactic antibodies to other Fc∈R$^+$ cells, such as eosinophils, mast cells and platelets (Capron *et al.* 1986). Moreover, the prolific expression of Fc∈RII on the surface of a variety of effector cell types begged the question of which receptor positive cells mediated which effector function of IgE *in vivo*. Hence, definitive elucidation of the specific effector mechanism operating against adult worms is difficult. It is apparent that these studies have failed to demonstrate conclusive evidence for the role of ADCC mechanisms operating against adult schistosomes *in vivo*.

The third approach in the study of effector mechanisms acting against adult worms *in vivo*, involves the histological examination of infected rat tissues; this technique has provided the most convincing evidence for effector cell reactivity during schistosomiasis. Irrespective of the rosetting and transfer data, ultrastructural analysis of the cellular response in the livers of infected rats 17 to 19 days post-exposure has revealed that macrophages *are* present in large numbers in the primary anti-schistosome foci. They appear to contain a striking amount of electron-dense material, which is probably attributable to schistosomal pigment (Bentley, Carlisle & Phillips, 1981). Thus, it is evident that macrophages recruited to the site of inflammation could directly influence schistosome viability *in vivo* by their characteristic cytotoxic (superoxide dismutase) and phagocytic properties. However, since macrophages were not identified in direct contact with the adult worm surface *in vivo*, it would be presumptious to assume a role for macrophages in the immune attrition of parasites in the infected rat.

Despite the low numbers of mast cells detectable in the granulomatous liver lesions surrounding parasites at days 17 to 19 post-infection (Bentley, Carlisle & Phillips, 1981), this effector cell is capable of augmenting IH reactions through the release of vasoactive amines, as well as chemotactic factors of anaphylaxis, such as ECF-A and the interleukins 4 and 5 (Plaut *et al.* 1989). It is well documented that antigen-induced degranulation of basophils and mast cells is initiated by cross-linking of IgE on the cell membrane (Weltman & Senft, 1983; Siraganian, Hock & Levine, 1975), thereby indicating the existence of Fc∈R at the mast cell surface. Indeed, the high affinity receptor (Fc∈RI) is responsible for the binding of IgE via

the Fc region, and the subsequent triggering of mediator release through a calcium-dependent biochemical cascade upon IgE crosslinking (Sutton & Gould, 1993). Mediator release and the basic immunobiology of mast cells will be discussed in section 1.10.

More recent serological and histopathological analyses of schistosome infections in Fischer rats and C57BL/6 mice have documented the manifestation of a pronounced hepatic mastocytosis in the former, compared to a less extensive intestinal mastocytosis in the latter (Miller *et al.* 1994). Additionally, the degree of mast cell degranulation *in vivo* was evaluated by measuring the levels of rat mast cell proteinases (RMCP) I and II, and mouse mast cell proteinses (MMCP) I and II, in the hosts' bloodstream and parasitised tissues. These authors detected raised levels of RMCP I and II in the serum and livers of infected rats from day 21 post-infection. The levels appeared to peak at day 35 and then decline slowly until termination of the experiment. Thus, it was concluded that mast cell degranulation progressed from day 21 after exposure, one week prior to worm elimination in rats; a period associated with the accumulation of mast cells in the liver (Miller *et al.* 1994). Furthermore, comparative analysis of mast cell responsiveness in mice revealed that systemic and tissue MMCP content was elevated, but not to the same extent when compared to rats. Taken together, it seems that rats are able to mount a more significant mast cell response to liver worms than mice, and that parasite elimination in non-permissive hosts occurs coincident with RMCP I and II release. Moreover, schistosome attrition may be a consequence of the level of degranulation in rats, and/or may be induced by the manifestation of a hepatic mastocytosis rather than an intestinal mastocytosis.

It seems that mast cells may perform two distinct functions in the effector mechanism of immunity to schistosomes. Firstly, they may trigger the recruitment and subsequent extravasation of inflammatory cells and antibody into the hepatic tissue, thereby facilitating the destruction of worms lodged in the peri-portal venules. Secondly, they might enhance the killing activity of eosinophils and other effector cells by the release of a battery of chemokines and interleukins (Seder *et al.* 1991).

PART TWO: MAST CELLS

1.12 *Mast cells – basic biology and function*

In view of the study by Miller *et al.* (1994), the final chapter of this thesis focuses upon the activation of a rat basophilic leukemia (RBL) cell line with immune rat serum, and the subsequent degranulation of these cells upon stimulation with schistosome antigen preparations. Thus, the following section will outline the important features of mast cell biology, with regard to the requirements for mast cell activation, mediator release, regulation and function.

1.12.1 *The Fc∈RI*

The ability of mast cells to participate in IgE–mediated reactions is due to the presence of Fc receptors for IgE on their surface. When a multivalent allergen binds with Fc∈RI–associated IgE, it crosslinks receptor molecules on the plasma membrane, triggering degranulation of the cell which involves the rapid release of stored mediators and the secretion of cytokines that attract and activate other inflammatory cells (Seder *et al.*, 1991). Many studies have used a rat basophilic leukemia (RBL) cell line, as an analogous model of mast cell function, in order to characterise the receptor and to analyse the biochemical triggers for mast cell degranulation (Barsumian *et al.* 1981). The Fc∈RI in RBL–1 cells and normal rat mast cells is composed of four transmembrane polypeptides with the composition $\alpha\beta\gamma_2$, and while the α–chain binds the Fc portion of IgE, the β and γ–chains are required for insertion of the α–chain into the membrane and for signal transduction (Beaven & Metzger, 1993). The extracellular region of the α–chain contains two domains characteristic of the immunoglobulin superfamily (Sutton & Gould, 1993).

In most species, including the rat, the majority of total IgE (67%) in the body is found bound to the surface of cells (Chen & Enerback, 1992). The avidity of binding is high and the number of receptor sites per human mast cell ranges from 1×10^5 to 5×10^5 (Parker, 1984). After infection with *N. brasiliensis*, the density of Fc∈RI on mast cells increases along with enhanced production of IgE (Chen & Enerback, 1992). Malveaux *et al.* (1978) suggested that the increase in surface receptors was probably due to the induction of Fc∈RI synthesis as a result of elevated IgE titers. Thus, it is thought that the increased levels of IgE, together with augmented Fc∈RI expression serve to amplify the inflammatory response following infection.

As far as is known, monomeric IgE does not stimulate mediator release. Release

occurs when IgE receptor molecules are cross-linked by multivalent antigen and IgE, by aggregated IgE, by IgE and bivalent anti-IgE antibody, or by bivalent ant-receptor antibody (Metcalfe, Kaliner & Donlon, 1981). Additionally, the induction of exocytosis or degranulation is associated with a change in distribution of IgE receptors at the mast cell surface. Thus, the cross-linking of Fc∈RI induces their aggregation, and may explain the segumental secretion of mast cell granules in the immediate area of an antigen stimulus (Sullivan & Parker, 1976). Recently an additional molecule, identified as the memory marker CD45, has been associated with Fc∈RI-mediated degranulation of murine mast cells (Berger, Mak & Paige, 1994). Mutant mouse mast cells that do not express CD45 are unable to degranulate in response to IgE-dependent stimulation. However, their biochemical machinery for exocytosis appears intact, as they release mediators upon activation with the calcium ionophore A23187. The existence of a similar marker remains to be elucidated with respect to rat mast cells.

In addition to the Fc∈RI, mast cells also express receptors for IgG (FcγR; Lobell, Austen & Katz, 1994; Alber, Kent & Metzger, 1992) and complement (C3b; Sher & McIntyre, 1977). However it is debatable whether cross-linking of the FcγR triggers degranulation *in vivo*, since this reactivity has only been shown in transfected RBL cells.

IgE-independent exocytosis may be induced by naturally occurring agonists, such as adenosine triphosphate (ATP), chymotrypsin, phospholipase A and C, lymphokines and neuropeptide (Kuby, 1992). Although these responses show many similarities to IgE-dependent reactions in the composition of released products, they do not stimulate intracellular signalling events via the Fc∈RI.

1.12.2 *Mediator release*

The rapidity of mediator release by mast cells reinforces the importance of this effector cell in orchestrating IH responses. IgE-dependent or independent stimulation results in the release of histamine and other chemokines to the cell exterior 15 to 20 seconds after application of antigen/secretagogue (Kuby, 1992). As mentioned above, IgE-dependent signalling is via the transmembrane β and γ-chains of the Fc∈RI (Sutton & Gould, 1993). These early events require the induction of intracellular transduction pathways which operate in a biochemical cascade finally culminating in the exocytosis of potent mediators (Beaven & Metzger, 1993).

One of the most striking changes in intracellular metabolism is the rapid breakdown and conversion, or resynthesis of membrane phospholipids. Phosphatidic

acid (PA) is one of the early precursors of mast cell degranulation and its presence is demonstrable within 3 seconds of stimulation. Furthermore, this precursor has been reported to act as a calcium ionophore in model lipid membranes, thus providing a possible mechanism for the observed increases in calcium occurring during mast cell activation. Once PA is phosphorylated via the enzymatic action of phospholipase A_2, raised levels of phosphatidylinositol and phosphatidylcholine are observed; in addition, there is a later phase (30 to 60 seconds) of methylation. Of course all these events require optimal conditions for enzyme function: a temperature of 37°C, an influx/efflux of calcium and the presence of intracellular ATP. The cellular content of cyclic AMP rises 15 to 20 seconds after stimulation (Sullivan & Parker, 1976), presumably through the activation of adenylate cyclase. All these acute changes in mast cell metabolism contribute towards physical changes at the ultrastructural level. Initiation of the exocytosis is achieved by the fusion of the plasma membrane with the perigranular membranes immediately beneath the cell surface. As a result, communication channels are formed between the granules and the interstitial medium, yet although some granules are actually secreted, others appear to release their contents without ever leaving the cell (Langunoff, 1980).

Degranulation is a rapid transient process since the typical IgE–mediated reaction is maximal at 10 to 30 minutes. However, some allergic responses, like those in the skin and bronchi continue for many hours and are characterised by the increasing strength of the challenging dose of allergen. The mediators involved in these chronic responses may differ from those released in more transient IgE–mediated responses.

Upon stimulation the mast cells release a host of potent mediators; histamine, heparin, ATP, proteolytic enzymes, chemotactic factors for neutrophils and eosinophils (NCF, ECF–A tetrapeptides), serotonin, arachidonic acid metabolites (leukotrienes, prostaglandins), platelet activating factor (PAF), and a wide range of multifunctional cytokines (Seder *et al.* 1991; Plaut *et al.* 1989) that may regulate their own function as well as influence other cells within the microenvironment. All of these factors serve to amplify the inflammatory response within a localised area by increasing vascular permeability, and encouraging the extravasation of soluble mediators, which recruit and prime cytotoxic effector cells.

1.12.3 *Mast cell heterogeneity*

Morphological and functional heterogeneity of mast cells exists in rats as demonstrated by specific biochemical and histochemical characteristics; two functional cell sub–types appear to predominate. Mucosal mast cells (MMC) are

smaller and less granular than connective tissue mast cells (CTMC), and stain blue using the alcian blue staining procedure, compared to the granules of CTMCs which stain red (Enerback, 1966). However, the distinction is not absolute as CTMCs in the dermis exhibit varying amounts of blue and red stain, with some staining blue alone (Enerback, 1966).

Biochemical analysis of the granule constituents has revealed differences in the proteoglycan (heparin versus chondroitin sulphate) and serine protease (RMCP I versus RMCP II) content of CTMC compared to MMC. The apparent site–dependent expression of RMCP I and II indicated that each could be used as a marker for CTMC and MMC respectively (Gibson & Miller, 1986). However, further investigation found that the differences were not mutually exclusive, and that tissue site was not a reliable indicator of mast cell sub–types (Gibson *et al.* 1987). A later study cast further doubt upon the discrete nature of mast cell heterogeneity, as Huntley *et al.* (1990) described a population of mast cells expressing both proteases in the lungs and livers of rats, as determined by ELISA and paired immunofluoresence.

One possible explanation for this lack of clarity between mast cell sub–types is that the functional characteristics are reversible, and that CTMCs can transform into MMCs and vice versa. This supposition has been demonstrated *in vitro* using rat and murine bone marrow mast cells (BMMCs), which were derived from haemopoietic precursor cells in the presence of the T cell derived cytokine IL–3 (Haig, 1988). These cultured cells are considered to be the *in vitro* equivalent of MMCs, since they share functional, histochemical and biochemical characteristics (Schrader *et al.* 1981). Dayton *et al.* (1988) found that when murine BMMCs were cultured on 3T3–fibroblasts they underwent a change in proteoglycan histochemistry, consistent with a phenotype change from MMCs to CTMCs. It appeared that factors released from the 3T3–fibroblasts were initiating the transformation of cell type in this model.

Other signals are involved in the differentiation and maturation of rodent BMMCs *in vitro* namely the T cell dependent cytokines IL–3 and IL–4 (reviewed by Castells, Katz & Austen, 1992; Stevens & Austen, 1989), and stem cell factor (SCF; *c–kit* ligand), which induces the proliferation of rat mast cells *in vivo* (Tsai *et al.* 1991). Further information regarding the regulation of mast cell responses by cytokines is given below.

1.12.4 *Regulation of mast cell differentiation and function*

A convenient and quantitiative method for obtaining pure populations of rat

BMMCs was discovered by culturing haematopoietic progenitor cells in conditioned medium derived from lectin-activated T cells. These rat BMMCs resemble rat MMCs in their histochemical properties, histamine and serine proteinase content. The cytokine involved in the preferential growth and differentiation of progenitor cells to mast cells was identified as IL-3 (Haig, 1988), but IL-4 can act in synergy with IL-3 to stimulate increased growth of mast cells (Hamaguchi *et al.* 1987). Furthermore, IL-9 and IL-10 augment the proliferation of murine BMMCs *in vitro*, but do not support cell growth when added to the medium in isolation (Kitamura *et al.* 1993). Rat CTMCs, in contrast, are not maintained in culture with T cell supernatants (Levi-Schaffer *et al.* 1985), which therefore indicates a T cell independent mechanism for the differentiation and proliferation of CTMCs. Hence, it appears that the early events in the regulation and differentiation of mastocytosis are dependent upon IL-3 and IL-4. Furthermore, it is concluded that MMCs require T cell signalling mechanisms, whereas CTMCs develop in the absence of T cell factors.

Helminth-infected rodents invariably generate an IH reaction to invasive parasites, in which elevated IgE production, eosinophilia and mastocytosis are prominent features. Thus, the infection of mice/rats with *N. brasiliensis*, *Trichinella spiralis*, and *S. mansoni* provides an ideal opportunity to manipulate, and to observe the effects of specific cytokines upon effector cell populations and the outcome of infection. For example, Madden *et al* (1991) administered mAb against IL-3 or IL-4 to mice prior to exposure with *N. brasiliensis*. These authors found that the depletion of either of these Th2-associated cytokines resulted in a partially abrogated mastocytosis, which is a characteristic feature of infection. Upon simultaneous injection of anti-IL-3 and anti-IL-4 immunoglobulin the mastocytosis was reduced by approximately 90%, when compared to control animals. This *in vivo* evidence supports the conclusions drawn from earlier *in vitro* experiments. Further, although IL-10 is known to increase the expression of MMCP in mast cells (Ghildyal *et al.* 1993; 1992), a similar role for IL-9 and IL-10 in the regulation and maturation of mast cells *in vivo* has yet to be determined.

From Madden's study (1991) it is apparent that other factors must be involved in the development of a mastocytosis in mice and rats. Direct proof for the involvement of another signal was provided in a series of studies that used rats and mice deficient in mast cells (Ws/Ws and W/W^v, respectively). The W locus encodes for the *c-kit* receptor. Initially, it was shown that a factor, produced by endothelial cells and other stromal cells, was able to induce murine BMMC proliferation on the

NIH/3T3-fibroblast cell line without the addition of T cell derived growth factors (Fujita *et al.* 1988). Furthermore, it was shown that mouse mutants, homozygous for the Sl locus, could not produce SCF, which is a ligand for the *c-kit* receptor (Huang *et al.* 1990). Thus, binding of SCF to the *c-kit* receptor appeared to be an essential prerequisite for mast cell growth and development (Valent, 1994; Galli, Tsai & Wershil, 1992).

However, the action of SCF upon mast cells *in vivo* does not appear to be that simple, since the injection of recombinant soluble SCF (rSCF) increases the number of mast cells in tissues of both SCF-deficient mice and rats, whilst the soluble SCF in normal physiologic concentration seems to have a limited effect upon the generation of mast cells (Tsai *et al.* 1991). To investigate the role of SCF upon mastocytosis during infection with the helminth *T. spiralis* Grencis *et al.* (1993) ablated SCF function by administering a rat anti-murine *c-kit* mAb (ACK-2) to mice *in vivo*. FACscan analysis showed that blocking of the SCF receptor almost completely abrogated the mast cell hyperplasia normally observed after *T. spiralis* infection (12.3% Mac-1 positive cells compared to 58.5% in controls; MAC-1 is a member of the $\beta 2$ leukocyte integrin family). Thus, these results provided further support for the *in vitro* findings, since SCF appears to be an important requirement for the generation of intestinal mastocytosis, and for the development of resistance against *Trichinella* in mice. However, it must be noted that Mac-1 is also a marker for macrophages, and hence the relative contribution of mast cells to immunity in this model is difficult to assess from Grencis's findings. In this context, it is also worth mentioning that the effect of SCF upon other cell types is as yet unknown.

The effects of rat SCF and IL-3 alone, or in combination, upon the *in vitro* growth and serine proteinase content of rat CTMC or BMMC were also determined (Haig *et al.* 1994). By adding rSCF to *in vitro* cultures for 21 days, the growth of both mast cell types was stimulated to a greater extent than with IL-3 alone. As alluded to above, IL-3 was not effective in increasing the number of rat CTMCs in culture. Moreover, the addition of rSCF facilitated the production and release of serine proteases into the medium without cross-linking of the IgE receptors. These data seem to suggest a direct role for SCF in activating mast cell growth and sub-type differentiation, as defined by the ratio of RMCP I and II produced *in vitro*. In addition, it could be inferred that SCF acts as a secretagogue. However, caution should be exercised, as this *in vitro* culture system involves the long-term growth and stimulation of mast cells in high concentrations of SCF.

The mechanism by which a rat mast cell recognises its destination is dependent

upon the expression of β–integrins at the cell surface (Hamawy, Mergenhagen & Siraganian, 1994; Gurish *et al.* 1992). The expression of these molecules promotes intercellular cohesion between mast cells and those cells which have the appropriate receptor on their surfaces. The receptors for β–integrins are presumably located on the cells lining the high endothelial venules of the gut lymphoid tissue, since it is to this tissue that MMC are recruited following intestinal helminth infection (Grencis *et al.* 1993). Indeed, the ablation of rat β–integrin by mAb resulted in a significantly diminished intestinal inflammatory response in rats infected with *T. spiralis*, compared to untreated control animals (Bell & Issekutz, 1993).

Once attracted to the source of inflammation, mast cells bind IgE antibody and become sensitised. In view of this event, it is interesting to note that IgE may also be preferentially transported to the site of inflammation by an IL–4 inducible mechanism (Ramaswamy, Hakimi & Bell, 1994). This accumulation of tissue IgE may be a consequence of mast cells themselves, since they have been shown to store IgE and to release it during degranulation, or as a consequence of other effector cell types. Thus, the transport of IgE could be a mast cell autocrine function. Subsequent to the increase in IgE levels, the density of Fc∈RI is probably enhanced (Malveaux *et al.* 1978), and as a consequence mast cell activation amplified still further.

Several mast cell lines, as well as murine BMMCs, have been found to express mRNA for granulocyte macrophage–colony stimulating factor (GM–CSF), IFN–γ, IL–1, IL–3, IL–4, IL–6 and TNF–α upon stimulation via their Fc∈RI or by exposure to calcium ionophore A23187 (Seder *et al.* 1991; Plaut *et al.* 1989). Since IL–4 release is critical for the expansion of IH responses, the release of this protein by mast cells further polarises the inflammatory reactivity (Garside & Mowat, 1995; Williams *et al.* 1993), as well as exerting an autocrine effect. IL–4 is also known to induce isotype switching at the level of the germline (Xu & Rothman, 1994), thereby promoting IgE expression and release by B cells (Gauchat *et al.* 1993). Additionally, the release of mast cell mediators elicits the recruitment and activation of other cell types, for example eosinophils, neutrophils and platelets to the site of inflammation (Garside & Mowat, 1995).

Taken together, this evidence suggests that mast cells may play a key role in IH responses not only by producing vasodilatory mediators, but also by directly regulating IgE production and transportation independently of T cells.

1.12.5 *Mast cell function*

There is ample evidence for the contribution of mast cells to the pathogenesis of inflammation, as described above. However, perhaps the most striking mast cell response is seen in infections with multicellular parasites *N. brasiliensis*, *T. spiralis*, and *S. mansoni*. As discussed, mast cells release a battery of mediators upon stimulation, some of which (peroxidase, superoxide dismutase and various acid hydrolases) are normally associated with lysosomal enzymes. Such enzymes are highly cytotoxic, and so it may be inferred that mast cells represent potent effector cells in their own right. At least *in vitro* however, the direct killing of schistosomes by mast cells has not been demonstrated (Capron *et al.* 1978b). Instead, mast cells appear to potentiate eosinophil cytotoxicity against schistosome larvae via the secretion of ECF–A tetrapeptides (Capron *et al.* 1978b).

Numerous studies have attempted to prove the involvement of mast cells by histological assessment, by modifying their responsiveness experimentally (Ws/Ws rats or anti–SCF immunoglobulin), or by the functional analysis of mast cells and their products *in vivo* and *in vitro*. Substantial work has been done in the rat following the demonstration that RMCP II was secreted in serum and local intestinal secretions during the expulsion of *N. brasiliensis* and *T. spiralis* (Woodbury *et al.* 1984). The correlation between mastocytosis and worm expulsion strongly suggested a role for mast cells, and in particular MMCs, in protection against helminth infections. Confirmation of their importance came from experiments performed in mice, in which the capacity of the host to develop a mucosal mastocytosis, and to expel *T. spiralis* was blocked by injection of a mAb (ACK–2) against the *c–kit* receptor (Grencis *et al.* 1993).

Limited information exists on the occurrence of mastocytosis in mice following schistosome infection, since the Th2–type response is thought to mediate disease rather than cure. In rats, however, the mechanism of worm elimination is associated with IgE production and hepatic cellular infiltrates containing eosinophils, macrophages and mast cells (Bentley, Carlisle & Phillips, 1981). Histopathological examination of infected rat tissues suggested the possible involvement of mast cells in schistosome infection. However, the correlation between mastocytosis and worm elimination was recently clarified (Miller *et al.* 1994).

Apart from hypersensitivity or inflammatory reactions, mast cells may also participate in normal physiological processes. One of the most interesting findings is that heparin released by mast cells can enhance vascularisation at sites of inflammation. The growth of blood vessels, along with the effects of vasoactive amines increases permeability and blood flow, which will result in increased cell numbers at sites of infection (Paul, 1984).

PART THREE: THE SECRETORY ANTIGENS OF ADULT WORMS

From the preceding description of adult worm elimination, it should be apparent that its timing is coincident with the maturation of schistosomes in the hepatic portal system. This so-called "window of sensitivity" from day 28 post-infection onwards is associated with the worms themselves, rather than the period which needs to elapse before rats are able to expel parasites (Cioli, Blum & Ruppel, 1978). Furthermore, since parasites remain viable in non-permissive and permissive hosts prior to day 28, the inference is that the hepatic effector mechanism(s) in rats is triggered by antigens released from intact schistosomes, and not dead or dying worms.

In order to sustain normal physiologic function the liver-stage worm ingests approximately 30 000 to 300 000 host red blood cells per day (Lawrence, 1973). Once ingested the erythrocytes are subjected to a host of proteolytic enzymes, which participate in the process of digestion. Some of the products of digestion are absorbed by the lumenal cells, whilst the residual material is regurgitated into the host's bloodstream. Undoubtedly, the gut contents provide a potential source of schistosome immunogens, as the worm's digestive enzymes will be released along with the breakdown products of host blood components.

A second source of potentially antigenic material is the worm tegument. The surface of the parasite is continually exposed to the hostile environment of the host, and as such represents an important host-parasite interface. Tegumental membrane synthesis and turnover is a continual process, which results in the loss of surface components throughout the worm's residence *in vivo*.

Taken together, it is clear that the adult worm is a metabolically active source of potentially immunogenic products, with the tegument and gut functioning as the major tissues of molecule release/ secretion. For this reason the next section will focus upon the structure of the tegument and gut, describing those released antigenic products which have been characterised from them. Initially, however I shall discuss the use of the *in vitro* culture system as a method for obtaining excreted/ secretory (E/S) material.

1.13 *In vitro culture as a method for obtaining released material*

The bulk of published information on the released proteins of schistosomes has relied upon the incubation of labelled worms in a defined culture medium.

Characterisation of these E/S products has been accomplished by using different culture media: saline/Hank's balanced salt solution (Tendler *et al.* 1991; Pierce *et al.* 1983; Murrell, Vannier & Ahmed, 1974), DMEM (Lewis & Strand, 1991), or more complex culture media with added serum supplements (Atkinson & Atkinson, 1982; Kusel & MacKenzie, 1975; Kusel, MacKenzie & McLaren, 1975).

The amount of material released appears to depend upon the conditions of incubation, for example the length of incubation, the type of medium used and the method of antigen detection. This contention is supported by comparing the complexity of the released products identified by different workers. Atkinson and Atkinson (1982) who used a complex culture medium for long-term incubation of parasites (26 days) found that 75 polypeptides were shed into the supernatant, as detected by 2-D gel electrophoresis. This figure was revised by Lewis and Strand (1991) and Rotmans *et al.* (1981) who only identified 20 to 25 components. Although, these latter studies described products from short-term incubations (9h only), the culture medium used was not comprised of contaminating serum proteins that could mask the detection of true secretory material. In addition, Lewis & Strand (1991) observed the same pattern of antigen release from separate incubations, thereby supporting evidence for normal parasite maintenance during culture. Furthermore, the long-term culture regime used by Atkinson and Atkinson (1982) would probably not sustain worm viability even in the presence of foetal calf serum (FCS), since Wilson & Barnes (1974b) have shown that damage to the worm *in vitro* was incurred after 30 minutes of incubation alone. Thus, the adverse effects of culture upon the parasite would result in the release of somatic antigens, which would again limit the detection of E/S material.

Immunoprecipitation of the radiolabelled E/S products showed that most of the released products were immunogenic, compared to more complex preparations such as whole worm homogenates (Lewis & Strand, 1991; Pierce *et al.* 1983; Rotmans *et al.* 1981; Murrell, Vannier & Ahmed, 1974; Vannier *et al.* 1974). Additionally, it appeared that most of the antibody reactivity was directed against molecules released from the tegument membrane, rather than gut exudates (Lewis & Strand, 1991; Rotmans *et al.* 1981). However, caution must be exercised in interpreting these results, since the schistosomes may not regurgitate the residual products of digestion *in vitro* (Rotmans & Burgers, 1987), and hence it might appear that the tegument is the more active source of released immunogens.

Another major disadvantage with *in vitro* culture, is the paucity of material yielded from this laborious and expensive procedure. In view of this, investigations

into the immunochemical nature of secretions and excretions has been limited, since 50 000 to 100 000 worms have to be cultured for 48h to obtain as little as 15 to 20mg of soluble protein (Murrell, Vannier & Ahmed, 1974). It is apparent that such large scale production is not feasible as it would be difficult to ensure normal parasite maintenance in a medium lacking serum supplements. Nevertheless, several workers have demonstrated the allergenic character of E/S products using different experimental approaches: PCA (Vannier *et al.* 1974), the Prausnitz–Kustner test (Murrell, Vannier & Ahmed, 1974; Pierce *et al.* 1983) and the radioallergosorbent test (Pierce *et al.* 1983). All of these studies reported the relative allergenicity of adult worm E/S products compared to other soluble preparations. Undoubtedly, the E/S material used to sensitise animals contained gut and tegument membrane antigens. However, the methods of obtaining the components by freezing and thawing (Vannier *et al.* 1974), incubation in saline (Murrell, Vannier & Ahmed, 1974) or water (Pierce *et al.* 1983) make the data difficult to interpret. Such incubation conditions are extreme and probably result in the release of somatic antigens into the culture supernatant.

Immunisation of mice with released material from adult worms has met with some success. Tendler *et al.* (1991) injected released material into Swiss mice prior to a cercarial challenge. These authors reported up to 75% protection afforded to immunised mice compared to control animals. However, this figure was dependent upon the dose of E/S material, the route of cercarial challenge and the period between immunisation and challenge.

To conclude, it is evident that a large proportion of antigenic material is selectively shed from the tegument membrane of adult worms during *in vitro* culture (Lewis & Strand, 1991; Rotmans *et al.* 1981). Furthermore, these E/S products are highly immunogenic when compared to crude parasite fractions (Lewis & Strand, 1991; Rotmans *et al.* 1981; Murrell, Vannier & Ahmed, 1974; Vannier *et al.* 1974), and are composed of allergenic epitopes which elicit pronounced PCA and PK reactivity in both mice and rats (Pierce *et al.* 1983; Murrell, Vannier & Ahmed, 1974; Vannier *et al.*, 1974). Although, the released adult worm fractions have been used to immunise mice successfully, it is unclear whether somatic antigens could have contaminated the material used. Thus, the *in vitro* procedure must be viewed with some caution since the need to use protein-free medium precludes normal parasite maintenance after 30 minutes of culture (Wilson & Barnes, 1974b). Finally, elucidation of the immunochemical properties of proteins/polysaccharides is limited due to the paucity of material released.

It was not until recently that a more ideal method of parasite culture was developed. By surgically transferring adult male worms from donor mice to the mesenteric veins of naive recipients it was possible to maintain healthy schistosomes *in vivo* for an extended period of time (up to 8 weeks; Saunders *et al.* 1993). Using this technique the host's immune response could be used as a sensitive detector of released material. Furthermore, by transferring adult male worms larval development was bypassed, and hence antibody reactivity was directed against products released by mature parasites only. To demonstrate the viability of schistosomes following transfer, serum collected from recipient animals was tested for the presence of schistosome circulating anodic antigen (CAA), indicative of a viable prepatent infection (Deelder *et al.* 1980). Thus, it was concluded that this technique permitted the development of normal healthy parasites over a prolonged period *in vivo*, therefore negating the requirement for *in vitro* culture with its inherent disadvantages.

1.14 *The adult worm tegument*

1.14.1 *Structure and function*

The surface layer of schistosomes is comprised of a morphological syncitium, about 4.0μm in thickness, lacking lateral membranes associated with most cellular epithelia (McLaren, 1980). A number of scanning electron microscope studies have identified numerous spines and pits upon the surface of worms, and in particular male parasites, since they need to gain purchase along the lumen of mesenteric veins. These structures are interspersed with sensory papillae, which are evidence for the strong reliance upon tactile and perhaps chemical stimuli (Miller, Tulloch & Kuntz, 1972). The tegument is comprised of an apical membrane and a basal membrane; the former consists of two lipid bilayers. This formation was characterised following membrane treatment with uranyl acetate after routine fixation, revealing that the apical membrane possesses an unusual heptalaminate structure. Furthermore, the outer bilayer is less stable than the inner one, since in the absence of uranyl acetate fixation, the layers appear in a typical trilaminate form (McLaren & Hockley, 1977; Hockley & McLaren, 1973).

The tegument syncitium lacks nucleii and most organelles associated with synthesis, but it is connected to cell bodies lying beneath the syncitium through long narrow cytoplasmic connections. These subtegumental cells are the source of the metabolic machinery and give rise to the discoid bodies (DB) and membraneous

(multilammelar) bodies (MLB) described by Wilson & Barnes (1974a). Briefly, these inclusions are released from separate regions of the Golgi apparatus and are carried from the subtegumental cells to the tegument by a combination of fluid flow and diffusion (Wilson & Barnes, 1974a). Initially, DB were thought to be involved in the maintenance of the tegument, since they degenerated to release stored mucopolysaccharide which forms an integral component of the tegument cytoplasm (Wilson & Barnes, 1974b). However, upon isolation of the discoid granules from a membrane preparation it was revealed that a glycoprotein associated with these bodies was found in the fraction enriched in surface membrane (MacGregor, Kusel & Wilson, 1988), suggesting that these inclusions could participate in membrane formation. To characterise the function of the MLB, worms were treated with a series of chemical inhibitors. Treatment with ouabain resulted in the depletion of these inclusions and the subsequent degeneration of the tegument membrane. Thus, it may be inferred that the membraneous organelles store preformed membrane material.

For the most part, evidence for membrane synthesis and turnover has been demonstrated *in vitro*. By radiolabelling cultured parasites it was possible to determine the rate of protein and polysaccharide shedding from the tegument membranes. Subsequent fractionation of the tegument constituents or dissection of schistosomes for electron microscopy permitted the tracing of radiolabel within the parasite tissues at various times after labelling. Wilson & Barnes (1979) found that cationised ferritin label and ^{3}H-leucine migrated *en masse* from the pits and onto the tegumental spines, to be lost from the spines approximately 2 to 3h later. In addition, Kusel, MacKenzie & McLaren (1975) showed that rather than being released individually the radiolabelled membrane proteins were attached to membrane residues and shed into the medium as membrane fragments. However, evidence for the selective release of individual surface proteins was provided by Lewis and Strand (1991). These authors compared the proteins released from adult worms against a purified membrane preparation by 2-D SDS-PAGE electrophoresis and showed that although worms did shed tegument molecules they did not shed the components indiscriminately. Furthermore, the proposed rapid turnover of the schistosome surface was revised by Saunders *et al.* (1987) who demonstrated that the loss of acquired mouse glycoproteins from worms transferred into hamsters was slower than that reported in earlier *in vitro* studies (Wilson & Barnes, 1979; Kusel, MacKenzie & McLaren, 1975; Wilson & Barnes, 1974a). In fact, erythrocyte antigen was still detectable on the parasite surface after 7 days residence *in vivo*, thereby eliminating

rapid membrane turnover as a parasite evasion strategy. Instead, the implication was that worms were protected from the hostile environment of the host by the acquisition of host molecules at the parasite surface (Saunders, Wilson & Coulson, 1987).

1.14.2 *Molecules shed from the tegument*

The concept of the schistosome surface as a dynamic structure was supported by the demonstration of membrane antigen release *in vitro* (Lewis & Strand, 1991; Rotmans *et al.* 1981; Wilson & Barnes, 1979; Kusel, MacKenzie & McLaren, 1975b). This suggests that successful vaccination could be realised by delivery of tegument-associated molecules to naive animals prior to challenge. In adopting this immunisation strategy, Smith and Clegg (1985) used 2 surface antigens of Mw 155 and 53 kDa isolated from adult worms to vaccinate mice. These authors reported a small but significant level of resistance was conferred to mice against a challenge infection, thus validating the use of surface antigens within a vaccine regimen.

Two experimental approaches have been adopted in the identification of tegumental released material. The first and most widely used has been the generation of protective mAb, followed by the characterisation of the relevant target epitopes (Dissous *et al.* 1982; Grzych *et al.* 1982). The second is to identify antigens recognised uniquely by resistant hosts. In view of this strategy, chronic mouse serum (CMS), obtained from mice infected for 15 weeks with *S. mansoni*, was used to immunoprecipitate 4 major antigens of Mw 200, 38, 32 and 20 kDa. These molecules were identified as surface glycoproteins, since they lost their immunogenicity upon treatment with the oxidising agent sodium periodate (Omer-Ali *et al.* 1986). Although the antigens were not shown to be released from the parasite surface, the study did confirm the immunogenicity of surface glycoproteins.

Since a large proportion of the tegument-derived components are glycoproteins (Nyame, Cummings & Damian, 1988; Hayunga & Sumner, 1986; Samuelson & Caulfield, 1982; Murrell *et al.* 1978) it was not surprising that some of these components could elicit an effective immune response in mice and rats (Pearce & Sher, 1989; Omer-Ali *et al.* 1988; Omer-Ali *et al.* 1986; Dissous *et al.* 1982; Grzych *et al.* 1982). Grzych *et al.* (1982) adopted the alternative experimental rationale by producing a rat IgG2a mAb against adult worm incubation products, and more specifically a 38 kDa surface component. It was proposed that this epitope was the 38 kDa moiety described by Omer-Ali in mice.

A potential mechanism of molecule release was investigated by Pearce and Sher

(1989) who proposed that the 38 kDa antigen was shed from the schistosome surface following cleavage of its glycophosphatidylinositol (GPI) membrane anchor by phospholipase C (PLC) or phosphatidylinositol-specific PLC (PIPLC). Despite the fact that these experiments were performed upon 3h schistosomula, Pearce and Shers' study does provide an insight into the antigenic nature of the tegumental membrane and, in particular, the mechanism by which these products are released. With few exceptions the GPI linkages of proteins are sensitive to degradation with bacterial PIPLC; these enzymes act upon the junction between the phosphate and the hydrophobic diacylglycerol moiety (Low, 1989). Although it was anticipated that this treatment should result in the coincident release and solublisation of the attached protein, the shed GPI-anchored surface antigen was actually found to be associated with membrane vesicles and therefore insoluble (Pearce & Sher, 1989). Thus, it is implied that surface antigen loss *in vivo* is stimulated by contact between the schistosome and the GPI-specific enzyme, PIPLD, found in human serum (Pearce & Sher, 1989). Alternatively, the phosphatase activity may be endogenously expressed within the parasite tegument, since Rahman & Podesta (1982) demonstrated the presence of this enzyme in the MLB of schistosomes. The release of antigens in this way obviously provokes a significant antibody response in infected rats and mice, which supports their potential as vaccine candidates as well as demonstrating the protective capacity of carbohydrates released by schistosomula during infection.

Perhaps the most relevant study, with respect to the present thesis, was that of Verwaerde *et al.* (1987) who raised a rat IgE mAb against adult schistosome incubation products. This mAb was directed against a 26 kDa component, and conferred a significant level of protection (40-60%) by passive transfer to naive rats, compared to untreated animals. Not only is the molecule an allergen, it is also an E/S product of adult worms. However, as alluded to earlier, the incubation of adult worms in saline alone is unlikely to have been satisfactory for normal parasite maintenance, and therefore may have resulted in the degeneration of schistosome tissue. Thus, it is difficult to ascertain the true E/S character of this component without further analysis by immunolocalisation for example.

1.15 *The adult worm gut*

1.15.1 *Structure and function*

The syncitial digestive tract is tegumentary in origin and consists of a long coiling tube with numerous folds which ends blindly (Spence & Silk, 1970). The surface of

the luminal edge is thrown into loops which resemble microvilli, reflecting the absorptive function of this structure. These projections are extensions of the gut cytoplasm. The idea of the digestive system as a secretory tissue is supported by the presence of granules which are discharged from the Golgi apparatus. These electron dense secretory bodies are transported to the apical plasma membrane, where they fuse and release their products into the gut lumen.

The oesophagus is the most anterior portion of the digestive system, and is distinguishable morphologically from the rest of the worm gut, by the elongate cells lining this region. It has been suggested that the secretory granules produced in the posterior portion of the oesophageal gland may be responsible for the early digestion of host erythrocytes (Bogitsch & Carter, 1977). For example, the number of granules in the oesophagus diminishes when red blood cells are absent from the lumen (Bogitsch & Carter, 1977). However, the physiological significance of these granules is still speculative.

Since the gut is essentially a cul-de-sac, the parasite digests and absorbs soluble material from the ingested meal and then regurgitates the residual products, including parasite antigens, into the host bloodstream at regular intervals.

1.15.2 *Released products from the parasite gut*

The immunochemical nature of the gut secretions was analysed by Wilson & Barnes (1974a) who labelled adult worms with tritiated leucine and tritiated glucosamine, in order to characterise the proteins and polysaccharides released, respectively. These authors found that the material released from the caeca was composed of up to 72% of the exportable ^{3}H-glucosamine-containing polysaccharide and 23% of the ^{3}H-leucine containing protein, relative to the quantities shed from the tegument.

It has long been recognised that schistosomes ingest large quantities of host red blood cells daily and that in view of this observation the gut represents a metabolically active tissue. Evidence for the haemolytic activity of adult worms came from an early study by Timms and Bueding (1959). However, 12 years elapsed before Grant and Senft (1971) investigated the biochemical properties of the schistosome proteinase(s). Similar work by other groups reached a consensus that the proteinase activity required an acidic pH optimum (Deelder *et al.* 1977), the molecule had a Mw of approximately 27 (Sauer & Senft, 1972) to 32 kDa (Deelder *et al.* 1977) and an activity that appeared to be inhibited by N-ethylmaleimide, an inhibitor of thiol proteinases (Dresden & Deelder, 1979; Lindquist, 1986).

Moreover, the specific activity of this confirmed haemoglobinase (designated Sm32) is lower in male worms than in females (Timms & Bueding, 1959), which is presumably a consequence of the lower erythrocyte ingestion rate of males compared to females (Lawrence, 1973).

A histochemical study by Bogitsch and Dresden (1983) indicated that activity consistent with an acidic thiol proteinase could be localised to the gastrodermis of adult worms. Confirmation was provided by Chappell and Dresden (1987) who generated a mAb against Sm32 from the spleen cells of an *S. mansoni*-infected mouse. Furthermore, since these workers showed that anti-Sm32 antibodies were detected in the sera of humans infected with the parasite, it was suggested that this proteinase could act as an immunodiagnostic marker of infection.

