Modulation of T cell responses by the products of *Ascaris suum*.

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Abstract

Parasitic helminths infect over a billion people world-wide and contribute to one million deaths annually. Therefore, these parasites represent an enormous burden on human health and well-being. Amongst the most prevalent of helminth parasites is the round worm *Ascaris*. The development of protective immunity against helminth infections is characterised by eosinophilia, mastocytosis and elevated IgE antibody. These responses are known to be controlled by $CD4^+$ T helper type-2 (Th2) cells through the cytokines they secrete following activation. The features of helminth infections that promote the induction of Th2-type immune responses are unknown but it has been suggested that parasite-derived products may have some involvement. The experiments presented in this thesis analysed the immune response resulting from infection with *Ascaris suum* in the murine host and the contribution of the parasite's products in this process. It was found that infection with *Ascaris* resulted in the generation of a Th2-type response that was influenced by the host MHC haplotype. Furthermore the Th2-type response generated by infection could be modulated following exposure to the pseudocolomic fluid (ABF) but not with the major allergen of *Ascaris*, ABA-1. Immunomodulation by ABF was demonstrated further in its ability to suppress development of the Th1-associated delayed-type hypersensitivity reaction to a heterologous antigen. Immunomodulation by ABF did not require the presence of ABA-1 and was a stable phenomenon resistant to both heat and acid treatments. The future identification and analysis of the component of ABF responsible for this activity has important implications for the mechanisms by which helminth parasites modulate the immune response of their host, which has important implications for vaccine development and the control of pathology.

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Abbreviations

α	anti / alpha
β	beta
μCi	Micro Curie(s)
μg	Microgramme(s)
μl	Microlitre(s)
°C	Degree celcius
%	Percentage point(s)
^{3}H	Tritiated
ABF	*Ascaris* body fluid
APC	Antigen presenting cell
B cell	B lymphocyte
Con A	Concanavalin A
cpm	counts per minute
DTH	Delayed type hypersensitivity
dH_2O	Distilled, de-ionised water
ELISA	Enzyme linked immunosorbent assay
FCA	Freund's complete adjuvant
FCS	Foetal calf serum
FIA	Freund's incomplete adjuvant
g	Gramme(s) / centrifugal force
HAO	Heat aggregated ovalbumin

IFNγ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
kBq	Kilo Bequerel
kDa	Kilo Daltons
L	Litre(s)
LPS	Lipopolysaccharide
mA	Milli ampere(s)
mg	Milligramme(s)
ml	Millilitre(s)
mm	Millimetre(s)
mM	Millimolar
M	Molar
MLN	Mesenteric lymph node
MW	Molecular weight
OD	Optical density
OVA	Ovalbumin
p-value	Probability value
pABA-1	Parasite-derived *Ascaris* body antigen-1
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PLN	Popliteal lymph node
PPBS	Pharmacia recipe phosphate buffered saline

rABA-1	Recombinant *Ascaris* body antigen-1
rpm	Revolutions per minute
s.c.	Subcutaneously
SEM	Standard error of the mean
SPL	Spleen
T cell	T lymphocyte
TEMED	N,N,N',N-tetraethylmethylenediamine
Th	T helper lymphocyte
U	Units

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Background

Helminth infections, including gastrointestinal nematodes, schistosomes and filarial parasites infect more than one billion people world-wide and contribute to one million deaths annually (World Health Organisation 1967), and some helminthiases are identified amongst the seven infections listed for control or eradication by the World Health Organisation (World Health Organisation 1996). Furthermore, helminths impede the economic growth in both developed and developing countries through incapacitation of workers and infections of livestock. While helminth infections are in general not associated with high mortality rates, they can be seriously debilitating and pre-dispose hosts to secondary bacterial, viral or protozoal infections (Actor *et al.* 1993), or compromise host tolerance to self, food and/or other luminal antigens (Rocken *et al.* 1992a). Both these effects have potentially fatal consequences, so a thorough understanding of helminth parasite-induced effects upon the host immune system is essential for the future development of therapies and vaccines. Within the phylum Nematoda, ascaridid and filarial worms are closely related and several species of ascaridid are of importance to humans, (genera *Toxocara*, *Anisakis*, *Baylisascaris* and *Ascaris*) (Blaxter 1999). Of all the helminth infections known, one of the most prevalent, infecting one quarter of the world's population is the roundworm *Ascaris lumbricoides* (Crompton 1985), which flourishes in developing countries where poverty, inadequate sanitation, limited medical resources and poor health education persist.

1.1.1 The life cycle of *Ascaris*

There are two species of *Ascaris*, *A. lumbricoides* which infects humans, and *A. suum* found in pigs. Both species infect their host by the oral route and produce very similar disease, characterised by chronicity and malnourishment (Ogilvie and Savigny 1982). *Ascaris* spp. are, in general, highly host specific although reports exist of *A. lumbricoides* infecting orang-utans, dogs, cats and sheep (Crompton 1985). Instances of human infection however, appear to arise from a reproductively isolated population significantly different from those found in pigs (Anderson *et al.* 1993; Peng *et al.* 1998). The most

common species reported in the literature with respect to animal models is *A. suum*. *A. suum* is most prevalent in porcine hosts although it is possible to infect small laboratory rodents (i.e., guinea pigs, rats and mice) and rabbits. However, in the case of small animals it is not possible for infections to reach full patency, possibly due to the difference in anatomical size between rodents and the natural host (Pawlowski 1982). This means that studies of the parasite in laboratory rodents, as reported in this thesis, can only investigate the immune response generated by the early stages of infection. A diagrammatic summary of the *Ascaris* life cycle is illustrated in Figure 1.1.

Infection is initiated by the ingestion of infective eggs containing larvae in contaminated food (i.e., unwashed vegetables), contaminated drinking water or ingestion of soil by other means. Following survival of the acid stomach environment, the larvae hatch in the small intestine and penetrate the intestinal wall from where they migrate through the host tissues to the liver and then the lungs from where they emerge into the air spaces and pass into the alimentary tract for a second time. Subsequent to the second encounter with the stomach the larvae pass into the small intestine where maturation to the adult form occurs allowing mating and the production of eggs by females. The eggs as they appear in faeces contain undeveloped larvae, and develop to the infective form outside the host, given appropriate oxygen, shade and moisture conditions. The eggs are extremely resistant to adverse environmental conditions and chemicals and can remain viable for several years. The time scale from initial ingestion to egg production by the adult females in humans is approximately 60 days, (reviewed in Crompton 1985; Crompton 1989).

1.1.2 Physical manifestations of ascariasis

The physical effects associated with *Ascaris* infection in humans are linked to the discrete stages of the parasite's life cycle. Symptoms attributable to the larval stages are usually acute and affect both the connective tissues and the lungs. Respiratory symptoms begin 5-6 days post-infection as the newly hatched larvae migrate to the lungs and appear as pneumonitis or an asthmatic or type I hypersensitivity response, commonly referred to as

Loeffler's Syndrome (Coles 1985; Ogilvie and Savigny 1982). These disturbances can persist for up to 10-12 days before giving way to the digestive and nutritional disturbances generated by the adult worms (Coles 1985). The adult form of the parasite can cause harm in a variety of ways, either alone or in combination. Firstly, and most commonly, digestive and nutritional disturbances arise which are usually chronic. Examples of these include colic, nausea, vomiting, malnourishment, lactose intolerance and growth impairment. In direct contrast are the acute symptoms, occurring infrequently but are potentially life-threatening: intestinal, biliary and pancreatic obstructions and intestinal lesion ruptures. A third and final effect results from adults residing in abnormal locations such as the heart, nose, ears, lachrymal ducts or the reproductive tract.

As mentioned above, ascariasis is endemic in countries where poverty, inadequate sanitation, limited medical resources and poor health education are rife. Prevalence of the parasite is decreasing in areas where sanitation has been improved and living standards raised, but control can often only be achieved with regular treatments with anti-helmintic or "de-worming" medications which cause adult worm expulsion from a host's gastrointestinal tract (Crompton and Pawlowski 1985). However, these drug treatments only cure as opposed to prevent infections, and only by educating locals to the dangers of these parasites in conjunction with other control measures will limit the re-introduction of the parasite to a community. The development of vaccines may require an investigation of the invasion and establishment mechanisms employed by these parasites, and the immune responses associated with these activities.

1.1.3 The development of immunity

The development of protective immunity against helminth infections in laboratory animals requires the presence of $CD4^+$ T cells and is associated with the development of eosinophilia, mastocytosis and elevated IgE immunoglobulin antibody (Bancroft *et al.* 1994b; Finkelman *et al.* 1991; McKay *et al.* 1995; Ogilvie and Savigny 1982). The induction of such effector mechanisms has been shown to be controlled by $CD4^+$ T helper

type-2 (Th2) cells and the cytokines they produce, interleukins (IL)-3, IL-4, IL-5, IL-9, IL-10 and IL-13, whose expression is enhanced as a consequence of infection (Finkelman *et al.* 1991). The importance of such immune responses in protective immunity has been demonstrated *in vivo*, where the Th2 response results in clearance of infection, while the antithetical T helper type-1 (Th1) response leads to chronic disease (Else *et al.* 1992b; Else and Grencis 1991b).

Of the effector mechanisms induced, eosinophils and IgE have been reported to be central to the development of protective immunity against helminths (Sher and Coffman 1992). However, immunisation of mice with anti-IL-5 monoclonal antibodies, which reduces total eosinophil numbers, has been shown to have no effect immunity against *Schistosoma mansoni*, *Heligmosomoides polygyrus* or *Nippostrongylus brasiliensis* (Coffman *et al.* 1989; Finkelman *et al.* 1991). Furthermore, the value of increased serum IgE as a form of protection has been debated for some time now (Allen and Maizels 1996). Depletion of total IgE by immunisation with anti-IL-4 monoclonal antibodies did not alter resistance to *S. mansoni* in both mice and rats or block worm expulsion in mice infected with *N. brasiliensis* (Sher and Coffman 1992). However, epidemiological support for increased total IgE and protection has been provided by studies with *Schistosoma haematobium* and *S. mansoni*, where resistance to re-infection with these parasites was greater as levels of IgE increased (Hagan *et al.* 1991; Rihet *et al.* 1991). The protective effects of IgE have been shown to be blocked by increases in IgG4 antibodies which was associated with reduced parasite-induced pathology (Hagan *et al.* 1991; Hussain *et al.* 1992), thus highlighting the fine balance between protection and pathology in the host. Immunity to *A. lumbricoides* has also been shown to be influenced by the production of IgE; resistance to re-infection was associated with high levels of antigen-specific but not polyclonal IgE (Hagel *et al.* 1993; McSharry *et al.* 1999). Therefore, the development of protective immunity against helminths is not only subtle but more complex than initially anticipated.

1.2 The role of parasite products on the development of Th2 immune responses

The features of helminth infections that promote Th2 immune responses are unknown, but the induction of IgE and the development of hypersensitivity responses in the host suggested that parasite products may have some involvement (Ogilvie and Savigny 1982). Priming naive $CD4^+$ T cells with extracts of the microfilarial stage of *Brugia pahangi*, for example, has been shown to enhance IL-5 production upon antigen restimulation (Steel and Nutman 1998). While the culture of T cells from individuals with lymphatic filariasis with the antigens of *B. pahangi* and *Onchocerca volvulus* preferentially expanded pre-existing Th2-like cells (Mahanty *et al.* 1993).

Helminth products have also been reported to influence antibody production. Immunisation with excretory/secretory products of *N. brasiliensis* can induce the production of polyclonal IgE in uninfected mice (Uchikawa *et al.* 1993). The ability of *N. brasiliensis* products to enhance non-specific IgE has also been demonstrated with homogenate of adult worms (Stephan and Konig 1992). Similar effects upon antibody production have been reported with larval excretory/secretory products and the adult body fluid of *A. suum* (Lee and Xie 1995; Stromberg 1980). The potentiation of immunoglobulin production by helminth products involves antigen-specific antibody levels too; immunisation of uninfected mice with a membrane-associated antigen of *Schistosoma japonicum*, which is the pre-dominant target of IgE responses during infection, elicited IgE specific for the antigen (Waine *et al.* 1997). It was therefore proposed that this effect was due to the antigen containing signals in its primary amino acid sequence, secondary structure or tertiary structure, which directed the isotype switch to IgE.

1.3 Development of T cell immune responses

The discovery by Mosmann and colleagues in 1986 that murine $CD4^+$ T clones could be classified into two subsets based upon differences in cytokine secretion patterns

revolutionised understanding of immune responses and disease (Mosmann *et al.* 1986). The two populations described were referred to as T helper 1 (Th1) cells which produced IL-2, interferon (IFN)-γ and tumour-necrosis factor (TNF)-β, and T helper 2 (Th2) cells which secrete IL-4, IL-5, IL-6, IL-10 and IL-13 (Finkelman *et al.* 1997; Mosmann and Coffman 1989). It has been shown that not only do the cytokines produced by each subset act as autocrine growth factors, but they also influence the differentiation of $CD4^+$ into effectors of the same phenotype (Seder and Paul 1994). Furthermore, the cytokines produced by a given T cell subset can regulate the development and activity of cells of the other phenotype, as illustrated by the inhibition of Th2 cell proliferation by IFN-γ and the impedance of Th1 activation by IL-10 (Fiorentino *et al.* 1989; Gajewski and Fitch 1990).

The dichotomy described for cytokine production by the two subsets also occurs with respect to the effector mechanisms they induce. Th1 clones were shown to mediate delayed-type hypersensitivity response (i.e., cell-mediated immunity), while Th2 clones provided help predominantly for antibody responses i.e., humoral immunity (Boom *et al.* 1988; Cher and Mosmann 1987). Furthermore, differences in the antibody isotype induced by the clones were noted with the Th1 subset inducing IgG2a, whereas Th2 cells promoted IgE and IgG1 (Mosmann and Coffman 1989). Taken together, these data appeared to explain the differences in immune responsiveness that had been reported for some time to occur in diseases such as leishmaniasis and leprosy (Abbas *et al.* 1996).

The existence of these subsets within the $CD4^+$ T cell populations of humans was much more problematic. Early work on T cell clones derived from the peripheral blood of healthy donors resulted in populations with a mixed Th1 and Th2 response more commonly referred to as Th0 (Romagnani 1991). However, the demonstration that clones derived from patients with autoimmune disease, or those stimulated with bacterial antigens, produced IFN-γ, and those from patients with allergic disease, or stimulated with

excretory/secretory product of *Toxocara canis*, released IL-4 and IL-5, indicated that the selective expansion of discrete Th cell lineages did occur in humans (Romagnani 1994).

1.3.1 Cytokine influences on Th1/Th2 development

Both Th1 and Th2 cells have been shown to develop from the same $CD4^+$ precursor T cell that produces mainly IL-2 upon its initial encounter with antigen (Seder and Paul 1994). The differentiation of the precursor into either subset is influenced by the manner and environment in which it is stimulated. One of the major factors that contributes to this process is the cytokine micro-environment of the cell. IL-12, secreted by activated macrophages and dendritic cells is the principal Th1-inducing cytokine (Abbas *et al.* 1996; Chan *et al.* 1991). Activation of macrophages by microbial products tend to stimulate IL-12 production and induce Th1-type immune responses (Gazzinelli *et al.* 1994; Hsieh *et al.* 1993). The role of IL-12 in Th1 development was highlighted further by the inability of IL-12 defective mice to produce IFN-γ and develop Th1 effector responses (Magram *et al.* 1996). The influence of IL-12 is not only restricted to naive $CD4^+$ populations, but it can also act as a co-stimulus to antigen-activated Th1 cells to maximise their secretion of IFN-γ (Murphy *et al.* 1994). The role that IFN-γ itself plays in the development of Th1 cells is somewhat more controversial, the addition of neutralising IFN-γ antibodies to priming cultures has been shown to diminish the presence of IFN-γ-secreting T cells, suggesting IFN-γ is required for Th1 development (Macatonia *et al.* 1993). However, it has also been reported in a similar study by another group that neutralisation of endogenous IFN-γ in priming cultures has no effect (Seder and Paul 1994).

The development of Th2 effector populations depends upon the presence of IL-4 during priming of $CD4^+$ T cell precursors both *in vivo* and *in vitro* (Le Gros *et al.* 1990; Swain *et al.* 1990). The fact that IL-4 was necessary for the development of Th2 cells raised the

problem of the source of the cytokine during priming, because IL-4 was thought only to be produced by T cells post-differentiation. Mast cells, basophils and $CD4^+$ $NK1.1^+$ T cell subset have all been proposed as possible sources of IL-4 (Mosmann and Sad 1996). However, the discovery that T cells can produce tiny amounts of IL-4 following initial activation has raised the possibility that T cells may be self-sufficient with respect to Th2-effector differentiation and development (Schmitz *et al*. 1994).

1.3.2 Antigen presenting cells (APCs)

Early work investigating the differentiation of $CD4^+$ T cells suggested that the antigen presenting cell (APC) population present at priming may influence the Th1/Th2 fate. Gajewski and co-workers demonstrated that while Th1 cells responded optimally to purified adherent cells from the spleen, Th2 cells proliferated when splenic B cells were present (Gajewski *et al*. 1991). However, in a study using transgenic T cells and peptide antigen in order to control for differences in antigen uptake and processing, activated B cells and bone-marrow derived macrophages were shown to promote the proliferation of both Th subsets equally and that the exogenous cytokine present at priming decided the differentiating cells' fates (Duncan and Swain 1994), and has also been described for dendritic cells (Mosmann and Sad 1996). This issue was complicated further by the finding that APC-derived IL-6 was capable of polarising naive Th cells to the Th2 phenotype by stimulating IL-4 production in $CD4^+$ T cells (Rincon *et al*. 1997). Therefore, a role for APCs in Th1/Th2 development and polarisation cannot be overlooked, and, indeed, deserves serious consideration.

1.3.3 Costimulatory molecules

A further way in which APCs may influence Th1/Th2 differentiation is through the costimulatory molecules that are expressed on their cell surfaces. Effector T cells are triggered by the binding of their antigen-specific receptor and CD4 co-receptor to peptide/MHC complexes on the APC. However, in order for a naive T cell to proliferate and differentiate into the effector population, a second signal is required which is provided

by the APC costimulatory molecules. The best defined costimulatory molecules known are the structurally related B7-1 and B7-2, which bind CD28 on naive T cells and CTLA-4 on activated T cells (Lenschow *et al.* 1996). Interestingly, early data reporting that the Th2-associated cytokine IL-10 could act upon macrophage APCs to inhibit cytokine production by Th1 cells was shown to be due to a decrease in the expression of the B7 molecules (Ding *et al.* 1993; Fiorentino *et al.* 1991). The development of both Th subsets is dependent on costimulation with B7-1 or B7-2 and has been demonstrated in some systems to differentially regulate the process (Kuchroo *et al.* 1995; Lenschow *et al.* 1995; Seder and Paul 1994). High levels of costimulation have been reported to promote Th2 responses, probably because increasing the magnitude of initial T cell activation increases IL-4 production and the ensuing IL-4-mediated autocrine pathway of Th2 differentiation, while Th1 development, which depends more upon the presence of IL-12 in addition to some level of costimulation, is less influenced by the magnitude of the T cell response these molecules can invoke (Murphy *et al.* 1994).

The effects of costimulation during a gastrointestinal helminth infection have not been extensively studied. However, mice deficient for B7-2 have revealed that while interactions between CD28 and the B7-2 molecule are not necessary for the initiation of the Th2-associated response, they are required for its progression after the development of effector T cells (Greenwald *et al.* 1999). Other studies have investigated the role of CTLA-4 during helminth infections with varying results. CTLA-4, as mentioned above, is expressed on activated T cells and also binds the B7 molecules, albeit with a higher affinity. Blockade of B7 costimulation with anti-CTLA-4 antibodies has been reported by some groups to inhibit the development of optimal Th2 development but not protective immunity or the T cell memory response (Harris *et al.* 1999; Lu *et al.* 1994), while others have reported the only difference observed is an enhancement of the response normally seen (McCoy *et al.* 1997).

1.3.4 Antigen dose and ligand-TcR interactions

The effects of varying antigen dose during $CD4^+$ T cell priming and consequently Th1/Th2 differentiation has been acknowledged for some time, although a dichotomy exists regarding whether Th1- or Th2-type responses are evoked by high versus low doses of antigen. This effect was first reported by Parish and Liew, where rats were immunised with different doses of bacterial flagellin (Parish and Liew 1972). Upon antigen challenge, animals receiving very high or very low doses developed strong delayed-type hypersensitivity responses. In contrast, those administered with intermediate doses had high anti-flagellin antibody responses. Similar effects have also been reported in animals infected with *Leishmania major* and *Trichuris muris*, where infections with low parasite numbers resulted in the development of a Th1-type immune response (Bancroft *et al.* 1994a; Bretscher *et al.* 1992). In contrast, mice that were exposed to low doses of soluble protein antigens, via a mini-osmotic pump, developed a Th2-type response (Guery *et al.* 1996). Furthermore, glutaraldehyde polymerisation of OVA, (which increases molecular weight and thus antigen dose), resulted in the development of a Th1 response whereas the monomeric antigen promoted a Th0-like response (Gieni *et al.* 1996).

Therefore, the effects of antigen upon the Th1/Th2 decision may not be dose but its source and nature as Th1 responses were generated when parasite-derived immunogens were used while Th2 responses associated with soluble proteins. An alternative interpretation of these results, accounting for the fact that parasite-derived immunogens are unlikely to occur at low enough levels to induce a Th1 response during a natural infection, is that at high antigen doses Th1 clones are more susceptible than Th2 clones to activation-induced cell death. Therefore, high doses of parasite antigen may promote the expansion of Th2 cells in the absence of a Th1-mediated negative cross-regulation (Constant and Bottomly 1997).

The involvement of the T cell surface antigen receptor (TcR) in Th1/Th2 differentiation was investigated using peptide to prime purified $CD4^+$ T cells from TcR transgenic mice

in vitro (Constant *et al.* 1995). This eliminated the affinity of the TcR for the antigen as a potential variable allowing greater control over antigen dose. It was found that low doses of peptide gave rise to Th2-like cells while high doses led to Th1 cells. Therefore, high densities of MHC class II/peptide complexes on the surface of antigen presenting cells may promote Th1 cells through increased TcR engagement, while low densities favour Th2 cells.

Interactions between the TcR and its peptide/MHC ligand may also influence Th cell differentiation. Priming mice with the same antigen gave rise to a Th1 or Th2 type response upon challenge, depending on the MHC genotype of the animal (Murray *et al.* 1989). Although this suggested that the MHC genotype was influencing function through differences in TcR repertoire, studies in TcR transgenic mice with antigen had demonstrated similar effects. Immunisation of mice with an immunodominant peptide of the antigen also resulted in the same skewing of the immune response, thus demonstrating that the effect was determined by the peptide/MHC class II complex and its interaction with the TcR (Pfeiffer *et al.* 1995). By utilising altered peptide ligands (APLs), this study demonstrated that those peptides which bound well to MHC class II molecules and/or to the TcR stimulate development of Th1 cells, whereas peptides which bound less well favoured Th2 effectors.

It has been proposed that these differences in binding may influence Th1/Th2 development alter the signals delivered via the TcR after binding, either through signal intensity or types of altered signals (Rocken *et al.* 1992b). Not only have differences in TcR signalling intensity been proposed to influence Th1/Th2 differentiation directly, but it also affects the receptiveness of naive $CD4^+$ T cells to B7 costimulatory molecules, thus compounding the involvement of this route in the whole process (Tao *et al.* 1997).

1.4 Aims of the thesis

In the present study, experiments were carried out to investigate the effects on the immune system of infection with *A. suum* in mice. Previous research from this laboratory had shown that IgG and IgE antibody recognition profiles generated as a result of immunisation with *Ascaris*-derived products were influenced by host MHC haplotype (Kennedy *et al.* 1991; Tomlinson *et al.* 1989). Therefore, the contribution that host MHC had on other aspects of the immune response following *Ascaris* infection was investigated.

Increases in IgE immunoglobulin and the generation of hypersensitivity responses in the host following infection with helminths brought attention to the influences that the products of these parasites may be having on the generation of Th2 immune responses. This was reinforced by the findings that parasite products could deviate the response phenotype of naive $CD4^+$ T cells as well as preferentially expanding existing Th2 populations and enhancing the production of IgG1 and IgE (Mahanty *et al.* 1993; Steel and Nutman. 1998; Stephan and Konig 1992; Uchikawa *et al.* 1993). Experiments were designed to test whether exposure to the products of *Ascaris*, pseudoceolomic fluid (ABF) and the major allergen ABA-1, had any influence on immune response generated following parasite infection or could skew the immune response towards a Th2-type phenotype.

A final aspect of the immune response associated with helminth infection investigated was the capacity to modulate immune responsiveness in the host. Immunosuppression or potentiation is a common feature of many infectious diseases and is considered in some cases to be mediated by suppressor cell populations, the induction of hyperimmunoglobulinemia, or by the effects of parasite-derived products (Harnett and Harnett 1993; Ottensen *et al.* 1977; Playfair 1982; Poels and Niekerk 1977; Schulz *et al.* 1998) and can alter the host's ability to respond to heterologous antigens and pathogens (Actor *et al.* 1993; Price and Turner 1986). Similar activities had been reported with *Ascaris*-derived products in which exposure resulted in impaired T cell function and influenced immunoglobulin production to non-parasite antigens (Ferreira *et al.* 1995;

Stromberg 1980). The ability to modulate a heterologous immune response by ABF and rABA-1 was investigated and the initial steps were made towards elucidation of the mechanisms responsible.

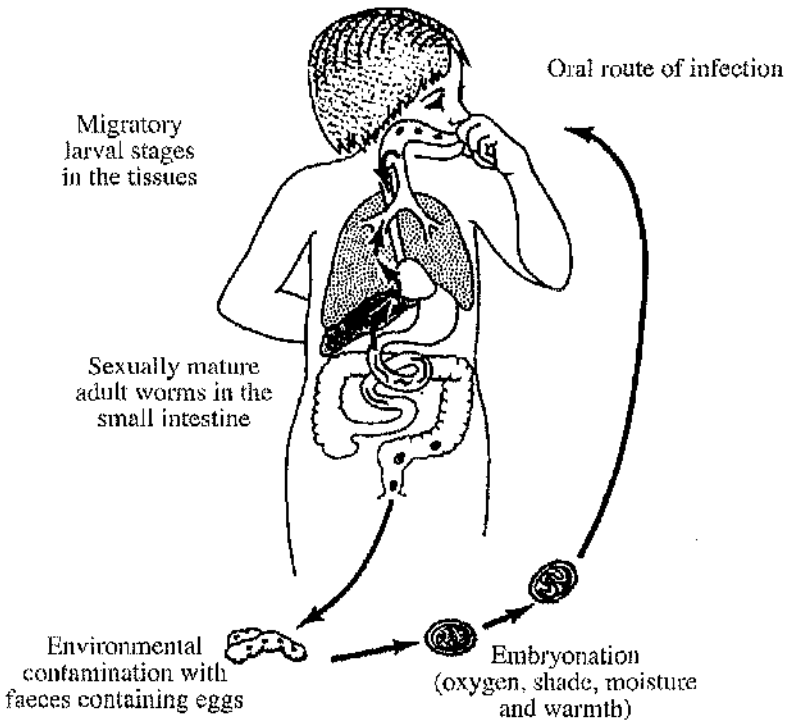


Figure 1: Life cycle of the helminth species *Ascaris*

CHAPTER 2

MATERIALS AND METHODS

2.1 Animals

Syngeneic female BALB/c mice were obtained from a breeding colony maintained at the Central Research Facility, University of Glasgow. Syngeneic female B10.S and B10.BR mice were purchased from Harlan Olac (Bicester, UK). IL-4 deficient mice (129 x C57BL/6 background) were a kind gift of Dr Allan Mowat, Department of Immunology, University of Glasgow. All animals were housed at the Joint Animal Facility, University of Glasgow under standard animal house conditions. Pelleted CRM breeder diet (Labsure Ltd.) and tap water were available *ad libitum*. All mice were used for experimental purposes when 6-8 weeks old.

2.2 Parasites

Adult *Ascaris suum* were obtained from local abattoirs within 24 hours of host death. The worms were washed thoroughly in tap water to remove debris from the host gut and the somatic fluids collected as described in section 2.4.1. Female worms were separated from the males and then stored in a 5% formalin/Nystatin solution, (see Appendix) at 4°C.

2.2.1 Parasite egg preparations

Uteri were removed from female worms and placed in a flask containing a 5% bleach solution. This was incubated for 15 minutes in an orbital shaker at 37°C in order to dissolve the uterine walls and release the unembryonated eggs. The eggs were washed eight times with dH_2O (see Appendix) for 5 minutes at 2000 rpm to remove all traces of bleach. To embryonate, eggs were incubated at 26°C, with 5% formalin/Nystatin, for 1 month and counted weekly to calculate total percentage embryonation. Upon reaching 70% embryonation the eggs were stored at 4°C in 5% formalin/Nystatin.

2.3 Parasitological techniques and infections of mice

Embryonated eggs were washed five times with dH_2O as in the previous section and resuspended in a 5ml volume. The eggs were counted and numbers adjusted to 15000 embryonated eggs/ml in a 50% dH_2O/50% 0.1% agarose working solution (see Appendix).

Animals were infected orally *via* a gavage needle with 0.2ml of the working solution which delivered 3000 embryonated *A. suum* eggs per dose.

2.4 Parasite antigen purification

2.4.1 Ascaris Body Fluid (ABF) preparation

The body walls of adult worms were pierced at one end and the body fluid squeezed into a siliconised petri dish. The body fluid was transferred into microfuge tubes and centrifuged at 13000 rpm for 5 minutes to pellet any cellular debris. The supernatant was carefully removed and the total protein concentration quantified using the Bradford Method (see section 2.5.4). The *Ascaris* Body Fluid (ABF) was then stored at -70°C until further use.

2.4.2 Recombinant ABA-1 purification

Recombinant ABA-1 (rABA-1) was produced by culturing *E. coli*, NovaBlue competent cells, (Novagen, Cambridge, UK), which had been transformed with a pGEX-1λT expression vector that contained cDNA coding for ABA-1. Preparation of the pGEX-1λT expression vector was carried out by Dr Joyce Moore, Wellcome Laboratories for Experimental Parasitology, University of Glasgow. rABA-1 was produced as a fusion protein with the glutathione S-transferase (GST) of *Schistosoma japonicum* separated by a thrombin cleavage site. The bacterial cells transformed in order to produce rABA-1 were named ΔR4 and are referred to as this subsequently. Throughout the purification process bacteria were cultured in the presence of 100μg/ml ampicillin (Sigma, Poole, UK), to select for the transformed population.

A single colony of ΔR4 was picked from a L-agar stock plate (see Appendix), added to 100ml of T-broth (see Appendix) and incubated 16-18 hours at 37°C in an orbital shaker. The culture was then added to 1L of T-broth and incubated further until the optical density, (OD), at 550nm was greater than 1.0. Isopropyl β-D-thiogalactopyranoside, (IPTG; Sigma), was added to 0.2mM to induce rABA-1 production and the cultures were

incubated for a further 3 hours. Cultures were harvested by centrifugation at 10000 rpm and 4°C for 10 minutes. The supernatants were discarded and the bacterial pellets stored at -20°C prior to recombinant protein purification.

The bacterial pellets were resuspended in a 40ml total volume of Pharmacia recipe PBS, (PPBS; see Appendix), and sonicated on ice at 10μM amplitude for 30 seconds with a 90 second cooling period. This cycle was repeated 30 times. The sonicate was centrifuged at 15000 rpm for 10 minutes at 4°C to pellet cellular debris. The supernatant was retained and a 20μl sample removed for SDS-polyacrylamide gel analysis.

The GST fusion protein was purified from other *E. coli* proteins using a GST binding chromatography column. 1ml of Glutathione Sepharose-4B slurry (Amersham Pharmacia Biotech, St Albans, UK), was prepared as per manufacturers instructions and added to a Poly-Prep® chromatography column (Bio-Rad Laboratories, Hemel Hempstead, UK; 731-1550). The column was equilibrated with 10 ml of PPBS prior to use. The sonicate supernatant was applied to the column and the flow-through collected, 20μl of which was retained for gel analysis. The column was washed with 15ml of PPBS to flush out unbound material and plugged. 25 units of thrombin (Sigma, T6759) in 500μl PPBS was mixed with the sepharose and incubated 16-18 hours at room temperature. The eluate was collected and PPBS added until five 500μl fractions had been collected from which 20μl was sampled for gel analysis. To remove the GST protein the sepharose was incubated with 500μl of glutathione elution buffer (see Appendix) for 10 minutes at room temperature. Five 500μl fractions were collected as before. All fractions and gel samples were stored at -20°C until further use.

The sepharose column was regenerated by washing the slurry with double bed volumes of 0.1M Tris-HCl/0.5M NaCl, pH 8.5, and 0.1M Na acetate/0.5M NaCl, pH 4.5, (see Appendix for both). This was repeated four times. As a final step the column was

equilibrated with 10ml of PPBS and stored at 4°C. Each column was used a maximum of three times.

The samples retained for gel analysis were run on 17.5% SDS-polyacrylamide gels. The fractions observed to contain the 14kDa ABA-1 protein were pooled and analysed on a scanning spectrophotometer to estimate the protein concentration.

2.4.3 Native protein purification

ABF was purified using a Pharmacia Superose 12 gel filtration column fitted to a BioRad Biologic Fast Performance Liquid Chromatography (FPLC) system. The Superose 12 column was prepared for use by isocratic flow for 50ml (2 column volumes) with dH_2O at 0.1ml $minute^{-1}$ to remove the storage solvent, typically 20% ethanol. The column was equilibrated by isocratic flow of FPLC buffer (see Appendix) for 50ml at 0.3ml $minute^{-1}$. All eluents were degassed and filtered using a 0.22μM sterile filter prior to use. ABF was centrifuged at 13000rpm for 10 minutes to remove fine debris before injection. The purification protocol involved isocratic flow of FPLC buffer for 2ml, injection of 300μl of ABF, followed by isocratic flow of buffer for 30ml. Flow rates of 0.3ml $minute^{-1}$ were used resulting in a back pressure in the range 160-180psi.

2.4.4 Dialysis and concentration of *Ascaris* proteins

Dialysis of proteins was carried out using Pierce Slide-A-Lyzer™ dialysis cassettes (Pierce & Warriner Ltd., Chester, UK) with 10000 MW cut-off membranes. Up to 3ml volumes were injected into the cassette using a hypodermic needle attached to a syringe. Samples were dialysed with constant stirring at 4°C against a large volume of PBS overnight. Volumes of proteins 10ml or above were concentrated using Centricon-10 centrifugal concentrators (Amicon Ltd., Stonehouse, UK) with a 10000 MW cut-off. Centrifugation at 3000 x g was employed in accordance with the manufacturers instructions. Concentration of small volumes, typically 3ml or less, was performed using Vivapore 2 concentrators

with a 7500MW cut-off (Vivascience Ltd., Binbrook, UK). Following dialysis and concentration protein estimations were carried out as described in section 2.5.4.

2.5 Biochemical techniques

2.5.1 SDS-Polyacrylamide gel electrophoresis

The Laemmli gel buffer system was used (Laemmli 1970) to cast 0.5mm thickness gels of 17.5% acrylamide, (see Appendix for buffers and solutions). Glass plates were cleaned with 70% ethanol and assembled according to the manufacturers instructions for the Hoefer Mighty Small SE245, (Hoefer Scientific Instruments, San Francisco, CA). The resolving gel once poured, was overlaid with water saturated butanol to achieve an even surface during polymerisation. Once polymerised the butanol was removed and the stacking gel poured with the correct well formers in place. Upon stack polymerisation the well formers were removed and the wells thoroughly washed with dH_2O. The gels were mounted in the electrophoresis apparatus, Hoefer Mighty Small II, SE250/SE260 and Tris-glycine electrophoresis buffer was added to the top and bottom reservoirs. Samples were then loaded into the wells. Electrophoresis was normally carried out at a constant current of 30mA for approximately 1 hour. Gel tanks were cooled by circulating water at 4°C to minimise any gel distortion due to heating during electrophoresis. Upon completion of electrophoresis, the gels were removed from the plates and the stacking gels discarded leaving the resolving gel ready for staining.

