Mucosal and Systemic Immunity of the mouse to the nematode *Trichinella spiralis*

BY

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MUCOSAL AND SYSTEMIC IMMUNITY OF THE MOUSE TO THE NEMATODE *Trichinella spiralis*

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

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Abstract

Gastrointestinal nematode infections result in a mucosal immunoglobulin response, but this response is not well defined and the role of mucosal immunoglobulins in producing the effects of immunity is not clear. *Trichinella spiralis* infections in mice were used to assess the immunoglobulin response, to determine strain differences and the influence of host genotype on the response, to determine the effects of oral immunization with crude antigens on the immunoglobulin response and on *Trichinella*, and to determine the mucosal response to the specific antigens, i.e. phosphorylcholine (PC) and cholinesterase. Following infection with *Trichinella* the predominate mucosal immunoglobulin was IgA, but IgG also occurred. This antibody response was correlated with a reduction in fecundity and worm size but not expulsion in BALB/c and five other strains of mice. Reduced fecundity and size of adult female worms and of total muscle larvae occurred in mice immunized by feeding with crude *Trichinella* antigens in combination with cholera toxin. These effects were associated with a 10 fold increase in specific mucosal IgA titer. PC was detected in *Trichinella* extracts, and following infection serum and mucosal immunoglobulin responses to PC were detected. Regulation of the subclasses differed: anti-PC IgG occurred following primary infection but decreased following challenge; IgM occurred in both primary and challenge

infections, but was slightly lower following challenge; bile IgA was similar to serum IgM. Host genotype influenced the response to PC, but differences were not associated with H-2 haplotype. Following infection, no antibody response to *Trichinella* cholinesterases was detected, in contrast to the positive response reported for other animal parasites. The lack of antigenicity was correlated with a low level of cholinesterase secretion and differences in characteristics of *Trichinella* cholinesterases such as the absence of the 7S form. The mucosal response to hexosaminidase appears to make this antigen a good candidate for oral immunization, but the inability of antibodies to neutralize its activity may limit its value. These results support a role for mucosal immunoglobulins in protection against helminth infections of the gastrointestinal tract which may be mediated either directly through antibody binding to the cuticle or orifices of the parasite, or indirectly by an enhanced rate of antigen presentation or stimulation of an inflammatory response.

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Abbreviations

APC	- antigen presenting cell
ATCI	- acetylthiocholine iodide
BSA	- bovine serum albumin
BTCI	- butyrylthiocholine iodide
BW284C51	- 1,5bis(4-allyldimethylammoniumphenyl)-pentan-3-one
CBB	-Coomassie Brilliant Blue
CFA	- complete Freunds adjuvant
CFW	- Swiss Webster mice
CHE	- cholinesterase
CON A	- concanavilin A
CT	- cholera toxin
CTB	- cholera toxin B subunit
EE AChE	- electric eel cholinesterase
ELISA	- enzyme linked immunosorbent assay
ES	- excretory/secretory products
ETOH	- ethanol
FCS	- fetal calf serum
GST	- glutathionine-S-transferase
H-2	- the murine major histocompatibility locus
HLA	- the human major histocompatibility locus
hr	- hour
HRP	- horse radish peroxidase
IFA	- incomplete freunds adjuvant
IFN	- interferon
ISO OMPA	- tetraisopropyl phosphoramide
KD	- kiloDalton
KLH	- keyhole limpet haemocyanin
L1	- larval stage prior to 1st moult
MHC	- major histocompatability
min	- minute
MW	- molecular weight
o/n	- overnight
OVA	- ovalbumin
P1	- designation of a porcine isolate of *Trichinella*
PA	- particulate antigen extract of *Trichinella*
PBS	- 0.02M phosphate buffered saline
PBST	- PBS + 0.05% Tween 20
PC	- phosphorylcholine
PC-BSA	- phosphorylcholine conjugated to BSA
PCCl	- phosphorylcholine chloride
PI	- post infection
PMSF	- phenylmethylsulfonyl flouride
RT	- room temperature
SA	- soluble antigen extract of *Trichinella*
SPA	- soluble/particulate antigen extract of *Trichinella*
SPS	- somatic polysaccharide extract of *Asperigillus*
Th1	- T helper 1 lymphocyte
Th2	- T helper 2 lymphocyte

General Introduction

Parasitism, as defined by J.F.A. Sprent, is an interaction between distinct organisms in which the parasite "obtains it's nourishment from the actual substance of the hosts body" (Sprent, 1963). Within this context a spectrum of host/parasite interactions have been described, ranging from near symbiosis (eg. *Hymenolepis* in rats) to extreme pathogenicity (eg *Schistosoma* in BALB/c mice). The interaction is dynamic, it is affected by characteristics of both the host and the parasite, and the results of infection vary between individuals.

The host response to infection is mediated through the cells and tissues of the immune system and protection is associated with a specific cellular or humoral response. The resistance or susceptibility of hosts vary within and between populations, predominantly as a function of host genotype, but also based on nutritional status and other factors. The influence of host genotype on the response to infection has recently been reviewed (Wakelin, 1988; Wassom and Kelly, 1990). All aspects of the response to infection are influenced by host genotype and the recognition of parasite antigens as foreign is genetically restricted. Antigens, phagocytosed by antigen presenting cells (APC's) are subject to proteolysis and expressed on the APC surface in conjunction with MHC class I or class II molecules. Within a population there are numerous alleles for the subunits of these molecules, and these polymorphic products

vary in their capacity to bind antigen. As a consequence, individual differences in antigen binding are a function of MHC haplotype. Genetic differences can also affect specific immune components (eg. absence of IgE in SJA/9 mice; Watanabe *et al.*, 1988) or have a more drastic effect such as the complete absence of functional B and T cells in SCID mice (Bosma and Carroll, 1991). Furthermore, genetic effects on resistance can be manifested through a system unrelated to the immune response such as the correlation between the sickle cell trait and infection with *Plasmodium*. Finally, experiments with different strains of *Trichinella* indicated that parasite genotype also influenced the response to infection, and that complex interactions occurred in experiments where both host and parasite genotypes were varied (Dick *et al.*, 1988; Wassom *et al.*, 1988; Bolas-Fernandez and Wakelin, 1990)

In addition to the influence of host genotype, specific parasite antigens also have a role in this interaction. Perhaps the best known example is the variant surface glycoprotein of *Trypanosoma*. Antigenic variation resulting from changes in surface glycoproteins protects the parasite during infection (Borst, 1991). A more recent example of specific antigen effects is the modulation of immunity by *Schistosoma* egg antigens (Sher *et al.*, 1991). Prior to egg production, the response to infection is polarized to the T helper 1 (Th1) pathway and is protective. With egg

production and the release of egg antigens a Th2 response is activated, which subsequently down regulates the Th1 response via interleukin 10 and exacerbates infection (Sher *et al.*, 1991).

The examples above illustrate the role of both host and parasite in this dynamic relationship. An understanding of the mechanisms which influence the final outcome of the host/parasite interaction may lead to the potential for manipulation of the response. For example, the observation that protection from the effects of *Vibrio cholerae* was dependent on the antibody response to cholera toxin (CT) led to the development of a vaccine which mediated protection by inducing a response to CT (Holmgren and Svennerholm, 1990). In another parasite system, protection in *Leishmania* infections was associated with a Th1 response and interferon gamma (γIFN) production (Liew, 1990). As a consequence, efforts to develop a vaccine for *Leishmania* have focused on the characterization of antigens which stimulate a Th1 response. These examples illustrate the importance of establishing the mechanism of immunity as a prerequisite to the development of a vaccine. It is also apparent that antigens which illicit a response are not necessarily protective as illustrated using the circumsporozoite antigen from malaria (Nussenzwieg and Nussenzwieg, 1989). Differences in the nature of the response and genetic restriction of antigen recognition may limit the potential

of specific purified antigens as vaccines. Indeed, the most effective vaccines produced have been crude whole cell preparations (Holmgren and Svennerholm, 1990).

To date, the majority of research has focused on the systemic response. Despite that fact that approximately 2 billion gastrointestinal helminth infections occur per year (Wakelin, 1988), limited information is available on the mucosal response to helminth infection. One 'barrier' to the study of intestinal immunity has been the phenomenon of oral tolerance (Kagnoff, 1982). The recent discovery of adjuvants which abrogate the tolerance response has stimulated renewed interest in this system. My thesis examined the mucosal response following infection of mice with *Trichinella spiralis*. As indicated in the following historical account, this host/parasite system provides an excellent model in which to assess the mucosal immune response, the influence of host genotype, the role of parasitic antigens and the potential for oral immunization.

Historical Introduction and the *Trichinella* life cycle

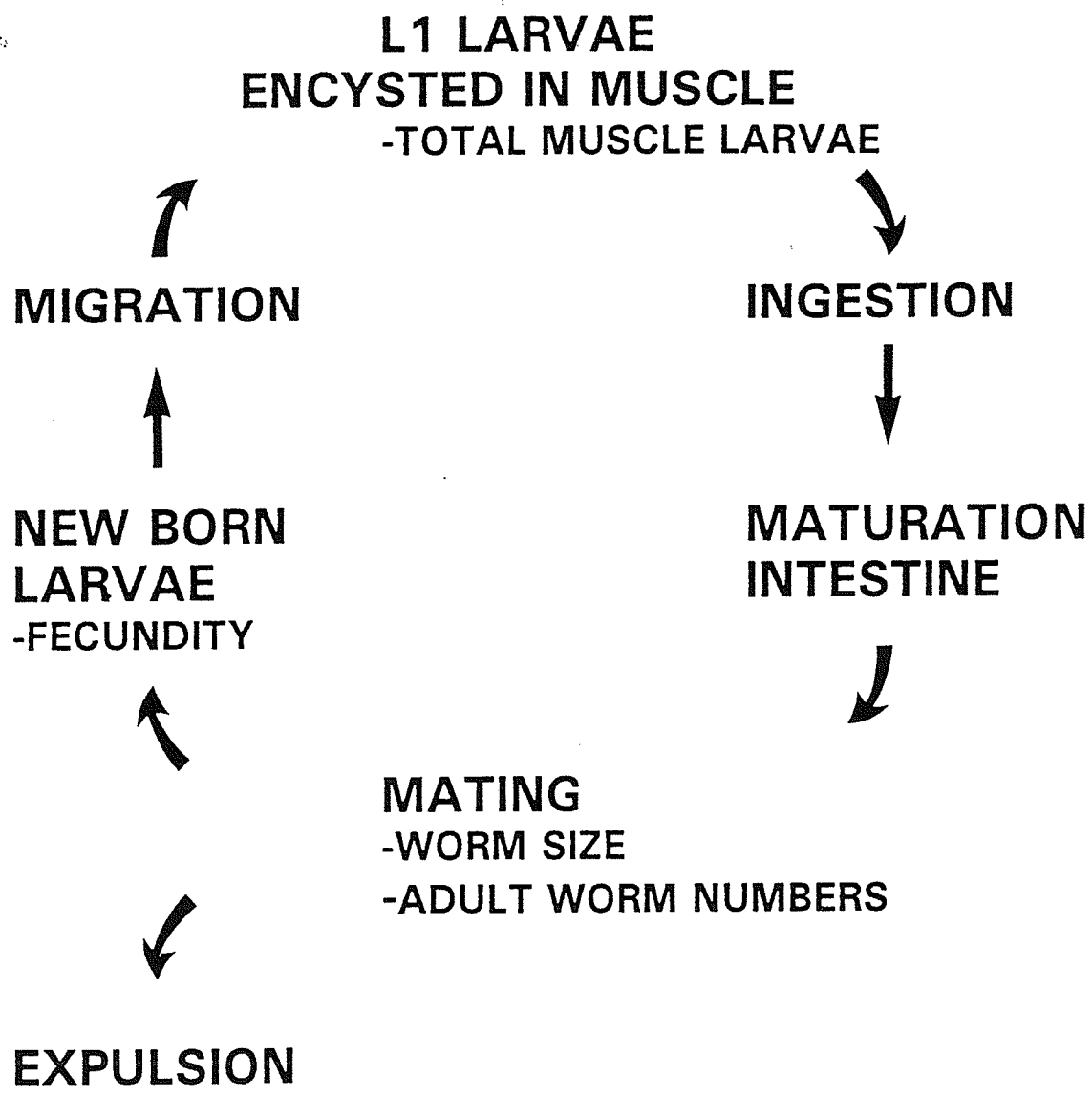
The initial discovery of *Trichinella spiralis* (Owen, 1835) is attributed to John Paget who noted granular cysts in the peripheral musculature of a cadaver which 'dulled his scalpel' during human anatomy classes at Guy's Hospital in London in 1835 (Owen, 1835). In the 150^{+} years since its description, *Trichinella* has been the focus of considerable research effort. Initially it was considered of little

importance and to have no pathological significance (Owen, 1835). This was proven incorrect by Zenker (Gould, 1970) who identified *Trichinella* as the causative agent in the death of milkmaid in 1860. A visit to the farm in question and an analysis of ham and sausage samples allowed Zenker to establish the mode of transmission (reviewed in Gould, 1970; Campbell, 1983). Experimental infections with larvae isolated from the autopsy were valuable in establishing the *Trichinella* life cycle. Numerous small and medium scale outbreaks of human trichinosis have been recorded since that time and continue to occur even today (Dick *et al.*, 1990).

Research in the mid to late 1800's established most aspects of the *Trichinella* life cycle (Figure 1) reviewed by Despommier (1983). Briefly, the encysted form which blunted Paget's scalpel is an L1 larvae which lives intracellularly in modified skeletal muscle fibres of most mammals. Transmission of *Trichinella* is by the consumption of infected meat. Cysts are digested in the stomach and the larvae penetrate the intestinal epithelium within a few hours post infection. They develop rapidly within an intraepithilial tunnel, moulting 4 times to achieve the adult stage in approximately 30 hours. Worms mate and within 5-6 days females begin to shed new born larvae. New born larvae are carried via the circulation to peripheral tissue, penetrate the musculature and develop to the infective L1 stage within 30 days.

Figure 1. The life cycle of *Trichinella spiralis*.

Variables measured in the response to infection included: adult worm numbers, fecundity, size and muscle larvae numbers.


L1 LARVAE
ENCYSTED IN MUSCLE
-TOTAL MUSCLE LARVAE
INGESTION
MATURATION
INTESTINE
MATING
-WORM SIZE
-ADULT WORM NUMBERS
EXPULSION
NEW BORN
LARVAE
-FECUNDITY
MIGRATION

To summarize, the life cycle has three phases: a long lived muscle phase, a short intestinal phase which varies in duration depending on host immune status and the host genotype, but generally lasting from 1 to 4 weeks, and a larval dissemination phase of a few weeks duration depending on the length of the intestinal phase.

The first serological evidence for a host response to *Trichinella* was the description of a high number of eosinophils in the blood of infected patients by T.R. Brown, a medical student at Johns Hopkins University Hospital (Brown, 1897). At the time these cells were not associated with a protective response. Eosinophilia was further studied following *Trichinella* infection of experimental animals (Opie, 1904), with similar results to that shown in humans. Antibody production was first demonstrated by Strobel in 1911 (reviewed in Kagan and Norman, 1970) and in 1916 the transfer of immune serum was used as treatment for trichinosis (Salzer, 1916). However, experiments by Schwartz (1917) put this idea to rest. Immunity to *Trichinella* was demonstrated experimentally by Ducas in 1921 using rats in which prior exposure protected against subsequent infection (reviewed in Sandground, 1929). These results were supported by McCoy (1931) who went on to demonstrate that vaccination of rats with *Trichinella* extracts would also protect against infection (McCoy 1935) and by Culbertson who demonstrated similar protection in mice (Culbertson 1942). The primary

effects of immunity described in these studies were an increased rate of expulsion from the gut and a reduction in the number of muscle larvae. These authors suggested that the effects were mediated in the intestine.

Convincing evidence for the role of the intestine in the induction of immunity was provided by Levin and Evans (1942). These authors demonstrated protective immunity in mice primed with irradiated larvae (which developed to the adult stage but produced no larvae) and challenged with normal larvae. Intestinal effects were also described by Rappaport and Wells (1951). In their experiments an increase in rate of expulsion and a decrease in adult worm size and fecundity occurred following challenge infections in mice primed with 100 larvae. Similar reductions in gut parameters were reported by Campbell (1955) following exposure to excretory/secretory antigen and by Kim (1957) following exposure to irradiated larvae.

Despite the early identification of a host response following infection and the clear biological effects of immunity on the intestinal stage of *Trichinella* the effector mechanisms in this response were not identified and continue to elude us to this day. Initially the serum antibody response was considered key in mediating the effects of immunity (McCoy, 1935). This view came into question because the success rate of passive immunization by serum transfer was very poor and few studies demonstrated protection.

Furthermore it was difficult to reconcile intestinal effects with a serum immunoglobulin response. Through the 1950's the focus shifted from the humoral response to the role of intestinal inflammation. This is reflected in the work of Larsh and co-workers. Initially, immune effects were attributed to antibody with a secondary effect due to inflammation (Larsh and Race 1954). With time this view shifted and the inflammatory response regulated by T-cell specificity was considered critical, while the humoral response was thought to have no role in expulsion (Larsh and Race, 1975). They hypothesized that the best model for the increased expulsion rate of the intestinal stage of *Trichinella* was that of delayed type hypersensitivity. However, the role of inflammation in the reduction in worm size and fecundity was not considered. Furthermore, evidence for a cellular response does not preclude a role for other factors in protection.

The production of a protective mucosal antibody response has been reported in other systems. Crandall and Crandall (1972) described a mucosal antibody response following infection with *Trichinella* and subsequently Jacqueline *et al.*, (1978,1981) reported that specific biliary IgA caused a reduction in *Trichinella* fecundity as measured by *in vitro* larval release. However, other factors have also been implicated. Since eosinophilia was first described (Brown 1897) a protective role for these cells has

been implied and the demonstration of *in vitro* killing of larvae by eosinophils (Mackenzie *et al.*, 1980) and major basic protein (Wassom and Gleich, 1979) lends support to this hypothesis. Immune effects have also been attributed to the specific IgE response in conjunction with intestinal cells of the myeloid lineage (Dessein *et al.*, 1981; Ahmad *et al.*, 1991), though studies in mice with a genetic defect which inhibits IgE production do not support a role for this immunoglobulin (Watanabe et al., 1988). Regardless of the mechanism, it is apparent that despite considerable experimental effort the immune effector mechanism(s) acting following *Trichinella* infection remain unclear.

Considering that the major effects of immunity occur in the intestine and the anti-fecundity role for mucosal immunoglobulins described by Jacqueline *et al.*, (1978, 1981), the mucosal immunoglobulin response has potential as an effector response in this system. Further support for an effector role for mucosal immunoglobulins is found in parallel research in our lab, in which a strong correlation between mucosal immunoglobulin production and the effects of immunity has recently been described (deVos *et al.*, 1992).

The mucosal response

Intestinal lymphoid cells occur in organized tissues (ie. Peyer's patches) as well as being distributed in diffuse lymphoid tissues. The Peyer's patches are specialized for the uptake and processing of antigens from the intestinal lumen. The surface is composed of specialized M-cells which mediated enhanced antigen uptake and transfer to the subepithelial APC's. The T cells which respond to antigen presented via this route produce cytokines (eg interleukin 5) which infuence differentiation of the mucosal B cells to produce a mucosal IgA response. Following stimulation these cells migrate to populate distant mucosal sites and develop to plasma cells.

Objectives

My thesis focuses on immunity to *Trichinella* following infection in mice, and in particular on the relationship between the mucosal immunoglobulin response and the effects of immunity on the life cycle of *Trichinella*. In this context the specific objectives were to i) assess the immunoglobulin response following infection, ii) determine the influence of host genotype on the immune response, iii) determine the effect of mucosal immunization with crude antigen preparations on the course of infection and the immunoglobulin response following challenge infections with *Trichinella*, iv) determine the antigenicity of phosphorylcholine and cholinesterase in infections with *Trichinella* and their relation to protection of the host against infection, and v) to speculate on the mucosal immune response in the *Trichinella*/mouse model based on data from this thesis and information from the literature.

Chapter summary and rationalization

In Chapter 1, antibody production in the serum, bile, intestinal lumen and faeces were determined following infection with *Trichinella*, exposure to CT, or a combination of *Trichinella* and CT. This study was designed to confirm previous observations on the response to *Trichinella* (Crandall and Crandall, 1972; Kozek and Crandall, 1972; deVos *et al.*, 1992). In addition, total immunoglobulin

levels, immunoglobulin subclass distribution and the response to CT were determined to compare the anti-*Trichinella* response with mucosal responses to other immunogens. In previous studies immunoglobulin kinetics were determined by sacrificing animals at fixed intervals following infection, which produced a composite picture. To reduce the variability inherent in this approach, a method was developed to assess faecal immunoglobulin levels which allowed sequential sampling over time from the same animals. Finally, characterization of the response following concurrent exposure to *Trichinella* and CT allowed an analysis of the effects of exposure to a combination of strong immunogens.

In Chapter 2, the influence of host genotype on immunoglobulin production and the effects on expulsion, fecundity, worm size and muscle larvae were examined by comparison of the response in 6 inbred strains of mice. In competent animals, strain differences have been reported for antigen recognition, amplification and effector phases of the response and these have been associated with the major histocompatibility (MHC) genes, as well as background genes. Differences in the susceptibility or resistance of hosts to infection have been attributed to each of the phases described (Wakelin, 1988). The influence of host genotype on the effects of immunity to *Trichinella* has been the subject of considerable research (Wakelin, 1988; Wassom and Kelly,

1990) and strain specific differences in susceptibility/resistance reported have been attributed to both H-2 and background genes. Considering the role of the intestine in immunity to helminth infection, it is surprising that these studies have not examined the influence of host genotype on the mucosal immunoglobulin response following infection. Evidence with other mucosal immunogens (eg. CT) indicates that mucosal and systemic responses are influenced in a similar manner (Elson and Ealding, 1987; Hirabayashi *et al.*, 1990). In Chapter 2, the influence of H-2 and background genes on the response to *Trichinella* were examined to determine if regulation of the mucosal and system responses was similar, as was described for CT. Furthermore, in previous research in our lab (deVos *et al.*, 1992), mucosal antibody production and the effects of immunity were found to be correlated in CFW mice. Therefore, the mucosal immunoglobulin response and the effects of immunity were examined to determine if the relationship observed in CFW mice also occurred in other resistant and susceptible strains.

In Chapter 3, the effects of immunity and the mucosal and serum immunoglobulin response were determined following challenge infections in mice which had been primed by oral immunization with different crude *Trichinella* antigen preparations using CT as adjuvant. As described earlier, numerous studies, dating back to McCoy (1935) report

attempts to immunize against *Trichinella*. Despite the role of the intestinal response in producing the immune effects, mucosal immunization was described in only two reports (Vernes, 1976; Tronchin *et al.*, 1979), and immune effects on the worm were only described in the former (Vernes, 1976). This lack of interest in oral immunization likely stems from the general observation that soluble antigens presented via the mucosal route induced tolerance rather than immunity (Wells, 1911; Kagnoff, 1982). However, as a byproduct of the development of an oral vaccine for cholera it was reported that in addition to its strong mucosal immunogenicity, CT abrogated oral tolerance to soluble proteins (Elson and Ealding, 1984b; Lycke and Holmgren, 1986) and functioned as a mucosal adjuvant for heterologous antigens. In this context, the adjuvancy of CT and the effects of oral immunization with crude antigens on *Trichinella* were determined following challenge infections. The use of crude *Trichinella* antigen preparations in this study was based on several observations from the literature. In general, the most effective vaccines have been whole cell preparations rather than specific proteins. For example, the oral cholera vaccine consists of a combination of heat killed *Vibrio cholerae* and the B subunit of CT (Holmgren and Svennerholm, 1990). Also, it is apparent from vaccine research on malaria that immunization with an antigenic epitope does not guarantee protection (Nussenzweig and Nussenzweig, 1989) and

that antigen mixtures may be more effective than single epitopes. Therefore, in the present study, which was designed to develop an oral immunization system and determine baseline data, mice were immunized with crude antigenic fractions with a demonstrated protective capacity by intra peritoneal injection. Finally, this study was designed to assess the relationship between the effects of immunity and mucosal immunoglobulin production to determine if the correlation observed following infection also occurred following oral immunization.

In Chapter 4, the presence of, and host response to *Trichinella* PC was examined. The unusual regulation of the immune response to PC has resulted in considerable research on this molecule. Although many of the pathogens containing PC occur in the intestine, reports on the mucosal response to PC are limited to a cursory analysis of IgA following *Nippostrongylus* infection (Pery et al., 1979a). The role of the response to PC is also not clear. Numerous studies report that anti-PC antibodies (Szu et al., 1983; Briles et al., 1989,1992) or immunization with PC-antigen (Lim and Choy, 1990) protected against exposure to infection with *Streptococcus*. However, others report no correlation between protection and the anti-PC response (Mitchell et al., 1976). In Chapter 4, the kinetics of the serum and mucosal immunoglobulin response, and the influence of host genotype on this response were determined following infection with

Trichinella.

In Chapter 5 the antigenicity of the enzyme cholinesterase (CHE) was determined. Although potential antigens have been described from *Trichinella*, with some exceptions, these antigens have not been characterized. In fact, a number of apparently independent antigens were shown to share a common immunodominant carbohydrate epitope (Denkers *et al.*, 1990). The identity of an antigen is of significance in determining the mechanism of a protective response. For example, glutathione S-transferase (GST) is being considered as an anti-*Schistosoma* vaccine since the discovery of inhibition of GST activity by anti-GST antibodies (Xu *et al.*, 1991). In Chapter 5, cholinesterase was selected as a candidate antigen based on its reported antigenicity in other nematode species and the protective potential which could result from inhibition of this activity. CHE was examined in further detail and compared with CHE's from other nematode species to determine if a general pattern of CHE activity occurred among nematodes. In this study *Trichinella* hexosaminidase was used a positive control.

In Chapter 6 the thesis results are summarized and the relationship between the mucosal immunoglobulin response and effects on the course of infection is reviewed. Finally, potential mechanisms for mucosal immunoglobulin effects are discussed.

Chapter 1 The Antibody Response

Abstract

The mucosal and systemic immunoglobulin response to *Trichinella spiralis* was examined following infection in Swiss Webster (CFW) mice. Total immunoglobulin levels were determined for IgA, IgG, and IgM in bile and intestinal lumen samples (LUM), and a method was developed to assess coproantibody levels. The major subclass in mucosal secretions was IgA. Following primary infection with *Trichinella,* specific IgA was detected in the faeces by Day 9 and IgA peaks occurred at Days 12 and 18, but only low levels of specific IgG and IgM were detected. The response was anamnestic following challenge infections and IgA remained the predominant subclass, but specific IgG occurred and total mucosal IgG levels increased. The kinetics of the response to *Trichinella* were compared to the response following oral exposure to CT. The initial phase of the response to CT was similar to that for *Trichinella*, occurring by Day 12, primarily in the IgA subclass. The coproantibody response differed, as only a single peak of anti-CT IgA production occurred and a peak of anti-CT IgG was detected by Day 18 following primary exposure. Other differences included the relative distribution of the response in the bile and LUM samples.

Introduction

Aspects of the mucosal immunoglobulin response to *Trichinella* have been described (Crandall and Crandall, 1972; Sinski *et al.*, 1983; VanLoveren *et al.*, 1988). In recent work in our lab, the kinetics, dose dependence and subclass distribution of the specific anti-*Trichinella* immunoglobulin response in the bile, LUM and serum of outbred mice was described and the immunoglobulin response was correlated with biological effects on the parasite in the gut phase of the infection (deVos *et al.*, 1992). Mucosal antibody responses have also been described for other nematode species (Miller, 1984), but differences in hosts, infection protocol and detection methods limit comparisons, and in most systems essential features of the response (eg. dose dependence and kinetics of immunoglobulin production) have not been examined.

Among nematodes, the mucosal response to *Nippostrongylus brasiliensis* in rats has been examined in detail. Biliary, intestinal, faecal, lung and serum responses in rats have been characterized (Poulain *et al.*, 1976; Sinski and Holmes, 1977; Brown *et al.*, 1981a,b; Wedrychowicz *et al.*, 1983, 1984, 1985) and correlations between mucosal titers and immune effects were observed. However, there is, as yet, no definitive evidence for a protective role of mucosal antibody in these infections.

In contrast, the role of the mucosal immunoglobulin

response has been clearly established for protection against viral (Taylor and Dimmock, 1988), bacterial (Lycke and *et al.*, 1989b) and protozoan (Underdown *et al.*, 1988) infections. Of these, the response to CT has been extensively characterized and provides the basis for much of our current understanding of the mucosal immunoglobulin response. CT is an enterotoxin produced by the bacteria *Vibrio cholerae*. This polymeric protein is composed of five 11.6 kD B subunits and a 28 kD A subunit composed of disulphide linked 21 kD and 7 kD sequences (Holmgren, 1981). The B subunits have high affinity for GM-1 ganglioside receptors present on mucosal epithelial cells. Following exposure, the toxin binds epithelial cells via the B subunit and the A subunit penetrates the cytoplasm. The A subunit catalyzes ADP-ribosylation of a stimulatory G protein, which maintains the G protein in its active form and consequently enhances adenylate cyclase activity (Moss and Vaughan, 1990). As a result, intracellular cAMP levels rise, inducing the fluid secretion responsible for the clinical cholera condition (Holmgren, 1981). Mucosal exposure to microgram quantities by feeding CT results in the production of IgA and IgG in the bile, LUM and faeces (Elson *et al.*, 1984; Elson and Ealding 1984a; Pierre *et al.*, 1988), as well as an IgA and IgG response in the serum, in an MHC restricted fashion (Elson and Ealding, 1987; Richardson and Kuhn, 1986). Primary exposure to CT produces life long memory in

mice and following challenge the response is anamnestic (Lycke and Holmgren, 1989).

The characteristic murine response to CT described above provides a model system for comparison of the mucosal response following infection with *Trichinella*. This system also provides a means to study interactions in the immune response following concurrent exposure to different antigens since both *Trichinella* and CT produce strong mucosal immune responses. Infection with *Trichinella* has been associated with immunosuppression of the host response to heterologous antigen (Faubert and Tanner, 1974; Faubert, 1976; Baltar *et al.*, 1991) and this effect has also been studied with combined exposure to *Trichinella* and CT (Ljungstrom *et al.*, 1980).

It is apparent that there are many gaps in our understanding of the mucosal response to *Trichinella*, and to helminths in general. The purpose of this study was to assess the mucosal immune response following *Trichinella* infection and compare it with the well characterized response to CT. In this context the objectives were i) to quantify mucosal IgA, IgG, and IgM concentrations following infection with *Trichinella*, ii) to develop a faecal sampling method to assess the mucosal immune response, iii) to assess the immunoglobulin response to *Trichinella* and CT in the bile, LUM and faeces of mice to determine similarities and differences in the mucosal response to these different

antigens, and iv) to assess interactions in the response following concurrent exposure to *Trichinella* and CT. Although immunosuppression was not the focus of this study, the experiments provided some observations on immunosuppression of the response to CT which are summarized in Appendix 1.

Materials and Methods

Experimental animals / Trichinella

Mice used were 6 wk old CFW (Swiss Webster: CRL COBS CFW) male mice (Charles River Laboratories, St Constant, Que, Canada). The P1 strain of *Trichinella* (Dick, 1983) was used. *Trichinella spiralis* was maintained in our laboratory by serial passage in CFW mice as described previously (Dick *et al.*, 1988). Briefly, to obtain infective L1 larvae, mice infected for >35 days were killed by cervical dislocation, skinned, eviscerated and homogenized in 200 ml of a 1% HCl/pepsin solution per mouse. The homogenate was digested by shaking at 200 RPM at 37°C for 2 hr in an incubator (Labline). The digest was removed, allowed to settle for 15 min, the lipid layer aspirated off and the larvae isolated by sieving through #80 and #230 Tyler sieves. The larvae, trapped in the 230 sieve, were washed into test tubes with a 0.8% saline solution and washed 5X with saline. Larvae were suspended in 10 ml of saline in a test tube by stirring with a magnetic stirrer and total larval numbers were determined as the mean number of larvae in 5 aliquots (10 μl) multiplied by the dilution factor (1000).

Infection and oral immunization

Mice were infected by feeding a fixed volume (between 0.15 and 0.25 ml) of larval suspension in saline with a blunt tipped 18 gauge feeding needle. For oral immunization with CT, mice were fed 10 μg of CT in 0.25 ml of 0.2M

bicarbonate buffer (pH 8.3) with a blunt tipped feeding needle as above.

Sampling

Terminal sampling

All mice were starved 16 hr prior to sampling to improve the recovery of bile. They were killed, skinned and a blood sample taken from the brachial vein/artery. The body cavity was opened, the gall bladder removed intact, rinsed in 0.02M phosphate buffered saline pH 7.2 (PBS) and blotted on a paper towel to eliminate serum contamination. The gall bladder was pierced in a microcentrifuge tube and the bile collected and stored on ice. The small intestine was ligated at the pylorus and caecum, removed, cut in two equal parts and 0.25 ml of sample buffer (PBS pH 7.2 + 0.05M EDTA, 1mg/ml Soybean Trypsin Inhibitor and 1mg/ml Na azide; Elson *et al.*, 1984) was washed through each section by applying gentle pressure along the gut. The LUM samples were pooled, 1/100 volume of 100 mM phenylmethylsulfonylflouride in 95% ETOH was added and samples were stored on ice for later processing.

The blood samples were held at RT for 1 hr to clot, the clot was ringed and samples were stored at 4°C overnight (o/n). Samples were then centrifuged at RT, the serum was removed and stored at -80°C and the clot discarded. Bile samples were centrifuged, the volume determined and the samples stored at -80°C. LUM samples were centrifuged and

the supernatant was stored at -80°C. The number of adult worms remaining in the pellet were counted and the pellet was discarded.

Faecal sampling

Mice were placed on paper towels in cages without shavings, and faecal pellets were collected after 1 hr. Samples were weighed and extracted immediately or stored at -70°C. Samples of 0.1 gm were weighed, placed into 1.5 ml microcentrifuge tubes, 1 ml (10 volumes w/v) of PBS was added, and the mixture incubated at room temperature 15 min. Samples were mixed, left to settle for 15 min, remixed until all material was suspended, then centrifuged at 13000 RPM for 10 min in a Hereaus Biofuge A microfuge (Baxter Canlab, Winnipeg MB). The supernatant was removed and stored at -70°C or immediately tested for immunoglobulins. Single faecal pellets (10-20 mg) were treated as above, while larger samples were centrifuged at 18000 RPM in a JA 20.1 rotor on a J2-21 centrifuge (Beckman, Palo Alto, CA). Protease activity of samples was measured using the nonspecific protease substrate Azocoll (Calbiochem, La Jolla, CA.). Twenty μl aliquots of faecal supernatants were added to 2.5 ml Azocoll (10mg/ml in PBS) and incubated at 37°C for 60 min. Samples were centrifuged and 200 μl aliquots were measured on a microplate reader at an O.D. of 540 nm. Protease activity was expressed as the change in O.D. 540 nm per min.

Enzyme linked immunosorbent assay (ELISA)

Antigen specific ELISA

Trichinella and CT specific immunoglobulins were measured as follows. Microtitre plates (Flow Laboratories, Mclean, Virginia) were coated with soluble *Trichinella* antigen at 1 μg/well or with CT at 0.5 μg/well, in carbonate buffer (pH 9.6) and incubated o/n. Plates were washed with PBS + 0.05% Tween 20 (PBST) and each well was blocked with 200 μl 0.5% gelatin in PBST for 3 hr. Plates were washed with PBST, 100 μl of test sample was added per well, and incubated for 2 hr. Plates were washed with PBST and 100 μl HRP labelled subclass (Fc) specific antimouse antibody (Sigma Chemical Co, St Louis MO.) was added to each well and incubated 2 hr. Plates were washed, developed for 30 min with HRP substrate (o-phenylenediamene + H_2O_2), stopped with 100 μl 2.5 N HCl, and read at 490 nm with a Bioteck EL308 microplate reader (Mandel Scientific, Rockwood, ON.).

Quantitative ELISA

Immunoglobulin concentration was determined by capture ELISA and compared to subclass standards. The protocol was similar to that described above with the following modifications. Plates were coated with 0.5 μg/well sheep anti-mouse IgA, IgG, or IgM. Test samples were serially diluted and compared with subclass standards (TEPC-15 (IgA), purified IgG and TEPC-183 (IgM) Sigma Chemical Co. St. Louis MO.) to determine concentrations.

Statistical Analysis

Treatment means were compared with t-tests with a significance level of $p<0.05$. For multiple comparisons of treatment means, Duncan's multiple range test was used with a significance level of $p<0.05$. All statistical analysis were performed with the SAS statistical analysis package as implemented by Computer Services, University of Manitoba, or the PC version of SAS, Department of Zoology, Universtiy of Manitoba.

Results

Mucosal subclass distribution

Groups of 6 CFW mice were infected with 10 or 150 P1 larvae on Day 0 and challenged with 10 or 150 larvae on Day 28. Bile and LUM samples were obtained from 6 mice per treatment on Day 6 1° and 6 mice per treatment on Day 6 2°. The concentrations ($\mu g/\mu l$) of IgA, IgG and IgM were determined (Table 1). The predominate subclass in both bile and LUM samples was IgA. The concentration of biliary IgA was approximately 100X the concentration of IgG or IgM. The LUM IgA concentration in primary infections and in the 10-10 challenge was also ~100X the IgG concentration. Although IgG concentrations increased in the 10-150 and 150-150 infections, IgA remained the predominate immunoglobulin (Table 1). LUM IgM was essentially undetectable in all treatments. The concentration of IgA in the bile increased with increased exposure to *Trichinella*, while LUM IgA levels remained unchanged (Table 1). Finally, although the LUM IgA concentration was lower than bile IgA, when factored for volume, total IgA in LUM samples was greater.

Table 1. Concentration (μg/μl) of IgA, IgG and IgM in bile and LUM samples from Day 6 of primary and challenge infections with Trichinella in CFW mice.

Infection Dose	Bile			LUM		
	IgA	IgG	IgM	IgA	IgG	IgM
Uninfected	0.431±0.211[a] (11.8±6.3)[b]	0.003±0.002 (0.08±0.06)	0.012±0.002 (0.33±0.09)	0.180±0.052 (90.3±26.2)	0.002±0.001 (1.38±0.54)	0 0
1° 10[c]	0.790±0.322 (17.2±7.97)	0.010±0.006 (0.20±0.10)	0.001±0.001 (0.01±0.03)	0.159±0.030 (79.6±15.0)	0.002±0.002 (1.00±1.06)	0.003±0.004 (1.25±2.16)
1° 150	0.678±0.230 (18.0±8.22)	0.001±0.002 (0.05±0.03)	0.004±0.004 (0.04±0.05)	0.102±0.036 (51.0±17.9)	0.001±0.003 (0.88±1.52)	0 0
2° 10-10	0.987±0.461 (19.4±8.52)	0.001±0.006 (0.18±0.07)	0.006±0.004 (0.12±0.07)	0.206±0.016 (103.0±7.8)	0.014±0.003 (6.98±1.54)	0 0
2° 10-150	1.127±0.502 (14.4±8.73)	0.004±0.002 (0.06±0.05)	0.010±0.006 (0.11±0.07)	0.093±0.037 (46.5±18.6)	0.072±0.040 (36.0±20.0)	0.001±0.001 (0.15±0.21)
2° 150-150	2.719±1.040 (48.7±28.9)	0.016±0.006 (0.27±0.09)	0.023±0.024 (0.29±0.28)	0.203±0.062 (101.7±31.2)	0.035±0.020 (17.3±10.2)	0.001±0.001 (0.25±0.43)

Immunoglobulin concentration was determined by ELISA and comparison with standard immunoglobulins of known concentration. Mice were primed on Day 0 and challenged on Day 28.

[a] Mean concentration ± standard deviation of original sample in μg/μl.

[b] Mean total immunoglobulin levels (in μg) ± standard deviation (= concentration x volume)

[c] Infection doses: 1° 10 = 10 larvae/mouse, 150 = 150 larvae/mouse.

2° 10-10 = 1°-2° 10 larvae, 10-150 = 1° 10 larvae 2° 150 larvae, 150-150 = 1°-2° 150 larvae.

Coproantibody Assay

Groups of 8 CFW mice were infected with 150 P1 larvae or fed 10 μg of CT on Day 0. Mice in the *Trichinella* treatments were challenged with 150 larvae on Day 28. Faecal samples were collected at 3 day intervals throughout the experiment, extracted, and the specific IgA or IgG response to *Trichinella* or CT was determined (Table 2). Control mice were negative for the response to either *Trichinella* or CT. The predominate subclass in the primary coproantibody response following infection with *Trichinella* was IgA (Table 2). This response was biphasic, with peaks at Day 12 and 18. The IgG response remained at a low level during the primary infection but increased similar to IgA following challenge infections (Table 2). The coproantibody response to CT was monophasic, with a peak of IgA production at Day 12, and an increase in IgG production by Day 15 (Table 2). Protease activity in faecal samples was assessed with the Azocoll assay. The mean proteolytic activity of faecal samples was 0.003±0.006 compared with 0.034±0.003 for LUM samples treated with protease inhibitors.

Table 2. Faecal antibody levels of mice over time following exposure to *Trichinella* or cholera toxin.

	Trichinella		Cholera Toxin	
Day	IgA	IgG	IgA	IgG
6	0.056±0.053		0.104±0.085	0.002±0.002
9	0.276±0.184		0.478±0.449	0.061±0.082
12	0.530±0.124		1.210±0.770	0.293±0.319
15	0.204±0.124	0.095±0.032	0.554±0.475	0.134±0.092
18	0.591±0.323	0.193±0.038	0.613±0.529	0.415±0.508
21	0.409±0.035	0.237±0.104		
27	0.298±0.261	0.105±0.077		
6PC*	0.482±0.322	0.465±0.421		

Values equal the mean OD490 results ± standard deviation of undiluted faecal samples from 8 mice as determined by ELISA.
* Day 6 post challenge

Immune Response to Trichinella and CT

Groups of 12 CFW mice were infected with *Trichinella* and/or fed 10 μg CT in different combinations. Faecal samples were collected at 3 day intervals from all mice. Four mice per treatment were sampled for bile, LUM and serum on Day 15 1°. The remaining mice were challenged with different combinations of *Trichinella* and CT and 8 mice per treatment were sampled on Day 6 2°.

Coproantibody response

The response to *Trichinella* and CT was determined in faecal samples of mice exposed to a combination of *Trichinella* and CT. The faecal antibody response to *Trichinella* is summarized in Figure 2. The response was similar to that in the preliminary experiments described above (Table 2). Following the primary infection anti-*Trichinella* antibody production was biphasic with an initial peak at Day 12 and a second broader peak from Day 18 to 24. There was no IgG response detected following primary infection but following challenge a response occurred (Fig. 2). The coproantibody response was dose dependent as no response (IgA or IgG) was detected following primary or challenge infections with 10 larvae (Fig. 2). Finally, the IgA and IgG anti-*Trichinella* response did not differ significantly in mice infected with *Trichinella* in the presence or absence of CT (Fig. 2).

Figure 2. Kinetics of anti-*Trichinella* IgA and IgG production in the faeces of mice exposed to combinations of CT and *Trichinella*.

Mice were exposed on Day 0 and challenged on Day 28. Bars represent mean ± standard error of ELISA results (O.D. 490) from undiluted faecal extracts from 8 mice per group (SE<0.01 not included in graph). Treatments were as follows: A= 10 μg CT, B= CT + 150 *Trichinella*, C = CT + 10 *Trichinella*, D = 150 *Trichinella*.

