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IMMUNOGENETICS OF *TRICHURIS MURIS* INFECTION

by

Kathryn J. Else, B.Sc.

Thesis submitted to the University of

Nottingham for the degree of

Doctor of Philosophy,

October 1989.

To my parents and Andy

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ABSTRACT

Investigations have been made into the genetic control of immunity to the nematode *Trichuris muris*. Both background genes and genes within the mouse major histocompatibility complex (MHC), H-2, were shown to influence the expulsion of *T. muris* with the former having the stronger influence. At least two genes within the H-2 complex determined response phenotypes, the effects of "resistance" or "susceptibility" alleles at I-A being modulated by resistance or susceptibility alleles at a D end locus/loci. Differential responsiveness within slowly responding mouse strains suggested that parasite-dependent effects were also important.

The primary antibody response to *T. muris* excretory/secretory (E/S) antigen, predominantly an IgG response, was also shown to be controlled by background and H-2-linked genes. In general, mouse strains less resistant to infection developed higher levels of IgG than more resistant strains of mice. However strains of mice possessing the $\underline{H\text{-}2}^q$ haplotype, irrespective of their genetic background, rapidly developed higher levels of IgG1 antibodies than strains of other haplotypes, $\underline{H\text{-}2}^q$ haplotype mice tending to be more resistant to infection. Recognition of two high molecular weight (MW) E/S antigens by IgG as revealed by immunoprecipitation was also found to be almost exclusively $\underline{H\text{-}2}^q$ restricted. This restriction may be partly quantitative but as such would operate *in vivo* due to the restriction on the ability to produce high levels of specific IgG. Both $\underline{H\text{-}2}^q$ restricted phenomena may be part of, but not absolute requirements for, protective immunity.

Parasite-induced effects on host immunity were also studied. Later larval and adult stages of *T. muris* were shown to be immunosuppressive, immunosuppression being long lasting and preventing the expulsion of subsequent infections.

Vaccination with E/S antigen was shown to protect strains of mice

which are slow to expel worms (poor–responder) or totally unable to expel worms (non–responder) from a primary infection with *T. muris.* However protection was slow to be expressed. Antigen recognition profiles of vaccinated strains of mice differed from their primary infection recognition profiles and included the recognition of the two high MW antigens shown to be H–2q restricted in a primary infection. Thus altering the mode or route of E/S antigen presentation may lead to shifts in responsiveness of H–2 genotypes to specific determinants and/or boost specific antibody levels sufficiently to reveal recognition of these antigens. Prior experience of a patent primary infection prevented vaccination protecting non–responder mice against subsequent infections. This inability was correlated with suppressed IgG1 antibody levels and failure to recognise three high MW antigens including the H–2q restricted antigens.

Using a panel of monoclonal antibodies raised against E/S antigen it was shown that E/S antigens, apparently including both immunogenic and immunosuppressive molecules, were localised to granules within the stichocyte cytoplasm of the adult *T. muris* stichosome.

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ABBREVIATIONS

AA	Anterior end antigen
AMA	Adult male antigen
APS	Ammonium persulphate
B10	C57BL/10 ScSn strain of mouse
BSA	Bovine serum albumin
C/A	Hydrocortisone 21-acetate
C.A.	Stichocyte antigen
C.E/S	Stichocyte excretory/secretory antigen
ConA	Concanavalin A (type IV)
c.p.m.	Counts per minute
CTAB	Cetyltrimethyl-ammonium bromide
d	Day
DMSO	Dimethylsulphoxide
EEA	Embryonated egg antigen
ELISA	Enzyme-linked immunosorbent assay
E/S	Excretory/secretory antigen
FACS	Fluorescence activated cell sorting
FCA	Freund's complete adjuvant
FCS	Foetal calf serum
FDA	Fluorescein diacetate
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-macrophage colony stimulating factor
H-2	The mouse major histocompatibility complex
HLA	Human leucocyte antigen
^{3}H-TdR	(Methyl-3H)thymidine
IFN-γ	Gamma interferon

Ig	Immunoglobulin
Il	Interleukin
i.p.	Intraperitoneal
i.v.	Intravenous
kDa	Kilodaltons
L1(L2,L3,L4)	First (second, third, fourth) stage larvae
LPS	Lipopolysaccharide
M	Molar
McAb	Monoclonal antibody
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
MW	Molecular weight
NS	Naive serum
OD	Optical density
O/N	Overnight
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
p.c.	Post challenge
PC	Phosphorylcholine
PCF	Pseudocoelomic fluid
p.i.	Post infection
PPO	2'5 diphenyloxazole
RT	Room temperature
rpm	Revolutions per minute
s.c.	Subcutaneous
SDS	Sodium dodecyl sulphate
S/N	Supernatant
SPL	Spleen
TBS	Tris buffered saline

TCA	Trichloro–acetic acid
TEMED	N,N,N',N'–tetramethylethylenediamine
T_H cell	Helper T lymphocyte
TNF	Tumour necrosis factor
Tris	Tris(hydroxymethyl)aminomethane
T.S.	Tolerant serum
UEEA	Unembryonated egg antigen
1D	One dimensional.

There are four things that make this world go round: love, energy, materials and information. We see about us a critical shortage of the first commodity, a near–critical shortage of the second, increasing shortage of the third, but an absolute glut of the fourth.

Robert A. Day

CHAPTER 1

INTRODUCTION

Despite considerable advances in the fields of immmunology and molecular biology parasites remain a continued and unacceptable threat to man and his domestic animals, contributing to the mortality and chronic diseases suffered by millions of people in the tropics and subtropics and causing significant economic loss. In 1947 Stoll reported that, out of all human infectious diseases, gastro-intestinal helminthiases were amongst the most prevalent and today the global picture remains very similar. Although needs are great there is still no readily available vaccine against any human parasite (reviewed by McLaren and Terry, 1989) and despite the availability of cheap, safe, effective chemotherapeutic agents for most of the major human helminth infections (Van den Bossche, 1978) the estimated 1,000 million cases of *Ascaris lumbricoides*, 500-800 million cases of *Trichuris trichiura*, over 600 million cases of hookworm (*Necator americanus* and *Ancylostoma duodenale*) and 300 million cases of filarial infections (mainly *Wuchereria bancrofti* and *Onchocerca volvulus*) (Anderson and May, 1982; Cooper and Bundy, 1988) testify to their ineffectiveness. Clearly attention needs to be paid to ways of targeting the available therapeutic agents to the most heavily infected individuals in order to benefit the community as a whole. However such approaches, even if successful, will solve only part of a much more complex problem; many parasites have evolved strategies by which they exist in nutritionally rich yet paradoxically immunologically hostile host enviroments. A greater understanding of protective immune responses and host-parasite interactions is required before the elimination of disease symptoms and/or the eradication of the infection can become a serious proposition.

1.1 *TRICHURIS TRICHIURA* IN MAN

The nematode *Trichuris trichiura* is one of the most ubiquitous of

human parasites, its prevalence exceeded probably only by that of *Ascaris lumbricoides* with which it is often coextensive (for recent reviews on human trichuriasis see Cooper and Bundy, 1987, 1988; Bundy 1988). The global incidence of trichuriasis, predominantly a disease of children, is wide, prevalence being over 70% in parts of Asia and Africa, and nearly 60% in the Carribean (Cooper and Bundy, 1988). Despite its prevalence, trichuriasis is often considered a harmless infection. Most *T. trichiura* infections are light (less than 100 worms) and thus often asymptomatic, infections reach clinical significance only when worm burdens are large. Although severe trichuriasis syndrome, characterised by the production of profuse mucus, bloody diarrhoea, tenesmus and rectal prolapse, with associated anaemia, finger clubbing and growth stunting (Fernán-Núñez, 1927; Jung and Beaver, 1951; Gilman *et al.*, 1983) is associated only with burdens exceeding 500 worms (Bundy, 1986), infections with fewer worms can still cause severe disease. Less severe forms of trichuriasis, associated with milder chronic dysentry and growth stunting are largely under-recognised and under-reported yet can have a considerable impact on public health; the harmfulness of trichuriasis lies in the chronic insidious nature of the disease (Cooper and Bundy, 1988). Community-based chemotherapeutic control programmes have indicated that *T. trichiura* is intrinsically more difficult to control than, for instance, *Ascaris*, worm populations recovering to pre-control levels after chemotherapy in approximately seven months. Such resistance to control is probably related to the high basic reproductive rate of *T. trichiura* populations (Bundy *et al.*, 1985*a*). Epidemiological aspects of trichuriasis in the Caribbean have been studied in detail by Bundy *et al.* (reviewed by Bundy, 1986; Cooper and Bundy, 1988; Bundy, 1988). Prevalence of *T. trichiura* in a St. Lucian community was found to rise rapidly with age, plateauing at 80-100% after the age of two to four years (Bundy *et al.*, 1987*a*), with distributions being typically overdispersed within

each age class (Bundy *et al.*, 1985*b*; 1987*a*). Although similar proportions of adults and children were infected the age-intensity population profile revealed that young children had larger worm burdens than adults. Studies of reinfection rates demonstrated that the rate of re-acquisition of infection also exhibited age-dependency (Bundy *et al.*, 1988) child age classes being reinfected faster than adults. Such age-dependency of infection intensity and re-infection rate may reflect a reduced exposure to infection in adults and/or their development of a partially effective acquired immunity (Anderson and May, 1985). Although the degree of exposure to *T. trichiura* is difficult to quantify, one potentially major route of childhood infection is provided by the ubiquitous habit of geophagia in endemic regions (Cooper and Bundy, 1987), infection occuring through the ingestion of embryonated eggs. Gilman *et al.*, 1983 showed a relationship between intense infection with *Trichuris* and deliberate soil eating, and Bundy (1988) also reported an increase in intensity of *T. trichiura* infection with increasing frequency of geophagia. Thus the peak in infection within the child age classes may be related to increased exposure.

Individuals have been shown to be predisposed to heavy or light infections with *T. trichiura*, there being a significant correlation between initial infection intensity and the intensity acquired by the same individual after drug treatment (Bundy *et al.*, 1987*b*). This again may reflect individual differences in exposure or susceptibility. Studies on the clustering of *T. trichiura* and *Ascaris lumbricoides* infections within households (Forrester *et al.*, 1988) revealed that heavily infected individuals were clustered in particular households. Such clustering could arise from members of the same household being exposed to similar concentrations of eggs and/or genetic similarities between family members influencing their ability to express a protective immune response. Indeed preliminary evidence, which correlates HLA-class II antigens with infection intensity, does suggest a role for host

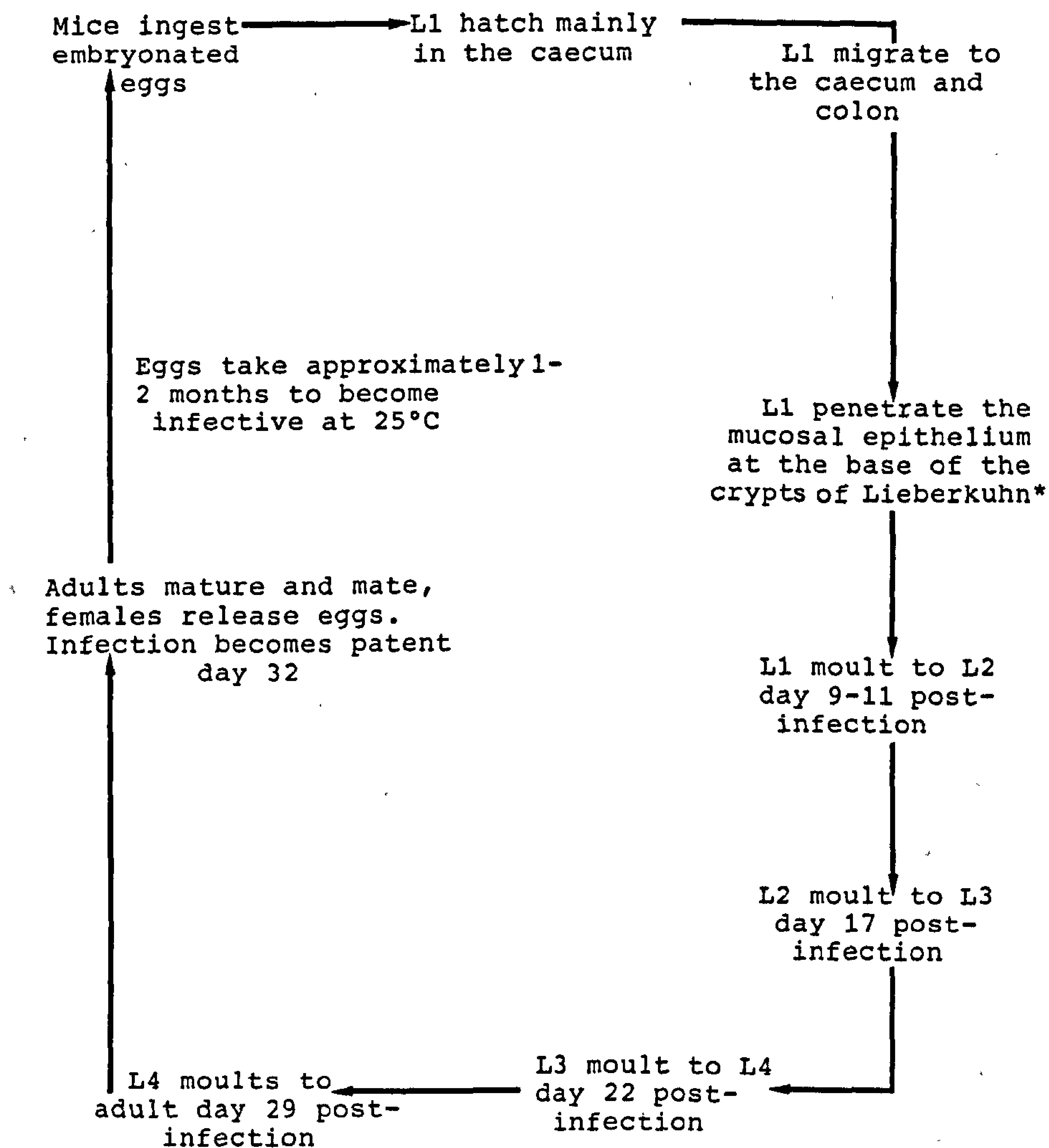
genetics in susceptibility (Bundy, 1988). Characterisation of the human immune response to *T. trichiura* is currently under investigation (D. A. P. Bundy, *pers. comm.*), initial studies indicating a marked age-dependency in antibody levels, with antigen-specific IgG levels rising in childhood, as does intensity of infection, then declining (Bundy, 1988). Thus although infected children have the ability to mount a humoral immune response to *T. trichiura* it is apparently of little value in protection. Immunoprecipitation studies currently in progress using ^{35}S-methionine labelled *T. trichiura* excretory/secretory antigen and *T. trichiura* human infection sera may reveal correlations between antigen recognition and resistance, or susceptibility, to infection. Such studies are obviously restricted by the availability of both human and parasite material. Indeed most of the information available on the interactions which occur between trichuroid nematodes and their hosts has come from the *T. muris*-mouse model. This model is not only convenient, enabling the analysis of immune responses to infection under controlled laboratory conditions, it is also a relevant model for human trichuriasis, *T. muris* and *T. trichiura* exhibiting considerable cross-reactivity (Roach *et al.*, 1988). Thus there is reason for optimism that elucidation of the intricate host-parasite interactions in the *T. muris*-mouse model will contribute towards an understanding of the chronicity of, and predisposition to, trichuriasis in man.

1.2 *TRICHURIS MURIS* IN THE LABORATORY MOUSE

The simple direct lifecycle of *T. muris* is shown in figure 1.1. Adult worms are attached to their host by the anterior part of their body which is embedded in a "tunnel" in the mucosa of the host's large intestine, the posterior regions protruding free into the lumen. The tunnel is a syncytium of enterocyte origin, occurring in the epithelial sheet only. The worm is

Figure 1.1 Life cycle of *Trichuris muris*

(adapted from Roach,1986)



* 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 caecum and colon 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.

thought to induce a syncytium about its head, probably through the secretion of digestive enzymes, feeding on the syncytial cytoplasm, then moving forward thus extending the syncytium (Lee and Wright, 1978).

The *T. muris*-mouse system provides a unique model in which to analyse immunological parameters involved in the development of both acute and chronic intestinal infections in the same host species and indeed within the same host strain. Strain variation in the outcome of infection is well documented and ranges from expulsion of the parasite well before maturity in some strains, to the development of patency in others (Worley *et al*, 1962; Campbell and Collette, 1962; Wakelin, 1967; 1970*b*; 1975*b*; Tomašovičová *et al.*, 1988). Variation in resistance to infection within an inbred strain has also been demonstrated, a proportion of DBA/2 mice failing to expel *T. muris* before the infection reached patency (Worley *et al.*, 1962; Lee and Wakelin, 1982*b*). Tomašovičová *et al.* (1988) recently described a DBA/2 strain of mouse uniformally non-responsive to infection. Mice used in these experiments were only 4 weeks old when infected thus providing an explanation for the apparent discrepancy in results compared to those of earlier workers; as pointed out by Worley *et al.* (1962) weanling DBA/2 mice are distinctly more susceptible to infection than adult mice. Wakelin (1970*b*; 1975*b*) suggested that the between strain variation in patterns of worm expulsion reflected genetically determined variation in the immunological capacity to respond to infection. The curious phenomenon of differential responsiveness within a genetically uniform strain of mouse was not explained.

The mechanisms by which *T. muris* is expelled from the mouse large intestine have been analysed in several studies (Selby and Wakelin, 1973; Wakelin and Selby, 1974*b*; 1976; Wakelin, 1975*a*, Lee *et al.*, 1983). Lymphoid cells or serum from immunised mice were shown by Selby and Wakelin (1973) to transfer immunity passively to *T. muris*, with immunity

being transferred most reliably with mesenteric lymph node cells and most effectively with serum. Lymphoid cells from the spleen did not transfer immunity. The immune expulsion of *T. muris* is thought to involve both antibody- and cell-mediated phases (Wakelin, 1975*a*) with a mesenteric lymph node T cell population implicated as the primary mediator of expulsion (Lee *et al.*, 1983). T cell mediated expulsion via local inflammatory changes, as suggested for the nematode *Trichinella spiralis* in the mouse (Larsh and Race, 1975; Wakelin and Wilson, 1979) appears not to apply to the expulsion of *T. muris*. No signs of gross inflammation have been reported and no correlations between the presence of mediators of inflammation, such as mast cells, and expulsion have been made (Lee and Wakelin, 1982*b*). Furthermore, Wakelin and Selby (1976) demonstrated that bone marrow-derived cells, other than lymphoid cells, were not apparently involved in the expulsion of *T. muris* and concluded that delayed hypersensitivity reactions were not of major importance in this system. Lee *et al.* (1983) postulated that effector T cells and worms interact more directly in the *T. muris* system than seen for *Trichinella spiralis*, the epithelial attachment and feeding site of this parasite making it potentially particularly vulnerable to the effects of intraepithelial lymphocytes thought to be derived from rapidly dividing T cell populations in the mesenteric lymph nodes and Peyer's patches. However, more sensitive and biologically more meaningful assays of mast cell activity, including quantifying serum levels of a protease released from mast cells may yet reveal a role for mast cells in the expulsion of *T. muris*.

The administration of cortisone acetate, an immunosuppressive agent, during early larval development is known to enable a primary infection to survive to patency in a normally resistant host (Wakelin, 1970*a*). This state of chemically induced tolerance has been shown to extend into subsequent infections after the removal of the primary infection by chemotherapy

(Wakelin and Selby, 1974*a*; Lee and Wakelin, 1982*a*) and as such provides a model for chronic trichuriasis in humans. Lee and Wakelin (1982*a*) have shown that serum from mice rendered tolerant to infection could passively transfer immunity to naive mice but mesenteric lymph node cells from such donors were ineffective. In addition immune mesenteric lymph node cells could not confer immunity to mice with an established adult infection but were effective against challenge infections, given on the day of cell transfer, after removal of the primary infection by chemotherapy. Control mesenteric lymph node cells in this experiment failed to restore immunocompetence to tolerant mice. On the basis of this work Lee and Wakelin suggested that chemically induced tolerance to infection involved a defect in an effector T cell population, perhaps operating at the level of antigen presentation. Chronic infections with *T. muris* have been shown to be associated with decreased humoral responsiveness to T dependent and T independent antigens and to render the host less able to respond to a primary infection with *Trichinella spiralis* (Lee and Wakelin, 1983). T cells from the mesenteric lymph nodes showed no reduction in responsiveness to the polyclonal activator phytohaemagglutinin and homing of activated lymph node cells from *T. spiralis* infected donors was not impaired.

The marked strain variation in the ability of mice to expel *T. muris* observed by Wakelin (1975*b*) indicates that the initiation and expression of resistance to infection is influenced by genetic factors. The availability of genetically defined strains of mice has led to much study of the genetic control of immunity to infection in a system which allows considerable scope for genetic manipulation and in which the immune mechanisms involved can be readily dissected.

1.3 IMMUNOGENETICS

Immunogenetics is a powerful tool by which to analyse the mechanisms of immunity at a molecular level. An understanding of the processes involved in B and T cell activation together with a knowledge of the nature and function of genes in the major histocompatibility complex (MHC) enable hypotheses to be made as to how a particular response status arises in terms of both resistance to infection and ability to generate T and/or B cell responses to particular antigenic determinants.

1.3.1 THE MAJOR HISTOCOMPATIBILITY COMPLEX (MHC)

The ability to discriminate between self and non-self resides in the recognition of cell surface structures (histocompatibility antigens) coded for by genes which form the so called major histocompatibility complex (MHC) found on chromosome 17 in the mouse. Only a brief description of the mouse MHC, together with its human counterpart, the human leucocyte antigen (HLA) system located on chromosome 6, is given here. For a more detailed description see Male *et al.*, 1987 Chapter 5. The organisation of the murine and human MHC is shown in figure 1.2. The mouse MHC consists of the H-2 complex and the T1a complex. A human equivalent of the latter complex is not known but may exist. Genes within the K and D regions of the H-2, the H-2K, H-2D and H-2L loci, code for class I MHC molecules found on nearly all nucleated cells. T cells expressing the CD8 cell surface marker, e.g. most cytotoxic T cells, mainly recognise antigen fragments in association with class I molecules. HLA-A, -B and -C loci code for class I molecules in man. Class I genes are also found in the mouse T1a complex, their products differing in tissue distribution and probably function from those within the H-2. The I region of the H-2

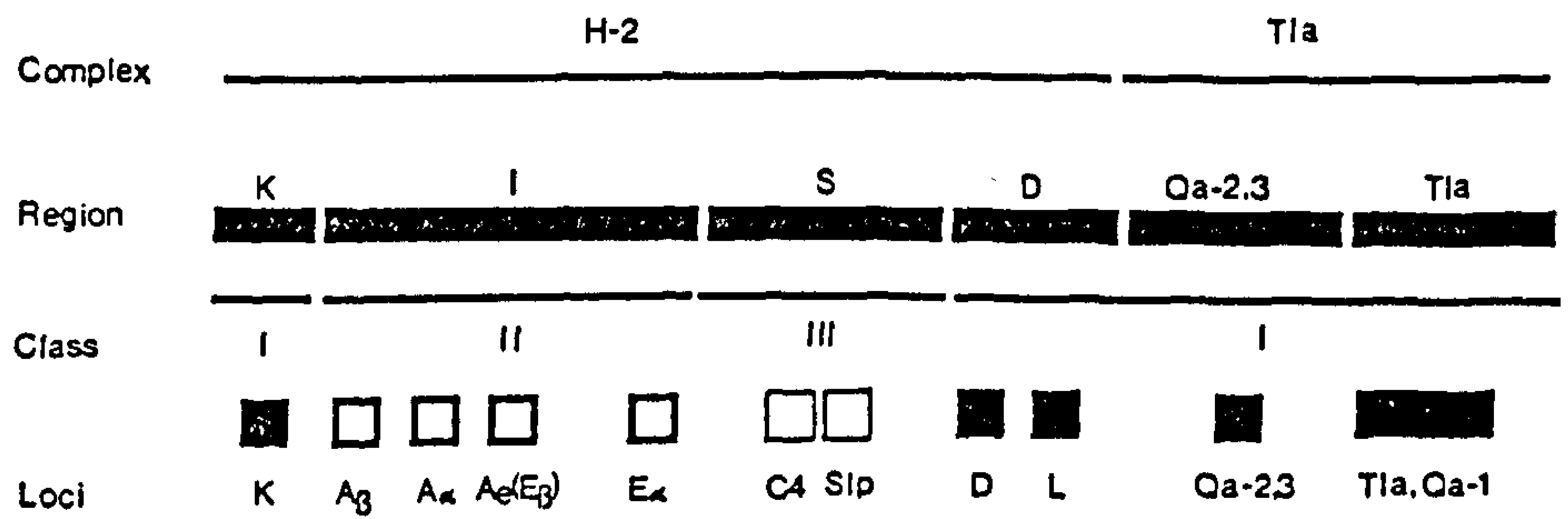
Figure 1·2 The murine and human MHC (adapted from Male et al, 1987)

■ Class I loci

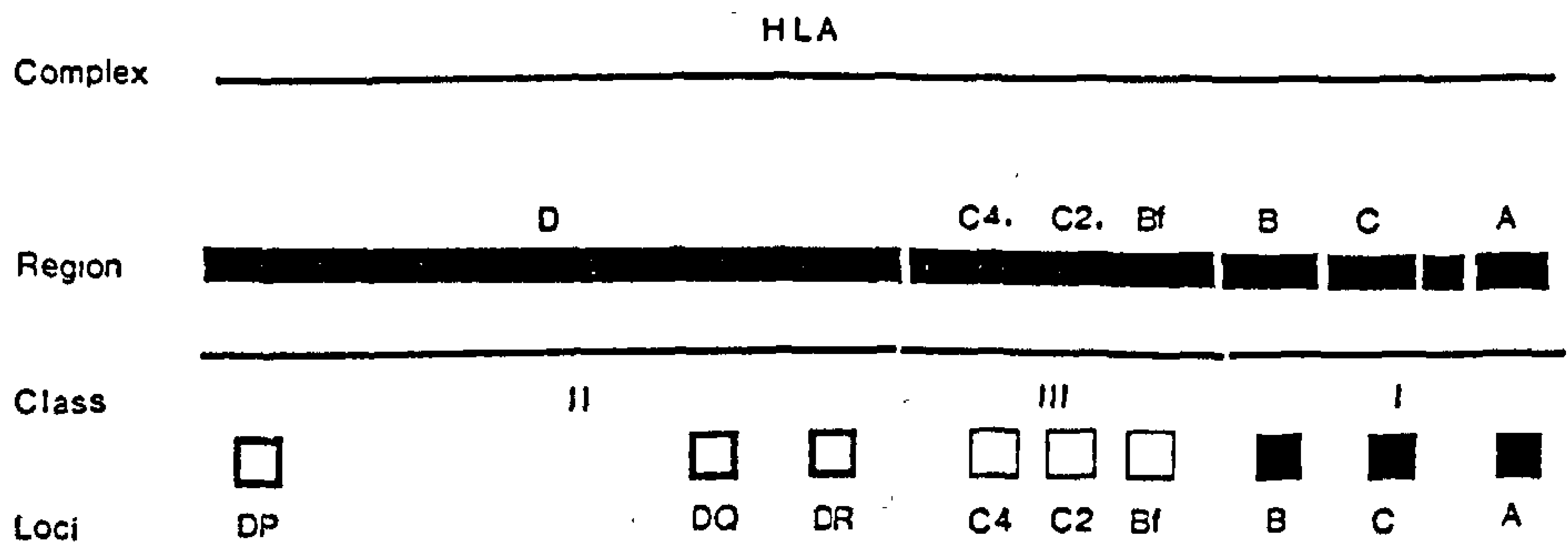
□ Class II loci

□ Class III loci (complement proteins)

Mouse - Chromosome 17



Human - Chromosome 6



complex (the HLA-D region in man) contains the class II (or Ir genes) encoded in the I-A and I-E subregions (HLA-DQ, HLA-DR and HLA-DP in man). The I-A subregion contains the A_β, A_α and the A_e (E_β) loci with the E_α locus lying in the I-E subregion. Products of the class II genes (Ia antigens) are expressed predominantly on B lymphocytes and other antigen presenting cells such as macrophages. T cells which recognise antigen in the context of class II molecules generally express the CD4 marker (e.g. T cells of the helper phenotype). The I-A region product is an $A_\beta A_\alpha$ dimer, the I-E region product being an $E_\beta E_\alpha$ dimer. Whilst all inbred mouse strains express I-A molecules, mice of the b, s, f and q haplotypes (see below) fail to express I-E molecules. The b and s haplotype strains fail to make E_α chains due to a deletion in the promoter region of the E_α gene but do express cytoplasmic E_β chains which are hence available for use in hybrid I-E molecules in F1 hybrids between b or s haplotype mice and strains expressing E_α. Mice of the f and q haplotypes fail to make both E_α and E_β chains. The E_α defect in f haplotype mice resides in the synthesis of an E_α mRNA of aberrant size whilst q haplotype mice have defects in RNA processing and mRNA stability (Jones *et al.*, 1981; Mathis *et al.*, 1983). The class III products of the mouse and human MHC are components of the complement system and bear little functional or structural similarities to the class I and class II molecules.

The major feature of the MHC is the extreme polymorphism of many of the class I and class II genes, excluding the E_α gene. The set of alleles expressed by an inbred mouse strain within the MHC defines its haplotype. Inbred strains which are more than 99% homozygous and that express only one allele of each histocompatibility antigen can be obtained by twenty or more brother-sister matings. Thus mice of, for instance, the $H\text{-}2^b$ haplotype express b alleles at all H-2 loci. H-2 congenic strains of mice differ from each other only at the H-2, whilst H-2 recombinant mouse strains are

identical except for a small number of genes within the H-2. The availability of such strains enables the influence of background genes, H-2 genes, and genes within the H-2, on the ability to mount a protective immune response to be studied in isolation.

1.3.2 ANTIGEN PRESENTATION

The recognition of antigen by B cells and T cells is markedly different. Whilst B cells bind free antigen, T cells only bind antigen which has been processed and presented to them in association with MHC molecules. In addition, as outlined in 1.3.1, cytotoxic T cells usually only recognise antigen in association with class I MHC molecules and are thus targeted against tumour cells and virally infected cells (Male *et al.*, 1987 Chapter 7). Helper T cells (T_H) are, in most cases, MHC-class II restricted, only recognising antigen which has been processed and presented to them by so called antigen presenting cells (e.g. macrophages, dendritic cells and B cells) in association with class II molecules (reviewed by Schwartz, 1985; Janeway *et al.*, 1988). Activated T_H cells subsequently interact with B cells (most B cell responses are T cell dependent, reviewed by Abbas, 1988), inflammatory cell precursors and other effector cell types, through the production of lymphokines, resulting in immune responses typical of those believed to be important in the control of many helminth infections (see Rothwell, 1989; Wakelin, 1988*b*). Current models of antigen presentation suggest that a single structure, a processed antigen-Ia complex, is recognised by the T cell receptor (Kappler *et al.*, 1981; Heber-Katz *et al.*, 1983; Babbitt *et al.*, 1985; reviewed by Marrack and Kappler, 1987). The three dimensional structure of the binding site of the class II molecule has not been described although it may well have features similar to those described by Bjorkman *et al.*, 1987 for the MHC class I molecule. These

workers described a single potential binding site envisaged as a groove with β-strands at the base and α-helices at the sides of a size allowing binding of peptides 8-20 amino acids long. This binding site has the capacity to bind many different peptides (Buus *et al.*, 1987*b*) with peptides competing for antigen presentation (Babbitt *et al.*, 1985; Guillet *et al.*, 1987). Immunodominant peptides presumably bind to the class II site with the greatest affinity. Thus MHC-restricted B and T cell responses to particular antigens in mice (Del Guidice *et al.*, 1986; Good *et al.*, 1986) and correlations between histocompatibility antigens and susceptibility to particular human diseases (Sasazuki *et al.*, 1977; Osoba *et al.*, 1979; Lamoureux *et al.*, 1985; Sterkers *et al.*, 1988) and parasite infections in mice (Blackwell *et al.*, 1980; Blackwell, 1983; Wassom *et al.*, 1979) may reflect limitations on the ability of a particular MHC molecule to bind certain antigens. Non-responsiveness to parasite antigens can arise at the level of the antigen presenting cell through the expression of inappropriate (non-responder) alleles at the MHC, non-responder MHC-encoded molecules failing to bind the antigen fragment in an immunologically relevant way (Rosenthal, 1978; Heber-Katz *et al.*, 1983; Buus *et al.*, 1987*a*). Alternatively the immunogenic complex generated might induce suppressor rather than helper T cells (Kapp *et al.*, 1974; Nagy *et al.*, 1981). Failure to synthesise one or both of the two chains of a class II molecule (Jones *et al.*, 1981) may also be associated with non-responsiveness. Quantitative defects in Ia molecule expression at the cell surface may dictate responder status (Matis *et al.*, 1982; Janeway *et al.*,1984) as may the affinity of the interaction between Ia and the peptide (Babbitt *et al.*, 1985). Binding of an immunodominant peptide to a class II molecule is a necessary but not the only prerequisite for an immune response to take place. The absence of T cells bearing receptors able to recognise a particular combination of antigenic peptide plus MHC class II molecule (i.e. a "hole" in the antigen-specific T cell

repertoire) can also account for functional non-responsiveness (Ishii *et al.*,1981; Thomas and Hoffman, 1982), "holes" arising, at least in part, through the deletion of T cells able to react with self in the thymus during T cell ontogeny (Marrack and Kappler, 1987; 1988; Blackman *et al.*, 1988).

1.3.3 T HELPER CELL SUBSETS

The above discussion has considered T_H cells as a single population of lymphocytes. Recently T_H cell clones have been separated into two distinct sets *in vitro* on the basis of the lymphokine profiles: T_H1 cells, also referred to as inflammatory $CD4^+$ T cells, produce interleukin (Il-2) and gamma interferon (IFN-γ) whilst T_H2 cells (helper $CD4^+$ T cells) produce Il-4 and Il-5. Il-3 and granulocyte-macrophage colony stimulating factor (GM-CSF) are apparently produced by both cell types (reviewed by Mosmann and Coffman, 1987; Bottomly, 1988; Coffman *et al.*,1988). The lymphokines produced by T_H1 cells are required to stimulate IgG2a antibody production (Snapper and Paul, 1987) and the involvement of T_H1 cells in delayed type hypersensitivity responses has been demonstrated (Cher and Mosmann, 1987). The lymphokines defining T_H2 cells (Il-4 and Il-5) are thought to be involved in the production of IgG1 and IgE (Vitetta *et al.*, 1985; Coffman and Carty, 1986), and in the generation of eosinophils and IgA production (Harriman and Strober 1987; Murray *et al.*, 1987; Coffman *et al.*, 1987) respectively. However at present it is uncertain as to whether the two distinct T_H cell lineages exist *in vivo* or whether they represent long term *in vitro* selection. Reports of short term T cell clones secreting both Il-2 and Il-4 (Glasebrook *et al.*, 1988) and the demonstration of the random coexpression of GM-CSF, IFN-γ, Il-3 and Il-4 (Kelso and Gough, 1988) would suggest that at least initially T_H cells are pluripotential with respect to lymphokine production but preferentially secrete a more restricted

panel of lymphokines according to their local enviroment. In subsequent chapters the T_H1 and T_H2 subsets are referred to. It should be borne in mind that their existence as separate lineages is still open to debate.

1.4 OBJECTIVES OF THIS STUDY

In the preceding subsection (1.3) the importance of genetically determined factors in the control of immunity was emphasised. Such factors represent one of several components which can affect a host's ability to respond to infection. Section One of this thesis was designed to analyse the effects of both H-2 and non-H-2 genes on the expulsion of *Trichuris muris* from the mouse, their role in the control of humoral immune responses to *T. muris* excretory/secretory (E/S) antigen and their overall contribution in the determination of responder/non-responder status of the host (for the purpose of this thesis "responder" mice are defined as those individuals able to expel *T. muris* before the primary infection reaches patency, "non-responder" mice harbouring mature adult worms). The decision to use E/S antigen as the target antigen in the serological studies was based on the fact that, although still consisting of a relatively heterogeneous mixture of antigens, it represents a more restricted set of potentially functional relevant antigens compared to worm homogenate (Roach, 1986; Pritchard, 1987).

Host determined parameters are clearly important in determining the outcome of infection, but they are not the only factors. Equally important are the constraints the parasite imposes on the host's ability to respond to infection. One of the paradoxes of chronic infections such as *T. muris* in certain strains of mice, and *T. trichiura* in man, is the long term survival of the parasite despite host immune responses. The evasion of host immunity by parasites is well documented (for reviews see Bloom, 1979;

Parkhouse, 1984; Wakelin, 1986; Mitchell, 1989*b*) and is achieved via numerous elaborate mechanisms. The work presented in Section Two was aimed at analysing the factors involved in the immunological predisposition of certain strains of mice to infection with *T. muris* and in the curious phenomenon of differential responsiveness within inbred mouse strains.

Finally the studies described in Section Three addressed the problem of the likely source of the E/S antigens of *T. muris*, components of which are known to be highly immunogenic (Wakelin and Selby, 1973; Jenkins and Wakelin, 1977; 1983).

CHAPTER 2

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2.1 GENERAL TECHNIQUES

2.1.1 MICE

Six to eight-week old inbred male mice were obtained from Harlan Olac Ltd., except C57BL/10, BALB/c, NIH and B10.G, and the outbred strains MF1 and CFLP, which were bred at the University of Nottingham (unless otherwise stated). All the animals were maintained under conventional animal house conditions receiving food and water *ad libitum.* Naturally acquired oxyurid infections were removed from homebred mice by the administration of 0.2mls piperazine citrate (tripiperazine dicitrate, Sigma) (12.5% w/v solution in water) on two occasions seven days apart. All the mice were given piperazine citrate in their drinking water (1g/l) until used.

2.1.2 CROSS BREEDING

Parental strains were obtained from Harlan Olac Ltd. Groups of two to three eight to ten-week old female mice were housed with one male mouse. Females were removed once pregnant and caged separately with bedding material. F1 hybrid mice were weaned at four weeks of age and used when six to ten-weeks old.

2.1.3 PARASITE

a) Maintenance of *Trichuris muris* and infection protocol

Stock infections were maintained in CFLP mice. Infective egg cultures were stirred on magnetic stirrers and embryonated eggs in four 50μl aliquots

were counted under low magnification and averaged. The desired concentration was obtained by adjusting the total volume of the egg culture to give the required number of viable eggs in 0.2ml. Stock mice were infected orally with approximately 400 embryonated eggs and received corticosteroid treatment (see 2.1.4) to enable the infection to reach patency. Cortisone-treated mice were given oxytetracycline hydrochloride (Terramycin, Pfizer Ltd.) in their drinking water at a concentration of 165mg/l.

On day 42 p.i. mice were killed by an overdose of chloroform (May and Baker) and the worms removed, washed in PBS (Appendix 2.6.2) and placed in sterile RPMI 1640 plus supplements (Appendix 2.6.1) with 500μg/ml of penicillin/streptomycin (Gibco) and fungizone (Gibco) at 2.5μg/ml for O/N collection of E/S products (see 2.2.1). Eggs released O/N by female worms were collected, washed and re-suspended in 0.25% formaldehyde (Eagle Scientific Ltd.) in distilled water. Gravid females were ground in a glass tissue homogenizer with a loose fitting plunger so as not to rupture the eggs, and the resulting suspension filtered through fine nylon mesh. The eggs were washed once and resuspended as described above. Eggs were incubated in tissue culture flasks (Nunc) in the dark at room temperature for at least 45 days, cultures being examined regularly for both egg development and contamination. Once eggs had embryonated, cultures were stored at 4°C.

b) Worm Counts

Mice were killed by an overdose of chloroform. The caecum and first 4 cms of the colon were removed from each mouse, placed in a petri dish and frozen at −20°C for at least 24 hours. Guts were thawed for counting in PBS in a petri dish, slit open and flushed through with PBS.

For early larval stage counts each gut was placed in fresh PBS in a

second petri dish and the caecum and colon scraped with curved forceps. The gut was then re-scraped in a third petri dish. Larvae were removed from the petri dishes as they were counted and each dish was scanned three times. The gut contents were also scanned for larvae, although few were usually present.

L4 and adult *T. muris* were counted as they were removed individually from the gut mucosa using fine forceps. Incomplete worms were only counted if an entire posterior end was present.

2.1.4 CORTICOSTEROID TREATMENT

Mice were injected subcutaneously (s.c.) with 0.05ml of hydrocortisone 21-acetate (C/A) (25mg/ml, Sigma) on days 7, 9, 11, 13 and 15 p.i. This regime prevents worm expulsion and allows the long-term survival of adult worms (Wakelin, 1967).

2.1.5 ANTHELMINTIC TREATMENT

The anthelmintic methyridine (Promintic, I.C.I) was injected s.c. at a dose of 500mg/kg body weight, mice being given three such treatments at 3 hour intervals. Mice sacrificed the day after treatment showed the drug to be effective in removing all larval and adult worms on the day of treatment.

2.1.6 IMMUNISATION

The test antigen preparation was added dropwise to, and emulsified with, an equal volume of Freund's Complete Adjuvant (FCA) (Sigma). Mice were immunised s.c. on day-10 and infected on day 0, along with controls

injected with PBS emulsified with FCA (Jenkins, 1977).

2.1.7 SERUM

Mice sacrificed for worm burden determination were bled by cardiac puncture. In all other cases mice were bled from the tail vein. Whole blood was centrifuged at 11,500g for 10 minutes and the sera obtained stored in 100μl aliquots at -40°C.

2.2 ANTIGEN PREPARATIONS

2.2.1 EXCRETORY/SECRETORY (E/S) ANTIGEN

Mice with patent *T. muris* infections were killed and the caeca and colons removed. After opening the guts and flushing out the gut contents with PBS, adult male and female *T. muris* were removed individually, washed in PBS and placed in sterile RPM1 1640 medium plus supplements, excluding FCS and L-glutamine, (Appendix 2.6.1) with 500μg/ml penicillin/streptomycin (Gibco) and fungizone (Gibco) at 2.5μg/ml. After 4 hours in this medium at 37°C worms were put into fresh medium and left O/N for the collection of E/S products. The 4 hour supernatant (S/N) was retained, containing considerable amounts of E/S products, centrifuged at 200g for 5 minutes to remove eggs, and filtered (0.22μm filter, Millipore).

The culture S/N from the O/N incubation was treated as above. Both 4 hour and O/N E/S samples were freeze-dried, reconstituted to no more than one quarter of the original volume with distilled water, and dialysed O/N using dialysis tubing with a 2kDa cut-off point (Sigma). Samples were

analysed for protein (see 2.3.7), aliquoted, and stored at −40°C.

2.2.2 ^{35}S-METHIONINE LABELLED E/S ANTIGEN

Metabolically labelled E/S antigen was prepared by incubating worms O/N, as above, in the presence of ^{35}S-methionine (Amersham) at 250μCi/ml (9.25 MBq/ml) in methionine-free medium. 25 worms were incubated per 1ml of medium. At the end of the culture period S/Ns were removed, centrifuged (11,500g for 5 min.), aliquoted and frozen directly at −40°C, without sterile filtering. E/S products were analysed for labelled protein (see 2.3.6) prior to use.

2.2.3 ADULT MALE ANTIGEN (AMA)

Adult male and female worms were incubated O/N at 37°C as in 2.2.1. Male worms were then removed, washed in PBS, homogenised on ice in a minimal volume of PBS using a glass tissue homogeniser and allowed to extract O/N at 4°C. The resulting suspension was centrifuged at 11,500g for 30 minutes. The S/N was removed, filtered (0.22μm filter, Millipore), analysed for protein (2.3.7) and stored at −40°C.

2.2.4 ANTERIOR END ANTIGEN (AA), STICHOCYTE E/S (CE/S) AND STICHOCYTE ANTIGEN (CA)

Adult *T. muris* were washed in PBS and the anterior portion of each worm severed from the posterior portion at the oesophageal-intestinal junction using fine forceps.

a) Anterior end antigen (AA)-anterior ends were homogenised on ice in a minimal volume of PBS using a glass tissue homogeniser and left to extract

O/N at 4°C. The suspension was then centrifuged at 11,500g for 30 minutes and the resulting S/N filtered using a 0.22μm Millipore filter, analysed for protein, aliquoted and stored at −40°C.

b) Stichocyte E/S (CE/S) - stichocytes were dissected out into PBS from the anterior ends of the worms using blunt ended glass rods. Individual cells were collected and placed in RPMI-1640 medium plus supplements, excluding FCS and L-glutamine, for O/N culture at 37°C. The resulting S/N was removed, centrifuged at 11,500g for 30 minutes, filtered (0.22μm Millipore paper), aliquoted and stored at −40°C.

c) Stichocyte antigen (CA) - Stichocytes were boiled for 5 minutes in reducing sample buffer prior to SDS-PAGE (see 2.3.2)

2.2.5 PSEUDOCOELOMIC FLUID (PCF)

Individual adult *T. muris* were placed in PBS in a Watchman's glass and the anterior end severed from the posterior end at the oesophageal-intestinal junction, thus releasing a dense fluid into the PBS. Approximately 200 worms were dissected in this manner before the PBS plus fluid was collected, centrifuged at 11,500g for 15 minutes and filtered (0.22μm Millipore filter). The resulting solution was analysed for protein, aliquoted and stored at −40°C prior to use.

2.2.6 UNEMBRYONATED EGG ANTIGEN (UEEA)

Unembryonated *T. muris* eggs (approximately $11x10^6$), obtained by maceration of female worms, were placed in 20% bleach for approximately 20 minutes to dissolve the egg capsule leaving the lipid membrane intact. Eggs were washed thoroughly to remove all bleach and sonicated for 5 minutes at 1.5 Amperes. The resulting suspension was centrifuged (11,500g

for 15 minutes) and the S/N filtered (0.22μm Millipore filter), analysed for protein, aliquoted and stored at -40°C.

2.2.7 EMBRYONATED EGG ANTIGEN (EEA)

Eggs obtained from an infective egg culture were treated as above. The approximate ratio of embryonated to unembryonated eggs in such a culture was 2:1

2.2.8 ^{125}I-LABELLED SURFACE PROTEIN

^{125}I-surface labelling was carried out using the Iodogen reagent (1,3,4,6-tetrachloro-3α, 6α-diphenylglycoluril, Pierce Chemical Co.) (Fraker and Speck, 1978) which promotes labelling of tyrosine residues (Zingales, 1984). Iodogen was dissolved in methylene chloride to give a working dilution of 1mg/ml. 50μl of this solution were dispensed into a series of small glass tubes and the solvent allowed to evaporate at RT, leaving a thin film of iodogen at the bottom of the tube. Iodogen coated tubes were rinsed with PBS to remove loose flakes of reagent prior to use.

One male and one female worm in PBS were added to each tube and the volume reduced to approximately 50μl. 10μl of potassium iodide solution (BDH, 11μg/ml in PBS) were added to each tube followed by 2μl (27μCi; 1MBq) of ^{125}I (Amersham). The reaction was allowed to proceed for 10 minutes at RT, rotating the tubes every 2 minutes. Samples were then diluted with PBS, tipped into universals and washed 6 times with large volumes of PBS. Worms were either homogenised in PBS, the homogenate being centrifuged at 11,500g for 10 minutes and the S/N removed, analysed for protein by trichloroacetic acid (TCA) precipitation (2.3.6), aliquoted and stored at 4°C, or, cultured O/N in RPMI-1640 medium, the E/S being

collected, centrifuged at 11,500g for 10 minutes and the S/N analysed for protein and stored as above. Worms from the O/N incubation were homogenised as above.

2.3 BIOCHEMICAL/IMMUNOLOGICAL TECHNIQUES

2.3.1 ENZYME-LINKED IMMUNOSORBANT ASSAY (ELISA)

Alkaline phosphatase method

The protocol for the ELISA is outlined below, and is essentially as described by Voller *et al.* (1979).

Assay plates were wrapped in foil during incubation periods to limit evaporation from sample wells and to allow the enzymic reaction to proceed in the dark once the photosensitive substrate was added. Optimal antigen concentrations and serum dilutions were as determined by Roach (1986). For all buffer recipes see Appendix (2.6.6).

Ninety-six-well flat-bottomed flexible assay plates (Falcon) were coated with E/S antigen (5μg/ml; 50μl/well) in 0.05M carbonate/bicarbonate buffer, pH 9.6, and left O/N at 4°C. Antigen was tipped off and the plate washed five times (three rapid washes followed by two three minute washes) with PBS containing 0.05% Tween 20 (Sigma). Plates were blocked with 3% bovine serum albumin (BSA, Sigma) in PBS/Tween, 100μl/well, for 1 hour at RT. After tipping off the blocking solution and washing twice in PBS/Tween, 50μl of test serum at a dilution of 10^{-2} in PBS/Tween were added to each well and incubated at RT for 1.5 hours. After three rapid and two three minute washes, alkaline phosphatase-conjugated sheep

anti-mouse IgG (whole molecule, Sigma), sheep anti-mouse IgG1 (Serotec), goat anti-mouse IgA (α chain specific, Sigma), goat anti-mouse IgM (μ chain specific) (Sigma), all diluted 1/1000 in PBS/Tween, or goat anti-mouse polyvalent IgGAM (Sigma) diluted 1/350 in PBS/Tween, was added (50μl/well) and incubated for another 1.5 hours at RT. Plates were washed five times, as described above, with PBS/Tween and the substrate p-Nitrophenyl phosphate, disodium hexahydrate tablets (Sigma) dissolved in diethanolamine buffer, pH 9.8 (Fisons) (1mg/ml; 1 tablet/5mls) added to the plates at 100μl/well. Plates were left for 0.5-1 hour at RT until a control positive serum sample, included on all plates, had reached a selected reference O.D. value. The enzymic reaction was read at 410nm on a Dynatech MR 700 Microplate reader.

Horse radish peroxidase method

Levels of specific IgG2a in serum samples were determined using the enzyme horse radish peroxidase conjugated to streptavidin. The method used was as outlined for the alkaline phosphatase-conjugated anti-immunoglobulin reagents except for the stages mentioned below. All stages were performed at RT except the initial antigen binding step, as before.

After incubation of the test sera for 1.5 hours and the subsequent washing steps, goat anti-mouse IgG2a (Southern Biotech), diluted 1/500 in 1% skimmed milk in PBS was added, 50μl/well, and left for 2 hours. Plates were washed five times, as before, prior to the addition of a biotinylated rabbit anti-goat IgG reagent (Kirkegaard and Perry), diluted 1/1000 in 1% skimmed milk in PBS and added at 50μl/well. Plates were left for 1 hour, then washed as above, followed by the addition of horse radish peroxidase-streptavidin diluted 1/500 in 1% skimmed milk in PBS (50μl/well). After a 1 hour incubation, and 5 washes in PBS/Tween (0.05%)

the peroxidase substrate 2.2'-azino-di-[3-ethyl]-benzthiazoline sulfonate (Kirkegaard and Perry) mixed 1:1 with hydrogen peroxide (H_2O_2) (Kirkegaard and Perry) was added at 100μl/well. The reaction was stopped after 4 minutes with 0.3M H_2SO_4, 50μl/well, and read at 410nm as before.

2.3.2 SDS POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

The protocol for the preparation, pouring and running of 10-20% SDS-polyacrylamide gradient gels using the method of Laemmli (1970), is outlined below. Recipes for stock acrylamide solutions, buffers, staining and destaining solutions are given in the Appendix (2.6.3).

a) Preparation of resolving and stacking gels.

Resolving gradient gel

	10% acrylamide solution	20% acrylamide solution
"Dense" acrylamide solution	-	11.0ml
"Light" acrylamide solution	16.7ml	-
Lower gel buffer pH 8.8	12.5ml	5.5ml
Distilled water	20.8ml	-
10% ammonium persulfate (APS)	50.0μl	15.0μl
TEMED (N,N,N',N',-tetramethylethylenediamine, Sigma)	50.0μl	15.0μl

The 10% and 20% acrylamide solutions were mixed as above without TEMED and degassed under a vacuum. TEMED was added to both solutions immediately before pouring into the gradient gel mixer, in which the 20% solution was gradually diluted by the 10% solution as the gel was poured between the gel plates. To exclude air during the polymerisation

process the gel was overlaid with 2-methyl-propan-1-ol (BDH), the gel surface being washed with distilled water after polymerization was complete.

Stacking gel

The stacking gel was made by mixing the following reagents:-

Light acrylamide solution	3.0ml
Upper gel buffer pH 6.8	5.0ml
Distilled Water	12.0ml
10% APS	90.0μl
TEMED	30.0μl

The solutions were mixed and degassed before TEMED was added. The stacking comb was placed between the two glass plates and the empty space filled with the stacking gel solution. After polymerization was complete the comb was carefully removed and the gel washed thoroughly with electrode buffer before overlaying with electrode buffer.

b) Sample preparation

The volume of sample containing 50–100μg of protein was boiled with an equal, or excess, volume of reducing sample buffer for 5 minutes in a water bath. Samples were centrifuged for 1 minute at 11,500g prior to loading on the gel. Samples were loaded into the wells using a micropipette. Gels were run at 10°C O/N at a constant voltage of 100V on an LKB 2001 vertical electrophoresis kit (LKB Instruments, Sweden). The voltage was increased to 400V the next morning until the dye front had migrated to the bottom of the gel.

c) Gel staining and destaining

Gels were removed from the glass plates and placed in 0.1% Coomassie Brilliant Blue stain (Sigma) O/N on a rocker. Gels were transferred to destain until protein bands were visible and background staining was low, and then dried down onto filter paper using a BioRad 443 Slab Dryer.

d) Elution of antigen from SDS-polyacrylamide gels

For temporary visualisation of protein bands, gels were placed in ice cold 0.25% potassium chloride solution (Fisons) for 1-5 hours. Opaque colourless protein bands were thus rendered visible. The required band was cut out from gels, equilibrated with equilibrating buffer (elution buffer plus 5mM dithiothreitol (Sigma) for 30 minutes, and the protein eluted by homogenisation of the band in elution buffer and extraction O/N at 4°C.

e) Autoradiography

^{125}I-labelled proteins were run at 50,000 cpm per lane following boiling in reducing sample buffer as previously described. Gels were stained O/N with Coomassie Brilliant Blue stain (Sigma), destained, and dried on to filter paper. After drying, gels were exposed to X-ray film (Fuji) in combination with an intensifying screen at -80°C until the film was suitably exposed.

2.3.3 WESTERN BLOTTING

Western blotting was carried out basically as described by Burnette (1981). *T. muris* E/S antigen was run on gradient gels as previously described. Nitrocellulose membrane (Schleicher and Schuell) was cut to fit

the gel and pre-soaked in distilled water followed by blotting buffer (Appendix 2.6.4). A Scotch-brite pad, soaked in blotting buffer, was placed on an open blotting cassette, followed by two sheets of 3mm filter paper wetted with blotting buffer. The gel was placed on the filter paper, and the nitrocellulose laid on top, avoiding entrapment of air bubbles. Lanes on the gel were marked on the nitrocellulose and a second wet filter paper and pad added. Finally the cassette was closed and the unit placed in blotting buffer in a blotting tank, with the nitrocellulose facing the anode. Blotting was performed for 2 hours at 10°C at 100-300mA. Once transfer of proteins to the nitrocellulose was complete, the marker lane and one sample lane were cut off and stained for two minutes in amido black stain (Sigma) (Appendix 2.6.4) and then destained to reveal the protein bands. After saturating any remaining protein-reactive sites by incubating the remaining nitrocellulose in 10% skimmed milk in PBS/Tween (0.05%) for one hour at RT on a rocker, the membrane was cut into strips and incubated with test sera (1/500 dilution in 10% skimmed milk in PBS/Tween) O/N at 4°C. Blots were washed three times prior to incubation with ^{125}I-rabbit anti-mouse polyvalent immunoglobulins (0.5μCi/strip, Amersham) for 2 hours. Blots were washed again, air dried and exposed to X-ray film (Fuji) at -80°C in combination with an intensifying screen.

2.3.4 IMMUNOPRECIPITATION

^{35}S-methionine labelled E/S antigen (2.2.2) was added to a series of eppendorf tubes to give 100,000cpm. A volume of 25μl of test serum was added and made up to a volume of 0.5ml with immunoprecipitation buffer pH7.4 (Appendix 2.6.5). The solutions were vortexed and left O/N at 4°C. 15μl of sheep anti-mouse IgGFc (Serotec), sheep anti-mouse IgG1 (Serotec) or sheep anti-mouse IgGAM and L chains (Serotec), were added to each

sample and left for 3 hours at RT. Immunoprecipitates were spun down at 11,500g and washed four times in immunoprecipitation buffer before boiling in 25μl of reducing sample buffer. Solubilised antigens were analysed by SDS-PAGE (10-20% gradient gels). Gels were fixed, fluorographed, dried down and exposed to X-ray film (Fuji) at -80°C.

2.3.5 FLUOROGRAPHY OF ^{35}S-METHIONINE GELS

Gels were fixed in destain for 30 minutes, then soaked in 20 times their volume of dimethylsulphoxide (DMSO) (Sigma) for 1 hour, followed by a second 4 hour immersion in fresh DMSO to ensure the removal of all water from the gels. Gels were then soaked in 22% 2'5 diphenyl oxazole (PPO) (Sigma) in DMSO O/N, and placed in water for 5 hours before drying down at 60°C and exposing to X-ray film (Fuji) at -80°C.

2.3.6 TRICHLOROACETIC ACID (TCA) PRECIPITATION FOR LABELLED PROTEIN

The amount of labelled protein obtained from both the ^{35}S-methionine and ^{125}I-labelling procedures was estimated using trichloroacetic acid (TCA) (BDH) precipitation.

a) ^{35}S-methionine labelled protein

10μl of the sample to be counted were placed in an eppendorf with 100μl ice cold 25% TCA. 50μl of foetal calf serum (FCS) (Gibco) were added and, after vortexing, the solution was left on ice for 10 minutes before filtering using a buchner funnel and flask onto a 4.25cm glass microfibre filter (Whatman) pre-wetted with ice-cold 8% TCA. The filter

was washed four times with ice-cold 8% TCA before airdrying. Once dry the filter was placed in a scintillation vial containing 5mls of scintillation fluid (Optiphase 'X') (Fisons) for counting on an Intertechnique SL 30 liquid scintillation spectrometer.

b) ^{125}I-labelled protein

100μl of 25% TCA was added to 10μl of sample followed by 30μl of FCS. After 20 minutes this mixture was spun at 11,500g for 1 minute and the resulting S/N removed. The cpm of the precipitate, S/N and 10μl of the original protein solution were determined using a gamma counter (Packard A 500c) and the percentage of labelled TCA precipitable protein calculated. Labelled antigens were stored at 4°C and used within 3 days of being prepared (half life of ^{125}I = 60 days).

2.3.7 LOWRY METHOD

Protein concentrations were estimated using a method modified from Lowry *et al.* (1951). A set of protein standards, ranging from 16 to 200μg/ml, were prepared using BSA (Sigma) in a total volume of 250μl of distilled water. Dilutions of test samples were made in the same total volume. 1.25ml of working reagent (Appendix 2.6.7) were added to each dilution and the dilutions incubated at 37°C for 5 minutes. 125μl of Folin and Ciocalteu's phenol reagent (Folin's reagent) (BDH), diluted 1:1 in distilled water, were added to each dilution and the samples vortexed and incubated at 37°C for a further 15 minutes. 100μl aliquots of each dilution were read in triplicate on a Dynatech MR 700 Microplate Reader at 630nm and the mean optical densities (O.D.) calculated. A calibration graph was constructed of O.D. versus total protein content, using the BSA standards,

and thus the total protein content of the test samples determined. Protein concentrations in mg/ml were then calculated.

2.3.8 SURFACE IMMUNOFLUORESCENCE

Adult male and female worms were isolated from the large intestines of mice and washed five times in PBS. 2-4 worms were placed in eppendorfs with the test solutions (infection sera, tolerant sera (TS) from mice with patent infections, or ascites) at an appropriate dilution. Controls (PBS plus 1% BSA) (Sigma) and naive serum (NS) were also included. After 1 hour at 37°C in the test solution worms were washed three times in PBS and 0.75mls fluorescein-conjugated affinity-purified goat anti-mouse immunoglobulins (IgGAM, heavy and light chains specific) (Cappel), at a 1/20 dilution in PBS, added to each eppendorf and left at 4°C in the dark for 30 minutes. Worms were washed, mounted in 50% glycerol (C.P. Pharmaceuticals) in PBS and examined under ultra violet illumination.

2.4 CELLULAR TECHNIQUES

2.4.1 PREPARATION OF CELL SUPSPENSIONS

Aseptic technique was used throughout, using sterile media, sterile filtered antigen preparations, sterile instruments and sterile disposables. Spleens and mesenteric lymph nodes (MLN) were removed from mice, stripped of excess fat and placed in RPMI 1640 medium plus supplements (Appendix 2.6.1). All materials were kept at RT throughout. Cell suspensions were made by pressing the tissue through a gauze into a petri

dish using a 5ml syringe plunger. The dish was inclined and left for 5 minutes to allow debris and cell clumps to settle, the cell suspension in the S/N then being removed and spun down at 1000rpm for 5 minutes. The cell pellet was loosened and 5mls ammonium chloride (Appendix 2.6.2) added to lyse red blood cells. After 30-45 seconds 15mls of medium were added, the cells transferred to clean universals and washed twice by centrifuging at 1000rpm for 5 minutes and resuspending in fresh medium. After the final wash cells were resuspended in 5mls of medium and cell viability assessed (2.4.2).

2.4.2 CELL VIABILITY

All cells take in fluorescein diacetate (FDA) (Sigma) but only living cells can hydrolyse FDA to give free fluorescein. Such cells fluoresce green when viewed under ultra violet illumination.

A stock solution of FDA (5mg/ml in acetone (May and Baker)) was prepared and stored at -20°C. Just prior to use this stock solution was diluted 1/50 in PBS and one volume added to nine volumes of the cell suspension (at an appropriate dilution). The average number of fluorescing cells was determined using a Neubauer haemocytometer and hence the number of cells/ml calculated.

2.4.3 LYMPHOCYTE PROLIFERATION ASSAY

The ability of spleen and MLN cells to respond to *T. muris* E/S antigen, and to mitogens, was determined by culturing cells *in vitro* in the presence of the test substance, followed by the addition of tritiated thymidine (^{3}H-TdR, Amersham International). Lymphocyte proliferation was thus assessed by the incorporation of ^{3}H-TdR into the DNA of dividing

cells.

Cells were recovered from mice and the cell suspension adjusted to $5x10^6$cells/ml (see 2.4.1 and 2.4.2). 100μl of medium were placed in each well of flat bottomed microtitre plates (Nunc) followed by 50μl of sterile filtered antigen at final concentrations of 1, 10, 50, 100 or 200μg/ml, 50μl of mitogen (sterile type IV-s Concanavalin A (Con A) (Sigma) at 1, 5, or 10μg/ml, or sterile-filtered lipopolysaccharide (LPS) from *Escherichia coli* serotype 055:B5 (Sigma) at 5, 10 or 25μg/ml), or 50μl of medium (in control wells). 50μl of the cell suspension ($2.5x10^5$ cells) were added to every well and the covered plate incubated at 37°C for 48 hours in a 5% CO_2 humidified incubator.

1.0μCi (0.037 MBq) ^{3}H-TdR in 50μl of medium was added to each well and the plate incubated O/N (18 hours). Cells were harvested onto glass fibre filter paper (Dynatech) using a Dynatech Multimash 2000 cell harvester. Filter paper discs were dried thoroughly before being placed in 5mls of scintillation fluid (Optiphase 'X', Fisons) in capped scintillation vials (Hughes and Hughes). Radioactivity per disc was determined by counting for 1 minute on an Intertechnique SL 30 liquid scintillation spectrometer.

2.4.4 CELL SEPARATION

a) Panning

To obtain B cell and T cell populations from spleen and MLN preparations cells were panned under sterile conditions using 90mm petri dishes coated O/N at 4°C with either rat anti-mouse thy-1 monoclonal antibody (Clone YTS 154.7) (Sera-lab) at 50μg/ml, or sheep anti-mouse IgGAM and L chains (Serotec) at 100μg/ml, 5mls/petri dish.

The anti-thy-1 and anti-IgGAM and L chain preparations were made

up in PBS, sterile filtered before use (0.22μm filter, Millipore), and retained after the O/N incubation for re-use. Coated dishes were washed for 3 minutes with warm sterile PBS and blocked with sterile 1% BSA in PBS at 4°C for 1 hour. After washing three times in sterile PBS, mixed lymphocyte suspensions, obtained as in 2.4.1, were pipetted onto the dishes at a concentration of $5x10^6$ cells/ml in RPM1 1640 medium plus supplements, 3mls per dish, and left for 2 hours at 4°C. Non-adherent cells were then collected and resuspended in medium followed by the adherent cells, recovered by vigorous pipetting with cold PBS. Viable B and T cells were counted (2.4.2) and resuspended at the required concentration in RPM1 1640 medium plus supplements. B cell, T cell and mixed B and T lymphocyte preparations were plated out at equal cell concentrations with *T. muris* E/S antigen as previously described (2.4.3). Up to $1x10^6$ cells per sample were reserved for Fluorescence Activated Cell Sorting (FACS) analysis to check the efficiency of the cell separation technique.

b) Separation of B and T lymphocytes using Immunomagnetic beads

Dynabeads M-450 (Dynal) coated with affinity purified sheep anti-mouse IgG were used to negatively select for T lymphocytes by removal of B cells. The protocol used is outlined below.

Beads were washed 3 times in sterile, cold RPMI medium (containing no FCS) and resuspended to approximately 7.5ml in RPMI. Cells, prepared as in section 2.4.1 were added, 10^8 cells per $4x10^8$ beads, i.e. a ratio of four beads to every one cell, up to a volume of 10-12mls in sterile universals. FCS was added to approximately 0.5-1%. Cells were rocked for 30 minutes at 4°C and then the volume of the mixture increased to 20ml with RPMI. A cobalt-samarium magnet (Dynal) was placed on the outside wall of the universal for 30 seconds to collect rosetted cells and free

Dynabeads on the inside wall of the vessel. The S/N, containing non-rosetted cells, was decanted into a fresh universal, and the cells washed 3 times in RPMI plus 10% FCS, counted, resuspended at $5x10^6$ cells/ml and plated out, all as outlined in section 2.4.3. Mixed lymphocyte populations were also plated out and cells from fractionated and unfractionated samples were prepared for FACS analysis (section 2.4.5) to determine the efficiency of the negative selection procedure.

Immunomagnetic beads were recovered by resuspending the rosetted cells to 5mls in RPMI and incubating at 37°C for 24 hours. Detached beads were then washed three times in sterile 0.1% BSA in PBS, resuspended to $4x10^8$/ml in 0.1% BSA/PBS plus 0.02% sodium azide, and stored at 4°C.

2.4.5 IMMUNOFLUORESCENT STAINING OF T-CELL SUBSETS PRIOR TO FLUORESCENCE ACTIVATED CELL SORTING (FACS) ANALYSIS

B, T and mixed lymphocyte suspensions were prepared as described (2.4.1, 2.4.4). Up to $1x10^6$ cells per sample were removed, placed in an eppendorf and pelleted by spinning for 10 seconds at 11,500g in a microcentrifuge. 50μl of rat anti-mouse monoclonal antibodies (rat anti-thy-1 monoclonal antibody (clone YTS 154.7, Sera-lab), anti-L3T4 monoclonal antibody, anti-Lyt 2-monoclonal antibody or a pooled anti L3T4 plus anti Lyt 2- monoclonal antibody preparation) or washing medium (RPMI 1640 medium (Gibco) containing 0.1% sodium azide, 0.5% BSA (Sigma), and 5% heat inactivated normal rabbit serum) were added to the cell pellet and the sample vortexed to resuspend the cells. After a 30 minute incubation on ice, cells were washed three times in washing medium, pelleted and 50μl of fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat IgG2b (Sera-lab; clone NORIG 7.16.2), diluted 1/20 in washing medium, were added. Cells were resuspended and incubated for a further 30

minutes on ice. After three more washes in washing medium the final pellet was resuspended in 200μl of washing solution. 200μl of buffered 1% paraformaldehyde (Eagle Scientific Ltd.) were added to fix the cells, and the samples were left at 4°C prior to FACS analysis.

2.5 HISTOLOGICAL AND IMMUNOHISTOCHEMICAL TECHNIQUES

2.5.1 WAX SECTIONS

Caeca and colons were removed from mice with adult *T. muris* infections, the guts opened and washed with PBS. Pieces of gut (approximately 1cm long) with adult male and female worms *in situ* were laid on filter paper to prevent curling, and placed in Carnoys fixative (Appendix 2.6.8) for 6 hours. The tissue was then transferred to 70% ethanol and stored.

Fixed tissues were placed in wire baskets and dehydrated using a HistoKinette (Hendrey, wax bath type c.6250) which passed the tissues through a graded alcohol series, two changes of xylene (Sigma) and finally into a wax bath. Tissues were embedded in the paraffin polymer Polywax (Difco Ltd.), mounted on blocks and sectioned at 5μm. Prior to staining, sections were rehydrated by passing through xylene and a graded series of alcohols.

2.5.2 FROZEN SECTIONS

Specimens of adult *T. muris in situ* and isolated worms, removed from the gut using fine forceps, were dried on tissue then orientated in an

embedding compound (Tissue-Tek II OCT compound, Miles Laboratories) within a plastic mould. A beaker of isopentane was cooled down by placing in liquid nitrogen, and the specimens added when the isopentane started to solidify, resulting in the immediate freezing of the samples. Samples were stored in covered universals at -80°C until required.

Prior to sectioning the plastic mould was cut away, the frozen sample mounted on a cork ring and the cork attached to the cryostat chuck using the embedding compound. Sections were cut using a Reichert-Jung Cryocut E at 4μm and left to dry O/N before fixing and staining.

2.5.3 JB4 SECTIONS

Fixation

Plastic pipettes were used throughout for all manipulations. The fixative, 2% paraformaldehyde in 0.05M sodium cacodylate buffer pH7.4, and wash buffer, 0.05M sodium cacodylate buffer, were placed on ice 1 hour before use. Adult male and female *T. muris* were extracted from the mouse caecum, placed on a slide in a drop of ice cold fixative and cut at the oesophageal-intestinal junction into anterior and posterior segments using a fresh razor blade. The two segments were transferred into tubes of ice cold fixative and held in an ice bath for 15-20 minutes only. Segments of the posterior regions of male and female worms were prepared separately, anterior ends were pooled. Worm segments were washed twice in wash buffer and stored in fresh buffer at 4°C until further processing (performed as soon as possible as the samples were only lightly fixed).

Processing, embedding and sectioning

Fixed worm segments were dehydrated through a graded alcohol series (70% alcohol for 1 hour, 90% alcohol for 1 hour, 4 changes in absolute alcohol over the next 6 hours) prior to placing in catalysed solution A (Appendix 2.6.8) O/N. Segments were transferred to fresh catalysed solution A the next morning. After 2 hours solution B (Appendix 2.6.8) was prepared, added to solution A in a ratio of 42mls A to 1ml B in a plastic beaker and the mixture agitated. Specimens were placed within a capsule, orientated, the resin, JB4 glycol methacrylate (Polaron Ltd., Watford) added and left for 1 hour.

Semi-thin (2μm) sections prepared with a Reichert Ultracut and diamond knife were collected on acid-cleaned glass slides and allowed to dry O/N before labelling. Immunoperoxidase staining was carried out as described in section 2.5.5 starting at the blocking of the endogenous peroxidases stage. No counterstain was used. Immunofluorescent staining of JB4 sections is described in section 2.5.4.

2.5.4 IMMUNOFLUORESCENCE

Wax sections

Wax sections were rehydrated and placed in a humidified chamber. Monoclonal antibody S/Ns or ascites diluted from 1/50 to 1/100 in PBS plus 0.1% w/v BSA (Sigma) were pippetted onto the sections along with appropriate controls (PBS/BSA, P3NS1 S/N). Sections were incubated for 30 minutes at RT, washed in PBS/BSA (0.1%) and fluorescein-conjugated affinity-purified goat anti-mouse immunoglobulins (IgGAM, heavy and light chain specific, Cappel) added at 0.1mg/ml. Sections in the humidified

chamber were placed at 4°C in the dark for 45 minutes before washing in PBS/BSA (0.1%) (Sigma) and mounting in 50% glycerol (CP Pharmaceuticals) in PBS. Sections were examined immediately under ultra violet illumination.

JB4 sections

JB4 sections were blocked for 1 hour using 0.02M glycine in PBS prior to incubation with the primary antibody (monoclonal antibody S/Ns used neat, test sera diluted 1/50 and 1/100, an anti-phosphorylcholine monoclonal antibody diluted 1/100 and 1/500, or appropriate control reagents) in a humidified chamber for 2 hours at RT. After thorough washing in PBS a fluorescein-conjugated rabbit anti-mouse IgG(H+L) (Miles-Yeda Ltd.), diluted 1/25 in PBS, was added and left for 1 hour at 4°C in the dark. Sections were washed thoroughly in PBS, air dried, and mounted as before prior to viewing under ultra violet illumination.

2.5.5 IMMUNOPEROXIDASE STAINING OF FROZEN SECTIONS

Air dried cryostat sections were fixed by incubating with filtered sodium sulphate-dried acetone (May and Baker) for 10 minutes. After the acetone had evaporated, tissue peroxidases were blocked with 0.7% 100 volume hydrogen peroxide (H_2O_2) (Fisons) in methanol (May and Baker) for 30 minutes in a humidified chamber. Slides were washed in distilled water, then TBS (Appendix 2.6.2) for 5 minutes. Sections were blocked with 5% normal rabbit serum or 1% BSA in PBS for 15 minutes. Slides were drained of the blocking agent and monoclonal antibody S/Ns applied neat, and in dilutions in TBS from 1/10 to 1/50. Test sera were used at a 1/50 dilution. Slides were incubated for 1-2 hours along with control slides

incubated with TBS, P3NS1 S/N, and irrelevant ascites. After 3, 5 minute washes in TBS, peroxidase-conjugated rabbit anti-mouse immunoglobulins (DAKO) diluted 1/50 was added to the sections and left for 1-2 hours. Slides were again washed using 3, 5 minute washes in TBS. To develop the reaction, sections were incubated with peroxidase substrate solution (Appendix 2.6.8). After a 5 minute incubation slides were washed in distilled water, counterstained using Harris' haemotoxylin, blued under tap water, dehydrated and mounted using DePeX (DPX) mounting medium (BDH Chemicals Ltd.).

2.5.6 ALKALINE PHOSPHOTASE STAINING OF FROZEN SECTIONS

The same method was used as for peroxidase staining (2.5.4) except the alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulins reagent (DAKO) was diluted 1/30 before use, and tissue phosphatases were blocked by adding levamisole (Sigma) to the alkaline phosphatase substrate (Appendix 2.6.8). Sections were mounted using Loctite UV adhesive 357.

2.6 APPENDIX

2.6.1 CULTURE MEDIA

RPMI 1640 Medium (for cell culture)

RPMI 1640 powdered medium	10.42g/l	(Gibco)
Sodium bicarbonate	2g/l	(Sigma)
L-glutamine	2mM	(Gibco)
Sodium Pyruvate	0.1mM	(Gibco)
HEPES	10mM	(Sigma)
Monothioglycerol	$7.5x10^{-5}$M	(Sigma)
Penicillin	100units/ml	(Gibco)
Streptomycin	100μg/ml	(Gibco)
Foetal Calf Serum	100ml/l	(Seratec)

Medium made up in double distilled deionised water, filter sterilised and stored at 4°C.

RPMI 1640 Medium (for worms)

RPMI 1640 powdered medium 10.42g/l (Gibco) plus supplements as above but without L-glutamine and FCS and with 5 times the above concentration of penicillin/streptomycin plus 2.5μg/ml of fungizone (Gibco)

2.6.2 GENERAL BUFFERS AND SOLUTIONS

Phosphate buffered saline (PBS) pH7.4

NaCl	9.0g	(Sigma)
Na_2HPO_4	2.84g	(BDH)
$NaH_2PO_4.2H_2O$	2.76g	(BDH)

Adjust pH and make up to 1 litre with distilled water.

Tris buffered saline (TBS) pH7.6

Tris	60.75g/l	(Sigma)
NaCl	90.0g/l	(Sigma)

Make up to 1 litre with distilled water and dilute 1 in 10 with distilled water before use.

Ammonium chloride/potassium reagent

NH_4Cl	8.29g	(BDH)
$KHCO_3$	1.0g	(Sigma)
EDTA	32.2mg	(BDH AnalaR)

Add distilled water up to 1 litre, filter sterilise (0.22μm filter, Millipore), aliquot and store at 4°C.

2.6.3 PAGE BUFFERS AND SOLUTIONS

Tris-glycine electrode buffer

Tris	15.15g	(Sigma)
Glycine	72.0g	(Sigma)
SDS (Sodium dodecyl sulphate)	5.0g	(Sigma)

Make up to 5 litres with distilled water.

Dense acrylamide solution

Acrylamide	29.2g	(Sigma)
NN'-Methylenebis acrylamide	0.8g	(Sigma)

75% glycerol to 100ml. Store in dark at 4°C.

Light acrylamide solution

Acrylamide	29.2g	(Sigma)
NN'-Methylenebis acrylamide	0.8g	(Sigma)

Make up to 100ml with distilled water. Store in dark at 4°C.

Lower gel buffer pH8.8

Tris	118.2g	(Sigma)
SDS	2.0g	(Sigma)

Dissolve in 200ml distilled water, adjust pH to 8.8. Make up to 500ml with distilled water.

Upper gel buffer pH6.8

Tris	30.2g	(Sigma)
SDS	2.0g	(Sigma)

Dissolve in 200ml distilled water, adjust pH to 6.8. Make up to 500ml with distilled water.

10% Ammonium persulphate (APS)

Ammonium persulphate	0.1g	(Sigma)

Make up to 1ml with distilled water. Make up fresh on day of use.

Reducing sample buffer

Upper gel buffer	12.5ml	
SDS	2.3g	(Sigma)
Glycerol B.P.	10.0ml	(CP Pharmaceuticals)
2-Mercaptoethanol	5.0ml	(Sigma)
1% Bromophenol blue	0.1ml	(Sigma)

Distilled water to 100ml.

Coomassie Brilliant Blue (Coomassie)

Coomassie brilliant blue	0.5g	(Sigma)
Methanol	125mls	(Fisons)
Glacial acetic acid	50mls	(BDH)

Distilled water to 500mls, filter before use.

Coomassie destain (also used as fixer)

Methanol	200ml	(May and Baker)
Glacial acetic acid	50ml	(BDH)

Distilled water to 500 mls.

0.25% KCl solution

KCl	2.5g	(Fisons)

Distilled water to 1 litre.

Equilibrating buffer

NH_4HCO_3	0.45g	(Fisons)
SDS	0.05g	(Sigma)
Dithiothreitol	0.08g	(Sigma)

Distilled water to 100ml.

Elution buffer

NH_4HCO_3	0.45g	(Fisons)
SDS	0.05g	(Sigma)

Distilled water to 100ml.

2.6.4 WESTERN BLOT BUFFERS AND SOLUTIONS

Blotting buffer

Glycine	57.6g	(Sigma)
Tris	12.0g	(Sigma)
Methanol	1.0litre	(May and Baker)
SDS	5.0g	(Sigma)

Distilled water to 5 litres.

PBS/Tween (0.05%)

Tween 20 0.25ml (BDH)
Polyoxyethylene sorbitan monolaurate

PBS (2.6.2) to 500mls.

Blocking buffer

Skimmed milk 10g (Marvel)

PBS/Tween as above to 100ml

Amido black stain

Amido black	0.1g	(Sigma)
Methanol	45.0ml	(May and Baker)
Glacial acetic acid	10.0ml	(BDH)

Distilled water to 100ml.

Amido black destain

Methanol	90.0ml	(May and Baker)
Glacial acetic acid	2.0ml	(BDH)

Distilled water to 100ml.

2.6.5 IMMUNOPRECIPITATION BUFFERS AND SOLUTIONS

Immunoprecipitation buffer pH7.4

Tris	0.605g	(Sigma)
NaCl	0.87g	(Sigma)
EDTA	0.372g	(BDH AnalaR)
Triton X-100	50μl	(Sigma)

Distilled water to 50mls, pH with 1M HCl, increase volume to 100mls with distilled water.

22% PPO in DMSO

2'5 diphenyloxazole (PPO) 88g (Sigma)

Dimethylsulphoxide (DMSO) (Sigma) to 400 mls.

2.6.6 ELISA BUFFERS AND SOLUTIONS

0.05M Carbonate/bicarbonate buffer pH9.6

Na_2CO_3	1.59g	(BDH AnalaR)
$NaHCO_3$	2.93g	(BDH AnalaR)
NaN_3 (optional)	0.2g	(Sigma)

Distilled water to 200mls, adjust pH with 1M HCl, distilled water to 1 litre.

Diethanolamine buffer pH9.8

Diethanolamine 97mls (Sigma)

Adjust pH with 1M HCl. Distilled water to 1 litre.

3% Bovine Serum Albumin

Bovine Serum Albumin (BSA) 0.3g (Sigma)

PBS/Tween (0.05%) to 10 mls.

2.6.7 LOWRY SOLUTIONS

2% Na_2CO_3 in 0.1M NaOH

Na_2CO_3	5g	(BDH AnalaR)
NaOH	1g	(BDH AnalaR)

Distilled water to 250 mls

0.5% $CuSO_4.5H_2O$

$CuSO_4.5H_2O$ 1.25g (BDH)

Distilled water to 250ml

1% Potassium sodium tartrate

$KNaC_4H_4O_6.4H_2O$ 2.5g (Fisons)

Distilled water to 250ml.

