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Immune modulation of B cell function by nematodes

by

Humphrey Nosayaba Ehigiator

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

at

Dalhousie University
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DALHOUSIE UNIVERSITY

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by Humphrey Nosayaba Ehigiator

in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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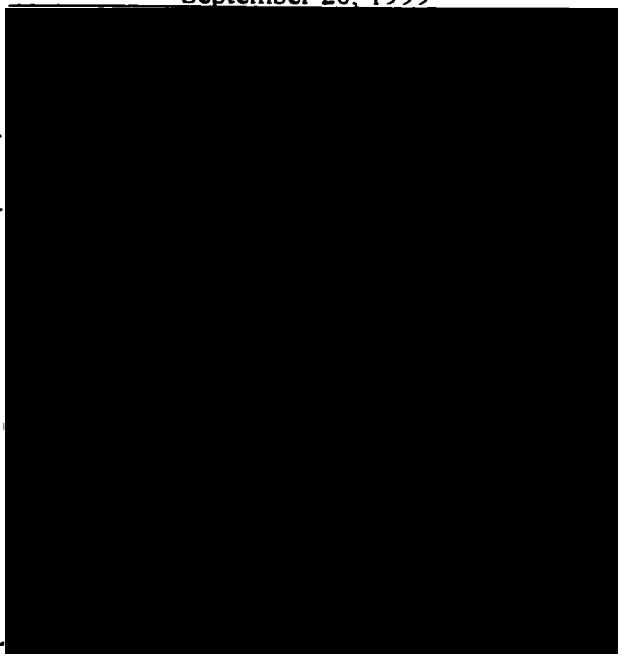
External Examiner

Research Supervisor

Examining Committee



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Author: Humphrey Nosayaba Ehigiator
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Department of Microbiology & Immunology
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Humphrey N. Ehigiator (Author)

dedication

To all those who supported me through this journey

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Abstract

In both natural infections and experimental models, the immune response associated with nematode infections is defined by the development of type 2 immune effector pathways. Of these, the increase in reaginic immunoglobulin has received a lot of attention because of its polyclonal nature and the role of such antibodies in allergic responses. However, the mechanisms involved in the development of this unique polyclonal response are still unclear. The focus of this thesis is to investigate the association of the increase in murine IgG1 and IgE, in response to the intestinal nematode *Nippostrongylus brasiliensis (Nb)*, with the induction of Ig isotype switch.

To allow for the investigation of this hypothesis both *in vivo* and *in vitro*, a reductive approach, involving the use of extracts of *Nb*, rather than infection, was adopted. The extract was first confirmed to induce a marked increase in IgE and IgG1 levels, similar to that induced by the infection. The extract did not affect the level of IgG2a in serum, showing that the effect was specific to IgE and IgG1 (type 2 associated Ig) rather than inducing a non-specific increase in all Ig isotypes. Assessment of the cytokine profile showed that the extract also induced significant increases in IL-4 and IL-13, both cytokines reported to be involved in the regulation of reaginic Ig isotypes. These results confirm that the extract, like infection, is a strong inducer of polyclonal type-2 responses, and a reliable model for investigating the regulation of nematode induced responses.

The extract induced the production of IgG1 when added to *in vitro* cultures of LPS-stimulated B cells. This provides evidence for the induction of class switch. It did not induce upregulation of IgG1 in naive (unstimulated) B cells, nor expand B cells in *in vitro* cultures. Analysis of DNA from the spleens of mice treated with the extract by DC-PCR demonstrated a marked increase in the occurrence of reaginic Ig switch region gene recombination in the cells *in vivo*. These results provide evidence that the marked increase in the Ig response associated with nematode infection is mediated by *de novo* switch DNA recombination, and not by the expansion of Ig memory B cells.

The production of elevated levels of Ig and the inability to induce expansion of B cells suggests that nematodes exert varying modulatory effects on B cell function. This focussed the research on the effects of *Nb* extract on B cell proliferation. It was found that not only did the extract not induce expansion of naive B cells, it significantly inhibited LPS-mediated B cell proliferation in a dose-dependent manner. The lymphocyte inhibitory effect of the extract was specific to B cells, as proliferation of T cells is not affected. The inhibitory activity does not interfere with early activation events but appears to target signaling pathways downstream of PKC. The inhibitory activity appears to be mediated by modulation of macrophage (accessory cell) activation. The elimination of macrophages from B cell preparations after pre-activation appears to render B cells resistant to the anti-proliferative effect of *Nb* extract upon restimulation in fresh media. Partial characterization of the factor(s) in *Nb* extract responsible for the inhibitory activity, showed that the factor is a protein with molecular weight of between 50 and 100 kDa.

List of abbreviations

ACK	Ammonium chloride potassium
ADCC	Antibody-dependent cell-mediated cytotoxicity
ANOVA	Analysis of variance
APC	Antigen presenting cell
AWH	Adult worm homogenate
BCA	Bicinchoninic acid (protein assay kit)
BCR	B cell receptor
bp	Base pairs
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CD40L	CD40 ligand
CFA	Complete Freund's adjuvant
C_H	Immunoglobulin heavy chain constant region
Con A	Concanavalin A
cRPMI	Complete RPMI 1640 medium
DC	Digestion circularization
DC-PCR	Digestion circularization polymerase chain reaction
DNA	Deoxyribonucleic acid
DPM	Disintegration per minute
ECP	Eosinophil cationic protein

EDN	Eosinophil-derived neurotoxin
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EPO	Eosinophil peroxidase
ES	Excretory-secretory
FACS	Fluorescence-activated cell sorter
FcεRII	Fc-epsilon receptor II
FCS	Fetal bovine serum
FIA	Freund's incomplete adjuvant
h	Hour
[^{3}H]-TdR	Tritiated thymidine
HEPES	*N*-2-hydroxyethylpiperazine-*N*-2ethanesulfonic acid
HRP	Horse-radish peroxidase
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IgE	Immunoglobulin E
IgG1	Immunoglobulin G1
IgM	Immunoglobulin M
IL	Interleukin
IL-13R	Interleukin-13 receptor
IL-4R	Interleukin-4 receptor
IL-4Rα	Interleukin-4 receptor alpha subunit

IRF-1	Interferon regulatory factor-1
Jak	Janus kinase
kDa	Kilodalton
JNK	c-Jun N-terminal kinase
KO	Knock out
LPS	Lipopolysaccharide
LWH	Larval worm homogenate
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MBP	Major basic protein
2-ME	2-mercaptoethanol
min	Minute
MLN	Mesenteric lymph node
mRNA	Messenger ribonucleic acid
nAChRe	Nicotinic acetylcholine receptor
Nb	*Nippostrongylus brasiliensis*
NFAT	Nuclear factor of activated T lymphocyte
NS	Not significant
OPD	o-phenylenediamine
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI 3-k	Phosphatidylinositol 3-kinase

PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PTK	Protein tyrosine kinase
PWM	Pokeweed mitogen
RBC	Red blood cells
rIL	Recombinant interleukin
RNA	Ribonucleic acid
RT	Reverse transcription
RT-PCR	Reverse transcription polymerase chain reaction
S	Switch region
SAC	*Staphylococcus aureus* cowan I
STAT	Signal transducers and activators of transcription
TBE	Tris-borate-EDTA
TBS	Tris-buffered saline
TE	Tris-EDTA
TGF-β	Transforming growth factor beta
Th	T helper
TMB	3.3'.5.5'-tetramethylbenzidine
TNF	Tumor necrosis factor
VDJ	Variable. diversity and joining region

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To everyone, thank you!

1.0 Introduction

Nematodes are among the most common parasites worldwide and are responsible for diseases of major importance in humans and domestic animals. Nematode infection occurs most commonly in the tropical regions of the world, where economic, social and environmental factors allow the nematodes to flourish (Warren 1988; Maizels et al 1993; Bundy 1997). Many of the parasitic nematodes inhabit the gastrointestinal tract but in some cases the adults live in tissues as diverse as the major visceral organs, lymphatic tissue and the eye. Most prevalent among the human nematode parasites are the genera, *Ascaris*, *Trichuris*, *Necator*, *Ancylostoma*, *Trichinella*, *Strongyloides*, *Brugia*, and *Onchocerca*. It has been estimated (Warren 1988; Bundy 1997) that over one-quarter of the world's population are infected with one or more of these nematodes. Although they generally provoke low mortality, these infections do contribute to significant morbidity (Chan et al 1994; Chan 1997), which can include diarrhea, anemia, joint and muscle inflammation, protein malnutrition, and iron deficiency among many other pathological events (Warren, 1988).

It is characteristic of many of these nematode infections, like other parasitic infections, that they are persistent. Re-infection occurs readily, often throughout the lifetime of the host. The chronicity of nematode infection is particularly damaging to children, causing growth retardation and impaired cognitive function (Nokes and Bundy 1994). This ability of nematodes to constantly re-infect their

hosts suggests the development of an inappropriate immune response to the nematodes. However, there is abundant evidence indicating that there is the generation of adequate immune recognition and response to parasite antigens by the host. However, the parasites appear to have evolved a unique relationship with the host, which has allowed them to modulate the host's response (Maizels et al 1993). This relationship between the nematode and the host has intrigued researchers for a number of years. In an attempt to understand the nature and regulation of the host-parasite relationship, researchers have developed experimental models to investigate the mechanisms involved in the generation of the immune responses associated with nematode infections. The contribution of these responses to controlling infection has also received significant interest.

The most commonly investigated experimental models include infection of rodents with either intestinal nematodes or filarial nematodes. In the former case, *Nippostrongylus brasiliensis (Nb)*, *Trichuris muris*, *Heligmosomoides polygyrus*, *Trichinella spiralis*, and *Ascaris suum* are the most common (note that infection of rodents with *Ascaris suum* leads to a limited, larval, infection since this is a parasite of swine that does not complete its life cycle in rodents). The filarial nematodes most used are *Brugia pahangi* and *Brugia malayi* infection in mice. The life span of these nematodes in their hosts vary considerably, ranging from two weeks in *N. brasiliensis* infection, to several weeks in *Heligmosomoides* and *Brugia* infections.

In both natural infections, and experimental models, the immune responses associated with nematode infections are defined by the development of type-2 associated responses. These are characterized by marked increases in the serum reaginic antibodies: IgE in all species, and IgG1 in mice (IgG2a in rats, IgG4 in humans). Blood and tissue eosinophilia, and local mast cell hyperplasia (Jarrett et al 1976; Jarrett and Miller 1982; Nawa and Korenaga 1983; Finkelman et al 1988a) are also characteristic of nematode infection as well as goblet cell and other mucosal cell effects in intestinal nematode infection (Nawa and Korenaga 1983; Arizono et al 1994; Nawa et al 1994). These responses are mediated by type-2 cytokines, which are induced by nematodes (Finkelman et al 1997; Urban et al 1998b; Bancroft and Grencis 1998). The significance of these responses to the host and to the nematode, is still a matter of controversy. In a number of reports, it has been demonstrated that inhibition of type-2 responses does not hinder the ability of the host to reject the worms (Crowle and Reed 1981; Madden et al 1991; Lawrence et al 1995, 1996; Urban et al 1998a). This observation suggests that the type-2 responses are not essential for the elimination of the worms from the host. Contrary to this view, other researchers (Else et al 1992; Pearlman et al 1993c; Finkelman et al 1988, 1994, 1997; Grencis 1997; Bancroft et al 1998; Mckenzie et al 1998; Urban et al 1998a,b; Bancroft and Grencis 1998) have shown that, in some other nematode infections, the type-2 responses are an important immune mechanism by which the host mediates the elimination of the worms. These

varying observations show both the uniqueness of different nematode infections and the lack of critical data regarding the parasite / host immunological interface.

However, all nematode infections are associated with the production of a dramatic polyclonal IgE response, most of which is not directed at the parasite. This has made them very important models in the investigation of the development and activities of IgE.

There have been a number of reports investigating the identification and purification of nematode antigens involved in the induction of the polyclonal reaginic immunoglobulin response (Marretta and Casey 1979; Lee and McGibbon 1993; Kamata et al 1995). Although a number of possible antigens have been reported, data from most of the studies suggest that the polyclonal IgE (and IgG1 in mice) response does not likely result from a single antigenic stimulation, but rather, the modulation of the response by a number of nematode factors which are able to mediate the induction of type-2 responses.

The increase in non-specific immunoglobulin levels suggests that nematodes have the ability to modulate B cell function *in vivo*. However, the mechanisms involved in the development of this unique polyclonal IgG1 and IgE response are unclear. In the recent past, there has been increasing interest in the regulation of this response by type-2 cytokines (IL-4 and IL-13), and in the related signal transduction pathways involved in this kind of response.

In this thesis project I chose to investigate how nematodes induce the

dramatic polyclonal IgG1 and IgE response, using the model of *Nb* infection in mice. This model is widely used in the literature as a strong inducer of type-2 responses and it exhibits all of the classical type-2 associated phenomenon including the dramatic IgE and IgG1 increase which is polyclonal in nature and mostly not directed to worm antigens (Jarrett et al 1976; Finkelman et al 1988a).

Since the production of both IgE and IgG1 involves the process of Ig heavy chain gene rearrangement during Ig class switching, and switch to both of these isotypes is regulated by IL-4, a hypothesis was developed that the production of the polyclonal IgE and IgG1 responses is mediated by the induction of *de novo* class switch by B cells, rather than an expansion of IgE and IgG1 memory B cells. To allow for the investigation of this hypothesis in both *in vivo* and *in vitro* systems, a reductive approach, involving the use of extracts of *Nb* rather than infection was adopted. Therefore, the first part of this thesis involves the establishment that the extract also induces polyclonal activation and isotype switch similar to the infection, as well as experimentation designed to address the hypothesis.

Besides the induction of immunoglobulin production in serum, nematodes have also been shown to modulate the host immune activities in other ways. This is a possible adaptation by the parasite to evade the host immune responses, thereby allowing the opportunity for continuation of the parasite life cycle. This modulatory activity has not been well characterized. Thus, the second section of

this thesis characterizes the effects of *Nb* infection on B cell proliferative responses and addresses the specific mechanism(s) by which *Nb* mediates these effects.

This introduction provides an overview of the significance of nematode infections, general information on why the immune responses to nematode infection has attracted the attention of biomedical scientists in the past, and the objectives of the study, presented in this thesis. The subsequent sections of this introductory chapter will provide background information on the biology of *Nb* and recent advances in the immunology of *Nb*, in the context of the immune responses associated with the nematode infection. In addition, the areas of B cell activation / proliferation and immunoglobulin class switch, regulation of IgE and IgG1 responses in mice, macrophage activation in the context of its interaction with B cells, and the effect of nematodes on the activation process, will be reviewed. The background information reviewed here is intended to provide the information that will be necessary for an understanding of the discussion that is to follow in the other chapters of this thesis.

2.0 Background.

2.1 Biology of *Nippostrongylus brasiliensis*

The biology of *Nb* has been extensively studied (reviewed by Haley, 1962). However, for the purpose of this thesis, the discussion will be restricted to the areas that are pertinent to fully appreciate the complexity of the nematode.

Nippostrongylus brasiliensis, is a small, slender, reddish worm, naturally a parasite of the small intestine of wild rats. For experimental purposes, the worm has been adapted to also infect mice (Wescott and Todd, 1966; Solomon and Haley 1966), and hamsters (Haley 1966). *Nb* belongs to the Phylum Nematoda, Order Strongylida, as do the human hookworms *N. americanus* and *A. duodenale*.

As with most nematodes, the structure of *Nb* is simple and characteristic of the order strongylida. It possesses a mouth at the extreme anterior end and an anus near the posterior tip, thus having a complete digestive tract. Distal to the mouth is the buccal cavity and the esophagous, which serve as both a pumping and glandular organ. This allows *Nb* to suck food into its intestine, a simple, tube-like structure constructed of a single layer of intestinal cells. Besides its digestive system, *Nb* also has efficient muscular, nervous, circulatory and reproductive systems, all of which are bathed in fluid of the body cavity. The structure of the worm is maintained by the body wall comprised of a thick and protective cuticle as well as underlying tissue, which together function as a hydrostatic skeleton of the worm. The cuticle of *Nb*, as with other nematodes, provides both structural

integrity to the parasite and protection from damage. The cuticle is structurally complex and very plastic. Haemoglobin in the cuticle is responsible for the red colour of the adult *Nb*, and it allows *Nb* to take up oxygen, which is diffusing from the host's mucosa into the gut lumen quickly, before the oxygen is removed by the host gut flora. This allows the worm to exploit areas in the intestine that are quite hypoxic (Lee 1965). The cuticle has been described to contain a number of antigenic proteins (Maizels et al 1982, 1983; Dorzok et al 1989). Because the extract preparation used in the experiments reported in this thesis is a whole worm homogenate, it contains proteins from the cuticle and other portions of the worm.

Thus, the structure of the nematode is quite simple: a robust, protective, yet flexible outer covering which provides an exo-skeletal-like structural integrity to the worm; underlying layers of nervous and muscular tissues; a pseudocoelomic cavity containing serum-like fluid; a central intestinal tract consisting only of a single cell thick mucosa-like tube; and reproductive organs. This information is provided to give a sense of the targets for the immune response. Obviously the cuticle, although the most visible target, is unlikely to be easily damaged, with the exception of the developmental process when the worms undergo a number of moults of the cuticle. On the other hand, as discussed in later sections, the single layer thick intestine, in an animal using hydrostatic force for locomotion and structural integrity, is a vulnerable, if more difficult, target.

The life-cycle and development of *Nb* was first described by Yokogawa

(Yokogawa 1922). A detailed review of the life-history was carried out subsequently (Haley 1962). Briefly, following mating by adult male and female *Nb* in the intestine of the host, eggs are laid by the female, and these are passed out with the faeces. In the external environment, the eggs hatch into first-stage larvae within 24 h. This process is believed to occur optimally at 22 to 30°C. The larvae undergo further development to the second-stage larvae, and subsequently to the third-stage larvae, within 3-4 days. This third-stage larvae is the infective (free-living) larval stage. The third-stage larvae have been described to contain proteins to which sera from infected hosts have had specific reactions, suggesting the involvement of the third-stage larvae in the generation of the specific immune responses associated with *Nb* infection. At this stage, the worm can survive extremes of conditions. The third-stage larvae are able to withstand temperatures ranging from 0 to 45°C and a lack of moisture for several days. They can also go for long periods without feeding, using stores accumulated during the active feeding phase of the second stage larvae. These features have ensured survival and adequate transmission of the worms in the wild.

In the wild, and in laboratory cultures, the larvae are negatively geotactic. In the wild they actively migrate up blades of grass, attaching with the posterior end and waving their anterior ends freely back and forth, pending interaction with host skin. The infective larvae enter the host by penetration of the host skin in natural infections, and by subcutaneous injection of experimental animal models.

Following penetration by either route, the larvae migrate actively via the bloodstream to the lungs within 24-36 hours. In the lungs, the larvae feed, grow and undergo the third molt into the fourth stage larvae. At this point they leave the blood by rupturing the capillary bed and enter the alveolar spaces in the lung. During this process, they have been shown to induce inflammatory responses characterized by pulmonary eosinophilic granulomas, mast cell hyperplasia, and cytokine production by alveolar macrophages (Egwang et al 1984, 1985; Ramaswamy et al 1991; Benbernou et al 1992). These inflammatory responses are believed to influence the developing immune response associated with *Nb* infection. The fourth stage larvae then migrate from the alveoli to the bronchi and up the trachea. They are then coughed up into the buccal cavity and swallowed. They are resistant to stomach acid and reach the small intestine, where they grow and then molt to the fifth stage (adult). Most of the worms reach the small intestine by 72 h after infection. The adults live in the small intestine by burrowing among the villi and crypts of the mucosa. They mature rapidly and begin to pass eggs 5-6 days after infection. Egg production continues for about 6 days and then worms are expelled from the gut of the host over a period of 2-3 days. The entire cycle, from penetration of the host by the infective larvae to expulsion of the adults, takes about 14 days (Ogilvie and Hockley 1968). Subsequent infection of an immunocompetent host is usually shorter, the worms being expelled within six days. This is due to the strong resistance that is developed by the host after the

primary infection (Keller 1970; Ogilvie and Jones 1971).

Both the lung phase and intestinal phase of infection are associated with some pathology. In the lung they include: petechial heamorrhage, bacterial infection and inflammation. In the gut they include: diarrhea, mucous secretion, villous atrophy, crypt hyperplasia and goblet cell hyperplasia (Haley 1962; Ramaswamy et al 1991).

2.2 Immunology of *Nippostrongylus brasiliensis*

The review of the immunology of *Nb* will focus on the polyclonal immune responses associated with *Nb* infection in rodents. Because of the similarities in the immune responses induced by *Nb* infection, and that of other intestinal nematodes, reference will also be made to them in order to present a concise review of the substantial volume of information available on this subject.

There has been increasing interest in understanding the mechanisms involved in the generation of the characteristic Th2 responses associated with nematode infections. The immune responses associated with *Nb* infection have served as a good model for these studies, since *Nb* is a strong inducer of these responses. Also, *Nb* serves as a good model system because it is easy to maintain in the lab and it has a short life-cycle of infection in the host. In this section, the cellular, and antibody responses during *Nb* infection will be discussed, as well as

the role of these responses in controlling the infection. The ability of *Nb* and other nematodes to modulate the host immune responses to other agents will also be discussed.

2.2.1 Cellular Responses

Infection of mice and rats with *Nb* provokes a range of cellular responses in the host. Early adoptive transfer and cell depletion experiments have demonstrated that the activation and differentiation of T cells, in particular, $CD4^+$ T cells, is central in the immune response induced in rodents infected with *Nb* (Katona et al 1988) and other nematodes (Lee et al. 1983; Urban et al 1991a; Koyama et al 1995). Based on the division of T helper cells into two groups (Th1 and Th2) depending on their cytokine profile (Mossmann and colleagues), the T cell response induced in mice and rats infected with *Nb*, is consistent with the Th2 pattern of response (Mosmann et al 1986; Mosmann 1991; Abbas et al 1996; Finkelman et al 1997). This response is characterized by increased production of the cytokines interleukin (IL)-4, IL-5, IL-6, IL-9, IL-10, and IL-13. This increased production is associated with a significant increase in the number of cells secreting these cytokines (Seder et al 1991; Sher and Coffman 1992; Urban et al 1992; Matsuda et al 1995), suggesting the induction of a shift in the differentiation of Th precursor cells to Th2 cells. Similar patterns of response have been reported in

rodents and humans infected with other nematodes, such as *Ascaris*, *Trichuris*, and *Trichinella* (Urban et al 1992; Mahanty et al 1993; Pearlman et al 1993a). These cytokines play a significant role in mediating the effector cell activities and antibody production associated with nematode infection.

The activation and preferential differentiation of precursor T helper cells into Th2 cells during *Nb* infection is thought to be primarily mediated by IL-4. IL-4 has been well demonstrated to be a key cytokine in Th2 cell development (Abehsira-amar et al 1992; Chatelain et al 1992; Kopf et al 1993; Benbernou et al 1993). In *Nb* infected mice, a dramatic increase in IL-4 secreting T cells has been observed in the spleen and in the peripheral lymph nodes (Street et al 1990; Seder et al 1991). Numerous studies have shown that in the absence of IL-4, (by anti-IL-4 antibody treatment, use of IL-4 KO mice, or IL-12 administration), there is a marked reduction in Th2 responses in mice infected with *Nb* (Madden et al 1991; Urban et al 1991b; Kopf et al 1993; Benbernou et al 1993; Finkelman et al 1994; Pearlman et al 1995). In contrast, IL-12 mediated Th1 responses appear to be inhibited in most nematode infections, including *Nb* (Manetti et al 1993; Svetic et al 1993; Uchikawa et al 1994; Matsuda et al 1995).

Besides IL-4, IL-13 has also been shown to contribute to the development of Th2 responses associated with nematode infections (Punnonen et al 1993; Defrance et al 1994; Bancroft et al 1998, McKenzie et al 1993, 1998b; Urban et al 1998a,b). In one of these studies, Urban and colleagues (Urban et al 1998a),

showed that the inhibition of IL-13 activity in mice deficient in the alpha chain of the IL-4R (a receptor subunit shared by both IL-4 and IL-13; Aversa et al 1993; Zurawski et al 1995) and in STAT6 (a transcription factor activated by both cytokines as part of the signaling cascade; Lin et al 1995; Takeda et al 1996; Kaplan et al 1996; Nelms et al 1999), resulted in the inability of mice to reject *Nb*. Bancroft and colleagues (Bancroft et al 1998) and Mckenzie and co-workers (Mckenzie et al 1998a) reported a similar finding in IL-13 KO mice infected with *T. muris*. Mckenzie and co-workers (Mckenzie et al 1998b) demonstrated the importance of IL-13 in Th2 cells development in a study with mice deficient in the IL-13 gene. They found a significant reduction in the levels of Th2 cytokines from T cells isolated from IL-13-deficient mice. Similarly, they also found that the level of IgE in the serum of IL-13-deficient mice was lower than control mice in response to *Nb* infection. Another cytokine that has been demonstrated to be involved in the development of Th2 cells is IL-6 (Rincón et al 1997). During *Nb* infection, IL-6 has been shown to play a role in the enhancement of T cell proliferative responses in *Nb* infected mice and a link has been made with these proliferative responses and Th2 differentiation (R. Liwski; J. Immunol. in press).

It has been suggested that a potential contributing factor to the preferential induction of Th2 response in nematode infection is the activation of B7-2 on antigen presenting cells (APC). This was demonstrated by Subramanian and co-workers (Subramanian et al 1997) who showed that helminth infected, anti-B7-2

antibody treated, mice exhibit impaired Th2 responses. Similarly, Tsuyuki and colleagues showed that blocking B7-2, and not B7-1 interaction with CD28 inhibited eosinophil infiltration, IgE production, and Th2 cytokine secretion in mice exposed to allergens (Tsuyuki et al 1997). However, in a study with B7-2-deficient mice infected with *H. polygyrus*, it was observed that B7-2 was not required at the initiation of the type-2 response, but is required for the progression of the response. This observation is supported by an earlier study, where it was shown that interaction of either B7-1 or B7-2 with CD28 was adequate to induce type-2 responses in mice infected with *H. polygyrus*. These findings suggest that preferential interaction between CD28 and B7-2 does not appear to be the only B7 costimulatory interaction that contributes to Th2 differentiation in nematode infections. Nonetheless, data from a number of studies show that the interaction between B7 molecules on APC and their CD28/CTLA4 counter-receptors on T cells, is essential for the development of Th2 responses in nematode infections (Lu et al 1994; Greenwald et al 1997; Harris et al 1999; Greenwald et al 1999). Thus, while IL-4 mediates the development of Th2 responses, other factors are involved in the cascade of events, which results in the responses associated with nematode infection.

The production of Th2 cytokines occurs during the early, as well as the late, phase of the infection. This fact has been demonstrated by a number of researchers in different infection models. For example, the presence of mRNA for some of

these cytokines in cells isolated from the mesenteric lymph node (MLN) has been reported within 48-72 hours of the worms reaching the intestine (Svetic et al 1993; Matsuda et al 1995; Lawrence et al 1996; Ishikawa et al 1998). The MLN are the nodes that drains the small intestine where the worms inhabit, suggesting that the Th2 responses associated with *Nb* infection is initiated at this site (Mayrhofer 1984; Auci et al 1992). From this site, they have been shown to migrate to other secondary lymphoid organs, like the spleen. In most nematode infections, the Th2 response in the spleen is usually detected 2-3 days after the development of the response in MLN (Lawrence et al 1996). In addition to the migration, there is evidence that activation and differentiation of the T helper cells also take place in the spleen (Seder et al 1991; Auci et al 1992).

The involvement of Th2 cytokines in the regulation of resistance to nematodes has been extensively investigated in a number of models. From various studies, a central role of IL-4 has been identified. This is clearly demonstrated in *T. muris* and *H. polygyrus* infections. For example, inhibition of IL-4 activities by either IL-4 gene knock-out or receptor blockade with antibody treatment, have been demonstrated to prevent expulsion of the worms from the host (reviewed in Finkelman et al 1997 and Bancroft and Grencis 1998). In like manner, the significance of IL-4 in the development of protective immune responses is further demonstrated by the ability of IL-4 to reverse chronic infections, and expulsion of the worms (Else et al 1994; Urban et al 1995). However, in *Nb* infection the role of

IL-4 in host protection is not as clear cut as in *T. muris* and *H. polygyrus* infections. A number of studies have shown that anti-IL-4, anti-IL-4R mAb treatments and lack of IL-4 functional gene (Lawrence et al 1996; Urban et al 1998b) do not effect the expulsion of *Nb*. These data, however, point to the involvement of another Th2 cytokine in the development of a protective response in *Nb* infection. In a recent study it was reported that IL-13, which has effects similar to IL-4, appears to play a prominent role in *Nb* expulsion (Urban et al 1998a; Mckenzie et al 1998a). A similar role for IL-13 has also been observed in *T. muris* infected mice (Bancroft et al 1998; Mckenzie et al 1998b). Besides these cytokines, other Th2 cytokines have been reported to contribute to a protective response in some infections but not in others (Finkelman et al 1997).

Infection with *Nb* also induces local mast cell hyperplasia, blood and tissue eosinophilia, (Arizono et al 1994; Kasugai et al 1995) and accumulation of other mucosal cells such as goblet cells at the site of infection as well as throughout the gut (Miller and Nawa 1979; Ishikawa et al 1993, 1994; Nawa et al 1994). These responses are mediated by the Th2 cytokines, IL-3, IL-4, IL-5 and IL-9 (Arizono et al 1994; Nawa et al 1994). A number of studies have demonstrated the dependence of these effector cell responses on the activities of these specific cytokines (Madden et al 1991). It has been suggested that much of the immuno-pathology associated with *Nb* infection, including hypersensitivity responses and inflammation in both the lung and the gastrointestinal tract is due to the activities

of both mast cells and eosinophils.

In *Nb* infection, the massive increase in the population of mast cells has been reported in the lungs/airway passage, in the intestine and, to a more limited extent, in the peritoneal cavity (Ramaswamy et al 1991). Similar findings have been reported in mice infected with *A. suum* (Miller and Jarrett 1971; Ramaswamy et al 1991). Due to this significant mast cell accumulation *Nb* infection has been used in many studies to generate the large populations of mast cells required to investigate their role in allergic and other immune responses. These mast cells bear high affinity IgE receptors, to which IgE molecules bind. When these molecules are cross-linked by nematode antigens, the cells become activated and release a number of preformed mediators, such as histamine, and newly formed mediators such as prostaglandins and leukotrienes. Besides these mediators, mast cells have been demonstrated, *in vitro*, to produce IL-4, and IL-13 (Gordon et al 1990; Burd et al 1995) which may contribute significantly to local, and potentially systemic immune responses. However, the levels of IL-4 and IL-13 in these studies have been quite low and it is questionable whether they have significance *in vivo*.

The role of mast cells in host defense has been the subject of detailed research in the last few years. Mast cells have been associated with tumor cell killing capacity, prevention of parasite re-infestation, as well as control of bacterial infection in mice (Nawa et al 1994; Church and Levi-Schaffer 1997). The support for their role in the immune responses to nematodes includes a correlation between

the hyperplasia of intestinal mucosal mast cells with the time of expulsion of various nematode infections (Miller and Jarrett 1971; Lee and Wakelin 1982) although these experiments were unable to confirm a direct cause and effect in this relationship. Indeed, Lee and Wakelin (1982) have suggested that mast cells have a limited role in nematode expulsion. This is based on their observation that about 20% of DBA-2 mice do not reject the parasite *T. muris*, however all DBA-2 mice have similar levels of mast cell hyperplasia. Thus, there is no correlation between mast cell hyperplasia and worm rejection in this model. Further to this, a number of studies have shown that W/Wv mice, which lack mast cells, are capable of rejecting nematodes, albeit at a slower rate than normal littermate controls (Mitchell et al 1983).

The increase in the population of blood and tissue eosinophils, associated with *Nb* infection, is mediated by IL-5 (Sanderson 1992; Sher and Coffman 1992). IL-5 is secreted primarily by Th2 cells. Other than its role in the induction of eosinophil growth and differentiation in mice, IL-5 also (i) augments B cell proliferation and differentiation (Takatsu 1997); and (ii) synergizes with IL-4 to enhance IgG1, IgE and with TGF-β to enhance IgA secretion in mice (Purkerson and Isakson 1992; reviewed in Takatsu 1997). In the blood, eosinophil levels have been reported to increase from about 2% to more than 50% of circulating white blood cells (Maizels et al. 1993) during nematode infection. Indeed circulating eosinophilia is a diagnostic indicator of nematode infection in persons returning

from high-risk areas of the world. As with mast cells, eosinophils have also been reported to play a significant role in the regulation of allergic responses. The work of Gleich and colleagues (Gleich et al 1979; Sanderson 1992; Kay et al 1994) has clearly shown that the late stage response of allergic asthma, which is responsible for most of the pathology associated with asthmatic events, is dependent on eosinophil accumulation and degranulation. The involvement of eosinophils in allergic responses is primarily a consequence of the potent granule proteins major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN), as well as partly a consequence of the release of arachidonic acid metabolites. A role for eosinophils in the immune response to nematodes has been suggested by the observation that mice treated with anti- eosinophil serum *in vivo* were more susceptible to nematode infection (Gleich et al 1979) and that peritoneal eosinophils kill newborn larvae of *T. spiralis* when cultured in the presence of anti-parasite antibody (Lee, 1991). Thus a theory has developed that eosinophils are engaged in anti-nematode activity by ADCC. There have been some suggestions (Capron et al 1984) that this ADCC is mediated by the low affinity FcεR on eosinophils binding to parasite bound IgE.

Contrary to reports suggesting a role for eosinophils in anti-parasite immunity, there have been a number of studies, which suggest that mast cells and eosinophils are not major players in worm rejection. For example, in a study by Madden and co-workers (Madden et al 1991), it was demonstrated that combined

anti-IL-3 and anti-IL-4 treatment failed to block the expulsion of *Nb* from infected mice, even though it inhibited intestinal mastocytosis. Moreover, the prevention of eosinophilia in mice infected with either *T. spiralis* or *H. polygyrus*, did not have any detectable effect on worm burdens (Herdon and Kayes 1992; Urban et al 1991b). In addition, although peritoneal eosinophils are capable of killing *T. spiralis* newborn larvae by ADCC, it was discovered that intestinal lamina propria eosinophils did not express this killing activity (Lee 1991), even though they would be the eosinophils in contact with the larvae as they migrate into the circulation. These conflicting reports suggest that the role of these cells in worm expulsion is not clearly understood. There is increasing evidence that expulsion of nematodes is a complex immune response, part of which involves a variety of inflammatory cells in the intestinal mucosa of infected animals, including mast cells, eosinophils and goblet cells. However, there is also clear evidence that this complex immune response is T helper ($CD4^+$) cell-dependent (Finkelman et al 1997).

2.2.2 The Antibody Response

The immunoglobulin response associated with *Nb* infection in mice is characterized by elevated serum levels of IgE and IgG1 (Lebrun and Spiegelberg 1987; Zakroff et al 1989). In all species IgE is consistently upregulated by

nematode infection but the other reaginic antibody differs. In rats it is IgG2a (Yamada et al 1993a: Benbernou et al 1993), not IgG1, and in humans it is IgG4 (Garraud et al 1995). The generation of this characteristic Th2 response is believed to involve polyclonal activation of B cells and subsequent differentiation to the antibody isotypes, under the regulation of IL-4 and IL-13. The central role of IL-4 in this process has been widely reported by numerous investigators (Finkelman et al 1991, 1997: Urban et al 1998b: Bancroft and Grencis 1998: Nelms et al 1999). IL-4 is a pleitropic cytokine produced mainly by Th2 cells (Mosmann 1991). However, the production of IL-4 by a number of other cell types has also been reported (Ben-Sasson et al 1989: Conrad et al 1990: Williams et al 1993: Seder and Paul 1994: Kullberg et al 1996: Falcone et al 1996.). IL-4 plays a critical role in the regulation of immune responses. Specifically, with IgE and IgG1 responses, IL-4 acts as the switch factor, mediating the Ig isotype switching process of activated B cells, from IgM-expressing to IgG1- and IgE-expressing B cells. This observation has been reported in several *in vitro*, (Coffman and Carty, 1986: Snapper and Paul, 1987) and *in vivo* (Finkelman et al 1986, 1990, 1991: Katona et al 1991) models.

The unique feature of the IgE and IgG1 response associated with *Nb* infection is that most of this immunoglobulin response is not directed against the parasite. Only a fraction (10-20%) is parasite-specific, and this fraction is usually detected 14-21 days after the total (non-specific) antibody response reaches its

peak level. In parallel with the level of IgE in the serum, Katona and colleagues (Katona et al 1983; Urban et al 1984) have shown that there is an increase in the number of cells, in both mesenteric lymph nodes and spleen, expressing surface IgE. Similar observations have also been made in mice infected with *Ascaris* (Urban et al 1989). The significance of the massive non-specific ("irrelevant") IgE response, associated with nematode infection, to the host is still a matter of contention. It has been speculated that nematodes mediate this irrelevant IgE response as an escape mechanism to inactivate nematode specific mast cell degranulation by overwhelming FcεRI occupancy. This would result in an evolutionary advantage to the nematode. A number of studies have demonstrated the irrelevance of antibodies in resistance of hosts to worm infection (Finkelman et al 1997). In one such study, Takai and co-workers showed that the classical interaction between cells and antibody is unnecessary for worm expulsion. This observation is due to the findings that FcγR KO mice are capable of expeling *T. spiralis* as in normal control mice (Takai et al 1994; Grencis 1997).

The potency with which *Nb* and other nematodes induce a polyclonal antibody response is amply demonstrated by experimentation in which nematodes enhance IgE responses to third party antigens, when the antigen is given concurrently with the nematode infection (Stromberg 1980; Lee and McGibbon 1993). This observation is significant in that it suggests that the influence of nematodes on immune responses is far reaching and may have profound effects on

developing immune responses to unrelated antigenic challenge. This is particularly relevant in developing countries where multiple infections with nematodes and other infectious agents are widespread.

2.3 Modulation of immune responses by nematodes

There is an increasing body of evidence from studies using concurrent exposure of the host to nematodes and third party antigens that a shift in the response towards the third party antigens occurs. This effect has been suggested to be based on the reciprocal regulation of Th1 and Th2 cells. Selective activation and/or differentiation of one or the other T cell subset has been shown to be important in determining the course of infection with intracellular protozoa and parasitic nematodes. For example, infection of mice with protozoans such as *Leishmania major* (Locksley et al 1991; Noben-Trauth et al 1999) or *Toxoplasma gondii* (Gazzinelli et al 1993) result in the generation of Th1 associated responses required for the control of these infections. On the other hand, infection with nematodes such as *Nb* (Urban et al 1992) *T. muris* (Else and Grencis 1991; Else et al 1994) and *H. polygyrus* (Urban et al 1991b) result in the development of Th2 responses. However, if an inappropriate Th2 response is induced in protozoan infected mice, either by Th2 cytokines or by nematode infection, the protozoal infection is exacerbated, resulting in host death (Heinzel et al 1989). Pearlman and

colleagues (Pearlman et al 1993b) demonstrated downregulation of the Th1 cytokine responses associated with Mycobacterial antigens by infection with the nematode *Brugia malayi.* Similar observation has also been reported with the helminth *Schistosoma mansoni* (Kullberg et al 1992).

Perhaps the most dramatic evidence of this ability of nematodes to mediate Th2 responses in animals immunized with antigens that usually induce a response with a Th1 bias, is the work of Ledingham and colleagues who showed that *Nb* infection of rats leads to markedly prolonged kidney allograft survival (Ledingham et al 1996).

This modulation of immune responses by mediating type-2 dominance is expressed at the level of cytokine production, skewing the balance to a response which is less effective against the parasite. This approach allows the parasite to use the host regulatory system to its advantage. For example, Yamaoka and colleagues observed promotion of the nematode *Dirofilaria immitis* infection by the enhancement of type-2 cytokines and suppression of type-1 cyokines (Yamaoka et al 1994). Similarly, in infection with *B. malayi*, the adult worms usually induce a rapid polarization of the immune response to that of a type 2 profile, from the type 1 profile stimulated by the first stage larvae (Lawrence et al 1994). This switch is surprising, as host resistance is not dependent on a type-2 response. Lawrence and co-workers (Lawrence et al 1995) reported that in mice deficient in IL-4 activity, the survival and fecundity of larval and adult worms is not affected.