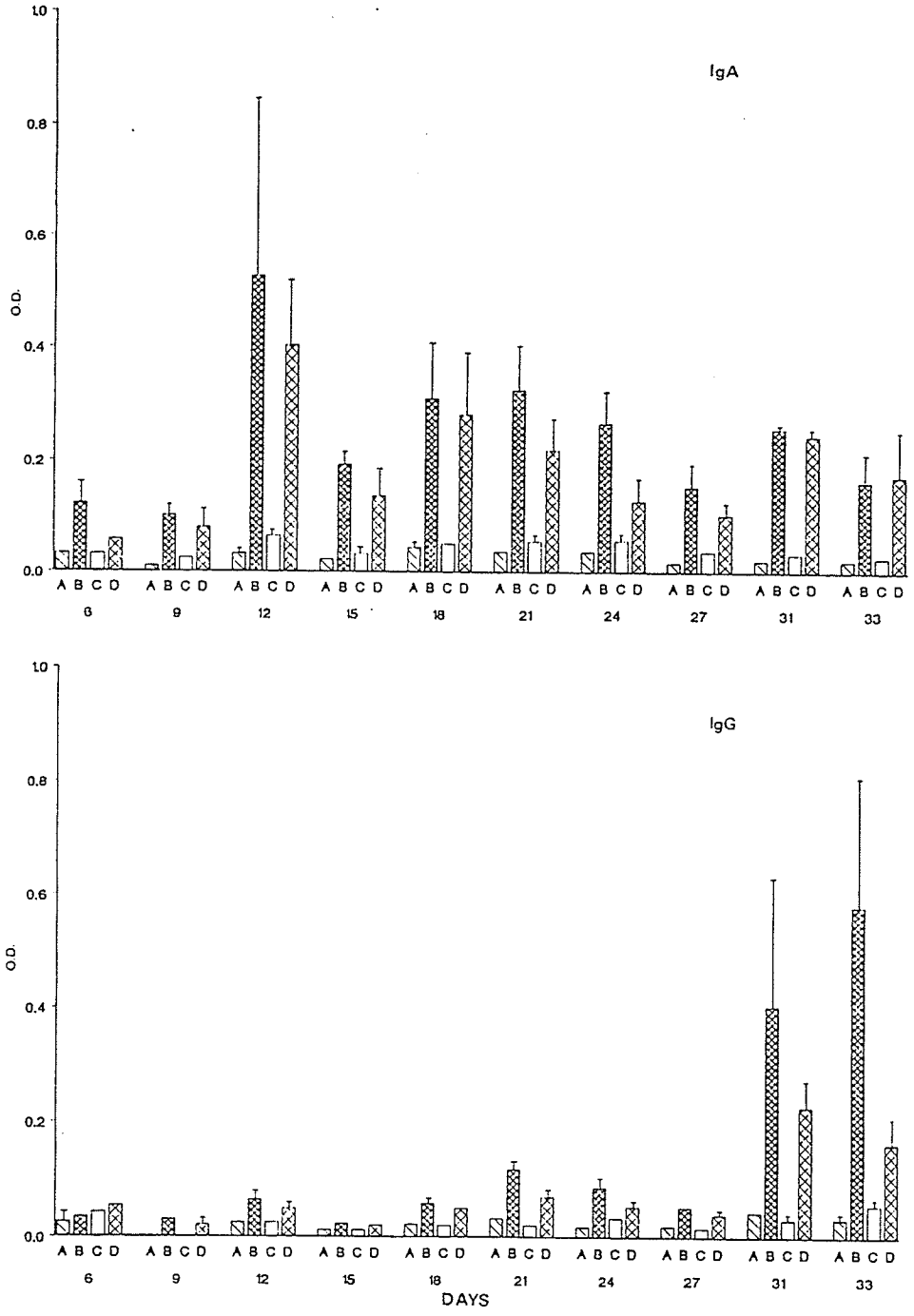
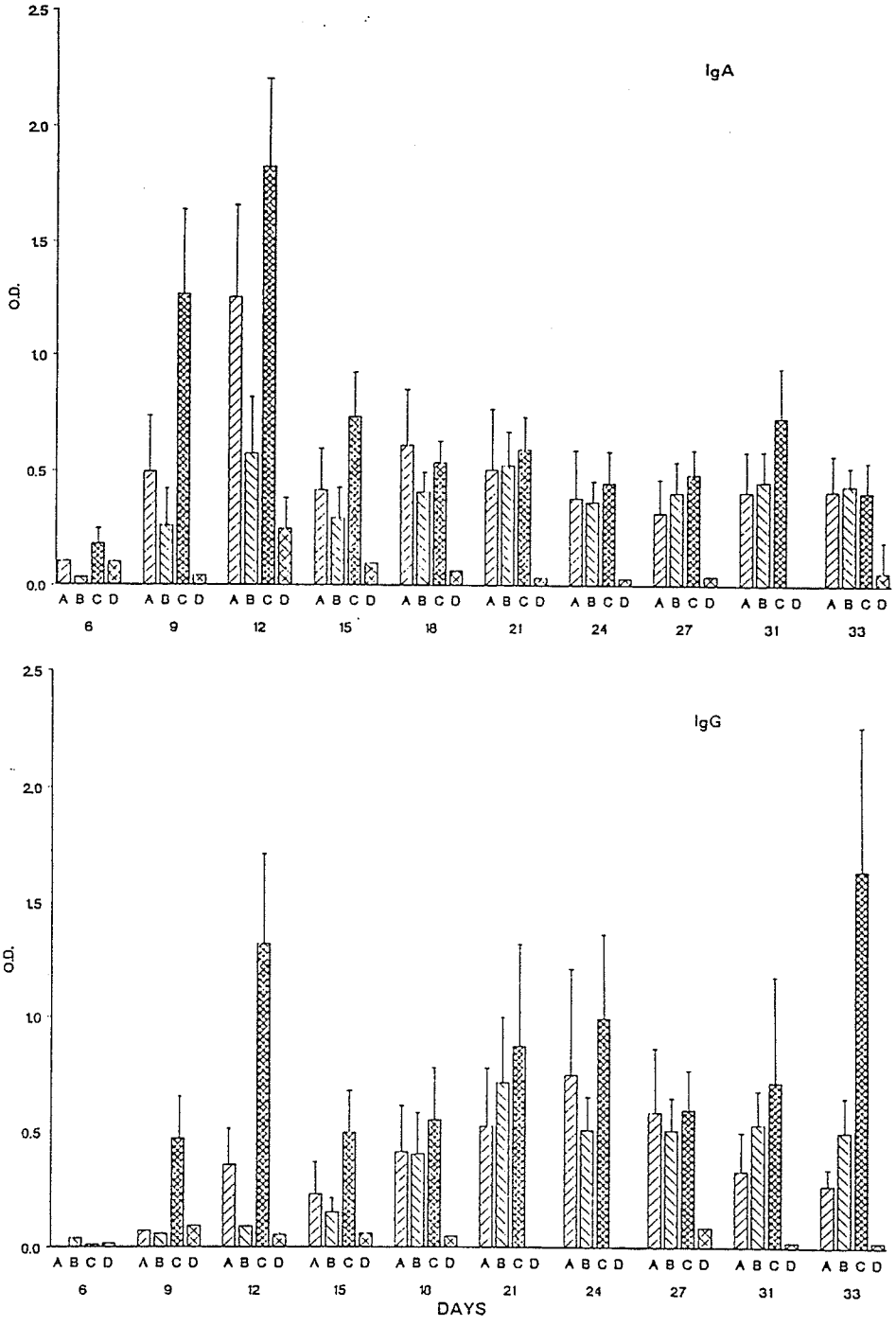

IgA
IgG
O.D.
1.0
0.8
0.6
0.4
0.2
0.0
A B C D
6
9
12
15
18
21
24
27
31
33
DAYS

Figure 3. Kinetics of anti-CT IgA and IgG production in the faeces of mice exposed to combinations of CT and *Trichinella*.

Mice were exposed on Day 0 and challenged on Day 28. Bars represent mean ± standard error of ELISA results (O.D. 490) from undiluted faecal extracts from 8 mice per group (SE<0.01 not included in graph). Treatments were as follows: A= 10 μg CT, B = CT + 10 *Trichinella*, C = CT + 150 *Trichinella*, D = 150 *Trichinella*.


IgA
IgG
O.D.
2.5
2.0
1.5
1.0
0.5
0.0
A B C D
6
9
12
15
18
21
24
27
31
33
DAYS

The faecal antibody response to CT is summarized in Figure 3. Specific anti-CT IgA and IgG were detected following primary exposure but not IgM. Specific IgA was detected by Day 9 and peaked by Day 12, while the IgG response was detected by Day 12 and peaked by Days 21-24 (Fig. 3). The faecal IgA response to CT was not significantly greater in mice with concurrent *Trichinella* infections but the kinetics of IgG production were significantly altered. A new peak of IgG occurred at Day 12 post infection but this peak did not occur in mice fed CT alone. Following challenge the IgG anti-CT response was also enhanced (Fig. 3).

Mucosal response

Trichinella

The biliary and LUM antibody responses to *Trichinella* determined on Day 15 1° and Day 6 2° are summarized in Table 3. On Day 15 1° the biliary IgA response in mice infected with 150 larvae (TS150) was significantly greater ($p<0.05$) than the response in mice infected with 10 larvae (TS10). Similarly, the LUM IgA response was greater in 150 larvae infections compared with infections of 10 larvae (Table 3), though the difference was not significant ($p<0.05$). Further, the Day 15 1° IgA response to *Trichinella* was similar in treatments with or without CT (ie. CTTS10 vs TS10 and CTTS150 vs TS150).

Table 3. The mucosal immunoglobulin response to *Trichinella* in mice infected with *Trichinella* in combination with exposure to cholera toxin.

Treatment		Bile IgA	LUM IgA	LUM IgG
Day 15 1°				
TS10		0.306±0.145^{b}	0.134±0.080^{a}	-
CTTS10		0.341±0.172^{b}	0.205±0.110^{a}	-
TS150		2.000±0.470^{a}	0.346±0.470^{a}	-
CTTS150		1.443±0.610^{a}	0.363±0.530^{a}	-
Day 6 2°				
1°	2°			
TS10	-TS10	1.060±0.361^{cde}	0.744±0.500^{bc}	0.071±0.039^{c}
	-CTTS10	1.065±0.540^{cde}	0.434±0.240^{bcd}	0.072±0.064^{c}
	-CT	0.453±0.183^{fg}	0.188±0.063^{def}	0.056±0.049^{c}
CTTS10	-TS10	1.007±0.510^{de}	0.604±0.311^{bcd}	0.081±0.040^{c}
	-CTTS10	0.647±0.394^{ef}	0.420±0.302^{cde}	0.066±0.038^{c}
	-CT	0.334±0.196^{fg}	0.071±0.045^{f}	-
TS150	-TS150	1.377±0.722^{bcd}	0.658±0.470^{bcd}	0.572±0.420^{a}
	-CTTS150	1.535±0.423^{abc}	0.896±0.417^{b}	0.647±0.498^{a}
	-CT	0.378±0.273^{fg}	0.107±0.067^{f}	0.068±0.038^{c}
CTTS150	-TS150	1.924±0.620^{a}	1.327±0.853^{a}	0.571±0.340^{a}
	-CTTS150	2.014±0.710^{a}	1.511±0.574^{a}	0.380±0.204^{ab}
	-CT	1.013±0.334^{de}	0.430±0.090^{de}	0.168±0.185^{c}

Values equal mean ± standard deviation of OD490 ELISA results from n = 4 (Day 15 1°) or n = 8 (Day 6 2°) mice per treatment. Dilutions were (1/1000) for bile samples and (1/10) for LUM samples.

Treatment designations were as follows: CT = 10 μg cholera toxin, TS10 = 10 *Trichinella* larvae, TS150 = 150 *Trichinella* larvae, CTTS10 = CT + 10 *Trichinella* larvae, CTTS150 = CT + 150 *Trichinella* larvae.

Differences among treatments within each box were analyzed by Duncan's multiple range test ($p<0.05$). Significant differences are indicated by differences in superscript letters. Treatment means with common superscripts are not significantly different.

On Day 6 2° the biliary and LUM IgA responses did not differ significantly ($p<0.05$) between treatments infected and challenged with 10 (TS10-TS10) or 150 (TS150-TS150) larvae (Table 3). The biliary and LUM IgA response in mice fed CT in combination with 10 larvae (CTTS10) in either the primary or challenge did not differ significantly from the response in mice infected with *Trichinella* alone (TS10, Table 3). In contrast, the biliary and LUM IgA response was significantly greater ($p<0.05$) in mice fed CT in combination with 150 larvae in the primary (CTTS150) and challenged with 150 larvae with (CTTS150) or without CT (TS150), than the response in treatments infected with 150 larvae without CT (TS150) and challenged with 150 larvae with or without CT (Table 3). The LUM IgG response was negative on Day 6 2° in all treatments with 10 larvae (TS10) but was positive in treatments with 150 larvae (TS150), and no significant differences were detected between mice primed and challenged with 150 larvae with or without CT (Table 3).

Cholera Toxin

The biliary and LUM antibody responses to CT determined on Day 15 1° and Day 6 2° are summarized in Table 4. There were no significant differences among treatments in the biliary or LUM responses to CT on Day 15 1°. In contrast, on Day 6 2° both the biliary and LUM responses to CT were significantly enhanced in mice fed CT with 150 larvae in the primary (CTTS150) and challenged with CT alone or CT

Table 4. The mucosal immunoglobulin response to CT in mice infected with *Trichinella* in combination with exposure to CT.

Treatment		Bile IgA	LUM IgA	LUM IgG
Day 15 1°				
CT		0.673±0.390[a]	1.755±0.676[a]	-
CTTS10		0.578±0.219[a]	1.247±0.565[a]	-
CTTS150		0.958±0.505[a]	1.407±0.383[a]	-
Day 6 2°				
1°	2°			
CT	-CT	0.626±0.461[c]	0.540±0.292[d]	0.065±0.031[c]
	-TS10	-	-	0.084±0.054[c]
	-TS150	-	-	0.883±1.119[ab]
CTTS10	-CT	0.975±0.398[bc]	0.993±0.494[c]	0.764±0.885[ab]
	-CTTS10	1.146±0.895[bc]	0.760±0.557[cd]	0.286±0.324[bc]
	-TS10	-	-	0.362±0.588[bc]
CTTS150	-CT	1.447±0.912[ab]	1.485±0.373[b]	0.510±0.550[abc]
	-CTTS150	1.742±0.437[a]	1.824±0.734[a]	0.608±0.688[abc]
	-TS150	-	-	0.991±0.656[a]

Values equal mean ± standard deviation of OD490 ELISA results for n = 4 (Day 15 1°) or n = 8 (Day 6 2°) mice per treatment. Dilutions were 1/1000 for bile and 1/10 for LUM samples

Treatment designations were as follows: CT = 10 μg cholera toxin, TS10 = 10 *Trichinella* larvae, TS150 = 150 *Trichinella* larvae, CTTS10 = CT + 10 *Trichinella* larvae, CTTS150 = CT + 150 *Trichinella* larvae.

Differences among treatments within each box were analyzed by Duncan's multiple range test ($p<0.05$). Significant differences are indicated by differences in superscript letters. Treatment means with common superscripts are not significantly different.

with 150 larvae (CTTS150) (Table 4). The LUM IgG response to CT was also enhanced in mice fed CT in combination with *Trichinella* larvae.

Discussion

Total Immunoglobulin

Previous studies from this lab and others reported a specific immunoglobulin response following infection with *Trichinella*. Quantitative analysis of LUM and biliary immunoglobulins in the present study indicated that the primary mucosal immunoglobulin was IgA. Prior to infection with *Trichinella* mucosal IgA concentrations were ~100 fold greater than IgG or IgM and although the IgG and IgM levels increased following infection, IgA remained the predominant immunoglobulin. The immunoglobulin concentration was within reported ranges, though the previously published concentrations of mucosal immunoglobulins varied considerably. Estimates of biliary IgA ranged from 0.2 to 1.2 mg/ml (Manning *et al.*, 1984; Delacroix *et al.*, 1985), while estimates of LUM IgA range from 0.004-2.28 mg/ml but generally were in the 0.1-0.2 mg range (Sinski *et al.*, 1983; Elson *et al.*, 1984). Reports on the levels of other immunoglobulins are controversial. Delacroix *et al.*, (1985) report equivalent IgA and IgG levels in the bile obtained from cannulated mice. In contrast, Manning *et al.*, (1984) reported IgG levels were 1/10 of IgA in rats and Renegar and Small, (1991) reported IgG levels were ~1/100 of IgA from whole gall bladders in mice. For LUM samples, a complete absence of the IgG and IgM antibody classes is often reported. Considering the level of protease activity in LUM

samples, despite the use of protease inhibitors, the absence of these immunoglobulins is not surprising.

Coprosampling

In the initial phase of this study and in previous studies from our lab and others, the kinetics of the mucosal response were determined by terminal sampling and analysis of biliary and LUM antibody responses (deVos *et al.*, 1992). Besides the prohibitive cost and excessive use of animals, this approach is subject to considerable variability since the overall kinetics are a composite of different individuals sampled at each time point. Clearly, sampling of the same individuals over time would produce a more accurate picture of the development of the mucosal immune response. Coproantibodies, which have been identified in viral (Lipton and Steigman, 1962), bacterial (Freter *et al.*, 1964) and helminth (Wedrychowicz *et al.*, 1983) infections of the intestinal tract, provide a convenient mucosal source for sequential analysis. A protocol described for the isolation and characterization of coproantibodies in intestinal secretions of mice required a 2 ml lavage treatment followed by an injection with pilocarpine, a cholinergic drug which increases intestinal motility and stimulates IgA secretion (Elson *et al.*, 1984). Stress induced by this method and the induction of IgA secretion by pilocarpine injection (Freier *et al.*, 1989) may produce immunoglobulin levels atypical of a normal response. Further, the lavage/pilocarpine treatment

may alter the normal course of infection of gastrointestinal nematodes. A noninvasive method has been reported for the analysis of rat coproantibodies (Wedrychowicz *et al.*, 1983) but it required 10 gm of faeces per sample, was labour intensive and required 48 hr to complete. The coprosampling method developed in the present study was noninvasive, simple, rapid and applicable to small samples (deVos and Dick, 1991). With this method the kinetics of mucosal immunoglobulin production following exposure to *Trichinella* and CT were determined.

Response to Trichinella

The results of the experiments on the faecal, bile and LUM mucosal anti-*Trichinella* response confirm and extend previous studies with *Trichinella*. Initial studies on this response reported an increase in surface immunoglobulin positive B-cells in the gut of rabbits (Crandall *et al.*, 1967), an increase in specific IgA and an anamnestic response in intestinal washes from hyperinfected mice (Crandall and Crandall, 1972; Kozek and Crandall, 1972). *Trichinella* specific IgA was also detected in the bile (Jacqueline *et al.*, 1977, 1981) and intestinal washes (Vanloveren *et al.*, 1988) of infected rats. Further, Peyer's patch size and total intestinal IgA in mice increased following infection (Sinski *et al.*, 1983). Finally, in a previous study from our lab, the mucosal immunoglobulin response to infection was dose dependent and correlated with

the timing of a reduction in fecundity and worm size (deVos *et al.*, 1992). In the present study anti-*Trichinella* antibodies occurred in biliary, LUM and serum samples of infected mice and an anamnestic response was detected following challenge infection, which corroborates the observations above. The kinetics of the anti-*Trichinella* IgA response in the faeces extend studies on the kinetics of the biliary and LUM response (deVos *et al.*, 1992). In primary infections the IgA response was biphasic while IgG levels remained near background. However, following challenge exposure an increase in specific faecal IgG was detected, similar to total LUM IgG levels described above. The origin of faecal IgG following challenge infections is not clear. Although it may be derived from serum leakage due to intestinal pathology, it has also been detected following infection with as few as 10 larvae (deVos *et al.*, 1992) for which negligible pathology would occur. Alternatively, in challenge infections the intestinal IgG B cell pool may be expanded as a component of the overall cellular increase in the anamnestic response.

Response to CT

As indicated in the Introduction, much of our current understanding of the mucosal immune response is based on studies with CT. In the present experiment the response to CT was examined as a positive control for mucosal antibody production and for comparison with the response following

Trichinella infection. Oral exposure to CT results in both a mucosal anti-CT immunoglobulin response which can be detected in the bile, LUM and faeces and a systemic response (Pierce and Gowans, 1975; Pierce *et al.*, 1978; Elson and Ealding, 1984a,b; Elson *et al.*, 1984). Following oral exposure to CT in rats, the cellular IgA anti-toxin response was increased 4-5 fold above background by Day 10 and by Day 5 following challenge the response was amplified 80 fold (Pierce and Gowans, 1975; Altorfer *et al.*, 1987). IgA was the predominant class of antibody in the mucosal response (Elson and Ealding, 1984a) while the serum response included IgG as well (Wilson *et al.*, 1990).

The results in the present experiment were generally similar to the reports above. Anti-CT antibodies were detected in the bile, LUM, faeces and serum of mice exposed to CT. Faecal IgA anti-CT production increased to a peak by Days 12-15 and an anamnestic response occurred following challenge infections, similar to the cellular response described above. Faecal IgG anti-CT peaked several days after the IgA peak. This difference in immunoglobulin kinetics suggests independent regulation. It is possible that the faecal IgG is derived from the systemic anti-CT pool which is rapidly increasing at Day 18 following infection. However, faecal IgG levels subsequently decrease while systemic levels remain high, indicating that if the source of faecal IgG anti-CT is the serum, transport to the

intestine must be regulated by exposure to CT.

CT / Trichinella comparison

The mouse immune response to CT and *Trichinella* were similar in many respects. High levels of IgA occurred in the LUM, the gall bladder and the faeces and high levels of specific IgG were detected in the serum. The initial peak of IgA in the faeces occurred by Day 12 post exposure, the response was dose dependent and a challenge with homologous antigens produced a strong response with similar kinetics in LUM, bile, faeces and serum. However, differences in kinetics also occurred. The IgA response following primary infection with *Trichinella* was biphasic with a second peak occurring by Day 18. The biphasic nature of the response may be generated by different *Trichinella* antigens during its development from larva to adult (Jungery and Ogilvie, 1982), which would be enhanced in the strong responder CFW mice used in this study. Overlap in life cycle stages is limited as a result of the rapid rate of expulsion, which may accentuate the production of two antibody peaks. The kinetics of faecal antibody production in relation to expulsion rate is dealt with further in Chapter 2. The production of faecal IgG following primary exposure to CT also differed from the IgG response following *Trichinella* infection, which was only detected following challenge infection.

Differences were also noted in the distribution of

specific IgA in the bile and LUM samples. In the response to *Trichinella*, IgA levels were proportionally higher in the bile compared to the LUM, whereas in the anti-CT response the proportion of IgA was higher in the LUM sample. Differences were most pronounced following primary exposures and were reduced or disappeared following challenge. The basis for these differences is not clear, but may relate to antigenic differences or the route of antigen presentation. In a number of systems the nature of the antigen determines the location of the response. For example, following oral exposure to *E. coli* O6, the biliary anti-LPS titer was higher than the milk titer, whereas the anti-fimbriae response had the opposite distribution (Wold *et al.*, 1989). Similarly, the tissue distribution of mucosal anti-I/II antibodies differed from the anti-CT response following oral exposure to antigen I/II of *Streptococcus* coupled to CT (Russell and Wu, 1991).

Comparison with other nematodes

Although comparisons can be made, there are problems in interpreting and comparing results among studies. The methods used for the identification and quantification of antibodies include indirect immunofluorescence (Kozek and Crandall, 1972), double immunodiffusion (Jacqueline *et al.*, 1981), radial immunodiffusion (Cypess *et al.*, 1977), indirect haemagglutination (Maclean *et al.*, 1986) and ELISA (Wedrychowicz *et al.*, 1984). These methods differ in the

information they provide, as well as in their sensitivity. In this context the response to other parasitic nematodes is described below.

The mucosal and serum immunoglobulin response following infection with the nematode *Nippostrongylus* has been studied in detail. A specific, dose dependent serum IgG response to *Nippostrongylus* was detected by Day 14 following subcutaneous injection of L3 larvae (Poulain *et al.*, 1976; Sinski and Holmes, 1977; Brown *et al.*, 1981a; Yamada *et al.*, 1992) and following challenge infections the serum IgG response was anamnestic (Brown *et al.*, 1981a). In initial studies on the mucosal response, total and specific IgA levels in intestinal secretions were determined. The specific IgA response to *Nippostrongylus* was dose dependent and occurred by Day 6 following infection. Following challenge exposure the response was anamnestic (Poulain *et al.*, 1976; Sinski and Holmes, 1977). In subsequent studies similar results were reported for biliary immunoglobulins (Brown *et al.*, 1981a,b). The kinetics of the faecal immunoglobulin response in rats have also been reported (Wedrycowicz *et al.*, 1983, 1984, 1985). In these studies coproantibodies were detected by Day 6 and peaked by Day 14 and following challenge the faecal response was anamnestic. The role of this response in host protection has not been studied in detail but Wedrycowicz *et al.*, (1985) reported an association between mucosal IgA levels and protection.

The mucosal response to *Heligmosomoides* has also been described (Cypess *et al.*, 1977). In these studies a slight increase in intestinal IgA was detected by Day 14 following a primary infection. Following challenge a significant increase in IgA was detected by Day 3. Intestinal IgG was also detected by Day 7 but this response was associated with intestinal pathology (Cypess *et al.*, 1977). The mucosal and systemic response to *Trichostrongylus* in gerbils was measured by passive haemagglutination (Maclean *et al.*, 1986). The specific serum response remained low following primary infections but increased significantly by Day 25 following challenge. An intestinal response was detected following primary infection, but the challenge response did not differ much by Day 25. The faecal response was detected by Day 14 of primary infections, but the increase following challenge was variable occurring by Days 10-14 depending on the antigen used for detection (McClean *et al.*, 1986).

As indicated above, the kinetics of mucosal immunoglobulin production have been described for a number of helminth species and a correlation between antibody production and expulsion of worms has been described (Wedrychowicz, 1985). Differences in components of the response may be attributed to life cycle differences. For example, in the *Nippostrongylus* life cycle infection occurs by penetration of the skin and the larvae follow a systemic migratory route to the lungs prior to reaching the

intestine. In the other nematodes described above and *Trichinella*, infection occurs by consumption of infected meat and the initial stages of infection are intestinal, the more common route of intestinal helminths. Despite these differences, it is apparent from this comparison that there are a number of similarities in the immunoglobulin response to gastrointestinal nematodes. Infection results in the production of a mucosal immunoglobulin response which can be detected in bile and LUM as well as in the faeces. In both *Nippostrongylus* and *Trichinella* the response is dose dependent and it appears to be anamnestic in all species.

Trichinella / CT interaction

There were some differences in the immunoglobulin response following exposure to a combination of *Trichinella* and CT compared with separate exposure to these immunogens. The kinetics of faecal anti-CT immunoglobulin production were altered. An IgG response was detected by Day 9 and peaked by Day 12, which did not occur in mice exposed to CT alone. This enhanced IgG response may be due to serum leakage, since *Trichinella* larval migration occurs at this time and the systemic response to CT had also developed. However, serum leakage is not the only source of intestinal IgG, as discussed above for the response to *Trichinella*. In addition to an enhanced faecal IgG response to CT, differences were also noted in the biliary and LUM responses. In mice primed with CT and 150 *Trichinella*

larvae, the anti-*Trichinella* response was significantly greater than in mice treated with larvae alone. Similarly, the mucosal response to CT was enhanced in mice exposed to a combination of CT and *Trichinella*. Enhancement of the immune response by CT will be discussed in detail in Chapter 3. Observations on immunosuppression based on the above experiments are summarized in Appendix 1.

It is clear from this study that infected mice produce a mucosal and systemic immunoglobulin response to *Trichinella*. The kinetics of mucosal antibody production were correlated with the course of infection in CFW mice and this temporal correlation provides indirect support for an effector role for the immunoglobulin response. This correlation is examined further in Chapter 2 by characterizing the relationship between mucosal immunoglobulin production and the effects of immunity in other resistant or susceptible strains of mice.

Chapter 2. The influence of host genotype.

Abstract

The influence of host genotype on the immune response following infection with *Trichinella spiralis* was examined. In BALB/c mice the immune effect on expulsion rate was dissociated from the effects on fecundity and worm size, the latter two being highly correlated with the mucosal immunoglobulin response. The mucosal immunoglobulin response and its effects on *Trichinella* were subsequently determined in five other inbred strains of mice: C57BL10 (B10), B10.BR, B10.D2, C3H/HeJ, and 129/J. Differences in the rate of expulsion and total muscle larvae numbers occurred between strains, and these were associated with H-2 and background gene effects. The reduction in fecundity was similar in all strains and the reduction in worm size was similar in all strains but 129/J. Although all strains had positive responses, mucosal and systemic immunoglobulin titers varied among strains, and these differences were influenced by both *H-2* and genetic background. The reduction in fecundity and size (except in 129/J) were associated with an increased IgA titer. In contrast, the rate of expulsion was not associated with mucosal IgA titers in most strains. These differences indicate independent regulation of the anti-fecundity and expulsion responses.

Introduction

Despite the significant differences in immune parameters observed between labs for *Trichinella* infections in mice (Culbertson 1942; Larsh and Hendricks, 1949; Rappaport and Wells, 1951) the role of host genotype in determining the immune response level was not considered experimentally until the late 1960's (Stefanski and Kozar, 1969). Host genotype effects were highlighted by Wakelin and Lloyd (1976) who described resistance in NIH mice, similar to that of outbred Swiss mice (Despommier *et al.*, 1977) which differed significantly from the weak response reported by Larsh (1970) for Swiss albino mice. Numerous studies since, clearly demonstrated a genetic basis for variation in murine susceptibility/resistance to *Trichinella* infection (Bell *et al.*, 1982b; Wakelin and Donachie, 1981; Wassom *et al.*, 1979).

A literature review of the influence of host genotype indicated the following consensus: The level of response to *Trichinella* is dependent on both the genetic background of the host (for this discussion background = genes coding outside the *H-2* complex) and on MHC genes (genes mapping within the *H-2* complex) (Wakelin, 1988). Despite this consensus there has been considerable debate on the relative contribution of *H-2* versus background genes in influencing the responses. Using panels of congenic mice differing only at the *H-2* locus, Wassom *et al.*, (1979) demonstrated

significant *H-2* dependent differences in total muscle larvae and rate of expulsion, and identified putative *H-2* response genes (*Ts1* and *Ts2*) (Wassom *et al.*, 1983a; Wassom and Kelly, 1990). However, by comparison of identical *H-2* loci on different genetic backgrounds, Bell *et al.*, (1984a) and Bell (1988) identified dominant non *H-2* genes (*Ise*) responsible for the expulsion phenotype.

Regardless of the controversy described above, it is clear that host genotype influences the murine response to *Trichinella*. What is the basis for host genotype effects in the response to a parasitic infection? The response to infection is a complex process which can arbitrarily be divided into recognition, amplification and effector phases each of which is subject to genetic influence:

1) Recognition: Recognition of foreign molecules as antigens is a genetically restricted process. The T cell receptor recognizes antigen physically associated with MHC molecules. Antigens, phagocytosed and degraded by antigen presenting cells (eg. macrophages) associate with MHC gene products and are expressed on the surface of the antigen presenting cell with the MHC molecule. Antigen presentation with MHC class I molecules stimulates a cytotoxic T cell response while association with class II molecules stimulates a T helper cell response. Genes for the class II molecules and for a portion of the class I protein occur at multiple loci within the *H-2* complex of the mouse (or of human *HLA*) and each

locus is highly polymorphic. The products of these multiple alleles vary in the binding capacity for different proteins (Chen and Parham, 1989). Since recognition by the T cell receptor requires both antigen and MHC product, the inability to recognize specific antigens can result from lack of MHC/antigen interaction. It has also been reported that expression of some MHC alleles results in the clonal deletion of specific T cells (Kappler *et al.*, 1988). In this case, the inability to recognize heterologous antigen would result from the loss of the specific T cell clones.

2) Amplification: Following antigen recognition the immune response develops by a regulated amplification of lymphocytes and other effector cells. Presentation of antigen in the presence of I-E class II molecules may stimulate T suppressor activity (Wassom et al., 1987). Since the expression of I-E is genetically regulated, the nonresponsiveness associated with certain strains may be a function of antigen specific T suppression. As discussed in Chapter 1, differences have also been reported with respect to nature of the T helper cell response to infection with *Leishmania* and *Schistosoma*. Distinct classes of T helper cells have been described and the response to *Leishmania* in resistant mice has been characterized as a Th1 response, compared with a Th2 response in sensitive mice. In this case, the influence of host genotype is mediated by differences in the path of amplification of the T cell

response, which in turn determines the resistance phenotype of the host.

3) Effector: A variety of genetic influences related to the effector phase of the response have been described. Effectors include antibodies, complement, acute phase proteins, inflammatory products (eg. histamine), products of the respiratory burst to name a few. Clearly a defect in the production of any of these could alter the resistance phenotype of a host. Indeed, variation in resistance has been reported in relation to mast cell and eosinophil activity, cytokine levels and macrophage activity.

Studies with *Trichinella* implicate genetic effects in all phases of the response. T cell dependence, demonstrated by infection in thymectomized and congenitally athymic mice (Walls *et al.*, 1973; Ruitenberg and Steerneberg, 1974) and transfer of immunity via mesenteric lymph node T cells (Wakelin and Wilson, 1979), implied T cell recognition of *Trichinella* antigens, a genetically regulated process. Also, the *in vitro* proliferation assay following *Trichinella* infections (Ottesen *et al.*, 1975) revealed strain specific differences in the kinetics and level of the T cell response (Wassom *et al.*, 1985, Zhu *et al.*, 1990). Differences in the antigen recognition profiles of serum immunoglobulins between mouse strains following infection also supports the influence of host genotype on antigen recognition (Kennedy *et al.*, 1991; Robinson *et al.*, 1991). With respect to

amplification, the susceptibility of B10.BR and mice with the *H-2*^{k} haplotype has been attributed to the development of a T suppressor response which would inhibit amplification of the response (Wassom *et al.*, 1987). Furthermore, genetic differences are implied in T helper or T inflammatory responses generated in different strains (Wassom and Kelly, 1990). Finally, the dominant effects of background genes proposed by Bell have been attributed to differences in the effector phase of the response (Bell *et al.*, 1982b; Bell *et al.*, 1984a,b).

The studies described above and others describing cell transfer (Wakelin and Donachie, 1981,1983) can be incorporated in a general hypothesis (Wassom *et al.*, 1984b; Wakelin, 1988): The response to *Trichinella* includes a number of independently regulated but interacting phases each of which is influenced by host genotype. Recognition and effector functions were attributed to different T-cell populations (genetic control mapped to separate loci) and nonlymphocyte effector cells (Wassom *et al.*, 1984b; Wakelin, 1988). This hypothesis provided a framework for the immune response to *Trichinella*, but the specific mechanisms by which worms were expelled, stunted, or by which fecundity was reduced were not directly addressed.

The initial characterization of the mucosal response to *Trichinella* in our lab, described in Chapter 1, utilized outbred mice (deVos *et al.*, 1992). Since host genotype can

affect the response, outbred mice were used to assess the response in genetically variable hosts as a baseline for comparison with infections in inbred mice. In CFW mice the mucosal immunoglobulin response was positively correlated with an increased rate of worm expulsion and reductions in worm fecundity and size (deVos *et al.*, 1992). In previous studies with *Trichinella* the reduction in fecundity following exposure to specific IgA isolated from bile and the increased duration of infection following bile duct ligation support an effector role for mucosal IgA following *Trichinella* infections (Jacqueline *et al.*, 1978, 1981). Differences in serum IgA levels were also implicated in differences between resistant and susceptible mouse strains (Almond and Parkhouse, 1986). Despite the potential of the local immunoglobulin response in producing immune effects, the influence of host genotype on the mucosal immunoglobulin response to *Trichinella* has not been examined.

Differences in the response of inbred strains of mice to *Trichinella* infections provide a model system in which to study the association between mucosal antibody production and the effects of immunity. The objectives of this study were i) to determine the relationship between mucosal antibody production and effects on expulsion, fecundity and size in BALB/c mice, ii) to assess the effects of immunity and the mucosal and systemic immunoglobulin responses in inbred mice and iii) determine the relationship between the

immunoglobulin response and the effects of immunity in these mice.

Materials and Methods

BALB/c mice were from Charles River (St. Constant, Que.) or from Jackson Laboratories (Bar Harbor, Maine). All other strains (Table 5) were obtained from Jackson Laboratories. *Trichinella* was isolated and maintained as described in Chapter 1.

BALB/c Mice

In initial experiments, BALB/c mice were infected with 150 larvae/mouse on Day 0, and challenged with 150 larvae on Day 28. Mice with primary and challenge infections were sampled on Days 6, 9 and 12 post infection and challenge with a modification of the methods described in Chapter 1. Briefly, mice were terminated and serum, bile and LUM samples were obtained. The intestine was slit open and incubated in saline at 37°C. Following a 2 hr incubation at 37°C adult female worms were picked from gut samples. The intestinal samples were incubated for a minimum of 4 hr and the total number of worms counted following incubation. Adult females picked at 2 hr were washed 3x in sterile saline and 3x in RPMI 1640. Worms were then incubated in 200 μl of RPMI 1640 supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin and 0.1 mg/ml streptomycin, in 96 well culture plates at 37°C with 5% CO_2. Following 24 hr of incubation the total number of newborn larvae released per female was counted using an inverted microscope.

Table 5. Characteristics of mouse strains including *H-2* haplotype and resistance to *Trichinella* infection.

			Resistance Phenotype*		
Strain	Desig**	*H-2*	Wassom	Wakelin	Bell
C57Bl/10J(B10)	B10	*b*	67	Weak	Slow
B10.BR/SgSn	BR	*k*	104	Weak	Slow
B10.D2	D2	*d*	-	Weak	Slow
129/J	J129	*b*	48	-	-
C3H/HeJ	C3H	*k*	_	Int.	-
BALB/cByJ	BALB	*d*	57	Int.	Slow

* Resistance phenotype ranked as follows:
Wassom - muscle larvae recovery expressed as percentage of recovery in C3HeB/FeJ, a susceptible strain (Wassom *et al.*, 1979, 1983a).
Bell - based on day at which adult worm recovery reduced by 95% following infection with 400-500 larvae. Strong <= 14 days, Intermediate 14-15 days, Weak = 18 days (Bell *et al.*, 1984b)
Wakelin - based on timing of expulsion following infection with 300-400 larvae. Rapid <=12 days, Slow >= 12 days (Wakelin, 1988).

** Designation used in this Chapter.

The adult female worms were fixed with hot 70% ETOH and cleared with 70% ETOH + 5% glycerine. Worms were mounted in glycerine and worm lengths were determined. Worms lengths were determined by two methods: 1) Worms were projected with a microfiche reader, drawn in outline on transparent film and lengths determined with a digitizing tablet (Jandel Scientific) or 2) worms were projected on screen with a photomicroscope and lengths determined with an image analysis program (Optimas).

Other strains

In the second experiment the immune response to *Trichinella* was determined for 6 defined mouse strains (Table 5). Strains were chosen to allow comparisons between congenic mice with similar genetic backgrounds differing only at the *H-2* locus and between mice with similar *H-2* haplotypes on different genetic backgrounds. Groups of mice were infected with 150 *Trichinella* and the kinetics of mucosal immunoglobulin production determined in faecal samples taken at 3 day intervals. Serum samples were taken at 6 day intervals. On Day 28 mice were challenged with 150 *Trichinella* and control mice were infected on the same day. Faecal samples were obtained from mice following challenge. Mice were sampled on Days 6 and 10 post infection/challenge as described above. In challenge infections the mean of total muscle larvae was determined as follows. Following sampling mice were skinned and eviscerated. They were

homogenized and digested as described in Chapter 1 and larvae were isolated from individuals. Total muscle larval numbers were estimated by counting 10 μl aliquots from 10 ml samples.

Specific anti-*Trichinella* immunoglobulin titers were determined by ELISA as described in Chapter 1. Immunoglobulin titers were defined as the reciprocal of the dilution at which the OD 490 was greater than 0.3. Samples were diluted serially and tested using standardized conditions. Antibody standards were included on each plate to determine plate to plate variation in antibody titer. The IgA standard was the myeloma protein TEPC-15 which bound to the phosphorylcholine fraction in *Trichinella*. The standard for the IgG and IgM responses was a serum pool from mice infected and challenged with 150 *Trichinella*. Immunoglobulin titers were determined for bile, intestinal lumen, and serum samples. The faecal response was determined with undiluted extracts.

Results

Response in BALB/c mice: Effect on Parasite

Adult worm recovery, fecundity and worm size data for BALB/c mice infected with 150 larvae/mouse are summarized in Table 6. Based on adult worm recovery BALB/c mice were intermediate to slow with respect to expulsion. The high variability for some treatment groups resulted from a small number of mice within treatments in which adult recovery was close to 100%. Expulsion levels were not significantly different ($p<0.05$) between days in the primary infections though the mean declined by Day 12 (Table 6). Comparison of primary and challenge infections indicated no reduction in challenge mice for Day 6 but a reduction (though not significant) in challenge groups on Day 9 (Table 6). Fecundity decreased significantly from Day 6 to Day 9 primary, but did not differ significantly between Days 9 and 12 primary or challenge. Fecundity was also significantly decreased ($p<0.05$) on Day 6 in challenge mice. Differences in worm size followed a similar trend to differences in fecundity (ie. a decrease with time, and reduction in challenge infections).

Host immune response

Mucosal and serum antibody response data are also presented in Table 6. *Trichinella* specific bile IgA was not detected on Day 6 of the primary infection but occurred on

Table 6. Expulsion rate, fecundity, worm length and mucosal and systemic immunoglobulin production in BALB/c mice following primary and challenge infections with 150 Trichinella larvae.

	DAY POST INFECTION					
	6		9		12	
	1°	2°[a]	1°	2°	1°	2°
Adult Recovery[b]	64.5±17.2 (8)[c]	68.5±43.7 (8)	82.3±56.4 (4)	47.8±33.7 (8)	42.3±58.4 (4)	14.3±16.4* (4)
24 hr larvae production (n=40-50)	85.8±17.9	42.8±14.5*	19.4±16.1*	15.9±8.0*	26.9±17.4[d]*	21.9±8.7[e]*
Length (mm) (n=30)	2.4±0.16	1.49±0.20*	1.84±0.18*	1.44±0.18*	1.27±0.17[f]*	1.49±0.16*
Bile IgA	0.11±0.05 (8)	1.07±0.04* (8)	1.38±0.16* (4)	1.05±0.12* (6)	1.04±0.74* (4)	1.23±0.15* (3)
Serum IgG	0.09±0.01 (8)	0.59±0.05* (8)	0.10±0.02 (4)	0.71±0.04* (8)	0.12±0.02 (4)	0.70±0.05* (4)

Note. For each variable, treatments were compared with t-tests. Significant differences ($p<0.05$) from Day 6-1° are indicated by *

[a] Mice were challenged on day 28.

[b] The high variability in expulsion on Days 6-2°, 9-1°2° and 12-1° resulted from single mice which showed low expulsion (eg day 9 1° ~90% infection dose).

[c] n. [d] n = 33. [e] n = 19. [f] n = 25.

Days 9 and 12 of the primary and Days 6, 9 and 12 of the challenge infections (Table 6). Anti-*Trichinella* IgA was temporally associated with the reduction in fecundity and worm size in every treatment. However the rate of expulsion was dissociated from the IgA response since expulsion was not significantly different between primary and challenge infections on Day 6, when a strong specific IgA response had occurred. Furthermore, in the mice in which adult recovery was virtually 100% (ie. no expulsion) fecundity was significantly reduced and specific IgA was detectable. This provides further support for a dissociation of the expulsion and anti-fecundity responses. Serum IgG anti-*Trichinella* was not detected by Day 12 of the primary infection, but at each sampling time in the challenge infection (Table 6). The effects of expulsion, size and fecundity were not temporally associated with the serum IgG response.