2.5.2 Sample preparation

Samples were added to an equal volume of non-reducing sample buffer. Molecular weights of bacterial products were estimated by reference to molecular weight marker proteins (Amersham Pharmacia Biotech, 17-0446-01) (MW range 14-94 kDa). 5μl of the reconstituted markers were added to 20μl of non-reducing sample buffer. All samples were heated to 100°C for 10 minutes in a Cetus DNA Thermal Cycler 480 (PE Applied Biosystems, Warrington, UK) and allowed to cool before loading onto the gel using a Gilson automatic pipette.

2.5.3 Gel staining

Protein in electrophoresed gels was visualised by staining with a 0.1% Coomassie Brilliant Blue R-250 solution (see Appendix) for 1 hour with shaking. The gels were destained in Coomassie Blue Destain (see Appendix) for 2-3 hours until a clear background was obtained. The gels were then dried onto cellophane membrane backing (Bio-Rad, 165-0963) on a slab gel dryer (Bio-Rad, 1125B) at 60°C for 1.5 - 2 hours.

2.5.4 Protein estimation (Dye-binding)

A quantitative estimation of total protein concentrations was determined using a modification of the Bradford Method (Bradford 1976). The assay is based on absorbance shift when the protein assay reagent binds protein in solution. The standard microtitre plate variation of the assay was used. Briefly, known protein concentrations ranging between 20-1500μg/ml were prepared by diluting a 2 mg/ml stock bovine serum albumin standard (Pierce & Warriner Ltd., Chester, UK) in PBS. Test samples were diluted 1/50, 1/100, 1/500 and 1/1000 in PBS. 5μl of the standards and test samples were added in triplicate to a 96 well microtitre plate and 250μl of Coomassie® protein assay reagent (Pierce Chemical Co, 23200) added. The absorbance at 595nm was read versus PBS using a MRX plate reader (Dynatech Laboratories, Billingshurst, UK). The net absorbance of each standard was obtained by subtracting the absorbance of PBS from each sample. A standard curve was constructed which was used to determine the protein concentration for the unknown samples.

2.5.5 Protein estimation (Spectrophotometric)

A second quantitative method was used to measure the protein concentration of recombinant ABA-1 purified from ΔR4 using a scanning spectrophotometer (Shimadzu, UV-1601). Samples to be measured were typically diluted 1/10 with PPBS (see Appendix) and absorbance scanned from 300nm through to 230nm. The absorbance value at 280nm, A_{280}, was multiplied by the dilution factor. The absorbance, A_{280}, of a 0.1% solution of ABA-1 was calculated using the ProtParam program accessed through the

ExPASy/SwissProt world wide web site (http://expasy.hcuge.ch/sprot/protparam.html). The program calculated the A_{280} of the 0.1% solution to be 0.738 based on the amino acid sequence of ABA-1. This value was used to divide the absorbance of the test sample thus expressing the protein concentration in mg/ml.

2.6 Adjuvant assisted immunisations

Aluminium hydroxide gel adjuvant, Alhydrogel® 2.0%, (Superfos Biosector, Denmark) and Freund's Complete (Sigma, F5881) and Incomplete (Sigma, F5506) adjuvants were used to immunise mice in association with various parasite and non-parasite derived antigens.

Antigens were adsorbed onto aluminium hydroxide particles, (Alum), by incubating with rotation at 37°C for 30 minutes. The Alum was centrifuged at 1000rpm for 5 minutes and the supernatants were retained for Bradford method protein estimation (see section 2.5.4). This was to ensure all of the protein antigens had adhered to the adjuvant. The solution was increased to the original volume by addition of sterile 0.9% saline to ensure each dose had an equivalent amount of Alum particles.

Freund's Complete and Incomplete adjuvant immunisation mixes were prepared by adding soluble antigens to an equivalent volume of adjuvant and mixed by either water bath sonication or high speed mixing with a Ultra-Turrax T-25 (Janke & Kunkel Laboratories) until a stable emulsion formed. An emulsion was deemed stable when a drop of the emulsion was dropped into ice cold PBS and could maintain its structure without dispersal.

2.7 Sample collection and preparation

2.7.1 Sera collections

Whole blood was collected by exsanguination *via* cardiac puncture following the sacrificing of mice. Clotting of blood was carried out at 4°C followed by centrifugation at 13500 rpm for 5 minutes to separate serum from the clot. Overlying serum was collected

by pipetting and transferred to a fresh microfuge tube. Sera samples were stored at -20°C until analysis.

2.7.2 Cell suspensions

To prepare suspensions of spleen and lymph node cells suitable for *in vitro* cytokine and proliferation assays mice were sacrificed and the spleens and lymph nodes of mice within experimental groups dissected out aseptically and pooled. Organs were dissociated by rubbing through a sterile stainless steel sieve using a sterile syringe plunger and collected into 10% FCS/RPMI medium (see appendix). Cells were washed by centrifugation at 1000 rpm for 5 minutes. Pellets were resuspended in 10% FCS/RPMI, passed through sterile Nitex mesh (Cadish & Sons, London, UK) to remove cellular debris and washed again. The suspensions were counted by light microscope using a haemocytometer and the cell suspensions adjusted to 1 x 10^7 cells/ml by diluting in 10% FCS/RPMI.

2.7.3 Thymidine incorporation assays

1 x 10^6 cells, prepared as described in 2.7.2, were cultured with 50μg/ml ABF, 50μg/ml rABA-1, 5μg/ml Concanavalin A, (Con A), (Sigma, C-5275), 5μg/ml lipopolysaccharide, (LPS), (Sigma, L-7770), 5μg/ml Purified Protein Derivative 289 of *Mycobacterium bovis*, (PPD), (Central Veterinary Laboratory, UK) 50μg/ml chicken Ovalbumin Grade V (OVA), (Sigma, A-5503) or medium alone in a final volume of 200μl, in triplicate in 96 well flat-bottomed microtitre plates (Greiner, Stonehouse, UK). All concentrations given for the stimuli are the final concentrations.

Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere for 72 hours. At this time point each well was pulsed with 0.5 μCi (18.5 kBq) of ^{3}H methyl thymidine (Amersham Pharmacia Biotech, TRK120) in a 25μl volume of 10%FCS/RPMI. Cells were incubated for a further 24 hours after which well contents were harvested onto a printed filtermat A (Wallac, UK) using a Wallac 1295-004 Betaplate™ 96-well harvester. Filtermats were allowed to dry completely and placed in sample bags to which 10 ml of

Betaplate Scint (Wallac, UK) was added. The amount of ^{3}H thymidine incorporated by cells was measured by a Wallac 1205 Betaplate™ liquid scintillation counter.

2.7.4 Cytokine production assays

Cells were prepared as described in 2.7.2. 7.5 x 10^6 cells were incubated in 24 well tissue culture plates (Greiner), with 50μg/ml rABA-1, 50μg/ml ABF, 5μg/ml Con A, 50μg/ml OVA or medium alone in a final volume of 1.5ml.

Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere for 24 or 72 hours. At these time points culture supernatants were harvested and centrifuged at 13500 rpm for 5 minutes to pellet cells. Supernatants were transferred into fresh microfuge tubes to prevent cellular contamination of the sample and stored at -20°C prior to cytokine quantification.

2.8 Immunological techniques

2.8.1 Enzyme Linked Immunosorbent Assays (ELISA)

To quantify cytokine concentrations in cell supernatants, capture sandwich ELISAs were performed. The supernatants tested were prepared as outlined in section 2.7.4. All buffers and solutions used are listed in the Appendix (section 2.13.6) as are the antibodies (section 2.13.8). Essentially, 96 well Immulon IV microelisa plates (Dynatech Laboratories) were coated overnight at 4°C with 2μg/ml of monoclonal capture antibody (PharMingen, San Diego, CA) in 0.1M carbonate buffer, pH 8.2 added at 50μl/well. Plates were washed twice for 1 minute with PBS/0.05% Tween-20 and pounded dry on paper towels. Non-specific protein binding sites were blocked by incubation with 200μl of PBS/10% FCS for 1 hour at 37°C. Plates were washed twice as before after which test samples (used neat) and standards diluted in PBS/10% FCS were added in triplicate to individual wells in a volume of 50μl and incubated at 37°C for 3 hours. Plates were washed 4 times and 1μg/ml of biotinylated detecting antibody (PharMingen) in PBS/10% FCS added at 50μl/well. Following a 1 hour incubation at 37°C, plates were washed 6 times and 100μl/well of streptavidin/peroxidase (SAPU, Carluke, UK) added at a 1/1000 dilution in PBS/10% FCS.

Following a final incubation at 37°C for 1 hour the plates were washed 8 times before addition of 100μl of TMB peroxidase substrate (Kirkegaard & Perry Laboratories, 50-76-06) to each well. The light absorbance was then read at 630nm with a reference filter at 405nm on a MRX ELISA reader (Dynatech Laboratories). Serial dilutions of recombinant cytokines were included as standards on each plate and the concentration in the test samples calculated from the resulting standard curve using the BIOLINX software package (Dynatech Laboratories).

2.8.2 Total immunoglobulin ELISAs

Total levels of IgG1, IgG2a and IgE immunoglobulins in the serum of individual mice were also determined by capture sandwich ELISA. Sera samples were prepared for analysis as described in section 2.7.1. Buffers, solutions and antibodies used are listed in the Appendix (sections 2.13.6 and 2.13.8). Essentially, 96 well microelisa plates were coated overnight at 4°C with 50μl/well of 4μg/ml (IgG1) or 2μg/ml (IgG2a, IgE) of monoclonal capture antibody (PharMingen) in 0.1M carbonate buffer pH 8.2. Plates were washed twice for 1 minute with PBS/0.05% Tween-20 and pounded dry on paper towels. Non-specific protein binding sites were blocked by incubation with 200μl of PBS/10% FCS for 1 hour at 37°C. Plates were washed twice as before after which test samples (diluted 1/100 with PBS/10% FCS for IgG1 and IgE and 1/1000 for IgG2a) and standards were added in triplicate to individual wells in a volume of 50μl and incubated at 37°C for 2 hours. Plates were washed 4 times and 2μg/ml of biotinylated detecting antibody (PharMingen) in PBS/10% FCS added at 50μl/well. This concentration of detection antibody was used for all three isotypes examined. Following a 1 hour incubation at 37°C, plates were washed 6 times and 100μl/well of streptavidin-peroxidase (SAPU) added at a 1/1000 dilution in PBS/10% FCS. Following a final incubation at 37°C for 1 hour the plate was washed 8 times before addition of 100μl of TMB peroxidase substrate (Kirkegaard & Perry Laboratories) to each well. The light absorbance was then read at 630nm with a reference filter at 405nm on a MRX ELISA reader (Dynatech Laboratories). Serial dilutions of purified antibody were included as standards on each plate and the

concentration in the test samples calculated from the resulting standard curve using the BIOLINX software package (Dynatech Laboratories).

2.8.3 Antigen-specific antibody ELISAs

Levels of antigen-specific IgG1 and IgG2a in test serum were determined by a modification of the capture sandwich ELISA. Sera samples were prepared for analysis as described in section 2.7.1. Buffers, solutions and antibodies used are listed in the Appendix (sections 2.13.6 and 2.13.8). 96 well microelisa plates were coated overnight at 4°C with 50μl/well of 5μg/ml ABF, rABA-1 or OVA diluted in 0.05M carbonate buffer pH 9.6. Plates were washed twice for 1 minute with PBS/0.05% Tween-20 and pounded dry on paper towels. Non-specific protein binding sites were blocked by incubation with 200μl of PBS/10% FCS for 1 hour at 37°C. The plates were washed twice with PBS/0.05% Tween-20 and blocked for 1 hour at 37°C with PBS/10% FCS, 200μl/well. Pooled murine sera from animals seropositive for ABA-1, ABF or OVA and pooled naive murine sera were diluted 1/50 with PBS/10% FCS and used as positive and negative controls respectively. Test samples were also diluted 1/50 prior to use. All samples and controls were added in triplicate to individual wells in a 50μl volume and incubated for 2 hours at 37°C. Plates were washed 4 times and 2μg/ml of biotinylated detecting antibody (PharMingen) in PBS/10% FCS added at 50μl/well. The same concentration of detection antibody was used for both antibody isotypes examined. Following a 1 hour incubation at 37°C, plates were washed 6 times and 100μl/well of streptavidin-peroxidase (SAPU) added at a 1/1000 dilution in PBS/10% FCS. Following a final incubation at 37°C for 1 hour the plate was washed 8 times before addition of 100μl of TMB peroxidase substrate (Kirkegaard & Perry Laboratories) to each well. The light absorbance was then read at 630nm with a reference filter at 405nm on a MRX ELISA reader (Dynatech Laboratories). The amount of antigen specific antibody in a sample was expressed in arbitrary units as a ratio relative to the positive control which was designated numerically as 1.00.

2.9 Heat aggregated ovalbumin preparation

Chicken Ovalbumin Grade V (OVA), (Sigma, A-5503), was dissolved in saline to give a 2% ovalbumin solution and incubated in a 100°C water bath for 1 hour to aggregate the OVA. The supernatant was removed carefully so as not to disturb the aggregated ovalbumin and fresh saline added to return the solution to its original volume. This was taken to yield a concentration of 20mg/ml. The solution was then aliquoted into 0.4ml volumes and stored at -20°C. Prior to use an aliquot of aggregated ovalbumin was added to 3.6ml of saline and placed in a water bath sonicator for 30 minutes, after which 50μl was injected subcutaneously into the foot pads of primed animals.

2.10 Delayed Type Hypersensitivity induction and measurement

Groups of animals were injected sub-cutaneously (s.c.) in the left hind footpad with 100μg of OVA emulsified in 50μl of FCA. In some groups ABF, rABA-1, pABA-1 or ABF minus pABA-1 were added to the OVA before emulsification. Animals were allowed to rest for 7 days before challenge immunisation with 50μl of a 2% heat aggregated ovalbumin (HAO) solution s.c. in the right hind footpad. Footpad thickness was measured before and 24 hours after antigen challenge immunisation using dial gauge microcalipers, 0-10mm in 0.1mm intervals (Röhn GB Ltd., Kingston-Upon-Thames, UK; POCO2T). The difference between the means of the two measurements gave an index of footpad swelling in millimetres which was used for group comparison. Naive animals injected with HAO in both footpads were always included as test controls for non-specific swelling.

2.11 Neutralising anti-interleukin-10 monoclonal antibody treatment *in vivo*

To investigate the effects of depleting the cytokine interleukin (IL)-10 on the generation of DTH immune responses *in vivo*, mice were immunised intra-peritoneally with either 200μg of a neutralising rat-anti-mouse IL-10 monoclonal antibody (clone name JES5-2A5) or 200μg of a rat IgG isotype control (Sigma, I-4131) two hours prior to antigen sensitisation.

Both antibodies were a kind gift of Professor Tim Mitchell, Division of Infection and Immunity, Institute of Biomedical and Life Sciences, University of Glasgow.

2.12 Statistical analysis

Results are presented as mean values (x) plus or minus the standard error of the mean (SEM) for groups of animals undergoing identical treatment. Differences between groups were analysed using a Student's *t*-test. As the Student's *t*-test assumes normal distribution within a population a standard Bonferonni correction was used to control for this factor in the experiments described where test groups generally consisted of 5 animals. The Bonferonni correction was generated by dividing the probability value (p) of 0.05, the assumed upper limit of significance in a *t*-test, by the total number of *t*-tests performed within a data set. This decreases the probability of significance for the data set whilst correcting for the small sample sizes.

2.13 Appendix

2.13.1 General solutions

(a) dH_2O

The distilled, de-ionised water used throughout was produced by a Milli-Q 50 water purification system (Millipore Ltd., Watford, UK).

(b) 0.9% (w/v) saline

NaCl	9g (Fisher Scientific, UK)

Increase volume to 1L with dH_2O.

(c) PO_4 stock

$Na_2HPO_4.12H_2O$	60g (BDH)
$NaH_2PO_4.2H_2O$	13.6g (Fisher Scientific, UK)
NaCl	8.5g

Increase volume to 1L with dH_2O.

(d) Phosphate Buffered Saline, PBS, pH 7.2

PO_4 stock	40ml

Increase volume to 1L with 0.9% saline and adjust pH to 7.2.

(e) 5% (w/v) sodium bicarbonate

$NaHCO_3$	5g (BDH)

Increase volume to 100ml with dH_2O and autoclave prior to use.

2.13.2 Bacterial culture media

(a) T-broth

Bacto-tryptone	12g (UniPath Ltd.)
Yeast extract	24g (UniPath Ltd.)

Glycerol	4ml (Fisher Scientific, UK)

Volume was increased to 900ml with dH_2O and the solution was autoclaved. Once the temperature of the solution was below 60°C, 100ml of sterile K phosphate was added.

(b) K phosphate

KH_2PO_4	23.1g (Fisher Scientific, UK)
K_2HPO_4	125.4g (BDH)

The volume was increased to 1L with dH_2O and autoclaved.

(c) L-broth

Bacto-tryptone	10g
Yeast extract	5g
NaCl	10g

The pH was adjusted to 7.2 with NaOH, the volume increased to 1L with dH_2O and the solution autoclaved.

(d) L-agar

1L of L-broth was made up as described above. 15g of Bacto-agar (Difco) was added to a 1L bottle prior to adding the L-broth.

2.13.3 Protein purification solutions

(a) 10X PBS (Pharmacia recipe)

NaCl	81.82g
KCl	2.01g (BDH)
Na_2HPO_4	14.34g
KH_2PO_4	2.45g

The pH was adjusted to 7.3 and the volume increased to 1L with dH_2O. The solution was stored at 4°C and made up to 1X PBS with dH_2O prior to use.

(b) Glutathione Elution Buffer

10mM Reduced glutathione	0.154g (Sigma, G4251)
50mM Tris-HCl, pH 8.0	50ml

The solution was stored at -20°C.

(c) 0.1M Tris-HCl/0.5M NaCl, pH 8.5

Tris-HCl	7.88g
NaCl	14.61g

The pH was adjusted to 8.5 with NaOH and the volume increased to 500ml with dH_2O. The solution was stored at 4°C.

(d) 0.1M Na Acetate/0.5M NaCl, pH 4.5

Na Acetate	4.10g (Sigma)
NaCl	14.61g

The pH was adjusted to 4.5 with HCl and the volume increased to 500ml with dH_2O. The solution was stored at 4°C.

(e) FPLC buffer

0.15M NaCl	4.38g
0.05M K_2HPO_4	4.35g

The pH was adjusted to 7.2 and the volume increased to 500ml with dH_2O. The solution was filtered and degassed prior to use.

2.13.4 Electrophoresis buffers and solutions

(a) Resolving gel buffer, pH 8.8

1.5M Tris	18.15g (BDH)
0.4% SDS	4 ml of 10% (w/v) stock

The pH was adjusted to 8.8 with HCl and the volume increased to 100 ml with dH_2O.

(b) Stack gel buffer, pH 6.8

0.5M Tris	6.05g
0.4% SDS	4 ml of 10% (w/v) stock

The pH was adjusted to 6.8 with HCl and the volume increased to 100 ml with dH_2O.

(c) 10% (w/v) APS

1g of ammonium persulphate (Sigma) was dissolved in 10ml of dH_2O. The solution was dispensed into 1ml aliquots and stored at -20°C until use.

(d) 17.5% Resolving gels

Resolving gel buffer	3.75ml
Acrylamide stock	8.75ml (ScotLab, UK, SL-9208)
(30% (w/v) Acryl : 0.8% (w/v) Bis)	
dH_2O	2.5ml
10% (w/v) APS	100μl
TEMED	10μl (Sigma, T9281)

(e) 4.75% Stacking gels

Stacking gel buffer	1.25ml
Acrylamide stock	750μl
dH_2O	3ml
10% (w/v) APS	50μl
TEMED	10μl

(f) 5X Tris-glycine electrophoresis buffer

Glycine	1440g (Sigma)
Tris-base	300g (BDH)
SDS	50g

Increase to 10L with dH_2O and dilute to 1X prior to use.

(g) Non-reducing sample buffer

10% (w/v) SDS	2ml
Glycerol	1ml
1M Tris, pH 6.8	625μl
1% bromophenol blue	100μl (Sigma)

Increase volume to 10ml with dH_2O and store in foil covered universals to protect from sunlight.

(h) Reducing sample buffer

As for non-reducing buffer but with the addition of 500μl of 2-mercaptoethanol (Sigma, M6250).

2.13.5 Tissue culture medium

Incomplete RPMI

RPMI 1640 powder	(Gibco BRL)
25mM Hepes	5.96g (Sigma)

The pH was adjusted to 7.2 using NaOH and the volume increased to 1L with dH_2O. The resulting solution was filter sterilised and then under sterile conditions the following added:

5mM L-Glutamine	11ml (Gibco BRL)
3.5% $NaHCO_3$	5.5ml
0.1M 2-Mercaptoethanol	0.55ml

The medium was then dispensed into 90ml aliquots and stored at 4°C. To complete the medium 10ml of heat inactivated Foetal Calf Serum, FCS, (Gibco BRL) was added.

2.13.6 ELISA buffers and solutions

(a) 0.1M carbonate coating buffer, pH 8.2

$NaHCO_3$	4.2g

The pH was adjusted to 8.2 and volume increased to 500ml with dH_2O.

(b) 0.05M carbonate/bicarbonate coating buffer, pH 9.6

Na_2CO_3	2.65g
$NaHCO_3$	2.1g

The pH was adjusted to 9.6 and the volume increased to 500ml with dH_2O.

2.13.7 Stains and fixatives

(a) Coomassie Blue R-250 stain

Coomassie Blue R-250	1 g (Sigma)

Volume was increased to 1L with Coomassie Blue destain.

(b) Coomassie Blue Destain

Methanol	2.5 L (Fisher Scientific, UK)
Acetic acid	1 L (Fisher Scientific, UK)
Glycerol	100 ml

The volume was increased to 10L with dH_2O.

2.13.8 Antibodies & standards used for ELISAs

Cytokine/ antibody ELISA	Capture monoclonal antibody (clone designation)	Detecting monoclonal antibody (clone designation)	Recombinant cytokine standard (Cat. No.)	Standard curve maximum concentration#
IL-4	11B11	BVD6-24G2	19231W	400U/ml
IL-5	TRFK5	TRFK4	19241W	100U/ml
IL-10	JES5-2A5	SXC-1	19281V	200U/ml
IFN-γ	R4-6A2	XMG1.2	19301U	1000U/ml
IgG1	G1-6.5	A85-1 R26-46* R8-140*	03001D	1μg/ml
IgG2a	R12-4	R19-15	03021D	0.4μg/ml
IgE	R35-72	R35-92	03131D	2μg/ml

# Indicates the starting concentration of the standard curve used for each ELISA. The standard was then diluted through seven doubling dilutions to create the curve.

* Indicates detecting antibodies that were combined in a cocktail for total IgG1 ELISAs. A85-1 was used for antigen specific IgG1 ELISAs only.

All monoclonal antibodies and recombinant standard proteins were purchased from PharMingen.

CHAPTER 3

THE IMMUNE RESPONSE TO *ASCARIS SUUM* INFECTION

3.1 Introduction

3.1.1 Helminth infection and immunity

Immunity to parasitic helminth infections is characterised by eosinophilia, mastocytosis and increased production of IgE immunoglobulin (Urban Jr. *et al.* 1992). These responses are controlled by an increase in the production of the Th2-associated cytokines interleukin (IL)-3 IL-4, IL-5, IL-9, IL-10 and IL-13 (Finkelman *et al.* 1997). The development of protective immunity has an absolute requirement for T lymphocytes, specifically the $CD4^+$ subset; for example, mice vaccinated against *Brugia pahangi* were unable to control a challenge infection as a result of treatment with a depleting anti-CD4 antibody (Bancroft *et al.* 1994b; McKay *et al.* 1995). Although $CD4^+$ T cells are vital to host protection, they do not appear to mediate their effects directly on the parasites, but through the induction of effector mechanisms by the cytokines they secrete following activation.

3.1.2 Cytokine production and the effect on host immunity to infection

The intrinsic importance of selective expansion of the $CD4^+$ T cell subsets and the development of protective immunity has been demonstrated in a variety of helminth infections. BALB/c mice vaccinated with the irradiated larvae of *B. pahangi* develop strong immunity to challenge infections which is characterised by high IL-5 and IL-9, moderate IL-4 and negligible amounts of IFN-γ in mitogen and antigen restimulated spleen cultures of these animals, accompanied by a significant elevation in parasite-specific IgE and peripheral blood eosinophilia (Bancroft *et al.* 1993). This response phenotype was also observed with *Trichinella spiralis* (Grencis *et al.* 1991).

The first description correlating $CD4^+$ Th subset activation and the development of protective immunity was in work by Else and Grencis with *Trichuris muris* (Else *et al.* 1992b; Else and Grencis 1991a). By infecting H-2 compatible strains of mice it was found that responder mice which rapidly cleared infection developed a Th2 phenotype with high levels of IL-3, IL-4, IL-5, IL-9 and low IFN-γ production. In contrast, those unable to

expel the parasite develop chronic infections showed the antithetical Th1 cytokine profile. Development of such responses also promoted differences in the character of the antibody response; responder strains generated high IgE and IgG1 titres while non-responders had high levels of IgG2a. Despite these differences in antibody production it was later shown that resistance to infection was not dependent on antibody and that infections could be controlled in its absence (Else and Grencis 1996).

Alterations of the adult worm expulsion phenotypes occurring naturally in mice as a result of infection were shown to be reversible by altering the cytokine profile of the host (Else *et al.* 1992a). AKR mice susceptible to *T. muris* infection were converted to a resistant phenotype by depleting IFN-γ with monoclonal antibodies or by administering a long-lasting IL-4 formulation made up of the cytokine molecule complexed to a non-neutralising anti-IL-4 monoclonal antibody. Furthermore, by blocking the functions of IL-4 by administering an anti-IL-4 receptor antibody, resistant strains became chronically infected and unable to expel the adult worms. IL-4 complexes have also been used in both normal and immunodeficient mice infected with *Nippostrongylus brasiliensis* or *Heligmosomoides polygyrus* (Urban Jr. *et al.* 1995). In both infections it was found that this treatment decreased adult worm fecundity and cured chronic infections but its effects were dependent upon the life cycle stage of the parasite at the time of administration.

3.1.3 Cytokine control of protective immune mechanisms

The studies discussed above highlight the importance of selective expansion of $CD4^+$ T cells with protection against infection. The differentiation of the T cells results in the secretion of specific sets of cytokines, which in turn affect the protective mechanisms invoked. The influence of cytokines on the development of anti-helminth protective immune mechanisms was first described for IgE production. IL-4 was originally shown to be essential for the antibody class-switching to IgE in studies investigating the effects of cytokine addition to *in vitro* cultures of murine B cells which had been stimulated with bacterial lipopolysaccharide in order to promote the proliferation and differentiation of the

cells into plasma cells (Coffman *et al.* 1986). The presence of IL-4 was demonstrated to induce the production of high levels of IgG1 and IgE at the expense of the IgG2a and IgG3 subclasses. Furthermore, IFN-γ, which is known to be an IL-4 antagonist, enhanced IgG2a and inhibited both IgG1 and IgE (Coffman and Carty 1986; Snapper and Paul 1987). This effect of IL-4 was also demonstrated during infections with either *N. brasiliensis* or *H. polygyrus*, where injection of anti-IL-4 and anti-IL-4 receptor antibodies blocked the IgE responses in both primary and secondary infections (Finkelman *et al.* 1988). More recently, IL-13, a cytokine similar in activity to IL-4, has also been shown to contribute to anti-parasite immunoglobulin responses (Bancroft *et al.* 1998).

Increases in total blood and tissue eosinophil numbers is another manifestation of helminth infections and has been associated with the development of debilitating pulmonary hyper-responsiveness in infected individuals (Hall *et al.* 1998; Ogilvie and Savigny 1982). In many helminth species this effect has been shown to have a direct relationship to IL-5, a cytokine shown to induce the *in vitro* differentiation of eosinophils from bone marrow pre-cursors (Finkelman *et al.* 1990). This was established *in vivo* by treating *N. brasiliensis* infected mice with neutralising anti-IL-5 antibodies which blocked both tissue and blood eosinophilia (Coffman *et al.* 1989).

Cytokine control of mucosal mastocytosis is, in contrast to IgE production and eosinophilia, a much more complex affair. Not only is IL-4 involved in IgE production but also it can induce mastocytosis in collaboration with IL-3, a cytokine that also enhances basophil production during *Strongyloides venezuelensis* infection (Lantz *et al.* 1998; Madden *et al.* 1991). More recently mastocytosis has been shown to be influenced by IL-9 from work using transgenic mice which over-express the cytokine (Faulkner *et al.* 1997). IL-9 transgenic mice have enhanced expulsion of adult *T. spiralis* which was attributed to an increase in enteric mastocytosis. This hypothesis was confirmed by treating the transgenics with anti-c-*kit* antibodies, which depressed the mast cell response and prevented worm expulsion.

3.1.4 Experimental design and aims

In light of the vast amount of data on protective immunity against helminth infections, the aims of the experiments presented in this chapter were to investigate the effects of infection with *Ascaris suum* in mice and to establish whether such effects were influenced by the number of infections an animal received. To address this groups of mice were given a single or multiple infective doses of *A. suum* eggs orally and the resulting cytokine and proliferation responses and antibody production measured. This would reveal if *Ascaris* infection of mice promoted the development of a Th2-type immune response as observed with other murine helminth infections. The effects of crude parasite antigens in *Ascaris* primed mice were also investigated by challenging infected animals with *Ascaris* body fluid (ABF) emulsified in Freund's Complete Adjuvant (FCA). This was to discover if exposure to such antigens could modulate the immune response normally observed in the mice as a result of infection.

Following on from this, previous studies in which mice were immunised with products of *Ascaris* indicated that the immune response generated was influenced by the host animal's Major Histocompatability Complex (MHC) haplotype (Tomlinson *et al.* 1989) therefore, the effects of host genetics upon the immune response associated with *Ascaris* infection in mice were also investigated. Groups of B10.S ($H\text{-}2^s$), B10.S(9R) ($H\text{-}2^{q4}$) and C57BL/10 ($H\text{-}2^b$) mice were given multiple oral immunisations with infective *A. suum* eggs. The resulting cytokine and proliferative responses and antibody production were measured to assess the effects of host genetics and strain background. Control animals for each strain used were uninfected.

3.2 Results

3.2.1 The immune response generated by *Ascaris suum* infection has a Th2 phenotype

The immune response resulting from infection with *Ascaris suum* was investigated in B10.S mice which had previously been shown to be high IgE responders following immunisation with the major allergen of *Ascaris*, ABA-1 (Tomlinson *et al.* 1989). Groups of 5 animals were orally immunised 1, 2 or 3 times with 3000 infective eggs of the parasite at weekly intervals. To investigate the effects of crude parasite antigens on primed mice a second group, which had received three infections, were challenged intra-peritoneally with 100μg of *Ascaris* body fluid (ABF) emulsified in Complete Freund's Adjuvant (CFA) one week after the last infection. Seven days after the completion of the infection programmes all animals were sacrificed in order to analyse cytokine responses and the production of serum immunoglobulins.

Figure 3.1 illustrates cytokine production in the culture supernatants of the mesenteric lymph node populations following restimulation with various stimuli *in vitro* for 72 hours. These populations had been pooled within test groups. Stimulation of cultures with the Con A mitogen indicated that the production of IL-4 increased with the number of infections an animal received. In contrast, IFN-γ levels were found to have decreased after one infection and were undetectable after two or more doses. Antigen-specific cytokine production was found at low levels with IL-4 in the mice given two or more doses of infective eggs. Challenge of primed mice with ABF emulsified in FCA reversed the cytokine profiles that had been observed for the group receiving the same number of infections following restimulation with Con A. IL-4 production was completely ablated while it was once more possible to detect IFN-γ. Neither cytokine was produced as a result of antigen-specific restimulation.

Antigen-specific immunoglobulin production was measured in the sera of the mice, the results of which are shown in Figure 3.2. Measurement of IgG1 specific for either ABF or rABA-1, the major allergen of *Ascaris*, showed that while one infection with the parasite had no effect upon production, two or more infections significantly enhanced the IgG1 levels when compared to the uninfected controls (p-value ≤ 0.006). It is also interesting to note that in contrast to cytokine production, challenge of mice with the ABF emulsified in FCA enhanced the levels of antigen-specific IgG1 further. This suggested that despite being presented in the context of a potent Th1 promoting adjuvant, ABF can continue to enhance the development of a Th2 immune response. Similarly to IgG1, the pattern of IgG2a production was also identical irrespective of antigen-specificity. Whereas one infection with *Ascaris* had no influence upon the levels of antigen-specific IgG1, with IgG2a this lead to a significant decrease in production. This depression of IgG2a was maintained at the same level as the number of *Ascaris* infections administered increased. However, when mice were challenged with ABF and FCA this resulted in an increase in production which was thought to reflect the effects of adjuvant-assisted immunisation.

Levels of total IgE antibody were also measured in the sera as shown in Figure 3.3. It was found that when the mice were infected once with the parasite it was not possible to detect any more IgE in the sera as was found in the uninfected controls. However, two consecutive infections resulted in a significant increase in production, which was maximal in the group receiving three infections (p-value ≤ 0.006). While levels of antigen-specific antibodies of the IgG sub-classes were found to be enhanced following ABF and FCA challenge production of total IgE decreased to levels similar to those observed in the double infected animals, indicating that the effects of ABF on boosting the immunoglobulin response did not extend to the polyclonal production of IgE.

3.2.2 The immune response against *Ascaris suum* is influenced by host major histocompatability complex (MHC) haplotype

In order to establish whether the MHC haplotype of a host could influence the development of the immune response following infection with *Ascaris*, groups of B10.S ($H\text{-}2^s$), B10.S(9R) ($H\text{-}2^{t4}$) and C57BL/10 ($H\text{-}2^b$) mice were orally immunised 3 times with 3000 infective eggs of the parasite at weekly intervals. Control animals for each strain were uninfected. Seven days after the third infection all animals were killed in order to analyse cytokine production and serum immunoglobulin levels arising from infection.

Cytokine production in the culture supernatants of mesenteric lymphocyte populations restimulated *in vitro* with Con A is shown in Figure 3.4. The lymphocyte populations used had been pooled within test groups. IL-4 production by the B10.S and B10.S(9R) animals showed a 6- and 4-fold increase in production respectively as a consequence of *Ascaris* infection. No IL-4 was detectable in either of the cultures from the C57BL/10 mice. IFN-γ levels were also assessed in the cultures. It was found in the B10.S mice that IFN-γ levels were completely ablated following parasite infection. This effect was even more striking in the B10.S(9R) strain where IFN-γ production was depressed by 94% from levels in the uninfected controls that were 10 times greater than those observed in the B10.S controls (B10.S 45.8U/ml; B10.S(9R) 461.8U/ml). In the C57BL/10 mice IFN-γ production was also completely inhibited by infection. Therefore, *A. suum* does modulate the balance of cytokines in these animals although this did not appear to be the case initially when IL-4 production was quantified.

Antigen-specific IgG1 and IgG2a production was measured in the sera of the mice as illustrated in Figure 3.5. All strains used showed a significant increase (p-value ≤ 0.008) in ABF- and rABA-1-specific IgG1 following *Ascaris* infection when compared to their appropriate controls. Of the infected animals the B10.S mice appeared to produce slightly higher levels of both antibodies in comparison to the other two strains but the differences

in production were not significantly different. Analysis of antigen-specific IgG2a levels revealed that production was very low and was not significantly altered by infection. The exception to this was in ABF-specific IgG2a in the B10.S(9R) mice which had unusually high levels of the antibody in the uninfected controls. Following infection in this strain the levels were significantly decreased (p-value ≤ 0.008) to levels that were equivalent to those found in the other strains.

3.3 Discussion

The data presented in this chapter demonstrate that infection with *Ascaris suum* promotes the development of a Th2-type immune response, as has been reported following infection with other helminth species (Finkelman *et al.* 1997; Urban Jr. *et al.* 1992). The effects of *Ascaris* infection were initially investigated in B10.S mice which had previously been described as a high IgE responder following immunisation with the products of the parasite (Kennedy *et al.* 1986; Tomlinson *et al.* 1989). Cytokine production in the mitogen-stimulated cultures of the draining mesenteric lymph nodes showed that infection was associated with an increase in the production of the Th2 promoter, IL-4 and a decrease in IFN-γ. This effect was shown to be dependent on the number of infections administered (Fig. 3.1). It was also found possible to reverse the observed cytokine profiles by challenging *Ascaris* primed animals with crude parasite antigen (ABF) emulsified in the Th1-promoting adjuvant FCA.