Working reagent

50ml 2% Na_2CO_3 in 0.1M NaOH (fresh on day of use)
1ml 0.5% $CuSO_4.5H_2O$
1ml 1% $KNaC_4H_4O_6.4H_2O$

2.6.8 HISTOLOGICAL AND IMMUNOHISTOCHEMICAL REAGENTS

Carnoy's Fixative

Absolute ethanol	60ml	(Fisons)
Chloroform	30ml	(May and Baker)
Glacial acetic acid	10ml	(BDH)

Peroxidase substrate

3,3, Diaminobenzidine tetrahydrochloride (DAB) 150mg (Sigma)

TBS to 300mls, filter (Whatman No. 4 filter), then add 0.015% of 100 volume H_2O_2.

Alkaline phosphatase substrate
(make up in a glass vessel)

N,N dimethyl formamide (DMF)	2-4mls	(Sigma)
Naphthol AS-TR phosphate	100mg	(Sigma)
1M Levamisole	2-5mls	(Sigma)
0.1M Tris pH8.2	500mls	(Sigma)

Store at 4°C. Add 100mg Fast Red TR salt (Sigma) to 100mls of above solution before use.

JB4 processing solutions

Solution A

2 hydroxyethyl methacrylate	80mls	(Polaron)
2 butoxyethanol	8ml	(Polaron)
Benzoyl peroxide	1gm	(Polaron)

Store at 4°C for no more than 2 weeks. Bring to RT before use.

Solution B

Polyethylene glycol 400	15mls	(Polaron)
N,N-dimethylamiline	1ml	(Polaron)

Mix together just prior to use.

SECTION ONE

GENETIC CONTROL OF IMMUNITY TO *TRICHURIS MURIS* IN MICE

CHAPTER 3

THE EFFECTS OF H-2 AND NON-H-2 GENES ON THE EXPULSION OF *TRICHURIS MURIS* FROM INBRED, CONGENIC AND H-2 RECOMBINANT MICE

Section 3.1 has been published in Parasitology (1988), 96, 543–550

3.1 THE EFFECTS OF H-2 AND NON-H-2 GENES ON THE EXPULSION OF THE NEMATODE *TRICHURIS MURIS* FROM INBRED AND CONGENIC MICE.

3.1.1 SUMMARY

Two groups of H-2 congenic mice were compared for their susceptibility to a primary infection with the nematode *Trichuris muris*. Mice of the BALB genetic background were markedly more resistant than mice of the B10 genetic background, as reflected by the rate of expulsion of *T. muris* from the large intestine. Within each of the two groups of H-2 congenic strains mice possessing the $\underline{H\text{-}2}^{k}$ haplotype (BALB/k, B10.BR) were more susceptible to infection than mice expressing other haplotypes; B10 background strains expressing $\underline{H\text{-}2}^{b}$ (B10) or $\underline{H\text{-}2}^{q}$ (B10.G) alleles were the most resistant of the four congenic strains studied. Differential resistance was observed within three of the four B10 congenic strains and this is discussed in terms of the rate of development of the protective immune response in relation to worm development. The results support the conclusion that both H-2-linked and non-H-2 genes play important roles in controlling the immune response which expels worms from the gut.

3.1.2 INTRODUCTION

Immune responses to infections with parasitic helminths show well-defined genetic control. For example genes within the mouse major histocompatibility complex (MHC), H-2, have been shown to play an important role in controlling levels of resistance to *Trichinella spiralis* (Wassom, David and Gleich, 1979) and it has been suggested that their

effects are exerted upon the T lymphocyte populations which mediate worm expulsion. Genes outside the MHC have also been shown to influence the relative degree of susceptibility or resistance to *T. spiralis* expressed by a given mouse strain (Wakelin, 1980; Wakelin and Donachie, 1980, 1981, 1983; Bell, McGregor and Adams, 1982*a,b*; Wassom, Brooks and Cypess, 1983*a*). These background genes may influence the development of the intestinal inflammatory responses which are the immediate effectors of protective immunity.

Studies on a parasite closely related to *T. spiralis*, *Trichuris muris*, a parasite of the mouse large intestine, have shown differences in the outcome of infection in a variety of mouse strains, ranging from total failure of *T. muris* to mature in certain strains, to the development of patency in others (Worley *et al.*, 1962; Campbell and Collette, 1962; Wakelin, 1975*b*; Tomašovičová *et al.*, 1988). These differences in susceptibility are known to be genetically determined differences in the ability of mice to bring about immune expulsion of the parasite (Wakelin, 1975*b*) but the relative contribution of background and MHC-linked genes have not been evaluated.

This present study was undertaken in order to investigate the effects of both H-2-linked and non-H-2-genes on the expulsion of *T. muris*.

3.1.3 MATERIALS AND METHODS

Animals

Six to eight-week-old inbred male mice obtained from Harlan Olac Ltd were used. The strains concerned and their haplotypes are listed in Table 3.1.1. NIH mice were used to provide baseline data, as the kinetics of *T. muris* infections in this strain are well established (Wakelin, 1975*b*).

Table 3.1.1 List of mouse strains with their H-2 haplotype and the abbreviations used in this paper

Strain	H-2 haplotype	Abbreviation used
BALB/c/Ola	d	BALB/c
BALB/B/Ola	b	BALB/B
BALB/K/Ola	k	BALB/K
C57BL/10ScSn/Ola	b	B10
B10.D2/n/Ola	d	B10.D2/n
B10.BR/Ola	k	B10.BR
B10.G/Ola	q	B10.G
NIH/Ola	q	NIH

Parasite

The maintenance of *T. muris* and the methods used for infection and examination of the experimental animals were as described by Wakelin (1967).

Statistical analysis

The significance of differences between mean worm recoveries from experimental groups was calculated using the Students *t* test. A value greater than P = 0.05 was considered non-significant.

3.1.4 RESULTS

Establishment and expulsion of infections in inbred and congenic strains

Mice were infected with 400 *T. muris* eggs on day 0, killed in groups of at least 5 mice at various intervals post-infection (p.i.) and their worms recovered.

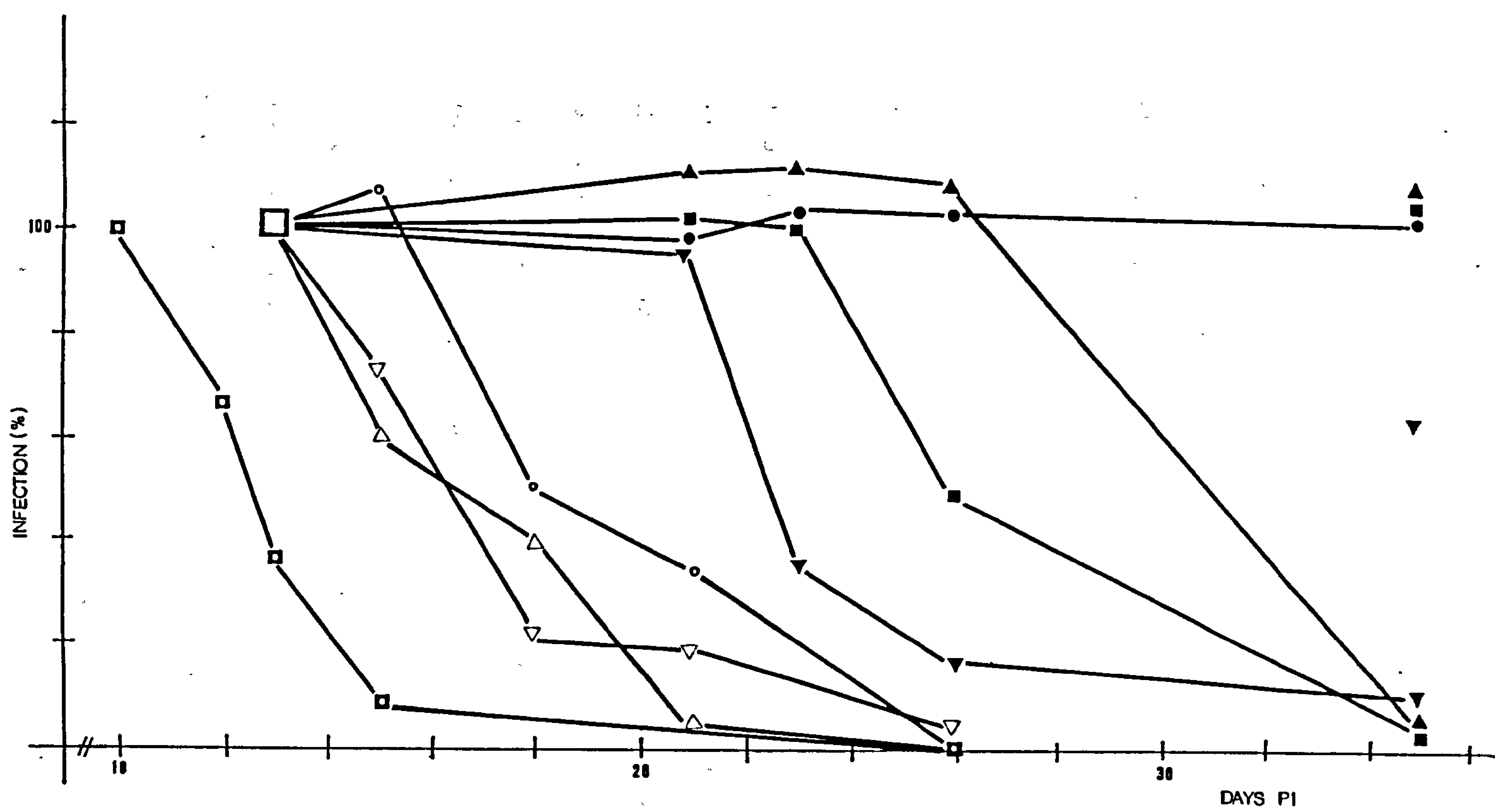
Table 3.1.2 Establishment of *Trichuris muris* in inbred strains of mice

		No. of worms recovered after infection (day 10–NIH; day 13 all other strains)	
Exp.	Strain	Mean	S.D.
1	B10.G	71.2	5.1
	B10.BR	72.2	4.8
2	B10	133.3	41.8
	B10.D2/n	122.2	25.9
	BALB/B	109.0	36.2
	BALB/c	83.0	22.3
	BALB/K	116.0	13.5
	NIH	132.0	31.6

The mean number of larvae established in each strain is given in Table 3.1.2; no significant difference in establishment was recorded between any of the strains studied in any one experiment. The time-courses of expulsion of *T. muris* from the strains studied are shown in figure 3.1.1. In terms of kinetics of worm expulsion the strains of mice formed three distinct groups. Expulsion was fastest in NIH mice and slowest in the B10 congenics, with the BALB congenics occupying an intermediate position. NIH mice had expelled 90% of their worm burdens by day 15 of infection. This value was not reached until day 20–25 in BALB background mice and day 35 in B10 background mice.

The influence of H-2 genes on the rate of expulsion was more readily discernible within the slowly responding B10 congenics than amongst the BALB congenic mouse strains. BALB/B (H-2^b) and BALB/c (H-2^d) mice initiated expulsion earlier (between days 13 and 15 p.i.) than mice of the BALB/K strain (H-2^k), where no decrease in worm burden from the day 13 mean was seen until after day 15 p.i. Indeed, the difference between the observed worm burdens of BALB/K and BALB/c mice on day 15 was significant at the 0.01% level. Worm expulsion was complete in all BALB congenic strains by day 26 p.i.

Figure 3.1.1 Time course of expulsion of *Trichuris muris* from NIH (◘—◘), BALB/c (△—△), BALB/B (▽—▽), BALB/K (○—○), B10 (▼—▼), B10.G (■—■), B10.D2/n (▲—▲) and B10.BR (●—●) mice infected with 400 T. muris eggs. Mean worm burdens are expressed as percentages of the day 10 (NIH) or day 13 (all other strains) mean burden: these times were chosen because it is known that worm burdens are stable up until that time (Wakelin, 1975b). Non-responder (NR) individuals harboured patent infections on day 35 p.i. the proportion of NR mice within each strain being B10.D2/n NR (4/7); B10.G NR (1/5); B10.BR NR (5/5); B10 NR (1/5). For inbred strains exhibiting differential responsiveness worm burdens on day 35 p.i. for the NR individuals are indicated by single points.


INFECTION (%)
100
10
20
30
DAYS PI

B10 mice (H-2^b) responded more rapidly than the other B10 congenic strains; worm expulsion began around day 21 p.i. and a significant reduction in worm numbers was seen by day 23 p.i. ($P<0.05$). B10.G mice (H-2^q) initiated expulsion between days 23 and 26 p.i., the first significant decrease in worm numbers being observed on day 26 p.i. ($P<0.05$). Mice of the strain B10.D2/n (H-2^d) showed no evidence of a protective immune response reflected by a reduced worm burden until after day 26 p.i., whilst B10.BR mice (H-2^k) appeared totally unable to expel *T. muris*, the patent worm burdens recovered on day 35 p.i. being equivalent to those recorded on day 13 p.i.

Within each of the B10 congenic strains studied, excluding B10.BR mice, a proportion of individuals was found to be unable to expel *T. muris* before the parasite reached patency. The proportion of non-responder individuals within each strain on day 35 p.i. varied from 1/5 (B10 and B10.G) to 4/7 (B10.D2/n).

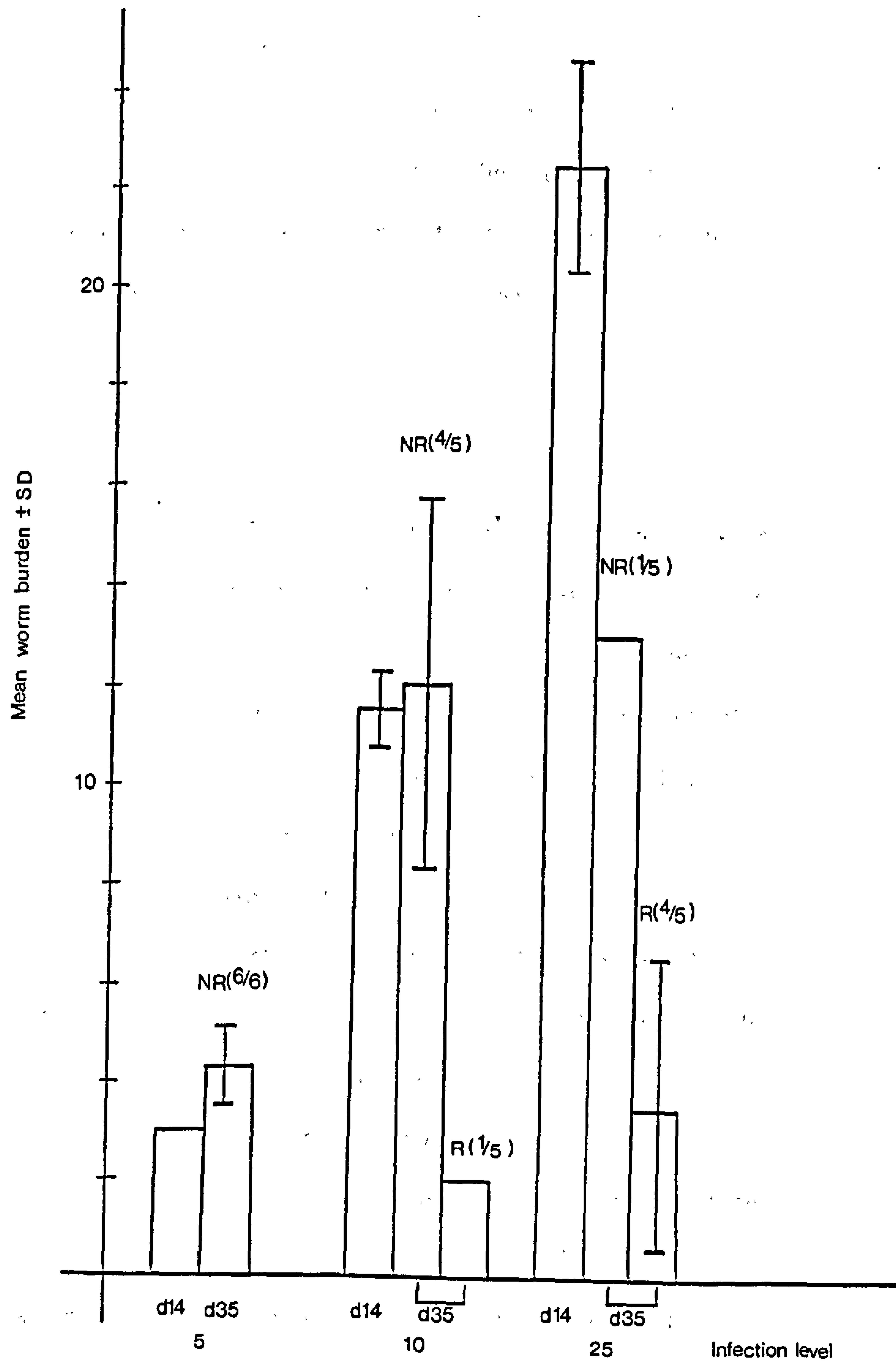
The reliability of the congenic strain expulsion data was confirmed in repeat experiments.

Survival of worms from low-level infections

Although from earlier data (Wakelin, 1973) it was not considered likely, it was thought necessary to investigate the possibility that the occurence of non-responsiveness in certain individual mice might reflect exposure to a level of infection inadequate to elicit a protective response. Differential infections were established in B10 mice in an attempt to identify whether a threshold level of infection existed.

Three groups, each of at least 8 mice, were infected on day 0 with the appropriate number of eggs to establish day 14 larval burdens of 25, 10 and 5 worms. Mice were killed on days 14 and 35 p.i. and the worms

Figure 3.1.2 Survival of different levels of infection with *Trichuris muris* in B10 mice. Mean worm burdens on day 14 and day 35 p.i. are shown ± standard deviations. Non-responder mice (NR) harboured patent infections on day 35 p.i. The proportions of NR and responder (R) mice within the population at each infection level on day 35 p.i. are given in parentheses.



Mean worm burden ± SD
20
10
NR(6/6)
NR(4/5)
R(1/5)
NR(1/5)
R(4/5)
d14
d35
5
10
25
Infection level

recovered. The results are shown in figure 3.1.2 from which it can be seen that it is not until burdens of fewer than 10 *T. muris* are established that the infection becomes subthreshold in all mice, allowing worms to reach patency. The percentage of non-responder mice within each group increased as the mean worm burden decreased, and all mice were non-responsive at a mean burden of 5 worms. Establishment of a burden of 10 worms resulted in 4/5 of the mice being unable to expel the parasite. At burdens of 25 worms all the mice initiated expulsion but 1/5 of the mice showed only a partial response, reducing, but not eliminating the infection.

3.1.5 DISCUSSION

The establishment and survival of helminth parasites in potential hosts is influenced by many factors. In natural host-parasite relationships, where there is physiological compatibility between the two species, one of the most important influences arises from the host's capacity to mount effective anti-parasite responses. In genetically heterogeneous host populations the expression of this capacity is variable between hosts. This effect is seen most clearly in experimental systems where uniform infections can be administered to genetically distinct strains of the same host species. Under these conditions variations in the outcome of infection reflect genetically determined variations in the host immune response.

The data presented here confirm the existence of marked host strain variation in susceptibility in mice infected with the large intestinal nematode *T. muris*, and additionally show that both H-2-linked and non-H-2 genes are involved in determining host response phenotype. Thus mice of the B10 genetic background responded comparatively poorly to infection in contrast to mice of the BALB background, and very much more slowly than NIH mice. In addition, distinctly different patterns of worm expulsion were

observed when H-2 congenic strains of the B10 and BALB backgrounds were used.

The influence of H-2-linked genes on the rate of worm expulsion was seen more clearly within the more slowly responding B10 congenics. Indeed, a gradation in responsiveness was seen from the relatively responsive B10 mice (H-2^{b}) through B10.G(H-2^{q}) and B10.D2/n(H-2^{d}) to the 100% non-responsive B10.BR (H-2^{k}) mice. In both B10 and BALB mice the H-2^{k} haplotype was associated with the lowest degree of responsiveness, and it appears that in circumstances where the B10 genetic background predisposes mice to a certain degree of susceptibility to *T. muris*, the presence of the H-2^{k} haplotype renders mice totally unable to expel *T. muris*.

It is striking that similar H-2 and non-H-2 effects upon worm expulsion have also been reported for the related nematode *Trichinella spiralis*. B10 background strains of mice were markedly more susceptible to this parasite than NIH, SWR and DBA/1 strains, retaining the intestinal worms for a comparatively long period and therefore acquiring heavier muscle larval burdens (Wakelin, 1980). Using the latter criterion, Wassom *et al.* (1979) showed that strains of mice sharing the H-2^{k} haplotype were much more susceptible to infection than strains expressing H-2^{s} and H-2^{q}, with other haplotypes occupying intermediate positions. In later work Wassom *et al.* (1984*b*) additionally showed that the greater susceptibility of H-2^{k} haplotype mice extended to their ability to expel the intestinal stages of *T. spiralis* as well as to regulate worm fecundity.

The relative importance of H-2-linked and non-H-2-linked genes in controlling immunity to the intestinal phase of *T. spiralis* has been the subject of controversy. Wakelin (1980), Wakelin and Donachie (1980, 1981), Bell and McGregor (1980) and Bell *et al.* (1982*a,b*) concluded that background genes were the primary regulating factors, whereas Wassom *et*

al. (1984*b*) placed greater emphasis upon the role of H-2 genes in controlling the immune response. A current consensus is that H-2-linked genes effect a 'fine-tuning' of anti-worm immunity within limits defined by the influence of the background genome, a view which is consistent with the data described here for infection with *T. muris.*

One hypothesis to explain the operation of H-2-linked and background genes in controlling immunity, put forward by Wassom, Krco and David (1987), was based on the fact that the H-2 congenic strains most resistant to *T. spiralis* (those that express b, s, f or q haplotypes) do not express I-E products at the surface of their antigen presenting cells. It was suggested that presentation of worm antigen in the context of I-A-coded molecules primed for effective immune responses whereas presentation in the context of I-E products (as would occur in susceptible k, r or p H-2 haplotype mice) preferentially induced suppressor T cells. As the genetically controlled differences in susceptibility that exist among strains of mice infected with *T. muris* appear to bear a striking resemblance to those that exist among strains of mice infected with the closely related parasite *T. spiralis*, it is conceivable that the above hypothesis is also applicable to the *T. muris*-mouse system. However, it is difficult to interpret within this framework the curious phenomenon of differential immune responsiveness to *T. muris* within the B10 congenic strains. As all members of a given inbred strain are genetically identical they should behave uniformly in their response to *T. muris.* The existence of differential responsiveness within inbred strains strongly suggests that parasite-induced effects may be responsible. For example, it may be that, after reaching a certain size, worms cease to be susceptible to effector mechanisms, variation in the rate of parasite growth may mean that individuals reach this size at different times; alternatively it may be that worms reach a stage at which they are capable of actively suppressing host immunity before a protective immune

response can be expressed. Differential responsiveness was seen only in the more slowly responding B10 congenic strains, there being 100% responsiveness within each of the BALB congenics. In addition the proportion of non-responders within any one strain was seen to increase as the time taken to mount an effective worm expulsion increased until 100% non-responsiveness was seen within the B10.BR strain. This correspondence between expulsion time and proportion of non-responders reinforces the view that a parasite-dependent influence is responsible.

Differential responsiveness to *T. muris* has previously been described in DBA/2 (H-2^d) mice (Worley *et al.* 1962; Lee and Wakelin, 1982*b*), a more or less fixed proportion of mice being unable to expel the worm during larval development. Roach (1986) showed that individual DBA/2 which failed to expel the parasite during a primary infection remained susceptible to a secondary infection after the primary infection had been cleared using an anthelmintic. Preliminary experiments suggest that the same is true in B10 congenic mice. If differential responsiveness was to be explained only in terms of rate of response it would be expected that mice unable to expel a primary infection should be resistant to a secondary infection when larval worms would be more susceptible to effector mechanisms. The inability of non-responder mice to expel a secondary infection must result from some continuing 'defect' in the host-protective response, although this clearly would have to be long-term to explain the results obtained by Roach (1986).

It is unlikely that the observed non-responsiveness reported here in B10 congenic strains resulted from a subthreshold infection being given to a proportion of mice, as high worm burdens of over a 100 worms were recovered from non-responders on day 35 p.i. Indeed the threshold experiment conducted in B10 mice showed that very small worm burdens (>10) are all that are necessary to stimulate a protective immune response,

a result compatible with data from other strains (Wakelin, 1973).

It is clear from this report that the H-2 complex plays a significant role in controlling the host immune response to *T. muris* as is known to be the case in other helminth infections (Wakelin, 1985). However, background genes clearly also influence the response, mouse strains sharing common H-2 alleles differing markedly in susceptibility.

The clear demonstration of background and H-2-linked genetic influences upon *T. muris* provide the basis for a more detailed investigation of variations in the humoral and cellular immune responses to *T. muris* which may produce a functional explanation for strain dependent variation. The close parallels between infection with *Trichuris* in mice and man provides some optimism that elucidation of this variation in the former will contribute towards an understanding of predisposition to infection in the latter (Bundy, 1986).

3.2 THE INFLUENCE OF GENES MAPPING WITHIN THE MAJOR HISTOCOMPATIBILITY COMPLEX ON RESISTANCE TO *TRICHURIS MURIS* INFECTIONS IN MICE

The experiments described above demonstrate the occurence of genetically determined variation in immune responsiveness to *T. muris*. Both MHC-linked and background genes contributed to this variation although the stronger influences were associated with the latter.

The natural extension of this work is to attempt to map genes which influence the expulsive response within the H-2 complex using H-2 recombinant strains of mice. This approach has been used extensively by a number of workers, notably Wassom and his colleagues, working with

Trichinella spiralis (Wassom *et al.* 1979; 1983*b*; 1984*a, b*; Wassom, 1985) and more recently *Heligmosomoides polygyrus* (*Nematospiroides dubius*) (Enriquez *et al.* 1988*b, c*), and Blackwell working on genetic control of host resistance to leishmaniasis (Blackwell *et al.* 1980; Blackwell, 1983; 1988).

Any correlations between particular alleles/loci and response status which emerge from such studies may be explicable at a molecular level in the light of a knowledge of the mechanisms involved in the development of immune responses to parasitic infection, in conjunction with an understanding of the way these mechanisms are genetically controlled (see Introduction 1.3).

Non-responsiveness to particular parasite antigens and hence potentially to parasite infection can arise by many mechanisms, including qualitative and quantitative variation in the expression of MHC class II gene products (Jones *et al.* 1981; Janeway *et al.* 1984), and the antigen specific repertoire expressed by the host's T cells (Guillet *et al.*, 1987). Mechanisms which may account for functional non-responsiveness are discussed in detail in the Introduction (1.3.2). Whilst the qualitative difference between responder and non-responder individuals, in terms of an expulsive response, in the *T. muris*-mouse model is clearly genetically determined, the precise components of the protective response that are deficient in non-responder mice are not known. The following experiment attempts to map H-2 genes involved in controlling resistance/susceptibility within the H-2 complex. The results are discussed in relation to those of Wassom working with the *T. spiralis*-mouse system.

3.2.1 EXPERIMENTAL DESIGN

A panel of H-2 recombinant and congenic strains of mice were

selected on the basis of alleles expressed at the K, D and I region loci of the H-2 complex. An F1 mouse strain resulting from a B10.BRxB10.G cross was also included in the experiment (see Table 3.2.1).

Table 3.2.1 Map of the H-2 complex showing the haplotype compositions for the congenic, recombinant and F1 strains of mice infected with *T. muris*

Strain	K	I				S	D
		A_β	A_α	$A_e(E_\beta)$	$E\alpha$		
B10.A(2R)	k	k	k	k	k	d	b
B10.A(4R)	k	k	k	k	(b)	b	b
B10.T(6R)	q	q	q	(q)	(q)	q	d
B10.A(3R)	b	b	b	b	k	d	d
B10.A(5R)	b	b	b	b	k	d	d
B10.AQR	q	k	k	k	k	d	d
B10	b	b	b	b	(b)	b	b
B10.BR	k	k	k	k	k	k	k
(B10.BRxB10.G)F1	kq	kq	kq	k(q)	k(q)	kq	kq

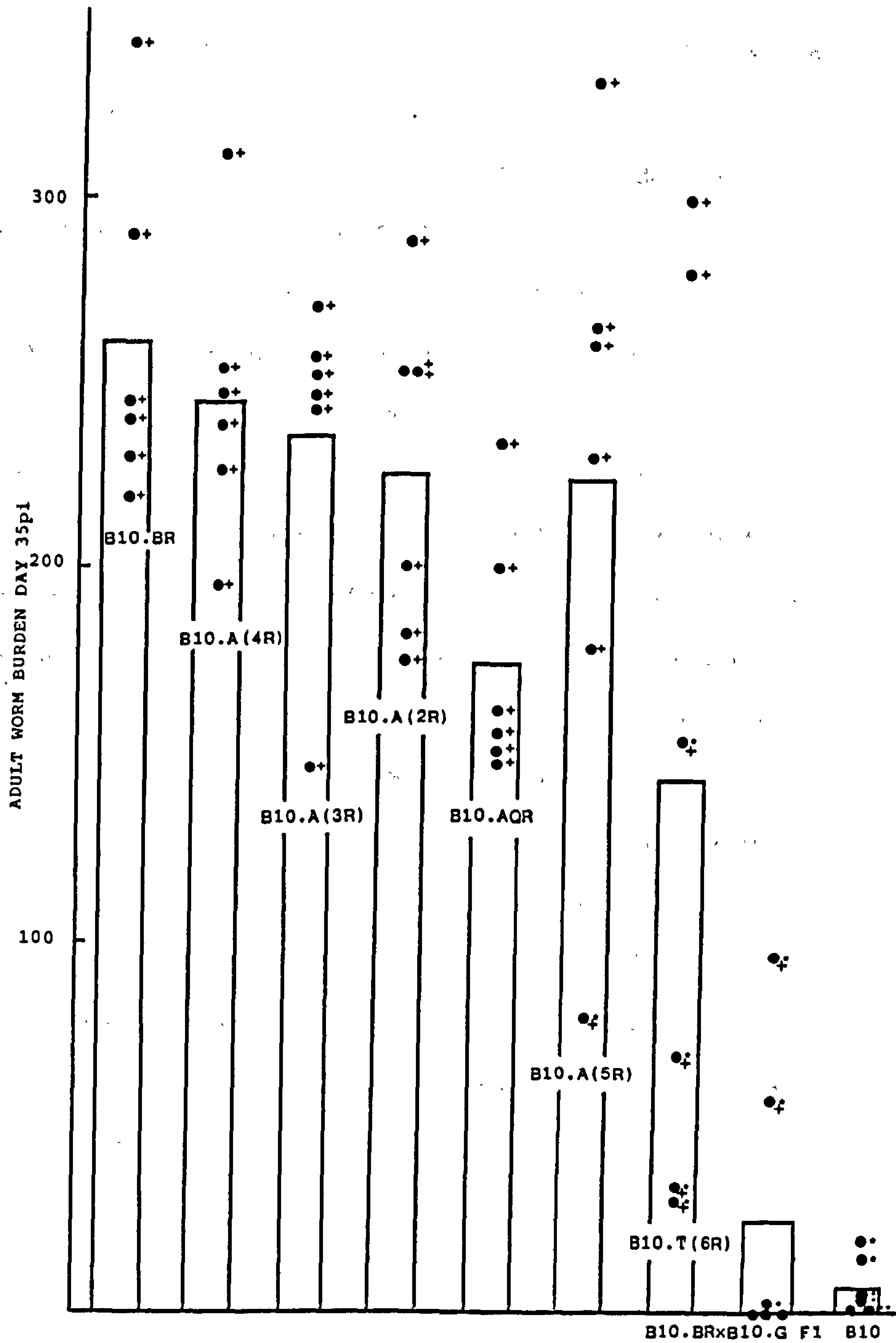
Six male mice were used per strain and were infected at six to eight weeks old with 400 embryonated *T. muris* eggs. Mice were bled individually from the tail vein on days 14, 21 and 28 p.i. and from the heart on day 35 p.i. when mice were sacrificed for adult worm burden determination. The serum obtained was subsequently used in a study of the genetic control of the humoral immune responses to *T. muris* E/S antigen (Chapter 4).

Adult worm burdens for each strain were compared, using the non-parametric Mann Whitney U test, with burdens recovered from B10.BR mice, a strain of mouse shown previously to be non-responsive to infection (Else and Wakelin, 1988). $P>0.05$ was considered non-significant.

3.2.2 RESULTS

Figure 3.2.1 shows the adult worm burdens recovered from individual mice on day 35 p.i. The data presents a rather complex picture, any

Figure 3.2.1 Adult worm burdens recovered from H-2 recombinant, congenic and F1 strains of mice on day 35 p.i. following infection with 400 *T. muris* eggs on day 0. Bar graphs represent the mean worm recovery for each strain. The B10.BR strain of mouse has previously been shown to be unable to expel a primary infection with *T. muris* (Else and Wakelin, 1988). The presence of stunted worms in individual mice is indicated by *; + = patent infection (see text).


ADULT WORM BURDEN DAY 35pi
300
200
100
B10.BR
B10.A(4R)
B10.A(3R)
B10.A(2R)
B10.AQR
B10.A(5R)
B10.T(6R)
B10.BRxB10.G F1
B10

patterns being somewhat obscured by the existence of differential responsiveness, typical of the slowly responding B10 genetic background mice, within some of the H-2 recombinant strains. Individual mice were classified as responders to infection only if they exhibited both a significant decrease in worm burdens from the burdens seen for B10.BR individuals on day 35 p.i. and stunting of worm growth such that infertile pre-adult stages of the parasite were present in the caecum/colon. Using this definition, only B10 and the (B10.BRxB10.G)F1 strains of mice were uniformally resistant to infection, showing significant reductions in worm burdens from B10.BR levels (P=0.001) and stunting of worm growth in individuals where a few worms remained. Two of the F1 mice harboured normal adult worms as well as stunted worms. In both cases, as has been observed before (Else and Wakelin, unpublished), the normal parasites were found in the first part of the colon, stunted worms being confined to the caecum. The only other mouse strain to show evidence of a protective immune response using the above definition was the B10.T(6R) strain, 4/6 mice harbouring stunted worms at levels significantly lower than the worm burdens recovered from B10.BR mice (P<0.01). B10.AQR mice had significantly reduced levels of adult *T. muris* compared to B10.BR mice (P<0.01) although these worms were not stunted. Worm burdens in B10.A(2R), B10.A(4R), B10.A(3R) and B10.A(5R) were not significantly different from B10.BR levels (P>0.05), although one B10.A(5R) individual did harbour some stunted parasites and showed a reduced worm burden.

3.2.3 DISCUSSION

The ability to resist primary infections with *Trichuris muris* is genetically controlled with both background (non-H-2) genes and genes within the mouse MHC influencing the outcome of infection (Else and

Wakelin, 1988). Within B10 congenic strains, mice expressing q and b alleles ("resistance" alleles) tended to be more resistant to infection than those expressing k or d alleles ("susceptibility" alleles). The data presented here confirms the involvment of H-2-linked genes in determining host response phenotype and enables hypotheses to be constructed as to how genes within the H-2 interact with each other.

B10.T(6R) mice were less resistant to infection than seen previously for B10.G mice (figure 3.1.1; Else and Wakelin 1988) where worm burdens were almost completely cleared except in one individual. Both these strains of mice possess q alleles at K and I-A loci and do not express I-E molecules. They differ at the D end of the H-2 however, B10.T(6R) possessing d alleles as opposed to q alleles (Table 3.2.1). It would seem therefore that the relative resistance to infection of mice expressing "resistance" q alleles at I-A, or that do not express I-E molecules, is subject to modulation by particular alleles at the D end of the H-2, especially d alleles ("susceptibility" alleles). The modulating effect of d in the D region was also seen in B10.A(3R) and B10.A(5R) strains of mice, both of which possess resistance b alleles at K and I-A but d alleles in the D region resulting in a non-responder phenotype. However, expression of the resistance allele b at the D end of the H-2 apparently cannot modulate the influence of susceptibility k alleles at K and I-A. For instance B10.A(2R) and B10.A(4R) strains of mice were both unable to expel *T. muris*. The possibility that lack of I-E expression correlates with resistance to infection, although suggested by the resistant B10 phenotype, was not borne out when other response phenotypes were examined. For instance B10.A(4R) do not express functional surface I-E molecules yet were unable to resist infection and the (B10.BRxB10.G)F1, although expressing $I\text{-}E^k$ molecules, were very resistant to infection.

The results suggest that response phenotypes may be determined by the

presence of resistance (q,b) or susceptibility (k,d) alleles at I-A, but that the influence of resistance alleles can be modulated by alleles at a D end locus/loci, especially susceptibility d alleles. A lack of appropriate recombinant haplotype strains with a crossover between K and I-A make it impossible to rule out the possibility that the H-2 K locus may influence response phenotype. Indeed B10.AQR mice, possessing susceptibility k and d alleles at I-A and D end loci respectively and resistance q alleles at K, had significantly lower worm burdens than B10.BR mice. However it is difficult to envisage how class I gene products might be involved as antigen is recognised in the context of class I molecules primarily by cytotoxic T cells (see Introduction 1.3.1). Cytotoxic T cells represent a subpopulation of T lymphocytes important in the killing of, for instance, virally infected cells, parasitised cells and tumour cells (Male *et al.*, 1987 Chapter 7) rather than in the development of immune responses against intestinal helminths.

The possible interaction between an I region gene and a gene at the D end of the H-2 complex described here bears many similarities to results obtained by Wassom and his colleagues working with *Trichinella spiralis* (Wassom *et al.* 1979; 1983*b*) and *Heligmosomoides polygyrus* (*Nematospiroides dubius*) (Enriquez *et al.* 1988*b*, *c*). However Wassom *et al* found that in both their model systems there was a correlation between strains of mice which did not express cell surface I-E molecules and resistance. The presentation of antigen to T cells in association with the $I\text{-}E^k$ molecule has been shown to induce a response which suppresses the ability of I-A-restricted T cells to proliferate *in vitro* (Baxevanis *et al.*, 1981) thus providing a mechanistic explanation for the observed relationship between $I\text{-}E^k$ expression and susceptibility to infection. As mentioned (3.1.5), Wassom *et al.* (1987) suggested that parasite antigens presented in the context of $I\text{-}E^k$ molecules activated T suppressor cells which preferentially suppressed an otherwise effective I-A mediated response.

Alternative hypotheses were also put forward for the association between the expression of I-E^k molecules and susceptibility to parasite infection including the induction of autoreactive T cells which could down regulate the I-A restricted response (Wassom *et al.*,1987; Wassom and Kelly, 1989). Autoreactive T cells, which recognise class II-like molecules on other T cells, possess multipotent immunoregulatory capabilities and have been shown to augment, suppress or contrasuppress antigen-specific B cell responses according to the types and proportions of other cells present in the culture (Quintáns *et al.*, 1986; Suzuki *et al.*, 1986; Suzuki and Quintáns, 1986). Thus the involvement of autoreactive T cells in the *Trichinella* system is an attractive hypothesis, providing an explanation for the dose dependent suppression of the anti-*Trichinella* response (Wassom *et al.*, 1984*a*). Thus at high infective doses the suppressive influence would dominate, down regulating the otherwise strong T helper cell response.

In the light of recent findings on the existence of two major functional helper T cell phenotypes, helper CD4$^+$ T cells (T_H2) and inflammatory CD4$^+$ T cells (T_H1) (Mossman *et al.* 1986; Mossman and Coffman, 1987; reviewed by Bottomly, 1988; see Introduction 1.3.3) the possibility is also raised that different T_H cell subsets are induced, according to the allelic form of the class II molecule with which parasite antigen is presented. T cell mediated inflammation is believed to be important in resistance to *T. spiralis* therefore it is possible that antigen presented in the context of I-E^k fails to activate inflammatory T_H1 cells, preferentially inducing the T_H2 cell type.

The concept of I-E regulated suppressor activity is supported by the work of Blackwell with *Leishmania donovani* in mice. She found that administration of a specific anti-I-A^d monoclonal antibody to non curing B10.D2/n (H-2^d) mice during infection resulted in the maintenance of high parasite loads whilst mice given anti-I-E^d monoclonal antibodies exhibited

some resolution of the parasite load (Blackwell and Roberts, 1987). However she also found that some non cure haplotypes did not express the I-E class II molecule (Blackwell, 1983). The work presented in this chapter likewise suggests that, although in some experimental systems the presence of I-E molecules certainly seems to be important in determining susceptibility, it is not a phenomenon which can be extended fully to all systems. In contrast to the findings of Wassom *et al.* (1987) with *T. spiralis*, crossing susceptible B10.BR mice ($\underline{H-2}^k$, $\underline{I-E}^k$) with resistant B10.G mice ($\underline{H-2}^q$, I-E$^-$) produced F1 mice which were resistant rather than susceptible to *T. muris* infection. I-E and I-A products are codominantly expressed in F1 animals hence antigen presenting cells of the above F1 mice will express four types of I-A molecules and cell surface $\underline{I-E}^k$ molecules. Clearly therefore the presence of $\underline{I-E}^k$ in the *T.muris*-mouse system is not an overriding factor in the determination of non-responsiveness, whereas the presence of some surface $\underline{I-A}^q$ molecules on antigen presenting cells of the F1 strain may contribute to the resistant phenotype observed.

The identification of genes involved in the control of anti-parasite responses is an important step in attempts to elucidate the immune mechanisms which operate in protective immunity to parasitic infections. Through correlations between haplotype, response status and expression, or lack of expression, of MHC class II molecules it is possible to speculate at a molecular level as to how the phenotypically expressed response is effected. Although such approaches are mainly restricted to experimental models they point the way towards a more fundamental analysis of genetically determined variation in responsiveness in man (Wakelin, 1986). For instance Bundy (1988) in a preliminary study on the association between human MHC (HLA) antigen frequencies and intensity of infection with *Ascaris lumbricoides* and *Trichuris trichiura* in a Caribbean population, revealed higher frequencies of a class II product DQw2 in the uninfected

population. As HLA-DQ antigens in general seem to be associated with restriction of cytotoxic T cells (Navarrete *et al.* 1985), Bundy suggested that this finding was of potential relevance to susceptibility to gut parasites.

Hirayama *et al.* (1987), studying the control of the immune response to *Schistosoma japonicum* antigen in humans, reported that HLA-DR and DQ molecules (comparable to the murine class II I-E and I-A molecules respectively) had distinct functions in immune regulation. They showed that the HLA-DR2 molecule from a non-responder haplotype (HLA-Dw12-DR2-DQw1) was required for the proliferative T cell response to *S. japonicum* antigen, but that this response was suppressed by suppressor T cells controlled by the DQw1 molecule in the non-responder haplotype.

It is clear that through the study of genetic control of immunity in experimental models, and in man, insights may be gained into the underlying mechanisms involved in protective immune responses to parasite infections.

3.3 SUMMARY POINTS

1. Background genes and genes within the mouse MHC were shown to influence the host primary immune response to *T. muris* as reflected by rate of expulsion of the parasite from the large intestine, the strongest effects being associated with the former.

2. Mice of the BALB genetic background were markedly more resistant to infection with *T. muris* than mice of the B10 genetic background. Within B10 congenic strains of mice, mice possessing k or d alleles ("susceptibility"

alleles) throughout the H-2 complex responded less well to infection than mice expressing other haplotypes. The presence of q or b alleles ("resistance" alleles) was associated with a relatively rapid expulsion of the parasite.

3. Differential resistance to infection within an inbred strain of mouse was observed in three of the four slowly responding B10 congenic mouse strains, strongly suggesting that later stages of the parasite were modulating the host immune response.

4. A panel of H-2 recombinant strains of mice was used in an attempt to map control of resistance to infection with *T. muris* within the H-2. Response phenotypes could be related to the presence of "resistance" (q,b) alleles or "susceptibility" (k,d) alleles at I-A under the modulating influence of "resistance" or "susceptibility" alleles, particularly d alleles, at a D end locus/loci.

5. Absence of I-E molecules correlated with resistance in some, but not all, strains studied. A (B10.BRxB10.G)F1 strain, which expressed $I\text{-}E^k$ gene products, was resistant to infection. Hence the expression of $I\text{-}E^k$ molecules is not an overriding factor in the determination of non-responsiveness to *T. muris* infection.

CHAPTER 4

GENETIC VARIATION IN THE HUMORAL IMMUNE RESPONSES OF INBRED, CONGENIC AND H-2 RECOMBINANT MICE TO *TRICHURIS MURIS*

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4.1 GENETIC VARIATION IN THE HUMORAL IMMUNE RESPONSES OF MICE TO THE NEMATODE *TRICHURIS MURIS*

4.1.1 SUMMARY

Genetically based differences in the antibody responses to the large intestinal nematode *Trichuris muris* were studied in two groups of H-2 congenic strains of mice that differed in their relative resistance to infection with this parasite. The primary response to parasite excretory/secretory (E/S) antigen was predominantly an IgG response with the strains forming two distinct groups, defined by their genetic background. The susceptible B10 genetic background mice had strikingly higher antibody levels than mice of the BALB background. Superimposed upon these background effects were clearly defined influences attributable to H-2-linked genes, strains which differed genetically only at H-2 loci exhibiting differences in the kinetics of the antibody response. Only B10.G and B10.BR mice showed any real increase in IgM levels post-infection. No IgA specific to E/S antigen was detected in the peripheral circulation of any strain at any time post-infection. Antibody responses to a 40-43kDa antigen revealed clear H-2-linked gene effects, with mice sharing the $\underline{H\text{-}2}^k$ haplotype (B10.BR, BALB/K) exhibiting considerably higher total antibody levels than mice expressing other haplotypes; mice of the $\underline{H\text{-}2}^d$ haplotype (BALB/c, B10.D2/n) responded very weakly to this antigen. A Western blot analysis of antigen recognition by antibody revealed similarities between the mouse strains in their total antibody responses to *T. muris* E/S antigen. However, immunoprecipitation studies showed that in general the more susceptible B10 congenic strains had wider spectra of antigen recognition than the BALB congenics. Strains sharing the same H-2 haplotype had dissimilar antigen

recognition profiles, but strains sharing the H-2[b] haplotype (B10, BALB/B) recognised a low MW antigen (20-23kDa) not recognised by any other strain, suggesting an exclusively H-2[b] restriction on the recognition of this antigen. These results support the conclusion that both H-2-linked and background genes play important roles in controlling the humoral immune response to *T. muris* infection.

4.1.2 INTRODUCTION

Genetically based differences in resistance to parasitic infections have been demonstrated in a wide range of host-parasite systems (Wakelin 1978). Relative resistance may be determined both by genes within the major histocompatibility complex (MHC) and by background genes. In the case of the large intestinal nematode *Trichuris muris* in the mouse, it is thought that genes linked to the mouse MHC (H-2) effect a fine tuning of anti-worm immunity within limits defined by the influence of the background genome (Else and Wakelin, 1988).

Expulsion of *T. muris* is known to involve at least two immunologically mediated components, an initial antibody-mediated phase and a subsequent lymphoid cell mediated phase (Wakelin 1975*a*), either or both of which could involve genetically controlled antigen recognition and be rate limiting. Alternatively, control may be exerted via the level and timing of antibody production during the first phase. Thus the range of variation in time of expulsion seen in inbred and congenic strains of mice (Wakelin 1975*b*) could reflect a variation in ability to produce the required level of antibody.

In this study the kinetics and specificity of the antibody response to *T. muris* antigens were studied in congenic strains of mice, to determine whether the ability of mice to make antibody responses to *T. muris* showed genetically determined variation and whether this ability could be correlated

with relative resistance or susceptibility.

4.1.3 MATERIALS AND METHODS

Animals

Congenic male mice, six to eight weeks old, obtained from Harlan Olac Ltd, were used. The strains concerned, their H-2 haplotypes, and their relative times of expulsion of *T. muris* are shown in Table 4.1.1.

Table 4.1.1 List of mouse strains with their H-2 haplotype, the abbreviations used in this paper and the time of worm expulsion in responder mice

Strain	Abbreviation	H-2 haplotype	Time of worm expulsion (days p.i.)
BALB/c/01a	BALB/c	d	13-26
BALB/B/01a	BALB/B	b	13-26
BALB/K/01a	BALB/K	k	15-26
C57BL/10ScSn01a	B10	b	21-35
B10.D2/n/01a	B10.D2/n	d	26-35
B10.BR/01a	B10.BR	k	No expulsion observed
B10.G/01a	B10.G	q	23-35

Parasite

The maintenance of *T. muris* and the methods used for infection and examination of the experimental animals were as described by Wakelin (1967). Mice were killed at various times post-infection (p.i) to establish the infection level and duration in each strain.

Serum

Groups of eight to ten mice were infected with 400 *T. muris* eggs on day 0 and subsequently bled from the tail vein weekly for a period of 9

weeks p.i. The sera obtained from the blood samples were stored at -20°C.

Antigen

Excretory/secretory (E/S) antigen

Adult male and female *T. muris* were removed individually from the large intestines of mice and placed in sterile RPMI-1640 medium supplemented with 500μg/ml of penicillin/streptomycin, and fungizone (GIBCO) at 2.5μg/ml. After washing for 2-3 hours in this medium at 37°C, worms were put into fresh medium and left O/N at 37°C for the collection of E/S products. The culture S/N was removed, centrifuged at 200g for 5 min. to remove eggs, and filtered (0.22μm filter, Millipore). After freeze-drying and reconstituting to one-quarter the original volume with distilled water, samples were dialysed O/N at 4°C before being analysed for protein, aliquoted, and stored at -20°C. Metabolically labelled E/S antigen was prepared by incubating worms as above in the presence of ^{35}S-methionine at 250μCi/ml of medium.

Adult male antigen (AMA)

Adult male and female worms were incubated O/N at 37°C as above. Male worms were then removed, washed in phosphate-buffered saline (PBS), homogenised in a minimal volume of PBS using a glass tissue homogeniser and allowed to extract O/N at 4°C. The resulting suspension was centrifuged at 1500g for 30 min. to remove particulate matter followed by ultracentrifugation of the S/N at 100,000g for 1 hour to remove insoluble material. The S/N was filtered (0.22μm filter, Millipore), analysed for protein, and stored at -20°C.

Protein estimation

Total protein concentrations were determined using a method modified from Lowry *et al.* (1951).

Enzyme-linked immunosorbent assay (ELISA)

Ninety-six-well-flat-bottomed plates (Falcon) were coated with E/S antigen (5μg/ml) in 0.05M carbonate/bicarbonate buffer, pH 9.6, and left O/N at 4°C. These antigen-coated plates were used in the ELISA essentially as described by Voller, Bidwell and Bartlett (1979). Briefly, plates were washed five times with PBS containing 0.05% Tween 20 (Sigma) and blocked with 3% bovine serum albumin (BSA, Sigma) in PBS/Tween for 1 hour. After two washes in PBS/Tween, 50μl of test serum at a dilution of 10^{-2} in PBS/Tween were added to each well and incubated at RT for 1.5 hours. After five further washes, alkaline phosphatase-conjugated sheep anti-mouse IgG (whole molecule)(Sigma), goat anti-mouse IgM (μ chain specific)(Sigma), goat anti-mouse IgA (α chain specific) (Sigma), all diluted 1/1000 in PBS/Tween, or goat anti-mouse IgGAM (Sigma) diluted 1/350 in PBS/Tween was added (50μl/well) and incubated for another 1.5 hours at RT. Plates were washed five times with PBS/Tween and the substrate *p*-nitro-phenylphosphate tablets (Sigma) dissolved in diethanolamine buffer, pH 9.8 (Fisons), added to the plates at 100μl/well. The enzymatic reaction was read at 410nm on a Dynatech MR700 Microplate Reader.

Elution of antigen from sodium dodecyl sulphate polyacrylamide gels

Proteins of adult male *T. muris* homogenate were separated by

SDS-PAGE. A 40-43kDa antigen band was cut out of gels after staining, homogenised in ammonium carbonate elution buffer (100mM NH_4HCO_3 (Fisons), 0.05% SDS (BDH Electron)) and left to extract O/N at 4°C. The resulting extract was used as antigen in ELISA.

Western blotting

Western blotting was carried out according to Burnette (1981). *T. muris* E/S antigen was boiled in the presence of SDS (BDH Electron) and 2-beta-mercaptoethanol (2-ME), and separated on 10-20% gradient SDS-polyacrylamide gels using the method of Laemmli (1970). Proteins were transferred from gels to nitrocellulose membrane (Schleicher and Schuell) electrophoretically. After saturating any remaining protein-reactive sites on the nitrocellulose with 10% skimmed milk in PBS/Tween (0.05%) the membrane was cut into strips and incubated with test sera (1/500 dilution in 10% skimmed milk in PBS/Tween) O/N at 4°C. After washing in PBS/Tween the strips were incubated with ^{125}I-rabbit anti-mouse polyvalent immunoglobulins (0.5μCi/strip, Amersham) for 2 hours. Strips were washed again, air dried, fixed to a support and exposed at -80°C to an X-ray film (Fuji) in combination with an intensifying screen.

Immunoprecipitation

Metabolically labelled E/S antigen was added to a series of Eppendorf tubes to give 100,000ct/min per tube. A volume of 25μl of test serum was added and made up to 0.5ml with immunoprecipitation buffer, pH 7.4. The solutions were vortexed and left O/N at 4°C. 15μl of sheep anti-mouse IgG Fc (Serotec) were added to each sample and left for 3 hours at RT. Immunoprecipitates were spun down at 11,500g and washed four times in

immunoprecipitation buffer before redissolving in reducing sample buffer for SDS-PAGE (10-20% gradient gels). Gels were fixed, fluorographed and dried down for exposure on X-ray film (Fuji) at -80°C.

4.1.4 RESULTS

Antibody responses to E/S antigen

All ELISAs were performed using pooled sera for each strain at each time-point p.i. The decision to use pooled sera was based on the knowledge that the range of individual variation within pools is relatively small even when using strains of mice which split into individuals which expel *T. muris* before day 35 p.i. and those which do not, such as the DBA/2 strain (Worley *et al.* 1962, Lee and Wakelin 1982*b*). For instance, in five samples from B10.BR individuals (day 35 p.i.) the mean optical density (OD) was 0.27±0.05, and the mean OD for 26 DBA/2 individuals (day 52 p.i.) was 1.27±0.18.

IgG and IgM levels to E/S antigen, as reflected by OD in ELISA, were followed from day 15 to day 61 p.i. for all strains except B10.D2/n mice, where the serological response was followed only to day 35 p.i. Each ELISA was performed at least three times using sera taken from mice in two separate experiments. Antibody response patterns for the strains of mice studied were found to be highly repeatable, typical results being shown in figures 4.1.1 (IgM) and 4.1.2 (IgG). Specific IgM levels (figure 4.1.1) showed little variation p.i. for all strains except B10.G, where the IgM response reached high levels well above the day 15 p.i. value by day 61 p.i., and to a lesser extent B10.BR.

In contrast, IgG levels (figure 4.1.2) rose sharply after infection in all strains studied except BALB/c mice, which were remarkably unresponsive to

Figure 4.1.1 Time course of the IgM–specific antibody response to *T. muris* E/S antigen in serum samples from infected B10.BR (●–●), B10 (▼–▼), B10.D2/n (▲–▲), B10.G (■–■), BALB/B (▽–▽), BALB/K (○–○) and BALB/c (△–△) mice. NS, mean naive serum OD for all strains.

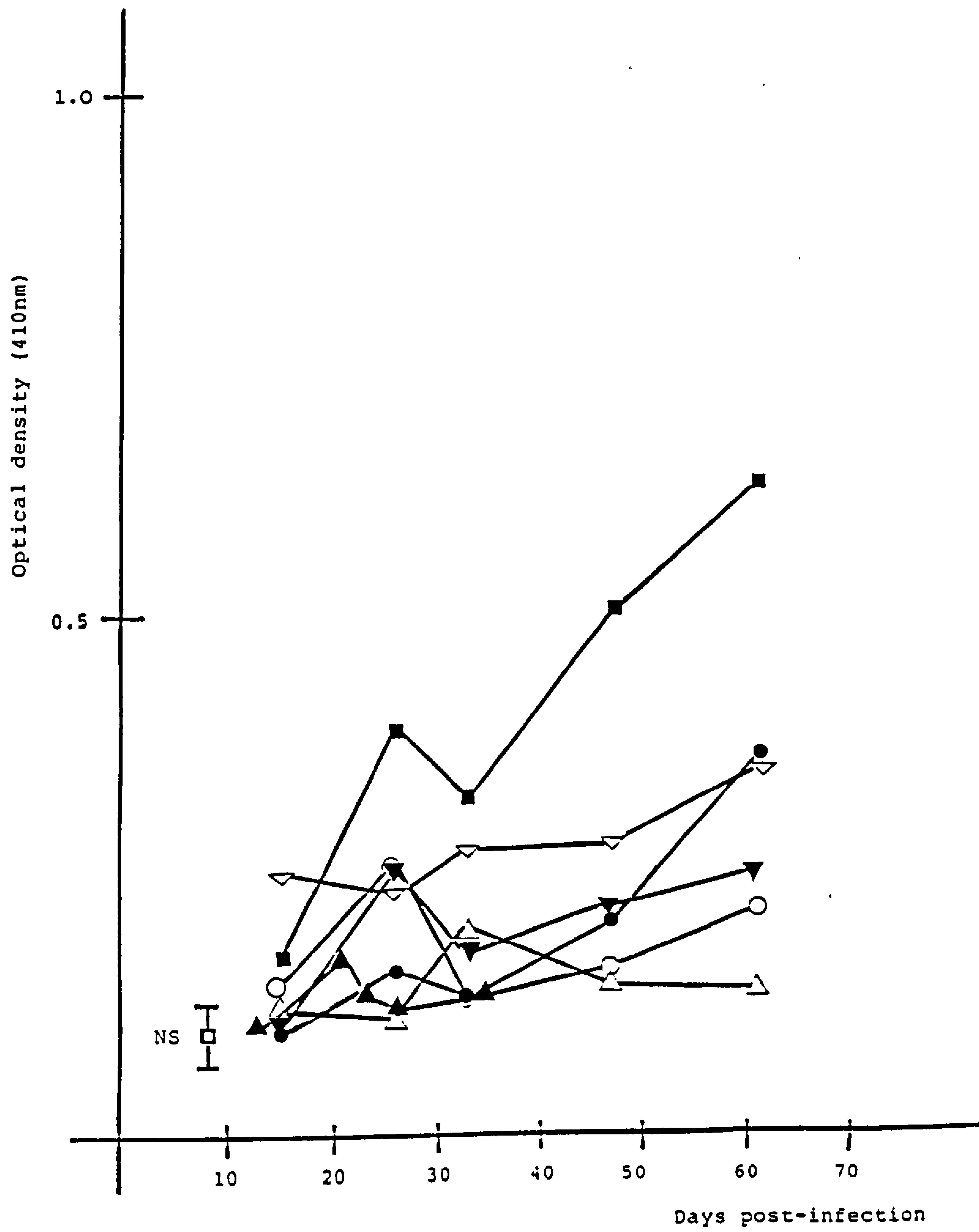
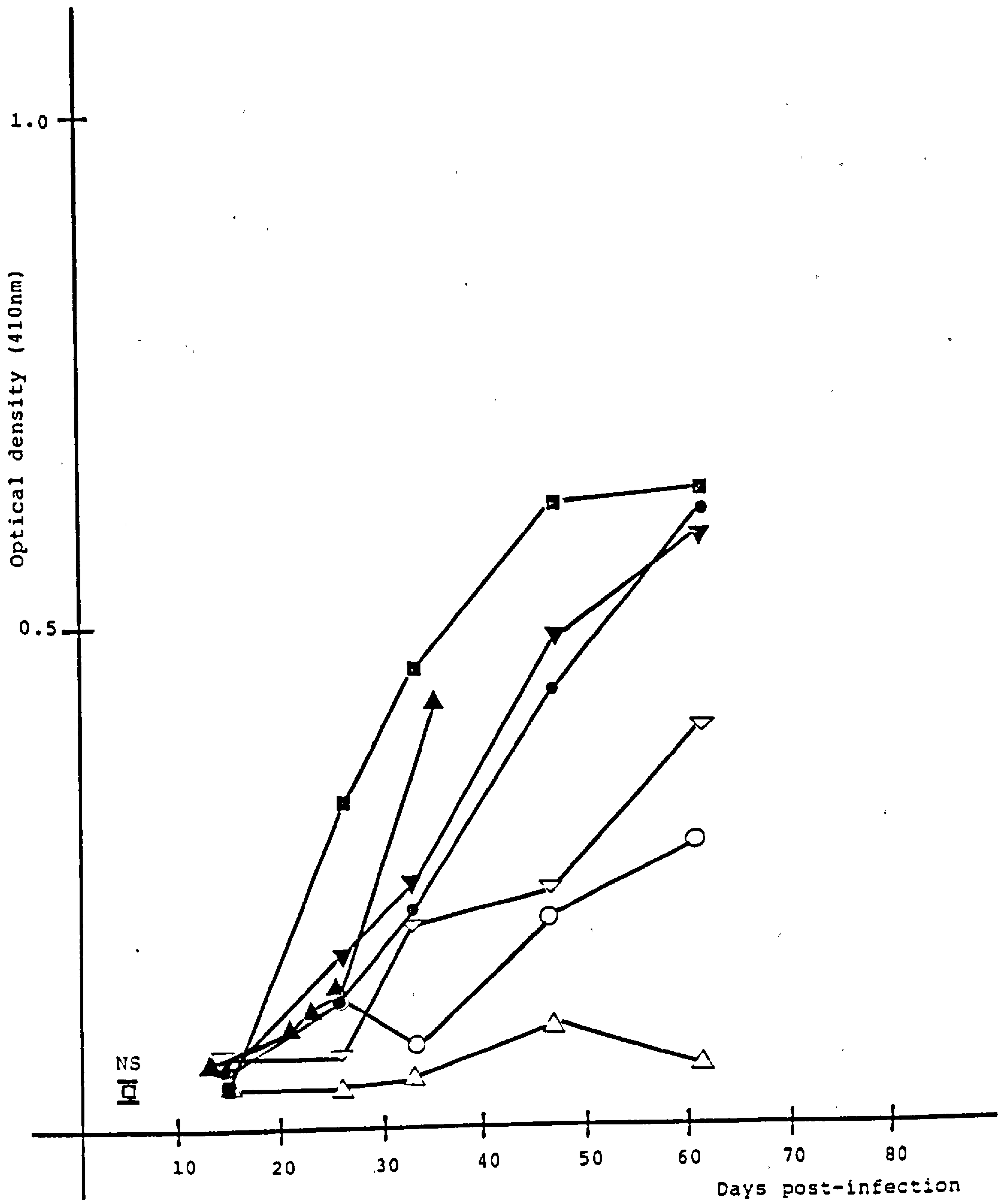

1.0
0.5
Optical density (410nm)
NS
10
20
30
40
50
60
70
Days post-infection

Figure 4.1.2 Time course of the IgG-specific antibody response to *T. muris* E/S antigen in serum samples from infected B10.BR (●—●), B10 (▼—▼), B10.D2/n (▲—▲), B10.G (■—■), BALB/B (▽—▽), BALB/K (○—○) and BALB/c (△—△) mice. NS, mean naive serum OD for all strains.



1.0
0.5
Optical density (410nm)
NS
10
20
30
40
50
60
70
80
Days post-infection

parasite E/S antigen in terms of their humoral immune response, and continued to rise irrespective of whether the parasite had been expelled from the gut. The strains of mice studied formed two distinct groups according to genetic background, with mice of the B10 genetic background exhibiting considerably higher IgG levels than mice possessing BALB background genes. Within both BALB and B10 congenic strains the influence of H-2-linked genes was evident, with strains which differed only at the H-2 having different levels of antibody response to *T. muris* E/S antigen. For instance B10.G mice ($H\text{-}2^q$) showed a more rapid IgG response, which reached a higher level than any other of the B10 congenic strains. Likewise BALB/B mice ($H\text{-}2^b$) exhibited higher IgG levels than BALB/K mice ($H\text{-}2^k$), and considerably higher levels than BALB/c ($H\text{-}2^d$) mice.

Specific IgA antibody was not detected in the peripheral blood of any strain at any time p.i.

Total specific antibody response to the 40-43kD antigen

The major constituent of AMA and E/S products is a single protein band of apparent MW 40-43kDa when reduced, 35-38kDa when non-reduced, as seen by one-dimensional SDS-PAGE (Roach, 1986). This protein also appears as a single major component of the surface antigens removed when worms are treated with cetyltrimethyl-ammonium bromide (CTAB), a cationic detergent (Roach, 1986). Thus it was deemed appropriate to study host strain variations in the humoral immune response to this specific antigen, it being considered likely that H-2-linked control of the antibody response would be more clearly discerned when a more restricted target antigen preparation than E/S antigen was used. The total antibody response to the 40-43kDa antigen was followed from day 13 p.i.

to day 47 p.i. in all strains except B10.D2/n, where the response was followed only to day 35 p.i. The results are shown in figure 4.1.3.

Mice sharing the $\underline{H-2}^k$ haplotype (BALB/K, B10.BR) exhibited considerably higher antibody levels than all other strains except B10.G mice ($\underline{H-2}^q$) which showed equivalent antibody levels. B10.D2/n and BALB/c mice (both $\underline{H-2}^d$) responded very weakly to the 43kDa antigen whilst $\underline{H-2}^b$ haplotype strains (BALB/B, B10) occupied an intermediate position between these two extremes, their antibody levels being far higher than those of the $\underline{H-2}^d$ haplotype strains.

Differential recognition of E/S antigen in congenic mouse strains

Western blotting

Recognition of E/S antigen by antibodies in sera collected on day 35 p.i. from all strains was analysed using the Western blotting technique. The results are shown in figure 4.1.4.

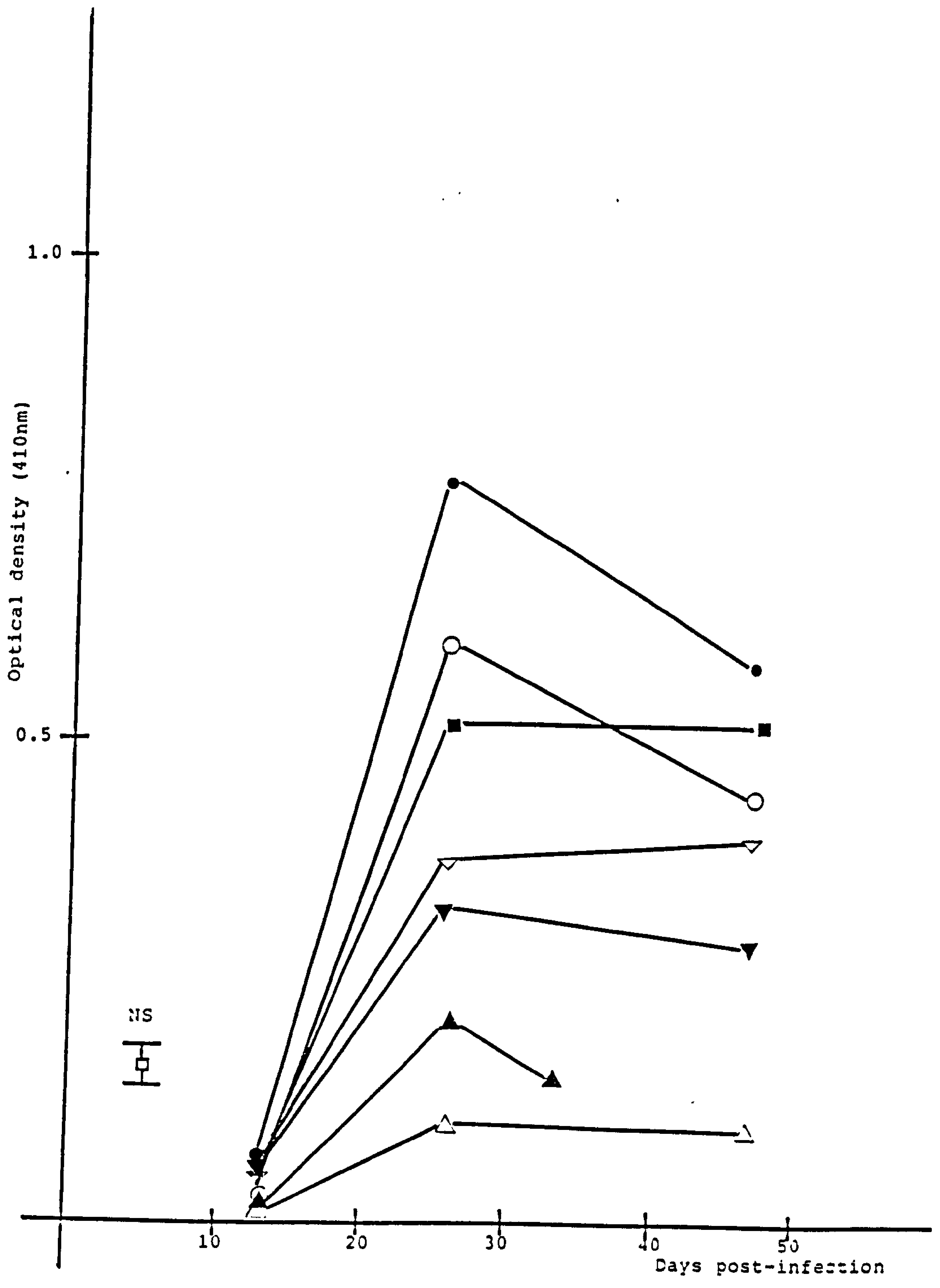
Patterns of antigen recognition were similar in all strains with three antigens of MW 50.7kDa, 43kDa and 23.4kDa being recognised by antibodies in the serum from every strain. Of these three antigens, serum from naive mice (lane 8) recognised the 50.7kDa antigen, but only weakly.

Additional experiments, using sera collected on day 47 p.i., day 54 p.i. and day 61 p.i. confirmed the apparent similarity between strains in their antigen recognition profiles as revealed by this technique.

Immunoprecipitation

Metabolic labelling and immunoprecipitation before electrophoretic analysis revealed qualitative differences between congenic mouse strains in

Figure 4.1.3 Time couse of the total specific antibody response to the 40-43kDa antigen of *T. muris* in serum samples from infected B10.BR (●–●), B10 (▼–▼), B10.D2/n (▲–▲), B10.G (■–■), BALB/B (▽–▽), BALB/K (○–○) and BALB/c (△–△) mice. NS, mean naive serum OD for all strains.



1.0
0.5
Optical density (410nm)
NS
10
20
30
40
50
Days post-infection

Figure 4.1.4 Autoradiograph of Western blot showing the antigen/antibody reactions detected when nitrocellulose-bound *T. muris* E/S antigen was incubated with sera taken day 35 p.i. from B10.G, B10, BALB/B, B10.BR, BALB/K, BALB/c and B10.D2/n mice (lanes 1–7 respectively) or naive serum from B10 mice (lane 8) and developed with ^{125}I-labelled anti-mouse IgGAM. Arrows indicate the three antigens of mol. wts. 50.7, 43 and 23.4 kDa recognised by antibodies in the sera from all strains.

Figure 4.1.4

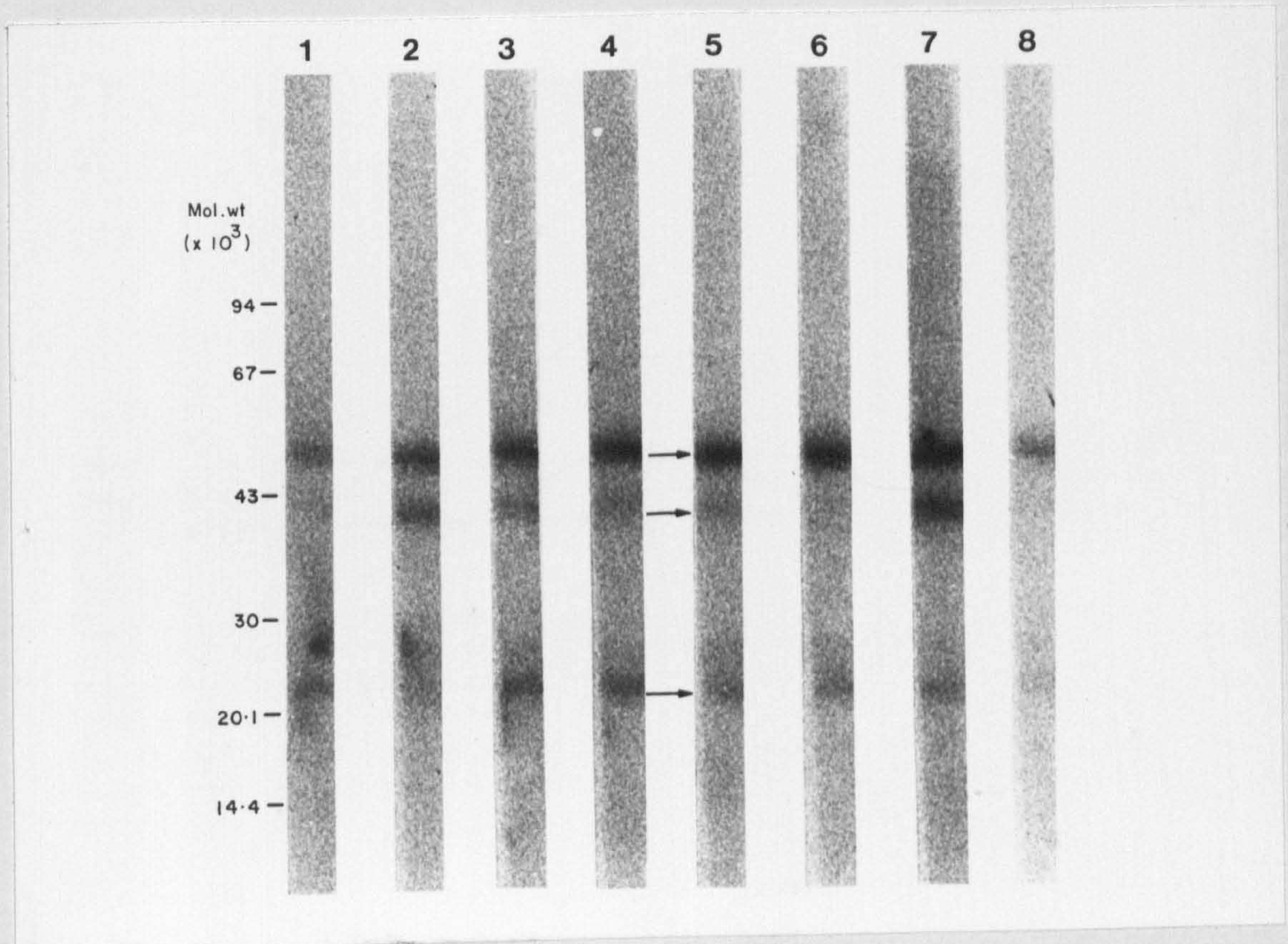


Figure 4.1.5 Fluorograph of SDS-PAGE of ^{35}S-metabolically labelled *T. muris* E/S antigen after immunoprecipitation with sera taken day 61 p.i. from B10.G, BALB/K, B10.BR, BALB/c, BALB/B and B10 mice (lanes 1-4, 6 and 7) and day 35 p.i. from B10.D2/n mice (lane 5). No antigens were immunoprecipitated with naive serum from B10.BR mice (lane 8). The arrow indicates the 20-23kDa antigen referred to in the text.

Figure 4.1.6 Fluorograph of SDS-PAGE of ^{35}S-metabolically labelled *T. muris* E/S antigen after immunoprecipitation with sera taken day 26 p.i. from B10.G, BALB/K, B10.BR, BALB/c, B10.D2/n, BALB/B and B10 mice (lanes 1-7). No antigens were immunoprecipitated with naive serum from B10.BR mice (lane 8).

Figure 4.1.5

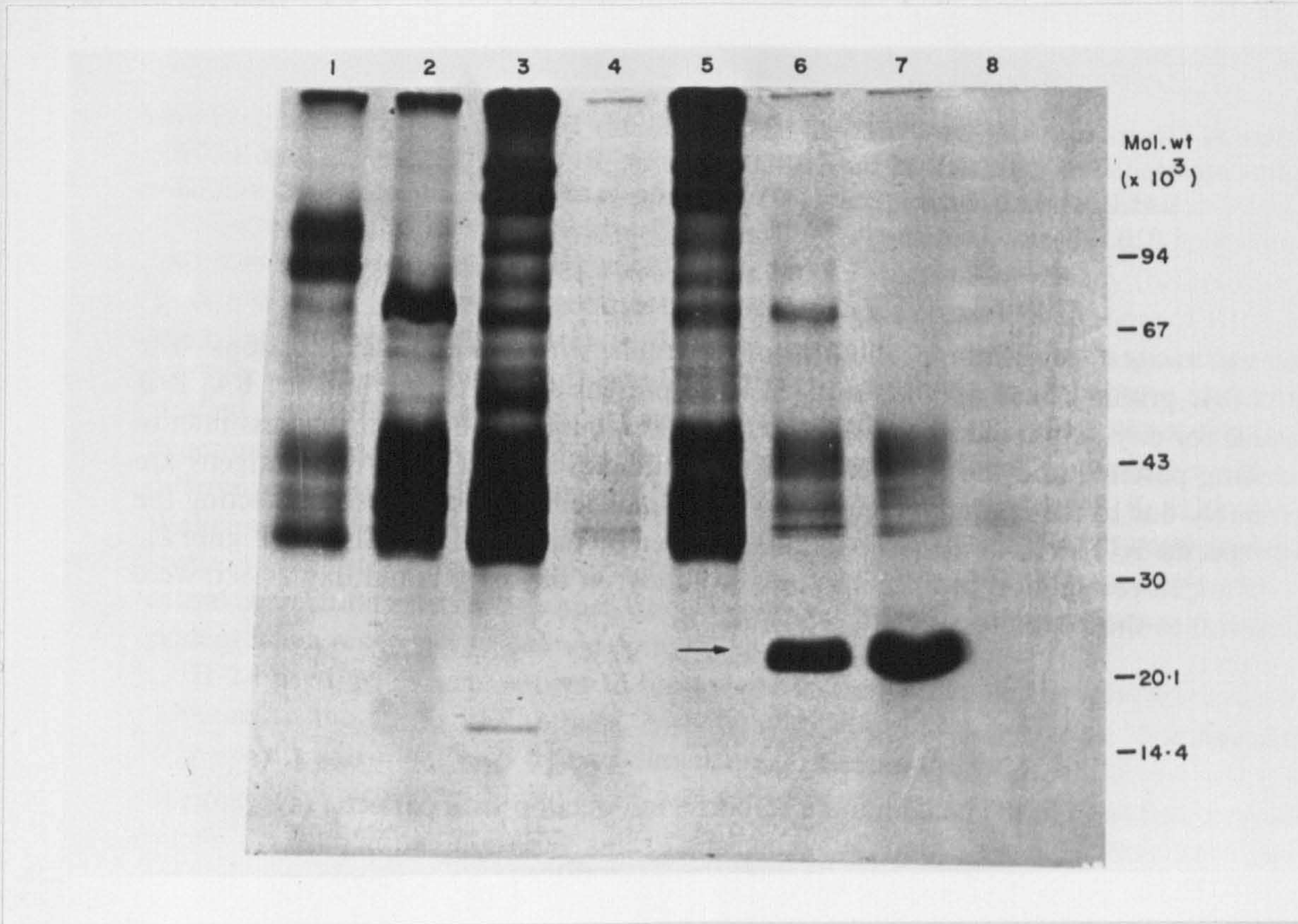
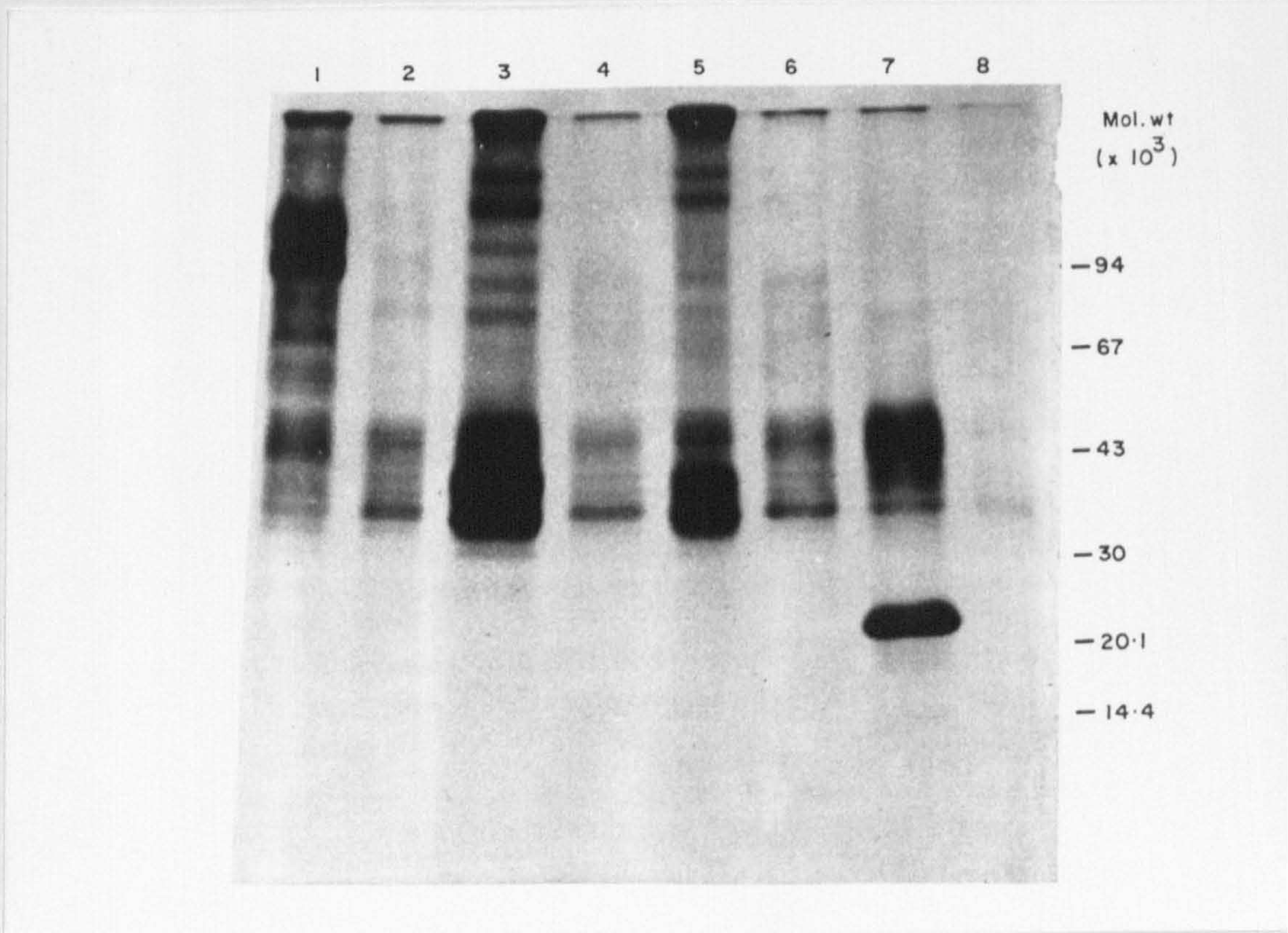


Figure 4.1.6



their IgG response to *T. muris* E/S antigen. The results are shown in figures 4.1.5 (day 61 p.i.) and 4.1.6 (day 26 p.i.).

