IMMUNOCHEMICAL AND BIOCHEMICAL ANALYSIS OF LARVAL SECRETED ANTIGENS FROM THE PARASITIC NEMATODES ASCARIS SUUM AND ASCARIS LUMBRICOIDES

THESIS
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BY

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PREFACE

This thesis consists of five chapters and includes part or all of the content of three manuscripts which have been published with one in preparation. Apart from a detailed general introduction, each chapter contains a short, specific introduction and summary.

References do not accompany each chapter and are presented together in a Reference section. Materials and Methods are also presented in a separate and clear chapter to avoid the need for repetition.

The figures presented in this thesis are represented by single numerals prefaced with the chapter number for instance Figure 1 of chapter 2 will be shown as Fig. 2.1.

AUTHORSHIP STATEMENT

This is to certify that this thesis is entirely the work of F. Qureshi and that he made a significant contribution to the scientific content and composition of joint papers that have been published or are in preparation.

Dr. M.W. Kennedy

ABBREVIATIONS

ABF	-	Adult *A.suum* body fluid
AL	-	*A.lumbricoides*
AS	-	*A.suum*
Cht or Cht A	-	Chymotrypsin A
Con A	-	Concanavalin A
CTMC	-	Connective tissue mast cells
GS-I	-	*Griffonia simplicifolia* lectin
HPA	-	*Helix Pomatia* agglutinin
IMMC	-	Intestinal mucosal mast cells
IVRS	-	*In vitro* - released secretions
L2	-	Developmental stage of Ascaris nematodes following first moult
L3/4	-	Heterogenous mixture of larvae following the second or third moults
Lch	-	*Lens culinaris* lectin
M a AS	-	Serum antibodies from *A. suum* - infected mice
M a Tcn	-	Serum antibodies from *T. canis* - infected mice
Mc Ab	-	Monoclonal antibodies
Mr	-	Relative apparent molecular weight (estimated by SDS-PAGE)
NMS	-	Serum from normal (uninfected) mice
NR	-	Non-reducing electrophoretic conditions (on SDS-PAGE)
N Rab S	-	Serum from normal (uninfected) rabbits
N Rat S	-	Serum from normal (uninfected) rats
PBS-A	-	Phosophate buffered saline (pH 7.5)
PCA	-	Passive cutaneous anaphylaxis
PNA	-	*Arachis hypogea* lectin
R	-	Reducing conditions in SDS-PAGE

Rab a ABF	-	Serum raised in rabbits against adult A.suum body fluid
Rab a AL	-	Serum antibodies from A.lumbricoides - infected rabbits
Rab a AS	-	Serum antibodies from A.suum - infected rabbits
Rab a Tcn	-	Serum antibodies from T.canis - infected rabbits
Rab a 14000	-	Serum antibodies from rabbits immunized against the Mr 14000 component of A.suum adult body fluid
Rat a AL	-	Serum antibodies from A.lumbriocoides - infected rats
Rat a AS	-	Serum antibodies from A.suum - infected rats
RIP	-	Radio-immunoprecipitation
RMCP I	-	Rat mast cell proteinase type I (mainly from CTMC)
RMCP II	-	Rat mast cell proteinase type II (mainly from IMMC)
SDS-PAGE	-	Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis
s/n	-	Supernatant
SOD	-	Superoxide dismutase
SRIP	-	Staphylcoccus aureus - mediated, radio-immunoprecipitation
UEA-I	-	Ulex europus agglutinin
WGA	-	Wheat germ agglutinin

GENERAL SUMMARY

There is a relatively small amount of experimental data regarding the *in vitro* - released secretory products (IVRS) of the parasitic roundworm nematodes of man and swine, *Ascaris lumbricoides* and *Ascaris suum* respectively. The importance of detailed biological examination of the IVRS from these nematodes is justified by the relative inadequacy, for serodiagnosis or prophylaxis, of alternative sources of nematode-derived material: for instance, cuticular surface molecules, or worm homogenates.

Here the IVRS from L2 and L3/4 stages of *Ascaris lumbricoides* and *Ascaris suum* were examined for the first time both immunochemically and biochemically to evaluate their usefulness for specific detection of ascariasis and for possible use in vaccination of animals or humans against Ascaris infection. The main techniques used involved the extrinsic (^{125}I) or intrinsic (^{35}[S]-methionine) labelling of parasite secretions followed by precipitations with either lectins or immune sera and final analysis of the precipitated nematode products by SDS-PAGE. Alternatively, radio-iodinated IVRS from *A.suum* were incubated with various proteinases and the products of the reactions also analysed by SDS-PAGE.

The first studies of radio-iodinated *A.suum* IVRS showed that the range and number of worm products were different as the parasites developed from the L2 stage to lung stages (L3/4) and that there was considerable, but incomplete stage-specificity of the repertoire of secretions produced. *S.aureus*-mediated radio-immunnoprecipitations (SRIP's) of *A.suum*-immunised rabbit antisera and *A.suum* IVRS showed that, except for one product from *A.suum* L3/4 IVRS, all the secretions of *A.suum* L2 and L3/4 worms are antigenic. The non-antigenic

component of L3/4 products was identified as rabbit serum albumin. Antisera from A.suum-immunised mice and rats also precipitated components of larval IVRS in SRIP's but different distinct sets of antigens from those recognized by infected rabbits. One possible reason for these differences could be attributed to the separate genetic composition of each species. Mouse anti-A.suum antisera failed to immunoprecipitate an Mr 14000 component of A.suum larval secretions, which was also found to be the major antigen of adult A.suum body fluid (ABF). As ABF is a commonly used source of material for immunological analysis of Ascaris infection, it could be advisable to look for alternative sources of target antigens that avoid an undue bias towards the Mr 14000 product.

Immunological crossreactions have been a major stumbling block for specific detection of nematode parasite infection of humans and animals. One of the ways in which crossreactions could lead to false positive results in serodiagnostic immunoassays is the possible occurence of similar antigens among the products of different species of parasites. The secreted products of A.suum and A.lumbricoides were examined by radio-iodination followed by SDS-PAGE.

The results showed that the secretions of larval stages of both species were very similar in SDS-PAGE profile except that IVRS from the larvae of the human roundworm apparently contained a secretion of Mr 17000 that was unique to these. SRIP's with sera from rabbits infected either by A.suum or A.lumbricoides and ^{125}I-labelled IVRS of the larval stages of these parasites followed by examination of the immunoprecipitates by SDS-PAGE, showed that there was complete crossreactivity of a given antiserum of one species and the antigens of the other, including the Mr 17000 antigen of A.lumbricoides. Additionally there was also extensive crossreactivity of antiserum from rabbits infected with the ascaridoidean parasite Toxocara canis

and the secreted antigens of both A.suum and A.lumbricoides. These findings advised against the use of IVRS from Ascaris species in possible serodiagnostic assays or in serological tests to differentiate between A.suum and A.lumbricoides as separate species. However it might be possible, by raising monoclonal antibodies (McAb) against Ascaris IVRS to find McAb's that differentiate between the Ascaris species and/or provide specificity in serodiagnostic assays.

Radio-iodination of IVRS from Ascaris species involved material collected from the supernatants of worm cultures in vitro, not necessarily representative of the products of living worms, but could have consisted of worm somatic components. One biochemical method that aid in confirming that, for the most part, living worms synthesised the IVRS is the technique of intrinsic (or metabolic) labelling of Ascaris larval products. A.suum and A.lumbricoides larval stages were labelled with the radioactive amino acid ^{35}[S]-methionine (^{35}[S]-met) and the products examined by SDS-PAGE alongside corresponding radio-iodinated secretions. The range in molecular weight and the quantity of intrisically labelled secretions exceeded those of ^{125}I-labelled IVRS and certain components of radio-iodinated IVRS were not apparently labelled by ^{35}[S]-met. These observations could be attributed to the bias of each labelling technique to certain amino acid and the heterogeneous distribution of these amino acids among the secreted glycoproteins of Ascaris worms. SRIP's of intrinsically labelled IVRS and sera from Ascaris infected rabbits, followed by SDS-PAGE analysis confirmed that the parasite secretions were antigenic. Additionally, it was found that an ^{35}[S]-met labelled Mr 17000 antigen of A.lumbricoides larvae was absent from the intrinsically labelled IVRS of A.suum and A.lumbricoides. However SRIP's of both sets of IVRS with antisera from rabbits infected with either the human or the porcine parasite showed that there was total reciprocal

crossreactivity. Moreover, SRIP's of ^{35}[S]-met labelled Ascaris IVRS and antiserum from T.canis infected rabbits showed that there was extensive crossreactivity. The results of intrinsic labelling, overall, confirmed that Ascaris larval stages actively manufacture and secrete antigenic proteins into the culture medium and support the results of immunochemical analysis of ^{125}I-labelled antigens.

Part of the intestinal immune response, in nematode infected animals involves the increase in production of intestinal mucosal mast cells (IMMC). It is possible that mast cell products could affect nematode secretions or surface molecules. This possibility was examined by incubating radio-iodinated antigens of A.suum L2 and L3/4 stages with one of the proteinases of rat IMMC, namely, RMCP II (which may be significant in expulsion phases of intestinal nematodes) and examining the products of the reaction by SDS-PAGE. Additionally, the radioactive worm antigens were incubated with a proteinase from rat mast cells of connective tissue (CTMC), namely RMCP) I which migrating nematodes could encounter in early migratory stages. The results of the enzyme digests showed that there was only limited proteinolysis of IVRS thus mitigating against the possibility that the mast cell proteinases would exert a direct effect on A.suum secretions in vivo. The use of other site specific enzymes, namely trypsin and chymotrypsin, on A.suum larval secretions showed that some components were apparently resistant to cleavage by these enzymes. The reason for resistance to proteinolysis is unknown, although it could be due to some feature of conformation and/or amino acid sequence of the nematode antigens. None of the secreted antigens of A.suum were resistant to cleavage by pronase, confirming that all are proteinaceous.

Further examination of ^{125}I-labelled L3/4 IVRS by precipitation with Sepharose-bound lectins and examination of the products by SDS-PAGE confirmed that the IVRS components were glycoproteins containing a variety of sugar groups including mannose, fucose and galactose. However only the host serum albumin component of IVRS appeared to contain a limited amount and range of sugar groups. Finally, the Mr 14000 entity of IVRS was found to contain apparently less sugar relative to other secretions. This finding is consistent with other data on a major allergen of ABF, allergen A, and supports the possiblity that the larval Mr 14000 antigen could be allergen A.

GENERAL INTRODUCTION

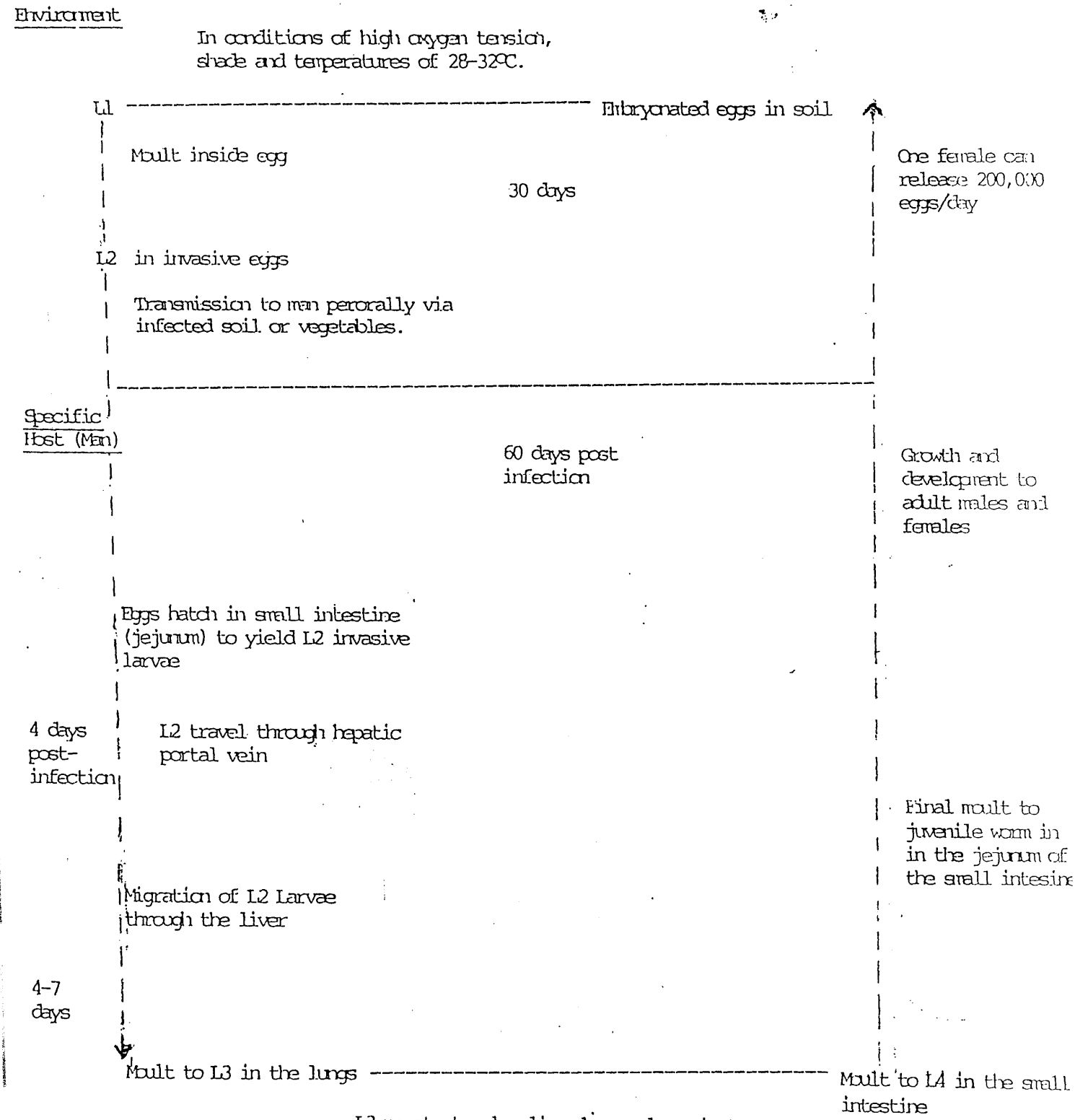
General Epidemiology of Ascaris Infection of Humans

Recent estimates of the number of people infected by the large roundworm of humans, Ascaris lumbricoides, indicate that the calculated figure might over 1000 million (Bruer, 1982;Davis, 1985), which represents approximately one quarter of the world's population. Ascaris would therefore be the most common nematode disease of man. People who become infected with the parasite often live in under-developed nations where there is poor sanitation, poverty, malnutrition, lack of funds for medical health care and a low level of knowledge among the endemic population of the way in which Ascaris infection is transmitted (Crompton, Nesheim and Pawlowski, 1985).

Transmission of infection occurs by the oral ingestion of soil contaminated with Ascaris eggs or with food that has been in contact with contaminated soil (Davis, 1985). The life cycle of the parasite is described in Fig. 1.1. Briefly, the larvae hatch in the small intestine of infected humans, and migrate through the lung and trachea and finally to the small intestine (Crompton and Pawlowski, 1985). Growth and development of worms occurs during the migratory phases. However the precise number and tissue location of the moults that take place are unknown. Maung (1978) suggests that two moulting steps take place in the egg for these to be fully infective and that previous investigators who considered that there was only one moult were misled by artefacts during light microscope examination of the eggs of Ascaris worms. However, until there is confirmation of this report this thesis will observe the usual theory of the occurrence of four moults of Ascaris worms in the infected human. Ascaris

Fig. 1.1

The Life Cycle of Ascaris Lumbricoides in Man



Based on Table drawn up by Crompton and Pawlowski, 1985.

infection does not persist in the host and the adult stages in the small intestine of infected hosts can remain there for 12 to 18 months before expulsion (Cord, et al, 1929).

The prevalence of ascariasis in some communities endemic for the parasite has been reported at levels over 90% (WHO, 1981; Jones, 1977; Arfaa and Ghadirian, 1977). However, prevalence data can be sometime based on small sample numbers and different surveys come up with different results. Additionally, prevalence data is often based on people who voluntarily go for treatment and the final figures could therefore be an underestimate (Crompton, 1987). The most commonly used method of Ascaris diagnosis involves the counting of embryonated Ascaris eggs in the faeces of infected individuals (Massoud et al, 1978; Vinayak et al, 1978; Hall, 1982). This method could conceivably miss out people infected with single male worm infections, immature worms and also those people in whom there were larval stages present, that is, in the prepatent period of infection (Seo, Cho and Chai, 1979).

The incidence of ascariasis seems to be dependent on several factors including frequency of infection and climate. Climatic factors appear to be important to the way in which the disease is transmitted to susceptible individuals. For instance, embryonation of eggs appears to be dependent on moisture content of soil in which the eggs are located, temperature, amount of oxygen, and also the amount of ultraviolet light available to the eggs from sunlight (Morishita, 1972; WHO, 1981).

The intensity of infection (the number of worms per infected

individual) might also be an important factor in the course that infection of Ascaris takes particularly in regard to the population regulation of the parasite and to the morbidity and, to a lesser extent, mortality, that can result from ascariasis (Anderson, 1985). It is now clear that mass chemotherapy might be ineffective at lowering the incidence of ascariasis since prevalence values can reach pre-chemotherapy levels in comparatively short periods of time usually, on average, six to twelve months (Arfaa and Ghadirian, 1977; Cho, 1977 Seo, et al, 1980; Hlaing, Saw and Lwin, 1987). It might be relevant that the frequency distribution of number of Ascaris worms infecting humans is overdispersed, in that only a few people in a given community apparently carry the largest number of worms and contribute considerably to the overall prevalence of infection (Seo, Cho and Chai, 1979; Croll, et al, 1982; Martin, et al, 1984; Hlaing, et al 1985; Schad and Anderson, 1985). It has been suggested that targeting of these individuals for selective and frequent chemotherapy could reduce the prevalence of infection with Ascaris worms (Anderson and May, 1982; Anderson and Medley, 1985). The reasons for the predisposition to infection with high numbers of worms is not known but could be genetic, social, behavioural or a combination of these (and possibly other factors). Studies in experimental animals infected with nematodes suggest that the genotype might be important in resistance, or otherwise, to nematode infection (Wakelin, 1985). There is no evidence to suggest that humans who become infected with Ascaris become resistant to re-infection therefore the importance of protective immunity to the incidence of ascariasis appears not to affect overall prevalence of the disease.

There are indications that in people with chronic or heavy infections of Ascaris the worms could contribute to malnutrition symptoms (Stephenson, 1980; Tripathy, et al, 1972; Brown et al, 1980). In chronically infected people, and even in some individuals infected with light worm burdens, there are signs that the worm infection might be at least in part responsible for various disorders of metabolism that could be associated with the presence of the worms in the small intestine and which appear to be reversed by deworming the afflicted people. For instance: protein energy malnutrition (Venkatachalam and Patwardhan, 1953; Gupta, 1980), vitamin A deficiency and iron deficiency, (Mahalanabis et al, 1979), steatorrhoea (Tripathy, et al, 1972), impaired retention of nitrogen and malabsorption of D-xylose (Tripathy, et al, 1972) were all remitted on treatment of Ascaris infected individuals with anthelminthics.

Polyparasitism of individuals in areas that are endemic for Ascaris might be another factor that might be important to the development of ascariasis in infected individuals possibly by decreasing or aggravating disease symptoms, particularly if the other parasites are also present in the intestine (Stephenson, 1980). Some studies seems to support the view that Ascaris infection itself could suppress the incidence of malaria in a community under study and that deworming could actually increase the incidence of malaria (Murray, et al, 1978). Studies have also shown that Ascaris infection of mice or immunisation with the perienteric fluid of adult worms could depress the antibody response to paratyphoid B (Komatsue, et al, 1979). The possibility of increase of incidence of disease in

Ascaris infected individuals, should be considered before parasite clearance programmes are undertaken.

The majority of chemotherapeutic agents against Ascaris exert their effect through interfering with the metabolic processes of adult Ascaris worms, partly due to the limitations of present diagnostic methods in only detecting infection with adult worm stages. In addition, the main anthelminthic drugs used to treat ascariasis might have harmful (for example: hypersensitive and neurotoxic) side effects (Davies, 1973) and it is for this reason that piperazine derivatives are being replaced with newer drugs. One of these is mebendazole, which impairs phosphorylation and glucose uptake of Ascaris worms (Borgers and De Nollin, 1975), but this could possibly be embriotoxic and cytotoxic to humans, as it is in animals (Delatour, et al, 1976). However, it should also be possible to target the larval stages of Ascaris worms for attack for vaccination puposes. These developmental stages of Ascaris are possibly capable of affecting the host organs by their mechanical action of tissue damage during the migratory phase and also due to the release of products including allergens, but this effect is arguably less serious than the effect of adult worm sojourn in the infected host and related complications such as intestinal obstruction (Louw, 1966; Blumenthal and Schultz, 1975; Katz, et al 1985). It has been suggested that the larval stages of Ascaris are capable of production and release in vitro of antigens that could have a protective effect in vivo (Guerrero and Silverman, 1971; Stromberg and Soulsby, 1977; Stromberg, 1979) and it is important to investigate these claims among other reasons in order to find a technique for the killing of worms in

an infected host which would not have harmful effects on the host.

Although present methods for clearing infected people of Ascaris are relatively efficient, there is evidence of complications occurring during attempts to surgically remove adult worms from infected people which can sometimes lead to potentially harmful results (Beaver, 1975; Blumenthal and Schultz, 1975; Lloyd, 1981). In addition, it has been suggested that people dying from massive anaphylaxis after chemotherapy and later found to be carrying worm burdens could have been subjected to helminthic anaphylactic syndrome (H.A.S) as a result of the release from nematodes of internal products especially allergens (Odunjo, 1970). Given the massive prevalence of ascariasis and the number of mass chemotherapy programmes in operation in developing nations, the final figure could be considerable. Therefore it could be more convenient to look for larval molecules that would terminate the larval stages of parasites before they developed to maturity.

The possible importance of IVRS to the immunobiology, immunodiagnosis and immunoprophylaxis for ascariasis will be presented in more detail later. It is important, for the basic reasons stated above, to examine the immunological and biochemical properties of IVRS molecules for factors which could help in the isolation and evaluation of the usefulness of these products.

General Pathology and Immunopathology of Ascariasis

The larval migratory stages of Ascaris worms, in their natural hosts, appears to involve physical penetration, by the parasites, through several organs in the host and invasion of the bloodstream of the infected animal prior to return to the small intestine (Ransom and Foster, 1919; Yoshida, 1919; Ransom and Cram, 1921; Douvres, Tromba and Malakatis, 1969). In experimentally infected hosts such as guinea pigs and some rodents, the larvae only apparently proceed to the lung stages of development (Crompton, Nesheim and Pawlowski, 1985) so that investigation of Ascaris immunopathological reactions in these animals only involves the larval stages of Ascaris. Nevertheless, the juvenile worms are able to stimulate considerable antibody and cellular responses in infected animals (See later sections) which might be of importance to the biological changes in the infected host.

The major organs of infected animals in which immunopathological changes have been examined in detail following infection with embryonated Ascaris eggs are the liver and lungs. The intestine of pigs or humans infected with Ascaris appears to be a major site for pathological manifestations particularly in regard to nutritional disturbances (reviewed by Crompton, 1985) but only the changes in host animals carrying larval parasites will be considered here. Several studies appear to indicate that the liver is the primary site for larval trapping and destruction particularly in animals infected at multiple intervals with A. suum (Fallis, 1944; Soulsby, 1961; Taffs, 1964a and b) although some larval destruction also seems to occur in the lungs (Soulsby, 1957 and 1961). In mice infected with Ascaris eggs, several pathological reactions caused by infection have been noted by Arean and Crandall, 1971. The trapping and destruction of

larvae appears to occur in the liver sinusoids. In this particular location, larval debris is surrounded by infiltrates of eosinophils, neutrophils and histiocytes which can combine with lymphocytes and macrophages to form granulomas. Local hypersensitivity reactions were suggested to have occurred in the liver of infected mice by the presence of amorphous acidophilic material around destroyed larvae.

In pigs infected with A. suum, the liver of some animals following dissection were found to contain white, "milk spot" , lesions which appear to result from larval migration (Nakagawa et al, 1983; Taffs, 1968). These were suggested to be immunologically mediated (Soulsby, 1957) and present in pigs that had developed resistance to infection following multiple infections (Roneus, 1966; Taffs, 1968). Complement fixation antibodies were also found to be present in higher levels in pigs with liver "milk spot" lesions than those without the lesions (Yoshihara,Nakagawa and Suda, 1987) and the higher antibody levels were also suggested to be related to increased resistance of the animals to Ascaris worm migratory stages. It appears that the formation of lesions in the liver of A. suum infected pigs required the exposure of these animals to the products of the parasites. This was shown in experiments in pigs where hepatic lesions were only found in animals that had been immunised with larval IVRS and egg hatching products before a challenge infection with A. suum eggs (Urban and Romanowski, 1985) suggesting that the metabolic turnover of larval antigens in vivo at the host-parasite interface stimulated components of the host animal immune system which might have eventually caused the pathological reactions in the liver. There are no reports, yet, of liver lesions occurring in humans infected with Ascaris although hepatomegaly has been described to occur (Phills, et

al, 1972) so it might be assumed that at least some inflammatory processes are operational in the organs of infected humans.

Some larvae of Ascaris worms which pass through the liver of infected animals and survive in the face of host attrition at that organ migrate to the lungs of individuals and often appear to cause considerable but transient pulmonary damage to the host animal (Phills, et al, 1972; Gelpi and Mustafa, 1967; Arean and Crandall, 1971; Loeffler, 1932). Lung stage Ascaris larval infection is the subject of considerable debate because the processes by which parasites might cause pulmonary distress by lung infiltration and/or release of worm products are still not clear along with knowledge of the extent to which the host immune response might be involved.

Two of the main types of lung changes which are suggested to occur in Ascaris infected individuals and could cause respiratory distress are Loeffler's syndrome and allergic asthma. Histopathological examination of the lungs of Ascaris infected mice (Arean and Crandall, 1971) shows that the host immune response might be involved in the disease of the lungs by local hypersensitivity reactions of the lungs of infected animals, with resulting infiltration of eosinophils and neutrophils and also increased mucus production in the brochi, alveolar sacs becoming filled with serous exudate, and bronchial spasm.

This disease state of Ascaris infected mice resembles symptoms that were recorded in a medical study of American students that had been maliciously infected with Ascaris eggs (Phills, et al, 1972).

The more seriously affected people were subject to severe respiratory failure 10 to 14 days after infection and had large and transient pulmonary infiltrates with eosinophilia in the lungs. Studies of pulmonary changes in humans infected with Ascaris (Engel, 1935; Mark, 1954; Beaaver and Danaraj, 1958; Gelpi and Mustafa, 1967; Phills, et al. 1972) suggested that these were representative of the syndrome of hypersinsitivity in the lungs with eosinophilic infiltration reported by Loeffler (Loeffler, 1932 and 1956) and proposed that these phenomena occur as a direct result of larval migration through the lungs of infected individuals. This latter theory was supported, among other studies, by the discovery of lung stage worms from the sputum and/or gastric washings of Ascaris infected people (Gelpi and Mustafa, 1967) and also from a report of self-infection with A. Lumbricoides eggs by an investigator who later developed the common clinical signs of Loeffler's syndrome, namely, a racking cough with mucoid and bloody sputum, wheezing, dyspnoea and chest pains (Koino, 1922). An attractive theory for the cause of Loeffler's syndrome was made by Phills, et al, 1972, who proposed that IgE and IgM antibodies were of prime importance in causing pulmonary infiltrates in people who were not continuously infected with Ascaris. According to this theory, IgE antibodies of the host, produced in response to Ascaris allergens, are suggested to be involved in hypersinsitivity reactions resulting in the release of vasoactive amines which increase vascular permeability, enabling the access of immune complexes IgM and Ascaris antigens into the lungs. An Arthus type reaction could then be induced resulting in pulmonary infiltrates with eosinophilia (Loeffler's syndrome).

There are indications that the severity of the symptoms of Loffler's syndrome in humans might be dependent on the frequency of infection of these people with Ascaris eggs. For instance Gelpi and Mustafa, 1967, noted that pneumonitis in Saudi Arabian individuals was seasonal and correlated with the onset of a short period of rainfall, following a long and dry summer. The above authors suggested that the humid and relatively cool climatic conditions were more favourable for transmission of ascariasis in Saudi Arabia, accounting for the intermittent transmission of Ascaris infection in areas of this country and this has been supported by evidence that high temperature (Ransom and Foster, 1920; Takasaki 1935) or low moisture content (Ransom and Foster, 1920) of soil containing Ascaris eggs could arrest embryonation. In direct contrast, there are few reports of Loffler's syndrome occurring in tropical locations in which frequent infection of people by Ascaris could be expected. The lack of such reports has been attributed to the possibility that the continuous infection of humans in tropical areas had made them tolerant to the parasitosis so that pulmonary reactions to Ascaris were not detected in these people (Spillman, 1975). The factors that might be responsible for such tolerance, if it exists, in addition to the immunological processes that underly the pathological changes in people infected either seasonally or in uninterrupted conditions have not been fully characterised. Suggestions that the negligible ocurrence of Loffler's syndrome in tropical areas was due principally to low grade infection, which could explain the low incidence of lung infiltrates, were counteracted by the results of experiments by Vogel and Minning, 1942, who were able to demonstrate the presence of eosinophilic pulmonary infiltrates of

transient duration in humans infected with low (6-45) numbers of A. lumbricoides eggs.

People continuously exposed to Ascaris have been suggested to show a lack of symptoms of lung ascariasis due to the presence of IgG or IgM antibodies with the same specificity for Ascaris allergens as IgE which could bind to allergen before it reached IgE bound to mast cells or basophils (Ogilvie and De Savigny, 1985). Such a possibility has been suggested to occur in some individuals with filarial (Otteson, et al, 1981; Hussain and Otteson, 1985) or schistosomal (Mazinque, et al, 1980; Hoffstetter et al, 1982) infection where the pathogenesis of the disease and immunopathological symptoms were suggested to be affected by the presence of "blocking" (usually IgG) antibodies. Another mechanism by which immediate hypersensitive reactions in the lungs of continuously infected people could be modulated has been suggested to involve, in some people, the occurrence of high levels of IgE that lack specificity for parasite derived allergens (Jarrett, Orr and Riley, 1971; Turner, Feddema and Quinn, 1979; Dessaint, et al, 1975) and that compete with worm allergen specific IgE for binding to mast cells (Stanworth , 1971; Stanworth, et al, 1967; Jarrett, Orr and Riley, 1971; Bazaral, Orgel and Hamburger, 1973). This latter proposed mechanism has been suggested to be less plausible than the former because it is suggested that the proportion of non-specific to specific IgE is too low to block all available mast cell receptors (Jarrett, Mackenzie and Bennich, 1980). Furthermore, the amount of IgE receptors on mast cells is dependent upon the total concentration of serum IgE so that elevated levels of IgE would apparently induce more binding sites on

mast cells (Weltman and Senft, 1981). However, the basic processes that induce various immunopathological aspects of larval ascariasis are still unclear so it is not possible to exclude the possibility that IgE without specificity for parasite allergens could be important in modulating the effects of ascariasis.

Usually in the absence of infection with Ascaris but after natural or experimental exposure to Ascaris allergens, some individuals are prone to symptoms of local and general anaphylaxis, manifested by urticaria, angioneurotic oedema, a pathological condition in the lung that appears similar to asthma and possibly severe gastric upset (Lloyd Jones and Kingscote, 1935; Sprent 1949; Andrews, 1962; Barry and O'Rourke, 1964; Lehmensick, 1960; Coles, 1975). It has been proposed that since the resultant symptoms of respiratory disease due to Ascaris migration and allergic asthma are similar, the majority of symptoms of immunopathology of larval ascariasis are of pulmonary origin and, additionally, there might be a relationship between allergic asthma and Ascaris infection.

There are several reports of asthma in experimental animals that had been sensitised previously with Ascaris and were sensitive to inhalation of Ascaris adult worm allergens (Cain, et al, 1980; Goodman and O'Neil, 1981; Richards, et al, 1983). Monkeys caught from the wild appear to be naturally sensitised to Ascaris, (Patterson, et al, 1976; O'Neil and Goodman, 1981) possibly due to previous infection of these animals in their natural habitat with parasites that produced antigens similar to those of Ascaris worms (Weiszer Patterson and Pruzansky, 1968). High proportions of wild

monkeys that were confirmed positive for Ascaris by skin test were induced to produce the symptoms of allergic asthma after inhalation of aerosols of Ascaris antigens (Weiszer, Patterson and Pruzansky, 1968; Patterson, et al, 1976).

In contrast, laboratory bred monkeys did not respond with pulmonary distress following challenge with Ascaris antigen aerosol but were able to respond to allergens after prior infection with Ascaris eggs (Patterson, Harris and Pruzansky, 1983; Richards, et al, 1983; Patterson and Harris, 1985). The most common physiopathological reactions to challenge of Ascaris allergen in sensitised animals were: narrowing of the bronchial lumen by contraction of bronchial smooth muscle, increased respiratory rate and increase in the period of respiratory exhalation (Patterson, et al, 1976; Cain, et al, 1980; Goodman, O'Neil, 1981 ; Richards, et al, 1983; Takishima, et al, 1985). These are also the common features of asthmatic symptoms in humans exposed to Ascaris allergens (Barry and O'Rourke, 1964; Lehmensick, 1960). It has been suggested from studies of Ascaris sensitised animals challenged with allergens, that elevated levels IgE possibly stimulated by larval allergens might be involved in hypersensitivity reactions to cause asthmatic attacks (Matsumura, 1987 ; Joubert, de Klerk and Malan, 1979). There is support for this theory from the observation that the inhalation of aerosolised histamine caused bronchial constriction in animals (Krell, 1976; Patterson and Talbot, 1972) and also that mast cells from monkeys infected with A. suum degranulated after contact with Ascaris antigens in vitro and released histamine (Pritchard, et al, 1983b) It appears that the similar cause and result of respiratory disease

due to migration through the lungs by Ascaris worms, and allergic asthma after inhalation of Ascaris allergens, indicate that the immunopathological changes in larval ascariasis are for the most part localised in the lungs.

Although the possible association of parasitic infection and asthma in humans is appealing, at present the evidence for such a correlation is contradictory and the answer is still unresolved. Tullis claimed in 1970 that parasite eggs were found in the faeces of 198 out of 201 asthmatics in a hospital in Canada and none in control patients without asthma. Several other studies also appeared to indicate that asthma is more common in areas of the world where the prevalence of helminth (including Ascaris) infection is high (Lasch, 1976; Kok and Robinson, 1976). In India, it has been suggested that most case of asthma could be attributed to infection with roundworms or hookworms (Chacko, 1970). However, a number of studies claim that there is no association of asthma with parasite infection (Van Dellen and Thomson, 1971; Salako and Sofowara, 1970; Wolstenholme, 1979) and the Tullis report was challenged on the bases that parasite eggs had been confused with, for instance, vegetable particles and/or light microsocpe artifacts (Farzan, 1970).

Although the evidence for association of Ascaris is sparse, there are two other possible associations between Ascaris infection of humans and asthma, namely that asthmatic people might control worm infections better than non-asthmatic people and therefore carry lower worm burdens (Grove and Forbes, 1975; Shield, Scrimgeour and Vaterlaws, 1980) or that worm infections could protect individuals

against asthma (Godfrey, 1975; Merrett, Merret and Cookson 1976). The latter theory suggests that parasite infections can reduce asthma in individuals, by stimulation of the production of high levels of IgE in these people that fill up sites on mast cells preventing the chances of reaction to other allergens that would be normally encountered,(as mentioned previously in the experimental model for the lack of lung changes in people continuously infected with Ascaris). However, there is evidence that instead of protecting infected individuals against asthma, Ascaris infection could be responsible for the potentiation of the IgE response in infected individuals against allergens unrelated to those of Ascaris, (Joubert, 1978; Turner, Feddema and Quinn, 1979; Joubert, et al, 1979; Joubert, et al, 1980). This feature of helminth infection was first shown in rats, sensitised with a particular allergen and then infected with Nippostrongylus brasiliensis (Jarrett and Stewart, 1972; Orr, Reilly and Doe, 1972). Asthmatic people from an African community that were positive for skin tests of Ascaris also gave a higher number of positive tests for a range of common, inhaled allergens than those who were negative in skin tests for the parasite (Joubert, Van Schalkwyk and Turner, 1980). Additionally, the same authors claimed that the heightened response to other allergens was dependent on the potentiation of the IgE response by parasite infection. In guinea pigs experimentally immunised with IVRS from lung stage Ascaris larvae, it was shown the larval products apparently potentiated the IgE response in guinea pigs to ovalbumin (Stromberg, 1980). This leaves open the possibility that larval allergens could also exert a potentiating effect in humans which could partly explain the

enhanced IgE reaction to inhaled allergens. Turner, Feddema and Quinn, 1979, discovered that the majority of the high levels of IgE was not specific for Ascaris adult perienteric fluid antigens or towards common environmental inhalant antigens and the specificity of this IgE is unknown, although the RAST test used by the investigators only includes high avidity IgE, so it is still possible that there was IgE produced against common inhalant allergens, but that it was of low avidity.

At present there is evidence that levels of IgE are important in lung pathological disorders in people infected by Ascaris worms although, more evidence is required to confirm that Ascaris, and possibly the larval stages or their products, could aggrevate the atopic condition in infected individuals. If true, this could be an important factor in justifying the need to control ascariasis.

Allergens of Ascaris

There are various reasons for investigating the allergens of nematodes generally and also those produced by Ascaris worms. One reason is that allergens produced by Ascaris could be significant in the onset of immunopathological reactions caused in humans during larval developmental stages of the parasite infection and investigation of Ascaris allergens could aid in the dissection of the disease processes in larval ascariasis. Additionally, it is known that nematode infection commonly leads to the production of high amounts of IgE by the infected animal. The investigation of

Ascaris allergens provides an ideal opportunity for examination of the immunological mechanisms that control the production of IgE in nematode infected animals. Moreover, it is important to judge if crossreacting allergens from other organisms are significant to the general immunopathology of ascariasis since people of areas endemic for ascariasis might be infected with different parasites containing similar allergens.