A number of studies (Madden et al 1991; Pritchard 1993; Kopf et al 1993; Finkelman et al 1997) have provided evidence that casts doubt on theories that the IL-4 mediated type-2 response is protective against all nematodes. Even in infections like *T. muris* where the importance of Th2 cytokines in protective immunity has been elegantly demonstrated (Grencis 1997), the story is complex. In the wild, for example, predominantly pregnant and lactating female mice bear *T. muris* to the adult stage and these animals are known to show exaggerated Th2 responses to antigenic challenge.

In addition to modulation of the dominant T cell subset, nematodes can modify immune responses in a number of ways. There is some evidence that nematodes suppress lymphocyte function. This suppressive effect is associated with both infections and products from the parasites. Generally, the effects are mediated either directly on the cells or by modifying other components of the immune response from having a positive to a negative effect. Products from the nematode *H. polygyrus* have been suggested to downregulate lymphocyte proliferation (Pritchard et al 1994). However, this nematode is "exceptional" in that it exhibits a marked in vivo suppressive effect, which includes suppression of IL-9 and IL-10 from MLN, and reduced mastocytosis (Behnke et al 1993). Unlike other rodent/nematode models, this model allows for chronic parasite survival (Robinson et al 1989). Extracts from *A. suum*, have been variously reported to increase (Xie and Lee, 1995) and decrease (Ferreira et al 1995) lymphocyte

proliferation.

Nematodes are also known to contain proteins that are capable of mimicking host regulatory proteins. It has been reported that nematodes use these proteins to modulate the host immune response away from that which would result in expulsion (Riffkin et al 1996). One such protein has been described in the intestinal nematodes *Nb* and *Trichostrongylus colubriformis*. It is a 30 kDa protein which shows functional activity similar to porcine vasoactive intestinal polypeptide (VIP) (Savin et al 1990; Foster and Lee 1996). VIP and the 30 kDa protein have been shown to decrease the amplitude of contraction of the rat intestine, allowing worm establishment. VIP has also been shown to modulate IL-2, IL-4, and IL-10 cytokine gene expression (Xin et al 1994; Wang et al 1996; Ganea 1996; Bellinger et al 1996). Other examples of mimicry exhibited by parasites, to possibly control the host immune response, involve the production of cytokine homologues. Grencis and Entwistle (1997) have reported the presence of an IFN-γ homologue in *T. muris*. Moreover, the nematode parasites *B. malayi*, *Wuchereria bancrofti*, and *Onchocerca volvolus* have been reported to secrete a homologue of human macrophage migration inhibition factor (Pastrana et al 1998). These cytokine homologues, like others, have the potential to modify host immune responses to promote parasite survival.

The use of cytokines as growth factors by parasites to boost their development has been reported in the protozoan parasites *Leishmania*

amazonensis (Mazingue et al 1989; Charlab et al 1990), *Trypanosoma brucei* (Olsson et al 1991) and *Trypanosoma cruzi* (Ming et al 1995; Riffkin et al 1996). Although this mechanism has not been reported in nematodes, it would not be surprising if they also utilize this system, considering their ability to develop other ways to modulate the host immune responses to favour their survival.

2.4 B cell activation / proliferation

The induction of the humoral immune response associated with B cells involves initial activation of the B cells, followed by proliferation and, subsequently, differentiation into mature Ig-secreting B cells. To understand how this activation process is regulated, numerous experimental systems, using a variety of stimuli both *in vivo* and *in vitro* have been employed. These include cross-linking of surface immunoglobulin with either anti-Ig antibody or *Staphylococcus aureus* cowan I (SAC); ligation of CD40 molecules with anti-CD40 antibodies; and mitogen (Lipopolysaccharide, LPS; Pokeweed mitogen, PWM) activation. These stimuli are capable of activating B cells in a polyclonal fashion, without the requirement for antigen engagement of the B cell receptor (BCR). B cell activation by both LPS and CD40 ligation are presented in this thesis and both of these are introduced below.

The most commonly used B cell mitogen in murine systems is bacterial

LPS (Ulevitch and Tobias 1995; Armitage and Alderson 1995). LPS does not require help from T cells to induce proliferation and antibody production in B cells. However, it has been demonstrated that for robust activation of B cells, secondary stimuli are required from accessory cells such as macrophages or dendritic cells. Soluble factors from these cells will also provide the additional accessory help (Corbel and Melchers 1983). Indeed, the addition of very few macrophages, or small amounts of soluble factors from activated macrophages, is sufficient to provide this help. Suppression of macrophage activity, by IL-4 (Hart et al 1991; Maizels et al 1993; Doherty et al 1993; MacDonald et al 1998; Nasarre et al 1998), will interfere with this accessory help for LPS-induced B cell proliferation. In B cell activation mediated by either anti-Ig or anti-CD40 antibodies, the role of macrophages is not well defined. Nonetheless, the involvement of cytokines from other sources, such as T cells and dendritic cells is well documented (Armitage et al 1993; Maliszewski et al 1993).

The signal transduction pathway that occurs in B cells upon stimulation with LPS, involves activation of protein kinase C (PKC; Rush and Waechter 1987; Gupta et al 1988), and normal translocation of the PKC from the cytosol into the cytoplasmic membrane but without Ca^{2+} mobilization (Chen et al 1986; Rosoff and Cantkey 1985). Further, Dearden-badet and Revillard (1993) reported the involvement of protein tyrosine kinases (PTK) in LPS activation by demonstrating the sensitivity of this activation to PTK inhibitors such as herbimycin A and

genistein. As have been demonstrated with B cells stimulated via the BCR, which initiates the phosphoinositide pathway, a recent report have shown that LPS induced B cell activation also involves the activation of a phosphatidylinositol 3-kinase (PI 3-kinase) dependent signaling pathway (Venkataraman et al 1999a). In their study, they observed that the addition of Wortmannin a PI 3-kinase specific inhibitor significantly blocked B cell proliferation induced by LPS. In addition to these signaling pathways, activation of the MAP kinase cascade and NF-κB activation have also been reported in LPS treated B cells (Liou et al 1994). The activation of these downstream events leads ultimately to proliferation of the cells.

The CD40 molecule on B cells is frequently ligated in *in vitro* B cell studies to investigate the mechanisms involved in the regulation of the development of T-dependent humoral immunity *in vivo* by CD40. CD40 is a 50 kDa transmembrane glycoprotein involved in B cell growth, differentiation, and survival (Maliszewski et al 1993; Armitage and Alderson 1995; Takatsu 1997). It is also expressed on dendritic cells, epithelial cells, and macrophages. *In vivo*, CD40 interacts with its ligand CD40L (found on T cells), to provide the second signal required for antigen-induced B cell activation. It is also involved in antibody class switching, germinal centre formation and the rescue of B cells from apoptosis (Aversa et al 1994; Wang et al 1995; Schauer et al 1996; Hsing et al 1997; Schauer et al 1998). B cell proliferation is induced *in vitro* by the use of a mAb to CD40 (Nomura et al 1995). Activation of B cells with anti-CD40 mAb has been demonstrated to

induce upregulation of surface molecules such as B7, LFA-1, ICAM-1, Fas, and CD23 (Noelle and Snow 1990; Takatsu 1997) as well as render the cells more sensitive to the activities of IL-4 and IL-5, thereby promoting B cell maturation (Aversa et al 1994). Proliferation of B cells, induced by anti-CD40 mAb has been reported to be enhanced by the addition of a number of cytokines (Maliszewski et al 1993; Armitage et al 1993). All these effects of CD40 on B cell function clearly demonstrate the pivotal role it plays in B cell responses.

The signal transduction pathway initiated when CD40 on B cells is ligated is thought to involve the Janus kinase 3 (Jak3) pathway. The Jak kinase is thought to be associated with the cytoplasmic domain of CD40, since this domain itself does not contain enzymatic activity (Hsing et al 1997). The signaling pathway has also been reported to involve the induction of PTKs and protein kinase C, but this is still controversial because of conflicting reports in different systems (Purkerson and Parker 1998). It is well established, however, that the mitogen activated protein kinase/extracellular signal related protein kinase (MAPK/ERK) and Jun kinase (JNK) pathways are activated by CD40 ligation. Unlike LPS activation, the activation of the ERK pathway by CD40 is independent of PKC (Li et al 1996) and is not inhibited by elevation of cAMP (Sevetson et al 1993). The activated signal transduction molecules of these pathways are coupled to the cytoplasmic domain of CD40 by TRAF-2. The downstream signaling trans-acting factors activated by CD40 ligation on B cells include NF-κB, NF-AT, and AP-1 (Francis et al 1995).

The recruitment of these factors results in gene transcription, ultimately causing proliferation of the cells.

In both of these activation systems, immunoglobulin production will follow activation and proliferation, but in the absence of exogenous cytokines, class switch to isotypes other than IgM will not occur.

2.5 Immunoglobulin class switch

The generation of secreted antibody responses other than that of the IgM isotype in response to antigens or mitogens has been clearly demonstrated, in both *in vivo* and *in vitro* studies, to involve the complex set of molecular events associated with the process of immunoglobulin class switch. This process allows activated B cells to change the heavy chain constant (C_H) region of the antibody being produced while leaving the variable (V), diversity (D) and joining (J) (VDJ) regions intact. This results in a modified effector function of the antibody while antigen binding remains unchanged. At the cellular level, this switching is manifested by the transition of B cells from expressing membrane IgM or mIgD (or secreting IgM; very little IgD is secreted) to expressing / secreting IgE, IgA or one of the IgG subclasses. Evidence from numerous *in vitro* studies has shown that this transition is mediated by a number of cytokines (Harriman et al 1993; Mandler et al 1993b; Fujieda et al 1995; Siebenkotten and Radbruch 1995; Stavnezer 1996).

They are often referred to as "switch factors" and are able to induce switch to specific Ig isotypes, while inhibiting switch to other isotypes. For example, the addition of IL-4 has been shown to induce switch to IgE and IgG1 and inhibit switch to IgG2a and IgG3 in *in vitro* cultures of murine B cells stimulated with LPS (Rothman et al 1988; Berton et al 1989; Kepron et al 1989; Chu et al 1992; Mandler et al 1993a, 1993b). Conversely, IFN-γ stimulates IgG2a isotype switch, but inhibits IgG1 and IgE production (Snapper and Paul 1987, Snapper et al 1989). Other identified switch factors include TGF-β, and IL-13 (Defrance et al 1994; Stavnezer 1996).

At the molecular level, the change in the class of antibody is mediated by a deletional DNA recombination event called switch recombination. The recombination process, which primarily occurs between two sites on the same DNA segment, brings together a downstream constant gene to the rearranged VDJ region, with the recombination occurring between regions of highly repetitive DNA called switch regions (Nikaido et al 1981). This recombination, as shown in sequencing analysis of the recombination sites, is not restricted to specific sequence sites, but occurs at multiple sites within the switch region (Obata et al 1981; Siebenkotten et al 1992). The DNA sequence between the 5′ end of the switch region of the Cμ locus and the 3′ end of the other C_H locus involved in the recombination, loops out and is deleted. The excised segment is then subsequently ligated to form an extrachromosomal circle.

In addition to the switch recombination event between Sμ and other isotype switch regions to generate isotype switch directly, there is substantial evidence that switching can also occur through sequential deletional and recombination events. This has been suggested since sequenced switch region junctions from chromosomal DNA indicate that recombination has occurred between more than two switch regions (Mills et al 1992; Siebenkotten et al 1992; Dunnick et al 1993; Stavnezer 1996). Additional evidence for the occurrence of sequential class switching has come from a number of studies, in which circular DNA obtained from actively switching B cells was found, upon sequencing, to contain switch region sequences of Sμ ligated to two different S sequences. The most common and most studied phenomenon of sequential class switching involves the switch to IgE. (Siebenkotten et al 1992; Mandler et al 1993b; Miller and Rothman 1998). A number of studies (Siebenkotten et al 1992; Mandler et al 1993b) have shown that switch to murine IgE is often, if not always, preceded by a Sμ-Sγ1 recombination. This has been documented by analyzing the sequences of the recombined switch region upstream of the Cε gene locus and finding both switch γ1 and switch ε elements, in most cases, in B cells which have switched to the IgE isotype.

The detection of extrachromosomal circles, associated with deletional DNA recombination, has been used as a marker of immunoglobulin class switch (Yoshida et al 1990; Mandler et al 1993b). In the recent past, however, other methods have been developed to more accurately assess the occurrence of switch

recombination. One of these, is the digestion-circularization polymerase chain reaction (DC-PCR), which directly assesses the level of switch by measuring the recombination event between the switch regions (Chu et al 1992, 1993; Mandler et al 1993a). The details of this technique are discussed later.

The C_H genes involved in the class switch process are located 3′ of the heavy chain VDJ gene loci. DNA sequencing studies revealed that, in mice, the C_H gene locus is 200 kb in size, and consists of eight C_H genes arranged in the following order: 5′ Cμ-Cδ-Cγ3-Cγ1-Cγ2b-Cγ2a-Cε-Cα 3′. In humans, the size of the C_H locus is 300 kb, and it contains nine functional genes and two pseudogenes (ψ), arranged in the order 5′ Cμ-Cδ-Cγ3-Cγ1-ψε -Cα1-ψγ-Cγ2-Cγ4- Cε-Cα2 3′. With the exception of Cδ, for which no switch region appears to exist, the switch regions are located upstream of each constant region. According to Siebenkotten and Radbruch (1995), all the switch regions in mice have been at least partially sequenced. They are 2-10 kb in size, highly repetitive, with a very high GC content. They contain repeats of the nucleotides GAGCT, TGGGG, and C/TAGGTTG, which have been suggested to play an essential role in the class-switch process (Obata et al 1981). These repeats are stretches of 40-80 bp in length, the exact size depending on the isotype of the switch region (Obata et al 1981; Siebenkotten et al 1992; Siebenkotten and Radbruch 1995). Located 5′ to each switch region is a promoter followed by a short sequence (small exon) called the I_H exon. Because of this arrangement, it has been suggested that the switch

regions represent intronic sequences, and this structure of switch regions is very well conserved.

In activated B cells, the recombination machinery is highly active, and appears to require two types of signals. One is the B cell activation signal required to turn on the recombination machinery. Agents such as anti-Ig antibody, LPS, and SAC, have been shown to provide this signal in *in vitro* experimentation. In the *in vivo* situation, ligation of CD40 on the surface of B cells by its ligand (CD40L), which is expressed on activated Th cells, is believed to be a significant source of the signal (Armitage et al 1993; Banchereau et al 1994). This interaction takes place in germinal centers of the secondary lymphoid organs and, in part, prevents B cells from undergoing apoptosis (Banchereau et al 1994; Xu et al 1994). The significance of the interaction between CD40 and CD40L in *in vivo* Ig class switch is demonstrated by defective class switching, and the absence of Ig isotypes other than IgM, in animals where this interaction is impaired (Fuleihan et al 1993; Kawabe et al 1994). Generally, the nature of the B cell activator is believed to influence the isotype specificity, depending on the cytokine milieu (Snapper et al 1991; Siebenkotten and Radbruch 1995).

The second type of signal involved in the regulation of Ig class switching involves cytokines. Cytokines appear to regulate isotype switch at the level of germline transcripts (Coffman et al 1993). The selective regulation of this process by cytokines is supported by evidence from a number of studies. For example, it

has been demonstrated that the addition of IL-4 to cultures of either LPS- or anti-CD40 antibody-stimulated B cells induces high levels of germline ε and γ1 transcripts that correlate with the amount of IgE and IgG1 induction, respectively (Xu et al 1994; Miller and Rothman 1998). In contrast, IL-4 has been shown to suppress the induction of γ2a germline transcripts by IFN-γ. Similarly, IFN-γ has been shown to suppress the IL-4-induced germline ε transcription (Berton et al 1989; Xu and Rothman 1994).

It is generally accepted that Ig class switching is preceded by the generation of the germline transcripts. However, in some studies, it has been demonstrated that cytokines can also influence switching to specific isotypes, without regulating levels of the corresponding germline transcripts (Gauchat et al. 1992; Stavnezer 1996; Shparago et al. 1996). This has raised the question as to whether these germline transcripts are a prerequisite for the switch recombination or an epiphenomenon of the event. However, data showing a strong correlation between transcript induction and class switching (Leung and Maizels 1992), and data from a number of gene targeting experiments strongly support a requirement for germline transcription for successful isotype switching. In fact, Siebenkotten and Radbruch (1995) suggested that germline transcripts are likely candidates for class-specific components of the putative enzyme, believed to be involved in switch recombination, called switch recombinase. The identity and characteristics of the protein components of the enzyme are still unknown. There is some

suggestion that the enzyme must perform several of the same functions (binding to DNA, cutting it, and ligating it) as the recombinase involved in VDJ recombination (Siebenkotten and Radbruch 1995).

In addition to germline C_H gene expression, DNA synthesis has been also suggested as a necessary condition for isotype switching of B cells. This observation was based on a number of studies that demonstrated an association between inhibition of DNA synthesis and inhibition of switching in *in vitro* cultures (Stavnezer 1996; Snapper et al 1997). However, some other studies have reported that the production of Ig does not necessarily require cell proliferation. Lycke and colleagues (Lycke and Strober 1989; Lycke et al 1990) and others have demonstrated that cholera toxin is able to induce switch of LPS stimulated B cells to IgG1, in the absence of cell proliferation. Similar findings have been reported by Dillehay and co-workers, who showed that retinoids differentially regulated LPS-induced proliferation and immunoglobulin secretion (Dillehay et al 1991). Retinoids potentiated Ig secretion while inhibiting LPS-induced B cell proliferation. At this point it is reasonable to assume, based on available evidence that certain factors involved in class switch require proliferation while others do not. This adds another level of complexity to our understanding of the regulation of class switch.

Nuclear factors have been identified as playing a role in the regulation of class switching. For example, studies in NF-κB/p50 knockout mice showed a 40-

fold reduction in the level of serum IgE (Snapper et al 1996). Furthermore, splenic B cells from these mice induced to switch in culture (with CD40, IL-4 and IL-5) transcribed germline ε RNA and underwent switch to IgE at a markedly reduced level compared to B cells from normal mice control (Snapper et al 1996). In contrast, the B cells from the p50 KO mice expressed normal levels of germline γ1 and germline α RNAs and minimally reduced level of switch to IgG1, relative to control B cells. Also, the Rel protein, c-Rel, is essential for class switch to IgE. B cells from mice deficient in the transactivation domain of c-Rel fail to switch to IgE, despite expressing the same levels of germline ε RNA as control B cells (Zelazowski et al 1997). In addition to these observations, NF-κB/p50 has also been shown to contribute to the regulation of some germline promoters and to the binding to the tandem repeats of Sγ (Snapper et al 1996; Stavnezer 1996). Taken together, this evidence shows that these nuclear factors have significant roles in the regulation of class switching at different stages depending on the isotype involved.

2.6 Regulation of IgE and IgG1 responses in mice

Understanding the mechanisms involved in the regulation of IgE and IgG1 responses in mice has been a significant research goal for several decades. This interest arises from the obvious role that reaginic antibodies play in allergic reactions, and the considerable clinical impact of the increasing level of allergic

asthma in the western world.

Observations by Isakson and colleagues as well as Coffman and colleagues contributed significantly to the current understanding of how IgE and IgG1 responses are regulated. Their studies revealed that the responses were mediated by T cell derived IL-4 (Isakson et al 1982; Coffman and Carty 1986; Coffman et al 1986). The landmark observation by Mossman and colleagues that $CD4^+$ T helper cells could be categorized into two subsets (Th1 and Th2) depending on the pattern of cytokines they produced, identified the IL-4-producing T cells as Th2 cells. Furthermore, the observation that Th1 and Th2 cells can crossregulate each other (by way of IFN-γ vs IL-4), suggested that the IgE and IgG1 antibody responses may be regulated by Th1 and Th2 cells.

Coffman and Carty (1986) first demonstrated that LPS-activated B cells, cultured in the presence of IL-4, secrete not only IgG1 but also IgE. This finding was supported by similar observations reported by other investigators, from both *in vivo* and *in vitro* studies (Finkelman et al 1988a, Lebrun et al 1989; Zakroff et al 1989; Yamada et al 1992). A very significant finding supporting the co-regulation of IgE and IgG1 by IL-4 was demonstrated by the finding that the production of IgE involved the process of sequential switching from Sμ (IgM) to Sγ1 (IgG1) and subsequently to Sε (IgE) (Lebman and Coffman 1988; Mandler et al 1993b). These findings suggested that both Ig isotypes were regulated by a similar mechanism. However, it is now known that some differences exist in the way the

isotypes are regulated. For example, induction of the responses by IL-4 is dose dependent, with the production of IgE requiring a higher amount of IL-4 than IgG1 (Snapper et al 1988; Spiegelberg 1990). At this high level of IL-4, it has been shown that the level of IgG1 could be inhibited. Also, it has been demonstrated that the regulation of IgG1 is not totally mediated by IL-4 (Snapper et al 1988; Kuhn et at 1991; Yamada et al 1993a; Kopf et al 1993; Bancroft et al 1998). The involvement of other factors has been described (Aversa et al 1993; McKenzie et al 1993).

Currently, the data suggest that IgE production in response to nematodes is regulated by IL-4. Experimentation using nematode infection and IL-4 activity blocking treatments has been shown to inhibit the production of non-specific IgE in the serum (Finkelman et al 1997; Bancroft and Grencis 1988; Urban et al 1998b). Similar experiments examining nematode-specific IgE responses induced by worm extracts yielded identical results. In addition, mice homozygous for a mutation in either the IL-4 or IL-13 gene are unable to mount type-2 associated immune responses (Kuhn et at 1991; Kopf et al 1993; Punnonen et al 1993; Smertz-Bertling and Duschl 1995; McKenzie et al 1998a,b). This observation has been further confirmed by the data reported in a recent publication showing that mice in which both the IL-4 and the IL-13 genes have been deleted do not mount Th2 mediated responses, with the exception of IL-5 and eosinophils (Mckenzie et al 1999). Also, several *in vitro* B cell culture studies have shown that IgE

production is dependent on IL-4, as the addition of either antibodies to IL-4, or the IL-4R, completely inhibits IgE production (Finkelman et al 1990).

In contrast, IgG1 production is not completely disrupted by these treatments, but shows reduced levels in comparison to control treatments (Bancroft et al 1998). This difference has been attributed to the involvement of IL-13 in IgG1 production. However, recent evidence has shown that IL-13 also plays a major role in IgE production, independent of IL-4. In fact, IL-13 appears to be able to induce germline IgE heavy chain gene transcription in purified B cells, in the presence of neutralizing anti-IL-4 antibody (Punnonen et al 1993). IL-13 has been described as a close relative of IL-4, activating a number of responses also stimulated by IL-4 (Aversa et al 1993; Punnonen et al 1993; Doherty et al 1993; Zurawski and de Vries 1994). Furthermore, IL-13 signals through a sub-unit of the IL-4 receptor (Aversa et al 1993; Zurawski et al 1995; Smertz-Bertling and Duschl, 1995). There is an increasing body of evidence that IL-13 plays a significant role in the regulation of IgG1 and IgE (Bancroft et al 1998; Emson et al 1998; Lai and Mosmann 1999; Mckenzie et al 1999). For example, in a recent study with IL-13 transgenic mice, a 10-100-fold higher levels of serum IgE than their littermate controls was observed upon nematode infection (Emson et al 1998). The total dependence of this response on IL-13 was confirmed in IL-4-deficient mice carrying IL-13 transgenes, which also responded with similar elevated IgE levels as the IL-13 transgenics. The involvement of IL-13 in IgG1

and IgE production is yet further evidence of the redundancy associated with cytokine regulation networks.

The activities of IL-4 and IL-13 are mediated via the activation of the JAK-STAT signaling pathway (Lin et al 1995). A number of studies have shown that both cytokines stimulate the activation of JAK1. In addition, IL-4 stimulates the activation of JAK3 (Callard et al 1996). The activation of these JAKs results in the phosphorylation of tyrosine residues within the cytoplasmic tail of the cytokine receptor, to which STAT proteins are recruited. The STAT molecule utilized by IL-4 and IL-13 has been identified as STAT6. A role for this molecule in the regulation of IgE and IgG1 responses has been demonstrated. Takeda and co-workers (Takeda et al 1996) and Urban and co-workers (Urban et al 1998a) for example showed the absence of an IgE or IgG1 response in STAT6 knockout mice infected with *Nb*, a strong inducer of the Th2 associated response. Moreover, Kaplan and co-workers (Kaplan et al 1996), Shimoda and colleagues (Shimoda et al 1996) and Berton and co-workers (Berton et al 1997) demonstrated the requirement for STAT6 in the generation of IL-4 mediated responses. These data clearly showed that the lack of the IgE and IgG1 response in the STAT6 KO mice was due to the inability of the cytokines to signal properly through their receptors, confirming the role of STAT6 in the regulation of IgE and IgG1 responses.

Although it is well established that IgE production is regulated by IL4, recent reports have suggested the possible involvement of an additional IL-4

independent, pathway for IgE production (Punnonen et al 1993; Morawetz et al 1996; Ferlin et al 1996; Emson et al 1998). This observation raises the possibility that nematodes may directly induce IgE and IgG1 responses. Finkelman and co-workers (Finkelman et al 1997) and others have suggested the possible involvement of phosphorylcholine and proteolytic enzymes in the generation of the type-2 responses associated with nematode infection (Blackburn et al 1992; Kamata et al 1995; Grigg et al 1996; Pritchard et al 1997). The effect of these factors may be direct or be mediated through the activation of factors like IL-4. This possibility is yet to be investigated. However, it is very likely that the IL-4 regulatory pathway still remains the primary pathway for the regulation of IgE and IgG1 production, more so as nematodes are able to induce polyclonal activation of IL-4 (reviewed in Miller and Rothman 1998).

While IL-4 induces the production of IgE and IgG1, the addition of IFN-γ to LPS/IL-4-activated murine B cells completely inhibits both IgE and IgG1 secretion (Breton et al 1989). This inhibitory effect of IFN-γ has been reported to involve the downregulation of STAT6 activation. (Venkataraman et al 1999b). Besides IFN-γ, other cytokines such as IL-2, IL-12, and IL-18, have been shown to regulate the production IgE and IgG1 (Nakanishi et al 1995; King et al 1995; Miller and Rothman 1998). These cytokines inhibit IL-4 mediated activation of the Ig response by enhancement of IFN-γ activity.

Another factor that has been shown to modulate the production of IgE is

associated with the $CD8^+$ T cells (Diaz-Sanchez and Kemeny 1991; Diaz-Sanchez et al 1993). Kemeny and colleagues, have demonstrated that the injection of mice with ricin, an agent known to be toxic specifically to $CD8^+$ T cells, that bear an increased number of ricin-binding glycoproteins on their surface, resulted in a significant increase in the level of IgE in rodents immunized with ova in alum. The effect was only detected when the $CD8^+$ T cells are depleted when the IgE response is already established. However, this regulatory activity of the $CD8^+$ T cell, has not been reported in *Nb* infection. It may be restricted to established IgE responses to soluble antigens (Kemeny et al 1994; Holmes et al 1996).

The role of CD23 (FcεRII) in IgE regulation is currently under review. Heyman and co-workers reported that IgE-Ag complexes could augment anti-BSA IgG-, IgM-, and IgE antibody responses by interacting with CD23 (Heyman et al 1993). They confirmed that this enhancement was mediated via FcεRII (CD23) since the enhancement in antibody responses was completely inhibited by monoclonal antibody to FcεRII. In support of this finding, Flores-Romo and colleagues, using anti-CD23 antibody demonstrated that CD23 upregulates IgE responses (Flores-Romo et al 1993). Contrary to these findings however, two studies in CD23-deficient mice (Fujiwara et al 1994; Stief et al 1994), and two studies in transgenic mice (Texido et al 1994; Cho et al 1997) have showed that CD23 has a negative regulatory effect on IgE and IgG1 production. Furthermore, evidence from a recent study by Payet and co-workers (Payet et al 1999) also

supports a negative regulatory effect of CD23 on IgE production. Payet and co-workers used transgenic mice in which CD23 was overexpressed on both B and T cells and they found that these mice consistently exhibited strongly suppressed IgE and IgG1 responses to antigen/alum immunization, *Nb* infections, or anti-IgD injection.

The reason for these contradictory findings is not clear but the evidence does point to a role for CD23 in the regulation of IgE production. The prevailing evidence supports a negative feedback mechanism able to impair further B cell differentiation/Ig production.

The discussion above clearly demonstrates that the regulation of the IL-4 mediated IgE and IgG1 response is multi-dimensional, involving both positive and negative regulatory elements. However, a positive regulatory effect appears to be dominant in the presence of IL-4.

2.7 Macrophage activation by LPS

Macrophages play a key role in the regulation of a number of immune responses. They are able to influence both innate and acquired aspects of the host immune responses. They influence immune responses, in a positive or negative manner, by their ability to act as antigen presenting cells, to produce chemokines and to produce cytokines such as IL-1, IL-6, IL-10, IL-12, IL-18, TNF-α, and

TGF-β. They also produce substantial amounts of arachidonic acid metabolites, which are known to modulate lymphocyte activation, as well as reactive oxygen and nitrogen intermediates, which are implicated in host defence against bacterial and parasitic infections (Celada and Nathan 1994; Abrahamsohn and Coffman 1995; Nasarre et al 1998).

The predominant theory for the activation of macrophages involves a two-step process: an initial priming step; and, subsequently a triggering step. A number of studies have shown that the priming signal is best provided by IFN-γ. Other agents such as LPS and TNF-α, provide the trigger signal (Celada and Nathan 1994). However, there are numerous studies, which have shown that macrophages can be adequately activated upon exposure to LPS alone (Weinstein et al 1993; Meng and Lowell 1997). The activation of macrophages by LPS and subsequent production of cytokines such as IL-1, IL-6, and TNF-α, allows macrophages to contribute positively to the stimulation of B cell proliferation and immunoglobulin production by LPS *in vitro*.

Exposure of macrophages to LPS via the LPS receptor (CD14; Kielian and Blecha 1995; Ulevitch and Tobias 1995) initiates a signal transduction cascade that leads to the induction of the activities associated with macrophages. This signaling pathway is not yet fully understood. CD14 is a 55 kDa glycoprotein that is attached to the macrophage membrane by a glycosyl-phosphatidylinositol-linkage. It lacks a transmembrane domain, and therefore lacks the ability to carry out

signaling functions (Ulevitch and Tobias 1995: Kielian and Blecha 1995). The crucial role of CD14 in LPS responses has been demonstrated in transgenic mice overexpressing CD14, and in CD14-deficient mice. In the former, the animals were hypersensitive to LPS (Ferrero et al 1993), while in the latter, they were unresponsive to LPS (Haziot et al 1996). The binding of LPS to CD14 on macrophages is facilitated through the activity of a serum protein, LPS-binding protein (LBP). This allows macrophages to be stimulated with picogram amounts of LPS (Ulevitch and Tobias 1995). However, it has been shown that macrophage activation by LPS can also occur independent of CD14, as anti-CD14 mAb treatment of macrophages failed to block all biological responses associated with macrophage activation (Weinstein et al 1993; Amura et al 1997). In addition, activation of macrophages by this pathway requires large amounts of LPS. B cells, which do not express CD14, can be activated by high doses of LPS (Amura et al 1997).

The early intracellular events activated by LPS in macrophages have been shown to involve the tyrosine phosphorylation of a number of proteins (Weinstein et al 1992) by PTKs. Surprisingly, the Src-family of protein tyrosine kinases, Hck, Fgr, and Lyn, are not involved in LPS-mediated signal transduction. This was demonstrated by the ability of macrophages from Hck, Fgr, and Lyn triple knock-out mice to induce similar cytokine secretion, nitrite production and tumouricidal activity as macrophages from normal mice (Meng and Lowell, 1997). This is

surprising because these PTKs constitute the major src-family kinases described in macrophages and have been associated with macrophage activation by other means.

The important role of PTK activities in macrophage activation by LPS is demonstrated by the fact that herbimycin is able to prevent LPS-induced biological responses (Weinstein et al 1992). Some of the major proteins that become tyrosine phosphorylated include MAP kinases p42 (ERK2), p44 (ERK1), and p38 (Han et al 1994; Hambleton et al 1996; Weinstein et al 1992). Additional evidence in support of the critical role of tyrosine phosphorylation of proteins in macrophage activation by LPS comes from a report that the tyrphostin family of tyrosine kinase inhibitors also blocks TNF-α and nitric oxide production as well as MAP kinase phosphorylation from macrophages in LPS treated mice (Novogrodsky et al 1994). There is also evidence that LPS activates the JNK pathway within minutes of macrophage stimulation (Hambleton et al 1996). Because MAP kinases are implicated in the regulation of a wide range of cellular responses, the activation of these kinases in LPS-stimulated macrophages is believed to be important in the development of anti-bacterial and anti-protozoal responses (Muller et al 1993). In addition to these events, other events that have been suggested to be involved in LPS responsiveness include activation of G proteins, phospholipase C, protein kinase A, and protein kinase C (Ulevitch and Tobias 1995).

The major downstream signaling trans-acting factors activated by LPS in

macrophages include the NF-κB family of proteins and IFN-γ regulatory factor 1 (IRF-1) (Muller et al 1993; Held et al 1999). NF-κB, in the cytoplasm of naive cells is bound to an inhibitory protein IκB. Activation of macrophages by LPS releases NF-κB from the complex and NF-κB subsequently translocated into the nuclues. In the nucleus, it binds to the κB element of the genes encoding cytokines such as IL-1, IL-6, IL-12 and TNF-α and activates the expression of these genes (Muller et al 1993; Schow and Joly 1997). The expression of these cytokine genes following macrophage activation is regulated primarily at the transcriptional level. IRF-1, for example, is essential for the induction of inducible nitric oxide synthase (iNOS) (Kamijo et al 1994). Reactive nitrogen intermediates have been identified as the major mechanism by which macrophages mediate their anti-bacterial and anti-parasitic activities. For example, nitric oxide (NO) production by macrophages, catalyzed by iNOS, has been reported to be the mechanism by which *T. gondii*, *L. major* and *T. cruzi* infection are cleared from infected animals (James 1995; Deckert-Schlüter et al 1996; MacMicking et al 1997). This is supported by studies in mice treated with iNOS inhibitors, and in iNOS knock-out mice, which are unable to clear their parasites, resulting in severe disease (MacMicking et al 1997).

These activities, and others associated with macrophages, are susceptible to the regulatory effects of IFN-γ and IL-4/IL-13. IFN-γ generally induces or

upregulates macrophage functions, alone or in combination with other stimuli (Celada and Nathan 1994; Held et al 1999), while IL-4 and IL-13 inhibit macrophage function (Hart et al 1991; Doherty et al 1993; Doyle et al 1994; Oswald et al 1998). For example, iNOS is induced by IFN-γ and suppressed by IL-4 and IL-13 (Doherty et al 1993; Doyle et al 1994; Held et al 1999).

Because of the prominent role of macrophages in the regulation of immune responses to microorganisms, they are a likely target for immune regulation by nematodes.

2.8 Objectives

This work represents part of an ongoing investigation into how nematodes modulate immune responses in a host. The substantial increase in "irrelevant" immunoglobulin suggests that nematodes have the ability to modulate B cell function *in vivo*. However, the mechanisms involved in the development of this unique polyclonal IgG1 and IgE response are unclear. In this thesis project I chose to investigate how nematodes induce the dramatic polyclonal IgG1 and IgE response, using the model of *Nb* infection in mice. To allow for the investigation in both *in vivo* and *in vitro* systems, a reductive approach, involving the use of extracts of *Nb* rather than infection was adopted. Therefore, the first part of this thesis involves the demonstration that the extract also induces polyclonal

activation and isotype switch similar to infection, as well as experimentation designed to address the hypothesis.

Besides the induction of immunoglobulin production in serum, nematodes have also been shown to modulate other aspects of host immune activities. This modulatory activity has not been well characterized. Thus, the second section of this thesis characterizes the effects of *Nb* on B cell proliferative responses and addresses the specific mechanism(s) by which *Nb* mediates these effects. Together, results of these studies will provide new insights into the mechanism by which nematodes induce the characteristic Ig response as well as modulate other aspects of the host immune responses.

3.0 Materials and Methods

3.1 Animals

Female nude (-/-) BALB/cBYJ mice and control littermates (+/+) were purchased from Jackson Laboratories (Bar Harbour, Maine). All mice were used at 8 to 14 weeks of age. Male Sprague Dawley (SD) rats (220-250g) used in the maintenance of *N. brasiliensis* and in the preparation of AWH, were purchased from Harlan Sprague Dawley (Indianapolis, Indiana). All animals were housed in the Carleton Animal Care Centre, Dalhousie University (Halifax, NS). They were maintained in compliance with the Canadian Council on Animal Care guidelines, with food and water provided *ad libitum*.

3.2 Laboratory maintenance of *Nippostrongylus brasiliensis*

Third stage (infective) larvae of *Nippostrongylus brasiliensis (Nb)* were obtained from Dr. Dean Befus (University of Alberta, Edmonton). The strain obtained was adapted to develop adequately in both mice and rats. The life cycle of *Nb* was maintained regularly by passage in SD rats.

Male SD rats were injected subcutaneously with 5000 infective larvae suspended in 500µl of phosphate-buffered saline (PBS). On day six post-infection, fresh bedding was placed in the rat cages. The next day faecal pellets, which contain *Nb* eggs, were collected from the cages. This allowed for the collection of only faeces passed between the sixth and seventh day post infection (faeces with

optimum egg number). The faecal pellets were soaked in tap water for one hour, following which they were mashed with a wooden applicator stick and mixed with activated charcoal (Fisher Scientific; Nepean, ON). The mixed faecal material was spread on the central area of damp 7 cm Whatman #1 filter papers (Whatman; Maidstone, England), leaving about a 1 cm perimeter of filter paper. The filter papers were placed on top of foam pads on petri dishes (20 cm x 100 cm) and the petri dishes were kept in a paper box with the floor overlaid with aluminum foil and wet paper towels. This created the humid environment required for the development of the parasite. After seven days incubation at room temperature, the eggs have hatched, developed to the infective (third) stage, and migrated to the edge of the filter paper.

For infection purpose, infective larvae were harvested from the faecal cultures with a Bearmann apparatus. The apparatus consisted of a glass funnel with tubing clamped tightly at the bottom attached, this was placed on a retort stand. The funnel was filled with warm tap water (40°C), ensuring that all air bubbles were eliminated from the tubing. Two pieces of gauze folded together and covered with kleenex were then placed into the funnel with the bottom completely immersed in water. A section of the filter paper cut with scissors and edges of the faecal material were placed on the immersed gauze, and then allowed to stand for at least 1 h at room temperature. During this time, the larvae actively migrate through the gauze to the bottom of the funnel, collecting at the point where the

tubing was clamped. The worms were collected in a 15 ml polypropylene tube (Fisher Scientific; Nepean, ON), by carefully releasing the clamp. The worms were allowed to settle in the tube for 30 min, and the supernatant was carefully removed by pipetting. The worms were then washed at least 6 times with sterile PBS supplemented with 100 U/ml of penicillin and 100 µg/ml of streptomycin (PBS-PS; Gibco BRL). After washing, a 20 µl aliquot of the larvae suspension was placed on a glass slide and the number of live larvae counted under a dissecting microscope. The worms were resuspended in PBS-PS to either 3,000 larvae/ml for mice infections (600 larvae/mouse) or 10,000 larvae/ml for rat infections (5000 larvae/rat).

