Genetic Control of the Antibody Response in Experimental and Human Ascariasis

by

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to mum and dad

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ABBREVIATIONS

ABF, *Ascaris* Adult Body Fluid

°C, degrees centigrade

CFA, Complete Freunds Adjuvant

cpm, counts per minute

ELISA, Enzyme Linked Immunosorbent Assay

epg, eggs per gram

ES, Excretory/Secretory

hr, hour(s)

HLA, human histocompatability leucocyte antigen

H-2, mouse MHC

IFA, Incomplete Freunds Adjuvant

Ig, immunoglobulin

i.p., intraperitoneally

kDa, kilo Dalton(s)

L2, second larval stage

L3/4, third/fourth larval stage

M, molar

mA, milliamp(s)

MHC, major histocompatibilty complex

min, minute(s)

mm, millimetre(s)

μm, micrometre(s)/micron(s)

Mr, relative molecular mass

nm, nanometre(s)

OD, optical density

PAGE, poly acrylamide gel electrophoresis

PCA, passive cutaneous anaphylaxis

ABBREVIATIONS (continued)

s, second(s)

SAPU, Scottish Antibody Production Unit

SDS, sodium dodecyl sulphate

TRIS, tris(hydroxymethyl) aminomethane

v/v, volume per volume

w/v, weight per volume

WLEP, Wellcome Laboratories for Experimental Parasitology

uv, ultra violet

SUMMARY

The immune response of both infected laboratory animals and humans from an endemic area to the parasitic nematode *Ascaris lumbricoides* was investigated. Initially, some biochemical characteristics of various components of the parasite were examined. The four parasite preparations were the adult body fluid (ABF), *Ascaris* body fluid alleregn-1 (ABA-1), excretory/secretory products from infective larvae kept in culture (L2 ES) and excretory/secretory products from lung stage larvae kept in culture (L3/4 ES). Biochemical studies showed that the preparations (except the ABA-1) were complex mixtures of glycoproteins. The ABA-1 is a 14kDa molecule with very little carbohydrate content.

The IgG antibody response to these preparations of different strains of mice and rats infected both with infective *Ascaris* eggs and parasite preparations in adjuvant were analysed by immunoprecipitation of radiolabelled antigen followed by SDS-PAGE analysis. Results showed that the IgG antibody repertoire was affected both by the MHC type of the animal and the mode of presentation of antigen. Similarly, the IgE response to ABA-1 was investigated in PCAs. Positive IgE responses to this molecule corresponded with positive IgG responses to it in strains of mice which possess the s allele at the I-A region of their H-2 and were infected with infective eggs. However, administration of ABA-1 in adjuvant altered the recognition patterns of the ABA-1 molecule by both IgG and IgE in mice and rats to that seen when generated in the context of infection.

The IgG antibody responses of humans from an area endemic for *Ascaris* in Nigeria were examined by ELISAs against all four parasite preparations. Considerable heterogeneity in the levels of response was displayed but no

correlations between these levels and any of the epidemiological data were discovered. Analysis of the antibody response in immunoprecipitations followed by SDS-PAGE analysis also revealed heterogeneity in the antigens recognised by individuals. Of particular interest was the restricted recognition of the ABA-1 which was also seen in humans from enedemic *Ascaris* areas in the Gambia and Karachi. IgG responses were also analysed by a Western blotting method. Human IgE responses were investigated in a blotting method and revealed that a wide range of parasite components are the target of an IgE response.

The antibody response to the surface of the two larval stages was examined by means of a quantitative fluorescent antibody labelling technique. These studies revealed stage specificity of antigen expression and were suggestive of surface shedding contributing to ES products. Investigations with human sera revealed considerable heterogeneity not only in antibody responses but within the parasite population itself.

CHAPTER 1

INTRODUCTION

INTRODUCTION

Ascaris lumbricoides is one of the best known parasites of man and has long been recognised as an infective agent. In 1683 when the organism was called *Lumbricus teres* it was described as "that common roundworm which children usually are troubled with" (Tyson, 1683). It remains a "usual" infection with an estimated total of 1008 million people being infected (WHO, 1981: Crompton, 1988). It is a world-wide problem too - in the last 15 years 153 out of the 218 recognised states or countries have reported ascariasis, the disease caused by infection with the parasite.

It is a remarkable infection, not only because of its cosmopolitan nature and the vast numbers of people it infects but also because of the sheer bulk of foreign material it presents to its host, the chronicity of infection and its ability to persist even in the face of chemotherapeutic control measures. This introduction will review the following aspects of nematode immunology with special reference to *Ascaris*;

a) the life history, epidemiology and clinical impact of infection in the field

b) the work carried out in pigs infected with *Ascaris suum*,

c) laboratory work relating to the immune response to parasite antigens and allergens in general, and

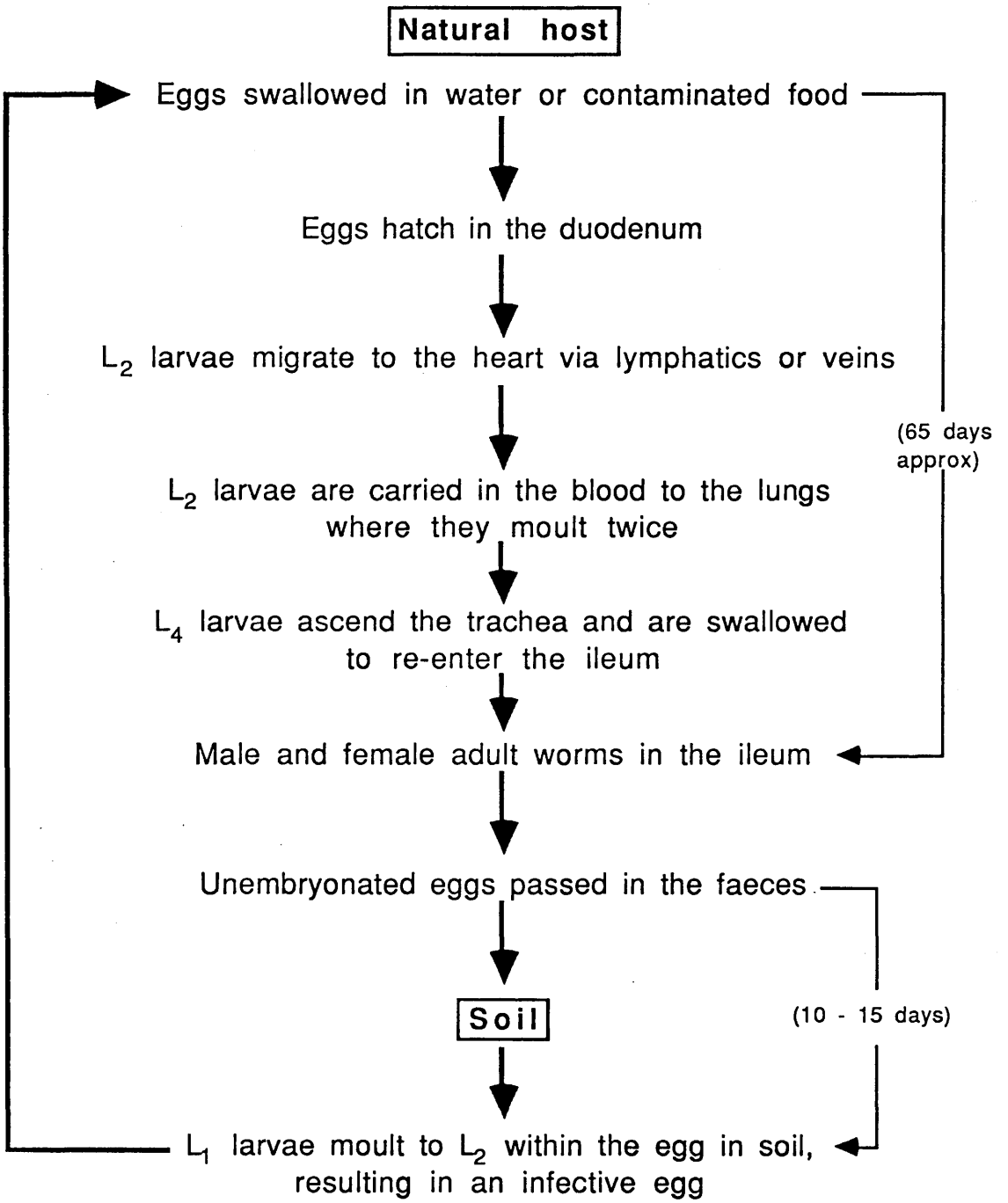
d) evidence for Major Histocompatibility Complex (MHC) control of such responses.

1.1 BIOLOGY

1.1.1 LIFE HISTORY

There are two conceptually separate populations and reservoirs of *A.lumbricoides*: the adult worms which parasitise humans and the eggs that contaminate the environment. A diagrammatic representation of the life cycle is shown in Figure 1.1. The eggs are voided in the faeces of people who harbour adult females, are elliptical in shape and measure 50-70 by 40-50μm. The requirements of embryonation are moisture, oxygen, shade, time and a favourable temperature, e.g., ten to fourteen days at 30-32°C or 45-55 days at 17°C . The zygote is enclosed in a complex eggshell made up of four layers (Wharton, 1980). The outer layer is a deposit of sticky mucopolysaccharide and is no doubt responsible for the well known adhesiveness of *A.lumbricoides* eggs to so many surfaces in the human environment (Kagei, 1983). The remaining three layers are secreted by the embryo from its own reserves (Wharton, 1980). Next to the mucopolysaccharide outer layer is a thin, proteinaceous membrane and the third inner layer which is the thickest, consists of protein and chitin and confers structural strength to the egg. The innermost layer, known as the acaroside layer, is selectively permeable and is largely responsible for the survival of the parasite under a wide range of conditions (Perry and Clark, 1982). This selective permeability increases in response to a rise in ambient temperature and is almost certainly why desiccation is a major hazard for the eggs. Under optimum

Figure 1.1:
The life cycle of *Ascaris*.



embryonation conditions (shaded soil, 28-32°C, humidity greater than 80% and available oxygen) the first larval stage, L1, is formed within about 10 days. After several days the first larval moult from L1 to L2 occurs and larvae can be seen coiled within the egg shell when viewed microscopically. Fully developed larva within the egg shell possess great powers of survival and are therefore able to withstand many chemical treatments and adverse climactic conditions. The longest recorded survival of embryonated eggs is 14 years (Krasnonos, 1978).

The establishment of infection in man depends upon the ability of viable invasive larvae to detect and respond to physiological stimuli in the host. Several factors including elevation of temperature to 37°C, an acidic pH range, the correct combination of carbonate and bicarbonate concentrations and reducing conditions stimulate the larvae to release a hatching fluid (Rogers, 1960). This contains various proteinases and glycosidases which find their substrates in the egg shell. Within a few hours of the eggs being swallowed, they hatch and release L2 larvae into the jejunum.

Newly hatched larvae must quickly penetrate the mucosa of the small intestine to enable survival and further development. They migrate through the mucosal tissues to the liver where a delayed second moult has been observed (Maung, 1978), illustrating that the developmental processes are not rigid events. The larvae reach the liver via the portal vessels 4 days post infection (p.i.), and continue on their migration to the lungs, which are reached 7 days p.i.. The moult from L2 to L3 occurs during this migratory stage. The larvae then leave the lungs and return to the small intestine via the bronchi, trachea and

oesophagus. The final moult occurs in the small intestine about 17 days p.i. at which time the sex of the parasite can be identified (Lee, 1972). Growth from the L4 to juvenile and finally to adult is very rapid and by 2 months p.i. males and females can attain lengths of up to 125mm and 142mm respectively (Seo and Chai, 1980).

Sexually mature male and female *A. lumbricoides* are large worms and are typically 200mm and 300mm respectively. Males are smaller and thinner and can also be recognised by the prominent curvature of the posterior end which houses the copulatory spicules (Crompton and Pawlowski, 1985). The worms move easily in the jejunum and their locomotion is effected by contraction of longitudinal muscles antagonised by a hydrostatic skeleton (Harris and Crofton, 1957). The cuticle of the adult is not an inert layer but is involved in the function of the hydrostatic skeleton and is a likely source of antigens as it forms a component of the host-parasite interface (Ogilvie and de Savigny, 1982). At least eight layers have been characterised and the general structure and functional morphology has been reviewed (Bird, 1971). The worms feed by pumping their food, usually thought to be chyme and intestinal debris. Growth, fecundity and longevity of *Ascaris* are probably heavily influenced by the nutritional quality of the host's dietary intake. This perspective, however, has not been adequately investigated (Bundy and Golden, 1987). Adult worms have an average life span of one year and females can produce 200,000 eggs daily (Elkins, Haswell-Elkins and Anderson, 1986). One advantage to the parasite of aggregation of the parasite population, (which will be discussed more fully later) is the increased probability of the eggs being fertilised. Where worm numbers are low or only one

male is present then unfertilised eggs are produced (Seo, Cho & Chai, 1979).

1.1.2 TRANSMISSION

A. lumbricoides is commonly described as a soil transmitted helminth because its eggs reach the soil in human faeces and subsequently contaminate the environment. Safe systems of sanitation and disposal would ultimately protect people from infection, but endemicity of the parasite is associated with entrenched poverty and the absence of basic sanitation. Add to this partnership the warm and wet climates which favour embryonation and which are the norm in many developing countries, and you have a recipe for high rates of contamination with infective eggs. In addition to soil and sewage as reservoirs of contamination, numerous household sites, items of food and everyday objects and surfaces have been shown to harbour infective eggs (Kagei, 1983). Some evidence suggests that eggs may be inhaled and thus swallowed as part of wind-borne dust particles (WHO, 1967; Kagei, 1983). The wide range of weight of worms, recovered as a result of treatment with appropriate drugs, indicates that infection occurs on a continuous basis (Martin, Keymer, Isherwood *et al*, 1983). This evidence contradicts earlier theories which suggested that an initial acquisition of a worm load in some way protected against subsequent infection (Jung, 1954).

Young children are especially at risk through playing on the ground in defecation areas and through the habit of pica which is common in young children (Wong, Bundy and Golden, 1988). All ages are at risk from ascariasis in regions where human excrement (night soil) is used as agricultural fertiliser. The experience in Israel whereby the banning of

waste water in irrigation systems led to a dramatic reduction in infection rate shows that a simple measure can have a drastic effect on the whole community (Shuval, Yekutiel and Fattal, 1985).

With its well documented ability to migrate through tissue there is a possibility that some *Ascaris* larvae may cross the placenta to become established in the human foetus. There have been several reported cases of this mode of transmission and while other explanations may be offered it remains a route to be given consideration (reviewed by Crompton, 1989). Other unusual (and ethically questionable) modes of transmission have been demonstrated experimentally. These include swallowing larvae recovered from guinea pig lungs (Yoshida, 1919) and penetration of slightly scratched skin by larvae which results in a mature infection with eggs subsequently being passed in the faeces (Urabe, 1957).

Normal transmission is highly efficient, as the rapid re-infection of individuals treated chemotherapeutically demonstrates (Croll, Anderson, Gyorkos *et al.*, 1982; Thein Hlaing, Saw and Lwin, 1987; Elkins, Haswell-Elkins and Anderson, 1988). Poor hygiene results in children becoming quickly re-infected and the rapidity with which the mean worm load, in child age classes, returns to its former level is a good measure of the transmission success or reproductive potential of *A.lumbricoides* within the treated community (Anderson and May, 1985a). Within as short a time as six months children can re-acquire their previous worm burden (Holland, Asaolu, Crompton *et al.*, 1989).There exists a whole science of mathematical modelling of rates of transmission, infection and re-infection. These mathematical techniques can help to clarify which of the many processes that influence transmission are of greatest

importance to the reproductive success (and hence abundance) of *Ascaris*. Ultimately such knowledge will be of assistance in the design of control programmes (Anderson and May, 1985a; Anderson and May, 1985b; Anderson and Medley, 1985).

1.1.3 DIAGNOSIS

Overt and distinctive clinical symptoms of ascariasis are the exception rather than the rule, and as nausea, lethargy, loss of appetite and weight loss are the accepted common symptons, it is easy to see why diagnosis of this parasite requires some effort. The definitive method for diagnosing ascariasis is the detection of eggs in the faeces. Values are usually expressed as eggs per gram of faeces (epg) or total egg production per day (EPD). Determination of such values is time-consuming and the techniques involve several different steps. A whole range of methods to preserve and detect *Ascaris* eggs in faeces are in use and their relative merits have been discussed (WHO, 1983). The Kato-Katz method has been selected in an attempt to ensure comparability between different workers and different studies (WHO, 1983). It has been shown that with increasing numbers of adult females there is a density dependent relationship between this statistic and epg values (Croll *et al.*, 1982) such that in a study in Iran there was a direct relationship between egg output per female worm and worm burden. However, when the worm burden exceeds 10-14 parasites per host the egg output per female declines rapidly and then remains approximately constant over heavier burdens. This relationship may be of value in estimating an individuals worm burden, but it is far from accurate (Elkins, 1987). Diagnosis is occasionally based on the collection of worms following chemotherapy (Seo and Chai, 1980) or on the reported history of passing worms (Gupta,

1985). The former method is very reliable, but time consuming and requires full co-operation from the community under study. The latter method may result in missing many infected individuals consequently underestimating the size of the problem.

A reliable serological test of a quantitative nature would be of real value, but this is currently unavailable. Various studies have shown the presence of antibodies specific to parasite antigens. Many virological diagnostic tests which are in current use are based on detecting antibodies generated in response to infection. Haswell-Elkins has shown that the total antibody score of individuals (as measured by intensity of bands in an immunoprecipitation) is predictive of epg values four months after the blood was taken which suggests that antibody levels reflect recently migrating larvae (Haswell-Elkins, Kennedy, Maizels *et al.*, 1989). This would not, however, be diagnostic of current infection with adult worms as antibody to parasite antigens has been detected in individuals who are persistently worm-free (personal observation). It is likely that antibodies are a reflection of exposure rather than infection. Studies on other parasites have suggested the possibility of a relationship between antibody isotype and the index of infection (Kwan Lim *et al*, *in press*; Ottesen, 1984).

One of the major problems in developing a serological diagnostic test is the difficulty encountered due to the cross-reactivity with antigens from other parasite species. This has been demonstrated for cross reactions between *Acaris* and *Toxocara canis* (Kennedy, Qureshi, Fraser *et al*, 1989), *Necator americanus* (Pritchard *et al.*, 1990) and is a persistent problem because polyparasitism is widespread in areas where

Ascaris is endemic. A simple test for detecting the presence of parasite antigens in faeces or urine would be invaluable for field diagnosis.

1.2 EPIDEMIOLOGY

1.2.1 PREVALENCE

Prevalence of infection is the proportion of people who are infected with the parasite. *Ascaris* is unevenly distributed in the many countries where it is entrenched, for example, prevalence values from more than 30 surveys in Nigeria (Holland and Asaolu, 1990) ranged from 0.9% to 98.2%. Values which have been reported from around the world include 90% in Central Iran (Arfaa and Ghadirian, 1977), 0% - 76% from different villages in rural Ghana (Annan, Crompton, Walters *et al.*, 1986), 49.6% in rural Malaysia (Bundy, Kan and Rose, 1988a), 6.9% in Guinea Bisau (Carstensen, Hansen, Kristiansen *et al.*, 1987) and 90% in Bangladesh (Martin *et al.*, 1983). Investigation of such prevalence values in the literature from around the world has drawn attention to possible trends and associations which may account for the observed patchiness:-

DEMOGRAPHIC FACTORS

Age is a major consideration with prevalence being very low in infants still dependent on breast feeding . After weaning prevalence increases dramatically to reach maximum values by as young as four years of age and these values remain high for older children and adult age classes (Annan *et al.*, 1986; Elkins *et al.*, 1986; Holland, Crompton, Taren *et al.*, 1987; Martin *et al.*, 1983; Robertson, Crompton, Walters *et al.*,

1989). Some studies have reported a decline in prevalence with age (Thein Hlaing, Saw, Aye *et al.*, 1984).

There are a number of reports which indicate that prevalence is higher in females (Cross, Clarke, Durfee *et al.*, 1975; Arfaa and Ghadirian, 1977; Elkins *et al.*, 1986). Haswell-Elkins (Haswell-Elkins, Elkins, Anderson , 1987) also noted that egg output, and antibody levels, decreased with age in males only. This may be due to cultural differences resulting in altered exposure to infection due to work patterns etc., as was considered to be the case in that study. To substantiate this there have also been some reports of prevalence being higher in males (Prakash, Chandra, Bhatnagar *et al.*, 1980).

;; ENVIRONMENTAL FACTORS

A review of African data shows that ascariasis is usually higher in rural than in urban communities (Crompton and Tulley, 1987). This also holds true for Korea (Seo and Chai, 1980). However, the movement of populations from a settled rural lifestyle into urban areas, at a rate faster than adequate housing and sanitation can be provided, probably favours the spread and increase of ascariasis in the shanty towns of cities in developing countries (Prost, 1987).

As mentioned earlier, embryonation processes are enhanced by a warm, moist climate. Prevalence in equatorial Congo is 73% and in arid Chad is 4% (Crompton and Tulley, 1987). Within the country of Cameroon, prevalence is less than 10% in dry parts of the country but reaches 60% in the humid zone (Carrie, 1982).

Investigation of prevalence data also often reveals evidence of associations with other parasites e.g. with hookworm (Annan *et al.*, 1986) and *Trichuris* (Haswell-Elkins *et al.*, 1987).

iii SOCIO-ECONOMIC FACTORS

Ascaris infections tend to cluster in families with one study showing that 82% of siblings of children with ascariasis are also infected (Williams, Burke and Hendley, 1974). It has been suggested that a child born into a large family is more likely to become infected than one born into a small family (Adekunle, Bammeke and Lucas, 1986; Thein Hlaing *et al.*, 1984).

Prevalence of ascariasis and other soil-transmitted helminthiases is significantly higher in children living in wooden or bamboo, rather than concrete, housing (Holland, Taren, Crompton *et al.*, 1988). The same pattern applies to those living in crowded conditions with poor water supplies and inadequate sanitation.

1.2.2 INTENSITY

Intensity is the single most important statistic to obtain. It is also the most difficult, because it is time-consuming and requires the co-operation of subjects. It is most usefully defined as the number of intestinal stages per host. There are different patterns of intensity of infection from different populations. In a study in Burma the average worm burden increased rapidly from birth to reach a mean of 17.3 adult worms in the 5-9 years age class followed by a decline to a mean of 4 worms per person in the oldest age class (Thein Hlaing *et al.*, 1984). In an Iranian study, the greatest intensity (an average of 30.6 worms per

person) was found in the 20-39 age group (Arfaa and Ghadiran, 1977). The convex pattern has been shown to be much more common (Bundy *et al.*, 1988a; Bundy, 1988b; Elkins *et al.*, 1986). Observed increases in intensity in childhood and decreases in adulthood are assumed to involve some form of age-related change in exposure or susceptibility. Generally, though, even when intensity declines with age (due to immunity ? Anderson, 1986), prevalence does not. After treatment with anthelmintics, prevalence returns more rapidly to its pre-treatment level than intensity (Arfaa and Ghadirian, 1977) and significant reductions in intensity may have only marginal effects on prevalence (Croll, 1982; Elkins *et al.*, 1986).

There are important reasons why understanding the factors that influence intensity are important - these are:

(1) morbidity is expected to be related to intensity.

(2) theoretical considerations show that regulation of parasite populations hinges on intensity (Anderson, 1982) and

(3) it is possible that efficient control measures could be devised if heavily infected people can be identified and given particular attention (Anderson, 1985a).

Measurement of average worm burdens in a population is not a useful statistic as the frequency distribution of worm numbers is aggregated or overdispersed. As long ago as 1931 it was observed that 50% of the worms were harboured by 5% of the population (Cort, 1931). In an Indian fishing village one quarter of the population harboured three quarters of the worms (Elkins, 1987). This overdispersion has been recorded for all age classes and both sexes (Anderson and May, 1985a; Bundy *et al.*,

1988a; Elkins *et al.*, 1986). An interesting slant here is that pica in children has also been shown to be overdispersed (Wong *et al.*, 1988). This may imply that the more geophagous children are disproporionately exposed to soil-borne infection but was not shown by the data in this study.

1.2.3 PREDISPOSITION

What makes the phenomenon of overdispersion even more striking is the observation that individuals seem, in some way, to be predisposed to harbouring no, a few or many worms. Studies where a community has been chemotherapeutically treated for ascariasis on two or more occasions, allowing time for re-infection, have shown that individuals normally return to the same level of worm burden (Bundy *et al.*, 1988a; Elkins, 1987; Haswell-Elkins *et al.*, 1987; Henry, 1988; Thein Hlaing *et al.*, 1987; Holland *et al.*, 1989).

Just as clustering of heavy worm burdens occurs in families, so too does predisposition. An explanation for this may be the genetic similarities that occur amongst family members and an ability to mount an immune response, focal transmission, or both (Forrester, Scott, Bundy *et al.*, 1988). MHC Class 1 frequencies reveal a statistically significant association between B14/Bw63 antigens and heavy *Ascaris* infection (Bundy, 1988b). Predisposition is also seen in other parasitic infections e.g. *Ancylostoma duodenale* and *Necator americanus* (Schad and Anderson, 1985) and *Trichuris trichiura* (Bundy, 1986).

There is a great need to understand what the cause of this predisposition is. Careful monitoring of nutritional status and

parasite-specific antibody as well as cellular responses, following drug treatment and during re-infection in patients who are predisposed to both heavy and light infections would yield interesting and useful data.

1.2.4 TREATMENT AND RE-INFECTION

A marked discrepancy exists between our knowledge of how to treat individuals and how to treat communities. Ascariasis has been successfully eradicated in Israel and Japan, but in both cases it was a lengthy, expensive process requiring excellent organisation and compliance. Treatment is straightforward but is useless in the absence of other control measures to eliminate re-infection. Indeed, intensities of infection are sometimes greater after treatment (Thein Hlaing *et al.*, 1987) and after an eleven month period following anthelmintic treatment the rebound population can comprise more massive parasites (Elkins *et al.*, 1989). Consequently, treatment every two or three months has been suggested to successfully control infection(Arfaa and Ghadirian, 1977).

Re-infection has been shown to occur at the rate of 20% of the previous load per month (Thein Hlaing *et al.*, 1987) with five to ten year old children being reinfected most quickly (Bundy, 1988b; Elkins *et al.*, 1986; Holland *et al.*, 1989; Thein Hlaing *et al.*, 1987). This age-dependency of re-infection rates may be due to increased exposure and/or increased susceptibility, in children.

1.3 CLINICAL EFFECTS OF *ASCARIS*

Pawlowski (1982) has defined clinical expression of ascariasis at different levels:

(1) Asymptomatic, with little pathological change as encountered in light infections in well nourished children.

(2) Asymptomatic or oligosymptomatic with some pathological changes where the host shows tolerance to the presence of *Ascaris*.

(3) Symptomatic due to pathological changes.

(4) Fatal.

Types (3) and (4) are clearly of major concern and the term morbidity should be restricted to cases where healthy days are lost, medical help is sought, or where specific drugs are used for treatment at home or in hospital.

1.3.1 ALLERGY AND HYPERSENSITIVITY REACTIONS.

It is well established that transient lung infiltration with blood eosinophilia and raised IgE levels are frequently caused by *Ascaris* larvae migrating through the lungs (Loffler, 1956). IgE levels are not elevated in helminth infections confined to the intestine so it is likely that IgE levels are directly associated with the tissue migratory phase (Radermecker, Bekhiti, Poncelet *et al.*, 1974; O'Donnell and Mitchell, 1980). A seasonal pneumonitis with eosinophilia has been reported in Saudi arabs and was shown to coincide with the transmission of *A. lumbricoides* during the rainy season (Gelpi and Mustafa, 1967). The symptoms reported here were probably worsened by previous sensitisation and indeed proved fatal in some cases.

The low incidence of Loffler's syndrome in many endemic areas has been attributed to the continuous transmission which is the norm where seasonal transmission is not marked (Arfaa and Ghadirian, 1977; Spillman, 1975). Bearing in mind the bias of ascariasis towards children, it is interesting to note that Jones (1977) recorded blood eosinophil levels which were higher in childhood, but decreased rapidly in parallel with declining intensity of infection. In addition, there was also an accompanying, but not necessarily related, increase in antibody levels. A study on infected pre-school children in Ethiopia revealed that their IgE levels were 28 times higher than equivalent Swedish children (Johansson, Melbin and Vahlquist, 1968) but this IgE was not all *Ascaris* specific.

Allergy in research workers handling *Ascaris* has been widely reported with three clinical conditions being recognised - respiratory, skin rashes and gatrointestinal disorders (reviewed by Coles, 1985).

1.3.2 ASCARIASIS AND HUMAN NUTRITION

A. lumbricoides is large, inhabits the small intestine and is in contact with the absorptive surface of the intestine. It is thus placed to disrupt host nutrition and such effects may be the most important aspect of the disease at a community level, especially for children.

ABSORPTION OF NUTRIENTS

The presence of adult worms in the small intestine has been associated with abnormalities of the mucosal surface, with marked hypertrophy of villi the most striking feature (Tripathy, Duque, Bolanos *et al.*, 1972). Similar results have been seen in pigs infected with *A. suum*

(Stephenson, Pond, Nesheim *et al.*, 1980). The implications of such observations for mal-absorption have been suggested by several studies e.g. (Venkatachlan and Patwardhan, 1953; Northrop, Lunn, Wainwright *et al*, 1987; Mahalanabis, Simpson, Chakrabarty *et al*, 1979)).

Whilst much of the data appears to suggest that ascariasis has no dramatic impact on nutrition, where it is borderline, such apparently small effects could tip the balance towards nutritional deficiency. Global distribution of protein energy malnutrition coincides indistinguishably with ascariasis and for both conditions children from 6 months to six years are most at risk.

Faecal nitrogen losses in children infected with *Ascaris* represent about 7% of daily nitrogen intake and these are reduced after de-worming (Brown, Gilman, Khatum *et al.*, 1980). Reduced fat absorption and reduced xylose absorption have also been shown in infected children and, again, improvements were seen after treatment (Tripathy, Gonzales, Lotero *et al.*, 1971; Tripathy *et al.*, 1972; Brown *et al.*, 1980). These effects seem to be dependent on density of infection, as the greatest fat and nitrogen losses were found in children harbouring at least 16 worms. Taren (Taren, 1986; Taren, Nesheim, Crompton *et al*, 1987) has also shown that ascariasis is associated with lower plasma vitamin A and β carotene values and has shown that the impaired absorption of fat may be related to this reduced level of vitamin A . Northrop *et al.*, (1987) have shown that *A. lumbricoides* is implicated in the lowering of plasma albumin levels, apparently through effects of the parasite on gastrointestinal function and mucosal integrity.

One consistent finding in pigs as well as in children is the reduction in ability of the infected host to digest lactose. Studies by Carrera *et al* (Carrera, Nesheim and Crompton, 1984) on Panamanian children showed that breath hydrogen production was higher in infected children following an oral challenge with lactose administered in milk, than uninfected children. Thirty days after anthelmintic treatment, no differences were seen. Similar work by Taren *et al.*, (1987) reinforces these results. In both studies levels of hydrogen produced were found to be significantly correlated with the intensity of infection. Lactose intolerance has been clearly demonstrated in infected children (Nesheim, 1989) and this illustrates a functional consequence of *Ascaris* infection. Control of *Ascaris* is important to ensure good utilisation of milk - this is particularly crucial where milk is given as a supplement to combat malnutrition. Gupta *et al.*, (Gupta, Mithal, Arora *et al.*, 1977) calculated that one year's supply of anthelmintics for a child would cost approximately 4% of one year's food ration.

ii EFFECTS ON GROWTH OF CHILDREN

Several studies have attempted to determine whether the prevalence of ascariasis contibutes to sub-optimal growth of children. Experimental infections in pigs with *A. suum* (Forsum, Nesheim and Crompton, 1981) clearly demonstrated that a reduction in growth rate of pigs was associated with infection with *Ascaris* and a slowing of growth rates in the pigs was observed about the time that the worms matured and began to release eggs. Growth depression was related to intensity of infection and a reduction in food intake was also observed in those pigs harbouring the heaviest infections. Studies on growth are very difficult

in children due to the long time span, the need to take socio-economic factors into account and the prevalence of polyparasitism.

Studies which have been completed have shown significant weight gain in children treated for ascariasis compared to untreated children (Gupta *et al.*, 1977; Willett, Kilama, Kihamia, 1979; Stephenson *et al.*, 1980; Foo, 1986). Such studies require regular (3-4 monthly) treatment and it appears that the rapid improvement in weight gain and skin fold thickness in children in a period immediately after treatment is "catch up" growth.

The mechanism whereby ascariasis results in reduced growth rates has not been determined. As already discussed, reductions in nutrient absorption have been shown in infected children, but it seems unlikely that this is the principle reason for slower growth rates. Animal studies have suggested that appetite and food intake are reduced by the presence of infection and that this effect may be significant in explaining differences in child growth. Infection, causing a mild anorexia combined with impaired absorption, might explain the differences in growth between infected and uninfected children.

1.3.3 SURGICAL COMPLICATIONS

Surgical complications of ascariasis are the most severe manifestations of the disease, often determining a picture of obstructive, inflammatory or perforated acute abdomen. Intestinal obstruction caused by adult *Ascaris* is one of the most common causes of laporotomy. It is the second most common cause in 2-4 year old children in Durban, Lishiu and Sao Paulo and the fifth most common cause in adults from China, Nigeria and

Burma (WHO, 1987). Such obstructions can be fatal. For example, in a two year old girl who, on post mortem examination, harboured almost 800 worms weighing 550 grammes (Baird, Mistrey, Pimsler *et al.*, 1986). Blumenthal and Schultz (1975) estimated that 0.2% of two to five year olds infected with *Ascaris* are presented at hospital in the southern states of the USA with intestinal obstructions.

Worms can perforate the intestinal wall and have been found free in the abdominal cavity , in the liver, pancreas and other organs (Odaibo and Awogan, 1988). Numerical analysis of the occurrence of intestinal obstructions is difficult and will almost always be an underestimate due to deaths occurring outside hospitals and without post mortem examinations.

1.3.4 HOST REGULATION OF *ASCARIS* INFECTION AND DEVELOPMENT

Jung (1954) suggested that a pre-existent intestinal infection with *Ascaris* may prevent the development of worms subsequently entering the body and that, incoming larvae fail in some way to mature until adults are virtually eliminated. In cases of massive, fatal infections where, e.g. 16 large adults and 778 small adults were found, it was suggested that this protective effect had failed in some way (Baird *et al.*, 1986). One consequence of de-worming is that rebound populations are greater than those previously seen (Elkins *et al.*, 1989; Thein Hlaing *et al.*, 1987) and this phenomena has been attributed to established worms inhibiting the development of large numbers of newly acquired larvae. Martin *et al.*, (1983) proposed that, normally, transmission occurs continually rather than at discrete intervals.

THE ROLE OF THE IMMUNE RESPONSE

Suppression of the immune system is likely to lead to increased numbers of infective stages successfully establishing themselves in the host. Elkins (1987) concludes that there is no evidence that acquired resistance mechanisms influence the proportion of young worms which become established in older people with long periods of past experience of *Ascaris*. Changes in intensity in older children and adults are probably due to alterations in exposure, as it seems unlikely that acquired resistance plays any role (Holland *et al.*, 1989). What is perhaps most fascinating is the observation that some individuals are persistently worm free in the face of massive infection potential. Elucidation of the mechanisms which prevent parasite establishment in such cases would be of great value and interest.

1.4 *ASCARIS SUUM*

A. suum and *Ascaris lumbricoides* are phylogenetically closely related but their precise status as strains of one species or reproductively isolated true species is controversial (Sprent, 1952; Kurimoto, 1974). In common usage the parasite which is found in pigs is named *Ascaris suum* and that which infects humans, *Ascaris lumbricoides*. This simple division breaks down, however, when studying the immunology and serology of human infection in an area where both types occur. Such is their close similarity that *A. suum* and its antigens tend to be used as a substitute for *A. lumbricoides* because it is more readily available. Attempts to resolve the relationship between the two has recently been reviewed by examining the morphology, chromosomes, physiology, biochemistry, antigenicity and courses of infection (Crompton, 1989).

Of particular interest to the work described here are differences in antigens of the two parasites. We have previously observed that a 17kDa antigen is found in excretory/secretory products from L2 larvae maintained *in vitro* (L2 ES) from *A. lumbricoides* but not in *A. suum*, although this may be quantitative rather than qualitative (Kennedy, Qureshi, Haswell-Elkins *et al*, 1987). Recent work by Christie *et al.*, (Christie, Dunbar, Davidson *et al*, 1990) has shown that the 14kDa molecule (*Ascaris* body fluid allergen-1, ABA-1) from *A. suum* and *A. lumbricoides* are identical in amino acid sequence to at least 41 residues. The molecular weight of the *A. suum* molecule has been determined by mass spectrometry to be 14,573-14,801. This emphasises the similarity between the two and further justifies the use of the porcine worm where the human one is unavailable.

If any real differences have been seen between the two it is in the courses of infection, with pre-patent periods being shorter in pigs for pig roundworm than for human roundworm in pigs (Takata, 1951; Galvin, 1968). Galvin also noted differences in larval migrations in rabbits, where more larvae reached the lungs from a human source whilst more larvae reached the intestine from pig roundworm. However, such differences may well be seen if the sources of worms, whether *A. suum* or *A. lumbricoides* were from geographically distinct sources.

The definitive experiment to show whether the two are the same species has not and, in all likeliehood, never will be done. It would require interbreeding larvae from pigs and man. If fertile hybrids were produced then it would be possible to claim species identity. This has been done successfully with different species of *Trichinella* from geographically distinct areas and hybridisation was demonstrated (Dick and Chadee, 1983).

1.4.1 *ASCARIS SUUM* IN PIGS

Studies of *A. suum* in pigs have been carried out with the purpose of reducing infection for commercial reasons. While care must be taken in directly applying results to *A. lumbricoides* in man, there are many valuable insights to be gained from these porcine studies.

In pigs, immunity to re-infection is acquired through repeated natural infection (Taffs, 1968). Many studies carried out have been concerned with the induction of a more rapid development of protection. Benkova (1982) immunised 4-week old piglets with ultra-sound disrupted L2 and L3/4 larvae and found that larval antigen, especially from the L2 stage generated a greater antibody response than did adult antigen. All

antigens generated some protective immunity as measured by reductions in larvae recovered from the lungs. Lunney *et al.*, also used altered infection to generate protection (Lunney, Urban and Johnson, 1986). Eggs were treated with ultra-violet (UV) light (which attenuates the larvae and prohibits maturation) and pigs were exposed to these on a contaminated feeding lot. When challenged with 10,000 infective eggs, both inbred and outbred swine developed greater than 80% protection. Analysis of peripheral blood cells showed that macrophage numbers were elevated and a marked increase in MHC class II antigen expression was observed, changes which preceded the peak serum antibody responses. Urban and Tromba (1982) also used UV attenuated eggs and found an 88% reduction in the number of larvae recovered from the lungs after challenge infection. Antibody responses to larval antigens were examined and very low titres to L3/4 ES and no anti-L2 ES specific antibody was found.

In a later study, Urban and Romanowski (1985) repeated protection studies with antigens from larval products and again found significant protection on challenge infection, however there were marked pathological responses to larvae migrating (and possible abbreviation of migration) in the liver. Later work (Urban, Alizadeh and Romanowski, 1988) examining where challenge infections are arrested revealed that continual exposure to eggs elicited effector components necessary to prevent larval migration from the intestine. While a regime of immunisation promoted resistance, this was at the cost of numerous "white spot" lesions in the liver.

Other, more complex, immunisation schedules have been attempted in the search for the basis of acquired protection, e.g. Rhodes *et al.* (Rhodes,

Baker, Christensen *et al*, 1988) incorporated L2 larval antigens and an adult intestinal aminopeptidase into liposomes. These were administered with a primary dose of eggs and subsequent challenge showed that protection against migrating larvae was conferred. However, continuous exposure to infection still confers better protection than all immunisation studies carried out to date. These studies are, however, informative of the various parasite antigens involved in generating protection. This is of value when considering the situation with *A. lumbricoides* in man, but a fundamental difference appears to remain between the two systems. Continuous exposure in pigs confers protection against subsequent challenge in all pigs, while this does not appear to occur in humans where only a small number of individuals appear to acquire (or possess inherently) protective immunity.

1.4.2 *ASCARIS SUUM* IN RODENTS

Rats, mice and guinea pigs are commonly used as laboratory hosts for *Ascaris* infection. As all infections in animals other than the definitive hosts, man and pigs, terminate in the lungs or on migration from them, such studies can only yield information about the larval stages.

The majority of studies on immunity in laboratory animals cite the liver as the major site of attrition (Soulsby, 1961). Histopathological examination shows the liver of such infected animals to be the focus for arrest and destruction (Bradbury, Percy and Strejan, 1974; Mitchell, Hogarth-Scott, Edwards *et al.*, 1976). Very low numbers of infective eggs can induce immunological responses as seen by increased numbers of immunoglobulin-bearing cells, levels of hepatobiliary IgA and resistance to challenge (Jeska and Stankiewicz, 1989).

Subsequent to infection the following immunological changes are seen in laboratory animals:

Eosinophilia, mediated by T lymphocytes, is observed within one week of a primary infection with *Ascaris* (Archer, Coulits, Jindira *et al.*, 1985; Mitchell, 1976b) and eosinophilia is more elevated and rapid after a second infection (Sugane, 1988). Mast cells also increase in number after infection but their appearance is slower than eosinophils with maximum numbers being seen at three weeks after a primary infection (Archer *et al.*, 1985).

Antibodies to parasite antigens are found as early as 5 days after primary infection. IgM antibodies are the first to be seen and a strong anti-phosphoryl choline response is detected in mice (Crandall and Crandall, 1971). Sugane (1988) showed the classical immune response in mice with IgG titres rising rapidly after the second and subsequent infections and reaching a higher and longer-lasting peak compared to primary infection. Significant increases in IgA production were also found 3 weeks post-infection in mice (Crandall and Crandall, 1971), but the role played by secretory IgA in resistance to *A. suum* infections in mice is unknown (Jeska and Stankiewicz, 1989).

Ascaris is particularly notable for eliciting a reaginic or IgE response (see Section 1.5 on *Ascaris* allergens). This antibody has usually been detected by passive cutaneous anaphylaxis (PCA) assays in laboratory animals or in PRIST or RAST assays in humans. Both crude extract of adult *Ascaris* and a purified protein Asc-1 (which has ABA-1 as a major constituent) caused mast cell degranulation and hence demonstrated the presence of specific IgE (Bradbury *et al.*, 1974). Only one immunisation

of L3/4 ES antigen of 60μg was required to generate IgE, but if two or more doses were given, as little as 3μg of antigen generated an IgE response detectable by PCA assay (Stromberg, 1979). In the same study less IgE was generated following intramuscular rather than intraperitoneal administration showing that the route of delivery of antigen is important. Another important aspect of infection with *Ascaris* is that it has been shown to potentiate IgE responses to other, unrelated antigens e.g. ovalbumin (Stromberg, 1980).

