IMMUNITY TO *TRICHURIS MURIS* IN THE MOUSE

by

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" THE HOTTEST PLACES IN HELL
ARE RESERVED FOR THOSE WHO,
IN A TIME OF GREAT MORAL CRISIS,
MAINTAIN THEIR NEUTRALITY "

Dante

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ABSTRACT

Quantitative and qualitative analyses of the serum antibody responses of NIH, C57BL/10, BALB/c, DBA/2 and CFLP mice infected with Trichuris muris have been made using ELISA and immunoprecipitation techniques. No correlation was found between specific serum antibody titres measured using T. muris E/S products and the time of onset of expulsion in the different mouse strains examined. However, there were some differences in the antigen recognition profiles of some sera as determined by immunoprecipitation analyses. In all the strains of mice examined significant increases in detectable specific serum antibody to the parasite E/S products occurred around day 15 to 20 post-infection and continued to rise, as measured up to at least day 40 and even up to day 65. Cortisone acetate treatment during larval development, in infected CFLP mice, in order to establish heavy adult worm burdens, did not reduce specific antibody titres to T. muris E/S products. In responding and tolerant DBA/2 mice there was no marked difference in either the kinetics of specific serum antibody production during primary and secondary infections, or in the antigen specificities of secondary infection sera. The "defect" in mechanism in the tolerant DBA/2 mice, which allows primary infections of T. muris to develop to patency, was shown to be permanent as secondary infections with the parasite could also establish in these animals.

An investigation was made of the phenomenon of tolerance in the DBA/2 model system and in the cortisone treated CBA mice. The capacity of MLNC from different groups of animals to produce IL-2 in vitro upon mitogen stimulation was investigated, on the basis that IL-2 deficit during antigen presentation may result in immune tolerance. Although no differences were found in the responding and tolerant DBA/2 cell

populations, there was an apparently synergistic interaction between cortisone administration and T. muris infection which dramatically reduced the IL-2 producing capacity of the MLNC. However, IL-2 cannot yet be ruled out as a factor in the inherent tolerance of a proportion of the DBA/2 population as IL-2 receptor expression by the 2 groups of cells assayed was not examined.

Basic analyses of the antigens of T. muris were performed. The major protein of adult male homogenate (AMA) was also the major protein of the excretory/secretory (E/S) products and the surface antigen preparations. In addition several common E/S and surface antigens were shown to have proteolytic enzyme activities against gelatin and/or casein.

The relationship between T. muris and Trichinella spiralis was examined in greater detail, and the m.wts. of the cross-reacting antigens were determined. Evidence suggested that the stichosomes of these worms may be the source of these antigens. Both Trichuris muris adults and Trichinella spiralis infective larvae each had common major E/S and surface antigens, indeed, both were shown to have surface proteases. These studies were extended to examine the possibility of cross-reactivity between Trichuris muris and T. trichiura; mouse infection sera and human infection sera respectively were able to cross-react with heterologous antigen preparations. The demonstration that anti-Trichinella spiralis 48 kD and 50/55 kD stichocyte antigen MoAbs also reacted with Trichuris trichiura adult homogenate in ELISA supports the suggestion that common stichocyte antigens may exist amongst the trichuroid nematodes Trichuris muris, Trichuris trichiura and Trichinella spiralis.

Monoclonal antibodies were produced against the E/S products

of Trichuris muris, which were characterized in terms of isotype and antigen specificities. Initial experiments indicated that one of the IgA MoAbs recognizing 34, 22, 20 and 18 kD E/S proteins may be effective in the passive transfer of immunity.

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ABBREVIATIONS

AA	anterior ends of worm
Ag	antigen
AH	adult homogenate
AMA	adult male homogenate
APS	ammonium persulphate
BBS	borate buffered saline
con A	concanavilin A (type IV)
coomassie	Coomassie brilliant blue
c.p.m.	counts per minute
CNBr	cyanogen bromide activated
CTAB	cetyltrimethylammonium bromide
CTMC	connective tissue mast cells
CTTL	cytotoxic T cell line
d	day
DMSO	dimethylsulphoxide
ELISA	enzyme-linked immunosorbent assay
E/S	excretory/secretory
FCA	Freunds complete adjuvant
FCS	foetal calf serum
g	gramme (weight), gravity (centrifugal force)
HAT	hypoxanthine, aminopterin, thymidine
HMT	hypoxanthine, methotrexate, thymidine
HS	human serum
IEL	intraepithelial lymphocytes
Ig	immunoglobulin
IMLNC	immune mesenteric lymphnode cells
i.p.	intra-peritoneal
IS	immune/infection serum
i.v.	intra-venous
kD	kilodaltons
L	litre
L_1(L_2,L_3 and L_4)	first (second, third and fourth) larval stage
LPS	lipopolysaccharide
M	molar
MHC	major histocompatibility complex
MS	mouse serum
m.wt.	molecular weight
NS	naive serum

O.D.	optical density
o/n	overnight
PAG	polyacrylamide gel
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pen/strep	penicillin/streptomycin
PHA	phytohaemaglutinin
RIA	radioimmunoassay
RT	room temperature
s.c.	sub-cutaneous
SDS	sodium dodecyl sulphate
TBS	Tris buffered saline
TCA	trichloroacetic acid
TEMED	tetraethylmetheylene diamine
Tris	Tris (hydroxymethyl) amino methane
TS	tolerant serum

CHAPTER 1

GENERAL INTRODUCTION

It is widely acknowledged that parasitic diseases are major factors contributing to the high rates of mortality and morbidity in the populations of the developing countries. Their elimination and/or control, so that they are no longer a major health problem, is justifiable on both humanitarian and economic grounds. If disease, malnutrition, low income, illiteracy and underemployment are taken as the foremost manifestations of poverty, an improvement in any of the above manifestations would result in a positive change in the others (see Brandt, 1980); the reverse is also the case. In the developed world, parasitic diseases affecting humans are now secondary in importance compared with bacterial and viral infections. Nonetheless, gastro-intestinal helminths, and gut nematodes in particular, remain amongst the most prevalent of human infections (Peters, 1978; Warren and Mahmood, 1984) and are a significant cause of disease in the developing world.

Parasitic diseases impose further economic burdens, in both temperate and tropical regions, as a result of infections in domestic animals, and here again gut nematodes are of major importance. In temperate countries the burden is primarily one of lowered productivity, from decreased food conversion, or of increased production costs, from anthelmintic treatment and the use of preventative practices such as rotational grazing. In many tropical countries the effects of gastrointestinal parasitism can be much more severe; in addition to dramatic reductions in productivity, the effects may be such as to seriously reduce longevity and fecundity of domestic livestock (Griffiths, 1978).

Trichuroid nematodes are common members of the gastro-intestinal parasite populations of both man and domestic animals.

Compared with other species their biology and host-parasite relationships are relatively little studied and their importance is controversial. Almost nothing is known of immunological aspects of infections in these hosts, but some data have been gained from studies of laboratory models. This thesis is concerned with extending these studies to throw further light on immune responses to this group of worms.

1.1 TRICHURIS TRICHIURA IN MAN

Nematodes of the family Trichuridae infect a variety of mammalian hosts, with Trichuris trichiura (Fig. 1.1) infecting the large intestine of man, producing chronic infections in many individuals. Infection with T. trichiura is generally coextensive in distribution with Ascaris lumbricoides, which is currently perceived to be the most common gastro-intestinal nematode infection of man, infecting approximately 33% of the world's population (Peters, 1978; Warren and Mahmood, 1984). According to data collected from recent mass surveys throughout the tropical world, however, Bundy (1986) now suggests that trichuriasis is at least as prevalent as ascariasis particularly in children. The high prevalence of these nematodes persists despite the availability of safe and effective chemotherapy. Trichuris populations recover to pre-control levels after chemotherapy in approximately 6 months, while recovery for Ascaris takes at least a year, and hookworm several years (Bundy et al., 1985c; Croll et al., 1982; Anderson and Medley, 1985). Comparison of the estimates for the basic reproductive rates (R_o) of these nematodes, indicates that Trichuris has a higher R_o than either Ascaris or hookworm, and this could be the reason why it is intrinsically more resistant to control. Indeed, high levels of infection with this

nematode are not restricted to tropical developing countries (Silverston, 1962; Paggi and Berlinguer, 1964; Croll and Gyorkos, 1979).

Pathology arising as a result of Trichuris infection is thought to be related to the intensity and chronicity of infection, and only attains clinical significance with relatively large worm burdens (Kuori and Valdes-Diaz, 1952; Ramsey, 1962; Cooper and Bundy, 1986). High worm burdens are characterised by the production of profuse mucus and chronic bloody diarrhoea, with abdominal pain, tenesmus and rectal prolapse (Jung and Beaver, 1951; Beck and Beverley-Burton, 1968). Cooper and Bundy (1986) also demonstrated that, in at least one Caribbean community, where 84% of 2-6 year old children examined had T. trichiura infections, 10-15% had colitis and growth stunting associated with intense trichuriasis, with 4% exhibiting severe trichuriasis syndrome, including rectal prolapse. These findings are contrary to published medical statistics, which suggest that severe trichuriasis is rare. The discrepancy between the statistical records and the observed prevalence in the communities examined is attributed to under-reporting, morbidity in particular has tended to be under-estimated, as only the most severe cases are recogized by, or reported to, the health services.

The most recent work on the epidemiology and chemotherapeutic control of T. trichiura has been carried out in the Caribbean by Bundy and colleagues (reviewed Bundy, 1986), where this nematode is now the most prevalent helminth infection (Grell and Watty, 1976; Grell et al., 1981; Tikasingh, 1981). Prevalence of T. trichiura infection was found to rise rapidly with age, attaining a plateau of between 80% to 100% after the age of 2 to 4 years, and remaining

high and relatively constant throughout adulthood (Bundy et al., 1986). However, the age-intensity profile indicated that worm loads rose rapidly to a peak in the child-age classes but declined to a low level in adult groups. A pattern of overdispersed distribution was observed in T. trichiura infections within age groups, a characteristic feature of many helminth infections in humans (Anderson, 1982). In fact, Trichuris showed a greater degree of aggregation in the population studied than did Ascaris. The greater degree of aggregation of T. trichiura would increase the probability of worm mating, and may thus be a contributing factor to the worm's high basic reproductive rate, enhancing the parasite's population stability (Croll et al., 1982). Paradoxically this could also facilitate control by selective therapy of the most intensely infected individuals (Anderson and May, 1982). However, it is not known whether the overdispersion observed is a result of heterogeneity in exposure rather than of individual host differences in resistance/susceptibility.

An additional consideration is the effect that a chronic infection with these relatively large nematodes may have on the immunocompetence of the host, due to the secretion/excretion of copious quantities of soluble antigens, in particular in relation to other potential intestinal infections. In mice it has been shown that animals with chronic infections of T. muris have a marked reduction in in vivo humoral responses to both T-dependent and T-independent antigens (Lee and Wakelin, 1983). It was suggested that the highly antigenic material released into the local environment of the worm may have affected the hosts ability to respond normally to other antigens through some form of antigenic competition. However, the phenomenon of antigenic competition/antigen induced suppression

(Kerbel and Eidinger, 1971) is not well understood (Taussig, 1977), and studies involving continuous antigenic presentation as would exist in chronic trichuriasis have yet to be resolved. In this context, the presence of circulating antigens, persistent antigen stimulation and immune complex formation, may all play a role in immunodepression of the host, in addition to causing considerable pathology (reviewed by Theofilopoulos and Dixon, 1980). However, there has been little investigation of these manifestations during trichuriasis. There is evidence that susceptibility to Entamoeba histolytica infection is enhanced in Trichuris-infected humans and laboratory animals (Jung and Beaver, 1951; Knight and Chew, 1974). However, histological studies of the interaction between E. histolytica and T. muris in mice suggested that amoebic tissue invasion usually occurred at the actual site of worm attachment (Knight and Chew, loc. cit.). The observed persistence of the induced amoebic infection, thus appeared to be a direct result of mucosal tissue damage rather than a result of general reduction in immunocompetence of the host during a chronic worm infection.

To-date there is little information concerning immune responses to T. trichiura infections in man, or of immunologically-mediated mechanisms of resistance per se. Indeed, there are insufficient data to be able to determine whether or not any form of resistance develops against this parasite. The observed decline in the intensity of worm infections in adults may be due to a reduction in exposure to infection. Such information, and the development of immunodiagnostic tests, would facilitate epidemiological studies, aid in the analysis of data and ultimately contribute to the implementation of effective control programmes.

Based upon available data, several hypotheses could be put forward to explain the persistence of chronic infections in humans :

i) Worms survive in individuals unable to develop effective resistance ("non-responders").

ii) Worms are able to survive the development of an immune response.

iii) Worms survive by suppression of the immune response.

These possible explanations are all speculative at present and are not necessarily mutually exclusive. Obviously only restricted immunological investigations of infected humans are possible, as neither human nor parasite material for *in vitro* experimentation is readily available. It is hoped that *in vivo* and *in vitro* investigations of immunity to *T. muris* in the mouse, under controlled laboratory conditions, may help lead the way to an understanding of the situation which exists in human infections with *T. trichiura*. Investigations of gastrointestinal helminths using laboratory models have been useful not only for obtaining information relating to specific host-parasite relationships, but have also provided valuable data on the mechanism of gastrointestinal immunity in infected hosts (Owen, 1982).

1.2 TRICHURIS MURIS IN THE LABORATORY MOUSE

Trichuris muris in the mouse has a direct life cycle, which is summarized in Fig. 1.2. Unlike many other gut nematodes, which are lumen dwelling, trichuroid nematodes are in intimate contact with the host throughout infection. The anterior end of *T. muris* remains embedded in the epithelium of the gut throughout the life

of the parasite, as it tunnels through the caecum and colon of its host (Fig. 1.3, Lee and Wright, 1978). The "tunnel" within which the nematode is contained has been shown to be a cytoplasmic syncytium, and the worm is believed to feed on the syncytial cytoplasm.

Heavy infections of T. muris are expelled from most strains of laboratory mice before reaching patency (Wakelin, 1967, 1975b). However, outbred Schofield and inbred DBA/2 mice are exceptions in that the parasite is able to develop to patency in a proportion of the populations of each strain (Pike, 1963; Wakelin, 1975b; Lee and Wakelin, 1982a). Patent infections may also be obtained by infection with a small number of eggs, such that the number of worms developing is below the "threshold" required to elicit an expulsive immune response (Wakelin, 1973). Low level infections in mice nonetheless stimulate immunity as measured by a reduction in the time of onset of expulsion of a subsequent infection. The development of immunity as a result of a low-level infection is dependent on the persistence of the immunizing population for at least 14 days, and is markedly reduced if the larvae are removed by anthelmintic treatment on day 14 (Wakelin, loc. cit.). The administration of immunosuppressive drugs to mice during larval development in an infection, results in a heavy patent worm burden (Campbell, 1963, 1968; Wakelin, 1970; Lee and Wakelin, 1982b, 1983). Lactating female mice are also more susceptible to infection (Selby and Wakelin, 1975). These mice failed to expel T. muris at the normal time but expulsion did occur once lactation had ended. In a survey of wild house mice (Behnke and Wakelin, 1973) it was found that the majority of mice had small worm burdens, an observation consistent with a situation of repeated low level infection. However, some female

mice harboured larger mature worm burdens. It was suggested that in these mice pregnancy and/or lactation may have suppressed the immune response, allowing the accumulation of worm burdens in excess of the "threshold", above which there is usually expulsion.

In mice which are able to mount an expulsive immune response to this parasite, the time of onset of expulsion varies according to the strain of mice involved (Wakelin, 1970b, 1975b; Lee, 1982). Out-bred mice have a greater variation in worm burdens and show less uniformity in the time of onset of expulsion (Wakelin, 1975b). The expulsive response, once initiated in individual mice is completed within 24 hours, with the time of onset of expulsion varying slightly between individuals of each particular strain, although this variation is not as great in the inbred strains. NIH mice have the most rapid expulsion of all the strains examined so far, and are able to expel T. muris within 15 days post-infection. Other inbred strains such as CBA/Ca and C_3H/He are slower in mounting an immune response controlling expulsion, which does not occur until day 20-23 and day 23-28 respectively (Lee, 1982). Factors responsible for the variation in expulsion time are thought to be under direct genetic control, and a rapid expulsive response has been shown to be inherited as a dominant characteristic (Wakelin, 1975b). Populations of Schofield strain outbred mice were shown to have a bimodal variation in the ability to expel T. muris before patency, which was found to be independent of the size of the infection experienced. The proportion of mice unable to achieve expulsion (non-responders) was relatively constant in various populations of the strain, but could be increased by selective breeding from mice of known status (Wakelin, 1975b).

The mechanisms involved in the expulsion of T. muris from the

caecum and colon of the mouse have been examined in a number of studies (Wakelin and Selby, 1973, 1974, 1976; Wakelin, 1975a, 1978). Immunity to T. muris can be passively transferred with serum or lymphoid cells taken from actively immunized animals. The greatest degree of protection was achieved with serum, but immunity was transferred most consistently by cells. However, the level of immunity transferred was not comparable to that obtained by active immunization in either case. Immune mesenteric lymph node cells (IMLNC) were effective in the transfer of immunity, but lymphoid cells from the spleen were not, suggesting that cells primed against T. muris are localized in the mesenteric lymph node (MLN). The antibody mediated phase of expulsion has been shown to precede the lymphoid cell-mediated phase, and nylon wool non-adherent T cells, but not adherent B cells, were the effective component of IMLNC in the transfer of immunity (Lee, Wakelin and Grencis, 1983). Lee and Wakelin (1982a) have examined the presence of mucosal mast cells (MMC) during T. muris infections, and compared MMC accumulation in "responding" and "non-responding" DBA/2 mice (the former are able to expel an infection of T. muris during larval development, the latter are not), early rejecting NIH and late rejecting CBA/Ca strains. They found no temporal correlation between MMC accumulation in the gut and expulsion of T. muris, although this does not formally rule out any causal relationship between the two. MMC accumulation during the expulsion of secondary infections was also examined. MMC numbers in both NIH and CBA/Ca did not rise higher than control levels for at least 3 days after the expulsion of the challenge infection. Again this does not rule out the possibility of the participation of MMC in the expulsive immune response, as MMC which may have degranulated will not be observed by staining histological sections of the

gut. Indeed, Miller et al. (1983) demonstrated that a specific rat MMC protease (RMCP II) can be detected in the gut lumen of rats within minutes of an i.v. antigen challenge of Nippostrongylus brasiliensis primed animals. The appearance of RMCP II systemically was associated with a depletion of the jejunal concentration of RMCP II and of intestinal MMC. If mouse MMC are found to possess a specific protease, analagous to RMCP II of rat MMC, then measurement of systemic levels of the released specific protease would be a more reliable means of monitoring the secretory activities of MMC in vivo.

It is believed that a mouse model tolerant to a chronic infection of T. muris provides an opportunity to examine factors operating in chronic trichuriasis and may help illuminate the situation occurring in chronic human infections with T. trichiura. Wakelin (1970a) demonstrated that the administration of cortisone for a short period during a primary infection of T. muris in Schofield mice allowed the establishment of an adult population of worms; if this infection was cleared by anthelmintic treatment, a secondary infection would also survive to maturity. This condition has been interpreted as a state of chemically-induced tolerance. Lee and Wakelin (1982b) also demonstrated cortisone-induced immunotolerance to T. muris in CBA/Ca mice. Tolerant mesenteric lymph node cells (TMLNC) will not transfer immunity to naive mice but tolerant serum (TS) will (Lee, 1982). Transfer of IMLNC to a tolerant mouse does not cause the expulsion of the existing infection, however, if the patent infection in tolerant mice is cleared with anthelmintic and IMLNC are given at the same time as a secondary infection, the latter is expelled. Naive MLNC, on the other hand, given at the same time as a secondary infection are ineffective and the infection establishes.

Hence it has been suggested that the tolerance observed is a result of a defect in an effector mechanism involving the priming of MLN T cells. It also seems to be the case that an established adult infection is more difficult to remove by immunological means than a developing larval infection. Subsequent experiments revealed that mice harbouring chronic infections were less able to respond to a primary infection with Trichinella spiralis or to produce humoral responses to lipopolysaccharide (LPS) and sheep red blood cells (SRBC) (Lee and Wakelin, 1983). However, MLN T cells from tolerant mice were as responsive to the polyclonal activator phytohaemagglutinin (PHA) as those of naive mice. Furthermore, homing of activated MLNC from infected donors to the intestine of animals harbouring chronic infections was not impaired.

The expulsion of Trichuris muris from the large intestine does not involve a marked inflammatory response and myeloid components have not been shown to be involved in expulsion (Wakelin and Selby, 1976). The restoration of the ability to expel infections in irradiated mice was found to require primarily the replacement of a lymphoid cell type present in MLNC; expulsion was not restored (within 10 days) by the transfer of bone marrow and no synergistic effect was seen when both bone marrow and MLNC were transferred. The inhibitory effect of irradiation on delayed hypersensitivity responses in immunized mice is known to be due to the destruction of the macrophage precursor pool, an effect which is rapidly reversed by the transfer of bone marrow (Youdin et al., 1973). The failure of the bone-marrow to restore challenge expulsion within 10 days thus appeared to confirm that bone-marrow-derived cells other than lymphoid cells were not involved in the expulsion of T. muris, since the differentiation of such cells would have been expected within

this time (Youdin et al., loc. cit.). Trichuris muris is quite different from Trichinella spiralis in this respect, in the latter inflammation has an important role to play in the expulsion of the parasite (Wakelin and Wilson, 1979). Alizadeh and Murrell (1984) also demonstrated the requirement of bone marrow cells and mast cells (MC) for the immune expulsion of T. spiralis in MC-deficient W/W^v mice. Indeed, helminth infections are often associated with marked intestinal inflammation, reflected both in cellular changes in the mucosa itself, and in altered physicochemical changes in the intestinal lumen (see Wakelin, 1978). It is widely believed that the immediate cause of worm expulsion in many infections lies more in these inflammatory changes than in the immune responses which precede them (reviewed by Larsh and Race, 1975; Miller, 1984).

As an overt inflammatory response does not appear to be necessary for the expulsion of Trichuris muris from the large intestine of the mouse, other effector mechanisms have been proposed. One possibility is a more direct interaction of effector T cells with the parasite (Lee, 1982). Intraepithelial lymphocytes (IEL), thought to be derived from rapidly dividing T cell populations in the MLN and Peyer's patches, may be of importance in immunity to T. muris. The attachment and feeding site of the worm may render it particularly susceptible to IEL, either as a result of environmental changes mediated by these cells, or the direct ingestion of cytotonic granules by the parasite. These may cause extensive damage to the intestinal tract of the worm and could significantly contribute to its expulsion.

1.3 ANTIGENS OF TRICHURIS MURIS

Active immunization, resulting in an accelerated expulsion of a challenge infection of T. muris, has been demonstrated using various antigen preparations of the worm (Wakelin and Selby, 1973). A reduction of up to 92% of the larval worm burdens was obtained in some instances, and the most effective preparations were found to be either the soluble extract of homogenates of the anterior portion of the nematode containing the stichosome (SA) (see Fig. 1.4) or excretory/secretory (E/S) products collected by in vitro culture of T. muris. Jenkins and Wakelin (1977) partially fractionated the protective antigens from SA and E/S, which were recognized by rabbit antisera raised against crude whole worm extract, and concluded that one of the protective immunogens was a protein believed to originate in the stichosome. The isolation and identification of antigens which elicit protective immunity against T. muris would facilitate analysis of the immune mechanisms which underlie such responses. These "target antigens" may be used to determine which immunological events ultimately cause the expulsion of T. muris, from its host. The latter could be examined in greater detail in vivo and in vitro, in the absence of "background" responses, which may be triggered by various other parasite molecules during the course of infection, or may be present in antigen preparations, such as crude worm homogenates or E/S products, used in previous investigations.

The stichosome is only found in the trichuroid nematodes and its ultrastructure has been partially described in the L_1 larvae and adults of Trichinella spiralis (Bruce, 1970, 1974; Despommier, 1974; Despommier and Muller, 1976; Despommier, 1983), in the adults of Capillaria hepatica (Wright, 1972) and in the adults of T. muris

(Sheffield, 1963; Wright, 1972), T. myocastoris and T. vulpis (Wright, loc. cit.; Sheffield, loc. cit.). Generally the stichosome consists of a row of cells (stichocytes) which partially or completely enclose the posterior portion of the oesophagus. The latter is separated from the stichosome by a membranous sheath continuous with the pseudocoelomic membranes, which enclose the entire stichosome and oesophagus together. The relatively large stichocytes contain numerous granules, abundant cytoplasmic reticulum and large numbers of mitochondria (Wright, loc. cit.; Sheffield, loc. cit.), suggesting that they are active secretory cells. The cytoplasm of the stichocytes also contains many fine anastomising canaliculi, and a direct duct-like connection between the stichocyte and the oesophagus has been reported for both larval and adult Trichinella spiralis (Richels, 1955; Bruce, 1970; Despommier, 1974). In T. muris only the canaliculi have been observed. Wright (1972) suggests that there is undoubtedly a connection between the stichocytes and the oesophageal lumen, but as a result of the sampling problems with electron microscopy this single small structure in the relatively large stichocyte has not yet been recorded. It is probable that secretory material from the stichocytes is passively pulled from the intracellular collecting ducts as the oesophageal lumen expands, and may be passed out of the mouth or into the intestine.

Investigations concerned with the stichosome and its secretions have progressed much further with Trichinella spiralis than with Trichuris spp. Analysis of the contents of the secretory granules of the stichocytes of Trichinella muscle larvae (Despommier and Muller, 1976), showed that the cells usually contained one of two types of granules; the α-granules, approximately 800 nm in diameter with a prominent inclusion and a granular matrix, and the β-

granule, about 600 nm with a homogeneous matrix. The granules may be separated by density gradient centrifugation and at least 4 unique antigens were attributed to each of the granule types. Antigens from both types of granule also cross reacted totally with those present in the E/S products of L_1 muscle larvae kept alive in in vitro culture. Silberstein and Despommier (1984) generated a series of MoAbs using splenic lymphocytes from T. spiralis infected mice, in order to study the antigens of the infective L_1 larvae. Three of these MoAbs were isolated and were found to have specificities for antigens of molecular weights of 37 kD, 48kD and 50/55 kD, which possessed different abilities to induce protection. The 48 kD antigen induced a high level of protection at biologically relevant doses (1.0 and 0.1 µg protein/mouse); the 50/55 kD induced a lower level of protection than the 48 kD, but was still effective at similar doses; the 37 kD antigen elicited protection only at a dose of 50 µg protein/mouse, and was not as effective as unpurified secretory antigen at any dose. The 48 kD and 50/55 kD antigens were shown to be significant components of the larval secretions (12% and 5% respectively), but were only relatively minor components of the subcellular fractions of homogenised worms. The 37 kD antigen represented a major component of the cytosol subcellular fraction but was essentially absent from the soluble portion of the large subcellular fraction and from the secretions. Immunocytolocalization studies showed that the 48 kD antigen was located in the β-stichocytes, the lining of the gut and the surface of the cuticle. The 50/55 kD antigen was detected in the α-stichocytes and was occasionally observed in the gut or on the cuticle. The 37 kD antigen was detected only in the pseudocoelom. Both α and β stichocytes, containing the 50/55 kD and 48 kD antigens respectively, secrete their

granule contents into the host during the first 30 hours of the enteral phase of infection. These data were the first account of antigens purified from a nematode, which produced a strong protective immune response after *in vivo* immunization.

Gamble and Graham (1984) also used a MoAb to isolate 49 kD and 53 kD antigens from *in vitro* derived E/S products of *Trichinella* infective L_1 larvae, the antigens sharing a common epitope. When these affinity-isolated antigens were used in an ELISA for the immunodiagnosis of trichinosis in pigs, all false positives with sera from healthy swine were eliminated, and all *T. spiralis*-infected swine were detected. The antigenic determinant recognised by this MoAb was believed to be unique to *T. spiralis* as there was no cross reaction with other common swine parasites such as *Trichuris suis*. Isenstein *et al.* (1985) have also described the development of an enzyme immunoassay for the surveillance of trichinellosis in swine, using antigens isolated by immuno-affinity chromatography from the large-particle fraction of muscle larvae, as described by Despommier and Laccetti (1981). Gamble (1985) used the 49 kD and 53 kD antigens to immunize mice and found that the reduction of muscle larvae was at a level comparable to priming with crude E/S antigens. These affinity-isolated antigens also induced the accelerated expulsion of adult worms from the intestines of mice, and reduced the fecundity of remaining females. The protective antigens were localized in the stichocytes by immunoperoxidase staining techniques, and it is plausible to suggest that the 49 kD and 53 kD described by Gamble and Graham (1985) are similar or identical to the 48 kD and 50/55 kD antigens described by Silberstein and Despommier (1984).

Durham, Murrell and Lee (1984) isolated antigen fractions from whole muscle larval extract by gel filtration and ion exchange chromatography, and assayed these for allergenicity by a footpad swelling test in mice. IgE antibody levels in rats immunized with the fractions were then determined by passive cutaneous anaphylaxis tests in rats. By these methods an allergenic fraction from T. spiralis muscle larvae, which was found to be a single glycoprotein (F1-b allergen) with a molecular weight of 45 kD, was obtained. Rats immunized with this antigen had fewer intestinal worms than control animals at 24 hours and 7 days after oral challenge with T. spiralis larvae. However, the source of the F1-b allergen was not determined.

1.4 NEMATODE ANTIGENS AS TARGETS OF THE HOST IMMUNE RESPONSE

There is specific cross-immunity between Trichuris muris and Trichinella spiralis (Lee, Grencis and Wakelin, 1982b) and infections with either parasite were effective in eliciting an accelerated onset of expulsion of a heterologous challenge. Accelerated expulsion could also be achieved by the administration of soluble crude worm antigen given 12 days before the heterologous challenge or by the adoptive transfer of MLNC from mice infected with the heterologous parasite. These results thus indicated a specific cross-immunity due to shared antigens and it was postulated that these shared antigens were derived from the stichosome.

To date there is little information available on the individual components comprising in vitro E/S products of T. muris, which elicit a protective immune response. It is possible that the stichocyte products make up a significant proportion of the E/S

material and it has been proposed that the antigens shown to elicit a protective immune response originate in the stichocyte (Jenkins and Wakelin, 1977). As stated previously, to evaluate the importance of data on the mechanisms of the immune response to infection, it is essential to identify the parasite molecules, which are the target of the protective immune response. So far there has been limited success in identification and purification of the target antigens of parasitic nematodes (reviewed by Silberstein, 1984; Almond and Parkhouse, 1985). In the case of T. spiralis, however, several groups have raised MoAbs against protective antigens from the infective muscle larvae of T. spiralis (Silberstein and Despommier, 1984; Gamble and Graham, 1985; Ortega-Pierres et al., 1984b) and the former two have utilized these MoAbs to isolate the protective antigens by affinity chromatography.

So far parasite antigens have been discussed in terms of their ability to elicit a protective immune response in the host. There is relatively little information with regard to the function of these antigens in the parasites themselves, and it is hoped that the localization of target antigens in the parasite may provide clues as to their function in vivo. Parasite antigens have been classified in a number of ways (Anders et al., 1982), and as the original classification was proposed by parasitologists the criteria used were based on life cycle stage and site of origin. Parasites may be conveniently divided into 3 compartments; the surface, the E/S products and the residual somatic antigens. Such divisions are arbitrary and are by no means exclusive. Indeed, it has been recognized for some time that surface molecules are present amongst the released components (Kusel et al., 1975; Philipp et al., 1980; Grencis et al., 1986). However, the utilization of recently

developed radiochemical labelling procedures has enabled precise analysis of the various antigenic components, and their sites of origin. Accordingly, the techniques used to investigate the antigenic composition and the dynamic nature of helminth surface structures, have been recently reviewed (Philipp and Rumjanek, 1984). Notwithstanding the considerable amount of information concerning parasite-derived products, with the E/S and surface antigens identified for the different life stages of many nematodes (Philipp *et al.*, 1980; Maizels *et al.*, 1982; Almond and Parkhouse, 1985), few have been classified functionally. However, many nematodes have been shown to secrete the enzyme acetyl cholinesterase (AChE) *in vitro*, and infective hosts may produce anti-nematode AChE antibodies (Rothwell *et al.*, 1976). The precise role of this enzyme in the survival of parasites is not known, although it has been suggested that it may act as a "biochemical holdfast" for adult worms in the GI tract (Ogilvie and Jones, 1971), possibly by paralysing the intestinal smooth muscle and interfering with mucus secretion by intestinal goblet cells (Philipp, 1984). Recent investigations have been undertaken to examine the proteolytic enzymes secreted by *Ancylostoma caninum* and *Necator americanus* in greater detail (Hotez and Cerami, 1983; McKean, Carr and Pritchard, 1986). Hotez *et al.* (1985) succeeded in isolating and purifying a proteolytic enzyme from adult *A. caninum*, which is believed to play a role in the histolytic and anti-clotting activities of the worm. The enzyme was found in both L_3 larvae and adults. If specific antibody were to be produced by the host against the enzyme released by the L_3 larvae, then this antibody may inhibit the proteolytic activity, and hence the feeding of adults. In support of this it has been observed, that adult hookworms obtained from dogs immunized against L_3 larvae

were found to have greatly diminished capacity to feed *in vivo* on host blood (Miller, 1971). The production of enzymes believed to be involved in the penetration of host tissues have been investigated in adult and larval *Strongyloides ratti* and larval *S. ransomi* (Wertheim *et al.*, 1983; Dresden *et al.*, 1985). Wertheim and colleagues found that the collagenolytic activity of the metabolites of adult worms appeared weak, whereas that of extracts of the adults was strong; it was suggested that the collagenase was active in adult females at the time of migration in the intestinal mucosa during oviposition. However, no collagenolytic activity was found to be exerted by the living larvae, for which skin penetration by enzymatic means has been postulated (Lewert and Lee, 1956); their extracts and metabolites were also examined and found to be negative. On the other hand, Dresden *et al.* (1985) isolated proteolytic enzymes from the L_3 larvae of *S. ransomi*, and made preliminary characterisations of 2 groups of these enzymes found in extracts of this parasite.

If the defined protective antigens of *T. muris* are found to originate in the stichosome then an investigation of their possible enzymatic capacity may give clues as to the role of the stichocytes in the survival of trichuroid nematodes. To date, there is no direct evidence to substantiate the belief that the stichosome in *T. muris* is the source of enzymes involved in extra-corporeal digestion, a role previously suggested for it (Lee and Wright, 1978). Concerning *Trichinella spiralis*, there is no information as regards the feeding of the worm, although as the adults contain only small amounts of glycogen (Ferguson and Castro, 1973), it has been assumed that their energy requirements are met by the ingestion of

material from their immediate environment. Speculations as to the function and the precise site of action of the stichosome secretions of T. spiralis have included the suggestion that they may be enzymes (Despommier, 1974; Despommier and Muller, 1976), as the immune responses directed against them have such profound influences on the establishment of an infection (Despommier, 1977). Worms developing in the immune host become stunted, are inhibited in their reproduction and are forced out of their enteral niche at an accelerated rate. It is reasonable to expect that a number of parasite antigens might induce these protective responses, which serve to reduce the number of migrating larvae in the host. Secreted products from both the larval and adult stages of T. spiralis in the gut, elicit a major portion of the protective responses in the host (Despommier, loc. cit.), and the effects upon the worm of the immune responses of the host, which act against these secretions, are reversible by transplanting adult worms from immune animals to non-immune recipients (Bell et al., 1979). The fact that the observed adverse effects on the worms are not permanent, has led to the suggestion that they may result from an inhibition of excretory/secretory products which function outside the worm, perhaps interferring in parasite nutrition, as originally put forward by Chandler (1937). Until recently, no enzymatic activities had been ascribed to any particular fraction of secretions obtained from any stage of T. spiralis (Despommier, 1983). However, Rhoads (1983) identified and purified a superoxide dismutase from T. spiralis muscle larvae, which was present in somatic extracts and was also excreted into culture fluids during in vitro culture of larvae for periods as short as 3 hrs and up to 72 hrs. The enzyme was believed to function as an essential defence mechanism against the highly destructive superoxide radical en-

countered either intracellularly, as a product of biological oxidation, or externally as a component of the host's immune system. A number of glycosidases and an acid phosphohydrolase of T. spiralis have also been characterised (Rhoads, 1985), these were present at high levels in extracts of the adult and muscle larvae, but were at considerably lower levels in the new born larvae. The latter enzymes were also detected in culture fluids collected after 15-20 hrs. incubation of both muscle larvae and adults, albeit at much lower activities.

Nimmo-Smith and Keeling (1960) examined the hydrolytic enzyme capabilities of extracts of adult T. muris, which were shown to hydrolyse a large number of substrates. The anterior ends of the worms were found to have appreciably higher proteolytic and esteratic activity than the posterior ends, but with few exceptions the enzymes encountered in the survey had acid pH optima, with relatively little activity above neutrality. It was not possible to distinguish between digestive and intracellular enzymes, and those examined were believed to be primarily concerned with the turnover of cellular constituents and metabolites of the worm. Jenkins (1970) carried out a morphological and histochemical study of T. suis, to localize various enzymes in the worm, and found the bacillary band and stichosome to be metabolically active. This study, however, did not include an examination of proteinases. T. suis differs in attachment to T. muris in that the foremost part of the anterior end of the worm is not enclosed within the epithelial tunnel, and a proportion of the worms found in infected caeca are always unattached. Jenkins (loc. cit.) concluded that T. suis ingested mucopolysaccharides and/or mucoproteins from the caecal lumen of the host, and this ingested material was then subjected to preliminary hydrolysis in the

oesophagus, the hydrolytic enzymes being located in the stichosome and the oesophageal wall.

1.5 SUMMARY

The aims of this thesis are :

i) To look at the mechanisms of expulsion of Trichuris muris from the large intestine of the mouse qualitatively and quantitatively. To determine which factors are responsible for the variation in the time of onset of expulsion of T. muris from different strains of mice, and for the lack of an expulsive response in a proportion of mice belonging to the DBA/2 strain.

ii) To investigate the antigens of T. muris, and to attempt to isolate and localize those responsible for eliciting a protective immune response. Localization of these antigens in the worm may then provide clues as to their function.

iii) To further examine the antigenic relationship between Trichuris muris and Trichinella spiralis, and determine the nature of those shared antigens which are responsible for heterologous immunization.

iv) To make an initial investigation of the possibility of there being shared antigens between Trichuris muris and Trichuris trichiura.

Monoclonal antibodies were produced to assist in these investigations; notably in the identification and isolation of the protective components within the E/S products.

Fig. 1.1

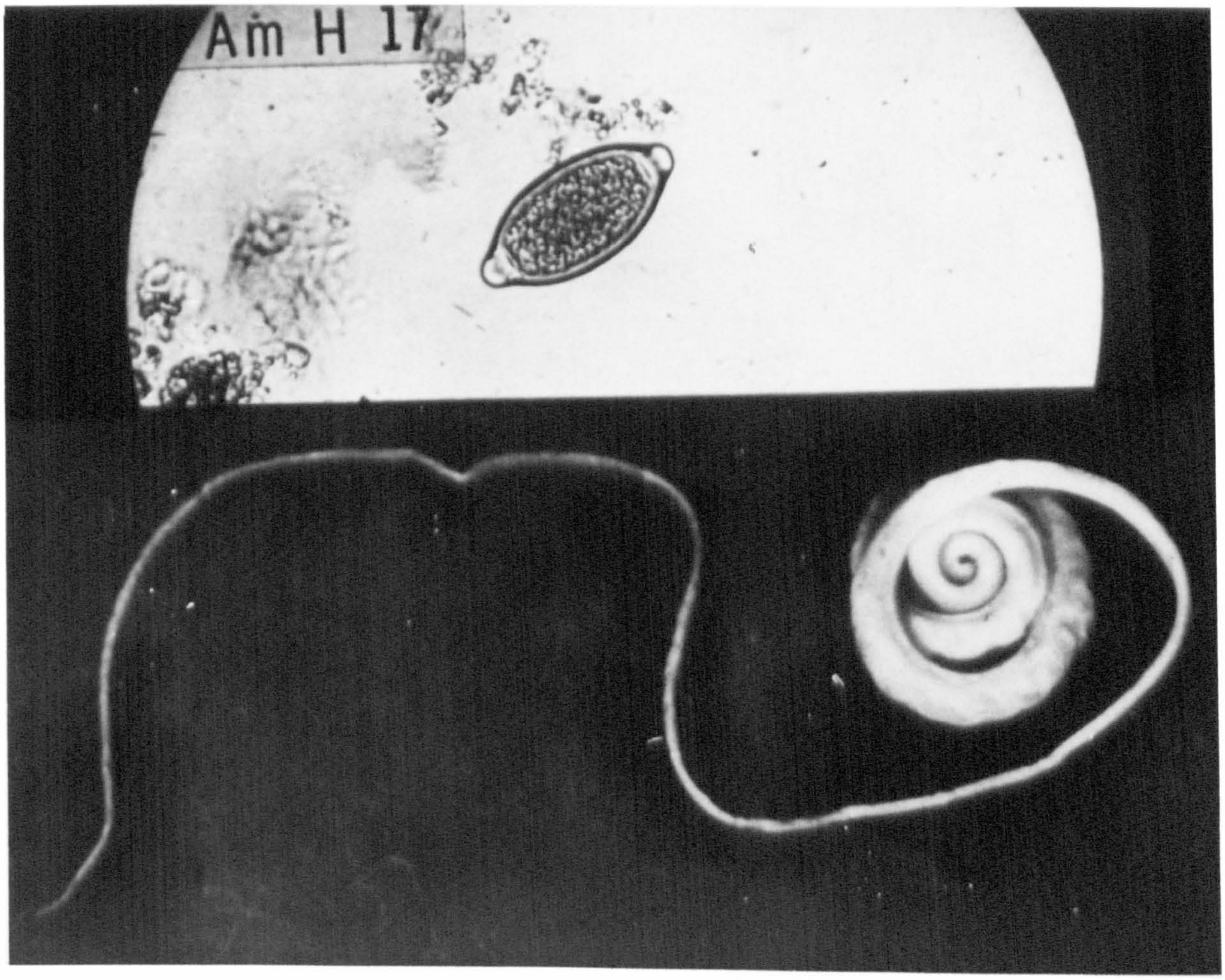
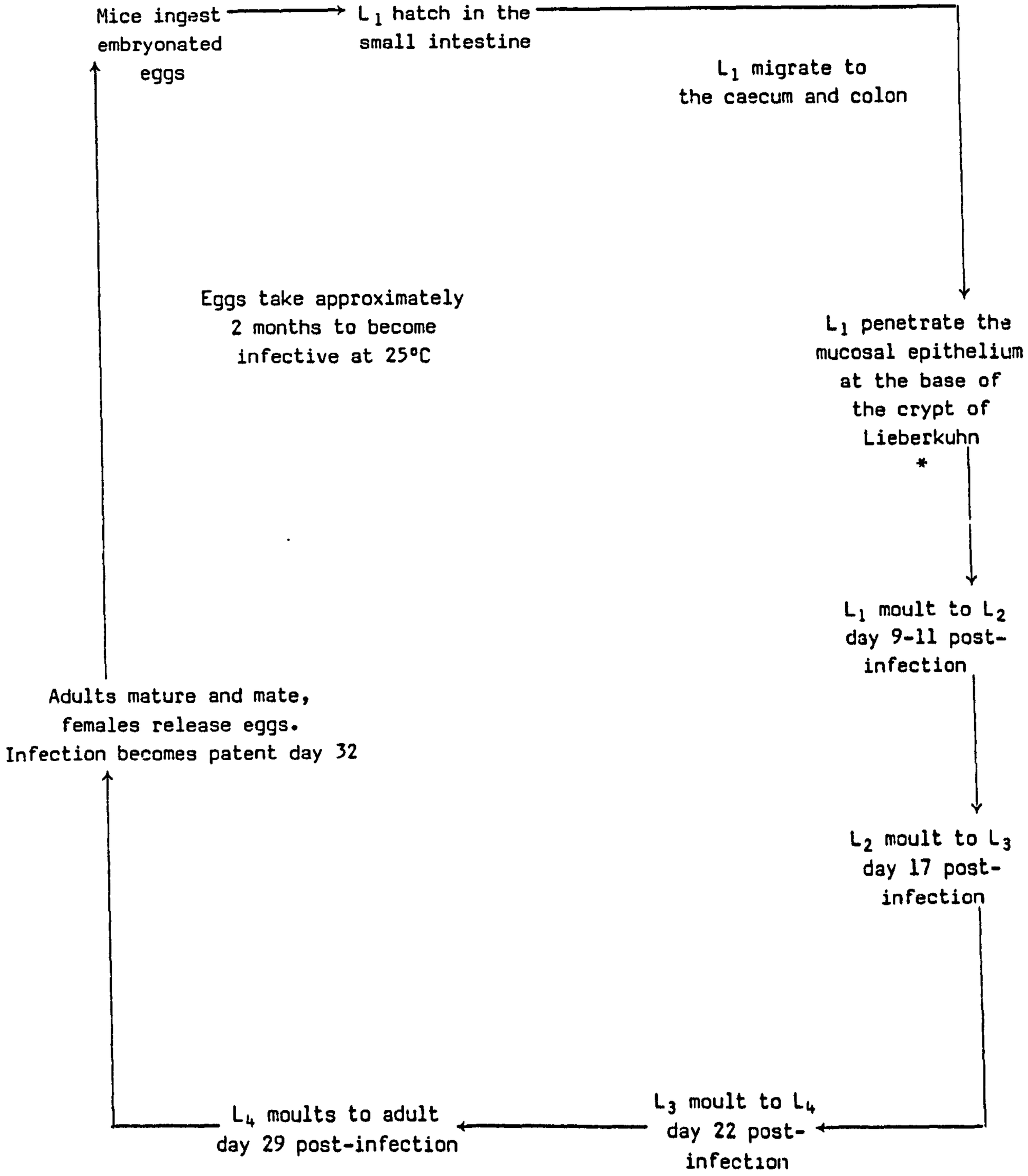


FIGURE 1.2

Life Cycle of Trichuris muris



* Larvae migrate up the crypts of Lieberkuhn during their development. At approximately day 10-15 post-infection they are found on the surface of the epithelium still completely embedded in the epithelial sheet. It is here that further growth and development occurs. At around day 15-20 post-infection the posterior end of the worm breaks free from the epithelium.

FIGURE 1.3 SCANNING ELECTRON MICROGRAPH OF TRICHURIS MURIS FEMALE IN THE LARGE INTESTINE OF A MOUSE

o - Oesophageal portion of worm (anterior end)

v - Vulva (note eggs clustered at opening)

p - Intestinal portion of worm (posterior end)

Reproduced from Lee and Wright 1978.

Fig. 1.3

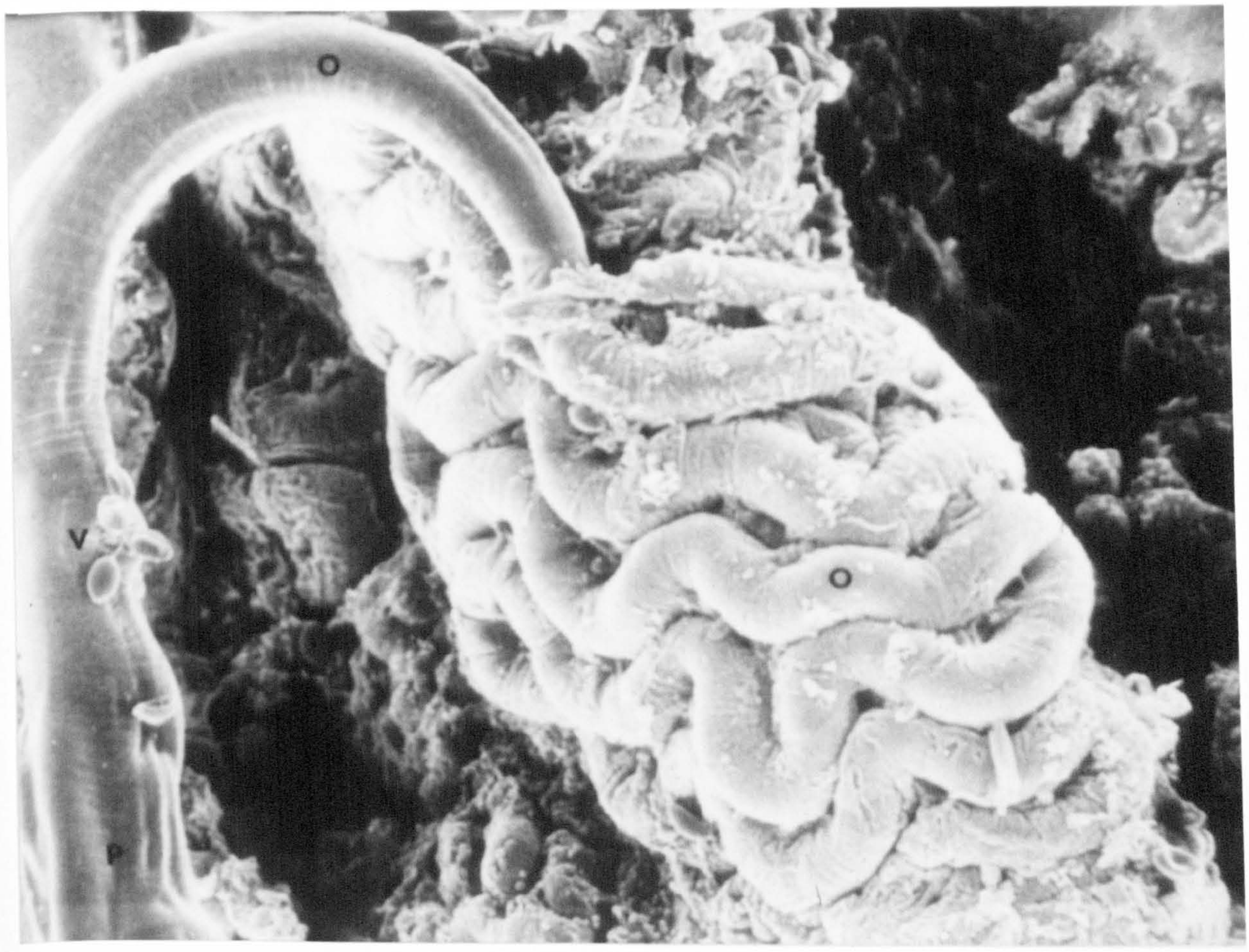


FIGURE 1.4

Trichuris muris (x 100)

A - anterior end of worm

J - stochosomal-intestinal junction

I - beginning of worm intestine

FIGURE 1.5

Trichuris muris (x 200)

S - stichocyte surrounding oesophagus

N - nucleus of stichocyte

Fig.1.4

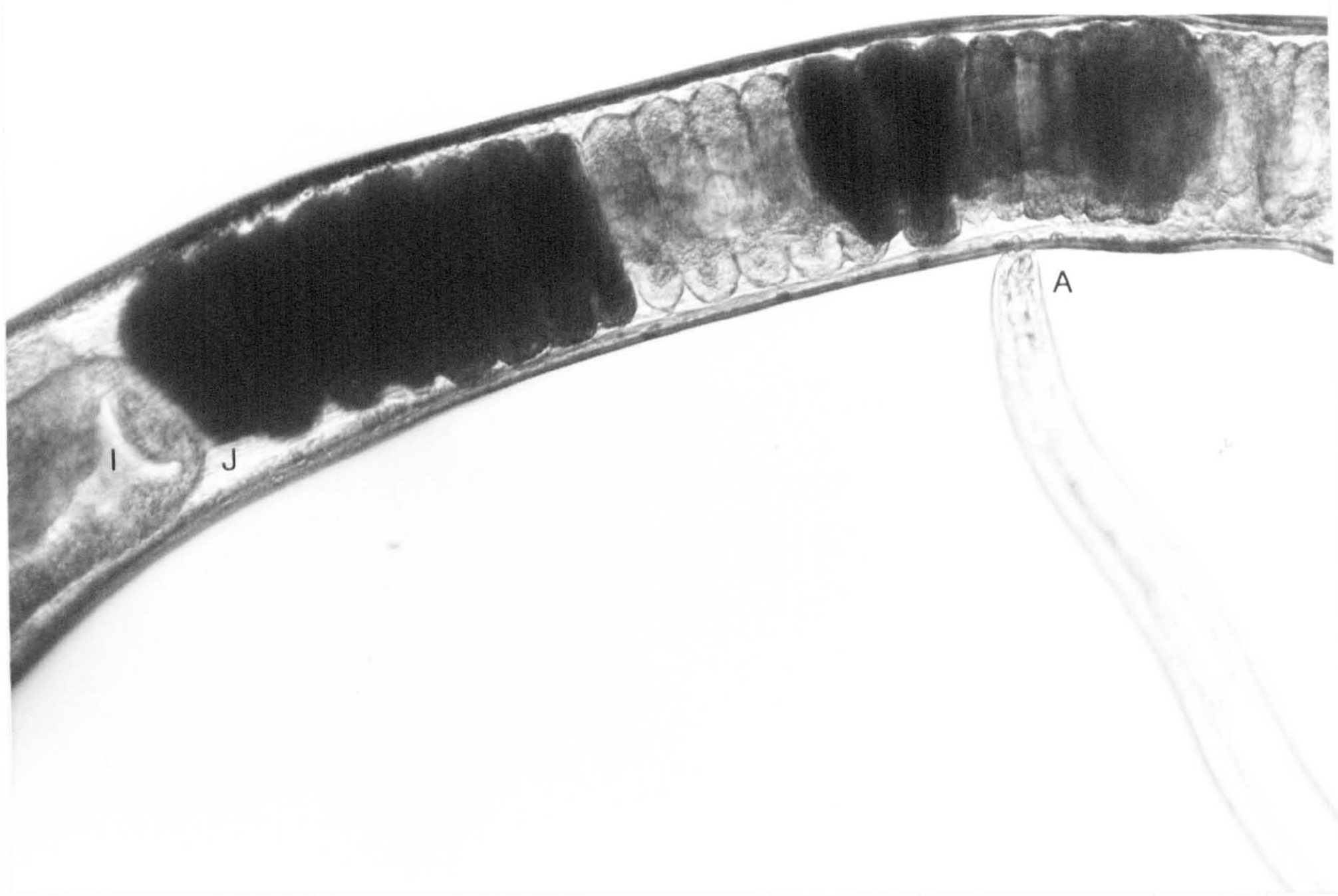
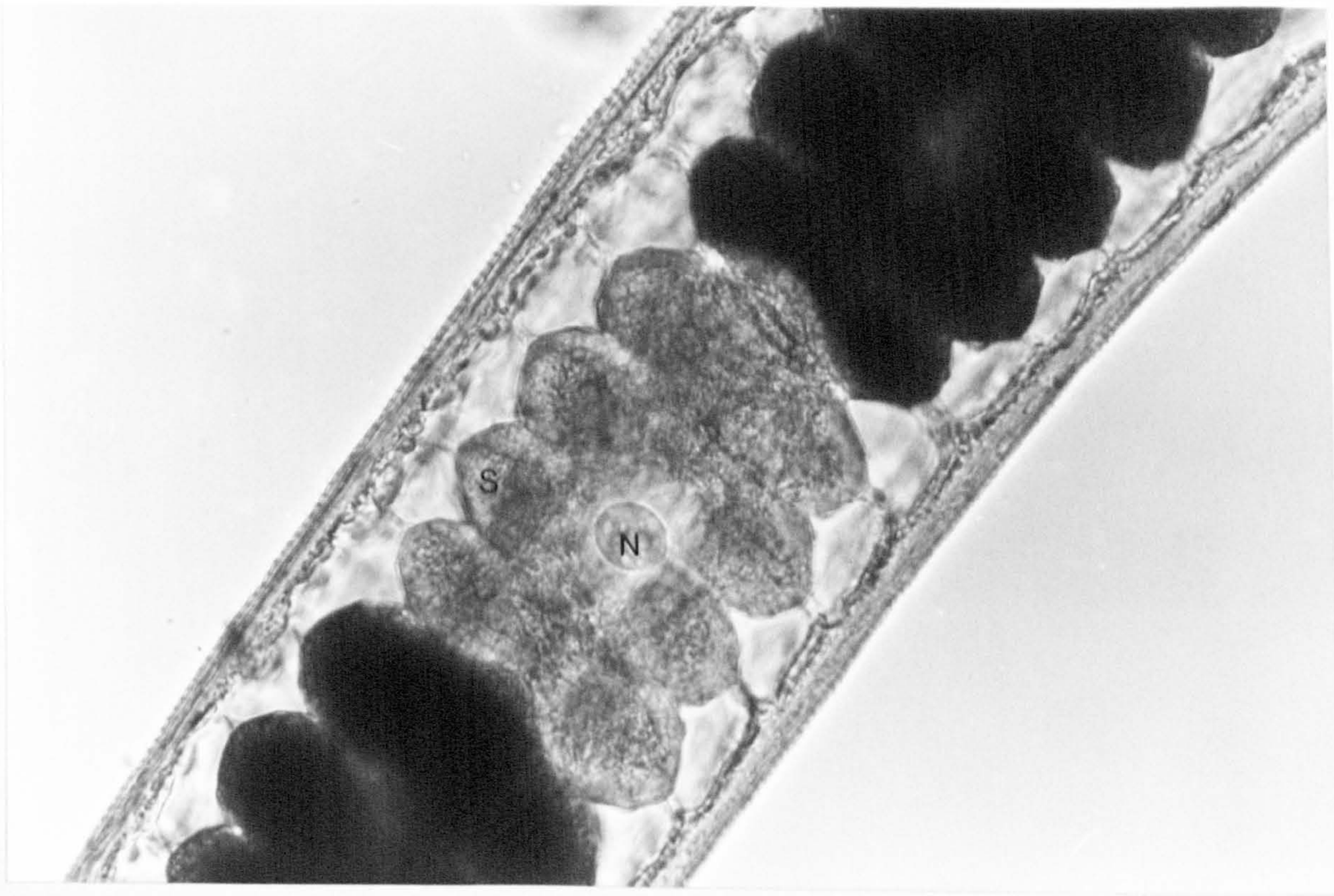


Fig.1.5



CHAPTER 2

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MATERIALS AND METHODS

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2.1 GENERAL TECHNIQUES

Mice :

DBA/2 and CBA/Ca inbred mice were obtained from Olac 1976 Ltd., BALB/c, C57Bl/10, NIH inbred and CFLP outbred mice were bred and maintained in the Department of Zoology, University of Nottingham, using animals obtained from Olac 1976 Ltd. Home bred animals were treated with 0.2 mls piperazine citrate (12.5% solution in water, Sigma) on 2 occasions seven days apart to clear any existing pinworm infections. All animals were kept on piperazine citrate water (3g/l) until used. Male mice were used in all experiments; they were 6-8 weeks old at the start of each experiment, unless otherwise stated.

Rats :

Male Wistar rats were obtained from the University of Nottingham, School of Agriculture, Sutton-Bonington, and housed under conventional animal house conditions.

Nematodes :

i) *Trichuris muris*

Maintenance and Infection :

Stock infections were maintained in CFLP mice. Mice were infected with approximately 400 *T. muris* embryonated eggs, and given 0.05 - 0.075 ml of cortisone acetate (25 mg/ml Cortistab, Boots) S.C. on days 7, 9, 11, 13 and 15 post-infection, according to the size of the mouse. Animals were given oxytetracycline hydrochloride (165 mg/l, Terramycin, Pfizer) in their drinking water from day 7, when cortisone acetate was first administered.

On day 40 post-infection mice were killed and the female worms were removed into phosphate buffered saline (PBS, see

Appendix). Worms were ground in a glass tissue homogenizer with a size smaller plunger, so as not to rupture the eggs. Ground worms were filtered through 2 thicknesses of fine nylon mesh, the eggs were washed once and finally resuspended in distilled water with 0.25% formalin and 250 µg/ml fungizone (Gibco).

The eggs were incubated at room temperature in the dark for at least 45 days, in used tissue culture flasks (Nunc). Egg cultures were inspected occasionally to check development and possible contamination. Once sufficient time had elapsed for embryonation of the eggs, the cultures were stored at 4°C.

Larval Worm Counts :

Mice were killed by an overdose of chloroform and the caeca and colons removed. The pieces of gut were placed in plastic petri-dishes and frozen at -20°C for at least 24 hours.

Guts were thawed for counting, and immediately after thawing the gut contents were flushed out with PBS. Each gut was then placed in fresh PBS in a petri-dish and the caecum and colon scraped with curved forceps. Worms were removed from the petri-dishes as they were counted and each dish was routinely examined twice. The gut contents were also scanned for the presence of larvae, but contained relatively few worms, if they had been flushed from the gut immediately after thawing.

ii) Trichinella spiralis

T. spiralis maintenance and recovery have been extensively described by Wakelin and Wilson (1977). Briefly, infective larvae were obtained from the muscles of stock mice, which had been infected for a minimum of 35 days. The tissue was digested for 1½ - 2 hrs in 0.5% pepsin and 0.5% HCl at 37°C. Adult worms were recovered from the

intestine by a Modified Baerman technique.

Antigen Preparations :

i) Adult Male Homogenate (AMA)

The caeca and colons of mice with patent infections of T. muris were removed and placed in PBS at room temperature. The guts were cut open and the contents flushed out. After placing the guts in fresh PBS the adult males were carefully removed using watchmaker's forceps, and placed in PBS at 4°C.

Collected worms were washed three times in PBS, and at this stage could then be frozen in a minimal amount of PBS. When a sufficient number of worms had been collected, they were homogenized in PBS using a glass tissue homogenizer, and allowed to 'extract' overnight at 4°C. The resulting suspension was then centrifuged at 1500 g for 30 minutes at 4°C to remove coarse particulate matter, followed by ultra-centrifugation of the supernatant at 100,000 g for 1 hour at 4°C, to remove insoluble material. The final resulting supernatant was filtered using 0.22 μm Millipore filter, analysed for protein (Section 2.1, protein estimation), aliquoted and stored at -40°C.

ii) Day 14 Larval Homogenate (d14 LH)

The caeca and colons of infected mice were removed at d14 post-infection, the guts were slit open and the gut contents thoroughly flushed out. The opened guts were then put in a large piece of fine mesh nylon gauze and placed in a 500 ml glass beaker with PBS at 37°C, so that the guts were just below the surface of the liquid. The beaker was then incubated in a water bath at 37°C for 2 hours and the larval worms collected by a modified Baerman technique.

Larval worms were collected from the bottom of the beaker and washed 3 times in fresh PBS. Worms could at this stage be frozen in a minimal amount of PBS until sufficient had been collected for homogenization. Larval worms were homogenized in a glass tissue homogenizer in PBS and left to extract o/n at 4°C. The resulting mixture was then centrifuged for 30 min at 10,000 g. The supernatant was filtered using a 0.22 μm sterile filter (Millipore) analysed for protein, aliquoted and stored at -40°C.

iii) Excretory/Secretory Antigen (E/S)

After the guts of mice with patent infections of T. muris had been removed, opened and the gut contents flushed out with PBS, they were placed in sterile RPM1 1640 plus supplements (see Appendix) with x5 usual concentration of penicillin/streptomycin, and fungizone (Gibco) at 250 μg/ml. Adult males and females were individually removed and placed in fresh medium, and 'washed' for 2-3 hours in this medium at 37°C. Worms were then put into a final change of medium and left overnight at 37°C for the collection of E/S products.

The following day the culture supernatant was removed and centrifuged at 200 g for 5 minutes at 4°C to remove eggs, then filtered (0.22 μm filter, Millipore), analysed for protein (Section 2.1) aliquoted and stored at -40°C.

iv) ^{35}S-methionine Labelled E/S and AMA

The procedure was the same as for the collection of non-labelled E/S products, but ^{35}S-methionine (Amersham) was added before the overnight incubation at 250 μCi per ml of medium. About 25 worms were used for 1 ml of medium. The following day the E/S products were analysed for labelled protein (Section 2.1 Antigen Preparation (vi)), aliquoted and frozen at -70°C.

The whole worms from the overnight incubation in medium containing ^{35}S-methionine were then homogenized in worm homogenization buffer (see Appendix) using a glass tissue homogenizer. The resulting suspension was spun at 10,000 g for 10 minutes and the supernatant analysed for labelled protein, aliquoted and stored at -70°C.

v) ^{125}I-labelled Surface Antigen (SA)

Labelling was performed essentially as in Philipp et al., 1980 using IODOGEN reagent (1,3,4,6-tetrachloro-3-alpha-6-alpha-diphenylglycouril, Pierce), which promotes labelling of tyrosine residues (Zingales, 1984). IODOGEN was dissolved in methylene chloride to give a working dilution of 200 µg/ml, 100 µl of this solution was added to a number of small glass test-tubes and evaporated to dryness in a fume cupboard at room temperature. Once coated these tubes could be stored in the dark in a desiccator at room temperature for several weeks.