3.3 *Nippostrongylus* adult worm extract preparation

A whole adult worm extract (adult worm homogenate; AWH) was prepared essentially as previously described by Nawa and co-workers (Nawa et al. 1981). Briefly, SD rats were infected by subcutaneous injection of 5000 3rd stage larvae of *Nb* in 0.5 ml PBS-PS. Rats were sacrificed 8 days later, the abdominal cavity was exposed and the adult worms recovered from the small intestine using a modified Baermann apparatus. This consisted of a beaker (600 ml) filled with 450 ml warm PBS-PS, two pieces of loose gauze which were placed over the beaker and pushed in to make contact with the PBS-PS and rubber bands were used to hold the gauze firmly in place. The exposed intestines with the worms were

placed on the gauze in the beaker, then allowed to stand for 2-3 hours in a 37°C water bath, during which time the adult worms actively migrate through the gauze to the bottom of the beaker. The worms were collected by carefully removing the PBS-PS and pipetting the worms into 50 ml polypropylene tubes (Fisher Scientific; Nepean, ON). The worms were washed at least 10 times with sterile PBS-PS. The last two washes were done in PBS alone. After washing, the worms were counted, transferred into a glass tube and homogenized in 1-2 ml PBS with a glass tissue homogenizer. The homogenate, in addition to 3-5 ml PBS used to rinse the homogenizer was transferred into 15 ml polypropylene tubes. To eliminate large particles, the homogenate was centrifuged at 1,000 x *g* for 15 min at 4°C. The supernatant was collected and then further clarified (to eliminate fine particles) by centrifugation at 15,000 x *g* for 30 min at 4°C. The homogenate was sterilized through syringe filters (0.22μm; Millipore Corp.), aliquoted into 1.5 ml microfuge tubes (Fisher Scientific; Nepean, ON) and stored at –20°C. No protease inhibitors were added at any stage of the procedure. Protein content of the extract was determined with the Bicinchoninic acid (BCA) protein assay kit (Pierce laboratory) according to the manufacturer's instruction.

3.4 *Nippostrongylus* larval worm extract preparation

To prepare the larval worm extract (larval worm homogenate; LWH), infective larvae were harvested from the faecal cultures as described above. The

larvae were washed extensively with sterile PBS-PS, counted and then homogenized in 1-2 ml PBS with a glass tissue homogenizer. The homogenate was centrifuged at 1,000 x *g* for 15 min at 4°C. The supernatant was collected and then further clarified (to eliminate fine particles) by two rounds of centrifugation at 15,000 x *g* for 30 min at 4°C. The homogenate was sterilized by passing it through syringe filters (0.22μm; Millipore Corp.), aliquoted and stored at –20°C. No protease inhibitors were added at any stage of the procedure. Protein content of the extract was determined with the Bicinchoninic acid (BCA) protein assay kit (Pierce laboratory) according to the manufacturer's instruction.

3.5 Heat and Proteolytic treatment of AWH

Heat treatment of AWH was carried out by placing 500 μl of AWH in a 1.5 ml microcentrifuge tube, which was then suspended in a beaker of boiling water for 5-10 minutes. The tube was cooled on ice and stored at -20°C. Before use, boiled AWH was always vortexed to ensure that it was in completely in solution.

Proteolytic treatment of AWH involved the incubation of AWH with the insoluble agarose matrix-bound enzymes, trypsin (10 U/ml) or chymotrypsin (10 U/ml) (Sigma-Aldrich Co) in 1.5 ml microfuge tubes for 2-3 h at 37°C. Prior to use, the enzymes were pre-washed with PBS. After treatment, the preparation was centrifuged to separate the insoluble bound enzymes from AWH. The supernatant (AWH) was then filter sterilized and stored at -20°C until used.

3.6 Fractionation of AWH

Fractions of AWH were prepared by centifugation in Centricon concentrators of various molecular weight cut-offs (Amicon Inc.). To prepare fractions of above / below 30 kDa and above / below 50 kDa, 500 ml AWH were placed in the concentrators with the appropriate molecular weight cut-off and centrifuged at 5000 x g for 30-60 min (4°C) in a fixed angle microcentrifuge. For fractions above / below 100 kDa, 500 ml AWH placed in the concentrators was centrifuged at 1000 x g for 1-2 h. To prepare the fraction that was >50 kDa but <100 kDa, the less than 100 kDa fraction was centrifuged through the 50 kDa cut-off concentrator. The retentate contained the greater than 50 kDa but less than 100 kDa fraction while the filtrate was made up of proteins less than 50 kDa. Separation with the centricons generates a filtrate portion (proteins below the molecular weight cut-off allowed to pass through the membrane filter) and a retentate portion (proteins of sizes greater than the molecular weight cut-off of the membrane filter). The retentate does contain some proteins of sizes below the molecular weight cut-off. This problem was greatly reduced in my preparations by re-diluting the retentate and re-centrifugation in new concentrators. This was performed three times. To maintain the fractions at the appropriate initial concentration, the final retentate was reconsistituted with PBS to the original volume of the AWH sample applied. All samples were filter sterilized before storage or use. The efficiency of the centricons to fractionate whole AWH was

determined to be adequate as assessed by running some of the fractions through SDS-PAGE and size exclusion columns (BioSep-SEC-S3000; Phenomenex, USA). It is important to note that this procedure is not designed to be 100% accurate but to give an idea of whether the depleted filtrate / retentate remains active.

3.7 *In vivo* treatment in mice

For *in vivo* experimentation, BALB/c mice were injected subcutaneously at the back of the neck with 200 µl (200 µg protein) of either AWH, LWH, boiled AWH, or killed Mycobacteria (Sigma-Aldrich Co.). Control animals were either untreated (naive) or injected with either PBS or Fruend's incomplete adjuvant (FIA; Sigma-Aldrich Co.). Prior to use, AWH, LWH and boiled AWH were emulsified in FIA (ratio 1:1). For *Nb* infections, mice were injected subcutaneously with 600 larvae in 200 µl of PBS. After injection, animals were separated into appropriately identified cages and monitored for the duration of the experiment.

3.8 Serum Collection

Serum was obtained from mice that received various *in vivo* treatments on a weekly basis. The procedure was kindly performed by either Heather Kearns or Dr. Tim Lee. Mice were bled through the retro-orbital plexus with heparinized

microcapillary tubes. These were then centrifuged in a haematocrit centrifuge and the serum obtained, and stored at -20°C until use. Serum samples were pooled at each time point within each group.

3.9 B cell Isolation

Spleen single cell suspensions were prepared in RPMI-1640 culture medium (without Bicarbonate) (ICN Laboratories, Irving, Ca.) supplemented with 10% fetal bovine serum (FBS; Gibco BRL), 20 mM HEPES (Amersham life sciences), 100 U/ml of Penicillin, 100 μg/ml of Streptomycin, L-glutamine and 50 μM 2-mercaptoethanol (Gibco BRL), hereafter referred to as complete RPMI (cRPMI). Mice were sacrificed by cervical dislocation and the spleens harvested. A spleen cell suspension was made by grinding the spleen with the barrel of a 5 ml syringe in a small petri dish containing 5 ml of cRPMI. The cell suspension was purged of red blood cells by hypotonic lysis with ACK lysing buffer. Spleen cells from nude mice consist mainly of B cells with a minimal T cell population. To enrich for B cells, the cell suspension after red blood cell lysis was washed 3x in cRPMI and adherent cells depleted by 2 rounds of incubation in 75 cm^2 tissue culture flasks (Falcon) for 1 h at 37°C. From control littermates, B cells were isolated as we have previously described (Lee and Xie, 1995). Briefly, T cells were depleted by treatment of the cell suspension with anti-Thy 1.2 mAb (Cedarlane Laboratories, Hornby, ONT.) for 1 hour at 4°C. The cell suspension

was centrifuged to remove excess antibody and then resuspended in cRPMI containing low-tox rabbit complement (Cedarlane Laboratories, Hornby, ONT.) and incubated for 1 h at 37°C. After this, the cell suspension was washed 3x in cRPMI by spinning at 200 x g for 10 min (4°C). Adherent cells were depleted as described above. This treatment consistently yielded a B cell purity of > 95% as determined by flow cytometry (using FITC-anti-B220 mAb for staining). The viability of the cells was determined by trypan blue exclusion.

3.10 B cell stimulation

For proliferation assays, B cells (2 x 10^5/well) were cultured in 96 well flat bottom plates (Nunc Inc, Denmark) in a total volume of 200 µl per well. Each well was stimulated with LPS (5 µg/ml; from *E. coli* serotype 055.55; Sigma-Aldrich Co.) in the presence or absence of AWH at different concentrations. In other experiments, B cells were also stimulated in proliferation assays with Hamster anti-mouse CD40 mAb (0.5 µg/ml; Pharmingen); pokeweed mitogen (PWM; 5 µg/ml; Sigma-Aldrich Co.); *Staphyloccocus aureus* cowan strain I (1:2000 final dilution in the well; Calbiochem, USA); Phorbol 12-myristate 13-acetate (PMA; 1 ng/ml; Sigma-Aldrich Co.) and Ionomycin (0.5 µg/ml; Sigma-Aldrich Co.). The cultures were incubated for 72 h at 37°C, followed by an additional 18 h after pulsing the cells with 1µCi/ml [^{3}H]-thymidine (ICN radiochemicals). In time course experiments, the cells were pulsed with ^{3}H-

thymidine at various times: 0, 24, 48 and 72 h. The cells were harvested onto glass fiber mats with a cell harvester (Skatron Instrument Ltd). Proliferation was assessed by measuring [^{3}H]-thymidine incorporation with a scintillation counter (Beckman). In all experiments, wells were set up in triplicate. Data is expressed as disintegrations per minute (dpm) ± standard deviation (SD) in each triplicate.

For cell viability assay, B cells (2 x 10^6) were cultured in 24 well plates and stimulated with LPS in the presence or absence of AWH. After 24 or 72 h of incubation, the cells were collected into centrifuge tubes, washed and resuspended in equal volumes of cRPMI. Cells in the different treatment groups were counted and viability determined by trypan blue dye exclusion.

3.11 *In vitro* stimulation of B cells for immunoglobulin production

Purified B cells (2 x 10^6/ml) were stimulated with 10 μg/ml LPS alone, or LPS in combination with AWH (20 μg/ml) or IL-4 (5 ng/ml; Genzyme Corp). The cells were incubated in 1 ml of cRPMI, in 24-well plates (Falcon) at 37°C. Culture supernatants were harvested 7 days later, centrifuged to eliminate cells and stored at -20°C until analyzed for IgG1 levels using antibody capture ELISA.

To determine the level of switch DNA recombination in *in vitro* B cell cultures, 3 x 10^6 cells were stimulated as stated above. The cells were set up in quadruplicate wells. Four to five days later, the cells in each of the quadruplicate wells were harvested and pooled, pelleted by centrifugation, washed twice with

PBS and then resuspended in digestion buffer for subsequent DNA isolation.

3.12 T cell stimulation

For T cell proliferation assays, spleen single cell suspensions (1 x 10^6/ml) were cultured in 96 well flat bottom plates in a total volume of 200ul per well. Each well was stimulated with either Concanavalin A (Con A; 5 µg/ml; Sigma-Aldrich Co.) or anti-CD3 mAb (hybridoma supernatant; 1:100 dilution in the well), in the presence or absence of AWH at different concentrations. The cultures were incubated for 72 h at 37°C, followed by an additional 18 h after pulsing the cells with 1µCi/ml [^{3}H]-thymidine (ICN radiochemicals). The cells were harvested onto glass fiber mats with a cell harvester (Skatron Instrument Ltd). Proliferation was assessed by measuring [^{3}H]-thymidine incorporation with a scintillation counter (Beckman). In all experiments, wells were set up in triplicate. Data is expressed as disintegrations per minute (dpm) ± standard deviation (SD) in each triplicate.

3.13 Isolation and stimulation of macrophages

Macrophages were isolated from spleen cell suspensions by placing 4 x 10^6/well spleen cells in 96-well flat-bottom plates and incubating the plates for 4 or 24 h, during which time macrophages adhere to the plate. Non-adherent cells wre discarded, and the cells left in the wells were washed with fresh cRPMI.

Following this, the cells were stimulated with LPS (5 μg/ml) alone or in combination with AWH. The cells were incubated for 48 h at the end of which supernatants were harvested and stored at -20°C until analyzed. The cytokine levels in the supernatants were assessed by cytokine sandwich (capture) ELISA.

3.14 Assessment of immunoglobulin levels in serum and culture supernatants by capture ELISA

The level of IgE, IgG1 and IgG2a in mouse serum and IgG1, IgG2a and IgM levels in B cell culture supernatants were determined by standard ELISA. Flat-bottom 96-well maxiSorb immuno-plates (Nunc Inc, Denmark) were coated with 2 μg/ml of the respective capture anti-immunoglobulin antibody (Goat anti-mouse IgG1, Goat anti-mouse IgG2a, Goat anti-mouse IgM; Cedarlane Laboratories Hornby, ONT), or Anti-mouse IgE mAb; Pharmingen) overnight at 4°C. The following day excess antibody was discarded and the plates were washed with phosphate-buffered saline containing 0.05% Tween 20 (BioRad) (PBS-Tween) three times for 1-3 min per wash. Wells were blocked with 200 μl of 1% gelatin (BioRad) in PBS-Tween 20 for 2 h at room temperature, following which they were washed three times with PBS-Tween. Next, the wells were seeded with 100 μl of either serum or B cell culture supernatants and appropriate standards, all diluted in PBS-Tween containing 1% gelatin. All dilutions of standards and samples were seeded either in duplicate or triplicate wells. Plates

were incubated overnight at room temperature. Following this, the plates were washed as before and then the appropriate secondary antibody conjugated to horse radish peroxidase (HRP: obtained from the same source as primary antibodies) was added to the wells (100 µl/well) at a 1:1000 dilution in PBS-Tween containing gelatin and incubated at room temperature for 1 h. At the end of the incubation period, the plates were then washed as before, followed by the addition of the substrate solution which consisted of o-phenylenediamine (OPD) substrate (Sigma-Aldrich Co.) 0.4 µg/ml in citrate buffer pH 5.0 (50 ml ddH_2O, 24.2 ml of 0.1 M citric acid and 25.8 ml of 0.2 M Na_2HPO_4). Hydrogen peroxide (0.04%: Sigma-Aldrich Co.) was added to the substrate solution just before use. 100 µl of the substrate solution was added per well. Plates were incubated in the dark for 30-60 min to allow for colour development. The reaction was stopped by the addition of 50 µl of 2.5 M H_2SO_4. Absorbance was read at a wavelength of 490 nm using a Titertek plate reader (ICN Laboratories).

3.15 Measurement of cytokine levels in spleen cells and macrophage culture supernatants by sandwich ELISA

Flat-bottom 96-well ELISA plates were coated with the respective capture anti-cytokine mAb: anti-mouse IL-4 (1 µg/ml); anti-mouse IL-6 (1 µg/ml): anti-mouse IL-10 (1 µg/ml): anti-mouse IL-12 (2 µg/ml): anti-mouse TNF-α (4 µg/ml) (all anti-cytokine mAb were purchased from Pharmingen): in 0.1 M carbonate

buffer (pH 9.6) 100 µl per well and incubated overnight at 4°C. Following the incubation, excess antibodies were discarded and the plates were washed with Tris-buffered saline (TBS) 3 times for 3 min per wash. Wells were blocked with 200 µl of 2 mg/ml BSA in TBS for 2 h at room temperature, after which they were washed three times with TBS-containing 0.05% Tween 20 (TBS/Tween). Next, 100 µl of standards and the culture supernatants were applied to the wells, all diluted in TBS-containing 1 mg/ml BSA. All dilutions of standards (obtained from Pharmingen) and samples were analyzed either in duplicate or triplicate. Plates were incubated overnight at 4°C. Following this, the standards and samples were discarded and the plates were washed with TBS/Tween as before. The appropriate biotinylated detection anti-cytokine antibody (Pharmingen) was added to the wells (100 µl per well) at 0.5 µg/ml diluted in TBS/Tween-containing 1 mg/ml BSA and incubated at room temperature for 1 h. Plates were then washed 6 times with TBS/Tween. After this, 100 µl of extravidin-peroxidase (Sigma-Aldrich Co.) diluted 1:1000 in TBS/Tween-containing 1 mg/ml BSA, was added to the wells, and incubated at room temperature for 30 min. The plates were washed 8 times with TBS/Tween, following which 100 µl of the detection substrate solution comprising a mixture, in equal proportion, of 3,3'5,5`-tetramethylbenzidine TMB (Reagent A) and Hydrogen peroxide H_2O_2 (Reagent B) (Kirkegaard Perry Laboratories) was added to each wells and the plates were stored in the dark until adequate colour development. The reaction was stopped by

the addition of 100 µl of 1 M H_3PO_4. Absorbance was read at a wavelength of 450 nm using a Titertek plate reader (ICN Laboratories).

3.16 Flow cytometry

The level of macrophage contamination in naïve B cells, or B cells pre-incubated with LPS was examined by direct staining for surface expression of CD45R (B220) and CD11b (Mac-1) using FITC-conjugated rat IgG2a anti-mouse CD45R and FITC-conjugated rat IgG2b anti-mouse CD11b (both antibodies were purchased from Cedarlane Laboratories, ON). B cells ($1x10^6$/ml) were washed once with PBS containing 3% fetal calf serum (staining buffer) and then incubated with 5 µg/ml of either FITC-rat anti-mouse CD45R or FITC-rat anti-mouse CD11b in the staining buffer for 1 h on ice. B cells used to determine background-staining levels were incubated with staining buffer alone. At the end of the incubation period, cells were pelleted by centrifugation and washed twice with staining buffer, and fixed in 1% paraformaldehyde in PBS (Sigma-Aldrich Co.). Cells were stored at 4°C in 6 ml tubes wrapped with aluminium foil to prevent exposure to light until read using a Becton Dickinson FACScan and Lysis II software (Becton Dickinson.NJ).

3.17 JAM (Just Another Method) Assay (Matzinger 1991)

B cells (3×10^7 at 3×10^6/ml) in cRPMI were placed into 25 cm^2 culture

flasks and stimulated with LPS (10 μg/ml) and incubated for 24 h at 37°C in the presence of [^{3}H]-thymidine (1 μCi/ml). The following day, the cells were pelleted by centrifugation, excess media was discarded, the cells washed twice with cRPMI, resuspended in media and then counted using a hemocytometer. The labelled cells (2 x 10^5/well) were placed in 96 well flat-bottom plates, stimulated with LPS (5 μg/ml) alone or in combination with different concentrations of AWH and incubated for 24, 48, and 72 h at 37°C. At the initiation of the culture, some cells were harvested onto glass filter mats to determine the thymidine incorporation at time zero (T_0). At the indicated times, cultures were harvested onto glass filter mats and the level of incorporated thymidine present at that time point (T_p) was determined by liquid scintillation counting. To determine the level of DNA damage, T_p was subtracted from T_0 and the product divided by T_0 and then expressed as a percentage.

$$\frac{T_0 - T_p}{T_0} \times 100\%$$

3.18 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

3.18.1 RNA Isolation

Total cellular RNA was isolated from spleen cells of mice injected with AWH, *Nb* or naive (untreated) mice using TRIZOL™ reagent (Gibco BRL). Ten million ($1x10^7$) spleen cells were pelleted by centrifugation in 15 ml tubes, culture

media was decanted and the cell pellets resuspended in 1 ml TRIZOL. The TRIZOL/cell lysates were mixed vigorously by pipetting up and down several times, and then transferred to 1.5 ml microfuge tubes which were then allowed to sit for 10 min at room temperature after which the tubes were stored at -70°C until processed. To further process the lysates, the lysates were thawed at room temperature, after which 200 µl Chloroform was added to the lysates, mixed vigorously and centrifuged at 12 000 x g for 15 min at 4°C to separate the aqueous and organic phases. The aqueous phase was carefully pipetted off and transferred to fresh 1.5 ml microfuge tubes. The RNA was then precipitated by the addition of 500 µl isopropanol, following which the RNA samples were pelleted by centrifugation at 12 000 x g for 10 min (4°C). The isopropanol was carefully pipetted out and discarded, and the pellet washed once with 1 ml volume of 75% ethanol by mixing a few times, then re-pelleted by spinning at 7500 x g for 5 min (4°C). The supernatant was pipetted out carefully, and the pellet allowed to air dry at room temperature for several hours. The RNA pellet was then resuspended in sterile dH_2O and incubated in a 55°C water bath for 10 min to facilitate solubilization. The RNA suspension was quantified and the purity determined based upon the absorbance readings at wavelengths 260 and 280 nm. The samples were then stored at -70°C until used in the reverse transcription reaction.

3.18.2 Reverse transcription of RNA

The reverse transcription reaction of each RNA sample was performed in 500 µl microfuge tubes with 1 µg of total RNA using murine-Moloney Leukemia Virus (M-MLV) reverse transcriptase (Gibco BRL). Each reaction mixture of 20 µl volume, contained 4 µl 5X first strand buffer (1X), 2 µl 0.1 M dithiothreotol (DTT: 0.01 M), 1 µl 10 mM deoxyribonucleotide triphosphates (dNTP: 0.5 mM), 1 µl 1 mg/ml random hexamer primers (1 µg), 1 µl M-MLV reverse transcriptase enzyme (200 units) and 11 µl RNA solution (1 µg). Using an automated thermal cycler (PTC-100; MJ Research Inc.), each RT reaction tube was incubated for 10 min at 20°C followed by another incubation period of 60 min at 37°C to allow for reverse transcription of the RNA samples. The reactions were stopped by denaturation of the enzyme at 94°C for 10 min. Following this, the tubes with the cDNA samples, were centrifuged briefly, and then stored at -70°C until used for PCR.

3.18.3 PCR amplification of cDNA for IL-4 and IL-13 mRNA

Polymerase chain reaction was performed on cDNA products (3 µl) for IL-4 and IL-13 sequences in 0.5 ml microfuge tubes in the presence of reaction mixtures made up of 5 µl of 10 X PCR buffer (2 M KCl, 1 M Tris pH 8.4, 1 M $MgCl_2$, 1 mg/ml BSA), 0.2 mM dNTPs, 0.5 µM each primer set and 2.5 units of *Taq* DNA polymerase (Gibco BRL). Each reaction was adjusted to a final volume of 50 µl

with dH_2O. The samples and reaction mixtures were centrifuged briefly, then placed in the wells of an automated thermal cycler and subjected to 35 cycles of 60 seconds denaturation at 94°C, 60 seconds annealing at 55°C, and 60 seconds extension at 72°C, at the end of which, the product was subjected to final incubation period of 5 min at 72°C to ensure complete product extension. Amplification of β-Actin sequences served as control transcripts for the reaction and for semi-quantitative purposes. PCR products were kept at -20°C until resolved by agarose gel electrophoresis.

The sequences for the primers used in the PCR were as follows:

β-Actin (5') 5'-CTGGAGAAGAGCTATGAGC-3'

β-Actin (3') 5'-TTCTGCATCCTGTCAGCAATG-3' (241 bp)

IL-4 (5') 5'-CGAAGAACACCACAGAGAGTGAGCT-3'

IL-4 (3') 5'-GACTCATTCATGGTGCAGCTTATCG-3' (203 bp)

IL-13 (5') 5'-ATGGCGCTCTGGGTGACTGCAG-3'

IL-13 (3') 5'-GAAGGGGCCGTGGCGAAACAGTTG-3' (350 bp)

To control for genomic DNA contamination of PCR products, all primers used for RT-PCR were designed as intron spanning primers.

3.19 Digestion Circularization Polymerase Chain Reaction (DC-PCR)

3.19.1 DNA Isolation

DNA was isolated by two methods, one for spleen cells harvested from *in*

vivo experimentation and the second for B cells harvested from *in vitro* class switch experimentation. Genomic DNA from spleen cells of naive (untreated) mice or mice injected with AWH, *Nb*, FIA, killed Mycobacteria, PBS (*in vivo* experimentation) was prepared by standard methods (Gross-Bellard et al. 1973; Strauss, 1994) with some modification. Thirty million (30 x 10^6) spleen cells isolated from each group of mice were pelleted by centrifugation in 15 ml tubes, culture media was decanted and the cell pellets were washed twice in ice-cold PBS. The pellets were lysed by resuspension in 0.3 ml of digestion buffer (0.5% SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8.0, 25 mM EDTA pH 8.0 and 0.25 mg/ml proteinase K (Sigma-Aldrich Co.). The lysates were mixed vigorously by pipetting up and down several times, and then transferred to 1.5 ml microfuge tubes and incubated overnight at 50°C. An equal volume (0.3 ml) of phenol/chloroform/isoamyl alcohol (25:24:1) mixture was next added to the lysates. They were then mixed by inversion and centrifuged at 12 000 x g for 10 min to extract the DNA from the samples. The DNA was carefully pipetted off and transferred to fresh 1.5 ml microfuge tubes. The DNA was then precipitated by the addition of ½ volume of 7.5 M ammonium acetate and 2 times the original volume of DNA of 100% ethanol, following which the DNA samples were centrifuged at 12 000 x g for 10 min (4°C). Recovery of DNA samples was very difficult because the samples were very viscous. DNA samples were carefully washed twice with 1 ml volume of 70% ethanol by mixing a few times, then

spinning at 10.000 x g for 5 min (4°C). The supernatant was pipetted out carefully, and the DNA samples were allowed to air dry at room temperature for few hours, following which they were then resuspended in Tris-EDTA (TE). The DNA samples were usually sheared by passing through 18, 21 and 23 gauge needles as well as incubated in 65°C water bath for 30-60 min to facilitate solubilization. DNA concentration and purity was determined by UV absorption at wavelengths 260 and 280 nm. The samples were then stored at 4°C until used in DC-PCR.

The second isolation method was used to prepare DNA from B cells harvested from *in vitro* cultures for class switch experimentation. Isolation of DNA was accomplished using DNAZOL® Reagent (Gibco BRL) essentially following the manufacturer's instructions. Ten to twenty million ($1\text{-}2 \times 10^7$) cells harvested from 24 well plates were centrifuged at 400 x g for 7 min in 15 ml centrifuge tubes to pellet the cells. One millilitre (1 ml) of DNAZOL was added to each sample tube and the cells lysed by gently pipetting the mixture, following which the lysates were transferred into 1.5 ml microfuge tubes. To precipitate DNA from the lysate, 0.5 ml of 100% ethanol was added to the samples, mixed by inversion, stored at room temperature for 5 min and pelleted by centrifugation at 4000 x g for 3 min at room temperature. Supernatant was carefully decanted, and the pellet washed twice with 1 ml volume of 95% ethanol by mixing a few times, then re-pelleted by spinning at 4000 x g for 2 min. The pellet (DNA) was allowed

to air dry at room temperature for about one hour. DNA was then dissolved in 0.3 ml of 8 mM NaOH by slowly passing the pellet through a pipette tip. After DNA samples were adequately solubilized, the pH of the DNA solution was adjusted to the original pH with 1 M HEPES. Quantification of samples was done by running them through 1.5% agarose gels and estimating the amount by comparing to known amounts of DNA. Samples were stored at 4°C until digested with the restriction endonuclease EcoR1.

3.19.2 Digestion and Circularization (DC) of DNA samples

Digestion of each DNA sample was performed in 1.5 ml microfuge tubes with 5-10 µg of DNA in 100 µl volume using EcoRI as the restriction endonuclease. Each reaction mixture contained 10 µl 10x React 3 buffer (1x final: Gibco BRL), EcoRI (2 U/µg DNA; Gibco BRL), DNA solution and the appropriate volume of ddH_2O required to adjust the volume to 100 µl. The reaction mixtures were then incubated overnight in a 37°C waterbath following which the enzyme was inactivated by incubation at 70°C for 20 min.

For ligation (circularization), 10-20 µl of digested DNA samples were placed in 1.5 ml microfuge tubes to which 20 µl of 5x T4 DNA ligase buffer (1x final: Gibco BRL), 2 µl (20 U) of T4 DNA ligase (Gibco BRL) and ddH_2O were added to a final volume of 100 µl. The reaction mixtures were incubated overnight in a 16°C waterbath. Ligated DNA samples were kept at -20°C until amplification

by PCR.

3.19.3 Amplification of ligated DNA by Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed on ligated DNA products for the detection of Sμ-Sγ1 recombination and nicotinic acetylcholine receptor (nAChRe) β subunit gene with appropriate primers in clean 0.5 ml microfuge tubes. Aliquots (5-20 μl) from the ligation reaction were placed into PCR tubes with the reaction mixture containing 5 μl of 10 x GeneAmp® PCR buffer II (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 2 μl (1.0 mM) of $MgCl_2$, 0.2 mM of each dNTP, 0.5 μM of each primer set and 2.5 U of AmpliTaq® DNA polymerase (AmpliTaq Gold™; Perkin Elmer Corp., Norwalk, CT). Each reaction was adjusted to a final volume of 50 μl with dH_2O. Amplification of the samples for the detection of Sμ-Sγ1 rearrangement and nAChRe gene was performed in an automated thermal cycler subjected to the following cycling parameters: 94°C for 9 min to activate the enzyme, initial 5 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 1 min, and extension at 72°C for 2 min; further 30 cycles of denaturation at 94°C for 1 min, stringent annealing at 68°C for 1 min, and extension at 72°C for 2 min; complete extension of PCR products at 72°C for 7 min. Amplification of nAChRe gene served as control for DNA preparation, restriction digestion, and ligation in the DC-PCR procedure and for semi-

quantitative purposes. PCR products were kept at -20°C until analyzed by agarose gel electrophoresis.

The primer sequences used in the PCR (initially published by Chu *et al.* 1992) were purchased from Gibco BRL and are as follows:

nAChRe (sense) 5'- AGGCGCGCACTGACACCACTAAG-3'

nAChRe (antisense) 5'-GACTGCTGTGGGTTTCACCCAG-3'

(generated a 753 bp PCR product from the digested and circularized genomic DNA template).

S μ (antisense) 5'-**GGCCGGTCGAC**GGAGACCAATAATCAGAGGGAAG-3'

S γ1 (sense) 5'-**GCGCCATCGATG**GAGAGCAGGGTCTCCTGGGTAGG-3'

(generated a 219 bp PCR product from the digested and circularized genomic DNA template). Bolded nucleotides are linker sequences containing *Sal* I and *Cla* I sites respectively, the remaining nucleotides represent mouse genomic sequences.

Primers upon receipt were reconstituted with sterile ddH_2O, aliquoted in autoclaved 500 µl microfuge tubes and stored at -20°C.

3.20 Agarose gel electrophoresis

PCR products from RT-PCR or DC-PCR were resolved through either 1.5% or 2.0% (w/v) agarose gel (high grade; Gibco BRL) in 0.5x tris-borate-EDTA buffer (TBE; 0.045 M Tris, 0.045 M boric acid, 0.01 M EDTA). Ethidium bromide (Sigma-Aldrich Co.) was added to the gel (at a final concentration of 0.5

μg/ml) to visualize the DNA. Twenty microlitres (20 μl) of PCR products was mixed with 2 μl of bromophenol blue / xylene cyanol DNA loading buffer (0.025% phenol blue, xylene cyanol and 30% (v/v) glycerol). One microgram (1 μg) of 100 bp DNA ladder (Gibco BRL) was used as a molecular weight marker to confirm amplicon size. Samples were loaded and the gel was run at 70-80V for 45-80 min. Gels were then photographed with a DS/34 Polaroid camera (Bio/Can) using a Foto/Prep I UV transilluminator (Bio/Can) or digitally captured with Micro Analyst® software (BioRad Laboratories, Hercules, CA.). For data presentation, photographs were scanned with a UMAX Astra 1200S scanner. For quantitation of PCR products, relative levels were assessed by densiometric analysis using Micro Analyst® software.

3.21 Sequencing of nAChRe PCR product

Nucleotide sequence of the nAChRe amplified gene product was determined by the dideoxy chain termination method using the sequenase® version 2.0 DNA sequencing kit (United States Biochemical). The procedure was performed by Ms. Mei Li. PCR product used for the sequencing reaction was purified with GENECLEAN II kit (BIO 101 Inc.). Nucleotide bases read off the autoradiograph were analyzed at the Genbank nucleotide sequence depository.

4.0 Results

4.1 Immune response to *N. brasiliensis* extract (AWH) in BALB/c mice

4.1.1 *N. brasiliensis* extract induces IgE and IgG1 production

Increased production of circulating immunoglobulin of the IgE and IgG1 isotypes in mice infected with nematodes have been widely reported (Urban et al 1998b). However, the mechanism involved in this unique response is still unclear. To investigate this, we adopted a reductive approach using an extract (Adult Worm Homogenate; AWH) of the intestinal nematode *N. brasiliensis (Nb)*. This approach allows for the establishment of an *in vitro* model.

To address the question of whether the extract will induce the characteristic immune response associated with *Nb* infection, female BALB/c mice were injected subcutaneously at the back of the neck with AWH (approximately 200 μg of protein, which is equivalent to about 200 worms) emulsified in FIA. Serum was obtained from these mice on a weekly basis over a three week period. The levels of three immunoglobulin isotypes (IgE, IgG1 and IgG2a) were assessed by capture ELISA. Since IgG2a is a type-1 immunoglobulin, the measurement of IgG2a levels will address the issue of whether AWH induces the type-2 Ig dominance seen in mice infected with *Nb*, or whether it induces Igs of all classes. Injection of mice with AWH resulted in a marked increase in the level of total IgE in the serum (fig. 1A). This increase was about 5-6 fold at three weeks post AWH injection. In mice injected with the vehicle (FIA) alone, no increase in IgE level was detected in

the serum. in fact, the baseline level at day 0 was maintained at all the time points examined.

In addition, a marked increase in the levels of IgG1 was also observed in the serum of mice injected with AWH (fig. 1B). The increase reached about 6 fold at 21 days post treatment with AWH. In some experiments, a booster dose of AWH (at the same level as the first dose) was administered four weeks later. In these animals, a continued increase in the level of IgG1 was also observed, reaching a peak of about 9-10 fold increase at seven weeks after the initial AWH injection (fig. 2). The kinetics and level of the increase after boosting with AWH do not resemble that expected from an antigen driven response, indicating that the AWH effect is non-specific stimulation rather than antigen driven. A decline from the peak level was observed in the serum samples obtained at eight weeks post treatment. The pattern of the increase in IgE and IgG1 levels in the serum of mice injected with AWH is very similar to the pattern observed in the serum of mice infected with *Nb* (Zakroff et al 1989). However, the final absolute levels were always higher in *Nb* infected than in AWH treated mice.

Nb like most nematodes, possesses a protein composition with a degree of specificity to the different developmental stages involved in the life cycle (Maizels et al 1983; Dorzok et al 1989). Therefore, it was of interest to determine if the increase in IgE and IgG1 levels observed in the serum of AWH treated mice was specific to the extract of the adult stage of *Nb*. To address this, mice were injected

Figure 1. AWH induces IgE and IgG1production in the serum of BALB/c mice. Female BALB/c mice in groups of three were injected subcutaneously at the back of the neck with AWH emulsified in Freund's incomplete adjuvant (100-200 μg protein in 200 μl volume). Control group was injected with the vehicle alone. Each group of mice was bled for serum each week for 3 weeks. Serum samples were assayed for IgE **(A)** and IgG1 **(B)** levels by capture ELISA. The data are from one experiment and are representative of three (IgE) and five (IgG1) independent experiments.

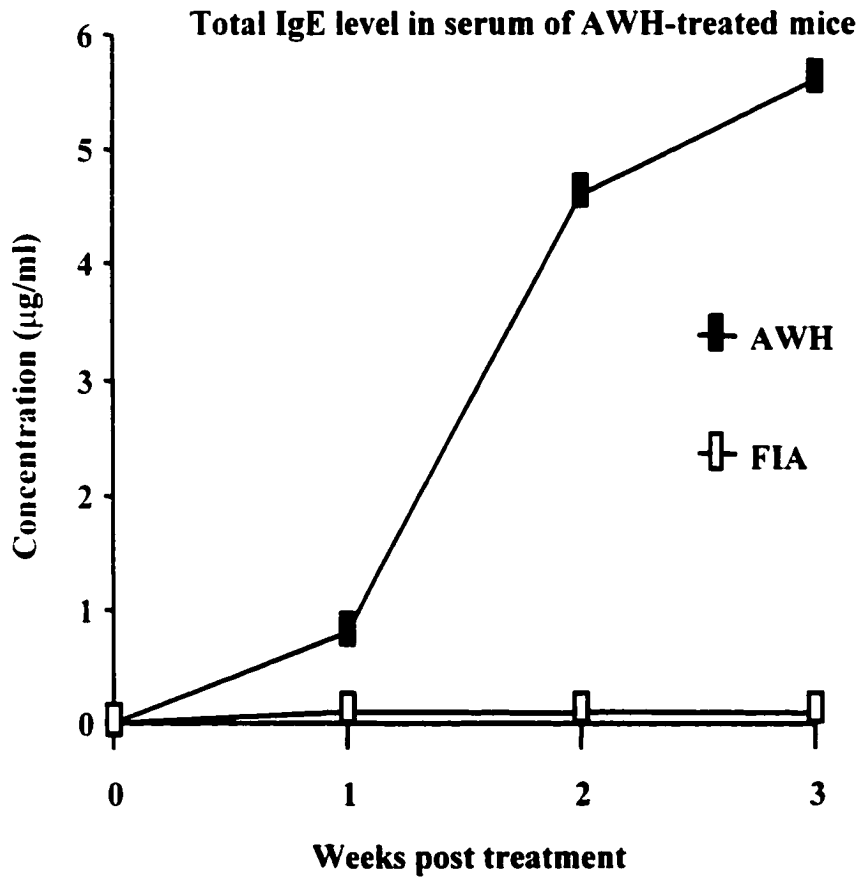

Total IgE level in serum of AWH-treated mice
Concentration (µg/ml)
6
5
4
3
2
1
0
AWH
FIA
0
1
2
3
Weeks post treatment

Figure 1A

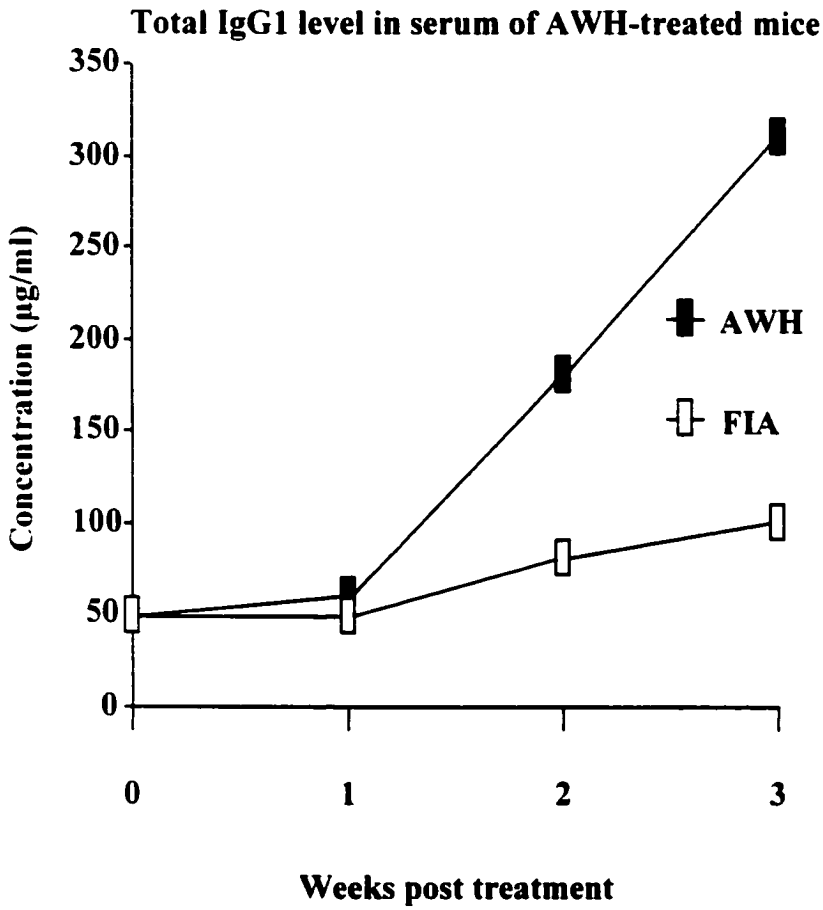

Total IgG1 level in serum of AWH-treated mice
Concentration (µg/ml)
350
300
250
200
150
100
50
0
0
1
2
3
Weeks post treatment
AWH
FIA

Figure 1B

Figure 2. Booster dose of AWH induces higher levels of IgG1 in mice serum. Female BALB/c mice in groups of three were injected subcutaneously at the back of the neck with AWH emulsified in Freund's incomplete adjuvant (approximately 200 μg protein in 200 μl volume). Control group was injected with the vehicle alone. At four weeks, mice were injected with a second dose of AWH as in the first. Mice were bled through the retro-orbital plexus for serum once a week for a total of eight weeks. Serum samples obtained were pooled within each group. Serum samples were assayed for IgG1 levels by capture ELISA. The data is presented as fold increase of IgG1 at the time point assayed over the level at the initiation of the experiment (week 0). The data are from one experiment and are representative of three such experiments.