As well as potentiating some responses, infection with *Ascaris* or with antigens of *Ascaris* may suppress immune responses to bystander antigens. Crude extracts from adults and ES products suppress antibody responses, especially the IgE response to hen egg white lysozyme (Komatsu, Nishimura, Sano *et al.*, 1979). Such suppression is thought to be mediated by T lymphocytes, however different subsets of T cells are involved in different aspects of the immune response. Studies with nude (hypothymic) mice indicate that natural resistance is not affected by lack of T cells (Mitchell, 1976*b*), whereas Brown *et al.* (1977) propose that T cells are important in the antibody responses which mediate acquired resistance.

1.5 *ASCARIS* ANTIGENS AND ALLERGENS

The first report in the literature of the antigenicity of *Ascaris* components was in 1929, when Canning demonstrated that various tissue fragments were antigenic in a precipitin ring test (Canning, 1929). Later studies using serum agar double diffusion showed different numbers of antigenic components from various stages of the parasite's life cycle

(Kagan, 1957). Only two antigens were detected in embryonated eggs and fourteen in the whole worm homogenate. However the limitations of this technique, in terms of lack of sensitivity, have been acknowledged. Absorption studies also showed that several antigenic components are common between different tissue fragments. Kagan also noted that antibody profiles varied between two different rabbits; immunoelectrophoretic analysis of *Ascaris* preparations found a total of 24 antigenic components as detected by infection serum (Tormo and Chordi, 1965). They described the perienteric fluid as having all but one of these components and two different saline extracts of homogenised worm displayed the components most strongly recognised by infection serum.

With the advent of gel filtration technology in the early 1970's, work began on characterising fractions of *Ascaris* by different separation techniques. Ambler and co-workers tested fractions in PCA assays and detected allergenic activity over a wide molecular weight range (Ambler, Doe, Gemmell *et al.*, 1972). Subsequent work isolated a 13-15,000 dalton molecule, named Allergen A, which was detected in both maintenance fluid and whole worm extracts (Ambler, Croft, Doe *et al.*, 1973a). Further studies characterised Allergen A as having a molecular weight of 14,000 daltons by gel filtration and sedimentation equilibrium co-efficient determination and 12,000 by amino acid analysis, an isoelectric point of 5-5.2 and less than 1% carbohydrate (Ambler, Miller,Johnston *et al.*, 1973b). The molecule proved to be very stable, resistant to heat at 100°C, hot acid and cold periodate. PCA activity was, however, affected by treatment of the molecule with proteinases such as pronase and subtilisin (Ambler, Miller and Orr, 1974).

At the same time Hussain and his group attempted to isolate and characterise *Ascaris* allergens. Initially, a major allergen in the 30-40,000 Mr range was described and named Asc-1 (Hussain, Strejan and Campbell, 1972). Further purification and characterisation revealed the molecular weight to be in the 17-19,000 range with the molecule dissociating into 9kDa subunits. Asc-1 was found to have a pI of 4.8-5 and contained nearly 9% carbohydrate (Hussain, Bradbury and Strejan, 1973). Kuo and Yoo (1977) also isolated an allergen from body fluid with a pI of 6.1, which they described as being different from both Allergen A and Asc-1. Tanaka *et al.* (Tanaka, Kawamura, Tohgi *et al.*, 1983)) detected a 15kDa protein, which they named Asp (*A. suum* protein), in serum from patients with helminth infections and showed that *A. lumbricoides* contained the same amount of Asp as *A. suum*. In addition the protein was found to have very little or no carbohydrate content. Greenspon (1986) purified and described an allergen of 18.1kDa with a pI of 5.7 and approximately 1% reducing sugars. Another group (McWilliam, Stewart and Turner,, 1987) found some allergenic activity at molecular weights of 600kDa (see results in chapter 5) and suggested that 10 allergens existed here and 6 allergens in the lower molecular weight region of approximately 16kDa. Sasagawa (1987) confirmed these results and showed some mitogenic activity in a 12-68kDa fraction.

A change to the theme of isolating lower molecular weight allergen(s) came when Stromberg identified a 67kDa molecule from L3/4 ES (Stromberg, 1979). This molecule was found to consist of 79% protein and 22% carbohydrate, to have allergenic activity as defined by PCA assay, and to induce significant protection in guinea pigs challenged with *A. suum*.

Work in this laboratory has shown that both infective and lung stage larvae kept in culture (L2 ES and L3/4 ES), excrete or secrete a large number of antigens (Kennedy and Qureshi, 1986). They range in molecular weight from 14-410kD as shown shown by SDS-PAGE analysis of radiolabelled protein. It was also shown that there are both common and stage-specific antigens. Treatment with reducing agent showed that one L2 ES molecule is labile, two L3/4 ES molecules are lost and the mobility of a further two is altered by reduction. Further work on characterisation of *Ascaris* antigens will be presented in Chapter 3.

1.6 IMMUNE RESPONSES TO PARASITE ANTIGENS

1.6.1

The ultimate aim of immunoparasitology is to find a cure by identifying the protective responses of the host which lead to rejection of the parasite. Secondary to that, but maybe more realistically practical, is in the clinical situation, where attempts must be made to ameliorate the immunopathological reactions associated with parasite infection.

Helminth parasites differ substantially from protozoan and microbial pathogens in that they typically present the host with a large, non-phagocytosable foreign object. Thus, the concept that immune and inflammatory responses are designed to kill the invader must be discarded. It must also be borne in mind that many nematode infections have both tissue and gastrointestinal stages and different immune strategies may apply to each location. At best, host responses can abbreviate infection, minimise re-infection and stunt parasite growth

but the effector mechanisms and molecules leading to parasite loss are, on the whole, unknown.

The literature conventionally recognises three life cycle stages of antigens. These are the surface of the parasite, the products excreted and/or secreted by the parasite and the somatic components. In practise it has been evident that antigens are not restricted to these compartments but that, for example, ES products may include antigens derived from the surface or secreted from internal organs, so that considerable "overlap" does occur.

From the point of view of parasite survival, any strategy must prevent the exposure of any antigens which could stimulate the development of protective immunity. Various mechanisms for escape have been suggested (Almond and Parkhouse, 1985):

1) the parasite totally changes its surface proteins before an immune response develops; this is one consequence of larval moulting.
2) materials are secreted which then function as a diversion to "mop up" antibodies against similar epitopes on the cuticular surface; such antibodies have the potential to damage the parasite.
3) parasite antigens are masked with host-derived components e.g. *Trichinella* (Parkeson and Despommier, 1974), schistosomes (reviewed by McLaren, 1984) and onchocerca (Greene, Taylor and Aikawa, 1981).
4) heterogeneity in the parasite, i.e. changes in larval stages and see Chapter 6.

The release of antigens by viable nematode parasites *in vivo* may provide the host with molecules suitable for uptake and processing by antigen

presenting cells. Where released molecules are identical to, or are cross-reactive with, surface antigen this has the potential to stimulate an immune response against the parasite surface. Conversely, some surface molecules have cryptic epitopes which are "hidden" in some way so that they are undetectable by, i.e. surface immunfluorescence assays. When such molecules are shed from the surface they then become immunogenic.

In the following overview of current knowledge on the role of various host responses to parasite antigens it must be borne in mind that, in most cases, immune components in serum may not be representative of host responses in the organ or tissue micro-environment where the parasites would be encountered.

1.6.2 INNATE RESISTANCE

Innate, non-specific resistance to gastro-intestinal infections is mediated by such factors as gastric acidity, mucus secretion, intestinal motility, activities of bile constituents and various ubiquitous enzymes. Because of the non-specific nature of such defences it is difficult to ascribe any efficacy to them. However, some important observations have been made i.e. where immunosuppression is seen to act at mucosal surfaces, survival times of *Nippostrongylus brasiliensis* are extended (McElroy, Szewczuk and Befus, 1983).

1.6.3 COMPLEMENT

Complement consists of a complex series of proteins which non-specifically assist specific and non-specific immune responses. Complement performs three major functions (1) activation of cells e.g.

macrophages (2) cell lysis and (3) opsonisation - enhancing phagocytosis of foreign particulate matter.

It appears that such functions would be ineffective in combatting parasite infection due, as already mentioned, to the sheer size of the infective organism. While the role of complement in parasitic diseases and the immune response to them has not been elucidated, some reports raise interesting possibilities. People infected with hookworm display elevated levels of complement fixing antibody, yet remain susceptible to infection (Behnke, 1987). Interactions of the classical complement pathway with *Onchocerca volvulus* mediated a host inflammatory response and chemotaxis of granulocytes towards the parasite, but no damage was seen (King, Spagnuolo and Greene, 1983). While mouse C3 could not be detected on the surface of infecting schistosomula, subsequent incubation *in vitro*, with fresh mouse serum led to effective deposition of mouse C3 on the schistosomula (Ruppel, McLaren, Diesfold *et al.*, 1984). It was suggested that schistosomula may, *in vivo*, evade the lytic activity of complement and complement-mediated cellular cytotoxicicty.

1.6.4 T LYMPHOCYTES

T lymphocytes play a major role in the immune response as antigen reactive cells and effector cells in cell-mediated immunity and they also cooperate with B cells in antibody production against some antigens. Within the T cell population there are many functional subsets and the role which they play in responding to parasites is complex but important to understand.

Parasite specific T-cell clones represent sensitive reagents for identifying parasite antigens and revealing specific immune effector

mechanisms. T-cell clones specific for antigens of *B. malayi*, *S mansoni* and *T. spiralis* have been reported (reviewed by Baldwin, Goddeeris and Morrison, 1988). These clones are being used to identify parasite antigen epitopes which can activate T-cells, including T helper cells involved in antibody production and activation of cell mediated immunity.

The effects of different T cell subsets have been noted in many parasite infections. While looking at immunisation against leishmaniasis T cells from intravenously immunised BALB/c mice were found to be either protective or ineffective when passively transferred. A different route of immunisation (subcutaneous) in the same strain of mouse generated T cells, which either accelerated disease or had no effect in protestion experiments (Liew, Hodson and Lelchuk, 1987).

When mice are infected with *Nippostrongylus brasiliensis* and immune events at mucosal surfaces examined, either T helper cell function is defective or T suppressor cells mediate an effect on mucosal sites which facilitate parasite survival (these may be the same mechanisms) (McElroy *et al.*, 1983). The possible role of suppressor T cells in disease manifestation in humans has been particularly examined for filariasis. The immune response to parasite antigens is much lower in patients with microfilaraemia than in those with other manifestations of filariasis. In the former group, 15 out of 17 patients showed increased numbers of T suppressor cells by phenotype, while in a group of patients with elephantiasis, only 6 out of 11 showed such elevated numbers (Piessens, Partono, Hoffman *et a.*l, 1982). When activated suppressor T cells were removed, improved reactivity to filarial antigens was seen, hence strengthening the argument that immunsuppression induced by filarial

parasites is a mechanism of survival. Ottesen (1984) also noted that the number of T suppressor cells and the ratio of these to helper T cells was abnormally high in most patients suffering from both filariasis and onchocerciasis.

Among the mechanisms by which T cells mediate their effect is the production of lymphokines, which control the activities of accessory cells. T cells activated by, for example *Trichinella spiralis* muscle larvae, release a wide variety of lymphokines (Grencis, Crawford, Pritchard *et al.*, 1986). This has recently been futher characterised (Pond, 1989); the T cells of a *Trichinella*-resistant mouse strain (AKR), produced more gamma-interferon than the susceptible B10.BR strain which produce mainly IL4. The same pattern was seen with *Leishmania major* infections in C57BL/6 and BALB/c mice (Heinzel, 1989). Again T, helper cells from the resistant C57BL/6 produced more gamma-interferon, while the susceptible BALB/c produced mainly IL4. Possible explanations for the differences between the strains will be examined in Section 1.7.

Where T cells are absent many immune functions are affected, e.g. in a schistosome infection of mice, reductions in immunoglobulin levels, macrophage and eosinophil cytotoxicity were observed in athymic animals (Capron, Capron, Abdel Hafez *et al.*, 1983).

1.6.5 B LYMPHOCYTES AND IMMUNOGLOBULIN RESPONSES

When stimulated by antigen B lymphocytes differentiate into antibody-forming plasma cells. For some antigens, this requires T cell co-operation (Howard and Paul, 1983). While immunoglobulin may be easier to detect and characterise than other immune components, it must be borne in mind that the levels of antibodies elicited may remain high for a

long period of time, thus it is not always possible to distinguish active from past infection.

It has long been thought that humoral resistance is an isotype-dependent phenomenon and many studies have sought to discover relationships between isotype levels and outcomes of infection. Work on IgG antibody subclasses in human filariasis, revealed that IgG4 immunoglobulin levels were 17 times higher in microfilaraemic patients than those with chronic lymphatic obstructive pathology (Hussain, Grogl and Ottesen, 1987). Microfilaraemic patients showed greater antibody reactivity to antigens of less than 68kDa, while elephantiasis patients had strong IgG_1 and IgG_3 responses to antigens over 68kDa. There appears to be correlations here between isotype response, recognition of antigens and disease status.

Work done on mice infected with *Trichinella spiralis* detected antibodies of IgG_1, IgG_2, IgG_3 and IgA to biochemically defined, stage-specific surface and secreted components of infective larvae, adults and new born larvae (Almond and Parkhouse, 1986a). Independent variation of the responses of each immunoglobulin isotype was seen, but specific anti-parasite responses did not reflect the total serum immunoglobulin levels in all classes. In this system close correlation was seen in resistant NIH mice between the production of IgA antibody to surface components of adult worms and accelerated expulsion. This was not seen in susceptible C3H mice.

using *D. viteae*
In another study, three mouse strains (C57BL/10, BALB/c and CBA/N) showed significant variations in total immunoglobulin levels and also in their ability to recognise radiolabelled antigen in immunoprecipitations

(Almond, Worms, Harnett *et al.*, 1987). No serological differences in specificity were seen in the resistant C57BL/10 strain so effective immunity, in this case, is not mediated by antibodies alone.

Identification of antigens recognised strongly by infected individuals has been pursued in the attempt to find out if these antigens confer any kind of protection. In studies on human schistosomiasis, it was shown that a somewhat higher proportion of resistant than of susceptible children recognised a 27kDa and a 24-25kda antigen (Butterworth, Capron, Cordingley *et al.*, 1985). There was, however, a marked heterogeneity in the response in different individuals to a variety of antigens. Some were recognised by most sera and others by only a few. Again, in filariasis, sera from amicrofilaraemic donors preferentially reacted with a 70/75 kDa antigen when up to 25 antigens were detected in a Western blot (Kurniawan, Busandari, Fuhrman et al, 1990). Some residents who are amicrofilaraemic, in areas of endemic brugian filariasis, develop immune reactions to a microfilarial stage-specific antigen that has previously been identified as a potentially "protective" parasite antigen in animal models of lymphatic filariasis (Kazura, Cicirello and Forsyth, 1986).

Antibodies can confer resistance in some cases. An anti *Taenia saginata* oncosphere monoclonal antibody has been shown to confer protection in calves against subsequent oral infection with *T. saginata* eggs (Harrison and Parkhouse, 1986).

1.6.6 CROSS-REACTIVITY BETWEEN PARASITE ANTIGENS

A major problem with examining antibody levels in parasite infection of humans is the occurrence of immunological or serological cross-

reactivity between parasite antigens. This is especially a problem with phosphoryl choline - an antigenic determinant found on many infectious agents. It is present on *T. spiralis*, *D. viteae* (Almond and Parkhouse, 1986b) *B. pahangi*, *B. malayi* (Maizels, Burke and Denham, 1987) and *Echinococcus granulosus* (Shepherd and McMnaus, 1987).

Cross-reactivity between other parasite antigens has also been described, i.e. between *Ancylostoma*, *A. lumbricoides* and *Schistosoma mansoni* (Correa-Oliviera, Dusse, Viana *et al.*, 1988). Cross-reactive components have been characterised in *A. suum* and *A. lumbricoides* with *Toxocara canis* (Kennedy *et al.*, 1989). Thus, care must be taken when looking at antibody levels and profiles so that cross-reactivities are accounted for in any conclusions drawn.

1.6.7 ROLE OF ACCESSORY CELLS

Eosinophils secrete cationic proteins when stimulated and this effects the damage they cause. Eosinophilia is a common event in nematode infections and has been associated with protection in schistosomiasis (Butterworth and Hagan, 1987). After treatment of a community, re-infection was significantly lower in those with high eosinophil counts. Eosinophils from such individuals have been shown to mediate damage to schistosomula *in vitro* (Capron *et al.*, 1983). Eosinophils, in the presence of serum, adhere to the surface of *T. spiralis* larvae within minutes, flatten and degranulate causing fatal damage to the larvae (MacKenzie, Jungery Taylor *et al.*, 1981).

Mucosal mast cell numbers are also elevated in intestinal nematode infections and are thought to be involved in immunity to such infections

(Befus, 1986). These function by the release of pharmalogical mediators and thus may have an effect on expulsion of nematodes from the gut.

The presence of human neutrophils in cultures of *T. canis* larvae lowers the rate of antigen release by the larvae (Williamson, Allardyce, Clemmett *et al.*, 1990). Again, degranulation of these cells has some inhibitory effect on the parasite.

1.6.8 PRESENTATION OF ANTIGENS

The route and mode of exposure of antigens into the immune system of an infected animal bears heavily on the resulting immune response. If BALB/c mice are immunised intravenously with *Leishmania*, then a protective subset of T cells is generated, but subcutaneous immunisation of the same strain of animals generated T cells which inhibited the protective effect and accelerated disease development (Liew *et al.*, 1987).

The role of adjuvants is discussed more fully in Chapter 4. The enhancement of protection by the use of adjuvants is well documented: e.g. *T. spiralis* larval extracts given in Freunds Complete Adjuvant resulted in a reduction of the number of adult worms in the intestine 8 days after a challenge infection (Bell and McGregor, 1980). Antigen alone was less effective in causing this rapid expulsion reaction. Grencis *et al.* (Grencis *et al.*, 1986) immunised mice with surface antigens of *T. spiralis* in Freunds Complete Adjuvant and challenge infections resulted in stunted adult worms compared to control mice.

1.7 GENETIC CONTROL OF THE IMMUNE RESPONSE

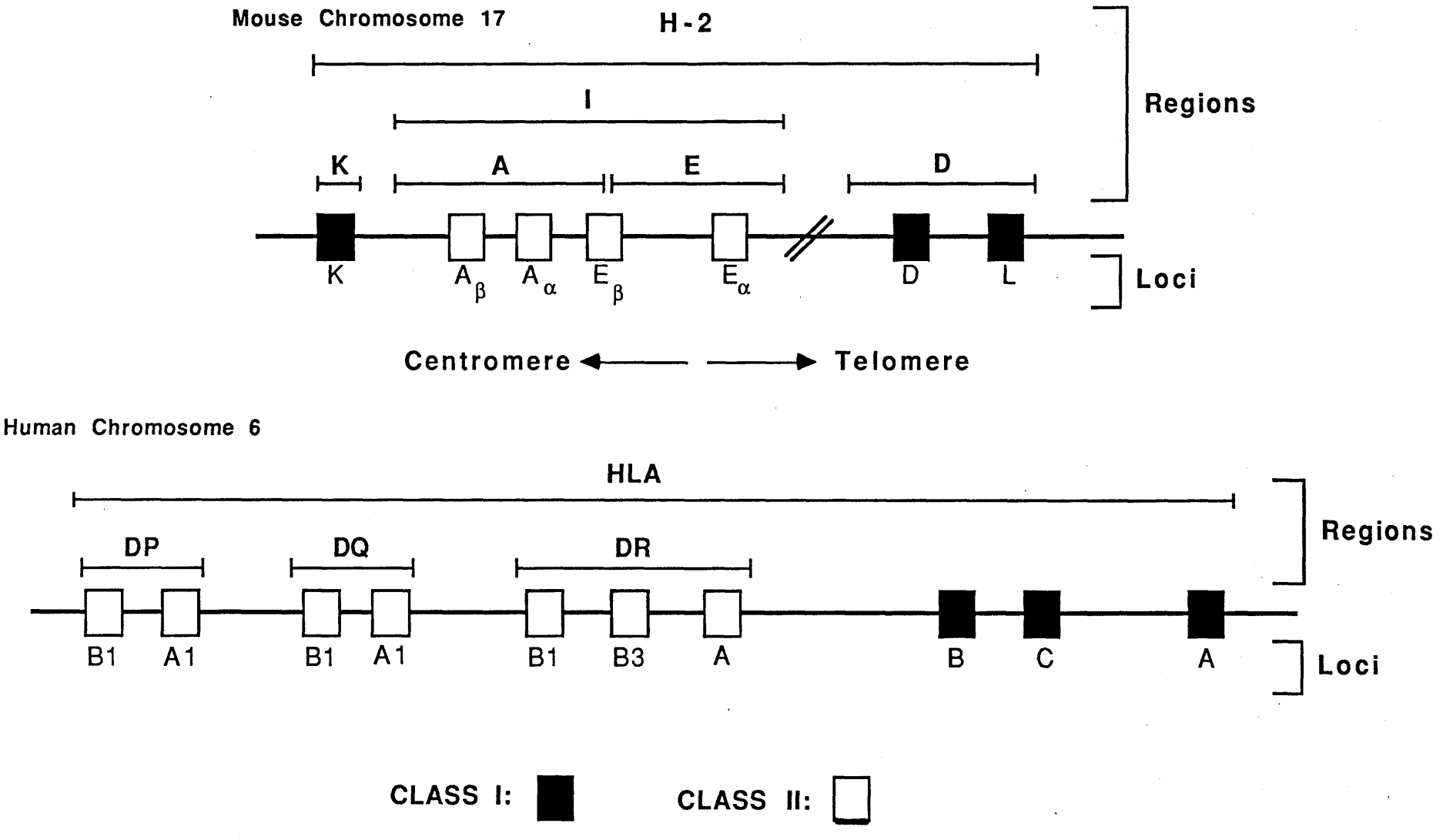
The phenomenon of predisposition to helminth infections (discussed in 1.2.3) may be the result of individual differences in exposure to infection or in protective immune responses or a combination of both. A major part of this thesis is concerned with genetic control of the immune response and this aspect of nematode infections will now be reviewed.

Many of the immune responses described in previous sections rely on the action of T lymphocytes. The T cell receptor (TCR) interacts with foreign antigen in association with the MHC gene products on the surface of antigen presenting cells. The cluster of closely linked genes of the MHC encode a variety of surface molecules which are central to the immune response. Class II region genes (see Figure 1.2) are associated with antigen presentation in antibody responses and T and B lymphocyte interaction. Class I molecules are involved in cytotoxic T cell killing of, for example, virus infected cells. As the principal effector mechanisms against helminth infections are T cell dependent, the influence of inherited MHC genes on the ability of the host to deal with infection could contribute to the predisposition to nematode infections mentioned above.

1.7.1 STUDIES IN MAN

Studies on the genetic factors influencing resistance or susceptibility to infection in humans are complicated ventures under field conditions. Because the populations concerned are outbred, large numbers of subjects are required and the acquisition of information about families presents great logistical problems. It is also difficult to differentiate environmental, social and age factors from genetic ones. The MHC genes in

Figure 1.2


Mouse Chromosome 17
H-2
I
K
A
E
D
Regions
K
A_β
A_α
E_β
E_α
D
L
Loci
Centromere
Telomere
Human Chromosome 6
HLA
DP
DQ
DR
Regions
B1
A1
B1
A1
B1
B3
A
B
C
A
Loci
CLASS I:
CLASS II:

man are called Human Leukocyte Antigens (HLA) and are associated with disease susceptibility more than any other known marker in man (Tiwari and Terasaki, 1985).

Despite these difficulties several studies have endeavoured to establish relationships between parasite infection and HLA antigens. Ottesen *et al.* demonstrated familial predisposition to filarial infection but were unable to show any correlation with HLA-A or -B locus specificities (Ottesen, Mendell, MacQueen *et al.*, 1981). Later work in Sri Lankans and Indians showed an association between HLA B15 and elephantiasis (Chan, Dissanayake, Mak *et al.*, 1984).

Significant differences were seen in a study of onchocerciasis patients, where those with the localised form of the disease had higher HLA-B35, Cw4, DR3, DR5 and DRw52 frequencies than an uninfected group in the same popultaion who had experienced the same exposure to infection (Brattig, Tischendorf, Reifegeste *et al.*, 1986). The uninfected group had higher HLA DR1 frequecies - possible evidence that different forms of onchocerciasis represent different immunological types of host responsiveness.

Early cases of *Schistosoma japonica* were shown to have an association with HLA B8 and advanced cases were significantly associated with HLA A1 and HLA B13 (Chong-gong, Qui-ying, Pan-yu *et al.*, 1984). Another parasitic disease where a particular stage or type of infection has shown HLA associations is amoebiasis. Examination of 31 Mexican mestizos, with amoebic abscesses of the liver showed that they had significantly higher frequencies of HLA Bw16 and HLA DR3 than uninfected individuals in the same community (Arellano, Isibasi, Miranda *et al.*, 1987)

One report by Bundy (1988b) describes the finding of higher frequencies of DQw2 antigen in uninfected people in the Carribean where *A. lumbricoides* and *T. trichuria* are prevalent. Investigation of Class I antigen frequencies also revealed a statistically-significant association between B14/Bw65 antigen and intense infection with either parasite. It is an uncommon antigen (0-6% frequency) and was found in this study only in heavily-infected individuals. These associations are reported as tentative and no futher developments have been reported in the literature.

Since the human population is so genetically heterogeneous there is great difficulty in attributing direct cause and effect to particular genes. The availibility of many inbred strains of mice provides an excellent model for dissection of genetic control of immune responses to parasite infection. Mouse MHC genes, known as the H-2 complex, and their Class II genes especially, exhibit close homology in structure and function with those of man. The HLA-DR molecule of man is homologous to the murineI-E dimer and HLA-DQ to I-A. Therefore, the use of animal models allows investigation of genetic factors which influence resistance and/or susceptibility to parasitic infection.

1.7.2 STUDIES IN MICE

Genetic factors controlling the immune response to parasite infections in mice have been the subject of an increasing number of studies - some of these studies are summarised in Table 1.4. I will review the work carried out to date on the system about which most is known on immunogenetics of nematode infections, *T. spiralis* in the mouse and then will review the work which has been carried out on *Ascaris*.

TABLE 1.1 **GENETIC CONTROL OF IMMUNE RESPONSE TO PARASITES AND THEIR ANTIGENS**

SPECIES	GENETIC FACTORS INVOLVED	COMMENTS	REFERENCE
Nematospiroides dubius	H-2 and non H-2	$H\text{-}2^{s}$ and $H\text{-}2^{q}$ best responders	Behnke & Robinson, 1985
Trichuris muris	H-2 and non H-2	antibody response to 40-43kDa antigen showed clear H-2 control ; $H\text{-}2^{k}$ strongest and $H\text{-}2^{d}$ weakest	Else & Wakelin, 1989
Leishmania donovani	non H-2	Lsh gene on chromosome 1 plays a vital role in resistance to the early course of infection	Blackwell, 1985
Schistosoma mansoni	H-2 and non H-2	response to 86kDa antigen restricted to $H\text{-}2^{d}$, $H\text{-}2^{k}$ and $H\text{-}2^{a}$: responses to other antigen show non H-2 control	Kee, Taylor, Cordingley et al., 1986
Dipetalonema viteae	non H-2	ability to respond rapidly inherited as a dominant trait	Storey, Wakelin & Behnke, 1985
Brugia malayi	H-2 and non H-2	internal adult antigens 24 & 66kDa recognised only by $H\text{-}2^{d}$: levels of response to phosphoryl choline and 15kDa adult surface antigen influenced by non H-2 genes	Kwan-Lim & Maizels in press

T. SPIRALIS

Mice of different inbred strains vary in the time of immune expulsion of *T. spiralis* from the gut. Using inbred and congenic mice it has been shown that some H-2^a strains expelled adult worms rapidly and had relatively low burdens of muscle larvae. However, mice of the H-2^a haplotype on a B10 background are slow responders so both H-2 and non H-2 genes were shown to be involved (Wakelin, 1980). Expulsion is thought to be mediated by mast cells and the response of mast cells was shown to be under genetic control. Responsiveness to infection, e.g. rapid expulsion in the rat, is inherited as a dominant trait and is controlled by non H-2 genes (Alizadeh and Wakelin, 1982). Studies on the response to the intestinal stage confirmed that whilst non H-2 genes exerted the major influence on worm expulsion, H-2 genes did have some effect, as demonstrated by the marked time differences of parasite survival among mice with slow responder B10 backgrounds (Wakelin and Donachie, 1983). Further investigation indicated that the response to adult parasites, the rapid expulsion response, and the anti-fecundity response are all under independent genetic control (Wassom, Wakelin, Brooks *et al.*, 1984). Immunisation with parasite antigens did not alter the patterns of response with slow responder C57Bl/10 mice showing no accelerated loss in subsequent infections. This was in contrast to NIH rapid responders which developed high levels of immunity (Wassom *et al.*, 1984).

One particularly interesting study showed that mice of H-2 haplotypes (s, b, q and f), which cannot express I-E heterodimers on their cell surfaces, are less susceptible to infection with *T. spiralis* (and *Nematospiroides dubius*, an unrelated parasite), than mice with haplotypes which do express the I-E molecule (Wassom, Krco and David, 1987). It is suggested that the suboptimal anti-parasite responses occur when relevant

parasite antigens are presented to T cells in the context of the I-E molecule.

ASCARIS

Initial characterisation of the antibody response to ES antigens of *Ascaris* in inbred mouse strains revealed that -

(1) no strain recognises all ES components

(2) with monthly infections, there is sequential recognition of antigens with time

(3) only animals with identical H-2 haplotypes have similar recognition patterns and

(4) when tertiary infections mounted by H-2 disparate strains appear to have the same recognition patterns the primary response patterns discriminate between them (Kennedy, Gordon, Tomlinson *et al.*, 1986).

To confirm that this restriction is the result of H-2 control, congenic mice were examined and mice with the same background genes, but different H-2 haplotypes, responded differently to the ES antigens (Tomlinson, Christie, Fraser *et al.*, 1989). Mouse haplotypes which display identical antibody recognition of *Ascaris* antigens have been found to differ in other immune effector mechanisms, i.e. IgE responses (Tomlinson *et al.*, 1989), eosinophilia (Vadas, 1982) and numbers of larvae reaching the lungs (Mitchell *et al.*, 1976). Thus while IgG antibody repertoires may be under H-2 control, other genes have an important role to play in the control of the immune response.

Observations that the ABA-1 molecule is only recognised by $H\text{-}2^{s}$ haplotypes and that MHC restriction only acts in the context of infection will be discussed in chapter 4.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 PARASITES

Adult *Ascaris suum* and *Ascaris lumbricoides* worms were obtained from infected pigs and humans respectively, the former from abbatoirs in the Glasgow and Edinburgh areas and the latter from Nigerian schoolchildren. The children were given 2 "Ketrax" tablets (active ingredient, Levamisole: ICI, Macclesfield) and the worms collected from faeces within the following 24 hrs. The worms were washed, stored in 2% formalin (37-41% Formaldehyde) at 4°C and transported to Glasgow within a few days.

The perienteric fluid (Adult Body Fluid, ABF) was collected by cutting the end of the worms and gently squeezing out the ABF soon after collection and microfuging the fluid (13,000g for 10mins) to remove any particulate matter. The supernatant was then dialysed against PBS (100g NaCl; 2.5g KCl; 14.37g Na_2HPO_4; 2.5g KH_2PO_4, all chemicals from BDH, made up to 1l with dH_2O, pH 7.2)for 24hrs then stored in aliquots at -70°C.

2.2 EMBRYONATION OF *ASCARIS* EGGS

Uteri were removed from mature female worms and disrupted in 5% Na Hyporchlorite (10-14% w/v chlorine) to dissolve the wall and release the eggs which were then washed 6 times in dH_2O (1,500g for 4 mins). The eggs were embryonated in 2% Formalin (37-41% formaldehyde) at 25°C in the dark and agitated once every week. After 3-4 weeks the embryonation rate was checked by microscopic examination under a x10 objective. Usually embryonation ranged from 30-50% of embryonated eggs out of the total number of eggs. When a satisfactory percentage developed the eggs

were washed as described above and stored under water at 4°C until required for hatching or infection.

2.3 HATCHING

Embryonated eggs were incubated in 5% Na Hyporchlorite at 37°C for 5-10 minutes until, when viewed under the microscope, the egg shells were seen to be disrupted, upon which they were washed 6-8 times as described in Section 2.2 to remove all traces of bleach and then hatched mechanically using a hand held glass tissue homogeniser (Jencons). Seven to ten strokes were applied until free swimming larvae were detected under x10 magnification. Larvae were separated from unhatched eggs and eggshell debris by means of an overnight Baermann (Baermann, 1917). This apparatus consists of a cotton wool plug at the surface of 12ml *Ascaris* medium (see Section 2.4)in a universal which is placed in a 37°C water bath overnight. The larvae migrate through the cotton wool and the infective or L2 stage larvae are collected and set up in culture (see Section 2.5).

2.4 PRODUCTION OF LUNG STAGE LARVAE OF *ASCARIS*

Adult, male New Zealand White rabbits of 2-3 kg (Interfauna) were infected orally with 50,000 infective (embryonated) eggs and after 7 days the lungs were removed, homogenised in a blender and incubated in PBS for 1 hr at 37°C. The homogenate was sieved through a 0.45mm Endecotts sieve (Gallenkamp) and the larvae collected. After several washes in PBS the larvae were washed in RPMI 1640 (Gibco) medium with the following additives (to 100ml of medium:- 1.2ml 200mM L-Glutamine, (Flow); 1ml 100mg/ml D-Glucose, (BDH); 200µl 200µg/ml glycyl-L-histidyl-L-lysine acetate salt, (Sigma G-1887); 10µl 5µg/ml Glutathione, (Sigma); 2ml 500IU Penicillin/500µg/ml Streptomycin, (Flow); 1ml 100mM sodium

pyruvate, (Flow); 1ml 7.5% sodium bicarbonate, (Flow); 2ml 250µg/ml Fungizone, (Flow); 0.25ml 10mg/ml gentamycin sulfate, (Sigma); 1 cephalexin Selectatab, (Mast Labs, MS10); 0.4 G.C. Selectab VCNT, (Mast Labs, MS6)) containing 200mM Chloramphenicol (Sigma C-0135). The larvae were set up in a Baermann overnight and then set up in culture.

2.5 COLLECTION OF EXCRETORY/SECRETORY PRODUCTS

L2 and L3/4 larvae were maintained at 37°C in a humidified atmosphere containing 5% CO_2 in RPMI 1640 medium plus additives as in Section 2.4. After 4-7 days the medium was collected and the larvae Baermannised to remove any dead larvae. The larvae were discarded after 14 days or whenever larval death exceeded 5%, whichever was the sooner. The culture medium containing excretory/secretory (ES) products was filter sterilised through a 0.22µm Millex GV low protein retention filter (Millipore) and stored at -20°C until required.

2.6 ULTRAFILTRATION OF EXCRETORY/SECRETORY PRODUCTS

Concentration of ES was carried out by ultrafiltration using cellulose triacetate membranes (Sartorious) in an Amicon apparatus or by centrifugal ultrafiltration with a nominal molecular weight cut-off of 10,000 (Centricon 10, Amicon, Danvers, MA) until the volume had reduced to 1/20th of the original. The ES was dialysed against PBS and the protein concentration determined. The ES was then aliquoted and stored at -70°C until used.

2.7 BIOSYNTHETIC LABELLING OF EXCRETORY/SECRETORY PRODUCTS

This was carried out by culturing the larvae as described above in ^{3}H-glucosamine (Amersham TRK 375) 100µCi/10ml RPMI 1640 or ^{35}S-methionine (Amersham SJ 1015) 250µCi/10ml RPMI 1640 methionine free. The cultures

were incubated for 3-5 days and the ES collected as described in Section 2.5

2.8 TREATMENT OF ANTIGENS

2.8.1

Autoclaving of antigens was carried out by adding 50μg of antigen to 8ml of 0.1M sodium acetate and autoclaving at 121°C for 20 mins. The antigen was then dialysed against PBS and the protein concentration re-determined by the Pierce method (Lane, Federman, Flora *et al.*, 1986).

2.8.2

Periodate treatment of antigens was carried out by adding 30-50μg of antigen in a volume of 300-500μl to 2ml of 0.1M sodium acetate containing 0.1M sodium periodate. This was left in the dark overnight at room temperature and then dialysed against PBS before re-determining the protein concentration as in 2.8.1.

2.8.3

Treatment of antigens by use of Endoglycopeptidase F enzyme or PNGaseF (Boeringer Mannheim 913 782) was carried out by incubating in a boiling water bath 40μl of ^{125}I labelled antigen for 3 minutes with 100μl of 1% Sodium Dodecyl Sulphate (SDS), 1.6% beta 2 mercaptoethanol(β-2-ME, Sigma). This was then mixed with 400μl of digestion buffer (0.2M sodium phosphate pH8.6, (May and Baker); 10mM 1,10 phenanthroline, (Sigma); 1.25% Nonidet (Sigma)) and 20μl of enzyme in 80μl of 50% glycerol (BDH), 2.5mM Ethylene diamintetracetic acid (EDTA, Sigma). This was then incubated for 24 hours at 37°C before use.

2.9 PURIFICATION OF *ASCARIS* BODY ALLERGEN - 1 (ABA-1)

ABA-1, formerly called "14K", (Christie, Dunbar, Davidson *et al.*, 1990) was immunoaffinity purified using antibody raised in rabbits as follows. Small quantities of ABA-1 were obtained by separation of ABF (unboiled), laced with ^{125}I-labelled ABF, on 1.5mm thick 20% acrylamide Sodium Dodecyl Sulphate gels (SDS-PAGE). Gels were vacuum dried at 60°C and the 14,000Mr region excised using an autoradiograph of the gels as a template. The gel matrix was re-hydrated in 25mM TRIS, 192mM glycine, 0.1% SDS buffer, and ABA-1 recovered by electoelution in the same buffer using a Bio-Rad (Richmond, CA) electroelutor with membrane caps having a nominal exclusion limit of 3500Mr. SDS was removed by dialysis against 10% methanol over 48 hours at 4°C using a 2000Mr exclusion limit dialysis membrane (Sigma) followed by passage through an AffinityPak Extracti-Gel D column (Pierce) to remove any remaining detergent. The protein sample was then concentrated and dialysed as described for the ES products.

2.10 RADIOIODINATION

Antigens were labelled (Markwell and Fox, 1978) with ^{125}I (Amersham IMS 30) catalysed by the IODO-GEN reagent, 1,3,4,6-tetrachloro-3a6a diphenyl glycoluril (Pierce Chemical Co., Rockford, IL). Briefly, 1-20μg of protein was labelled with 50 μCi of iodine (Amersham) and excess iodine consumed by the addition of saturated tyrosine before passing down a PD10 Sephadex G-25M de-salting column (Pharmacia, Uppsala). Fractions were collected and peak counts taken as assessed by a hand held gamma counter. To estimate the efficiency of the labelling triplicate samples of 2μl of antigen was added to 5μl of a normal serum. The percentage of radiolabel precipitable by 10% Trichloroacetic acid (TCA) was determined

after washing and aspiration of the supernatant leaving only the macromolecules which had precipitated.

2.11 IMMUNOPRECIPITATION

This was performed using a Protein A based assay whereby approximately 200,000 counts per minute (cpm) of radiolabelled antigen was incubated overnight with 2.5µl of test serum in duplicate. Immune complexes were captured on heat-killed, formalin fixed *Staphylococcus aureus* (Gibco). The resulting pellets were washed in PBS/0.5% Triton or a lithium wash buffer for the human immunoprecipitations to cut down on background radioactivity (10ml 10% SDS, 20ml 1M lithium chloride, 5ml Triton X-100, 50ml 1M TRIS, 29g sodium chloride made up to 1l with dH_2O) three times and the remaining radioactivity measured in an LKB 1272 Clini Gamma counter. The level of precipitation is expressed as the amount of iodine (cpm) precipitated by antibody as a percentage of the counts precipitable by TCA. Dried pellets were analysed by SDS-PAGE immediately or stored at -20°C until use.

2.12 SDS-PAGE ANALYSIS OF IMMUNOPRECIPITATION

This was carried out in a Pharmacia GE-2/4 LS slab gel apparatus according to the manufacturers instructions. Gradient gels (5-25%) consisted of 120mm separating gel and 15mm stack gel and were 0.7mm thick. Samples were prepared by addition of 40µl SDS-PAGE sample buffer (5g Sodium Dodecyl Sulphate, (BDH); 5ml 1M TRIS pH 7.5; 2ml 100mM phenylmethyl sulfonyl fluoride (PMSF, Sigma P-7626) in Isopropanol, (BDH); 1ml 100mM EDTA (Sigma); 10ml glycerol, (May and Baker); 2ml 0.2% Bromophenol Blue, (BDH); made up to 95ml with H_2O, pH 6.8) with 5%β-2-ME (Sigma) or 1mg/ml iodoacetamide (BDH) for reducing and non-reducing conditions respectively, added immediately before use. After mixing,

samples were incubated in a boiling water bath for 10min, microcentrifuged at 13000g for 5 min and the supernatants loaded onto gels. After electrophoresis (20mA per gel at constant current run for approximately 4 hr), gels were fixed in 25% methanol, 10% acetic acid, 1% glycerol and then dried at 80°C on a Biorad vacuum drier for 2 hr. Autoradiographs of the gels were exposed to film at -70°C with pre-flashed Fuji RX film. Apparent molecular weights were estimated by reference to standard marker proteins (Pharmacia 17-0446-01).

2.13 TWO-DIMENSIONAL ELECTROPHORESIS OF ANTIGENS

ABF and L3/4 ES were analysed by non-equilibrium pH gradient electrophoresis (NEPHGE) which resolves basic as well as acidic proteins (O'Farrell, Goodman and O'Farrell, 1977). The first dimension gels were poured to a height of 12cm in glass tubes (130mm x 2.5mm inside diameter) with the gel mixture (9.2M urea, 2% Nonidet P-40, 45% acrylamide/bisacrylamide (30% stock solution of 28.4% acrylamide and 1.6% bisacrylamide) and 2% Ampholines (pH 3.5-10 LKB 1809-121) ; gels were polymerised with 20µl 10% ammonium persulphate and 14µl TEMED per 10ml of gel mixture). Gels were overlayed with water and allowed to polymerise for 1 hr. Samples of approximately 2 x 10^6 cpm of antigen were loaded onto the gels and overlayed with 20µl of overlay solution (8M urea, 0.8% pH5-7 Ampholines (LKB, 1809-101) 0.2% pH 3.5-10 Ampholines, as before). The lower reservoir of the electrophoresis chamber was filled with 0.02M Na OH and the upper chamber with 0.01M phosphoric acid. Gels were electrophoresed with the cathode at the bottom and the anode at the top for a total of 1600-2000 volt hours. Gels were removed and equilibrated in equilibration buffer (10ml glycerol, 50ml 10% SDS, 6.25ml 1M TRIS pH 6.8, 1ml 2% Bromophenol blue, 27.75ml deionised water, 5% β-2-ME added immediately prior to use) for 2

hr. Gels could be stored at -20°C before use. Tube gels were laid across the top of 5-25% SDS-PAGE gels , sealed with 1% agarose and the second dimension gels run, fixed, dried and set up in an autoradiograph as described in Section 2.12.