Tubes were rinsed out with borate buffered saline (BBS, see Appendix) before use, to remove any loose flakes of IODOGEN reagent. Worms to be labelled were then put into the tubes in a minimal volume of BBS. Only 5 or 6 worms were used per tube so as to give each worm adequate contact with the bottom of the tube. 10 µl of potassium iodide solution (11 µg/ml in BBS) was added to each tube, followed by 100 µCi of ^{125}I (Amersham), and the volume in each tube made up to 100 µl with BBS. The reaction was allowed to proceed for 5 minutes at room temperature, after which the worms were washed in a saturated solution of L-tyrosine (Sigma) in BBS to remove excess ^{125}I. Worms were further washed with BBS until the supernatant was free of radioactivity. Worms were then homogenized in BBS or PBS with 2% Sodium Dodecyl Sulphate (SDS) and the solid

material was removed by centrifugation at 10,000 g for 10 mins. The resulting supernatant was analysed for labelled protein, aliquoted and stored frozen at -40°C.

vi) Surface Antigens removed with the Detergent CTAB

Surface antigens of adult worms were stripped from the cuticle of worms by the cationic detergent cetyltrimethylammonium bromide (CTAB, Sigma). CTAB antigen was obtained by incubating the worms in CTAB dissolved in RPM1 1640 medium (Sigma) to give a final concentration of 0.25% (w/v). Parasites were incubated for 1½ hours, but although alive when put into the medium containing CTAB worms began to die after approximately ½ hour. The incubation medium was collected, spun at 200 g for 5 mins to remove any eggs, and then dialysed overnight against PBS to remove the CTAB. The resulting solution was filtered, aliquoted and stored at -40°C.

vii) Trichloroacetic Acid Precipitation for Labelled Protein

Trichloroacetic acid (TCA) precipitation was used to estimate the amount of labelled protein obtained from either ^{35}S-methionine or ^{125}I-labelling procedures.

For ^{35}S-methionine labelled E/S products or AMA, 10 µl of the sample to be counted was diluted in 20 µl of PBS. Dilution of the sample helped to prevent any fat in the samples (especially AMA) from rapidly precipitating and sticking to the sides of the sample tube. 2 mls of ice-cold TCA (BDH) containing 2 mls hydrolysed casein (Sigma) was then added, the resulting precipitates were held on ice for 30 mins. The solutions containing the precipitates were filtered using a buchner funnel and flask onto 4.25 cm glass micro-fibre filters (Whatman), which were pre-wet with ice-cold 8% TCA. The filters were washed four times with ice-cold 8% TCA then finally

once with ethanol. After air-drying the filters were rolled up and put into a vial containing 6 ml of scintillation fluid (Optiphase 'X', Fisons) for counting on a Packard Tricarb liquid scintillation counter.

The above procedure could also be used for counting ^{125}I-labelled protein, with a final direct count of the filter paper on a gamma counter (Packard Auto Gamma 800 c counting system). However, a differential count of the percentage of labelled protein was usually performed as follows. 20 µl of protein solution was added to 30 µl of foetal calf serum (Gibco) and 50 µl of 25% TCA with 2% hydrolysed casein. This was spun at 10,000 g for 1 minute and the supernatant removed. The counts per minute (c.p.m.) of the precipitate, supernatant and 20 µl of the original protein solution could then be made using the gamma counter, and the percentage of labelled protein determined.

Antisera Preparations :

Large quantities of immune sera (IS) were raised in CFLP mice and three standard types of IS were prepared :-

i) IS d21 - CFLP mice were infected with 400 embryonated eggs of T. muris, killed with chloroform at 21 days post-infection and bled by cardiac puncture.

ii) IS AA - hyper-immune serum was raised against the anterior portion of adult T. muris (AA), severed from the posterior portion at the stichosomal-intestinal junction, using the following protocol of injections :-

	d0	d3	d10	d17	d24	d36
Antigen:	FCA+AA(50µg)	AA(100µg)	AA(100µg)	AA(100µg)	AA(100µg)	mice killed
Route:	S.C.	S.C.	S.C.	S.C.	S.C.	and bled

iii) IS/ES - hyper-immune serum was raised against adult T.muris E/S, using the following protocol of injections :-

	d0	d14	d28	d58	d65
Antigen:	FCA+E/S(50µg)	E/S(100µg)	E/S(100µg)	E/S(100µg)	mice killed
Route:	S.C.	S.C.	S.C.	S.C.	and bled

Blood was allowed to clot at room temperature in glass test-tubes for one hour, and was then left at 4°C for 4 hours to allow the clot to contract. The clots were then dislodged, and the tubes were centrifuged at 1500 g for 15 minutes at 4°C. The supernatant was then removed and spun for another 15 minutes. The serum was finally taken off and frozen in 0.5 ml aliquots at -40°C.

Protein Estimation :

Lowry Method

The method used was modified from Lowry et al. (1951). The following solutions were prepared :-

0.1 N NaOH/2% Na_2CO_3 - Lowry solution

1% $CuSO_4$

Folin reagent - diluted 1:1 with distilled H_2O

2% potassium tartrate

0.1 ml $CuSO_4$ and 0.1 ml 2% potassium tartrate were added to 9.8 mls of Lowry solution. 10 µl of test sample was added to 300 µl of this mixture and allowed to stand for 15 minutes, followed by 30 µl of diluted Folin reagent left for a further 15 minutes. A set of protein standards were prepared, using BSA in the range 0.25 to 2.0 mg/ml, and were assayed along with the test sample. The optical densities (O.D.) of the solutions were read on a spectrophotometer at 595 nm, and a calibration graph was drawn of O.D. versus protein

concentration using the BSA standards. The protein concentration of the test sample was read off the calibration graph.

Bio-rad Method

Protein concentrations were also measured using Bio-rad protein assay dye reagent (Bio-rad Laboratories, West Germany). The dye reagent was diluted 1:5 with distilled water and filtered. 100 µl of the sample to be tested was added to 5 mls of filtered reagent in a test tube and the solution vortexed. The optical density of the solutions were then read (595 nm) on a Unicam SP600 UV spectrophotometer.

Protein estimation was determined by comparing results with those from a standard graph of the O.D.s of known concentrations of bovine serum albumin (Sigma).

2.2 CELLULAR TECHNIQUES

Preparation of Cell Suspensions :

Organs (mesenteric lymph nodes or spleens) were removed from the mice under aseptic conditions, attached fat was trimmed off *in situ*, and the organs placed in serum-free RPM1 1640 medium plus supplements (see Appendix). All materials were kept at room temperature throughout the procedure.

Cell suspensions were made by pressing the diced organs through stainless steel gauze in a petri-dish, using a 5 ml syringe plunger. Cell clumps and debris were allowed to settle and the cells in the supernatant were washed twice by centrifuging at 200 g for 5 minutes and resuspending in fresh medium. Viable cells were then counted (see 2.2 Cell Viability).

Cell Viability :

Cell viability was assessed by the ability of live cells to

hydrolyse fluorescein diacetate (FDA, Sigma) to give free fluorescein. When viewed under an Olympus BHS microscope with an Olympus BH-RFL-W reflected light fluoresent attachment, live cells fluoresced green.

A stock solution of FDA (5 mg/ml in acetone) was prepared, and stored at -20°C. A working dilution of 1:50 stock solution in phosphate buffered saline (PBS, see Appendix) was freshly prepared on the day of use. Cell viability was then assessed in a solution containing 9 parts cell suspension (at an appropriate dilution) to 1 part FDA at working dilution. The average number of fluorescing cells was counted using an improved Neubauer haemocytometer.

Cell Separation :

Anti-thy 1.2 monoclonal antibody (Sera-lab) and low-tox M rabbit complement (Cedar Lane Laboratories) were used to obtain B cells from a mixed lymphocyte population. To do this under asceptic conditions the anti-thy 1.2 monoclonal antibody, and the rabbit complement, diluted 1 in 10,000 and 1 in 12 respectively in RPM1 1640 medium plus supplements and 10% FCS, were filtered at 0.22 μm (pyrogenic filter, Millipore). After washing the cell population to be treated was resuspended in the medium containing anti-thy 1.2 and left for 40 minutes at 4°C. The cells were then pelleted and resuspended in the diluted rabbit complement and left at 37°C for 40 minutes. Cells were then washed twice, and counted before being resuspended at the required concentration in RPM1 1640 medium plus supplements with 10% FCS.

Lymphocyte Proliferation Assay :

An *in vitro* assay measuring antigen-induced proliferation of lymphocytes was performed essentially as described by Corradin *et al.* (1977).

Cells were recovered from mice, and the cell suspension prepared was adjusted to 1 x 10^7 cells/ml. 50 µl of the suspension was aliquoted into the wells of flat-bottomed microtiter plates (Nunc). Antigen was added at various dilutions in a volume of 50 µl and the total volume per well was adjusted to 200 µl with RPM1 1640 medium plus supplements with 10% FCS.

The plates were incubated at 37°C (5% CO_2, 95% air, humidified) for varying periods of time.

Antigen-induced proliferation was assessed by the incorporation of tritiated thymidine (^{3}H-TdR 2 Ci/m mol., Amersham International) into the DNA of dividing lymphocytes. 0.5 µCi ^{3}H-TdR was added to each well in a volume of 50 µl, and cells were harvested onto glass fibre filter paper discs about 24 hrs later, using a Skatron Titertek cell harvester. The filter paper discs were dried and placed in 6 mls of scintillation fluid (Optiphase 'X', Fisons) in capped scintillation vials (Hughes and Hughes). Measurements of the amount of radioactivity in each disc were made using a Packard Tricarb liquid scintillation spectrophotometer.

IL-2 Assay :

This assay was essentially performed as described by Gillis (1978). Cell suspensions of the cells to be assayed were prepared as described previously, and adjusted to a concentration of 5 x 10^6 cells/ml, and 2 mls of the suspensions were added to each of the wells in a 24 well cluster plate (Nunc). Cells were incubated with medium alone, and were also stimulated with either concanavalin A (con. A type IV, Sigma) or antigen.

The supernatants from these cells were then plated out in doubling dilutions in round-bottomed microtitre plates, and incubated

for 24 hrs with 4 x 10^3 cells from the CTTL line (see Appendix). As a positive control CTTL were incubated with supernatant from EL-4 cells (see Appendix) treated with PMA. 0.5 μ Ci of 3H-TdR(2 Ci/m mol., Amersham) was added for the final 4 hrs of incubation. Cells were harvested onto glass fibre filter paper using a Skatron Titertek cell harvester, and processed for counting on a Packard Tricarb liquid scintillation counter as described in Section 2.2 Lymphocyte Proliferation Assay.

2.3 MONOCLONAL ANTIBODY PRODUCTION

Immune Cells :

BALB/C mice were given a primary infection of *Trichuris muris*, followed by 200 μg of adult E/S antigen given i.p. 14 days post-infection. The mesenteric lymph node (MLN) cells were then taken 3 days later.

Myeloma Cells :

P3NS1 cells (see Appendix) were harvested from actively dividing cultures when their viability was not less than 90%.

Feeder Cells :

Peritoneal exudate cells (PEC) from naive wistar rats were set up as feeder cells in flat-bottomed microtitre plates (Nunc), or 24 well cluster plates (Nunc), 24 to 48 hours before use.

To obtain PEC, 20 mls of serum-free RPM1 1640 medium plus supplements was injected into the peritoneal cavity using a 21 G needle under aseptic conditions. The abdomen was gently managed and the cells aspirated using the same needle. The cells were washed twice and thenplated out at 2.5 x 10^3 per microtitre well or 10^4 per cluster plate well.

Polyethylene Glycol :

Polyethylene glycol (PEG) 1500 (BDH) was prepared 24 hrs before the fusion process. 1 g of PEG 1500 was sterilized by autoclaving, and when still hot was mixed with 1 ml of warmed serum-free RPM1 1640 plus supplements, to give a 50% solution. The PEG was then gassed overnight at 37°C (5% CO_2, 95% air, humidified) to maintain a pH of 7.2 to 7.4.

Fusion :

The media and cells were maintained at room temperature throughout the fusion. The selective HMT medium plus 15% FCS (see Appendix), for plating out the fusion mixture was kept at 37°C. MLNs from immunized mice were used to make a cell suspension, and myeloma cells were harvested from tissue culture flasks. 10^8 MLNC and 10^7 myeloma cells were washed twice, separately, in 25 mls of serum-free RPM1 1640 plus supplements by spinning down cells at 200 g for 5 minutes and resuspending in fresh medium, after first loosening the cell pellet. The cells were then mixed and washed once more. All the supernatant was then removed by means of a pasteur pipette attached to a vacuum line, and the pellet of cells was loosened by gently tapping the tube.

0.8 ml of 50% PEG 1500 was added dropwise to the pellet, over 1 minute, mixing by continuous rotation of the universal. The suspension was incubated in the palm of the hand for a further minute, mixing thoroughly by gentle pipetting. 1 ml of serum-free RPM1 1640 medium plus supplements was then added dropwise over 1 minute, followed by 20 mls of the same medium over 5 minutes, mixing by rotation of the universal. The cells were then washed once by centrifugation at 200 g for 5 minutes and gently resuspended in 1 ml

of 15% HMT selective medium. The volume was made up to 24 mls with the same medium, and 100 µl of this added to each of 192 wells of 2 microtitre plates containing feeder cells. The plates were incubated at 37°C in humidified atmosphere of 5% CO_2 95% air.

Hybridoma Maintenance :

Microtitre plates containing fused cells were fed once a week and checked regularly for hybridoma colonies, which could be visible from day 7 onwards, on an inverted microscope. The medium from the wells was completely changed on at least two occasions before any screening of supernatants. Medium in the plates was removed by means of a pasteur pipette attached to a vacuum tap, and fresh medium could then be added. In this way materials released from dying cells and any antibody which may have been initially released into the cultures by immune cells could be washed away. When hybridoma colonies were of a reasonable size, 150 µl of supernatant was removed from the wells for screening in an ELISA against adult T. muris ES antigen. Colonies giving a positive result in the ELISA were transferred to 24 well cluster plates (Nunc) with feeder cells. The hybridomas were then grown up and cloned twice by limiting dilution.

Selected hybridomas were grown up in cluster dates until they could be maintained without feeder cells. They were then transferred to culture flasks and large numbers grown up for freezing down.

Cloning by Limiting Dilution :

Dilutions of the hybridomas to be cloned were prepared so as to give 100, 60 and 20 cells/ml medium. Cells were then plated out in 3 separate flat-bottomed microtitre plates, with feeder cells, in 50 µl volumes to give plates with 5, 3 and 1 cell per well. The number of wells supporting colonies per plate, was noted after 10 to

14 days incubation. If less than 35% of the wells on a plate contained colonies of hybridomas, then this was taken as 95% certain that the colonies were clones (Lefkovits and Waldman, 1984; Coller and Coller, 1986). Colony supernatants were again screened by ELISA to adult T. muris E/S.

Ascites Production :

BALB/C mice were given 0.5 ml of sterilized pristane (2,6,10, 14-Tetramethylpentadecane, Sigma) i.p., and left for at least two weeks before use. It has been shown that plasmacytoma cells grow better in mineral oil-treated hosts (Potter, et al., 1972), however the reasons as to why this is are still purely speculative.

Ascites fluids were produced by injection of 5 x 10^6 cultured cells i.p. into the pristane-primed mice. The first t apping of fluid could be from day 10 onward. Mice were anaesthetized with Trilene (ICI) and fluid drained from the abdomen with a 19 gauge needle. Mice were drained on alternate days on 2 occasions, or once only, and were always killed for the final tapping. The ascites fluids were centrifuged at 300 g at 4°C, then aliquoted and stored at -70°C.

For passage, cells isolated from the ascites fluid were resuspended in RPM1 1640 plus supplements to give no more than 10^7 per ml, and 0.5 injected i.p. into new mice. Cells were passaged twice in pristane-primed mice, and then passage was continued in untreated mice.

Cell Freezing and Thawing :

Cells were frozen down at 5 x 10^6 - 10^7 cells/ml of freezing mixture; 45% FCS, 45% RPM1 1640 medium, 10% Dimethylsulphoxide (DMSO, Sigma), or 95% FCS, 5% DMSO). The freezing mixture was filtered

through a 0.22 μm Millipore pyrogenic filter and kept at 4°C before use. Cells were frozen down in 1 - 1.8 ml aliquots in cryotubes (Nunc). Cells suspended in freezing mix and kept at 4°C were then kept overnight at -70°C before transferral to a liquid nitrogen store at -196°C.

Cells were rapidly thawed in water at 37°C, and taken to the laminar flow cabinet while a small amount of ice still remained in the tube. Cells were transferred to a sterile universal with a pasteur pipette, and diluted slowly, to double their volume, with RPM1 1640 plus supplements added dropwise. Medium was then added to fill the universal and the cells washed once by centrifugation at 200 g for 5 minutes, cell viability was then assessed.

Cells were set up in tissue culture flasks as an initial concentration of between 5×10^4 and 1×10^5 cells/ml.

2.4 HISTOLOGICAL TECHNIQUES

Preparation of Fixed Sections :

The caecum and colon were removed from mice with patent infections of *T. muris*, the gut was cut open and the contents flushed out. The opened gut was then laid on dampened filter paper, to prevent curling up, and put into Carnoys fixative (see Appendix) for 4-6 hours. After this time the gut was kept in 70% ethanol.

Fixed tissues, cut to measure 1 cm^2, were placed in wire baskets and dehydrated using a Histokinette (Hendrey Relays). The histological processor passed the tissues through three changes of 70% ethanol, followed by three changes of xylene, remaining in each solution for 1 hour.

Tissue was then embedded in the paraffin polymer Polywax (Difco Ltd.). Sections were cut at 5 μm.

Avidin-Biotin Immunoperoxidase :

i) Biotinylation of Antibodies

Biotinyl-N-hydroxysuccinamide (BNHS, Sigma) was made up in dimethylformamide (Sigma) at 7.2 mg/ml. 120 µg BNHS were used per mg protein to be biotinylated. Concentrated MoAb, either from ammonium sulphate precipitated ascites or culture supernatant was dialysed against 0.1 M bicarbonate buffer, pH 8 (all the ammonium sulphate must be removed as it will react with the BNHS). MoAb solutions were adjusted to 3 mg protein/ml, and 50 µl of BNHS solution was added per ml of MoAb solution. BNHS/MoAb solution was incubated a minimum of 2 hours at RT with stirring, alternatively the solution could be left overnight at 4°C. After biotinylation the MoAb solutions were dialysed against PBS, aliquoted, and stored frozen at -40°C. In order to check that there had been no loss of binding ability in the biotinylated MoAbs, they were tested in an ELISA before use in immunoperoxidase staining of histological sections.

ii) Immunoperoxidase Staining of Sections

Parafin embedded sections were rehydrated through a series of alcohols before being used in any staining procedure. Throughout staining, sections were kept in a humid container, and all solutions were added onto the slides carrying the sections using pasteur pipettes. The procedure used for staining was as follows :-

1. Sections were covered with PBS, which was left for 10 mins, then tipped off.

2. A $^{1}/_{10}$ dilution of 3% H_2O_2 was added and left 5 mins, then washed off using PBS. Sections were left in fresh PBS for 5 mins.[1]

3. 10% skimmed milk was used to 'block' the sections, this was left for 30 minutes then tipped off.

4. Biotinylated MoAbs were added to the sections and left for 40 mins. Sections were washed and left in fresh PBS for 10 mins.

5. Avidin peroxidase was added at a chosen dilution, left for 40 mins, washed off, and left in fresh PBS for 10 mins.

6. Peroxidase substrate[2] was added and left until desired colour change observed, to stop further reaction the substrate was washed from the sections.

[1] Before each new solution was added, the previous solution was tipped off and the area on the slide surrounding the sections was patted dry with tissue paper.

[2] Chloronapthol (CN, Sigma) was used as peroxidase substrate. 3 mg CN was dissolved in 100 µl of absolute alcohol, then added, with stirring, to 10 ml PBS. The resulting suspension was filtered and 0.1 ml of 3% H_2O_2 was added to the filtrate, to produce the peroxidase substrate solution.

2.5 BIOCHEMICAL/IMMUNOLOGICAL TECHNIQUES

Enzyme-linked Immunosorbent Assay (ELISA) Protocol :

Recipes for buffers are given in the Appendix. ELISAs were routinely performed using flexi microtitre plates from Falcon. Plates were wrapped in foil during assay incubation periods to limit evaporation from the sample wells and to exclude light when the substrate was added. The basic ELISA protocol used was as follows :-

1. Plates were coated o/n with antigen at 0.25 µg protein/well in 0.05 M carbonate/bicarbonate buffer pH 9.6, 50 µl/well.

2. Antigen was tipped off and plates were washed with PBS - Tween (0.05%). The first 3 washes were tipped off immediately, then 2 more washes were left on the plates for 3 mins each.

The PBS - Tween (0.05%) was applied to the plates using a squeezy bottle.

3. Plates were "blocked" with either 3% bovine serum albumin (BSA, Sigma) or 10% skimmed milk (Marvel Cadburys), 100 µl/well and left for 30 mins to 1 hr at RT.

4. The blocking solution was tipped off and the plates washed once.

5. The test sera or hybridoma supernatants were added 50 µl/well at the appropriate dilution in PBS - Tween (0.05%) and left for 1½ - 2 hrs at RT or 1 hr at 37°C.

6. Plates were washed as in step 2.

7. Anti-immunoglobulin phosphatase conjugate (Sigma) was added, 50 µl/well at the appropriate dilution in PBS - Tween (0.05%) and left for 1½ - 2 hrs at RT or 1 hr at 37°C.

8. Plates were washed as in step 2.

9. Substrate was added; p-nitrophenyl phosphate tablets (Sigma) were dissolved in diethanolomine buffer pH 9.8 (1 tablet/5 ml buffer) and added to the plates at 100 µl per well. Plates were left for ½ - 1 hr at RT or 37°C until there was an appropriate colour change, then read on a Titertek multi-scan spectrophotometer at 405 nm.

Polyacrylamide Gel Electrophoresis (PAGE) :

i) Stock Solutions for the Preparation of Gels

Stock Solution	Final Acrylamide Concentration		
Resolver Gel	15%	12%	10%
Acrylamide/Bisacrylamide (30:0.8)(Acryl./Bis.)	20.0 ml	16.0 ml	13.4 ml
1.5 M Tris-HCl pH 8.8 (Tris(hydroxy methyl)amino methane-HCl)	15.0 ml	16.0 ml	10.0 ml
distilled water	9.5 ml	13.5 ml	16.1 ml

	15%	12%	10%
10% SDS (Sodium Dodecyl Sulphate)	0.4 ml	0.4 ml	0.4 ml
10% APS (Ammonium Persulphate)	0.2 ml	0.2 ml	0.2 ml
TEMED (N,N,N¯-Tetramethylethylenediamine)	40 µl	40 µl	40 µl
Stacker Gel			
Acrylamide/Bisacrylamide (30:0.8)	1.3 ml		
0.5 M Tris-HCl pH 6.8	2.5 ml		
distilled water	6.1 ml		
10% SDS	0.1 ml		
10% APS	0.1 ml		
TEMED	30 µl		

See Appendix for making up solutions used.

ii) Gradient Gels

The same stock solutions were prepared as with single percentage gels but in addition a solution of "heavy" acrylamide/bisacrylamide (30:0.8) was made, by dissolving the latter in 75% glycerol. The following solutions were then prepared, with TEMED added just before the gel pouring.

	10% Gel Solution	20% Gel Solution
Acryl./Bis. (30:0.8)	8.35 mls (in water)	10 mls ("heavy")
1.5 M Tris-HCl pH 8.8	6.25 mls	5
H_2O	10.4 mls	-
10% APS	50 µl	50 µl
10% SDS	200 µl	200 µl
TEMED	20 µl	15 µl

The above solutions were sufficient to pour one gel, and were added to a gradient gel mixer, in which the 20% solution is gradually diluted by the 10% solution as the gel is poured. The general protocol for making gels is the same for single percentage and gradient gels (see PAGE protocol).

iii) PAGE Protocol

The solutions were mixed without the polymerization accelerators, APS and TEMED, and deoxygenated using a vacuum pump for about 2 minutes. APS and TEMED were then added and the gel was poured between the prepared plates of the gel apparatus (LKB). To obtain a smooth upper surface on the gel, and to exclude air during the polymerization process, the gel was overlayed with propan-1-ol. Once the gel had polymerized, the propan-1-ol was washed off with distilled water. The stacking gel was then poured on top of the resolving gel, a comb was inserted into the stacking gel before polymerization to form the sample wells. Samples were loaded into the wells and the gels were run at 8°C overnight at a constant voltage of 50 V on LKB 2001 vertical electrophoresis unit powered by a Shandon Southern "Vokam" transformer. The following morning the voltage was increased to 250 V and the gel was taken off once the proteins had migrated to a sufficient distance from the end of the gel. Gels were stained in comassie blue for a minimum of 3 hours, and then destained until protein bands were clearly visible. Gels were reduced in size after staining and destaining, and could be returned to original size by leaving in 10% acetic acid. Gels were dried down on a Biorad 443 Slab dryer.

iv) Silver Staining of PAGE

All equipment was thoroughly cleaned with a surface-active detergent, decon 90 concentrate (5% solution, Decon Laboratories Ltd.), following manufacturers instructions. All gel solutions and staining solutions (see Appendix) were made up with double-distilled (dd) water. Gloves should be worn throughout for handling equipment and gel. The staining protocol was as follows :

1. After electrophoresis the gel was transferred to 200 ml of 50% methanol/10% glacial acetic acid, and shaken gently for 2 hours.

2. The previous solution was poured off and 200 ml of 5% methanol/10% glacial acetic acid added. The gel was shaken gently for 2 hours.

3. The previous solution was poured off and the gel was soaked in 10% glutaraldehyde, shaking intermittently for 30 mins.

4. The gel was rinsed in dd H_2O and washed in 400 ml dd H_2O for $2\frac{1}{2}$ hours.

5. The water was drained off and 100 ml of silver stain (freshly made) was added, shaking for 15 mins.

6. The stain was drained off and gel developed in developing solution (freshly made) until bands were sufficiently stained.

7. Developing was stopped by draining the gel. Gel can then be stored in 2.5% glacial acetic acid.

Western Blotting :

After samples had been run on a gel, the separated proteins were transferred to nitrocellulose (Scheicher and Schnell) using the following procedure.

The nitrocellulose was cut to size and the position of the lanes on the gel were marked. LKB Blot apparatus was used and the tank filled with blotting buffer (see Appendix). The nitrocellulose, pre-soaked in blotting buffer, was laid on the gel, ensuring that there were no air bubbles between the two, and they were then sandwiched between 2 sheets of 3 mm filter paper and the blotting pads from the blot apparatus. The unit was then fitted into the tank

with the nitrocellulose towards the anode, and electrophoresed for 2 hours at 8°C at 100-300 mA (150 V).

After the transfer of proteins to the nitrocellulose, the marker lane and one sample lane were cut off and stained for 2 minutes in amido black stain, then destained until protein bands were visible. The remaining nitrocellulose was then blocked in blocking solution for 1 hour at room temperature on a rocking platform. After cutting the nitrocellulose into strips containing the various sample lanes, these were then incubated overnight at room temperature in the test sera diluted in TBS. The blots were washed several times in TBS, then incubated for 1-2 hours with the appropriate anti-immunoglobulin sera, usually ^{125}I-rabbit anti-mouse polyvalent immunoglobulins (10^{-3} dilution, Amersham). Blots were then washed again and air dried. Dried blots were exposed on Kodak X, OMAT film, separated from the film by an acetate sheet, at -70°C until bands were visible on developed film.

Immunoprecipitates :

Serum and antigen were first spun at 11,500 g for 5 minutes to bring down any insoluble material which was discarded. ^{125}I- or ^{35}S-labelled antigen was added to a series of eppendorf tubes to give around 100,000 cpm per tube. Serum, supernatants or ascites fluids to be tested were then added to the tubes at 25,200 and 10 µl respectively. The volume in each tube was then made up to 0.5 mls with immunoprecipitation buffer, the solutions vortexed and left to stand overnight at 4°C.

The following day either 15 µl of sheep anti-mouse immunoglobulin (SAM IgGAM and L chains, Serotech) or rabbit anti-mouse immunoglobulin (courtesy Dr. M. Robinson) or 30 µl of 50% protein-A

sepharose (Sigma) in buffer was added and the samples left for 3 hours at room temperature. Immunoprecipitates were then spun down at 11,500 g and washed four times in immunoprecipitate buffer. After the final wash they were redissolved in reducing sample buffer for PAGE. Gels were dried down, ^{125}I gels could be directly exposed on Kodak X OMAT film at -70°C, but ^{35}S gels had to be fluorographed first.

Fluorography of ^{35}S-methionine Gels :

Gels were fixed in destain solution (see Appendix) for ½ hour. Gel was then washed in dimethylsulphoxide (DMSO, Sigma) for 1 hour, followed by fresh DMSO for ½ hour, to ensure that all water had been removed from the gel. Gel was then soaked in 22% 2'5 diphenyl oxazole (PPO, Sigma) in DMSO for 4 hours. The scintillant, PPO, was precipitated in the gel, by placing the gel in water, and left in water overnight. The following day the gel was dried down at 60°C (as fluors tend to be heat labile) and then exposed on Kodak X OMAT film at -70°C.

Single Radial Immunodiffusion (SRID) :

Serum or supernatant immunoglobulin concentration was estimated by SRID (Mancini, Carbonara and Heremans, 1965). 1% agarose (Electron, BDH) and 3% Polyethylene glycol 6000 (BDH) in 0.2 M TRIS (Hydroxymethylaminomethane, Sigma) were heated in a boiling water bath until dissolved. The agarose/PEG solution was then cooled to 57°C before adding to the various antisera. The latter were rapidly vortexed and then poured evenly into custom made plastic Mancini plates (Miles) on a levelling table. The antisera used, sheep anti-mouse IgG, IgA and IgM (Serotech) were used at 2 µl of antiserum per

cm^2 of plate.

When the agarose had set, 3 mm diameter wells were cut into the plates and the plugs removed using a pasteur pipette attached to a vacuum line. Wells were filled with 5 µl of the appropriate sera, and the plates covered and left for at least 48 hours at room temperature. The IgM plates took longer for the precipitin rings to develop, and were left for up to 5 days. Each plate had wells containing a series of dilutions of a calibrated mouse reference serum (WAS 05, Serotech), from which the calibration graphs of log Ig concentration vs precipitation ring diameter could be plotted. Plates were dried then stained with coomassie blue (see Appendix).

Immunoadsorbent Columns :

i) Coupling of Antibody to Cyanogen Bromide Activated Sepharose-4B

Cyanogen bromide activated sepharose-4B (CNBR-sepharose, Pharmacia) was prepared for coupling according to the manufacturers instructions. 3 g of dried powder (makes approximately 10.5 mls of swollen gel) was used for each column. The powder was suspended in 1 mM HCl (200 ml/g of powder) to remove additives. Preparations of MoAbs at 0.5 mg/ml in coupling buffer ($NaHCO_3$ (0.1 M) containing NaCl (0.5 M) were added to the swollen gel (10 mls/3 g gel) and left overnight on a roller at 4°C.

The following day the mixture was centrifuged (200 g for 5 mins), the supernatant was removed, and the O.D. at 280 nm was measured to determine the percentage of antibody coupled to the column. Excess uncoupled antibody was removed by 4 washes of the gel with coupling buffer. Any remaining active groups on the CNBr-sepharose were blocked with 1 M ethanolamine pH 7.6, rolling at 4°C

for one hour. The coupled CNBr-sepharose was then washed alternately with 3 cycles of 0.1 M Tris-HCl pH 8 followed by 5% acetic acid pH 2.6. The gel was equilibrated with 0.1 M Tris-HCl pH 8 for 2 hrs at 4°C on a roller, and stored in fresh buffer at 4°C until required.

ii) Preparation and Use of the Column

The coupled CNBR-sepharaose was packed into a 15 ml LKB column under gravity, and pre-eluted with 0.2 M glycine pH 2. The column was then washed with 0.1 M Tris pH 8.0 containing 1 M NaCl, the high salt blocking any potential non-specific binding, followed by washing in 0.1 M Tris pH 8, to remove the salt. All washes were carried out using 30-40 mls of solution, each wash taking around 1 hr. The sample was then loaded onto the column in 0.1 M Tris pH 8.0, with a protein concentration at no greater than 1 mg/ml, and allowed to recirculate overnight at 4°C.

The following day the column was washed with 0.1 M Tris pH 8.0 to remove any unbound sample, followed by 0.1 M Tris pH 8.0 containing 1 M NaCl, to disrupt any non-specific binding. Washing with 0.1 M Tris pH 8.0 then removed the salt from the column, and the bound sample was finally eluted using 0.2 M glycine pH 2.0. 2 ml fractions were collected, 0.5 ml of 0.1 M Tris was added to each fraction tube to buffer the acid as the fractions came off the column. The column was again washed with 0.1 M Tris pH 8.0 to remove the acidic glycine buffer. All procedures were carried out in a cold room at 4°C and the columns were stored at 4°C inbetween use.

APPENDIX

CELL CULTURE MEDIA :

RPM1 1640 Medium plus Supplements

RPM1 1640 powdered medium		10.42 g/litre		Gibco
Sodium bicarbonate		2.0 g/litre	20 mM	Sigma
Glutamine			2 mM	Gibco
Sodium pyruvate			0.1 mM	Gibco
HEPES			10 mM	Sigma
Monothioglycerol			7.5×10^{-5}M	Sigma
Penicillin/streptomycin	penicillin	100 units/ml		Gibco
	streptomycin	100 µg/ml		
Foetal calf serum,FCS (heat inactivated)		100 mls/litre	10%	Serotech
or				
Myoclone FCS		50, 100 and 150 mls/litre	5, 10 and 15%	Gibco

(used with P3NS1 cells and hybridomas)

Medium was made up with double distilled deionized water, prepared in 5-10 litre batches, filter sterilized and stored at 4°C until use.

HMT Selective Medium

RPM1 1640 medium plus supplements used with additional constituents, as listed below :

Hypoxanthine	13.6 mg/litre	10^{-4} M	Sigma
Thymidine	2.42 mg/litre	10^{-5} M	Sigma
Methotrexate	4.54 mg/litre	10^{-5} M	Lederle

During the initial selection of hybridomas after cell fusion, HMT medium contained 15% myoclone FCS. Once hybridomas were well established in tissue culture the amount of FCS was gradually reduced to 5%.

CELL LINES :

P3NS1 (NS1/1.Ag 4.1 from P3K)

The P3NS1 plasmacytoma line was obtained from the Cancer Research Campaign Labs., University of Nottingham. This cell line was derived from the P3K line originally isolated from BALB/c mice. The P3NS1 line is able to produce kappa light chains, which are not normally secreted. However, after fusion the plasmacytoma light chains may be incorporated into the secreted hybridoma immunoglobulin. The P3NS1 cells lack the enzyme hypoxanthine guanine ribosyltransferase (HGRT) and are azaguanine (6-thioguanine) resistant. Such mutants cannot grow in medium containing methotrexate or aminopterin supplemented with hypoxanthine and thymidine (HMT or HAT medium) as they are unable to utilize the salvage pathway.

The P3NS1 line was routinely maintained in continuous culture in tissue culture flasks (Nunc). Every 6 months the line was maintained for several generations in medium containing 6-thioguanine (Sigma), a purine analogue, to eliminate any HGRT revertants. Cells were always at least two passages removed from medium containing 6-thioguanine before being used in a fusion.

EL-4

A C57 BL/6N lymphoma which produces interleukin 2 (IL-2) upon stimulation with phorbol myristate acetate (PMA, Sigma). Cells were maintained in continuous culture in tissue culture flasks (Nunc) in RPMI 1640 medium plus supplements and 10% FCS. Cells were split every 2-3 days.

CTTL (Cytotoxic T-Lymphocyte Line)

A C57 BL/6 cytotoxic T cell line, dependent on IL-2. Cells

were maintained in continuous culture in tissue culture flasks (Nunc), in RPM1 1640 medium plus supplements and 10% FCS, and an additional supplement of PMA stimulated EL-4 conditioned medium, as a source of IL-2. Cells were split every 2-3 days.

GENERAL BUFFERS AND SOLUTIONS :

Borate Buffered Saline 0.1 M pH 8.3

Boric acid	6.18 g/litre	BDH
Sodium tetraborate (Borax)	9.54 g/litre	BDH
NaCl	4.38 g/litre	Sigma

Made up in distilled water.

Carnoy's Fixative

Tissues fixed for 3-6 hours.

Absolute ethanol	60 ml	Fisons
Chloroform	30 ml	Fisons
Glacial acetic acid	10 ml	BDH

Immunoprecipitation Buffer pH 7.4

Tris (Tris(hydroxymethyl) amino methane)	10 mM	12.1 g/litre	BDH Electron
EDTA (Ethylene diamine tetra acetic acid)	1 mM	0.3 g/litre	Sigma
NaCl		9 g/litre	Sigma
Tween 20	0.05%		Sigma

Made up in distilled water, pH with 1 M HCl

EDTA dissolves once the pH of the solution is brought below 8.

Worm Homogenization Buffer pH 8.3

Contains enzyme inhibitors, made up in 10 mM Tris-HCl

*L-1-tosylamide-2-phenyl-ethyl-chloromethyl ketone	50 µg/ml	Sigma
+N-α-p-tosyl-L-lysine-chloromethyl ketone-HCl	25 µg/ml	Sigma
*Phenyl methyl sulphonyl fluoride	1 mM	1.74 mg/ml Sigma
Sodium dodecyl sulphate		2% BDH Electron

The enzyme inhibitors are made up at a X 100 concentration,

* in acetone and + in water, then diluted in the buffer.

BUFFERS FOR ELISA :

0.05 M Carbonate Buffer pH 9.6

Na_2CO_3	1.59 g/litre	BDH AnalaR
Na H CO_3	2.93 g/litre	BDH AnalaR
Na N_3 (optional)	0.2 g/litre	Sigma

Buffer made up in distilled water, and pH adjusted, final volume 1 litre.

Diethanolamine Buffer pH 9.8

$MgCl_2.6H_2O$	0.101 g/litre	BDH AnalaR
Diethanolamine	97 mls	Fisons

Adjust pH with 1 M HCl, add make up to volume of 1 litre with distilled water.

PhosphateBuffered Saline (PBS) pH 7.4

NaCl	8.00 g/litre	Sigma

KCl	0.20 g/litre	Fisons
$Na_2HPO_4.12H_2O$	1.15 g/litre	BDH AnalaR
KH_2PO_4	0.20 g/litre	BDH AnalaR

pH adjusted, final volume 1 litre with distilled water.

PBS/Tween (0.05%)

PBS made up and 0.05% v/v Tween added

Tween 20	0.5 ml/litre	Sigma

(Polyoxyethylene sorbitan monolaurate)

PAGE BUFFERS AND SOLUTIONS :

Resolving Gel Buffer : 1.5 M Tris-HCl pH 8.8

Tris (Tris(hydroxymethyl) amino methane)	18.1/100 mls	BDH Electron

pH with 1 M HCl, make up to final volume of 100 mls with distilled water.

Stacking Gel Buffer : 0.5 M Tris-HCl pH 6.8

Tris	6.0g/100 mls	BDH Electron

pH with 1 M HCl, final volume 100 mls, made up with distilled water.

Reservoir Buffer : Tris/SDS/Glycine pH 8.3

Tris	0.25 M	3.03 g/litre	BDH Electron
SDS (Sodium dodecyl sulphate)	1%	14.40 g/litre	BDH Electron
Glycine	1.92 M	1.00 g/litre	Sigma

Dissolve in about 200 mls of distilled water, then adjust pH to 8.3. Make volume up to 1 litre. Usually about 5 litres of reservoir buffer prepared.

Sample Buffer (for Reducing Gels)

SDS	2%	2.3 g/100 ml	BDH Electron
2ME (2-beta-mercapto ethanol)	5%		Sigma
Glycerol	10%		Sigma
BPB (Bromophenol biue)	1%		Sigma

For non-reducing gels the 2ME is not added.

For non-reducing gels leave out 2ME from sample buffer. Reduced samples were warmed for 5 minutes in sample buffer at 80-100°C before being loaded onto the gel.

10% Sodium Dodecyl Sulphate (SDS)

SDS	10 g/100 ml	BDH Electron

Make up in distilled water.

10% Ammonium Persulphate (APS)

APS	0.5 g/5 mls	BDH

Make up in distilled water, fresh on day of use.

Acrylamide / Bis-acrylamide (30:0.8)

Acrylamide	30 g/100 ml	BDH Electron
Bisacrylamide	0.8 g/100 ml	BDH Electron

Make up in distilled water. Stored in dark, 4°C, not longer than one month.

PAGE STAINING SOLUTIONS :

Coomassie Brilliant Blue (Coomassie)

Coomassie brilliant blue	0.1%	Sigma
Methanol	25%	Fisons

Glacial acetic acid	10%	BDH

Made up to 500 mls with distilled water, and filtered before use.

Destain (also used as fixer)

Methanol	50%	Fisons
Glacial acetic acid	10%	BDH

Made up in distilled water.

Silver Stain (made fresh on day of use)

Concentrated NH_4OH (~30%)	1.4 ml	Fisons
0.36% NaOH (made fresh)	21 ml	BDH AnalaR
19.4% Ag NO_4	4 ml	BDH AnalaR

Make up to 100 mls in double distilled (dd) H_2O.

Silver Stain Developer (made fresh on day of use)

5% citric acid (made fresh)	1 ml	BDH AnalaR
10% formaldehyde (made fresh)	1.9 ml	

Made up to 1 litre in dd H_2O.

STAINING ON NITROCELLULOSE :

Amido Black Stain

Amido Black	0.25%	Sigma

Dissolve amido black in 5:5:1 solution of methanol:water: glacial acetic acid.

Destain

Methanol	45%	Fisons
Glacial acetic acid	5%	BDH
Distilled water	50%	

WESTERN BLOT BUFFERS AND SOLUTIONS :

Blotting Buffer

Glycine	1	14.4 g/litre	Sigma
Tris	0.25 M	3 g/litre	AnalaR BDH
Methanol	25%		May and Baker

Make up 4 litres with distilled water.

Tris Buffered Saline (TBS) pH 7.4

Tris	10 mM	AnalaR BDH
NaCl	9 g/litre	Sigma
Na N_3	0.1%	Sigma

Make up 500 ml of X 10 concentration, and dilute with distilled water.

TBS/Tween

Make up TBS as above and add 0.05% Tween 20 (Sigma).

TBS Blocking Buffer

Make up TBS/Tween as above and add 3% Bovine Serum Albumin (BSA, Sigma).

CHAPTER 3

THE DEVELOPMENT OF IMMUNOSORBENT ASSAY SYSTEMS FOR THE DETECTION OF ANTIBODY SPECIFIC FOR ANTIGENS OF TRICHURIS MURIS

INTRODUCTION

High levels of specificity and sensitivity can be obtained when immunological reactions are used as the basis for assays or detection procedures involving biological molecules; the labelling of antibodies or antigens with various markers has been particularly useful in this respect. Fluorescent dyes have been used for this purpose for many years, and immunofluorescence (Nairn, 1976) is now one of the best established diagnostic methods in microbiological and in clinical immunology laboratories. However, immunofluorescence is time consuming, is not easy to perform in an automated manner and the reading of results is usually subjective and non-quantitative. Radio-isotopes have been used as labels in immunoassays, the so-called radio-immunoassay (RIA) techniques (see Sönksen, 1974), which have a very high level of sensitivity and reproducibility and are amenable to automation for large scale processing. However, the use of radio-isotopes poses some problems : the costly reagents have a short shelf-life, complex equipment is required for reading the results, and special safety measures have to be observed in the handling and the disposal of the reagents. These factors have meant that isotopic assays have been restricted to more sophisticated centres of the affluent world. In contrast, enzyme-labelled reagents are cheap to prepare, safe, and highly stable with a long shelf life; yet enzyme linked immuno-sorbent assay (ELISA) approaches the sensitivity of RIA and gives objective results, which can be determined visually or with relatively simple equipment. Since the introduction of the ELISA by Engvall and Pearlmann (1972), there has been a mass of work published in which this technique has been employed; evidence indeed of its broad

applicability and potential. The flexibility of the system is also apparent in the numerous variations in the methodology employed in basic ELISA procedures. Finally, the "multiscan" spectrophotometers, which have relatively recently been brought on the commercial market can read a whole ELISA micro-plate of 96 sample wells in times ranging from 30 to 60 seconds; another advantage over the RIA, where individual sample wells must be cut from the micro-plate and each one monitored on a γ-radiation counter for at least 1 minute.

The main stages of an indirect immunosorbent assay such as ELISA or RIA are :-

1. Absorption of antigen to the solid phase.
2. Wash.
3. Addition of antibody - incubation.
4. Wash.
5. Addition of enzyme-labelled or I^{125}-labelled anti-immunoglobulin - incubation.
6. Wash.
7. Addition of enzyme substrate.
8. Determination and expression of results.

This chapter describes the development of ELISA and RIA as a means of measuring specific antibody responses of mice infected with Trichuris muris and also as a means of screening hybridoma supernatants in the course of production of monoclonal antibodies specific to this parasite.

There are some excellent recent reviews on the development and use of ELISAs in various areas of research (Voller et al., 1979; McLaren et al., 1981; Voller and De Savigny, 1981; Wardley and Crowther, 1982). It should be noted, however, that certain initial

investigations are necessary to be able to determine the optimum protocol for each particular system in which an ELISA or RIA is to be used.

The initial step in immunosorbent assays is the adsorption of one of the reactants to a solid phase, this allows the separation of immunologically reacted from unreacted material during the test, and it is imperative that the solid phase should take up an adequate amount of material in a reproducible manner. It is probable that variability at this stage is the major factor in determining the precision of all solid phase immunoassays (Denmark and Chessum, 1978). Several types of polystyrene and polyvinylchloride (PVC) plates were tested for use in an ELISA. PVC plates in particular tend to have a high uptake of immunological reagents, however, these plates may also yield higher non-specific "backgrounds" (Voller et al., 1979). Flat-bottomed micro-titre plates were used in the present work as round-bottomed plates are not suitable when reading results with a "through-the-plate" spectrophotometer. There appears to be uniform agreement that satisfactory adsorption of most protein and lipoprotein is obtained with solutions in 0.05M carbonate buffer pH 9.6 (Voller et al., loc. cit.; Wardley and Crowther, 1982). Most adsorption is completed in 1 to 2 hours at 20-25°C, however, for convenience, overnight coating at 4°C was used. Several antigen preparations of T. muris were tested for their suitability in an indirect ELISA and RIA, whole adult male antigen (AMA), posterior ends of adult males (PAM), anterior ends of adult males and females (AA) and excretory/secretory products of adult worms (E/S). These antigens were tested at various dilutions using reference sera and reference enzyme conjugated or I^{125}-labelled anti-immunoglobulin sera. As many of the antigen preparations used were relatively

crude, non-specific "background" reactions obtained when naive sera are used in the assays may be relatively high, and it is important to determine to what extent these reactions may be taking place.

The washing of the plates after the incubation of the different reagents may be done in many ways, but it is essential that the washing completely removes any unadsorbed reagents which would interfere in subsequent assay procedures. Plates were washed using phosphate buffered saline with 0.05% Tween 20 (a non-ionic detergent), the individual micro-titre wells were flushed with a jet of fluid from a washing-bottle. The wash procedure was repeated 5 times, the first 3 washes were followed by an immediate emptying of the plate, the final 2 washes were left for 3 minutes each before being tipped off.

In immunosorbent assays for large molecular weight substances, such as serum immunoglobulins, there is often a tendency for materials in the samples being assayed to attach in a non-specific manner to the solid phase. This problem is largely avoided by making a solution of the test sample in a buffer containing a wetting agent (e.g. Tween 20), which prevents non-specific attachment. A number of dilutions of the sample should also be tested in order to determine at what dilution the non-specific "background" given with naive serum is negligible. The immunosorbent assay plate may also be "blocked" with a solution of inert protein, which will adsorb non-specifically to any available binding sites on the solid phase, before adding the test sera. Alternatively the blocking protein may be included in the wash buffer with the wetting agent.

Alkaline phosphatase conjugates were used in the ELISAs performed because of the availability of a substrate in a convenient

tablet form; tablets of p-nitrophenyl phosphate were stored at -20°C, and freshly made up into a working solution (which was not significantly photosensitive) each time an assay was performed. The yellow product of the enzyme reaction was assessed photometrically at 405 nm. Although peroxidase conjugates are less expensive than alkaline phosphatase conjugates, care has to be taken to choose a substrate oxidised by H_2O_2 that has adequate solubility. A number of substrates are available, but the differences in the dose response curves using these are quite large (Voller et al., 1979). Also many of the peroxidase substrates are photosensitive and some are mutagenic.

As far as incubation times are concerned, higher reaction temperatures (37°C) reduce the reaction times needed, but a moist chamber is needed at 37°C to avoid evaporation from the plates. Generally in the ELISAs and RIAs performed here, incubation times of test sera and conjugate were 1½ to 2 hours, and the substrate ½ to 1 hour, either at room temperature or 37°C, and a reference positive and negative sera were always included on test plates. It was not necessary to stop the alkaline phosphatase reaction in ELISA by the addition of concentrated NaOH as the multiscan sphectrophotometer used to read the plates completed the reading in a short enough time.

To summarize, having decided on certain criteria to carry out an ELISA (or RIA) for the detection of specific antibody to T. muris antigens (see Chapter 2), the following aspects were investigated :-

i) The choice of antigen.

ii) The selection of a micro-titre plate for the solid phase.

iii) The optimum concentrations of antigen and test sera to use in the assays.

RESULTS

3.1 THE USE OF DIFFERENT ANTIGEN PREPARATIONS OF *TRICHURIS MURIS* WITH VARIOUS INFECTION SERA IN RIA AND ELISA

As described in Chapter 1, the passive transfer of immunity to *T. muris* in mice has been demonstrated using sera from infected animals. However, nothing is known of the antigenic specificity of the immunoglobulins in sera responsible for the passive transfer of immunity. Using RIA the antibody titres of naive and infection sera (tolerant serum - TS - from d35 tolerant mice), reacting with 3 different antigen preparations of *T. muris* were examined. The following antigen preparations were used :-

i) posterior ends of adult males (PAM)

ii) anterior ends of adult males and females (AA)

iii) whole adult male antigen (AMA)

As all these antigen preparations are homogenate extracts they are relatively crude, thus the amount of non-specific "background" binding obtained with naive serum, as opposed to the specific binding of antibodies in infection sera (d35 TS) was examined for each of the preparations (Fig. 3.1.1). An excess of protein (6 µg/well) was used to coat the micro-plates used in the immunoassays as the optimum concentration of antigen required had not yet been determined; sera were used at dilutions of 10^{-1}, 10^{-2} and 10^{-3}.

Active immunization against infection using the aforementioned antigen preparations has been demonstrated (Wakelin and Selby, 1973). The degree of protection obtained, as determined by a reduction in worm burdens when mice were given a subsequent infection, was greatest using the AA preparation. Indeed, the degree of protection obtained

with AA was equivalent to that obtained with E/S antigens (Wakelin and Selby, loc. cit.). E/S antigens were used in RIA with immune sera raised in mice immunized with either AA or E/S antigens (designated ISAA and ISE/S respectively), and the antibody titres for these sera were determined (Fig. 3.1.2). Again the micro-titre plates were coated using an excess of antigen (6 µg/well) and sera used at 10^{-1}, 10^{-2} and 10^{-3}dilution.

When PAM, AA and AMA preparations were used in RIA, d35 TS was found to have highest specific antibody titres against AA. This indicates that tolerant serum, which passively transfers immunity to infection with T. muris (Lee, 1982), has higher titres of specific antibodies recognising antigens present in the anterior portions of adults than those present in the posterior portions. The non-specific "backgrounds" obtained with naive sera used against these antigen preparations were all of similar magnitude, although those obtained using PAM were slightly higher. Non-specific binding decreased as the dilution of the naive serum was increased. Dilution of sera to 10^{-2}gave the greatest ratios of tolerant sera : naive sera (TS : NS) antibody titres for all antigen preparations used, a prozone effect, due to steric inhibition of antibody binding when sera are too concentrated was observed with dilutions of 10^{-1}. Both ISAA and ISE/S had high specific antibody titres against E/S antigens. As with d35 TS the greatest ratio of IS : NS was obtained using 10^{-2} dilutions of sera.

3.2 EXAMINATION OF ANTIGEN BINDING CAPACITIES OF MICRO-TITRE PLATES PRODUCED BY VARIOUS MANUFACTURERS

Plastics vary in their binding capacities when used in immunosorbent assays, and polystyrene and polyvinylchloride (PVC) are the

usual plastics used in the manufacture of micro-titre plates for use in ELISA or RIA, due to their relatively high binding capacities. Significant variability is also found between plates produced by different manufacturers (Kenny and Dunsmoor, 1983), probably as a result of differences in the processing of the plastics to make the plates. Micro-titre plates from the following manufacturers were examined for their suitability in ELISA, using E/S antigens of T. muris (at 1 µg and 3µg/well) adsorbed to the solid phase, and various sera; immune serum raised against E/S products (ISE/S), d21 infection serum (IS d21) and naive serum :-

i) Dynatech
ii) Nunc (high binding)
iii) Nunc (medium binding)
iv) Falcon (flexi plates)

There were greater differences in the antibody titres obtained for the high titre ISE/S using the 4 types of micro-titre plate in ELISA than those determined for the low titre IS d21 (Fig. 3.2). Titres for ISE/S determined on the Falcon plates were approximately double those obtained using some of the other plates, however, the non-specific "background" obtained with naive sera was also greater on these plates. E/S antigen used at 1 µg and 3 µg/well gave comparable results, as regards the determination of antibody titres for both sera tested on all of the plates. The prozone effect was noticeable with ISE/S when using Dynatech and Nunc (high-binding) plates with the more concentrated sera (10^{-1} and $10^{-1.5}$ dilutions). However, dilutions of sera to 10^{-2} removed any prozone effect which may have been present and gave optimal IS : NS ratios. The Falcon plates, which gave the highest O.D. readings, had the lowest IS : NS

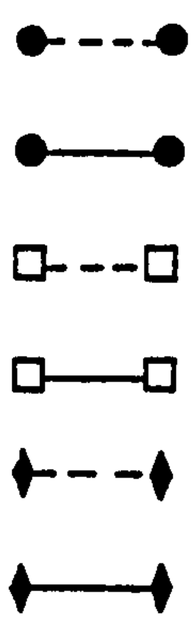
ratios for both ISE/S and IS d21. Indeed, the Nunc medium-binding plates with the lowest O.D. values for the ISE/S sera, had the highest IS : NS ratios for this serum. All the plates examined gave reasonable O.D. readings for the high and low titre immune sera tested, and would have been suitable for use in ELISA. However, the plates with the low backgrounds for naive sera would probably be more suitable for serological tests, where non-specific binding of naive sera is often a problem, and the Falcon plates with higher O.D. values may be preferable for use in hybridoma screening assays, where the background obtained with P3NS1 myeloma supernatants is insignificant, and the concentration of specific antibody in hybridoma supernatants may be very low. A final factor to be considered in the choice of plates was the cost and thus the Falcon plates were selected for routine use in the laboratory as they were the most economical purchase. The plates are also flexible, and are readily cut into individual sample wells as is required for reading results in RIAs.

3.3 CHECKERBOARD TITRATIONS USING IMMUNE SERA AND T. MURIS E/S ANTIGENS ON FALCON MICRO-TITRE PLATES

Naive serum, d21 infection serum (low specific antibody titre) and immune serum raised against E/S products (high specific antibody titre), designated NS, IS d21 and ISE/S respectively, were used at ½ log dilutions from 10^{-1} to $10^{-4.5}$ against T. muris E/S antigen at 0.25 µg, 0.5 µg, 1 µg and 3 µg/well. 0.25 µg/well used in ELISA gave results comparable to 3 µg/well. Using sera dilutions of 10^{-1} and $10^{-1.5}$ with antigen at 0.5 µg, 1 µg and 3 µg/well a prozone was observed, although this was not the case when antigen was used at 0.25 µg/well. Dilution of sera in the range $10^{-1.5}$ to 10^{-3} gave

reasonable ratios of IS : NS, however, for the low titre IS d21, end point titres were reached at dilutions greater than 10^{-3} and were already quite low at 10^{-2}. Hence, to maintain sensitivity when using low titre sera it is preferable not to use dilutions much greater than 10^{-2}.

FIGURE 3.1.1 RIA USING DIFFERENT ANTIGEN PREPARATIONS OF T. MURIS WITH NAIVE AND DAY 35 TOLERANT SERUM FROM CFLP MICE



CFLP d35 TS + AA

CFLP NMS + AA

CFLP d35 TS + AMA

CFLP NMS + AMA

CFLP d35 TS + PAM

CFLP NMS + PAM

d35 TS - Day 35 tolerant serum
NMS - Naive mouse serum
AA - Anterior ends of adult T. muris homogenate
AMA - Whole adult male T. muris homogenate
PAM - Posterior ends of adult male T. muris homogenate

TABLE 3.1.1 RATIO OF c.p.m., TS:NMS AT DIFFERENT DILUTIONS

Antigen	Dilution of Serum		
	10^{-1}	10^{-2}	10^{-3}
AA	2.41	2.79	3.13
AMA	1.81	3.21	2.57
PAM	1.38	1.96	1.85

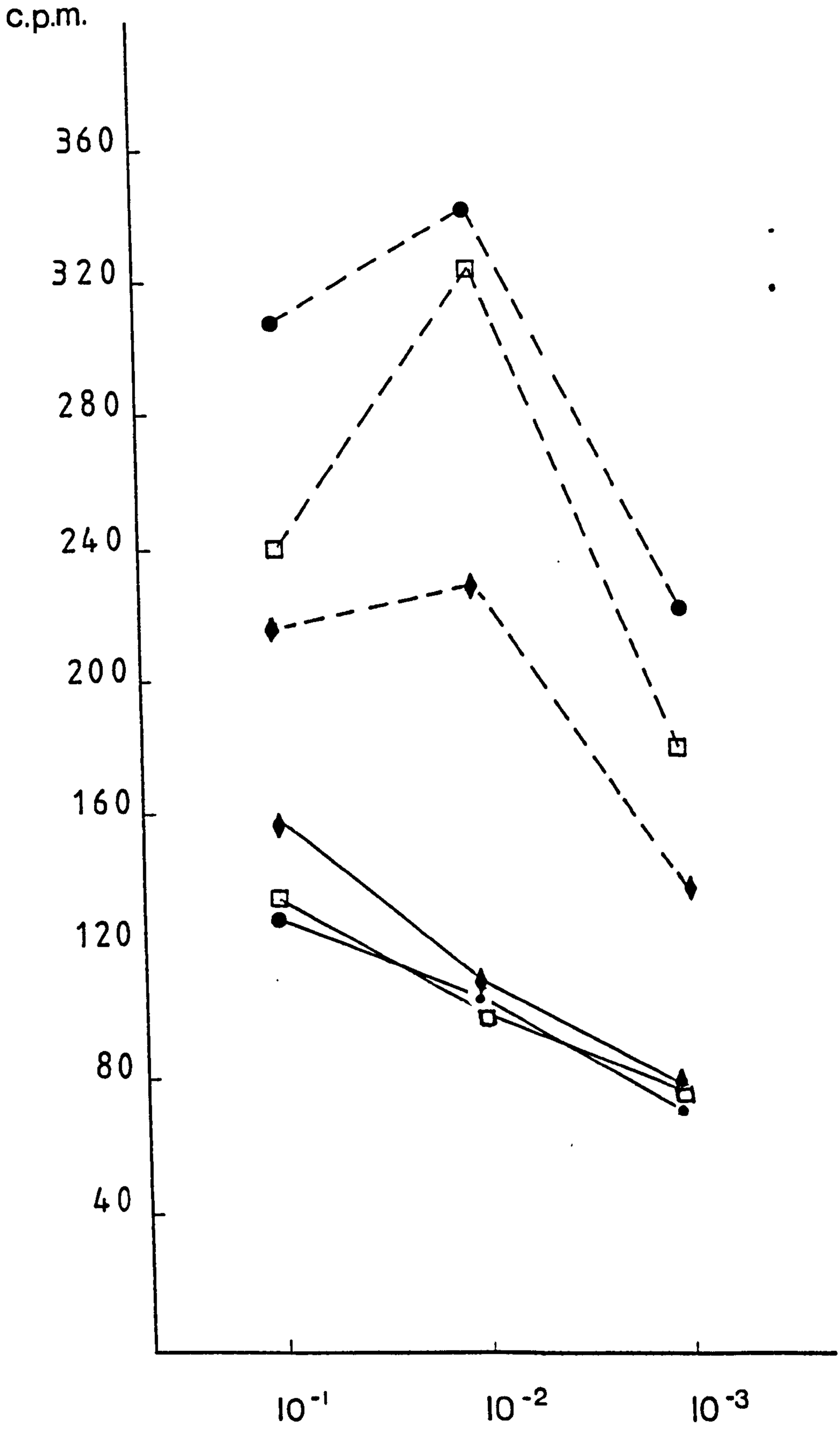
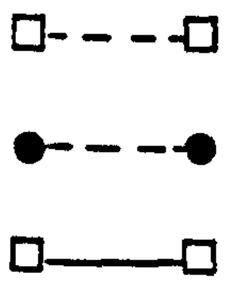

c.p.m.
360
320
280
240
200
160
120
80
40
10^{-1}
10^{-2}
10^{-3}
Serum Dilution

FIGURE 3.1.2 RIA USING ISAA AND ISE/S AGAINST T. MURIS E/S



□- - -□ ISAA

●- - -● ISE/S

□——□ NMS from control animals for ISAA

●——● NMS from control animals for ISE/S

ISAA - Immune sera raised against anterior ends of T. muris

ISE/S - Immune sera raised against T. muris E/S products

NMS - Naive mouse serum

TABLE 3.1.2 RATIOS OF c.p.m., IS:NMS AT DIFFERENT DILUTIONS

Serum	Dilution				
	neat	0.5	10^{-1}	10^{-2}	10^{-3}
ISAA	7.74	—	7.58	14.26	10.09
ISE/S	—	10.87	9.73	11.58	4.93

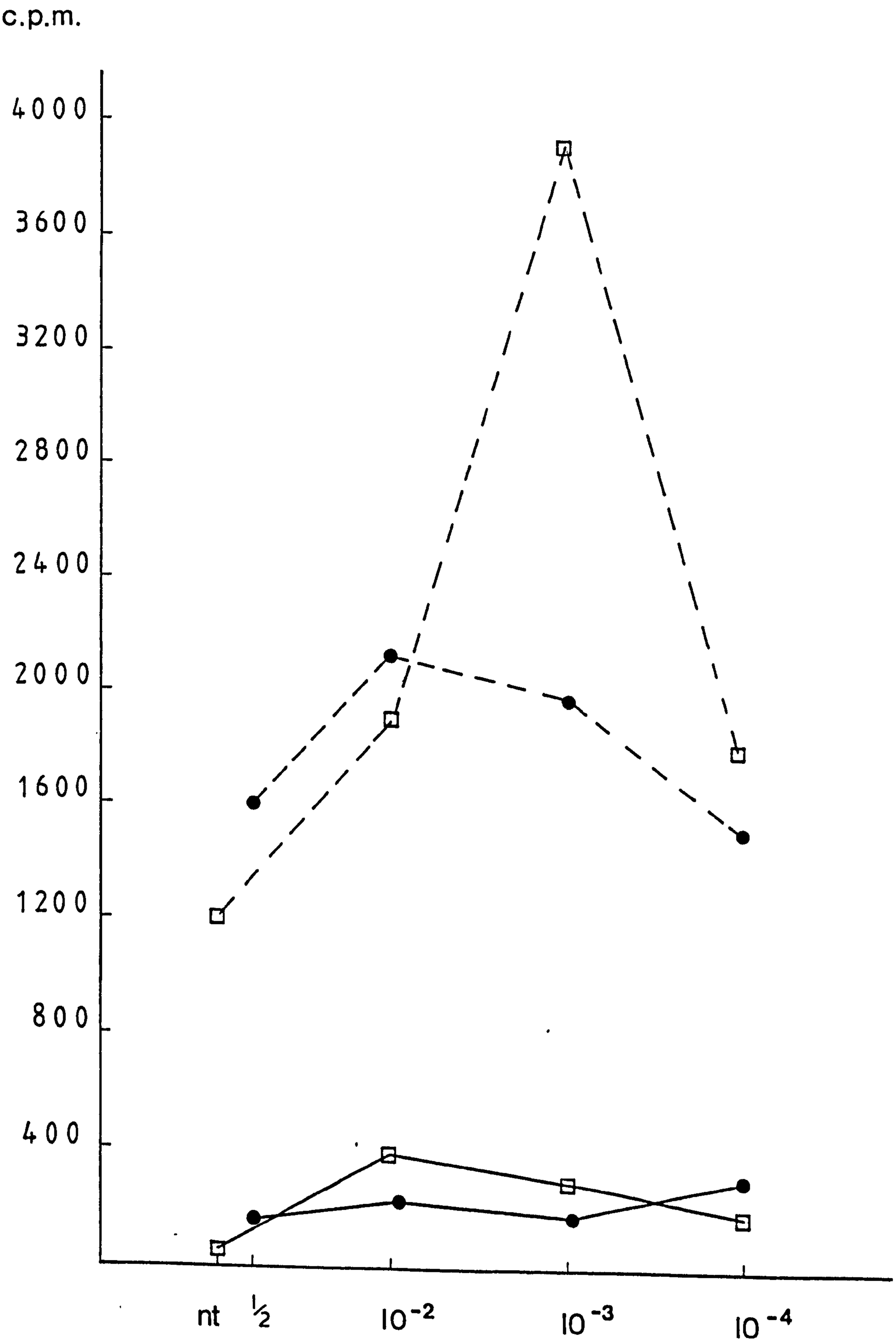
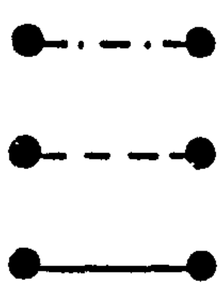

c.p.m.
4000
3600
3200
2800
2400
2000
1600
1200
800
400
nt
½
10-2
10-3
10-4
Serum Dilution

FIGURE 3.2 ANTIGEN BINDING CAPACITIES OF MICRO-TITRE PLATES PRODUCED BY DIFFERENT MANUFACTURERS



ISE/S - Immune serum raised against T. muris E/S products (high titre serum)

ISd21 - Day 21 infection serum (low titre serum)

NMS - Naive mouse serum

Microtitre plates from Dynatech, Nunc (high and medium binding) and Falcon tested using T. muris E/S antigen at 3 µg and 1 µg/well.