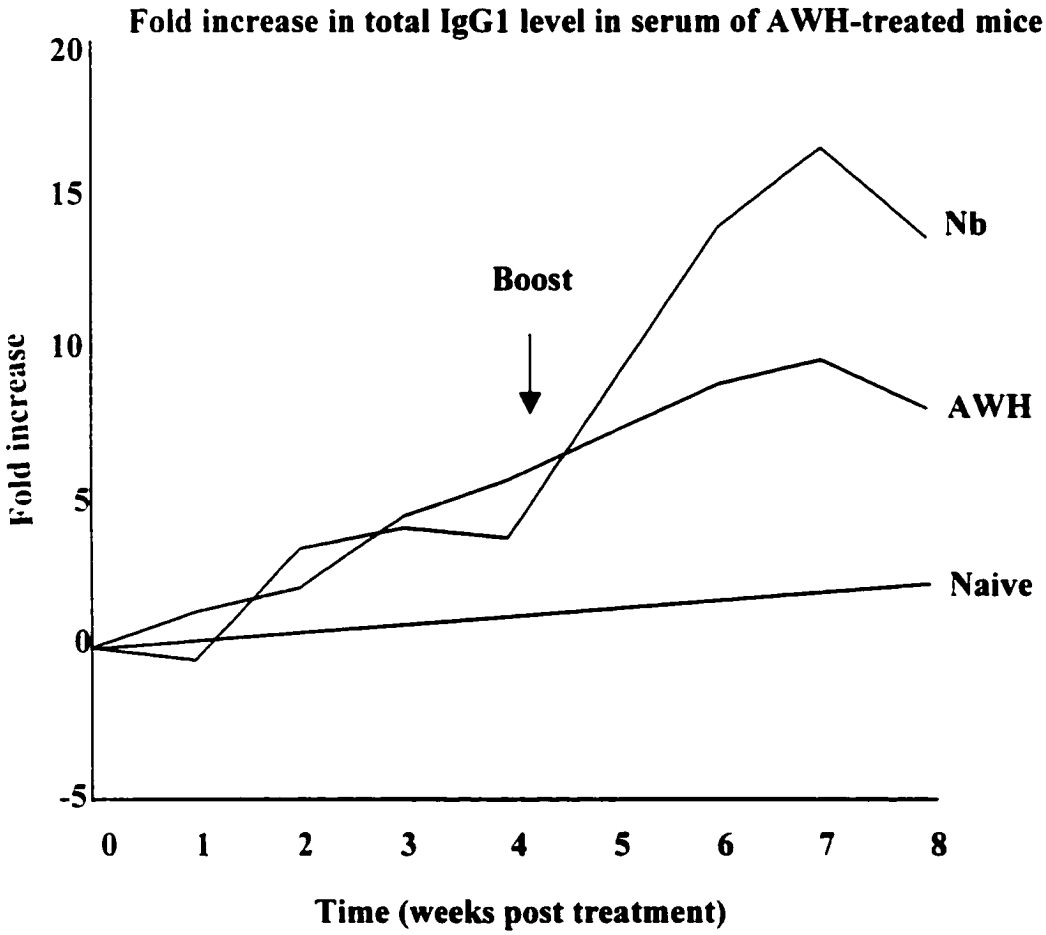

Fold increase in total IgG1 level in serum of AWH-treated mice
20
15
10
5
0
-5
Fold increase
Boost
Nb
AWH
Naive
0
1
2
3
4
5
6
7
8
Time (weeks post treatment)

Figure 2

with an extract of the infective larvae (L3) of *Nb* (LWH), and the levels of IgE and IgG1 present in the serum assessed as stated previously. At three weeks post treatment, LWH also induced a significant increase ($p<0.001$) in total IgE levels similar to the level of IgE induced by AWH ($p>0.05$) (fig. 3A). LWH also induced a significant increase ($p<0.01$) in IgG1 levels (fig. 3B). These data suggest that the factor(s) responsible for the increase in IgE and IgG1 level is not specific to the adult stage of the nematode. To determine whether an intact protein factor was responsible for the increased IgE and IgG1 levels, mice were injected with AWH denatured by boiling and the level of IgE and IgG1 assessed at three weeks post treatment. As shown in fig. 4A, denaturation of AWH by boiling resulted in ablation of the polyclonal IgE response. There was, however, a slight increase, although not significant ($p>0.05$), in IgG1 levels in the serum of mice injected with the denatured AWH over the level observed in mice that received the vehicle (FIA) alone. However, this increase was always lower than that observed in the serum of AWH-treated mice (fig. 4B).

Nb is a well characterized inducer of type-2 immune response (Finkelman et al 1997; Urban et al 1998b). To verify whether the induction of increased IgE and IgG1 levels by AWH is specific to these type-2 immunoglobulins, the levels of IgG2a, a type-1 immunoglobulin isotype, was also examined. The level of IgG2a in the serum of the AWH injected mice was similar to that observed in the mice that were injected with the vehicle control (fig. 5). These observations show that

Figure 3. LWH induces IgE and IgG1 production in the serum of BALB/c mice. Female BALB/c mice in groups of three were injected subcutaneously with LWH or AWH both emulsified in Freund's incomplete adjuvant. The third group was injected with the vehicle alone. Mice in each group were bled for serum at 14 and 21 days post treatment. Serum samples obtained were pooled within each group. Serum samples were assayed for IgE and IgG1 levels by capture ELISA. Data shown is the IgE **(A)** and IgG1 **(B)** levels at 21 days post treatment. Results are expressed as mean ± SD of triplicate wells and are representative of three experiments (** $p < 0.01$; *** $p < 0.001$, one-way ANOVA).

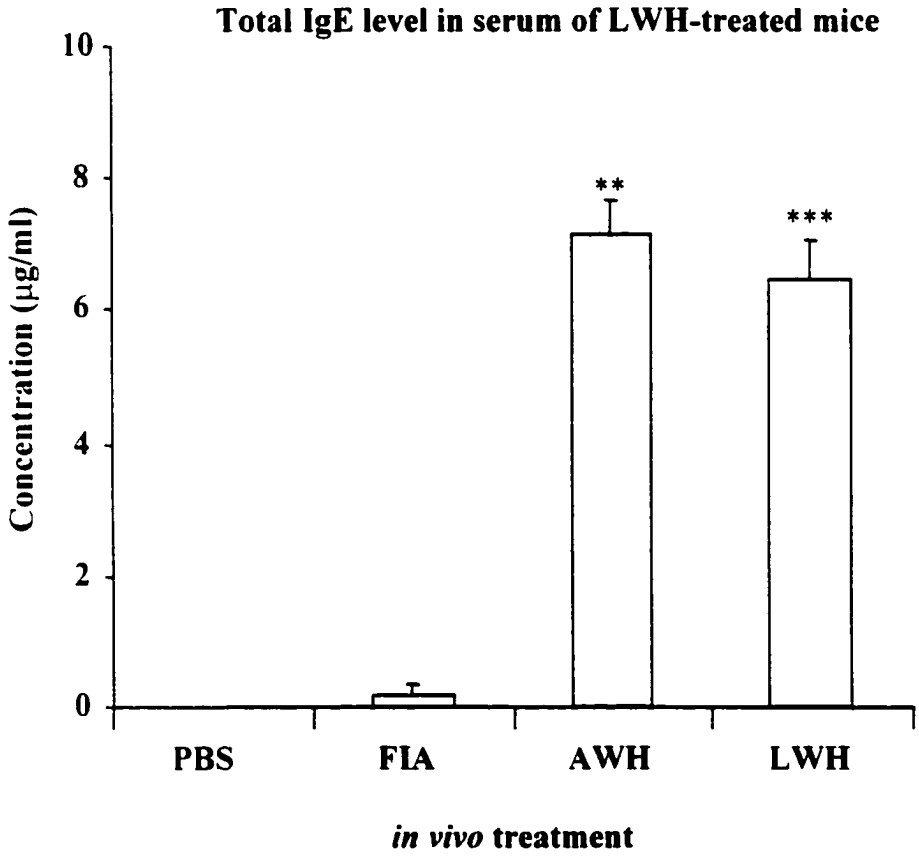

Total IgE level in serum of LWH-treated mice
Concentration (μg/ml)
10
8
6
4
2
0
**
***
PBS
FIA
AWH
LWH
in vivo treatment

Figure 3A

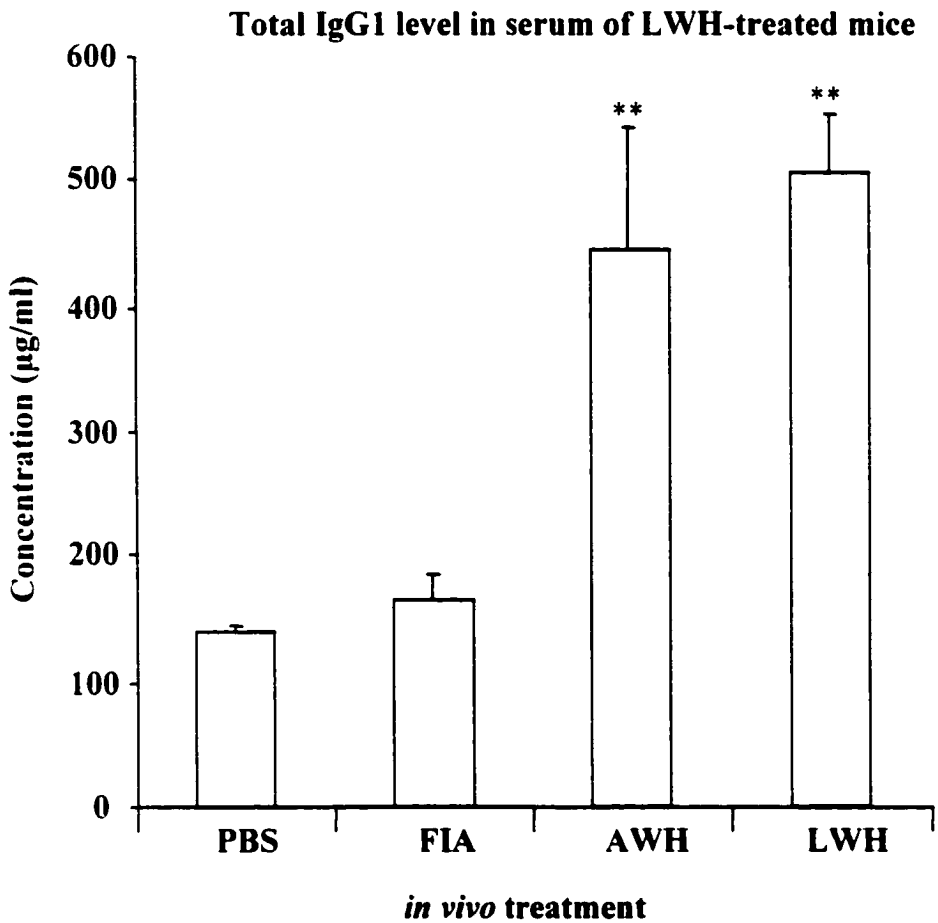

Total IgG1 level in serum of LWH-treated mice
600
500
400
300
200
100
0
Concentration (μg/ml)
**
**
PBS
FIA
AWH
LWH
in vivo treatment

Figure 3B

Figure 4. IgE and IgG1 levels in the serum of mice injected with boiled AWH. Female BALB/c mice in groups of three were injected subcutaneously with Boiled AWH or untreated AWH both emulsified in Freund's incomplete adjuvant. The third group was injected with the vehicle alone. Mice in each group were bled for serum at 14 and 21 days post treatment. Serum obtained was pooled within each group. Serum samples were assayed for IgE and IgG1 levels by capture ELISA. Data shown is the IgE **(A)** and IgG1 **(B)** levels at 21 days post treatment. Results are expressed as mean ± SD of triplicate wells and are representative of three experiments (fig. 4A ** $p < 0.003$, two-tailed, unpaired Student's T-test; fig 4B * $p < 0.05$, one-way ANOVA).

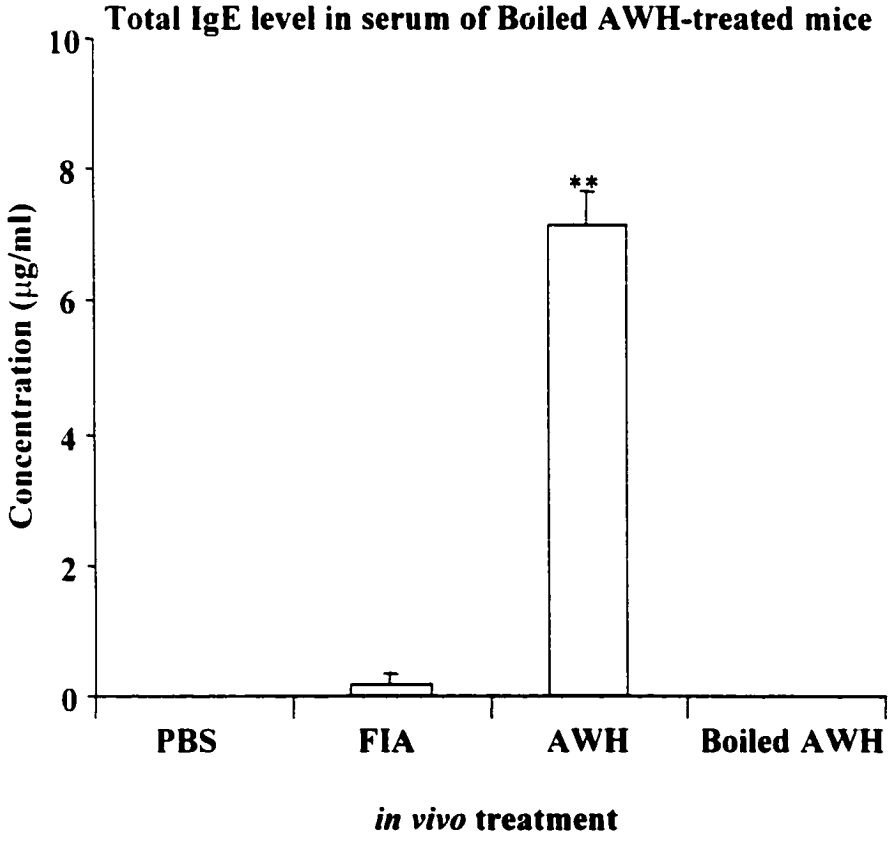

Total IgE level in serum of Boiled AWH-treated mice
Concentration (μg/ml)
10
8
6
4
2
0
**
PBS
FIA
AWH
Boiled AWH
in vivo treatment

Figure 4A

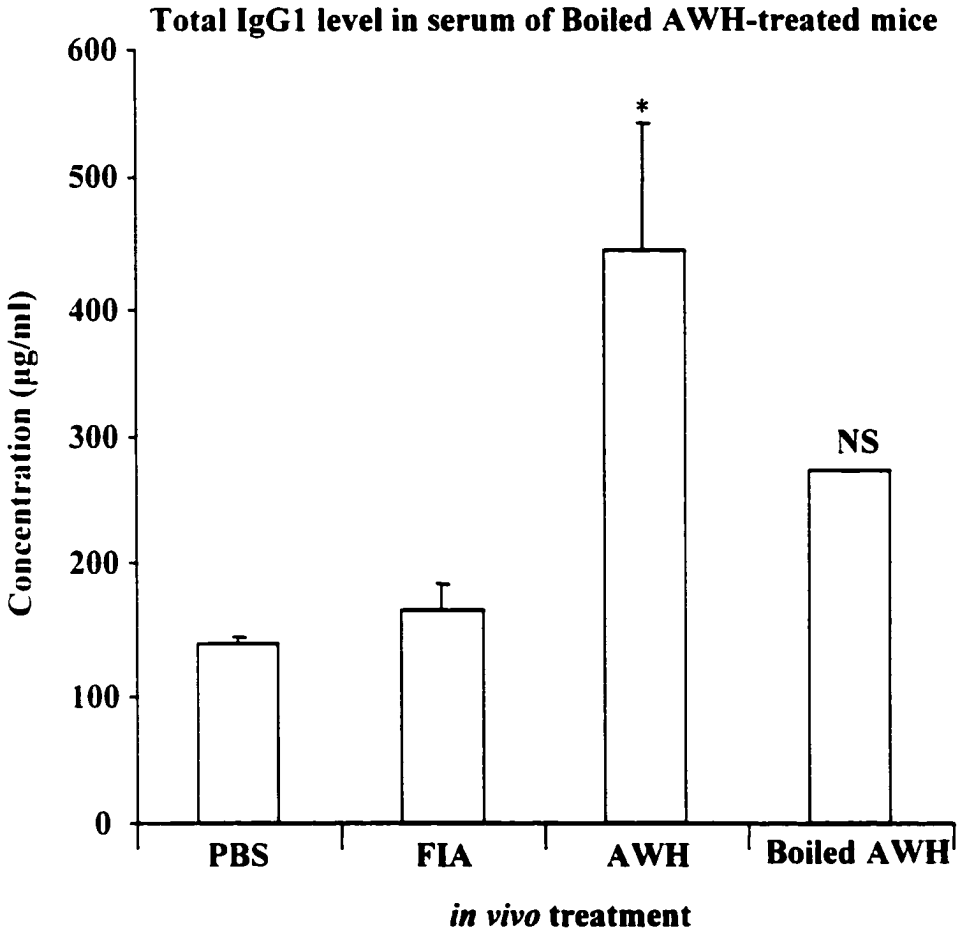

Total IgG1 level in serum of Boiled AWH-treated mice
600
500
400
300
200
100
0
Concentration (µg/ml)
*
NS
PBS
FIA
AWH
Boiled AWH
in vivo treatment

Figure 4B

Figure 5. Serum IgG2a levels is unaffected by AWH treatment.

Serum was obtained from mice treated as in figure 1. The level of IgG2a in the serum was determined by capture ELISA. The data are from one experiment and are representative of four such experiments.

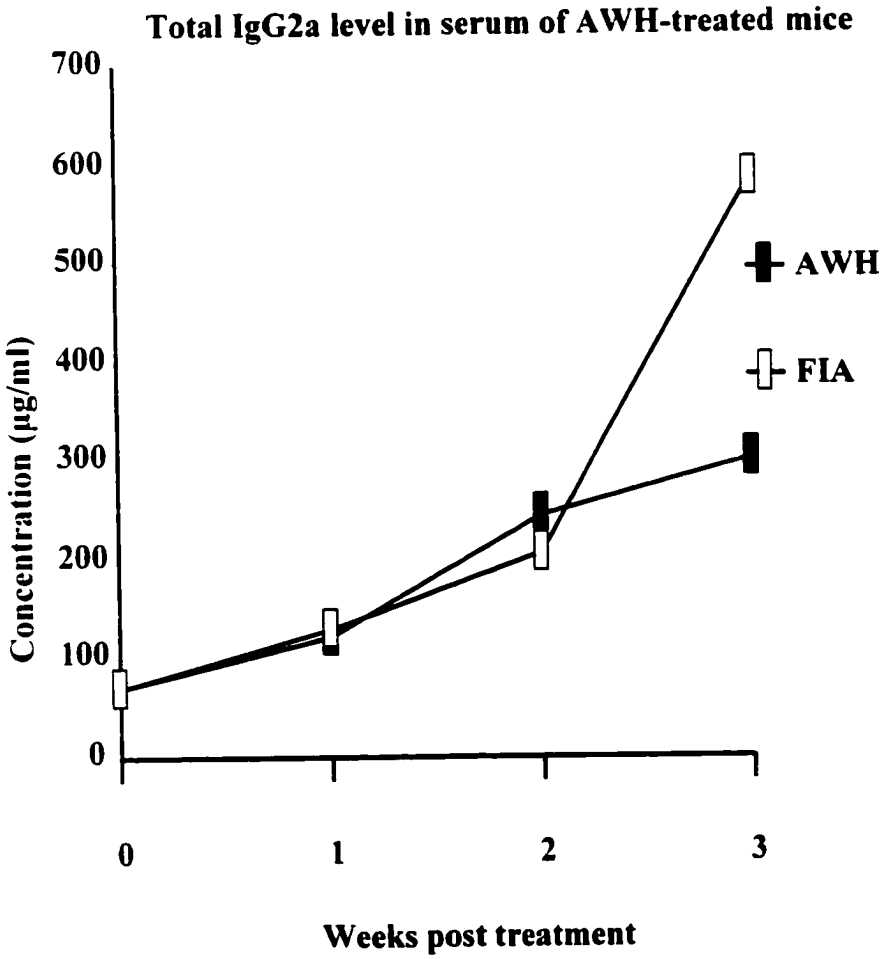

Total IgG2a level in serum of AWH-treated mice
700
600
500
400
300
200
100
0
Concentration (µg/ml)
AWH
FIA
0
1
2
3
Weeks post treatment

Figure 5

AWH induces an immune response that is biased toward type-2 immunoglobulins, similar to our observations with *Nb* infection.

The kinetics of the boosting response and the evidence from the lack of increase in total IgE with boiled AWH suggest that the increase in type-2 immunoglobulins is not simply a response to a complex antigen. To confirm this, mice were injected with killed *Mycobacteria* in the same vehicle as AWH, and the levels of IgE, IgG1 and IgG2a assessed. The Ig response to *Mycobacteria* is characterized by increased IgG2a levels. As expected, mice injected with *Mycobacteria* showed higher levels of IgG2a in the serum than control animals, from 150 μg/ml to about 450 μg/ml. In the same experiment, mice injected with AWH showed the same level of IgG2a as naive (untreated) or FIA injected mice (fig. 6A). In contrast to IgG2a, IgE was not detected in the serum of mice injected with *Mycobacteria* but it was detected in significant amounts in the serum of mice injected with AWH, reaching a level of 20 μg/ml (fig. 6B). Also, the level of IgG1 measured in the serum of *Mycobacteria*-treated mice was not elevated over the mice injected with the vehicle control or the serum of naive (untreated) mice (fig. 6C). These data provide strong evidence that the increased type-2 immunoglobulin response associated with AWH is specifically induced by the extract and not just a response to the injection of a complex antigen mixture.

Figure 6. Increased IgE and IgG1 response is specific to AWH treatment. BALB/c mice in groups of three were injected with killed *Mycobacteria* (Myc) or AWH emulsified in Freund's incomplete adjuvant (vehicle). The third group received vehicle alone. Mice in each group were bled for serum at 14 and 21 days post treatment. Serum samples obtained were pooled within each group. Serum samples were assayed for IgG2a, IgE and IgG1 levels by capture ELISA. Data shown are the IgG2a **(A)**, IgE **(B)** and IgG1 **(C)** levels at 21 days post treatment. Results are expressed as mean ± SD of triplicate wells and are representative of three experiments (fig. 6A and C, *** $p < 0.001$, NS = not significant $p > 0.05$, one-way ANOVA; fig 6B *** $p < 0.0004$, two-tailed, unpaired Student's T-test).

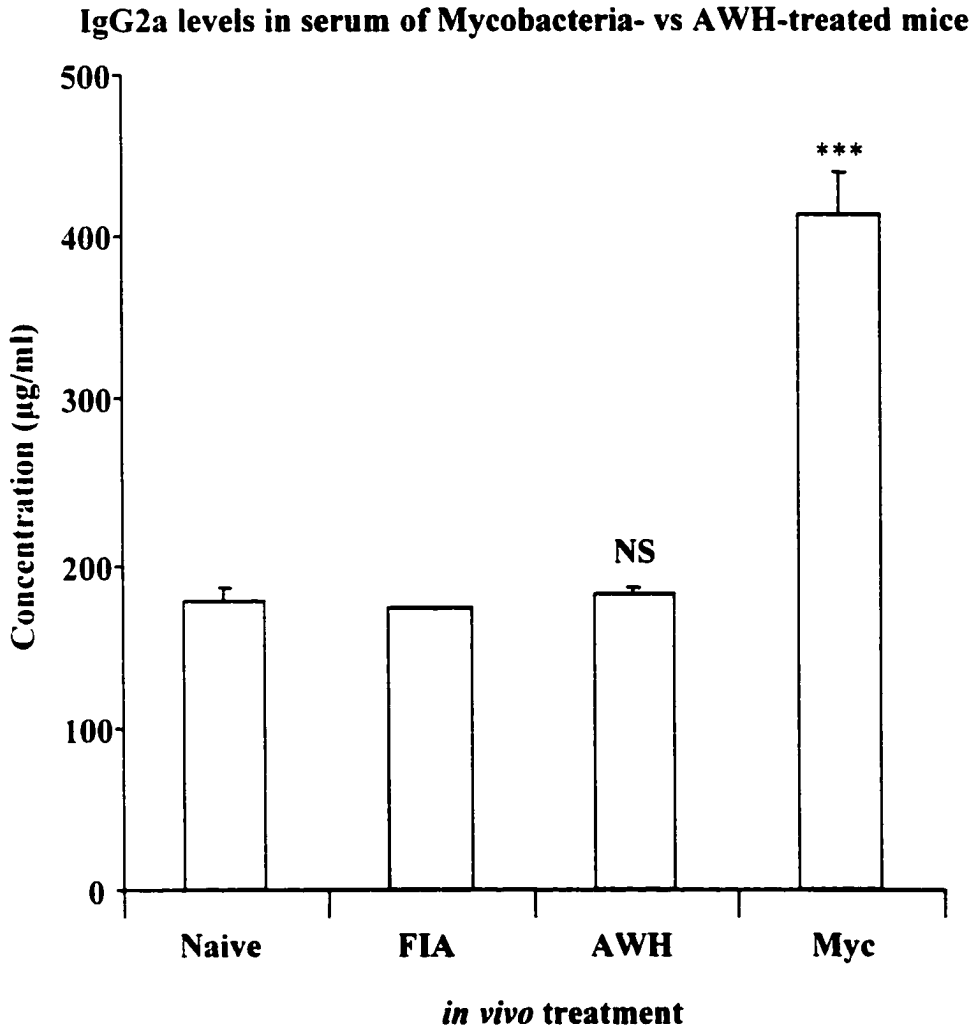

IgG2a levels in serum of Mycobacteria- vs AWH-treated mice
500
400
300
200
100
0
Concentration (µg/ml)
***
NS
Naive
FIA
AWH
Myc
in vivo treatment

Figure 6A

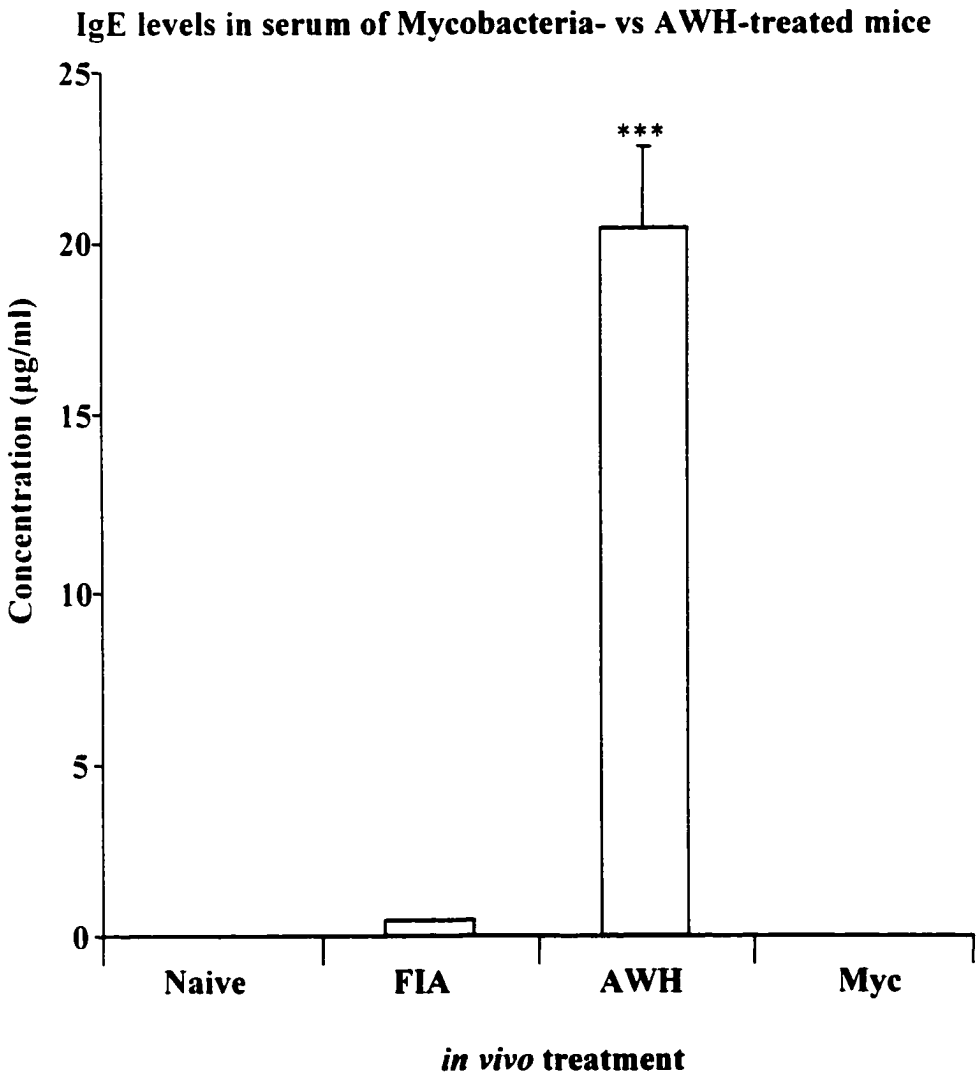

IgE levels in serum of Mycobacteria- vs AWH-treated mice
25
20
15
10
5
0
Concentration (µg/ml)
***
Naive
FIA
AWH
Myc
in vivo treatment

Figure 6B

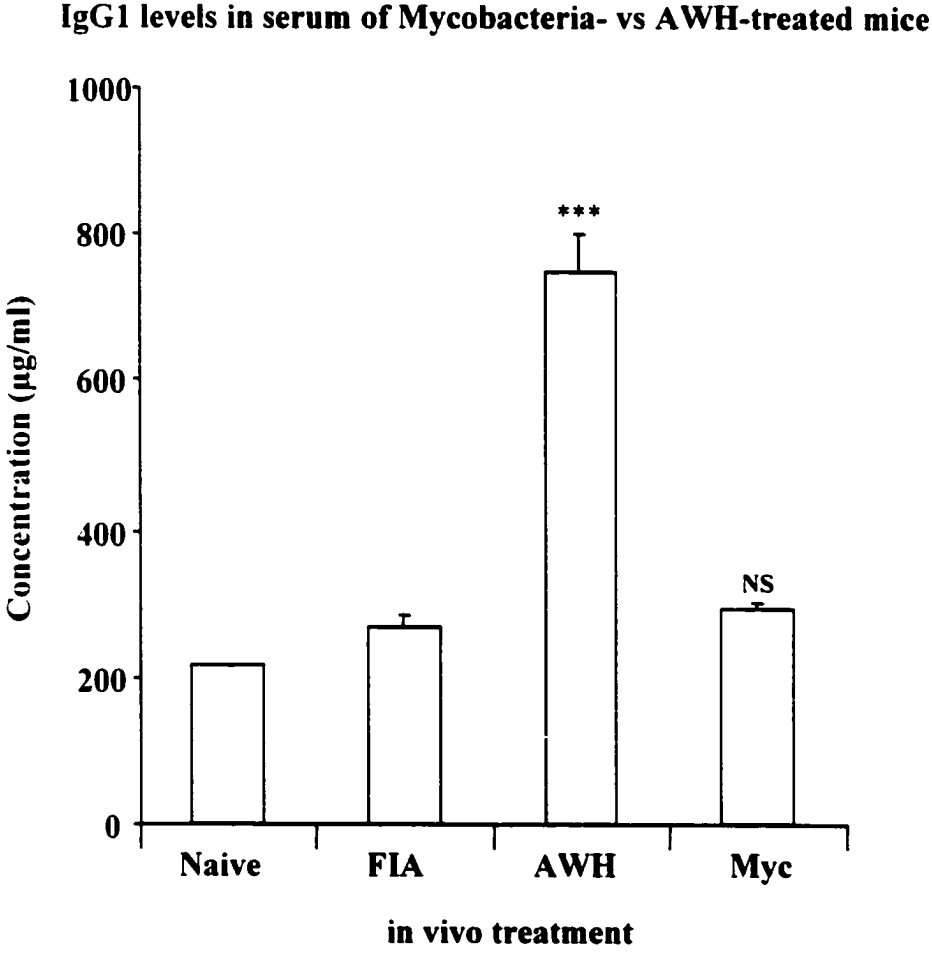

IgG1 levels in serum of Mycobacteria- vs AWH-treated mice
1000
800
600
400
200
0
Concentration (µg/ml)
***
NS
Naive
FIA
AWH
Myc
in vivo treatment

Figure 6C

4.1.2 AWH induces IL-4 and IL-13 production

Increased IgG1 and IgE levels are associated with IL-4 and IL-13 gene transcription and protein secretion. In nematode infections the levels of mRNA for these cytokines has been demonstrated to be increased in both spleen and mesenteric lymph node cells. This observation has been reported during both early and late phases of nematode infection (Svetic'et al 1993; Uchikawa et al 1994; Arizono et al 1994; Matsuda et al 1995; Lawrence et al 1996; Ishikawa et al 1998). If our hypothesis regarding the increase in IgE and IgG1 activity induced by AWH is correct we would expect that the increased levels of these immunoglobulins would likewise be associated with an increase in the levels of these cytokines. To investigate this possibility, spleen cells from mice treated with AWH were analyzed for IL-4 and IL-13 mRNA expression and for IL-4 protein secretion. For mRNA analysis, total RNA was isolated and reverse transcribed to cDNA with random hexamers. Sequences of IL-4 and IL-13 genes were amplified in a PCR reaction with primers specific for these cytokines. β-actin expression served as a control for equal cDNA loading and for a basis of semi-quantitative analysis of the PCR products. IL-4 mRNA expression was detected in spleen cells from both *Nb* and AWH treated mice (fig. 7A). In agreement with the data regarding levels of IgE and IgG1 in serum, the cytokine mRNA level was higher in *Nb* infected animals than in AWH treated mice. IL-4 mRNA was not detectable in the spleen cells of naive (untreated) mice.

To assess whether the IL-4 mRNA is translated and IL-4 is secreted, spleen cells isolated from mice, two and three weeks post treatment, were stimulated with con A *in vitro*. Supernatants were collected and analyzed for IL-4 levels by ELISA. IL-4 was detected in the supernatants of con A-stimulated spleen cells from both *Nb* and AWH treated mice (fig. 7B), but not in supernatants of cells from naive control mice. The level was significantly higher ($p<0.001$) in *Nb* infected (620 pg/ml) than in AWH injected mice (110 pg/ml).

IL-13 mRNA was detected in spleen cells from both *Nb* and AWH treated mice (fig. 7C) but not in spleen cells from naive, control mice. Similar to IL-4 mRNA expression, the level of IL-13 mRNA expression was higher in *Nb* infected animals than in AWH treated mice. We did not analyze for IL-13 protein production due to lack of ELISA reagents.

To assess whether the observed increase in IL-4 mRNA and protein, as well as IL-13 mRNA, in spleen cells from AWH-treated mice was induced specifically by the injection of AWH, and not a non-specific effect of treatment with complex antigen mixture, mice were injected with *Mycobacteria* as described above. The level of IL-4 in the culture supernatants of con A stimulated spleen cells from *Mycobacteria* injected mice was compared to the level in the supernatants of con A stimulated spleen cells from AWH injected mice. Since both AWH and *Mycobacteria* preparations are in a similar vehicle, to assess the effect of the specific agents, this data was normalized for the vehicle. As shown in fig. 8,

Figure 7. IL-4 and IL-13 are induced by AWH. Spleen cells were isolated from mice 14 or 21 days post treatment with AWH or *Nb*. For assessment of IL-4 (**7A**) and IL-13 (**7C**) mRNA expression, total cellular RNA was isolated with TRIZOL, and was reverse-transcribed into single-stranded cDNA using M-MLV reverse transcriptase and random hexamers as described in the Materials and Methods. The resulting cDNA template was used in a PCR reaction using mouse primers specific for either IL-4 or IL-13. β-Actin mRNA levels were also determined by RT-PCR to control for equal RNA loading. PCR amplicons were resolved on a 1.5% agarose gel with ethidium bromide staining. Molecular weight marker is the 100 bp DNA ladder.

For assessment of protein secretion **(7B)**, Spleen cells isolated from naive (untreated), AWH or *Nb* treated mice 14 or 21 days post treatment were stimulated with con A (5 μg/ml) for 24 or 48 hours. Culture supernatants were then analyzed by ELISA for IL-4 levels as described in the Materials and Methods. Results are expressed as the mean concentration of IL-4 (pg/ml) ± the standard deviation of three replicate wells (*** $p < 0.001$, one-way ANOVA). ND = Below detection limit (15 pg/ml). Results are representative of three experiments.
Nv = Naïve (untreated); Nb = *N. brasiliensis;* AWH = *Nb* extract. Negative control lanes in 7C are: no enzyme in RT reaction; no RNA in RT reaction; no cDNA in PCR; no primers in PCR controls.

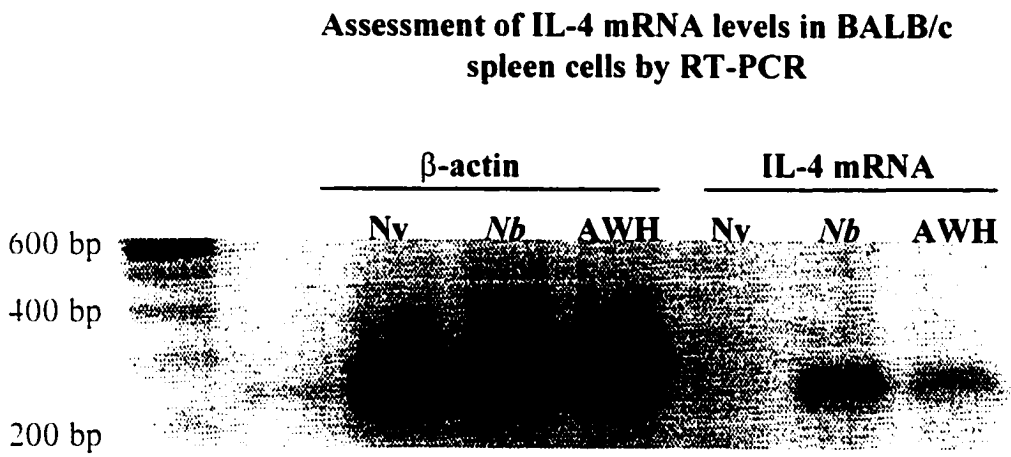

Assessment of IL-4 mRNA levels in BALB/c spleen cells by RT-PCR
β-actin
IL-4 mRNA
Ny
Nb
AWH
Ny
Nb
AWH
600 bp
400 bp
200 bp

Figure 7A

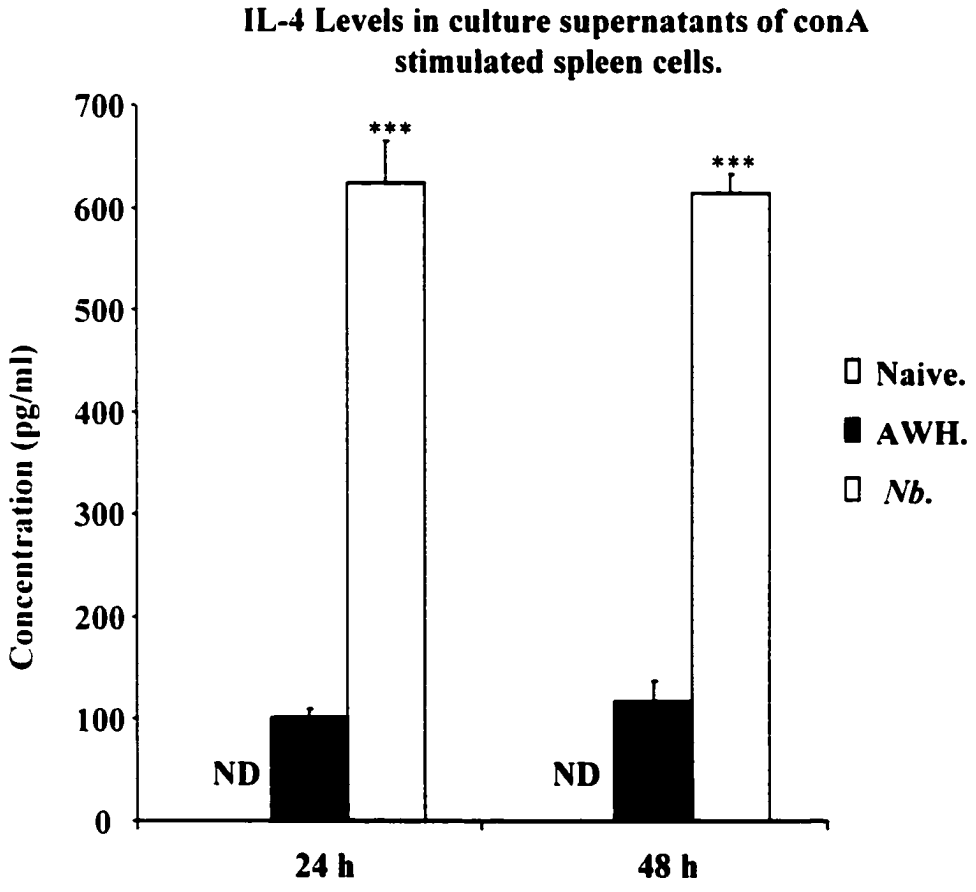

IL-4 Levels in culture supernatants of conA stimulated spleen cells.
Concentration (pg/ml)
700
600
500
400
300
200
100
0
***
***
ND
ND
24 h
48 h
Naive.
AWH.
Nb.