2.14 ENZYME LINKED IMMUNOSORBENT ASSAYS

These were carried out on Immulon B 96 well plates (Dynatech) and all volumes were 50µl. See Table 2.1 for concentrations of protein used for coating the plates and the dilutions of serum and conjugates in each assay. Briefly, the plates were coated overnight at 4°C in coating buffer (1.5g sodium carbonate , 2.93g sodium bicarbonate, 0.2g sodium azide made up to 1l with dH_2O, pH 9.6) and then washed three times with wash buffer (1.07g di-sodium hydrogen ortho-phosphate, 0.39g sodium di-hydrogen ortho-phosphate, 8.5g sodium chloride made up to 1l with dH_2O 0.05% Tween 20 (Sigma P-1379) pH 7.2). Subsequent incubation steps were carried out at 37°C for 1 hr. Dilutions of serum and conjugates (all Sigma) were made up in wash buffer containing 0.25% Bovine Serum Albumnin (Sigma A-9647) and 0.1% sodium azide. The chromogen used was p-nitrophenyl phosphate (Sigma 104-105), one 5mg tablet/5ml diethanolamine buffer (9.7ml diethanolamine (BDH), 0.5mM magnesium chloride, 0.2g sodium azide and 800ml distilled water, pH 7.2) and the development of colour was stopped after 15-60 mins with the addition of 50µl 3M NaOH. The plates were read at 405nm on a Titretek automatic ELISA reader (Flow Labs, Irvine).

2.15 IMMUNOFLUORESCENCE

L2 or L3/4 larvae were washed 3 times in ice-cold PBS/0.1% sodium azide and left in a final volume of approximately 100µl. Test serum was added to give a final dilution of 1/30 and incubated on ice for 30 min. The

TABLE 2.1

ELISA SPECIFICATIONS.

SPECIES	Antigen	Concentration of antigen used to coat plate- µg/ml	Dilution of test serum	Dilution of Alkaline Phosphatase conjugate	Sigma catalogue number
				Goat anti-rabbit IgG whole molecule	A-8025
Rabbit	ABF	6	1/500	1/500	
	ABA-1	4	1/500	1/500	
	L3/4ES	8	1/500	1/100	
				Goat anti-mouse IgG whole molecule	A-5153
Mouse	ABF	4	1/500	1/500	
	ABA-1	2	1/500	1/500	
	L3/4ES	0.5	1/100	1/250	
				Goat anti-human IgG gamma chain specific	A-6029
Human	ABF	4	1/100	1/500	
	ABA-1	0.5	1/100	1/500	
	L2ES	1	1/100	1/500	
	L3/4ES	0.5	1/100	1/250	

larvae were washed three times and the appropriate fluorescein isothyocyanate conjugated antibody added to a final dilution of 1/30 (Scottish Antibody Production Unit: donkey anti-rabbit IgG S076-201, sheep anti-human IgG S074-201). After 30 min incubation on ice the larvae were washed a final three times. The parasites were viewed by a x40 objective under ultra violet light and the fluorescence quantified by photon counting using a Leitz MPV Compact 2 microscope photometer linked to Ernst Leitz Wetzlar, Program MPV-C2 MS DOS Version 1.2. The areas selected for measurement avoided any edge fluorecence and the area measured was kept constant in each experiment. The fluorescence of one of the brightest specimens was used to standardise the photometer at the arbitrary value of 650 and all values were automatically background subtracted.

2.16 **ANIMALS**

2.16.1

MICE - all mice were supplied by Harlan Olac (Bicester, UK) except for B10 HTT which were bred in our own animal house from Olac stock. A list of the different strains used and their H-2 haplotypes is found in Table 2 (Klein, Figueroa and David, 1983). Mice were infected with 2,000 infective stage *Ascaris* eggs at 0, 28, 56 and 84 days (where a quaternary infection was given). They were bled at 28 days after a primary infection and 14 days after all subsequent infections. Where mice were immunised with antigen in the context of adjuvant, the regime was as follows: on day 0 the antigen (5µg of ABF, ABA-1, L2ES or L3/4ES) was emulsified with Freunds Complete Adjuvant (FCA, Gibco) and administered in 0.1ml subcutanously. The same quantity and volume of antigen was then given on day 28 in freshly prepared Incomplete Freunds Adjuvant (IFA, Gibco) subcutanously and then intravenously in PBS on day

TABLE 2.2

STRAINS OF MICE USED AND THEIR MHC (H-2) HAPLOTYPES.

Strain	Haplotype	K	A-beta	A-alpha	E-beta	E-alpha	D
Classical							
BALB/c	d						
NIH	q						
CBA/Ca	k						
B10.R111	r						
NZW	z						
B10.HTG	g						
B10.AKM	m						
B10.A2G	a						
B10.M	f						
B10.BR	k	k	k	k	k	k	k
C57Bl/10	b	b	b	b	b	b	b
SJL	s	s	s	s	s	s	s
Recombinant							
B10.A5R	i5	b	b	b	b/k	k	d
B10.S9R	t4	s	s	s	s/k	k	d
B10.A4R	h4	k	k	k	k/b	b	b
ATL	t1	s	k	k	k	k	d
ATH	t2	s	s	s	s	s	d
B10.A2R	h2	k	k	k	k	k	b
B10.AQR	y1	q	k	k	k	k	d
B10.D2	g1	d	d	d	d	d	b
B10HTT	t3	s	s	s	s/k	k	d

56. Blood was collected by exsanguination 14 days after the final administration.

2.16.2

RATS - Table 2.3 shows the strains of rats used and their MHC types. The WLEP strain of rat has been inbred in our own animal house since 1979 for more than 15 generations whilst the other strains were supplied by Harlan Olac (Bicester, UK). Rats were infected with 4,000 infective stage *Ascaris* eggs at 0, 28 and 56 days and bled out 14 days after tertiary infection. For the immunisation with antigen in adjuvant the same protocol was used as described for mice. The quantities of antigen given in this case were 50μg ABF, 20μg L2ES and 50μg L3/4ES administered in 0.2ml.

2.16.3

RABBITS - Half Sandy Lops (National Institute for Medical Research, London) were used to generate antisera. Antiserum to infection was raised by infecting with 3,000 infective eggs every 28 days. R10 was the rabbit for *A.suum*; R19 for *A.lumbricoides* and R1 for *Toxocara canis* (20,000 embryonated eggs/infection).

Antiserum to ABA-1 or L3/4ES was generated by giving 100μg of the protein intramuscularly in 0.3ml FCA (Gibco) on days 0 and 7. Twenty-eight days later protein was injected subcutanously in IFA (Gibco). Tertiary boosts consisted of 100μg antigen given intravenously in PBS. Since ABA-1 and other parasite products are potent allergens, rabbits were given 0.3ml of 20mg/ml antihistamine "Vetibenzamine" (Ciba Geigy) prior to their final boost. Animals were bled at various time points after the final boost. Optimal antibody titres were usually found to occur at day 7 after tertiary immunisation as assessed by ELISA.

TABLE 2.3

STRAINS OF RATS AND THEIR HAPLOTYPES

Rat Strain	RT1 Haplotype
WLEP	u
PVG	c
PVG-RT1^{l}	l
PVG-RT1^{u}	u

2.17 PASSIVE CUTANEOUS ANAPHYLAXIS

The backs of normal, inbred WLEP rats were shaved with clippers (model A5, blade no.80, size 40, Oster, Milwaukee, Wisconsin) and 100µl of test serum or dilutions thereof, injected intradermally. Twenty-four hours later rats received an intravenous injection of 50µg ABA-1 containing 5mg Evans Blue (Sigma) in 1ml PBS. Between thirty and forty-five minutes later positive reaction spots were recorded. Normal serum controls were used in each rat and each assay point was carried out in duplicate on separate animals. Sera used in these experiments were pools from at least 3 animals per experimental group.

2.18 DETECTION OF WESTERN BLOTTED IgE BINDING ANTIGENS

SDS-PAGE gels (as described previously) were run with 100µg of ABF laid across the top of the gel in one long well with separate end wells for molecular weight markers (200,000cpm) and radiolabelled ABF (200,000cpm) as described in Section 2.12. Antigen was blotted onto nitrocellulose Hybond N (Amersham) in a TransBlot tank (Biorad) at 4°C for 4 hrs at 200mA, 60V or overnight at 100mA, 30V in blotting buffer (9.09g TRIS, 43.2g glycine, 600ml methanol, 3.03g SDS made up to 3l with dH_2O, pH adjusted to 8.3). The blot was then air dried and cut into strips and the two marker tracks set aside. The remaining strips were blocked in 10% skimmed milk in blotting buffer on a rocker for 1 hr at room temperature. After 3 x 5 min washes the strips were incubated in a 1/40 dilution of test serum overnight, washed again and then incubated in 200,000cpm/strip of Pharmacia's PRIST isotope - ^{125}I labelled anti-human IgE for 4 hr. After a final 5 x 5 min washes the strips were dried and then re-assembled along with the marker tracks before being set up in an autoradiograph as described in Section 2.12.

2.19 DETECTION OF WESTERN BLOTTED IgG BINDING ANTIGENS

This was carried out in the same way as described in Section 2.18 with the following changes; the strips of blotted nitrocellulose were incubated in test sera for 3 hr. After washing the strips were incubated in 200,000 cpm/track of Pharmacia's IgG RAST reagent - ^{125}I mouse anti human IgG.

2.20 LECTIN AFFINITY CHROMATOGRAPHY

Radiolabelled antigen (400,000 cpm) was incubated rotating at 4°C for 21 hr with 50µl of immobilised lectin beads. Unbound antigen was washed away in three washes with a 0.1M solution of nonspecific sugar (see Table 2.4) in running buffer (4.09g sodium chloride, 0.605g TRIS , 10mg manganese chloride, 74mg calcium chloride, 2.5ml Triton X-100 , 0.5g sodium azide made up to 500ml with dH_2O, pH adjusted to 7.4 with 1M HCl) by adding 100µl, rotating for 3 min and then microfuging at 13,000g for 3 min. The supernatant was removed and bound glycoconjugates were removed by one wash with the specific eluting sugar. The pellet was retained to examine for any strongly binding glycoconjugates and to check the efficiency of the washing. The supernatants and pellets were then analysed by SDS-PAGE.

2.21 ELECTRON MICROSCOPY WORK

This was carried out in the Electron Microscopy Unit of the Zoology Department at Glasgow University by Dr. Max Huxham. Live L3/4 worms in culture were fixed by adding equal volumes of 4% paraformaldehyde and 0.05% gluteraldehyde in 0.12M sodium phosphate buffer, pH 7.2 for 30min at 4°C. This was replaced with 100% paraformaldehyde for 1hr at 4°C. The worms were then washed three times in 0.12M sodium phosphate buffer pH 7.2, 5% sucrose overnight. They were then dehydrated for 2hr each in 30,

TABLE 2.4

LECTIN AFFINITY CHROMATOGRAPHY

LECTIN *	SIGMA Catalogue no.	BINDING SPECIFICITY	SPECIFIC ELUTING SUGAR	NON-SPECIFIC ELUTING SUGAR
Lentil	L-4018	D-mannose, glucose	methyl mannoside	galactose
Concanavalin A	C-7511	D-mannose, glucose	methyl mannoside	galactose
Tetragonolobus Purpureas	L-3257	L-fucose	L-fucose	galactose
Wheat Germ Agglutinin	L-6257	N-acetyl glucosamine	N-acetyl glucosamine	galactose
Helix Pomatia	L8639	N-acetyl galactosamine	N-acetyl galactosamine	mannose
Arachis Hypogea	L-2507	N-acetyl galactosamine	N-acetyl galactosamine	mannose

* immobilised on cross-linked beaded sepharose from Sigma

all sugars used at 0.1M concentration in running buffer

Reference - Maizels, R.M., Gregory, R.F., Kwan-Lim, G. and Selkirk, M.E., 1989.

50, 70, 90 and 100% alcohol, then dried at 100% overnight. They were incubated for 2hr each in 2:1, 1:1, 2:1 alcohol:Lowicryl mix at 4°C and neat Lowicryl, with 2 changes. Lowicryl was then polymerised for 2 days at room temperature using indirect UV(360nm) light. 70nm sections were cut on a Reichert Ultracut E ultramicrotome using a diamond knife and the sections then mounted onto gold 700 mesh grids. Sections were pre-adsorbed using 1% BSA in PBS for 10 min, washed x 2 with PBS and then incubated in antibody diluted 1/25 with PBS for 2 hr at room temperature. Sections were washed x 3 in PBS for 10 min. They were then incubated in undiluted Protein-A conjugated to 20nm colloidal gold, stabilised using BSA, for 1hr at room temperature. After x 3 washes in PBS, and air drying they were examined after staining with uranyl lead citrate. Micrographs taken at 20,000 magnification and negatives at 50,000 magnification.

2.22 DATA HANDLING

Data was analysed using the Statworks Version 1.1 and CricketGraph Versions 1.0 and 1.2.1 progammes on an Apple Macintosh computer.

CHAPTER 3

CHARACTERISATION OF *ASCARIS* PRODUCTS

3.1 INTRODUCTION

Ascaris and other gastro-intestinal nematodes secrete or excrete a large number of parasite products into their environment i.e. their host. In any attempt to prevent or eliminate these disease-causing organisms several aspects of infection must be characterised e.g. :-

(1) the biochemistry and function of the parasite products

(2) the role of these products in invasion of the parasite and its evasion or induction of immunity

(3) the potential use of such products for diagnosis or prevention of disease e.g. the 47kDa and 50/55kDa antigens from *T. spiralis* ES have been shown to induce protection in mice (Silberstein and Despommier, 1984). Also, the degree of developmental retardation and stunting of adult *Heligosomoides polygyrus* worms could be correlated with the titre of antibody specific for larval ES but not with antibody reacting with cuticular or internal somatic antigens (Ey, 1988a).

The results presented in this chapter are largely concerned with the first of these approaches.

During infection with *Ascaris* the host is potentially exposed to a variety of parasite products. Materials shed from the parasite surface, moulting and the release of excreted or secreted somatic products together make up that which is conventionally termed ES materials. They can be harvested from the supernatant during culture of the parasite *in vitro* and are potential sources of both diagnostic and protective antigens. Both infected humans and experimental animals generate high antibody responses to ES products (reviewed by Ogilvie and de Savigny, 1982; van Knapen, van Leusden, Polderman *et al.*,1983; Almond, Parkhouse, Chapa-Ruiz *et al.*, 1986c; Kennedy and Qureshi, 1986; Haswell-Elkins *et al.*, 1989). Because

larvae migrate through different tissues and organs ES products are found systemically and can be detected in sites remote from infection. In *Ascaris* ES can be obtained from infective (L2) larvae *in vitro* (L2 ES) and from lung (L3/4) stage larvae *in vitro* (L3/4 ES). Both are complex mixtures with probably few molecular species in common and the composition of ES changes with the development of the parasite (Kennedy and Qureshi, 1986).

L2 ES is difficult to produce in appreciable quantities in the laboratory, does not stain well with e.g. Coomassie or silver stains and can be difficult to label with ^{125}I or ^{35}S methionine. Antibody to it, however, is present in large quantities in serum from people and experimental animals infected with the parasite (see Chapters 4 and 5). L3/4 ES is produced in much more appreciable quantities, radio- and biosynthetically labels well and stains much more readily with Coomassie or silver stains.

In terms of supply, ABF (adult worm body fluid) is, in contrast to ES materials, much more abundant - an adult female would yield 2-3ml with a protein concentration of 4.2mg/ml. ABF is a complex mixture whose major constituent is the Mr 14kDa ABA-1 protein which comprises 60-70% of the total protein content of the body fluid (Kennedy and Qureshi, 1986). The degree of exposure to the host of the components of ABF is unclear. It is likely that the host immune system only encounters ABF when an adult worm is damaged in some way and subsequently releases fluid from its pseudoceolom. Individuals from an endemic population do have antibodies specific for ABF components - these may be the result of adult worms damaged in some way by the immune system or may be due to exposure to molecules coming from other as yet unknown sources.

Most work in this laboratory has concentrated on the ABA-1 molecule because of its relative abundance, restricted recognition of it by experimental animals (Chapter 4) and humans (Chapter 5) and its allergenic properties (Chapters 4 and 5). Isolation of this molecular species and its purification (see 2.9) have allowed its use in various assays as a pure molecule (e.g. when investigating its allergenic properties) and in sequencing.

Basic biochemical knowledge of these products is invaluable in determining their biological function and in understanding the nature of the host's response to them and whether this can be exploited in any way.

3.2 RESULTS

3.2.1 CHARACTERISATION OF ABF PROTEINS

The profiles of the protein content of ABF from *A. suum* and *A. lumbricoides* were compared by Coomassie staining the two sources of ABF after separation on an SDS-PAGE gel (Figure 3.1). It is common practice to dialyse ABF after collection and before storage but this carried the risk of losing small polypeptides. No real differences between the profiles of dialysed and non-dialysed body fluids were, however, found at least within the operating range of the SDS-PAGE used here. The quantity of ABF loaded onto the gel in Figure 3.1 allows comparison of minor components e.g. in the 20-30kDa and higher Mr ranges. The profiles of ABF from the two sources appear to be very similar. Figure 3.2 is a Coomassie stain of *A. suum* and *A. lumbricoides* ABF run reduced and non-reduced. Minor differences can be seen here between the two sources of ABF. Non-reduced *A. suum* ABF has several high molecular weight proteins which are seen only very faintly in the *A. lumbricoides* preparation and these disappear under reducing conditions. A band of approximately 18kDa is seen in both preparations - this resolves into 4 bands on reduction for *A. suum* and only 2 bands for *A. lumbricoides*. An extra band is seen in reduced *A. suum* ABF at approximately 120kDa - this may well be a reduction product of the high molecular weight species seen in non-reduced preparations.

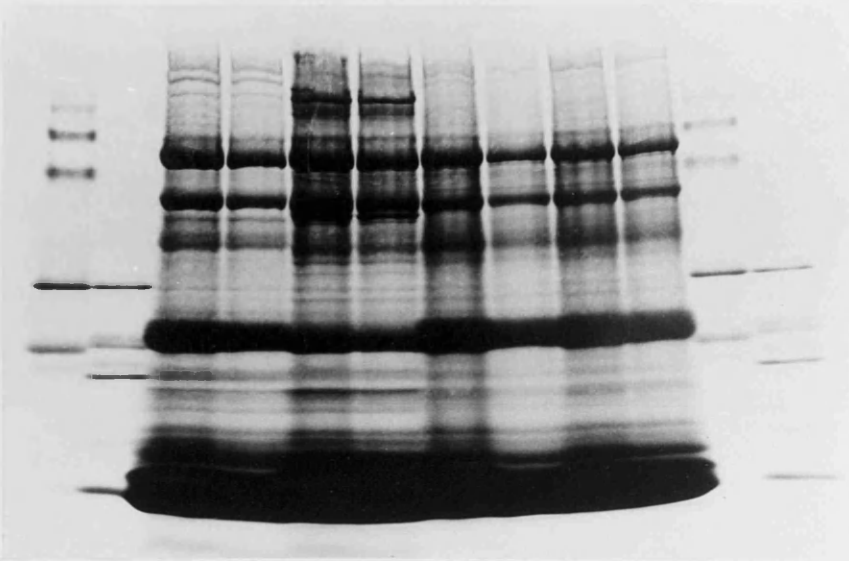
3.2.2 COMMON MOLECULES IN ANTIGEN PREPARATIONS

It is already apparent that ABF, L3/4 ES and L2 ES carry some molecular species in common, but it is important to establish the extent of this e.g. if a molecule is common to larval and adult stages, it would not be useful for diagnosis of the presence of adult worms in an individual. (Comparison of L2 and L3/4 stage-specific or common molecules

Figure 3.1 Comparison of *Ascaris suum* and *Ascaris lumbricoides* Adult Body Fluid. Coomassie stained 5-25% SDS-PAGE gel run non-reduced. Tracks loaded as detailed in figure. a) High Molecular Weight markers - in ascending order 43, 67, 140, 232, 440 and 669 Mr. Tracks b) Low molecular weight markers 14, 20, 30, 43 and 67 Mr.

D - ABF dialysed before use, ND ABF not dialysed before use.

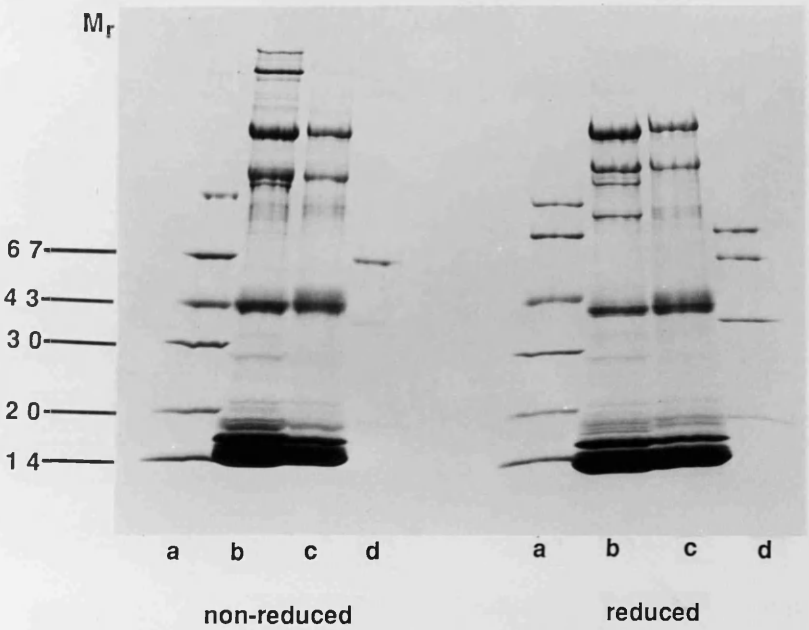
Comparison of *A. suum* ABF and *A. lumbricoides* ABF- dialysed and non-dialysed



a - High MW markers
b - Low MW markers
c - *A. suum* ABF (ND)
d - *A. suum* ABF (D)
e - *A. lumbricoides* ABF (ND)
f - *A. lumbricoides* ABF (D)

Figure 3.2 Comparison of *A. suum* and *A. lumbricoides* ABF run on a Coomassie stained 5-25% SDS-PAGE gel. Tracks on left hand side were run under non-reducing conditions, tracks on right hand side were run under reducing conditions.

Comparison of *A. suum* and *A. lumbricoides* ABF - reduced and non-reduced



a - Low MW Markers
b - *A. suum* ABF
c - *A. lumbricoides* ABF
d - High MW Markers

will be considered in Chapter 6). The question can be partially answered for ABF and L3/4 ES by immunoprecipitation of each preparation with antisera to the other (Figures 3.3 and 3.4). One common molecule is certainly the ABA-1. This molecule is abundant in ABF and found in both L2 and L3/4 ES preparations. Other molecules appear not to be common to both ABF and L3/4 ES.

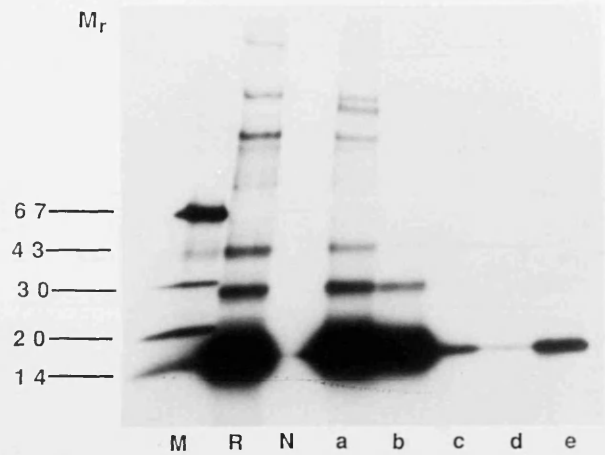
3.2.3 2-DIMENSIONAL GELS

Non-equilibrium pH gels (NEPHGE) were run as described in 2.13. This procedure allows for separation of molecules over a wide range of isoelectric points than can immuno electro focussing (IEF) 2-dimensional gels. Radiolabelled ABF and L3/4 ES were analysed by this method and autoradiographed as shown in Figure 3.5a and b. Figure 3.5a shows that ABF separates out in 2 dimensions into several species. The ABA-1 appears to have at least 2 species of different pI. A "ladder" of monomers, dimers and trimers of the ABA-1 is seen at the acidic (right hand) side of the gel. The 17kDa molecule can be seen at the basic (left hand) side of the gel.

Figure 3.5b shows the separation of L3/4 ES according to pI and Mr of its component molecules. Again, 2 species in the 14kDa region are discernible and other higher Mr components also separate out along the pH gradient. As both gels in Figure 3 were run at the same time, under identical conditions, it is possible that the ABA-1 excreted/secreted by the parasite is one of two or more isomers of the molecule whereas the whole range is found in the ABF.

Figure 3.3 Autoradiograph of immunoprecipitated ^{125}I labelled *A. suum* ABF analysed under non-reducing conditions on a 5-25% SDS-PAGE gel. The ABF was precipitated with various anti *Ascaris* sera as detailed in the figure.

Various Rabbit Sera versus ^{125}I ABF

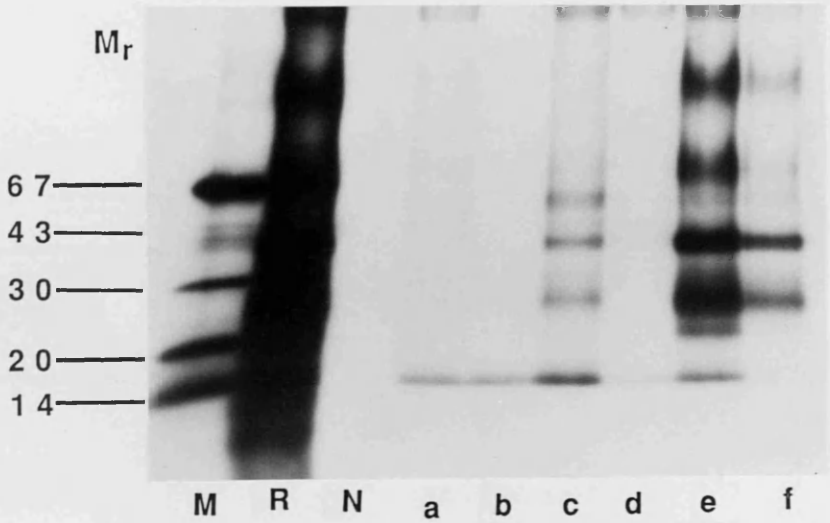


M - Markers
R - Reference ^{125}I ABF
N - Normal Rabbit Serum

a - Rabbit anti ABF serum
b - Rabbit anti ABA-1 serum
c - Rabbit anti *A. suum* serum
d - Rabbit anti *A. lumbricoides* serum
e - Rabbit anti L3/4 ES serum

Figure 3.4 Autoradiograph of immunoprecipitated ^{125}I labelled *A. suum* L3/4 ES analysed under non-reducing conditions on a 5-25% SDS-PAGE gel. The L3/4 ES was precipitated with various anti *Ascaris* sera as detailed in the figure.

Various Rabbit Sera versus ^{125}I L3/4 ES



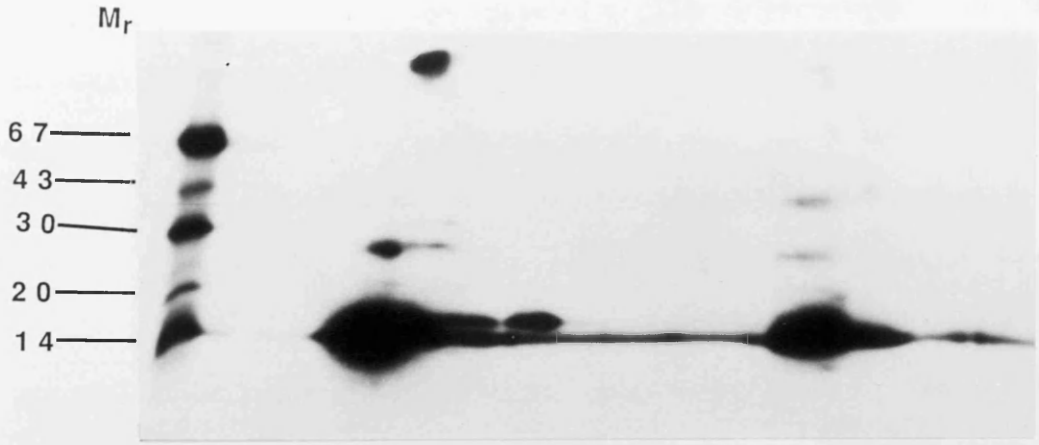
M - Markers
R - Reference
N - Normal Rabbit Serum

a - Rabbit anti ABF serum
b - Rabbit anti ABA-1 serum
c - Rabbit anti *A. suum* serum
d - Rabbit anti *A. lumbricoides* serum
e - Rabbit anti L3/4 ES serum
f - Rabbit anti *T. canis* serum

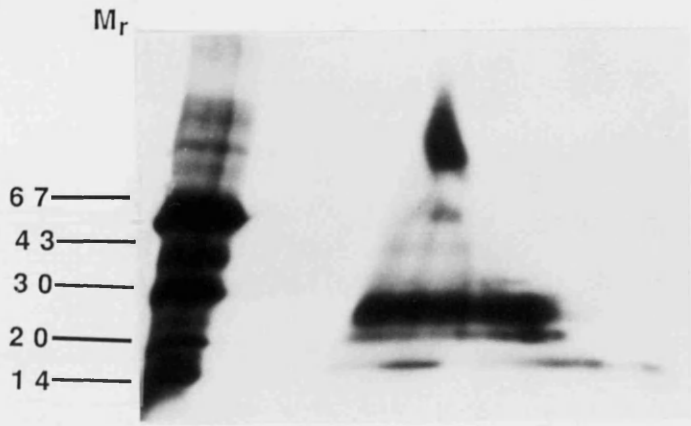
Figure 3.5a Autoradiograph of ^{125}I *A. suum* ABF run on non-equilibrium pH tube gel, subsequently analysed under reducing conditions on a 5-25% SDS-PAGE gel.

Figure 3.5b Autoradiograph of ^{125}I *A. suum* L3/4 ES run on non-equilibrium pH tube gel, subsequently analysed under reducing conditions on a 5-25% SDS-PAGE gel.

2-D NEPHGE gel of ^{125}I ABF



2-D NEPHGE gel of ^{125}I L3/4 ES



3.2.4 LECTIN BINDING

Radiolabelled L2 ES, L3/4 ES and ABF were incubated with Sepharose-bound lectins as described in 2.20. The sugar-binding specificities of the lectins used are listed in Table 2.4. Lectin binding of radiolabelled L3/4 ES is shown in Figure 3.6. L2 ES results are not shown as no apparent binding to any of the lectins was detected. The supernatant from the washes was collected and a sample run in the tracks marked W. This represents both the excess antigen loaded in the experiment and molecules which did not bind to the lectins. The specifically eluted molecules from the lectins can be seen in the tracks labelled E. L3/4 antigens which bound to lectins but were not eluted (possibly due to high affinity binding or suboptimal elution conditions) are visualised in the tracks labelled P. Some mannose or glucose residues are present on molecules in the 20-30kDa range as shown by elution from Concanavalin A and the 25kDa molecule is also possibly characterised by N-acetyl galactosamine as shown by elution from *Helix pomatia* lectin. N-acetyl galactosamine is also present on a 40kDa molecule. These results are confirmed by the elution of the 25kDa and 40kDa molecules from *Arachis hypogea* lectin which also specifically binds N-acetyl galactosamine.

Lectin binding specificities for radiolabelled ABF are shown in Figure 3.7. The apparent ABA-1 binding in this figure is due to over-exposure of the ABA-1 from the alternate tracks of washed species. A shorter exposure of the autoradiograph confirmed this but other species were then too faint to identify. Concanavalin A binding shows that there are either mannose or glucose residues on several components; at Mr of 22kDa, 40kDa and several higher masses. N-acetyl glucosamine residues are present on several components as shown by the molecules eluted, shown in track h.

Figure 3.6 Autoradiographs showing lectin binding specificities of ^{125}I L3/4 ES. Lectins are Con A - Concanavalin A; Lentil; HP - *Helix Pomatia*; TP - *Tetragonolobus purpureas*; WGA - Wheat Germ Agglutinin; AH - *Arachis hypogea*.

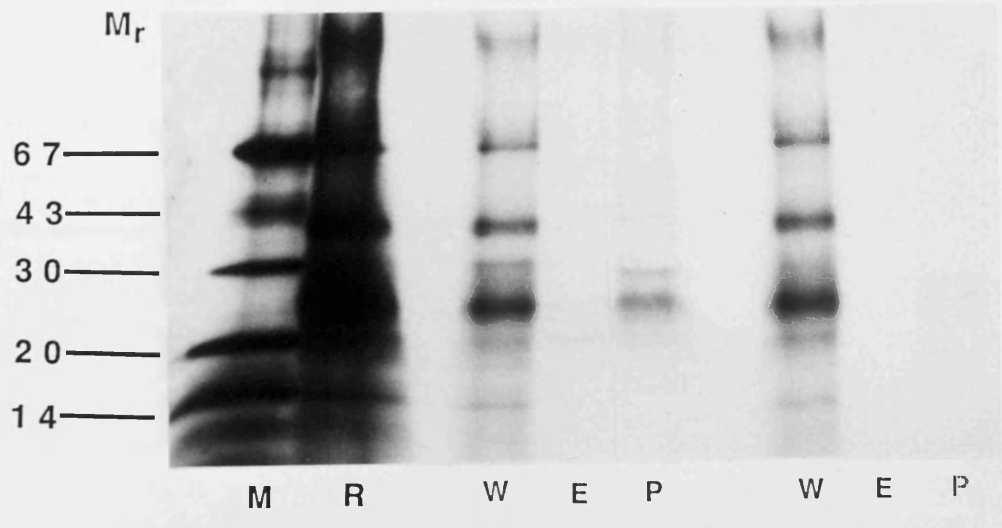
W tracks are the washes from the beads.

E tracks are what has been eluted by the specific sugar.

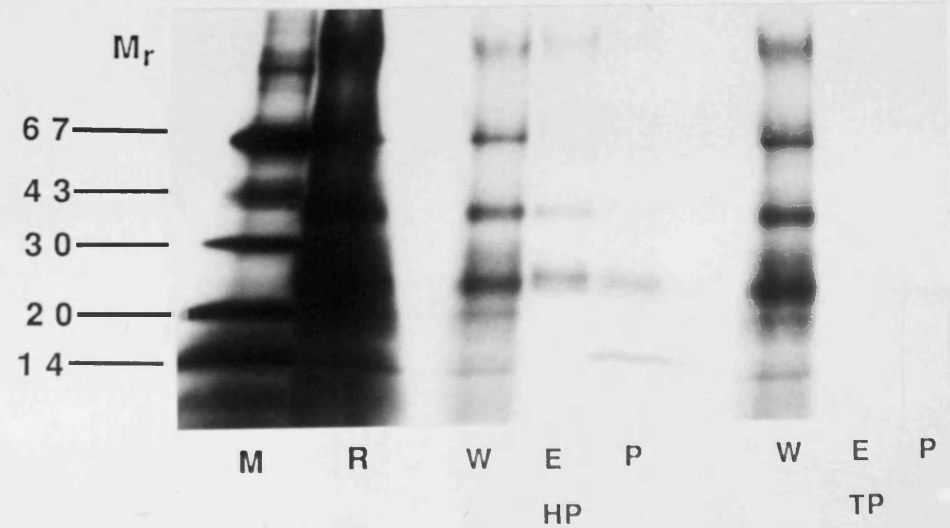
P tracks represent what was left on the beads after washing and elution.

Samples run under non-reducing conditions on a 5-25% SDS-PAGE gel.

Lectin binding specificities of ^{125}I L3/4 ES



M - markers
R - reference ^{125}I L3/4 ES



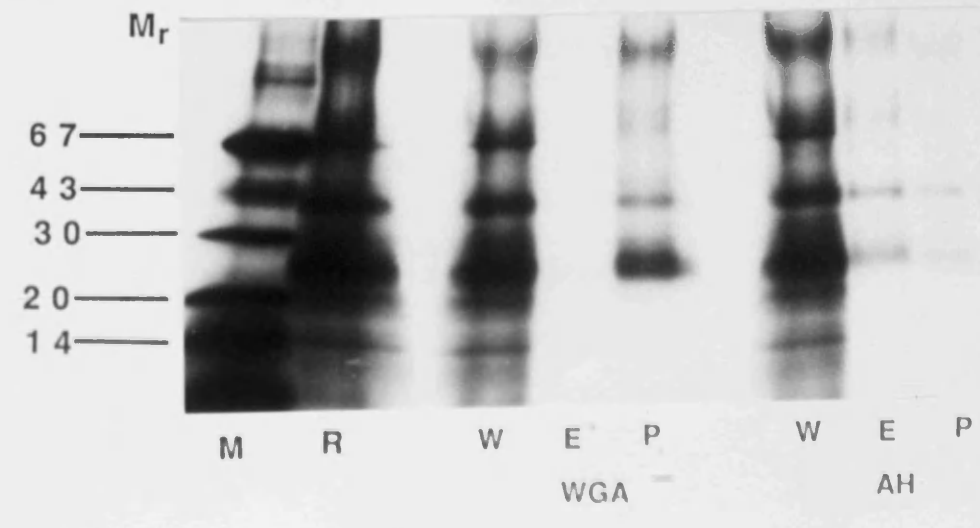
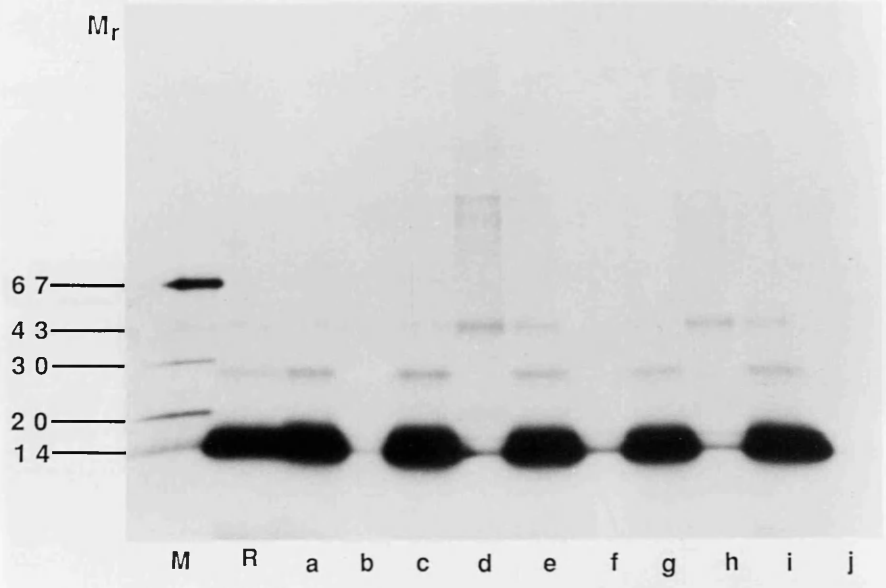


Figure 3.7 Autoradiograph showing lectin binding specifities of ^{125}I ABF. Lectins are Lentil; Con A - Concanavalin A; HP - *Helix pomatia*; WGA - Wheat Germ Agglutinin; TP - *Tetragonolobus purpureas*.

Tracks are as detailed in the figure. Samples run under non-reducing conditions on a 5-25% SDS-PAGE gel.

Lectin Binding Specificities of ^{125}I ABF



M - Markers
R - Reference ^{125}I ABF

a - Lentil wash
b - " elute
c - Con A wash
d - " elute
e - HP wash
f - " elute
g - WGA wash
h - " elute
i - TP wash
j - " elute

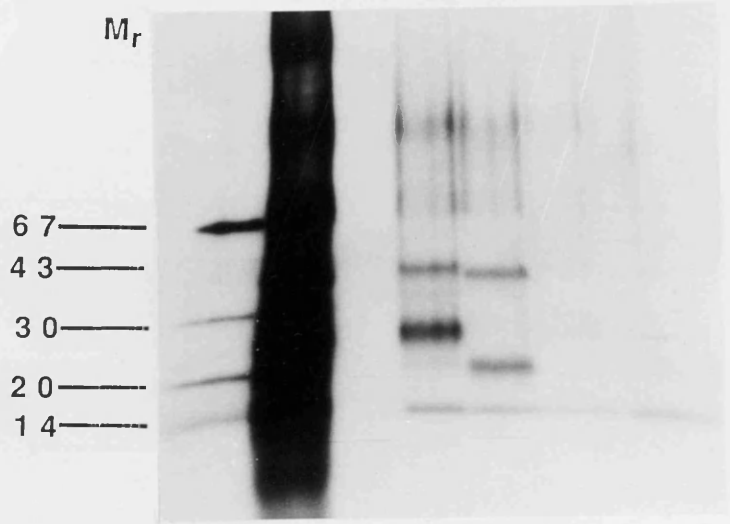
3.2.5 GLYCOPEPTIDASE DIGESTION

The enzyme glycopeptidase F (PNGaseF) cleaves N-glycanes between asparagine residues and the carbohydrate chain (Tarentino, Gomez and Plummer, 1985). The extent and rate of de-glycosylation of glycoproteins depends on the nature of the molecule under treatment. The presence of SDS, heating and the addition of detergents are each advantageous to deglycosylation. The optimal quantity of the enzyme was determined in a titration immunoprecipitation where increasing volumes of the enzyme were employed to digest the same quantity of ES. Each trial was then immunoprecipitated with rabbit anti-L3/4 ES sera and the smallest volume which effected digestion chosen from the autoradiograph of the gel the products were analysed on (not shown). Figure 3.8 is an autoradiograph of radiolabelled, PNGaseF digested L3/4 ES immunoprecipitated with rabbit anti-L3/4 ES serum and rabbit anti-*Ascaris* infection serum. While the anti-infection tracks are too faint to show any results the anti-L3/4 ES serum tracks show either a change in antigen recognition due to removal or alteration of one of the epitopes on the 25kDa molecule and the exposure of a new antigenic epitope on a 20kDa molecule. A more likely explanation is that digestion of the 25kDa molecule has resulted in a lowering of molecular mass. The modification of the 25kDa molecule is the only one observed with this enzyme using radiolabelled antigen but other ES components not recognised by this serum may be recognised by other sera, or other glycosidases may reveal other glycosylated molecules.

Periodate treatment of parasite products can also be used to examine carbohydrate structures. The effect of such treatment on recognition by antibody from infected humans in ELISAs is examined and discussed in Chapter 5.