Other strains

As described above, the expulsion response was dissociated from the effects on fecundity and size in BALB/c mice and the latter effects were temporally correlated with the IgA response. The following experiments were designed to examine this relationship in a number of defined strains of mice to determine if the association between the mucosal immunoglobulin response and the effects on fecundity and size was a general phenomenon. Furthermore these experiments

were designed to assess the role of the *H-2* complex and background genes on the rate of expulsion and on fecundity, worm size and the mucosal immunoglobulin response to infection.

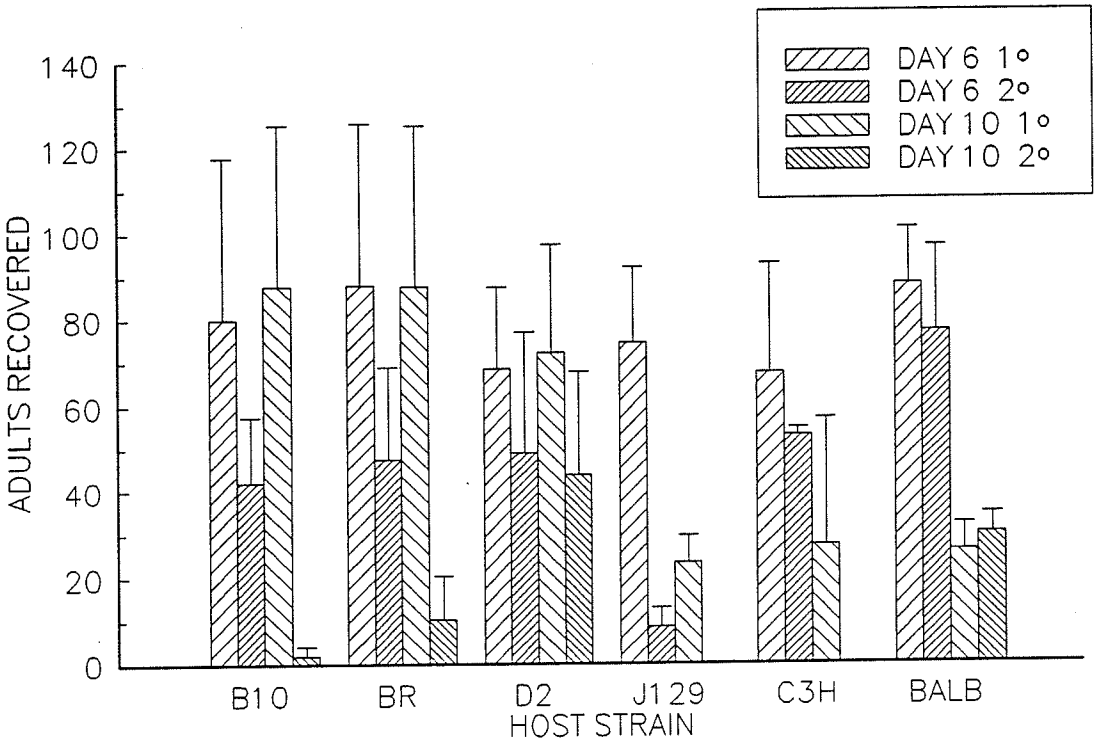
Effect on Trichinella

Expulsion

Adult worm recovery from the intestine on Days 6 and 10 primary and challenge is shown in Figure 4. There were clear differences in the rate of expulsion between strains. In the primary infection the number of intestinal worms recovered on Day 10 did not differ from the Day 6 level in the three strains with a common B10 background (B10, B10.BR, and B10.D2) or in C3H, while in the other strains tested (BALB/c, J129) intestinal worm counts were reduced on Day 10 (Fig. 4). Adult worm recovery was reduced in all strains on Day 6 following challenge compared with Day 6 primary, but these differences were only significant in the B10 and 129/J strains ($H\text{-}2^b$). By Day 10 following challenge, expulsion was complete in J129 and C3H strains and worm numbers were also significantly reduced in B10 and B10.BR mice compared with Day 6 and Day 10 primary and Day 6 challenge (Fig. 4). In contrast adult worm numbers in the D2 and BALB strains (both $H\text{-}2^d$) were not significantly reduced from Day 10 primary levels.

Figure 4. Adult worm recovery in 6 strains of mice infected and challenged with 150 *Trichinella* larvae.

Bars represent mean ± standard deviation of the total number of adult worms recovered from mice on Days 6-1°, 6-2°, 10-1° and 10-2°. Treatment designations were : B10 = C57BL/10, BR = B10.BR, D2 = B10.D2, J129 = 129/J, C3H = C3H/HEJ, BALB = BALB/c. For each strain, differences between days were analyzed by pairwise t-tests, and the results for each comparison are included in Table form below the Figure. n = 4 mice per treatment.



Pairwise t-tests

Strain	6-1°/ 10-1°	6-2°/ 10-2°	6-1°/ 6-2°	10-1°/ 10-2°
B10	NS[a]	SIG	SIG	SIG
BR	NS	SIG	NS	SIG
D2	NS	NS	NS	NS
J129	SIG	SIG	SIG	SIG
C3H	NS	SIG	NS	NS
BALB	SIG	SIG	NS	NS

[a] NS = not significant at $p<0.05$. SIG = significant at $p<0.05$.

Fecundity

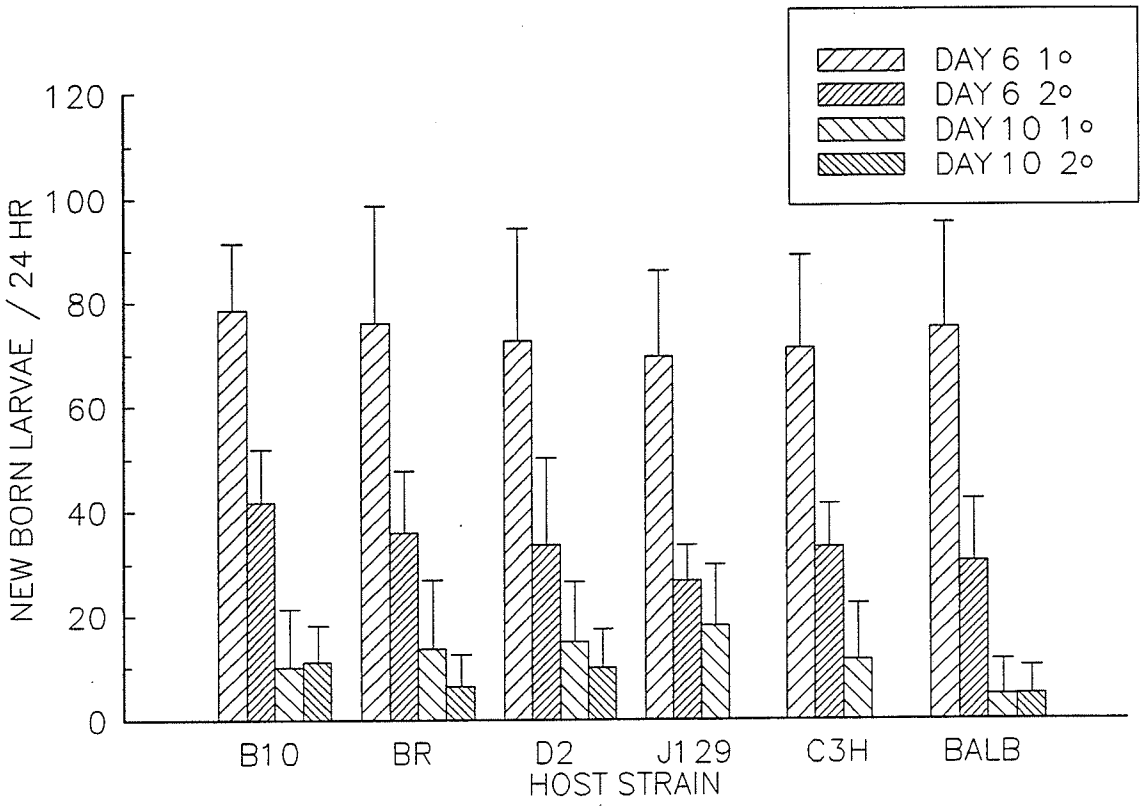
Fecundity, measured as 24 hr *in vitro* larval release, was determined on Days 6 and 10 of the primary and challenge infections (Fig. 5). Complete expulsion of C3 and 129/J by Day 10 of challenge precluded *in vitro* analysis for this day. There were no significant differences between strains on Day 6 primary and for all strains *in vitro* larval release was reduced by ~50% on Day 6 challenge compared with Day 6 primary and by 80-90% on Day 10 primary and challenge (Fig. 5). Differences occurred between strains at Day 10 primary and Days 6 and 10 challenge but these differences did not cluster by haplotype or background.

Size

Adult female worm length was determined for 30 worms from each treatment (Fig. 6). Complete expulsion from C3H and 129/J on Day 10 challenge precluded these strains from analysis. Worm length followed a similar pattern in all mouse strains except 129/J. A significant reduction in worm size occurred by Day 10 compared with Day 6 following primary infection in all strains but B10.BR (Fig. 6). Worms were also significantly shorter on Day 6 following challenge compared with Day 6 primary. On Day 6 primary the largest mean size occurred in C3H and BR mice, which share the $H\text{-}2^k$ haplotype. No similar haplotype clustering was evident on

Figure 5. Adult worm fecundity (newborn larvae / 24 hr) in 6 strains of mice infected and challenged with 150 *Trichinella* larvae.

Bars represent mean ± standard deviation of the newborn larvae / 24 hr, from mice on Days 6-1°, 6-2°, 10-1° and 10-2°. Treatment designations were : B10 = C57BL/10, BR = B10.BR, D2 = B10.D2, J129 = 129/J, C3H = C3H/HEJ, BALB = BALB/c. For each strain, differences between days were analyzed by pairwise t-tests, and the results for each comparison are included in Table form below the Figure. n = 40-50 worms per treatment.



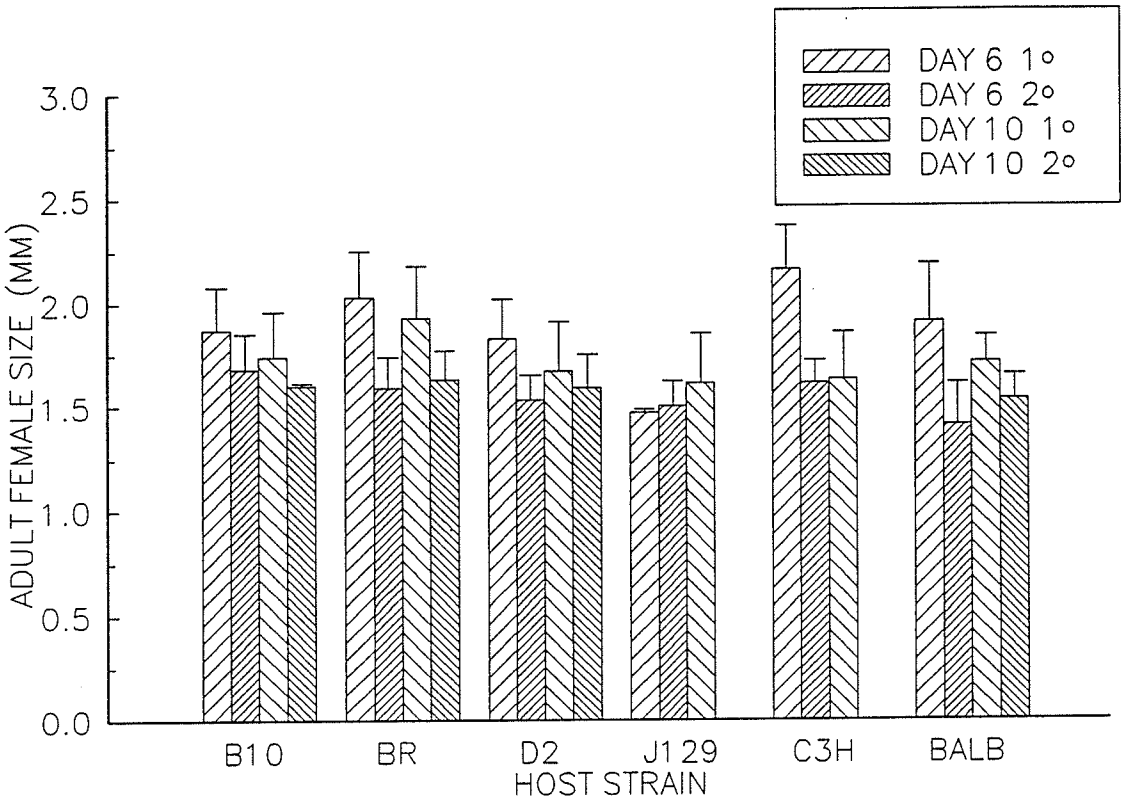
Pairwise t-tests

Strain	6-1°/ 10-1°	6-2°/ 10-2°	6-1°/ 6-2°	10-1°/ 10-2°
B10	SIG[a]	SIG	SIG	NS
BR	SIG	SIG	SIG	SIG
D2	SIG	SIG	SIG	SIG
J129	SIG	-	SIG	-
C3H	SIG	-	SIG	-
BALB	SIG	SIG	SIG	NS

[a] NS = not significant at $p<0.05$. SIG = significant at $p<0.05$.

Figure 6. Adult worm size (mm) in 6 strains of mice infected and challenged with 150 *Trichinella* larvae.

Bars represent mean ± standard deviation of size of adult worms (mm) from mice on Days 6-1°, 6-2°, 10-1° and 10-2°. Treatment designations were : B10 = C57BL/10, BR = B10.BR, D2 = B10.D2, J129 = 129/J, C3H = C3H/HEJ, BALB = BALB/c. For each strain, differences between days were analyzed by pairwise t-tests, and the results for each comparison are included in Table form below the Figure. n = 30 worms per treatment.



Pairwise t-tests

Strain	6-1°/ 10-1°	6-2°/ 10-2°	6-1°/ 6-2°	10-1°/ 10-2°
B10	SIG[a]	NS	SIG	NS
BR	NS	NS	SIG	SIG
D2	SIG	NS	SIG	NS
J129	SIG	-	NS	-
C3H	SIG	-	SIG	-
BALB	SIG	SIG	SIG	SIG

[a] NS = not significant at $p<0.05$. SIG = significant at $p<0.05$.

any of the other sample days. In contrast to the patterns described above, worms in the 129/J mice were significantly smaller than in the other strains at Day 6 of the primary infection, and did not differ significantly between primary and challenge infections.

Muscle Larvae

Total muscle larvae recovery from primary infections is reported in Figure 7. B10 mice had higher muscle larvae recoveries regardless of haplotype than the other strains though the only significant difference ($p<0.05$) occurred with the 129/J strain which had the lowest recorded recovery. Muscle larvae recovery was not correlated with *H-2* haplotype in this experiment.

Immunoglobulin responses

Faecal response

The *Trichinella* specific antibody response was determined from faecal samples taken at 3 day intervals from 8 mice for each of the 6 strains examined. Antibody production against time for all strains is presented in Figure 8. From this plot it is apparent that the specific response is initiated at different times in different strains. For instance, a response is detectable in the B10.D2 and C3H mice by Day 9 in comparison with the 129/J response on Day 12, the B10.BR and B10 responses on Day 15 and the BALB/c response on Day 18.

Figure 7. Total muscle larvae from primary infections in 6 strains of mice infected and challenged with 150 *Trichinella* larvae.

Bars represent mean ± standard deviation of total muscle larvae from mice on Days 6-2° and 10-2°. Treatment designations were : B10 = C57BL/10, BR = B10.BR, D2 = B10.D2, J129 = 129/J, C3H = C3H/HEJ, BALB = BALB/c. n = 8 mice per treatment.

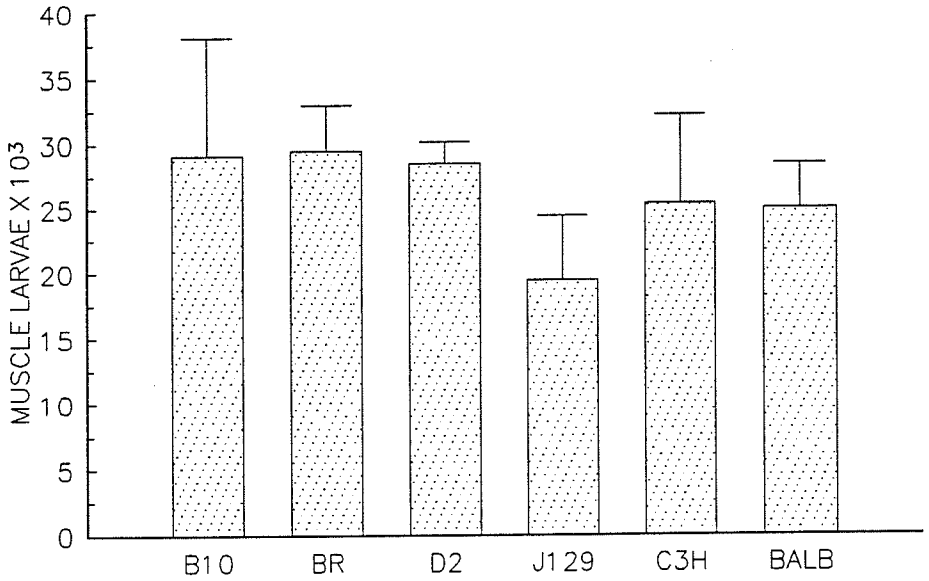
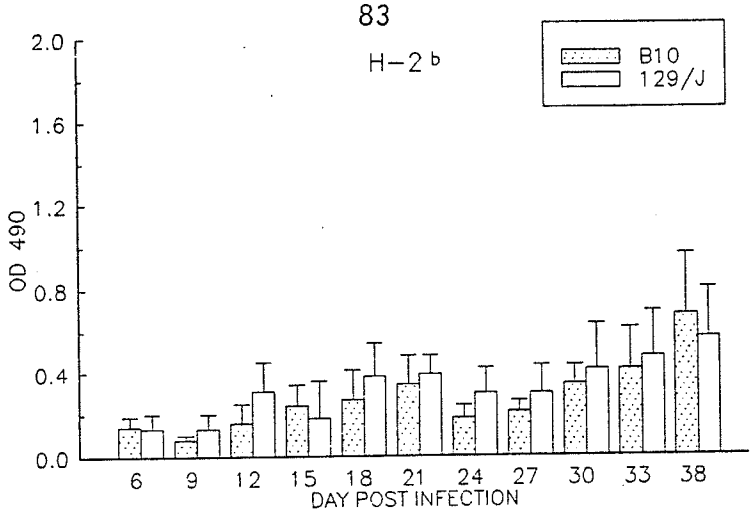
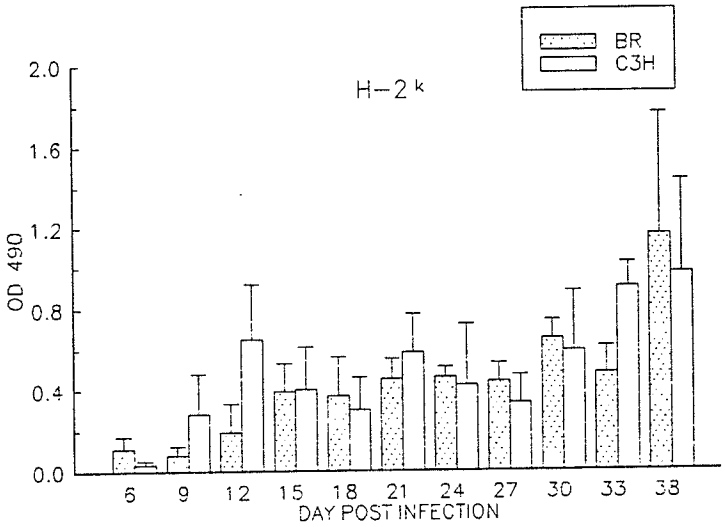
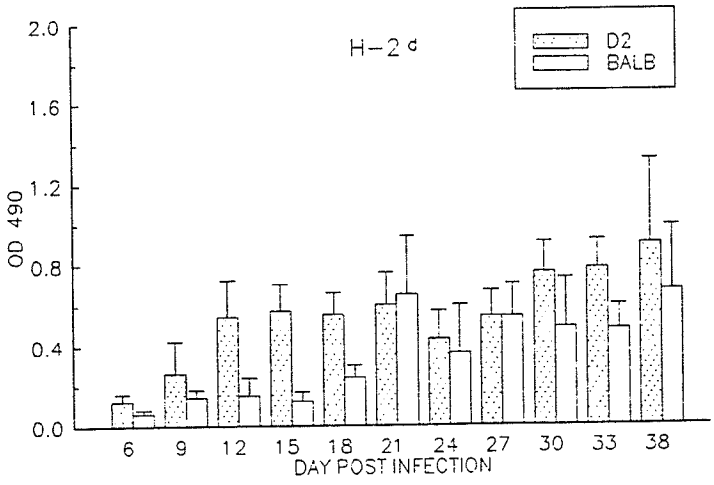

MUSCLE LARVAE X 10³
40
35
30
25
20
15
10
5
0
B10
BR
D2
J129
C3H
BALB

Figure 8. The anti-*Trichinella* IgA response in the faeces of 6 strains of mice.

Strains were separated based on *H-2* haplotype. Mice were infected on Day 0 and challenged on Day 28. Bars represent the mean ± standard deviation of the OD 490 ELISA result for undiluted faecal samples from 8 mice (except Day 38 = 4 mice).


H-2 b
B10
129/J
OD 490
2.0
1.6
1.2
0.8
0.4
0.0
6 9 12 15 18 21 24 27 30 33 38
DAY POST INFECTION


H-2 k
BR
C3H
OD 490
2.0
1.6
1.2
0.8
0.4
0.0
6 9 12 15 18 21 24 27 30 33 38
DAY POST INFECTION

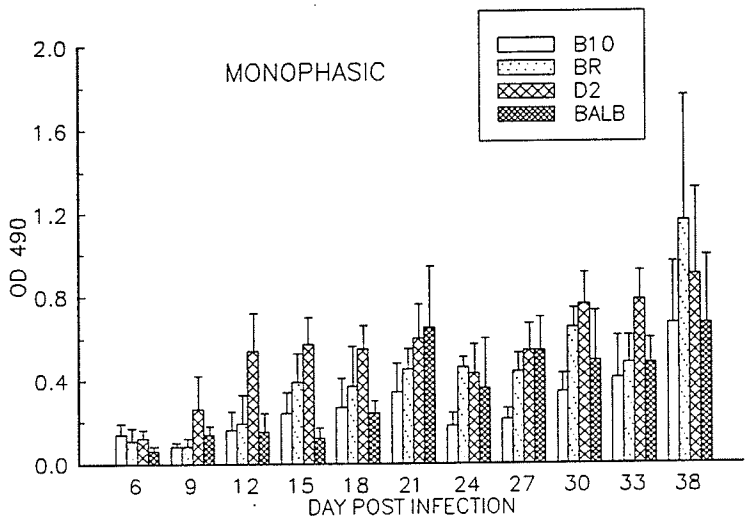

H-2 d
D2
BALB
OD 490
2.0
1.6
1.2
0.8
0.4
0.0
6 9 12 15 18 21 24 27 30 33 38
DAY POST INFECTION

Furthermore, in two strains the primary response was biphasic (C3H, 129/J) with peaks at Day 12 and 21 (C3H) and Day 12 and 18-21 (129/J), while in the other strains the response was monophasic (Fig. 9). Following challenge (Day 28) IgA levels increased in all strains (Fig. 8). Comparison of strains reveals similarities within haplotypes for the general pattern of the response. The exception to this was the early phase of the *H-2*^{d} haplotype response in which antibody production was initiated more rapidly in B10.D2 than in BALB/c mice. A specific IgG was not detected in the primary response but did occur following challenge.

Mucosal response

The anti-*Trichinella* immunoglobulin response on Day 6 and 10 of the primary and challenge infection in the bile and LUM of the 6 strains of mice is summarized in Table 7. The LUM response was negative in the primary infection (titer < 10), but a specific response was detected following challenge infections. The biliary response was negative on Day 6 of the primary (titer < 100) but increased by Day 10 (titer: 250 - 2500). The titer on Day 6 post challenge was significantly greater than the titer on Day 6 of the primary infection, ranging from 1500 (B10) to > 10000 (B10.D2 and C3H). On Day 10 post challenge the titer was >10000 in 4 of 6 strains, and >1000 in the 2 remaining strains.

Figure 9. The faecal anti-*Trichinella* IgA response described in Figure 8 separated by strain to illustrate the monophasic and biphasic response types.


MONOPHASIC
B10
BR
D2
BALB
OD 490
2.0
1.6
1.2
0.8
0.4
0.0
6 9 12 15 18 21 24 27 30 33 38
DAY POST INFECTION

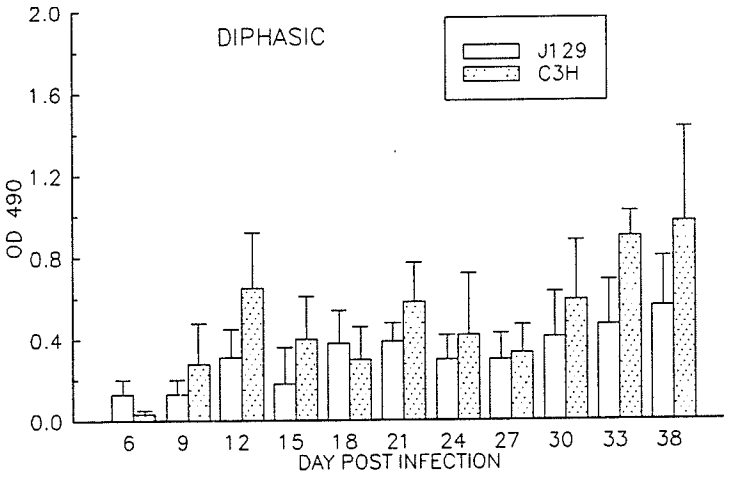

DIPHASIC
J129
C3H
OD 490
2.0
1.6
1.2
0.8
0.4
0.0
6 9 12 15 18 21 24 27 30 33 38
DAY POST INFECTION

Table 7. Mean biliary and mucosal anti-*Trichinella* IgA titre in 6 strains of mice infected with 150 *Trichinella* larvae.

	6-1°	10-1°	6-2°	10-2°
BILE IgA				
BR	<2	2.44±0.27	3.92±0.38	4.04±0.44
B10	<2	<2.39	3.14±0.47	3.71±0.26
D2	<2	3.35±0	4.27±0.27	4.43±0
C3H	<2	2.63±0.24	4.04±0.44	4.27±0.27
BALB	<2	2.88±0.21	3.47±0.40	4.43±0
J129	<2	2.51±0.32	3.35±0.25	3.19±0.28
LUM IgA				
BR	<0.69	<1	1.95±.34	2.07±0.39
B10	<0.69	<1	<1	2.07±0.39
D2	<0.69	<1	1.96±0.48	2.51±0.36
C3H	<0.69	<1	1.95±0	1.84±0.39
BALB	<0.69	<1	1.12±0.21	2.29±0.50
J129	<0.69	<1	1.12±0.21	1.16±0.27

Values equal the geometric mean ± standard deviation of the IgA titer for n = 4 mice per treatment. Strain designations as in Table 5.

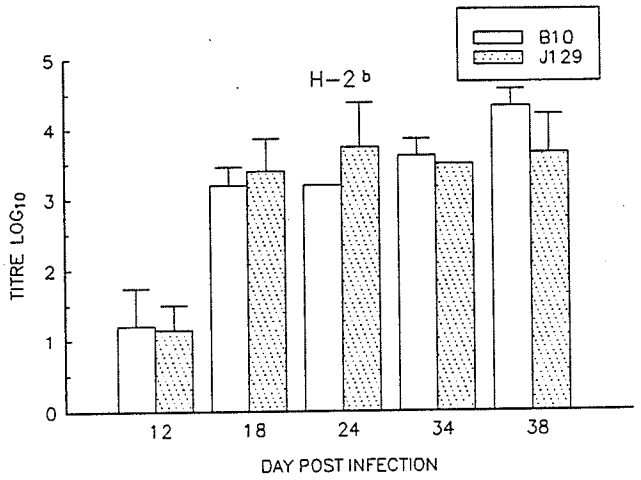
The lowest bile titer occurred in the B10 and 129/J strains, both of which have the *H-2*^{b} haplotype, and these strains also had the lowest LUM IgA titer. Amongst all isolates B10.D2 had highest specific titer for both LUM and bile samples.

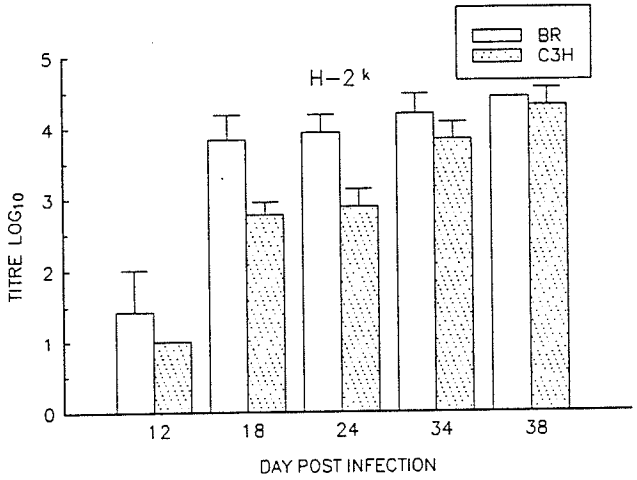
Serum Response

The anti-*Trichinella* serum immunoglobulin titer was determined on Days 12, 18 and 24 of the primary infection and Days 6 and 10 following challenge for IgG and IgM. Serum IgA titers were not determined as a result of the interference in the assay due to high IgM and IgG titers. The kinetics of IgG production were similar in all strains: a response was detected by Day 12, which increased by Day 18 to 24, and again following challenge (Fig. 10). However, the magnitude of the response differed in some strains. The primary response in B10.BR was greater than that observed in other B10 mice indicating an *H-2* influence, but also greater than in C3H which supports a role for background genes. Primary differences also occurred between the *H-2*^{d} strains (B10.D2 and BALB/c) which provides further evidence for background gene effects. Following challenge (Days 34 and 38) titers increased and variability between strains was limited. The kinetics of *Trichinella* specific IgM production differed from the IgG response. In primary infections high IgM titers occurred by Day 12, increased slightly at Day 18, and decreased by Day 24 (Fig. 11).

Figure 10. The serum IgG response over time in 6 strains of mice following infection with *Trichinella*.

Graphs were separated by haplotype. The strains were as listed in Table 5. Bars represent the mean IgG titer ± standard deviation from n = 8 mice (except Day 38 = 4 mice)


B10
J129
H-2 b
TITRE LOG10
0
1
2
3
4
5
12
18
24
34
38
DAY POST INFECTION


BR
C3H
H-2 k
TITRE LOG10
0
1
2
3
4
5
12
18
24
34
38
DAY POST INFECTION

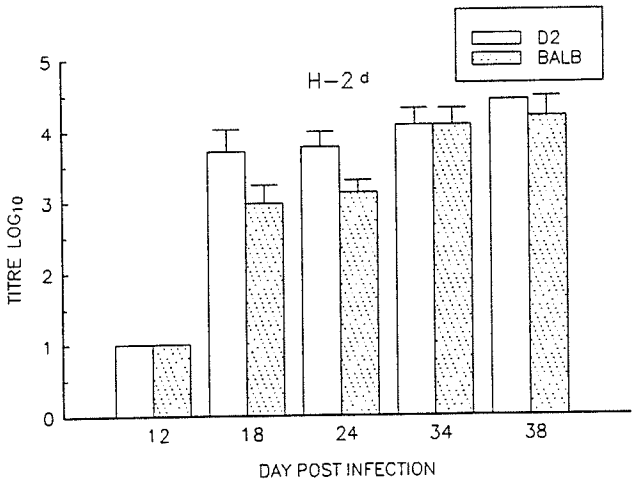
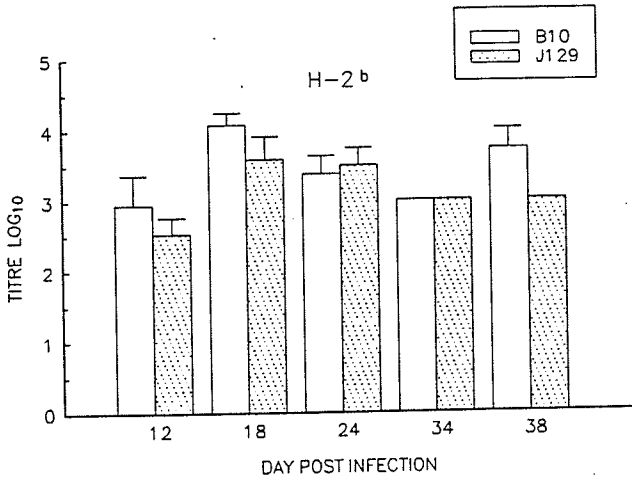
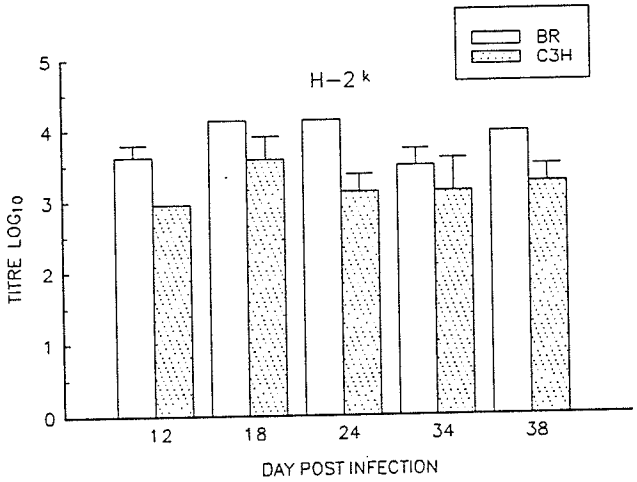
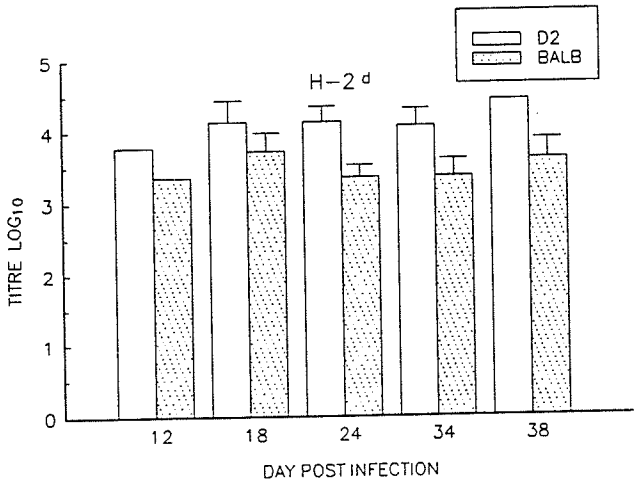

D2
BALB
H-2 d
TITRE LOG10
0
1
2
3
4
5
12
18
24
34
38
DAY POST INFECTION

Figure 11. The serum IgM response over time in 6 strains of mice following infection with *Trichinella*.

Graphs were separated by haplotype. The strains were as listed in Table 5. Bars represent the mean IgM titer ± standard deviation from n = 8 mice (except Day 38 = 4 mice)


B10
J129
H-2 b
TITRE LOG10
0
1
2
3
4
5
12
18
24
34
38
DAY POST INFECTION


BR
C3H
H-2 k
TITRE LOG10
0
1
2
3
4
5
12
18
24
34
38
DAY POST INFECTION


D2
BALB
H-2 d
TITRE LOG10
0
1
2
3
4
5
12
18
24
34
38
DAY POST INFECTION

In all strains there was no increase in IgM titer by Day 6 post challenge, but a slight increase occurred by Day 10 post challenge in most strains. For all haplotypes the response was greater in mice sharing the B10 background than in mice with different genetic backgrounds (Fig. 11) and the titer in the B10 $H\text{-}2^k$ and $H\text{-}2^d$ strains was greater than in the $H\text{-}2^b$ strain.

Discussion

The influence of host genotype on the immune response to *Trichinella* has been examined in considerable detail (reviewed in Wakelin, 1988; Wassom and Kelly, 1990). Strain differences in the response to *Trichinella* described previously were used in this study to assess the relationship between the immunoglobulin response and the effects of immunity. Initially the response was examined in BALB/c mice and subsequently in 5 other strains. In addition, the influence of host genotype on the response to *Trichinella* was determined by comparison of specific immunoglobulin titers, and effects on the biology of the parasite among different strains of mice. Despite the considerable effort to date (Wakelin, 1988; Wassom and Kelly, 1990), the role of host genotype in regulating the kinetics and titer of the mucosal immunoglobulin response following helminth infection has not previously been described.

Reports on influence of genotype on the response to *Trichinella* are controversial because of different interpretations of the effect of worm dose (Bell and Liu, 1988; Wassom and Kelly, 1990). Wassom *et al.*, (1984a) reported that with increasing dose, haplotype specific differences in suppression of expulsion occurred which were correlated with a dose dependent suppression of lymphocyte proliferation. In contrast Bell and Liu (1988) reported that

differences in expulsion were optimized at higher doses and that effects on expulsion resulted from a temporal shift in onset, and not from suppression of the response. Zhu *et al.*, (1990) also reported that lymphocyte proliferation was not suppressed with increasing antigen dose. However, these apparent differences are not mutually exclusive. Regardless of the mechanism, expulsion of worms is suppressed or delayed. Furthermore lymphocyte proliferation is strain dependent (Wassom *et al.*, 1984a) and different strains were examined by Zhu *et al.*, (1990). In my study, mice were infected at a low dose (150 larvae/mouse) to limit the potential for immunosuppression.

Immune effects and immunoglobulin: BALB/c mice

Initial experiments in our lab utilized outbred CFW mice as the host for *Trichinella* infection. Based on expulsion rates and muscle larvae counts this strain was a rapid responder to *Trichinella* (deVos *et al.*, 1992). The increased rate of expulsion and the reduction in fecundity and worm size following challenge infections were highly correlated with IgA titer in the bile and intestinal lumen (deVos *et al.*, 1992). It was interesting to note that several inbred strains derived from CFW mice (Rice and O'Brien, 1980) were also rapid responders to *Trichinella*, including SJL and SWR (Wassom *et al.*, 1979; Wakelin, 1988), NIH (Wakelin and LLoyd, 1976), and NFR and NFS strains (Bell *et al.*, 1982a,b).

In my study, the immunoglobulin response and effects of immunity were assessed in BALB/c mice to determine if the correlation between immune effects and the mucosal immunoglobulin response described above, also occurred in a slow responding strain. In BALB/c mice, worm expulsion from the gut was slower in both primary and challenge infections than in CFW mice (deVos et al., 1992), similar to previous reports (Wakelin, 1978). This delay in expulsion occurred despite a specific IgA response. In some mice which expressed a specific IgA response, the recovery of adult worms was 95%. The absence of expulsion in IgA positive mice suggested that the mucosal immunoglobulin response had no significant role in the expulsion phenomenon in *Trichinella* infections. In contrast, the reduction in worm size and fecundity following infection in BALB/c mice were similar to that observed in CFW mice and occurred in conjunction with a *Trichinella* specific mucosal IgA response. It was not possible to dissociate the effects on worm size and fecundity from the mucosal immunoglobulin response. Based on these observations, this relationship was reexamined in BALB/c and 5 other strains of mice.

Influence of host genotype on the effects of immunity

Expulsion of worms from the intestine

The influence of *H-2* and background genes on the production of an expulsion response has been described for *Trichinella* and other gastrointestinal nematodes. Both *H-2*

and background gene effects have been reported for *Trichinella* (Bell *et al.*, 1982b; Bell and Liu, 1988; Dick *et al.*, 1988; Wassom *et al.*, 1983b; Wakelin, 1978), *Trichuris muris* (Wakelin, 1975; Wakelin 1978; Else and Wakelin, 1988; Else et al., 1989,1990a) and *Heligmosomoides polygyrus* (Sitepu *et al.*, 1985; Enriquez *et al.*, 1988ab). For each of these systems *H-2* response genes have been mapped to similar *I* and *D* regions (Wassom et al., 1979; Else et al., 1990a; Enriquez et al., 1988b) but the response phenotype of the different strains is not the same for each of the parasite species (Behnke and Wahid, 1991).

In the present study the rate of expulsion of *Trichinella* following primary infections was associated with genetic background. However, analysis beyond Day 10 of infection would likely reveal *H-2* differences between B10 congenics. Following challenge infections, expulsion rates correlated with *H-2* haplotype, being slowest in $H\text{-}2^{d}$ mice and more rapid in $H\text{-}2^{b}$ and $H\text{-}2^{k}$ strains. However, even in these treatments, background genes had an effect since expulsion was greater in both 129/J and C3H/He than in the B10 strains having similar haplotypes. Similar interaction of *H-2* and background genes have been reported in previous studies with different strains as described above (Wassom *et al.*, 1985, Wassom and Kelly, 1990).

The variability in the BALB/c expulsion response between this experiment and the first experiment in this

Chapter is of interest. The two groups of mice were obtained from different sources, the first from Charles River (CRL) and the second from Jackson Laboratories. Though originating from the same stock, BALB/cAn (CRL) and BALB/cByJ (Jackson) have been separated since 1961 and genetic differences have been described (Potter, 1985). These differences further support the importance of host genotype in determining the expulsion response and highlights the difficulty in comparing strains from different sources.

Fecundity of adult female worms

Although an antifecundity effect had been inferred in early studies (Rappaport and Wells, 1951) the effects of immunity on fecundity were revealed by quantitation of larval production in the *in vitro* larval release assay (Despommier *et al.*, 1977). For primary infections in CFW mice, larval release occurred by Day 5, peaked at Day 6, then gradually declined, ending by Day 12 (Despommier *et al.*, 1977; Silberstein and Despommier, 1985a). In immunized or previously infected CFW mice, larval production also began at Day 5, but the mean was reduced >50% by Day 6, and continued to decline throughout the course of infection (Despommier *et al.*, 1977; Silberstein and Despommier, 1985a,b; deVos *et al.*, 1992). The recovery of fecundity following transplantation to naive hosts indicated that the reduction in fecundity in the course of infection is a function of host immunity and not simply senescence of the

worms (Kennedy and Bruce, 1981).

Genetic influences on fecundity have been reported for *Trichinella* and other nematode infections. For *Trichinella*, strain differences in the antifecundity response implicate both *H-2* and background genes (Bell *et al.*, 1982ab; Wassom *et al.*, 1984ab; Dick *et al.*, 1988) but the high variability reported make comparisons difficult. For example, Bell *et al.*, (1982a) report 40 larvae/day on Day 6 primary in B10.D2 mice, compared with 83 larvae/day for pooled B10 mice in the same paper. Similarly, in the NIH strain larval release was reported as 80.9±3.9/day and 63.4±7.3/day in the same paper (Kennedy, 1980). Fecundity reported for *Heligmosomoides* and *Trichuris* is based on estimates of egg production per gram faeces, and the effects on fecundity are determined after several weeks of infection, in contrast to the response to *Trichinella* which occurs by Day 10 of infection. Genetic control of the anti-fecundity response for *Heligmosomoides* (Sitepu *et al.*, 1985; Brindley *et al.*, 1986; Behnke and Wahid, 1991) and *Trichuris* (Else *et al.* 1990a) was associated with both *H-2* and background genes. It was suggested that effects on expulsion and fecundity in *Heligmosomoides* were regulated independently (Brindley *et al.*, 1986). However, Behnke and Wahid (1991) reported an association between fecundity and expulsion, as was suggested for *Trichuris* infections (Else *et al.* 1990a).