The high levels of IgE that characterise the infection of animals with nematodes (see later this chapter) could arise either by the intrinsic biological properties of the allergen itself or by the way the allergen is presented to the immune system of the host. It was proposed that the allergen itself contained the properties that were necessary to induce and IgE response and also that there would be common features in allergens which would support this view, such as charge, size, specific sugar group-protein bonds and relative resistance to proteinolysis (Berrens, 1971). However, although some similarities have been shown between several allergens of different sources there are apparently no unifying features of allergens. The allergens of some nematodes, either in purified form or in the form of whole worm extracts, are not able to stimulate the immunised host to produce as much IgE as that of living worms (Strejan and Campbell, 1968; Hussain, Strejan and Campbell, 1972; Jarrett, 1978; O'Donnell and Mitchell, 1978). One explanation for these results comes from studies of antibody responses in animal immunised with the hapten dinitrophenol (DNP) conjugated to Ascaris extract, which showed that the main antibody isotype stimulated in immunised animals was IgE (Strejan and Marsh, 1971) whilst conjugation of DNP to, for instance,

Levan (a T cell independent agent) produced a predominent IgM response (Klaus and Humphrey, 1974), and an IgG response was elicited by DNP conjugated to a T dependent carrier protein (North and Dresser, 1977). The carrier appeared to be important for the induction of the IgE response in the immunised animals and it is interesting to speculate that the worm surface *in vitro* could be important, as a carrier entity, to the presentation of allergens to the host immune response. Several factors probably combine to produce the high IgE response in nematode infected animals. These could include the manner in which the allergen is presented to the host animal by the worms, possible induction of the development of certain T cell subsets by the worms (see later this chapter) and the production by T cells of lymphokine(s) that may induce the selective proliferation of IgE-producing B cells (Suemara and Ishizaki, 1979; Suemara, *et al*, 1980).

The allergens derived from Ascaris worms have been examined for IgE responses in infected animals, including humans, and have also been characterised at the molecular level with estimations available of molecular size, isoelectric charge and also the carbohydrate to protein ratio. Early experiments on Ascaris allergens were based on the products derived either from whole adult worm extracts (WWE) or from the body fluid (ABF) of adult Ascaris parasites. There are varying estimates of molecular size, electrophoretic mobilities and also number of allergens that are present in Ascaris parasites. Hogarth-Scott (1967) showed that fractions of ABF derived after gel chromatography were able to induce allergenic reactions in sensitised animals. The allergenic activity of the perienteric fluid ranged in

molecular weight from 10000 to 50000. Hussain, Bradbury and Strejan (1973) suggested that the major allergen purified from A. suum adult worm extract was of molecular weight 17-19000 and made up of two non-covalently bound subunits with 8.6% carbohydrate. The aforementioned authors called the allergen Asc-1. Greenspon, et al 1986) also found that the major allergen of WWE of A. suum was of apparent molecular weight 9000. However, these results are in contrast with those obtained by various other investigators. For instance, Ambler, et al (1973b) obtained an allergenic fractions from WWE A. suum of approximate molecular weight 14000 which also contained a lower carbohydrate content than Asc-1 (1% glucose and 0.5% hexosamine). This glycoprotein, suggested to be the major allergen of A. suum adult worms, was termed allergen A and also appeared to be released from adult parasites maintained in vitro in saline (Ambler, et al, 1973a) Later investigations of the biochemical properties of allergen A (Ambler, Miller and Orr, 1974) included periodate treatment of the allergen prior to PCA tests which apparently made little difference to the allergenic properties of the glycoprotein and it was concluded that carbohydrates were not implicated in the induction of elevated IgE levels by allergen A.

The major allergen of perienteric fluid as opposed to crude WWE was apparently found to have the isoelectric point (pI) 6.1 (Kuo and Yoo, 1977), in contrast to allergen A and Asc-1 (pI 5.0-5.2 and 4.8-5.0 respectively). One suggestion for the apparent heterogeneity of biochemical properties of allergens was postulated to be due to the presence, among Ascaris products, of an allergenic unit that could be present among molecules of different molecular weights and biochemical

composition (Dandeu and Lux, 1978; Hussain, Strejan and Campbell, 1972; Ambler, et al, 1973b). However, there are other suggestions, for instance, the variety of techniques employed to investigate the allergens could have contributed to the observed differences. Alternatively, the differences could be attributed to heterogeneous worm strains (Coles, 1985). O'Donnell and Mitchell (1978) were able to show that a pure fraction of ABF, of molecular weight 360000 and pI 8.2-8.4 (Da.S1), elicited a significant IgE response in sensitized guinea pigs. This is different to all the other allergens derived from adult parasites but the authors observed that rapid breakdown of Da.S1 occurred unless ABF was freshly prepared. It is possible that aggregation of a small molecular weight unit containing allergenic epitopes could also explain the relatively high molecular weight of Da.S1.

It was shown that an allergen produced by migrating stages of Ascaris larvae in infected rats could be identical to that of Asc-1 from the adult parasite and was apparently able to induce the host production of allergen specific IgE (Bradbury, Percy and Strejan, 1974). Additionally, the liver and lungs of the infected animals developed the lesions that were also present in pigs infected with A. suum and, by inference, in humans infected with A.lumbricoides (Bradbury, Percy and Strejan, 1974). These lesions appear to be immunologically mediated and it would be interesting to speculate on the importance of larval allergen(s) to the host-parasite interactions during early stages of infection as well as later stages when adult

worms would presumably secrete allergens *in vivo*. Stromberg (1979b) found that the IVRS of L3/4 *A. suum* worms were capable of inducing high levels of IgE and IgG1 in guinea pigs. The levels of IgE were equivalent to those achieved by intravenous immunisation of living L2 larvae into guinea pigs (Dobson, Morseth and Soulsby, 1971) and by immunisation procedures involving successive re-infection of guinea pigs with Ascaris eggs (Khoury, Stromberg and Soulsby, 1977). IgE and IgG1 might be protective in guinea pigs against infection with Ascaris (Khoury, Stromberg and Soulsby, 1977). Therefore it is conceivable that, *in vivo*, the production of allergen(s) by worms and release of IVRS could trigger protective immune mechanisms that also lead inadvertently to damage of host tissue.

The possible existence of similar or identical allergens between different nematode species could also be significant to the immunopathology of ascariasis, particularly in regard to the allergic manifestations of the disease, following infection/sensitsation by Ascaris infection in individuals who live in areas endemic for infection with more than one parasite. Additionally, there is also concern for laboratory workers who might be exposed to a number of allergens (Coles, 1985). There are various lines of argument to support this view. For instance, Ball, Voller and Taffs (1971) showed that there was apparent allergenic crossreactivity between crude adult worm extract components of *A. suum*, *Toxocara canis* and *Necator americanus* and *Haemonchus contortus* from skin tests of individuals with a know history of infection with any of these. Other investigators have also apparently found crossreactivity between the allergens of *A. suum* (usually adult parasites) and those of other

parasites including N. brasiliensis adult worms and Ostertagia circumcincta third stage larvae, on the basis of gastro-intestinal disturbances in people already sensitised to Ascaris (Coles, 1975). RAST tests with antisera from people with human sera reactive with Ascaris allergens have shown crossreactivity with allergens from various nematode species including larval and adult extracts of N. brasiliensis, H. contortus and T. canis (O'Donnell and Mitchell, 1978; McWilliam, Stewart and Turner, 1987).

Caution has to be applied to the experiments involving human sera because the past history of infection of humans with certain parasites is difficult to fully define. Additionally, it is also possible that non-nematode organisms could contain antigens that crossreact with those of Ascaris. The importance of crossreactivity of allergens to the immunobiology of larval ascariasis is not yet known although there appears to be evidence that component(s) of Ascaris IVRS might have the capacity to potentiate the IgE response in infected hosts to allergens unrelated to those of Ascaris (Stromberg and Soulsby, 1977; Marretta and Casey, 1979; Stromberg, 1980). This activity of nematode products has also been noted for animals infected with either N. brasiliensis (Jarrett and Stewart, 1972) or Fasciola hepatica (Jarrett, 1972) and might have potentially serious consequences for people infected with Ascaris, in that, infections proceeding through larval stages could possibly enhance the respose of individuals to common (possibly inhaled) allergens (Joubert, Van Schalkwyk and Turner, 1980).

Antibody Responses to A.suum

Experimentally infected animals have been often used to study the antibody responses that are induced following nematode infection, often to study the role of antibodies in acquired resistance to parasite infection (reviewed by Wakelin, 1976). The most common antibody types that are induced in infected animals are IgE, IgG and IgM, with levels of the first two types of antibody present in greatly increased overall serum concentration (Orr and Blair, 1969; Jarrett and Stewart, 1972; Dobson, Morseth and Soulsby, 1971; Stromberg, 1980) although some nematodes that inhibit the small intestine also induce higher than normal levels of IgA, which is the predominant antibody in the gastro-intestinal tract (Cypess, Ebersole and Moliniari, 1977; Sinsky and Holmes, 1978; Poulain, Luffau and Pery, 1976). The effect that antibodies have on the length of time that the nematode infection persists in the infected host is not known, but it is possible that antibodies could be important in concerted action with certain immune cells to inhibit the growth and development of nematode larvae. For example, studies indicate that macrophage mediated killing of nematodes might require IgM or IgE (Ogilvie et al, 1980) and that IgG or IgE antibodies could be required for the killing of nematodes by eosinophils (Capron and Capron, 1980). The role of these antibodies and cells has been established in in vitro studies. Although there are indications that similar activities might take place in vivo (Grove, Warren and Mahmoud, 1977; Chaicumpa, Jenkin and Fisher, 1977; Chaicumpa and Jenkin, 1978; Gleich, Olson and Herlich, 1979) there is no conclusive evidence of such activity.

In the general immune response to the experimental infection of

animals with A. suum, no exhaustive investigations have been made regarding particular antibody isotypes which might be important in protection against ascariasis. Various animals have been experimentally infected with A. suum for the examination of resulting antibody responses, including dogs, (Lewis et al, 1982), monkey, (Goodman and O'Neil, 1981), rodents (Crandall and Crandall, 1967 and 1971) and pigs (Taffs, 1958). The first two species of animals are induced by Ascaris infection to produce raised amounts of IgE, and have been mainly used to examine the pathological processes which cause allergic asthma (see later section this chapter). The possible role of Ascaris allergens and parasite-specific IgE generally in the induction of hypersensitivity states in immunologically primed animals will be described later. The predominant antibody response in rats infected with A. suum was shown apparently to be of IgG antibodies (Crandall and Crandall, 1967) although IgE has also been reported to be present in raised amounts of these animals, apparently in response to an allergen released from larvae in vivo (Bradbury, Percy and Strejan, 1974). In contrast, the major antibody response in Balb/c mice infected with A. suum involved IgM antibodies which were produced by the animals five to six days after infection and rose to maximum levels after two to three weeks (Crandall and Crandall, 1971). Following the decline of IgM levels, there was an increase of levels of other imunoglobulins, particularly IgG_1, (although not in the same amounts as IgM). The levels of IgM antibody correlate with the presence of antibody producing cells in major lymphoid tissues and at sites of local immunoglobulin production, such as the liver and lungs of infected mice (Crandall and Crandall, 1971).

A significant amount (50%) of the IgM produced for A. suum infected mice was shown to have specificity for phosphorylcholine (Pc), a hapten which has sometimes been found to be associated with phospholipids of eukaryotic cell membranes (Kaplan and Volanakis, 1974), the phospholipid portions of some lipoproteins, (Leon and Young, 1971) with bacterial and fungal C-polysaccharide (Abernathy and Avery, 1941; Tomasz, 1967; Brundish and Baddiley, 1968; Volanakis and Kaplan, 1971; Pepys and Longbottom, 1971; Anderson, Stroud and Volanakis, 1978) and also in parasites or parasite products (Pery et al, 1974; Pery et al, 1979; Frayha and Smyth, 1983; Mitchell et al, 1976; McWilliam, Stewart and Turner, 1987). The significance of a potent IgM antibody response to Pc is not yet known, but it appears that these antibodies are not of protective value to mice infected with A. suum (Brown et al, 1977). However, it has been suggested that the reason for the high IgM response in infected mice is due to the inhibition, by Pc bearing antigens, of the proliferation of B cell precursors other than those that would produce IgM of low affinity for the antigens. (Gutman and Mitchell, 1977; Mitchell and Lewers, 1977). The effect of this selective inhibition was presumed to protect the parasite against a potent multi-isotype antibody response. Pc does not appear to be present on the cuticle of lung stage Ascaris larvae but it has been observed to be associated with membranous structures inside the larvae, including the worm intestine (Gutman and Mitchell, 1977). Contact of larval Pc bearing antigens with the host immune response would then presumably occur through the IVRS of these worms, or after a larval moult (Gutman and Mitchell, 1977). Unfortunately, to date there is no evidence that Ascaris arval IVRS moulting products contain Pc.

It is difficult to assume that antibody and cellular responses elicited by experimental Ascaris infection of rodents totally reflect those that are induced in the natural hosts. In the former animals, the worms are not natural parasites for the animals and do not complete their life cycle, since the larvae presumably die after proceeding to the lung stage of development (Lloyd and Soulsby, 1985). Presumably, only the immune responses to liver and lung stage larvae are examined in rodents infected with Ascaris.

In the natural host of A. suum, namely the pig, two peaks of antibody production were noted in animals given a single experimental infection with A. suum; the first after two weeks, and the second at times between five and eight weeks after infection, attributed to the induction of antibody production in the host animals, first by migrating larvae and then by adult worms (Taffs, 1964a). The isotypes of antibodies produced against infection were not stated by the author but were thought to provide a measure of the host animals' ability to resist infection, and high concentrations of some antibodies were suggested to reduce the life span of larvae in vitro, by forming precipitates with worm antigens (making them ineffective) on sites on the larval surface that could be used by the parasites for feeding, and/or by helping to target immune effector cells and complement components to the worm surface (Taffs, 1961 a and b). It is unclear if humans who are infected on multiple occasions with A. lumbricoides similarly acquire resistance to infection that is suggested to occur for pigs.

Most studies of the human antibody responses against infection

with Ascaris have had to rely on naturally infected individuals, living in areas endemic for a number of parasite infections, usually whose past history of infection with Ascaris was unknown and who might have been infected simultaneously with more than one parasite. Nevertheless several reports have been made on the antibody responses of individuals who were positive for Ascaris on the basis of either immunological tests (for example the RAST assay) or by the faecal excretion of Ascaris eggs. The main antibody response of humans, to infection with Ascaris, is thought to be of the IgE type (Johansson, Mellbin and Vahlquist, 1968; Orren and Dowdle, 1975; Purtilo et al, 1976; Joubert, Van Schalkwyk and Turner, 1980). Johansson, Mellbin and Vahlquist (1968) discovered that the IgE levels in Ascaris infected Ethiopian school children were increased by fifteen to twenty-fold, in comparison to presumed uninfected children. Although levels of other antibody isotypes were also increased, particularly IgG and IgD, IgM and IgA were not present in elevated levels. Increased levels of IgE were also found in the serum of five Ascaris-infected humans, using the RAST test (O'Donnell and Mitchell, 1980). However, only one of these individuals had elevated amounts of other antibodies, namely, of the IgG type. The authors suggested that IgG antibodies in infected humans are short-lived and are induced by migrating Ascaris larvae, or as a result of larval moulting.

Malicious infection of four adult males with embryonated Ascaris eggs resulted in increased levels of IgM in two of the individuals, and of IgE levels in three patients (Phills et al, 1972). The two patients with the highest levels of IgM and IgE also had the most notable clinical symptoms, including severe transient pulmonary

lesions, resulting in respiratory distress, probably due, in part, to larval migration through the lungs, but the levels of IgE and IgM were also thought to contribute to the severity of the lung disorder. Additionally, the patients with the most increased levels of antibodies carried the lowest adult worm burden, supporting the suggestion of Taffs that the antibody response to Ascaris by infected animals is of protective importance. However, it is more likely that the overall interaction of the antibody and cellular responses in Ascaris infected individuals are more important to the final course of the disease, particularly at the larval stages than any single component of the immune response.

The Cellular Immune Response to Ascaris and Protective Immunity

One of the main scientific interests in the cellular immune response of animals experimentally infected with intestinal nematodes involves the possible role that this particular host response plays, along with antibodies, in acquired resistance to infection with these parasites. Two general manifestations of protective immunity in nematode infected animals are "self cure" and "spontaneous cure". The former phenomenon occurs when adult worms established in the intestine of the animal are expelled from this site, undamaged, following the growth and development of larvae from subsequent infection (Love, Ogilvie and McLaren, 1976; Bell, McGregor and Despommier, 1979; Bell and McGregor, 1980; Alizadeh and Wakelin, 1982). The latter event can occur during a single infection with the parasite and might be under the control of T cells (Dineen and Wagland, 1966; Jarrett, Jarrett and Urquhart, 1968; Nawa and Miller, 1979; Love, Kelly and

Dineen, 1974). Many of the studies of factors that stimulate protective immunity involve host-parasite systems in which spontaneous cure occurs. It is likely that protective immunity in infected animals involves a combination of immune response components including antibodies (Ogilvie and Jones, 1968; Neilson, 1969; Wakelin, 1976; Love, Ogilvie and McLaren, 1976; Miller, 1980; Dobson, 1982; Williams and Behnke, 1983), lymphocytes (Ogilvie and Jones, 1968; Kelly and Dineen, 1972; Love, Ogilvie and McLaren, 1976; Wakelin and Lloyd, 1976; Crum, Despommier and McGregor, 1977) and non lymphoid effector cells. The formation of protective immunity could also include physiological changes in the intestine of the parasitised animal that could be detrimental to the parasite, for instance, increase in intestinal permeability (Castro, 1982; Murray, Jarrett and Jennings, 1971; Miller et al, 1983; King and Miller, 1984) and/or biological composition or rate of secretion of mucus at the site of adult parasite establishment (Miller, Huntley and Wallace, 1981; Lee and Ogilvie, 1981). Most of these events have been suggested to be under the regulatory control of T cells which are thought to be important if not vital to the development of resistance to infection with intestinal nematodes (Mitchell et al, 1976; Crum, Despommier and McGregor, 1977; Jacobson and Reed, 1976; Nawa, Parish and Miller, 1978; Wakelin and Wilson, 1977 and 1979). For example, effective immunity to N. brasiliensis infection can be transferred to lymphocyte deficient (X-irradiated) or athymic mice by a T cell enriched cell population from infected syngeneic mice (Mitchell et al, 1976; Jacobson and Reed, 1976; Crum, Despommier and McGregor, 1977; Wakelin and Wilson, 1979; Nawa, Parish and Miller, 1978).

The role of the T cells in immune responses against Ascaris infection is still unclear. There is the possibility that parasites could influence the proliferation of T cell subpopulations that would be present in the infected host animal to facilitate their own survival. This phenomenon appears to occur during the infection of mice with T. spiralis (Faubert, 1976, and 1982) where the products of newborn larvae might stimulate T cell mediated immunosuppression, and possibly also by a similar mechanism in mice infected with the nematode Nematospiroides dubius (Pritchard, Ali and Behnke, 1984).

T cell proliferation has been discovered to be stimulated by products of continuous cultures of Plasmodium falciparum (Ballet et al, 1981) while soluble fractions of Schistosoma mansoni egg antigens were able to activate antigen specific T suppressor cells (Rocklin, Tracy and Kholy, 1981). A crude extract of adult A. suum worms was also found to contain a substance that was highly mitogenic for human T cells (Sasagawa, Suzuki and Fujikura, 1987) but there is no information yet regarding the types of T cell stimulated by Ascaris derived mitogen.

Immunosuppression of the antibody response appears to occur in humans (Levinas, 1965) and animals (Crandall and Crandall, 1976; Komatsu et al, 1979) infected with Ascaris. The mechanism of the immunosuppression is not known. Spleen cells from Ascaris infected guinea pigs apparently contained suppressor cells which could, after passive transfer, increase the number of larvae that migrated to the lungs of infected animals (Khoury, Stromberg and Soulsby, 1977) although these suppressor cells were not investigated further.

Johnstone, Leventhal and Soulsby, 1981, proposed that the selective removal of suppressor T cells from mice, later infected with Ascaris, decreased the number of tissue migrating stages of larvae in infected animals suggesting that suppressor T cells were important to the survival of tissue migratory stages of Ascaris worms.

There are suggestions that perhaps the most important step in the expulsion of worms from the small intestine involves local anaphylaxis (Rothwell and Dineen, 1972; Wakelin and Donachie, 1981; Alizadeh and Wakelin, 1982). In experiments where attempts were made to confer resistance to T.spiralis or N.brasiliensis with immune lymphocytes to irradiated recipient animals, it was shown that a population of bone marrow cells was also necessary for immunity to infection with these parasites (Murray, Jennings and Armour, 1970; Dineen and Kelly, 1973; Wakelin and Wilson, 1977 and 1980; Wakelin and Donachie, 1981). Two types of cells, among others, have been found to be prominent at the site of intestinal inflammation and are thought to be recruited for action at the mucosa by T cells. These are intestinal mucosal mast cells (IMMC) (Urquhart et al, 1965; Wells, 1962; Befus, Johnstone and Bienenstock, 1979) and eosinophils (Wells, 1962; Ritchie et al, 1966; Rothwell and Dineen, 1972; Kelly and Ogilvie, 1972).

Eosinophilia and raised levels of IgE are two of the characteristic host responses to infection with helminth parasites, including larval stages (Ansari and Williams, 1976; Ackerman et al, 1981; Sugane and Oshima, 1982; Phillips et al, 1977). Eosinophilia has been suggested to be under the control of T cells in some helminth infected animals (Basten and Beeson, 1970; Walls et al, 1971; Phillips et al, 1977; Wakelin and Donachie, 1983) but two types of

esinophilia were apparently induced in mice infected with T.canis. These were either dependent or independent of T cells (Sugane and Oshima, 1982). Eosinophils have been proposed to contribute to the acquired resistance to nematode infection by possible action as killer cells in vitro, acting through antibody (IgE or IgG) (Butterworth et al, 1977; Butterworth et al, 1979; Kazura and Aikawa, 1980; Capron and Capron, 1980) or complement dependent mechanisms (Ramalho-Pinto, McLaren and Smithers, 1978; Anwar, Smithers and Kay, 1979). However, there is no direct evidence at present to suggest that eosinophils are capable of killing nematodes in vivo.

In humans infected with A. lumbricoides, or animals experimentally infected with A. suum, one of the most frequently observed feature of the cellular response is peripheral and local eosinophilia; the latter apparently occurring at host tissues through which larval migration occurs (Gelpi and Mustafa, 1967; Vogel and Minning, 1942; Phills et al, 1972; Arean and Crandall, 1971). Possible T cell control of eosinophilia was examined in A. suum infected mice where it was discovered that eosinophilia is thymus dependent (Nielsen, Fogh and Andersen, 1974). However, no equivalent studies have been performed in Ascaris infected pigs. In pigs experimentally infected with A.suum eggs, peripheral blood eosinophils were present in high numbers at four to seven days after infection, presumably at the same time that early stages of larval migration were occurring (Rhodes, Keralis and Staudinger, 1982). Secondary infection of pigs resulted in the increase of eosinophil numbers up to levels equivalent to 44% of white blood cells (Rhodes, Keralis and Staudinger, 1982) although it was not established if there was a

concurrent decrease in the number of larvae developing to adult stages.

Archer et al, 1985, examined the possibility that the Ascaris parasite itself, during the course of infection, could induce the increased production of certain immune effector cells. The authors observed that a single intraperitoneal (i.p.) injection of adult A. suum cuticle into mice caused a local eosinophilia within three days reaching maximum levels after two weeks and also an increase in the number of connective tissue mast cells (CTMC) three weeks after injection. The eosinophils apparently degranulate and release their contents on to the worm cuticle, in the presence of immune serum, but do not affect the integrity of the cuticle. Treatment of cuticle sections with collagenase prior to immunization of the rats drastically diminished eosinophilia and mast cell hyperplasia suggesting that some substance closely associated with, or contained in, Ascaris collagen is responsible for inducing these events. Archer et al, 1985, suggested that the molecules involved in induction of high numbers of eosinophils might have been present on the outer surface of the cuticle and could have interacted with antibody components of immune serum which appear to be required for eosinophil binding to the surface of some nematodes.

Experiments have shown that killed, intact nematode larvae were able to induce eosinophilia in rats but that this effect was not achieved with larvae that were ground up before administration to these animals (Basten, Boyer and Beeson, 1970). Presumably, the intact cuticle was necessary to induce increases in eosinophil numbers. It

is still not known, however, if the larval cuticle of Ascaris worms fulfils the same role as that of the adult cuticle. However, the observations that larval stages can induce high levels of eosinophilia (see earlier this chapter) do not rule out the possibility that either the cuticle or IVRS of Ascaris parasites could be, at least in part, responsible for eosinophilia. Infection of mice with the ascaridoid worm, T.canis, or immunization with IVRS was observed to induce peripheral eosinophilia in these animals (Sugane and Oshima, 1984). Since most of the components of T.canis L2 IVRS are shed from the cuticular surface of the worms (Maizels, De Savigny and Ogilvie, 1984), this would suggest involvement of the larval cuticle of these worms in the induction of eosinophilia.

Examination of the body fluid of adult A.suum worms revealed that there were components of ABF that were chemotactic factors for both eosinophils and neutrophils (ECF and NCF, respectively) (Tanaka, Baba and Torisu, 1979). The former chemotactic factor was of apparent molecular weight 30000, whilst NCF was present as two components of different molecular weights. Both factors were distinct from Ascaris allergens as determined from PCA tests. There was no information available on the possibility that these factors were eventually directed to the cuticle of adult worms or if larval stages of Ascaris produce factors chemotactic for either eosinophils or neutrophils. However, it might be relevant that extracts of Anisakis larvae also appear to contain a factor that is chemotactic for eosinophils (Tanaka and Torisu, 1978).

Protective Immunity to Ascaris Infection

There are no indications that there is sterilizing immunity to Ascaris infection in animals that have been experimentally infected with the parasite. Nevertheless, researchers often refer to acquired resistance to Ascaris infection, in the context of reduced worm burden, usually in the lungs of immunized animals following challenge infection. These experiments will be described in greater detail below. The main criterion used for judgement of degree of protection has often involved counting the number of larvae in particular experiments that reach the liver or lungs of immunized animals, after a fixed period of time following challenge infection, by examination of the number that emerge through organ tissue and a Baerman apparatus (Lloyd and Soulsby, 1985; Urban and Tromba, 1982 and 1984). The percentage protection against re-infection can be calculated by the formula: $1-\frac{a}{b} \times 100$

where a = number of larvae recovered from immunized animals and b = number of larvae recovered from non-immunized infected animals (from Khoury, Stromberg and Soulsby, 1977). Both the numbers of larvae that reach particular organs of the animals or that migrate through the Baerman apparatus might be variable in different animals so care is needed to evaluate the degree of protective immunity that has been apparently conferred to animals (Lloyd and Soulsby, 1985). Moreover, the mechanisms by which protective immunity is formed in immunized animals have still not been fully characterized.

In broad terms, attempts to characterise protective responses to A.suum in experimentally infected animals have used four types of worm

derived material for immunization of the animals, either singly or in combination, namely: infective eggs, infective eggs previously irradiated with ultra-violet light, worm extracts from larval or adult stages, antibodies and/or immune cells, or larval IVRS.

Taffs, 1964a, found that pigs that were re-infected with A. suum were able to establish "self-cure" by expulsion of the first population of adult worms that had been established in the small intestine. This phenomenon was also associated with a decrease in faecal egg counts and the presence of maximal levels of antibodies in serum (Taffs, 1964a). The latter phenomenon was considered by Taffs to be a major contributory factor to the protective immunity of pigs from Ascaris infection. Support for this possibility comes from observations that hyperimmune serum or colostrum from pigs that had been multiply infected with A. suum apparently induced partial protection to recipient piglets against challenge A. suum infection (Kelley and Nayak, 1965a and b). However, a later study did not confirm that hyperimmune serum did any more than sensitize animals to infection and did not confer more protection to recipient pigs than would be provided by re-infection (Rhodes et al, 1986). Other studies also questioned the importance of the humoral antibody response to protection against Ascaris infection of pigs and suggested that it was unimportant (Lunney, Johnson and Urban Jr., 1985). More success at conferring protective immunity against Ascaris to animals was achieved by passive transfer of serum or cells from hyperimmunized guinea pigs to recipient guinea pigs (Khoury, Stromberg and Soulsby, 1977). The antibody isotypes thought to be the most successful were IgG1+IgE given together, or IgG2, the former antibody types were suggested to

induce local hypersensitivity reactions and the latter to promote the binding of leucocytes to A.suum larvae (Khoury, Stromberg and Soulsby, 1977). Both events could make the local environment unfavourable for worm survival (see earlier, this chapter).

IgG1 and IgE antibodies are also thought to be important in the acquired immunity of mice to Ascaris infection (Brown, Crandall and Crandall, 1977). This was investigated using a mutant substrain of CBA/H mice, CBA/N, that is unable to produce antibody against T independent antigens and whose antibody response to T dependent antigens is decreased; both events due to a genetic defect with the phenotype xid (Scher, 1982). Infection of CBA/N mice with A.suum, followed by a challenge infection with the parasite resulted in the formation of protective immunity in these animals, although to a lesser degree that CBA/H mice and amounts of IgE and IgG1 in CBA/N mice were decreased in comparison to CBA/H mice (Brown, Crandall and Crandall, 1977). The above mentioned authors concluded that levels of IgG1 and IgE in infected animals were important to the development of host immunity.

The importance of the cellular immune response to the protective response against Ascaris infection was investigated in experiments where guinea pigs were injected with pooled cells from mediastinal hepatic or mesenteric lymph nodes and challenged with Ascaris (Khoury, Stromberg and Soulsby, 1977). Hepatic node cells alone were able to confer a reasonably high degree of protection to infected animals which the other two cell populations could not. This was suggested to be possibly due to a higher amount of antigen specific effector cells

and antigen reactive lymphocytes in the hepatic lymph nodes than in mediastinal or mesenteric nodes (Khoury, Stromberg and Soulsby, 1977).

The liver has been considered to be a major protective organ in animals that have been infected on more than one occasion with Ascaris (Soulsby, 1961; Taffs, 1964a and b). This has been suggested to correspond in guinea pigs with larval moulting occurring at the liver stage of parasitosis, which might result in the release of larval products that could be powerful mediators of host immune response (Khoury, Stromberg and Soulsby, 1977). However, some studies. have indicated that immunity at the level of the small intestine against early larval stages might also be significant in protection of multiply infected animals, and prevent larvae from reaching the liver of infected animals (Lunney, Johnson and Urban Jr., 1986) as suggested by the relatively few liver lesions in "protected" as opposed to non-immune pigs. The protective mechanisms in the A. suum resistant pigs are thought to be due to intestinal anaphylaxis (Urban Jr., 1986). Further evidence of the involvement of intestinal immunity in resistance to nematodiasis (in this case, non-specific) came from evidence of high levels of resistance to A. suum infection by pigs that were experimentally infected with transmissible gastro-enteritis virus followed by a single challenge infection with the parasite (Gaafar. Dugas and Symensma, 1973).

Tromba (1978a and b); and Urban Jr. and Tromba, (1982) asserted that high levels of protective immunity were apparently activated in pigs that were given U.V. attenuated A. suum eggs. The degree of protection was quantified by the significantly fewer larvae or adult worms that were present in the lungs and intestines of immunized animals

Immune responsiveness was detected in *in vitro* peripheral blood lymphocyte (PBL) assays with the various Ascaris worm antigens that might have been present at the interface with the host animals *in vivo*, namely, hatching fluid antigens from embryonated eggs (Ea) and culture supernatants from L2/L3 and L3/L4 worms. The antigen responsive PBL's did not contain immunoglobulin and were suggested to be T cells.

Attempts to stimulate resistance in various animals to challenge infection with *A.suum* using extracts of Ascaris larvae (Souslby, 1963; Crandall and Arean, 1965; Guerrero and Silverman, 1969) or adult worms (Berger and Wood, 1964; Bindseil 1969) have had varying degrees of success. In marked contrast to some other researchers who have failed to elicit reasonable levels of protection using homogenate extracts of *A.suum* larvae, it was claimed, by Benkova, 1982, that the material derived from ultrasound treatment of intact larvae was capable of eliciting high values of protective immunity (89% for L2 extract and 80% for L3 extract). This material presumably contained cuticular, as well as somatic antigens and it appeared that the products of L2 larvae were capable of inducing the highest levels of protection as well as stimulating the highest levels of circulating antibodies in immunized animals. Nevertheless, this is one of the few accounts of very high levels of protection using worm somatic products.

Stromberg and Soulsby, 1977, reported that L2, L3 or L4 larval homogenate extracts were ineffective at inducing significant protection in guinea pigs whilst adult worm extracts were able to

induce reasonable levels of protective immunity. In addition the highest levels of protection were induced in guinea pigs immunized with the products of larvae that were able to develop to L4 stages during *in vitro* maintenance; L3 larval secretory material was ineffective. Therefore it was considered that the moulting products of L3 larvae that had developed to the L4 stage *in vitro* were of relevance to the protection of immunized animals.

Further examination of *A. suum* L3/4 IVRS suggested that this material consisted only of a single product which stimulated resistance in guinea pigs to re-infection with Ascaris (Stromberg, 1979a). The basic assertion that the IVRS of L3/4 larvae consists of a single entity has not been supported by the results of later experiments performed by Urban and Romanowski, 1985, who find that the larval IVRS of L3/4 and IVRS L2 worms that had developed in L3 *in vitro* consist of numerous components visualized by SDS-PAGE and Coomassie Blue staining of the gels for protein. These products were not characterized in detail using immunochemical or biochemical techniques. Ascaris larval (L2/3 and L3/4) IVRS and egg hatch supernatants were capable of inducing reasonable levels of protective immunity (62%) in pigs when administered to these animals simultaneously after a primary infection of egg hatch products. This experiment attempted to reconstruct the presumed *in vivo* situation with migrating larvae releasing IVRS and promoting the establishment of protective immunity. The most effective immunization regime with IVRS involved the use of L3/4 IVRS to immunize pigs followed by a second infection with U.V irradiated eggs, after one week. This method gave 80% protection against a challenge infection (Urban and

Romanowski, 1985). The relatively low values of protection achieved in the above animals immunized with larval IVRS could be partly attributed to the lack of involvement of intestinal immunity as deduced by the relatively high number of liver lesions in pigs immunized with IVRS then challenged with Ascaris eggs, showing that the liver was the main protective organ in these pigs.

Evidence of sterilizing protective immunity to A. lumbricoides infection in humans is scarce, although there is the possibility of non-sterilizing immunity (Cohen, 1974) which might exist in pigs multiply infected with A. suum either naturally or during the course of experiments (See earlier, this chapter). Very few experimental infections of humans have been performed to evaluate the possibility of acquired resistance to ascariasis, presumably due to ethical and moral considerations. Additionally humans in Ascaris endemic areas are presumably infected with different doses of Ascaris, at varying intervals and, moreover, might be the hosts to a number of different parasites simultaneously, which could presumably have a synergistic or antagonistic effect on the course or longevity of Ascaris infection (Stephenson, 1980). Nevertheless, it has been shown that in areas endemic for Ascaris, the prevalence of infection appears to be of two types, either at the same level throughout different age groups (Chowdhury and Schiller, 1968; Arfaa and Ghadirian, 1977; Elkins, Haskwell-Elkins and Anderson, 1986) or, a more common finding, decreasing prevalence with age (Hlaing et al, 1984; Hpay et al, 1970; Tu et al, 1970). The latter pattern of prevalence is suggested to be an indication of protective immunity but, among other factors, it could also be argued that the social behaviour of adults is less likely to

lead to the ingestion of Ascaris eggs (Crompton and Pawlowski, 1985). Another factor which has been suggested to be important in acquired immunity to ascariasis is the frequency with which people are infected, that is, either seasonally or continuously throughout the year.

An immunological study of ascariasis in New Guinea showed that in the location with the highest infection rates in childhood, the serum concentrations of antibodies to Ascaris were also the highest (Jones, 1977). Adults in this community also had high concentrations of antibody but low egg counts. It was suggested that the inhabitants of this community were continuously infected but that adults had acquired some degree of immunity to re-infection which involved the termination of infection before worms reached the mature stages of development (Jones, 1977). Further apparent evidence for the existence of protective immunity in humans was provided by studies which indicated that lung lesions in seasonally infected humans (caused by migrating worms) were more pathologically severe than those of people that were continuously infected with the parasite (Gelpi and Mustafa, 1967). The latter community had apparently developed tolerance to infection with the parasite and to larval migration, whereas the interrupted transmission of infection to the former community had not apparently allowed the formation of protective immunity. This is supported by observations that there were few reports of Loeffler's syndrome in individuals from tropical regions which would be expected to be endemic for ascariasis (Spillman, 1975). This is supported by consistent findings of low worm burdens in communities who are in constant contact with Ascaris, even in the high risk groups, namely, children (Jung, 1954; Spillman, 1975).

Some reports apparently indicate that there is no resistance to re-infection by Ascaris exists in humans. These are based on data of the prevalence and intensity of Ascaris infection in humans and indicate that after chemotherapeutic deworming of communities, the prevalence and average intensity return to the pretreatment levels. Arfaa and Ghadirian, 1977, reported that the people of several Iranian communities endemic for ascariasis after deworming, expelled both mature and immature worms simultaneously at times investigated throughout the year. This suggested that the transmission of ascariasis was a continuous process. Overall, it is possible that some individuals or communitites might become resistant to Ascaris infection whilst other do not. The reasons for such disparity are not known.

The Larval IVRS of Ascaris

The products of nematodes can be classified into three rough functional and possibly overlapping groups. These are respectively: cuticular surface molecules, somatic products and in vitro released molecules. Secreted or in vitro released products of Ascaris are those which are derived from the culture supernatants of living larvae that are maintained in vitro in defined culture medium. Little is known at present of the IVRS of Ascaris larval or adult stages. However, investigations of the IVRS of other nematodes and their biological relevance to those parasites might provide valulable insights into the functions, if any are forthcoming, of Ascaris IVRS. In regard to the immunobiological manifestations of Ascaris infection, there are several possibilities, either singly or jointly, through which larval Ascaris allergens could be presented at the interface with the infected host, namely through larval moulting, after larval death and subsequent release of internal worm components, or by

secretion.

One of the most convenient experimental model systems which could be used to investigate the functions of Ascaris IVRS is the parasitic nematode Toxocara canis. The migratory route of T.canis newly hatched larvae in experimentally infected mice involves the liver, lungs and finally general circulation of the animals (Olson, 1962). In humans, infection occurs by ingestion of soil containing embryonated eggs of T.canis (Glickman and Schantz, 1981) and the development of T.canis is limited to the infected (L2) larval stage in these individuals (Glickman and Schantz, 1981). The pathological manifestations of the disease include clinical disorders such as hepatomegaly and transient pneumonitis (Glickman, Schantz and Cypess, 1979), peripheral eosinophilia (Sugane and Oshima, 1980) and raised levels of IgE against Toxocara antigens (Sugane and Oshima, 1983). Investigation of the products of T.canis worms which could contribute to the immunopathology of the resulting disease led to the in vitro culture of T.canis infective worms. These parasites have been found to survive in vitro for long periods in defined medium, during which they produce and release quantities of IVRS (de Savigny, 1975).

Experimental immunological and biochemical studies of the IVRS of infective stage T.canis worms have revealed a number of interesting features. Maizels, de Savigny and Ogilvie (1984) discovered that the components of IVRS were restricted in number, possibly derived from the worm surface and antigenic in RIP's with antisera from T.canis infected mice. At least some of the products of IVRS could have been released from the worms by surface turnover, an event which appears to

to be dependent on active metabolic processes in the worms (Smith et al, 1981) and which also appears to be a mechanism of generating IVRS in T.spiralis and T.pseudospiralis (Almond, McLaren and Parkhouse, 1986).