Subsequently, another protein associated with the worm gut, but distinct from Sm32, was identified (Ruppel *et al.* 1985; Klinkert, Ruppel & Beck, 1987). This molecule had an apparent Mw of 31 kDa and demonstrated sequence homology with mammalian cathepsin B (Klinkert, Ruppel & Beck, 1987). Furthermore in mice, as well as humans, antibodies against the Sm31 kDa antigen were detectable during prepatency, around 28/40 days after infection.

The reported immuogenicity of the Sm32 and Sm31 antigens, together with their localisation in the gut, and the appearance of specific antibodies in the serum early after infection, emphasises their potential in immunodiagnosis of clinical infections. In view of this supposition both Sm31 and Sm32 were extensively studied as putative serodiagnostic agents (Chappell *et al.* 1990; Chappell, Hackel & Davis, 1989; Ruppel, Breternitz & Burger, 1987a). Although, there was no significant correlation between proteinase antibodies, egg output, and age of the infected individual, the ELISA was rapid and yielded minimal day-to-day variation. Since the sera of individuals infected with other schistosomes *S. japonicum* (Ruppel *et al.* 1987c) and *S. haematobium* cross-reacted with these proteinases (Ruppel *et al.* 1987b) the importance of these ELISAs in the field was elevated further.

By cloning Sm32 (Davis, Nanduri & Watson, 1989) and Sm31 (Klinkert *et al.* 1988; Gotz *et al.* 1992) it has become possible to produce recombinant antigens, thereby facilitating their use as serodiagnostic agents, since fusion proteins can be generated in relative abundance. With the development of this molecular technology it has also become possible to produce a relatively pure recombinant product for use in biochemical analyses. Gotz and Klinkert (1993) expressed both Sm32 and Sm31 genes in insect cells and used the products to assess their relative proteolytic activities. From this study it was judged that Sm32 was not the purported

haemoglobinase detected by Timms and Bueding (1959), since Sm31 not only shared more extensive homology with cathepsin B, but was also able to utilise haemoglobin as a substrate. Of course, further work is required to clarify and confirm these observations.

Using a different experimental approach, a mAb (31–3B6) was generated from mice which had been immunised with an emulsion of schistosomula and Complete Freund's Adjuvant (CFA) (King *et al.* 1987). Administration of the 31–3B6 mAb to BALB/c mice resulted in a 30 to 66% reduction in the recovery of adult worms, compared to control animals. Biochemical analyses identified the target epitope as a glycoprotein of Mw 68 kDa, composed of approximately 25% carbohydrate (King *et al.* 1987). Additionally, it was found that extensive periodic acid treatment did not diminish the binding of 31–3B6 to the purified antigen, indicating that the mAb bound to an epitope on the peptide portion of the antigen, and not to its carbohydrate moiety. The secretory nature of this molecule was confirmed by immunolocalisation studies, which showed that SmW68 was prominent in the gastrodermis and the tegument of adult male worms, but not in subtegumental cells (Blanton *et al.* 1991). From this description, it was concluded that the antigen was excreted from the gut to become attached on the parasite surface. However, the immunofluorescence observed at the schistosome surface could be due to non-specific binding of the secondary antibody or alternatively, evidence for the existence of shared determinants between the gut product and a tegumental-derived molecule. However the absence of subtegumental staining dismisses this latter suggestion.

The vaccine potential of SmW68 in human schistosomiasis was evaluated by ELISA, using serum obtained from a chronically-infected Egyptian community (King *et al.* 1989). These workers found that the preliminary studies were successful, since they showed that human serum antibody titers against SmW68 were correlated inversely with egg burden.

Other products released by healthy, feeding parasites include the proteoglycans, circulating anodic antigen (CAA), and circulating cathodic antigen (CCA). These antigens are detected in the circulation of mice, hamsters and human hosts early after infection (Deelder *et al.* 1980). Ultrastructural examination using mAbs specific for the carbohydrate epitopes of both CAA and CCA have shown that the antigens are present in the gastrodermis of adult worms, and more specifically in the Golgi apparatus, cytoplasmic vesicles, and in the luminal surface coat (de Water, Fransen & Deelder, 1986). Furthermore, the oesophagus of these worms exhibited negligible levels of CAA reactivity, whilst CCA reactivity was undetectable.

CAA was found in the serum of infected hamsters, mice, and human hosts (Saunders *et al.* 1993; Quian & Deelder, 1982; Deelder *et al.* 1980; Deelder *et al.* 1976) as well as being regularly excreted by regurgitation during *in vitro* culture (Rotmans & Burgers, 1987; Deelder *et al.* 1976). Further characterisation of this antigen revealed that it was a proteoglycan with a glucosamine-like monosaccharide composition, supporting the hypothesis that the antigen is a mucin or mucus glycoprotein-component. Moreover, it is heat-stable, TCA-soluble, and can be purified readily from an adult worm soluble preparation by TCA-treatment and subsequent ion-exchange chromatography (Nash, Prescott & Neva, 1974). CAA is thought to contribute towards worm physiology by acting as a protective shield, thereby preventing the degradation of the parasite gut by digestive enzymes, the low pH environment, or host components such as antibodies or complement (Deelder *et al.* 1980).

Likewise, the second circulating antigen CCA is TCA-soluble, heat stable and can be purified from the TCA-soluble fraction of adult worms antigens by diethylaminoethyl ion-exchange chromatography (Carlier *et al.* 1980). However, CCA is neutral or positively charged at pH 8.2 and is detected in the serum and urine of infected hamsters (Deelder *et al.* 1980; Deelder *et al.* 1976). CCA is believed to perform the same parasite-protective role as described for CAA. Additional support for this hypothesis was given by Van Dam (1995) who found that red blood cell ingestion had no effect upon the kinetics of CAA and CCA production.

The demonstration that CAA and CCA were present in human serum, as detected by haemagglutination assay, focused attention upon the potential of these proteoglycans as markers of infection (Deelder *et al.* 1989a). Antibody capture ELISAs were designed to detect small quantities of CAA (Deelder *et al.* 1989b; deJonge *et al.* 1988) and CCA (deJonge *et al.* 1990) from infected human sera. These assays provide a sensitive detection system with the lower detection limits of 3ng (CAA) and 0.2ng (CCA) per ml of TCA-soluble adult worm preparation. Using this technique, levels of CAA were found to be lower than the levels of CCA in the serum and urine of infected patients (5ng/ml compared to 50ng/ml, respectively). However, despite the small amounts of circulating antigens, the serum levels of CAA and CCA were strongly correlated with egg excretion; yet no correlation was found between egg excretion and anti-CAA and anti-CCA immunoglobulin levels as determined by ELISA (Deelder *et al.* 1989a; Deelder *et al.* 1989b).

To summarise, it is evident that gut-derived antigens are of major importance as

immuno-serodiagnostic targets, since they seem to be produced during pre-patent and patent infections as well as stimulating the production of antibodies within the the first 4 weeks of infection in experimental animals and humans. ELISAs have been developed to exploit this potential in the field, with workers reporting the assays' continuing success. Additionally, the epitopes they present, whether carbohydrate (CAA and CCA) or peptide (SmW68, Sm32 and Sm31) in origin, appear to be shared between schistosome species, thus providing further justification for their development. However, the financial and logistical limitations imposed by governments of endemic countries make the wide-spread used of these ELISAs unlikely in the near future.

1.16 *Aims of this study*

From the preceding introduction it is evident that the inbred laboratory rat is able to expel adult schistosomes from the hepatic portal system around day 28 post-infection onwards. It should also be apparent that during rat schistosomiasis, IgE titers are elevated concomitant with the recruitment of mast cells to the liver and their subsequent degranulation. The escalation and timing of these immunological events, coincident with parasite expulsion, implicates IgE-mediated mast cell responses in the immune attrition of adult worms. The trigger for this antibody-dependent response is the viable adult parasite, since the schistosomes appear healthy prior to day 28. Thus, the main aim of this study was to characterise the allergens released by healthy parasites, and to investigate their involvement in stimulating IgE production and mast cell degranulation.

Chapter two describes the identification of the E/S products of adult worms. The surgical transfer of mature schistosomes from donor mice to naive recipients permitted *in vivo* culture of parasites for up to 8 weeks. Providing the worms were recovered intact, it was assumed that the recipient animals had mounted an antibody response only against the released products of healthy schistosomes. Soluble parasite protein fractionated by SDS-PAGE was probed with serum taken from recipient animals, thus enabling the detection of scarce E/S products. Immunocytochemistry of frozen parasite sections using affinity purified antibody, facilitated the immuno-localisation of antigens to the sites of release.

The work presented in Chapter three addresses the phenomenon of spontaneous cure in rats. Serum and tissues were collected from rats at weekly intervals post-infection, in order to characterise the IgE reactivity and to check for egg deposition.

Additionally, liver worms were recovered to permit clearer interpretation of the data, as regards causal correlations between worm elimination and the nature of the IgE-mediated response. Immunopurified IgE from the rat serum was used to probe adult worm material in western blots, in order to characterise the allergens released by mature schistosomes.

Finally, Chapter four describes the correlation between mast cell-bound specific-IgE and the stimulation of mast cell degranulation by schistosome allergen. A functional RBL-serotonin release assay was developed to measure the quantity of systemic IgE in rat infection serum, and the effect of different antigen preparations upon mast cell responsiveness. Moreover, it was hoped that this assay would identify those allergenic parasite components involved in stimulating mast cell degranulation.

CHAPTER TWO

The antigens released *in vivo* by adult male *Schistosoma mansoni*.

2.1 INTRODUCTION

Successful development of a vaccine against *Schistosoma mansoni* necessitates the investigation and detailed characterisation of protective immune responses elicted by such a parasite infection and identification of the relevant antigens involved. In endeavours to dissect the protective immune mechanism elicited by worm antigens, workers have established that live-attenuated parasites consistently induce a high level of resistance in mice to a homologous challenge (60-70%; Dean, 1983) when compared to vaccination with non-living worm preparations (Smith & Clegg, 1985; Horowitz, Smolarsky & Arnon, 1982). However, Soisson *et al.* (1992) did report a significant level of protection, between 38.6 and 82.9%, achieved in mice upon challenge, following subcutaneous injection with rIrV-5 (immunogenic fragment of myosin) in the form of protein micelles or proteosomes with the outer membrane protein of meningococcus (OMP). An explanation for the discrepancy observed between live and dead vaccines was provided by the results of Mountford, Coulson & Wilson (1988). They postulated that parasites sequestered in the skin draining lymph nodes of vaccinated mice released significant quantities of antigenic material, priming schistosome-specific lymphocytes with protective Th1 characteristics (Mountford *et al.* 1992; Pemberton *et al.* 1991). The subsequent recruitment and infiltration of leukocytes into the lungs arms that organ against invading challenge schistosomula (Aitken, Coulson and Wilson, 1988). Since the target of the immune effector response is the live parasite, it is reasonable to assume that only antigens released by intact parasites will participate in the reactivation of primed Th lymphocytes. In support of this theory Mountford, Harrop and Wilson (1995) used an *in vitro* T-cell blastogenesis assay to confirm the immunogenicity of schistosomula-derived fractions released by cultured parasites.

In addition, several workers have associated secretory antigens with the induction of protective, cell-mediated immune responses in rats (Damonneville *et al.* 1986b; Auriault *et al.* 1985; Auriault *et al.* 1984). Active immunisation of rats with schistosomula-released products (SRP) afforded between 46 and 83% resistance, whilst the passive transfer of anti-SRP serum conferred up to 83% protection (Damonneville *et al.* 1986b).

The potential sites of antigen release from live schistosomes vary according to the developmental stage of the parasite: the partially shed glycocalyx and acetabular glands of cercariae, the tegumental membrane and gastrodermis of lung-stage and adult worms. The major host-parasite interface is the syncitial tegument, and as

such is considered an important target for vaccine-induced immunologic attack (Pearce & Sher, 1989; Harn *et al.* 1987; Kelly *et al.* 1985; Payares *et al.* 1985a; Grzych *et al.* 1982). With respect to the tegument's secretory nature, Pearce and Sher (1989) found that 6 molecules of Mw 38, 32, 18, 13, 11 and 9 kDa were released/shed from the surface of radiolabelled 3h-old schistosomula following 1-2h of *in vitro* culture. Three of the 6, the 38, 32 and 18 kDa molecules, served as targets for antibody from vaccinated/chronically-infected mouse serum. Monoclonal antibody to the 38 and 32 kDa glycophosphatidylinositol-linked molecules, previously identified by Harn *et al.* (1987) and Dissous, Grzych and Capron (1982) respectively, conferred significant levels of protection against infection. These data support the concept that such shed molecules may possess unique functional advantages as vaccine immunogens.

Adult worms are metabolically active. They release/secrete macromolecules from epithelial surfaces, such as the gut and tegument (Lewis & Strand, 1991; Payares *et al.* 1985a; Rotmans *et al.* 1981; Wilson & Barnes, 1979) as part of their normal activities. This propensity means that the mature schistosome represents a rich source of potential immunogens (Lewis & Strand, 1991; Pierce *et al.* 1983; Rotmans *et al.* 1981; Murrell, Vannier & Ahmed, 1974; Vannier *et al.* 1974). The humoral response of the host exhibits an early and strong reactivity against a number of gut-associated antigens (Chappell & Dresden, 1988; Deelder *et al.* 1980; Nash, 1978). However, the molecules released by adult *S. mansoni* have received most attention for their use as immunodiagnostic markers of pre-patent and patent infection (Chappell *et al.* 1990; Ruppel *et al.* 1987b; Quian & Deelder, 1982), rather than vaccine candidates.

The importance of released material in establishing protective immunity in the attentuated vaccine model has been discussed. More recently a correlation between increased anti-adult worm IgE titers and the phenomenon of acquired immunity in man has been demonstrated (Dunne *et al.* 1992a; 1992b). The IgE-reactivity appeared to be directed against a subset of adult schistosome proteins, with the specific response dominated by an antigen of Mw 22 kDa. If it is the released components of living parasites that influence the expression of acquired immunity, as in the irradiated-vaccine model, then the identification of excreted/secreted adult worm material could be of major interest.

Currently, a large body of information is available about tegumental proteins and gut-associated products of adult worms, yet the full extent of antigen release by mature parasites has not been investigated comprehensively. Previous studies have

described the difficulties in maintaining adult parasites successfully *in vitro*. Rapid deterioration of schistosome tissue is observed following the culture of adult worms for more than 3–4 hours in media lacking supplements (Wilson & Barnes, 1974b), with the subsequent release of somatic antigens obscuring the presence of genuine secretory products (Carlisle, Weisberg & Bentley, 1983). In addition, the low and inconsistent yield of material released into the medium by parasites, in conjunction with the variety of *in vitro* incubation procedures adopted by different workers (Lewis & Strand, 1991 Murrell *et al.* 1974) have led to extensive but disparate analyses of released material. No study has characterised the proteins released *in vivo* by adult worms alone.

Problems associated with *in vitro* culture have led to the development of an *in vivo* culture system in mice, with which to identify and locate the scarce secretory products of adult male worms. Briefly, samples of ten 4–week–old male parasites, obtained from donor mice by portal perfusion, were introduced into the hepatic portal vein of naive recipients by surgical transfer (Saunders *et al.* 1993; Saunders *et al.* 1987), allowing the worms to function normally without the limitations of an *in vitro* environment. Worms recovered by portal perfusion from recipient mice at eight weeks post–transfer were examined to ensure that they had not incurred damage whilst resident in the host. Providing the whole complement of ten male worms was recovered intact, it was assumed that the recipient animal had been exposed to material released from healthy parasites. Hence, the antibody response was used as a sensitive detector of scarce antigenic secretory products. *In vivo* culture excludes interference from larval and/or dead schistosome proteins, and the male–only transfer circumvents the egg–induced pathology associated with disease, thereby permitting the extension of experimental study up to eight weeks.

In the present study, this surgical transfer technique was used to identify the products released by adult worms *in vivo*. Serum samples, taken from the recipient mice at weekly intervals post–transfer, were used to detect released material by western blotting. The origin of each dominant antigen, within parasite tissue, was resolved by the combination of antibody elution with immunocytochemistry of frozen worm sections. This system exhibits a key advantage over conventional *in vitro* methods of secretory antigen identification, since the antibody response of recipient mice provides a detection technique of exquisite sensitivity, in which artefactual release of antigens from dead or moribund worms is eliminated.

2.2 MATERIALS AND METHODS

2.2.1 *Parasites and Hosts*

A Puerto Rican strain of *S. mansoni* was maintained by routine passage through albino *Biomphalaria glabrata* and outbred LACA mice. C57BL/6 mice were infected with male cercariae, providing adult worms for male-only soluble worm antigen preparation (SWAP) and the worm transfer experiments.

The sex of the cercariae was determined by polymerase chain reaction (PCR) following the method of Gasser *et al.* (1991). The PCR reaction mix contained 10mM dNTPs, 20μM of each primer W1a and W1b, 10mM Tris–HCl pH 8.8, 50mM KCl, 1.5mM $MgCl_2$, 0.1% non–ionic detergent and 2.5U Taq DNA polymerase (Promega, Southampton, England). The samples were spun in a microfuge for 20 seconds at 1000 g and overlaid with 50μl mineral oil (Sigma, Poole, England). The reaction was performed at 95°C (60 s)$^{-1}$ denaturing, 50°C (80 s)$^{-1}$ annealing and 72°C (80 s)$^{-1}$ extension, for 40 cycles. An 8μl aliquot of the amplified DNA from each tube was mixed with 2μl DNA loading buffer (containing 0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll 400) and subjected to 2% agarose gel electrophoresis carried out in 0.5x Tris buffered EDTA (TBE) at a constant voltage. The ethidium bromide stained gels were transilluminated (302nm) and photographed. Snails shedding female, or male and female (mixed–sex) cercariae were identified by the presence of a 500 base pair band, these snails were discarded.

2.2.2 *Surgical transfer of adult worms*

The surgical transfer of adult worms from donor C57BL/6 mice to recipient mice of the same strain has been described in detail by Saunders *et al.* (1993). Four week old schistosomes were recovered from donor C57BL/6 mice by portal perfusion with heparinised minimal essential medium (Gibco, Paisley, Scotland) containing 10mM HEPES (MEMH) and 4 USP ml^{-1} of porcine heparin (Sigma). Male *S. mansoni* were maintained in MEMH containing 10% normal mouse serum. Perfusion to transfer times were no longer than 45 minutes. Samples of ten male worms were surgically transferred into the superior mesenteric vein of eight naive recipients. Every seven days following transfer, samples of blood were obtained from recipient mice by tail bleeding (60 μl), the serum separated, and centrifuged at 10 000 g for 5 minutes. The serum was aliquotted and stored at –20°C. After 8 weeks the mice were perfused to determine worm burden.

2.2.3 *Antigen preparations*

Total soluble preparation. Adult male worms were recovered from C57BL/6 hosts, 6 weeks post-infection, by portal perfusion with heparinised MEMH. The worms were washed 4 times in MEMH (without heparin) and debris removed. The worm bodies were stored at -80°C in Hank's Balanced Salt Solution (HBSS; Sigma) with protease inhibitors 1 (350μg/ml Pepstatin A, 250μg/ml Leupeptin, 20mg/ml Tosyl-1-Lysine chloromethyl ketone dissolved in double distilled water) diluted 1:250, and 2 (350mg/ml Tosylamide-2-phenyl-ethylchloromethylketone, 8.7mg/ml Phenyl-methyl-sulphonyl fluoride dissolved in ethanol) diluted 1:500. The soluble worm fraction was obtained by thawing the worms at room temperature (RT) and sonicating for 5min on ice (21 kHz at 6.5μm amplitude). This sonicate was centrifuged at 105 000 g for 1h at 4°C. The supernatant was removed and stored at -80°C. Protein content for all preparations was determined by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as the standard.

In vitro culture supernatant. Worms from a mixed-sex infection were perfused and washed as described above. The worms were resuspended in 20ml fresh MEMH, decanted into a sterile culture flask (Nunclon) and incubated for 3 hours at 37°C, 5% CO_2. Following culture the parasites were poured into sterilin tubes and spun in a bench top centrifuge for 20 seconds at 1000 g. The parasite free culture supernatant was removed and concentrated to 1ml in a stirred ultrafiltration cell containing a 3kDa cut-off Diaflo membrane (Amicon, Beverley, USA), under N_2 at a pressure of 40 psi. The concentrated supernatant was stored at -80°C with protease inhibitors 1 and 2. The worm bodies were washed 4 times with HBSS and stored under the same conditions. These male and female worms were subsequently used for a tegument membrane preparation.

Tegument membrane preparation. The tegument membrane was removed and purified following the method of Roberts *et al.* (1983). Male and female worms stored in HBSS and protease inhibitors were thawed initially at RT then on ice. The HBSS was replaced with 3ml Tris buffered saline (TBS;10mM Tris HCl, 0.85% NaCl) pH 7.4. Worms were vortexed for 5x1 second pulses and placed on ice before this procedure was repeated. The vortex supernatant (V1) was removed and centrifuged at 1000 g for 30 min, the pellet (S2P) resuspended in 2ml double distilled water and left on ice for 10 minutes (osmotic shock step). Finally, this solution was centrifuged at 1000 g for 30 min, the supernatant taken off and the pellet (S2P/Os)

composed of purified tegument membrane, resuspended in TBS and protease inhibitors.

Oesophageal, gonad and gut preparations: Male worms perfused from C57BL/6 mice were washed four times with MEMH (without heparin) and poured into a petri dish. Using a pair of watch-maker's tweezers and iris scissors, worms were carefully sectioned into 3 portions (figure 2.1). The male worm oesophageal preparation was derived from worm tissue lying just above the ventral sucker. The male gonad preparation was derived from tissue lying between the points A and B (the gonads having been identified in this region). The remainder of the worm, tissue lying behind point B, was used to provide a preparation depleted in oesophageal and gonad material.

Approximately 500 male worms were dissected in total. The different tissues were placed in HBSS and stored in protease inhibitors at −80°C. Upon thawing, all preparations were sonicated on ice for 5 min and the soluble fraction isolated, as described above. Several preparations were made on different days. The soluble fractions were pooled for each tissue and concentrated in a stirred ultrafiltration cell. The samples were stored at −80°C with protease inhibitors.

2.2.4 *Western blotting*

Proteins were separated electrophoretically under reducing conditions by 1−D SDS−PAGE according to the method of Laemmli (1970). Male−only SWAP was boiled for 2 minutes in sample buffer (4 volumes of sample: 1 volume of 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). Male SWAP was loaded at 80μg of protein/1cm of trough comb. Molecular weight standards (Bio−Rad, Hemel Hempstead, England) were treated in the same way as the antigen preparation. Resolved fractions were electroblotted onto PVDF membrane (Millipore, Watford, England; catalogue number IPVH 151 50), using procedures based on those of Towbin *et al.* (1979), 20v per gel for 16 hours, 4°C. The non−specific sites on the membrane were blocked with blocking buffer, 2% BSA, 4% normal goat serum, 0.3% Tween 20 in PBS (150mM NaCl, 1.5mM KH_2PO_4, 8mM Na_2HPO_4, 2.6mM KCl) for 1 hour at room temperature. The blotting procedure followed the protocol

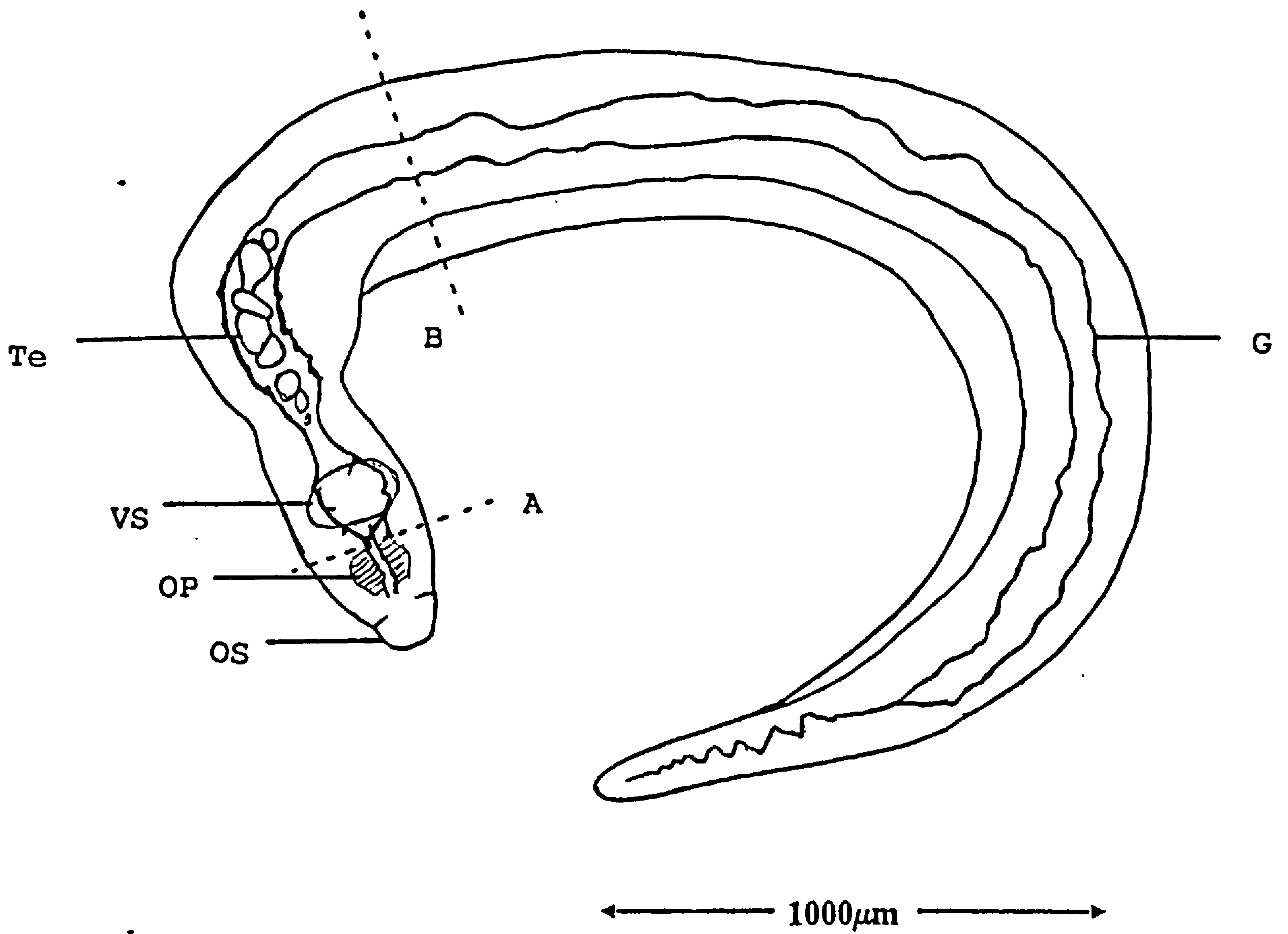


Figure 2.1 The gross structure of adult male *S. mansoni* with incision points A and B labelled.
Oral sucker (OS), ventral sucker (VS), oesophageal passage (OP), male worm testes (Te), gut (G).

described by Roberts *et al.* (1987). Briefly, 5mm strips of membrane were cut, sealed in plastic bags and left incubating with 2ml of each test serum, diluted 1:1000 in blocking buffer, on a rotating windmill for 16 hours at 4°C. Normal mouse serum (NMS), and chronic mouse serum (CMS) obtained from mice infected with male cercariae for twelve weeks, were used as negative and positive controls respectively. Both control sera were diluted 1:1000 with blocking buffer. After five washes in blot wash buffer (150mM NaCl, 1.5mM KH_2PO_4, 8mM Na_2HPO_4, 2.6mM KCl, 0.3%(v/v) Tween 20) over 45 min, each strip was incubated for 1.5 h at RT with the secondary antibody, goat anti-mouse IgG (Fab specific) peroxidase conjugate (Sigma), diluted 1:2000. The strips were washed as before, and finally developed with diaminobenzidine (DAB) substrate (Sigma). The developed strips showing the proteins recognised by the worm transfer serum (WTS) over the 8 week experimental period were scanned by an optical densitometer (Bio-Rad). The change in intensity of each band with time was calculated from the arbitary values obtained from the densitometry readings. From these figures the strength of the antibody response against each of the antigens over the 8 week experimental period could be determined.

In order to determine the primary sites of antigen release from adult worms a selection of antigen preparations: oesophageal, gonad, material depleted of gonad and oesophageal components, purified tegumental membrane, culture supernatant, female-derived SWAP and male-only SWAP (described in section 2.2.3) were loaded at equal protein concentrations (20μg/well) on SDS-PAGE gels. The electrophoretic procedure was carried out as above. The fractionation patterns were visualised by staining the proteins with Coomassie blue solution. The gels were fixed with 15% tri-chloroacetic acid (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.

To demonstrate the relative immunogenicity of the various preparations, the samples were electrophoresed by SDS-PAGE and then electroblotted onto PVDF membrane as described above. The different preparations were probed with WTS, week 8 (diluted to 1:1000 with blocking buffer); all washes and incubations with primary and secondary serum are also described in the previous paragraph. It was assumed that the antibody response to each immunogen would be enhanced by enriching for proteins in defined tissues. By visualising and comparing the intensity of each band in each preparation it was possible to assess the source of secretion for each antigen. Thus, these experiments gave a preliminary indication of the major

sources of adult worm secretory antigens.

2.2.5 *Antibody elution from western blots*

The location of the excretory/secretory (E/S) proteins in the tissues of adult schistosomes was determined by using oligospecific polyclonal antibody eluted from western blots to probe parasite sections in immunofluorescence studies. Male SWAP, reduced by heating in sample buffer (section 2.2.4), was loaded along the length of a trough comb at a concentration of 80μg of protein/ 1cm. The electrophoresis and transfer procedures were followed as above. The following antibody elution technique was modified from the "antibody select" method described by Hall *et al.* (1984) and Coppell, Smith & Peterson (1993). The antigen-coated PVDF membrane was blocked and incubated with week 8 test serum diluted 1:1000 overnight at 4°C. Two 5mm strips were cut from either side of the blot. These were washed and developed with the secondary antibody and DAB substrate. The remaining blot was left incubating with the primary serum at 4°C. After five washes the blot and strips were realigned and the dominant bands, as revealed by the adjacent strips, excised from the main body of the blot. Each band was cut into 1mm pieces, placed in a 1.5ml eppendorf tube containing 400μl of elution buffer, 200mM Glycine-HCl pH 2.8, and agitated for 10 minutes. The samples were spun on a microfuge for 60 seconds at 1000 g, the supernatant removed and neutralised with 1M Tris-HCl, pH 9.0. The specificity of the eluted antibody (diluted 1:25) for each of the dominant antigens was tested by reprobing a fresh blot.

2.2.6 *Immunocytochemistry on parasite sections*

The immunocytochemical procedure used in the present study was based on methodology developed by Riengrojpitak *et al.* (1989). Adult male *S. mansoni* worms were perfused from C57BL/6 mice as described. They were washed thoroughly in MEMH, embedded in OCT compound (Tissue-Tek, London, England) and plunged into liquid nitrogen before storing at -70°C. The tissue was sectioned (7μm thickness) onto alcohol-cleaned slides at -28°C using a cryostat (Slee, London, England), air-dried and fixed for 1 min in absolute acetone. The slides were left to dry at room temperature for 30 min and stored at -20°C.

Before staining, the sections were thawed for 30 min and fixed again in absolute acetone for a further 10 min. The fixed samples were washed 3 times for 30 min with PBS before treatment. Sections were blocked by incubating with undiluted normal goat serum for 60 min. All incubations were carried out in a humid box. The

eluted antibody was then applied undiluted. Normal mouse serum and WTS collected 8 weeks post-transfer, served as negative and positive controls respectively. Both were diluted 1:500 with blocking buffer. The sections were probed overnight at 4°C with the primary serum. Following 3 washes in PBS and a further 10 min blocking step with normal goat serum, the secondary antibody, FITC-conjugated goat anti-mouse Ig (Nordic, Tilburg, The Netherlands) diluted 1:60, was applied and incubated in the dark. Finally, the samples were washed 3 times with normal goat serum and mounted in Citifluor (Agar Scientific, Stanstead, Essex, England). The stained parasite sections were examined under a Nikon fluorescence microscope and photographed on Kodak ektachrome, ASA 400.

2.3. RESULTS

2.3.1 *Surgical transfer*

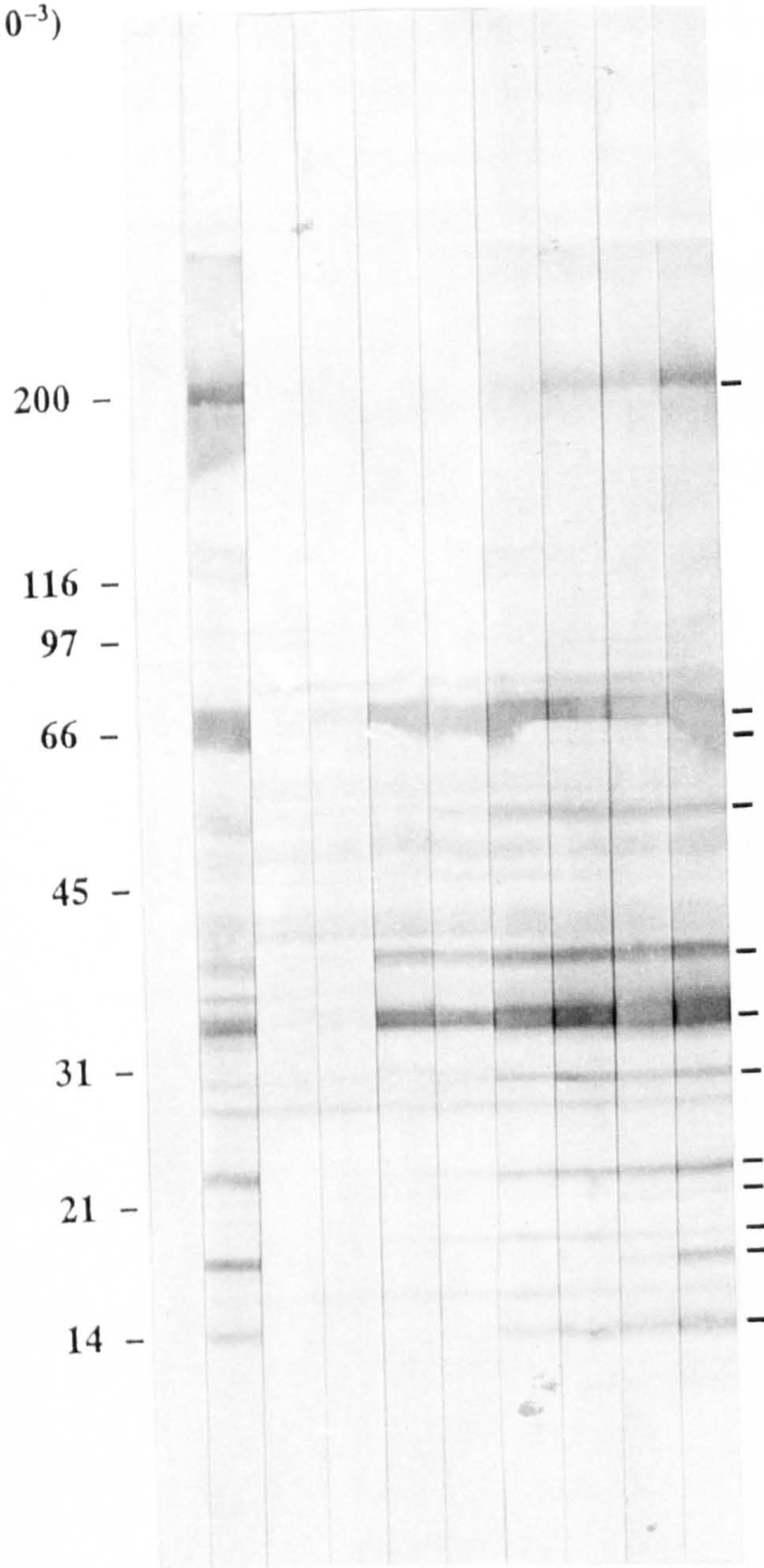
Virtually all mice survived the surgery and remained healthy throughout the experiments. To ensure that each recipient had harboured ten live male worms, over the experimental period of 8 weeks, mice were perfused and the worm burden determined. Providing ten worms were recovered intact it was assumed that the serum collected from the host was against male schistosome E/S products, and not somatic antigens leaked by dead or dying parasites. Ten viable adult worms were recovered from one mouse, number 5.

The transfer of worms to naive animals has subsequently been repeated using five C57BL/6 mice as recipients. Following portal perfusion eight weeks later ten male worms were recovered intact from four of the five mice. Since serum from one mouse (number 5) was used in the present study it was necessary to confirm whether this WTS provided an accurate representation of the humoral response to transferred parasites. Qualitative analysis of WTS from all mice against male worm protein was carried out by western blotting. The results indicated that the pattern of antigen recognition by antibody from each mouse was identical.

2.3.2 *The antibody response*

The detection of released schistosome protein was carried out by western blotting of male-only SWAP. Twelve schistosome-specific antigens, molecular weights (Mw) 208, 67, 62, 53, 38, 32, 30, 24, 22, 19, 18, and 14 kDa, were identified (indicated by the bars in figure 2.2). Upon analysis of the western blot it

Figure 2.2 Reactivity of the worm transfer serum (WTS) against fractionated adult male SWAP, as detected by western blot analysis.
Each lane was probed with the following sera (diluted 1:1000):
A, normal mouse serum (NMS); **B**, chronic mouse serum (CMS); lanes **1–8** were incubated with WTS from mouse #5, weeks 1–8 respectively.
The 12 immunodominant antigens of interest are indicated by bars (see text for Mw).


Mw
(x10⁻³)
A B 1 2 3 4 5 6 7 8
200
116
97
66
45
31
21
14

appeared that the protein at 32 kDa possessed the same relative Mw as a gut protease described by Deelder, Reinders and Rotmans (1977). The similarity between the molecules was confirmed by probing fractionated SWAP with polyclonal antibody to the proposed haemoglobinase Sm32, kindly donated by Dr. Rotmans, State University of Leiden, The Netherlands.

The immunogenicity of each antigen appeared to differ across the experimental course of 8 weeks with the lower molecular weight antigens detected later than the higher molecular weight proteins. Densitometry revealed that the antibody response to each band fell into one of two categories. Category one: the 67, 38, 32 and 22 kDa antigens were detected 1–2 weeks post-transfer. The antibody titer increased rapidly reaching a plateau 4 weeks later (figure 2.3a). Category two: proteins at 208, 62, 53, 30, 24, 19, 18 and 14 kDa were detected following a lag phase of 3–6 weeks. The antibody titer increased slowly until termination of the experiment 8 weeks later (figure 2.3b).

2.3.3 *Origin of the E/S antigens*

Coomassie-stained SDS-PAGE gels of the different antigen preparations. Soluble antigen preparations were loaded at equal concentrations on the same gel allowing qualitative comparison of the antibody response between samples. A graphic representation of the coomassie-stained gels is shown in figure 2.4. The pattern of fractionated proteins visualised between each defined soluble preparation did not appear significantly different. In general, the preparations were composed of a complex mixture of many molecules. The majority of the proteins were of higher molecular weights, with dominant bands at 65, 58, 47, 40 and 30 kDa. Conversely the proteins released following *in vitro* culture of adult worms showed a dramatically different banding pattern. The 65 kDa molecule was present in much larger quantities in the culture fraction. In addition there was a heavily stained band at 12 kDa. The tegument membrane preparation contained a protein, molecular weight 22 kDa, which did not appear in the other samples.