In my study anti-fecundity effects were similar in all

strains at each time period. Fecundity was reduced by ~70% on Day 10 following the primary infection and by 50% on Day 6 of challenge infections. The percent reduction in fecundity on Day 10 primary in B10.BR and C3H was greater than previously reported (Dick *et al.*, 1988), as was the reduction on Day 6 challenge in B10.D2, while the reduction in B10.BR fecundity on Day 6 was similar (Bell *et al.*, 1982a). The basis for these differences is not clear, though the variability even within labs described above may be a factor. The treatments in this experiment were designed to utilize a common infection source for the intestinal stages of both primary and challenge infections and each step was standardized to minimize error. Furthermore, since random samples were recounted independently, experimental bias is likely not a factor. However, it is clear from these results that the reduction in fecundity occurs in the absence of an effect on expulsion and that these effects are regulated separately.

Size of adult female worms

Another biological variable which is affected in the immune response is worm size. Worm length has been reported in a number of publications and was shown to vary temporally (Rappaport and Wells, 1951) similar to fecundity, increasing to a maximum by Days 6-8 and subsequently declining. The reduction in worm length is a function of the host response since 'stunted' adult worms at Day 10 of infection grow to

the size of Day 6 primary worms when transferred to naive recipients (Kennedy and Bruce, 1981). Reported worm lengths vary from 2.0 to 3.3 mm (Rappaport and Wells, 1951; Grencis *et al.*, 1985) in different strains. Despite this variability between labs and strains, the influence of host genotype on the reduction in worm length has not been reported. For other nematodes, Sitepu *et al.*, (1985) reported a significant reduction in both male and female *Heligmosomoides* length in high responder mice compared with random bred and low responder mice. Immune effects on the growth of *Trichuris* have also been implied, but quantitative data were not provided (Else *et al.*, 1990a). In the latter study, worm stunting occurred in conjunction with the expulsion and antifecundity responses. However, influence of the *H-2* locus on size has not been reported.

In my study, the effects on worm length followed a similar pattern in all strains except 129/J. Worms were reduced in size on Day 10 compared with Day 6 of the primary infection and following challenge a pronounced reduction in worm length occurred by Day 6. Although worm length varied among strains, a clear association with either *H-2* haplotype or genetic background was not evident, similar to the effect on fecundity discussed above. The stunting observed in the 129/J strain remains an enigma. Although the 129/J strain was a strong responder with respect to expulsion, worm length was ~55% that reported in the strong responder NIH

strain (Kennedy and Bruce, 1981). The possibility of experimental error was reduced since worms from all strains were batch processed and measured without knowledge of their source. Regardless, the similarities in the reduction in fecundity and size in the 5 other strains provides further indirect support for the hypothesis of a common mechanism for these responses.

Total muscle larvae

The mean of total muscle larvae has been used extensively as an indicator of protection in experiments with *Trichinella* (McCoy, 1931; Culbertson, 1942; Wassom *et al.*, 1979). Muscle larvae recovery is influenced by the rate of expulsion, the anti-fecundity response and the anti-newborn larvae response. Furthermore, muscle larvae recovery represents the success of infection since this stage is transmitted to the next host. The initial association of resistance/susceptibility with the *H-2* haplotype was based on differences in muscle larvae recovery (Wassom *et al.*, 1979; 1983a) and in these studies *I* and *D* region genes were implicated in the response to *Trichinella*.

In the present study, based on fecundity and expulsion, the predicted muscle larvae recovery in the C3H and 129/J strains would be lower than in the other strains. As predicted, the larval recovery in 129/J was the lowest among all strains and similar to levels described previously (Wassom *et al.*, 1983a). Larval recovery in the BALB/c

strains was intermediate (~25000) but greater than that observed previously (Wassom *et al.*, 1983b), while the B10 strains were most susceptible. Recovery in B10.BR mice (~30000) was similar to, but recovery in B10 was greater than previously described Wassom *et al.*, (1979,1983b). Comparative data for B10.D2 and C3H/HeJ were not reported. The comparison between B10 and 129/J suggests that the response is influenced to a greater extent by background genes.

Influence of host genotype on the Immunoglobulin Response

Serum Immunoglobulin Response

As described in Chapter 1, a specific mucosal and systemic immunoglobulin response occurs following infection with *Trichinella*. Although specific anti-*Trichinella* precipitins were described as early as 1911 (reviewed in Kagan and Norman, 1970) the influence of host genotype on immunoglobulin production has been described for a only a few strains of mice. The first studies utilized Biozzi mice which were selected for high or low antibody responsiveness to sheep erythrocytes (Biozzi *et al.*, 1975). Differences between strains were minimal following infection with 300 larvae, though resistance was greater in the high antibody producing mice at doses of 50 larvae (Perrudet-Badoux *et al.*, 1975,1978; Ruitenberg *et al.*, 1980). Almond and Parkhouse (1986) reported higher serum IgA titers in strong responder NIH mice than in susceptible C3H/He mice but only

minor differences for the other isotypes, and for a number of isotype/antigen combinations the response was greater in the C3H mice. Bolas-Fernandez and Wakelin (1989,1990) compared the response to several strains of *Trichinella* in NIH and B10 mice and found the total antibody response to be generally higher in B10 than in NIH mice, in contrast to lymphocyte proliferation which was higher in the NIH mice. Pond *et al.*, (1989) reported that the antibody response in susceptible B10.BR mice was greater for all isotypes except IgG_{2a} than the response in more resistant AKR mice. B10.BR mice also had a higher level of interleukin 4, while splenic IFN-gamma levels were higher in the more resistant AKR mice. Results from studies with other nematodes are somewhat contradictory. Following infection with *Trichuris* high serum immunoglobulin titers were associated with susceptibility to infection (Else and Wakelin, 1989) but in later experiments high serum titers were associated with the strong responder $H\text{-}2^q$ haplotype (Else and Wakelin, 1990b). Increased serum antibody levels in susceptible mice have also been reported for infections with *Heligmosomoides polygyrus* (Monroy and Enriquez, 1992).

My results for the serum immunoglobulin response were generally similar to those for *Trichinella* described above. In all strains examined, specific IgG was detectable by Day 12, increasing by Day 18 to 24 and following challenge. Differences in IgG titers among strains occurred in primary

infections, but generally disappeared following challenge. Although differences occurred between congenic mice, such as the Day 18 response in B10 strains, differences were also observed between strains with the same *H-2* haplotype on different backgrounds. This is particulary evident comparing the response in B10.BR with that in C3H/HeJ and these results corroborate the observations of Pond *et al.*, (1989) who compared B10.BR mice with AKR as described above. It is apparent from the above that both *H-2* and background genes influence the serum immunoglobulin response.

Mucosal immunoglobulin response

Host genotype effects on mucosal immunoglobulin responses following infection with *Trichinella* or other gastrointestinal helminths have not previously been described. However, the mucosal immunoglobulin response to CT provides a model for comparison. Following exposure to CT, both immunoglobulin production (Elson and Ealding, 1987; Hirabayashi *et al.*, 1991) and intestinal fluid accumulation (Richardson and Kuhn, 1986) correlated with *H-2* haplotype, and the genes which influenced this response mapped to the I-A region. Strong responder strains had the $H\text{-}2^{b}$ and $H\text{-}2^{q}$ haplotypes while $H\text{-}2^{d}$, $H\text{-}2^{k}$, and $H\text{-}2^{s}$ strains had low antibody titers (Elson and Ealding, 1987).

Following infection with *Trichinella*, strain differences occurred in the kinetics of faecal antibody production in my experiments. However, these differences

were not associated with a specific *H-2* haplotype genetic background. The early phase of the B10.D2 response differed from the B10 and B10.BR responses suggesting *H-2* influences, but it also differed from the response in BALB/c mice which share the $H\text{-}2^d$ haplotype. Strain differences also occurred in the biliary and intestinal mucosal response and an *H-2* relationship was apparent. For example, low IgA titers in the bile were associated with the $H\text{-}2^b$ haplotype following challenge while both $H\text{-}2^k$ and $H\text{-}2^d$ strains had high IgA titers. However, a significant response was detected in all strains, and titer differences between strains were not as great as reported for the response to CT described above.

The faecal IgA response following primary infections in C3H and 129/J strains was diphasic, similar to that described for CFW mice in Chapter 1, and these strains also had the highest rate of expulsion. The primary antibody response in the remaining strains was monophasic. In chapter 1 it was hypothesized that the two peaks in the faecal response in CFW mice may result from changes in antigen types in the course of the *Trichinella* life cycle. In this chapter, the correlation of expulsion rate with the type of response provides further support for this hypothesis. An increased rate of expulsion would reduce the temporal overlap of antigenic types, and produce a response with distinct phases. In slow responding strains, exposure would be continuous, overlap in antigenic type would occur, and

the response would be less distinct or monophasic.

Summary

Immune effects and the Immunoglobulin response

What is the relationship between the effects of immunity on expulsion and the production of a specific immunoglobulin response? The initial observations with BALB/c mice indicated that strain differences in the rate of expulsion were not correlated with mucosal IgA production. Similar results occurred in the other strains. For example, despite a similar biliary IgA response on Day 10 primary, expulsion in B10 mice differed significantly from expulsion in C3H mice. Furthermore, mucosal antibody titers in susceptible B10.BR mice were greater than in resistant 129/J mice. This interpretation, in combination with the serum IgA data of Pond *et al.*, (1989) and the results of Bolas-Fernandez *et al.*, (1990) contradict the suggestion by Almond and Parkhouse (1986) that mucosal IgA may be fundamental to the expulsion observed in NIH mice.

In contrast to the expulsion response, there was a strong temporal correlation between the reduction in the fecundity and size of female worms, and the mucosal IgA response. The reduction in fecundity by Day 10 primary and Day 6 challenge was associated with an increase in *Trichinella* specific biliary IgA production in all strains examined. Furthermore, specific IgA was not detected on Day 6 primary when no effect on fecundity occurred. These

observations suggest that the effects on expulsion and fecundity are regulated independently.

The independence of the expulsion response from the reduction in fecundity and size has been suggested previously. Rappaport and Wells (1951) described stunting of worms following challenge in the absence of an increased expulsion and suggested that 'immunity in trichinosis may be manifested in several ways'. Similar results were reported by Campbell (1955), Kim (1957), and Despommier and Wastmann (1969) following vaccination experiments. The suggestion of multiple mechanisms was reiterated by Larsh (1967) who hypothesized that the worm stunting resulted from antiworm antibodies, but that expulsion was a delayed type hypersensitivity response. Comparison of *in vitro* larval release and expulsion (Bell *et al.*, 1982b; Bell *et al.*, 1983; Wassom *et al.*, 1984b) also led to the conclusion that these were independent responses under separate genetic control.

In the studies above, immune effects were attributed to antibodies of mucosal origin which could act to inhibit nutrient uptake and interfere with reproduction by occlusion of parasite orifices. This hypothesis was based on several observations including immunoprecipitation following *in vitro* incubation with immune serum (Oliver-Gonzalez, 1940), the distribution of antibody binding on adult worms as revealed by immunoflouresence labelling (Jackson, 1959),

transfer of immunity from infected mothers to sucklings (Perry, 1974), reduced *in vitro* larval release following the addition of specific biliary IgA to cultures (Jacqueline *et al.*, 1978, 1981), and the prolonged intestinal phase of infection in rats following bile duct occlusion (Jacqueline *et al.*, 1981). The association of a specific mucosal immunoglobulin response with the reduction in fecundity in my experiments provides further support for an effector role for mucosal IgA.

Influence of host genotype

Differences in the effects of immunity and the host immunoglobulin response in this chapter clearly demonstrate that host genotype has a significant influence on the response following infection. It is apparent that these genotype effects are related to both *H-2* haplotype as well as genetic background. For the three nematode species discussed above, mapping studies with panels of congenic mice revealed two loci within the *H-2* complex, one associated with the *I* region (*I-E*) and another with the *D* region which influenced the response to infection (Wassom *et al.*, 1979; Else *et al.*, 1990a; Enriquez *et al.*, 1988b). Although these similarities would suggest common mechanisms in the response, significant differences also occur. The reported correlation between *I-E* expression and susceptibility to infection (Wassom *et al.*, 1987; Robinson *et al.*, 1989) was not apparent in *Trichuris* (Else *et al.*,

1990a) and *Heligmosomoides* infections (Behnke and Wahid, 1991). Furthermore, although the expulsion and fecundity responses were considered independent in *Heligmosomoides* infection (Sitepu *et al.*, 1985), similar to *Trichinella* in this and previous studies (Wassom *et al.*, 1984b; Bell *et al.*, 1983) subsequent analysis suggested a correlation of these effects in *Heligmosomoides* (Behnke and Wahid, 1990) and *Trichuris* (Else *et al.*, 1990a) infections.

Although strain differences in serum and mucosal immunoglobulin titers were detected in my study, positive immunoglobulin responses were recorded for all strains. In comparison, the influence of *H-2* genes on the immunoglobulin response to CT was much greater (Elson and Ealding, 1987). This difference is not unexpected, since the influence of H-2 genes occurs at the level of antigen recognition (reviewed in Benaceraff and Katz, 1975; Schwartz, 1986). *Trichinella* infection presents the host with numerous different antigens, increasing the probability of successful interaction with the *H-2* products which regulate antigen presentation. Therefore an immune response is expected, although the antigens recognized may vary between strains, as illustrated by strain and individual differences in antigen recognition profiles of mice following infection (Kennedy *et al.*, 1991; Robinson *et al.*, 1991). By comparison, CT presents a small number of potential epitopes, reducing the probability of a *H-2*/antigen

interaction and consequently differences between strains are accentuated. Genetic influences on the amplification phase of the immune response could also influence immunoglobulin levels following infection. For instance, responses directed to the Th2 pathway would stimulate greater antibody production than would a Th1 type response, and these differences have been reported for mice infected with *Trichinella* (Pond *et al.*, 1989).

From the above, it is apparent that an understanding of genotype influences is critical in the development of antigens for vaccination. These influences are particularly relevant for purified antigens, since antigen specific differences would affect *H-2* recognition, as well as the pathway of helper cell amplification. In combination, these observations suggest vaccination with crude or mixed antigens would limit host genotype effects.

Chapter 3. Oral Immunization.

Abstract

The effects of oral immunization on the immunoglobulin response to *Trichinella*, rate of expulsion, fecundity, size, and the mean of total muscle larvae were determined in CFW mice fed *Trichinella* antigen in the presence or absence of cholera toxin. The mean of total muscle larvae was reduced by 36% in mice infected with *Trichinella* larvae in combination with CT. In mice fed soluble, particulate or soluble/particulate antigens in combination with CT on Day 0, 14 and 21, and challenged with *Trichinella* larvae on Day 28, there was a significant reduction in adult worm fecundity (50%), worm size (20-30%) and the mean of total muscle larvae (75%), but no apparent effect on the rate of expulsion on Day 6 post challenge. On Day 6 following challenge with 150 *Trichinella* larvae, specific anti-*Trichinella* immunoglobulin titers were significantly greater in the bile and LUM of mice fed antigen plus CT than in control mice or mice fed antigen alone. The specific serum IgG titer was enhanced following challenge with *Trichinella* larvae in treatments fed soluble, particulate or soluble/particulate antigen with CT. The response was also enhanced in the particulate, but not in soluble or soluble/particulate antigen treatments without CT. Despite the strong serum response in the particulate treatment, the mean of total muscle larvae was 3X greater than in the particulate plus CT treatment.

Introduction

It is clear from the first two chapters, and from previous studies that infection with *Trichinella* results in an immunoglobulin response which is correlated with the effects of immunity (Crandall and Crandall, 1972; Van Loveren *et al.*, 1987; deVos *et al.*, 1992). Both the mucosal and systemic immunoglobulin responses have been implicated as immune effectors in the anti-*Trichinella* response. As a consequence, numerous studies have reported attempts to immunize mice with antigenic preparations from Trichinella, and these are summarized in Table 8. It is apparent from this summary that the majority of experiments used intra-peritoneal or subcutaneous injection of antigen in combination with adjuvant. Considering that three of the four major effects of immunity to *Trichinella* occur within the intestine, it is surprising that only two related studies report oral immunization (Vernes, 1976; Tronchin *et al*, 1979).

Given the role of the mucosal response in viral (Taylor and Dimmock, 1985) and bacterial (Pierce *et al*, 1978) systems and the intestinal effects correlated with mucosal IgA in *Trichinella* described above, oral immunization with *Trichinella* antigen has potential as a vaccination strategy for intestinal helminths.

Table 8. Summary of reported immunization for experimental trichinosis.

Antigen	Adj.	Type	Effects	Authority
Crude ML extract, 3x	No Adj.	IP	Dec. ML	McCoy, O.R. 1935
ML powder, 8x	CBS	IP	Dec. ML	Culbertson, J.T. 1942
ES	No Adj.	SC	Dec. ML,WL	Campbell, C.H. 1955
ES 6 or 12 x	No Adj.	IP	Dec AW,ML	Chipman, P.B. 1957
ES	No Adj.	ORAL	Dec. ML	Vernes, A. 1976
Crude	CFA	IP	Dec. AW,ML,FEC	Despommier *et al.*, 1977
LPF	CFA	IP	Dec. ML	Despommier and Lacceti, 1981a
Fractionated LPF	CFA	IP	Dec. ML	Despommier and Lacceti, 1981b
ES	No Adj.	ORAL	no worm effect	Tronchin *et al.*, 1979
37K	CFA	IP	Dec. ML	Silberstein and Despommier, 1984
48K	CFA	IP	"	"
50/55K	CFA	IP	"	"
48K	CFA	IP	Inc. Survival	Silberstein and Despommier, 1985b
50/55K	CFA	IP	"	"
ES	CFA	IP	Dec. ML, Fec	Gamble, 1985a
AWE	CFA	IP	Dec. AW, ML	"
49K	CFA	IP	Dec AW,ML,Fec	Gamble, 1985b
53K	CFA	IP	Dec AW,ML,Fec	"
CTAB	CFA	IP	Dec. AW,ML,Fec,WL	Grencis *et al.*, 1986
Crude NBL	CFA	IP	Dec. ML	Marti *et al.*, 1987
Group II Ag	CFA	IP	Dec. ML, Fec	Denkers *et al.*, 1990
9D4, 6D8-E3, 6-B1-G10 (mAb purified Ag)	CFA	IP	Dec. AW, ML, Fec	Zhu and Bell, 1990

Antigens: ML = muscle larvae; ES = excretory/secretory; LPF = large particle fraction; AWE = adult worm extract; CTAB = cetyltrimethylammonium bromide; NBL = new born larvae; Group II = antigens which induce dominant response later in infection; ##K = various antigens of specific MW.
Adjuvant: CBS = Carbolized salt solution; CFA = Complete Freunds adjuvant
Type: IP = intra peritoneal; SC = sub cutaneous
Effects: Dec. = decrease; Inc. survival = increase host survival of infection; AW = adult intestinal worms; ML = muscle larvae; WL = worm length; Fec = fecundity.

However, antigens vary in mucosal immunogenicity, the majority of proteins being weakly immunogenic, requiring large immunizing doses and often producing oral tolerance rather than stimulation (Chiller and Glasebrook, 1988). As indicated in the first chapter, CT is an exceptionally immunogenic protein which when given in combination with unrelated antigens enhances both systemic and mucosal immune responses to these proteins (Elson and Ealding, 1984ab; Lycke and Holmgren, 1986). This adjuvant effect has been described for haptens coupled to CT (Azcona-Olivera *et al*, 1992; Silbart and Keren, 1989), purified proteins mixed with CT (Elson and Ealding, 1984b; Lycke and Holmgren, 1986; Wilson *et al*, 1990), CT bound to viral particles (Liang *et al*., 1989), CT mixed with viral haemagluttinins (Hirabayashi *et al*., 1991) and CT mixed with crude protozoan extracts (Bourguin *et al*., 1991). CT enhanced stimulation of both mucosal and systemic anti-*Trichinella* immunoglobulin responses would provide a model to determine the effects of oral immunization on the course of infection with *Trichinella*.

The purpose of this study was i) to determine the effect of oral immunization on the rate of expulsion, fecundity, size and total muscle larvae in mice, ii) to determine the effect of oral immunization on the mucosal and systemic immunoglobulin response to *Trichinella*, iii) and to assess the relationship between the effects of immunity and

the immunoglobulin response. The adjuvant effect of CT for the anti-*Trichinella* immunoglobulin response and the effect on the *Trichinella* life cycle was determined in natural infections and fed antigen treatments and the antigenicity of different *Trichinella* protein extracts was assessed.

Materials and Methods

Animals

Mice used were 6 wk old CFW male mice (Charles River Laboratories, St Constant, Que, Canada). The P1 strain of *Trichinella* (Dick, 1983) was used. *Trichinella* was maintained in our laboratory by serial passage in CFW mice. Procedures for the isolation of worms and infection of mice were as described in Chapter 1 and 2.

Antigen Preparation and feeding

Antigens were prepared from L1 muscle larvae. Larvae were suspended in 4 volumes of 1% sucrose in Tris-HCl pH 7.4. Homogenates were prepared by grinding with a motor driven teflon pestle in a ground glass homogenizer on ice until larvae were completely disrupted (15 min). The homogenate was centrifuged at 100,000g for 1 hr. The supernatant was considered the soluble antigen fraction (SA). The pellet was resuspended in the above buffer, and centrifuged at 10000g for 30 min. The supernatant was considered the particulate antigen fraction (PA). Soluble/particulate antigen (SPA) was prepared by centrifugation of the original homogenate at 10000g for 30 min. The protein concentration of these fractions was determined with the BioRad protein assay, the samples aliquoted and stored at -70°C.

Sample collection

Prior to treatment, control serum samples were obtained

from mice by tail bleeding. Serum samples were obtained at 6 day intervals. Mucosal sampling methods were as described in Chapters 1 and 2. Briefly, mice were starved 12 hr, killed, a serum sample taken from the brachial vein/artery, the gall bladder removed and bile collected, and an intestinal lumen sample obtained by flushing the gut with 0.5 ml PBS + protease inhibitors. For infected mice adult worm recovery was determined as described in Chapter 2.

For Experiment IIb adult females collected within 2 hr of sampling were cultured 24 hr in RPMI 1640 supplemented with 10% fetal calf serum and the antibiotics penicillin and streptomycin (as in Chapter 2). Fecundity was measured as the number of newborn larvae released in 24 hr, as described in Chapter 2. In addition, the potential for further larval production was assessed qualitatively. Worms were scored by the number of developing embryos in the posterior third of the worm and the number of larvae in the uterus: type 0 = <10 large embryos and no larvae in uterus; type 1 = ~10 embryos and <20 larvae in uterus; type 2 (normal) = 100^{+} embryos and >20 larvae in uterus. Worms were then fixed with hot 70 % ETOH, cleared in 5% glycerine in 70 % ETOH and mounted on slides. Worms were projected and drawn with a microfiche reader and lengths were determined from drawings with a digitizing board using Sigma Scan software (Jandel Scientific).

ELISA

Immunoglobulin subclass distribution and levels were determined by ELISA as described in Chapter 1 and 2. Briefly, microtiter plates (Corning 25805-96) were coated o/n with 50 μl of 5 μg/ml CT or 10 μg/ml *Trichinella* antigen per well in carbonate buffer (pH9.6). Plates were blocked with 150 μl/well 0.5% gelatin in PBST and all subsequent steps were as described, using 50 μl volumes for samples, second antibody, substrate and stop buffer. Titer was defined as the reciprocal of the dilution at which the optical density was 0.3 (1.5 S.D. greater than untreated bile samples), and was determined by analysis of doubling or tripling dilutions. The minimal dilution for bile samples was 1/100. Mouse anti-*Trichinella* antibodies of known titer were included in each assay for comparison between plates. Titer values were log transformed, the mean and standard deviation calculated and treatment differences were assessed by t-tests ($p<0.05$ significant).

Experimental design

Experiment I: *Trichinella* larvae plus CT

In the initial experiments describing the mucosal antibody response (Chapter 1) mice were fed CT in conjunction with *Trichinella* infection to assess interaction in the immunoglobulin response. The mean of total muscle larvae from these experiments is reported here to illustrate the CT adjuvant effect. Briefly, groups of mice were infected on Day 0 and challenged on Day 28. Mice were

sampled 6 days following challenge infection (Day 34) to determine total muscle larvae numbers.

Experiment IIa: Fed antigen plus CT (preliminary)

A preliminary experiment was undertaken to determine the adjuvant effect of CT for fed *Trichinella* antigen. CFW mice were fed 0.5 or 0.05 mgs of soluble antigen (SA) in the presence or absence of CT on Days 0, 14, and 21. On Day 28, mice were infected with 150 P1 larvae and sampled 34 days later to determine muscle larvae burden.

Experiment IIb: Fed antigen plus CT

Based on the preliminary experiments, the adjuvant effect of CT for *Trichinella* antigens was examined in a large scale experiment. Eight groups of 20 mice were fed *Trichinella* antigen preparations with or without CT as described below. Mice were fed on Days 0, 14 and 21. Four mice from each group were sampled on Day 28. The remaining 16 mice in each group were infected with 150 larvae on Day 28. Eight mice per group were sampled on Day 34 (Day 6 post infection) to assess the effects on adult worms in the gut, and the remaining 8 were sampled on Day 68 (Day 40 post infection) to determine worm burden in the muscle. Mice fed bicarbonate buffer or CT alone were used as controls in this experiment. Serum samples were obtained at 6 day intervals to determine the kinetics of the systemic immunoglobulin response.

Results

Trichinella larvae plus CT

Total muscle larval numbers for the treatment groups are shown in Figure 12. Over all treatments, muscle larvae numbers were reduced by 36% in mice infected with *Trichinella* larvae in combination with CT compared with mice infected with *Trichinella* larvae only.

Fed antigen plus CT

The effect of oral immunization with soluble *Trichinella* antigen on total muscle larvae and the adjuvant effect of CT were assessed in a preliminary experiment (Table 9). A reduction of total muscle larvae occurred in mice fed antigen without CT, but the greatest reduction (80%) occurred in the treatments fed soluble antigen with CT. Based on this observation the following large scale oral immunization experiment was undertaken.

Biological effects

The effects of oral immunization on the rate of expulsion, worm fecundity and worm size were determined on Day 6 following challenge with *Trichinella* larvae, during the intestinal phase of infection, and the effects on the mean of total muscle larvae were determined on Day 40 PI. A comparison of treatment means for the rate of expulsion revealed no significant differences ($p<0.05$) among all

Figure 12. The mean of total muscle larvae recovery on Day 34 from 6 groups of 4 mice exposed to different combinations of 150 *Trichinella* and 10 μg of CT on Day 0 and challenged on Day 28.

Bars indicate the mean $\pm$ standard deviation for the following treatments A=1°(*Trichinella*)/2°(*Trichinella*); B=1°(CT-*Trichinella*)/2°(*Trichinella*); C=1°(*Trichinella*)/2°(CT); D=1°(CT-*Trichinella*)/2°(CT); E=1°(*Trichinella*)/2°(CT); F=1°(CT-*Trichinella*)/2°(CT-*Trichinella*). Treatment means were compared by t-tests, and significant differences ($p<0.05$) from group A are indicated by an asterix.

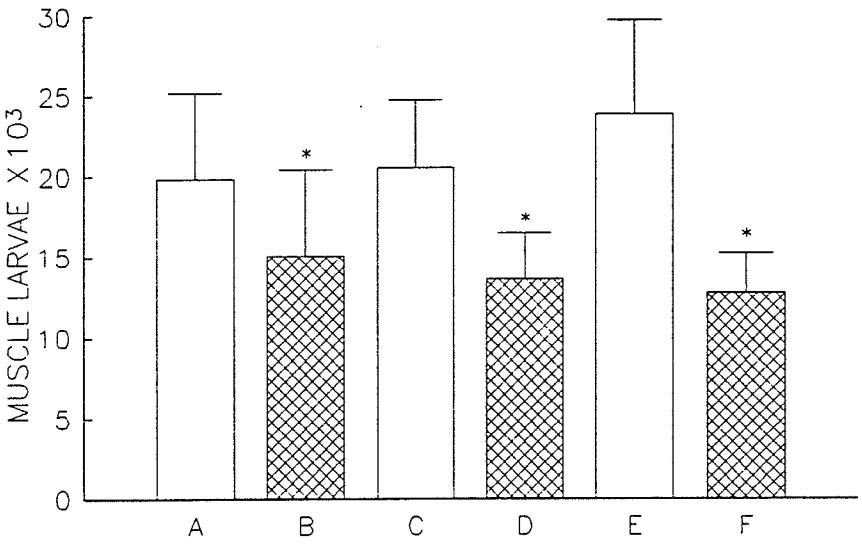

MUSCLE LARVAE X 10³
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Table 9. The effect of oral immunization on total muscle larvae recovery, and the adjuvant effect of CT for fed *Trichinella* antigen.

Treatment[a]	Muscle Larvae x 10^3	Percent of Control
Control	16.1±7.6	
SA- 50 μg	8.6±0.9	53
SA- 500 μg	10.4±0.3	65
CT - 10 μg	16.8±9.2	104
SAC - 50 μg	8.5±1.0	53
SAC - 500 μg	3.3±1.5	20

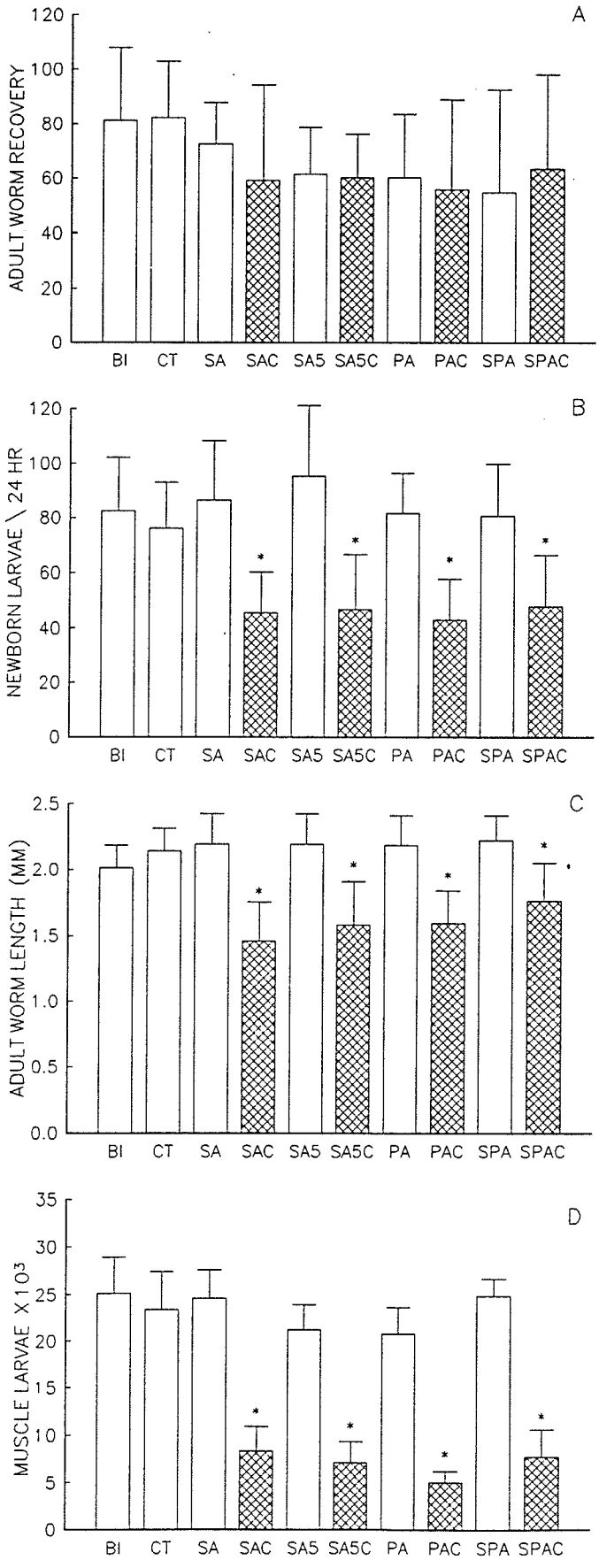
[a] Treatment designations were as follows: Control = mice fed saline; SA mice fed 50 or 500 μg soluble antigen; CT = mice fed 10 μg cholera toxin; SAC = mice fed 50 or 500 μg SA + CT. n = 2 mice per treatment

treatments in the number of adult worms recovered from the gut regardless of the antigen type or the presence/absence of the CT (Fig. 13A). In contrast, the fecundity of adult female worms, determined by *in vitro* larvae release, was reduced by ~50% in mice fed soluble, particulate or soluble/particulate antigens in combination with CT. Mean fecundity in these treatments was significantly lower ($p<0.05$) than fecundity in control mice fed CT or buffer alone and challenged with larvae (Fig. 13B) and also significantly lower than fecundity in treatments fed soluble, particulate, or soluble/particulate antigens without CT. The fecundity in these latter treatments was not significantly ($p<0.05$) lower than fecundity in control mice (Fig. 13B).

Adult female worm size was also significantly ($p<0.05$) reduced (20-30%) in mice fed soluble, particulate or soluble/particulate antigens with CT compared with control treatments. These observations contrast with mean worm size in mice fed these antigens without CT, which did not differ significantly ($p<0.05$) from control mice (Fig. 13C). A qualitative assessment of the adult worms following 24 hr in culture in the *in vitro* larval release assay indicated that the majority of worms from mice exposed to soluble, particulate, or soluble/particulate antigens with CT were type 0 and type 1 worms while in control treatments or in treatments fed antigen without CT the majority were type 2.

Figure 13. Effects of oral immunization in mice fed *Trichinella* antigen on Days 0, 14, 21 and challenged with *Trichinella* larvae on Day 28.

Mice were sampled on Day 6 following larval challenge to determine A) Adult worm recovery B) fecundity and C) worm length, and on Day 40 following larval challenge to determine D) the mean of total muscle larvae. Bars represent mean $\pm$ standard deviation for adult worm recovery, *in vitro* larval release, adult worm length and the mean of total muscle larvae respectively. Treatment means were compared with the BI control group by t-tests, and significant differences ($p<0.05$) from BI are indicated by an asterix (*). n = 8 mice per treatment, except BI and CT for which n = 4. Treatment designations were as follows: BI= bicarbonate buffer, no antigen; CT = 10 μg cholera toxin; SA = 1 mg soluble antigen; SAC= SA + CT; SA5= 0.5 mg SA; SA5C= SA5 + CT; PA= 1 mg particulate antigen; PAC= PA + CT; SPA = 1 mg soluble/particulate antigen; SPAC= SPA + CT.


A
ADULT WORM RECOVERY
120
100
80
60
40
20
0
BI CT SA SAC SA5 SA5C PA PAC SPA SPAC
B
NEWBORN LARVAE \ 24 HR
120
100
80
60
40
20
0
*
BI CT SA SAC SA5 SA5C PA PAC SPA SPAC
C
ADULT WORM LENGTH (MM)
2.5
2.0
1.5
1.0
0.5
0.0
*
BI CT SA SAC SA5 SA5C PA PAC SPA SPAC
D
MUSCLE LARVAE X 103
35
30
25
20
15
10
5
0
*
BI CT SA SAC SA5 SA5C PA PAC SPA SPAC

The mean of total muscle larvae was determined on Day 40 following challenge with *Trichinella* larvae. The means of total muscle larvae in treatments immunized with soluble, particulate or soluble/particulate antigens with CT were reduced by ~75% which was significantly lower ($p<0.05$) than the mean of total muscle larvae in control mice (Fig. 13D) or mice fed soluble, particulate, or soluble/particulate antigens without CT. The latter did not differ significantly ($p<0.05$) from control mice (Fig. 13D). The lowest mean of total muscle larvae amongst treatments without CT occurred in the particulate antigen treatment while the treatment with particulate antigen with CT had the lowest mean for all treatments, though this was not significantly lower than the other treatments which included CT.

Anti-Trichinella immunoglobulin response

The *Trichinella* specific immunoglobulin response was determined 7 days after the third oral immunization (ie. Day 28). Titers were considered positive as follows: bile >250; LUM >30; serum >50. A positive response was detected in the bile of 1 mouse fed soluble antigen without CT (Table 10). Positive bile and LUM responses were detected in only 2 of 14 mice following oral immunization with soluble, particulate or soluble/particulate antigens with CT (Table 10). A positive serum IgG response was detected in 5 of 20 mice fed *Trichinella* soluble, particulate or soluble/particulate

Table 10. Mucosal and serum immunoglobulin responses to Trichinella antigen determined 7 days after final antigen feeding in mice fed combinations of antigen and cholera toxin.

TREATMENT	BILE IgA	LUM IgA	SERUM IgG
BI[a]	<2 (0/4)	<1 (0/4)	<1.39 (0/4)
SA	<2 (0/4)	<1 (0/4)	1.62±0.45 (1/4)
SAC	<2 (0/3)	<1 (0/3)	2.20±0.92 (2/3)
SA5	<2 (0/4)	<1 (0/4)	1.46±0.15 (1/4)
SA5C	2.30±0.60 (1/4)	1.12±0.23 (1/4)	2.60±1.10* (3/4)
PA	<2 (0/4)	<1 (0/4)	1.69±0.60 (1/4)
PAC	2.77±0.56 (1/3)	1.46±0.66 (1/3)	3.10±1.49* (2/3)
SPA	2.08±0.15 (1/4)	<1 (0/4)	1.99±0.74 (2/4)
SPAC	<2 (0/4)	<1 (0/4)	1.87±0.41* (3/4)

Note. Mice were immunized on Days 0, 14 and 21 and sampled on Day 28. Results are presented as geometric mean titer ± standard deviation. Significant differences ($p<0.05$) from control mice fed no antigen were determined by t-tests and are indicated by *. The number of positive samples in each treatment are included in brackets.

[a] Treatment designations are: BI = Bicarbonate buffer, no antigen; SA = 1 mg soluble antigen; SAC = SA + 10 µg CT; SA5 = 0.5 mg SA; SA5C = SA5 + 10 µg CT; PA = 1 mg particulate antigen; PAC = PA + 10 µg CT; SPA = 1 mg soluble/particulate antigen; SPAC = 1 mg SPA + 10 µg CT.

antigen without CT, or control mice fed no antigen, while positive responses were detected in 10 of 14 mice fed soluble, particulate, or soluble/particulate antigen with CT (Table 10). The serum IgG titers in mice fed 0.5 mg soluble antigen or particulate antigen with CT were significantly higher ($p<0.05$) than titers in mice fed these antigens without CT (Table 10).

On Day 6 following challenge with *Trichinella* larvae, the IgA titer in bile and LUM samples from mice fed soluble, particulate or soluble/particulate antigen without CT did not differ significantly ($p<0.05$) from the response in control mice or mice fed CT alone (Table 11). The mean bile IgA titer in mice fed soluble, particulate, or soluble/particulate antigen with CT and challenged with larvae was approximately 10 fold higher ($p<0.05$) than in control mice, or mice fed these *Trichinella* antigens without CT and challenged with larvae (Table 11). The LUM response was also enhanced, but not to the same degree (Table 11). Positive responses following infection were detected in bile samples from 27 of 31 and LUM samples from 19 of 31 mice fed soluble, particulate, or soluble/particulate antigens with CT compared with 1 of 40 positive bile samples and 0 of 40 positive LUM samples from mice fed these different antigens without CT, fed CT alone, or not fed antigen (Table 11).

Table 11. Mucosal and serum anti-*Trichinella* immunoglobulin responses on Day 6 following challenge with larvae in mice orally immunized with combinations of *Trichinella* antigens and cholera toxin.

TREATMENT	BILE IgA	LUM IgA	SERUM IgG
BI[a]	2.16±0.11 (0/4)	<1 (0/4)	<1.39 (0/4)
CT	2.02±0.05 (0/4)	<1 (0/4)	<1.39 (0/4)
SA	2.11±0.23 (1/8)	<1 (0/8)	<1.39 (0/8)
SAC	3.04±0.51* (7/8)	1.30±0.51 (3/8)	2.18±0.65* (6/8)
SA5	2.11±0.23 (0/8)	<1 (0/8)	1.61±0.38 (2/7)
SA5C	3.5±0.86* (7/8)	1.43±0.49 (4/8)	2.15±0.69* (5/8)
PA	2.19±0.16 (0/8)	<1 (0/8)	2.03±0.31* (3/8)
PAC	3.07±0.45* (6/7)	1.54±0.40 (6/8)	2.90±0.88* (7/8)
SPA	2.16±0.14 (0/8)	<1 (0/8)	<1.39 (0/8)
SPAC	3.01±0.68* (7/8)	1.65±0.50 (6/8)	2.64±0.79* (7/8)

Note. Mice were immunized on Days 0, 14, and 21, challenged with *Trichinella* larvae on Day 28, and sampled on Day 6 following challenge. Results are presented as geometric mean titer ± standard deviation. Significant differences ($p<0.05$) from control mice fed no antigen were determined by t-tests and are indicated by *. The number of positive samples in each treatment are included in brackets.

[a] Treatment designations are: BI = Bicarbonate buffer, no antigen; CT = 10 μg cholera toxin; SA = 1 mg soluble antigen; SAC = SA + CT; SA5 = 0.5 mg SA; SA5C = SA5 + CT; PA = 1 mg particulate antigen; PAC = PA + CT; SPA = 1 mg soluble/particulate antigen; SPAC = 1 mg SPA + CT.

On Day 6 PI the serum IgG titer in mice fed soluble, particulate, or soluble/particulate antigen with CT and challenged with *Trichinella* was also significantly greater than the titer from challenged control mice (Table 11). In mice fed these antigens without CT, the response in soluble and soluble/particulate antigen treatments did not differ from controls, but the titer in the particulate antigen treatment was significantly ($p<0.05$) enhanced (Table 11).

Serum IgG titers increased following infection in all treatments, through the course of the experiment (Fig. 14). On Day 12 post infection and throughout the experiment, serum IgG titers were significantly higher ($p<0.05$) in mice fed soluble, particulate, or soluble/particulate antigens with CT compared with control mice (Fig. 14). In mice fed soluble or soluble/particulate antigens without CT and challenged with *Trichinella*, titers were not significantly different from control mice which were not immunized prior to challenge. In contrast, mice fed particulate antigen without CT had a significantly enhanced IgG response compared with control mice. This difference was detectable at Day 6 post infection (Table 11) and continued throughout the experiment (Fig. 14). Serum results at Day 40 post infection were similar to Day 24 (data not shown).

Figure 14. Serum IgG responses to *Trichinella* antigen on Days 12, 18, and 24 following challenge infection with *Trichinella* larvae in mice fed antigen on Days 0, 14 and 21 and infected on Day 28.

Bars represent geometric mean $\pm$ standard deviation of the anti-*Trichinella* IgG titer. Treatment means were compared with the BI control by t-tests, and significant differences ($p<0.05$) from BI are indicated by an asterix (*). n = 8 mice per treatment, except BI and CT for which n = 4. Treatment designations were as follows: BI = bicarbonate buffer, no antigen; CT = 10 μg cholera toxin; SA = 1 mg soluble antigen; SAC = SA + CT; SA5 = 0.5 mg SA; SA5C = SA5 + CT; PA = 1 mg particulate antigen; PAC = PA + CT; SPA = 1 mg soluble/particulate antigen; SPAC = SPA + CT.