Figure 4.1.5 shows the antigen recognition profiles using sera taken day 61 p.i. In general, mice of the B10 genetic background, particularly B10.G, B10.BR and B10.D2/n, recognised a wider range of antigens than mice of the BALB genetic background, the MW of antigens recognised varying from 30 to 136kDa. B10 mice, however, recognised fewer antigens than both BALB/B and BALB/K mice. The BALB/c recognition profile was little different from naive serum.

Mouse strains sharing the $\underline{H\text{-}2^b}$ haplotype (B10, BALB/B - lanes 6 and 7) recognised a low MW antigen (20-23kDa) which was not recognised by any other strain studied.

Immunoprecipitations using sera taken day 26 p.i. showed similar, though less intense antigen recognition profiles for most strains, with two notable exceptions - the BALB/K profile lacked a 79kDa antigen band present at day 61 p.i., and the BALB/B profile for day 26 p.i. did not reveal the 20-23kDa antigen band. Both the less intensive banding patterns and the apparent lack of antibodies against these two antigens are probably due to the immunoprecipitation technique being less sensitive in detecting the low specific IgG levels in the peripheral blood seen on day 26 p.i. by ELISA (figure 4.1.2).

Antigen recognition profiles for total specific antibody on day 61 p.i. and day 26 p.i. were identical to those seen for IgG.

4.1.5 DISCUSSION

Genetic variation in the host immune response to infection with parasitic helminths is a well-documented phenomenon (Wakelin 1978). This variation can manifest itself in a number of host parameters, one of the

easiest of which to measure is the antibody response to infection. Although there are many papers concerned with the analysis of genetic variation in antibody responses *per se* (Rivera-Ortiz and Nussenzweig 1976; Jungery and Ogilvie 1982; Storey, Behnke and Wakelin 1987), the genetic control of the specific antibody response to infection is poorly understood. Progress has been made possible in this field by the recent development of procedures such as immunoprecipitation of antigen-antibody complexes, the reaction of antibodies with nitrocellulose-bound antigens, and the ELISA. In this study the combined use of these three techniques has provided evidence for the involvement of both H-2-linked and background genes in the control of the humoral immune responses of mice to the nematode *T. muris*.

Analysis of the kinetics of the antibody response in congenic mice revealed that the primary antibody response to *T. muris* E/S antigen is predominantly an IgG response, although B10.G mice, and to a lesser extent B10.BR, also showed elevated IgM levels. No IgA was detected in any strain of mouse p.i. This does not necessarily mean that IgA is unimportant in the protective immune response to *T. muris*, but rather may merely reflect low levels of IgA in the peripheral circulation. A study of intestinal IgA levels may reveal a role of IgA in the expulsion of *T. muris*. Indeed, IgA monoclonal antibodies have been shown to passively transfer immunity to this parasite (Roach 1986).

Mice of the B10 genetic background exhibited much higher levels of IgG antibodies to E/S antigen than mice possessing BALB background genes, the pattern of responses in mice of these backgrounds being highly reproducible. Striking differences in antibody levels between mice of identical genetic backgrounds suggested that, in addition to these background genetic effects, H-2-linked genes may also play a role in the control of the antibody response to *T. muris*, superimposed upon the background effects.

B10 congenic strains of mice are known to be significantly more

susceptible to *T. muris* (Else and Wakelin 1988) than the BALB congenics under study, therefore the higher specific antibody levels observed in the former may in part reflect the longer retention of the parasite within the large intestine. However, the differences in the antibody levels observed cannot totally be explained by differences in rate of expulsion of *T. muris*, as they do not account for the differences in antibody levels seen between BALB/B and BALB/c mice, which exhibit similar kinetics of expulsion of *T. muris*; neither do they explain the fact that when sera from NIH mice, which expel *T. muris* very rapidly (before day 15 p.i.), were tested in a similar experiment, antibody levels were similar to those of B10.G mice even though the parasite was retained within the gut for a significantly shorter period. Equally it is unlikely that the observed differences in antibody levels between the strains studied resulted from the establishment of different levels of infection. A threshold experiment conducted previously in MF1 mice showed there to be no significant difference in specific antibody levels in mice infected with 50 or 400 *T. muris* eggs (Else and Wakelin, unpublished observations). There is no simple correlation between antibody levels in a host strain and the time course of infection. That is not to say, however, that no causal correlation exists in any host-parasite combination; such correlations may only be evident when responses to defined epitopes can be determined.

H-2-linked genes were shown to have a marked influence on the humoral immune response to the 40-43kDa antigen. Although this band may prove to have multiple components when analysed by two-dimensional electrophoresis, it is a single entity as seen by one-dimensional SDS-PAGE and forms the major constituent protein of E/S. Mice sharing the $\underline{H\text{-}2}^k$ haplotype (B10.BR, BALB/K) responded considerably better to this antigen in terms of total antibody levels than all other strains studied, with BALB/c and B10.D2/n mice (both $\underline{H\text{-}2}^d$) responding only very weakly. The

differences in antibody levels observed between strains of identical haplotypes (k-k, d-d, b-b) were insignificant in comparison with the very considerable differences in antibody levels seen between strains of mice possessing different haplotypes. The greater antibody response seen in mice of the H-2^k haplotype may suggest that the 40-43kDa antigen is presented more efficiently to helper T cells than in mice of other haplotypes, thus allowing a greater response by the B cells which effect the functional anti-40-43kDa response. Since the H-2^k haplotype has been shown to be associated with relative susceptibility to *T. muris* (Else and Wakelin 1988) the importance of the anti-40-43kDa response in terms of protective immunity remains to be determined.

Deelder, Claas and de Vries (1978) studied the influence of two different H-2 haplotypes (H-2^b and H-2^k) on an experimental infection with *Schistosoma mansoni*. Their results suggested that different H-2-linked immune response genes had a considerable influence on the immune response against *S. mansoni*, the more susceptible C3H.B10 (H-2^b) mice exhibiting higher antibody titres than C3H/Sn (H-2^k) mice. The high mortality observed in C3H.B10 mice was interpreted as the consequence of a less effective cellular immune response. It is therefore conceivable that the high susceptibility of mice sharing the H-2^k haplotype to *T. muris*, and the high anti-40-43kDa antibody levels they exhibit, may similarly reflect poor anti-40-43kDa cellular immune responses.

A Western blot analysis of antigen recognition failed to reveal any evidence to suggest that H-2-linked genes exercised significant control of the antibody repertoire in response to *T. muris* infection, the same three antigens being recognised by the sera from all strains, regardless of H-2 haplotype. However, a complementary study, involving immunoprecipitation, possibly a more realistic way of analysing antigen recognition, as the antigen-antibody reaction occurs with the antigen in its native state,

revealed differences between strains in their antigen recognition profiles. Three of the four B10 background strains studied exhibited wider antigen recognition profiles than strains of the BALB genetic background. BALB/c mice, which expel a primary infection with *T. muris* relatively rapidly compared to the B10 congenics, had recognition profiles similar to those for naive serum. Mouse strains sharing the $\underline{H-2}^b$ haplotype (B10, BALB/B) recognised a low MW antigen (20-23kDa) which was not recognised by any other strain. A more exhaustive study involving other strains of the $\underline{H-2}^b$ haplotype would have to be performed to confirm this apparent $\underline{H-2}^b$ restriction in the recognition of the 20-23kDa antigen, a finding which implicates H-2-linked genetic control of the antibody repertoire to *T. muris* antigens. However, pairs of mice sharing the same H-2 haplotype showed dissimilar total recognition profiles.

It therefore appears that antibody recognition of *T. muris* antigen is H-2-linked but that additional factors are also involved in determining the recognition profile. In this respect the control of antibody responses to *T. muris* is similar to that of many other species (Rivera-Ortiz and Nussenzweig 1976; Kee *et al.* 1986). Although there are some instances where H-2-linked control appears to play a dominant role, e.g. in the response of mice to infection with *Ascaris suum* (Kennedy *et al.* 1986), such a clear discrimination between strains in the specificity of antibody responses to nematode antigens is rare (Wakelin 1985).

Many studies which have shown marked H-2-linked and background genetic influences of antigen recognition (e.g. those of Deelder *et al.* (1978) with *Schistosoma mansoni*; Pond, Wassom and Hayes (1988) with *Trichinella spiralis*; and Gibbens, Harrison and Parkhouse (1986) with *Taenia taeniaeformis*) have also, as described here for *T. muris*, found a correlation between greater responsiveness (titre and/or profile) and greater susceptibility to infection. There may of course be many explanations for

such a correlation, including diversion of immune responses and production of blocking antibodies, but it is a phenomenon which may have considerable relevance to the development of infections in man. If the correlation between high antibody levels, wide antigen recognition profiles and susceptibility demonstrated with *T. muris* can be extended to infections with *T. trichiura* in man it may provide useful serological markers for studying the epidemiology of trichuriasis and aid the identification of susceptible individuals in control programmes.

4.2 THE INFLUENCE OF GENES WITHIN THE H-2 COMPLEX ON THE HUMORAL IMMUNE RESPONSE TO *T. MURIS* EXCRETORY/SECRETORY (E/S) ANTIGEN

The outcome of a parasitic infection may be critically determined by the level and specificity of the immunoglobulin response and the pattern of isotype expression elicited in the host. The level of response may determine either resistance or susceptibility, and the isotype dictate the range of effector function available. The results presented in section 4.1 demonstrate that the primary antibody response to *T. muris* E/S antigen is predominantly an IgG response, and that both background and H-2-linked genes influence this response in a qualitative and quantitative manner. In order to analyse further the role of the H-2-linked genes concerned, and to identify the contribution of the IgG1 isotype to the total *T. muris*-specific antibody response, a panel of H-2 recombinant mouse strains were used for a detailed serological study. The choice of the IgG1 isotype was determined by the fact that many metazoan parasites induce high levels of serum IgG1 during the course of infection (Chapman *et al.*, 1979*a*, *b*;

Pritchard *et al* 1983; Pond *et al.* 1988). IgG1 has been shown to be capable of mediating antibody-dependent cellular cytotoxicity by eosinophils resulting in parasite damage (Ramalho-Pinto *et al.* 1979).

4.2.1 EXPERIMENTAL DESIGN

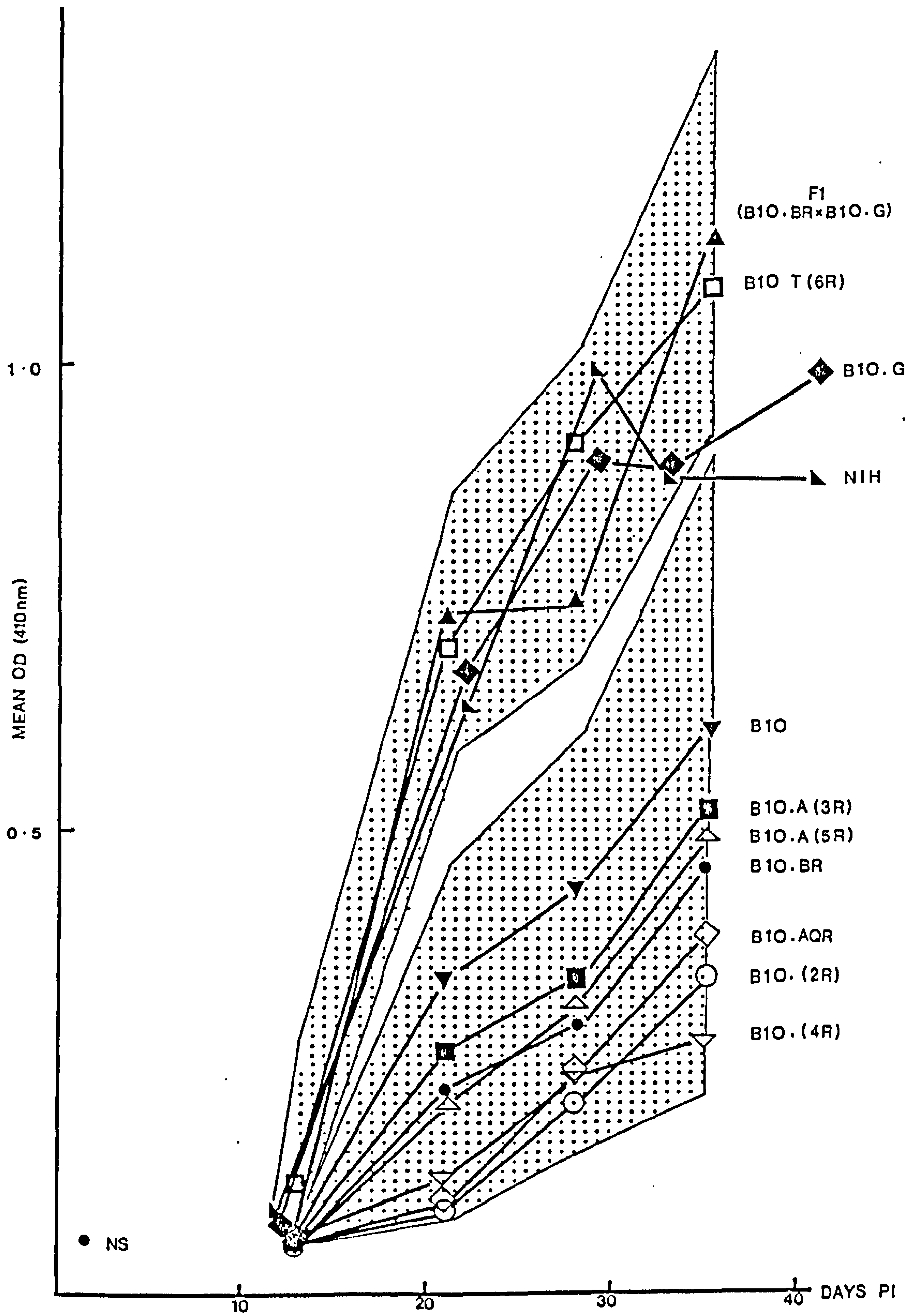
The panel of H-2 recombinant strains of mice was as used in Chapter 3.2 (Table 3.2.1). The sera obtained from these mice was used in the ELISA and immunoprecipitation studies described in this section of Chapter 4. IgG and IgG1 antibody responses to E/S antigen were determined by ELISA. Immunoprecipitation studies were performed using a sheep anti-mouse IgGFc antibody and 10-20% gradient gels. Significant differences in antibody levels between strains were determined using Analysis of Variance.

4.2.2 RESULTS

IgG and IgG1 antibody response to E/S antigen

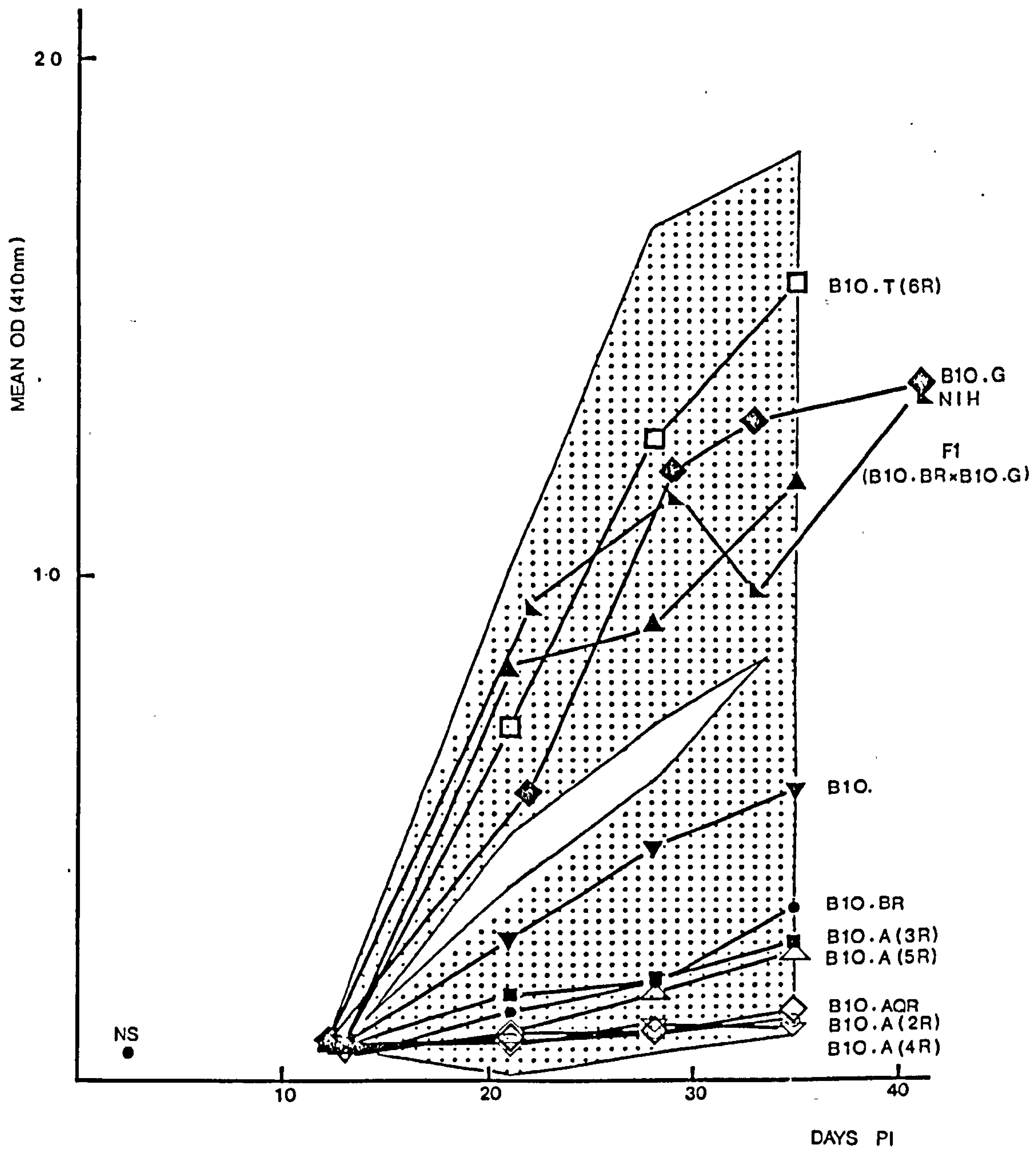
The IgG and IgG1 antibody levels (reflected by optical density (OD) in ELISA) for the strains studied are shown in figures 4.2.1 and 4.2.2 respectively. As can be seen, the specific IgG1 response resembled the pattern of response for total specific IgG antibody except that the majority of the H-2 recombinant strains studied showed a smaller increase, if any, in IgG1 antibody post-infection. Strains of mice formed two distinct groups as determined by their IgG and IgG1 response patterns, B10.T(6R) and (B10.BRxB10.G)F1 mice having significantly higher levels ($P<0.05$) of both antibodies than any other strain studied. Within each of the two groups, antibody levels did not differ significantly between strains. The division into

Figure 4.2.1 Time course of the IgG-specific antibody response to *T. muris* E/S antigen in serum samples from infected congenic and H-2 recombinant strains of mice. The shaded area represents the range of standard deviations from the mean optical densities calculated from six individual values for each strain at each time point post infection. Antibody levels in serum from B10.G and NIH mice were determined using pooled sera (at least five individuals per strain). N.S., naive mouse serum. O.D., optical density.



F1
(B10·BR×B10·G)
B10 T(6R)
B10.G
NIH
B10
B10.A (3R)
B10.A (5R)
B10·BR
B10·AQR
B10· (2R)
B10·(4R)
NS
MEAN OD (410nm)
1·0
0·5
10
20
30
40
DAYS PI

Figure 4.2.2 Time course of the IgG1-specific antibody response to *T. muris* E/S antigen in serum samples from infected congenic and H-2 recombinant strains of mice. The shaded area represents the range of standard deviations from the mean optical densities calculated from six individual values for each strain at each time point post infection. Antibody levels in serum from B10.G and NIH mice were determined using pooled sera (at least five individuals per strain). N.S., naive mouse serum. O.D., optical density.



MEAN OD (410nm)
2·0
1·0
NS
10
20
30
40
DAYS PI
B10.T(6R)
B10.G
NIH
F1
(B10.BR×B10.G)
B10.
B10.BR
B10.A(3R)
B10.A(5R)
B10.AQR
B10.A(2R)
B10.A(4R)

high and low responder strains with respect to IgG and IgG1 production was apparent early on during the course of the primary infection, a clear separation being evident by day 21 p.i. Pooled post-infection sera from B10.G and NIH strains of mice followed the pattern of response seen for B10.T(6R) and (B10.BRxB10.G)F1 mice. Individual variation in OD within a strain was relatively small in most cases except for the day 35 p.i. B10 values where a large standard deviation from the mean value was observed.

Differential recognition of ^{35}S-methionine labelled E/S antigen by H-2 recombinant strains of mice

The profile of antigens recognised by IgG antibodies in sera from H-2 recombinant mouse strains and (B10.BRxB10.G)F1 mice, as determined by immunoprecipitation, are shown in figures 4.2.3 and 4.2.4 respectively. Most strains recognised a number of antigens within the 30-50kDa region. Heterogeneity in the antibody responses to specific antigens between strains suggested the existence of H-2-linked control. For instance two high MW antigens (90-95kDa and 105-110kDa) were only recognised by B10.T(6R) and (B10.BRxB10.G)F1 strains of mice (figure 4.2.3 lane 3, figure 4.2.4 lanes 1-5), although in the example shown there is some evidence of the presence of antibodies against the latter antigen in the B10 serum sample. However sera from five other B10 individuals did not reveal recognition of antigens in this MW range. B10.A(3R), B10.A(5R), B10.AQR and B10.BR recognised an antigen resolving at approximately 97kDa and a doublet resolving around 92kDa (fig. 4.2.3 lanes 4, 5, 6 and 8). Although clearly resolving within a similar MW range to the 90-95kDa and 105-110kDa antigens, these antigens were judged to be distinct from the two high MW antigens (see Chapter 7). A 20-23kDa antigen was only recognised by sera from B10 mice (figure 4.2.3 lane 7), although one individual from each of

Figure 4.2.3 Fluorograph of SDS-PAGE of ^{35}S-metabolically labelled *T. muris* E/S antigen after immunoprecipitation with sera taken day 35 p.i. from individual B10.A(2R), B10.A(4R), B10.T(6R), B10.A(3R), B10.A(5R), B10.AQR, B10 and B10.BR mice (lanes 1-8). Lane 9, B10.BR naive serum. Arrows indicate the 90-95kDa, 105-110kDa and 20-23kDa antigens referred to in the text. Worm burdens are also given, where known, beneath the lane numbers. s = stunted; + = patent infection

Figure 4.2.4 Fluorograph of SDS-PAGE of ^{35}S-metabolically labelled *T. muris* E/S antigen after immunoprecipitation with sera taken day 35 p.i. from individual (B10.BRxB10.G)F1 mice (lanes 1-5). Antigen recognition profiles for B10.G, B10 and B10.BR mice are shown in lanes 6-8. Lane 9, naive serum from (B10.BRxB10.G)F1 mice. Arrows indicate the 90-95kDa and 105-110kDa antigens referred to in the text. Worm burdens are also given, where known, beneath the lane numbers. s = stunted; + = patent infection.

Figure 4.2.3

1	2	3	4	5	6	7	8	9
182+	311+	70s	257+	179+	162+	4s	245+	

MW (kDa)

94

67

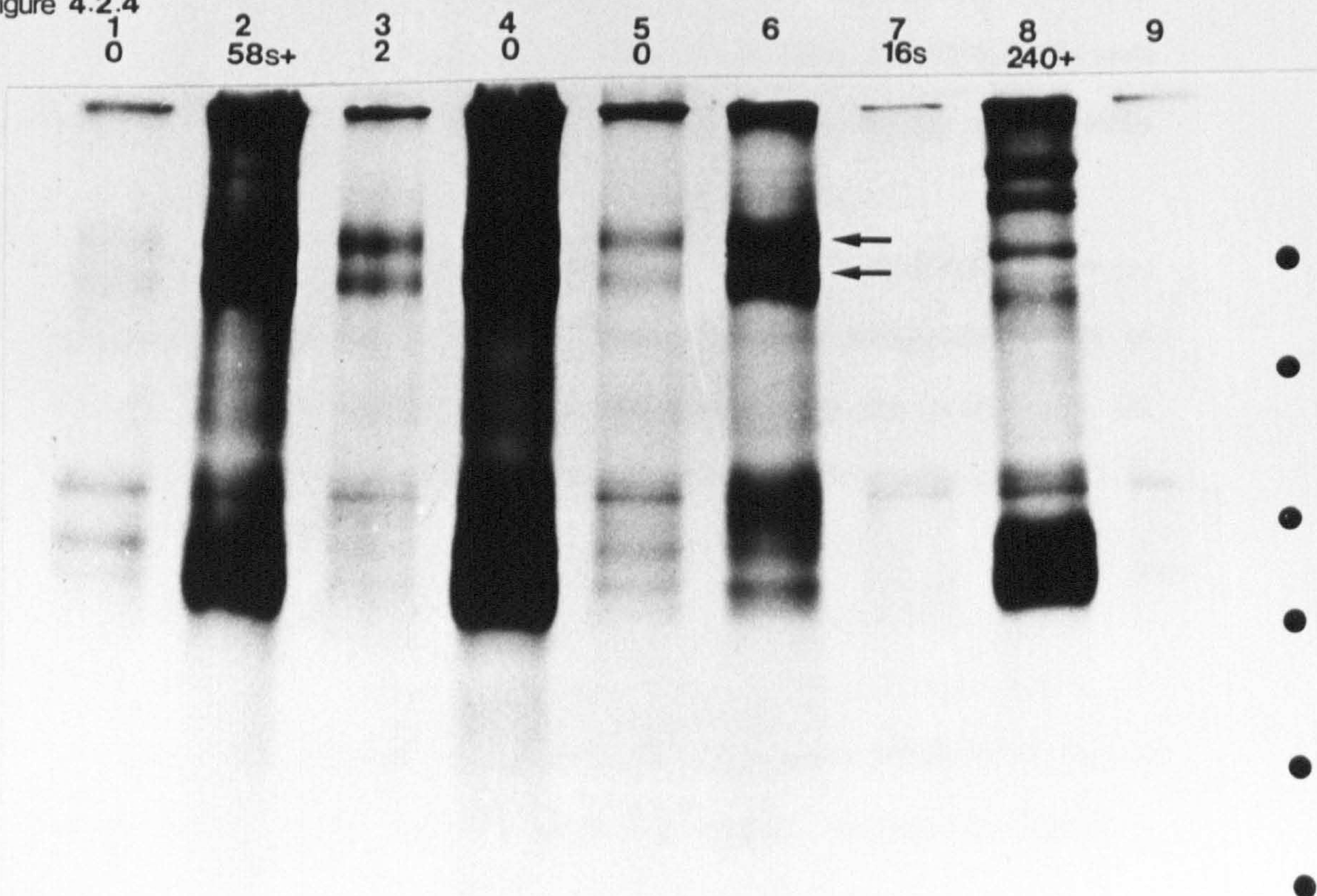
43

30

20·1

14·4

Figure 4.2.4



the B10.A(5R) and B10.A(3R) strains of mice also recognised this antigen (data not shown), perhaps suggesting the involvement of I-A^b. Marked quantitative differences, reflected in the intensity of bands on the autoradiographs, and in some cases qualitative differences in antibody response to individual antigens occurred within inbred strains. For instance figure 4.2.3 shows very strong recognition of a 20-23kDa antigen by an individual of the B10 strain of mouse (lane 7), whereas recognition of this antigen is barely detectable in a different individual of the same strain (figure 4.2.4, lane 7), although visible on the original autoradiograph. Considerable quantitative variation was also seen with respect to IgG antibodies produced against the two high MW antigens (90-95kDa, 105-110kDa) by individuals of the (B10.BRxB10.G)F1 strain (figure 4.2.4, lanes 1-5). Qualitative variation in antigen recognition was illustrated by the B10.A(4R) mouse strain where four of the five individuals displayed antigen recognition profiles similar to those seen for naive serum (e.g. figure 4.2.3, lane 2), however one individual showed considerable antigen recognition, particularly within the 30-50kDa MW range (data not shown). Also, as mentioned, only one out of five individuals of both the B10.A(5R) and B10.A(3R) strains of mice appeared to recognise a 20-23kDa antigen (data not shown).

Antigens recognised by individuals of the (B10.BRxB10.G)F1 mouse strain (figure 4.2.4 lanes 1-5) were identical to those recognised by one of the parental strains, B10.G (lane 6), but dissimilar to the profile seen for the other parent, B10.BR (lane 8), except within the 30-50kDa range.

4.2.3 DISCUSSION

The results presented here confirm the influence of MHC-linked factors on the antibody responses to *T. muris* E/S antigen. Through the use of a

panel of H-2 recombinant mouse strains significant differences between certain strains of mice in their qualitative and quantitative responses to *T. muris* E/S antigen were apparent.

There has been a considerable amount of work on the genetic control of antibody responses to a variety of purified proteins and hapten-protein conjugates (Vaz and Levine 1970; Rathbun and Hildemann 1970; Urba and Hildemann 1978; Tite *et al.* 1987). Studies concerned with the genetic control of antibody responses to parasite antigens have focused on protozoal antigens and include the work of Del Giudice *et al.* (1986) on the control of antibody responses in mice to the immunodominant repetitive epitope of the main *Plasmodium falciparum* sporozoite surface antigen, the circumsporozoite protein, and the work of Taylor *et al.* (1988) who studied the influence of MHC-linked factors on immunity to *P. yoelii* and isotype expression. In comparison only a limited number of studies have considered how antibody responses to helminth antigens are regulated. Kaji *et al.* (1983) suggested that the gene(s) regulating the IgE antibody response to *Schistosoma japonicum* mapped in the I-E subregion of the mouse MHC, and Kee *et al* (1986), using mRNA *in vitro* translation products of adult worms as target antigens in immunoprecipitation experiments, provided evidence for the genetic control of the antibody response to *S. mansoni*. Kennedy *et al.* (1986) tentatively ascribed the control of the antibody repertoire in response to *Ascaris* infection to the H-2 complex.

The involvement of MHC-linked Ir genes in the control of antibody responses to parasite antigens revealed by the studies of Kaji *et al.* (1983), Kee *et al.* (1986) and Kennedy *et al.* (1986) is consistent with the work presented here. MHC-linked Ir gene control of antibody responses is exerted at the level of the T cell - B cell interaction, helper T cells having an obligatory role in antibody responses to most antigens (Abbas, 1988). These cells only recognise processed antigen fragments presented to them by

antigen presenting cells, such as macrophages and dendritic cells, or B cells, in association with MHC class II gene products present at the antigen presenting cell or B cell surface (Benacerraf, 1978; reviewed by Unanue, 1984; Blackman *et al.*, 1988; Janeway *et al.*, 1988). Thus in order for an antigen to stimulate helper T cells which then enable B cells to produce antibody, it must give rise to peptides able to bind to the MHC class II gene products of that individual, and the host must have T cells bearing receptors able to recognise that combination of antigenic peptide plus MHC (Marrack and Kappler, 1987; Introduction 1.3.2). Thus the phenomenon of MHC-restricted antibody responses arises. Although, as mentioned, B cell responses to many antigens are known to be under MHC-linked Ir gene control, it is unusual to see clear differences between mouse strains in the specicifity of the antibody response to complex nematode antigens (Wakelin, 1985). However, through the use of a panel of H-2 recombinant strains of mice an apparent $\underline{H\text{-}2}^{q}$ restriction on rapid, high level IgG and IgG1 antibody responses to *T. muris* E/S antigen and the recognition of two high MW E/S antigens (90-95kDa, 105-110kDa) by IgG antibody was revealed, only B10.T(6R) and (B10.BRxB10.G)F1 mice exhibiting these antibody response patterns. It is likely that the relevant MHC product is $\underline{I\text{-}A}^{q}$, as B10.T(6R) mice lack cell surface I-E molecules, as do the NIH and B10.G mouse strains (both $\underline{H\text{-}2}^{q}$) which showed similar antibody response patterns to those seen for the B10.T(6R) and (B10.BRxB10.G)F1 strains of mice, as revealed by ELISA (figs. 4.2.1 and 4.2.2) ,and immunoprecipitation (figs. 4.1.5, 4.1.6, 4.2.3 and 4.2.4 - data for NIH not shown). A more detailed discussion of the apparent MHC restricted antibody responses to *T. muris* E/S antigen and their possible relevance with respect to resistance to infection is given in Chapter 5.

A number of inbred strains of mice have previously been described where individuals belonging to the same strain did not behave uniformly

with respect to their ability to expel the infection (Worley *et al.*, 1962; Lee and Wakelin, 1982*b*; Else and Wakelin, 1988). The term "differential responsiveness" has been used to describe this phenomenon which provides a unique opportunity to investigate the factor(s) involved in determining the different states of responsiveness. Differential responsiveness in the ability to expel *T. muris* was observed within certain of the H-2 recombinant mouse strains used in this, and the preceding chapter (see figure 3.2.1, e.g. B10.T(6R)). However no correlation was found between individual antigen recognition profiles and/or antibody levels and resistance to infection. Roach (1986) reported similar findings when analysing the humoral immune responses of responder and non-responder DBA/2 mice. Indeed, as mentioned in section 4.1.5, a number of workers studying different host-parasite systems have shown that, if anything, poor responder strains tend to have higher levels of specific antibody than more resistant strains of mice (Pond *et al.*, 1988; Else and Wakelin, 1989).

Immunoprecipitation studies revealed qualitative and quantitative differences in antibody responses between, and in some cases within strains. Between strain variation presumably reflected the influence of different alleles at H-2 loci, however the reason for variation in antigen recognition between individuals of the same strain is not known although a similar phenomenon has been reported by other workers. For example heterogeneity in the antibody response of mice belonging to the same inbred strain to certain antigens has previously been reported by Kee *et al.* (1986). Immunoprecipitations of *in vitro* translation products of mRNA from adult *Schistosoma mansoni* using sera from individual mice showed that antibody responses to particular antigens varied considerably within a strain. The reasons for the inconsistency in response could not be explained. Similarly, Davern *et al.* (1987) reported considerable variation between individuals within a strain in their antibody responses to a *Schistosoma japonicum*

protein, (Sj.26).

In order to determine whether the ability to produce high levels of IgG antibody to *T. muris* E/S antigen early on during the course of infection was inherited as a dominant or recessive trait, the antibody responses of an F1 hybrid mouse strain resulting from crossing a low level antibody producing strain B10.BR ($\underline{H-2}^k$) with the rapid, high level antibody producing B10.G ($\underline{H-2}^q$) strain were analysed. Results suggested dominant inheritance of high responsiveness, the antibody responses of F1 mice being indistinguishable from those of the high responder parental strain with respect to both kinetics and level of the IgG and IgG1 responses and the antigen recognition profiles. I-E and I-A gene products are codominantly expressed in F1 animals, hence the ability of these F1 mice to produce rapid high level specific antibody responses and to recognise the 90-95kDa and 105-110kDa antigens may be explained by the presence of $A_\beta^qA_\alpha^q$ molecules at the cell surface in addition to $A_\beta^kA_\alpha^k$ and the two hybrid molecules $A_\beta^kA_\alpha^q$ and $A_\beta^qA_\alpha^k$. The antibody responses of hybrid mouse strains from high x low-responder crosses have been studied in other systems. High responsiveness to 2,4,6-trinitrophenyl conjugated to autogenous mouse serum albumin was found to be inherited as a recessive trait (Urba and Hildemann, 1978) whilst Tite *et al.* (1987), analysing antibody responses to human basement membrane collagen, found F1 (high x low-responder) hybrids to be far lower responders than the high responder parent, although they were significantly more responsive than the low-responder parental strain. Kee *et al.* (1986) reported both dominant and recessive responses towards individual antigens in F1 animals chronically infected with *Schistosoma mansoni*.

Although MHC-linked Ir gene control of antibody responses to antigens is most readily assayed in experimental models, work done in the field also suggests that MHC-linked control occurs. For instance Osoba *et al.* (1979)

suggested that antibody production to *Plasmodium falciparum* antigens was controlled by Ir genes within the HLA complex. The restricted ability of circumsporozoite (CS)-derived vaccines against *P. falciparum* to protect human volunteers (Ballou *et al.*, 1987; Herrington *et al.*, 1987) and the failure of synthetic peptides from the CS protein to stimulate T cell proliferation in up to 40% of adult Gambians living in a region endemic for *P. falciparum* (Good *et al.*, 1988*c*) would also seem to testify to the existence of MHC-restricted T cell responsiveness in the field. The demonstration that the human whipworm, *Trichuris trichiura* and the mouse trichuroid *T. muris* possess shared or related antigens (Roach *et al.*, 1988) suggests that *T. muris* in the mouse will prove to be a realistic model in which to study the genetic control of humoral and cellular immune responses to *T. trichiura* in man.

4.3 SUMMARY POINTS

1. The primary antibody response to *T. muris* E/S antigen was shown to be predominantly an IgG antibody response under the control of both background and H-2-linked genes.

2. A comparison of the antibody responses of congenic strains of mice revealed that, in general, strains of mice less resistant to infection developed higher level IgG responses than strains of mice which expelled *T. muris* more rapidly.

3. Analysis of the levels of specific IgG1 antibody in H-2 recombinant strains of mice showed the response pattern for this isotype to be similar to

that seen for total specific IgG.

4. Immunoprecipitation analyses revealed that both background and H-2-linked genes influenced the recognition of ^{35}S-methionine labelled E/S antigen by IgG antibodies.

5. An apparent H-2^{q} (??I-A^{q}) restriction on the development of a rapid high level IgG (IgG1) antibody response and the recognition of two high MW antigens (90-95kDa, 105-110kDa) was demonstrated by analysing the humoral immune responses of a panel of H-2 recombinant strains of mice.

CHAPTER 5

MHC-RESTRICTED ANTIBODY RESPONSES TO *TRICHURIS MURIS* EXCRETORY/SECRETORY (E/S) ANTIGEN

5.1 INTRODUCTION

It is now generally recognised that helper T cells regulate virtually every aspect of the specific B cell response to the majority of antigens (reviewed by Coffman *et al.*, 1988; Abbas, 1988). B cell responsiveness to most T cell dependent antigens is controlled by Ir genes linked to the MHC loci which encode the class II histocompatibility molecules (Ia molecules) necessary for antigen presentation to T helper cells (reviewed by Unanue, 1984).

The existence of Ir genes was discovered through the stimulation of the host immune system with relatively simple antigens, such as synthetic polypeptides, or antigens administered in low doses (Benacerraf and McDevitt, 1972). In both cases the antigen confronts the responding T cells with only a few determinants. When more complex antigens, or higher antigen doses are used, it is likely that strains not responding to some determinants will respond to others such that the overall phenotype is one of responsiveness. Thus, non-responsiveness to individual antigenic determinants is lost in the general heterogeneity of the response (Marušic *et al.*, 1982). Most parasite derived molecules are complex entities, therefore it is not surprising that there have been few reports of H-2 restricted responses to these antigens. The work of Kennedy *et al.* (1986) is one exception to this. Analysis of the antibody repertoire to *Ascaris suum* infective (L2) larval E/S antigen revealed that mouse strains sharing MHC haplotypes had identical antigen recognition profiles. However in this study only two strains of each haplotype ($\underline{H\text{-}2}^q$ and $\underline{H\text{-}2}^d$) were used.

The results presented in Chapter 4 provide evidence for the existence of $\underline{H\text{-}2}^q$ ($\underline{I\text{-}A}^q$?) restricted antibody responses to *T. muris* E/S antigen, but this correlation was based on the antibody response patterns of just four

strains of mice possessing q alleles at the H-2 complex, three of which had identical background genes (B10.T(6R), B10.G and a (B10.BRxB10.G)F1 hybrid strain). In order to confirm or refute the apparent H-2^{q} restriction on the antibody responses to *T. muris* E/S antigen a more exhaustive study of the humoral responses of mouse strains of the H-2^{q} haplotype was undertaken. Strains of mice possessing the H-2^{q} haplotype on four different genetic backgrounds (NIH, DBA, B10 and SWR) were studied. In addition to analysing antibody response patterns, expulsion phenotypes (where not previously described) were established. The functional significance of the rapid high level IgG (IgG1) antibody response and antibody specificities seen in H-2^{q} mouse strains was also investigated by examining the capacity of immune serum from (B10.BRxB10.G)F1 hybrid mice (H-2$^{q/k}$) to transfer resistance to infection to normally non-responsive B10.BR (H-2^{k}) mice.

5.2 EXPERIMENTAL DESIGN

5.2.1 DETERMINATION OF EXPULSION PHENOTYPE AND SEROLOGICAL ANALYSIS

Six to eight week old mice of the DBA/1, SWR (both H-2^{q}) and B10.BR (H-2^{k}) strains of mice were purchased from Harlan Olac Ltd. B10.G (H-2^{q}) and (B10.BRxB10.G)F1 (H-2$^{q/k}$) were bred locally at Nottingham. Serum from the NIH mouse strain (H-2^{q}) was obtained from mice used in Chapter 3, section 3.1, the expulsion phenotype for this strain being well-established (Wakelin 1975*b*; Else and Wakelin, 1988). B10.T(6R), B10.AQR, B10.A(2R) and C57BL/10 (B10) serum samples were also used in section 4.2 and are included here for comparative purposes.

At least five mice per strain were killed on days 11, 18, 21, 23, 28 and 35 p.i., their worm burdens determined and individual serum samples collected. Only two mean worm burden determinations were made for the B10.BR mouse strains, on d11 and d35 p.i., this strain being unable to expel a primary *T. muris* infection (Else and Wakelin, 1988). Serum samples for days 21 and 28 p.i. were obtained by tail bleeding in this case. Specific IgG, IgG1 and IgM antibody levels in the sera were examined by ELISA. Antigen recognition profiles for IgG antibody were determined by immunoprecipitation. 10–20% gradient gels were used throughout.

5.2.2 PASSIVE TRANSFER OF IMMUNITY

60 female (B10.BRxB10.G)F1 mice were infected with 400 *T. muris* eggs from which 114.25±15.4 larvae established as seen on d13 p.i. Mice were killed on d33 p.i. and their sera collected and pooled. All individuals harboured either no or a few stunted worms, i.e. they were resistant to infection. Antibody levels in the F1 pooled serum, as determined by ELISA, are shown in table 5.1.

Table 5.1 Antibody levels in the pooled (B10.BRxB10.G)F1 hybrid mouse strain immune serum assessed for its capacity to protect the normally non-responder B10.BR mouse strain against a primary infection of 400 *T. muris* eggs

	O.D. (410nm)			
	IgG	IgG1	IgM	IgA
Pooled (B10.BRx B10.G)F1 serum d33 p.i	1.223	1.383	0.519	0.068
(B10.BRxB10.G)F1 naive serum	0.085	0.101	0.055	0.136

The antibody recognition profile for the pooled F1 serum was identical to that seen before for individual male F1 sera (figure 4.2.4). Recipient

male B10.BR mice were infected with 400 *T. muris* eggs and injected with 0.25, 0.5 and 0.5 mls of pooled F1 immune serum i.p. on days 0, 1 and 3 p.i. respectively. Control mice were injected with PBS. Mice were killed, bled individually, and their worm burdens determined on days 15 and 35 p.i.

5.2.3 STATISTICS

The significance of differences between worm burdens recovered from experimental groups was calculated using the Mann-Whitney U test. A value greater than P=0.05 was considered non-significant.

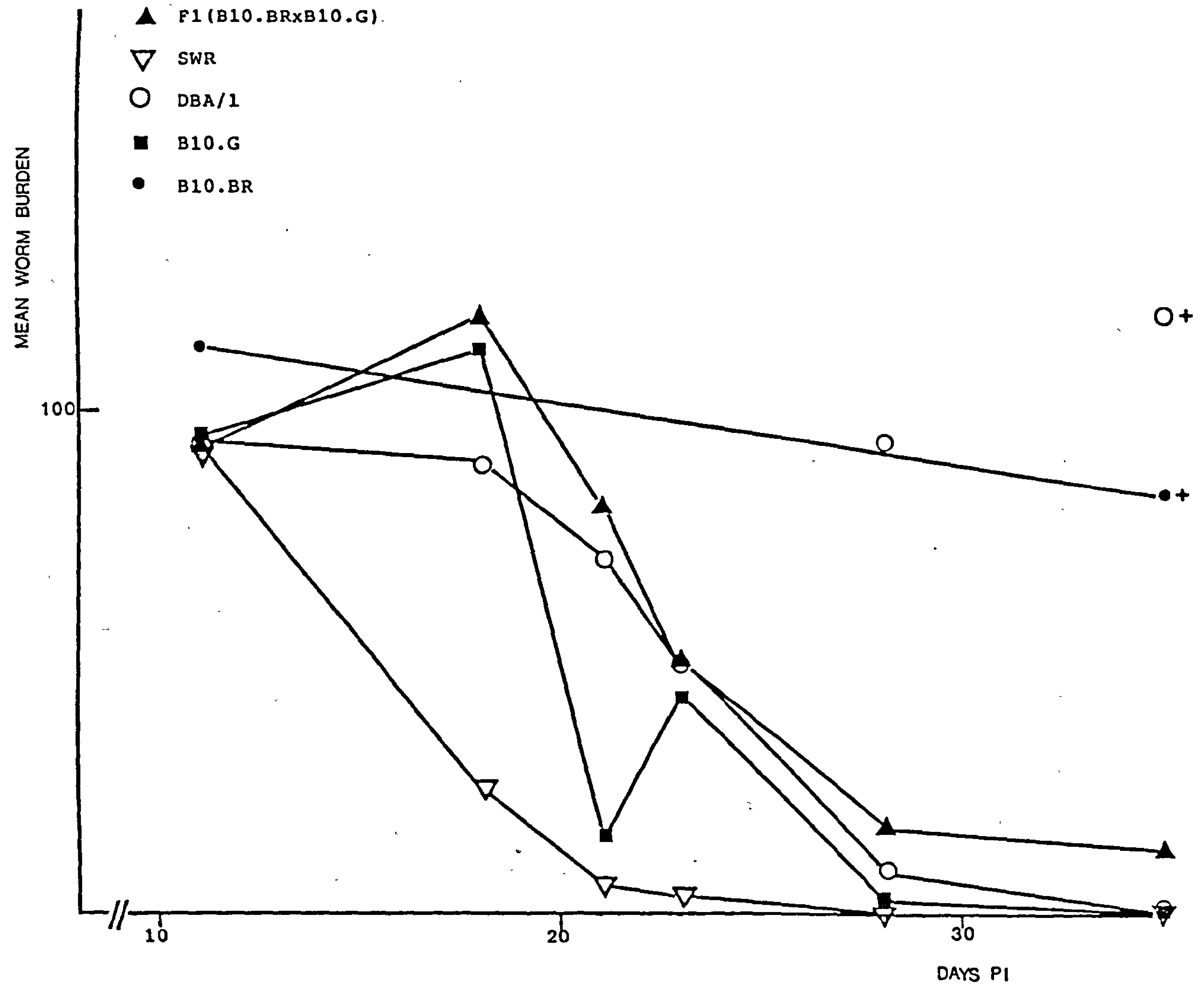
The significance of differences in antibody levels between strains post infection was determined using Analysis of Variance. $P>0.05$ was considered non-significant.

5.3 RESULTS

5.3.1 TIME COURSE OF EXPULSION OF *T. MURIS* FROM H-2^q HAPLOTYPE STRAINS OF MICE

The time course of expulsion of *T. muris* from each of the five strains studied is shown in figure 5.1. Only mean worm burdens are shown (for at least five individuals per strain per time point), standard deviations being omitted for clarity. The degree of variation between individual worm burdens within a group is shown in Table 5.2 where all mean worm burdens and their standard deviations are given. No significant differences in the number of larvae recovered on d11 p.i. were observed between any of

Figure 5.1 Time course of expulsion of *T. muris* from (B10.BRxB10.G)F1, SWR, DBA/1, B10.G and B10.BR strains of mice. Only mean worm burdens (for at least five individuals per strain per time point) are shown, standard deviations being omitted for clarity. Mean worm burdens plus standard deviations are given in Table 5.2. No significant differences in establishment of larvae in any strain was seen on d11 p.i. The DBA/1 mouse strain exhibited differential responsiveness and was divided into responder and non-responder populations from d28 p.i. onwards. Responders were identified on the basis of harbouring a reduced worm burden (less than the d11 mean minus two standard deviations) and showing evidence of worm stunting. 4/6 DBA/1 individuals harboured patent infections on d35 p.i. + = patent infection.


F1(B10.BRxB10.G)
SWR
DBA/1
B10.G
B10.BR
MEAN WORM BURDEN
100
10
20
30
DAYS PI
+
+

the strains studied (P>0.05).

Table 5.2 Mean worm burdens plus/minus standard deviations (for at least five individuals per strain per time point) for H-2^q haplotype strains of mice and the B10.BR mouse strain. The time course of expulsion of *T. muris* from these strains is illustrated in figure 5.1. Individuals of the DBA/1 strain of mouse were divided into responders (R) and non-responders (NR) from d28 p.i. onwards, responders harbouring reduced worm burdens and showing evidence of worm stunting (see text).

	Day post-infection					
	11	18	21	23	28	35
F1	93.0±15.3	119.7±24.2	81.5±42.9	50.5±50.6	17.3±14.7	12.7±15.3
B10.G	94.8±13.4	113.4±32.6	15.4±15.6	43.6±38.0	3.0± 4.5	0.4± 0.55
B10.BR	114.0±25.6	-	-	-	-	84.0±50.5
SWR	94.2±12.9	25.6±38.0	6.0±10.2	3.6± 8.0	0.0±0.0	0.0±0.0
DBA/1(R)	93.8±28.8	90.8±33.3	72.0±46.7	50.4±50.7	8.5±12.0	1.0±1.4
DBA/1(NR)					90.0±44.4	120.8±55.1

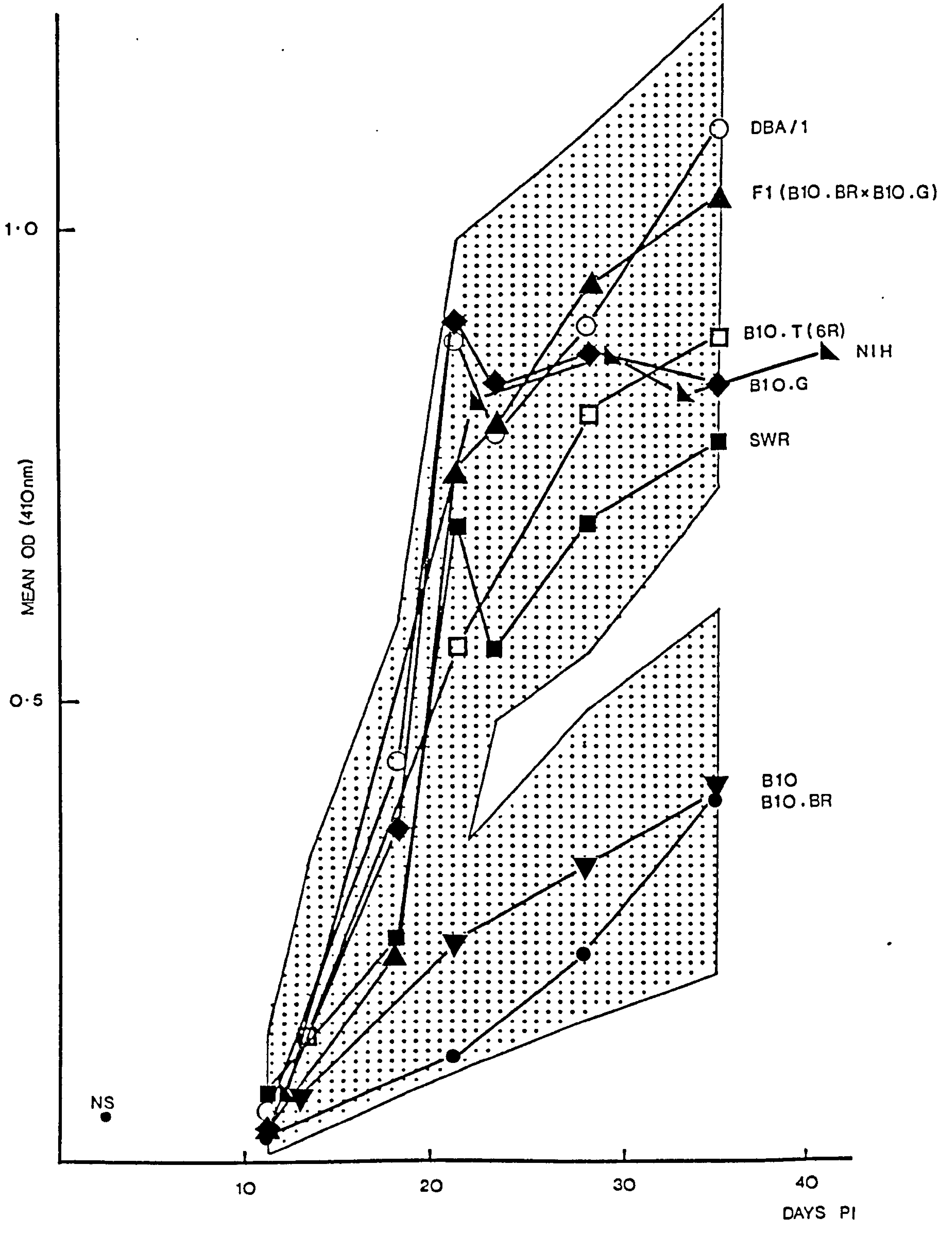
The SWR strain expelled worms the fastest, a significant decrease in worm burdens being seen on d18 p.i. (P<0.025). B10.G mice showed a significant decrease in worm numbers on d21 p.i. (P<0.01). Although 3/6 (B10.BRxB10.G)F1 mice had lost nearly all their worms by d23 p.i., a significant decrease in worm burdens was not observed until d28 p.i. because of the large standard deviation seen on d23 p.i. Unlike SWR, B10.G and (B10.BRxB10.G)F1 mice which all became uniformally resistant to infection, few if any worms remaining by d28 p.i., the DBA/1 strain of mouse displayed differential responsiveness, 4/6 individuals being unable to expel the parasite before the infection reached patency. Individuals were divided into responders and non-responders from d28 p.i. onwards, responders being identified on the basis of harbouring worm burdens less than the d11 mean minus two standard deviations and displaying evidence of worm stunting. Although it is clear from the standard deviations that the two response phenotypes became apparent before day 28 (see d23 values), no separation was possible before this time as similar wide variation occured in B10.G and (B10.BRxB10.G)F1 mice. However these latter strains were uniformly resistant on day 28. 6/6 B10.BR mice harboured patent infections

on d35 p.i. and so were classified as non-responders. However 2/6 individuals showed a reduction in worm burdens from the d11 mean resulting in a large standard deviation on d35 p.i.

5.3.2 ANTIBODY RESPONSES OF H-2^{q} AND NON-H-2^{q} MOUSE STRAINS TO E/S ANTIGEN AS DETERMINED BY ELISA

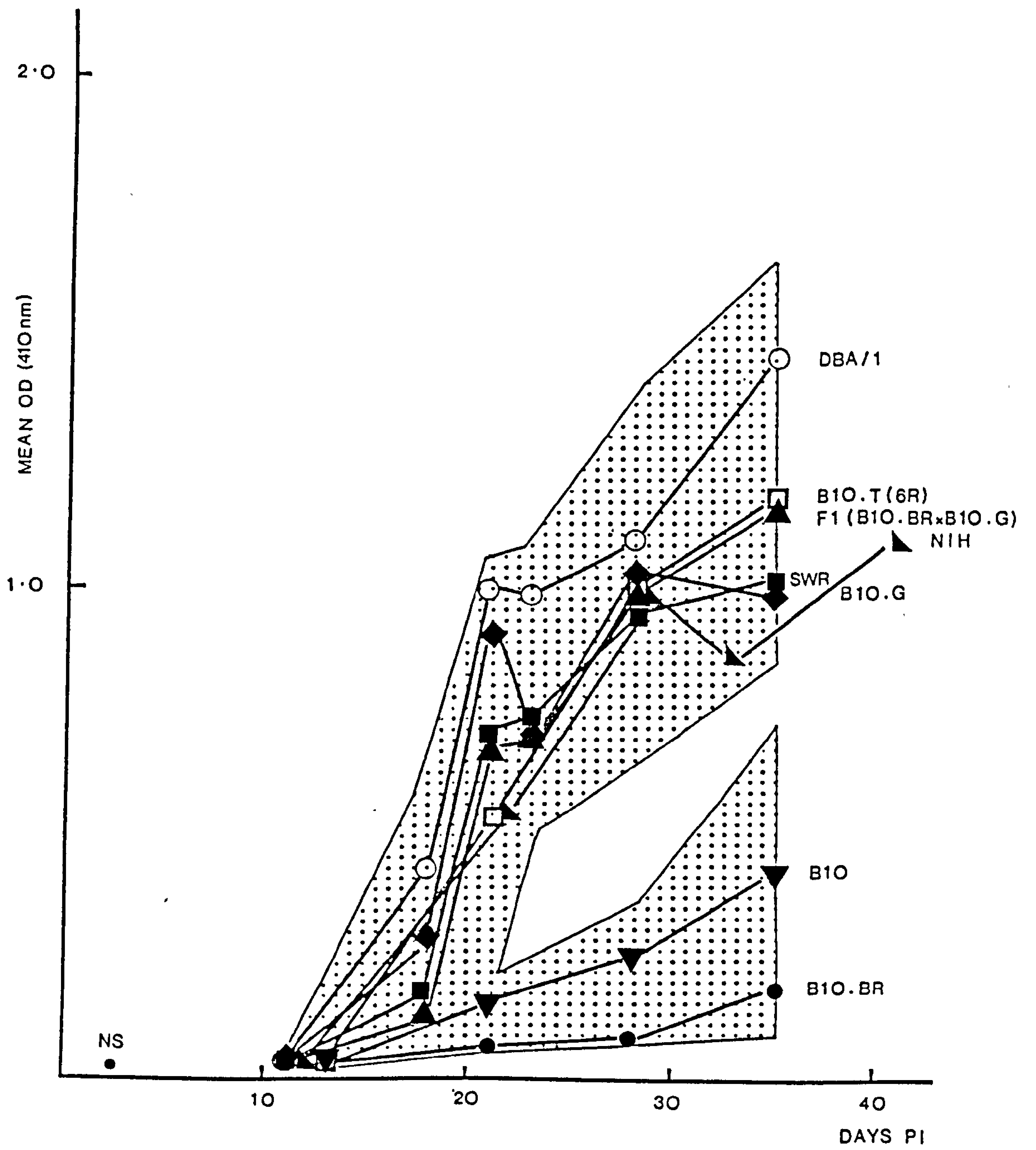
Figure 5.2 shows the specific IgG antibody responses to *T. muris* E/S antigen of the five strains of mouse under study. NIH, B10.T(6R) and B10 response patterns are included for comparison. Mean optical densities, reflecting specific antibody levels, represent values for at least five individuals at each time point. In most cases standard deviations were small in comparison to the mean, although as noted before (Chapter 4.2.2) individuals of the B10 strain of mouse showed considerable variation in levels of specific antibody, especially later on in infection. Two distinct groups of mice could be identified by d21 p.i. on the basis of levels of IgG antibody to E/S antigen. Strains of mice possessing q alleles throughout the H-2 (DBA/1, (B10.BRxB10.G)F1, NIH, B10.G and SWR) and B10.T(6R) mice (q alleles at K and I-A) rapidly developed levels of specific IgG antibody very significantly higher than non-H-2^{q} mouse strains (B10, B10.BR). There was no significant difference between levels of specific IgG antibody produced by the H-2^{q} haplotype strains as a group, although there was some suggestion that the highest specific IgG producing strain, DBA/1, may have had IgG levels significantly higher than the lowest specific IgG producer, SWR. Antibody levels continued to rise, or were maintained at these high levels, after the parasite had been expelled from resistant strains/individuals. Although antibody levels of the B10 and B10.BR mouse strains also continued to rise during the course of infection, and post-infection with respect to the B10 individuals, the levels never

Figure 5.2 Time course of the specific IgG antibody response to *Trichuris muris* E/S antigen in serum samples from DBA/1, (B10.BRxB10.G)F1, B10.G, SWR and B10.BR mice. Serum samples from B10.T(6R), B10 and NIH mice are included for comparative purposes. The shaded area represents the range of standard deviations from the mean optical densities calculated from at least five individual values for each strain at each time point post infection. N.S., naive mouse serum. O.D., optical density.



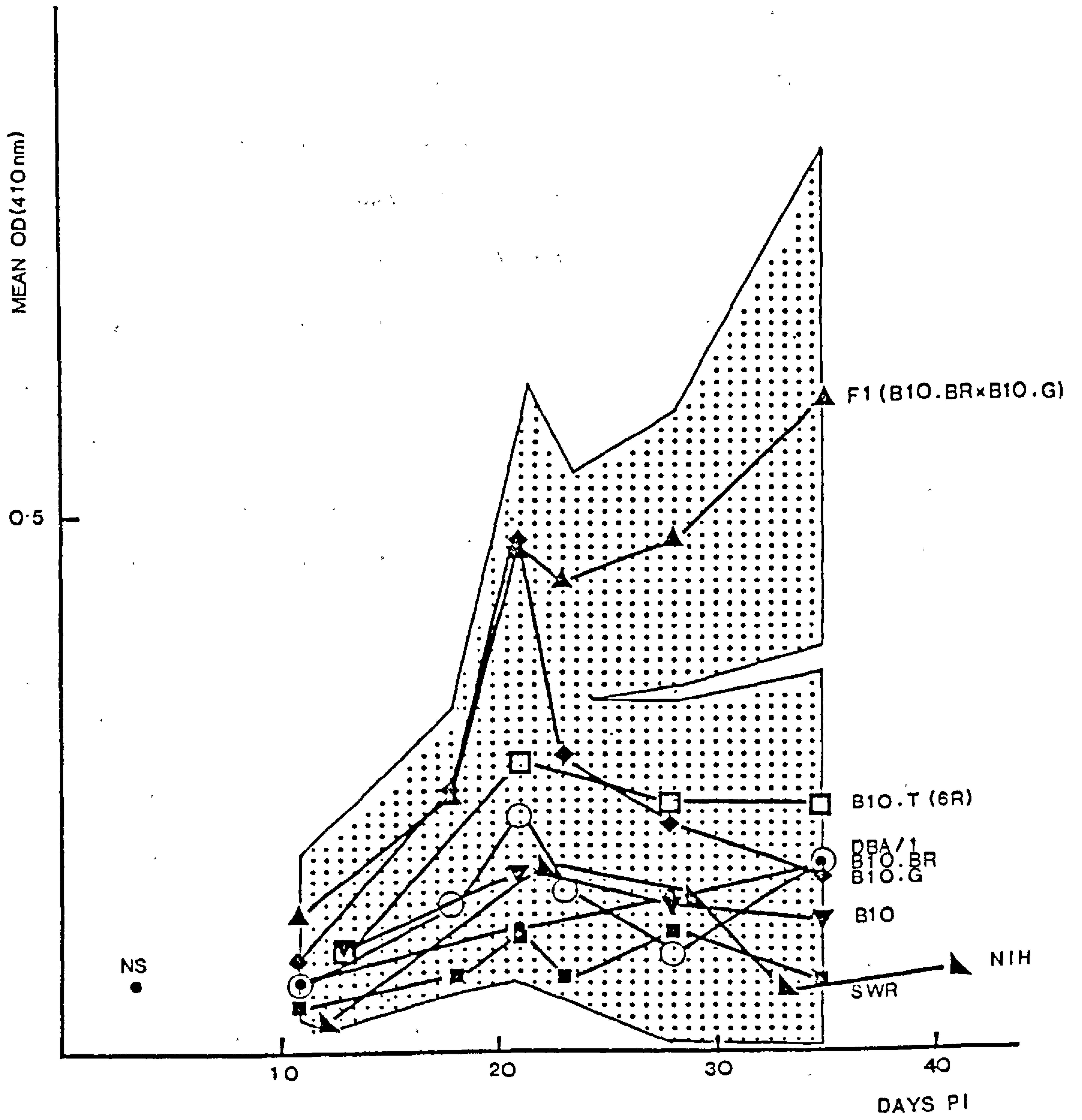
DBA/1
F1 (B10.BR×B10.G)
B10.T(6R)
NIH
B10.G
SWR
B10
B10.BR
1·0
0·5
MEAN OD (410nm)
NS
10
20
30
40
DAYS PI

Figure 5.3 Time course of the specific IgG1 antibody respose to *Trichuris muris* E/S antigen in serum samples from DBA/1, (B10.BRxB10.G)F1, B10.G, SWR and B10.BR mice. B10.T(6R), B10 and NIH serum samples are included for comparative purposes. The shaded area represents the range of standard deviations from the mean optical densities calculated from at least five individual values for each strain at each time point post infection. N.S., naive serum. O.D., optical density.



2·0
1·0
MEAN OD (410nm)
NS
10
20
30
40
DAYS PI
DBA/1
B10.T(6R)
F1 (B10.BR×B10.G)
NIH
SWR
B10.G
B10
B10.BR

Figure 5.4 Time course of the specific IgM antibody response to *Trichuris muris* E/S antigen in serum samples from DBA/1, (B10.BRxB10.G)F1, B10.G, SWR and B10.BR mice. B10.T(6R), B10 and NIH serum samples are included for comparative purposes. The shaded area represents the range of standard deviations from the mean optical densities calculated from at least five individual values for each strain at each time point post infection. N.S., naive serum. O.D., optical density.


MEAN OD(410nm)
0·5
NS
F1 (B10.BR×B10.G)
B10.T (6R)
DBA/1
B10.BR
B10.G
B10
NIH
SWR
10
20
30
40
DAYS PI

approached those of the H-2^{q} strains as seen on day 35 p.i.

The IgG1 antibody responses to *T. muris* E/S antigen of DBA/1, (B10.BRxB10.G)F1, SWR, B10.G and B10.BR mice are shown in figure 5.3, with the IgG1 response patterns of B10.T(6R), B10 and NIH mice included for comparison. Again two distinct groups of mice were apparent by d21 p.i., the H-2^{q} haplotype strains of mice developing a rapid high-level IgG1 response unlike the non-H-2^{q} strains (B10, B10.BR) where specific IgG1 levels rose only gradually post-infection. Figure 5.4 shows the specific IgM responses to *T. muris* E/S antigen of the H-2^{q} and non-H-2^{q} strains of mice under study. Response patterns were quite distinct from those seen for IgG and IgG1. SWR and B10 mice showed little variation in IgM levels post-infection, whilst individuals of the B10.BR strain showed a small and gradual increase. Circulating specific IgM rose slightly up until d21 p.i. and then declined in NIH mice, whilst the DBA/1 mouse strain showed a slight peak in IgM levels on d21 p.i. IgM levels also peaked markedly on d21 p.i. in B10.G mice. Levels of IgM rose slightly and then plateaued in B10.T(6R) mice whilst individuals of the (B10.BRxB10.G)F1 hybrid strain developed high levels of IgM by d21 p.i. Although the d35 mean optical density value for this strain was greater than the d21 value, considerable variation between individuals was seen at this time point.

5.3.3 ANTIGEN RECOGNITION PROFILES OF H-2^{q} AND NON-H-2^{q} STRAINS OF MICE

Figures 5.5 and 5.6 show the capacity of IgG antibody in sera, taken on d35 p.i. for all strains except SWR, where serum samples were taken on d28 p.i., to precipitate ^{35}S-methionine labelled *T. muris* E/S antigen. Antigen recognition profiles are for individual serum samples. Individuals of most strains possessed IgG antibodies which recognised a number of antigens

Figure 5.5 Fluorograph of SDS-PAGE of ^{35}S-metabolically labelled *T. muris* E/S antigen after immunoprecipitation with IgG antibodies in sera taken d35 p.i. from DBA/1 (lanes 1 and 6), B10.BR (lanes 2 and 7), B10.G (lanes 3 and 8), and (B10.BRxB10.G)F1 (lane 4) individuals and d28 p.i. from an SWR individual (lane 5). No antigens were precipitated with naive serum from (B10.BRxB10.G)F1 mice (lane 9). Worm burdens are also given beneath the lane numbers; + = patent infection, s = stunted worms present. Arrows indicate the 90-95kDa and 105-110kDa antigens referred to in the test.

Figure 5.6 Fluorograph of SDS-PAGE of ^{35}S-metabolically labelled *T. muris* E/S antigen after immunoprecipitation with IgG antibodies in sera taken d35 p.i. from B10.G, (B10.BRxB10.G)F1, DBA/1 and B10.BR individuals (lanes 1, 2, 4 and 5 respectively) and d28 p.i. from an SWR individual (lane 3). B10.T(6R) and B10.AQR d35 p.i. antigen recognition profiles are shown in lanes 6 and 8 (see chapter 3.2). Lane 7 shows recognition of antigen by IgG antibodies in serum taken d26 p.i. from NIH mice (see chapter 3.1). Lane 9, (B10.BRxB10.G)F1 NS. Worm burdens are also given beneath the lane numbers; + = patent infection, s = stunted worms. Arrows indicate the 90-95kDa and 105-110kDa antigens referred to in the text.

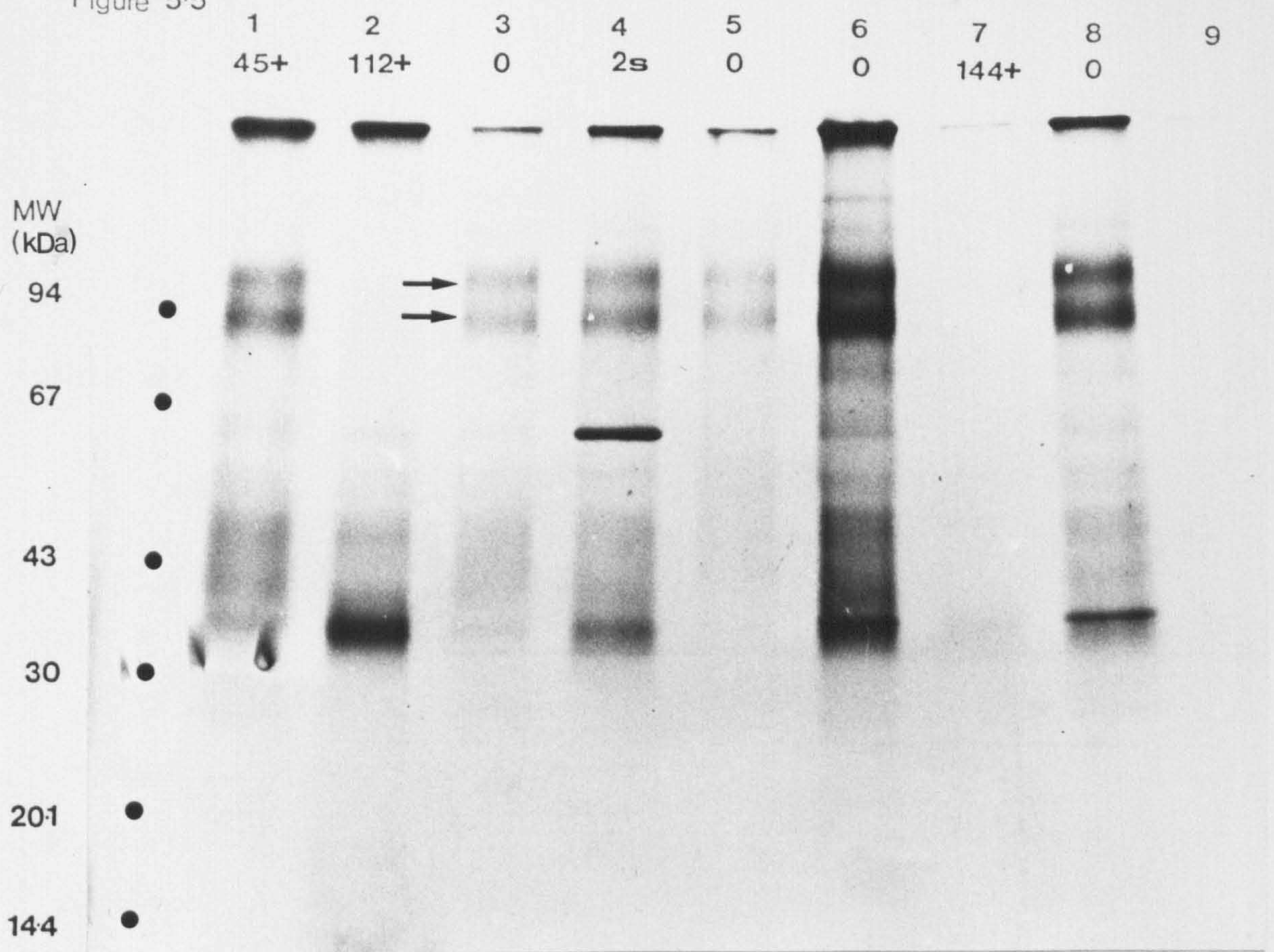


Figure 5·5
1
45+
2
112+
3
0
4
2s
5
0
6
0
7
144+
8
0
9
MW
(kDa)
94
67
43
30
20·1
14·4

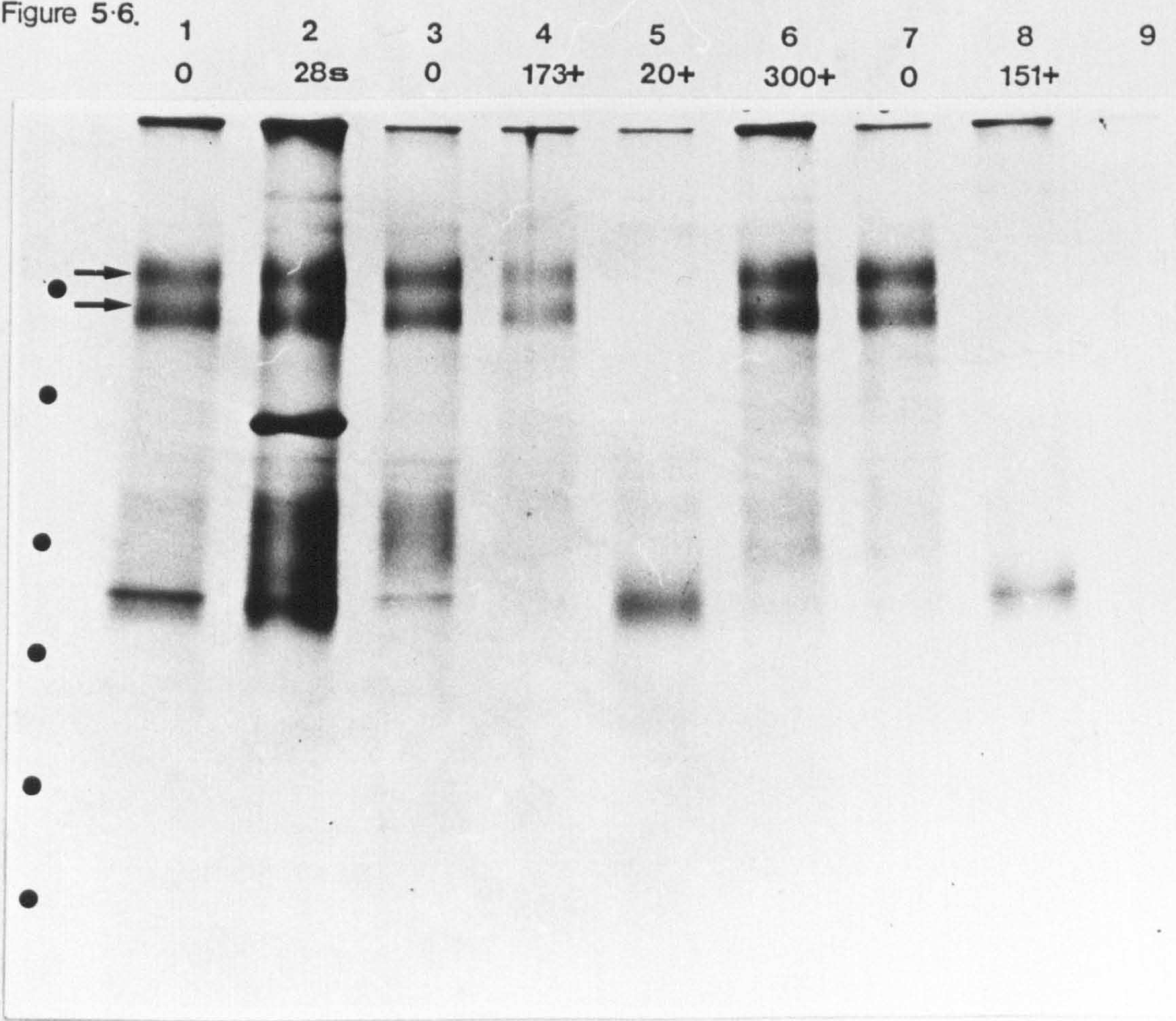


Figure 5·6.
1
0
2
28s
3
0
4
173+
5
20+
6
300+
7
0
8
151+
9

Figure 5.7 Fluorogaph of SDS-PAGE of ^{35}S-metabolically labelled *T. muris* E/S antigen after immunoprecipitation with IgG antibodies in sera taken d21 p.i. from B10.G, (B10.BRxB10.G)F1, SWR, DBA/1 and B10.BR individuals (lanes 1-5 respectively). Antigen recognition profiles for B10.T(6R), B10.AQR and B10.A(2R) (see chapter 3.2) on d21 p.i. are shown in lanes 6-8 respectively. Lane 9, (B10.BRxB10.G)F1 N.S. Worm burdens, where known, are also given beneath the lane numbers. Arrows indicate the 90-95kDa and 105-110kDa antigens referred to in the text.