Western blotting. After the protein had been electroblotted, the antigen fractions were incubated with the WTS enabling the site of release for each antigen to be determined (figure 2.5). For example, it was assumed that a tegumental membrane antigen would be more abundant in a preparation enriched for tegumental membrane components. Hence, the IgG response against this antigen would be enhanced in the respective sample lane. The dominant 67 kDa protein was present in all preparations with the exception of the isolated tegumental membrane fraction. The 32 kDa

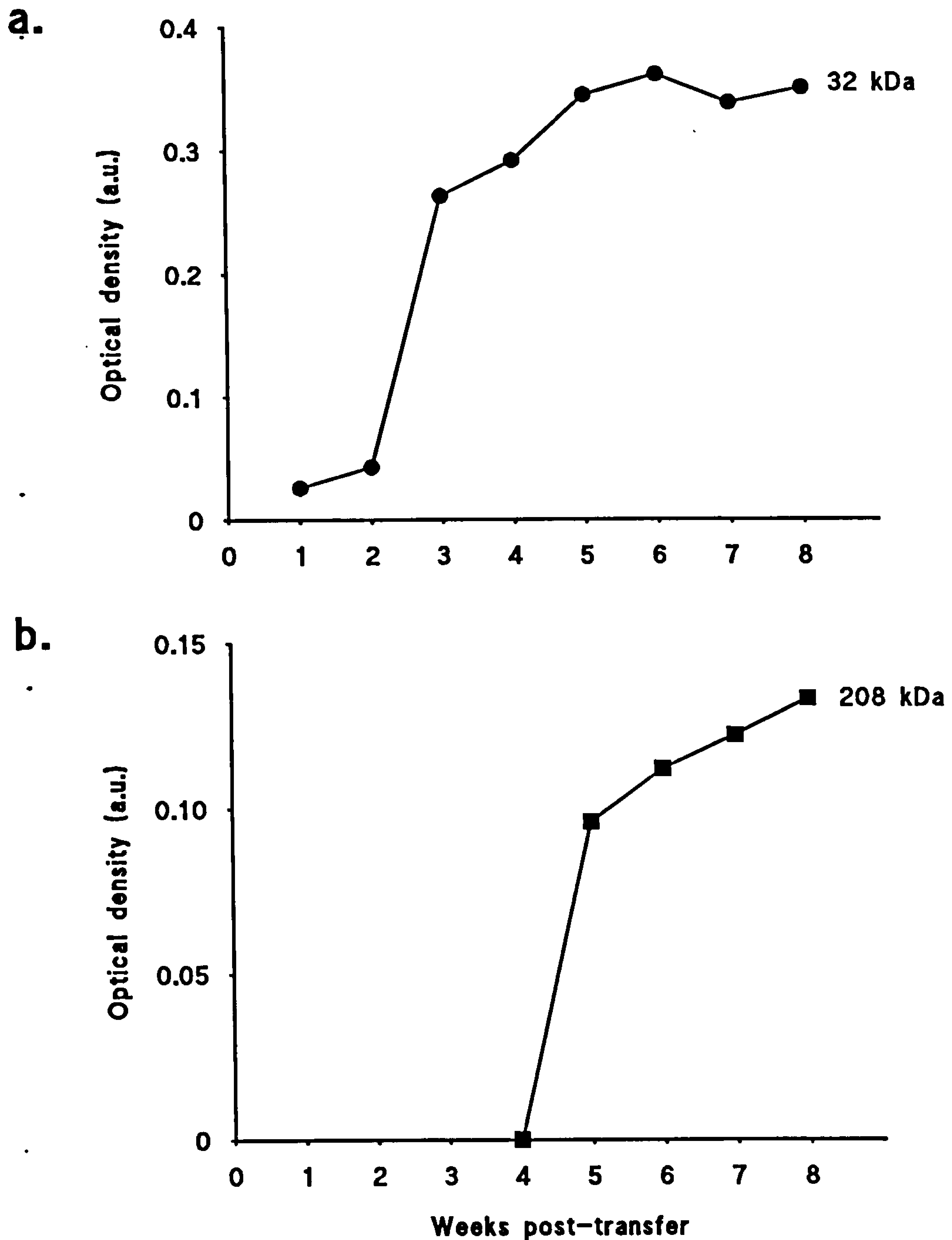


Figure 2.3 The nature of the recipient host's IgG response, directed against individual E/S products, has been grouped into two categories dependent upon the intensity of antigen/antibody binding (see text for details).

a. The level of antibody rises rapidly following worm transfer and reaches a plateau 4 weeks later. This pattern is indicative of a category I response.

b. The antibody response is detected following a lag phase of 3–6 weeks, with IgG levels increasing slowly until termination of the experiment. This pattern is indicative of a category II response.

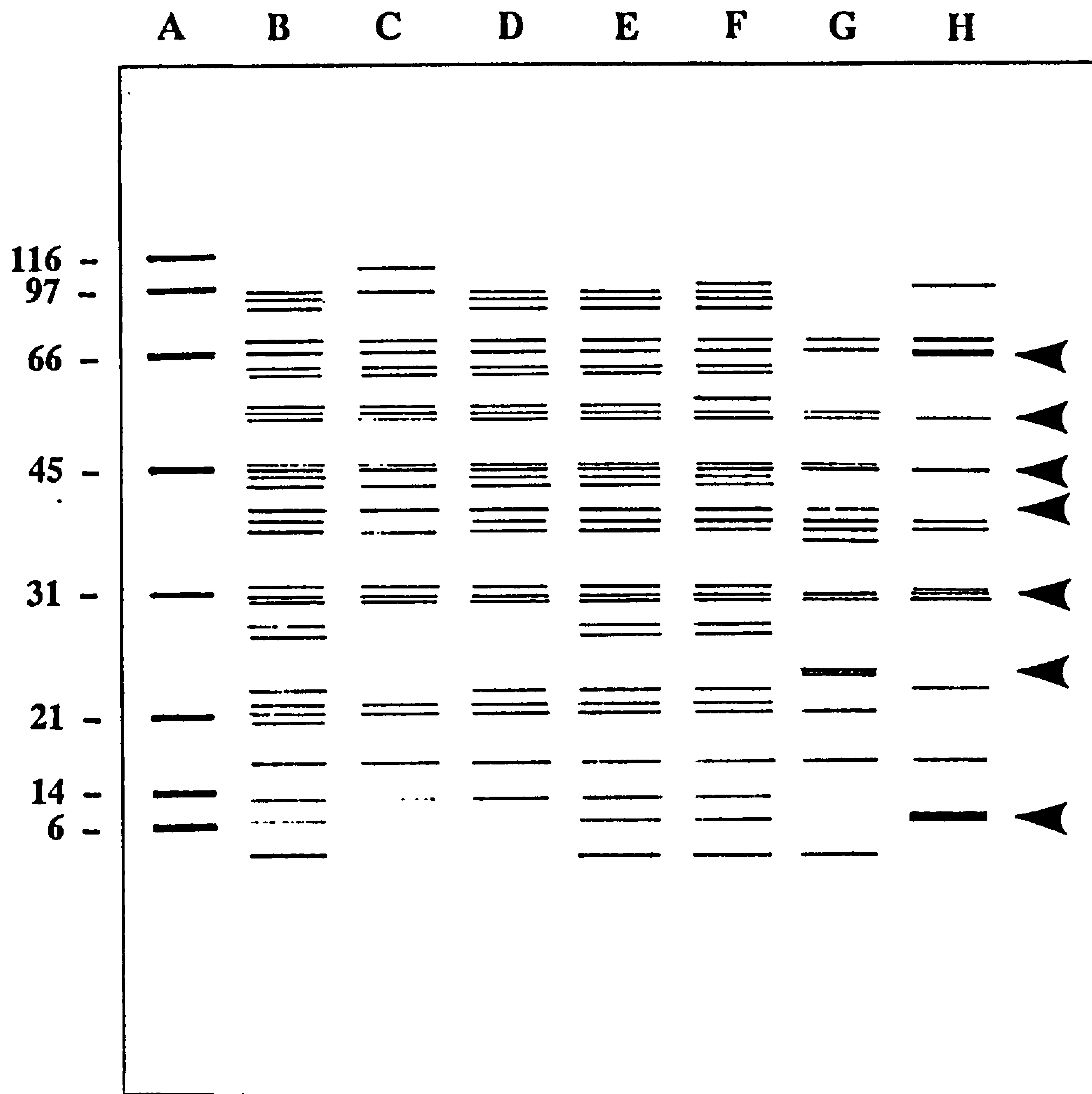


Figure 2.4 Graphic representation of Coomassie–stained gels defining the fractionation pattern of proteins from different soluble antigen preparations.

The samples were loaded at the same protein concentration (20μg/well) and the lanes represent:

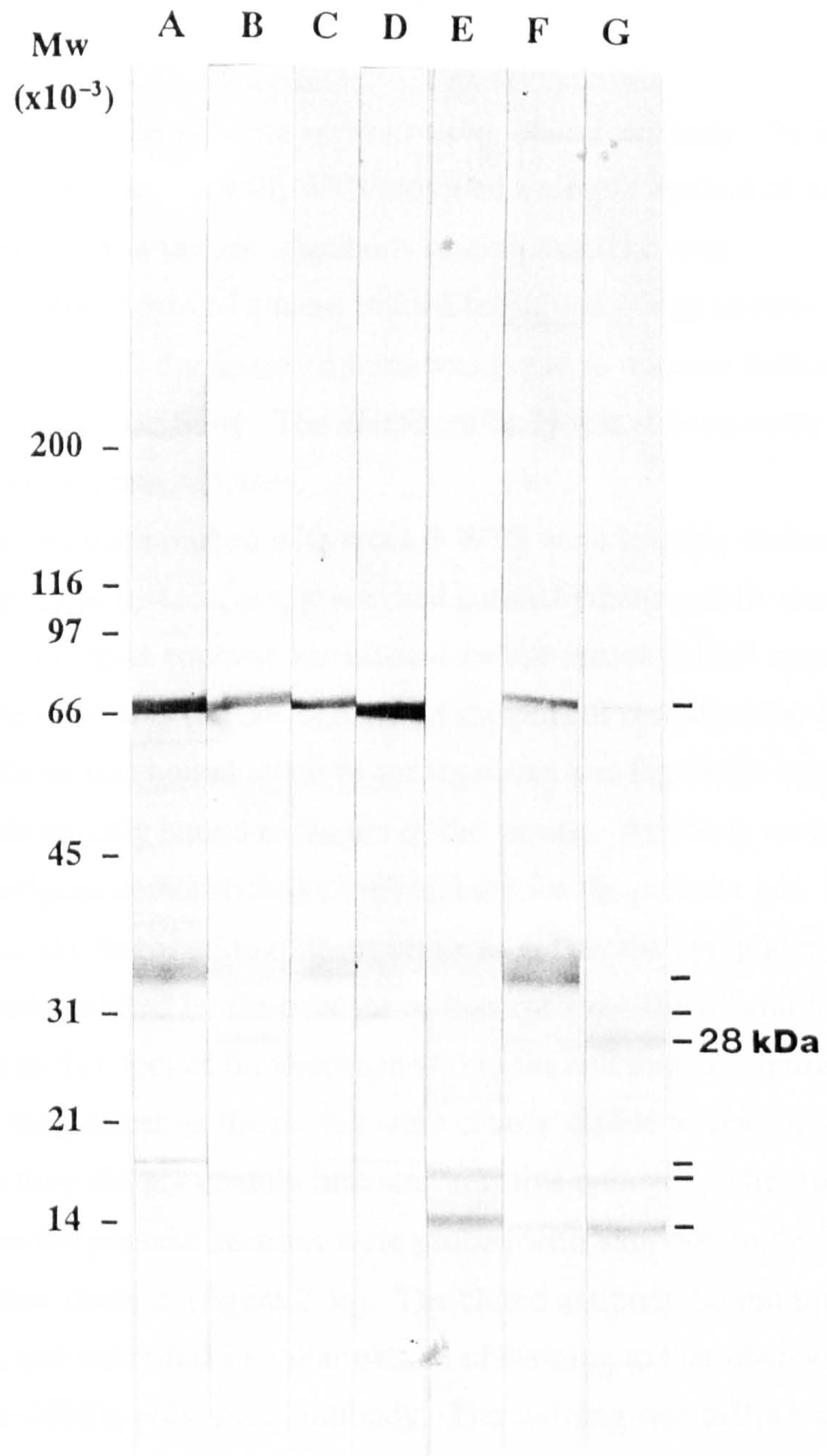
A, Mw markers (x10^{-3}); B, male–only SWAP; C, oesophageal material; D, gonad material; E, parenchymal preparation (rear portion); F, female–derived SWAP; G, purified tegumental membrane; H, adult worm culture supernatant. Proteins of interest are indicated by arrows.

Figure 2.5 Western blotting analysis of different antigen preparations probed with WTS week 8, diluted 1:1000.

The antigen preparations were loaded at the same protein concentration (20μg/well) in the following order:

A, male–only SWAP; **B**, soluble oesophageal fraction; **C**, soluble gonad preparation; **D**, soluble parasite material depleted of oesophageal and gonad components (rear); **E**, isolated tegumental membrane fraction; **F**, female–only SWAP; **G**, adult worm *in vitro* culture supernatant.

Antigens of interest are represented by bars.


Mw
(x10⁻³)
A
B
C
D
E
F
G
200
116
97
66
45
31
21
14
28 kDa

antigen, of the same relative molecular weight as the proposed haemoglobinase, was prominent in the female-derived SWAP and male-only SWAP lanes. A previously unidentified 28 kDa antigen appeared in the oesophageal preparation and, to a lesser extent, in the female SWAP and culture supernatant. Lower molecular weight antigens 24, 18 and 14 kDa were detected in the tegument membrane extract and culture supernatant.

Immunocytochemistry on parasite sections using eluted antibody. Probing the various antigen preparations with WTS provided a simple method of locating the E/S products within parasite tissues. Antibody elution together with immunocytochemistry proved a more refined technique. Oligospecific polyclonal antibody for 8 of the 12 dominant proteins was found to relocate faithfully to fresh blots confirming its specificity. The eluted antibody was subsequently used to probe cryostat sections of parasite tissue.

The parasite sections probed with week 8 WTS were brightly stained (figure 2.6a). The tegument surface, gut, gonad and parenchymatous cells were strongly immunogenic. In direct contrast, the normal mouse serum did not appear to react with any worm structures (figure 2.6b). The samples of eluted antibody were grouped into those that bound either to the tegument and tegument membrane or those that preferentially bound to the gut of the worms. Antibody to the 67, 53, 38 and 32 kDa antigens demonstrated a high affinity for the parasite gut. Furthermore, it appeared that the distribution of these antigens within the cytoplasm of the cells differed, as distinguished by the patterns of fluoresence. The 67 and 53 kDa proteins were exposed as tiny foci of fluorescence within the cell matrix (figure 2.6c–d). Under higher magnification the nucleii were clearly visible as regions of negative staining since they did not contain immuno-reactive epitopes. The fluorescence observed when the parasite sections were probed with antibody to the 38 and 32 kDa proteins was less distinct (figure 2.6e). The eluted antibody bound intensely to the gastrodermis, and exhibited a similar pattern of staining to that observed with Rotmans' anti-32kDa polyclonal antibody. The staining was diffuse and hence distinct foci of fluorescence were not demonstrable.

Antibody eluted from the lower molecular weight bands 24, 19, 18 and 14 kDa bound to the tegument of the schistosomes (figure 2.5f). The tegument membrane of the worm was bright and sharp with the contours of the tegumental pits clearly visible. Once more the pattern of binding was non-uniform with a gradation in fluoresence from the basal membrane to the apical membrane of the syncitial

Figure 2.6 Immuno–localisation of secretory proteins within adult male schistosome tissue using affinity–purified antibody for each antigen of interest. Examples of cryostat sections probed with the following antibody are shown:

a) WTS week 8, positive control, magnification x40.

b) NMS, negative control, magnification x40.

c) eluted antibody against the 67 kDa antigen, magnification x100.

d) eluted antibody to the 53 kDa antigen, magnification x100.

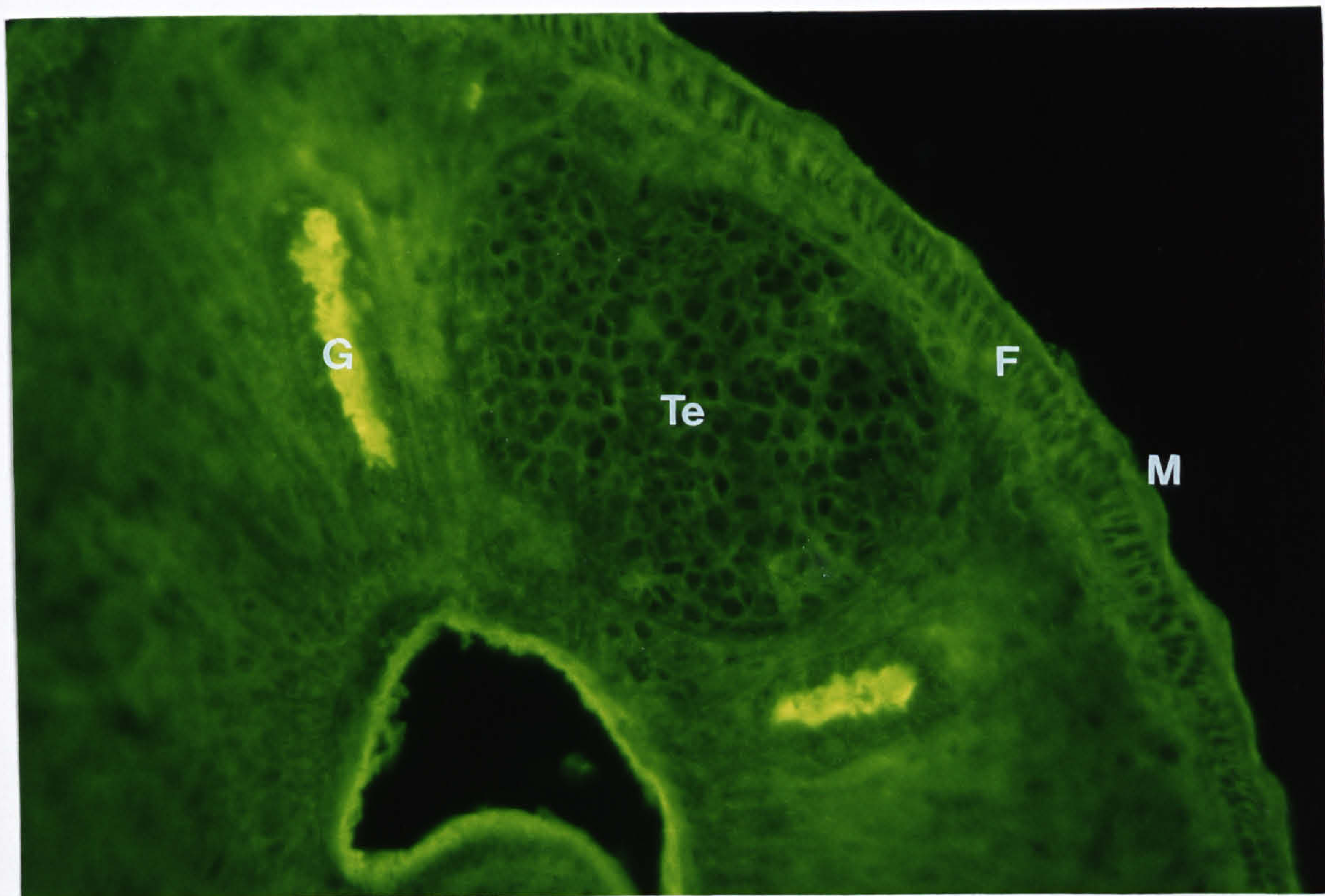
e) affinity–purified antibody to the 32 kDa antigen, magnification x100.

f) eluted antibody to the 19 kDa antigen, magnification x40.

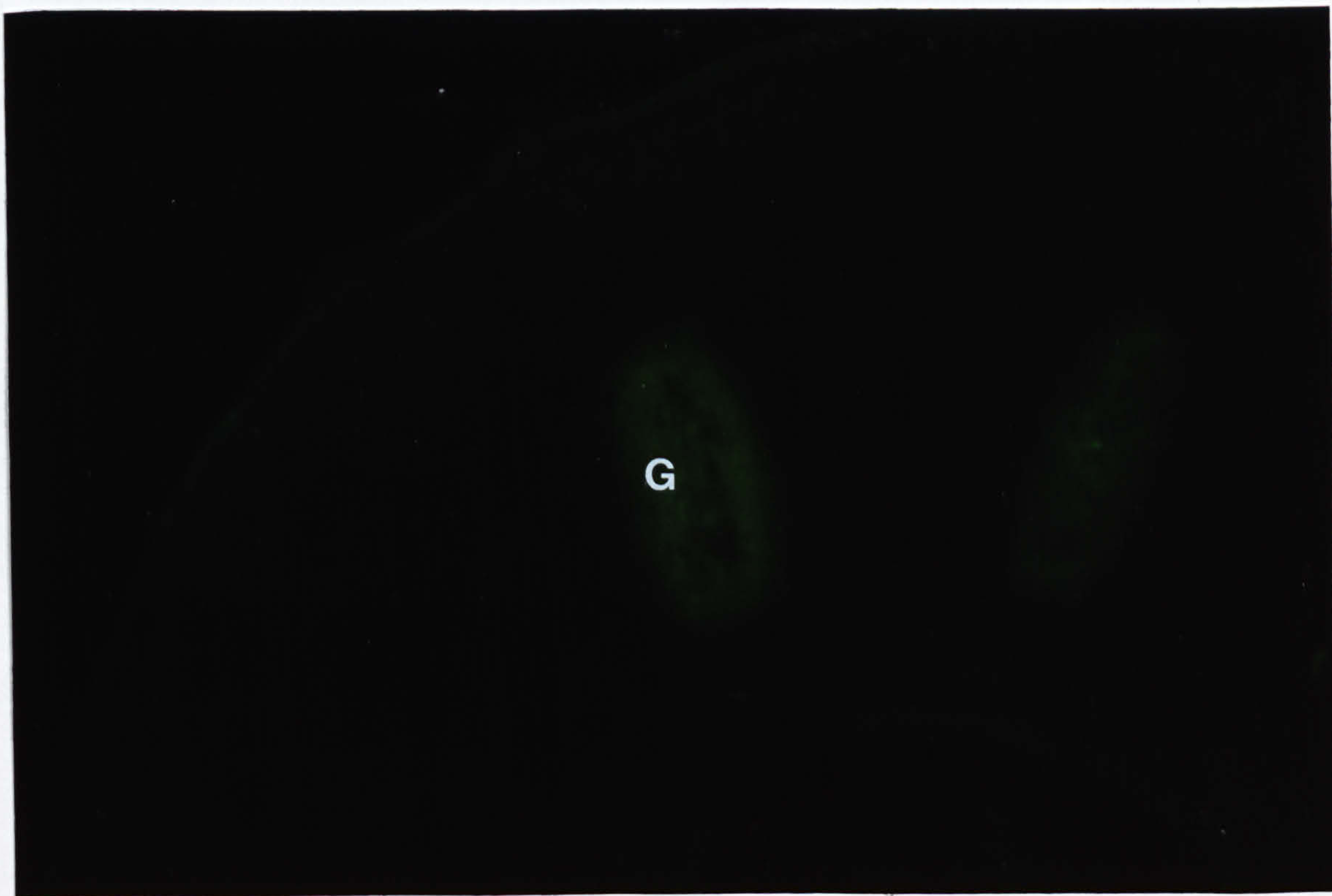
g) eluted antibody against the 14 kDa antigen, magnification x100. All sections were photographed under oil.

KEY: Gut (G); muscle fibres (F); tegument membrane (M); subtegumental cell (St); male worm gonads (Te).

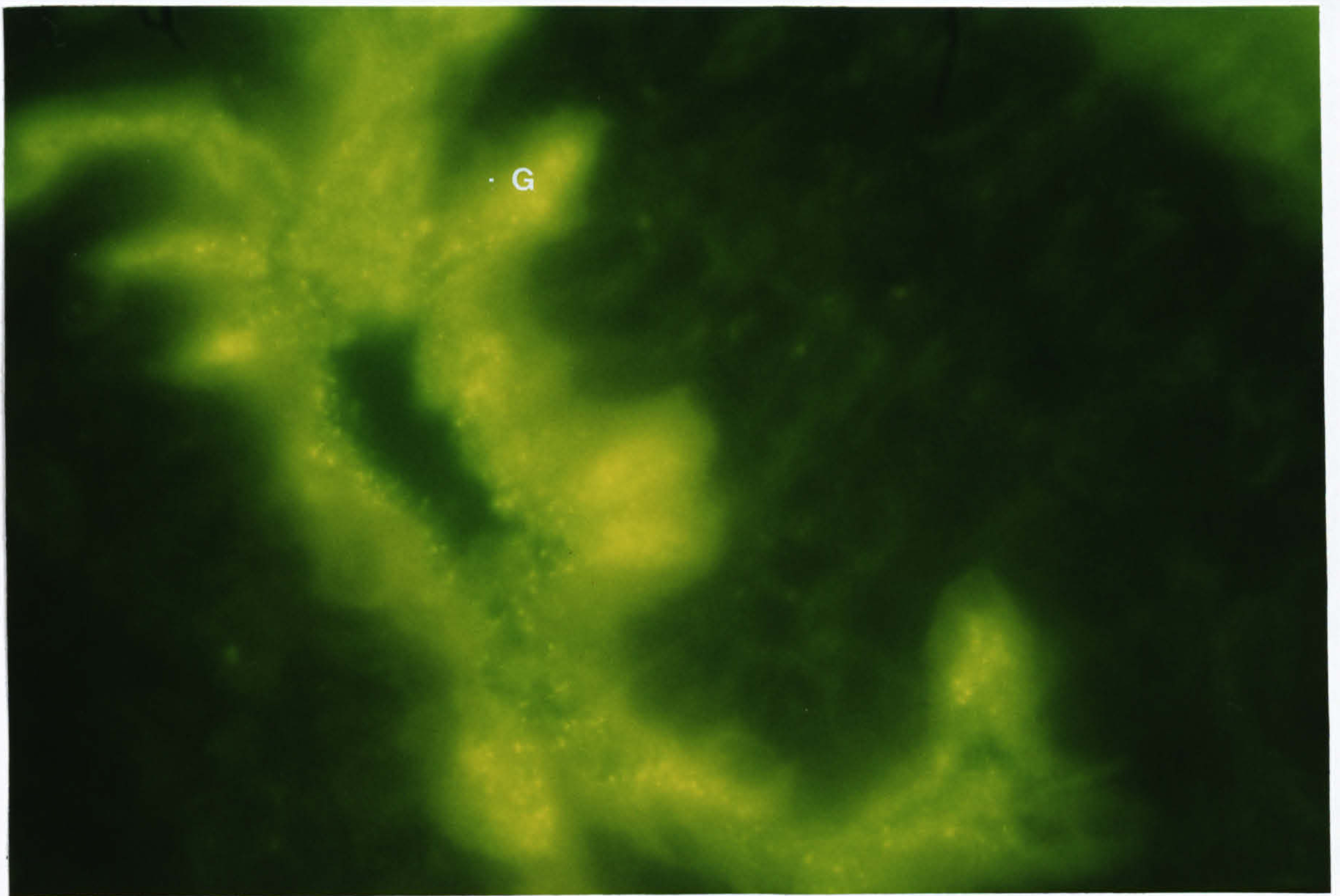
2.6a



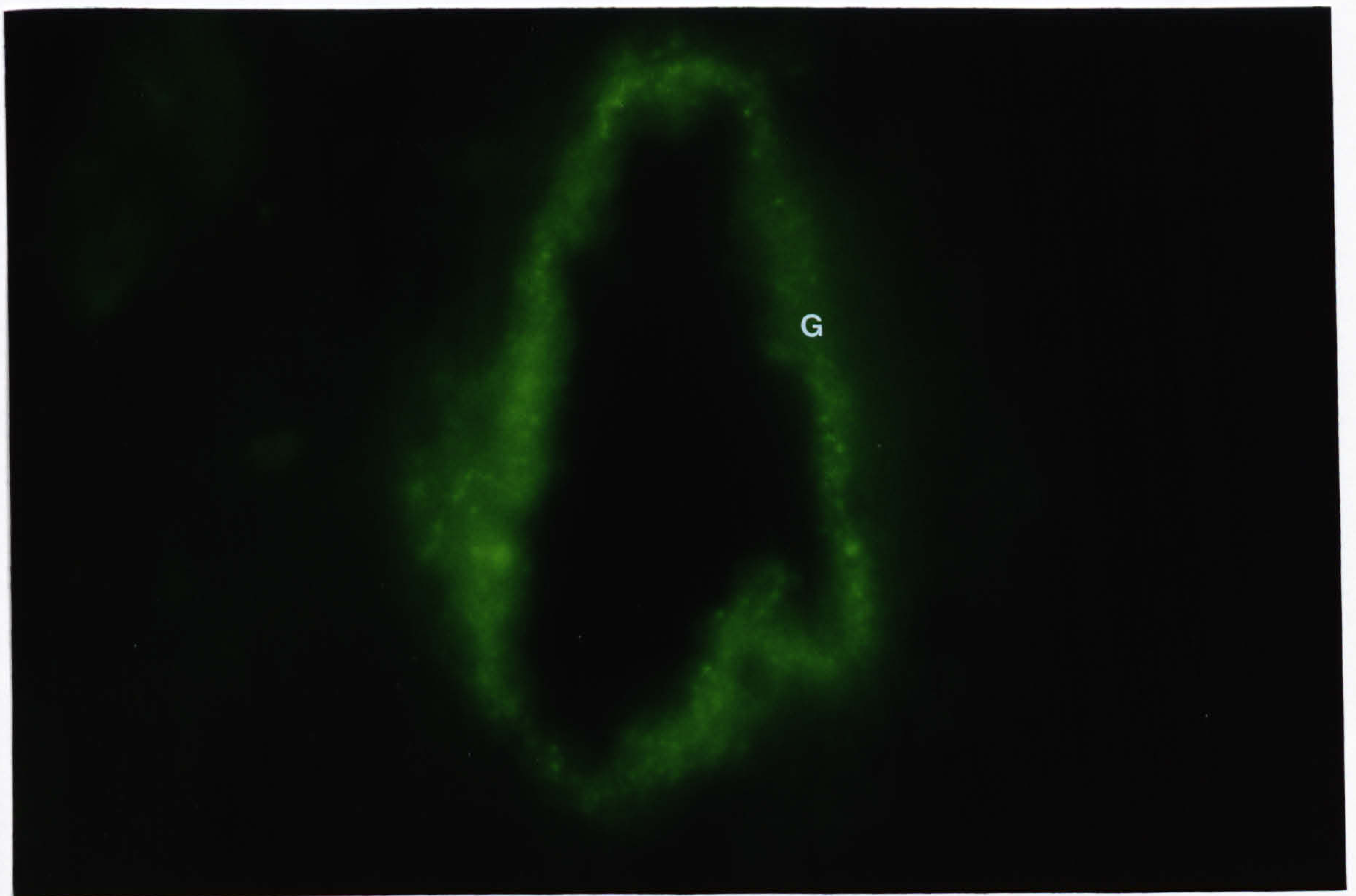
2.6b



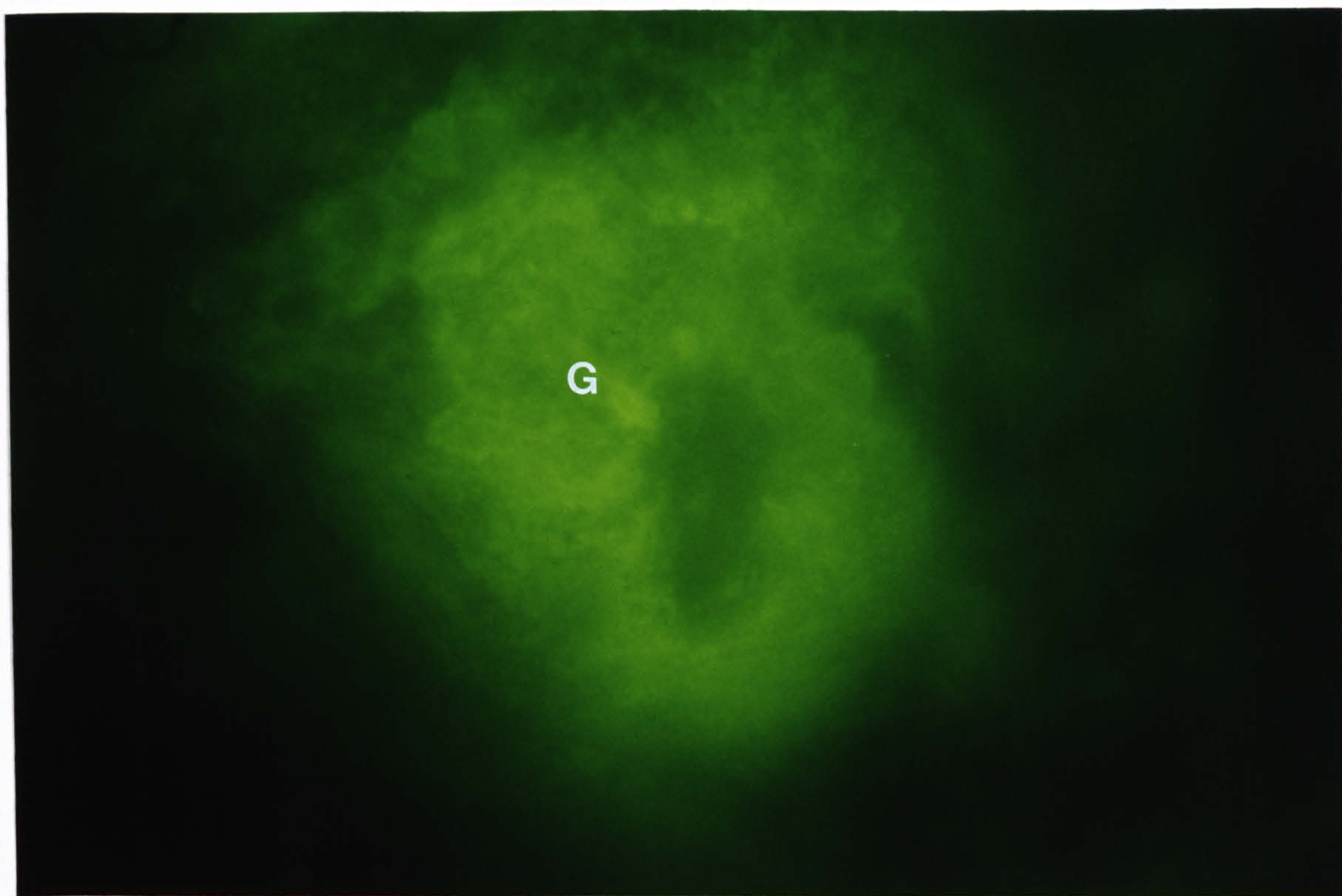
2.6c



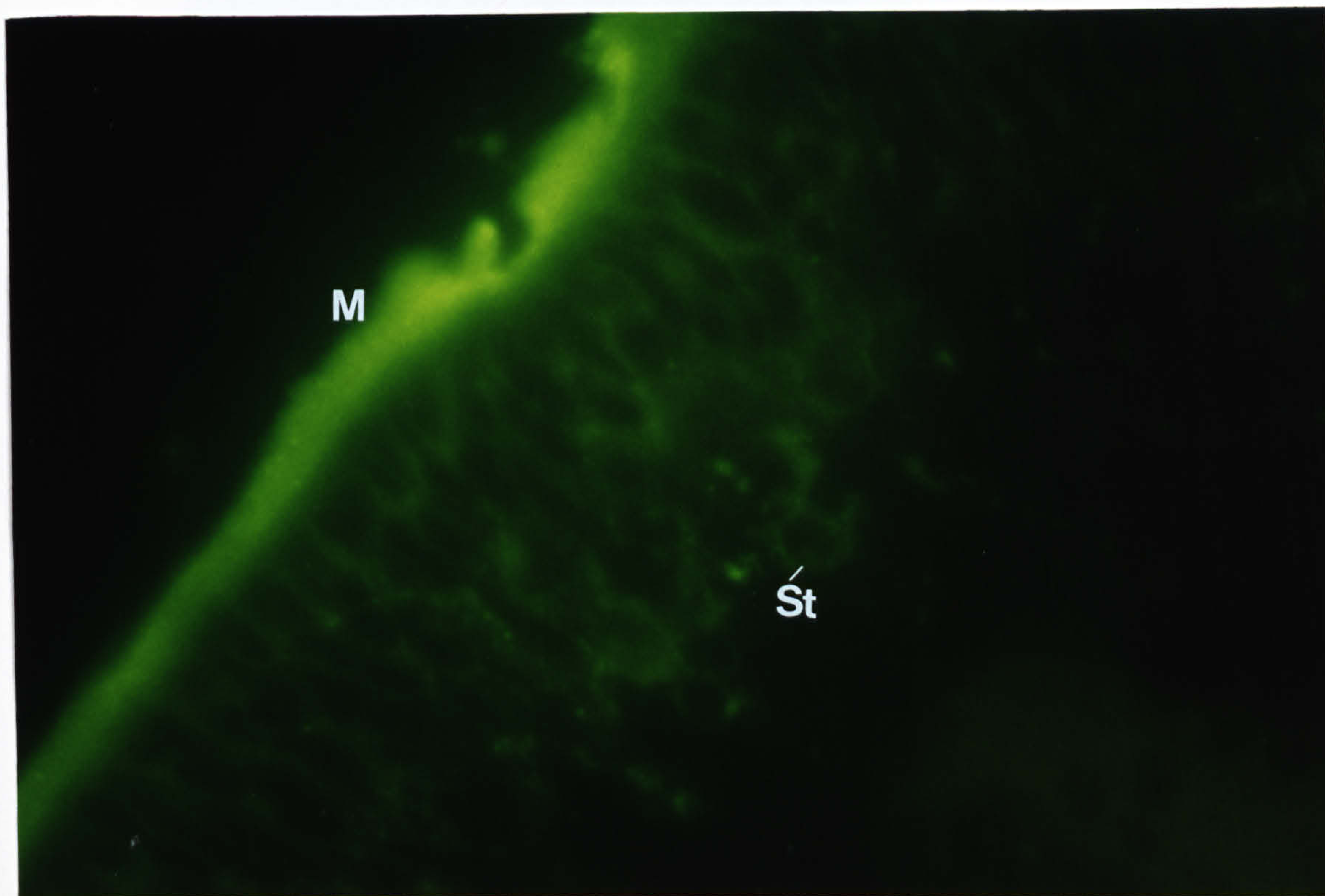
2.6d



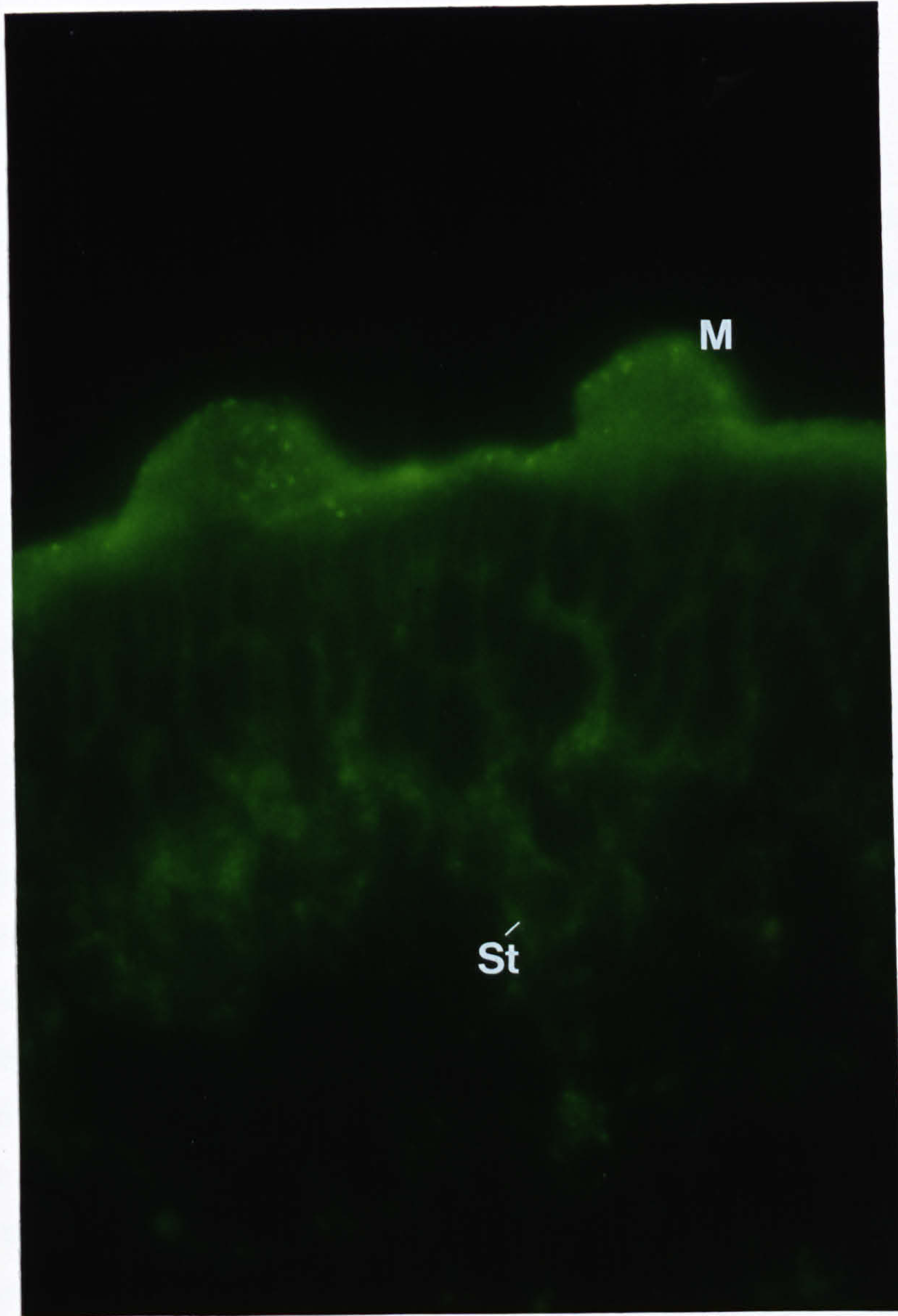
2.6e



2.6f



2.6g



tegument. Beneath the basal membrane lies the circular and longitudinal muscles. Their surfaces were strongly stained creating a reticulum network of fluorescence. Subtegumental cells, embedded amongst the parenchymal cells, were evident at intervals along the dorsal surface of the worm when probed with the anti–14kDa oligospecific polyclonal antibody (figure 2.5g). Under higher magnification it was difficult to determine if the 14 and 19 kDa antigens were localised at the surface of each subtegumental cell or present within the cell matrix.