Figure 3.8 Autoradiograph of ^{125} I L3/4 ES, digested with Endoglycopeptidase F (PNGase F) and immunoprecipitated with rabbit anti-L3/4 ES serum (tracks a), and rabbit anti-*A. suum* serum (tracks b). Precipitates run under non-reducing conditions on a 5-25% SDS-PAGE gel.

PNGase F Digest of L3/4 ES
Immunoprecipitated with Rabbit Sera



M - Markers
R - Reference ^{125}I L3/4 ES
N - Normal Rabbit Serum

a - Rabbit anti L3/4 ES serum
b - Rabbit anti *A. suum* serum

– no PNGase F
+ with PNGase F

3.2.6 ELECTON MICROSCOPY STUDIES

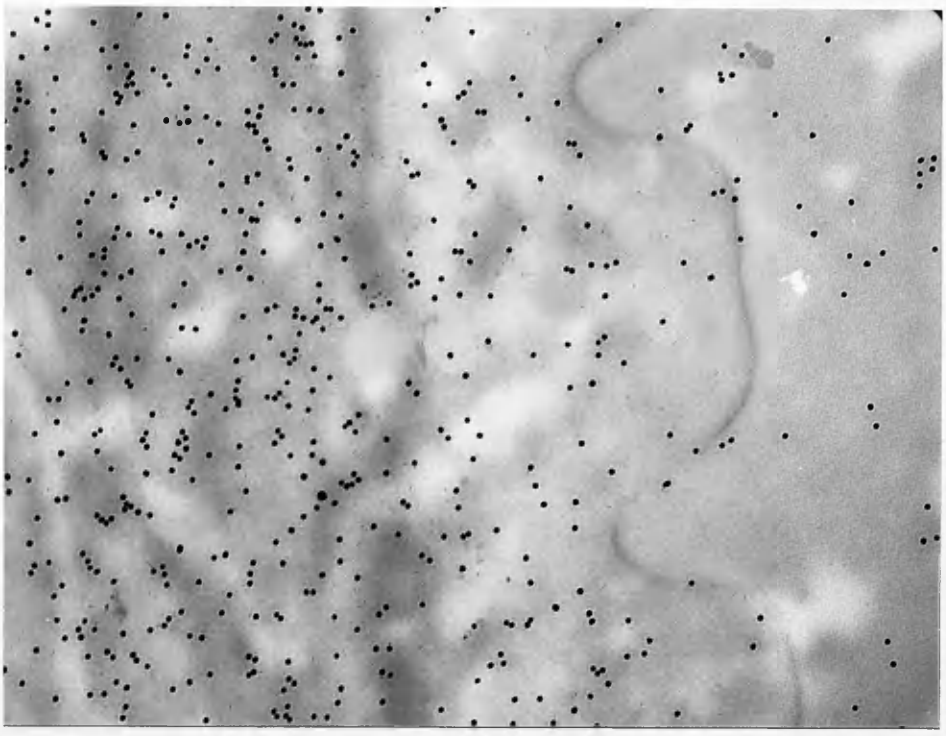
L3/4 *A. suum* were treated as descibed in 2.21. Figure 3.9a is an electron micrograph of a section of an L3/4 larva probed with anti-ABA-1 serum. The homogeneous distribution of the gold particles shows that the ABA-1 molecule is distributed fairly evenly throughout the larval tissue. The control (using normal rabbit serum, not shown) shows very sparse, random distribution of the gold particles. In contrast the anti-L3/4 ES serum almost exclusively recognises surface components as shown in Figure 3.9b. In the control, recognition of the surface is sparse and few gold particles are seen. This result corresponds well with those achieved with the same sera on the surface of living larvae as detected in an indirect immunofluorescence test (Chapter 6).

3.2.7 *IN VITRO* LABELLING OF L3/4 LARVAE WITH ^{35}S METHIONINE

Biosynthetic labelling was carried out *in vitro* as described in 2.7 and the resulting preparation immunoprecipitated with a panel of rabbit sera as shown in Figure 3.10. The labelled bands are the ES products which label as they are synthesised by the parasites maintained in culture. Comparing Figure 3.10 with Figure 3.4 it is clear that many molecules which are recognised by antibody from infected rabbits (track c in Figure 3.4 and track b in Figure 3.10) label with both ^{125}I and ^{35}S and thus must contain both tyrosine and methionine residues. Some ES products are only seen in the methionine labelling e.g. the 22kDa and 70kDa molecules.

Figure 3.9a Electronmicrograph of a section of an L3/4 larvae, probed with anti-ABA-1 rabbit serum, binding visualised with protein A conjugated to 20nm colloidal gold particles.

Figure 3.9b Electronmicrograph of a section of an L3/4 larvae, probed with anti-L3/4 ES rabbit serum, binding visualised with protein A conjugated to 20nm colloidal gold particles.



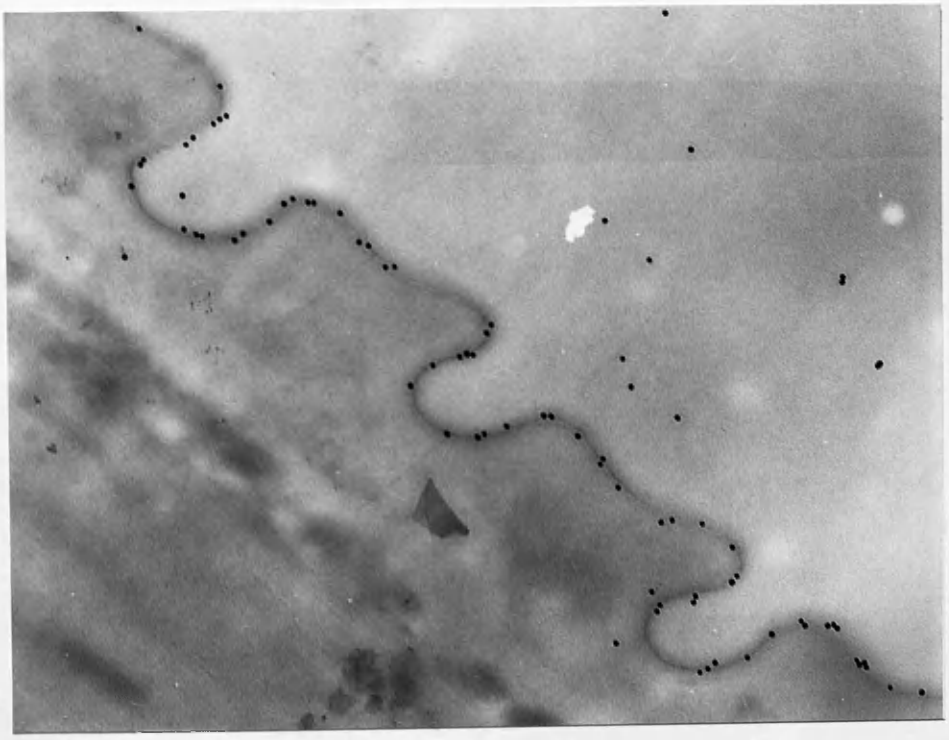
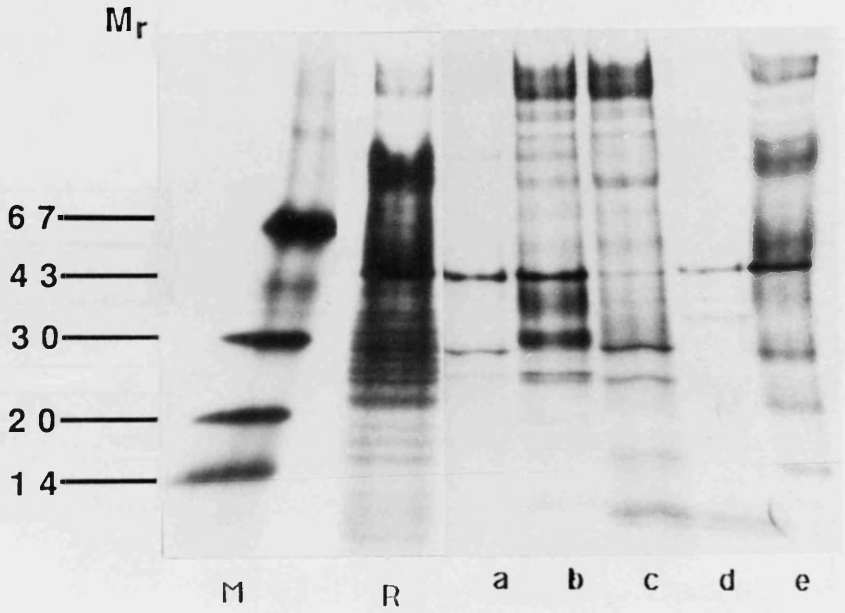


Figure 3.10 Autoradiograph of ^{35}S labelled L3/4 ES immunoprecipitated with various anti-*Ascaris* sera as detailed in the figure. Precipitates were analysed under non-reducing conditions on a 5-25% SDS-PAGE gel.

Rabbit sera versus ^{35}S L3/4 ES



M Markers
R Reference ^{35}S L3/4 ES

a Normal Rabbit Serum
b anti A. suum
c anti ABF
d anti ABA-1
e anti L3/4 ES

3.3 DISCUSSION

ES products are thought to be released from nematodes from several sources e.g. from stichocyte cells in *T. spiralis* (Despommier and Muller, 1976), from secretory glands at the anal end and excretory pores of *T. canis* (Hogarth-Scott, 1966), shed from the surface (Philipp, Parkhouse and Ogilvie, 1980; Ortega-Pierres, Chayen, Clark *et al.*, 1984). The electron microscopy studies suggest that at least some of the the L3/4 ES is derived from the surface of the L3/4 larvae although it may be that excreted antigens have not been fully processed i.e. glycosylated and hence are not recognised by specific antibody in the soma of the larvae. The ABA-1 is distributed throughout the larval tissue and is the most abundant protein of the worm. It consists of less than 1% carbohydrate (see Section 1.5 and J. Christie, personal communication) and thus little if any glycosylation of the protein would take place after transcription.

The results show that ES moleculesare glycoproteins with both protein and carbohydrate antigenic determinants. The L3/4 ES labels readily with both ^{125}I and ^{35}S, with the latter label showing many more components when analysed by SDS-PAGE. The biosynthetic labelling also shows that many components are newly synthesised in culture and rapidly released. With *Heigmosomoides polygyrus* exsheathed larvae, only one antigen is released in the first 10 hours of culture and other antigens released subsequently (Ey, 1988b).

The apparent stage specificity of ES molecules is related to developmental changes and this has been observed with other helminths e.g. a major 23kDa protein of *Onchocerca* larvae is synthesised almost exclusively by infective third stage larvae (Bianco, Robertson, Kuo *et al.*, 1990) and

biosynthetic labelling of *T. spiralis* secreted proteins demonstrated stage-specificity in ES products (Parkhouse and Clark, 1983). Proteinases are present in ES from both larval stages of *Ascaris* (Knox and Kennedy, 1988). The sets of released proteinases were found to be specific to each stage but some activities were indistinguishable between the two. The proteinases are antigenic as demonstrated by inhibition of activity by antibody from infected animals. These characteristics suggest that a possible function could be tissue digestion as the larvae migrate through the host. This migration might be stopped or impaired when proteinases are inactivated by antibody binding, as it appears they can be (Knox and Kennedy, 1988). The proteinases may also inactivate non-specific host defences (see 1.6.2)

Collection of adult ES would be difficult technically but it would be worthwhile to compare with ES from larval stages and with ABF too. Investigation of the effect of adult ES on the host, particularly in the intestine may yield information as to why such a large, foreign object is tolerated in the gut.

Regarding the presence of carbohydrate groups, as shown by the lectin binding and enzyme de-glycosylation work, their presence may account for some of the recognition of ES products by infected rabbits who apparently reconise all components of ES. This may be explained by the fact that the rabbit immune system recognises many cross-reactive carbohydrate epitopes presented on otherwise unrelated polypeptide backbones which are found on larval ES products e.g. *T. canis* (Maizels, Kennedy, Meghji *et al.*, 1987). Thus recognition of a molecule by antibody does not necessarily indicate a specific immune response to that parasite product.

Examination of lectin binding could be extended to examine the larval surface and hence if any carbohydrate epitopes are exposed there. This was performed for *Brugia malayi* surface proteins and while a 29kDa molecule stripped from the surface was bound by Concanavalin A, no binding was seen on the intact larvae (Maizels *et al.*, 1989) hence that particular epitope is cryptic.

The relatively crude comparison of ABF products from Scottish pigs and Nigerian humans does reveal minor differences but it is not possible to say how reflective of phylogenetical differences these are. Suffice to say, these results (and others) are taken to be sufficient justification for the use of *A. suum* ABF and other parasite products in place of the corresponding *A. lumbricoides* ones when access to these is limited.

CHAPTER 4

ASCARIS IN LABORATORY ANIMALS

4.1 INTRODUCTION

4.1.1 GENETIC CONTROL OF IMMUNE RESPONSE IN INFECTION AND ARTIFICIAL IMMUNISATION

Genetic variation in the host immune response to infection with respect to resistance to infection with parasitic helminths is a well documented phenomenon (Ackert, 1942; Wakelin, 1978). The most accessible of the large number of host parameters one could measure to monitor the immune response is the antibody response to infection. Genetic control of the specificity of antibody responses to infection is poorly understood and for investigations of this type the mouse is the species of choice because of the extensive knowledge of its H-2 and the availibility of a large number of inbred, congenic and recombinant inbred strains. Despite the complexity of parasites, and the antigens they present to their hosts, combined with the complexity of the immune responses they elicit, it is surprising that inheritance of variations in the immune response are often simple, suggesting that relatively few genes are involved. Much of the work has focussed on the major histocompatibility complex and its control of responses to infection - as much because the MHC is so well characterised as because of its importance. For example, a gene mapping between the S and D loci of the H-2 influences susceptibility to infection with *Trichinella spiralis* and has been designated Ts-2 (Wassom *et al.*, 1983). A second locus, Ts-1, maps to the A_B locus (Wassom, Brooks, Babbish *et al.*, 1983). When the d allele is expressed at the Ts-2 locus, strains of mice expressing s,q,f or b alleles at Ts-1 are rendered more susceptible to infection.

The haplotypes b,s,q and q are most resistant to *T. spiralis* and are also I-E deficient (Wakelin, 1985). Conversely, the k,r and p haplotypes which

have functional I-E molecules are most susceptible to *T. spiralis*. It has therfore, been proposed that presentation of worm antigen in the context of I-A rather than I-E products may elicit an effective immune response, whereas, in the context of I-E, presented antigen may preferentially induce suppressor T cells.

Resistance to infection can be mediated by non-MHC genes as demonstrated by Skamene *et al.* (Skamene, Gros, Forget, *et al.*, 1984) who showed that the resistance/susceptibility trait to *Mycobacterium lepraemurium* is controlled by the Bcg/Ity/Lsh locus on chromosome 1. Moreover, Bell *et al.* (Bell, Adams and Ogden, 1984) demonstrated that the rapid expulsion mechanism of *T. spiralis* in mice is controlled by a single gene, Ihe-1.

There are many examples where H-2 and non H-2 genes work in concert to control responses to infection. For instance, the antibody responses of various H-2 recombinant mice on B10 and BALB backgrounds to 5 epitopes of *M. tuberculosis* has been shown to be dependent on both H-2 and background genes (Ivanyi and Sharp, 1986). These workers also showed that the mechanism of control was at the level of I-A restricted antigen presentation to T_H cells. Thus, the most common means of genetic control seems to be a combination of H-2 and non-H-2 genes (see Table 1.1; Gibbens, Harrison and Parkhouse, 1986).

What significance, then, does this polygenic control of antigen recognition have on resistance to infection, the issue of most importance? The most commonly quoted direct correlation between antigen recognition and resistance was made using *Schistosoma japonicum* in 129/J mice (Mitchell, Beall, Cruise *et al.*, 1985). These mice showed high levels of resistance to first or repeated infections with *S. japonicum*. Even after 6

infections 50% of the mice remained healthy with no or very few worms. A 26kDa antigen of the parasite (Sj26) was not immunoprecilitated by sera from resistant mice so the obvious question was whether or not immune responses to Sj26 were directly involved in the expression of resistance to *S. japonicum*. These results may, in part, be due to some mice having a "leaky" portal system whereby schistosomes have increased access to, or residency times in, the lungs. This would increase opportunities for expression of anti-schistosome immune responses (Mitchell, 1989).

In the literature, in connection with *A. suum* the most commonly reported antibody isoptype has been IgE (see following section). While its importance is without doubt, the role and function of the IgG response in ascariasis has been largely ignored.

Previous work (Kennedy *et al.*, 1986; Kennedy, Tomlinson, Fraser *et al.*, 1990) has shown that only the $H\text{-}2^{5}$ haplotype recognises ABA-1. The results in this Chapter describe a further analysis to determine which locus/loci of the H-2 control this recognition. The immunopreciptations described have all been carried out with Protein A, a reagent which previous work in this laboratory has shown that even with addition of a broad spectrum anti immunoglobulin reagent, the apparent recognition profiles are not affected (L.Tomlinson, personal communication).

4.1.2 ROLE OF IgE IN THE IMMUNE RESPONSE TO HELMINTHS.

The production of IgE antibodies ("reagin") is a hallmark of helminth infection. Both parasite-specific and non-specific IgE is produced as a result of helminth infection. Other features of such infection are the occurrence of immediate hypersensitivity reactions and a proliferation of

hypersensitivity associated cells i.e. mast cells and eosinophils (Jarrett and Miller, 1982). A unique characteristic of IgE from all mammalian species so far studied is the capacity for prolonged tissue fixation mediated by the Fc portion of the IgE molecule.

The most common laboratory test for the presence of antibody specific IgE is the passive cutaneous anaphylaxis test (PCA) which was first developed by Ovary (1952). It can be used both to look for antigen-specific IgE and to investigate the allergenicity of a substance under investigation (Hogarth-Scott, 1967; Murrell, Vannier and Ahmed, 1974).

One question which has been asked and investigated for many years is what makes any given molecule an allergen. Studies of structure and biochemistry of many defined allergens have not led to any consensus (Aas, 1978; Lind and Lowenstein, 1988). Helminths produce allergens which are among the most active known in the stimulation of IgE. Allergens have been detected in homogenates of *N. brasiliensis*, *Toxocara* and *Toxascaris* (Hogarth-Scott, 1967). A schistosome glycoprotein (which functions as a proteolytic enzyme) is a potent allergen (Senft, Weltman, Goldgraber *et al.*, 1979). *Ascaris* has long been recognised as a source of allergens (Ogilvie and De Savigny, 1982) with allergens described from 14kDa (Ambler *et al.*, 1973a; Christie *et al.*, 1990) to 360kDa (O'Donnell and Mitchell, 1978). It is also well established that both the mode of presentation (discussed below) and the previously discussed genetic make-up of individuals influence which parasite products induce formation of antigen specific IgE antibody (Levine and Vaz, 1970). They also recorded that only minute doses of allergen are required to induce IgE production.

Infection with helminths often induces a massive increase in IgE levels with only a proportion of the IgE being parasite specific (Jarrett, Haig and Bazin, 1976). One frequently observed effect of such elevation of IgE levels by parasite allergens is the potentiation of IgE responses to unrelated antigens. For example *Ascaris* culture fluid (ACF, equivalent to L3/4 ES) was able to potentiate an IgE response to ovalbumin in guinea pigs (Stromberg, 1980). Conversely, a factor from T cells from *N. brasiliensis* infected mice had the ability to selectively suppress the IgE response to dinitrophenyl-ovalbumin primed cells to homologous antigen at certain time points (Hirashima, Yodoi and Ishizaka, 1980).

An understanding of the kinetics of IgE production is important and a classic study by Jarrett *et al.* (1976) elucidated several important features. During primary infection with *N. brasiliensis* the potentiated IgE response to egg albumin (EA) and elevation of total IgE levels occurred synchronously, rising to a peak on days 12-14 (p.i.). In contrast IgE specific for parasite antigens peaked 2-3 weeks p.i. when both total and potentiated response had largely declined. Following a second infection, total IgE levels rose rapidly, peaked at 6 days p.i. and levels of secondary specific IgE also rose. The total IgE rose by a much greater factor than parasite specific IgE and then declined much more rapidly. The EA response is not repotentiated. These rapid changes with time have also been recorded by others. Stromberg (1980) showed that the secondary IgE response to *Ascaris* ACF (L3/4 ES) had declined by 8 days p.i.. Watanabe and Kobayashi (1988), looking at the sensitivity of PCAs in the *Nippostrongylus* system showed that PCA reactions were suppressed in the early and late stages after infection. In an investigation of soluble (T cell?) factors which potentiate/mediate the IgE response, the formation of

the factor appeared to be transient i.e. IgE was present on day 8 but none could be detected by day 14 p.i..

The induction of IgE production is thought to be distinct from the induction of IgG/IgM production with different T helper cells and B memory cells involved in the processes (Ishizaka, 1976). Jarrett and Miller (1982) suggested a scheme whereby T cells bearing Fcε receptors and activated by allergens produce IgE binding factors which regulate the differentiation of IgE B cells and may also provide activating and proliferative signals to other cells including mast cells, macrophages and eosinophils which possess Fcε receptors. In this way the prominent proliferation and activation of these various cell types may be part of an intricately orchestrated response in which IgE may play the central role. The most recent work in this realm has focussed on the role of interleukins (Watanabe, Katakura, Kabayerashi *et al.*, 1988). The mechanisms of functional IgE deficiency in SJA/9 mice appear to relate to a defect of a subset of T cells which induce IL4. The production of IL4 in some way impairs the induction of Fcε receptor bearing lymphocytes.

4.1.3 PRESENTATION OF ANTIGENS

The importance of the mode of presentation of antigens to the host is assumed but not often considered in detail. The use of adjuvants to non-specifically enhance an immune response to antigen is very common. Antibody responses to antigen in adjuvant are greater, more prolonged and frequently consist of immunoglobulin isotypes in different proportions than produced by antigen or infection alone. Many studies of immunity to parasites and their antigens have relied on adjuvant immunisation protocols e.g. Bell and McGregor (1980) used *T. spiralis* extract in

Freunds Complete Adjuvant (FCA) for their studies on rapid expulsion; Grencis *et al.* (1986) stripped surface molecules from *T. spiralis* larvae and used this in FCA to immun ise mice; to generate antisera to cyst fluid of *Echinoccocus granulosus* Shepherd and McManus (1987) immunised their rabbits with cyst fluid in FCA.

The use of FCA (and other adjuvants) with antigens in inducing IgE responses has been well characterised. The use of adjuvant has been described as essential for inducing an IgE response in some systems - the argument being that adjuvant plays a role in activating macrophages (Jarrett, 1978).

4.2.1 ANALYSIS OF RECOGNITION OF ABA-1 BY H-2 RECOMBINANT MICE

Having already established that the H-2^{s} haplotype confers the ability to recognise the ABA-1 molecule, in order to further define where the focus of control is, the following analysis was carried out. Sera from 12 different H-2 recombinant mouse strains infected 3 times with *A. suum* were immunoprecipitated with radiolabelled ABF, purified ABA-1 or L3/4 ES. The percentage of radiolabelled ABA-1 precipitated by antibody in the individual sera is shown in Table 4.3. The strains with detectable IgG antibody levels to the protein were SJL, ATH, B10.HTT and B10.S9R. Autoradiographs of the SDS-PAGE analysed immunoprecipitates of the sera with L3/4 ES are shown in Figure 4.1. The strains which recognise the ABA-1 molecule are ATH, SJL and B10.HTT. If the autoradiograph is exposed for longer or if quaternary infection serum is used, the B10.S9R strain also recognises the ABA-1 molecule (not shown). Thus the four strains which recognise the ABA-1 in an IgG detection assay are SJL, ATH, B10.HTT and B10.S9R.

4.2.2 EFFECT OF MODE OF PRESENTATION ON RECOGNITION OF PARASITE ANTIGENS BY MICE

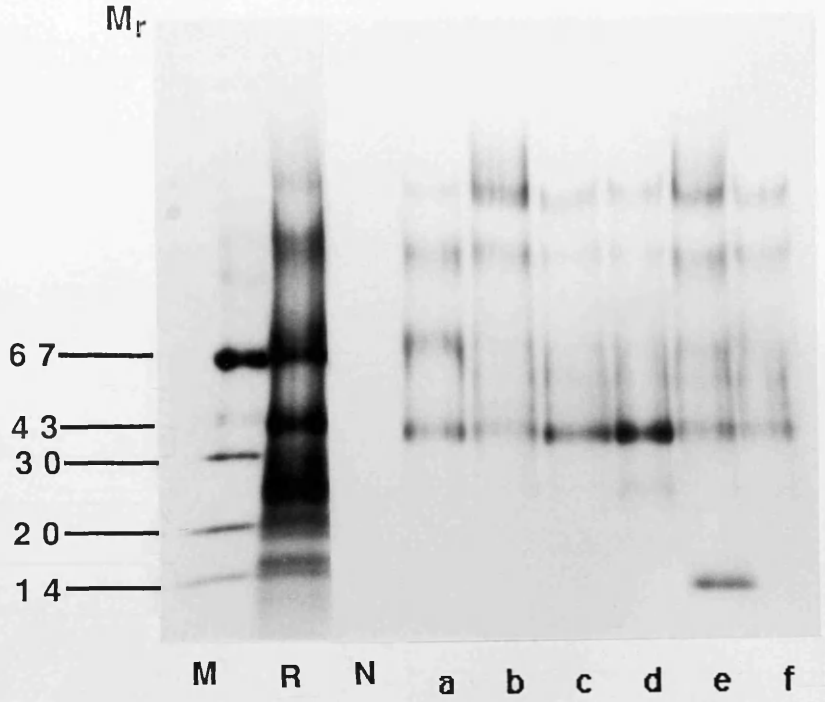
Comparisons of sera generated by immunising mice by infection with *Ascaris* or exposure to purified antigen in adjuvant were made. Figure 4.2 is an autoradiograph of radiolabelled L3/4 ES immunoprecipitated with C57BL/10 mouse sera and analysed by SDS-PAGE. Looking at responses to infection, IgG antibody (to some components) was only detectable after the second infection and the complete recognition repertoire is seen after three infections (previous work in this laboratory had shown that the complete repertoire of recognition was seen by the third infection and subsequent

Figure 4.1a Autoradiograph of ^{125}I L3/4 ES immunoprecipitated with tertiary infection sera from the strains of mice indicated in the figure.

Figure 4.1b Autoradiograph of ^{125}I L3/4 ES immunoprecipitated with tertiary infection sera from the starins of mice indicated in the figure.

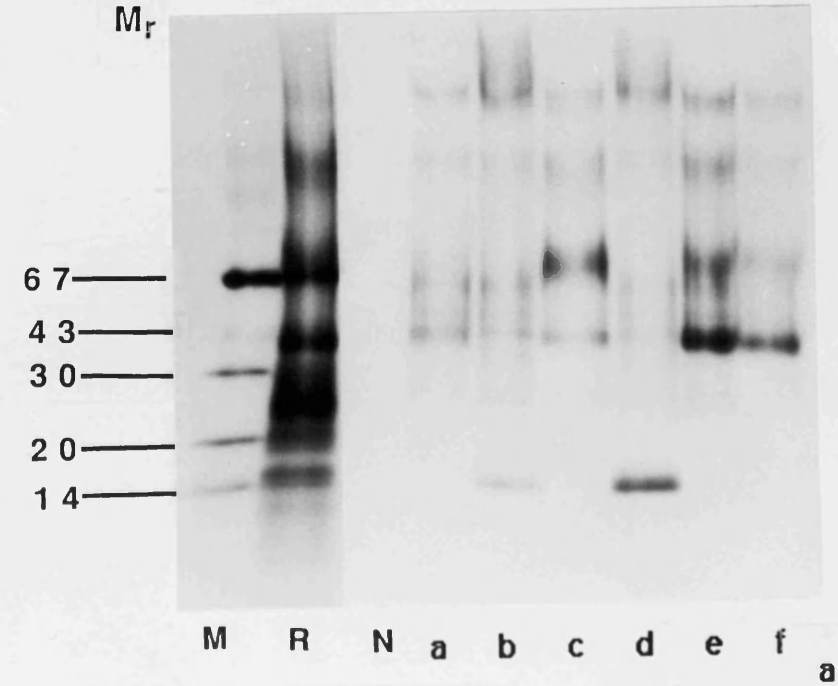
Immunoprecipitates in both figures were analysed under non-reducing conditions on 5-25% SDS-PAGE gels.

Congenic mice versus ^{125}I L3/4 ES



M - markers
R - reference ^{125}I L3/4 ES
N - normal mouse serum

a - B10.A5R
b - B10.S9R
c - B10.A4R
d - ATL
e - B10.HTT
f - C57.BL10

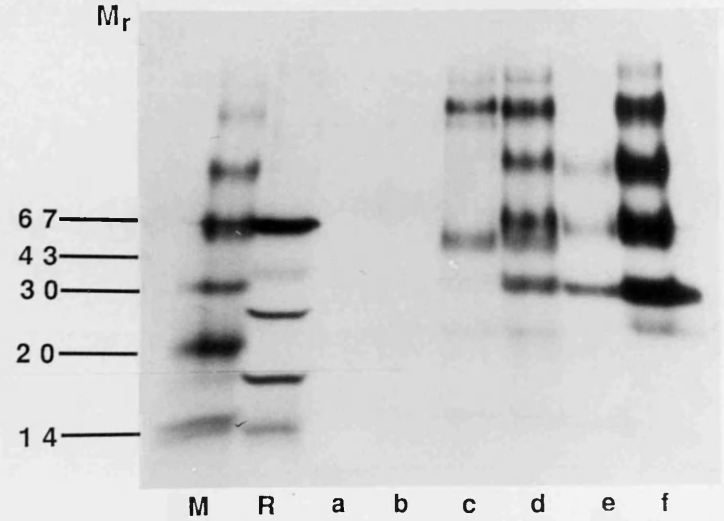


M - markers
R - reference ^{125}I L3/4 ES
N - normal mouse serum

a - B10.AQR
b - SJL
c - B10.D2
d - ATH
e - B10.BR
f - B10.A2R

Figure 4.2 Autoradiograph of ^{125} I L3/4 ES immunoprecipitated with sera from C57 BL10 mice. Mice were either infected with infective *Ascaris* eggs or with L3/4 ES in adjuvant. Mice were bled at the times indicated in the figure. Precipitates were analysed under non-reducing conditions on a 5-25% SDS-PAGE gel.

C57 mouse - infection and adjuvant serum versus ^{125}I L3/4 ES



M - Markers
R - Reference ^{125}I L3/4 ES

a - C57 Normal Mouse Serum
b - day 14 1^{o} Infection
c - day 14 2^{o} Infection
d - day 14 3^{o} Infection
e - day 14 1^{o} Adjuvant
f - day 14 3^{o} Adjuvant

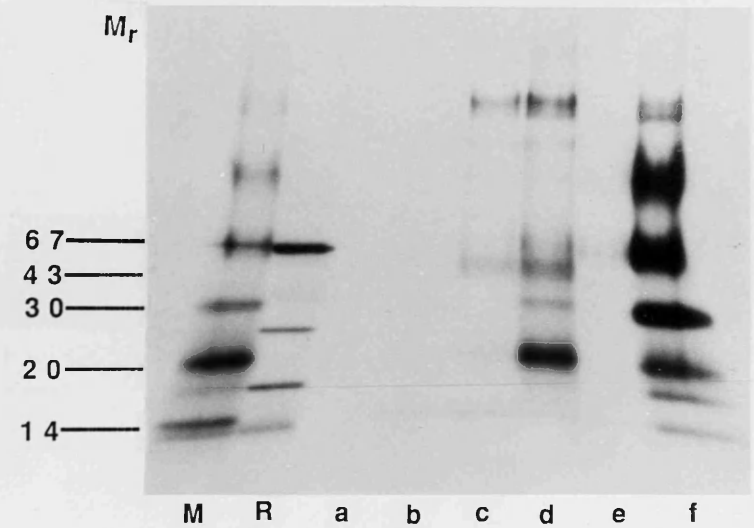
infections did not alter this). In contrast, infection of L3/4 ES in adjuvant generates a much more rapid antibody response with several components being recognised after one immunisation. The final recognition profile of serum generated by exposure to antigen in adjuvant, was however, identical to the infection-generated profile with only minor quantitative differences i.e. the 28kDa molecule is recognised more strongly after the third exposure to antigen in adjuvant than after the third exposure to infection.

The result of the same experiment performed in BALB/c mice is shown in Figure 4.3. Again it can be seen that infection serum recognises a few components faintly after the second infection and the recognition repertoire is stronger after the third infection with infective larvae. In contrast to the C57BL/10 sera, only one component (67kDa) is recognised very faintly after one immunisation with L3/4 ES in adjuvant. After three immunisations the antibody level is much greater and three molecules are recognised which are not in the context of infection, ABA-1, 17kDa and 90-100kDa.

Figure 4.4 is the equivalent autoradiograph for the same experiment in B10.S mice. Again, in the context of infection, antigen specific IgG antibody begins to be detectable after the second infection. After the third the antibody levels are higher and the detectable repertoire greater. Note the stong recognition of ABA-1 (track d). One immunisation of L3/4 ES in adjuvant is sufficient to generate detectable antibody to several components. After three immunisations in adjuvant the antibody repertoire is similar with only one new component (90-100kDa) seen. What is most striking is the absence of any recognition of the ABA-1.

Figure 4.3 Autoradiograph of ^{125}I L3/4 ES immunoprecipitated with sera from BALB/c mice. Mice were either infected with infective *Ascaris* eggs or with L3/4 ES in adjuvant. Mice were bled at the times indicated in the figure. Precipitates were analysed under non-reducing conditions on a 5-25% SDS-PAGE gel.

Balb/c mouse - infection and adjuvant serum versus ^{125}I L3/4 ES



M - Markers

R - Reference ^{125}I L3/4 ES

a - Balb/c Normal Mouse Serum

b - day 14 1^{o} Infection

c - day 14 2^{o} Infection

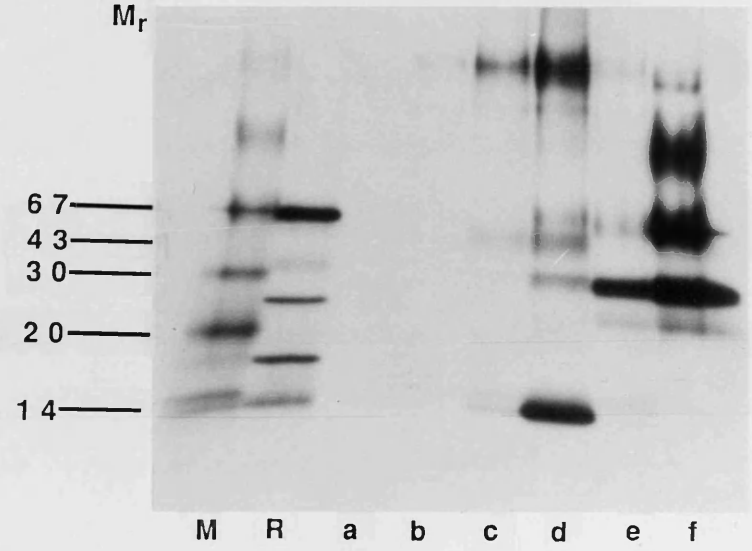
d - day 14 3^{o} Infection

e - day 14 1^{o} Adjuvant

f - day 14 3^{o} Adjuvant

Figure 4.4 Autoradiograph of ^{125}I L3/4 ES immunoprecipitated with sera from B10.S mice. Mice were either infected with infective *Ascaris* eggs or with L3/4 ES in adjuvant. Mice were bled at the times indicated in the figure. Precipitates were analysed under non-reducing conditions on a 5-25% SDS-PAGE gel.

B10s mouse - infection and adjuvant serum versus ^{125}I L3/4 ES



M - Markers
R - Reference ^{125}I L3/4 ES

a - B10s Normal Mouse Serum
b - day 14 1^{o} Infection
c - day 14 2^{o} Infection
d - day 14 3^{o} Infection
e - day 14 1^{o} Adjuvant
f - day 14 3^{o} Adjuvant

4.2.3 EFFECT OF MODE OF PRESENTATION ON RECOGNITION OF PARASITE ANTIGENS BY RATS

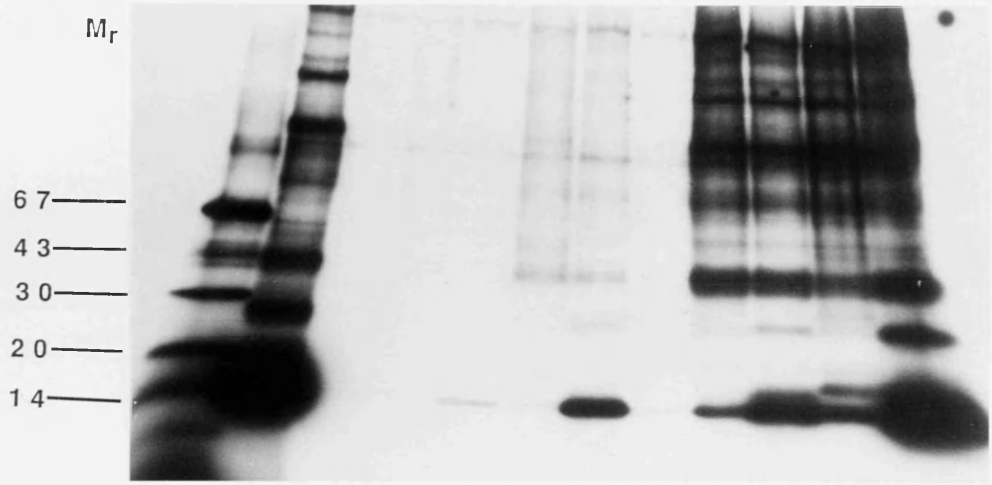
Comparisons were also made between sera from rats exposed to infective L2 eggs on three separate occasions and those immunised with purified antigen in adjuvant. Figure 4.5 is an autoradiograph of radiolabelled ABF immunoprecipitated with various infection and adjuvant generated rat sera. All the sera were collected 14 days after the third exposure to either infective eggs or antigen in adjuvant. Both the $RT1^u$ haplotypes, WLEP and PVG-$RT1^u$ recognise ABA-1 in the context of infection. When ABF is presented in adjuvant, all strains recognise the ABA-1.

When the antigen used in the assay is L2 ES (Figure 4.6) both WISTAR and $RT1^u$ again recognise ABA-1 in the context of infection. The PVG and PVG-$RT1^1$ strains recognise many other components of L2 ES - the infection and adjuvant recognition profiles for these strains appear to be identical. The components in the 30-50kDa range recognised by $RT1^u$ strains in infection are not recognised when presented in adjuvant. PVG-$RT1^u$ adjuvant-immunised response to ABA-1 is no greater than in infection and the faint recognition of ABA-1 by WLEP in infection is lost when presented in adjuvant.

The corresponding autoradiograph for L3/4 ES antigen is shown in Figure 4.7. The repertoire of infected PVG, PVG-$RT1^1$ and PVG-$RT1^u$ rats is similar to, but not as full as the antibody repertoire of adjuvant immunised rats. (90-100kDa antigen(s) is recognised by infected sera this is seen when the autoradiograph is exposed for a longer time). The faint recognition of ABA-1 by WISTAR rats is amplified in the adjuvant immunised serum and more components are recognised.

Figure 4.5 Autoradiograph of ^{125} I ABF immunoprecipitated with sera from rats infected either with infective *Ascaris* eggs or ABF in adjuvant. Rats were bled 14 days after a tertiary infection. WISTAR strain is our own WLEP and tracks d are PVG-RT1 L, tracks e PVG-RT1 U. Precipitates were analysed under non-reducing conditions, run on a 5-25% SDS-PAGE gel.

Infection and Adjuvant Immunised rat serum versus ^{125}I ABF

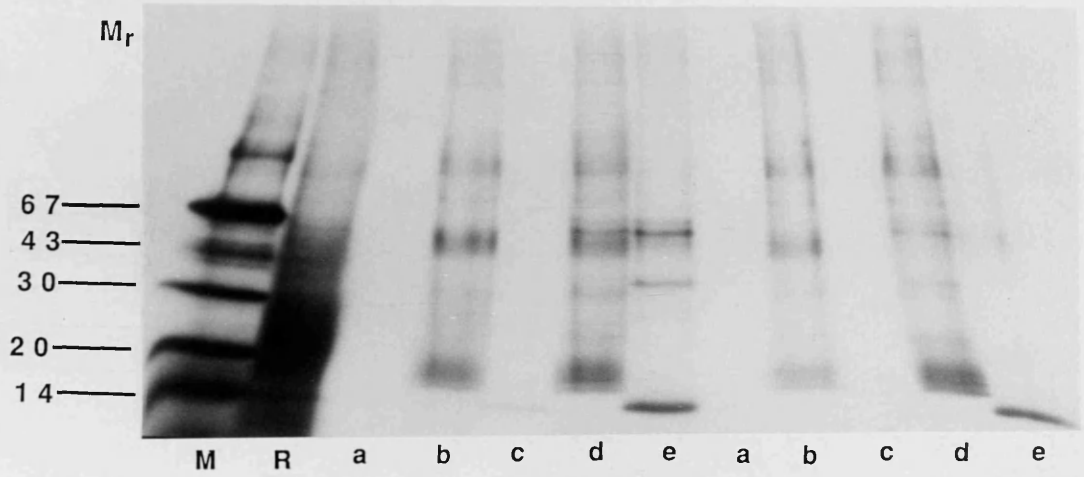


M - markers
R - reference ^{125}I ABF

a - Normal Rat
b - PVG
c - WISTAR
d - RT1^{l}
e - RT1^{u}

Figure 4.6 Autoradiograph of ^{125} I L2 ES immunoprecipitated with sera from rats infected either with infective *Ascaris* eggs or ABF in adjuvant. Rats were bled 14 days after a tertiary infection. WISTAR strain is our own WLEP and tracks d are PVG-RT1 L, tracks e PVG-RT1 U. Precipitates were analysed under non-reducing conditions, run on a 5-25% SDS-PAGE gel.