Figure 7B

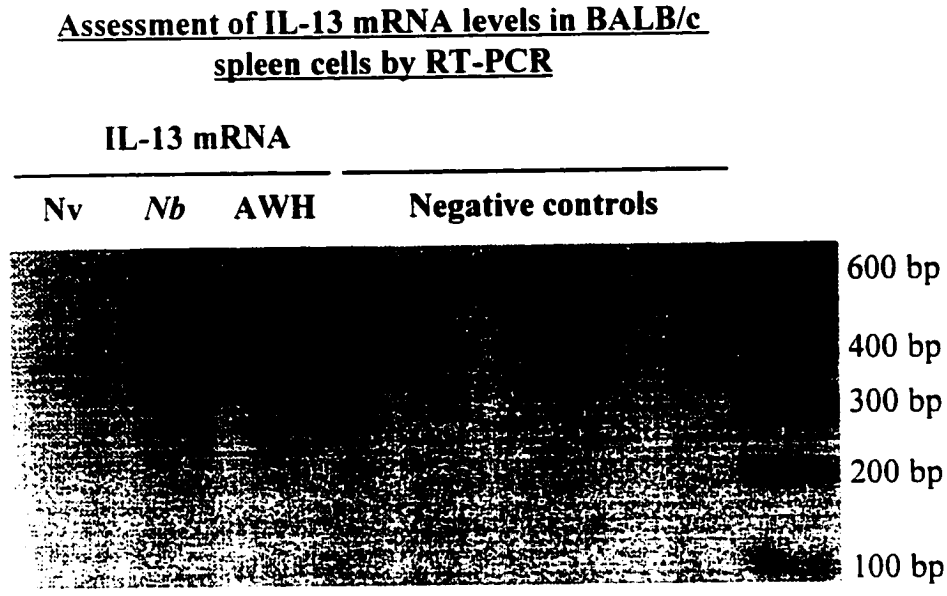

Assessment of IL-13 mRNA levels in BALB/c spleen cells by RT-PCR
IL-13 mRNA
Nv
Nb
AWH
Negative controls
600 bp
400 bp
300 bp
200 bp
100 bp

Figure 7C

substantial levels of IL-4 (>120 pg/ml) were observed in the culture supernatants of con A stimulated spleen cells from AWH injected mice whereas < 20 pg/ml of IL-4 was present in the supernatants from *Mycobacteria* treated mice. This data mirrors the immunoglobulin levels observed in the serum of both groups of animals shown earlier in figure 6. These data confirm that the IL-4 and IL-13 mRNA and IL-4 protein induced in the spleen cells of mice treated with AWH are mediated specifically by the extract and are not simply a "response to antigen" effect.

4.1.3 AWH induces IgG1 production in *in vitro* B cell culture

Since it was observed in the *in vivo* experimentation above that AWH induces an increase in IgE and IgG1 levels in mouse serum, the next step was to address the mechanism by which this effect is mediated. A first step was to reproduce the *in vivo* results *in vitro*. To determine whether AWH could induce B cells in culture to produce IgE and IgG1 purified B cells were isolated from the spleen of either normal or nude BALB/c mice and stimulated with LPS, then incubated in the presence of AWH for 7 days. Supernatants from these cultures were assayed for IgE and IgG1 levels by capture ELISA. Due to the level of detection sensitivity of this assay, only IgG1 could be detected with complete confidence. As a positive control, LPS-stimulated B cell cultures were incubated with IL-4. B cells stimulated with LPS, secrete significant amounts of IgM, but in

Figure 8. Increased IL-4 response in spleen cell cultures is specific to AWH treatment *in vivo*. BALB/c mice in groups of three were injected with killed *Mycobacteria* (Myc) or AWH emulsified in Freund's incomplete adjuvant (FIA: vehicle). The third group received vehicle alone. Spleen cells isolated from each group of mice 14 or 21 days post treatment were stimulated with con A (5 μg/ml) for 24 or 48 hours. Culture supernatants were then analyzed by ELISA for IL-4 levels as described in the Materials and Methods. Data presented has been normalized for vehicle. Results are expressed as the mean concentration of IL-4 (pg/ml) ± the standard deviation of three replicate wells. Results are representative of two separate experiments.

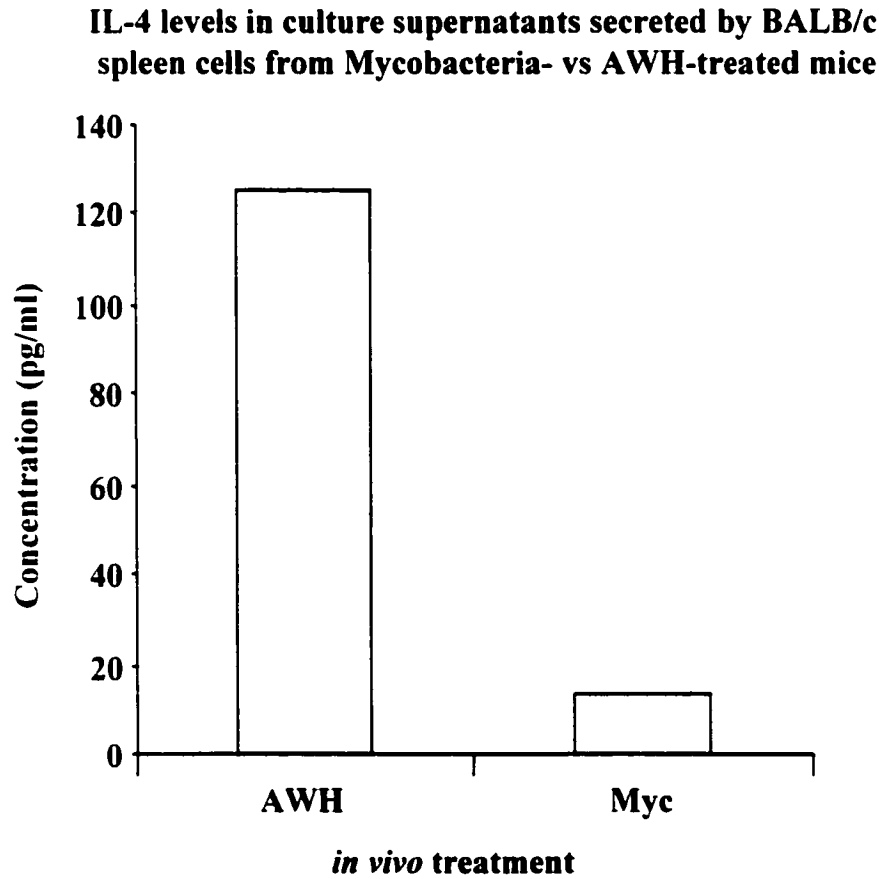

IL-4 levels in culture supernatants secreted by BALB/c spleen cells from Mycobacteria- vs AWH-treated mice
Concentration (pg/ml)
140
120
100
80
60
40
20
0
AWH
Myc
in vivo treatment

Figure 8

the presence of IL-4, the cells undergo class switch due to rearrangement of the heavy chain constant region genes to become IgG1 or IgE secreting B cells. IL-4, the switch factor of IgG1 and IgE, induced an increase in IgG1 levels in the B cell culture supernatants (fig. 9A). AWH also induced an increase in IgG1 levels in cultures of LPS stimulated B cells (fig. 9A). This increase was not observed in cultures of naive B cells stimulated with AWH alone, neither did LPS alone induce IgG1 production in B cells, in the absence of AWH. In addition, the induction of increased IgG1 levels in the B cell culture was associated with a decrease in the levels of IgM in the culture supernatants (fig. 9B). These data suggest that AWH, like IL-4, induce LPS stimulated B cells to undergo class switch from IgM to IgG1 producing cells.

4.1.4 Mechanisms involved in the increased IgG1 levels in AWH treated mice

The data in figures 1a-b, 2 and 9a clearly show that AWH increases specific immunoglobulin levels. Although we are hypothesizing that this results from increased *de* novo class switch it could be due, in part, to either an upregulation of antibody production by memory cells or expansion of IgG1-producing B cells. To investigate the possible mechanisms, we first addressed the question of whether the increase in IgG1 production after AWH treatment in mice, correlates with an increase in the number of IgG1 switched cells. To do this, we adopted a molecular

Figure 9. Influence of AWH on immunoglobulin production in *in vitro* B cell culture. Highly purified naive B cells isolated as described were stimulated in culture containing AWH (20 μg/ml) alone, 10 μg/ml LPS alone, or LPS in combination with AWH (20 μg/ml) or IL-4 (5 ng/ml). Culture supernatants were harvested 7 days later and then analyzed for IgG1 **(A)**, and IgM **(B)** levels using antibody capture ELISA as described in the Materials and Methods. Results are expressed as the mean concentration ± the standard deviation of three replicate wells and are representative of six separate experiments (fig. 9A, *** $p < 0.001$, one-way ANOVA).

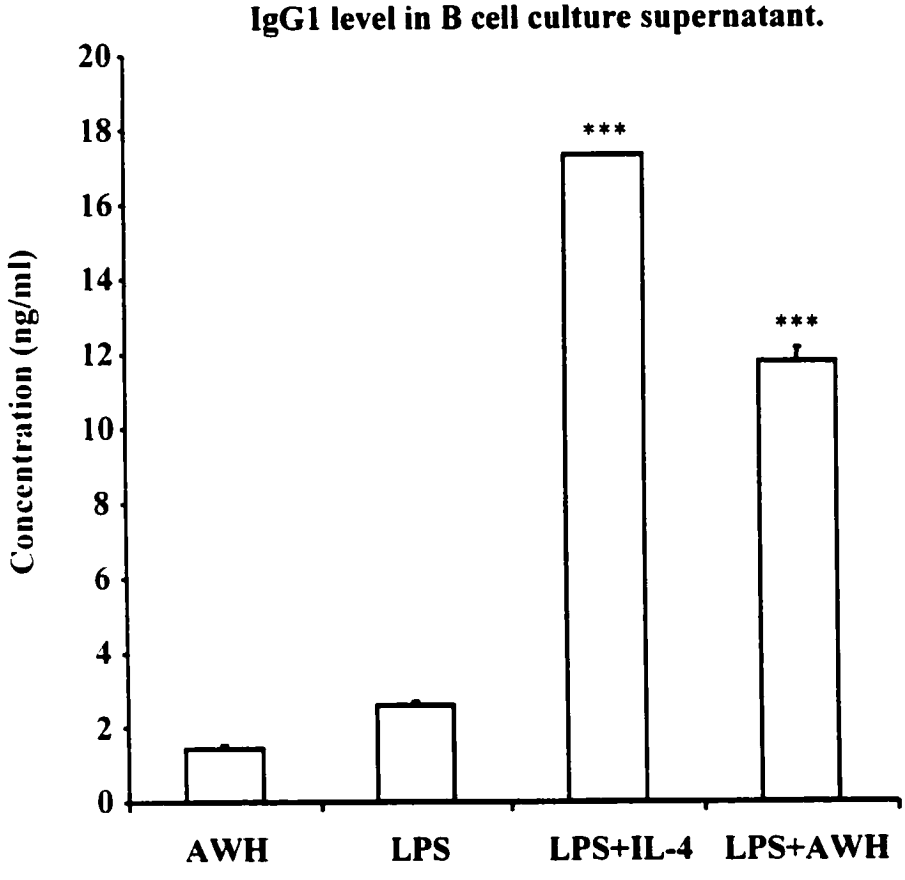

IgG1 level in B cell culture supernatant.
Concentration (ng/ml)
20
18
16
14
12
10
8
6
4
2
0
***
***
AWH
LPS
LPS+IL-4
LPS+AWH

Figure 9A

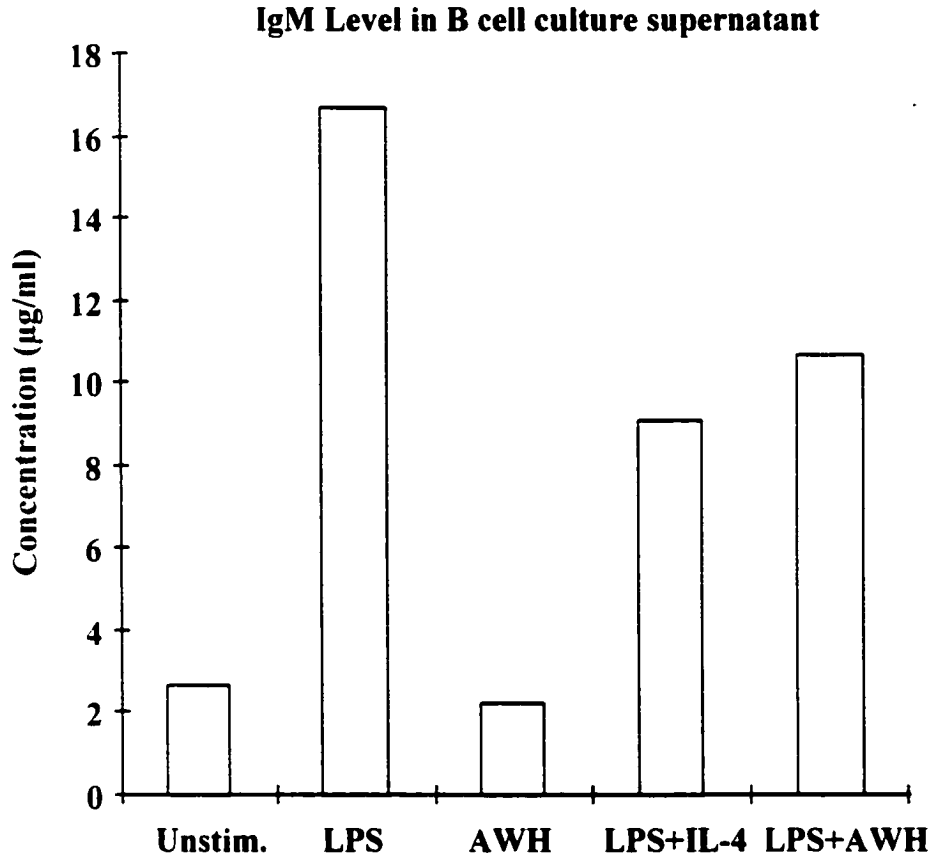

IgM Level in B cell culture supernatant
Concentration (µg/ml)
18
16
14
12
10
8
6
4
2
0
Unstim.
LPS
AWH
LPS+IL-4
LPS+AWH

Figure 9B

approach that allowed for the assessment (semi-quantitatively) of switched DNA recombination events. Before employing this technique to address the possible mechanisms involved in the increase in IgG1 levels observed in *Nb* or AWH-treated mice, the technique was first optimized. This was carried out by amplifying DNA for the nicotinic acetylcholine receptor (nAChRe) gene from naive BALB/c spleen cells using the primers described in the materials and method section. This gene is present in all cells and thus allows for quantitation of the Sμ-Sγ1 PCR amplicon.

In this experiment, genomic DNA isolated from BALB/c spleen cells was digested with EcoR1, ligated with T4 DNA Ligase and then amplified. The PCR product for nAChRe upon gel electrophoresis showed that the amplicon was between 700 and 800 bp in size (fig. 10). This product is similar in size to the 753 bp product previously reported (Chu et al 1992). To confirm the identity of the amplicon, the amplicon was sequenced and 117 nucleotide bases read from the autoradiograph. This partial sequence was queried to Genbank and the report analysis showed a 100% match to mouse nicotinic acetylcholine receptor beta subunit gene (fig. 11). As further confirmatory evidence that the DC-PCR technique had adequately generated the expected amplicon, we examined the amplicon for the location of the one EcoR1 restriction site. To do this, the amplicon was first purified from the PCR reagents with a Gibco PCR product purification kit, digested with EcoR1 and then electrophoresed on a 2% agarose

gel. The expected sizes of the two EcoRI fragments would be 600-700 bp and 50-150 bp. Fig. 12 shows the results of the restriction digest. A large fragment of about 650 bp in size is clearly visible. The smaller fragment could not be adequately resolved using these gel techniques.

Having demonstrated the specificity of DC-PCR in the amplification of nAChRe gene, examination of genomic DNA from an IgG1-producing hybridoma (TSI-18; positive control) and an IgG2a-producing hybridoma (IB4; negative control; hybridomas were obtained from Dr. A. Issekutz, Dalhousie University, Halifax), was undertaken. This experiment was designed to confirm the specificity of the DC-PCR technique for IgG1 class switch. The TSI-18 hybridoma, by its clonal nature, is homogenous for switched γ1. The IB4 hybridoma does not have γ1 rearrangement but has undergone γ2a rearrangement. Using previously published primers (Chu et al 1992) and DC-PCR, the presence of an amplicon in DNA from TSI-18 similar to the 219 bp product for switched γ1 DNA reported previously using these primers, was detected. This amplicon was not detected by DC-PCR from IB4 hybridoma (fig. 13). This observation confirms that DC-PCR can specifically assess switch DNA recombination, allowing us to assess whether increased class switch is a mechanism involved in the increased type-2 Ig production associated with nematode infection or nematode products.

To ascertain whether the increase in IgG1 production *in vivo* after AWH treatment is mediated, at least in part, by an increase in the number of IgG1

Figure 10. Detection of nAChRe gene in naive spleen cells by DC-PCR. Genomic DNA was isolated from spleen cells of naive mice as described in the Materials and Methods. DNA was digested with EcoR1, ligated with T4 DNA Ligase and amplified by PCR using primers specific for the mouse nicotinic acetylcholine receptor gene. Each lane represents template from the spleen of an individual mouse used in the reaction. PCR amplicons were resolved on a 1.5% agarose gel with ethidium bromide staining. Results are representative of ten experiments.

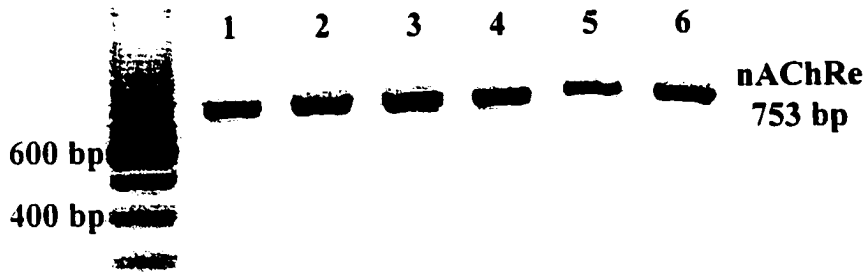


Figure 10

Figure 11. Sequencing analysis of nAChRe PCR product. The amplicon was excised from the gel, purified and then re-amplified. The resultant product was gene cleaned and then sequenced as described in the Materials and Methods. Nucleotide bases read off the autoradiograph were analyzed at the Genbank nucleotide sequence depository.

```
>gb|J04699|MUSACHRBB Mouse nicotinic acetylcholine receptor beta subunit\
          (nAChRE) gene, complete cds.
          Length = 9722

 Minus Strand HSPs:

Score = 585 (161.6 bits), Expect = 4.3e-41, P = 4.3e-41
Identities = 117/117 (100%), Positives = 117/117 (100%), Strand = Minus

Query:   117 CTGTGATGTCCGTTAGATAGTCAGCAACAACAAAATGAGTCGGCATCTGAATGTGAGCC
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  5787 CTGTGATGTCCGTTAGATAGTCAGCAACAACAAAATGAGTCGGCATCTGAATGTGAGCC

Query:    57 GGTGTTCAGAGGAAGTCTGAGAAATGGCTGGGTGAGAGAGATTTGCATACAAATGGC 1
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  5847 GGTGTTCAGAGGAAGTCTGAGAAATGGCTGGGTGAGAGAGATTTGCATACAAATGGC 5
```

Figure 11

Figure 12. Restriction analysis of nAChRe PCR product with EcoR1. PCR product amplified from DNA isolated from spleen cells of *Nb* infected mice and TSI-18 hybridoma were purified and then subjected to digestion with EcoR1 (+EcoR1) in 25 µl reaction volume for 2-3 hours at 37°C. As control, the template was incubated in reaction buffer without the enzyme (-EcoR1). Digestion products were resolved on a 2% agarose gel with ethidium bromide staining.

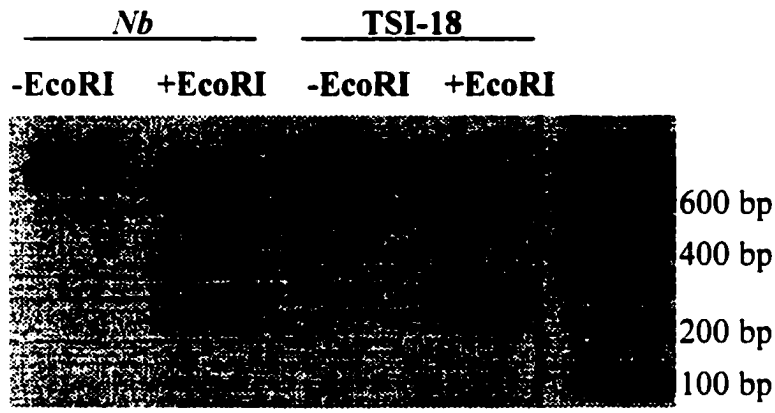


Figure 12

Figure 13. Detection of IgG1 switch recombination (Sμ-Sγ1) in TSI-18 hybridoma by DC-PCR. Genomic DNA was isolated from TSI-18 and IB4 hybridomas as described in the Materials and Methods. The DNA was digested with EcoR1, ligated with T4 DNA Ligase and amplified by PCR using primers specific for the recombined switch regions. nAChRe levels in TSI-18 were also determined by DC-PCR to control for equal template loading and allow for semi-quantitation (comparison) of the Sμ-Sγ1 products. PCR amplicons were resolved on a 1.5% agarose gel with ethidium bromide staining. Results are representative of three experiments.

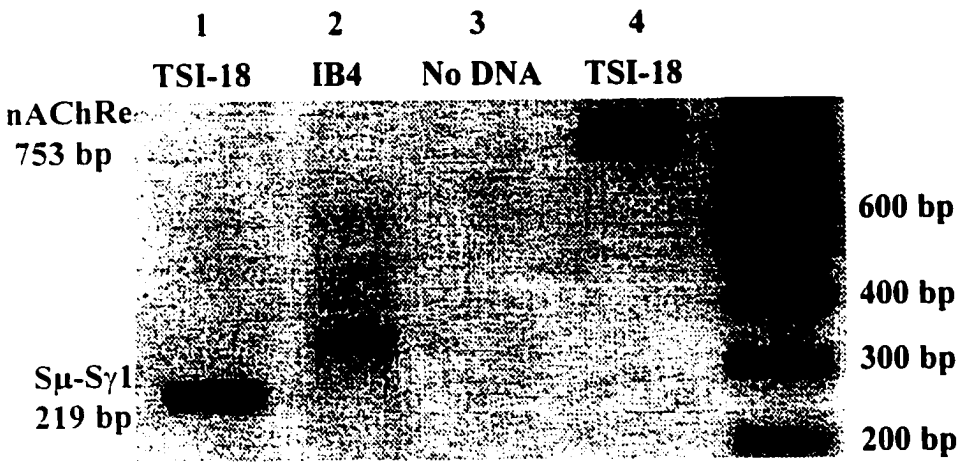

1
2
3
4
TSI-18
IB4
No DNA
TSI-18
nAChRe
753 bp
Sμ-Sγ1
219 bp
600 bp
400 bp
300 bp
200 bp

Figure 13

switched cells, genomic DNA isolated from the spleen cells of mice injected with either AWH or *Nb* was examined for IgG1 switch region recombination events as above. Control mice were injected with vehicle (PBS or FIA) alone. Amplicons with a size of 219 bp, similar in size to the amplicons detected by DC-PCR of the IgG1 secreting hybridoma, were detected after DC-PCR was performed on spleen cells from *Nb* infected animals (fig. 14). This indicates a significant level of switched (to IgG1) B cells in the spleens of these animals. An amplicon of much smaller intensity was seen after DC-PCR from control mice. The presence of an amplicon in these mice was not unexpected since normal mice constitutively produce IgG1 in response to environmental challenge. The animals treated with AWH showed amplicons of similar size, and nearly the same intensity as the *Nb* treated animals indicating a dramatic increase in switched B cells over control animals. nAChRe levels were also determined by DC-PCR to control for equal template loading and allow for semi-quantitation of the Sμ-Sγ1 products. These data suggest that the increase in total IgG1 levels observed in the serum of mice injected with either *Nb* or AWH, is associated with increased DNA recombination, resulting in increased switched B cells. Because we were unsuccessful at sequencing the amplicon, the identity of the product was verified by endonuclease restriction analysis with EcoR1. The process is expected to generate two fragments of 141 and 78 bp. As shown in fig. 15, the amplicon was adequately digested by the restriction enzyme. However, these small pieces are not visible on

the gel as they could not be resolved adequately due to insufficient amount.

As with IgG1 levels in the serum, we also assessed the extent of IgG1 switch recombination in *Mycobacteria* treated mice in comparison to the level observed in AWH treated mice. Fig. 16 demonstrates that there is no higher level of switched γ1 DNA in the *Mycobacteria* treated mice than in the vehicle alone controls. This observation confirms the earlier conclusion that AWH induction of increased IgG1 level is a specific effect, rather than a non-specific stimulation of immunoglobulin response.

The observation that an increase in reaginic antibody induced by nematodes is not associated with expansion of IgG1 switched cells determined by DC-PCR, confirms that upregulation of antibody production by IgG1 and IgE memory B cells is not the major mechanism by which nematodes and nematode extracts mediate increased type-2 immunoglobulin production. However, it remains possible that the observed effect is due to the expansion of a population of committed IgG1 memory B cells rather than the *de novo* class switch of B cells. Therefore we examined whether there was expansion of IgG1 memory B cells *in vitro* in the presence of AWH. To address this, B cells from control mice were stimulated with LPS and cultured in the presence of AWH. If AWH mediates its effects by expansion of an IgG1 memory population, a significant increase in proliferation should be observed. Since higher levels of IgG1 are found both *in vivo* and under these *in vitro* conditions, this experiment appropriately addresses

Figure 14. AWH induced IgG1 production is associated with increase in the number of IgG1 switched cells. Genomic DNA was isolated from spleen cells of mice treated with either AWH, *Nb* or FIA as described in the Materials and Methods. DNA was digested with EcoR1, ligated with T4 DNA Ligase and amplified by PCR using primers specific for the recombined IgG1 switch regions. nAChRe levels were also determined by DC-PCR to control for equal template loading and for semi-quantitation of the Sμ-Sγ1 products. PCR amplicons were resolved on a 1.5% agarose gel with ethidium bromide staining. Results are representative of six experiments.

Detection of IgG1 switch recombination in BALB/c spleen cells by DC-PCR

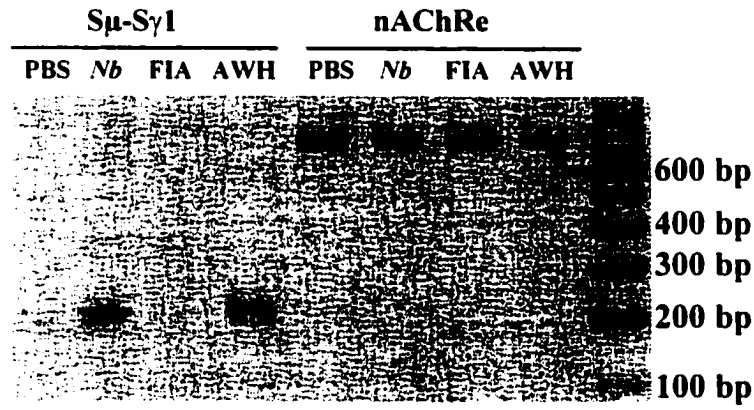


Figure 14

Figure 15. Restriction analysis of Sμ-Sγ1 PCR product with EcoR1. PCR products amplified from DNA isolated from spleen cells of *Nb* infected mice and TSI-18 hybridoma were purified and then subjected to digestion with EcoR1 (+EcoR1) in 25 μl reaction volume for 2-3 hours at 37°C. As control, the template was incubated in reaction buffer without the enzyme (-EcoR1). Digestion products were resolved on a 2% agarose gel with ethidium bromide staining.

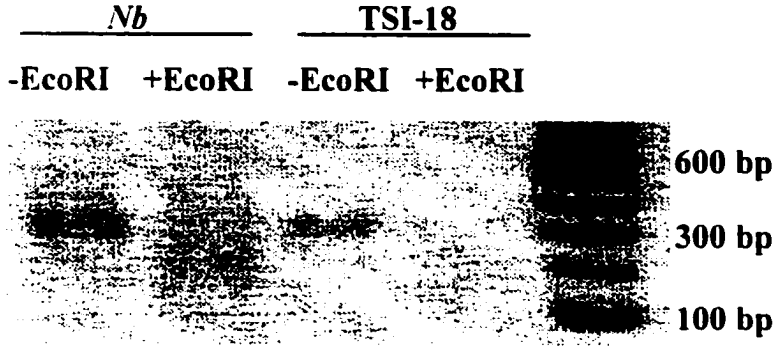


Figure 15

Figure 16. AWH induces higher level of IgG1 switch DNA recombination than killed *Mycobacteria*. Genomic DNA was isolated from spleen cells of mice treated with either AWH, or killed *Mycobacteria* (Myc) as described in the Materials and Methods. DNA was digested with EcoR1, ligated with T4 DNA Ligase and amplified by PCR using primers specific for the recombined IgG1 switch regions. nAChRe levels (upper panel) were also determined by DC-PCR to control for equal template loading and for semi-quantitation of the Sμ-Sγ1 products (lower panel). PCR amplicons were resolved on a 1.5% agarose gel with ethidium bromide staining. Results are representative of three experiments.

Detection of higher levels of IgG1 switch recombination in the spleen cells from AWH treated than from Myc treated mice.

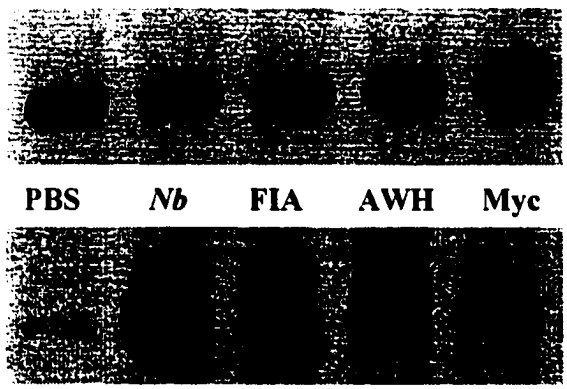


Figure 16

the issue of expansion of memory cells. Proliferation was assessed by thymidine incorporation. As expected, LPS itself induced significant proliferation. This proliferative response was significantly inhibited ($p<0.001$) when the cells were cultured in the presence of AWH (fig. 17). Further, AWH alone did not demonstrate stimulatory activity when added to B cells. This inability of AWH to either stimulate naive B cell proliferation or enhance LPS induced B cell proliferation confirms that the increase in type-2 immunoglobulin production due to AWH or *Nb*, is not primarily mediated by expansion of memory B cells by nematode products. These data provide strong evidence that the polyclonal IgG1 and IgE response induced by nematodes is due to large-scale induction of *de novo* class switch of B cells.

4.2 Characterization of the inhibitory effect of AWH on B cell proliferation mediated by LPS

4.2.1 AWH inhibits LPS-induced murine B cell proliferation

The data presented in fig. 17 showed that AWH does not mediate the expansion of B cells but rather inhibits B cell proliferation. This was a very interesting observation, which indicates that nematodes may mediate immunomodulatory effects on B cells in a variety of manners. In the first experiment to follow up this interesting finding, an examination was made as to whether the effect is dose dependent. Therefore, AWH was added concurrently to

Figure 17. AWH does not expand B cells. B cells from BALB/c were stimulated in culture containing 5 μg/ml LPS alone, or LPS in combination with AWH [10 μg/ml or 50 μg/ml]. After 72 hours of incubation at 37°C, the cultures were pulsed with [^{3}H] thymidine and the cells assayed for incorporation 18 hours later. Data shown are expressed as mean disintegration per minute (dpm) of triplicate wells ± standard deviation and are representative of seven experiments (*** $p < 0.001$, one-way ANOVA).

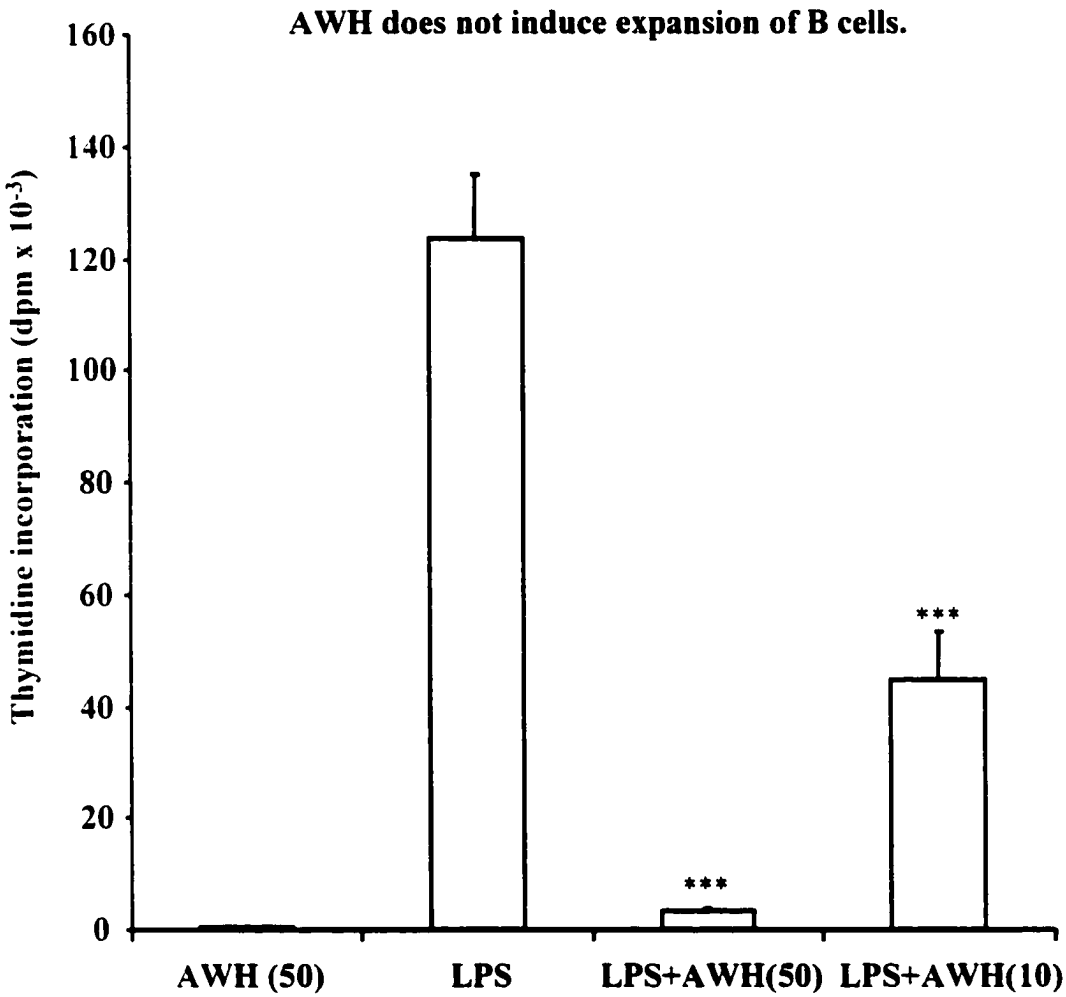

AWH does not induce expansion of B cells.
Thymidine incorporation (dpm x 10-3)
160
140
120
100
80
60
40
20
0
***
***
AWH (50)
LPS
LPS+AWH(50)
LPS+AWH(10)

Figure 17

LPS-stimulated B cells, at concentrations ranging from 0.032 μg/ml to 40 μg/ml. After 72 h in culture, the cells were pulsed with tritiated thymidine for an additional 18 h. After this, proliferation was measured by thymidine incorporation. As expected, addition of LPS to the culture induced marked proliferation of B cells. However, when AWH was added at the initiation of culture with LPS, the proliferative response was significantly inhibited ($p<0.001$) (fig. 18). The maximum inhibition was always greater than 90% and inhibition occurred in a dose dependent fashion (fig. 18). To assess the temporal nature of this inhibition, cells were pulsed at different times (24, 48 and 72 h) post culture initiation and harvested 18 h later. Fig. 19 shows a representative result of ten such experiments. As expected, the proliferation in response to LPS is maximal at 72 h (which is why this time point was initially chosen for assessment of AWH activity). However, thymidine incorporation in LPS stimulated cells was evident even at 24 h. The AWH mediated inhibition of LPS mediated proliferation could be seen at all time points tested, even as early as 24 h.

To ascertain whether the inhibitory effect of AWH on proliferation was specific to B cells, splenic T cells were stimulated with either con A (5 μg/ml) or anti-CD3 mAb (1:10 dilution) and cultured in the presence of AWH. In contrast to the effect on LPS-stimulated B cells, AWH had no inhibitory effect on the proliferative response of T cells to both T cell mitogens (fig. 20).

Figure 18. AWH inhibitory effect on LPS-induced B cell proliferation is dose-dependent. Purified splenic B cells (from BALB/c nude mice) were activated with LPS (5 μg/ml) alone, or in the presence of various concentrations of AWH. After 72 hours of incubation at 37°C, the cultures were pulsed with [^{3}H]TdR and DNA synthesis assessed by measuring [^{3}H]TdR incorporation with liquid scintillation counting 18 hours later. Data shown are expressed as mean disintegration per minute (dpm) of triplicate wells ± standard deviation. The results are from a single experiment and are representative of ten separate experiments (** $p < 0.01$; *** $p < 0.001$, NS = not significant, $p > 0.05$, one-way ANOVA).