2.4. DISCUSSION

Materials released by living parasites have received considerable attention because of their potentially important role in the immunobiology of infection (McKerrow & Doenhoff, 1988) and their ability to generate significant protection against several species of helminth (Britton *et al.* 1993; Yamada *et al.* 1991; Tendler *et al.* 1991). Several studies have attempted to identify and characterise the diverse range of schistosome released products secreted by the developing parasite, from cercaria (McKerrow & Doenhoff, 1988), to the schistosomulum (Harrop & Wilson, 1993) and finally the adult worm (Lewis & Strand, 1991; Atkinson & Atkinson, 1982; Rotmans *et al.* 1981; Kusel, Mackenzie & McLaren, 1975; Murrell *et al.* 1974), thereby creating an understanding of worm biology and facilitating the development of a vaccine. All of these investigations have relied upon methods of *in vitro* culture to obtain sufficient quantities of E/S products for characterisation yet, as alluded to previously the viability of worms during such culture regimes may be compromised (Rotmans & Burgers, 1987; Wilson & Barnes, 1974b) and as a consequence somatic contaminants released into the surrounding medium (Carlisle *et al.* 1983).

In the present study we have used an *in vivo* culture system which involved the transfer of live adult worms from donor mice to the hepatic portal system of naive recipients of the same inbred strain. Surgical transfers carried out using this technique permitted the parasites to maintain normal physiological function, as revealed by the presence of circulating anodic antigen (CAA; indicative of a live schistosome infection) in the serum of recipients (Saunders *et al.* 1993). In addition, serum samples were collected from the recipient animals at weekly intervals post–transfer and the WTS used in western blotting studies to monitor the complex subset of polypeptides released *de novo* into the host bloodstream by healthy schistosomes.

2.4.1 *The surgical transfer of adult schistosomes*

The success of the surgical transfer technique has been proven prior to this study by several workers using monkeys (Smithers, Terry & Hockley, 1969), mice (Boyer, Ketchum & Palmer, 1976; Saunders *et al.* 1993) and hamsters (Cioli, 1976; Saunders, Wilson & Coulson, 1987) as recipient hosts. In all studies the physiological status of the transferred worms has been either assumed or inferred from parasite survival and egg-deposition. More recently, the systemic presence of the well-defined gut secretory proteoglycan CAA (DeWater *et al.* 1986), released by healthy, feeding parasites has provided a diagnostic marker for viable infections (Saunders *et al.* 1993). Confirmation of normal worm function during residence in the host was verified following careful examination of recovered worms under a light microscope. Saunders concluded that the system of worm transfer worked well, particularly with male schistosomes since it avoided egg-induced pathology permitting long-term studies to be undertaken. In addition, the technique allowed the characterisation of secretory antigens released into the host circulation.

The objective of the study was firstly, to identify by molecular weight, the proteins that induced an IgG response and secondly, to locate their site of secretion from the tissues of adult worms. This knowledge has provided a basis for the succeeding chapters three and four, in which the IgE response mounted against parasites by infected laboratory rats has been investigated. The WTS has defined a subset of scarce secretory products released by adult worms, so the response may be used as a template against which the molecules identified by the rat infection serum can be analysed.

It is plausible that degrees of cross-reactivity exist between the adult worm proteins detected and those molecules released by larval stages; this phenomenon has been widely documented for a variety of antigens (Porchet *et al.* 1994; Riengrojpitak *et al.* 1989; Payares *et al.* 1985(a); Atkinson & Atkinson, 1982). By separating sonicated extracts of cercariae, schistosomula, and adult worms using 2-D SDS-PAGE, Miller, Rekosh and LoVerde (1989) calculated that adult worms shared 60% of the polypeptides of schistosomula. Irrespective of the antigenic similarity between life-stages, the fundamental purpose of my study was to determine the nature of the proteins of interest.

Since the transferred parasites reside in the mouse for a moderate length of time, the host's excretory system could limit the detection of worm secretory products; the liver functions as an effective detoxification mechanism for foreign and non-essential systemic material. Phagocytosis and enzymatic degradation of schistosome

antigens by hepatic Kupffer cells not only reduces the amount of antigen available for immune processing, but may also alter the character of the native protein, reducing it to a series of smaller immunogenic peptide units. Thus, the distortion of schistosome proteins by protease digestion may induce the production of antibody to a series of peptides that do not resemble the more complex quaternary epitopes of the intact molecule.

2.4.2 *IgG production in response to E/S material*

The transfer of mature worms permitted the production of antibody against released proteins from adult male schistosomes to be examined over an extended period of study. Previous publications have adopted different approaches to detect the release of secretory products. However the techniques used have not been standardised and use different incubation media in conjunction with different methods of detection. The low and inconsistent yield of antigen, obtained by laborious and expensive *in vitro* culture procedures, limits many investigations into the immunochemical nature of worm secretions. According to Murrell *et al.* (1974) one adult worm releases approximately 0.2–0.4μg protein/worm/48hr. *In vitro* systems of culture also result in the restricted release of antigens as shown in the present study (figure 2.5) (Rotmans and Burgers, 1987; Kusel & MacKenzie, 1975). Despite these inherent disadvantages *in vitro* studies have been used to demonstrate the antigenic heterogeneity of worm secretions (Rotmans *et al.* 1981; Murrell *et al.* 1974), and furnished our knowledge of tegumental membrane turnover (McLaren, 1980; Wilson & Barnes, 1974a; Kusel *et al.* 1975).

The range of E/S polypeptides readily available as targets for the immune system differs between studies. Atkinson and Atkinson (1982) maintained radiolabelled adult parasites in serum–supplemented medium for up to 2 weeks. Fluorographic 2–D analysis of the released proteins revealed that 74 polypeptides were commonly secreted by male and female worms. Lewis and Strand (1991) performed a similar investigation immunoprecipitating the immunogenic fractions with schistosome–infected human serum and then resolving the antigens by 2D SDS–PAGE. They detected an extensive range of antigens from 220 – 20 kDa; eight of the immunoprecipitated glycoproteins identified were greater than 60 kDa. However in the present study only 3 higher Mw proteins (208, 67, 62 kDa) elicited an antibody response. Moreover, Lewis characterised additional lower molecular weight polypeptides consistent with the western blotting data reported here. Direct comparisons of this nature are limited since the Lewis study obtained E/S parasite

material by *in vitro* culture for 9h only and used serum from schistosome-infected humans to immunoprecipitate antigens. The incorporation of radiolabel in both the Lewis and Atkinson studies may also misrepresent the true extent of protein release by adult worms, as the identification of antigens may be restricted to those that contain methionine (Lewis & Strand, 1991) or leucine (Atkinson & Atkinson, 1982). Thus, proteins not containing these amino acids were not demonstrable by immunoprecipitation.

Care must be taken in correlating the appearance of schistosome-specific IgG in the host bloodstream to the kinetics of antigen release from adult worms. The development of this arm of the humoral response is dependent upon many rate-limiting factors: a) the amount of antigen released, b) the initial processing of antigen by antigen presenting cells (APCs), c) co-stimulatory interactions between T and B cells, and d) the efficacy of antigen-specific B cell clonal expansion and antibody production. Furthermore, with the advent of sensitive western blotting detection protocols, such as enhanced chemiluminescence (ECL), it is possible to identify scarce antigenic products. However, in the present study the less sensitive DAB development reagent was used and as a consequence may have limited the early detection of proteins immediately post-transfer. To minimise this shortcoming, the efficacy of the DAB method was optimised by loading high concentrations of male SWAP onto SDS-PAGE gels prior to electroblotting, thereby maximising the capture of schistosome-specific IgG present in the serum.

The magnitude of the IgG response against each antigen was determined by measuring the absorbance of each antigen band by optical densitometry as a function of time post-transfer. Two categories of antibody response predominated. The first category, encompassing proteins identified 1-2 weeks post-transfer, suggested that the molecules were released in larger quantities from the adult worm than the second group of antigens. However, as alluded to above the rate of protein release by worms was difficult to assess, primarily because the function of hepatic Kupffer cells could have obscured the nature of secretion. Despite this caveat, high levels of antibody were directed against the 67, 53, 38 and 32 kDa proteins early post-transfer. It appeared that the production of antibody reached a plateau 4-5 weeks post-transfer, since the levels of specific-IgG did not increase.

Conversely, the second group of antigens, comprised of most of the lower molecular weight proteins, did not induce a detectable IgG response as quickly as the 67, 53, 38 and 32 kDa proteins. The following explanations could account for the different antibody responses observed. The antigen in category two responses could

be: a) processed less effectively by APCs, b) comprised of less immunogenic epitopes than the higher molecular weight fractions, or c) released in scarcer quantities than category one antigens.

The parasites recovered intact from the recipient hosts did not appear to be affected by the period spent *in vivo*. Hence, the intense humoral immune response mounted against the secretory products did not influence their survival. It is not surprising that the host's immune response is directed against released antigens that can be recognised without causing harm to the adult worm, since mature parasites can live within a susceptible host for many years. Furthermore, the proteins released are highly immunogenic but not host protective, supporting "Waksman's postulate" that molecules crucial for worm viability are effectively hidden from the infected animal's immune system (discussed by Sher, 1988).

2.4.3 *Tissue localisation of the E/S products*

The secretory nature of the antigens was confirmed by determining their site of release from the tissues of adult worms using two different experimental approaches. The first technique involved the careful dissection of adult male schistosomes into 3 portions (figure 2.1). This procedure allowed enrichment of soluble proteins specific for regions of potential secretory activity. By equating the protein concentration of each sample it was possible to compare fractionation patterns between different tissue preparations. In general, there was a high level of homology between the banding patterns seen on coomassie–stained gels of soluble material, with the exception of the culture supernatant and the tegumental membranes. These two protein mixtures were less complex, containing fewer polypeptides (13 and 16 respectively) compared to male–only SWAP which was comprised of 30 distinguishable bands. The proteins were also transferred to PVDF membrane and incubated with WTS to identify antigenic fractions. Since the concentration of protein used in this experiment was limited by the amount of protein released during *in vitro* culture (one quarter of that transferred in the initial western blotting study), some of the 12 immunodominant antigens were not demonstrable in the male–only SWAP. Thus, the overall reactivity of the immunoblot was reduced and the 53 and 24 kDa proteins were too scarce to bind detectable levels of antibody.

The results showed that there were varied patterns of IgG binding between the various soluble preparations (figure 2.5). The most striking differences were seen in the tegumental membrane and culture supernatant fractions. Weak antigenic reactivity was demonstrated against the 32, 30, 24 and 19 kDa proteins isolated from

schistosome tegumental membrane, and hence it is suggested that these proteins originate from the membrane of adult worms. Payares *et al.* (1985a) has described a similar range of proteins: Mw 32, 25, 20 and 15 kDa associated with the tegument of adult worms. Antigens were isolated, purified and the 32 and 20 kDa products shown to be consistently immunogenic when used to actively immunise rabbits, rats and mice (Payares *et al.* 1985b). Another study by Pearce and Sher (1989) described a subset of immunogenic surface antigens Mw 38, 32 and 18 kDa, that were released from 3h schistosomula cultured *in vitro* post-transformation. Immune recognition of the 18 kDa protein appeared to occur only in vaccinated mice and not in infected mice. However, infected mice did produce antibody to a molecule of 20 kDa, which is of comparative Mw to the 19 kDa described here. However, the similarity between Pearce's surface immunogens and the adult worm fractions identified in the present study is debatable, since Pearce's radiolabelled 38 and 32 kDa molecules were not demonstrable in the secretions from older lung stage parasites. However Pearce and Shers' study emphasises the potential role of released products, and more specifically epitopes shed from the surface of parasites, in the development of the humoral response.

The culture supernatant was comprised of faint bands at 67, 32, 30, 28, 24, 18 and 14 kDa. Since the binding of IgG was so similar between the membrane and culture supernatant it is proposed that cultured parasites release most of their soluble immunogenic products from the tegument. This finding was also supported by Rotmans *et al.* (1981) and Lewis and Strand (1991) who found that the gut contributed very little to the products released *in vitro*, although they did not identify/characterise the individual molecules. Furthermore, following a pulse-chase incubation with adult worms Wilson and Barnes (1979) showed evidence for the more prolific secretion of exportable leucine-containing protein from the tegument (67–80%), compared to the gut (20–33%). Hence, it is plausible that the 3h incubation of schistosomes used in the present study restricts the identification of antigens to more abundant products released from the tegument. The implication is that *in vitro* maintenance of worms limits their secretory potential by either, discouraging them from opening their oesophagous to the less favourable environment, or because the adults no longer feed actively and therefore are less likely to release the products of digestion (Rotmans & Burgers, 1987). In addition, studies on cultured adult worms maintained under unfavourable conditions have indicated that the parasites respond immediately by attempting to replace their outer membrane (Hockley & McLaren, 1973). This phenomenon will occur even in

certain basic culture media not supplemented with serum and is accompanied by the more frequent appearance of membraneous bodies in the tegument cytoplasm. The bodies, participants in membrane replacement (McLaren, 1980; Wilson & Barnes, 1974a; Hockley & McLaren, 1973), align themselves beneath and in close apposition to the double outer membrane. It seems likely therefore, that in the present study and the *in vitro* labelling experiments described above, the worms may be attempting to shed their surface macromolecules in order to repair membrane damage.

A 28 kDa antigen, previously unidentified by WTS, was detected within the culture supernatant preparation. This protein appeared to originate from the oesophageal secretions as it was also present in the soluble oesophageal sample. This observation was supported further by its absence from the gut-enriched, gonad and tegumental membrane preparations. An oesophageal-derived molecule of similar Mw has been partially cloned by Dr. Agnew and co-workers (personal communication). This protein is detected in the caudal region of the oesophagous, and is present within the cells of the oesophageal gland. At a subcellular level it appears to be contained within secretory vesicles, indicating that it is released into the narrow lumen of the oesophagous. These workers propose that this molecule possesses anti-coagulant properties.

The Sm32 protease described by Sauer and Senft (1972), and later by Deelder, Reinders and Rotmans (1977), possessed the same relative molecular weight as the 32 kDa antigen reported here. The 32 kDa protein identified by the WTS was a ubiquitous component appearing in all preparations, but expressed to a greater extent in the male-only SWAP and female-derived soluble material. It was anticipated that as a major enzyme believed to be involved in the digestion of erythrocytes (Timms & Beuding, 1959) it should be found in large amounts in the parasite gut. As female schistosomes *in vivo* ingest 8.5 times more erythrocytes than do males (Lawrence, 1973) the female-only SWAP represented a more metabolically active source of gut products, compared to the other samples. Thus, the intense binding of IgG from the WTS to this protein in the female SWAP, in comparison to the other preparations, suggested that it could represent a gut-derived antigen.

A narrow band at 30 kDa was demonstrable in the sample depleted of oesophageal and gonad material (rear), male-only SWAP and to a lesser extent in the culture supernatant. This moiety could be related to the antigen of 31 kDa described by Dresden and Deelder (1979) and Lindquist *et al.* (1986). Sm31 or Cathepsin B is a thiol proteinase (Dresden, Payne & Basch, 1982) and has been proposed as a prospective candidate in the diagnosis of murine and human

schistosome infections (Chappell *et al.* 1990; Klinkert *et al.* 1988). More recently it has been studied at a molecular level being expressed in a baculovirus transfer vector (pAcDZI) and insect cells (Gotz *et al.* 1992; Felleisen & Klinkert, 1990; Klinkert *et al.* 1989).

Gotz and Klinkert (1993) provided evidence that Sm31 was the enzyme responsible for the breakdown of haemoglobin, casting doubts upon the identity of Sm32 as the major haemoglobinolytic enzyme of *S. mansoni*. If this result proves to be correct, then the work described here would need to be repeated with a monospecific serum or monoclonal antibody directed against Sm31. Both Sm32 and Sm31 have been traced to the gastrodermis of adult worms by immunocytochemistry, thereby confirming their proposed secretory origin (Chappell & Dresden, 1987).

Finally, the absence of the highly immunogenic 67 kDa product within the tegumental membrane preparation is suggestive of its dominance as a gut secretory product. King and co-workers investigated a glycoprotein of similar molecular weight (SmW68), produced by all life stages of the parasite following murine *S. mansoni* infections (King *et al.* 1987). Active immunisation of BALB/c mice with 20μg of purified SmW68 conferred up to 66% protection to a challenge infection (King *et al.* 1987). In view of this, King demonstrated that a significant IgM response to this antigen occurred in chronically infected humans; the antibody titer increased with age showing an inverse correlation ($r = -0.28$, $p < 0.04$) with intensity of infection (King *et al.* 1989). Ultrastructural localisation of the antigen in the tissues of adult worms was performed using specific polyclonal anti-serum raised in SmW68-vaccinated mice and a mAb directed against purified SmW68 (Blanton *et al.* 1991). The anti-SmW68 immunoglobulin bound specifically to the guts of male and female worms, consistent with the predicted origin of the 67 kD protein, identified in the present study.

The 67 kDa antigen also possesses a similar Mw as the tegument-associated enzyme alkaline phosphatase (Mw 66 kDa). Initially identified by (Payares, Smithers & Evans, 1984) its abundance in tegumental membrane preparations is commonly used as an indicator of membrane purity (Lewis & Strand, 1991; Roberts *et al.* 1983). However, the possibility that the 67 kDa molecule described in this chapter is alkaline phosphatase is unlikely, since the transferred parasites remained undamaged whilst resident in the host. Furthermore, previous publications have not described this membrane-associated antigen as a released product.

The western blotting technique provided preliminary results regarding the origin of antigen release. To verify and expand upon the conclusions drawn, the cellular distribution of each antigen was determined by probing cryostat sections of adult male schistosomes with affinity-purified oligospecific polyclonal antibody for each protein of interest. Eight out of the twelve antibodies were successfully eluted from western blots. The relative paucity of antibody to the 208, 62, 30 and 22 kDa antigens meant that the retrieval of immunoglobulin from these specific proteins was comparatively ineffective.

In this study, four of the eluted fractions reacted with their respective antigenic targets in the schistosome gut; the remaining four bound to the tegument of adult worms. Distribution of the target epitopes supported the data obtained from the western blotting study. In addition, patterns of reactivity were revealed at a cellular level, permitting a more detailed description of the secretory source.

Eluted antibody directed against the 67, 53, 38 and 32 kDa antigens relocated to the cells lining the lumen of the gut. Moreover, a differential distribution of antigenic epitopes was revealed within the cell matrix. Antibody to the 67 and 53 kDa antigens was evident as packets of intense reactivity indicating that the antigens are synthesised and stored within vesicles ready for transport to the gut lumen. The staining pattern of the anti-67 kDa eluted antibody is comparable to the results presented by Blanton *et al.* (1991). All evidence suggests that the 67 kDa antigen is King's SmW68 molecule. In order to corroborate this observation it would be necessary to obtain MAb 31-3B6 and use it to probe schistosome sections alongside the affinity-purified anti-67 kDa immunoglobulin in a competition assay. If the specific reactivity of the eluted antibody to the 67 kDa protein was blocked it would suggest that the monoclonal antibody was directed against the same epitope. Comparisons between the two reactivities could then be made more accurately using a standardised immunocytochemical approach.

The subcellular source of the 38 and 32 kDa antigens was less distinct. Since it was possible to identify the negatively stained cell nucleii under high magnification it appeared that the target epitopes were distributed within the cell cytoplasm. However, unlike the 67 and 53 kDa proteins their inclusion in secretory vesicles was debatable since reactivity was not aggregated into foci of fluorescence. Therefore, the 38 and 32 kDa products may represent constituent cytoplasmic components not involved directly in the process of digestion. Alternatively, the antigens may be generated in such abundance that the limited resolving power of the light microscope cannot distinguish individual pockets of reactivity. It is noteworthy that the

proposed haemoglobinase, Sm32, also displayed the same cellular distribution pattern as the 38 and 32 kDa proteins described in the present study. Thus, it may be surmised that the latter antigens share epitopes with each other as well as "haemo^hglobinase".

The reactivity of the cytoplasm of the cells with all 4 gut-defined antibodies could delineate the intracellular sites of protein synthsis (ER, Golgi, vesicles). Using the eluted antibodies it should be possible to examine the sites of protein synthesis and transport mechanisms at a molecular level by immuno-gold electron microscopy.

Oligospecific polyclonal antibody to the 24, 19, 18 and 14 kDa proteins immuno-localised to the tegument, sunken subtegumental cell bodies and the zone in between. The dorsal regions of male worms exhibited more intense fluorescent staining compared to the ventral surface. It is worth emphasising that the dorsal region of the male parasite is highly convoluted and formed into large bulbous tubercules, with pits expressed along it's entirety. These tubercules allow the mature male to gain purchase along the mesenteric veins. Thus, it is plausible that the dorsal side is continually exposed to mechanical wear during the parasite's residence in the host bloodstream, thereby establishing this surface as a richer source of released antigens, compared to the worm's ventral region. The enhanced binding of antibody to this site appears to validate this supposition.

The pattern of tegumental staining corresponds to the events involved in membrane synthesis, as described by other workers. Membraneous vesicles, pre-synthesised in the sub-tegumental cell bodies are translocated to the surface of adult worms, via cytoplasmic connections, where they participate in the process of membrane replacement (Wilson and Barnes, 1974a; Hockley & McLaren, 1973). The cell bodies, cytoplasmic tubules (which run between the cell bodies and the tegument) and the antigenically dense tegumental layers were all clearly differentiated in this study. The characteristic honeycomb appearance was probably due to the negative reaction of the longitudinal muscle fibre cytoplasm (Riengrojpitak *et al.* 1989). Hence, a clear distinction between the staining of the muscle fibre membranes, cytoplasmic connections and interstitial ground substance cannot be made. Selective reactivity of the antibody is suggested since other cell membranes are not stained, for example the gonads and parenchymal cells. Hence, it is proposed that the antibody is directed against specific tegumental products and not moieties expressed upon cell membranes in general. However, a certain degree of cross-reactivity must exist between the target epitopes and other common

constitutive components, since unfractionated WTS was directed against all cell types including the non-secretory tissues (parenchymal cells). Furthermore, a gradation in the intensity of staining was observed from the inner basal membrane to the apical membrane of the tegument surface. This polarisation probably reflects the accumulation of higher densities of protein transported through the syncitial cell layer of the tegument to the outer membrane.

In conclusion, the culture of adult parasites *in vivo* has facilitated the analysis of worm proteins released during a schistosome infection. This technique has many advantages over *in vitro* methods of culture since: 1) the antibody response is used as a sensitive detector of scarce released products, 2) the transfer of adult male worms prevents egg-induced pathology, thereby permitting the study of E/S material over an extended time-course, 3) the host's bloodstream provides an ideal environment for parasite maintenance, 4) the release of significant amounts of somatic contaminants from dead or dying parasites is precluded.

It is interesting to note that after the transfer of adult worms, the first antibodies detectable two weeks post-infection were mostly directed against the gut epithelium. As the time-course progressed additional reactivity was expressed against the tegumental membrane. The early response to gut-associated antigens in comparison to tegumental antigens might be the result of very immunodominant epitopes on the gut-derived products, or of the relatively large amounts of antigens which are released into the host circulation rapidly post-transfer. The high excretion rate of gut-associated antigens means that the worms exhibit high metabolic activity *in vivo* compared to *in vitro* culture, since in the present study most of the immunogens detected *in vitro* were associated with the tegument.

Our results are not consistent with the observations made by Lewis and Strand (1991). These workers concluded that immunogens released by adult worms were not associated with the tegumental membrane. However, the results obtained in the present show that the tegumental membrane preparation was comprised of four immunodominant molecules Mw 24, 19, 18 and 14 kDa, clearly defined by IgG from the WTS. Furthermore, it was established that the 18 and 14 kDa fractions were released *in vitro*. An explanation for the secretory potential of the parasite surface was provided by the work of Pearce and Sher (1989) who showed that the 38, 32 and 18 kDa glycoproteins, released from cultured schistosomula, were detected following cleavage of the glycophosphatidylinositol membrane anchor. This accumulation of data supports the view that the adult worm surface represents an immunologically

active site and that some of those products might be released/shed into the host bloodstream during infection by virtue of their biochemical properties.

Despite the immunogenicity of the surface membrane it was obvious that antibody–dependent effector responses directed against the adult worm were ineffectual in mediating parasite death. Transferred adult worms remained viable throughout the period of study, reflecting the redundancy of such mechanisms in triggering parasite attrition and in inducing the development of immunity to schistosomes in mice. However, it is accepted that no attempt was made to demonstrate the adhesion of IgG (WTS) to live worms in this chapter. Given the knowledge that parasite surface antigens are nearly always immunogenic (Pearce & Sher, 1989; Dissous *et al.* 1987; Payares *et al.* 1985b; Grzych *et al.* 1982), it is not surprising that defence mechanisms, for example: antigenic variation, membrane turnover and host molecule acquisition, would be a major priority for worm survival.

As the WTS has defined a variety of E/S moieties, the IgG response may be used as a template against which the antigens identified by rat infection serum can be compared in chapter three.

CHAPTER THREE

The magnitude and kinetics of the anti-schistosome IgE response in rats

3.1. INTRODUCTION

It is known that helminth infections induce remarkable specific and non-specific IgE antibody responses in humans and animals (Dunne *et al.* 1992a; Dunne *et al.* 1992b; Rousseaux-Prevost *et al.* 1978; Rousseaux-Prevost *et al.* 1977; Sadun & Gore, 1970). The most commonly used parasite in studies of the IgE stimulating effect of helminths has been the nematode *Nippostrongylus brasiliensis*. The elimination of these parasites from the rat is contingent upon the dramatic increase in IgE antibody titers associated with infection. Exposure of rats to *N. brasiliensis* in the laboratory induces an early non-specific rise in serum IgE levels, followed two weeks later by an increase in specific and protective IgE (Jarrett and Miller, 1982). The protective effects of IgE are also evident in the *Trichinella spiralis* model, where it has been associated with the rapid expulsion (RE) of worms from rats (Ahmad *et al.* 1991). In the Ahmad study functional IgE was isolated from whole infection serum and passively transferred, by intra-peritoneal injection, into rats which had been primed 3 days earlier with an intravenous injection of thoracic duct lymphocytes (TDL), derived from infected rats. The results showed that IgE from immune serum, collected 28 days post-infection and administered to naive animals in conjunction with TDL, contained sufficient reaginic activity to promote the onset of RE in infected animals, compared to non-reaginic fractions which had no effect.

Immunoglobulin E is an essential component in mediating immediate hypersensitivity reactions. It interacts, via the Fc region of the molecule, with a highly specific Fc epsilon receptor (Fc∈RI) which is present on mast cells, basophils and eosinophils (Gounni *et al.* 1994; Lee, Swieter & Befus, 1986). These immune cells are believed to be the principal effectors involved in initiating worm death; either directly through release of toxic proteases, or indirectly via the release of a variety of inflammatory cell mediators, such as IL-4, IL-10, Il-5 and TNF-α (Plaut *et al.* 1989).

With respect to the present study, IgE has been implicated in the clearance of schistosomes from infected rat hosts (review by Capron *et al.* 1992). Following a primary exposure of laboratory rats to *Schistosoma mansoni* cercariae, the host is able to eliminate the majority of this intial parasite population, from 28 days post-infection onwards. It is well documented that worm expulsion follows a critical event around the fourth week of parasite development (Cioli, Blum & Ruppel, 1978), a period associated with the onset of feeding and sexual maturation. If schistosomes are transferred from a non-permissive rat host to the mesenteric veins of a

permissive hamster host, up until four weeks post–infection, the surviving parasites demonstrate the ability to recover and persist in the mesenteries of the susceptible recipients (Cioli, Knopf & Senft, 1977). This result is in agreement with the concept that worm development within the rat host is essentially normal during the first month following infection. These workers suggested that rats were either denying the maturing parasites sufficient or specific nutrients for normal development (arthrepsia), or that the host was causing irreparable damage to the invading worm through an effective immune response. Knopf, Nutman and Reasoner (1977) challenged the arthrepsia theory by showing that as the infection dose increased the number of schistosomes resident in the livers of rat hosts also increased. Hence crowding, resulting in the subsequent depletion of nutrient levels associated with higher worm burdens, did not impose restrictions upon the establishment of new worms in the liver. Experiments on athymic (Phillips *et al.* 1983) and thymectomised animals (Cioli & Dennert, 1975), together with the adoptive transfer of purified thymic lymphocytes (Phillips *et al.* 1975), have confirmed the immune and T–cell dependence of host resistance, in addition to establishing the rat host as a representative model with which to elucidate the protective immune mechanisms involved in parasite killing.

The adoptive transfer of antibodies can induce resistance, and anti–μ treatment to deplete B cells reduces immunity (Capron *et al.* 1983; Bazin *et al.* 1980; Phillips *et al.* 1975). *In vitro* assays have been used as a powerful investigative tool to study the relationship between antibody production in rats (particularly the anaphylactic antibody IgG2a and IgE) and subsequent effector cell activation. Macrophages, eosinophils, and more recently platelets, have all been shown to induce cytotoxic reactions against schistosomula, in the presence of immune rat serum (IRS). Depletion of IgE from IRS leads to a significant decrease in anti–schistosome cell cytotoxicity, establishing the protective effect of IgE *in vitro* (reviewed by Capron *et al.* 1992; Capron & Capron, 1986; Capron & Dessaint, 1985). This proposed method of *in vitro* attack, involving the activation of effector cells by anaphylactic antibody (antibody–dependent cell cytotoxicity mechanism, ADCC), remains unclarified with respect to the *in vivo* situation. Thus, any extrapolation made between the two experimental systems should be viewed with caution.

A recent *in vivo* study has been carried out by Miller *et al.* (1994) in which they compared the serum, and the immunopathology between livers and intestines of *S. mansoni*–infected rats and mice. They described the manifestation of a pronounced hepatic mastocytosis in infected rats, from day 28 to 35 post–infection with *S.*

mansoni, coincident with the expulsion of parasites. Furthermore, the elimination of schistosomes occurred alongside the systemic release of rat mast cell protease II (RMCP II) from mucosal mast cells (MMCs). Significantly, in the murine infection model, C57BL/6 mice did not exhibit an increase in mast cell recruitment to the liver. In the permissive mouse host the adult worms successfully migrated to the hepatic portal system, where they matured and persisted until termination of the experiment. This difference in hepatic pathology between rat and murine infections suggests a possible role for MMCs in the development of protective immunity against schistosomes.

If hepatic MMC reactivity is responsible for schistosome death in rats, either directly or indirectly, then the preceding activation phase must be dependent upon the binding of parasite-specific IgE to Fc∈RIs. Elevated levels of RMCP II in tissues and serum, from day 28 onwards, revealed that mast cell degranulation was triggered by the cross-linking of this surface-bound schistosome-specific IgE with parasite antigen. Following infection, this reaginic antibody must exceed basal titers in order to raise the sensitisation state of mast cells from day 28 onwards, and hence it is apparent that serum IgE plays a pivotal role in the proposed inflammatory mechanism. Around day 28 post-infection the migrating schistosomulum undergoes a substantial morphological change to the adult worm. This transition stage is demarcated by changes in sexual and behavioural development, and more importantly, antigenic profile. It is proposed that if developing worms release unique, and or large amounts of antigen into their immediate environment (the blood, liver and upper mesenteries of the rat) a subset of these secretory proteins could act as allergens stimulating IgE production, with the consequent sensitisation of tissue mast cells.

The aim of this chapter was to demonstrate the presence of schistosome allergens in the bloodstream of infected rats. In addition this study has evaluated the total serum IgE and IgG responses induced following infection of laboratory rats with male-only parasites, or cercariae of both sexes (mixed-sex). Total and schistosome-specific serum IgE and IgG levels were measured prior to, and subsequent to, worm elimination. Serum RMCP II release was also determined by immunoassay, for both test sera, as an indirect measure of the extent of mast cell degranulation during infection. In addition, IgE antibody was isolated from infection serum, using an immunopurification technique, and used to probe adult male-only, and female-derived soluble worm antigen preparations (SWAP) by western blotting. Finally, I shall discuss the findings with respect to the extent and rate of worm

elimination from rats.

3.2 MATERIALS AND METHODS

3.2.1 *Parasite and host*

A Puerto Rican strain of male-only and mixed sex *S. mansoni* parasites were routinely maintained in the laboratory following procedures described in section 2.2.1.

Specific pathogen-free (SPF), female F344 rats, 8–10 weeks of age (Harlan Laboratories, UK) were used throughout this study. Rats were anaesthetised by an intra-peritoneal injection of Hypnorm/Hypnovel (0.33ml/100g).

3.2.2 *Infection of rats and collection of antisera/ tissues*

Serum and tissue samples were collected from infected rats, in an attempt to characterise the magnitude and nature of the protective, schistosome-specific, immediate-type hypersensitivity response elicited against adult parasites. In all cases, blood samples were allowed to clot at room temperature (RT) for 1h and at 4°C for a further 2h. The blood was then spun at 4°C for 10 min at 1000 g, the serum removed, aliquotted into eppendorf tubes, and stored at −20°C.

Expt.1. In a pilot study, three F344 rats, each weighing approximately 150g, were infected percutaneously, via the shaved abdomen, with 2000 male *S. mansoni* cercariae. These infected rats were terminally bled, by cardiac puncture, on days 21, 28 and 35 post-infection (n=1). An naive SPF rat was used to obtain normal serum (NRS) for subsequent serological assays.

Expt. 2. Six F344 rats were infected, as above, with 2000 male and female *S. mansoni* cercariae. On days 21, 28, 35 and 42 post-infection, one rat was terminally bled by cardiac puncture. A further two rats (# 2 and 3) were bled at each time point, via the tail vein, under light halothane anaesthesia.

Expt.3. Six F344 rats (# 1–3 and 6–8) were infected percutaneously with 2000 male *S.mansoni* cercariae. In addition, four rats (# 4,5,9 and 10) were infected with 2000 male and female cercariae. On day 28 post-infection rats # 1–5 were terminally bled by rupture of the jugular vein. Adult schistosomes were recovered by portal

perfusion (Smithers & Terry, 1965) and the worm burden determined for each rat. Pieces of liver and jejenum were taken from each rat, weighed, fixed in 2% paraformaldehyde for histological study, or frozen in 5ml bijou vials (Bibby Sterilin, Stone, UK) at −70°C. In order to check for the presence of eggs, fresh liver samples were digested for 6h at 37°C with 20mg trypsin in 10ml of PBS (section 2.2.4). The tissue debris was separated by sieving (mesh 180μm), and the remaining solution viewed under a dissecting microscope in a glass petri dish.

The remaining animals (# 6–10) were bled from the tail vein, under light halothane anaesthesia, on days 28 and 35 post–infection, and sacrificed on day 42 for serum collection, quantitative perfusion, and tissue sampling, using the above procedures.

Vaccinated rat serum (VRS) and serum from *Nippostrongylus brasiliensis* infected rats served as positive control samples in subsequent serological assays. Samples of VRS were taken from frozen stores collected by Mrs S. Roberts (Roberts *et al.* 1988). Briefly, F344 rats were vaccinated with four inoculations of purified schistosome tegument membrane, and were bled by cardiac puncture 21 days post–challenge with 1000 mixed *S. mansoni* cercariae. *Nippostrongylus braziliensis* rat infection serum was a gift of Mr. G. Newlands, Moredun Research Institute, Edinburgh. All sera were stored at −20°C.

3.2.3 *Antigens*

Soluble adult worm antigen preparations (SWAP) were prepared using mature male parasites or schistosomes of both sexes (mixed–sex), following the procedures described in section 2.2.3. Briefly, C57BL/6 inbred mice were infected with 200 male, or male and female *S. mansoni* cercariae. Adult worms recovered by portal perfusion 7 weeks after infection were washed thoroughly with HBSS (Sigma, Poole, UK) and then freeze–thawed once, prior to sonication. This whole worm homogenate was centrifuged at 105, 000 **g** for 1h at 4°C and the resulting supernatant collected and stored at −70°C.

A soluble egg preparation (SEA) was made following the method of Dunne *et al.* (1984). The SWAP and SEA protein concentrations were determined by the method of Lowry *et al.* (1951).

3.2.4 *ELISA assay for antigen–specific IgG*

Schistosome–specific IgG titers of serum collected from rats infected with male–only or mixed–sex parasites, were quantified by ELISA. To permit comparisons

between plates, control sera were included on every plate, diluted from 1:500 for the IgG ELISA. In addition, all ELISAs were repeated on separate plates on a different occasion.

The wells of 96-well microplates (Nunc-Immunoplate, Life Technologies, Paisley, UK) were coated at 4°C overnight with 2.5μg/ml of SWAP or SEA mixture (100μl/well) diluted with 0.1M carbonate buffer (pH 9.5). After washing the plates once with PBS, each well was blocked for 1h at 37°C with a blocking agent containing 3% heat-inactivated normal goat serum (NGtS; Sigma, Poole, UK). Equal volumes of serum obtained from infected rats (# 6-10; experiment 3; section 3.2.2) on days 28, 35 and 42 post-exposure were pooled for each time point to obtain 3 test serum samples. These were diluted to 1:500 in the blocking agent, and serially diluted in triplicate across the plates. Negative and positive controls, NRS and VRS respectively, were included on each plate under the same conditions. Antibody not bound to the antigen was removed by washing the wells three times with 0.05% Tween 20 in PBS. The plates were incubated in humid chambers at 37°C for 3h, washed as described and then incubated with horseradish peroxidase-conjugated (HRP) goat anti-rat IgG (Serotec, Oxford, UK) (diluted to 1:1000) for 2h at 37°C. After a further wash, a mixture of 0.04% o-phenylenediamine, 0.003% H_2O_2 in phosphate-citrate buffer was added to each well (250μl/ well) and the colour allowed to develop for 30min. The reaction was stopped by adding 50μl of 12.5% sulphuric acid. The absorbance at 492nm was measured with a microplate reader (Dynatech, UK), and the specific absorbance of the test and positive control samples was calculated by subtracting the absorbance of NRS wells.

3.2.5 *Antibody capture ELISAs to determine total IgE in the infection sera.*

The level of IgE in all samples from each experiment (section 3.2.2) was assessed using the following ELISA protocol. The method for this assay was essentially the same as section 3.2.4. with the following modifications. In order to capture total IgE from rat infection serum ninety-six well microtiter plates were coated with mouse anti-rat IgE monoclonal antibody (500ng/ml, 100μl/well; MARE-1; Serotec) diluted in PBS alone (pH 7.2) at 4°C for 16h. Following a wash step with PBS, the non-specific binding sites were blocked with 1% normal mouse serum (NMS), 3% normal rabbit serum (NRbS; Sigma) diluted in PBS. The incubation was performed for 2h at 37°C in a humid chamber. The well contents were flicked out and the plate blotted thoroughly with absorbent paper. Duplicate samples of test serum (diluted to 1:10 in blocking buffer) for each time point (section 3.2.2) were serially diluted

across the plate in blocking agent. NRS, VRS and *N. braziliensis* infection serum controls were also run in duplicate (diluted to 1:10) on each plate. Incubation with the test sera was carried out in a humid chamber for 6h at 37°C. Plates were then washed and incubated for a further 16h at 4°C, with HRP-conjugated MARE-1 (diluted to 1:1000, 100μl/well; Serotec) for detecting serum IgE. After a further wash a mixture of 0.04% o-phenylenediamine, 0.003% H_2O_2 in phosphate-citrate buffer was added to each well (250μl/ well) and the colour allowed to develop for 50min. The reaction was stopped by adding 50μl of 12.5% sulphuric acid. The absorbance at 492nm was measured with a microplate reader (Dynatech, UK), and the specific absorbance of the test and positive control samples was calculated by subtracting the absorbance of NRS wells.

3.2.6 *Dot blot assay to quantify schistosome-specific IgE*

Schistosome-specific serum IgE is difficult to detect due to overwhelming titers of blocking IgG in rat infection serum. To overcome this problem a dot-blot assay was developed, since PVDF membrane binds high concentrations of schistosome antigen.. Thus, it was possible to limit the extent of steric hindrance by the IgG isotype. Mixed sex SWAP was applied to Immobilon-P PVDF membrane (Millipore, Chester, UK) using a Bio-Dot SF Blotting apparatus (Bio-Rad Laboratories, Hemel Hempstead, England). Essentially, three sheets of precut filter paper and one sheet of Immobilon-P were moistened in blot wash buffer (150mM NaCl, 1.5mM KH_2PO_4, 8mM Na_2HPO_4, 2.6mM KCl, 0.03% Tween 20), excess liquid being removed by blotting onto absorbent paper before the membrane was placed inside the sealing gasket. The sample template was placed on top of the membrane and sealed under a strong vacuum. Each well was loaded with 50μg of SWAP in 200μl of blot buffer; unused wells were filled with buffer alone to ensure no cross contamination between samples. Protein was gently filtered through the apparatus, the membrane removed and the non-specific sites on the Immobilon blocked with blocking buffer: 2% NMS, 5% NRbS, 0.03% Tween 20 in PBS for 1h at RT. Washing between incubations was carried out five times at 5min intervals with 0.03% Tween 20 in PBS. Control and test sera from experiment 1 were diluted 1:1, 1:2, 1:4, 1:8, 1:16 (200μl volume), and incubated with protein spots, in sealed plastic bags, for 16h at 4°C. Following a further wash step, each piece of membrane was incubated for 2h at RT in peroxidase-labelled MARE-1 (diluted to 1:1000 with blocking buffer). The samples were washed as before, and finally developed by enhanced chemiluminesence (ECL; protocol and reagents from Amersham, UK).

The optical density of each sample was quantified by reading the blots on an Enhanced Laser Densitometer (Pharmacia LKB, St. Albans, UK). The test serum values were calculated by subtracting the figure obtained for the secondary antibody control.