Modifications in the production of antigen-specific immunoglobulins were also noted with respect to increasing rounds of parasite infection. Both ABF and rABA-1-specific IgG1 required a minimum of two infections before a significant increase in production could be detected (Fig. 3.2). Again, this effect was directly proportional to the number of infections. In contrast, specific IgG2a significantly decreased, an effect associated with increased IL-4 (Snapper and Paul 1987). It is interesting to note that while challenge with ABF and FCA ablated the production of IL-4 it further increased the production of both ABF and rABA-1-specific IgG1, indicating that the parasite antigen is still capable of driving this aspect of the Th2 response despite being presented in the context of a potent adjuvant. Furthermore, levels of antigen-specific IgG2a were significantly increased as a result of challenge emphasising the capacity of the adjuvant to promote Th1 responses. Analysis of total serum IgE production revealed that, like antigen-specific IgG1, mice had to be infected twice in order to exhibit differences in the levels detected (Fig. 3.3). Challenge with ABF and FCA depressed production but this was still significantly different from the levels observed in the uninfected controls.

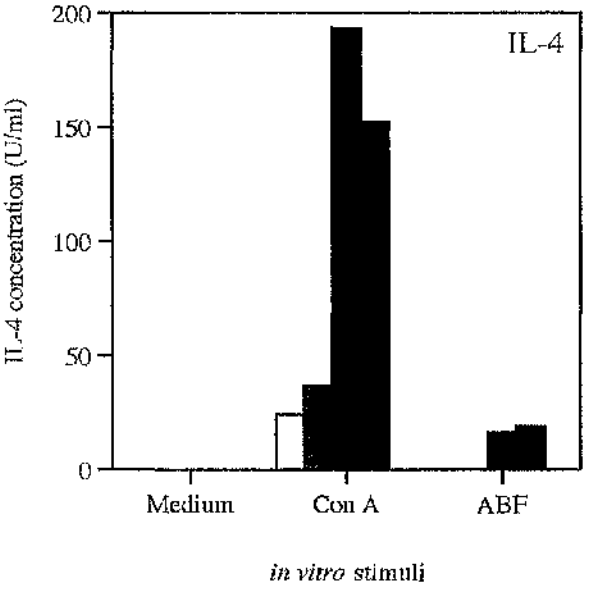
As described earlier, host genetics can have considerable influence on whether the immunity induced is protective (Else *et al.* 1992b; Else and Grencis 1991b). This was investigated for *Ascaris* infection as illustrated in Figures 3.4 and 3.5. Measurement of cytokine production by the mesenteric lymphocytes as a result of mitogen restimulation *in vitro* suggested that host genetics could be influencing immune responsiveness. IL-4 production was highest in B10.S mice, and approximately ten-fold less in the B10.S(9R) strain. C57BL/10 animals did not produce IL-4 at all. IFN-γ was also measured in the cultures and it was found that infection resulted in a 94% decrease in production in the B10.S(9R) animals and a complete ablation in both B10.S and C57BL/10. The effect in C57BL/10 was of particular interest as it indicated that while the effects of infection did not appear outwardly to promote Th2 development, as indicated by the lack of IL-4 production, the parasite was still altering the Th1/Th2 balance by blocking the production of Th1-associated cytokines.

This point was reinforced by the antigen-specific antibody responses. All the strains exhibited a significant increase in ABF and rABA-1-specific IgG1 following infection when compared to the appropriate uninfected controls. The response appeared to be slightly higher in the B10.S animals but this was not statistically different from the levels found in the other strains. Antigen-specific IgG2a was similarly low in all the groups with the exception of the naiveB10.S(9R) mice which had exceptionally high responses. This was, however, reduced to levels similar to those found in the other animals following infection.

Figure 3.1

Multiple infections with *A. suum* influences the cytokine profiles of murine mesenteric lymphocytes. IL-4 and IFN-γ protein production was assessed in the culture supernatants of mesenteric lymphocytes from B10.S mice following *in vitro* restimulation for 72 hours. Mice were immunised orally with 3000 infective eggs of *A. suum* as detailed below at weekly intervals. Controls were left unimmunised. Group 5 animals were challenged one week after the final *A. suum* infection with 100μg ABF emulsified in FCA intra-peritoneally. The animals were sacrificed 7 days post-challenge or final infection and the mesenteric lymphocytes within groups pooled. Cytokine production was measured using capture sandwich ELISAs.

Group	Week 1	Week 2	Week 3	Week 4
1 □	-	-	-	-
2 ■	-	-	-	1 x *A. suum*
3 ■	-	-	1 x *A. suum*	1 x *A. suum*
4 ■	-	1 x *A. suum*	1 x *A. suum*	1 x *A. suum*
5 ■	1 x *A. suum*	1 x *A. suum*	1 x *A. suum*	100μg ABF + FCA


IL-4
IL-4 concentration (U/ml)
200
150
100
50
0
Medium
Con A
ABF
in vitro stimuli

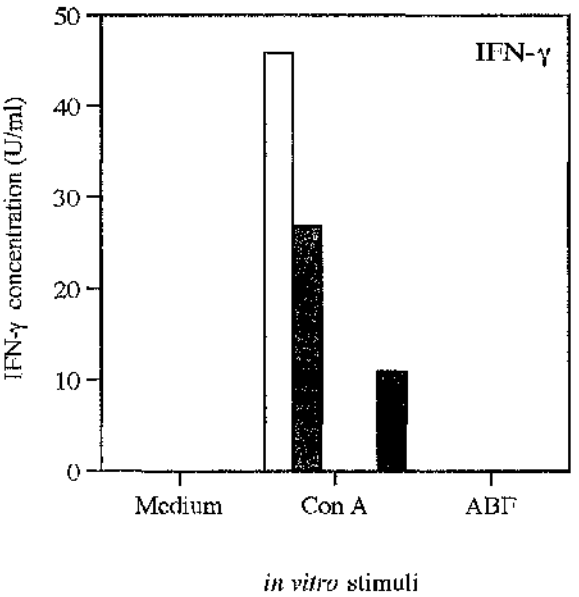
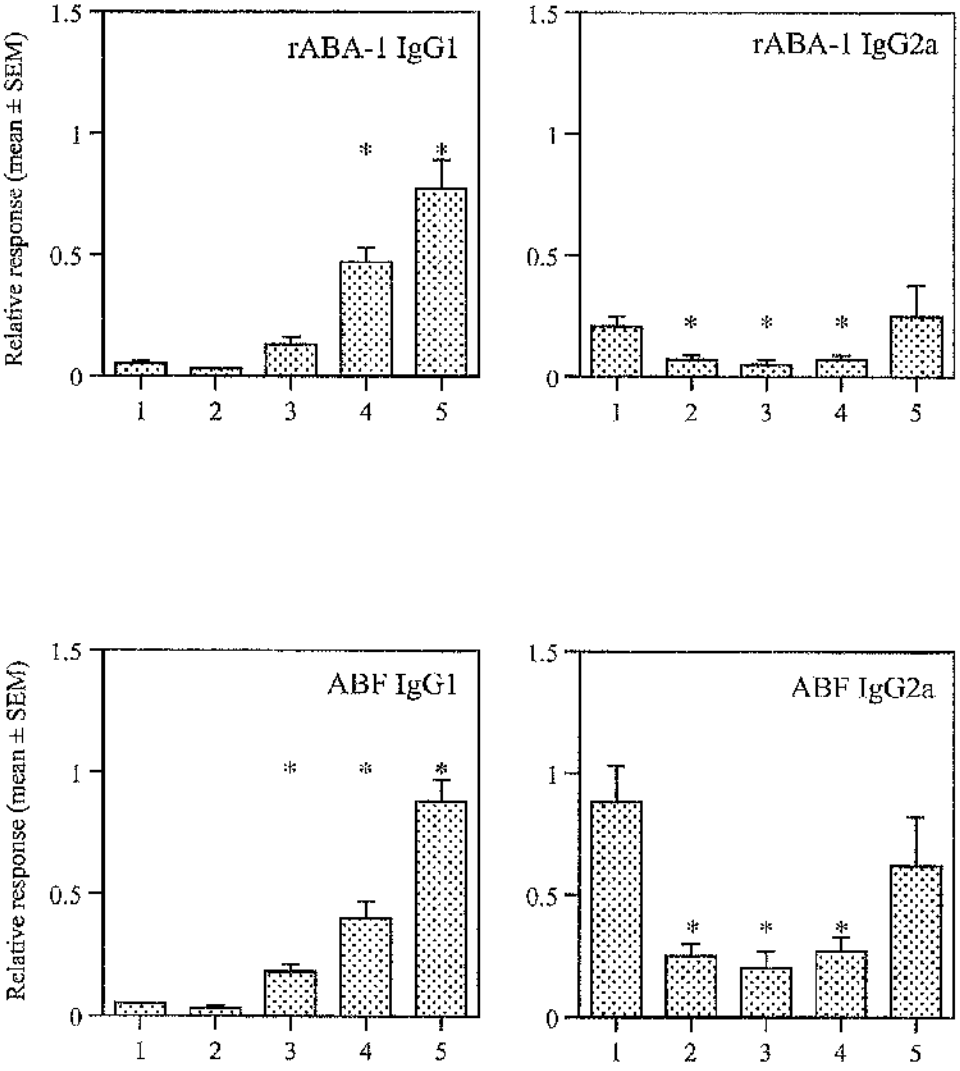

IFN-γ
IFN-γ concentration (U/ml)
50
40
30
20
10
0
Medium
Con A
ABF
in vitro stimuli

Figure 3.2

Multiple infections with *A. suum* influences the production of antigen-specific antibody. B10.S mice received 3000 infective eggs of *A. suum* orally as detailed below at weekly intervals. Controls were left unimmunised. Group 5 animals were challenged one week after the final *A. suum* infection with 100μg ABF emulsified in FCA intra-peritoneally. The animals were sacrificed 7 days post-challenge or final infection and the sera collected. Antigen-specific immunoglobulins were measured by ELISA. Data are expressed as the amount of antigen-specific antibody in a sample relative to the positive control in arbitrary units. Each data point represents the mean ± SEM for an experiment performed in triplicate.

* represents a p-value ≤ 0.006 when compared to group 1 and group 2 using a Student's *t*-test.

Group	Week 1	Week 2	Week 3	Week 4
1	-	-	-	-
2	-	-	-	1 x *A. suum*
3	-	-	1 x *A. suum*	1 x *A. suum*
4	-	1 x *A. suum*	1 x *A. suum*	1 x *A. suum*
5	1 x *A. suum*	1 x *A. suum*	1 x *A. suum*	100μg ABF + FCA


rABA-1 IgG1
rABA-1 IgG2a
ABF IgG1
ABF IgG2a
Relative response (mean ± SEM)
1.5
1
0.5
0
1
2
3
4
5
*

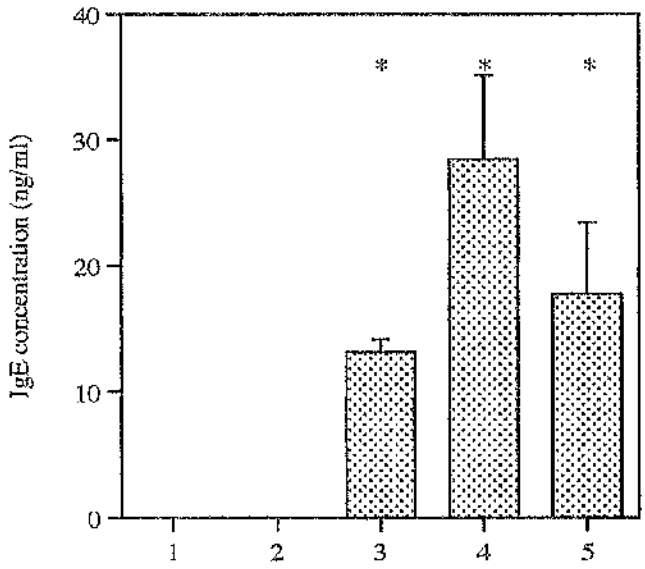


Figure 3.3

Total serum IgE concentrations in mice receiving multiple *A. suum* infections. B10.S mice received 3000 infective eggs of *A. suum* orally as detailed below at weekly intervals. Controls were left unimmunised. Group 5 animals were challenged one week after the final *A. suum* infection with 100μg ABF emulsified in FCA intraperitoneally. The animals were sacrificed 7 days post-challenge or final infection and the sera collected. Total IgE was measured by ELISA. Each data point represents the mean ± SEM for an experiment performed in triplicate.

* represents a p-value ≤ 0.006 when compared to group 1 and group 2 using a Student's *t*-test.

Group	Week 1	Week 2	Week 3	Week 4
1	-	-	-	-
2	-	-	-	1 x *A. suum*
3	-	-	1 x *A. suum*	1 x *A. suum*
4	-	1 x *A. suum*	1 x *A. suum*	1 x *A. suum*
5	1 x *A. suum*	1 x *A. suum*	1 x *A. suum*	100μg ABF + FCA

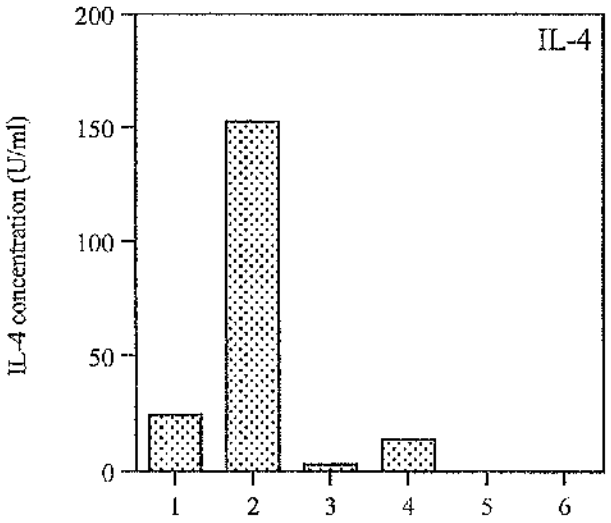
Figure 3.4

Host MHC haplotype influences the cytokine profile produced following *Ascaris* infection. IL-4 and IFN-γ production was assessed in the culture supernatants of mesenteric lymphocytes from B10.S, B10.S(9R) and C57BL/10 mice following *in vitro* restimulation for 72 hours. Mice were immunised orally with 3000 infective eggs of *A. suum* as detailed below at weekly intervals. Controls were left unimmunised. The animals were sacrificed 7 days after the final infection and the mesenteric lymphocytes within groups pooled. Cytokine production was measured using capture sandwich ELISAs.

Group	Strain	MHC haplotype	Treatment
1	B10.S	H-2^s	-
2	B10.S	H-2^s	3 x *A. suum*
3	B10.S(9R)	H-2^{q}	-
4	B10.S(9R)	H-2^{q}	3 x *A. suum*
5	C57BL/10	H-2^b	-
6	C57BL/10	H-2^b	3 x *A. suum*

MHC haplotype	K	A_β	A_α	E_β	E_α	S	D	L
H-2^s	s	s	s	(s	s)	s	s	s
H-2^{q}	s	s	s	s/k	k	d	d	d
H-2^b	b	b	b	b	b	b	b	b

Parentheses represent inability to express the H-2E heterodimer because of the defective E_α^s chain gene. The B10.S(9R) strain will express the I-E molecules as the E_α^k gene product is expressed normally and assembles with the $E_\beta^{s/k}$ chain on the cell surface.


IL-4
IL-4 concentration (U/ml)
200
150
100
50
0
1
2
3
4
5
6

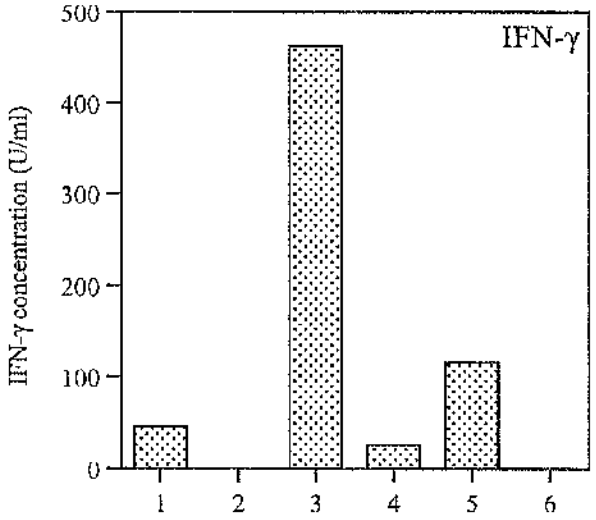

IFN-γ
IFN-γ concentration (U/ml)
500
400
300
200
100
0
1
2
3
4
5
6

Figure 3.5

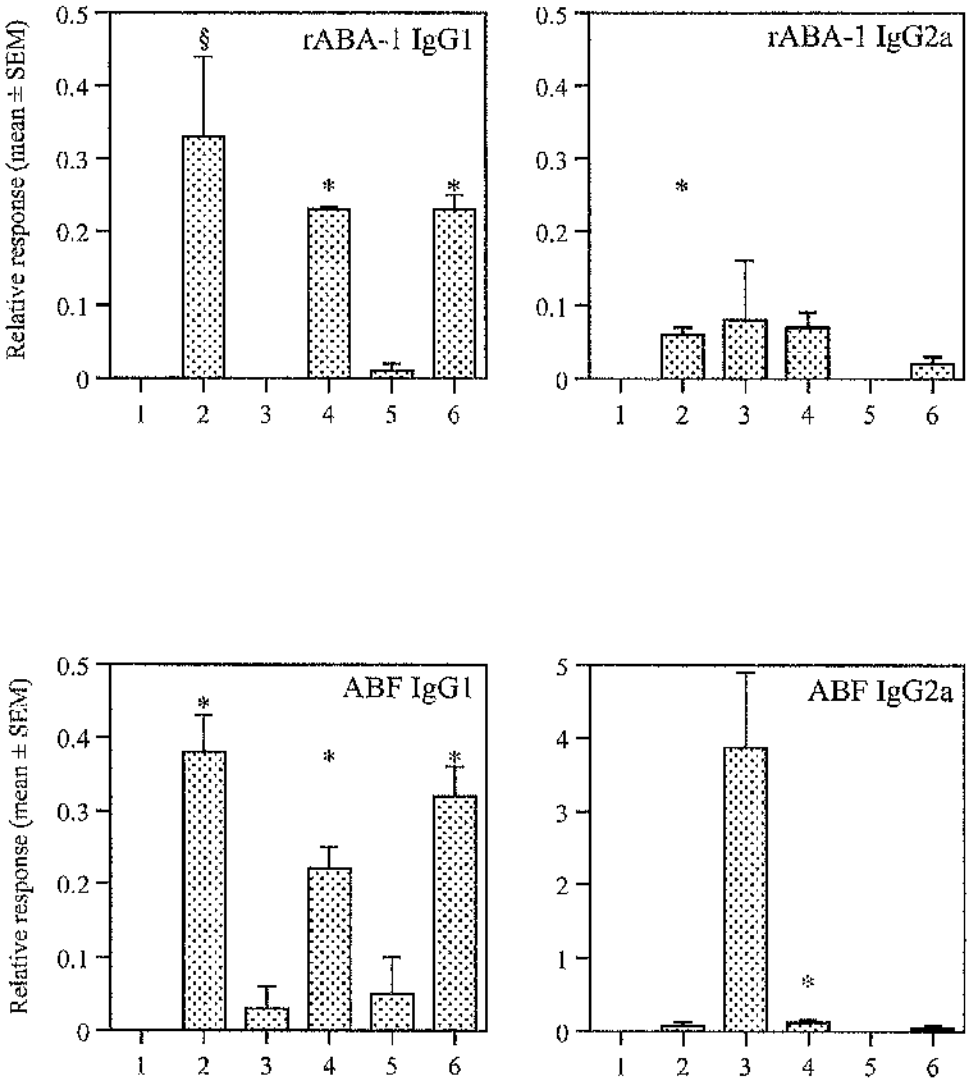
Host MHC haplotype influences the antigen-specific immunoglobulin production following *Ascaris* infection. Groups of B10.S, B10.S(9R) and C57BL/10 mice were immunised orally with 3000 infective eggs of *A. suum* as detailed below at weekly intervals. Controls were left unimmunised. The animals were sacrificed 7 days after the final infection and the sera collected. Antigen-specific immunoglobulins were measured by ELISA. Data are expressed as the amount of antigen-specific antibody in a sample relative to the positive control in arbitrary units. Each data point represents the mean ± SEM for an experiment performed in triplicate.

* represents a p-value ≤ 0.008 and § approaching 0.008 when uninfected controls were compared to the *Ascaris* infected animals of the same strain using a Student's *t*-test.

Group	Strain	MHC haplotype	Treatment
1	B10.S	H-2^s	-
2	B10.S	H-2^s	3 x *A. suum*
3	B10.S(9R)	H-$2^{s/q}$	-
4	B10.S(9R)	H-$2^{s/q}$	3 x *A. suum*
5	C57BL/10	H-2^b	-
6	C57BL/10	H-2^b	3 x *A. suum*

MHC haplotype	K	A_β	A_α	E_β	E_α	S	D	L
H-2^s	s	s	s	(s	s)	s	s	s
H-$2^{s/q}$	s	s	s	s/k	k	d	d	d
H-2^b	b	b	b	b	b	b	b	b

Parentheses represent inability to express the H-2E heterodimer because of the defective E_α^s chain gene. The B10.S(9R) strain will express the I-E molecules as the E_α^k gene product is expressed normally and assembles with the $E_\beta^{s/k}$ chain on the cell surface.


rABA-1 IgG1
rABA-1 IgG2a
ABF IgG1
ABF IgG2a
Relative response (mean ± SEM)
Relative response (mean ± SEM)
§
*
0
0.1
0.2
0.3
0.4
0.5
1
2
3
4
5
6

CHAPTER 4

THE IMMUNE RESPONSE TO THE *ASCARIS* ALLERGEN, ABA-1

4.1 Introduction

4.1.1 Parasite products and Th2 response development

Helminth infections in humans and animals are characterised by the development of strong Th2 responses that can induce pathology reminiscent of that observed in atopic disorders. The features of the infection causing this bias in the T cell response are unknown, but suggestions include both the site and route of antigen presentation, activation of cells of the non-specific immune response, modification by a physical component of the parasite or dose (Liew *et al.* 1985; Yang *et al.* 1991). The induction of high levels of IgE immunoglobulin and the development of hypersensitivity reactions in the host (Ogilvie and Savigny 1982) have drawn attention to the role of parasite products in the development of Th2 responses. For example, priming naive $CD4^+$ T cells from healthy, normal individuals with filarial-derived antigens *in vitro* leads to enhanced IL-5 production upon antigen restimulation, demonstrating that the antigens can cause deviation of a developing immune response (Steel and Nutman 1998). Furthermore, filarial antigens have been shown to be capable of preferentially expanding existing Th2-like populations in human peripheral blood *ex vivo* (Mahanty *et al.* 1993).

Exposure to parasite somatic antigens has also been shown to influence immunoglobulin production. B lymphocytes from *Nippostrongylus brasiliensis*-infected mice exhibit enhanced production of IgG and IgE during *in vitro* culture in the presence of adult worm homogenate (Stephan and Konig 1992). This has also been reported in humans where normal peripheral blood mononuclear cell cultures exposed to recombinant proteins of *Onchocerca volvulus* produced antigen-specific and polyclonal IgG4 and IgE (Garraud *et al.* 1995). The ability to induce antigen-specific and polyclonal immunoglobulin extends to parasite excretory-secretory (E/S) products too. E/S from adult *N. brasiliensis* can promote the production of non-specific IgE that is utilised by parasites as a protective mechanism diverting the host defences (Uchikawa *et al.* 1993). This effect has also been demonstrated with the E/S of *Dirofilaria immitis*, which in addition to enhancing IgE

production can also stimulate Th2 cytokine production and the up-regulation of the low affinity IgE receptor, FcεIII / CD23 (Yamaoka *et al.* 1994).

4.1.2 Parasite products and host protection

Besides influencing the differentiation of naive $CD4^+$ T cells and immunoglobulin production, parasite products can be employed to induce host protective immunity. One approach described for *Leishmania major*, was to induce tolerance in susceptible mice to an antigen of the parasite shown to be the focus of the early immune response, resulting in the development of a healer phenotype (Julia *et al.* 1996). Alternatively, immunisation of hosts with the adult homogenates or the E/S products of *Trichinella spiralis* or *Heligmosomoides polygyrus* either alone or together can protect against subsequent infections (Monroy *et al.* 1985; Silberstein and Despommier 1984). In the case of *H. polygyrus*, however, the development of protection was dependent upon the route of antigen presentation, an effect that has also been demonstrated with *Leishmania* antigens (Liew *et al.* 1985; Monroy *et al.* 1985; Yang *et al.* 1991).

4.1.3 Experimental design and aims

As discussed, parasite antigens not only influence the development and differentiation of $CD4^+$ T cells but can also aid the generation of protective immunity. Therefore, it was of considerable interest whether similar effects could be observed with the products of *A. suum*, particularly the ABA-1 allergen. ABA-1 was selected not only because it has been well characterised but it was also readily available as a purified recombinant protein and in an enriched form from the parasite (Kennedy *et al.* 1991; McReynolds *et al.* 1993; Tomlinson *et al.* 1989). Mice infected orally with *A. suum* eggs on three occasions were challenged with recombinant ABA-1 (rABA-1) either solubilised in PBS, emulsified with Freund's Complete Adjuvant (FCA) or adsorbed to aluminium hydroxide adjuvant (alum) intra-peritoneally. Control animals were challenged with PBS, FCA or alum alone. The

resulting cytokine, proliferative and serum immunoglobulin responses indicated whether rABA-1 had similar effects to *Ascaris* body fluid (ABF) in *Ascaris* primed mice.

Another possibility following on from this was that priming with rABA-1 prior to an *Ascaris* infection could modify immune responsiveness, which had been described in other systems (Else *et al.* 1992a). To investigate this, mice were immunised with rABA-1 intra-peritoneally either solubilised in PBS, emulsified with FCA or adsorbed to alum. Control groups were also included for each of the adjuvants. All groups then received multiple doses of *A. suum* infective eggs orally before being sacrificed to assess the cytokine production, proliferation and immunoglobulin production.

While the preceding experiments investigated the influence that immunisation with *Ascaris* products had on the immune response induced by parasite infection it was not known whether exposure to the products themselves could skew the immune response towards a Th2-type phenotype. This was addressed by evaluating the immune responses of mice primed intra-peritoneally with rABA-1 solubilised in PBS, emulsified with FCA or adsorbed to alum and challenged 3 weeks later with rABA-1, in soluble form, emulsified in Freund's Incomplete Adjuvant (FIA), or adsorbed to alum. Control groups were immunised with adjuvant or PBS alone.

4.2 Results

4.2.1 Effect of rABA-1 challenge on the immune response in *Ascaris suum* infected mice

The data presented in the previous chapter demonstrated that challenge of *Ascaris*-infected mice with the parasite body fluid, ABF, and adjuvant depressed the anti-*Ascaris* immune response. However, it was not known whether the ABA-1 allergen of *Ascaris*, which comprises approximately 50% of the total ABF protein (Kennedy and Qureshi 1986), is the active principle. To investigate this, groups of B10.S mice were infected orally with 3000 *A. suum* eggs on three occasions at weekly intervals. Seven days after the third infection the animals were challenged with 100μg of recombinant ABA-1 (rABA-1) solubilised in PBS, emulsified with Freund's Complete Adjuvant (FCA) or adsorbed to aluminium hydroxide adjuvant (alum) intra-peritoneally. Control groups were challenged with PBS, FCA or alum alone. The mice were sacrificed one week post-challenge in order to analyse the cytokine and proliferative responses of the mesenteric lymph node (MLN) and spleen (SPL) lymphocyte populations. In addition, serum immunoglobulin production was measured.

Figure 4.1 illustrates the cytokine levels, as measured by capture sandwich ELISA, in the culture supernatants of MLN and SPL cells that had been restimulated *in vitro* for 72 hours. The lymphocyte populations used were pooled within test groups. The Th2-associated cytokine IL-4 was not detectable in any of the culture supernatants, but differences were observed in IL-5 production. In the MLN cultures, it was found that challenge with alum alone (group 5) induced the highest production of IL-5 following stimulation with Con A. Challenge with rABA-1 adsorbed to the alum resulted in a 50% decrease in the IL-5 detected, whereas the other groups challenged with rABA-1 exhibited increased secretion relative to the controls. In the SPL population, no major differences between the groups were found but IL-5 production was approximately three fold less than that by the MLN cells. IL-5 could not be detected in either population following culture with rABA-1, and ABF-specific IL-5 production was found only in the MLN cultures of

the FCA-rABA-1, alum control and alum-rABA-1 challenged (groups 4 – 6). Analysis of IFN-γ production revealed that very little of this cytokine was produced by MLN cells as a result of Con A stimulation with the exception of animals challenged with alum and rABA-1. This was also found when the MLN cells were cultured with rABA-1. Systemic production of IFN-γ measured in the SPL occurred in all the cultures but was reduced in the FCA- and rABA-1-challenged animals. Furthermore, rABA-1-specific IFN-γ was secreted at high levels following challenge with rABA-1 irrespective of adjuvant used.

The ability of the MLN cells to proliferate following *in vitro* restimulation are shown in Figure 4.2. All groups responded well to stimulation with the T cell mitogen, Con A, particularly those challenged with alum alone or mixed with rABA-1 (groups 5 and 6). This suggested that immunisation with the Th2 promoting adjuvant could enhance proliferation non-specifically. An identical pattern of responsiveness was also observed when cells were cultured in the presence of ABF. Levels of rABA-1-specific proliferation were enhanced in all the rABA-1-immunised animals and was most pronounced in the alum and rABA-1 challenged group. Similar results were found in the spleen (data not shown).

Measurement of antigen-specific immunoglobulin production in the mice, (Fig. 4.3), revealed that both rABA-1- and ABF-specific IgG1 were greatest when the mice were challenged with rABA-1 mixed with FCA or alum (groups 4 and 6). Equally, when rABA-1-specific IgG2a was measured, an identical pattern was observed, with groups 4 and 6 producing significantly more of the antibody in comparison to the PBS controls (group 1). ABF-specific IgG2a levels approached a significant difference from group 1 only when the animals were challenged with FCA and rABA-1. Demonstrating in this instance the ability of FCA as a Th1 response-promoting adjuvant that favours the production of IgG2a antibody isotype.

4.2.2 Effects of priming with rABA-1 upon responses to a subsequent infection with *A. suum*

It is known that in some systems exposure to parasite products prior to a homologous infection can modify the immune response observed (Else *et al.* 1992a). Therefore, mice were primed with rABA-1 to investigate if a similar effect could be observed in mice infected with *A. suum*. Briefly, groups of B10.S mice were immunised with 100μg of rABA-1 intra-peritoneally either solubilised in PBS, emulsified with FCA or adsorbed to alum. Control groups were also included for each of the adjuvants. Mice were rested for one week before an infection programme with *A. suum*. Animals were immunised on a weekly basis with 3000 *A. suum* eggs three times. One week after the final infection the animals were sacrificed to assess the cytokine production and proliferation of the MLN and SPL populations which had been pooled within groups and to collect sera for measurement of immunoglobulins.

Cytokine production following *in vitro* restimulation of the MLN and SPL populations is illustrated in Figure 4.4. IL-4 was not detectable in any of the cultures but differences in IL-5 were found. In both the MLN and SPL populations IL-5 was only detected when populations were stimulated with Con A. IL-5 production was greatest in the MLN when mice were primed with soluble rABA-1 (group 2), while all the other groups produced 2 – 3 fold less. In the SPL, however the differences in production were not as marked between the groups. When IFN-γ production was measured a dampening in the levels detected generally was found when mice were primed with the antigen as opposed to challenged. As described in section 4.2.1, in the MLN the highest levels of Con A-induced IFN-γ was observed in the mice immunised with rABA-1 adsorbed to alum. In the SPL, non-specific production of IFN-γ was maximal not only in the rABA-1 and alum primed mice but also in the alum, PBS and FCA-rABA-1 immunised groups. Antigen-specific IFN-γ was detected following restimulation with rABA-1. This was at low levels in the MLN, while

in the SPL the highest production was found in FCA controls, reflecting the Th1-nature of the adjuvant (Grun and Maurer 1989), along with the alum and rABA-1 primed.

Examination of both the MLN (Fig. 4.5) and SPL (data not shown), proliferative responses indicated that all primed groups responded well to culture with the Con A mitogen with the exceptions of those primed with FCA-rABA-1 or alum-rABA-1. The levels of antigen-specific proliferation were considerably lower in comparison to the Con A cultures but differences were found. Although the alum and rABA-1-primed group's cultures responded poorly following mitogenic stimulation, these were the highest responders following stimulation with rABA-1 and ABF.

Priming mice with rABA-1 prior to an *Ascaris* infection affected the production of antigen-specific immunoglobulin as shown in Figure 4.6. Exposure to the allergen generally resulted in an overall increase in both rABA-1 and ABF-specific IgG1 apart from the FCA and rABA-1 immunised group's ABF-specific IgG1 which was lower. Levels of rABA-1-specific IgG2a were low with little difference between the groups. Although a high response was noted in the FCA controls, reflecting the Th1-promoting nature of the adjuvant, there was a high degree of variability within the group.

4.2.3 Effect of immunisation with rABA-1 in adjuvants

While the priming and challenge of *A. suum* infected mice with rABA-1, had been shown to influence the immune response associated with infection, the effects of the allergen in a naive, uninfected host were not fully established. This was resolved by immunising groups of B10.S mice intra-peritoneally with either 100μg of rABA-1 solubilised in PBS, emulsified with FCA or adsorbed to alum. The animals were rested for 21 days before intra-peritoneal challenge with 20μg of rABA-1, in soluble form, emulsified in Freund's Incomplete Adjuvant (FIA), or adsorbed to alum. Control groups were immunised with adjuvant or PBS alone. One week post-challenge the mice were sacrificed to investigate

cytokine and proliferative responses in the MLN and SPL populations which were pooled within groups and antibody production in the sera.

Figure 4.7 illustrates the cytokine production in the 72 hour culture supernatants of the MLN and SPL populations. Neither IL-4 nor IL-5 could be detected in any of the cultures and while IFN-γ production was detectable this was at very low levels. In the spleen, Con A-induced IFN-γ was detectable only in the cultures of mice immunised with alum-rABA-1 and following culture with rABA-1. Culturing the SPL cells with rABA-1 revealed that IFN-γ production was greatest in the soluble rABA-1 immunised. Both groups exposed to FCA (3 and 4), and the alum controls secreted low levels of the cytokine while alum and rABA-1 resulted in no IFN-γ at all. Non-specific IFN-γ was produced in all the MLN cultures apart from the mice receiving rABA-1 in PBS. Addition of rABA-1 to FCA resulted in an increase in Con A-stimulated production but this was still not as great as the levels observed with the alum controls. Antigen-specific IFN-γ was detectable in only a few groups following culture with rABA-1, the strongest response being noted in the soluble rABA-1 immunised animals.

Analysis of the proliferative activity by the MLN population *in vitro*, (Fig. 4.8), showed that all groups proliferated strongly following Con A stimulation. Immunisation with the FCA or alum adjuvants alone or with rABA-1 suppressed the ability of the MLN population to proliferate when compared to those receiving PBS alone or mixed with rABA-1 following restimulation with the allergen. In the SPL, however, exposure to rABA-1 with adjuvant boosted this response (data not shown). ABF-specific proliferation yielded similar results for both the MLN and SPL cells. Immunisation with FCA with or without rABA-1 promoted the strongest responses. Although these differences in the antigen-specific response were found, overall the ability of the populations to respond was considerably lower than the non-specific responses found with Con A.