The possible involvement of IVRS in toxocaral immunopathology has been investigated generally using the products of L2 T.canis larvae. It was shown that two separate fractions of IVRS from the parasites, collected from column chromatography, were able to induce peripheral blood eosinophilia in mice, one of high molecular weight (Fr 1) and one of low molecular weight (Fr 2) (Sugane and Oshima, 1983). Fr 1 of IVRS also activated complement, was antigenic and also allergenic (Sugane and Oshima, 1983). These findings seem to infer that many of the pathological effects of T.canis infection in infected animals and perhaps humans could be activated by larval secretions, which could conceivably be an analogous situation to that of ascariasis, except that in the latter situation, larval moulting and the IVRS of different developmental stages could also be important to the pathogenesis of tissue lesions and other manifestations of the disease. Experiments in animals infected under laboratory conditions appear to support the view that T.canis worms released IVRS in vivo at least similar to those produced in vitro (Parsons, Bowman and Grieve, 1986).

IVRS have been found circulating in the serum of infected animals (Matsumura et al, 1984; Maizels, de Savigny and Ogilvie, 1984) and humans (Matsumura et al, 1984) and also localized in tissue sections of the liver of infected mice (Parsons, Bowman and Grieve, 1986), by anti-T.canis IVRS antibodies, in patterns that suggested larval migration. In addition, granulomas in the liver of infected

animals, that would be encountered during larval migration, appeared to contain IVRS material. It is interesting to consider that at least some of the pathology of toxocariasis (and by analogy, ascariasis), perhaps also in humans, could be attributed to physical damage to tissue by larval migration and by the release of IVRS by larvae.

Although the IVRS of nematodes could be relevant to the symptoms of the resulting disease in the infected individuals, the parasite itself could have various purposes in producing and releasing IVRS products. The production of IVRS by worms might be a natural part of parasite behaviour which could be important in the maintenance of worms in vivo. The evasion of the immune response by parasites is also a viable property of parasite IVRS. Various suggestions have been made on how worms manage of avoid total destruction by host animals. Perhaps one of the best known methods by which a parasite evades the host immune system comes from a non-nematode parasite, namely trypanosomes, which produce a surface and secreted glycoprotein that can be changed by a variant in subpopulations of trypanosomes allowing the parasite to keep one step ahead of the host's immune system (Vickerman, 1969; 1978; Tetley, Vickerman and Moloo, 1981; Borst and Cross, 1982).

The action of metabolically dependent turnover of surface antigens from nematodes in vitro in particular T.canis infective stage larvae, has been suggested to be a possible worm mechanism to avoid the host immune response (Maizels, de Savigny and Ogilvie, 1984). Attempts to bind fluorescein-bound antibodies to the surface of

T.canis larvae failed at 37°C due to the release of these surface molecules and antibodies from the worm surface (Smith et al, 1981). The process of surface turnover might occur to prevent antibodies and immune cells in vivo from adhering to the surface of the worms. This could also be the case for T.spiralis worms, where shedding of surface molecules was shown to occur during in vitro culture. Moreover, the rate of surface turnover appeared to be substantially increased when immune serum was added to worm cultures (Philipp, Parkhouse and Ogilvie, 1980).

There are a number of alternatives to the function of IVRS as "immunosorbent" for evasion of the host immune system. For instance it is possible that the process of release of surface molecules into the culture medium involves the exposure of new parasite product epitopes to the host immune system which diverts or at least causes a delay in the antibody response of the host animal. This was shown to occur for T.spiralis, where monoclonal antibodies raised against the surface antigens of each of newborn, infective and adult larvae reacted with epitopes of detergent solubilised surface antigens which are not present in living larvae (Ortega-Pierres et al, 1984) suggesting that the shedding of surface molecules in vivo could expose new antigenic determinants to the host animal which could delay or divert the host immune system because the host animal would need to make antibodies against the new epitopes.

Other possible functions of IVRS could involve factors which might be important to the growth and development of larvae in vivo. Newly hatched Ascaris larvae appear to go through a set pattern of migration through host tissues before return and final establishment in the

small intestine as sexually mature adult males and/or females. During the migratory route that the larval stages follow, penetration of host tissues takes place. It is reasonable to assume that enzymes, possibly lytic enzymes, could be secreted by the worms in order to break down cell walls and/or associated proteins. Enzymes such as non-specific esterases, thiol- and carboxyl proteinases have been found in extracts of adult Ascaris worms and recent exciting results show the presence of proteolytic activity in the IVRS of Ascaris larval stages (Knox and Kennedy, 1988). Additionally, the types of enzymes released were stage specific, which could comply with the differing requirements of tissue migrating stages (Matthews, 1977). Moreover, non-specific esterases have been found to be localised, among other internal worm structures, at excretory canals, via which route, these could reach the host-parasite interface (Lee, 1962).

The adult stages of the dog hookworm, Ancylostoma caninum secrete a proteinase that is able to cleave fibrin and elastin, the latter when derived from the extracellular matrix of rat vascular smooth muscle (Hotez and Cerami, 1983). The proteinase was suggested to have major properties that could be relevant to Ascaris larval adaptation in the infected host animal. First, it was suggested that the hookworm proteinase could be used in extra-corporeal feeding by the worms of the blood of from capillaries following proteinase mediated rupture of the small intestine (Hotez et al, 1985). Additionally this proteinase is suggested to inhibit blood coagulation and also aid in the rapid ingestion of intestinal material by worms (Hotez and Cerami, 1983; Hotez et al, 1985). Moreover it was found that L3 hookworm larvae were able to produce the proteinase although it was not

confirmed if the proteinase was actually secreted by the larvae. This finding suggests the possibility that hookworm larvae could use the proteinase for penetration through the skin of the host animal which might also be the case for the larvae of Schistosoma mansoni (McKerrow et al, 1983). Knox and Kennedy (1988) also included the above possibilities in their report and also a possible additional function for larval proteinases namely enzymatic cleavage of antibodies bound to the larval epicuticle shown to occur for another nematode (Auriault et al 1981).

Another enzyme which might be important to the establishment of nematodes in vivo is superoxide dismutase (SOD). This enzyme has been proposed to fulfil an important function for worms in vivo, that is to protect these organisms from superoxide radical ions (O^{-}_{3}) that could be produced in vivo by immune cells such as eosinophils (McCord, Keele and Fridovich 1969) and/or to protect worm produced superoxide ions that could be produced by aerobic metabolic processes occurring inside the parasites (Sanchez Moreno et al 1987). SOD. is produced and secreted as a dimer of Mr 36000 from infected larvae of T. spiralis (Rhoads, 1983) and has also been found in the whole adult worm extracts of several ascaridoid nematodes including Toxocara cati and Toxascaris leonina with the highest levels observed in T. canis and A. suum adult worms (Sanchez-Moreno et al, 1987). It could be imagined that larval stages of Ascaris worms would find it useful to neutralize the effects of cells such as eosinophils which appear to figure so prominently in the immune response to migrating parasites (see earlier, this section). In conclusion, it seems that parasites have evolved various strategies whereby they can co-exist with the host

animal in such a manner that the host response is not sufficient to result in the complete destruction of the parasites. However one potentially harmful effect of ascariasis could be suppression of the antibody response to other pathogens. Crandall and Crandall (1976) have reported the immunosuppression of antibody responses in mice infected with Ascaris eggs. There is limited larval development in these animals so it is possible that larval products could contribute to the suppressive effect with potentially serious consequences.

Importance of IVRS in Immunodiagnosis and Immunoprophylaxis

Among the different immunochemical methods used to investigate the surface of parasites, labelling with a radioactive isotope, followed by radio-immunoassay and analysis by SDS-PAGE (Kusel, MacKenzie and McLaren, 1975; Kusel et al, 1975; Parkhouse, Philipp and Ogilvie, 1981; Maizels, De Savigny and Ogilvie,1984) has several attractive properties. On occasion there is not sufficient in vitro secreted material from important human parasites for use in techniques such as immuno-electrophoresis or Ouchterlony gel double diffusion (Irving and Howell, 1982), and it is more convenient to use radiolabelling due to the property that technique has of allowing the labelling of minute quantities of antigens (Markwell and Fox, 1978) and the ease with which parasite macromolecules can be radiolabelled (Maizels, de Savigny and Ogilvie, 1984; Parkhouse and Ortega-Pierres, 1984). There are also a number of other attractive features of the above technique which can be exploited for use on the IVRS of nematodes in general, and also specifically for the larval stages of A.suum. For instance, radiolabelling of nematode produces and direct analysis in

radio-immunoassays and SDS-PAGE can circumvent requirement for purification of individual or collective secreted components (Philipp, Parkhouse and Ogilvie, 1980; Cabrera and Parkhouse, (1986). Second, each of the radiolabelled secreted molecules can be tested for antigenicity with serum from animals infected with the nematode species, and studies of the time course of antibody response to worm antigens could also be made (Philipp, Parkhouse and Ogilvie, 1980; Jungery and Ogilvie, 1982). Also, it could be possible to examine the molecules secreted by larvae during growth and development in vitro by different life cycle stages and to examine any immunological or biological similarity between them (Parkhouse and Clark, 1983; Urban Jr. and Romanowski, 1985). Moreover, it might also be possible to examine crossreactions and the biological aspects of crossreactivity between the antigens of different nematode species using radiolabelled molecules from different species, by RIP with antiserum from animals infected with one of the relevant species and examination of the products of the immunoassay by SDS-PAGE (Dissanayake and Ismail, 1980; Forsyth et al, 1981; Klenk, Geyer and Zahner, 1984; Cabrera and Parkhouse, 1986). Fifth, the specificity of antibodies and of certain antibody isotypes for components of worm IVRS could be measured in RIP's involving radiolabelled worm secretions. Finally, radiolabelled products that might be unique to the parasite could indicate which components of IVRS would be potentially suitable for immunodiagnosis of the helminth infection (Kaushal, Hussain and Otteson, 1982 Philipp et al, 1984).

At present there is a shortage of serological methods for the specific diagnosis of several helminth infections of humans including

A. lumbricoides. Those assays that usually claim high sensitivity for a particular nematode often do not measure other criteria which could be important to diagnosis, namely: specificity, crossreacitivity, precision and predictive value (WHO, 1975; Ogilvie and de Savigny, 1982). Part of the problem in devising good serodiagnostic methods for detecting nematode infections could be attributed to the use of non-specific and/or undefined antigens in various detection assays (Kagan, 1963; Kagan and Norman, 1974).

Possibly the most commonly used set of antigens in a number of helminth diagnostic assays are soluble extracts of whole worm homogenates or components purified from these (Kagan, 1963; Ogilvie and de Savigny, 1982). These antigens presumably comprise antigens from the worm surface, secretions and also from inside the parasite. There might be components derived from inside the parasites which might not be exposed to the host immune response in vivo except on parasite death, making it improbable that they will be good serological antigens. Additionally, there are the possibilities that internal proteinases in the homogenate extracts might degrade worm specific antigens and also that non-specific molecules could compete in immuno-assays with specific components (Ogilvie and de Savigny, 1982). Moreover, there is the major problem that the use of crude extracts for diagnosis could lead to the compromise of the diagnostic test due to crossreactivity between nematode antigens.

Studies have shown that the products of nematode parasites recovered from in vitro culture might be superior to other whole worm homogenate molecules for several reasons. First, if secreted products

are functional antigens, and necessary for parasite maintenance in vivo, these might be more specific for infection of animals and also present in the systemic circulation where these could stimulate antibody responses (Kaushal, Hussain and Otteson, 1982; Prasad, Kharat and Harinath, 1983; Malhotra and Harinath, 1984). In light infections, IVRS could be presented to the host animals in a slow and continuous manner, which could be analogous to the way parasite antigens would be presented to the host animal immune system using adjuvant (Bradbury, Percy and Strejan, 1974). Conveivably, the immune response against functional secreted components, such as enzymes, could be protective, therefore it could be worthwhile to immunize experimental animals with IVRS from a given nematode and to use the antibodies and/or immune cells from the immunized animals in protection experiments.

In vitro culture of living worms in defined medium should theoretically provide worm antigens that are not associated with host derived antigens or components of the host immune system (de Savigny, 1975; Stromberg, Khoury and Soulsby, 1977). Synthetic media have been used for the in vitro maintenance of some nematodes and allow the relatively simple collection of worm secretions without purification steps (de Savigny, 1975; Stromberg, Khoury and Soulsby, 1977; Speiser and Gottstein, 1984; Nicholas, Stewart and Walker, 1986). Parasite secretions collected in this way could be then directly used in solid phase assays for detection of parasite antigen specific antibodies and ideally without interference of the assays by somatic worm components (de Savigny, Voller and Woodruff, 1979; van Knapen et al, 1983; Maizels, de Savigny and Ogilvie, 1984; Speiser and

Gottstein, 1984; Nicholas, Stewart and Walker, 1986).

Use of IVRS in diagnostic assays has several advantages over the use of antibodies from infected individuals, including the possibility that antibody-based detection of parasitosis could face problems of false negative or positive results. The former could be generated in individuals infected with a nematode but with a lag period between infection and detection of specific circuating antibodies (Ogilvie and de Savigny, 1982). The latter results could be misleading because parasite specific antibodies could still be found in the serum of test individuals even after the infection had been terminated (Cypess et al, 1977; de Savigny Voller and Woodruff, 1979; Malhotra and Harinath, 1984). Alternatively, false positive results could also be found if different parasites with shared or similar antigens had infected the individual undergoing the diagnostic test. In contrast,it would be expected that nematode antigens could be detected concurrently with infection and would not persist in the circulation after infection had ended (des Moutis et al, 1983; Matsumura et al, 1984). Additionally, IVRS appears to be of enhanced species specificity and is proposed to be less crossreactive in immuno-assays than other comparable worm antigens (see earlier).

The arguments in favour of the use of IVRS in for serodiagnostic and immunophrophylatic use could also apply to the molecules secreted in vitro by Ascaris worms in defined medium (Stromberg and Soulsby, 1977; Stromberg, 1979a; Urban and Romanowski, 1985). As yet no methodical attempts have been made to use IVRS in immunodiagnosis of ascariasis although a limited amount of success has been claimed using an Ascaris derived enzyme (Mukerji et al, 1980), antigen from the

Moult of L3 Ascaris to the L4 stage *in vitro* (Stromberg, 1979, b) and also affinity purified components from adult Ascaris worms (Welch, Dobson and Chopra, 1986). The production of secretions by Ascaris worms *in vitro* could provide a good opportunity for the examination of the host-parasite interface of larval stages and the infected host, something which is relatively inaccessible from *in vitro* studies where it could be expected that larval surface and IVRS molecules could be bound to immunoglobulin and/or effector cells (Premaratne, Parkhouse and Denham, 1984). The major assumptions regarding IVRS from Ascaris worms would be that these are identical to the material produced *in vivo* by worms and are at least related to the material on the worm surface, thereby avoiding the need for separate investigation of surface molecules. Given these conditions it should be possible to investigate parasite secretions at the host-parasite interface that are responsible for the immunopathological manifestations of larval ascariasis, as well as the components of IVRS that contribute to the evasion of host immunity by worms and those that could be used in protection (namely, functional antigens). Eventually it is hoped that knowledge will be gained about important IVRS molecules to establish the basis for vaccine production against this widespread disease.

GENERAL MATERIALS AND METHODS

Parasite Maintenance and Products

Ascaris suum and Ascaris lumbricoides

Adult A. suum were obtained from the abattoir, Springburn, Glasgow, Scotland. These parasites were collected from the intestines of newly slaughtered pigs. A. lumbricoides adult worms were collected from two geographically separate locations. India and the West Indies. The worms from India were donated by Drs. M. R. Haskwell-Elkins and D B Elkins (present address Imperial College, London) who recovered them from the faeces of the members of a fishing community in Tamil Nadu, up to 48 hours after administering them with 10 mg/ml body weight of pyrantel pamoate (Mexin Pharmaceuticals, Bombay, India). Adult female parasites were placed in 4% formalin, packed in ice and flown to Glasgow, where they arrived in 7 days. Female adult A. lumbricoides from the Caribbean were donated by Dr. D.A.P. Bundy (present address Imperial College, London). These worms were recovered from the faeces of humans in St. Lucia, Jamaica, following the treatment of individuals with 100 mg mebandazole (Vermex, Janssen Pharmaceuticals, Belgium) and despatched to Glasgow by airfreight, within 24 hours of collection, in 2% formalin (containing 125 μ/ml nystatin) and packed on ice. Transit time was approximately 12 days.

Both A suum and A. lumbricoides worms were dissected for removal of uteri which were then suspended in a 50% solution of sodium hypochlorite (GPR 30169, BDH, Poole, Dorset, England) to liberate Ascaris eggs. The eggs were washed with deionized water at room temperature ten times by centrifugation on a mistral 4L centrifuge, at 1000g, for ten minutes each wash and finally resuspended, for embryonation, in 2% formaldehyde

containing 125 units/ml of the antimycotic agent nystatin (Peadiatric Oral Susplension; Squibb Ltd., London, England). Thirty days at 25°C in a wide-neck, 1 litre capacity, conicla flask were sufficient for full embryonation. This was judged by light microscopy, following hatching of the eggs by coverslip pressure, which revealed the presence of cast cuticles around the larvae indicating that moulting had occurred and that the larvae were at the infective L2 stage.

Routing artificial hatching of A. suum and A. lumbricoides embryonated eggs was performed by the following method. The eggs were washed by centrifugation on a Mistral 4L centrifuge, at 1000g, ten times with deionized water for then minute durations then resuspended in a solution of 50% sodium hypochlorite to decoat the eggs. This decoating reaction was performed at room temperature. Light microscope examination of hypochlorite-suspended eggs at regular intervals indicated if the egg coats were thin enough to facilitate artificial hatching. The eggs were then washed ten times, as above, with deionized water and five times with PBS-A. The eggs were hatched in 1-2ml aliquots using the shear forces generated by the action of the mortar and pestle of a tissue culture homogenizer (Product No: TKW-300-050 N, Gallenkamp, East Kilbride, Scotland). The extent of hatching was observed by light microscopy. Following the artificial hatch larvae were placed directly on a Baerman apparatus, to separate living larvae from dead and dying larvae and egg debris, and left for 4-5 hours at 37°C to allow migration through a sterile cotton wool plug into Ascaris culture medium.

This medium was a defined serum-free medium, RPMI-1640, modified according to the methods of Stromberg, Khoury and Soulsby (1977) and later by Urban and Douvres (1984). Namely; the concentrations of sodium pyruvate and giuthatione were adjusted to 1mM and 16μM respectively and a tripeptide, 1-glycyl-1-histidyl-1-lysine acetate tetrahydrate (Product No: G 1887, Sigma, Poole, Dorset, England) was added at a concentration of 400ng/ml, glucose was added at 1mg/ml and antibiotic protection was provided by penicillin, 100 iu/ml, streptomycin, 100 μg/ml and gentamicin, 125 μg/ml.

Both L2 and L3/4 larvae were cultured in this medium at 37°C at concentrations of 20000 larvae/ml for L2 larvae and 1000 larvae/ml for L3/4 worms. Whilst the worms were maintained in vitro, they released secretions into the culture medium. These are in vitro released secretions (IVRS). IVRS was collected in the formof culture supernatant following centrifugation of the worms in their culture medium in 10 ml sterile tissue culture tubes (Flow Laboratories, Ayrshire, Scotland) at 1500g for 10 minutes at room temperature. the IVRS material was filter sterilized with Millex GV low protein retention filters (Product No:SLGV 025 BS, Millipore Filters, Molsheim, France) and frozen at -70°C

Usually, in vitro cultures of L2 Ascaris worms contained few dead or moribund larvae and were maintained for one week without the need for further Baermann purification. Cultures maintained for more than 7 days were Baermannised when larval deaths exceeded 5% of the total number of larvae in vitro as viewed by light microscopy by a random count of 200 larvae. L2 larvae were rarely maintained in vitro for more than 3 weeks and L3/4 larvae were commonly cultured for 2 weeks. Observations of SDS-PAGE analysis of radio-iodinated L2 and L3/4 IVRS showed that the

secretions collected from worm cultures older than 7 days were usually identical to those collected earlier. The experiments described in this thesis involved IVRS from five different L2 cultures and at least seven L3/4 cultures. There was no discernable difference in the SDS-PAGE profiles of IVRS from different larval cultures. Each experiment on IVRS was performed in triplicate or on multiple occasions.

Ascaris L3/4 Extraction from Infected Rabbits

The technique used for recovery of lung stage Ascaris worms from infected rabbits was based on that used by Urban Jr. and Doubres (1981). New Zealand White male rabbits of 2-2.5 kg mass and 6 weeks old were routinely infected with 50000 fully embryonated Ascaris eggs. Seven days later, after which time larvae should have migrated to the lungs of the animals, the rabbits were killed by injection of 2.5 ml of the anaestheitic sodium pentobarbitone (May and Baker Ltd., Dagenham, England) into the marginal ear vein. the lungs were removed by dissection and sliced into pieces of approximately, on average, $3mm^3$ to facilitate removal of long stage larvae. The lung fragments were layered on to a 60-mesh Endecott sieve (250μm seive. Endecotts, London. England) and suspended in PBS-A that had been warmed beforehand to 37°C and contianed the antibiotics:penicillin, 100 iu/ml, streptomycin, 100 μg/ml and gentamicin, 125 μg/ml. The larvae were allowed to migrate through lung tissue into the solution for 2-3 hours. following ten washes by sedimentation in the same solution the larvae were placed on a Baerman apparatus as described for L2 larvae and eventually maintained in vitro in the modified meduim RMPI-1640 also described earlier.

Collection of Adult A suum Body Fluid (ABF)

Adult A suum worms were collected in distilled water in sealed containers immediately after their removal from the intestines of slaughtered infected pigs (Abbatoir, Springburn, Glasgow, Scotland). The worms were thoroughly washed with deionised water, followed by 10 washes with PBS-A containing the following antibiotics: vancomycin, 3 μg/ml; colistin sulphate, 7.5 μg/ml; nystatin, 125 units/ml and trimethoprim, 5 μg/ml (all from Mast Laboratories, Merseyside, England). Additional antibodic cover was provided by cephalexin, 40 μg/ml and gentamicin, 5μg/ml (Sigma Chemical Co., Poole, Dorset, England).

Petri dishes containing the worms were kept on ice and in an operating fume hood, to avoid possible inhalation of A suum body fluid allergens whilst the worms were slit at anterior, posterior and central positions with a sharp seeker used for animal dissections. The ABF was collected from all three areas with a pasteur pipette and pooled along with the ABF of several other worms. ABF was then placed in 1.5 ml centrifuge tubes (Eppendorf tubes, product No: 72.690, Sarstedt Ltd., Leicester, England) and centrifuged at 13000g for 30 minutes, at 4°C on a Micro Centaur centrifuge (Cat. No: 41137-2182, MSE Scientific Instruments Ltd., Sussex) to separate ABF from worm organ fragments and cellular matter. The supernatant (ABF) was removed and immersed in liquid nitrogen then immediately stored at -70°C.

Toxocara canis

Adult T. canis were collected by dissection of infected dogs in London (Dr. R. M. Maizeis, Imperial College, and Dr. M. W. Kennedy) and

also in Glasgow (donations also by Dr. H. V. Smith, Department of Bacteriology, Stobhill Hospital, Glasgow). The Toxocara1 worms were kept in 2% formalin with 125 units/ml nystatin. Given adequate aeration the eggs developed to full embryonation in the uterus of gravid female worms after 30 days at room temperature. The embryonated eggs were collected after dissection and prepared for infection of rabbits as described for Ascaris eggs.

Antisera

Animal Maintenance

NIH mice were maintained before and after infection with A suum eggs in cages of dimensions 48cm x 15cm x 13cm (M3 cages, North Kent Plastics Ltd., Kent). Cage bottoms were covered to a depth of 2.5cm of wood shavings (Bygates, Midlothian, Scotland). Wistar inbred rats were kept in cages of dimensions 38cm x 25cm x 18cm and New Zealand and White or Lops rabbits in cages of 48cm x 61cm x 18cm. All the animals were kept in a light: dark cycle of 12 hours: 12 hours, respectively. Mice and rats were fed on Oxoid diet (A & J Beveridge Ltd., Edinburgh) and rabbits on Diet 18 rabbit food (J. Stewart, Larbert, Scotland).

Five week old male NIH mice were purchased from Olac 1976 Ltd., Hull. 20-30 mice were used for the experiments described in this thesis. These were rested for one week then infected orally with 2000 A suum eggs in 50% agarose, administered through a blunt needle attached to a 1ml plastic syringe. The infection regime was continued at 28 day intervals. One week after the secondary infection, the mice were killed and cardiac blood collected and pooled. The serum was collected, after

the blood had clotted, by centrifugation at 3000g in 50ml centrifuge tubes at 4°C and removal of the supernatant. Serum was split into 20µl and 50µl aliquots in 1.5ml centrifuge tubes and immersed in liquid nitrogen then stored at -40°C. Individual aliquots were used when required. Later experiments with pooled serum from other A suum infected animals confirmed the reproducibility of the results. (F. Qureshi, not shown; L. Tomlison and M. W. Kennedy pers. comm.).

Twelve week old Wistar rats, originally purchased from Olac Ltd., Hull were bred at the Wellcome Laboratories for Experimental Parasitology, Glasgow. Ten rats were used for the experiments described in the thesis and the results were reproducible on repeating experiments several times (also confirmed by L. Tomlinson and M.W. Kennedy pers. comm.). The rats were infected orally at 28 day intervals by the same procedure discribed for NIH mice, bled for serum seven days after administering the secondary infection and the serum pooled and collected again as described for mouse serum.

Antiserum against A suum and A. lumbricoides (from India) was prepared in six week old Sandy Lops rabbits (Hyline Rabbits Ltd., Northwich Cheshire) which were also used for the preparation of other antisera described below. These animals were infected with 3000 fully embryonated eggs delivered to the stomach via umbilical canulae (35 cm length, size 8FG, Hospital Management Supplies, East Kilbride, Scotland) and bled from the ear at weekly intervals. The serum was collected from blood, 2 weeks after quaternary infection, as described for infected mice. The serum from single animals infected with either A suum or A. lumbricoides was used continuously in the experimetts described in this thesis. The reproducibility of the results is confirmed by infection,

subsequently, of different rabbits with Ascaris (M. W. Kennedy, pers. comm.)

Antiserum was raised against ABF by M. W. Kennedy using the following method: 1mg of ABF was combined with Freund's incomplete adjuvant (Cat no: 660-5720, Gibco,) and injected in an intramuscular location in a Sandy Lops rabbit. Seven days later the animal was given an identical injection. 60 days after the second injection 1mg of ABF was combined with Freund's incomplete adjuvant and injected at several locations on the back of the rabbit. Blood was collected from the rabbit 14 days later and the serum from this used in the experiments described in this thesis. Antiserum prepared against the Mr 14000 component of ABF was also prepared by M. W. Kennedy using the following method: ABF was electrophoresed on a 1.5mm thick, 20% homogenous SDS-PAGE gel and stained for protein with Coomassis Blue (Product no: B 0630, Sigma Poole, Dorset). Following this procedure, the gel was destained and washed in distilled water three times for ten minutes each. The Mr 14000 band was located and the appropriate part of the gel was cut out using a scalpel blade. This gel portion was ground, in liquid nitrogen, using a mortar and pestle and then mixed with #Freund's complete adjuvant (Cat No: 660-5721, Gibco, Paisley, Scotland) and the emulsion was injected into several sites on the back of a Snady Lops rabbit. Forty days later, the rabbit was again injected with an emulsion of Mr 1400 component (in the gel matrix) and Freund's incomplete adjuvant (Cat No: 660-5720, Gibco, Paisley, Scotland) at several sites on its back then blood was collected for serum 19 days later and used for experiments described later. Experiments performed by M. W. Kennedy and colleagues (pers. comm.) with other rabbits in which other rabbits have been immunized against ABF or the Mr 14000

component of ABF confirms that the results obtained for this thesis can be reproduced.

Antiserum was raised against T. canis in a Sandy Lops rabbit infected initially with 20000 fully embryonated eggs creating a situation where there was persistent parasitism. Serum was collected from these animals at 35 days after infection then at weekly intervals thereafter. Antiserum raised in goats against whole rabbit serum was bought from Nordic, Berks., England (Product No: GAR/Alb). Sheep anti-rabbit whole serum was purchased from Serotec, Oxon., England (Product No: AAS 09).

Radiolabelling of Nematode Surface and Secretions

Radio-iodination of Parasite In Vitro Released Secretory (IVRS) Products

The methods for labelling were based on those used by Markwell and Fox, 1978). Namely, IODO-GEN (1,3,4,6;tetrachloro,3a,6a diphenyl glycoluril) (Pierce Chemical Co.; 28600, Illinois, USA) was dissolved in chloroform at a concentration of 2 mg/ml, then dried to the inside of 1.5 ml centrifuge tubes (Sarstedt Ltd.; 72.690, Leicester, England). 200 ul of PBS-dialysed IVRS materials were added to these tubes.

Following the addition of 200 uCi of ^{125}I (West of Scotland Radionuclides Dispensary, Western Infirmary, Glasgow, Scotland), the mixture was agitated occasionally on ice for 10 min. Unbound ^{125}I was reacted with a solution of saturated L-tyrosine in PBS-A, added to 10% v/v of the total volume for 5 min. on ice.

The contents of the centrifuge tubes were added to "Exocellulose" desalting columns (Pharmacia; 20440) to allow the separation of radiolabelled macromolecules from unbound iodide.

Fractions representing the radiolabelled material were pooled and were kept frozen at -20°C until use.

Biosynthetic Labelling of Ascaris Larval Products

Endogenous sources of L-methionine were depleted from 20,000 Ascaris L2 or 5,000 L3/4 larvae during the *in vitro* maintenance of these parasites in L-methionine-free Minimal Eagles Medium, (MEM) Glasgow Modification, purchased from Flow Laboratories; 16-222-49, Ayrshire, Scotland. Supplements were added as mentioned previously which were required for larval maintenance. Four days later, the medium containing IVRS products of the worm, was removed and replaced by fresh medium containing 1 mCi of L-^{35} [S]-methionine (Amersham; SJ.204, Buckinghamshire, England). The larvae were cultured *in vitro* at 37°C in 5% CO_2: 90% N_2: 5% O_2 for 3 days after which the supernatant containing radioactive IVRS material was removed and filtered through 0.22 um pore size, low protein-retention filter (millipore slgv 025 BS, Molsheim, France). Following overnight dialysis against PBS-A, the intrinsically-labelled IVRS was stored at -20°C.

Detection of Parasite-Specific Antigenic and Carbohydrate Components Radio-immunoprecipitation

The antigens of radiolabelled in vitro released secretory (IVRS) products were examined by radio-immunoprecipitation (Maizels, De Savigny and Ogilvie, 1984). 2.5 ul of antiserum were added to LP3 tubes (Luckhams, England) containing 50 ul of 0.5% Triton X-100 in PBS-A. 50-200,000 cpm of radiolabelled IVRS products were added to each tube and the mixture left overnight at 4° C. To select for IgG (Kessler, 1975). 50 ul of Staphylocuccus aureus suspension (Pansorbin Cells, Standardized; Cambridge Bioscience, Cambridge, England) were added. The mixture was left for 1 hour at room temperature with occasional agitation followed by 2 hr at 4°C.

The S.aureus pellets with antibody bound to labelled antigen, absorbed on to their protein A, were washed 3 times by resuspension in 0.5% Triton X-100 in PBS-A, followed by centrifugation at 1,500 rpm for 15 min. at 4°C on a Mistral (MSE) 4L general purpose centrifuge.

In order to co-precipitate a wider range of antibody classes than IgG, 2.5 ul of an anti-immunoglobulin antiserum were added to the mixture of antigen and test antiserum. After incubating at room temperature for 1.5 hr, 50 ul of the S.arueus suspension were added. The washing procedure followed that for absorption by S.aureus.

Lectin-Mediated Precipitation

The following lectins conjugated to Sepharose 4B (50-250 um) were purchased from MACOM (Hamilton, Scotland, U.K.); Bandeiraea simplicifolia lectin, GS-I A-2401 (lactose of galactose-binding); concanavalin A; Con-A, A-1104 (Mannose); Lens culinaris lectin, LcH (Mannose); Ulex europus lectin, UEA-I, A-2101 (N-acetyl-glucosamine); Helix pomatia agglutinin, HPA, A-3601 (N-acetyl-galactosamine); Arachis hypogea

lectin, PNA, A-2301 (lactose or galactose).

0.5 ml of these lectins were washed six times by centrifugation (1000g, 4°C) for 5 min in the incubation buffer. This was Tris-HCl pH 7.0 containing 15mM NaCl, 1.0mM $CaCl_2$ dihydrate, 1.0mM $MnCl_2$ tetrahydrate, 0.5% Triton X-100 and 0.1% sodium azide. An equal volume of this buffer was added to lectin to make a 50% solution.

100 ul of the appropriate lectin was transferred to duplicate LP3 tubes and 200-500 x 10^3 cpm of radio-iodinated parasite material added. Following a 1 hour incubation at room temperature, with frequent agitation, the complex was washed three times by centrifugation, (1000g, 4°C, 5 min). To reduce background counts, the contents of each tube were transferred to new LP3 tubes and three more washes by centrifugation were performed.

To one half of the duplicate tubes, the appropriate specific inhibiting carbohydrate was added at optimum molarity for elution of lectin bound material. The tubes were left overnight at 4°C with frequent agitation for the first hour. The concentrations of sugar used were as follows: for GS-I, 0.2M galactose: con-A, 0.1M -methyl mannopyranoside; LcH, 0.1M -methyl mannopyranoside; UEA-I, 0.05M L-fucose; WGA, 0.2M N-acetyl glucosamine; HPA, 0.2M N-acetyl galactosamine; PNA, 0.2M galactose.

The supernatant was removed and combined with an equal amount of sample buffer containing 1 mg/ml iodoacetamide then immersed for 10 min in a water bath at 100°C, before submitting the samples to SDS gel electrophoresis. The pellet bound material was extracted by the addition of 40 ul of the abovementioned sample buffer.

Meanwhile, the supernatants and lectin pellets from the other half of the remaining control tubes, containing no eluting sugar, were

Proteinases and Parasite Secretions

Radio-iodinated IVRS of A. suum were incubated at fixed amounts (estimated as 2mg and 28ng for L2 and L3/4 respectively) with a range of concentrations of proteolytic enzymes. Rat Mast Cell Proteinases Type I (registry no:82599-73-3) and type II (registry no: 82599-74-4) were recovered from specific types of rat mast cells, purified and donated to Wellcome Parasitology by S. Gibson and H.R.P. Miller (Moredun Research Institute, Edinburgh). 0.4 - 250ug/ml RMCP I or II were reacted in Eppendorf tubes with above mentioned amounts of ^{125}I labelled IVRS in PBS-A (pH 7.5)for 2 hours. The reactions were terminated by boiling the samples with an equal amount of SDS-PAGE sample buffer containing 5mM Tris (pH 7.5), 0.1mM ethylene diamine tetracacetic acid (EDTA), 1% glycerol and 0.1% bromphench blue. A number of proteinase inhibitors purchased from Sigma Chemical Co. Ltd., Dorset, England had been added to modify this sample buffer, namely: 0.2mM phenylmethylsulphonyl fluoride (PMSF, product No: P-7626) in isopropanol, 5mM pepstatin A (product no: P-4265), 100mM 1, 10 - phenanthroline (P-9375), 5mM leupeptin (L-2884), 5mM antipain (A-6271), 25 mg/ml N-tosyl-l-lysine chloromethyl ketone (TLCK, product no T7254) and 25mg/ml N-tosyl-l-phenylalanine chloramethylketone (TPCK, product no:T 4376). The samples were then immersed in a boiling water bath for 10min. and the products of the enzyme reaction were analyzed by SDS-PAGE.

Treatment of radio-iodinated IVRS with trypsin (EC 3.4.21.3, cat. no: 109 819, Boehringer Mannheim, East Sussex, England) or chymotrypsin (EC 3.4.21.1, cat. no: 103 306, Boehringer Mannheim) was performed at 37°C for 2 hr in an incubation buffer containing 100mM Tris/HCl,pH 8.0, and 50mM $CaCl_2$. The reaction products were treated and analyzed as described above.

identically treated and similarly analysed by SDS-PAGE in order to evaluate the degree of lectin specificity for secreted parasite sugars.

Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Gel Casting 0.7mm, 5.25% gradient gels

Gels were cast using the method suggested by the manufacturers (Pharmacia Ltd., Milton Keynes, England). Glass cassettes were prepared for the gels in the following way; each cassette comprised two glass plates of dimensions 14 cm X 18 cm X 2.7 mm (Pharmacia; 19-4904-01) separated by spacers measuring 14 cm X 0.5 cm X 0.7 mm (Pharmacia; 19-4929-01) and sealed at the shorter sides with waterproof adhesive tape.

The cassettes were than inserted into a casting stand which was kept in the vertical position on a levelling table.

Since gel formation was inhibited by the presence of oxygen, buffer B (see section on Buffers and Culture Media) was pumped into the bottom of the casting stand, using a peristaltic pump (LKB Bromma 2132 Microperpex Peristaltic Pump 2132-002; LKB, Sweden).

Solutions of 5% and 25% polyacrylamide with the appropriate amount of 10% ammonium persulphate were poured into two separate compartments of a mixing chamber (Pharmacia GM-1, 19-0485-01) and allowed to mix using the accompanying stirrer (Pharmacia GM-a, 19-0485-01), whilst being pumped into the bottom of the casting stand using the peristaltic pump set at 10 ml/min.

After the solutions had been pumped through the gel mixer, the

dead space below the gel cassettes at the bottom of the casting stand was filled with 50% glycerol in water containing 0.1% w/v bromophenol blue which was denser than the polyacrylamide solutions.

Sample Loading

The products of either ^{125}I or intrinsic labelling were made up to a volume of 20 µl with deionized water in 1.5 ml centrifuge tubes. After the addition of an equal amount of sample buffer (Maizels et al, 1984), these samples were immersed in a boiling water bath for 10 min. before transfer on to 5-25% SDS-polyacrylamide gels.

The pellets from S. aureus immunoprecipitations with or without adsorbed anti-immunoglobulin antiserum had 20 µl of deionized water added to them followed by an equivalent amount of sample buffer. After resuspension of the pellet using a vortex mixer, the samples were placed in a boiling water bath for 10 min. then centrifuged at 13,000 g on a Micro Centaur centrifuge for 5 min. The supernatants were loaded on to 5-25% SDS-polyacrylamide gels for analysis (Maizels et al, 1984).

Fixing and Drying

Gels were removed from their glass cassettes and fixed in 25% methanol, 7.5% glacial acetic acid and 1% glycerol.

After 30 minutes at room temperature, the gels were removed from the fixing solution and dried onto filter paper (Maizels et al, 1984) using a Bio Rad Slab Gel Dryer Model 1125B (Bio Rad Laboratories, Hertfordshire, England).