Dynatech Plates

O.D.

Ag 3µg/well

2.5
2.0
1.5
1.0
0.5

Ag 1µg/well

2.5
2.0
1.5
1.0
0.5

1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5

-log 10 Dilution

Nunc (high binding) Plates

O.D.

Ag 3µg/well

2.5
2.0
1.5
1.0
0.5

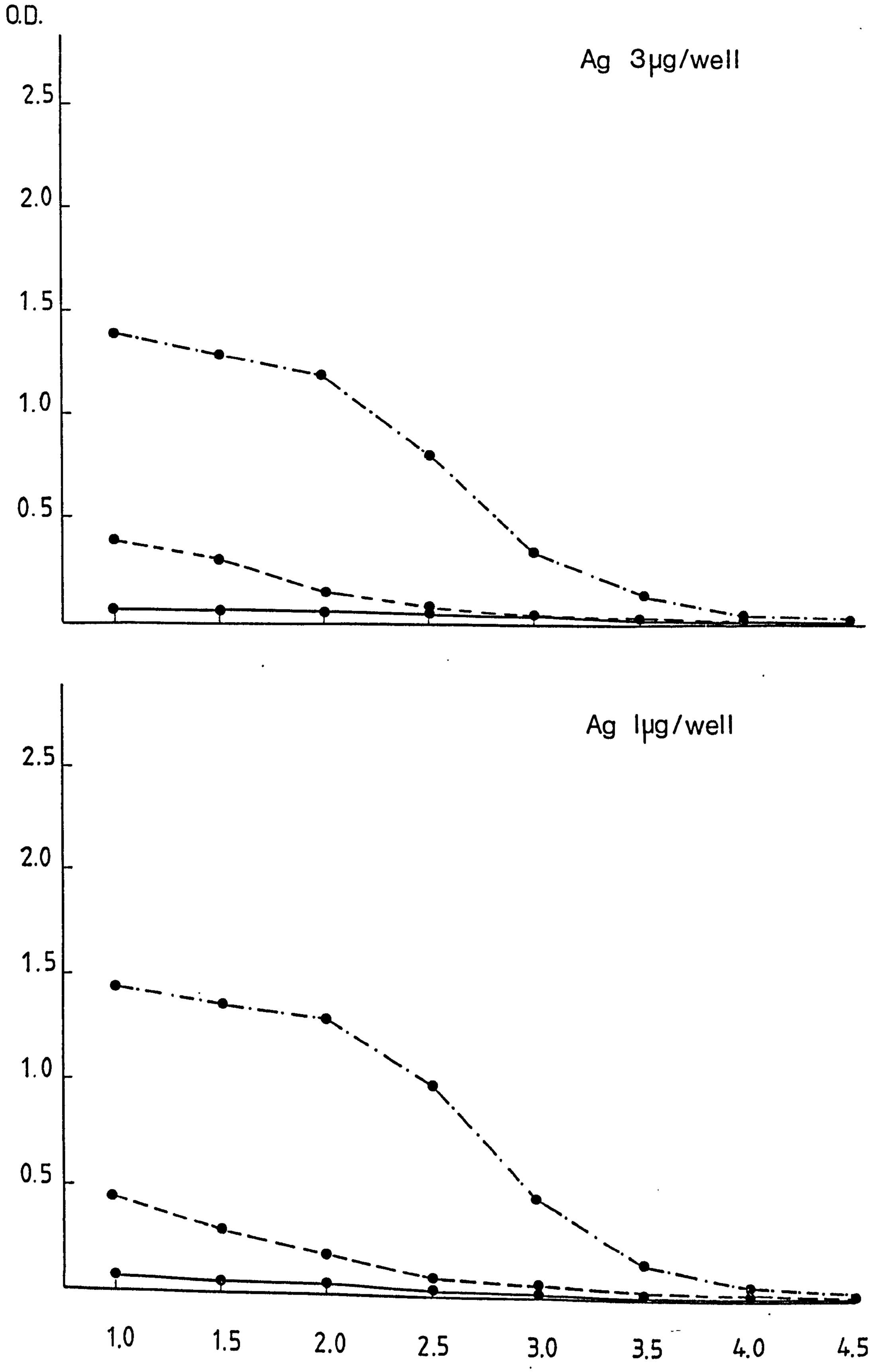
Ag 1µg/well

2.5
2.0
1.5
1.0
0.5

1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5

-log 10 Dilution

Nunc (medium binding) Plates



Falcon (flexi) Plates

O.D.

Ag 3µg /well

Ag 1µg /well

-log 10 Dilution

TABLES 3.2 RATIO OF O.D., IS:NMS AT DIFFERENT DILUTIONS

Dynatech Plate

Serum	Antigen µg/well	Dilution of Serum							
		10^{-1}	$10^{-1.5}$	10^{-2}	$10^{-2.5}$	10^{-3}	$10^{-3.5}$	10^{-4}	$10^{-4.5}$
ISd21	1	8.8	8.0	5.3	3.5	2.3	1.9	1.1	1.2
	3	9.9	8.2	5.1	2.7	1.7	1.0	1.0	1.0
ISE/S	1	11.0	15.6	17.5	18.1	12.6	6.4	3.2	1.0
	3	12.3	16.8	16.0	15.8	10.8	3.4	1.0	1.3

Nunc (high binding) Plate

Serum	Antigen µg/well	Dilution of Serum							
		10^{-1}	$10^{-1.5}$	10^{-2}	$10^{-2.5}$	10^{-3}	$10^{-3.5}$	10^{-4}	$10^{-4.5}$
ISd21	1	6.5	6.4	5.2	2.8	2.3	1.5	1.3	1.3
	3	8.9	5.9	3.7	3.0	2.1	1.1	1.1	1.1
ISE/S	1	8.8	13.2	16.3	13.7	11.5	4.6	2.7	1.4
	3	9.7	13.3	13.6	14.3	13.0	4.0	2.5	1.2

Nunc (medium binding) Plate

Serum	Antigen µg/well	Dilution of Serum							
		10^{-1}	$10^{-1.5}$	10^{-2}	$10^{-2.5}$	10^{-3}	$10^{-3.5}$	10^{-4}	$10^{-4.5}$
ISd21	1	7.9	5.6	4.5	2.3	2.4	1.9	1.3	1.0
	3	7.0	5.8	3.3	1.7	1.1	1.2	1.6	1.4
ISE/S	1	25.7	26.0	31.6	30.3	19.4	5.4	2.8	1.3
	3	23.7	26.6	25.1	22.4	11.5	3.8	2.1	1.5

Falcon (flexi) Plate

Serum	Antigen µg/well	Dilution of Serum							
		10^{-1}	$10^{-1.5}$	10^{-2}	$10^{-2.5}$	10^{-3}	$10^{-3.5}$	10^{-4}	$10^{-4.5}$
ISd21	1	4.1	3.7	2.5	1.9	1.7	1.4	1.3	1.0
	3	5.3	4.2	2.7	1.8	1.2	1.0	1.0	1.0
ISE/S	1	9.5	12.3	11.8	12.3	8.9	4.3	1.9	1.1
	3	9.7	10.3	11.3	12.3	7.4	3.4	1.5	1.1

FIGURE 3.3 ELISA CHECKERBOARD TITRATIONS USING IMMUNE SERA WITH T. MURIS E/S PRODUCTS

●-·-● CFLP ISE/S

●- - -● CFLP ISd21

●——● NMS

ISE/S - Immune serum raised against E/S products

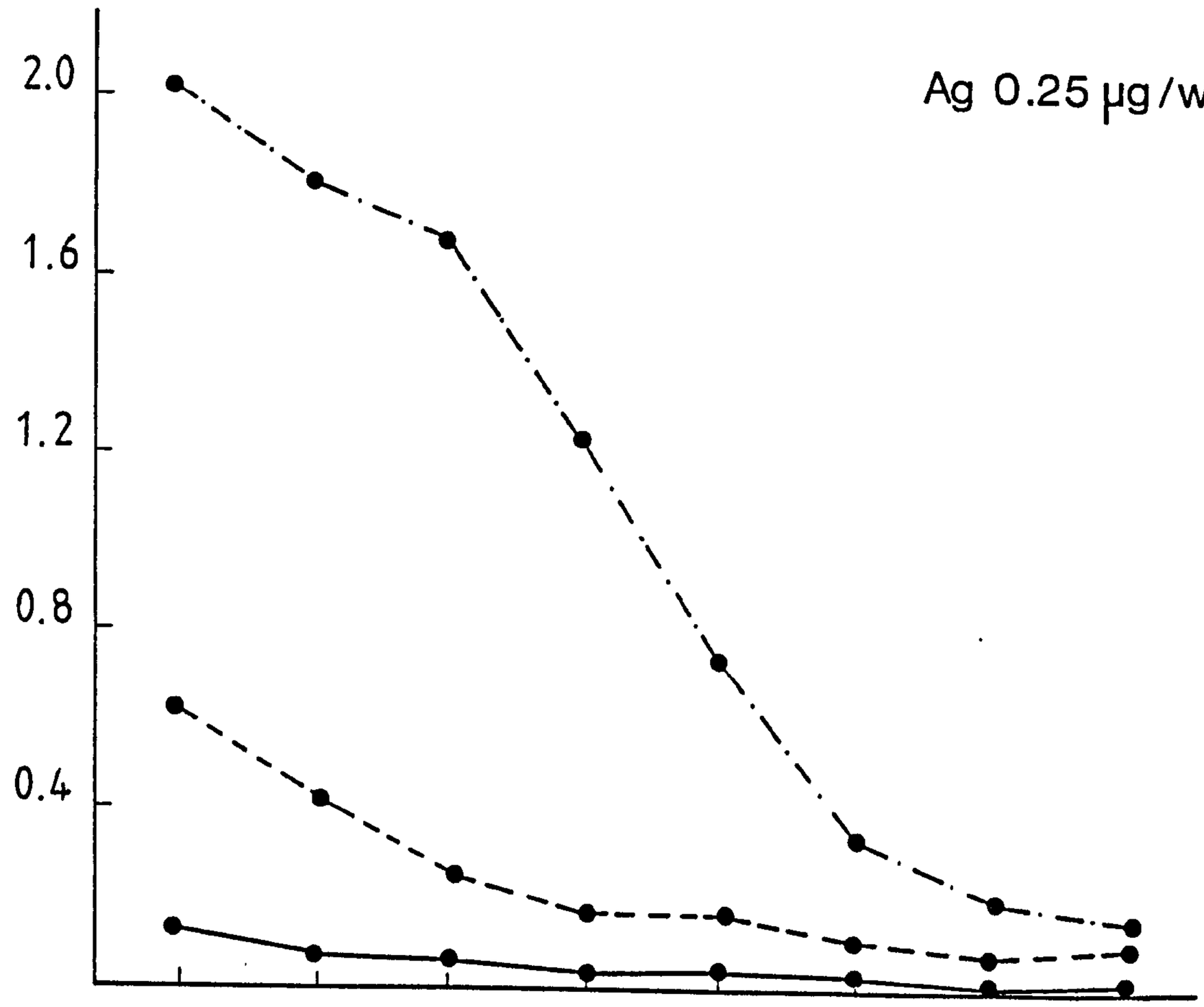
ISd21 - Day 21 infection serum

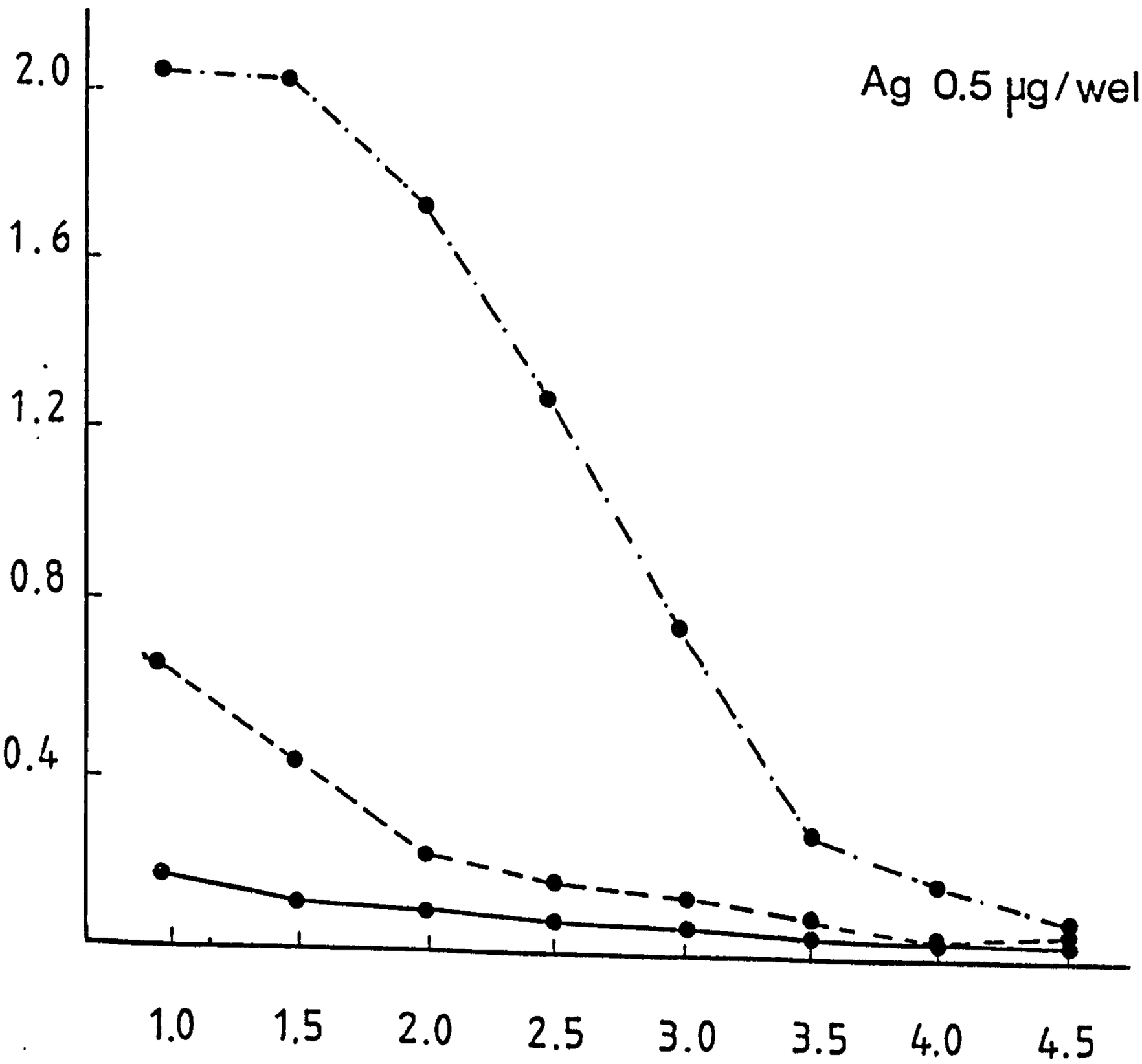
NMS - Naive mouse serum

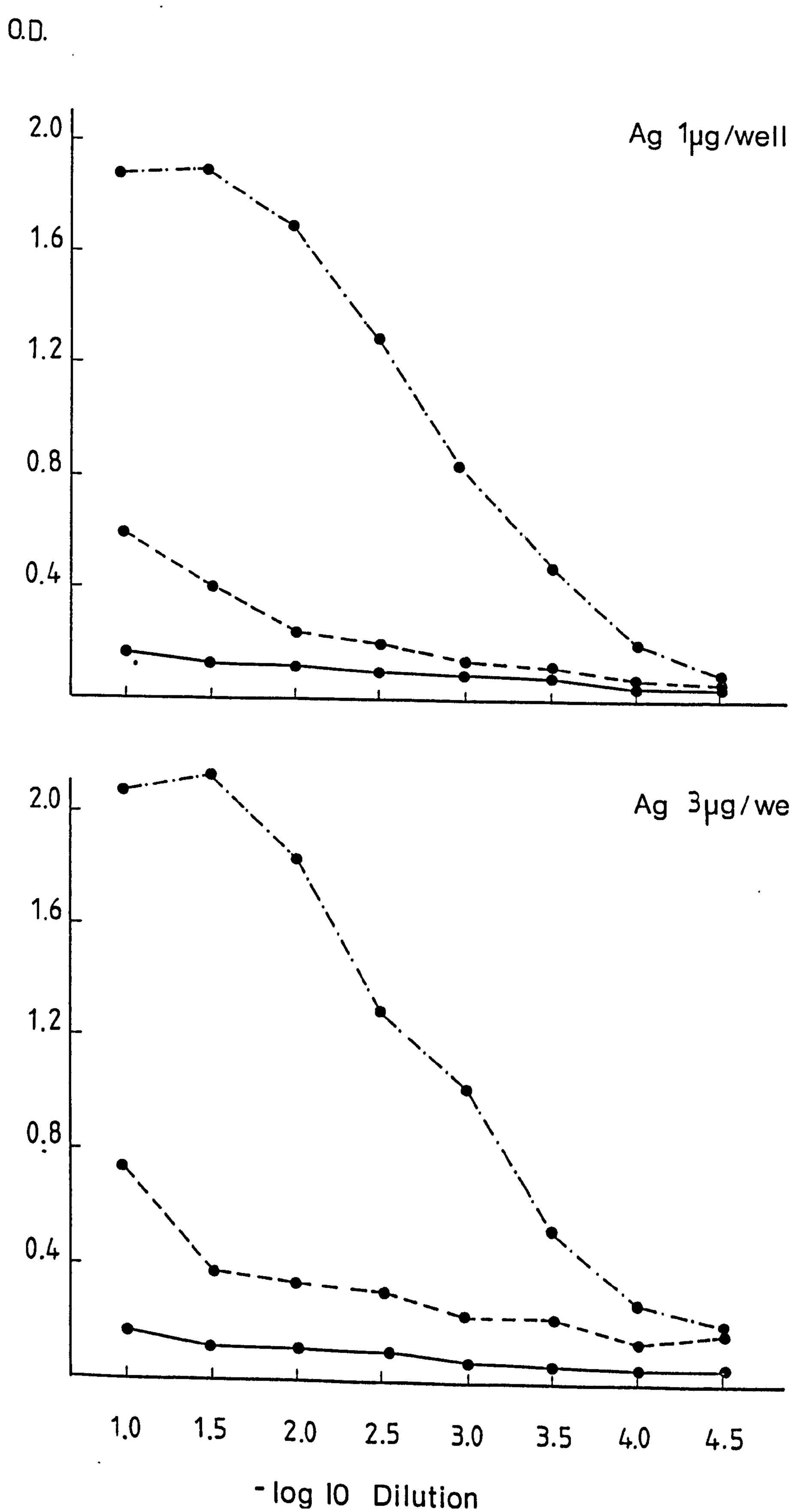
TABLE 3.3 RATIOS OF O.D. IS:NMS IN CHECKERBOARD TITRATIONS WITH IMMUNE SERA AND T. MURIS E/S PRODUCTS

Serum	Antigen µg/well	Dilution of Serum 10^{-1}	$10^{-1.5}$	10^{-2}	$10^{-2.5}$	10^{-3}	$10^{-3.5}$	10^{-4}	$10^{-4.5}$
ISd21	0.25	4.8	5.1	3.7	3.5	3.5	3.0	4.8	3.9
	0.50	3.9	4.2	2.5	1.8	1.9	1.5	0.3	1.3
	1.00	3.5	3.0	2.1	1.9	1.6	2.4	0.6	1.0
	3.00	4.4	3.7	3.5	3.1	3.7	4.0	3.5	3.2
ISE/S	0.25	15.8	21.3	24.3	23.8	16.7	9.8	11.2	5.2
	0.50	12.3	19.4	17.9	14.6	10.7	5.5	1.5	2.0
	1.00	11.0	14.3	14.3	12.5	10.0	5.4	2.6	1.3
	3.00	13.0	20.4	18.6	13.0	16.5	11.6	7.0	3.9

O.D.


2.0
1.6
1.2
0.8
0.4
Ag 0.25 µg/well


2.0
1.6
1.2
0.8
0.4
Ag 0.5 µg/well
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5


O.D.
2.0
1.6
1.2
0.8
0.4
Ag 1µg/well
Ag 3µg/well
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
- log 10 Dilution

DISCUSSION

The anterior portion of T. muris contains the stichosome and the oesophagus of the worm. The stichocytes are thought to be highly active secretory cells, and as such, may be responsible for the production of a large proportion of the immunogenic material which is released by the parasite into the host. This could explain the observation that d35 TS has high specific antibody titres against the AA antigen preparation in particular. Immune sera raised against either the AA preparation or the E/S antigens have high specific antibodies against E/S products as measured in either RIA or ELISA. This suggests that a significant proportion of the E/S products collected in vitro may originate from the anterior portion of the worm. Active immunization with adult E/S products results in a high degree of protection against subsequent infection with the parasite (Wakelin and Selby, 1973); E/S antigens are thus an obvious "starting material" in the search for target immunogens, which elicit an effective immune response in the host. Although E/S antigens are a heterogenous group of molecules, they are a more selective representation of the parasite antigens which are likely to come into contact with the host, and therefore elicit an effective immune response, than antigens obtained by homogenizing whole worms, or portions of worms. Hence, in initial investigations of antibody responses in mice infected with T. muris, and in the screening of hybridoma supernatants, the E/S products of the worm were used; the AA antigen preparation also gave good results in terms of high antibody titre, when used in detection or stimulation, but the dissection of the parasites involved in the preparation of this antigen was very laborious.

A survey of the available literature shows that most ELISAs used to assay responses against parasitic nematodes or parasites in general have involved the use of relatively heterogeneous mixtures of antigens such as whole worm homogenates or E/S products adsorbed to the solid phase (Voller and De Savigny, 1981). Relatively few nematode antigens have actually been isolated and purified, and are thus simply not available for use in immunoassay, although Silberstein (1984) has described the use of various partially purified antigen preparations of Trichinella spiralis in ELISA.

A modified ELISA, which does not involve any antigen purification procedures, has been developed for the detection of antibodies specific for the surface antigens of parasitic nematodes (Schroeder, 1985). The assay was used to detect the quantity of surface-specific IgG antibodies in sera of rats infected with Nippostrongylus brasiliensis; intact worms were randomly sorted into groups of 30, placed in glass tubes and kept on ice during antibody and conjugate incubations. The general protocol was similar to that used in standard immunoassays with incubations and subsequent washings. However, after the washings following the conjugate incubation the worms were transferred to clean glass tubes prior to the addition of the substrate, this was found to eliminate background activity considerably. The worms were kept on ice throughout the procedure as a number of parasitic nematodes are known to shed surface antigens in a temperature-dependent manner (Smith et al., 1981). This immunoassay method may be used for the detection of antibodies specific for surface antigens of other parasitic helminths provided the parasite lacks intrinsic alkaline phosphatase activity under the assay conditions. One advantage of this assay is that there is no possibility for the alteration of antigen conformation by binding to

a solid phase, as it has been suggested that antigen conformation on solid phase may frequently be different from that in solution (Vaidya et al., 1985). The most likely consequence of this phenomenon is that immobilization of a soluble antigen on the surface of a plastic plate may significantly alter its conformation and thus expose an epitope/epitopes which otherwise would not be presented when the antigen is in solution. Vaidya et al. suggested that antibodies which have been produced in response to antigens present in solution, should not be screened by techniques which immobilize the antigen directly to a plastic surface, as the possibility exists that some of these antibodies may not be detected in such assays as a result of the aforementioned conformational changes.

Plastic plates are by far the most popular solid phase used in immunoassays since they make procedures extremely simple. However, plastics may have some important limitations : i) they are immunoreactant consumptive ii) the immunological properties of the bound reagent may be altered and iii) the rate of antibody-antigen interactions is slower than in solution or with particulate solid phases (hours instead of minutes), due to the necessity for the free immunoreactant to diffuse to the solid phase (Tijssen, 1985). Nonetheless, ELISA and RIA are the most convenient assay systems available for large scale screening of samples, and many workers have successfully selected MoAbs and assayed specific antibody titres of test sera using these procedures; obviously the advantages outweight the aforementioned disadvantages.

Non-covalent adsorbtion is most frequently used to coat the solid phase with antigen and it was also used in the assays performed with Trichuris muris antigens. However, up to 68% of non-covalently

adsorbed antigens may be desorbed during the test (Engvall et al., 1971; Lehtonen and Viljanen, 1980). In each step some of the loosely bound antigen detaches and competes for the immunoreactant added, leading to decreased detectability and increased variations in assays. To minimise this problem the immunoreactant for immobilization must be used at the appropriate concentration and plates must be extensively washed after every subsequent step (Lehtonen and Viljanen, loc. cit.). Protein adsorbtion to plastic surfaces is generally attributed to non-specific hydrophobic interactions, and it is independent of the net charge of the protein, although binding is different and characteristic for each protein (Cantarero et al., 1980). Up to a certain limit, a constant amount of the protein is adsorbed to plastic surfaces, which is independent of the input. The protein molecules at this limit become equidistantly distributed on the surface, and the failure to exceed this coverage is due to steric hindrance (Butler, 1981). With an excess of protein more adsorbtion occurs than dictated by the binding capacity due to the formation of multiple layers, stacked on the protein monolayer by protein-protein interactions. Such secondary interactions are not very stable and interfere with the immunoassay. Hence, it is important to determine the minimum optimal concentration of antigen to be adsorbed to the plate, that will give consistent results without a loss of sensitivity.

Another factor to be taken into consideration when using parasite antigens in immunoassays is the often heterogenous nature of the antigens to be adsorbed onto the solid phase. It has been recognized that components of antigenic mixtures are likely to compete with each other for the limited sites on plastic surfaces (Pesce et al., 1977; Cantarero et al., 1980; Tijssen, 1985). At small concentrations of pure proteins, the amount bound to the

plastic is proportional to the concentration of the protein in solution, and at saturation a constant amount of protein is bound regardless of concentration (Cantarero et al., 1980). When adsorption with mixtures of proteins is attempted the bound proteins are not adsorbed in proportions similar to those of the solution (see Tijssen, 1985). Kenny and Dunsmoor (1983) examined the use of antigenic mixtures in the ELISA in order to determine the nature of the competition involved. They observed significant competition when mixtures of proteins were used as coating antigens, and found that a component present at 1% or less in the mixture was essentially undetectable unless excessive amounts of conjugate were used. The important factor was the ratio of competitor to antigen, and not the absolute amounts of components. In indirect ELISA for the measurement of serum antibody they found that the use of antigenic mixtures gave significantly lower antibody titres when the desired antigen was less than 1% of the total protein coated. It was concluded that the use of mixed or crude antigens in ELISA presents significant problems concerning the sensitivity and specificity of tests.

E/S products, even in the crudest form, tend to be mixtures of relatively few antigens (De Savigny, 1975; Jenkins and Wakelin, 1977; Crandall and Zam, 1968), thus problems encountered in using such preparations in immunoassays are likely to be much reduced compared to those encountered using whole worm homogenates. Also, the antigens present in E/S products are a more representative selection of antigens likely to be encountered by the host than crude homogenates, and are thus more likely to be relevant in the search for specific antibodies which are effective in a protective immune response. The E/S products of Trichuris muris are known to be effective in active immunization, and satisfactory results were obtained using this

preparation in ELISA and RIA; specific antibody was measured in a number of different sera from both infected and immunized animals. However, the factors mentioned previously, as regards the use of antigen mixtures in ELISA should always be kept in mind when interpreting results.

Kenny and Dunsmoor (1983) also examined the use of different polystyrene micro-plates from various manufacturers in ELISA and distinguished essentially 2 types of plate : one which adsorbs albumin poorly and the other which adsorbs albumin well. Plates which bound albumin well were best suited for use in ELISA with mixtures of antigens, and tended to give higher O.D. values for test sera, however the "backgrounds" observed with such plates were also higher. Interestingly, the Falcon flexi plates used in ELISA with T. muris E/S antigens gave the highest O.D. readings for test sera, and high background values for naive sera, although their adsorption of albumin was not tested.

Obviously for consistency the same type of plates should be used in all the assays performed; however as plates may exhibit significant variability between lots, it is also advisable to include standard reference sera in each assay if possible. Chessum and Denmark (1978) have described an "edge-effect" on assay plates, due to a variation in the surface characteristics of sample wells at the edges of plates compared to those of the centre, which affects antigen binding. Shekarchi et al. (1984), however, found that well to well variation was more significant than the "edge-effect". In the immunoassays performed with the T. muris antigens no "edge-effects" were observed and test samples were always assayed in duplicate or triplicate as a precaution against individual well variability.

To summarize, it is probable that variability in the adsorption of antigen to the solid phase is the major factor which determines the precision of all solid phase immunoassays. The 3 most important variables for the adsorption of antigen to the solid phase are temperature, time and concentration of antigen. Using Trichuris muris E/S antigens the most convenient and reliable method of coating assay plates to be used in ELISA was overnight at 4°C. 5 µg/ml (0.25 µg per sample well) was chosen as a suitable concentration of antigen for coating the assay plates. This concentration contained sufficient antigen to saturate binding sites on the assay plates as determined by checkerboard titrations and still gave high O.D. values for test sera, comparable to those obtained with higher concentrations of antigen. "Background" values obtained with naive sera were also at an acceptable level using this concentration of antigen. With assay plates prepared in the aforementioned manner, serum dilutions of 10^{-2} were found to give readings for antibody titres which were consistently outside the prozone, and sensitivity of the ELISA for immune sera with low specific antibody titre was maintained.

Studies of various workers so far suggest that the potential of the ELISA has not yet been fully realised. Reported results between laboratories are too variable to be able to suggest that ELISAs will have universal applicability without considerable adaptation to meet the requirements of different host-parasite systems. The main problem appears to be the production of suitable specific antigens, which will increase the sensitivity of the technique and remove or reduce the influence of cross-reacting antibodies and non-specific reactions.

Nevertheless, despite these reservations, ELISA is widely

used for studies on both human and veterinary parasites. In the diagnosis of helminth infections the ELISA has been used on a large scale only for the diagnosis of Trichinella spiralis in pigs (Ruitenberg et al., 1976), but preliminary observations have been carried out on a considerable number of other animal parasites, which include the nematodes Dirofilaria immitis and Toxocara canis (reviewed by Sinclair, 1982). The Trichinella spiralis system is the most studied system with regard to the application of ELISA for serodiagnosis and it was also the first helminth infection to be studied with ELISA (Ljungstrom et al., 1974). Considerable effort has been expended on investigating the reasons why false reactions, in particular false positive reactions occur in ELISAs performed. Clinard and his co-workers (1978) reported that 15% of 265 pigs sent for slaughter gave false positive reactions, and suggested that the specificity of the test might be increased by fractionation and purification of the antigen used in the test. They also fractionated positive and false positive sera with DEAE-cellulose and found that activity of the positive sera appeared in the IgG class fractions, while the activity of the false positive sera appeared in the IgM class fractions. In further investigations Clinard (1979) revealed that pig sera giving false positive reactions contained 2 immunoglobulin classes, an IgG fraction which could be adsorbed with T. spiralis larvae and an IgM component which was unaffected by adsorption. Neither fraction precipitated with T. spiralis antigen in the Ouchterlony test or counter-immunoelectrophoresis. The IgM factor was observed to increase with age of the animal, which was in agreement with the results of a study on the influence of age and husbandry on the extinction values obtained by ELISA for the sera from uninfected pigs (Taylor et al., 1978). It was found that these values increased significantly

with age and that specific pathogen free pigs had lower extinction values than commercially reared pigs of a comparable age.

The use of ELISA in the diagnostic serology of tropical parasitic diseases has been reviewed (Voller et al., 1976; Voller and De Savigny, 1981) as has the use of ELISA in veterinary research and diagnosis (Wardley and Crowther, 1982). Although most of the new methods used in diagnostic serology have more than enough sensitivity, achieving acceptable specificity is still the major problem. An increased effort needs to be devoted to the preparation, purification and standardisation of parasitic antigens for use in the new generation of immunoassays, and the development of in vitro cultivation techniques would contribute greatly to rapid advances as should the use of MoAbs for antigen purification by affinity chromatography and other procedures.

Ultimately, the reliable diagnosis of parasitic infection may depend on immunoassays for the detection of parasite antigens in clinical specimens, such as serum or urine. RIA and ELISA could be employed in this way and the use of monoclonal antibodies in such assays could enhance sensitivity and defined specificity. Antigen immunoassays could provide definitive parasitological diagnosis whereas antibody assays provide at best only a presumptive diagnosis. In reference to the diagnosis of trichinellosis, Garcia and his co-workers (1979) detected antigen in the sera of rats infected 48 hours previously, however this phenomenon has not yet been reported to occur in pigs.

CHAPTER 4

HUMORAL AND CELLULAR RESPONSES TO INFECTION WITH TRICHURIS MURIS

INTRODUCTION

Immunity to Trichuris muris may be transferred to naive animals either passively with serum or adoptively with mesenteric lymph node cells (MLNC) taken from infected mice (Selby and Wakelin, 1973). Serum taken from CFLP mice 21 days post-infection (Wakelin, 1975a) or from "tolerant" mice (Lee, 1982) has been successful in passive transfer experiments, but there is no information as regards parasite-specific antibody titres and isotypes, their antigen specificities or the time course of antibody production in any of the strains of mice examined in this model system. Further investigation of the immune cells responsible for the adoptive transfer of immunity has revealed that nylon wool-adherent B cells had no effect, whereas enriched T cell populations consistently transferred considerable protection to naive mice (Lee et al., 1983). However, the time course of appearance of specific antigen reactive cells in the MLN of infected animals of different strains of mice has not been investigated. As discussed in Chapter 1, different mouse strains vary in the time taken for the onset of expulsion of an infection with T. muris, differences in the time of appearance of T. muris-specific cells in the MLN of these mice could therefore possibly be correlated with differences in parasite expulsion.

The humoral responses to nematode infections have been examined in various systems in an attempt to assess the importance and function of parasite-specific antibody in the immunity expressed against these worms. Anti-worm antibodies are not thought to act in isolation (Wakelin, 1978), but to act in concert with other components of the immune system to ultimately produce a protective immune response. The passive transfer of serum from Trichinella spiralis-infected

animals has been examined by a number of workers and has yielded variable results (see Grencis and Wakelin, 1983; Silberstein, 1984). In the rat model, it has been suggested that IgA producing lymphoblasts are directly involved in the expulsion of adult T. spiralis from the gut (Despommier et al., 1977), and that immune T cells have a 'helper' function in promoting the formation of protective B cells. In the mouse model, Wakelin and Wilson (1979) suggested that the T cells act not as antibody-helper cells but are involved in the generation of inflammatory changes in the intestinal environment that are detrimental to worm survival. Crandall and Crandall (1972) emphasized the complexity of the immune response to T. spiralis infection by demonstrating that there were changes in immunoglobulin levels, in production of antibodies of different isotypes and specificities and in local intestinal antibody responses, but the significance of these responses in resistance to infection was not investigated. More recently Almond and Parkhouse (1986) examined immunoglobulin class-specific responses to biochemically defined antigens of T. spiralis, and observed a close correlation in resistant NIH mice between the production of serum IgA to surface components of adult worms and accelerated expulsion of this stage of the parasite from the gut. Susceptible C3H mice, however, failed to synthesize IgA antibodies to the same surface antigens. Nematospiroides dubius, usually produces chronic primary infections in mice, but animals immunized with repeated infections of the parasite, interspersed by anthelmintic treatment, developed host protective antibodies in their serum, as demonstrated by passive transfer to naive recipients (Williams and Behnke, 1983). The increase in the protective activity of the serum was correlated with a ten-fold concurrent rise in IgG_1 levels, hence the suggestion that the host

protective antibodies were of the IgG_1 class. Indeed, Pritchard et al. (1983) examined the role of IgG_1 hypergammaglobulinaemia in immunity to N. dubius, and confirmed that the immunological activity of immune sera could be exclusively accounted for by their IgG_1 content. Circulating immunoglobulins have also been shown to be involved in protective immunity to the intestinal stage of Nippostrongylus brasiliensis in the rat (Jones et al., 1970).

A comparison has been made of systemic and local immunoglobulin responses in several intestinal nematode infections. Irradiated Haemonchus contortus larval vaccine protects adult sheep against a challenge infection but is ineffective in lambs; in adult sheep protection against a challenge infection was shown to be associated with raised levels of abomasal mucus IgA and serum IgG (Duncan et al., 1978). Vaccination of lambs, however, did not stimulate either abomasal IgA or serum IgG. In Nematospiroides dubius infected mice, rapid elevation of specific IgA in intestinal perfusate was demonstrated, which correlated with early events in the parasite life cycle (Cypess et al., 1977). After infection with Nippostrongylus brasiliensis larvae, intestinal haemagglutinating antibodies appear rapidly once the worms have reached the gut, whereas serum antibodies are detected very much later after the worms have been expelled (Poulain et al., 1976). However, when a minimal immunizing dose of larvae was given, although local antibodies appeared in the gut, no serum antibodies were detected until after the animals were challenged with a large dose of larvae. Sinski and Holmes (1977) also examined systemic and local IgA and IgG production in rats infected with N. brasiliensis and found an almost ten-fold increase in mucosal anti-worm IgA during the 12 days following infection with the parasite (adult worms are usually expelled between

day 12-16 post-infection), whilst serum IgG levels increased more gradually and were not so closely correlated with parasite expulsion. The elimination of adult N. brasiliensis is thought to occur in two stages, first worms are "damaged" by antibodies and then they are expelled by an immune mediated inflammatory reaction in the intestine; Poulain et al. (1976) and Sinski and Holmes (1977) both suggested that local IgA may be involved in the first stage of expulsion.

Although immune responses against filarial nematodes are likely to involve effector mechanisms which differ from these seen in gastrointestinal infections, there are nevertheless some interesting points of comparison regarding serological responses.

Investigations of filarial nematode infections have concentrated on the relationship between the presence or absence of microfilariae and parasite specific antibody in the serum of infected animals. The immune response of high and low responder mice subcutaneously implanted with adult females of Dipetalonema vitae revealed that in C57BL/10 mice (high responders ie. have short low level microfilaraemias) immunity was associated with IgM antibodies to the surface of the microfilariae. In contrast, no such antibodies were found in BALB/c (low responders ie. have long lasting high-level microfilaraemias) and no surface specific IgG was found in either strain (Storey et al., 1986). Thomson et al. (1981) investigated microfilaraemia and antibody responses in CBA/H and CBA/N mice. The latter are a mutant strain of CBA/H with a defect in B cells resulting in reduced antibody response to T-independent antigens, but with normal T cell function. CBA/H mice were shown to have an efficient clearance of injected microfilariae of Brugia malayi, in

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The antigen specificities and isotype of the antibodies actually effective in immune clearance were not investigated, but antibodies reactive with the surface of microfilariae, as demonstrated in CBA/H mice,

contrast to a prolonged microfilaraemia in CBA/N mice. The differences in the antibody responses to infection in the two strains of mice were consistent with an antibody dependent mechanism of clearance; the CBA/N mice had a delayed IgG and a deficient IgM response in comparison to CBA/H mice, have been implicated in the control of microfilaraemia in human infections with Brugia malayi (McGreevy et al., 1980) and with other filarial infections (Weiss, 1978).

There is widespread evidence that protective responses to intestinal nematodes are specific in their initiation, involving antibodies and T cells, but non-specific in their expression, involving myeloid cells and other biologically active factors in an inflammatory response. In the systems investigated immunity against helminths almost without exception has been shown to be T cell dependent (Mitchell, 1980), whatever the ultimate effectors involved. The T cell mediated intestinal inflammation which often accompanies infection with intestinal nematodes is associated with profound structural, physiological and biochemical changes in the environment of the worm and these changes play a major role in worm expulsion (Larsh and Race, 1975; Wakelin, 1978, 1980).

Many immunological changes are associated temporally with Trichinella spiralis infection, including the infiltration of inflammatory cells in tissues surrounding parasites, alterations in lymphocyte responsiveness and alterations in the immunoglobulin content of sera and tissue fluids (reviewed by Despommier, 1977; Wakelin and Denham, 1983). The expulsion of adult worms from the intestine is thought to be the result of immune-mediated inflammatory processes (Wakelin and Denham, loc. cit.); furthermore (Grencis

et al., 1985) demonstrated that L3T4 positive T lymphoblasts were responsible for the transfer of immunity to T. spiralis in mice and proposed that these immune cells within the MLN proliferated in response to infection releasing a variety of lymphokines which would be involved in the amplification, recruitment and differentiation of inflammatory cell types and antibody producing cells.

Moderate levels of infection with Trichuris muris (approximately 400 embryonated eggs) induce no obvious gross inflammation in either the large or small intestine, thus severe inflammation is unlikely to play any significant role in the expulsion of T. muris from the large intestine. Lee et al. (1983) postulated a direct interaction between effector T cells and worms in this system. The epithelial attachment and feeding site of T. muris could render the parasite particularly susceptible to the effects of intraepithelial lymphocytes (IEL), thought to be derived from rapidly dividing T cells in Peyers patches and the MLN. Local environmental changes mediated by the cells or the direct ingestion of cytotoxic granules could affect worm nutrition and significantly contribute to parasite expulsion from the host.

The experiments described in this chapter provide further information on the humoral response to T. muris in various strains of mice. Serum antibody has been investigated as an indicator of immune responsiveness for both enteral and parental dwelling nematodes and their different developmental stages; there does seem to be a correlation between serum and mucosal responses with certain gut dwelling nematodes, although the isotypes and relative titres of the antibodies involved may differ. The strains of mice investigated were selected for their differences in responsiveness to T. muris in-

fections. The so-called "fast-responding" NIH strain is able to expel the worms within 15 days compared with "intermediate" strains such as BALB/c and CFLP which usually expel worms before day 22 and the "slow" responding C57BL/10 strain which may expel worms between day 27 to 34 post-infection. The timing of the appearance of serum antibody to adult E/S product and day 14 larval homogenate (d14LH) was examined in these strains to determine whether there was any correlation between serum antibody titres and the onset of expulsion of the parasite from the gut. The antigen specificities of infection sera from these strains was also investigated by immunoprecipitation of ^{35}S-methionine-labelled T. muris E/S and AMA. The kinetics of antibody production were examined in "tolerant" CFLP mice given cortisone acetate (C/A) during larval development and compared to those in untreated mice infected with T. muris. Serum from tolerant mice (TS), ie. animals with heavy patent infections, does transfer immunity, but it is not known whether antibody titres or production kinetics are affected by C/A treatment, or indeed the antigen specificities of TS compared with those of serum from untreated infected animals.

The importance of T cells in immunity to T. muris has been firmly established, and the variance in the time of onset of expulsion of worms in the different strains of mice could also possibly be related to the time at which antigen reactive lymphocytes appear in the MLN of these animals. Slow responding strains may simply take longer to generate specific effector cells. In vitro lymphocyte proliferation of MLNC from infected animals in response to T. muris E/S was thus examined, in an attempt to determine the time at which specific antigen reactive lymphocytes appeared in the MLNs of these mice.

RESULTS

4.1 SPECIFIC SERUM ANTIBODY TITRES TO TRICHURIS MURIS E/S ANTIGENS THROUGHOUT THE COURSE OF PRIMARY AND SECONDARY INFECTIONS IN NIH, BALB/c AND C57BL/10 MICE

NIH, BALB/c and C57BL/10 mice were used as representatives of "fast", "intermediate" and "slow" responding strains respectively. 12 mice from each strain were infected with 400 embryonated eggs of T. muris and bled from the tail until around 60 days post-infection. Serum samples were aliquoted and stored frozen at -20°C. 2 mice from each strain were killed on day 10 post-infection and the larval worm burdens were counted in order to determine the levels of infection achieved. Faecal samples were examined after day 35 to check whether patent infections had developed as a result of the primary infections given. Secondary infections were given approximately 70 days after the primary infections, and mice were then bled up to day 14 post-infection. The serum samples collected were tested in an ELISA (see Chapter 2) against T. muris E/S antigens using sheep anti-mouse IgG, A and M alkaline phosphatase conjugate.

As can be seen in Figure 4.1, significant levels of specific antibody against adult T. muris E/S products were not detectable in the sera of infected mice, at a level above the "background" obtained with naive mouse serum, until around day 20 post-infection in all 3 strains examined. The specific antibody titres then continued to rise as measured up to day 50 in BALB/c mice and day 65 in NIH and C57BL/10 mice, although the expulsion times of these strains are day 15-20, before day 15 and before or around day 34 respectively. When secondary infections were given there was a halt to the previously steadily increasing antibody titres observed, in the NIH mice

the fall in titre over the next 14 days was marked from the first day of the secondary infection.

4.2 SPECIFIC ANTIBODY TITRES TO T. MURIS DAY 14 LARVAL HOMOGENATE

NIH, C57BL/10, untreated CFLP and C/A treated CFLP post-infection sera from experiments 4.1 and 4.3 were tested in ELISA (at 10^{-2} dilution) against day 14 LH using goat anti-mouse IgG, A and M alkaline phosphatase conjugate, results are given in Figure 4.2.1. Background readings obtained using naive sera were relatively high with this antigen preparation. Titres measured for infection sera from NIH mice and CFLP mice given cortisone acetate were below background values with the exception of the samples for day 24 and day 27 respectively. Values for untreated CFLP infection sera fluctuated, the highest value was recorded on day 17. Sera from C57BL/10 mice gave the highest titres with a peak around day 24, although the titres were relatively low they did remain above background values up to day 60.

Sera with high specific antibody titres for T. muris adult E/S antigens reacted strongly with day 14 larval homogenate (Figure 4.2.2, IS E/S, IS AA and TS), and day 21 CFLP infection serum which has a relatively low specific antibody titre to adult E/S antigens had lower reactivity against day 14 LH.

4.3 SPECIFIC SERUM ANTIBODY TITRES TO T. MURIS ADULT E/S ANTIGENS IN "TOLERANT" AND IMMUNE CFLP MICE

30 CFLP mice were divided into 3 groups of 8; naive mice, mice infected with T. muris (400 embryonated eggs) and given cortisone acetate (C/A) (see Chapter 2) on day 7, 9, 11, 13 and 15

post-infection, and mice infected with T. muris only. Animals were bled from the tail up to day 41 post-infection, sera from individual naive and individual untreated infection animals were each pooled, whereas sera from cortisone acetate treated mice were kept individually. Finally the collected sera were tested at 10^{-2} dilution in an ELISA against T. muris E/S antigens using goat anti-mouse IgG, A and M alkaline phosphatase conjugate, results given in Figure 4.3

There was no significant rise in detectable specific serum antibody titres until around day 20 in either mice given an untreated infection or in those given an infection with cortisone acetate. The titres measured for the individual mice given C/A tended to be higher than the values obtained for the pooled untreated infection sera, and all titres remained considerably above background right up to day 41.

4.4 IMMUNOPRECIPITATION OF ^{35}S-METHIONINE LABELLED T. MURIS E/S PRODUCTS AND AMA BY INFECTION AND IMMUNE SERA

Autoradiographs of precipitated antigens are given in Figures 4.4.1 and 4.4.2, tables giving the apparent m.wts. in kD of the precipitated proteins are numbered 4.4.1 and 4.4.2 respectively. Although the late post-infection sera of NIH, C57BL/10 and BALB/c mice all gave high optical density (O.D.) values in ELISA against T. muris E/S products, they differed in their ability to immunoprecipitate labelled E/S antigens. NIH sera in particular precipitated very little labelled E/S material, although bands of proteins were just visible at around 104, 88, 39, 36 and 34 kD. CFLP day 21 post-infection serum also precipitated only a limited amount of labelled antigen compared to the other sera tested, the m.wts of the antigens recognized were almost identical to those identified for the NIH serum, with an additional protein at 134 kD. No qualitative

differences were observed in the antigen specificities of the DBA/2 sera which recognized antigens with apparent m.wts ranging from 17 to 136 kD. The C57BL/10 and BALB/c late post-infection sera had similar antigen recognition patterns, although the C57BL/10 serum precipitated more labelled E/S protein, the antigens identified were in the range of 17 to 136 kD. However, the C57BL/10 serum strongly precipitated a protein of apparent m.wt 60 kD which was not identified with the NIH, BALB/c, DBA/2 or ISE/S sera, but was precipitated by IS AA and TS. In addition C57BL/10 and DBA/2 infection sera identified a 20 kD band which was only otherwise identified by the IS AA immune serum. The IS AA serum also precipitated an 118 kD antigen not identified by the IS E/S.

In immunoprecipitation studies using labelled T. muris AMA less material was precipitated using equivalent volumes compared to experiments using labelled E/S antigens, reflecting the greater amount of "background" material present in AMA preparations. The marker used in Figure 4.4.2 was the Necator americanus homogenate preparation in which only 3 proteins (67, 45 and 35 kD) were identified, hence the molecular weights quoted for the precipitated antigens may not be within the same accuracy as reported for other gels. IS AA precipitated molecules of weights ranging from 85 to 12 kD, however very little material was precipitated by IS E/S, bands were visible at 85 and 80 kD and just identifiable at 54 and 52 kD, IS d21 was similar in precipitation pattern to IS E/S. The tolerant serum (TS d40) identified a large number of antigens m.wts ranging from 85 to 22 kD as did the two DBA/2 sera, again as observed with precipitation of E/S antigens no qualitative differences were detectable in the latter two sera. The T. trichiura infection serum did precipitate a 43 kD antigen which was not identified by naive human serum.

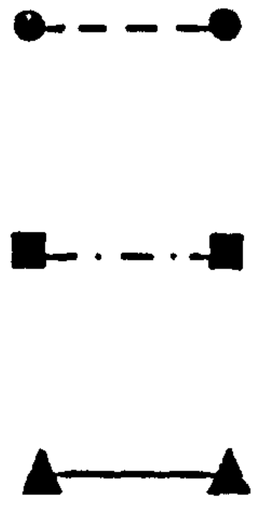
4.5 IN VITRO LYMPHOCYTE PROLIFERATION IN RESPONSE TO T. MURIS E/S ANTIGENS

Initial in vitro lymphocyte proliferation experiments were performed with T. muris adult male homogenate antigen (AMA) (unpublished data), however this preparation was found to be toxic to cells at 100 µg/ml (protein measured in Bio-rad assay). Lower concentrations of 50 and 10 µg/ml were examined but using 10 µg/ml proliferation of immune cells from day 14 post-infection NIH mice was at the most only double that of the cells incubated in medium alone. The same amount of proliferation in response to AMA was observed for naive cells.

E/S products are preferable for use in in vitro proliferation experiments not only because of the aforementioned toxicity of AMA but as mentioned previously the former are a more selective preparation of host-parasite interface antigens. Lymphocyte proliferation assays were performed as described in Chapter 2 using C57BL/10 ("slow" responder), CBA/Ca ("intermediate" responder) and DBA/2 ("differential" responders) MLNC. MLNC were used in preference to spleen cells as the former are capable of transferring immunity to T. muris the latter are not. The DBA/2 mice were divided into responding and tolerant mice following a primary infection, the immune cells used in the assay were taken at day 14 post-secondary infection, and the proliferation of these cells compared with that of naive cells. The CBA/Ca and C57BL/10 MLNC examined were from naive mice. All results are given as ratios of counts per minute (c.p.m) of experimental group (with E/S antigen) to control group (medium alone) in Figure 4.5 Although the background values, c.p.m. obtained with cells cultured in medium alone, were relatively high, T. muris E/S products increased the proliferation of the lymphocytes, as

determined by c.p.m., in a dose dependent manner. The groups tested have similar ratios increasing from 1.4 or 1.5 with 10 µg/ml E/S antigen to 2.6, 2.9 and 3.5 with 100 µg/ml with the exception of the naive C57BL/10 mice. In the latter the ratio increases to 4.4 with 50 µg/ml E/S products and 7.8 with 100 µg/ml, the "slow" responding naive C57BL.10 MLNC appear more susceptible to the mitogenic effects of T. muris E/S antigens.

FIGURE 4.1 SPECIFIC SERUM ANTIBODY TITRES TO TRICHURIS MURIS E/S ANTIGENS THROUGHOUT THE COURSE OF PRIMARY AND SECONDARY INFECTIONS IN NIH, BALB/c AND C57BL/10 MICE



NMS = naive mouse serum

Secondary infection given at day 70 after primary infection.

↑ approximate time of expulsion of T. muris from mice

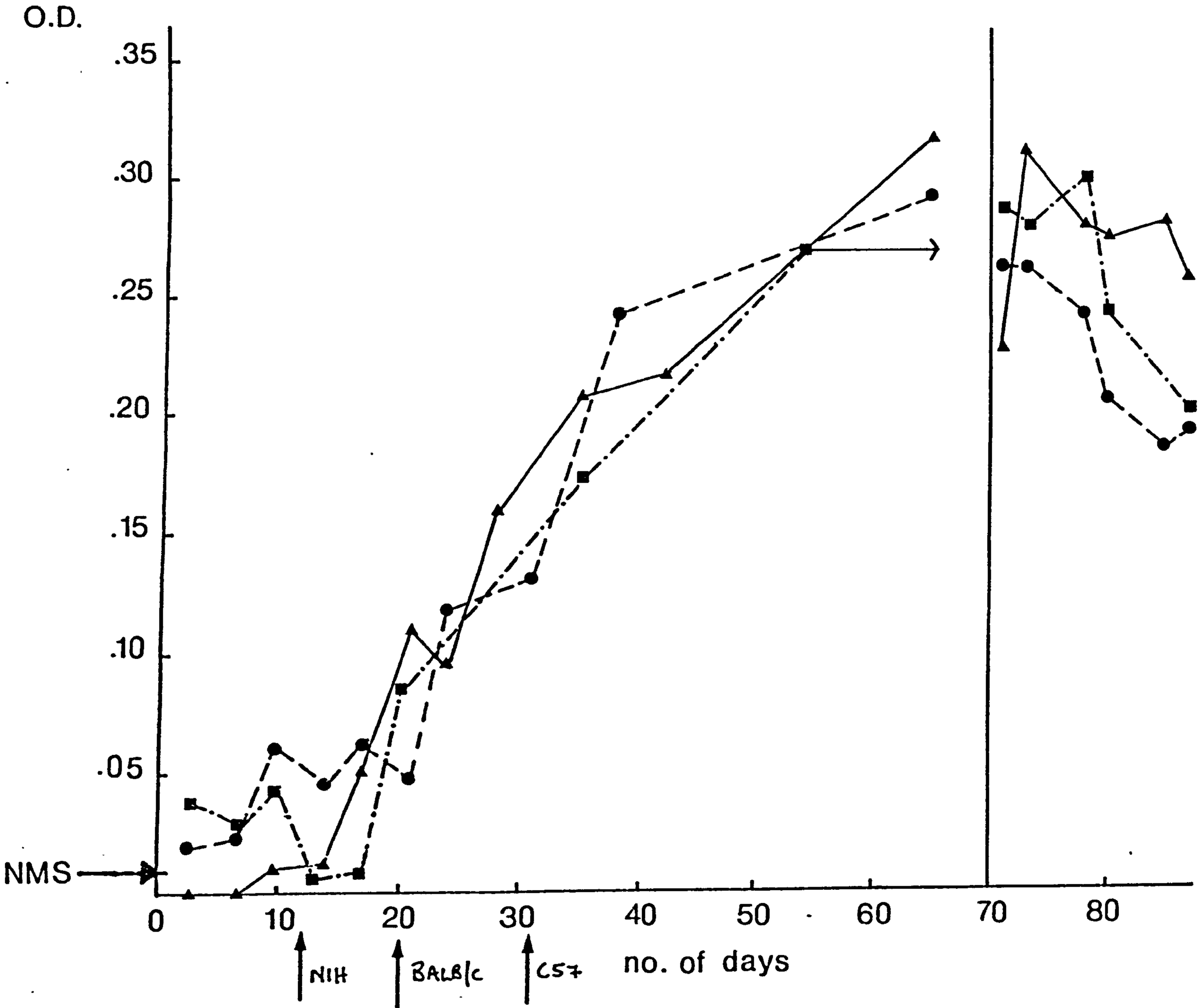
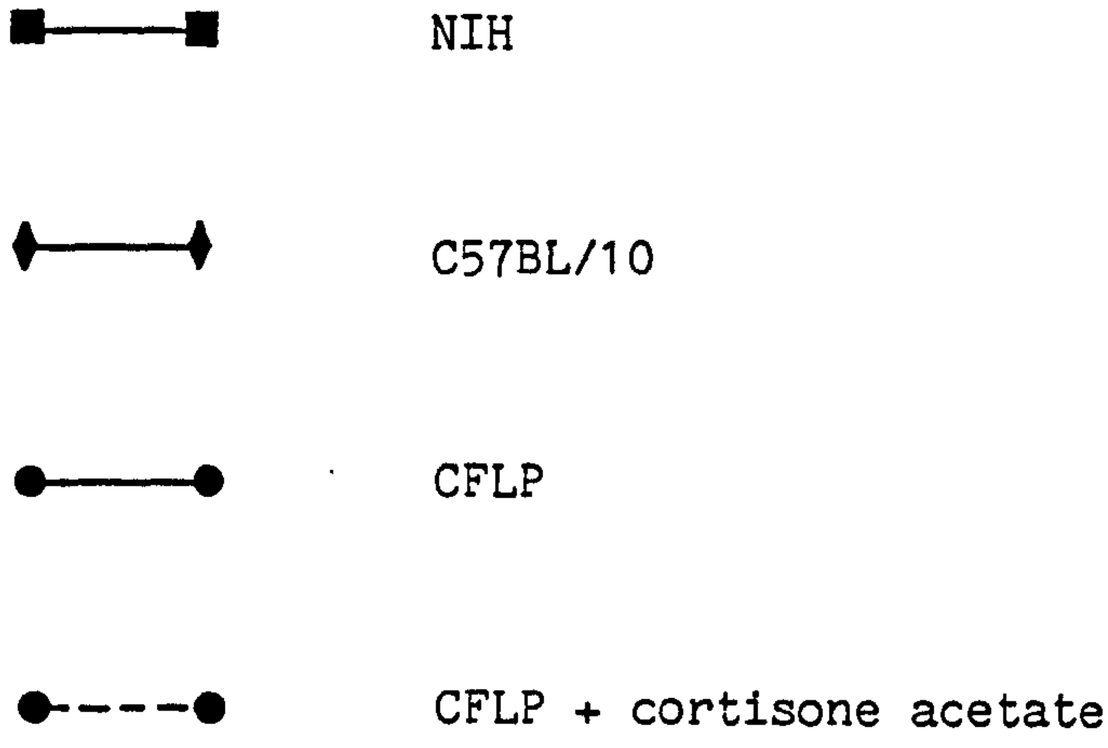

O.D.
.35
.30
.25
.20
.15
.10
.05
NMS
0
10
20
30
40
50
60
70
80
NIH
BALB/c
C57
no. of days

FIGURE 4.2.1 SPECIFIC SERUM ANTIBODY TITRES TO DAY 14 LARVAL HOMOGENATE IN INFECTION SERA FROM NIH, C57BL/10, CFLP AND CORTISONE TREATED CFLP MICE



NMS = naive mouse serum

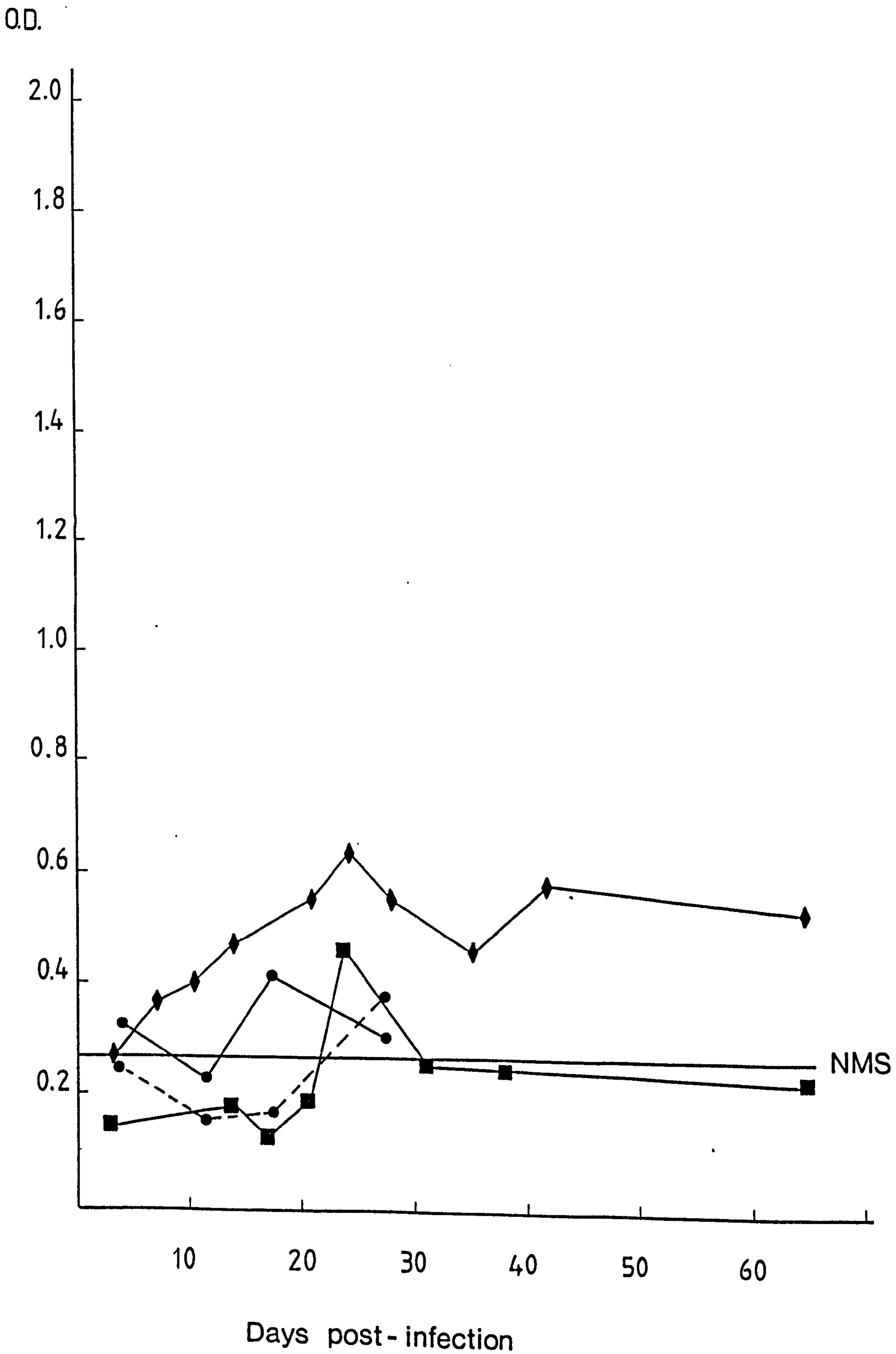

O.D.
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
NMS
10
20
30
40
50
60
Days post-infection

FIGURE 4.2.2 ANTI-TRICHIURIS MURIS SERA AND MoAbs AND ANTI-TRICHINELLA SPIRALIS MoAbs IN ELISA WITH DAY 14 TRICHURIS MURIS LARVAL HOMOGENATE

anti-Trichinella spiralis MoAbs against 48 kD and 50/55 kD muscle larvae stichocyte antigens.