Infection and Adjuvant Immunised rat serum versus ^{125}I L2 ES

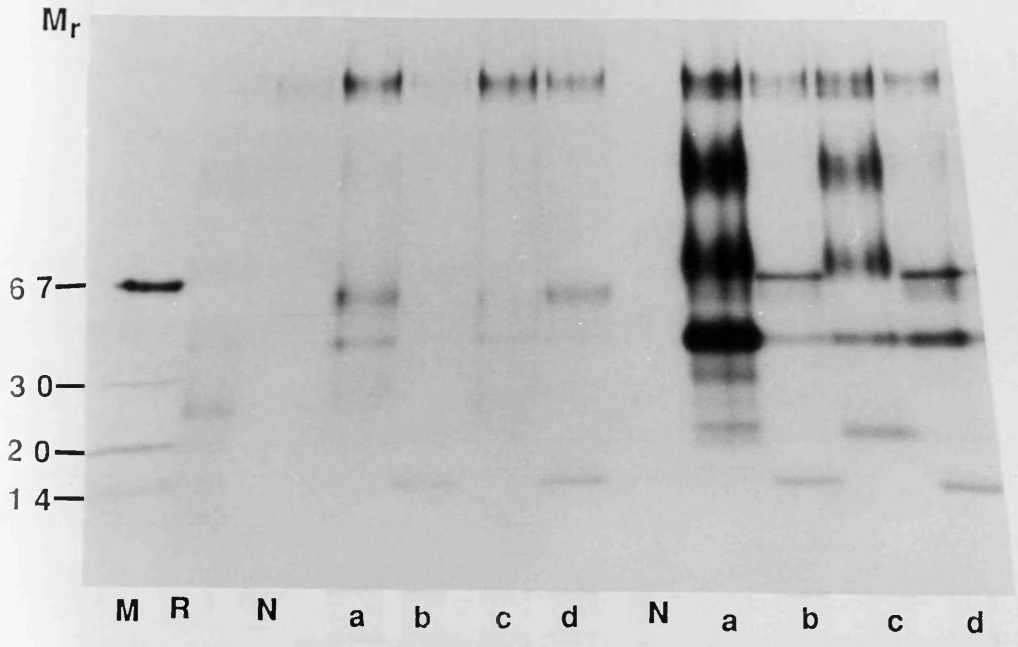


M - markers
R - reference ^{125}I L2 ES

a - Normal Rat
b - PVG
c - WISTAR
d - RT1^{l}
e - RT1^{u}

Figure 4.7 Autoradiograph of ^{125}I L3/4 ES immunoprecipitated with sera from rats infected either with infective *Ascaris* eggs or ABF in adjuvant. Rats were bled 14 days after a tertiary infection. WISTAR strain is our own WLEP and tracks d are PVG-RT1 L, tracks e PVG-RT1 U. Precipitates were analysed under non-reducing conditions, run on a 5-25% SDS-PAGE gel.

Infection and Adjuvant Serum from Rats versus ^{125}I L3/4 ES



M - Markers
R - Reference ^{125}I L3/4 ES
N - Normal Rat Serum

a - PVG
b - WISTAR
c - $RT1^L$
d - $RT1^U$

Purified ABA-1 was immunoprecipitated by this panel of sera and the quantity of antigen precipitated by each one recorded in Table 4.1. These numerical values most closely resemble the pictorial analysis of Figure 4.5 - the immunoprecipitation of ABF.

4.2.4 IgE RESPONSE OF RATS

This was measured in PCA assays and the results are presented in Table 4.1. The WLEP infected rats generate the strongest IgE responses to ABA-1 and the PVG-RT1^u rat generates a weak but detectable IgE response to this allergen. Sera from rats immunised with ABF in adjuvant do not contain any ABA-1 specific IgE.

4.2.5 IgE RESPONSES IN MICE

Results of detection of ABA-1-specific IgE in infected and adjuvant-immunised mice by PCA are presented in Table 4.2. The quantity of IgE in sera from adjuvant-immunised BALB/c mice is greater than in sera from infected BALA/c mice. The IgE response of B10.S mice is lower than when the allergen is presented in adjuvant rather than in the context of infection. C57BL/10 mice generate a low level of IgE to ABA-1 presented in adjuvant but none when it is presented to the mouse in the context of infection. NIH mice are unable to recognise ABA-1 with IgE when they are immunised with adjuvant but generate appreciable quantities when ABA-1 is seen in the context of infection.

Out of the panel of recombinant congenic mice, only four strains generate IgE specific for ABA-1 when infected three times with infective eggs - SJL, ATH, B10.S9R and B10.HTT (Table 4.3). The SJL result, however, was interesting as previous work in this laboratory (Tomlinson *et al.*, 1989) had not detected any ABA-1 specific IgE in SJL infection serum. To further

Table 4.1

INFECTED AND ADJUVANT-IMMUNISED RAT SERUM - PCA AND % TCA RESULTS VERSUS ABA-1

RAT STRAIN	INFECTION SERUM		ADJUVANT IMMUNISED SERUM	
	PCA titre	% TCA precipitated	PCA titre	% TCA precipitated
PVG	0	1.02	0	14.13
WLEP	256	23.63	0	25.19
PVG-RT1L	0	1.11	0	32.93
PVG-RT1U	1	23.68	0	49.45

Infection generated serum and serum generated by adjuvant immunisation with ABF - produced as described in 2.16.2. PCA titres determined in duplicate on separate rats given 50 microgrammes of ABA-1 24 hr after serial dilutions of test serum was injected intradermally. Radiolabelled ABA-1 was immunprecipitated with test sera and the percentage preciptated recorded.

Table 4.2 **INFECTED AND ADJUVANT IMMUNISED MOUSE SERUM - PCA AND % TCA RESULTS VERSUS ABA-1**

MOUSE STRAIN	INFECTION SERUM		ADJUVANT IMMUNISED SERUM	
	PCA titre	% TCA precipitated	PCA titre	% TCA precipitated
BALB/c	32	0.82	64	1.27
B10.S	64	16.53	4	37.5
C57/BI10	0	1.45	2	29.6
NIH	64	0.85	0	20.8

Serum generated as described in 2.16.1 with infective L2 eggs and ABF in adjuvant respectively.
PCA titres determined as described in 2.17 and % TCAs as in 2.11

Table 4.3

RECOMBINANT AND CONGENIC MICE PCA AND % TCA VERSUS ABA-1

MOUSE STRAIN	PCA TITRE	% TCA PRECIPITATED
NMS	0	0.93
B10.BR	0	1.19
B10.A2R	0	1.06
B10.A4R	0	1.08
B10.AQR	0	1.05
B10.A5R	0	1.21
SJL	256*	10.82
ATH	256	24.8
B10.S9R	128	4.26
ATL	0	0.74
C57/BL10	0	1.46
B10.D2	0	1
B10.HTT	64	3.87

* see Table 4.4

investigate this all other available batches of SJL infection serum were tested with the same batch of allergen and under the same conditions - the results are presented in Table 4.4.

Table 4.4

VARIABLE SJL INFECTION SERUM PCA RESULTS

Date of PCA	Nature of serum	PCA titre
22/3/90	tertiary infection	256
1/5/90	tertiary infection	64
1/6/90	quaternary infetcion	0
	secondary infection	64
3/7/90	tertiary infection	0
	quaternary infection	256

All serum collected 14 days after infection, each PCA carried out with different batches of sera

4.3 DISCUSSION

How is the observed selective recognition of the ABA-1 molecule explained? Clearly, mice with an H-2^{s} and rats with an RT1^{u} haplotype are able to process and present the molecule in such a way that B cells are activated to make antibody to it. The lack of response in infection by other haplotypes can thus be explained in the context of some defect in the animal's ability to present this particular molecule to T cells. This phenomenon is sometimes described as immune blind spots or "holes" in the repertoire (Benacerraf and McDevitt, 1972; Schwartz, 1986) and is thought to have three possible causes.

The first is that if a V region gene from either the B or T cell receptor is missing from the gene pool, the animal will be unable to express the specific MHC antigen the V region codes for (Epstein, Sham, Womack *et al.*, 1986). This may account for the non-responsiveness of some strains to some of the *Ascaris* components (i.e. those not seen by any strain) but is unable to account for the selective recognition by MHC congenics. Congenic animals, by definition, have the same background genes and thus the gene pool of receptors for B10 and BALB congenics is the same. Thus the fact that the H-2^{s} and RT1^{u} haplotypes can respond to ABA-1 in infection means that the necessary V genes are present.

The second possible explanation is that the observed lack of response to certain components is caused by Ir genes. Immune responses against protein antigens involves the activation of MHC-restricted T cells by processed fragments of the peptide complexed to the Class II (Ia) molecules on the surface of the presenting cells. As there does not appear to be any difference in the way that antigen is processed by different strains of

mice (and presumably rats) (Friedman, Zerubavel, Gitler *et al.*, 1983) it is likely that differences occur at the level of association of processed antigen with Ia molecules. It has been shown that polymorphisms in these Ia molecules correlate with resposiveness to many antigens and hence the failure to recognise an antigen represents a failure of the antigenic peptide to bind to the Ia molecule (Benacerraf, 1978). The products of some MHC alleles cannot form complexes with particular antigens (Babbitt, Allen, Matsueda *et al.*, 1985; Buus, Setto, Colon *et al.*, 1987) which renders individuals with such alleles unresponsive to that specific antigen. This explanation may be what is happening in the *Ascaris* model. It can be envisaged that the only Ia molecule which results in T cell activation, when complexed with the ABA-1 molecule, is that encoded by the $H\text{-}2^{s}$ or $RT1^{u}$ haplotypes.

The third possible explanation is that of self-tolerance where presented parasite antigens may resemble host components. To prevent mounting an immune response to self, animals are tolerised by the process of thymic education to self components. Thus, if a combination of foreign antigen and Ia molecule being presented to a T cell resembles self, no response is induced (Vidovic and Matzinger, 1988). This still leaves the question of the $H\text{-}2^{s}$ and $RT1^{u}$ haplotypes being the only ones to recognise the ABA-1 as the T cell receptor and immunoglobulins are encoded by non-MHC genes.

Having established that only the $H\text{-}2^{s}$ haplotype in mice recognises the ABA-1 molecule the next point to establish was which alleles control the recognition. Reference to Table 2.2 and the four strains which recognise the ABA-1 protein indicates that SJL mice have s genes at all alleles of the H-2. ATH has d at the D locus so the D locus does not control recognition. All mice of the $H\text{-}2^{s}$ haplotype cannot express functional I-E

molecules so the remaining possibilities are the I-A or K regions. The ATL mouse has s at its K region and does not recognise ABA-1 so the control region for recognition of ABA-1 by IgG and IgE in the context of infection is the I-A region.

Mouse IgG responses to *Ascaris* antigens are controlled by several factors including the genetic make-up of the mouse and the mode of presentation of antigen to the host's immune system. Presentation of parasite antigen in adjuvant can affect the rate at which the antibody response is generated (C57BL/10 mice, Figure 4.2); increase the number of components recognised by the antibody repertoire (BALB/c mice, Figure 4.3) and cause absence of recognition of major antigens seen in the context of infection (B10.S mice, Figure 4.4). With regard to specific recognition of ABA-1, this is generated in C57BL/10 and NIH mice.

There are conflicting results presented in Figure 4.2 and the non-recognition by adjuvant sera of ABA-1 and the appreciable response recorded in Table 4.2. Also, the failure of B10.S mice exposed to ABA-1 in adjuvant to recognise ABA-1 shown in Figure 4.4, and their recognition of it recorded in Table 4.2 appears contradictory. One possible explanation is that the sera analysed in Figures 4.2 and 4.4 were generated by immunisation with L3/4 ES and then immunoprecipitated with L3/4 ES. These ES preparations may have contained low levels of ABA-1, too low to generate an IgG response or too low to be detected in the labelled preparation, or both. In contrast, the mouse sera used for the experiment reported in Table 4.2 were generated by immunising mice with purified ABA-1 and then using this to immunoprecipitate purified radiolabelled ABA-1. Thus there would have been no limitation on availibility of ABA-1 either

at the presentation or precipitation stage. Hence the dose of antigen may also play a factor in the overall response generated.

The IgE system is also characterised by complex and varied patterns of response. Presentation of ABA-1 in adjuvant fails to elicit an IgE response except in BALB/c mice (Table 4.2). Presentation of ABA-1 in the context of infection generates an IgE response in BALB/c, NIH, SJL and some B10 congenic mice (Tables 4.2 and 4.3). The positive SJL response was unexpected because previous work in this laboratory had shown that no IgE response was generated to ABA-1 by this strain. Subsequent repititions with different batches of infection sera did not serve to clarify the situation (Table 4.4). Until the results of a time course experiment (currently in hand) are known, one can only speculate as to the basis of these discrepant results. One possibility is that the kinetics of the IgE response characterised by rapid increases and decreases in IgE levels in helminth infection, especially secondary and subsequent infections is the cause of these results (Jarrett *et al.*, 1976). Infections may have been faulty but sera are pools of at least three animals and other results from the same sera i.e. IgG responses detected in immunoprecipitations did not differ from those expected.

How far do the IgG and IgE responses of mice correlate? Table 4.2 shows that they are not related and indeed all combinations of low and high, IgG and IgE responses are seen. This adds weight to previous suggestions that IgE responses are under quite separate control from IgG responses (Jarrett, 1978).

Rat IgG responses, as detected by protein A-mediated precipitation of antibody bound to antigen in the *Ascaris* system can be described thus:

Response to ABF components (Figure 4.5) is amplified by presentation of those components in adjuvant (except for WLEP rats who display little amplification). Response to L2 ES (Figure 4.6) is much the same from both sets of sera. This may be due to exposure to large quantities of L2 ES over several days as the L2 larvae migrate through tissue during the course of infection. As L3/4 larvae do not develop further and are terminated in the lungs of the rat, the animal is probably not exposed to large quantities of L3/4 ES. Hence presentation of a large amount of L3/4 ES which is released slowly (the adjuvant acting as a depot for antigen) means that antibody titres are greater than in infection sera (Figure 4.7).

Rat IgE responses to ABA-1 as described in Table 4.1 are very high for the WLEP strain and low, but consistently detectable, for the PVG-RT1^u. As both strains have the RT1^u haplotype the level of IgE response is apparently controlled by background genes. Presentation of ABA-1 in adjuvant is not conducive to IgE formation specific for the allergen as none of the adjuvant immunised sera contain IgE.

Correlation between IgE and IgG responses to ABA-1 in the context of infection is good with IgG response matching IgE response in both WLEP and PVG-RT1^u strains. However, this relationship no longer holds with the different mode of presentation of allergen in adjuvant.

The general increase in antibody responses generated by antigen preparations presented to the host in the context of adjuvant has often been suggested as a potential means of inducing protection or as a model for vaccine development. However, as these studies show, there are often discrepant responses generated by antigen in adjuvant rather than in the

context of infection and it may be that the efficacy of such responses could also be altered. Another factor to consider is the current prohibition on the use of some adjuvants in humans due to the pathology caused.

CHAPTER 5

ASCARIS IN HUMANS

5.1 INTRODUCTION

In 1942 some work by Ackert reviewed what was known about resistance to helminth infections. As a result of observations on helminth infection in both negroes and caucasians in southern states of the US he concluded that some kind of "racial immunity" existed whereby their genetic make-up rendered negroes more naturally resistant to infection than caucasians. Since then many different studies have shown not so much racial immunity to parasites but, certainly, immunity in some individuals alongside others who are infected when both are exposed to the same environment. Much recent work on these differences has focussed on relationships between the gene cluster which controls immune responses (HLA) and the character of infection or particular disease manifestations caused by helminth infection.

Much of this type of research has focussed on schistosomiasis. One of the first reports linking certain HLA types and disease was a report on schistosome infected Egyptian school children with similar egg counts. Hepatosplenomegaly - a common complication in schistosomiasis - was related to the presence of HLA-A1 and HLA-B5. It was shown that of those who had schistosomiasis and the haplotype HLA-A1, they were 29 times more likely have hepatosplenomegaly, those with HLA-B5 had a relative risk of 18.9 and those who had both haplotypes were 55.6 times more likely to have hepatosplenomegaly than others (Salam, Ishaac and Mahmoud, 1979). Other work on response to a defined antigen - Sj26 from *S. japonicum* - identified two genes as controlling immune responsiveness (HLA-DR) and immune suppression (HLA-DQ). Analysis showed that HLA-DQ is epistatic to HLA-QR in the control of immune responsiveness to schistosome antigen in humans (Hirayama, Matsushita, Kikuchi *et al.*, 1987).

The only report which has linked *Ascaris* infection and HLA type is that by Bundy (1988b). Higher frequencies of DQw2 were reported for uninfected people in a Carribean community among which ascariasis is endemic. Class I antigen frequencies also revealed an association between B14/Bw65 antigen and intense infection with both *Ascaris* and *T. trichuris*.

In contrast to the many studies on the epidemiology of ascariasis, little work has been done on the immunology of *Ascaris* infections in humans. One study from Papua New Guinea and Timor showed that positive haemagglutination tests (which detected the presence of antibodies but were not quantitative) reflected the overall endemicity of *Ascaris* infection but had no value as a diagnostic aid. However, one community did show significant negative correlations between detected antibody titre and eosinophil counts. It was suggested that high titres in adults resulted from continuous exposure to infective eggs and that declining egg counts and infection rates with age may be the result of an unidentified immune mechanism (Jones, 1977).

Probably the only study to date that combines epidemiology with some basic immunology was carried out in a fishing village in South India where the prevalence of ascariasis is 96% (Haswell-Elkins *et al.*, 1989). Radiolabelled L3/4 ES was immunoprecipitated with sera from individuals in the village. When the precipitates were analysed on SDS-PAGE gels the intensity of antigen precipitated by IgG antibody was measured by semi-quantitatively scoring 8 different bands with reference to negative European sera and positive rabbit infection sera. A significant relationship between intensity of precipitated ES antigens and host age was observed with 5-9 year olds showing both the heaviest banding and the heaviest infections. The total antibody "scores" were predictive of epg

values 4 months after the blood samples were taken but the antibody was not shown to be protective. These results were taken to support the hypothesis that the degree of exposure to infective stages of *Ascaris* is a major determinant of the convex age-intensity profile observed in the community (also, Haswell-Elkins *et al.*, submitted).

The other major Ig isotype of interest in ascariasis is IgE which has the unique property of binding reversibly, with high affinity, to specific (Fcε) membrane receptors on basophils and mast cells. The presence of IgE has long been associated with helminth infections (Rackemann and Stevens, 1927). Suggestions as to the role of IgE in these infections is a continuing source of debate. Several lines of evidence suggest a protective role for IgE in helminth infections (reviewed by Geha, 1984):
(1) the observed high serum IgE levels in endemic areas
(2) IgE dependent binding of macrophages and eosinophils to schistosomes which leads to their death. IgE also mediates mast cell discharge which is thought to play a role in expulsion of gastrointestinal helminths.
(3) IgE deficient rats have 2-3 times more *Trichinella* larvae encysted in their muscles than control litter mates.

One study which looked at both IgG and IgE responses to *Wuchereria bancrofti* antigens showed that "dual recognition" was the rule rather than the exception for each individual's immune response to the parasite antigens i.e. IgG and IgE responses usually occurred together. However, the relative magnitudes of IgG and IgE responses differed among three clinical groups (Hussain and Ottesen, 1985). More commonly, studies have looked only at IgE and often sought associations between IgE levels and alllergic disease. In a study of adult asthmatics in the Highland area of Papua New Guinea where IgE levels are high and ascariasis endemic, no significant

difference was found in the incidence of IgE antibodies to *Ascaris lumbricoides* in asthmatic compared to control subjects (Turner, Baldo and Anderson, 1975). A later study by the same group on a similar population showed that IgE specific for *Ascaris* antigens contributed only a minor fraction to the total serum IgE (Turner, Feddema and Quinn, 1979). They hypothesised that in the same way as experimental animals (Jarrett, 1978), humans infected with helminths produce IgE specific for unrelated, bystander antigens. With the development of more accurate assays for the determination of specific IgE i.e. the radio allergosorbent or RAST test, results from such tests correlated well with skin reactions induced by the same *Ascaris* allergen (Joubert, Van Schalkwyk and Turner, 1980).

Immunological analysis of a population which has been well characterised in epidemiological terms has never really been performed and a limited investigation is descibed here. The Nigerian sera used in this chapter (and Chapter 6) came from a study which was carried out by Holland *et al.* (1989). The study population was the children of St. Peter's Primary School, Ile Ife, Nigeria and the study was carried out with the permission and co-operation of the staff and parents. 766 children aged from 5-16 years participated in the study and were given an anthelmintic on three occasions - March 1987, October 1987 and March 1988. Worm burdens and epg values were determined for each child at each time point and blood samples were taken in October 1987. On entry to the UK these samples were screened for Hepatitis B and HIV antibodies and any positive sera removed. The remaining 102 samples were used in the following studies.

The study was the first one to examine reinfection rates and predisposition in an African population. The results show that the children were quickly reinfected and that the strong trend was for them to

return to their previous type of infection i.e. some children were persistently un-infected with *Ascaris* while others had, within the 6 month reinfection periods, returned to very heavy worm burdens. A return visit was made to the school in May 1990 and worms and sera collected from 6 of the pupils who, in the original study, had carried very heavy worm burdens. These follow-up sera were also examined in a limited way.

The sera from Karachi are courtesy of Dr. J.M. Blackwell of the London School of Tropical Medicine and Hygiene where the sera are being HLA typed. The Gambian sera are also the subject of HLA typing studies and were kindly donated by Adrian Hill of the Department of Molecular Medicine of the John Radcliffe Hospital in Oxford, UK. We have no parasitological data on either group of people except that they come from areas where ascariasis is endemic.

5.2 RESULTS

5.2.1 ENZYME LINKED IMMUNOSORBENT ASSAYS ON SERA FROM NIGERIAN DONORS.

ELISAs were developed for human sera against the four antigens ABF, ABA-1, L2 ES and L3/4 ES - the various concentrations of antigen, serum and conjugate used are recorded in Table 2.1. All 102 Nigerian serum samples, along with a normal European serum sample as negative control were assayed in the four different ELISA systems. Assays were repeated and correlation coefficients between different tests on the same antigen were commonnly around 0.9 e.g. the ABF tests in Figure 5.1a. Comparing optical density (OD) values of ABF and ABA-1 assays showed that there was some correlation between the two readings (Figure 5.1b) - presumably reflecting the abundance of ABA-1 in ABF.

Relationships between OD values of individual samples with host-age, epg counts and worm burdens were sought but no clear associations were found between any of these factors. To illustrate the heterogeneity of the results, the OD values of all four antigens for 9 of the 18 individuals who had no worms on each of the three de-worming occasions are shown in Figure 5.2a. It can be seen that there are no obvious patterns of antibody levels against any of the four antigens. High anti-ABF responses are common, but were very low for some donors e.g. 283 and 551. Generally, quantitative antibody responses to these antigens show no distinguishing features. The same is true for individuals who were persistently heavily infected i.e. who had at least 10 worms on reach of the occasions they were given anthelmintics - Figure 5.2b. Overall the antibody responses tend to be higher and an individual's reponse to the four antigens of similar levels but, again, nothing conclusive can be drawn from these results.

Figure 5.1a Plot of OD values from an ABF ELISA of Nigerian donors carried out on 1/9/88 versus OD readings from a similar ELISA carried out on 13/1/89. Coefficient of correlation (r value) = 0.897.

Figure 5.1b Plot of OD values from an ABF ELISA of Nigerian donors versus the OD readings of an ABA-1 ELISA with the same individuals. Coefficient of correlation (r value) = 0.449.

Figure 5.1a

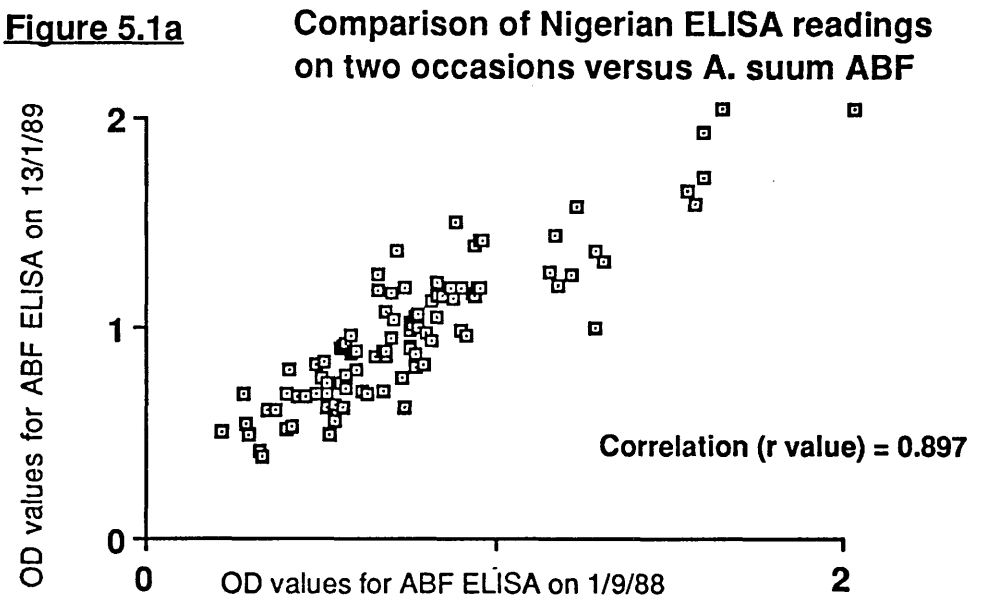


Figure 5.1b

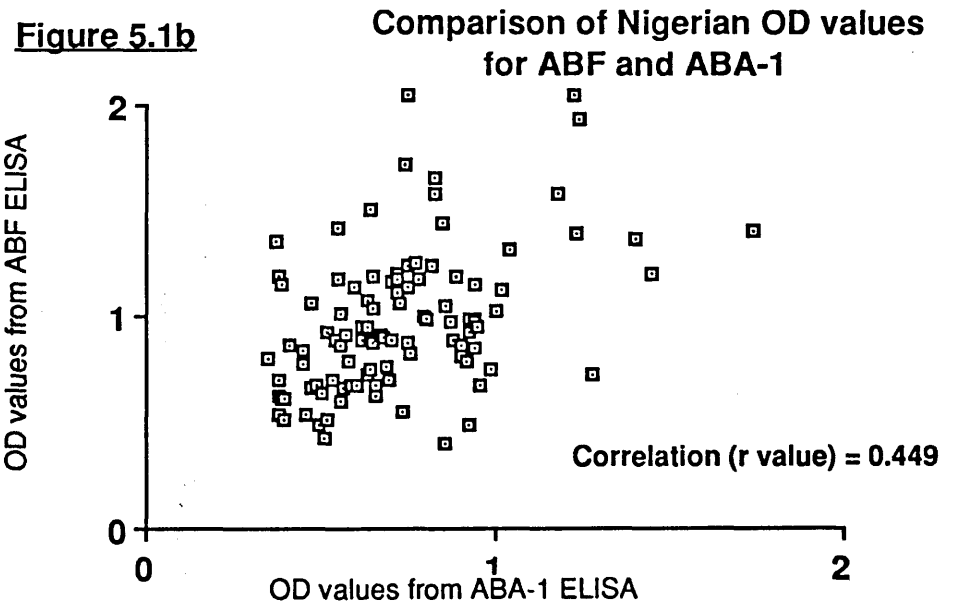


Figure 5.2a ELISAs of the four antigens (ABF, ABA-1, L2 ES and L3/4 ES) were carried out on sera from each of the Nigerian donors. The OD values from the four assays for nine individuals who were worm-free on each of three occasions are shown. N represents a normal European individual.

Figure 5.2b ELISAs of the four antigens (ABF, ABA-1, L2 ES and L3/4 ES) were carried out on sera from each of the Nigerian donors. The OD values from the four assays for nine individuals who harboured at least 10 worms on each of three occasions are shown. N represents a normal European individual.

Figure 5.2a

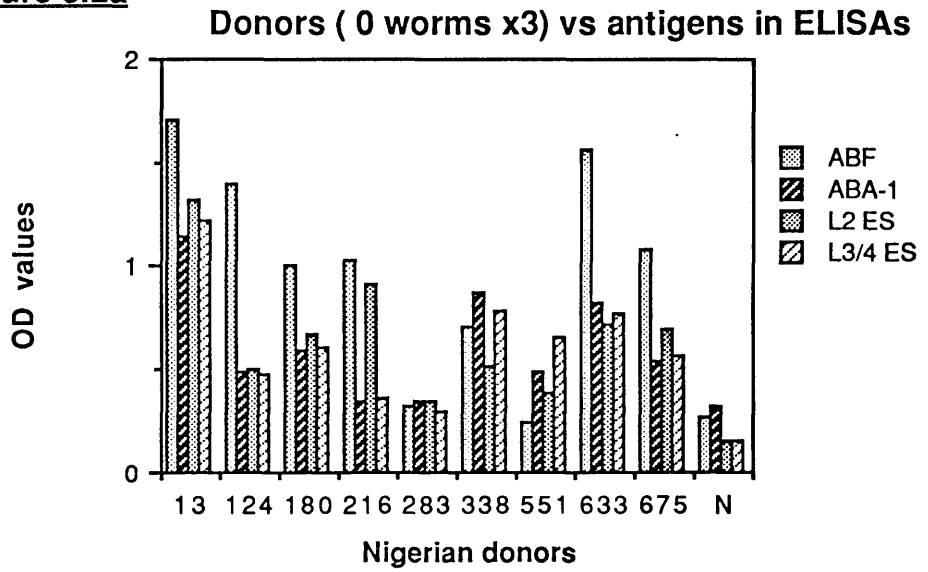
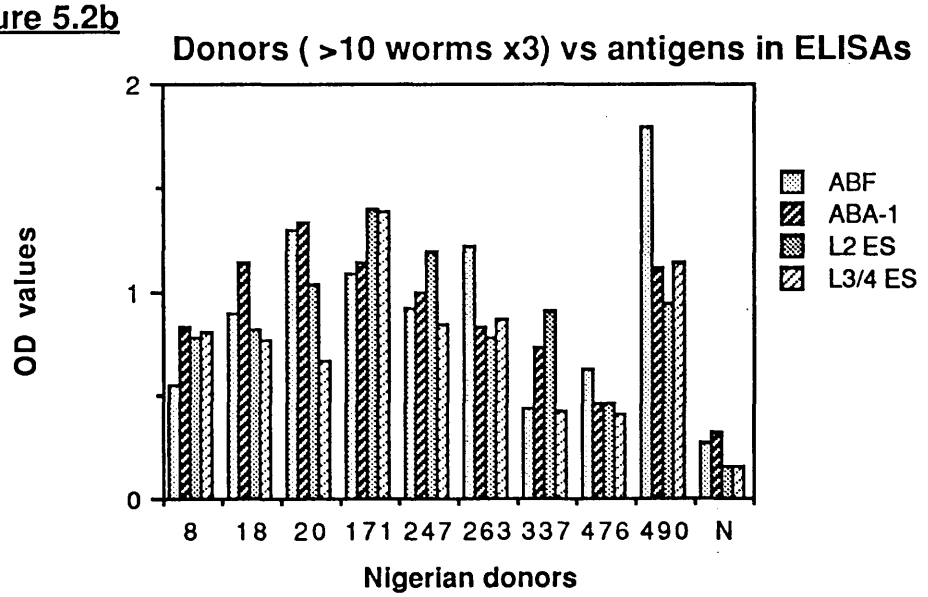


Figure 5.2b



A list of some of the donors and their worm burdens is shown in Table 5.1.

5.2.2 AUTOCLAVE AND PERIODATE TREATMENT OF ANTIGENS IN ELISAS.

In an attempt to investigate the nature of the determinants recognised by antibodies from infected individuals, the antigen preparations were autoclaved (where heat denatures or unfolds polypeptides) or periodate treated (to disrupt carbohydrate determinants). The four antigens were treated as described in 2.8 and ELISAs performed as before. The OD values for the first 8 donors in the series are shown for each of the four antigens in their normal, periodate treated and autoclaved forms. The ABA-1 results (Figure 5.3a) show a heterogeneous response by the donors. Donor 13's response to the three forms of the antigen is almost identical while for donors 3, 18 and 20 the response to periodate treated and autoclaved antigens could almost be additive to give the reponse to the untreated antigen. In every case the antibody response to the normal ABA-1 is greatest but it does not appear that this is significant.

For ABF (Figure 5.3b) the antibody response to the untreated antigen is greatest with the periodate treatment consistently having the most marked effect in terms of reducing antibody recognition of the antigen. Autoclave treatment also reduces the specific recognition but not to the same extent as does the periodate treatment.

The antibody response to L2 ES as detected in the ELISAs are generally quite low (Figure 5.3c). Periodate treatment of the antigen usually causes a slight increase and autoclave treatment a greater increase. Thus, hidden antigen determinants must be revealed or steric hindrance reduced in some way.

Table 5.1

WORM BURDENS OF SOME NIGERIAN DONORS

DONOR	MARCH '87	OCT '87	MARCH '88	MAY '90
8	2	4	11	
13	0	0	0	
18	22	13	20	2
20	0	0	13	
124	0	0	0	
171	38	12	16	25
180	0	0	0	
181	22	28	7	
188	21	12	0	
190	4	4	2	
200	6	4	0	
207	2	2	0	
210	4	2	6	
211	0	0	0	
216	0	0	0	
235	7	4	8	
244	23	24	0	
247	103	33	12	64
249	4	4	4	
254	43	9	-	

Figure 5.3a ABA-1 was used as the antigen in an ELISA in three forms ABA-1 N, normal ABA-1; ABA-1 P, periodate treated ABA-1; ABA-1 A, autoclave treated ABA-1. The OD values of sera from seven Nigerian individuals are shown along with those of a normal European individual.

Figure 5.3b ABF was used as the antigen in an ELISA in three forms ABF N, normal ABF; ABF P, periodate treated ABF; ABF A, autoclave treated ABF. The OD values of sera from seven Nigerian individuals are shown along with those of a normal European individual.

Figure 5.3a

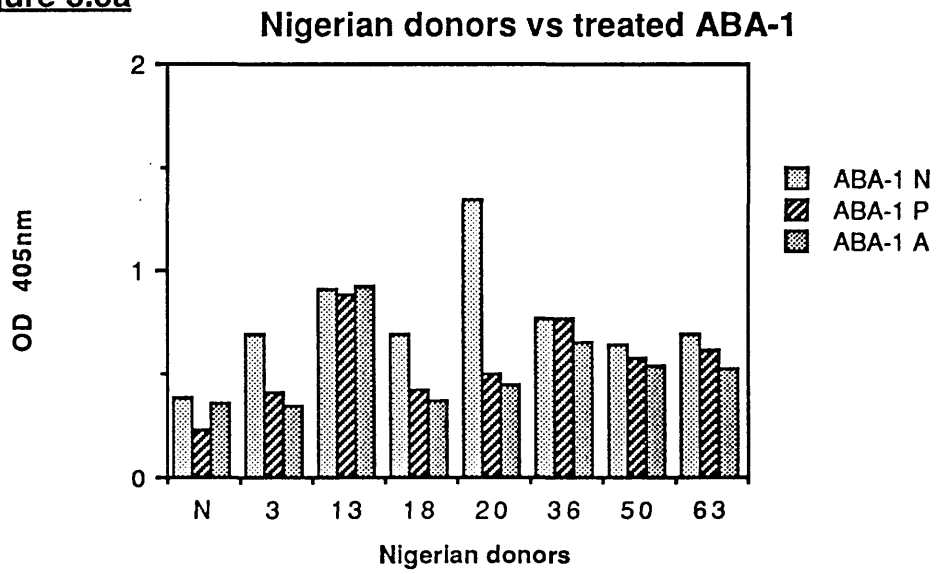


Figure 5.3b

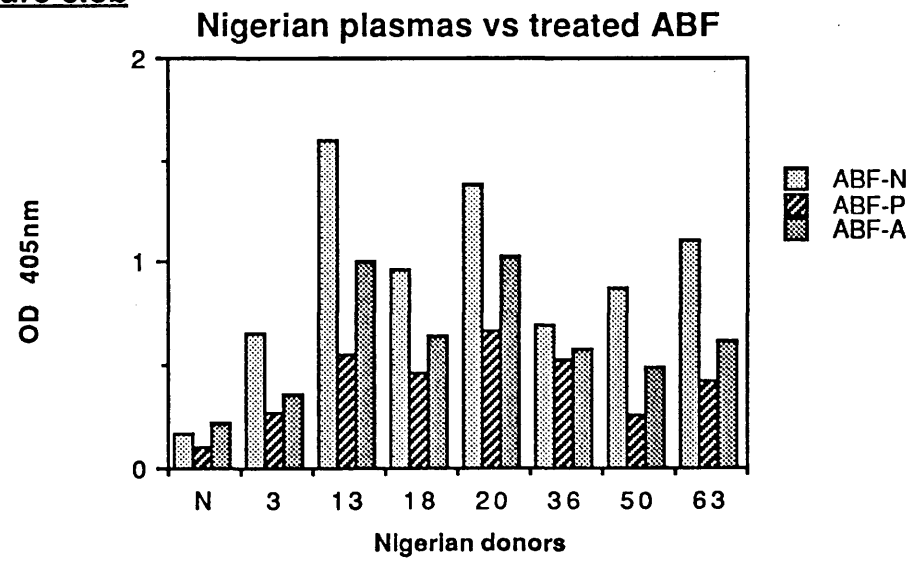


Figure 5.3c L2 ES was used as the antigen in an ELISA in three forms L2 ES N, normal L2 ES; L2 ES P, periodate treated L2 ES; L2 ES A, autoclave treated L2 ES. The OD values of sera from seven Nigerian individuals are shown along with those of a normal European individual.

Figure 5.3d L3/4 ES was used as the antigen in an ELISA in three forms L3/4 ES N, normal L3/4 ES; L3/4 ES P, periodate treated L3/4 ES; L3/4 ES A, autoclave treated L3/4 ES. The OD values of sera from seven Nigerian individuals are shown along with those of a normal European individual.

Figure 5.3c

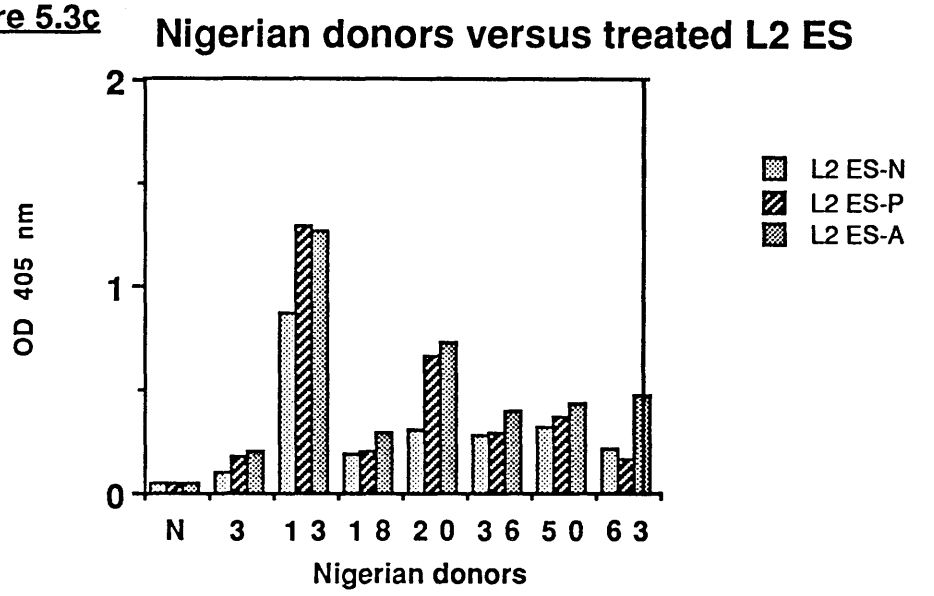
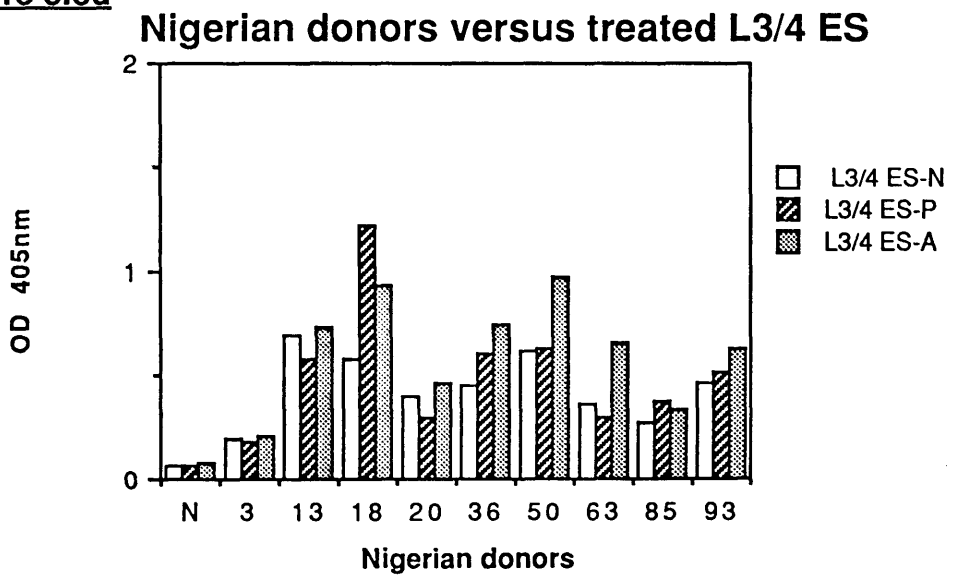


Figure 5.3d



The pattern for L3/4 ES is very similar to that of L2 ES (Figure 5.3d) with autoclaved antigen especially causing an increase in specific antibody recognition. For donor 18, a dramatic increase in the antibody response is seen when the the L3/4 ES is periodate-treated. Overall, these results reflect the heterogeneity of response not only in level but also in the sites of binding of antibody to the antigen.

To add weight to the debate about species identity between *A. suum/A. lumbricoides*, when *A. suum* ABF ELISA values are compared with A. lumbricoides values from identical systems, the correlation is 0.944 (Figure 5.4). This is despite the fact that the sera concerned come from individuals with widely differing antigen recognition patterns, which strengthens the argument for antigen similarity.

5.2.3 IMMUNOPRECIPITATION OF RADIOLABELLED *ASCARIS* ANTIGENS BY HUMAN SERA.

Radiolabelled antigens were immunoprecipitated with the series of sera from Nigerian donors. Figure 5.5 is an autoradiograph of the SDS-PAGE analysis of such an immunoprecipitation of ABF by sera from selected Nigerian donors. Recognition of some of the higher molecular weight components seems to be fairly universal but interesting differences are seen at lower molecular weights. Donor 200 recognises 15kDa and 20kDa components, 210 and 235 also see the 15kDa while 207 precipitates a 17kDa component and 211 (and possibly also 247) recognises ABA-1 at 14kDa. This diversity of recognition of antigens in the 14-20kDa range suggests polymorphic species (and their recognition by individuals) of the ABA-1 molecule.

Figure 5.4 Plot of the OD values of an ELISA of sera from the Nigerian donors with *A. suum* ABF versus the OD values of an ELISA of the same individuals with *A. lumbricoides*. The coefficient of correlation (r value) = 0.944.