The antigen-specific and total immunoglobulin responses generated by immunisation with rABA-1 are shown in Figures 4.9 and 4.10 respectively. By exposing mice to rABA-1 either in a soluble form or mixed with an adjuvant resulted in a significant increase (p-value ≤ 0.005), in the production of rABA-1-specific IgG1, IgG2a and ABF-specific IgG1 in comparison to the PBS immunised controls. The groups that had been immunised with either adjuvant and rABA-1 also showed a significant increase when compared to the appropriate controls. Levels of ABF-specific IgG2a, although increased by the presence of rABA-1, were not significantly different from the PBS or adjuvant controls apart from the FCA and rABA-1 group, which was probably due to adjuvant influence. When the total levels of IgG1, IgG2a and IgE were assessed it was found that immunisation with alum and rABA-1 promoted the highest production of IgG1 and IgE, while the production of IgG2a was equivalent in all the animals. The effects of alum and rABA-1 together on IgG1 and IgE production was found to be significantly greater (p-value ≤ 0.005) than when the animals were immunised with either component separately.

4.3 Discussion

The results of the experiments presented in this chapter show that immunisation with the recombinant ABA-1 allergen of *A. suum* does influence the immune response associated with infection, albeit weakly. By challenging mice with rABA-1 post-infection with *Ascaris*, it was possible to test whether the allergen had similar effects as had been described for ABF in the previous chapter (Fig. 3.1). This was assessed through antibody production and cytokine and proliferative responsiveness *in vitro*. Infection with helminth parasites is associated with increased production of the Th2-associated cytokine IL-4 (Urban Jr. *et al.* 1992), but no IL-4 protein was measurable in the cell cultures of any of the experiments performed. The precise reason for this was unknown although possibilities include that it was rapidly bound upon secretion by cells expressing its receptor on their surface or by secreted soluble receptors. Despite this inability to detect IL-4 differences were found in the secretion of IL-5, another cytokine associated with these infective agents (Coffman *et al.* 1989).

The highest levels of IL-5 production in the draining mesenteric lymph node populations were found in the Con A-stimulated cultures of infected animals which had been challenged with alum adjuvant alone (Fig. 4.1). Interestingly, challenge of mice with rABA-1 adsorbed to the adjuvant decreased the production of the cytokine by approximately half. Systemic production of IL-5 was found only when cultures were stimulated with Con A. There were no major differences between the groups' splenocyte cultures with respect to IL-5 secretion although the levels detected were three-fold less than those found in the MLN. However, by priming mice with rABA-1 prior to infection this decrease in splenic IL-5 was abolished (Fig. 4.4). Production of the Th1 cytokine IFN-γ was also found and differences noted. High levels of IFN-γ were found in the Con A and rABA-1-stimulated spleen cultures of the mice challenged with allergen post-infection (Fig. 4.1). In the rABA-1 stimulated cultures, exposure of mice to the allergen resulted in a huge increase in IFN-γ when compared to those infected and challenged with

PBS. In the MLN, this was true only of those challenged with alum and rABA-1. In contrast, priming with the allergen before infection (Fig. 4.4) resulted in substantial decreases in IFN-γ detected. This suggested that ABA-1 may be similar to the house dust mite allergen, Der p 1, which can reduce IFN-γ mRNA expression and the frequency of IFN-γ secreting cells modulating the balance between IL-4 and IFN-γ in favour of a Th2 response profile (Comoy *et al*. 1998).

Alterations in the proliferative responses were also noted following immunisation with the recombinant allergen. *Ascaris* infected mice challenged with FCA or PBS alone or mixed with allergen exhibited reduced levels of non-specific activity following culture with the Con A mitogen (Fig. 4.2). A similar effect was noted for both the rABA-1 and ABF-specific responses. In contrast, allergen priming yielded strong non-specific proliferative responses (Fig. 4.5), although mice exposed to rABA-1 emulsified in FCA were still low responders. Interestingly, the other group showing a drop in the Con A stimulated response was the rABA-1 and alum primed mice although analysis of the antigen-specific responses showed that these animals were the highest responders. This demonstrates that while priming with the allergen depresses non-specific proliferation it does not diminish the enhanced antigen-specific response.

The production of antigen-specific immunoglobulin was reduced slightly when mice were primed as opposed to challenged with rABA-1 in the context of *Ascaris* infection (Fig. 4.6). As expected in both experiments, inclusion of rABA-1 in the immunisation mix increased the levels of all rABA-1 and ABF-specific antibody isotypes measured. While no significant differences in production were noted for the mice primed with allergen, statistical significance was found in the ABA-1 challenged animals as shown in Figure 4.3. Challenging infected animals with rABA-1 emulsified in FCA resulted in a significance increase in both ABF-specific IgG1 and IgG2a and rABA-1-specific IgG2a. While this reflected the immune response biasing properties of FCA regarding levels of the Th1-

associated IgG2a isotype (Grun and Maurer 1989), it was interesting that this group developed a significant increase in ABF-specific IgG1.

Taken together these data reveal that exposure to the ABA-1 allergen of *Ascaris* can influence the immune response generated by infection although the effects that were observed were not as marked as those seen with ABF. Furthermore, comparing the effects of immunisation before and after infection demonstrated that both approaches could influence the immune response, albeit different aspects.

The immunisation with rABA-1 with or without adjuvant was used to investigate whether the parasite product could deviate the immune response in naive, uninfected mice in favour of a Th2-type response as had been described in other systems (Steel and Nutman 1998). Figure 4.7 illustrates the cytokine production that was found in the resulting spleen and mesenteric lymph node cultures. Not only did immunisation with rABA-1 alone not induce IL-4, it was also impossible to detect IL-5, but differences did occur in the production of IFN-γ. In both MLN and SPL cultures it was found that immunisation with PBS soluble rABA-1 produced the highest levels of rABA-1-specific IFN-γ. Examination of proliferation following re-stimulation with Con A or ABA-1 in these cultures (Fig. 4.8), revealed that immunisation with either FCA or alum mixed with rABA-1 decreased responsiveness in comparison to the PBS controls and soluble rABA-1 immunised groups. These data suggested that while adjuvant-assisted immunisation with rABA-1 had no major influence upon the proliferation and cytokine production there was a possible influence of contaminating endotoxin in the soluble rABA-1 immunised and PBS control groups.

While analysis of proliferation and cytokine production in the mice showed only slight differences, a different picture was seen with antibody production. Significant increases in antigen-specific immunoglobulin production were found irrespective of whether

immunisation was assisted by adjuvant (Fig. 4.9). Furthermore, total levels of IgG1 and IgE were increased significantly by immunisation with alum and rABA-1. This illustrates that while rABA-1 is not a significant Th2-promoting parasite product with respect to cytokine production it has a significant effect upon immunoglobulin production.

Figure 4.1

Cytokine production by mesenteric lymphocytes and splenocytes of *Ascaris suum*-infected mice challenged with rABA-1. B10.S mice were infected three times at weekly intervals with 3000 infective eggs of *A. suum*. The animals were challenged 7 days after the last infection, as detailed below. All groups were sacrificed one week later. The mesenteric lymphocytes and splenocytes were pooled within groups and cultured *in vitro* with various stimuli as indicated for 72 hours. Cytokine production was measured in the culture supernatants using capture sandwich ELISAs. ◊ indicates detection limit of assay.

Group	Infections	Immunisation
1 □	3 x *Ascaris suum*	PBS control
2 ■	3 x *Ascaris suum*	PBS + 100μg rABA-1
3 ■	3 x *Ascaris suum*	FCA control
4 ■	3 x *Ascaris suum*	FCA + 100μg rABA-1
5 ■	3 x *Ascaris suum*	Alum control
6 ■	3 x *Ascaris suum*	Alum + 100μg rABA-1

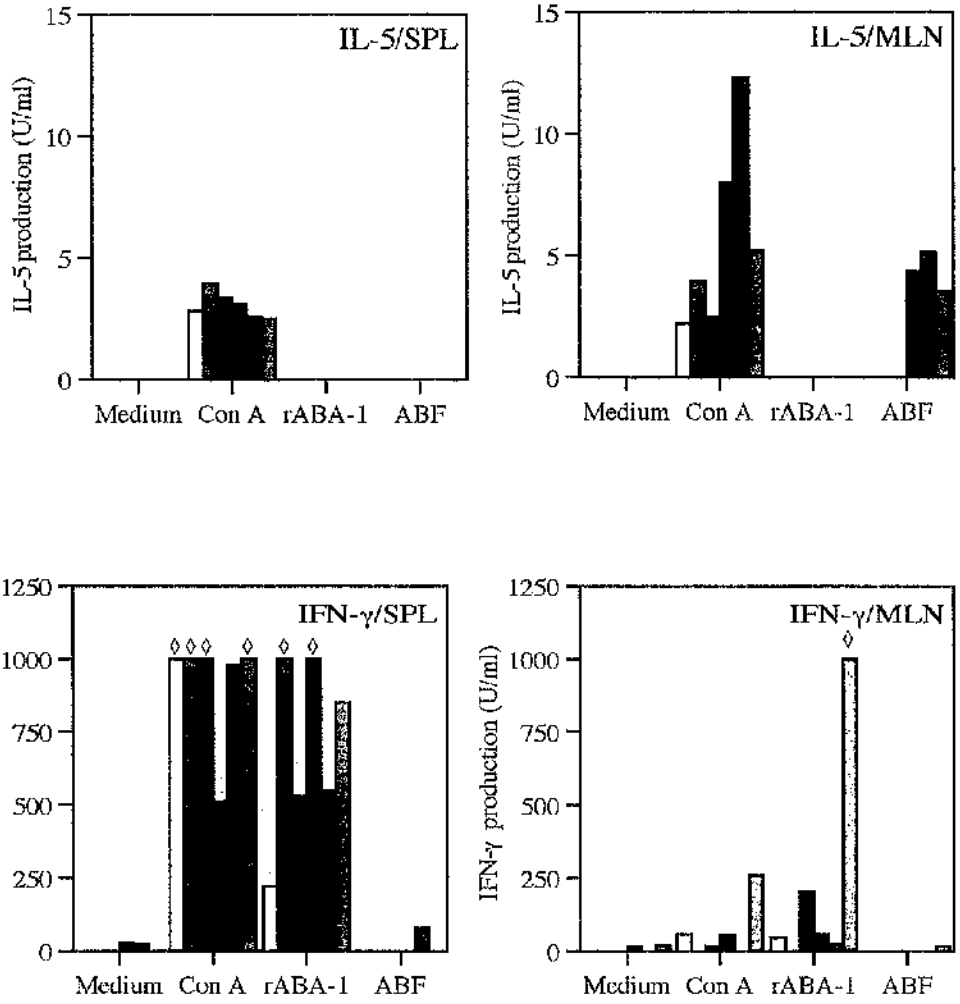

IL-5/SPL
IL-5/MLN
IL-5 production (U/ml)
IFN-γ/SPL
IFN-γ/MLN
IFN-γ production (U/ml)
0
5
10
15
250
500
750
1000
1250
Medium
Con A
rABA-1
ABF

Figure 4.2

Proliferation responses in *A. suum* infected mice following challenge with rABA-1. B10.S mice were infected three times at weekly intervals with 3000 infective eggs of *A. suum*. The animals were challenged 7 days after the last infection as detailed below. All groups were sacrificed one week later. The mesenteric lymphocytes were pooled within groups and cultured *in vitro* with various stimuli as indicated for 72 hours. Cultures were pulsed with 0.5μCi of ^{3}H thymidine and incubated for a further 24 hours before harvesting. Each data point represents the mean uptake of thymidine ± SEM for an experiment performed in triplicate.

Group	Infections	Immunisation
1	3 x *Ascaris suum*	PBS control
2	3 x *Ascaris suum*	PBS + 100μg rABA-1
3	3 x *Ascaris suum*	FCA control
4	3 x *Ascaris suum*	FCA + 100μg rABA-1
5	3 x *Ascaris suum*	Alum control
6	3 x *Ascaris suum*	Alum + 100μg rABA-1

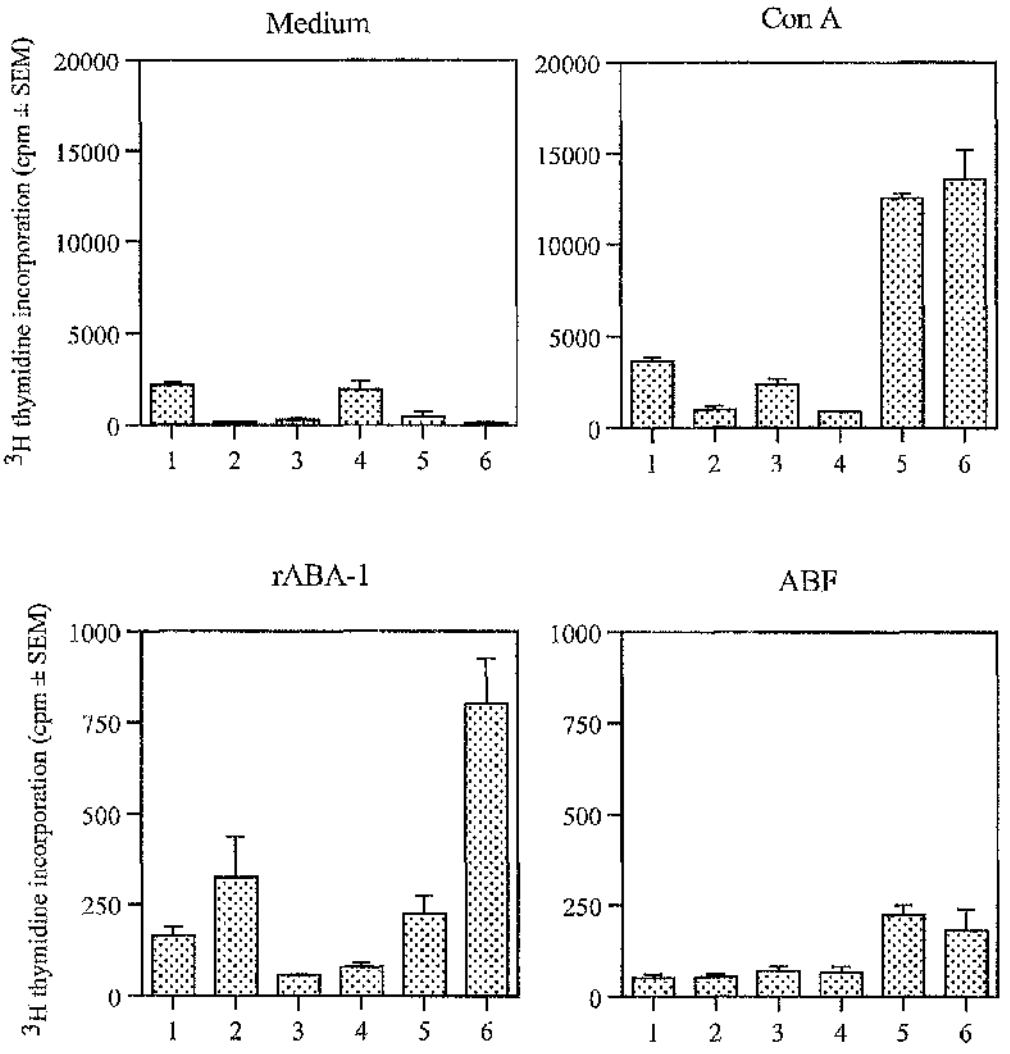

Medium
Con A
rABA-1
ABF
^{3}H thymidine incorporation (cpm ± SEM)
20000
15000
10000
5000
0
1000
750
500
250
1
2
3
4
5
6

Figure 4.3

Antigen-specific antibody production in *A. suum* infected mice following challenge with rABA-1. B10.S mice were infected three times at weekly intervals with 3000 infective eggs of *A. suum*. Animals were challenged 7 days after the last infection as detailed below. All groups were sacrificed one week later and sera collected. Antigen-specific antibodies were measured by ELISA. Data are expressed as the amount of antigen-specific antibody in a sample relative to the positive control in arbitrary units. Each data point represents the mean ± SEM.

* represents a p-value ≤ 0.005 and § approaching 0.005 when compared to group 1 using a Student's *t*-test.

Group	Infections	Immunisation
1	3 x *Ascaris suum*	PBS control
2	3 x *Ascaris suum*	PBS + 100μg rABA-1
3	3 x *Ascaris suum*	FCA control
4	3 x *Ascaris suum*	FCA + 100μg rABA-1
5	3 x *Ascaris suum*	Alum control
6	3 x *Ascaris suum*	Alum + 100μg rABA-1

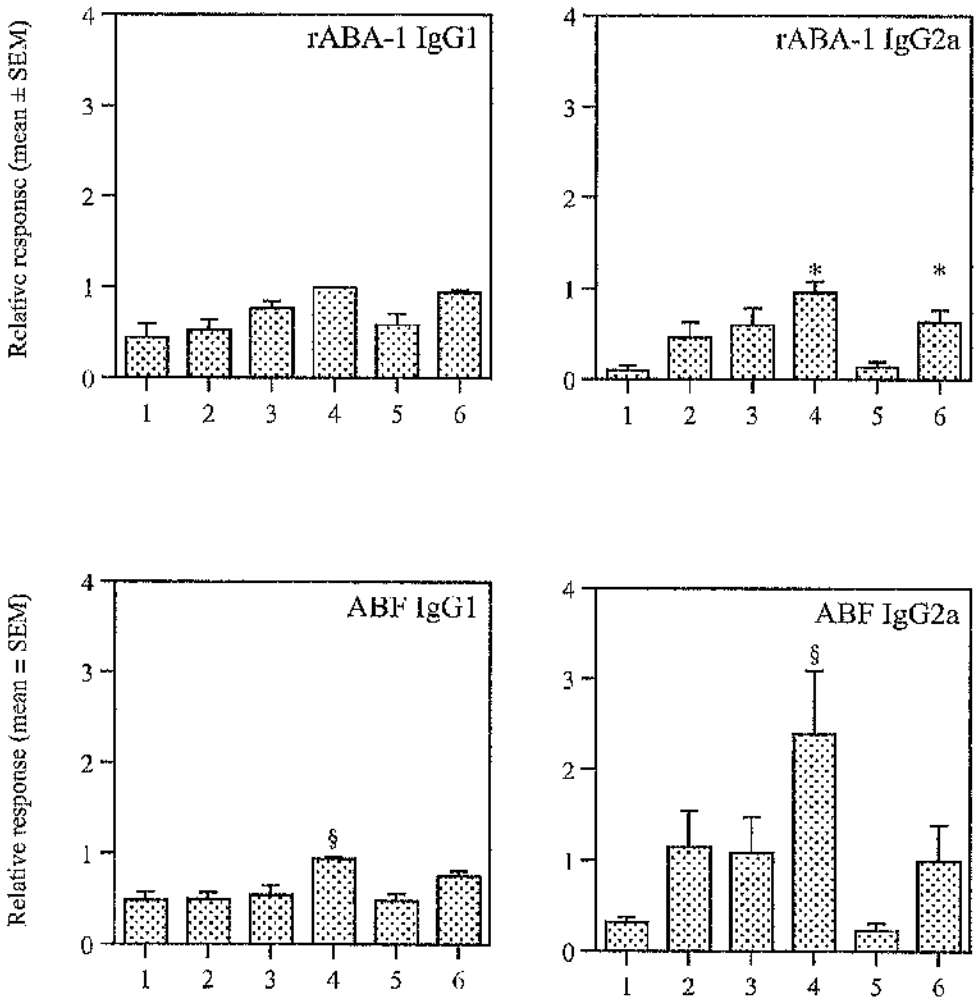

Relative response (mean ± SEM)
rABA-1 IgG1
rABA-1 IgG2a
ABF IgG1
ABF IgG2a
*
*
§
§
0
1
2
3
4
1
2
3
4
5
6

Figure 4.4

Cytokine production by mesenteric lymphocytes and splenocytes of rABA-1-primed mice infected with *A. suum*. B10.S mice were immunised with rABA-1 as detailed below . One week later the animals were infected with 3000 infective eggs of *A. suum*. The mice were infected a total of three times at weekly intervals. All groups were sacrificed one week later. The mesenteric lymphocytes and splenocytes were pooled within groups and cultured *in vitro* with various stimuli as indicated for 72 hours. Cytokine production was measured in the culture supernatants using capture sandwich ELISAs.

Group	Immunisation	Infections
1 □	PBS control	3 x *Ascaris suum*
2 ■	PBS + 100μg rABA-1	3 x *Ascaris suum*
3 ■	FCA control	3 x *Ascaris suum*
4 ■	FCA + 100μg rABA-1	3 x *Ascaris suum*
5 ■	Alum control	3 x *Ascaris suum*
6 ■	Alum + 100μg rABA-1	3 x *Ascaris suum*

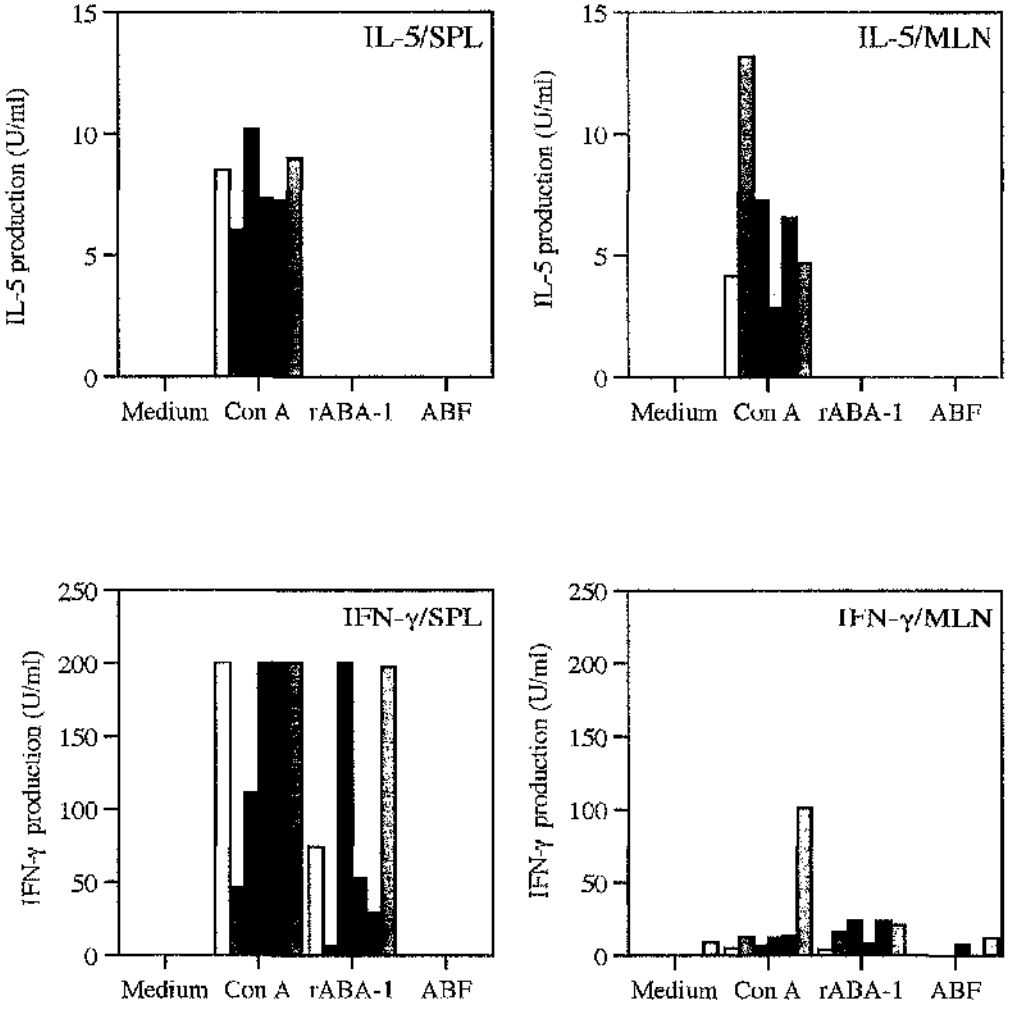

IL-5/SPL
IL-5/MLN
IFN-γ/SPL
IFN-γ/MLN
IL-5 production (U/ml)
IFN-γ production (U/ml)
15
10
5
0
250
200
150
100
50
Medium
Con A
rABA-1
ABF

Figure 4.5

Proliferative responses of the mesenteric lymphocytes of mice primed with rABA-1 prior to *A. suum* infection. B10.S mice were immunised with rABA-1 as detailed below. One week later the animals were infected with 3000 infective eggs of *A. suum*. The mice were infected a total of three times at weekly intervals. All groups were sacrificed one week later. The mesenteric lymphocytes were pooled within groups and cultured *in vitro* with various stimuli as indicated for 72 hours. Cultures were pulsed with 0.5μCi of ^{3}H thymidine and incubated for a further 24 hours before harvesting. Each data point represents the mean uptake of thymidine ± SEM for an experiment performed in triplicate.

Group	Immunisation	Infections
1	PBS control	3 x *Ascaris suum*
2	PBS + 100μg rABA-1	3 x *Ascaris suum*
3	FCA control	3 x *Ascaris suum*
4	FCA + 100μg rABA-1	3 x *Ascaris suum*
5	Alum control	3 x *Ascaris suum*
6	Alum + 100μg rABA-1	3 x *Ascaris suum*

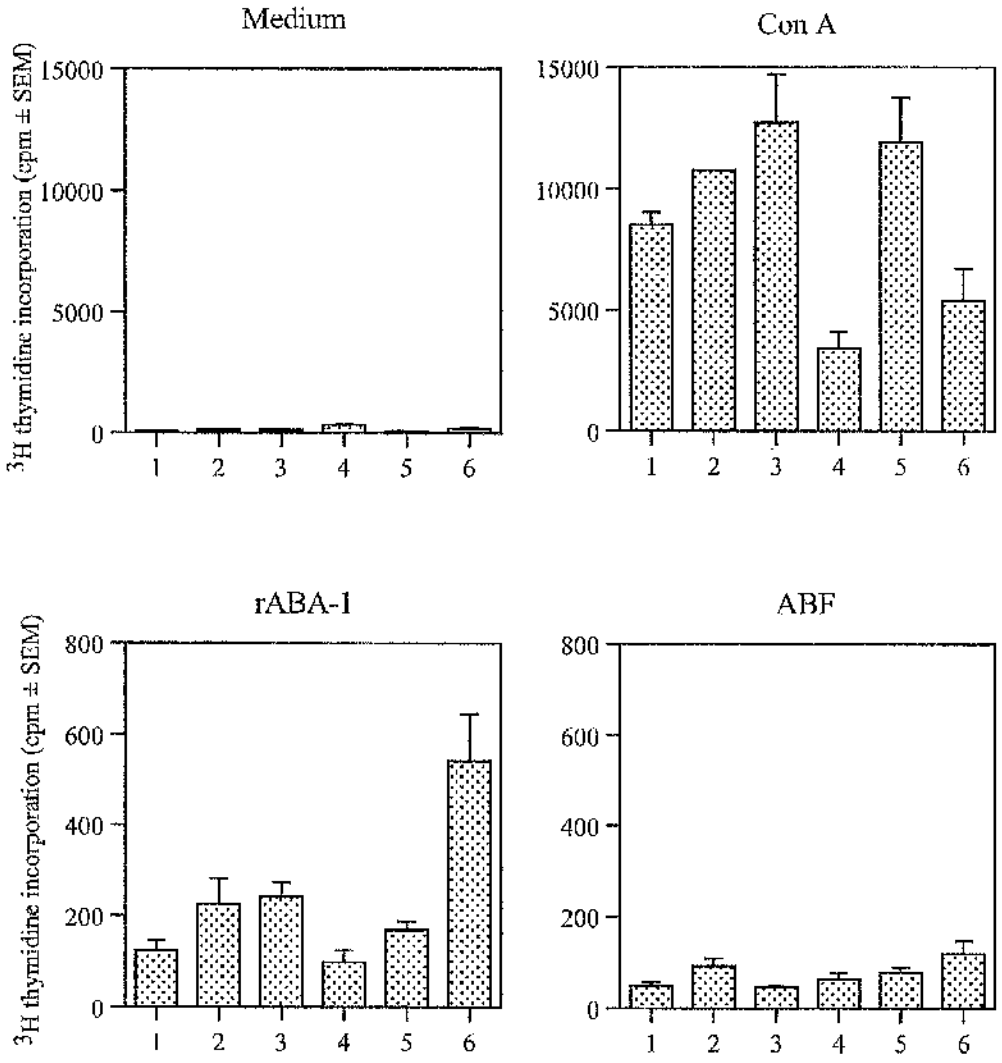

Medium
Con A
rABA-1
ABF
^{3}H thymidine incorporation (cpm ± SEM)
15000
10000
5000
0
800
600
400
200
1
2
3
4
5
6

Figure 4.6

Antigen-specific antibody production in rABA-1-primed mice infected with *A. suum*. B10.S mice were immunised with allergen as detailed below. One week later the animals were infected with 3000 infective eggs of *A. suum*. The mice were infected a total of three times at weekly intervals. All groups were sacrificed one week later and sera collected. Antigen-specific antibodies were measured by ELISA. Data are expressed as the amount of antigen-specific antibody in a sample relative to the positive control in arbitrary units. Each data point represents the mean ± SEM.

Group	Immunisation	Infections
1	PBS control	3 x *Ascaris suum*
2	PBS + 100μg rABA-1	3 x *Ascaris suum*
3	FCA control	3 x *Ascaris suum*
4	FCA + 100μg rABA-1	3 x *Ascaris suum*
5	Alum control	3 x *Ascaris suum*
6	Alum + 100μg rABA-1	3 x *Ascaris suum*

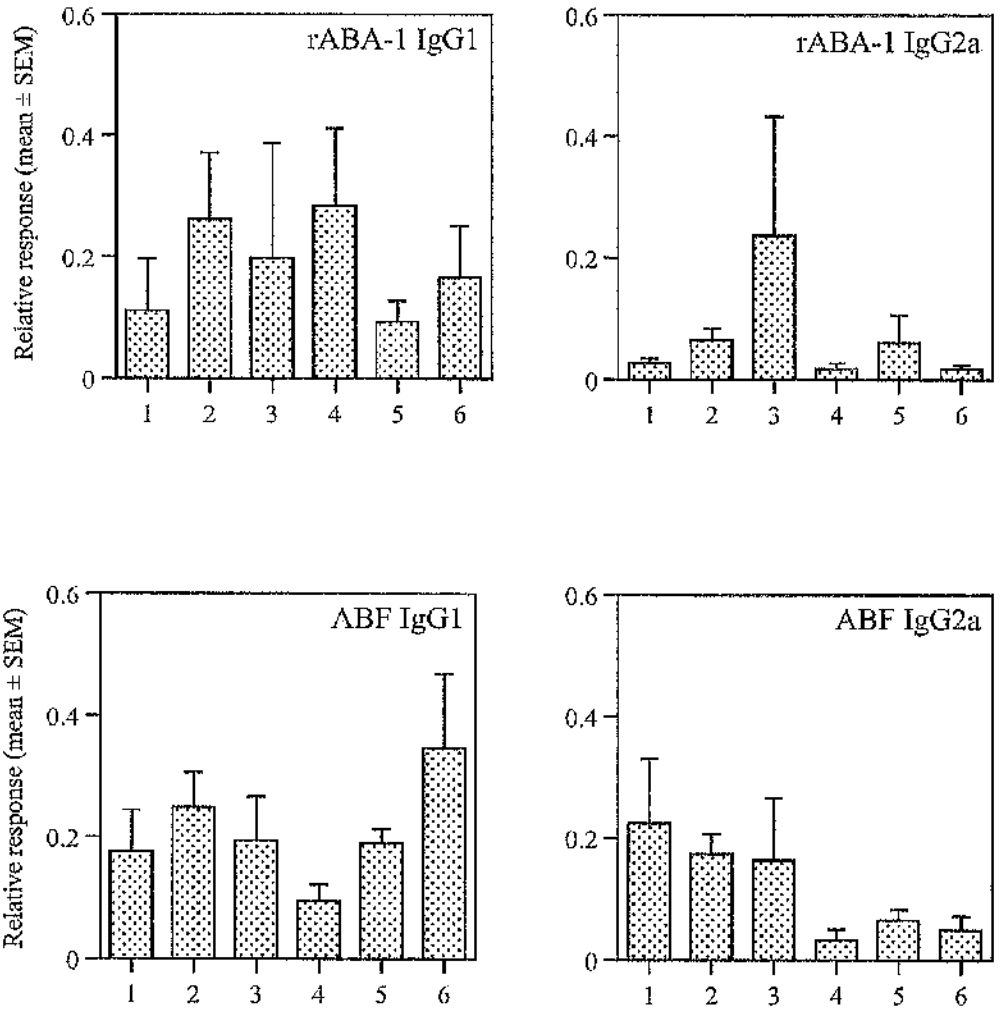
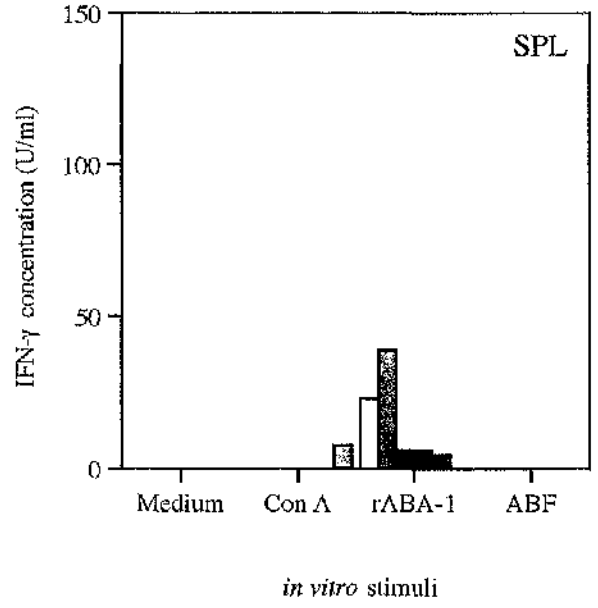

rABA-1 IgG1
rABA-1 IgG2a
ABF IgG1
ABF IgG2a
Relative response (mean ± SEM)
0.6
0.4
0.2
0
1
2
3
4
5
6

Figure 4.7

IFN-γ production by mesenteric lymphocytes and splenocytes of rABA-1-immunised mice. B10.S mice were sensitised and three weeks later challenged with rABA-1 as detailed below. All groups were sacrificed one week post-challenge. The mesenteric lymphocytes and splenocytes were pooled within groups and cultured *in vitro* with various stimuli as indicated for 72 hours. IFN-γ protein production was measured in the culture supernatants using capture sandwich ELISAs.

Group	Sensitisation	Challenge
1 □	PBS control	PBS control
2 ■	PBS + 100μg rABA-1	PBS + 20μg rABA-1
3 ■	FCA control	FIA control
4 ■	FCA + 100μg rABA-1	FIA + 20μg rABA-1
5 ■	Alum control	Alum control
6 ■	Alum + 100μg rABA-1	Alum + 20μg rABA-1


150
100
50
0
SPL
IFN-γ concentration (U/ml)
Medium
Con A
rABA-1
ABF
in vitro stimuli

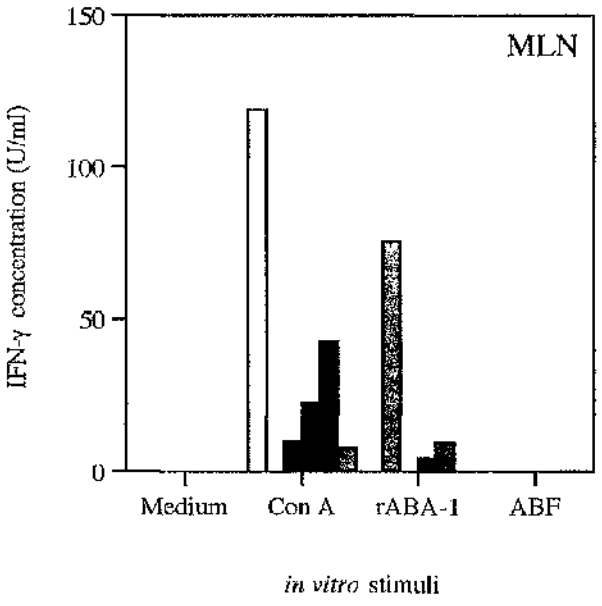

150
100
50
0
MLN
IFN-γ concentration (U/ml)
Medium
Con A
rABA-1
ABF
in vitro stimuli

Figure 4.8

The effects of immunisation with rABA-1 upon proliferation. B10.S mice were sensitised and three weeks later challenged with rABA-1 as detailed below. All groups were sacrificed one week post-challenge. The mesenteric lymphocytes were pooled within groups and cultured *in vitro* with various stimuli as indicated for 72 hours. Cultures were pulsed with 0.5μCi of ^{3}H thymidine and incubated for a further 24 hours before harvesting. Each data point represents the mean uptake of thymidine ± SEM for an experiment performed in triplicate.