anti-Trichuris muris sera and MoAbs

E12 and F11 - T. muris MoAbs

TSd63 - Day 63 CFLP Tolerant Serum

IS E/S - CFLP immune serum raised against adult E/S products

IS AA - CFLP immune serum raised against the anterior ends of adult worms

ISd21 - CFLP day 21 infection serum

NMS - naive mouse serum

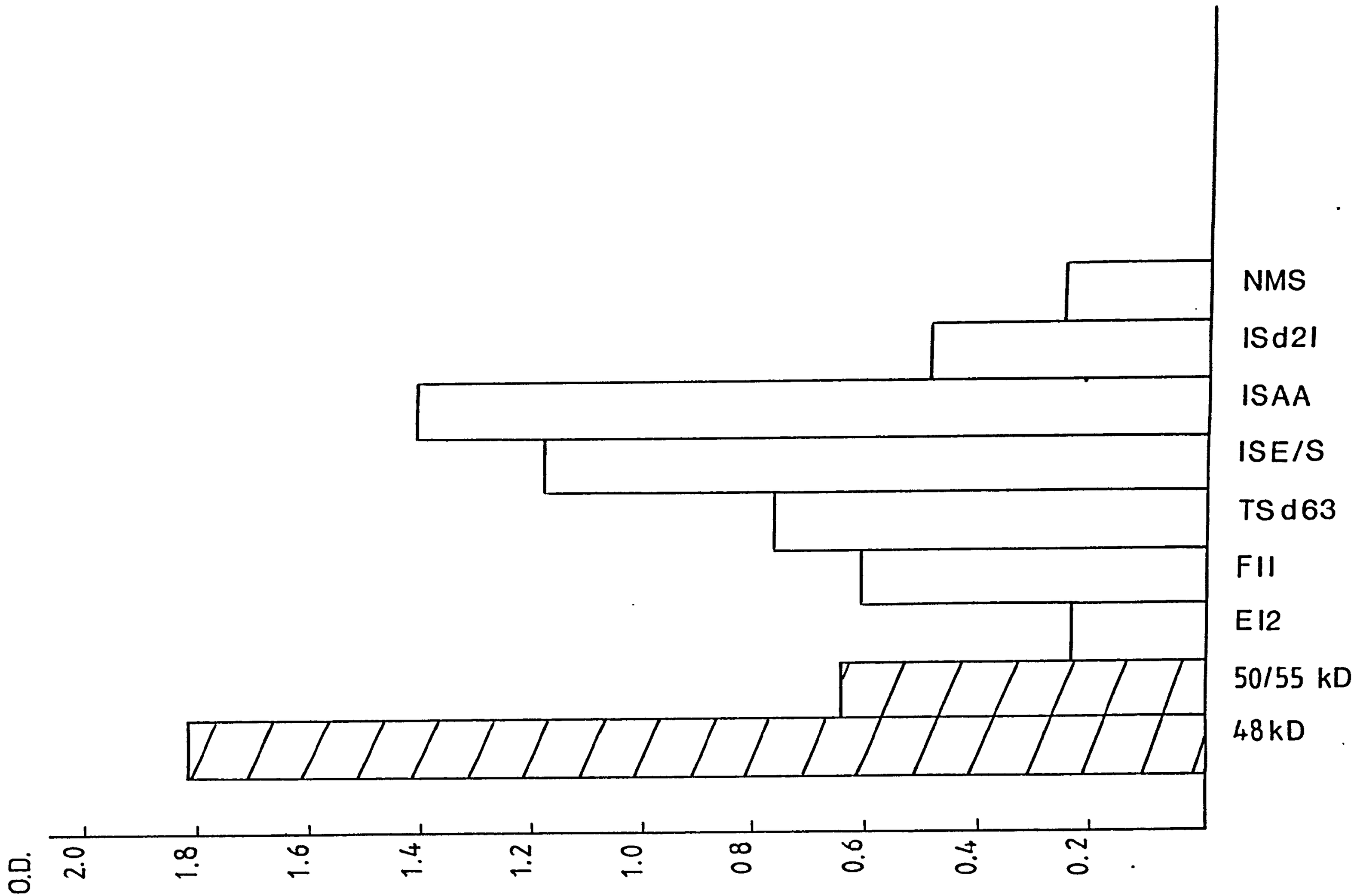

NMS
ISd21
ISAA
ISE/S
TSd63
FII
E12
50/55 kD
48kD
O.D.
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2

FIGURE 4.3 SPECIFIC SERUM ANTIBODY TITRES TO T. MURIS ADULT E/S PRODUCTS IN "TOLERANT" AND IMMUNE CFLP MICE

"Tolerant" mice infected and given cortisone acetate during larval development (numbered 1 to 8)

Mice given infection only

Naive mouse serum (NMS)

X^1 - Immune serum raised against T. muris E/S products (IS E/S)

X^2 - Immune serum raised against T. muris anterior ends (IS AA)

X^3 - Standard of day 21 infection sera (ISd21)

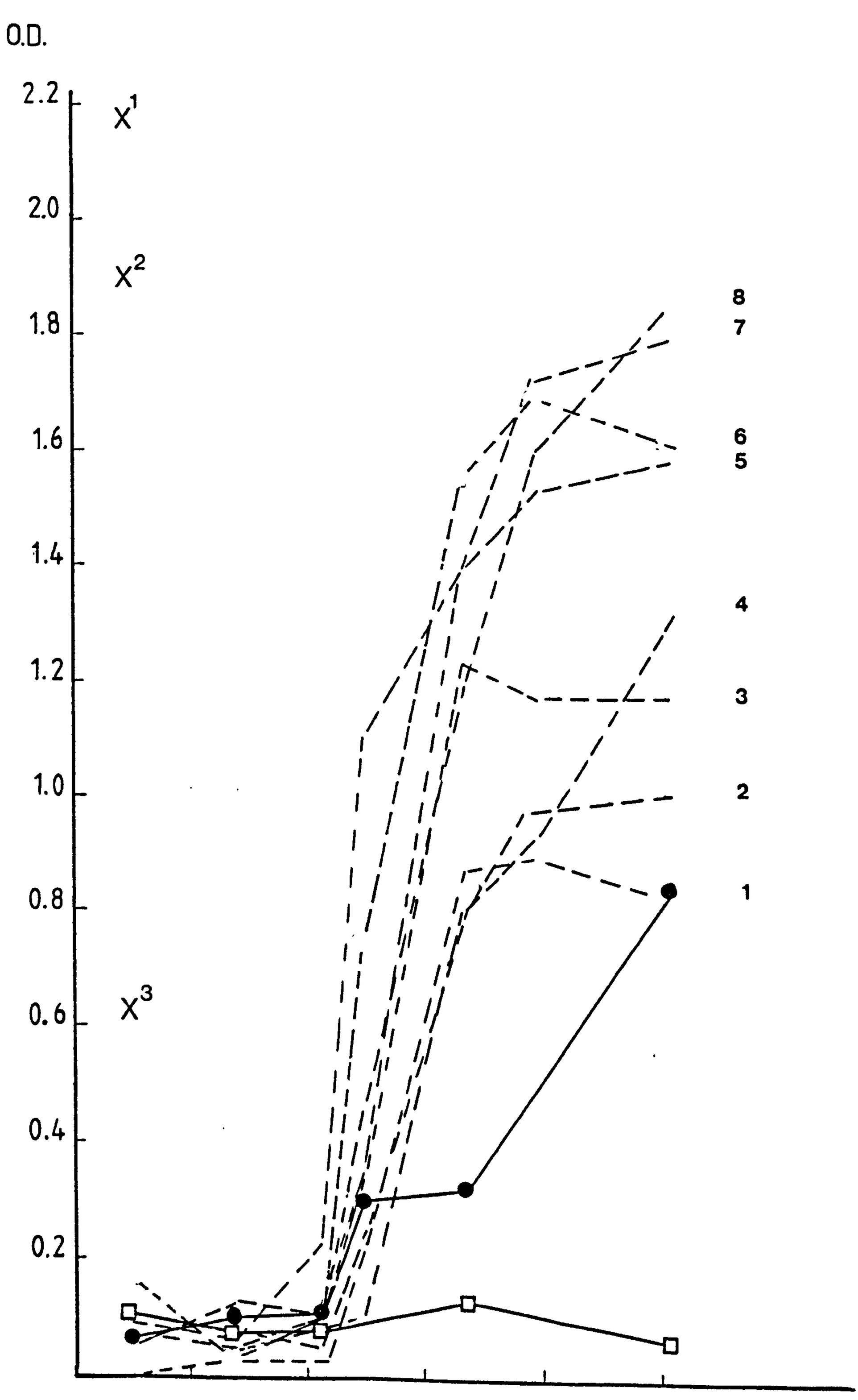

O.D.
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
X1
X2
X3
8
7
6
5
4
3
2
1
8
16
24
32
40
Days post-infection

FIGURE 4.4.1 IMMUNOPRECIPITATION OF ^{35}S-METHIONINE LABELLED T. MURIS E/S PRODUCTS 15% gel

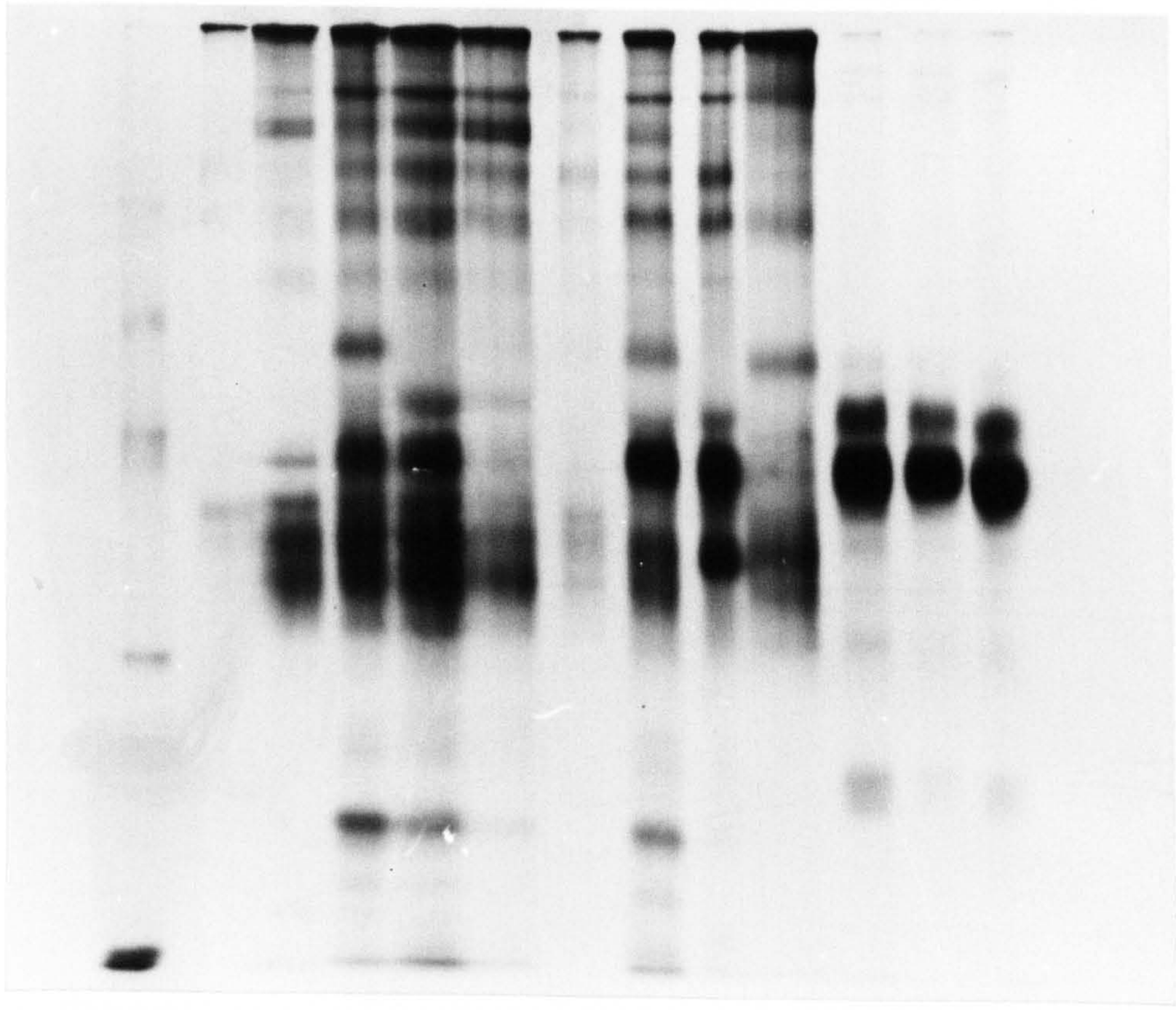
lane

1 Marker : 92 kD, 69 kD, 46 kD, 30 kD, 14.3 kD
2 NIH day 65 post-primary infection serum
3 BALB/c day 54 post-primary infection serum
4 C57BL/10 day 65 post-primary infection serum
5 DBA/2 tolerant serum, day 14 post-secondary infection
6 DBA/2 responder serum, day 14 post-secondary infection
7 CFLP day 21 primary infection serum
8 IS AA - immune serum raised against anterior ends of worms
9 IS E/S - immune serum raised against E/S products
10 CFLP tolerant serum (TS) day 40
11 MoAb E12
12 MoAb E15
13 MoAb E51

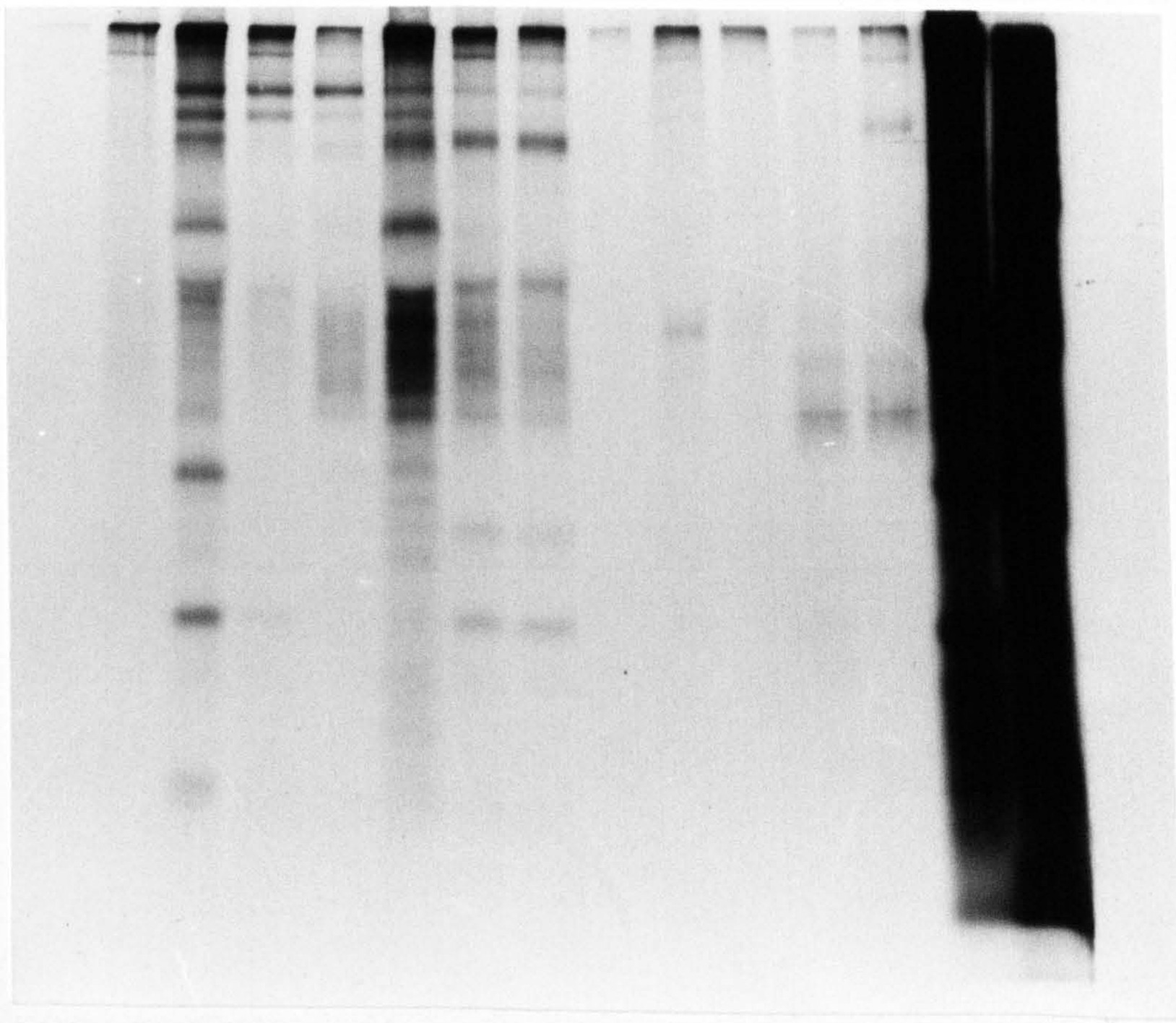
FIGURE 4.4.2 IMMUNOPRECIPITATION OF ^{35}S-METHIONINE LABELLED T. MURIS AMA 15% gel

lane

1 IS AA - immune serum raised against anterior ends of worms
2 IS E/S - immune serum raised against E/S products
3 CFLP day 21 primary infection serum
4 CFLP tolerant serum day 63
5 DBA/2 tolerant serum, day 14 post-secondary infection
6 DBA/2 responder serum, day 14 post-secondary infection
7 Naive mouse serum (NMS)
8 Human serum, T. trichiura infected individual (70 worms)
9 Naive human serum (NHS)
10 T. muris AH - not precipitated
11 Necator americanus AH - not precipitated (used as marker)



1 2 3 4 5 6 7 8 9 10 11 12 13



1 2 3 4 5 6 7 8 9 10 11

TABLE 4.4.1 ^{35}S-METHIONINE LABELLED T. MURIS E/S PRODUCTS PRECIPITATED BY DIFFERENT SERA

NIH (d65 1°)	BALB/c (d54 1°)	C57BL/10 (d65 1°)	DBA/2 (d14 2°)	ISd21	ISAA	ISE/S	IS (d40)	E12 (MoAb)
	136	136	136	(134)	136	136	136	
	124	124	124					
	118				118		(118)	
(104)	(104)	107	107	104	104	107	(104)	
(88)	(93)	91	91	(91)	93	91	91	
	(76)	76	76			(76)		
		60			60		60	
		(52)	52		48	49	(46)	48
	43	44	44		44	44	(44)	43
39	40	40	40	39			39	
36	37	37	37	37	37	36	36	(34)
(34)	34	34	34	34	32			
	30							
		(27)	(27)				29	
		20	20		20			
		(19)	(19)					19
	(17)	(17)	(17)		(17)			18

TABLE 4.4.2 ^{35}S-METHIONINE LABELLED T. MURIS AMA PRECIPITATED BY DIFFERENT SERA

ISAA	ISE/S	ISd21	IS (d40)	DBA/2 (d14 2°)	T. trichiura human serum no2. (70 worms)
85	85	85	85	85	
80	80	80	80		
75		(73)	73	73	
61					
58	(54)		58		
49	(52)	(48)	48	49	
47					
44			45	43	43
41			41	41	
39			37	39	
34		(34)	34	36	
29			29	33	
			27		
			25	24	
			22		
(19)				18	
(12)					

FIGURE 4.5 IN VITRO LYMPHOCYTE PROLIFERATION IN RESPONSE TO T. MURIS E/S PRODUCTS

Ratios experiment : control

	CBA/Ca Naive	C57BL/10 Naive	DBA/2 Naive	DBA/2 Responding	DBA/2 Tolerant
control cpm	28,620	14,016	30,004	21,725	22,224
10 µg/ml E/S	1.4	1.5	1.5	1.5	1.4
50 µg/ml E/S	2.3	4.4	2.3	2.4	2.3
100 µg/ml E/S	2.6	7.8	2.9	3.5	2.9
5 µg/ml con A	1.6	2.6	2.5	3.8	3.5

DISCUSSION

The results of the ELISAs using adult T. muris E/S antigens indicate that specific serum antibody titres of infected animals do not temporally correlate with the different parasite expulsion times of the mouse strains examined. The serum antibody response is apparently delayed in relation to the initiation of the expulsive immune response in the gut of NIH and BALB/c strains, and significant titres are not detectable until around day 20 post-infection even though expulsion occurs before day 15, or between day 15 and 20 respectively in these strains. The kinetics of the response recorded for the "slow" responding C57BL/10 mice (worms expelled between day 27 and 34 post-infection) were similar to those of the previously mentioned "fast" and "intermediate" responding strains. The immune response which expels T. muris from the large intestine in most strains of mice is directed against the developing larvae, however, adult E/S products can be used to actively immunize animals against a subsequent infection (Wakelin and Selby, 1973). Hence, it may be assumed that at least some of the protective antigen(s) responsible for eliciting an expulsive immune response against this nematode are common to the larvae and the adults. A crude day 14 larval homogenate was prepared and used in ELISA with NIH and C57BL/10 infection sera and the results compared with those obtained using adult E/S products in order to verify whether or not any significant systemic antibody was detectable much before day 20, and whether any such response was only detectable with larval antigens and not adult E/S products. As can be seen from Figures 4.1 and 4.3 there do not appear to be any significant titres of antibody either to d14 LH or to adult E/S in the systemic circulation much before day 20 in any of the strains

examined. It is interesting to note that although a small peak of antibody to larval homogenate was seen in the NIH sera at day 27 post-infection, no such antibody was present after day 30, a striking contrast to the persistent high antibody titres against adult E/S measured in the NIH sera up to day 65. Higher titres of antibody to the larval antigens were detected in the C57BL/10 sera and these persited until day 65, however they were not as great as those obtained with the same sera against adult E/S antigens. Thus, it appears that the antibodies present in mice long after a primary infection with T. muris which bound to some product in adult E/S material giving high O.D. values in ELISA, did not bind to the day 14 larval homogenate preparation.

Observations showing a delayed systemic antibody response to T. muris infections are similar in some aspects to those reported for Nippostrongylus brasiliensis infections in rats where serum antibodies were detected very much later after the time at which worms had been expelled (Poulain et al., 1976; Sinski and Holmes, 1977). The lack of temporal correlation between local and systemic antibody responses to gastrointestinal nematode infections emphasizes the major role played by the gut associated lymphoid tissue (GALT) in expulsion of these parasites. Serum from infected animals, however, has been successfully used to transfer immunity against both these nematodes, (Jones et al., 1970; Selby and Wakelin, 1973), and it has been shown that the time at which infection serum is taken for use in passive transfer experiments is a major factor determining whether or not immunity is transferred. Nonetheless, day 21 CFLP T. muris infection serum has been consistently successful in passive transfer of immunity despite the relatively low specific antibody titres to either adult E/S or larval homogenate sometimes found in serum at this point post-

infection. It is also noteworthy that consistent transfer of immunity has been recorded with serum taken on day 20 post-infection from NIH mice (unpublished observations, Jenkins, 1977; Selby and Wakelin, 1973), and serum taken prior to this time did not transfer immunity.

Another notable feature of systemic antibody titres after T. muris infections is the maintenance of relatively high specific antibody titres against adult E/S products long after the worms have been expelled. Snider and Underdown (1986) examining the quantitative and temporal antibody responses in sera and gut secretions of mice infected with Giardia muris also reported that serum IgA and IgG anti-G. muris antibody remained at high levels for up to 10 weeks following parasite clearance, but offered no explanation for this phenomenon. Persistent antigen in the lymphoid tissue of the previously infected mice is a possibility, antigen can remain for several months in the B cell areas of lymph nodes (Klein, 1982).

The E/S products used in the ELISAs performed although less complex than AMA are still a relatively heterogenous mixture and perhaps a more purified preparation of protective antigen will have to be used to determine whether any significant differences exist in the quantitative aspects of the serological responses in the different strains of mice. Qualitative differences in the production of different immunoglobulin isotypes recognizing the worm antigens may exist, as was demonstrated with mice infected with Trichinella spiralis (Almond and Parkhouse, 1986). In the latter system expulsion of worms was correlated with the production of serum IgA in resistant NIH mice. However, as only limited titres of total specific serum antibody of IgG, A and M classes were detectable in T. muris infected mice before

day 20, it is unlikely that the measurement of the production of a particular isotype could be correlated with the onset of expulsion occurring in any of the strains examined here. In order to determine whether there is any temporal correlation between the quantitative antibody responses and the onset of parasite expulsion in the different strains of mice an investigation of local mucosal responses may be preferable to examining the appearance of systemic antibody for the T. muris/mouse system.

The abundant literature on the mechanisms of action of cortico steroids on the immune system (reviewed by Dupont and Wybran, 1985) provides inconsistent results, mostly due to variation in methodology, but suggests that antibody production is relatively resistant to treatment. The systemic antibody responses of cortisone acetate (C/A) treated CFLP mice infected with T. muris were not adversely affected quantitatively, indeed, most of the individual sera from these animals had specific antibody titres much greater than the pooled sera from untreated infected mice. Serum from tolerant animals (ie. with patent infections following C/A treatment) passively transfers immunity (Lee, 1982) so the observation of high specific antibody titres is not so surprising; the infected mice are probably responding to the continual release of antigenic material from the persistent worms. Lee and Wakelin (1983) reported the reduced humoral responsiveness of tolerant mice with chronic T. muris infections to T-dependent and T-independent antigens and suggested some form of antigen induced suppression may be responsible. The high levels of specific antibody in these animals certainly confirms a continued stimulation of the humoral response during infection, first detectable in the serum around day 15 to 20 post-infection and continuing to rise as measured up to day 43. However, this response

alone was not able to effect expulsion of the established adult worms, even when the suppression of the immune system with C/A had ceased. On the basis of adoptive transfer experiments it was suggested that the inability of "tolerant" (C/A treated) mice to expel larval T. muris was due to a defect in the priming of effector T cells (Lee, 1982). IMLNC can only adoptively transfer immunity to a secondary infection in tolerant mice if given at the same time as the infection, and are ineffective against an established adult population. The persistance of adult worms may be dependent on some form of suppression of the immune response by parasite product(s) but a more detailed analysis of the adult E/S antigens and their activities is necessary before this may be determined.

Early and late post-infection sera from CFLP, NIH, C57BL/10, BALB/c mice, secondary infection sera from DBA/2 mice and immune sera raised against E/S products and anterior ends of worms (IS E/S and IS AA) recognized a wide range of in vitro labelled E/S antigens with m.wts ranging from 136 to 17 kD. Qualitative and quantitative differences were observed in the amounts of labelled proteins precipitated, notably the limited amounts of E/S antigens precipitated by the late (day 65) post-infection NIH serum and day 21 CFLP serum compared with the other sera tested, and the selective recognition of 60 and 20 kD proteins by late post-infection C57BL/10 serum, IS AA and day 40 tolerant serum (d40 TS) and C57BL/10, secondary infection DBA/2 sera and IS AA respectively. IS AA also identified a 118 kD protein not precipitated by IS E/S. However, further investigations on the source and nature of the identified E/S antigens are needed before the importance of these differences may be determined and related to other data on the ability of sera to passively transfer immunity to T. muris infection and the "responsiveness" of the different mouse

strains.

Only preliminary experiments were performed to look at in vitro lymphocyte proliferation in response to T. muris E/S products. As can be seen by the results presented here (Table 4.5) the concentrations of antigen used in these experiments (10 μg to 100 μg/ml, protein estimation by bio-rad method) produced dose dependent responses in the proliferation of cells from naive animals implying mitogenicity of the E/S products. Trichinella spiralis muscle larvae antigen (E/S and homogenate) used at the same protein concentrations does not have a mitogenic effect on naive lymphocytes (Grencis, pers. comm.). Perhaps at lower concentrations of T. muris E/S antigen the mitogenic activity might not be present, but if this activity is a persistent feature of these products then the possibility that it is an inherent property of T. muris E/S material must be examined. Bacterial contamination of the E/S products is thought to be unlikely, worms were washed in several changes of medium and cultured with high concentrations of penicillin and streptomycin, the collected culture supernatants were then filtered sterile before being stored in aliquots at -40°C. It would be interesting to determine whether the E/S products are mitogenic for B or T cells, if the former are affected then the possibility of bacterial contamination, perhaps with LPS does arise.

In the light of the possible mitogenic activity of T. muris E/S products, it would be interesting to reconsider the factors which may be responsible for the reduced immune responsiveness observed in mice with chronic infections of this parasite (Lee and Wakelin, 1983). Mice harbouring chronic infections were less able to respond to a primary infection with Trichinella spiralis or to produce humoral responses to T-dependent and T-independent antigens.

Mitogen-like substances have been described for several parasitic protozoa in which general immunosuppression of the host is a feature of chronic infections with these organisms, the parasite mitogens are thought to cause the polyclonal hypergammaglobulinaemia observed during such infections. Protozoa for which mitogens have been described included Plasmodium falciparum (Gabrielsen and Jensen, 1982) and Trypanosoma spp. (Mansfield et al., 1981). There has been much speculation about the immunomodulation observed in experimental and clinical african trypanosomiasis and it seems that different species of trypanosomes induce different types of suppressor cell populations and the genetic background of the host may play a role in the development of immunosuppression. In T. brucei infections Mansfield et al. (loc. cit.) proposed that the primary event behind polyclonal B cell activation and generalized immunosuppression was either the antigen non-specific stimulation of T_H and T_S populations by the trypanosomes or the polyclonal exhaustion of B and T cell clones by trypanosome mitogen coupled with the generation of T_S cell and suppressor macrophages. As regards nematodes, Wadee and Piessens (1986) have reported that the microfilariae of Brugia malayi contain a T cell mitogen which acts on human $T4^+$ cells (helper phenotype) but not on $T8^+$ cells (suppressor phenotype) or on B cells. $T4^+$ lymphocytes stimulated with filarial antigen augmented the in vitro production of immunoglobulins by autologous B lymphocytes. Hence this was suggested as a mechanism by which polyclonal hypergammaglobulinaemia was stimulated in persons exposed to or infected with lymph dwelling filarial parasites, and implies that the filarial parasites may secure their survival by diverting the hosts immune system to react with antigens irrelevant to the host-parasite balance. The Trichuris muris system may differ from the aforementioned systems in that hypergamma-

globulinaemia has not been described during chronic infections. Lee and Wakelin (1982b) examined the possibility that T_S cells may be present in the MLN of mice with chronic T. muris infections, but found little evidence for their existence. However studies on other cell populations such as those of the spleen were not performed so no firm conclusions were made as regards the presence of T_S cells.

The existence of a possibly mitogenic substance within adult E/S products, which may be responsible for the suppression of host immuno-responsiveness observed during chronic infections with the parasite, is an interesting possibility. This could also explain the apparent resistance of established adults to expulsion as compared to larval worms. Lee (1982) reported that a secondary infection given to "tolerant" mice, once the primary infections had been cleared with anthelmintic, could be prevented from establishing if IMLNC were given at the same time as the infection. However, if the cell transfer was delayed until the adults were established the worms could not be expelled. Preliminary investigations have been made on the stability of adult T. muris infections, established under the C/A regime or by using a below "threshold" infection (less than 20 adult worms establish) in C57BL/10 mice, to a superimposed larval challenge (unpublished observations). At day 14 post-secondary infection substantial numbers of larvae were found in many of the guts of "tolerant" mice and in those of mice with below threshold primary infections alongside the infections with established adults. The adult worms appeared to be able to prevent the development of an immune response which normally results in the accelerated expulsion of a secondary infection. To summarize, these observations add further support to the suggestion that adult worms may actively suppress the immune response by some as yet unidentified immune modulatory

substance(s); released mitogens may non-specifically exhaust B and/or T cell populations thus preventing the usually relatively rapid response to establishing larvae and diverting the immune system from attacking established adult parasites.

CHAPTER 5

DIFFERING IMMUNE RESPONSIVENESS TO INFECTION WITH TRICHURIS MURIS WITHIN INBRED DBA/2 MICE : AN INVESTIGATION OF TOLERANCE

INTRODUCTION

It has been reported by earlier workers (Pike, 1963; Lee and Wakelin, 1982a) that mice from inbred DBA/2 populations do not respond uniformly to an infection with Trichuris muris. A more or less fixed proportion of mice from DBA/2 populations are unable to expel T. muris during larval development and the worms reach patency in these animals. Thus DBA/2 mice may be divided into two sub-populations on the basis of the outcome of a primary infection, one group, referred to as the "non-responders" which cannot expel worms, and the remainder (the majority) which are able to expel the parasite, the "responders". This differing immune responsiveness within an inbred strain of mice offers a unique opportunity to examine the mechanisms at work in the expulsion of T. muris, and to perhaps determine the factor(s) responsible for these two states of responsiveness.

Lee and Wakelin (1982a) examined the accumulation of mucosal mast cells (MMC) in a primary infection with T. muris in DBA/2 mice and compared it with that observed in NIH (rapid responders, expel worms before day 15) and CBA/Ca (slow responders, expel worms before day 23). The kinetics of MMC accumulation, as measured by staining the cells in situ, were similar in all the groups and no differences were observed between responding and non-responding DBA/2 mice. On the basis of these results it was suggested that although there may be a relationship between MMC accumulation in the large intestine of mice and expulsion of T. muris, the two events were not temporally correlated and the relationship did not necessarily appear to be cause and effect in nature.

No other work has been carried out with this system and the experiments described in this chapter provide further insight into the immune responsiveness of "responding" and "non-responding" DBA/2 mice to infection with T. muris. The possibility that a difference exists in the quantitative and/or qualitative humoral responsiveness of the two groups has been examined. Serum antibody titres were measured during primary and secondary infections and the specific antigen recognition patterns of the different sera were examined by immunoprecipitation with ^{35}S-methionine labelled E/S and AMA.

The state of immunological "non-responsiveness" is often referred to as tolerance; Roitt, Brostoff and Male (1985) defined tolerance as : "the acquisition of non-reactivity to certain antigens which would normally be expected to excite an immunological response." Current concepts on tolerance and tolerance induction have been excellently reviewed by Klein (1982) and Roitt, Brostoff and Male (1985), however, Malkovsky and Medawar (1984) have since suggested that immunological tolerance may be a consequence of IL-2 deficit during the recognition of antigen by T cells. Zinkernagel and Doherty (1974) originally put forward the concept of "dual recognition". According to this concept T cell receptors recognise antigen in association with molecules coded by MHC genes. There are a few exceptions to this rule, such as the recognition of MHC molecules as allo-antigens, but the question asked by Malkovsky and Medawar (loc. cit.) was : "is immunological tolerance MHC-restricted as most positive immune responses appear to be ?". It appears so, according to most experiments done so far to test this. If the same molecule is recognized in the context of the appropriate MHC as antigen or tolerogen, then there must be another regulatory

principle determining which of the alternatives will predominate. After a series of experiments by various workers Malkovsky and Medawar put forward the following hypotheses, which are not mutually exclusive :

i) Antigenic exposure in the relative absence of IL-2 may be a paralysing experience for T lymphocytes, making them unable to react to the same antigen in future (clonal anergy).

ii) This experience may be fatal for some immunocompetent cells (clonal deletion).

iii) This experience is neither paralysing nor fatal for the immunocompetent cells but switches on or increases suppressor cell activities, which could mask the presence of cells responsible for a normal immune response to the antigen.

Of course, tolerance to an antigen may not necessarily be complete but rather involve the deletion of some aspects only of the immune response. With the aforementioned hypotheses in mind the ability of MLN and splenic lymphocytes from responding and tolerant DBA/2 mice to produce IL-2 in vitro was investigated. Lymphocytes from CBA/Ca mice given cortisone acetate during larval development were also examined for their ability to produce IL-2, in order to compare the factors which may be operating in mice with chemically induced tolerance with those at work in inherently tolerant DBA/2 mice. In the CBA/Ca system the development of a patent infection depends on the suppression of the immune system during larval development; once adult worms have established they are not expelled by the responses which normally develop during larval infection. Hence the inability to expel worms is dependent on the rate of responses in

the light of worm growth. To determine whether this phenomenon was important in the DBA/2 system responding and tolerant mice were given secondary infections of T. muris; in all other strains examined expulsion of a secondary infection is extremely rapid and complete within 5 days. However, this was not the case with tolerant DBA/2 mice. Hence some explanation for the state of tolerance to T. muris infection within the DBA/2 population not based on the rate of development of an immune response must be found.

RESULTS

5.1.1 The Production of Antibody to T. muris E/S Antigens during Infection in DBA/2 Mice

31 mice were infected with 400 embryonated eggs of T. muris and bled from the tail until day 35 (3 animals were killed on day 14 to check the level of infection). The parasite takes approximately 35 days to reach patency, hence after this period mice were divided into 2 groups on the basis of presence or absence of eggs in faecal samples (15 positive, 13 negative), and designated non-responding (tolerant) and responding mice respectively. Primary infections were cleared using methyridine (1 g/kg body wt., Mintic, ICI) and left for a further week before being given secondary infections of 400 embryonated eggs of T. muris. Mice were bled up to day 14 after the secondary infections. Serum (at a dilution of 10^{-2}) was assayed in ELISA against adult T. muris E/S products using sheep anti-mouse Ig G, A and M alkaline phosphatase conjugate.

As can be seen in Figure 5.1.1 the antibody titres measured for the "mixed" group of DBA/2 showed no significant rise until around day 20, and continued to rise up to day 35. In this experiment the antibody titre of the pooled sera from the tolerant group was slightly

higher than that of the responding group as measured at day 45 post-primary infection, and remained so into the secondary infection.

5.1.2 Establishment of a Secondary Infection of T. muris in DBA/2 Mice

Mice were infected, segregated and primary infections cleared as described in Section 5.1.1, mice were killed on day 14 after the secondary infection with 400 embryonated eggs of T. muris. Five naive mice, were also infected with 400 eggs of T. muris and killed on day 14 post-infection to act as challenge controls. Larval worm counts were performed on each of the guts (see Chapter 2) and the results are shown in Figure 5.1.2.

5.2 IMMUNOPRECIPITATION OF ^{35}S-METHIONINE-LABELLED E/S PRODUCTS AND ADULT HOMOGENATE OF T. MURIS BY DBA/2 SERA

Sera from infected responding and tolerant DBA/2 mice (day 14 post secondary infection)were used to immunoprecipitate ^{35}S-methionine labelled E/S products and AMA antigen. Protein-A sepharose (Sigma) was used to isolate bound antigen from unreacted material. Protein-A sepharose binds to all classes of mouse IgG. Hence the profiles of antigen specificities seen in Figures 4.4.1 and 4.4.2 (pg 109) represent the IgG responses of the mice. The sera from the responding and tolerant DBA/2 mice precipitated the same labelled E/S and AMA antigens, the m.wts of the antigens are given in Tables 4.4.1 and 4.4.2 (pg 110).

5.3 INTERLEUKIN-2 (IL-2)PRODUCTION BY MESENTERIC LYMPH NODE CELLS FROM T. MURIS INFECTED DBA/2 MICE

Three groups of 5 DBA/2 mice were used; naive, responding and tolerant. The latter 2 groups were given secondary infections of 400 embryonated eggs of T. muris, after their primary infections had been cleared with mintic, and were killed on day 14 post-infection along

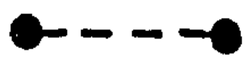
with the naive mice. The MLNC of these animals were cultured overnight with either T. muris E/S antigen or con A, the supernatants were removed the following day and tested (in doubling dilutions $^1/_2$ to $^1/_{128}$) in an IL-2 assay (see Chapter 2). EL-4 cell supernatants were used as positive controls in IL-2 assays, these cells can produce 5,000 times as much IL-2 as optimally stimulated mouse spleen cells. The assay results are presented as figures which correspond to the reciprocal of the dilution of supernatant producing proliferation of the IL-2 dependent CTTL cells equivalent to 50% of the maximum proliferation obtained with EL-4 supernatant, these figures are given in Table 5.3. There was no difference in the abilities of MLNC from responding or tolerant DBA/2 mice to produce IL-2 when stimulated with con A, and the levels produced were equivalent to those produced by stimulated naive cells.

5.4 IL-2 PRODUCTION BY MLN AND SPLEEN CELLS FROM CHEMICALLY INDUCED TOLERANT CBA/Ca MICE

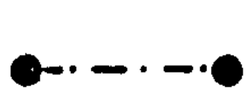
The IL-2 production of cells taken from CBA/Ca mice made tolerant to T. muris infection by cortisone acetate (C/A) administration during larval development was compared with that of cells from untreated mice infected with T. muris, cells from naive mice and cells from naive mice given cortisone acetate. Animals infected with T. muris were given 400 embryonated eggs, animals given C/A were injected s.c. on day 7, 9, 11 and13 and all animals were killed on day 13. MLNC and spleen cells from each of the 4 groups were cultured overnight either with medium only or medium plus con A. Supernatants were removed the following day and tested in IL-2 assays (using doubling dilutions, neat to $^1/_{64}$), results are expressed as described in Section 5.3, and are given in Table 5.4.

FIGURE 5.1.1 THE PRODUCTION OF ANTIBODY TO T. MURIS E/S ANTIGENS DURING INFECTION IN DBA/2 MICE

non-segregated DBA/2 population



responding DBA/2 mice



tolerant DBA/2 mice

naive mouse serum (NMS)

Secondary infections were given on day 55 post-primary infections, after treatment with anthelmintic.

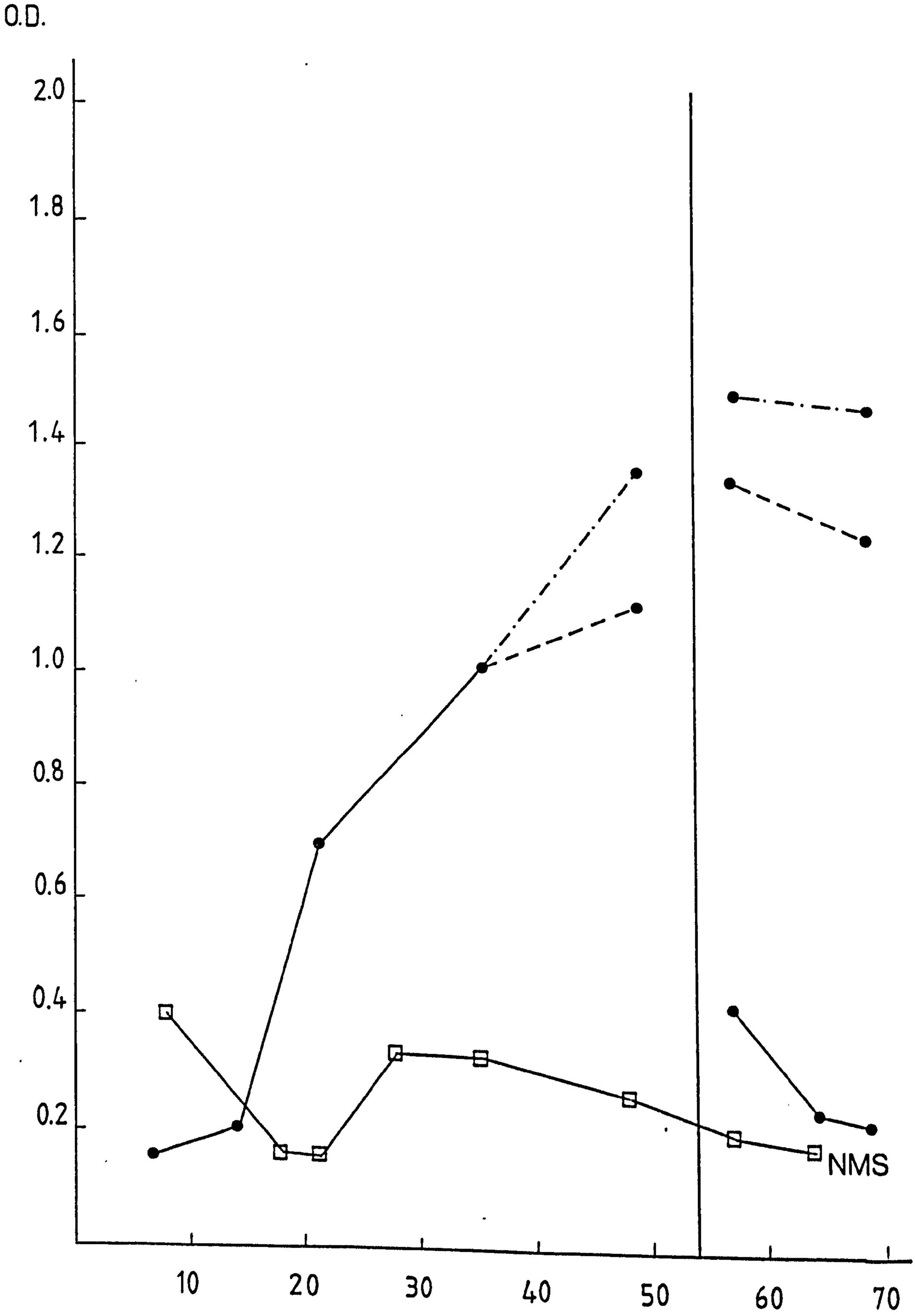
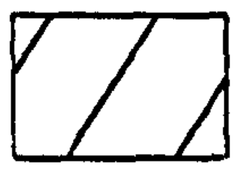

O.D.
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
NMS
10
20
30
40
50
60
70
Days post - infection

FIGURE 5.1.2 ESTABLISHMENT OF A SECONDARY INFECTION OF T. MURIS IN DBA/2 MICE

challenge control, day 14 primary infection

responding mice, day 14 secondary infection



tolerant mice, day 14 secondary infection

standard error bars are shown on the histogram.

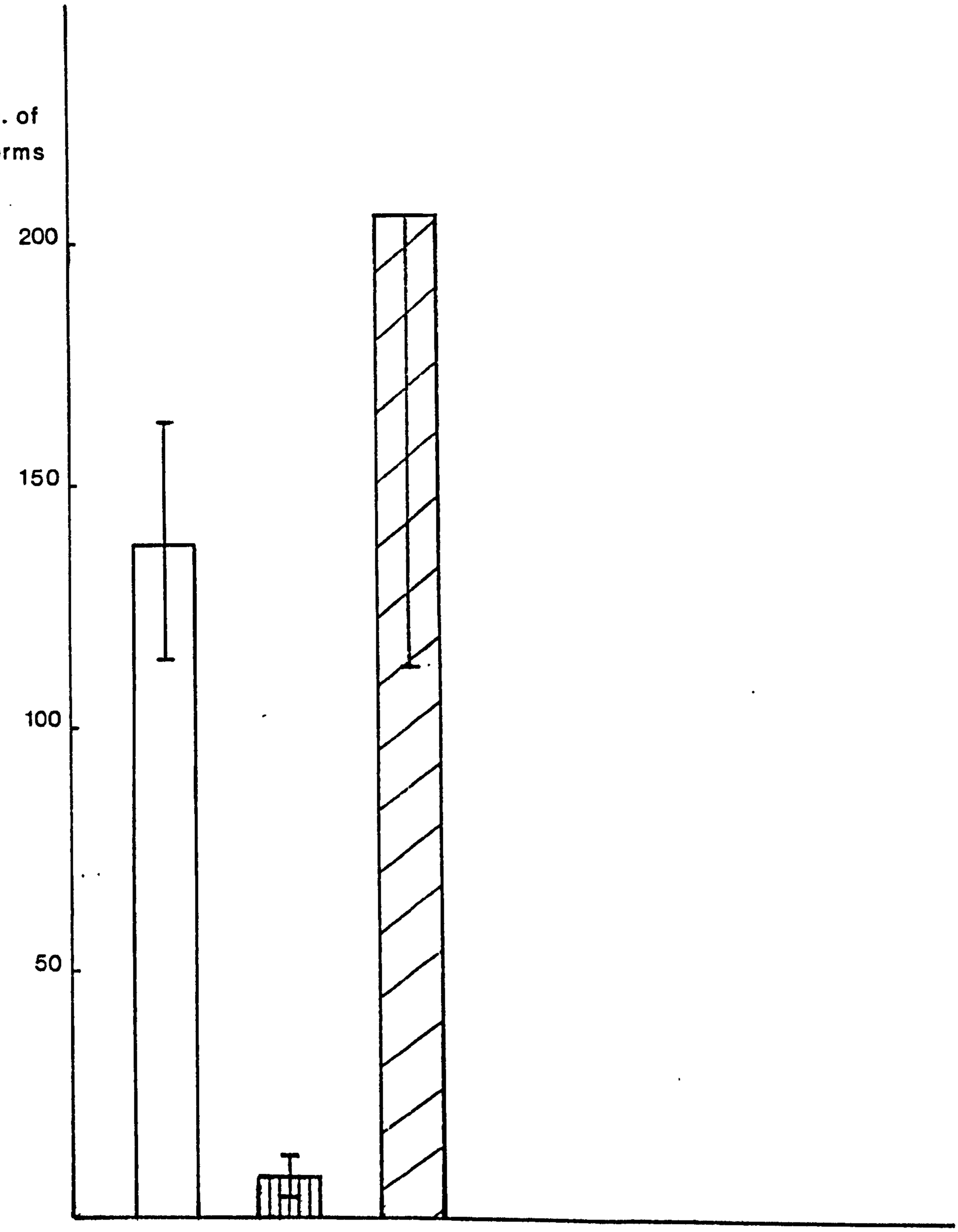

no. of
worms
200
150
100
50

TABLE 5.3 IL-2 ASSAY WITH DBA MLNC SUPERNATANTS; RECIPROCAL DILUTIONS EQUIVALENT TO 50% MAX. PROLIFERATION VALUES

EL-4 supernatants, 50% max. proliferation c.p.m. = 5915

Mice	Medium	100 µg E/S	5 µg con A
Naive	< 2	< 2	32
Responding	< 2	< 2	32
Tolerant	< 2	< 2	32

TABLE 5.4 IL-2 ASSAY WITH CBA/Ca MLN AND SPLEEN CELL SUPERNATANTS; DILUTIONS EQUIVALENT TO 50% MAX. PROLIFERATION VALUES

EL-4 supernatants, 50% max. proliferation c.p.m. = 5917

Mice	MLNC		Spleen Cells	
	Medium	Medium + con A	Medium	Medium + con A
Naive	< 2	8	< 2	32
Naive + C/A	< 2	8	< 2	4
T. muris	< 2	8	< 2	16
T. muris + C/A	-	< 2	< 2	4

DISCUSSION

The ELISA results (Figure 5.1.1) showed that a significant antibody response developed against T. muris E/S antigens in the tolerant DBA/2 mice, and that in these mice the antibody titre was slightly higher than that of the responding groups when measured at day 48 post-infection. The kinetics of the responses in DBA/2 mice are similar to those which have been observed in all the other strains of mice examined (see Chapter 4) and it is between days 15 and 20 that serum antibody is first detectable in the sera of infected mice. As was observed in other strains which expel T. muris during larval development, the specific serum antibody titres of infected mice remain high for some time after the parasite has been expelled, responding DBA/2 mice expel worms before day 20 but anti-T. muris E/S antibody titres increased up to day 48 as measured in this experiment. The possibility that there were qualitative differences in the antigen specificities of antibodies in the infection sera from responding and tolerant DBA/2, was examined by the immunoprecipitation of labelled E/S products (p 109 , Figures 4.4.1 and 4.4.2). The bands representing the precipitated antigens were denser when serum from tolerant mice was used, which reflects the higher titres of antibody in this serum, however all the antigens precipitated by the serum from responding mice were also found to have been precipitated by the serum from tolerant mice (both sera were taken on day 14 post-secondary infection) with no differences between the two.

The establishment of worms during a secondary infection in tolerant and responding DBA/2 mice was examined after the primary infections had been cleared with anthelmintic. The mice which were

tolerant to primary infections with T. muris were also unable to expel the developing larvae of the secondary infection, the responding mice however, showed an accelerated expulsion of the secondary infection and only a few larvae were present in the large intestine of these animals at day 14. Expulsion of secondary infections of T. muris is usually extremely rapid and completed within 5 days at the most with no surviving larvae (Lee, 1982); however, as seen with the responding DBA/2 perhaps a few larvae do survive in "privileged" sites in the intestine. The inability of the tolerant mice to expel a secondary infection with this parasite must be a result of some permanent "defect" in the mechanism by which expulsion is usually effected against T. muris infections; the same "defect" not being present in the responding DBA/2.

The capacity of T lymphocytes from the MLN of responding tolerant and naive DBA/2 mice to produce IL-2 in vitro was investigated. T. muris E/S antigen failed to stimulate IL-2 production at a detectable level in any of these groups, and possibly the antigen concentration used was too high. The lymphocytes were stimulated with con A and the IL-2 in the supernatants was then assayed revealing that the MLN T cells from these mice all had equivalent capacities to produce IL-2. Hence the "defect" in the tolerant mice did not appear to be a result of a reduced capacity to produce IL-2, which could have lead to the tolerization of T cells if antigens were presented to the cells in the relative absence of IL-2 (Malkovsky and Medawar, 1984). Infection with T. muris did not reduce the capacity of the T cells from tolerant or responding mice to produce IL-2 compared with cells from naive animals. However, cells which are producing IL-2 do not necessarily express IL-2 receptors and T cells without IL-2 receptors would of course not be

stimulated by this lymphokine. In order to determine conclusively whether the tolerance observed in a proportion of DBA/2 mice is a result of IL-2 response deficit during parasite antigen presentation, perhaps because of a lack of IL-2 receptors on T cells responsible for eliciting the expulsive immune response against T. muris, the responsiveness of the T cell populations to IL-2 in the presence of T. muris antigens would also have to be investigated.

In the tolerant mouse model (Lee and Wakelin, 1982b), cortisone acetate (C/A) given to mice during larval development of T. muris resulted in the establishment of patent T. muris infections. The major targets of corticosteroids in processes concerned with T cell activation are thought to be the helper T cells and IL-2 production, the effects on earlier steps such as Ia presentation on monocytes (shown to be inhibited by corticosteroids in animal models) and IL-1 production (depressed in vitro by corticosteroids) are still under investigation (reviewed by Dupont and Wybran, 1985). Indeed, many of the mechanisms of action of corticosteroids are still incompletely understood, the existence of specific cytoplasmic receptors has been established for many years and there is preliminary evidence which suggests that several peptidic hormone like products are modulated by corticosteroids via their cytoplasmic receptors and behave as secondary messengers which are either inhibited or activated by the drug.

MLN and spleen cells were taken from naive or T. muris infected CBA/Ca mice which were either untreated or given C/A and their capacity to produce IL-2 in vitro when stimulated with con A was examined. The ability of naive spleen cells to produce IL-2 was dramatically affected by C/A administration, T. muris infection alone

also slightly reduced IL-2 production (although this may not have been a significant reduction), and the effect of C/A administration during T. muris infection was equivalent to that observed when naive mice were given C/A only. The results obtained for MLN T cells were different from those for the splenic T cells, where C/A alone produced the most dramatic reduction in the capacity to produce IL-2, C/A did not reduce the capacity of naive MLNC to produce IL-2 and neither did T. muris infection alone. However, when C/A was administered during parasite infection there was a synergistic interaction which virtually wiped out the relative IL-2 producing capacity of the MLN T cells. The dramatic effect of C/A on the ability of splenic lymphocytes to produce IL-2 could be related to the route of injection of the drug which was given s.c. Hence, the spleen is likely to be a more immediate target than the GALT and would receive a higher dose of C/A. Yet C/A was effective against GALT both functionally and in terms of size. The data presented here suggests that the chemically induced tolerance to T. muris infection achieved by C/A administration during larval development is a result of a synergistic interaction between the parasite and the C/A which results in a deficit of IL-2 in the MLN at the time of parasite antigen presentation. T cells are central in the activation and regulation of the various components involved in the expulsive immune responses described for most nematode infections (Wakelin, 1978; Mitchell, 1980), hence tolerization of T cells specific for parasite antigens responsible for eliciting an expulsive immune response would allow indefinite parasite survival unless this situation could later be reversed.

To summarize, an immunological basis for the paradox of differing immune responsiveness within the inbred DBA/2 strain has

not yet been identified, although the lack of responsiveness in a proportion of these mice has now been shown to be a persistent feature also observed in secondary infections thus ruling out the hypothesis that the state of tolerance is based on the rate of development of an immune response to T. muris infection. The responding and tolerant mice within this strain have more or less identical humoral responses to parasite antigens as regards quantitative responses measured in an ELISA against T. muris E/S products and qualitative responses regarding antigen specificities determined by immune precipitation of labelled E/S antigens. No differences were found in the capacities of MLNC from these animals to produce IL-2 in vitro, however the presence of IL-2 receptors on the T cells of these populations was not examined. The mechanism by which C/A administration produces tolerance in CBA/Ca mice thus appears to be different to the inherent "defect" in mechanism which results in tolerance in a proportion of DBA/2 mice as can be determined by investigations performed so far.

CHAPTER 6

CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST *TRICHURIS MURIS* EXCRETORY/SECRETORY PRODUCTS

INTRODUCTION

MONOCLONAL ANTIBODY TECHNOLOGY

The derivation of cell lines capable of permanent production of specific antibody was first reported by Kohler and Milstein in 1975. They were originally investigating fusions of mutant myeloma cell lines, in order that they might gain insight into the processes by which immunoglobulin gene expression was regulated. However, their successful fusion of mouse myeloma cells with spleen cells from immunized animals to produce hybrid cell lines *in vitro*, and the realization that these lines secreted antibodies of unprecedented specificity against the immunizing antigen, revolutionized the tools of biotechnology. The wide range of applications of MoAbs in different medical disciplines is reflected, not only by the vast number of papers published in the last 10 years, but also by the enormous interest of commercial companies.

There are excellent reviews available on the production and applications of MoAbs (Staines and Liew, 1980; Milstein, 1980; Yelton and Scharff, 1980; WHO, 1982; Sinkovics and Dreesman, 1983; Staines, 1983), thus the basic principles will be discussed only briefly here. MoAbs are chemically homogeneous reagents, produced against single antigenic determinants in potentially unlimited quantities without using pure antigens for immunization. Each MoAb is a unique protein with its own biochemical and immunochemical characteristics, a situation quite different from conventional polyclonal antisera, which are composed of antibodies of different classes, affinities and specificities. However, despite these advantages, several technical problems had to be solved, in order to realize the full potential of monoclonal technology. The expression of immuno-

globulins in hybrid cells is codominant, ie. both myeloma-coded and spleen cell-coded polypeptide chains are produced. This problem was overcome by using variant myeloma lines from the original Kohler-Milstein P3/X63/Ag 8 line, obtained from BALB/c mice which had the disadvantage of producing indigenous immunoglobulins. As hybrids tend to lose chromosomes (usually those coding for heavy chains are lost first), an unstable clone may be lost as it grows. Cultures are assayed to select desired variants until a stable HL (heavy plus light chain) variant secreting clone is isolated. Fusion with mutant myelomas producing only a k light chain (eg. P3/NS1/1.Ag 4.1 (P3NS1), although the light chains are not usually secreted), or no indigenous immunoglobulin at all (eg. X63/Ag 8.653 Sp2/0 and NS0/U) makes it easier to derive the desired HL clones (WHO, 1982). Myeloma cells have a very low spontaneous fusion frequency (1 in 10^6 - 1 in 10^7 cells) and most of the early attempts to fuse mouse myeloma cells used inactivated Sendai virus. However, this was not very successful, presumably because most mouse myeloma cells do not have receptors for the virus. It is common practice now to use polyethylene glycol (PEG) as a fusing agent (Pontecorvo, 1975; Davidson and Gerald, 1976); it is inexpensive and easily available and fusion frequency is usually increased to around 1 hybrid /2 x 10^5 lymphocytes. Careful attention must be paid to the conditions used to expose cells to PEG for it can be highly toxic. Myeloma cells selectively fuse with B lymphocytes, hence it is not necessary to purify the B cells before fusion, and as many as 10% of immunoglobulin secreting hybrids may secrete specific antibody. Since the relative proportion of specific antibody secreting cells in the spleen is at best 1%, there appears to be strong selection in favour of spleen cells that give rise to hybrids secreting desired antibodies. The low frequency of fusion is a continuing problem in

the production of MoAbs, hence the frequency of antibody forming cells in the lymphocyte population to be used is routinely increased by immunizing the donor animals.

After the fusion process the few cells which have formed viable hybrids must be selected from among the many unfused cells. The unfused lymphocytes do not survive long in tissue culture, and a technique developed by Littlefield (1964) is the one most widely used to select for the growth of hybrids as opposed to myeloma cells. Variant mouse myeloma cells lacking the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT) are available, and these cells are unable to utilize exogenous hypoxanthine or guanine to synthesize purines. Hence, if these cells are grown in the presence of aminopterin or methotrexate, which block endogenous synthesis of purines and pyrimidines, the HGPRT negative myeloma cells die. By using selective medium, containing hypoxanthine and thymidine and either aminopterin or methotrexate (HAT or HMT medium), the hybrid cells are the only cells which survive after the fusion process, as they are able to utilize the HGPRT enzyme of the fused lymphocyte.

The labour involved in the production of MoAbs intensifies as cultures are expanded, and there is a limit to the number of colonies which can be selected from the initial screening. Hence, it is very important to use appropriate screening techniques at an early stage of selection. On average 50% of initially selected hybrids may be lost due to instability, poor growth or cessation of secretion during subsequent handling, up to and including growth in ascitic tumours (WHO, 1982).

MONOCLONAL ANTIBODIES IN IMMUNOPARASITOLOGY

The antigenic heterogeneity of parasites has been a major factor responsible for the relatively slow development of immunoparasitology. Parasites present a great range of antigens to the host, a fact which often makes interpretation of experimental work difficult, and which is a serious barrier to understanding the mechanisms of protective immunity. The definition of parasite antigens, perhaps with the help of MoAbs, is an essential early step in the understanding of the immune response to parasites, together with the immunopathology of parasitic infections, and in the development of effective and safe vaccines. In the area of immunodiagnosis the absence of defined antigens also creates problems as a result of the lack of specificity of assays. The role of MoAb technology in immunoparasitology has been relatively recently reviewed, (Rowe, 1980; Mitchell, 1981; WHO, 1982) and 5 broad areas of application have been defined :

1. As effector molecules of anti-parasite immunity.
2. As antigen detecting probes.
3. In the isolation and purification of parasite antigens.
4. In the development of immunodiagnostic assays.
5. In the induction of anti-idiotype responses.

The production of MoAbs to the E/S products of Trichuris muris has been described in Chapter 2. Mesenteric lymph node cells (MLNC) used in the fusions with myeloma cells were taken from mice, which had been given a primary infection of T. muris and a boost of E/S antigen 3 days before the fusion. These MLNC were used rather than spleen cells from immunized mice, in the hope of obtaining hybridomas which would secrete functionally active MoAbs to relevant antigens encount-

ered by the host during parasite infection. Appleby et al. (1985) also used MLNC in the production of MoAbs against either Schistosoma mansoni or Nematospiroides dubius. Greatly improved yields of positive clones (ie. those producing specific anti-parasite antibodies) were obtained using the MLNC compared with the results from using splenic cells from infected animals as a source of B cells for fusion. The concept of taking lymphocytes for fusions from local lymph nodes draining areas of infection was clearly demonstrated to be valid in the case of the latter two parasites. In the production of MoAbs to T. muris E/S products using MLNC, the proportion of IgA secreting hybridomas was found to be very high, whereas most workers using spleen cells in fusions usually obtained IgG or IgM secreting hybridomas.

In this chapter the characterisation of the MoAbs produced against T. muris E/S products is described. As explained previously, screening hybridoma supernatants in an ELISA using E/S antigens was considered a more reliable means of detecting MoAbs to functional worm antigens encountered by the host during infection; crude worm homogenate, contains a larger amount of "background" material, unlikely to be involved in the stimulation of protective immunity. The MoAbs produced were identified in terms of isotype and antigen specificity. Some MoAbs were also used in initial experiments to investigate the passive transfer of immunity, and in the affinity isolation of antigens to be used in active immunization. Attempts were also made to localize the antigens recognized by the MoAbs in the nematode using immunoperoxidase techniques on a variety of histological sections, however, these have so far been unsuccessful.

RESULTS

A series of MoAbs to the E/S antigens of adult T. muris were produced and their isotypes determined by RIA. The MoAbs were adsorbed onto the solid phase and screened using specific ^{125}I-labelled anti-isotype antibodies, the results were as follows :

Istotype	MoAb
IgM	E8
IgG_1	E12, E15, E51
IgA	A2, A16, C7, F5, F11

The MoAbs were characterized in terms of E/S antigen specificities using western blot analyses (photographs not shown) and immunoprecipitation studies using ^{35}S-methionine labelled worm proteins (Figures 6.1, 6.2, 6.3 and 6.4, see also pg 109 Figure 4.4.1 and pg 202 Figure 8.2.1). For immunoprecipitation with IgG_1 MoAbs protein A sepharose could be used to separate the antibody and bound antigen from the unreacted material, but polyvalent rabbit anti-mouse immunoglobulin serum (RAM Ig) was used to secondarily precipitate the IgM and IgA MoAbs. Some of the MoAbs produced by hybridomas isolated from the same second limiting dilution cloning appeared to share the same isotype and antigen specificities ie. they were probably derived from the same hybrid clone. Indeed, E12, E15 and E51, A2 and A16, and F5 and F11 appeared to be producing only 3 different MoAbs, so the total number of cloned hybridomas producing different specific anti- T. muris MoAbs was in fact 5. A summary of MoAb antigen specificities is given in Table 6.5.