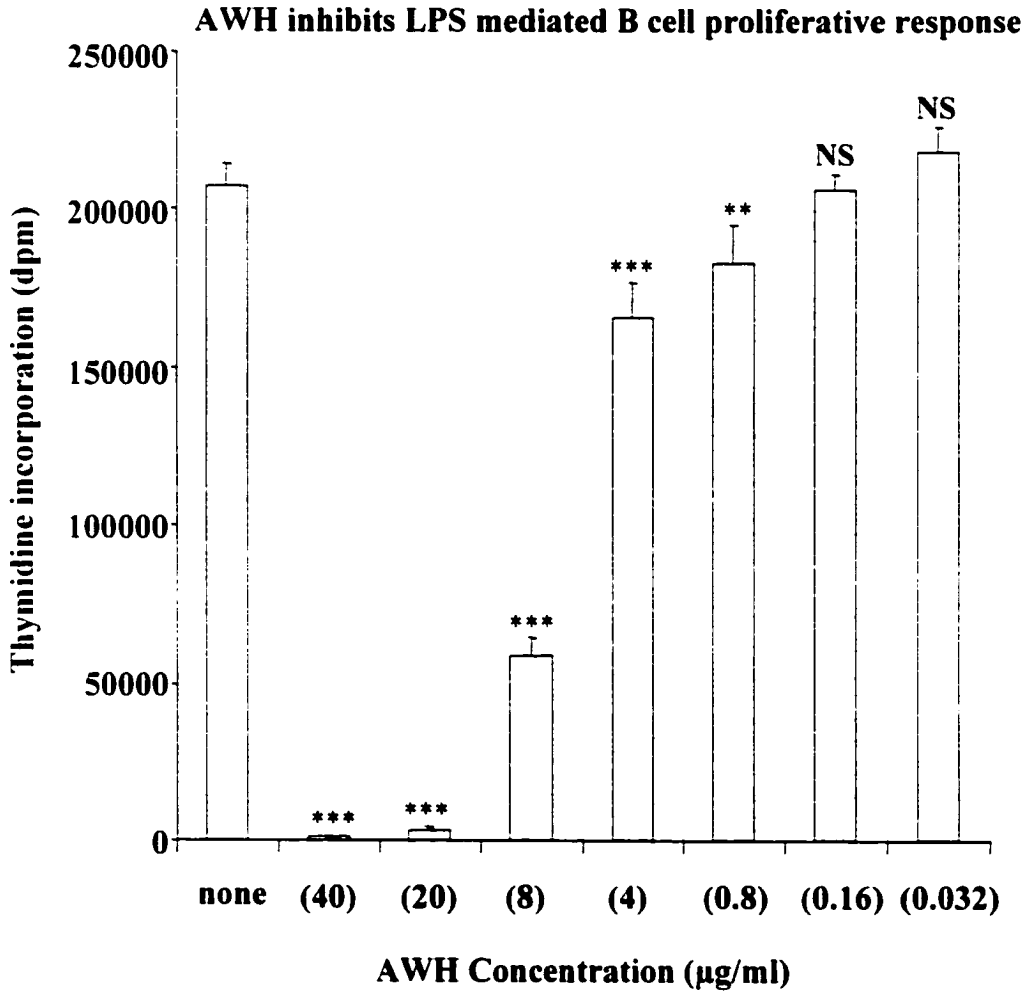

AWH inhibits LPS mediated B cell proliferative response
Thymidine incorporation (dpm)
250000
200000
150000
100000
50000
0
***
***
***
***
**
NS
NS
none
(40)
(20)
(8)
(4)
(0.8)
(0.16)
(0.032)
AWH Concentration (µg/ml)

Figure 18

Figure 19. Time course study of the inhibitory effect of AWH. Purified B cells were activated with LPS (5 μg/ml) alone, or in the presence of AWH added at a concentration of either 4 or 20 μg/ml. The cultures were pulsed with [^{3}H]TdR at the days indicated and DNA synthesis assessed by measuring [^{3}H]TdR incorporation with liquid scintillation counting 18 hours later. Data shown are expressed as mean disintegration per minute (dpm) of triplicate wells ± standard deviation. The results are from a single experiment and are representative of five separate experiments. (*** $p < 0.001$, NS = not significant, $p > 0.05$, one-way ANOVA).

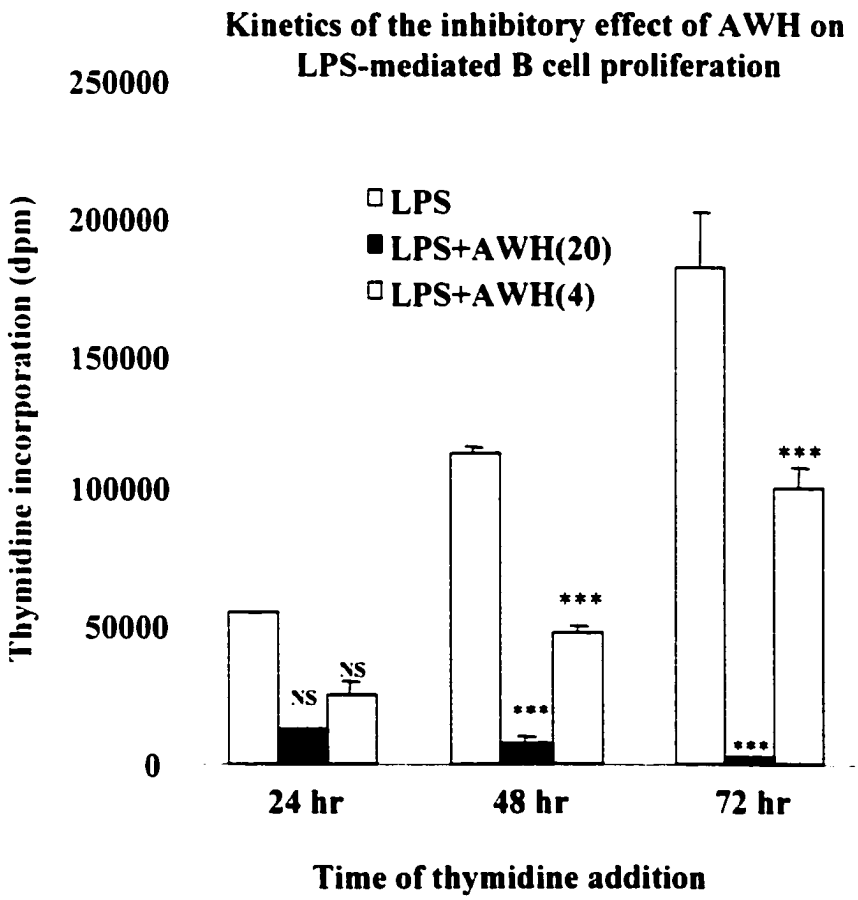

Kinetics of the inhibitory effect of AWH on LPS-mediated B cell proliferation
250000
200000
150000
100000
50000
0
Thymidine incorporation (dpm)
LPS
LPS+AWH(20)
LPS+AWH(4)
NS
NS
***
***
***
24 hr
48 hr
72 hr
Time of thymidine addition

Figure 19

Figure 20. AWH has no inhibitory effect on the proliferative response of spleen cells to various T cell activators. Spleen cells were activated with con A (5 μg/ml) or anti-CD3 mAb (final dilution of 1:10), alone or in the presence of AWH. The cultures were pulsed with [^{3}H]TdR after 72 hours and DNA synthesis assessed by measuring [^{3}H]TdR incorporation with liquid scintillation counting 18 hours later. Data shown are expressed as mean disintegration per minute (dpm) of triplicate wells ± standard deviation. The results are from a one experiment and are representative of five independent experiments (NS = not significant, $p > 0.05$, one-way ANOVA).

AWH has no inhibitory effect on spleen cell proliferation induced by various T cell activators.

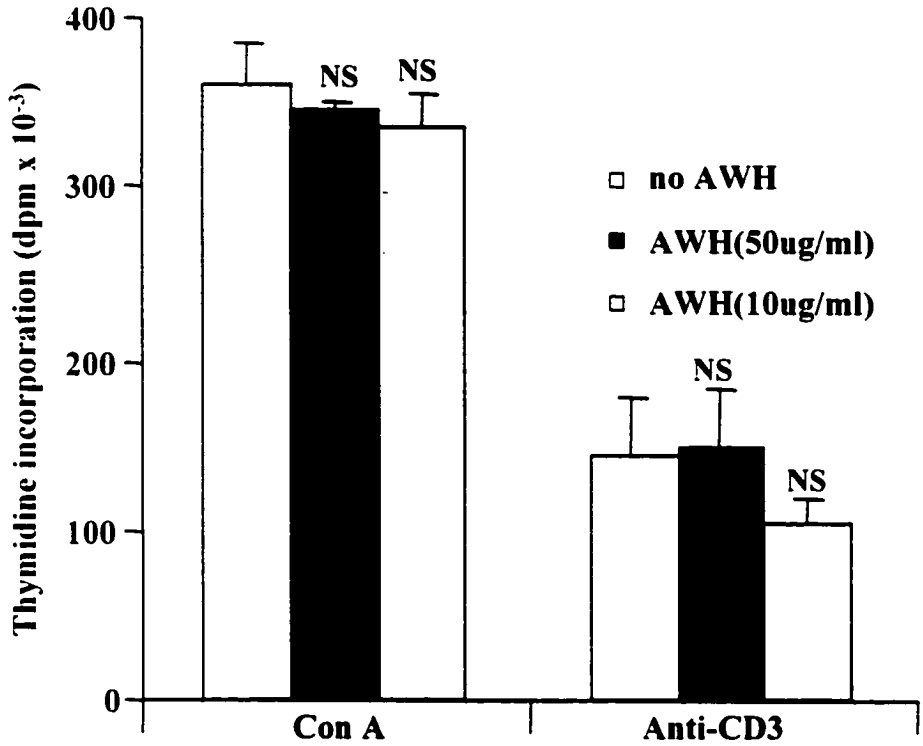


Figure 20

These data confirm that the inhibitory activity of AWH is not non-specific and suggests that B lymphocytes, may be uniquely sensitive to the effects of AWH.

4.2.2 AWH is not toxic to B cells

Because AWH does not reduce T cell proliferation, it is clear that AWH does not mediate its effects on B cells by non-specific cellular cytotoxicity. However, it is possible, although highly improbable, that AWH is uniquely toxic to B cells. To address the question whether AWH mediates its inhibition by inducing B cell death, B cells were cultured as above, and cell count and viability was assessed at 24 and 72 h of culture by trypan blue exclusion. As expected, both unstimulated B cells, and the B cells stimulated with LPS alone, exhibited a decrease in cell numbers by 24 h. Initially, 2 x 10^6 cells were plated in each well. In the experiment shown in fig. 21, the mean recovery for the untreated cells, and the LPS treated cells was 0.7 x 10^6 and 1.2 x 10^6 cells respectively. The addition of AWH at 40 μg/ml to LPS stimulated cultures did not significantly change ($p>0.05$) the B cell numbers in comparison to LPS stimulation alone (1.0 x 10^6 vs 1.2 x 10^6). This observation confirms that AWH is not toxic to stimulated B cells in culture. Interestingly, when these cultures were examined, only the LPS stimulated cultures showed high levels of blast cells versus resting cell morphology. By 72 h, there was no difference between cell counts in AWH treated and control cultures, indicating no late stage toxicity (data not shown). To

provide additional evidence that AWH does not induce death of B cells. LPS stimulated B cells co-incubated with AWH were assessed for DNA fragmentation (apoptosis) by the JAM assay. As shown in fig. 22, the percent DNA damage, as a measure of apoptosis, at the three time points tested was the same in B cells stimulated with LPS alone and those stimulated with both LPS and AWH. These data show that AWH does not enhance apoptosis of LPS-stimulated B cells, further supporting the earlier observation that AWH is not toxic to B cells and does not mediate its inhibitory activity by inducing cell death.

4.2.3 Inhibitory activity of AWH is not specific to LPS-mediated B cell proliferation

To ascertain whether the AWH effect is LPS specific, mitogens using different signaling pathways were tested. B cells were stimulated with anti-CD40 mAb, pokeweed mitogen (PWM) or *Staphylococcus aureus* cowan I (SAC). The proliferative response was then assessed in the presence or absence of AWH. AWH also inhibited the proliferative response of B cells to these activators (fig. 23). However, the extent of the inhibitory effect (percent inhibition) was variable among these different B cell activators. B cell proliferation mediated by anti-CD40 was the least susceptible, followed by SAC and then PWM, which was inhibited to approximately similar levels as LPS stimulated B cells. This

Figure 21. Total cell yield in AWH-containing, LPS-stimulated B cell cultures. Two million (2×10^6) B cells were stimulated with LPS (5 μg/ml) alone or in the presence of AWH (40 μg/ml). After 24 hours of incubation at 37°C, the cells were harvested, cell number and viability were assessed by trypan blue exclusion. The untreated group represents cells that were neither stimulated with LPS nor AWH. The data shown is representative of four similar experiments (NS = not significant $p > 0.05$, one-way ANOVA).

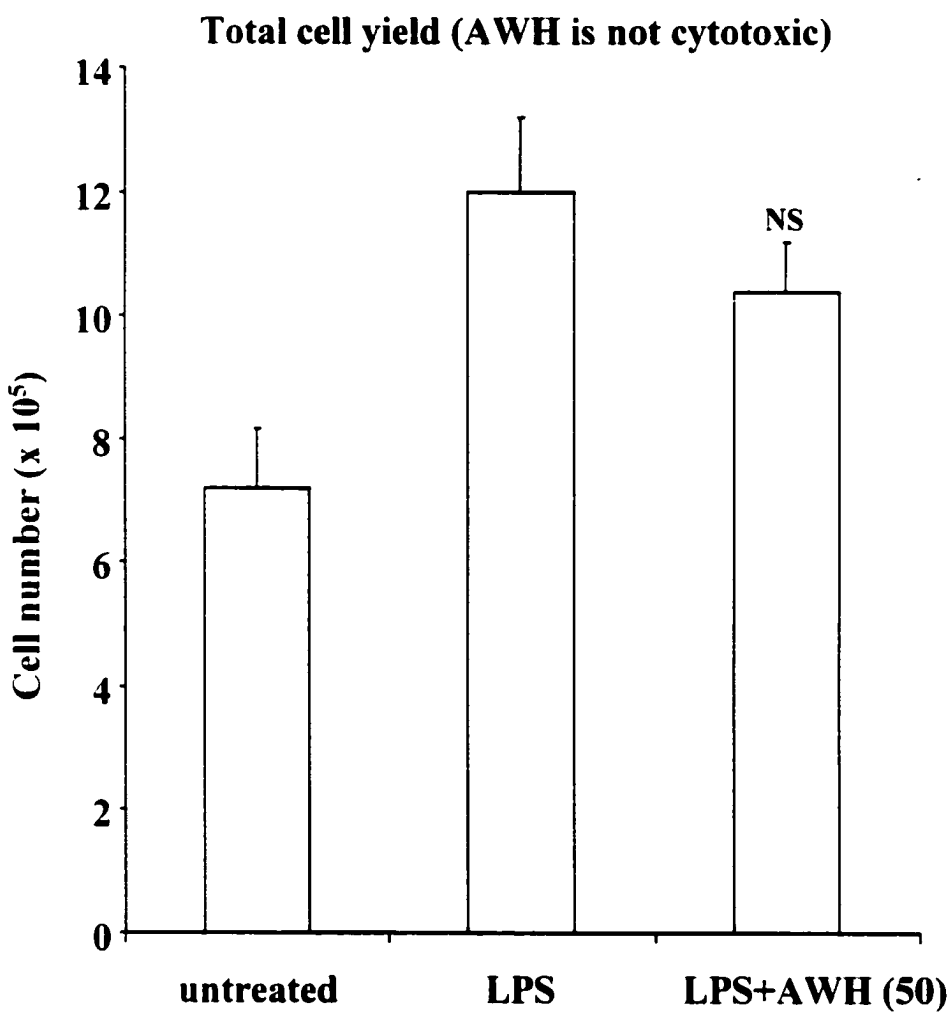

Total cell yield (AWH is not cytotoxic)
Cell number (x 10^5)
14
12
10
8
6
4
2
0
NS
untreated
LPS
LPS+AWH (50)

Figure 21

Figure 22. AWH does not induce apoptosis of LPS-stimulated B cells. B cells were labeled by stimulating with LPS in the presence of [^{3}H]-thymidine for 18-24 h in a flask. Labeled cells were subsequently restimulated with LPS in the presence or absence of AWH. At the end of 24, 48 and 72 h, cells were harvested onto glass filter mats. Total incorporated DNA was measured by scintillation counting. Data is expressed as percent DNA damage, which is indicative of loss of [^{3}H]-thymidine between initiation of culture and time of harvest. The data shown is representative of four similar experiments (NS = not significant $p > 0.05$, one-way ANOVA).

AWH does not induce apoptosis of LPS-stimulated B cell.

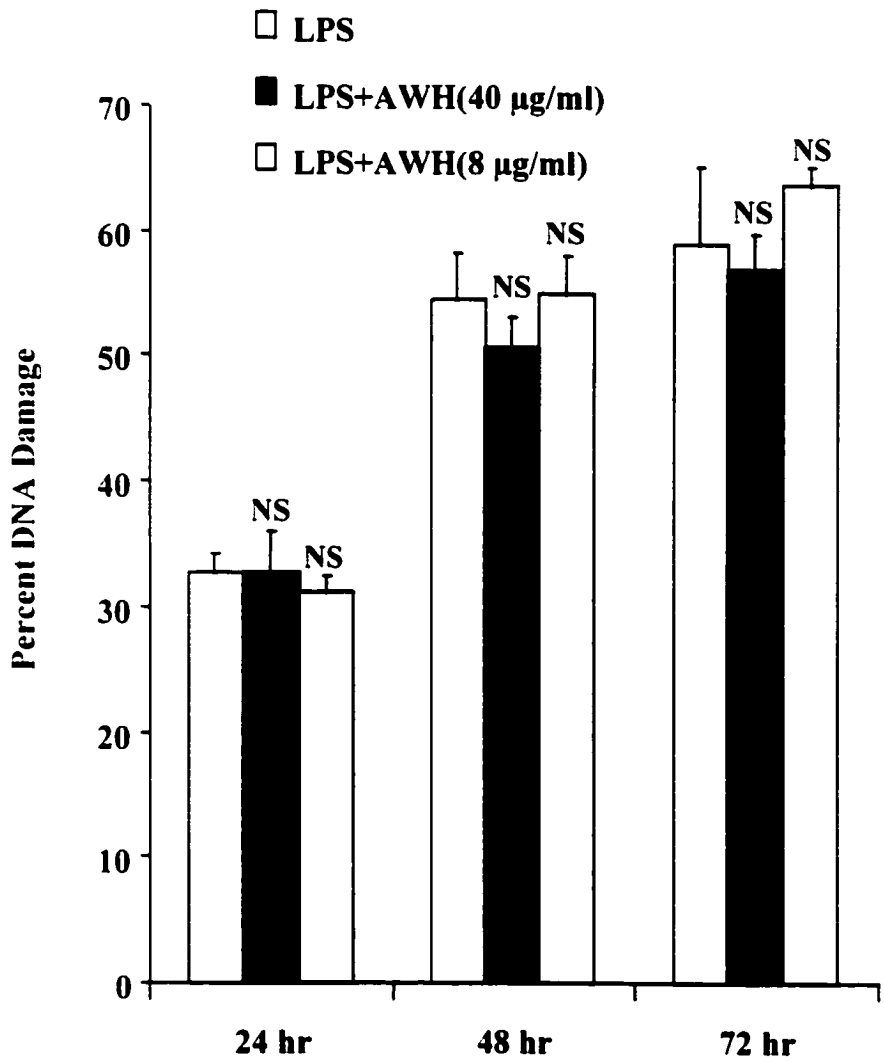


Figure 22

Figure 23. AWH inhibits B cell proliferative response to other stimuli. B cells were stimulated with LPS (5 μg/ml), anti-CD40 mAb (0.5 μg/ml), pokeweed mitogen (PWM: 5 μg/ml) and *S. aureus* cowan I (SAC 1:2000 dilution) respectively, with or without AWH (40 μg/ml). The cultures were pulsed with [^{3}H]TdR at the days indicated and DNA synthesis assessed by measuring [^{3}H]TdR incorporation with liquid scintillation counting 18 hours later. Data are expressed as mean percentage inhibition of proliferation ± standard deviation. The data shown is representative of four similar experiments.

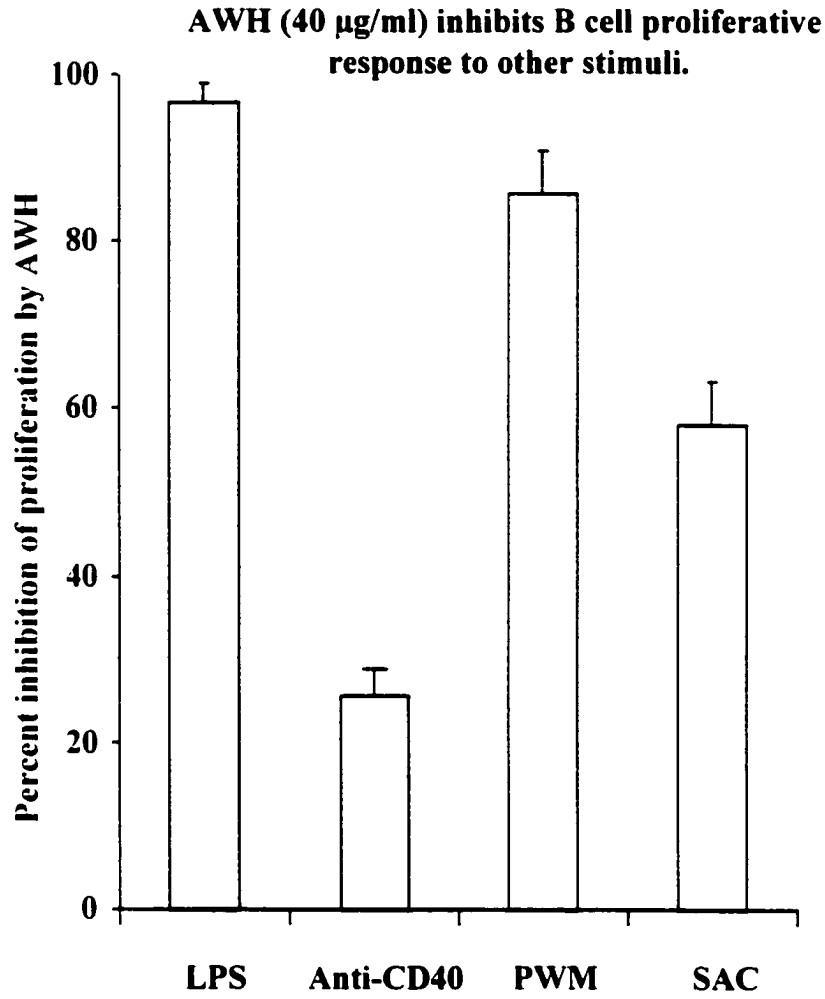

AWH (40 µg/ml) inhibits B cell proliferative response to other stimuli.
Percent inhibition of proliferation by AWH
100
80
60
40
20
0
LPS
Anti-CD40
PWM
SAC

Figure 23

observation shows that the inhibitory activity of AWH on B cell proliferation is not restricted to LPS mediated B cell proliferation and also that AWH appears to affect a downstream signaling mechanism required by all B cell activators. This is supported by the observation that AWH also inhibited the proliferative response of B cells stimulated with the combination of the pharmacological B cell activating agents PMA (PKC activator) and Ionomycin (stimulator of calcium influx) (fig. 24). This shows that the target signaling pathway of AWH inhibitory activity involves a pathway downstream to initial signaling events and to PKC activation and calcium mobilization. Whether these downstream events are B cell associated or accessory cell associated is unclear.

4.3 Mechanism of AWH inhibitory activity

4.3.1 AWH does not interfere with early activation events of B cells

To address the mechanism involved in the inhibitory effect of AWH on LPS stimulated B cell proliferation, we assessed whether the effect of AWH was directly on B cells, or by blocking LPS from binding to its receptor on B cells. AWH was added to cultures of B cells at 24, 48, and 72 h after the addition of LPS. As shown in fig. 25, the inhibition of proliferation was observed when AWH was added 24 and 48 h after LPS. The level of inhibition when AWH is added at 24 h after LPS was comparable to that observed when added simultaneously. The inhibitory effect decreased when AWH was added at 48 h, and a minimal

inhibitory effect on proliferation was detected when AWH was added at 72 h. In addition, cells pre-incubated with AWH for 4 h and washed, proliferated adequately, in response to re-stimulation with LPS (fig. 26). These data indicate that AWH does not act by interfering with LPS binding to its receptor or other early activation events, but seems to alter the ability of LPS-stimulated B cells to progress into the cell cycle.

To further confirm that AWH is not blocking LPS binding to B cells, the possibility of AWH binding directly to LPS and thus competing for LPS binding to its receptor was examined. Cells were stimulated with excessively large amounts (100 μg/ml) of LPS in the presence of AWH. Fig. 27 confirms that (i) increasing the concentration of LPS does not stimulate B cell proliferation beyond the level observed when 5 μg/ml of LPS was used; and (ii) AWH exhibited the same inhibitory activity on B cell proliferation even in cultures stimulated with a significant excess of LPS. These data provide evidence that AWH does not interfere with early binding effects of LPS and that the effects appear at a later stage.

4.3.2 AWH inhibitory activity appears to be mediated by modulation of macrophage (accessory cells) activation

For adequate stimulation of a B cell proliferative response to LPS, additional signals from accessory macrophages are essential (Corbel and Melchers

Figure 24. AWH inhibitory effect targets signaling pathway downstream of PKC. Purified B cells were activated with PMA (10 ng/ml) in combination with Ionomycin (1μg/ml) in the presence of different concentrations of AWH. After 72 hours of incubation at 37°C, the cultures were pulsed with [^{3}H]TdR and DNA synthesis assessed by measuring [^{3}H]TdR incorporation with liquid scintillation counting 18 hours later. Data are expressed as mean disintegration per minute (dpm) of triplicate wells ± standard deviation. The results are from one experiment and are representative of three separate experiments (* $p < 0.05$, *** $p < 0.001$, NS = not significant $p > 0.05$, one-way ANOVA).

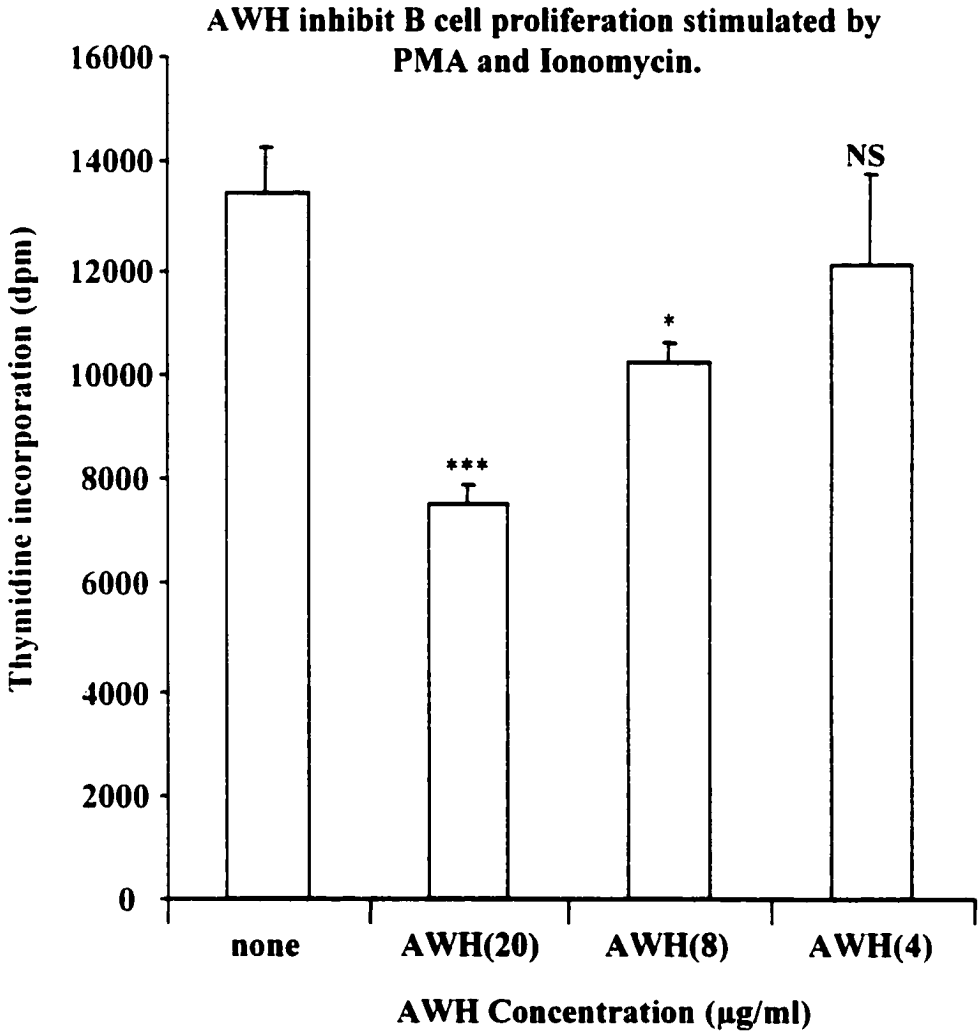

AWH inhibit B cell proliferation stimulated by PMA and Ionomycin.
Thymidine incorporation (dpm)
16000
14000
12000
10000
8000
6000
4000
2000
0
***
*
NS
none
AWH(20)
AWH(8)
AWH(4)
AWH Concentration (µg/ml)

Figure 24

Figure 25. Effect of AWH when added at different times post LPS stimulation of B cells. B cells were stimulated with LPS (5 μg/ml) and cultured in the presence of different concentrations of AWH added at the initiation of the culture (0 h). 24. 48 or 72 h after LPS stimulation. After 72 hours of incubation at 37°C (from the initial time of LPS stimulation) the cells were pulsed with [^{3}H]TdR for additional 18 hours after which the cells were harvested and [^{3}H]TdR incorporation determined with a liquid scintillation counter. The data are expressed as mean disintegration per minute (dpm) of triplicate wells ± standard deviation. The results are from a single experiment and are representative of five separate experiments.

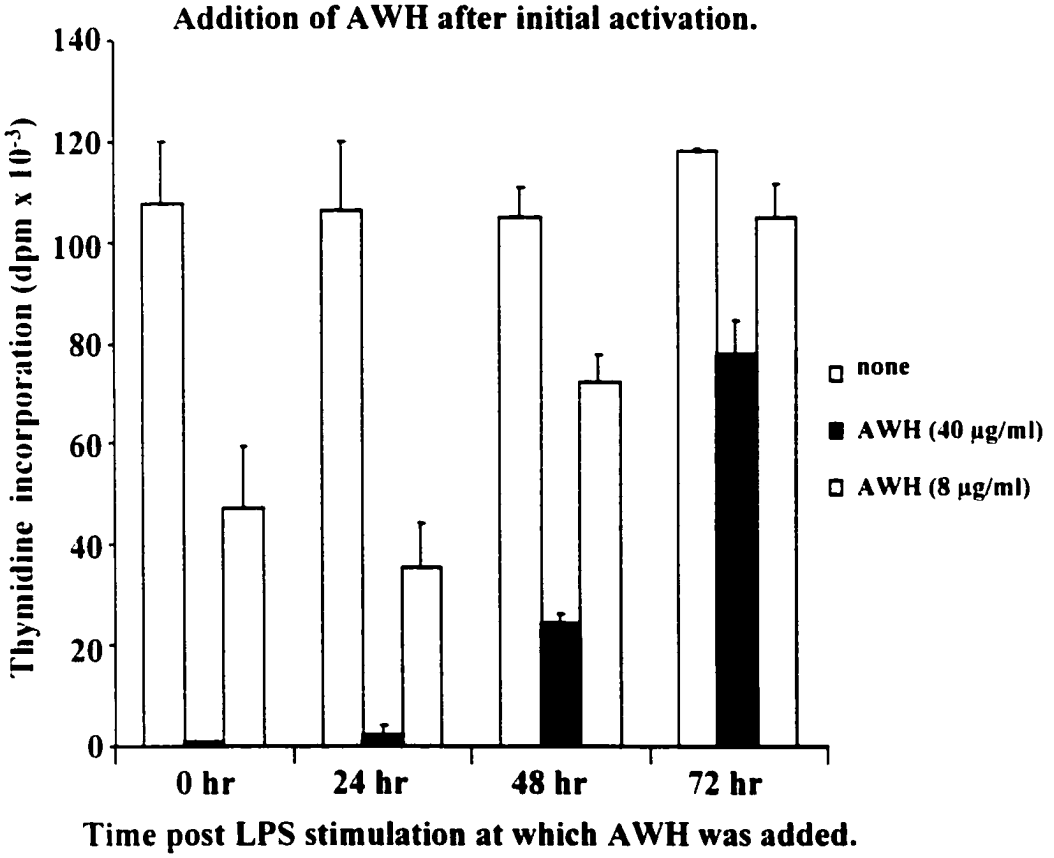

Addition of AWH after initial activation.
Thymidine incorporation (dpm x 10⁻³)
140
120
100
80
60
40
20
0
0 hr
24 hr
48 hr
72 hr
Time post LPS stimulation at which AWH was added.
none
AWH (40 µg/ml)
AWH (8 µg/ml)

Figure 25

Figure 26. AWH does not block LPS binding to its receptors. B cells were pre-incubated with AWH (10 or 50 μg/ml) or without AWH for 4 hours at 37°C. washed in fresh media and then stimulated with LPS. After 72 hours of incubation the cells were pulsed with [^{3}H]TdR for an additional 18 hours after which the cells were harvested and [^{3}H]TdR incorporation determined with a liquid scintillation counter. The data are expressed as mean disintegration per minute (dpm) of triplicate wells ± standard deviation (NS = not significant $p > 0.05$. one-way ANOVA). Results from one of three experiments are shown.

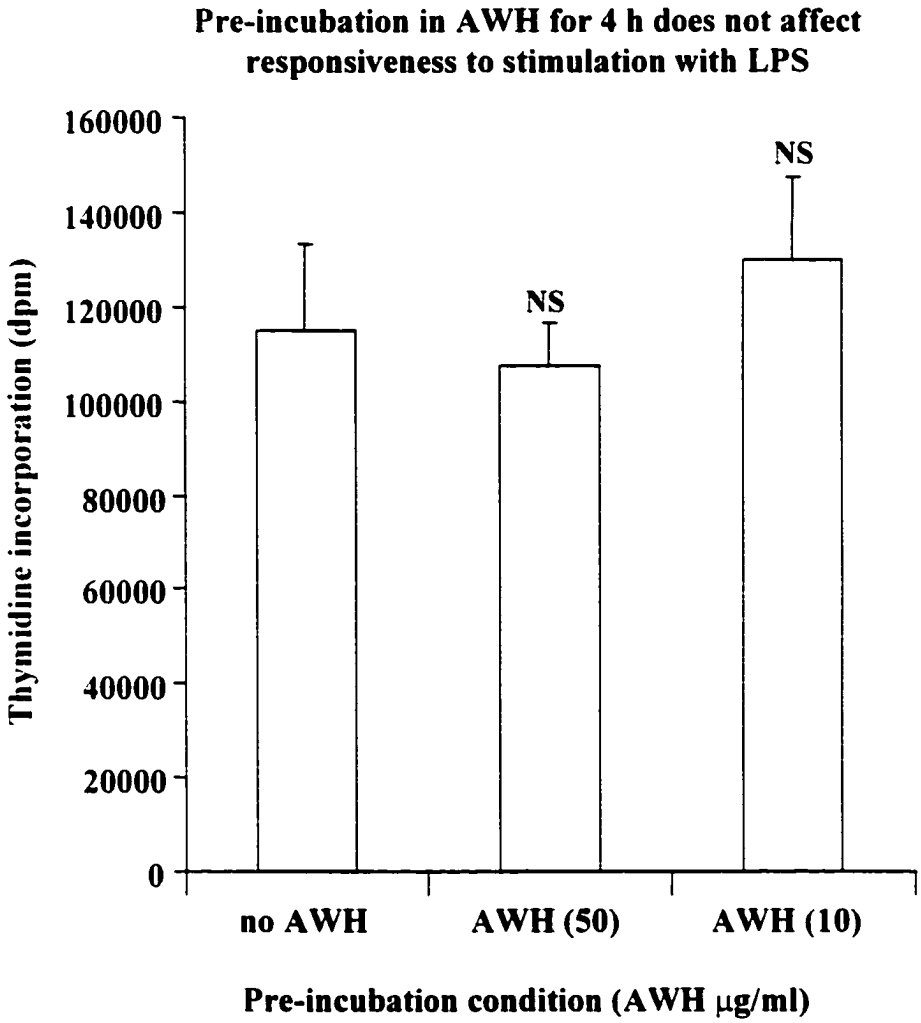

Pre-incubation in AWH for 4 h does not affect responsiveness to stimulation with LPS
160000
140000
120000
100000
80000
60000
40000
20000
0
Thymidine incorporation (dpm)
NS
NS
no AWH
AWH (50)
AWH (10)
Pre-incubation condition (AWH μg/ml)

Figure 26

Figure 27. AWH still exhibit inhibitory activity in the presence of higher concentration of LPS. B cells were stimulated with LPS (100 μg/ml) and cultured in the presence of different concentrations of AWH added at the initiation of the culture. After 72 hours of incubation at 37°C, the cells were pulsed with [^{3}H]TdR for additional 18 hours after which the cells were harvested and [^{3}H]TdR incorporation determined with a liquid scintillation counter. The data are expressed as mean disintegrations per minute (dpm) of triplicate wells ± standard deviation (*** $p < 0.001$, one-way ANOVA). Results from one of five experiments are shown.

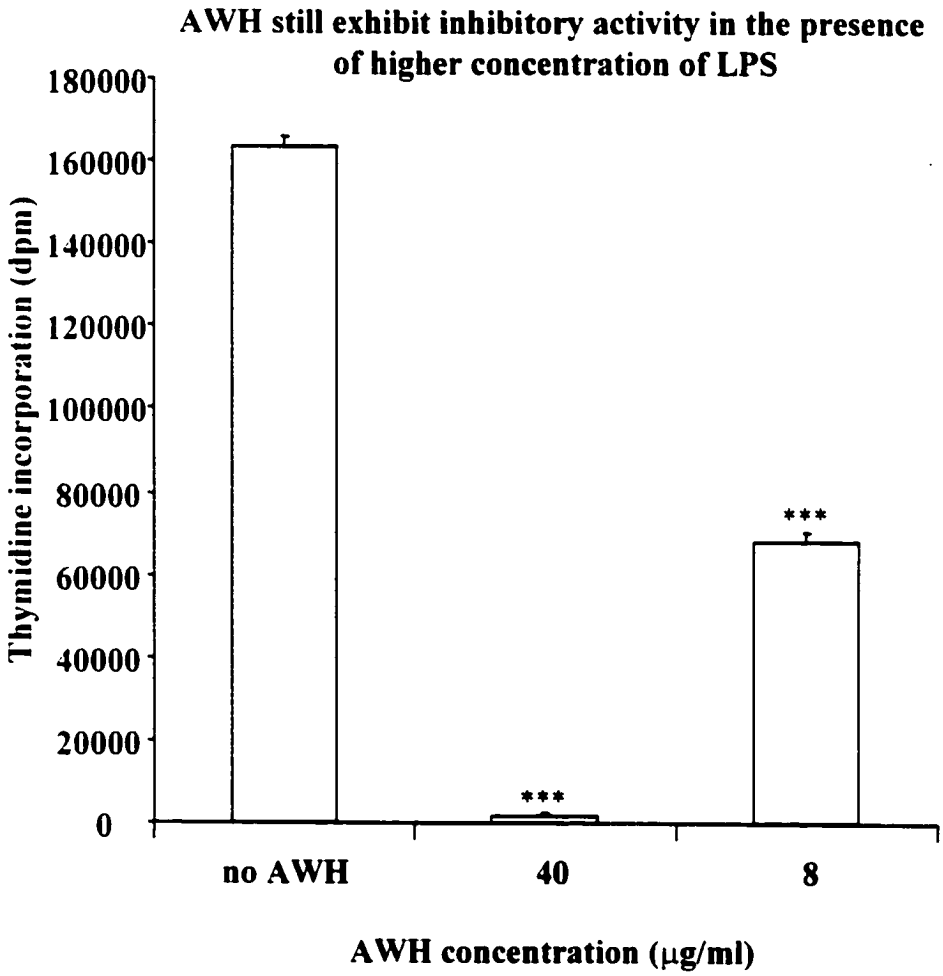

AWH still exhibit inhibitory activity in the presence of higher concentration of LPS
Thymidine incorporation (dpm)
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
***
***
no AWH
40
8
AWH concentration (μg/ml)

Figure 27

1983). Since AWH can have an effect 24 h after LPS activation of B cells, it is possible that AWH could be mediating its inhibitory effect by modulating macrophage function, potentially blocking late activation signals or inducing late suppressive signals. To address this issue, B cells (with adherent macrophages) were pre-incubated in the presence of LPS (10 μg/ml) for 24 h in a flask. Non-adherent cells harvested from this flask were then passed through a B cell enrichment column to remove any remaining macrophages and to remove dead cells. This procedure essentially eliminated the macrophages in the B cell preparation as shown by FACS analysis (fig. 28). Following this process, the cells are washed in fresh media and then stimulated with LPS in the presence or absence of AWH in 96 well plate. Fig. 29 reveals that absence of macrophages during AWH treatment reversed the inhibitory effect of AWH on LPS mediated B cell proliferation, restoring it to about 80-90% of the level observed in B cells stimulated with LPS alone. The incomplete reversal of the AWH inhibitory effect following removal of macrophages, may be attributed to the few contaminating macrophages, which are still capable of mediating the inhibitory effect. As few macrophages are required to initiate the co-stimulatory effect they provide for LPS mediated B cell proliferation (Corbel and Melchers 1993), so few macrophages could also mediate the inhibitory effect. When B cells are stimulated with LPS for 24 h, then incubated with AWH in the presence of adherent cells (macrophages), inhibition occurs. However, if the adherent macrophages are purged from the B

Figure 28. Assessment of macrophage levels in B cells after removal procedure. B cells were activated with LPS (10 μg/ml) in a flask for 24 h. The non-adherent population was removed and then washed in fresh media. To eliminate more macrophages and dead cells, cells were passed through a B cell enrichment column. This column contains anti-Thy1 and anti-mac1 antibodies to eliminate T cells and macrophages rescpectively. Cells were then stained with buffer alone (no antibody; -ve control) (brown), 3 μg/ml FITC-conjugated rat anti-mouse CD11b (green) or FITC-conjugated rat anti-mouse CD45R (purple). No antibody isotype control was used. Flow cytometric analysis was performed with a Becton Dickinson FACScan and Lysis II software. The x-axis represents fluorescence intensity and the y-axis shows the relative cell number.