IMMUNOCHEMICAL CHARACTERISATION OF THE *IN VITRO* RELEASED SECRETIONS (IVRS) PRODUCED BY LARVAL STAGES OF *ASCARIS SUUM*

Summary

The *in vitro* released secretions (IVRS) of *Ascaris suum* L2 and L3/4 worms were examined by radiolabelling with ^{125}I, followed by radio-immunoprecipitations and SDS-PAGE. The multi-component IVRS of L2 or L3/4 parasites (the latter worms were recovered from the lungs of infected rabbits) appeared to be of different apparent molecular weights (Mr) for each developmental phase, and most were antigenic with antiserum from *A. suum* infected animals. However, a major component of Mr 67000, from *A. suum* L3/4 worms, was not recognised by antibodies from infected rabbits, and was subsequently found to be rabbit serum albumin. Moreover, this component appeared to have been processed by the worms, and could have been internalized by these parasites prior to release *in vitro*.

Antisera from *A. suum* infected rats and mice recognised fewer products of IVRS than the antibodies of infected rabbits. Mouse antibodies, in particular, were notable for not recognising the Mr 14000 antigen that is apparently secreted by L2 and L3/4 larvae. These findings could be important to the study of various immunological aspects of ascariasis. The differences in host antibody response to *A. suum* might have a genetic basis, but this possibility was not investigated further. The antisera, described above, from different species of infected animals, were also used to examine the antigenicity of radio-iodinated body fluid products from adult Ascaris parasites (ABF). This material was shown, in general, to be inferior to IVRS as a target antigen for antibody responses. Additionally, the major antigen of ABF was found to be of Mr 14000, which was identical to the antigen of this molecular weight from larval IVRS. Moreover,

immunoprecipitation of A. suum L2 and L3/4 antigens, with antiserum from rabbits infected with the nematode Toxocara canis, showed that the Mr 14000 antigen was the only IVRS component that was not apparently recognised by this antiserum.

A.suum L2 and L3/4 larval IVRS, or perhaps individual components, such as the Mr 14000 antigen, might eventually be of importance in devising methods for the detection of Ascaris infestation in humans and animals. However, the immediate implications of this study are that previous claims, stating that the IVRS of L3/4 worms (Stromberg, 1979, a, and b) was a single product which had several immunological properties, should be more thoroughly investigated. In addition, animals which are to be experimentally infected for the immunochemical analysis of Ascaris antigens should be carefully chosen for an immune response to these components.

Introduction

Immunological studies involving the characterisation of antibody or cellular responses of Ascaris infected animals to A.suum have frequently used extracts of ABF (O'Donnell and Mitchell, 1980; Rhodes, Keralis and Staudinger, 1982; Tanaka, Baba and Torisu, 1979), or extracts of the homogenates of adult worms (Crandall and Crandall, 1967 and 1971; Jones, 1977; Laubach, 1985; Sasagawa, Suzuki and Fujikura, 1987) as target antigens. However the in vitro released secretions (IVRS) of nematodes have been recently receiving increasing attention as an alternative source of antigens, mainly for serodiagnosis and immunoprophylaxis, due to the several possible advantages that the secretory products might offer over the alternative worm antigens (Gen. Intro.). One of the arguments for the use of IVRS in the detection of parasite specific antibody responses, in infected hosts, is that IVRS could be produced by living parasites. In contrast, worm homogenates, and perhaps also ABF, may contain components that might not be the subjects of host antibody response, except perhaps following worm death. Other supporting arguments for the use of secretory molecules have been previously presented (Gen. Intro.).

Among the range of antigens produced by A.suum worms, some are capable of stimulating animals to produce high levels of IgE (Gen. Intro.). ABF has been the usual source of these allergens for immunochemical study, (Gen. Intro.) but it was suggested that the larvae of Ascaris were also capable of the secretion of allergen, in vivo, (Bradbury, Percy and Strejan, 1974) and in vitro (Stromberg, 1979 a and b, 1980). The in vitro released allergen of A. suum L3/4

worms has been described by Stromberg as the single secreted component of these worms, and is of relative approximate molecular weight 67000. Moreover, this secretion apparently has several biological properties, in addition to its capacity to induce high levels of IgE in infected guinea pigs (Stromberg, 1979 a and b), for example, as a factor that potentiates the IgE response of animals to antigens unrelated to those of Ascaris worms (Stromberg, 1980). Furthermore, it is also claimed that the single secreted molecule was apparently protective in guinea pigs, administered with IVRS followed by infection with A. suum infected eggs (Stromberg, 1979 a). It is important, to the study of the immunobiology of larval ascariasis, that these findings are confirmed. One of the most convenient techniques for the analysis of parasite-derived material is to radiolabel the IVRS of larvae, apply various immunochemical techniques to the radioactive parasite products, and analyse the products by SDS-PAGE. These techniques have been successfully used to examine the products of other nematodes (Gen. Intro.).

The study described in this chapter involved an immunochemical investigation of the IVRS released from A. suum larvae of L2 and L3/4 developmental stages. The secretions of these larvae appear to be specific to individual stage of worm development, although this stage specificity is not total, and also highly antigenic with antiserum from A. suum infected rabbits. Additionally, lung stage larval IVRS appeared to be represented as several components, by SDS-PAGE analysis, thereby questioning Stromberg's assertion that several immunobiological properties could be ascribed to a single A. suum IVRS molecule.

Results

A. suum Larval IVRS

Culture supernatants from A.suum L2 and L3/4 larvae, maintained in vitro, were radio-labelled with ^{125}I, using IODO-GEN, and analysed by SDS-PAGE (Figs. 3.1 and 3.2). IVRS from L2 worms comprised a limited number of components of the following Mr: 14000, a band of diffuse appearance spanning 20-28000, 60000, and also an entity of Mr 225000, that was only visible in gel autoradiographs of longer exposure time. The secretions of L2 worms were not apparently affected by treatment with β-2 mercaptoethanol.

Radio-labelled IVRS of pulmonary stage larvae was of radically different appearance on SDS-PAGE (Fig. 3.2) than that of the earlier developmental stage and lung stage worms also appeared to secrete a greater number of products. L3/4 IVRS contained two components that were of equivalent Mr to those of L2 worms, namely, of Mr 14000 and 225000. The remaining L3/4 secretions were observed at Mr 25500, 41000, 67000, 118000 and 410000, respectively. In contrast to the situation for infective (L2) larvae, two components of lung stage larval secretions were reducible to entities of lower Mr, namely, those of Mr 225000 and 410000. Reducing conditions also altered the mobility, in SDS-PAGE, of the Mr 410000 molecule and one half of an Mr 67000 moiety. This latter component co-migrated in gels with bovine serum albumin, from the radio-iodinated molecular weight standards, even following β-2 mercaptoethanol treatment, when the migration properties of serum albumin are altered so it gives the impression of having increased in molecular weight.

Antigenicity of A. suum Larval Secretions, and Crossreactions with Anti T.canis Antiserum

Antisera, from rabbits infected at multiple intervals with A. suum infected eggs, or the nematode parasite, Toxocara canis, were used in SRIP's, along with the IVRS from A. suum second and lung stage larvae. The immunoprecipitates were analysed by SDS-PAGE (Figs. 3.3 and 3.4). Antisera from uninfected rabbits, from the same batch as the infected rabbits, were used as experimental controls for non-specific precipitation of Ascaris secretions, and/or to detect possible crossreactivity of A. suum antigens, with antibodies from those animals which may have been previously infected with parasites during the time that they were reared, prior to experimental infection with Ascaris.

The results show that all the radio-labelled sécretions of second stage A. suum larvae are antigenic with the infected rabbit antiserum (Fig. 3.3). Lung stage larval IVRS was also antigenic, with the notable exception of a major ^{125}I labelled product of Mr 67000 (Fig. 3.4). Antiserum from uninfected rabbits did not perceptibly precipitate any secretions of L2 and L3/4 larvae. Additionally, the antiserum from T. canis infected rabbits recognised the majority of the radio-labelled IVRS products of A. suum L2 and L3/4 larval stages, with the exception of the Mr 14000 component.

A. suum L3/4 IVRS Contains Host Serum Albumin

The failure of rabbit anti-A. suum antiserum to precipitate an Mr

67000 molecule of L3/4 IVRS and the alteration in SDS-PAGE mobility under reducing conditions suggested the possibility that this molecule was serum albumin, possibly derived from the host animals from which the L3/4 worms were originally recovered. This possibility was investigated in RIP's with ^{125}I labelled lung stage worm secretions and two separate antisera, namely, anti-rabbit serum albumin, or, anti-rabbit whole serum (Fig. 3.5). The results were identical, and an Mr 67000 entity was precipitated by either antiserum, along with unexpected components of lower Mr. The above immunoprecipitations were repeated with increasing amounts of purified unlabelled rabbit serum albumin to compete with the radio-labelled form of this molecule, and all of the precipitates were analysed by SDS-PAGE (also in Fig. 3.5). The precipitation of albumin, from L3/4 IVRS, and also the lower Mr molecules, was effectively prevented by the unlabelled albumin, showing that L3/4 IVRS contains rabbit serum albumin.

Species-Specific Recognition of A. suum Larval Antigens

Experimentally infected animals have been used to investigate various features of Ascaris immunology, including host animal antibody and cellular responses (Gen. Intro.). Differences of host response to Ascaris antigens could therefore be important, in the choice of animals for the production of, for instance, Ascaris specific antisera which could be used in diagnosis or for protection of animals against further infection with Ascaris. Serum from Lops rabbits, Wistar rats or Balb/c mice, given at least tertiary infections with *A. suum*, were reacted in SRIP's with ^{125}I labelled *A. suum* IVRS from L2 or L3/4 worms and the immunoprecipitates analysed by SDS-PAGE (Figs. 3.6 and

3.7, respectively). Each species of infected animals appears to recognise a unique set of IVRS bands on SDS-PAGE, but rat and mouse antibodies only precipitate a limited subset of the available antigens, and Balb/c mouse antisera, in particular, do not recognise the Mr 14000 molecules from the IVRS of either infective or lung stage parasites. Rabbit antibodies, in contrast, precipitated all of the labelled products of L2 larvae and most of the components of L3/4 larval secretions, except for host serum albumin.

Arguably, the use of S. aureus as an immunosorbent could have introducted an isotype bias into the above assays. Anti-IgG antisera were therefore used in repeat immunoassays; otherwise identical to those described above. Anti-IgG should precipitate most isotypes through immunoglobulin light chain crossreactions, but the results (not shown) indicated that there were no significant qualitative or quantitative differences in the SDS-PAGE profiles of precipitates analysed after anti-IgG immunoprecipitation.

Adult A. suum Body Fluid (ABF) Antigens

The relative complexity, and antigenicity of ABF, in comparison to A. suum larval secretions, was investigated by an SRIP of radio-iodinated ABF with serum from A. suum infected rabbits. The precipitates were analysed by SDS-PAGE (Fig. 3.8).

Although A. suum does not normally develop to adult stages in rabbits, the antibodies of infected rabbits, presumably raised against larval antigens, recognise several components of ABF, suggesting that there might be similarities between ABF and juvenile worm secretions.

Apparently, the dominant antigenic molecule of ABF, is of Mr 14000, judging by the abundance of this molecule in SDS-PAGE gels, that were treated with the dye Coomassie Blue, (M. W. Kennedy, pers. comm.) and also by the gel profiles of radio-iodinated ABF antigens. In addition, there are two other major antigens of Mr 30000 and 41000, and minor antigens or Mr 58000, 60000 and 78000, respectively.

Species-Specific Recognition of ABF Antigens

SRIP's were performed with radio-labelled ABF and antisera from A.suum infected rabbits, mice, or rats, that had been previously employed in immunoprecipitations of larval Ascaris IVRS. Analysis of the ABF precipitates by SDS-PAGE (Fig. 3.9) showed that the rat antibodies did not precipitate ABF products quantitatively as well as those of infected rabbits, but did apparently recognise an Mr 14000 molecule. Infected mice, in contrast, might be classed as non responders to the antigens of the perienteric fluid of adult A. suum. The results of the above precipitations, are presented in Table 3.1, as percentages of acid precipitable radioactive counts for ^{125}I labelled ABF, L2 or L3/4 IVRS. Using this criterion, larval IVRS material and particularly L3/4 IVRS, are generally better target antigens for the antiserum of infected animals, than ABF. Additionally, the difference, in quantity of antigen precipitated, between uninfected and infected host animals, was also the greatest for larval antigens, except for the reaction of ABF with rabbit antiserum.

The heterogeneous antibody responses to the Mr 14000 component of ABF, and perhaps also of A. suum larval stages, by rats, rabbits, or

mice, might reflect their level of responsiveness to this entity, considering that it is apparently the major antigen of ABF. This particular component might also be similar to the Mr 14000 antigen of larval stages, although confirmation is required of any biological similarity.

The Mr 14000 Secretion of Larval A. suum and from ABF are Identical

The identity of the Mr 14000 secretion of juvenile A. suum and of the ABF product was confirmed by a radio-immunoprecipitation involving antiserum from A. suum infected rabbits and ^{125}I labelled A. suum L2 IVRS, in the presence of increasing amounts of unlabelled ABF. The precipitates were analysed by SDS-PAGE (Fig. 3.10). ABF would presumably contain the Mr 14000 component, that could compete with radio-labelled Mr 14000, of parasite origin, for binding to the test antiserum. The results clearly demonstrate that there was efficient inhibition, by unlabelled material, of the L2 A. suum Mr 14000 antigen.

The cold target inhibition experiment, described above, was repeated with A.suum L3/4 IVRS (Fig. 3.11) and the results were essentially the same, namely, the ^{125}I labelled Mr 14000 component of A. suum IVRS was inhibited from binding to the anti-A. suum antiserum and was, therefore, absent from the gel profile of the immunoprecipitates.

Discussion

During tissue migratory phases in Ascaris infected hosts, each individual developmental stage of larvae might pose unique pathological and immunological challenges to the infected animal. Immunochemical characterisation of the antigens produced by the worms at different phases of development may allow insights into the biology of ascariasis. The first accounts were presented here, of immunochemical studies on the radio-iodinated secretions of A. suum larval stages. Gel analysis shows that A. suum infective (L2) larvae apparently secrete a different set of molecules to those of lung stage worms. If the secretions of Ascaris worms were ascribed with some biologically important function, the different gel profiles of the IVRS from separate life cycle stages could concur with the particular immunopathological symptoms displayed by individuals with liver or lung larval Ascaris (Gen. Intro.).

The origins of the molecules collected from the supernatants of A. suum juvenile worms in vitro, and nominated as IVRS, are uncertain, although there are several possibilities, including the excretory and anal pores of the nematodes, and also the worm surface. Examination of the radio-labelled surface of IVRS antigens of A. suum L2 larvae apparently indicates that at least several components of larval IVRS are derived from the parasite cuticle, reflecting the situation for T. spiralis and T. canis (Gen. Intro.). For pulmonary stage worms, IVRS could also, additionally, consist of products released in vitro following larval moulting.

These findings, presented here, show that radio-iodinated A. suum

L3/4 IVRS contains a number of entities of distinct Mr, also including two components of Mr 67000. One of these components is a major labelled element of radio-labelled IVRS, and was shown to be rabbit serum albumin, the other is of parasite origin and recognised by antibodies from Ascaris infected rabbits. Biological assays were not performed on the latter molecule, so it is not possible to investigate the claims regarding this molecule. However, the IVRS of L3/4 worms does not consist of a sole product and hence invites a re-examination of the data concerning the properties of the IVRS component investigated by Stromberg (Intro., this chapter).

Host animal albumin has been found on the surface of several filarial nematodes (Forsyth, Copeman and Mitchell, 1984; Maizels et al. 1984; Philipp et al, 1984) and a mechanism can be conceived for Ascaris whereby rabbit albumin is absorbed to the surface of lung stage worms and then subsequently shed during the normal course of surface turnover. This possibility is supported by evidence that surface molecules have been released by nematodes in vitro as constituents of IVRS (Maizels et al 1984). However, the possibility that albumin could have been ingested by the worms, and then excreted into the in vitro culture supernatant, cannot be ruled out. The rabbit albumin could have been acquired, by worms, from the host animal bloodstream, and/or damaged host tissue, during the migration phase of these parasites.

Cold target inhibition experiments showed that apparent fragments of albumin were also present in the IVRS of A. suum L3/4 worms, along with the intact molecule, which was the predominant form. These findings suggested that the albumin may have been processed by

proteolysis, possibly by worm enzymes, either inside the worms, or by enzymes released from the parasites *in vitro*. It is not known if some, or all the components of IVRS are derived by a proteolytic event, but the SDS-PAGE profile of Ascaris IVRS remains unchanged after the inclusion of proteinase inhibitor in larval cultures (illustration not shown).

Therefore, it is likely that host albumin is internalised by L3/4 Ascaris worms, and then later expressed from the worm as a feature of IVRS, possibly involving the worm surface as an intermediate site. The absorption of host albumin, and possibly other host substances, such as blood group antigens to the surface of nematodes (Dean and Sell, 1972; Leventhal and Soulsby, 1976; Smith *et al*, 1981; Goldring *et al*, 1976) might provide a mechanism for reducing the antigenicity of the worm cuticle, thereby reducing the binding of antibody and phagocytic cells to the parasites.

Considering the potent antigenicity of *A. suum* L3/4 IVRS, it is surprising that host animal immunoglobulin was not found among the secretory products of these worms. However, it is possible that the rabbits infected with *A. suum*, for the purpose of harvesting lung stage parasites, might not have been able to raise an antibody response of sufficient magnitude by the time that larvae had reached the appropriate stage of development (approximately seven days). Alternatively, antibodies bound to larval antigens may have been removed from the surface of parasites, by surface turnover, occurring during the washing procedures that preceded *in vitro* culture of the juvenile worms. The worms are highly mobile at these stages and could

be prodigiously producing IVRS. It is possible that rabbit albumin could be bound to the surface of Ascaris L3/4 larvae by mechanisms that differ from antibodies. Of possible significance is the observation that albumin has the capacity to link to membranes in a number of different ways, for example, through sulphydryl groups (Peters and Reed, 1977).

There appear to be clear differences in the recognition of radio-labelled A. suum L3/4 and L2 secretions, by antisera from different species of animal infected with the nematode. Antibodies from infected rats and mice recognise only a subset of the range of radio-labelled Ascaris antigens that are precipitated by rabbit antiserum, and there could be a number of explanations for these observations. One of these is that S. aureus, used for the immunoprecipitations can only bind to certain IgG subtypes (Kessler, 1975) and the observed species specific differences might be due to bias of the immunoassays towards this antibody isotype. However, no additional antigens of IVRS were detected in gels of immunoprecipitations, in which particular antisera had been used to precipitate a wider range of antibody isotypes than IgG. Other reasons for the heterogeneity of the infected animal response, could include the possibility that the antibody response could have taken longer to develop in mice and rats.

Alternatively, the quantitative, and/or qualitative differences in the exposure, to the host animal, of worm antigens could have been different in each animal. These latter propositions become unlikely when it is considered that the animals were administered with multiple infections of A. suum, and that the larvae, in each case, developed to the lung stage. Moreover, the SDS-PAGE profiles of antigens

recognised by the antibodies of each animal were unique to these species, for the secretions of infective or pulmonary larvae.

The overall conclusion that can be drawn from the disparity shown by Ascaris infected hosts to A. suum secreted products is that the genetic differences of the species might be the most important factor. This could be of relevance to the use of animals to investigate various biological aspects of experimental Ascaris infection, and also to investigations of the antibody responses of genetically heterogeneous humans to infections with Ascaris, which could have implications for the possible predisposition of individuals to infection with Ascaris (Hlaing et al, 1984; Elkins, Haskwell-Elkins and Anderson, 1986). Studies of the antibody responses of different strains of mice to infection with several nematodes have suggested that the histocompatability (H-2) loci in these animals might be important for the specific recognition of nematode antigens (Mitchell et al, 1976; Wakelin, 1980 and 1985; Jungery and Ogilvie, 1982; Wassom et al, 1983). However, it is also important to note that strain specific immune responses to nematode infestation might also involve genes that are separate from the histocompatability complex (Mitchell et al, 1976; Vadas, 1982; Wassom et al, 1983; Sugane and Oshima, 1984). Nevertheless, different strains of mice were infected with A. suum and the resulting antibody responses studied by SRIP's, followed by SDS-PAGE analysis (Kennedy et al, 1987). the H-2 locus was implicated as the major controlling factor of the heterogeneous antibody responses to A. suum antigens. It was also interesting to note that, of the experimentally infected mouse strains used, only SJL (of H-2 haplotype H-2s) mice recognised the Mr 14000 antigen of A. suum larvae. The

Balb/c (H-2d) mice used in the analysis of species specific immune responses in this chapter did not apparently raise an antibody response to the range of Ascaris secretions of Mr lower than Mr 60000, including the Mr 14000 antigen, and would not, from these results, be of value in investigating some immunological properties of the latter molecule.

The stage specificity of the molecules of A.suum L2 and L3/4 IVRS is clear, but not absolute. Cold target inhibition experiments with the Mr 14000 component of ABF worms revealed that this component is also secreted by infective and lung stage worms. This was supported by evidence that A. suum infected rabbits and rats recognised this dominant radio-iodinated molecule of adult worms. In addition, it was shown that antiserum from rabbits infected with the ascaridoid nematode Toxocara canis recognised all the secreted antigens of A. suum larval stages except for that of Mr 14000. The lack of recognition of this antigen, by the otherwise apparently highly crossreactive antiserum, along with the apparent relative abundance of the Mr 14000 antigen in ABF, and the ease with which ABF can be drawn from adult worms, makes this substance an attractive source of the Mr 14000 antigen. The Mr 14000 component could be used for the immunisation of suitable animals, in order to produce monoclonal antibodies for use in serodiagnostic tests for larval and adult A. suum infection.

Apparently large amounts of the Mr 14000 molecule in the perienteric fluid of adult A.suum worms and also in larvae (results of analysis of A. suum larval homogenates not shown) meant that Mr 14000

could have been released into the culture medium *in vitro* by even small numbers of dead or dying worms, and might have been mistaken as a secretion, following radio-iodination. This possibility may also apply to the other components of larval IVRS. However, the culture supernatants that were chosen for radio-iodination were from worm cultures in which the percentage of dead or dying worms was minimal, and it is suggested that the ^{125}I labelled products of Ascaris larval stages are secreted, but contamination of IVRS by body fluid cannot be disregarded.

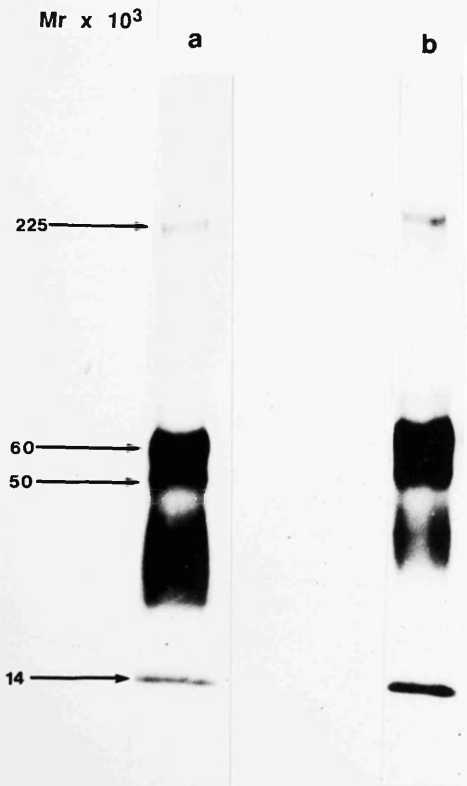
The perienteric fluid of adult Ascaris worms is a source of potent allergens, which have still not been fully analysed by biochemical means. Accounts of the relative molecular weights of major body fluid allergens vary considerably, but encompass the range 6000-600000 (Hussain, Bradbury and Strejan, 1973; Kuo and Yoo, 1977; O'Donnell and Mitchell, 1978; Greenspon *et al*, 1986; McWilliam, Stewart and Turner, 1987). An allergen was characterised from ABF and was also in the above molecular weight range, namely of Mr 14000, and named Allergen A, (Ambler *et al*, 1973, a and b). This component was also apparently secreted by adult worms *in vitro* (Ambler *et al* 1973, b). If the product of Mr 14000 from adult worms analysed in this thesis is found to be Allergen A, then it is suggested that this product is also produced and secreted by the juvenile worms of *A. suum*. However, no further information is available on the nature of the larval Mr 14000 although it would be interesting to perform Passive Cutaneous Anaphylaxis (PCA) tests in animals whose antibodies recognise this antigen in order to investigate any allergenic activity of larval Mr 14000 molecules.

It is reported that the predominant antibody response to Ascaris infection is of the IgE type (Tsuji et al, 1977; O'Donnell and Mitchell, 1980). The latter authors concluded that the human IgG response to Ascaris is relatively rare and transient, and that these individuals could be producing IgG due to re-infection with the parasite, or due to larvae being in tissue phases. Therefore, it was concluded the IgG was not useful for diagnostic purposes. The target antigen used in these studies was ABF. We have shown that the apparent dominant antigen of ABF is of Mr 14000. If it is shown to be an allergen, it is not surprising that there is a bias towards IgE in the assays performed. However, in addition, it appears that only a fraction of the IgE produced by infected individuals is specific for Ascaris antigens (Turner, Feddema and Quinn, 1979). This discovery, compounded with the procedural difficulties involved in diagnostic assays involving IgE, underline the importance to find a source of antigens that are highly antigenic, with IgG. In contrast to ABF, IVRS appear to have some of the necessary properties for use in serodiagnosis, especially the secretions of L3/4 worms which are the subjects of a high antibody response in infected rabbits.

The Ascaris worms of man and pig appear to be closely related, (Gen. Intro.). However, little is known of the relationship of the two types of parasite at the antigenic level and it is important to undertake immunochemical study to establish the degree of similarity of the secreted molecules of both types of parasite, so that, if there is true homology, the antigens of A. suum can be considered as

analogous to those produced by the human parasite.

Fig. 3.1 Secretions of *A. suum* L2 Larvae



a) *A. suum* L2 IVRS (NR)
b) *A. suum* L2 IVRS (R)

Fig 3.1

^{125}I-labelled secretions of A.suum L2 larvae were boiled in the presence of iodoacetamide (non-reducing conditions track (a)) or with β-2 mercaptoethanol (reducing conditions Track (b)) and analyzed by SDS-PAGE.

Fig. 3.2 Secretions of *A. suum* L3/4 Larvae

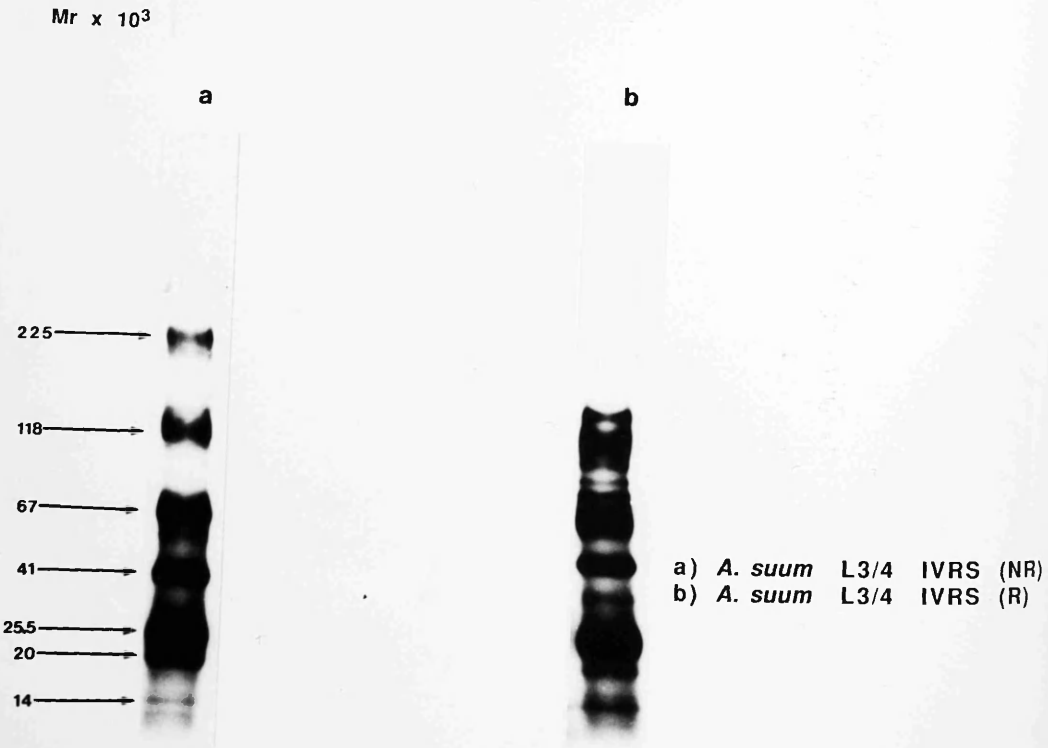
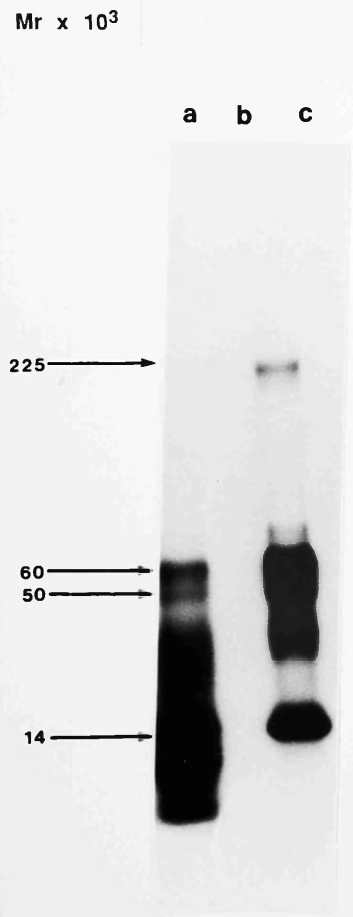


Fig. 3.2

^{125}I-labelled A.suum L3/4 IVRS products were boiled in the presence of iodocetamide, (a) or β-2 mercaptoethanol, (b) and analyzed by SDS-PAGE.

Fig. 3.3 All *A. suum* Second Stage Larval Secretions are Antigenic

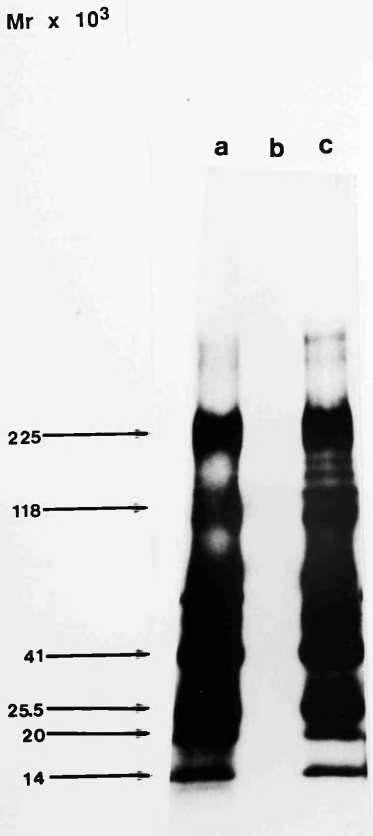


a) *A. suum* L2 IVRS
b) N. Rab.S
c) Rab. a. *A. suum*

Fig. 3.3

All L2 IVRS components are antigens. Radio-iodinated A.suum L2 secretions were immunoprecipitated with serum from uninfected rabbits (b), or with serum from rabbits infected at multiple intervals with A.suum eggs (c). The immune complexes were adsorbed to S.aureus and analyzed by SDS-PAGE. Track (a) shows L2 secretions not reacted with rabbit serum.

Fig. 3.4 Antigens of *A. suum* L3/4 Larvae

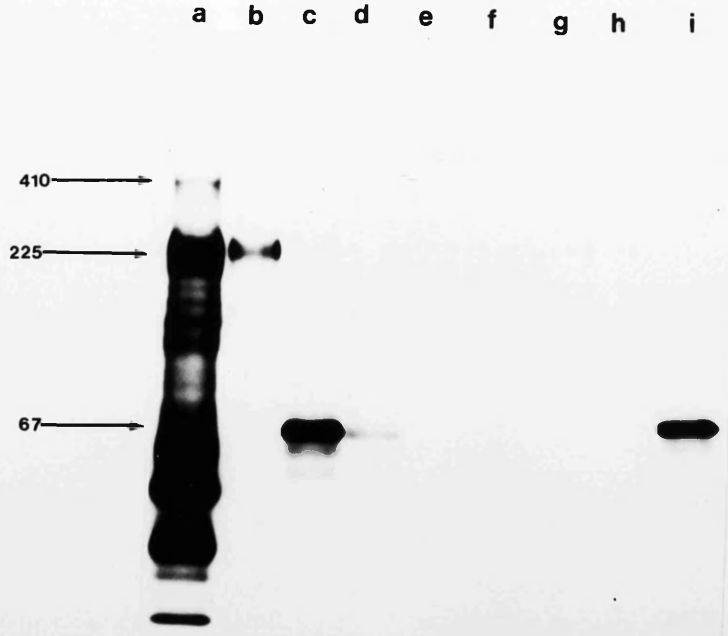


a) *A. suum* L3/4 IVRS
b) N.Rab. S.
c) Rab. a. *A. suum*

Fig. 3.4

Most L3/4 secretions are antigens. ^{125}I-labelled A.suum L3/4 IVRS products were immunoprecipitated with serum from uninfected rabbits (b) or from rabbits multiply infected with A.suum (c). The immunoprecipitates were adsorbed to S.aureus and analyzed by SDS-PAGE. An SDS-PAGE profile of L3/4 IVRS components is shown in track (a).

Fig. 3.5 Presence of Host Albumin in *A. suum* L3/4 IVRS

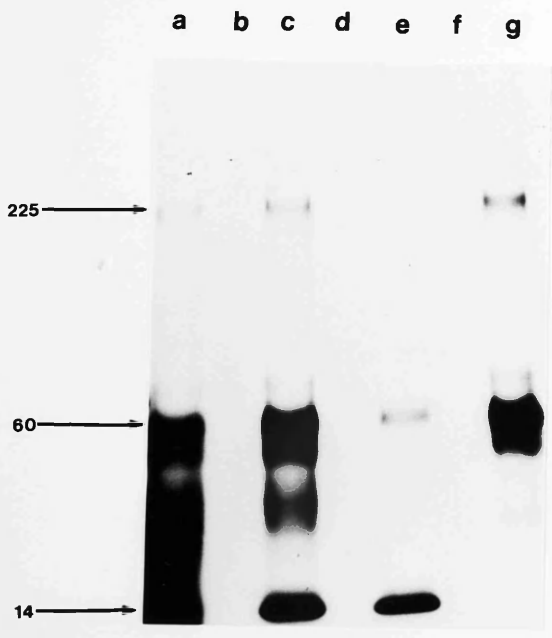


a) *A. suum* L3/4 IVRS
b) N. Sheep S
c) Sh. a Rab. W.S.
d-g) Sh. a Rab. W.S. + increasing amts. albumin
h) N. Goat S.
i) Goat a. Sh. W.S.

Fig. 3.5

Host albumin is present in A.suum L3/4 secretions. ^{125}I-labelled A.suum L3/4 IVRS were reacted in SRIP with sheep serum raised against rabbit whole serum (c-g) and also with the following amounts of unlabelled rabbit serum albumin: 5 µg/ml (d), 50 µg/ml (E), 0.5 mg/ml (f) or 5 mg/ml (g). Reactions of IVRS with serum from uninfected sheep or goats are represented in (b) and (h) respectively. Reactions of L3/4 IVRS with sheep or goat sera raised against whole rabbit serum are shown in (c) and (i) respectively. The products of the SRIP's were analyzed by SDS-PAGE. Radio-iodinated A. suum L3/4 secretions are shown in track (a).

Fig. 3.6 Species Differences in Immune Recognition of L2 Secretions

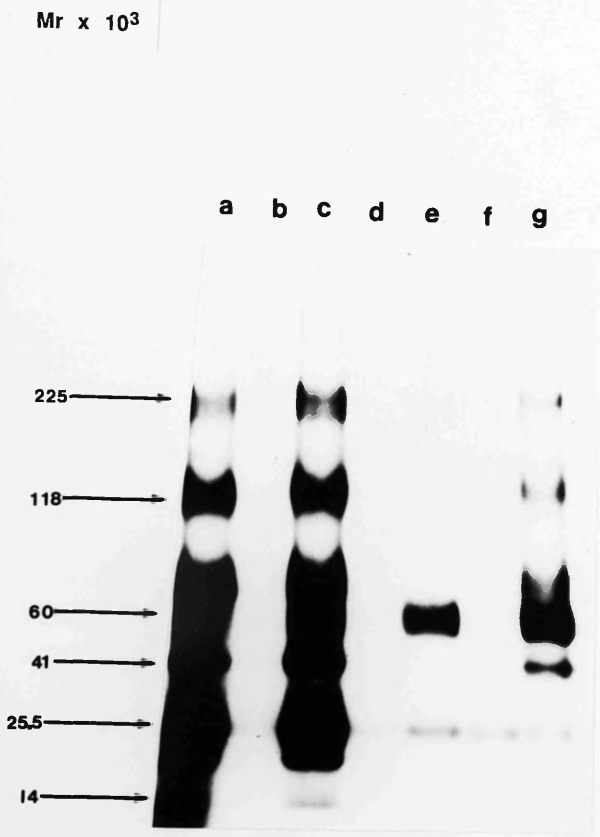


a) *A. suum* L2 IVRS
b) N. Rab. S.
c) Rab. a. *A. suum*
d) N. Rat S
e) Rat a. *A. suum*
f) N.M.S.
g) M.a. *A. suum*

Fig. 3.6

Species differences in immune recognition of L2 IVRS. A.suum L2 IVRS, radiolabelled with ^{125}I (a), were reacted with sera from animals given at least secondary infection with A.suum including lops rabbits (4 infections),(c), Wistar inbred rats (infected twice) (e) and NIH mice (infected twice) (g). Reactions of L2 IVRS with normal rabbit, rat and mouse sera are shown in tracks (b), (d) and (f) respectively. Immune precipitates were adsorbed to S.aureus and analyzed by SDS-PAGE.