6.6 PASSIVE TRANSFER OF IMMUNITY TO INFECTION WITH T. MURIS IN CBA/Ca MICE USING SPECIFIC ANTI-E/S ANTIGEN MoAbs PRODUCED IN VITRO

Hybridoma culture supernatants (from cells grown in HMT medium with 5-10% FCS) were stored frozen at -20°C. 1 ml of test supernatant was given i.p. on day -1, day 1, day 2 and day 5 post-infection with 400 embryonated eggs of T. muris. Estimated antibody titres of the supernatants using Mancini were E8 < 13 µg/ml, E12, E15, and E51 ~ 22 µg/ml, F5 and F11 ~ 76 µg/ml and C7 ~ 107 µg/ml. Mice were killed on day 14 post-infection, the caecum and colon of each mouse was removed and frozen for subsequent counting of larval worm burdens. Results are given in Figure 6.6.

Passive transfer experiments with culture supernatants did not give consistent results, but F5 and F11 supernatants did transfer immunity on a number of occasions in CBA/Ca mice, and A2 and A16 supernatants did so on one occasion in NIH mice, as determined by the accelerated expulsion of a primary infection. The obvious cause of the inconsistency is the variability in the amount of antibody given in supernatants. Preliminary passive transfer experiments using supernatants were only intended as indicators for the initial selection of the most promising MoAbs worthy of immediate more intensive examination. MoAbs may also be produced in tumour-bearing BALB/c mice ie. ascites production, and the concentration of MoAb is often 1000 times more concentrated than that of in vitro spent medium (in the latter the concentration is usually in order of 10 µg/ml but may be increased to perhaps 50 µg or 100 µg/ml (WHO, 1982)), however, this is not always the case. The main advantage of using spent medium MoAb is that the protein impurities are largely controlled as they are components of the medium, indeed in the in vitro system used the MoAb is the only antibody of mouse origin present in the supernatant. In contrast ascites fluid always contains other immunoglobulins and the MoAb is not likely to be much better than 90% of the total antibody.

However for passive transfer experiments as long as the appropriate controls are used this is not an important consideration.

6.7 ACTIVE IMMUNIZATION IN CBA/Ca AND NIH MICE USING F11 AND E12 MoAb AFFINITY ISOLATED ANTIGENS

F11 and E12 MoAbs coupled to CNBr-activated sepharose-4B were used to affinity purify T. muris E/S antigens (see Chapter 2). Concentrated preparations of isolated antigens were examined by SDS-PAGE (pg 173 Figure 7.2), reduced samples of F11 antigen (Ag) and E12 Ag contained major proteins of apparent m.wts. 33 and 21 kD, and 48, 41 and 28 kD respectively. Antigens were given to two groups of 5 NIH and 5 CBA/Ca mice emulsified in Freunds complete adjuvant (FCA, Seralab). A volume of 0.1 ml i.p. was given 14 days and 7 days prior to infection with 400 embryonated eggs of T. muris the total amount of protein received per mouse being approximately 1 μg. For each antigen test group, two groups of 5 mice were included as positive and negative controls using total E/S products and PBS in FCA respectively. Results are given in Figure 6.8, but the experimental groups for the NIH mice given F11 Ag are not included as the negative control figures were unusually low, hence the data could not be assessed as regards the success of the immunization. CBA/Ca mice were killed day 14 post-infection, NIH mice were killed day 10 post-infection.

Initial results suggested that the F11 Ag was successful in active immunization against T. muris infections in CBA/Ca mice. However, E12 Ag had different effects when used in immunization in CBA/Ca and NIH mice. In the former the infection level appeared to be higher in animals given E12 Ag than in the negative controls, in the latter the larval worm burdens were reduced, albeit not signifi-

cantly. Of course, with the limited amounts of antigens initially isolated by affinity chromatography, only limited experiments were possible. The antigen doses given were very low, and the worm burdens within groups of animals in these experiments showed unusually high variability. Hence, only tentative suggestions may be made about the potential of the MoAb affinity isolated antigens in active immunization until further experiments are performed.

6.8 IMMUNOPEROXIDASE LOCALIZATION OF E/S ANTIGENS RECOGNIZED BY MoAbs

Several intial antigen localization studies were performed using Carnoy's fixed sections of adult T. muris in the gut. MoAbs were biotinylated and tested for retention of their binding abilities in ELISA, using the aforementioned antibodies with avidin-peroxidase conjugate (see Chapter 2). Areas of blue staining due to the oxidation of the chloronapthol substrate (CN) were observed, but there was no difference between control sections (incubated with P3NS1 supernatant or ascites as appropriate) and MoAb test sections. The bacillary band of T. muris appears to selectively bind avidin as do the mucosal mast cells (MMC), as shown in Figures 6.9.1 and 6.9.2, hence the oxidation of CN in these areas.

Indirect assays using non-coupled MoAbs with sheep anti-mouse Ig peroxidase conjugate were also performed, but again failed to localize the antigens recognized by the MoAbs in the sections used. Assays using different incubation times and concentrations of reagents were investigated but none of these were successful.

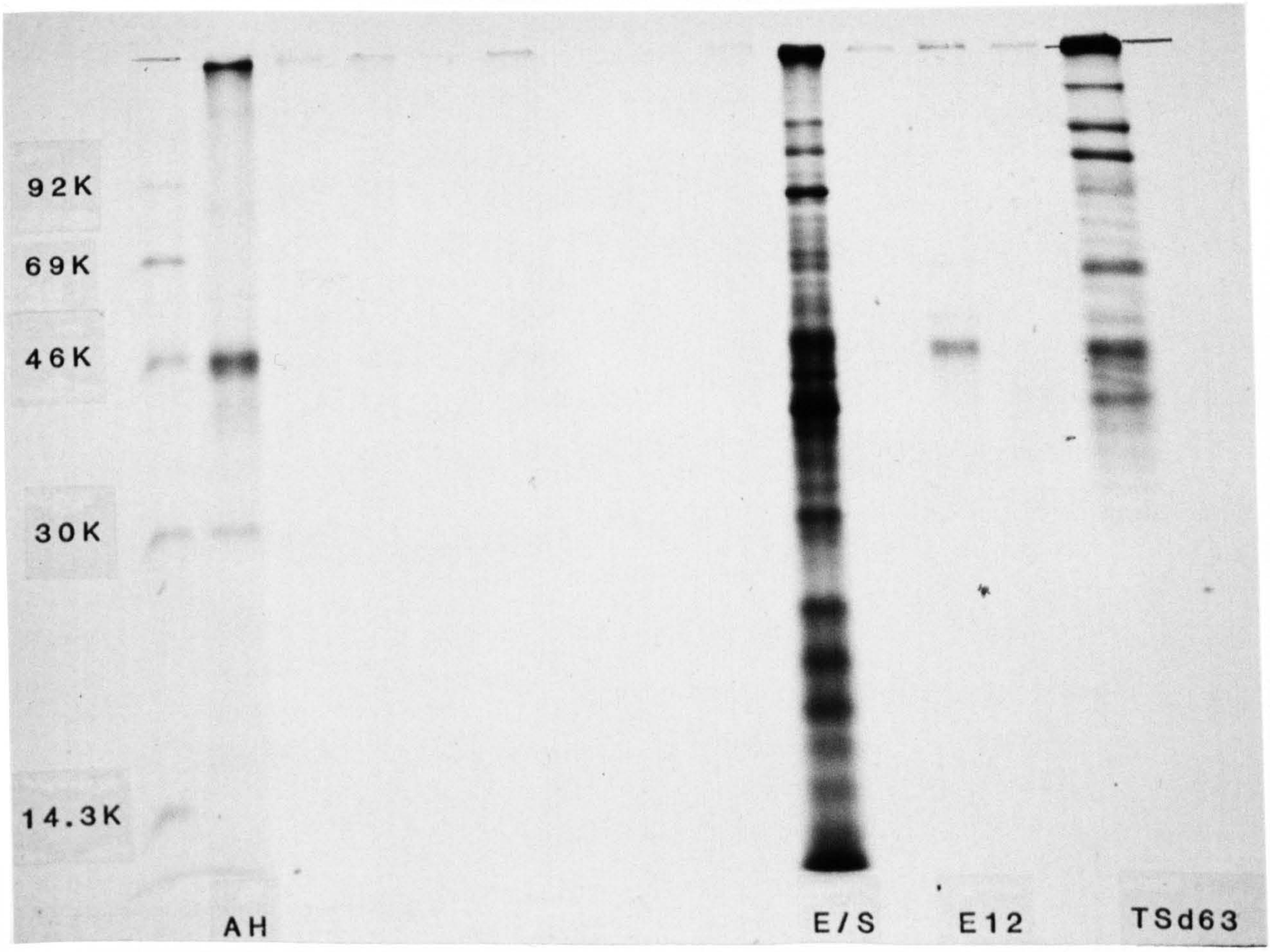
IMMUNOPRECIPITATION OF ^{35}S-METHIONINE LABELLED T. MURIS AH AND E/S ANTIGENS 15% gels

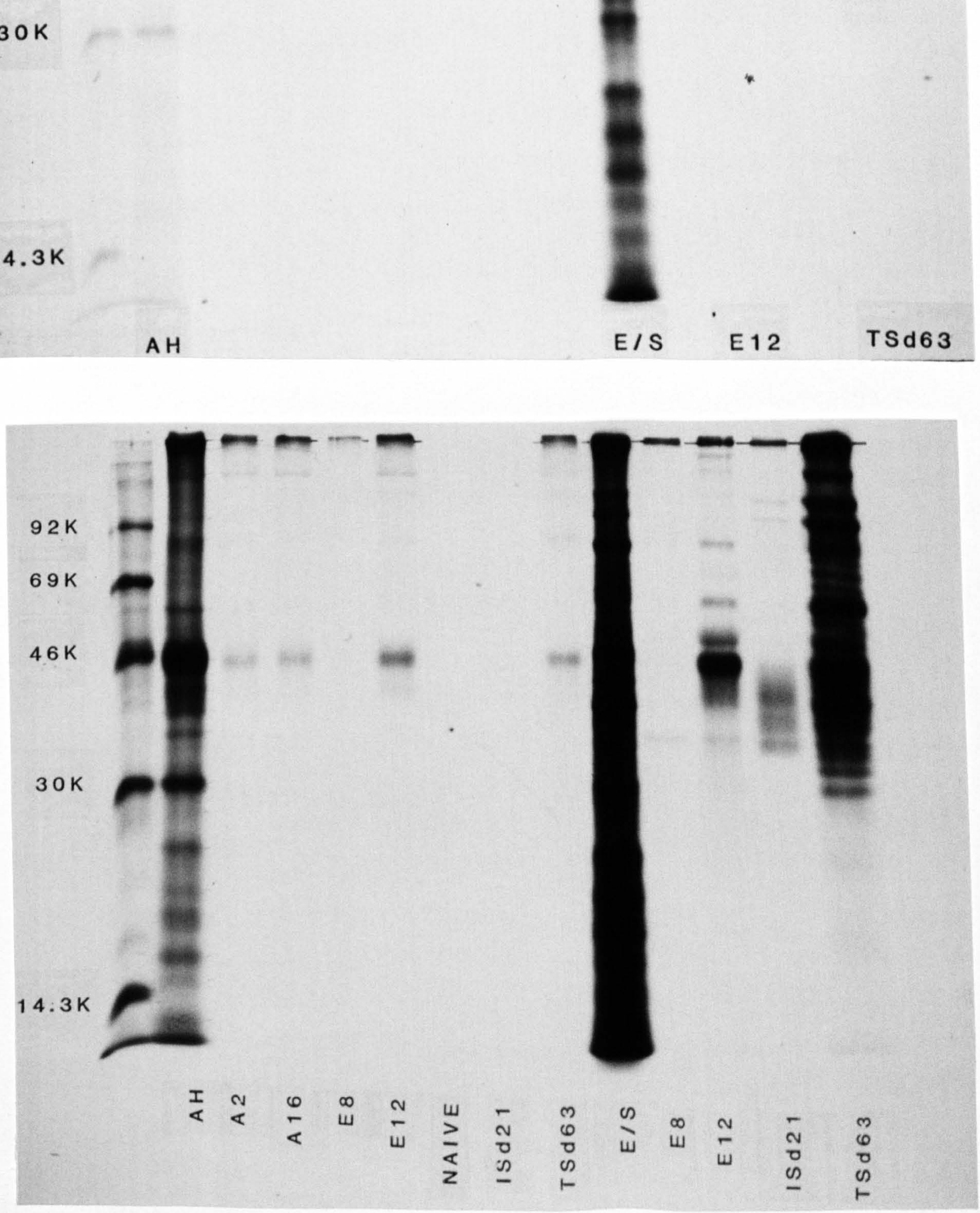
FIGURE 6.1

AH - adult homogenate (not precipitated)
E/S - E/S products (not precipitated)
E12 - IgG_1 MoAb + E/S

FIGURE 6.2

AH - adult homogenate (not precipitated)
A2 - IgA MoAb + AH
A16 - IgA MoAb + AH
E8 - IgM MoAb + AH
E12 - IgG_1 MoAb + AH
NAIVE - naive mouse serum + AH
Isd21 - day 21 infection serum + AH
TSd63 - day 63 tolerant serum + AH
E/S - E/S products (not precipitated)
E/8 - IgM MoAb + E/S
E12 - IgG_1 MoAb + E/S
ISd21 - day 21 infection serum + E/S
Tsd63 - day 63 tolerant serum + E/S


92K
69K
46K
30K
14.3K
AH
E/S
E12
TSd63


92K
69K
46K
30K
14.3K
AH
A2
A16
E8
E12
NAIVE
ISd21
TSd63
E/S
E8
E12
ISd21
TSd63

IMMUNOPRECIPITATION OF ^{35}S-METHIONINE LABELLED T. MURIS E/S ANTIGENS 15% gels

FIGURE 6.3

F5 - IgA MoAb + E/S

A16 - IgA MoAb + E/S

E51 - IgG_1 MoAb + E/S

E/S - E/S products (not precipitated)

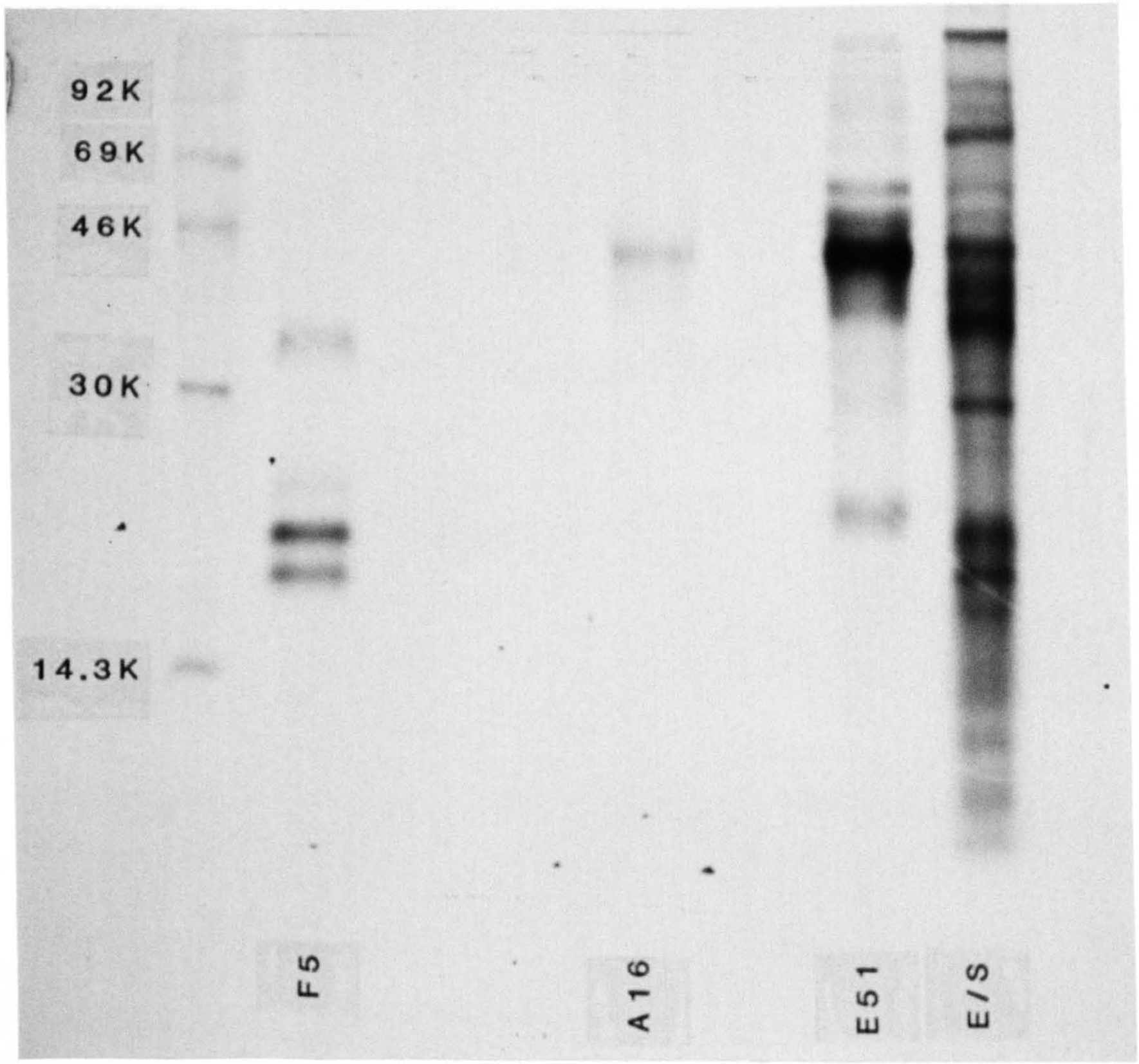
FIGURE 6.4

E/S - E/S products (not precipitated)

F11 - IgA MoAb + E/S

F5 - IgA MoAb + E/S

A16 - IgA MoAb + E/S


92K
69K
46K
30K
14.3K
F5
A16
E51
E/S

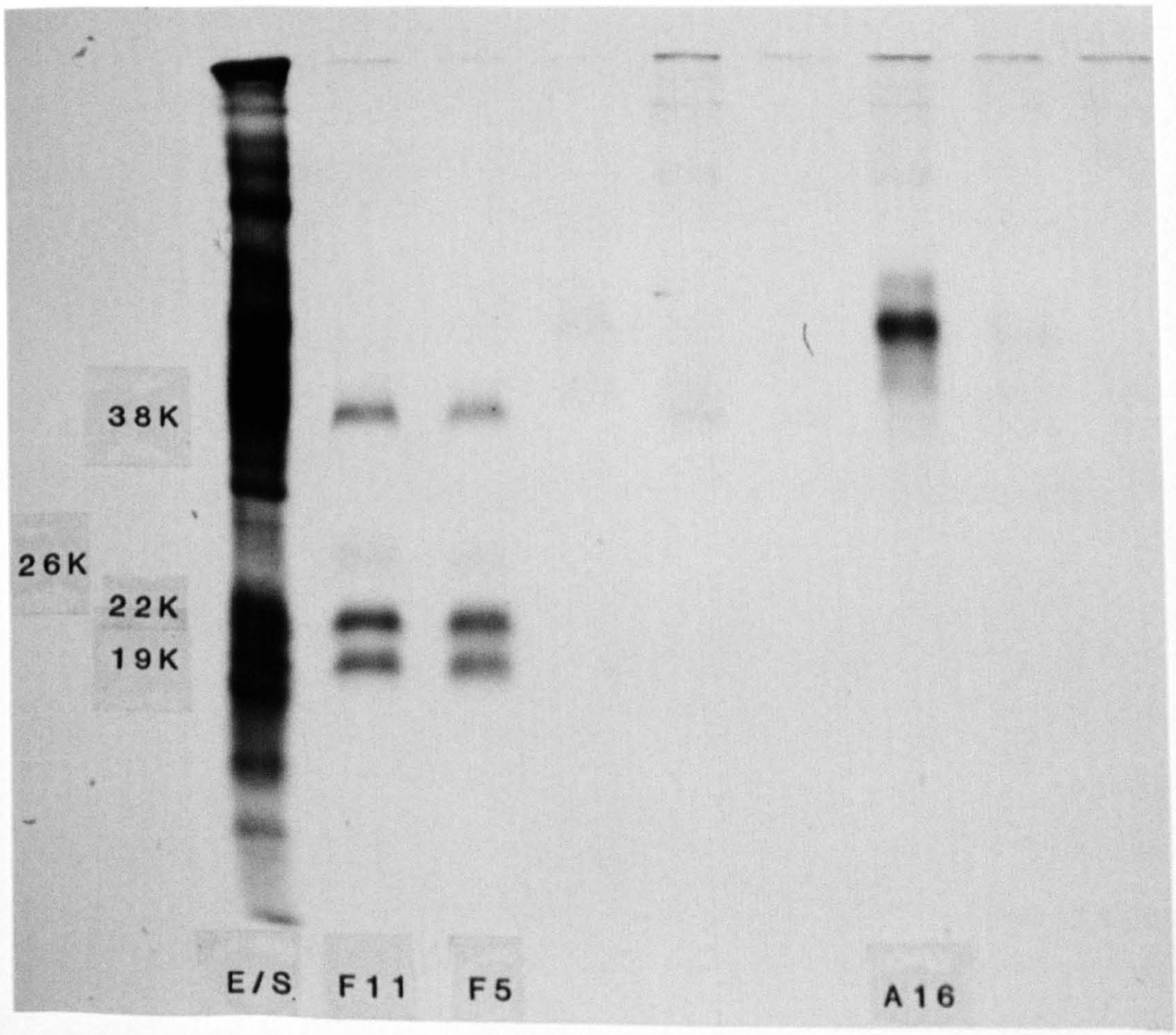

38K
26K
22K
19K
E/S
F11
F5
A16

TABLE 6.5 ANTIGEN SPECIFICITIES OF ANTI- T. MURIS MoAbs

^{35}S-methionine labelled E/S products (major proteins)	Immunoprecipitation										Western Blotting (using reduced E/S products)				
	Isd21	E8	E12	E15	E51	A2	A16	F5	F11	C7	E8	E12	E15	E51	A16
			(136)	129											
			(128)	125		123									
111	(109)		111			111	111								
99	(99)														
			88												
83				82											
68											68				
			65												
62				61											
				56	55						(55)	55	55	55	
			50	48											
48															
	45		45		46	45	45				44	45	45	45	45
43	42				43					(43)					
			40			40	40								
39										(38)					
	38														
37				37											
36		36	36												
	35			35				34	34						
32															
29															
24															
								(22)	(22)						
21					(21)										
			(19)					20	20						
18			18	18				18	18						
				17											
16															
15															
13															

M.wts. in kD

FIGURE 6.6 PASSIVE TRANSFER OF IMMUNITY TO INFECTION WITH T. MURIS IN CBA/Ca MICE USING MoAbs PRODUCED IN VITRO

■ Control animals given P3NS1 supernatant or HMT medium with 10% FCS

□ Test animals given hybridoma supernatants containing MoAbs :

E12, E15, E51 - IgG_1

C7, F5 F11 - IgA

E8 - IgM

All hybridomas were grown in HMT medium with 5-10% FCS, myelomas (P3NS1) were grown in RPMI 1640 with 5% FCS

lane		
1	P3NS1	
2	E15	(IgG_1)
3	E12	(IgG_1)
4	E8	(IgM)
5	P3NS1	
6	HMT	
7	F5	(IgA)
8	F11	(IgA)
9	C7	(IgA)
10	E51	(IgG_1)

Standard errors are shown on the histogram. According to Student's t test the larval worm burdens are significantly reduced compared with control infections when F5 and F11 IgA MoAbs were given to the mice $p < 0.01$ and $p < 0.05$ respectively.

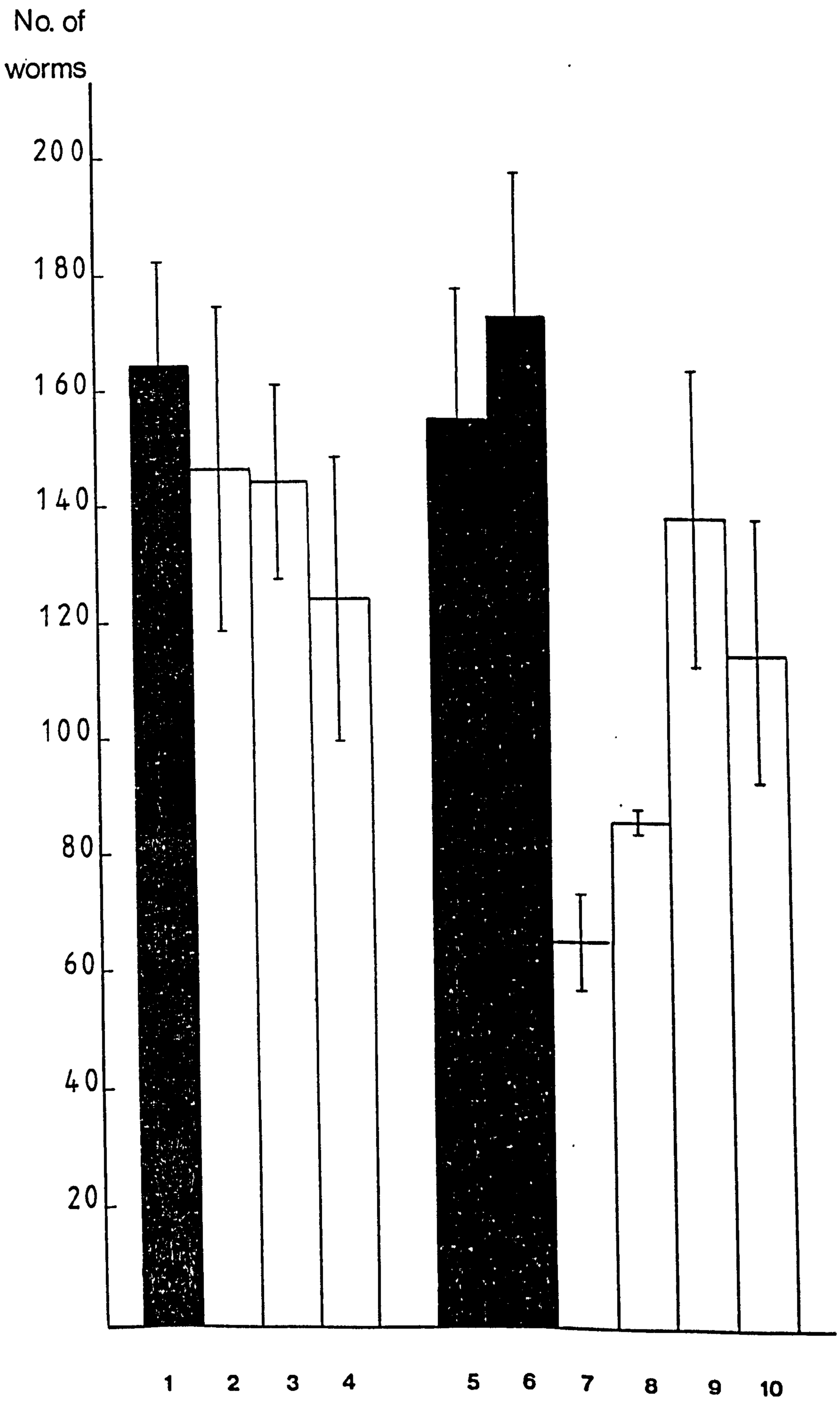
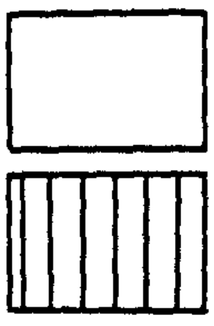

No. of worms
200
180
160
140
120
100
80
60
40
20
1
2
3
4
5
6
7
8
9
10

FIGURE 6.7 ACTIVE IMMUNIZATION AGAINST T. MURIS INFECTION IN CBA/Ca AND NIH MICE USING F11 AND E12 MoAb AFFINITY ISOLATED ANTIGENS



NIH mice

CBA/Ca mice

lane	
1	E12 Ag
2	PBS (-ve control)
3	E/S products (+ ve control)
4	E12 Ag
5	PBS
6	E/S products
7	F11 Ag
8	PBS
9	E/S products

The results were analysed by Students t test. In the CBA/Ca mice given F11 Ag larval worm burdens were significantly less than those of the corresponding PBS control ($p < 0.05$), as were those of the mice immunized with total E/S products ($p < 0.01$). However, the larval worm burdens of CBA/Ca mice immunized with E12 Ag were not reduced. The latter mice appeared to have larval worm burdens significantly above those of the PBS control group, however, the worm burdens of the corresponding mice immunized with E/S products did not have significantly reduced worm burdens. Although active immunization in NIH mice with E/S and E12 antigens reduced larval worm burdens compared to the corresponding PBS control group, the reduction was only significant ($p < 0.1$) with the E/S products.

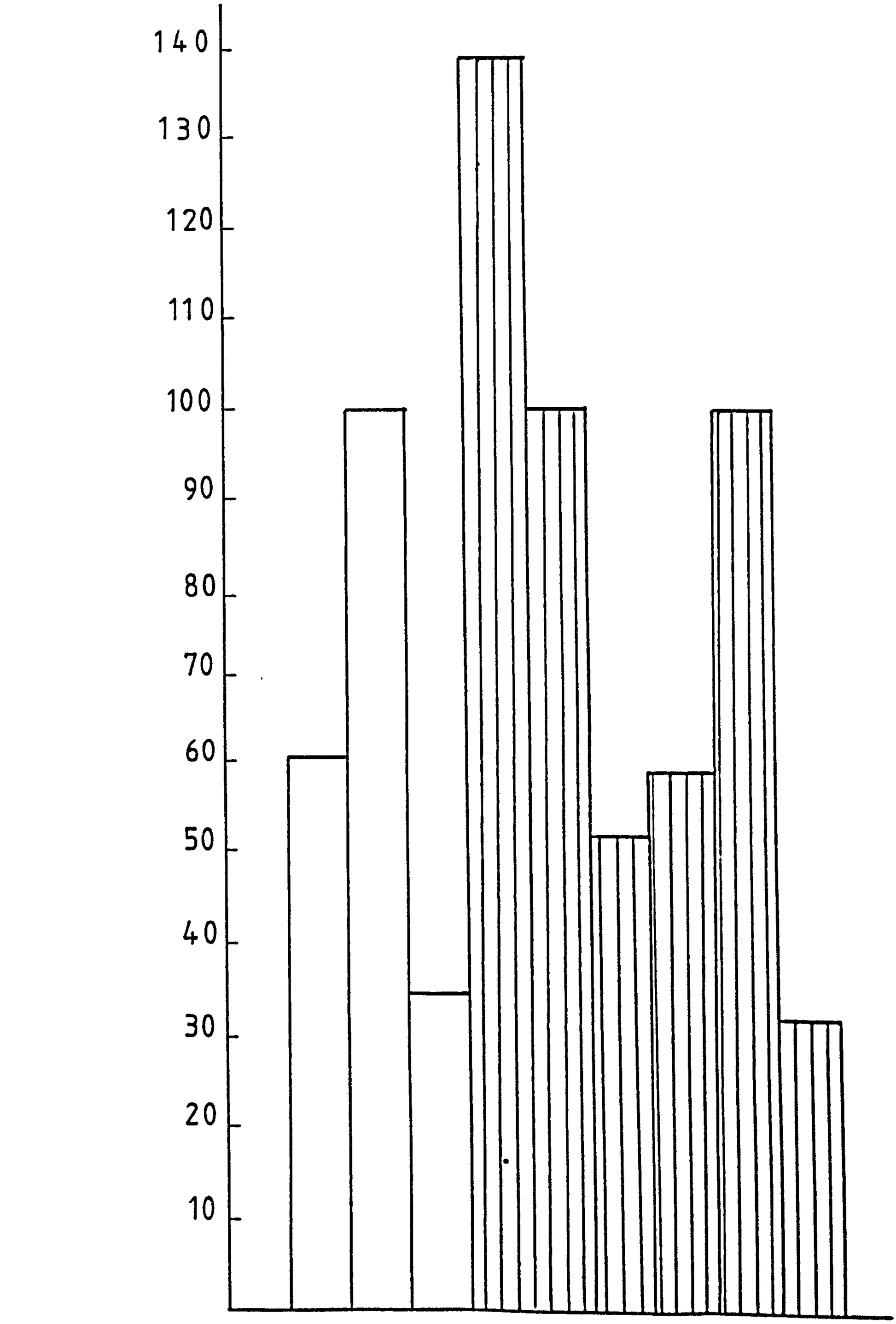

No. of worms
as % of control
140
130
120
110
100
90
80
70
60
50
40
30
20
10
1
2
3
4
5
6
7
8
9

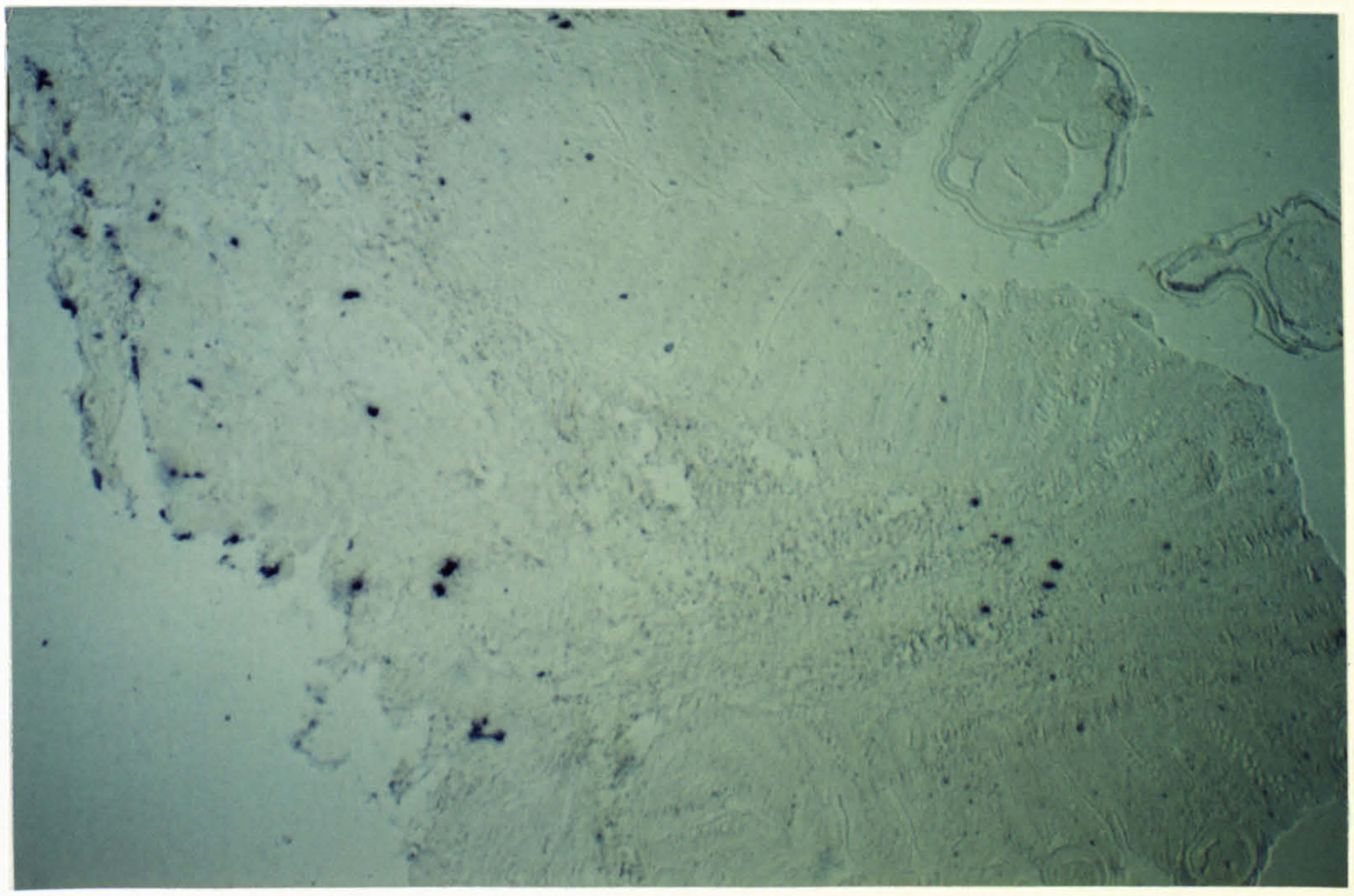
FIGURE 6.8 AVIDIN/BIOTIN IMMUNOPEROXIDASE STAINING OF HISTOLOGICAL SECTIONS OF T. MURIS IN SITU IN THE LARGE INTESTINE

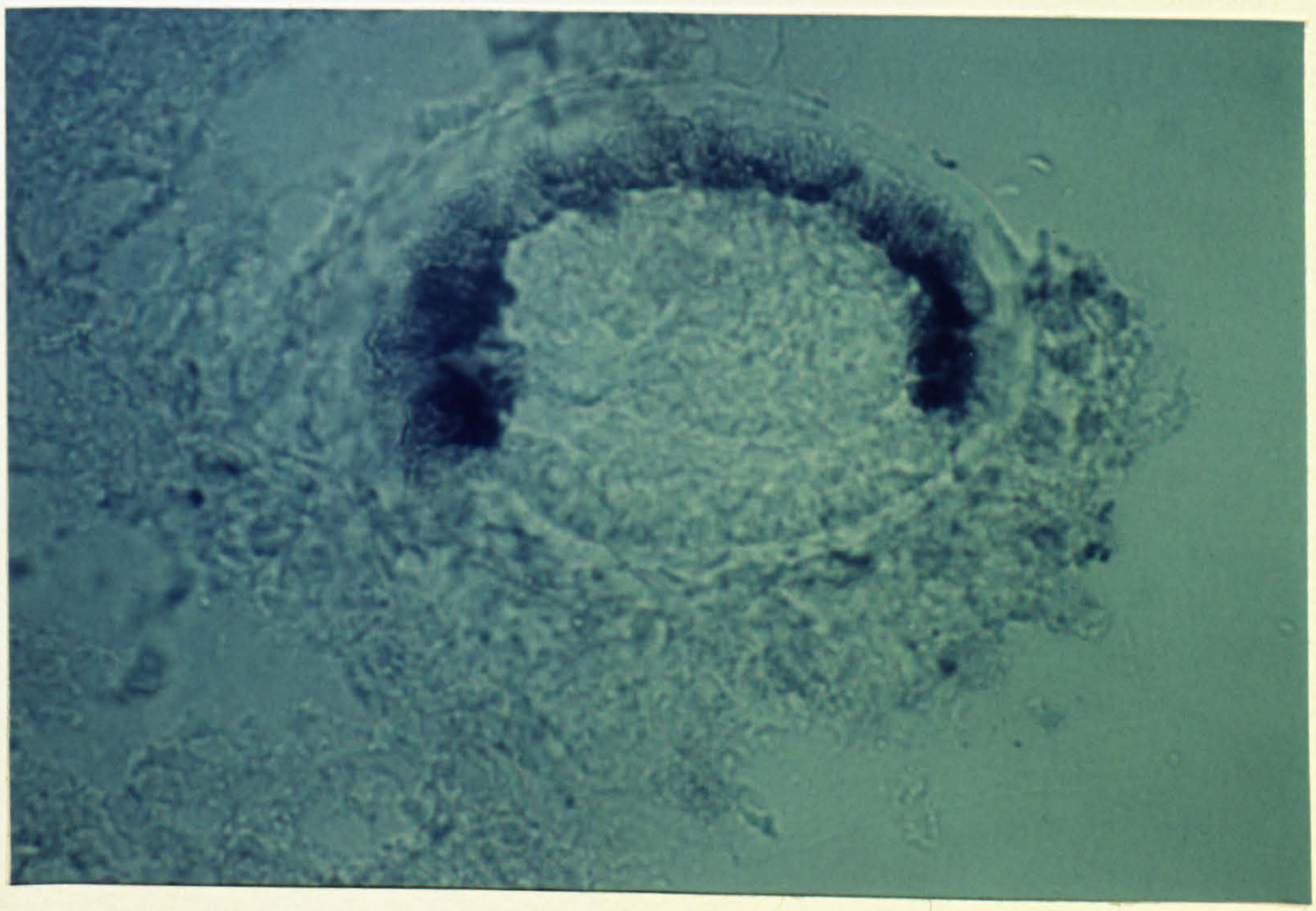
TOP

MMC of gut and bacillary bands of T. muris (on right) stained purple/blue with chloronapthol

BOTTOM

Bacillary band of T. muris stained purple/blue with chloronapthol.





DISCUSSION

The anti- T. muris MoAbs all precipitated a number of proteins from ^{35}S-methionine labelled E/S products. E12, E15 and E51 MoAbs gave the largest number of reduced protein bands on the autoradiographs, the major antigens recognized had approximate m.wts. of 88/82, 65/61, 56/50, 48/43, 35, 18 and 17 kD. In contrast, E8, A2 and A16 precipitated only 36 kD and 111, 45 and 40 kD proteins respectively. There appear to be many E/S proteins with similar m.wts. in the region 30 to 46 kD and it should be born in mind that the apparent m.wts. referred to may only be accurate to within 5 or 10 kD. Several hypotheses may be put forward to explain the apparently multispecific recognition of antigens by the anti- T. muris MoAbs. Many of the E/S antigens may share common epitopes, a situation which may be likened to that reported for Trichinella spiralis infective larvae surface antigens, which are thought to differ in being monomeric or dimeric, glycosylated or non-glycosylated, but sharing most of a common polypeptide "backbone" of antigen (Parkhouse et al., 1981; Clark et al., 1982). Indeed, Ortega-Pierres et al. (1984a) isolated a MoAb (NIM-M1) which precipitated all four surface-labelled molecules of the infective larvae. It is also possible that the anti- T. muris MoAbs cross-react with dissimilar epitopes. Ghosh and Campbell (1986) discussed the frequent cross-reactions observed with MoAbs and attributed them to either partial epitope identity or to irrelevant interactions involving additional binding capacity of the antibody. The majority of such interactions were found to fall into the latter category and were most commonly detected when one of the antigens had a high epitope density and the antibody was multivalent. Hence a spurious interaction of low intrinsic affinity was

amplified by local concentration effects. The E12, E15 and E51 group and the A2 and A16 group of MoAbs both appear to recognize the same major antigen with an apparent m.wt. in the range 43 to 48 kD, although the two groups of antibodies have different overall antigen specificities. Presumably the MoAbs recognized different epitopes on this antigen, which differed in their occurrence on other E/S antigens.

There were differences in the antigen specificities of MoAbs as determined by immunoprecipitation of labelled antigen and western blot analyses. In the western blots reduced proteins were transferred to nitrocellulose paper which was then probed with the MoAbs, both the reduction process and/or the binding of proteins to the nitrocellulose could have altered the configuration or availability of epitopes for the binding of antibody. In contrast, in immunoprecipitation experiments the labelled E/S antigens were not reduced until after their isolation using the MoAbs. The two techniques also differed in their sensitivity. In western blotting the emphasis was on the detection of antibodies binding to cumulative E/S proteins (collected during in vitro culture of worms) which were transferred to nitrocellulose paper after PAGE. However, with immunoprecipitation analyses the ability of antibodies to precipitate newly synthesized antigens, labelled during in vitro incubation with ^{35}S-methionine, was investigated, even proteins present in minute quantities were thus detectable if they bore specific epitopes.

The antigen specificities of the F5 and F11 MoAbs were distinct from those of the other MoAbs. These MoAbs precipitated a group of low m.wt. antigens of 34, 20 and 18 kD with another protein band faintly visible with apparent wt. of 22 kD. The F5 and F11 hybridoma

supernatants containing these IgA MoAbs gave the only significant successful results in the initial passive transfer experiments. Whether their effectiveness in passive transfer was because of the antigen specificities of these MoAbs and/or their IgA isotype cannot be determined at this stage. The A2 and A16 IgA MoAbs were also found to be successful in passive transfer of immunity on one occasion but this result was not repeated in subsequent experiments. Passive transfer of immunity with the MoAbs produced has not yet been thoroughly investigated and the failures observed in initial experiments could be the result of an insufficient amount of antibody being transferred. There appears to be a threshold concentration of specific antibody which must be attained for successful passive transfer of immunity against T. muris, albeit the "threshold" may vary depending on the strain of mouse and the antibody used. However, once approximate "threshold" concentrations have been determined for the experimental system being used and the MoAbs are produced in sufficient quantities, standardization of passive transfer experiments should be possible. Further investigations are also needed on the efficacy in the passive transfer of immunity of MoAbs with different isotypes, which recognize the same antigens, ie. a comparison of E12, E15 and E51 (IgG_1) to A2 and A16 (IgA), both groups recognizing an antigen of apparent m.wt. 43 to 48 kD. If the passive transfer of immunity to T. muris using IgA F5 and F11 MoAbs can be confirmed this would be the first report of passive transfer of immunity to gut nematodes using IgA antibodies.

There are some excellent recent reviews on the mucosal immune response to parasite infection (Befus and Bienenstock, 1982; Miller, 1984; Allardyce and Bienenstock, 1984) and indeed the role of IgA in particular in mucosal immunity (see Newby, 1984; Underdown and Schiff,

1986). Designing in vivo experiments to investigate the function of IgA in immune responses against gastrointestinal parasite infections is difficult. In passive transfer experiments whether antibody reaches the gut and directly affects parasite survival cannot readily be determined, and although there have been a number of demonstrations that recovery from mucosal infection parallels the timing and magnitude of the secretory antibody response they do not provide insight as to whether IgA participates in curing the host. In passive transfer intravenously injected IgA could not be expected to provide direct mucosal protection except in the upper bowel of animals with an efficient hepatobilary transport pathway (rats, rabbits and chickens), because of the limited access from blood to the mucosa. In most experiments involving transfer of immunity against gut nematode infections whole serum has been given i.p., and some degree of protection against virtually every laboratory model of nematode infections has been achieved (Wakelin, 1978). Passive protection against Nippostrongylus brasiliensis in rats has been shown to be active against the luminal dwelling adults (Miller, 1980), but protective antibodies generated by surgically transplanted adult worms in serum donors, as well as protective antibodies derived from larval infections, were predominantly IgG_1 (Jones et al., 1970). Lethal irradiation of recipient rats abrogated passive protection with immune serum, and it was suggested that antibody per se did not cause expulsion but was instrumental in inducing morphological damage and reduced fecundity of worms. Repeated or drug abbreviated infections in mice stimulated high levels of protective antibodies against Nematospiroides dubius (Dobson, 1982; Williams and Behnke, 1983) and Pritchard et al. (1983) demonstrated that the activity of such serum was exclusively associated with IgG_1. The histotrophic larvae of N. dubius were most susceptible

to the serum antibodies (Behnke and Parish, 1981) although effects on adults (male/female ratios, fecundity and stunting) were all correlated with quantity and quality of serum given and the antibody titres of the passively protected mice (Dobson, 1982). In the Trichuris muris/mouse system the adult worm anterior ends are embedded within the mucosal epithelium and the larvae, against which passive transfer with immune serum is effective, are completely embedded in the epithelial sheet of the large intestine up to day 15-20 post infection. This intimate association of T. muris with its host, compared to that of entirely luminal dwelling nematodes, is believed to render these worms more susceptible to the effector mechanisms of the host immune response, but as yet no particular isotype of immunoglobulin has been implicated as the one with major responsibility for the success of passive transfer.

IgA is the major isotype of immunoglobulin secreted locally in the gastrointestinal tract, at least in monogastric animals, but has not been shown directly to affect nematode survival in the gut. Increases in mucosal IgA have been observed during infection with Nippostrongylus brasiliensis in rats (Poulain et al., 1976; Sinski and Holmes, 1977) and Nematospiroides dubius in mice (Cypess et al., 1977), indeed, IgA was thought to be part of the expulsion effector mechanism against Nippostrongylus brasiliensis adult worms; protection against Haemonchus contortus challenge infections in vaccinated sheep was also associated with raised abomasal mucus IgA levels (Duncan et al., 1978). Involvement of IgA producing lymphoblasts in the expulsion of adult Trichinella spiralis from rats has been suggested (Despommier et al., 1977), but this has not been shown to be the case in mice (Wakelin and Wilson, 1979). However, Almond and Parkhouse (1986) did find a close temporal correlation between the production of

serum IgA antibodies against the surface components of adult worms in resistant NIH mice and the accelerated expulsion of this stage of the parasite from the gastrointestinal tract. It should be borne in mind that Serum IgG may often be found in intestinal secretions, albeit this isotype does not have the ability of IgA to resist proteolysis, and considerable amounts of IgG_1 may be secreted locally in ruminants (Miller, 1984).

The prime biological function of IgA is to protect the host from invading organisms that initiate their assault from outside the mucosal surface. The means by which IgA antibodies carry out this task are the subject of much discussion, but briefly IgA has been implicated in the inhibition of antigen uptake, bacterial adherence to epithelia, complement activation and deposition and neutrophil chemotaxis; it acts as a blocking antibody, neutralizes toxins and viruses and resists proteolysis (reviewed by Befus and Bienenstock, 1982). In addition neutrophils, macrophages and lymphocytes have all been demonstrated to express surface receptors for IgA. Although IgA has been found to inhibit neutrophil activities such as bactericidal activity and perhaps phagocytosis, IgA dependent monocyte mediated antibacterial activity has been demonstrated. The functions of the reported IgA Fc receptor bearing lymphocytes, some of which were T cells, some B cells and others non-T and non-B distinguishable lymphocytes have not been clearly elucidated, but the cells tend to be more common in mucosal tissues. The alteration of immunoglobulin levels especially IgA and the presence of specific antibodies in serum or secretions of animals infected with parasites have been investigated, as mentioned previously, as has the presence of IgA and other cytoplasmic immunoglobulin positive cells in intestinal tissues, draining lymph nodes and the thoracic duct (reviewed by Befus and Bienenstock,

1982; Miller, 1984). Such observations have indicated that the IgA system is activated in many intestinal nematode infections but an evaluation of the role of the IgA system based on the studies performed has been difficult. However, Befus and Bienenstock (1982) suggested that IgA antibody is relevant in the control of the uptake of parasite antigens, their clearance from the circulation and in the modulation of the activities of various cells in the inflammatory events associated with infections. The latter may involve the enhancement of cytotoxic functions or the minimization of potentially pathologic reactions. Nonetheless, many Fc-mediated effector mechanisms are not available in secretions, where IgA mainly appears to resist proteolytic attack of invading organisms such as bacteria and prevent their attachment to the mucosal wall; most potential pathogens being disarmed by non-specific factors in secretions. Although IgA may limit the systemic immune response during times of health by clearing antigens from the blood, it is less than certain whether IgA plays a role in limiting the course of a major infection once underway (Underdown and Schiff, 1986). Trichuris muris has more intimate contact with its host than many other entirely luminal dwelling gut nematodes, indeed, its attachment site within the epithelial cells may render it more susceptible to immune effector mechanisms. Whether IgA is of particular importance either directly or indirectly in the expulsion of worms from the gut may only be determined by further investigations, in which the anti- T. muris IgA MoAbs produced should be extremely useful.

In order to determine which E/S antigens were indeed functional in the stimulation of a protective immune response the antigens recognized by the MoAbs were isolated by affinity chromatography and examined in active immunization experiments. Preliminary investi-

gations were made with F11 and E12 affinity isolated antigens, m.wts. of the major reduced proteins present (identified by silver staining after PAGE) were 33 and 21 kD and 48, 41 and 28 kD respectively. Affinity isolation of the antigens was time consuming and only small amounts of protein were available for the initial immunization experments (~ 1 µg per mouse). Silberstein and Despommier (1984) found that 0.1 to 1 µg of 48 kD and 50/55 kD stichocyte antigens were effective in active immunization against Trichinella spiralis. These small quantities of antigens were referred to as "biologically relevant" doses, ie. based on the amount and rate at which material was released from infective larvae in vitro, the quantity of specific antigen which would be released into the host with a particular level of infection was calculated. Only tentative suggestions as to the potency of the E12 and F11 antigen preparations can be made on the basis of the preliminary experiments reported here. The F11 Ag (33 and 21 kD proteins) did appear to be effective in active immunization in CBA/Ca mice, although the E12 Ag (48, 41 and 28 kD proteins)did not significantly reduce worm burdens in CBA/Ca or NIH mice some NIH mice in the test group did have markedly reduced worm burdens. It appears that the effectiveness of the antigens in active immunization may vary according to the strain of mouse used. In addition, minimal doses of antigens effective in active immunization were not determined, and possibly either antigen used at a higher dose may give protection against subsequent infection. Until these "threshold" quantities of antigens effective in active immunization in the different strains of mice have been determined those functional antigens which elicit a protective immune response in the T. muris/ mouse system cannot be identified.

Attempts to localize the antigens recognized by the MoAbs in

T. muris by immunoperoxidase staining techniques have so far been unsuccessful. The techniques used may have been lacking in sensitivity and/or the important antigens may have been destroyed by the fixation process used in the preparation of the histological sections. Localization experiments need to be attempted using cryostat sections of nematodes and perhaps higher density staining techniques such as avidin-biotin-peroxidase complex (ABC) (Hsu, Raine and Fanger, 1981). The ABC method involves an application of biotin-labelled secondary antibody followed by the addition of avidin-biotin-peroxidase complex, the availability of binding sites on the complex being created by the incubation of a relative excess of avidin with biotin-labelled peroxidase. A 'lattice' complex is formed containing several peroxidase molecules and binding of this complex to the biotin moieties associated with secondary antibody results in high staining intensity. Interestingly, avidin (a basic glycoprotein m.wt. 68 kD found in egg white) was observed to bind to mucosal mast cells (MMC) and T. muris bacillary band with the biotin-avidin-horseradish peroxidase staining technique used (Figures 6.9 and 6.10). Tharp et al. (1985) reported that conjugated avidin bound to connective tissue mast cells (CTMC) in both tissues and cell suspensions. In the absence of prior fixation, however, the mast cells were not identified with the conjugated avidin, although granules released from these cells were stained with this labelled glycoprotein. Electron microscopic studies confirmed that the avidin bound specifically to the individual mast cell granules rather than to other cellular structures and both rodent and human CTMC were readily stained with avidin conjugated to horseradish peroxidase. The observations reported here as regards MMC binding of avidin-peroxidase, suggest that the mast cell granules of MMC have similar avidin binding properties to those of CTMC. Tharp et al.

(loc. cit.) found conjugated avidin staining to be a reliable histochemical method for enumerating CTMC in both tissues and cell suspensions; it appears that this technique could also be used for the identification of MMC. Finally, if localization of T. muris E/S antigens recognized by specific MoAb in these nematodes is not successful using cryostat sections and high density staining techniques, it is possible that the antigens concerned only bind the MoAbs after excretion or secretion from the parasite. The specific binding epitopes may not be present or may be unavailable for binding on the antigens present in the worm tissues.

CHAPTER 7

ANTIGENS OF TRICHURIS MURIS

INTRODUCTION

In the search for target antigens, which elicit a protective immune response in the host, studies in recent years have concentrated on the so-called viable host/parasite interfaces, ie. surface and excretory/secretory antigens. Studies of antibody-mediated cell adherence to parasites (reviewed by Ogilvie et al., 1980; McLaren, 1980) brought to the forefront the importance of the nematode surface as a target of functional immune responses. The concept that the acellular multilayered cuticle was an inert covering providing little immune responsiveness was finally dismissed, as the surfaces of these parasites were analysed in far greater detail (reviewed by Maizels et al., 1982). The release of material is also an important stimulus for the initiation of host immunity to large extracellular parasites. The majority of antigens must be presented to lymphoid cells in the context of the host's MHC gene products to stimulate an immune response, and thus molecules released by the parasite are more likely to be available for processing by the antigen presenting cells than are bound, surface molecules. To date active immunization against a nematode using isolated surface proteins has been achieved only with Trichinella spiralis in the mouse (Grencis et al., 1986). In these experiments surface proteins were stripped from the cuticle of live muscle larvae using the cationic detergent cetyltrimethylammonium bromide (CTAB). However, immunoprecipitation experiments confirmed that the surface of the muscle larvae shared antigenic epitopes with antigens contained within and secreted by the stichosome of the worm. Thus, with this system, as with others, the distinction between "surface" and "secreted" antigens is blurred. Although the majority of parasites are known to elicit antibody responses to their surface

antigens, no other workers have yet isolated preparations of purified surface antigens which have been successfully used in active immunization against a subsequent infection. In contrast E/S products have been successfully used in active immunization with several nematodes, eg. Trichuris muris, Trichinella spiralis and Ancylostoma caninum (Jenkins and Wakelin, 1977; Silberstein and Despommier, 1984; Thorsen, 1956b; reviewed by Almond and Parkhouse, 1985). E/S products even in crude form tend to be mixtures of relatively few antigens compared to worm homogenates (De Savigny, 1975; Jenkins and Wakelin, 1977; Crandall and Zam, 1968) thus simplifying any purification steps which may be necessary, and facilitating standardization of antigens. E/S products also usually contain biologically relevant and functional antigens, hence their species specificity tends to be higher (Fife, 1971). Despite this, in most cases the E/S products are still a relatively heterogeneous mixture of antigens, as was the aforementioned CTAB preparation of Trichinella spiralis surface antigens; few workers have actually identified or purified parasite molecules which elicit a protective immune response. Indeed, Silberstein and Despommier (1984) have the only recorded success to-date; they isolated 48 kD and 50/55 kD glycoproteins from the stichosome of the infective L_1 larvae of Trichinella spiralis and the 48 kD protein in particular was highly potent when used in active immunization.

Many workers have made a clear distinction between surface and secreted antigens but, as has been shown with T. spiralis (Philipp.et al., 1980; Parkhouse et al., 1981; Silberstein and Despommier, 1984; Grencis et al., 1986), Toxocara canis (Maizels et al., 1984) and Nippostrongylus brasiliensis (Maizels et al., 1983) this division is by no means exclusive. Evidence that antigens of nematodes presumed to be of surface origin may be shed from the cuticle in in vitro

culture was first provided by Vetter and Klaver-Wesseling (1978), using ex-sheathed infective larvae of Ancylostoma caninum. The larvae were kept at either 0°C or 20°C in the presence of sodium azide, incubated with serum from infected dogs and fluoroscein isothiocyanate labelled anti-dog IgG immunoglobulin. While the larvae remained at a low temperature they showed an intense fluorescence, but fluorescent particles were shed into the culture fluid when the temperature was raised to 20°C or the azide was not present. Eventually all fluorescence was released from the surface of the worms. Smith et al. (1981) also observed temperature dependent and anti-metabolite sensitive in vitro release of antibodies bound to the surface of second stage larvae of Toxocara canis. As the antibodies used in the experiment had been raised to larval E/S components, this suggested the presence of surface antigens in the E/S material. Iodinated culture fluids of infective larvae of Trichinella spiralis have been shown to contain Ags also present on the larval surface (Parkhouse et al., 1981) and, conversely, surface iodinated larvae and adult worms released radiolabelled surface antigens into the culture medium (Philipp et al., 1980). A significant proportion of iodinated material (10 - 25%) was released into the culture medium over 24h, the presence of serum doubled the rate of release, and a neutrophil-rich cell population raised the shedding of labelled proteins to 50-70%. If it is accepted that the radiolabelling procedure did not affect surface protein stability, the implication from these experiments is that surface antigens are turned over on the cuticle and released into the environment in vivo. Indeed, soluble products (whether secreted or released) may play a crucial role in the parasite's capacity to modulate or escape the mechanisms of host immunity (Svet-Moldavsky et al., 1970; Faubert, 1976, 1982; Behnke

et al., 1983; Pritchard et al., 1984; Piessens et al., 1986). There is also a very practical importance attached to the study of excretory/secretory antigens, their presence in the host's circulation may be a far more reliable indicator of infection than any test for circulating antibody in the host.

The relatively recent development of techniques using radioisotopes in the labelling of worm proteins has provided much more detailed information as regards the identification of surface and E/S antigens. The major advantage of using radioisotopes is the amplification achieved when visualising antigens using autoradiography, hence, paucity of material is no longer a serious disadvantage. Surface and secretory antigens have been identified for the different life cycle stages of several nematodes (reviewed by Maizels, 1982; Philipp and Rumjaneck, 1984; Almond and Parkhouse, 1985), and labelled antigens have been used as probes to assay humoral immunity in infected hosts. For many purposes the selective labelling of molecules combined with radiometric assays, circumvents the need for antigen purification. Trichinella spiralis, in particular, has been extensively studied in this way (Philipp et al., 1980; Parkhouse et al., 1981), and remarkable stage specificity of the surface proteins of each stage of T. spiralis was demonstrated using standard cell surface radiolabelling methods. The E/S products of T. spiralis have also been metabolically labelled in in vitro cultures using ^{35}S-methionine, and again produced unique stage-specific profiles (Parkhouse and Clark, 1983). This might be expected as the exposure of stage-specific surface proteins reflects changes in internal metabolism. It could be that the expression of stage-specific surface antigens allows this parasite to survive the course of a primary infection by "keeping ahead" of the immune response that

rapidly eliminates a secondary infection.

Surface proteins of nematodes are readily radiolabelled using techniques originally developed for labelling proteins in solution and at mammalian cell surfaces, despite the fact that the nematode cuticle is very different from the mammalian cell membrane. The methodology used to label surface proteins of helminths has been recently reviewed by Philipp and Rumjaneck (1984). Direct evidence that radioactive iodine is exclusively associated with the cuticle after iodination can be obtained by viewing autoradiographs of sections of radiolabelled worms under the electron microscope. However, published evidence as regards the localization of the I^{125} radiolabel after using Bolton Hunter reagent, lactoperoxidase catalysed or chloramine-T-mediated iodination is limited for some of the techniques. It is advisable to examine afresh the merits of a given iodine labelling technique with each new nematode species or developmental stage under investigation. IODOGEN is the most recently developed reagent for protein iodination, and has been shown to be surface restricted when used to label cells and various organisms. Evidence of cuticular localization with nematodes has been provided in the case of Onchocerca gibsoni (Forsyth et al., 1981) and adult worms and L_3 of Dipetalonema viteae (Bashong and Rudin, 1982). The great diversity of helminth surfaces that can be selectively labelled with this reagent, the high specific activity of the products, the mildness of the oxidative reaction involved and the technical simplicity of the procedure, make it the most convenient of the methods of surface iodination used so far.

An important technical point, as regards the surface labelling techniques involving iodination is that there is a bias towards polypeptides containing lysine and tyrosine residues. Polysaccharide and

glycolipid antigens, for example, may escape labelling altogether and consequently be excluded from any analyses performed. This is thought to be a possible explanation for the occasional difficulties encountered in attempts to identify antigens from worms known to be immunogenic. In the case of the infective larvae of Nippostrongylus brasiliensis, antibody to this stage of the parasite is known to be a potent mediator of cell adherence to the surface (Mackenzie et al., 1980), however, these antibodies reacted poorly with surface labelled preparations (see Maizels et al., 1982). It is possible to label surface glycoproteins and glycolipids, but the procedure is relatively complex and there is only one recorded attempt to systematically characterize these molecules in helminths so far (Samuelson and Caulfield, 1982).

The technique for metabolically labelling proteins using ^{35}S-methionine was also originally adapted from work involving in vitro cell culture, and has been used successfully to identify the E/S products of a number of nematodes eg. Trichinella spiralis, Toxocara canis and Brugia pahangi (Parkhouse and Clark, 1983; Maizels et al., 1984; Parkhouse et al., 1985). The technique is straightforward in that as long as the worms can be cultured in vitro the radioisotope is simply added to the culture medium. However, as with surface labelling of proteins with iodine, in metabolic labelling of proteins with ^{35}S-methionine important polysaccharide and glycolipid antigens may be missed in subsequent analyses. Metabolic labelling of carbohydrates using ^{3}H-glucosamine has been described in in vitro cell culture (Leonard et al., 1982), hence it is feasible that if this isotope was added to the in vitro culture medium of nematodes, newly synthesized carbohydrates could be labelled with ^{3}H, provided the worms metabolize glucosamine. This would provide a means of

labelling carbohydrates as easy to perform as the procedure for labelling newly synthesized proteins, and both moieties could thus be analysed.

In the Trichuris muris/mouse system little is known of the parasite antigens which actually stimulate the expulsive immune response in the host. Lee (1982) attempted some preliminary work on antigen investigation using SDS-PAGE analysis and staining gels with coomassie brilliant blue. However the latter stain is not very sensitive and unless antigens are present insufficient quantity (~ 1-5 µg per protein band) they will not be observed. Only one band was stained in the E/S products, this was around 43 kD, and relatively few bands were visible in adult male homogenate, but the major band was also around 43 kD. In this chapter the basic protein composition of T. muris adults and day 14 larvae has been investigated using SDS-PAGE. AMA, E/S and surface proteins of adult worms and homogenates of day 14 larvae have been separated by PAGE and stained with coomassie blue and the highly sensitive silver stain. Iodinated surface proteins, ^{35}S-methionine metabolically labelled proteins and ^{3}H-glucosamine metabolically labelled carbohydrates have been examined in immunoprecipitation analyses with various sera.