Figure 5.4 Comparison of ELISA results of Nigerian donors from two sources

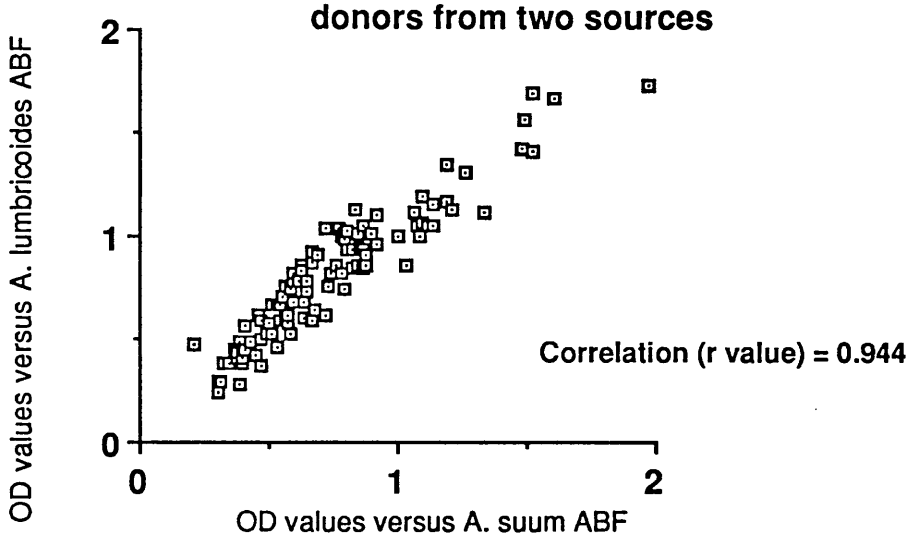


Figure 5.5 Autoradiograph of ^{125} I ABF immunoprecipitated by sera from some Nigerian donors as indicated in the figure. The precipitates were analysed under non-reducing conditions on a 5-25% SDS-PAGE gel.

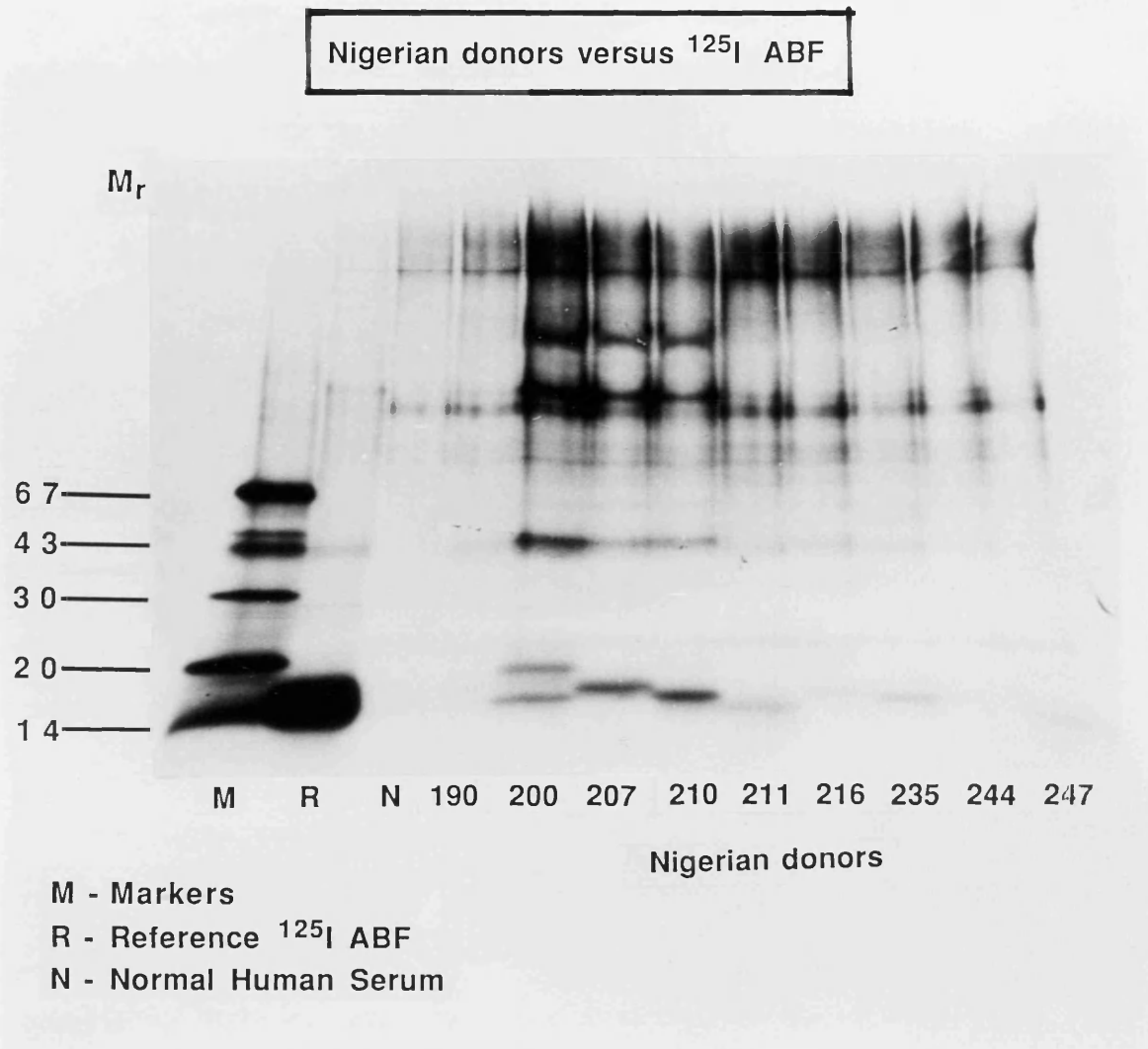

Nigerian donors versus ^{125}I ABF
M_r
6 7
4 3
3 0
2 0
1 4
M
R
N
190
200
207
210
211
216
235
244
247
Nigerian donors
M - Markers
R - Reference ^{125}I ABF
N - Normal Human Serum

Figure 5.6 illustrates the results of an immunoprecipitation of ^{125}I L2 ES with Nigerian sera. The smearing of the reference antigen and fairly indistinct bands of the precipitated antigen are characteristic of L2 preparations, possibly due to the low tyrosine content of L2 ES components. ABA-1 is present in small amounts and is recognised by donor 273 (a persistently worm-free individual). Donor 254 also has high OD values in the L2 ES ELISA as well as showing one of the most intense banding patterns in L2 ES gels.

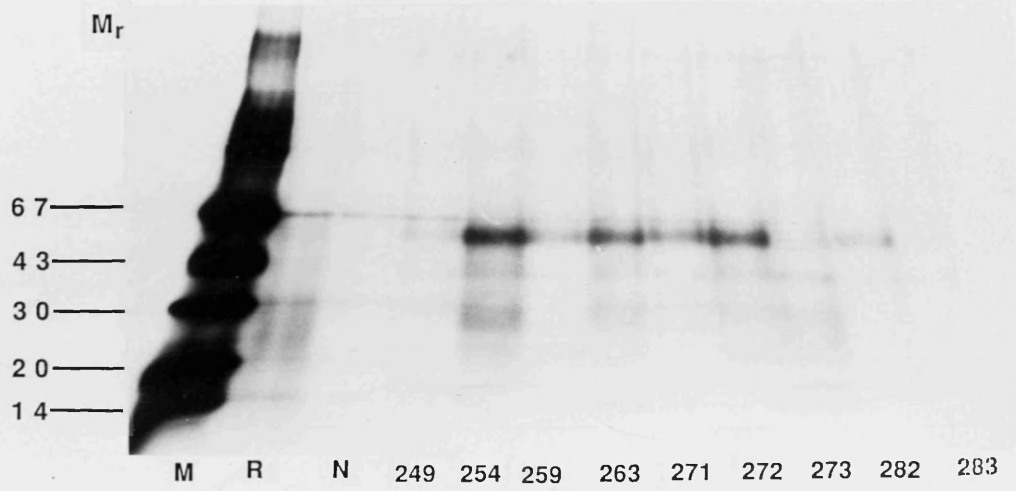
Figure 5.7 shows the results of another group of sera this time using radiolabelled L3/4 ES as a target antigen. The recognition of the approximately 43kDa antigen was almost universal throughout the donors but the intensity of recognition varied enomously. The 17kDa molecule was more abundant in the reference antigen than ABA-1 and there was non-specific recognition of the former. Only individuals 28 and 63 recognise the ABA-1 - over the whole population recognition of ABA-1 (as determined by immunoprecipitations) was around 12%.

Figure 5.8 compares the recognition profiles of the same individuals in 1987 and 1990. There are some differences between the profiles of individuals e.g. donor 20's serum from 1990 recognises 18 and 20kDa components of L3/4 ES which are not seen by the serum from 1987; serum from donor 490 taken in 1987 recognises the ABA-1 molecule but this recognitiom is not present in the 1990 serum.

When Nigerian *A. lumbricoides* material (ABF, L2 ES and L3/4 ES was available and used in immunoprecipitations, the same results were seen as those for *A. suum* products.

Figure 5.6 Autoradiograph of ^{125} I L2 ES immunoprecipitated by sera from some Nigerian donors as indicated in the figure. The precipitates were analysed under non-reducing conditions on a 5-25% SDS-PAGE gel.

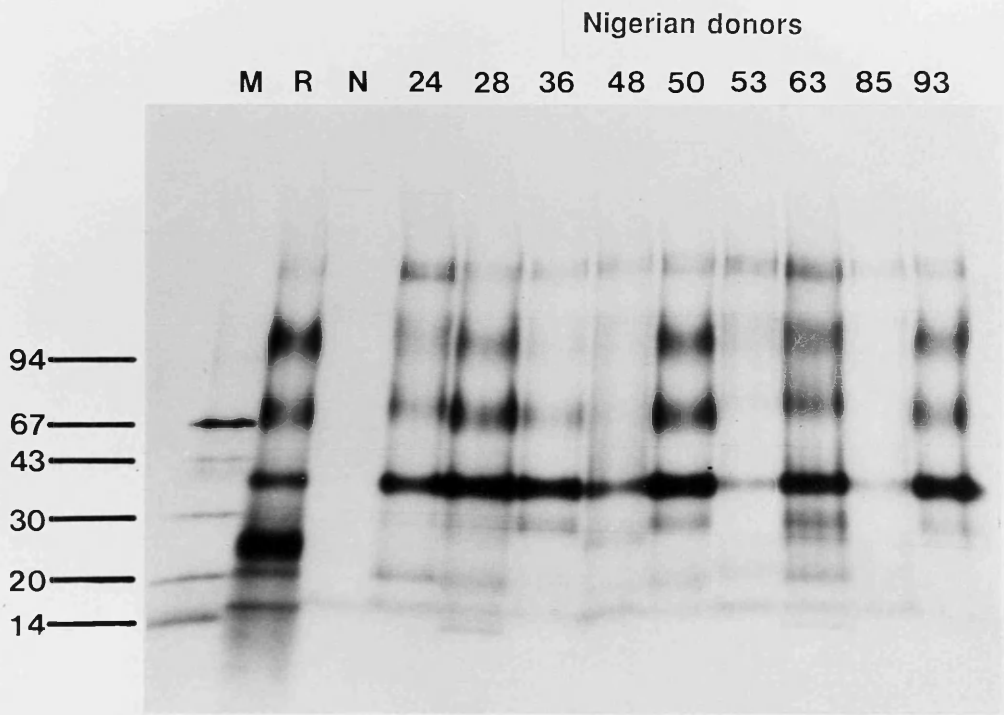
Nigerian donors versus ^{125}I L2 ES



M - Markers
R - Reference ^{125}I L2 ES
N - Normal Human Serum

Figure 5.7 Autoradiograph of ^{125}I L3/4 ES immunoprecipitated by sera from some Nigerian donors as indicated in the figure. The precipitates were analysed under non-reducing conditions on a 5-25% SDS-PAGE gel.

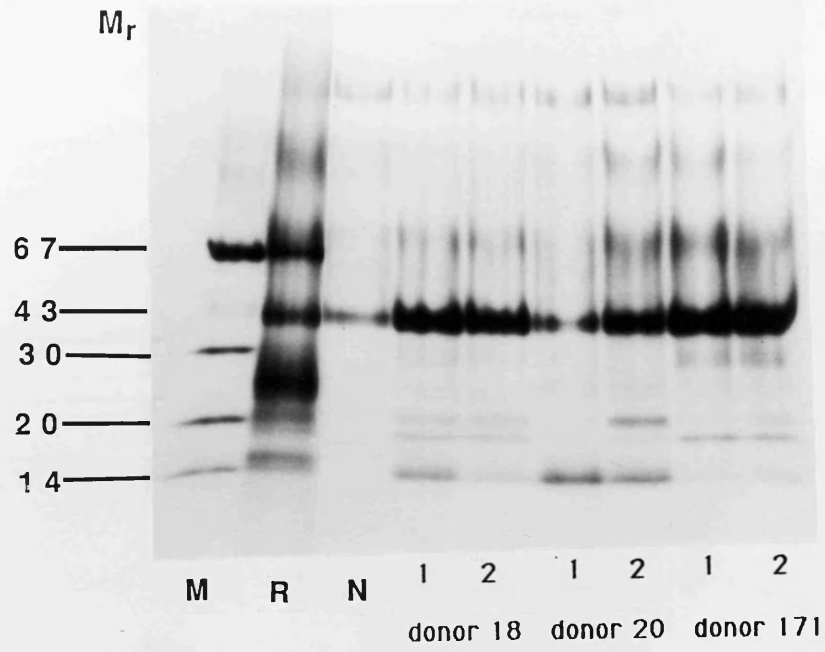
M - Markers
R - Reference ^{125}I L3/4 ES
N - Normal Human Serum



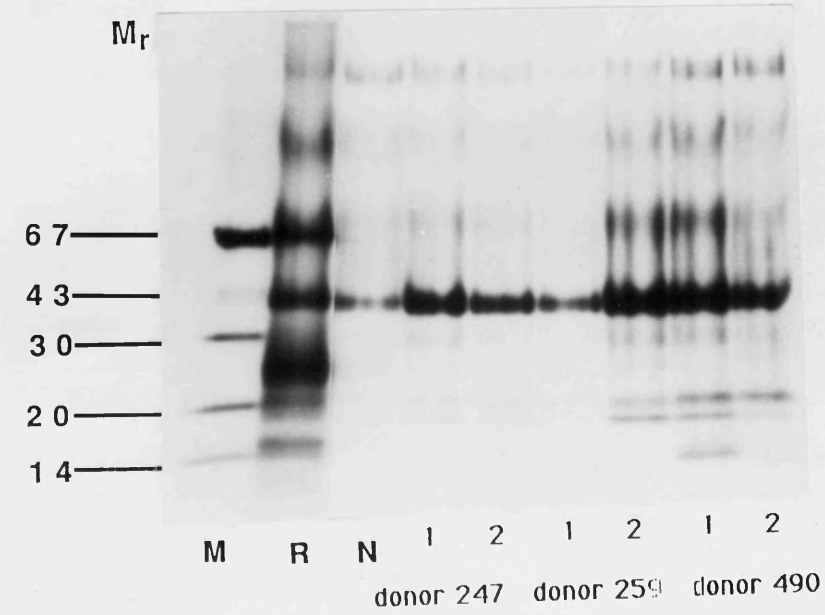
Nigerian donors versus ^{125}I L3/4 ES

Figure 5.8 Autoradiograph of ^{125}I L3/4 ES immunoprecipitated by sera from some Nigerian individuals as indicated in the figure. Sera in tracks 1 were collected in October 1987 and sera in tracks 2 were collected in May 1990.

Nigerian sera from two time points versus ^{125}I L3/4 ES



1 - October 1987 2 - May 1990



M - markers
R - reference ^{125}I L3/4 ES
N - normal human serum

Sera from other areas where *Ascaris* is endemic show the same patterns of heterogeneous recognition as seen in the Nigerian population. Figure 5.9 shows ABF immunoprecipitated by donors from Karachi. There is little general recognition of the higher molecular weight components as seen in the Nigerian donors but some individuals do recognise these components. There is differential recognition of the 43kDa molecule and ABA-1. Out of a series of 89 samples, 14 individuals recognise ABA-1.

The differential recognition of the 43kDa and ABA-1 molecules is also seen in an immunoprecipitation with L3/4 ES (Figure 5.10). Recognition of ABA-1 and the 43kDa molecules appears to be independent although some individuals do see both e.g. donor 30 and some neither e.g. donor 29.

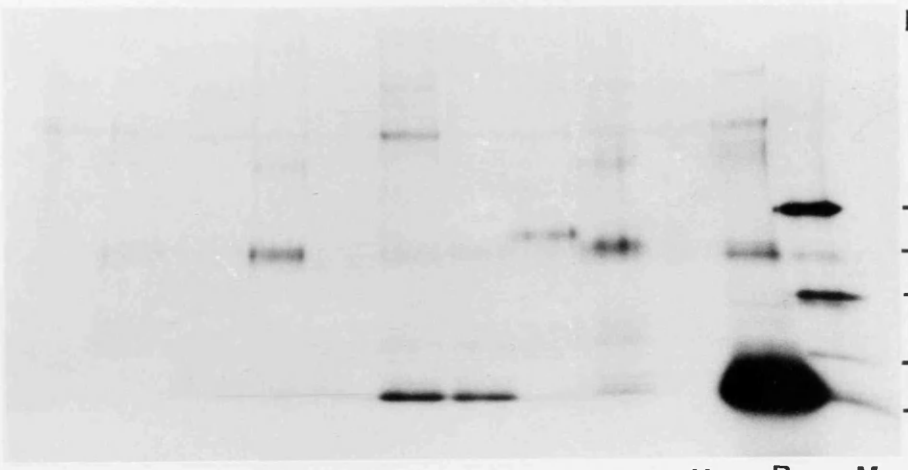
Preliminary studies were begun on a population of 300 adults and 200 children from the Gambia (where the prevalence of *Ascaris* is unknown), HLA typing to HLA-DR and HLA-DQ is being carried out in parallel with this study. An immunoprecipitation with some of the adults is shown in Figure 5.11. Fewer individuals recognise ABA-1 e.g. only 1388 very faintly here but the same differential recognition of the 43kDa component is seen.

5.2.4 IMMUNOPRECIPITATION OF ^{35}S LABELLED L3/4 ES.

As described in Chapter 3, *in vitro* labelling of L3/4 ES visualises a different set of ES components. Figure 5.12 shows an immunoprecipitation of ^{35}S methionine-labelled L3/4 ES by selected Nigerian individuals. Despite the larger number of ES products labelled by this method the number and range of antigens precipitated is not any greater than for the radioiodination method. The normal serum precipitates a lot of components (more than some of the donors). The 23kDa, 30kDa and 40kDa molecules are

Figure 5.9 Autoradiograph of ^{125} I ABF immunoprecipitated by sera from some Karachi donors as indicated in the figure. The precipitates were analysed under non-reducing conditions on a 5-25% SDS-PAGE gel.

Karachi Donors versus ^{125}I ABF

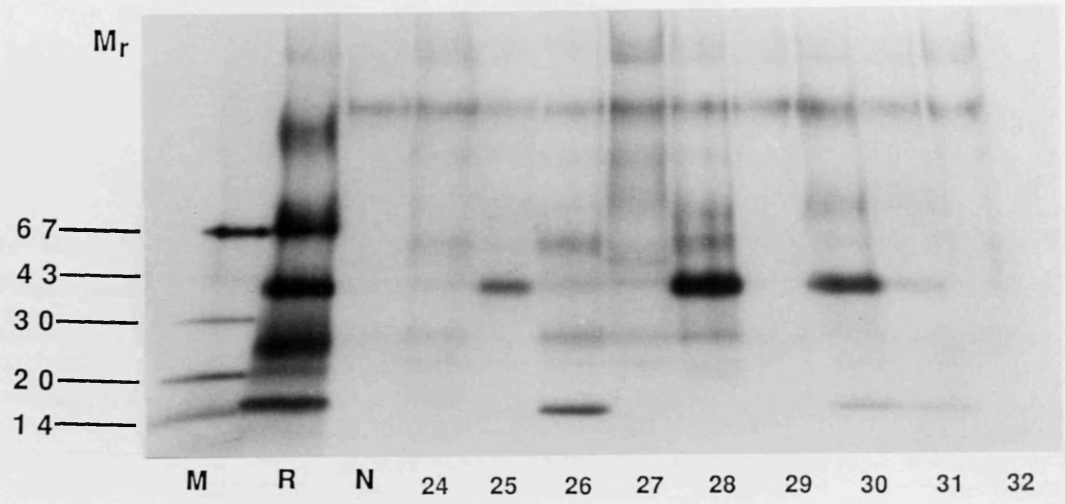


Karachi donors

M - Markers
R - Reference ^{125}I ABF
N - Normal Human Serum

Figure 5.10 Autoradiograph of ^{125}I L3/4 ES immunoprecipitated by sera from some Karachi donors as indicated in the figure. The precipitates were analysed under non-reducing conditions on a 5-25% SDS-PAGE gel.

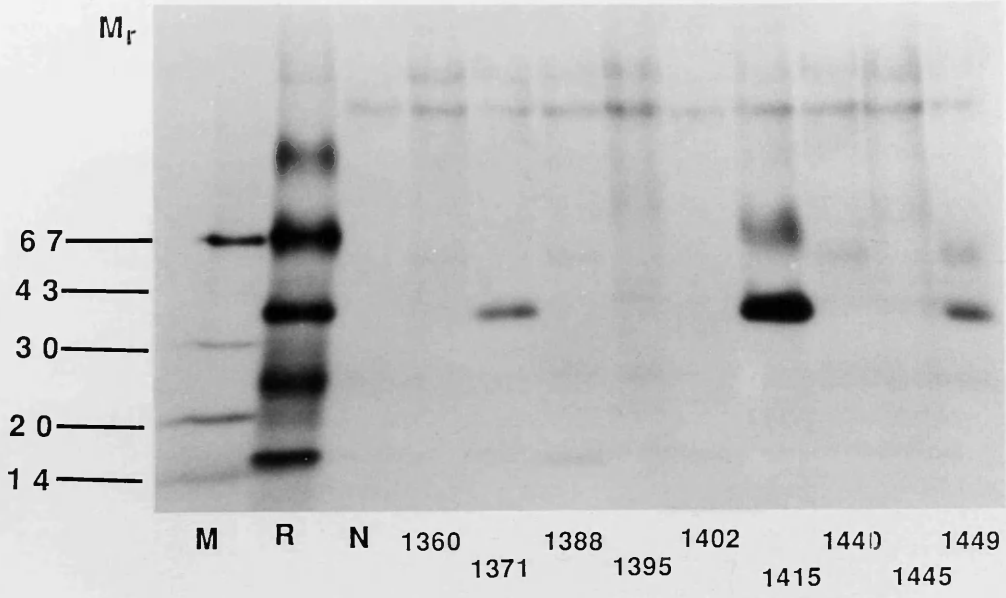
Karachi donors versus ^{125}I L3/4 ES



M - Markers
R - Reference ^{125}I L3/4 ES
N - Normal Human Serum

Figure 5.11 Autoradiograph of ^{125}I L3/4 ES immunoprecipitated by sera from some Gambian donors as indicated in the figure. The precipitates were analysed under non-reducing conditions on a 5-25% SDS-PAGE gel.

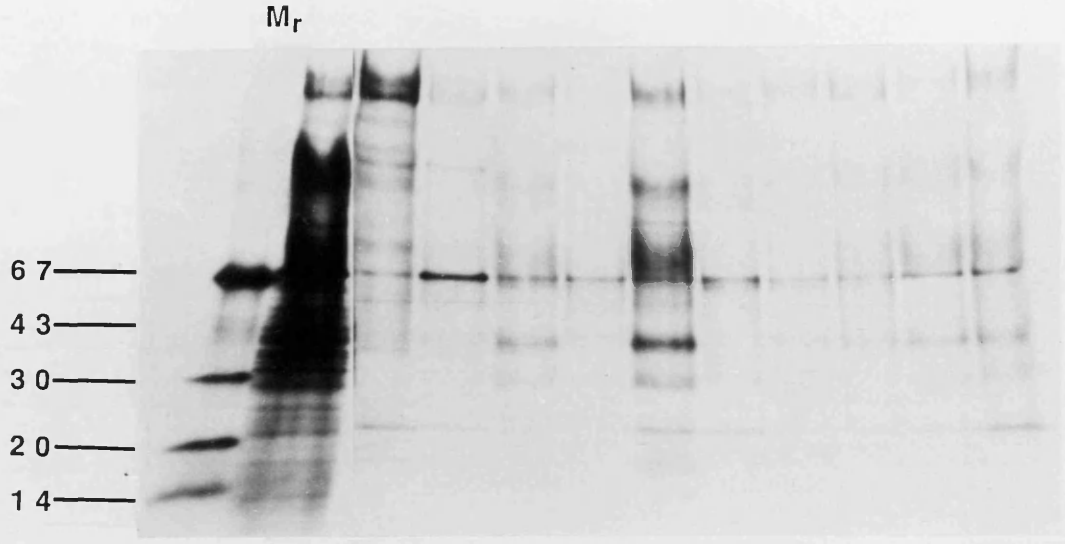
Gambian serum versus ^{125} I L3/4 ES



M - markers
R - reference ^{125} I L3/4 ES
N - Normal Human Serum

Figure 5.12 Autoradiograph of ^{35}S L3/4 ES immunoprecipitated by sera from some Nigerian donors as indicated in the figure. The precipitates were analysed under non-reducing conditions on a 5-25% SDS-PAGE gel.

Nigerian donors versus ^{35}S L3/4 ES



M Markers
R Reference ^{35}S L3/4 ES

differentially recognised in this system. ABA-1 is not labelled and hence not detected by this method.

5.2.5 WESTERN BLOTTED ABF, PROBED WITH ANTI-IgG.

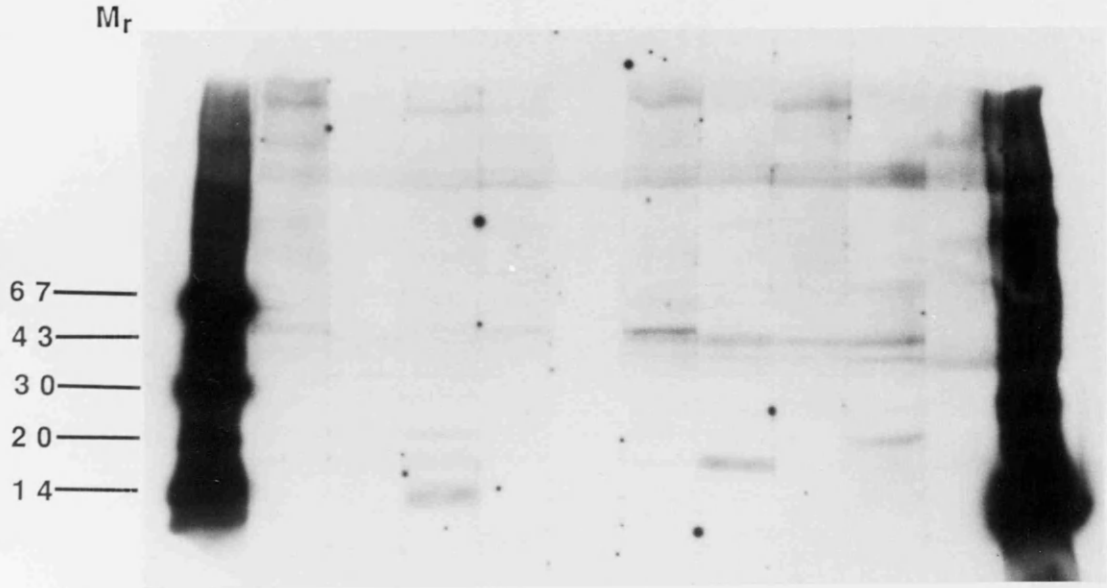
A different detection method for the presence of IgG-specific antibodies is to blot the antigen onto nitrocellulose, incubate in test serum and then, after washing, probe for the presence of IgG using a radiolabelled anti-IgG reagent. As this method uses large quantities of antigen, only ABF was used because of the limited availibility of the other preparations. The autoradiograph of a typical blot is shown in Figure 5.13. There is some non-specific recognition as illustrated by the bands in the normal serum track, although no binding of the radiolabelled probe to antigen on nitrocellulose was detected on the strip in which the serum incubation step was omitted (track labelled -). Some recognition patterns are similar to those seen in the immunoprecipitations e.g. donor 28's recognition of ABA-1, donor 85's recognition of the 20kDa molecule. On the whole, however, the blots did not show as full a repertoire of recognition as seen with the immunoprecipitations, especially in the 14-20kDa range. This could be due to the orientation of antigen binding to the nitrocellulose which would result in the antigenic epitopes not being available for antibody binding.

5.2.6 WESTERN BLOTTED ABF, PROBED WITH ANTI-IgE.

Figure 5.14 shows an autoradiograph of a western blot of ABF treated in the same way as the anti IgG blot but probed instead with a radiolabelled anti-IgE reagent. The normal serum tracks were completely clean although two *Ascaris* laboratory workers (Jackie Christie and Eleanor Fraser) showed IgE responses, one to the ABA-1 molecule and the other to higher molecular

Figure 5.13 Autoradiograph of Western blotted ABF, detected with serum from the Nigerian donors indicated and then probed with a ^{125} I anti-human IgG reagent. The marker and reference tracks were run on the gel with radiolabelled reagents and the nitrocellulose tracks not included in incubation steps but added again before exposure to film.

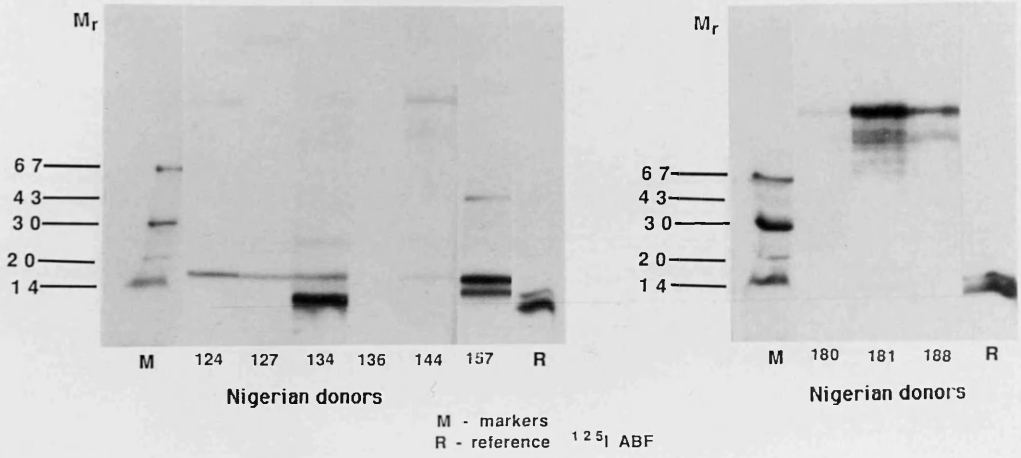
ABF Western blot versus Nigerian donors probed with ^{125}I anti-human IgG



M Markers
R Reference ^{125}I ABF

Figure 5.14 Autoradiograph of Western blotted ABF, detected with serum from the Nigerian donors indicated and then probed with a ^{125}I anti-human IgE reagent. The marker and reference tracks were run on the gel with radiolabelled reagents and the nitrocellulose tracks not included in incubation steps but added again before exposure to film.

Western Blot of ABF probed with human serum, detected with ^{125}I anti-human IgE



M - markers
R - reference ^{125}I ABF

weight markers, similar to donor 188. ABA-1 is a major target for recognition by IgE, occasionally the 43kDa molecule and other, higher molecular molecules. The recognition of the approximately 10kDa molecule by e.g. donor 134 is found with another few donors but the nature or function of this molecule(s) is unknown.

5.3 DISCUSSION

The humoral arm of the human immune response to infection with *Ascaris lumbricoides* has been investigated in this work by means of ELISA tests. These have been developed against antigens which in some senses can be thought of as reflecting the different life-cycle stages of the parasite; the two larval stages (L2 ES and L3/4 ES) and the adult stage (ABF). The ELISA method reflects comparative levels of antigen specific antibody (not total levels because some antigenic epitopes may be bound to the ELISA plate) but, apart from responses to the purified ABA-1, doesnot indicate the specificity of the recognition to different parasite components. It is interesting to note that even donors who are persistently worm-free have antibodies to ABF. There are a few components found in all stages, notably ABA-1, so the explanation for this must be that these individuals (obviously exposed to infective eggs continually) are exposed to these antigens in the larval stages but terminate the development of the parasite before it reaches the adult, intestinal stage. It may be that dead and disintegrating larvae release components which would not be released by viable larvae.

The results presented here do not reveal any relationships between total antibody levels and epidemiological data such as epg values or worm burden. The age range of the sample size is too small (5-16 years) to draw any conclusions and it would be interesting to assay a population encompassing all age classes to see if there were any age-related changes in antibody levels or recognition of the different antigens - and whether these change for individuals over time.

The ELISA studies with the periodate treated antigen (which disrupts some or all carbohydrate epitopes depending on the severity of the treatment) and the autoclaved antigen (where heat denatures or unfolds polypeptides, thereby again affecting epitope structure of antigenic determinants) revealed some interesting observations. The observation that periodate treatment can halve the binding of antibody to ABA-1 (Figure 5.3a) is most interesting when biochemical studies on this molecule have shown it to contain less than 1% carbohydrate. It could be that what little carbohydrate is present forms a major antigenic epitope of the molecule. However, not all sera respond in this way so there would appear to be differential recognition at the epitope level. This is borne out by results from the other antigens with no clear patterns suggesting that differences at the epitope level are important. Monoclonal antibodies would be of great value here in determining the nature of epitopes which are recognised by antibodies and if this recognition corresponds to protection of any sort. Such work has been carried out on *Toxocara canis* and 5 out of 8 monoclonal antibodies developed were found to be against carbohydrate determinants common to several otherwise distinct molecules (Maizels *et al.*, 1987).

The technique of precipitating radiolabelled antigen with serum and then analysing such precipitates an SDS-PAGE gels allows for a qualitative analysis of the antibody responses to *Ascaris* infection in that one can visualise which antigens are being recognised by the various antibodies in any one serum sample. As shown for the Nigerian, Karachi and Gambian populations, considerable heterogeneity exists in the nature of individual responses to the antigens and the effect is therefore, probably, universal. It is also possible to distinguish "strong" recognition i.e. dark bands on autoradiographs from "weak" where faint bands are seen. This

semi-quantitaive analysis was carried out rigorously by Haswell-Elkins *et al.* (1989) on 8 different bands seen in immunoprecipitation of ^{125}I L3/4 ES. Some correlations were seen between the sum of these subjective values and the worm burden of individuals four months after the blood samples were taken. Identifying all eight antigens which were used in Haswell-Elkins' analysis on the L3/4 ES gels described here was not easy. Some of the higher molecular weight antigens were either apparently universally recognised by individuals from an endemic area or not apparent in our preparations. The answer to these problems may lie in the different analytical conditions used in the two studies and the different geographical sources of the sera.

The common feature of these techniques is that while the ELISAs detect humoral antibody quantitatively and the immunoprecipitates qualitatively, both techniques detect circulating antibody. Antibody bound to antigen in immune complexes will not be detected by either of these methods. Treatment of serum to dissociate any immune complexes present may well reveal new levels and specificities of antibody in the serum of infected individuals. The antibody detected will reflect exposure to antigen but not the recency of this exposure and the antibody levels detected are those "in excess" of those involved in active immune responses and may not be protective antibodies (if these exist). Some workers on other parasite systems have developed antigen capture ELISAs where a single antigen of the parasite can be detected (Barsoum, Colley and Kamal, 1990). This method requires selecting an antigen which is the target of an immune response, circulates in the infected individual and is only present when infection is current.

The immunoprecipitates do demonstrate the differential recognition of parasite antigens, also seen by Haswell-Elkins *et al.*(1989) and this is highly reminiscent of the patterns seen with different strains of mice (Chapter 4). This leads on to the question of the factors which influence recognition of particular antigens and the role this plays in the immune response. The explanation of differences in the genetic control of the immune response is a real possibility, especially in the light of the animal work. The results of the typing of the Karachi and Gambian populations will provide an opportunity to investigate if the recognition of any of the parasite antigens are under HLA control. It will be intersting to compare any associations between recognition of parasite antigens and HLA antigens and compare with the tentative reporting of HLA associations in ascariasis in the Carribean (Bundy, 1988b).

A comparison of the relative merits of the two parasite-specific IgG detection techniques - immunoprecipitation (which also detects other classes of antibody) and western blotted antigen detection of IgG - would seem to favour the former method. It uses much smaller quantities of both serum and antigen - major considerations when both of these can be in very short supply - and allows for a greater repertoire of antigen recognition to be visualised. The advantages of immuno-blots, however, are that they are quicker to perform and there is no possibilty of tracks merging into each other. The apparently more limited recognition repertoire observed using blots may be due to the manner of binding of antigen to the nitrocellulose.

The same argument about reduction in available epitopes obviously applies to the IgE blots as well but here there is no comparable method which allows identification of the parasite components which elicit an IgE

reponse. RAST assays allow for determination both of total IgE levels and determination of levels of IgE directed against particular allergens. This blotting method , while it uses relatively large amounts of serum uses less than a PCA assay would and is more informative as to which components elicit an IgE response. The work shown here confirms the previously untested assumption that ABA-1 is an allergen in humans. While IgG and IgE recognition of ABA-1 does often occur in the same individual, some appear to have only an IgG or only an IgE response to the molecule. Whether this is related to predisposition to atopy, is a result of the way the molecule is presented to the individual or is also under direct HLA control is interesting to speculate.

CHAPTER 6

ASCARIS SURFACE STUDIES

6.1 INTRODUCTION

The cuticle of nematodes is a complex extracellular structure composed primarily of proteins with lipid and carbohydrate (Bird, 1980). The cuticle can be divided structurally into three zones - cortical, median and basal - with an outermost region termed the epicuticle. This has the superficial appearance of a classical cellular plasma membrane under transmission electron microscopy, but it is no longer considered to be a homologous structure (Kennedy, Foley, Kuo *et al.*, 1987). The cuticle presumably plays an important role in maintaining the integrity of the parasite against attack from various immune effector mechanisms. The result of a breakdown in this integrity was amply demonstrated when infective and new-born *T. spiralis* were killed by antibodies and eosinophils; the larvae were seen to have burst, due to the high internal pressure of the nematode, with external organs extruding through the presumably weakened or damaged cuticle (Mackenzie, Jungery, Taylor *et al.*, 1980).

The surface has the following properties of potential immunological relevance. First, the epicuticle can undergo structural changes between moults and has been identified as a major source of ES products (reviewed by Phillip and Rumjaneck, 1984). A 30kDa molecule found on the surface of *Brugia* species is also found in ES preparations of the worm (Devaney, 1988). In *T. canis* surface antigens, as detected by either immunfluorescence (Smith, Quinn, Kusel *et al.*, 1981) or radiolabelling (Maizels, De Savigny and Ogilvie, 1984) were shown to be shed from the surface of infective larvae and found in ES preparations. Secondly, Smith *et al.* (1981) showed that surface antigens were not shed at 2°C or in the presence of antimetabolites but, at 37°C, there was complete loss of

surface labelled antigen in 3 hours. Also, exposure of epitopes on the surface of living parasites is much more restricted than for ES antigens and an epitope common between two species might be exposed on one but not on the other (Kennedy, Maizels, Meghji *et al.*, 1987; Maizels *et al.*, 1987).

The selective nature of surface labelling amplifies the few components on the surface labelled by any agent and allows a direct analysis of their antigenic nature by conventional immunochemical techniques. Radio-labelling is a commonly used method for surface studies of parasites (Pritchard, McKean and Rogan, 1988; Selkirk, Neilson, Kelly *et al.*, 1989: Devaney, 1988; Maizels *et al.*, 1984; Maizels *et al.*, 1987). The two most common methods of surface labelling are the Bolton-Hunter method and the IODO-GEN method - the bias of these techniques is towards polypeptides containing lysine and tyrosine respectively. While labelling with such extrinsic agents has provided important details on surface-associated proteins and glycoproteins, the distinction between which epitopes are or are not exposed on the surface can be lost.

In an attempt to characterise surface molecules, some studies have homogenised larval cuticles and extracted molecules from the homogenate (Devaney, 1988). Others have removed antigens by making use of the properties of detergents to remove only surface-associated molecules e.g. cetyl trimethyl ammonium bromide (CTAB) (Pritchard, Crawford, Duce *et al.*, 1985). This detergent selectively stripped some surface molecules from *N. dubius* which did not represent the full range found in ES and homogenised larval preparations. This could be due to the fact that the other antigens are somatic or normally secreted from pores; that the detergent has not fully stripped the epicuticle or that the the surface is composed

of a limited number of antigens. The opposite problem with antigen stripping is that it can be too harsh and sub-suface compoents may also be removed.

In contrast to radio-labelling and detergent stripping, immunofluorescence (which examines the host-parasite interface directly) focusses only on the surface exposed epitopes and is not biased by the biochemical nature of the epitopes.

Surface studies of different larval stages have demonstrated strict stage-specificity e.g adults and infective larvae of *T. spiralis* were originally found to display distinct sets of surface antigens by the differential recognition of the surfaces by antibody from rats (Mackenzie *et al.*, 1980). Newly hatched infective *T. canis* larvae bind various *Toxocara* monoclonals very poorly but by day 2 of *in vitro* culture antibody binding is increased considerably (Maizels *et al.*, 1987). While this does not demonstrate stage specificity it does illustrate that the larval changes rapidly after hatching and that antigens recognised at later times of the stage are not expressed immediately and thus the surface is not an inert, unchanging entity.

Work on *A. suum* by Fetterer and Urban (1988) looking at soluble proteins from the cuticles of the different developmental stages revealed considerable heterogeneity between the cuticles. Proteins from the L2 cuticle differed from adult and other larval stages in both the number and molecular weights of protein bands. The amino acid composition of proteins was similar for adult and L3/4 stages but glycine and proline were present in lower amounts in proteins from the L2 stage. These results suggest that there are stage-specific differences in cuticular

proteins from *A. suum*, the greatest difference being seen between L2 and the other stages. The hypothesis is examined here with regard to antibody reconition of surface antigens on the L2 and L3/4 stages - these are distinct from but possibly reflective of the cuticle proteins.

As with all immunparasitological studies , one of the major questions to ask is whether it is possible to i dentify surface antigens which are involved in immune recognition and whether exploitation of such recognition to generate immunity is possible. Some surface molecules which are antigenic have been identified e.g. the 29/30kDa antigen from *Brugia* (Devaney, 1988; Maizels *et al.*, 1989) and a 65kDa antigen from *N. dubius* (Pritchard *et al.*, 1985). Antibodies to sub-surface structural elements have also been detected (Pritchard *et al.*, 1988) and it is suggested that antibody with specificity against superficial cuticular epitopes may remove these or alter the surface in some way such that an immune response against deeper lying elements is generated. While the real problem appears to be getting through the surface, the inclusion of both surface and sub-surface epitopes in any recombinant vaccine may induce antibody to effectively destroy the nematode at the cuticular level.

Are these surface characterised molecules recognised by infected animals and humans? The antigenicity of surface-labelled filarial proteins in infected human patients and laboratory hosts has been well documented (Maizels *et al.*, 1989) and the well-characterised 29/30kDa antigen is strongly recognised by infected individuals. Investigations of the humoral immune response of *Ascaris* infected humans to the surface of infective larvae are described in the following work.