Fig. 3.7 Species Differences in Immune Recognition of L3/4 Secretions

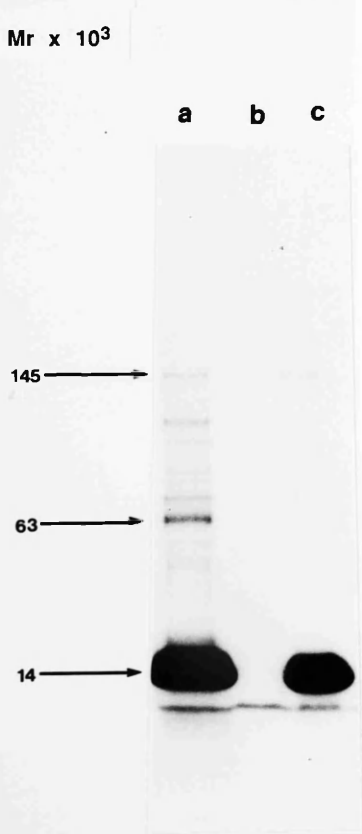


a) *A. suum* L3/4 IVRS
b) N. Rab. S.
c) Rab. a. *A. suum*
d) N. Rat S
e) Rat a. *A. suum*
f) N.M.S.
g) M.a. *A. suum*

Fig. 3.7

Species differences in immune recognition of L3/4 secretions. Radio-iodinated L3/4 secretions of A.suum larvae were reacted in SRIP with sera from animals given at least secondary infection with A.suum. This includes lops rabbits (infected 4 times) (c), Wistar inbred rats (infected twice) (e) and NIH mice (infected twice) (g). Serum from uninfected rabbits, rats or mice were also reacted in SRIP's with the target antigen, (b), (d) and (f), respectively. The immunosorbates of the SRIP's were analyzed by SDS-PAGE. Radio-iodinated A. suum L3/4 secretions are shown in track (a).

Fig. 3.8 The Major Antigen of ABF is of Mr 14000

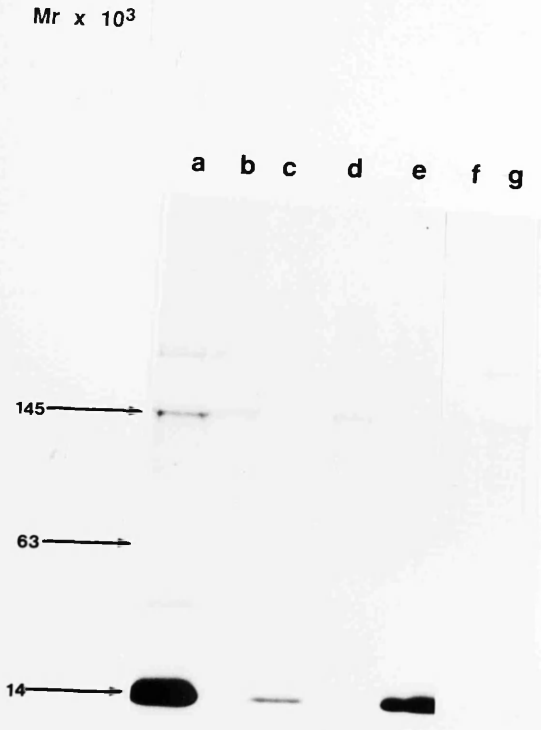


a) ABF
b) N. Rab. S.
c) Rab. a. *A. suum*

Fig. 3.8

The major antigen of ABF is of Mr 14000. ^{125}I-labelled perienteric fluid (ABF) from adult A.suum worms, was reacted with serum from rabbits infected four times with A.suum (c) or from uninfected rabbit serum (b) in SRIP's. The resulting immunoadsorbates were analyzed by SDS-PAGE. Radioactive ABF, unreacted with serum, is shown in Track (a).

Fig. 3.9 Non-recognition of the ABF Mr 14000 Component by Mouse Anti-*A. suum* Antibodies



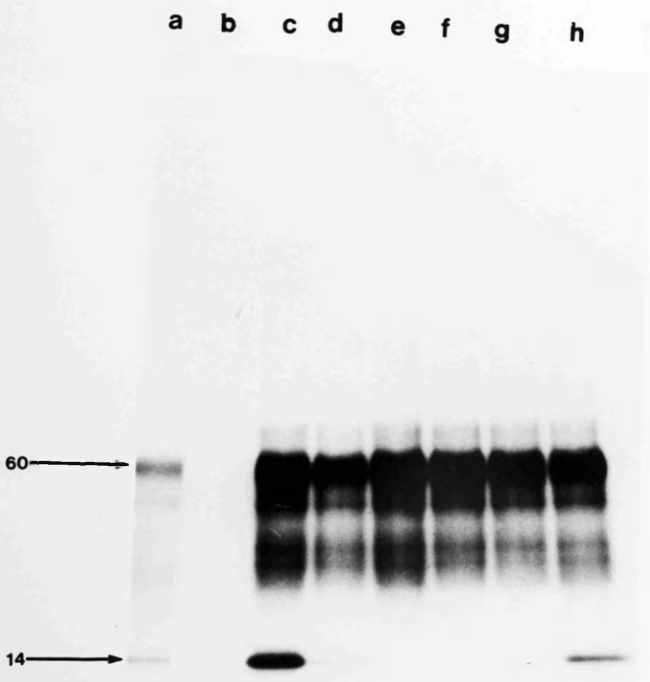
a) ABF
b) N. Rab. S.
c) Rab. a. *A. suum*
d) N. Rat S
e) Rat a. *A. suum*
f) N.M.S.
g) M.a. *A. suum*

Fig. 3.9

Mouse Anti-A.suum Antibodies do not recognize the Mr 14000 component of ABF. ^{125}I - labelled ABF (a) was reacted in immunoprecipitations with sera from animals multiply infected with A.suum, namely, lops rabbits infected four times (c), Wistar inbred rats given two infections (e) and NIH mice given secondary infections (g) The resultant immunoprecipitates were absorbed to S-aureus and analyzed by SDS-PAGE. Reactions of target antigens with sera from uninfected rabbits, rats or mice are shown in tracks (b), (d) and (f) respectively.

The results are also presented in tabulated form in Table 3.1.

Fig. 3.10 Mr 14000 Antigen is Present in L2 Secretions of *A. suum*



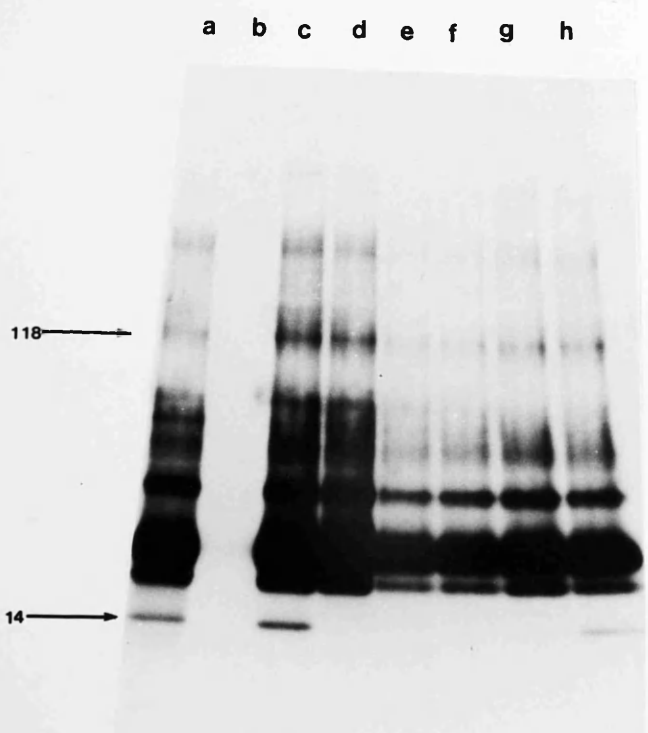
a) *A. suum* L2 IVRS
b) N. Rab. S.
c) Rab. a. *A. suum*
d-g) Rab. a *A. suum* + increasing amts. unlabelled ABF
h) Rab. a. *A. suum*

Fig. 3.10

The A.suum Mr 14000 antigen is present in several stages of parasite development. ^{125}I-labelled A.suum IVRS (a) were combined with serum from rabbits given quaternary infections with A.suum; in the presence of the following quantities of unlabelled ABF: none (c and h), 0.1 µl (d), 0.25 µl (e), 1.0 µl (f) and 2.5 µl (g).

Pre-infection rabbit serum reactions with radiolabelled ABF are shown in track (b). The immunoprecipitations were mediated by S.aureus and immunoprecipitate examined by SDS-PAGE. The concentration of protein in ABF was estimated at 2.5 mg/ml (M.W Kennedy, pers. comm.).

Fig. 3.11 Mr 14000 Antigen is Present in L3/4 Secretions of *A. suum*



a) *A. suum* L3/4 IVRS
b) N. Rab. S.
c) Rab. a. *A. suum*
d-g) Rab. a *A. suum* + increasing am unlabelled ABF
h) Rab. a. *A. suum*

Fig. 3.11.

The A.suum Mr 14000 antigen is present in several stages of parasite development. Radio-iodinated secretions of A.suum lung-stage larvae (a) were reacted with serum from rabbits given quaternary infections of A.suum in the presence of the following quantities of unlabelled ABF, namely; none (c and h), 0.1 µl (d), 0.25 µl (e), 1.0 µl (f) or 2.5 µl (g). The reaction of uninfected rabbit serum with target antigen is shown in track (b). The SRIP immunosorbates were analyzed by SDS-PAGE.

Table. 3.1 Species- Specific Recognition of Ascaris Larval and Adult Parasite Products

TEST ANTIGENS \ SERUM DONORS	Normal Mice (NIH)	A.sum - infected mice	Normal Rats (Wistar)	A.sum Infected Rats	Normal Rabbits (lops)	A.sum Infected Rabbits
A.sum L2 IVRS	2.9±1.8	10.4±3.5	2.3±1.1	10.5±3.0	3.2±1.0	36.6±7.2
A.sum L3/4 IVRS	3.2±1.6	18.3±5.1	2.7±1.5	9.3±2.8	4.1±0.7	54.6±10.1
ABF	2.4±0.2	2.8±0.4	2.3±0.1	5.7±2.4	2.7±0.1	43.3±10.2

Legend to Table 3.1

70 - 200 x 10^3 cpm of the appropriate ^{125}I-labelled antigens were reacted with antisera from normal (uninfected) or A.sum - infected animals in an SRIP. The results are tabulated as the mean percentage of TCA precipitable counts from triplicate tubes (mean % TCA ± standard deviation). The SRIP pellet s/n's were analyzed by SDS-PAGE (Figs. 3.6, 3.7 and 3.9 for A.sum L2 and L3/4 IVRS and ABF respectively.

COMPARISONS AND IMMUNOLOGICAL CROSSREACTIONS BETWEEN THE *IN VITRO* RELEASED SECRETIONS (IVRS) OF *ASCARIS SUUM* AND *ASCARIS LUMBRICOIDES* LARVAL STAGES

Summary

The extent to which A. suum is responsible for the epidemiology and pathology of A. lumbricoides infections of humans is not known. Various methods have been used to identify biological characteristics unique to either type of roundworm (see following Introduction) but the subject remains unresolved.

In this report, the IVRS from larvae of A. lumbricoides (from the West Indies and India respectively) and A. suum (Glasgow) were examined by SDS-PAGE, following radio-iodination, and also after immunoprecipitation of radioactive IVRS. The antisera used was obtained from rabbits infected with either Indian A. lumbricoides or A. suum (Glasgow).

L2 and L3/4 secretions from the human parasite were found to be stage-specific and homologous to those of the pig roundworm, but it was also possible, by comparison of SDS-PAGE antigen profiles to separate the products of A. suum from A. lumbricoides. Despite these differences, however, there was complete crossreactivity between A. suum-specific antiserum, and the radiolabelled antigens of the human roundworm (and vice versa) demonstrating that the immunological methods employed here are insufficient for the species-specific detection of ascariasis. However, these techniques are worthwhile to provide the basis for studies of the immunobiology of naturally and experimentally-infected subjects.

Introduction

The surface and IVRS molecules of filarial nematodes, in general,

provide a good example of th serological crossreactivity between, and within, helminths species that could interfere with the specific diagnosis of these diseases in man (Forsyth et al, 1981; Maizels et al, 1983; Klenk, Geyer and Zahner, 1984) and perhaps, that of other nematode infections in general. Extensive crossreactions, possibly attributable to shared or similar antigens are found between the filarid species, Brugia timori and Brugia malayi (Maizels et al, 1983) and also within the different life cycles examined for each individual species (adults, microfilariae and L2 larvae respectively). Despite these problems, there may be minor components within the range of either larval surface or secretions which are completely stage or species-specific. This justifies attempts to search for such components among the secretions of Ascaris larvae which could then be used, after purification, for serological investigation.

Epidemiological and biochemical studies of A. suum and A. lumbricoides (Denham, 1984;Eaton, 1985; Taffs, 1985; Kurimoto, 1974) have suggested there are grounds for the discrimination of the two parasites. This was made uncertain by conflicting evidence from the examination of Ascaris morphology (Sprent, 1952; Lysek, 1963; Weise, 1973). Therefore there is no unambiguous method for evaluation of the phylogenetic status of both worm types.

Immunochemical studies have suggested that the IVRS of nematodes may be of greater value in serodiagnosis of specific parasite infestation than somatic extracts of worms (See Chapter 3). There is reason, therefore, in employing the secretions of both A. lumbricoides

and A. suum to investigate the antigenic relationship of these roundworms.

Here, the secretions of L2 and L3/4 stages of A. suum and A. lumbricoides were examined at the molecular level, primarily with the use of radioimmunoassays, followed by SDS-PAGE. There was a wide ranging similarity between the secreted antigens of both types of parasites but there are also differences, the significance of which can be examined in further studies.

Additionally, differences in gel profile were shown for antigens from West Indian and Indian isolates of A. lumbricoides, which may be representative of genetic differences in these geographically distinct parasites. Finally the homology of the pig and human parasite antigens may be exploited, for use of the more readily available worm A. suum in clarifying the host-parasite relationship in human ascariasis.

Results

Second stage larval secretions

The supernatants, collected separately from A. suum and A. lumbricoides infective (L2) larvae in vitro, were radioiodinated and examined by SDS-PAGE (Fig. 4.1). The IVRS of A. suum have been analysed this way and described previously (Chapter 3). A. lumbricoides L2 secretory products are also of similar range in relative apparent molecular weight (Mr), namely, from 14-225,000. The main components of human Ascaris IVRS are of Mr 14,000, 17,000, 20-30,000 and 60,000 respectively. The molecule of Mr 17,000 appeared to be represented only in the SDS-PAGE profile of the human parasite.

Reducing conditions did not significantly alter the gel profile of secretions from either nematode.

Antigenicity of, and antibody crossreactions between, L2 secretions and Ascaris-infected animals

Infective larval IVRS of the human and porcine nematodes were radiolabelled with ^{125}I and reacted with antiserum from rabbits infected with A. suum or A. lumbricoides in an S. aureus-mediated radioimmunoprecipitation (SRIP). The products of the reaction were analysed by SDS-PAGE (Fig. 4.2). One immediate observation is that all the radiolabelled molecules secreted from the human and pig roundworms, are antigenic. In addition, there was complete reciprocal recognition of Ascaris antigens, that is, antibodies specific for infection by one parasite recognised the radiolabelled antigens of the other.

The non-relatedness of the Mr 17,000 component of A. lumbricoides to the Mr 14,000 of A. suum

In addition to the facts stated above, the close migration of Mr 17,000 to the Mr 14,000 band prompted enquiry into the possibility that the latter component was a modified form of the former. This was carried out by performing an SRIP of radiolabelled A. lumbricoides L2 IVRS with two different antisera raised in rabbits; one with a specificity for the Mr 14,000 molecule of adult A. suum worms (prepared by Dr. M.W. Kennedy; see Materials and Methods, Chapter 2) and one which recognises most of the secretions of Ascaris, excluding the Mr 14,000 molecule. The latter antiserum is raised from T. canis infection of rabbits.

The resultant precipitates were analysed by SDS-PAGE (Fig. 4.3)

and there are two major findings of interest. Firstly, the Mr 14,000 component of A. lumbricoides is similar to that of the pig parasite. Secondly, there is no apparent homology between the Mr 14,000 and 17,000 components of the human nematode, confirmed by rabbit anti-T. canis antibodies. Bands of diffuse appearance in the SDS-PAGE profiles of L2 secretions from both types of parasite did not facilitate further molecular comparisons to be made. This is less problematic when lung-stage larval stages are examined.

Secretions from pulmonary stage larvae of both human and pig roundworms were radiolabelled with ^{125}I and examined by SDS-PAGE in Fig. 4.4. The range of Mr of larval products from A.lumbricoides was identified to that of A.suum (Chapter 3), that is, from Mr 14,000 to 410,000. Some A.lumbricoides IVRS components were similar in Mr to those from the pig nematode, namely, those of Mr 14,000; 67,000; 225,000 and finally, 410,000 (not visible in this figure). Additionally eachworm has elements of IVRS that are unique to it; an Mr 28,000 molecule is present among the products of the human worm and those of Mr 25,500 and 118,000 are apparently specified to A.suum lung-stage larvae.

Under reducing conditions, certain of the components of both human and porcine nematode secretions are broken down, apparently, into smaller fragments.

Antigenicity of, and crossreactivity between, the secretions of lung-stage larvae

Radioiodinated IVRS from A. suum and A. lumbricoides were reacted in a SRIP with antisera from rabbits infected with either nematode and the resulting precipitates analysed by SDS-PAGE (Fig. 4.5). The situation was similar to that for the earlier developmental larval stage examined, in that most of the secretions of pulmonary stage human worms were antigenic, except for the major labelled Mr 67,000 molecule of host origin. Extensive crossreaction is shown for the reaction of rabbit anti-A. suum antibodies against A. lumbricoides, and vice versa.

Effect of different geographical location on the IVRS from A. lumbricoides larvae

Previous findings regarding A. lumbricoides focussed on worm and worm products which were derived from individuals in India. A. suum parasites were recovered from pigs in Glasgow. The presence of secretions apparently unique to the human nematodes could have been attributed to polymorphism of IVRS depending on the geographical area from which the parasites were recovered. Such polymorphism could have accounted for some, or all, of the differences in SDS-PAGE profiles of A. lumbricoides antigens, in comparison to A. suum. An examination was made, therefore, of the ^{125}I-labelled IVRS from L2 and L3/4 larval stages of Indian A. lumbricoides or those from a Caribbean location by SDS-PAGE (Fig. 4.6). Differences between the two isolates are apparent at the level of L2 secretions, where the tropical parasites display a large diffuse band spanning the area from Mr 32,000 to

75,000. Bands of similar appearance are found in the SDS-PAGE profiles of Indian worms from Mr 35,000 to 55,000, and Mr 28,000 respectively. The latter component is also found in the IVRS of infective juvenile A. suum worms.

Extensive similarities are found among the secretions of lung-stage A. lumbricoides larvae from both different locations in apparent molecular weights of secretory products although Caribbean worms contain, in noticeable amounts, an Mr 17,000 component which may, or may not, be homologous to the Mr 17000 component of infective worms. This molecule does not appear to be present in detectable amounts in the culture supernatants of Indian L3/4 worms.

Reducing conditions for infective and lung-stage IVRS from A. lumbricoides (Fig. 4.7) revealed no differences in the SDS-PAGE profiles of infective larvae. In contrast, some products of L3/4 larvae were broken down into entities of lower Mr.

Crossreactivity between Caribbean A. lumbricoides IVRS and anti-A. lumbricoides (India) antibodies

L2 and L3/4 A. lumbricoides larval products from Indian and West Indian worms were radioiodinated and precipitated with antiserum from rabbits infected with Indian A. lumbricoides in an SRIP (Fig. 4.8). THere was almost complete crossreaction of the infection-induced antibodies with the target antigens. An important observation was that the Mr 17,000 components of both infective and lung-stage larval IVRS were precipitated by the antiserum.

Discussion

This study reports that the secretions of *A. lumbricoides* larvae from Caribbean and Indian sources are similar to those of *A. suum* in respect to limited stage-specificity and apparent immunological homology of certain components. Also, there are differences in the SDS-PAGE profiles of the porcine and human parasites which may possibly be representative of species and/or strain specific molecules in IVRS.

There is a possibility that some of the heterology of *Ascaris* secretions could be attributed to a number of factors. First, adult *A. lumbricoides* worms from the West Indies or South East India, were harvested from infected individuals following treatment with anthelmintic drugs and the worms were transported to Scotland over a seven day period whereas *A. suum* worms were retrieved from slaughtered pigs and dissected soon after, for (unembryonated) eggs. Arguably, the time taken to transport the human worms, along with possibly high ambient temperatures could have been sufficient to allow partial embryonation of eggs inside the adult worms. This, in turn, could have led to the death of some larvae due to lack of oxygen in the eggs. *A. lumbricoides* eggs from Caribbean and Indian worms took longer to proceed to full embryonation *in vitro* and in fewer numbers than those of *A. suum* (Glasgow). The percentage of total eggs embryonated was at an average of 46%.

The *Ascaris* larvae from which culture supernatants were collected for biochemical analysis may have conceivably previously undergone a selection process. Only those L3/4 larvae which had migrated to

within the lungs of experimentally-infected rabbits by seven days, and which could subsequently traverse through a Baermann apparatus, would be included in the results. Likewise, the products of L2 larvae were harvested from worms which had migrated through similar sets of sterile cotton wool plugs. However despite the reservations stated above, several deductions can still be made regarding the homologies and differences between A. suum and A. lumbricoides.

Most of the findings regarding A. lumbricoides considered the secretions of Indian isolates and it was shown that the larval stages (L2 and L3/4) produced molecules that were recognised by the antibodies of infected hosts mainly rabbits. There was, however, one exception. An Mr 67,000 molecule, prominent in pulmonary stage radioiodinated IVRS, was not a target for A. lumbricoides-specific antibodies. In A. suum, this situation was investigated and an Mr 67,000 component of lung-stage worms was shown to be rabbit serum albumin (Chapter 3). No further information regarding this molecule is available for A. lumbricoides but the similarity in molecular weight to this component of A. suum in addition to the above situation, would suggest that similar mechanisms exist in the human parasite for intake and/or processing of this molecule, followed by its release among worm secretions (Discussion, Chapter 3). Further methods are required for the confirmation of the identity of this prominently radiolabelled molecule, such as cold-target inhibition of SRIP's of radioactive A. lumbricoides IVRS with non-radioactive rabbit serum albumin, which was used to identify this host component in A. suum L3/4 (Chapter 3).

Comparison of the SDS-PAGE profiles of infective and lung-stage

larval secretions of A. suum and the human roundworms, revealed the presence of an Mr 17,000 component among the products of L2 A. lumbricoides derived from both Indian and Caribbean worms and the pulmonary stage secretions of the Caribbean parasites, which was not apparent among the radiolabelled secretions of the pig parasite. However, antibodies from A. suum-infected rabbits precipitate the Mr 17,000 component among the majority of other antigens of the human roundworms, possibly indicating that epitopes on this antigen are distributed among one or more of the secretions of A. suum. Alternatively, the Mr 17,000 antigen may be secreted in vivo by A. suum larvae, but not in vitro. The possibility of the Mr 17,000 molecule being a modified form of the Mr 14,000 component was explored by use of antibodies specific for the latter larval products, but was no indication that there was immunological homology between the two secretions. This would imply that the Mr 17,000 component is specific for A. lumbricoides but that amino acid and possibly carbohydrate residues are not unique to the nematode.

Crossreactivity of anti-A. lumbricoides antibodies with A. suum antigens (and vice versa) extended to virtually all of the ^{125}I-labelled parasite products that could be visualised by SDS-PAGE. This fact testifies to the difficulty involved in isolating components of IVRS for species-specific diagnosis of ascariasis in humans.

The similarity in gel profile of A. suum and A. lumbricoides secretory products would seem to argue in favour of true homology. This was supported by the finding that the Mr 14,000 antigen of the porcine worms was homologous to that secreted by the human worms.

Confirmation by other means is required to show the degree of similarity in some of the other antigens secreted by both sets of worms. The situation could be clarified, possibly by purification of individual secreting components and subsequent amino acid sequencing. Bearing in mind the remarkable reciprocal crossreactivity displayed by Ascaris-specific antisera against heterologous Ascaris antigens, a high degree of polypeptide sequence homology is anticipated. However, the contribution of homologous carbohydrate residues to the above crossreactions cannot be ignored. Lectins could be used to identify sugar groups on radiolabelled IVRS from both types of parasite larval stages, and indicate if these are similar.

Immunochemical analysis of secreted or surface products, has been a useful technique for revealing strain- or species-specific differences in closely related parasites such as Trichinella spiralis and Trichinella pseudospiralis (Almond, McLaren and Parkhouse, 1986) and also the human blood flukes Schistosoma mansoni and Schistosoma haematobium (Norden and Strand, 1984). When applied to the secretions of Caribbean and Indian A. lumbricoides larvae, the infective larval products were different in SDS-PAGE profile, whereas those of L3/4 larvae were almost identical. However, using antibodies from rabbits infected with the Indian isolate of the human nematode, extensive crossreactions were shown and no significant distinguishing features highlighted, although the lung-stage larval products of Caribbean A. lumbricoides contained an Mr 17,000 molecule not present in corresponding Indian worms.

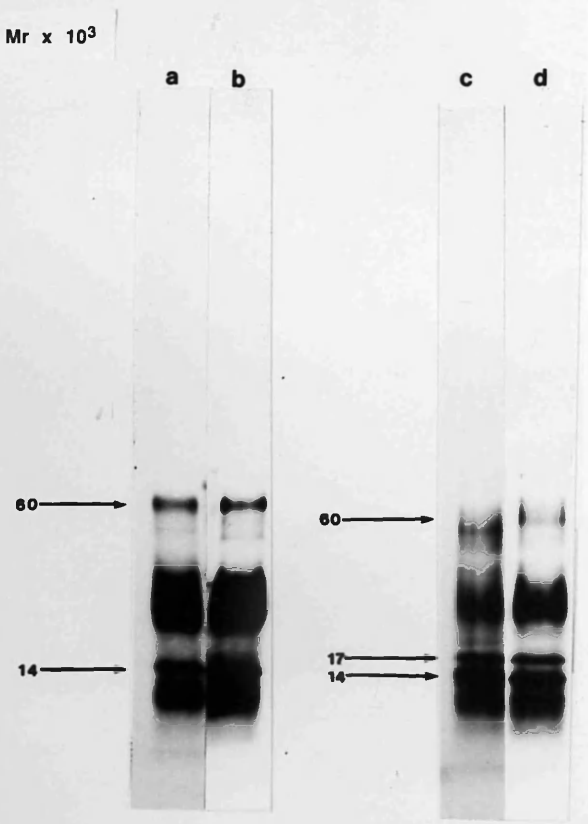
Starch gel electrophoresis of proteins from geographically separate adult A. suum populations in America (Leslie et al, 1982)

revealed only limited polymorphism of enzymatic and non-enzymatic loci, which would support the results for A. lumbricoides above. A similar study of 14 protein loci of purified extracts from adult A. suum and of A. lumbricoides worms (Nadler, 1987) discovered that the only difference between the worms was for the locus encoding the enzyme superoxide dismutase (SOD), which may be partly responsible for the removal in vivo, of superoxide (O_3^-) ions, which could be potentially cytotoxic to parasites (Gerasimov, Kasatkina and Darmova, 1979; Paul and Barrett, 1980; Rhoads, 1983). There is no knowledge as yet of the presence of SOD on the surface or in the secretions of Ascaris larvae, therefore there is a need to investigate such enzymic activity in the culture supernatants of juvenile worms. However the worms might only secrete the above enzyme in vivo along with other IVRS components. Further, evaluation of the activities of SOD in vitro showed that it was a potentially useful tool for the taxonomic differentiation of the ascarid nematodes Toxocara canis and Toxascaris leonina (Sanchez-Moreno, Garcia-Ruiz and Monteolivia, 1987).

It would be of interest to if biochemical method based on study of enzyme loci could be applied for the phylogenetic classification of A. suum and A. lumbricoides. Also, it could be of relevance to explore the possibility that there are genetically different populations of Ascaris depending on country of origin and to probe the heterologous phenotypes and eventually genotypes, since immunoprophylactic measures may be more effective on one strain than another. Additionally, it may become possible to isolate components from Ascaris secretory or surface molecules that are truly species and/or strain specific.

The close biological homology of A. suum and A. lumbricoides is advantageous in allowing for the use of antigens from the former nematode, both in preliminary diagnostic study (Macfarlane and Shephard, 1984) and also in building up an understanding of the process that combine and contribute to the disease of ascariasis.

Fig. 4.1 **Secretions of Human and Porcine *Ascaris* Infective Larvae**

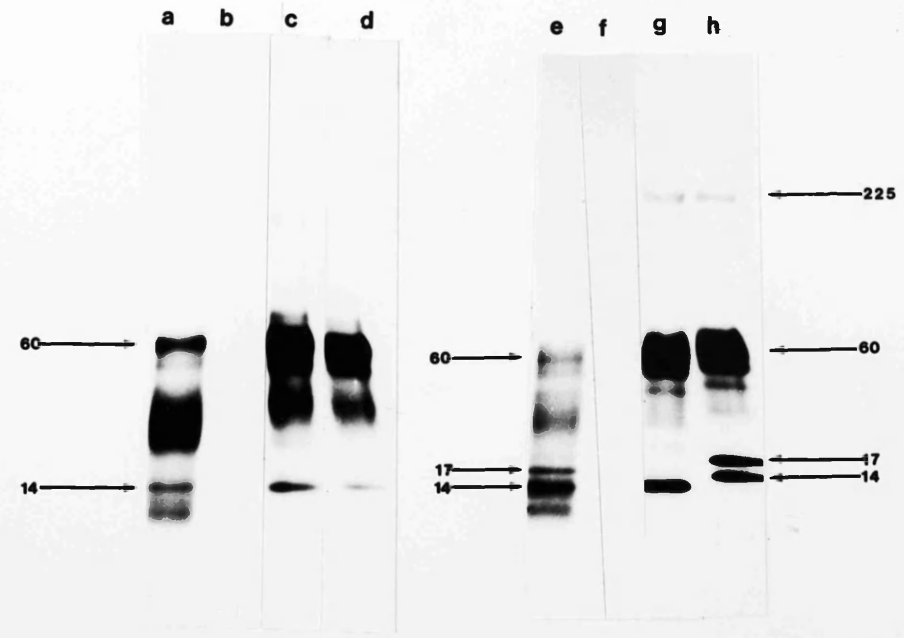


a) *A. suum* L2 IVRS (NR)
b) *A. suum* L2 IVRS (R)
c) *A. lumbricoides* L2 IVRS (NR)
d) *A. lumbricoides* L2 IVRS (R)

Fig. 4.1

Secretions of human and porcine Ascaris infective larvae. A.suum (from Glasgow) and A.lumbricoides (from India) L2 IVRS material was radio-labelled with ^{125}I and examined by SDS-PAGE under non-reducing (a and c) and reducing (b and d) conditions.

Fig. 4.2 Antigens of Human and Porcine *Ascaris* Infective Larvae



a) *A. suum* L2 IVRS
b) N. Rab. S.
c) Rab. a. *A. suum*
d) Rab. a. *A. lumbricoides*
e) *A. lumbricoides* L2 IVRS
f) N. Rab. S.
g) Rab. a. *A. suum*
h) Rab. a. *A. lumbricoides*

Fig. 4.2

Antigens of human and porcine Ascaris infective larvae. A.suum (Glasgow) and A.lumbricoides (India) L2 IVRS were radio-iodinated and reacted in an SRIP with the following antisera:uninfected rabbit serum (b and f), serum from rabbits given secondary infections of A.suum (c and g) or serum from rabbits given secondary infections of A.lumbricoides (d and h). The resultant immunoprecipitates were examined by SDS-PAGE. ^{125}I-labelled A.suum and A.lumbricoides L2 IVRS are shown in (a) and (e), respectively.

Fig. 4.3 The Mr 14000 Component of *A. suum* IVRS is not Immunologically Similar to the Mr 17000 of *A. lumbricoides* L2 IVRS

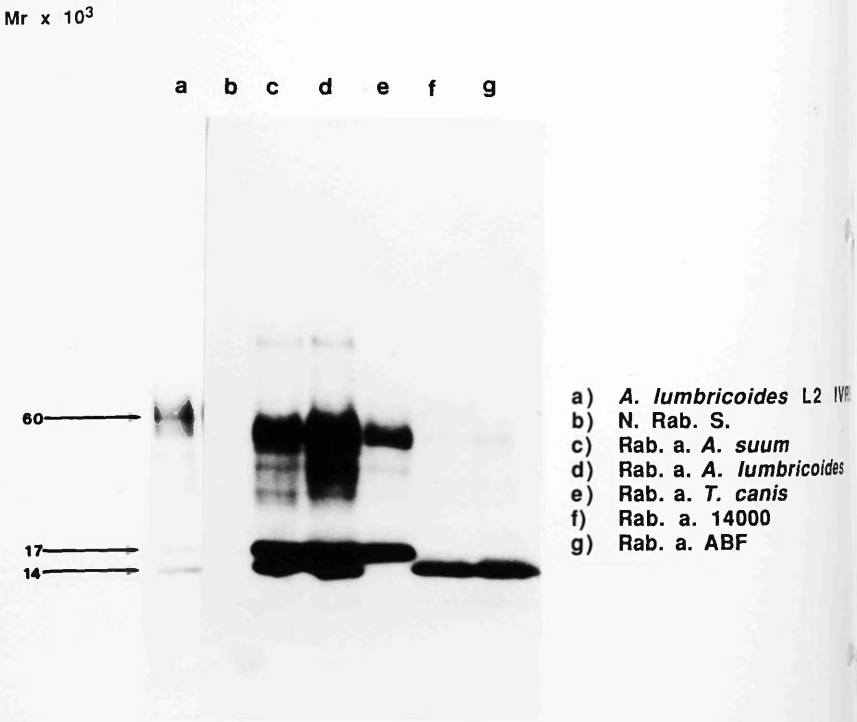


Fig. 4.3

Non-homology of the Mr 14000 A.suum IVRS component and the Mr 17000 secretion of A.lumbricoides L2 IVRS. A.lumbricoides (India) L2 IVRS were radio-iodinated and reacted in an SRIP with the following antisera: uninfected rabbit serum (b), serum from rabbits given multiple infections of A. suum (c), A. lumbricoides (d), or infected on a single occasion with T. canis (e). and also sera raised in rabbits against the Mr 14000 Ascaris IVRS component (f) and ABF (g). The products of the immunoprecipitations were analyzed by SDS-PAGE. ^{125}I-labelled A. lumbricoides L2 IVRS is shown in (a).

Fig. 4.4 Comparison of the L3/4 IVRS of *A. suum* and *A. lumbricoides*

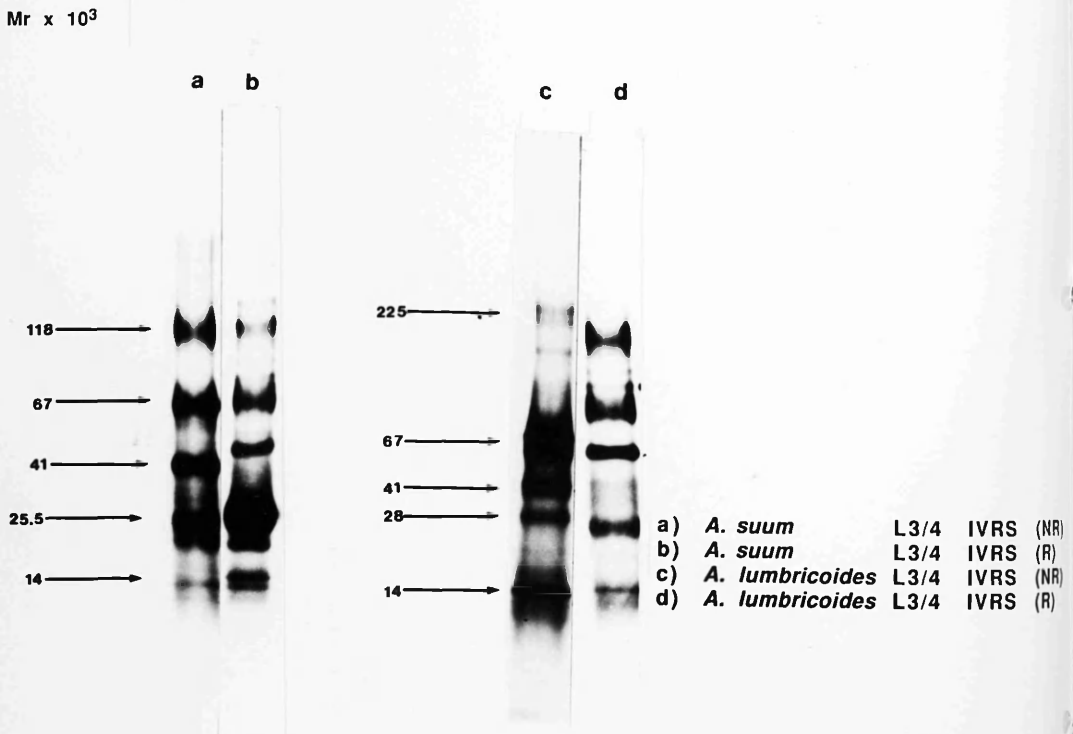


Fig. 4.4

Comparison of SDS-PAGE profiles of IVRS from A.suum and A.lumbricoides. The secretions of human and porcine L3/4 worms were radiolabelled with ^{125}I and examined by SDS-PAGE under non-reducing (a and c) and reducing (b and d) conditions.

Fig. 4.5 Antigenicity and Crossreactivity of Anti-Ascaris Sera and IVRS of *A. suum* and *A. lumbricoides* L3/4

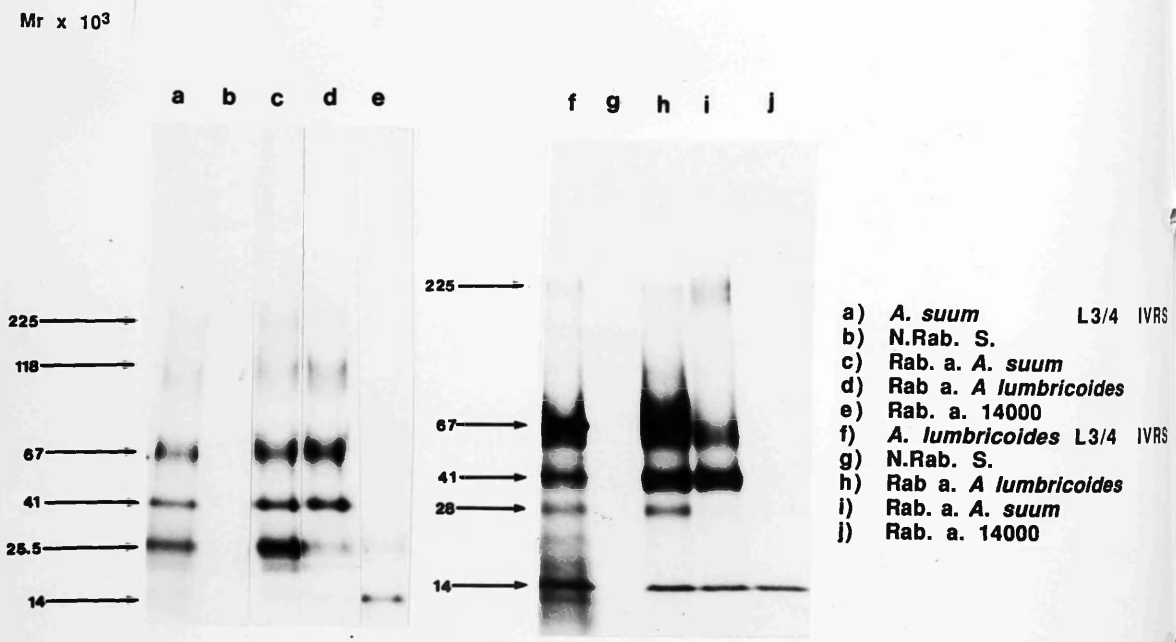
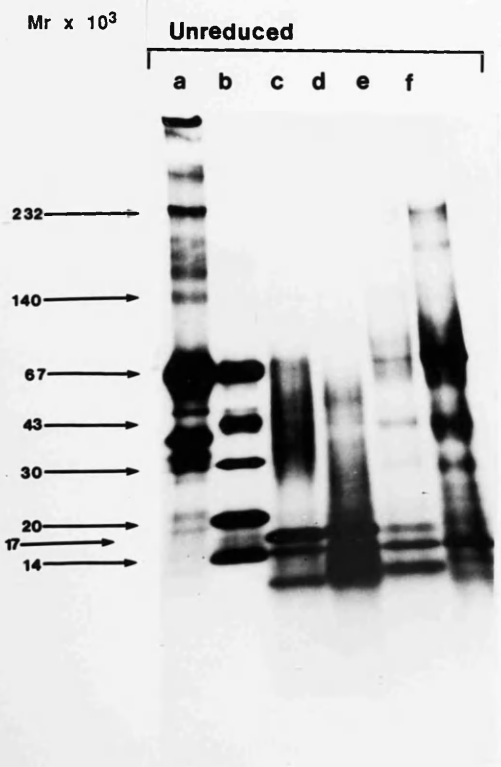


Fig. 4.5

Antigenicity and Crossreactions of anti-Ascaris sera and IVRS products of A.suum (Glasgow) and A.lumbricoides (India) L3/4 parasites. ^{125}I-labelled A.suum and A.lumbricoides L3/4 IVRS were reacted in an SRIP with antisera from rabbits infected multiply with A.lumbricoides (India) (d and h) and A.suum (c and i), sera from rabbits immunized with the Mr 14000 component of ABF and Ascaris IVRS (e and j) and sera from uninfected rabbits (b and g). The products of the SRIP were analyzed by SDS-PAGE. Radio-iodinated A. suum and A. lumbricoides L3/4 IVRS are shown in tracks (a) and (f) respectively.