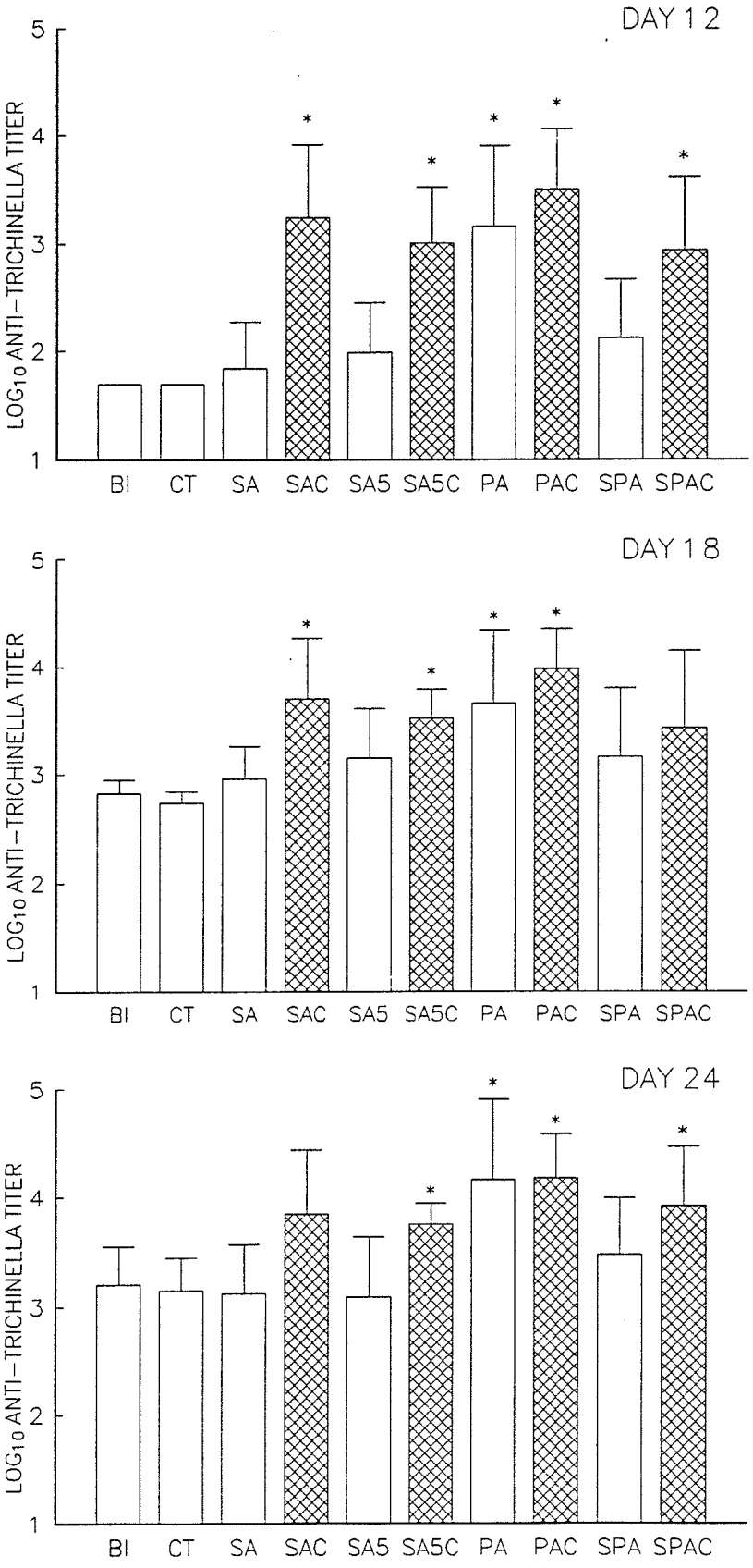

DAY 12
DAY 18
DAY 24
LOG10 ANTI-TRICHINELLA TITER
5
4
3
2
1
*
BI
CT
SA
SAC
SA5
SA5C
PA
PAC
SPA
SPAC

Table 12. Mucosal anti-CT titers in mice fed combinations of Trichinella antigen and CT.

	DAY 28*		DAY 34**	
	BILE	LUM	BILE	LUM
CT	nd.+	nd.	2.68±0.13++	1.48±0.34
SAC[a]	2.39	1.16±.23	3.05±0.47	1.36±0.52
SA5C	3.07±0.14	1.95	3.22±0.55	1.74±0.60
PAC	2.79±0.48	1.81±0.61	3.24±0.49	1.71±0.53
SPAC	3.07±0.25	1.72±0.41	2.94±0.46	1.90±0.44

Titers were determined 7 days after the third antigen feeding (Day 28) and 6 days after infection with 150 Trichinella (Day 34).
* n = 4. ** n = 8.
[a] Treatment designations were as follows: CT = 10 μg cholera toxin; SAC = 1 mg soluble antigen +10 μg; SA5C = 0.5 mg SA +CT; PAC = 1 mg particulate antigen + CT; SPAC = 1 mg soluble/particulate antigen + CT.
+ nd. = not determined.
++ value = geometric mean titer ± S.D.

Anti-CT immunoglobulin response

No cross reactivity was detected between *Trichinella* antigens and CT. The mucosal response in mice sampled on Days 28 and 34 (6 days after infection with *Trichinella*) is summarized in Table 12. On Day 28, 10 of 13 bile samples and 11 of 14 LUM samples were positive and on Day 34, 31 of 35 bile and 25 of 36 LUM samples were positive. The mucosal response, though variable, did not differ significantly between antigenic treatments or between Days 28 and 34 except for the soluble antigen + CT (SAC) combination in which the Day 28 bile response was low. The serum IgG response to CT on Days 28 and 34 (Table 13) did not differ significantly between treatments or days. Titers remained essentially unchanged in mice sampled throughout the course of the experiment to Day 68 (Table 13).

Table 13. Serum IgG titer of anti-CT antibodies in mice fed combinations of Trichinella antigen and cholera toxin determined 7 days after antigen feeding (Day 28) and at intervals following infection.

	Day following initial antigen exposure					
Treatment*	28[a]	34	40	46	52	68
CT[a]	nd.[+]	3.35±0.26[++]	3.13±0.44	3.18±0.40	3.13±0.44	3.13±0.44
SAC	2.9±0.65	3.09±0.42	3.53±0.22	3.60±0.62	3.40±0.47	3.46±0.45
SA5C	3.35±0.14	3.01±0.30	3.55±0.26	3.53±0.29	3.64±0.42	3.49±0.29
PAC	3.40±0.14	3.43±0.20	3.81±0.40	3.60±0.44	3.64±0.40	3.73±0.46
SPAC	3.35±0.26	3.35±0.15	3.66±0.25	3.46±0.37	3.45±0.54	3.24±0.34

* n = 7 to 8 except where noted. [a] n = 4.
+ nd = not determined.
++ Geometric mean titer ± S.D.
[a] Treatment designations were as follows: CT = 10 μg cholera toxin; SAC = 1 mg soluble antigen +10 μg; SA5C = 0.5 mg SA + CT; PAC = 1 mg particulate antigen + CT; SPAC = 1 mg soluble/particulate antigen + CT.

Discussion

The focus of this study was to determine the potential for oral immunization with *Trichinella* antigens and the adjuvant effect of CT for fed antigen and infection with *Trichinella* larvae. It is clear from these experiments that antigen feeding with CT enhances the immune response and protects the host.

CT Adjuvancy

The adjuvant effect of CT for mucosal antigens was first described by Elson and Ealding (1984ab). In these studies feeding a combination of CT and KLH induced a serum and mucosal antibody response to KLH and reversed tolerance to KLH produced by feeding KLH alone. As outlined in the Introduction, this adjuvant effect has been utilized in a number of systems, though the means by which the response is enhanced remains unclear. CT has been shown to act synergistically with interleukins 4 and 5 *in vitro* to promote switch differentiation of B-cells and enhance intestinal IgA production (Lycke and Strober, 1989; Lycke *et al.*, 1990; Lycke *et al.*, 1991ab). The addition of CT in macrophage cultures also enhanced macrophage antigen presentation (Lycke *et al.*, 1989a) and this response was subsequently correlated with an increase in cell-associated interleukin 1-alpha (Bromander *et al.*, 1991). Similar results were obtained with splenic adherent cells as antigen presenting cells in a culture system (Hirabayashi *et al.*,

1992). It has also been demonstrated that oral exposure to CT and heterologous antigen results in a long term intestinal memory to the antigen which can be activated following challenge in the absence of CT (Vajdy and Lycke, 1992). Although these studies imply a specific cellular enhancement, the adjuvant effect of CT has also been attributed to an enhancement of intestinal permeability, as revealed by enhanced uptake of flourescently labelled dextran (Lycke *et al.*, 1991a). Regardless of the mechanism, it is clear that antigen feeding with CT results in an enhanced immunoglobulin and T cell response (Lycke and Holmgren, 1986; Clarke *et al.*, 1991).

Trichinella larvae plus CT

In this study an adjuvant effect of CT for *Trichinella* infections and fed antigens has been clearly established. Infections with larval *Trichinella* combined with CT resulted in an enhancement of the mucosal immunoglobulin response (Chapter 1) and a 36% reduction in muscle larvae compared with controls. The effect of CT on the murine response to live worm infections and the success of CT as an adjuvant in other systems (eg. influenza haemagglutinin (Hirabayashi *et al.*, 1991) and *Toxoplasma* extracts (Bourguin *et al.*, 1991)) suggested that CT might enhance the immune response of mice to *Trichinella* extracts. Since fed antigen would be more uniformly distributed along the intestine compared to larval infections which are known to be localized (Dick and Silver

1980) exposure to lymphoid tissue via Peyers patches would increase. Further, since CT enhanced intestinal permeability (Lycke *et al.*, 1991a) and increased antigen presentation (Bromander *et al.* 1991) it was hypothesized that oral immunization with *Trichinella* antigens plus CT would enhance the immune response.

Fed antigen plus CT

Effect on Trichinella

Oral immunization with different preparations of *Trichinella* antigen plus CT as an adjuvant resulted in a 20-30% reduction in worm size and a 50% reduction in fecundity on Day 6 and an ~75% reduction in the mean of total muscle larvae on Day 40. In contrast, mice fed *Trichinella* antigen without CT or fed CT alone did not show a decrease in worm size, fecundity or muscle larvae numbers. These results differ from reports on oral immunization with ES antigens of *Trichinella*, in which a significant reduction in muscle larvae and macrophage spreading index was observed (Vernes, 1976). Since antigen doses were similar, differences are likely not a function of concentration but may be attributed to differences in antigenicity between ES products and larval extracts. However, common antigenic determinants occur between these antigen preparations (Denkers *et al.*, 1990). In a subsequent study immunization with ES antigen produced an increase in intestinal mast cells numbers and altered eosinophilia (Tronchin *et al.*, 1979). However,

comparisons with the present study were not possible since muscle larvae numbers were not reported and no specific immune response was detected.

The decrease in fecundity, size, and muscle larvae numbers for *Trichinella* in the present study were comparable to results obtained following intra-peritoneal injection of antigens; worm size (Campbell, 1955), fecundity (Denkers *et al.*, 1990; Silberstein and Despommier, 1985ab; Zhu and Bell, 1990) and muscle larvae (Denkers *et al.*, 1990; Silberstein and Despommier, 1985ab) were reduced. Silberstein and Despommier (1985a) proposed that the reduction in muscle larvae was a direct result of a reduction in fecundity. The results above corroborate this observation since the high proportion of type 0 and 1 female worms in the present experiment indicate that larval production would be further reduced on subsequent days and therefore the overall reduction in larval production was likely > 50%.

Effects on adult worm numbers in the intestine are not as clear. Although a number of studies report an increased rate of expulsion following intra-peritoneal immunization (Campbell, 1955; Silberstein and Despommier, 1985a) others reported that the increase was dependent on host strain (Zhu and Bell, 1990) or did not occur (Denkers *et al.*, 1990). The separation of the size and fecundity effects from expulsion in the present experiment supports observations in Chapter 2 in which these two variables were dissociated in BALB/c mice

and other strains. Furthermore, as in the previous chapter, effects on size and fecundity were highly correlated with the mucosal IgA response.

Anti-*Trichinella* immunoglobulin response

Following oral immunization and/or infections, the mucosal and systemic immunoglobulin response was determined as an indicator of host responsiveness. The mucosal IgA titer prior to infection with larvae (Day 28), was lower than that reported for the defined antigens KLH or OVA (Elson and Ealding, 1984b; Wilson *et al.*, 1990) but was comparable to the results reported for *Toxoplasma* (Bourguin *et al.*, 1991). This difference is not surprising since the adjuvant effect of CT is related to concentration of the antigen (Wilson *et al.*, 1989). In this study, crude extracts were used at 0.5 and 1 mg (ie. pure antigenic components would be in the ng range) compared with 5-25 mg of purified antigen used in experiments with OVA and KLH (eg. Elson and Ealding, 1984; Wilson *et al.*, 1989,1990). A significant increase in titer occurred after challenge with larvae in orally immunized mice. Clearly mice fed *Trichinella* antigen with CT were primed, and the absence of a similar response in mice fed antigen without CT indicated that CT functioned as an adjuvant. Furthermore, a significant reduction in the mean of total muscle larvae occurred in a preliminary experiment in which mice were fed 50 μg of soluble antigen with CT (10 μg). This indicates that the level of antigen

exposure required to produce protective immunity does not necessarily result in the production of a detectable specific mucosal antibody response prior to a challenge infection with *Trichinella* larvae. Furthermore, the concentration of purified antigen required to prime a protective response in mice is in the μg range, similar to the concentration used to immunize systemically (eg. Denkers *et al.*, 1990).

The enhanced systemic immunoglobulin response of mice fed antigen plus CT corroborates previous work with pure proteins and bacterial extracts (Elson and Ealding, 1984b; Wilson *et al.*, 1989; Russell and Wu, 1991). A significant enhancement of anti-*Trichinella* IgG in the serum occurred by Day 28 in mice fed different *Trichinella* antigens + CT, and was maintained throughout the experiment. The response was also enhanced in mice fed particulate antigen without CT in contrast to feeding with soluble and soluble/particulate preparations, which did not enhance the serum response. Why the particulate preparation is more antigenic is not clear. It is known that differences in the immunogenicity of proteins are related to their capacity to bind mucosal surfaces (De Aizpurua and Russell-Jones, 1988), but this binding capacity is not the only prerequisite for immunogenicity, since CTB which binds GM-1 receptors was less immunogenic than CT toxin which has the same binding capacity (Wilson *et al.*, 1990). Various other factors

including antigen size, shape, particulate nature and lipophilicity have been shown to affect intestinal uptake (O'Hagan *et al.*, 1987). Further characterization of the antigenic components in the crude extracts may resolve this issue.

Immune effectors

It is tempting to attribute the immune effects observed during the intestinal phase of infection and ultimately the reduction in muscle larvae, to the mucosal IgA response. *Trichinella* specific IgA isolated from the bile of infected rats caused a reduction in *in vitro* larval release (Jacqueline *et al.*, 1978) and diversion of the rat bile duct increased the length of the gut stage of infection (Jacqueline *et al.*, 1981). Despite the strong correlation, the data must be interpreted with caution. In addition to B-cells, mucosal 'defense' includes helper, cytotoxic and suppressor T-cells, a significant population of gamma-delta T cells, as well as a variety of nonspecific protective cell types. Since oral immunization with KLH and CT as adjuvant stimulated a specific T-lymphocyte response which included both $CD4^+$ and $CD8^+$ cells (Clarke *et al.*, 1991), factors in addition to the mucosal immunoglobulins may have a role in producing protection. Further, results from feeding particulate antigen indicate that the systemic immunoglobulin response does not influence the muscle larvae burden. Particulate antigen without CT enhanced the systemic

response but the mean of total muscle larvae was not significantly different from the other antigen treatments without CT in which the serum response was not enhanced. It appears that the response we observed and the biological effects of exposure to P1 antigen plus CT result from a combination of lymphocyte and granulocyte effectors, of which IgA is only one.

The anti-CT response

The response to CT was measured in this study as a positive control for oral immunization. In addition, differences in the response to CT in combination with antigen could provide information on immune interaction. As indicated in Chapter 1 and summarized in Appendix 1, in some combinations, concurrent exposure to *Trichinella* and CT resulted in suppression of the immune response to CT. One hypothesis for the immunosuppressive effects of infection is the secretion of immunosuppressive molecules by the parasite (Ljungstrom *et al.*, 1980). A similar hypothesis for suppression of the anti-PC response has been suggested (Baltar *et al.*, 1991). In the present study the serum and mucosal anti-CT titers were similar to previous reports (Wilson *et al.*, 1990; Schmucker *et al.*, 1988) and the serum IgG anti-CT titer, though variable, did not differ significantly among treatments or with time. The anti-CT response of mice fed CT alone was not significantly different from the response of mice fed CT and *Trichinella*

antigen. Therefore, there was no evidence for immunosuppression by any of the three extracts fed in this study.

It is apparent from this study that the mucosal adjuvant properties of CT can be exploited to produce protection against infection with a gastro-intestinal helminth in association with a 10 fold increase in specific mucosal IgA. However, the mechanism of protection is not clear. While the production of an IgA response is indicative of stimulation of the Th2 T cell pathway, other cell types may also be stimulated. The *Trichinella*/CT system provides a model to assess this mechanism. Furthermore, since CT also has adjuvant properties for non protein antigens, this system provides a model to assess the oral vaccine potential of other components, including the carbohydrates.

Chapter 4. The antiphosphorylcholine response

Abstract

The presence of the PC determinant in *Trichinella* and the influence of *Trichinella* infection on the anti-PC immunoglobulin response were determined. The PC specific IgA myeloma, TEPC-15, bound *Trichinella* extracts in ELISA and Western blot experiments. These reactions were inhibited in the presence of 1mM phosphorylcholine chloride (PCCl), indicative of the presence of PC in *Trichinella*. Cross reactivity of serum and mucosal samples with *Asperigillus* extracts and the synthetic conjugate PC-BSA provided evidence for an anti-PC response in mice infected with *Trichinella*. The kinetics of the serum and mucosal anti-PC immunoglobulin response were determined following infection. Serum IgA, though positive for PC was a minor fraction of the serum response. In primary infections a small proportion of the IgG response (up to 25%) was PC positive, but following challenge this proportion dropped to <5%. The PC specific proportion of serum IgM peaked near 70% in the primary and 60% following challenge. The bile anti-PC IgA response was similar to serum IgM, peaking at near 60% in the primary, but dropping to ~30% following challenge. The mucosal and serum response was also determined in the 6 mouse strains described in Chapter 2. Although differences occurred between strains, an MHC influence was not apparent.

Introduction

Antigens recognized in the immune response are not limited to enzymatic or structural proteins. This is illustrated by the carbohydrate antigens for which an increasingly important role has been described in bacterial, protozoan and helminth infections. This is also the case for *Trichinella* in which a carbohydrate antigen is the dominant epitope after the fourth week of infection in mice and men (Denkers *et al.*, 1990). Another epitope which has been the subject of considerable attention is phosphorylcholine.

PC is a ubiquitous immunogenic determinant present in a diversity of infectious agents (Potter, 1972; Lal and Ottesen, 1989). Several observations contributed to the discovery of the antigenicity of PC. The C-polysaccharide extract of *Pneumococcus* was shown to precipitate with myeloma derived antibodies (Cohn, 1967) and the subsequent identification of PC as a component of C-polysaccharide (Brundish and Bailey, 1968) and inhibition of the C-polysaccharide/myeloma protein precipitation reactions with PCCl (Leon and Young, 1970; Potter and Lieberman, 1970) identified PC as an immunogenic determinant. Soon after it was recognized that a number of independent PC specific myelomas from BALB/c mice (Leon and Young, 1971) expressed a common (T15) idiotype (Potter and Lieberman, 1970) and this led to an interest in the regulation of the response to PC.

Precipitation of extracts of *Lactobacillus* (Potter and

Lieberman, 1970), fungal determinants from *Aspergillus* (Potter, 1972) and extracts of the nematode *Ascaris* (Potter, 1970; Crandall and Crandall, 1971) revealed a common antigenic determinant which was identified as PC by inhibition of precipitation with PCCl. In addition to *Ascaris*, PC determinants were reported from numerous parasitic species including the nematodes *Nippostrongylus brasiliensis* and *Haemonchus contortus* (Pery *et al.*, 1974, 1979a,b), *Trichinella spiralis* and *Heligmosomoides polygyrus* (Brown and Crandall, 1976), *Dipetalonema viteae* (Gualzata *et al.*, 1986), *Strongyloides ratti* and *Toxocara canis* (McWilliam *et al.*, 1986) as well as the trematode *Fasciola hepatica* (Sloan *et al.*, 1991) and a variety of protozooans, trematodes and nematodes (Lal and Ottensen, 1990).

Although PC has been reported from a large number of parasites, the antibody response to this determinant has been studied in only a few. In *Ascaris* infections a T-independent, T15 idiotype restricted serum anti-PC IgM response occurred which had similar kinetics in both primary and challenge infections (Crandall and Crandall, 1971; Brown and Crandall, 1976; Mitchell *et al.*, 1976). Following infection with *Trichinella* the anti-PC titer was lower than for *Ascaris* (Brown and Crandall, 1976). Plaque assay studies indicated a peak of anti-PC B cells at Day 12 following infection with *Trichinella*, which subsequently declined (Ubeira *et al.*, 1987a). Infection with *Nippostrongylus*

produced a serum IgM and lower IgG anti-PC response and an intestinal IgA response which occurred in adult rats but only in primary infections (Pery *et al.*, 1975; Brown *et al.*, 1981a,b). The tissue distribution of PC was similar in these species, occurring internally, but not on the cuticle (Crandall and Crandall, 1971; Gutman and Mitchell, 1977; Pery *et al.*, 1979b; Choy *et al.*, 1991).

In contrast to the response following parasitic infection, the systemic response to PC-protein conjugates has been described in detail. The response to PC-protein is comprised of a number of independent immunoglobulin types designated group I and group II antibodies. Group I and II immunoglobulins differ in isotype distribution, idiotype, kinetics and specificity for PC (Chang *et al.*, 1982a,b,1984), which in combination indicates independent regulation. Group I antibodies were PC specific, predominantly of the IgM class and had similar kinetics in primary and challenge exposure, in contrast to the group II antibodies which were specific for the PC-nitrophenyl linkage, were anamnestic following challenge exposure and were predominantly of the IgG class (Chang *et al.*, 1982a,b,1984). However, the mucosal response to PC-protein has not been studied.

While it is apparent that PC antigens induce a response, the role of this response in immunity to infections is controversial. Szu *et al.*, (1983) reported

that the anti-PC response was effective in protecting against pneumococcal infection. This was corroborated by Briles *et al.*, (1989) who identified a series of PC specific monoclonal antibodies which could transfer protection to infection. Lim and Choy (1990) reported that following injection with a T-independent form of PC-positive *Trichinella* antigen, mice were protected from infection with *Pneumococcus*. Furthermore, Pery *et al.*, (1975) reported that mice with a strong anti-IgA response were resistant to infection with *Nippostrongylus*. However, Mitchell *et al.*, (1976) found no correlation between the level of the anti-PC response and resistance to infection with *Ascaris*. More recently, it was also reported that the induction of tolerance to PC-BSA enhanced resistance to infection with *Fasciola* (Sloan *et al.*, 1991), which led to the suggestion that an anti-PC response may be detrimental rather than beneficial.

It is clear that despite considerable research effort, questions and controversies remain regarding the antigenicity of, and immune response to PC. These include the mechanisms of isotype and idiotype regulation of anti-PC antibodies, the biochemical nature of helminth PC, the role of T-cells in the response to PC, or the protective function of an anti-PC response. With the exception of *Nippostrongylus*, information on the mucosal anti-PC response is lacking. In this context the objectives of the following

study were i) to determine the presence of PC in *Trichinella*, ii) to assess the serum and mucosal immune response to PC, and iii) to determine the influence of host genotype on the response to PC in mice infected with *Trichinella*.

Materials and Methods

Mice used in these experiments were outbred CFW mice (Charles River) and the inbred strains described in Chapter 2 (Table 5). The P1 isolate of *Trichinella* was used.

Prior to determining the anti-PC immunoglobulin response the presence of the PC determinant in *Trichinella* extracts was determined in PC specific ELISA experiments and by Western blotting as described below.

PC-ELISA and Western Blotting

The PC-specific antibody response following infection was assessed by a modification of the ELISA methods described in Chapters 1 and 2. Briefly, plates were coated o/n with 100 μl of the appropriate antigen in carbonate buffer (SPS, PC-BSA, *Trichinella* antigen: 5 μg/ml), washed and blocked. Dilutions of test samples (serum, bile, faeces) were prepared and 1mM PCCl was added to inhibit PC/anti-PC reactions. Subsequent steps were similar to the previous ELISA's. The proportion of the immunoglobulin response specific to PC was determined by comparison of the reaction in the presence and absence of PCCl.

The presence of PC was also determined by Western blotting. SDS-PAGE was performed by the methods of Laemmli (1970) with a Protean II apparatus (Biorad) and transfer to nitrocellulose membranes was as described by Towbin *et al.*, (1979) with a TransBlot apparatus (BioRad). Briefly, samples were prepared in buffer (80 mM Tris, 3% SDS, 15% sucrose,

0.001% Bromophenol Blue and 5% β-mercaptoethanol), denatured by boiling 3 min, and loaded on 0.75 mm 5-15% polyacrylamide gradient gels with a 3% stacking gel. Gel running buffer was 0.025 M Tris, 0.2M glycine, 0.1% SDS, and electrophoresis conditions were constant current (20 mA) for 3.5-4 hr. Gels were stained with Coomassie Brilliant Blue (0.2% CBB, 25% isopropyl alcohol, 10% glacial acetic acid), destained (25% isopropyl alcohol, 10% glacial acetic acid) and photographed. For protein transfer, following electrophoresis gels were equilibrated in protein transfer buffer (0.025 M Tris, 0.192 M Glycine) for 30 min, and transferred to nitrocellulose by electrophoresis at a constant voltage (70 V) for 2 hr. Blots were dried, blocked with 3% BSA in PBS for 2 hr, washed with PBS-T, incubated with TEPC-15 (1/5000 in 3% BSA) for 2 hr, washed with PBS-T, and incubated with alkaline phosphate conjugated goat anti-mouse IgA (1/1000 in 3% BSA) for 2 hr. Blots were washed and incubated in 9 ml alkaline phosphatase reaction buffer (0.1 M NaCl, 0.1 M Tris, 5 mM $MgCl_2$) + 1 ml nitro blue tetrazolium (1 mg/ml in H_2O) and 0.1 ml 5-bromo-4-chloro3-indolyl phosphate (5 mg/ml in dimethylformamide). Reactions were stopped by incubation in 0.1 M EDTA, and blots were dried and photographed.

The molecular weight of the PC-BSA conjugate was estimated by comparison with the standard. Molecular weight standards were plotted against acrylamide percentage

(determined by migration distance) on $\log_{10}$ graph paper. The molecular weight (MW) of PC-BSA was estimated by interpolation from the linear portion of this plot.

Asperigillus SPS

In initial experiments to determine the presence of anti-PC antibodies, a somatic polysaccharide (SPS) extract of *Asperigillus* was also used as a PC positive antigen for ELISAs. The SPS extract isolated by the hot phenol method of Westfhal *et al.*, (1952), has been shown to contain the PC determinant (Baldo and Fletcher, 1977). To eliminate the possibility of cross reactivity which could occur via binding of SPS epitopes other than PC, a specific protein-PC conjugate was synthesized as follows.

Synthesis and Characterization of PC-BSA

PC-BSA was synthesized by the method of Rifai and Wong (1986). In this reaction, p-aminophenylphosphorylcholine was converted to p-isothiocyanatophenyl phosphorylcholine by reaction with thiophosgene and this reagent was coupled directly to BSA. Thiophosgene was redistilled prior to use. In the first stage of this synthesis, 4 ml of 0.2 mM p-aminophenylphosphorylcholine (Sigma Chemical Co, St. Louis) in 0.1 M $NaHCO_3$ was mixed with 24 μl of thiophosgene in 5 ml of chloroform and stirred at RT for 1 hr. The solution was then transferred to a 15 ml centrifuge tube and the phases left to separate. The organic phase was discarded, and the aqueous phase was then re-extracted twice with 5 ml of fresh

chloroform to remove residual thiophosgene. The aqueous phase was then gently bubbled with N_2 for 15 minutes to remove residual chloroform and used immediately in the coupling reaction with BSA. The entire reaction product (~4 mls) was mixed with 5 mls of BSA (20 mg/ml in 0.3 M NaCl - 0.1 M $NaHCO_3$ pH 9.5) for 6 hr with gentle stirring at room temperature. The solution was separated on 10 ml Sephadex G25 desalting columns and the OD 280 positive fraction was then dialized for 48 hr against 4 x 4l of ddH_2O, lyophilized and stored at -70°C.

The presence of PC in the products of this synthesis was assessed as follows. The absorption spectrum (200-300nm) of the reaction products was determined with a Spectronic 601 (Milton Roy) spectrophotometer and compared with the BSA spectrum. Reaction products were then separated on 5-15% SDS-PAGE gels, transferred to nitrocellulose membranes, and probed with TEPC-15 (PC specific) myeloma protein.

Kinetics of the anti-PC response

The immunoglobulin subclasses and kinetics of the anti-PC immunoglobulin response were determined for serum and bile samples from CFW mice. Samples were taken from normal and infected mice. Forty mice were infected with 150 *Trichinella* on Day 0 and 4 mice were sampled on Days 6, 12, 18 and 28 of a primary infection. The remaining mice were challenged on Day 28 and 4 mice were sampled on Days 29, 30, 31, 34, 40 and 46. Serum and bile samples were obtained as

described in Chapter 2. The percentage of the anti-*Trichinella* response specific for the PC determinant was determined by comparing ELISA values (as described above) in the presence and absence of 1mM PCCl. For IgA determinations serum was diluted 1/10 and bile was diluted 1/250, while for IgG and IgM serum was diluted 1/1000.

Influence of host genotype

The serum and mucosal response to PC was also determined in the 6 strains of mice described in Chapter 2. The proportion of serum IgM and faecal IgA specific for PC were determined in ELISA by inhibition with PCCl as described above. The preparation of faecal extracts was as described in Chapter 1 and undiluted faecal samples were used to determine the PC response.

Results

PC-BSA synthesis

A number of methods were used to assess the synthesis of PC-BSA. The UV spectrum (200-300 nm) of PC-BSA differed from the BSA spectrum, having significantly greater absorbance in the 200-300 nm range. A PC specific response was detected in ELISA tests with TEPC-15, which was inhibited with 1mM PCCl. SDS-PAGE analysis revealed an increase in apparent MW of PC-BSA compared with BSA (Fig. 15). PC-BSA occurred as a broad band with an approximate MW of 75 KD. Based on an estimated MW of 320 per PC molecule and a 9 KD increase in MW of PC-BSA relative to BSA, the BSA molecules are substituted with an average of 27 PC molecules. PC-BSA was detected with TEPC-15 following Western transfer to nitrocellulose while no reaction occurred with BSA (Fig. 15). These experiments also revealed higher MW PC-positive proteins which likely are BSA complexes produced in the reaction.

PC in Trichinella extracts

The presence of PC in *Trichinella* extracts was initially detected by ELISA with TEPC-15 which was inhibited with PCCl (Table 14). It was also detected in Western blots of *Trichinella* antigen probed with TEPC-15 in which a number of PC specific bands occurred (Fig. 15).

Figure 15. Photograph of 1) SDS-PAGE separation of PC-BSA and *crude Trichinella* antigen on a 5-15% gradient gel, and 2) Western blot of the above probed with TEPC-15.

Designations were as follows: H = high molecular weight marker (Approximate molecular mass in KD: 205, 116, 97, 66, 45, 29); L = low molecular mass marker (KD: 97, 66, 45, 31, 21, 14); A = 3μg BSA; B = 1.5 μg BSA + 1.5 μg PC-BSA; C = 3 μg PC-BSA; D = 3 μg crude *Trichinella* antigen.

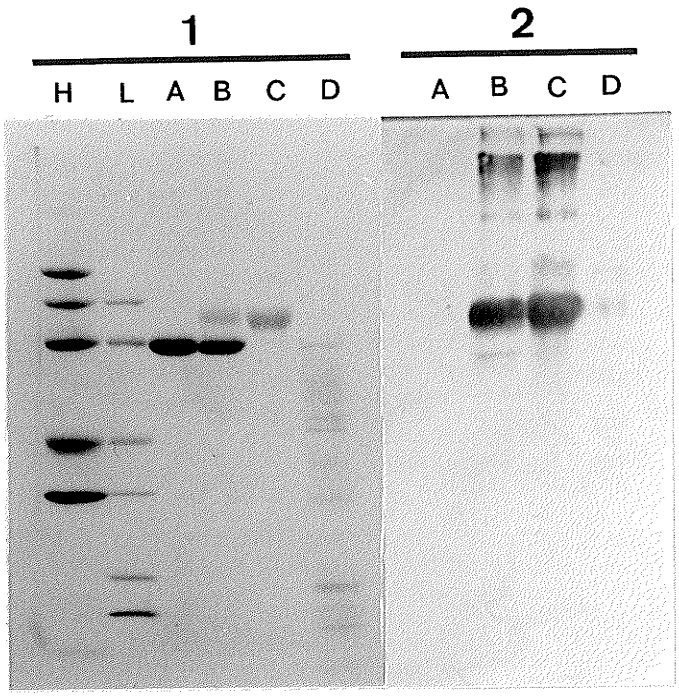

1
2
H
L
A
B
C
D
A
B
C
D

Table 14. PC specific crossreactivity of TEPC-15 and immunoglobulins from mice following infection with *Trichinella*.

		Antigen		
Antibody	PCCl	*Asperigillus*	PC-BSA	*Trichinella*
TEPC-15	-	1.84±0.01	0.93±0.02	1.44±0.05
TEPC-15	+	0.08±0.03	0.04±0.01	0.05±0.07
Trichinella				
Serum IgM	-	1.00±0.03	0.84±0.26	1.22±0.06
Serum IgM	+	0.357±0.01	0.24±0.13	0.55±0.02
Bile IgA	-	0.38±0.03	0.36±0.01	1.14±0.48
Bile IgA	+	0.11±0.06	0.04±0.04	0.71±0.26
Faecal IgA	-	0.57±0.07	0.61±0.25	0.36±0.11
Faecal IgA	+	0.13±0.05	0.07±0.03	0.20±0.14

Antibody response reported as mean OD 490 ± standard deviation of ELISA results in presence and absence of PCCl.

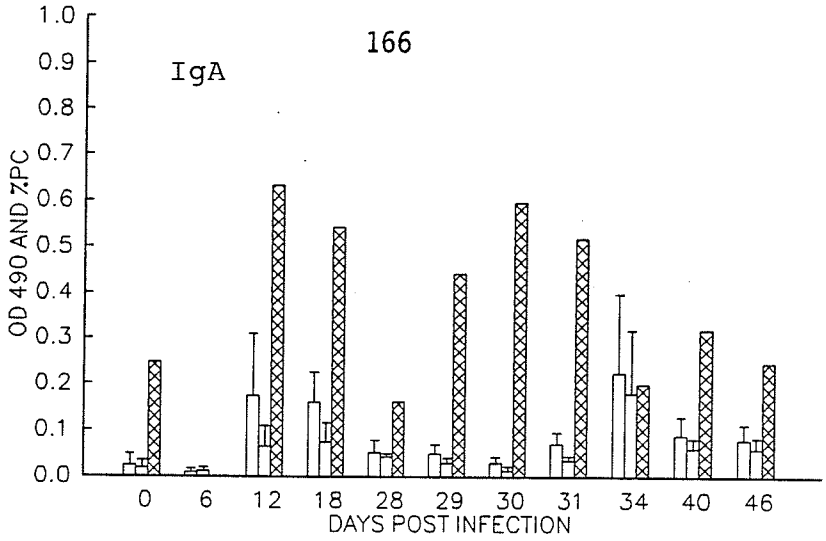
Anti-PC immunoglobulin response

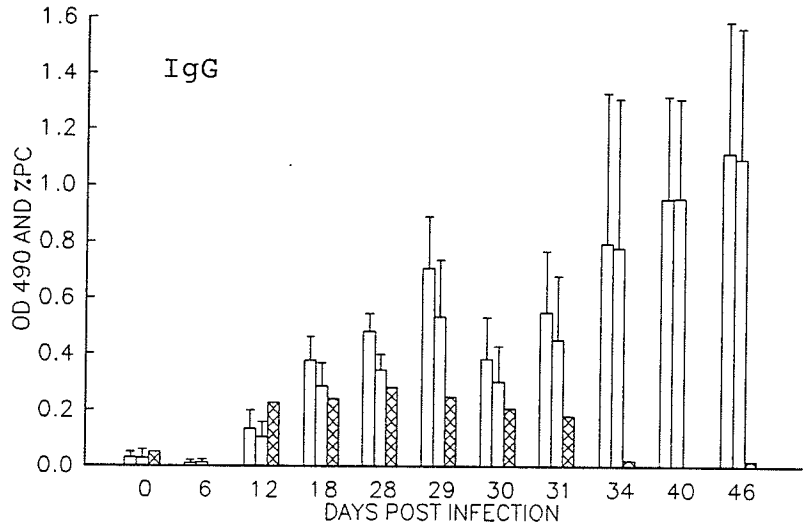
Initial experiments to establish the production of an anti-PC immunoglobulin response in mice infected with *Trichinella* were based on cross reactivity of anti-*Trichinella* antibodies with PC rich antigen preparations from *Asperigillus*. Normal and immune serum, bile and faecal extracts were tested in ELISA for cross reactivity with these antigens and the effects of the addition of PCCl as inhibitor (Table 14). In further experiments the PC-specific substrate PC-BSA (described above) was used as the target antigen in ELISA's. These results indicate a PC specific response which was inhibited by PCCl (Table 14).

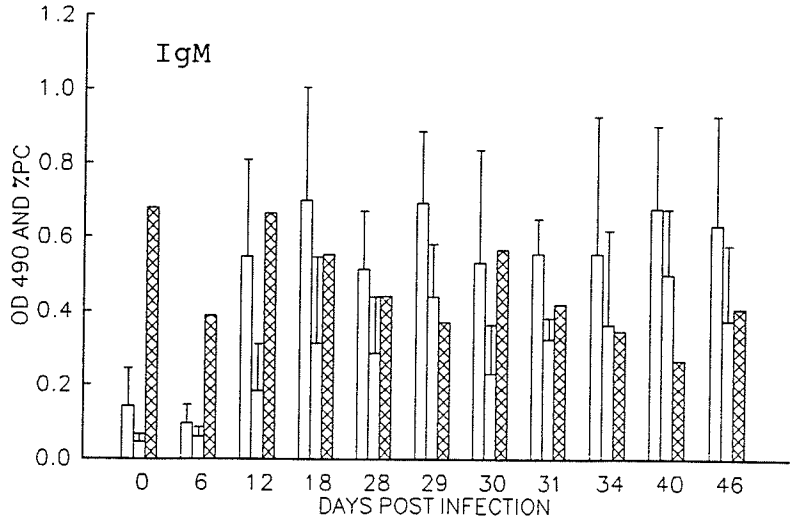
Having established that infection with *Trichinella* resulted in the production of anti-PC antibodies, subsequent experiments were designed to determine the anti-PC immunoglobulin subclasses and the kinetics of anti-PC antibody production. In the serum anti-PC response, PC specific antibodies occurred in the three subclasses tested (Fig. 16). For serum IgA, up to 50% of *Trichinella* specific response was inhibited by PCCl (Fig. 16) in both primary and challenge infections. However, IgA accounts for only a minor fraction of the serum response as indicated by low absorbance values (OD 490) compared with the IgG and IgM samples which were diluted 100-fold more. In uninfected mice serum IgG anti-PC was negligible.

Figure 16. Serum IgA, IgG, and IgM response to PC over time in mice infected with *Trichinella*. Mice were challenged on Day 28.

For each day the first and second bars (open) equal the mean ± standard deviation of the anti-*Trichinella* response of undiluted faecal samples in the absence and presence of PCCl respectively. The hatched bar x100 equals the percentage difference in the response as a measurement of the proportion of the response due to PC.


IgA
OD 490 AND %PC
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 6 12 18 28 29 30 31 34 40 46
DAYS POST INFECTION


IgG
OD 490 AND %PC
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 6 12 18 28 29 30 31 34 40 46
DAYS POST INFECTION

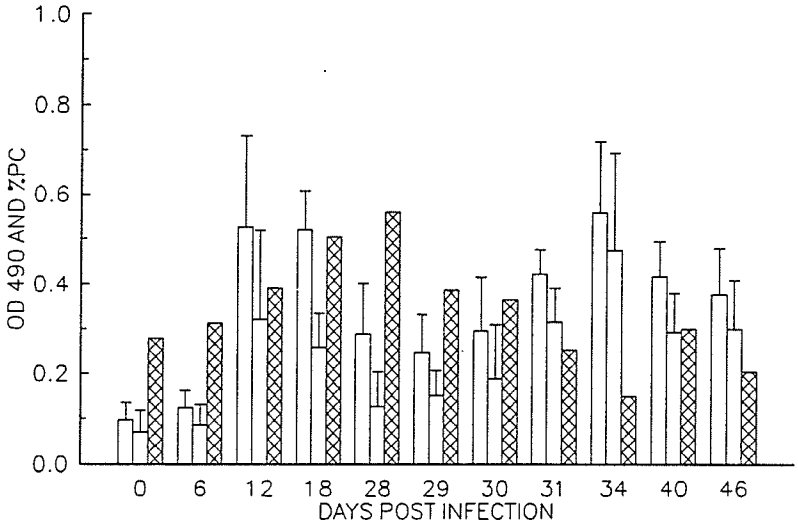

IgM
OD 490 AND %PC
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 6 12 18 28 29 30 31 34 40 46
DAYS POST INFECTION

Following infection the IgG anti-PC response was a minor component of total anti-*Trichinella* antibody, peaking at ~25% by Day 28 of the primary infection (Fig. 16). Following challenge infections the anti-PC response dropped to <5% of the total IgG anti-*Trichinella* antibody by Day 6. For IgM, prior to infection anti-PC antibodies accounted for 65% of the cross reactivity with *Trichinella* antigen (Fig. 16). In primary infections the IgM anti-PC response peaked by Day 12 at approximately 65% of the total anti-*Trichinella* response, then declined to ~35% by Day 28 (Fig. 16). Following challenge the anti-PC proportion increased but not to the level observed in the primary infection (Fig. 16). Prior to infection, anti-PC antibodies accounted for approximately 25% of the background cross reactivity of bile IgA (Fig. 17). Following infection the proportion rose to ~60% by Day 28. Following challenge infections there was an initial decrease to Day 6 post challenge (Day 34) and subsequently a slight increase by Day 40, but not to the level observed in primary infections (Fig. 17).

The faecal anti-PC immunoglobulin response was determined over time for a number of strains of mice (listed in Chapter 2) infected with *Trichinella*. The proportion of the response specific for PC was determined by inhibition studies with PCCl (Fig. 18). The different strains had some features in common.

Figure 17. The bile IgA response to PC in mice infected with *Trichinella spiralis*. Mice were challenged on day 28.

For each day the first and second bars (open) equal the mean ± standard deviation of the anti-*Trichinella* response of undiluted faecal samples in the absence and presence of PCCl respectively. The hatched bar x100 equals the percentage difference in the response as a measurement of the proportion of the response due to PC.

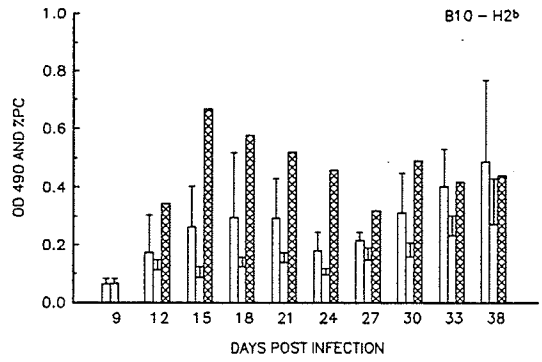

1.0
0.8
0.6
0.4
0.2
0.0
OD 490 AND %PC
0
6
12
18
28
29
30
31
34
40
46
DAYS POST INFECTION

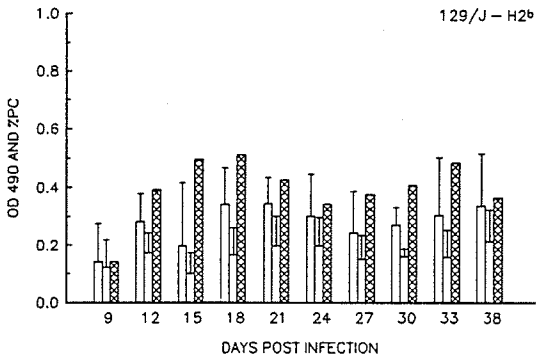
In the primary infection the proportion of the response attributable to PC peaked at 50-80% and generally at a lower percentage following challenge infections (Fig. 18). Comparison among strains also revealed some differences. The anti-PC response was greater in BR mice ranging from 60-80% in the primary, though declining to 50% by Day 10 following challenge. In BALB/c mice the response was delayed by 6 days, increasing by Day 18. The differences suggest an influence of *H-2* haplotype (compare among B10 congenics) as well as background gene effects.