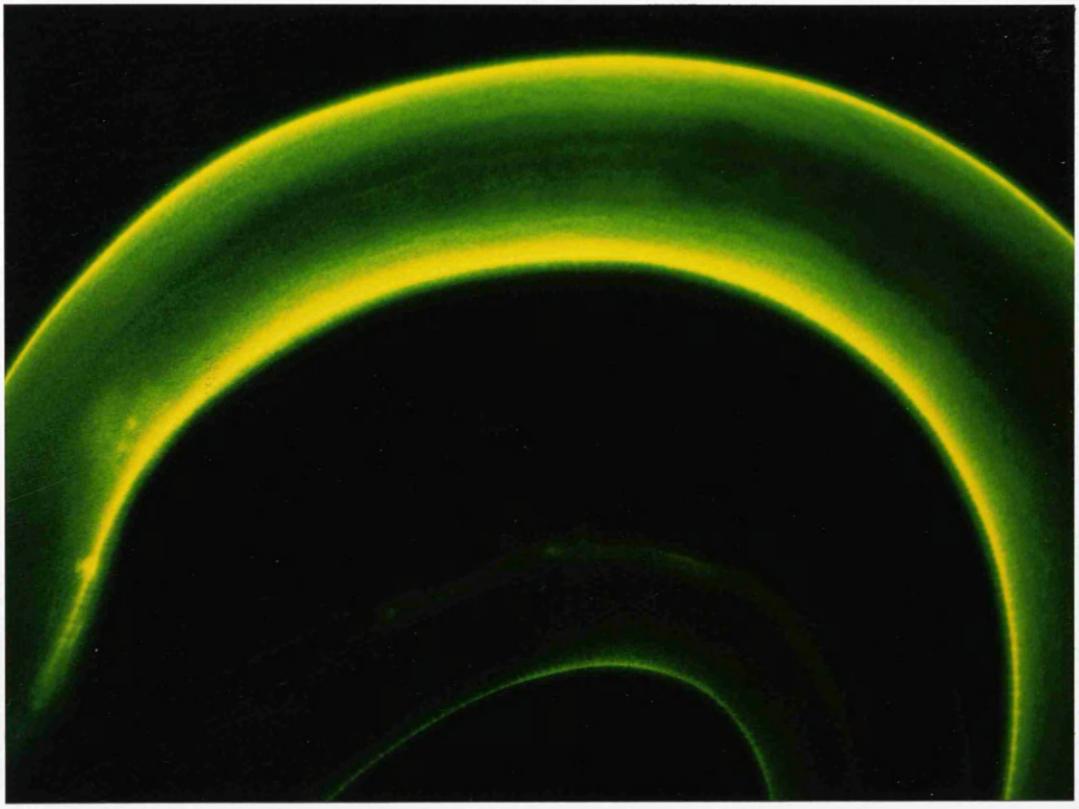
6.2 RESULTS

6.2.1 TIME COURSE OF L2 AND L3/4 SURFACE SHEDDING.

L2 and L3/4 were fluorescently labelled with anti-*Ascaris* infection serum as described in 2.15 but with no sodium azide present in any of the washing or incubation stages so that metabolic processes would not be inhibited. Photometer readings were taken at 0, 5 and 8 hours after the labelling, the larvae being kept at room temperature. The readings were not particularly informative but qualitative changes could be seen in the fluorescence with time. Figure 6.1a is a photograph of an L3/4 larvae at 0 hours and Figure 6.1b an L2 larvae at 0 hours. The fluorescence (and hence level of antibody binding) is evenly distributed over the surfaces of the two larval stages. Due to the diameter of the L3/4 larvae the fluorescence appears much brighter at the edge of the larvae - this was avoided when measurements were taken. The L2 larva in Figure 6.1b probably moved during exposure which accounts for the lack of clarity of the parasite outline. Five hours later, larvae from the same cultures were examined - Figure 6.1c shows an L3/4 larva at this time and some changes can be seen. The fluorescence is less even and, especially at the edges, blebs or areas of very bright fluorescence are seen. The fluorescence of the L2 at 5 hours is still quite even (Figure 6.1d) but a few slightly brighter areas can be seen on the parasite. By 8 hours the L3/4 larvae have lost most of their fluorescence (Figure 6.1e). They displayed the distinctive pattern of the edges of the posterior end fluorescing quite brightly and a very few patches of brightness along the edge of the parasite. At 8 hours, the infective larvae are still fairly bright but the fluorescence was considerably more granular. These patches of brightness may be areas where the labelled molecules have aggregated together or may indicate an area where, relative to the whole parasite, no shedding has taken place.

Figure 6.1a Photograph of L3/4 larvae with a primary layer of 1/30 rabbit anti-*Ascaris* sera and a secondary layer of 1/30 fluorescein conjugated goat anti-rabbit antibody. The photograph was taken at 0hr with Kodak Ektachrome film P1600 at x40 magnification with an exposure time of 2 minutes.

Figure 6.1b Photograph of an L2 larva with a primary layer of 1/30 rabbit anti-*Ascaris* sera and a secondary layer of 1/30 fluorescein conjugated goat anti-rabbit antibody. The photograph was taken at 0hr with Kodak Ektachrome film P1600 at x40 magnification with an exposure time of 2 minutes.



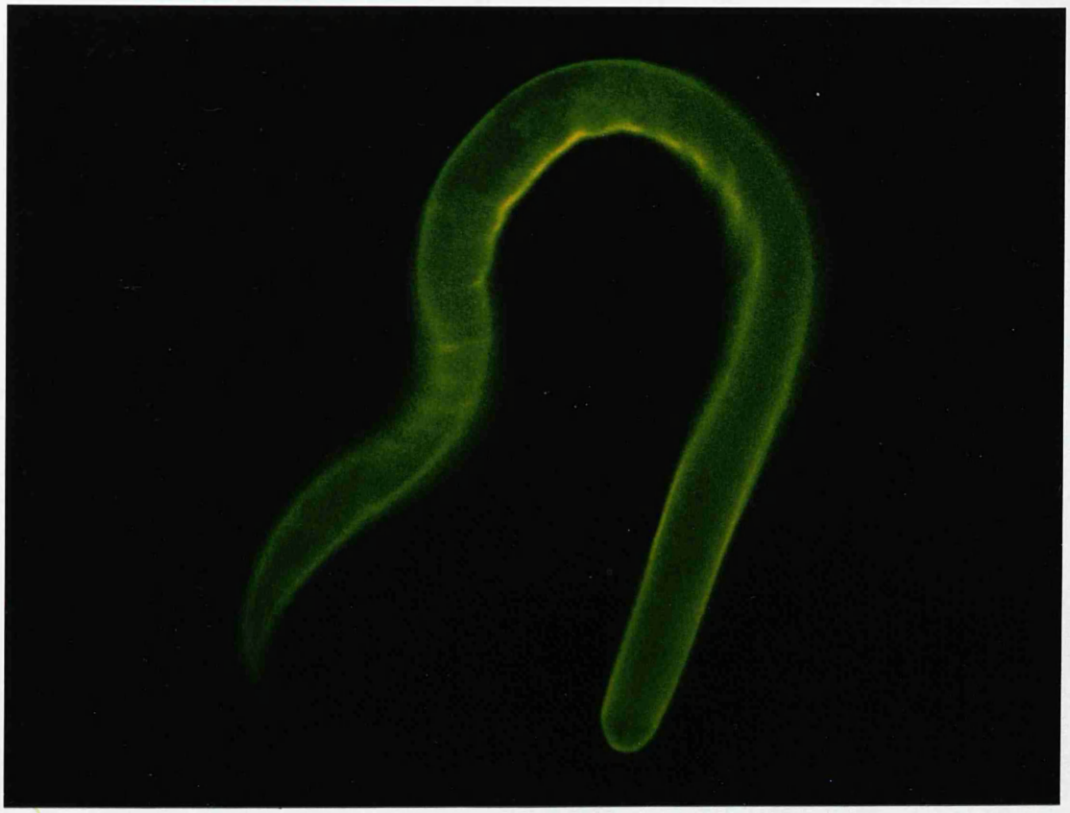
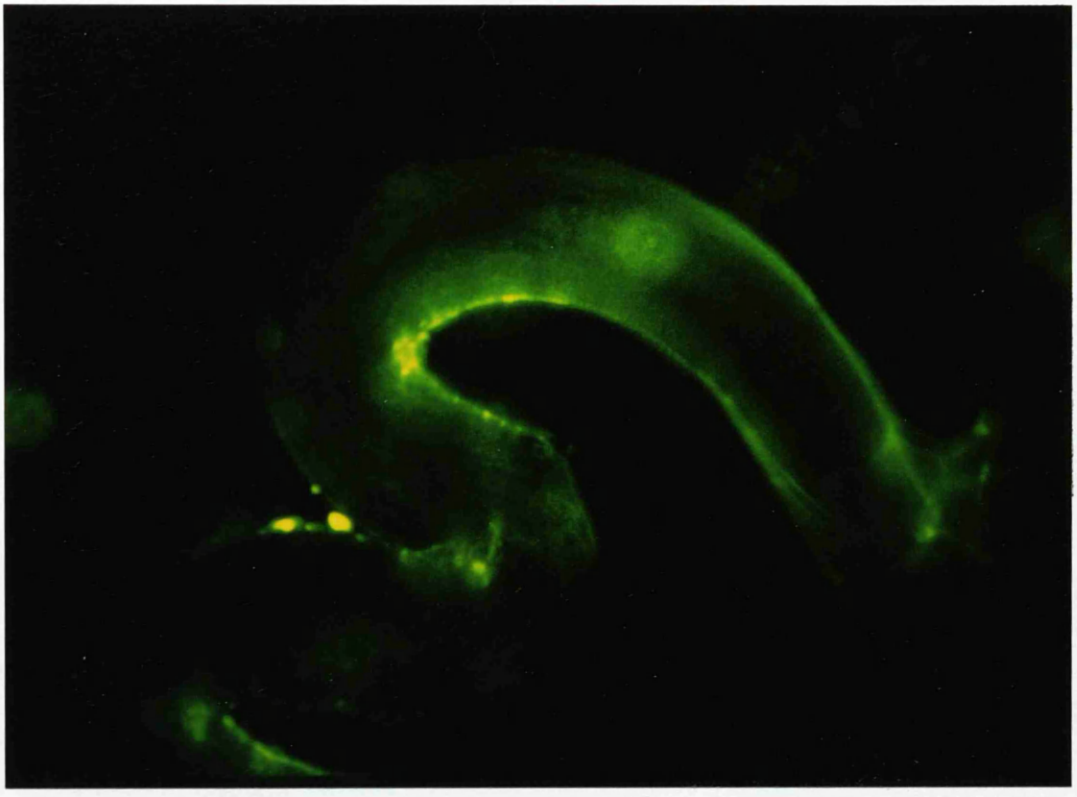


Figure 6.1c Photograph of an L3/4 larva with a primary layer of 1/30 rabbit anti-*Ascaris* sera and a secondary layer of 1/30 fluorescein conjugated goat anti-rabbit antibody. The photograph was taken at 5hr with Kodak Ektachrome film P1600 at x40 magnification with an exposure time of 2 minutes.

Figure 6.1d Photograph of an L2 larva with a primary layer of 1/30 rabbit anti-*Ascaris* sera and a secondary layer of 1/30 fluorescein conjugated goat anti-rabbit antibody. The photograph was taken at 5hr with Kodak Ektachrome film P1600 at x40 magnification with an exposure time of 2 minutes.



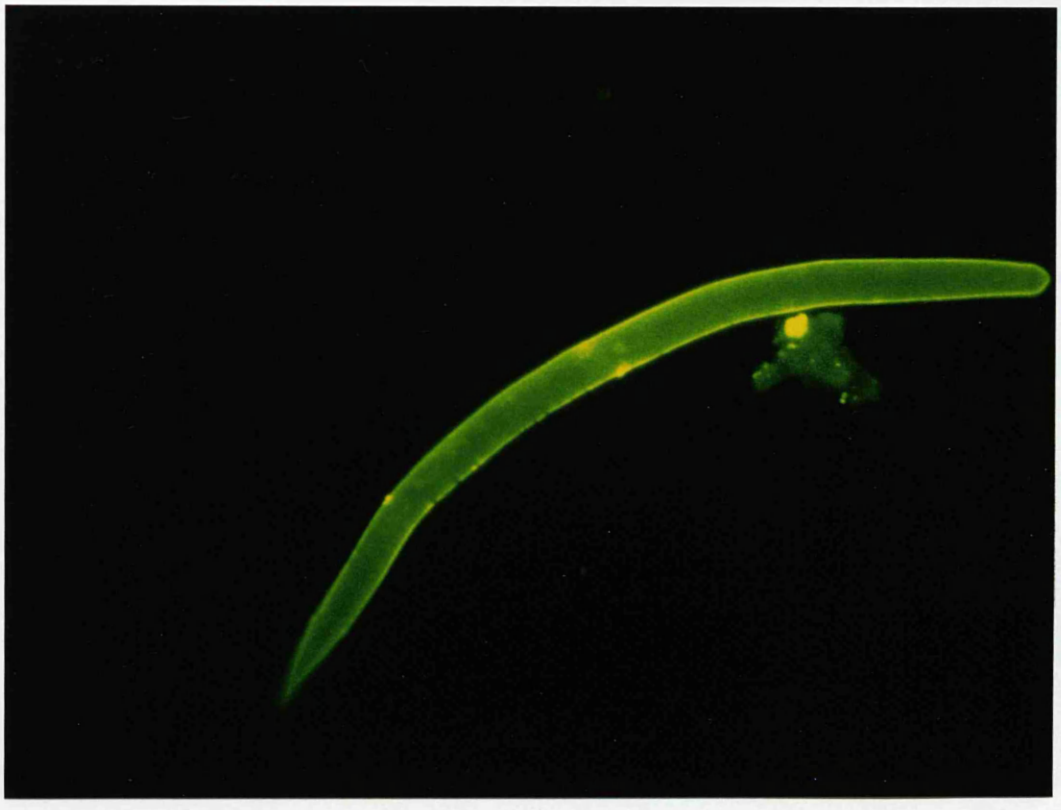
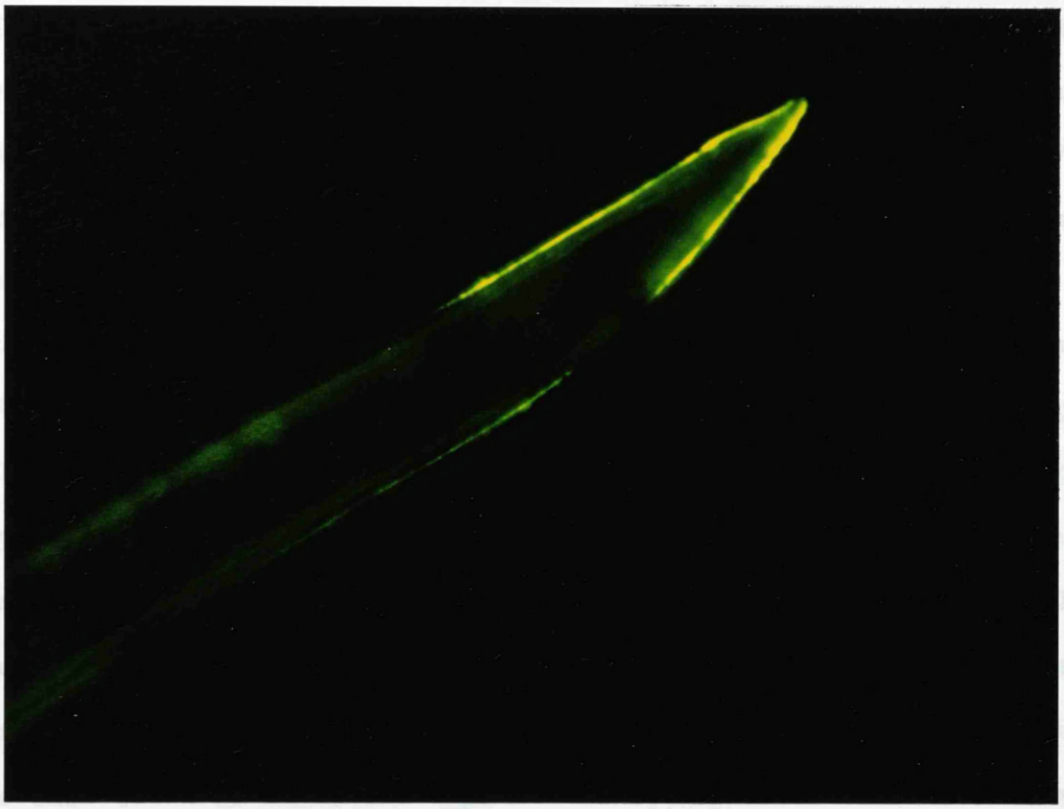
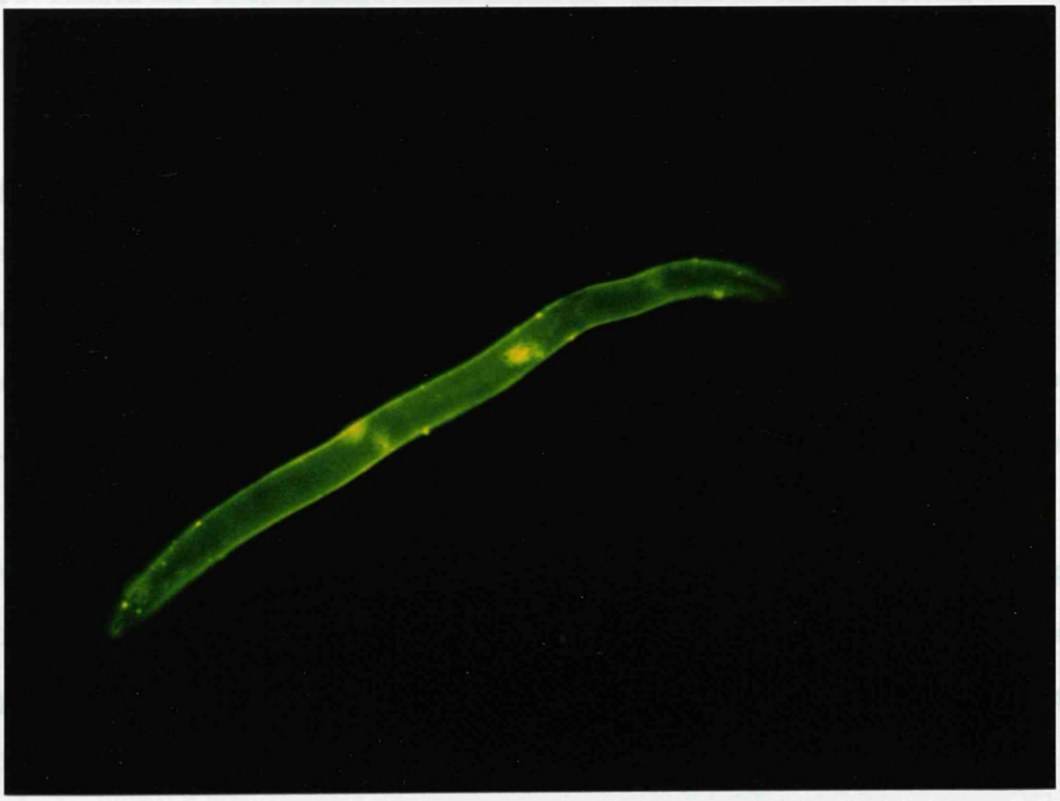


Figure 6.1e Photograph of an L3/4 larva with a primary layer of 1/30 rabbit anti-*Ascaris* sera and a secondary layer of 1/30 fluorescein conjugated goat anti-rabbit antibody. The photograph was taken at 8hr with Kodak Ektachrome film P1600 at x40 magnification with an exposure time of 2 minutes.

Figure 6.1f Photograph of an L2 larva with a primary layer of 1/30 rabbit anti-*Ascaris* sera and a secondary layer of 1/30 fluorescein conjugated goat anti-rabbit antibody. The photograph was taken at 8hr with Kodak Ektachrome film P1600 at x40 magnification with an exposure time of 2 minutes.





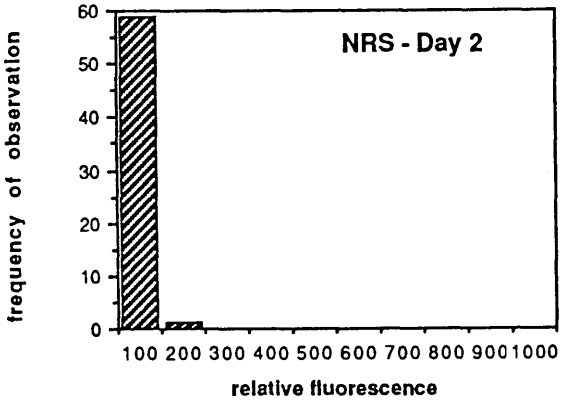
6.2.2 TIME COURSE TO DETERMINE OPTIMAL TIME FOR EXPRESSION OF ANTIGEN.

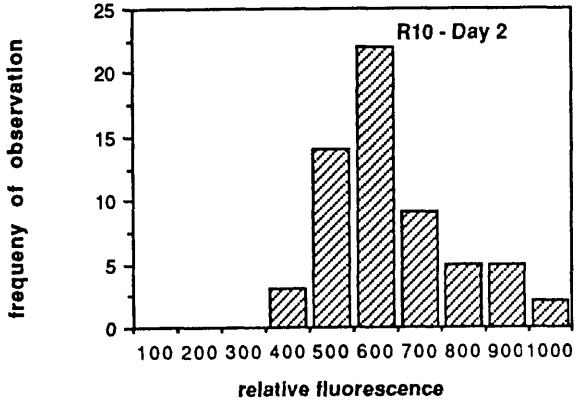
Just as newly hatched *T. canis* larvae fail to express some surface antigens recognised by specific antibodies (Maizels *et al.*, 1987) so, initial studies had shown that the surface of newly hatched L2 *Ascaris* larvae was not recognised by antibody from anti-infection (R10) serum while the surface of larvae cultured for 24 hours was (not shown). To determine the optimal time for expression of stage-specific antigen, a bulk culture of L2 larvae was hatched and the expression of antigen on days 2, 4, 7 and 10 after the initiation of the culture determined by quantifying the level of antibody binding by means of measuring relative fluorescence. All incubations were carried out in the presence of sodium azide to inhibit surface turnover. New cultures of L2 were hatched from the same batch of infective eggs at the appropriate times to ensure that there was always a day 2 culture with which to compare the other time points. These day 2 time points always showed a similar level and range of fluorescence - only the first is shown in Figure 6.2 and 6.3. The larvae were incubated with rabbit anti-*Ascaris* infection serum in Figure 6.2 and rabbit anti-L3/4 ES serum in Figure 6.3.

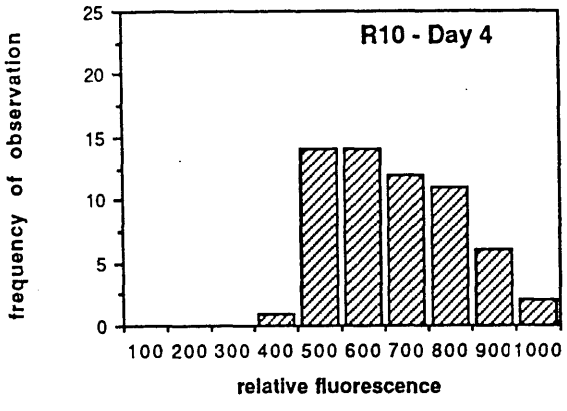
Figure 6.2 shows that the range of recognition of antibody for the larval surface (i.e. the relative fluorescence readings) increased with time so that the normal distribution of recognition at day 2 changes to a broad spread with a very heavy bias for lower values by day 10. Thus the majority of the surface antigens recognised by the rabbit in infection are no longer present or available in this culture system by day 7. If development of L2s to L3s occurs *in vitro* this would appear to happen to only a small proportion of the larvae as the rabbit would also have been exposed to the L3/4 surface and thus would recognise any L3 larvae in the culture.

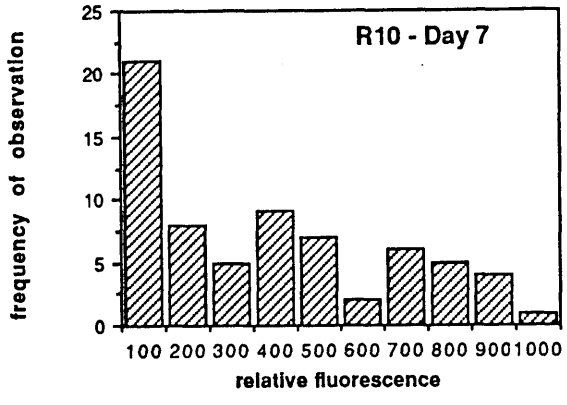
Figure 6.2 A time course of L2 larvae detected by binding of antibody from a 1/30 dilution of rabbit anti-*Ascaris* sera with a second antibody layer of 1/30 fluorescein conjugated goat anti-rabbit . Bound antibody was quantified by photon counting and the frequencies of observation in groups of 100 arbitrary units plotted for days 2, 4 , 7 and 10 of the same *in vitro* L2 culture.

Figure 6.2 Time course of L2 heterogeneity









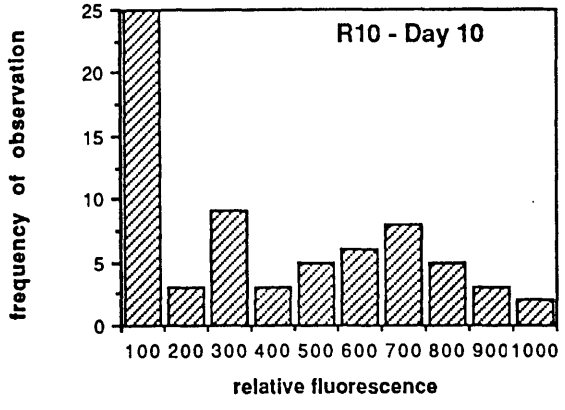
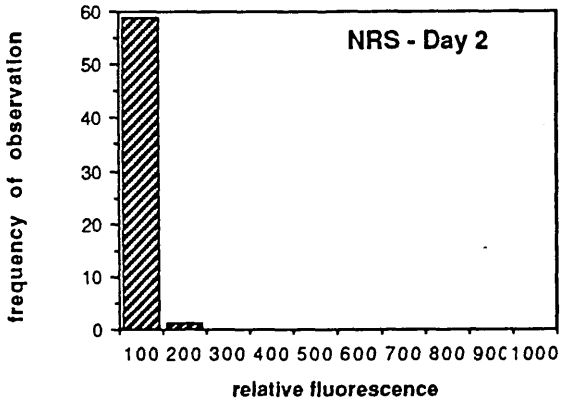
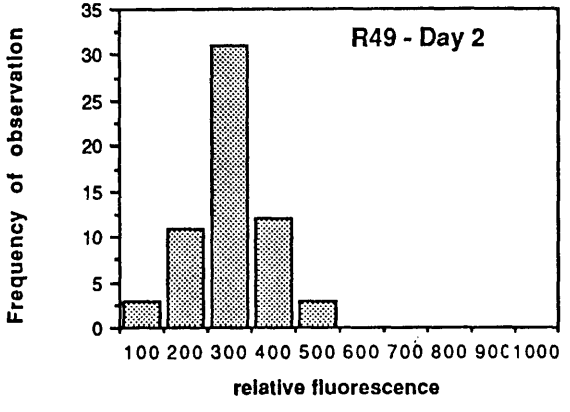
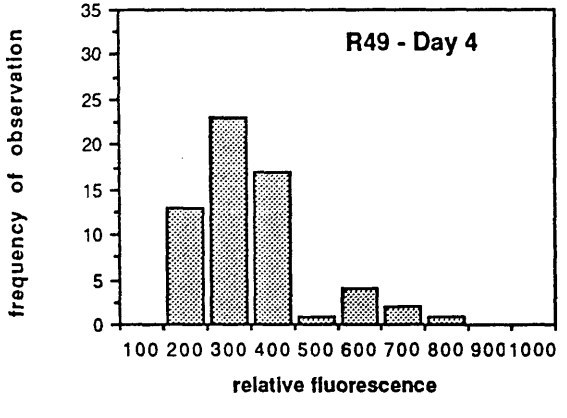


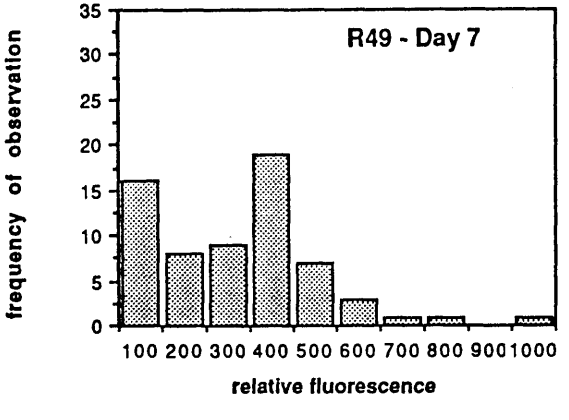
Figure 6.3 A time course of L2 larvae detected by binding of antibody from a 1/30 dilution of rabbit anti-L3/4 ES sera with a second antibody layer of 1/30 fluorescein conjugated goat anti-rabbit . Bound antibody was quantified by photon counting and the frequencies of observation in groups of 100 arbitrary units plotted for days 2, 4 , 7 and 10 of the same *in vitro* L2 culture.

Figure 6.3 Time course of L2 heterogeneity









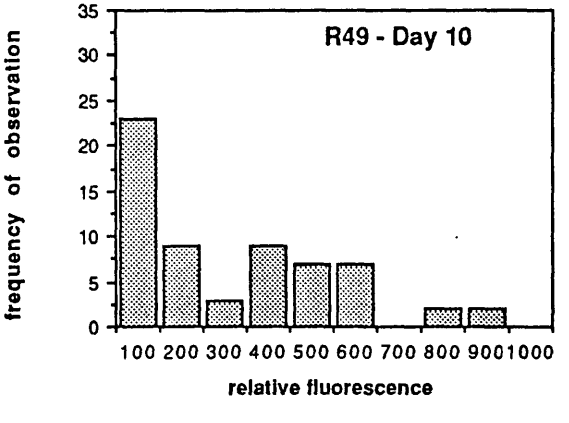


Figure 6.3 again shows that at day 2 there is quite a narrow range of recognition, this time at the lower end of the scale. While the majority of larvae remain poorly recognised by the serum, by day 10 a significant number are well recognised. This may be due to L2s developing to L3 larvae in culture. In the infected host the L2 larvae would have been arrested or developed into L3 or L4 larvae by around day 7.

On the basis of these results, day 2 L2 larvae were used in all subsequent experiments. A similar, less rigorous study was carried out for L3/4 larvae and day 7 after harvaesting from rabbit lungs chosen as the time point for subsequent immunofluorescent investigations.

6.2.3 DIFFERENTIAL RECOGNITION OF L2 AND L3/4 LARVAE BY ANTI-*ASCARIS* SERA.

Two cultures of L2s (2 day and 17 day) and a 7 day culture of L3/4 larvae were incubated in various rabbit sera as a primary layer for the indirect fluorescence test. R10 is rabbit anti *Ascaris* infection serum; R42, anti-ABF; R45, anti-ABA-1 and R49, anti-L3/4 ES. The mean relative fluorescence readings of more than 40 observations are shown in Figure 6.4. Both L2 and L3/4 are recognised strongly by the anti-infection serum. The L2s fluorescence is more intense than that of the L3/4 larvae (compare Figures 6.1a and 6.1b which were photographed under identical exposure times and magnification). The anti-ABF serum recognises the L2 larvae much more strongly than the L3/4s, suggesting that few antigens on the L3/4 surface are shared with body fluid from the adult. In contrast, the surface of L2s appears to share many antigens. The ABA-1 molecule does not appear to be expressed on the surface of either of the larval stages as demonstrated by the readings for the R45 serum - these are similar to readings generated by normal rabbit serum. The highest readings for L3/4 larvae are recorded

Figure 6.4 L2 and L3/4 larvae from *in vitro* cultures were incubated in various rabbit sera.

NRS - Normal Rabbit Serum

R10 - rabbit anti-*Ascaris* infection serum

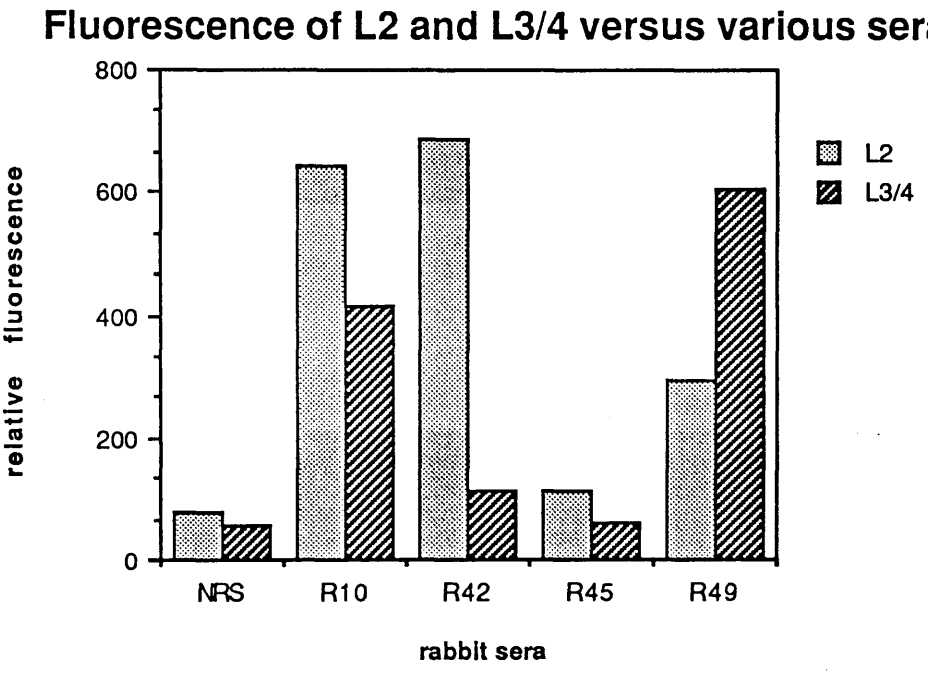
R42 - rabbit anti-ABF serum

R45 - rabbit anti-ABA-1 serum

R49 - rabbit anti-L3/4 ES serum

Bound antibody was detected by fluorescein conjugated goat anti-rabbit antibody and quantified by photon counting. The mean relative fluorescence of more than 40 observations is indicated.

Figure 6.4


Fluorescence of L2 and L3/4 versus various sera
800
600
400
200
0
relative fluorescence
NRS
R10
R42
R45
R49
rabbit sera
L2
L3/4

for the R49 serum - indicating that L3/4 ES preparations and the surface of L3/4 larvae have epitopes in common and, in all likliehood, ES molecules may be surface molecules.

6.2.4 EXAMINATION OF LARVAL AND SPECIES STAGE-SPECIFICITY.

The different levels of recognition by the L2 and L3/4 larval stages were examined further to determine whether the antigens expressed on the surface of these developmental stages were specific to those stages. *T canis* L2 larvae were also examined as they are closely related phylogenetically to *Ascaris* and are the developmemtal equivalent of infective *Ascaris* larvae. Incubations of larvae were performed with serial dilutions of anti-*T. canis* and anti-*A. suum* sera and the results of the fluorescence readings are presented in Figure 6.5. It can be seen that only homologous serum antibody bound to the surface of *A. suum* L2 (Figure 6.5c) but anti-*Ascaris* antibody bound to infective *T. canis (Figure 6.5a)*. This specificity also broke down when L3/4s was examined as the anti-*T. canis* serum recognised some surface antigens from this developmental stage (Figure 6.5b).

6.2.5 STAGE-SPECIFICITY EXAMINED BY ADSORBED SERA.

The binding of anti-*Ascaris* infection serum (R10) and anti-L3/4 ES serum (R49) to L2 and L3/4 larvae was examined before and after the sera had been absorbed for 1 hour against culture derived larvae of both stages.

Figure 6.6 shows the recognition of L2 larvae by R10 serum. Figure 6.6a is a typical distribution of relative fluorescence readings for L2 larvae and anti-infection serum. Figures 6.6b and 6.6c show the same batch of larvae recognised by serum which has been absorbed against L2 and L3/4 larvae respectively. Figure 6.6b shows that absorption was not complete as there

Figure 6.5 Antibody binding to the surfaces of infective and lung stage *Ascaris suum* and infective *Toxocara canis* (Figures 6.5c, 6.5b and 6.5a respectively). Larvae were probed with with normal rabbit serum at a dilution of 1/20 , rabbit anti-*Ascaris* and rabbit anti-*Toxocara* sera at serial dilutions. Bound antibody was detected by fluorescein conjugated goat anti-rabbit antibody and quantified by photon counting. The relative fluorescence values are the means of at lest 40 observations.

Figure 6.5a

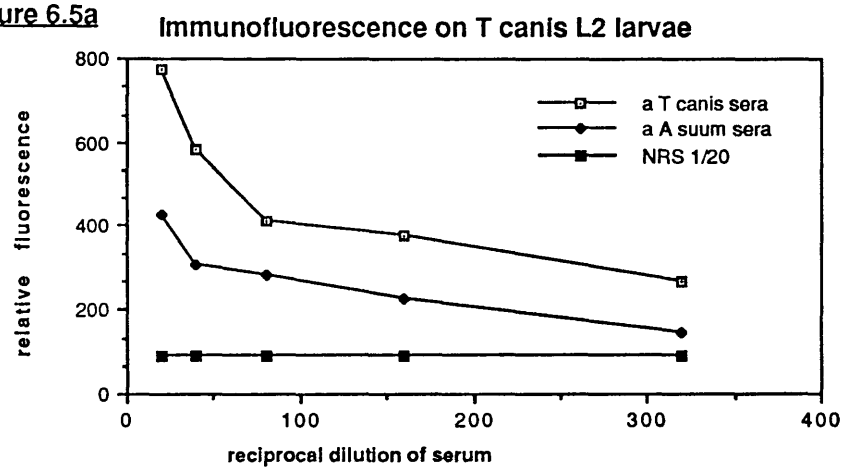


Figure 6.5b

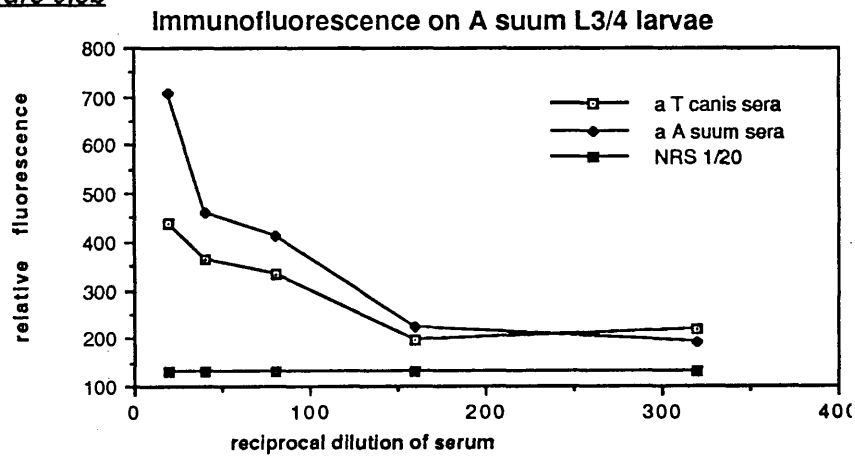


Figure 6.5c

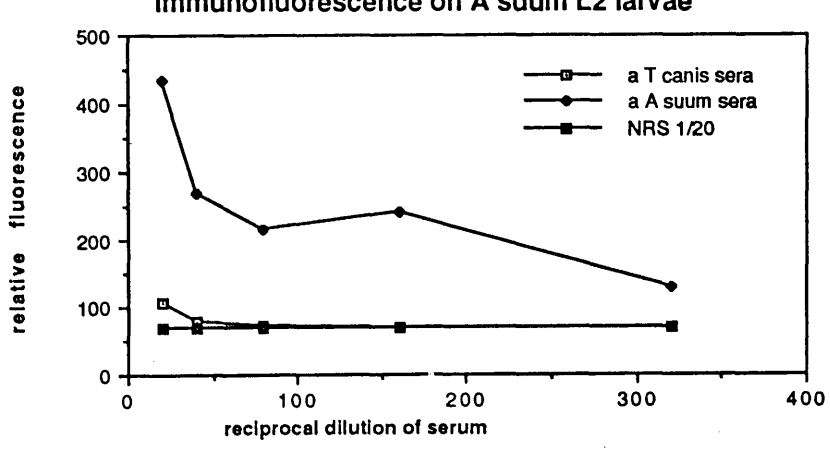


Figure 6.6a L2 larvae were probed with R10 (anti-*Ascaris*) serum, bound antibody detected by fluorescein conjugated goat anti-rabbit antibody and quantified by photon counting. The frequency of observation of values within groups of 100 arbitrary fluorescence units is shown.

Figure 6.6b L2 larvae were probed with R10 (anti-*Ascaris*) serum absorbed against L2 larvae, bound antibody detected by fluorescein conjugated goat anti-rabbit antibody and quantified by photon counting. The frequency of observation of values within groups of 100 arbitrary fluorescence units is shown.

Figure 6.6c L2 larvae were probed with R10 (anti-*Ascaris*) serum absorbed against L3/4 larvae, bound antibody detected by fluorescein conjugated goat anti-rabbit antibody and quantified by photon counting. The frequency of observation of values within groups of 100 arbitrary fluorescence units is shown.