Fig. 4.6 *A. lumbricoides* Larval Secretions from Indian and Caribbean Parasites

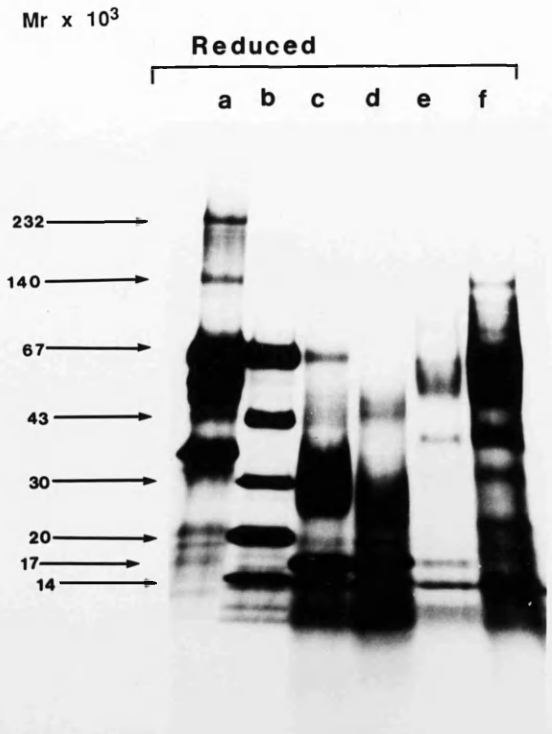


a) High M. Wt. Markers
b) Low M. Wt. Markers
c) *A. lumbricoides* L2 IVRS (Caribbean)
d) *A. lumbricoides* L2 IVRS (India)
e) *A. lumbricoides* L3/4 IVRS (Caribbean)
f) *A. lumbricoides* L3/4 IVRS (India)

Fig. 4.6

Comparison of secretions from A.lumbricoides larvae of Indian and Caribbean origins. ^{125}I-labelled IVRS material of Indian L2 and L3/4 Ascaris worms (d and f, respectively) and Caribbean L2 and L3/4 parsites (c and e, respectively) were analyzed by SDS-PAGE. Radio-iodinated molecular weight markers for high and low molecular weight ranges are shown in (a) and (b) respectively.

Fig. 4.7 *A. lumbricoides* Larval Secretions from Indian and Caribbean Parasites



a) High M. Wt. Markers
b) Low M. Wt. Markers
c) *A. lumbricoides* L2 IVRS (Caribbean)
d) *A. lumbricoides* L2 IVRS (India)
e) *A. lumbricoides* L3/4 IVRS (Caribbean)
f) *A. lumbricoides* L3/4 IVRS (India)

Fig. 4.7

Comparison of secretions from A.lumbricoides larvae of Caribbean or Indian origin. Radio-iodinated IVRS from Caribbean L2 and L3/4 parasites (c and e, respectively) and Indian L2 and L3/4 worms (d and f, respectively) were examined by SDS-PAGE under reducing conditions. ^{125}I-labelled HMW and LMW markers were also electrophoresed under reducing conditions (a and b, respectively).

INTRINSIC LABELLING OF THE LARVAL PRODUCTS FROM *ASCARIS SUUM* AND *ASCARIS LUMBRICOIDES*

Summary

Larval stages of the nematodes A. lumbricoides and A. suum were intrinsically labelled, in vitro, with the radioactive amino acid ^{35}[S]-methionine (^{35}[S]-met). These parasites were shown to actively synthesise products, some of which were secreted into the surrounding culture medium labelled with the radio-isotope. The main results obtained from experiments with metabolically labelled molecules were similar to those using ^{125}I-labelled parasite material. For example, ^{35}[S]-met labelled A. suum products are apparently stage-specific, in regard to worm development, antigenic in SRIP's with antiserum from A. suum infected animals, and also crossreact with antibodies from rabbits infected with T. canis or A. lumbricoides. However, there are also differences in the SDS-PAGE profiles of intrinsically and ^{125}I-labelled molecules from A. suum and the human roundworm, including the absence of Mr 1400 and 67000 ^{125}I-labelled components, among others, from the array of ^{35}[S]-met labelled products. Additionally, metabolically labelled antigens appear to be more numerous than the corresponding ^{125}I-labelled components. Some of the disparity of the two labelling techniques might be a reflection of the frequency with which tyrosine or methionine residues are found in larval products.

Examination of the ^{35}[S]-met labelled products from A. suum larvae revealed considerable homology of Mr with the IVRS of A. lumbricoides. These results are encouraging for the continued use of the pig parasite as an experimental equivalent of the human roundworm. However, an Mr 17000 component was apparently unique to the human parasites, although antiserum, raised in rabbits against A. suum,

precipitated this component in SRIP's. Nevertheless, this molecule may eventually become valuable, for diagnostic purposes, in the differentiation of human and pig roundworm infestation.

Introduction

Experiments on Ascaris IVRS (Ch. 3, 4, 6 and 7) have involved ^{125}I-labelled larval products and were conducted under the basic assumption that these secretions were derived from living parasites and in the form that was evident by SDS-PAGE analysis, and not from precursor molecules of different Mr. In addition, there is doubt about the extent to which Ascaris larval somatic components, from dead or dying worms, might be labelled with ^{125}I, along with actual secretions, although attempts were made to keep larval deaths *in vitro* to the lowest levels (Ch. 3). Intrinsic labelling of Ascaris larval products might be of help in determining which molecules of radio-iodinated IVRS are actively synthesised by worms. This technique, when used on nematodes, depends upon the depletion, from larvae in culture, of a chosen amino acid, followed by the addition of the same constituent in a radio-labelled from (Sugane, Howell and Nicholas, 1985; Almond, McLaren and Parkhouse, 1986; Meghji and Maizels, 1986). Most, if not all, newly-synthesised worm proteins should contain the radio-label.

There are several reasons why intrinsically labelled IVRS could be equally as useful as radio-iodination of the examination of Ascaris immunobiology. First, only living parasites should be capable of incorporating a radio-labelled amino acid into newly synthesised proteins. Second, radio-iodination of Ascaris secretions using the

IODO-GEN reagent assumed that only proteinaceous components would be labelled (Markwell and Fox, 1978) although other substances, such as lipids, and/or glycolipids might also be capable of non-specific uptake of ^{125}I (R. M. Maizels, pers. comm.) during radio-iodination of IVRS. Metabolic labelling, on the other hand, using a specific amino acid, should make it probable that only larval products examined by SDS-PAGE would be proteins. Third, examination of ^{125}I-labelled secretions, from human and pig Ascaris worms (Ch. 4) identified possible sources of difference in the components released by both types of nematodes in vitro, which could have been representative of strain and/or species. This heterogeneity of IVRS gel profiles may be of importance in the analysis of the immunobiology of host infection by these parasites, and intrinsic labelling could be of use in determining if both sets of worms actively synthesise different sets of secretions in vitro. In addition, intrinsic labelling could be of use in establishing which components of radio-iodinated Ascaris IVRS are of parasite origin and which are host derived, since host derived molecules would not be synthesised by worms. This only applies to L3/4 larvae, which are collected from the lungs of experimentally infected rabbits, (see Ch. 2 for method and references) and might have ingested or have bound to their cuticle host serum components, such as albumin (Ch. 3).

Results

Intrinsically-labelled Secretions from the Larval Stages of A.suum

^{35}[S]-met labelled IVRS were collected from A. suum infective stage (L2) larvae and examined by SDS-PAGE along with corresponding radio-iodinated molecules (Fig. 5.1). The major biosynthetically labelled worm products were present in a range, of Mr, from 11000 to 129000. Apart from the above mentioned two entities, the remaining components were of approximate Mr; 15000, 18200, 20500, 32700, 44000, 60000, 80500 and 82500 respectively, and, therefore, more numerous than the ^{125}I-labelled products. Three prominent products of ^{125}I-labelling were absent from the array of biosynthetically labelled molecules, namely, those of Mr 14000, 20-28000 and 225000. Reducing conditions did not alter the gel profile of ^{35}[S]-met labelled L2 secretions.

Metabolically labelled L3/4 molecules were also analysed by SDS-PAGE (Fig. 5.2) where these were found to be present in larger number than corresponding radio-iodinated products. The major components of metabolic labelling were of the following Mr: 12500, a quadruplet of bands in the range 15000-18500; Mr 22000, 35000, 41000, 60000, 81000, 110000 and 225000, respectively. Four components of Mr: 14000, 25500, 67000 and 118000, which are significant in gel profiles of ^{125}I-labelled IVRS, were not present among biosynthetically labelled products.

One component of ^{35} [S]-met L3/4 labelled IVRS (Mr 225000) was broken down into elements of lower molecular weights.

Metabolically Labelled Larval Scretions of A.lumbricoides

Earlier experiments have suggested that there might be a molecular basis for distinguishing between A. lumbricoides and A. suum IVRS (Ch. 4). ^{35}[S]-met labelling of the human roundworm larval secretions was performed to further investigate this possibility. Intrinsically labelled L2 worm products were examined by SDS-PAGE (Fig. 5.3) and compared to ^{125}I-labelled L2 components. The major ^{35}[S]-met labelled product is of Mr 17000; the remaining components are of Mr 11800, 20000, 27500, 32500, 35500, 41000, 43000, 55000, 60000, 69000, 80500, 88000, 133000. Three distinct secretions, labelled by the latter technique, were absent from the gel profiles of intrinsically labelled IVRS, namely, those of Mr 14000 and 20-28000 and 225000. Reducing conditions did not apparently break down any L2 secretions.

Metabolically labelled A. lumbricoides L3/4 products were analysed by SDS-PAGE (Fig. 5.4) alongside radio-iodinated lung stage larval IVRS. ^{35}[S]-met labelled secretions ranged in Mr from 12500 to 250000, with major components of Mr: 17000, 22000, 26000, 41000, 53000, 69000, 123000, 155000, 183000 and 225000. However, four components, prominent in ^{125}I-labelled IVRS, are absent from the above array of secretions; these are of Mr: 14000, 28000, 67000 and 118000. Reducing conditions apparently affected the Mr 225000 component of intrinsically labelled worm products so that it was broken down to elements of lower Mr. It is important to note that each Ascaris species synthesises and secretes components that are apparently unique to it. Expecially notable is the Mr 17000 molecule which is the major metabolically labelled product of A. lumbricoides

infective larvae, and also secreted, as a minor labelled product, by L3/4 worms, but not found in the IVRS of larval A. suum stages.

Antigenicity and Reciprocal Reactivity of Intrinsically Labelled A.suum and A.lumbricoides IVRS with Rabbit Anti-Ascaris Antisera.

^{35}[S]-met labelled secretions from L2 and L3/4 larval stages of human and pig roundworms were reacted in SRIP's with antisera, from rabbits infected with the eggs of either species. The precipitates of the reactions were examined by SDS-PAGE, for A.suum secretions in Figs. 5.5 and 5.6 and A.lumbricoides secretions in Figs. 5.7 and 5.8.

The results show that all the radio-labelled components of Ascaris IVRS are antigenic and also that the antigens of one Ascaris species are almost entirely crossreactive with antibodies from rabbits infected with the other worm species. Extensive crossreactions were also found between the major products of both pig and human roundworm secretions, from L2 and L3/4 stages and antisera from rabbits infected with the ascaridoid nematode T. canis. Both the above sets of results support previous data obtained using radio-iodinated secretions (Ch. 3 and 4).

Discussion

The results, presented above, provide the first accounts of ^{35}[S]-met uptake by A. suum and A. lumbricoides larval stages and the apparent incorporation of this radio-label into the secretions of these worms. However, the possibility still remains that at least some of the labelled molecules, in the culture supernatants of Ascaris

larvae, originated from the tiusses and/or body fluid of parasites that used radio-labelled methionine in the synthesis of certain products, but released these *in vitro* after death. This possibility cannot be entirely disregarded, and could have contributed to the differences with ^{125}I-labelled products, although the percentage of larval deaths were minimal, (approximately 5%, on average). It is unlikely, therefore that worm somatic products were represented, at a notable level, in metabolically labelled IVRS. Additionally, the antigenicity of the metabolically labelled products, shown in SRIP's with antibodies from Ascaris-infected rabbits, supports the case for these being actively synthesised, then secreted, by worms *in vitro*, as well as *in vivo*, since secreted, and not somatic products of nematodes, would presumably be the primary target of rabbit antibody responses (Discussion, Ch. 3).

The main results obtained with metabolically labelled IVRS were similar to those for radio-iodinated secretions, (Ch. 3) in terms of antigenicity, and immunological crossreactivity, of the products of one Ascaris species, and antiserum from rabbits infected with the other type of parasite. Moreover, gel analysis showed several apparently common features between the radio-iodinated and ^{35}[S]-met labelled secretions, of a given worm developmental stage, on the basis of identical Mr, although identical Mr is not proof of homology. However, the appearance of major IVRS products at the same Mr from the same parasite species argues for the possibility that these entities might be homologous. Differences were also detected between ^{35}[S]-met and ^{125}I-labelled secretions, on analysis by SDS-PAGE and autoradiography. Using *A. suum* as an example, some products of radio-

iodination were absent from corresponding gel profiles of intrinsically labelled material. This included the Mr 14000 antigen from L2 and L3/4 larvae, and the Mr 67000 component from lung stage worms. Additionally, the ^{35}[S]-met labelled molecules were present in, apparently, greater number than the secretions labelled by the other technique.

The non-appearance of certain components, that were radio-labelled with ^{125}I, from the gel profile of metabolically labelled secretory products might be attributed to several possible factors. For instance, some molecules could be missing from the array of ^{35}[S]-met labelled products due to larvae stopping the production of these proteins before the end of their maintenance time *in vitro*, which was, on average, eight days (Ch. 2). Alternatively, larvae could have accumulated certain proteins in concentrations large enough to discourage renewed synthesis of these components, by the time radio-isotopic methionine was added to the larval cultures *in vitro*. These products might not then be visible in autoradiograms of gels containing the products of metabolic labelling. The Mr 14000 element is apparently present as an ^{125}I-labelled Ascaris secretion, as well as in perienteric fluid and secretions of the adult worm stage (Ch. 3, Ambler, *et al*, 1973 a and b). IODO-GEN mediated radio-iodination of ABF shows that the Mr 14000 component also takes up the most radiolabel, relative to other ABF constituents and appears to be present in the largest concentration, (Ch. 3) judging by this method. These observations might be relevant to the lack of labelling of this particular component by ^{35}[S]-met, if it exists inside larvae in proportion to the apparent amount in adult worms. The above arguments

might not apply to the major Mr 67000 molecule of radio-iodinated Ascaris lung stage larval IVRS, because it is likely that this product is rabbit serum albumin (Ch. 3).

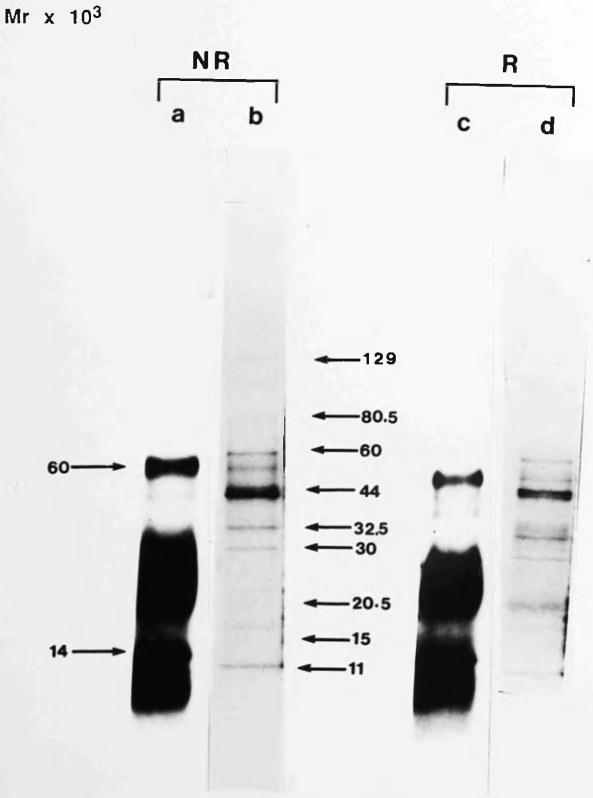
One of the most likely explanations for the disparity of SDS-PAGE profiles of ^{125}I and ^{35}[S]-met labelled secretions, including the apparent greater number of products labelled by the latter method, is that there is a bias of each radio-labelling technique for one particular amino acid. Those proteins that contained multiple tyrosine residues, but little, or no methionine, in their amino acid sequence would be labelled with radio-iodide and not with ^{35}[S]-methionine. Similarly, proteins with high methionine, and low tyrosine content might be labelled with ^{35}[S] - met but not with ^{125}I. The problem of appropriate choice of target amino acids, for investigation of parasite secretions was also highlighted by Irving and Howell (1982), who labelled the IVRS of liver stage juvenile Fasciola hepatica with ^{14}[C]-leucine, ^{35}[S]-met or ^{14}[C]-isoleucine (^{14}[C]-ile). Gel analysis showed that an Mr 27000 component was a minor product of radio-labelling with the first and second reagents, but a major product of labelling with ^{14}[C]-ile, presumably because it contained relatively high amounts of isoleucine, but relatively few residues of leucine and methionine. One technique that could be used in alleviating the problem of labelling with a single isotope, involves the labelling of parasite products simultaneously with two radioactive amino acids instead of one. This method has been successfully performed on adult Necator americanus products, using both ^{3}[H]-leucine and ^{35}[S]-methionine, at the same time, (Carr and Pritchard, 1986) and could be easily used for the study of Ascaris

IVRS. It is possible that another technique could be useful for probing the relative amounts of certain amino acids in Ascaris IVRS, namely the treatment of radioactive secretions with site-restricted proteinases and analysing the products by SDS-PAGE. This technique could facilitate the choice of radio-labelling reagent for Ascaris secretions.

The situation for the metabolically labelled products of A.lumbricoides, in regard to homology, and heterology, with radio-iodinated products was similar to that of A. suum, described above. Additionally, there were also indications of ^{35}[S]-met labelled molecules that were apparently specific to either A. suum or the human roundworm larvae, but were totally crossreactive with polyclonal antiserum from rabbits infected with the heterologous species. This was also found to be the case when ^{125}I-labelled secretions were examined previously (Ch. 4). From the data presented, in this chapter, it appears that there is a major ^{35}[S]-met labelled secretion of Mr 17000 from infective A. lumbricoides larvae, that is unique to human worms only, as in ^{125}I- labelled L2 culture fluid. The possible significance of this secretion to the specific diagnosis of ascariasis has been discussed earlier (Ch. 4) and it is also shown here to be a major difference in the SDS-PAGE profiles of metabolically labelled L2 A. suum and A. lumbricoides IVRS. There is a high apparent level of homology between the metabolically labelled secretions of the human and pig roundworm, on the basis of Mr, in SDS-PAGE profiles of labelled secretions, and in gels of SRIP's, with antibodies from Ascaris infected rabbits. The overall degree of similarity of the IVRS from two species of Ascaris, using two different methods of

radio-labelling, augurs well for the continued use of the pig worm, and its secretions in the investigation of immunological aspects of human ascariasis.

Fig. 5.1 Intrinsically Labelled *A. suum* L2 Larval Secretions

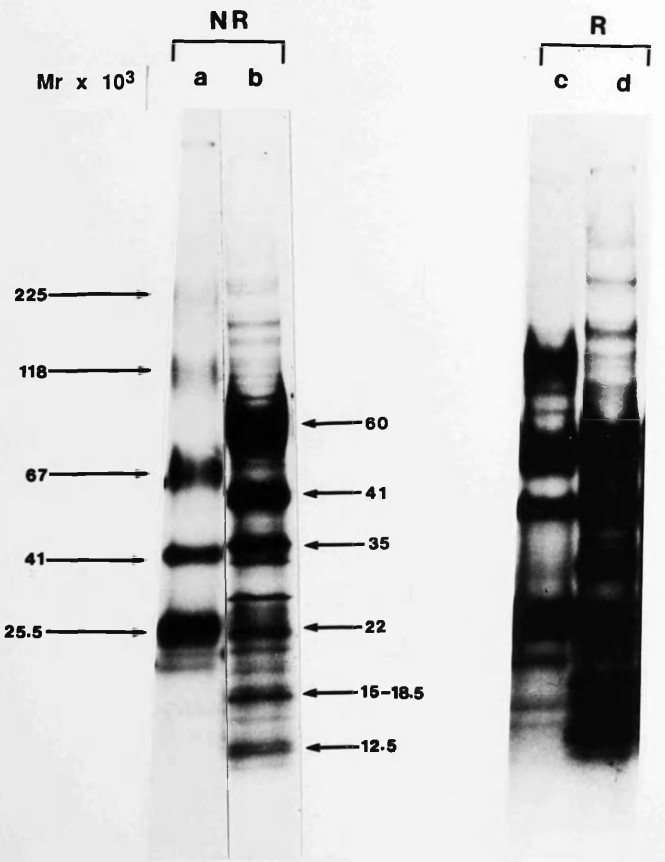


a) *A. suum* L2 IVRS (12
b) *A. suum* L2 IVRS (35
c) *A. suum* L2 IVRS (12
d) *A. suum* L2 IVRS (35

Fig. 5.1

Metabolically labelled A.suum L2 secretions. The IVRS products of A.suum L2 larvae were labelled extrinsically with ^{125}I (a and c) or intrinsically with ^{35}[S]-met (b and d) and analyzed by SDS-PAGE. Tracks (c) and (d) represented reducing conditions.

Fig. 5.2 Intrinsically Labelled *A. suum* L3/4 Larval Secretions

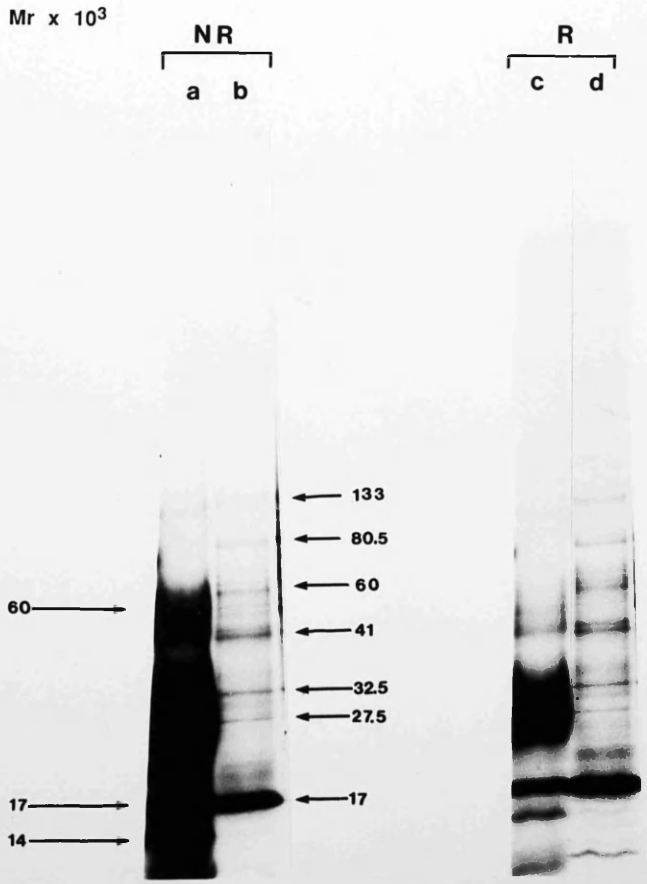


a) *A. suum* L3/4 IVRS (125
b) *A. suum* L3/4 IVRS (35 S
c) *A. suum* L3/4 IVRS (125
d) *A. suum* L3/4 IVRS (35 S

Fig. 5.2

Intrinsically labelled L3/4 secretions of A.suum.IVRS from A.suum L3/4 larvae were labelled either intrinsically with ^{35}[S]-met (b and d) or extrinsically with ^{125}I (a and c) and analyzed by SDS-PAGE. Tracks (c) and (d) represented reducing conditions.

Fig. 5.3 Intrinsically Labelled *A. lumbricoides* L2 Larval Secretions



a) *A. lumbricoides* L2 IVRS (^{125}I)
b) *A. lumbricoides* L2 IVRS (^{35}S)
c) *A. lumbricoides* L2 IVRS (^{125}I)
d) *A. lumbricoides* L2 IVRS (^{35}S)

Fig. 5.3

Metabolically labelled A.lumbricoides L2 IVRS. IVRS from infective stage A.lumbricoides larvae were labelled intrinsically with ^{35}[S]-met (b and d) or labelled with ^{125}I (a and c). Samples in tracks (c) and (d) were electrophoresed under reducing conditions.

Fig. 5.4 Intrinsically Labelled *A. lumbricoides* L3/4 Larval Secretions

a) *A. lumbricoides* L3/4 IVRS (^{125}I
b) *A. lumbricoides* L3/4 IVRS (^{35}S
c) *A. lumbricoides* L3/4 IVRS (^{125}I
d) *A. lumbricoides* L3/4 IVRS (^{35}S

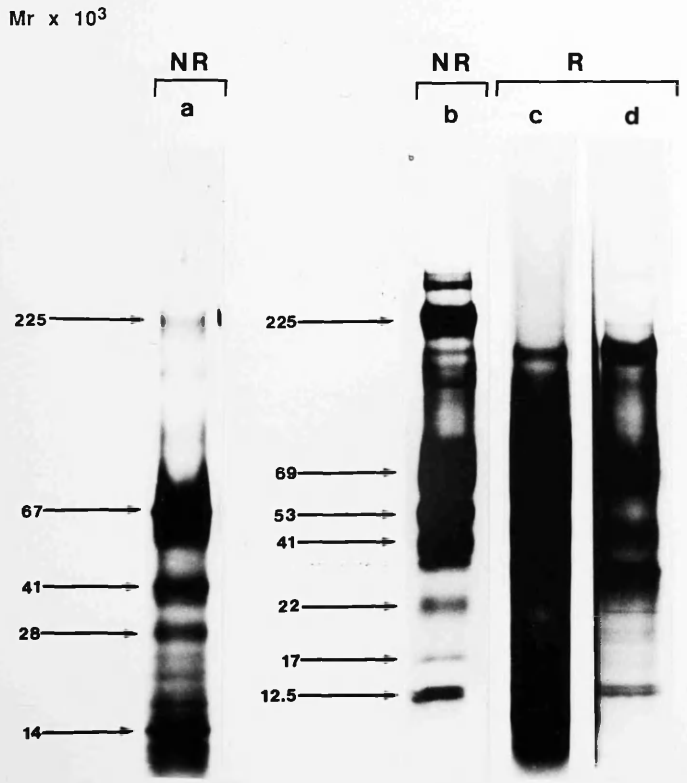
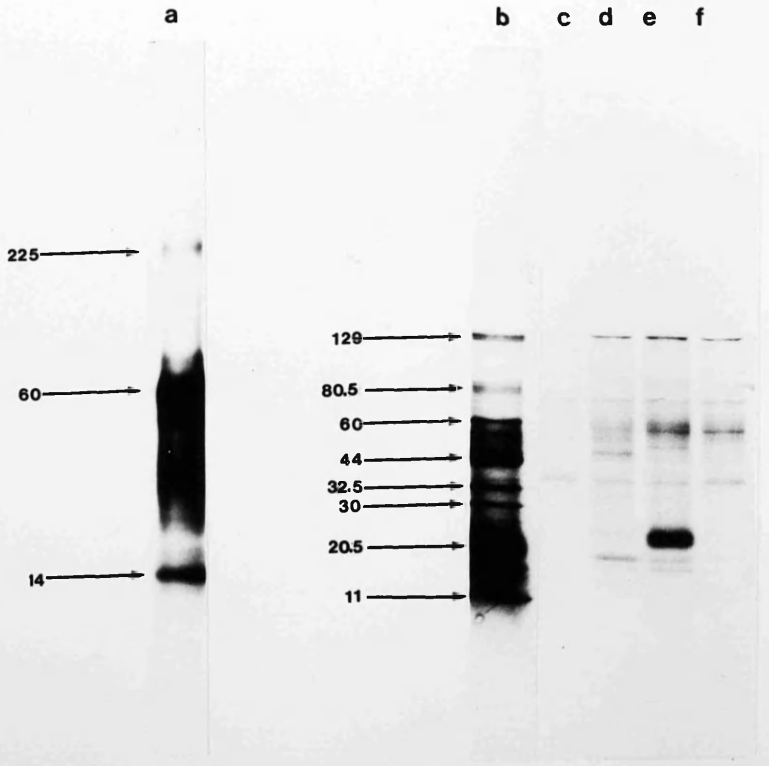


Fig. 5.4

Intrinsically labelled A.lumbricoides L3/4 secretions, IVRS of pulmonary stage A.lumbricoides larvae were labelled intrinsically with ^{35}[S]-met (b and d) & extrinsically with ^{125}I (a and c). Samples in tracks (c) and (d) were subjected to electrophoresis under reducing conditions.

Fig. 5.5 Antigens of Intrinsically Labelled *A. suum* L2 Larval Secretions

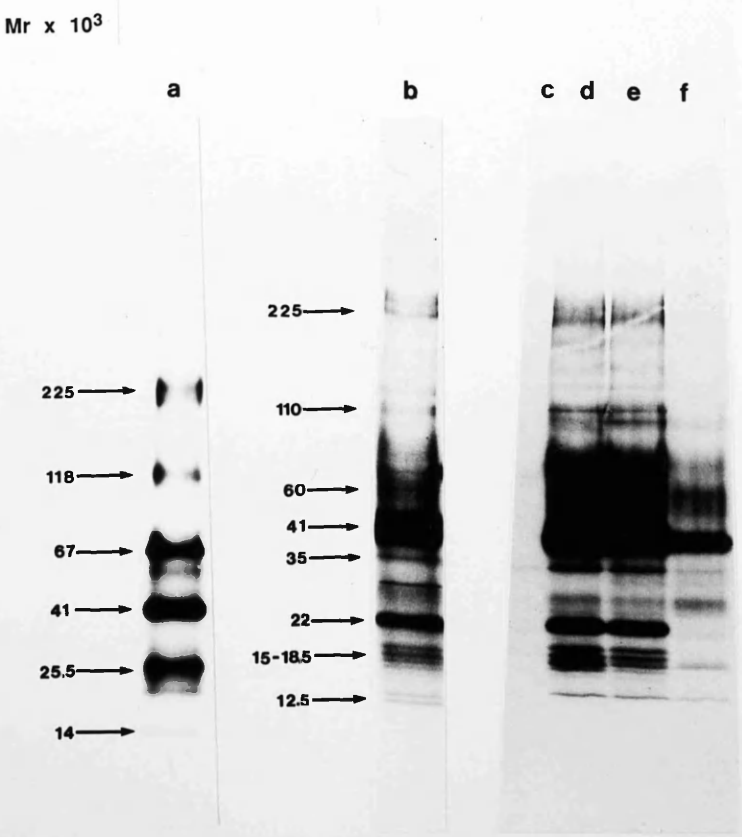


a) *A. suum* L2 IVRS (^{125} I)
b) *A. suum* L2 IVRS (^{35} S)
c) N. Rab. S.
d) Rab. a. *A. suum*
e) Rab. a. *A. lumbricoides*
f) Rab. a. *T. canis*

Fig. 5.5

Intrinsically labelled A.suum L2 secretions are antigenic. The IVRS of A.suum L2 larvae labelled with ^{35}[S]-met were reacted in immunoprecipitations, mediated by S.aureus, with the following antisera: uninfected rabbit serum (c), serum from rabbits multiply infected with A. suum (d), A. lumbricoides (e), or infected on a single occasion with T. canis (f). The immunosorbates were analyzed by SDS-PAGE. ^{125}I and ^{35}S-met labelled A. suum L2 IVRS are shown in tracks (a) and (b) respectively.

Fig. 5.6 Antigens of Intrinsically Labelled *A. suum* L3/4 Larval Secretions

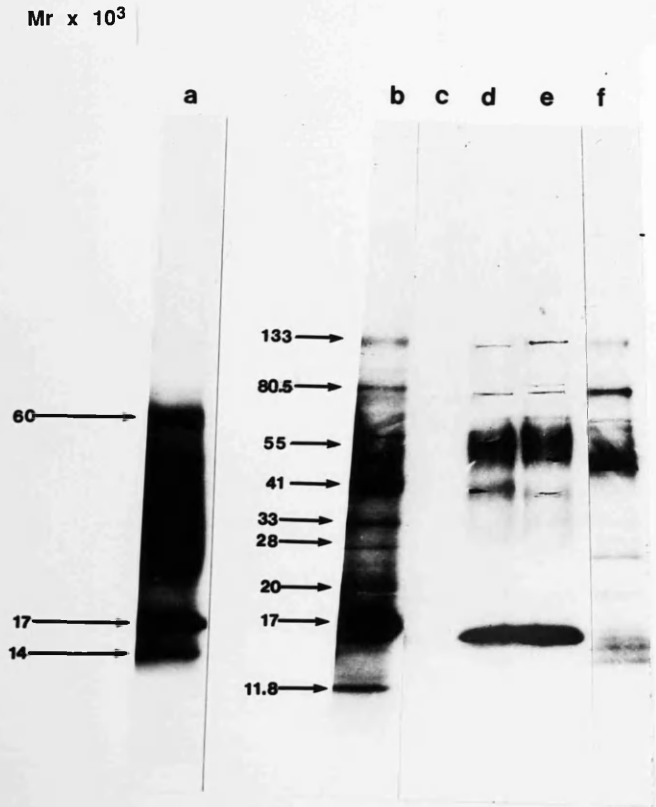


a) *A. suum* L3/4 IVRS (^{125} I)
b) *A. suum* L3/4 IVRS (^{35} S)
c) N. Rab. S.
d) Rab. a. *A. suum*
e) Rab. a. *A. lumbricoides*
f) Rab. a. *T. canis*

Fig. 5.6

Secreted antigens of intrinsically labelled A.suum L3/4 larvae. The IVRS of ^{35}[S]-met labelled A.suum pulmonary stage worms were reacted in immunoprecipitations, mediated by S.aureus, with sera from rabbits multiply infected with A. suum (d), A. lumbricoides (e), or infected on a single occasion with T. canis (f). Reactions of ^{35}S-met labelled IVRS with uninfected rabbit serum are shown in (c). The immunosorbates were analyzed by SDS-PAGE. ^{125}I and intrinsically labelled A. suum L3/4 IVRS are shown in tracks (a) and (b) respectively.

Fig. 5.7 Antigens of Intrinsically Labelled
A. lumbricoides L2 Larval Secretions



a) *A. lumbricoides* L2 IVRS (^{125}I)
b) *A. lumbricoides* L2 IVRS (^{35}S)
c) N. Rab. S.
d) Rab. a. *A. suum*
e) Rab. a. *A. lumbricoides*
f) Rab. a. *T. canis*

Fig. 5.7

Antigenicity of metabolically labelled A.lumbricoides L2 secretions. A.lumbricoides L2 secretions labelled with ^{35}[S]-met were reacted with the following sera in an SRIP: uninfected rabbit serum (c), serum from rabbits multiply infected with A. suum (d), A. lumbricoides (e), or infected on a single occasion with T. canis (f). The resultant immunoadsorbates were analyzed by SDS-PAGE. ^{125}I and ^{35}S-met labelled A. lumbricoides L2 IVRS are shown in tracks (a) and (b) respectively.