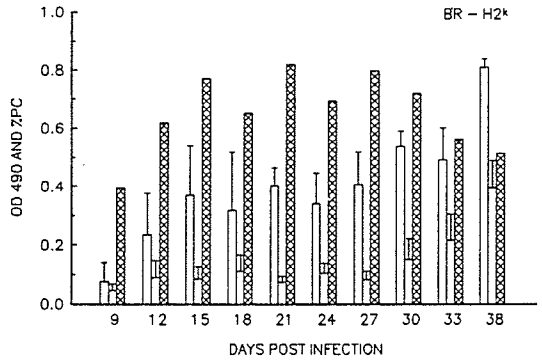
To further examine genetic differences in the anti-PC response, the proportion of the serum IgM anti-*Trichinella* response attributable to anti-PC was determined in the primary infection for the strains of mice described above (Table 15). The proportion of the response specific for PC varied among strains and with time. Early in the primary infection the PC specific proportion of the response ranged from 22-35% in the B10 strains, but was higher in BALB/c (60%) and C3H (80%). By Day 24 the response was similar, at 50-60%, in all strains (Table 15). The differences amongst strains did not group by *H-2* haplotype, but were similar among the B10 congenic strains, indicative of a role for genetic background in the regulation of this response.

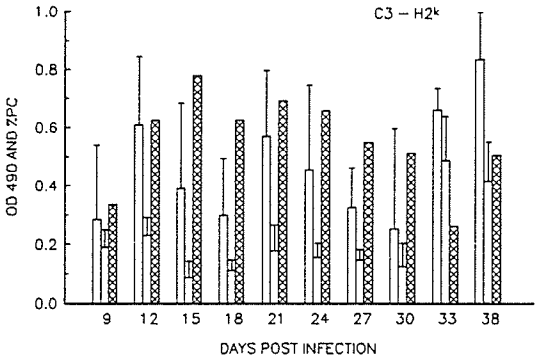
Figure 18. The PC specific response over time in the faeces of six strains of mice infected with *Trichinella*. Mice were challenged on Day 28.

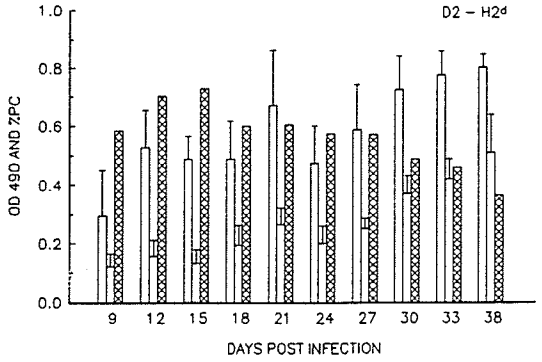
Strain designations as in Table 5. For each day the first and second bars (open) equal the mean ± standard deviation of the anti-*Trichinella* response of undiluted faecal samples in the absence and presence of PCCl respectively. The hatched bar x100 equals the percentage difference in the response as a measurement of the proportion of the response due to PC.


B10 – H2b
OD 490 AND ZPC
1.0
0.8
0.6
0.4
0.2
0.0
9 12 15 18 21 24 27 30 33 38
DAYS POST INFECTION


129/J – H2b
OD 490 AND ZPC
1.0
0.8
0.6
0.4
0.2
0.0
9 12 15 18 21 24 27 30 33 38
DAYS POST INFECTION


BR – H2k
OD 490 AND ZPC
1.0
0.8
0.6
0.4
0.2
0.0
9 12 15 18 21 24 27 30 33 38
DAYS POST INFECTION


C3 – H2k
OD 490 AND ZPC
1.0
0.8
0.6
0.4
0.2
0.0
9 12 15 18 21 24 27 30 33 38
DAYS POST INFECTION


D2 – H2d
OD 490 AND ZPC
1.0
0.8
0.6
0.4
0.2
0.0
9 12 15 18 21 24 27 30 33 38
DAYS POST INFECTION

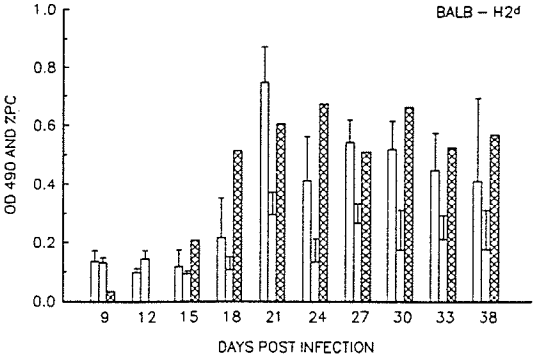

BALB – H2d
OD 490 AND ZPC
1.0
0.8
0.6
0.4
0.2
0.0
9 12 15 18 21 24 27 30 33 38
DAYS POST INFECTION

Table 15. Serum IgM response following primary infections in different inbred strains of mice.

Strain	Day Post Infection		
	12	18	24
BR	0.232±0.107	0.306±0.034	0.642±0.071
B10	0.369±0.148	0.471±0.090	0.614±0.115
D2	0.301±0.092	0.237±0.093	0.574±0.077
C3H	0.669±0.148	0.467±0.231	0.548±0.061
BALB	0.801±0.209	0.345±0.145	0.628±0.098
J129	0.394±0.090	0.490±0.077	0.604±0.029

Values equal the mean percentage reduction ± standard deviation in response to *Trichinella* following incubation with PCCl, compared with non PCCl controls.
Strain designations as in Table 5.

Discussion

PC antigen

My results confirm previous reports on the presence of PC-antigens in *Trichinella spiralis* (Choy *et al*, 1991). In ELISA experiments, TEPC-15 binding of *Trichinella* extracts was inhibited by 1mM PCCl. Inhibition with PCCl has been used extensively to determine the specificity and avidity of immunoglobulins and other PC binding proteins including C-reactive protein (Leon and Young, 1970) for the PC determinant. Inhibition of binding with 1mM PCCl is a generally used as a standard to determine the specificity of anti-PC antibodies (Potter and Lieberman, 1970). PC determinants were also identified in *Trichinella* extracts in western blots using TEPC-15 as a specific probe.

PC determinants have been reported from a number of nematodes and are distributed in a similar fashion in those studied. Immunofluoresence analysis of *Ascaris*, *Nippostrongylus* and *Trichinella* revealed that PC did not occur on the cuticle but was present on internal structures in these nematodes (Gutman and Mitchell, 1976; Pery *et al.*, 1979b; Choy *et al.*, 1991). Western blots of *Dipetalonema* (Gualzata *et al.*, 1986), *Onchocercha* (Garate *et al.*, 1990) and a number of protozoa, nematodes and trematodes (Lal and Ottesen, 1990) revealed multiple PC positive bands. Although Lim and Choy (1990) reported purification of a single PC bearing protein from *Trichinella*, subsequent studies also

revealed multiple PC determinants (Choy *et al.*, 1991). The biochemical nature of nematode PC has been examined in a number of studies. Pery *et al.*, (1979b) reported that PC from *Nippostrongylus* occurred in a polysaccharide fraction similar to PC from *Pnuemococcus* (Brundish and Baddiley, 1968) and *Epidermophyton* (Baldo *et al.*, 1977). However, McWilliam *et al.*, (1986) reported that nematode PC's were predominantly associated with lipoproteins. This may explain why the recovery reported by Pery *et al.*, (1979b) was only ~10%.

Anti-PC antibody

Having confirmed the presence of the PC determinant studies were undertaken to assess the immune response to *Trichinella* PC. The presence of anti-PC antibody in mice following *Trichinella* infection was detected as cross reactivity in ELISA experiments with extracts from bacteria and other parasites. Inhibition of this response with 1mM PCCl provided further support for the PC specificity of the cross reactive immunoglobulin response. PC-BSA was synthesized for use as a specific target antigen which allowed PC binding in the absence of other potential cross reactive determinants present in crude bacterial or nematode extracts. The combined results of these experiments revealed the presence of anti-PC antibodies following infection with *Trichinella*. The PC specificity of these immunoglobulins was confirmed by PCCl inhibition and by recognition of PC in a

variety of antigenic contexts (ie. bound to BSA, in SPS extracts and in nematode extracts). However, these studies provide only indirect evidence that the anti-PC antibodies were in response to *Trichinella* infection. To control other potential sources of PC stimulation, serum samples from uninfected mice were obtained throughout the experiment and background anti-PC levels were determined. The anti-PC response in these mice remained relatively constant, unlike the response in the infected mice which provides further evidence for an antibody response to *Trichinella* PC.

Serum response

The anti-PC response following infection with *Trichinella* was first reported by Crandall and Crandall (1971) and Brown and Crandall (1976). These studies focused on the response to *Ascaris* and the anti-PC response following infection with *Trichinella* was included for comparative purposes. The antibody response to *Trichinella* PC was significantly lower than the response to *Ascaris* PC. The anti-PC response following a primary infection was also determined in a plaque assay using PC-sheep red blood cells as target antigen (Ubeira *et al.*, 1987a). By Day 12 following primary infections a small peak in PC specific B cell activity was detected which declined by Day 28. Although the anti-PC response was characterized further in subsequent papers, the focus was on the immunosuppressive effects of *Trichinella* infection on the response to injected

Trichinella antigen, and the kinetics of the anti-PC response following infection were not described further (Ubeira *et al.*, 1987a, 1987b).

The PC specific proportion of the serum IgA, G and M and biliary IgA response to *Trichinella* was determined by PCCl inhibition for samples taken at intervals following primary and challenge infections. In the primary infections a significant proportion of the serum IgA and IgM response were inhibited by PCCl while the IgG response was only slightly reduced. The anti-PC IgM response in the primary infection corroborates the previous study with the related species *Trichinella nelsoni* described above (Ubeira *et al.*, 1987). Anti-PC responses were detected in both the IgM and IgA classes following challenge infections. The timing of the response was similar to the primary infection, the magnitude decreased and the proportion of PC specific IgG decreased to <5%. These characteristics are indicative of a lack of maturation in the challenge response.

Crandall and Crandall (1971) reported that IgM was the primary class of anti-PC antibody following infection with *Ascaris*. They also reported that the magnitude and timing of the challenge response did not differ from the primary response (Crandall and Crandall, 1971; Brown and Crandall, 1976) and similar results were reported by Mitchell *et al.*, (1976). Similarly, Pery *et al.*, (1979b), reported a predominate serum IgM anti-PC response in rats infected with

Nippostrongylus. However, the serum anti-PC response was not determined following challenge with *Nippostrongylus*. The serum responses in these other species are similar to the response following *Trichinella* infection described above, and as described later, are comparable to the Type I response to PC-protein conjugates.

Mucosal response

The PC specific bile IgA response was also determined in the present study. The kinetics of this response were generally similar to the serum IgM response. In the primary infection the proportion of PC specific bile IgA increased to Day 28, declined following challenge, and rose slightly by Day 40 following challenge infections. This response showed some similarities to the mucosal response following *Nippostrongylus* infection in rats (Perry *et al.*, 1979a; Brown *et al.*, 1981a,b), where the response occurred in adult rats, peaked by Day 7 of the primary infection and was significantly reduced in magnitude following challenge infections. Finally, in the present study, faecal IgA anti-PC production followed a similar pattern to the bile IgA response. The proportion of PC specific IgA increased to a peak in primary infections which was generally greater than the proportion detected following challenge infections. The similarity between the kinetics of the mucosal IgA and serum IgM anti-PC responses suggests that affinity maturation also does not occur in the mucosal anti-PC response. This implies

that class switching to IgA in the mucosal system can occur in the absence of affinity maturation. This possibility is discussed in the comparison with the response to conjugated PC later in this chapter.

Influence of host genotype

In Chapter 2 both MHC and background genes were shown to influence the systemic and mucosal responses to *Trichinella* antigens. The influence of host genotype on the response to PC was assessed in the present study. Comparison of the response among genetically distinct strains of mice revealed differences among strains but these differences were not associated with specific MHC haplotypes. The response to *Ascaris* PC showed a similar lack of MHC influence (Brown and Crandall, 1976) as did the group I response to PC-KLH (Bruderer *et al.*, 1988) described below. The influence of MHC genes occurs at the level of T cell recognition (described in more detail in Chapter 2), and MHC differences should not influence the response to T independent antigens. Therefore, the lack of an MHC effect in this study provides further support for the contention that *Trichinella* PC antigens produce a T-independent response (Lim and Choy, 1990).

Comparison with PC conjugates

There are some similarities between the anti-PC response following infection with *Trichinella* and the complex immune response observed following immunization with

PC-protein conjugates. The primary response following immunization with PC-protein conjugates is dominated by T15 idiotype positive antibodies, primarily of the IgM isotype, but also includes some IgA and IgG_3 (Chang *et al.*, 1982ab). These antibodies, which crossreact with pneumococcal C polysaccharide (Pn-PC) and which are inhibited by PCCl, are considered the 'Group I' response. Following challenge with PC-protein the secondary group I response has the same kinetics as the primary and there is no apparent affinity maturation or mutation of the antibodies (Chang *et al.*, 1982a,b; Chien *et al.*, 1988; Feeney *et al.*, 1988). However, following challenge a distinct group of antibodies were also detected. These 'Group II' antibodies do not bind Pn-PC and are inhibited by nitrophenyl-PC (the PC coupling bond) but not by PCCl (Chang *et al.*, 1982a,1984). Fine structure analysis revealed somatic mutation and affinity maturation, indicative of a secondary response and the immunoglobulins were predominately of the IgG_{1a} and IgG_{2a} isotypes (Chang *et al.*, 1982a; Bruderer *et al.*, 1988; Stenzel-Poore and Rittenberg, 1989).

The group I and group II antibody responses are independent, originating from separate B-cell pools (Chang *et al.*, 1982b,1984; Quan and Quintans, 1981; Bruderer *et al.*, 1988). The predominance of the Group I response in primary infections has been attributed to the presence of an expanded anti-PC B cell pool prior to exposure (Feeney *et*

al., 1988). The precursor level has been shown to be influenced by prior exposure to PC via the microbial fauna of the intestine or respiratory infections (Bruderer *et al.*, 1988). However, studies have also shown that T15 gene rearrangements are enhanced in pre B cells (which do not express surface immunoglobulin) in the absence of antigenic stimulation (Klinman and Stone, 1983), and it has been suggested that the group I precursor bias reflects an evolutionary history of exposure to PC antigens. The Group II precursors occur at low frequency prior to immunization, but expand and mature following primary and secondary exposure. The capacity of the group II response to affinity mature provides a mechanism by which these antibodies predominate following secondary exposure (Feeney *et al.*, 1988; Stenzel-Poore and Rittenberg, 1989).

The anti-PC response following infection with *Trichinella* described above, as well as other nematodes (Brown and Crandall, 1976; Pery *et al.*, 1979a; Brown *et al.*, 1981a,b) is similar to the group I response. The antibodies cross react with C-polysaccharide, are primarily of the IgM isotype in the serum and have similar kinetics in primary and challenge infections. The mucosal anti-PC response in biliary and faecal samples is also similar to the group I response, which implies that class switching to IgA occurs in the absence of affinity maturation. Similar results have been observed following PC-protein immunization in which the

group I response includes IgA antibodies (Chang *et al.*, 1982a). Previous studies have also shown that a significant proportion of the PC specific B-cells in the Peyer's patches of nonimmunized mice were committed to the IgA class (Cebra *et al.*, 1991). Furthermore, proposed mechanisms for class switching to IgA in the mucosal system do not require affinity maturation as a prerequisite (Strober and Sneller, 1988; Cebra *et al.*, 1991).

As yet, the lack of affinity maturation in the group I response is not understood. Mitchell and Lewers, (1976) hypothesized that affinity maturation was inhibited directly by the PC moiety. This theory, which was based on the reported toxicity of PC (Weltzien, 1973), suggests that B-cells with high affinity PC binding would be exposed to an elevated local PC concentration which would make them 'susceptible to inhibition if not suicide', consequently restricting affinity maturation. However this theory cannot accommodate the group II response described above. The high affinity of these antibodies for the PC ligand would also produce a high local PC concentration, and yet affinity maturation occurs in this response. Other theories suggest that group I antibodies are subject to negative idiotypic regulation which restricts maturation (Fung and Kohler, 1980) or that group I precursors are less susceptible to somatic mutation (Feeney *et al.*, 1988). Regardless of the mechanism, as a result of this regulation, the infected host

has the capacity to produce a rapid response following exposure to PC via the expanded precursor pool, but the affinity and isotype distribution of these immunoglobulins would remain the same. Consequently, although it may expand the precursor pool, oral or systemic immunization with PC antigens would likely not produce an anamnestic anti-PC response.

This does not imply that PC has no potential for vaccination. In both primary and challenge infections with *Trichinella*, a component of the mucosal antibody response is specific for PC, and as described in Chapters 2 and 3 the mucosal antibody response is correlated with protection in *Trichinella* infections. Further, anti-PC antibodies have a demonstrated protective capacity in some systems (Briles *et al.*, 1992). Finally, since the anti-PC response does not appear to be influenced by the MHC haplotype, it may have broad applications.

Chapter 5. The response to cholinesterase and hexosaminidase in mice infected with *Trichinella*.

Abstract

The antigenicity of specific components from extracts of *Trichinella* muscle larvae was examined. Cholinesterases were partially purified and characterized. The enzyme occurred in multiple molecular forms which differed in size, kinetic properties, substrate utilization and interaction with inhibitors. *Trichinella* CHE's differed from other nematode CHE's, lacking the 7S form which occurs in *Caenorhabditis elegans* and *Stephanurus dentatus*. CHE activity in homogenates prepared from *C. elegans* for comparison confirmed that the differences were not artifacts of the extraction method. No antibody response was detected to *Trichinella* CHE's by ELISA, heat denaturation or double diffusion. The lack of an antibody response may be associated with the low level of CHE secretion, or the absence of the 7S form. The humoral response to the enzyme hexosaminidase was confirmed by immunoprecipitation experiments and a mucosal anti-hexosaminidase response was detected by immunoprecipitation and ELISA. The mucosal response to hexosaminidase makes this enzyme a potential candidate to be used for oral immunization.

Introduction

It is apparent from Chapters 1 and 2 and from numerous previous studies (e.g. Crandall and Crandall, 1972; Van Loveren *et al.*, 1988) that a specific antibody response to *Trichinella* occurs following infections. As indicated in Chapter 2, this response is correlated with effects on worm fecundity and worm size. Furthermore, the results in Chapter 3 indicated that immunization with crude antigens protects against subsequent infections, and from Chapter 4 it was apparent that following challenge infections the response to PC would not be anamnestic though it may be protective. Do *Trichinella* enzymes have potential as antigens to protect the host from infection by immunization? This is dependent on the nature of the specific antigens recognized by immunoglobulins in the response, since characteristics of the antigen may determine the mode of protection. For example, the glutathione transferase enzyme (GST) of *Schistosoma* is recognized as an antigen following infection. Immunization with GST produces a protective immune response (Balloul *et al.*, 1987) by antibody mediated inhibition of enzymatic activity (Xu *et al.*, 1991). Protective antibodies which bind structural components utilize different inhibitory mechanisms. In this chapter, *Trichinella* CHE's were examined as potential antigen candidates. Hexosaminidase, an enzyme for which antigenicity has been documented (Rhoads, 1988), was used as a positive control

for comparison in these experiments.

The CHE's catalyze the hydrolysis of choline esters, including the neural transmitter acetylcholine (Silver, 1974). In higher vertebrates two CHE'S are recognized based on substrate specificity and interactions with inhibitors. Acetylcholinesterase preferentially hydrolyses acetylcholine and is inhibited by eserine while butyryl (pseudo) cholinesterase preferentially hydrolyses butyrylcholine and is resistant to inhibition by Iso Ompa (Silver, 1974; Taylor, 1991). Each of these enzymes occurs in multiple molecular forms (Taylor, 1991). Amongst invertebrates and lower vertebrates, CHE's also occur in multiple molecular forms differing in activity and interaction with inhibitors (Silver, 1974; Johnson and Russell, 1983; Fournier *et al.*, 1988; Stieger *et al.*, 1989). However, these properties do not fit the acetyl/pseudo cholinesterase model described above (Taylor, 1991) and for this discussion the general term cholinesterase will be used.

CHE activity has been reported from a number of helminths. Amongst parasitic nematodes CHE activity has been characterized from *Stephanurus dentatus* (Rhoads, 1981), *Brughia malayi* (Rathuar *et al.*, 1987) and *Necator americanus* (Pritchard *et al.*, 1991). While only a single secretory form was detected in *S. dentatus*, multiple forms were detected in the latter two parasites and anti-CHE antibodies were detected for all three. The antigenicity of CHE from a

number of intestinal nematodes had been reported previously (Ogilvie *et al.*, 1973) though the enzyme was not characterized in these species. Antigenicity was correlated with the level of *in vitro* CHE secretion by the parasites (Ogilvie *et al.*, 1973).

CHE has also been studied in detail in the free living nematode *Caenorhabditis elegans*. Three CHE classes were characterized based on size, Km, inhibition by carbamates and activity with the substrate butyrylthiocholine (BTCI) and three genetic loci were identified (Johnson and Russell, 1983; Johnson *et al.*, 1988). CHE's from the plant parasitic nematodes *Meloidogyne* (Chang and Opperman, 1991) and *Heterodera* (Chang and Opperman, 1992) also occurred in multiple forms, but the size distribution differed from *C. elegans*. The nonsecreted CHE's of *S. dentatus* had a similar size distribution to that of *C. elegans* and Western blot analysis revealed multiple molecular forms of CHE in somatic extracts of *N. americanus* (Pritchard *et al.*, 1991). The antigenic CHE of the digenean parasite *Schistosoma mansoni* was a 7.5S molecule (Tarrab-Hazdai *et al.*, 1984). In contrast to the above, information on *Trichinella* CHE is limited to anecdotal reports of the absence of CHE following *in vitro* culture of *Trichinella* (McClaren, 1976).

Hexosaminidase (β-N-acetyl-D-hexosaminidase) is a hydrolytic enzyme which catalyzes the release of terminal N-acetyl amino sugars from glycoproteins. In contrast to the

CHE literature, comparative studies of hexosaminidase are rare. The majority of published information on hexosaminidase relates to the lysosomal storage diseases of humans (Tay Sachs, Sandhoffs etc) resulting from deficient hexosaminidase activity. However, the enzyme has been isolated from a number of animals and plants and the hexosaminidase gene (*nag a*) has been cloned from the cellular slime mold *Dictyostelium*. Hexosaminidase activity in *Trichinella* has been studied in some detail (Rhoads, 1985; Rhoads, 1988). In *Trichinella* the enzyme is a glycoprotein with an apparent molecular mass of 100 KD, is separated into multiple forms by isoelectric focusing and is antigenic in natural infections (Rhoads, 1988)

The purpose of this study was to identify *Trichinella* antigens and determine their specific activity. The CHE and hexosaminidase activities were partially characterized and the antigenicity of these molecules was determined following infection.

Materials and Methods

Protein Extraction

Larvae were suspended 1:3 (larvae:buffer) in 0.1 M phosphate buffer, pH 7.5 and homogenized by grinding on ice for 15 min in a ground glass homogenizer with a motor driven teflon pestle. The homogenate was centrifuged at 31,000 RPM for 1 hr in a Ti50 rotor with an L8-55 ultracentrifuge (Beckman). The supernatant (S1) was kept on ice, and the pellet was rehomogenized in the same buffer with a Kinematica tissue blender. The homogenate was centrifuged as above, the supernatant stored on ice (S2), and the pellet rehomogenized as above in an equal volume of the same buffer + 1% Triton X-100. The homogenate was centrifuged as above, the supernatant (S3) stored and the pellet rehomogenized as above in an equal volume of the Triton buffer. This final homogenate was centrifuged, the supernatant (S4) kept, and the pellet discarded. Hexosaminidase was extracted in a similar manner using 0.1 M Tris, 0.85% NaCl pH 8, in place of 0.1M phosphate buffer.

Characterization of CHE and hexosaminidase

Concanavalin A precipitation

Con A was added at a 1 mg:1 mg ratio to a 1 mg/ml extract and incubated 14 hr at 4°C. The Con A suspension was tested for activity in the standard CHE or hexosaminidase assays, then centrifuged in a microfuge. The supernatant and pellet were tested for activity to determine interaction

with Con A. Similar assays were performed in the presence of 0.5M methyl-alpha-D-mannopyranoside, which inhibits specific Con A binding by interaction with its binding site.

Sucrose density centrifugation

Sucrose gradients were prepared in 0.1M phosphate buffer, pH 7.5, including 1% Triton X-100 for those samples containing the detergent. Samples (500 μl) were loaded on 8ml 5-20% linear gradients, and centrifuged for 18 hr at 4°C in an SW 41Ti Rotor on an L8-55 ultracentrifuge. β-galactosidase (16S), catalase (11.3S) and alkaline phosphatase (6.1S) were included as molecular weight standards. Sedimentation coefficients were calculated by comparison with the molecular weight markers as described by Martin and Ames (1961).

Enzyme Assays

Protein concentrations were determined by the BioRad protein microassay, using bovine serum albumin (BSA) as a protein standard. CHE activity was determined by a modification of the method of Ellman *et. al.* (1961). In the standard assay, 990 μl of assay solution (0.3mM 5,5-dithio-bis-(2-nitrobenzoic acid), 0.1mM acetylthiocholine iodide (ATCI), 0.1M phosphate, pH 7.5) was added to 10 μl of sample, and the change in optical density (O.D.) over time at 412 nm was assayed using a Milton Roy Spec 601. In the microassay, 200 μl of assay solution was added to 10 μl of sample in a 96 well microtiter plate, and the activity was

calculated by comparing the O.D. (410 nm) before and after incubation at 37°C, using a BioTek EL308 Microplate Reader. BTCI was substituted for ATCI in the same assay solution to determine the rate of BTCI hydrolysis. B-galactosidase activity was measured by the change in O.D. at 410 nm in microtiter plates in which 200 μl assay solution (0.1 M phosphate, pH 7.5, 20 mM o-nitrophenyl B-D-galactopyranoside, 10 mM NaCL, 1 mM $MgCl_2$ and 0.1 M B-mercaptoethanol) was added to 10 μl sample. Alkaline phosphatase activity was measured by the change in O.D. at 410 nm in microtiter plates in which 200 μl assay solution (0.1 M Tris/HCl, pH 8.5, 1 mM sodium-p-nitrophenyl phosphate) was added to 10 μl of sample. Catalase activity was determined by measuring the change in O.D. over time at 240 nm in a Milton Roy Spec 601, following the addition of 1 ml assay solution (0.05 M phosphate buffer, pH 7.0, 0.02% H_2O_2) to 10 μl sample. Hexosaminidase activity was assayed by the method of Opheim and Touster (1978). Briefly, *Trichinella* extracts were incubated in 100 μl of reaction buffer (80 mM sodium citrate pH 5.0 + 0.1 mg/ml BSA + 1 mM p-nitrophenyl-N-acetyl-B-glucosaminide) in microtiter plates at 37°C for a fixed time. One hundred μl of stop buffer (133 mM glycine, 67 mM NaCl and 83 mM Na_2CO_3 pH 10.7) was added and the optical density was determined at 410 nm with a BioTeck microplate reader. Activity was calculated based on an extinction coefficient of 16600 and units were defined by

the release of nitrophenyl in μmol/mg protein/minute.

Inhibition

The inhibition of CHE activity was determined as follows. Samples were incubated with the specific inhibitors Eserine, 1,5bis(4-allyldimethylammoniumphenyl)-pentan-3-one (BW284c51), and tetraisopropyl phosphoramide (Iso Ompa) at various concentrations. Inhibition was calculated as a percentage of the activity of positive controls, as measured in the microassay.

Antigenicity of cholinesterase and hexosaminidase

Cholinesterase

A number of methods were used to determine the antigenicity of *Trichinella* CHE. In these experiments the immunoglobulin response to electric eel acetylcholinesterase (Sigma Type III) was used as a positive control. Antibodies to EE AChE were raised in mice by intra-peritoneal injection. Mice were injected with antigen (50 μg in 100 μl PBS) at a 1:1 ratio with complete freunds adjuvant (CFA), and challenged with a similar dose in incomplete FA 14 days later. Serum samples were obtained by tail bleeding.

The immunoglobulin response was determined by ELISA. The ELISA method was as described in Chapter 1 with the following modifications. Plates were coated with *Trichinella* antigen at 1 μg/well or with EE AChE at 0.25 μg/well in 100 μl of carbonate buffer o/n. Plates were blocked with 3% BSA in PBS for 2 hr and all subsequent steps were 1 hr. Cross

reactivity between *Trichinella* CHE and EE AChE was also assessed in the above ELISA's.

A modified ELISA method was also utilized to determine the presence of anti-CHE antibodies. Plates were coated with 0.5 μg/well sheep antimouse IgG or IgA in carbonate buffer o/n. Plates were washed with PBST, blocked for 2 hr with 0.5% gelatin in PBST, washed with PBST and dilutions of serum and bile samples were added. Plates were incubated 2 hr, washed with PBST and dilutions of *Trichinella* antigen or EE AChE were added. Following o/n incubation plates were washed with PBST, CHE substrate (described above) was added and the reaction monitored as the change in absorbence at 410 nm.

The antigenicity of *Trichinella* CHE was also assessed in double diffusion experiments. *Trichinella* antigen and EE AChE were tested in double diffusion experiments with anti-Trichinella and anti-EE AChE samples by the method of Ouchterlony (1968). Precipitin reactions were detected by staining with Amido Schwarz and CHE activity in precipitates was determined by specific staining as described by Karnovsky and Roots (1964). CHE antigenicity assessed by heat denaturation studies as described by Michaeli *et al.*, (1969). Briefly, *Trichinella* antigen or EE AChE were incubated with serum or mucosal samples for 1 hr, then heated to 60°C for 30 min. CHE activity was then determined by the microassay described above. Protection from heat

denaturation was expressed as the percentage of control CHE activity, as was compared with the reduction in CHE activity in the absence of antibodies.

Hexosaminidase

Hexosaminidase antigenicity was assessed by immunoprecipitation with samples from mice infected with *Trichinella*. In serum immunoprecipitation experiments, *Trichinella* extracts were incubated o/n at 4°C with positive and control serum at various concentrations. A 10 μl aliquot was removed following incubation and the sample was then centrifuged at 13000 RPM for 5 min in a microcentrifuge. The supernatant was removed and the pellet resuspended in PBS. The hexosaminidase activity of the mixture (10 μl aliquot), the supernatant, and the resuspended pellet was determined as described above. Immunoprecipitation was determined as the percentage reduction in hexosaminidase activity in the supernatant fraction relative to control samples. The presence of anti-hexosaminidase antibodies was also determined by a modification of the ELISA for EE AChE described above. The HEX enzyme substrate was substituted for the CHE substrate to determine the presence of anti-HEX antibodies.

Results

Characterization of cholinesterase and hexosaminidase

Approximately 86% of the *Trichinella* CHE activity detected in crude homogenates was solubilized with a combination of phosphate and detergent (Triton X-100) buffers. The protein concentration and enzyme activity for a typical extraction are summarized in Table 16. Activity with the substrate BTCI was 60% that determined with ATCI. Extractions under high salt (1M NaCl) conditions released no further activity. Low levels of ES CHE activity were also detected (Table 16). Hexosaminidase activity was also determined for Tris/saline and detergent soluble extracts. Hexosaminidase occurred in both Tris/saline and detergent extracts and the specific activity recovered (Table 16) was similar to that described previously (Rhoads, 1988).

The phosphate and detergent extracts were fractionated by sucrose density gradient centrifugation (Fig. 19). Separation of the phosphate extract produced peaks of activity at 13S and 5.3S, of approximately equal magnitude, and a small peak at 7S. The majority of the CHE activity in the detergent extract was 13S, with a minor 5.3 S peak, and no 7S activity (Fig. 19). The absence of the 7S peak was surprising, since it has been reported from other nematodes (Johnson and Russell, 1983; Kolson *et al.*, 1985; Chang and Opperman, 1991).

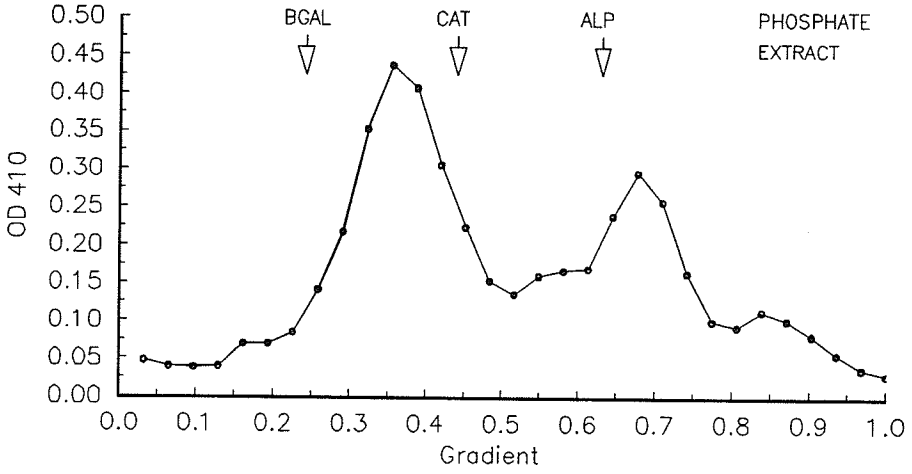
Table 16. Typical extraction of cholinesterase and hexosaminidase from Trichinella.

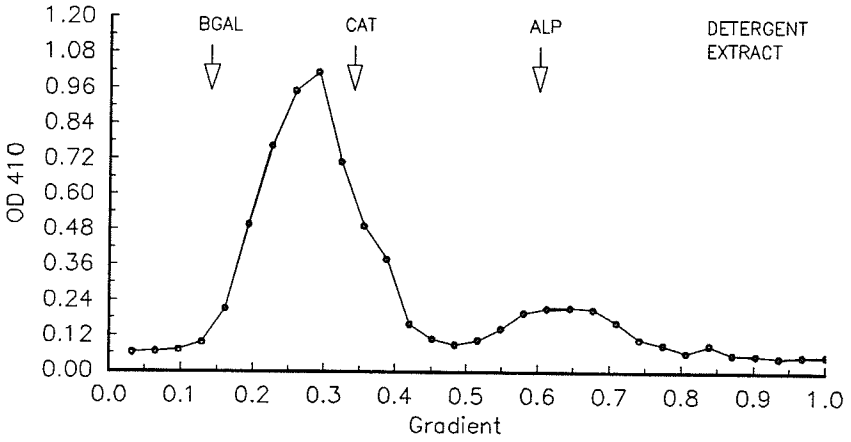
Extraction	Protein (mg)	Specific Activity[a]		Total Units	% Rec.[b]	Hex[c] mU
Homogenate	82	1.0×10^{-2}	0.82			
S1: 0.1M PO_4	60	5.0×10^{-3}	0.294	35.8		104.4
S2: 0.1M PO_4	9.9	8.0×10^{-3}	0.080	9.7		123.9
S3: 0.1M PO_4+ 1% TX-100	20.7	1.2×10^{-2}	0.248	30.2		194
S4: 0.1M PO_4 + 1% TX-100	8.7	1.0×10^{-2}	0.087	10.6		156
				Total	86.3	
Excretory/ Secretory	1.0	3.5×10^{-3}	0.045			

[a] Specific activity: 1 unit = 1 μmole ATCI hydrolysed/min/mg protein.
[b] Recovery of cholinesterase in extracts as a percentage of activity in crude homogenate.
c Extracts substitute 0.1M Tris + 0.85% saline for phosphate; Hexosaminidase activity: 1 mU = 1 nmol nitrophenyl released/min/mg protein.

Figure 19. Cholinesterase activity profile following sucrose density gradient centrifugation of the phosphate and detergent extracts of *Trichinella*.

Activity is reported as the change in OD 410 in the microplate assay compared with controls. Sedimentation coeffecients were determined by comparison with standards indicated on the profiles: BGAL = β-galactosidase (16S); CAT = catalase (11S); ALP= alkaline phosphatase (6.1S). Results were presented as a proportion of the gradient, from 0 (density = 20%) to 1 (density = 5%).


BGAL
CAT
ALP
PHOSPHATE
EXTRACT
OD 410
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
Gradient


BGAL
CAT
ALP
DETERGENT
EXTRACT
OD 410
1.20
1.08
0.96
0.84
0.72
0.60
0.48
0.36
0.24
0.12
0.00
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
Gradient

To confirm that the absence of this peak was not an artifact of our extraction procedure, *C. elegans* homogenates were prepared and the CHE size distribution determined as described above. The profiles were similar to that described previously (Johnson and Russell, 1983) including the presence of the 7S peak. Centrifugation of the detergent extracts in the absence of detergent produced similar profiles (data not shown). In profiles produced for both the phosphate and detergent extracts using BTCI as substrate, the 5.3S peak was reduced in magnitude to a greater extent than the 13S peak. Activity of the 5.3S and 13S peaks were 30% and 60% respectively of the activity with ATCI as substrate. The hexosaminidase activity in Tris/saline extracts separated on similar sucrose gradients occurred in a single peak at approximately 6.5S.

The kinetic constants of the 5.3S and 13S forms and interactions with specific CHE inhibitors were examined. Both forms showed inhibition at high substrate concentrations (Fig. 20), typical of CHE's (Silver, 1974). The two forms differed slightly in apparent Km (Table 17) and differences were also observed in the interaction with specific inhibitors (Table 17). The 5.3S form was more sensitive to Iso Ompa than was the 13S form, while the 13S form was more sensitive to both Eserine and BW284c51 than was the 5.3S form.

Figure 20. Specific CHE activity of the 13S and 5.3S forms extracted from *Trichinella* at varying concentrations of ATCI.

Each point represents the mean ± standard deviation of the specific activity (1 unit = 1μmole ATCI hydrolysed/min/mg protein) determined in triplicate microplate CHE assays for these extracts.

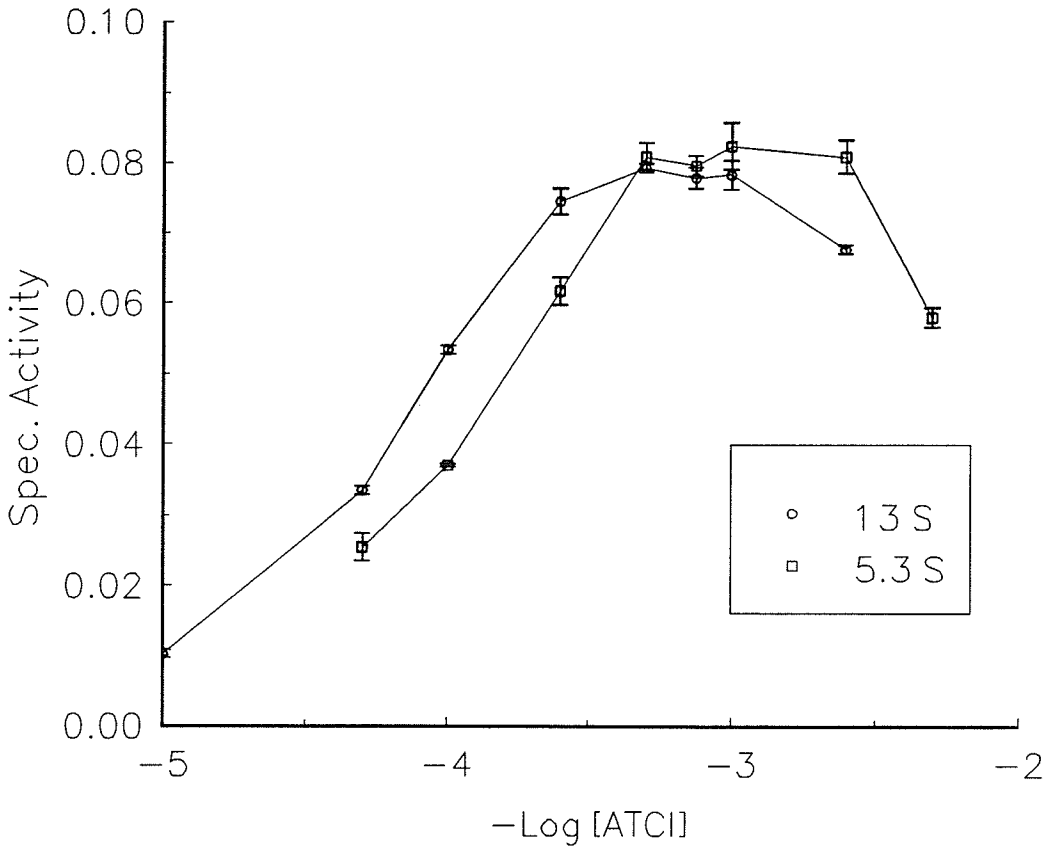

0.10
0.08
0.06
0.04
0.02
0.00
Spec. Activity
−5
−4
−3
−2
−Log [ATCI]
13 S
5.3 S

Table 17. Kinetic and inhibitory parameters of partially purified cholinesterase fractions from *Trichinella* larvae.

Fraction	Km[a]	Inhibitor[b]		
		Eserine	BW284c51	Iso Ompa
5.3S	140μM	5.5×10^{-8}	1.5×10^{-7}	5.5×10^{-4}
13S	80μM	1.5×10^{-8}	1.5×10^{-8}	4.8×10^{-3}

[a] Activity determined with acetylthiocholine iodide. Apparent Km determined from Eadie-Hofstee plots.

[b] Molar concentration of inhibitor producing 50% inhibition as compared to untreated controls.

The glycoprotein nature of the *Trichinella* CHE and hexosaminidase was assessed by precipitation with Concanavilin A (Con A). Following overnight precipitation with 1 mg/ml Con A, and centrifugation, the supernatant CHE activity of both the phosphate and detergent extracts was reduced by 88-89% and the Tris/saline hexosaminidase activity was reduced by 98% (Table 18). For both CHE and hexosaminidase the enzymatic activity was concentrated in the Con A precipitate and precipitation of CHE and hexosaminidase was inhibited by 0.5M Methyl-alpha-d-mannopyranoside in the reaction solution.

Antigenicity of cholinesterase and hexosaminidase

Cholinesterase

The antigenicity of *Trichinella* CHE was assessed in a number of experiments. Initial experiments were based on the reported cross reactivity of anti-*Schistosoma* antibodies with EE AChE. Antisera from mice infected with *Trichinella*, though strongly positive with *Trichinella* antigen, did not cross react with EE AChE in ELISA experiments (Table 19). Furthermore, control serum having a high anti-EE AChE titer did not cross react with *Trichinella* antigen extracts (Table 19). No cross reactivity was detected in double diffusion experiments and CHE staining of double diffusions with

Table 18. Precipitation of CHE and hexosaminidase with Concanavalin A.

Sample	Con A[a]	Con A+ m-manno[b]
S1-CHE		
sup	12.7%[c]	98%
ppt	conc[d]	np[e]
S3-CHE		
sup	11.4%	89%
ppt	conc	np
S1-HEX		
sup	2.0%	50%
ppt	conc	np

Sample designations as in Table 16.
[a] Concanavalin A, 1 mg/ml
[b] Con A + 0.5M methyl mannopyranoside.
[c] Percentage of enzymatic activity remaining in supernatant following centrifugation compared with controls.
[d] conc = enzymatic activity concentrated in pellet following centrifugation.
[e] np = no precipitate, no activity measured.