3.2.7 *Measurement of serum RMCP II*

Evidence for mast cell degranulation *in vivo* was provided by an immunoassay designed to detect small quantities of rat mast cell protease (RMCP II) in infected rat serum. The protocol and reagents used have been described by Huntley *et al.* (1990). Briefly, plates were coated, in a humid chamber, with a monoclonal mouse anti-RMCP II antibody, diluted to 1μg/ml with 0.1M carbonate buffer (pH 9.6) (50μl/well), for 24h at 4°C. The coated plates were washed six times with 0.05% Tween 20/PBS, as in subsequent wash steps. Non-specific binding was minimised by incubating with 4% BSA in Tween 20/PBS, for 30min at RT (100μl/well), followed by one wash with Tween 20/PBS. The RMCP II standard was diluted to 2μg/ml with normal rat serum, then diluted further with 4% BSA/Tween 20/PBS to make up working standards at 0.25, 0.5, 1, 2, 4, 8, 10 and 12ng/ml. Standards and unknown sera were run in duplicate on each plate (50μl/well) and incubated for 1.5h at RT. Following a wash step, sheep anti-RMCP II peroxidase conjugate was diluted 1:400 with PBS/Tween 20/BSA (50μl/well), and incubated for 1h at RT. Plates were washed carefully and developed with 0.04% o-phenylenediamine, 0.003% H_2O_2 in phosphate-citrate buffer (50μl/well). The reaction was stopped, after 10min, with 2.5M H_2SO_4 (25μl/well). The absorbance values were read at 492nm.

3.2.8 *Purification of antibody from the A2 and B5 hybridomas*

Two research groups have recently described successful affinity purification of IgE from rat and human serum using anti-IgE monoclonal antibodies bound to Sepharose (Ahmad *et al.* 1991; Rihet *et al.* 1992); techniques used in this study were combined from both publications. A2 and B5 mouse anti-rat IgE hybridoma cell lines were gifts of Dr. D.H. Conrad, Subdepartment of Immunology, The John Hopkins University, Baltimore. Both lines were ∈-chain specific; the antigenic site for B5 antibody is in the Fab region of the IgE heavy chain molecule and A2 antibody reacts with the IgE Fc region. Initially, both A2 and B5 cell lines were routinely maintained in RPMI containing 10% FCS, 1% L-glutamine and supplementary antibiotics (Gibco, Paisley, UK). Mouse anti-rat IgE monoclonal antibody (mAb) released by the cultured hybridoma cell lines was then purified using

FPLC. Large volumes of supernatant were collected from both A2 and B5 lines and stored at -20°C.

Unsuccessful attempts to purify B5 by fast protein chromatography (FPLC; columns and accessories from Pharmacia LKB), using affinity columns protein A and G, prompted the need for an alternative purification protocol. It was decided to concentrate on purification of the A2 antibody, as the B5 antibody appeared to be binding to the superose beads non-specifically. A sequence of procedures was used for the separation of antibody from the medium. Firstly, a 50% saturated ammonium sulphate (SAS) precipitation was carried out for 16h at 4°C, on 300ml of thawed supernatant. The precipitate was dissolved on ice in 20ml PBS, and dialysed against three x 5 litre volume changes of PBS (pH 7.2) for 36h at 4°C. Purification by ion exchange chromatography, as described by Conrad *et al.* (1983), was modified for FPLC using initial runs of 200μl of A2 antibody solution. Finally, 25ml of the dialysed A2 antibody preparation was mixed with 25ml of MES buffer (pH 5.5; Sigma) and loaded onto a Mono S cation exchange column at 1ml/min, using a 50ml superloop. Protein bound to the column was eluted with a two step linear gradient going from 0 to 1M NaCl in MES buffer (pH 5.5).

An ELISA was developed to detect mouse anti-rat IgE antibody present in elution fractions from FPLC. Incubation times and wash regimes were as described in section 3.2.5. Plates were coated with 1μg/ml of purified rat myeloma IgE (kappa; 100μl/well; Serotec) diluted in PBS alone (pH 7.2) for 4h at 16°C. Non-specific binding was limited by incubating plates with a blocking agent (3% NRbS in 0.05% Tween 20/PBS; 200μl/well) for 2h at 37°C in a humid chamber. Neutralised elution fractions containing the mouse anti-rat IgE mAb (A2) were diluted 1:1 with blocking buffer and incubated for 6h at 37°C. MARE-1 (50ng/ml, 100μl/well) was serially diluted across the plate in duplicate rows. The mAb was of a known concentration and therefore used as a standard. Following a final wash step with 0.05% Tween 20/PBS, the rabbit anti-mouse IgG peroxidase conjugate (diluted to 1:1000 in 3% NRbS/0.05% Tween 20/PBS; 100μl/well; Serotec) was aliquotted into each well and the plates incubated for a further 16h at 4°C. The detection step with the OPD/H_2O_2 substrate was as described elsewhere (section 3.2.4). The absorbance was read at 492nm with a microplate reader (Dynatech). By comparing the intensity of each test well against the serially diluted MARE-1 standard it was possible to calculate the amount of A2 retrieved from the Mono S column.

A peak, identified by ELISA as A2 antibody, was collected in 5 x 1ml fractions and desalted by dialysing the sample against 3 x 5 litre volumes of PBS for 36h at

4°C. The A2 sample was then diluted with an equal volume of MES binding buffer and passed again over the column, using the procedure described. Antibody purity of each elution fraction was estimated by SDS–PAGE and spectrophotometric protein evaluation, using the calculation:

Protein concentration (mg/ml) = $1.55 \times A_{280} - 0.77 \times A_{260}$ (Hudson & Hay, 1989)

3.2.9 *Mini–column design and serum IgE purification*

The immunosorbent (A2–Sepharose 4B) used in the design of the mini–columns was prepared by coupling purified A2 anti–rat IgE monoclonal antibody to cyanogen bromide–activated Sepharose 4B (Pharmacia). Briefly, the purified 5ml sample of A2 retrieved from the Mono S column, was dialysed overnight at 4°C in a large volume of coupling buffer (CB; 0.1M $NaHCO_3$, 0.5M NaCl, pH 8.3). The pH was checked and adjusted further by addition of 3ml of CB. This antibody solution was incubated with 2ml of activated Sepharose gel for 2h at RT, on a rotating windmill. After washing with CB, the remaining reactive groups were blocked with 0.1M Tris/HCl, 0.5M NaCl, 0.05% sodium azide (pH 8.0) at 4°C.

Mini–column design was based on a method first published by Rihet *et al.* (1992). Approximately 200μl A2–Sepharose 4B was packed in 0.5ml microfuge tubes, previously prepared by removing the tube bases and plugging them with siliconised glass wool. The columns were washed with 10 volumes of PBS (1% BSA; pH 7.2) and incubated for 2h at RT, with 100μl of day 35 test serum from experiment 3 (diluted 1:1 in 1% BSA/PBS). BSA was added to inhibit non–specific binding of the infection serum to the sepharose beads. After washing with 10 volumes of 1% BSA/PBS, bound material was eluted. The efficiency of recovery of functional IgE, following different elution regimes, was carefully assessed. Basic elution was favoured; pre–elution with 4 volumes of 10mM phosphate buffer (1% BSA) (pH 8.0) was followed by four incubations of 10 min using 1 volume of 100mM triethylamine, 1M NaCl, 1% BSA (pH 11.5). Hence, fractions of 200μl were collected, including the void volume. The eluates were neutralised with 40μl of 0.5M Tris/HCl (pH 6.8) and the specific–IgE titer for each fraction was evaluated by ELISA (see section 3.2.5.). Finally, the column was washed with 10 volumes of PBS (1% BSA) and stored at 4°C in PBS containing 0.02% sodium azide.

3.2.10 *Western blotting*

Male–only and mixed–sex SWAP (approx 3.5mg/ml in final concentration) were

added to sample buffer containing 2% SDS (section 2.2.4), and heated for 3min in boiling water. The two reduced mixtures were loaded on the same gel at equal protein concentrations, using a dual trough comb, and SDS-PAGE was carried out according to the method of Laemmli (1970) using 6-16% gradient gel. The separated proteins were electroblotted on to a PVDF membrane (Millipore, Chester, UK), using procedures based on those of Towbin *et al.* (1979; section 2.2.4).

Immunostaining of the blot was carried using the same procedure employed for section 2.2.4 with the following modifications. The membrane was cut into 5mm strips, and blocked with 2% NMS, 5% NRbS, 2% BSA, 0.03% Tween 20 in PBS, or 5% NGtS, 2% NRbS, 2% BSA, 0.03% Tween 20 in PBS, for strips incubated with rat or mouse infection serum, respectively. The mouse serum used (WTS) was as described in section 2.2.2. The strips of membrane were then washed and incubated with either peroxidase-labelled MARE-1 (diluted 1:1000 in blocking buffer; Serotec), for detecting IgE reactivity in the rat serum, or peroxidase-labelled goat anti-mouse IgG (diluted to 1:1000 in the respective blocking buffer; Fab specific; Sigma) for characterising the IgG response from the WTS. This final step was carried out in sealed bags on a rotating windmill for two hours at RT. The detection was performed by enhanced chemiluminescence (ECL detection kit; Amersham, Amersham, England) according to the manufacturer's instructions. All steps were carried out in a darkroom. Blots were exposed to the ECL reagents for 1 min. The reaction and the subsequent detection of antigen/antibody complexes relied upon the oxidation of luminol by the peroxidase-antibody conjugate. The emission of light from the oxidised luminol was detected on light sensitive film (Hyper-ECL film; Amersham). The reaction was visualised by placing the film in Kodak developer D19 for approximately 1 minute. Finally the film was fixed in unifix (1 volume unifix: 2 volumes deionised water; Kodak).

3.3 RESULTS

3.3.1 *Serum IgE levels in rats following infection*

a. *Preliminary observations*

The first experiment was designed as a pilot study to observe total IgE titers in serum collected from rats infected with male schistosomes. Time points were chosen at intervals of seven days prior to, and subsequent to, worm elimination. Total serum IgE levels following exposure of rats to 2000 male cercariae are shown in figure

3.1a. NRS contained negligible IgE reactivity when compared to IgE titers measured in rat infection serum and *N. braziliensis* infection serum. Systemic IgE levels in the schistosome-infected rat serum were detectable at day 21 post-infection rising rapidly between days 28 and 35 (2.1 fold increase, day 35). Infection with the nematode *N. braziliensis* induced an elevated IgE response in rats, exceeding the antibody response observed in *S. mansoni* infections.

The presence of schistosome-specific IgE in the infected rat serum, was demonstrated by dot blot analysis. Using this technique, it was possible to coat a larger surface area of membrane with up to 100μg schistosome protein/cm^2, thereby reducing steric hindrance from overwhelming titers of blocking antibodies (figure 3.1b). The OD values showed that parasite-specific IgE titers increased progressively from day 21 to 35 (1.8 fold increase, per week). Furthermore, an enhanced IgE response to adult worm antigen was induced following vaccination of rats with tegumental membrane (VRS). The technique was limited by non-specific binding of the secondary antibody, as demonstrated by the high background levels of anti-adult worm IgE detected in NRS.

b. *IgE production following single and mixed-sex infections of rats*

A second experiment was conducted using serum from rats infected with parasites of both sexes. Total IgE levels in serum collected from rats infected with male-only cercariae (experiment 1, section 3.2.2.), or mixed-sex parasites (experiment 2, section 3.2.2.) were measured. Once again, IgE was not detectable in samples of NRS compared to the relatively high titers found in the schistosome infection serum and positive controls (figure 3.2). In serum from rats infected with male and female worms, an IgE response was detected at day 21 post-infection, with elevated titers measured at day 28 (2.2 μg/ml), increasing rapidly up until day 35 (5.28 μg/ml). Furthermore, the level of total serum IgE in the mixed-sex infected rats, at day 35 post-infection, is equivalent to the value measured in the *N. braziliensis* infected rat serum, used as a control.

In contrast, the IgE levels detected by ELISA from the serum of male-only infected rats were at substantially lower levels than in the animals infected with mixed-sex worms. The comparative IgE values for male-only and male and female schistosome infected rats, measured at day 35 post-infection, were 0.725 μg/ml and 5.28 μg/ml respectively. The total IgE titer appeared to increase between days 28 and 35, but not noticeably, relative to the mixed-sex infection serum.

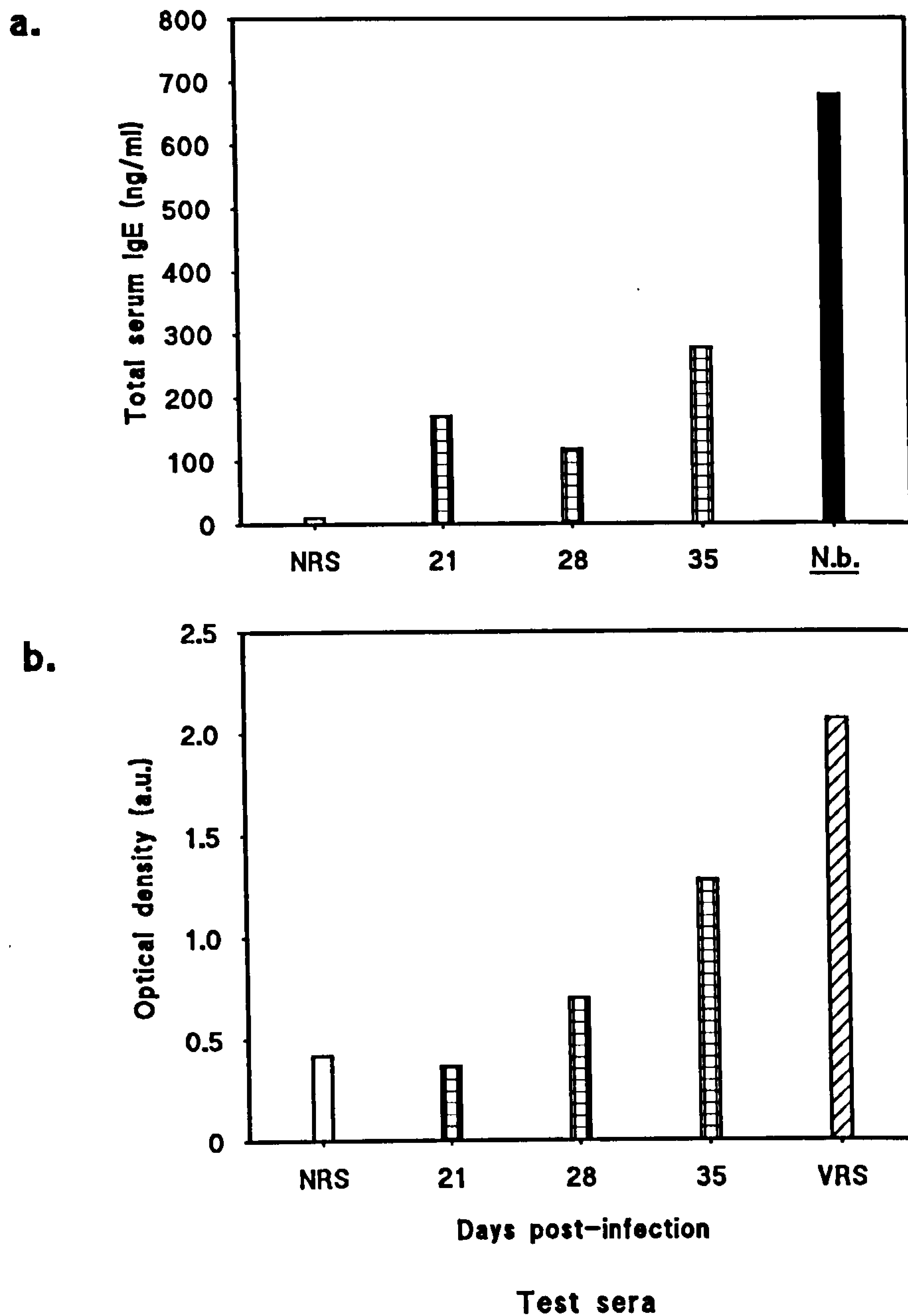


Figure 3.1a. Total serum IgE detected by antibody capture ELISA in the serum of rats infected with 2000 male *S. mansoni* cercariae (from experiment 1) on days 21, 28 and 35 post-infection. Positive and negative control sera are represented by N.b. (*N. braziliensis* rat infection serum); and NRS (normal rat serum) respectively.

Figure 3.1b. Determination of schistosome-specific IgE levels by dot blot analysis, using serum taken from rats infected with 2000 male-only parasites. The OD values of IgE from non-infected (NRS) and vaccinated rats (VRS) are also shown. One rat was sacrificed at each time point.

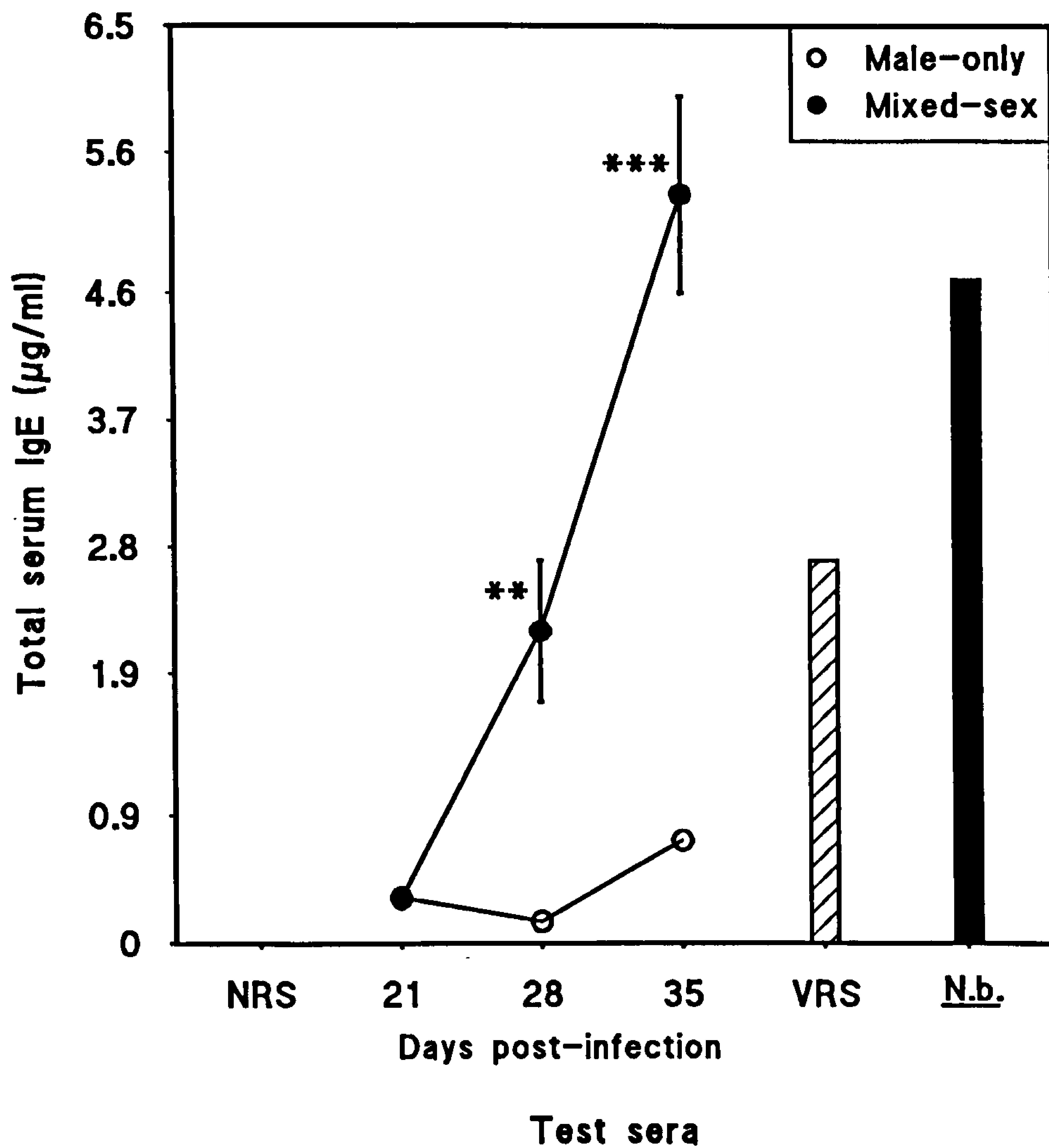


Figure 3.2. Total serum IgE titers present in serum taken from rats infected with male-only worms or parasites of both sexes, from experiments 1 and 2, respectively. NRS (normal rat serum) and N.b. (serum taken from rats infected with *N. braziliensis*). Data for experiment 2 is shown as the mean ± standard error (SE) of 3 animals at days 21, 28 and 35 post-infection.

Points from experiment 2 significantly different from the day 21 value are indicated with asterisks ($p < 0.05$ **, $p < 0.01$ ***).

c. *Further evidence for an enhanced IgE response to mixed-sex infections*

The final experiment was designed to confirm and expand upon the data obtained from experiments 1 and 2. Rats were infected with 2000 male-only or mixed-sex cercariae, and bled at weekly intervals as described (section 3.2.2.). As before, serum collected from schistosome-infected rats contained greater titers of IgE than NRS (figure 3.3). Total serum IgE titers (following exposure of rats to male-only cercariae) rose little until day 35 post-infection. From day 35 onwards there was rapid production of antibody, with no significant difference in the amount of IgE between male-only and mixed-sex test sera at day 42.

The pattern of IgE production, by animals infected with mixed-sex parasites, supported preliminary observations. The amount of IgE antibody present in the serum of these individuals was higher at day 35 ($p \leq 0.1$) than male-only schistosome infected rats. Furthermore, this IgE response was augmented, from day 28 onwards, in the animals infected with cercariae of both sexes, giving a convex shape to the response curve. This enhanced IgE production continued until termination of the experiment at day 42.

3.3.2 *Serum levels of parasite-specific IgG*

Following infection of rats with male-only parasites, schistosome-specific serum IgG showed similar patterns of increase and overall absorbance values against the three antigen preparations tested (figures 3.4a-c). The OD data at day 28 was above basal levels (> 0.6 units), but remained at low levels over the next seven days. However, from day 35 the IgG levels rose dramatically (up to 1.7 units), as described previously for total serum IgE.

Generally, rats infected with male and female cercariae were able to mount an earlier antibody response of greater magnitude than the male-only schistosome-infected hosts. The OD values taken at day 28 after infection were similar to the male-only schistosome infected group. At day 35 there was a greater antibody response to all three antigen preparations, noticeably more marked against mixed-sex SWAP (figure 3.4b) and SEA (figure 3.4c) compared to the antibody reactivity from the male-only infection serum. From days 35 to 42 post-exposure there was a less pronounced increase in IgG responsiveness to male (figure 3.4a.), mixed-sex SWAP and SEA than earlier time points.

3.3.3 *Release of RMCP II following infection*

A sensitive immunoassay was employed to detect levels of circulating RMCP II

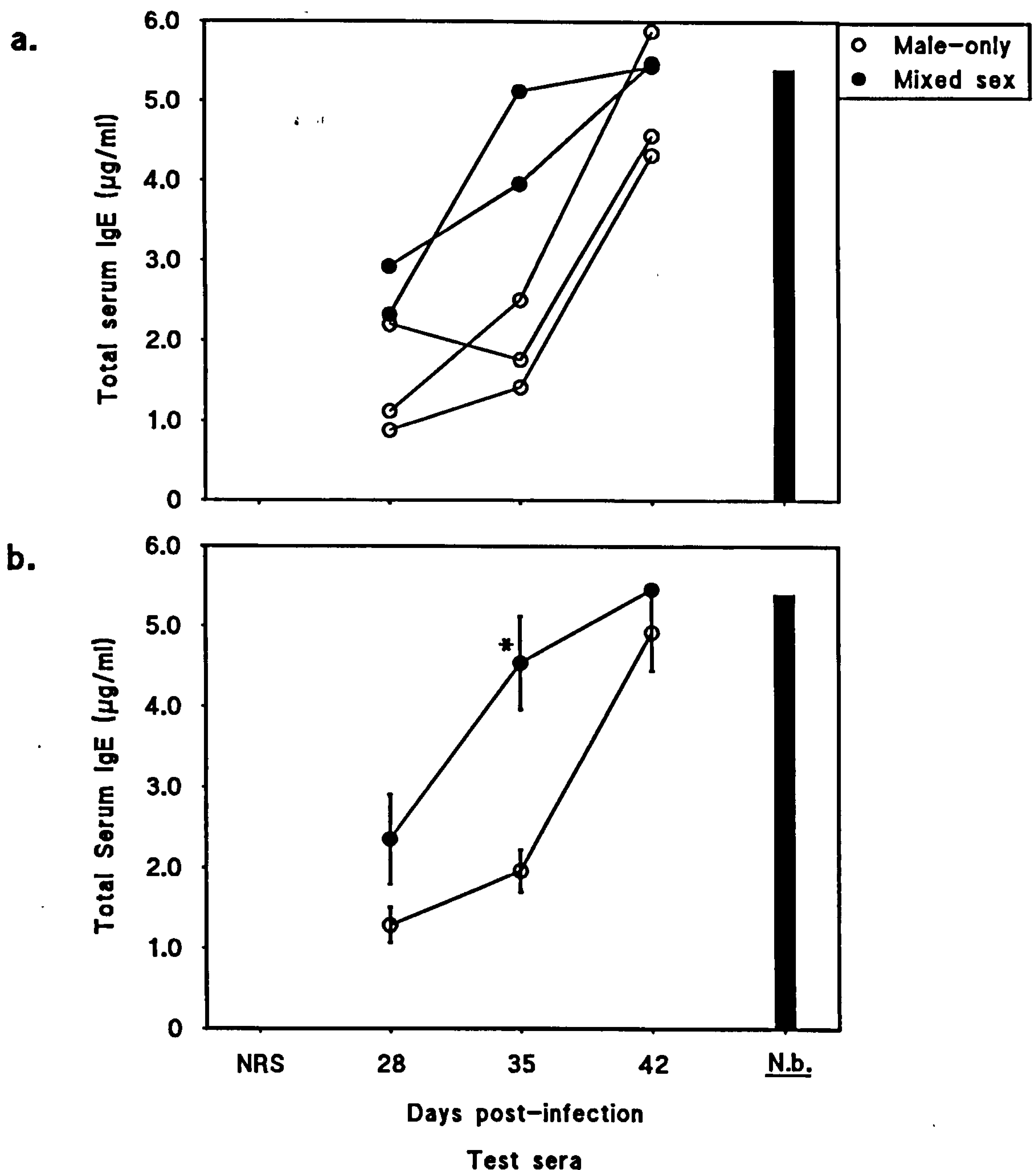


Figure 3.3a. Total serum IgE response made by individual rats against male–only parasites and schistosomes of both sexes at days 28, 35 and 42 post–infection. NRS (normal rat serum) and N.b. (serum collected from rats infected with *N. braziliensis*).

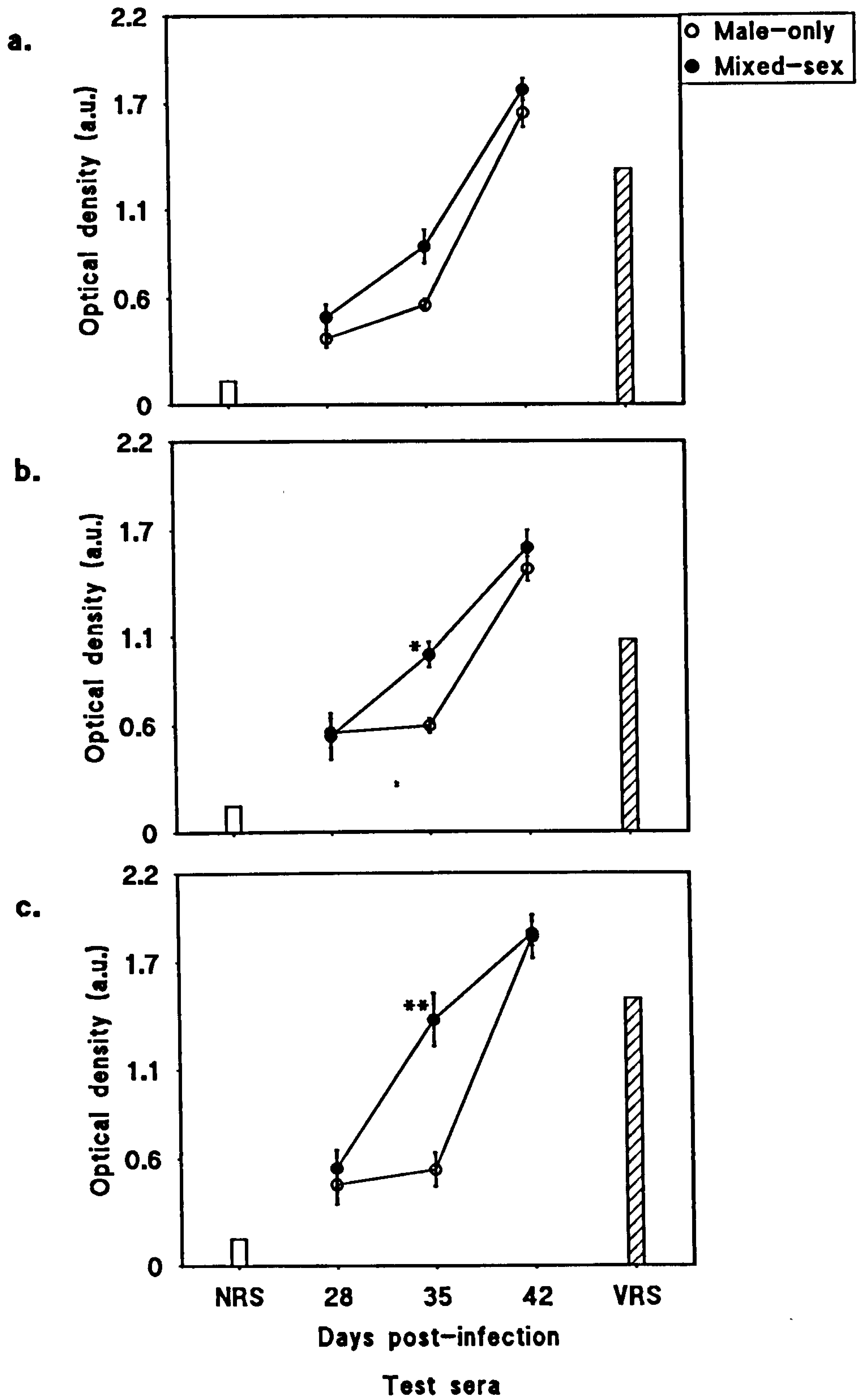
Figure 3.3b. The summarised data from figure 3.3a. Values for experiment 3 are given as the mean ±SE of 3 male–only infected animals and 2 rats infected with male and female worms at days 28, 35 and 42 post–infection.

Values which are significantly different between the two schistosome infected groups of rats, for each time–point shown, are represented by an asterisk ($p < 0.1$ *).

Figure 3.4a–c. Evaluation of schistosome–specific serum IgG responses from male–only and mixed–sex infected animals. All sera used were taken from experiment 3 (described in section 3.2.2.). IgG reactivity was measured against: **a.** male–only SWAP, **b.** SWAP from both sexes, **c.** SEA soluble egg antigen. NRS, normal rat serum and VRS, vaccinated rat serum were used as negative and positive controls, respectively.

Values for experiment 3 are given as the mean ±SE of 3 male–only schistosome–infected animals and 2 rats infected with male and female worms at days 28, 35 and 42 post–infection.

Points significantly different between the male–only parasite–infected group and the mixed–sex parasite–infected animals are indicated with asterisks ($p < 0.1$ *, $p < 0.05$ **).


a.
b.
c.
Optical density (a.u.)
2.2
1.7
1.1
0.6
0
Male-only
Mixed-sex
*
**
NRS
28
35
42
VRS
Days post-infection
Test sera

released *in vivo*, following infection of rats with schistosomes. The protease levels in serum give an indirect measurement of mast cell participation during infection, and more importantly of worm elimination. The pattern of RMCP II release differed between both groups of infected animals (figure 3.5). Protease production was high at day 28, in the serum of rats infected with male and female parasites, but was not sustained. Levels declined slightly at day 35, and then fell rapidly over the next week (from 0.773μg/ml, day 35 to 0.353μg/ml, day 42). This early production of RMCP II appears in male-only infected animals, but at a lower level than in mixed-sex infections. From day 28 slightly higher levels of RMCP II release were detected, these then declined until termination of the experiment at day 42.

3.3.4 *Worm elimination*

Experiment three was designed to address two hypotheses. Firstly, was the sudden increase in IgE production, from day 28 in mixed-sex infections attributable to egg production by mature ovipositing females? Secondly, since single-sex infections elicited a delayed and much reduced antibody response, what was the fate of male-only worms in rats?

On day 28, rats #1-5 were sacrificed, the hepatic portal system perfused, and the adult worms recovered and counted (Table 3.1, third column). There was no significant difference between the number of worms recovered from male-only and mixed-sex infected rats at day 28 post-infection. Furthermore, no eggs were found in sampled liver tissue from animals infected with both sexes, the female worms appeared severely stunted in growth and sexually immature, when viewed at high magnification. Differences were observed in total serum IgE titers and RMCP II release between both groups, as described above in sections 3.3.1-3. (data is summarised in Table 3.1).

Rats #6-10 were perfused on day 42, the worm burden determined and liver pieces examined for the presence of eggs, as described. It is apparent that worm elimination had occurred since the number of parasites recovered fell dramatically between days 28 and 42 post-infection. The data revealed no significant difference in the numbers of worms recovered from the two experimental groups of rats. Examination of the trypsin-digested livers, revealed the absence of deposited eggs following mixed-sex schistosome infections of rats. These observations were further supported by the lack of development, and sexual immaturity of surviving female parasites. At day 42, augmented levels of circulating IgE were measured in both single-sex and mixed-sex infected rats. In addition, there was a decrease in

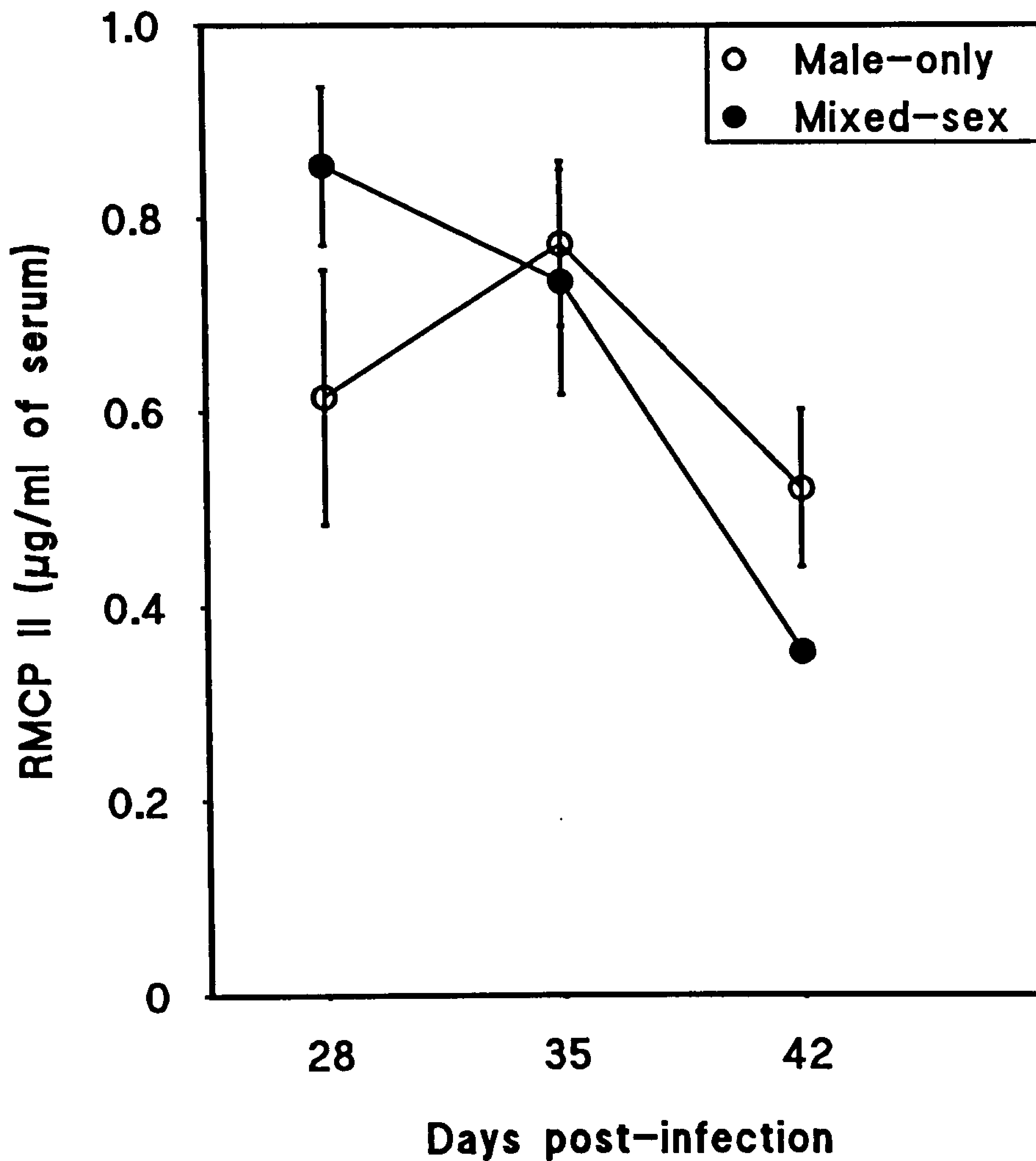


Figure 3.5. Measurement of RMCP II released into the host circulation, following infection of rats with male-only and mixed-sex parasites, as a direct indication of mast cell degranulation.

Values are given as the mean ±SE of 3 male-only infected animals and 2 rats infected with both male and female parasites.

Time post-infection (days)	Male-only (♂) or mixed-sex infection (M)	Worms recovered	Total serum IgE (μg/ml)	RMCP II release (ng/ml)
28	♂	168.3 (±42.7)	1.29 (±0.22)	0.691 (±0.08)
	M	174.5 (±14.5)	2.35 (±0.55)	0.854 (±0.07)
35	♂	N.D.	1.96 (±0.26)	0.773 (±0.08)
	M		4.54 (±0.58)	0.734 (±0.11)
42	♂	30 (±18.5)	4.92 (±0.48)	0.521 (±0.08)
	M	41.5 (±14.5)	5.46 (±0.019)	0.353 (±0.01)

Table 3.1. The worm population recovered from rats infected with male-only (n=6) and mixed-sex schistosomes (n=4), on days 28 and 42 post-infection, the time-points associated with the onset and decline of worm expulsion. The total serum IgE and systemic RMCP II means are also included. The values in brackets represent the SE around the mean.
N.D., not done.

released RMCP II, indicating a reduction in mast cell degranulation.

3.3.5 *Immunopurification of IgE from rat infection serum*

Serum IgE was immunnopurified from whole infection serum, the fractions collected (including void volume and washes) and tested by ELISA (see section 3.2.8.) for functional rat IgE activity. Figure 3.6 shows the profile of IgE recovered from the mini-columns following incubation with day 35 mixed-sex serum. The binding of IgE to the A2-sepharose beads was very efficient, as demonstrated by the limited amount of IgE detected in the waste fractions. Most of the bound antibody was eluted from the columns in the first two fractions (#16 and 17). Total IgE levels in the whole serum and immunopurified fractions was measured by ELISA (section 3.2.5) and compared (data not shown). Results showed that 31.5% of the serum IgE was recovered in the immunopurified fractions, 14.85% in the void volume, and 53.65% remained unaccounted for.

Data from subsequent passes of serum over the columns was consistent with previous values. Thus, eluted fractions (#16 and 17) from six successive immunopurification runs were pooled in preparation for Western blotting.

3.3.6 *Schistosome-specific IgE responses*

To circumvent the problem of blocking antibodies, as alluded to previously, total IgE was isolated from rat infection serum by an immunopurification technique (sections 3.2.9 and 3.3.5). This fractionated serum, containing functional IgE antibody, was used to probe strips coated with adult female-only or male-only schistosome protein. Relevant controls were included: NRS, VRS (IP) and unfractionated day 35 serum from male-only and mixed sex infections. In addition, the mouse anti-adult worm IgG response (worm transfer serum, WTS; section 2.2.2), to the aforementioned parasite proteins, was used as a template for the identification of secretory antigens, against which rat IgE responses could be compared and characterised.