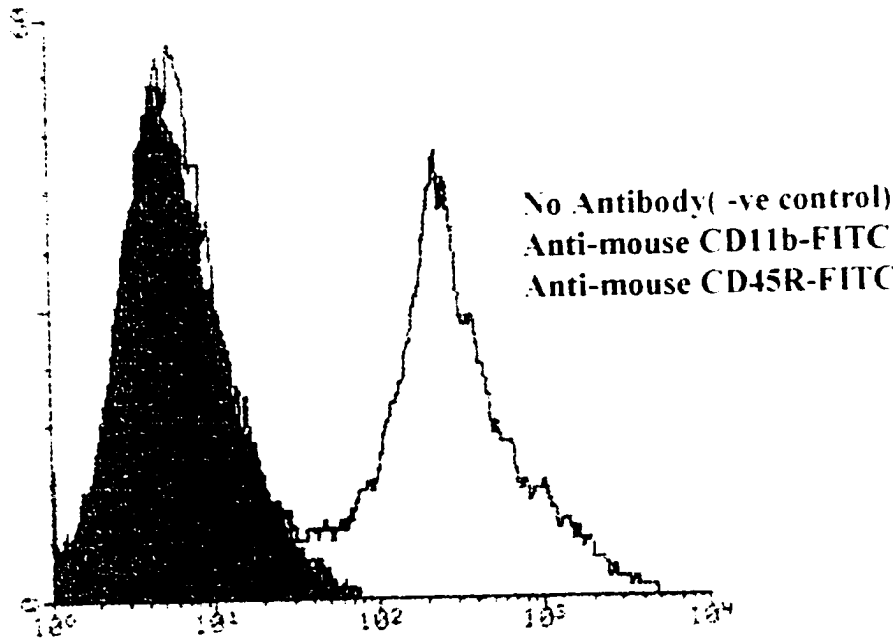

No Antibody(-ve control)
Anti-mouse CD11b-FITC
Anti-mouse CD45R-FITC

Figure 28

Figure 29. Removal of macrophages from B cells reverses the inhibitory effect of AWH. B cells were activated with LPS (10 μg/ml) in a flask for 24 h. The non-adherent population were removed and then washed in fresh media. To eliminate more macrophages and dead cells, the cells were passed through a B cell enrichment column. The cells were then re-stimulated with LPS (5 μg/ml) in the presence or absence of AWH in 96 well plates. After 72 hours of incubation at 37°C, the cells were pulsed with [^{3}H]TdR for additional 18 hours after which the cells were harvested and [^{3}H]TdR incorporation determined with a liquid scintillation counter. The data are expressed as mean disintegration per minute (dpm) of triplicate wells ± standard deviation (* $p < 0.05$, NS = not significant $p > 0.05$, one-way ANOVA). The results are from one experiment and are representative of five separate experiments.

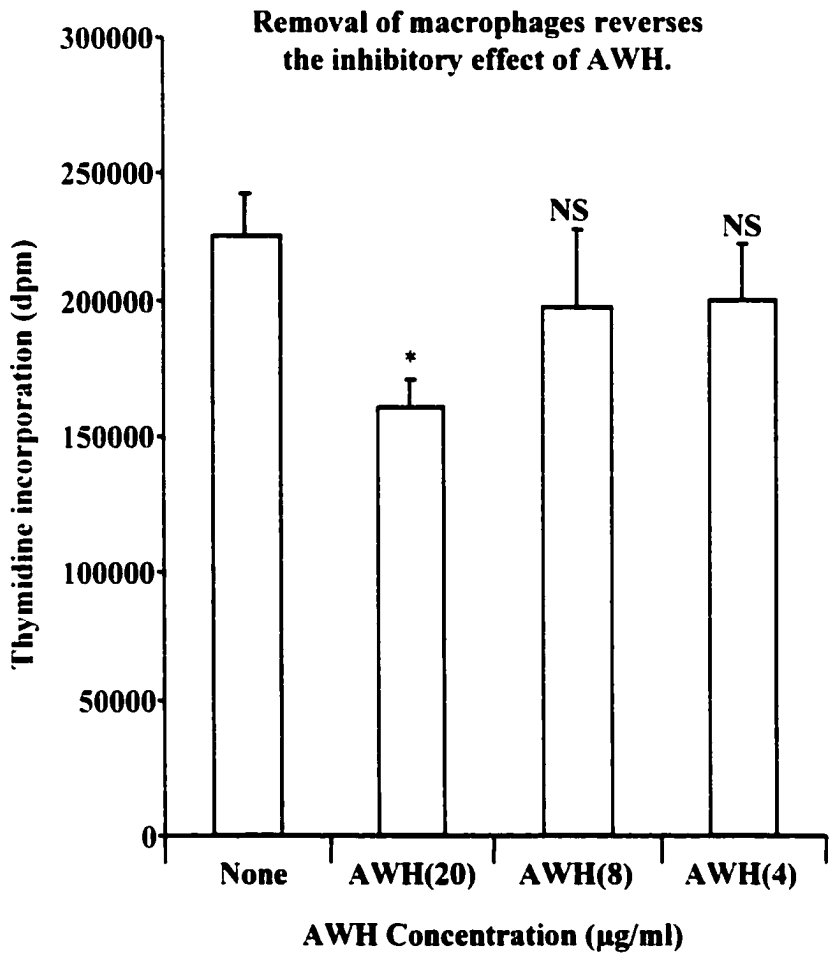

Removal of macrophages reverses the inhibitory effect of AWH.
300000
250000
200000
150000
100000
50000
0
Thymidine incorporation (dpm)
NS
NS
*
None
AWH(20)
AWH(8)
AWH(4)
AWH Concentration (µg/ml)

Figure 29

cell preparation before the addition of AWH. inhibition does not occur. These data suggest that AWH mediates inhibition of B cell proliferation indirectly through accessory cells.

4.3.3 AWH suppresses cytokine production by macrophages

To examine the effect AWH might be having on macrophage accessory cell function. macrophage cytokine secretion in response to LPS stimulation was examined. To address this possibility. splenic macrophages were plated in 24 or 96 well plates and then stimulated with either LPS alone or LPS in combination with AWH. Supernatants were collected 24 and 48 h later and analyzed for known pro and anti-inflammatory cytokines (IL-6. IL-10. IL-12 and TNF-α) by ELISA. In control cultures all of these cytokines were induced in significant amounts. However. in the presence of AWH. the levels of all these cytokines were significantly reduced ($p<0.01$) by approximately 30-40% (fig. 30 A-D).

4.3.4 Inhibitory activity of AWH is not mediated through reduction of cytokines

To test a possible hypothesis that the inhibition of LPS-mediated B cell proliferation is due to the reduced level of cytokines in the cultures. IL-6 and TNF-α were added back to the culture and the ability of the cytokines to restore the proliferative response of B cells stimulated with LPS in the presence of AWH

Figure 30. AWH suppresses cytokine production by macrophages. 24 or 96 well plates were coated with splenic macrophages by incubating spleen cells in the plates for 4 hours and then washing out the non-adherent population. Adherent cells (macrophages) were stimulated with LPS alone or in combination with different concentration of AWH. Supernatants were harvested 24 or 48 h later and then analyzed for IL-6 **(A)**, IL-10 **(B)**, IL-12 (p70) **(C)** and TNF-α **(D)** levels by ELISA. Results are expressed as the mean concentration (pg/ml) ± the standard deviation of three replicate wells (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA). Results are representative of four experiments.

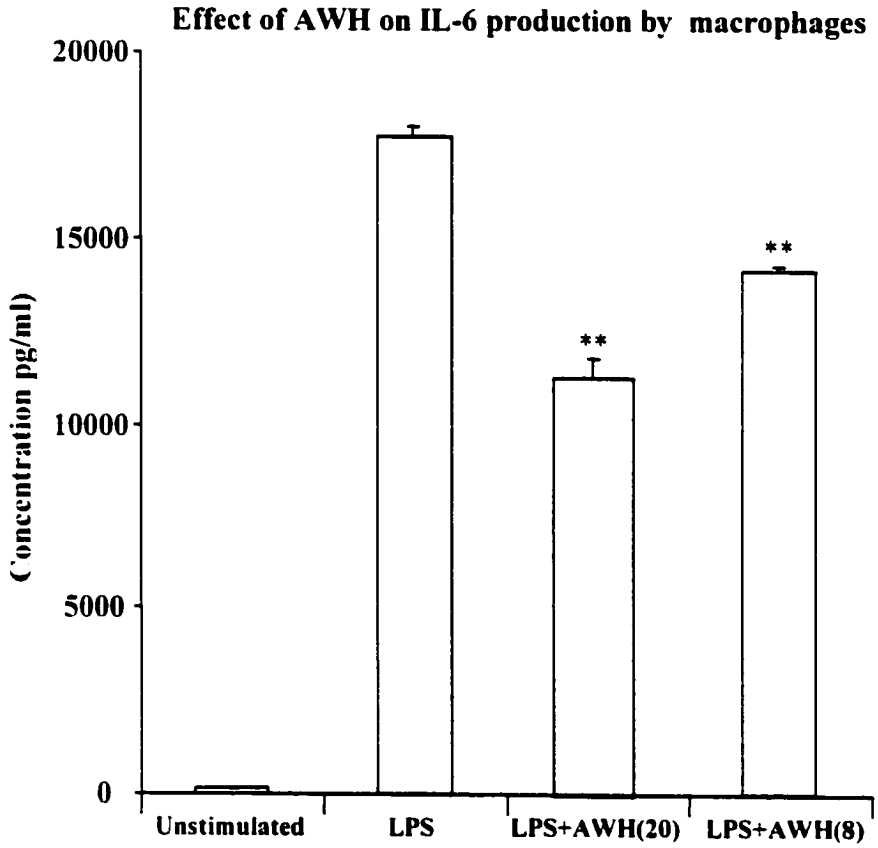

Effect of AWH on IL-6 production by macrophages
Concentration pg/ml)
20000
15000
10000
5000
0
**
**
Unstimulated
LPS
LPS+AWH(20)
LPS+AWH(8)

Figure 30A

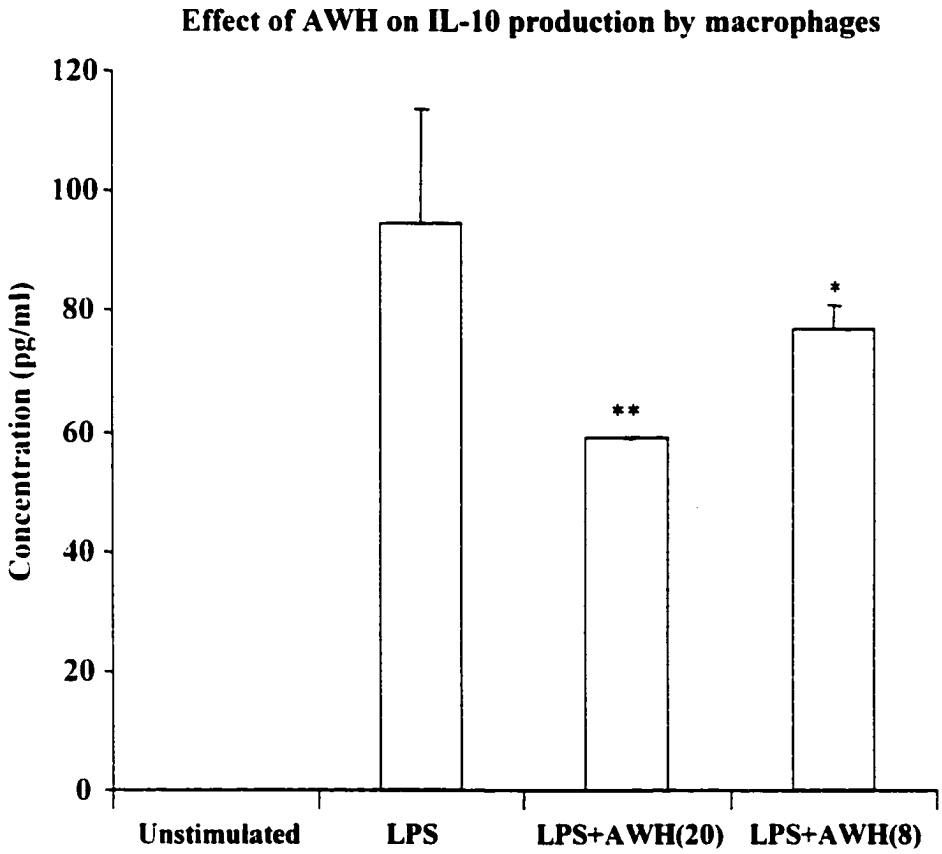

Effect of AWH on IL-10 production by macrophages
Concentration (pg/ml)
120
100
80
60
40
20
0
**
*
Unstimulated
LPS
LPS+AWH(20)
LPS+AWH(8)

Figure 30B

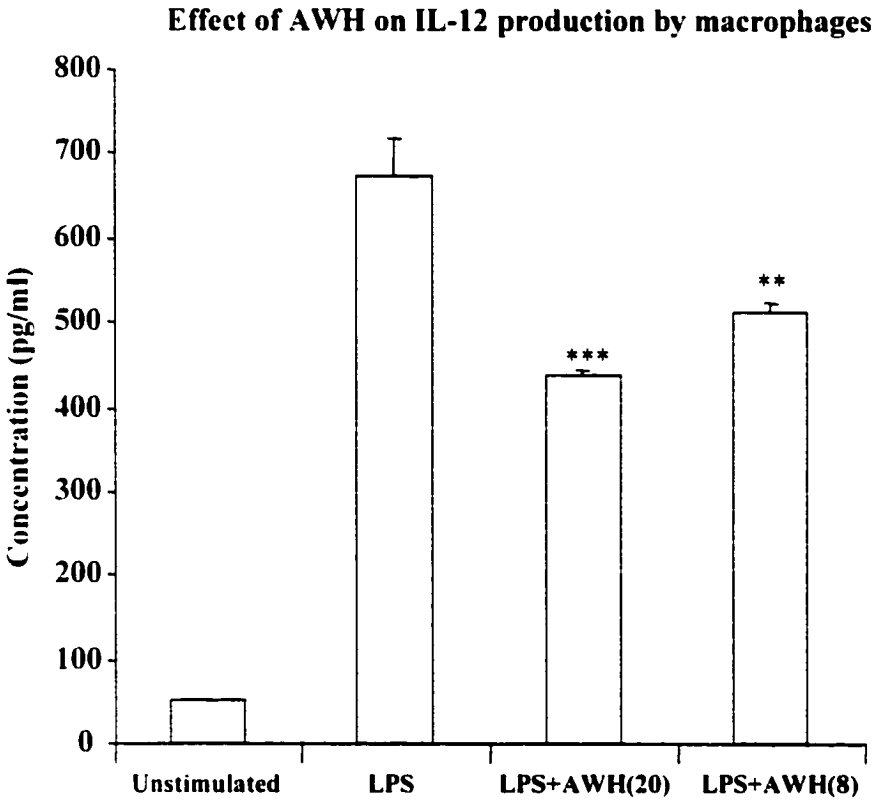

Effect of AWH on IL-12 production by macrophages
Concentration (pg/ml)
800
700
600
500
400
300
200
100
0
***
**
Unstimulated
LPS
LPS+AWH(20)
LPS+AWH(8)

Figure 30C

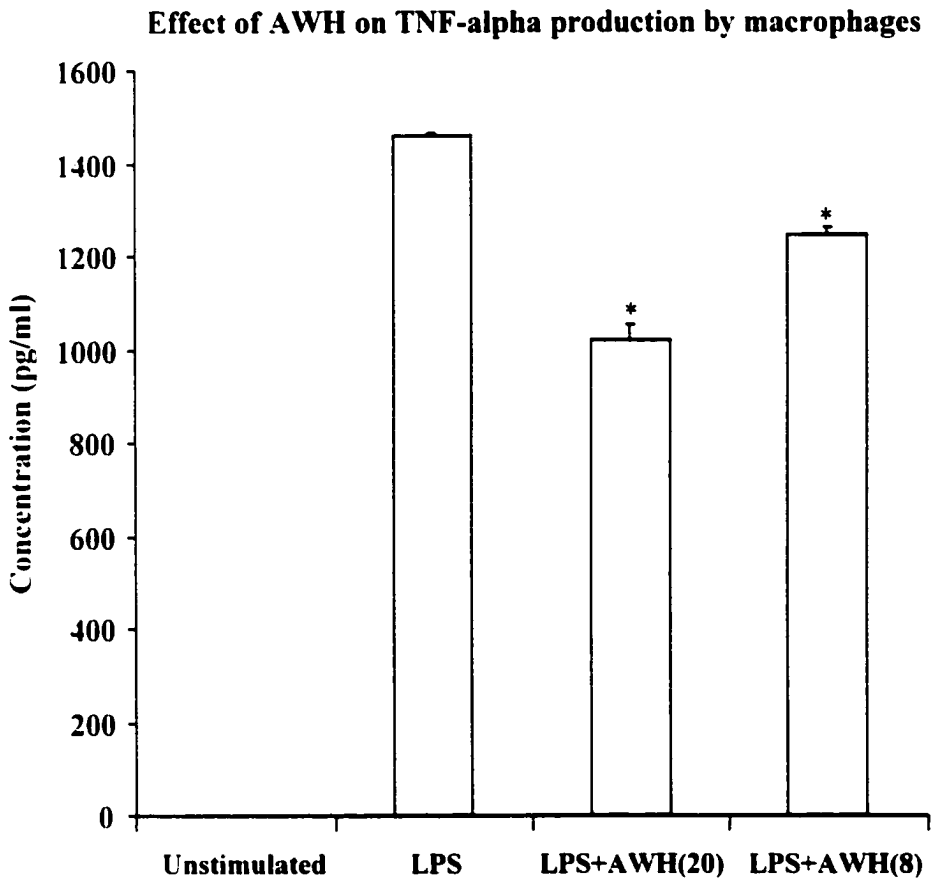

Effect of AWH on TNF-alpha production by macrophages
Concentration (pg/ml)
1600
1400
1200
1000
800
600
400
200
0
*
*
Unstimulated
LPS
LPS+AWH(20)
LPS+AWH(8)

Figure 30D

assessed. The amount of the cytokines added was to make up the difference in their levels as a result of AWH inhibition. The addition of exogenous IL-6 and TNF-α, cytokines that have been shown to be required for B cell proliferation (Clark et al 1996; Takatsu 1997; Rieckmann et al 1997), did not restore the proliferative response of B cells stimulated with LPS in the presence of AWH (fig. 31). This indicates that the inhibition of B cell proliferation by AWH is not due to the reduction of secretion of these growth favouring cytokines by macrophages.

4.3.5 Inhibitory effect of AWH on LPS-induced B cell proliferation is reversed by anti-CD40 monoclonal antibody

Since the addition of exogenous cytokines did not affect the inhibition mediated by AWH, it is possible that macrophages produce an inhibitory cytokine. Anti-CD40 mAb have been demonstrated to reverse cytokine mediated inhibition of B cell proliferation (Marcelletti 1996) and the general induction of apoptosis of B cells (Wang et al 1995; Schauer et al 1998). Thus, the sensitivity of AWH mediated inhibition of proliferation to anti-CD40 treatment was examined. B cells stimulated with LPS and AWH were cultured in the presence or absence of 0.5 μg/ml anti-CD40 mAb. The cells were pulsed after 72 h and thymidine incorporation assessed 18 h later. As shown in fig. 32, simultaneous addition of anti-CD40 to LPS-stimulated B cells significantly increased ($p<0.001$) the proliferation in an additive manner. When added to cultures of B cells stimulated

Figure 31. Effect of exogenous addition of IL-6 and TNF-α on the inhibitory activity of AWH. B cells were activated with LPS (5 μg/ml) alone, or in the presence of various concentrations of AWH alone or in combination with IL-6 (2 ng/ml) or TNF-α (1 ng/ml). After 72 hours of incubation at 37°C, the cultures were pulsed with [^{3}H]TdR and DNA synthesis assessed by measuring [^{3}H]TdR incorporation with liquid scintillation counting 18 hours later. Data are expressed as mean disintegration per minute (dpm) of triplicate wells ± standard deviation (*** $p < 0.001$, NS = not significant $p > 0.05$, one-way ANOVA). The results are from one experiment and are representative of three separate experiments.

Exogenous IL-6 and TNF-alpha does not restore LPS-induced proliferation in AWH containing B cell cultures.

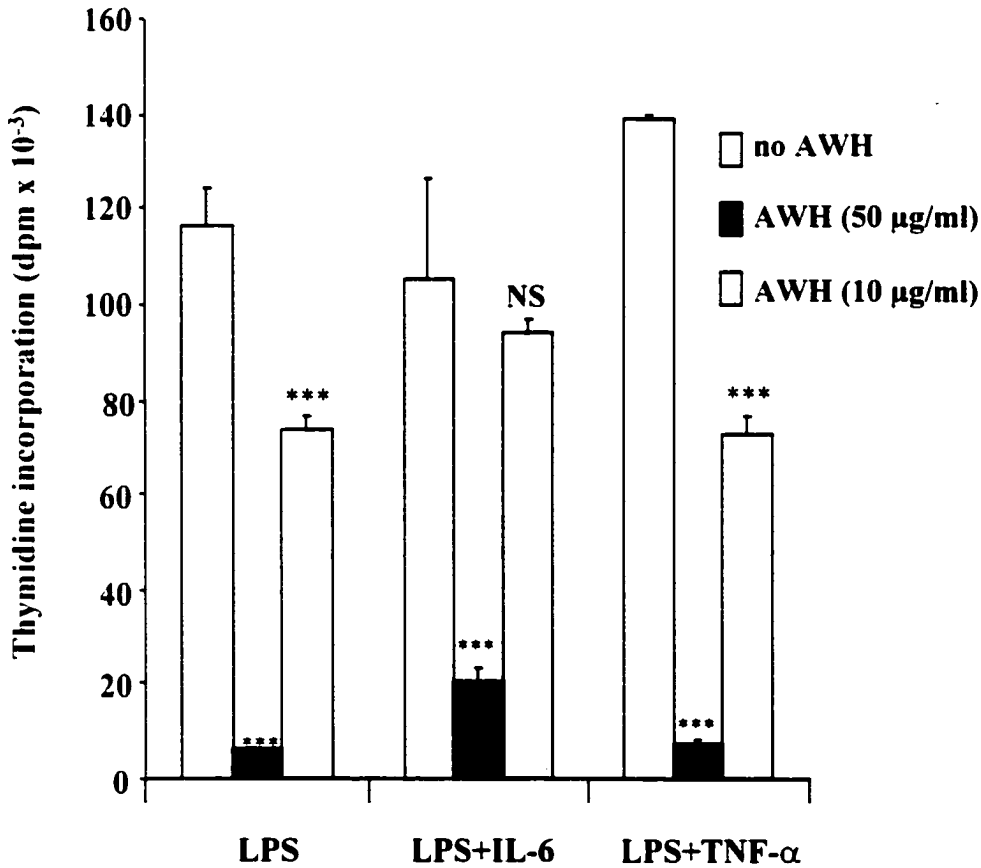


Figure 31

Figure 32. Anti-CD40 mAb restores the proliferative response of LPS-stimulated B cells from the inhibitory activity of AWH. B cells were activated with LPS (5 μg/ml) alone, or in the presence of various concentrations of AWH alone or in combination with 0.5 μg/ml Hamster anti-mouse CD40 antibody added at the initiation of the culture. Hamster serum was used as control for the antibody. After 72 hours from the initiation, the cultures were pulsed with [^{3}H]TdR and DNA synthesis assessed by measuring [^{3}H]TdR incorporation with liquid scintillation counting 18 hours later. Data are expressed as mean disintegration per minute (dpm) of triplicate wells ± standard deviation (*** $p < 0.001$, one-way ANOVA). The results are from one experiment and are representative of five separate experiments.

Anti-CD40 mAb restores the proliferative response of LPS-stimulated B cell from the inhibitory activity of AWH.

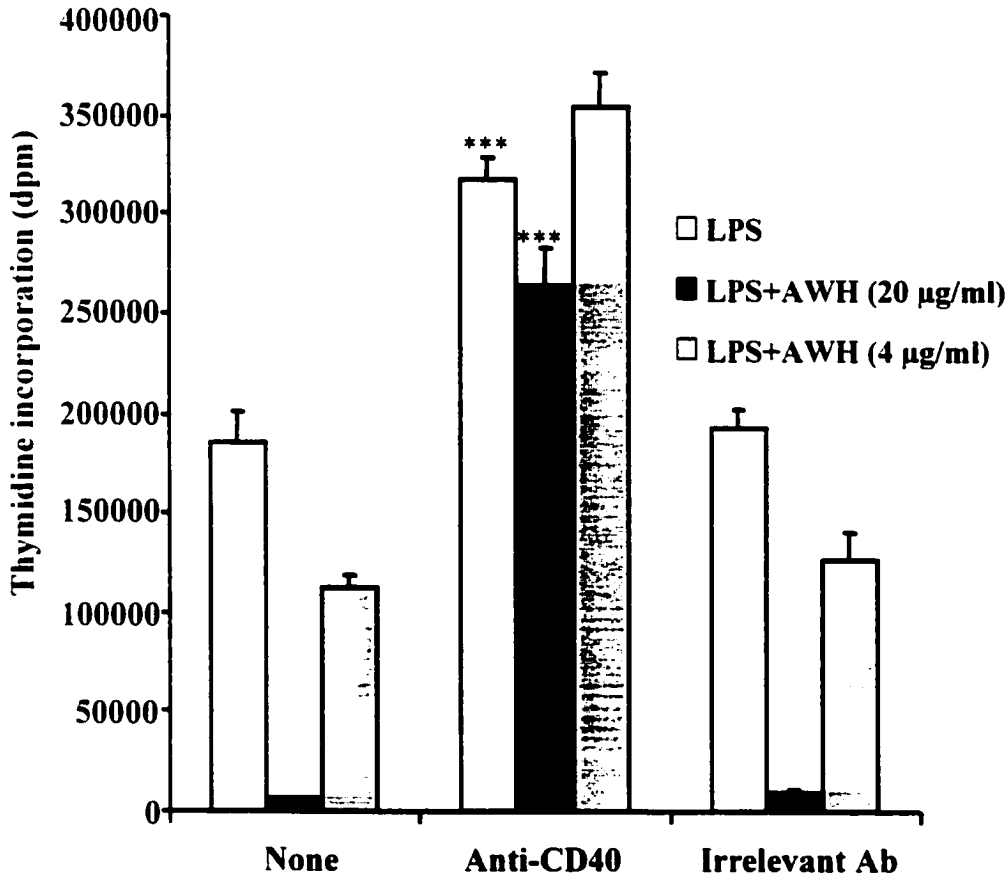


Figure 32

with LPS in the presence of AWH. anti-CD40 completely reversed the inhibitory effect of AWH on B cell proliferation. Control antibody did not affect the activity of AWH. These data provide strong evidence that the effect seen by AWH may be induced by inhibitory cytokine secretion by accessory macrophages. The data also demonstrate that ligation of CD40 is sufficient to rescue LPS stimulated B cells from death as a result of the inhibitory activity of AWH.

4.4 Characterization of the factor responsible for the inhibitory activity of AWH

Preliminary experimentation was carried to determine the nature and approximate molecular weight of the factor mediating the inhibitory activity of AWH on LPS-induced B cell proliferation.

4.4.1 *Nb* larvae do not contain the factor, which mediates B cell inhibition.

Because of the changes in protein composition associated with the developmental life cycle of nematodes (Maizels et al 1983; Dorzok et al 1989), the effect of extract from the infective larvae (3rd stage larvae) of *Nb* was examined. This was to ascertain whether the inhibitory effect of *Nb* extract was specific to the adult stage. To do this, B cells stimulated with LPS were cultured in the presence of extracts from either the infective larvae of *Nb* (LWH), or the adult stage (AWH) and proliferation assessed as described previously. Data from this experiment

reveal that unlike AWH, LWH had no inhibitory effect on the proliferative response of B cells to LPS, even at the highest concentration of 20 μg/ml (fig. 33), a level at which AWH inhibited B cell proliferation by greater than 90%. This observation confirms that the factor responsible for the inhibitory activity of AWH is specific to the adult stage in the developmental life cycle. This will aid in the future characterization of this factor since the larvae and the adult share many constituents, which can be eliminated from consideration as candidate molecules.

4.4.2 Inhibitory factor is proteinaceous in nature

To ascertain the nature of the factor, AWH was subjected to denaturation by boiling or with the proteolytic enzymes, trypsin and chymotrypsin (bound to an insoluble agarose matrix). The effect of the treated AWH on LPS-induced B cell proliferation was then assessed. Fig. 34A confirms that boiling AWH ablated the inhibitory effect of AWH on LPS-induced B cell proliferation, consistent with the factor being proteinaceous in nature. In addition, proteolytic cleavage of AWH by trypsin or chymotrypsin resulted in a reduction of the inhibitory effect on LPS-induced B cell proliferation by 80% and 75%, respectively (fig. 34B). This observation provides further support that the factor mediating this effect is proteinaceous in nature.

Figure 33. Inhibitory effect of *Nb* extract is specific to the adult stage. B cells were activated with LPS (5 μg/ml) alone, or in the presence of various concentrations of extract of the adult stage (AWH) or extract of the larvae stage (LWH). After 72 hours of incubation at 37°C, the cultures were pulsed with [^{3}H]TdR and DNA synthesis assessed by measuring [^{3}H]TdR incorporation with liquid scintillation counting 18 hours later. Data are expressed as mean disintegration per minute (dpm) of triplicate wells ± standard deviation (*** $p < 0.001$, NS = not significant $p > 0.05$, one-way ANOVA). The results are from a single experiment and are representative of three separate experiments.

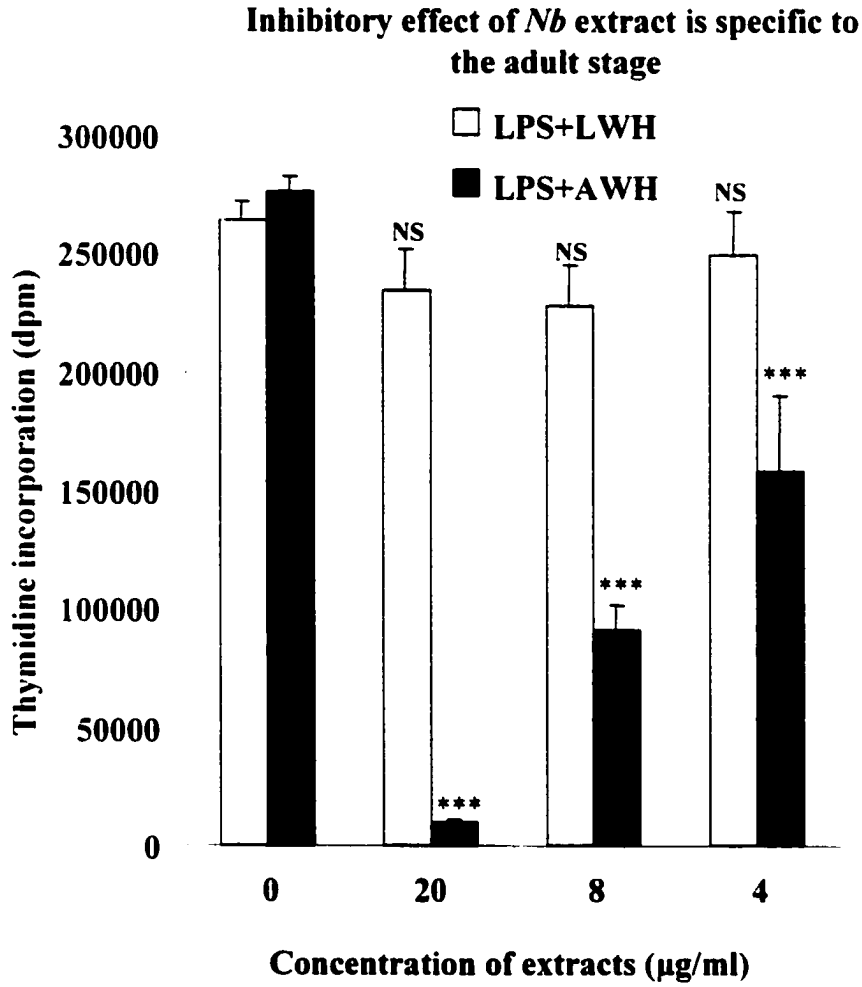

Inhibitory effect of Nb extract is specific to the adult stage
LPS+LWH
LPS+AWH
300000
250000
200000
150000
100000
50000
0
Thymidine incorporation (dpm)
NS
NS
NS
***
***
***
0
20
8
4
Concentration of extracts (µg/ml)

Figure 33

Figure 34. Heat and Proteolytic enzyme treatment reverses the inhibitory effect of AWH on LPS-induced B cell proliferation. B cells were stimulated with LPS (5 μg/ml) and cultured in the presence of AWH (10 μg/ml) that had been either heat-treated by boiling (100°C) for 5-10 min **(A)** or digested with the proteolytic enzyme, trypsin bound to an insoluble agarose matrix (10 U/ml) **(B)**. The culture was incubated for 72 hours at 37°C and then pulsed with [^{3}H]TdR for additional 18 hours after which the cells were harvested and [^{3}H]TdR incorporation determined with a scintillation counter. The data are expressed as mean disintegration per minute (dpm) of triplicate wells ± standard deviation (** $p < 0.01$, *** $p < 0.001$, NS = not significant $p > 0.05$, one-way ANOVA). The results are from a single experiment and are representative of five separate experiments.

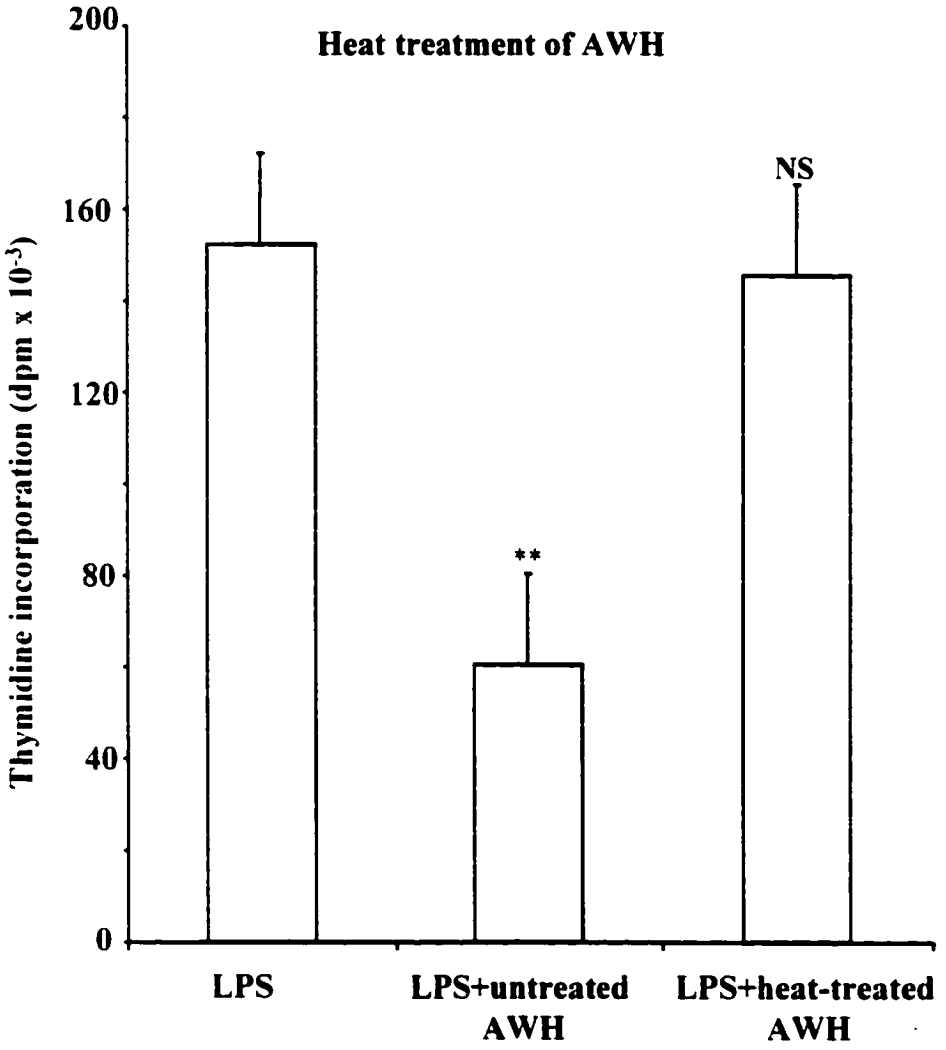

Heat treatment of AWH
Thymidine incorporation (dpm x 10-3)
200
160
120
80
40
0
NS
**
LPS
LPS+untreated AWH
LPS+heat-treated AWH

Figure 34A

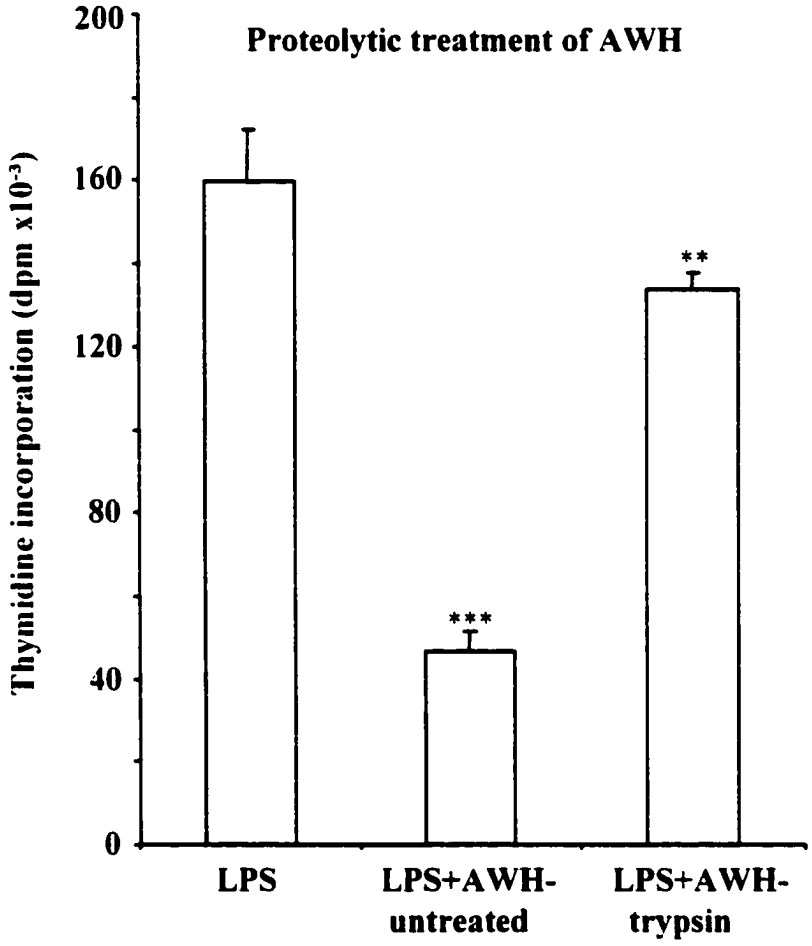

Proteolytic treatment of AWH
Thymidine incorporation (dpm x10⁻³)
200
160
120
80
40
0
***
**
LPS
LPS+AWH-untreated
LPS+AWH-trypsin

Figure 34B

4.4.3 Molecular size of the factor

In an effort to determine an approximate molecular size of the active factor in AWH. Centricon concentrators with various molecular weight cut-off points of 10 kDa. 30 kDa. 50 kDa and 100 kDa were used to prepare the appropriate fraction. To adjust the concentration of fractions back to their concentration in whole AWH. fractions were always resuspended back to the original volume after fractionation. Initial experiments showed that the fraction containing molecules of less than 100 kDa fraction exhibited similar inhibitory effects as the whole (unfractionated) AWH. The fraction containing molecules of less than 50 kDa and the fraction containing molecules of greater than 100 kDa showed no inhibitory effects (fig. 35). These data indicate that the approximate molecular size of the factor responsible for the inhibitory activity of AWH was between 50 kDa and 100 kDa. To confirm this. the fraction containing molecules of less than 100 kDa fraction was passed through a 50 kDa cut-off Centricon concentrator. generating a fraction (the retentate) with molecules of a molecular size greater than 50 kDa. but less than 100 kDa and a second fraction (filtrate) with proteins less than 50 kDa. The effect of these fractions were then assessed. Data from this experiment confirm that the greater than 50 kDa but less than 100 kDa fraction inhibited B cell proliferation stimulated by LPS. like whole AWH (fig. 35). A serendipitous internal control for the fractionation comes from the fact that the *Nb* hemoglobin. which is red in colour in the visible spectrum (Sharpe and Lee 1981). co-migrated

with the active fraction of AWH. This molecule is thought to be between 65-75 kDa in size and its absence in fractions above or below this molecular weight range (fig. 36). adds an additional level of confidence to the fractionation technique.