Group	Sensitisation	Challenge
1	PBS control	PBS control
2	PBS + 100μg rABA-1	PBS + 20μg rABA-1
3	FCA control	FIA control
4	FCA + 100μg rABA-1	FIA + 20μg rABA-1
5	Alum control	Alum control
6	Alum + 100μg rABA-1	Alum + 20μg rABA-1

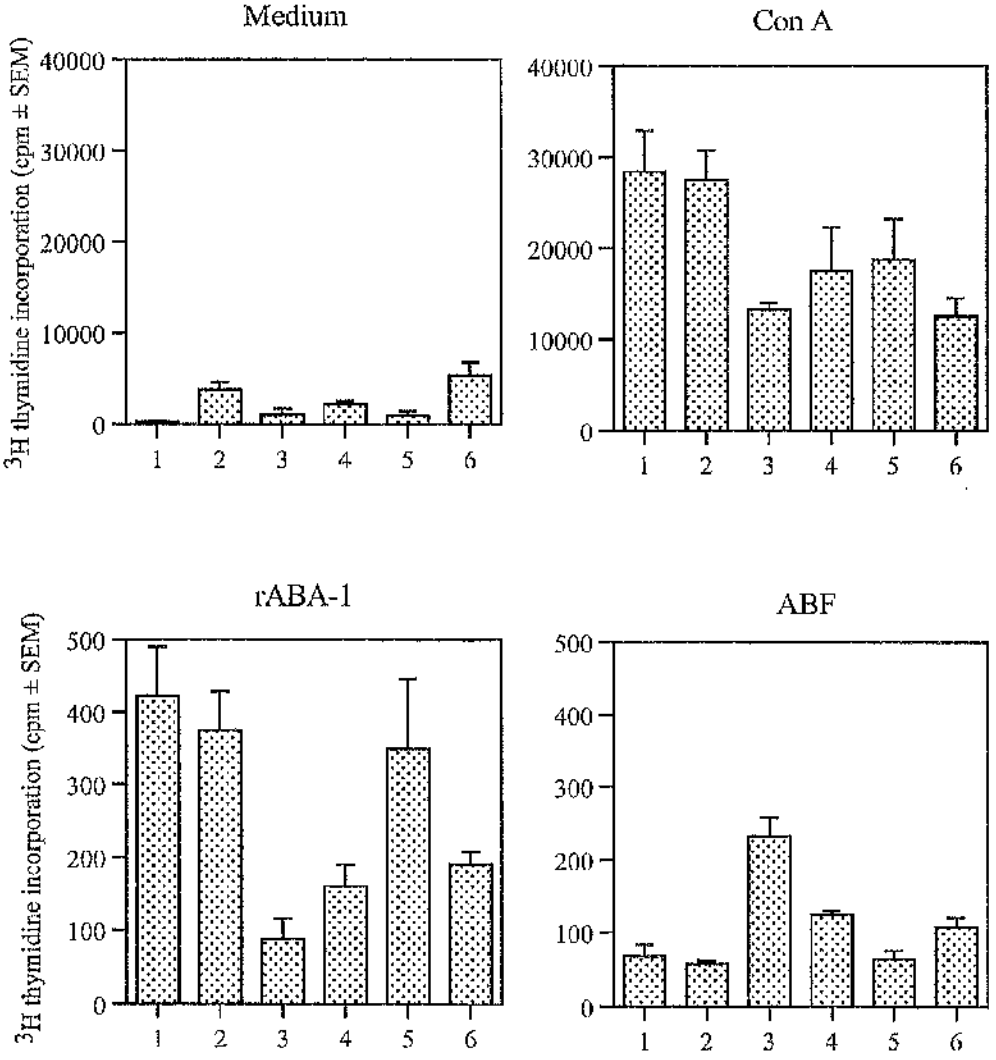

Medium
Con A
rABA-1
ABF
^{3}H thymidine incorporation (cpm ± SEM)
40000
30000
20000
10000
0
500
400
300
200
100
1
2
3
4
5
6

Figure 4.9

Antigen-specific antibody production in rABA-1-immunised mice. B10.S mice were sensitised and three weeks later challenged with rABA-1 as detailed below. All groups were sacrificed one week later and sera collected. Antigen-specific antibodies were measured by ELISA. Data are expressed as the amount of antigen-specific antibody in a sample relative to the positive control in arbitrary units. Each data point represents the mean ± SEM.

* represents a p-value ≤ 0.005 when compared to group 1 (PBS controls), § when compared to group 2 (FCA controls) and # when compared to group 5 (Alum controls) using a Student's *t*-test.

Group	Sensitisation	Challenge
1	PBS control	PBS control
2	PBS + 100μg rABA-1	PBS + 20μg rABA-1
3	FCA control	FIA control
4	FCA + 100μg rABA-1	FIA + 20μg rABA-1
5	Alum control	Alum control
6	Alum + 100μg rABA-1	Alum + 20μg rABA-1

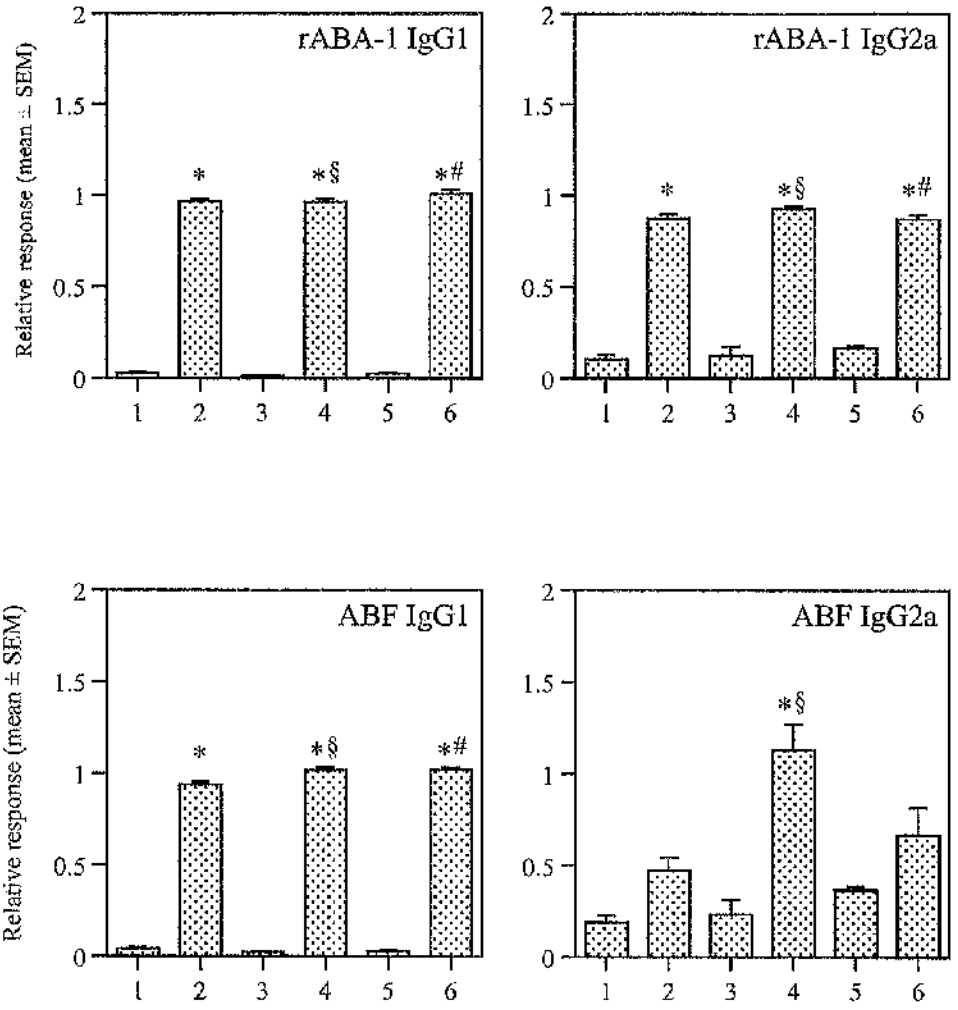
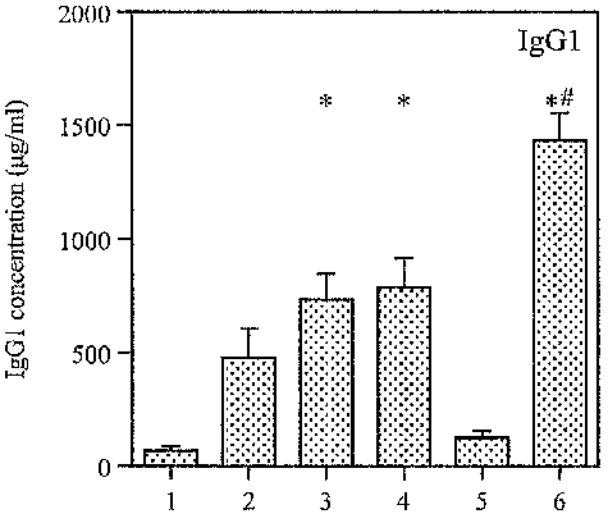

rABA-1 IgG1
rABA-1 IgG2a
ABF IgG1
ABF IgG2a
Relative response (mean ± SEM)
Relative response (mean ± SEM)
2
1.5
1
0.5
0
*
*§
*#
1
2
3
4
5
6

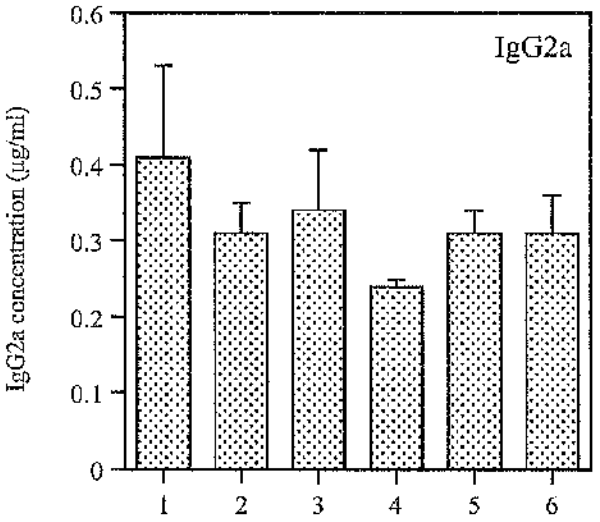
Figure 4.10

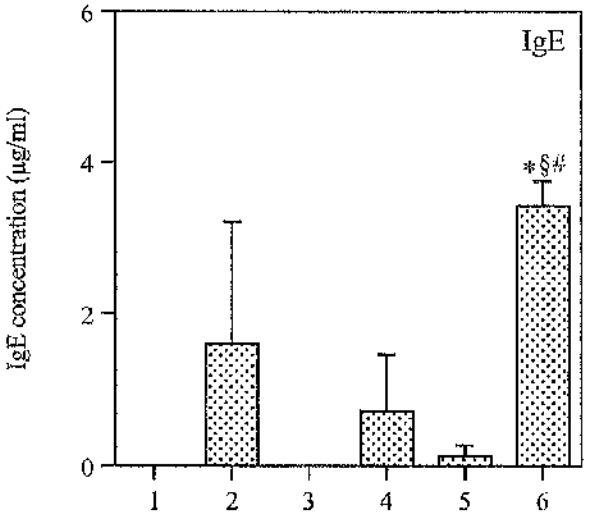
Total antibody production in rABA-1-immunised mice. B10.S mice were sensitised and challenged three weeks later with rABA-1 and adjuvants as detailed below intra-peritoneally. All groups were sacrificed one week later and sera collected. Total IgG1, IgG2a and IgE levels were measured by ELISA. Each data point represents the mean ± SEM.

* represents a p-value ≤ 0.005 when compared to group 1 (PBS controls), § when compared to group 2 (FCA controls) and # when compared to group 5 (Alum controls) using a Student's *t*-test.

Group	Sensitisation	Challenge
1	PBS control	PBS control
2	PBS + 100µg rABA-1	PBS + 20µg rABA-1
3	FCA control	FIA control
4	FCA + 100µg rABA-1	FIA + 20µg rABA-1
5	Alum control	Alum control
6	Alum + 100µg rABA-1	Alum + 20µg rABA-1


IgG1
IgG1 concentration (µg/ml)
2000
1500
1000
500
0
*
*
*#
1
2
3
4
5
6


IgG2a
IgG2a concentration (µg/ml)
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6


IgE
IgE concentration (µg/ml)
6
4
2
0
*§#
1
2
3
4
5
6

CHAPTER 5

IMMUNOMODULATION BY THE PRODUCTS OF *ASCARIS*

5.1 Introduction

5.1.1 Immunomodulation and infection

A feature that is common to many infectious diseases is modulation of the host immune response which can be either specific or non-specific. Early studies suggested that immunomodulation was mediated by a suppressor cell population, particularly macrophages or T lymphocytes. Suppressor macrophages were shown to mediate their effects non-specifically interfering with mitogen responses or primary antibody responses to both T cell dependent and independent antigens (Cunningham and Kuhn 1980). Conversely, suppressor T cells which were more specific in their activities were proposed by some to be important in determining disease outcome (Playfair 1982). In recent years, the concept of suppressor T cell populations has been replaced by one of regulatory T cells following the establishment of the T helper 1 (Th1)/Th2 model for $CD4^+$ T cells. Many studies have described such regulatory populations that are associated with, but are distinct from, Th2 cells (Bridoux *et al.* 1997; Groux *et al.* 1997; Powrie *et al.* 1996). For example, $CD4^+$ T cells, which develop in an IL-10 rich environment, give rise to a subset that secretes high levels of IL-10 and little IL-2 and IL-4 (Groux *et al.* 1997). These T regulatory 1 (Tr1) cells can inhibit the development of colitis in immunodeficient mice and the *in vitro* activation of T cells and have been described in humans and mice. Another subset of $CD4^+$ T cells expressing low levels of the transmembrane molecule CD45RB which could also inhibit colitis and T cell mediated autoimmune disease was described which secreted high levels of Transforming Growth Factor β (TGF-β) (Bridoux *et al.* 1997; Powrie *et al.* 1993a; Powrie *et al.* 1996). Further studies by other groups investigating oral tolerance have highlighted the importance of these $CD4^+$ TGF-β-secreting or T helper 3 (Th3) cells in the regulation of mucosal immune responses (Fukaura *et al.* 1996; Neurath *et al.* 1996).

The development of a Th2 response due to helminth infection has been demonstrated to modulate responsiveness to both non-parasite antigens and other pathogens in infected

animals and humans (Curry *et al.* 1995; Hermanek *et al.* 1994; Price and Turner 1986). Although this can leave the host vulnerable to secondary infections (Actor *et al.* 1993), it can also confer protection against an otherwise fatal disease (Yan *et al.* 1997). How the generation of such responses down-regulates protective immunity to other pathogens, such as protozoan parasites, is unclear but has been linked to the innate chronicity of the helminth itself.

5.1.2 Helminth product mediated influences

The ability to alter immune reactivity has also been described following exposure to parasite antigens and excretory-secretory (E/S) products. Parasite products mediate their effects through a variety of mechanisms. The suppression of B and T cell proliferation described during infection (Ottensen *et al.* 1977; Piessens *et al.* 1980), can be mediated by parasite products through altering levels of intracellular signalling molecules (Harnett and Harnett 1993), targeting signalling pathways (Deehan *et al.* 1998), or inducing suppressor populations (Allen and MacDonald 1998). Conversely, with some parasite species, the products may enhance proliferation non-specifically, which in the case of *H. polygyrus*, occurs independently of the MHC, suggestive of superantigen-like properties (Robinson *et al.* 1994). Helminth products with protease activity or which inhibit host proteases have been described in the literature; these have a diverse effect on the immune response ranging from disruption of the MHC class II antigen processing pathways (Bennett *et al.* 1992), and modulation of T cell effector functions (Hamajima *et al.* 1994), to cleavage of essential B and T cell surface molecules (Schulz *et al.* 1998). Furthermore, parasites can secrete human cytokine homologues, creating the potential to divert a developing immune response in their favour (Pastrana *et al.* 1998).

Products derived from *Ascaris suum* have also been shown to be immunomodulatory. Soluble antigen obtained from third stage *A. suum* larval culture medium and adult body fluid have been demonstrated to potentiate IgE antibody response to ovalbumin in rodents (Lee and McGibbon 1993; Stromberg 1980). However, the role of the adult worm

homogenate in IgE production is contentious with some groups claiming it boosts IgE levels whilst others claim the reverse (Lee and McGibbon 1993; Soares *et al.* 1992). T cell function impairment has also been reported in connection with *Ascaris* extracts demonstrating that the effects of this parasite are not exclusive to immunoglobulin production (Ferreira *et al.* 1995).

5.1.3 Experimental design and aims

As described, immunomodulation is a common feature of many infections and can affect a host's ability to respond to heterologous antigens and pathogens. With helminth infections the parasite products can mediate these effects, therefore, it was decided to investigate whether the products of *Ascaris*, ABF and ABA-1, behaved similarly by analysing their effects on the development of the delayed-type hypersensitivity (DTH) response against chicken egg ovalbumin (OVA) in mice. The DTH reaction was measured using the footpad swelling test and is primed for by sub-cutaneous immunisation with OVA emulsified in Freund's Complete Adjuvant (FCA) in the left hind footpad. Animals were challenged one week later with soluble heat-aggregated OVA (HAO) in the opposite rear footpad. This gives rise to an inflammatory response usually visible at 16 to 24 hours post-challenge which has been reported to be mediated by T helper 1 $CD4^+$ cells (Cher and Mosmann 1987).

Firstly, in order to establish whether the *Ascaris* antigens, ABA-1 and ABF, could modulate the immune response normally observed in the OVA-DTH model in the mouse, animals were sensitised with OVA either alone or mixed with ABF or rABA-1 and challenged with HAO. Further groups were primed and challenged with *Ascaris* products alone to control for the ability of the parasite antigens to induce DTH responses themselves. A control group consisting of 3 animals was immunised in both feet with HAO at the time of test challenge to compensate for non-specific swelling resulting from the procedure.

Subsequently, the aims of the remaining experiments presented in this chapter were to elucidate the mechanisms involved in the any immunomodulatory activities of the *Ascaris* products and identify components that were directly involved.

To investigate whether the dose of ABF used at priming was important for modulating the OVA-specific DTH response, mice were sensitised with OVA alone or mixed with varying doses of ABF. Mice were challenged with HAO and the resulting DTH response measured. One week post-challenge, mice were killed to analyse cytokine, proliferative and serum immunoglobulin responses, which was performed in all the experiments presented.

While the two previous experiments examined the effects of *Ascaris* antigens during the sensitisation phase of the DTH model it was of interest if a similar phenomenon occurred in an established immune response. Therefore, mice were primed with OVA and challenged 7 days later with HAO mixed with ABF. The resulting DTH and immune responses were compared to animals primed with OVA alone or mixed with ABF and challenged with HAO only, thus indicating the stage in an immune response where the influence of ABF was maximal.

In order to identify the components of ABF with immunomodulatory activities, ABF was applied to a gel filtration column which separated the body fluid into parasite derived ABA-1 (pABA-1) and two ABF protein peaks, named peak 1 and peak 2 respectively. Mice were sensitised with OVA alone or in addition to the unfractionated ABF, pABA-1, fractionated ABF with the pABA-1 removed ABF peak 1 or ABF peak 2. Challenge occurred seven days later with HAO. This experiment addressed firstly whether pABA-1 was similar to rABA-1 in activity, secondly, did the effects of ABF require the presence of pABA-1 and thirdly, could the ability to deviate the immune response be assigned to a particular subset of ABF's proteins.

Previous studies on immunosuppressive proteins produced by another gastrointestinal nematode described the effects of extreme heat and pH on the stability of the immunomodulatory properties of the proteins (Pritchard *et al.* 1994). A similar approach was employed to investigate the stability of ABF's proteins that had displayed these characteristics. ABF that had received heat or acid treatments prior to use were mixed with OVA and used to immunise mice. Seven days later the mice were challenged with HAO to investigate if the proteins in ABF were susceptible to such treatments. While this set of experiments was designed to investigate the role of the protein molecules of ABF in immunomodulation, they did not rule out the influence that other groups of molecules e.g., glycolipids or carbohydrates, may have in this model.

The final set of experiments addressed the issue that ABF may mediate its effects by promoting the production of Th2 cytokines in the naive, uninfected host. Mice deficient for the Th2 associated cytokine IL-4 and control animals were treated systemically with a neutralising monoclonal antibody against IL-10 or an isotype control prior to immunisation with OVA alone or mixed with ABF. Challenge occurred 7 days later with HAO. These experiments investigated the influence of both IL-4 and IL-10 in the modulation of DTH responsiveness and whether they mediated their effects alone or in synergy, which had been described in another model system (Powrie *et al.* 1993b).

5.2 Results

5.2.1 *Ascaris* products modulate the immune response against a heterologous antigen

In order to investigate whether *Ascaris* products could modulate the footpad DTH response against OVA, experiments were carried out in BALB/c mice. Groups of 5 animals were sensitised in the rear footpad with 100μg OVA, either alone or mixed with 500μg ABF protein or 100μg rABA-1. To investigate whether the *Ascaris* products induced a DTH response themselves, further groups were immunised with 500μg ABF or 100μg rABA-1 alone. All antigens were emulsified in Freund's Complete Adjuvant (FCA) and administered in a 50μl volume. Mice were challenged 7 days later in the opposite rear footpad with either 100μg heat-aggregated OVA (HAO), 250μg soluble ABF or 100μg soluble rABA-1. A control group consisting of 3 animals was immunised in both feet with HAO at the time of test challenge to compensate for non-specific swelling resulting from the procedure. Footpad thickness was measured before and 24 hours after antigen challenge. The difference between the means of the two measurements gave an index of footpad swelling in millimetres, which was used for group comparison. The results of this experiment are illustrated in Figure 5.1a.

Sensitisation and challenge with OVA alone resulted in a pronounced thickening of the challenge footpad (group 1) indicative of DTH generation. Mixing of OVA with the recombinant form of the *Ascaris* allergen, ABA-1, at priming (group 3) had no significant effect on the development of the OVA-specific DTH response. In contrast, when animals were sensitised with OVA mixed with ABF (group 2), a complete ablation of the OVA-specific response occurred (p-value ≤ 0.001). Identical effects following sensitisation with the *Ascaris* antigens were also observed when this experiment was performed in B10.S mice (Figure 5.1b), suggesting that the immunomodulatory properties of ABF are not MHC restricted as the strains used had disparate haplotypes (BALB/c is H-2^d while B10.S is H-2^s). To confirm this finding, further experiments encompassing a wider range of host haplotype would be required. Marked differences in responsiveness between the mouse

strains were found following sensitisation and challenge with ABF or rABA-1 alone. While B10.S mice did not generate DTH responses against ABF and rABA-1, BALB/c mice developed strong responses, which although not as great as the response to OVA alone were not significantly different. The reasons for the development of such a response in BALB/c and not B10.S mice are unknown and would require further investigation.

Cytokine production by the draining popliteal lymph node (PLN) cells were analysed by capture sandwich ELISA, the results of which are shown in Figure 5.2. DTH is an inflammatory response, associated with the production of cytokines such as IFN-γ (Cher and Mosmann 1987). All groups produced IFN-γ following restimulation with the T cell mitogen Concanavalin A (Con A) and this was greatest in those mice sensitised with OVA alone or mixed with ABF (Figure 5.2a). Of particular interest were the levels of OVA-specific IFN-γ, which were maximal in the mice exposed to OVA alone or in addition to rABA-1 during priming. While rABA-1 had no effect on the OVA-specific IFN-γ, the presence of ABF during sensitisation resulted in a decrease in the production of the cytokine. Other helminth antigens have been reported to alter immune responsiveness by promoting the production of Th2 associated cytokines, e.g., IL-4, IL-5 (Mahanty *et al.* 1993; Steel and Nutman 1998). Therefore the decrease in OVA-specific IFN-γ observed following sensitisation with ABF could have been due to the *Ascaris* antigens promoting the production of these cytokines at the expense of IFN-γ. However, no IL-4 or IL-5 could be detected in any of the supernatants. Interestingly, differences were found in the levels of IL-10, a cytokine suggested to mediate the balance between inflammation and humoral immunity (Fiorentino *et al.* 1991; Mosmann and Moore 1991; Rousset *et al.* 1992). Figure 5.2b illustrates that IL-10 production was comparable in the mitogen restimulated cultures from animals sensitised with OVA alone or mixed with rABA-1, but a doubling occurred following exposure to ABF during priming. OVA-specific IL-10 production occurred slightly when OVA was mixed with rABA-1 but this was minimal when compared to

priming with OVA and ABF. Interestingly, no OVA-specific IL-10 was produced as a result of exposure to OVA alone.

The proliferative responses of the PLN cell cultures following antigen restimulation *in vitro*, are shown in Figure 5.3. All groups responded well following stimulation with Con A. Stimulation with either ABF or rABA-1 promoted proliferation in those groups sensitised with the *Ascaris* antigens. The OVA-specific proliferative response was identical in the groups that had been primed with OVA alone or mixed with rABA-1. However, in contrast to that which had been observed with DTH responsiveness and cytokine production, exposure to ABF during sensitisation boosted the OVA-specific proliferative response.

A final aspect of the immune response that was investigated was whether ABF modulated the associated humoral response. Antigen-specific IgG1 and IgG2a and total IgE titres were measured in the sera samples collected 7 days post-challenge, the results of which are shown in Figures 5.4 and 5.5. The pattern of production of OVA-specific antibodies was similar for both the IgG sub-classes. As seen previously, exposure to rABA-1 during priming had no effect on the course of OVA-specific immunity, while ABF significantly reduced the levels of IgG2a (p-value ≤ 0.007) and to a level approaching significance for IgG1. An unexpected finding was that mice which had been immunised and challenged with rABA-1 alone, produced large amounts of OVA-specific IgGs. While ABF-specific antibodies were detected in all the groups immunised with either of the *Ascaris* antigens, rABA-1-specific production occurred only in the groups exposed to the allergen. Measurement of total IgE production revealed that the highest titres were seen in those groups 2 and 3 that were immunised with ABF or rABA-1 in conjunction with OVA and HAO challenged. Surprisingly, IgE levels in groups which had been immunised with the *Ascaris* antigens were equal to or less than the titres in animals immunised and challenged with OVA alone.

5.2.2 Immunomodulation by ABF is dose-dependent

In order to investigate whether the ABF-mediated modulation of the OVA-specific DTH response was affected by the amount of ABF, BALB/c mice were immunised as in section 5.2.1 with 100μg OVA, alone or mixed with varying doses of ABF. Seven days after immunisation, mice were challenged with 100μg HAO and DTH responsiveness was measured. One week post-challenge, mice were killed in order to analyse cytokine and proliferative responses in the PLN population along with immunoglobulin production in the serum. The results of this experiment are shown in Figures 5.6 – 5.9.

The development of the OVA-specific DTH response was suppressed significantly (p-value $\leq$ 0.001), when mice were exposed to 500 or 250μg ABF during priming. Administration of 50μg ABF did not alter response significantly (Figure 5.6). The priming dose of ABF influenced the proliferative responses of the PLN populations also, as shown in Figure 5.7. Increasing the amount of ABF at priming resulted in an increased depression of the non-specific proliferative response following Con A restimulation. This was in contrast to culturing with OVA where decreasing the dose of ABF increased the ability to depress the response. In another model of helminth product mediated immunosuppression (Hartmann *et al.* 1997), the ability to suppress proliferative responses has been attributed to a general dampening of antigen- or mitogen-specific responses. To investigate if this was the case with ABF, the PLN cells were cultured with both Con A and ABF simultaneously. In doing so the ability of the cells to proliferate actually increased, suggestive that the mechanism through which ABF suppresses proliferation, albeit unknown, is specific.

As illustrated in Figure 5.8, ABF dose also affected the production of cytokines. Although no IL-4 or IL-5 could be detected in the culture supernatants, there were differences in IFN-γ levels. Non-specific IFN-γ production was greatly enhanced in mice which were immunised with 250μg ABF (group 3; 1000U/ml) in comparison to the groups exposed to 500μg (group 2; 356U/ml) or 50μg (group 4; 132U/ml). As observed with the OVA-

specific proliferative responses, when the dose of ABF used to immunise decreased so the suppression of OVA-specific IFN-γ production increased. The effect of ABF dosage on the production of antigen-specific antibody production is shown in Figure 5.9. Both OVA-specific IgG1 and IgG2a levels are decreased if mice are exposed to ABF during OVA priming and the degree of suppression is directly proportional to the dose of ABF used.

5.2.3 Parasite-derived ABA-1 cannot influence the immune response against OVA and does not contribute towards the effects of ABF

While the data in section 5.2.1 indicated that the recombinant form of the *Ascaris* allergen, ABA-1, was unable to modulate the OVA-specific DTH response, it was unknown if this were also true of parasite-derived ABA-1 (pABA-1). This was an important issue as pABA-1 constitutes approximately half of the total protein in ABF and it was possible that pABA-1 may be post-transcriptionally modified or have a different tertiary structure from the recombinant protein which conferred immunomodulatory properties. To address this, ABF was applied to a gel filtration column that separated and allowed the collection of proteins according to molecular weight. This enabled the isolation of pABA-1 from the other protein components of ABF. BALB/c mice were immunised as before with 100μg OVA alone or in addition to 250μg of the unfractionated, complete ABF, 100μg pABA-1 or 250μg fractionated ABF with the pABA-1 removed. All groups were challenged seven days later with 100μg HAO. The resulting DTH responses were assessed and seven days after challenge the mice were killed to analyse the cytokine, proliferative and antibody responses. The results, shown in Figure 5.10, indicate that exposure to pABA-1 (group 3), like rABA-1, during OVA sensitisation does little to interfere with the development of the DTH response. Furthermore, the ability of ABF to modulate the response was not dependent upon the presence of the allergen (group 4). The suppression seen with both fractionated ABF and complete ABF was statistically significant (p-value = 0.001).

Examination of the PLN proliferative responses, as depicted by Figure 5.7, revealed that exposure to pABA-1 during OVA priming did not alter the ability of the population to

respond to culture with OVA *in vitro* (group 5). Interestingly, the mice primed with fractionated ABF from which the pABA-1 had been removed (group 6), showed depression of the OVA-specific response greater than that observed for the equivalent dosage of the unfiltered ABF (group 3). A further effect of immunisation with fABF was an ablation of the ABF-specific proliferative response, revealing that while pABA-1 did little in modulating the immune response, it contributed to ABF's mitogenicity. Removal of pABA-1 did not impede fABF (group 6), in suppressing the production of OVA-specific IFN-γ or IgG1 and IgG2a, Figures 5.8 and 5.9 respectively. However, decreases in ABF-specific IFN-γ were noted, along with a drop in ABF- and rABA-1-specific IgG1 titres.

5.2.4 The ability of ABF to suppress the DTH response cannot be assigned to a particular fraction of the *Ascaris* antigens

By gel fractionating ABF, as discussed previously, its component molecules can be separated. The proteins segregate into three main groups as illustrated in the FPLC trace shown in Figure 5.11. The two peaks not containing pABA-1 were collected separately and were used to immunise BALB/c mice, in an attempt to identify the immunomodulatory components. Mice were immunised with OVA alone or in addition to 250μg unfractionated ABF (nABF), 100μg pABA-1, 250μg FPLC-purified ABF peak 1 (peak 1) or 250μg FPLC-purified ABF peak 2 (peak 2) as before. Mice were challenged seven days later with 100μg HAO. It was found that by sensitising mice to OVA mixed with peak 1 or peak 2 the development of the OVA-specific DTH response was significantly depressed (Figure 5.12, p-value $\leq$ 0.005). Therefore, the immunomodulatory activity is present in both of the isolated ABF peaks.

Measurement of cytokine production by the PLN cell populations (Figure 5.13), revealed that non-specific production of IFN-γ was greatly enhanced when mice were sensitised with OVA mixed with either nABF, pABA-1 or ABF peak 2. Immunisation with OVA and ABF peak 1 did not augment the production of non-specific IFN-γ as well as the other

Ascaris antigens, although the ABF-mediated suppression of OVA-specific IFN-γ was most pronounced with this fraction. No IL-4, IL-5 or IL-10 was detectable in any of the culture supernatants. These data are presented in Figure 5.13. The proliferative responses, shown in Figure 5.14, indicated that the presence of nABF, pABA-1 and ABF peak 2 at OVA sensitisation increased OVA-specific reactions above the levels observed in mice immunised with the antigen alone. Administration of ABF peak 1 had no effect upon the OVA-specific response. Identical effects were also seen with respect to rABA-1- and ABF-specific reactivity. Examination of antigen-specific antibody production indicated that the OVA-specific IgG2a response generated in these animals was depressed only when the mice were immunised with either normal ABF or ABF peak 2, (groups 2 and 5, Figure 5.15). Furthermore, levels of both ABF- and rABA-1-specific immunoglobulins were considerably reduced when mice were immunised with either peak 1 or 2 in comparison to the unfiltered ABF.

5.2.5 ABF-mediated immunosuppression is dependent on the timing of ABF exposure

While the data presented in the previous sections demonstrated that exposure to ABF during priming resulted in a dampening of the Th1-associated heterologous DTH response, it was unknown if the *Ascaris* antigens had similar effects upon an established response. Therefore groups of BALB/c mice were immunised with either 100μg OVA, alone or mixed with 250μg ABF as before. Those animals immunised with OVA alone were challenged one week later with 100μg HAO mixed with 250μg ABF and the OVA and ABF primed were exposed to HAO alone. As illustrated in Figure 5.16, ABF significantly depressed (p-value ≤ 0.01) the DTH response if exposure occurred during antigen priming but it had no effects when administered at antigenic challenge. This demonstrates that there is a small window of opportunity during which ABF can mediate its effects.

The timing of exposure to ABF did not alter the ability of lymphocyte cultures to proliferate in response to Con A, (see Figure 5.17). While the OVA-specific proliferative

response was depressed by priming in the presence of ABF, this effect was greater in animals that had been exposed to the antigens during antigen challenge. The response following restimulation with ABF was greatly reduced in the animals challenged in comparison to those primed with the ABF. This however reflects the point that priming with ABF was adjuvant-assisted. Non-specific production of IFN-γ, like proliferation, was unaffected by the timing of ABF exposure; in both instances, as illustrated in Figure 5.18, levels were enhanced following exposure to ABF. However, OVA-specific IFN-γ was only suppressed by the presence of ABF at priming, while exposure during challenge enhanced production. Similar to ABF-specific proliferation, ABF-specific IFN-γ was greater in the primed animals but not in the challenged, again, this was probably due to adjuvant. IL-4 and IL-5 were undetectable in any cultures and no differences were observed regarding the production of IL-10 (data not shown). The antigen-specific antibody responses are shown in Figure 5.19. While levels of OVA-specific IgG2a decreased slightly as a result of sensitisation with ABF, they remained constant if exposure occurred at challenge only.

5.2.6 Effect of heat and chemical treatments on ABF and the implications this for immunosuppression

Previous work on the E/S products of *H. polygyrus* investigated the effects of extreme heat and pH on the stability of the immunomodulatory properties of the parasite proteins (Pritchard *et al*. 1994). It was decided to use this rationale for the basis to investigate the stability the proteins of ABF which had displayed these characteristics. Aliquots of ABF were either heated to 100°C for 30 minutes in a thermal cycler block or acid treated by lowering the pH to 2 by addition of 3.6M H_2SO_4, before neutralisation with 7M NaOH until pH7 was reached. BALB/c mice were immunised with 100μg of OVA alone or mixed with 250μg of untreated ABF, chemically treated ABF or heated ABF. Seven days later the mice were challenged with 100μg of HAO and one week post-challenge the animals were killed to analyse cytokine, proliferative and antibody responses.

The data presented in Figure 5.20 shows that treating ABF with extremes of heat or pH has no major effect on the immunosuppressive effects with respect to the DTH response. However, the ability of ABF to enhance the production of IFN-γ following Con A restimulation was maintained only in ABF that had been chemically treated. Sensitisation with the heated ABF resulted in a suppression of the cytokine's production greater than in the mice immunised with OVA alone (Figure 5.18). Levels of IFN-γ secreted due to culture with OVA were suppressed by all the forms of ABF used but was most pronounced when the heated ABF was used. Both heating and acid treatment of ABF lead to a reduction in levels of ABF-specific IFN-γ. Interestingly, this was not the case when ABA-1-specific production was assessed, where priming with untreated ABF resulted in the lowest levels of production.