Preliminary experiments have been performed to investigate the enzymatic activity of these parasite antigens. If the function of target antigens could be determined, it would aid in understanding the actual effector mechanisms of immunity involved in parasite expulsion. The results of immunization experiments with various antigen preparations of T. muris (Selby and Wakelin, 1973; Jenkins and Wakelin, 1977), investigation of the structure of the stichosome (Sheffield, 1963; Wright, 1972; Lee and Wright, 1978) together with other data presented in this thesis, all implicate the stichosome

as the major site for the synthesis of material which makes up a significant proportion of the E/S products. Earlier workers put forward the hypothesis that stichosomal products may be involved in worm feeding ie. enzymes secreted for the external (and/or internal) digestion of host products (Lee and Wright, loc. cit.). It is also possible that such secretions are involved in the process by which the parasite tunnels through the gut epithelium by enzymatic digestion of host tissue. A stylet has been observed in some species of Trichuris, hence the tunnelling of certain of these nematodes could be via mechanical means. Of course, a combination of enzymatic and mechanical means could also be used. The production of proteases by several nematodes with life cycle stages which burrow through host tissues has been examined by various workers, with emphasis on the ability to digest collagen, the latter being a major structural protein in most animals (Hotez and Cerami, 1983; Wertheim et al., 1983; Dresden et al., 1985; Hotez et al., 1985; McKean, Carr and Pritchard, 1986). An initial investigation has been made of T. muris proteases using SDS-PAGE in which the substrates were incorporated into the gel, hence the proteases were identified by negative staining. Collectively it was considered that such analyses of worm antigens and their enzymatic activity should lead to a greater understanding of the host-parasite relationship within the T. muris/mouse system, and contribute to the identification and isolation of protective antigens.

RESULTS

SDS-PAGE was used to separate and identify coomassie blue and silver stained protein components of T. muris antigen preparations according to their molecular weights (T. trichura AH and Trichinella

spiralis preparations were also examined); gel photographs are given in Figures 7.1, 7.2 and 7.3. Tables numbered to correspond with figures give the molecular weights (in kD) of major/important proteins which have been identified. Underlining of m. wts indicates the relative amount of a particular molecule within a sample and brackets indicate only faint visibility. Samples run on gels were reduced (boiled for 5 mins in the presence of β-mercaptoethanol to destroy disulphide bonds) unless otherwise stated. In order to examine which proteins were antigenic, as determined by the induction of specific host antibodies, various sera were used in immunoprecipitation studies with in vitro ^{35}S-methionine-labelled worm proteins (Figures 7.4, 7.5 also see p 202/4, Figures 8.2.1 and 8.4.1). Precipitated antigens were separated from unreacted labelled material using protein A-sepharose which binds to IgG antibodies. Preliminary investigations as regards labelling carbohydrate moieties using ^{3}H-glucosamine were performed, Figure 7.6 shows profiles of labelled carbohydrate containing antigens, and some labelled antigens were immunoprecipitated by T. muris immune serum.

The proteolytic activity of T. muris antigen preparations was investigated (Figures 7.7, 7.8, 7.9 and 7.10). The methodology, based on that originally used by Hanley et al. (1966) with starch gels, is not given in Chapter 2, so will be briefly described here. Solutions of substrates (5% gelatin or 10% evaporated milk (Carnation)) were prepared then incorporated into the resolver gel solution mixtures (see Chapter 2) so that they constituted 10% of the total volume of the final solution. Gels were electrophoresed at 3.5°C (overnight) to ensure that there was no enzymatic digestion of the substrates during the protein separation, all samples applied to the gels were non-reduced. After SDS-PAGE the marker lanes were removed and stained

immediately with coomassie blue, the remaining sample lanes were washed for 1 hr in 2% Triton X-100 (non-ionic detergent) on an orbital shaker at RT in order to remove the SDS (anionic detergent). SDS reversibly denatures enzymes and the enzymes recover activity as soon as the detergent diffuses out of the gel (Lacks and Springhorn, 1980). Following rinsing in TRIS-HCl pH 7.4 for ½ hr. gels were placed in appropriate buffers (0.05 M TRIS-HCl pH 7.4 or pH 9.0, 0.05 M glycine-HCl pH 5.0 or 3.0) and incubated for 48 hrs at 37°C. Finally gels were negatively stained with coomassie blue.

FIGURE 7.1 COOMASSIE STAINED GEL OF TRICHURIS spp. ANTIGEN PREPARATIONS 15% gel

lane

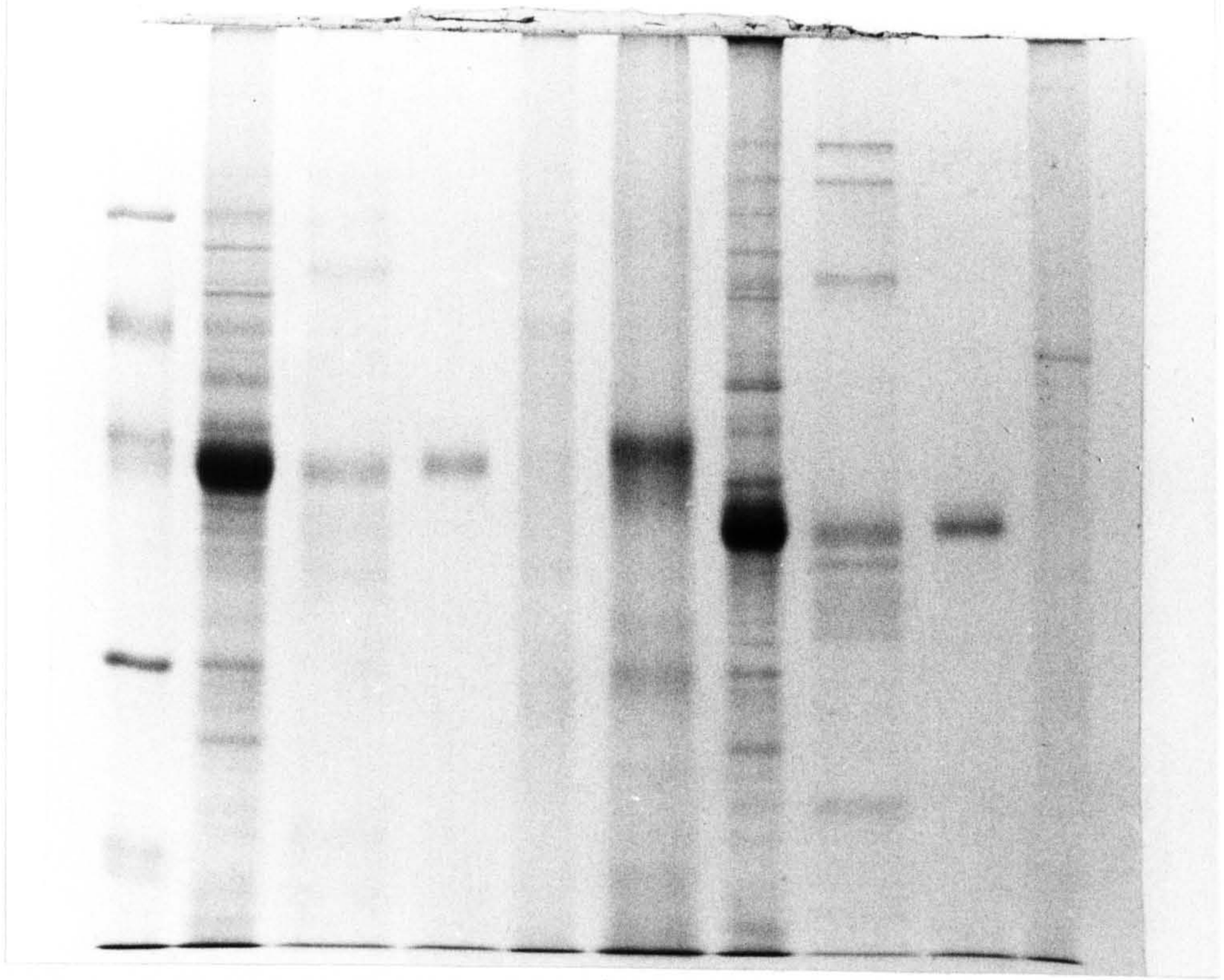
1 Markers : 94 kD, 67 kD, 43 kD, 30 kD and 20 kD
2 T. muris AMA (reduced)
3 T. muris E/S products (reduced)
4 T. muris CTAB stripped surface antigen (reduced)
5 T. muris day 14 larval homogenate (d14LH) (reduced)
6 T. trichiura AH (reduced)
7 T. muris AMA (non-reduced)
8 T. muris E/S products (non-reduced)
9 T. muris CTAB stripped surface antigen (non-reduced)
10 T. muris d14 LH (non-reduced)

FIGURE 7.2 SILVER STAINED GEL OF TRICHUROID NEMATODE ANTIGEN PREPARATIONS 15% gel

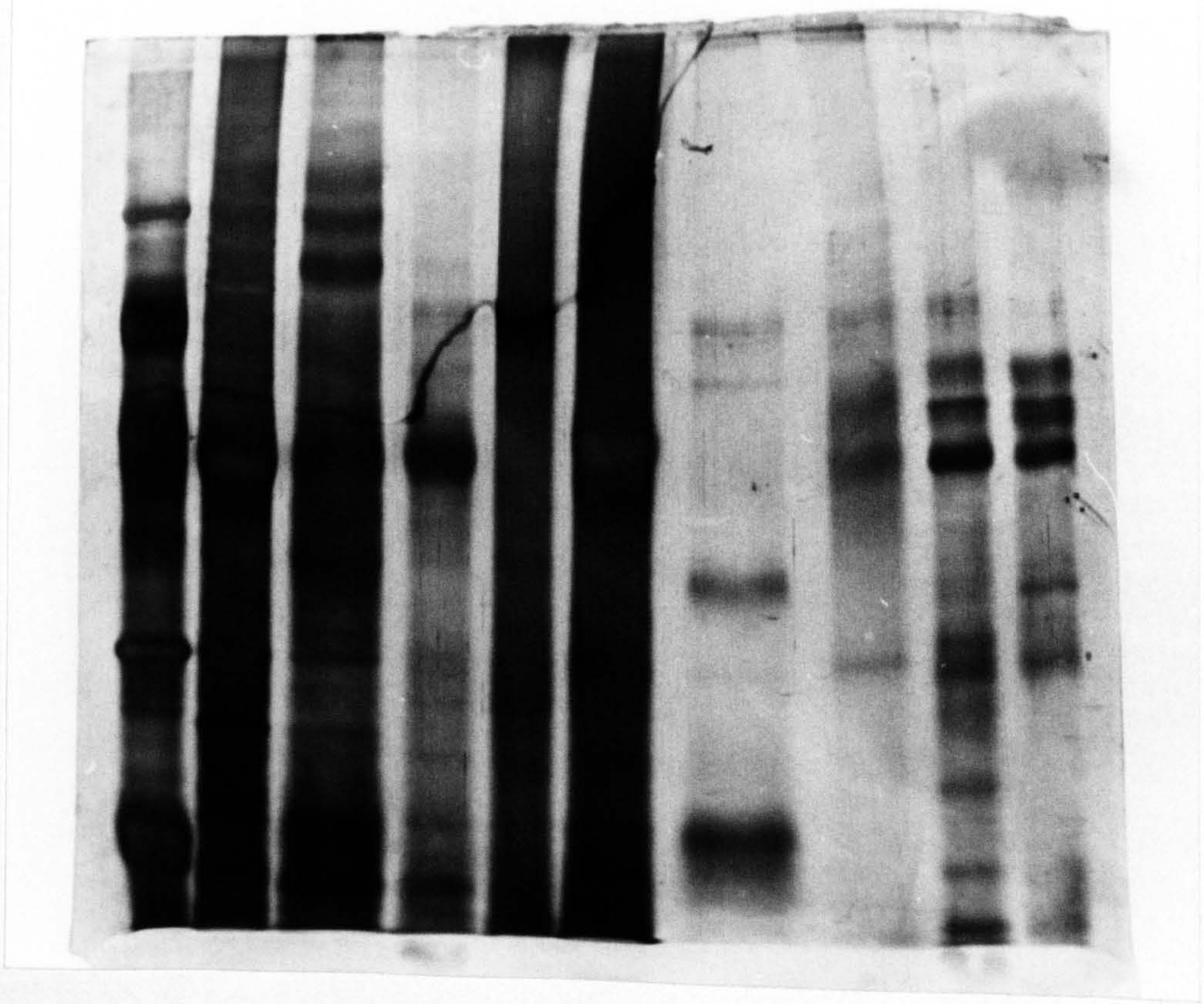
lane

1 Markers : 94 kD, 67 kD, 43 kD, 30 kD and 20 kD
2 T. muris AMA
3 T. muris E/S products
4 T. muris CTAB stripped surface antigen
5 T. muris d14 LH
6 T. trichiura AH
7 anti-T. muris MoAb F11 affinity purified antigen
8 anti-T. muris MoAb E12 affinity purified antigen
9 Trichinella spiralis (infective larvae) CTAB stripped surface antigen
10 Trichinella spiralis (infective larvae) E/S products

Where antigen preparations were run in both this silver gel and the coomassie stained gel (Figure 7.1) the same amounts of proteins were loaded on each gel.



1 2 3 4 5 6 7 8 9 10



1 2 3 4 5 6 7 8 9 10

TABLE 7.1 MAJOR PROTEIN BANDS VISIBLE ON COOMASSIE STAINED GEL OF TRICHURIS spp. ANTIGEN PREPARATIONS

I. muris AMA		I. muris E/S		I. muris CTAB S.A.		I. trichiura AH
(red)	(non-red)	(red)	(non-red)	(red)	(non-red)	(red)
lane 2	lane 7	lane 3	lane 8	lane 4	lane 9	lane 6
	128		127			
	112		112			
		(109)				
	102					
95		(92)				
86	86					
	78	78	79			
73	75					
64						
54	55					
46	46					46
41	41	41		42		
39	40					
37	38	(38)	38		38	39
	35	(35)	36			
	32		35			(32)
	30		31			
29	29					29
25	25		23			(25)
	18					(20)

TABLE 7.2 MAJOR PROTEIN BANDS VISIBLE ON SILVER STAINED GEL OF TRICHUROID NEMATODE ANTIGEN PREPARATION

	Trichuris muris						
AMA	E/S	CTAB S.A.	d14 LH	F11 Aq	E12 Aq	CTAB S.A.	E/S
lane 2	lane 6	lane 4	lane 5	lane 7	lane 8	lane 9	lane 10
	112						
	97						
	81						
			66	(66)	(66)	(66)	(66)
				(64)	(64)	(64)	(64)
				(54)		54	54
					48	47	47
42	42	42			41	41	41
	36				36		33
	34			33	28	28	29
	22			21		22	
	19			19		19	
						17	

FIGURE 7.3 SILVER STAINED GEL OF TRICHURIS spp. ANTIGEN PREPARATIONS 15% gel

lane

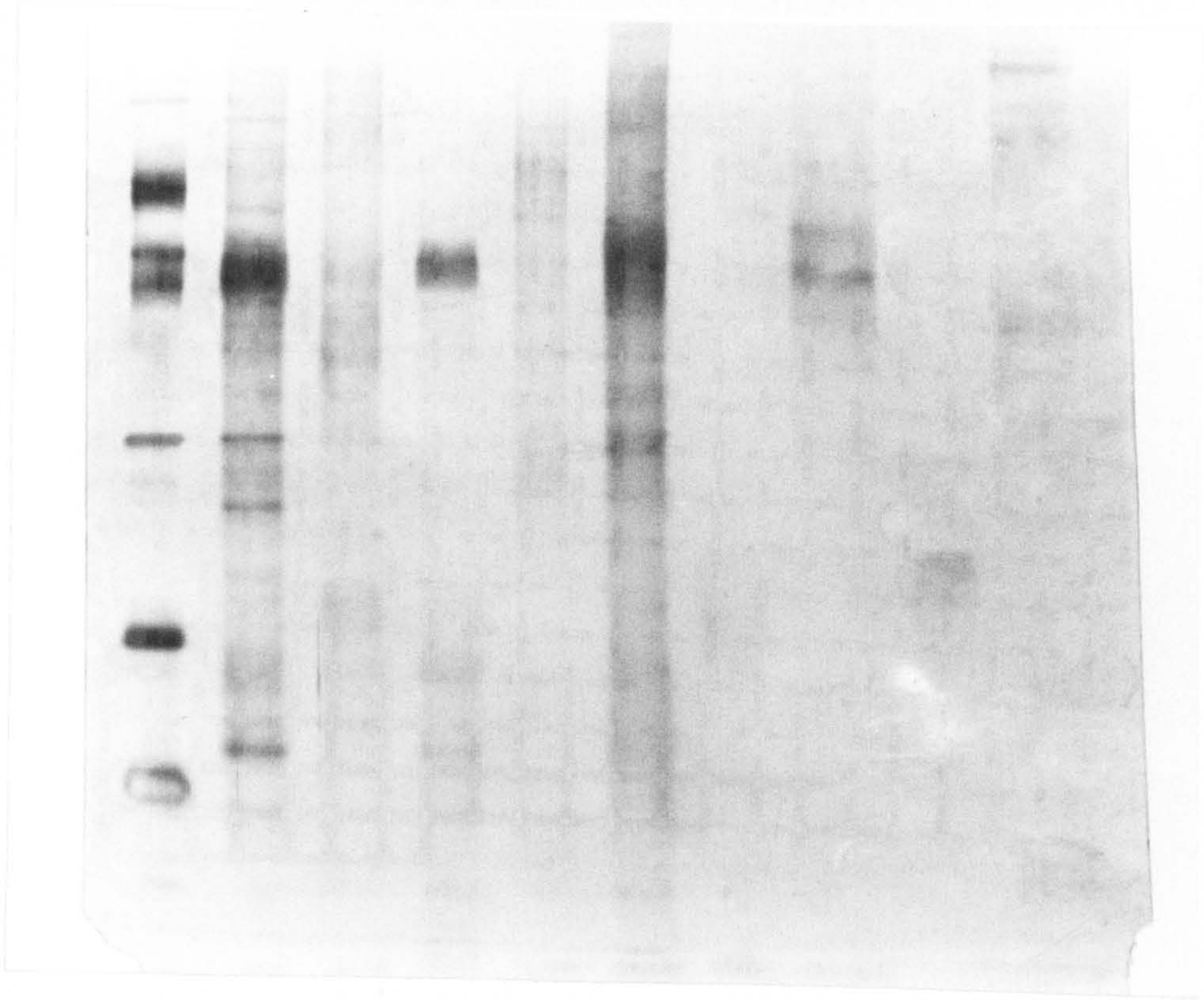
1 Markers : 94 kD, 67 kD, 43 kD, 30 kD, 20.1 kD and 14.4 kD
2 T. muris AMA
3 T. muris E/S products
4 T. muris CTAB S.A.
5 T. muris d14 LH
6 T. trichiura AH

Amount of proteins loaded were approximately $^{1}/_{10}$ of those used in silver stained gel in Figure 7.2, with exception of lane 4.

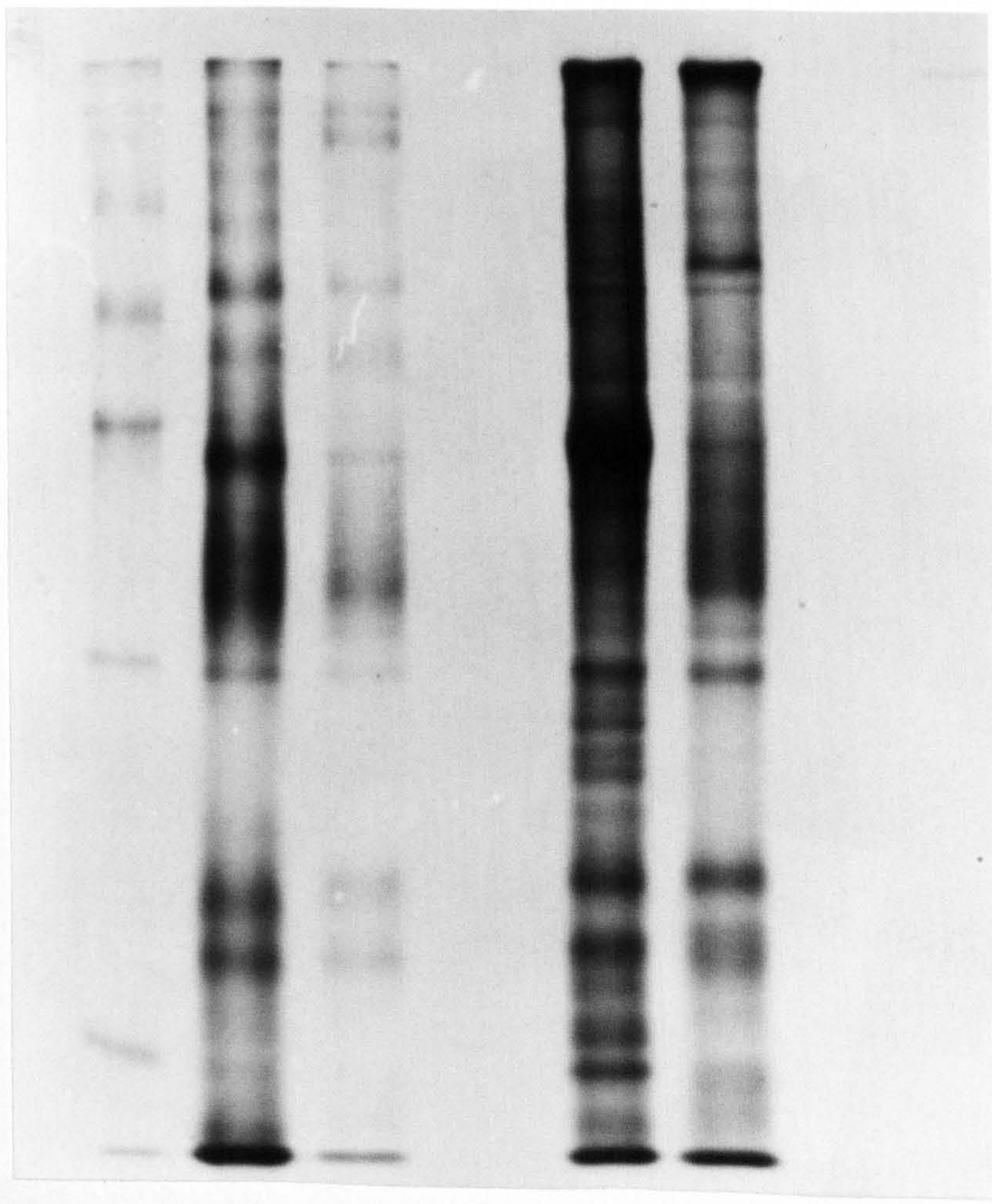
FIGURE 7.4 ^{35}S-METHIONINE LABELLED T. MURIS ANTIGEN PREPARATIONS 12% gel

lane

1 Markers : 92 kD, 69 kD, 46 kD, 30 kD, 14.3 kD
2 E/S products
3 Anterior end products (AA)
4 AMA
5 Anterior end homogenate (AAH)



1 2 3 4 5 6



1 2 3 4 5

TABLE 7.4 MAJOR PROTEINS OF ^{35}S-METHIONINE-LABELLED T. MURIS ANTIGEN PREPARATIONS

E/S Products	AA	AMA	AAH
lane 2	lane 3	lane 4	lane 5
(~ 136)	(~ 136)		
(~ 127)	(~ 127)		
(~ 106)	(~ 106)		
82			
73	73		
59	59		
44	44	45	45
40	40		
36			
34	34		34
31	31		32
29	29		30
			22
19	19		19
17	17		17
			16
14			13

FIGURE 7.5 IMMUNOPRECIPITATION OF ^{35}S-METHIONINE-LABELLED T. MURIS PROTEINS WITH INFECTION AND IMMUNE SERA

12% gel

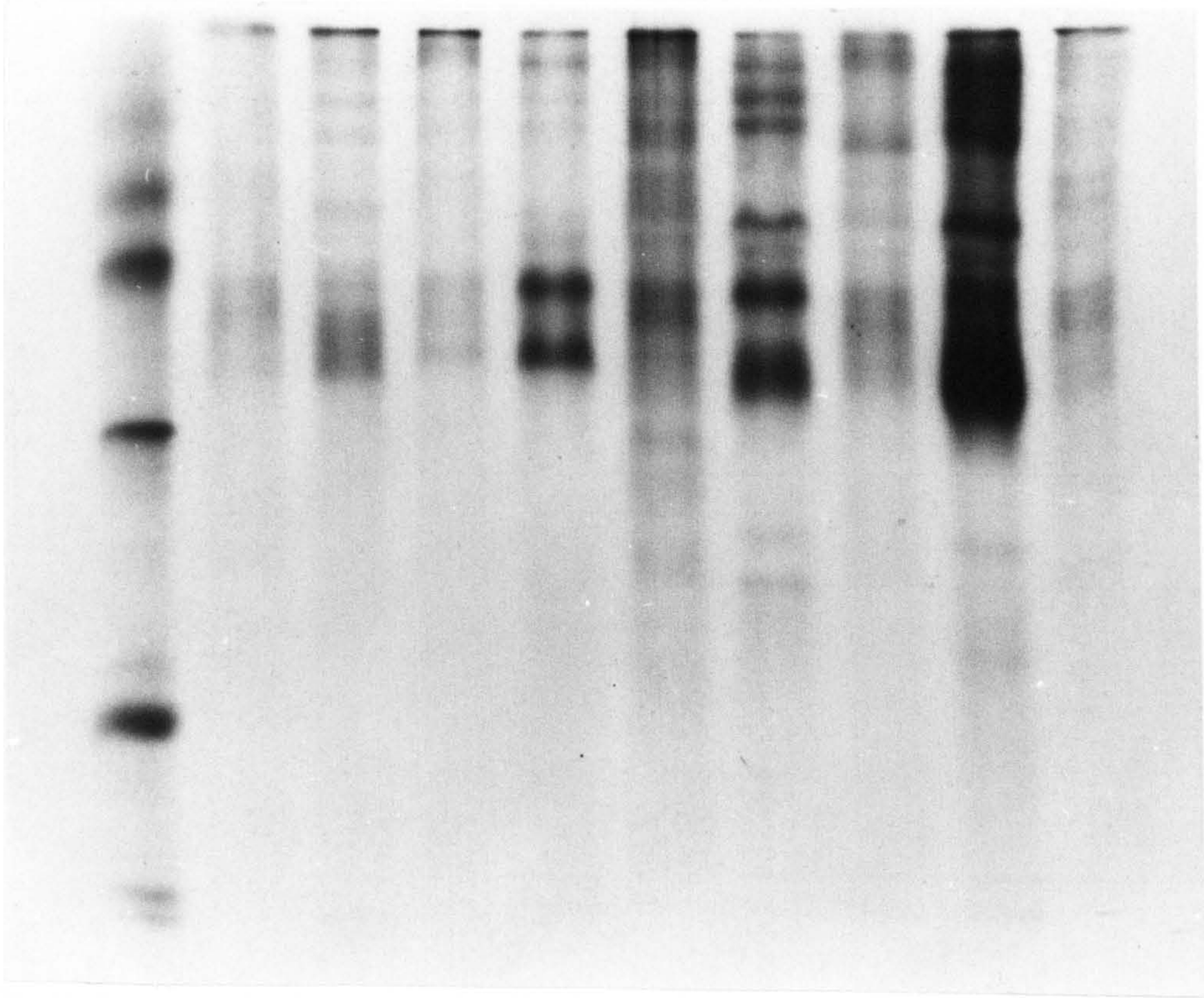
lane

1 Markers : 92 kD, 69 kD, 46 kD, 30 kD, 14.3 kD
2 AMA + ISd21
3 E/S + ISd21
4 AMA + IS E/S
5 E/S + IS E/S
6 AMA + IS AA
7 E/S + IS AA
8 AMA + tolerant serum d40 (TSd40)
9 E/S + TSd40
10 AMA + naive serum

FIGURE 7.6 ^{3}H-GLUCOSAMINE LABELLING OF CARBOHYDRATE MOIETIES ON T. MURIS ANTIGENS 12% gel

lane

1+2 Markers : 92 kD, 69 kD, 46 kD, 30 kD, 14.3 kD
3 E/S products
4 AMA
5 E/S + IS AA
6 AMA + IS AA
7 E/S + rabbit anti-*Trichinella spiralis* serum (rab. *T. sp*)



1 2 3 4 5 6 7 8 9 10

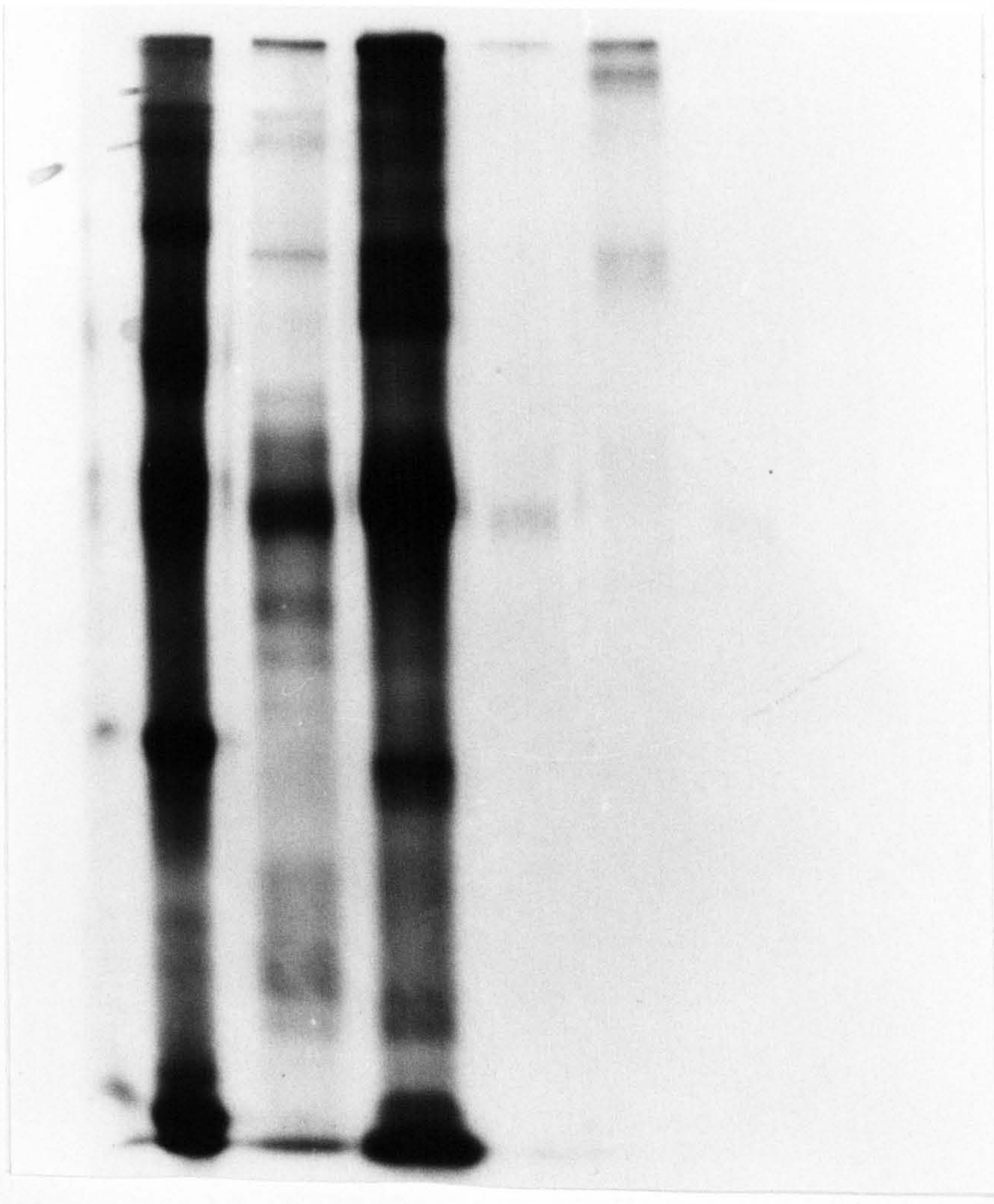


TABLE 7.5 MAJOR ^{35}S-METHIONINE-LABELLED T. MURIS E/S ANTIGENS IMMUNOPRECIPITATED WITH INFECTION AND IMMUNE SERA

E/S+ISd21 lane 3	E/S+ISE/S lane 5	E/S+ISAA lane 7	E/S+TSd40 lane 9
121	121	118	118
91	(91)	104	
	89	89	87
(85)			
60	(60)		
	54	54	54
47		(51)	50
44	44	43	46
42			42
		39	41
	37	36	
35		34	35
		(33)	31
			22
		19	17

TABLE 7.6 ^{3}H-GLUCOSAMINE LABELLING OF CARBOHYDRATE MOIETIES ON T. MURIS ANTIGENS

E/S Products lane 3	AMA lane 4	E/S+ISAA lane 5	AMA+ISAA lane 6	E/S+rab T.sp lane 7
133			150	
124	91			
89			89	
	75			
61				
56				
44	44	44		(44)
37				
34				
	28			
23				
19				
17				

GELATIN PROTEASES

FIGURE 7.7 T. MURIS E/S PRODUCTS AND AMA ACTIVITY AT pH 3,5,7 and 9 12% gel

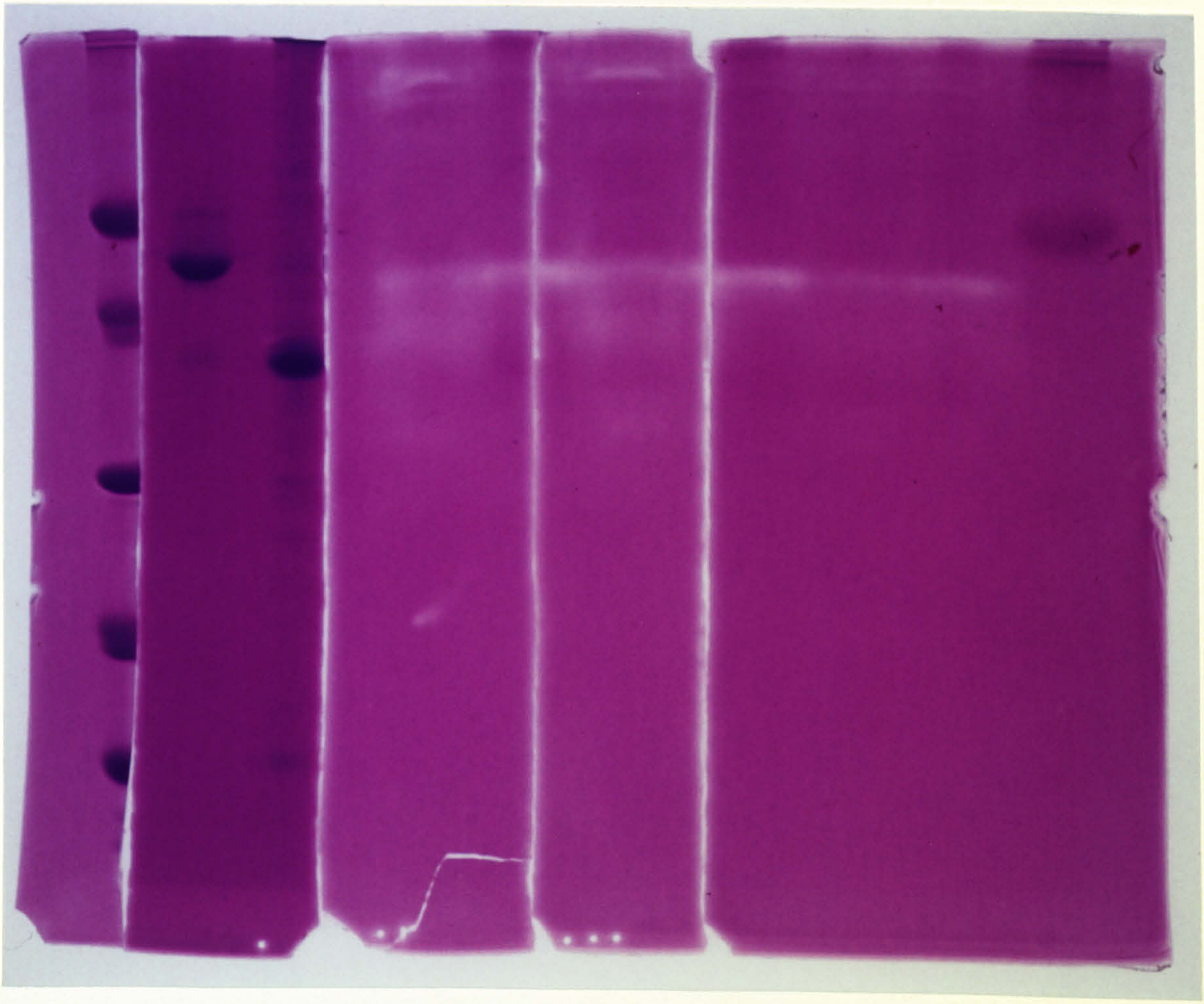
lane

1 Markers : 67 kD, 43 kD, 30 kD, 20.1 kD and 14.4 kD
2 E/S products, pH3
3 AMA, pH3
4 E/S products, pH5
5 AMA, pH5, AMA, pH7
6 E/S products, pH7
7 AMA, pH9
8 E/S, pH9
9 AMA, pH9
(10 Marker : 67 kD)

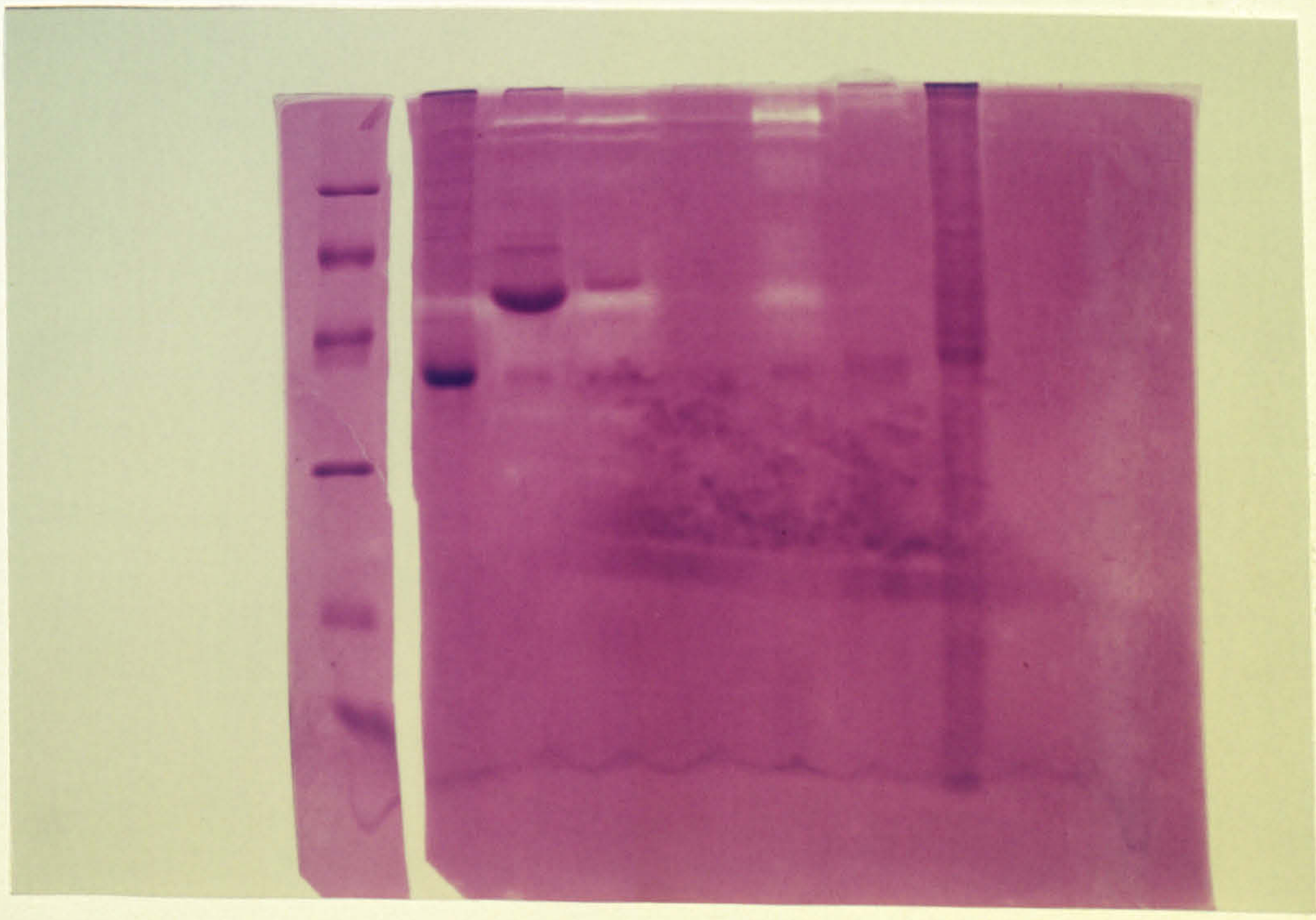
FIGURE 7.8 T. MURIS E/S PRODUCTS, AMA AND CTAB STRIPPED SURFACE PROTEINS AT pH 7.4 12% gel

lane

1 Markers : 94 kD, 67 kD, 43 kD, 30 kD, 20.1 kD, 14.4 kD
2 AMA
3 E/Sc (with contaminating protein)
4 E/S
5 CTAB stripped surface antigen



1 2 3 4 5 6 7 8 9 10



1 2 3 4 5

GELATIN PROTEASES

TABLE 7.7 T. MURIS E/S PRODUCTS AND AMA AT pH 3,5,7 and 9

pH 5		pH 7.0			pH 9.0		
E/S lane 4	AMA lane 5	AMA lane 5	E/S lane 6	AMA lane 7	AMA lane 7	E/S lane 8	AMA lane 9
133			133				
132			132				
58	58	58	58	58	58	58	58
44			44				
41			41				
33			33				

TABLE 7.8 T. MURIS E/S PRODUCTS, AMA AND CTAB STRIPPED SURFACE PROTEINS AT pH 7.4

AMA lane 2	E/S lanes 3 and 4	CTAB S.A. lane 5
	133	133
	124	124
		111
		95
51	51	51
	49	
	40	
	34	
	29	

CASEIN PROTEASES

FIGURE 7.9 TRICHUROID NEMATODE PRODUCTS ACTIVITY AT pH 7.4

12% gel

lane

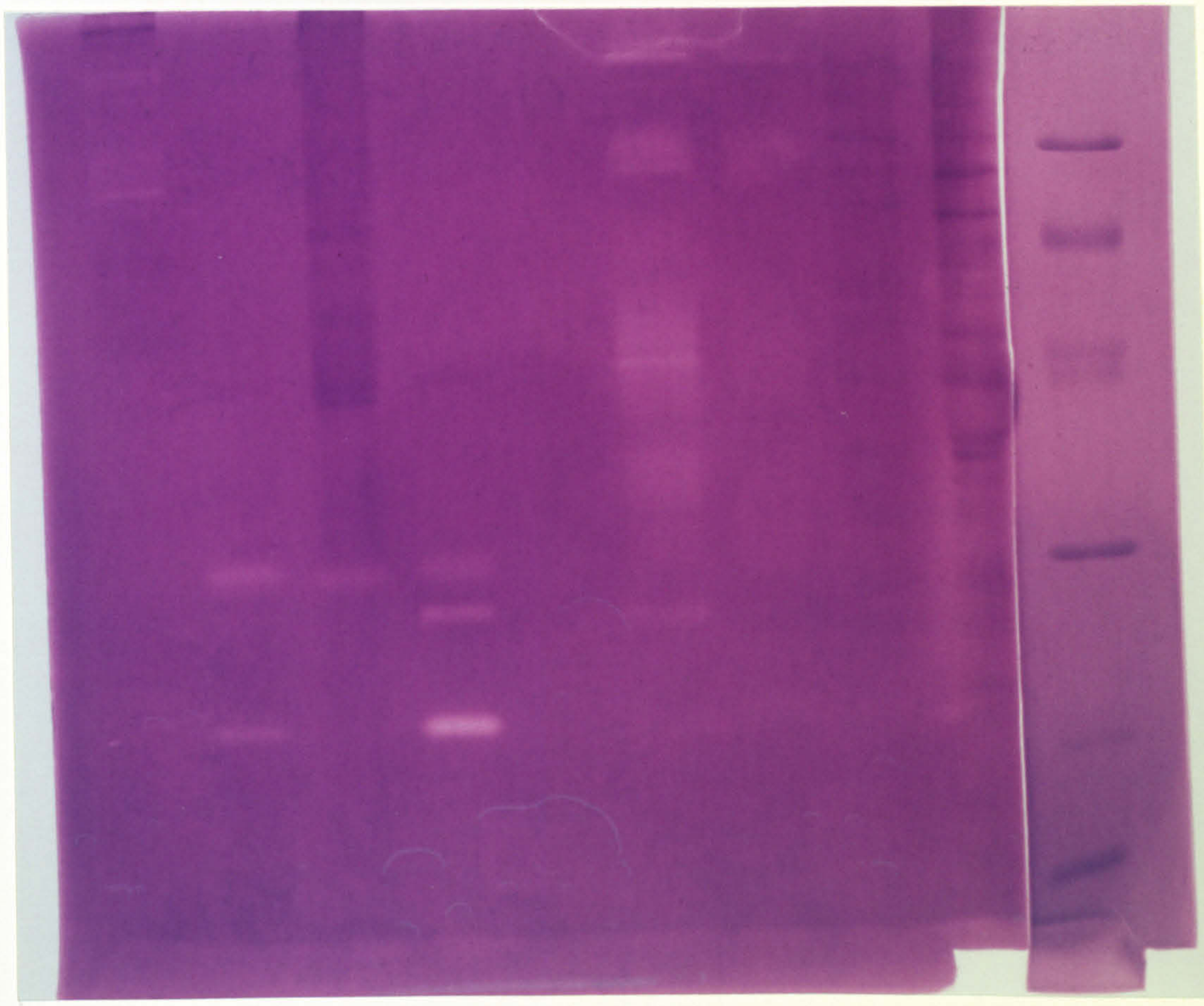
1 Markers : 94 kD, 67 kD, 43 kD, 30 kD, 20.1 kD and 14.4 kD
2 Trichinella spiralis infective larvae E/S products
3 T. spiralis LH
4 T. spiralis CTAB stripped larval surface antigen
5 Trichuris muris E/S products
6 T. muris AMA

FIGURE 7.10 TRICHUROID NEMATODE PRODUCTS ACTIVITY AT pH 7.4

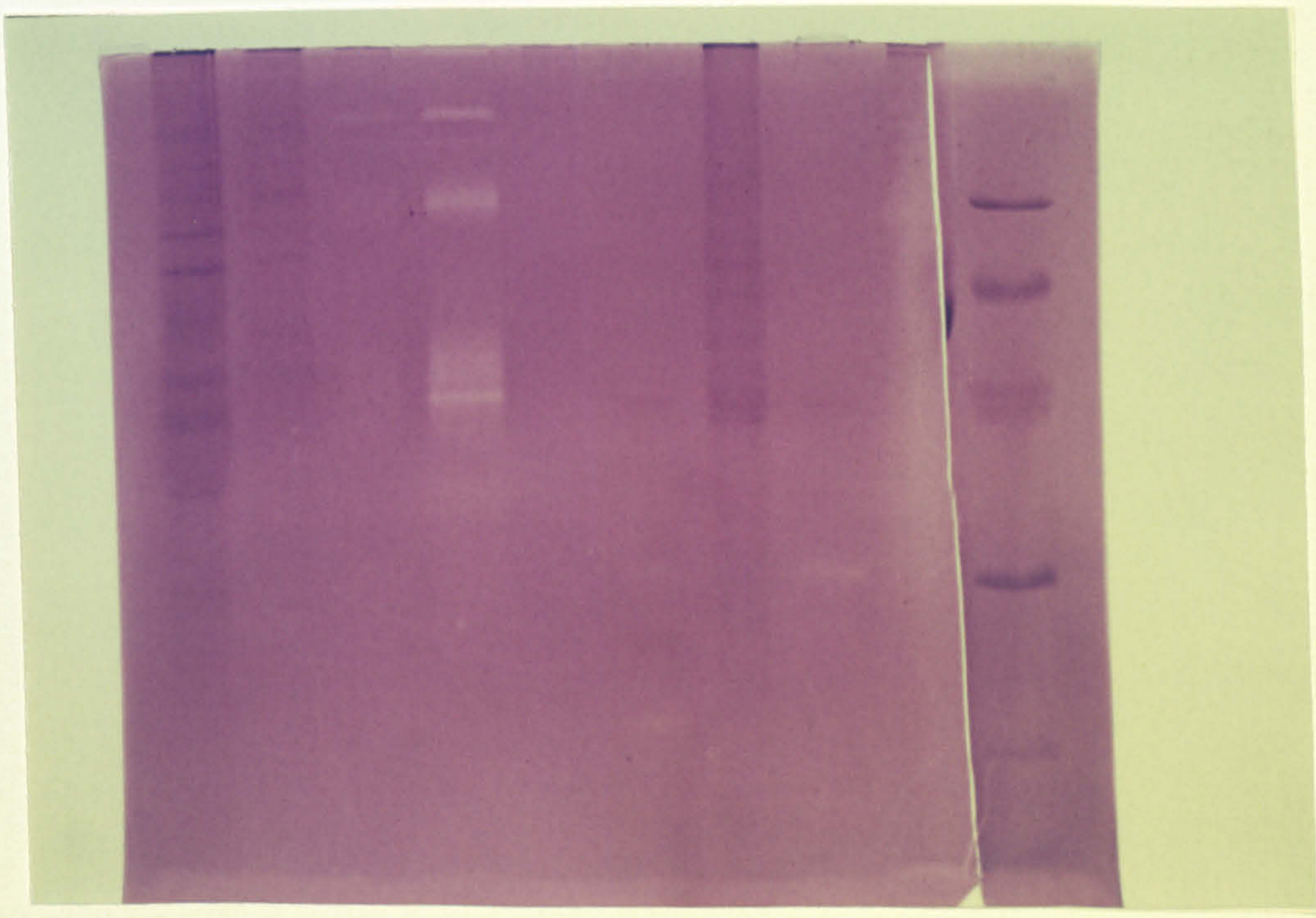
12% gel

lane

1 Markers : 94 kD, 67 kD, 43 kD, 30 kD and 20.1 kD
2 T. muris AMA
3 T. muris E/S products
4 Trichinella spiralis CTAB stripped surface antigen
5 T. spiralis infective larvae E/S



2 3 4 5 6 1



2 3 4 5 1

CASEIN PROTEASES

TABLE 7.9 TRICHUROID NEMATODE PRODUCTS ACTIVITY AT pH 7.4

Trichinella spiralis Infective Larvae			*Trichuris muris*
E/S lane 2	LH lane 3	CTAB Surface Aq lane 4	E/S lane 5
			121
			91
			78
			46
			41
			37
			34
			32
27	27	27	
		25	25
20		20	

TABLE 7.10 pH 7.4

T. muris E/S lane 3	*T. spiralis* CTAB Surface Aq lane 4	*T. spiralis* E/S lane 5
119		
107		
88		
54		
45		
43		
	29	29
	21	

DISCUSSION

The separation and identification of proteins present in adult T. muris antigen preparation confirmed the heterogeneity of the whole worm homogenate preparation (AMA) compared with the E/S products. Nonetheless, the E/S products did contain at least 8 consistently identifiable major proteins in reduced and non-reduced samples as determined using coomassie staining. The reduction process destroys disulphide bonds hence reducing any polymerized proteins into consituent peptide chains, however, disulphide bonds responsible for holding together the tertiary structures of proteins are also destroyed, molecules become "straightened out" and are retarded in their movement through the pores of the gel matrix, thus the apparent increase in the molecular weights of proteins in reduced compared with non-reduced samples.

The major protein identified in AMA, E/S products and CTAB stripped surface antigens was of apparent molecular weight 41/42 kD when reduced, 38 kD when non-reduced, however, although present in all the adult antigen preparations examined this protein was not found to be a major constituent in the crude day 14 larval homogenate preparation. Of course, only preliminary examinations have been made of the day 14 larvae and it could be that the preparation made contained insufficient material to be able to readily identify any larval protein constituents amongst the contaminants, only one protein around 66 kD was in fact identified. The adult E/S products contained a group of proteins with molecular weights in the range of 30 to 46 kD, which were often only just distinguishable from each other, distinct higher m.wt. bands around 127, 112

and 79 kD and 2 distinct lower m.wt. bands around 23 and 19 kD. The possibility of different proteins of similar m.wts. coming to lie in the same position in single dimension PAGE should always be borne in mind, and of course, possession of the same apparent m. wts. does not make proteins identical. A more detailed analysis of the constituent proteins could be made using two-dimensional gel electrophoresis. Proteins are initially separated on a pH gradient in the first dimension followed by separation according to m.wt. in the second dimension. However, for current purposes as regards the T. muris / mouse system separation in a single dimension was adequate.

Many more constituent proteins were detected in adult worm E/S products as a result of the highly sensitive technique of in vitro labelling with ^{35}S-methionine. Newly synthesized proteins were detectable in labelled E/S products even when in minute quantities, in contrast detection of E/S proteins by staining alone allowed identification of only the major cumulative proteins. Most of the newly synthesized proteins apparent in labelled E/S products from intact adult worms were also identified in the products of cultures containing the anterior ends of the worms only (Figure 7.4). This suggests that the anterior ends of the worms (containing the oesophagus, stichosome,bacillary band and cuticle) are responsible for the production of a major proportion of the total E/S products of these nematodes. As observed with the stained cumulative proteins (Figure 7.1) a blur of protein bands was evident on the autoradiographs of labelled E/S and AA preparations in the

region 30 to 44 kD. Immunoprecipitation studies with ISE/S and ISAA immune sera and labelled E/S products (Figure 7.5 and Figure 9.2.2 (pg 219)) and with these sera and labelled AA antigens (Figure 8.2.1 (pg 202)) do suggest that both preparations contain identical antigens as determined by specific antibody recognition in addition to identification by m.wt. alone. The approximate m.wts of the *in vitro* released E/S antigens which originated from the anterior ends of the worms appeared to be 121-128, 115-118, 98-105, 89-91, 46, 43, 37, 33 and 20 kD. Labelling of surface proteins of *T. muris* via IODOGEN method was attempted (data not shown) but results were variable. Successful labelling of proteins was apparently dependent on the number of worms placed in the IODOGEN coated tubes. If too many worms were present the surface proteins were not labelled, perhaps due to inadequate contact between the worm surfaces and the reaction tubes. Nonetheless a minimum of 10 surface proteins were identified by ^{125}I-labelling, with a large amount of material in the m.wt. range of 30 to 44 kD.

Most ^{35}S-methionine labelled *T. muris* E/S products were antigenic and were precipitated by infection and immune sera raised against the worms. Both ISE/S and ISAA had particular affinities for proteins of apparent m.wts. 43/44 and 36/37 kD (Figure 7.5), although only ISAA strongly precipitated a 54/59 kD protein (see also Figure 9.2.2 (pg 219)). Tolerant serum (TS) from mice with patent *T. muris* infections precipitated a large amount of material and all these proteins were also precipitated by ISE/S and ISAA as determined

by identification using m.wts., although the quantities of antigens precipitated differed. In contrast day 21 infection serum (ISd21), which had relatively low specific antibody titres in ELISA against adult T. muris E/S products, precipitated a relatively small amount of labelled protein, a blur of bands was evident with weights ranging from 35 to 47 kD; proteins with m.wts. 121, 91 and 60 kD were also evident. ^{125}I-labelled surface antigens were also precipitated by immune sera (data not shown), ISAA precipitated a large amount of material in the region 35 to 44 kD.

Immunoprecipitation studies have demonstrated that T. muris E/S products collected in vitro contain a large proportion of proteins newly synthesized and released by the anterior ends of these nematodes, most of these proteins were antigenic. Comparing the identified newly synthesized proteins with those of cumulative preparations raises questions as to the origins of these proteins. The major 41/42 kD cumulative protein of AMA, E/S and CTAB stripped surface antigen preparations (Figure 7.1) appears to be the 43/44 kD newly synthesized protein precipitated by infection and immune sera. Whether this protein is a stichocyte product, released and then adsorbed onto the cuticle of the worms, or is synthesized in the hypodermis and transported to the cuticle surface, from where it may then be shed in vitro, cannot yet be determined. Although this protein did not appear as a major protein band in the labelled anterior end (AA) products (Figure 7.4) it was precipitated by ISAA from labelled E/S proteins (Figures 7.5 and 9.2.2 (pg219)) and by ISE/S from labelled AA products (Figure 8.2.1 (pg 202)). Notably, T. muris died after a short period in CTAB detergent (approximately 20-30 mins), so the nematodes must be very sensitive to the rapid stripping of surface proteins, mainly a 41/42 kD molecule, by this detergent. In contrast

Trichinella spiralis infective larvae and Nematospiroides dubius adults survive for up to 3 hours in CTAB detergent (Dr. Crawford, pers. com.) and do not appear to be deleteriously affected. T.muris survived very well in RPMI 1640 medium for several days, as long as the spent medium was regularly changed and the 41/42 kD protein was the major cumulative protein collected in the spent culture medium.

^{3}H-glucosamine labelling of carbohydrate moieties was successful, although the labelling of precipitated antigens did not appear to be as effective as when the constituent proteins were labelled as determined by the visualization of antigen bands on autoradiographs after immunoprecipitation analyses. Nonetheless, antigens were identified in immunoprecipitation studies eg. ISAA precipitated a carbohydrate labelled molecule of apparent m.wt. 44 kD (a ^{35}S-methionine-labelled protein of similar m.wt. was also precipitated by this sera). Whether the carbohydrate moiety is part of the recognized epitope or is merely present on an antigen which has been recognized by some other epitope(s), perhaps entirely protein, it is not possible to determine by these studies alone.

Studies using labelled antigens with other nematodes have concentrated mainly on the identification of surface labelled material and it is only relatively recently that workers have begun to use ^{35}S-methionine for in vitro labelling of E/S products (reviewed by Maizels et al., 1982; Almond and Parkhouse, 1985). The general conclusions drawn from the surface labelling studies are that only a limited number of proteins (1 to 10) dominate the surface of each developmental stage in each species of nematode, the stage specificity of antigens varies with different nematodes, and surface proteins are nearly always antigenic in the host. Not only is the cuticle anti-

genically active but it is now conceived to be a dynamic structure capable of alteration and development even between moulting events. Trichinella spiralis has been the most extensively investigated nematode, and the surface proteins demonstrate remarkable stage specificity. In the infective larvae 4 proteins of different m.wts. were detected (Parkhouse et al., 1981) but these were all closely related structurally and it was suggested that they shared a common origin. The molecules differed in being monomeric or dimeric, glycosylated or non-glycosylated, but shared most of the polypeptide backbone of the antigen. According to the number of different E/S antigens precipitated by individual anti-Trichuris muris MoAbs (Chapter 6) the sharing of common epitopes also seems to be the case in this system. Finally, the surface of Trichinella spiralis is not a homogeneous covering, as demonstrated by the binding of a MoAb to the surface of the adult male copulatory bell only (Ortega-Pierres et al., 1986). Parkhouse and Clark (1983) also analysed the E/S products of T. spiralis by metabolic labelling with ^{35}S-methionine and found them to be stage-specific, the stage specificity of surface and secreted proteins was taken as a reflection of changes in the worms metabolism as their needs and environment change. As mentioned previously the division of nematode antigens into E/S or surface components is by no means exclusive, and surface molecules of Trichinella spiralis and Nippostrongylus brasiliensis are released in in vitro culture. Toxocara canis in particular is a prolific producer of E/S antigens in vitro (De Savigny, 1975) and common antigens have been found on the larval surface and in the E/S material (Maizels et al., 1984). The true origin of the surface released antigens has not yet been established, but it is known that specific E/S antigens are prolifically released from glands at the excretory and anal pores

of toxocaral larvae (Hogarth and Scott, 1966), and these may be transiently deposited on the cuticle for later modification and turnover.

Unfortunately, partly due to the difficulty in obtaining large numbers of T. muris larvae, investigations on the production and release of E/S and surface antigens have been restricted to adult worms only. It is not possible to speculate on the degree of stage specificity demonstrated by this nematode but common antigens must exist between the developing larvae and the adults as immunity is expressed against larvae following active immunization with adult E/S products. Day 21 and day 27 infection sera (Figure 8.4.1, pg 204) exposed to stages up to L_3 and L_4 larvae respectively, do recognize a wide range of labelled adult E/S and AA antigens, notably 124/122, 101, 91/88, 62, 52, 46, 42, 39 and 35 kD proteins with the strongest bands at 39 kD, and had relatively high specific antibody titres for adult E/S products as measured in ELISA. In contrast, earlier infection sera had very low specific antibody titres to the adult parasite antigen preparations, as determined in ELISA and by immunoprecipitation studies, however circulating specific antibodies to day 14 larval homogenate were also not detectable in ELISA. Hence the lack of reactivity of the early infection sera could not be put down to a lack of shared antigens between the early larval stages, L_1 and L_2, and the adult E/S products, but was a reflection of the delayed serum antibody response compared with the local GALT response. The question as to whether any of these shared antigens, and indeed which E/S antigens, are stichocyte derived may only be answered by localization studies using the anti-T. muris MoAbs, but so far such studies have not been successful.

Initial studies as regards the proteolytic activity of T. muris

E/S products have yielded some interesting data. The proteases detected could be differentiated on the basis of whether they could digest gelatin (monomer of collagen) and/or casein (major protein of milk). Nimmo-Smith and Keeling (1960) found proteolytic activity of adult worm extracts to be greatest around pH 3 with higher activity in the anterior ends of the worm, as there was relatively little activity above neutrality it was thought unlikely that the detected enzymes, even if they could be shown to be secreted into the parasites immediate·environment, could be responsible for significant tissue digestion. These enzymes were thus suggested to be primarily concerned with the turnover of cellular constituents and metabolites. In contrast the proteases detected in the E/S products seemed to operate within a wide pH range with apparently optimal activity around pH 7. Indeed, at very acid pH 3 the proteins appeared to be fixed in the gel and no enzymatic activity was detected. Distinct gelatin proteases were detected at around 133 and 51/58 kD, and ^{35}S-methionine labelled E/S antigens of apparent m.wts. 54 kD were strongly preciptated by ISAA and d40 TS. Some enzymatic activity was evident at around 44 to 41 kD, 33 kD and 29 kD but digestion bands were not clear. As regards casein proteases the major bands of digestion produced by T. muris E/S products were at 121, 91, 41 kD and 25 kD, with more diffuse regions of digestion around 91 to 78 kD, 46 to 37 kD and 34 to 32 kD. In the E/S and CTAB stripped surface antigens of Trichinella spiralis, proteolytic enzymes which digested casein were detected in the former preparation with apparent m.wts. 27 and 20 kD and in the latter with m.wts of 27, 25 and 20 kD. The 20 kD digestion band observed with the CTAB surface antigen preparation was particularly strong. Hence, both these trichuroids have proteolytic enzymes, which function at mammalian physiological pH, and potentially gut pH values, in their E/S products.

Indeed, with Trichinella spiralis infective larvae the E/S proteases collected in vitro were shown to be present in the CTAB stripped surface protein preparation. Only a limited examination of surface proteases of Trichuris muris was carried out, but there appeared to be proteolytic activity in gelatin gels, the enzymes having apparent m.wts. of 133, 124 and 51 kD, these digestion bands were also observed with the E/S preparation. Repeated freezing and thawing of antigens was observed to considerably reduce enzymatic activity, and additional experiments need to be performed to confirm the results obtained so far on surface and E/S proteases in trichuroid nematodes. The possibility that the trichuroid E/S proteases, which may be secondarily adsorbed onto the nematode cuticle after release from other production sites in the parasite, or may be directly released from the cuticle after synthesis at this site, are involved in digestion and/or penetration of host tissues is an interesting one. Further investigations in this area particularly as regards the source of these enzymes in the parasites will produce useful data. It has been accepted for some time that the cuticle of nematodes is an extremely active structure, indeed, enzymes and haemoglobin have been located in it (Lee, 1972) and adults of Brugia pahangi have been shown to take up nutrients via their surfaces (Chen and Howells, 1979; Howells and Chen, 1981). The demonstration of surface proteases on Trichinella spiralis infective larvae and Trichuris muris adults serves to add further confirmation to the concept of an active nematode surface.

To summarize, the antigens of T. muris were divided into "compartments" for investigation which were by no means exclusive. Worm homogenate (AMA), E/S products and surface stripped material were all examined. The major protein of AMA, 41/42 kD, was also the major protein found within E/S products and surface stripped material.