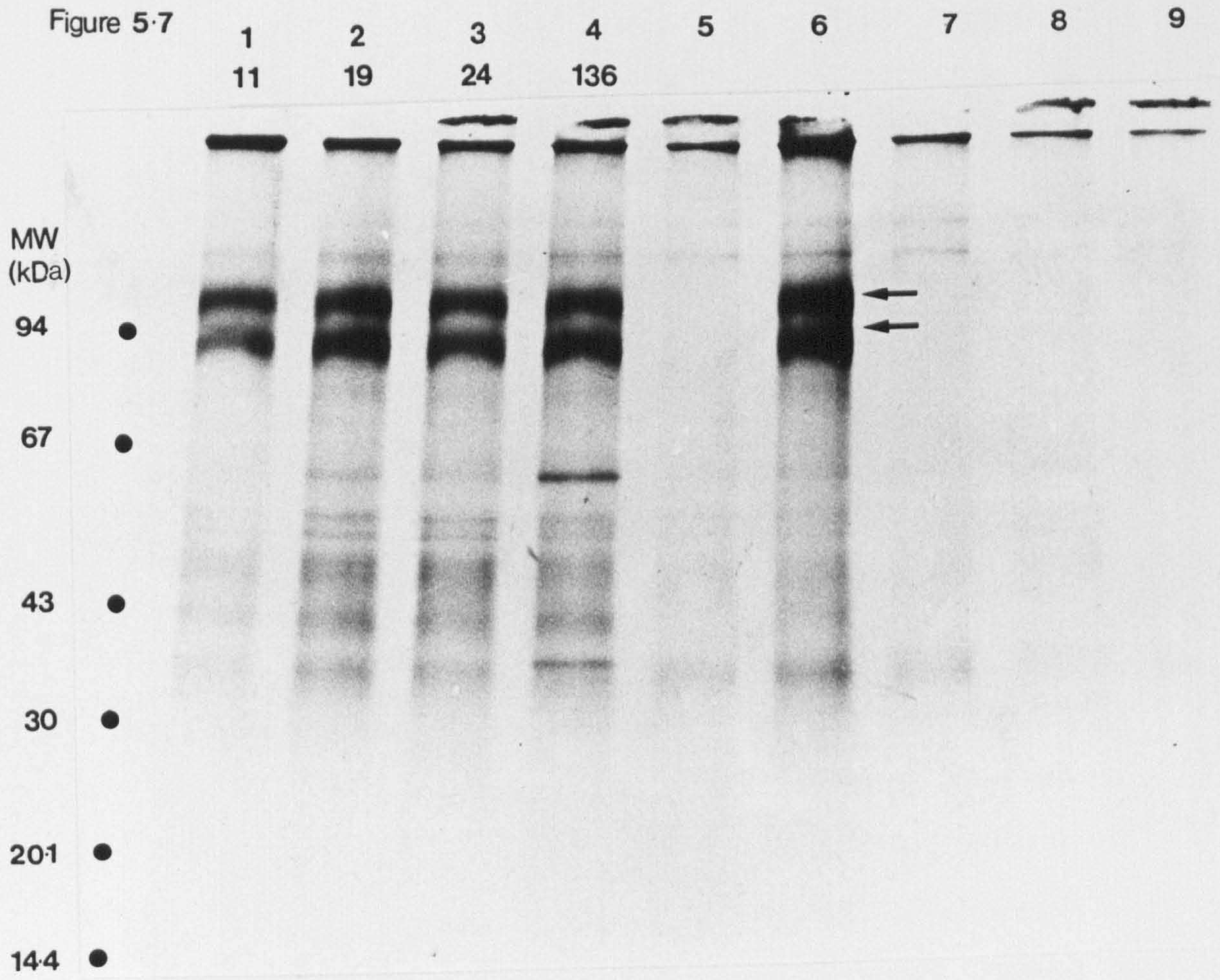


Figure 5·7

within the 30-50kDa region, although the antigen recognition profile for one B10.BR individual, shown in figure 5.5 (lane 7), was little different from naive serum (lane 9). Such a limited recognition profile for B10.BR mice was unexpected based on previous experience (Chapter 4, figures 4.1.5, 4.1.6, 4.2.3 and 4.2.4) and illustrates further the existence of individual variation in antigen recognition within inbred strains discussed in Chapter 4. More antigens were precipitated by the B10.BR sera used in figure 5.5 (lane 2) and figure 5.6 (lane 5) but even here the banding pattern was far less intense than previously seen. The most striking feature revealed by the immunoprecipitation studies performed using individual serum samples from $\underline{H-2}^q$ ($\underline{I-A}^q$) (DBA/1, B10.G, (B10.BRxB10.G)F1, SWR, NIH and B10.T(6R)) and non-$\underline{H-2}^q$ (B10, B10.BR, B10.AQR) strains of mice was that two high MW antigens (90-95kDa, 105-110kDa), were recognised by IgG antibodies only in serum from individuals of $\underline{H-2}^q$ haplotype strains or strains possessing q alleles at I-A (antigen recognition profiles for individuals of non-$\underline{H-2}^q$ strains are represented in lanes 2 and 7 in figure 5.5, and lanes 5 and 8 in figure 5.6). Immunoprecipitations using sera from all individuals within each mouse strain were subsequently performed (data not shown) and revealed the uniform ability of individuals possessing q alleles at I-A to recognise the two high MW antigens. Although only three strains of mice expressing haplotypes other than $\underline{H-2}^q$ were studied here, previous work (Chapter 4, figures 4.1.5, 4.1.6, 4.2.3 and 4.2.4) has shown that recognition of the the 90-95kDa and 105-110kDa antigens by IgG antibody is apparently restricted to strains of mice possessing q alleles at I-A.

Figures 5.5 and 5.6 show the antigen recognition profiles for strains of mice using sera taken late (d28 or d35 p.i.) during the course of the infection. However the ELISA data presented here revealed that high levels of IgG (and IgG1) antibodies were present in the peripheral circulation of mouse strains expressing q alleles at I-A by d21 p.i. The antigens

recognised by IgG antibodies in serum samples taken at this time are shown in figure 5.7. As can be seen, B10.G, (B10.BRxB10.G)F1, SWR, DBA/1 and B10.T(6R) individuals (all I-A^{q}, lanes 1-4 and 6) already possessed IgG antibodies against the 90-95kDa and 105-110kDa antigens by d21 p.i., strains of mice not expressing q alleles at I-A (B10.BR, B10.AQR and B10.A(2R), lanes 5,7 and 8) again being negative.

It is possible that the apparent I-A^{q} restriction on the recognition of the two high MW antigens is a quantitative artefact. Thus the I-A^{q} restriction on the development of a high level IgG response as seen by ELISA would mean that only these strains of mice would possess adequate levels of specific IgG to reveal precipitation of the two high MW antigens. However, when immunoprecipitations were performed using B10 (H-2^{b}) and BALB/c (H-2^{d}) sera in volumes from two to four times that used for H-2^{q} mouse strains in an attempt to equalise serum samples for specific antibody, no additional antigens were precipitated than when a standard volume of 25μl was used. Also the antigen recognition profiles for the B10.BR and B10 mouse strains (both non-H-2^{q}), using sera taken on d61 p.i. (Chapter 4, figure 4.1.5) when specific IgG levels were similar to seen for B10.G mice (H-2^{q}) as seen by ELISA (figure 4.1.2), did not reveal recognition of the two high MW antigens. From these results it would seem unlikely that the I-A^{q} restricted antibody responses were reflections of antibody titre. However evidence presented in the Appendix to this chapter suggests that there is a quantitative component involved in the observed I-A^{q} restriction, at least for some mouse strains.

Apart from the consistent recognition of the two high MW antigens by all strains of mice of the H-2^{q} haplotype, the other striking feature revealed by immunoprecipitation was the recognition of an antigen of approximately 65kDa by certain individuals, in particular two (B10.BRxB10.G)F1 mice, where the banding pattern was very intense

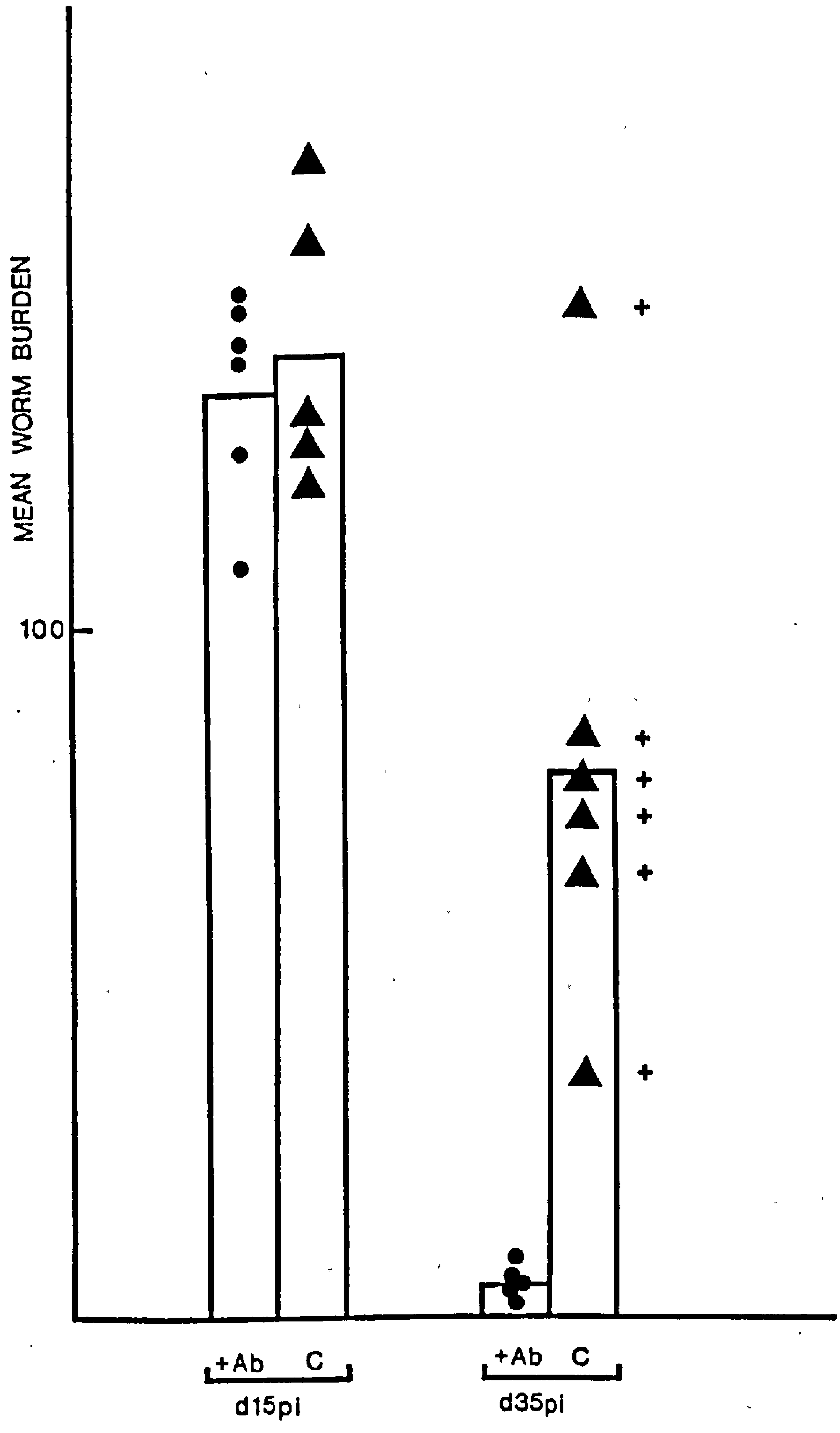
(figures 5.5 and 5.6). However, subsequent immunoprecipitations revealed that this antigen was also recognised by naive serum, implying that the response was infact non-specific.

No differences in antibody levels or antigen recognition profiles were observed between responder and non-responder DBA/1 individuals. For instance, figure 5.5 shows antigen recognition profiles for a resistant individual (lane 6) and an individual harbouring a patent infection (lane 1). A non-responder DBA/1 antigen recognition profile is also shown in figure 5.6 (lane 4). Although the banding pattern was more intense for the resistant individual, qualitatively the recognition profiles were identical.

5.3.4 CAPACITY OF IMMUNE SERUM CONTAINING HIGH LEVELS OF SPECIFIC IgG (IgG1) FROM (B10.BRxB10.G)F1 MICE ($H\text{-}2^{q/k}$) TO TRANSFER RESISTANCE TO THE NON-RESPONDER PARENTAL STRAIN B10.BR ($H\text{-}2^{k}$)

Figure 5.8 shows the effect of transferring immune serum from the resistant, high IgG (IgG1) producing (B10.BRxB10.G)F1 mouse strain to the normally non-responder, low level IgG (IgG1) producing, parental mouse strain B10.BR. No significant difference in worm burdens was observed between experimental and control groups on d15 p.i. ($P>0.05$). However, by d35 p.i. mice receiving (B10.BRxB10.G)F1 immune serum had significantly fewer worms ($P<0.01$) than control mice on day 35 p.i., mice harbouring less than ten worms all of which were stunted in appearance. Control mice on d35 p.i. showed a significant reduction in worm burdens ($P<0.025$) from d15 control values, reflecting the existence of a degree of resistance to the primary infection. However, although some stunted worms were present in control mice all infections were patent, in contrast to the infections of mice injected with immune serum where no mature adult

Figure 5.8 The fate of a primary infection (400 eggs) in non-responder B10.BR mice which had received serum from resistant (B10.BRxB10.G)F1 mice containing high levels of specific IgG (IgG1) antibodies. This serum also contained IgG antibodies which recognised the 90-95kDa and 105-110kDa antigens referred to in the text. B10.BR mice were injected intra-peritoneally with 0.25mls, 0.5mls and 0.5mls of serum on days 0, 1 and 3 p.i. respectively. Control mice received identical injections of PBS. At least 5 mice per group were killed on days 15 and 35 p.i. and their worm burdens assessed. + = patent infection, +Ab = B10.BR mice receiving (B10.BRxB10.G)F1 serum, C = control mice.



MEAN WORM BURDEN
100
+Ab
C
d15pi
+Ab
C
d35pi

females were found. The increase in resistance to a primary infection described here for the B10.BR strain of mouse compared to that previously reported (Else and Wakelin, 1988) may reflect the fact that the mice used in this experiment were eight weeks older than the standard 6-8 weeks when infected. This phenomenon is discussed in more detail in Chapter 6.2 where a similar effect of B10.BR age on the ability to express some resistance to infection is described.

5.4 DISCUSSION

The results presented in this chapter support the correlation between the expression of q alleles at the H-2 and resistance to infection (Else and Wakelin, 1988) and confirm the influence of background genes on the ability to express protective immunity, SWR, DBA/1 and B10.G mouse strains (all $H\text{-}2^q$) exhibiting differences in the kinetics of worm expulsion. The significant decrease ($P<0.01$) in worm burdens on d21 p.i. observed for B10.G mice represents a slightly faster rate of expulsion than previously reported for this strain (Else and Wakelin, 1988) and may reflect the difference in the origin of the B10.G mice used in the two studies, mice in the earlier study being purchased from Harlan Olac Ltd., whereas mice in the present study were bred locally at Nottingham. The importance of background genes in defining the limits within which the H-2 genes operate is illustrated by the differential responsiveness seen within the DBA/1 mouse strain, 4/6 mice harbouring mature parasites on d35 p.i. This phenomenon, first described for the related DBA/2 strain (Worley *et al.*, 1962; Lee and Wakelin, 1982*b*), suggests that the DBA background genome imposes limits such that expulsion is not initiated before the immunomodulatory stages of

the parasite have developed (Else *et al.*, 1989) even though resistant q alleles are present throughout the H-2.

The results presented in this chapter also support the suggestion of an apparent $\underline{H\text{-}2}^q$ ($\underline{I\text{-}A}^q$) restriction on the development of rapid high level IgG (IgG1) antibody responses to *T. muris* E/S antigen and the recognition of two high MW antigens (90-95kDa, 105-110kDa) first indicated during the analysis of the humoral immune responses of H-2 recombinant mouse strains in Chapter 4. MHC restricted responses are rarely observed for complex foreign antigens as such antigens have many epitopes each of which may be restricted by different MHC alleles. Thus it is unlikely that any one mouse strain is unable to respond to any of these epitopes. Most previous demonstrations of MHC-restricted antibody responses to foreign molecules have involved simple antigens such as the *Plasmodium falciparum* circumsporozoite repetitive epitope, the antibody response to which has been shown by Del Giudice *et al.* (1986) to be exclusively $\underline{I\text{-}A}^b$ restricted. Rathbun and Hildemann (1970) reported a similar phenomenon studying antibody responses to the 2,4,6-trinitrophenyl hapten (TNP) conjugated to mouse serum albumin, the $\underline{H\text{-}2}^b$ haplotype being associated with high antibody responsiveness even on diverse strain backgrounds. It was surprising therefore to see a clear division into high and low antibody producing strains to as complicated an antigenic mixture as parasite E/S products, high responsiveness being correlated with a particular haplotype ($\underline{H\text{-}2}^q$), with an $\underline{H\text{-}2}^q$ ($\underline{I\text{-}A}^q$) restriction on the recognition of two high MW E/S antigens (90-95kDa, 105-110kDa). This phenomenon is explicable in a number of ways. For instance if recognition by IgG antibody of the two high MW antigens in E/S by $\underline{H\text{-}2}^q$ mice is related to the development of the rapid high level IgG (IgG1) antibody response to E/S antigen seen in $\underline{H\text{-}2}^q$ strains, rather than just being coincidental, then it may be that only a limited number of epitopes expressed by the 90-95kDa and 105-110kDa

antigens (immunodominant sites) are recognised by T cells so that failure to respond to one or two immunodominant sites leads to low responsiveness. Alternatively negative signals may be generated, "suppressor" epitopes turning off the response to the rest of the molecule. Such hypotheses have been considered by Berzofsky *et al.*, (1987) in relation to vaccine design. More simply, the two high MW antigens may possess highly repetitive structures thus confronting T cells with only a few determinants.

IgG1 was the only subclass of IgG antibody analysed in this study. Although levels of the other IgG subclasses to *T. muris* E/S antigen should also be determined (see Appendix to this chapter), a knowledge of the IgG1 regulatory mechanism by interleukin 4 (Il-4) (reviewed by Snapper *et al.*, 1988) leads to the prediction that levels of the other IgG subclasses would be low.

Il-4 has potent effects on cells of most haematopoietic lineages, in particular promoting the selective secretion of murine IgG1 and IgE (Vitetta *et al.*, 1985; Coffman and Carty, 1986) and inhibiting the production of IgG2a, IgG2b, IgG3 and IgM by LPS-stimulated B cells (Snapper and Paul, 1987; Snapper *et al.*, 1988). Another lymphokine, gamma interferon (IFN-γ) is associated with the selective stimulation of IgG2a production and inhibition of IgG1 secretion by LPS-stimulated B cells (Snapper and Paul, 1987; Snapper *et al.*, 1988). Thus Il-4 and IFN-γ reciprocally regulate immunoglobulin isotype secretion. If regulation of the IgG1 antibody response by Il-4 can be extended to the *T. muris*-mouse system, the high IgG1 antibody levels observed here for H-2 strains of mice would imply that Il-4 levels are also high, and hence levels of other IgG subclasses low. Interestingly ,the two major subsets of T_H cells designated T_H1 and T_H2 (Mosmann *et al.*, 1986; Mosmann and Coffman, 1987; see Introduction 1.3.3), identified and defined on the basis of their lymphokine expression patterns, can be distinguished on the basis of whether they secrete Il-4 or

IFN-γ, T_H1 producing IFN-γ (and Il-2) whilst T_H2 produce Il-4 (and Il-5). This implies that there are two distinct pathways of B cell activation; the T_H2 pathway utilising Il-4 and Il-5 and the T_H1 pathway utilising Il-2 and IFN-γ (reviewed by Coffman *et al.*, 1988). Coffman *et al.*, 1988, suggested that the T_H2 pathway stimulated the optimum combination of responses for dealing with multicellular parasites whilst the set of responses invoked by the T_H1 pathway was important for dealing with viral or tumour antigens. The high IgG1 levels of the $\underline{H\text{-}2}^q$ haplotype strains of mice infected with *T. muris* raises the possibility that the T_H2 pathway is more efficiently induced when E/S antigen is presented in combination with $\underline{I\text{-}A}^q$ cell surface molecules than when other alleles are present at I-A, the *in vivo* production of IgG1 depending perhaps on the relative activities of the two T_H cell populations during parasitic infection.

The $\underline{H\text{-}2}^q$ ($\underline{I\text{-}A}^q$) restricted antibody responses reported in this chapter have immunogenetic interest but may also be important in the determination of resistance. Strains of mice expressing the $\underline{H\text{-}2}^q$ haplotype tend to be resistant to infection with *T. muris* (figure 5.1) suggesting that the rapid development of high levels of IgG (IgG1) and/or the recognition of the 90-95kDa and 105-110kDa antigens may be involved in protective immunity. However some members of the DBA/1 strain were unable to expel the parasite before the infection reached patency yet all DBA/1 individuals had identical IgG (IgG1) antibody responses. Therefore if antibody is important in resistance, it cannot be the sole component in the protective immune response. Similar conclusions were made by Davern *et al.* (1987) studying antibody responses to Sj26, a glutathione-S-transferase (GST) enzyme of *Schistosoma japonicum* worms, data suggesting that antibodies to Sj26 *alone* were not responsible for resistance. Likewise Butterworth *et al.* (1985) concluded that if antibodies were relevant to the expression of immunity by humans to *Schistosoma mansoni* they were not a limiting factor.

The capacity of serum from (B10.BRxB10.G)F1 mice to transfer immunity to the B10.BR mouse strain reported here suggests that high level IgG (IgG1) antibody responses and/or recognition of the 90-95kDa and 105-110kDa antigens do have some role in determining resistance to *T. muris*, although the F1 serum also contained moderate levels of IgM antibodies. To conclusively demonstrate a role for IgG1 antibodies in transferring protective immunity it would be necessary to immunochemically purify this IgG isotype from the serum and inject it into B10.BR mice.

Expulsion of *T. muris* is known to involve both an antibody-mediated and a cell-mediated phase (Wakelin, 1975*a*). It may be that antibody-mediated parasite damage renders the worm susceptible to cellular effector mechanisms. IgG1 purified from immune serum to *Heligmosomoides polygyrus* (*Nematospiroides dubius*) has been shown to be anti-parasitic when administered *in vivo*, leading to a significant reduction in worm numbers (Pritchard *et al.*, 1983). The mechanisms of action of IgG1 suggested included mediation of cellular adhesion to *H. polygyrus* akin to that described by Ramalho-Pinto *et al.*, (1979), studying murine *Schistosomiasis mansoni* and/or the neutralisation of vital metabolic antigens present in parasite E/S products. It is possible to envisage how such mechanisms could also operate in the *T. muris*-mouse system, the 90-95kDa and 105-110kDa antigens perhaps representing enzymes vital for parasite survival. Indeed the existence of casein proteases in *T. muris* E/S products within the 90-121kDa MW range has been demonstrated by Roach (1986). Thus transferring immune serum from a resistant to a non-responder strain of mouse, as reported here, might enable the recipient to mount an effective immune response, antibodies in the transferred serum neutralising important parasite metabolic products and/or potentially immunosuppressive molecules. Similarly Behnke and Parish (1979) suggested that immune serum raised against *H. polygyrus* contained factors which

facilitated the expression of a second component in worm expulsion not normally effective in a primary infection.

In order to explain the fact that some resistant mouse strains (e.g. BALB congenics, Chapter 3.1; Else and Wakelin, 1988) do not exhibit high IgG levels to *T. muris* E/S antigen (Chapter 4.1; Else and Wakelin, 1989) it is necessary to hypothesise that the cellular immune responses to *T. muris* may be potent enough in some strains to result in expulsion of the parasite without prior antibody attack. Likewise the fact that all DBA/1 individuals have high level antibody responses to E/S antigen even though some are unable to expel the worm suggests that the defect in the protective immune response lies in the cellular arm of the two stage attack. This defect presumably must be induced by the parasite in inbred strains of mice exhibiting differential responsiveness in order to explain the observed differences in response status within genetically uniform strains.

In order to determine the importance of the anti-90-95kDa and anti-105-110kDa IgG responses in protective immunity it would be interesting to vaccinate mice with those two high MW antigens. Butterworth *et al.*, 1985, suggested that two *Schistosoma mansoni* adult worm *in vitro* translation products recognised more extensively by resistant than susceptible children would be suitable candidates for testing as "protective" antigens following cloning and expression in *Escherichia coli*.

The MHC restricted antibody responses demonstrated to *T. muris* E/S antigens are potentially important in the context of man. It is not known if genetic factors are involved in regulating the specificity of the antibody response to *T. trichiura* in man although recent studies have suggested that susceptibility to infection is partly under genetic control (Bundy, 1988). It is possible that an MHC restriction, as demonstrated in the *T. muris*-mouse model, could limit the capacity of certain vaccinated individuals (should a vaccine be developed) to develop an effective antibody and/or cell mediated

response. It may be possible to overcome MHC-linked non-responsiveness by modifying the antigen but, in animals unresponsive to synthetic peptides, secondary responses to these peptides, using peptide-carrier conjugates require the same conjugate as that used to induce the primary response (Green *et al.*, 1968). Therefore individuals vaccinated with antigen plus carrier molecule may not gain from the boosting effect after a natural infection with *T. trichiura*.

5.5 SUMMARY POINTS

1. An apparent $\underline{H\text{-}2}^{q}$ ($\underline{I\text{-}A}^{q}$) restriction on the development of a rapid, high level IgG (IgG1) antibody response to *T. muris* E/S antigen has been demonstrated.

2 Recognition of two high MW antigens (90-95kDa, 105-110kDa) by IgG antibodies was also found to be an $\underline{H\text{-}2}^{q}$ ($\underline{I\text{-}A}^{q}$) restricted phenomenon.

3. Serum from resistant (B10.BRxB10.G)F1 hybrid mice ($\underline{H\text{-}2}^{q/k}$) containing high levels of IgG (IgG1) antibodies specific for *T. muris* E/S, and IgG antibodies which recognised the 90-95kDa and 105-110kDa E/S antigens was effective in transferring protection to the normally non-responsive B10.BR mouse strain as seen on d35 p.i.

4. It is suggested that the IgG responses described for $\underline{H\text{-}2}^{q}$ strains may be part of, but not an absolute requirement for, protective immunity, antibody-mediated parasite damage facilitating a subsequent cellular attack in some strains of mice.

APPENDIX - CHAPTER 5

The following experiments were carried out in collaboration with Dr. D. L. Wassom at the University of Wisconsin-Madison, U.S.A.

A5.1 INTRODUCTION

Chapters 3, 4 and 5 have described experiments indicating the apparent importance of particular alleles at certain H-2 loci both in determining the overall response phenotype to *T. muris* infection and in the control of the kinetics, level and specificity of the antibody response to *T. muris* E/S antigen. The work described in this appendix was performed in an attempt to support and extend the earlier observations through the use of a wider selection of H-2 recombinant mouse strains and additional serological analysis.

A5.2 EXPERIMENTAL DESIGN

The H-2 recombinant mouse strains studied are shown in Table A5.1 together with their haplotypes. Of particular interest were B10.K, B10.BR, B10.MBR and B10.RKQ1, whose response phenotypes would perhaps indicate whether resistance "q" alleles at the D end of the H-2 were capable of up-regulating the effects of susceptibility k alleles at I-A, and B10.T(6R) and B10.Q, where an increase in susceptibility of B10.T(6R) over B10.Q would suggest that d alleles at D end loci could down regulate the effects of q alleles at I-A. Such a situation was suggested by the results summarised in Chapter 3.2 but the appropriate q haplotype control strain could not be obtained at the time of the experiment and so conclusions were drawn by comparing response phenotypes of strains used in other experiments. As far as possible strains of mice were age matched although due to the large numbers of strains used in some cases mice were slightly

older than the standard age of 6-8 weeks when infected.

Table A5.1 Map of the H-2 complex showing the haplotype composition for the congenic and H-2 recombinant strains of mice infected with *T. muris*. Brackets indicate that the allele is not expressed; ●, undefined. B10 = C57BL/10 strain of mouse.

Strain	K	I				S	D
		A_β	A_α	$A_e(E_\beta)$	E_α		
B10.K	k	k	k	k	k	k	k
B10.BR	k	k	k	k	k	k	k
B10.Q	q	q	q	q	q	q	q
B10	b	b	b	b	b	b	b
B10.D2	d	d	d	d	d	d	d
B10.MBR	b	k	k	k	k	k	q
B10.RKQ1	k	k	k	k	(b)	●	q
B10.T(6R)	q	q	q	(q)	(q)	q	d
AKR/J	k	k	k	k	k	k	k
AKR-Fv-1^b	k	k	k	k	k	k	k

However B10.MBR, B10.RKQ1, B10.T(6R) and B10.Q were almost identical in age (9-11 weeks on day 0). Mice were infected with approximately 400 embryonated *T. muris* eggs on day 0 from which 203.7±43.7 larvae established, as seen on day 14 p.i. in B10.BR mice. Mice were killed in groups of 6 wherever possible, on days 21, 28 and 35 p.i. Because of restricted numbers of mice in certain strains fewer individuals were sacrificed at the earlier time points and no day 21 p.i. kill was made for B10.RKQ1 and B10.MBR mice. Worm burden determinations for AKR/J and AKR-Fv-1^b were made on day 35 p.i. only. The day 21 p.i. time point was selected to control for any differences in infectivity between strains, it being known that mice of the B10 genetic background, with the exception of the B10 strain, do not initiate expulsion until after this time point (Else and Wakelin, 1988). Blood samples were taken individually from mice in groups of 6 in most cases, on days 14, 21, 28 and 35 p.i., by tail bleeding, or at autopsy, as appropriate. Levels of IgG and IgG1 in the sera were determined by ELISA (alkaline phosphatase method). Levels of IgG2a

were also analysed by ELISA (peroxidase method). In addition the antigen recognition profiles for IgG in a random selection of serum samples from all strains on days 21 and 35 p.i. were examined by immunoprecipitation using ^{35}S–methionine labelled E/S antigen and 10–20% gradient gels.

Statistics

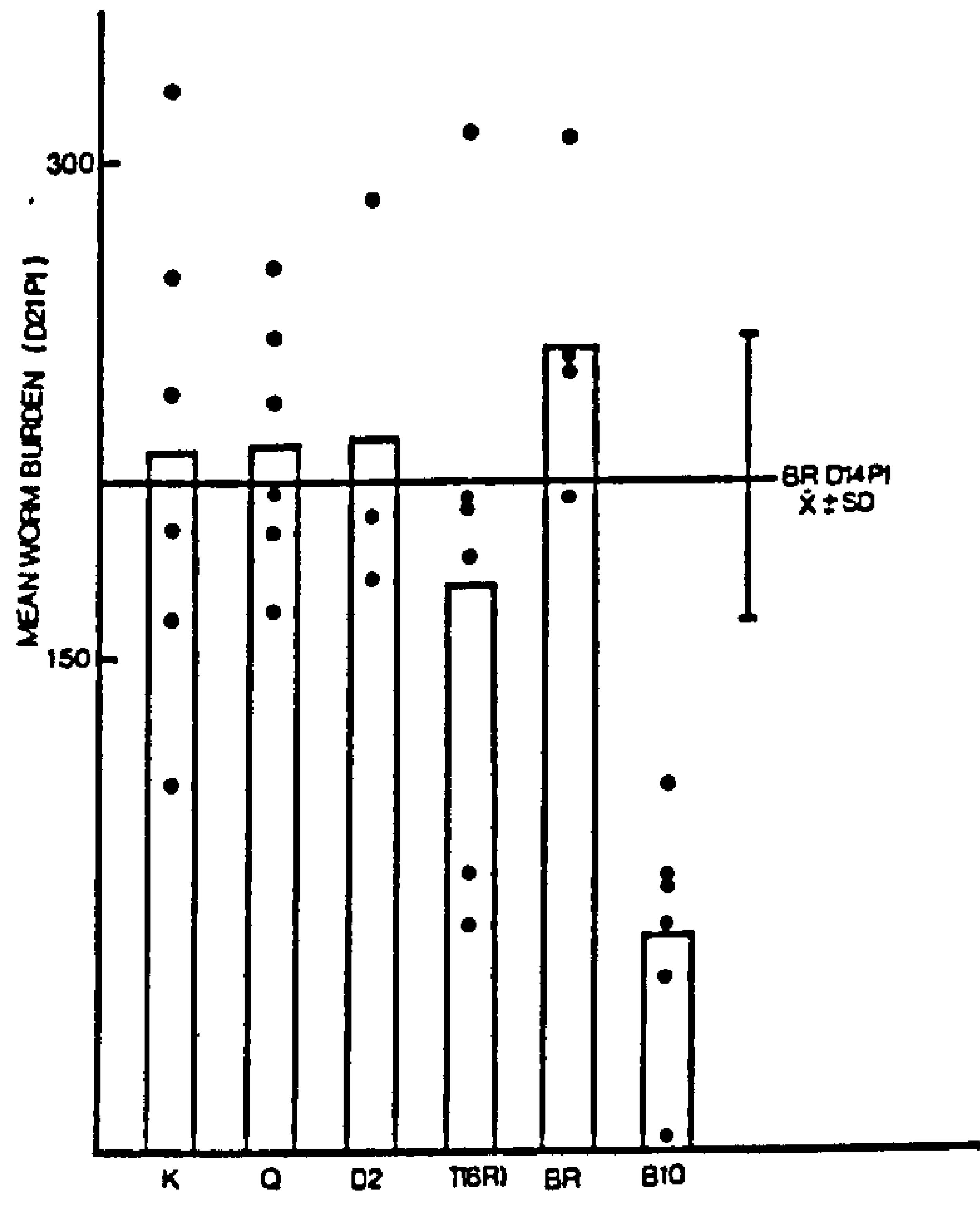
The significance of differences between worm burdens recovered from experimental groups was calculated using the Mann–Whitney U test. A value greater than P=0.05 was considered non–significant.

A5.3 RESULTS

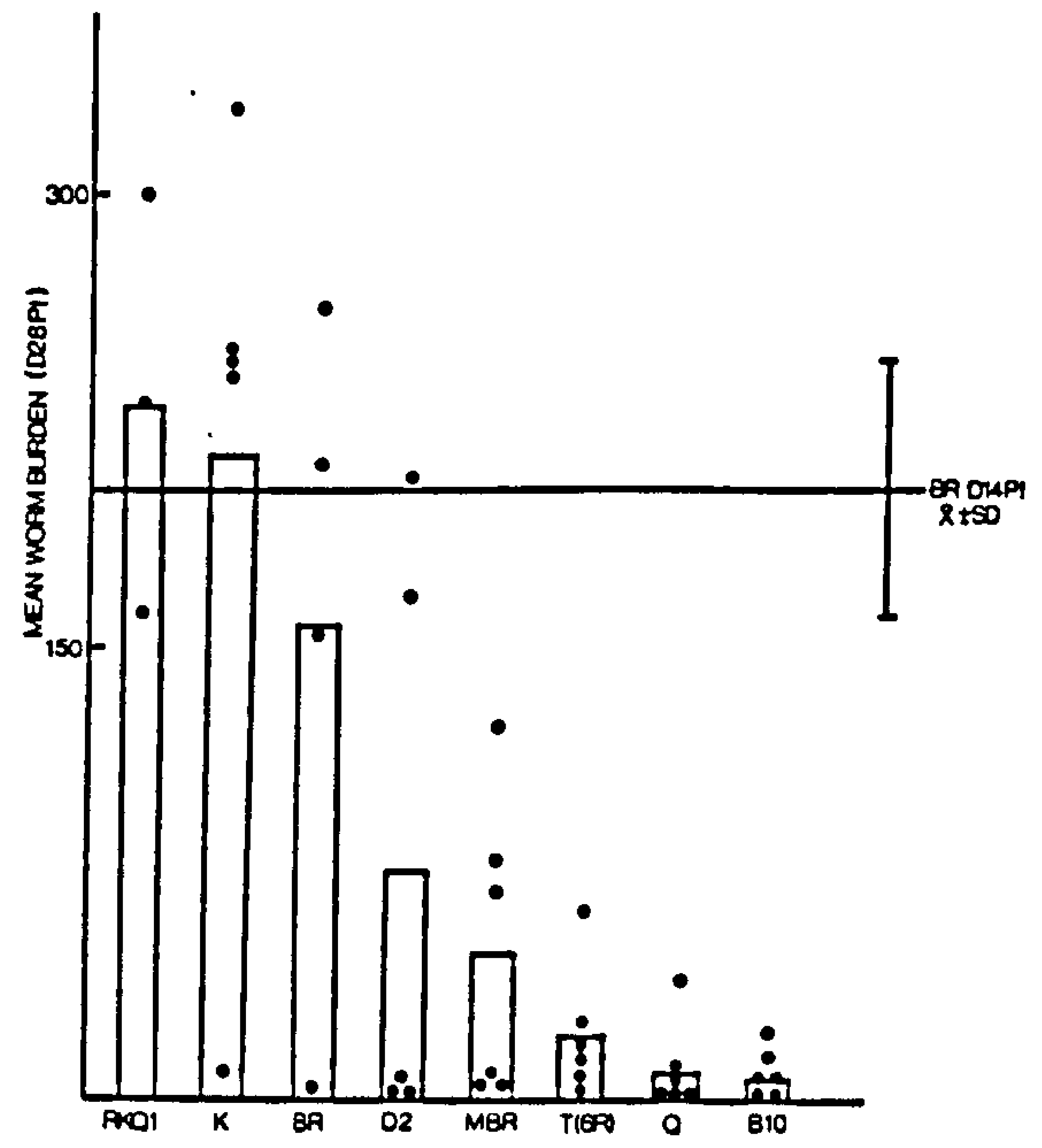
Establishment and expulsion of infections in H–2 recombinant and congenic strains of mice.

Figure A5.1 shows the worm burdens recovered from mice on days 21, 28 and 35 p.i. in relation to the day 14 p.i. mean for the B10.BR mouse strain. The presence of mature worms and/or stunted worms on day 35 p.i. is indicated by '+' and 's' respectively. The number of day 21 larvae recovered from all strains assessed except B10 mice did not differ significantly from B10.BR day 14 p.i. levels although two B10.T(6R) individuals showed reduced worm burdens. A very significant reduction from B10.BR day 14 levels was seen in B10 mice (P=0.001). The day 28 p.i. worm burdens recovered from B10.RKQ1, B10.K and B10.BR individuals were not significantly different from the establishing day 14 p.i. levels seen in B10.BR mice. However significant decreases were observed in B10.D2

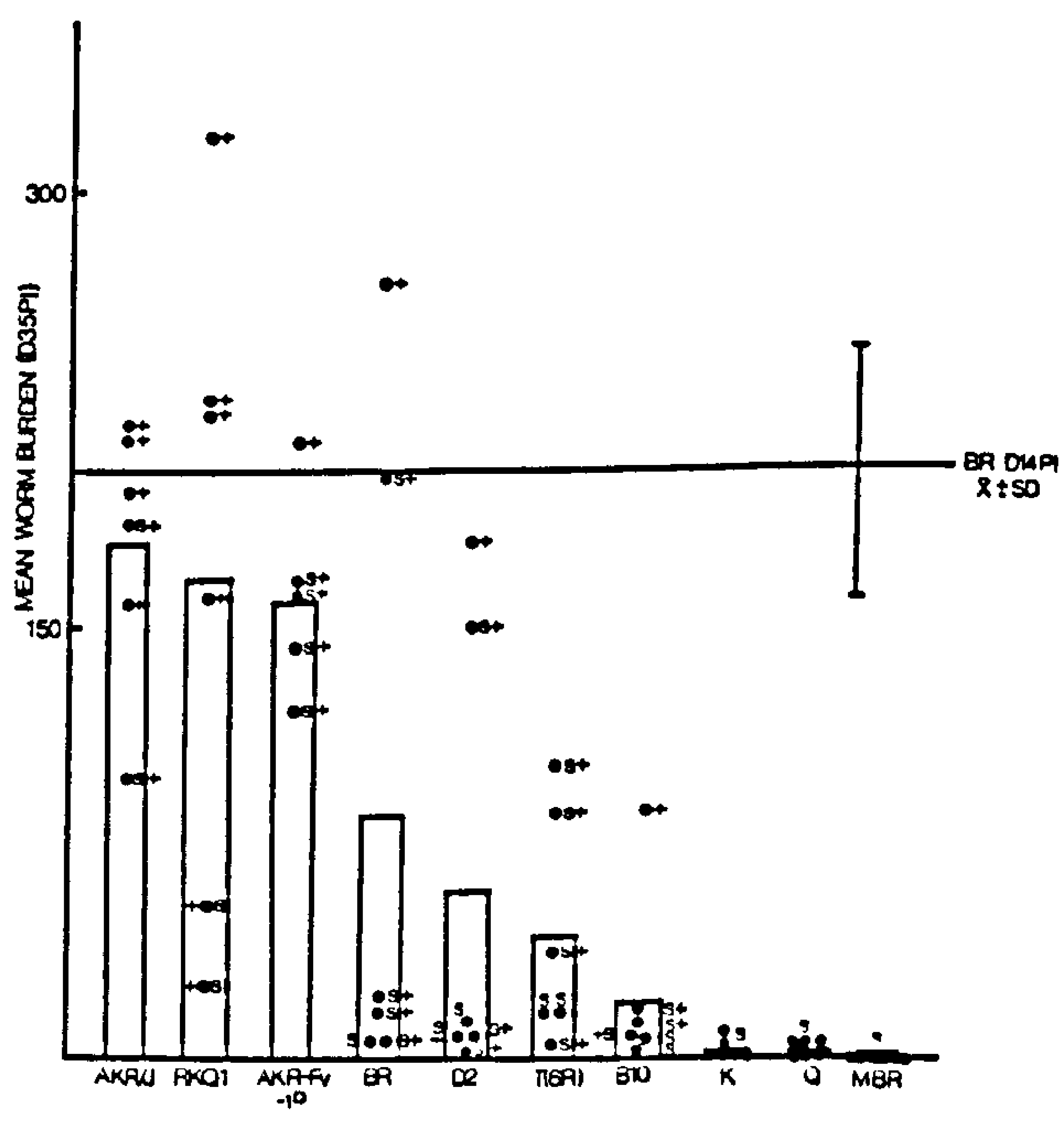
Figure A5.1 Worm burdens recovered from H-2 recombinant and congenic strains of mice on day 21 p.i., day 28 p.i. and day 35 p.i. (K, B10.K; Q, B10.Q; D2, B10.D2; T(6R), B10.T(6R); BR, B10.BR; B10, C57BL/10; RKQ1, B10.RKQ1; MBR, B10.MBR. Bar graphs represent the mean worm recovery with individual worm burdens indicated by dots. The horizontal line shows the mean number of larvae established in B10.BR mice on day 14 p.i. The presence of mature adult worms on day 35 p.i. is indicated by +; s indicates the presence of at least some stunted worms. B10.K, B10.Q and B10.MBR individuals all harboured either no worms or stunted worms on day 35 p.i. Day 21 p.i. worm burdens were not determined for B10.RKQ1 and B10.MBR. The number of worms harboured by AKR/J and AKR-Fv-1^b was determined on day 35 p.i. only.



MEAN WORM BURDEN (D21PI)
300
150
BR D14PI
X̄ ± SD
K
Q
D2
T16R)
BR
B10



MEAN WORM BURDEN (D28PI)
300
150
BR D14PI
X̄ ± SD
RKQ1
K
BR
D2
MBR
T(6R)
Q
B10



MEAN WORM BURDEN (D35PI)
300
150
BR D14PI
X̄ ± SD
AKR/J
RKQ1
AKR-Fv-1b
BR
D2
T16R)
B10
K
Q
MBR

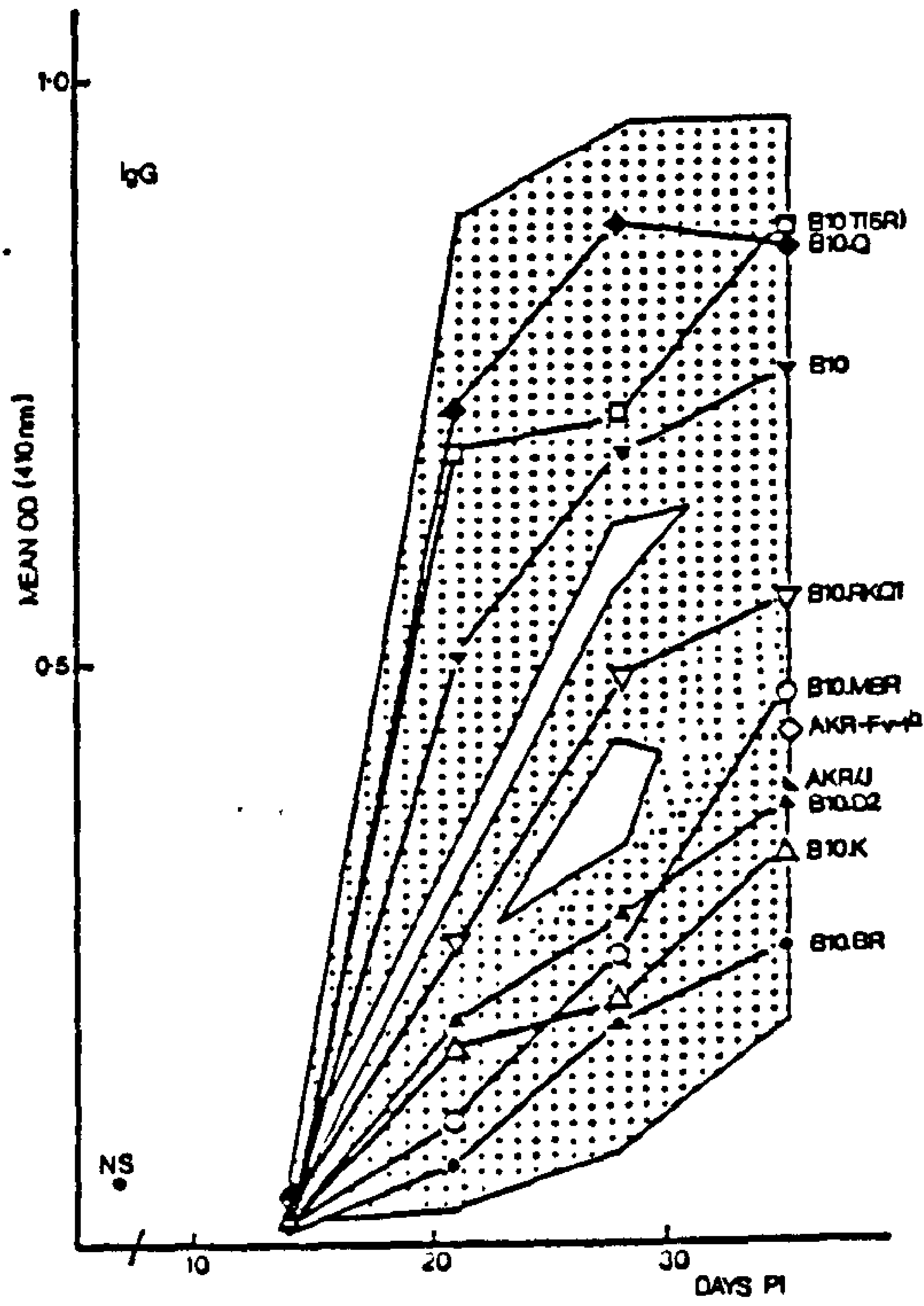
($P<0.05$), B10.MBR, B10.T(6R), B10.Q and B10 mice (all $P=0.001$) with individuals of the latter two strains having almost totally eliminated the infection by day 28 p.i. At day 35 p.i. all strains except B10.RKQ1 and AKR/J exhibited a significant reduction in worm burdens compared with day 14 p.i. B10.BR levels, although the decrease seen in AKR-Fv-1^b mice was only just significant and all individuals harboured some mature adult worms. AKR/J and AKR-Fv-1^b day 35 p.i. burdens were not significantly different ($P>0.05$). The reduction in day 35 p.i. worm burdens in B10.BR mice from the day 14 p.i. levels was significant at the 5% level, in B10.D2 at the 1% level and in all other strains at the 0.1% level. B10.T(6R) individuals had significantly higher numbers of adult worms on day 35 p.i. than B10.Q mice as did B10.RKQ1 mice compared to mice of the B10.MBR strain (both $P=0.001$). B10.MBR mice were significantly more resistant than B10.BR individuals on day 35 p.i. ($P=0.001$). B10.K individuals, most harbouring over 200 worms on day 28 p.i., had almost completely expelled the infection by day 35 p.i.

Antibody responses to E/S antigen

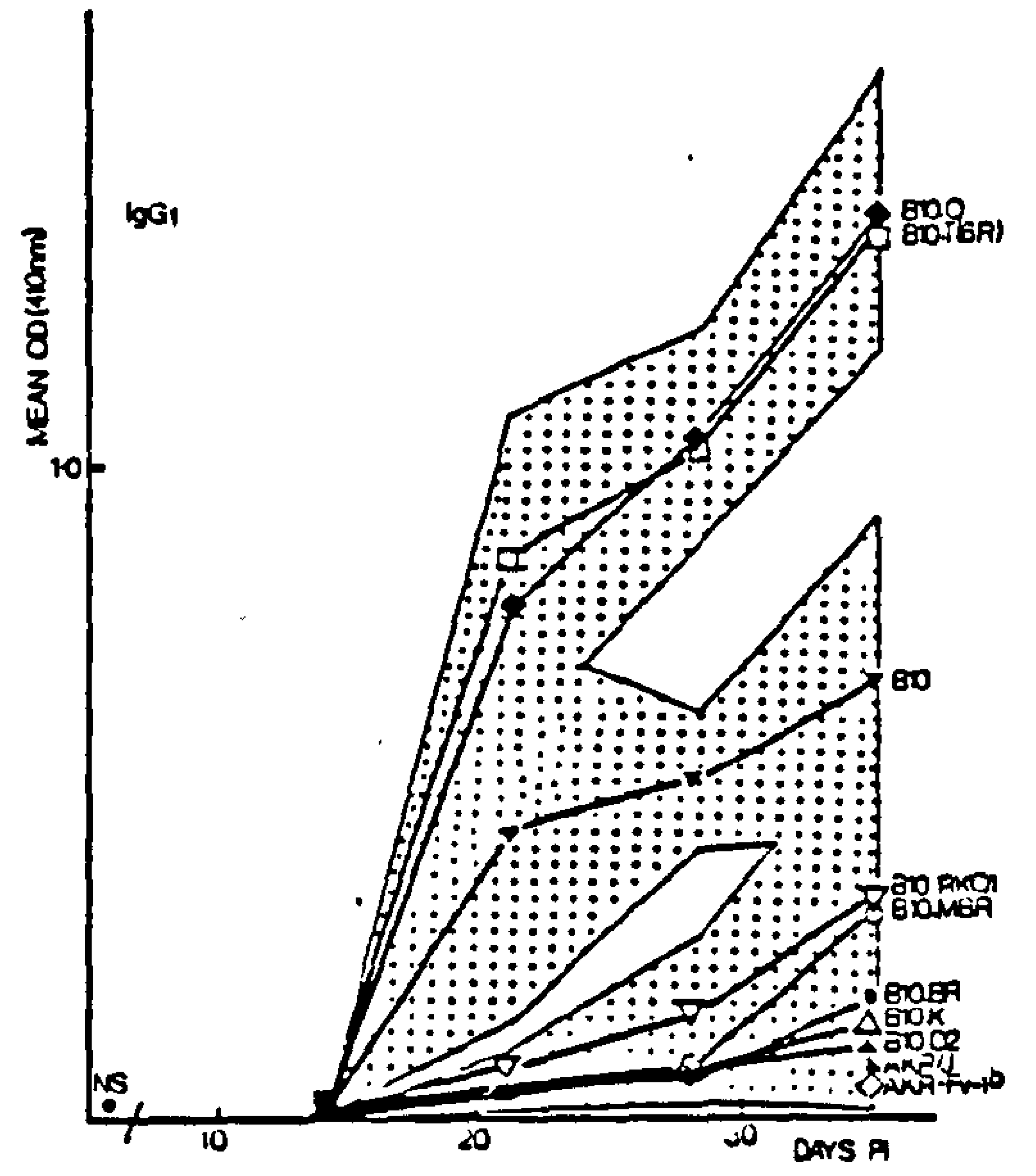
The specific IgG, IgG1 and IgG2a antibody responses to E/S antigen for the strains studied, from day 14 to day 35 p.i., are shown in figure A5.2.

B10.T(6R) and B10.Q mice developed a rapid high level IgG response which reached levels considerably higher than all other strains except individuals of the B10 strain where a good IgG response was also seen. The IgG1 response patterns revealed a dramatic rise in this isotype in B10.T(6R) and B10.Q individuals unequalled by any other strain. B10 mice possessed higher levels of specific IgG1 than all other strains except B10.T(6R) and B10.Q. High levels of IgG2a were reached in several strains other than

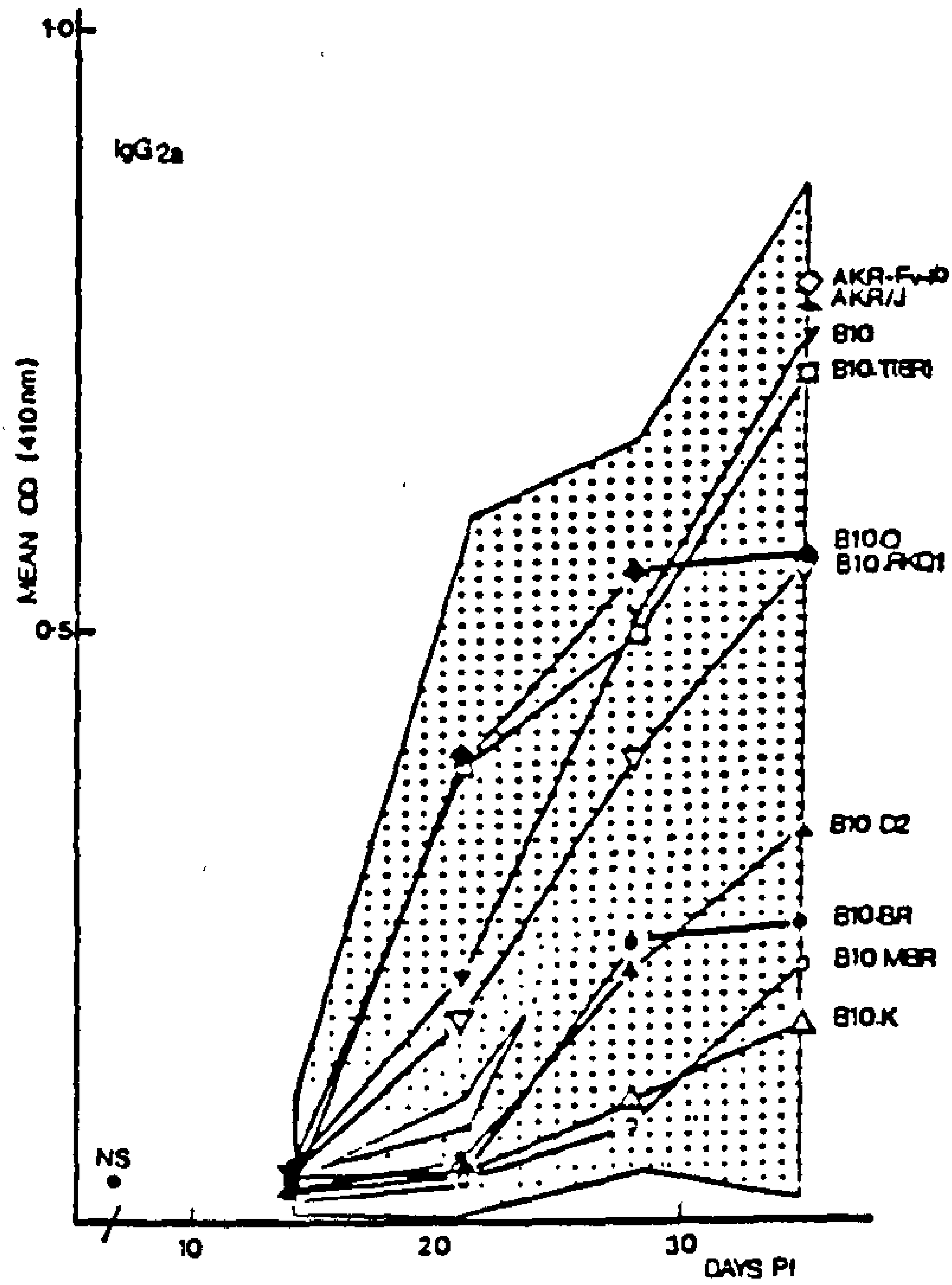
Figure A5.2 Time courses of the specific IgG, IgG1 and IgG2a antibody responses to *Trichuris muris* E/S antigen in serum samples taken from B10.K, B10.MBR, B10.BR, B10.D2, B10.RKQ1, B10.Q, B10.T(6R) and B10 mice. Antibody levels in AKR/J and AKR-Fv-1^b mice on day 35 p.i. are also shown. The shaded area represents the range of standard deviations from the mean optical densities calculated from at least five individual values for each strain at each time point p.i. except for B10.BR on days 21 and 28 p.i. where only four serum samples were taken. N.S., naive serum; O.D., optical density.



IgG
1·0
0·5
MEAN OD (410 nm)
NS
10
20
30
DAYS PI
B10.T(6R)
B10.Q
B10
B10.RKD1
B10.MBR
AKR-Fv-1b
AKR/J
B10.D2
B10.K
B10.BR



IgG1
1·0
MEAN OD (410 nm)
NS
10
20
30
DAYS PI
B10.Q
B10.T(6R)
B10
B10.RKD1
B10.MBR
B10.BR
B10.K
B10.D2
AKR/J
AKR-Fv-1b



IgG2a
1·0
0·5
MEAN OD (410 nm)
NS
10
20
30
DAYS PI
AKR-Fv-1b
AKR/J
B10
B10.T(6R)
B10.Q
B10.RKD1
B10.D2
B10.BR
B10.MBR
B10.K

B10.T(6R) and B10.Q by day 35 p.i. Interestingly mice of the AKR genetic background (AKR/J and AKR-Fv-1^b) showed a high level IgG2a response but had negligible IgG1. A similar phenomenon was also seen for B10.RKQ1 individuals. All three strains were poor or non-responders to infection.

Differential recognition of E/S antigen

Incubation of sera with metabolically labelled E/S antigens prior to SDS-PAGE was used to study the IgG antigen recognition profiles of the H-2 recombinant strains of mice (data not shown). All strains recognised a number of antigens in the 30-50kDa MW range with antigen recognition profiles being otherwise fairly restricted. The dominant feature revealed by the immunoprecipitation studies was the recognition by five individuals of both the B10.Q and B10.T(6R) strains of the two high MW antigens (90-95kDa and 105-110kDa) referred to in Chapter 5. However profiles for six B10 individuals revealed that, in addition to uniformally recognising a 20-23kDa antigen referred to in Chapter 4, 3/6 mice also recognised the two high MW antigens and of the remaining three mice, two possessed antibodies against the 105-110kDa antigen. 1/6 individuals of the B10.RKQ1 strain also showed weak recognition of the two high MW antigens.

A5.4 DISCUSSION

The data reported here both support and extend earlier observations on the effect of H-2 genes on the expulsion of *T. muris* from mice. In general, strains of mice used previously (Chapter 3) behaved as before

although the B10 strain expelled worms more rapidly, a considerable reduction in worm burdens being seen on day 21 p.i. whereas earlier work reported the first significant decrease to occur after day 21 p.i. (Else and Wakelin, 1988). B10.T(6R) individuals were slightly more resistant than indicated earlier, where 2 individuals failed to show any reduction in worm burdens (Chapter 3.2) and the B10.BR mouse strain also showed a greater resistance to infection than reported in Chapter 3. This is a phenomenon that has arisen in a number of experiments and is discussed in Chapter 6.2. Mice of the B10.K strain, like B10.BR, possess k alleles throughout the H-2. Both strains have identical genetic backgrounds and differ only in the origin of their k alleles, the donor strains being CBA/J and C57BR/cd respectively (Klein *et al.*, 1983). On day 28 p.i. neither strain showed a significant decrease in worm burden but by day 35 p.i. an almost 100% reduction in worm burdens was seen in the B10.K mice, this strain being significantly more resistant than B10.BR mice (P=0.001). It is difficult to explain this difference in responsiveness, although the fact that the B10.K individuals killed on day 35 p.i. were about 2 weeks older than the B10.BR mice may perhaps be important. The massive expulsion seen in the B10.K strain between day 28 p.i. and day 35 p.i. is also hard to explain in the context of the work presented in Chapter 6, (Else *et al.*, 1989), where it is suggested that if expulsion is not initiated by around day 21 p.i. worms cannot be expelled later due to the production of parasite-derived immunomodulatory factors. However it is possible to envisage the existence of strain variation in susceptibility to immunomodulation and thus the time point beyond which no expulsion can occur due to suppression of host immunity also becomes genetically determined.

The identical behaviour of AKR/J and AKR-Fv-1^b, two strains which differ only in I-J expression (AKR-Fv-1^b being I-J^k positive, Hayes *et al.*, 1984), suggests that I-J^k expression is not important in the induction of

non-responsiveness to *T. muris* infection. A similar situation was reported in Chapter 3.2 where B10.A(3R) and B10.A(5R) behaved identically despite differing in I-J expression. The functional significance of I-J molecules expressed on subpopulations of T cells interacting within the suppressor circuit (Möller, 1985), is unclear. However a correlation between $\underline{I\text{-}E}^k$ expression, $\underline{I\text{-}J}^k$ expression and susceptibility to *Trichinella spiralis* infection (Wassom *et al.*, 1987) has been demonstrated giving rise to speculation that susceptible $\underline{I\text{-}E}^k$ positive mice are actively immunosuppressed during infection by a mechanism involving the induction of $\underline{I\text{-}J}^k$ positive suppressor cells.

The significantly greater susceptibility of B10.RKQ1 compared to B10.MBR, evident on days 28 and 35 p.i., was particularly interesting in the light of their H-2 compositions (Table A5.1). Both strains possess k alleles at I-A and q alleles at D. They differ at K, at I-E, B10.RKQ1 being unable to express I-E molecules, and perhaps also at the S locus (B10.RKQ1 being undefined). If it is assumed tht the K locus is not important in the determination of resistance (it is difficult to envisage how class I molecules might be involved as discussed in Chapter 3.2.3) and that I-E molecules play a minor role (as indicated in Chapter 3.2) then one has to postulate that the two strains differ at some important locus (or loci) lying between S and D, the possession of q alleles at this D end locus/loci in B10.MBR significantly up-regulating the effects of k alleles at I-A. Likewise the disparate response phenotypes of B10.T(6R) and B10.Q suggest that the presence of d alleles at the hypothetical D end locus/loci can greatly down-regulate the effects of q alleles at I-A (see Chapter 3.2). Interestingly the genes for tumour necrosis factor (TNF-α) and lymphotoxin (LT; TNF-β) map close to the H-2 D in the mouse MHC (Müller *et al.*, 1987). TNF-α and TNF-β, produced by macrophages and T lymphocytes respectively, have a variety of effects on cells of the immune system including activation of neutrophils, macrophages and T lymphocytes (see

Beutler and Cerami, 1986; Playfair, 1988). Thus their genes are perhaps candidates for both the Ts-2 gene, important in the *Trichinella* system and mapped to the right of S in the S-D interval of the H-2 (Wassom *et al.*, 1983*b*) and the hypothetical gene(s) involved in the *T. muris* system. Indeed such hypotheses have been put forward (Wassom and Kelly, 1989) and are being tested (D.L. Wassom, *pers. comm.*; R.K. Grencis, *pers. comm.*).

B10.T(6R) and B10.Q developed early high levels of specific IgG and IgG1 antibodies as seen before for $\underline{H\text{-}2}^{q}$ strains (Chapter 5). However individuals of the B10 strain were also good IgG producers, possessing levels of IgG similar to the two $\underline{H\text{-}2}^{q}$ haplotype mouse strains. This high level IgG response was not reflected in the IgG1 response of B10 mice, B10.Q and B10.T(6R) having dramatically more specific IgG1 than any other strain, thus supporting the hypothesis of an $\underline{H\text{-}2}^{q}$ restriction on the ability to rapidly develop high levels of specific IgG1 (Chapter 5). No such restriction was apparent when the IgG2a antibody response to E/S antigen was analysed. Although B10.T(6R) and B10.Q were both good IgG2a producers so were other non-$\underline{H\text{-}2}^{q}$ strains including B10, B10.RKQ1, AKR-Fv-1^{b} and AKR/J. The prediction made in Chapter 5 that there would be a reciprocal relationship between IgG1 and IgG2a antibody levels because of the regulatory mechanisms involving Il-4- and IFN-γ-producing T_H2 and T_H1 cell subsets is partly supported by the ELISA data presented here. For instance AKR/J and AKR-Fv-1^{b} possessed negligible specific IgG1 but exhibited high levels of IgG2a on day 35 p.i. However both B10.T(6R) and B10.Q produced high levels of both isotypes indicating that both T_H1 and T_H2 cell subsets had been activated in these strains and that the inhibitory effects of Il-4 on IgG2a secretion and IFN-γ on IgG1 secretion by LPS-stimulated B cells observed *in vitro* (Snapper *et al.*, 1988) are not absolute. Interestingly, in general, slow/non-responder strains of mice (expressing $\underline{k}$ alleles at I-A and I-E), including the three strains which

harboured the highest mean worm burdens on day 35 p.i. (AKR/J, AKR-Fv-1^b, B10.RKQ1) produced very little IgG1 compared to the more resistant ($\underline{H-2}^q$, $\underline{H-2}^b$) mouse strains. In contrast AKR/J, AKR-Fv-1^b and B10.RKQ1 developed relatively high levels of IgG2a, as did the $\underline{H-2}^q$ and $\underline{H-2}^b$ strains of mice. Zakroff *et al.*, (1989) reported that four different helminth infections all induced increases in serum IgG1 (and IgE), and usually a decrease in IgG2a. However alterations in the presentation of parasite antigens induced higher IgG2a than IgG1 serum levels suggesting that both T_H1 and T_H2 cells had receptors for parasite antigens but that the anatomical location of the parasite infections favoured the induction of Il-4 secreting T helper cells. Rather than preferentially activating one or the other T helper cell subset via the type of antigen presenting cell available at the site of antigen introduction it is equally conceivable that presentation of antigen in combination with a particular allelic form of a class II molecule favours the induction of T_H1 or T_H2. A more efficient induction of the T_H2 pathway by E/S antigen in combination with $\underline{I-A}^q$ was suggested in Chapter 5. This hypothesis can now be extended in the light of the IgG2a data presented here. In the *T. muris*-mouse system E/S antigen presented in combination with $\underline{I-A}^k$ and/or $\underline{I-E}^k$ may activate T_H1 cells leading to IgG2a production by B cells whilst presentation of antigen in the context of $\underline{I-A}^q$ may stimulate both types of T helper cell, though perhaps favouring the expansion of T_H2 cells. Exceptions inevitably arise. For instance the B10.BR strain ($\underline{H-2}^k$) produces little IgG2a (or IgG1) and levels of IgG2a in B10.MBR ($\underline{I-A}^k$, $\underline{I-E}^k$) are also low.

The non-responder status of the three high IgG2a producing strains raises the question of the relevance of this isotype in resistance. It may be that the development of high levels of an isotype irrelevant to protective immunity (here IgG2a), through the activation of the "wrong" subset of helper T cell and in the absence of a relevant isotype (IgG1), contributes

to the poor/non-responder phenotype observed. IgG2 antibodies are the major complement fixing antibodies in the mouse (Klaus *et al.*, 1979) and IgG2a is also a potent mediator of antibody dependent cell-mediated cytotoxicity (ADCC) by macrophages (Johnson *et al.*, 1985). Complement fixation, opsonization, and macrophage-mediated ADCC are thought to be primarily involved in host defense mechanisms against, for instance, viruses (Coffman *et al.*, 1988) rather than parasitic nematodes where IgG1 mediated ADCC by eosinophils (Ramalho-Pinto *et al.*, 1979) and immune responses involving IgE are believed to play a critical role in many cases (reviewed by Rothwell, 1989). Exceptions to the correlations between a good IgG1 response and resistance to infection (e.g. B10.MBR, a resistant strain which produces very little IgG1 or IgG2a) and between high IgG2a production and poor-responsiveness (e.g. B10.BR, only weakly responsive to infection yet producing little IgG2a or IgG1) serve to underline the complexity of the system, there being no one overriding mechanism in the development of resistance to infection.

The work presented in Chapter 5 suggested that the recognition of the two high MW antigens (90-95kDa, 105-110kDa) was an exclusively $\underline{H-2}^{q}$ restricted phenomenon. Experiments performed to control for the possibility that the apparent restriction was really a reflection of the associated $\underline{H-2}^{q}$ restriction on the production of high levels of specific IgG (IgG1) antibody suggested that this was not the case. Analysis by immunoprecipitation of sera from the present experiment revealed that, as before, individuals expressing q alleles at I-A recognised the two high MW antigens. However, in addition, a number of individuals of the B10 mouse strain and one B10.RKQ1 individual possessed antibodies against one or both of these antigens, immediately suggesting that the unusually high specific IgG response seen for B10 mice in this experiment was being reflected in the visualisation of the high MW antigens on the autoradiographs. Although

levels of antibody to E/S antigen as seen by ELISA may not necessarily reflect levels of antibody to the two high MW antigens they may give some indication as to the importance of antibody titre in the $\underline{H-2}^q$ restricted phenomenon. The B10.RKQ1 individual which recognised the 90-95kDa and 105-110kDa antigens did have the highest O.D. in ELISA on day 35 p.i. However the B10 individual which failed to recognise either antigen did not have the lowest of the B10 O.D. values and day 21 p.i. sera from B10.Q and B10.T(6R) mice contained adequate levels of specific antibodies to reveal recognition of the two high MW antigens even though O.D.s in ELISA were lower than the day 35 p.i. values for all B10, and all but one B10.RKQ1 individuals. Clearly any correlation between antibody levels to E/S antigen and recognition of the two high MW antigens is not absolute. These results do imply however that some component of the apparent $\underline{H-2}^q$ restriction is titre-based, at least for certain mouse strains. The IgG antibodies produced by B10 mice to the two high MW antigens may well be directed against different epitopes from those recognised by mouse strains of the $\underline{H-2}^q$ haplotype. Thus the $\underline{H-2}^q$ restriction reported in Chapter 5 would still be valid at the epitopic level, and, if antibody titre is important, would probably operate *in vivo* due to the almost exclusive $\underline{H-2}^q$ restriction on the ability to produce high levels of specific IgG early on post-infection.

A5.5 SUMMARY

1. Results from the expulsion experiments presented in Chapter 3 and in this appendix suggest that certain alleles present at a locus/loci mapping to the D-end of the H-2 complex are able to strongly modulate the effects of alleles expressed at I-A in the determination of response phenotypes. In particular:

a) the expression of q alleles in this region can dramatically up-regulate the effects of k alleles at I-A (e.g. B10.MBR)

b) the presence of b alleles in this region cannot up-regulate the effects of $I\text{-}A^k$ (e.g. B10.A(4R))

c) d alleles are capable of down-regulating the effects of q alleles at I-A (e.g. B10.T(6R))

d) d alleles can also down-regulate the effects of expressing b alleles at I-A (e.g. B10.A(3R), B10.A(5R)).

2. The $H\text{-}2^q$ restriction on the early development of high levels of IgG1 to E/S antigen was again apparent in this study. No such restriction was demonstrated for specific IgG2a and was not reflected as clearly as seen previously in the total specific IgG response patterns.

3. High levels of specific IgG2a, in the absence of a good IgG1 response to E/S antigen, may contribute to the poor/non-responder status of certain strains and arise through the preferential activation of T helper cells secreting IFN-γ as opposed to Il-4. Such preferential activation may, in some cases, be related to the allelic form of the class II MHC molecules with which parasite antigens are presented.

4. Sera from five individual B10.Q and B10.T(6R) mice (both strains possessing q alleles at I-A) contained antibodies against two high MW antigens, the recognition of which was felt to be H-2^{q} restricted and unrelated to antibody titre (Chapter 5). However a proportion of individuals of the B10 mouse strain also recognised these two antigens, probably reflecting their unusually high specific IgG response. This suggests that at least some component of the H-2^{q} restriction is in fact quantitative, although the restriction is probably still valid at the epitopic level and would operate *in vivo* due to the almost exclusive H-2^{q} restriction on the ability to produce high levels of specific IgG early on during the course of infection.

SECTION TWO

MODULATION OF HOST IMMUNITY BY LATER LARVAL AND ADULT STAGES OF *TRICHURIS MURIS*

CHAPTER 6

STUDIES ON THE IMMUNOLOGICAL PREDISPOSITION TO TRICHURIASIS IN MICE

Section 6.1 has been published in Parasitology (1989), 98, 275-282.

The results presented in section 6.1.4 for the DBA/2 strain of mouse were taken from Roach (1986).

6.1 HOST PREDISPOSITION TO *TRICHURIASIS*: THE MOUSE-*T.MURIS* MODEL

6.1.1 SUMMARY

Predisposition to trichuriasis in mice is reflected in the inability of certain strains, or certain individuals within strains, to express protective immunity. Poor responders fail to expel worms and harbour chronic patent infections. The mechanisms underlying this phenomenon were studied in poor responder mice challenged after abbreviated or prolonged primary infections. Mice exposed to a complete primary infection were fully susceptible when challenged after the removal of the primary infection by anthelmintic. Failure to expel either infection suggests (a) that non-responsiveness to a primary infection does not reflect an inability to expel worms of a certain size, i.e. is not a consequence of the speed of the immune response in relation to parasite growth and (b) that non-responsiveness is long-lasting. Challenge after abbreviation of primary infections at different stages of worm development showed that persistence of larvae beyond day 21 was critical in determining poor response to infection. By inference the same conclusion can be drawn about the inability of such mice to expel primary infections. Serological analysis suggested a relationship between low antibody levels, restricted antigen recognition profiles and resistance to infection. It is suggested that the later stages of parasite development are immunosuppressive; the implications for human trichuriasis are discussed.

6.1.2 INTRODUCTION

The nematode *Trichuris trichiura* is one of the most ubiquitous of gastro-intestinal helminths of man (Peters, 1978; Warren and Mahmoud, 1984), chronic trichuriasis being associated with considerable pathology (Bundy, 1986). The distribution of worms in the human population is typically overdispersed (Bundy *et al.*, 1985*b*), individuals apparently being predisposed to high or low intensity of infection and to a correspondingly high or low rate of acquisition of infection (Bundy, 1986). Such predisposition may have a nutritional, behavioural and/or immunological basis but the relative contributions of each component are hard to determine.

Predisposition to trichuriasis also occurs in mice infected with *Trichuris muris*. Under experimental conditions, in which nutritional and behavioural factors are controlled, the importance of genetically determined variation in immune responsiveness can easily be demonstrated (Else and Wakelin, 1988). In the majority of mouse strains expulsion of a primary infection is complete by the fourth week of the infection and the mice are thereafter resistant to challenge. Some strains, e.g. DBA/2, show an intermediate condition in which a proportion of mice fail to expel the worm of a primary infection (Worley *et al.*, 1962; Wakelin, 1975*b*) , and other strains are completely unresponsive (Else and Wakelin, 1988). Both major histocompatibility complex (MHC-)-linked and background genes contribute to this variation, but the strongest influences upon protective immunity are associated with the latter. Thus, all mice of the B10 genetic background show weak protective immunity, assessed by the time of worm expulsion, but distinct effects of different MHC (H-2) haplotypes are evident, certain haplotypes being associated with slower expulsion times than others. As the time taken to initiate expulsion increases, there is a corresponding increase in the proportion of individual mice within a strain that fail to respond at

all; in B10.BR ($\underline{H-2}^k$) no expulsion occurs and all mice develop patent infections. Indeed burdens in excess of 200 adult worms have been recovered on day 84 after infection with 400 eggs (Else and Wakelin, unpublished observations) suggesting that mice of this strain are unable to mount a protective response to *T. muris* at any point during the natural life-span of the parasite (12-14 weeks). The phenomenon of differential responsiveness between and within congenic strains of the B10 background implicates H-2-linked genes but does not explain what determines whether an individual can or cannot expel a primary infection. It is clear, however, that the phenomenon must arise from some aspect of the individual host-parasite interaction and may therefore be parasite-induced or reflect some characteristic property of *T. muris* itself. For example it has been suggested (Else and Wakelin, 1988) that when worms reach a certain stage of development they become capable of suppressing host immunity, or alternatively, that at a certain size they become insusceptible to host immunity. Resolution of this point is important because it may help to explain the generation of the conditions which lead to the chronic infection and persistent susceptibility to reinfection characteristic of trichuriasis in predisposed humans.

6.1.3 MATERIALS AND METHODS

Animals

Six- to eight-week-old inbred male C57BL/10ScSn/Ola (B10), B10.BR/Ola, DBA/2/Ola and BALB/c/Ola mice, obtained from Harlan Olac Ltd. were used.

Parasite

The maintenance of *T. muris* and the methods used for infection and examination of the experimental animals were as described by Wakelin (1967).

Anthelmintic

The anthelmintic methyridine (Promintic, ICI) was injected subcutaneously (s.c.) at a dose level of 500mg/kg body weight. This drug is known to result in the expulsion of all worms within a few hours of administration (Wakelin, 1970*a*).

Cortisone treatment

Mice were injected s.c. with 1.25mg hydrocortisone 21-acetate (Sigma) on days 7, 9, 11, 13 and 15 post-infection. This regime prevents worm expulsion and allows the long-term survival of adult worms (Wakelin, 1967).

Serum

Groups of at least 7 mice were infected with 400 *T. muris* eggs on day 0 and subsequently bled from the tail vein weekly during the course of chemically abbreviated primary and challenge infections. The sera obtained from the blood samples were pooled for each experimental group and stored at -20°C.

Antigen

Excretory/secretory (E/S) antigen. Adult male and female *T. muris* were removed individually from the large intestines of mice and placed in sterile RPMI 1640 medium supplemented with 500μg/ml penicillin/ streptomycin and fungizone (Gibco) at 2.5μg/ml. After washing for 2-3 hours in this medium at 37°C, worms were put into fresh medium and left O/N at 37°C for the collection of E/S products. The culture S/N was removed, centrifuged at 200g for 5 min to remove eggs and filtered (0.22μm filter, Millipore). After freeze drying and reconstituting to one quarter the original volume with distilled water, samples were dialysed at 4°C before being analysed for protein, aliquoted and stored at -20°C.

Metabolically labelled E/S antigen was prepared by incubating worms as above in the presence of ^{35}S-methionine at 250μCi/ml medium.

Protein estimation

Total protein concentrations in samples were determined using a method modified from Lowry *et al.*, (1951).

Enzyme-linked immunosorbent assay (ELISA)

96-well flat-bottomed plates (Falcon) were coated with E/S antigen (5μg/ml, 50μl/well) in 0.05M carbonate/bicarbonate buffer, pH9.6, and left O/N at 4°C. These antigen coated plates were used in ELISA essentially as described by Voller, Bidwell and Bartlett (1979). Test serum was used at a dilution of 10^{-2} in PBS containing 0.05% Tween 20 (Sigma). The conjugate used was alkaline phosphatase-conjugated goat anti-mouse IgGAM (Sigma) diluted 1 in 350 in PBS/Tween, and the subtrate, *p*-nitro-phenylphosphate

tablets (Sigma) dissolved in diethanolamine buffer, pH 9.8 (Fisons).

Immunoprecipitation

Metabolically labelled E/S antigen was added to a series of eppendorf tubes to give 100,000 cpm/tube. A volume of 25µl of test serum was added and made up to 0.5ml with immunoprecipitation buffer, pH 7.4. The solutions were vortexed and left O/N at 4°C. The following day 15µl of sheep anti-mouse IgG1 (Serotec) was added to each sample and left for 3 hours at RT. Immunoprecipitates were spun down at 11,500g and washed 4 time in immunoprecipitation buffer before redissolving in reducing sample buffer for SDS-PAGE (10-20% gradient gels). Gels were fixed, fluorographed and dried down for exposure on X-ray film (Fuji) at -80°C.

Statistical analysis

The significance of differences between the mean worm recoveries from experimental groups was calculated using the Student's *t* test. A value greater than P=0.05 was considered non-significant.

6.1.4 RESULTS

The fate of challenge infections in B10.BR, DBA/2 and BALB/c mice

Two experiments were carried out to investigate the fate of challenge infections in (a) non-responsive individuals of the DBA/2 strain and (b) in the non-responder B10.BR strain after a primary infection had been removed using an anthelmintic. In the first experiment the response of these animals was compared with that of responder DBA/2 mice and, in the

second, with that of BALB/c mice, a strain in which expulsion of a primary infection is completed in all individuals by day 26 p.i.

Mice were infected with 400 *T. muris* eggs on day 0. Individuals which failed to expel the parasite before patency was reached (non-responders) were identified by the presence of parasite eggs in the faeces on day 41 p.i. Primary infections were removed from non-responders using Promintic and the faeces re-examined the following day to confirm anthelmintic efficacy. All mice were challenged at least 7 days after anthelmintic treatment, along with the appropriate challenge control, killed 12 days post-challenge for B10.BR and BALB/c mice and 14 days post-challenge for DBA/2 mice, and their larvae recovered. The results are shown in fig. 6.1.1 (DBA/2 mice) and fig. 6.1.2 (B10.BR and BALB/c mice).

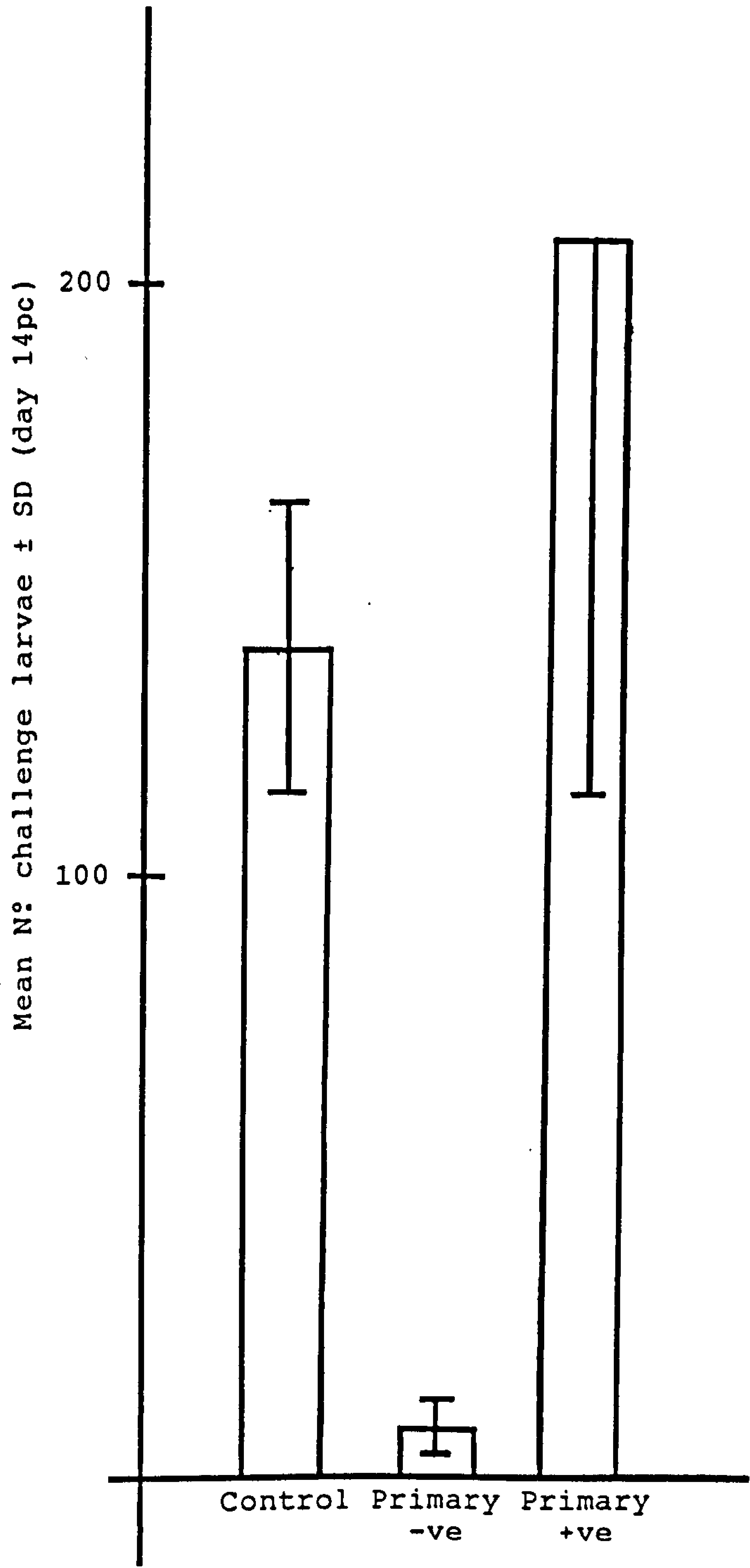
On the basis of the faecal egg counts on day 41 the DBA/2 mice were divided into individuals which had expelled the primary infection (13/28) and those that had not (15/28). The mice which had failed to respond to the primary infection were also unable to expel the developing larvae of the secondary infection, whilst responder individuals were all resistant to challenge.