As seen in Figure 5.17, proliferative responses following restimulation with either Con A or rABA-1 were identical in all the ABF sensitised mice as was the ability to suppress OVA-specific responsiveness. Acid treatment of ABF lead to a decrease in the ABF-specific response, but this decrease was most marked in the mice that received the heat treated ABF. Analysis of the levels of antigen-specific antibody in the sera, indicated that while the production of OVA-specific IgG2a was depressed following exposure to untreated ABF, priming with either heated ABF or acid treated ABF could reduce this response further. All groups immunised with the *Ascaris* antigens produced similar amounts of ABA-1 and ABF-specific IgG1, but ABA-1-specific IgG2a was greatest in the heated ABF immunised and ABF-specific IgG2a was enhanced in those administered untreated ABF (Figure 5.19).

5.2.7 Effect of *Ascaris* antigens on the OVA-specific response in IL-4 deficient mice

As described in the section 5.2.1, modulation of the OVA-specific DTH response by ABF was associated with alterations in cytokine production when compared to mice immunised with OVA alone. Products of other helminth species have been reported to promote the production of Th2 cytokines in naive hosts (Mahanty *et al.* 1993; Steel and Nutman 1998), to determine whether ABF mediated its effect by a similar manner mice deficient for the Th2 associated cytokine IL-4 (IL-4 KO), were immunised with OVA alone or with ABF. As a result of the increase in IL-10 production described in section 5.2.1 following exposure to ABF during OVA sensitisation, mice were treated with a neutralising monoclonal antibody against IL-10 prior to sensitisation to investigate if this affected ABF's ability to suppress the DTH response. Groups of IL-4 KO and B10.BR wild-type (WT) controls were immunised as previously with 100μg OVA alone or mixed with 250μg ABF emulsified in a 50μl volume with FCA. Seven days after sensitisation all groups were challenged with 100μg of HAO and the DTH response assessed. The mice were sacrificed one week post-challenge to measure the animals' antibody, cytokine and proliferative responses.

Immunisation of IL-4 KO mice with OVA alone resulted in the development of a strong DTH response similar to those observed in the wild-type controls. While addition of ABF to OVA at immunisation resulted in ablation of DTH in the WT animals this was not seen in the IL-4 deficients which only exhibited a slight diminution of the response. Treatment with neutralising IL-10 antibodies prior to immunisation with OVA and ABF had no discernible effect on the IL-4 KO mice and although a slight DTH response was seen in the control group this was still significantly lower in comparison to immunisation with OVA alone. These results are shown in Figure 5.21.

The presence of ABF at immunisation lead to a 30% decrease in OVA-specific IFN-γ production in the B10.BR wild-types with α-IL-10 treatment ablating production totally. In the IL-4 KOs no OVA-specific IFN-γ could be detected following sensitisation with ABF and in the KOs immunised with OVA alone the levels were considerably less than that detected in the wild-type animals (Figure 5.22). Interestingly, treatment of the IL-4 KO mice with α-IL-10 antibody resulted in a 90% increase in the production of OVA-specific IFN-γ. As found in the other experiments presented no IL-4 or IL-5 was measurable and IL-10 production was below the assay detection limit.

The proliferative responses by the PLN populations in these mice following restimulation *in vitro* with antigen are presented in Figure 5.23. The data shows that both wild-type and IL-4 KO animals responded well following culture with the Con A mitogen, except for the WT mice treated with α-IL10 prior to sensitisation with OVA and ABF where the response was decreased. When the populations were restimulated with OVA, an enhancement of the antigen-specific response in WT animals was seen as reported in previous sections when OVA was presented in association with ABF. This was not observed in the IL-4 KOs and treatment with α-IL-10 ablated this response in the WTs. No differences were noted in the ability to respond due to culture with rABA-1, which was in contrast to ABF-induced proliferation. While WT mice responded strongly if exposed to ABF during priming, IL-4 KOs did not. Furthermore, where injection with α-IL-10 abolished this response in the WTs it promoted ABF-specific proliferation in the IL-4 deficient mice.

Differences were also observed in antigen-specific immunoglobulin production (Figure 5.24). OVA-specific IgG1 levels were equivalent to those seen in the HAO controls for the IL-4 KOs and addition of ABF or α-IL-10 did little to influence the production in the

WTs. The reverse was recorded for OVA-IgG2a where the IL-4 KOs had increased relative responses in comparison to the WT controls. This pattern of reversed responses was also found for both the rABA-1- and ABF-specific antibodies. The most marked differences in production occurred in ABF-specific production. In the case of IgG1 the WT mice responses were double that of the KOs and when IgG2a levels were assessed the difference was approximately three times greater in the KOs in comparison to the WTs. As with OVA-specific production treatment of the mice with α-IL-10 had little influence on the rABA-1- or ABF-specific responses.

5.3 Discussion

The data presented here demonstrate that exposure to ABF during priming for the OVA-specific DTH response results in a suppression of the heterologous response upon antigen challenge. This ability to suppress the response was found to occur in both BALB/c and B10.S mice indicating that ABF could mediate its effects independently of the MHC, which had been reported for other parasite products with immunomodulatory activities (Fig. 5.1, (Robinson *et al.* 1994)). This was in contrast to other murine immune responses resulting from exposure to *Ascaris* products that have been shown to be tightly controlled by MHC genetics (Kennedy *et al.* 1986; Tomlinson *et al.* 1989).

Analysis of the DTH reaction in mice primed for OVA in the presence of the ABA-1 revealed that the allergen, which contributes approximately 50% of the total protein in ABF (Kennedy and Qureshi 1986), did not afford the same immunosuppressive activity as described for ABF. This response occurred with both recombinant and native forms of the molecule, indicating that even if the parasite-derived form differs from the recombinant protein in some way (i.e., protein folding or post-transcriptional modification), then this does not confer modulatory activities which the recombinant molecule does not possess. Most significantly, these results suggested that the effects of ABF were not dependent upon the allergen. This point was confirmed by removing pABA-1 from ABF, where upon sensitisation of mice with the remaining ABF components still resulted in depressed DTH responses (Fig. 5.10).

There are a variety of candidates that may be the active component in ABF. One possibility is phosphorylcholine, against which antibodies are generated during *Ascaris* infection (Mitchell 1976), as phosphorylcholine has been shown to a major component of immunomodulatory products of the filarial nematodes *Acanthocheilonema vitae* and *Brugia malayi* which are known to interfere with polyclonal activation of lymphocytes (Deehan *et al.* 1998; Harnett and Harnett 1993; Lal *et al.* 1990). Alternatively, carbohydrate residues may be important as has been shown with soluble egg antigens of

Schistosoma mansoni (Velupillai *et al.* 1997), where removal of glycan units from the antigens lead to a decrease in the IL-10 production associated with early infection. A final possibility is that both lipid and glycan work in conjunction and two such molecules with biological activity have been described for *Ascaris suum* (Lochnit *et al.* 1998). Although the identity of the active component is unknown, heat and acid treatments of ABF indicate that with respect to immunomodulation at least, this component is very stable and resistant to harsh environmental conditions (Fig. 5.20).

Differences in *in vitro* cytokine production by PLN cells provided clues to the mechanisms of the immunosuppression. Mice primed with OVA and ABF produced IFN-γ at considerably lower levels than those given OVA alone or mixed with ABA-1 when stimulated with OVA. No IL-4 protein could be detected in any of the culture supernatants, this may be due to it being rapidly bound by cells either through cell surface or secreted soluble receptors. Culturing cells in the presence of blocking monoclonal antibodies against the IL-4 receptor would test this possibility. Interestingly, the decrease in IFN-γ did coincide with the production of OVA-specific IL-10 in cultures of the OVA and ABF treated animals (Fig. 5.2). IL-10 was first reported as being a Th2 cell subset activity that inhibited Th1 cell cytokine synthesis and its presence had also been shown to decrease contact hypersensitivity responses in mice (Ferguson *et al.* 1994; Fiorentino *et al.* 1989). Therefore ABF may modulate the Th1-associated DTH response by inducing aTh2 response through the production of IL-10, which decreased the associated pathology as a consequence.

Modifications of the proliferative response were not as marked as those observed for cytokine production (Fig. 5.3). Popliteal proliferative responses were similar in most groups following stimulation with a variety of antigens. Interestingly, the OVA-specific response was enhanced slightly as a result of sensitisation in the presence of ABF. Alterations in the ability of host cells to respond to antigen is frequently associated with

parasite antigens (Deehan *et al.* 1998; Harnett and Harnett 1993; Hartmann *et al.* 1997; Robinson *et al.* 1994), and can occur through a general dampening of the antigen- or mitogen-specific immune response. However, mitogen-specific responses were unaffected following exposure to ABF and co-culturing with both Con A and ABF resulted in enhanced mitogenic activity indicating that an overall lowering in responsiveness was not responsible in this instance (Fig. 5.7).

Mice immunised with OVA and ABF produced significantly lower levels of OVA-specific IgG in comparison to the OVA controls and OVA plus ABA-1 group (Fig. 5.4). The OVA and ABF group also developed lower titres of rABA-1-specific IgG2a but were capable of producing ABF-specific antibodies of both isotypes. This again highlighting that while ABF can modulate immunity to heterologous antigen this does not appear to be at any cost to the immune response generated against the parasite antigens themselves.

Taken together this data suggests that ABF can modulate the Th1-associated DTH response possibly by inducing the development of a Th2-type response. Immunising IL-4 deficient mice which had been treated with neutralising anti-IL-10 monoclonal antibodies allowed the assessment of the DTH response in mice that were lacking key Th2-promoting cytokines. Although IL-4 protein was not detectable in the cultures, the contribution of the cytokine could not be overlooked as not only is IL-4 crucial to Th2 development and helminth products are strong promoters of Th2 cytokine production, but IL-4 has been demonstrated to synergise with IL-10 to inhibit cell-mediated immunity *in vivo* (Powrie *et al.* 1993b).

IL-4 deficient mice exposed to ABF at priming did generate DTH responses but which were slightly depressed in comparison to the OVA controls (Fig. 5.21). Pre-treatment with anti-IL-10 antibodies did not affect the footpad swelling measured in the KO mice and while it permitted some swelling in the wild-type controls, the response was still significantly lower than the OVA controls. Cytokine analysis revealed that wild-type and

IL-4 KO mice sensitised with OVA and ABF had either reduced or no production of IFN-γ following *in vitro* stimulation with OVA. Furthermore, OVA-specific IFN-γ production was increased in the anti-IL-10 antibody treated IL-4 deficients, suggestive of IL-10 involvement, however, the wild-type mice that were treated in this way produced no OVA-specific IFN-γ at all. Antigen-specific IgG1 and IgG2a levels were respectively lower and higher in the IL-4 KO mice relative to the wild-type controls reflecting the importance of IL-4 in immunoglobulin production as had been expected. Therefore, this data indicates that while both IL-4 and IL-10 are influential to the immunomodulation seen with ABF the cytokines are responsible for different aspects of the response.

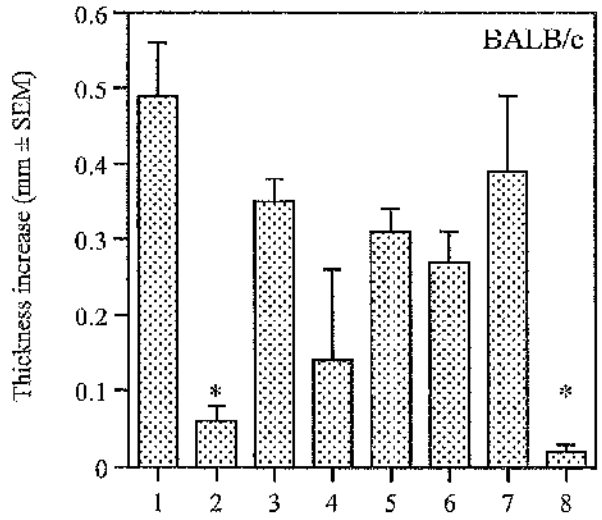
Finally further factors that influence the modulation of the DTH response by ABF are the dose of parasite product used at sensitisation, as mentioned earlier, and whether exposure occurs during the primary or recall phase of the anti-OVA immune response. Suppression of the DTH response was found to occur in a dose dependent manner, with lower doses (e.g., 50μg) failing to inhibit its development (Fig. 5.6). Similar effects of dosage were also noted with respect to the inhibition of antigen-specific cytokine and immunoglobulin production (Figs. 5.8 and 5.9). Following on from this, while mice that were exposed to ABF during primary or sensitisation phase of the DTH response suppressed the cell-mediated response, no effects were detectable when exposure occurred during the antigenic challenge or recall (Fig 5.16). This illustrates that while ABF is a potent immunomodulator it is quite specific in the ways in which it mediates its effects.

Figure 5.1

***Ascaris* antigens suppress the development of the OVA-specific DTH response in mice.** BALB/c and B10.S mice were sensitised subcutaneously in the left hind footpad with antigens as indicated below emulsified in FCA. Controls left unimmunised. All groups were challenged 7 days later in the opposite foot. Thickness increase represents the mean difference between footpad size before and 24 hours post challenge within a test group.

* represents a p-value ≤ 0.001 when compared to group 1 using a Student's *t*-test.

Group	Sensitisation	Challenge
1	100µg OVA	100µg HAO
2	100µg OVA + 500µg ABF	100µg HAO
3	100µg OVA + 100µg rABA-1	100µg HAO
4	100µg OVA + 500µg ABF	250µg soluble ABF
5	100µg OVA + 100µg rABA-1	50µg soluble rABA-1
6	500µg ABF	250µg soluble ABF
7	100µg rABA-1	50µg soluble rABA-1
8	unimmunised	100µg HAO


BALB/c
Thickness increase (mm ± SEM)
0.6
0.5
0.4
0.3
0.2
0.1
0
1 2 3 4 5 6 7 8
*
*

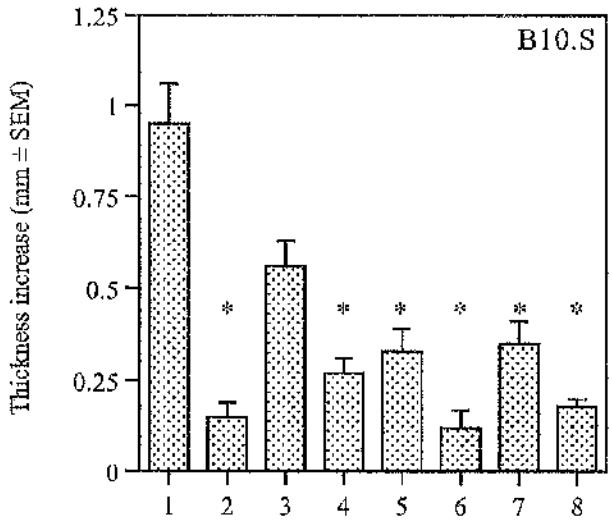
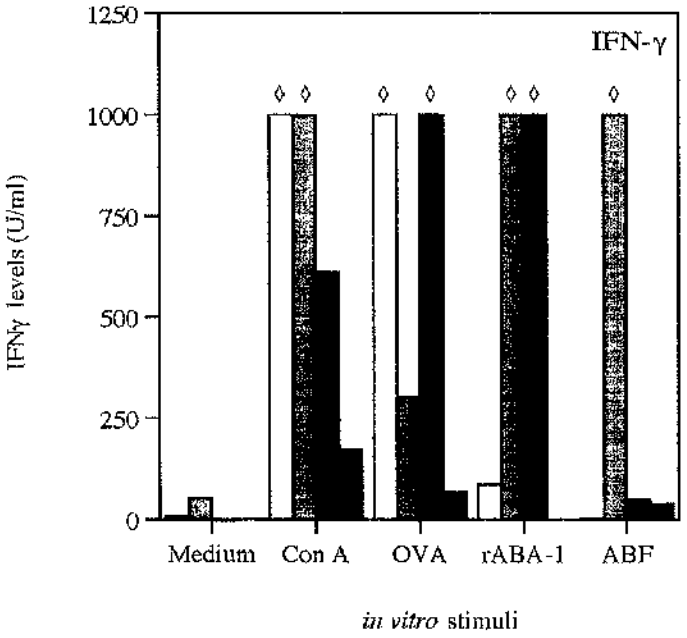

B10.S
Thickness increase (mm ± SEM)
1.25
1
0.75
0.5
0.25
0
1 2 3 4 5 6 7 8
*
*
*
*
*
*

Figure 5.2

Cytokine production by popliteal lymphocytes of mice exposed to *Ascaris* antigens during sensitisation to OVA. IFNγ and IL-10 protein levels were assessed in the culture supernatants of popliteal lymphocytes from BALB/c mice following *in vitro* restimulation for 72 hours. Mice were sensitised with antigens as detailed below emulsified in FCA in the left hind footpad. Controls were unimmunised. All groups were challenged 7 days opposite foot. Animals were sacrificed 7 days post-challenge and the popliteal lymphocytes within groups pooled. Cytokine production was measured using capture sandwich ELISAs. ◊ indicates detection limit of assay.

Group	Sensitisation	Challenge
1 □	100μg OVA	100μg HAO
2 ■	100μg OVA + 500μg ABF	100μg HAO
3 ■	100μg OVA + 100μg rABA-1	100μg HAO
4 ■	unimmunised	100μg HAO


IFN-γ
1250
1000
750
500
250
0
IFNγ levels (U/ml)
Medium
Con A
OVA
rABA-1
ABF
in vitro stimuli

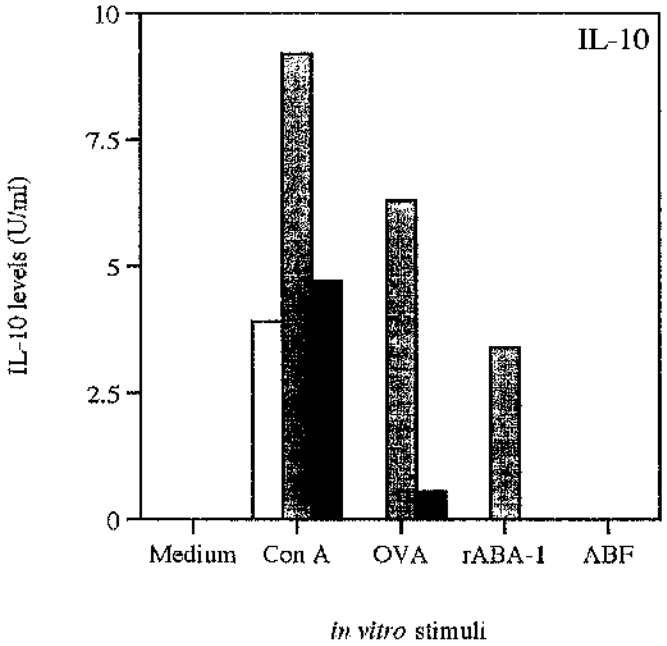

IL-10
10
7.5
5
2.5
0
IL-10 levels (U/ml)
Medium
Con A
OVA
rABA-1
ABF
in vitro stimuli

Figure 5.3

***Ascaris* antigens influence antigen-specific proliferation in OVA primed mice.** BALB/c mice were primed with antigens as indicated below emulsified in FCA and administered in the left hind footpad. Controls were unimmunised. All groups were challenged 7 days later in the opposite footpad. Animals were sacrificed one week later. The popliteal lymph nodes were pooled within groups and cultured *in vitro* with various stimuli as indicated for 72 hours. Cultures were pulsed with 0.5μCi of ^{3}H thymidine and incubated for a further 24 hours before harvesting. Each data point represents the mean uptake of thymidine ± SEM for an experiment performed in triplicate.

Group	Sensitisation	Challenge
1	100μg OVA	100μg HAO
2	100μg OVA + 500μg ABF	100μg HAO
3	100μg OVA + 100μg rABA-1	100μg HAO
4	unimmunised	100μg HAO

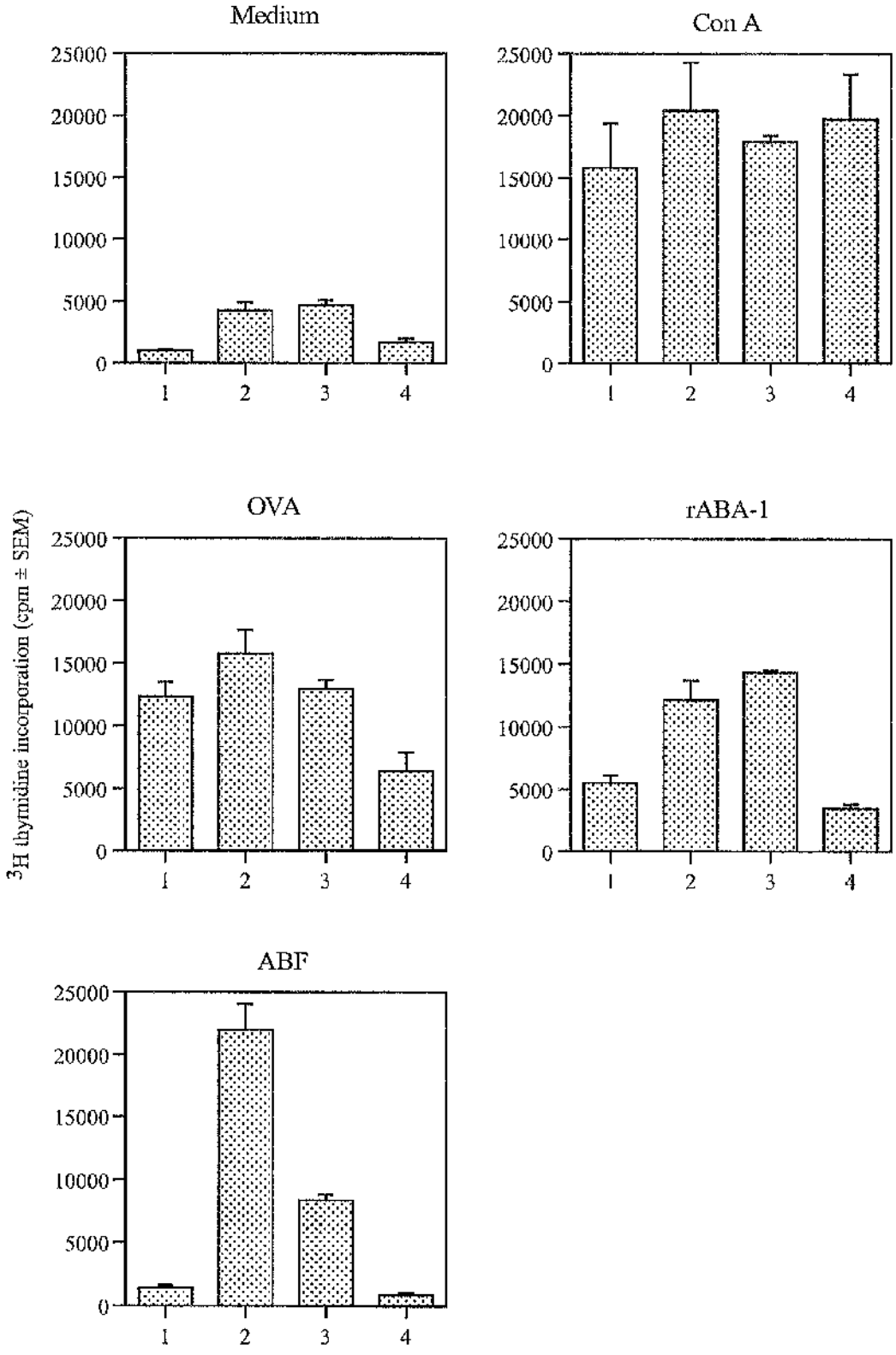
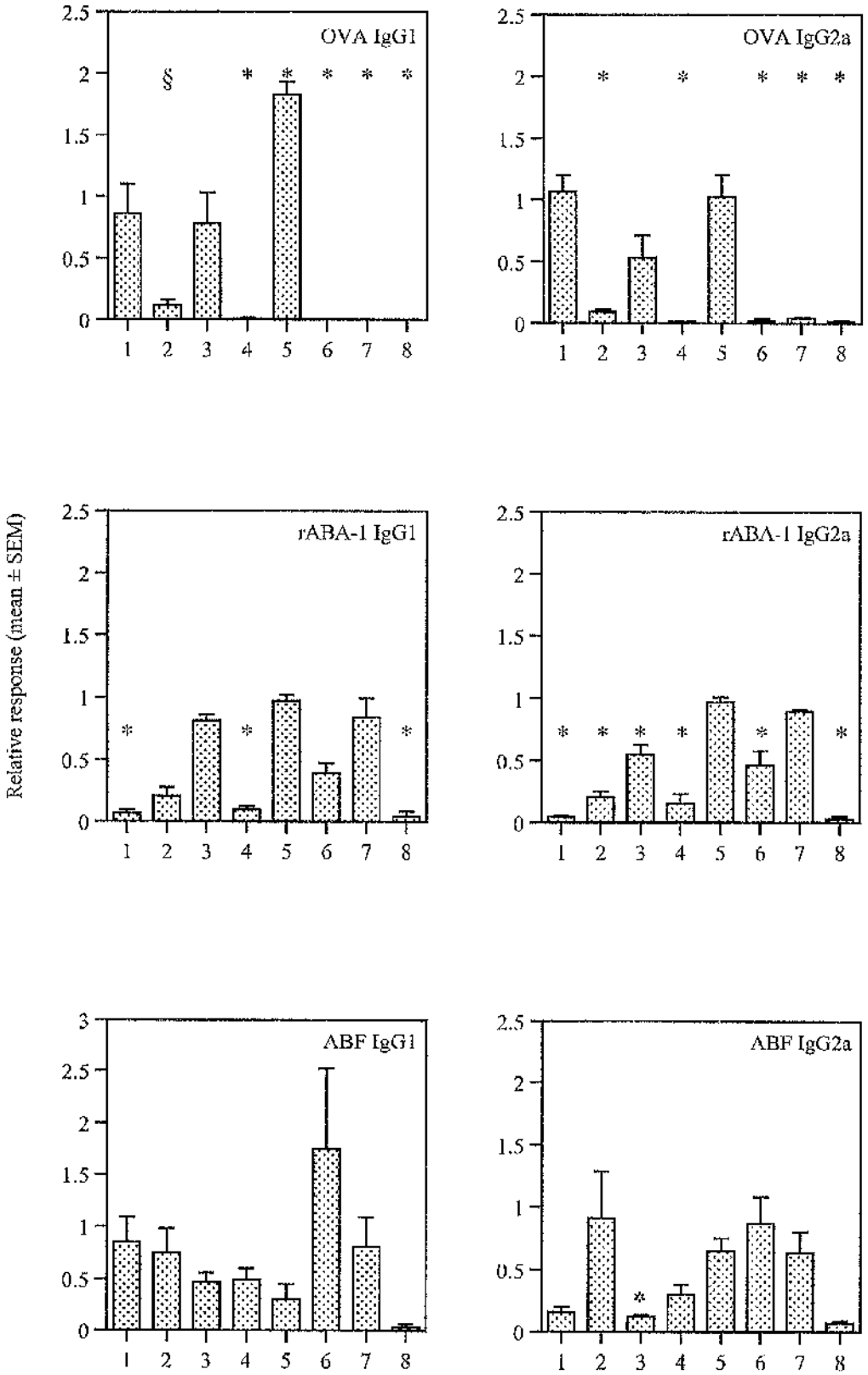

Medium
Con A
OVA
rABA-1
ABF
^{3}H thymidine incorporation (cpm ± SEM)
25000
20000
15000
10000
5000
0
1
2
3
4

Figure 5.4

Exposure to parasite antigens during OVA priming influences the production of antigen-specific immunoglobulins. BALB/c mice were sensitised with antigens detailed below emulsified in FCA via the left hind footpad. Controls were unimmunised. All groups were challenged 7 days later. Mice were sacrificed one week later and sera collected. Antigen-specific antibodies were measured by ELISA. Data are expressed as the amount of antigen-specific antibody in a sample relative to the positive control in arbitrary units. Each data point represents the mean ± SEM for an experiment performed in triplicate.

* represents a p-value ≤ 0.007 and § approaching 0.007 when compared to group 1 (OVA-specific), group 6 (ABF-specific) or group 7 (rABA-1-specific) using a Student's *t*-test.

Group	Sensitisation	Challenge
1	100μg OVA	100μg HAO
2	100μg OVA + 500μg ABF	100μg HAO
3	100μg OVA + 100μg rABA-1	100μg HAO
4	100μg OVA + 500μg ABF	250μg soluble ABF
5	100μg OVA + 100μg rABA-1	50μg soluble rABA-1
6	500μg ABF	250μg soluble ABF
7	100μg rABA-1	50μg soluble rABA-1
8	unimmunised	100μg HAO


OVA IgG1
OVA IgG2a
rABA-1 IgG1
rABA-1 IgG2a
ABF IgG1
ABF IgG2a
Relative response (mean ± SEM)
§
*
0
0.5
1
1.5
2
2.5
3
1 2 3 4 5 6 7 8

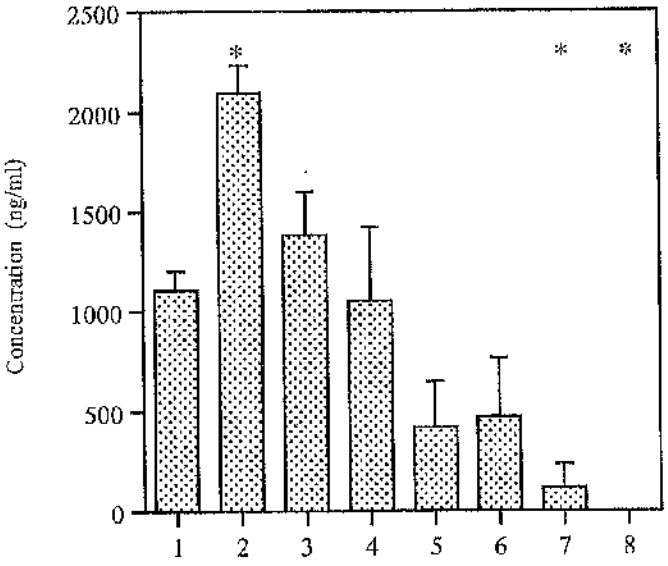


Figure 5.5

Total serum IgE concentrations in animals exposed to *Ascaris* antigens during OVA sensitisation. BALB/c mice were sensitised in the left hind footpad with antigens detailed below emulsified in FCA. Controls were left unimmunised. All groups were challenged 7 days later in the opposite foot. Animals were sacrificed one week post-challenge and sera collected for analysis by ELISA. Each data point represents the mean ± SEM for an experiment performed in triplicate.

* represents a p-value ≤ 0.008 when compared to group 1 using a Student's *t*-test.

Group	Sensitisation	Challenge
1	100µg OVA	100µg HAO
2	100µg OVA + 500µg ABF	100µg HAO
3	100µg OVA + 100µg rABA-1	100µg HAO
4	100µg OVA + 500µg ABF	250µg soluble ABF
5	100µg OVA + 100µg rABA-1	50µg soluble rABA-1
6	500µg ABF	250µg soluble ABF
7	100µg rABA-1	50µg soluble rABA-1
8	unimmunised	100µg HAO

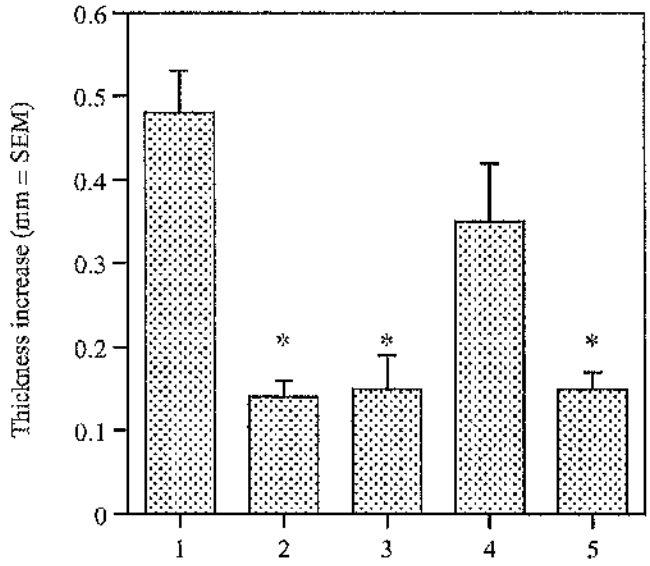


Figure 5.6

ABF suppresses the OVA-specific DTH response in a dose dependent manner. BALB/c mice were sensitised with antigens as detailed below emulsified in FCA via the left hind footpad. Controls were unimmunised. Challenge occurred one week later in the opposite foot. Thickness increase represents the mean difference between footpad size before and 24 hours post challenge within a test group.

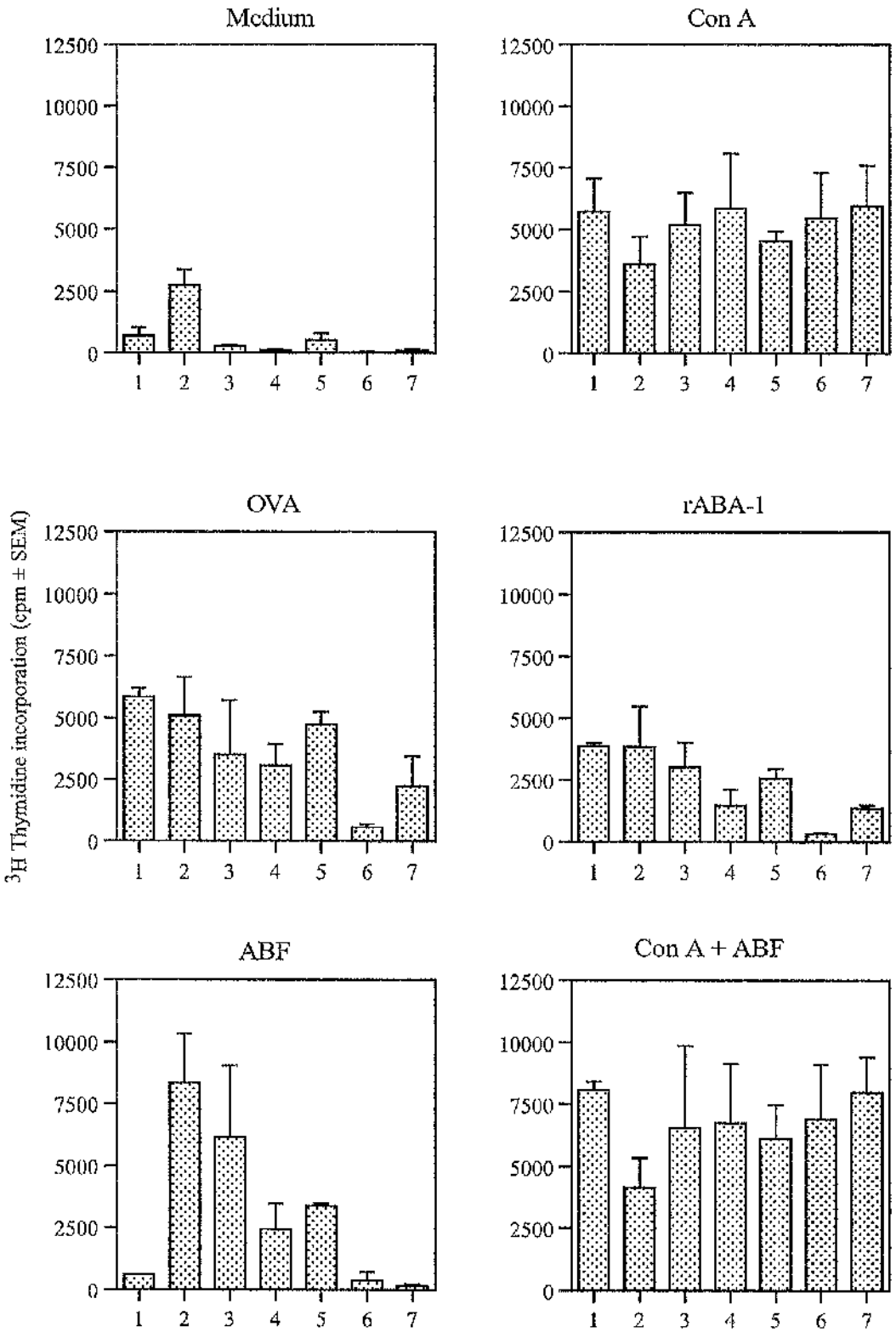
* represents a p-value ≤ 0.001 using a Student's *t*-test.