Table 19. Antibody response to *Trichinella* (P1) extracts and electric eel acetylcholinesterase (EE AChE).

Antigen Sample	Immunoglobulins		
	anti-P1 IgG[a]	anti-P1 IgA[b]	anti-EE AChE IgG[c]
S1[d]	2.900	0.170	0.080
S3[e]	0.730	0.120	0.010
5.3S	2.590	0.684[f]	0.040
13S	2.490	1.119[f]	0.050
ES[g]	2.300	0.135	0.010
EE AChE	0.010	0.040	1.160

Values = OD 490 results from ELISA.

[a] 1/100 dilution of serum from infected mice. [b] 1/10 dilution of intestinal lumen samples from infected mice. [c] 1/100 dilution of serum from EE AChE injected mice. [de] antigen extracts described in Table 16. [f] 1/2 dilution of intestinal lumen samples from infected mice.
[g] excretory/secretory antigen.

Trichinella antigen and anti-*Trichinella* antisera were also negative, indicative of a lack of anti-CHE antibodies. Furthermore in a modified ELISA, EE AChE activity was detected with anti-EE AChE antibodies while *Trichinella* CHE was not detected with biliary or serum anti-*Trichinella* antibodies. Finally, anti-CHE activity was assessed in heat denaturation experiments. While incubation of EE AChE with polyclonal anti-EE AChE protected against heat denaturation at 60°C, incubation of CHE positive *Trichinella* extracts with biliary, IL or serum anti-*Trichinella* antibodies or anti-EE AChE antibodies did not protect against heat denaturation (Table 20). In combination these experiments indicate that *Trichinella* CHE is not antigenic in natural infections.

Hexosaminidase

The presence of serum anti-hexosaminidase antibodies in mice infected with *Trichinella* was confirmed by immunoprecipitation experiments. As indicated in Table 21, serum antibodies from infected mice precipitated >80% of the *Trichinella* hexosaminidase activity. In contrast, although hexosaminidase activity was concentrated 3-fold in precipitates with immune bile compared with controls, due to endogenous biliary hexosaminidase the activity in the supernatant was not reduced (Table 21).

Table 20. The effect of heat denaturation on cholinesterase activity.

Antibody Source	ANTIGEN	
	SA	EE AChE
PBS-RT	0.139[a]	1.627
PBS-60°C	0.009	0.000
Normal-RT	0.638	0.732
Normal-60°C	0.010	0.011
anti-P1-RT	1.542	0.915
anti-P1-60°C	0.013	0.015
anti-EE AChE-RT	1.053	1.857
anti-EE AChE-60°C	0.001	1.677

Note. *Trichinella* extracts (SA) or electric eel acetylcholinesterase (EE AChE) were incubated with PBS, control, anti-P1 or anti-EE AChE serum at room temperature or 60°C for 30 minutes.

[a] Cholinesterase activity expressed as OD 410 nm determined in the modified Ellman assay.

Table 21. Immunoprecipitation and modified ELISA to determine antigenicity of hexosaminidase.

	ANTIBODY SOURCE		
	PBS CONTROL	NORMAL MICE	INFECTED MICE
Serum IgG			
Sup	0.49±0.01[a]	0.45±0.10	0.07±0.05
PPT	np[b]	0.06±0.01	1.18±0.16
Bile IgA			
Sup	2.77±0.17	2.65±0.03	2.86±0.15
PPT	np	0.15±0.01	0.48±0.18
Mod. ELISA	0±0	0.07±0.01	0.22±0.01

Soluble antigen (Table 16) was incubated with PBS, normal, or Immune serum or bile. Following centrifugation, hexosaminidase activity was determined in the supernatant and precipitate.
For the modified ELISA, plates were coated with sheep anti-IgA and incubated with PBS, normal or immune serum, then incubated with soluble antigen and the hexosaminidase activity determined.
[a] Hexosaminidase activity reported as OD 410nm in the microassay modification of the enzyme reaction.
[b] np = no precipitate

To provide further evidence for biliary anti-*Trichinella*-hexosaminidase antibodies a modified ELISA similar to that described above for CHE was utilized. A threefold increase in hexosaminidase activity was detected with immune bile compared with bile from normal mice. In combination with the precipitation results this is indicative of a biliary anti-hexosaminidase response.

Discussion

Antigenicity of cholinesterase and hexosaminidase

CHE: The purpose of these experiments was to identify and characterize antigens from *Trichinella*. CHE was selected as a potential candidate based on its reported antigenicity in several other nematode systems (Ogilvie *et al.*, 1973; Rathuar *et al.*, 1987 etc.). However, the experiments in this study indicated that following infection, *Trichinella* CHE's did not induce an antibody response and that *Trichinella* CHE's are not cross reactive with antibodies to EE AChE. Cross reactivity with antibodies raised against EE AChE was demonstrated for *B. malayi* CHE (Rathuar *et al.*, 1987), as well as for *Schistosoma mansoni* (Tarrab-Hazdai *et al.*, 1984). In the latter study, antibodies to EE AChE mediated a cytotoxic effect on schistosomulae. However, antigenic similarity does not always occur among CHE's from different species since heat denaturation studies indicated no cross reactivity in the immune response to the secretory CHE's of several closely related nematode species (Rothwell *et al.*, 1973). In this study, we did not detect cross reactivity between EE AChE and crude or partially purified extracts of *Trichinella*, by ELISA or heat denaturation.

Antigenicity of CHE's in infected hosts has been demonstrated for a number of nematodes (Ogilvie *et al.*, 1973; Rhoads, 1981; Rathuar *et al.*, 1987, Pritchard *et al.*, 1991). I found no evidence for antigenicity of CHE in

Trichinella infected mice by ELISA, precipitation, heat denaturation, or staining of double diffusion gels. Comparison of several gastrointestinal nematodes indicated a correlation between the level of CHE secretion and antibody production (Ogilvie *et al.*, 1973). While it is difficult to compare the secretory products because of the way in which activity was expressed, the *Trichinella* secretory activity is low, and this may account for the lack of antigenicity. The lack of antigenicity may also relate to differences in *Trichinella* CHE's, in particular, the lack of the 7S form.

Hexosaminidase: Precipitation of hexosaminidase by serum from infected mice, reported previously by Rhoads, (1988) was assessed as a positive control in the above experiments. The results in the present experiments corroborate and extend this report in which a serum immunoglobulin response to hexosaminidase was described (Rhoads, 1988). In the present study the immunoprecipitation and modified ELISA experiments also indicated a mucosal antibody response to hexosaminidase. The mucosal response indicates that antigens are presented to the mucosa, suggesting that the host is exposed to hexosaminidase during the intestinal phase of *Trichinella* infection. Intestinal exposure is likely since immunocytochemical staining of *Trichinella* sections indicated the presence of hexosaminidase on the cuticular surface and hexosaminidase activity was also detected in *in vitro* culture samples of

Trichinella larvae (Rhoads, 1988).

Characterization of cholinesterase and hexosaminidase

The lack of comparative information on CHE's from nematodes made further characterization of *Trichinella* CHE of interest. The characteristics of CHE's from a number of nematodes are summarized in Table 22. From this summary it is apparent that there is considerable variability among nematode CHE's. In most species, CHE's occur in multiple molecular forms, but the distribution and kinetics of these forms varies. A 12S or 13S CHE occurs in *C. elegans*, *T. spiralis*, *Meloidogyne* and *Heterodera*. Similar Km values were reported for the latter three species, but this contrasts with a significantly lower Km in *C. elegans*. A 7S CHE was reported from *C. elegans*, *Meloidogyne*, *Heterodera* and *Stephanurus* but was not detected in *T. spiralis*. This CHE is unusual, having a low Km (~10 nM), and is significantly more resistant to inhibition by carbamates and organophosphates (Kolson and Russell, 1985). The lower density CHE's (5.3S in *C. elegans* and *T. spiralis*; 5.8S in *S. dentatus*) were not detected in the plant parasitic nematodes *Meloidogyne* and *Heterodera*. The proportion of the molecular forms within species also varied.

Table 22. Characteristics of nematode cholinesterases.

Species	Source[a]	Size	Km(μM)	BTCI[b]	Reference
B. malayi	ES	100+200kDa		33%	Rathuar *et al.*, 1987
	ext.			25%	Rathuar *et al.*, 1987
C. elegans	ext.	13S A[c]	12	70%	Johnson and Russell, 1983
		7S B/C	67/16[d]	25%	Johnson and Russell, 1983 + Kolson and Russell, 1985
		5.3S A/B	15/80	64/18%	Johnson and Russell, 1983
Heterodera	ext.	7S C[c]	10[d]		Chang and Opperman, 1992
		12S A	47.5		Chang and Opperman, 1992
		12S A	120		Chang and Opperman, 1992
Meloidogyne	ext.	7S B/C[c]	32/8[d]		Chang and Opperman, 1991
		8S A	35		Chang and Opperman, 1991
		12S A	24		Chang and Opperman, 1991
		13S A	100		Chang and Opperman, 1991
M. *apri*	ext.		100	6.7%	Reiner *et al.*, 1978
N. americanus	ES	30-220 kDa		2.7%	Pritchard *et al.*, 1991
	ext.	30-220 kDa		24%	Pritchard *et al.*, 1991
S. dentatus	ES	5.8S	560	12.5%	Rhoads, 1981
	ext.	7S	16[d]		Kolson and Russell, 1985
T. spiralis	ext.	13S	80	60%	This study
		5.3S	140	30%	This study

[a] Source of sample: ES = excretory secretory product, ext. = worm extract.

[b] Activity with BTCI as a percentage of ATCI activity

[c] Class designations based on *C. elegans* (Johnson and Russell, 1983)

[d] Km in nM for Class C cholinesterases.

For example, in *C. elegans* and *T. spiralis* detergent extracts the 13 S form occurred at a higher concentration than the 7S form, but the 7S form predominated in *Heterodera* and *Stephanurus* extracts.

Differences in hydrolysis of BTCI were also noted. Hydrolysis of ATCI and BTCI substrates are used to differentiate vertebrate CHE's, since hydrolysis of BTCI by acetylcholinesterase occurs at 1/60 the rate of ATCI hydrolysis (Johnson and Russell, 1983). The rate of BTCI hydrolysis by nematode CHE's varied from 6-30% for several gastrointestinal parasites (Ogilvie *et al.*, 1973), 12.5% for *S. dentatus* secretory CHE, 25-33% for *B. malayi* secretory CHE (Rathuar *et al.*, 1987) and 3 and 24% respectively for secretory and somatic CHE from *N. americanus* (Table 22). The rates for *Trichinella* also varied (30 and 60%) as did the rates reported from *C. elegans* (18-75%). Despite the variability in activity, it is clear that hydrolysis of BTCI occurs at a greater relative rate for nematode CHE's compared with vertebrate acetylcholinesterase.

A key characteristic of CHE activity is the inhibition of enzyme activity at high substrate concentration (Silver, 1974). Inhibitory substrate concentrations reported previously were 10 mM for *N. brasiliensis* (Sanderson *et al.*, 1967), 10 mM for *Metastrongylus apri*, (Reiner *et al.*, 1978) and 20 mM for *S. dentatus* (Rhoads, 1981). Both the 5.3 and 13S forms in *Trichinella* showed the typical bell shaped

activity curve (Silver, 1974), and were inhibited at lower substrate concentrations (0.5-1 mM ATCI). The Km values for the 5.3S (140μM) and 13S (80μM) CHE's from *Trichinella* were similar to that reported for *M. apri* (100μM) (Reiner *et al.*, 1978), but less than that reported for the secretory CHE from *S. dentatus* (560 μM) (Rhoads, 1981). These values were also similar to those reported for the 12S form of *Heterodera* and the 13S form of *Meloidogyne*. The Km values for *C. elegans* class A and class B CHE's were 10 and 80 μM respectively (Johnson and Russell, 1983) but differences in the substrates limited direct comparisons with *C. elegans*.

The vertebrate acetyl and butyryl CHE's can be distinguished by differences in the concentration of specific inhibitors required for a 50% reduction in enzyme activity (Silver, 1974). Differences were also observed in specific inhibition of the CHE classes from *C. elegans*, but the nematode CHE's do not fit the vertebrate acetyl/butyryl CHE model (Johnson and Russell, 1983). For example, inhibition of vertebrate butyrylcholinesterase with Iso Ompa occurs at a 100-1000 fold lower concentration of inhibitor than inhibition of the CHE's from *C. elegans* (Johnson and Russell, 1983). The inhibition of CHE activity by eserine, BW284c51 and Iso Ompa in *Trichinella* was generally similar to that observed for class A and B CHE's from *C. elegans*, with some minor differences. Among other nematodes, the *S. dentatus* secretory CHE was more resistant to both eserine

and BW284c51 inhibition (Rhoads, 1981) than our results for *T. spiralis* CHE's, while CHE's from the plant parasitic nematodes *Meloidogyne arenaria* and *M. incognita* were more sensitive to eserine inhibition (Chang and Opperman, 1990).

From the above it is clear that there is considerable variability in the activity of CHE's of nematodes. However, the role of multiple CHE forms in nematodes remains unclear. Elimination of type A or B CHE by mutation or double mutants with type C in *C. elegans* are phenotypically normal, while AB double mutants, though abnormal, remain viable (Johnson *et al.*, 1988). Multiple forms of CHE are, therefore, not essential for survival in *C. elegans*, as apparently in other invertebrate groups. For instance, *Drosophila* CHE is encoded by a single gene (Fournier *et al.*, 1988), and in other insects CHE's occur in limited forms (Belzunces *et al.*, 1988). Perhaps the variability in the distribution of nematode CHE's reflects different evolutionary pressures. It is interesting to note that in the nematicide resistant plant parasite *Heterodera* the organophosphate resistant 7S CHE predominates (Chang and Opperman, 1992). It is also worth noting that *Trichinella* is in the class aphasmida which is considered evolutionarily distant from the other nematodes. The absence of a detergent soluble 7S form may reflect this evolutionary relationship. A more complete analysis of the nematode CHE's including other aphasmid species would be of considerable value to assess the

evolutionary history of CHE in this group. Finally, the considerable differences in the properties and antigenicity of CHE's amongst nematodes described above indicates that information from model systems such as *C. elegans* must be interpreted with caution.

In contrast to CHE, hexosaminidase has potential as a specific antigen. The serum and mucosal antibody response indicate that this molecule is recognized as an antigen during infections. Further, preliminary experiments (Rhoads, 1988) indicated that injection of *Trichinella* hexosaminidase may partially protect against infection. These studies also suggest that the antigenic fraction may be a carbohydrate moiety, similar to the immunodominant carbohydrate described by Denkers *et al.*,1990, though cross reactivity has not been demonstrated. *Trichinella* hexosaminidase or its carbohydrate components are ideally suited for analysis as antigens in the CT/*Trichinella* system described in Chapter 3.

The objective of developing a response to enzymatic antigens is to inhibit enzymatic activity, and as a consequence disrupt an essential metabolic pathway. This approach is illustrated by the protective effects of the inhibition of glutathionine transferase activity in schistosomes (Xu *et al.*, 1991). However, my study demonstrated that the enzymatic activities of both CHE and hexosaminidase were not inhibited by antibody binding. Thus, while antibody mediated inhibition of enzymatic activity may

be a successful strategy for some enzyme systems, one cannot automatically assume that an antibody response will inhibit enzyme activity.

The deflection of the response from recognizing critical epitopes on enzymes may represent a parasite strategy to enhance survival by modulation of the immune response. A similar interpretation has been suggested for the immunodominance of a carbohydrate moiety in the group II response to *Trichinella* (Denkers et al., 1990). As indicated above, preliminary work by Rhoads (1988) suggested that a significant proportion of the serum response to hexosaminidase recognized a carbohydrate moiety and therefore did not affect enzymatic activity. Further characterization of this enzyme, and immunization with antigen treated to remove the carbohydrate components may help resolve this enigma.

Chapter 6. General Discussion and Summary

This Chapter summarizes the key points of my thesis and concludes with a discussion on the relationship between the effects of immunity and the mucosal immunoglobulin response.

i) The antibody response

Analysis of the antibody response in Chapter 1 reconfirmed and extended previous observations on the response to *Trichinella*. The predominant mucosal immunoglobulin was IgA. Specific anti-*Trichinella* IgA was detected in the bile, LUM and faeces of infected mice and mucosal immunoglobulin production was temporally correlated with the course of infection. Minimal levels of mucosal IgG were detected in primary infections, but following challenge IgG levels increased. Comparisons with the response to unrelated mucosal immunogens and to other gastrointestinal helminths revealed a number of common features in the response. The predominant mucosal immunoglobulin in all systems was IgA, which was usually detected between 7 and 14 days following primary exposure and was anamnestic following challenge. However, differences in the level of IgG and the kinetics and tissue distribution of immunoglobulin production indicated that antigen specific influences on the immunoglobulin response also occurred. Finally, interactions occurred in the response following concurrent exposure to the two strong immunogens, *Trichinella* and CT. When fed in combination some synergism was apparent, as the response to both was slightly increased. However, faecal and serum

analyses for IgA revealed that under certain conditions, infection with *Trichinella* suppressed the serum or mucosal response to CT (see Appendix 1).

ii) The influence of host genotype

Initially, the response to *Trichinella* was examined in BALB/c mice to determine if the correlation between the immunoglobulin response and the effects of immunity reported for strong responder CFW mice also occurred in a more susceptible strain. The study was then expanded to assess this relationship in a number of strains of mice and to determine if these effects were influenced by *H-2* or background genes. The expulsion response was dissociated from the production of mucosal IgA in a number of strains, while the reduction in fecundity was temporally correlated with the specific IgA response in all strains. It is also apparent from these studies that both *H-2* and background genes influence the immunoglobulin response to *Trichinella*.

iii) Oral immunization

The reduction in the mean of total muscle larvae in mice infected with *Trichinella* in combination with CT and the enhanced response following oral immunization with *Trichinella* antigens and CT reported in Chapter 3, demonstrated the role of CT as an oral adjuvant. Oral immunization with antigen plus CT resulted in a reduction in worm size and fecundity, in contrast with treatments fed antigen alone, which did not differ significantly from

controls. In addition, the mucosal immunoglobulin response was enhanced in mice immunized in combination with CT but not in mice immunized without CT. However, the serum response differed between antigens, being greater in mice fed the particulate antigen. The relationship between the effects of immunity and the immunoglobulin response is discussed below.

iv) The antigenicity of phosphorylcholine

The anti-PC response described in Chapter 4 was similar to the Type I anti-PC response to PC-protein conjugates. While the predominant serum immunoglobulin was IgM, a primary IgG response occurred, but it was down regulated following challenge infections. The kinetics of the mucosal IgA response were similar to that described for serum IgM. Comparison with the Type I response to PC-protein suggests a lack of affinity maturation and limits the potential for an anamnestic IgA response to PC following challenge. However, a mucosal anti-PC response occurred in both primary and challenge infections, which may have a protective function as seen in other systems (Briles *et al.*,1992). If PC is a protective antigen, the lack of an MHC influence on the anti-PC response enhances its potential in genetically diverse hosts.

v) The antigenicity of cholinesterase

It is apparent from studies in Chapter 5 that *Trichinella* CHE's were not antigenic in the course of

infection in mice, in contrast with hexosaminidase which was tested as a positive control. Characterization of CHE activity revealed differences in size distribution and an overall low level of CHE production in *Trichinella* ES products, which may have contributed to the lack of antigenicity. A review of several nematode CHE's indicates that generalizations from the *C. elegans* model should be interpreted with caution. The positive mucosal response to hexosaminidase indicates that this enzyme may be a good candidate antigen for mucosal immunization as described in Chapter 3. However, a positive immunoglobulin response does not ensure protection, since antibodies to CHE and hexosaminidase did not inhibit enzymatic activity. This problem may be resolved with the development of methods to direct the antibody response against the catalytic site.

Effects of immunity and the immunoglobulin response

The theme of this thesis is the relationship between the anti-*Trichinella* immunoglobulin response of mice and the effects of this response on the course of infection.

Numerous reports suggest an effector role for mucosal immunoglobulins. The effects of immunity were shown to be mediated during the intestinal phase of infection (Levin and Evans, 1942). Immunoglobulin precipitates were detected at the orifices of adult worms isolated from immune mice (Jackson, 1959). Bile IgA had a demonstrated anti-fecundity effect *in vitro* (Jacqueline et al., 1978) and the effects of

bile duct ligation in rats suggested an *in vivo* effect as well (Jacqueline et al., 1981). The strong responder phenotype in NIH mice was associated with an enhanced serum IgA response (Almond and Parkhouse, 1986). A effector role was demonstrated for IgA antibodies in protection against viral and bacterial infection and the degranulation of protective cell types, particularly eosinophils and neutrophils, was shown to be mediated by high affinity IgA receptors (Abu-Ghazaleh *et al.*, 1989; Mazengra and Kerr, 1990). Furthermore, it is clear from experiments described above and from previous studies (deVos et al., 1992), that a temporal correlation exists between the immunoglobulin response and the reduction in fecundity and size of adult worms in the intestinal phase of infection.

In a previous study, immune effects in challenge infections following low dose primary infections (as low as 10 larvae) were associated with a biliary IgA response (deVos et al., 1992). In the present study, the reduction in fecundity remained associated with the mucosal immunoglobulin response in 6 strains of mice varying in resistance/susceptibility traits (Chapter 2), providing further correlative support for this relationship. Since the antibody response and the effects of immunity were not dissociated by low doses, or in mouse strains with varying immune response, the experiments in Chapter 3 were designed to examine this relationship following oral immunization. As

was observed with live worm infections, the effects of immunity produced by priming with *Trichinella* antigens could not be dissociated from the mucosal immunoglobulin response in this study. In all treatments, the reduction in the fecundity and size of adult female *Trichinella* was associated with a ten fold increase in the specific IgA response in the bile.

From the foregoing discussion it appears that mucosal immunoglobulins protect against infection with *Trichinella*. However, specific antibodies are only one of a number of potential effectors produced following infection. Although our understanding of mucosal immunity is far from complete, the following is a brief description of the mucosal immune system. Mucosal lymphoid cells occur diffusely throughout the intestine, as well as in discrete organized tissues (Peyer's patches). The immunobiological events in the activation of B and T cells have been described in detail (Mestecky and McGhee, 1987). Briefly, antigens are taken into the Peyer's patches via epithelial cells specialized for rapid antigen passage (M cells), are transported to antigen presenting cells (predominantly macrophages) and presented to T and B cells (O'Hagan *et al.*, 1987). T cell activation in the Peyer's patch is polarized to the Th2 pathway, and results in the production of the cytokines interleukin 4, 5 and 10, characteristic of a Th2 response. These cytokines influence the development of specific B-

cells, favouring the production of IgA, as well as stimulating differentiation and mobilization of granulocytes. The T lymphocyte response becomes polarized as a function either of its microenviroment or of antigen determined differences in presentation. Once polarized, stimulation of the alternative Th pathway is suppressed by cytokines from the active Th subset (Pearce and Sher, 1991). Observations with *Leishmania* and *Schistosoma* led to the hypothesis that protection from infection was associated with a Th1 response and Th1 cytokines, and that susceptibility was associated with the Th2 response (Scott *et al.*, 1988; Sher *et al.*, 1991). However, this generalization does not apply to some gastrointestinal nematode infections in which the Th2 response and Th2 cytokines were associated with protection (Urban *et al.* 1992). To date, the protective cell types have not been established for *Trichinella* infections. Cell transfer studies and analysis of the response in rats implicated a Th2 pathway (Korenaga et al., 1989), but Pond et al., (1989) demonstrated that resistance was correlated with an increased γIFN (Th1) response, and that both were correlated with genotype. More recently however, Kelly et al., (1991) reported that the response to *Trichinella* was compartmentalized, and that the mucosal response was predominantly Th2.

What role does mucosal antibody production have in the

Trichinella/mouse model? The Th2 bias apparent in the mucosal lymphoid tissue favors the production of an IgA response and mucosal immunoglobulins have a demonstrated potential for direct effects. For example, cuticular antibody binding and the formation of immunoprecipitates at parasite orifices may be responsible for the decrease in size and fecundity of worms as a result of nutrient stress, and inhibition of the release of new born larvae. Cytokines produced in the Th2 response also stimulate mast cell and eosinophil proliferation. The high affinity IgA receptors present on eosinophils provide a mechanism for IgA degranulation and enhancement of an inflammatory response following infection. Furthermore, following challenge exposure, the secondary IgA response may enhance the rate of antigen uptake in the Peyer's patch. Following primary exposure, *Trichinella* specific lymphocytes populate the diffuse intestinal lymphoid tissue. These cells are activated by antigen within hours following challenge infections, and IgA levels increase. IgA specific receptors on the luminal surface of M-cells (Weltzin *et al.*, 1989) which mediate the enhanced uptake of IgA/antigen complexes could increase the rate of antigen passage in the Peyer's patches, and thus enhance the rate of the secondary response.

In summary, mucosal IgA likely plays a significant role in the mucosal response to helminth infections. Whether this

role is direct, through binding to parasite surfaces and the subsequent production of immunoprecipitates, or indirect via other cellular processes such as increased rate of antigen presentation or enhancement of the inflammatory response, or both, remains to be resolved.

Literature Cited

Abu-Ghazaleh, R.I., T. Fujisawa, J. Mestecky, R.A. Kyle and G.J. Gleich. 1989. IgA mediated eosinophil degranulation. Journal of Immunology 142:2393-2400.

Ahmad, A., C.H. Wang and R.G. Bell. 1991. A role for IgE in intestinal immunity. Expression of rapid expulsion of *Trichinella spiralis* in rats transfused with IgE and thoracic duct lymphocytes. Journal of Immunology 146:3563-3570.

Almond, N.M. and R.M.E. Parkhouse. 1986. Immunoglobulin class specific responses to biochemically defined antigens of *Trichinella spiralis*. Parasite Immunology 8:391-406.

Altorfer, J. S.J. Hardesty, J.H. Scott, and A.L. Jones. 1987. Specific antibody synthesis and biliary secretion by the rat liver after intestinal immunization with cholera toxin. Gastroenterology 93:539-549.

Azcona-Olivera, J.I., M. Abouzied, R.D. Plattner, W.P. Norred and J.J. Pestka. 1992. Generation of antibodies reactive with fumonisins B_1, B_2, and B_3 by using cholera toxin as the carrier-adjuvant. Applied and Environmental Microbiology 58:169-173

Baldo, B.A., T.C. Fletcher and J. Pepys. 1977. Isolation of a peptidopolysaccharide from the dermatophyte *Epidermophyton floccosum*, and a study of its reaction

with human C-reactive protein and a mouse anti-phosphorylcholine myeloma serum. Immunology 32:831-842.

Balloul, J.M., P. Sondermeyer, D. Dreyer, M. Capron, J.M. Grzych, R.J. Pierce, D. Carvallo, J.P. Lecocq and A. Capron. 1987. Molecular cloning of a protective antigen of schistosomes. Nature 326:149-153.

Baltar, P., J. Leiro, M.T. Santamarina, M.C. Porto and F.M. Ubeira. 1991. Specific immunosuppression by *Trichinella*: fine specificity and effect on lymphocyte function in vivo. Parasitology 10:411-418.

Behnke, J.M. and F.N. Wahid. 1991. Immunological relationships during primary infection with *Heligmosomoides polygyrus* (*Nematospiroides dubius*): H-2 linked genes determine worm survival. Parasitology 103:157-164.

Bell, R.G., D.D. McGregor and L.S. Adams. 1982a. *Trichinella spiralis*: Characterization and strain distibution of rapid expulsion in inbred mice. Experimental Parasitology 53:301-314.

Bell, R.G., D.D. McGregor and L.S. Adams. 1982b. *Trichinella spiralis*: Genetic basis for differential expression of phase-specific intestinal immunity in inbred mice. Experimental Parasitology 53:315-325.

Bell, R.G., D.D. McGregregor, M.C. Woan, and L.S. Adams. 1983. *Trichinella spiralis*: Selective intestinal immune

deviation in the rat. Experimental Parasitology 56:129-142.

Bell, R.G., L.S. Adams and R.W. Ogden. 1984a. A single gene determines rapid expulsion of *Trichinella spiralis* in mice. Infection and Immunity 45:273-275.

Bell, R.G., L.S., Adams, and R.W. Ogden. 1984b. *Trichinella spiralis*: genetics of worm expulsion in inbred and F1 mice infected with different worm doses. Experimental Parasitology 58:345:355.

Bell, R.G. 1988. Genetic analysis of expulsion of adult *Trichinella spiralis* in NFS, C3H/He, and B10.BR mice. Experimental Parasitology 66:57-65.

Bell, R.G. and W.M. Liu. 1988. *Trichinella spiralis*: Quantitative relationships between intestinal worm burden, worm rejection, and the measurement of intestinal immunity in inbred mice. Experimental Parasitology 66:44-56.

Belzunces, L.P., J. Toutant and M. Bounais. 1988. Acetylcholinesterase from *Apis molifera* head. Evidence for amphiphilic and hydrophilic forms characterized by Triton X-114 phase separation. Biochemical Journal 255:463-470.

Benacerraf, B. and D.H. Katz. 1975. The nature and function of histocompatibility-linked immune response genes. In: Immunogenetics and Immunity (ed. B. Benecerraf). pp 117-178. University Park Press,

Baltimore Maryland.

Biozzi, G., C. Stiffel, D. Mouton and Y. Bouthillier. 1975. Selection of lines of mice with high and low antibody response to complex immunogens. In: Immunogenetics and Immunodeficiency. (ed. B. Benacerraf) p. 180-227. University Park Press, Baltimore.

Bolas-Fernandez, F. and D. Wakelin. 1989. Infectivity of *Trichinella* isolates in mice is determined by host immune responsiveness. Parasitology 99:83-88.

Bolas-Fernandez, F. and Wakelin. 1990. Infectivity, antigenicity and host responses to isolates of the genus *Trichinella*. Parasitology 100:491-497.

Borst, P. 1991. Molecular genetics of antigenic variation. Immunoparasitology Today 12:A29-A33.

Bosma, M.J. and A.M. Carroll. 1991. The SCID mouse mutant: Definition, characterization, and potential uses. Annual Review of Immunology 9:323.

Bourguin, I., T. Chardes, M. Mevelec, J.P. Woodman and D.P. Bout. 1991. Amplification of the secretory IgA response to *Toxoplasma gondii* using cholera toxin. FEMS Microbiology Letters 81:265-272.

Brindley P.J., S. He, P. Sitepu, W.A. Pattie and C. Dobson. 1986. Inheritance of immunity in mice to challenge infection with *Nematospiroides dubius*. Heredity 57:53-58.

Briles, D.E., C. Forman, J.C. Horowitz, J.E. Volanakis, W.H. Benjamin Jr., L.S. McDaniel, J. Eldridge and J. Brooks. 1989. Antipneumococcal effects of C-reactive protein and monoclonal antibodies to pneumococcal cell wall and capsular antigens. Infection and Immunity 57:1457-1464.

Briles, D.E., C. Forman and M. Crain. 1992. Mouse antibody to phosphocholine can protect mice from infection with mouse-virulent human isolates of *Streptococcus pneumoniae*. Infection and Immunity 60:1957-1962.

Bromander, A., J. Holmgren and N. Lycke. 1991. Cholera toxin stimulates Il-1 production and enhances antigen presentation by macrophages in vitro. Journal of Immunology 146:2908-2914.

Brown, A.R. and C.A. Crandall. 1976. A phosphorylcholine idiotype related to TEPC 15 in mice infected with *Ascaris suum*. Journal of Immunology 116:1105-1109.

Brown, P.J., J. Charley-Poulain and P. Pery. 1981a. *Nippostrongylus brasiliensis* infection in the rat: I. Production of bile IgA and serum IgG antibodies by adult rats. Veterinary Immunology and Immunopathology 2:343-352.

Brown, P.J., J. Charley-Poulain and P. Pery. 1981b. *Nippostrongylus brasiliensis* infection in the rat: 2. A comparison of production of antibodies in bile and serum by baby rats, lactating rats and adult rats.

Veterinary Immunology and Immunopathology 2:475-482.

Brown, T.R. 1897. Studies on trichinosis, with especial reference to the increase of the eosinophilic cells in the blood and muscle, the origin of these cells and their diagnostic importance. Journal of Experimental Medicine 3:315-347.

Bruderer, U., R. Aebersold, K. Blaser and C.H. Heusser. 1988. Characterization of the Group I and Group II antibody response against PC-KLH in normal and T15 idiotype-suppressed BALB/c mice. Immunology 64:385-390.

Brundish, D.E. and J. Baddiley. 1968. Pneumococcal C-Substance, a ribitol teichoic acid containing choline phosphate. Biochemical Journal 110:573-582.

Campbell, C.H. 1955. The antigenic role of the excretions and secretions of *Trichinella spiralis* in the production of immunity in mice. Journal of Parasitology 41:483-491.

Campbell, W.C. 1983. Historical Introduction. In: Trichinella and Trichinosis. (ed. W.C. Campbell). p. 1-30. Plenum, New York.

Cebra, J.J., A.C. Logan and P.D. Weinstein. 1991. The preference for switching to expression of the IgA isotype of antibody exhibited by B lymphocytes in Peyer's patches is likely due to intrinsic properties of their microenviroment. Immunologic Research 10:393-395.

Chang, S. and C.H. Opperman. 1991. Characterization of acetylcholinesterase molecular forms of the root-knot nematode, *Meloidogyne*. Molecular and Biochemical Parasitology 49:205-214.

Chang, S. and Opperman. 1992. Separation and characterization of *Heterodera glycines* acetylcholinesterase molecular forms. Journal of Nematology 24:148-155.

Chang, S.P., M. Brown and M.B. Rittenberg. 1982a. Immunological memory to phosphorylcholine. II. PC-KLH induces two antibody populations that dominate different isotypes. Journal of Immunology 128:702-706.

Chang, S.P., M. Brown and M.B. Rittenberg. 1982b. Immunological memory to phosphorylcholine. III. IgM includes a fine specificity population distinct from TEPC-15. Journal of Immunology 129:1559-1562.

Chang, S.P., R.M. Perlmutter, M. Brown, C.H. Heusser, L. Hood, and M.B. Rittenberg. 1984. Immunological memory to phosphocholine. IV. Hybridomas representative of group I (T15-like) and Group II (non-T15-like) antibodies utilize distinct V_H genes. Journal of Immunology 132:1550-1555.

Chen, B.P. and P. Parham. 1989. Direct binding of influenza peptides to class I HLA molecules. Nature 337:743-

Chien, N.C., R.R. Pollock, C. Desaymard and M.D. Scharff.

1988. Point mutations cause the somatic diversification of IgM and IgG2a antiphosphorylcholine antibodies. Journal of Experimental Medicine 167:954-973.

Chiller, J.M. and A.L. Glasebrook. 1988. Oral tolerance and the induction of T cell unresponsiveness. Monographs in Allergy 24:256-265

Chipman, P.B. 1957. The antigenic role of the excretions and secretions of adult *Trichinella spiralis* in the production of immunity in mice. Journal of Parasitology 43:593-598.

Choy, W.F., M.H. Ng and P.L. Lim. 1991. *Trichinella spiralis*: Light microscope monoclonal antibody localization and immunochemical characterization of phosphorylcholine and other antigens in the muscle larva. Experimental Parasitology 73:172-183.

Clarke, C.J., A.D. Wilson, N.A. Williams and C.R. Stokes. 1991. Mucosal priming of T-lymphocyte responses to fed protein antigens using cholera toxin as an adjuvant. Immunology 72:322-328.

Cohn, M. 1967. Natural history of myeloma. Cold Spring Harbor Symposium of Quantitative Biology 32:211-221.

Crandall, R.B., J.J. Cebra and C.A. Crandall. 1967. The relative proportion of IgG, IgA and IgM containing cells in rabbit tissues during experimental trichinosis. Immunology 12:147-158.

Crandall C.A. and R.B. Crandall. 1971. *Ascaris suum*: Immunoglobulin responses in mice. Experimental Parasitology 30:426-437.

Crandall, R.B. and C.A. Crandall. 1972. *Trichinella spiralis*: Immunologic response to infection in mice. Experimental Parasitology 31:378-398.

Culbertson, J.T. 1942. Active immunity in mice against *Trichinella spiralis*. Journal of Parasitology 28:197-202.

Cypess, R.H., J.L. Ebersole and J.A. Molinari. 1977. Specific antibody levels in the intestinal perfusates of *Heligmosomoides polygyrus*. International Archives of Allergy and Applied Immunology 55:496-503.

De Aizpurua, H.J. and G.J. Russell-Jones. 1988. Oral vaccination. Identification of classes of proteins that provoke an immune response upon oral feeding. Journal of Experimental Medicine 167:440-451.

Delacroix, D.L., G.N. Malburny and J.P. Vaerman. 1985. Hepatobiliary transport of plasma IgA in the mouse: contribution to clearance of intravascular IgA. European Journal of Immunology 15:893-899.

Denkers, E.Y., D.L. Wassom, J. Krco and C.E. Hayes. 1990. The mouse antibody response to *Trichinella spiralis* defines a single, immunodominant epitope shared by multiple antigens. Journal of Immunology 144:3152-3159.

Despommier, D.D. and B.S. Wastmann. 1969. *Trichinella*

spiralis: Immune elimination in mice. Experimental Parsitology 24:243-250.

Despommier D.D., W.C. Campbell, and L.S. Blair. 1977. The in vivo and in vitro analysis of immunity to *Trichinella spiralis* in mice and rats. Parasitology 74:109-119.

Despommier, D.D. and A. Laccetti. 1981a. *Trichinella spiralis*: proteins and antigens isolated from a large particle fraction derived from the muscle larva. Experimental Parasitology 51:279-295.

Despommier, D.D. and A. Laccetti. 1981b. *Trichinella spiralis*: Partial characterization of antigens isolated by immuno-affinity chromatography from the large particle fraction of the muscle larvae. Journal of Parasitology 67:332-339.

Despommier, D.D. 1983. Biology. In: Trichinella and Trichinosis. (ed. W.C. Campbell). p. 75. Plenum, New York.

Dessein, A.J., W.L. Parker, S.L. James and J.R. David. 1981. IgE antibody and resistance to infection. I. Selective suppression of the IgE antibody response in rats diminishes the resistance and the eosinophil response to *Trichinella spiralis* infection. Journal of Experimental Medicine 153:423-436.

deVos, T. and T.A. Dick. 1991. A rapid method to determine the isotype and specificity of coproantibodies in mice

infected with *Trichinella* or fed cholera toxin. Journal of Immunological Methods 141:285-288.

deVos, T., G. Danell and T.A. Dick. 1992. *Trichinella spiralis*: Kinetics and dose dependence of the mucosal immunoglobulin response in mice. Experimental Parasitology (in press).

Dick, T.A. and B.B. Silver. 1980. Intestinal distribution of *Trichinella spiralis* in rats. Journal of Parasitology 66:472-477.

Dick, T.A. 1983. Species and intraspecific variability. In: *Trichinella* and Trichinosis. (ed. W.C. Campbell) p.31-73. Plenum Press, New York.

Dick, T.A., D.A. Dougherty, and D.L. Wassom. 1988. *Trichinella spiralis* infections of inbred mice: Genetics of the host response following infection with different *Trichinella* isolates. Journal of Parasitology 74:665-669.

Dick, T.A., T. deVos and J. Dupouy-Camet. 1990. Identification of two isolates of *Trichinella* recovered from humans in France. Journal of Parasitology 76:41-44.

Ellman, G.L., K.D. Courtney, V. Andres and R. Featherstone. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology 7:88-95.

Else, K. and D. Wakelin. 1988. The effects of H-2 and non-

H-2 genes on the expulsion of the nematode *Trichuris muris* from inbred and congenic mice. Parasitology 96:543-550.

Else, K.J. and D. Wakelin. 1989. Genetic variation in the humoral responses of mice to the nematode *Trichuris muris*. Parasite Immunology 11:77-90.

Else, K.J., D. Wakelin, D.L. Wassom and K.M. Hauda. 1990a. The influence of genes mapping within the major histocompatibility complex on resistance to *Trichuris muris*. Parasitology 101:61-67.

Else, K.J., D. Wakelin, D.L. Wassom and K.M. Hauda. 1990b. MHC-restricted antibody responses to *Trichuris muris* excretory/secretory (E/S) antigen. Parasite Immunology 12:509-527.

Else, K.J., L. Hultner and R.K. Grencis. 1992. Cellular immune responses to the murine nematode parasite *Trichuris muris* II. Differential induction of Th-cell subsets in resistant versus susceptible mice. Immunology 75:232-237.

Elson, C.O. and W. Ealding. 1984a. Generalized systemic and mucosal immunity in mice after mucosal stimulation with cholera toxin. Journal of Immunology 132:2736-2741.

Elson, C.O. and W. Ealding. 1984b. Cholera toxin feeding did not induce oral tolerance in mice and abrogated oral tolerance to an unrelated protein antigen. Journal of Immunology 133:2892-2897.

Elson, C.O., W. Ealding, and J. Lefkowitz. 1984. A lavage technique allowing repeated measurement of IgA antibody in mouse intestinal secretions. Journal of Immunological Methods 67:101-108.

Elson, C.O. and W. Ealding. 1987. Ir gene control of the murine secretory IgA response to cholera toxin. European Journal of Immunology 17:425-428.

Enriquez, F.J., J.L. Zidian and R.J. Cypess. 1988a. *Nematospiroides dubius*: Genetic control of immunity to infections of mice. Experimental Parasitology 67:12-19.

Enriquez, F.J., B.O. Brooks, R.J. Cypess, C.S. David, and D.L. Wassom. 1988b. *Nematospiroides dubius*: Two H-2 linked genes influence levels of resistance to infection in mice. Experimental Parasitology 67:221-226.

Faubert, G.M. and C.E. Tanner. 1974. The suppression of sheep rosette-forming cells and the inability of mouse bone marrow cells to reconstitute competence after infection with the nematode *Trichinella spiralis*. Immunology 27:501-505.

Faubert, G.M. 1976. Depression of the plaque-forming cells to sheep red blood cells by the new born larvae of *Trichinella spiralis*. Immunology 34:485-489.

Feeney, A.J., S.H. Clarke and D.E. Mosier. 1988. Specific H chain junctional diversity may be required for non-

T15 antibodies to bind phosphorylcholine. Journal of Immunology 141:1267-1272.

Fiorentino, D.F., A. Zlotnik, P. Vieira, T.R. Mosmann, M. Howard, K.W. Moore and A. O'Garra. 1991. Il-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. Journal of Immunology 146:3444-3451.

Fournier, D., J. Bride, F. Karch and J. Berge. 1988. Acetlycholinesterase from *Drosophila melanogaster*. Identification of two subunits encoded by the same gene. FEBS 238:333-337.

Freier, S., M. Eran and I. Alon. 1989. A study of stimuli operative in the release of antibodies in the rat intestine. Immunological Investigations 18:431-447.

Freter, R., S.P. De, A. Mondal, D.L. Shrivastava and Sunderman, F.W. 1964. Coproantibody and serum antibody in cholera patients. Journal of Infectious Disease 115:83-87.

Fung, J. and H. Kohler. 1980. Late clonal selection and expansion of the TEPC-15 germ-line specificity. Journal of Experimental Medicine 153:1262-1273.

Gamble, H.R. 1985a. Comparison of immune effects in mice immunized with *Trichinella spiralis* adult and larval antigens. Journal of Parasitology 71:680-682.

Gamble, H.R. 1985b. *Trichinella spiralis* : Immunization of mice using monoclonal antibody affinity-isolated

antigens. Experimental Parasitology 59:398-404.

Garate, T., M.M. Kliks, Z. Cabrera and R.M.E. Parkehouse. 1990. Specific and cross-reacting antibodies in human responses to *Onchocerca volvulus* and *Dracunculus medinensis* infections. American Journal of Tropical Medicine and Hygiene 42:140-147.

Gould, S.E. 1970. History. In: Trichinosis in man and animals. (ed. S.E. Gould). p. 3-18. C.C. Thomas, Springfield, Ill.