Cumulative E/S proteins with apparent m.wts of 127, 112, 79 kD, a number of proteins between 46 and 30 kD and 23 and 19 kD bands were identified. Newly synthesized E/S proteins detected by in vitro labelling were more numerous but major bands had m.wts of 128-121, 118-115, 105-98, 91-89, 46, 43, 37, 33 and 20 kD. Most of the labelled proteins were precipitated either by immune serum raised against E/S products (ISE/S) and/or immune serum raised against the anterior ends of the worms (ISAA). Both sera had strong affinity for labelled E/S proteins of m.wts. 44/43 and 37/36 kD, and ISAA also strongly precipitated a 54/59 kD protein. Many of the E/S antigens could also be labelled with ^{3}H-glucosamine, and were apparently glycoproteins, this included a 44 kD protein. Serum from animals which had only been exposed to larval T. muris, L_3 and L_4 stages did precipitate a wide range of labelled adult E/S antigens notably 124/122, 101, 91/88, 62, 52, 46, 42, 39 and 35 kD proteins with the strongest band at 39 kD. Finally proteolytic activity was demonstrated in E/S products and surface stripped material of both T. muris adults and Trichinella spiralis infective larvae. Both nematodes had common E/S and surface enzymes, in the former these were of apparent m.wt. 133, 124 and 51 kD, in the latter these were 27 and 20 kD. Interestingly, the m.wts. of the T. muris proteases are similar to the m.wts. of some of the labelled antigens which were precipitated by immune and infection sera raised against T. muris adults and L_3 and L_4 larvae. Hence, the possibility arises that the immune expulsion of T. muris from its host before patency may involve the specific inhibition of burrowing and/or feeding of the nematodes, which results from the recognition of common adult and larval functional enzymes.

CHAPTER 8

A FURTHER INVESTIGATION OF ANTIGENS SHARED BETWEEN TRICHURIS MURIS AND TRICHINELLA SPIRALIS AND OF REACTIONS BETWEEN TRICHURIS MURIS E/S PRODUCTS AND IMMUNE SERA FROM OTHER NEMATODE INFECTIONS

INTRODUCTION

Specific cross-immunity between Trichuris muris and Trichinella spiralis has been described by Lee et al. (1982), infection with either parasite was effective in eliciting accelerated expulsion of a secondary heterologous infection. Accelerated expulsion of a heterologous infection was also achieved by the prior administration of soluble crude worm antigen or by adoptive transfer of mesenteric lymph node cells taken from mice infected with the heterologous parasite. This specific cross-immunity indicated shared antigens between Trichuris muris and Trichinella spiralis and it was postulated that these shared antigens were derived from stichocyte granules.

The stichosome is thought to be the major source of E/S antigens of both species and as a structure common to trichuroid nematodes, possibly serving similar functions within this group, the probability that the shared antigens are stichocyte products is reasonably high. The stichocyte products from Trichinella spiralis muscle larvae have been examined in great detail (Despommier and Muller, 1976). The stichocytes have been shown to contain 2 types of granules designated α and β and antigens from both types of granule are extremely effective when used in immunization against subsequent infection. In the Trichuris muris mouse system both E/S antigens and extracts from the anterior ends of worms (containing the stichosome) have been successfully used in immunization experiments (Wakelin and Selby, 1973), but actual stichosome granule antigens have not been isolated as a pure preparation. In preliminary serological analyses Lee (1982) found that antisera raised in rabbits against Trichinella spiralis or Trichuris muris antigens reacted with the heterologous

antigen preparations in immunodiffusion and immunoelectrophoresis, but the source and nature of these antigens were not determined. Further investigations have now been made, which include the immunoprecipitation of ^{35}S-methionine labelled T. muris E/S products with rabbit and mouse anti-Trichinella spiralis immune sera and anti-T. spiralis MoAbs (kindly donated by Drs. Silberstein and Despommier, New York), in order to determine the nature of the shared antigens of these trichuroid nematodes.

Lack of specificity is a notorious problem in the use of ELISAs in immunodiagnosis of parasite infections (Voller and De Savigny, 1981). However, as this problem is largely dependent on the quality of antigens used in the tests, it may be overcome by the use of more highly purified antigen preparations such as the so-called host-parasite interface antigens, E/S products and surface antigens. Of course, the possibility of shared antigens between parasites should also be considered when performing such tests with sera from individuals with various concurrent infections. E/S products even in their crude form are much less complicated antigen mixtures than homogenate preparations, thus simplifying any purification steps which may be necessary and facilitating the standardization of antigen. Indeed, in ELISAs performed with T. muris E/S products and anterior end homogenates the background readings obtained with naive sera were considerably reduced when compared with those obtained using whole male worm homogenates (AMA). The species specificities of nematode E/S products are thought to be relatively high (Fife, 1971) as they are usually biologically relevant and functional antigens. In the knowledge that the cross-reactivity between T. muris and Trichinella spiralis has been shown to be the result of as yet unidentified shared antigens (possibly E/S antigens), the cross-

reactivity of Trichuris muris E/S products in ELISA and immuno-precipitation experiments with immune serum from mice infected with other less closely related nematodes has also been examined.

RESULTS

8.1 PROTEINS OF TRICHURIS MURIS AND TRICHINELLA SPIRALIS

Proteins present in different antigen preparations of adult Trichuris muris and infective Trichinella spiralis larvae were separated by SDS-PAGE and stained with coomassie blue (Figure 8.1), only a few proteins were identifiable in the non-concentrated E/S products of either parasite using this stain. The major protein in the T. muris E/S products had an apparent m.wt. of 49 kD, the proteins stained in the T. spiralis E/S products had m.wts of 50 and 44 kD (Table 8.1). Early E/S antigen preparations for both parasites contained a "contaminating" protein of apparent m.wt 71 kD, which was not found in any of the later preparations. Interestingly, the surface antigen preparations for both nematodes removed using the cationic detergent cetyltrimethylammonium bromide (CTAB) contained the same proteins as the E/S products.

The coomassie and silver stained gels in Chapter 7 (pg 173 Figures 7.1 and 7.2) identified more proteins in the antigen preparations of these nematodes. T. muris E/S contained a number of proteins with apparent m.wts. in the range 127 to 19 kD, T. spiralis E/S proteins had m.wts in the range 66 to 29 kD (pg173 Tables 7.1 and 7.2) and both worms had a major E/S protein with m.wt. 41/42 kD (apparently 49/50 kD in Figure 8.1). T. spiralis CTAB and E/S antigens were very similar although the former contained some lower

m.wt proteins at 22, 19 and 17 kD which were not identified in the E/S products. As well as a major protein, m.wt 42 kD, a number of other proteins were stained in the T. muris CTAB preparation (pg 173 Figure 7.2). However, bearing in mind the sensitivity of the silver stain these proteins could be E/S products released during the CTAB incubation (1½ hrs), perhaps from live worms in the initial 20 minutes and subsequently from the dying/dead worms, and may not have been associated with the nematode surface at any time. The T. spiralis E/S and CTAB preparations had other proteins with similar m.wts to those found in T. muris E/S products besides the 41 kD molecule, notably with m.wts of 33 kD and 22 and 19 kD respectively. Of course, similar m.wts alone do not mean that proteins are identical or necessarily related.

8.2 IMMUNOPRECIPITATION OF ^{35}S-METHIONINE-LABELLED TRICHURIS MURIS ANTIGENS

In order to obtain labelled anterior end products (AA) live worms were bisected at the stichosomal-intestinal junction and the anterior ends of the worms containing the stichosome and the oesophagus were then put into culture medium containing ^{35}S-methionine. In immunoprecipitation studies AA and E/S bound antigens were separated from unbound proteins using protein A-sepharose 4B.

Trichuris muris immune sera precipitate many antigens produced by the anterior end of this nematode (Figure 8.2.1, m.wts of proteins given in Table 8.2.1. ISAA precipitated the same material as tolerant serum from mice with heavy patent infections of T. muris, the m.wts of these antigens ranging from 15 to 121 kD (lanes 2 and 5). IS E/S and day 21 sera (lanes 3 and 4) did precipitate some material but the bands on the autoradiograph were very faint, this may indeed reflect

low specific antibody titres for AA material in these sera, indeed AA make up only a proportion of the total E/S. In contrast IS E/S had relatively high specific antibody to total T. muris E/S products used in ELISA, as did IS AA, day 21 infection serum however had low specific antibody titres to E/S products in ELISA. IS E/S serum also precipitated less labelled protein than ISAA when reacted with total labelled E/S antigens as opposed to the AA preparation (pg 177 Figure 7.5).

Trichinella spiralis primary infection serum (mice exposed to adult and larval stages of the nematode) precipitated a number of labelled T. muris AA antigens (Figure 8.2.1, lane 6), the prominent proteins visible had m.wts of 121, 109 and 47 kD, together with a blur of protein bands in the range 35 to 43 kD and a faintly visible antigen with m.wt 56 kD. The same T. spiralis serum when reacted with labelled Trichuris muris E/S products precipitated two prominent antigens with m.wts of 56 and 49 kD (Figure 8.1.2 lane 7). The aforementioned 47 kD and the latter 49 kD are possibly the same protein, which if present in greater quantities in the labelled E/S material than the labelled AA material could result in the larger band observed when the Trichinella spiralis infection serum was reacted with these two preparations (in Figure 8.4.1 the m.wt of the major T. muris protein precipitated by the T. spiralis serum was apparently 47 kD). Alternatively, there are two different antigens of similar m.wts in the T. muris E/S and AA preparations which are both recognised by the T. spiralis primary infection serum. A 56 kD antigen was recognised by the T. spiralis infection serum in the Trichuris muris AA and E/S labelled antigens.

Anti-Trichinella spiralis rabbit serum precipitated a protein

of apparent m.wt 45 kD from labelled Trichuris muris adult male homogenate (AMA) (Figure 8.2.2, reacted material isolated using protein A-sepharose 4B) and anti-Trichinella spiralis 48 kD MoAb precipitated a definite band with an approximate m.wt of 47 kD. Surprisingly there seemed to be considerable "background" material precipitated from the AMA preparation by the MoAb. Perhaps the epitope recognised by the anti-48 kD MoAb is also found on many of the labelled AMA products or more likely the binding site of the antibody is able to react with epitope(s) with similar configurations to the 48 kD epitope present on other AMA proteins. The results of immunoprecipitation studies using labelled Trichuris muris E/S products and anti-Trichinella spiralis 48 and 50/55 kD MoAbs are given in Section 8.4.1, the anti-48 kD MoAb did not precipitate any protein with a m.wt between 42 and 52 kD.

8.3 ELISA USING T. MURIS DAY 14 LARVAL HOMOGENATE WITH ANTI-T. MURIS AND ANTI-TRICHINELLA SPIRALIS ANTIBODIES

The ELISA (Figure 4.2.2) was performed using 10^{-2} dilutions of sera and goat anti-mouse IgG, A and M alkaline phosphatase conjugate. The day 14 larval homogenate (LH) was a crude antigen prepared from washed larvae collected by a modified Baermann technique. The results indicate not only that sera raised against adult T. muris antigens (IS E/S and IS AA) react strongly with day 14 larvae, but that the anti-Trichinella spiralis 48 kD MoAb was the strongest reacting antibody with this preparation.

8.4.1 Immunoprecipitation of ^{35}S-methionine labelled Trichuris muris E/S Products by Anti-T.muris and Anti-Trichinella spiralis Antibodies and Sera from Mice infected with Non-trichuroid Nematodes

The autoradiograph presented in Figure 8.4.1 shows pre-

cipitated T. muris E/S antigens isolated using protein A-sepharose, Table 8.4.1 gives the apparent m.wts of the precipitated proteins. The "background" obtained with naive serum consists of a blur of material, from a band at 42 kD up to a band at 35 kD, with a faint band at 124 kD. All the sera from mice with non-trichuroid nematode infections also precipitate these bands, albeit some do so more strongly. Trichinella spiralis primary infection sera precipitated a considerable amount of a T. muris E/S protein of m.wt 47 kD, however, the anti-T. spiralis 48 kD MoAb in addition to the background material seen with naive serum, only precipitated a 52 kD antigen and a low m.wt protein around 25 kD. All precipitated protein bands seen with the anti-T. spiralis 50/55 kD MoAb are very faint and little material above the background seen with naive serum was evident.

8.4.2 ELISA using Trichuris muris E/S Products with Infection Sera from other Nematode Infections

The same infection sera used in Section 8.4.1, with the exception of the anti-T. muris sera, were used in ELISA against T. muris E/S products. In addition, a day 21 T. muris infection serum was included which has relatively low specific antibody titres. If the results obtained in ELISA (Figure 8.4.2) are compared with the corresponding immunoprecipitation data, it appears that the high O.D. values obtained with the heterologous sera at 10^{-1} dilution are for the most part a result of non-specific binding to the solid phase. Non-specific binding is particularly problematic when sera have high immunoglobulin levels, as in immune sera. Dipetalonema vitea has the highest O.D. ratio, together with the Trichinella spiralis double infection sera, at 10^{-1} dilution. The former is known to have greatly elevated levels of serum IgM (Dr. Storey, pers. com.). Most .

of the non-specific binding was greatly reduced at higher serum dilutions, but if the dilution was too great the specific binding of the low titre T. muris serum ie. the day 21 infection serum and the cross-reacting Trichinella spiralis serum was not detectable above background O.D.s. However, the non-trichuroid infection sera did appear to show some cross-reaction with Trichuris muris E/S products in ELISA, this result is comparable to observations made in immunoprecipitation studies (Section 8.4.1), although the proteins precipitated by these infection sera were also precipitated by naive serum.

FIGURE 8.1 PROTEINS OF TRICHURIS MURIS AND TRICHINELLA SPIRALIS

10-20% gradient gel

lane

1 Markers : 94, 67, 43, 30, 20.1 and 14.4 kD

2 Trichuris trichiura adult homogenate (T. t. AH)

3 Trichuris muris adult male homogenate (T. m. AMA)

4 Trichuris muris adult E/S products with contaminating protein (T. m. E/Sc)

5 Trichuris muris adult E/S products (T. m. E/S)

6 Trichuris muris CTAB stripped surface antigen (T. m. CTAB S.A.)

7 Trichinella spiralis larval homogenate (T. sp. LH)

8 Trichinella spiralis larval E/S products with contaminating protein (T. sp. E/Sc)

9 Trichinella spiralis larval E/S products (T. sp. E/S)

10 Trichinella spiralis CTAB stripped surface antigen (T. sp. CTAB S.A.)

FIGURE 8.2.1 IMMUNOPRECIPITATION OF ^{35}S-METHIONINE LABELLED T. MURIS ANTIGENS FROM THE ANTERIOR END OF THE WORM (AA) AND E/S PRODUCTS 12% gel

lane

1 Markers : 92, 69, 46, 30 and 14.3 kD

2 T. m. IS AA + T. m. AA

3 T. m. IS E/S + T. m. AA

4 T. m. CFLP day 21 post-infection serum + T. m. AA

5 T. m. CFLP tolerant serum day 40 + T. m. AA

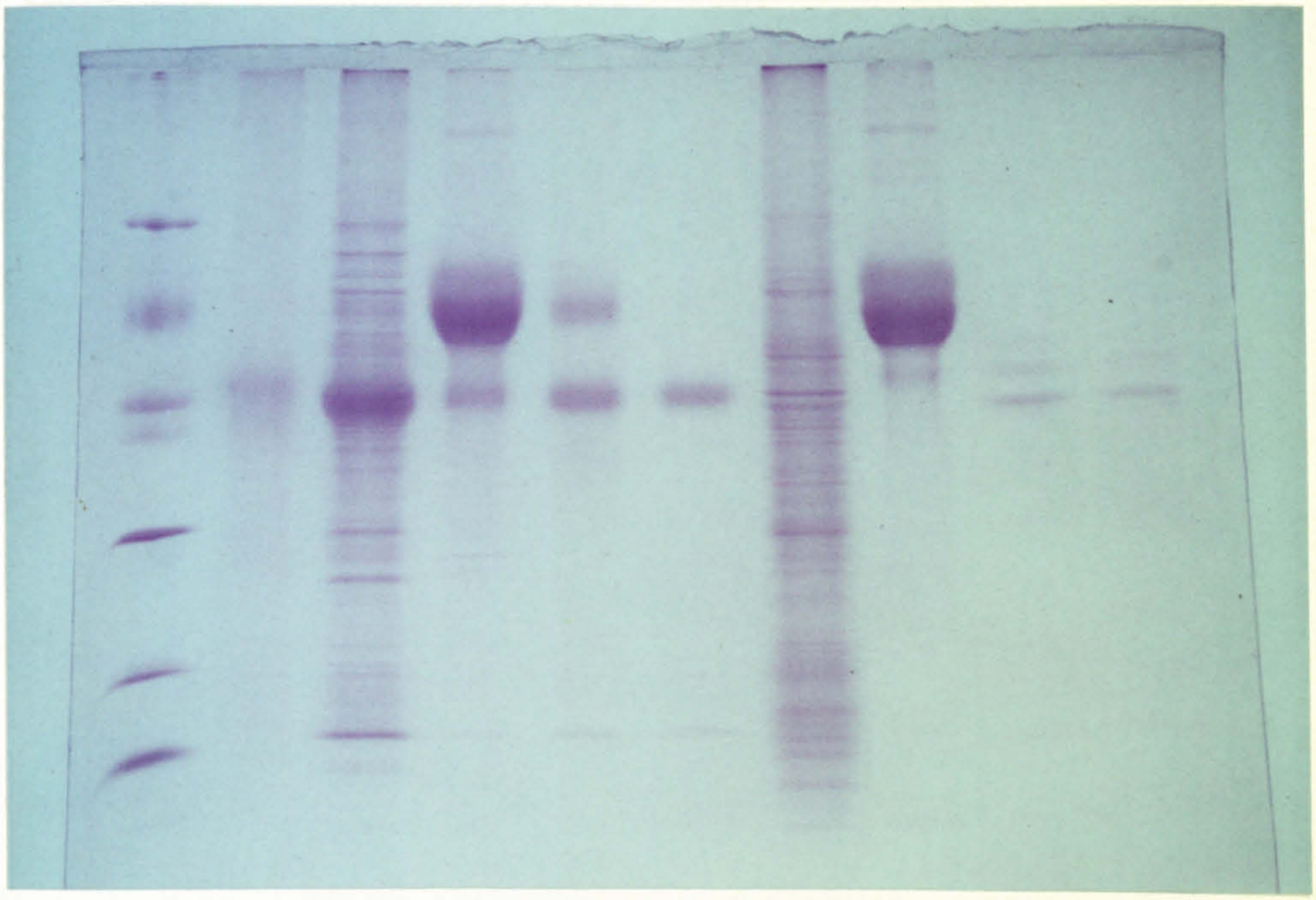
6 T. sp. primary infection serum + T. m. AA antigens

7 T. sp. primary infection serum + T. m. E/S antigens

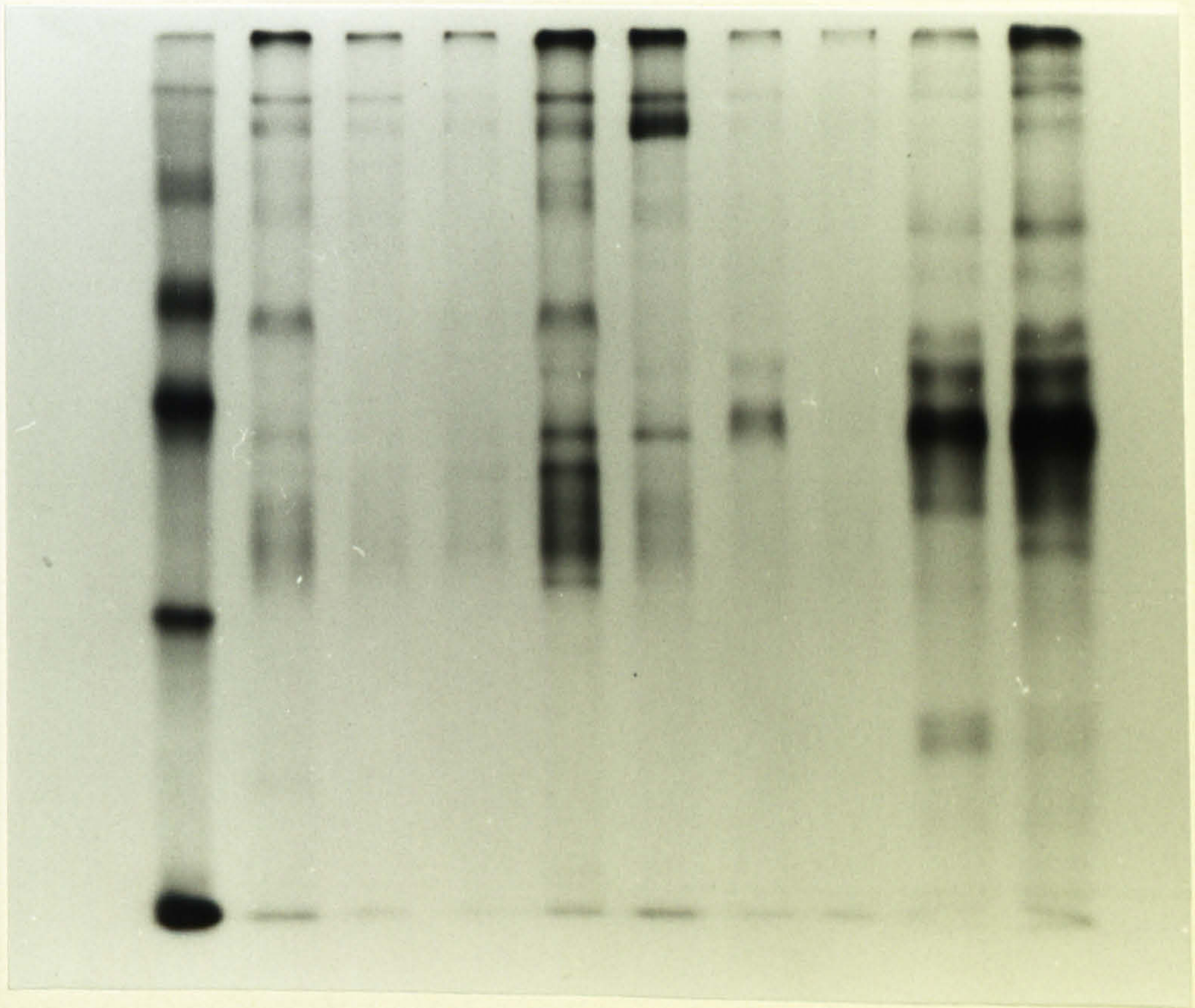
8 Naive mouse serum (NMS) + T. m. E/S

9 Anti-T. m. MoAb E15 + T. m. E/S antigens

10 Anti-T. m. MoAb E12 + T. m. E/S antigens



1 2 3 4 5 6 7 8 9 10



1 2 3 4 5 6 7 8 9 10

TABLE 8.1 PROTEINS OF TRICHURIS MURIS AND TRICHINELLA SPIRALIS

lane 2	lane 3	lane 4	lane 6	lane 9	lane 10
Trichuris trichiura	T. muris	T. muris	T. muris	Trichinella spiralis	T. spiralis
(AH)	(AMA)	(E/Sc)	(CTAB S.A.)	(Larval E/S)	(CTAB S.A.)
		71			
52				50	50
	49	49	49		
				44	44

TABLE 8.2.1 ^{35}S-METHIONINE LABELLED AA AND E/S ANTIGEN PRECIPITATED BY T. MURIS AND TRICHINELLA SPIRALIS IMMUNE SERA

IS AA+AA	IS ES+AA	d21+AA	TS+AA	T.sp.1°+AA	T.sp.1°+E/S
121[a]	121	121	121	121	121
109	109	109	109	109	(109)
98	(98)	(98)			
			94	(94)	
88	(88)		89	(89)	
65		65	65	(86)	
(54)			56	(56)	56
47		47	47	47	49
43	43	43	43	(43)	
38			38	38	
35		35	35	35	
31	31		31		
18					
			15		

Molecular weights given in kD

FIGURE 8.4.1 IMMUNOPRECIPITATION OF ^{35}S-METHIONINE LABELLED TRICHURIS MURIS E/S PRODUCTS 12% gel

lane

1 Markers : 92, 69, 46, 30 and 14.3 kD

2 T. m. CFLP d27 primary infection serum

3 T. m. CFLP d27 serum from tolerant (C.A treated) mice

4 T. sp. primary infection serum

5 Nematospiroides dubius, hyperimmune serum

6 Necator americanus, day 21 post-infection serum

7 Dipetalonema viteae, day 35 C57 BL/10 infection serum

8 Anti-T. sp. 48 kD MoAb

9 Anti-T. sp. 50/55 kD MoAb

10 Naive mouse serum (NMS)

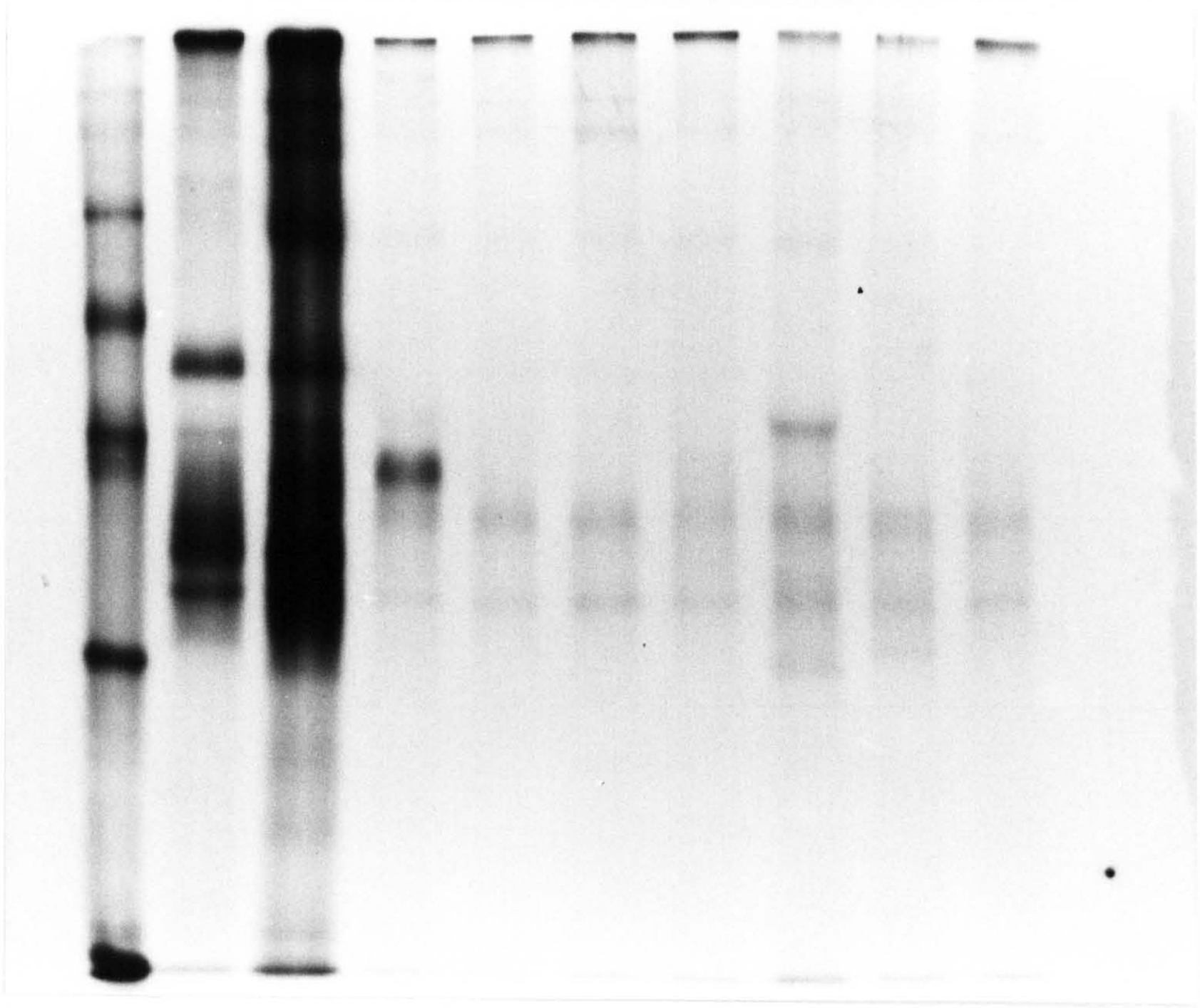
FIGURE 8.2.2 IMMUNOPRECIPITATION OF ^{35}S-METHIONINE LABELLED TRICHURIS MURIS AMA 12% gel

lane

1 Markers : 92, 69, 46, 30 and 14.3 kD

2 Anti-T. sp. rabbit serum

3 Anti-T. sp. 48 kD MoAb



1 2 3 4 5 6 7 8 9 10

TABLE 8.4.1 ^{35}S-METHIONINE-LABELLED T. MURIS E/S PRODUCTS PRECIPITATED BY OTHER NEMATODE INFECTION SERA AND ANTI-TRICHINELLA SPIRALIS MoAbs

CFLP T. muris sera		Trichinella spiralis serum and MoAbs			Naive serum	Nematospiroides dubius serum	Necator americanus serum	Dipetalonema viteae serum
d27	d27+C/A	Primary infn.	MoAb 48kD	MoAb 50/55kD				
(133)		(133)	(133)					
(122)	124	(124)	(124)	(124)	(124)	(124)	124	
(114)		(114)				(114)	114	(114)
	109							
(101)	101							
(91)	88	(88)	(88)	(88)			(88)	88
62	62							
52	52	(52)	52					
(46)	46	47						
42	42	42	42	42	42	42	42	42
39	39	(37)	37					
35	35	35	35		35	35	35	35
27	31			30				
	24		25					

Molecular wts. given in kD

TABLE 8.4.2 ELISA USING T. MURIS E/S PRODUCTS WITH SERA FROM MICE WITH OTHER NEMATODE INFECTIONS

O.D. Ratios test serum : naive serum.

	Serum				
Dilution	Trichuris muris	Trichinella spiralis	Nematospiroides dubius	Necator americanus	Dipetalonema vitae
10^{-1}	6.8	8.2	5.3	2.8	8.2
10^{-2}	6.2	3.5	2.9	2.4	3.4
10^{-3}	1.7	1.7	1.5	1.6	1.9
10^{-4}	0.7	1.0	0.8	0.9	1.4

Sera :

Trichuris muris - day 21 primary infection serum from CFLP mice.

Trichinella spiralis - immune serum from secondary infection in C57BL/10 mice.

Nematospiroides dubius - hyper immune serum.

Necator americanus - day 21 infection sera from susceptible BALB/c mice (expel worms by day 16, only get L_4 in gut d7 to d16)

Dipetalonema viteae - day 35 C57BL/10 infection serum, adults dead, mice amicro filaraemic

DISCUSSION

There is no doubt from the results presented that serum from Trichinella spiralis infected mice precipitates newly synthesized proteins produced and released into in vitro culture medium by anterior ends of adult Trichuris muris nematodes. The highest titres of specific antibodies were for two high m.wt proteins with approximate weights of 121 and 109 kD and a protein with an apparent m.wt of 47 kD. The latter antigen was also evident in T. muris E/S products and was again precipitated by Trichinella spiralis primary infection serum, together with a 56 kD protein, which was present to a lesser extent in precipitated AA material. Experiments with rabbit serum raised against T. spiralis antigens revealed that this immune serum also precipitated a protein with an apparent m.wt of 45 kD from Trichuris muris E/S products.

Paradoxically, anti-Trichinella spiralis 48 and 50/55 kD MoAbs raised against the stichocyte granule antigens of the muscle larvae of this nematode did not precipitate the 47 kD or the 56 kD Trichuris muris AA and E/S antigens precipitated by Trichinella spiralis infection serum. Although the anti-T. spiralis 48 kD MoAb did precipitate a number of proteins from Trichurus muris E/S products, none of these appeared to be particularly strongly recognised as only faint bands were visible on the autoradiographs produced; no proteins were precipitated with m.wts between 42 and 52 kD. MoAbs recognize only a single epitope on a particular antigen, it may therefore be that as far as the 47 kD T. muris antigen is concerned, the epitope recognized by the anti-Trichinella spiralis 48 kD MoAb is not present or is unavailable for binding in any sufficient quantity. The same may be true for the 56 kD T. muris E/S protein not precipitated by the

anti-50/55 kD MoAb. If antigen-antibody binding is only weak the washing of the antigen bound by immunoglobulin to protein A-sepharose 4B may dislodge the antigen, which would thus be removed in washing along with unbound material. In contrast Trichinella spiralis infection serum contains a range of specific antibodies with various affinities capable of reacting with different epitopes on a particular antigen. Hence, any common epitopes between Trichinella spiralis 48 kD and Trichuris muris 47 kD antigens would be recognized by the Trichinella spiralis infection serum; binding of antigen-antibody complexes is also likely to be stronger because of this. However, the anti-T. spiralis 48 kD MoAb did precipitate a protein with approximate m.wt 47 kD from ^{35}S-methionine labelled adult male Trichuris muris homogenate (AMA), other material also appeared to be precipitated but there was a definite band at 47 kD. The possibility thus arises that the 47 kD T. muris antigen, synthesized by the anterior end of the nematode, perhaps within the stichocytes, is altered upon secretion into the in vitro culture medium, such that the anti-Trichinella spiralis 48 kD MoAb no longer recognizes the appropriate epitope.

Nonetheless, the data presented here does suggest that the shared antigens responsible for the cross-immunity between Trichuris muris and Trichinella spiralis are synthesized and then secreted by the stichocytes of these worms. The "shared" antigens themselves are not necessarily identical in each nematode but appear to exhibit some homology in that they have similar m.wts ie. the 47 and 56 kD proteins of adult Trichuris and the 48 kD and 50/55 kD highly immunogenic stichocyte granule antigens of Trichinella muscle larvae. In addition the precipitation of the Trichuris antigens by heterologous sera does suggest shared epitopes between the two sets of antigens.

It is also of interest that major adult Trichuris and larval Trichinella E/S antigens, m.wts 49 kD and 50 and 44 kD respectively, were the major proteins found in the surface antigen preparations of these nematodes removed with detergent. However, whether the E/S proteins are adsorbed onto the worms surfaces after secretion or are newly synthesized by the hypodermal cells of the cuticle is not known. In addition Trichinella spiralis infection sera strongly recognized 121 and 109 kD Trichuris muris AA antigens, which were only slightly visible as bands on autoradiographs when the same serum was reacted with the E/S products.

To what extent the 47 kD and 56 kD adult Trichuris muris antigens are present in the larvae is mostly speculation at present. Nonetheless, it is likely that homologous antigens exist in the larvae, as in cross-protection experiments with the two nematodes the expulsive immune response in Trichuris infected animals is effected against the developing larvae. Interestingly, the anti-Trichinella spiralis 48 kD MoAb had high affinity for crude day 14 Trichuris larval homogenate in ELISA. In addition T. muris day 21 post-infection serum (which passively transfers immunity) did not have particularly high titres of specific antibody against adult 47 kD antigen as observed in immunoprecipitation studies. This suggests that although there must be some homology between the stichocyte products of Trichuris muris adults and larvae which elicit an expulsive immune response against the parasite, they are not identical as regards antigenic epitopes.

Finally, ELISA using "crude" T. muris E/S products may have only low specificity and give extensive cross reactions when immune sera from mice with other non-trichuroid nematode infections are

used. This was mainly thought to be the result of high levels of immunoglobulins in the immune sera tested which may have adhered to the ELISA solid phase. This problem was reduced to some extent by increasing the dilution of the sera tested in the assay. However, if T. muris infection sera have low specific antibody titres then the O.D. values obtained with such sera may not be much higher than naive background levels. As mentioned previously, the most important factor determining the sensitivity and specificity of an ELISA is the antigen used. Hence, in order to improve the diagnostic potential of ELISA using T. muris antigens a more purified preparation of E/S products would have to be used.

CHAPTER 9

CROSS-REACTIVITY BETWEEN HUMAN WHIPWORM *TRICHURIS TRICHIURA* AND THE MOUSE TRICHUROIDS, *TRICHURIS MURIS* AND *TRICHINELLA SPIRALIS*

INTRODUCTION

The possibility that there may be shared antigens between Trichuris muris and Trichuris trichiura has not previously been directly examined. Considering the cross-immunity which exists between T. muris and Trichinella spiralis, which is thought to be due to shared stichosomal antigens (Lee et al., 1982a; Chapter 8 this thesis), there is a reasonable probability that shared antigens also exist within the genus Trichuris itself. If this is the case, then there may be useful applications for the immunodiagnosis of trichuriasis, and perhaps the isolation of shared protective antigens for possible future use in vaccination against Trichuris infection could be considered. Hence, cross-reactivity between T. muris and T. trichiura infection sera and heterologous antigen has been investigated. Human infection sera from Jamaica and a sample of freeze dried T. trichiura collected from a patient following anthelmintic treatment were kindly donated by Dr. Bundy (Imperial College, London). A selection of human sera were tested in ELISA against T. muris E/S products and were also examined for their ability to immunoprecipitate ^{35}S-methionine-labelled E/S antigens. The T. trichiura antigen preparation (T. t. AH) was extremely crude as the dried male and female adult worms were contaminated with a considerable amount of faecal matter. However, with the limited amount of material available an investigation of coomassie and silver stained protein profiles of T. trichiura homogenate following SDS-PAGE was attempted. T. trichiura AH was used in ELISA with T. muris infection sera and immune sera raised against E/S products and anterior ends of the parasite (AA). Anti-T. muris MoAbs and anti-Trichinella spiralis 48 kD and 50/55 kD MoAbs (donated by

Drs. Silberstein and Despommier, New York) were also examined for reactivity in ELISA against the human trichuroid nematode.

RESULTS

9.1 SDS-PAGE PROTEIN PROFILES OF T. TRICHIURA ADULT HOMOGENATE

Although the T. trichiura antigen preparation made from adult worms was contaminated with a considerable amount of human faecal material, an attempt was made to determine whether or not any major proteins were present which might show some homology with those present in T. muris AMA. Gels are given on pgs 173,175 and 203 (Figures 7.1, 7.2, 7.3 and 8.1). The major protein observed in T. trichiura AH was of apparent molecular weight 44 kD when reduced, the non-reduced protein had a m.wt of around 41 kD. Faint protein bands were also visible on the coomassie stained gel (pg 173 Figure 7.1) with m.wts 32, 29, 20 and 18 kD. Unfortunately the gels stained with silver contained either too much protein (pg 174 Figure 7.2) or too little protein (pg 175, Figure 7.3) for bands to be clearly distinguishable, but even so it would not be possible to easily distinguish between parasite derived or contaminating proteins. Interestingly the major protein in adult T. muris AMA, E/S and CTAB stripped surface material is of approximate m.wt 41/42 kD, reduced and 38 kD non-reduced.

9.2.1 ELISA using T. muris E/S Antigens and Human T. trichiura Infection Sera

Based on the ELISAs performed with mouse sera (Chapter 3), a "checkerboard" investigation was carried out in order to determine

the optimum antigen concentration and serum dilution to use in ELISA with human sera. Human sera tends to give high backgrounds in ELISA and bovine serum albumin (BSA) was not an effective blocking agent, hence, as an alternative 10% (w/v) dried skimmed milk powder was used (made up fresh in distilled water). T. muris E/S antigen was used at 0.25, 0.5 and 1 µg/well, and human sera were tested in ten-fold dilutions from 10^{-2} to 10^{-5}. Naive mouse serum as well as immune mouse sera raised against T. muris E/S (IS E/S) and against the anterior ends of adult worms (IS AA) were includedin the test as reference standards. The following human sera were tested :

Serum	Worm Burdens			Age
	Trichuris	Ascaris	Hookworm	
Naive	No history of parasite infection			22 yrs
72	2	0	0	18-24 mo
139	0	0	0	18-24 mo
33	2	0	0	2-4 yrs
46	1016	6	0	2-4 yrs
113	433	+	0	5-10 yrs
204	0	0	0	5-10 yrs
23	523	3	0	11-30 yrs
53	0	0	0	11-30 yrs
67	0	0	0	over 30 yrs
107	33	4	0	over 30 yrs

ELISA results are presented in Tables 9.2.1 as O.D. ratios test sample : naive serum. The optimal results as regards maximal ratios which correlate with serum reactivity were obtained using sera at 10^{-3} dilution with T. muris E/S at either 0.25 µg/well or 0.5 µg/well. When antigen was used at 0.25 and 0.5 µg/well the O.D. ratios were comparable as similar background values for the naive serum

tested were obtained. However, the ratios for the human sera tested in ELISA with T. muris E/S antigen at 1 µg/well are disproportionately high. The background O.D. values for this group were as little as $^{1}/_{25}$ of the corresponding O.D.s obtained with antigen at 0.25 and 0.5 µg/well. Hence the two sets of data should not be directly compared.

Interestingly, human sera 46 and 113 from patients with relatively high current T. trichiura worm burdens (1016 and 433 respectively) consistently gave the highest O.D. ratios in ELISA with T. muris E/S antigen. The patients were in the 2-4 yr and 5-10 yr age groups respectively. The only other patient serum tested with a relatively high worm burden (523 worms) was number 23. However, the O.D. ratios obtained with this serum in ELISA were comparable to those obtained with sera from patients with no current worm burdens. Serum 23 was from the 11-30 yrs age group and the possibility does arise that a different level of humoral responsiveness may exist in adults and children. However, many more sera would have to be tested before any hypotheses on this subject are made.

9.2.2 Immune Precipitation of ^{35}S-methionine-labelled T. muris E/S Products by T. trichiura Infection Sera

Labelled antigens reacting with the test sera were separated from unreacted material using protein A-sepharose, hence the profiles seen in the autoradiographs (Figure 9.2.2) are those of antigens precipitated by IgG antibodies. Immune mouse sera, IS E/S and IS AA precipitated a range of antigens (the apparent m. wts. of which are given in Table 9.2.2), these antigens included most of those which were precipitated by any of the human sera, as far as may be determined by molecular weights alone. The background obtained with the naive human sera was greater than that with the

naive mouse sera, with the latter a very faint band is just visible around 34 kD, but with the former faint bands are visible in the region 36 to 53 kD. Despite this the jamaican sera showed greater reactivity ie. precipitated more antigens in greater quantity than the naive human serum. 72 and 139 apparently had the lowest antibody titres of all the jamaican sera and very little material was precipitated by these sera from individuals recorded as having 2 and 0 worms respectively. Their lack of antibody could be related to the fact that these individuals were the lowest age group tested, 18-24 months, and either had not yet experienced any sizeable T. trichiura infection, or were not producing specific antibodies which cross reacted with T. muris E/S, which can be seen with other sera. The distortion seen in the gel lanes of each of the human sera is due to overloading of proteins in the affected region, around 43 to 66 kD, this is most likely to be due to immuno-globulin heavy chains. The two sera which consistently gave the highest O.D. values in ELISA, 46 and 113 (1016 and 433 worms respectively) also precipitated the greatest amounts of labelled E/S antigen, heavy bands can be seen for a number of higher m.wt E/S Ags, notable bands in common between 46 and 13 having m. wts of approximately 137, 118, 91 and 75 kD, and lower m.wt. Ags with apparent weights of 43, 40, 32 and 27 kD. However, serum 23 (523 worms) precipitated similar amounts of material to sera with 33, 2 or 0 worms, it also gave relatively low O.D.s in ELISA.

9.3 ELISA USING T. TRICHIURA ADULT HOMOGENATE (T.t.AH) WITH INFECTION AND IMMUNE SERA RAISED AGAINST MOUSE TRICHUROIDS

ELISA was performed with early infection sera (10^{-2} dilutions) for CFLP infected mice and infected CFLP given cortisone acetate

which had antibody titres ranging from high to low for T. muris E/S antigens, and also late post-infection sera of NIH and C57BL/10 mice which had high antibody titres against T. muris E/S antigens (figure 9.3.1). The background O.D.s obtained with naive sera were relatively high as a result of the crudeness of the antigen preparation used. Notably the infection sera with the highest antibody titres against T. trichiura adult homogenate (AH) were the late post infection C57BL/10 sera, the NIH sera had some detectable antibody against T. trichiura AH but nothing comparable to the former.

Anti-T. muris immune sera raised in mice against E/S antigens and anterior ends of worms, IS E/S and IS AA respectively and anti-Trichinella spiralis 48 kD MoAb had very high affinities for Trichuris trichiura AH (Figure 9.3.2). Serum from CFLP mice with patent T. muris infections (TS d63) showed appreciable affinity for T. trichiura AH, and some reaction was also obtained with anti-T. muris E/S MoAb F11 and anti-Trichinella spiralis 50/55 kD MoAb.

TABLE 9.2.1 ELISA USING T. MURIS E/S ANTIGEN WITH SERA FROM T. TRICHIURA INFECTED INDIVIDUALS, O.D. RATIOS OF TEST SERUM:NAIVE SERUM

	Antigen = 0.25 µg/well Serum Dilution				Antigen = 0.5 µg/well Serum Dilution				Antigen = 1 µg/well Serum Dilution			
Mouse Sera	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Naive O.D.	0.164	0.157	0.076	0.063	0.184	0.052	0.075	0.014	0.105	0.041	0.038	0.041
ISE/S	17.4	7.3	2.7	1.9	15.8	24.6	3.2	1.1	3.0	2.9	3.5	1.2
ISAA	13.7	8.8	4.9	1.7	14.9	32.9	5.8	1.3	1.7	2.3	1.6	1.0
Human Sera (worm burden)												
Naive O.D.	0.364	0.062	0.056	0.064	0.252	0.041	0.079	0.053	0.095	0.002[a]	0.003[a]	0.016[a]
72(2)	1.1	1.0	1.1	0.9	1.3	1.2	0.5	0.7	1.6	9.5	4.3	8.0
139(0)	1.5	1.45	0.9	1.0	1.9	2.4	0.8	1.4	7.0	30.0	20.7	2.0
33(2)	2.4	2.0	0.8	0.6	2.7	4.3	1.0	1.5	2.4	49.5	14.0	2.1
46(1016)	4.5	5.8	2.0	1.0	7.0	11.7	2.3	2.0	1.2	32.5	10.0	1.1
113(433)	2.7	4.4	1.4	1.6	5.1	11.3	1.4	1.5	1.2	1.0	0.7	0.6
204(0)	1.2	2.4	1.3	1.1	2.5	4.4	0.7	1.1	1.2	2.5	3.7	2.3
23(523)	0.6	1.3	1.1	1.1	1.4	1.1	0.1	0.3	0.3	3.0	4.3	1.9
53(0)	0.8	1.7	0.9	0.8	2.1	3.2	0.3	0.4	0.3	9.5	1.0	0.1
67(0)	0.8	0.9	0.5	0.7	1.8	2.4	0.4	0.3	1.4	22.5	14.0	0.1
107(33)	1.8	2.8	0.8	0.7	2.8	3.5	0.7	1.0	1.4	35.0	25.7	3.1

Ratios of 2.5 and over underlined for 0.25 and 0.5 µg/well

[a]Unusually low background O.D. values resulted in high test:naive ratios which did not reflect the degree of positive reaction observed with the human sera at these dilutions with antigen at 1 µg/well.

FIGURE 9.2.2 IMMUNOPRECIPITATION OF ^{35}S-METHIONINE-LABELLED *T. MURIS* E/S PRODUCTS BY *T. TRICHIURA* INFECTION SERA 12% gel

lane

1 Markers : 92, 69, 46, 30 and 14.3 kD

2 Naive mouse serum (NMS)

3 Immune mouse serum raised against *T. muris* E/S products (IS E/S)

4 Immune mouse serum raised against *T.* muris AA (IS AA)

5 Naive human serum (NHS)

6 Jamaican serum no. 72 (*T. trichiura* = 2)

7 Jamaican serum no. 139 (*T. trichiura* = 0)

8 Jamaican serum no. 33 (*T. trichiura* = 2)

9 Jamaican serum no. 46 (*T. trichiura* =1016)

10 Jamaican serum no. 113 (T. trichiura = 433)

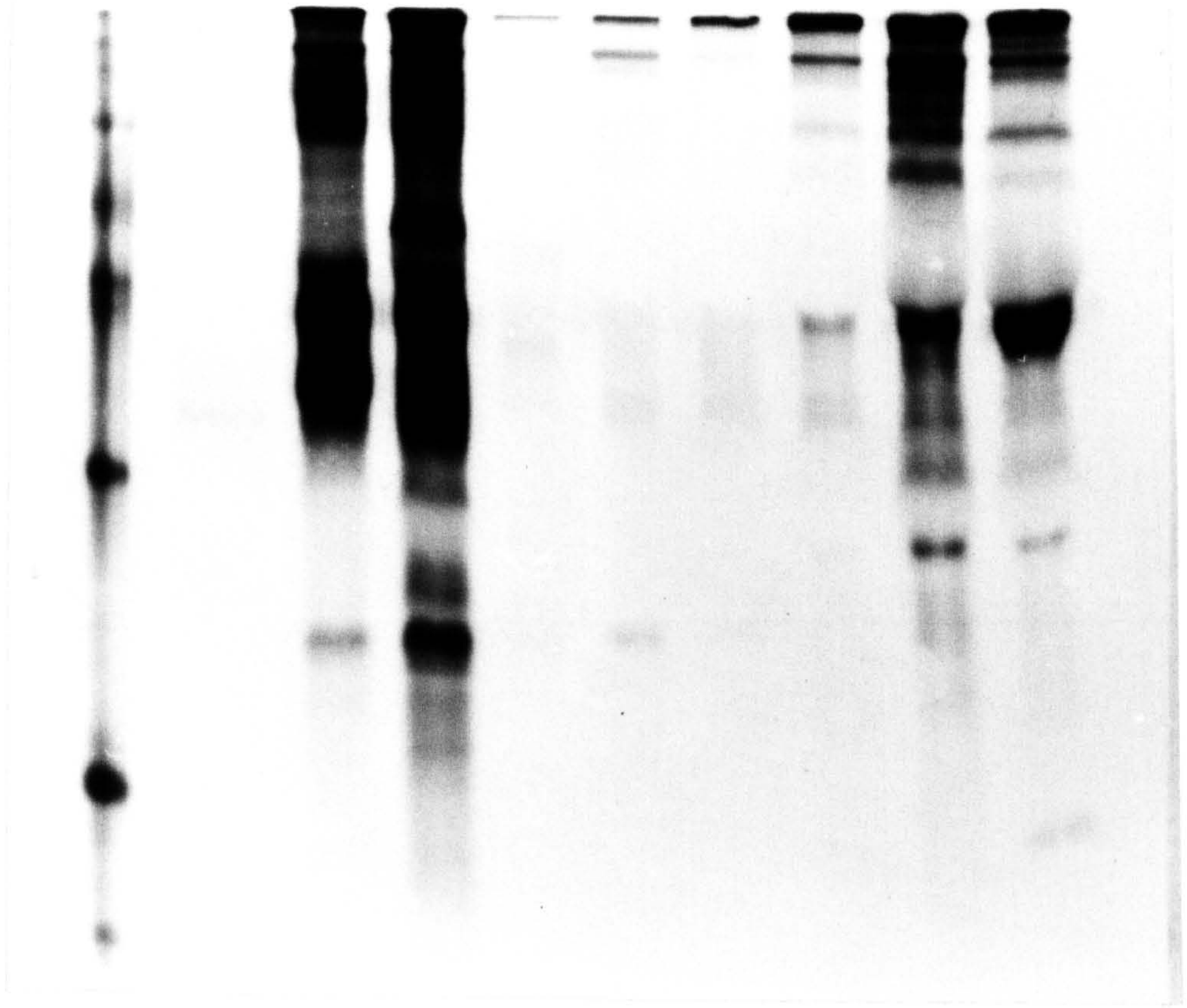
11 Jamaican serum no. 204 (*T. trichiura* = 0)

12 Jamaican serum no. 23 (*T. trichiura* = 523)

13 Jamaican serum no. 53 (*T. trichiura* = 0)

14 Jamaican serum no. 67 (*T. trichiura* = 0)

15 Jamaican serum no. 107 (*T. trichiura* = 33)



1 2 3 4 5 6 7 8 9 10

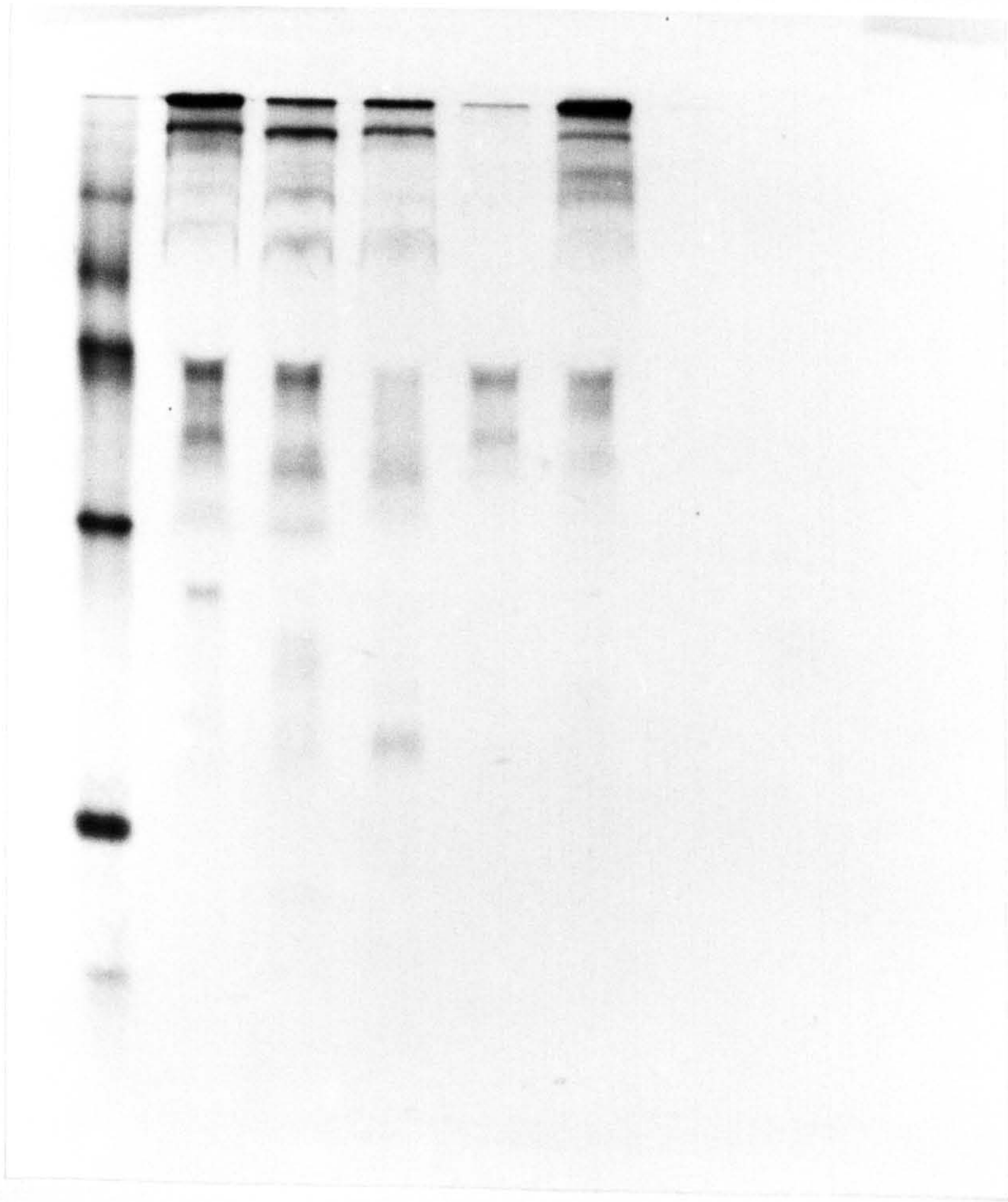


TABLE 9.2.2 IMMUNOPRECIPITATION OF ^{35}S-METHIONINE-LABELLED T. MURIS E/S PRODUCTS BY T. TRICHIURA SERA

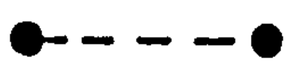
NMS	MS IS E/S	MS IS AA	NHS	HS 72	HS 139	HS 33	HS 46	HS 113
				(2)[a]	(0)	(2)	(1016)	(433)
								136
	128	128	(132)	132	(132)	132	127	127
(115)	115	115		115		(115)	118	118
	105	105					101	
	91	91				91	91	91
		59				(75)	75	(75)
	46	46						
	43	43	(55)					
	37	37						
		34				43	43	43
(34)	33	32	(40)	(41)	(41)		40	
		29					38	38
		24		(35)		(35)		
		21					32	32
	20	20					27	27
		19					25	
		17		22			22	
								12

HS 204	HS 23	HS 53	HS 67	HS 107
(0)	(523)	(0)	(0)	(33)
131				131
(109)	(109)			109
(95)	95	(95)		95
	77	77		(77)
43	43	(43)	43	43
39	(40)			40
37			37	(37)
34	34	34		
28		28		
25				
	22			
	18			
	17			

a - T. trichiura worm burdens given in brackets below the test sera

FIGURE 9.3.1 ELISA USING T. TRICHIURA AH WITH INFECTION SERA AGAINST T. MURIS

CFLP untreated sera



CFLP treated with C/A sera

C57BL/10 sera

NIH sera

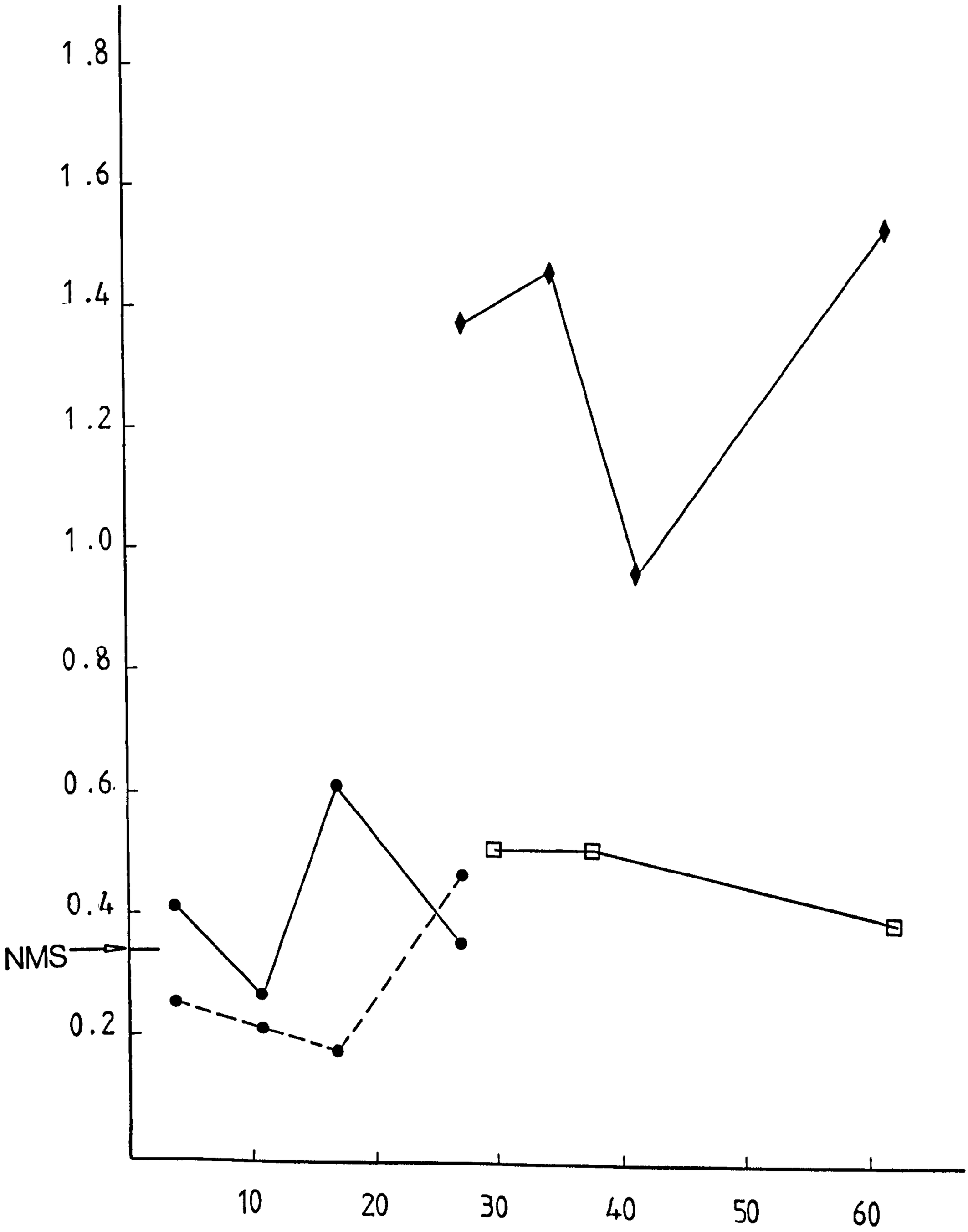
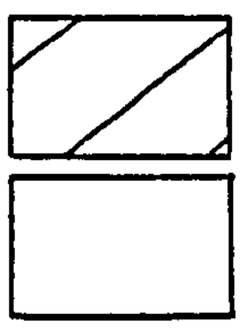

O.D.
1 .8
1 .6
1 .4
1 .2
1 .0
0.8
0.6
0.4
NMS
0.2
10
20
30
40
50
60
Days post - infection

FIGURE 9.3.2 ELISA USING T. TRICHIURA AH WITH ANTI-T. MURIS AND TRICHINELLA SPIRALIS SERA



anti-Trichinella spiralis MoAbs

anti-Trichuris muris sera

NMS

48 kD - MoAb against Trichinella spiralis 48 kD β-stichocyte granule antigen of infective larvae

50/55 kD - MoAb against T. spiralis 50/55 kD - α stichocyte granule antigen of infective larvae

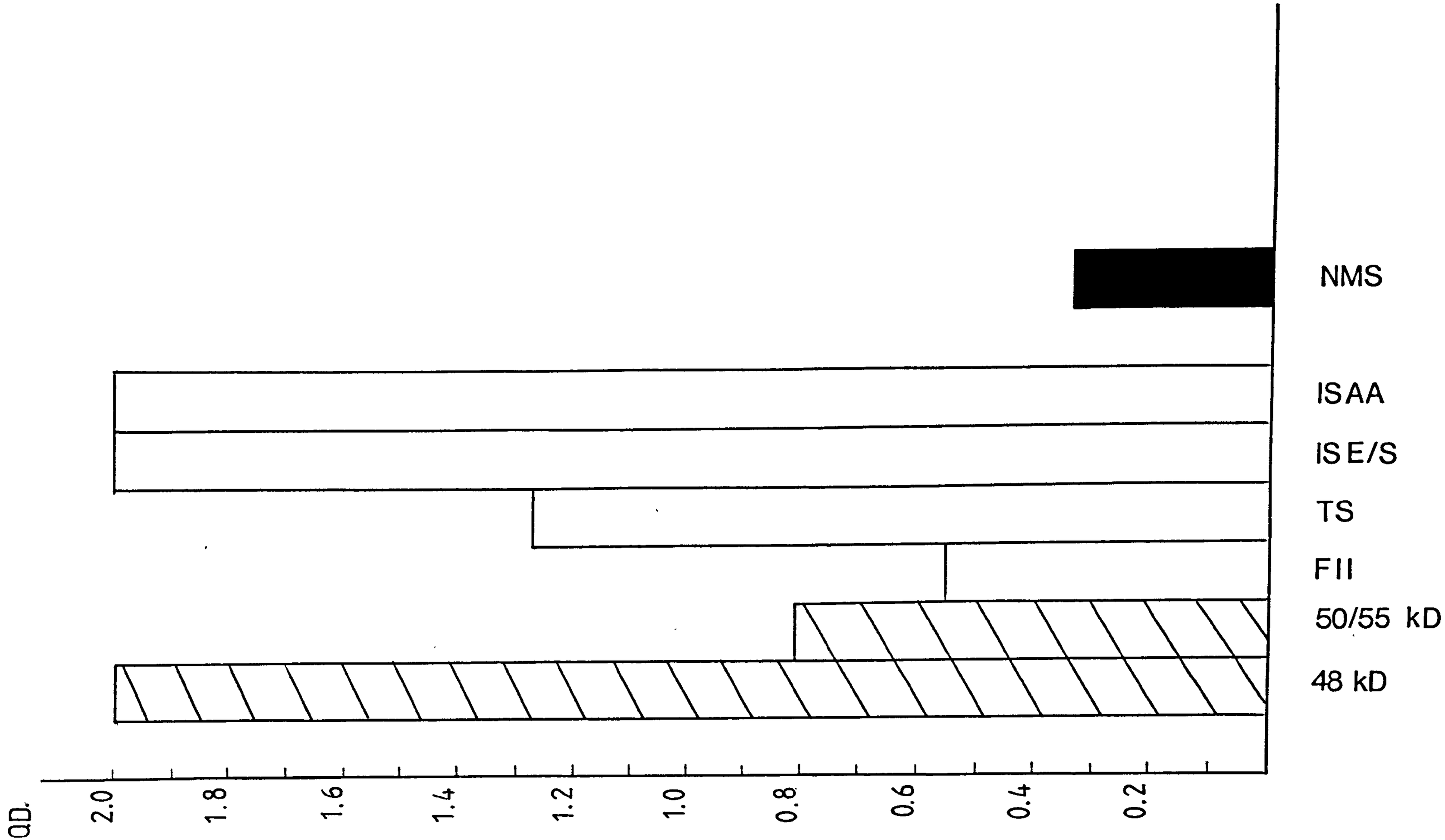
F11 - MoAb against adult Trichuris muris E/S products

TS - tolerant serum

ISE/S - immune serum raised against adult E/S products

ISAA - immune serum raised against anterior ends of adult worms

NMS - naive mouse serum


NMS
ISAA
ISE/S
TS
FII
50/55 kD
48 kD
OD.
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2

DISCUSSION

Bearing in mind the crudeness of the T. trichiura antigen preparation and its contamination with faecal matter SDS-PAGE analyses of this material nonetheless provided some interesting information. It appears that the major protein of T. trichiura adult homogenate (T. t. AH) has a similar molecular weight and tertiary structure to that of a major protein found in adult T. muris E/S, AMA and CTAB detergent stripped surface proteins. This major T. muris protein had an apparent m.wt of 41/42 kD when reduced and 38 kD when non-reduced compared with that of the T. trichiura protein which had an approximate m.wt of 44 kD reduced and 41 kD non-reduced. The 41/42 kD T. muris protein was not found in day 14 larval homogenate preparations. Similar m.wts of course do not mean that proteins are identical. However, the observations reported here, in particular the immunoprecipitation of labelled T. muris E/S antigens with approximate m.wts of 43 and 40 kD by the jamaican sera, do allow the tentative suggestion that a possibly homologous major protein may be present in adult T. trichiura and adult T. muris. Indeed, two of the sera from patients with the highest worm burdens (no 46 and 11, 1016 and 433 worms respectively) precipitated the greatest amounts of 43 kD T. muris E/S antigen.