Group	Sensitisation	Challenge
1	100µg OVA	100µg HAO
2	100µg OVA + 500µg ABF	100µg HAO
3	100µg OVA + 250µg ABF	100µg HAO
4	100µg OVA + 50µg ABF	100µg HAO
5	unimmunised	100µg HAO

Figure 5.7

The influence of ABF dose and pABA-1 on proliferative responses of OVA-primed mice. BALB/c mice were sensitised with antigens as detailed below emulsified in FCA in the left hind footpad. Controls were unimmunised. Challenge occurred one week later in the opposite foot. Seven days post-challenge the animals were killed. Popliteal lymph node cells were pooled within groups and cultured *in vitro* with various stimuli as indicated for 72 hours. Cultures were pulsed with 0.5μCi of ^{3}H thymidine and incubated for a further 24 hours before harvesting. Each data point represents the mean uptake of thymidine ± SEM.

Group	Sensitisation	Challenge
1	100μg OVA	100μg HAO
2	100μg OVA + 500μg ABF	100μg HAO
3	100μg OVA + 250μg ABF	100μg HAO
4	100μg OVA + 50μg ABF	100μg HAO
5	100μg OVA + 100μg pABA-1	100μg HAO
6	100μg OVA + 250μg fractionated ABF	100μg HAO
7	unimmunised	100μg HAO


Medium
Con A
OVA
rABA-1
ABF
Con A + ABF
^{3}H Thymidine incorporation (cpm ± SEM)
12500
10000
7500
5000
2500
0
1
2
3
4
5
6
7

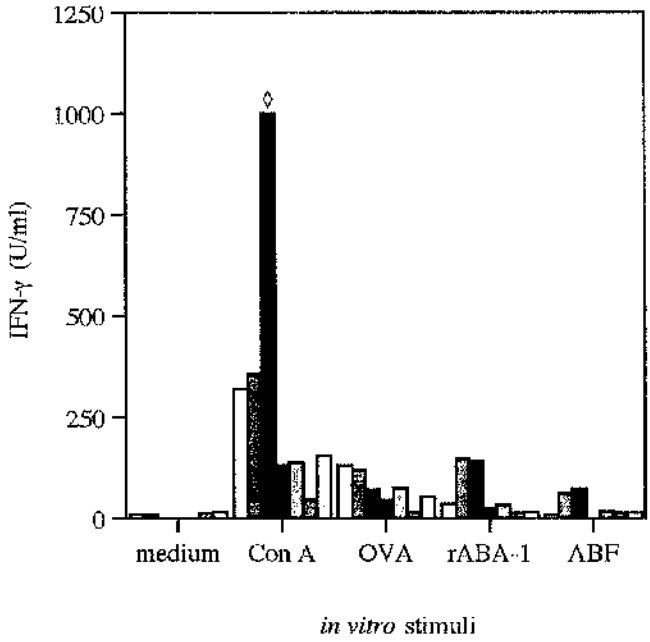


Figure 5.8

The influence of ABF dose and pABA-1 on IFN-γ production in OVA-primed mice. BALB/c mice were sensitised with antigens detailed below emulsified in FCA in the left hind footpad. Controls were unimmunised. Challenge occurred one week later in the opposite foot. Seven days post-challenge the animals were killed. Popliteal lymph node cells were pooled within groups and restimulated *in vitro* with various stimuli as indicated for 72 hours. IFN-γ protein levels were measured using capture sandwich ELISAs. ◊ indicates detection limit of assay.

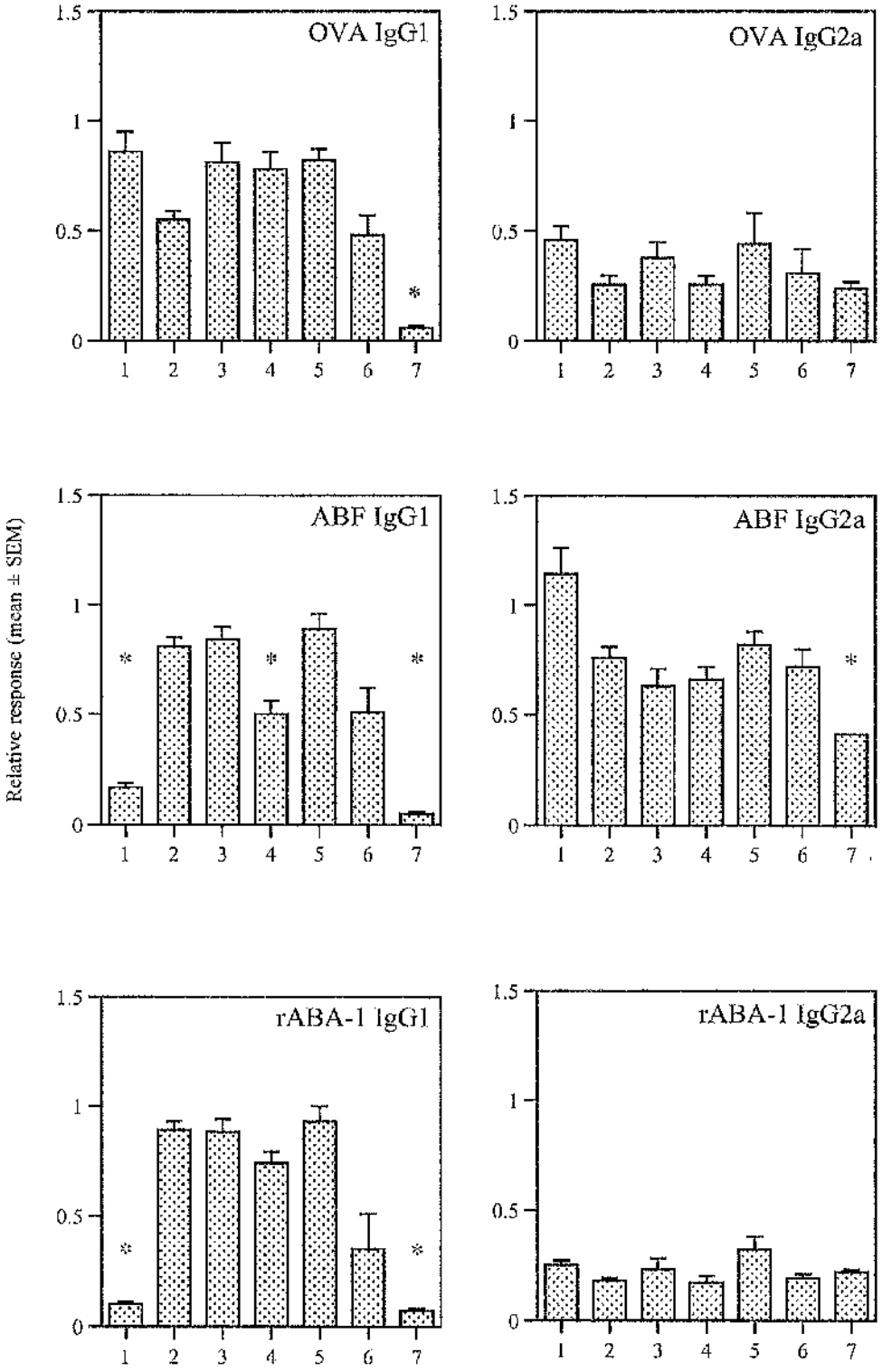
Group	Sensitisation	Challenge
1 □	100μg OVA	100μg HAO
2 ▒	100μg OVA + 500μg ABF	100μg HAO
3 ■	100μg OVA + 250μg ABF	100μg HAO
4 ■	100μg OVA + 50μg ABF	100μg HAO
5 ■	100μg OVA + 100μg pABA-1	100μg HAO
6 ▒	100μg OVA + 250μg fractionated ABF	100μg HAO
7 □	unimmunised	100μg HAO

Figure 5.9

The influence of ABF dose and pABA-1 on antigen-specific antibody production in OVA-primed mice. BALB/c mice were sensitised with antigens as detailed below emulsified in FCA in the left hind footpad. Controls were unimmunised. Challenge occurred one week later in the opposite foot. Mice were sacrificed seven days post-challenge and sera collected. Antigen-specific antibodies were measured by ELISA. Data are expressed as the amount of antigen-specific antibody in a sample relative to the positive control in arbitrary units. Each data point represents the mean ± SEM for an experiment performed in triplicate.

* represents a p-value ≤ 0.008 when compared to group 1 (OVA-specific), group 2 (ABF-specific) or group 5 (rABA-1-specific) using a Student's *t*-test.

Group	Sensitisation	Challenge
1	100μg OVA	100μg HAO
2	100μg OVA + 500μg ABF	100μg HAO
3	100μg OVA + 250μg ABF	100μg HAO
4	100μg OVA + 50μg ABF	100μg HAO
5	100μg OVA + 100μg pABA-1	100μg HAO
6	100μg OVA + 250μg fractionated ABF	100μg HAO
7	unimmunised	100μg HAO


OVA IgG1
OVA IgG2a
ABF IgG1
ABF IgG2a
rABA-1 IgG1
rABA-1 IgG2a
Relative response (mean ± SEM)
1.5
1
0.5
0
1 2 3 4 5 6 7
*

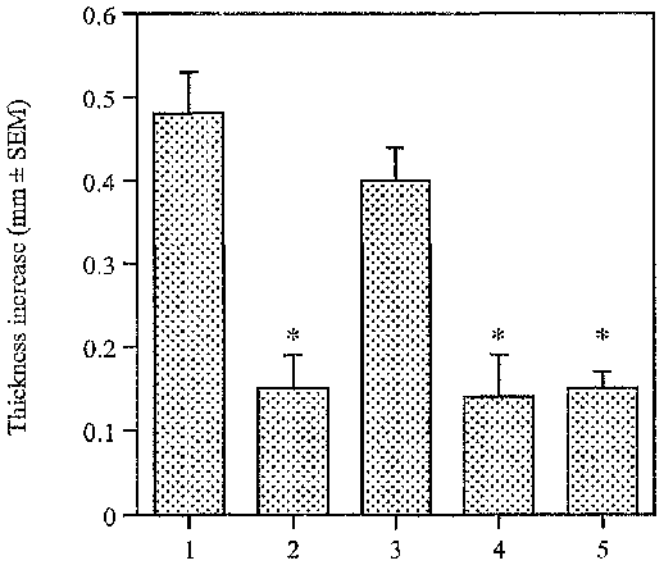


Figure 5.10

The suppression of the OVA-specific DTH response by ABF does not require the presence of pABA-1. BALB/c mice were sensitised in the left hind footpad with antigens detailed below emulsified in FCA. Controls were unimmunised. All groups were challenged one week later in the opposite foot. Thickness increase represents the mean difference between footpad size before and 24 hours post challenge within a test group.

* represents a p-value ≤ 0.001 when compared to group 1 using a Student's *t*-test.

Group	Sensitisation	Challenge
1	100µg OVA	100µg HAO
2	100µg OVA + 250µg ABF	100µg HAO
3	100µg OVA + 100µg pABA-1	100µg HAO
4	100µg OVA + 250µg fractionated ABF	100µg HAO
5	unimmunised	100µg HAO

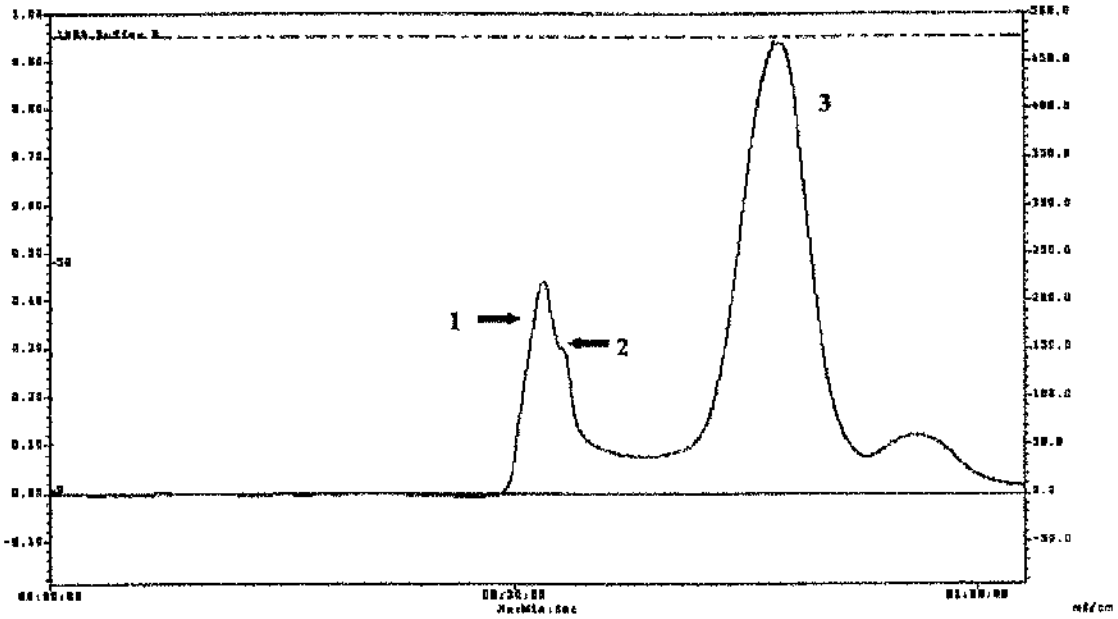


Figure 5.11

Separation of ABF proteins by gel fractionation.

In order to investigate the effects of parasite-derived ABA-1 and other body fluid proteins in isolation ABF was applied to a gel filtration column which separated the proteins according to molecular weight. A typical read-out trace from the procedure is illustrated. The proteins of ABF were separated into three main groups as indicated. FPLC-purified peak 1, FPLC-purified peak 2 and parasite-derived ABA-1 are indicated on the trace by 1, 2 and 3 respectively.

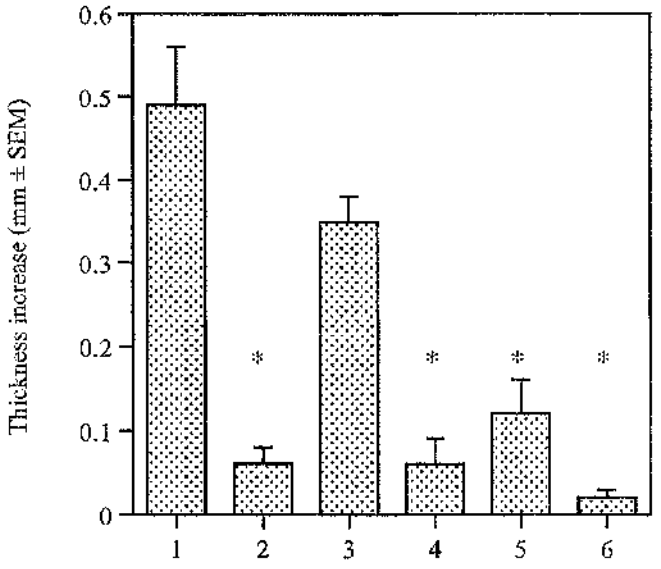


Figure 5.12

Both FPLC-purified fractions of ABF suppress the OVA-specific DTH response. BALB/c mice were immunised in the left hind footpad with antigens detailed below emulsified in FCA. Controls were unimmunised. All groups were challenged 7 days later in the opposite foot. Thickness increase represents the mean difference between footpad size before and 24 hours post challenge within a test group.

* represents a p-value ≤ 0.005 when compared to group 1 using a Student's *t*-test.

Group	Sensitisation	Challenge
1	100µg OVA	100µg HAO
2	100µg OVA + 250µg ABF	100µg HAO
3	100µg OVA + 100µg pABA-1	100µg HAO
4	100µg OVA + 250µg ABF peak 1	100µg HAO
5	100µg OVA + 250µg ABF peak 2	100µg HAO
6	unimmunised	100µg HAO

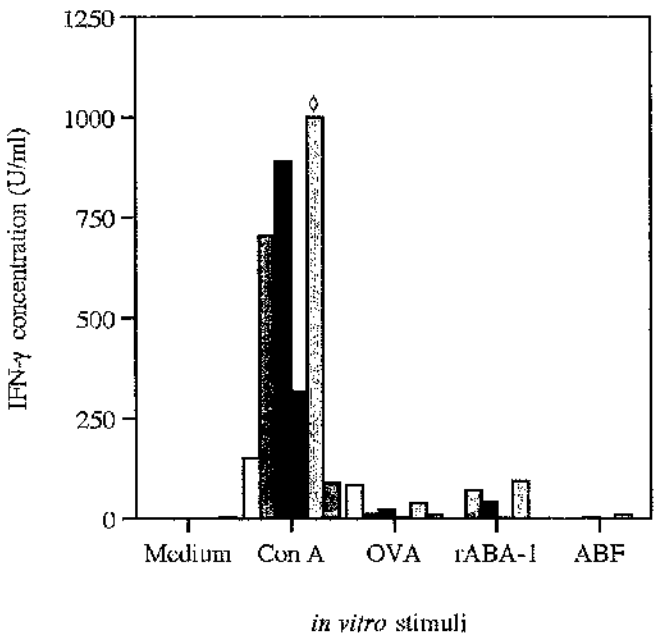


Figure 5.13

IFN-γ production in the popliteal lymph node cell cultures of mice exposed to FPLC-purified fractions of ABF during OVA priming. BALB/c mice were immunised in the left hind footpad with antigens as detailed below emulsified in FCA. Controls were unimmunised. All groups were challenged 7 days later in the opposite foot. Animals were sacrificed one week later. The popliteal lymph nodes were pooled within groups and cultured *in vitro* with various stimuli as indicated for 72 hours. IFN-γ production was measured in the culture supernatants using capture sandwich ELISAs. ◊ indicates detection limit of assay.

Group	Sensitisation	Challenge
1 □	100μg OVA	100μg HAO
2 ▒	100μg OVA + 250μg ABF	100μg HAO
3 ■	100μg OVA + 100μg pABA-1	100μg HAO
4 ■	100μg OVA + 250μg ABF peak 1	100μg HAO
5 ■	100μg OVA + 250μg ABF peak 2	100μg HAO
6 ▒	unimmunised	100μg HAO

Figure 5.14

Proliferation of popliteal lymph node cells following immunisation with FPLC-purified fractions of ABF during OVA priming. BALB/c mice were immunised in the left hind footpad with antigens detailed below emulsified in FCA. Controls were unimmunised. All groups were challenged 7 days later in the opposite foot. Animals were sacrificed one week later. The popliteal lymph nodes were pooled within groups and cultured *in vitro* with various stimuli as indicated for 72 hours. Cultures were pulsed with 0.5μCi of ^{3}H thymidine and incubated for a further 24 hours before harvesting. Each data point represents the mean uptake of thymidine ± SEM for an experiment performed in triplicate.

Group	Sensitisation	Challenge
1	100μg OVA	100μg HAO
2	100μg OVA + 250μg ABF	100μg HAO
3	100μg OVA + 100μg pABA-1	100μg HAO
4	100μg OVA + 250μg ABF peak 1	100μg HAO
5	100μg OVA + 250μg ABF peak 2	100μg HAO
6	unimmunised	100μg HAO

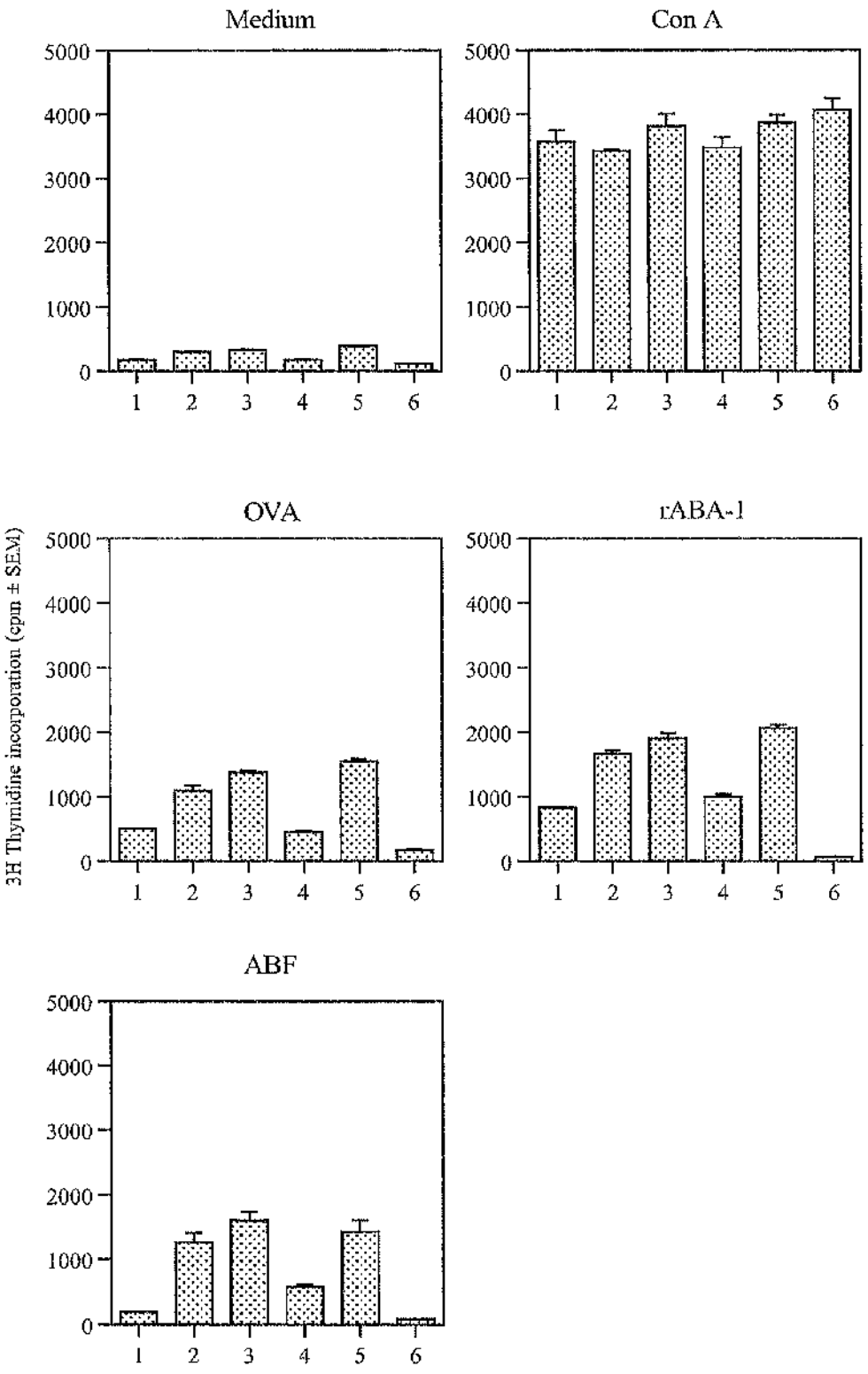
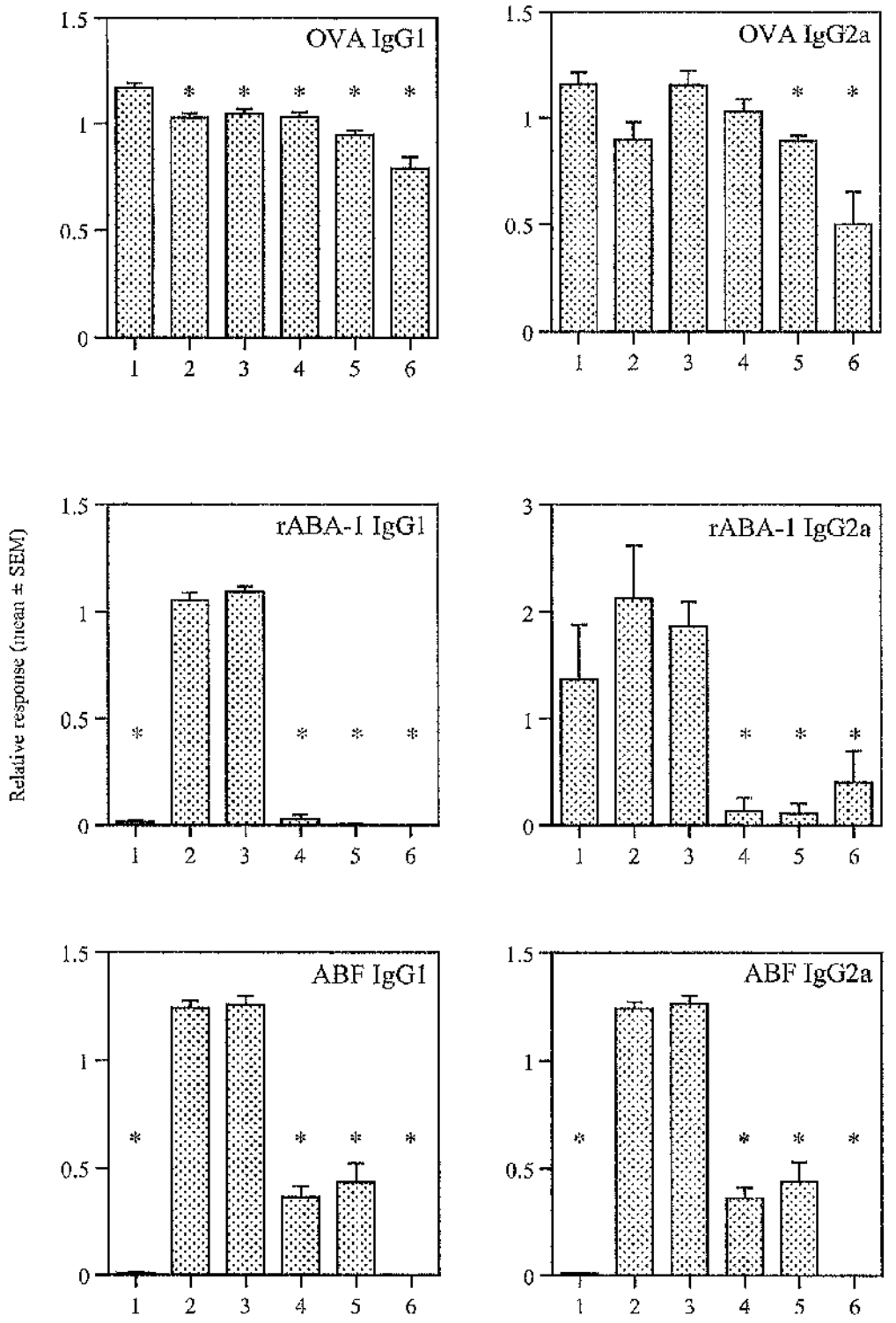

Medium
Con A
OVA
rABA-1
ABF
3H Thymidine incorporation (cpm ± SEM)
5000
4000
3000
2000
1000
0
1
2
3
4
5
6

Figure 5.15

Antigen-specific antibody production following exposure to FPLC-purified fractions of ABF during OVA priming. BALB/c mice were immunised in the left hind footpad with antigens detailed below emulsified in FCA. Controls were unimmunised. All groups were challenged 7 days later in the opposite foot. Mice were sacrificed one week later and sera collected. Antigen-specific antibodies were measured by ELISA. Data are expressed as the amount of antigen-specific antibody in a sample relative to the positive control in arbitrary units. Each data point represents the mean ± SEM.

* represents a p-value ≤ 0.001 when compared to group 1 (OVA-specific) and group 2 (rABA-1- and ABF-specific) using a Student's *t*-test.

Group	Sensitisation	Challenge
1	100µg OVA	100µg HAO
2	100µg OVA + 250µg ABF	100µg HAO
3	100µg OVA + 100µg pABA-1	100µg HAO
4	100µg OVA + 250µg ABF peak 1	100µg HAO
5	100µg OVA + 250µg ABF peak 2	100µg HAO
6	unimmunised	100µg HAO


OVA IgG1
OVA IgG2a
rABA-1 IgG1
rABA-1 IgG2a
ABF IgG1
ABF IgG2a
Relative response (mean ± SEM)

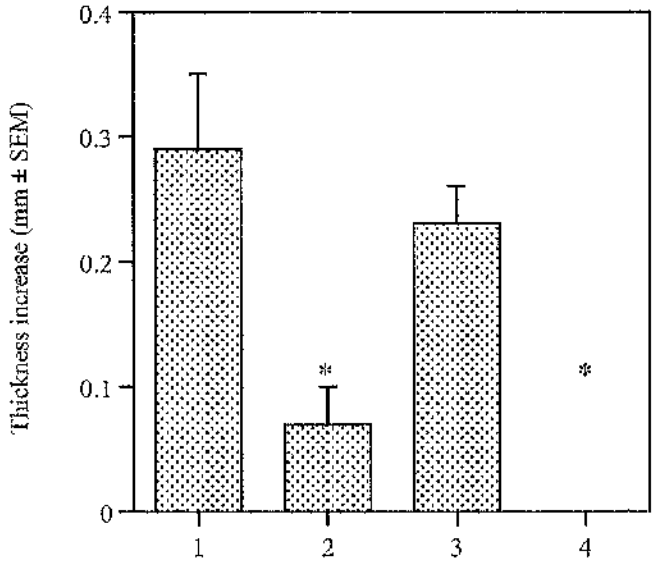


Figure 5.16

Suppression of the OVA-specific DTH response by ABF is dependent upon the timing of exposure to the parasite antigens. BALB/c mice were sensitised with antigens as detailed below emulsified in FCA via the left hind footpad. Controls were unimmunised. Challenge occurred 7 days post-sensitisation. Thickness increase represents the mean difference between footpad size before and 24 hours post challenge within a test group.

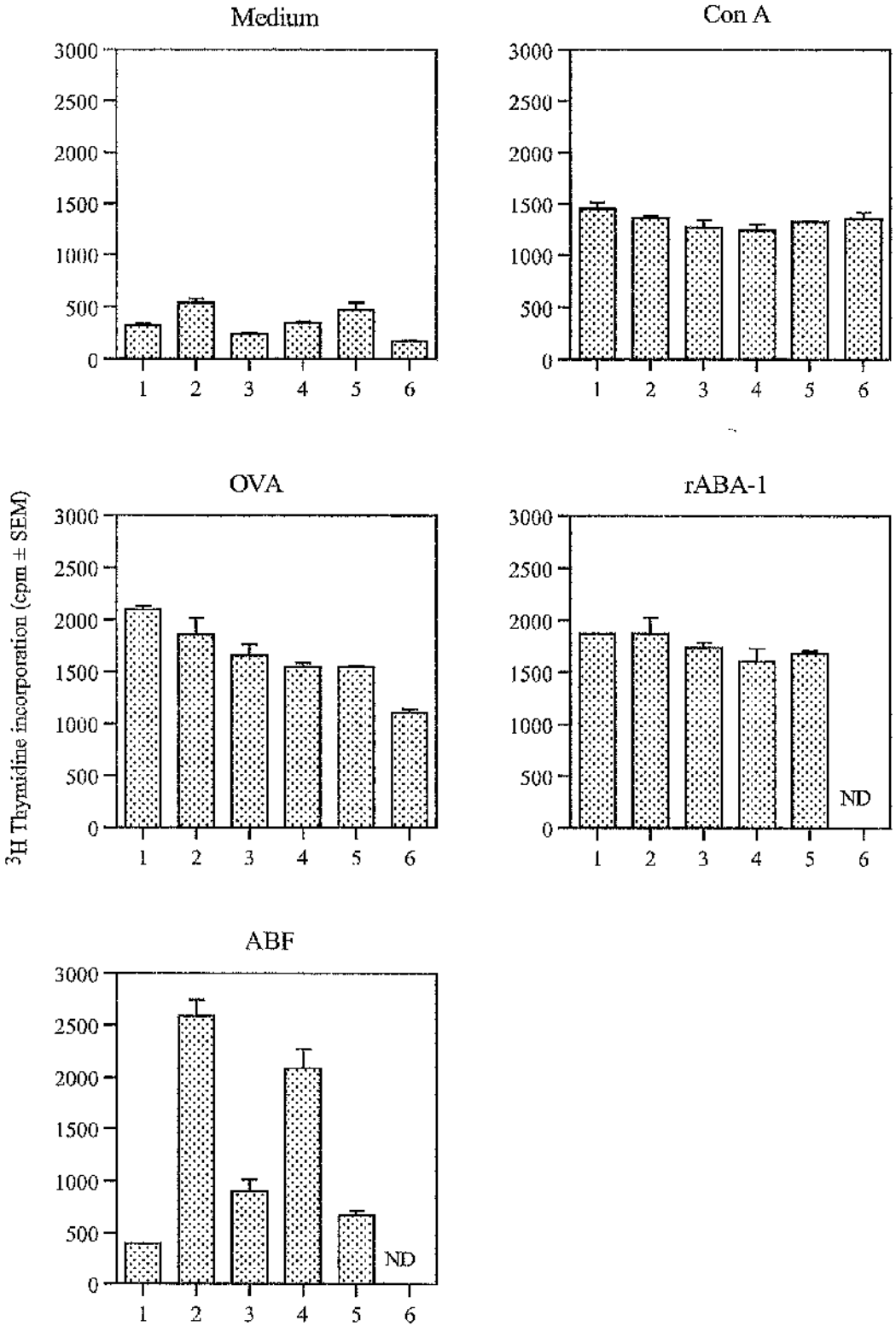
* represents a p-value ≤ 0.01 when compared to group 1 using a Student's *t*-test.

Group	Sensitisation	Challenge
1	100µg OVA	100µg HAO
2	100µg OVA + 250µg ABF	100µg HAO
3	100µg OVA	100µg HAO + 250µg soluble ABF
4	unimmunised	100µg HAO

Figure 5.17

Timing of exposure to ABF and heat or chemical treatments of the antigens modulates proliferation in popliteal lymph populations. BALB/c mice were primed with antigens detailed below emulsified in FCA in the left hind footpad. Controls were unimmunised (group 6). Groups were challenged 7 days post-sensitisation. Mice were killed one week later. Popliteal lymphocytes were pooled within groups and cultured *in vitro* with stimuli as indicated for 72 hours. Cultures were pulsed with 0.5μCi of ^{3}H thymidine and incubated for a further 24 hours before harvesting. Each data point represents the mean uptake of thymidine ± SEM for an experiment performed in triplicate. ND indicates experiment not done.

Group	Sensitisation	Challenge
1	100μg OVA	100μg HAO
2	100μg OVA + 250μg ABF	100μg HAO
3	100μg OVA + 250μg heated ABF	100μg HAO
4	100μg OVA + 250μg acid treated ABF	100μg HAO
5	100μg OVA	100μg HAO + 250μg soluble ABF
6	unimmunised	100μg HAO


Medium
Con A
OVA
rABA-1
ABF
^{3}H Thymidine incorporation (cpm ± SEM)
3000
2500
2000
1500
1000
500
0
1
2
3
4
5
6
ND

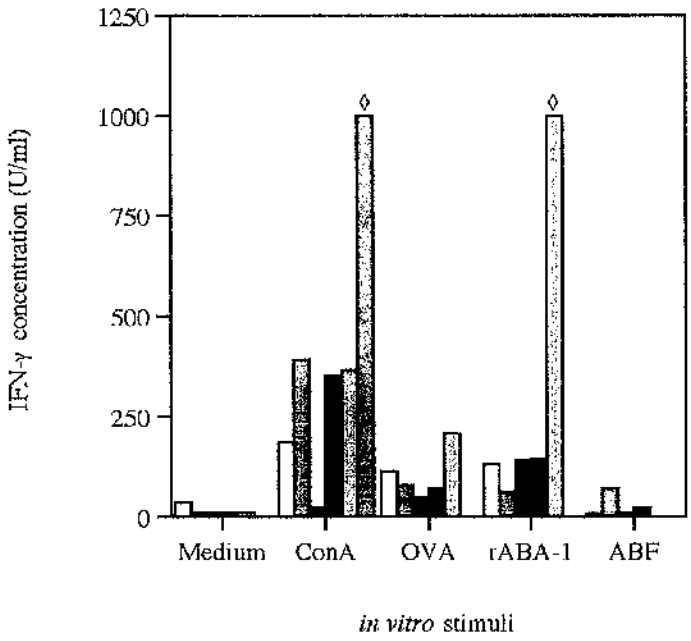


Figure 5.18

Timing of exposure to ABF and heat or chemical treatments of the antigens affects *in vitro* IFN-γ production by the popliteal lymph populations. BALB/c mice were primed with antigens as detailed below emulsified in FCA in the left hind footpad. Controls were unimmunised. Challenge occurred 7 days post-sensitisation. Animals were killed one week later and popliteal lymphocytes were pooled within groups for culture *in vitro* with stimuli as indicated for 72 hours. Culture supernatants were harvested and IFN-γ protein levels were assessed by capture sandwich ELISA. ◊ indicates detection limit of assay.