The mouse IgG response against both male and female SWAP was very intense (figure 3.7). The WTS (strips 1k and 2k) and chronic mouse serum (CMS; lanes 1c and 2c) detected a similar profile of sixteen immunodominant proteins, varying in Mw from 208kD to 12kD (some of these are described in chapter 2, section 2.3.2). As explained, each of the antigens represented an immunogenic product released by live worms. Immunoblots probed with WTS and CMS showed an increase in non-specific binding of mouse IgG to female antigen compared to male-only protein

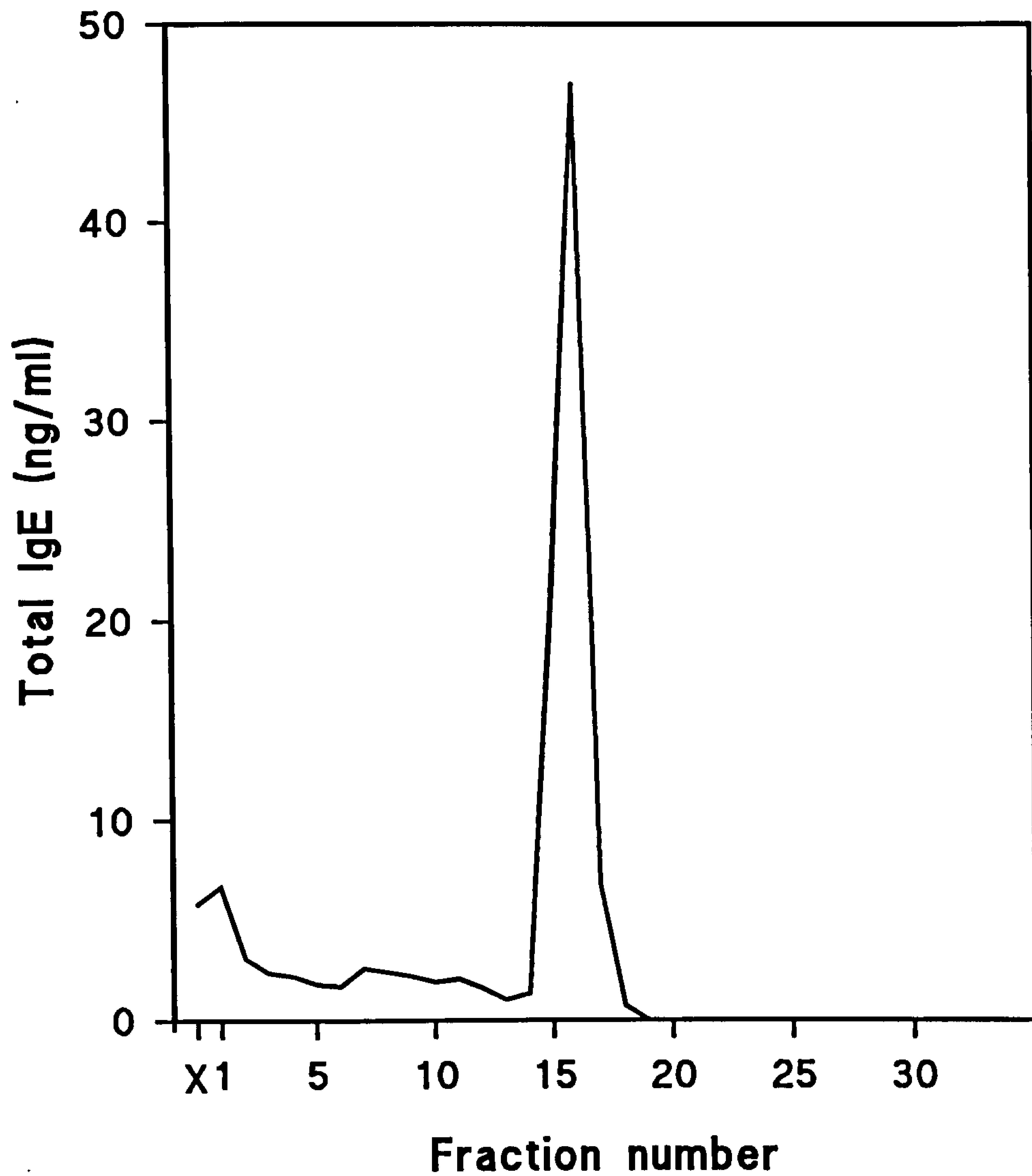


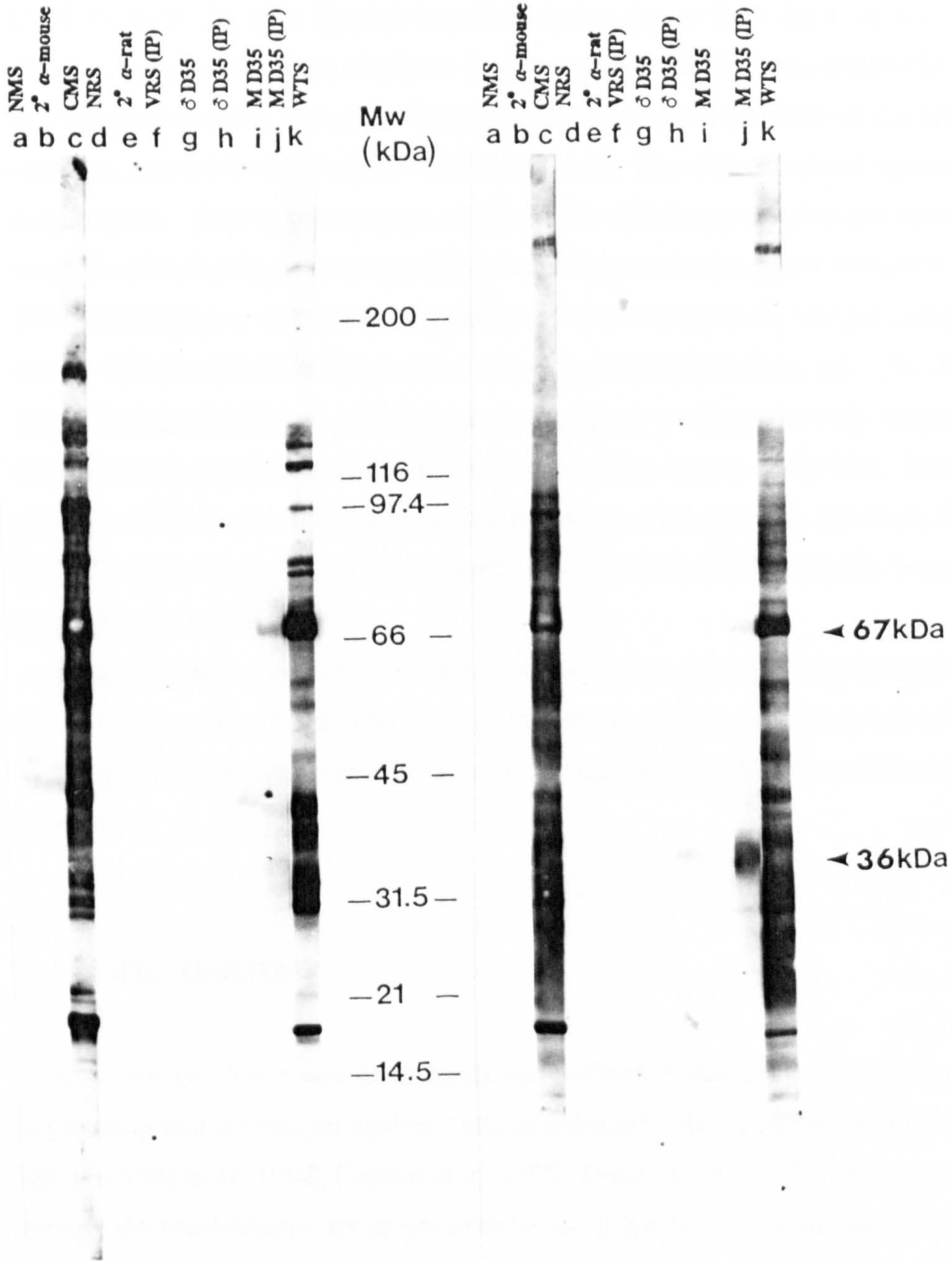
Figure 3.6. The profile of IgE antibody recovered from the mini-columns following incubation with day 35 mixed-sex serum. All fractions represent IgE recovered from the column after each wash/elution step, as follows: fraction **X** void volume, fractions **1–10** the PBS/BSA pre-washes, fractions **11–14** pre-elution, fractions **15–19** the basic elution step and fractions **20–30** represent IgE recovered during the final series of PBS/BSA washes.

Figure 3.7. Western blot analysis to define the range of male and/or female adult worm antigens detected by schistosome–specific IgG and IgE antibody, from mice and rats respectively, . Male proteins detected by the various sera are shown in the left–hand blot (**#1**), and the female antigens by the right–hand blot (**#2**). Both antigen preparations were loaded at the same protein concentration.

Several mouse sera were used to probe antigen–coated strips as follows: lanes **1a & 2a**, NMS; **1b & 2b**, goat anti–mouse 2° antibody control; **1c & 2c**, CMS; **1k & 2k**, WTS.

Whole (**W**) and immunopurified (**IP**) rat serum was used to probe the following strips: **1d & 2d**, NRS; **1f & 2f**, VRS (**IP**); **1g & 2g**, day 35 male–only infection serum (**W**); **1h & 2h**, day 35 male–only infection serum (**IP**); **1i & 2i**, day 35 mixed–sex infection serum (**W**); **1j & 2j**, day 35 mixed–sex infection serum (**IP**).

Lanes **1e** and **2e** were probed with secondary antibody (MARE–1) alone.


#1. Male-only SWAP
#2. Female-only SWAP
NMS
2° α-mouse
CMS
NRS
2° α-rat
VRS (IP)
δ D35
δ D35 (IP)
M D35
M D35 (IP)
WTS
a b c d e f g h i j k
Mw
(kDa)
200
116
97.4
66
45
31.5
21
14.5
67kDa
36kDa

(lanes 2c and 2k, compared to lanes 1c and 1k). These results will be interpreted in the following discussion (section 3.4.).

The rat schistosome–specific IgE signal was greater in immunopurified fractions (1f & 2f, 1h & 2h, 1j & 2j) than the whole serum (lanes 1g & 2g, 1i & 2i). This observation provides clear evidence for the presence of blocking antibodies in unfractionated serum samples. Significantly, the overall intensity of the IgE response against female–only SWAP was greater than that observed against male–only protein. Two major antigens at 67 and 36–38kD appeared to dominate. Both were identified using immunopurified male–only and mixed–sex infection serum. The 67kD antigen, analogous to a protein of similar molecular weight recognised by mouse IgG, bound more IgE in the male–only preparation (lane 1j). The 36–38kD was only identified by day 35 IP mixed–sex serum on the male–only strips, in contrast to its recognition by all three IP sera on the female–only blot. Enhanced anti–36–38kD reactivity was exhibited by the parasite–specific IgE from IP mixed–sex infection serum. Furthermore, both the 67 and 36–38kD antigens were identified by rat IgG (data not shown).

This qualitative data not only corroborates earlier evidence for elevated levels of IgE following mixed–sex infections of rats, compared to male–only infections, but also demonstrates the promotion of schistosome–specific IgE titers following infection.

3.4 DISCUSSION

Over the last few years, both direct and indirect evidence has accumulated suggesting that protection against various helminth infections can be attributed to IgE (Gounni *et al.* 1994; Capron *et al.* 1992; Dunne *et al.* 1992a; Ahmad *et al.* 1991), though the mechanisms are as yet undefined. It has been demonstrated that helminth infection provides the one stimulus capable of inducing a high level and long–lasting IgE response in man and laboratory animals (Ogilvie, 1964), compared to the muted transient nature of the IgE response to non–helminth or conventional antigens, seen for example against egg–albumin (EA) (Jarrett & Bazin, 1974). The most conclusive data implicating IgE antibody in protective immunity against helminths, has been derived from studies carried out on several parasite models: *N. braziliensis*, *T. spiralis* and *S. mansoni* infections of rats. In these parasite models protective immunity manifests itself in the dramatic expulsion of invading parasites from

mucosal tissue. Thus, the rat model provides an ideal opportunity to elucidate the synergistic roles of antibody and effector mechanisms in protecting the host against helminth infection. Yet, the IgE response represents a very small proportion of the total immunoglobulin production; there is 10^5 times more IgG than IgE in the circulation of an uninfected host. Moreover the anaphylactic reactivity of IgE is regulated by the action of specific blocking antibodies that compete with IgE for antigen binding. The most effective blocking isotype in man is IgG4 which has been shown to mute specific anti–schistosome IgE responses by competitive inhibition (Rihet *et al.* 1992). The combination of these factors make the study and characterisation of schistosome–specific IgE responses difficult.

Our findings have shown that rats infected with mixed–sex cercariae generated an earlier and stronger anti–adult worm IgE response than male–only infected rats. Parasite–specific IgG titers also increased dramatically in both infection regimes compared to uninfected control animals. Serum IgG responses in rats infected with mixed–sex schistosomes were measured against mixed–sex SWAP, male SWAP and SEA; the IgG titers rose rapidly from day 28 post–infection onwards. In contrast, the rapid increase in anti–schistosome IgG titers occurred later in male–only infected rats, rising from day 35 onwards. There was no evidence of eggs, or egg–induced hepatic pathology, following infection with mixed–sex parasites. This observation was further supported by the recovery of sexually immature female worms. It was concluded that induction of elevated immunoglobulin titers was attributable to the enhanced production of excreted/secreted antigenic macromolecules by female worms from mixed–sex infections and/or was a consequence of worm death. Hence, the attrition of maturing parasites or earlier migratory stages, may potentiate allergic reactivity by increasing the amount of antigen exposed to the host's immune system.

Paradoxically, the systemic release of RMCP II was coincident with the onset of IgE production, yet by day 42 post–infection IgE titers were high, in contrast to RMCP II levels which fell to low levels. However, it must be emphasised that mast cell degranulation is a transient phenomenon, and hence the lowering of RMCP II release implies that mast cells are no longer recruited nor stimulated by allergen after day 35 post–infection. Furthermore, rats infected with male–only parasites were able to eliminate the majority of the primary parasite population, despite the induction of a delayed and lower antibody and effector cell response. These observations suggest that IgE and IgE cell–mediated immune responses are either: a) not directly involved in schistosome expulsion and require the involvement of an alternative or synergistic effector mechanism or b) can cause worm expulsion even

when operating at comparatively low levels.

In addition, IgE isolated by immunopurification from day 35 serum samples, was used in western blotting to identify two dominant proteins, the 67kD and the 36–38kD allergens. The intensity of the anti-female worm IgE response was much stronger than the allergenic reactivity demonstrated against male-only proteins. These observations corroborate earlier evidence, confirming that non-specific and specific antibody production is potentiated by the release of large quantities of allergenic material from female parasites.

3.4.1 *Potentiation of the total IgE response by parasite antigens*

In this present study we have reported the induction of elevated titers of IgE antibody following infection of rats with male-only or mixed-sex cercariae. Total serum IgE levels were detected above the basal level from day 21, until termination of the experiment at day 42. Similarly, Rousseaux-Prevost and co-workers (1977) collected serum periodically for fourteen weeks from four strains of rats infected with *S. mansoni*. Generally, the pattern of total IgE production over the extended time course following infection was similar for each of the 4 strains. IgE antibody was detected by radioimmunoassay (RIA) from day 10 post-infection, remaining high from day 20 to 30, with the maximum binding obtained between days 31 and 52 (Rousseaux-Prevost *et al.* 1977; Rousseaux-Prevost *et al.* 1978).

This characteristic increase and subsequent persistence of total serum IgE was later addressed and confirmed by Reiner and Zahner (1986). A conflicting pattern of schistosome-specific IgE reactivity was measured by percutaneous anaphylaxis (PCA), demonstrating an infection dose-dependent response. A low infection dose of 1000 *S. mansoni* cercariae stimulated persistent titers of schistosome-specific IgE, in contrast to a high infection dose, of 10 000 cercariae per rat, which induced transient levels. Since differences in the specific and total serum IgE levels have been described in various parasite infections, for example *N. brasiliensis* in rats (Yamada *et al.* 1992), *S. mansoni* in mice (Rousseaux-Prevost *et al.* 1980) and rats (Rousseaux-Prevost *et al.* 1978) the disparity described between PCA-detectable and total IgE was not surprising. An explanation for the discrepancy was proposed by Orr and Blair (1969) who found that infection of rats with *N. braziliensis* caused a dramatic increase in the circulating levels of a pre-induced IgE antibody response to egg-albumin (EA) or conalbumin. The authors inferred that helminths, in addition to stimulating a parasite-specific response, were also able to influence the production of quite unrelated IgE. Their observation was soon confirmed and

expanded. Potentiated reagin responses to conventional antigens, such as EA and keyhole limpet haemocyanin (KLH), were only enhanced if the anti–EA/anti–KLH IgE response was established prior to helminth infection. This characteristic potentiation of the reagin response in rats was attributed to the secretion of an IgE–stimulating substance from live helminths (Lopes *et al.* 1990; Jarrett & Stewart, 1973). However, attempts to reproduce the effect by the injection of worm extracts or worm culture fluids high in allergen content into rat hosts were unsuccessful (Jarrett and Miller, 1982).

Further evidence for the occurrence of this non–specific potentiated IgE response in rats infected with *S. mansoni* was demonstrated by Rousseaux–Prevost and colleagues (1978). They found that the amount of parasite–specific IgE antibody was very small, relative to the total amount of IgE. These results suggest that total and specific IgE responses are regulated independently during helminth infections.

It is debatable whether outdated techniques such as PCA and the Prausnitz–Kustner test (PK) are precise enough tools with which to measure the relatively small quantities of circulating parasite–specific IgE. However, the presence of overwhelming titers of blocking antibodies, of the same antigenic specificity as IgE, have been shown to interfere with the accurate determination of parasite–specific IgE levels by conventional ELISA. Several clinical studies have implicated cross–reactive antibody of the IgG4 isotype in the prevention of the expression of immunity in young children by the ability of the immunoglobulin to inhibit IgE–binding to *S. mansoni* antigens (Demeure *et al.* 1993; Dunne *et al.* 1992a; Rihet *et al.* 1992; Hagan *et al.* 1991; Boctor and Peter. 1990; Butterworth *et al.* 1987). Recently, as a consequence of widely available monoclonal antibody and immunochemistry technologies, the development of affinity purification systems has permitted the immunopurification of small amounts of IgE from the complex milieu of proteins found in infection serum (Rihet *et al.* 1992; Ahmad *et al.* 1991). By adapting and applying this technique to our model it has been possible to deplete infection serum of competing IgG antibodies and successfully characterise, by immunoblot analysis, the relative amounts of parasite–specific IgE antibody present in male–only and mixed–sex infection serum.

Finally, with regard to the experimental model used, the occurrence of this potentiated antibody response suggests that enhancement of polyvalent non–specific IgE responses could be minimised by improving animal isolation procedures, restricting animal house access and thereby controlling exposure of rats to contaminating environmental allergens.

3.4.2 *The effect of elevated serum IgE titers upon worm expulsion*

Following infection of rats by *S. mansoni*, the significant increase in non-specific and specific IgE antibody titer has been associated with the rejection of invading parasites from host tissue. In order to verify the existing *in vitro* evidence for an IgE-dependent mechanism of worm elimination, we have investigated the apparent parallel relationship between worm expulsion and elevated IgE production. Infected rats were perfused at days 28 and 42, time points associated with the onset and decline of parasite elimination respectively. Our results are partly in agreement with those of Rousseaux-Prevost *et al.* (1977) who found that the highest IgE level was obtained on day 42, approximately 2 weeks after the onset of parasite elimination (Cioli, Blum & Ruppel, 1978).

Conversely, our results showed that the delayed and much lower total serum IgE response to male-only schistosomes, relative to mixed-sex infections, was not correlated to a reduction in the efficiency of parasite expulsion. However, it is conceiveable that small quantities of circulating specific-IgE antibody are sufficiently effective in mediating a protective effector response against adult worms. Furthermore, it is anticipated that effector cell-bound IgE would be provide a more realistic indication of host-protective antibody, since cross-linking of IgE at the mast cell surface triggers the immediate hypersensitivity (IH) response. The function of unbound systemic IgE is not known. Alternatively, Kigoni and co-workers (1986) who studied the early and late skin phase reaction to *S. mansoni* in rats, found that while IgE-suppressed animals cured a first infection as rapidly as control rats, they were subsequently less resistant to reinfection by schistosomes. These workers concluded that IgE-dependent mechanisms of resistance were not essential for the expulsion of parasites after *initial* exposure to cercariae. Obviously it is difficult to analogize the skin phase reaction to the present study of adult worm immunogenicity, however Kigoni's work does indicate that IgE could operate more effectively against a challenge infection. With respect to Kigoni's observations and those described in the present study, investigations into the IgE reactivity exhibited against a secondary infection and the subsequent rate of worm elimination may also be of interest.

The finding that worm elimination from infected animals was not inhibited by lower levels of serum IgE, nor enhanced by higher levels of IgE implies that this isotype may not play a significant role in worm expulsion as purported. Following the exposure of rats to mixed-sex cercariae, parasites promoted elevated titers of IgE antibody when compared to unisexual infections. However, it would be spurious to

suppose that increased levels of total serum IgE are indicative of elevated titers of parasite-specific antibody or an enhanced ability to expel worms. Mixed-sex infections may only be more effective in stimulating pre-induced non-specific polyvalent reactivity, the 'potentiated reagin response' discussed above, and not schistosome-specific titers. If this interpretation was correct, serum from mixed-sex infected rats and male-only infected rats would contain equivalent levels of parasite-specific IgE, and as a consequence both groups would be equally effective in eliminating parasites. This argument will be resolved in the following chapter which deals with the quantification of parasite-specific IgE titers.

3.4.3 *The potential role of effector cell (Fc∈RI⁺) responses in mediating protection*

If IgE alone is not a crucial component in the induction of the self-cure phenomenon, the worm elimination data presented here indicates the existence of a more complex mechanism(s) involving alternative or synergistic cell-mediated responses acting irrespective of/in concert with IgE-dependent mechanisms.

Miller *et al.* (1994) reported the manifestation of an extensive hepatic mastocytosis with release of RMCP II from day 28 post-infection with *S. mansoni*, coincident with parasite expulsion. This recruitment of mast cells to the liver was not observed in permissive mouse hosts. Following Miller's findings, it is hypothesised, for the purposes of this study, that mast cells are the principal effector cell population involved in the precipitation of worm death and that their activation requires the active participation of IgE antibody.

The IgE-triggered release of mast cell mediators in response to antigen is thought to be the primary event in IH reactions. However, recent *in vitro* and *in vivo* evidence suggests otherwise. Data from previous studies, proposing that rat IgG2a could interact with the rat high-affinity IgE receptor (Fc∈RI), was confirmed and expanded by the work of Benhamou *et al* (1994) who investigated the ability of rat IgG to bind to and activate the rat Fc∈RI present on the high secreting mast cell line RBL-2H3, and rat peritoneal mast cells. Of all the rat subclasses only cross-linked IgG2a was able to trigger the cells into releasing detectable levels of histamine. Despite conclusive evidence for IgE-independent stimulation of mast cells one caveat remained; upon addition of monomeric IgE the authors found that the IgE antibody successfully competed against the IgG isotypes for available Fc∈RI binding sites on the mast cells, preventing IgG-induced degranulation (Benhamou *et al.* 1994).

The mouse model was used to confirm previous observations, with regard to IgG

activation of mast cells via their Fcγ or Fc∈ receptors and their ability to generate immediate hypersensitivity (IH) reactions. Oettgen *et al.* (1994) actively immunised IgE–deficient mice with bovine gamma globulin and ovalbulmin (OVA), these mice then exhibited full–blown systemic anaphylaxis, clearly demonstrating that IH responses could be triggered independently of IgE antibody. However, it is debatable whether IgE–independent mechanisms of mast cell activation are significant following helminth infections of rats, as the animals produce elevated IgE titers that would compete against weakly bound IgG, or more specifically IgG2a (Benhamou *et al.* 1994).

Further putative evidence for the existence of alternative or synergistic systems of parasite attrition is given by our findings that show the systemic release of RMCP II by activated mast cells does not correlate with the levels of serum IgE. From these results we deduce that the cross–linking of IgE may not be a crucial factor in triggering mast cell degranulation, as discussed, or alternatively mast cell activation and subsequent degranulation could be an 'all or nothing' response (Hide *et al.* 1993); that is to say, once a population of mast cells is committed to releasing their contents more cells must be recruited to perpetuate the IH response. If mast cells are not attracted to the source of inflammation, after the initial burst of degranulation, the release of mediators, and noteably RMCP II, will wane.

Furthermore, an explanation for the reported rise in IgE titers, from day 28 following infection with *S. mansoni*, might be the observation that mast cells release B–cell stimulatory cytokines, for example IL–4 which directly regulates IgE production independently of T–cells (Schroeder *et al.* 1994; Gauchat *et al.* 1993; Williams *et al.* 1993; Plaut *et al.* 1989; Finkelman *et al.* 1988). In addition, mast cells have also been implicated in enhancement of eosinophil antibody–dependent cellular–cytotoxicity (ADCC) by the production of various soluble factors, such as eosinophil chemotactic factor (ECF), eosinophil activating factor (EAF) and IL–5 (Kung *et al.* 1995; Mazza *et al.* 1991; Plaut *et al.* 1989; Capron *et al.* 1978b).

The role of eosinophil–mediated mechanisms of parasite attrition have been demonstrated *in vitro* (reviewed by Capron *et al.* 1992; Capron & Dessaint, 1985; Capron *et al.* 1980). Schistosomula are killed following eosinophil activation by anaphylactic antibodies, both IgE and IgG2a, with the transfer of these cells from immune rats conferring significant resistance against a challenge infection (Capron *et al.* 1984). Thus, it is reasonable to speculate that following the IgE–dependent degranulation of hepatic mast cells from days 21 to 28 post–infection (Miller *et al.* 1994) the local and systemic release of inflammatory mediators (ECF) attracts

circulating eosinophils (Kung *et al.* 1995), thereby accounting for the appearance of eosinophil-mediated inflammatory reactions associated with adult schistosomes in the liver (Phillips *et al.* 1983; Bentley *et al.* 1981; Knopf, 1979). Hence the significant increase in IgE production, after mast cell degranulation, may be associated with eosinophil activation. This eosinophilia may compensate for the apparent redundancy in mast cell function, with the activation of eosinophils and subsequent release of eosinophil peroxidase (EPO), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and major basic protein (MBP) directly contributing to worm death (Khalife *et al.* 1985).

Furthermore, as the high affinity Fc∈RIs are not able to distinguish between non-specific and specific IgE, the significant increase in unrelated IgE responses may sterically inhibit the binding of specific IgE at the mast cell surface. This would account for the observed decline in systemic release of RMCP II which occurs with the appearance of an augmented reaginic response. This latter supposition could be further interpreted as evidence for the existence of a potential feed-back mechanism, limiting the pathology caused by the intensive production of inflammatory mediators into the host's environment. Alternatively, the apparent reduction in RMCP II release may be attributed to a fall in levels of circulating allergen as worms die and the schistosome antigen becomes immune-complexed, hypothetically 'using up' the available stimulatory proteins. However, this explanation is weak since parasite death would result in the extensive release of somatic antigen into the circulation.

In summary, the presence of mast cells in the liver of infected rats, coincident with the onset of parasite elimination, may be critical in orchestrating the effector arm of the immune response rather than participating in the direct killing of schistosomes.

3.4.4 *Attempts to characterise allergens from mixed-sex and male-only schistosome infections of rats*

It is well documented that in the mouse model it is the onset of egg deposition which triggers a switch from the protective Th1 mediated immune response to the Th2 phenotype (Chensue *et al.* 1992; Gryzch *et al.* 1991). In this model elevated levels of serum IgE, with the aggregation of immune cells, for example eosinophils, around live eggs in the liver, are indicative of the damaging Th2 response induced by the potent secretions emanating from schistosome eggs. If mice are exposed to a high infection dose, greater than 200 cercariae per mouse, the egg-induced hepatic pathology, associated with the Th1-Th2 switch, culminates in the death of the

animal 8 weeks later.

In contrast, upon KOH and enzymatic digestion of infected rats' livers we found no indication of egg deposition or egg–induced pathology. This observation was accompanied by the recovery of sexually immature, unpaired female worms. It is concluded that the production of IgE following infection reported here, was promoted and potentiated by the prolific release of allergens from female worms. The finding that mixed–sex parasites stimulate a more rapid IgE response of greater magnitude than male–only schistosomes, has not been demonstrated prior to this study. A gender–specific phenomenon was briefly described by Ritchie and colleagues (1963) who exposed rats and mice to schistosome cercariae and then observed the growth and survival of male and female parasites. They suggested that the natural defences of the rat were more effective against female schistosomes. This disparity may be explained by the ability of the mature female worm to ingest a larger blood meal than the male (Lawrence, 1973) and to regurgitate more residual products of digestion. By virtue of its larger appetite and secretory potential the female schistosome may be considered as a more potent source of antigenic material, and hence a more effective inducer of, and target for, the immune response. More specifically, we may surmise that the augmented release of these products of digestion from female worms, not excluding surface antigens, is correlated with the potentiation of IgE antibody production by B cells. In addition, it is noteworthy that helminths may produce a secretion(s) that selectively promotes polyvalent non–specific IgE antibody production by B cells, thus potentially minimising parasite–specific stimulation of the protective immediate hypersensitivity reaction (Lopes *et al.* 1990; Jarrett and Stewart, 1973).

Subsequent to these observations, we have attempted to characterise the parasite–specific IgE–mediated response by isolating IgE from day 35 (D35) serum samples, via immunopurification, and using these fractions to probe for allergens by western blotting analysis. Two dominant proteins were identified, with Mw of 67 and 36–38kDa. The intensity of the anti–female worm IgE response directed against these two proteins was much stronger than the allergic reactivity demonstrated against the same male–only antigens. Thus, the western blotting study supported data from the IgE ELISAs, indicating that the promotion of non–specific and specific antibody followed the enhanced release of allergenic material from female worms.

We have also reported the enhanced non–specific binding of the CMS and WTS to female worm products. This smeared effect on western blots is characteristic of antibody directed against carbohydrate epitopes present in the antigen preparations

(unpublished observation, R. Harrop). Glycoproteins and carbohydrates are known to separate less readily into discrete bands by SDS–PAGE than proteins. Complexes may form between carbohydrate and proteins which prevent entry into the gel/poor resolution (Hames & Rickwood, 1990). Thus the high background staining may be caused by antibody binding to immunogenic carbohydrates/glycoproteins which, due to their poor fractionation results in a broad smear. If this supposition is correct, it suggests that female worms not only produce larger quantities of E/S protein but also a comparative abundance of carbohydrate products, relative to unisexual male infections. Hence, sugar moieties may also function as effective allergens, increasing the diversity of potential immunogens and promoting elevated titers of serum IgE. This discussion point will be expanded upon in chapter four.

The characterisation of schistosome allergens has been the objective of several groups. Pierce and co–workers employed the radioallergosorbent (RAST) and PK tests to determine the relative potency of adult worm–released products and whole worm homogenate in *S.mansoni* infections of both humans and Fischer rats (Pierce *et al.* 1983). They reported a marked difference between the ranges of allergenic material recognised by humans and rats. During the rat infection one major allergen was found, a glycoprotein Mw of about 100–150 kDa, compared to the situation in man where the authors described several important allergenic fractions having a Mw distribution of between 20 and 150 kDa. Pierce suggested that since few worms reach maturity in the rat infection, rats are not exposed to the whole range of schistosome allergens, particularly those released by live eggs. In the present study the isolated IgE fractions did not bind a high Mw product, however the western blotting technique was restricted to the detection of proteins, not carbohydrate epitopes.

The results presented in this chapter are not in agreement with Harris *et al.* (1973) who used infected Wistar rat sera and the PK test to assay the allergenicity of different fractions. Allergenic heterogeneity was demonstrated within their antigen fractions, 12 out of 35 fractions possessing reactivity with IgE. However, the detection of allergens in the present study by western blotting is not comparable to the PK test. Moreover, the Fischer rat used in my experiments is a relatively low IgE responder and minor allergenic proteins may not be detectable. The limited amount of serum IgE was also compounded by the immunopurification technique which is not 100% effective, thus resulting in the recovery of approximately 80ng of IgE from 50μl of infection serum. Furthermore, schistosome–specific IgE reactive with scarce parasite allergens may be lost by denaturation or non–specific binding to the mini–

column immunosorbent. This inconsistency could be remedied, providing the availability of infection serum was not limited, by immunopurifying IgE from larger volumes of test serum, or by using high IgE responder strains, such as Brown-Norway rats.

The observation that the excretory/secretory (E/S) products from adult worms, particularly females, induce IgE production is further supported by evidence from Vannier *et al.* (1974) and Pierce *et al.* (1983) who investigated the allergenicity of a variety of adult worm preparations. Both groups concluded that the soluble material released after freezing and thawing the adult worms (Vannier *et al.* 1974) or following the incubation of adult parasites in water (Pierce *et al.* 1983) contained most of the allergic material reacting with sera from singly infected rats. However both techniques relied upon osmotic and temperature changes to induce worm vomiting. Such extremes exert internal and external stresses on the parasites, possibly resulting in the contamination of antigen preparations with somatic immunogens (Carlisle *et al.* 1983; Wilson and Barnes, 1974b). In view of this, the incubation product undoubtedly contained both tegumental and gut-derived antigens, but the relative importance of E/S material in stimulating IgE responses was difficult to assess.

In addition to the allergenic potency of adult worm E/S products, rat IgE directed against schistosomula-released products (SRP) is cytotoxic for *S. mansoni* schistosomula *in vitro* (Auriault *et al.* 1984; Auriault *et al.* 1985). However, the young larva seems to lose its susceptibility to *in vitro* effector mechanisms within a period of 24h, limiting the relative importance of protective anti-larval IgE-dependent cytotoxicity reactions *in vivo*. Following immunisation of rats with defined antigens of SRP, the 22 and 26 kDa molecules (Damonneville *et al.* 1986a) and whole SRP-antigen preparation (Damonneville *et al.* 1986b), significant levels of protection were observed. SRP-antigen reduced the worm burden in Brown-Norway rats by 83%, with passive transfer of anti-SRP serum successfully protecting recipient animals. More specifically, the protective *in vivo* effect was attributed to the IgE antibody response induced against serine proteases of SRP (Verwaerde *et al.* 1986). However, it must be noted that these series of experiments used secretory products of 3h schistosomula and not adult worms.

Further to this discussion, the 67 kDa protein identified by anti-schistosome IgE appears analogous to the product recognised by WTS (section 2.4). In the previous chapter this 67 kDa antigen was found, by immunocytochemistry, to be derived from the cells lining the schistosome gut. In order to confirm that these two antigens

represent an identical product, it would be necessary to probe schistosome fractions with rat infection serum and WTS, following separation of adult worm proteins by 2-D gel electrophoresis.

The 36–38 kDa antigen, however, is not identified by WTS, suggesting that this protein is either somatic in origin, or does not induce the production of antibody in mice. The former implies that the proteins are released following worm damage, and that the augmented IgE response could be attributed to the subsequent increase in systemic schistosome antigen levels following worm death. If this hypothesis is correct, an increase in the amount of circulating antigen may be more important in eliciting IgE production and IgE-dependent effector mechanisms than the release of scarce allergenic E/S products from live schistosomes. However as discussed, evidence from *in vivo* and *in vitro* studies have demonstrated the relative potency of released products from schistosomula in enhancing the killing ability of IgE-mediated immune responses against schistosomula. The 36–38 kDa antigen possesses the same Mw as a 38 kDa molecule described by Omer–Ali and colleagues (1988; 1986). Omer–Ali found that antibody from infected mouse serum was directed against carbohydrate epitopes on the antigen (Omer–Ali *et al.* 1988). More importantly, mouse mAb raised against the 38 kDa conferred protection against a homologous challenge when passively transferred to laboratory rats (Grzych *et al.* 1982). It was surmised that antibody directed against surface carbohydrate could transfer protection to naive animals. The western blotting results presented here showed that IgE bound with a higher affinity to the 38 kDa molecule in the female-derived SWAP than the male-only SWAP. In general, the paired female worm appears more allergenic and, in view of the work by Omer–Ali *et al.* (1988), together with the high background of antibody reactivity against the female products, the data indicates that the allergenicity resides in the carbohydrate moieties of the adult worm.

Alternatively, the failure of WTS to characterise the 36–38 kDa antigen may be due to a failure of the mouse's immune system to respond effectively to, and process, the allergen. It is known that there are inherent differences in the initial cell-mediated responses of permissive mouse and non-permissive rat hosts to schistosomes, with respect to T cell involvement. Antigen-induced proliferation of lymphocytes from the two rodent species revealed differences between both spleen and T cell derived responses of mice and rats (Mendlovic *et al.* 1987). In rats, thymocyte proliferation *in vitro* was 3x greater than splenocyte proliferation, whereas mouse thymocytes did not proliferate as dramatically as mouse splenocytes

upon antigenic stimulation. As a consequence there may be a relatively low level of T and B cell co-operation following *S. mansoni* infection of mice, compared to rats. In the rat model, interaction between these two cell populations is essential in stimulating the elevated antibody response to circulating parasite antigens (Phillips *et al.* 1991; Capron *et al.* 1983; Phillips *et al.* 1983; Cioli & Dennert *et al* 1975; Phillips *et al* 1975). Furthermore, the lower capacity of infected mouse thymocytes to proliferate compared to splenocytes, suggests that the preceding immune events, the recognition and presentation of antigens by antigen presenting cells (APCs) to T cells, are either different, or are not as effective when compared to the laboratory rat. The nature/relative abundance of the APCs in conjunction with the site of antigen presentation could be crucial in this respect. However, to date no data exists on the comparative induction mechanisms operating during a schistosome infection of rats and mice. It is also interesting to note that since the thymus functions as a repository of immunological memory the enhanced thymocyte responsiveness to schistosome antigens in the rat may result in the more rapid elimination of challenge infections. However, it must be emphasised that Mendlovic's results are based upon the assumption that there is two-way traffic between the thymus and the periphery in rats, which would account for the observed high thymocyte responsiveness.

4.4.6 *Concluding remarks*

In conclusion, the investigations presented in this study have demonstrated the enhanced production of serum IgE following infection of laboratory rats with *S. mansoni*. Rapid induction of the IgE response appears to be contingent upon the early and/or enhanced secretion of allergens from female worms. In particular this allergic reactivity seems to be directed against two dominant proteins of Mw 67 and 36–38 kDa. The former appears analogous in molecular weight to the antigen defined by WTS in the previous chapter, thereby supporting our postulate that protective immunoglobulin production is stimulated by the release of E/S material from living adult worms. The 36–38 kDa macromolecule, which is not identified by WTS, could be released as a consequence of worm death during the earlier migratory phase of the parasite, and hence could be of somatic origin. Since the mouse is a permissive host parasite attrition is limited in comparison, thus the immune system may not encounter large amounts of this antigen.

The fall in RMCP II levels after day 35 suggests that mast cells are no longer recruited to the site of inflammation and/or that their surface receptors are not cross-linked by schistosome allergen. However, since IgE production increases up until

termination of the experiment it may also be inferred that an alternative/synergistic IgE–dependent effector mechanism is involved in the killing of adult parasites. Moreover, the release of ECF, EAF, IL–5 and IL–4 by mast cells lends further credence to the theory that mast cells function as potentiators of humoral and effector responses, intensifying the inflammatory reaction against invading worms.

In this chapter, the parasite–derived factors which stimulate specific and non–specific IgE responses have been characterised in part, but have not been directly linked to the triggering of mast cell degranulation. Hence, the complex role of IgE antibody and mast cells will be investigated by measuring the levels of degranulation from sensitised mast cells, following stimulation with various soluble worm preparations, as a function of time post–infection. The connection between worm expulsion and IgE–dependent mast cell degranulation will be explored further in the next chapter.

CHAPTER FOUR

Mast cell responsiveness to schistosome allergens

4.1. INTRODUCTION

The potent ability of IgE to activate effector cell populations is widely accepted. Both the high affinity Fc∈RI and the low affinity Fc∈RII/CD23 receptors, are expressed on a variety of bone-marrow derived inflammatory cell populations, namely, mast cells, eosinophils, macrophages and platelets. Evidence for the *in vitro* killing of opsonised schistosome larvae by activated eosinophils, macrophages and platelets has been demonstrated by many workers (Gounni *et al.* 1994; Pestel *et al.* 1988; Capron & Capron, 1986); the release of proteases, nitric oxide and a series of highly toxic and highly cationic proteins: eosinophil cationic protein (ECP), eosinophil derived neurotoxin (EDN) and major basic protein (MBP) mediate parasite attrition. As schistosome larvae become gradually less susceptible to immune attack *in vitro* over a 24h period (Auriault *et al.* 1981) it is difficult to establish a role for IgE-dependent effector mechanisms *in vivo*. Thus, caution must be exercised when extrapolating between *in vitro* killing and the occurrence of infection and immunity *in vivo*.

Many intestinal helminth infections of mice and rats stimulate increased populations of infiltrating monocyte and polymorphonuclear cells to the site of inflammation, in which a mucosal mastocytosis is a prominent feature. The focal release of mast cell mediators induces vasodilation, and chemotaxis of other effector cell populations, generating an upregulation in inflammatory cell activity around the gut-invasive parasite. Furthermore it is possible that proteases, such as rat mast cell proteases (RMCP I and II), exert a deleterious effect upon the parasite, either making it more susceptible to immune attack, or causing irreparable damage. In some infections, notably *Trichinella spiralis* in mice, there is evidence that the immune-mediated inflammation is a cause of worm expulsion and that mast cells are functionally involved in this event (Grencis *et al.* 1993; Huntley, 1992; Lee, Swieter & Befus, 1986). Since the maturation of mast cells is dependent upon the production of stem cell factor and its subsequent recognition by the c-kit ligand on the surface of progenitor mast cells, c-kit deficient animals provide an *in vivo* model with which to determine the direct role of mast cells in parasite elimination. *Nippostrongylus braziliensis* infections of c-kit deficient mice (W/W^v) and rats (W^s/W^s) produced a defective intestinal mastocytosis coincident with the delayed expulsion of parasites from the gut (Crowle & Reed, 1981; Arizono *et al.* 1993). The induction of this mast cell hyperplasia in the gut is believed to be under the control of T helper (Th) cell-derived cytokines, including IL-3, IL-4, IL-9 and IL-10 (reviewed by Kitamura *et*

al. 1993; Ghildyal *et al.* 1992; Madden *et al.* 1991). Contrary to the study by Crowle and Reed (1981), Madden found that the administration of anti–IL–3 and anti–IL–4 monoclonal antibodies to *N. braziliensis*–infected mice, resulted in a much reduced intestinal mast cell hyperplasia (85–90%), with no effect upon worm expulsion kinetics. These experiments suggested that intestinal mastocytosis following helminth infection was predominantly dependent upon IL–3 and IL–4, but that intestinal mastocytosis was not necessary for the expulsion of *N. braziliensis* from the intestines of mice. The apparent disparity between the rat and mouse model, following helminth infection, reveals profound differences in the expression and induction of the protective immune response in each animal.