All the B10.BR mice (9/9) were egg positive on day 41 p.i. and after anthelmintic treatment and challenge, developed larval burdens equivalent to those in the challenge controls. All the BALB/c mice (8/8) were egg negative on day 41 p.i. and were completely resistant to challenge.

Factors influencing susceptibility to challenge infection

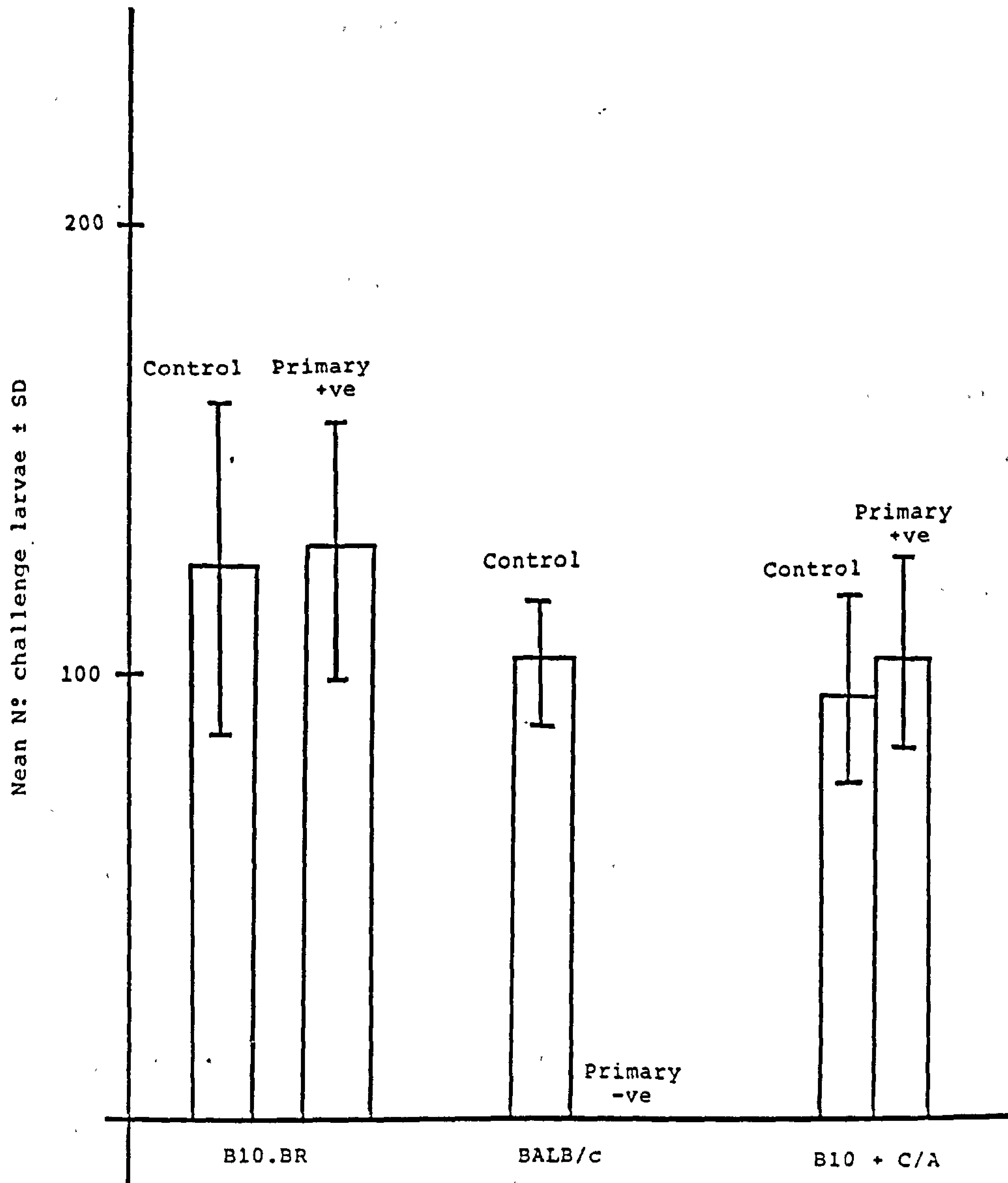
The above experiments show that the failure of certain mice (DBA/2 non-responders and B10.BR) to respond protectively to *T. muris* is expressed in both primary and challenge infections, thus eliminating parasite size, and insusceptibility to immunity, as a factor. Three experiments were

Figure 6.1.1 The fate of challenge infections (400 eggs) in responder (-) and non-responder (+) DBA/2 mice. All mice were given a primary infection of 400 eggs, challenged on day 55 p.i. and killed 14 days later. Adult worms were removed from non-responder individuals by chemotherapy 7 days before challenge. Non-responder mice were identified by the presence of eggs in the faeces after the parasite had reached patency (approximately 35 days p.i.)



Mean N° challenge larvae ± SD (day 14pc)
200
100
Control
Primary
-ve
Primary
+ve

Figure 6.1.2 The fate of challenge infections (400 eggs) in responder (BALB/c), non-responder (B10.BR) and cortisone-treated B10 mice (B10+C/A). BALB/c and B10.BR mice were given a primary infection of 400 eggs, challenged on day 54 p.i. and killed 12 days later. B10.BR do not expel a primary infection and the adult worms were removed by chemotherapy 12 days before challenge. BALB/c mice expel the primary infection within 26 days. Survival of the primary infection (400 eggs) in B10 mice was extended by treatment with cortisone given on days 7, 9, 11, 13 and 15 p.i.; the adult worms were removed by chemotherapy 7 days before challenge on day 48 p.i. Mice were killed 13 days post-challenge.



Nean Nº challenge larvae ± SD
200
100
Control
Primary
+ve
Control
Primary
-ve
Control
Primary
+ve
B10.BR
BALB/c
B10 + C/A

then carried out to investigate whether such non-responsiveness might therefore be a consequence of parasite-induced changes in host competence. The design of these experiments was based upon the knowledge that strains in which parasites are expelled before day 26 p.i. express strong immunity to challenge. Two experiments were carried out with C57BL/10 (B10) mice in which primary expulsion is complete in the majority (80%) of individuals by day 26 and the third was carried out in B10.BR in which no individuals expel worms. In B10 mice the aim of the experiments was to assess immunity to challenge after prolongation of a primary infection to extend host-parasite contact; in B10.BR mice the aim was to assess immunity to challenge after curtailment of the primary infection, to reduce the period of host-parasite contact. Curtailment of infection is easily achieved by chemotherapy; two approaches were taken to achieve prolonged infection in B10 mice, namely corticosteroid treatment (Exp. 1) and subthreshold infection (Exp. 2).

Experiment 1. Nine male B10 mice were infected with 400 *T. muris* eggs on day 0 and injected with cortisone on days 7, 9, 11, 13 and 15 p.i. The faeces of all mice contained parasite eggs on day 39 p.i. Promintic was administered on day 40 p.i. and 3 mice were killed the following day to confirm that all the worms had been removed. A challenge infection of 400 eggs was given 7 days later, mice killed 13 days post-challenge and their worms recovered. The results are as shown in fig. 6.1.2. As can be seen, challenge larval burdens recovered from mice which had had patent primary infections were equal to those of the challenge controls.

Experiment 2. Primary infections of approximately 10 worms were established in 4 groups, each of at least 6 male B10 mice. This infection was terminated using Promintic on various days p.i. as shown in Table

6.1.1, to expose the mice to different stages of parasite development. One mouse/group was killed 1 day after treatment to confirm anthelmintic efficacy. The remaining mice were challenged together with controls on day 56 p.i., killed 14 days later and the number of challenge larvae counted. The results are shown in fig. 6.1.3.

Table 6.1.1 Protocol for the abbreviation of low level infections in B10 mice, showing the number of larval stages experienced by each group.

Group	Duration of immunising infection (days)	Larval stages experienced
1	8	L1
2	15	L1, L2
3	21	L1, L2, L3
4	54	L1, L2, L3, L4, adult
5	naive-challenge control	-

Group 1 (primary infection terminated on day 8 p.i.) showed a 33% reduction in challenge larval burdens from control values ($P<0.05$) and group 2 (terminated on day 15 p.i.) a 45% reduction ($P<0.05$). However, mice in group 3 (terminated on day 21 p.i.) showed a division into responder and non-responder individuals as reflected by the fate of the secondary infection, the responder individual being over 99% immune to challenge whilst the non-responder challenge infection levels were not significantly different from control values ($P>0.05$). As expected 4/5 mice in group 4 (terminated on day 54 p.i.) harboured patent infections on day 53 p.i., as assessed by the presence of eggs in the faeces, and these individuals had challenge worm burdens similar to control values. The single individual in this group which eliminated the primary infection showed over 98% immunity to challenge.

Experiment 3. The previous experiment in B10 mice suggests that either the level of antigenic stimulation or exposure to stage-specific antigens,

Figure 6.1.3 The fate of challenge infections in B10 mice which had experienced low level primary infections (10 worms) terminated at different stages post-infection, as shown in parentheses for each group. In group 4, mice were divided into those which were positive for eggs on day 53 p.i. (indicated by +) and an individual which was egg negative (-), i.e. that had expelled the primary infection. All mice were challenged with 400 eggs on day 56 p.i. and killed after 14 days.

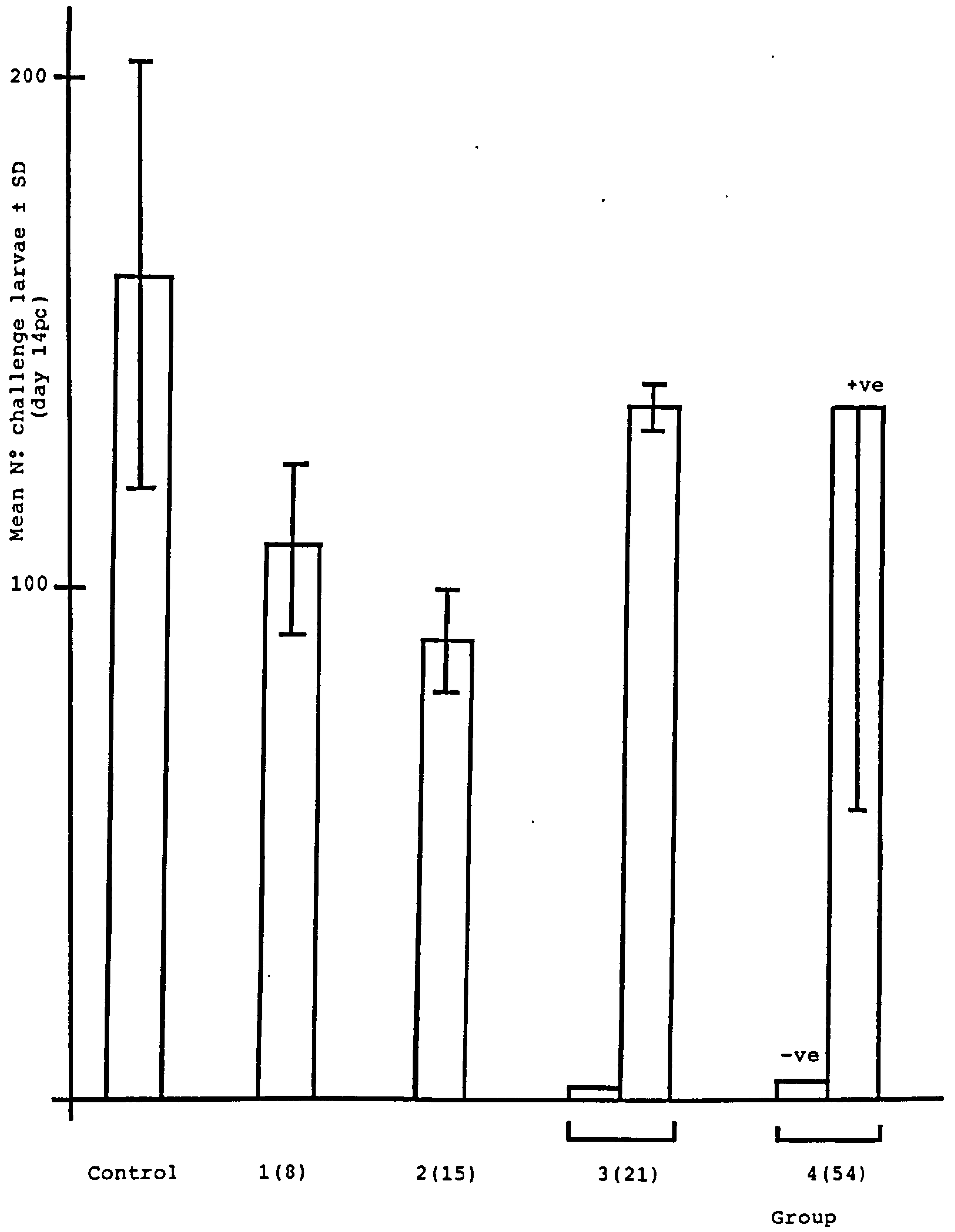
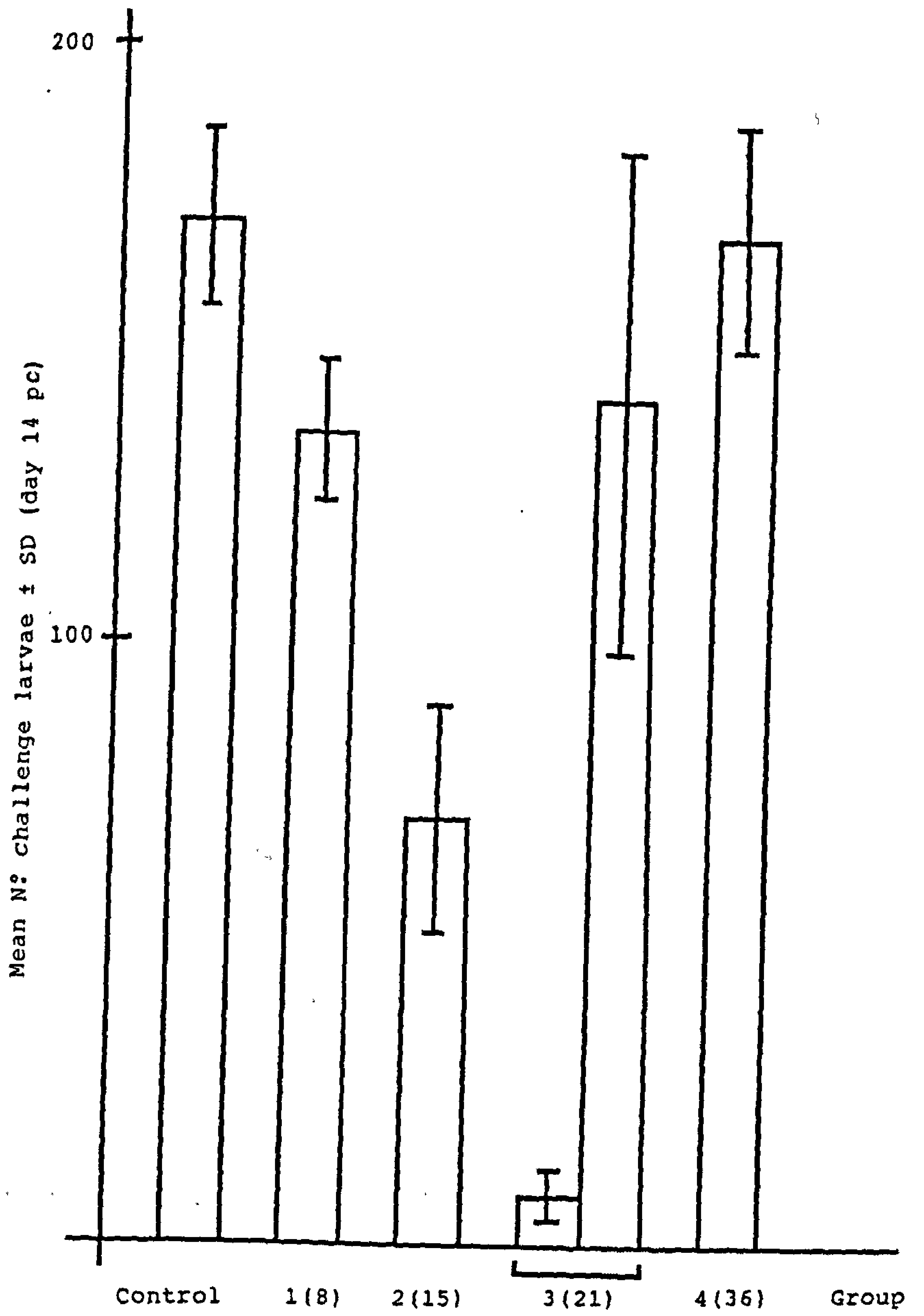

Mean Nº challenge larvae ± SD (day 14pc)
200
100
+ve
-ve
Control
1(8)
2(15)
3(21)
4(54)
Group

Figure 6.1.4 The fate of challenge infections in B10.BR after chemical abbreviation of the primary infection established by infection with 400 eggs. The duration of the primary infection is given in brackets for each group. All mice were challenged with 400 eggs (day 43 p.i) and killed after 14 days.



200
100
Mean N° challenge larvae ± SD (day 14 pc)
Control
1(8)
2(15)
3(21)
4(36)
Group

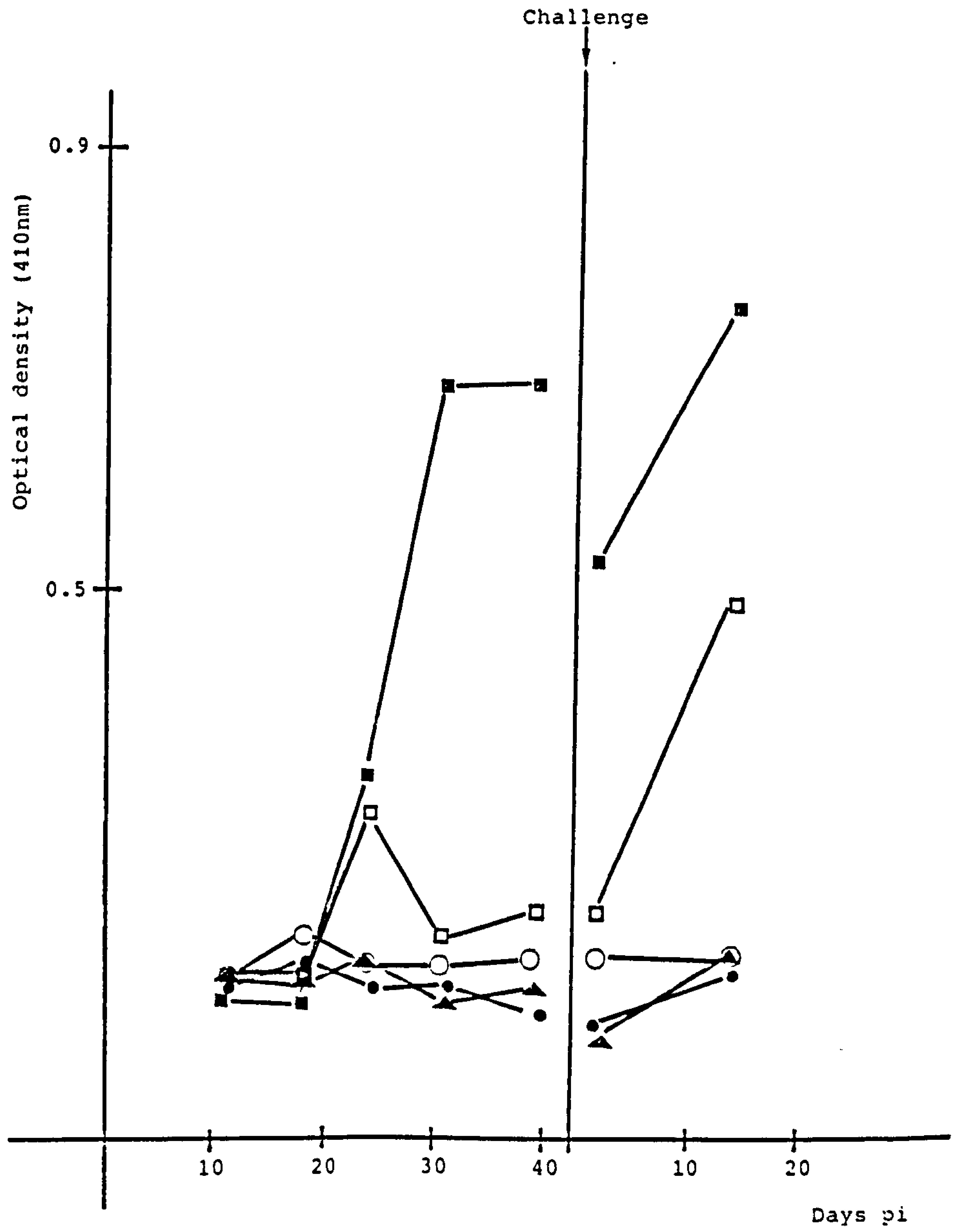
associated with the persistence of the parasite beyond 21 days is critical in determining whether the host can or cannot expel primary and challenge infections. In an attempt to discriminate between these two possibilities the abbreviation of a primary infection experiment was conducted in the non-responsive B10.BR strain using a higher infection level, 400 eggs being administered from which approximately 150 larvae established as seen on day 14 p.i. The protocol outlined in Table 6.1.1 was followed except that the group 4 primary infection was ended on day 36 p.i. All groups were challenged on day 43 p.i., killed on day 14 post-secondary infection and the number of challenge larvae present counted. As shown in fig 6.1.4, group 1 (terminated on day 8 p.i.) exhibited a 21% decrease in challenge larvae from control values ($P<0.002$) and group 2 (terminated on day 15 p.i.) a 59% decrease ($P<0.001$). However, group 3 in which the primary infection was terminated on day 21 p.i., showed a division into individuals which were nearly 100% immune or which had challenge larval burdens not significantly different from control values ($P>0.05$). Group 4, in which the primary infection was terminated on day 36 p.i. had challenge infection levels equivalent to those of the challenge controls ($P>0.05$).

Analysis of immune responses to prolonged or abbreviated infections in B10 and B10.BR mice.

To provide information concerning the level and specificity of antigen recognition during the induction of non-responsiveness, sera from the B10 and B10.BR mice used in the above experiments were analysed by ELISA and immunoprecipitation using adult E/S antigen as the target antigen preparation.

The results of a typical ELISA performed with sera from the B10.BR experiment are shown in fig. 6.1.5. As can be seen, groups 1 and 2, both

Figure 6.1.5 Time-course of the total specific antibody response to *Trichuris muris* excretory-secretory antigen in serum samples from B10.BR mice which had experienced chemically abbreviated primary infections of 400 eggs. The duration of the primary infection for each group is given in parentheses below. ○–○, Group 1 (8); ▲–▲, group 2 (15); □–□, group 3 (21); ■–■, group 4 (36); ●–●, group 5 (naive/challenge control).



Challenge
0.9
0.5
Optical density (410nm)
10
20
30
40
10
20
Days pi

Figure 6.1.6 Fluorograph of SDS-PAGE of ^{35}S metabolically labelled *Trichuris muris* excretory-secretory (ES) antigen after immunoprecipitation with sera taken on days 14 and 2 post-challenge (2°) from B10.BR mice which had experienced chemically abbreviated primary infections of 400 eggs. All mice were challenged with 400 eggs on day 43 p.i. The duration of the primary infection for each group is given in parentheses: group 1 (8), group 2 (15), group 3 (21), group 4 (36). NS, naive serum.

Figure 6.1.7 Fluorograph of SDS-PAGE of ^{35}S metabolically labelled *Trichuris muris* excretory-secretory (ES) antigen after immunoprecipitation with sera taken on days 24 and 38 post-primary infection (1°) from B10.BR mice which had experienced chemically abbreviated infections of 400 eggs. The duration of the primary infection for each group is given in parentheses: group 1 (8), group 2 (15), group 3 (21), group 4 (36). NS, naive serum.

Figure 6.1.6

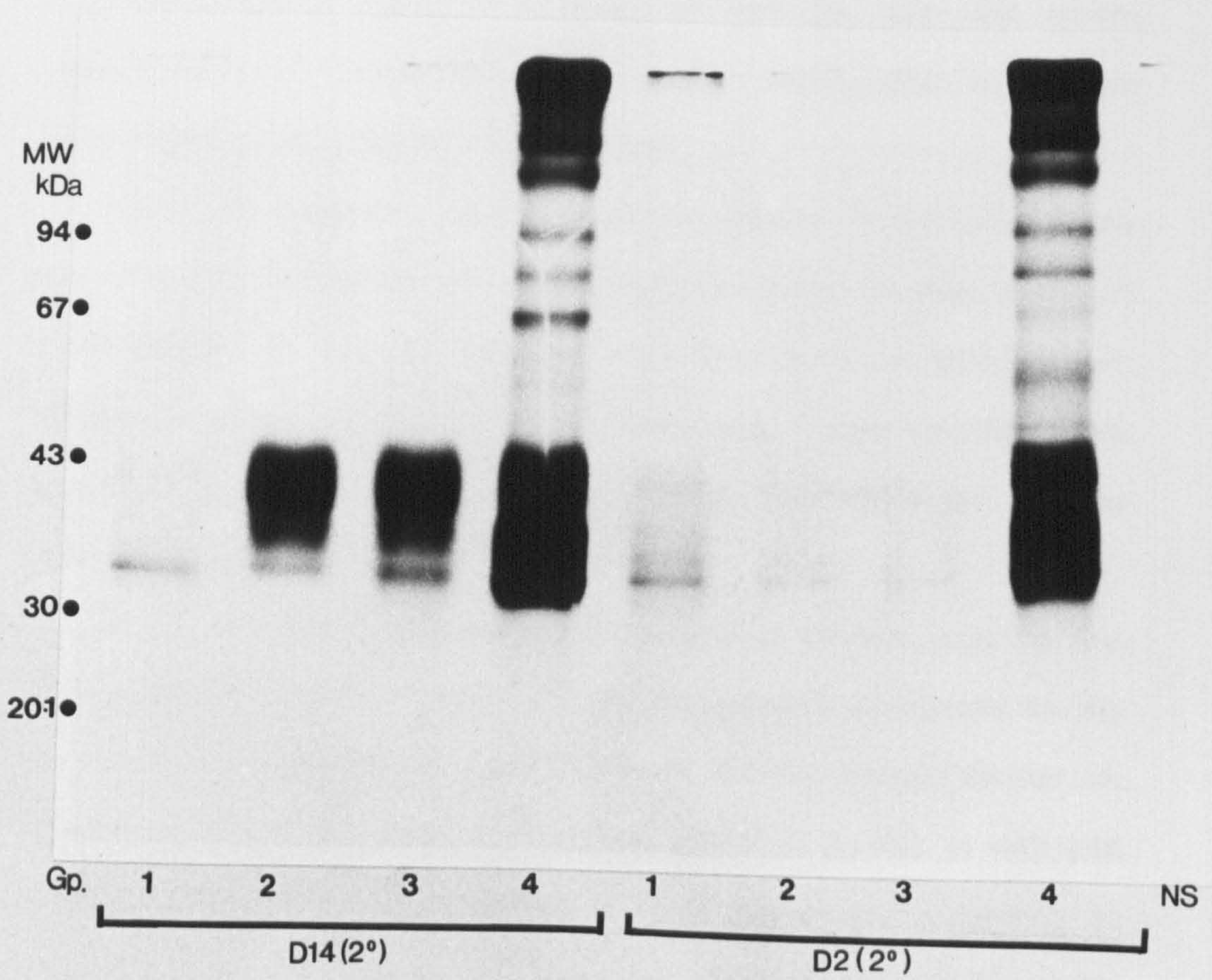
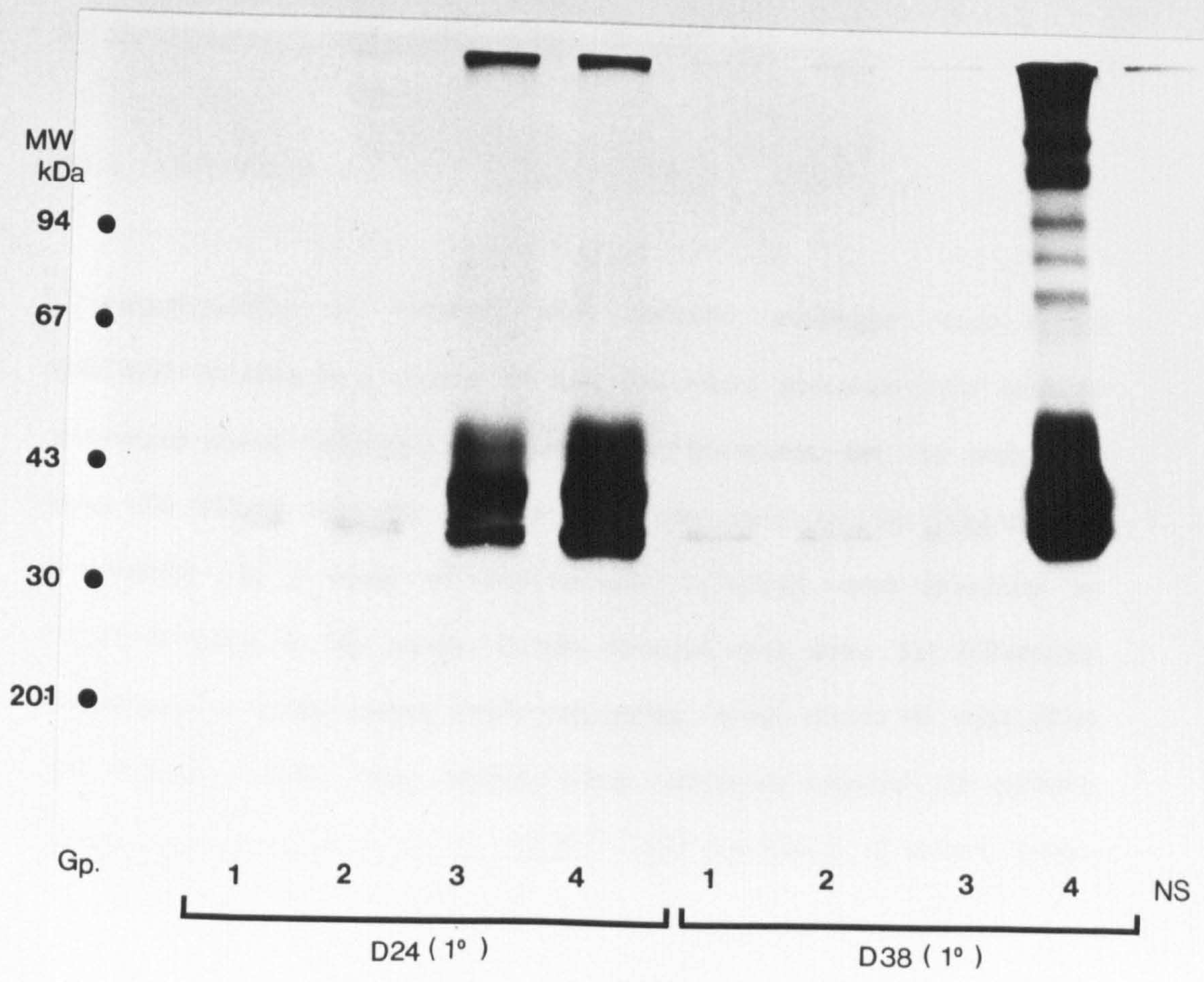


Figure 6.1.7



of which showed a degree of immunity to challenge, had total specific antibody levels no different from naive levels. Very similar results were achieved using sera from the B10 experiment.

Fig. 6.1.6 shows the antigen recognition profiles of antibodies in the sera from B10.BR groups 1-4. Tested sera was taken on days 2 and 14 post-challenge. As can be seen, only sera from group 4 mice (primary infection terminated on day 36 p.i.) exhibited wide antigen recognition, the sera from the other groups recognising antigens only within the restricted molecular weight range of 30-50kDa.

Fig. 6.1.7 shows the antigen recognition profiles for sera taken on days 24 and 38 post-primary infection. Profiles for groups 3 (terminated on day 21 p.i) and 4 (terminated on day 36 p.i.) are virtually identical on day 24, but by day 38 p.i. the group 4 recognition profile is as wide as seen post challenge. Sera from group 3 showed markedly less antigen recognition on day 38 p.i. than on day 24 p.i. This may reflect a decrease in specific antibody circulating in the peripheral blood following the removal of worms using Promintic 17 days earlier.

6.1.5 DISCUSSION

Predisposition to infection with parasitic nematodes may reflect individual variation in a number of host-determined parameters, for example nutritional status, behaviour or immune responsiveness, but can also arise from the effects that the parasite itself may have on the host-parasite relationship. In a study of host genetic influences upon immunity to *Trichuris muris* in the mouse, it was observed that there was differential responsiveness within certain slowly-responding inbred strains of mice (Else and Wakelin, 1988). Thus, whereas some individuals expelled the primary infection, others failed to do so and harboured populations of mature worms

at patency. The correspondence between time taken to expel worms and the proportion of non-responsive individuals strongly suggested that parasite-induced effects might be responsible for the phenomenon and two possibilities were proposed. The first was that, after reaching a certain size, worms cease to be susceptible to effector mechanisms. Variation in the rate of parasite growth would result in individual worms reaching this size at different times in different individuals, thus resulting in mouse-mouse variation. If this was the case it would be expected that mice unable to expel a primary infection would be resistant to a challenge infection after the primary infection had been removed using an anthelmintic. However, this was found not to be the case in the first experiment described here. Non-responsive DBA/2 mice, the strain in which differential responsiveness was first described (Worley *et al.*, 1962; Wakelin, 1975*b*, Lee and Wakelin, 1982*b*), not only failed to expel a primary infection, but also remained fully susceptible to challenge despite the removal of the primary infection. Those individuals which did respond to the primary infection also expressed immunity to the challenge. Similarly, B10.BR mice, which were uniformly non-responsive to the primary infection, remained non-reponsive to challenge. These results imply that the inability of non-responsive individuals to expel *T. muris* is not a consequence of worm size.

The second explanation proposed for differential responsiveness was that, after a certain period of infection, the host response becomes suppressed by the parasite, either as a consequence of increasing antigenic exposure, or of exposure to stage-specific antigens. This possibility was studied in three experiments in which the normal infection patterns of B10 and B10.BR were manipulated to increase or decrease worm survival. In the former, normally a responsive strain, establishment of an adult infection, by restricted immune suppression or by the use of subthreshold infection levels, induced unresponsiveness to a subsequent challenge. There is little evidence

that the effects of interfering with the host primary immune response by short-term use of immunosuppressive drugs such as cortisone extends into a secondary infection except for *T. muris*, where this phenomenon has been demonstrated previously (Wakelin, 1970*a*; Wakelin and Selby, 1974*a*). Lee and Wakelin (1982*a*) suggested that the inability of cortisone treated CBA/Ca mice to expel a challenge infection after removal of the primary infection resulted from some continuing defect in a T-cell population. It is tempting to speculate that this defect could be induced by the later stages of the parasite normally not seen by the host unless the host is immunosuppressed or the infection is subthreshold. The abbreviation of subthreshold infections in B10 mice followed by challenge gave results suggesting that either the level of antigenic stimulation or the survival of the parasite beyond 21 days is critical in determining whether or not the host is unable to expel both primary and challenge infections. However, a similar experiment using B10.BR mice demonstrated that it is the persistence of the parasite beyond day 21 p.i. which is critical. If suppression of host immunity does not occur one would expect the degree of immunity to challenge stimulated by the primary infection to increase as the duration of the primary infection increased. This was indeed the case for groups 1 and 2, terminated on days 8 and 15 p.i. respectively. However, if the infection was terminated on day 21, some individuals were susceptible to challenge, and mice which had experienced adult infections were all susceptible to the secondary infection. These results suggest that the observed differential responsiveness within inbred strains (Else and Wakelin, 1988) is due to suppression of host immunity by larval stages which persist beyond day 21. Hence, strains of mice which express protective immunity before day 21 (for instance BALB congenics and NIH mice) do not exhibit differential responsiveness (Else and Wakelin, 1988).

The hypothetical immunomodulatory factors produced by the later larval

stages of *T. muris* have not been identified, nor is the mechanism through which they act known. A number of possibilities can be proposed. It may be that there is a stage specific antigen which elicits cell populations with suppressor activities, capable of down-regulating protective immunity. If such an antigen exists then its activity is clearly one that causes a long-term change in responsiveness, akin to tolerance, and dose-dependent effects may therefore also be involved. Alternatively, the parasite may release mitogenic factors in a stage restricted manner, which produce a polyclonal activation and exhaustion of lymphocyte populations. Certainly adult *T. muris* E/S antigen preparations are mitogenic (Roach, 1986; Else and Wakelin, unpublished observations) and the ELISA and immunoprecipitation data presented here support the idea that there is a correlation between low specific antibody levels and resistance to infection, although the high antibody levels to E/S antigen and wide antigen recognition profiles seen for groups susceptible to challenge may merely reflect a prolonged exposure to parasite antigens rather than representing responses to irrelevant antigen.

Implications for human trichuriasis are far-reaching. Any delay in the initiation of a protective immune response, whether determined by genetic variation in immunity or by behavioural factors, through the acquisition of low-level infections, may leave the host exposed to immunosuppressive stages of the parasite resulting in the build up of heavy, debilitating, chronic infections.

6.2 THE SURVIVAL OF CHALLENGE INFECTIONS TO DAY 35 IN B10.BR MICE

In section 6.1 the ability to express acquired immunity to challenge infections was assessed on the basis of larval worm burdens. Challenge infections in good responder strains are known to be expelled from the host within a few days (Wakelin, 1973) therefore the presence of challenge larval burdens equivalent to control levels on or around day 13 p.c. was felt to be indicative of an inability to express acquired immunity. On this basis B10.BR mice were shown to be unable to expel both primary and secondary infections. Although it has been shown that in mice which have had patent primary infections, established through the administration of cortisone, challenge infections do survive until patency is reached 35 days later (Wakelin and Selby, 1974*a*), it was felt necessary to confirm the inability of non-responder B10.BR mice to expel secondary infections by following the fate of a challenge infection to day 35 p.c. It is theoretically possible that there is acquired immunity to reinfection in non-responder strains but that this immunity is slow to be expressed.

6.2.1 EXPERIMENTAL DESIGN

25 male B10.BR and 11 male NIH mice were given a primary infection of 400 *T. muris* eggs. Worm burdens of 119.8±28.8 and 109.8±45.1 were present in B10.BR and NIH mice on day 15 p.i. and day 10 p.i. respectively. Faecal samples were examined for the presence or absence of eggs on day 42 p.i. Individuals harbouring patent infections were treated with Promintic on day 43 p.i. and their faeces rechecked for eggs on day 48 p.i. to confirm the efficacy of the anthelmintic. All mice were

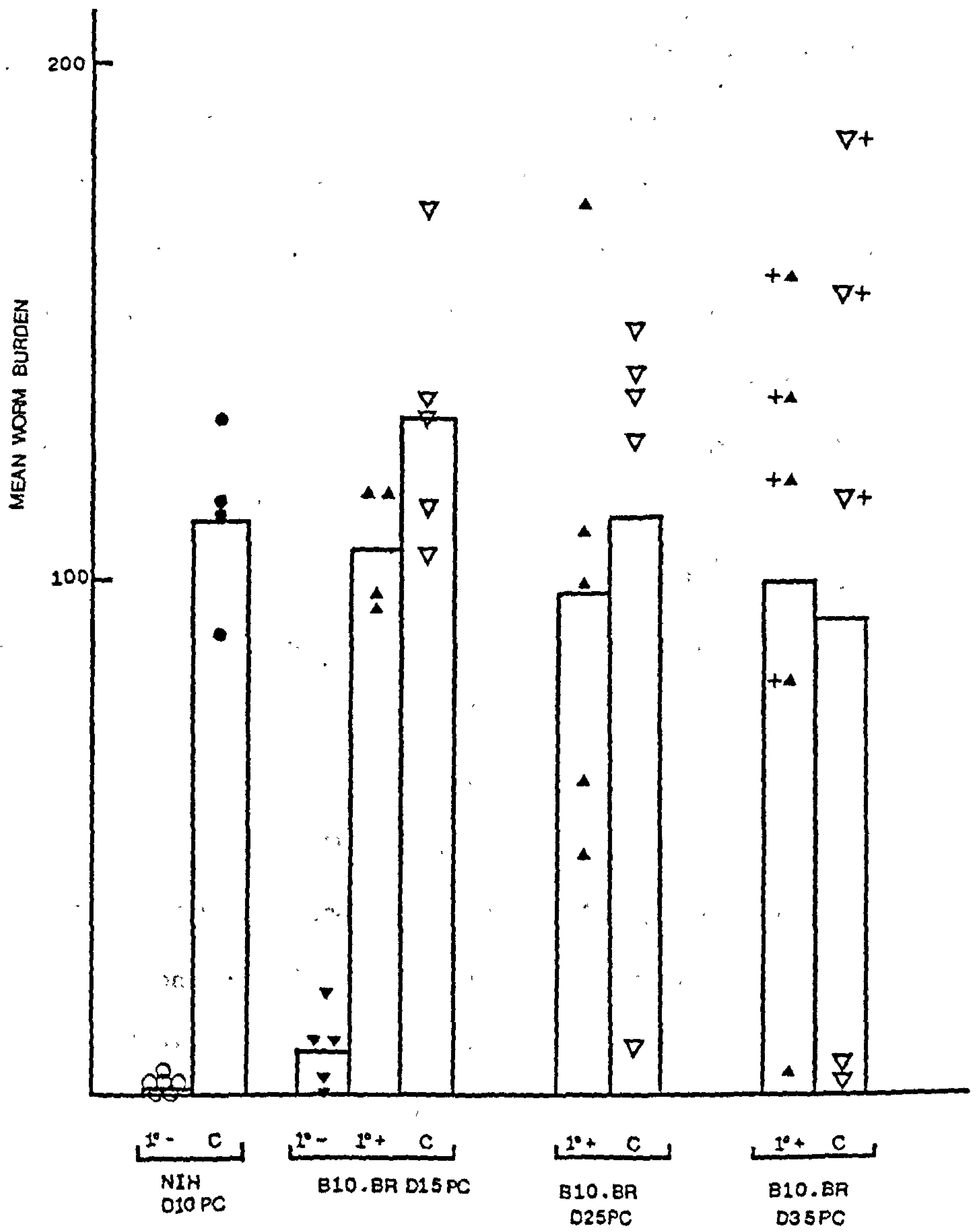
challenged with 400 *T. muris* eggs on day 50 p.i. along with appropriate challenge controls. Mice were killed on day 10 p.c. for NIH and on days 15, 25 and 35 p.c. for B10.BR mice, serum samples were taken and worm burdens determined. The specific IgG and IgG1 antibody responses to E/S antigen of B10.BR and NIH mice were analysed by ELISA. E/S antigen recognition profiles for total specific antibody were studied by immunoprecipitation using 10-20% gradient gels. The significance of differences between worm burdens recovered from the experimental groups was calculated using the Mann-Whitney U test. $P>0.05$ was considered non-significant.

6.2.2 RESULTS

The survival to day 35 of challenge infections in B10.BR mice

Examination of faecal samples on day 42 post primary infection revealed that a proportion of the B10.BR mouse strain (6/20) had expelled the primary infection, a situation not observed before for the normally uniformally non-responsive B10.BR strain of mouse. One mouse was sacrificed to determine whether the primary infection had really been expelled or whether adult female worms were simply not producing eggs. No worms were recovered. The remaining 14 individuals, harbouring patent infections, were treated with anthelmintic before challenge. All (6/6) individuals of the NIH strain of mouse were egg negative on day 42 p.i. Fig. 6.2.1 shows the challenge worm recoveries for NIH mice, B10.BR individuals which were negative for parasite eggs in the primary infection ($1^{\circ}-$), B10.BR individuals which had harboured patent primary infections ($1^{\circ}+$) and worm burdens for challenge control mice. NIH mice, which had all expelled the primary infection were uniformally resistant to challenge

Figure 6.2.1 The fate of challenge infections in the responder NIH strain and responder and non-responder individuals of the B10.BR strain of mouse. All NIH individuals expelled the primary infection, being egg-negative (indicated by $1^{\circ}-$) on day 42 p.i. 6/20 B10.BR individuals also expelled the primary infection ($1^{\circ}-$), all other B10.BR individuals harbouring patent primary infections ($1^{\circ}+$) on day 42 p.i. Mice harbouring adult worms from the primary infection were treated with an anthelmintic prior to challenge (400 eggs) on day 50 p.i. Mice were killed day 10 post-challenge for NIH and day 15, day 25 and day 35 post-challenge for B10.BR mice. +, individuals harbouring patent infections on day 35 post-challenge.



200
100
MEAN WORM BURDEN
1° - C
NIH
D10 PC
1° - 1° + C
B10.BR D15 PC
1° + C
B10.BR
D25PC
1° + C
B10.BR
D35PC

when examined on day 10 p.c. B10.BR individuals resistant to the primary infection were also relatively resistant to challenge, as seen on day 15 p.c., whilst individuals unable to expel a primary infection harboured worm burdens not significantly different to control levels on days 15, 25 and 35 p.c. ($P>0.05$). Individual B10.BR mice were found to be more variable in their ability to expel *T. muris* than previously seen (Else and Wakelin, 1988). For instance, one challenge control individual had substantially reduced its worm burden by day 25 p.c. and two individuals had only a few stunted, immature worms left on day 35 p.c. In addition, one individual non-responsive to the primary infection was able to expel most worms from the challenge infection by day 35 p.c. and the other individuals in the 1°+/challenge group on day 35 p.c. were found to harbour some stunted worms as well as fully mature adult parasites. Although the variability within control B10.BR mice made interpretation of results less easy, it was clear that individuals unable to expel a primary infection were at least as likely to harbour patent secondary infections as challenge control mice. Fig. 6.2.2 shows the IgG antibody levels to *T. muris* E/S antigen in NIH and B0.BR mice post challenge. Mice which had expelled the primary infection and resisted reinfection, 1°-/2° (all NIH individuals and a subpopulation of B10.BR mice), had considerably higher levels of specific IgG compared to challenge control mice on day 10 p.c. (NIH) or day 15 p.c. (B10.BR). Likewise B10.BR individuals which had not expelled the primary infection and had remained non-responsive to challenge, (1+/2°), had IgG antibody levels elevated over control values, even on day 35 p.c. when levels of antibodies in control mice had risen as the primary infection proceeded. Where variation in the ability to expel the challenge infection occured within B10.BR individuals no significant correlation could be made with specific IgG antibody levels. Although individual B10.BR mice which expelled the primary infection (1°-/2°) had slightly higher IgG levels on day

Figure 6.2.2 Time course of the specific IgG antibody response to *Trichuris muris* E/S antigen in serum samples from NIH mice challenged after expelling a primary infection (▼, 1°–/2°) and B10.BR mice which either resisted the primary infection (▲, 1°–/2°) or which harboured patent primary infections (●, 1°+/2°). ○, B10.BR challenge control; △, NIH challenge control; N.S., naive serum.

Figure 6.2.3 Time course of the specific IgG1 antibody response to *Trichuris muris* E/S antigen in serum samples from NIH mice challenged after expelling a primary infection (▼, 1°–/2°) and B10.BR mice which either resisted the primary infection (▲, 1°–/2°) or which harboured patent primary infections (●, 1°+/2°). ○, B10.BR challenge control; △, NIH challenge control; N.S., naive serum.

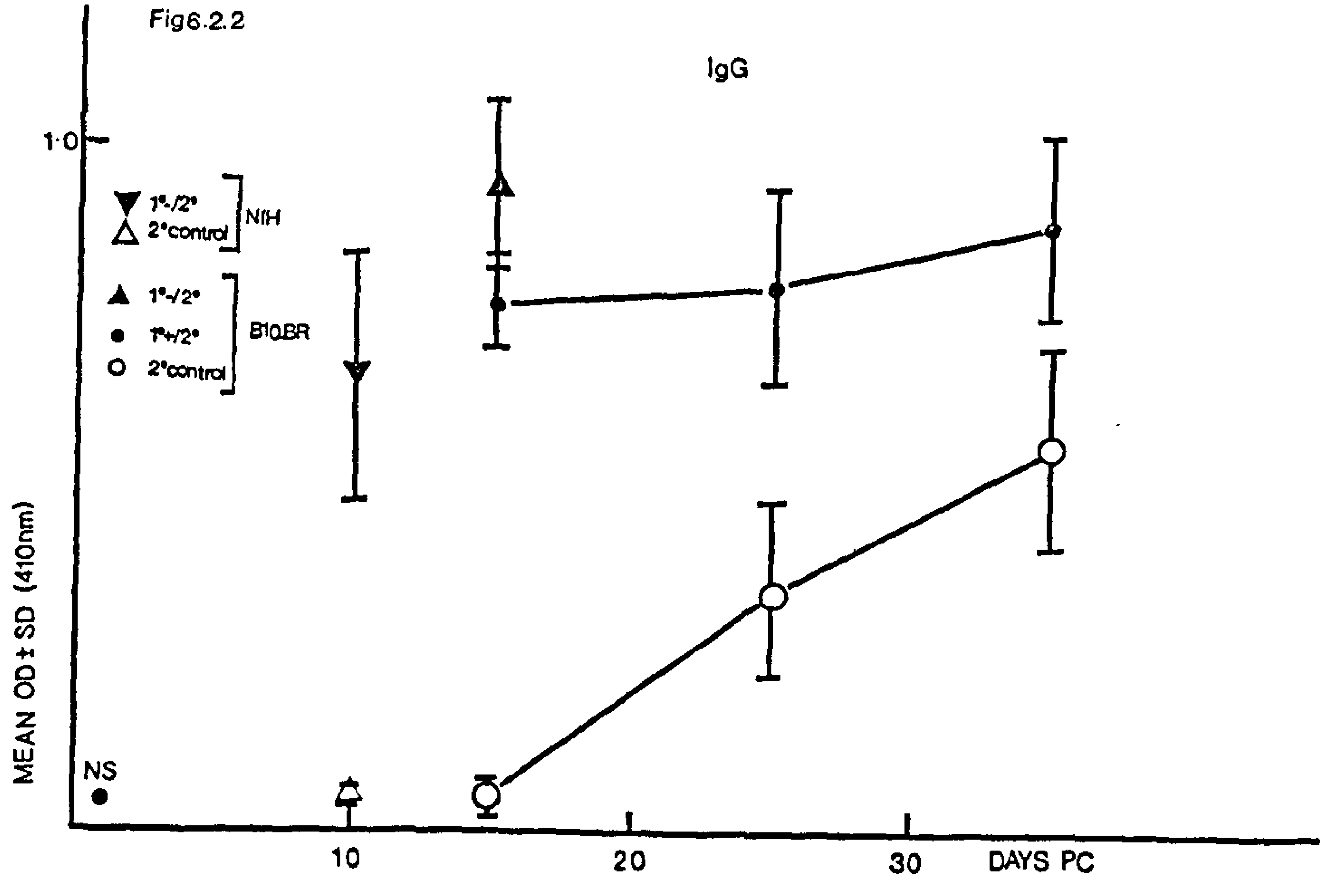


Fig6.2.2
IgG
1·0
1°-/2°
2°control
NIH
1°-/2°
1°+/2°
2°control
B10.BR
MEAN OD ± SD (410nm)
NS
10
20
30
DAYS PC

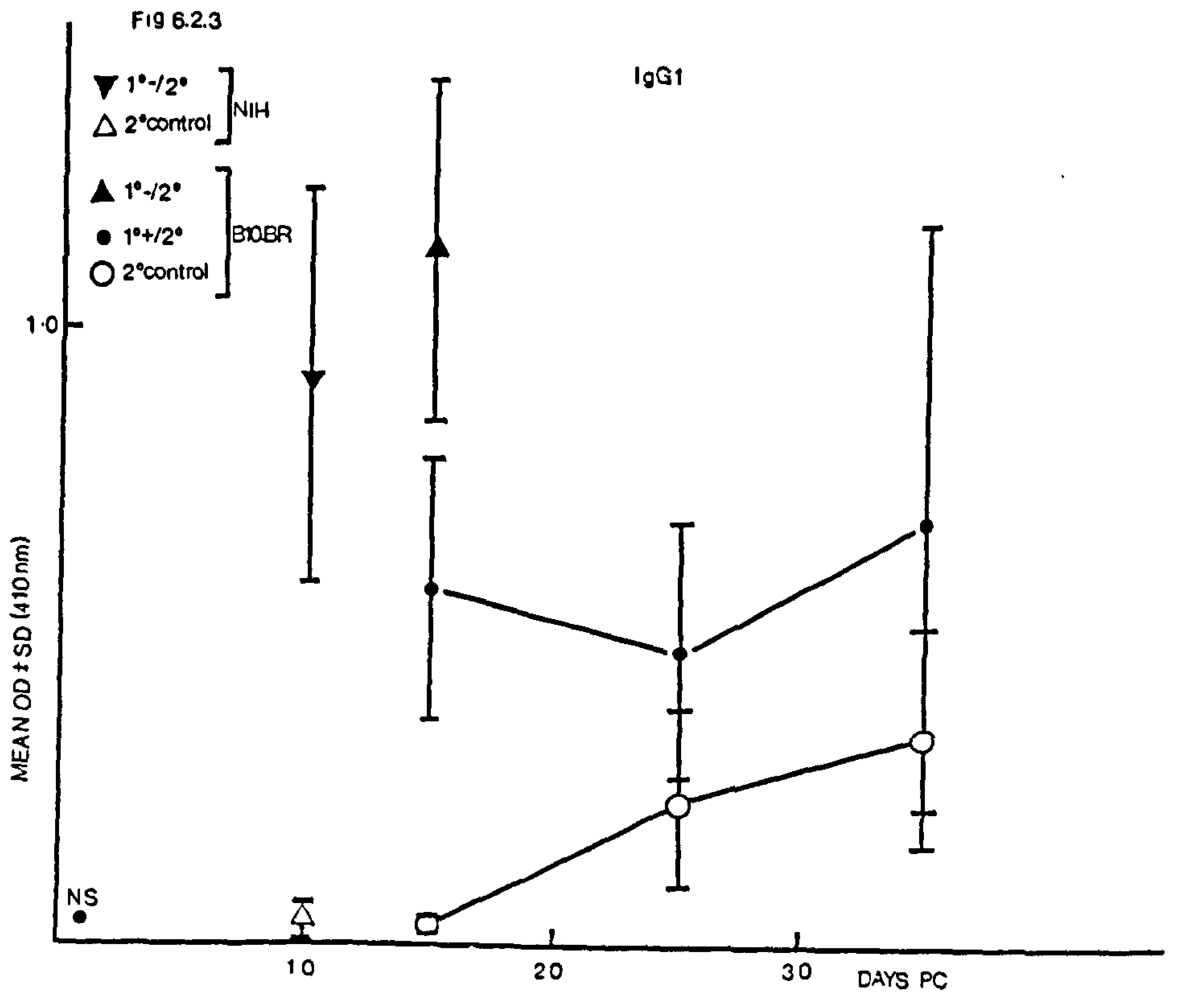


Fig 6.2.3
IgG1
1°-/2°
2°control
NIH
1°-/2°
1°+/2°
2°control
B10.BR
1·0
MEAN OD ± SD (410nm)
NS
10
20
30
DAYS PC

Figure 6.2.4 Fluorograph of SDS-PAGE of ^{35}S-metabolically labelled *T. muris* E/S antigen after immunoprecipitation with individual mouse sera taken day 10 post-challenge (NIH) or day 15 post-challenge (B10.BR). Lanes 1-3, NIH individuals resistant to primary infection and challenge (1°-/2°); lanes 4-6, NIH challenge controls (2°c); lane 7, B10.BR individual egg-positive in primary infection and challenged (1°+/2°); lane 8, B10.BR challenge control (2°c); lane 9, naive NIH serum. Worm burdens are also given beneath the lane numbers.

Figure 6.2.5 Fluorograph of SDS-PAGE of ^{35}S-metabolically labelled *T. muri* E/S antigen after immunoprecipitation with individual mouse sera taken day 15 post-challenge from B10.BR mice. Lanes 1-3, individuals non-responsive to primary infection and challenged (1°+/2°); lanes 4-6, challenge control mice (2°c); lanes 7-8, individuals resistant to primary infection and challenged (1°-/2°); lane 9, naive mouse serum. Worm burdens are also given beneath the lane numbers.

Figure 6.2.4

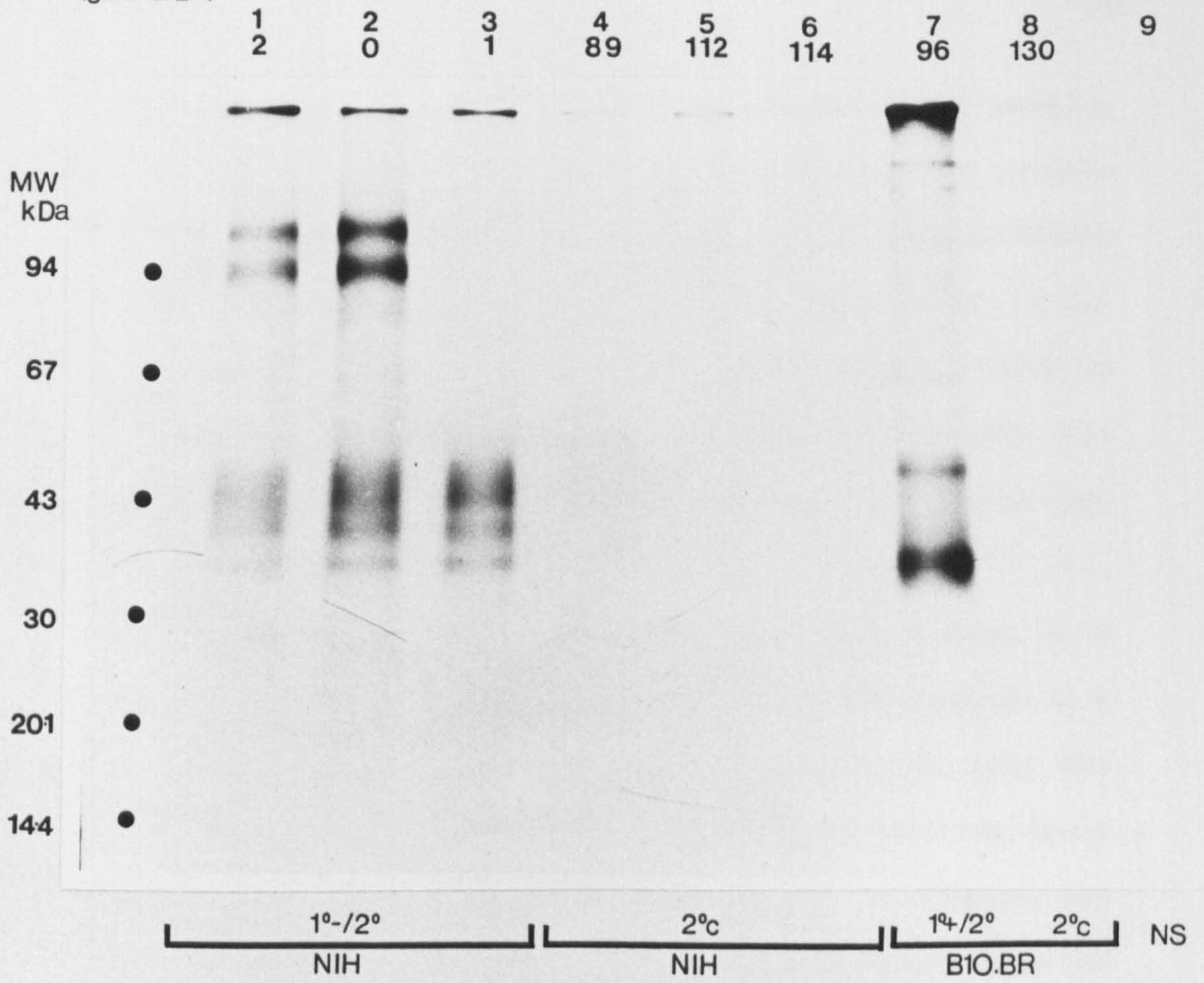


Figure 6.2.5

1 116
2 94
3 116
4 132
5 113
6 171
7 3
8 0
9

1°+/2°
2°c
1°-/2°
NS

B10.BR

15 p.c. than B10.BR individuals non-responsive to the primary infection, small sample sizes (n=5, n=4) prevented the significance of this elevation being determined. Specific IgG1 levels after challenge in individual B10.BR mice resistant to primary infection were considerably higher on day 15 p.c. than in individuals unable to expel the primary infection. Individuals non-responsive to the primary infection had higher levels of IgG1 than challenge control mice, although day 35 values were very variable (fig. 6.2.3).

The antigen recognition profiles for individual mice resistant to a primary infection (NIH and a group of B10.BR mice), non-responsive to a primary infection (B10.BR mice) and challenge control mice, using sera taken day 10 p.c. (NIH) or day 15 p.c. (B10.BR), are shown in figures 6.2.4 and 6.2.5. Profiles are for total specific antibody. As expected, sera from mice which had experienced a primary infection precipitated more E/S antigens than sera from control mice for both NIH (day 10 p.c.) and B10.BR (day 15 p.c.) strains of mice (fig. 6.2.4), although one NIH individual (lane 3) showed only very weak recognition of the 90-95kDa and 105-110kDa antigens (not visible on photograph, but evident on original autoradiograph). The day 35 p.c. profiles for B10.BR individuals could only be distinguished from day 35 challenge control profiles by the intensity of the banding patterns (data not shown). Individual B10.BR mice unable to expel the primary infection had identical antigen recognition profiles on day 15 p.c. to individuals which had become resistant to the primary infection (figure 6.2.5, lanes 1,2,3 (1°+/2°) and 7,8 (1°-/2°)).

6.2.3 DISCUSSION

The results presented in section 6.2.2 demonstrate that B10.BR mice which have experienced a patent primary infection are at least as likely to

develop patent secondary infections, following the removal of the primary infection using an anthelmintic, as challenge control mice. Differential responsiveness was observed within the B10.BR strain of mice, a phenomenon reported previously for other B10 congenics (Else and Wakelin, 1988), but not observed before for the normally uniformally non-responsive B10.BR strain. Mice used in this experiment were slightly older when they received the primary infection than routinely used, being 9-10 weeks old rather than 6-8 weeks old. Challenge control mice, which also exhibited variability in responsiveness were 16-17 weeks old when infected. Although mice are assumed to be immunologically mature by 6-8 weeks old it may be that older mice are able to mount a protective response slightly earlier than their younger counterparts resulting in some individuals expelling the parasite before the later larval and adult stages of the parasite are able to induce suppression of host immunity. Alternatively, continual inbreeding of the B10.BR strain may have led to a slight change in response kinetics such that immunity is expressed in some individuals before the parasite has developed to its later immunosuppressive stages. Certainly a change in response status of the B10.BR mouse strain has been observed recently in parasite systems other than the *T. muris*-mouse model (D.L. Wassom, pers. comm.).

After a primary infection both responder and non-responder B10.BR populations exhibited specific IgG antibody levels to challenge infections far higher than those seen for challenge control mice and recognised a wider range of antigens early on post challenge. No correlations between antibody levels or antigen recognition patterns and responder/non-responder status were established, as reported in Chapters 4 and 5, although there was some suggestion that the non-responder population of B10.BR mice on day 15 p.c. might have had significantly depressed IgG1 antibody levels to E/S antigen. This finding is discussed in more detail in Chapter 7.

The results presented in this chapter demonstrate that certain mice with non-responder phenotypes do possess the ability to mount effective anti-parasite responses in the absence of immunomodulation by later larval and adult stages of *T. muris*. Similar findings have been reported by Enriquez *et al.* (1988*a*) working with *Heligmosomoides polygyrus* (*Nematospiroides dubius*) where it was demonstrated that the stages in the parasite's life history to which the host was exposed, as well as the numbers of parasites present in each of these stages, were particularly important in influencing the ability of the host to resist challenge infections. Using a six-day abbreviated immunising infection susceptible strains were shown to possess the ability to mount effective anti-parasite responses in the absence of immunomodulation by adult worms. The association between the presence of adult *H. polygyrus* in the small intestine and suppression of host immunity reported by Enriquez *et al.*, 1988*a*, and others (Jacobson *et al.*, 1982; Behnke *et al.*, 1983) is consistent with the work presented here for *T. muris* which demonstrates a relationship between the presence of later larval and adult parasite stages in the gut and an inability to expel a challenge infection.

6.3 SUMMARY POINTS

1. Mice exposed to a patent primary infection were fully susceptible when challenged after the removal of the primary infection using an anthelmintic and the challenge infection reached patency in B10.BR mice.

2. Challenge after abbreviation of primary infections at different stages of worm development showed that poor response to infection was associated

with the persistence of primary infection larvae beyond day 21.

3. It is suggested that host immunity is suppressed by the later stages of the parasite (L3, L4, adult), this immunosuppression being long lasting and leading to long term interference with the ability of the host to mount a protective immune response.

4. After a primary infection both responder and non-responder B10.BR populations and the responder NIH mouse strain exhibited specific IgG antibodies to challenge infections far higher than seen for challenge control mice and recognised a wider range of antigens early on post challenge. Analysis of the specific IgG1 antibody response to E/S antigen indicated that the population of B10.BR mice unable to expel both primary and challenge infections might have had significantly depressed IgG1 antibody levels on day 15 p.c. compared to individuals of the same strain resistant to primary and challenge infections.

CHAPTER 7

GENETICALLY-DETERMINED INFLUENCES ON THE ABILITY OF POOR-RESPONDER MICE TO RESPOND TO VACCINATION AGAINST *TRICHURIS MURIS*

7.1 INTRODUCTION

An ideal vaccine should mimic the immunological stimulus associated with the natural infection. Side-effects evoked should be minimal and the vaccine cheap, stable and easy to administer (Steward and Howard, 1987). At present there is no vaccine against any human parasitic disease (reviewed by McClaren and Terry, 1989) yet vaccines against other pathogens are available and have had a considerable impact on public health. Laboratory animals can be successfully vaccinated with crude parasite antigens but for obvious reasons such an approach is not feasible for humans. Likewise, the immunopotentiating chemicals, or adjuvants, with which many parasite antigens are formulated in order to selectively boost the immune response cannot be used in man because of their inflammatory side effects. For instance the classical Freund's complete adjuvant (FCA) routinely used in laboratory models, is so toxic that it cannot even be used for veterinary vaccines. Adjuvants currently being examined for use in vaccines against parasitic infections have been reviewed by Bomford (1989). The rapid advances made recently in the fields of molecular biology and biochemistry suggest that anti-parasite vaccines suitable for human use will most probably come from recombinant DNA or synthetic peptide technology. Indeed, candidate recombinant (Ballou *et al.*, 1987) and synthetic (Herrington *et al.*, 1987) malaria vaccines have already been tested in human and non-human primates, albeit with limited success. Such vaccines have many advantages over the use of crude parasite antigens, including the potential to present only those antigens that elicit protective immunity. This is particularly important when considering parasites which appear to produce immunosuppressive molecules such as *Heligmosomoides polygyrus* (*Nematospiroides dubius*) (Pritchard and Behnke, 1985; Monroy *et al.*,

1989). As already indicated, research into the development of anti-parasite vaccines for use in humans is most advanced with respect to protozoal diseases, in particular malaria (Ballou *et al.*, 1987; Herrington *et al.*, 1987; Good *et al.*, 1987; Kabilan *et al.*, 1988; reviewed by Good *et al.*, 1988*a*, *b*; Bomford, 1989). In contrast the development of vaccines against human helminth infections lags far behind, although potential components of anti-schistosome vaccines have been suggested (Mitchell, 1989*a*) and purified antigens from the nematode *Trichinella spiralis* have been shown to induce strong protective immunity in mice (Silberstein and Despommier, 1984).

Mice vaccinated with *T. muris* adult male homogenate, stichosome extract and E/S antigen show high levels of immunity to challenge (Wakelin and Selby, 1973; Jenkins and Wakelin, 1977, 1983). These studies were carried out using the NIH strain of mouse, a strain known to be highly resistant to a primary *T. muris* infection (Wakelin, 1975*b*). Although most studies concerned with the immunogenicity of different parasite preparations are carried out in genetically resistant host strains, a clear-cut vaccinating effect in genetically susceptible hosts would be of much greater significance, providing a more stringent test for vaccine efficacy. For instance, Mitchell and Munoz (1983) succeeded in vaccinating genetically susceptible mice against a chronic primary infection with *H. polygyrus* by injecting adult worms intraperitoneally using pertussigen as adjuvant, and Mitchell and Handman (1983) vaccinated mice of high genetic susceptibility against cutaneous leishmaniasis using intraperitoneal injections of frozen and thawed *Leishmania tropica major*-infected macrophages with *Corynebacterium parvum* as adjuvant. Mice of the B10 genetic background are known to be considerably less resistant to *T. muris* infection than mice of the BALB or NIH background (Else and Wakelin, 1988) with the B10.BR mouse strain being completely unable to expel a primary infection. The inability of these

mice to express an effective immunity to infection has been attributed to stage-specific parasite suppression of the host response (Else *et al.*, 1989). This chapter describes experiments designed to determine the influence of the host's genetically determined response status on the ability to express resistance after vaccination. Good (NIH or BALB/c), poor (B10) and non-responder (B10.BR) strains of mice were immunised with E/S antigen at a dose ten times greater than the minimum dose required to reduce worm burdens by 80-90% in resistant NIH mice (Jenkins, 1977). In view of the evidence that the later larval and adult stages of *T. muris* are immunosuppressive (Chapter 6; Else *et al.*, 1989), mice exposed to adult worm infections being unable to expel challenge infections before they reach patency, the ability of good and non-responder strains to respond to vaccination after exposure to a primary infection was also assessed. These experiments were designed to monitor the potency of presumptive immunomodulatory parasite-derived factors in the face of a potentially protective crude parasite-derived vaccine.

In all experiments the IgG and IgG1 antibody responses of mice to E/S antigen were analysed by ELISA and the total antibody antigen recognition profiles assessed by immunoprecipitation in an attempt to correlate the ability of mice to respond to vaccination with specific immunological parameters.

7.2 EXPERIMENTAL DESIGN

Experiments 1 and 2: the ability of good (NIH), poor (B10) and non-responder (B10.BR) strains of mice to express resistance after vaccination.

Strains of mice which respond well (NIH), poorly (B10) or not at all (B10.BR) to a primary infection were injected subcutaneously (s.c.) with 100μg E/S antigen in FCA ten days before challenge with 400 *T. muris* eggs. Control mice received PBS in FCA s.c. prior to infection. Mice were killed in groups of at least 5 individuals at suitable time points post challenge (p.c.), according to the primary infection response phenotype of each mouse strain. Serum samples were collected from individual mice and worm burdens were assessed.

Experiment 3: the ability of good and non-responder strains to respond to vaccination after exposure to a primary infection.

Good responder (BALB/c) and non-responder (B10.BR) strains of mice were divided into three groups of at least 5 individuals. One group received a primary infection of 400 *T. muris* eggs before being injected with 100μg E/S in FCA s.c. and challenged ten days later (day 58 p.i.) with 400 eggs (1°v.2°). A second group was vaccinated as above then challenged (v.2°) and the third group received PBS in FCA s.c. before infecting with 400 eggs (PBS 1°). The faeces of mice receiving a primary infection prior to vaccination were examined for the presence of eggs on day 42 p.i. and all groups of mice were treated with an anthelmintic on day 44 p.i. to remove adult worms from B10.BR mice in the (1°v.2°) group and to act as a drug

control in the other groups of mice. Faeces were examined on day 47 p.i. and two mice were sacrificed to ensure that the absence of eggs reflected the removal of all worms and not merely suppression of egg production or the survival of a residual male population. The appropriate groups of mice were vaccinated on day 48 p.i., challenged on day 58 p.i. and killed in groups of at least 5 individuals at various times post challenge for worm burden determination and serum collection. In addition a group of B10.BR individuals were vaccinated with E/S or PBS in FCA and bled 25 days later in order to assess the antibody response to vaccination in the absence of infection.

ELISAs and Immunoprecipitation studies

The antigen recognition profiles for total specific antibody were revealed by incubating sera with ^{35}S-methionine labelled E/S antigen followed by the addition of sheep anti-mouse IgGAM. 10-20% gradient gels were used throughout. ELISAs were performed for various antibody isotypes namely IgG, IgG1 and, in some experiments, IgM.

Statistical Analysis

Significant differences in worm burdens between groups were determined using the Mann-Whitney U test. $P>0.05$ was considered non-significant.

7.3 RESULTS

The ability of good (NIH), poor (B10) and non-responder (B10.BR) strains of mice to express resistance after vaccination.

Experiment 1

Figure 7.1 shows the worm burdens recovered from vaccinated (v) and control (c) mice on day 9 p.c. for NIH, day 35 p.c. for B10.BR and day 19 p.c. and day 35 p.c. for B10 mice. Worm burdens for control groups on day 9 p.c. (NIH), day 35 p.c. (B10.BR) and day 19 p.c. (B10) did not differ significantly from each other (P>0.05). Vaccinated NIH mice had significantly lower worm burdens on day 9 p.c. (P=0.001) than control mice. Immunised B10.BR individuals harboured no or a few stunted worms on day 35 p.c. in contrast to control mice which had patent infections (indicated by +). Immunised B10 mice on day 19 p.c. showed great heterogeneity in their response to infection, 5/12 individuals having worm burdens significantly reduced (P<0.01) from control values, but 7/12 mice still harbouring more than 100 worms. By day 35 p.c. all immunised B10 mice (10/10) had reduced their worm burdens to less than 10 worms. As expected from previous work (Else and Wakelin, 1988), differential responsiveness was observed in B10 control mice; 4/6 mice had almost totally eliminated the primary infection whilst one individual harboured a considerable number of stunted immature worms, and one mouse a patent infection, although stunted worms (within the caecum) were also present.

Figures 7.2 and 7.3 show the antigen recognition profiles (total specific antibody) for vaccinated and control B10 mice using sera taken on day 19 p.c. and day 35 p.c. respectively. Vaccinated mice are represented by lanes

Figure 7.1 The ability of good (NIH), poor (B10) and non-responder (B10.BR) strains of mice to eliminate an infection of 400 *T. muris* eggs following immunisation with 100μg E/S antigen in FCA, s.c. Worm burdens were assessed on day 9 p.c. for NIH mice, day 35 p.c. for B10.BR mice and days 19 and 35 p.c. for B10 mice. v = mice immunised with E/S; c = control mice injected with PBS; + = patent infection.

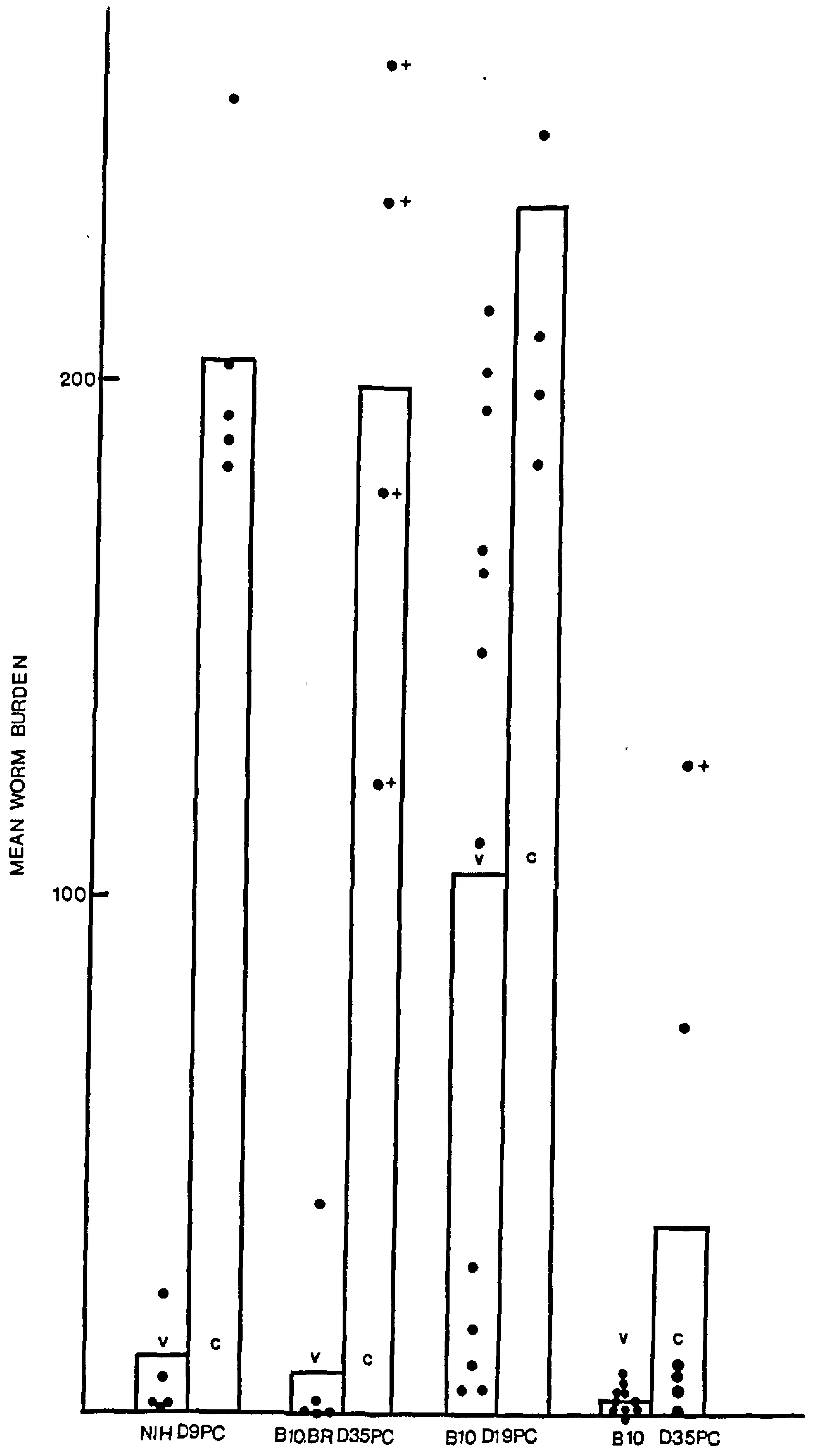

MEAN WORM BURDEN
200
100
V
C
NIH D9PC
B10.BR D35PC
B10 D19PC
B10 D35PC

Figure 7.2 Fluorograph of SDS-PAGE of ^{35}S-metabolically labelled *T. muris* E/S antigen after immunoprecipitation with sera taken day 19 p.c. from vaccinated (100μg E/S in FCA) B10 individuals (B10v) (lanes 1-4) and control mice (B10c) (PBS in FCA) (lanes 5-8). The naive serum (NS) profile is shown in lane 9. Arrows indicate the 80-85kDa, 90-95kDa and 105-110kDa antigens and the 20-23kDa antigen mentioned in the text.

Figure 7.3 Fluorograph of SDS-PAGE of ^{35}S-metabolically labelled *T. muris* E/S antigen after immunoprecipitation with sera taken day 35 p.c. from vaccinated (100μg E/S in FCA) B10 mice (B10v) (lanes 1-7). An example of a day 35 p.i. control mouse profile (B10c) (controls injected with PBS in FCA) is given in lane 8. No antigens were immunoprecipitated with naive serum (NS) (lane 9).