Grencis, R.K., J. Riedlinger and D. Wakelin. 1985. L3T4-positive T lymphoblasts are responsible for transfer of immunity to *Trichinella spiralis* in mice. Immunology 56:213-218.

Grencis, R.K., C. Crawford, D.I. Pritchard, J.M. Behnke and D. Wakelin. 1986. Immunization of mice with surface antigens from the muscle larvae of *Trichinella spiralis*. Parasite Immunology 8:587-596.

Gualzata, M., N. Weiss, and Ch. H. Heusser. 1986. *Dipetalonema viteae*: Phosphorylcholine and non-phosphorylcholine antigenic determinants in infective larvae and adult worms. Experimental Parasitology 61:95-102.

Gutman G.A. and G.F. Mitchell. 1977. *Ascaris suum*: Location of phosphorylcholine in lung larvae. Experimental Parasitology 43:161-168.

Hirabayasi Y., S.I. Tamura, Y. Suzuki, T. Nagamine, C.

Aizawa, K. Shimada, and T. Kurata. 1991. H-2-unrestricted adjuvant effect of cholera toxin B subunit on murine antibody responses to influenza virus haemagglutinin. Immunology 72:329-335.

Hirabayasi Y., S.I. Tamura, K. Shimada, and T. Kurata. 1992. Involvement of antigen-presenting cells in the enhancement of the in vitro antibody responses by cholera toxin B subunit. Immunology 75:493-498.

Holmgren, J. 1981. Actions of cholera toxin and the prevention and treatment of cholera. Nature 292:413-417.

Holmgren, J. and A. Svennerholm. 1990. Development of oral vaccines against cholera and entertoxinogenic *Escherichia coli* diarrhea. Scandinavian Journal of Infectious Diseases Supplement 76:47-53.

Jackson, G.J. 1959. Flourescent antibody studies of *Trichinella spiralis* infections. Journal of Infectious Disease 105:97-117.

Jacqueline, E., A. Vernes, D. Bout and J. Biguet. 1978. *Trichinella spiralis*: Facteurs immunitaires inhibiteurs de la production de larves II. Experimental Parasitology 45:42-54.

Jacqueline, E., J. Crinquette, D. Bout, J. Barrois and A. Vernes. 1981. *Trichinella spiralis* in rats: in vivo effects of the bile and in vitro action of secretory IgA from bile. Annales de Parasitologie (Paris)

56:395-400.

Johnson C.D. and R.L. Russell. 1983. Multiple molecular forms of acetylcholinesterase in the nematode *Caenorhabditis elegans*. Journal of Neurochemistry 41:30-46.

Johnson, C.D., J.B. Rand, R.K. Herman, B.D. Stern and R.L. Russell. 1988. The acetylcholinesterase genes of *C. elegans*. Identification of a third gene (*ace-3*) and mosaic mapping of a synthetic lethal phenotype. Neuron 1:165-173.

Jungery, M. and B.M. Ogilvie. 1982. Antibody response to stage-specific *Trichinella spiralis* surface antigens in strong and weak responder mouse strains. J. Immunol. 129:839-843.

Kagan, I.G. and L. G. Norman. 1970. The serology of Trichinosis. In: Trichinosis in man and animals. (ed. S.E. Gould). p. 222-268. C.C. Thomas, Springfield, Ill.

Kagnoff, M.F. 1982. Oral tolerance. Annals of the New York Academy of Sciences 392:248-265.

Kappler, J.W., U. Staerz, J. White and P.C. Marrack. 1988. Self-tolerance eliminates T cells specific for Mls-modified products of the major histocompatability complex. Nature 332:35-40.

Karnovsky, M. and L. Roots. 1964. A "direct-coloring" thiocholine method for cholinesterases. Journal of

Histochemistry and Cytochemistry 12:219-221.

Kelly, E.A.B., E.S. Cruz, K.M. Hauda and D.L. Wassom. 1991. IFN-gamma and IL-5 producing cells compartmentalize to different lymphoid organs in *Trichinella spiralis* infected mice. Journal of Immunology 147:306-311.

Kennedy, M.W. 1980. Effects of the host immune response on the longevity, fecundity and position in the intestine of *Trichinella spiralis* in mice. Parasitology 80:49-60.

Kennedy, M.W. and R.G. Bruce. 1981. Reversibility of the effects of the host immune response on the intestinal phase of *Trichinella spiralis* in the mouse, following transplantation to a new host. Parasitology 82:39-48.

Kennedy, M.W., D.L. Wassom, A.E. Mcintosh and J.C. Thomas. 1991. H-2 (I-A) control of the antibody repertoire to secreted antigens of *Trichinella spiralis* in infection and its relevance to resistance and susceptibiltiy. Immunology 73:36-43.

Kim, C.W. 1957. Immunity to *Trichinella spiralis* in mice infected with irradiated larvae. Journal of the Elisa Mitchell Society 73:308-317.

Klinman, N.R. and M.R. Stone. 1983. Role of variable region gene expression and enviromental selection in determining the antiphosphorylcholine B cell repertoire. Journal of Experimental Medicine 158:1948-1961.

Kolson D.L. and R.L. Russell. 1985. A novel class of acetylcholinesterase revealed by mutations in the nematode *Caenorhabditis elegans*. Journal of Neurogenetics 2:93-110.

Korenaga, M., C.H. Wang, R.G. Bell, D. Zhu and A. Ahmad. 1989. Intestinal immunity to *Trichinella spiralis* is a property of $OX8^-$ $OX22^-$ T-helper cells that are generated in the intestine. Immunology 66:588-594.

Kozek, W.J. and R.B. Crandall. 1972. Intestinal and serum immunoglobulins and antibodies in mice infected with *Trichinella*. In: Trichinellosis. (ed. C.W. Kim). p. 157-164.

Kullberg, M., E.J. Pearce, S.E. Hieny, A. Sher and J. Berzofsky. 1992. Infection with *Schistosoma mansoni* alters Th1/Th2 cytokine responses to a non-parasite antigen. Journal of Immunology 148:3264-3270.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227:680-685.

Lal, R.B. and E.A. Ottesen. 1989. Phosphocholine epitopes on helminth and protozoal parasites and their presence in the circulation of infected human patients. Transactions of the Royal Society of Tropical Medicine and Hygiene 83:652-655.

Larsh, J.E. and J.R. Hendricks. 1949. The probable explanation for the difference in the localization of

adult *Trichinella spiralis* in young and old mice. Journal of Parasitology 35:101-106.

Larsh, J.E. and G.J. Race. 1954. A histopathological study of the anterior small intestine of immmunized and nonimmunized mice infected with *Trichinella spiralis*. Journal of Infectious Diseases 94:262-272.

Larsh, J.E. 1967. The present understanding of the mechanism of immunity to *Trichinella spiralis*. American Journal of Tropical Medicine and Hygiene 16:123-132.

Larsh, J.E. 1970. Immunology. In: Trichinosis in man and animals. (ed. S.E. Gould) pp 129-146. C.C. Thomas Publisher. Springfield Ill.

Larsh, J.E. and G.J. Race. 1975. Allergic inflamation as a hypothesis for the expulsion of worms from tissues: A review. Experimental Parasitology 37:252-266.

Leon, M.A. and N.M. Young. 1970. Six mouse IgA myeloma proteins with phosphorylcholine specificity. Federation Proceedings 29:437.

Leon, M. and N.M. Young. 1971. Specificity of phosphorylcholine of six murine myeloma proteins reactive with *Pneumococcus* C polysaccharide and B-lipoprotein. Biochemistry 10:1424-1429.

Levin, A.J. and T.C. Evans. 1942. The use of roentgen radiation in locating an origin of host resistance to *Trichinella spiralis* infections. Journal of

Parasitology 28:477-483.

Liang, X., M.E. Lamm and J.G. Nedrud. 1989. Cholera toxin as a mucosal adjuvant. Glutaraldehyde treatment dissociates adjuvanticity from toxicity. Journal of Immunology 143:484-490.

Liew. F.Y. 1990. Regulation of cell-mediated immunity in Leishmaniasis. Current Topics in Microbiology and Immunology 155:53-64.

Lim, P.L. and W.F. Choy. 1990. A thymus-independent (type I) phosphorylcholine antigen isolated from *Trichinella spiralis* protects mice against pneumococcal infection. Immunology 69:443-448.

Lipton, M.M. and A.J. Steigman. 1962. Human coproantibody against polioviruses. Journal of Infectious Diseases 112:57-66.

Ljungstrom, I., J. Holmgren, G. Huldt, S. Lange and A. Svennerholm. 1980. Changes in intestinal fluid transport and immune responses to entertoxins due to concomitant parasitic infection. Infection and Immunity 30:734-740.

Luft, B.J. and C.W. Kim. 1989. T-cell dysfunction during acute murine Trichinellosis. In Trichinellosis. (ed. C.E. Tanner, A.R. Martinez and F. Bolas). p.153-158. CSIC Press, Madrid, Spain.

Lycke, N. and J. Holmgren. 1986. Strong adjuvant properties of cholera toxin on gut mucosal responses to

orally presented antigens. Immunology 59:301-308.

Lycke, N. and J. Holmgren. 1987. Long-term cholera antitoxin memory in the gut can be triggered to antibody formation associated with protection within hours of an oral challenge immunization. Scandinavian Journal of Immunology 25:407-412.

Lycke, N. and J. Holmgren. 1989. Adoptive transfer of gut mucosal antitoxin memory by isolated B cells 1 year after oral immunization with cholera toxin. Infection and Immunity 57:1137-1141.

Lycke, N., A. Bromander, L. Ekman, U. Karlsson and J. Holmgren. 1989a. Cellular basis of immunomodulation by cholera toxin in vitro with possible assocation to the adjuvant function in vivo. Journal of Immunology 142:20-27.

Lycke, N., A. Bromander, and J. Holmgren. 1989b. Role of local IgA antitoxin-producing cells for intestinal protection against cholera toxin challenge. International Archives of Allergy and Applied Immunology 88:273-279.

Lycke, N. and W. Strober. 1989. Cholera toxin promotes B cell isotype differentiation. Journal of Immunology 142:3781

Lycke, N., E. Severinson and W. Strober. 1990. Cholera toxin acts synergistically with IL-4 to promote IgG1 switch differentiation. Journal of Immunology 145:3316-

3324.

Lycke, N., U. Karlsson, A. Sjolander and K.E. Magnusson. 1991a. The adjuvant action of cholera toxin is associated with an increased intestinal permeability for luminal antigens. Scandinavian Journal of Immunology 33:691-698.

Lycke, N., E. Severinson and W. Strober. 1991b. Molecular effects of cholera toxin on isotype differentiation. Immunologic Research 10:407-412.

MacKenzie, C.D., M. Jungery, P.M. Taylor and B.M. Ogilvie. 1980. Activation of complement, the induction of antibodies to the surface of nematodes and the effect of these factors and cells on worm survival in vitro. European Journal of Immunology 10:594-601.

Maclean, J.M., M. Abebe, H. Wedrychowicz and P.H. Holmes. 1986. Local and systemic antibody responses in gerbils following vaccination with irradiated or non-irradiated *Trichostrongylus colubriformis* larvae. Journal of Helminthology 60:263-278.

Manning, R.J., P.G. Walker, L. Carter, P.J. Barrington and G.D.F. Jackson. 1984. Studies on the origins of biliary immunoglobulins in rats. Gastroenterology 87:173-179.

Marten, R.G. and B.N. Ames. 1961. A method for determining the sedimentation behaviour of enzymes: Application to protein mixtures. Journal of Biological Chemistry

236:1372-1379.

Marti, H.P., K.D. Murrell and H.R. Gamble. 1987. *Trichinella spiralis* : Immunization of pigs with newborn larval antigens. Experimental Parasitology 63:68-73.

Mazengra, R.L. and M.A. Kerr. 1990. The specificity of the IgA receptor purified from human neutrophils. Biochemical Journal 272:159-165.

McClaren, D.J. 1976. Sense organs and their secretions. In: The organization of nematodes. (ed. N.A. Croll). pp 139-162. Academic Press, London.

McCoy, O.R. 1931. Immunity of rats to reinfection with *Trichinella spiralis*. American Journal of Hygiene 14:484-494.

McCoy, O.R. 1935. Artificial immunization of rats against *Trichinella spiralis*. American Journal of Hygiene 21:200-213.

McWilliam, A.S., G.A. Sterwart and W. Allen. 1986. Phosphorylcholine bearing components of some helminthic parasites: Localization to parasite lipoproteins. Comparative Biochemistry and Physiology 85B:627-633.

Mestecky, J., and J.R. McGhee. 1987. Immunoglobulin A (IgA): Molecular and cellular interactions involved in IgA biosynthesis and immune response. Advances in Immunology 40:153-245.

Michaeli, D., J. Pinto, E. Benjamini and F.P. deBuren.

1969. Immunoenzymology of acetylcholinesterase-I. Substrate specificity and heat stability of acetylcholinesterases and of acetylcholinesterase-antibody complexes. Immunochemistry 6:101-109.

Miller, H.R.P. 1984. The protective mucosal response against gastrointestinal nematodes in ruminants and laboratory animals. Veterinary Immunology and Immunopathology 6:167-259.

Mitchell, G.F., R.S. Hogarth-Scott, R.D. Edwards, H.M. Lewers, G. Cousins and T. Moore. 1976. Studies on Immune responses to parasite antigens in mice. I - *Ascaris suum* larvae numbers and antiphosphorylcholine response in infected mice of various strains and in hypothymic nu/nu mice. International Archives of Allergy and Applied Immunology 52:64-78.

Mitchell, G.F. and H.M. Lewers. 1976. Studies on immune responses to parasite antigens in mice. IV. Inhibition of an anti-DNP antibody response with the antigen, DNP-Ficoll containing phosphorylcholine. International Archives of Allergy and Applied Immunology 52:235-240.

Monroy, F.G. and F.J. Enriquez. 1992. *Heligmosomoides polygyrus*: a model for chronic gastrointestinal helminthiasis. Parasitology Today 8:49-54.

Moss, J. and M. Vaughan. 1990. ADP-Ribosylation of guanyl nucleotide binding regulatory proteins by bacterial

toxins. Advances in Enzymology 61:303-379.

Nussenzweig, V. and R.S. Nussenzweig. 1989. Rationale for the development of an engineered sporozoite malaria vaccine. Advances in Immunology 45:283-334.

Ogilvie, B.M., T.L.W. Rothwell, K.C. Bremner, H.J. Schnitzerling, J. Nolan and R.K. Keith. 1973. Acetylcholinesterase secretion by parasitic nematodes --I. Evidence for secretion of the enzyme by a number of species. International Journal of Parasitology 3:589-597.

O'Hagan, D.T., K.J. Palin and S.S. Davis. 1987. Intestinal absorption of proteins and macromolecules and the immunological response. CRC Critical Reviews in Theraputic Drug Carrier Systems 4:197

Oliver-Gonzalez, J. 1940. The in vitro action of immune serum on the larvae and adults of *Trichinella spiralis*. Journal of Infectious Disease 67:292-300.

Opheim, D.J. and O. Touster. 1978. Alpha-1-fucosidase from rat liver lysosomes. Methods in Enzymology 50:505-510.

Opie, E.L. 1904. An experimental study of the relation of cells with eosinophile granulation to infection with an animal parasite (*Trichina spiralis*). American Journal of Medical Science 127:477-493.

Ottesen, E.A., T.K. Smith and C.H. Kirkpatrick. 1975. Immune response to *Trichinella spiralis* in the rat I. Development of cellular and humoral responses during

chronic infection. International Archives of Allergy and Applied Immunology 49:396-410.

Ouchterlony, O. 1968. Handbook of immunodiffusion and immunoelectrophoresis. Ann Arbor Science Publishers Inc. Ann Arbor, Michigan. 215 pp.

Owen, R. 1835. Description of a microscopic entozoon infesting the muscles of the human body. Transactions of the Zoological Society of London 1:315-323.

Pearce, E.J. and A. Sher. 1991. Functional dichotomy in the $CD4^+$ T cell response to *Schistosoma mansoni*. Experimental Parasitology 73:110-116.

Perrudet-Badoux, A., R.A. Binigha and G. Biozzi. 1975. *Trichinella* infestation in mice genetically selected for high and low antibody production. Immunology 29:387-390.

Perrudet-Badoux, A., R.A. Binigha and Y. Boussac-Aron. 1978. *Trichinella spiralis* infection in mice. Mechanism of the resistance in animals genetically selected for high and low antibody produciton. Immunology 35:519-522.

Perry, R.H. 1974. Transfer of immunity to *Trichinella spiralis* from mothers to offspring. Journal of Parasitology 60:460-465.

Pery, P., A. Petit, J. Poulain and G. Luffua. 1974. Phosphorylcholine bearing components in homogenates of nematodes. European Journal of Immunology 4:637-639.

Pery, P., G. Luffau, J. Charley, A. Petit, P. Rouze and S. Bernard. 1979a. Phosphorylcholine antigens from *Nippostrongylus brasiliensis*. I - Anti-phosphorylcholine antibodies in infected rats and location of phosphorylcholine antigens. Annales of Immunology (Institute Pasteur) 130C:879-888.

Pery, P., G. Luffau, J. Charley, A. Petit, P. Rouze and S. Bernard. 1979b. Phosphorylcholine antigens from *Nippostrongylus brasiliensis*. II - Isolation and partial characterization of phosphorylcholine antigens from adult worm. Annales of Immunology (Institute Pasteur) 130C:889-900.

Pierce, N.F. and J.L. Gowans. 1975. Cellular kinetics of the intestinal immune response to cholera toxoid in rats. Journal of Experimental Medicine 142:1550-1563.

Pierce, N.F., W.C. Cray and B.K. Sircar. 1978. Induction of a mucosal antitoxin response and its role in immunity to experimental canine cholera. Infection and Immunity 21:185-193.

Pierre, P., A. Langendries and J.P. Vaerman. 1988. Cholera toxin neutralization: a comparison of purified serum IgG and biliary secretory IgA antibodies. Immunological Letters 18:51-56.

Pond, L., D.L. Wassom and C.E. Hayes. 1989. Evidence for differential induction of helper T cell subsets during *Trichinella spiralis* infection. Journal of Immunology

143:4232-4237.

Potter, M. 1970. Mouse IgA myeloma proteins that bind polysaccharide antigens of enterobacterial origin. Federation Proceedings 29:85-91.

Potter, M. and R. Lieberman. 1970. Common individual antigenic determinants in five of eight BALB/c IgA myeloma proteins that bind phosphoryl choline. Journal of Experimental Medicine 132:737-751.

Potter, M. 1972. Antigen binding myeloma proteins in mice. Annals of the New York Academy of Sciences 190:306-321.

Potter, M. 1985. History of the BALB/c family. Current Topics in Microbiology and Immunology 122:1-5.

Poulain, J., G. Luffau and P. Pery. 1976. *Nippostrongylus brasiliensis* in the rat: Immune response in serum and intestinal secretions. Annales Immunologie (Institute Pasteur) 127C:215-224.

Pritchard, D.I., K.V. Legget, M.T. Rogan, P.G. McKean and A. Brown. 1991. *Necator americanus* secretory cholinesterase and its purification from excretory-secretory products by affinity chromatography. Parasite Immunology 13:187-199.

Quan Z.S. and J. Quintans. 1981. Alterations of clonal profile. III. T15 ontogenetic advantages are not sufficient for establishing idiotyopic dominance in adoptive transfer. Jouranl of Experimental Medicine 154:1475-1488.

Rathuar, S., B.D. Robertson, M.E. Selkirk and R.M. Maizels. 1987. Secretory acetylcholinesterase from *Brugia malayi* adult and microfilarial parasites. Molecular and Biochemical Parasitology 26:257-265.

Rappaport, I., and H.S. Wells. 1951. Studies in trichinosis I. Immunity to reinfection in mice following a single light infection. Journal of Infectious Disease 88:258-253.

Reiner, E., M. Skrinjaric-Spoljar, M. Kralj and S. Krvavica. 1978. Kinetic properties of the cholinesterase in *Metastrongylus apri* (Nematoda): substrate hydrolysis and reactions with organophosphorous compounds. Comparative Biochemistry and Physiology 60C:155-157.

Renegar, K.B., and P.A. Small. 1991. Passive transfer of local immunity to influenza virus infection by IgA antibody. Journal of Immunology 146:1972-1978.

Rhoads, M.L. 1981. Cholinesterase in the parasitic nematode, *Stephanurus dentatus*. Journal of Biological Chemistry 256:9316-9323

Rhoads, M.L. 1985. Glycosidases of *Trichinella spiralis*. Molecular and Biochemical Parasitology 16:137-148.

Rhoads, M.L. 1988. Purification, characterization and immunochemical studies of B-N-acetyl-D-hexosaminidase from the parasitic nematode *Trichinella spiralis*. Molecular and Biochmical Parasitology 31:57-70.

Rice, C.M., and S.J. O'Brien. 1980. Genetic variance of

laboratory outbred Swiss mice. Nature 283:157-161.

Richardson, S.H. and R.E. Kuhn. 1986. Studies on the genetic and cellular control of sensitivity to enterotoxins in the sealed adult mouse model. Infection and Immunity 54:522-528.

Rifai, A. and S.S. Wong. 1986. Preparation of phosphorycholine-conjugated antigens. Journal of Immunological Methods 94:25-30.

Robinson, M. and C.S. David. 1989. The genetics of the immune response to *Trichinella spiralis* antigens in the mouse. Advances in Experimental Biology and Medicine 251:329-340.

Robinson, M.W., C.J. Krco, T.G. Beito and C.S. David. 1991. Genetic control of the immune response to *Trichinella spiralis*: recognition of muscle larval antigens. Parasite Immunology 13:391-404.

Rothwell, T.L.W., B.M. Ogilvie and R.J. Love. 1973. Acetylcholinesterase secretion by parasitic nematodes - II. *Trichostrongylus* spp. International Journal of Parasitology 3:599-608.

Ruitenberg, E.J. and P.A. Steerenberg. 1974. Intestinal phase of *Trichinella spiralis* in congenitally athymic (nude) mice. Journal of Parasitology 60:1056-1057.

Ruitenberg, E.J., A. Perrudet-Badoux, Y. Boussac-Aron and A. Elgersma. 1980. *Trichinella spiralis* infection in animals genetically selected for high and low antibody

production. International Archives of Allergy and Applied Immunology 62:104-110.

Russell, M.W. and H. Wu. 1991. Distribution, persistence, and recall of serum and salivary antibody responses to peroral immunization with protein antigen I/II of *Streptococcus mutans* coupled to the cholera toxin B subunit. Infection and Immunity 59:4061-4070.

Sanderson, B.E. 1969. Acetylcholinesterase activity in *Nippostrongylus brasiliensis* (Nematoda). Comparative Biochemistry and Physiology 29:1207-1213.

Sandground, J.H. 1929. A consideration of the relation of host-specificity of helminths and other metazoan parasites to the phenomena of age resistance and acquired immunity. Parasitology 11:227-255.

Salzer, B.F. 1916. A study on an epidemic of fourteen cases of Trichinosis with cures by serum therapy. Journal of the American Medical Association 67:579-580

Schmucker, D.L., C.K. Daniels, R.K. Wang and K. Smith. 1988. Mucosal immune response to cholera toxin in ageing rats. I. Antibody and antibody-containing cell response. Immunology 64:691-695.

Schwartz, B. 1917. Serum therapy for Trichinosis. Journal of the American Medical Association. 69:884-886.

Schwartz, R.H. 1986. Immune response (Ir) genes of the murine major histocompatibility complex. Advances in Immunology 38:31-201.

Scott, P., P. Zatovitz, R.L. Coffman, E. Pearce and A. Sher. 1988. Immunoregulation of cutaneous Leishmaniasis. T cell lines that transfer protective immunity or exacerbation belong to different T helper subsets and respond to distinct parasite antigens. Journal of Experimental Medicine 168:1675-1684.

Sher, A., D. Fiorentino, P. Caspar, E. Pearce and T. Mosmann. 1991. Production of Il-10 by $CD4^+$ T lymphocytes correlates with downregulation of the Th1 cytokine synthesis in helminth infection. Journal of Immunology 147:2713-2716.

Silbart, L.K. and D.F. Keren. 1989. Reduction of intestinal carcinogen by carcinogen-specific secretory immunity. Science 243:1462-1464.

Silberstein, D.S. and D.D. Despommier. 1984. Antigens from *Trichinella spiralis* that induce a protective response in the mouse. Journal of Immunology 132:898-904.

Silberstein, D.S. and D.D. Despommier. 1985a. Effect on *Trichinella spiralis* of host responses to purified antigens. Science 227:948-950.

Silberstein D.S. and D.D. Despommier. 1985b. Immunization with purified antigens protects mice from lethal infection with *Trichinella spiralis*. Journal of Parasitology 71:516-517.

Silver, A. 1974. The biology of cholinesterases. North-Holland Publishing Co. New York. 596p.

Sinski, E. and P.H. Holmes. 1977. *Nippostrongylus brasiliensis*: Systematic and local IgA and IgG immunoglobulin responses in parasitized rats. Experimental Parasitology 43:382-389.

Sinski, E., E.L. Jeska and B. Bezubik. 1983. Enteral and parenteral responses in mice after primary infection with *Trichinella spiralis* (Nematoda). Journal of Parasitology 69:645-653.

Sitepu, P., C. Dobson and P.J. Brindley. 1985. Immunization and immunosuppression in mice reared for high or low immune responsiveness against *Nematospiroides dubius*. International Journal of Parasitology 15:277-281.

Sloan, T., D. Dooge and P. Joyce. 1991. Identification of phophorylcholine containing antigens of *Fasciola hepatica* - successful tolerization against this epitope in experimental animals. Parasite Immunology 13:447-455.

Sprent, J.F.A. 1963. Parasitism. University of Queensland Press, Queensland, Australia 143 pp.

Stefanski, W. and M. Kozar. 1969. Degree of resistance of some mouse strains to *Trichinella spiralis* infection. Wiadomosci Parzytologiczne 15:571-575.

Stenzel-Poore, M.P. and M.B. Rittenberg. 1989. Clonal diversity, somatic mutation, and immune memory to phosphocholine-keyhole limpet hemocyanin. Journal of

Immunology 143:4123-4133.

Stieger, S., R. Gentinetta and U. Brodbeck. 1989. Cholinesterases from flounder muscle. Purification and characterization of glycosyl-phosphatidylinositol-anchored and collagen-tailed forms differing in substrate specificity. European Journal of Biochemistry 181:633-642.

Strober, W. and M.C. Sneller. 1988. Cellular and molecular events accompanying IgA B cell differentiation. Monographs in Allergy 24:181-190.

Szu, S.C., S. Clarke and J.B. Robbins. 1983. Protection against Pneumococcal infection in mice conferred by phosphocholine-binding antibodies: Specificity of the phosphocholine binding and relation to several types. Infection and Immunity 39:993-999.

Tarrab-Hazdai, R., F. Levi-Schaffer, M. Smolarsky and R. Arnon. 1984. Acetylcholinesterase of *Schistosoma mansoni*: antigenic cross-reactivity with *Electrophorus electricus* and its functional implications. European Journal of Immunology 14:205-209.

Taylor, H.P. and N.J. Dimmock. 1985. Mechanism of neutraliztion of influenza virus by secretory IgA is different from that of monemeric IgA or IgG. Journal of Experimental Medicine. 161:198-209.

Taylor, P. 1991. The cholinesterases. Journal of Biological Chemistry 266:4025-4028.

Towbin, H., T. Staehelin and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proceedings of the National Acadamey of Sciences USA 76:4350-4354.

Tronchin, G., E. Dutoit, A. Vernes and J. Biguet. 1979. Oral immunization of mice with metabolic antigens of *Trichinella spiralis*: effects on the kinetics of intestinal cell response including mast cells and polymorphonuclear eosinophils. Journal of Parasitology 65:685-691.

Ubeira, F.M., J. Leiro, M.T. Santamarina, T.G. Villa and M.L. Sanmartin-Duran. 1987a. Immune response to *Trichinella* epitopes: the antiphosphorylcholine plaque-forming cell response during the biological cycle. Parasitology 94:543-553.

Ubeira, F.M., J. Leiro, M.T. Santamarina, and M.L. Sanmartin-Duran. 1987b. Modulation of the anti-phosphorylcholine immune response during *Trichinella spiralis* infections in mice. Parasitology 97:583-592.

Underdown, B.J., D.L. Skea, G.M. Loney and D.P. Snider. 1988. Murine giardiasis and mucosal immunity: A model for the study of imunity to intestinal protozoan parasites. Monographs in Allergy 24:287-296.

Urban, J.F. Jr., K.B. Madden, A. Svetic, A. Cheever, P.P. Trotta, W.C. Gause, I.M. Katona and F.D. Finkelman.

1992. The importance of Th2 cytokines in protective immunity to nematodes. Immunological Reviews 127:205-220.

Vajdy, M. and N.Y. Lycke. 1992. Cholera toxin adjuvant promotes long-term immunological memory in the gut mucosa to unrelated immunogens after oral immunization. Immunology 75:488-492.

VanLoveren, H., A.D.M.E. Osternaus, J. Nagel, H.J. Schuurman and J.G. Vos. 1987. Detection of IgA antibodies and quantification of IgA antibody producing cells specific for ovalbumin or *Trichinella spiralis* in the rat. Scandinavian Journal of Immunology 26:377-381.

Vernes, A. 1976. Immunization of the mouse and minipig against *Trichinella spiralis*. In: Biochemistry of parasites and host-parasite relationships. (H. Van den Bossche ed.) p 319. Elsevier/North Holland Biomedical Press, Amsterdam.

Wakelin, D. 1975. Genetic control of immune responses to parasites: immunity to Trichuris muris in inbred and random-bred strains of mice. Parasitology 71:51-60.

Wakelin, D. and M. Lloyd. 1976. Immunity to primary and challenge infections of *Trichinella spiralis* in mice: a re-examination of conventional paramaters. Parasitology 72:173-182.

Wakelin, D. 1978. Genetic control of susceptibility and resistance to parasitic infection. Advances in

Parasitology 16:219-308.

Wakelin, D. and M.M. Wilson. 1979. T and B cells in the transfer of immunity against *Trichinella spiralis* in mice. Immunology 37:103-109.

Wakelin, D. and A.M. Donachie. 1981. Genetic control of immunity to *Trichinella spiralis*. Donor bone marrow cells determine responses to infection in mouse radiation chimaeras. Immunology 43:787-792.

Wakelin, D. and A.M. Donachie. 1983. Genetic control of immunity to parasites: influence of H-2 linked genes on immunity to the intestinal phase of infection. Immunology 48:343

Wakelin, D. 1988. Helminth infections. In: Genetics of resistance to bacterial and parasitic infection. (eds. D.M. Wakelin and J.M. Blackwell) pp 153-224. Taylor and Francis Ltd. London.

Walls, R.S., R.L. Carter, E. Leuchars and A.J.S. Davies. 1973. The immunopathology of trichiniasis in T-Cell deficient mice. Clinical and Experimental Immunology 13:231-242.

Wang, C.H, M. Korenaga, F.R. Sacuto, A. Ahmad and R.G. Bell. 1990. Intraintestinal migration to the epithelium of protective, dividing anti-*Trichinella spiralis* $CD4^+$ OX22- cells requires MHC class II compatibility. Journal of Immunology 145:1021-1028.

Wassom, D.L. and G.J. Gleich. 1979. Damage to *Trichinella*

spiralis newborn larvae by eosinophil major basic protein. American Journal of Tropical Medicine and Hygeine 28:860-863.

Wassom, D.L., C.S. David and G.J. Gleich. 1979. Genes within the major histocompatibility complex influence susceptibility to *Trichinella spiralis* in the mouse. Immunogenetics 9:491-496.

Wassom, D.L., B.O. Brooks, J.G. Babish and C.S. David. 1983a. A gene mapping between the S and D regions of the H-2 complex influences resistance to *Trichinella spiralis* infections of mice. Journal of Immunogenetics 10:371.

Wassom, D.L., B.O. Brooks, R.H. Cypess, and C.S. David. 1983b. *Trichinella spiralis*: role of non-H-2 genes in resistance to primary infection in mice. Experimental Parasitology 55:153-158.

Wassom, D.L., D.A. Dougherty, C.J. Krco and C.S. David. 1984a. H-2-controlled dose-dependent suppression of the response that expels adult *Trichinella spiralis* from the small intestine of mice. Immunology 53:811-818.

Wassom, D.L., D. Wakelin, B.O. Brooks, C.J. Krco and C.S. David. 1984b. Genetic control of immunity to *Trichinella spiralis* infections of mice. Hypothesis to explain the role of H-2 genes in primary and challenge infections. Immunology 51:625-631.

Wassom, D.L., C.J. Krco and C.S. David. 1985. Genetic control of host resistance to *Trichinella spiralis* infections of mice. In Trichinellosis 6. (ed. C.W. Kim) pp 129-139. State Universtiy of New York Press, Albany.

Wassom, D.L., C.J. Krco and C.S. David. 1987. I-E suppression and susceptibility to parasite infections. Immunology Today 2:39-41.

Wassom, D.L., D.A. Dougherty, and T.A. Dick. 1988 *Trichinella spiralis* infections of inbred mice: immunologically specific responses induced by different *Trichinella* isolates. Journal of Parasitology. 74:283-286.

Wassom, D.L. and E.A.B. Kelly. 1990. The role of the major histocompatibility complex in resistance to parasite infections. Critical Reviews in Immunology 10:31-52.

Watanabe, N., K. Katakura, A. Kobayashi, K. Okumura and Z. Ovary. 1988. Protective immunity and eosinophilia in IgE-deficient SJA/9 mice infected with *Nippostrongylus brasiliensis* and *Trichinella spiralis*. Proceedings of the National Acadamy of Sciences 85:4460-4462.

Wedrychowicz, H., J.M. Maclean and P.H. Holmes. 1983. The detection and measurement of coproantibodies to *Nippostrongylus brasiliensis* in rats following primary infection. Parasite Immunology 5:277-287.

Wedrychowicz, H., J.M. Maclean and P.H. Holmes. 1984.

Secretory IgA responses in rats to antigens of various developmental stages of *Nippostrongylus brasiliensis*. Parasitology 89:145-157.

Wedrychowicz, H., J.M. Maclean and P.H. Holmes. 1985. Some observations on a possible role of lung and fecal IgA antibodies in immunity of rats to *Nippostrongylus brasiliensis*. Journal of Parasitology 71:62-69.

Wells, H.G. 1911. Studies on the chemistry of anaphylaxis III. Experiments with isolated proteins, especially those of the hen's egg. Journal of Infectious Diseases 9:147-171.

Weltzien, H.U. 1973. Slow-reacting hemolytic phosphatides: benzylated lysolecithins. Biochemica Biophysica Acta 311:6-14.

Weltzin, R., P. Lucia-Jandris, P. Michetti, B.N. Fields, J. P. Kraehenbuhl and M.R. Neutra. 1989. Binding and transepithelial transport of immunoglobulins by intestinal M cells.: demonstration using monoclonal IgA antibodies against enteric viral proteins. Journal of Cell Biology 108:1673-1685.

Westfhal, O., O. Luderitz and F. Bister. 1952. Uber die extraktion von bakterien mit phenol/wasser. Zeitschrifte fur Naturforschgang 7b:148-155.

Wilson, A.D., C.R. Stokes, and F.J. Bourne. 1989. Adjuvant effect of cholera toxin on the mucosal immune response to soluble proteins. Scandinavian Journal of

Immunology 29:739-745.

Wilson, A.D., C.J. Clarke and C.R. Stokes. 1990. Whole cholera toxin and B subunit act synergistically as an adjuvant for the mucosal immune response of mice to keyhole limpet haemocyanin. Scandinavian Journal of Immunology 31:443-451.

Wold, A.E., U.H. Dahlgren, L.A. Hanson, I. Mattsby-Baltzer and T. Midvedt. 1989. Difference between bacterial and food antigens in mucosal immunogenicity. Infection and Immunity 57:2666-2673.

Xu, C.B. C. Verwaerde, J.M. Gryzch, J. Fontaine and A. Capron. 1991. A monoclonal antibody blocking the *Schistosoma mansoni* 28-kDa glutathione S-transferase activity reduces female worm fecundity and egg viability. European Journal of Immunology 21:1801-1807

Yamada, M., M. Nakazawa, I. Kamata and N. Arizono. 1992. Low-level infection with the nematode *Nippostrongylus brasiliensis* induces significant and sustained specific and non-specific IgE antibody response in rats. Immunology 75:36-40.

Zhu, D., X.F. Lu and R.G. Bell. 1990. Antigen-specific lymphocyte proliferative responses in inbred mice after *Trichinella spiralis* infection. Journal of Parasitology 76:85-92.

Zhu, D. and R.G. Bell. 1990. *Trichinella spiralis* : Murine strain variation in response to monclonally defined,

protective, nonstage-specific antigens. Experimental Parasitology 70:330-343.

Appendix 1. Immunosuppression of the response to CT in mice infected with *Trichinella*.

Methods and Results: Groups of 8 CFW mice were fed 10 μg CT 5 days prior to, or 5 days after infection with 10 or 150 *Trichinella* larvae. The mucosal response to CT was monitored by faecal sampling and the systemic response by serum sampling. The experimental results are summarized in Appendix 1A. The faecal and serum responses to CT were suppressed in 7 of 8 mice infected 5 days previously with 150 *Trichinella* (TS150 Day -5) but this effect was dose dependent since no suppression was observed in mice infected with 10 *Trichinella* (TS10 Day -5). The suppression was long term since no response was detected up to Day 50 post exposure. Eight weeks post exposure, mice were challenged with identical treatments as in the primary. Suppression was maintained in the bile and serum of 5 of 7 mice suppressed in the primary infection (TS150 Day -5). The values for the Day 15 post challenge treatments were increased by 2 positive responses and this was reflected in the standard deviations. The mean ELISA value of the serum response for the 5 of 7 suppressed mice was 0.061±0.053, compared with 2.852±0.083 for the responder mice. Similarly, the mean faecal IgA response for the suppressed mice was 0.118±0.133 compared with 0.871±0.339 for the responder mice.

In mice fed CT 5 days prior to infection with

Trichinella (TS150 Day +5) there was no apparent suppression of the faecal immunoglobulin response, but the serum response was suppressed at Day 15 and Day 50 after primary exposure. However, following challenge at 8 weeks both the faecal and serum responses to CT were anamnestic, similar to a normal challenge exposure (TS150 Day +5) which suggested that despite the serum suppression observed following initial exposure, immunological memory was established. The faecal and serum response to *Trichinella* were not affected by exposure to CT prior to or following infection.

Appendix 1A. Faecal and serum immunoglobulin response to CT in mice infected with *Trichinella* 5 days prior to or after exposure to 10 μg CT.

	PRIMARY		CHALLENGE
	15	50	15
TS150 Day -5*			
Faecal IgA	0.078±0.071**	0.082±0.031	0.306±0.412
Serum IgG	0.081±0.105	0.083±0.032	0.758±1.290
TS150 Day +5			
Faecal IgA	0.906±1.056	0.130±0.080	1.045±0.818
Serum IgG	0.048±0.051	0.138±0.081	1.913±1.338
TS10 Day -5***			
Faecal IgA	1.939±0.899	0.589±0.598	2.125±0.541
Serum IgG	0.791±0.570	0.500±0.368	2.462±0.854

Serum samples were tested at 1/1000 dilution and faecal samples were undiluted.

* Day relative to 10 μg CT feeding (=Day 0). Designations as in Table 1.
** Mean ± SD of OD 490 ELISA result for n=8 mice.
*** Not significantly different from mice exposed to CT only ($p<0.05$).

Interpretation: The effects of *Trichinella* infection on the immune response to CT were described previously (Ljungstrom *et al.*, 1980). It was reported that anti-CT immunoglobulin production was inhibited when the immunization schedule began five days after infection with *Trichinella*. This effect was attributed to physiological effects of *Trichinella* infection (ie. production of suppressor substances by *Trichinella*) or to the development of suppressor T cells which inhibited the primary anti-CT response (Ljungstrom *et al.*, 1980). *Trichinella* induced suppression of the anti-SRBC response was also attributed to 'suppressive substances' (Faubert, 1976), as was the suppression of the anti-PC response following *Trichinella* infection (Baltar *et al.*, 1991).

The inhibition of the mucosal and systemic anti-CT response in mice fed CT following infection in the present experiment corroborate the results of Ljungstrom *et al.*, (1980). Although the mechanism of suppression was not examined in this study, the effect of different *Trichinella* antigen preparations on the response to CT is discussed in more detail in Chapter 3. Furthermore, the response was dose dependent since infection with 10 larvae had no effect on the response to CT.

In mice exposed to CT 5 days prior to infection the mucosal anti-CT response was normal but the systemic response was suppressed. This differs from the Ljungstrom

results in which no suppression of the serum response was observed when CT exposure began 5 days prior to *Trichinella* infection. However, in their studies mice were exposed to CT 3 times prior to sampling. This exposure protocol produces a challenge response and a strong serum anti-CT response was also detected on Day 15 following challenge exposure in the present study. The development of a normal primary mucosal response was expected, since mice were exposed 5 days prior to infection with *Trichinella*, which is sufficient time to develop the local immune response. In contrast, the serum response to CT would occur during the migratory/dissemination phase of the parasite life cycle.

Immunosuppression of the response to heterologous antigen by infection with *Trichinella* has been attributed to this life cycle stage (Faubert, 1976) but the mechanism by is not clear. Following CT exposure, primed intestinal lymphocytes migrate to target tissues and proliferate in response to antigen (Pierce *et al.*, 1978). Suppression can affect either of these processes. Previous reports describing the suppression of Con-A stimulated splenic lymphocyte proliferation following *Trichinella* infection support an effect at the level of proliferation (Luft and Kim, 1989).

Why is the response anamnestic following challenge in immunosuppressed mice? In the present experiment the mucosal antibody response indicated that mice were primed following

initial exposure. Following the initial exposure to CT, both mucosal and systemic sites are populated with anti-CT memory cells and a significant circulating memory pool also occurs (Lycke and Holmgren, 1989). Following challenge, antigenic stimulation of the memory cells would result in proliferation regardless of the site. This was reported to have occurred within two days of immunization (Lycke and Holmgren, 1987), prior to the potential suppressive effects of *Trichinella* infection which occurred 5 days after CT exposure. Therefore, a serum response is expected following challenge.

While immunosuppression by *Trichinella* is a well documented occurrence, mechanisms regulating this response are unknown, although there is some support for T cell involvement (Luft and Kim, 1989). As indicated above, suppression of the anti-CT response was attributed to "*Trichinella* substances" or T suppressor cells. Recent reports describing aspects of T cell stimulation in response to *Trichinella* provide some related information. Lymphocyte transfer experiments in rats identified a specific population of T helper cells (OX 22^-) which populated the intestine, proliferated following infection and protected naive hosts (Korenaga *et al.*, 1989; Wang *et al.*, 1990). In mice the T helper response was compartmentalized following infection, with a predominant Th2 response in the mucosal lymphoid tissue compared with a systemic Th1 response (Kelly

et al., 1991).

Negative interaction between T helper subclasses was demonstrated in a number of systems. For instance, following infection with *Schistosoma mansoni* in susceptible hosts, the initial Th1 response was inhibited by the development of the Th2 response to egg antigens (Pearce and Sher, 1991). This effect has been attributed to the Th2 specific interleukin IL-10 which has been shown to possess Th1 suppressive activity (Fiorentino *et al.*, 1991). Similarly, murine resistance or susceptibility to infection with *Leishmania* was correlated with the amplification of specific T helper subsets (Scott *et al.*, 1988; Liew, 1990).

The specific T helper cell response following *Trichinella* infection may function in the same way, and suppression of heterologous immune responses would occur as a result of interleukin mediated down regulation of a T helper subclass. Similar down regulation has been described for the response to heterologous antigens following infection with *Schistosoma mansoni* (Kullberg *et al.*, 1992). Further experimentation is required to address this possibility.