The conjecture that IgE–mediated immune responses are paramount in controlling intestinal parasite infections is consistent with the observation that circulating IgE can be preferentially transported to the site of inflammation (Ramaswamy *et al.* 1994). The results from Ramaswamy's study imply that an increase in the concentration of serum IgE at the site of infection could potentiate the priming of effector cells, for example mast cells, prior to specific stimulation with allergens.

In the previous chapter the appearance of elevated serum IgE titers in the rat, coincident with the onset of worm elimination from day 28 post–infection onwards, was described. Similarly, Miller *et al.* (1994) reported the manifestation of a pronounced hepatic mastocytosis in the non–permissive rat host around day 28, compared to the delayed recruitment of mast cells to the intestine in the permissive mouse host. They proposed that the accumulation of mucosal mast cells (MMCs) in the livers of infected rats could induce parasite expulsion, explaining the disparity in the expression of immunity between the two animal hosts. Causal correlations between worm expulsion and mastocytosis cannot be based purely upon histological data. However, by measuring levels of the mucosal mast cell–specific serine protease (RMCP II), which is released during mast cell degranulation, it was possible to monitor mast cell activity as a function of time post–infection. The results showed that the period of worm clearance coincided with the release of RMCP II, supporting histological evidence for the putative role of MMCs in the protective immune response to schistosomes in rats. This data suggests that the concurrent increase in mast cell numbers and degranulation, from day 28 onwards, is associated with the expression of immunity to adult parasites in the rat.

From the preceding account, it is apparent that the timing of mast cell recruitment and their subsequent degranulation, correlates with the onset of worm expulsion. In view of this association, it is of interest to characterise the allergenic molecules of

schistosomes that initiate the cross-linking of mast cell-bound IgE. Thus, in this study a rat basophilic leukemia mast cell (RBL-2H3 cell line) serotonin release assay was developed in order to evaluate the extent of mast cell degranulation upon stimulation with soluble worm antigen preparations (SWAP). The antigen-specificity of the response was established by sensitising the cells with serum taken from rats exposed to *S. mansoni*. Tritiated serotonin, added to the culture medium, was incorporated by RBL-2H3 cells *in vitro*, and subsequently released following the cross-linking of surface IgE bound to the Fc∈RIs. The amount of radiolabel recovered in the media, from allergen-stimulated mast cells, was quantified by reading the β-emission from test samples on a scintillation counter, and the extent of degranulation calculated as a percentage of the positive control. As IgE-independent mast cell degranulation was negligible, the assay was used as an indirect measure of schistosome-specific IgE levels. Furthermore, it was not necessary to remove potential blocking immunoglobulins by immunopurification since the Fc∈RI reactivity was largely specific for the ∈-heavy chain of IgE. The results support *in vivo* evidence for the active participation of MMCs in hepatic immunity following schistosome infections of rats.

4.2. MATERIALS AND METHODS

4.2.1. *Parasite and hosts*

A Puerto Rican strain of *Schistosoma mansoni* and male-only parasites were routinely maintained in the laboratory following the procedures described in section 2.2.1.

F344 female rats, 8-10 weeks of age and approximately 150g each, were used throughout this study.

4.2.2 *Cells*

Rat basophilic leukemia (RBL) cells from the 2H3 subline were a gift from Dr. Birgit Helm, University of Sheffield, Sheffield, England. Culture and cell passage methods were as described in section 3.2.8. for the A2 cell line. The RBL-2H3 cell line will release histamine, IL-4 and other cell mediators when subjected to an immunologic challenge (Barsumian *et al.* 1981; B. Helm, unpublished observations).

4.2.3. *Source of sensitising antibody*

RBL cells were sensitised with rat infection serum from experiment 3 (see section 3.2.2.), prior to direct antigenic stimulation with schistosome immunogens. In summary, F344 rats were infected percutaneously, via the shaved abdomen, with either 2000 male-only *S. mansoni* cercariae or 2000 cercariae of both sexes. Rats were bled from the tail vein, or by cardiac puncture, on days 28, 35 and 42 post-infection. All sera were aliquotted and stored at -20°C until use. Total serum IgE titers from uninfected and infected rats were determined; the methods and results are discussed in chapter 3. Rat serum was pooled for each time point, day 28, 35 and 42, and used subsequently in mast cell assays.

Initial optimising assays used pooled stores of vaccinated rat serum (VRS) to sensitise mast cells. The vaccination procedure is summarised in section 3.2.2. In addition, aliquots of VRS were heat-inactivated by incubating the samples at 56°C, in a water bath for 45min. Exposure at this temperature selectively denatures serum IgE, compared to the relatively heat-stable immunoglobulins: IgG, IgM and IgA.

4.2.4. *Antigen*

Male-only SWAP and SWAP made from schistosomes of both sexes was prepared using the procedures described in section 2.2.3. In addition, female-only SWAP was included in the serotonin-release assays. Female worms were recovered, by portal perfusion, following mixed-sex schistosome infections of C57BL/6 mice. Mature ovipositing females were removed from the perfusion medium, using a mounted needle, after a brief 10min incubation on ice. The parasites were frozen/thawed once (in the presence of protease inhibitors I and II, section 2.2.2.), prior to sonication and centrifugation at 105 000 g for 1h at 4°C. The supernatant, containing the soluble protein fraction, was removed, aliquotted, and stored with protease inhibitors (PIs) at -70°C. The protein content of each preparation was determined using the method of Lowry *et al.* (1951).

4.2.5. *Periodate treatment of soluble antigen*

Mixed-sex SWAP, containing N- and O-linked glycoproteins, was treated with sodium periodate, which oxidises the carbon-carbon linkages and thus alters the carbohydrate structure (Dunne *et al.* 1992a). Briefly, 50μg of SWAP was incubated on ice, in the presence of 20mM sodium periodate, for 10min. The preparation was then aliquotted and stored at -20°C. Protein denaturation, following such stringent oxidising conditions, was a possible outcome. The integrity of the treated sample

was tested by loading treated and untreated preparations on a 6–16% gradient gel. The samples were electrophoresed by SDS–PAGE, according to the method of Laemmli (1971), the separated proteins fixed, and stained with Coomassie blue (section 2.2.5.).

4.2.6. *The Serotonin Release Assay*

Cultured RBL–2H3 cells were grown to confluence, in RPMI medium containing 10% FCS, 1% L–glutamine and supplementary antibiotics (Life Technologies, Paisley, UK). Cells were harvested by incubating the adherent cell line briefly with 0.25% trypsin, in PBS (pH 7.2), centrifuging at 2 500*g* for 8min, removing the supernatant and resuspending the cells in 2ml of fresh RPMI, with supplements. Trypan blue solution was added to 50μl of cell culture suspension and the viable cells counted using a haemocytometer. The cells were plated into 24 well polyvinyl plates (Nunc, Denmark) at a density of 2 x 10^5/well, in 400μl of RPMI (5 x 10^6 cells in 10ml). Ten μl of tritiated serotonin, 1mCi/ml (specific activity DuPont NEN Division, Dreiech, Germany; catalogue no. NET–498), was added to each well using sterile techniques. Finally, cells were sensitised by adding rat infection serum (minimum dilution to 1:40), and incubating for 24h at 37°C. The contents were flicked into a designated radiolabel disposal sink, and the wells washed once with 500μl of PIPES buffer per well (0.1M NaCl, 0.005M KCl, 0.025M PIPES, 0.005M glucose, 40μl of 1M $MgCl.6H_2O$, 1ml of 1M $CaCl_2$, pH 7.4), on a shaking table (Flow Laboratories, UK) at 37°C for 10min. Again, the well contents were flicked into the sink, excess buffer removed by blotting the plate, and antigen added in 400μl of PIPES buffer/well (0.1% lipid free BSA). The positive control wells were incubated with 0.5% triton–X in PIPES buffer, which lysed the monolayer to release the total serotonin content of the cultured RBL–2H3 cells. The plates were incubated on a shaking table for 15min at 37°C, and the reaction stopped by putting the plates on ice. Supernatant was removed from each well (200μl), added to scintillation fluid (4ml/vial; Ultima–Gold; Canberra Packard, Pangbourne, UK), shaken well and read on a β–scintillation counter (Canberra Packard). The release of serotonin from each of the test samples, following IgE–mediated mast cell degranulation, was calculated as a percentage of the positive control (stimulation index).

4.2.7. *Mast cell western assay*

Mixed–sex SWAP was also presented to the RBL–2H3 cells bound to PVDF membrane (Millipore, Watford, UK). Protein was applied to Immobilon–P PVDF

membrane using a Bio-Dot SF blotting apparatus (Bio-Rad Laboratories, Richmond, CA), following the procedure detailed in section 3.2.6. Wells were loaded with 2, 4, 8 and 16μg of SWAP in 200μl of blot buffer (see section 3.2.5.); unused wells were filled with buffer alone to ensure no cross contamination between samples. Each piece of antigen-coated membrane was then cut from the blot, and stored at −20°C, in a sealed plastic bag, until use. RBL-2H3 cells were sensitised following the serotonin release assay described above. Antigen stimulation was provided by the SWAP-coated pieces of membrane, each blot carefully orientated so that the antigen lay directly on top of the adherent cells. Each test was carried out in triplicate and the incubation time extended to 25min at 37°C. In addition, Immobilon alone was added to control wells and serotonin release measured, to assess the effect, if any, of adding membrane to sensitised mast cells.

4.3 RESULTS

4.3.1. *Optimisation of the serotonin release assay*

The effects of altering serum dilution and antigen concentration upon the RBL-2H3 cell response were investigated in two separate studies. To avoid depleting the limited quantities of serum from male-only and mixed-sex cercariae infections (experiment 3, section 3.2.2.) abundant stocks of VRS were used in the first series of optimising experiments.

Standardisation of the assay was an essential prerequisite, hence the first step was to assess the ability of various serial dilutions of VRS to activate cultured mast cells. The experiment was repeated three times, and the results shown represent values taken from one study. Figure 4.1a shows that the extent of degranulation by mast cells is dependent upon the concentration of sensitising antibody. The level of IgE-independent mast cell degranulation is negligible, indicated by the stimulation index value for the wells containing cells only. Similarly, when RBL-2H3 cells are incubated with a low dilution of NRS (1:10), containing basal titers of serum IgE (section 3.3.), the degree of serotonin release barely rises above the cells alone level. In contrast, incubation with a 1:10 dilution of VRS results in a 3 fold increase in the release of radiolabel from antigen-stimulated cells. As the VRS is serially diluted, mast cell degranulation declines gradually at first then more rapidly from a dilution of 1:40 onwards. The cells appear to become saturated with parasite-specific IgE at very low concentrations of test serum, since there was no significant

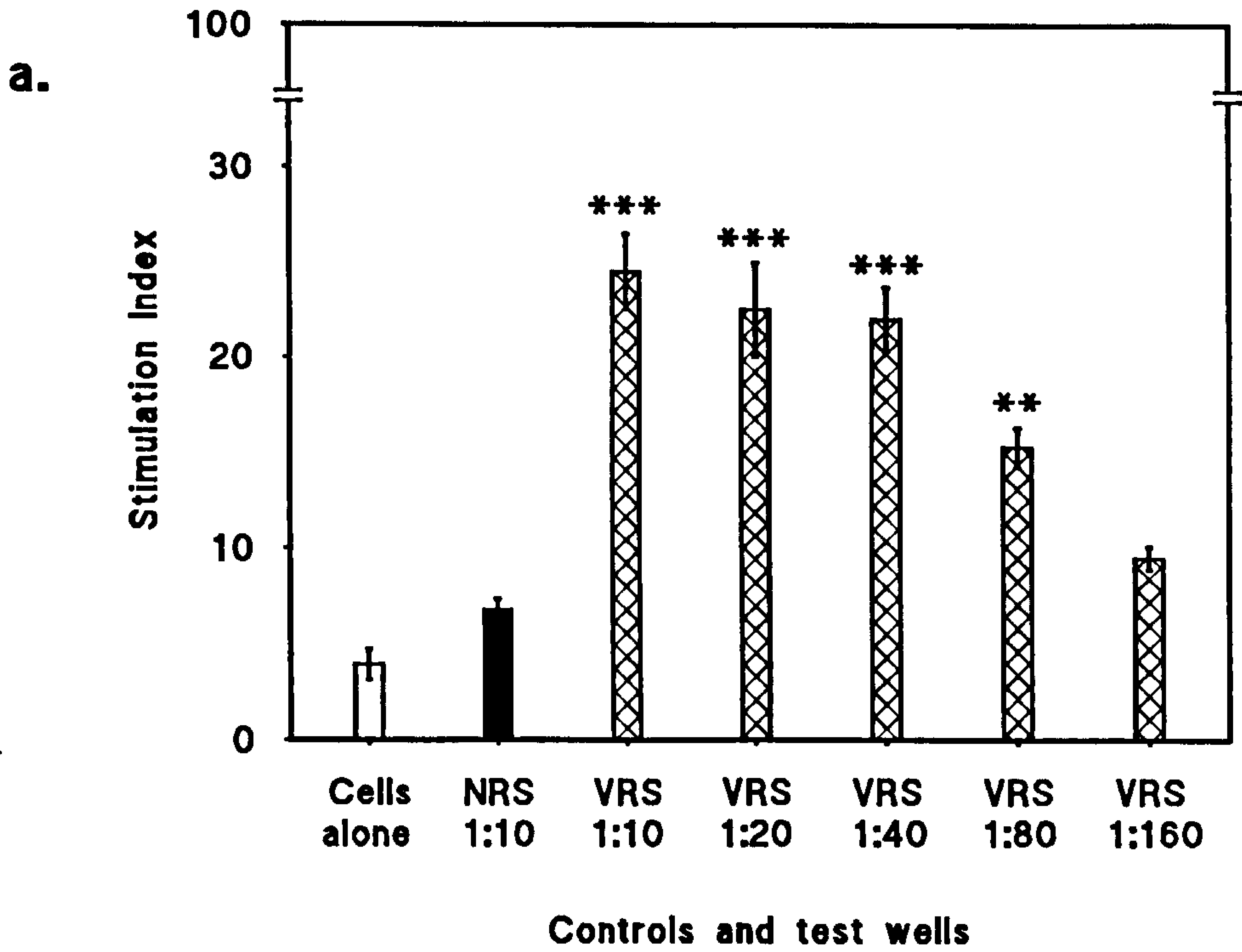
Figure 4.1a Tritiated serotonin release from RBL–2H3 cells sensitised with serial dilutions of vaccinated rat serum (VRS) and a 1:10 dilution of normal rat serum (NRS). Each well was stimulated with 4μg of mixed–sex SWAP, with the exception of the cells alone control.

Figure 4.1b The relationship between increasing concentration of mixed–sex SWAP (with protease inhibitors I and II) and the release of radiolabel from sensitised mast cells.

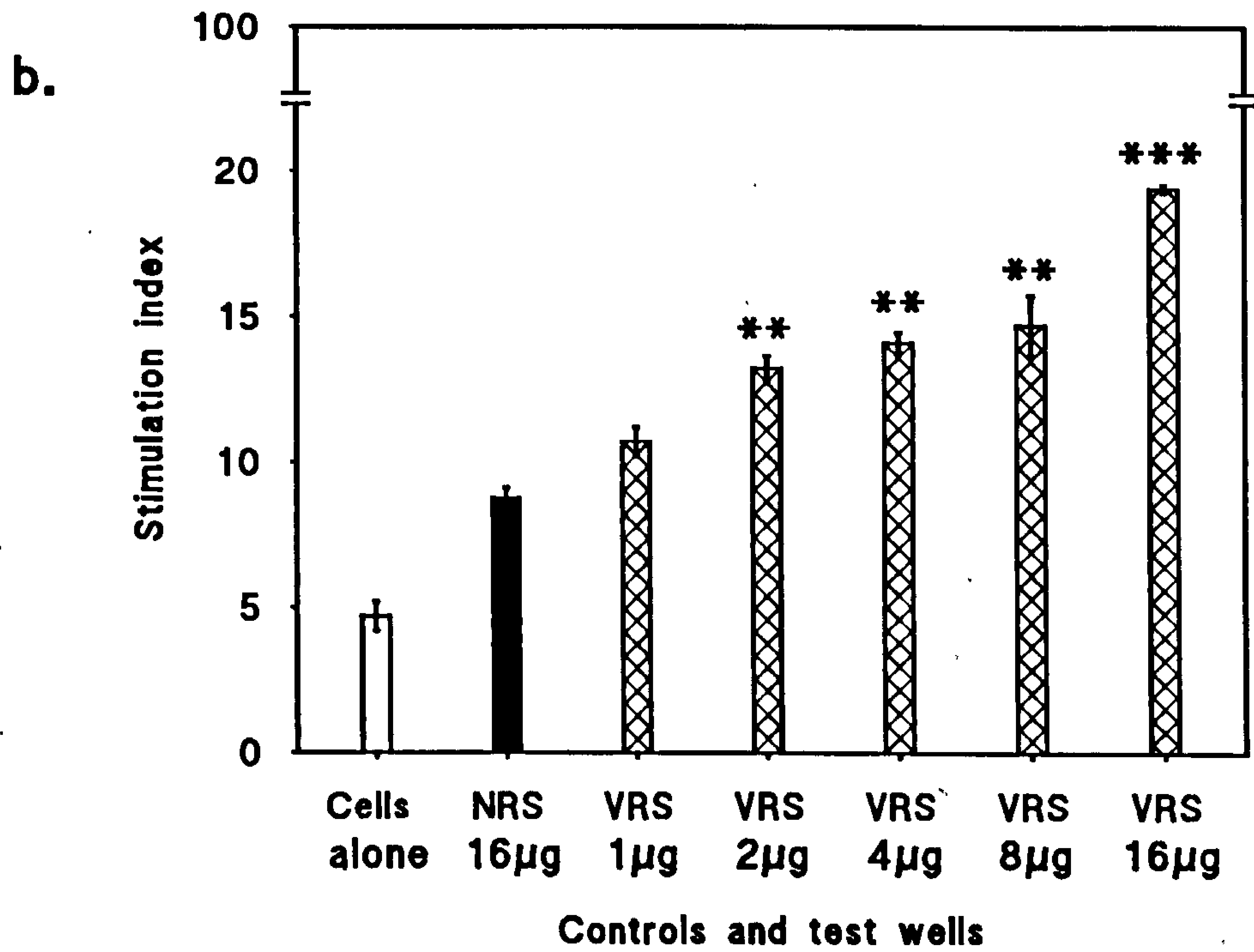
The results shown in both figures are means and standard errors (SE) of pooled test wells from one experiment. Each figure is representative of three replicate experiments performed.

Asterisks represent test values that are significantly different from the NRS control (** $p < 0.05$ and *** $p < 0.01$).

a.



b.



reduction in serotonin release after incubation with VRS at a 1:40 dilution compared to a 1:10 dilution. The data also establishes that serotonin release by mast cells may be used as an indirect evaluation of schistosome-specific IgE titers present in infection serum. All subsequent assays were performed using a standard dilution of 1:40 for both control and test serum samples.

It was also necessary to standardise another variable, namely the concentration of schistosome protein added to sensitised mast cell cultures. Cultured RBL-2H3 cells were incubated with VRS (diluted to 1:40) and tritiated serotonin prior to stimulation with different amounts of soluble parasite protein. This experiment was performed on three separate occasions and the findings shown represent results taken from one experiment (figure 4.1b.). Cells stimulated with 1μg/well of schistosome protein respond by degranulating above the level of the NRS control. In comparing these two values, it is noteworthy that the degree of mast cell degranulation obtained with the negative serum control was measured in response to 16μg/well of protein. Furthermore, upon addition of 2μg/well of soluble parasite antigen to cells sensitised with VRS, the quantity of released radiolabel rose significantly above the stimulation index of the serum control ($p < 0.05$), demonstrating the exquisite sensitivity of the assay. It appeared that the degree of serotonin release was dependent upon the strength of the antigen stimulus. Successive experiments were carried out using a protein concentration of approximately 4μg/well, as determined by the method of Lowry *et al.* (1951).

4.3.2. *Evidence for the IgE-dependency of the serotonin release assay*

The phenomenon of IgE-independent anaphylaxis *in vitro* and *in vivo* has been described recently by Benhamou *et al.* (1994) and Gauchat *et al.* (1993) respectively. Both groups suggest that the IgG2a isotype may be involved in the induction of mediator release by mast cells. To investigate the competence of IgE in mediating mast cell degranulation, with respect to schistosome infections of rats, the functional IgE was destroyed by heat-inactivation (HI) and the heat-treated serum samples used to sensitise RBL-2H3 cells. The data shown in figure 4.2 reveal that IgE-depleted VRS (7.2%) does not activate mast cells to the same extent as whole VRS (17.5%), $p < 0.01$. More dramatically, the basal levels of degranulation obtained in the NRS (5.9%) and cells alone (5.1%) treatments are not significantly different from the heat-inactivated test wells.

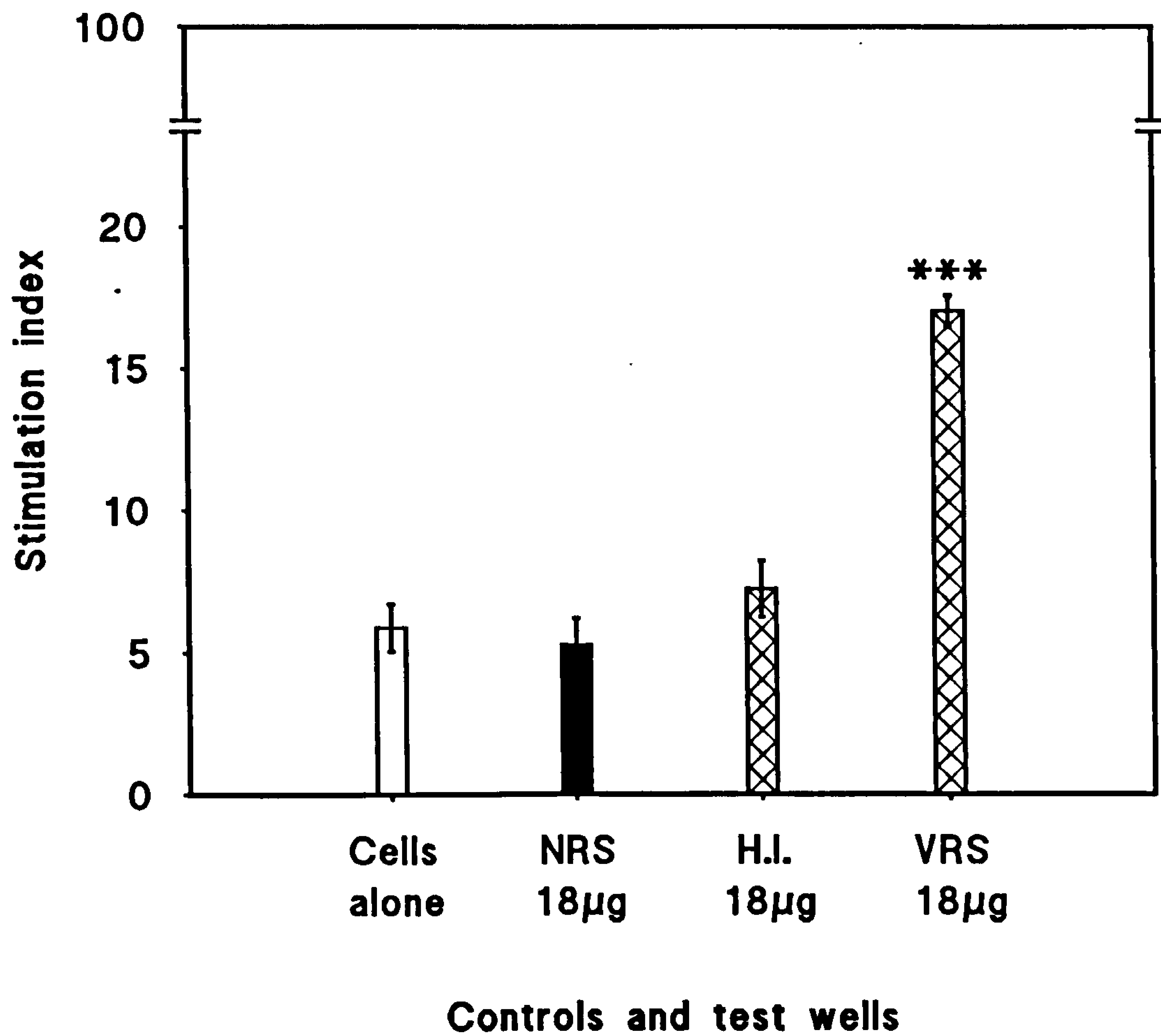


Figure 4.2 The extent of serotonin release stimulated by mixed-sex SWAP following sensitisation with VRS, heat-inactivated serum (HI); including the relevant controls. The HI serum exhibits a reduced ability to prime mast cells prior to activation with schistosome antigen, compared to NRS (*** $p < 0.01$).

4.3.3. *The effect of protease inhibitors (PI) upon serotonin release by the RBL-2H3 cell line*

Despite optimising the assay for antigen concentration and sensitising serum dilution, the extent of mast cell degranulation in response to schistosome protein never rose above a stimulation index value of 29%. The mixed-sex SWAP used to stimulate the cells contained protease inhibitors I and II (PIs; section 2.2.3.) at 1:250 and 1:500 dilutions respectively. It was of concern that their presence, even at such low concentrations per well, could have exerted a detrimental effect upon the enzymes involved in intracellular signal transduction, thereby influencing the mechanism of cell degranulation. To test our postulate RBL-2H3 cells were sensitised with VRS prior to allergenic stimulation with SWAP, with/without PIs (denoted by +/- respectively). As before, the protein concentration added to each well was standardised allowing direct comparisons to be made between the test wells. Figure 4.3 shows the relative levels of serotonin release triggered by different concentrations of parasite antigen. The response of NRS sensitised cells stimulated with SWAP +PIs is not significantly different from the values obtained with SWAP -PIs, and the cells alone, indicating that PIs have no effect upon cell membrane integrity. Similarly, PIs did not appear to inhibit the responsiveness of mast cells sensitised by VRS, since the antigen dose-dependent curve, observed in the preliminary optimisation studies was evident with both antigen preparations. Serotonin release from mast cells stimulated with greater protein concentrations +PIs (8μg/well) was slightly reduced when compared to cells activated with SWAP -PIs, but not to any significant degree. In conclusion, the presence of PIs in SWAP has no deleterious effect upon cell membrane integrity and/or cytoplasmic enzymes involved in the triggering of mast cell degranulation.

4.3.4. *Mast cell degranulation in response to three antigen preparations*

FcϵRI reactivity is largely specific for the ϵ-heavy chain of IgE. Thus, the presence of high affinity surface FcϵRIs on cultured RBL-2H3 cells provides an opportunity to determine the levels of schistosome-specific IgE in rat infection serum, by circumventing the problem of blocking antibody. As a consequence, release of serotonin from mast cells was negatively correlated with the dilution of sensitising antibody (figure 4.1a).

Mast cells were sensitised with rat infection serum from experiment 3 (section 3.2.2.), prior to stimulation with three soluble antigen preparations: SWAP derived from mixed-sex adult worms, female-only SWAP, and male-only SWAP. The

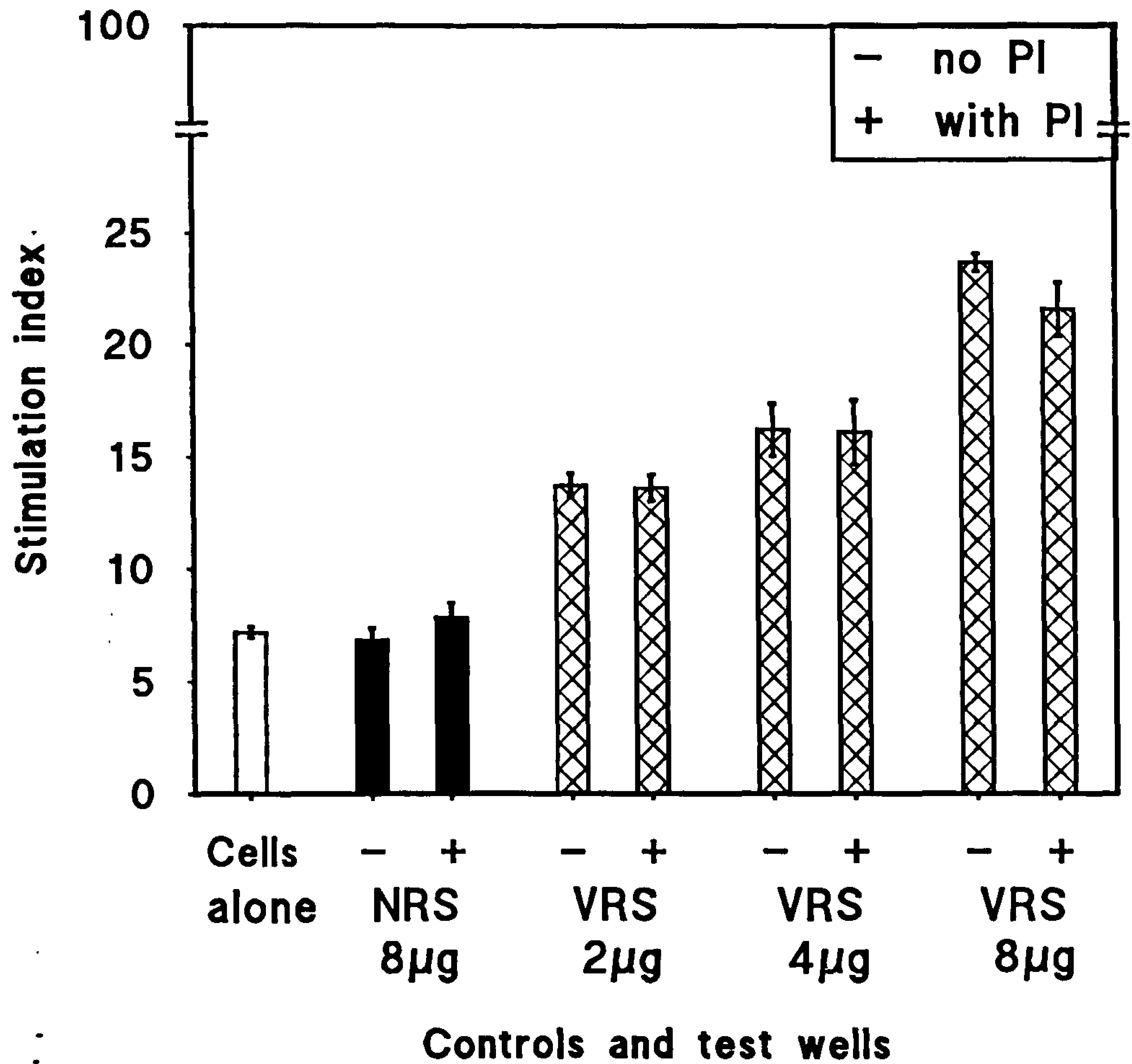


Figure 4.3 The ability of mixed-sex SWAP with or without protease inhibitors (PI; I and II), to trigger degranulation by sensitised mast cells. RBL-2H3 cells were primed with NRS and VRS, diluted to 1:40 with RPMI+.

serum dilution and protein concentrations were standardised for each well, as described above, and the intensity of mast cell degranulation measured for both test sera as a function of time post-infection (figure 4.4a–c). The basal levels of serotonin release observed with the cells alone and NRS negative controls reveal that mast cell degranulation is dependent upon the presence of schistosome-specific IgE in the sensitising rat serum. Each figure shows the enhanced ability of mixed-sex serum to activate cultured mast cells, compared to male-only serum. Generally, there is a significant difference in radiolabel release between the two test sera at days 28 ($p < 0.01$) and 35 ($p < 0.05$ and $p < 0.01$) post-infection, confirming the pattern of total serum IgE production characterised in the previous chapter. Furthermore, by day 42 both sera are equally effective in mediating mast cell degranulation. The degree of mast cell degranulation was enhanced when cells were sensitised with day 28 mixed-sex serum, indicating the expression of higher levels of schistosome-specific IgE at an earlier time-point, compared to male-only test serum.

Stimulation with mixed-sex antigen promoted a greater degree of serotonin release from activated RBL-2H3 cells (figure 4.4a), where the day 42 value was 27%, compared to 18% with female-only SWAP (figure 4.4b), and 16% with male-only SWAP (figure 4.4c). The response to male SWAP by mast cells sensitised with mixed-sex serum is limited in comparison to stimulation of these cells with the other preparations (figure 4.4c).

In addition, the data show that day 42 male-only infection serum can induce similar mast cell responsiveness to female proteins as day 42 mixed-sex serum, revealing the existence of shared epitopes between female and male antigens or, the delayed release of male antigens into the host bloodstream. Finally, the observation that mixed-sex SWAP generally mediates greater serotonin release than male-only or female-only SWAP, suggests that a combination of male and female allergens is necessary to stimulate an optimal effector cell response.

4.3.5. *The potential allergenic reactivity of carbohydrate moieties present in SWAP*

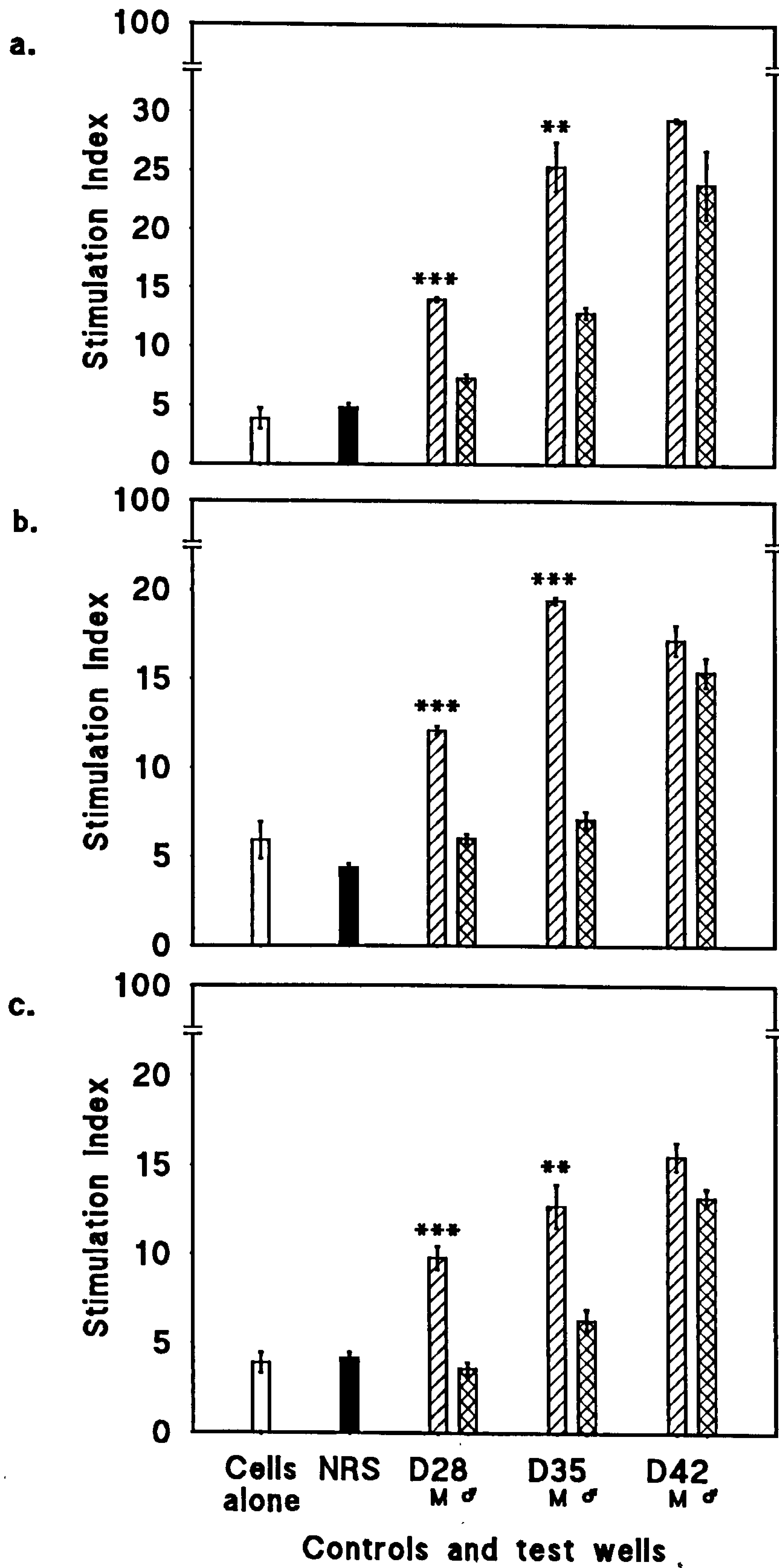
In the previous chapter it was suggested that the smeared appearance of western blots loaded with female-only SWAP compared to male-only SWAP, was consistent with the observation that sugars present in antigen preparations effect the specific binding of anti-schistosome immunoglobulins to parasite protein (Hames & Rickwood, 1990). Since the serotonin release assay is an indirect measurement of schistosome-specific IgE responses, as alluded to previously, the technique can be used to assess the ability of carbohydrate-depleted antigen samples to elicit

Figure 4.1a Tritiated serotonin release from RBL–2H3 cells sensitised with serial dilutions of vaccinated rat serum (VRS) and a 1:10 dilution of normal rat serum (NRS). Each well was stimulated with 4μg of mixed–sex SWAP, with the exception of the cells alone control.

Figure 4.1b The relationship between increasing concentration of mixed–sex SWAP (with protease inhibitors I and II) and the release of radiolabel from sensitised mast cells.

The results shown in both figures are means and standard errors (SE) of pooled test wells from one experiment. Each figure is representative of three replicate experiments performed.

Asterisks represent test values that are significantly different from the NRS control (** $p < 0.05$ and *** $p < 0.01$).


a.
b.
c.
Stimulation Index
100
30
25
20
15
10
5
0
***
**
Cells alone
NRS
D28
D35
D42
M ♂
Controls and test wells

schistosome-specific IgE-dependent mast cell degranulation. Treatment of complex antigen mixtures with sodium periodate results in the oxidation of single sugars, and has been used successfully to abrogate the carbohydrate reactivity of various schistosome antigen preparations (Dunne *et al.* 1992a). However, the technique involves particularly stringent conditions, creating two possible caveats. Firstly, could the protein be adversely affected by an extreme oxidative environment and secondly would the presence of trace amounts of sodium periodate inhibit degranulation by mast cells in this sensitive cell culture assay? To test the former, two samples of SWAP, one oxidised using 25mM of sodium periodate and the other untreated, were electrophoresed and stained for the presence of proteins (data not shown). The resolution of separated bands did not differ between treated and untreated antigen samples.

An assay was developed to evaluate the effectiveness of an oxidised antigen preparation in triggering serotonin release from cultured mast cells, compared to an untreated antigen mixture. Serum collected at day 42, following exposure of rats to mixed-sex and male-only cercariae, was used to sensitise mast cells prior to antigenic stimulation with the untreated and treated worm preparations. Equal protein concentrations of both mixtures were introduced to cultures of activated RBL-2H3 cells. The first bar in figure 4.5 shows that there was no adverse effect exerted upon the adherent mast cell population when cells were incubated for 15mins with 25mM sodium periodate alone. Basal levels of serotonin release were also obtained for the NRS controls. In addition, oxidation of the antigen samples did not appear to reduce/enhance the level of degranulation by the NRS-sensitised RBL-2H3 cell line. By contrast, mast cells sensitised with mixed-sex infection serum exhibited an approximate 2 fold reduction in mast cell responsiveness when stimulated with the oxidised preparation, compared to an untreated mixture ($p < 0.001$). Moreover, mast cells sensitised with male-only infection serum were not affected to the same extent, displaying no significant difference between treated and untreated test wells. If we assume that oxidation selectively depletes the carbohydrate content of SWAP, the observed decline in mast cell degranulation with treated SWAP may be attributed to modified immunoglobulin/sugar interactions.