Figure 7·2

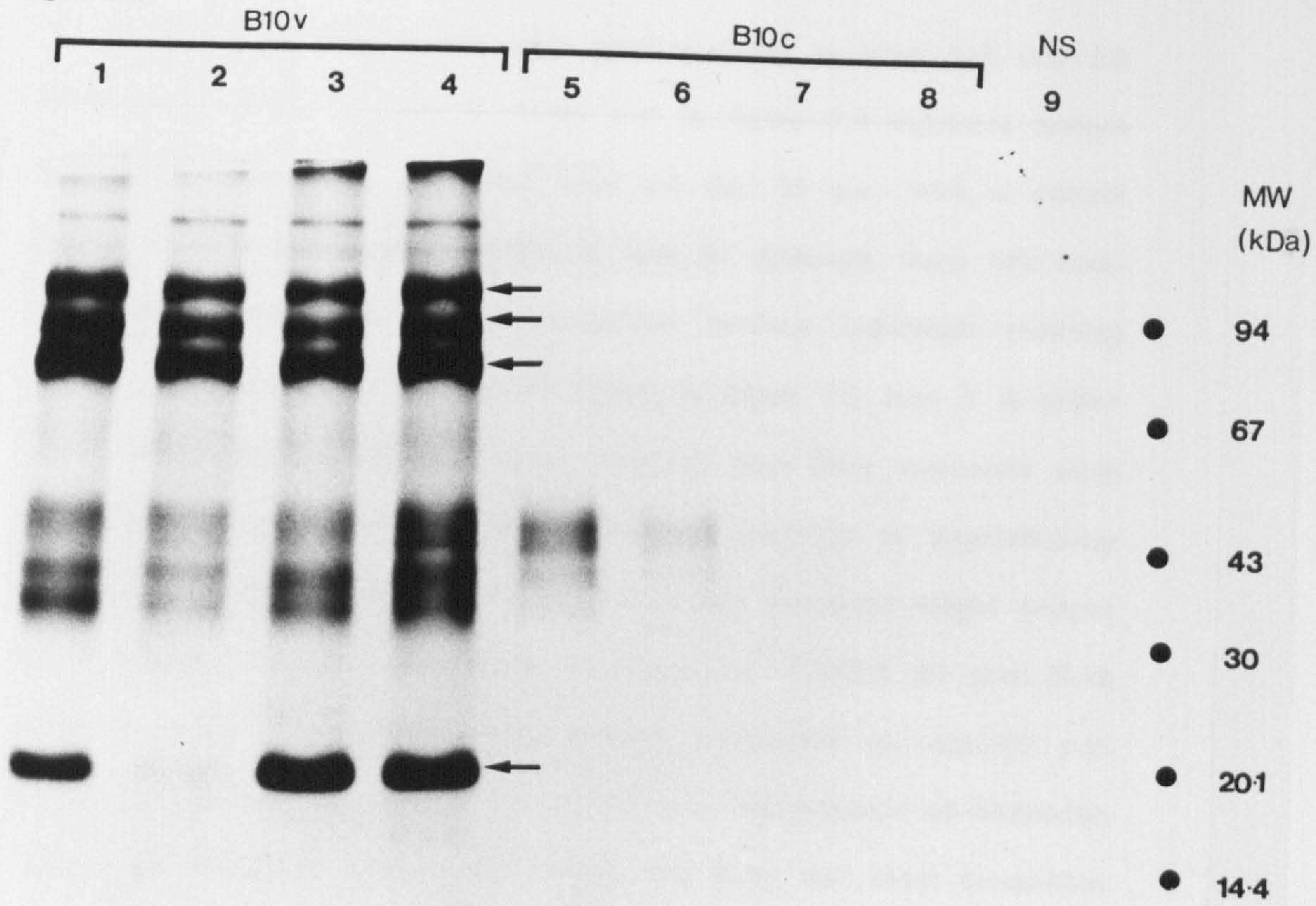
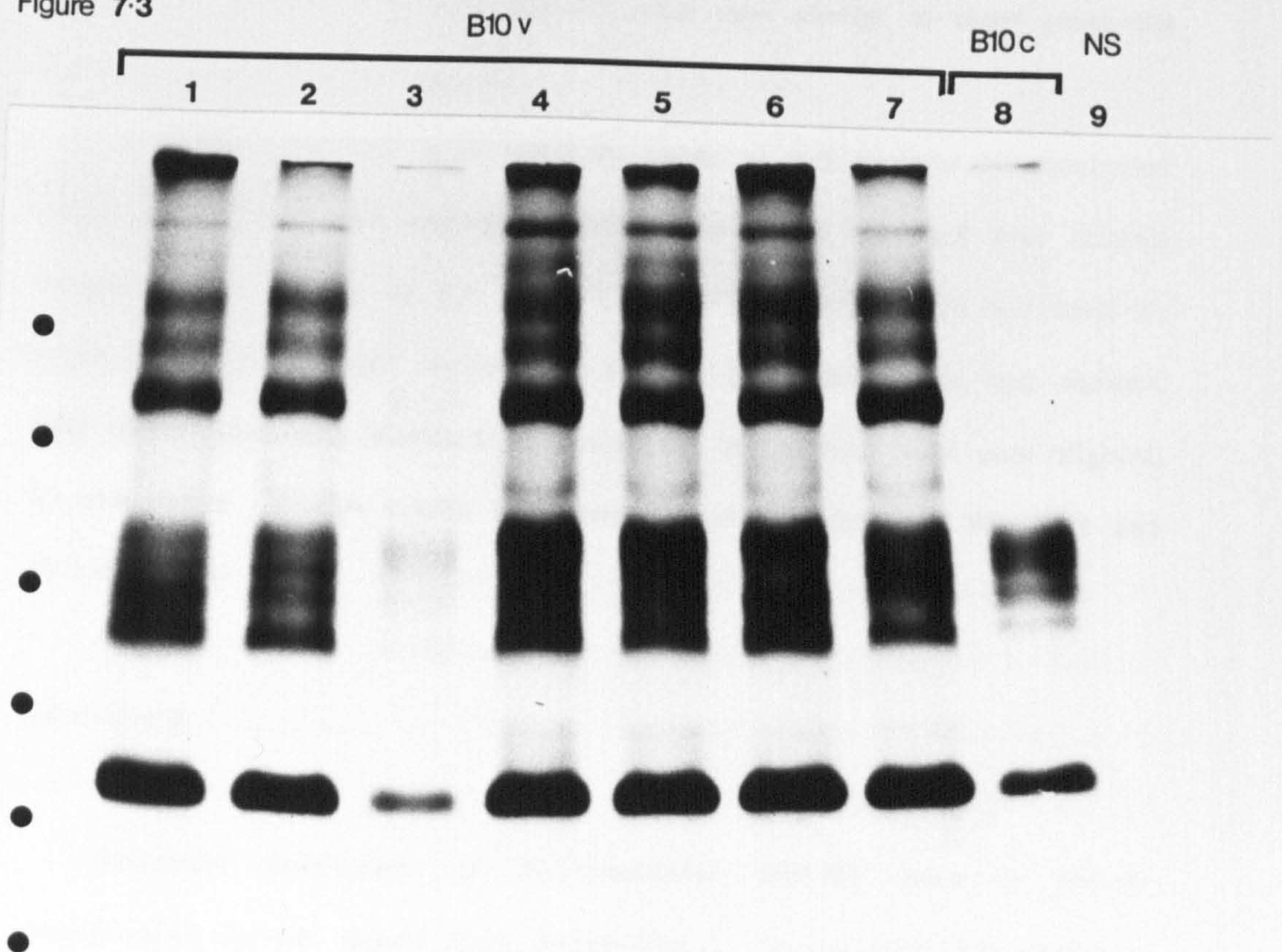


Figure 7·3



1-4 in figure 7.2 with control mice profiles shown in lanes 5-8 and the naive serum profile in lane 9. Lanes 1-7 in figure 7.3 represent antigen recognition profiles for vaccinated mice on day 35 p.c. with a control profile in lane 8 and naive serum in lane 9. Although there was some quantitative variation in antigen recognition between individuals receiving identical treatments, e.g. the profile shown in figure 7.3 lane 3 is rather weak, recognition profiles were infact identical. Sera from vaccinated mice recognised in particular a triplet of antigens resolving at approximately 80-85kDa, 90-95kDa and 105-110kDa and a low molecular weight antigen of 20-23kDa referred to previously in Chapter 4. Profiles for sera from infected control mice showed little antigen recognition on day 19 p.c. (figure 7.2 lanes 5-8) although by day 35 p.c. the presence of antibodies against the 20-23kDa antigen was evident and there was some recognition of antigens in the 43kDa region (figure 7.3 lane 8). No antigens were recognised by naive serum (lane 9 in both figures). The antigen recognition profiles recorded for NIH and B10.BR mice were similar to those presented for experiment 2 of this chapter.

In ELISA IgG and IgG1 antibody levels to E/S antigen in vaccinated NIH, B10 and B10.BR individuals were seen to be elevated over control values although by day 35 p.c. in B10 and B10.BR mice there had been an appreciable rise in both isotypes in control individuals (data not shown). IgM levels were also elevated in vaccinated mice, but only very slightly. Representative ELISA results are presented in experiment 2 for NIH and B10.BR mice.

Experiment 2

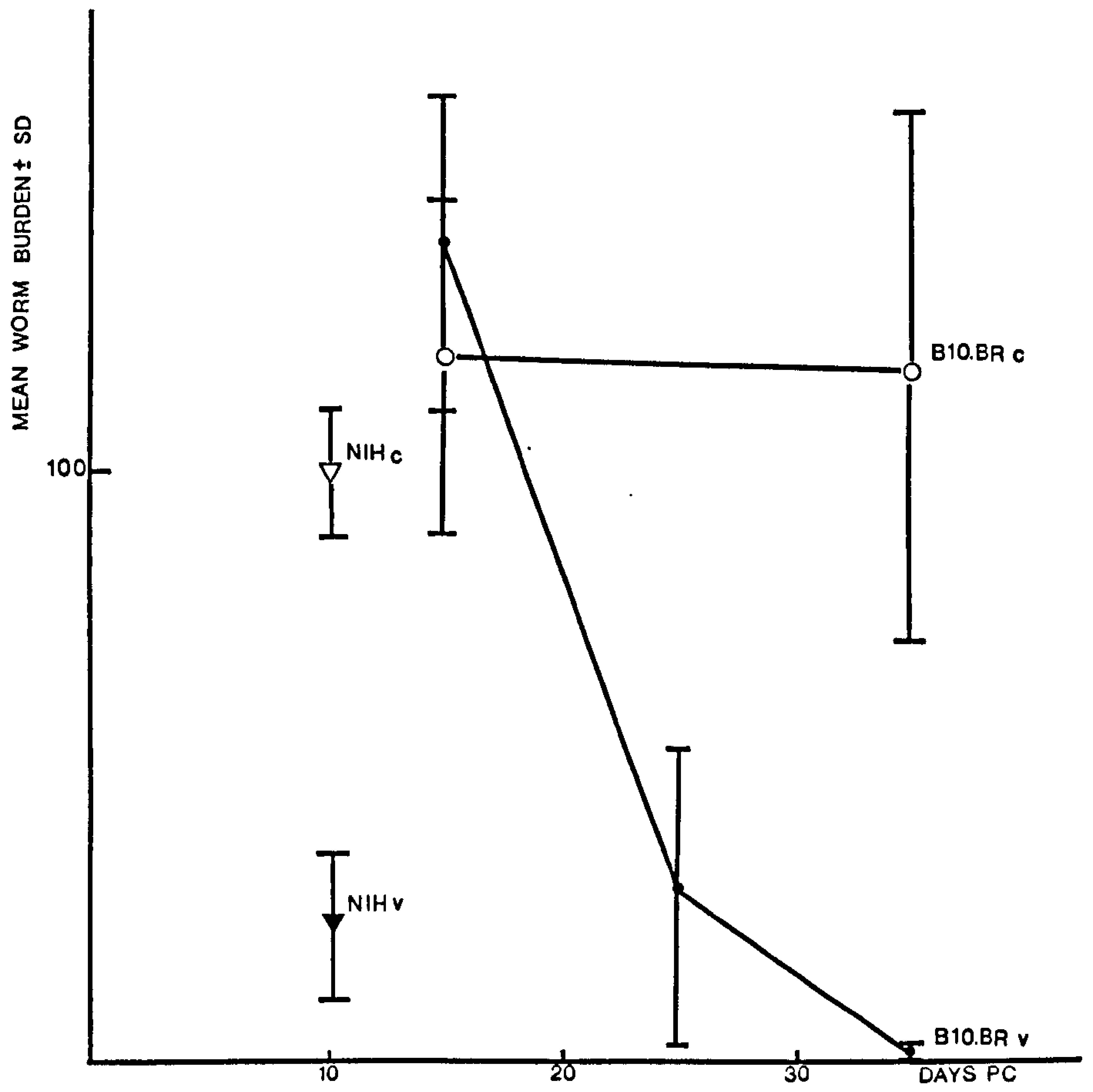
Successful vaccination of non-responder B10.BR mice is clearly demonstrated by the results from experiment 1, worms from the challenge

infection being lost by day 35 p.c. It was considered important to determine how rapidly the protection conferred by vaccination was expressed and therefore a repeat experiment was performed with the worm burdens of vaccinated B10.BR mice being assessed on days 15, 25 and 35 p.c. Mice of the NIH strain were also included to control for the loss of worms from a good responder strain, worm burdens being assessed on day 10 p.c. for vaccinated and control mice.

Figure 7.4 shows the time course of expulsion of *Trichuris muris* from B10.BR mice which had been immunised with 100μg E/S in FCA s.c. ten days before infection with 400 *T. muris* eggs. The worm burdens for vaccinated and control B10.BR mice on day 15 p.c. were not significantly different ($P>0.05$) although the larvae recovered from vaccinated mice were considerably stunted. However, by day 25 p.c. the mean worm burden for vaccinated B10.BR mice had fallen to approximately 21% of the day 15 p.c. levels and by day 35 p.c. very few, if any, worms remained in vaccinated B10.BR individuals, none of these worms being mature. In contrast, on day 35 p.c. all control B10.BR mice harboured numbers of mature adult worms not significantly different ($P>0.05$) from the numbers present on day 15 p.c. Although the day 15 p.c. and day 35 p.c. B10.BR control worm burdens were not significantly different from each other, five control mice killed on day 25 p.c. harboured significantly fewer worms ($P<0.01$) than seen on day 15 p.c. (data not shown). The existence of subpopulations of resistant B10.BR mice makes interpretation of the vaccination data less easy, but for reasons outlined in the discussion the protection seen to be conferred to B10.BR mice by vaccination is believed to be real. Vaccinated individuals of the NIH mouse strain, used as a positive control, exhibited a significant reduction in worm burdens ($P<0.01$) on day 10 p.c.

Analysis of the antigen recognition profiles for vaccinated and control

Figure 7.4 Time course of expulsion of *Trichuris muris* from B10.BR mice which had been immunised with 100μg E/S in FCA s.c. 10 days before infection with 400 *T. muris* eggs. ● , vaccinated B10.BR mice (B10.BRv); ○ , control B10.BR mice (B10.BRc) injected with PBS in FCA ten days prior to infection; ▼, NIH mice injected s.c. with E/S in FCA (NIHv); ▽, control NIH mice (NIHc) injected with PBS in FCA.



MEAN WORM BURDEN ± SD
100
NIH c
NIH v
B10.BR c
B10.BR v
10
20
30
DAYS PC

Figure 7.5 Fluorograph of SDS-PAGE of ^{35}S-metabolically labelled *T. muris* E/S antigen after immunoprecipitation with sera taken days 15, 25 and 35 p.c. from vaccinated (100μg E/S in FCA) B10.BR individuals (BRv) (lanes 1–3), days 15 and 35 p.i. from control B10.BR mice (BRc) (PBS in FCA) (lanes 4 and 5), day 10 p.c. from vaccinated NIH mice (NIHv) (lanes 6 and 7) and day 10 p.i. from a control NIH individual (NIHc) (lane 8). No antigens were precipitated with naive serum (NS) (lane 9). Arrows indicate the 80–85kDa, 90–95kDa and 105–110kDa antigens referred to in the text.

Figure 7·5

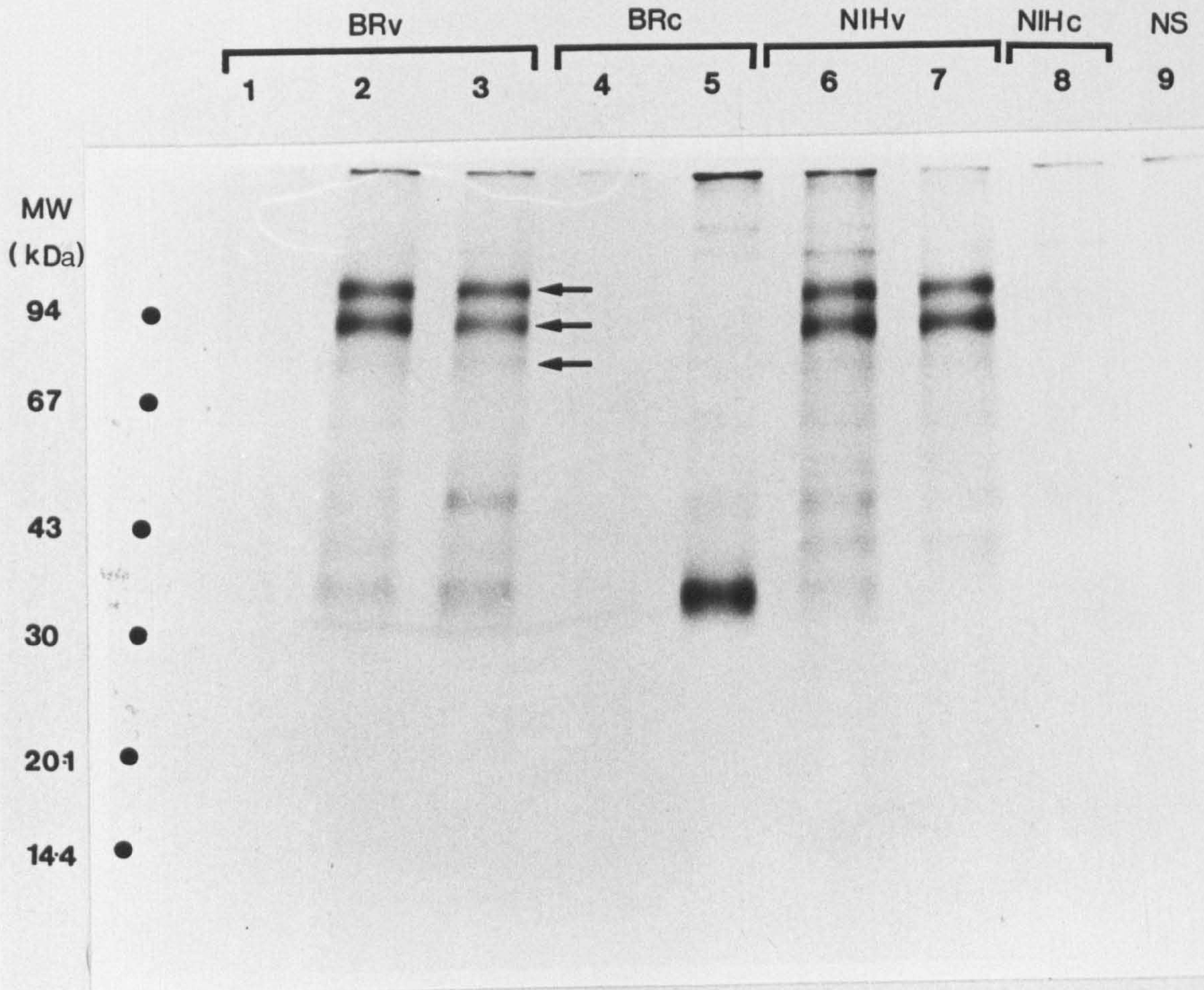


Figure 7.6 IgG1 antibody responses to *T. muris* E/S antigen in serum samples from individual B10.BR and NIH mice vaccinated s.c. with 100μg E/S in FCA (B10.BRv, NIHv) and from control individuals injected with PBS in FCA (NIHc, B10.BRc). N.S. = naive mouse serum; O.D. = optical density.

Figure 7.7 IgG antibody responses to *T. muris* E/S antigen in serum samples from individual B10.BR and NIH mice vaccinated s.c. with 100μg E/S in FCA (B10.BRv, NIHv) and from control individuals injected with PBS in FCA (NIHc, B10.BRc). N.S. = naive mouse serum; O.D. = optical density.

Figure 7·6

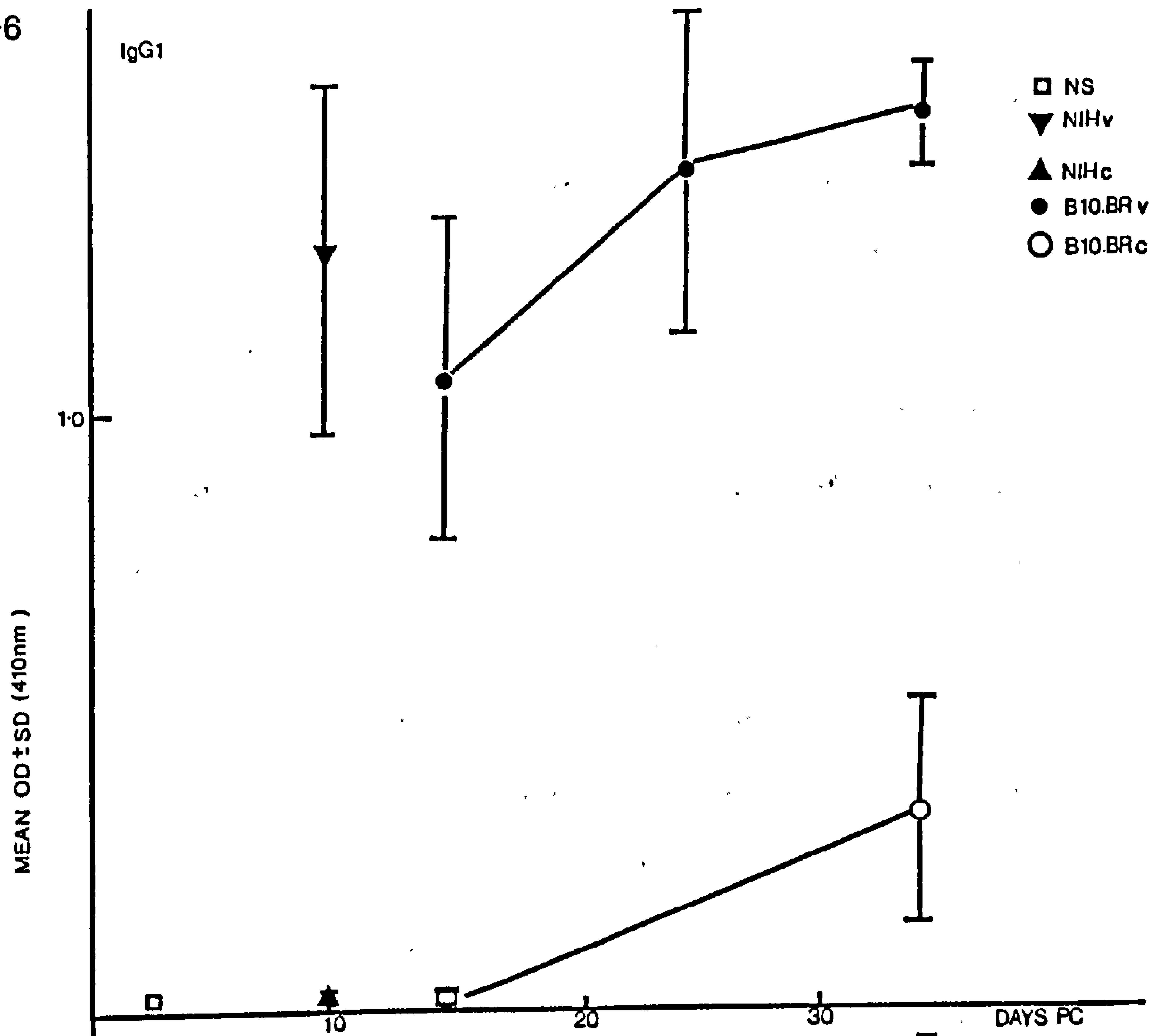
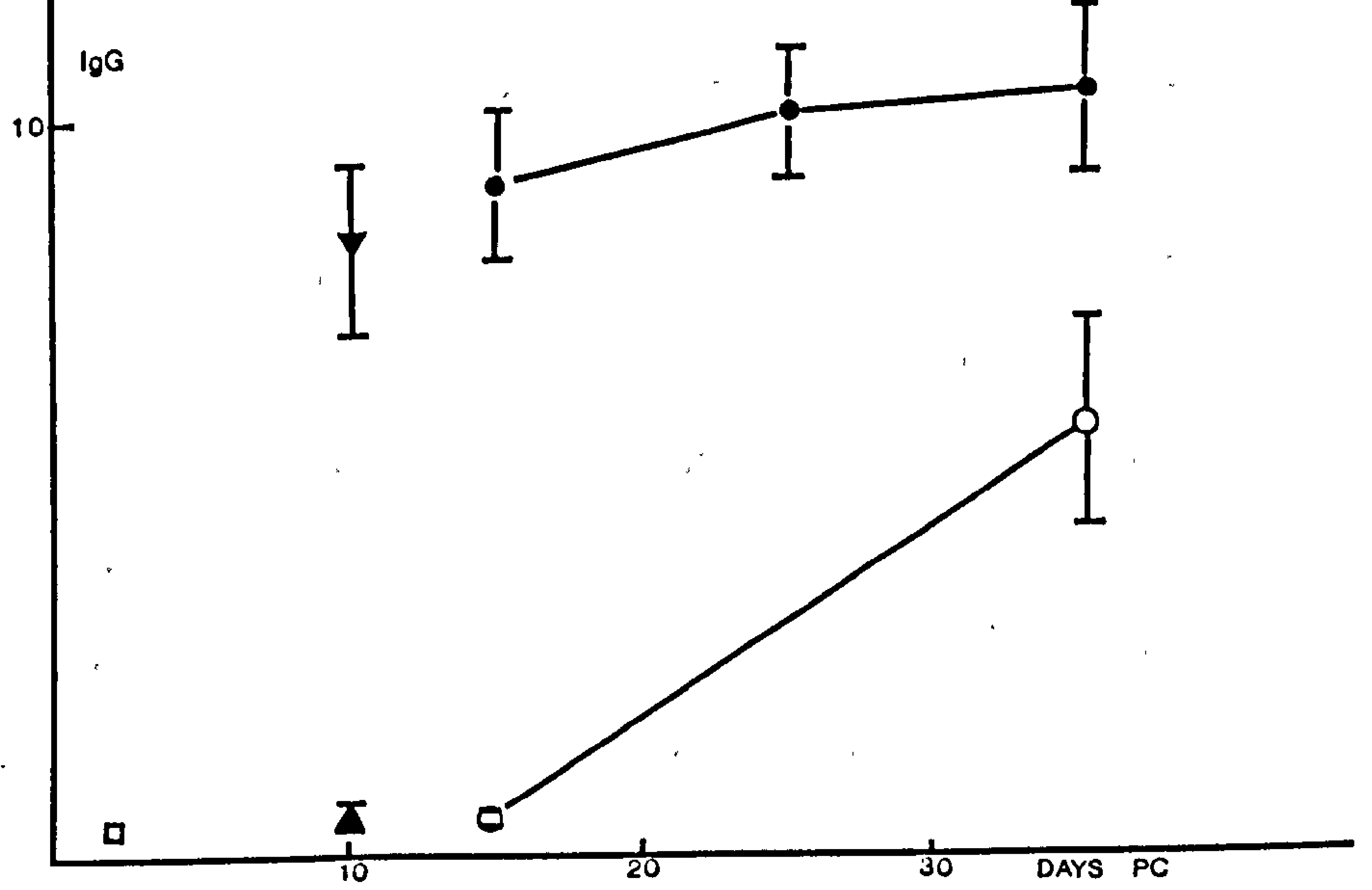


Figure 7·7



B10.BR and NIH mice (figure 7.5) revealed that vaccinated mice of both strains recognised the three high MW antigens (80-85kDa, 90-95kDa, 105-110kDa) (lanes 2 and 3 B10.BR, lanes 6 and 7 NIH) recognised by vaccinated B10 mice (figures 7.2 and 7.3) although here the 80-85kDa antigen was only weakly visualised. Other individuals of the B10.BR strain recognised this antigen more strongly (data not shown). Although none of the three high MW antigens were apparently immunoprecipitated by sera taken day 15 p.c. from vaccinated B10.BR individuals as shown in lane 1 of figure 7.5 (these antigens were apparent by days 25 and 35 p.c, figure 7.5 lanes 2 and 3), analysis of the day 15 p.c. recognition profiles for sera taken from other vaccinated B10.BR individuals did reveal the presence of antibodies against these antigens. Such individual variation in the development of the antibody response to particular antigens has been demonstrated previously (see Chapter 4). Sera from control infected mice for both strains did not recognise the three high MW antigens and showed more limited recognition profiles, especially early on during the course of infection (day 10 p.c. NIH, lane 8; day 15 p.c B10.BR, lane 4). The levels and kinetics of the IgG and IgG1 antibody responses to E/S antigen expressed by vaccinated and control NIH and B10.BR individuals were examined by ELISA. The results are shown in figures 7.6 (IgG1) and 7.7 (IgG). As can be seen, vaccinated mice of both strains had significantly higher levels of specific IgG and IgG1 than did control mice. Levels of IgG1, and particularly IgG, rose in control individuals as the infection proceeded, but even on day 35 p.c. when mature adult worms were present, antibody levels were still well below those seen for vaccinated mice.

The ability of good and non-responder strains of mice to respond to vaccination after exposure to a primary infection

Experiment 3

Figure 7.8 shows the mean worm burdens recovered from vaccinated good (BALB/c) and non-responder (B10.BR) mouse strains after prior exposure to a primary infection. Mice from each strain were divided into three groups; one group received a primary infection of 400 eggs (from which 149.8±46.0 worms established in BALB/c mice, and 167.3±33.4 worms in B10.BR mice on days 11 and 15 p.i. respectively) prior to vaccination and challenge (1°v.2°), a second group was vaccinated before infecting with 400 eggs (v.2°), and the third group served as a vaccination control group being injected with PBS in FCA prior to infection (PBS1°). As can be seen, both vaccinated groups of BALB/c mice, irrespective of prior exposure to infection, had significantly lower worm burdens on day 11 p.c. than the vaccination control mice ($P<0.01$), with mice receiving a primary infection before vaccination harbouring significantly fewer worms than mice only vaccinated before infection ($P<0.01$). In contrast B10.BR individuals experiencing a patent primary infection before vaccination were unable to expel the challenge infection, 6/6 mice harbouring mature adult worms on day 35 p.c. at levels very significantly higher ($P<0.01$) than in the other two groups. Establishment of worms at day 15 p.c. in the challenge infection was also significantly higher ($P<0.05$) in the (1°v.2°) group. As reported earlier in this chapter (expt. 2, figure 7.4), B10.BR mice which had been vaccinated but given no initial infection showed no significant decrease in worm burdens from control levels on day 15 p.c. ($P>0.05$). However by day 25 p.c. a significant decrease ($P<0.01$) from the day 15 p.c. larval burdens was evident, and by day 35 p.c. all worms had been

Figure 7.8 Time course of expulsion in immunised (100μg E/S in FCA) B10.BR mice. Mice were divided into three groups; group 1 experienced a full primary infection for 43 days. Adult worms were removed using an anthelmintic before mice were immunised s.c. ten days before challenge on day 58 p.i. (○ ; 1°v.2°); group 2 were immunised then given a primary infection (△ ; v.2°); group 3 served as vaccination controls, being injected with PBS in FCA prior to infection (▲ , PBS.1°). Good responder BALB/c mice were likewise divided into 3 groups; group 1 = 1°v.2° (●), group 2 = v.2° (▽), group 3 = PBS.1° (▼). Worm burdens were assessed on day 11 p.c. for BALB/c mice and on days 15, 25 and 35 p.c. for B10.BR mice. + = patent infection.

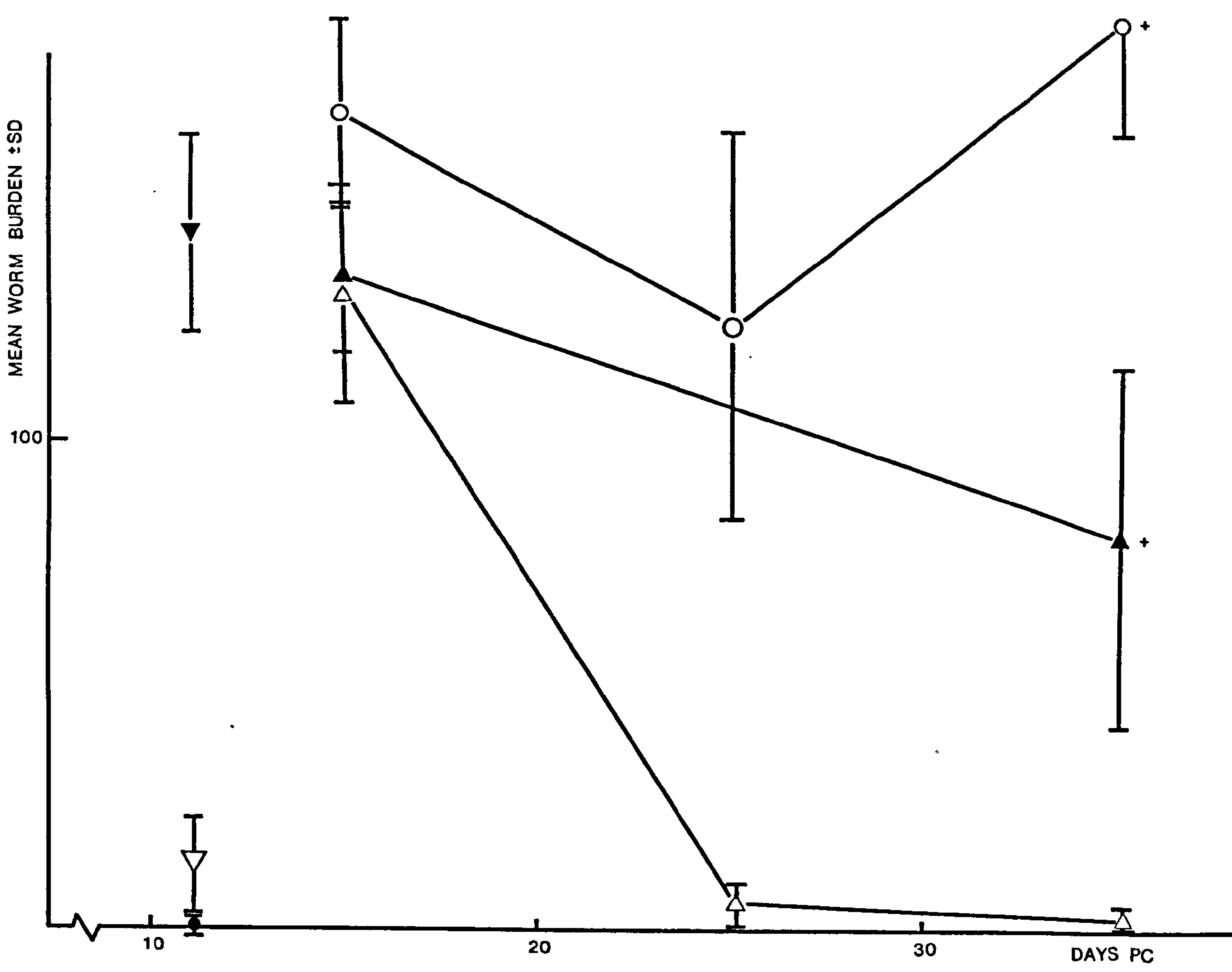

MEAN WORM BURDEN ±SD
100
10
20
30
DAYS PC
+
+

Figure 7.9 Fluorograph of SDS-PAGE of ^{35}S-metabolically labelled *T. muris* E/S antigen after immunoprecipitation with sera taken day 11 p.c. from a BALB/c individual receiving a primary infection prior to vaccination with E/S in FCA and challenge (1°v.2°, lane 1), an individual vaccinated then infected (v.2°, lane 2) and an individual injected with PBS in FCA prior to infection (P.1°, lane 3). Lanes 4 and 5 show the antigen recognition profiles of uninfected individual B10.BR mice using serum taken day 25 post injection with E/S in FCA (v) and PBS (P) in FCA respectively. Lanes 6, 7 and 8 represent the antigen recognition profiles for B10.BR individuals using day 15 p.c. serum samples from mice given a primary infection before vaccination and challenge (lane 6), mice vaccinated then infected (lane 7) and mice injected with PBS in FCA before infection (lane 8). Lane 9 shows the naive serum profile (NS). Arrows indicate the 80-85kDa, 90-95kDa and 105-110kDa antigens (e.g. lane 4), and the 107kDa antigen and the 92kDa antigen doublet (lane 6) referred to in the text.

Figure 7.10 Fluorograph of SDS-PAGE of ^{35}S-metabolically labelled *T. muris* E/S antigen after immunoprecipitation with sera taken day 15 p.c. (lanes 1-3), day 25 p.c. (lanes 4 and 5) and day 35 p.c. (lanes 6 and 7) from individual B10.BR mice. Lanes 1, 4 and 6 represent profiles of individuals from the (1°v.2°) group, lanes 2, 5 and 7 represent profiles of individuals from the (v.2°) group and lane 3 shows the antigens precipitated using serum from a mouse in the (P.1°) group. No antigens were precipitated using naive B10.BR serum (NS) (lane 9). Arrows indicate the 80-85kDa, 90-95kDa and 105-110kDa antigens (e.g. lane 5), and the 107kDa antigen and the 92kDa antigen doublet (e.g. lane 4) referred to in the text.

Figure 7·9

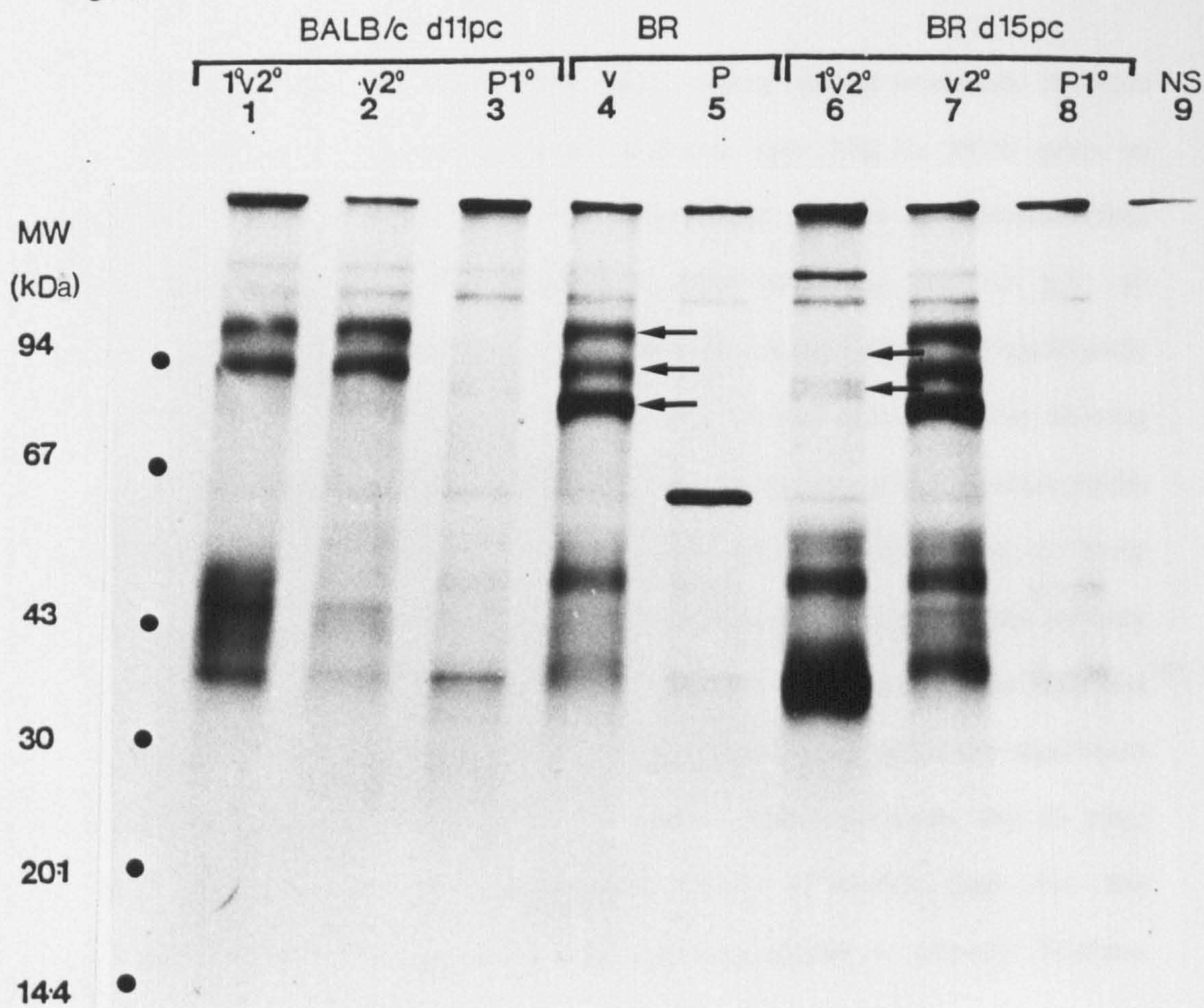
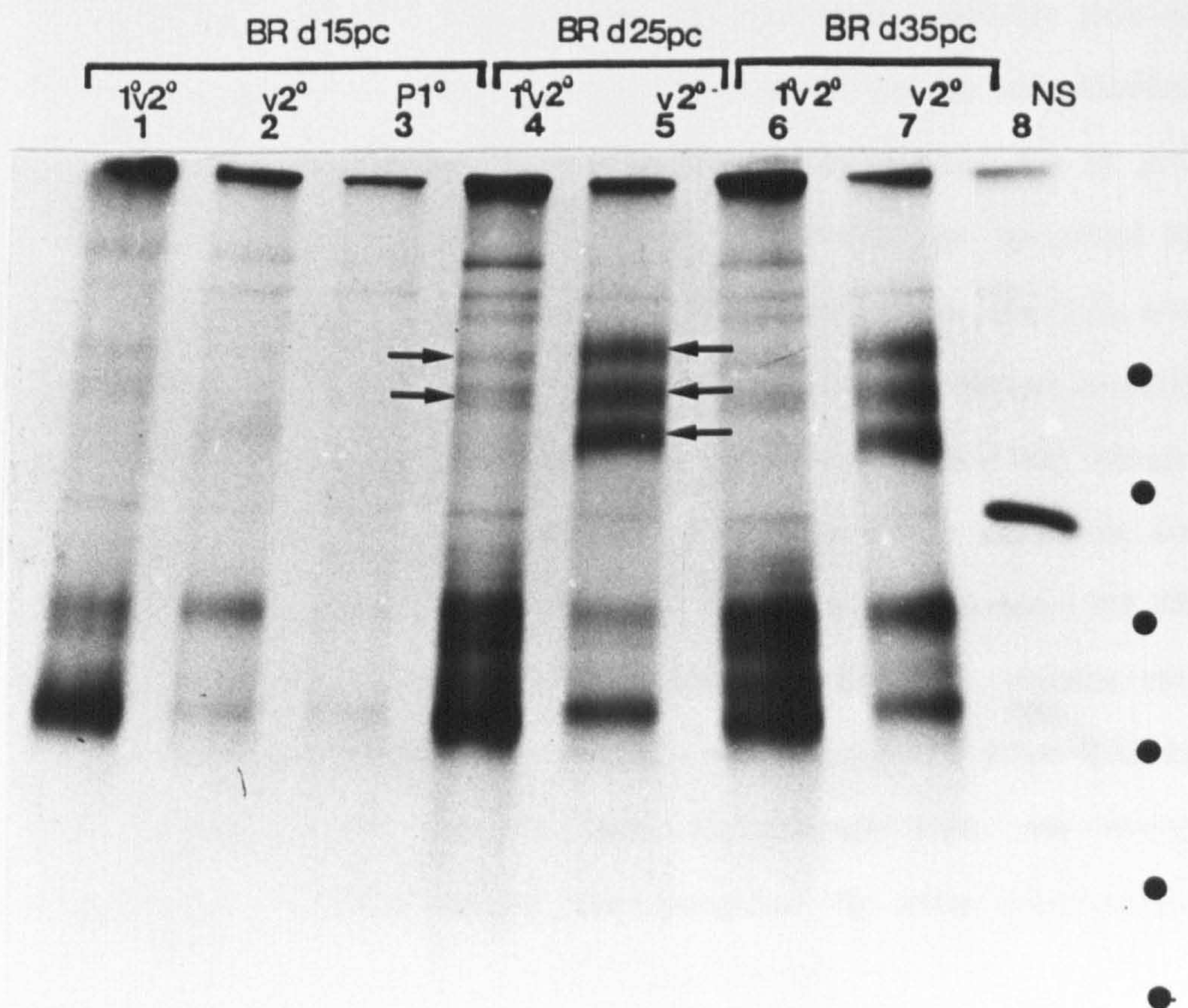


Figure 7·10



lost except in one individual where three stunted worms remained. As seen in experiment 2 five control mice (injected with PBS in FCA prior to infection) killed on day 25 p.c. were apparently resistant to infection (data not shown). However the six control mice killed on day 35 p.c. all harboured mature adult worms. These day 35 control mice had significantly lower worm burdens (P<0.025) than on day 15 p.c. therefore also showing some immunity to a primary infection, although as mentioned, mature adults as well as stunted immature parasites were present. The ability of these non-responder B10.BR mice to expel a significant proportion of the primary worm burden in this experiment may reflect their greater age at infection than the standard 6-8 weeks (see Chapter 6.2). Even with the significant reduction in worm burdens in the vaccination control group by day 35 p.c., worm burdens were still significantly higher (P<0.01) than for the vaccinated group of mice which had not experienced a primary infection before vaccination.

Figures 7.9 and 7.10 show examples of antigen recognition profiles for the BALB/c and B10.BR mice used in this experiment . BALB/c mice of the (1*v.2*) and (v.2*) groups (figure 7.9 lanes 1 and 2) had identical antigen recognition profiles, antibodies in serum samples taken day 11 p.c. recognising 2 high MW antigens (90-95kDa, 105-110kDa) also recognised by vaccinated B10 mice (figs 7.2 and 7.3), vaccinated B10.BR (fig 7.5) and NIH mice (fig. 7.5). BALB/c individuals injected with PBS before infection did not recognise these antigens (lane 3). Identical BALB/c antigen recognition profiles were seen using sera from three other individuals for each of the three treatment groups. Vaccinated, uninfected B10.BR individuals (figure 7.9 lane 4) recognised the three high MW antigens also seen by vaccinated B10 mice in expt. 1, whilst individuals given PBS in FCA but no infection did not (lane 5), although there was strong recognition of a 65kDa antigen also recognised by some naive serum

samples e.g. figure 7.10 (lane 8) and hence judged to be non-specific. The most striking feature revealed by the immunoprecipitation studies for B10.BR mice was that individuals in the (v.2*) group all recognised the 80-85kDa, 90-95kDa and 105-110kDa antigens whereas individuals in the (1*v.2*) group did not, and recognition profiles for individuals in the (PBS1*) group were very limited, especially early on post-challenge. For example, figure 7.9 lanes 6,7 and 8 show the antigen recognition profiles for a (1*v.2*) individual, a (v.2*) individual and a (PBS1*) individual all on day 15 p.c. Although the (1*v.2*) profile revealed the presence of an antigen at 107kDa and a doublet resolving around 92kDa, these antigens appeared to lie between the three high MW antigens recognised by (v.2*) individuals. These (1*v.2*) antigens were only weakly recognised and no antigen corresponding to the 80-85kDa antigen of (v.2*) individuals was ever immunoprecipitated. Subsequent immunoprecipitations using day 15 p.c. sera from 4 other B10.BR individuals from all 3 groups (1*v.2*, v.2*, PBS1*) revealed similar profiles although those of (v.2*) mice for the three high MW antigens were far weaker in most cases than that shown in figure 7.9 (lane 7), e.g. figure 7.10 (lane 2). Clearly quantitative variation between individuals receiving identical treatments exists in the antibody response to the three high MW antigens, something supported by the profiles shown in figure 7.5 (expt. 2) where the three antigens were only apparent on days 25 and 35 p.c. in vaccinated B10.BR mice and the 80-85kDa antigen was only weakly recognised. Typical antigen recognition profiles for sera taken on day 25 p.c. for (1*v.2*) and (v.2*) B10.BR mice are shown in figure 7.10 lanes 4 and 5, with day 35 p.c. serum profiles for (1*v.2*) and (v.2*) individuals in lanes 6 and 7 and the naive serum profile in lane 8. Again, (1*v.2*) individuals failed to recognise the 80-85kDa, 90-95kDa and 105-110kDa antigens seen by (v.2*) mice. Subsequent analysis of two more day 25 p.c. individuals and four more day 35 p.c. individuals for all three experimental

groups confirmed the apparent failure of (1*v.2*) mice to recognise the three high MW antigens. The day 25 and day 35 p.c. profiles also showed more clearly than the day 15 p.c profiles that the antigens recognised by (1*v.2*) individuals did not appear to resolve at the same MWs as the high MW antigens recognised by antibodies in serum from (v.2*) mice.

Figures 7.11 and 7.12 show the kinetics and levels of the IgG and IgG1 antibody responses to E/S antigen for the three treatment groups for both B10.BR and BALB/c mice. IgG and IgG1 antibody levels stimulated by vaccination and by injection of PBS in FCA in the absence of infection are also shown for B10.BR mice. The IgG response (figure 7.11) for BALB/c and B10.BR mice was elevated in both (1*v.2*) and (v.2*) groups to values well above those seen in the (PBS1*) group. Vaccinated uninfected B10.BR mice also had high IgG levels, comparable to those in both (1*v.2*) and (v.2*) groups on day 15 p.c. Levels of specific IgG in (1*v.2*) and (v.2*) B10.BR mice remained considerably higher than in the (PBS1*) group, even though levels had risen slightly by day 35 p.c. in this latter group. Figure 7.12 shows the IgG1 antibody response patterns to E/S antigen. The BALB/c IgG1 response was identical to that seen for IgG with (1*v.2*) and (v.2*) mice having antibody levels elevated well above those of mice in the (PBS1*) group. In contrast the IgG1 responses of B10.BR mice, in particular those of (1*v.2*) individuals, were very different from those seen for total IgG. IgG1 levels were elevated, as were IgG, in the (v.2*) and vaccinated uninfected groups to levels considerably higher than the (PBS1*) group, even on day 35 p.c. However mice in the (1*v.2*) group had IgG1 levels markedly lower than (v.2*) values on both day 15 and day 25 p.c. (IgG levels expressed by mice in these 2 groups were very similar, figure 7.11). Even on day 35 p.c., when the IgG1 levels of mice in the (1*v.2*) group had started to recover, the mean level was still well below that seen for the (v.2*) group.

Figure 7.11 IgG–specific antibody responses to *T. muris* E/S antigen in serum samples from BALB/c and B10.BR individuals in 3 treatment groups. The (1° v.2°) group received a primary infection of 400 *T. muris* eggs prior to vaccination with 100μg E/S in FCA s.c. followed by challenge (△ BALB/c, ■ B10.BR); the (v.2°) group was vaccinated as above then infected with 400 eggs (▽ BALB/c, ● B10.BR) and mice in the (PBS.1°) group were injected with PBS in FCA prior to infection (□ BALB/c, ▲ B10.BR). Antibody levels in serum from vaccinated uninfected B10.BR mice (▼) and B10.BR individuals injected with PBS in FCA (○) are also shown. N.S. = naive serum; O.D. = optical density.

Figure 7.12 IgG1–specific antibody responses to *T. muris* E/S antigen in serum samples from BALB/c and B10.BR individuals in 3 treatment groups. The (1° v.2°) group received a primary infection of 400 *T. muris* eggs prior to vaccination with 100μg E/S in FCA s.c. followed by challenge (△ BALB/c, ■. B10.BR); the (v.2°) group was vaccinated as above then infected with 400 eggs (▽ BALB/c, ● B10.BR) and mice in the (PBS.1°) group were injected with PBS in FCA prior to infection (□ BALB/c, ▲ B10.BR). Antibody levels in serum from vaccinated uninfected B10.BR mice (▼) and B10.BR individuals injected with PBS in FCA (○) are also shown. N.S. = naive serum; O.D. = optical density.

Figure 7·11

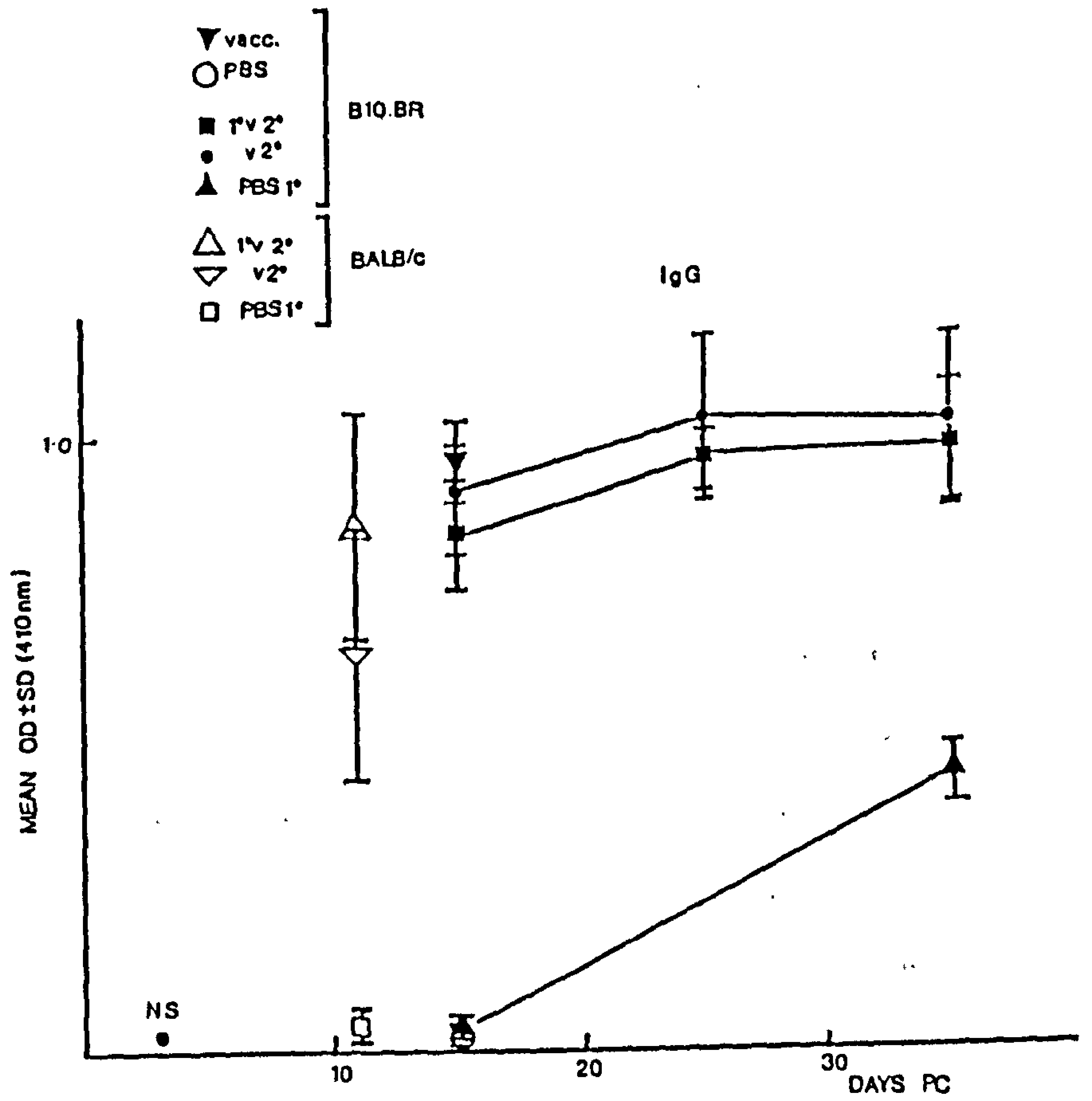
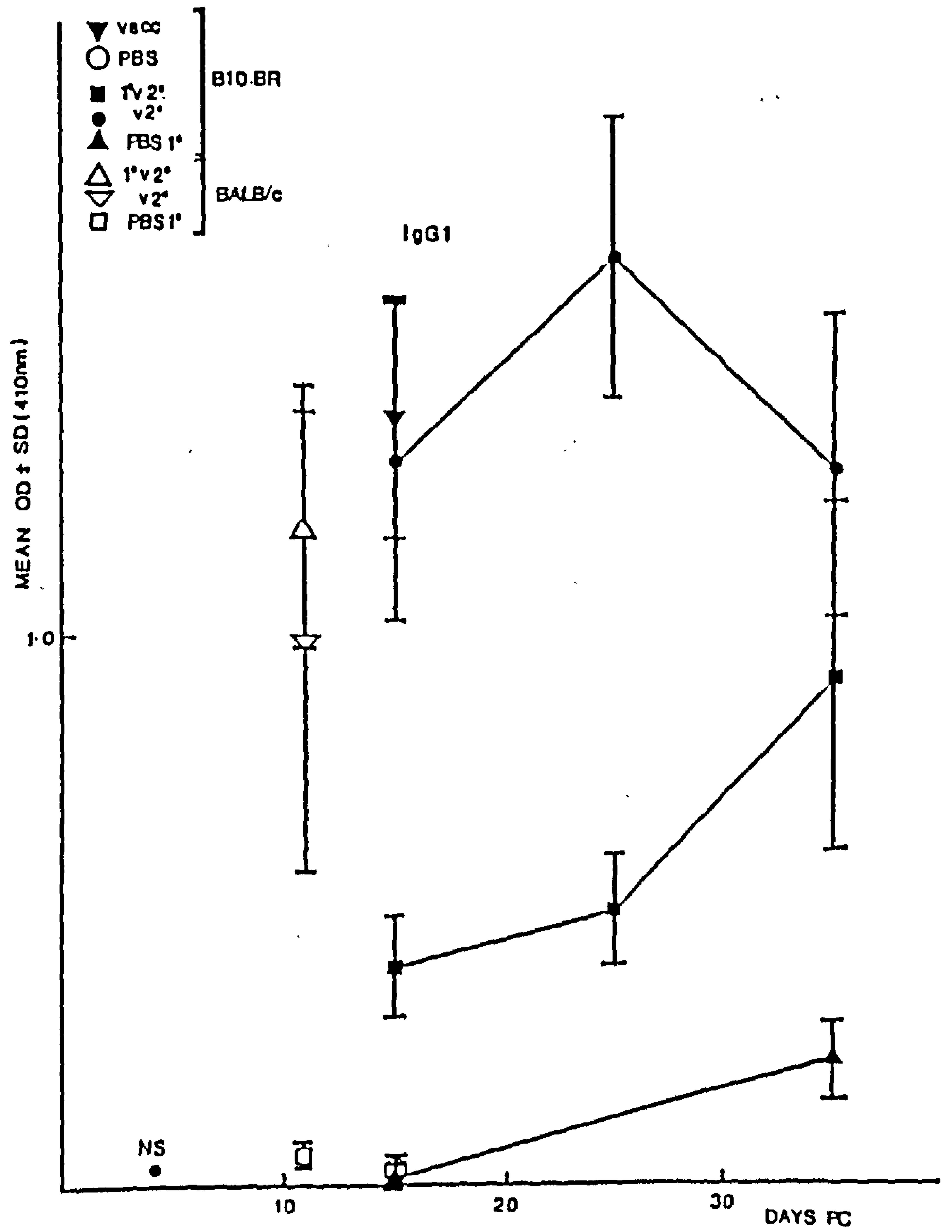


Figure 7·12



Antibody response patterns were highly repeatable, ELISAs carried out on three separate occasions producing similar results.

7.4 DISCUSSION

Later larval and adult stages of *Trichuris muris* are believed to be immunosuppressive (Else *et al.*, 1989), an ability common to many protozoan and metazoan parasites including *Trypanosoma cruzi* (Kierszenbaum *et al.*, 1989), *Ostertagia ostertagi* (Cross and Klesius, 1989), *Brugia malayi* (Piessens *et al.*, 1980; Wadee and Piessens, 1986), *Taenia taeniaeformis* (Leid *et al.*, 1986) and the well-documented *Heligmosomoides polygyrus* (*Nematospiroides dubius*) (Behnke *et al.*, 1983; Pritchard *et al.*, 1984; Pritchard and Behnke, 1985; Monroy *et al.*, 1989), immunosuppression being achieved through a variety of mechanisms. In the case of *T. muris*, mice which have experienced an adult primary infection are unable to expel a challenge infection (Chapter 6) raising the possibility that adult stages may release immunomodulatory factors, something which has been suggested for *H. polygyrus* (Pritchard *et al.*, 1984; Pritchard and Behnke, 1985). Paradoxically, whilst factors associated with adult *T. muris* are apparently immunosuppressive, crude antigen preparations including whole-worm antigen (Wakelin and Selby, 1973) and E/S antigen (Jenkins and Wakelin, 1977; 1983) are very immunogenic. Clearly the fact that antigens may be both immunosuppressive and immunogenic should be borne in mind in the development of vaccines. Previous studies on the ability of adult *T. muris* antigen preparations to stimulate protective immunity were performed in responder strains of mice. A more stringent test of the ability of any parasite product to protect against infection or disease is its use in

susceptible hosts (Mitchell, 1985). Experiments 1 and 2 of this chapter demonstrate that E/S antigen can protect strains of mice which usually respond only poorly (B10), or not at all (B10.BR) to a primary infection. However, in contrast to responder NIH mice, which showed significant reductions in worm burdens in vaccinated individuals on days 9 or 10 p.c., protection was slow to be expressed in B10 and B10.BR mice, 7/12 B10 individuals still harbouring more than 100 worms on day 19 p.c. and the first significant decrease in the worm burdens of vaccinated B10.BR mice not being seen until day 25 p.c. In both experiments 2 and 3 day 25 p.c. control B10.BR mice harboured significantly lower worm burdens ($P<0.01$) than seen on both day 15 p.c. and day 35 p.c., thus representing subpopulations of B10.BR mice naturally resistant to infection. The existence of such differential responsiveness within B10.BR mice, thought to be related to the age of the host (see Chapter 6.2), makes interpretation of the vaccination data less easy, any reductions in worm burdens in vaccinated mice perhaps reflecting natural resistance to infection rather than a direct result of vaccination. However previous experience (Chapter 6.2) has suggested that the proportion of resistant B10.BR individuals within any one population is small (6/20). The results of experiments 1, 2 and 3 presented in this chapter all revealed a significant reduction ($P<0.01$) in the day 35 p.c. worm burdens of vaccinated B10.BR mice relative to control values. This suggests that B10.BR mice can be protected against infection by vaccination; it is highly unlikely that in all three experiments the vaccinated individuals killed on day 35 p.c. were all members of resistant subpopulations of B10.BR mice. IgG and IgG1 levels to E/S antigen were considerably elevated over control values in all vaccinated mice. The injection of E/S antigen in FCA resulted in an apparent change in the antigen recognition profiles of B10 and B10.BR mice (experiments 1 and 2) and BALB/c mice (experiment 3) compared to mice experiencing only a

primary infection. In particular, two high MW antigens (90-95kDa and 105-110kDa), recognised by $H\text{-}2^q$ haplotype strains of mice in a primary infection (Chapter 5), were recognised by all vaccinated strains of mice, including these three non-$H\text{-}2^q$ strains. Three antigens, resolving at approximate MWs 74, 92 and 97kDa are known to be recognised by IgG antibodies in pooled primary infection serum from B10.BR mice on day 26 and day 61 p.i. (Else and Wakelin, 1989; Chapter 4, figures 4.1.5 and 4.1.6). Although these three antigens clearly resolve within a similar region to the two high MW antigens recognised by $H\text{-}2^q$ haplotype mouse strains and the vaccinated B10, B10.BR, NIH and BALB/c mice making differentiation between the antigens difficult, the two sets of antigens were judged to be distinct. This was determined both by calculation of MWs and direct comparison of bands on the autoradiographs (e.g. Chapter 4 figure 4.1.5 lanes 1 (B10.G-$H\text{-}2^q$) and 3 (B10.BR-$H\text{-}2^k$)) and through the use of 5-20% rather than 10-20% gradient gels to give a clearer separation of antigens in the high molecular weight regions. Thus altering the mode and/or route of presentation of E/S antigen seems to lead to a change in reponsiveness of H-2 genotypes to specific determinants. A similar phenomenon has been reported by Cohen *et al.* (1979) in the proliferative response of mouse T cells to determinants on ungulate insulins. The immunopotency of defined determinants on these molecules was found to be regulated by a number of factors including the immune response genes of the immunised mice and the mode of presentation of insulin, on cells or in Freund's complete adjuvant (FCA). In the present case it may be that the increase in immunoglobulin levels provoked by the addition of FCA to the antigen, a phenomenon reported previously by Barth *et al.* (1965), allows the visualisation of the high MW antigens, i.e. a quantitative defect during the primary infection has been overcome by vaccination (see Chapter 5) rather than the presentation of E/S in FCA leading to a qualitative change

in the antigens recognised by antibody. Another possibility is that the route by which the host is exposed to antigen may be critical in determining antigen recognition profiles and in obtaining protective immunity. The principle that the same antigen administered via different routes can provoke distinctive, and in some cases antagonistic, immune responses is well documented, and may reflect differential antigen presentation by cells residing at the sites of antigen introduction, e.g. Mitchell *et al.* (1984) and Liew *et al.* (1985*a*) both working with *Leishmania major* and Jenkins (1977) with *T. muris*. Liew *et al.* (1985*a*) showed that the responsiveness of BALB/c mice to i.v. immunisation with 150,000-rad irradiated or heat killed *L. major* promastigotes could be completely suppressed by prior s.c. injection of the same vaccine.The sequence of events after s.c. immunisation which lead to the inhibitory effect was unclear but appeared to involve a population of splenic T cells (Liew *et al.*, 1985*b*).

Thus the paradoxical situation whereby adult *T. muris* E/S antigen is immunosuppressive when presented to the host at the gut level but highly immunogenic (even though presumably containing immunomodulatory factors) when injected s.c. in FCA may be explained by the involvement of antigen presenting cells residing at the site of antigen introduction, the display of antigen on inappropriate antigen presenting cells resulting in inactivation rather than activation of specific T cells (Jenkins and Schwartz, 1987) or indeed the activation of a T helper cell subset irrelevant to, or detrimental to, the expression of protective immunity. The apparent ability of vaccinated non-$H\text{-}2^q$ mouse strains to recognise antigens recognised only by $H\text{-}2^q$ strains in a primary infection is in accordance with other work demonstrating that through the appropriate use of adjuvants or carrier proteins it is possible to overcome genetic restrictions on the recognition of specific antigens. For instance Del Giudice *et al.* (1986) showed that the strict $H\text{-}2^b$ restriction observed when mice were immunised with a

carrier-free synthetic polymer of the *Plasmodium falciparum* circumsporozoite repetitive epitope could be overcome through the use of a carrier protein.

The boosted IgG1 levels and recognition of the 80-85, 90-95 and 105-110kDa antigens seen for poor (B10) and non-responder (B10.BR) strains may be related to the protection conferred. For instance there may be a critical window of time early in infection during which the presence of antibodies to these antigens is required to render the parasite susceptible to subsequent cellular attack before the immunomodulatory stages of the parasite develop. The results of experiment 3 demonstrated that the ability to protect B10.BR mice by vaccinating with E/S in FCA prior to infection could be abrogated by experience of a patent *T. muris* infection before vaccination. B10.BR mice which had experienced mature adult worms prior to vaccination and challenge were completely unable to eliminate a challenge infection in contrast to BALB/c mice given a primary infection (which was expelled before patency) then vaccinated and challenged. There was also some evidence that experience of a patent primary infection before vaccination and challenge enabled more larvae to establish. The inability of vaccination to protect mice with previous experience of mature adult worms was correlated with suppressed IgG1 levels, particularly on days 15 and 25 p.c., and lack of recognition of the three high MW antigens. As mentioned earlier, there are obvious difficulties in distinguishing between antigens of similar MWs, particularly when comparing between separate experiments, and the restricted antigen recognition profiles revealed for individual serum samples from control infected B10.BR mice on day 35 p.i. (e.g. figure 7.5 lane 5) compared to those seen previously for B10.BR infection sera as early as day 26 p.i. (Chapter 4, figures 4.1.5 and 4.1.6, lane 3) did not help interpretation. The more restricted antigen recognition profiles reported in this chapter may reflect the use of individual as opposed to pooled

serum and/or quantitative differences between individuals in antibody levels produced against these antigens. Whatever the problems with identifying differences between antigens lying within a restricted MW range, the results from this experiment show clearly that mice in the (1*v.2*) group had recognition profiles on days 25 and 35 p.c. different from mice vaccinated and infected without prior exposure to adult worms, only the latter being protected by vaccination. The importance in resistance of the early development of high levels of IgG1 antibody to E/S antigens has been indicated previously by the high levels of IgG1 produced by the relatively resistant H-2^q strains of mice (Chapter 5, figure 5.3) and by the considerably higher IgG1 levels seen in B10.BR mice resistant to both primary and challenge infections on day 15 p.c. compared to those individuals susceptible to both primary and challenge infections (Chapter 6, figure 6.2.3). Thus adult stages of *T. muris* may exert their immunomodulatory effects in B10.BR mice by suppressing the specific IgG1 response to E/S antigen and/or blocking the recognition of the 80-85, 90-95 and 105-110kDa antigens. The mechanisms by which IgG1 antibodies could contribute to protective immunity are discussed in Chapter 5. Total specific IgG antibody levels to E/S antigen in (1*v.2*) and (v.2*) mice were similar, even though IgG1 levels were depressed in the former group. This suggests that levels of a different IgG isotype were increased in (1*v.2*) mice compared to (v.2*) mice, this isotype (IgG2a?) perhaps being irrelevant to the development of protective immunity (see Appendix to Chapter 5) and so also contributing to the non-responder status of (1*v.2*) mice.

Although many parasites are known to be immunosuppressive, most mechanisms of immunosuppression have not been fully elucidated. *Trypanosoma cruzi* has been shown to release soluble mediators which suppress Il-2 receptor expression by human lymphocytes leading to suppressed lymphocyte proliferation and hence inhibition of an effective

immune response (Kierszenbaum *et al*, 1989). Also, schistosome-derived inhibitory factors of mast cell degranulation (Mazingue *et al.*, 1980) and cytotoxic T cell activity (Mazingue *et al.*, 1983; 1986) have been identified. Enhancement of the number and function of suppressor T cells has been indirectly implicated in the modulation of host immunity by a number of parasitic nematodes including *H. polygyrus* (Pritchard *et al.*, 1984) and *Brugia malayi* (Piessens *et al.*, 1980). A partially pure larval antigen of *Ostertagia ostertagi* has been shown to suppress antibody production, although whether this suppression was via the inductive phase of antibody production e.g. macrophage processing of antigens, or the productive phase, including T and B cell activity, was not determined (Cross and Klesius, 1989). Low molecular weight immunosuppressors secreted by adult *H. polygyrus* are known to inhibit the proliferation of mitogen and E/S stimulated mouse spleen lymphocytes from normal and infected mice (Monroy *et al.*, 1989), and a proteinase inhibitor isolated from the larval stage of the cestode *Taenia taeniaeformis* has been shown to inhibit endogenous Il-2 generation in murine lymphocytes and Il-1 induced proliferation of murine thymocytes (Leid *et al.*, 1986). In the light of these documented immunosuppressive mechanisms it is possible to speculate as to how adult *T. muris* worms suppress the IgG1 antibody response to vaccination and challenge and block the recognition of the three high MW antigens in B10.BR mice. For instance IgG1 antibody production is believed to be under the control of Il-4 (Vitetta *et al.*, 1985; Snapper *et al.*, 1988), therefore suppressed IgG1 levels could result from depressed Il-4 secretion or suppressed Il-4 receptor expression. Alternatively a defect may arise at the level of antigen processing by antigen presenting cells, or suppressor T cells may be induced by the adult parasite antigens which suppress the potential B cell response. It is also possible that adult parasite antigens are recognised by cytolytic T cells in a class-II-restricted fashion resulting in

the selective killing of antigen-specific B cells and hence specific suppression of the antibody response (Shinohara *et al.*, 1988; Lanzavecchia, 1989). In contrast to the down regulation of the protective immunity induced by s.c injection by prior exposure to adult stages of *T. muris* involving suppressed IgG1 production, evidence from the *L. major* vaccination model (Liew *et al.*, 1985*b*) suggested that the inhibitory effects conferred by s.c. rather than i.v. immunisation involved Lyt 1^+2^-, $L3T4^+$ T cells that mediated cutaneous DTH and actually helped antibody synthesis yet prevented the induction and expression of protective T cells.

Whatever the mechanism by which *T. muris* adults modulate the host immune response it is evident that protection of poor responder strains of mice by vaccination is possible, but only if mice have not experienced the immunomodulatory stages of the parasite in a previous infection. If the mouse model can be extended to the human situation implications for immunisation strategies, should they become possible, in regions where the human parasite *T. trichiura* is endemic are obvious; vaccination programmes should be targetted at the younger age classes, before individuals become heavily infected, in order to elicit appropriate immune responses in the relative absence of adult worm immunomodulatory factors.

7.5 SUMMARY

1. Poor (B10) and non-responder (B10.BR) strains of mice were protected against infection by vaccination with 100μg E/S in FCA s.c. Protection was slow to be expressed compared to vaccinated good responder strains of mice.

2. Vaccination of B10 and B10.BR mice with E/S in FCA s.c. boosted their IgG and IgG1 antibody responses to E/S antigen and altered their antigen recognition profiles, three high MW antigens (80-85kDa, 90-95kDa and 105-110kDa) being recognised by antibodies in sera from vaccinated mice which were not recognised by antibodies in sera from mice receiving just a primary infection.

3. The mechanisms by which adult *T. muris* E/S antigen is on the one hand immunosuppressive, when presented at the gut level, and immunogenic when presented s.c. in FCA, may involve different populations of antigen presenting cells residing at the site of antigen introduction which preferentially stimulate effector or suppressor T cell subsets.

4. As the recognition of the 90-95 and 105-110kDa antigens was previously shown to be $\underline{H\text{-}2}^{q}$ restricted during a primary infection (Chapter 5), altering the mode or route of presentation of E/S antigen can apparently lead to shifts in responsiveness of H-2 genotypes to specific determinants and/or boost specific antibody levels sufficiently to reveal the recognition of these antigens.

5. B10.BR individuals which had experienced a patent primary infection could not be protected against challenge infections by vaccination and this inability was correlated with suppressed levels of IgG1, but not total IgG, antibodies to E/S antigen early on post-challenge compared to vaccinated infected individuals which had not seen an adult primary infection, and lack of recognition of three high MW antigens (80-85kDa, 90-95kDa and 105-110kDa) recognised by antibodies in sera from vaccinated infected mice.

6. It is suggested that the rapid development of high levels of IgG1

antibodies and the recognition of the three high MW antigens may be important in protective immunity and that immunomodulation of host immunity by *T. muris* is achieved, at least in part, by the suppression of specific IgG1 levels, possibly the production of an irrelevant IgG isotype (?IgG2a), and prevention of the recognition of the three high MW antigens, the former and latter perhaps being related phenomena.

SECTION THREE

ANTIGENS OF *TRICHURIS MURIS*

CHAPTER 8

ANTIGENS OF T. *MURIS*: IMMUNOHISTOCHEMICAL LOCALISATION USING MONOCLONAL ANTIBODY PROBES AND ABILITY TO CONFER PROTECTION *IN VIVO*.

8.1 INTRODUCTION

Monoclonal antibodies (McAbs) have proved to be powerful tools in the analysis, characterisation, isolation and purification of nematode antigens. This is exemplified by studies on the antigens of *Trichinella spiralis* where panels of McAbs have been raised against different life stages of *T. spiralis* (Ortega-Pierres *et al.*, 1984*b*; Silberstein and Despommier, 1984; Gamble and Graham, 1984). These monoclonals have been used to isolate and purify *Trichinella* antigens which have then been examined for their ability to confer protection *in vivo* (Silberstein and Despommier, 1984; Gamble, 1985), to passively transfer protective immunity (Ortega-Pierres *et al.*, 1984*a*) and to localise antigens on the surface and within parasite tissues (Silberstein and Despommier, 1984; Gamble and Graham, 1984; Capó *et al.*, 1986; Ortega-Pierres *et al.*, 1984*b*; 1986; McLaren *et al.*, 1987). Of particular interest was the work of Silberstein and Despommier (1984) who identified two highly protective polypeptides of MW 48kDa and 50-55kDa in muscle larvae and localised them to the β- and α-stichocytes respectively as well as on the cuticle surface and gut lining. This work thus supported the earlier work of Despommier and Müller (1970*a*, 1976) suggesting that protective muscle larvae antigens were derived primarily from the secretary granules of the stichosome. In contrast to the extensive studies on *T. spiralis* antigens work is limited on the antigens of *Trichuris muris*, a closely related trichuroid nematode. Crude vaccination experiments using antigens derived from the anterior (oesophageal) region of adult worms have suggested that the stichosome is, as in *T. spiralis*, a source of protective antigens (Wakelin and Selby, 1973; Jenkins and Wakelin, 1977). However the informative antigen localisation studies (*loc. cit.*) performed for *T. spiralis* have not been applied to the *T. muris*-mouse system.

A panel of McAbs to *T. muris* E/S has been produced and characterised with respect to isotype and antigen specificity (Roach, 1986). One of these McAbs, of the IgA isotype, was capable of passively transferring immunity to infection, and an affinity isolated antigen preparation, using this McAb was used successfully in active immunisation experiments (Roach, 1986).

The logical progression from this work was to characterise the McAbs' target antigens within the tissues of the nematode and thus identify the source(s) of protective antigens. This has been attempted at the light microscope level using a variety of immunohistochemical techniques on three types of tissue sections; wax, frozen and JB4 sections, retention of tissue morphology being perhaps greatest in the latter type of tissue section. The protein composition of parasite components localised within the worm were investigated and their ability to confer protection *in vivo* analysed in a series of vaccination experiments.

8.2 EXPERIMENTAL DESIGN

8.2.1 IMMUNOHISTOCHEMISTRY

Preliminary studies on the localisation of antigens within the tissues of adult *T. muris* recovered on day 42 p.i. were performed using wax sections. The presence of surface antigens was also investigated using live adult worms recovered from mice with patent infections. In both cases McAbs bound to the parasite were visualised using a fluorescein-conjugated goat anti-mouse-immunoglobulins reagent (Cappel). Subsequent studies were carried out using frozen sections in collaboration with Dr. R. K. Grencis at

Manchester University and JB4 sections in collaboration with Dr. D. J. McLaren at Mill Hill, London. In both cases bound antibody was visualised by immunoperoxidase labelling using peroxidase-conjugated rabbit anti-mouse immunoglobulins (DAKO), by alkaline phosphatase labelling using phosphatase-conjugated rabbit anti-mouse immunoglobulins (DAKO), or by immunofluorescence using a fluorescein-conjugated rabbit anti-mouse IgG (H+L) (Miles-Yeda Ltd.).

McAbs were used in the form of tissue culture S/Ns. Naive (NS) and tolerant serum (TS) (from mice with patent infections) were used throughout as negative and positive controls. An anti-phosphorylcholine McAb diluted 1/100 and 1/500 was included in some experiments to control for the recognition of phosphorylcholine determinants by the anti-*T. muris* E/S McAbs. Other control slides, included routinely, were treated with PBS, an irrelevant McAb, or tissue culture S/N from the myeloma line P3NS1 used for production of the McAbs. Initially a range of dilutions was tested, for both S/Ns and sera. Subsequently S/Ns were applied neat to all sections and sera diluted 1/50 in PBS before use.

A brief summary of the biochemical specificities of the probes tested on sections of the parasite is given in Table 8.1. The F11 McAb was found to successfully passively transfer immunity to infection and F11 affinity isolated antigens were effective in actively immunising mice against *T. muris*.

Table 8.1 Isotype and antigen specificities of the three McAbs used in the immunohistochemical studies presented in this chapter. Antigen specificities were determined by immunoprecipitation using ^{35}S-methionine labelled adult E/S antigen (Roach, 1986). MWs are in kDa.

McAb	Isotype	Antigen Specificity
E12	IgG1	111, 88, 65, 50, 45, 40, 36, 18
A16	IgA	111, 45, 40
F11	IgA	34, 20, 18

Results of the immunohistochemical studies presented are from several replicate experiments carried out on separate occasions. Staining patterns are illustrated by results obtained using JB4 sections where retention of tissue morphology was high. Descriptions of antigen localisation patterns for all experiments, using wax, frozen and JB4 sections, are also given.

8.2.2 VACCINATION EXPERIMENTS

Certain worm components localised by the McAbs were isolated as described in Chapter 2.2. The vaccination protocol was as outlined in Chapter 2.1.6.

8.2.3 STATISTICS

The significance of differences in worm burdens between vaccinated and control groups of mice were determined using the Mann-Whitney U test. A value greater than P=0.05 was considered non-significant.

8.2.4 ANTIGENS OF *T. MURIS*

The protein compositions of various antigen preparations were investigated using 10-20% SDS polyacrylamide gradient gels and Coomassie blue staining (Chapter 2.3.2). ^{125}I-surface labelling of proteins was carried out as in Chapter 2.2.8.

8.3 RESULTS

8.3.1 IMMUNOFLUORESCENT LABELLING OF LIVE WORMS AND IMMUNOFLUORESCENCE USING WAX SECTIONS

Intense fluorescence of the adult male and female worm cuticle was observed after worms were incubated with TS and the McAbs E12 and A16, bound antibody being visualised using a fluorescein-conjugated affinity-purified goat anti-mouse immunoglobulins reagent. However control worms, treated with NS or PBS showed similar fluorescence suggesting that the adult worms may have already been coated with antibody on removal from the gut or perhaps that the cuticle was autofluorescing.

Staining of wax sections using E12 and A16 revealed recognition of the external cuticle, a region within the eggs of adult female worms, the gut lining and the stichosome. Control sections showed negligible fluorescence. However retention of tissue morphology was not good and it was impossible to determine which layer of the egg was being recognised by the McAbs or whether the fluorescence of the stichosome was due to recognition of stichocyte contents.