Figure 6.6a

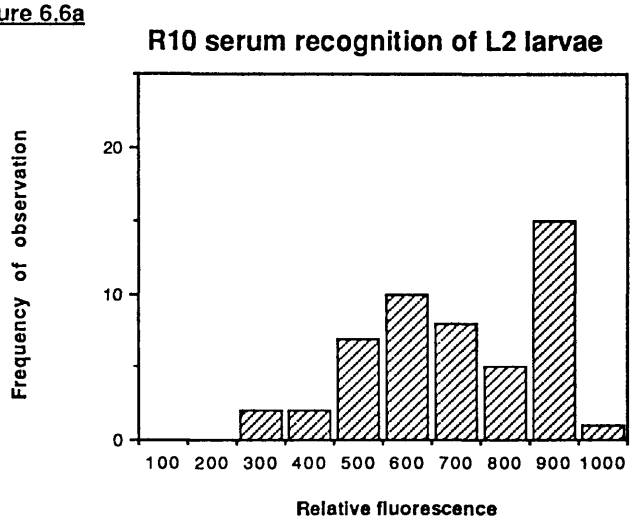


Figure 6.6b

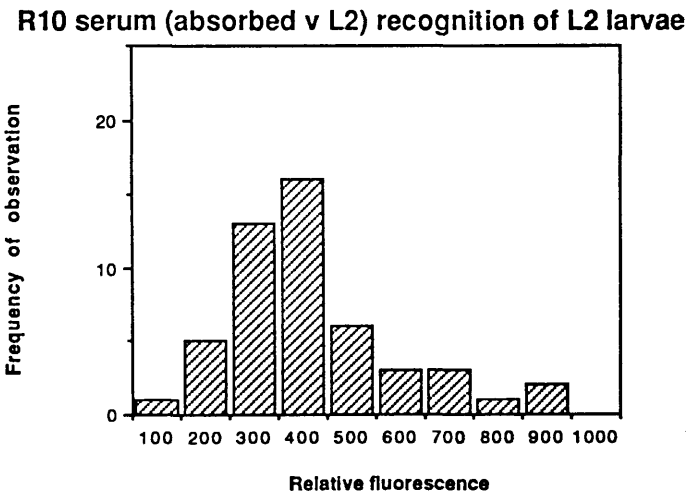
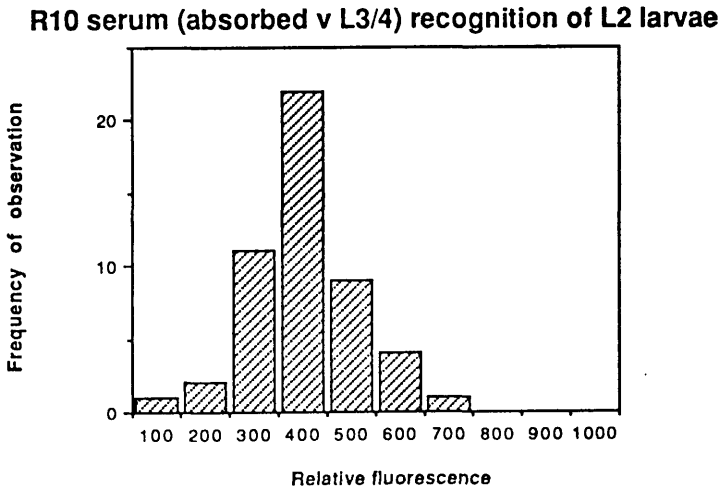


Figure 6.6c



was still recognition of the surface of L2 although the level of recognition had been reduced. Figure 6.6c shows that L2 and L3/4 larvae must share some epitopes recognised by the R10 serum as the level of recognition has been reduced. There are, however, stage specific antigens as not all recognition was removed.

Figure 6.7 shows the results from the same protocol, this time carried out with R49 serum. The initial recognition of the L2 larvae appears high but these are relative figures (mean of approximately 500), independent of the previous figure (mean of approximately 800). The effect of absorbing the R49 serum against L2 larvae reduces the overall recogniton of L2 larvae in the fluorescence as shown in Figure 6.7b and, similarly, absorption against L3/4 larvae reduces recognition but more substantially than with L2. Thus these results reinforce the hypothesis that there are both stage specific and and common antigens on the surfaces of the larvae.

The experiments were repeated with L3/4 larvae and Figure 6.8 illustrates the results for the R10 serum. Absorption of R10 serum with L2 larvae reduces the recognition of the L3/4 surface but, not markedly. Absorption against L3/4 larave also mediates a reduction in recognition but, as one might expect a much greater reduction, it is possible that absorption was not complete due to too few L3/4 larvae being present so that there was an excess of antibody and hence significant amounts of anti-L3/4 surface antibody remained.

The results for the R49 serum recognition of L3/4 larvae are much more convincing (Figure 6.9). Absorption against L2 larave did not reduce antibody recognition of the L3/4 surface at all whereas absorbtion against L3/4 larvae reduced it considerably. This is clear evidence for stage-

Figure 6.7a L2 larvae were probed with R49 (anti-L3/4 ES) serum, bound antibody detected by fluorescein conjugated goat anti-rabbit antibody and quantified by photon counting. The frequency of observation of values within groups of 100 arbitrary fluorescence units are shown.

Figure 6.7b L2 larvae were probed with R49 (anti-L3/4 ES) serum absorbed against L2 larvae, bound antibody detected by fluorescein conjugated goat anti-rabbit antibody and quantified by photon counting. The frequency of observation of values within groups of 100 arbitrary fluorescence units are shown.

Figure 6.7c L2 larvae were probed with R49 (anti-L3/4 ES) serum absorbed against L3/4 larvae, bound antibody detected by fluorescein conjugated goat anti-rabbit antibody and quantified by photon counting. The frequency of observation of values within groups of 100 arbitrary fluorescence units are shown.

Figure 6.7a

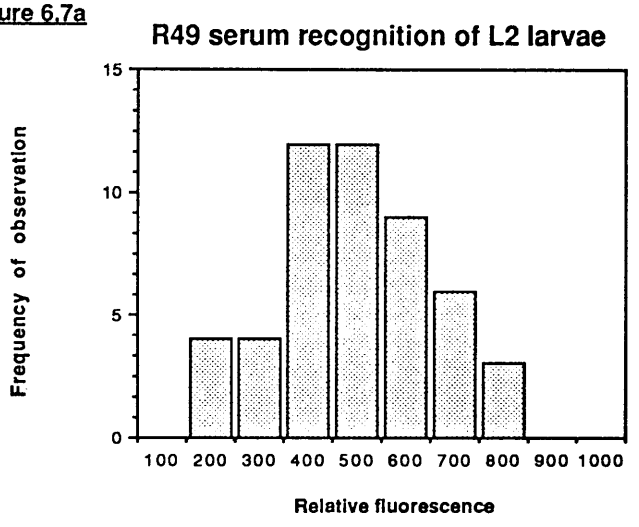


Figure 6.7b

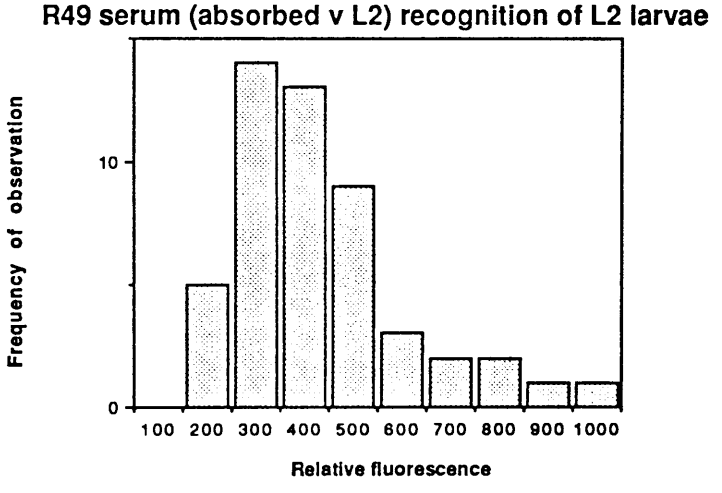


Figure 6.7c

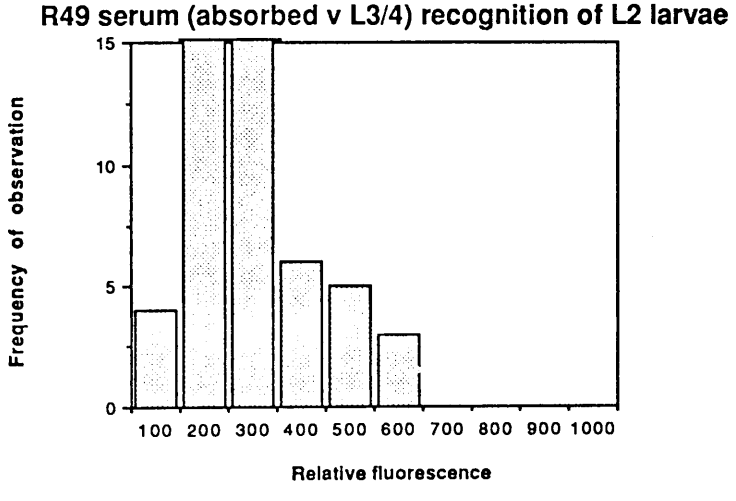


Figure 6.8a L3/4 larvae were probed with R10 (anti-*Ascaris*) serum, bound antibody detected by fluorescein conjugated goat anti-rabbit antibody and quantified by photon counting. The frequency of observation of values within groups of 100 arbitrary fluorescence units are shown.

Figure 6.8b L3/4 larvae were probed with R10 (anti-*Ascaris*) serum absorbed against L2 larvae, bound antibody detected by fluorescein conjugated goat anti-rabbit antibody and quantified by photon counting. The frequency of observation of values within groups of 100 arbitrary fluorescence units are shown.

Figure 6.8c L3/4 larvae were probed with R10 (anti-*Ascaris*) serum absorbed against L3/4 larvae, bound antibody detected by fluorescein conjugated goat anti-rabbit antibody and quantified by photon counting. The frequency of observation of values within groups of 100 arbitrary fluorescence units are shown.

Figure 6.8a

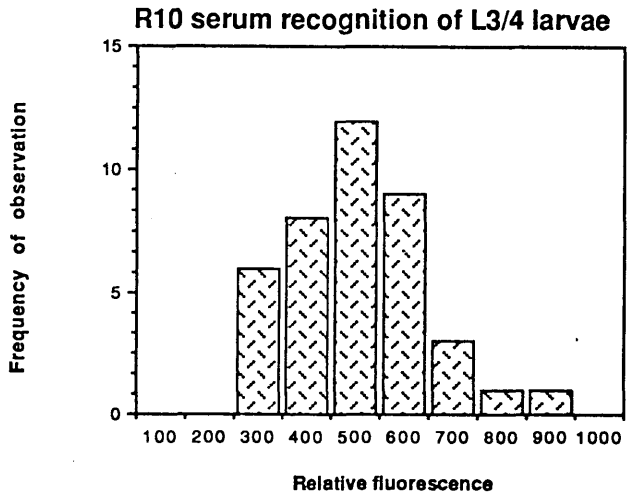


Figure 6.8b

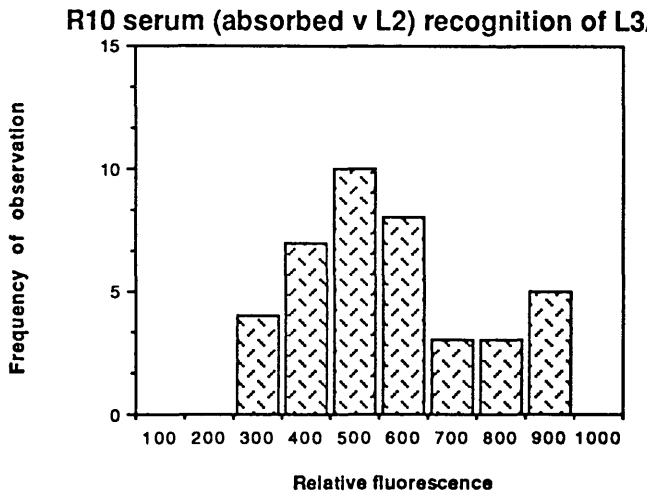


Figure 6.8c

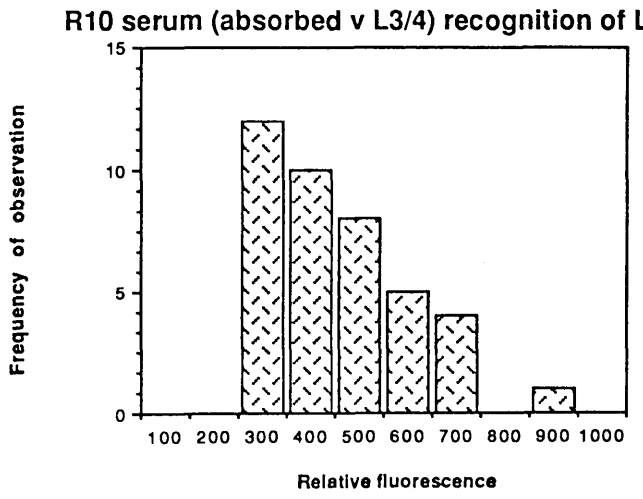


Figure 6.9a L3/4 larvae were probed with R49 (anti-L3/4 ES) serum, bound antibody detected by fluorescein conjugated goat anti-rabbit antibody and quantified by photon counting. The frequency of observation of values within groups of 100 arbitrary fluorescence units are shown.

Figure 6.9b L3/4 larvae were probed with R49 (anti-L3/4 ES) serum absorbed against L2 larvae, bound antibody detected by fluorescein conjugated goat anti-rabbit antibody and quantified by photon counting. The frequency of observation of values within groups of 100 arbitrary fluorescence units are shown.

Figure 6.9c L3/4 larvae were probed with R49 (anti-L3/4 ES) serum absorbed against L3/4 larvae, bound antibody detected by fluorescein conjugated goat anti-rabbit antibody and quantified by photon counting. The frequency of observation of values within groups of 100 arbitrary fluorescence units are shown.

Figure 6.9a

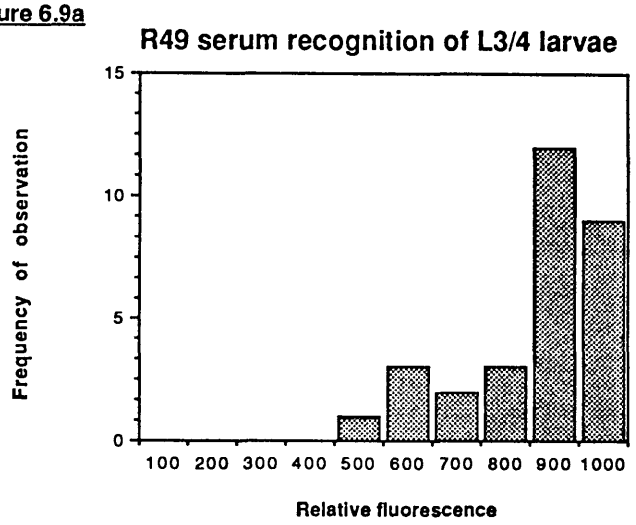


Figure 6.9b

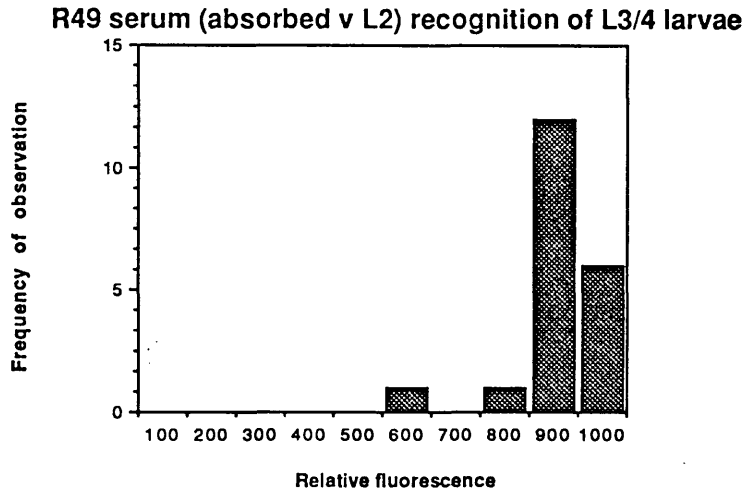
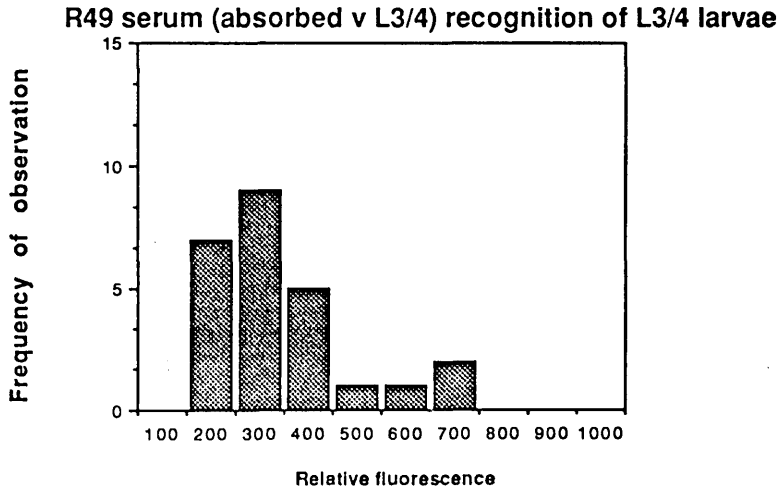


Figure 6.9c



specific antigens on the surface of developmental stages of *Ascaris* larvae.

6.2.6 HETEROGENEITY OF SURFACE ANTIGEN EXPRESSION.

All the fluorescence studies described so far on the surface of *Ascaris* larvae have employed rabbit sera. These sera were generated by repeatedly infecting the rabbits with infective eggs (R10) or immunising with parasite antigen in adjuvant (R42, R45, R49). Thus the rabbits (which make antibody to most if not all parasite components) display uniform recognition patterns. Investigation of sera from infected humans yielded quite different results. Figure 6.10 shows the results of incubating infective *A. suum* larvae with serum from different Nigerian individuals. Very different patterns of recognition are observed. Donor 13's recognition of larvae covers the whole spectrum from weak to strong. Donor 18's recognition is reminiscent of R10's recognition of infective larvae with an apparently normal distribution of relative fluorescence readings while donor 20 recognises the majority of larvae very strongly indeed. These experiments were performed twice, similar results were obtained on each occasion and only one set of results is shown. These results shift the focus of attention away from the nature of the recognition by the serum to the nature of the parasites themselves.

6.2.7 ANTIBODY RECOGNITION OF HOST FOR HOST LARVAE.

In all the previous studies the larvae were the product of eggs from porcine worms from several pigs collected in abbatoirs. In 1990 worms and serum were collected from selected individuals from the Nigerian population. Eggs were removed from the worms, embryonated and larvae hatched. The two different serum samples (from 1987 and 1990) were used as the primary layer in fluorescence assays. Figure 6.11 shows the results of

Figure 6.10 L2 larvae were probed with sera from three Nigerian donors 13 (Figure6.10a), 18 (Figure 6.10b) and 20 (Figure 6.10c). Bound antibody was detected by fluorescein conjugated sheep anti-human antibody and quantified by photon counting. The frequency of observations within groups of 100 arbitrary fluorescence units are indicated.

Heterogeneity of surface antigen expression

Figure 6.10a

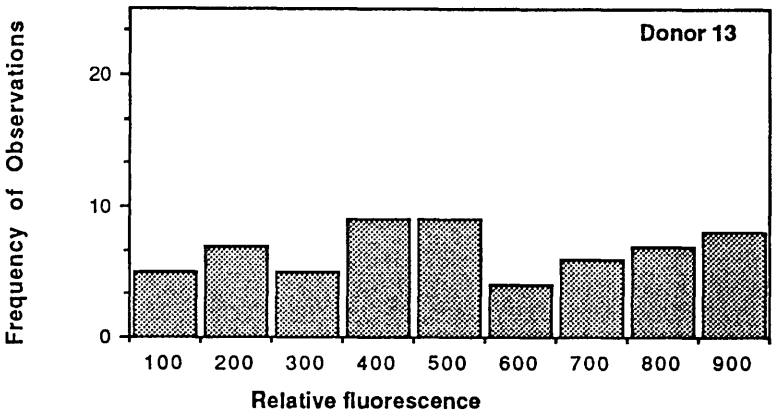


Figure 6.10b

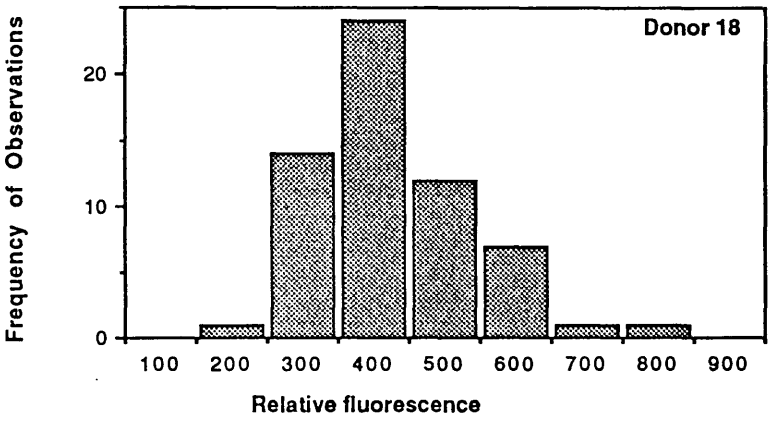


Figure 6.10c

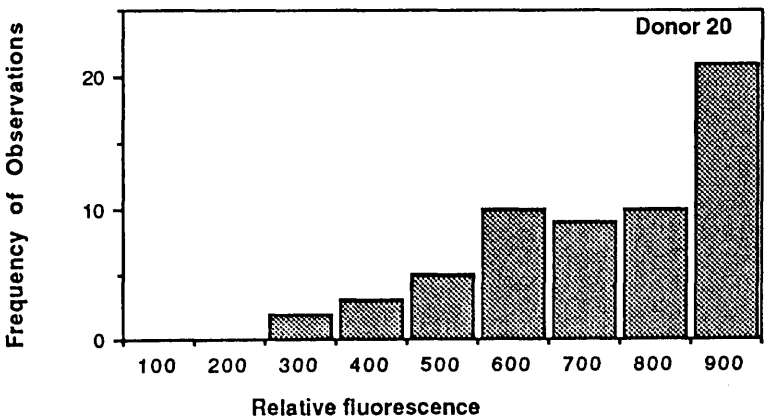


Figure 6.11a L2 larvae embryonated from worms collected from Nigerian donor 171 in May 1990 were probed with serum from donor 171 collected in 1987. Bound antibody was detected by fluorescein conjugated sheep anti-human antibody and quantified by photon counting. The frequency of observations within groups of 100 arbitrary fluorescence units are indicated.

Figure 6.11b L2 larvae embryonated from worms collected from Nigerian donor 171 in May 1990 were probed with serum from donor 171 collected in 1990. Bound antibody was detected by fluorescein conjugated sheep anti-human antibody and quantified by photon counting. The frequency of observations within groups of 100 arbitrary fluorescence units are indicated.

Figure 6.11a

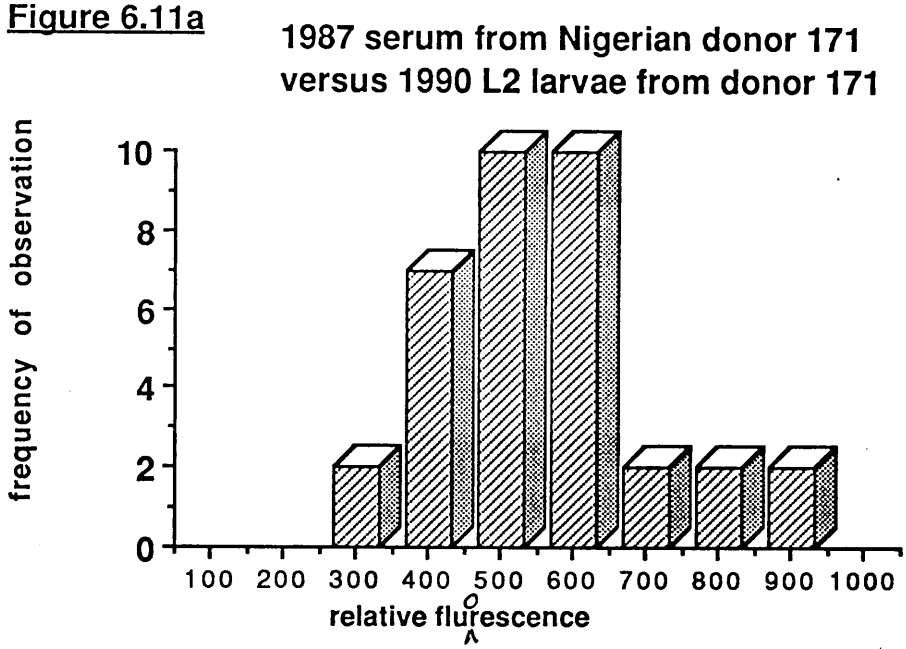
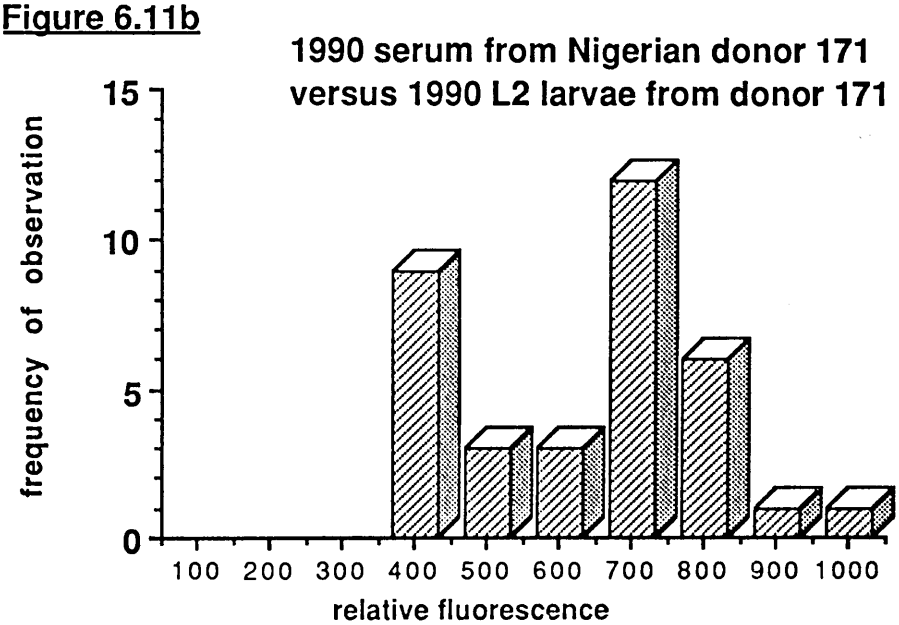


Figure 6.11b



such an experiment for donor 171 - the donor of both the serum and the worms. The 1990 serum appears to have a bi-modal pattern of recognition with a large number of larvae being fairly strongly recognised with a second population being less well recognised. The 1987 serum from the same person recognises these larvae too with the relative fluorescence readings giving a fairly normal distribution.

Figure 6.12 shows the results of the same experiment, this time with donor 247. The recognition of the 1990 serum is lower than the 1987 serum. As this individual is one of the most persistently heavily infected he is likely to be constantly exposed to larval stages and the lower level of recognition may imply less protection against these infective larvae.

Figure 6.12a L2 larvae embryonated from worms collected from Nigerian donor 247 in May 1990 were probed with serum from donor 247 collected in 1987. Bound antibody was detected by fluorescein conjugated sheep anti-human antibody and quantified by photon counting. The frequency of observations within groups of 100 arbitrary fluorescence units are indicated.

Figure 6.12b L2 larvae embryonated from worms collected from Nigerian donor 247 in May 1990 were probed with serum from donor 247 collected in 1990. Bound antibody was detected by fluorescein conjugated sheep anti-human antibody and quantified by photon counting. The frequency of observations within groups of 100 arbitrary fluorescence units are indicated.

Figure 6.12a

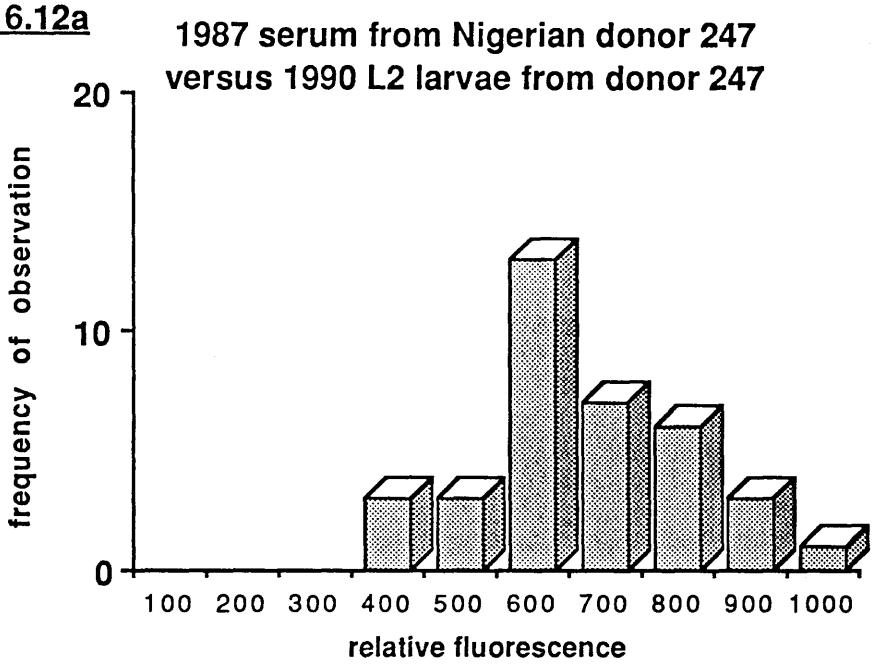
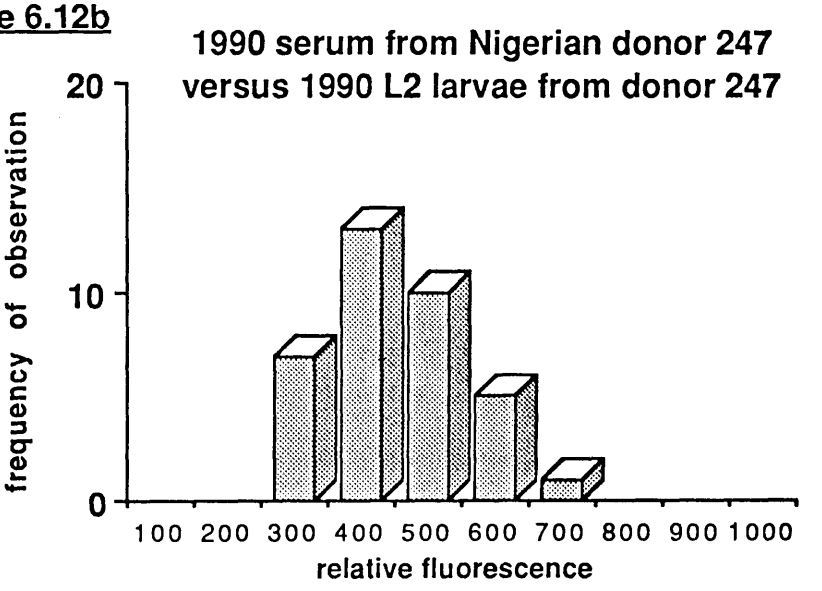


Figure 6.12b



6.3 DISCUSSION

Surface antigens of parasites are currently the focus of attention due to the possibility of identifying any molecules which may be the target of naturally acquired immunity in the hope of exploiting them in artificial immunisation. These studies on the surface of the larval stages of *Ascaris suum* have revealed several features of the surface of this parasite which may be useful in further characterisation the humoral immune response to infection with the parasite.

The time-course of labelled larvae, looking for changes in patterns of antigen binding, demonstrated that the loss of surface antigens bound by specific antibody is much more rapid in the L3/4 stage. The relatively bright and even fluorescence shown by the L2 even after 8 hr is indicative of a slow turnover of surface molecules at this stage. This would seem to suggest that shedding of surface antigens is not a mechanism of immune evasion at this stage, although this may be different *in vivo*.. It could also be an explanation for the low yield of ES from L2 larvae when cultured *in vitro*. This is in contrast to *T. canis* L2 larvae which have been shown to lose labelled surface molecules rapidly and produce copious quantities of ES (Smith *et al*, 1981).

The time course to determine the optimal time point in the rapid larval development for examining stage-specific surface antigens by immunofluorescence resulted in choosing infective larvae day 2 after hatching. Larval heterogeneity increased considerably after day 4 - this could well be the result of cumulative asynchrony of development after the synchronous start of hatching or may be the result of *in vitro* artefacts in parasite physiology. That the preparations of infective eggs are the

products of 6-8 adult female worms must also be considered as a source of heterogeneity. Heterogeneity of the L3/4 larvae (to be expected because both L3 and L4 are present) was apparent from the time of recovery from the lungs of rabbits. Although the larvae were derived from one infection, their passage in their host would have resulted in increasingly staggered development of the larvae from L2 to L3 and then L4 in the lungs. Use of rabbit sera as a primary layer may have obscured some of the larval heterogeneity due to the known ability of rabbits to recognise most, if not all, exo- and somatic antigens of the parasite giving the expectation that surface antigens would be similarly recognised. Also, the rabbit sera was generated by repeated infections of eggs or exposure to antigen in adjuvant so that the repertoire of recognition would be expected to be more complete and uniform..

The examination of stage-specificty by means of probing *Toxocara and Ascaris* larvae with both homologous and heterologous sera allowed for the identification of stage-specificity for the infective larvae of *Ascaris* where only homologous serum antibody bound to the surface. The reverse was not true as the surface of infective *Toxocara* larvae was recognised by both homologous and heterologous sera. The restricted recognition also broke down with the *Ascaris* lung stage larvae whose surface was recognised by *Toxocara* infection serum. The reasons for this are unknown but it does demonstrate both stage-specific recognition of larval surfaces and cross-reactivity between two closely related species. The latter problem, and how to get round it, has been the subject of investigation. ES antigens of *Ascaris* are cross-reactive as demonstrated by cross immunoprecipitations analysed by SDS-PAGE (Kennedy *et al.*, 1989) and such recognition possibly causes problems when attempts are made to develop tests for diagnosis of toxocariasis (Lynch, Wilkes, Hodgen *et al.*, 1988).

These results are reminiscent of the conclusions reached by Fetterer and Urban (1988) as a result of their studies on the composition of the cuticles of the various life cycle stages of *Ascaris*. That is that the L2 cuticle is the most different from all the other stages. What would be of real value in dissecting at the molecular level what is happening between the epicuticle and the host is the availability of *Ascaris* antigen-specific monoclonal antibodies. This would hopefully allow identification of surface-exposed epitopes and analysis of their efficacy in generating a protective immune response. The observation that there is no ABA-1 on the surface of either larval stage tempts speculation as to its origin as a genuinely secreted molecule rather than a shed surface molecule; that the molecule is released into larval cultures from dead larvae or the possibility that its antigenic epitope(s) is on the surface hidden from immune attack.

Investigations with sera from infected humans revealed two principal discoveries. First, that the distribution of fluorescence levels are different for different people and secondly, that the larvae themselves are heterogeneous. The first observation could almost be expected from the results looking at antibody responses to ES and somatic antigens which showed heterogeneity in both the quantity and quality of recognition of antigens (see Chapter 5). The possible reasons for this are as previously considered; affinity of antibody binding, duration of exposure to infection, recency of exposure, different antibody responses to stage-specific and common antigens and the highly likely role of MHC control of the immune response as seen in laboratory animals (Tomlinson *et al.*, 1989).

What, then, of the heterogeneity of the parasites? One explanation could be metabolic differences reflected in the synthesis of surface antigens. This has been suggested to explain the intra-clonal heterogeneity in larvae of *S. mansoni* (Jones and Kusel, 1989) and while *Ascaris* larvae do not constitute a clone, this is a possibility.

Another possibility is that the surface antigens are polymorphic - this has been shown for *Onchocerca lienalis* (Bianco, Robertson, Kuo *et al.*, 1990). Biochemical genetic studies of isoenzymes have revealed high levels of polymorphism and heterozygosity in natural parasite populations of endoparasitic helminths (Nadler, 1987). Thus other parasite components may also be polymorphic.

A third explanation is that of differential gene (and hence antigen) expression. In this hypothesis both expressivity (the degree or extent to which a genotype is expressed in an individual) and penetrance (the proportion of individuals with a given genotype which exhibit the phenotype associated with that genotype) must be considered. The role of these mechanisms in expression of surface antigens in parasites is not known but incomplete penetrance of expression of surface-exposed epitopes of the free-living nematode *Caenorhabditis elegans* has been shown (Politz, Chin and Herman, 1987).

The explanation for the heterogeneity of the antigen expression on the larval parasite surface may be any one of, none of, or any combination of these and other mechanisms. Certainly, differential antigen expression would be of selective advantage to members of parasite populations which face genetic variability in their host population, especially with respect

to their immune responses, and heterogeneity in acquired immunity due to previous exposure as is the case in ascariasis.

CHAPTER 7

GENERAL DISCUSSION

7 GENERAL DISCUSSION

The work presented here explores sveral aspects of immune responses to the parasitic nematode *Ascaris* , looking especially at antibody responses to components of the parasite and the control of such responses.

Initial characterisation of the products of the nematode is vital to determine the nature of the molecules the immune system is responding to. The various products examined - ABF, L2 ES and L3/4 ES - are all complex mixtures of glycoproteins. The affinity of lectins for some of the components has revealed the presence of sugars on molecules which are also precipitated by anti-infection sera, hence are antigenic. Whether the sugars are involved in immune recognition or whether such recognition is of protein epitopes is not clear. If the former were the case, as with anti-phosphoryl choline responses, then cross-rectivity would be a factor in responses to parasite antigens and also a potential problem in any application of immune recognition in diagnosis of infection.

The instability of L2 ES is an interesting phenomenon and further investigation into this mixture would be worthwhile. Relatively small quantities of ES are produced by *in vitro* cultures (perhaps a reflection of the slow surface turnover described in chapter 6) and the poor definition of components released in culture by Coomassie and silver staining along with indistinct profiles in autorads of radiolabelled preparations raise many questions. The instability of this preparation may be a reflection of its function *in vivo* to evade immune responses against the larval products (and hence the larval surface?). This may be by ES products being rapidly altered or broken down after release. However, antibody to L2 ES is detected at some level in all individuals from an

endemic area whether they harbour adult worms or not. Thus the continuous exposure to infective eggs in a contaminated environment (and presumably some larvae hatching and beginning to develop) ensures that the host is exposed to parasite antigens. A major question is why some individuals are apparently able to terminate larval development in the tissues and remain persistently free from infection with adults in the gut while others, in the same environment, are persistently infected and permissive of larval development and migration.

It would also be worthwhile investigating the nature of adult ES and determining whether it is the focus of immune responses in the intestine where it survives happily for up to one year.

The focus of work on the ABA-1 molecule is the result of several factors: its abundance in the ABF and presence in ES products, the relative ease of purification of the molecule and the clear cut restricted recognition of it. This latter has been shown to be under H-2 control in mice and this work has shown that this control of recognition to be effected by s alleles at the I-A region. Similarly, of the strains so far examined, only rats with an $RT1^{u}$ haplotype make antibody to this molecule. Thus when only a proportion of individuals in the human populations studied make antibody to ABA-1, HLA control of recognition is a real possibility and the results of typing of the Karachi and Gambian sera will be of great interest.

Further sequencing work on ABA-1 should allow the production of peptide fragments and hopefully the identification of antigenic epitope(s). Such identification may also allow elucidation of the structural interaction between MHC molecules and the antigenic fragment.

The animal work has also shown that the mode of presentation of parasite products to the host has a determining effect on subsequent immune recognition. For example, presentation of antigens in adjuvant can affect the level, kinetics and specificity of antibody responses to various components. New recognition repertoires can be generated and recognition of products in the context of infection lost when presented in adjuvant. Such results should be borne in mind when attempting to extrapolate laboratory results to the field situation.

The differential recognition of *Ascaris* products by different mouse and rat strains is apparently reflected in the heterogeneity of antibody recognition profiles seen in individual people from areas where *Ascaris* is endemic. Again the factors which possibly affect the development of such immune responses are many: level of contamination of the environment; hygiene habits; age; cultural differences in contact with contaminated areas; previous exposure to infection; recency of exposure; whether exposure is continuous or intermittent; genetic make-up of an individual; level of polyparasitism and cross-rectivity between parasites etc etc. Analysis of the role of these different factors would be complex and time consuming but some studies would be feasible. Following the antibody recognition repertoire of individuals from early childhood would be of value in determining whether antibody levels or specificities are age dependent and if any particular patterns are associated with heavy or light infections and how this progresses with time. Another valuable study would be to compare two endemic areas, one where transmission of infective eggs is continuous and one where it is intermittent due to climactic conditions. Again, it would be interesting to note any differences in antibody profiles between the two populations and whether in the population exposed to infection occasionally, if resistance to

establishment of adult worms correlated with any particular immune responses.

The lack of relationship between any of the epidemiological data (worm burdens on three occasions and epg values) from the Nigerian population and any of the immunological assays performed on the sera (ELISAs, immuno-precipitations, Western blots detecting parasite specific IgG and IgE) suggests that no such simple relationships exist. Elucidation of such a relationship would be of value in e.g. non-invasive field diagnosis where identification of infected individuals could be achieved without having to treat the whole population with anthelmintic drugs. Possibilities for this could be an ELISA which assays for a parasite component whose presence or whose concentration is reflective of infection (circulating ABA-1?) in serum, urine or faeces or identification of recognition patterns of parasite antigens in blots or precipitations which indicate infection - the characterisation of adult ES may be valuable here.

The high levels of IgE in people with *Ascaris* and other helminth infections has long been recognis ed and the cause and function of the presence of this isotype long debated. This work has shown that in rats and mice the mode of presentation of an allergen affects the subsequent IgE response with presentation of ABA-1 in adjuvant failing to generate allergen-specific IgE in rats and mediating different responses to those generated in the context of infection in mice. The IgE response of mice infected with infective eggs to ABA-1 as detected in PCAs appears to correlate with IgG responses detected by immunoprecipitations. The variations in IgE responses of different batches of anti-*Ascaris* SJL infection sera raise some interesting questions. Whether these differing

results are the outcome of differences in the kinetics of IgE responses where rapid rises and declines in reagin levels have been observed or due to differences in the infections or the mice themselves is not known.

The Western blots demonstrating which parasite components are the target of IgE responses reveal the number and wide range of molecular weights of such components. Whether such responses are indicative of atopic disease is not known for these individuals and whether all these components are allergens i.e. that they cause an allergic response or are merely the target of IgE antibody is not known.. One laboratory worker (Jackie Christie) has experienced atopic responses to the ABA-1 molecule which she has a detectable IgE response to, while another (Eleanor Fraser) has IgE responses to higher molecular weight components but no clinical symptoms to any *Ascaris* material.

The surface studies have exploited the advantages of the immunofluorescent technique which detects antibody binding to surface exposed epitopes only. Radiolabelling and detergent stripping of surface molecules would also be useful in examining the surface of *Ascaris* larvae but may be misleading in terms of which molecules and epitopes are exposed on the surface. The results described in this work strongly suggest that the surface of *Ascaris* larvae contributes in large measure to ES components harvested from larvae in culture. There is also evidence of stage specific surface molecules, this phenomenon may be a mechanism of escaping immune attack whereby immune responses to L2 larvae would be ineffective against subsequent L3/4 larvae.

The fluorescence studies revealed the extent of heterogeneity of parasites, at least in terms of their surface components. This is another quality which will allow for immune evasion of at least a proportion of larvae, thus allowing the development and establishment of adult worms in individuals whose immune response fails to terminate the development and migration of all larvae.

Thus, these studies have demonstrated the importance of both the mode of presentation of parasite antigen and the genetic constitution of the host on the resultant immune response to parasite components and the parasite itself. However, this work has also shown that, due to the complexities of the *Ascaris* life cycle and associated products and the apparent success in evading the immune response, immune reactions often do not parallel protective immunity and the presence of an immune response does not imply lack of disease.

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