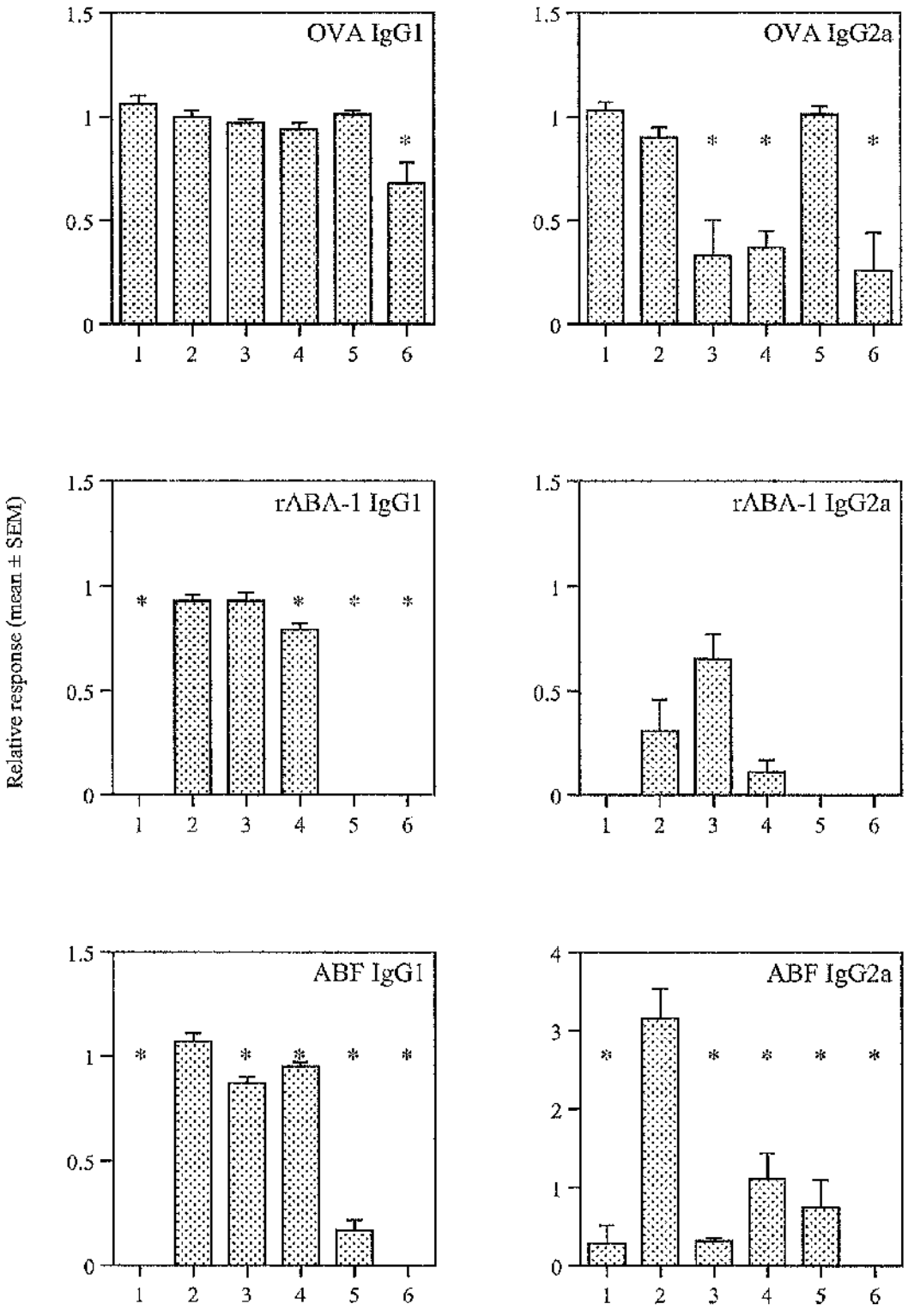
Group	Sensitisation	Challenge
1 □	100μg OVA	100μg HAO
2 ▒	100μg OVA + 250μg ABF	100μg HAO
3 ■	100μg OVA + 250μg heated ABF	100μg HAO
4 ■	100μg OVA + 250μg acid treated ABF	100μg HAO
5 ■	100μg OVA	100μg HAO + 250μg soluble ABF
6 ▒	unimmunised	100μg HAO

Figure 5.19

Timing of exposure to ABF and heat or chemical treatments of the antigens influences production of antigen-specific immunglobulins. BALB/c mice were primed with antigens detailed below emulsified in FCA in the left hind footpad. Controls were unimmunised (group 6). Challenge occurred 7 days post-sensitisation. Animals were killed one week later and sera collected. Antibody levels were assessed by ELISA. Data are expressed as the amount of antigen-specific antibody in a sample relative to the positive control in arbitrary units. Each data point represents the mean ± SEM for an experiment performed in triplicate.

* represents a p-value ≤ 0.01 when compared to group 1 (OVA-specific) and group 2 (rABA-1- and ABF-specific) using a Student's *t*-test.

Group	Sensitisation	Challenge
1	100µg OVA	100µg HAO
2	100µg OVA + 250µg ABF	100µg HAO
3	100µg OVA + 250µg heated ABF	100µg HAO
4	100µg OVA + 250µg acid treated ABF	100µg HAO
5	100µg OVA	100µg HAO + 250µg soluble ABF
6	unimmunised	100µg HAO


OVA IgG1
OVA IgG2a
rABA-1 IgG1
rABA-1 IgG2a
ABF IgG1
ABF IgG2a
Relative response (mean ± SEM)

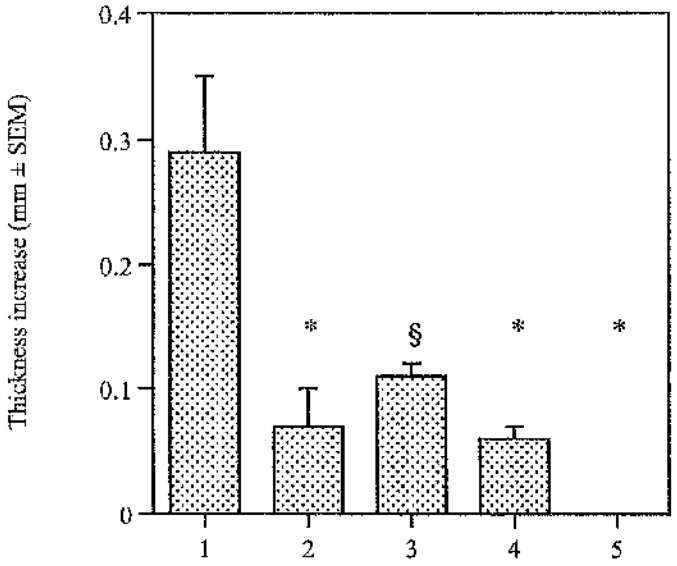


Figure 5.20

The ability to suppress the OVA-specific DTH response by ABF is unaltered by heating or chemically treatment prior to use. BALB/c mice were sensitised with antigens detailed below emulsified in FCA in the left hind footpad. Controls were unimmunised. All groups were challenged one week later in the opposite foot. Thickness increase represents the mean difference between footpad size before and 24 hours post challenge within a test group.

* represents a p-value ≤ 0.01 and § approaching significance when compared to group 1 using a Student's *t*-test.

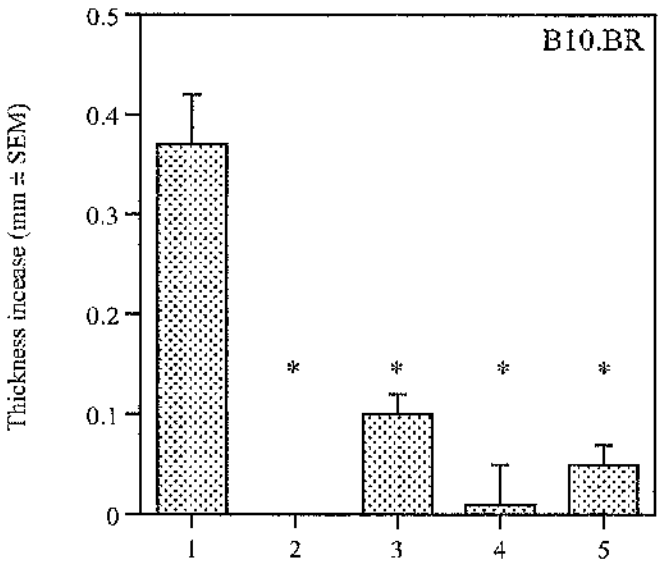
Group	Sensitisation	Challenge
1	100µg OVA	100µg HAO
2	100µg OVA + 250µg ABF	100µg HAO
3	100µg OVA + 250µg heated ABF	100µg HAO
4	100µg OVA + 250µg acid treated ABF	100µg HAO
5	unimmunised	100µg HAO

Figure 5.21

The influence of ABF on the development of the OVA-specific DTH response in IL-4 deficient and anti-IL-10 monoclonal antibody treated mice. B10.BR controls and IL-4 deficient mice were sensitised with antigens detailed below emulsified in FCA in the left hind footpad. Controls were unimmunised. In addition groups were treated with neutralising rat-anti-mouse IL-10 monoclonal antibodies or rat isotype control antibodies two hours pre-sensitisation. All groups were challenged one week later in the opposite foot. Thickness increase represents the mean difference between footpad size before and 24 hours post challenge within a test group.

* represents a p-value ≤ 0.001 when compared to group 1 using a Student's *t*-test.

Group	Sensitisation	Challenge	Antibody treatment
1	100μg OVA	100μg HAO	200μg isotype control
2	100μg OVA + 250μg ABF	100μg HAO	200μg isotype control
3	100μg OVA + 250μg ABF	100μg HAO	200μg anti-IL-10
4	250μg ABF	100μg HAO	-
5	unimmunised	100μg HAO	-


B10.BR
Thickness increase (mm ± SEM)
0.5
0.4
0.3
0.2
0.1
0
*
*
*
*
1
2
3
4
5

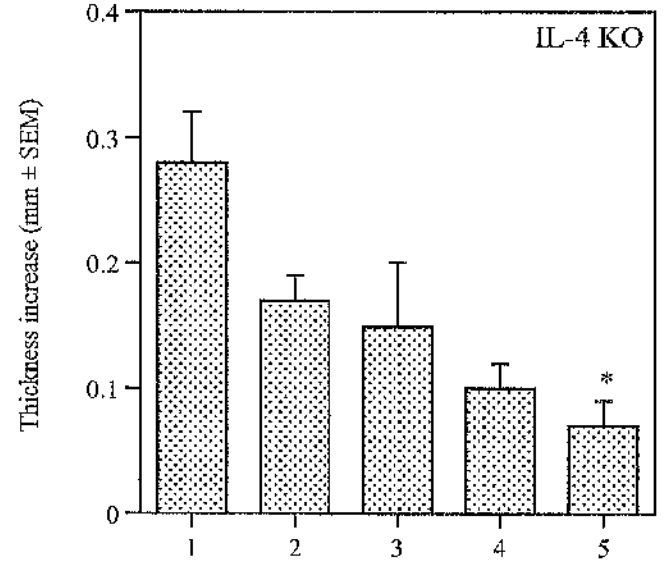
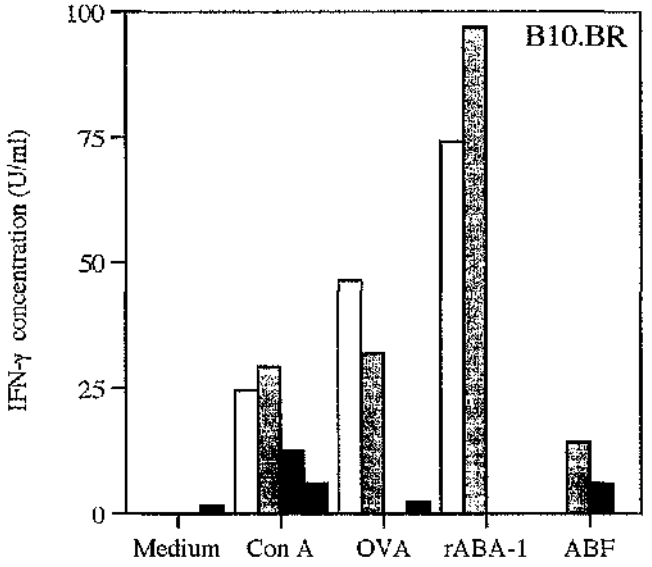

IL-4 KO
Thickness increase (mm ± SEM)
0.4
0.3
0.2
0.1
0
*
1
2
3
4
5

Figure 5.22

The influence of exposure to ABF during OVA sensitisation on IFN-γ production in IL-4 deficient and anti-IL-10 monoclonal antibody treated mice. B10.BR controls and IL-4 deficient mice were sensitised with antigens detailed below emulsified in FCA in the left hind footpad. Controls were unimmunised. In addition, animals were immunised with neutralising rat-anti-mouse IL-10 monoclonal antibodies or rat isotype control antibodies two hours pre-sensitisation. All groups were challenged one week later in the opposite foot. Seven days post-challenge the mice were killed and the popliteal lymph node cells from mice within groups pooled and cultured for 72 hours *in vitro* with various stimuli as indicated. Culture supernatants were measured for IFN-γ production by capture sandwich ELISA.

Group	Sensitisation	Challenge	Antibody treatment
1 □	100μg OVA	100μg HAO	200μg isotype control
2 ▒	100μg OVA + 250μg ABF	100μg HAO	200μg isotype control
3 ■	100μg OVA + 250μg ABF	100μg HAO	200μg anti-IL-10
4 ■	unimmunised	100μg HAO	-


B10.BR
IFN-γ concentration (U/ml)
100
75
50
25
0
Medium
Con A
OVA
rABA-1
ABF

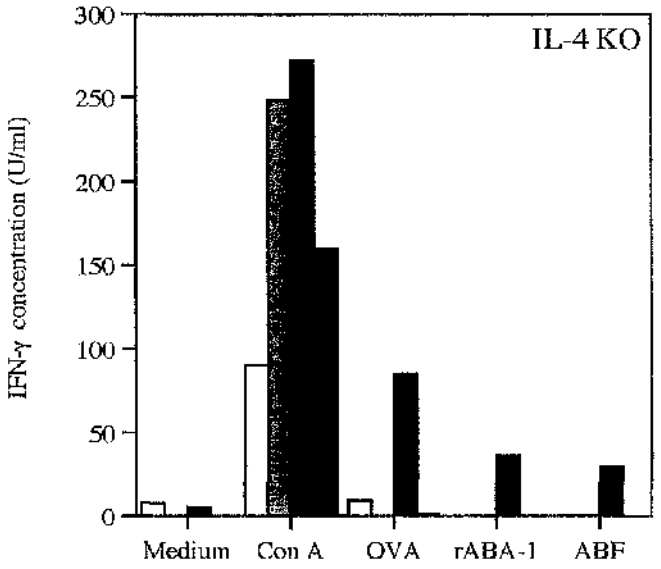

IL-4 KO
IFN-γ concentration (U/ml)
300
250
200
150
100
50
0
Medium
Con A
OVA
rABA-1
ABF

Figure 5.23

The influence of exposure to ABF during OVA sensitisation on proliferative responses in IL-4 deficient and anti-IL-10 monoclonal antibody treated mice. B10.BR controls and IL-4 deficient mice were sensitised with antigens as detailed below emulsified in FCA in the left hind footpad. Controls were unimmunised. In addition, animals were immunised with neutralising rat-anti-mouse IL-10 monoclonal antibodies or rat isotype control antibodies two hours prior to sensitisation. All groups were challenged one week later in the opposite foot. Seven days post-challenge the mice were killed and the popliteal lymph node cells from mice within groups pooled and cultured for 72 hours *in vitro* with various stimuli as indicated. Cultures were pulsed with 0.5μCi of ^{3}H thymidine and incubated for a further 24 hours before harvesting. Each data point represents the mean uptake of thymidine ± SEM for an experiment performed in triplicate.

Group	Strain	Sensitisation	Challenge	Antibody treatment
1	B10.BR	100μg OVA	100μg HAO	200μg isotype control
2	IL-4 KO	100μg OVA	100μg HAO	200μg isotype control
3	B10.BR	100μg OVA + 250μg ABF	100μg HAO	200μg isotype control
4	IL-4 KO	100μg OVA + 250μg ABF	100μg HAO	200μg isotype control
5	B10.BR	100μg OVA + 250μg ABF	100μg HAO	200μg anti-IL-10
6	IL-4 KO	100μg OVA + 250μg ABF	100μg HAO	200μg anti-IL-10
7	B10.BR	unimmunised	100μg HAO	-
8	IL-4 KO	unimmunised	100μg HAO	-

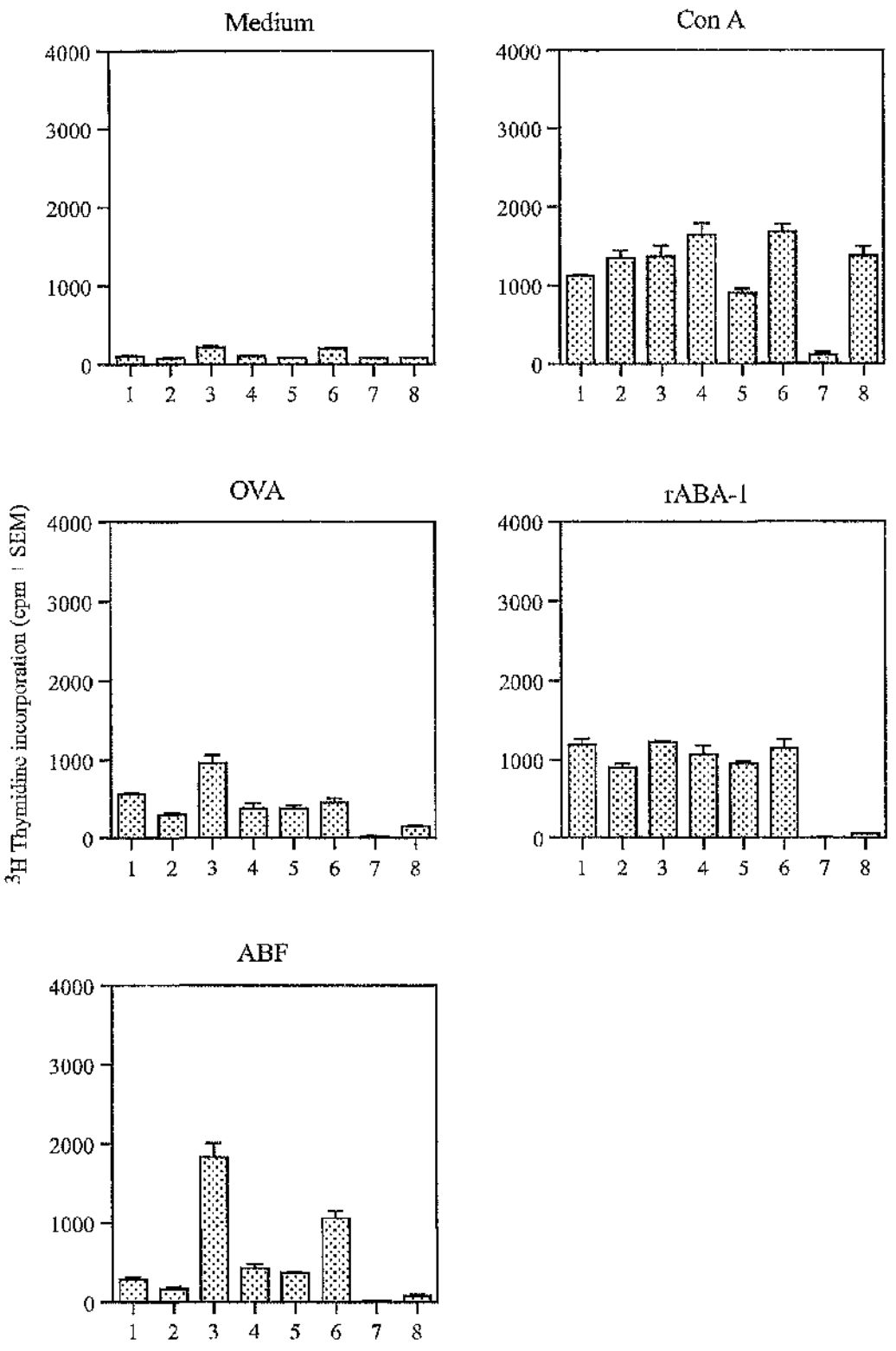
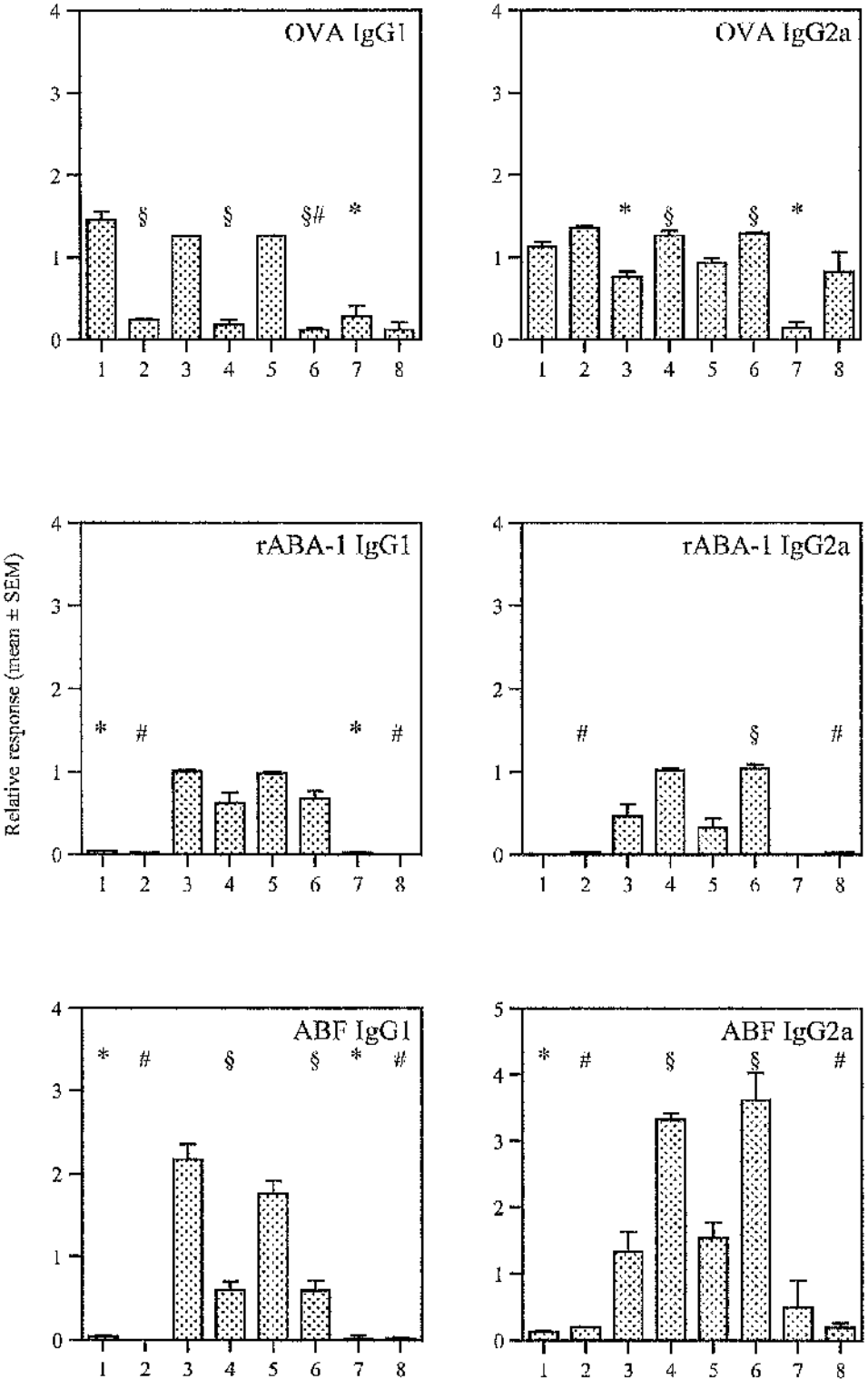

Medium
Con A
OVA
rABA-1
ABF
^{3}H Thymidine incorporation (cpm ± SEM)
4000
3000
2000
1000
0
1 2 3 4 5 6 7 8

Figure 5.24

The influence of exposure to ABF during OVA sensitisation on antigen-specific antibody production in IL-4 deficient and anti-IL-10 monoclonal antibody treated mice. B10.BR controls and IL-4 deficient mice were sensitised with antigens as detailed below emulsified in FCA in the left hind footpad. Controls were unimmunised. In addition, animals were immunised with neutralising rat-anti-mouse IL-10 monoclonal antibodies or rat isotype control antibodies two hours pre-sensitisation. All groups were challenged one week later in the opposite foot. Seven days post-challenge the mice were killed and the sera collected. Antigen-specific antibodies were measured by ELISA. Data are expressed as the amount of antigen-specific antibody in a sample relative to the positive control in arbitrary units. Each data point represents the mean ± SEM for an experiment performed in triplicate.

* and # represents a p-value ≤ 0.005 when compared to groups 1 and 2 (OVA-specific) and groups 3 and 4 (ABF- and rABA-1-specific) respectively using a Student's *t*-test. § represents a p-value ≤ 0.005 when control mice are compared to IL-4 deficient mice which have undergone identical treatment.

Group	Strain	Sensitisation	Challenge	Antibody treatment
1	B10.BR	100µg OVA	100µg HAO	200µg isotype control
2	IL-4 KO	100µg OVA	100µg HAO	200µg isotype control
3	B10.BR	100µg OVA + 250µg ABF	100µg HAO	200µg isotype control
4	IL-4 KO	100µg OVA + 250µg ABF	100µg HAO	200µg isotype control
5	B10.BR	100µg OVA + 250µg ABF	100µg HAO	200µg anti-IL-10
6	IL-4 KO	100µg OVA + 250µg ABF	100µg HAO	200µg anti-IL-10
7	B10.BR	unimmunised	100µg HAO	-
8	IL-4 KO	unimmunised	100µg HAO	-


OVA IgG1
OVA IgG2a
rABA-1 IgG1
rABA-1 IgG2a
ABF IgG1
ABF IgG2a
Relative response (mean ± SEM)

CHAPTER 6

DISCUSSION AND CONCLUSIONS

Discussion and conclusions

The aims of the experiments presented in this thesis were (1), to elucidate the nature of the immune response generated as a consequence of *A. suum* infection, (2), to investigate the effects of immunisation with *Ascaris* products on the development of such responses and (3) to establish whether *Ascaris* products had immunomodulatory properties in the context of a heterologous immune response.

The data presented in chapter 3 described the effects of *Ascaris* infection in the murine host. The natural host for *A. suum* is the pig and the adult form of the parasite is reported on average to be 200 – 300mm in length (Crompton 1989). Therefore, due to anatomical constraints it is not possible for infections of mice to reach full patency, although the larvae can survive to the L3 to L4 moult in the lungs. Thus, infections of mice with *Ascaris* can only provide data on the immune response generated as a consequence of infection and not the development of protective immunity. Nevertheless the immune response of mice to *Ascaris* can provide information on the antigens relevant to human infections (Kennedy *et al.* 1986).

Infection with *Ascaris* was found to promote the generation of a Th2-type immune response, characterised by an increase in production of the Th2-associated cytokine IL-4 and a decrease in the Th1-associated IFN-γ and these differences in cytokine production became more pronounced as the number of infections increased. This was similar to findings that had been reported for other helminth species (Finkelman *et al.* 1991). It was noted that the initial increase in IL-4 was not as striking as the approximately 50% reduction in IFN-γ, thus highlighting the dominance that IL-4 has in inducing Th2 responses over the Th1-polarising cytokine and that once the threshold for IL-4 has been reached there is a progressive increase in its production (Abbas *et al.* 1996; Hsieh *et al.* 1993). The production of antigen-specific immunoglobulins reflected the differences that had been observed with cytokine secretion where levels of Th1-associated IgG2a

significantly decreased after only one infection while a significant increase in Th2-associated IgG1 developed after two or more. Both total and antigen-specific (data not shown for the latter) serum IgE production increased in direct proportion to the number of infections, but the contribution that the antigen-specific IgE made to the total amount of IgE produced was not estimated. This is a point which should be addressed in future studies as protective immunity in humans has been shown to be associated with an increase in antigen-specific IgE and a decrease in polyclonal IgE production (Kennedy *et al.* 1990; McSharry *et al.* 1999).

The selective expansion of $CD4^+$ T cell subsets and the development of a protective or susceptible response phenotype has been described in a variety of infectious diseases such as leismaniasis, leprosy and trichuriasis (Abbas *et al.* 1996; Else and Grencis 1991b; Locksley and Scott. 1991). The influence of host genetics has also been demonstrated in the antigen recognition profiles generated following immunisation with *Ascaris*-derived products (Kennedy *et al.* 1990; Tomlinson *et al.* 1989). Therefore, the effects of host genetics on the immune response generated following *Ascaris* infection was investigated. It was found that mice of the B10.S ($H\text{-}2^s$) strain produced large amounts of IL-4 as a result of infection while the levels in B10.S(9R) ($H\text{-}2^{t4}$) and C57BL/10 ($H\text{-}2^b$) secreted little or none respectively, while IFN-γ was undetectable in both B10.S and C57BL/10 and greatly reduced (94%) in the B10.S(9R). Analysis of antigen-specific antibody production revealed that all the strains had significantly more IgG1 following infection and that IgG2a was low in all groups apart from the uninfected B10.S(9R) which had secreted the greatest amount of IFN-γ. This variability in immune responsiveness was surprising in light of the fact that all the animals were on a B10 background. However, a possible explanation for this phenomenon may lie in the MHC loci of the mice. Antibody responses against the products of *Ascaris* are known to be restricted by the I-A element of the MHC particularly $I\text{-}A^s$, which all the strains used expressed (Kennedy *et al.* 1991; Kennedy *et al.* 1990). It is also known that expression of the $I\text{-}E^k$ molecule, which occurs in B10.S but not in

B10.S(9R) and C57BL/10, is associated with susceptibility to infection (Wassom *et al.* 1987). Therefore, if expression of I-E^k were to make B10.S(9R) and C57BL/10 mice more susceptible to infection one would expect these mice to have diminished IL-4 secretion and enhanced IFN-γ, as was observed. Alternatively, these differences could arise due to polymorphisms in the structural genes and promoters of MHC-encoded cytokines such as tumour necrosis factors α and β and IL-1. It should however be noted that this is not the only possible explanation for the influence of host genetics on immune responsiveness. A study using a TcR transgenic system analysed the cellular components in which genetic susceptibility to disease may arise. This work revealed that APCs play little or no part in determining Th1/Th2 balance and that this ability lies within the T cells themselves (Hsieh *et al.* 1995).

The features of helminth infection that bias the immune response to a Th2 phenotype are unknown, but the associated production of IgE and hypersensitivity responses has focussed attention on parasite products (Ogilvie and Savigny 1982). Challenge of *Ascaris*-primed mice with ABF emulsified in FCA (Chapter 3) abolished the production of IL-4 in favour of IFN-γ and depressed total IgE levels. In contrast, this enhanced antigen-specific immunoglobulin production further. It was of considerable interest to examine whether these effects could also be observed by using the major allergen of *Ascaris*, ABA-1, which is thought to be equivalent to the Allergen A molecule first described in the early 1970s (Ambler *et al.* 1973a; Ambler *et al.* 1973b; Christie *et al.* 1990; Kennedy *et al.* 1986). Although exposure to ABA-1 did have some influence on the immune response generated by infection, the effects were not as marked as those observed with ABF. Following on from this, the ability of rABA-1 to directly stimulate Th2 responses *in vivo* was examined by immunising naive animals with the allergen mixed with various adjuvants. The data obtained from this experiment revealed that rABA-1 was not a significant Th2-promoting parasite product on its own. It is not known whether the ability to stimulate Th2-type

responses directly is a characteristic of ABF and this is an issue that should be addressed in future studies.

An extension of the work investigating whether the products of *Ascaris* can promote Th2 responses would be to assess their contribution to the generation of allergic responses in the host respiratory tract during infection. An immediate hypersensitivity response, similar to asthma, (Loeffler's syndrome) occurs frequently in individuals intermittently infected with *Ascaris* and is associated with the migration of larval stages of the parasite through the lung tissues (Ogilvie and Savigny 1982). It is not known whether a physical property of infection, e.g., lung blockade by the larvae, is the principal cause of the allergic response, or if there is an additional requirement for parasite products. The development of pathological immune responses in the lungs of mice can be studied, for example, by injecting antigen-coated sephadex beads via the tail vein, from where there is a rapid embolisation to the microvasculature of the lungs. This approach has been used successfully in other models of lung disease (Chensue *et al.* 1989; Chensue *et al.* 1995).

As immunisation with the products of *Ascaris* did not appear to induce Th2-type responses it was decided to assess the effects of the products on the development of a heterologous immune response. Helminth infections have been shown to break T cell tolerance which may trigger autoimmune disease, interfere with the induction of oral tolerance, increase susceptibility to secondary infections and modulate the immune response to non-parasite antigens and other pathogens (Actor *et al.* 1993; Curry *et al.* 1995; Hermanek *et al.* 1994; Rocken *et al.* 1992a; Shi *et al.* 1998). Similar effects have also been reported following exposure to parasite antigens and E/S products (Allen and MacDonald 1998; Deehan *et al.* 1997; Harnett and Harnett 1993; Ottensen *et al.* 1977).

Priming mice with OVA alone or in the presence of the *Ascaris* products revealed that while both rABA-1 and pABA-1 had no influence on the development of the Th1-type DTH response against OVA, exposure to ABF resulted in a total inhibition of the response

and was not influenced by the host MHC haplotype. Analysis of cytokine production in the draining lymph nodes revealed that immunisation with ABF resulted in a marked decrease in OVA-specific IFN-γ and was accompanied by an increase in both mitogen and OVA-stimulated IL-10. Although no IL-4 was detected in the cultures, the cytokine was thought to contribute towards ABF's modulatory activities because mice deficient for the cytokine developed DTH responses. Additionally, the effects of ABF were found to be dependent on dose; immunisation with less than 250μg had no effect, the active factor was resistant to heat and acid treatments, and was only influential if administered during a primary immune response. Perhaps the most interesting discovery was that immunomodulation by ABF did not require the presence of ABA-1. In an attempt to identify the immunomodulatory component(s), ABF was applied to a gel filtration column that separated proteins according to molecular weight. This produced two ABA-1-free fractions of ABF which both suppressed the development of a DTH response, unlike ABA-1 on its own.

A natural continuation of this project would be to further investigate and hopefully identify the component(s) responsible for the immunomodulatory activities of ABF. Possible candidates include two recently described glycolipid molecules that have been reported to have biological activity (Lochnit *et al.* 1998). There are a number of ways in which this could be carried out. Proteinase treatment, modifications of carbohydrate groups by periodate oxidation or deglycosylation of ABF are examples of approaches that could identify the nature of the component involved. A second approach could be to investigate the effects that High Pressure Liquid Chromatography (HPLC) purified peaks of ABF had on addition to APC assays, which is a system where the proliferation of primed T cells cultured with APCs of the same haplotype is assessed upon addition of soluble proteins. Alternatively, 2-D gel analysis proteomics could be used to investigate the role of polypeptide based components. Electrophoresis of ABF in a 2-D gel system would give fine separation of the component molecules that could then be Western blotted onto nitro-cellulose and extracted for use in an APC assay. Once components have been identified

which stimulate the proliferation of primed cells, mass spectrometry can be used to identify the activity, this approach, however, relying upon there being pre-existing appropriate knowledge in data bases. The ability to isolate such a component and study its effects both *in vitro* and *in vivo* could possibly have long-term implications for vaccine design and the treatment of allergic disease.

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