The optimum ELISA protocols for human and mouse sera were slightly different, as naive human sera tended to give higher backgrounds than naive mouse sera particularly at low dilutions. This was possibly due to high levels of serum immunoglobulins in the human sera which tend to bind to the solid phase in ELISA. Bovine serum albumin (BSA), a non-reactive protein is often used as a blocking agent in ELISAs as Tween 20 alone is often not adequate to

prevent non-specific binding. However, in ELISA with human sera, a 10% solution of dried skimmed milk powder was found to be more effective than BSA, and optimal test sera:naive sera ratios were obtained using serum dilutions of 10^{-3}. Kenna et al. (1985) also found casein to be a more effective blocking agent than serum albumin or gelatin in ELISAs with low titre rabbit serum and suggested its effectiveness was probably due to its heterogeneity compared with the homogeneity of the other agents used. Proteins differ considerably in the ability with which they bind to solid phases (Cantarero et al., 1980), hence a mixture of proteins differing in size, amino acids, degree of glycosylation and sulphation and surface charge are more likely to be able to block available non-specific binding sites on the solid phase than a homogeneous protein solution. Kenna et al. (1985) also noted high binding of human IgG to the solid phase, and could only block this binding with casein if the antimicrobial agent thimerosal was included in the casein buffer and if tween 20 in the wash buffer was also replaced by casein/thimerosal. It was suggested that not only did human IgG have a greater affinity for the solid phase than casein, hence casein must be applied at least in the wash buffer immediately before the test sera were added, but that human sera may contain antibodies which bind to microbial contaminants present in the buffer which in turn compete successfully with casein for binding to the solid phase. The observation that the rabbit sera did not need inclusion of thimerosal in the casein buffer was thought to be due to the fact that experimental animals are kept in a relatively microbe free environment and so are not naturally exposed to, and do not produce antibodies against, the same range of different organisms as humans. Immunoassay problems due to high binding of human immunoglobulins to the solid phase have been reported by several groups

(Hamilton et al., 1979; Djurup et al., 1983; Schønheyder and Anderson, 1984), but not by others (eg. Bullock and Walls, 1977). This discrepancy may be at least in part due to variability between different assays in the ratios of specific to non-specific antibody binding, and no particular problem was found here with the human sera using skimmed milk as a blocking agent and diluting sera to 10^{-3}.

The results with ELISA and immunoprecipitation studies using human sera and T. muris E/S antigens showed that T. trichiura infected individuals may possess specific antibodies reacting with T. muris E/S antigens. Those sera giving the highest O.D.s in ELISA (46 and 113) tended to produce the strongest bands on the immunoprecipitation autoradiographs. There were particular affinities for antigens with apparent m.wts of 127, 118, 91, 75, 43, 40, 32 and 27 kD, antigens of similar molecular weights are also seen to be precipitated by immune mouse sera raised against T. muris E/S products and T. muris anterior ends (IS E/S and IS AA respectively). However, the presence of specific serum antibody against T. muris E/S cannot necessarily be used as an indicator of the presence or absence of current infections with T. trichiura or as an indication of worm burden. Although serum 46 (1016 worms) and serum 113 (433 worms) had high specific antibody titres and precipitated a considerable amount of labelled T. muris E/S, serum 23 (523 worms) gave results similar to those obtained with sera from individuals with 33, 2 and 0 worms. Most of the Jamaican sera from individuals with no current T. trichiura infections (or at least egg negative) showed reactivity above that obtained with the naive serum from England, even if this was not always seen in the ELISAs, it was clearly evident in the immunoprecipitation studies. The Jamaican sera which precipitated the smallest amounts of labelled T. muris E/S antigens were 72 and 139 (2 and 0 worms respectively)

and were from the youngest age group tested, 18-24 months. It could be that these individuals had not yet been exposed to T. trichiura infection at levels experienced by other individuals tested or do not make the same specific antibodies, in either quantity or quality, as the older individuals. Antigen specificity of the sera did vary slightly with all the individuals tested, but certain proteins were recognized by most individuals. It appears that people do have circulating anti-T. trichiura antibodies for some time after the worms have been cleared, as was seen in the T. muris/mouse model (Chapter 4), and these antibodies are able to cross-react with T. muris E/S products.

ELISAs using crude T. trichiura AH with T. muris infection and immune mouse sera, anti-T. muris and anti-T. spiralis MoAbs also produced interesting results. The high backgrounds observed with naive mouse sera were probably due to the crudeness of the antigen preparation and its contamination with non-worm material. Despite the high backgrounds, late C57BL/10 post-infection sera had relatively high antibody titres for T. trichiura AH, this sera also had high titres for T. muris E/S products (although the values obtained for the two ELISAs do not correlate exactly). In contrast the late NIH sera, which had high titres of antibody against T. muris E/S, only has just detectable activity against T. trichiura AH at around day 30 and 40, which then declines. The implication being that the late C57BL/10 and NIH sera are recognizing different antigens within the T. muris E/S and T. trichiura AH antigens, this is not surprising considering the persistence of worms in infected C57BL/10 mice, which may sometimes survive to patency, compared with the rapid expulsion of larvae by NIH before day 15. Of greater interest is the high affinity of anti-T. muris IS E/S and IS AA for T. trichiura AH,

which implies cross reaction between T. muris E/S products originating from the anterior ends of the worms ie. from the stichocytes, with the antigens of adult T. trichiura. As if to confirm this, anti-T. spiralis 48 kD MoAb also had very high affinity for T. trichiura AH, although that of the anti-T. spiralis 50/55 kD MoAb was somewhat less. As mentioned previously the 48 kD and 50/55 kD antigens of T. spiralis muscle larvae are located in the α and β granules respectively. There was some reactivity between anti-T. muris MoAbs and T. trichiura AH, but the antigens recognized by these antibodies have not yet been localized in T. muris. The infection serum from tolerant CFLP mice with large patent worm burdens showed considerable affinity for T. trichiura AH, suggesting that the antigenic material being released into these animals during a patent adult infection with T. muris has some immunological relationship to material found in T. trichiura AH.

To summarize, there is little doubt that cross-reactivity exists between circulating antibodies in T. trichiura infected humans and T. muris infected mice for heterologous antigen preparations. Considering the marked affinity of mouse immune sera raised against T. Muris E/S products and anterior ends for T. trichiura AH, together with the immunoprecipitation of labelled T. muris E/S antigens by T. trichiura infection sera, it is likely not only that the cross reacting antigens are in the E/S products of these worms, but that they originate from the stichocytes of these nematodes. The great avidity of the anti-T. spiralis 48 kD MoAb, and to a lesser extent that of the 50/55 kD MoAb, for T. trichiura AH lends credence to the suggestion that all three trichuroids, Trichuris muris, T. trichiura and Trichinella spiralis share common stichocyte antigens.

SUMMARY DISCUSSION

The investigations described in this thesis were concerned with the mechanisms of expulsion of Trichuris muris from the caecum and colon of the mouse, and an examination of the parasite itself in terms of characterization, localization and isolation of protective antigens known to be components of the E/S products of the nematode. In relation to the identification of the protective antigens of T. muris, the antigenic relationship between T. muris and Trichinella spiralis was examined in greater detail, as these nematodes share antigens which are effective in heterologous immunization (Lee et al., 1982b). It has been suggested that the stichosome of T. muris is the major source of the highly immunogenic components found in E/S products, but to date stichocyte antigens have been isolated only from Trichinella spiralis muscle larvae (Despommier and Muller, 1976), the purified 48 kD β granule and 50/55 kD α granule stichocyte antigens being highly effective in active immunization against subsequent infection with this parasite (Silberstein and Despommier, 1984). Experiments to determine whether shared stichosomal antigens are responsible for the cross immunity between T. spiralis and Trichuris muris have been performed and these studies were extended to make an initial investigation of potential cross reactivity between the human parasite T. trichiura and T. muris resulting from shared stichosomal antigens. MoAbs were produced against the E/S products of T. muris in order to aid in these investigations, and were characterized in terms of isotype and antigen specificities. However, localization of the recognized antigens in the parasite itself have not yet been successful.

Initial experiments involved quantitative and qualitative

assays of the humoral immune responses of various mouse strains which differ in the time at which parasite expulsion occurs. No significant quantitative differences were observed in the overall kinetics of the specific serum antibody responses measured in ELISAs using total T. muris E/S products, and antibody titres could not be correlated with expulsion times in NIH, C57BL/10, BALB/c, CFLP or DBA/2 mice. Although some qualitative differences were observed in immunoprecipitation studies with labelled E/S antigens, until the antigens concerned can be characterized in terms of their function in the worm and perhaps other activity in the host, the significance of the aforementioned differences in antibody specificities cannot be determined. The MoAbs produced may be useful investigative tools in such areas. As regards temporal correlation of humoral responses and expulsion of the parasite, measurements of locally secreted specific antibodies may be more fruitful than measurements of systemic specific antibody.

The differential immune responsivenes within the inbred DBA/2 strain to infection with T. muris, which results in the establishment of patent worm burdens in a proportion of these mice, offers a unique opportunity to examine the mechanisms at work in the expulsion of T. muris. The humoral responses of responding and tolerant DBA/2 mice were examined in greater detail, and the overall kinetics of detectable serum responses in both groups after infection were found to be similar to those measured for the other mouse strains tested. In addition, sera from both groups of mice had the same range of antigen specificities for T. muris E/S components as determined by immunoprecipitation analyses. The "defect" in mechanism in the tolerant DBA/2 mice, which prevents the development of an expulsive immune response to a primary infection was found to be permanent, and was not depend-

ent on the rate of development of an expulsive immune response in relation to the rate of worm growth. Initial experiments to find an alternative explanation for the state of immune tolerance to T. muris infection existing within the DBA/2 population, involved an investigation of the capacity of MLNC from both groups of mice to produce IL-2 in vitro upon mitogenic stimulation. These experiments were based on the hypothesis put forward by Malkovsky and Medawar (1984) that IL-2 deficit during antigen presentation resulted in immune tolerance. Although no differences were found in the capacities of MLNC from responding and tolerant DBA/2 mice to produce IL-2 in vitro, the possibility remains that there may have been differences in IL-2 receptor expression in these cell populations. Similar experiments concerning IL-2 production were carried out with the chemically induced tolerant mouse model (Lee and Wakelin, 1982b, 1983), in which cortisone acetate administration during larval development in a primary infection with T. muris in CBA/Ca mice results in the establishment of a heavy adult worm burden. As in DBA/2 mice this tolerance is permanent and secondary infections also establish, following clearance of primary infections. In this system the induction of tolerance is dependent on the inhibition of the immune response during the development of the larval stages of the primary infection. Once the adults have established, the mice are unable to expel the parasite even in the absence of immunosuppressive drugs ie. the induction of tolerance is dependent upon suppression at a critical time in the development of the primary immune response against developing larvae, but once induced leaves the host permanently unresponsive. MLNC were taken during the period of cortisone administration and were assayed in vitro for IL-2 production in response to mitogen. Although neither cortisone administration or T. muris infection alone

reduced the IL-2 producing capacity of the cells, there was an apparently synergistic interaction between the drug and the parasite which caused a dramatic decrease in the relative IL-2 producing capacity of the MLN T cells from animals given both treatments. Hence the basic mechanisms responsible for the induced tolerance in CBA/Ca mice and the inherent tolerance of the DBA/2 mice do differ. However, IL-2 has been shown to have a role in the former and may yet be shown to play a role in the latter, albeit by a different mechanism.

In order to analyse the humoral and cellular mechanisms of the immune response against T. muris in greater detail, and to be able to relate these to strain variations in either the time of onset or the lack of an expulsive response, the characterization and isolation of functional parasite antigens which elicit a protective response is required. The supposition that a major proportion of the E/S products of this parasite originate from the anterior end of the nematode, has been confirmed in this thesis, hence implicating the stichosome as an important secretory organ in T. muris, although the function of the stichosomal products cannot yet be determined. Of the major metabolically-labelled anterior end (AA) parasite products, those precipitated by immune sera raised against E/S material and AA homogenate, had apparent m.wts of 43/44 and 36/37 kD (reduced). In addition ISAA also strongly precipitated a 54/59 kD protein. SDS-PAGE of T. muris antigen preparations demonstrated that the major protein of adult male homogenate (AMA), 41/42 kD (reduced), was also the major protein in the E/S products and CTAB-stripped surface material. Whether the latter protein is a stichocyte product adsorbed onto the cuticle or is actually synthesized by the cuticle hypodermis to be transported onto the worm surface, cannot be determined by these studies alone. However, several of the anti- T. muris MoAbs produced do recognize this major

protein, as determined by immunoprecipitation studies with labelled E/S antigens. Localization of recognized antigens in the nematode using MoAbs would undoubtedly assist in identifying the source of these molecules.

Investigations were also carried out on the proteolytic activity of T. muris E/S products, as other workers have found proteolytic enzymes in tissue penetrating nematodes which are thought to be involved in burrowing and/or feeding of the parasite (Hotez and Cerami, 1983; Wertheim et al., 1983; Dresden et al., 1985; Hotez, et al., 1985). It has previously been suggested that the stichosomal products of T. muris may be involved in the digestion of host tissues (Lee and Wright, 1978) and the investigations performed confirmed that T. muris does excrete/secrete proteolytic enzymes in vitro which can digest casein and/or gelatin at mammalian physiological pH. The m.wts of the T. muris proteases able to digest the latter proteins corresponded to those of some of the labelled E/S products precipitated by a) anti-T. muris immune sera raised against E/S products and anterior ends of worms; b) infection sera from animals exposed to only larval worm stages (up to L_3 and L_4, d21IS and D27IS respectively); and c) tolerant sera from mice with patent adult infections. Of those recognized by various host sera, those E/S proteases of m.wts 133, 121, 91, 58/51 and 41 kD are the most prominent. Proteases of m.wts 133, 124 and 51 kD were also stripped from the worm surface using detergent. The presence of surface enzymes, whether adsorbed onto the cuticle after production at another site or whether produced within the cuticle itself, is an interesting observation and affords another example of a nematode with a physiologically active surface (see Lee, 1972; Chen and Howells, 1979; Howells and Chen, 1981). A number of different proteases (approximately 10) were detected in the

adult T. muris E/S products. These may all have related functions, but possibly operate under slightly different conditions, or alternatively these enzymes may have widely differing functions in the survival and maintenance of worms in the host. Nonetheless, an important consideration is that immunoprecipitation studies allow the suggestion that adult and larval T. muris may possess common functional proteolytic enzymes, recognition of which by the host may be an integral part of the mechanism by which this parasite is expelled before reaching patency in most of the laboratory mouse models examined. An analagous situation has been described for Ancylostoma caninum, where a proteolytic enzyme isolated from adult worms, and believed to play a role in the histolytic and anti-coagulant activities of the nematode, was also found to be present in L_3 larvae (Hotez et al., 1985). Indeed, adult hookworms obtained from dogs immunized against L_3 larvae were found to have greatly diminished capacity to feed on host blood in vivo (Miller, 1971). The final site of attachment of adult T. muris compared with adult A. caninum could render the former more susceptible to the effector mechanisms of the specific immune response against common larval/adult functional antigens.

Heavy infections of adult Trichuris muris, which establish following immunosuppressive drug treatment of mice during larval development, are able to survive in mice for up to 9 weeks without stimulating an expulsive immune response. When primary infections of tolerant mice were cleared with anthelmintic, secondary infections could be prevented from establishing if IMLNC were given to the animals at the same time as the secondary infection (Lee, 1982). Delay of the adoptive transfer of immune cells until the secondary infection had established resulted in inability to remove the adult worms. Hence, adult worms appear to have immunomodulatory capabil-

ities, which make them more difficult to remove from the gut than developing larval worms. In addition mice with chronic worm burdens have depressed humoral responses to T-dependent and T-independent antigens (Lee and Wakelin, 1983). Interestingly, in the present work, initial in vitro lymphocyte proliferation experiments suggested that adult T. muris E/S products have mitogenic activity. Hence, the possibility arises that adult T. muris may actively suppress the immune response by some as yet unidentified immune modulatory substance(s). Released mitogens may non-specifically exhaust B and/or T cell populations, thus preventing the usual rapid response to developing larvae, and may also divert the immune system from attacking established adult parasites.

A number of cross-reacting antigens of T. muris and Trichinella spiralis have been identified in terms of their m.wts. T. spiralis infection sera precipitated 121, 109 and 47 kD, and 56 and 47 kD antigens respectively from Trichuris muris E/S and AA products. In addition anti-Trichinella spiralis rabbit serum precipitated a 45 kD molecule from Trichuris muris E/S products. Paradoxically anti-Trichinella spiralis 48 kD and 50/55 kD MoAbs did not precipitate any significant quantity of Trichuris muris E/S products. The 47 and 56 kD proteins of T. muris may have some homology with the 48 and 50/55 kD stichocyte products of Trichinella spiralis muscle larvae, but may differ in the presence and/or availability of the MoAb binding epitopes. In addition the observation that anti- T. spiralis infection sera precipitated 56 and 47 kD Trichuris muris AA products does suggest that homologous antigens may originate from the stichosome. These antigens are not necessarily identical but exhibit homology in m.wt and some binding epitopes. It is also interesting that the major adult Trichuris muris and Trichinella spiralis infective larval

E/S proteins were also stripped from the surfaces of these worms with detergent. Indeed, the 48 kD β stichocyte product of T. spiralis has been demonstrated to be present in the gut lining and on the surface of the muscle larvae (Silberstein and Despommier, 1986). In addition both T. spiralis infective larvae and Trichuris muris adults have been shown to possess surface proteases, whether these are stichocyte products secondarily adsorbed onto the cuticle, or are synthesized at the cuticle site remains to be determined. The high affinity of anti- Trichinella spiralis 48 kD MoAb for day 14 Trichuris muris larval homogenate in ELISA suggests the presence of a homologous protein in the trichuroid larvae, probably a stichocyte product. However, this protein must differ in terms of specific binding epitopes from the adult stichocyte products, thought to be present in in vitro collected E/S and AA material; perhaps stichocyte products are altered upon secretion in T. muris.

Cross-reactivity was demonstrated between circulating antibodies in T. trichiura infected humans and T. muris infected mice for heterologous antigen preparations. The marked affinity of mouse immune sera raised against T. muris E/S products and anterior end homogenate for T. trichiura adult homogenate in ELISA, together with the immunoprecipitation of labelled T. muris E/S antigens by T. trichiura infection sera, makes it likely not only that the cross-reacting antigens are found within the E/S products of these worms, but that they originate from the stichocytes of these nematodes. The great avidity of the anti- Trichinella spiralis 48 kD MoAb, and to a lesser extent the 50/55 kD MoAb, for Trichuris trichiura adult homogenate lends credence to the suggestion that all three trichuroids, Trichuris muris, T. trichiura and Trichinella spiralis share common stichocyte antigens, albeit these antigens are not all present in

comparable developmental stages of the worms.

Finally, a number of MoAbs were produced to Trichuris muris E/S products which were characterized in terms of isotypes and antigen specificities. Preliminary experiments were performed to assess their ability to passively transfer immunity and to allow isolation of purified E/S antigens for use in active immunization studies. More extensive experiments in these areas, together with studies to localize the recognized antigens in the nematode and the use of purified antigens in in vitro assays, in order to characterize them in terms of function and activity, will lead to a more comprehensive understanding of the host-parasite relationship in this model system.

REFERENCES

ALIZADEH, H. AND MURRELL, K.D. (1984). The intestinal mast cell response to Trichinella spiralis infection in mast cell deficient W/W^{V} mice. J. Parasitol., 70:767.

ALLARDYCE, R.A. AND BIENENSTOCK, J. (1984). The mucosal immune system in health and disease, with an emphasis on parasitic infection. Bull. WHO, 62:7.

ALMOND, N.M. AND PARKHOUSE, R.M.E. (1985). Nematode antigens In: Current Topics in Microbiology and Immunology, 120. Parasite Antigens in Protection, Diagnosis and Escape, p 173. Ed. Parkhouse, R.M.E.

ALMOND, N. AND PARKHOUSE, R.M.E. (1986). Immunoglobulin class specific responses to biochemically defined antigens of Trichinella spiralis. Parasite Immunol., 8:391.

ANDERS, R.F., HOWARD, R.J. AND MITCHELL, G.F. (1982). Parasite antigens and methods of analysis. In: Immunology of Parasitic Diseases. Eds. Cohen, S. and Warren, K.S. 2nd ed, Blackwell Scientific, Oxford.

ANDERSON, R.M. (1982). The population dynamics and control of hookworm and roundworm infections. In: Population Dynamics of Infectious Diseases, p 67. Ed. Anderson, R.M. Chapman and Hall.

ANDERSON, R.M. AND MAY, R.M. (1982). Population dynamics of human helminth infections : control by chemotherapy. Nature 297:557.

ANDERSON, R.M. AND MEDLEY, G.F. (1985). Community control of helminth infections of man by mass chemotherapy. Parasitology, 90:629.

APPLEBY, P., HAWKINS, S., PRITCHARD, D.I., YEPEZ MULIA, L. AND CATTY, D. (1985). Improved methodology for the production of monoclonal antibodies against parasites. Immunology Letters, 9:225.

BASHONG, W. AND RUDIN, W. (1982). Comparison of surface iodination methods by electron microscope autoradiography applied in vitro to different life-cycle stages of Dipetalonema vitea (Filaroidea). Parasitology, 85:559.

BECK, W.J. AND BEVERLEY-BURTON, M. (1968). Pathology of Trichuris, Capillaria and Trichinella infections. Helminthological Abstracts, 37:1.

BEFUS, A.D. AND BIENENSTOCK, J. (1982). Factors involved in symbiosis and host resistance at the mucosal-parasite interface. In: Progress in Allergy, 31:76. Ed. Karger, S.

BEHNKE, J.M., HANNAH, J. AND PRITCHARD, D.I. (1983). Nematospiroides dubius in the mouse : evidence that adult worms depress the expression of homologous immunity. Parasite Immunol., 5:397.

BEHNKE, J.M. AND PARISH, H.A. (1981). Transfer of immunity to Nematospiroides dubius : co-operation between lymphoid cells and antibodies in mediating worm expulsion. Parasite Immunol. 3:249.

BEHNKE, J.M. AND WAKELIN, D. (1973). The survival of Trichuris muris in wild populations of its natural host. Parasitology, 67:157.

BELL, R.D., McGREGOR, D.D. AND DESPOMMIER, D.D. (1979). Trichinella spiralis : Mediation of the intestinal component of protective immunity in the rat by multiple phase-specific, anti-parasite responses. Exp. Parasitol. 47:140.

BRANDT, W. (1980). North-South, a programme for survival: report/ independent commission on international development issues. Pan Books (Pan World Affairs).

BRUCE, R.G. (1970). Structure of the oesophagus of the infective juvenile and adult Trichinella spiralis. J. of Parasitol. 56:540.

BRUCE, R.G. (1974). The formation and utilization of stichosome secretory globules in Trichinella spiralis In: Trichinellosis, p 49. Ed. Kim, C. Intext Educational Publishers, New York.

BULLOCK, S.L. AND WALLS, K.W. (1977). Evaluation of some of the parameters of the enzyme-linked immunosorbent assay. J. Inf. Diseases, 136 (suppl.) 5279.

BUNDY, D.A.P. (1986). Epidermiological Aspects of Trichuris and trichuriasis in Caribbean communities, (in press).

BUNDY, D.A.P., THOMPSON, D.E., COOPER, E.S., GOLDEN, M.H.N. AND ANDERSON, R.M. (1985). Population dynamics and chemotherapeutic control of Trichuris trichiura infection of children in Jamaica and St. Lucia. Trans. Roy. Soc. Trop. Med. Hyg., 79:759.

BUTLER, J.E. (1981). The amplified ELISA : principles of and applications for the comparative quantitation of class and subclass antibodies and the distribution of antibodies and antigens in biochemical separates. Methods Enzymol., 73:482.

CAMPBELL, W.C. (1963). Spontaneous cure in Trichuris muris infections in albino mice and its suppression by cortisone. J. Parasitol., 49:628.

CAMPBELL, W.C. (1968). Effect of anti-inflammatory agents on spontaneous cure of Trichinella and Trichuris in mice. J. Parasitol., 54:452.

CANTARERO, L.A., BUTLER, J.E. AND OSBORNE, J.W. (1980). The adsorptive characteristics of proteins for polystyrene and their significance in solid-phase immunoassays. Anal. Biochem., 105:375.

CHANDLER, A.C. (1937). Studies on the nature of immunity to intestinal helminths. VI. General resume and discussion. Am. J. Hyg., 26:309.

CHEN, S.N. AND HOWELLS, R.E. (1979). The uptake of dyes, monosaccharides and amino acids by the filarial worm Brugia pahangi. Parasitology, 78:343.

CHESSUM, B.S. AND DENMARK, J.R. (1978). Inconstant ELISA (letter) Lancet, 1:161.

CLARK, N.W.T., PHILIPP, M. AND PARKHOUSE, R.M.E. (1982). Non-covalent interactions result in the aggregation of surface antigens of the parasitic nematode Trichinella spiralis. Biochem. Journal, 206:27.

CLINARD, E.H., SAUNDERS, G.C. AND LEIGHTY, J.C. (1978). Prospects for use of the ELA test in control of trichinellosis in swine. In: Trichinellosis, Proceedings of the 4th Int. Conf., p 501. Eds. Kim, C. and Pawlowski, Z.S.

CLINARD, E.H. (1979). Identification and distribution of swine serum immunoglobulins that react with Trichinella spiralis antigens and may interfere with the ELA for trichinosis. Am. J. Vet. Res., 40:1558.

COLLER, H.A. AND COLLER, B.S. (1986). Poisson statistical analysis of repetitive subcloning by the limiting dilution technique as a way of assessing hybridoma monoclonality. In: Methods in Enzymology 121, Hybridoma Technology and Monoclonal Antibodies Eds Langone, J.J. and Van Vunakis, H.

COOPER, E.S. AND BUNDY, D.A.P. (1986). Trichuriasis in St. Lucia. In: Diarrhoea and Malnutrition in Children. p 91. Eds. McNeish, A.S. and Walker-Smith, J.A. Butterworths, London.

CORRADIN, G., ETLINGER, V.M. AND CHILLER, J.M. (1977). Lymphocyte specificity to protein antigens. I. Characterization of the antigen induced in vitro T cell independent proliferative response with lymph node cells from primed mice. J. Immunol., 119:1048.

CRANDALL, R.B. AND CRANDALL, C.A. (1972). Trichinella spiralis : immunologic response to infection in mice. Exp. Parastiol., 31:378.

CRANDALL, R.B. AND ZAM, S.G. (1968). Analysis of excretory-secretory products of Trichinella spiralis larvae by disc-electrophoresis and immunodiffusion. Am. J. Trop. Med. Hyg., 17:747.

CROLL, N.A., ANDERSON, R.M., GYORKOS, T.W. AND GHADIRIAN, E. (1982). The population biology and control of Ascaris lumbricoides in a rural community in Iran. Trans. Roy. Soc. Trop. Med. Hyg. 76:187.

CROLL, N.A. AND GYORKOS, T.W. (1979). Parasitic disease in humans : the extent in Canada. Canadian Med. Association J., 120:310.

CYPESS, R.H., EBERSOLE, J.L. AND MOLINARI, J.A. (1977). Specific antibody levels in the intestinal perfusates of Nematospiroides dubius infected mice. Int.Archs.Allergy Appl.Immunol., 55:496.

DAVIDSON, R.L. AND GERALD, P.S. (1976). Improved techniques for the induction of mammalian cell hybridization by polyethylene glycol. Somatic Cell Genetics, 2:165.

DENMARK, J.R. AND CHESSUM, B.S. (1978). Standardization of enzyme-linked immunosorbent assay (ELISA) and the detection of Toxoplasma antibody. Med. Lab. Sciences, 35:227.

DE SAVIGNY, D. (1975). In vitro maintenance of Toxocara canis larvae and a simple method for the production of toxocara ES antigen for use in serodiagnostic tests for visceral larva migrans. J. Parasitol., 61:781.

DESPOMMIER, D.D. (1974). The stichocyte of Trichinella spiralis during morphogenesis in the small intestine of the rat. In: Trichinellosis, p 239. Ed. Kim, C. Intext Educational Publishers, New York.

DESPOMMIER, D.D. (1977). Immunity to Trichinella spiralis. Am. J. Trop. Med. Hyg., 26:68.

DESPOMMIER, D.D. (1983). Stichosome biology, p 107. In: Trichinella and Trichinosis Ed. Campbell, W.C. Plenum Press, New York/London.

DESPOMMIER, D.D. AND LACCETTI, A. (1981). Trichinella spiralis : Partial characterization of antigens isolated by immuno-affinity chromatography from the large-particle fraction of the muscle larvae. J. Parasitol. 67:332.

DESPOMMIER, D.D., McGREGOR, D.D., CRUM, E.D. AND CARTER, P.B. (1977). Immunity to Trichinella spiralis II. Expression of immunity against adult worms. Immunology, 33:797.

DESPOMMIER, D.D. AND MULLER, M. (1976). The stichosome and its secretion granules in the mature muscle larvae of Trichinella spiralis. J. Parasitol., 62:775.

DJURUP, R., SØNDERGAARD, I., MINUVA, U. AND WEEKE, B. (1983). A nylon ball solid-phase radioimmunoassay for specific antibodies in human sera. Application to measurement of IgG antibodies to pollen allergens. J. Immunol. Methods, 62:283.

DOBSON, C. (1982). Passive transfer of immunity with serum in mice infected with Nematospiroides dubius : influence of quality and quantity of immune serum. Int. J. Parasitol., 12:207.

DRESDEN, M.H., REGE, A.A. AND MURRELL, K.D. (1985). Strongyloides ransomi : Proteolytic enzymes from larvae. Exp. Parasitol., 59:257.

DUNCAN, J.L., SMITH, W.D. AND DARGIE, J.D. (1978). Possible relationship of levels of mucosal IgA and serum IgG to immune unresponsiveness of lambs to Haemonchus contortus. Vet. Parasitol., 4:21.

DUPONT, E. AND WYBRAN, J. (1985). Mechanisms of action of corticosteroids. Int. J. Immunotherapy, 1:135.

DURHAM, C.P., MURRELL, K.D. AND LEE, C.M. (1984). Trichinella spiralis : Immunization of rats with an antigen fraction enriched for allergenicity. Exp. Parasitol., 57:297.

ENGVALL, E. AND PEARLMANN, P. (1972). Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labelled anti-immunoglobulin in antigen-coated tubes. J. Immunol., 109:129.

FAUBERT, G.M. (1976). Depression of the plaque forming cells to sheep red blood cells by the newborn larvae of Trichinella spiralis. Immunology, 30:485.

FAUBERT, G.M. (1982). The reversal of the immuno-depression phenomenon in trichinellosis and its effect on the life cycle of the parasite. Parasite Immunol., 4:13.

FERGUSON, J.D. AND CASTRO, G.A. (1973). Metabolism of intestinal stages of Trichinella spiralis. Am. J. Physiol., 225:85.

FIFE, E.H. (1971). Advances in methodology for immunodiagnosis of parasitic diseases. Exp. Parasitol., 30:132.

FORSYTH, K.P., COPEMAN, D.B., ABBOT, R.F., ANDERS, R.F. AND MITCHELL, G.F. (1981). Identification of radioiodinated cuticular proteins and antigens of Onchocerca gibsoni microfilariae. Acta Tropica, 38:329.

GABRIELSEN, A.A. Jr. AND JENSEN, J.B. (1982). Mitogenic activity of extracts from continuous cultures of Plasmodium falciporum. Trop. Med. Hyg., 31:441.

GAMBLE, H.R. (1985). Trichinella spiralis : Immunization of mice using monoclonal antibody affinity-isolated antigens. Exp. Parasitol., 59:398.

GAMBLE, H.R. AND GRAHAM, C.E. (1984). Monoclonal antibody-purified antigen for the immunodiagnosis of trichinosis. Am. J. Vet. Res., 45:67.

GARCIA, V.G., OSORIO, M.R. AND CASTRO, J.G. (1979). Application de la tecnica micro-ELISA de dobles anticuerpos en la investigacion de antigenos en la trichinosis experimental de la rata. Revista Iberica de Parasitologia, 39:55.

GHOSH, S. AND CAMPBELL, A.M. (1986). Multispecific monoclonal antibodies. Immunology Today, 7:217.

GILLIS, S., FERM, M.M., OU, W. AND SMITH, K.A. (1978). T cell growth factor : parameters of production and quantitative microassay for activity. J. Immunol., 120:2027.

GRELL, G.A.C., DESAI, P., WATTY, E., MULLER, R. AND SERGEANT, G.R. (1981). A survey of parasites in primary school children in Dominica, W.I. West Indian Med. J., 25:166.

GRELL, G.A.C. AND WATTY, E. (1976). Analysis of infections and infestations in Dominica W.I. West Indian Med. J., 25:166.

GRENCIS, R.K., CRAWFORD, C., PRITCHARD, D.I., BEHNKE, J.M. AND WAKELIN, D. (1986). Immunization of mice with surface antigens from the muscle larvae of Trichinella spiralis. Parasite Immunol., 8:587.

GRENCIS, R.K., RIEDLINGER, J. AND WAKELIN, D. (1985). L3T4-positive T lymphoblasts are responsible for transfer of immunity to Trichinella spiralis in mice. Immunology, 56:213.

GRENCIS, R.K.G. AND WAKELIN, D. (1983). Immunity to Trichinella spiralis in mice factors involved in direct anti-worm effects. Wiadomosci parazytologiczne T.XXIX,NR 4-6, 387.

GRIFFITHS, R.B. (1978). Veterinary aspects. In: The Relevance of Parasitology to Human Welfare Today - Symposia for BSP Vol. 16. Eds. Taylor, A.E.R. and Muller, R. Blackwell Scientific Publications.

HAMILTON, R.G., SOBOTKA, A.K. AND ADKINSON, JR. (1979). Solid phase radioimmunoassay for quantitation of antigen-specific IgG in human sera with ^{125}I-protein A from Staphylococcus aureus. J. Immunol., 122:1073.

HANLEY, W.B., BOYER, S.H. AND ANUGHTON, M.A. (1966). Electrophoretic and functional heterogeneity of pepsinogen in several species. Nature, 209:996.

HOGARTH-SCOTT, R.S. (1966). Visceral larva migrans - an immunofluorescent examination of rabbit and human sera for antibodies to the E/S antigen of the second stage larvae of Toxocara canis, Toxocara cati and Toxascaris leonina (Nematoda). Immunology, 10:217.

HOTEZ, P.J. AND CERAMI, A. (1983). Secretion of a proteolytic anticoagulant by Ancylostoma hookworms. J. Exp. Med., 157:1594.

HOTEZ, P.J., LE TRANG, N., McKERROW, J.H. AND CERAMI, A. (1985). Isolation and characterization of a proteolytic enzyme from the adult hookworm Ancylostoma caninum. J. Biological Chem., 260:343.

HOWELLS, R.E. AND CHEN, S.N. (1981). Brugia pahangi : feeding and nutrient uptake in vitro and in vivo. Exp. Parasitol., 51:42.

HSU, S-M, RAINE, L. AND FANGER, H. (1981). Use of Avidin-Biotin-Peroxidase complex (ABC) in immunoperoxidase techniques. A comparison between ABC and unlabelled antibody (PAP) procedures. J. Histochem. Cytochem., 29:577.

ISENSTEIN, R.S., MARKS, H.M., WEBERT, D.W., JUDKINS, J.C., PARKER, C.M. AND DESPOMMIER, D.D. (1985). Development of an enzyme immunoassay for surveillance of trichinellosis in swine. In: Trichinellosis (I.C.T.6) Ed. Kim, C.W. State Univ. of N.Y. Press.

JENKINS, T. (1970). A morphological and histochemical study of. Trichuris suis (Schrank 1788) with special reference to the host-parasite relationship. Parasitology, 61:357.

JENKINS, S.N. AND WAKELIN, D. (1977). The source and nature of some functional antigens of Trichuris muris. Parasitology, 74:153.

JONES, V.E., EDWARDS, A.J. AND OGILVIE, B.M. (1970). The circulating immunoglobulins involved in protective immunity to the intestinal stage of Nippostrongylus brasiliensis in the rat. Immunology, 18:621.

JUNG, R.C. AND BEAVER, P.C. (1951). Clinical observations on Trichocephalus trichuris (whipworm) infestation in children. Pediatrics, 8:548.

KENNA, J.G., MAJOR, G.N. AND WILLIAMS, R.S. (1985). Methods for reducing non-specific antibody binding in enzyme-linked immunosorbent assays. J. Immunol. Methods, 85:409.

KENNY, G.E. AND DUNSMOOR, C.L. (1983). Principles, problems and strategies in the use of antigenic mixtures for the ELISA. J. Clin. Microbiol., 17:655.

KERBEL, R.S. AND EIDINGER, D. (1971). Further studies of antigenic competition. III. A model to account for the phenomenon based on a deficiency of cell to cell interaction in immune lymphoid cell populations. J. Exp. Med., 133:1043.

KLEIN, J. (1982). Immunology - The Science of Self-Nonself Discrimination. John Wiley and Sons, Inc.

KNIGHT, R. AND CHEW, L.H. (1974). The interaction between Entamoeba histolytica and Trichuris muris infections in mice. Am. J. Trop. Med. Hyg., 23:590.

KOHLER, G. AND MILSTEIN, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495.

KUORI, P. AND VALDES-DIAZ, R. (1952). Present views on the pathogenicity of Trichuris trichiura. Revista Cubana Med. Trop. Parasitol., 8:37.

KUSEL, J.R., MACKENZIE, P.E. AND McLAREN, D.J. (1975). The release membrane antigens into culture by adult Schistosoma mansoni. Parasitology, 71:247.

LACKS, S.A. AND SPRINGHORN, S.S. (1980). Renaturation of enzymes after polyacrylamide gel electrophoresis in the presence of Sodium dodecyl sulfate. J. Biological Chem., 255:7467.

LARSH, J.E. AND RACE, G.J. (1975). Allergic inflammation as a hypothesis for the expulsion of worms from tissues : a review. Exp. Parasitol., 37:251.

LEE, D.L. (1972). Structure of the helminth cuticle. Adv. Parasitol., 10:347.

LEE, T.D.G., GRENCIS, R.K.G. AND WAKELIN, D. (1982). Specific cross-immunity between Trichinella spiralis and Trichuris muris : immunization with heterologous infections and antigens and transfer of immunity with heterologous immune mesenteric lumph node cells. Parasitology, 84:381.

LEE, T.D.G. (1982). Immunological consequences of infection of the mouse colon with Trichuris muris. PhD Thesis, University of Nottingham.

LEE, T.D.G. AND WAKELIN, D. (1982a). The use of host strain variation to assess the significance of mucosal mast cells in the spontaneous cure response of mice to the nematode Trichuris muris. Int. Arch. Allergy Applied Immunol., 67:302.

LEE, T.D.G. AND WAKELIN, D. (1982b). Cortisone-induced immunotolerance to nematode infection in CBA/Ca mice. I. Investigation of the defect in the protective response. Immunology, 47:227.

LEE, T.D.G. AND WAKELIN, D. (1983). Cortisone-induced immunotolerance to nematode infection in CBA/Ca mice. II. A model for human chronic trichuriasis. Immunology, 48:571.

LEE, T.D.G., WAKELIN, D. AND GRENCIS, R.K.G. (1983). Cellular mechanisms of immunity to the nematode Trichuris muris. Int. J. Parasitol., 13:349.

LEE, T.D.G. AND WRIGHT, K.A. (1978). The morphology of the attachment and probable feeding site of the nematode Trichuris muris (Schrank, 1788) Hall, 1916. Canadian J. Zoology, 56:1889.

LEFKOVITS, I. AND WALDMAN, H. (1984). Limiting dilution analysis of the cells of the immune system. I. The clonal basis of the immune response. Immunology Today, 5:265.

LEHTONEN, O.P. AND VILJANEN, M.K. (1980). Antigen attachment in ELISA. J. Immunol. Methods, 34:61.

LEONARD, W.J., DEPPER, J.M., UCHIYAMA, T., SMITH, K.A., WALDMANN, T.A. AND GREEN, W.C. (1982). A monoclonal antibody that appears to recognize the receptor for human T-cell growth factor; partial characterization of the receptor. Nature, 300:267.

LJUNGSTROM, I., ENGVALL, E. AND RUITENBERG, E.J. (1974). Proceedings: ELISA, enzyme linked immunosorbent assay - a new technique for serodiagnosis of trichinosis. Parasitology, 69:xxiv.

LEWERT, R.M. AND LEE, C.L. (1956). Quantitative studies on the collgenase-like enzymes of cercariae of Schistosoma mansoni and the larvae of Strongyloides ratti. J. Inf. Diseases, 99:1.

LITTLEFIELD, J.W. (1964). Selection of hybrids from matings of fibroblasts in vitro and their presumed recombinants. Science, 145:209.

LOWRY, O.N.J., ROSEBROUGH, A.L., FARR, R.J. AND RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biological Chem., 193:265.

LUMSDEN, D.L. (1975). Surface ultra structure and cytochemistry of parasitic helminths. Exp. Parasitol., 37:267.

MACKENZIE, C.D., JUNGERY, M., TAYLOR, P.M. AND OGILVIE, B.M. (1980). Activation of complement, induction of antibody to the surface of nematodes and the effect of these factors and cells on survival in vitro. Eur. J. Immunol., 10:594.

MAIZELS, R.M., DESAVIGNY, D. AND OGILVIE, B.M. (1984). Characterization of surface and excretory-secretory antigens of Toxocara canis infective larvae. Parasite Immunol., 6:23.

MAIZELS, R.M., MEGHJI, M. AND OGILVIE, B.M. (1983). Restricted sets of parasite antigens for the surface of different stages and sexes of the nematode parasite Nippostrongylus brasiliensis. Immunology, 48:107.

MAIZELS, R.M., PHILIPP, M. AND OGILVIE, B.M. (1982). Molecules on the surface of parasitic nematodes as probes of the immune response in infections. Immunological Rev., 61:109.

MALKOVSKY, M. AND MEDAWAR, P.B. (1984). Is immunological tolerance (non-responsiveness) a consequence of interleukin-2 deficit during recognition of antigen ? Immunology Today, 5:340.

MANCINI, G., CARBONARA, A.O. AND HEREMANS, J.F. (1965). Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry, 2:235.

MANSFIELD, J.M., LEVINE, R.F., DEMPSEY, W.L., WELLHAUSEN, S.R. AND HANSEN, C. (1981). Lymphocyte functions in experimental african trypanosomiasis. iv. Immunosuppression and suppressor cells in athymic nu/nu mice. Cell. Immunol., 63:210.

McGREEVY, P.B., RATIWAYANTO, S., TUTI, S., McGREEVY, M.M. AND DENNIS, D.T. (1980). Brugia malayi: relationship between anti-sheath antibodies and amicrofilaraemia in natives living in an endemic area of South Kalimantan, Borneo. Am. J. Trop. Med. Hyg., 29:553.

McKEAN, P.G., CARR, A. AND PRITCHARD, D.I. (1986). Further studies of the antigens of Necator americanus. Abstracts of B.S.P. Spring Meeting p48.

McLAREN, D.J. (1980). Schistosoma mansoni : The parasite surface in relation to host immunity. In: Tropical Medicine Research Studies Series Ed. Brown, K.N. Research Studies Press, John Wiley and Sons.

McLAREN, M., LILLYWHITE, J.E. AND AU, A.C.S. (1981). Indirect enzyme linked immunosorbent assay (ELISA) : Practical aspects of standardization and quality control. Med. Lab. Sci., 38:245.

MILLER, H.R.P. (1980). Expulsion of Nippostrongylus brasiliensis from rats with serum. I. The efficacy of sera from singly and multiply infected donors related to time of administration and volume of serum injected. Immunity, 40:325.

MILLER, H.R.P. (1984). The protective mucosal response against gastrointestinal nematodes in ruminants and laboratory animals. Vet. Immunol. Immunopathol., 6:167.

MILLER, H.R.P., WOODBURY, R.G., HUNTLEY, J.F. AND NEWLANDS, G. (1983). Systemic release of mucosal mast-cell protease in primed rats challenged with Nippostrongylus brasiliensis. Immunology, 49:471.

MILLER, T.A. (1971). Vaccination against the canine hookworm diseases. Adv. Parasitol., 9:153.

MILSTEIN, C. (1980). Monoclonal antibodies. Scientific American, 243:56.

MITCHELL, G.F. (1980). T cell dependent effects in parasitic infection and disease. In: Progress in Immunology, 4 Ed. Fougereau, M. and Dausset, J., Academic Press, London.

MITCHELL, G.F. (1981). Hybridoma antibodies in immunodiagnosis of parasitic infection. Immunology Today, 2:140.

NAIRN, R.C. (1976). Fluorescent Protein Tracing Churchill Livingstone, Edinburgh.

NEWBY, T.J. (1984). Protective immune responses in the intestinal tract, p 143. In: Local Immune Responses of the Gut Eds. Newby, T.J. and St okes, C.R. CRC Press.

NIMMO-SMITH, R.H. AND KEELING, J.E.D. (1960). Some hydrolytic enzymes of the parasitic nematode Trichuris muris. Exp. Parasitol., 10:337.

OGILVIE, B.M. AND JONES, V.E. (1971). Nippostrongylus brasiliensis: a review of immunity and the host parasite relationship in the rat. Exp. Parasitol., 29:138.

OGILVIE, B.M., PHILIPP, M., JUNGERY, M., MAIZELS, R.M., WORMS, M.J. AND PARKHOUSE, R.M.E. (1980). The surface of nematodes and the immune response of the host. In: The Host Invader Interplay. Ed. Van den Bossche, H. p 99. Elsevier/North-Holland, Biomedical Press, Amsterdam.

ORTEGA-PIERRES, G., CHAYES, A., CLARK, N.W.T. AND PARKHOUSE, R.M.E. (1984a). The occurrence of antibodies to hidden and exposed determinants of surface antigens of Trichinella spiralis. Parasitology, 88:359.

ORTEGA-PIERRES, G., CLARK, N.W.T. AND PARKHOUSE, R.M.E. (1986). Regional specialization of the surface of a parasitic nematode. Parasite Immunol., 8:613.

ORTEGA-PIERRES, G., MACKENZIE, C.D. AND PARKHOUSE, R.M.E. (1984b)*. Protection against Trichinella spiralis induced by a monoclonal antibody that promotes killing of newborn larvae by granulocytes. Parasite Immunol., 6:275.

OWEN, D. (1982). Common parasites of laboratory rodents and lagomorphs. Laboratory Animals Handbook, 1. London.

PAGGI, L. AND BERLINGUER, G. (1964). Elimintiasi intestinalie condizioni ambientali di una zona agricola della provincia di Roma (Charchitti) Parassitologia, 6:151.

PARKHOUSE, R.M.E. AND CLARK, N.W.T. (1983). Stage specific secreted and somatic antigens of Trichinella spiralis. Mol. Biochem. Parasitol., 9:319.

PARKHOUSE, R.M.E., CLARK, N.W.T., MAIZELS, R.M. AND DENHAM, D.A. (1985). Brugia pahangi : labelling of secreted antigens with ^{35}S-methionine in vitro. Parasite Immunol., 7:665.

PARKHOUSE, R.M.E., PHILIPP, M. AND OGILVIE, B.M. (1981). Characterization of surface antigens of Trichinella spiralis infective larvae. Parasite Immunol., 3:339.

PESCE, A.J., FORD, D.J., GAIZUTIS, M., et al. (1977). Binding of protein to polystyrene in solid-phase immunoassays. Biochim. Biophys. Acta, 492:399.

PETERS, W. (1978). Medical aspects - comments and discussion. In: The Relevance of Parasitology to Human Welfare Today. Eds. Taylor, A.E.R. and Muller, R. Blackwell Scientific Publications, Oxford.

PHILIPP, M. (1984). Acetylcholinesterase secreted by intestinal nematodes : a reinterpretation of its putative role of "biochemical holdfast" (letter). Trans. Roy. Soc. Trop. Med. Hyg., 78:138.

* ref. in text to infective larvae

PHILIPP, M., PARKHOUSE, R.M.E. AND OGILVIE, B.M. (1980). Changing proteins on the surface of a parasitic nematode. Nature, 287:538.

PHILIPP, M. AND RUMJANECK, F.D. (1984). Antigenic and Dynamic properties of helminth surface structures. Molec. Biochem. Parasitol., 10:245.

PIESSENS, W.F., WADEE, A.A. AND KURNIAWAN (1986). Regulation of immune responses in lymphatic filariasis. Ciba Foundation Symposium, No. 127. 15th May. (in press).

PIKE, E.H. (1963) Bionomic, blood and ^{51}chromium investigations of Trichuris muris and studies with two related species. PhD Thesis, Columbia University.

PONTECORVO, G. (1975). Production of mammalian somatic cell hybrids by means of polyethylene glycol treatment. Somatic Cell Genetics, 1:397.

POTTER, M., PUMPHREY, J.G. AND WALTERS, J.L. (1972). Growth of plasmacytomas in mineral oil conditioned peritoneal environment. J. Natl. Cancer Instit. (Washington), 49:305.

POULAIN, J., LUFFAU, G. AND PERY, P. (1976). Nippostrongylus brasiliensis in the rat : immune response in serum and intestinal secretions. Ann. Immunol. (Inst. Pasteur) 127c:215.

PRITCHARD, D.I., ALI, N.M.H. AND BEHNKE, J.M. (1984). Analysis of the mechanism of immunodepression following heterologous antigenic stimulation during concurrent infection with Nematospiroides dubius. Immunology, 51:633.

PRITCHARD, D.I., WILLIAMS, D.J.L., BEHNKE, J.M. AND LEE, T.D.G. (1983) The role of IgG_1 hypergammaglobulinaemia in immunity to the gastrointestinal nematode Nematospiroides dubius. The immunochemical purification, antigen-specificity and in vivo antiparasite effect of IgG_1 from immune serum. Immunology, 49:353.

RAMSEY, F.C. (1962). Trichuris dysentery syndrome. West Indian Med. J., 11:235.

RICHELS, I. (1955). Histologische Shidien zu den problem der Zellkonstanz : Untersuchungen zur mikroskopischen Anatomie im Lebenszyklus von Trichinella spiralis. Zentralbl. Bakteriol. Parasitenk. Krankh. Hyg. (I. Orig.), 163:46.

RHOADS, L. (1983). Trichinella spiralis : Identification and purification of superoxide dismutase. Exp. Parasitol., 56:41.

RHOADS, M.L. (1985). Glycosidases of Trichinella spiralis Molec. Biochem. Parasitol., 16:137.

ROITT, I.M., BROSTOFF, J. AND MALE, D.K. (1985). Immunology. Gower Medical Publishing, London.

ROTHWELL, T.L.W., ANDERSON, N., BREMNER, K.C., DASH, K.M., LE JAMBRE, L.F., MERRITT, G.C. AND NG, B.K.Y. (1976). Observations on the occurrence and specificity of antibodies produced by infected host against the acetylcholinesterase present in some common gastrointestinal nematodes. Vet. Parasitol., 1:221.

ROWE, D.S. (1980). The role of monoclonal antibody technology in immunoparasitology. Immunology Today, 1:30.

RUITENBERG, E.J., STEERENBERG, P.A. AND BROSNI, B.J. (1976). Evaluation of the reliability of immunoenzymatic techniques for serodiagnosis in Trichinella spiralis infections In: Immunoenzymatic Techniques. p 149 Amsterdam.

RUITENBERG, E.J. AND VAN KNAPEN, F. (1977). The enzyme-linked immuno-sorbent assay and its application to parasitic infections. J. Inf. Diseases, 136 (supplement) : S267.

SAMUELSON, J.C. AND CAULFIELD, J.P. (1982). Loss of covalently labelled glycoproteins and glycolipids from the surface of newly transformed schistosomula of Schistosoma mansoni J. Cell Biol., 94:363.

SCHØNHEYDER, H. AND ANDERSEN, P. (1984). Effects of bovine serum albumin on antibody determination by the enzyme-linked immuno-sorbent assay. J. Immunol. Methods, 72:251.

SCHROEDER, L.L. (1985). An ELISA to detect antibody specific for surface antigens of parasitic nematodes. J. Immunological Methods, 83:135.

SELBY, G.R. AND WAKELIN, D. (1973). Transfer of immunity against Trichuris muris in the mouse by serum and cells. Int. J. Parasitol, 3:717.

SELBY, G.R. AND WAKELIN, D. (1975). Suppression of the immune response to Trichuris muris in lactating mice. Parasitology, 71:77.

SHEFFIELD, H.G. (1963). Electron microscopy of the bacillary band and stichosome of Trichuris muris and Trichuris vulpis. J. Parasitol., 49:998.

SHEKARCHI, I.C., SEVER, J.L., LEE, Y.J., CASTELLANO, G. AND MADDEN, D.L. (1984). Evaluation of various plastic microtiter plates with measles, toxoplasma and gamma globulin antigens in enzyme-linked immunosorbent assays. J. Clin. Microbiol., 19:89.

SILBERSTEIN, D. (1984). Studies on antigens of Trichinella spiralis PhD. Thesis. Columbia University.

SILBERSTEIN, D.S. AND DESPOMMIER, D.D. (1984). Antigens from Trichinella spiralis that induce a protective response in the mouse. J. Immunol., 132:989.

SILVERSTON, N.A. (1962). Whipworms in a Cambridgeshire village. British Med. J., 2:1726.

SINCLAIR, I.J. (1982). Review of application of ELISA in helminthology. In: Current Topics in Veterinary Medicine and Animal Science, 22:35.

SINKOVICS, J.G. AND DREESMAN, G.R. (1983). Monoclonal antibodies of hybridomas. Rev. Inf. Diseases, 5:9.

SINSKI, E. AND HOLMES, P.H. (1977). Nippostrongylus brasiliensis: Systemic and local IgA and IgG immunoglobulin responses in parasitized rats. Exp. Parasitol., 43:382.

SMITH, H.V., QUINN, R., KUSEL, J.R. AND GIRDWOOD, R.W.A. (1981). The effect of temperature and antimetabolites on antibody binding to the outer surface of second stage Toxocara canis larvae. Mol. Bioch. Parasitol., 4:183.

SNIDER, D.P. AND UNDERDOWN, B.J. (1986). Quantitative and temporal analyses of murine antibody response in serum and gut secretions to infection with Giardia muris. Infection and Immunity, 52:271.

SONKSEN, P.H. (Ed.) (1974). RIA and saturation analysis. Brit. Med. Bull., 30.

STAINES, N.A. (1983). Monoclonal antibodies. In: Biochemical Research Techniques, No. 6. Ed. Wrigglesworth, J.M.

STAINES, N.A. AND LEW, A.W. (1980). Whither monoclonal antibodies ? Immunology, 40:287.

STOREY, N., BEHNKE, J.M. AND WAKELIN, D. (1986). Immunity to Dipetalonema viteae (Filaroidea) infections in resistant and susceptible mice. Acta Tropica., 35:86 (in press).

SVET-MOLDAVSKY, G.J., SHADHIJAN, G.S., CHEMYAKHOISKAYA, I.Y., MKHEIDZE, D.M., LITOVCHENKO, T.A., OZERETSKOVSKAYA, N.N. AND KADAGHIDZE, Z.G. (1970). Inhibition of skin allograft rejection in Trichinella infected mice. Transplantation, 9:69.

TAUSSIG, M.J. (1977). Antigenic competition. In: The Antigens, Vol. IV. Ed. Sela, M. Academic Press, N.Y.

TAYLOR, S.M., KILPATRICK, D. AND KENNY, J. (1978). The influence of age and husbandry of pigs on evaluation of the enzyme-linked immunosorbent assay (ELISA) for T. spiralis infection (author's transl.) Zentralbl. Veterinaermed., 25:282.

THARP, M.D., SEELIG, JR., L.L., TIGELAAR, R.E. AND BERGSTRESSER, P.R. (1985). Conjugated avidin binds to mast cell granules. J. Histochem. Cytochem., 33:27.

THEOFILOPOULOS, A.N. AND DIXON, F.J. (1980). Immune complexes in human disease. Am. J. Pathology, 100:529.

THOMPSON, J.P., CRANDALL, R.B., CRANDALL, C.A. AND NEILSON, J.T. (1981). Microfilaremia and antibody responses in CBA/H and CBA/N mice following injection of microfilariae of Brugia malayi. J. Parasitol., 67:728.

THORSEN, R.E. (1956a). Proteolytic activity in extracts of the esophagus of adults of Ancylostoma caninum and the effect of immune serum on this activity. J. Parasitol., 42:21.

THORSEN, R.E. (1956b). The stimulation of acquired immunity in dogs by injections of extracts of the esophagus of adult hookworms. J. Parasitol., 42:501.

TIJSSEN, P. (1985). Practice and theory of enzyme immunoassays. Lab. Techniques in Biochemistry and Molecular Biology, 15. Eds. Burdon, R.H. and Van Knippenberg, P.H.

TIKASINGH, E.S. (1981). Human parasites in the commonwealth Caribbean. Mimeographed document of the Caribbean Epidemiology Centre/Pan American Health Organisation. Trinidad.

UNDERDOWN, B.J. AND SCHIFF, J.M. (1986). Immunoglobulin A : Strategic defense initiative at the mucosal surface. Ann. Rev. Immunol., 4:389.

VAIDYA, H.C., DIETZLER, D.N. AND LADENSON, J.H. (1985). Inadequacy of traditional ELISA for screening hybridoma supernatants for murine monoclonal antibodies. Hybridoma, 4:271.

VETTER, J.C.M. AND KLAVER-WESSELING, J.C.M. (1978). IgG antibody binding to the outer surface of the infective larvae of Ancylostoma caninum. Z. Parasitenkd., 56:147.

VOLLER, A., BARTLETT, A. AND BIDWELL, D.E. (1976). Enzyme immunoassays for parasite diseases. Trans. Roy. Soc. Trop. Med. Hyg., 70:98.

VOLLER, A., BIDWELL, D.E. AND BARTLETT, A. (1979). The enzyme-linked immunosorbent assay (ELISA). A guide with abstracts of microplate applications. Dynatech Europe, Guernsey.

VOLLER, A., DE SAVIGNY, D. (1981). Diagnostic serology of tropical parasitic diseases. J. Immunol. Methos., 46:1.

WADEE, A.A. AND PIESSENS, W.F. (1986). Microfilariae of Brugia malayi contain a T cell mitogen. Am. J. Trop. Med. Hyg., 35:141.

WAKELIN, D. (1967). Acquired immunity to Trichuris muris in the albino laboratory mouse. Parasitology, 57:515.

WAKELIN, D. (1970a). Studies on the immunity of albino mice to Trichuris muris. Suppression of immunity by cortisone acetate. Parasitology, 60:229.

WAKELIN, D. (1970b). The stimulation of immunity and the induction of unresponsiveness to Trichuris muris in various strains of laboratory mice. Z. Parasitenk., 35:162.

WAKELIN, D. (1973). The stimulation of immunity to Trichuris muris in mice exposed to low level infections. Parasitology, 66:181.

WAKELIN, D. (1975a). Immune expulsion of Trichuris muris from mice during a primary infection:analysis of the components involved. Parasitology, 70:397.

WAKELIN, D. (1975b). Genetic control of immune responses to parasites : immunity to Trichuris muris in inbred and random bred strains of mice. Parasitology, 71:51.

WAKELIN, D. (1978). Immunity to intestinal parasites. Nature, 273:617.

WAKELIN, D. (1980). Genetic control of immunity to parasites. Infection with Trichinella spiralis in inbred and congenic mice showing rapid and slow responses to infection. Paraiste Immunol., 2:85.

WAKELIN, D. AND DENHAM, D.A. (1983). The immune response, p 265. In: Trichinella and Trichinosis Ed. Campbell, W.C. Plenum Press, New York.

WAKELIN, D. AND SELBY, G.R. (1973). Functional antigens of Trichuris muris. The stimulation of immunity by vaccination of mice with somatic antigen preparations. Int. J. Parasitol., 3:711.

WAKELIN, D. AND SELBY, G.R. (1974). Thymus-dependency of the immune response of mice to a primary infection with the nematode Trichuris muris. Int. J. Parasitol., 4:657.

WAKELIN, D. AND SELBY, G.R. (1976). Immune expulsion of Trichuris muris from resistant mice : suppression by irradiation and restoration by transfer of lymphoid cells. Parasitology, 72:41.

WAKELIN, D. AND WILSON, M.M. (1977). Transfer of immunity to Trichinella spiralis in the mouse with MLNC : time of appearance of effective cells in donors and expression of immunity in recipients. Parasitology, 74:215.

WAKELIN, D. AND WILSON, M. (1979). T and B cells in the transfer of immunity to Trichinella spiralis in mice. Immunology, 37:403.

WARDLEY, R.C. AND CROWTHER, J.R., (1982). (Eds) The ELISA:Enzyme-linked immunosorbent assay in veterinary research and diagnosis. Current topics in veterinary medicine and animal science, 22. Martinus Nijhoff Publishers. The Hague/Boston/London.

WARREN, K.S. AND MAHMOUD, A.A.F. (1984). Tropical and geographical medicine. McGraw-Hill, New York.

WEISS, N. (1978). Studies on Dipetalonema viteae (Filaroidea). I. Microfilaraemia in hamsters in relation to worm burden and humoral immune response. Acta Trop., 35:137.

WERTHEIM, G., LUSTIGMAN, S., SILBERMAN, H. AND SHOSHAN, S. (1983). Demonstration of collaginase activity in adult Strongyloides ratti (Nematoda:Strongyloididae) and its absence in the infective larvae. J. Helminthology, 57:241.

W.H.O. (1982). Properties of the monoclonal antibodies produced by hybridoma technology and their application to the study of diseases. Eds. Houba, V. and Chan, S.H. UNDP/WORLD BANK/WHO.

WILLIAMS, D.J. AND BEHNKE, J.H. (1983). Host protective antibodies and serum immunoglobulin isotypes in mice chronically infected or repeatedly immunized with the nematode parasite Nematospiroides dubius. Immunology, 48:37.

WRIGHT, K.A. (1972). The fine structure of the esophagus of some trichuroid nematodes. I. The stichosome of Capillaria hepatica, Trichuris myocastoris and Trichuris vulpis. Canadian J. Zoology, 50:319.

YELTON, D.E. AND SCHARFF, M.D. (1980). Monoclonal antibodies. American Scientist, 68:510.

YOUDIN, S., STUTMAN, O. AND GOOD, R.A. (1973). Studies of delayed hypersensitivity to L. monocytogenes in mice : nature of cells involved in passive transfers. Cell. Immunol., 6:98.

ZINGALES, B. (1984). Genes and Antigens of Parasites : A laboratory manual. 2nd edition. p 333. SPRTTD, UNDP/World Bank/WHO.

ZINKERNAGEL, R.M. AND DOHERTY, P.C. (1974). Immunological surveillance against altered self components by sensitised T lymphocytes in lymphocytic choriomenigitis. Nature, 251:547.