Fig. 5.8 Antigens of Intrinsically Labelled
A. lumbricoides L3/4 Larval Secretions

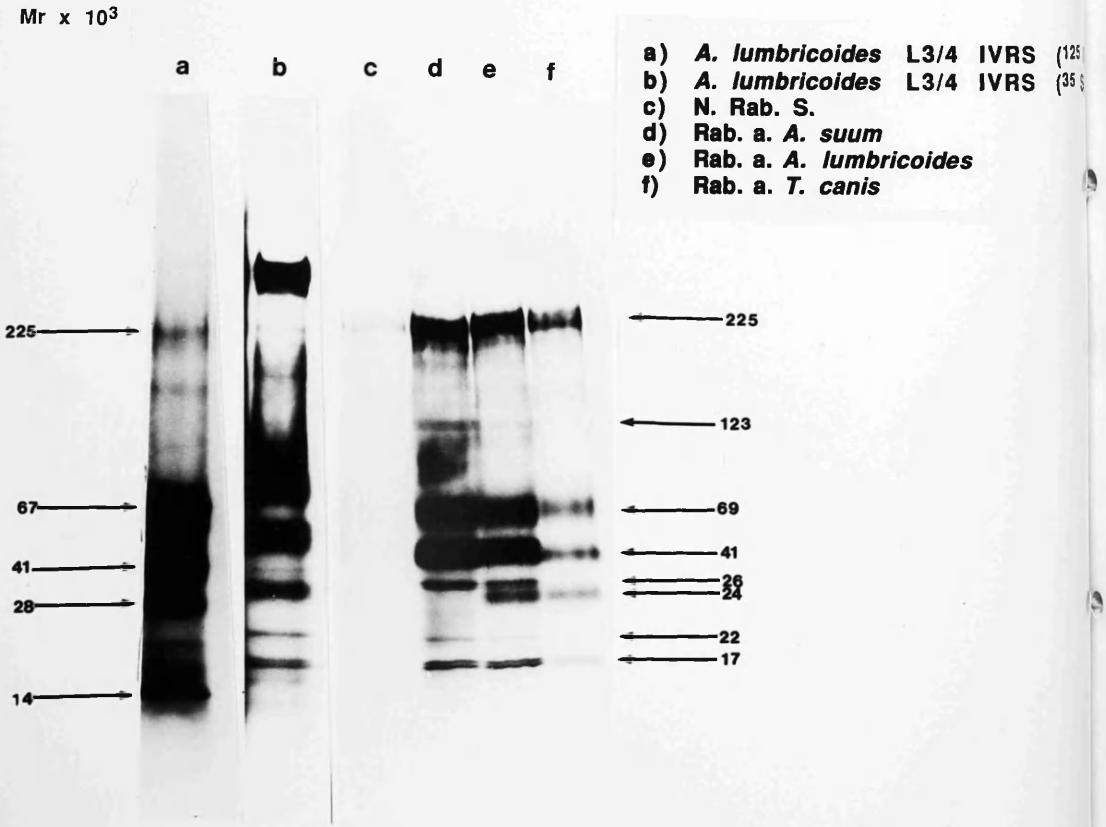


Fig. 5.8

Antigenicity of intrinsically labelled A.lumbricoides L3/4 IVRS. The secretions of pulmonary stage A.lumbricoides larvae were labelled with ^{35}[S]-met and reacted in SRIP's with sera from rabbits multiply infected with A. suum (d), A. lumbricoides (e), or infected on a single occasion with T. canis (f) or serum from uninfected rabbits (c). The resultant immunoadsorbates were analyzed by SDS-PAGE. Radio-iodinated or intrinsically labelled A. lumbricoides L3/4 secretions are shown in tracks (a) and (b) respectively.

RESISTANCE OF NEMATODE SECRETIONS TO RAT MAST CELL PROTEINASES *IN VITRO*

Summary

The secretion of rat mast cell proteinase type II, RMCP II, by mucosal mast cells is associated with the expulsion phase of intestinal nematode infection in rats. (Woodbury et al, 1984). The mode of action of the enzymes is unknown. One possibility is the inactivation of worm secretions (IVRS) by proteinase cleavage. This was examined by exposing the ^{125}I-labelled products of Ascaris suum larval stages to a range of proteinases, including RMCP I and II, and analysis of the reaction products by SDS-PAGE and autoradiography. All of the iodinated IVRS components were digested by pronase and were, therefore, proteinaceous. Unexpectedly, components of A.suum secretions were found to be resistant to the action of two mammalian enzymes, trypsin and ohymotrypsin. Additionally, the products of the nematode were extensively resistant to both RMCP I and II, although one component was suceptible to proteolysis by these. These results would seem to argue against a direct action of these mast cell enzymes against nematode products, in vivo.

Introduction

Infection of the rat with intestinal nematodes, such as Nippostrongylus brasiliensis and Trichinella spiralis, typically results in the accumulation of large numbers of intestinal mucosal mast cells (IMMC) at the site of infection (Woodbury and Miller, 1982; Miller et al, 1983; Woodbury et al, 1984). However, larvae from earlier migratory stages, through the host liver and lungs, may be subject to the action of an antigenically and biochemically different type of mast cell (Befus et al, 1982; Austen, 1984; Lee et al, 1985). These cells are localised in the peritoneal cavity and connective tissue of rats and are collectively known as connective tissue mast cells (CTMC).

Among other differences, the two types of cells differ in the type of serine proteinase that they contain (Beinenstock et al, 1982; Barrett and Metcalfe, 1984; Jarrett and Haig, 1984). Rat mast cell proteinase type I (RMCPI) is apparently bound, by ionic interactions, to heparin proteoglycan in the granules of CTMC, (Gibson and Miller, 1986) whereas rat mast cell proteinase type II (RMCP II) exists in a soluble form in IMMC (Woodbury, Gruzenski and Lagunoff, 1978) and is a specific biochemical marker for these cells.

RMCP II is released in considerable amounts during the expulsion of intestinal nematodes, (Woodbury et al, 1984). The function of this enzyme is unclear, but there are several possibilities, including general tissue degradation and repair (Seppa, Vannanen and Korhonen, 1979) and activation or inactivation of vasoactive peptides (Seppa, 1980). There is also the additional possibility, not hitherto examined, for direct attack by RCMP I and II on the two functional compartments

of nematodes that may be accessible to attack by immune effector cells (and their products) namely, the parasite surface, or secretary molecules.

Here, the possible actions of RMCP I and II on the IVRS of A.suum larval stages; were examined, along with the action of two mammalian enzymes, trypsin and chymotrypsin, that may be encountered in host animals by different developmental stages of the parasite (Martzen, Geise and Peanasky, 1986). The IVRS of A.suum are assumed to be identical to those released by worms in vivo, and have been examined in detail previously (Chapter 3) and may be of importance in the establishment, and maintenance, of ascariasis. Conceivably, degradation of IVRS by one, or more, above enzymes could influence the pathology of Ascaris infection as well as limiting the effectiveness of worm survival in vivo.

This study reveals that A.suum secretions are highly resistant to clearage by RMCP I and II, and also demonstrates that there are components of IVRS that are not hydrolyzed by either of the classic site-specific enzymes, chymotrypsin and trypsin. A direct enzymatic action on the products of A.suum in vivo is, therefore, deemed unlikely. Speculations are also made regarding the possible existence of novel amino acid sequences, and mechanisms of avoidance of enzyme hydrolysis by nematode products.

Results

Sensitivity of Ascaris IVRS to Pronase.

Previous evidence has shown that all of the radioiodinated secretions of Ascaris L2 larvae and most of the secretions of lung-stage worms, except for the Mr 67000 component, are antigenic and, presumably, of worm origin (Chapter 3). It was also assumed that the radiolabelled products from Ascaris were proteinaceous but evidence that lipids could bind to the radiolabel in a non-specific manner (R. M. Maizels pers. comm.) made it necessary to investigate the composition of IVRS. This was performed by incubation of radioiodinated A. suum L2 (Fig. 6.1) or $L^{3}/_{4}$ (Fig 6.2) IVRS with pronase. This is a mixture of endo-and exoproteinases from Streptomyces griseus, with a broad specificity for polypeptide hydrolysis. The products of the reaction were resolved on SDS-PAGE and confirmed that all the radiolabelled A. suum secretions were proteinaceous and, therefore, hydrolyzed by pronase.

Resistance of Some Ascaris IVRS to Cleavage by Site-Restricted Proteinases

Trypsin and chymotrypsin both cleave polypeptides at the carboxyl side of lysine and arginine residues (L. Stryer 1981). Radioiodinated A. suum L2 and L3/4 IVRS were incubated with trypsin and the products of the reaction examined by SDS-PAGE. A. suum infective-stage secretions were found to be sensitive to the action of this enzyme (Fig. 6.3). In contrast, L3/4 IVRS exhibited some resistance to proteolysis (Fig. 6.4) and two components, respectively of Mr 20000 and 41100, were apparently unaffected by trypsin action. The gradual enzymatic degration of the Mr 118000 component of L3/4 products was shown by the use of successively higher concentrations of

the proteinase.

Digest products of the enzymatic reaction between ^{125}I-labelled A.suum L2 IVRS and chymotrypsin were analysed by SDS-PAGE (Fig. 6.5). The majority of these products were subject to proteolysis by the enzyme, except for one of Mr 20,000.

Restricted Cleavage of A.suum IVRS by RMCP I and II

Both RMCP I and II have a specificity for cleavage of polypeptides which is similar to that for chymotrypsin. The reaction products of RMCP II with ^{125}I radiolabelled A.suum L2 or L3/4 ES, were analysed by SDS-PAGE, (Fig. 6.6. and 6.7, respectively). Apparently, only the Mr 20-28,000 component was a substrate for hydrolysis for L2 secretory products (Fig. 6.6) and L3/4 IVRS components were all resistant to proteolysis by RMCP II. Identical results were obtained for the CTMC proteinase, RMCP I, incubated with either A.suum infective larval products (Fig. 6.8), or those from the pulmonary stages (Fig. 6.9). The Mr 20-28,000 L2 secretion was hydrolyzed apparently into fragments too small to be resolved by SDS-PAGE.

Discussion

The expulsion of intestinal nematodes from infected animals may involve an antibody response, possibly including an increase in serum, IgE levels, (Ogilvie and Love, 1974; Wakelin, 1976) and also a non-specific cellular immune response involving, for example, basophilia, eosinophilia and mucosal mastocytosis (Kelly and Ogilvie, 1972; Askenase, 1980).

Mastocytosis is a common event in intestinal nematodiasis of rats (Mayrhofer and Fisher, 1979; Mitchell, 1979; Askenase, 1980) and may be under T-cell control (Nawa and Miller, 1978; Mitchell, 1979). The resultant production and release, by IMMC, of RMCP II, (Woodbury and Miller, 1982; Miller et al, 1983) indicates that these may influence the onset and severity of intestinal inflammation. There is also an additional possibility which has not been previously examined, namely, that RMCP II could hydrolyze, the surface, or secretory products, of the parasites.

RMCP II has a number of structural and mechanistic features which are unusual among serine proteinases, including unusual substrate specificity, requiring leucine and phenylalanine adjacent to the site of cleavage (Yoshida et al, 1980) probably resulting from a less polar binding site (Woodbury et al, 1978) and less disulphide bonds at the active site (Segal et al, 1971) than similar enzymes.

Evidence suggests that RMCP II may belong to a sub family of serine proteinases, which are likely to be associated with lytic processes that are the end products of target cell attack by immune effector mechanisms (reviewed by Reid, 1986). These enzymes include

proteinases from cytotoxic T and (Lobe et al, 1986) helper-killer T-cells, (Pasternak and Eisen, 1985, Pasternak et al, 1986) natural killer cells (Young and Cohn, 1986) and factor D of complement (Johnson, Gagnon and Reid, 1984; Niemann et al, 1984).

Common features within the family of serine esterases (including RMCP II) extend to primary amino acid sequence and substrate specificity and the possibility exists that these enzymes may be involved in regulated, complement-like "cascade" systems that eventually have a lytic effect on target cells. (Reid, 1986; Tschopp, Masson and Stanley, 1986). This hypothesis is supported by observations of the cleavage of complement components C3, C4 and C5 by RMCP I, or RMCP-like enzymes, (Schwartz et al, 1983; Gervasoni et al, 1986).

The results of this study on the treatment of A.suum antigens with various proteinases are summarized in Tables 6.1 and 6.2. A general observation is that components of the secretions of this nematode are resistant to cleavage by the enzymes used, and especially to the action of RMCP I and II.

Conceivably, A.suum secretions may have been selected, during worm evolution, for resistance to host enzymes, including mast cells proteinases. Although it seems apparent that RMCP I and II may not play a role in the degradation of nematode secretions, too little is known regarding the biochemistry and function of these productions for this possibility to be overlooked. The targets for proteolysis may involve molecules that could be conserved, in amino acid sequence, and important for parasite viability in vivo.

The failure by mast cell, and other host proteinases used, to hydrolyze all of the secretions from L2 and L3/4 stages of A.suum, may be due to several important factors. One reasonable assertion involves the production of enzyme inhibitors by the worms. Substances that curtail the activity of chymotrypsin and elastase have been localised to A.suum larvae, eggs and adult stages (Martzen and Peanasky, 1985; Martzen et al, 1985; Martzen, Geise and Peanasky, 1986). Moreover, adult worm extracts of this worm were found to contain inhibitory activity specific for the gut enzyme leucine aminopeptidase (Zenka and Prokopic, 1985). However, in the digests performed in this study, the considerable excess of enzyme in relation to the minute amounts of radiolabelled parasite material would, presumably overcome the action of inhibitory agents present among IVRS.

The possibilities exist, that in resistant Ascaris secretions, specific enzyme cleavage sites may be either absent; or made less accessible to proteinases by some feature of IVRS conformation, or a predominance of carbohydrate. Ascaris products are glycosylated, (Chapter 7) but the extent to which this feature can affect the hydrolysis of these molecules is not known. It is interesting to note that the Mr 118,000 component of A.suum L3/4 IVRS is only partially degraded, by trypsin, into a product of lower Mr, suggesting that this product is not broken down further due to the lack of access, to the enzyme, of certain lysine and arginine residues, or to the absence of these amino acids.

Deductions can be made regarding the amino acid sequences of

A. suum that are susceptible to enzymatic hydrolysis. For instance, the results suggest that the majority of A. suum L2 IVRS contain a preponderance of lysine or arginine residues (trypsin specificity) and also any of tyrosine, tryptophan and phenylalanine (chymotrypsin specificity). Also present may be the sequences required for cleavage of the Mr 20-28,000 component of L2 IVRS by RMCP I and II. This antigen is the only secretion from A. suum L2 and L3/4 worms that is the target of the mast cell proteinases. For this reason, as well as others mentioned earlier, there is speculation regarding the possible biological function(s) of RMCP II and I.

RMCP II, but not its counterpart in CTMC, is found to degrade naturally occurring type IV collagen in vitro (Sage, Woodbury and Bornstein, 1979). This is a matrix of collagen like fibrils that is uniquely associated with basement membranes, (Kefalides, 1975), including that which underlies the epithelial cells of the gastrointestinal mucosa. Perhaps the most plausible in vivo function for RMCP II, is the hydrolysis of those collagenous chains, which could head to a disruption of mucosal epithelial cell arrangement. This, in turn, could cause the influx of scrum components and effector cells into the intestinal lumen (Murray, 1972; Sinski and Holmes, 1978; Castro, 1982: Lee and Ogilvie, 1982). This occurrence may initiate a series of events that could eventually lead to the expulsion of the intestinal nematode.

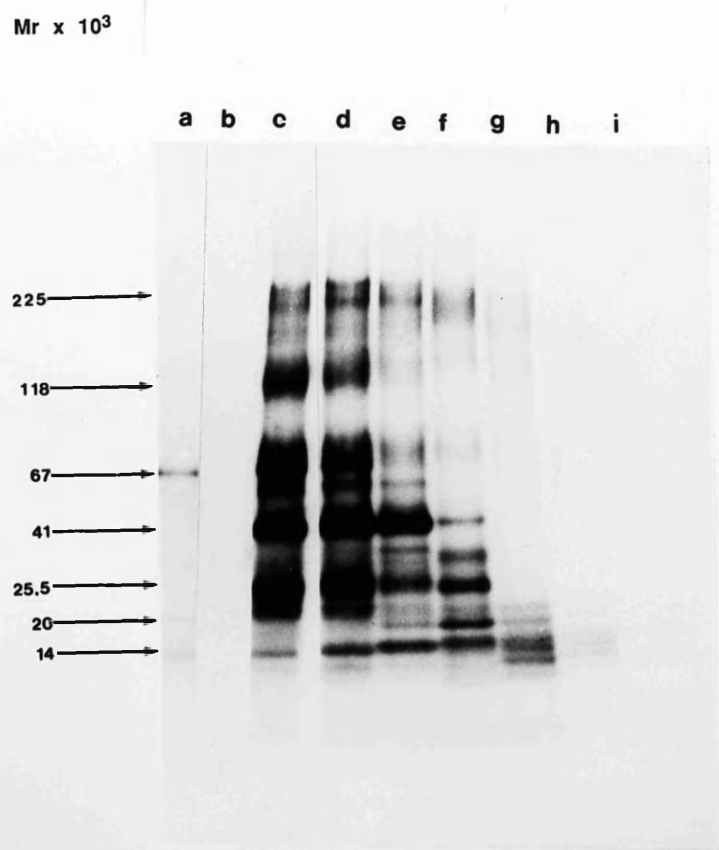
Fig. 6.1 *A. suum* Infective Larval Secretions are Proteinaceous

a) Low M. Wt. Markers
b) Low M. Wt. Markers + pronase
c) *A. suum* L2 IVRS
d-i) *A. suum* L2 IVRS + increasing amts pronase

Fig. 6.1

A.suum infective larval secretions are proteinaceous. A.suum L2 IVRS were radiolabelled with ^{125}I and incubated with pronase at the following concentrations: none (c), 2.5 µg/ml (d), 25 µg/ml (e), 250 µg/ml (f), 2.5 mg/ml (g), 12.5 mg/ml (h), 25 mg/ml (i). The enzyme reactions took place in water at 37°C, for 30 mins and the products examined by SDS-PAGE. Low molecular weight markers (LMW) labelled with ^{125}I were reacted with 25 mg/ml pronase (b) and untreated radioiodinated markers are shown in track (a).

Fig. 6.2 *A. suum* Pulmonary Stage Larval Secretions are Proteinaceous

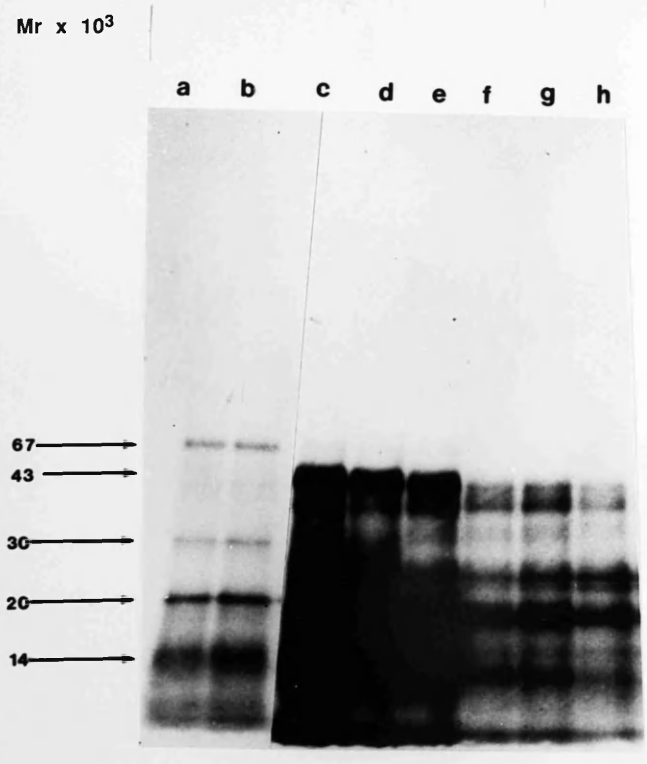


a) Low M. Wt. Markers
b) Low M. Wt. Markers + pronase
c) *A. suum* L3/4 IVRS
d-i) *A. suum* L3/4 IVRS + increasing amts pronase

Fig. 6.2

A.suum pulmonary stage larval secretions are proteinaceous. IVRS collected from L3/4 A.suum larvae were radio-iodinated and mixed with pronase at the following concentrations: none (c), 2.5 µg/ml (d), 25 µg/ml (e), 250 µg/ml (f), 2.5 mg/ml (g), 12.5 mg/ml (h) and 25 mg/ml (i). ^{125}I-labelled Low molecular weight markers were also incubated with 25 mg/ml pronase (b). All the enzymic reactions took place in water at 37^{o}C for a period of 30 mins. The products were analyzed by SDS-PAGE. Untreated radiolabelled markers are shown in track (a).

Fig. 6.3 **Limited Proteinolysis of *A. suum* L2 Secretions by Trypsin**

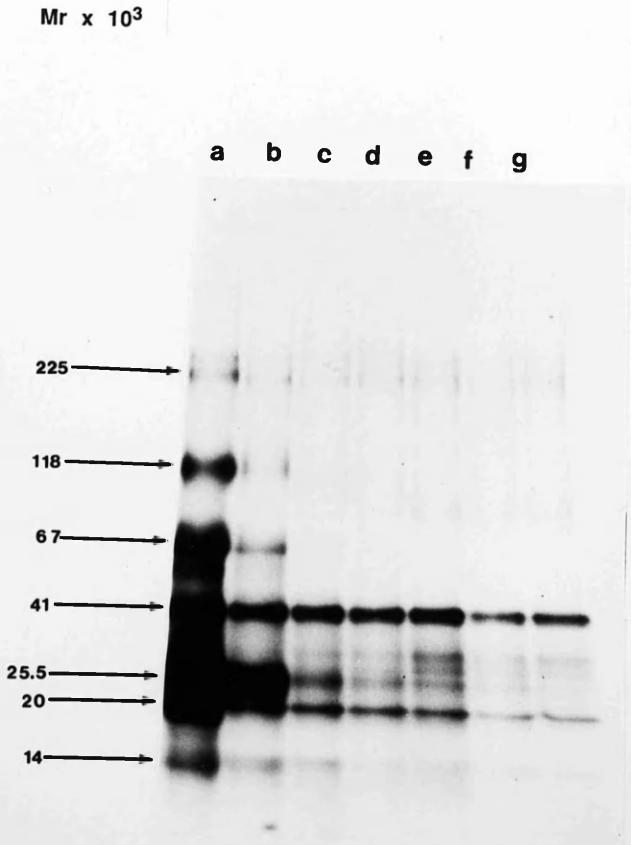


a) Low M. Wt. Markers
b) Low M. Wt. Markers + trypsin
c) *A. suum* L2 IVRS
d-h) *A. suum* L2 IVRS + increasing a
trypsin

Fig. 6.3

Resistance of A.suum infective larval secretions to cleavage by trypsin ^{125}I-labelled A.suum L2 IVRS were incubated with the following concentrations of trypsin: none (c), 1 mg/ml (d), 2 mg/ml (e), 4 mg/ml (f), 5 mg/ml (g) and 10 mg/ml (h). LMW were radio-iodinated and also mixed with 10 mg/ml of the enzyme (b). The enzyme reactions were performed in 100 mM Tris HCl (pH 8.0) containing 50 mM $CaCl_2$, at 37 C for 2 hr, and the products examined by SDS-PAGE. Untreated LMW markers are shown in (a).

Fig. 6.4 Some *A. suum* L3/4 Secretions are Cleaved by Trypsin

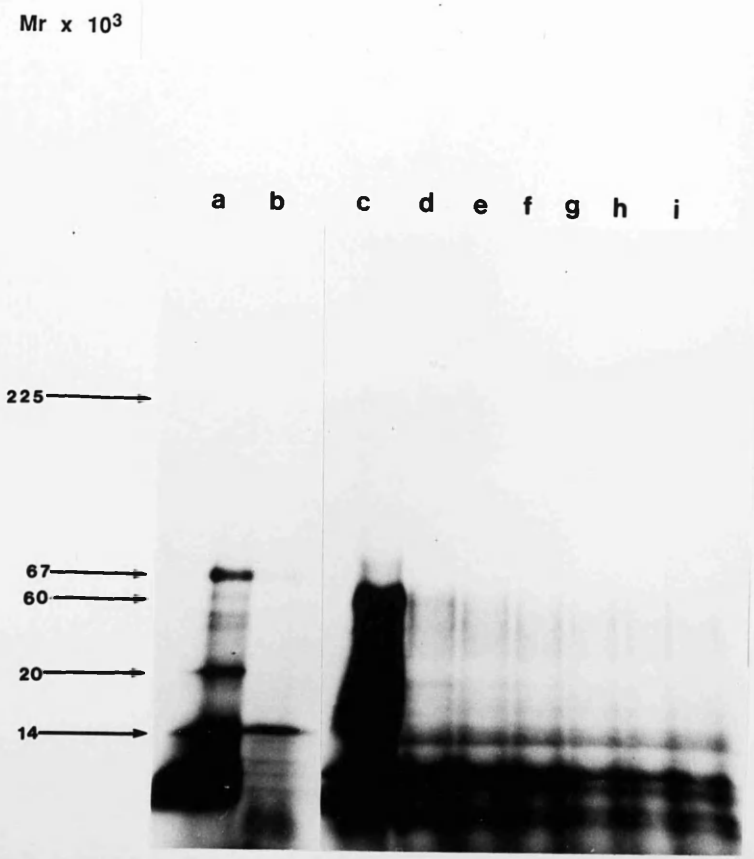


a) *A. suum* L3/4 IVRS
b-g) *A. suum* L3/4 IVRS + increasing amts trypsin

Fig. 6.4

Some A.suum lung-stage larval secretions are cleaved by trypsin. The IVRS of A.suum L3/4 larvae were radio-iodinated and mixed with the following concentrations of trypsin: none (a) 10 µg/ml (b), 50 µg/ml (c), 100 µg/ml (d), 500 µg/ml (e), 1 mg/ml (f) and 5 mg/ml (g). The enzyme reactions were performed in 100 mM Tris HCl (pH 8.0) containing 50 mM $CaCl_2$, at 37 C for 2 hr, and the products examined by SDS-PAGE.

Fig. 6.5 Resistance of *A. suum* L2 Secretions to Cleavage by Chymotrypsin

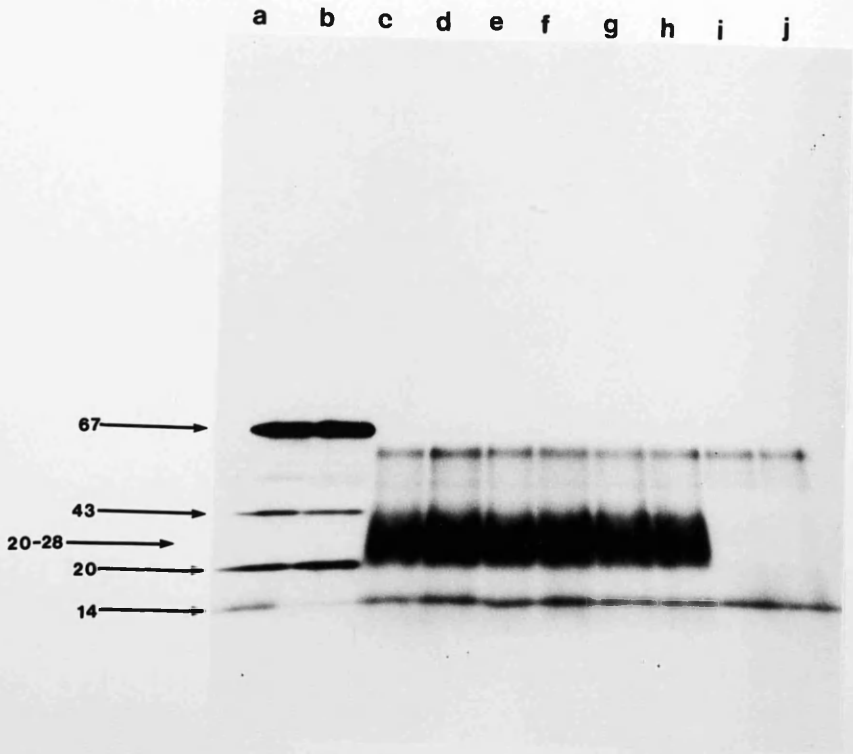


a) Low M. Wt. Markers
b) Low M. Wt. Markers + cht A
c) *A. suum* L2 IVRS
d-i) *A. suum* L2 IVRS + increasing amts cht A

Fig. 6.5

Resistance of A.suum L2 IVRS to cleavage by chymotrypsin. Radio-iodinated A.suum L2 IVRS were reacted with chymotrypsin, at the following concentrations: none (c), 1 mg/ml (d), 2 mg/ml (e), 3 mg/ml (f), 4 mg/ml (g), 5 mg/ml (h) and 10 mg/ml (i) LMW were labelled with ^{125}I and also mixed with 10 mg/ml of the enzyme (b). Untreated markers are shown in (a). The enzyme reactions were performed in 100 mM Tris HCl (pH 8.0) containing 50 mM $CaCl_2$, at 37 C for 2 hr, and the products examined by SDS-PAGE.

Fig. 6.6 Limited Proteinolysis of *A. suum* L2 Secretions by RMCP II

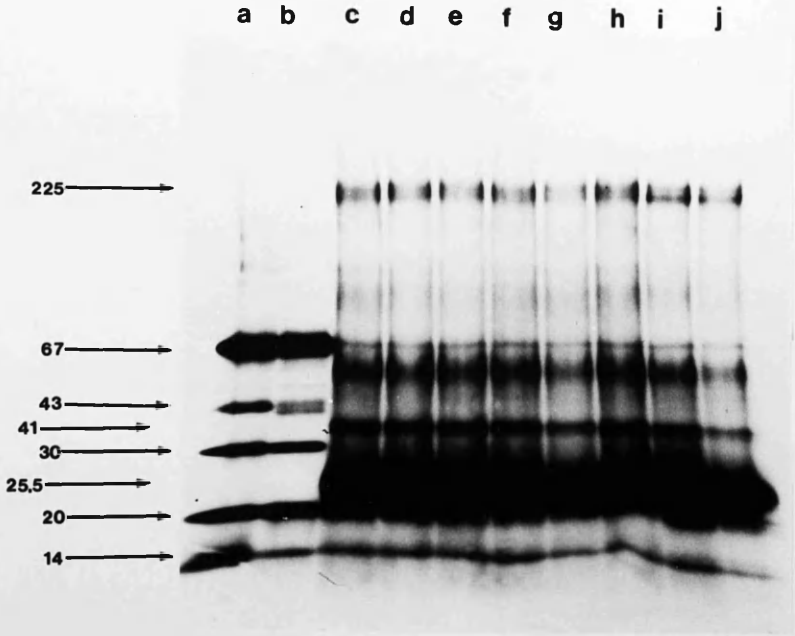


a) Low M. Wt. Markers
b) Low M. Wt. Markers + RMCP II
c) *A. suum* L2 IVRS
d-j) *A. suum* L2 IVRS + increasing amts RMCP II

Fig. 6.6

Limited proteinolysis of A.suum L2 secretory material by RMCP II. A.suum L2 IVRS were radio-iodinated and incubated with the following concentrations of RMCP II: none (c), 0.4 µg/ml (d), 2 µg/ml (e), 4 µg/ml (f), 20 µg/ml (g), 40 µg/ml (h), 200 µg/ml (i) and 1 mg/ml (j). ^{125}I-labelled LMW were also reacted with 1 mg/ml RMCP II (b). Untreated LMW are shown in track (a). The enzyme reactions were performed in PBS-A at 37^{o} for 2 hrs and the products analyzed by SDS-PAGE.

Fig. 6.7 All *A. suum* L3/4 Secretory Components are Resistant to Cleavage by RMCP II

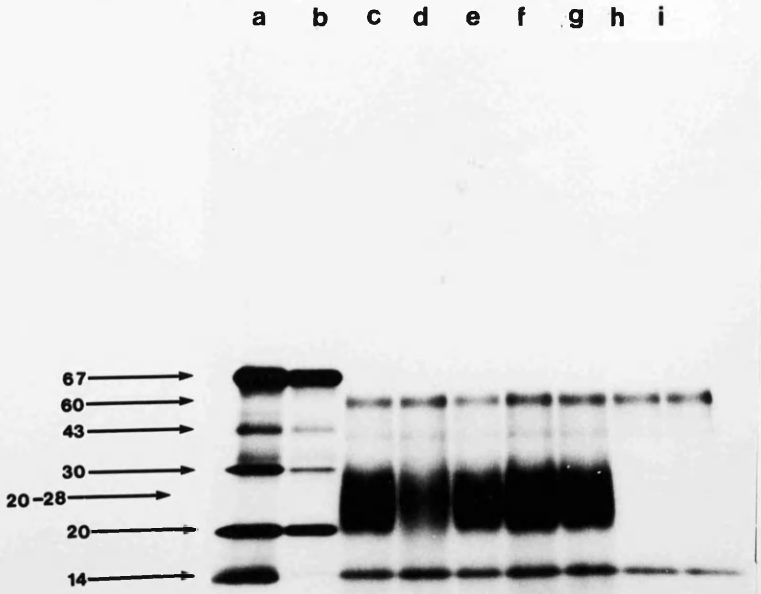


a) Low M. Wt. Markers
b) Low M. Wt. Markers + RMCP.II
c) *A. suum* L3/4 IVRS
d-j) *A. suum* L3/4 IVRS + increasing amts RMCP II

Fig. 6.7

All A.suum L3/4 secretory components are resistant to cleavage by RMCP II. A.suum L3/4 IVRS were radiolabelled with ^{125}I and incubated with the following concentrations of RMCP II: none (c), 0.4 µg/ml (d), 2 µg/ml (e), 4 µg/ml (f), 20 µg/ml (g), 40 µg/ml (h), 200 µg/ml (i) and 1 mg/ml (j). LMW were labelled with ^{125}I and also incubated with 1 mg/ml of RMCP II (b). Untreated ^{125}I-labelled markers are shown in (a). The enzyme reactions were performed at 37°C, in PBS-A, for 2 hrs and the products analyzed by SDS-PAGE.

Fig. 6.8 Limited Proteinolysis of *A. suum* L2 Secretions by RMCP I

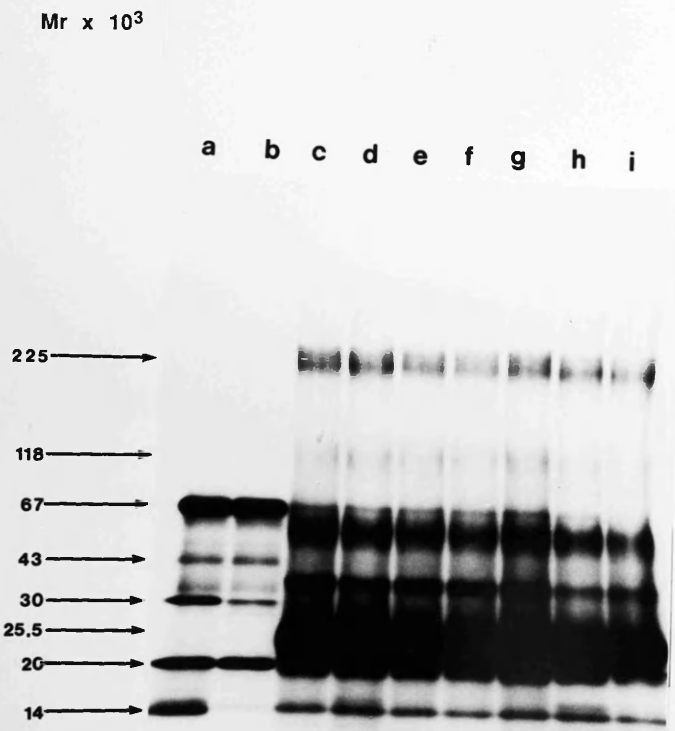


a) Low M. Wt. Markers
b) Low M. Wt. Markers + RMCP I
c) *A. suum* L2 IVRS
d-i) *A. suum* L2 IVRS + increasing amts RMCP I

Fig. 6.8

Limited cleavage of A.suum infective larval secretions by RMCP I. A.suum L2 IVRS were radiolabelled with ^{125}I and incubated with the following concentrations of RMCP I: none (c), 0.4 µg/ml (d), 2.0 µg/ml (e), 4 µg/ml (f), 10 µg/ml (g), 50 µg/ml (h) and 250 µg/ml (i). Radioiodinated markers were also reacted with 50 µg/ml of RMCP I (b). Untreated markers are shown in (a). The enzyme reactions were performed in PBS-A at 37°C for 2hrs and the products examined by SDS-PAGE.

Fig. 6.9 All *A. suum* L3/4 Secretory Components are Resistant to Cleavage by RMCP I



a) Low M. Wt. Markers
b) Low M. Wt. Markers + RMCP.I
c) *A. suum* L3/4 IVRS
d-i) *A. suum* L3/4 IVRS + increasing amts RMCP I

Fig. 6.9

Resistance of A.suum pulmonary larval IVRS to the action of RMCP I. ^{125}I-labelled A.suum L3/4 IVRS were incubated with the following concentrations of RMCP I: none (c), 0.4 µg/ml (d), 2 µg/ml (e), 4 µg/ml (f), 10 µg/ml (g), 50 µg/ml (h) and 250 µg/ml (i). Radio-iodinated LMW were also incubated with 50 µg/ml of RMCP I. The enzyme reactions were performed in PBS-A at 37^{o}C for 2 hrs and the reaction products examined by SDS-PAGE. Untreated ^{125}I-labelled LMW are shown in (a).

Table 6.1 Components of A.suum L2 IVRS have differing susceptibilities to proteinolysis

COMPONENTS OF A.SUUM L2 IVRS

Enzyme	14000	20-28000	40000
Pronase	-	-	-
Trypsin	-	-	-
Cht A	-	+	-
RMCP II	+	-	+
RMCP I	+	-	+

Legend to Table 6.1

Approximately 100-200 x 10^3 cpm aliquots of radio-iodinated A.suum L2 IVRS (representing approx. 2 - 4 ng of material were reacted with different amounts of the above enzymes. Resultant SDS-PAGE analysis and autoradiography indicated which components of IVRS were susceptible to proteinolysis.

- refers to susceptibility to proteinolysis

+ refers to resistance to proteinolysis

Table 6.2 Components of A.suum L3/4 IVRS have differing susceptibilities to proteinolysis

	COMPONENTS OF A.SUUM L3/4 IVRS						
Enzyme	14000	25,500	41000	67000	118000	225000	410000
Pronase	-	-	-	-	-	-	-
Trypsin	+	-	+	-	+/-	+	NV
RMCP II	+	+	+	+	+	+	+
RMCP I	+	+	+	+	+	+	+

Legend to Table 6.2

Approximately 100 - 200 x 10^3 cpm aliquots of radio-iodinated A.suum L3/4 IVRS (representing approx. 28-56 ng of material) were reacted with different amounts of the above enzymes. Resultant SDS-PAGE analysis and autoradiography indicated which components of IVRS were susceptible to proteinolysis.

- refers to susceptibility to proteinolysis

+ refers to resistance to proteinolysis

+/- refers to partial susceptibility to proteinolysis

NV - component not visible in SDS-PAGE autoradiographs

PARTIAL CHARACTERISATION OF THE CARBOHYDRATE GROUPS ON THE GLYCOPROTEINS OF *A. SUUM* IVRS

Partial Characterisation of the Carbohydrate Groups on the Glycoproteins of A.suum IVRS

Summary

The presence of carbohydrate on the IVRS of Ascaris larval stages could be relevant to the structure and function of these products. Additionally, similar sugar groups on nematode parasite secreted antigens might contribute to immunological crossreactions that could reduce the likelihood of specific detection of ascariasis. However, little is known of the biochemical nature or identity of carbohydrate groups among the products of Ascaris larvae. Here the radio-iodinated secretions of L3/4 A.suum larvae were examined for the presence of sugar groups in lectin-mediated precipitations with lectins linked to Sepharose beads, followed by SDS-PAGE analysis of the Sepharose bound lectin material. Seven different lectins were used in the experiments.

The results showed that all of the secreted products of A.suum pulmonary stage larvae are glycoproteins. Moreover, most individual components contained a range of sugar groups. The only exception was a product of Mr 67000 which might not be of parasite origin (Chapter 3). Finally, the Mr 14000 component of L3/4 IVRS was found to be lightly glycosylated in comparison to other secretions, which was also the case for an Mr 14000 product of adult A.suum worms which is an allergen (Ambler et al, 1973a and b; Ambler et al, 1974).