4.3.6. *Mast cell western blots*

The final series of experiments addressed mast cell responsiveness to individual excretory/ secretory antigens, previously identified by western blotting in chapters two and three. The RBL serotonin-release assay provided an opportunity to assess

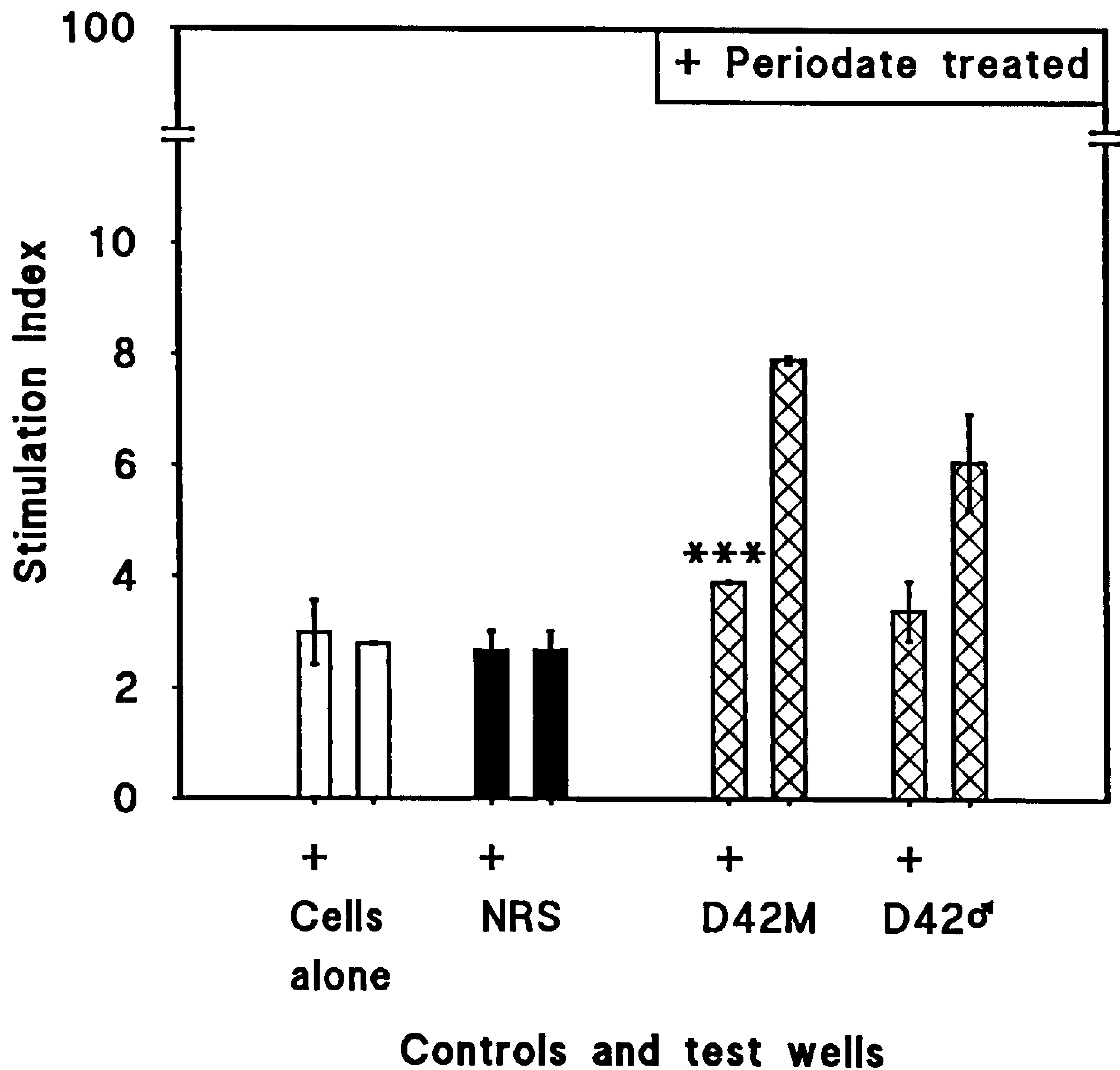


Figure 4.5 The stimulative effect of periodate-treated female SWAP upon the degree of serotonin release from mast cells, compared to the level of stimulation obtained with an unoxidised preparation. RBL-2H3 cells were sensitised with day 42 serum from mixed-sex (M) or male-only (♂) schistosome infections of rats.

Significant differences between untreated and treated wells for each test serum are represented by asterisks (*** p <0.01).

the relative allergenicity of a subset of parasite proteins. To evaluate each degranulative response, the whole SWAP was fractionated by electrophoresis. Initially, the direct electro-elution of proteins from SDS-PAGE gels was carried out in an attempt to isolate and purify specific regions of soluble antigen. However, no protein was recovered using this technique. An alternative methodology was considered, involving the electrophoresis of SWAP, followed by the electro-transfer of the separated proteins onto PVDF membrane and the careful excision of specific bands. Using this fractionation technique each potential allergen could in principle, be introduced to a population of sensitised mast cells.

To investigate the effectiveness of this latter approach a series of optimising experiments was designed using circular discs of PVDF membrane (Immobilon-P) blotted with increasing concentrations of whole mixed-sex SWAP. In addition, samples of PVDF membrane alone were also included, as a control for any toxic effects exerted upon the adherent cultured cells. The experiment was repeated on two separate occasions, each data point represents the mean of three replicates (figure 4.6). The amount of radiolabel released from cells alone was around 5%, in contrast to the extent of degranulation obtained with cells and Immobilon-P discs, which gave an approximate value of 11%, with extensive well to well variation. The result implies that toxic factors released by the PVDF matrix *in vitro*, either influence cell membrane integrity and/or intracellular signalling mechanisms by acting as a non-specific stimulator of mast cell degranulation (in a similar manner to calcium ionophores); both effects would induce non-specific efflux of serotonin. The control wells with cells and PVDF membrane alone now represented a new basal level of degranulation elicited by toxic factors, against which all other data was compared. Adherent cells sensitised with NRS and presented with schistosome protein attached to pieces of Immobilon-P, underwent extensive degranulation when compared to the preceding negative controls. This observation supports evidence for the toxic effect of PVDF membrane upon mast cells. It also shows that membrane loaded with antigen augments the degranulative response, although not significantly compared to the cells alone wells.

When RBL-2H3 cells sensitised with VRS were exposed to increasing concentrations of protein stimulus *in vitro*, the mast cell response rose accordingly. However, the mean test values were not significantly different compared to the NRS control, demonstrating a decline in sensitivity upon stimulation with antigen-coated Immobilon-P relative to the data presented in earlier sections. The extent of degranulation by pre-sensitised cells stimulated with 16μg/well was markedly

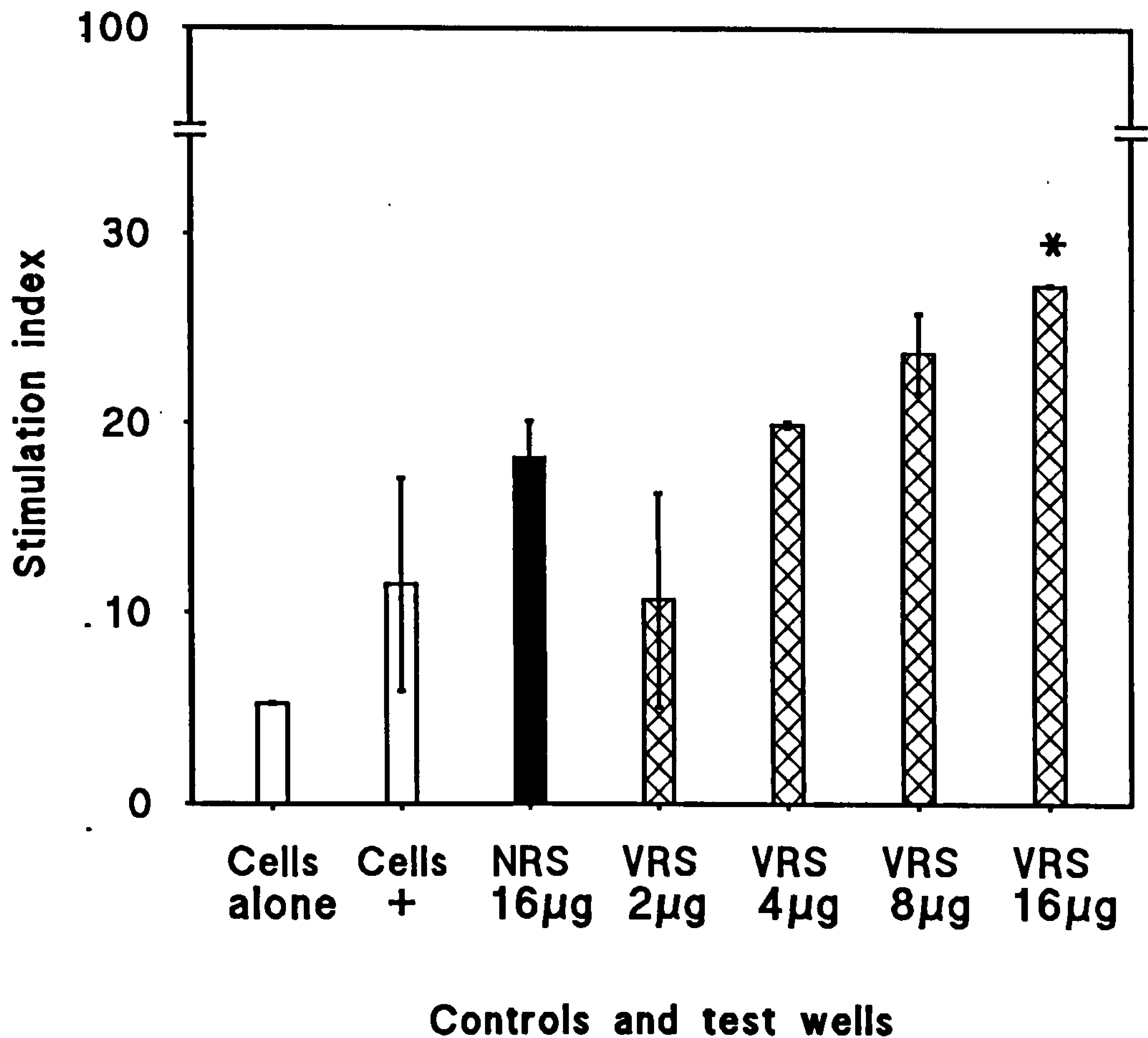


Figure 4.6 Levels of tritiated serotonin release from cultured mast cells stimulated with increasing concentrations of antigen-coated PVDF membrane. The relative toxicity of the membrane to the cells was measured by presenting discs of PVDF, without antigen, to RBL-2H3 cells alone (labelled cells +). Cells were primed with NRS or VRS diluted to 1:40 with RPMI+.

Significant differences between test wells and the NRS control are indicated by the asterisk (* $p < 0.05$).

greater than the negative serum control. Hence, effective allergenic stimulation of primed mast cells by antigen-coated membrane requires a protein concentration equal to or in excess of 16μg/well.

4.4. DISCUSSION

Traditionally, the measurement of specific-IgE titers in rat serum has been carried out using the passive cutaneous anaphylaxis (PCA) assay. This assay, while sensitive and IgE-specific, has the disadvantages of being laborious to perform, requiring the use of animals and large amounts of serum for sufficient replicates and doses to be tested. More recently reagents have become widely available for IgE ELISA. However, measurement of IgE in sera containing high levels of IgG can be a problem because of potential cross-reactivity between the anti-IgE reagents and IgG, and competition between IgE and high affinity IgG, particularly the IgG_4 isotype (Rihet *et al.* 1992), for antigen bound to the ELISA plate.

In the present study the optimisation of a rat basophilic leukemia (RBL) cell serotonin release assay was designed to replace the *in vivo* PCA assay and specific-IgE ELISA. This sensitive *in vitro* system has also been used to characterise mast cell responsiveness to a cocktail of schistosome antigens. Briefly, it was found that mast cell activation and degranulation was dependent upon the concentration of sensitising antibody, the strength of the allergic stimulus and the cross-linking of surface bound antigen-specific IgE. Finally, rat serum collected from animals exposed to mixed-sex cercariae was more effective in priming cultured mast cells to a subsequent antigenic stimulus, than male-only infection serum. Furthermore, mediator release from the RBL-2H3 cell line was potentiated when sensitised cells were stimulated with mixed-sex SWAP, compared to the degree of degranulation elicited by female-only or male-only SWAP, suggesting that a combination of male and female allergens triggered an optimal effector response.

4.4.1. *Validity of the serotonin release assay*

RBL cell lines are extensively used as *in vitro* models for mast cell and basophil function. The RBL-2H3 cell line used in this assay was derived from the sub-cloning of the high responder RBL-IV (HR^+) cell line (Barsumian *et al.* 1981). RBL-2H3 cells are easily maintained *in vitro* either in suspension or as an adherent monolayer, and have high affinity Fc epsilon receptors ($Fc\epsilon RI$) and cell

differentiation markers specific for mast cells expressed on their surfaces (Barsumian *et al.* 1981). Hence, it has been established that the RBL–2H3 cell line is functionally homologous to rat bone marrow–derived mast cells (BMMC). This propensity allows the subline to be used as a model with which to address fundamental questions about the nature of allergic reactivity to schistosomes in rats.

The RBL serotonin release assay has been used successfully, prior to this study, to quantify antigen–specific murine IgE as a direct correlate of the PCA assay (Kawabata and Babcock, 1993). Results obtained by Kawabata and Babcock from comparison studies, using 16 serum samples, showed a good correlation ($R^2 = 0.94$) between the RBL serotonin release assay and rat PCA.

In addition, it is now well established that mast cells and basophils carry on their surface receptors capable of interacting with IgG. In rats, IgG2a is considered to be involved in mediator release (Froese, 1984), with more recent evidence of this interaction provided by Benhamou and co–workers who confirmed that this cross–linked isotype was able to bind to, and activate histamine release, via the rat Fc∈RI on the RBL–2H3 cell line (Benhamou *et al.* 1994). The nature of mast cell activation, initiated by the cross–linking of immunoglobulin bound to FcR, appears to be more complex than supposed. The implication is that antigen–complexed IgG present in rat infection serum may compete with IgE for the Fc∈RI on RBL–2H3 cells and on mast cells *in vivo*. Furthermore, it has been reported that RBL–2H3 cells transfected with FcγRII, degranulate upon cross–linking with IgG (Daeron *et al.* 1995). However, an analogous role for this receptor has not been demonstrated *in vivo*. The data shown in the present study demonstrate that depletion of serum IgE, by heat–inactivation, has a dramatic effect upon mast cell responsiveness, resulting in a decrease in the ability of heat–treated VRS samples to prime mast cells (Kawabata and Babcock, 1993). It is concluded that antibody competition between IgE and IgG for the Fc∈RI is limited, and hence does not affect the RBL serotonin–release assay compared to the conventional ELISA. Thus, this technique provides a completely IgE–specific method of IgE quantification. Moreover the assay is sensitive detecting IgE in serum dilutions of 1:800 – 1:1000 (Cook *et al.* 1993) and requires serum volumes of only 10μl per sample.

4.4.2. *Mast cell activation*

As established, mast cells secure non–specific and specific IgE at their surface via the high affinity Fc∈RI. This process is known as activation or sensitisation. Cytofluorometric measurements of IgE and IgE receptors on individual,

microscopically identified mast cells, indicate that the peritoneal mast cells of normal, naive pathogen-free rats contain a significant number of IgE molecules with unknown specificity. On average 60-70% of the receptors available for binding are occupied by IgE in normal rats (Chen *et al.* 1992). During infection with the nematode worm *Nippostrongylus braziliensis*, associated with elevated serum IgE, increased numbers of IgE receptors were found on the surface of rat peritoneal cells 2-3 days following infection. This upregulation of Fc∈RI preceded the increased binding of serum IgE (40% on day 15 post-infection) to peritoneal mast cells (Chen & Enerback, 1992). This observation is consistent with the findings of Malveaux *et al.* (1978) who found that synthesis of the Fc∈RI was promoted and the surface density of receptors augmented upon elevated IgE production. However, results obtained in the present study show that despite the extensive binding of IgE to mast cells, RBL-2H3 activation and degranulation is limited. The levels of degranulation, expressed by the stimulation index, never rose above 29% implying that serotonin release is partial, or that not all mast cells respond to allergic stimulation. Since Chens' studies do not discriminate between non-specific and specific IgE the sub-optimal levels of serotonin release reported in this chapter could be explained by the binding of non-specific IgE to Fc∈RIs, limiting mast cell responsiveness to specific schistosome antigens.

The kinetics of the ligand-IgE interaction has also been shown to affect the extent of mast cell degranulation. The rate of ligand dissociation from aggregated IgE at the cell surface is slower than the rate of ligand dissociation from unclustered IgE (Posner *et al.* 1992). This data is further supported by a series of experiments carried out by Dr. Christine Pullar which show that a cultured population of RBL-2H3 cells only partially degranulates (a stimulation index of 25%) under optimal stimulation conditions, with a gradual reduction in serotonin release observed following the addition of increasing amounts of sensitising IgE to the medium (Dr. Christine Pullar, personal communication). An alternative explanation for this apparent saturation effect has been provided by Hide and co-workers (1993) who have shown that the release of inflammatory mediators from mast cells is an "all-or nothing" response. By stimulating permeabilised and non-permeabilised rat peritoneal mast cells with a calcium ionophore they demonstrated the existence of a threshold sensitivity, set at the level of individual cells. With a suboptimal stimulus, the population was comprised of totally degranulated cells or fully replete cells. Although the conditions used by Hide *et al.* (1993) involved non-immunological mechanisms of mast cell activation, the demonstration of partial exocytosis from a

population of RBL cells, following IgE cross-linking, could be indicative of threshold reactivity. However degranulation *in vivo* may be further complicated by the complex environment of the gut or liver. Tissue size and/or the presence of other IgE-binding inflammatory cell populations may mute the binding of IgE to mast cells and hence reduce the extent of IgE cross-linking upon stimulation with the appropriate allergens.

For the purposes of this study however RBL cells have been sensitised to schistosome allergens following incubation with rat infection serum containing parasite-specific IgE. The degree of activation was dependent upon the working dilution of sensitising serum (figure 4.1a) and hence the titer of IgE. Furthermore, the findings are in agreement with Chen and Enerback (1992) who found a correlation between elevated IgE titers and increased binding of IgE to peritoneal mast cells. At the molecular level, the apposition of two or more Fc∈RI initiates a calcium-dependent biochemical cascade, involving the phosphorylation of hydroxy amino acids and tyrosines, through topological modifications in the receptors themselves (reviewed by Beaven and Metzger, 1993). As the number of binding sites occupied by specific IgE rises, the probability of receptor aggregation increases, upon stimulation with the appropriate allergen. Hence, the extent of IgE-dependent mast cell degranulation is directly regulated by the number of cross-linked activation sites; it is the strength of the subsequent IgE-mediated intracellular signal transduction mechanism that determines the relative magnitude of the secretory response. As discussed, serum taken from rats infected with schistosomes was able to effectively sensitise RBL-2H3 cells prior to allergic stimulation, compared to the negligible capacity of NRS to activate cultured mast cells. This difference is related to the levels of schistosome-specific IgE in each of the sera. From chapter 3 it has been shown that the rat infection serum contained substantially greater titers of circulating IgE when compared to the background levels of IgE detected in the NRS. In addition, the elevated levels of serotonin- release exhibited by mast cells activated with serum collected from rats infected with mixed-sex worms, compared to male-only infection serum, can also be explained in terms of relative abundance of specific IgE. Titers of total serum IgE present in mixed-sex infection serum were shown to be two fold greater than the male-only serum samples at days 28 and 35 post-infection. It is apparent that the mechanism of mast cell degranulation requires a dynamic process of activation involving reaginic serum components. Moreover, exocytosis in this *in vitro* model is not triggered by an alternative IgE-independent event.

4.4.3. *Mast cell degranulation in response to schistosome allergens*

The RBL serotonin-release assay has provided evidence for the IgE-dependency of mast cell degranulation, and has been used as a quantitative measure of serum specific-IgE levels. However, the assay's potential as a test for the allergenicity of different antigens, with respect to infection, has been limited. Work presented recently by Birgit Helm, Sheffield, illustrated the boundless possibilities available to researchers in developing the RBL assay as a screen for sera, against which various allergens could be analysed (Dr. Birgit Helm, unpublished observations). Amongst the common antigens identified as effective allergens, such as cat dander and dust mite allergen (DerPI), were the schistosome cercarial proteases. A large amount of literature has accumulated demonstrating the allergenic capacity of parasite proteins. As yet it is impossible to interpret results from different laboratories since groups use a variety of techniques to purify allergens, different methods of antisera production and varied starting material. However, Horowitz *et al.* (1982) also determined the level of anti-schistosome IgE in mouse serum using the RBL degranulation assay described here. These authors used the assay for comparison of various groups of anti-sera collected from mice immunised with different antigen doses or adjuvant. They found that high levels of specific IgE were elicited against a schistosome cercarial sonicate, and concluded that the assay was an effective method of quantifying anti-schistosome IgE reactivity in the presence of antibody of other classes. Hence, the adaptation of the serotonin release assay has provided a sensitive standard with which defined or prospective parasite antigens can be investigated.

As described in this chapter, mast cells sensitised with rat infection serum degranulate readily in response to small amounts of soluble schistosome material. It may be concluded that crude adult worm preparations act as effective inducers of IgE-mediated mast cell reactivity. As elevated titers of total and specific IgE are associated with mixed-sex schistosome infections, the objective was to demonstrate the allergic potency of mixed-sex antigen preparations relative to male-only proteins. The experiments were performed keeping the sensitising serum dilution constant whilst varying the nature of the antigenic stimulus. In this chapter I have shown that the extent of exocytosis by RBL-2H3 cells was enhanced in response to mixed-sex SWAP. Lower, though significant, levels of degranulation were induced upon addition of female-only SWAP to pre-activated RBL cells. Thus, since there was no evidence of egg-deposition in rats infected with mixed-sex schistosomes, the data suggests that proteins released by female parasites constitute a major stimulant of anti-parasite effector responses. It appears that the presence of mature

female worms during an infection enhances IgE production through the release of unique proteins or greater quantities of allergen. As anticipated, the stimulation index values were markedly lower following stimulation of RBL-2H3 cells with male-only SWAP than results obtained with the mixed-sex and female-only SWAP, thus demonstrating reduced ligand content or allergenicity.

As discussed, the probability of FcεRI aggregation at the mast cell surface is promoted via the cross-linking of anchored IgE to appropriate parasite allergens. This interaction is further augmented if the strength of the allergic stimulus is increased. The rate of allergen release by female worms must be considered when attempting to rationalise this data. Antigen secretion by female parasites may be more rapid, supported by the appearance of an early positive response to mixed-sex and female SWAP from day 28 post-infection, compared to male-only SWAP. This former observation could be attributed to the earlier development of female worms, for example their enhanced capacity to ingest blood cells relative to their male counterparts (Lawrence, 1973). Alternatively, there may be qualitative or quantitative differences in the array of proteins released by adult female worms. Hence, recognition of mixed-sex infections by the rat's humoral immune response would be more rapid and the magnitude of antibody production enhanced, in contrast to unisexual infections. As a consequence, worm attrition would occur earlier inducing a further augmentation of antibody production as mixed-sex parasites degenerated and released large quantities of somatic antigens into the host bloodstream. There appears to be no recognition of this gender-specific immune phenomenon by other researchers. However, Ritchie and colleagues did record the relative growth and survival of male and female worms in Sprague-Dawley rats and showed that the natural defences of the rat appeared more effective against female worms (Ritchie, Garson & Knight, 1963).

Evidence for elevated levels of glycoproteins in female SWAP, compared to male SWAP, was demonstrated following western blotting studies described in chapter three. Upon probing female antigen with mouse infection serum an increase in the intensity of background staining was observed, relative to the appearance of the male-only immunoblots probed with identical sera. R. Harrop (unpublished observation) had reported this smearing effect in western blotting studies of cercarial protein; he suggested that the increase in indiscriminate non-specific binding was due to the presence of oligosaccharides in the soluble protein preparations. Since carbohydrate does not possess the same electrophoretic properties as proteins under denaturing conditions, glycoproteins do not form discrete banding patterns.

Antibodies from the infection serum bind to this heterogeneous mixture of carbohydrate and, since the method of detection is effectively limited to the identification of proteins, antibody bound to sugar moieties presents a less distinct pattern of binding (Hames & Rickwood, 1990). If it is assumed that this conjecture is correct, we may suppose that female worms contain/release an abundance of carbohydrate epitopes, compared to male-only infections. Thus mixed-sex schistosome infected mice/rats will produce higher titers of immunoglobulin against these products.

To determine the role of sugars, in the triggering of an effector cell response, female-only SWAP was treated with sodium periodate, which oxidises the carbon-carbon linkages of single sugars and alters the carbohydrate structure. These oxidised preparations have been used to stimulate degranulation of mast cells *in vitro*. By modifying the oligosaccharide epitopes the potential of the treated samples to stimulate serotonin release was halved. Moreover, mast cells incubated with serum from unisexual cercarial infection exhibited a reduction in exocytosis, but not to the same degree as RBL cells activated with serum from a normal, bisexual schistosome infection (figure 4.5). It is apparent that rats infected with male-only schistosomes produce lower levels of anti-oligosaccharide antibody than rats infected with mixed-sex parasites. Thus, it may be surmised that carbohydrate released by adult female parasites is able to provide an effective allergic stimulus during infection. In a clinical study of schistosomiasis, Dunne *et al.* (1992a) found that alteration of the carbohydrate epitopes in oxidised SWAP and SEA, did not compromise IgE reactivity against treated preparations, as measured by ELISA. However, any correlations made between the data presented here and Dunne's study are only putative, since rat and man represent separate models of schistosomiasis.

As far as is known, monomeric IgE alone does not trigger mediator release. Release occurs when IgE receptor molecules undergo cross-linking. The cross-linking may be produced by multivalent antigen and IgE, by aggregated IgE, by IgE and bivalent anti-IgE, or by bivalent anti-receptor antibody (Metcalfe, Kaliner and Donlon, 1981). In addition, lectins such as ricin, wheat germ agglutinin, or branched sugars such as dextran, which are known or suspected to bind to the sugar moiety of the receptor, probably stimulate histamine release by this mechanism (Kazmierczak & Diamant, 1978; Metcalfe *et al.* 1981). Oligosaccharide groups expressed on polypeptides determine, to some extent, the definitive structure of glycoproteins, for example intracellular matrix/adhesion proteins. The carbohydrate moieties are often repeated along the length of the proteins and therefore present multivalent targets for

the mast cell-bound IgE. The N-linked carbohydrates are prediposed to recognition by IgE, since the oligosaccharide structure is formed into open branches. In contrast, the O-linked sugars are aligned in a string of monosaccharide residues which are less accessible for specific antibody binding. Workers studying the olive tree pollen (Ole e I; Batanero *et al.* 1994) and the wheat and barley allergens (CM16 and CMb; Sanchez-Menge *et al.* 1992) have ascribed much of the IgE-reactivity to the expression of carbohydrate moieties. Both research groups have shown reduced binding capacity of sensitising IgE from human serum to the deglycosylated forms of both these glycoproteins in ELISAs, compared to the reactivity demonstrated against the native molecules. The affinity of IgE for Ole e I was further attributed to the epitopes defined by N-linked carbohydrates (Batanero *et al.* 1994). Similarly, in the present study it has been suggested that carbohydrates and/or glycoproteins contribute towards stimulating mast cell degranulation. Thus, successive investigations should focus on the characterisation of sugar determinants, in addition to protein/IgE interactions.

The investigations discussed above have concentrated upon the release of serotonin from cell populations stimulated with complex mixtures of soluble adult worm proteins. The definitive goal of my study was to identify individual allergens, by molecular weight, from a cocktail of schistosome components. Initially unfractionated mixed-sex SWAP was transferred onto PVDF membrane (section 4.2.7.) and regions of protein-coated blot cut out and added to an adherent population of serum-activated RBL-2H3 cells. A simple concentration (dose)-response pattern was not established at low protein concentrations. It appeared that PVDF membrane exerted a toxic effect upon sensitised and unsensitised mast cells inducing partial exocytosis and masking IgE-dependent reactivity. Furthermore, it is possible that the polarised presentation of antigen spatially inhibited optimal IgE-ligand interaction, as aggregation of FcεRIs was restricted to the upper surface of the adherent cells. In contrast, presentation of soluble material to mast cells promotes effective binding of allergen to cultured RBL-2H3 cells at all available surfaces.

Solutions, such as those adapted for T cell western blots should be investigated, for example DMSO-dissolved membrane or finely cut pieces of antigen-coated blot. These techniques would promote and maintain intimate contact between the PVDF membrane and activated cells. However, the use of detergent should be sparing and the sample subject to thorough washing prior to use in the cell assay.

In summary, the RBL serotonin–release assay has been used successfully to: a) investigate the nature of the IgE–dependent effector cell response, b) quantify schistosome–specific IgE levels in rat infection serum, c) assess the relative allergenicity of various antigen preparations. The technique is simple to perform, reliable and reproducible, however its potential as an investigative tool has not been exploited until recently. Nevertheless, care must be taken in extrapolating the conclusions drawn from *in vitro* data to the situation *in vivo*, since the *in vitro* model is an over–simplified, artificial system. Further study involving the infection of mast cell deficient rats (W^sW^s) with *S. mansoni*, and the abrogation of stem cell factor (SCF), a cytokine connected with the maturation of mast cell progenitors, should provide insights into the direct role of mast cells in worm elimination.

CHAPTER FIVE

Concluding Discussion

It is well documented that the inbred laboratory rat is able to expel adult *S. mansoni* worms from the hepatic portal system around day 28 post–infection onwards. The prominent features of this infection in rats are the elevated production production of IgE, both specific and non–specific (Capron *et al.* 1992), and the manifestation of a pronounced hepatic mastocytosis, coincident with worm elimination (Miller *et al.* 1994). The concomitant timing of these events suggests that adult worm attrition could be a consequence of IgE–mediated mast cell activation and degranulation. Thus, the work presented in this thesis has focused upon the participation of IgE and IgE–dependent mast cell degranulation in mediating parasite clearance from laboratory rats.

Furthermore, since it is apparent that the schistosome stimulates these specific responses, and that this reactivity is directed at the live parasite, the present study has attempted to characterise the material released from healthy adult schistosomes. Moreover, in view of the enhanced production of schistosome–specific IgE during infection it was of interest to identify the allergenic components of the adult parasite. In the ensuing discussion I will outline the salient features of this study and include ideas for future work. Finally, I shall speculate upon the role of IgE and mast cells in mediating parasite attrition in the experimental rat model.

The IgE–mediated elimination of schistosomes from rats

The working hypothesis used in this study was that mast cell–bound antibodies of the IgE class participate in the elimination of a primary schistosome infection from rats. To test this hypothesis experimentally, rats were exposed to *S. mansoni* cercariae of one sex (male), or of both sexes (mixed–sex). Blood samples were collected at weekly intervals from day 21 to 42 post–infection, thereby permitting the quantitation of serum IgE and RMCP II levels by ELISA, and determination of antigen specificity by western blotting (the characterisation of the allergic response will be discussed later in this section). By counting the number of adult worms recovered, following portal perfusion of both experimental groups, it was possible to correlate the extent of parasite elimination with levels of IgE and mast cell degranulation *in vivo*.

All infected rats showed elevated titers of specific and non–specific IgE, relative to naive animals, as detected by two independent assays: 1) IgE ELISA using anti–rat IgE mAb (Chapter three), and 2) a functional assay based on antigen–specific degranulation of RBL–2H3 cells, determined by ^{3}H–serotonin release (Chapter four). The level of IgE antibody in the mixed–sex parasite infection serum was high,

almost twice the level of specific IgE antibody in serum from male-only schistosome-infected rats at days 28 and 35 post-infection. It should be emphasised that although these data relate to serum IgE they probably indicate a concomitant high level of mast cell-bound IgE which might contribute towards the protective effect.

The major discrepancy, noted in Chapter three, was the lack of correlation between all three variables measured, namely IgE reactivity, levels of systemic RMCP II, and the extent of parasite death. Enhanced titers of total serum IgE detected in mixed-sex schistosome-infected rats did not appear to result in greater degrees of worm attrition as measured by the number of parasites recovered, compared to unisexual infection. The most significant difference in IgE levels between the experimental groups occurred at day 35 post-infection. Thus, the more rapid, augmented allergic response generated by mixed-sex infections could have important repercussions upon the timing of worm elimination. Conversely, it is also plausible that small quantities of IgE could be equally effective as enhanced levels of IgE in mediating expulsion. Furthermore, serum IgE is not a direct indicator of protective antibody, since free immunoglobulin is unlikely to exert a cytotoxic action upon liver worms.

Since RMCP II levels fall as serum IgE increases it may be inferred that IgE production does not reflect the state of activation of tissue mast cells. Similarly, the presence of systemic RMCP II does not necessarily indicate the degree of hepatic mast cell degranulation as the protease may be sequestered locally in the tissue after release. Thus, the ability of IgE to sensitise effector cell populations may be a more reliable indicator of schistosome-specific IgE reactivity and mast cell activation. Cytofluorometric analysis of Fc∈RI$^+$ cells in the tissue of interest would provide an ideal experimental approach, based upon studies by Chen and Enerback (1992). However, the levels of RMCP II released *in vivo* from degranulating mast cells do not negate a role for this effector cell in mediating worm expulsion (Chapter three). Thus, the technique described above would confirm this observation and provide an accurate evaluation of cytophilic IgE.

Using the RBL-serotonin release assay (Chapter four) it was possible to quantify parasite-specific IgE as a direct correlate of schistosome-specific mast cell degranulation *in vitro*. Mast cell degranulation was promoted upon pre-incubation of the RBL-2H3 cells with increasing concentrations of infection serum, prior to stimulation with schistosome antigen (SWAP). However, in the absence of specific IgE, mast cells did not release their contents. Thus, it was concluded that mast cell

activation and subsequent degranulation was dependent upon the quantity of sensitising IgE and the aggregation of specific IgE by parasite allergens.

The results obtained in Chapter four revealed that mast cells were primed with very low levels of sensitising serum, with serotonin release eventually reaching a plateau upon the addition of increasing amounts of antibody. Thus, it is suggested that mast cells are readily activated by IgE–cross–linking and that they operate at a low threshold of sensitivity. Alternatively, since the infection serum only contains 10–20% specific IgE (Rousseaux–Prevost *et al.* 1978), the observed plateau may simply reflect the steric hindrance of parasite–specific IgE binding by overwhelming titers of non–specific antibody.

Since serotonin release is a direct measure of parasite–specific IgE levels this assay was used to quantify the levels of specific IgE in the serum from rats infected with mixed–sex or male–only cercariae. As anticipated, mast cells pre–incubated with mixed–sex schistosome infection serum released more serotonin upon the addition of SWAP than cells sensitised with male–only schistosome infection serum. Over the longitudinal time course, the parasite–specific IgE levels increased from day 28 to 42 post–infection. This pattern of production supports the data obtained from the IgE–ELISAs performed in Chapter three. In fact, the schistosome–specific reactivity in mixed–sex serum, as determined by RBL–serotonin release assay, was raised above the level of that obtained for male–only infection serum at days 28 and 35, reinforcing earlier observations.

To address the issue of IgE–dependent immunity in rats a number of experimental approaches can be undertaken. Histological examination of infected rat tissues would provide further information regarding the time of influx, location, and size of the infiltrating effector cell population(s). Of interest is a paper by Kermanizadeh, Hagan and Crompton (1995) who describe the selective staining of eosinophil and mast cell populations in the livers, intestines and spleens of mice. At a molecular level, Bell and Issekutz (1993) have shown that suppression of the α–4 integrin family of adhesion molecules by mAbs, injected into rats following exposure to *Trichinella spiralis*, significantly reduces the expression of immunity. Theoretically it should also be possible to produce a mAb directed against those molecules that are involved in mast cell/eosinophil recruitment, for example VLA–4 and VCAM (Das *et al.* 1995). Targeting of such ligands/receptors would permit dissection of the cells' role in protective immunity. In addition the mAb could be used to observe the effect(s) of abrogating target cell function during the induction phase of infection, or late following cell recruitment to the site of inflammation. Finally, investigations to

elucidate the *direct* role of mast cells would be possible by ablating the growth factor for mast cells (SCF) using a polyclonal sheep anti-rat SCF antibody or, by infecting Ws/Ws (mast cell deficient) rats and their syngeneic littermates with *S. mansoni* cercariae.

The secretory antigens and allergens of adult worms

Successful vaccination against parasites not only requires the induction of a protective immune response, but is also contingent upon the nature of the antigen selected. Most research has concentrated on the identification of parasite antigens which are the targets of putative humoral protective responses. The target antigens of these responses often display similar characteristics. For example, many schistosome surface antigens are glycoconjugates in which the carbohydrate portions are most immunogenic , while heavily gamma or UV irradiated cercariae appear to stimulate antibodies recognising polypeptide epitopes (Omer-Ali *et al.* 1986). The majority of the well-defined antigenic targets of protective humoral immunity appear to be on the migratory stage of the parasite, the skin-stage or lung-stage schistosomulum. The adult worm, once established, appears to represent a relatively immunologically inert target, reflecting the capacity of this stage to evade surface-directed antibody-dependent effector mechanisms, an adaptation needed to maintain chronic infection. However, by culturing adult worms *in vivo,* as described in Chapter 2, it is evident that the mature parasite secretes/excretes 12 dominant antigenic moieties, ranging in Mw 14-208 kDa. More importantly, 4 of these molecules have been immuno-localised to the tegumental membrane of the worm. Excretory/secretory antigens may offer a distinct advantage for immunisation, in that parasites may not have evolved mechanisms for evading immune responses directed against them. The use of antigens released from the membrane for immunisation purposes would polarise an antibody-dependent response against the vulnerable host-parasite interface.

It was proposed that if the schistosome-specific IgE response was involved in parasite elimination, then it would be of interest to define the molecular targets of the reaginic antibody. Rat infection serum which had been affinity-purified for IgE was used to probe fractionated soluble SWAP by western blotting; two allergenic moieties were identified by this method, with Mw of 67 and 36-38 kDa. The 67 kDa antigen appeared analogous to a molecule of the same molecular weight detected by WTS, thus indicating that it was secretory in nature. However, the 36-38 kDa antigen was not detected by the WTS. In addition the IgE from mixed-sex

schistosome infections appeared to bind strongly to this fraction, in comparison to the relatively weak IgE response exhibited by male–only infection serum. This observation could be attributed to the enhanced release of the 36–38 kDa product from female parasites, or the unique expression of this moiety by female schistosomes. As the male worm induces an IgE response against the 36–38 kDa epitope this latter explanation seems unlikely.

Further support for the relative allergenicity of female worms compared to male worms was provided by the RBL–serotonin release assay (Chapter four). Using this assay the extent of serotonin release was correlated with the amount of allergen introduced to the sensitised mast cells *in vitro*. Thus, since the data showed that female SWAP was a more effective trigger of mast cell degranulation than male–only SWAP, for equal amounts of protein, it was confirmed that female parasites contained more allergens than their male counterparts.

Of further interest was the demonstration of increased background staining on the female–only SWAP western blot, compared to the lanes loaded with male–only SWAP. As carbohydrate does not form discrete banding patterns when separated by SDS–PAGE this heterogeneous specific antibody reactivity was ascribed to the presence of immunogenic sugar moieties. To investigate this supposition further, periodate–treated and untreated SWAP were used to stimulate degranulation by sensitised mast cells as an indication of their relative allergenic capacities. The findings demonstrated that the modification of carbohydrate structure by periodate–oxidation significantly reduced the extent of serotonin release by mast cells, compared to stimulation with untreated SWAP. The sensitivity of the parasite epitopes to periodate confirmed the carbohydrate nature of the allergenic molecules. These observations are in agreement with Pierce and co–workers (1983) who found that most of the allergenic material released from adult worms cultured *in vitro* was bound by Con A–sepharose, thereby indicating that the major allergens were glycoproteins. Further characterisation of the oligosaccharides could be performed by incubating female SWAP with either N–, or O–deglycosidase enzymes.

Man and rat

The relevance of the experimental rat model to human immunity has been tentative until recently. However, two series of studies in human populations have demonstrated a prominent, if not conclusive role for IgE–mediated effector mechanisms in the expression of acquired immunity against schistosomes in man. Observing the rate of reinfection after treatment in a community exposed to *S.*

haematobium in the Gambia, Hagan and his coworkers found that in subjects over the age of 15 years a lack of reinfection was positively correlated with the existence of a specific IgE antibody response to schistosome antigens (Hagan *et al.* 1991). Similarly, an investigation on the rate of reinfection of a study population in Kenya demonstrated that acquired immunity to *S. mansoni* was positively correlated with IgE production, and that protective IgE was directed against adult worm proteins (Dunne *et al.* 1992b). Furthermore, susceptibility to reinfection was associated with the expression of so-called 'blocking' antibodies, mainly of the IgG4 isotype, which exhibited the same antigenic specificity as protective IgE antibody (Demeure *et al.* 1993; Rihet *et al.* 1992). Since it appears that similarities exist between IgE-mediated parasite expulsion in rats and the expression of acquired immunity in human schistosomiasis it seems reasonable to suggest that studies using rats as experimental models could shed light upon the phenomenon of immune resistance to schistosomes in human populations.

The general biological significance of anaphylactic antibody responses have only been considered, for the most part, in the context of allergy. The continuous existence of IgE in phylogeny together with the results presented in this study, and data on clinical schistosomiasis, appears to implicate IgE as an important factor in protective immunity against parasites. However, the dangers in implementing a vaccine that preferentially selects an IgE-mediated effector response are numerous. By stimulating allergic responses, augmentation of inappropriate IgE reactivity to environmental antigens might be possible. The immunopathological consequences of initiating such a response far outweigh its protective capacities. Nevertheless, work has shown that protective IgE and IgA effector responses induced in rats by a single dose of recombinant Sm28GST confer significant resistance (up to 60%) against homologous challenge (Grezel *et al.* 1993).

In conclusion, since it appears that the requirements for mast cell activation are fulfilled during infection, namely the concomitant rise in IgE levels, the recruitment of mast cells to cellular foci adjacent to the impacted worms, it is implied that IgE-mediated mast cell degranulation could represent an important schistosomicidal mechanism, especially in view of the timing of these events. Additionally, this study has shown that cross-linking of mast cell-bound IgE appears to be dependent upon the oligosaccharide content of worm products. Since a direct role for mast cells in evoking parasite attrition has not been demonstrated conclusively, prospective work should address this issue. Finally, it is hoped that if worm expulsion is mediated by

humoral immunity, the 67 and 36–38 kDa allergens could represent potential vaccine candidates in rats.

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