8.3.2 IMMUNOPEROXIDASE AND IMMUNOFLUORESCENT LABELLING OF JB4 SECTIONS.

Sections of the anterior stichosomal region of male and female worms, the posterior region of females (severed from the anterior end at the oesophageal-intestinal junction) and the posterior ends of males were incubated with McAb S/Ns, positive and negative sera and other control reagents.

Examples of the results obtained by immunoperoxidase labelling are shown in figures 8.1 (A, B and C), 8.2 (A, B and C) and 8.3 (A, B, C and D).

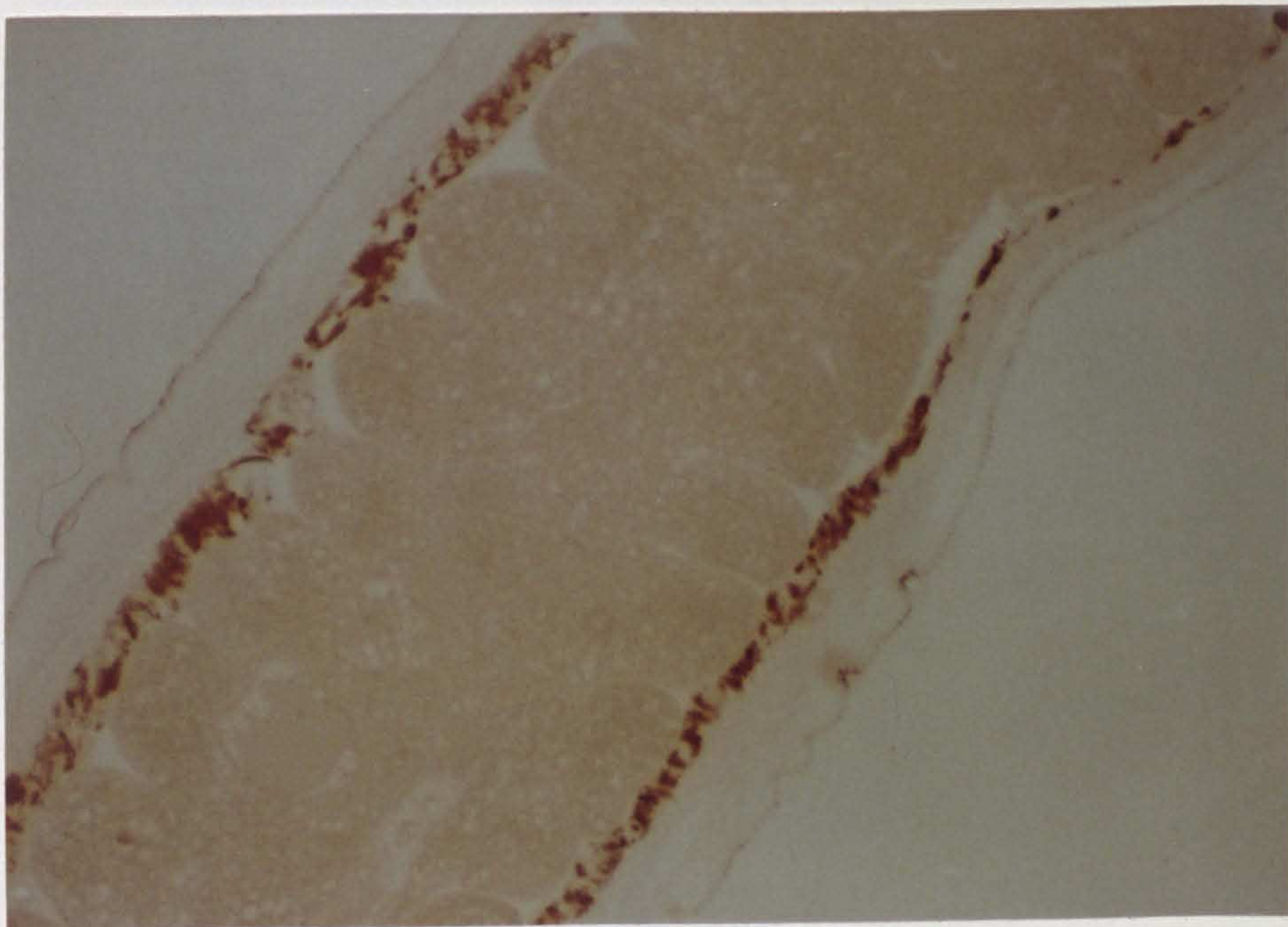
Figure 8.1 shows staining patterns for anterior ends of worms. 8.1A represents the staining obtained with E12, 8.1B with N.S. and 8.1C with TS. All three McAbs, E12, A16 and F11 showed reactivity with the stichocytes, the granular appearance illustrated in 8.1C being typical of the staining pattern. F11 reacted less strongly with the stichocyte granules than the other two McAbs but was none the less clearly positive. Incubation of sections with A16, E12 and TS produced a layer of reactivity on the outside of the cuticle suggesting the recognition of an epitope expressed by surface antigens. The non-contractile regions of the body wall muscle stained intensely with the three McAbs and TS, but low level reactivity was also seen with NS implying that this recognition was non-specific. Interestingly, sections through the more anterior, smaller stichocytes, which had a more vacuolated appearance than the larger posteriorly situated stichocytes, were stained less intensely by E12, A16, F11 and TS than the larger stichocytes, perhaps suggesting some sort of regional localisation of antigens within the stichosome. No staining of the bacillary cells was observed.

Staining of sections through the posterior regions of female worms showed the major site of reactivity for A16, E12 and TS to be the inner lipid layer of the egg (fig. 8.2A - A16, B - NS and C - E12). The unembryonated egg material also stained positive, particularly with E12 and TS, but the egg shell was negative in all cases. No staining was detected with F11 or NS. Other tissues within the posterior regions of adult female worms also showed reactivity with E12, A16 and TS as illustrated in fig. 8.3A-D. Figure 8.3A reveals the localisation of antigens by A16 (also seen by E12 and TS) to the developing oocytes and the outer cuticle. Strikingly

Figure 8.1 JB4 sections of the stichosome of *Trichuris muris*. 8.1 A (x700) was incubated with McAb E12 followed by peroxidase-conjugated rabbit anti-mouse immunoglobulins (Ig). The section was developed using the substrate 3,3 diaminobenzidine tetrahydrochloride. Section 8.1 B (x700) was incubated with naive serum (NS) and 8.1 C (x3500) with tolerant serum (TS) then treated as in 8.1 A. Granules within the stichocytes stained densely with E12 and TS and a fine layer of reactivity on the cuticle suggested that there was recognition of surface antigens also. The dense staining of the body wall inner muscle seen with E12 and TS was judged to be nonspecific, NS also recognising this region, albeit less strongly.

Figure 8·1

A



B

C

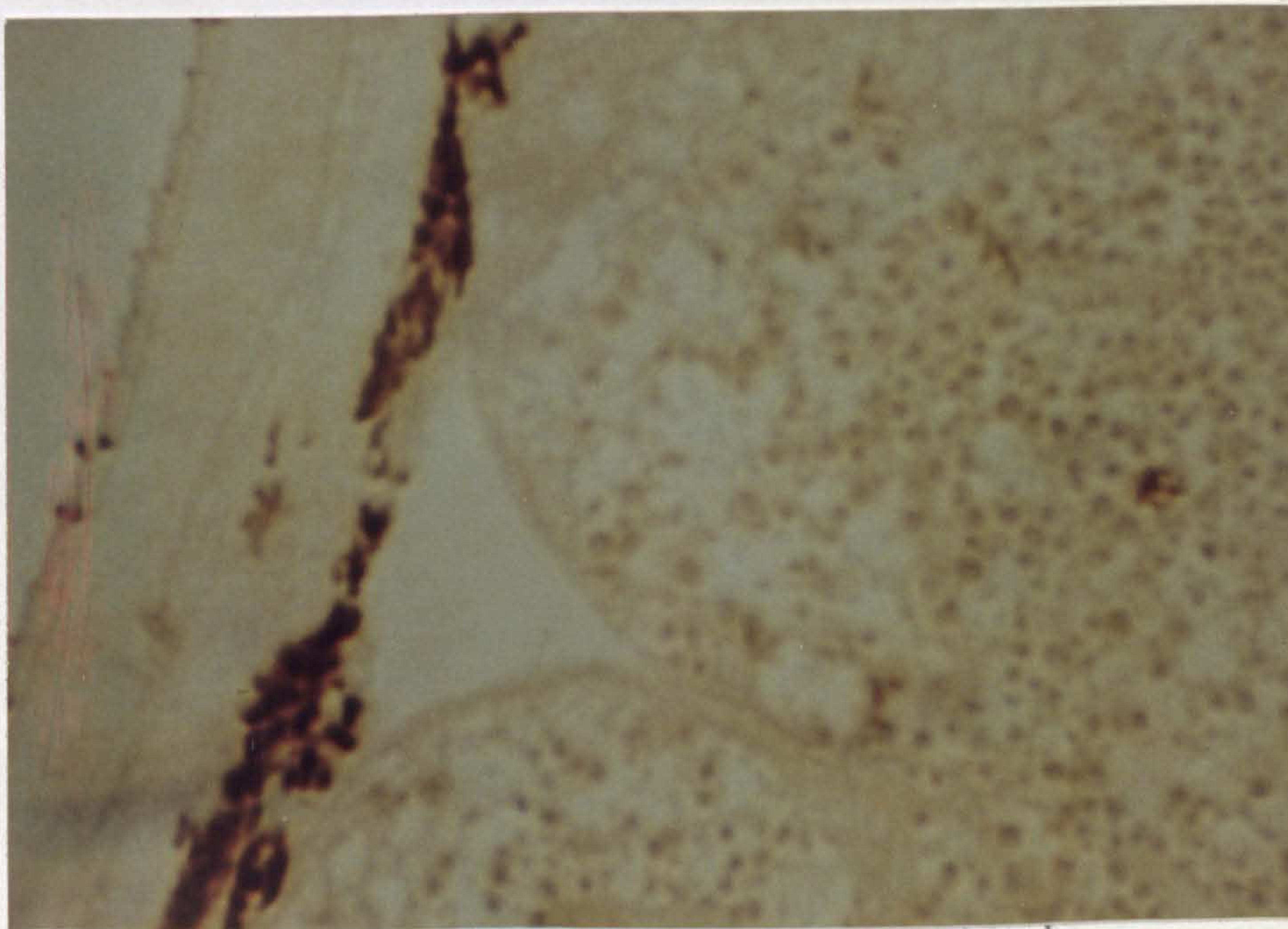
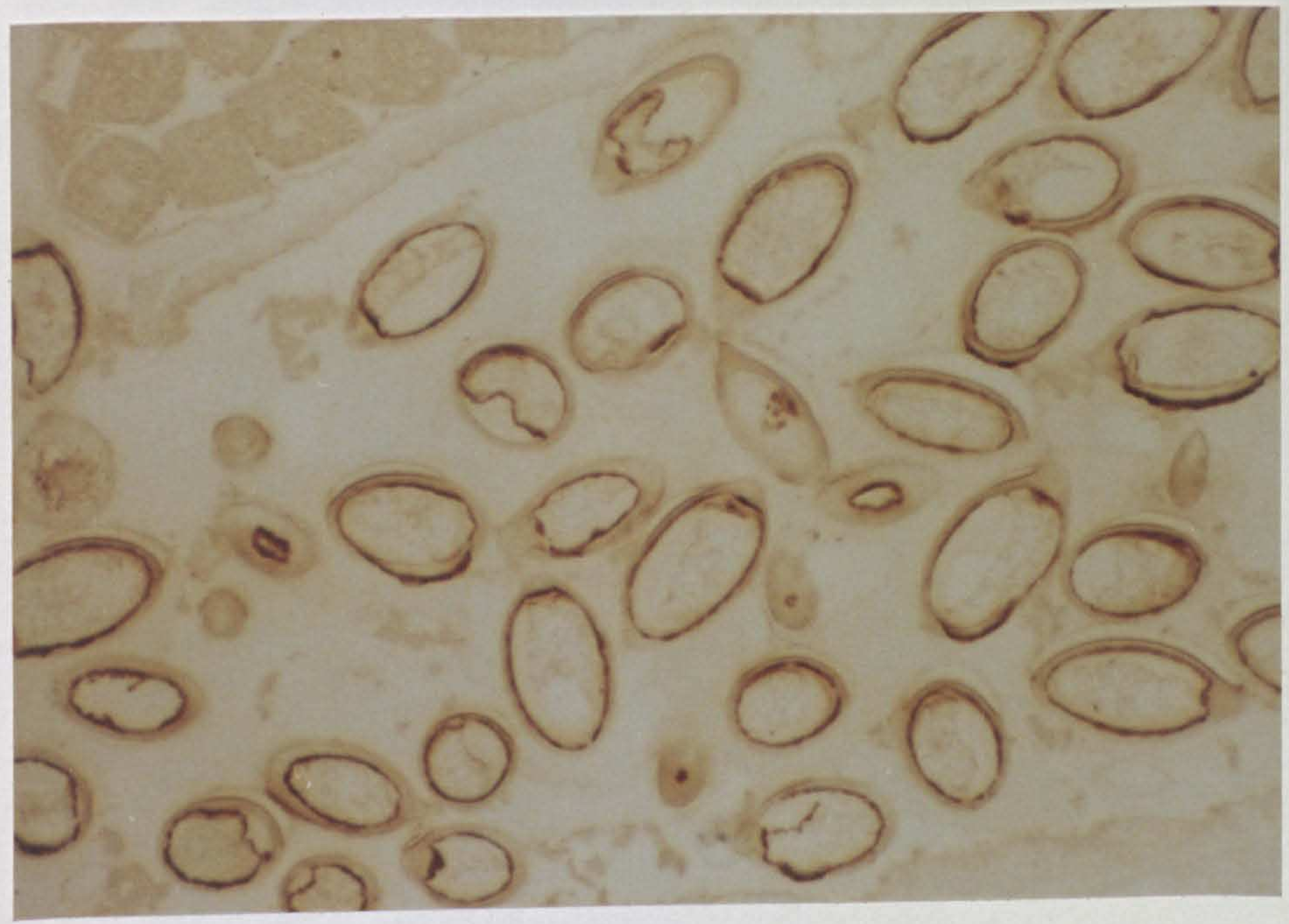


Figure 8.2 A, B and C. JB4 sections through the posterior region of a female worm showing unembryonated *T. muris* eggs within the oviduct. Section 8.2 A (x700) was treated with McAb A16, 8.2 B (x700) with NS and 8.2 C (x3500) with McAb E12. The McAbs showed reactivity with the lipid layer of the egg and with the unembryonated egg material. The egg shell was unstained.

Figure **8·2**

A



B

C

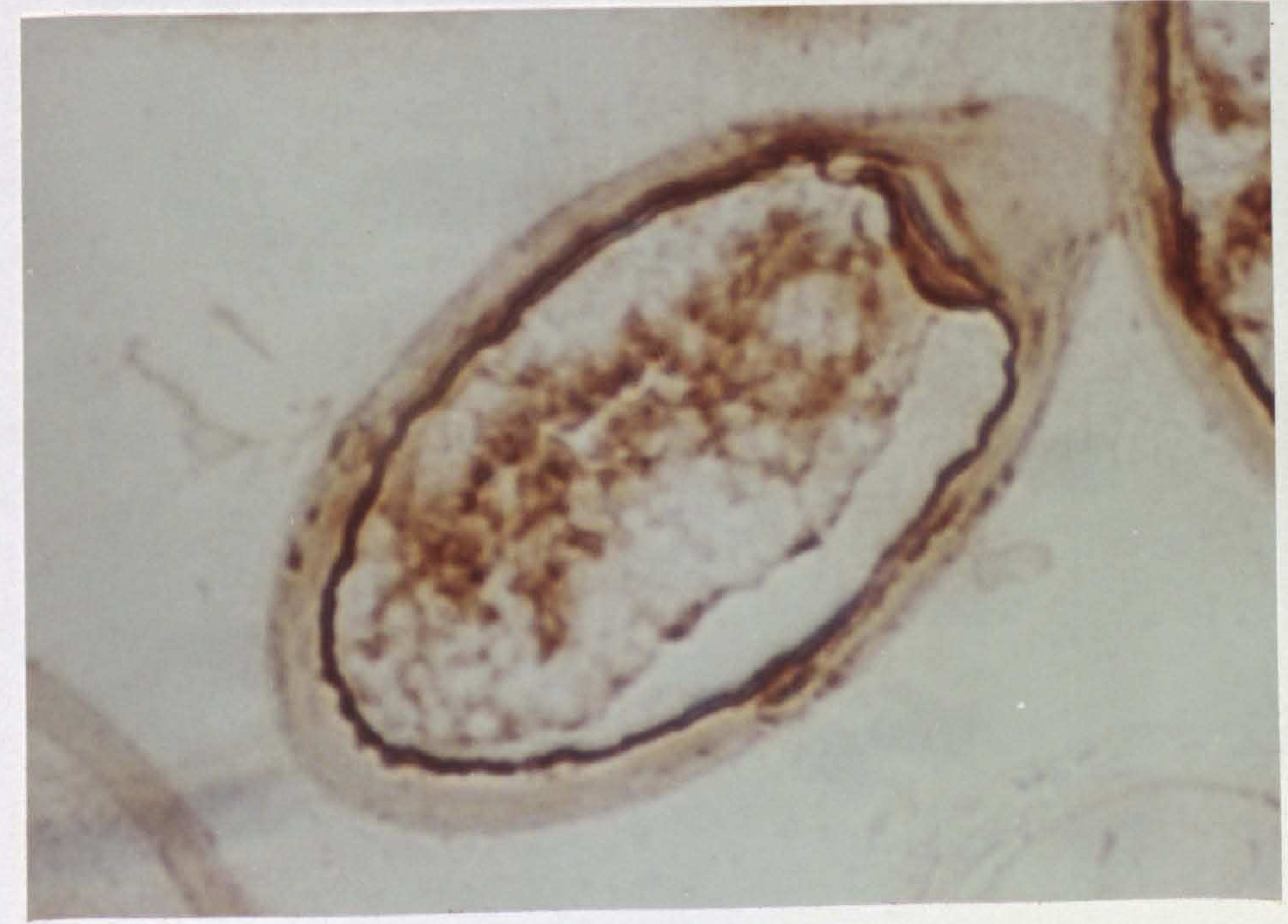
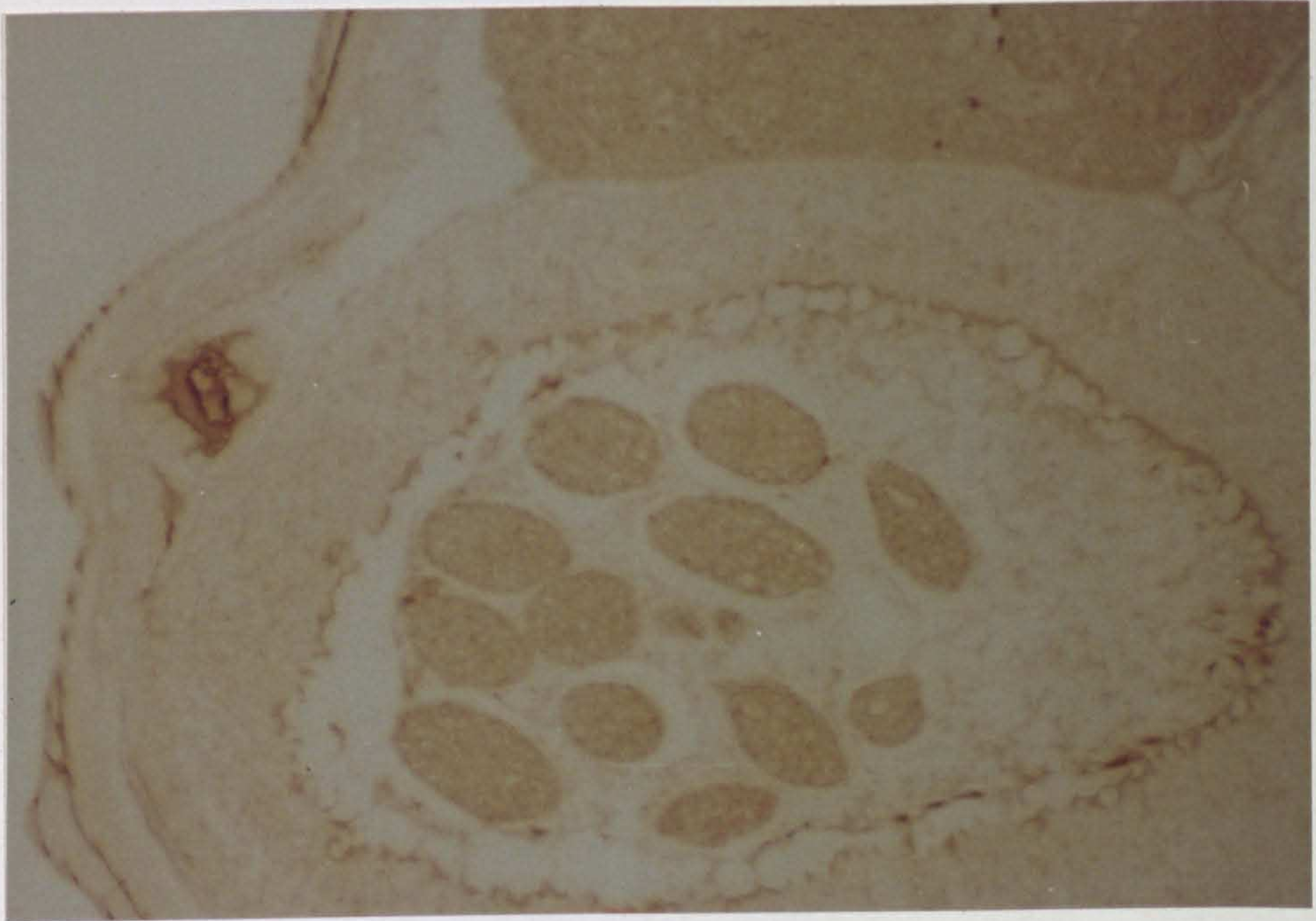


Figure 8.3 A, B, C and D. JB4 sections through the posterior region of adult female worms. Section 8.3 A (x700) was incubated with McAb A16, 8.3 B (x700) with NS and 8.3 C (x3500) and 8.3 D (x700) with E12.

8.3 A reveals recognition by A16 of the oocyte cytoplasm, the oviduct wall being relatively unstained. There was again evidence of reactivity with the outer cuticle and material within a 'duct'-like structure was also densely stained. This structure is shown at a higher magnification in 8.3 C, reacted this time with E12. Only the inner muscle layer stained with NS (8.3 B). 8.3 D shows positive staining by E12 of the gut lining and the pseudocoelomic fluid.

Figure 8·3

A



B

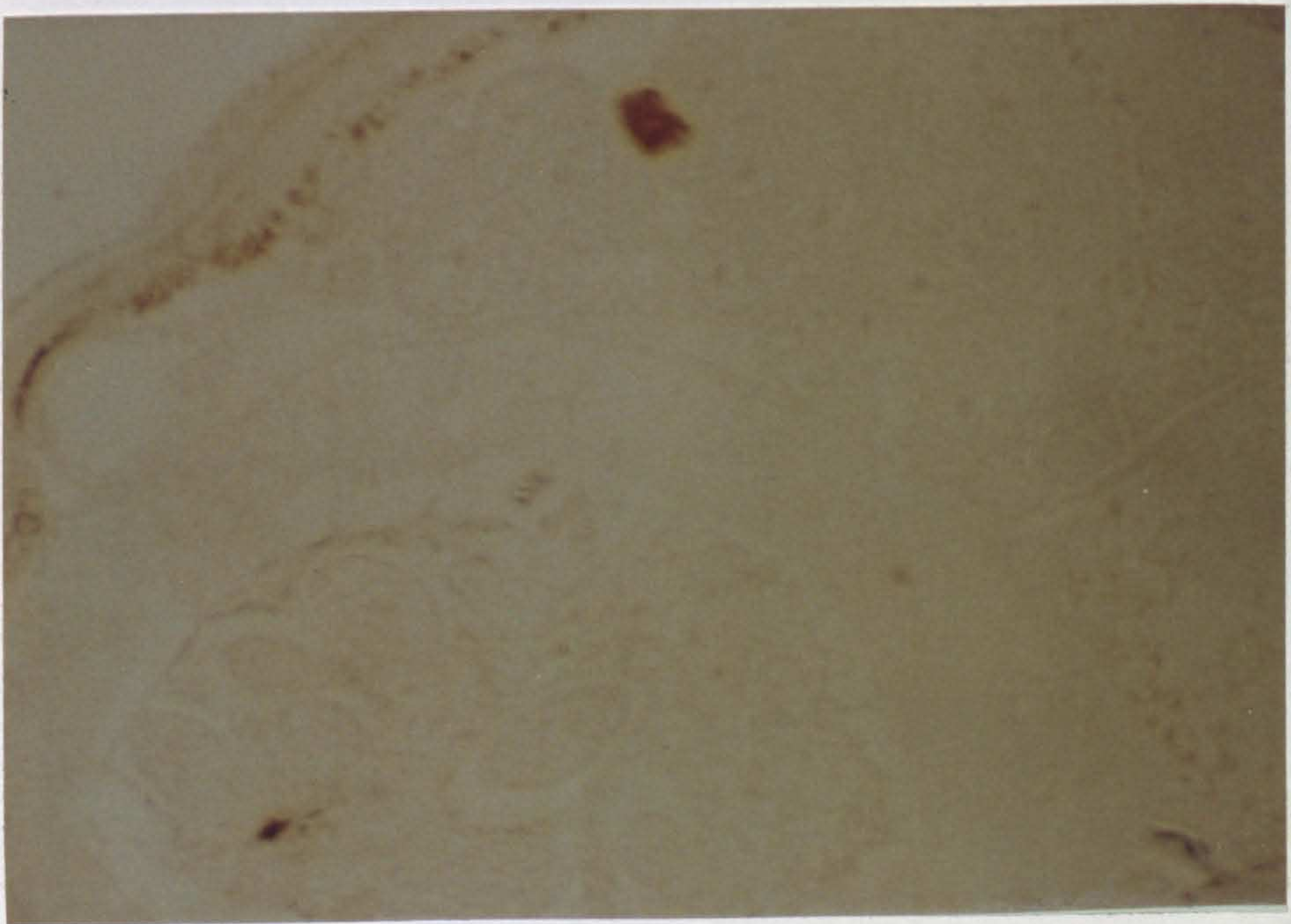
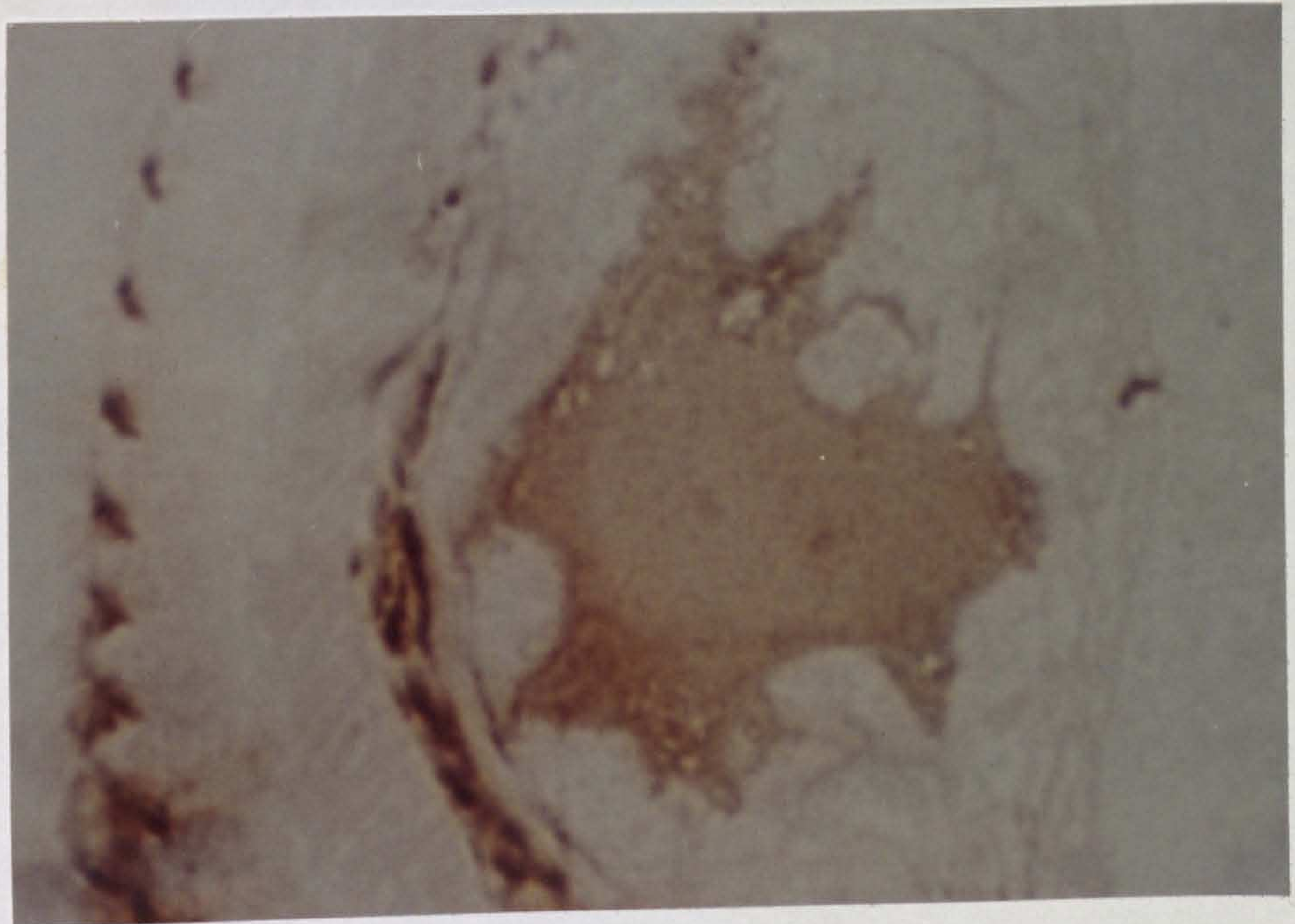


Figure 8·3

C



D

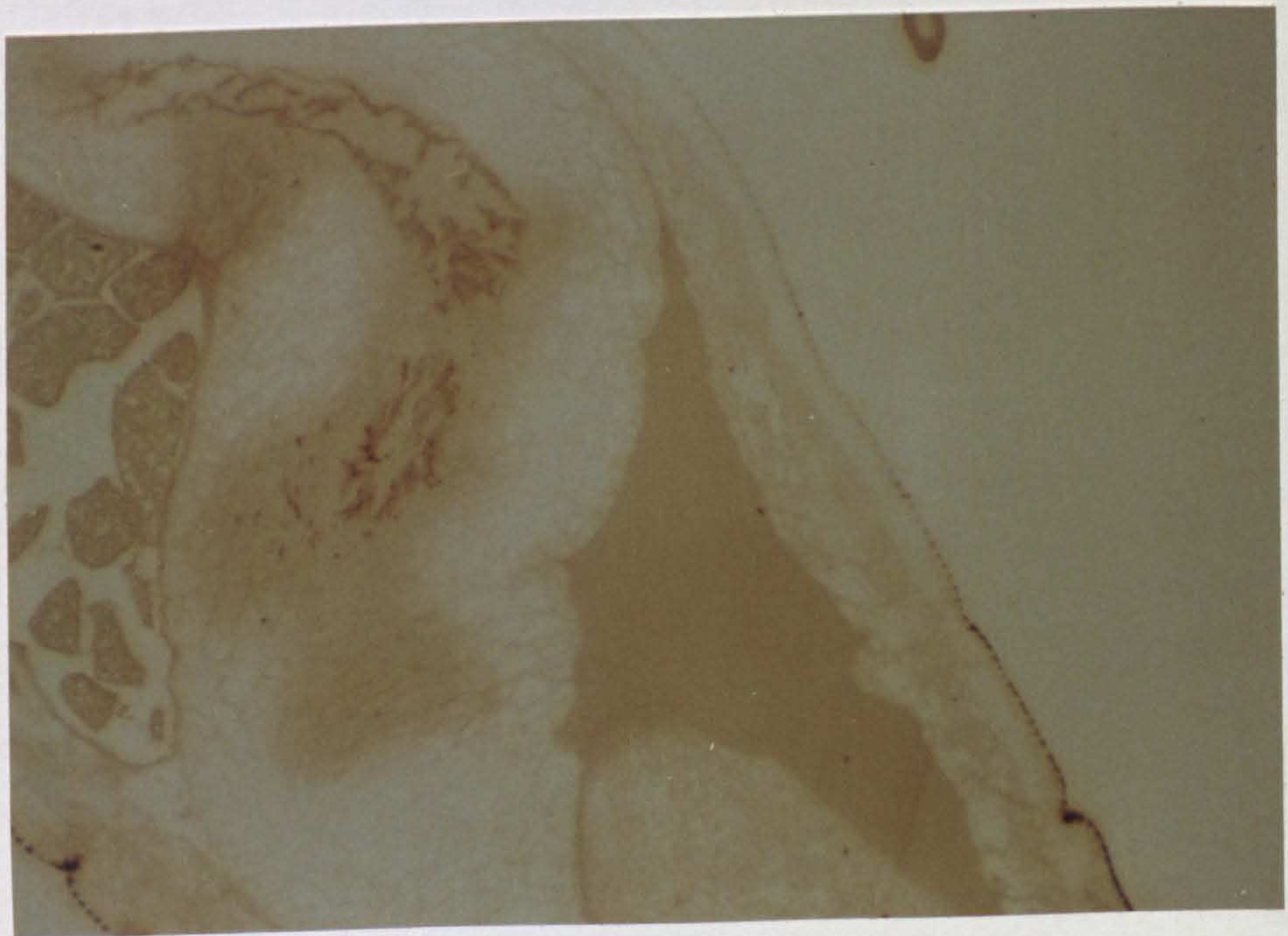
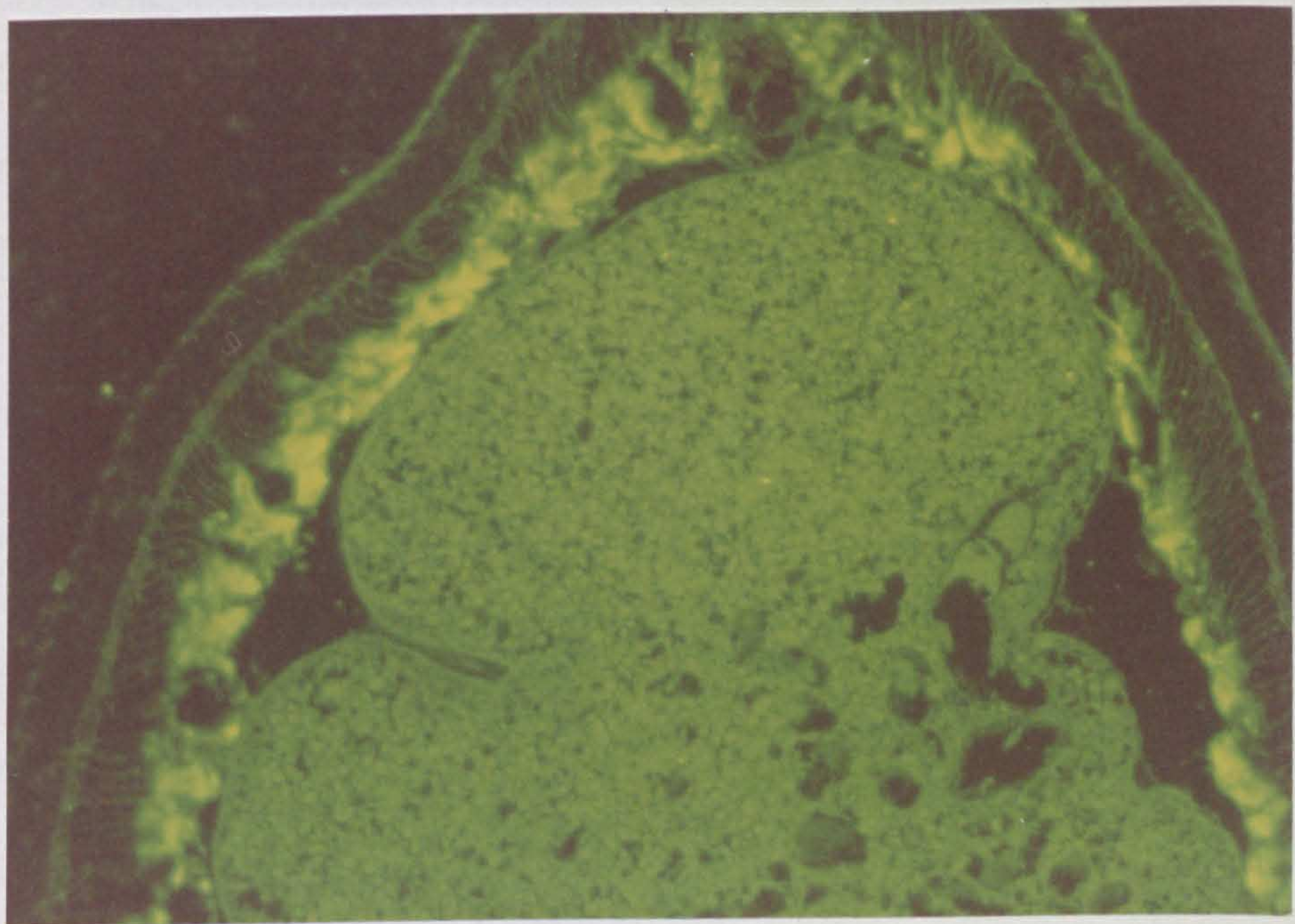


Figure 8.4 A, B, C and D. JB4 sections through the stichosome of adult *T. muris* worms. 8.4 A (x1250) was treated with TS followed by fluorescein-conjugated rabbit anti-mouse IgG (H+L) as was 8.4 B (x1250). In both examples intense fluorescence of granules within the stichocytes was observed. Cuticular fluorescence was also observed as was non-specific fluorescence of the inner muscle layer. This muscle layer was the only region to fluoresce in sections treated with NS (8.4 C x700). 8.4 D (x1250) shows the recognition by the F11 McAb of granules within the stichocytes. Although the fluorescence was less intense than seen for TS (and E12 and A16) there was a distinct reaction. No cuticular fluorescence was seen with F11.

Figure 8·4

A



B

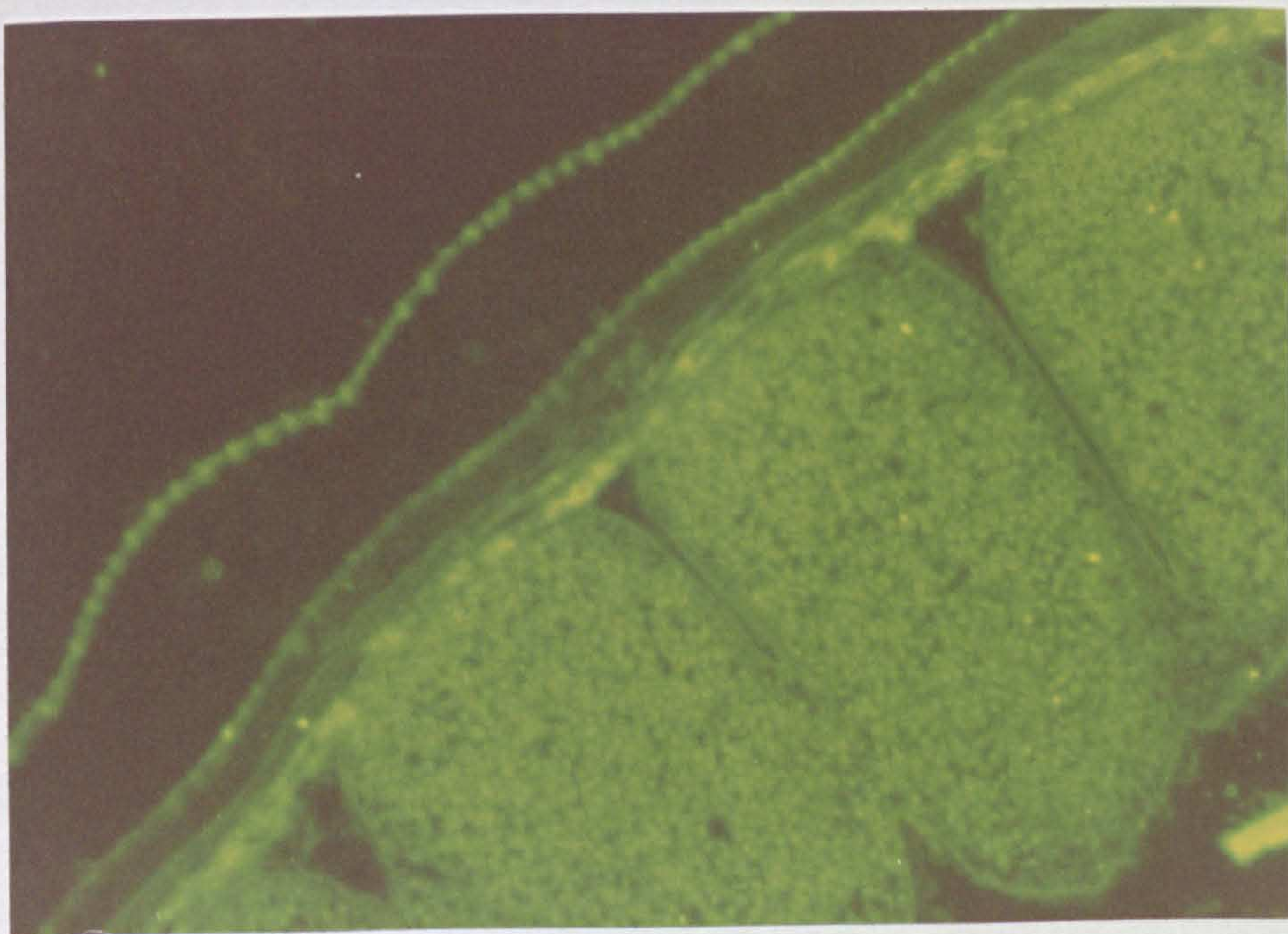
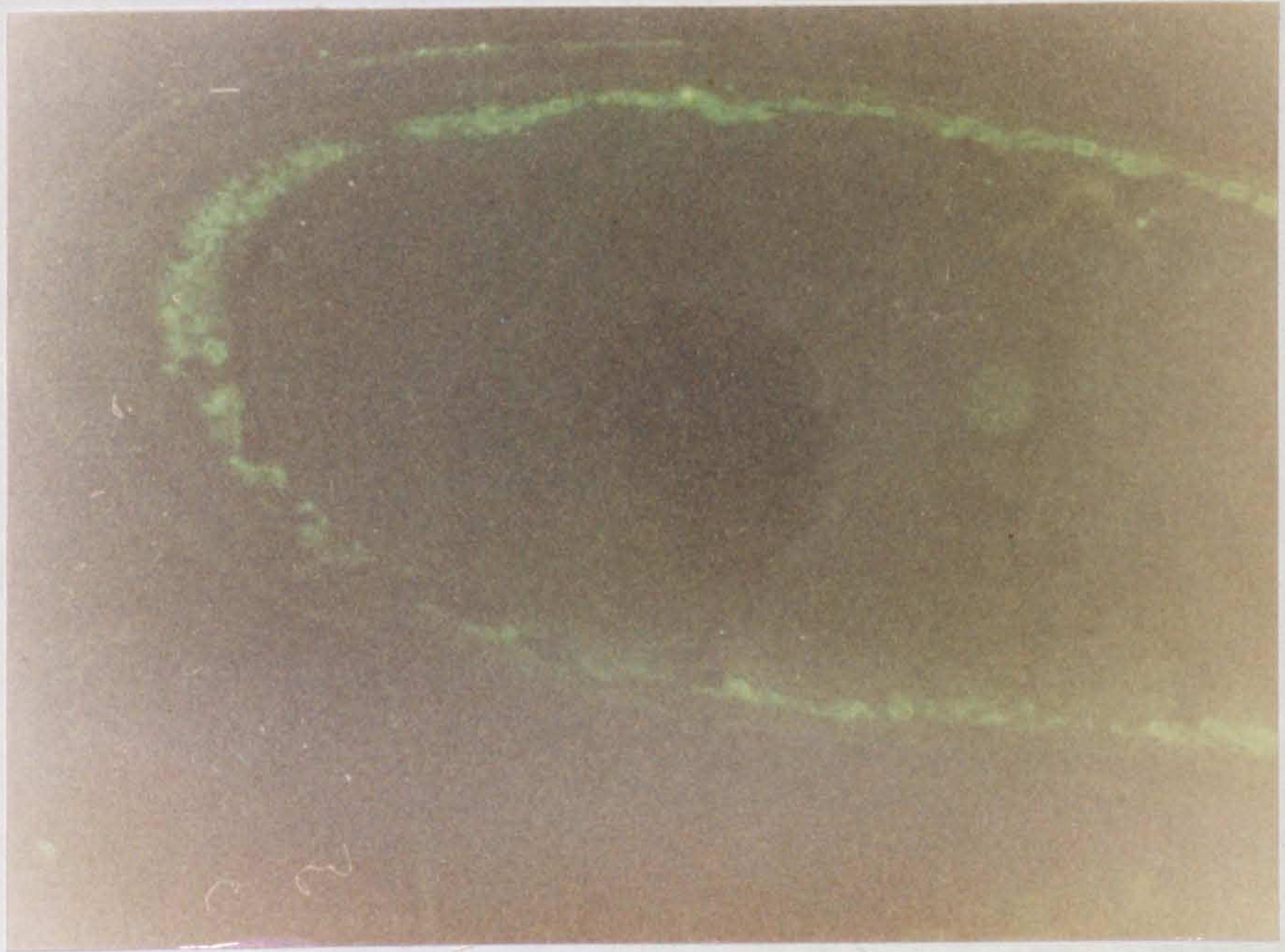


Figure 8·4

C



D

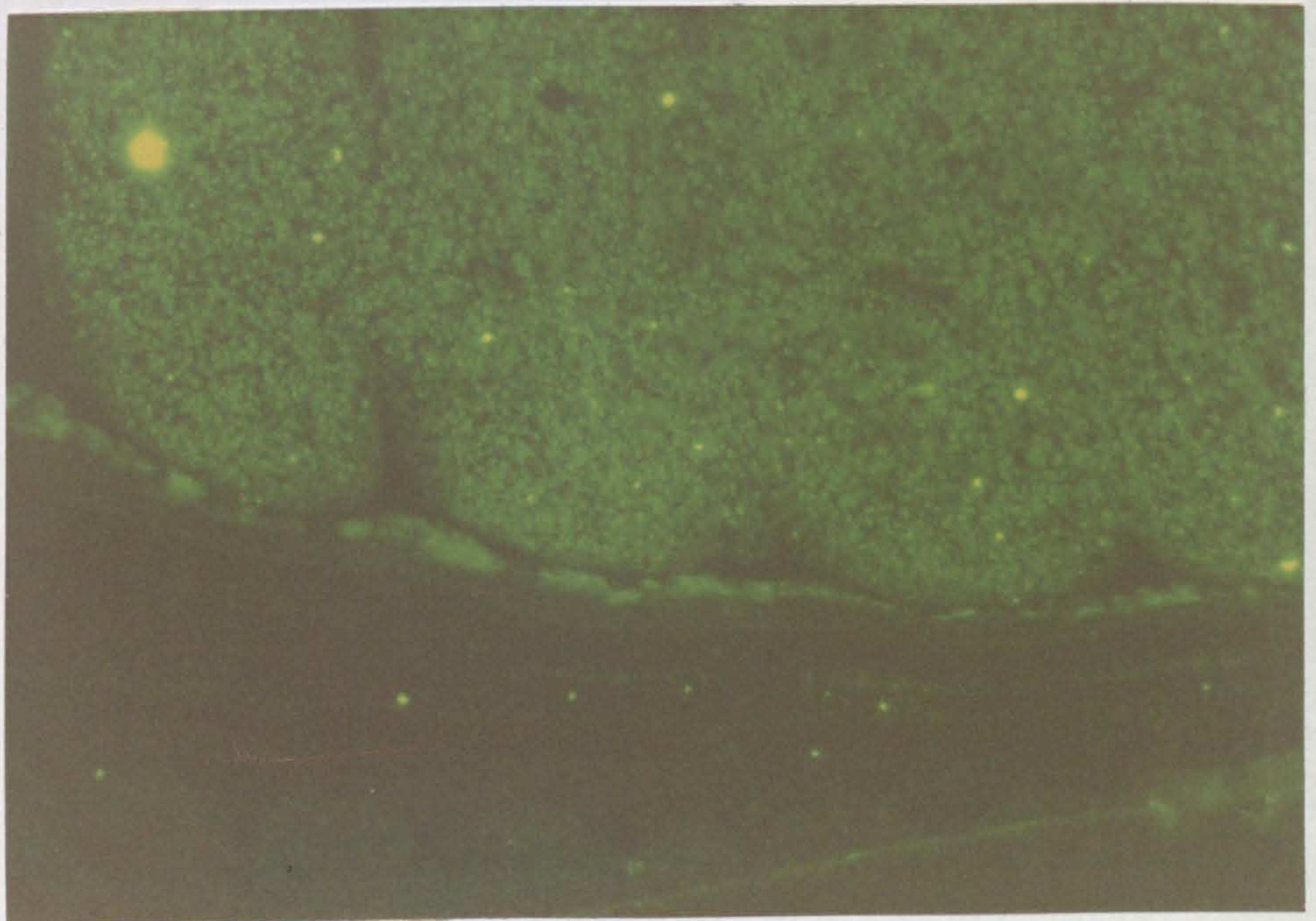
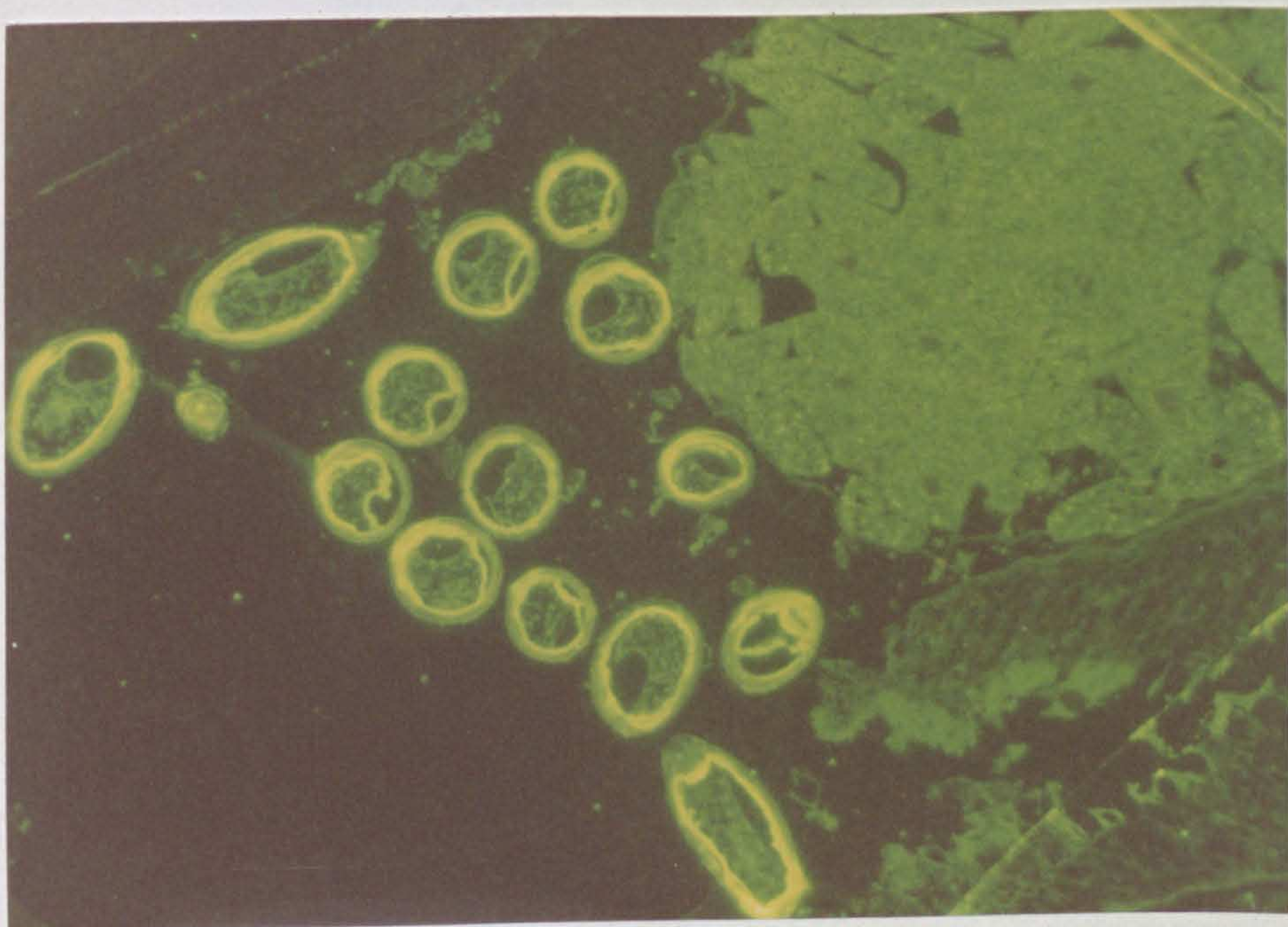


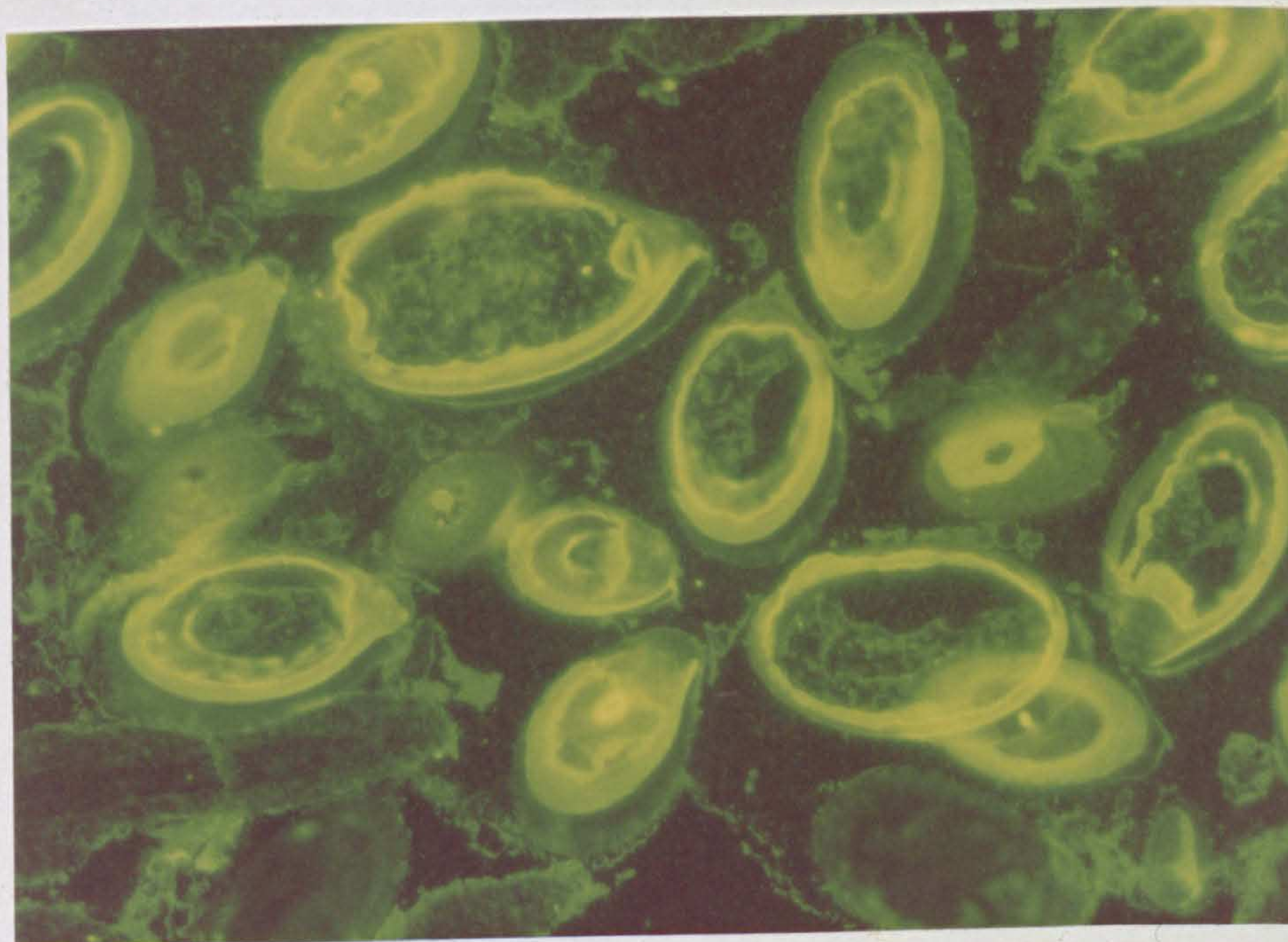
Figure 8.5 A, B and C. JB4 sections through the posterior regions of female worms showing strikingly bright fluorescence of the egg lipid layer following treatment with E12 (8.5 A x700; 8.5 B x1250). Unembryonated egg material also fluoresced. No fluorescence was detected in the negative controls, e.g. 8.5 C (treated with PBS) (x1250).

Figure **8·5**

A



B



C

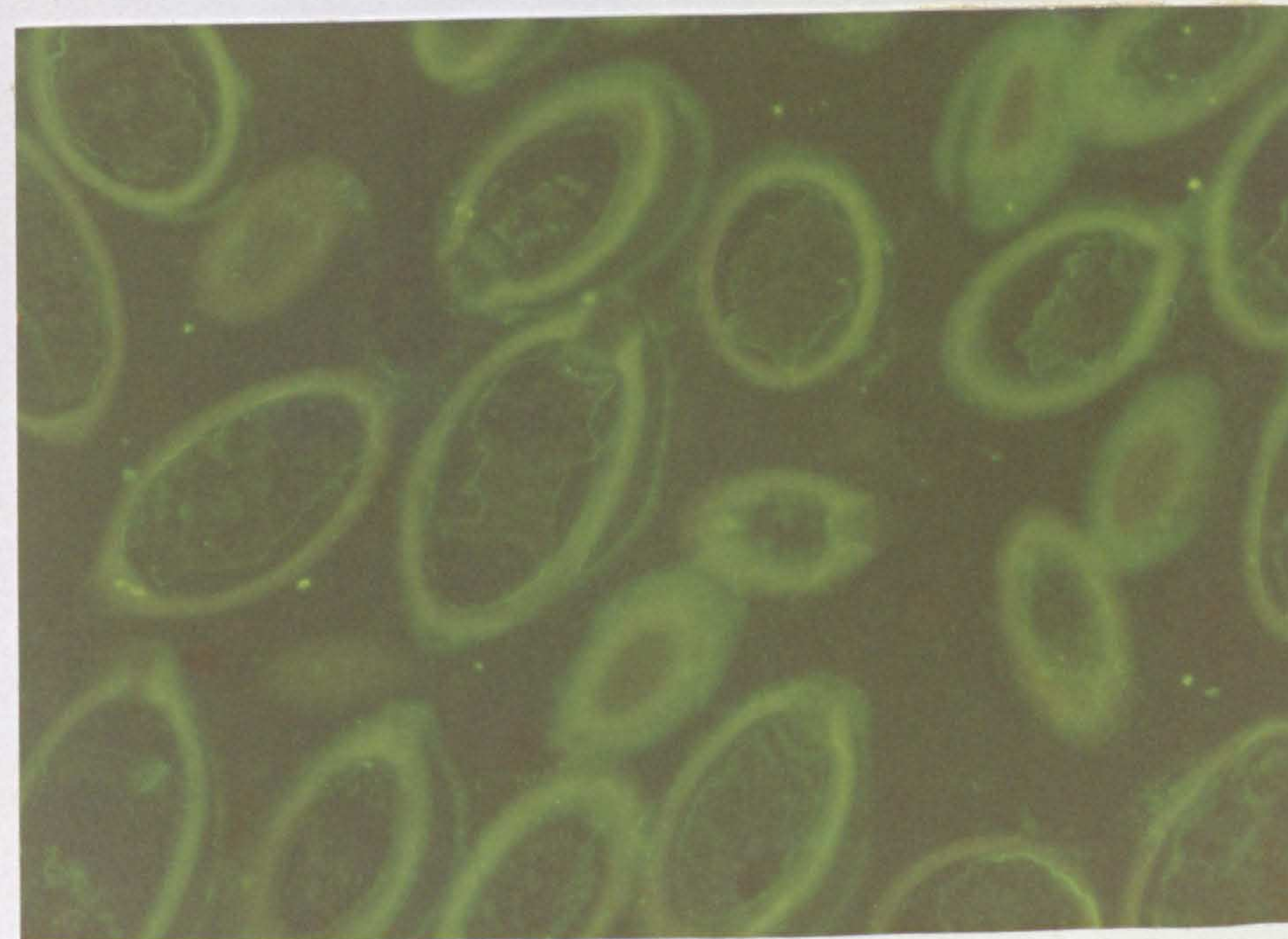
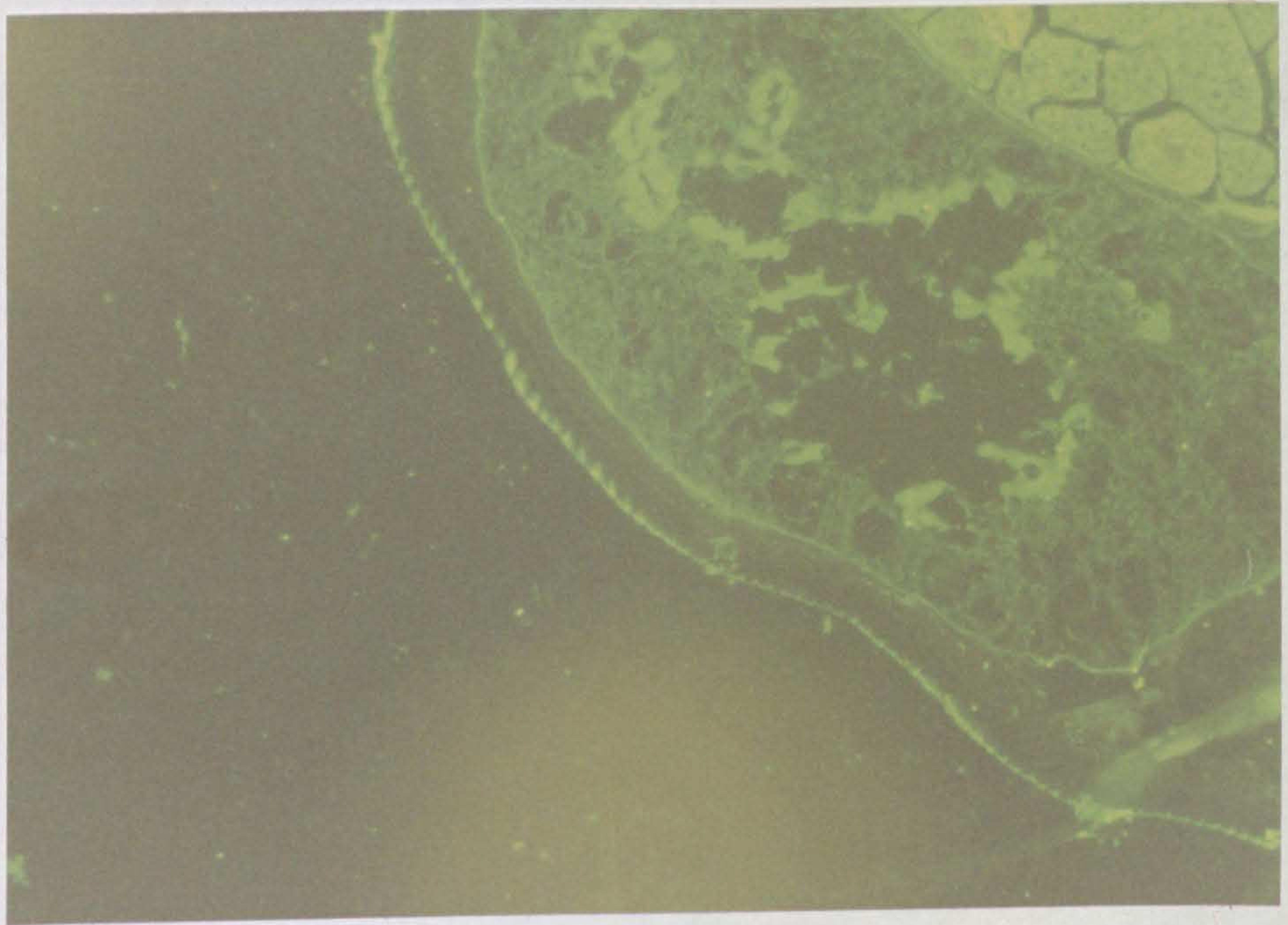


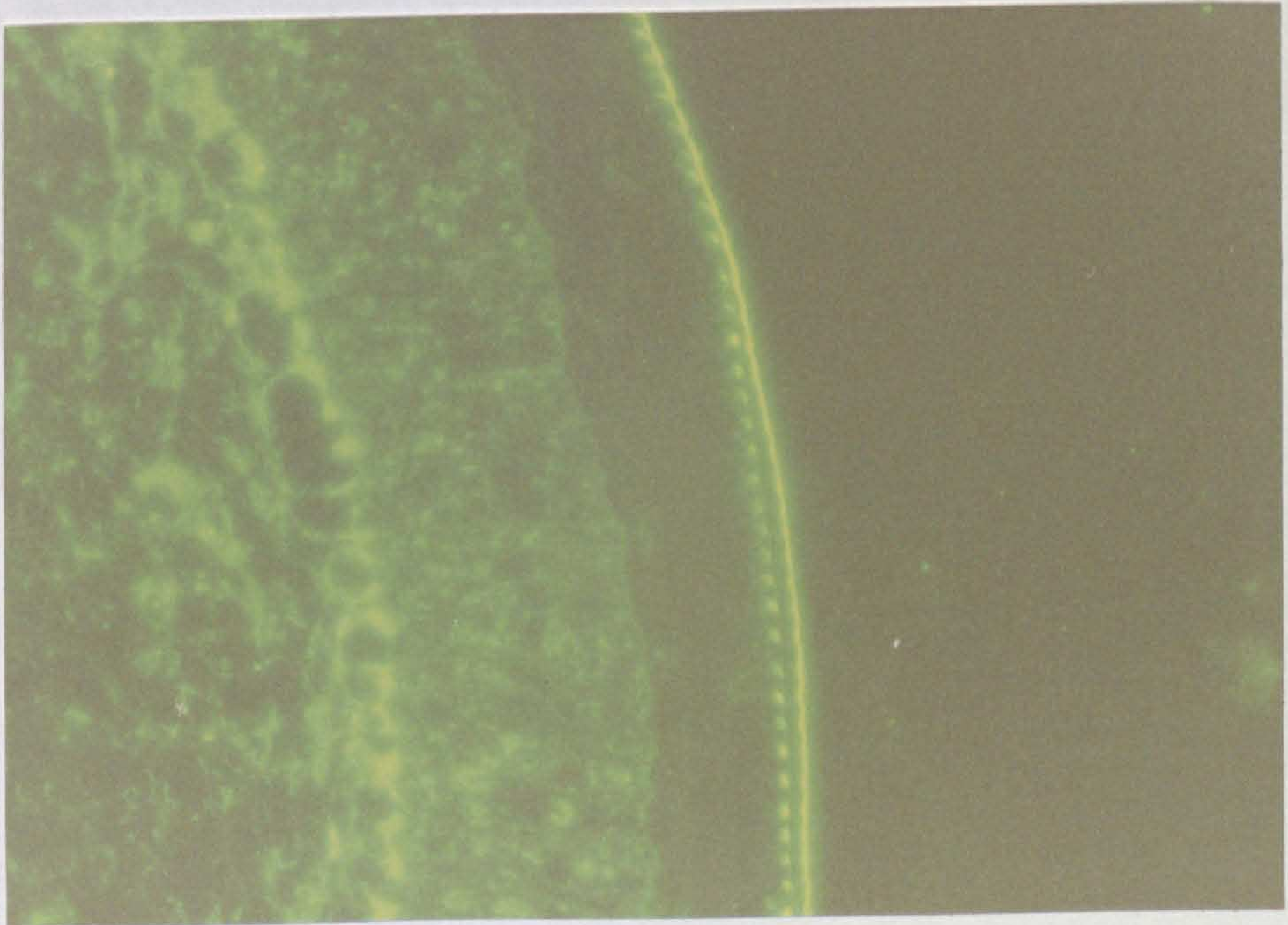
Figure 8.6 A and B. JB4 sections through the posterior regions of adult female worms. 8.6 A (x700), labelled with E12, shows evidence of reactivity with the gut lining, the outer cuticle and the cytoplasm of developing oocytes. 8.6 B (x1250) reveals the intense cuticular fluorescence seen after incubation of the section with A16 followed by fluorescein-conjugated rabbit anti-mouse IgG (H+L).

Figure 8·6

A



B



strong staining of material within a discrete duct-like structure was also observed with both A16 (8.3A) and E12 (8.3C). Figure 8.3D shows positive staining by E12 of the gut lining and the pseudocoelomic fluid. Similar staining was seen for A16 and TS. The inner muscle layer was the only region of posterior female worms to stain positively with NS (8.3B). No structures were stained with F11 (data not shown).

No specific staining was apparent when the 3 McAbs or TS were incubated with sections through the posterior regions of male worms, there being recognition by A16, TS, E12 but also NS of the inner muscle layer and sperm cells (data not shown).

Localisation of antigens within *T. muris* by immunofluorescence supported the results obtained by the immunoperoxidase studies. Examples of the staining patterns are shown in figures 8.4 (A, B, C and D), 8.5 (A, B and C) and 8.6 (A and B).

The stichocyte granules fluoresced intensely with TS (8.4 A, B), E12 and A16. Less intense, but still positive fluorescence was seen with F11 (8.4 D). Granules within the stichocytes were not recognised by NS (8.4C) and the cuticle was negative with both NS and F11. The lack of cuticular fluorescence with all negative controls, and F11, demonstrated that the strong fluorescence seen with A16, E12 and TS did not represent autofluorescence and also indicated that the dark staining of the cuticle seen by immunoperoxidase labelling did not merely represent refraction. Intense, non-specific fluorescence of the inner muscle layer was seen with all *T. muris* McAbs, TS, NS and an anti-phosphorylcholine monoclonal antibody (anti-PC McAb). The anti-PC McAb did not recognise the stichocyte granules although there was some indication of very weak cuticular fluorescence (data not shown).

Figure 8.5 A, B and C illustrate the localisation of antigens recognised by E12 (figure 8.5 A and B) and by A16 and TS to the lipid layer of the

egg. No fluorescence was detected using NS or F11, but there was some suggestion that the anti-PC McAb showed very low level reactivity with unembryonated egg material. The lipid layer was completely negative. A negative control section, to which PBS had been added rather than an antibody, is shown in figure 8.5 C. E12, A16 and TS also showed reactivity with the unembryonated egg material e.g. figure 8.5 A and B (E12), the gut epithelium and developing oocytes e.g., figure 8.6 A (E12), and the outer cuticle e.g. figure 8.6 B (A16). The anti-PC McAb showed low-level reactivity with the gut epithelium (not shown). The duct-like structure containing densely stained material as seen by immunoperoxidase labelling was only represented on one section. As before the material was recognised strongly by the McAb A16 (data not shown). The reactivity of E12, A16 and TS with tissues in the posterior regions of female worms was lost when the McAbs were preabsorbed with E/S antigen. Preabsorption of the A16 McAb with E/S antigen also removed all reactivity with the stichocytes.

8.3.3 IMMUNOPEROXIDASE AND ALKALINE PHOSPHATASE LABELLING OF FROZEN SECTIONS.

Sections of adult male and female worms removed from the mouse large intestine, and sections of worm *in situ* within the gut tissue, were incubated with a number of *T. muris* McAbs and the control reagents listed in 8.2.1 excluding the anti-PC-McAb.

Posterior regions of the worms produced identical staining patterns to those described for JB4 sections after staining with E12, A16 and TS. However the F11 McAb also showed some reactivity with tissues within the posterior regions of female worms using frozen sections, although background staining was also high. Attempts to repeat this apparent demonstration of immunoreactivity, using JB4 sections, failed even though

the experiment was repeated five times. In all cases no staining of tissues within the posterior regions of female worms was observed whilst the stichocytes consistently stained positive demonstrating that the lack of reactivity was not due to low McAb concentrations in the S/Ns. Sections of worms *in situ* within the mouse gut stained as seen for sections of isolated worms but the surrounding tissue also stained positive, presumably a problem of using anti-mouse Ig reagents on mouse tissue. However, although mouse tissue showed reactivity on control slides, worm tissue was consistently unstained.

Frozen sections of stichosomal tissue failed to preserve the stichocyte structure as well as JB4 sections making it difficult to localise antigens recognised by the McAb probes, although there was some indication of a granular staining pattern within the cells of the stichosome as seen before.

8.3.4 *Phosphorylcholine ELISAs*

Although no strongly positive labelling of *T. muris* tissues was observed using an anti-phosphorylcholine (PC) McAb on JB4 sections the possibility that the anti-*T. muris* E/S McAbs were recognising PC determinants was investigated in two ways:

1. Various *T. muris* antigen preparations were used as target antigens for the anti-PC McAb in ELISA.
2. McAb S/Ns were tested for reactivity to PC by ELISA using PC-BSA as the target antigen.

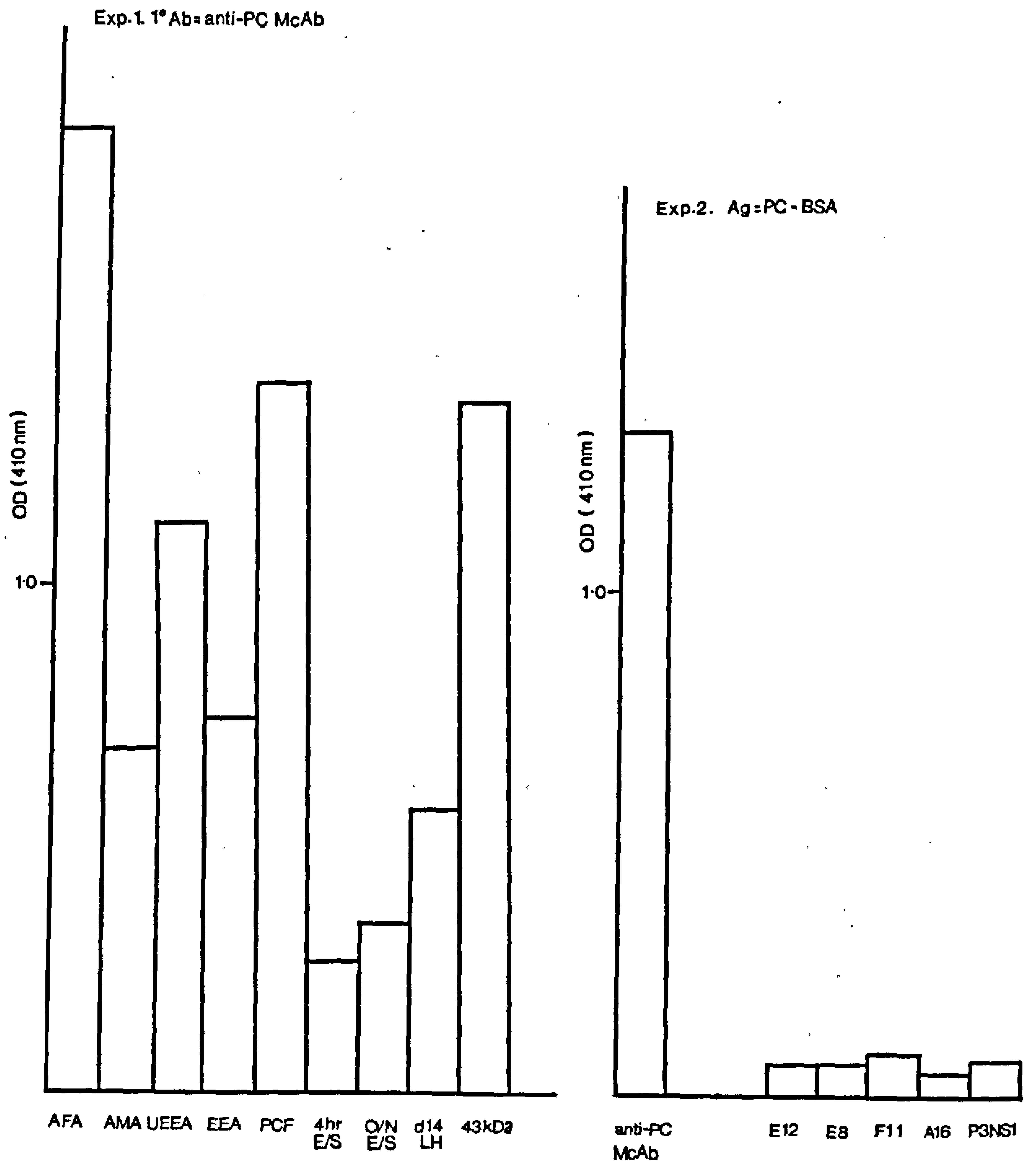
The results are shown in figure 8.7. In experiment 1 antigen preparations were used at a concentration of 5μg/ml, 50μl/well and the anti-PC McAb diluted 1/1000. As can be seen all the antigen preparations showed some reactivity with the anti-PC McAb with adult female antigen (AFA), pseudocoelomic fluid (PCF), the 43kDa entity and unembryonated

Figure 8.7

Exp. 1 Recognition of phosphorylcholine (PC) determinants expressed by *T. muris* antigen preparations by an anti-PC McAb. All target antigens in ELISA were used at 5μg/ml and the anti-PC McAb was diluted 1/1000.
AFA, adult female antigen; AMA, adult male antigen; UEEA, unembryonated egg antigen; EEA, embryonated egg antigen; PCF, pseudocoelomic fluid; 4hr and O/N excretory/secretory (E/S) antigen; d14 LH, day 14 larval homogenate; 43kDa, dominant protein band of AMA and E/S antigen of MW 43kDa (reduced) eluted from a polyacrylamide gel.

Exp. 2 Screening of anti-*T. muris* McAbs against PC determinants using PC-BSA at 10μg/ml as the target antigen in ELISA.

McAbs S/Ns were used neat and diluted from 1/10 to 1/1000. The anti-PC McAb was diluted from 1/100 to 1/5000. Results are given for neat *T. muris* McAb S/Ns and the anti-PC McAb at 1/1000.



Exp.1. 1° Ab = anti-PC McAb
OD (410 nm)
1·0
AFA
AMA
UEEA
EEA
PCF
4hr E/S
O/N E/S
d14 LH
43kDa
Exp.2. Ag = PC-BSA
OD (410 nm)
1·0
anti-PC McAb
E12
E8
F11
A16
P3NS1

egg antigen (UEEA) exhibiting the highest reactivity.

In experiment 2 the *T. muris* McAbs were tested for their ability to recognise PC-BSA (10μg/ml, 50μl/well) in ELISA. Results, using neat McAb S/Ns and dilutions from 1/10 to 1/1000, suggested that the McAbs did not recognise PC determinants. Antibody concentrations in the S/Ns were not determined therefore it is possible that the lack of recognition seen by ELISA reflected very low antibody levels in the S/Ns. However the ELISA is a very sensitive technique and would be expected to reveal some reactivity to PC determinants if the intense reactivity of the S/Ns observed in the immunohistochemical studies represented anti-PC activity. Screening of the S/Ns against PCF and E/S by ELISA showed high level reactivity of A16 and E12 S/Ns with the former and strong recognition by A16, E12 and F11 S/Ns of the latter.

8.3.5 PROTEIN COMPOSITION OF WORM TISSUES RECOGNISED BY ANTI-*T. MURIS* McAbs

Where possible worm components with which the test McAbs showed reactivity were extracted, isolated and analysed by SDS-PAGE. A preliminary investigation into the contribution of surface shed material to the contents of E/S was also made by ^{125}I-surface labelling using the Iodogen reagent. The results are shown in figures 8.8 A and B and 8.9 A and B.

Figures 8.8 A and B show the protein compositions of PCF, stichocyte E/S, solubilised whole stichocytes and embryonated egg antigen (EEA) as revealed by Coomassie blue staining of 10-20% SDS-polyacrylamide gels, samples being run under reducing conditions. AMA and adult E/S were also run for comparison. Figure 8.8B lane 2 shows the protein profile for the posterior ends of male worms.

Figure 8.8

A. Coomassie - stained 10-20% gradient SDS-polyacrylamide gel of *T. muris* antigen preparations run under reducing conditions.

Lane	
1	MW markers: 94, 67, 43, 30, 20.1 and 14.4kDa
2	Pseudocoelomic fluid 1
3	Pseudocoelomic fluid 2
4	Stichocyte E/S
5	Embryonated egg antigen
6	Adult male antigen
7	Adult worm E/S antigen

Amounts of protein run were only determined for AMA (100μg) and E/S (100μg). In all other cases the maximum volume that could be loaded on a gel was run as protein concentrations were expected to be low. In fact PCF was protein rich, typical protein concentrations from two further extractions being 1.6mg/ml and 1.0mg/ml.