Introduction

In mammalian cells, carobhydrate groups attached to cellular proteins may have a number of important functions. These could include control of the processing and transfer of nascent glycoproteins through the endoplasmic reticulum and Golgi networks to their final destination in the cell (Waechter and Lennartz, 1976; Robbins et al, 1977; Blobell et al, 1979). Those glycoproteins which are positioned in the plasma membrane of the mammalian cell are orientated so that the carbohydrate portions are exposed at the lumenal face of the lipid bilayer, whilst the polypeptide portion lies at the cytoplasmic side (Steck, 1974).

Little is known concerning the identify and orientation of sugar groups attached to the surface and/or IVRS proteins of parasitic nematodes. At present, the results of studies on some nematodes suggest that there may be differences in the way that carbohydrate groups of glycoproteins are orientated in mammalian cells and certain parasites. For instance, studies on the cuticular surface products and secretions of T.canis L2 larvae, maintained in vitro, showed that all the examined parasite products were glycoproteins. However use of monoclonal antibodies (McAb's) directed against T.canis products showed that some McAb's were specific for carbohydrate epitopes which were not available for antibody binding on the surface of living worms, but only on turnover of the worm surface (Kennedy et al, 1987b; Maizels et al, 1987). This suggested that the carbohydrate epitopes were buried in the cuticle of larvae and only exposed following metabolic turnover of the nematode surface. Similar results were achieved with the surface molecules of T.spiralis infective larvae, two of which are adherent to lentil lectin (Parkhouse, Philipp and

Ogilvie, 1981). A McAb made against solubilised surface molecules, which was found to be specific for carbohydrate groups of the glycoproteins failed to bind to the surface of live worms (Ortega-Pierres et al, 1984).

Lectins are bivalent or mutivalent carbohydrate binding proteins that can bind saccharides in a specific manner (Barondes, 1981). Although immunoglobulins have this property, they are not grouped together with lectins, presumably because they fall into a different category. Lectins are often used experimentally as tools for the investigation and characterisation of entities that contain sugar groups. Here, it was confirmed that all the components of A.suum L3/4 IvRS contain carbohydrate groups and are, therefore glycoproteins.

Results

Examination of the carbohydrate groups of A.suum L3/4 IVRS

The radio-iodinated IVRS molecules of pulmonary stage larvae were reacted with Sepharose-bound lectin. Following extensive washing with lectin buffer containing the appropriate eluting sugar and supernatants analysed by SDS-PAGE.

Figure 7.1 illustrates the different specificities of Ascaris secretions displayed by the variety of lectins. It is relevant to note that Helix pomatia agglutinin (HPA) precipitates the largest quantity of IVRS material. This lectin is specific for α/or B-N-Acetyl-Galactosamine which is commonly found in N-linked glycoproteins.

Among the agglutinating proteins used for the IVRS lectin-mediated those lectins that displayed the same affinity for particular carbohydrates gave rise to the same SDS-PAGE profile.

The results of all the lectin specificities for the molecules of IVRS are presented in tabulated form in Table 7.1.

The action of concanavalin A (Con A) and peanut agglutinin (PNA) on A.suum L3/4 IVRS antigens

Precipitations were performed using con A (specific for mannose) or PNA (specific for lactose or galactose) with A.suum L3/4 secretions labelled with ^{125}I. IVRS material eluted from the lectin complex was analysed by SDS-PAGE. Control precipitations without specific sugar were performed and examined on the same gels.

Figure 7.2 illustrates that some secretory material was not eluted from lectin-ES pellets and that there was also some apparent non-specific elution of secretory components from lectin-agarose pellets. Con A does not precipitate a prominent component of IVRS, namely that of Mr 25500 apparent molecular weight, showing that this product does not apparently contain detectable amounts of mannose. PNA lectin apparently recognises lactose and/or galactose residues on most components of IVRS.

Recognition of A.suum pulmonary larval ES material by Griffonia simplicifolia (GS-I) and Ulex europus agglutinin (UEA-I)

A.suum L3/4 secretory molecules were labelled with radio-iodine (^{125}I) and reacted with GS-I or UEA-I bound to Sepharose beads. The resultant material released from lections was analysed by SDS-PAGE.

The results (Figure 7.3), demonstrate that α-L-fucose residues, for which UEA-I is specific, are present on two major fractions of IVRS of Mr 25500 and 11800 respectively. In contrast, GS-I, which has an affinity for α-D-galactose, binds to three major secretions of Mr25500, 67000 and 11800. Both lections precipitate the Mr 14000 component as a minor band.

Distribution of N-Acetyl-Galactosamine among the IVRS molecules of A.suum L3/4 larvae

HPA lectin, linked to Sepharose, was combined with the ^{125}I-labelled molecules of A.suum L3.4 IVRS. The IVRS products eluted by the specific sugar, N-Acetyl Galactosamine, were analysed by SDS-PAGE.

Figure 7.4 shows that all the major iodinated ES products apparently contain the HPA-specific carbohydrate groups including a component of Mr 67000.

Discussion

The IVRS products of A. suum have been characterised earlier (Chapters 3-6) and have also been shown to be proteinaceous (Chapter 6). However, it was not known if IVRS entities were glycoproteins or if sugar groups contained on the glycoproteins were those commonly found on mammalian glycoproteins. This report was intended to clarify the situation. All the radio-iodinated secretions of A. suum L3/4 larval IVRS were found to be glycosylated with various different sugar groups present on each of the secreted components. It was notable that each of the IVRS products of the parasite were precipitated by the whole battery of lectins used, indicating that various different sugar groups were present on each component. These are: mannosyl and galactosyl residues and additionally, L-fucose, N-acetyl glucosamine and N-acetyl galactosamine. Additionally, as judged by the intensity of analytical SDS-PAGE autoradiographs following lectin mediated precipitations, the components of IVRS were glycosylated with different amounts of each type of sugar (Table 7.1).

Previous reports on Ascaris glycoproteins have focused on those components which induced hypersensitive responses in sensitized hosts (Gen. Intro.). An Mr 14000 allergen of A. suum has been biochemically characterised and varying estimates have placed the amount of carbohydrate on this product as between 1 and 8% (Ambler et al, 1973b; Hussain, Bradbury and Strejan, 1973; Dandeu and Lux, 1978). The findings presented here show that the Mr 14000 component of IVRS is present as a faint band in the SDS-PAGE gels of lectin mediated precipitations and as such may contain some carbohydrate but perhaps of low overall percentage of Mr.

Stromberg (1979 a and b) has suggested that the major product of A.suum L3/4 IVRS was of Mr 67000 and had several interesting biological properties and contained 22% carbohydrate (Stromberg, 1979 a and b). The major Mr 67000 entity examined in Chapter 3 was apparently immunologically identical to rabbit serum albumin and contradicted Stromberg's findings (Chapter 3, Discussion). Additionally, the Mr 67000 component of radio-iodinated L3/4 IVRS examined here was found to contain only α-D-mannosyl and D-galactose and was different from other IVRS components in that it did not contain the whole range of sugar groups associated with other IVRS products.

There was some degree of non-specific elution from the Sepharose-lectin beads of lectin-bound material as well as some non-specific binding of radiolabelled IVRS products to the lectin matrix. However the number of washing steps taken to eliminate non-specific interactions and the differences in the SDS-PAGE profile of experimental and control tracks in the gel autoradiographs indicated that the majority of IVRS molecules precipitated in the experiments were specific.

Immunological crossreactions between parasitic nematodes appear to be considerable (Gen. Intro.). Some of these crossreactions might be due to similar carbohydrate groups present among the products of Ascaris and other nematodes. The IVRS products of T.canis L2 larvae have been shown to contain mannose or N-acetyl galactosamine but both types of carbohydrate were not apparently present together on any individual secretion (Meghji and Maizels, 1986). Studies with McAb's

raised against T.canis infection and specific for carbohydrate have shown, in RIP's of radio-iodinated IVRS followed by SDS-PAGE analysis, that some McAb's apparently recognise carbohydrate epitopes common for several components of IVRS (Maizels, et al, 1987). The results of RIP's with some of the above panel of McAb's and radio-iodinated IVRS of T.cati indicated that there was extensive crossreactivity, some of it perhaps due to common carbohydrate epitopes occurring on the products of both species (Kennedy et al, 1987 b).

Three of the lectins used in the lectin-mediated precipitations with A.suum L3/4 IVRS have some reactivity with blood group determinants (Lloyd and Kabat, 1968), namely HPA (blood group A), UEA (blood group H) and GS-1 (blood group B). These lectins are able to precipitate components of A.suum IVRS but do not provide conclusive proof of the presence of blood group-like antigens among the secretions of A.suum larvae, since it is possible that these lectins have precipitated carbohydrate groups that are similar but not identical to those of blood group determinants. These results could be of importance to the accurate serodiagnosis of ascariasis since it is conceivable that false positive results could arise in some Ascaris antigen based diagnostic assays, by virtue of certain anti-blood group antibodies crossreacting with blood group-like sugar groups contained on parasite antigens.

Future study of carbohydrate groups on Ascaris secretions might consider the contribution of sugar groups of crossreactions of nematodes and consider the chemical depletion of sugar groups from IVRS glycoproteins to examine the possibility that this may decrease observed crossreactivity in parasite detection assays. This

possibility is favoured by the result that the addition of carbohydrate to newly-synthesised parasite polypeptides may not be essential to the antigenicity of these products (Carr and Pritchard, 1986).

Fig. 7.1 All *A. suum* L3/4 Secretions are Glycoproteins

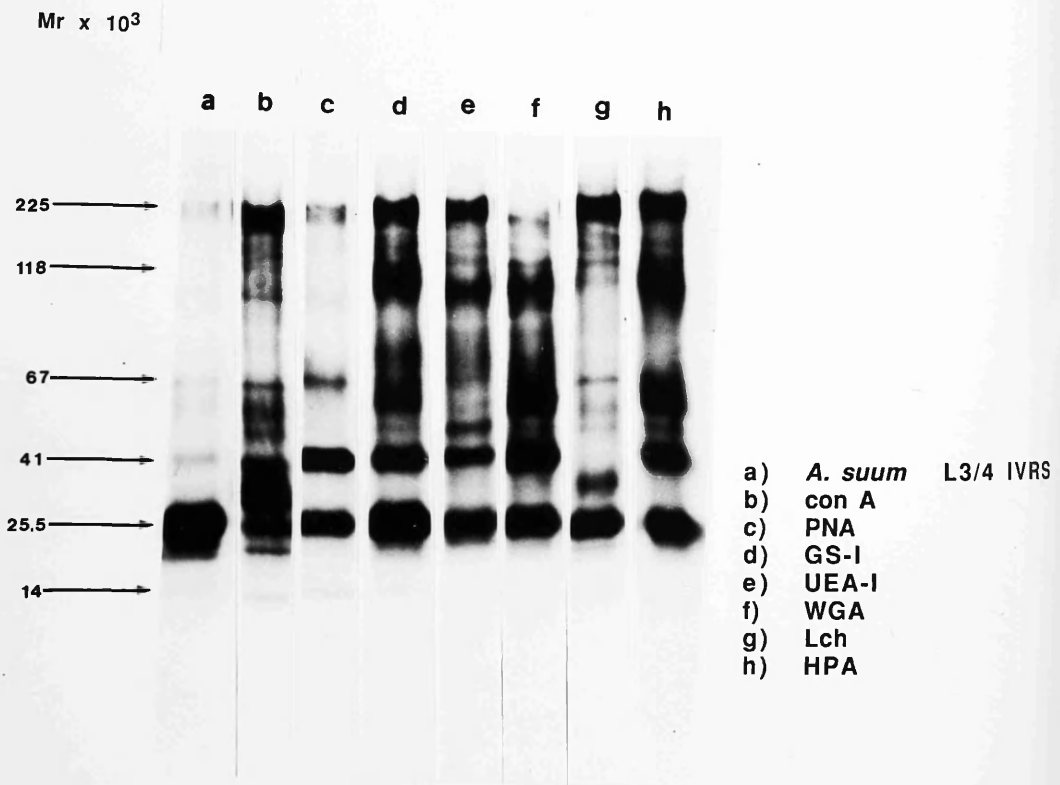
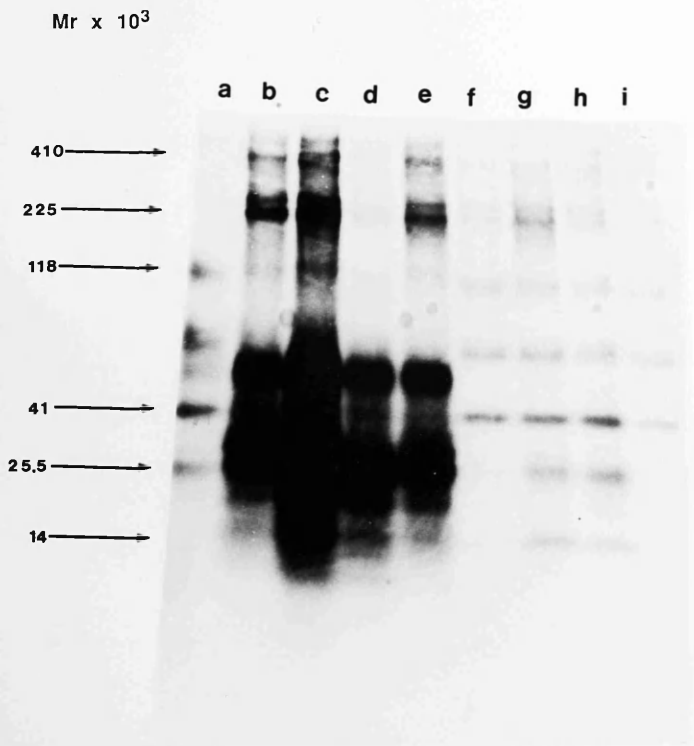


Fig. 7.1

Carbohydrate components of A.suum IVRS. 1×10^5 - 2×10^5 cpm of ^{125}I-labelled A.suum L3/4 IVRS were reacted with each of the following lectins bound to Sepharose: Con A (b), PNA (c), GS-1 (d), UEA-I (e), WGA (f), Lch (g) and HPA (h). Specific sugars were added to the lectin-IVRS pellets and the supernatants analyzed by SDS-PAGE. Radio-iodinated AS L3/4 IVRS are shown in track (a).

Fig. 7.2 Presence of Mannosyl and Galactosyl Residues in *A. suum* Secreted Glycoproteins

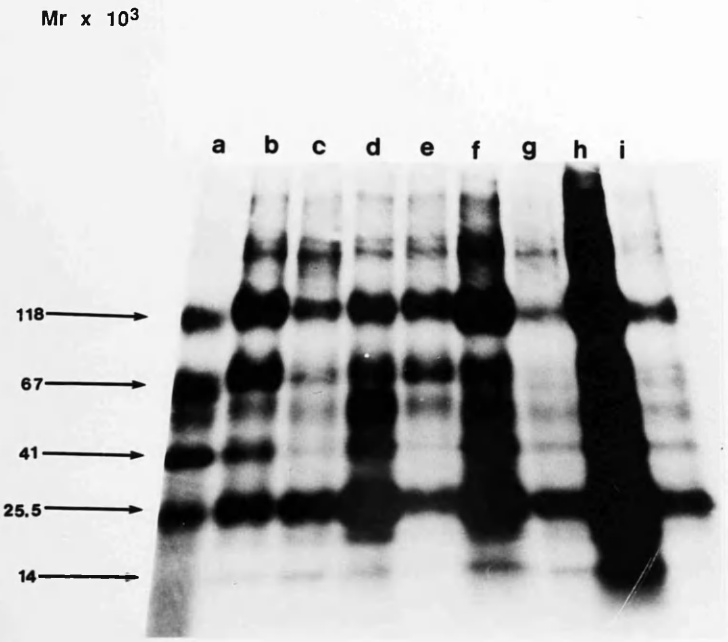


a) *A. suum* L3/4 IVRS
b) con A sugar s/n
c) con A pellet extract
d) con A control s/n
e) con A control pellet extract
f) PNA sugar s/n
g) PNA pellet extract
h) PNA control s/n
i) PNA control pellet extract

Fig. 7.2

Mannose and galactose residues in A.suum IVRS glycoproteins. 1 x 10^5 - 2 x 10^5 pm of radio-iodinated A.suum L3/4 IVRS were reacted with Sepharose-bound lectins, con A (b - e) or PNA (f - i). Specific eluting sugars were added to half of the samples, and eluted material (supernatants) were collected (b) and (f). Residual lectin pellet bound material was also extracted (c) and (g). To the remaining (control) samples, no sugar was added but supernatants were collected from these (d) and (h). Residue lectin-bound material was also extracted from Sepharose-lectin pellets (e) and (i). The IVRS material liberated from lectins was analyzed by SDS-PAGE. ^{125}I-labelled A.suum L3/4 IVRS are shown in (a).

Fig. 7.3 Presence of Fucosyl Residues in *A. suum* Secreted Glycoproteins

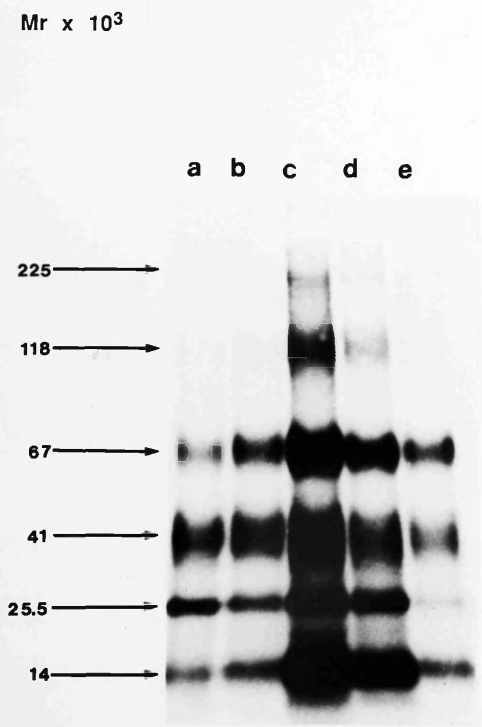


a) *A. suum* L3/4 IVRS
b) GS-I sugar s/n
c) GS-I pellet extract
d) GS-I control s/n
e) GS-I control pellet extract
f) UEA-I sugar s/n
g) UEA-I pellet extract
h) UEA-I control s/n
i) UEA-I control pellet extract

Fig. 7.3

Fucose residues are present in A.suum secreted glycoproteins. 1 x 10^5 - 2 x 10^5 cpm of radio-iodinated A.suum L3/4 IVRS were reacted with Sepharose-linked lectins GS-I (b - e) and UEA-I (f - i). Specific eluting sugars were added to half the samples and eluted material (pellet supernatants) were analyzed (b) and (f). Residual lectin pellet-bound macromolecules were also analyzed (c) and (g). No sugar was added as eluting agent to the remaining sample. Supernatants from these samples (d) and (h) and lectin pellet extracts (e) and (i) were also examined, as for all other samples by SDS-PAGE. Radio-iodinated A.suum L3/4 IVRS are shown in (a).

Fig. 7.4 Presence of N-acetyl Galactosamine in *A. suum* IVRS



a) *A. suum* L3/4 IVRS
b) HPA sugar s/n
c) HPA pellet extract
d) HPA control s/n
e) HPA control pellet extract

Fig. 7.4

Presence of N-acetyl galactosamine in A.suum IVRS. A.suum L3/4 IVRS were radiolabelled with ^{125}I and reacted with Sepharose-bound HPA. Specific sugars was added to some of the samples and supernatant (b) and lectin-pellet extracts (c) taken from these samples were analyzed by SDS-PAGE. Supernatant (d) and pellet extracts (e) from control samples to which sugar was not added, were also analysed by SDS-PAGE. Radio-iodinated A.suum L3/4 IVRS are shown in track (a).

TABLE 7.1

LECTIN REACTIVITY WITH ASCARIS SUUM L3/4 IVRS

	A. SUUM L3/4 COMPONENTS (Mr x 10^3)							
LECTIN	14	25.5	41	67	118	225	410	LECTIN SPECIFICITIES
CON A	+	+++	+	+	+	++	NV	MANNOSE
LCH	+	++	+	+	+	++	NV	MANNOSE
PNA	+	–	+	+	+	++	NV	LACTOSE AND/OR GALACTOSE
GS-I	+	++	++	+	+	+	NV	LACTOSE AND/OR GALACTOSE
UEA-I	+	++	++	–	++	++	NV	FUCOSE
WGA	+	++	++	–	++	–	NV	N-ACETYL GLUCOSAMINE
HPA	+	++	++	–	++	++	NV	N-ACETYL GALACTOSAMINE

Legend to Table 7.1

100-200x10^3 cpm of *A. suum* L3/4 IVRS were precipitated with Sepharose linked lectins as described in General Materials and Methods (Ch. 2). The resulting reactivity of lectins with individual components of IVRS was judged by band intensities in SDS-PAGE autoradiographs and scored as above. NV indicates that the band was not visible on autoradiographs.

GENERAL DISCUSSION

General Discussion

At present, both specific diagnosis of Ascaris infection or the protection of individuals against the disease have not been given special importance and inadequate procedures have been adopted (Gen. Intro). The IVRS of Ascaris nematodes could become the best source of antigens to fulfil the criteria for specific diagnosis and/or vaccination purposes in ascariasis (Gen. Intro.). However prior to proceeding to the uses of Ascaris IVRS, it was necessary to investigate some biological properties of these secretions including evaluation of the ability of IVRS products to elicit an antibody response in the infected host animal. The antigens which are also good immunogens would be useful for diagnostic assays, particularly if these antigens were species specific and did not crossreact with the antigens of other nematodes in various immunological assays.

From the viewpoint of finding an IVRS product that could protect animals against further infection with Ascaris, it would be interesting to find a product of Ascaris larvae that would be vital or important to the survival of the parasites (Gen. Intro.). Such products, if found, could also be relevant to the immunopathological consequences of ascariasis, which can be distressing to the infected individual at both early and later stages of Ascaris worm development.

Allergens produced by Ascaris worms may be important in the immunobiology of ascariasis but little is known of the biochemical or immunochemical nature of these products and only the allergens of

Ascaris adult worms have been examined in any detail. Nevertheless, allergenic molecules produced by Ascaris larval stages may be important in the immunopathological symptoms in individuals infected with Ascaris, particularly in regard to liver and lung disease in these people occurring as a direct result of parasite migration and possible release into the above organs of parasite products. It is hoped that the results of the experiments reported in this thesis will provide the basis for further study on the functions and possible uses of Ascaris IVRS.

Stimulation of an IgE response in the host by parasite derived allergens could be an important step in inducing the host immune response, especially the inflammatory response, which is significant in the immunopathology of Ascaris infection (Gen. Intro.). Suggestions have been made that Asc 1, an allergen produced by adult Ascaris worms, was also produced by larval stages of Ascaris in guinea pigs infected with the parasite (Hussain, Strejan and Campbell, 1972; Hussain, Bradbury and Strejan, 1973) and stimulated the reaginic response in these animals. A search for Ascaris larval IVRS components secrete by infective and lung stage worms which shared Mr with known Ascaris allergens led to the preliminary study of radio-iodinated L2 and L3/4 Larval IVRS (Ch. 3). On the basis of electrophoretic mobility in SDS-PAGE, there were two components of Mr 14000 and 225000, which were apparently present in the IVRS of both L2 and L3/4 worms. The use of an antiserum raised against the Mr 14000 component of ABF in radio-immunoprecipitation assays followed by SDS-PAGE indicated that the Mr 14000 components of L2 and L3/4 IVRS were immunologically related and probably homologous. This particular

secretion may be analogous to the allergen present in the body fluid and culture supernatants of adult A.suum worms first characterised by Ambler's group and referred to as Allergen A (Ambler et al, 1973a and b; Ambler, Miller and Orr, 1974). This is further supported by reports on the molar ratio of the amino acids of both Allergen A and the Mr 14000 product of Ascaris larvae, which have been found to be very similar (M.W.Kennedy, pers. comm.).

Further possible indication of the similarity of the Mr 14000 product with Allergen A was provided by the examination of L3/4 IVRS, using lectins, to identify the relevant carbohydrate group contained on the secreted components. It was shown that N-Acetyl glucosamine and N-Acetyl galactosamine were present on the Mr 14000 secretion (Ch. 7) but apparently in relatively small quantity, judging by the relative intensity of the gel autoradiographic band representing this antigen, following lectin precipitations and SDS-PAGE analysis of radiolabelled IVRS. Ambler et al (1974) showed that Allergen A was also not highly glycosylated. Moreover, as for Allergen A, the larval Mr 14000 is remarkably resistant to the action of various proteinases, (Ch. 6) in view of the relatively large concentrations of enzyme used against radiolabelled IVRS from liver and lung-stage worms. The resistance of Allergen A to chymotrypsin, trypsin and elastase was suggested to arise from the conformation of this molecule which could have restricted the access of proteinases to their recognition sites (Ambler, Miller and Orr, 1974). Perhaps the remaining molecules of Ascaris IVRS, which also display resistance to proteinase action, may also share some particular feature of conformation with the Mr 14000 component which may be relevant to the preservation of IVRS function (Ch. 4).

Little is known of the Mr 225000 product which appears in the SDS-PAGE profiles of both L2 and L3/4 worms (but may not necessarily be identical in both developmental stages). However, the use of anti-Mr 14000 antiserum in RIP's followed by SDS-PAGE suggested that this secretion was not immunologically related to the Mr 14000 product. Additionally, there are no reports of an allergen of Mr 225000 either produced or secreted by Ascaris worms.

Although elevated levels of IgE might be important to the allergic manifestations of ascariasis, in humans who may be frequently re-infected with Ascaris, the IgG response could be a major factor in reducing pathological symptoms of the disease (Gen. Intro.). Such IgG-mediated amelioration of disease symptoms might occur for individuals suffering from filariasis, by virtue of IgG with a specificity for certain antigens blocking the progression of these molecules to primed mast cells (Otteson et al, 1981; Ogilvie and De Savigny, 1982; Hussain and Otteson, 1985). The results of SRIP's (using antibodies from animals experimentally infected with Ascaris) followed by SDS-PAGE analysis apparently indicated a potent IgG response to the molecules of IVRS produced by Ascaris L2 and L3/4 parasites from rabbits and rats infected with A.suum.

In humans, it has been suggested that the IgG class of antibodies are only found in detectable amounts in those individuals carrying migratory stages of the A.lumbricoides, (O'Donnell and Mitchell, 1980) therefore their effect may only be on migrating larvae and production of these antibodies by the infected host might be decreased as some worms go on to further developmental phases. These observations

suggest the possibility that this antibody isotype might be important to those lung stages of ascariasis. It is of interest that people continuously infected with Ascaris throughout the year show fewer signs of pulmonary distress in general than those intermittently infected with the parasite (Gen. Intro).

Serum IgG from rats and rabbits infected with A.suum was found to react, in SRIP's, with the Mr 14000 element of IVRS from lung and second stage worms (Ch. 3) which might be an allergen. It would be interesting if humans infected with Ascaris and who raise an IgG response to the Mr 14000 component could be correlated with have less severe symptoms of larval ascariasis. The importance of the IgG response to ascariasis in humans still needs to be fully investigated and warrants a detailed knowledge of past history of infection of particular individuals, including contact with parasites that could secrete antigens or allergens that are similar to those of Ascaris larvae (Turner, Fisher and McWilliams, 1980).

It has been shown, albeit with a small number of individuals, that host antibody responses and pathological symptoms of ascariasis are heterogeneous in different humans (Gen. Intro). Different genetic species of animals (and strains of mice and rats) infected with Ascaris also precipitate different sets of Ascaris IVRS antigens as judged by RIP's followed by SDS-PAGE (Ch. 3; Kennedy et al, 1987a). Some animals do not produce detectable antibody against some components of Ascaris IVRS, including the Mr 14000 component (Ch.3, Kennedy et al, 1987a) so it would not be worthwhile using these animals to measure an immunological effect that requires detectable amounts of antibody to be raised against these worm products.

The importance of genetic factor in disease has recently come to prominence in regard to Ascaris. Differences in immune response to disease might be due to the particular genetic composition of these individual animal species. It is likely that the transplantation antigens could be important in the heterogeneity of antibody response against parasite antigens (Gen. Intro.).

It can be argued that studies of experimentally infected animals could be useful to provide an experimental model for the situation where humans are infected with Ascaris and examination of different parameters of infection could be undertaken using animals, for instance, effect on antibody response to Ascaris of frequency of infection, or number of eggs ingested. Additionally it could be possible to examine the importance of genotype of the animals on the type of antibody responses induced against Ascaris infection. In particular, there is a range of mouse strains commercially available that contained differences at the histocompatability (H-2) locus, providing a useful opportunity to examine the effect of different H-2 type on the response to infection with Ascaris. Further, some strains of mouse may be naturally less resistant to Ascaris than others and study of the immunological responses of these animals to Ascaris could provide some information on the basis for resistance to infection. The criterion for resistance could be related to numbers of worms migrating through the liver or lungs of infected animals (Gen. Intro.). Studies of infected animals could be eventually important to discover if genetic factors (perhaps transplantation antigens) are important to Ascaris resistance so that a basis could be provided for study of humans naturally infected with Ascaris.

Certain people of a given population, endemic for ascariasis, apparently carry a large worm burden whilst most individuals carry a low number of adult worms (Gen. Intro.). It would be interesting from several viewpoints if genetic factors were important to the large worm burdens carried by some humans (Gen. Intro.) but mainly for the hope that strategies could be devised to trace "wormy" humans and regularly deworm them (Gen. Intro.) thus reducing, in general, the overall chance of infection in the communities in which wormy individuals reside.

The extent to which infection of humans with A.suum could affect the immunopathology or immunobiology, in general, of Ascaris infestation of humans is unknown. There are reports that A.suum can infect humans and develop to the adult forms in the intestines of infected people (Ch. 4). This point is notable because it would indicate that there could be some biological similarity between this nematode and A.lumbricoides. Three sets of experiments in this thesis indicated that there was similarity of secreted antigens between A.suum and A.lumbricoides. The first demonstrated the wide-ranging crossreactivity between the larval antigens of both parasites after the gel analysis of SRIP's using anti-Ascaris antisera (Ch.4) and this was later confirmed for, at least, one of the components of IVRS, namely the Mr 14000 molecule which was found to be immunologically very similar between the two types of parasite. This would also suggest that this product of A.suum worms is identical to that of human roundworms. The SDS-PAGE profiles of radio-iodinated secretions from both types of parasite indicate that a number of infective stage larval antigens and the majority of L3/4 stage antigens are of similar Mr. Moreover, there was wide-ranging crossreactivity between the

antigens of A.suum and antisera raised in animals against A.lumbricoides infection (and vice versa; Ch. 4). Together, these findings can indicate that both sets of worms may be secreting the same types of antigens and therefore would indicate the usefulness of A.suum as a readily available organism for the examination of the biology of the human roundworm. However there is no certainty that molecules of the same Mr are indeed identical and homology of A.suum and A.lumbriocoides antigens will have to be examined by other means, for instance amino acid sequencing.

Biosynthetic labelling, with radioactive ^{35}[S]-methionine, of the products of human and pig roundworm larvae has also indicated the large degree of homology between the secretions of pig and human roundworm larvae, (Ch. 5) as judged by this particular method of radiolabelling. However, in addition, this method revealed some differences between A.suum and A.lumbriocoides IVRS, including an Mr 17000 molecule that was synthesised apparently only by L3/4 larvae of A.lumbriocoides. This would provide support for the results of radio-iodination studies (Ch.4) and also indicate that the differing molecules between the two types of larvae are synthesised continuously and not derived from higher Mr precursor molecules. Although the Mr 17000 component is similar in molecular weight to Asc-1, (which was an allergen of Mr 18000) it is not yet possible to speculate to the biochemical properties of this secretion, however,

from the results of metabolic labelling, the Mr 17000 component appears to contain methionine (Ch. 5) whereas Asc 1, from evaluation of molar ratios of amino acids in this glycoprotein appears to contain few methionine residues (M. W. Kennedy, pers. comm.). Also, the Mr 17000 product is not apparently related to the Mr 14000 component of both A.suum and A.lumbricoides, so it is not a modified form of the molecule of lower Mr (Ch. 4 and 5). However, as yet, there is no reason to exclude this molecule from a role in the pathological symptoms of human ascariasis, or from denying its ability to stimulate production of serum IgE in the infected host.

The Mr 17000 component of human roundworm IVRS may eventually become of interest in the specific immunodiagnosis of A.lumbricoides infestation, providing that a test can be devised that will allow for the specific detection of such molecules. In the case of two species of Toxocara, T.canis and T.cati, the production of monoclonal antibodies (McAb) against the surface products of T. canis allows differentiation between the two species (Kennedy et al, 1987 b). This technology could also be applied to attempts to differentiate between the two species of Ascaris.

A useful technique for examining homologies between secretions and possible processing events leading to the production of IVRS involves enzymatic treatment of radio-iodinted secretions, followed by SDS-PAGE analysis of the products of the enzyme reactions (Meghji and Maizels, 1986). This technique would indicate if a given component of lower Mr was derived from one of higher Mr because it would become

clear on SDS-PAGE when the radiolabelled products could be examined from reactions with increasing amounts of enzyme (Fig. 8.1). The resulting autoradiograph would show increasing amounts of the lower Mr product and corresponding decreases in the amount of higher Mr product(s). Examination of the secretions of A.suum demonstrated that none of the products of either L2 and L3 worms was derived in this manner, using a variety of proteinases (Ch. 6). Moreover, it was shown that the products of A.suum larval stages were relatively resistant to the effects of these proteinases. This observation could relate to the functions of IVRS but no further guesses can be made since biological identity has so far been ascribed to only one components of Ascaris larval IVRS, namely the host serum albumin molecule in L3/4 secretions. (Ch. 3).

The resistance of Ascaris larval secretions to mast cell proteinases suggested that the direct action of mast cell proteinases against Ascaris IVRS in vivo was unlikely, as the majority of A.suum IVRS components do not appear to be cleaved by the two proteinases from rat CTMC and IMMC respectively (Ch. 6). Despite observations that mast cells isolated from the lungs of Macaca monkeys are capable of degranulating, in vitro, after contact with Ascaris antigens, (Pritchard et al, 1983 b) it is still difficult to establish the relevance of mast cells to the viability of Ascaris larvae or adult worms in a given host animal.

The radio-iodinated products of Ascaris larvae incubated with various enzymes are proteinaceous (Ch. 6). The use of lectins also confirmed that these were glycoproteins (Ch. 7). However, the significance of glycosylation is not clear. There are suggestions, as

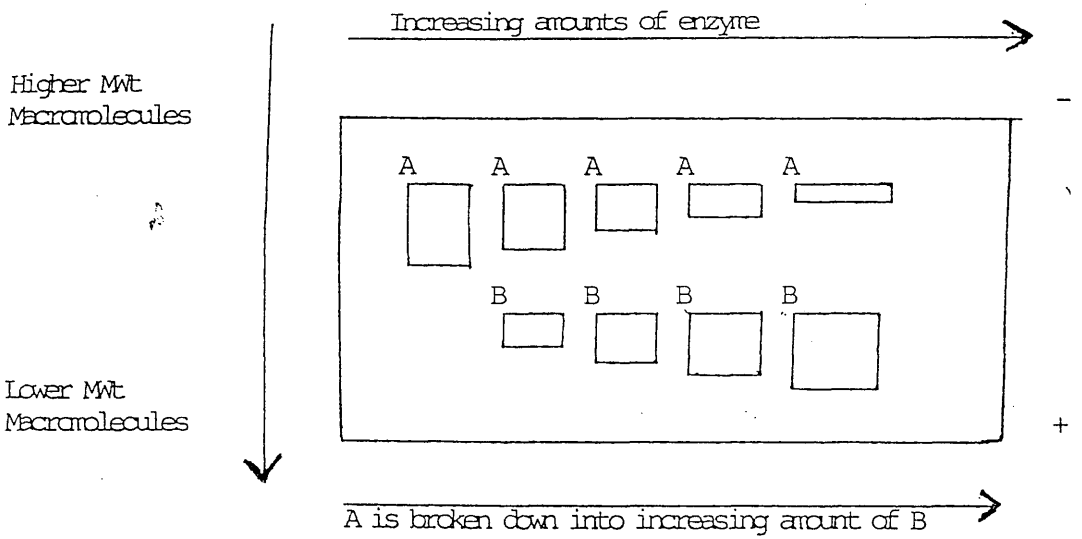
yet unconfirmed that the post translation addition of carbohydrate to proteins might be important to their allergenicity (Berrens and Bleumik, 1965; Berrens, 1970). Additionally there is the possibility that these carbohydrate components function in order to protect the IVRS components from proteolysis of the host inflammatory response *in vivo* (Discussion, Ch. 6). This is difficult to prove, however, since some components susceptible to proteolysis are also glycosylated (Ch. 6 and 7). At present, it is still not possible to evaluate the importance of glycosylation to IVRS. However, the polypeptide portions of IVRS molecules may be more important for the diagnosis of ascariasis and the vaccination of animals against Ascaris infection (Gen. Intro; Ch. 7).

One of the most important future studies that should be performed is the amino acid sequencing of the larval IVRS, for a number of reasons. At the basic level, the sequence of certain IVRS components could be compared to that of proteins of known function, such as enzymes. If significant homology is found, possible function could then be ascribed to individual secretions. Additionally, the amino acid sequence of Ascaris IVRS molecules could be compared to the proteinaceous molecules from the secretions of other nematodes, in particular those of *T.canis* and *T.cati* which are members of the same class of parasite as *A.suum* and *A.lumbricoides*. This comparison can help in deciding if apparent crossreactions between nematode products are due to actual homology of amino acid sequence similarity. The use of a chemical agent such as periodate to strip off carbohydrate groups from radio-iodinated IVRS, and the subsequent reaction of these radiolabelled molecules in SRIP's, can confirm if

these sugar groups contribute to the antigenicity of IVRS and to the crossreactions. These investigations could also lead to the discovery of at least one element of IVRS, whether a major or minor component that will be eventually revealed as truly species specific. Also, elucidation of the complete amino acid sequences of allergens of A.suum juvenile worms, and then computer aided projection of structural conformation might give an insight into the biochemistry of allergens in general and also, possibly, reveal features that might be common to other nematode allergens. This research could then help in clarifying the nature of those molecules which could be responsible for many of the immunopathological features of Ascaris infection.

Although various features of A.suum and A.lumbricoides have been revealed in this thesis, perhaps one of the most exciting developments was the finding that the immunogenetic composition of the host's genome could affect the recognition of the components of IVRS from these parasites. Recent advances have been made in this field for Ascaris infection by Kennedy's group (Kennedy et al, 1987 a) and it is hoped that the study of antibody responses to IVRS from humans in areas endemic for ascariasis, and on whom tissue-typing has been performed will give further insights into the biology of this widespread and important disease.

Fig. 8.1 Proteinolytic Derivative of a Protein from one of a Higher Molecular Weight



Theoretical diagrammatical representation of an autoradiograph of an SDS-PAGE gel containing the reaction products of a protein 'A' and increasing amount of an imaginary limited site - specific proteinase, yielding increasing amounts of product 'B'.

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