B. Coomassie - stained 10-20% gradient SDS-polyacrylamide gel of *T. muris* antigen preparations run under reducing contitions.

Lane	
1	MW markers: 94, 67, 43, 30, 20.1 and 14.4kDa
2	Posterior end of male worms
3	Stichocyte E/S
4	Solubilised whole stichocytes
5	Adult male antigen (100μg protein)
6	Adult worm E/S (60μg protein)

Protein concentrations were not determined for samples run in lanes 2, 3 and 4. Maximum volumes which could be loaded on gels were run in all three cases.

Figure 8·8A

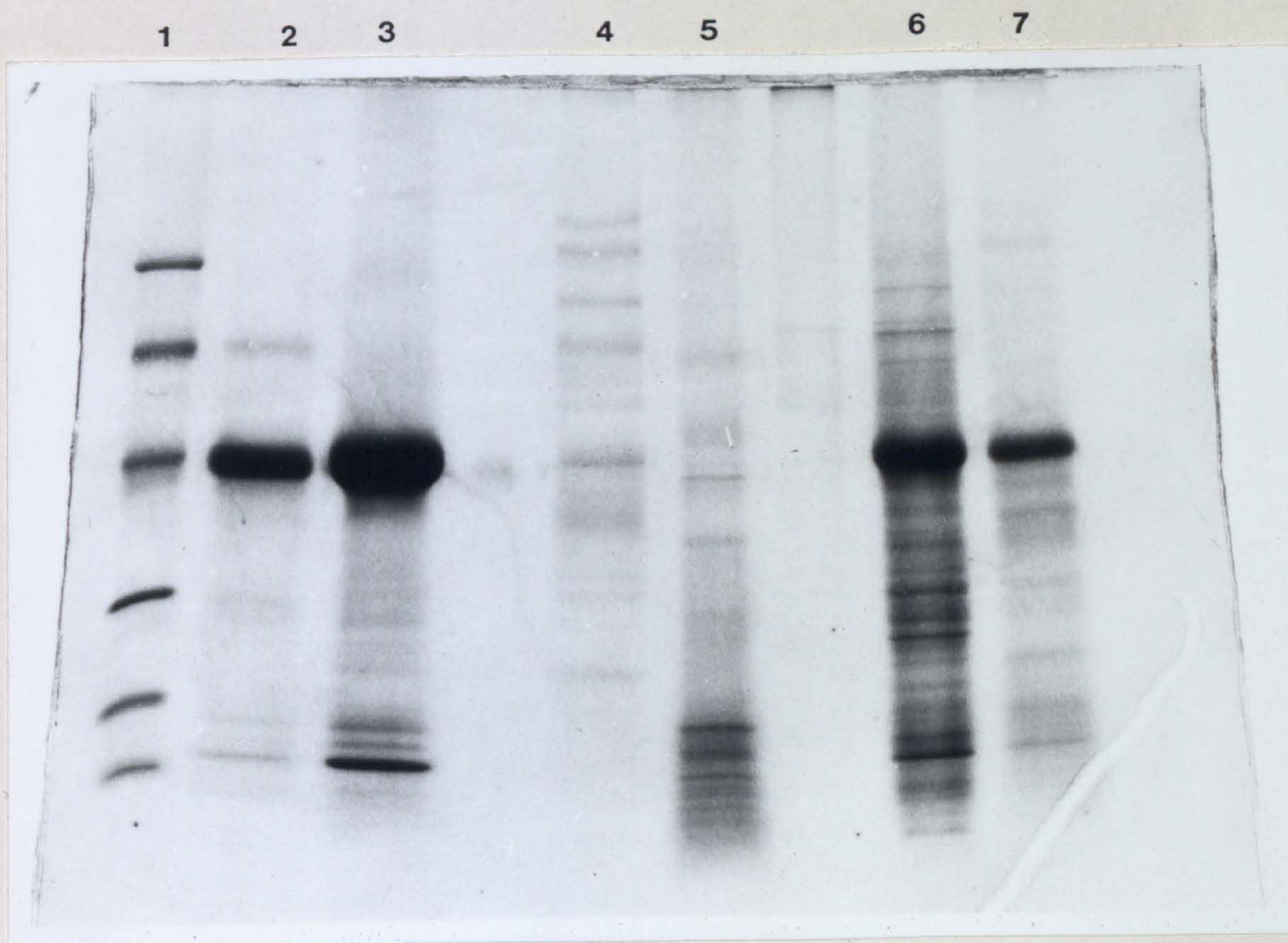


Figure 8·8B

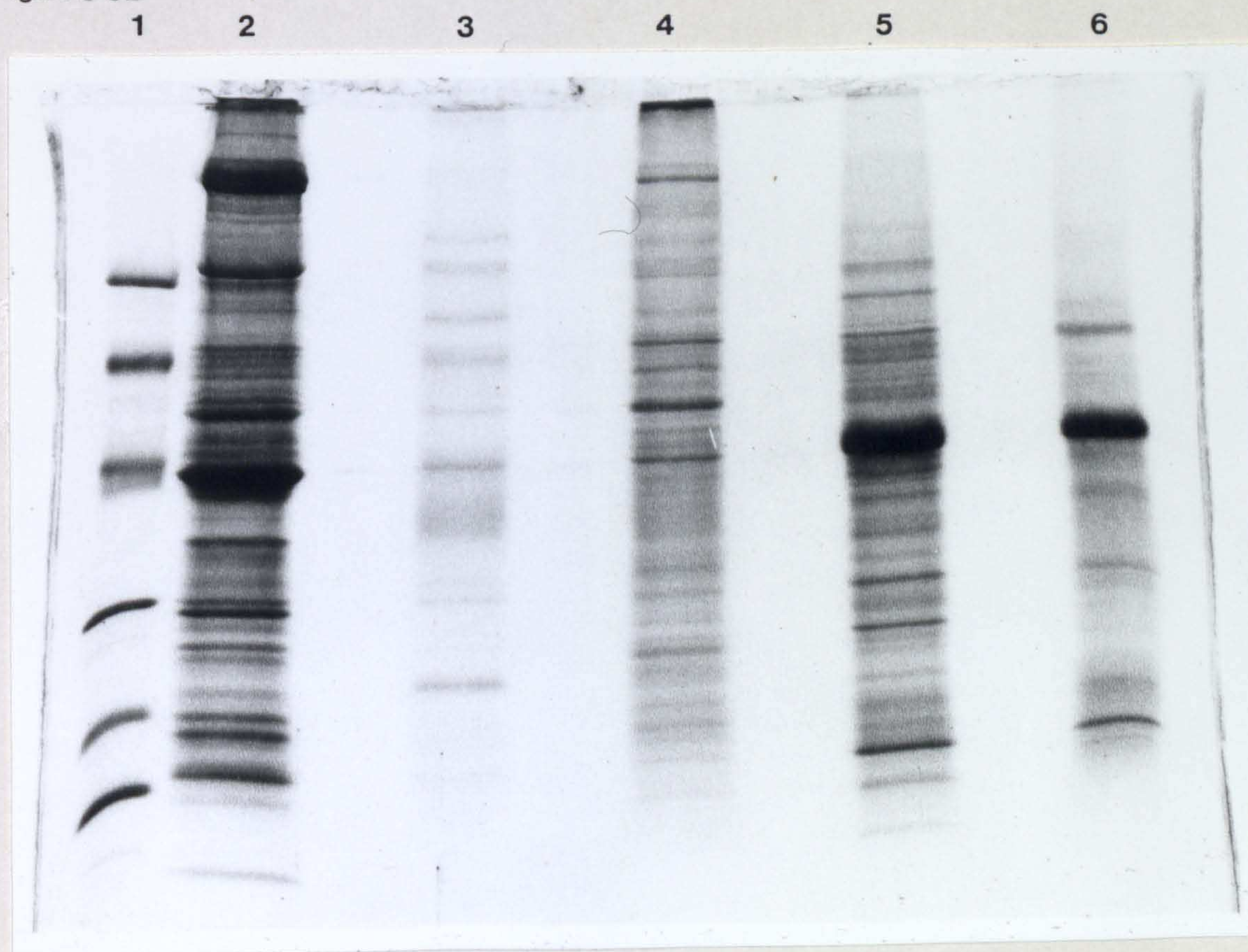


Figure 8.9

A. Coomassie-stained 10-20% gradient SDS-polyacrylamide gel of *T. muris* antigen preparations run under reducing conditions.

Lane	
1	MW markers: 94, 67, 43, 30, 20.1 and 14.4kDa
2	43kDa band eluted from gel
3	Adult male antigen
4	Embryonated egg antigen (10μl)
5	Embryonated egg antigen (40μl)
6	Embryonated egg antigen (80μl)

The protein concentration of the embryonated egg antigen preparation was not determined. Increasing volumes (10μl, 40μl, 80μl) of the preparation were run, plus sample buffer, in lanes 4, 5 and 6.

B. Autoradiograph of ^{125}I-surface labelled adult male and female worms and their E/S profiles. (10-20% gradient gel; all samples run under reducing conditions).

Lane	
1	MW markers: 94, 67, 43, 30, 20.1 and 14.4kDa
2	Profile of E/S products obtained by culturing ^{125}I-surface labelled male and female adult *T. muris* O/N in RPMI and collecting the S/N the following day (12,810 cpm)
3	^{125}I-surface labelled male and female adult *T. muris* homogenate prior to O/N culture (12,810 cpm)
4	^{125}I-surface labelled male and female adult *T. muris* homogenate after O/N culture (12,810 cpm)
5	as in lane 3 (30,000 cpm)
6	as in lane 4 (50,000 cpm)

Figure 8·9A

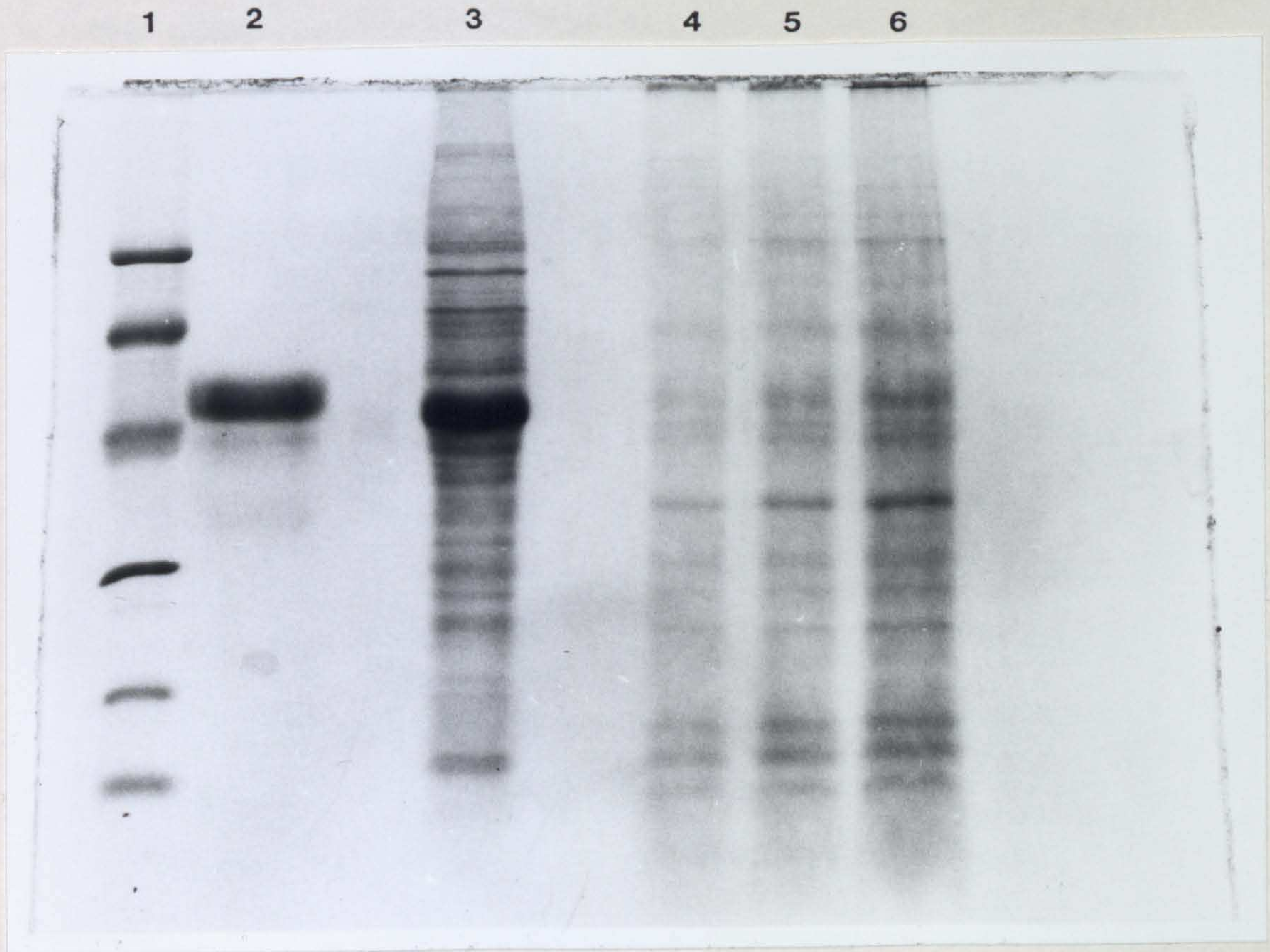
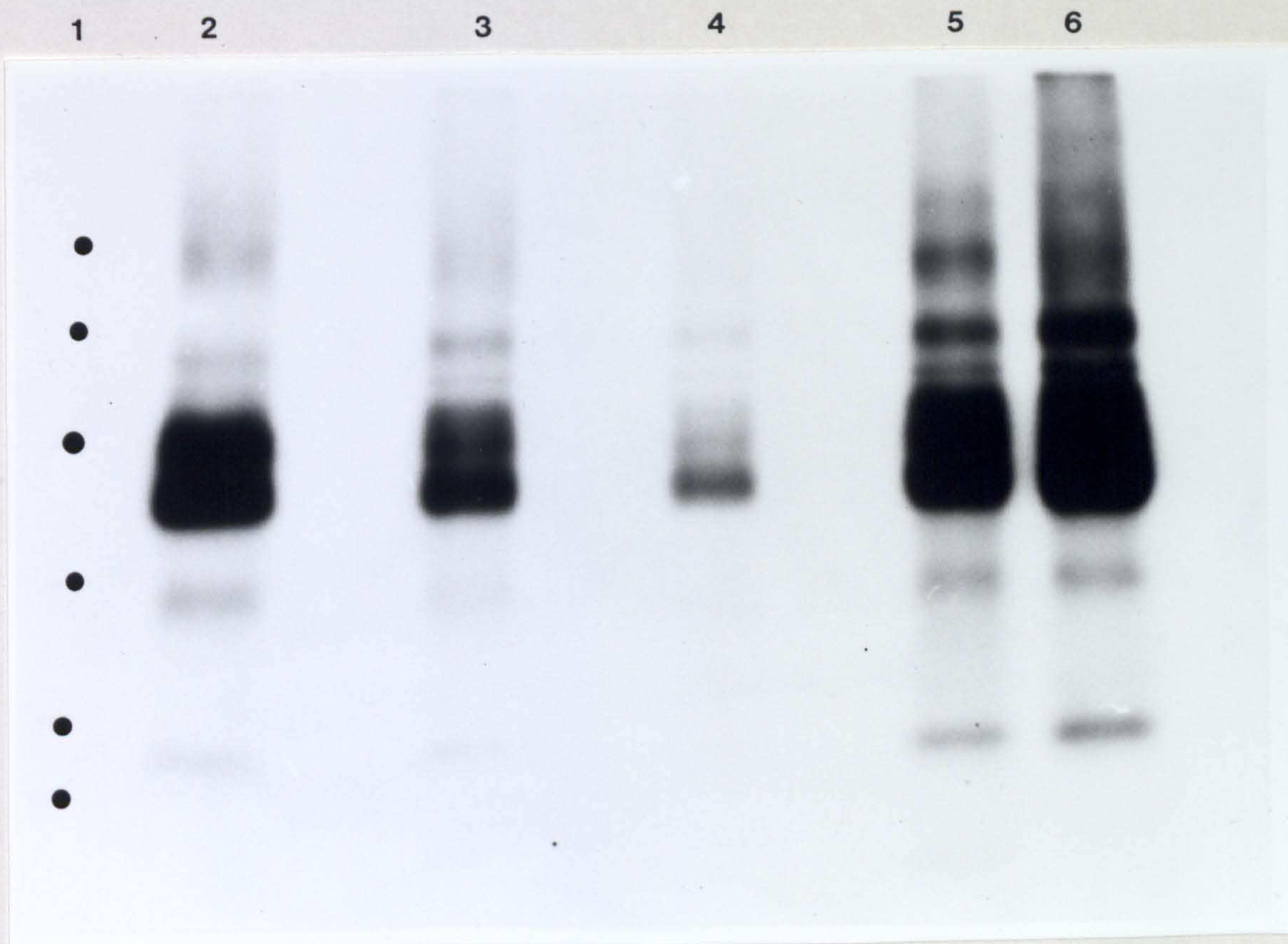


Figure 8·9B



The protein composition of PCF (*fig.* 8.8A, lanes 2 and 3) was remarkably restricted in its protein profile, one broad band resolving around 43kDa (2D gel electrophoresis might reveal that this single band has multiple components) and a triplet of low MW proteins resolving around 15-17kDa. The faint band observed around 67kDa in the first PCF extraction (lane 2) was felt to be a contaminant, no similar band being apparent in 3 other PCF preparations. Stichocyte E/S, obtained by dissecting out stichocytes and culturing them O/N at 37°C in RPMI followed by removal of the S/N, also had a fairly restricted protein profile, ten clear bands being visible on Coomassie stained gels (figs. 8.8 A lane 4 and 8.8 B lane 3). MWs ranged from 21kDa to 110kDa with one band resolving in the 43kDa region. Comparison of the protein profiles seen for stichocyte E/S and solubilised whole stichocytes (fig. 8.8 B lanes 3 and 4 respectively) revealed some common bands although solubilised whole stichocytes, as expected, had a far more heterogeneous protein profile. The protein composition of EEA shown in figure 8.8 A lane 5 is more clearly represented in figure 8.9 A lanes 4, 5 and 6. Many bands were visualised, including three low MW proteins (approximately 13, 15 and 17kDa), a strong band at 37kDa and bands around 43kDa. The AMA profile is shown in figure 8.9 A lane 3 for direct comparison. Figure 8.9A lane 2 represents an eluted 43kDa protein band rerun on a gel to test the efficacy of the elution procedure. The band is seen to resolve at a slightly higher MW than the 43kDa band in AMA (lane 3) perhaps due to a more complete unfolding of the protein structure following reboiling in reducing sample buffer. A second protein band, resolving just beneath the major band, has also been eluted in this example.

Figure 8.9 B represents a preliminary study on the contribution of surface shed proteins to the composition of E/S antigen. Surface protein iodination was carried out by the Iodogen method, iodogen catalysing the

radioiodination of tyrosine residues. Although iodination biases labelling towards proteins containing tyrosine residues it is still a valuable technique in the study of surface proteins. Six ^{125}I-surface labelled components of adult worms, resolving at MWs 89, 65, 48, 40, 30 and 18kDa were released into the culture medium during *in vitro* O/N incubation, although this could reflect breakdown rather than surface turnover (fig. 8.9 B lane 2). Homogenates of ^{125}I-surface labelled worms both before (lanes 3 and 5) and after (lanes 4 and 6) O/N culture in RPMI revealed similar banding patterns to the E/S profile with additional bands in the higher MW regions (above 48kDa). This is most clearly seen by referring to lanes 5 and 6, insufficient cpms being present in the volumes loaded in lanes 3 and 4 to clearly reveal all the bands.

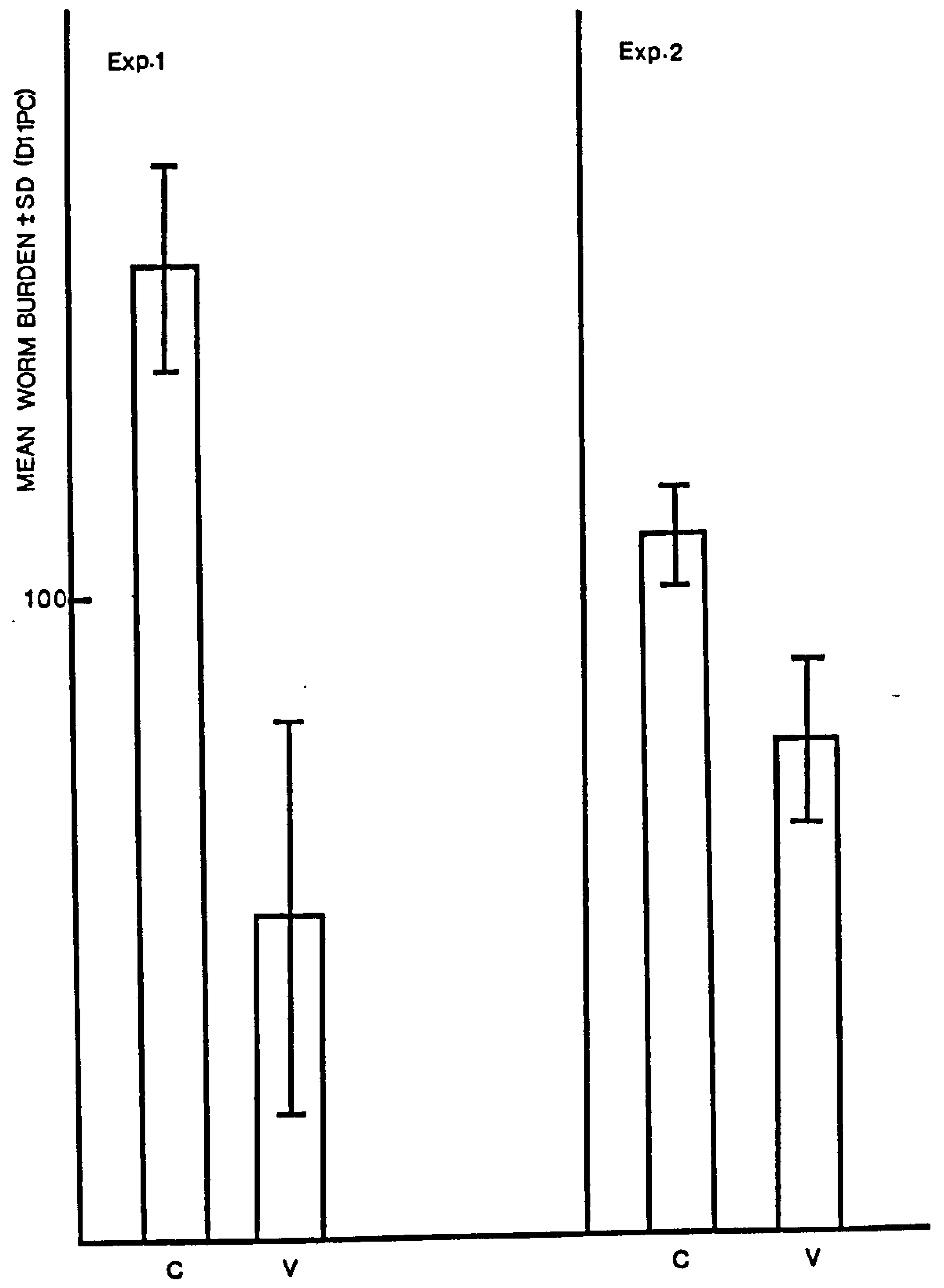
8.3.6 CAPACITY OF *T. MURIS* ANTIGEN PREPARATIONS TO INDUCE PROTECTION IN VIVO

Immunohistochemical studies revealed recognition by certain McAbs and TS of particular parasite components which were then isolated and tested for their ability to protect *in vivo.*

Pseudocoelomic fluid (PCF)

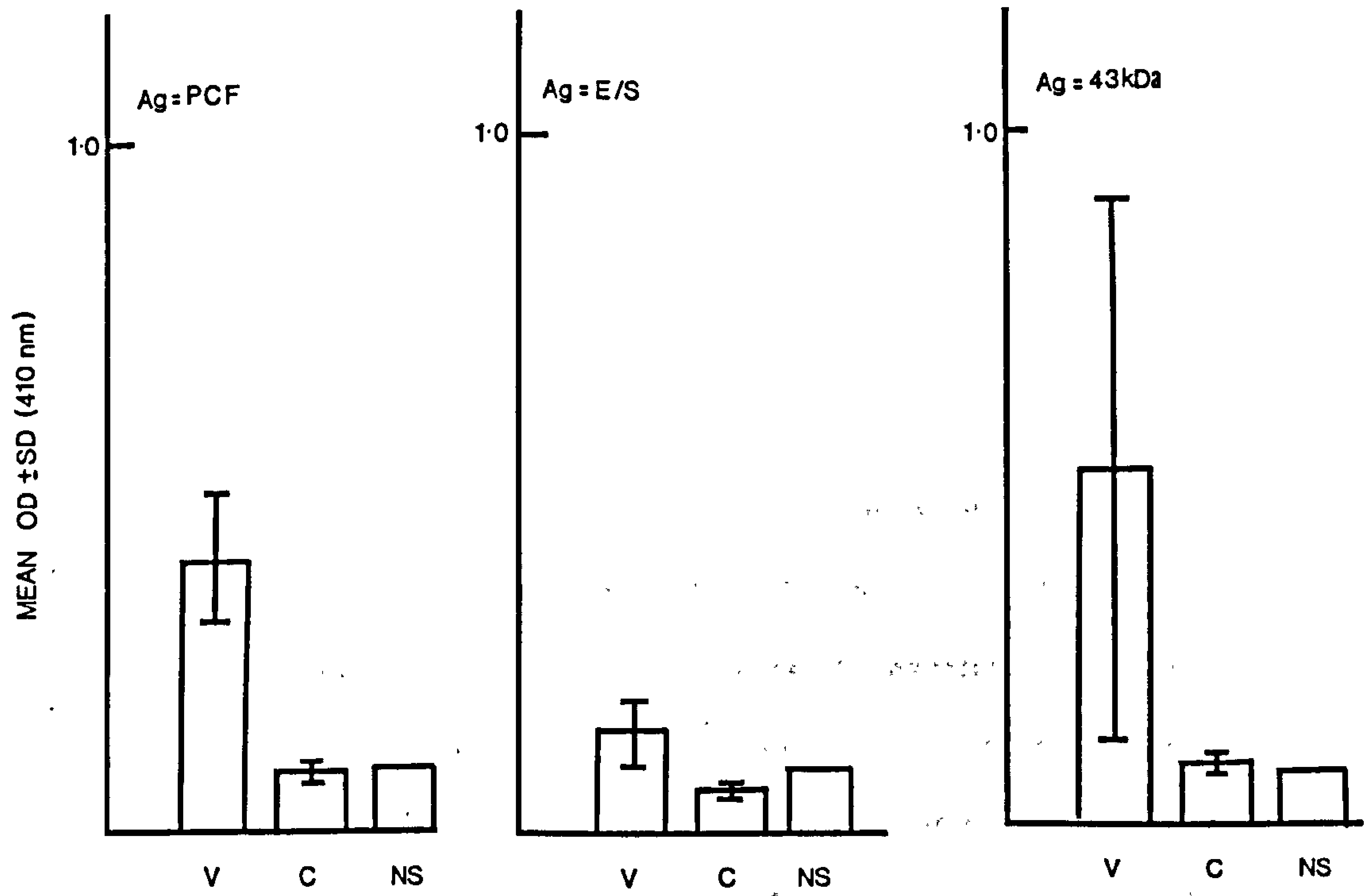
Two experiments were carried out in BALB/c mice using 6 individuals in vaccinated and control groups. In experiment 1 135μg PCF in FCA was injected s.c. into mice. Control individuals received PBS in FCA. In experiment 2 100μg PCF in FCA was used as vaccine. Worm burdens were assessed on day 11 p.c. in both experiments. The results are shown in figure 8.10. In both experiments vaccination with PCF led to a significant reduction (P=0.001) in the larval worm burden recovered on day 11 p.c.

Figure 8.10 Vaccination of BALB/c mice with PCF in FCA injected s.c. Vaccinated mice were injected with PCF 10 days prior to infection and killed day 11 p.c. for worm burden determination. In exp. 1 vaccinated mice (V) received 135µg PCF in FCA and in exp. 2 100µg PCF in FCA. Control mice (C) were injected with PBS in FCA. In both experiments vaccinated mice harboured worm burdens very significantly lower (P=0.001) than control mice.



Exp.1
Exp.2
MEAN WORM BURDEN ± SD (D11PC)
100
C
V
C
V

Figure 8.11 IgG antibody response of mice vaccinated with PCF to PCF, E/S and a 43kDa protein eluted from a polyacrylamide gel, as seen by ELISA. All target antigen preparations were used at 5μg/ml. Vaccinated mice (V) showed slightly elevated antibody responses to all three antigen preparations, especially PCF and the 43kDa protein. C = control sera from mice injected with PBS in FCA; NS = naive serum.


MEAN OD ±SD (410 nm)
Ag=PCF
1·0
V
C
NS
Ag=E/S
1·0
V
C
NS
Ag=43kDa
1·0
V
C
NS

Vaccinated mice showed a 67% and a 29% decrease from control values in experiments 1 and 2 respectively, the difference in efficacy of the vaccine perhaps reflecting the different amounts of protein injected. Interestingly, when sera from vaccinated and control mice were tested against PCF, E/S and an eluted 43kDa band in ELISA (fig 8.11, all target antigens used at 5μg/ml), mice injected with PCF showed raised IgG antibody levels to both PCF and the 43kDa extract, presumably reflecting the predominance of the 43kDa entity in PCF as revealed by SDS-PAGE (fig. 8.8 A). Antibody levels to E/S in vaccinated mice were only slightly elevated, probably reflecting the greater heterogeneity of proteins in this antigen preparation (including a 43kDa entity) compared to both PCF and the 43kDa extract.

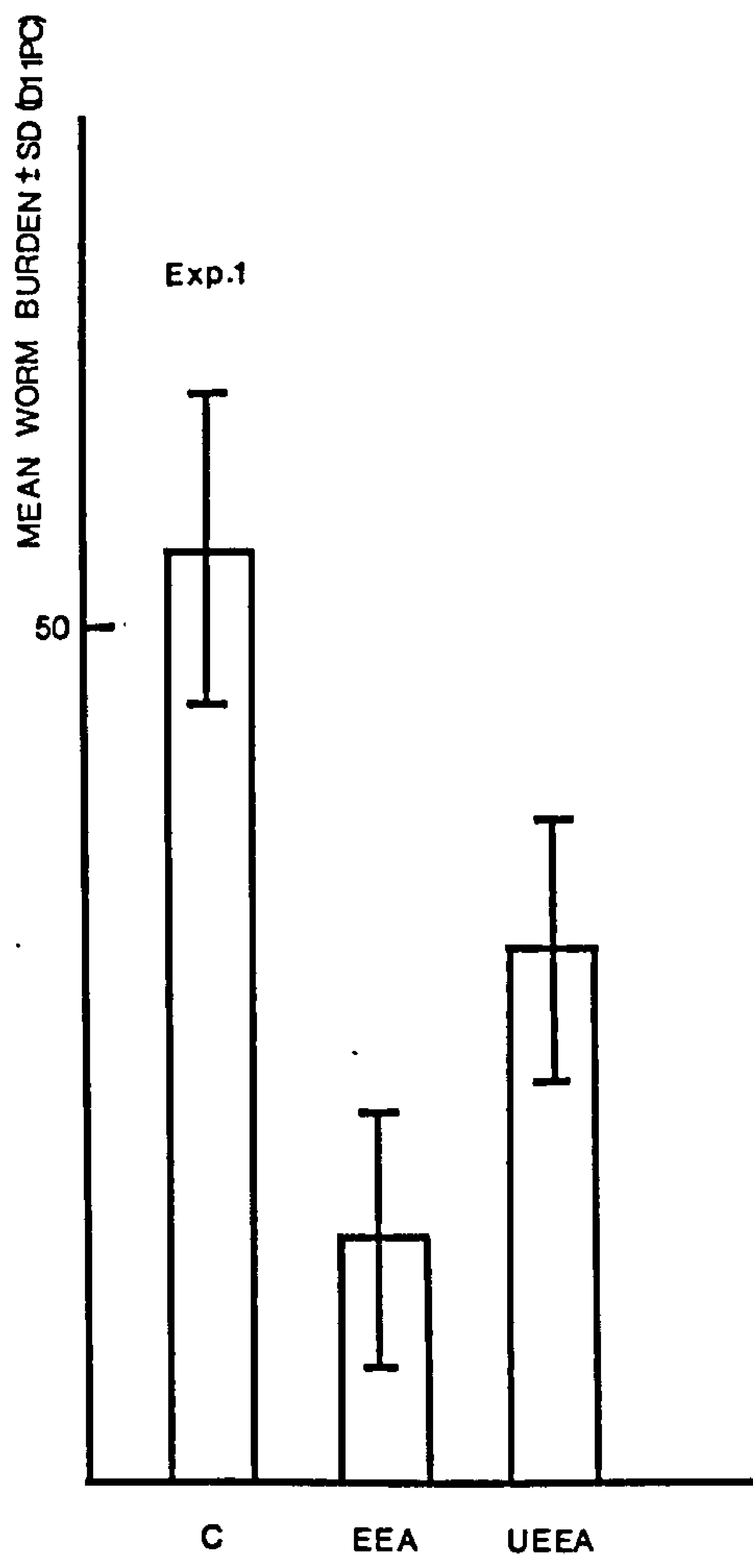
Embryonated and Unembryonated egg antigen (EEA and UEEA)

Figure 8.12 shows the effect on worm burden recovery of injecting EEA or UEEA in FCA into BALB/c mice. "EEA" contained approximately a 2:1 ratio of embryonated to unembryonated eggs. In experiment 1 10μg of EEA or UEEA in FCA was injected into experimental mice, control mice receiving PBS in FCA (at least 5 mice per group). Although establishment was low in the control group (54.2±9.2) both vaccinated groups showed significantly lower day 11 worm burdens, mice given EEA exhibiting a 74% reduction from control values ($P<0.01$) and the UEEA group showing a 43% decrease ($P<0.01$). In experiment 2 EEA in FCA was injected into BALB/c mice at a higher dose (67μg protein) resulting in an almost 100% reduction in worm burdens from control values ($P<0.01$).

43kDa antigen

The major component of AMA and adult E/S antigen is a protein of

Figure 8.12 Vaccination of BALB/c mice with embryonated or unembryonated egg antigen (EEA or UEEA). In exp. 1 experimental groups received 10μg EEA or UEEA in FCA 10 days prior to infection. Control mice (C) were injected with PBS in FCA. Both egg preparations caused a significant reduction in the day 11 worm burdens compared to control mice (both $P<0.01$). In exp. 2 an almost 100% reduction from control values was seen in mice injected with 67μg EEA in FCA on day 11 p.c.


MEAN WORM BURDEN ± SD (D11PC)
Exp.1
50
C
EEA
UEEA

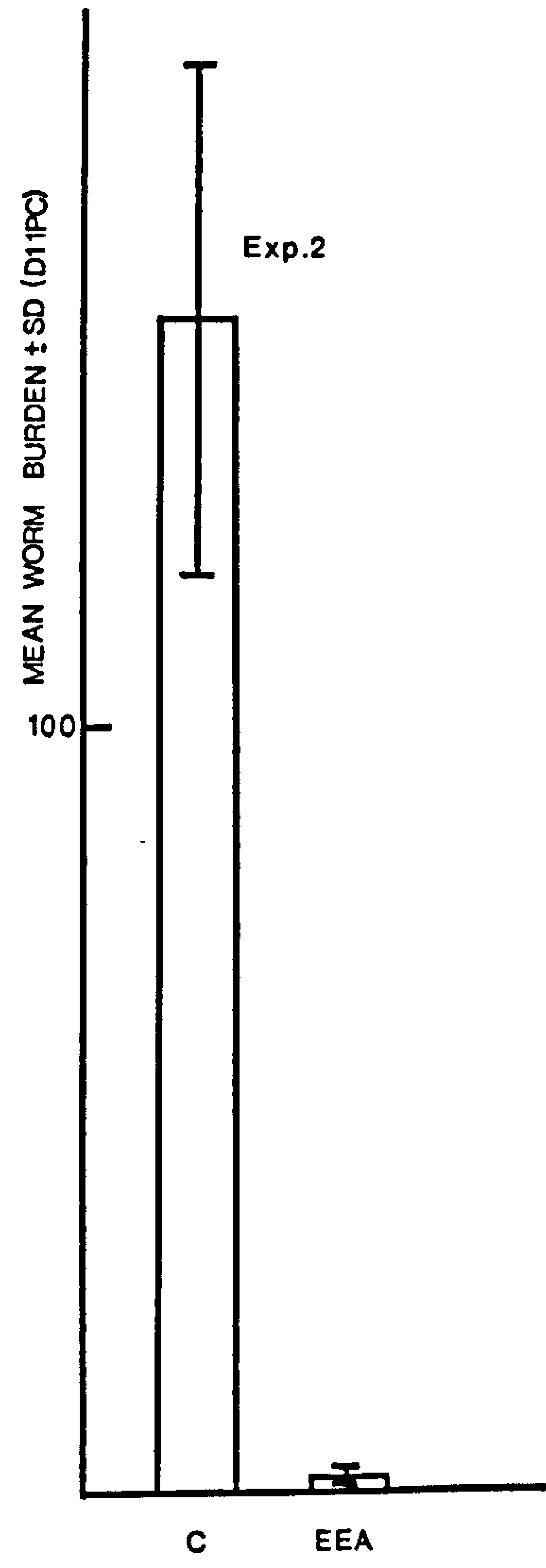
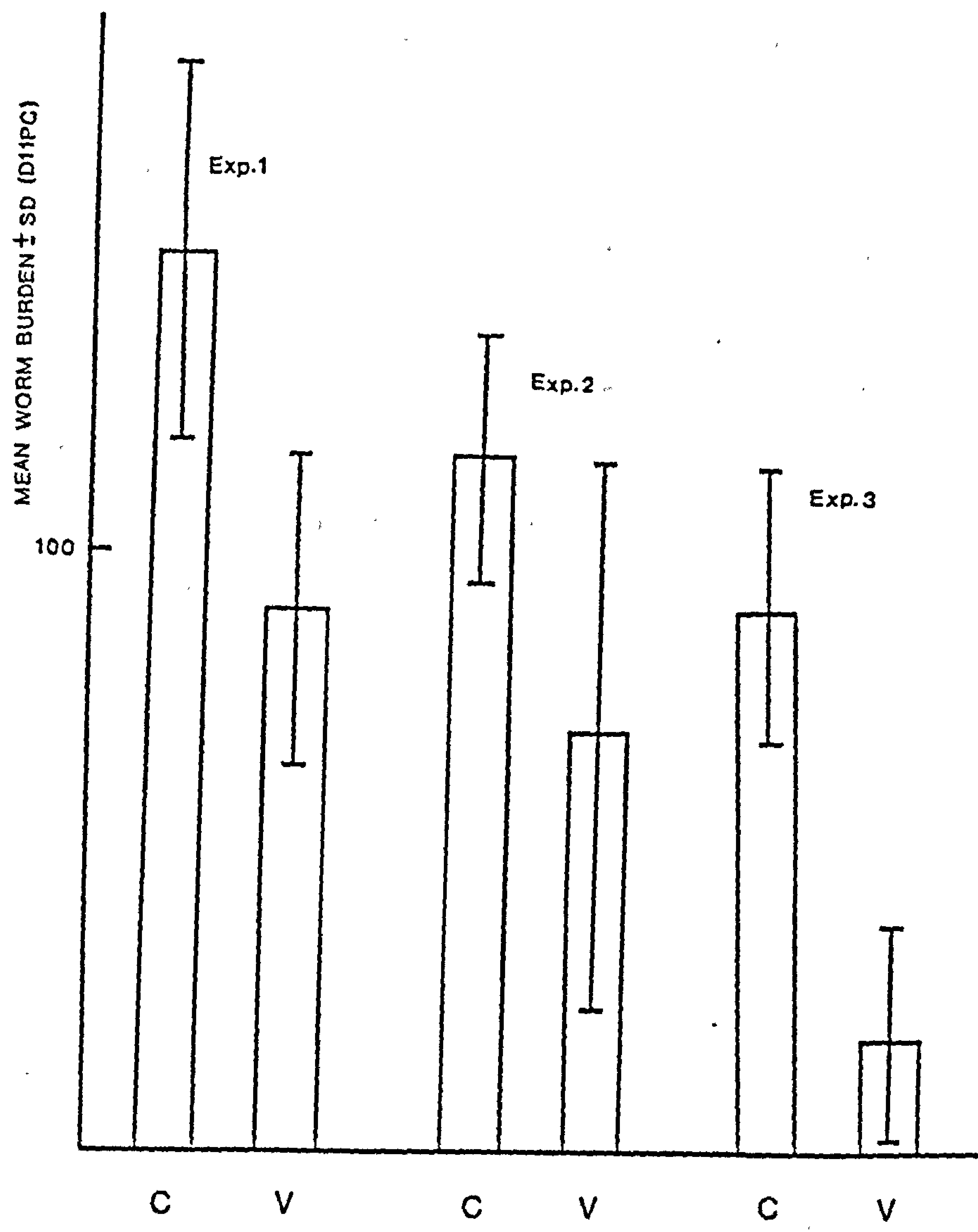

MEAN WORM BURDEN ± SD (D11PC)
Exp.2
100
C
EEA

Figure 8.13 BALB/c mice were injected with 10μg of the 43kDa protein in FCA on three separate occasions (V). In all experiments control mice (C) received PBS in FCA, all mice were infected 10 days after vaccination, and worm burdens were determined on day 11 p.c. Results were variable, particularly in experiment 2, but in all cases vaccinated mice harboured significantly fewer worms than the control mice ($P<0.025$, exp. 1; $P<0.025$, exp. 2; $P=0.001$, exp. 3).


MEAN WORM BURDEN ± SD (D11PC)
100
Exp.1
Exp.2
Exp.3
C
V
C
V
C
V

approximate MW 43kDa appearing as a single broad band on Coomassie stained 1D gels. This protein was eluted from the gel matrix after separation of adult male homogenate by SDS-PAGE and used in a series of vaccination experiments in BALB/c mice. The results of 3 experiments are shown in figure 8.13. At least 5 mice per group were used in experiments 1, 2 and 3. In all experiments mice received 10μg protein in FCA, control mice being given PBS in FCA. Results were variable, particularly in experiment 2 where 3/8 mice harboured day 11 larval burdens similar to control values. However in all experiments a significant reduction in worm burdens from control values was seen. In experiment 1 the decrease was 40% ($P<0.025$), in experiment 2 39% ($P<0.025$) and in experiment 3 79% ($P=0.001$). The greater efficacy as a vaccine of the same amount of the 43kDa protein in experiment 3 compared to experiments 1 and 2 may be related to the lower number of worms establishing in control mice.

8.4 DISCUSSION

Trichuroid nematodes are characterised by the possession of a stichosome consisting of a row of large glandular cells, or stichocytes which partially or completely enclose the oesophagus. The fine structure of the oesophagus, described by a number of authors (Chitwood and Chitwood, 1937; Sheffield, 1963; Jenkins, 1970; Wright, 1972), suggests that the stichocytes may be secretory in function, intracellular collecting ducts leading to the oesophagus being described in some cases (Chitwood and Chitwood, 1937; Wright, 1972). Perhaps the most detailed studies of the trichuroid stichosome have been performed for *Trichinella spiralis* muscle larvae (Despommier and Müller, 1970*b*; 1976). Here 45-55 stichocytes were

described, each being connected to the lumen of the oesophagus by a duct and containing one of two types of secretory granule. The posterior 10–13 stichocytes contained α–granules (α–stichocytes) whilst the more anterior β–cells contained β–granules. Complete cross reactivity between the E/S products released by muscle larvae and antigens of the α– and β–granules suggested that at least some of the components released by the larvae originated from the stichocytes. A 50–55kDa antigen and a 48kDa antigen, located in the α– and β–stichocytes respectively, have been shown to be highly protective *in vivo* (Silberstein and Despommier, 1984). Gamble and Graham (1984) isolated antigens of slightly different MWs from the E/S products of *T. spiralis* muscle larvae which were none the less also protective *in vivo* (Gamble, 1985). These two studies indicate the importance of the stichocyte secretions in the search for candidate molecules to be used as vaccines (see Pritchard, 1986; 1987).

The stichosome of *Trichuris muris* has long been thought to be the source of protective E/S antigens although the evidence is only circumstantial, obtained through the vaccination studies of Wakelin and Selby (1973) and Jenkins and Wakelin (1977) using antigens from the anterior (oesophageal) region of adult worms, and by direct comparison with work done on the closely related trichuroid nematode *T. spiralis* with which *T. muris* shows specific cross immunity (Lee *et al.*, 1982; Roach *et al.*, 1988).

The immunohistochemical investigations reported in this chapter demonstrate clearly that McAbs raised against adult *T. muris* E/S antigen recognise granules within the stichocytes of the stichosome. One of these McAbs (F11) has been shown to passively transfer immunity to infection and protection has also been conferred using F11 affinity isolated antigen (Roach, 1986). Interestingly the F11 McAb failed to recognise tissues within the posterior regions of both male and female worms and showed no evidence of reactivity with the cuticle using JB4 sections. In contrast some

reactivity of the F11 McAb with tissues within the posterior regions of female worms was observed using frozen sections. The reason for these disparate results is not known - despite many attempts to demonstrate reactivity of F11 with tissues within the posterior regions of female worms using JB4 sections, no staining was ever observed. Lack of specific recognition of posterior male tissues was common to all McAbs tested and sera from mice tolerised to infection (TS), but both E12, A16 and TS showed considerable reactivity with certain structures within the posterior of female worms as well as the stichocytes and cuticle. E12 and A16 failed to reliably transfer passive immunity and displayed distinct antigen specificities to the F11 McAb as seen by immunoprecipitation, E12 and A16 recognising the same major antigen of around 43kDa whilst F11 immunoprecipitated 3 lower MW antigens (34, 20 and 18kDa) (Roach, 1986). Staining of the stichocyte granules was strongest with E12 and A16, F11 showing weaker but still quite distinct reactivity. This is probably a reflection of different antibody concentrations in the S/Ns, antibody titres not being determined. The potency of the F11 McAb in conferring relative resistance to infection may be related to its restricted specificity, recognition of only the stichocyte contents and/or recognition of different types of stichocyte granules to E12 and A16. Reassuringly the antigen specificities of the 3 McAbs coincided with the MWs of proteins detected in stichocyte E/S by SDS-PAGE. Interestingly all McAbs tested and TS showed a lower level of reactivity with the contents of the smaller more anteriorly situated stichocytes, which were of a more vacuolated appearance, than with the larger more posterior cells perhaps indicating a regional localisation of antigens to particular stichocytes within the stichosome. The somewhat enigmatic bacillary band, an anterior area of cuticular plugs each with an underlying pore chamber and columnar bacillary cell, has been described by several workers (Wright, 1963; Sheffield, 1963; Jenkins, 1969). Its function remains a mystery,

Wright (1963) suggesting an osmoregulatory role, Sheffield (1963) an excretory function and Jenkins (1969) proposing that the bacillary band might be concerned with the synthesis of secretory material. Disappointingly the McAb probes used in this study failed to localise antigens to the bacillary cells. Preston *et al.* (1986) have previously reported an increased affinity of host anti-parasite antibodies for the surface plugs of the bacillary cells, these plugs being lost around the time of parasite expulsion (day 60 onwards). The functional significance of these observations is not fully understood. In her studies of the surface properties of the developing stages of *T. muris*, Preston reported a considerable degree of stage specificity with no major changes in surface antigenicity occuring after the final moult (day 25-30 p.i.). The generalised fluorescence recorded over the entire cuticle of day 20 and older nematodes following incubation in antiserum taken later in the infection supports the results obtained in this study where TS (taken day 42 p.i.) and two of the McAbs showed reactivity with the outside of the cuticle of day 42 p.i. adult worms. The dark staining pattern on the outer cuticle revealed by immunoperoxidase labelling could potentially represent refraction. However immunofluorescence studies also revealed that E12, A16 and TS displayed reactivity with cuticular antigens. Although at the light microscope level it was impossible to localise surface antigens further, it is tempting to speculate that the antigens recognised were located in the epicuticle, as was demonstrated by McLaren *et al.* (1987) for *Trichinella spiralis*. Using a characterised panel of McAbs raised against stage specific surface antigens of the three life cycle stages of *T. spiralis* (Ortega-Pierres *et al.*, 1984*b*) and electron microscopy they localised surface antigens to the epicuticle of muscle larvae. In addition they demonstrated that antigens present in the epicuticle of muscle-stage larvae were also present in the stichosome and intestinal brush border microvilli and that muscle larval antigens also existed in the stichosome of adult parasites. Silberstein and

Despommier (1984) have also shown that the surface antigens of muscle larvae include stichocyte-derived proteins, both the 48kDa and the 50-55kDa stichocyte polypeptides being present on the surface of the worm. A similar phenomenon is reported here with the McAbs E12 and A16, both raised against *T. muris* E/S, which of course may contain surface shed moietes, recognising epitopes expressed by both stichocyte and cuticular antigens, as well as antigens found in the posterior regions of female worms including the gut lining. If the stichocyte and surface antigens recognised by the McAbs do not merely share determinants but have a common origin (presumably the metabolically active stichocytes) it is necessary to hypothesise as to how the stichocyte antigens are transferred and inserted into the outer cuticle. It is perhaps easier to envisage the adsorption of antigens contained in stichocyte secretions onto the cuticle after their release into the exterior via the mouth and anus. Certainly the reactivity of E12 and A16 with the gut lining of *T. muris* could easily reflect the release of stichocyte secretions into the gut lumen.

The importance of nematode surface antigens in the stimulation of protective immune responses is illustrated by the work of Grencis *et al.* (1986) where it was demonstrated that relatively pure surface antigens of *Trichinella spiralis*, obtained by cetyltrimethyl-ammonium bromide (CTAB) stripping, were capable of generating protective host responses *in vivo*, reflected by a reduction in intestinal worm burdens, fecundity, worm length and muscle larval burdens. The active shedding of surface antigens (Vetter and Klaver-Wesseling, 1978; Philipp *et al.*, 1980; Smith *et al.*, 1981; Maizels *et al.*, 1984; Pritchard *et al.*, 1985) raises the question as to whether surface antigens or surface shed antigens are responsible for the generation of protective immunity *in vivo*, cuticular antigens making a substantial contribution to the contents of E/S products (Philipp and Rumjaneck, 1984; Pritchard, 1986; Lightowlers and Rickard, 1988). It should

also be borne in mind that the shedding of surface antigens could be parasite-protective rather than stimulating host protective responses, representing a mechanism by which bound antibody and/or adherent host cells could be removed from the parasite surface before exerting a damaging effect. Shed surface antigens may also divert the host protective response away from the parasite surface by stimulating immune responses at sites away from the parasite (Smith *et al.*, 1981; Philipp and Rumjaneck, 1984; Pritchard, 1987; Lightowlers and Rickard, 1988). The presence of a 43kDa entity on the surface of *T. muris* has been demonstrated by CTAB stripping (Roach, 1986). Preliminary studies on the shedding of *T. muris* cuticular molecules suggested that a restricted number of surface proteins were present in the adult (as seen by ^{125}I-surface labelling - Iodogen method), as has been reported for *T. spiralis* (Philipp *et al.*, 1980). These molecules included a protein of approximate MW 43kDa and most were released into the medium during *in vitro* culture. Of course this does not necessarily reflect turnover *in vivo*, release of surface components during *in vitro* culture perhaps representing some form of degradation rather than active shedding. The 43kDa protein of *T. muris* is assumed to be of stichocyte origin however its dominant presence in E/S could reflect surface shedding of a cuticular protein. The two of course are not incompatible due to the uncertain origin of surface released antigens - E/S antigens from the stichocytes may be deposited on the cuticle prior to being shed. The importance of the 43kDa protein in protecting the host against *T. muris* infection is indicated by the vaccination experiments carried out using 43kDa antigen preparations eluted from polyacrylamide gels after separation of AMA proteins by SDS-PAGE. A significant degree of protection was conferred in all experiments. Thus the 43kDa antigen is a potential target for gene cloning and synthetic peptide synthesis.

The localisation of antigens by McAb probes within worm tissues

posterior to the oesophageal–intestinal junction revealed that unlike male worm tissues, which showed little specific reactivity with the McAbs or TS, regions within the posterior of female worms showed considerable immunoreactivity. In particular a layer within the egg shell of mature females, identified as the lipid layer by comparison with electron micrographs of the egg shell of *T. muris* (Preston and Jenkins, 1984), stained intensely after incubating with E12, A16 and TS. As discussed earlier, F11 failed to react with any tissue other than the stichocytes. Although both embryonated and unembryonated egg antigen preparations were shown to be protective in vaccination experiments, it is difficult to see how egg–specific antigens could contribute to the development of a protective immune response. The reactivity of E12, A16 and TS with the lipid layer of the egg shell, and the potency of egg material in protecting mice against challenge infections almost certainly reflects the presence of epitopes also expressed by stichocyte and/or cuticular antigens. Thus if any anti–stichocyte activity was absorbed out from the McAb S/Ns prior to use the lipid layer may no longer fluoresce. Indeed preabsorption of the McAb S/Ns and TS with E/S antigen did remove all specific reactivity with all worm tissues. Interestingly a preliminary study using sera from *T. trichiura*–infected individuals revealed recognition of the *T. muris* egg lipid layer by *T. trichiura* positive serum samples. Control sera including high titre anti–hookworm serum (donated by D. I. Pritchard) showed no reactivity. Such cross reactivity, already demonstrated by Roach *et al.* (1988) may suggest a role for immunohistochemistry in immunodiagnosis of infection.

E12, A16 and TS displayed reactivity with pseudocoelomic fluid (PCF) within the body cavity of female worms. The lack of labelling of male worm sections may merely reflect less fortuitous sectioning. PCF was found to be protective when administered as a vaccine although a protective role

for PCF-specific antigens *in vivo*, as for egg-specific antigens is hard to envisage. Even if damaged worms leak PCF which thus gains access to the host immune system, immune responses directed against PCF-specific antigens would be unable to gain access to the interior of undamaged worms due to the resilient nematode outer cortex. 1D SDS-PAGE of PCF revealed a very restricted protein composition, consisting of one broad band resolving at approximately 43kDa, plus a triplet of low MW proteins. Mice immunised with PCF produced IgG antibodies to the 43kDa protein as seen by ELISA. Thus the recognition of PCF by E12, A16 and TS, and the capacity of PCF to protect, may reside in the dominancy of the 43kDa antigen in this protein-rich fluid, 1D SDS-PAGE already demonstrating the presence of a 43kDa protein in stichocyte secretions and at the cuticle surface (Roach, 1986). Silberstein and Despommier (1984) localised a 37kDa antigen of *T. spiralis* to the pseudocoelom but this antigen was only protective at doses fifty times greater than used for the highly protective 48kDa and 50-55kDa antigens. Perhaps the best documented study of nematode body fluid antigens involves *Ascaris suum* where a number of antigens, including potent allergens, have been identified (Kuo and Yoo, 1977; O'Donnell and Mitchell, 1980). The recognition by E12, A16 and TS of the contents of a duct-like structure within female worms is puzzling. In appearance the structure resembled an excretory duct but only one such 'duct' was revealed in transverse sections and sections through male worms showed no such structure, although this could reflect a sampling problem. There is no recognised conventional excretory system recorded for trichuroids (Jenkins, 1973) therefore the apparent excretory duct visualised here, and containing antigenic material, perhaps warrents further study.

The prominent sites of non-specific reactivity with the inner muscle regions of the body wall exhibited by test and control reagents may be related to the fact that this part of the somatic musculature is the main site

of glycogen storage in nematodes. Certainly histochemical tests for glycogen produce similar staining patterns to the non-specific antibody reactivity revealed by immunohistochemistry (T. Jenkins, *pers. comm.*).

The staining patterns produced by E12, A16 and TS on sections of adult females were reminiscent of those revealed by the immunoperoxidase studies on the filarial nematode *Brugia malayi* by Wenger *et al.*, (1988). In this study phosphorylcholine-containing antigens were localised in female and male worms in egg-bearing regions (especially the egg membranes and inner uterine lining) and the intestinal lining respectively. The presence of phosphorylcholine determinants on intestinal membraneous structures and the lining of the intestinal tract but not the cuticle has been shown for *Ascaris suum* (Gutman and Mitchell, 1977) and *D. viteae* (Gualzata *et al.*, 1986; 1988). In the latter study immunolabelling also revealed intense staining of the pseudocoelomic cavity due mainly to anti-PC antibodies, labelling being reduced after the absorption of PC-specific antibodies from test sera. Phosphorylcholine-containing antigens are present in a variety of organisms including nematodes. The presence of phosphorylcholine determinants has been demonstrated for *Ascaris suum* (Crandall and Crandall, 1971; Gutman and Mitchell, 1977), *Nippostrongylus brasiliensis* (Péry *et al.*, 1974), *Toxocara canis* (Sugane and Oshima, 1983), *Brugia malayi* and *Brugia pahangi* (Maizels *et al.*,1987) and *D. viteae* (Gualzata *et al.*, 1986; 1988). In order to control for the possibility that the anti-*T. muris* E/S McAbs had reactivity against PC they were tested against PC-BSA in ELISA. No reactivity was observed. In addition immunofluorescence on JB4 sections using an anti-PC McAb as primary antibody revealed only low level reactivity with the gut lining, the inner muscle region, and perhaps the cuticle and unembryonated egg material, the stichocytes and egg lipid layer being unlabelled. Thus the immunolabelling of structures within *T. muris* by E12, A16 and F11 does not apparently represent an anti-PC response even

though a number of *T. muris* antigen preparations, including egg antigen, PCF and the 43kDa antigen where shown to express PC determinants by ELISA. The presence of PC determinants on the 43kDa antigen may explain the weak bands revealed around this MW following immunoprecipitation of metabolically labelled E/S antigen with NS. The apparent contradiction in results whereby the use of an anti-PC McAb as primary antibody in immunolabelling studies failed to produce any strong labelling even though internal structures of *T. muris* clearly do express PC determinants as seen by ELISA, may reflect a greater accessibility to PC determinants in worm homogenates and tissue extracts as opposed to *in situ* as is the case for tissue sections, and/or the greater sensitivity of ELISA over immunohistochemistry.

Specific cross immunity between *T. spiralis* and *T. muris* is well documented (Lee *et al.*, 1982; Roach *et al.*, 1988) and it has long been speculated that the stichocytes are the source of any shared antigens. Stichocyte antigens of *T. spiralis* muscle larvae have been shown to be highly protective *in vivo* (Silberstein and Despommier, 1984) and the recognition by anti *T. muris* E/S McAbs, one of which is protective, of granules within the stichocytes of *T. muris* adults suggests that in parallel with *T. spiralis*, the stichosomal cells of *T. muris* contain functional antigens. Little is known about the function of stichocyte antigens (reviewed by Pritchard, 1987) although the stichosome is generally thought to be involved in feeding and localised tissue digestion through the secretion of enzymes (Jenkins, 1970). Nimmo-Smith and Keeling (1960) identified proteolytic activity in extracts of *T. muris* but felt that the enzymes were probably involved in turnover of cellular constituents and metabolites rather than representing secreted enzymes. Jenkins (1970) demonstrated the presence of esterase activity in the stichocytes of *T. suis* using histochemical techniques and Wright (1963) detected a phosphatase enzyme in the

bacillary cells of *Capillaria hepatica*. Roach (1986) identified proteases in *T. muris* E/S antigen by incorporating enzyme substrates into gels and electrophoresing E/S antigen samples O/N. It is relatively easy to envisage the stichosome acting as a source of antigenic material capable of eliciting protective immunity if indeed it is the origin of enzymes required for burrowing and/or feeding. Equally it seems likely that the membrane-bound secretory granules within the stichocytes of *T. spiralis* muscle larvae (Despommier and Müller, 1970*b*; 1976) are similar to the membrane bound secretory granules described by Wright (1972) in the stichocyte cytoplasm of two species of *Trichuris*, and that are apparently recognised strongly by McAbs raised against *T. muris* E/S as demonstrated by the immunohistochemical studies presented here. The basis of the specific cross immunity between *T. muris* and *T. spiralis* demonstrated by Lee *et al.* (1982), and between *T. muris*, *T. trichiura* and *T. spiralis* (Roach *et al.*, 1988) may well lie in the possession of common functional antigens derived from the stichocyte granules and if so may provide a target for immune attack against both *T. spiralis* and *T. trichiura* in man.

8.5 SUMMARY POINTS

1. Three McAbs, E12, A16 and F11, raised against *T. muris* E/S antigen have been shown to recognise granules within the stichocyte cytoplasm of adult *T. muris*.

2. E12 and A16 also showed reactivity with cuticular antigens and tissues within the female worm, in particular the lipid layer of the egg.

3. The F11 McAb, shown previously to transfer passive immunity and to display antigenic specificity distinct from E12 and A16, showed reactivity only with the stichocyte granules (JB4 sections). It is suggested that the specificity of F11 may be related to its ability to protect *in vivo*, neither E12 nor A16 reliably transferring immunity.

4. E12, A16 and F11 did not recognise PC determinants as seen by ELISA.

5. The presence of epitopes expressed by both stichocyte and cuticular antigens may reflect a common origin (the metabolically active stichocytes?) or merely shared determinants.

6. A major 43kDa protein of E/S, and AMA, also known to be on the surface of worms, may be shed during *in vitro* culture thus making a substantial contribution to the E/S products. A protein band at MW 43kDa has been demonstrated in stichocyte E/S perhaps suggesting a common origin for cuticular and stichocyte antigens, and a 43kDa protein eluted from polyacrylamide gels after the separation of adult male *T. muris* proteins by SDS-PAGE, has been shown to be protective *in vivo*.

CHAPTER 9

SUMMARY DISCUSSION

In any intricate host-parasite relationship many factors may influence the outcome of infection, the basis for an ability to resist infection perhaps residing in, for instance, behavioural factors, the nutritional status and/or the immunological status of the host. Equally important in the development of host-parasite relationships are the constraints the parasite imposes on the host through the modulation of immunity. In this thesis investigations have been made into two aspects of the interactions between the nematode parasite *Trichuris muris* and its host, the mouse.

Section One is concerned with the effects of host genes on the expulsion of *T. muris* and their role in the control of the humoral immune responses to this parasite.

Section Two attempts to dissect the mechanisms by which *T. muris* modulates the host's ability to express protective immunity and to assess the influence of the host's genetically determined response status on the ability to express resistance after vaccination.

The final section addresses the problem of the likely source of the immunogenic and immunosuppressive components of *T. muris* excretory/secretory (E/S) antigen using a panel of monoclonal antibodies raised against E/S antigen (Roach, 1986).

Wakelin (1975*b*) demonstrated the importance of genetically determined differences in the ability of mice to expel *T. muris* although an evaluation of the relative contributions of major histocompatibility complex (MHC), or H-2-linked genes, and background (non-H-2) genes was not made. The importance of both these sets of genes in the control of host immunity is well documented for the related parasite *Trichinella spiralis* (reviewed by Wassom, 1985; Wakelin, 1988*a*; Wassom and Kelly, 1989). In close parallel with the *T. spiralis* system the work presented in Section One demonstrates that both H-2 and non-H-2 genes influence the expression of protective immunity to *T. muris* infection, with H-2 linked genes effecting a fine

tuning of anti-worm immunity within the limits defined by the genetic background. For example, all strains of mice possessing BALB background genes were markedly more resistant to infection than mice of the B10 genetic background. However, within both these groups of H-2 congenic mice expression of q or b alleles at the H-2 was associated with stronger protective immune responses than the expression of "susceptibility" d or k alleles. Through the use of a panel of H-2 recombinant strains of mice control of resistance to *T. muris* infection was mapped to loci within the H-2. Response phenotypes were found to be related to the presence of "resistance" (q,b) or "susceptibility" (k,d) alleles at I-A, under the strong modulatory influence of certain alleles at a locus/loci lying between the S and D loci of the H-2 complex. In particular resistance q and susceptibility d alleles were associated with marked up- and down- regulation of the effects associated with the expression of $I\text{-}A^k$ and $I\text{-}A^q$ gene products respectively. These results bear a striking resemblance to those reported for the *T. spiralis*-mouse system.

Analysis of the humoral immune responses to *T. muris* E/S antigen by ELISA and immunoprecipitation revealed the influence of both background and H-2-linked genes with the more resistant BALB congenics expressing lower level antibody responses than the more slowly responding B10 congenics. The antibody response to E/S antigen was predominantly an IgG response. An exclusive $H\text{-}2^q$ restriction on the ability to rapidly develop high levels of specific IgG1 antibodies was revealed by studying the responses of inbred, congenic and H-2 recombinant strains of mice including seven strains expressing q alleles at I-A on four different genetic backgrounds. There was also an almost exclusive $H\text{-}2^q$ restriction on the ability of mice to recognise two high MW antigens (90-95kDa, 105-110kDa) this latter phenomenon, at least in part, probably reflecting the restriction on the ability to produce high levels of specific IgG antibody. As such, this

restriction would operate *in vivo* and may contribute to the generally good responder status of H-2^q haplotype mice perhaps by mediating parasite damage prior to cellular attack. However within the DBA/1 strain of mouse a differential ability to expel *T. muris* was observed yet all individuals expressed antibody responses typical of H-2^q strains of mice, therefore clearly a good IgG1 response is not the only requirement in protective immunity and the defect in non-responder DBA/1 individuals, genetically identical to their responder counterparts, may lie in modulation by the parasite at the T cell subset level. Interestingly, several non-responder mouse strains, all expressing k alleles in the I region of the H-2 complex produced strong specific IgG2a responses and negligible IgG1 in contrast to the more resistant I-A^q expressing strains which produced high levels of both isotypes, especially IgG1. This suggests that IgG2a is perhaps irrelevant to protection and the production of IgG2a in the absence of a strong IgG1 response may be a contributory factor in the non-responder status of certain mouse strains. It is possible to speculate at the molecular level as to how the preferential production of IgG2a or IgG1 might arise through the reciprocal regulation of IgG2a and IgG1 by IFN-γ and Il-4, produced by T_H1 and T_H2 cells respectively, discussed in the Appendix to Chapter 5. Thus, presentation of antigen in association with I-A^k molecules might activate T_H1 cells resulting in responses irrelevant to and possibly detrimental to protective immunity, whilst antigen presented in the context of I-A^q may stimulate both T helper subsets though possibly favouring the expansion of T_H2 cells. This would lead to the secretion by B cells of specific IgG1 which may contribute positively to resistance to infection.

Genetically slow- (B10) and non-responder (B10.BR) strains of mice can be protected against infection by vaccination with E/S antigen in Freund's complete adjuvant as demonstrated in Section Two. However protection was slow to be expressed compared to vaccinated

good-responder mouse strains (BALB/c, NIH). Analysis of the antibody responses of vaccinated B10 and B10.BR mice revealed boosted IgG and IgG1 antibody levels compared to control mice and a change in their antigen recognition profiles, three high MW antigens (80-85kDa and the two H-2^q restricted molecules, the 90-95kDa and 105-110kDa antigens) being recognised by antibodies in sera from vaccinated but not control mice. The ability to overcome genetic restrictions on antigen recognition by altering the mode and/or route of antigen presentation, thus perhaps shifting responsiveness of MHC genotypes to particular determinants or boosting specific antibody levels sufficiently to reveal recognition of critical antigens is clearly important with respect to vaccine design. The polymorphism displayed at MHC class I and II loci within the human population makes single peptide vaccines undesirable. Some members of the population would almost certainly possess MHC molecules unable to bind the antigen prior to presentation to T cells or MHC molecules that could bind antigen but in such a way that presentation of antigen would stimulate T cells that generate responses irrelevant to protective immunity. As pointed out by Blackwell (1989) a successful vaccine should contain a battery of peptide epitopes to compensate for the genetically diverse MHC class I and class II molecules with which they are presented to T cells. The problems of genetic restrictions on the recognition of specific antigens can also be overcome by the appropriate use of adjuvants or carrier proteins as suggested by the results presented in Chapter 7 in accordance with those of other workers (e.g. Del Giudice *et al.*, 1986).

Differential responsiveness to infection was observed within the slowly responding B10 congenic strains of mice and is a phenomenon described earlier for DBA/2 mice (Worley *et al.* ,1962; Lee and Wakelin, 1982*b*; Roach, 1986). Uniform behaviour within a group of genetically identical individuals is expected unless factors such as parasite-induced effects are

also present. The series of experiments described in Section Two involving the extension or abbreviation of exposure to the parasite in poor- and non-responder strains indicated that the later larval and adult stages of *T. muris* were indeed apparently immunosuppressive, suppression of host immunity during primary infection also affecting the fate of subsequent infections. The mechanisms by which *T. muris* modulates host immunity are not known. However the ability to protect the non-responder B10.BR mouse strain against infection by vaccination was abrogated by prior exposure of mice to adult stages of *T. muris*. This was correlated with depressed IgG1 levels, possibly the production of an irrelevant Ig isotype (IgG2a?) and failure to recognise the three high MW antigens recognised when B10.BR mice were vaccinated and challenged without previous experience of the parasite. Thus one way in which *T. muris* may interfere with the development of protective immunity is by suppressing IgG1 production, although whether suppression is exerted at the level of the B cell, T cell or antigen presenting cell is open to speculation. Other mechanisms of immunomodulation may also operate. For instance Roach (1986) reported that adult *T. muris* E/S antigen was mitogenic. Investigations into the mitogenicity of this antigen preparation have been made and the results are presented in the appendix to this chapter. Experiments performed using naive mixed lymphocyte populations from the spleen and mesenteric lymph nodes, and cell populations fractionated into B and T lymphocytes suggested that adult E/S antigen probably contained both B and T cell mitogens with lymphocytes from the non-responder B10.BR mouse strain perhaps being more susceptible to the mitogenic effects than cells from more resistant mouse strains. Similar findings have been reported by Roach (1986) for the poor responder B10 strain of mouse and suggest that the polyclonal activation and exhaustion of lymphocyte populations by mitogens, implicated as a parasite evasion strategy by many workers (e.g. Greenwood, 1974;

Hudson *et al.*, 1976), may represent a mechanism by which *T. muris* suppresses host immunity.

The protective components of adult E/S antigen, and indeed the parasite-derived immunomodulatory factors, have long been felt to originate in the stichocyte cells of the stichosome, in parallel with observations made for the related parasite *Trichinella spiralis* (Silberstein and Despommier, 1984; Gamble and Graham, 1984). The immunohistochemical studies described in the final section of this thesis, using a panel of monclonal antibodies (McAb) raised against adult *T. muris* E/S antigen (Roach, 1986) demonstrated the clear recognition by the McAb probes of granules within the stichocyte cytoplasm. One of these McAb probes, shown to transfer passive immunity *in vivo* (Roach, 1986) was specific for the stichocyte granules only whilst the other McAbs also recognised antigens present in the lipid layer of the egg and cuticular antigens, presumably reflecting common epitopes, or, in the latter case, a common origin. This would suggest that at least some of the host-protective antigens contained within adult E/S products are stichocyte-derived with the specific cross immunity between the three trichuroid nematodes *T. muris*, *T. trichiura* and *T. spiralis* (Lee *et al.*, 1982; Roach *et al.*, 1988) residing in the possession of common stichocyte antigens.

To gain a complete understanding of the intricate interactions which occur between host and parasite it is necessary to consider all aspects of the host-parasite relationship. Two aspects have been addressed in this thesis, namely the influence of host genetics on the outcome of infection and in controlling the humoral immune responses to E/S antigen, and the modulatory effects the parasite may have on the development of host immunity. Little attention has been paid to the cellular mechanisms governing the development of host immunity to *T. muris* although T cells are known to play a central role in resistance to infection (Lee *et al.*,

1983). Future studies should be aimed at examining the mechanisms by which T lymphocytes regulate an acute intestinal infection through the generation of protective immune responses and their involvment in the suppression of host immunity observed during chronic intestinal infection.

APPENDIX - CHAPTER 9

A9.1 INTRODUCTION

The data in this appendix are from a series of experiments performed to investigate the reported mitogenicity of *T. muris* E/S antigen (Roach, 1986). Results are presented from experiments in which naive mesenteric lymph node (MLN) and spleen (SPL) cell preparations, and cell populations fractionated into T and B lymphocytes (by panning techniques and immunomagnetic separation - see Mason *et al.*, 1987; Funderud *et al.*, 1987) were incubated with antigen and the degree of proliferation measured.

A9.2 RESULTS

Table A9.1 Proliferative responses of spleen (SPL) and mesenteric lymph node (MLN) cells

Pooled cells (2 mice/group) from naive and immune (day 74 p.i.) BALB/c mice, at $5x10^6$ cells/ml, 50μl/well, were incubated for 48 hours with 2 hour or O/N E/S antigen, pulsed for 6 hours with ^{3}H-TdR, then harvested (see Chapter 2.4.3). Absolute values are given for the control values, the other results are expressed as a ratio of experimental cpm/control cpm.

Cell population	Antigen concentration (μg/ml)	2 hour E/S	O/N E/S
	0	2660	2660
Naive	1	1.4	1.1
SPL	10	1.8	1.4
cells	50	6.4	3.7
	100	9.8*	5.9*
	200	12.9	5.4
	0	2002	2002
	1	1.4	1.3
Naive	10	2.4	1.6
MLN	50	5.1	2.6
cells	100	7.7*	2.9*
	200	7.9	1.7
	0	3575	3575
	1	1.1	1.6
Immune	10	2.4	2.1
SPL	50	9.9	9.7
cells	100	18.1	17.6
	200	23.9	16.2
	0	2506	2506
	1	1.4	1.3
Immune	10	2.2	2.3
MLN	50	6.6	4.5
cells	100	11.1	6.7
	200	12.8	5.1

*see A9.3

Table A9.2 Proliferative responses of naive spleen cells after removal of T cells by panning

Pooled spleen cells from 3 naive BALB/c mice were incubated on anti-Thyl coated plates at 4°C (see Chapter 2.4.4). The cell S/N was removed and the T-cell depleted population (BSPL) incubated with a pooled 4 hour and O/N E/S preparation at $5x10^6$ cells/ml, 50μl/well. An unpanned mixed splenic lymphocyte population was treated similarly (BTSPL). After 48 hours the cells were pulsed O/N with ^{3}H-TdR before harvesting. The percentage of T cells remaining in the T cell-depleted population was determined by FACS analysis using an anti-L3T4 plus anti-Lyt2-monoclonal antibody (McAb) preparation. Control cell samples, to which no anti-T cell marker McAbs were added, indicated the level of background fluorescence.

FACS analysis

	% T cells	Control
BSPL	12.9	7.6

Cell proliferations. Absolute values are given for the control values, the other results being expressed as a ratio of experimental cpm/control cpm.

Antigen concentration (μg/ml)	BSPL	BTSPL
0	51	52
10	8.8	11.3
50	11.5*	20.9
100	5.9	16.7

* see A9.3

Table A9.3 Immunomagnetic depletion of B cells from naive MLN preparations prior to incubation with antigen

Pooled MLN cells from 5 naive BALB/c and 6 naive B10.BR mice were incubated with Dynabeads M-450 (Dynal) coated with sheep anti-mouse IgG (see Chapter 2.4.4). The cell S/N was removed and the B cell-depleted population (TMLN) incubated with a pooled 4 hour plus O/N E/S preparation, AMA, ConA or LPS, at $5x10^6$ cells/ml, 50µl/well. A mixed MLN lymphocyte preparation (BTMLN) was treated similarly. After 48 hours the cells were pulsed O/N with ^{3}H-TdR prior to harvesting. The percentage of T cells in the B cell-depleted populations were determined by FACS analysis using an anti-thyl McAb (for BALB/c only). Control samples, to which no anti-thyl McAb was added indicated the level of background fluorescence.

FACS analysis

	% T cells	control
BALB/c TMLN	70.9	2.6
BALB/c BTMLN	50.4	1.1

Cell proliferations. Absolute values are given for the control values, the other results being expressed as a ratio of experimental cpm/control cpm.

Antigen	Antigen Concentration (µg/ml)	BTMLN B10.BR	BTMLN BALB/c	TMLN B10.BR	TMLN BALB/c	BTSPL B10.BR
	0	7306	4076	600	105	6657
	1	1.5	1.2	0.9	0.9	1.5
E/S	10	5.5**	2.8**	5.1	3.3	5.8
	50	12.2	9.4	14.2*	12.4*	13.6
	100	12.2	12.8	14.8	13.0	13.6
	1	13.0	34.2	139.5	1164.0	10.4
ConA	5	17.0	38.7	125.9	-	16.7
	10	12.3	26.8	190.4	75.0	10.1
	0.5	1.3	1.2	0.9	0.9	1.0
AMA	5.0	1.3	1.3	0.8	0.8	0.9
	50.0	1.7	1.7	1.8	0.9	1.1
	5	16.2	7.4	7.3	2.7	16.1
LPS	10	15.3	9.2	6.2	3.5	17.0
	25	17.1	9.0	4.4	2.2	16.9

**, * see A9.3

A9.3 DISCUSSION

The results presented in Table A9.1 indirectly indicate that adult E/S antigen might contain B cell mitogens (see * values for E/S at 100μg/ml). Naive, mixed lymphocyte populations from both the spleen and mesenteric lymph nodes (MLN) were stimulated by E/S. However spleen cells proliferated to a greater extent in the presense of E/S antigen than MLN cells, the spleen containing a higher percentage of B cells than the MLNs. It is possible that the mitogenicity of E/S antigen resides in bacterial contamination of the E/S products. However E/S products collected after incubating worms for just 2 hours in medium stimulated naive cells to a greater extent than E/S collected after O/N culture yet presumably would be less likely to contain bacterial contaminants. Naive T cell-depleted spleen cell populations (B_{SPL}) proliferated in response to E/S antigen (Table A9.2, e.g. * values for E/S at 50μg/ml) as did naive B cell-depleted MLN cell populations (T_{MLN}) (Table A9.3, e.g. * values for E/S at 50μg/ml). However cell depletions were never 100% making a clear interpretation of the results difficult. Also the efficiency of the depletion methods was monitored by staining for T cells only. Ideally percentages of both B and T cells in the fractionated cell populations should have been determined. Thus the B cell-depleted MLN cell population from BALB/c mice (Table A9.3) contained 70.9% T cells but the number of B cells remaining in the other 29.1% of cells was not estimated. Even though the limited proliferation of the B cell-depleted MLN cells observed after culturing with LPS would suggest that the depletion process had in fact removed most of the B cell activity, until purer B and T cell lymphocyte populations can be obtained it is only possible to speculate as to the identity of the parasite-derived mitogens' target lymphocyte population. Interestingly the proliferation of

unfractionated naive B10.BR MLN cells in the presence of E/S antigen was greater than that seen in unfractionated naive BALB/c cells, particularly at lower antigen concentrations (Table A9.3, e.g. ** values for E/S at 10μg/ml). This suggests that a contributory factor to the non-responder status of B10.BR mice may be a greater susceptibility of their lymphocytes to the mitogenic effects of adult E/S antigen. Roach (1986) reported similar findings with the poor responder B10 strain of mouse. It is possible that B10 background genes influence lymphocyte sensitivity to mitogenic factors in E/S. Gao *et al.*, 1988, reported that the action of a substance produced by splenic T cells from mice with chronic *Trypanosoma cruzi* infection, which inhibited the induction of delayed-type hypersensitivity to a range of antigens, was genetically highly restricted. Although all three inbred strains of mice tested produced the suppressor substance (SS) after *T. cruzi* infection only one was sensitive to its suppressive action. The genetic restriction of the SS activity was apparently not related to the H-2 gene complex nor susceptibility to infection. In contrast, the sensitivity of lymphocytes from mice of the B10 genetic background to the mitogenic effects of adult *T. muris* E/S may contribute to their poor responder status. Parasite-derived factors, mitogenic for B and T cells have been identified for a number of parasites including *Trypanosoma rhodesiense* (Campbell *et al.*, 1982), *T. brucei* (Esuruoso, 1976) and *Brugia malayi* (Wadee and Piessens, 1986). Parasite mitogens are thought to result in the polyclonal hypergammaglobulinaemia observed in such parasite infections, the elevated serum immunoglobulins including anti-parasite antibodies and a large proportion of antibodies directed against irrelevant or non-parasite antigenic determinants (see Mitchell, 1979).

The polyclonal activation of B cells by, for instance, malaria and trypanosome parasites may act as a parasite evasion mechanism causing generalised immunodepression (Greenwood, 1974; Hudson *et al.*, 1976).

Diamantstein *et al.*, 1976, suggested that mitogenic stimulation of B cells in the absence of a particular antigen leads to a temporary loss in their ability to respond to that antigen. Thus, as was suggested by Hudson *et al.* (*loc. cit.*), the polyclonal activation of B cells in the presence of a continuous trypanosome infection could result in the depletion of antigen-reactive B cells and hence immunodepression. Although hypergammaglobulinaemia during chronic *T. muris* infection has not been described, humoral responses to T-dependent and T-independent antigens are depressed in mice with chronic worm burdens (Lee and Wakelin, 1983). Thus the polyclonal activation and exhaustion of lymphocyte populations by mitogenic factors may be a mechanism by which *T. muris* suppresses host immunity.

A9.4 SUMMARY POINTS

1. Adult *T. muris* E/S antigen was found to contain mitogenic components.

2. E/S antigen stimulated proliferation of naive B cell-depleted and T cell-depleted cell populations suggesting that both B and T cell mitogens were present.

3. The production of mitogenic factors may represent a mechanism by which *T. muris* suppresses host immunity, via polyclonal activation and exhaustion of lymphocyte populations.

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