Figure 35. Determination of the molecular weight of the inhibitory factor in AWH. B cells were stimulated with LPS (5 μg/ml) alone, or in the presence of whole (unfractionated), and different fractions of AWH prepared as described in materials and methods. After 72 hours of incubation at 37°C, the cultures were pulsed with [^{3}H]TdR and DNA synthesis assessed by measuring [^{3}H]TdR incorporation with liquid scintillation counting 18 hours later. Data are expressed as mean disintegration per minute (dpm) of triplicate wells ± standard deviation. The results are from a single experiment and are representative of three separate experiments.

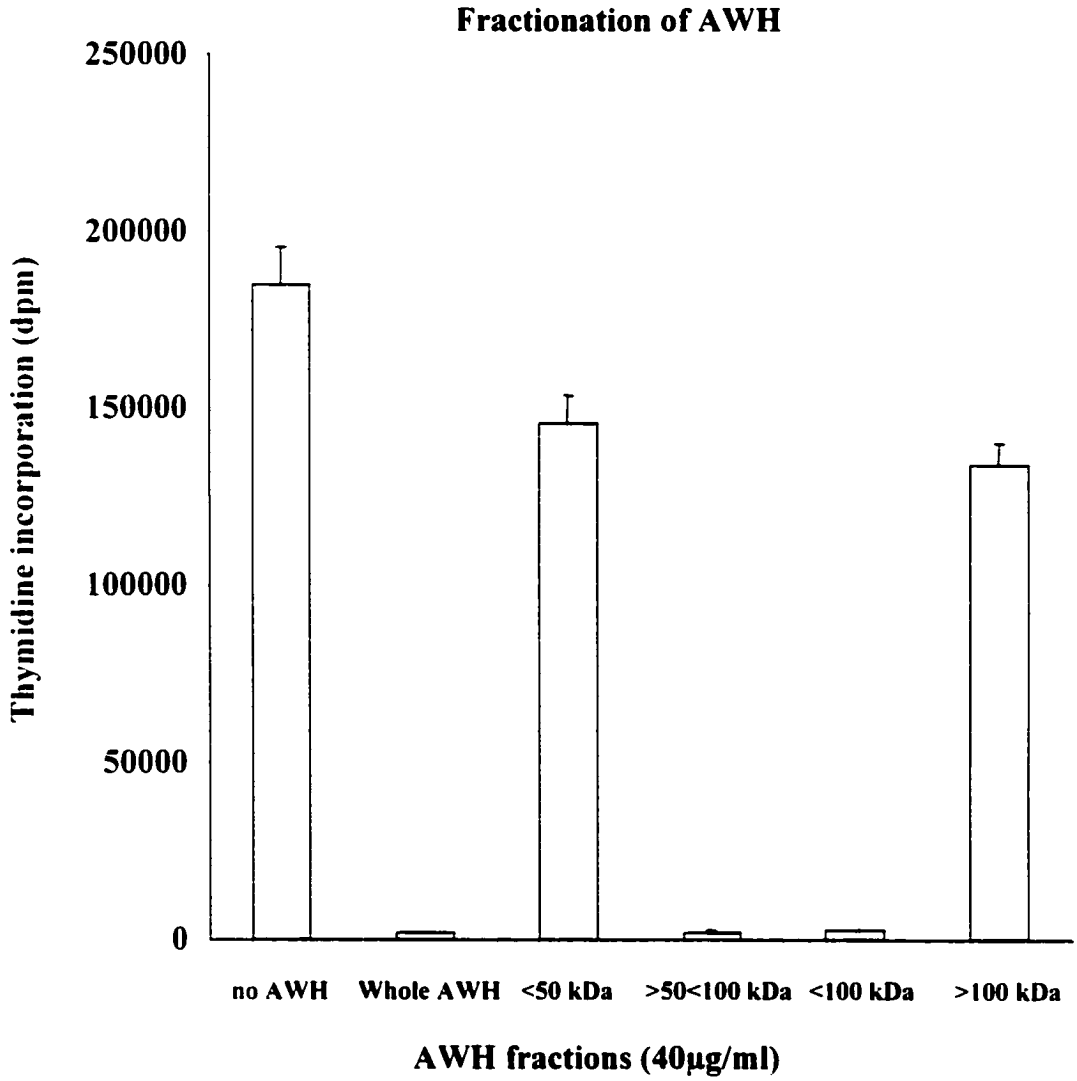
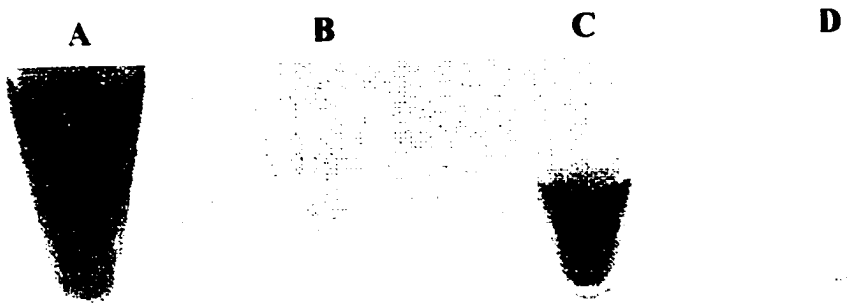

Fractionation of AWH
250000
200000
150000
100000
50000
0
Thymidine incorporation (dpm)
no AWH
Whole AWH
<50 kDa
>50<100 kDa
<100 kDa
>100 kDa
AWH fractions (40µg/ml)

Figure 35

Figure 36. Colour differentiation of AWH fractions. Picture representation of the fractions obtained from centrifugation through the Centricon concentrators.

AWH fractions



A = Whole AWH
B = < 50kDa AWH fraction
C = > 50 < 100 kDa AWH fraction
D = > 100 kDa AWH fraction

Figure 36

5.0 Discussion

5.1 Induction of type 2 associated immune responses by AWH

The immune response induced in mice infected with nematodes is defined by the characteristic type-2 cytokines, IL-4, IL-5, IL-6, IL-13, which in turn mediate the marked increases in serum IgE, IgG1, eosinophils, mast cells (reviewed in Sher and Coffman 1992; Urban et al 1998a; Mckenzie et al 1998a) and other mucosal cells such as goblet cells (Miller and Nawa 1979; Nawa and korenaga 1983) associated with the infections. The polyclonal immunoglobulins generated in response to the worms are not only directed at worm proteins (Jarrett and Bazin 1974; Jarrett and Miller 1982; Finkelman et al 1988a). In fact, it has been demonstrated that only a minor portion (less than 20%) of the IgE induced in the serum of *Nb* infected animals are specific to the worm antigens (Jarrett et al 1976; Jarrett and Miller 1982). The increase in immunoglobulin level suggests that *Nb* have the ability to modulate B cell function *in vivo*. However, the mechanism involved in the development of this unique polyclonal IgG1 and IgE in the serum is unclear. To investigate how *Nb* induces the dramatic polyclonal IgG1 and IgE response, a reductive approach, involving the use of nematode extracts was adopted. This approach provides the opportunity to assess the modulatory effect of *Nb* in both *in vivo* and *in vitro* systems. This approach to investigating the modulatory effect of *Nb* has not received significant attention in the past. Previous studies (Watanabe and Kobayashi 1988; Watanabe et al 1988; Yamada et

al 1991 and 1993a; Garraud et al 1995; Kamata et al 1995) have focused primarily on parasite-specific responses, such as examining serum samples from infected animals for the level of parasite-specific immunoglobulin. The observations reported in this thesis focus on the non-specific (polyclonal) responses induced by *Nb* extract, with the objective of investigating modulatory effects of *Nb* on B cell function.

In order to determine whether the extract possesses the ability to induce the characteristic T helper (Th) type-2 responses associated with *Nb* infection, mice were injected with the *Nb* extract, AWH, emulsified in a non-inflammatory vehicle (FIA). This vehicle was chosen because, unlike FCA, which enhances humoral immune responses when used as vehicle for antigen administration (Marretta and Casey 1979; Beck and Spiegelberg 1989), the injection of FIA does not influence immunoglobulin production. It does allow, however, the extract to be released into the host system slowly enough to influence the immune system. The dose of the extract administered was equivalent to 200 worms, a number of larvae that have been demonstrated to also induce the characteristic Th type-2 responses.

Injection of mice with as few as six infective *Nb* larvae has been demonstrated to induce measurable IL-4 production from the mesenteric lymph nodes and spleen of infected mice (Lawrence et al 1996). Similar data has also been obtained in rats infected with as few as ten infective *Nb* larvae (Yamada et al 1993b). In other parasite systems, however, infectious dose has proven to be

important in the generation of robust type 2 responses (Pauwels et al 1979; Marcelletti & Katz, 1992; Bancroft et al 1994; Ferreira et al 1995). Thus, it was important in my experimentation that mice were injected with the appropriate dose of AWH, to ensure that the mouse immune system is exposed to adequate amounts of the active factors in the extract to achieve the non-specific effects that I wished to study. This observation has been reflected in previous studies, in which mice were injected with various concentrations of extracted worm proteins ranging from 100 to 500μg, depending on the purity and immunogenicity of the proteins (Marcelletti & Katz, 1992; Lee and McGibbon 1993; Pearlman et al 1993a; Ferreira et al, 1995). In these studies, administration of these doses of worm extracts preferentially induced type-2 associated immune responses. The treatment regime in my experimentation induced very dramatic increases in the levels of total IgE and IgG1 in the serum of treated mice.

This is the first report of the induction of polyclonal IgE and IgG1 in mouse serum by an extract of *Nb*. Earlier reports by Uchikawa and co-workers (Uchikawa et al 1993) demonstrated the induction of a significant increase in IgE in the serum of rats injected with an excretory/secretory (ES) product of *Nb* intraperitoneally, for six consecutive days. However, previous attempts to induce similar IgE responses by the injection of rodents with adult worm extracts of *Nb* were unsuccessful (Ogilvie 1967; Orr et al 1971; Kojima and Ovary 1975). The difference in the outcome of these experiments and the observation reported in this

thesis, could be attributed to the difference in the preparation of the extracts and/or the concentration of the extracts injected into the rodents. It is worth mentioning at this point that preparation of ES products without significant bacterial contamination is extremely difficult, if not impossible. High level antibiotic treatment can limit bacterial growth in cultures but the presence of these drugs in the resulting ES complicates the interpretation of the experiment. Indeed, this was the main reason that whole worm extracts, rather than ES products were used for this project.

In most other studies, the investigation focused on the parasite-specific antibody response, using the excretory-secretory product of *Nb* as an antigen (Day et al 1979; Yamada et al 1991; Yamaoka et al 1994; Kamata et al 1995), or have examined the polyclonal IgE responses in rodents in response to *Nb* infection (Jarrett and Bazin 1974; Jarrett et al 1976; Zakroff et al 1989). This is not to say that other nematode extracts have not been linked with type-2 responses. The generation of Th2 responses have been reported in animals immunized with soluble extracts from other nematodes such as *Brugia malayi* (Pearlman et al 1993a & 1993b; Pearlman et al 1995), *Toxocara canis* (Del Prete et al 1991) and *Ascaris suum* (Strejan and Surlan 1977; Stromberg 1979; Lee and McGibbon 1993).

It has also been demonstrated that nematode extracts can potentiate specific immunoglobulin responses to third party antigens. For example, Lee and

McGibbon (1993) and others (Marretta and Casey 1979; Stromberg 1980) have reported the potentiation of IgE responses to ovalbumin. In these studies, mice were given an intraperitoneal injection of ovalbumin, concurrent with a subcutaneous injection of the nematode extract, resulting in a marked increase in anti-ovalbumin IgE response, but decreased anti-ovalbumin IgG response. The data from these experiments demonstrated that the presence of the extracts at the initiation of the response was critical for the development of the potentiated anti-ovalbumin IgE response.

The use of nematode extracts in the *in vitro* and *in vivo* setting has resulted in some contradicting findings. For example an extract from *A. suum* was demonstrated by one group (Soares et al 1987; Soares et al 1992; Ferreira et al 1995) to exhibit suppressive effects on both T cell proliferation and IL-2 production, as well as immunoglobulin (IgE and IgG) responses in mice. This observation is contrary to my findings with the nematode *Nb* and demonstrates the diversity of response that could be associated with a large diversity of nematode parasites.

It is important to compare the response with the *Nb* extract found in this work (and that of others) to that resulting from infection with the nematodes themselves. Although the increase in IgE levels due to AWH was lower than one would expect in mice injected with infective *Nb* larvae (Lebrun and Spiegelberg 1987; Zakroff et al 1989; Urban et al 1995; Nakanishi et al 1995), the levels are

remarkable considering the fact that the extract was delivered as a single injection subcutaneously compared to the continuous exposure to the large amounts of worm products. Some of the products may have short half-lives *in* vivo and the continuous production of these products in an infection would facilitate the expansion of immune responses much more so than one single injection of AWH. Moreover, there is evidence from another nematode (*Trichinella*; Maizels et al 1993; Riffkin et al 1996) that the site of immunomodulatory product production is important in the observed effects. Lunjgstrom and Huldt (Lunjgstrom and Huldt 1977) have shown that only the intestinal stage of the parasite will delay skin graft rejection. Given this, it is clear that there must be potent immunomodulatory factors in AWH for their effects to be observed under such conditions as applied in this experimentation.

It was very interesting to observe that the pattern of the increase in IgE and IgG1 responses mirrored the situation seen with infection. As shown in the group of mice that were exposed to a booster dose of AWH, the level of IgG1 increased continuously, peaking at seven weeks post treatment with a decline at week eight, similar to mice that were infected with live worms. However, in mice that receive an infection, there was an initial decline in the level of IgG1 in the serum at four weeks post infection, but not in the serum of AWH-treated mice. This difference could be attributed to the fact that by this time point (28 days), the worms would have been expelled. *Nb* adult worms are expelled from mice 10-14 days after the

infection (Wescott and Todd 1966). Thus, the mice are no longer exposed to worm products. In this study, it is reasonable to assume that AWH emulsification in FIA allows for the slow release of the extract and therefore a longer exposure to worm products. Alternatively, it is possible that the absence of the gastrointestinal inflammatory response associated with nematode infections (Urban et al 1993; Stadnyk and Kearsey, 1996) frees the AWH mediated effect from possible negative regulatory elements. The level and pattern of the response is indicative of a polyclonal (non-specific) effect rather than an antigen-driven response. In some ways this response is more closely related to a superantigen response than an antigen response.

Interestingly, this polyclonal response appears to be restricted to the type-2 associated immunoglobulins IgE and IgG1 (in mice). The level of the type-1 associated immunoglobulin, IgG2a, was not affected by injection of AWH. IgG2a levels were comparable to those detected in the sera of mice injected with the vehicle alone. In one experiment, the level of IgG2a was lower in AWH treated mice. Whether this points to an ability of AWH to cause a reduction in the level of IgG2a is unclear and will have to be the subject of future studies. This is an interesting preliminary finding in that it has been shown that infection can lead to a downregulation of this immunoglobulin isotype (Lebrun and Spiegelberg 1987; Finkelman et al 1988; Zakroff et al 1989). Thus, it is not unreasonable to suggest that, given the appropriate conditions, AWH may induce a similar effect.

It is well documented that helminths preferentially upregulate type-2 responses while downregulating type-1 associated responses. The fact that AWH induces marked increases in IgE and IgG1 levels, but does not affect the level of IgG2a, confirms that the effect of AWH on the immune response of injected mice is restricted to these type-2 associated immunoglobulins, and not a general upregulation of immunoglobulin levels. This is an important finding since it provides evidence of the nature of the AWH effect *in vivo*. The non-specific proliferation of B cells and the direct induction of antibody production seen in LPS activation (Snapper and Paul 1987; Lebrun et al 1989) do not appear to be occurring here. The response to AWH is clearly more complex. This view is supported by reports (Grzych et al 1991; Del Prete et al 1991; Bancroft et al 1993; Mahanty et al 1993; Yamaoka et al 1994) which have shown that injection of rodents with helminth products preferentially induces type-2 associated cytokine responses as indicated by the production of IL-4, IL-5, IL-9, and IL-13. Clearly nematode extracts initiate both non-specific and specific type-2 responses.

The generation of humoral immune responses to nematode infection (Lebrun and Spiegelberg 1987; Finkelman et al 1988; Zakroff et al 1989) or nematode antigenic immunization (Lee and McGibbon 1993; Kamata et al 1995), involves activation, proliferation, maturation and class switch (Kepron et al 1989). To produce IgE and IgG1 immunoglobulin responses this process is regulated by the cytokines IL-4 (Finkelman et al 1986 and 1988; Katona et al 1991; Madden et

al 1991; Kopf et al 1993; Urban et al 1993) and IL-13 (Punnonen et al 1993; Punnonen et al 1994; Mckenzie et al 1993, 1998a, 1998b; Barner et al 1998). These cytokines act as switch factors for activated B cells to mature and undergo class switch to produce IgE and IgG1 responses (Lebman and Coffman 1988; reviewed in Finkelman et al 1990; Mckenzie et al 1993, 1998a and 1998b). The importance of these cytokines in the generation of the type-2 associated immunoglobulin responses is well documented in infection of rodents with the nematode parasites *Nb* (Benbernou et al 1993; Kopf et al 1993; Urban et al 1993, 1995, 1998), *H. polygyrus* (Urban et al 1995; Finkelman et al 1997), *T. muris* (Else et al 1994; Bancroft et al 1998), *B. malayi* (Lawrence et al 1995) and *T. spiralis* (Pond et al 1989; Wakelin et al 1994). In contrast to IgE and IgG1, the induction of IgG2a response in mice is regulated by IFN-γ as a switch factor (Snapper and Paul 1987; Snapper et al 1988). As stated earlier, this response is downregulated in most nematode parasite infection (Lebrun and Spiegelberg 1987; Finkelman et al 1988; Zakroff et al 1989).

IL-4 and IFN-γ cross-regulate the responses generated by either cytokines (Mosmann 1991; Abbas et al 1996). IFN-γ inhibits the production of IgG1 and IgE in various systems (Coffman and Carty 1986; Sher et al 1992; Urban et al 1993). This observation has been demonstrated in mice injected with various nematode parasites. For example, the injection of IFN-γ or IL-12 in mice infected with helminths (Urban et al 1993; Oswald et al 1994; Finkelman et al 1994, 1997;

King et al 1995; Bancroft et al 1997; reviewed in Bancroft and Grencis 1998) significantly inhibits IgE and IgG1 responses, while enhancing IgG2a responses. Conversely, IL-4 inhibits IFN-γ mediated IgG2a production (Mosmann 1991; Finkelman et al 1994). This observation suggests a possible mechanism for the marked increase in IgE and IgG1 levels demonstrated in the serum of AWH treated mice, while no effect was seen in IgG2a levels. An IL-4 mediated mechanism is supported by the finding of high IL-4 levels in the culture supernatants of con A-stimulated spleen cells from AWH injected mice. This IL-4 preference is not a non-specific response to antigen since spleen cells from Mycobacteria injected mice did not exhibit such a type-2 preference. Mycobacteria is known to induce type-1 rather than type-2 responses (Beck and Spiegelberg 1989; Chensue et al 1994).

Clearly the immunoglobulin response induced by AWH is not merely a response to the injection of a complex antigen. This is supported by the fact that the injection of mice with killed Mycobacteria, in the same vehicle as AWH, did not induce the characteristic IgE and IgG1 response associated with AWH. In agreement with previous reports (Beck and Spiegelberg 1989), killed Mycobacteria induced a significant increase in IgG2a levels. Furthermore, the injection of mice with denatured AWH, did not induce the marked increase in IgE levels. Previous experiments in our laboratory have shown that denatured extracts from *A. suum* are sufficient to produce detectable specific anti-Ascaris antibody as

assessed by western blotting. However, they are not sufficient to cause a marked increase in total IgE in the serum as assessed by ELISA (T. Lee, personal communication). In this thesis, the boiled extract was sufficient to induce a slight increase in IgG1 levels. The difference in the IgE and IgG1 response suggests that perhaps a lower threshold is required to induce IgG1 response or alternatively, that some of the proteins are able to reconfigure in such a manner as to induce IgG1 in the serum. In summary, the fact that killed Mycobacteria elevated only IgG2a levels and not IgE and IgG1 levels and that the injection of denatured AWH did not cause a marked increase in immunoglobulin levels, confirms both the type-2 specificity and the active immune modulation associated with the AWH induced immunoglobulin response.

Nb, like most nematodes, possesses a protein composition with a degree of specificity to the different developmental stages involved in the life cycle (Maizels et al 1982, 1983; Goyal and Hanna 1988; Dorzok et al 1989; Healer et al 1991). However, both larval and adult stages of *Nb* have been reported to share many proteins (Maizels et al 1982; Goyal and Hanna 1988; Dorzok et al 1989). This is reflected in our data showing that an extract from the infective larvae of *Nb* (LWH) induced the production of both IgE and IgG1 in the serum of treated mice. Our data reveal that the active factor(s) is not specific to *Nb* adult worms. The IgE and IgG1 response induced by LWH was at the same level as that observed in the serum of mice treated with AWH. This observation suggests that the factor

responsible for the induction of the immunoglobulin response is likely present at the same level in both the larval and adult stages of the nematodes. In addition, because of the similar levels of IgE and IgG1 induced by LWH and AWH, it is reasonable to presume that the protein involved in the generation of the immunoglobulin response will not be the haemoglobin protein. This is the most abundant protein present in the adult stage of *Nb* (Lee 1965). It gives adult *Nb* its characteristic red colour, and is restricted to the adult stage alone (Sharpe and Lee 1981). There is some evidence that the enzyme acetylcholinesterase is present throughout the life cycle of *Nb* (Sanderson & Ogilvie 1971). However it is difficult to imagine that this molecule has the potential to induce the marked polyclonal IgE and IgG1 responses observed here.

It is well established that the production of IgE and IgG1 immunoglobulin isotypes in both *in vivo* and *in vitro* systems requires the presence of the cytokines IL-4 and IL-13 (Snapper and Paul 1987; King and Nutman 1992; Sher and Coffman 1992; Nakanishi et al 1995; Bancroft et al 1998; Lai and Mosmann 1999). These cytokines have always been associated with increases in the level of these immunoglobulins in mice serum (Katona et al 1988; Pearlman et al 1993a; Matsuda et al 1995; Finkelman et al 1997). To determine whether AWH would increase the production of these type-2 cytokines *in vivo*, cytokine mRNA and protein levels from spleen cells of AWH treated mice were examined. As expected, mRNA for both cytokines were detected in spleen cells isolated from

AWH treated mice. The levels were significantly lower than the mRNA levels observed in spleen cells from mice infected with *Nb* larvae. No mRNA for these cytokines was amplified from naive (control) mice, even though the reaction was amplified over 40 cycles. This confirms that the mRNA detected was stimulated by the AWH treatment *in vivo*. The mRNA levels here can be expected to be reflective of the situation *in vivo*, as the cells were treated *ex vivo*. It is possible that *in vitro* re-stimulation might affect the stability of the message (Ramaswamy et al 1994). mRNA have been reported to undergo degradation over time in culture conditions. This possibility must always be considered when RT-PCR data is discussed. However, there is no reason to believe that degradation in the spleen cell cultures of untreated mice would be greater than in AWH treated mice and thus such degradation would only reduce the apparent increase in IL-4 transcription in these cultures. Thus, a substantial increase in mRNA for IL-4 was seen in the AWH treated cultures even in the face of such potential degradation.

The detection of IL-13 mRNA in the spleen cell cultures from mice injected with AWH was of significant interest. It demonstrates the full spectrum of type-2 cytokines that regulate IgE and IgG1 production. This observation is of importance in light of the increasing evidence detailing the significant role of IL-13 in the regulation of type-2 associated immunoglobulin responses (Punnonen et al. 1993; Bancroft et al. 1998; McKenzie et al. 1998b; Urban et al. 1998a).

One criticism often leveled at mRNA levels as an assessment of cytokine

activity is the fact that the presence of mRNA does not always mean increased cytokine secretion. It is usually the case that increased transcription of cytokines leads to increased translation and increased secretion. However, some leukocytes can store cytokines before secretion (Gordon and Galli 1991). In addition, there is evidence of increased mRNA presence as a result of mRNA protection from degradation (increased mRNA stability) rather than increased transcription (Greenberg and Bender 1997). To address this, either transcription can be directly assessed by nuclear runoff assays or secretion can be measured by ELISA. In this study the latter technique was chosen. Cytokine secretion was assessed by stimulating spleen cells from AWH treated mice with the T cell mitogen, con A, and collecting the supernatant. The secretion results followed the same pattern as the mRNA, confirming the IL-4 and IL-13 activation in these cells. This finding is important as it indicates an increased secretion of these cytokines *in vivo*. This increase in IL-4 production by spleen cells did not occur in response to injection of mice with killed Mycobacteria, a control complex antigenic mixture.

A number of investigators have demonstrated type-2 cytokine mRNA and protein from spleen and mesenteric lymph node cells of mice infected with different nematode parasites (Svetic'et al 1993; Uchikawa et al 1994; Arizono et al 1994; Matsuda et al 1995; Stadnyk 1995; Lawrence et al 1996; Stadnyk and Kearsey 1996). The increase in cytokine mRNA and protein indicates that AWH, like live worms, stimulates both transcription and translation of the cytokines. In

both mRNA and protein profile, AWH induced lower levels than infection with *Nb* larvae. As stated previously, this difference could be due to a large number of differences between the single subcutaneous AWH injection and the fulminant parasitic infection. One such factor may be the migratory activity of the worm. Migration allows for greater contact with a variety of cells through the developmental path. Also, the possibility exists that there is a relationship between responses initiated during the lung and intestinal stages of *Nb* infection with the response observed in the spleen. This will not be the situation in mice injected subcutaneously with AWH.

IL-1 and IL-6 have been reported from alveolar macrophages in *Nb* infected animals (Egwang et al 1984, 1985). Since IL-6 is a differentiation factor for the development of type-2 cytokines (Rincón et al 1997), it is therefore a possible contributing factor to the higher level of type-2 responses generated in *Nb* infected mice than that seen in mice injected subcutaneously with AWH. Also, cells from the mesenteric lymph nodes develop the type-2 responses associated with nematode infections prior to cells in the spleen (Uchikawa et al 1994; Matsuda et al 1995; Lawrence et al 1996; Ishikawa et al 1998). It is unlikely that proteins in AWH home preferentially to these mucosal sites and the responses initiated there may have profound effects on the developing responses in the spleen. These would not be reflected in AWH induced responses. Taken together, it is reasonable to suppose that these factors are sufficient to mediate the difference in

cytokine levels and the subsequent difference in the serum levels of IgG1 and IgE between both treatments.

This study demonstrated that the injection of AWH, like *Nb* infection, induces a type-2 immunoglobulin response *in vivo* as shown by the marked elevation in both IgE and IgG1 levels and the no effect on the levels of IgG2a in the serum. Before addressing the possible mechanism(s) by which AWH mediated its effect, an investigation was initiated regarding whether AWH can reproduce the above effect in an *in vitro* system. The data presented here demonstrate that AWH also induced the production of IgG1 in B cell cultures stimulated with LPS. Neither LPS nor AWH alone, when added to B cells, induced an increased production of IgG1, confirming that the increase in IgG1 levels is not due to an enhanced production of immunoglobulins from memory B cells. This, and other data, is consistent with an increase in the number of switched B cells.

In congruence with the findings presented in this thesis, an extract of the intestinal nematode parasite *H. polygyrus* has recently been reported to stimulate the production of IgG1 in naive spleen cell culture *in vitro* (Robinson and Gustad 1996). However, this study did not examine direct effects on B cells since it examined IgG1 production of whole spleen cells in culture. This did not rule out the possible involvement of T cells. In fact, the data showed that the extract mediated its effect by stimulating T cells to produce a factor that induced the generation of IgG1 production in the culture. The data from my experimentation,

however, suggests a more direct effect of AWH on B cell differentiation that is T cell independent. This observation is supported by the fact that the purity of the B cells used in the *in vitro* cultures in my study was greater than 95 percent as shown by FACS analysis. Furthermore, similar results were observed when B cells from T cell deficient mice were used in the *in vitro* cultures.

The observation that AWH also induced the production of IgG1 in *in vitro* culture of pure B cells was very exciting, as it confirmed the activity of AWH we observed in the *in vivo* experimentation and suggested a possible mechanism of action. LPS induces naive B cells in culture to proliferate and mature into IgM secreting cells (Coffman et al 1986; Snapper and Paul 1987), (with some slippage to $\gamma 3$, the next C_H region on the mouse Ig gene), but it will not induce increased production of isotypes such as IgE, IgG1, or IgG2a, in the absence of an appropriate switch factor (Finkelman et al 1990). The production of IgG1 requires the switch factors IL-4 and IL-13 (Miller and Rothman 1998). Therefore, the production of IgG1 in the culture supernatant of purified B cells stimulated with LPS and AWH suggests that AWH has class switching activity. This remarkable finding was supported by the fact that neither LPS nor AWH alone had this effect, indicating that AWH does not activate B cells but affects a change in their differentiation. Furthermore, the observation that AWH decreased the levels of IgM in culture, while increasing IgG1 levels argues strongly for the induction of class switch of the LPS-stimulated B cells by AWH. Others (Kepron et al 1989;

Snapper and Finkelman 1990; Snapper et al 1991) have reported an association between reduction in IgM levels and class switch to IgE and IgG1 by IL-4. This reduction in IgM levels is believed to be due to the immunoglobulin class switch of IgM-bearing B cells to IgG1 B cells (Kepron et al 1989). Also, IgG2a level is known to be inhibited in the presence of IL-4 *in vitro*, as IL-4 cross-regulates IFN-γ activity, the switch factor for IgG2a (Coffman and Carty 1986; Snapper and Paul 1987). The *in vivo* data also supports this fact. Our observation of increased IgG1 in cultures of B cells stimulated with LPS in combination with AWH is in agreement with earlier reports that switch factors require prior activation of B cells for its activity to be mediated (Snapper and Mond 1993; Stavnezer 1996).

The *in vitro* activity of AWH reported here suggests strongly that AWH contains a switch factor, or that it mediates the production of a switch factor by the B cells themselves (or by the small number of accessory cells present to ensure adequate LPS stimulation of B cells). Evidence exists that some nematodes contain cytokine-like molecules (Maizel et al 1993; Riffkin et al 1996). For example an IFN-γ homologue has been reported in the intestinal nematode parasite *T. muris* (Grencis and Entwistle, 1997). Similarly, Pastrana and co-workers have also reported the secretion of a homologue of human macrophage migration inhibitory factor by the filarial nematode parasites, *B. malayi*, *Wuchereria bancrofti*, and *Onchocerca volvolus* (Pastrana et al 1998). These findings demonstrate that nematodes have the capacity to produce cytokine-like factors.

These cytokine homologues may have the potential to modify host immune responses to promote parasite survival.

This observation that AWH may induce class switch *in vitro* suggests a possible mechanism for the observations made in this study in whole animal experimentation. However, there are alternate mechanisms by which AWH could exert its effect to increase the levels of IgE and IgG1 *in vivo*. The increase in immunoglobulin levels *in vivo* could be as a result of; i) an upregulation of antibody production by committed plasma cells or ii) an expansion of IgG1 B memory cells.

A report of Robinson and Gustad (1996) which showed that adult worm products of *H. polygyrus* induced the production of IgG1 from murine lymphocytes *in vitro* might support the first alternate mechanism. However, in this thesis, AWH alone did not markedly induce the production of IgG1 from B cells *in vitro*, it required prior activation by LPS, a characteristic of a switch factor (Mandler et al 1993b; Snapper and Mond 1993; Stavnezer 1996). This characteristic is also exhibited by cholera toxin (Woogen et al 1987; Lycke and Strober 1989; Lycke et al 1990; Kim et al 1998) and retinoids (Dillehay et al 1991) known to promote B cell isotype differentiation only after B cell activation.

Robinson and co-workers (Robinson et al 1994, 1995), Lee and Xie, (1995) have shown that some nematodes can have B and T cell mitogenic activity suggesting a capability of inducing an expansion of memory B cells (the second

alternative above). However, this possibility is not supported by the data presented in this thesis. Data obtained from the *in vitro* proliferation assay showed that AWH does not expand B cells either from naive, AWH or *Nb* treated mice. In fact, AWH induced a significant inhibition of B cell proliferation.

A more convincing hypothesis is that the increase in immunoglobulin levels in the serum of AWH treated mice is the result of an increase in the number of IgG1 switched B cells, mediated by *de novo* class switch. Based on the increased production of IgG1 in LPS stimulated B cells exposed to AWH observed in our *in vitro* study, and the fact that it did not stimulate IgG1 production when added alone, a hypothesis that immunoglobulin class switch is the main mechanism by which AWH mediates the marked increase in total IgG1 levels *in vivo* is more tenable.

To evaluate whether the increase in total IgG1 by AWH *in vivo* was, in fact, due to wholesale class switch, we adopted the digestion-circularization polymerase chain reaction (DC-PCR) technique, first described by Chu and colleagues (Chu et al 1992). This technique is specifically designed to examine immunoglobulin class switch and its reliability in assessing the extent of immunoglobulin heavy chain gene recombination events has been well reported (Mandler et al 1993a; Purkerson and Isakson 1994; Nakanishi et al 1995; Shparago et al 1996; Zelazowski et al 1997). Because of the PCR component, the DC-PCR technique allows for the detection of a very low levels of DNA switch recombination, unlike the qualitative

Southern blotting technique, which can only measure cases where large amounts of DNA recombination have occurred (Chu et al 1993). Furthermore, the DC-PCR technique allows for the generation of a positive signal from switched DNA only, not from germline DNA. A major advantage of DC-PCR is that it generates a single amplicon of consistent size (Chu et al 1992; Chu et al 1993). This is not the case with other methods (Southern blotting or direct PCR) of detecting DNA switch rearrangement (Shapira et al 1991; Mills et al 1992). Direct PCR by amplifying across the switch region, for example, results in the generation of many amplicons with different sizes. The generation of the different size amplicons in direct amplification across the switch region arises from the random manner in which the recombination breakpoints occur within the large repetitive DNA switch regions of Sμ / S target, prior to deletion. Using primers located 5' of Sμ and 3' of target S region, amplification of the recombined Sμ / S target switch segment will thus yield different amplicons because of the many different ways these complex regions can be cut and recombined. In addition, because of the large size of S regions, direct priming across the recombined S region results in products in the kb range, in contrast to the much smaller products generated by DC-PCR. With DC-PCR, the formation of the small single amplicon allows for the comparison of the relative levels of switch recombination if the proper controls are incorporated. This and the other advantages mentioned above informed the choice of DC-PCR over the other techniques.

The DC-PCR technique involves the use of primers specific to regions upstream and downstream of μ and γ1 (in case of class switch to IgG1) switch regions (S). Gene rearrangement during immunoglobulin class switching is associated with the deletion of DNA between the Sμ region and the switch region of a downstream isotype (Sμ-Sγ1 for IgG1) (Dunnick et al 1993). This brings the S regions together and results in the ability to cut this switch (+ flanking) region out of the immunoglobulin gene DNA with a targeted restriction endonuclease (EcoRI). This particular region will have no internal EcoRI digestion sites. These fragments are then subjected to re-circularization under dilute concentrations (favours circularization over linear polymerization), to yield circular DNA pieces. This process brings together the 5' Sμ and 3' Sγ1 ends of the restriction fragments bearing the recombined switch region, resulting in the formation of DNA circles with one EcoRI site at the ligated ends. Using the appropriate primers designed to anneal to sequences just outside of the switch regions, the sequence is amplified by PCR across the ligated restriction sites. Amplifying this sequence allows for the generation of the uniform-sized fragment regardless of the sequence of the S region (Chu et al 1992; Chu et al 1993). It is important to note that the generation of the PCR product only occurs in DNA pieces where switch recombination has occurred. In the absence of switch, the switch regions, which are several kilobases apart exist on different fragments. These fragments lack the appropriate sequences to which the primer anneals during amplification. A schematic representation of

the digestion circularization polymerase chain reaction (DC-PCR) technique is shown in figure 37.

In assessing the level of switch between treatment groups, signals from the nicotinic acetylcholine receptor β sub-unit gene (nAChRe) were used as control. The gene is also digested with EcoRI and the fragment ligated in the same fashion as the Ig switch recombination. The fragment is then amplified with the appropriate primers as shown in figure 37. Two copies of this gene are present in all cells (including switched or unswitched B cells). DNA rearrangement is not required for the amplicon to be produced. (There are no internal EcoRI digestion sites between the primer target sites). This gene can be used to quantitate the DC-PCR since every cell will have two copies. Thus, switched vs non-switched DNA can be compared to this standard. This gene also serves as a control for DNA preparation, restriction digestion, and ligation (Chu et al 1992; Chu et al 1993). This control is not available in the other techniques. To ensure the adequacy of DC-PCR as a reliable technique to measure the extent of class switch, spleen cells from naive mice were examined for consistency of the nAChRe signal. A further control was the IgG1-producing hybridoma (TSI-18), examined for both nAChRe and Sμ-Sγ1 signals. The use of DNA from TSI-18 hybridoma provided a positive signal for IgG1 class switch with which to compare the level of immunoglobulin class switch in the different treatment groups in this thesis.

In all naive mice examined, an amplicon of 753 base pairs, the expected

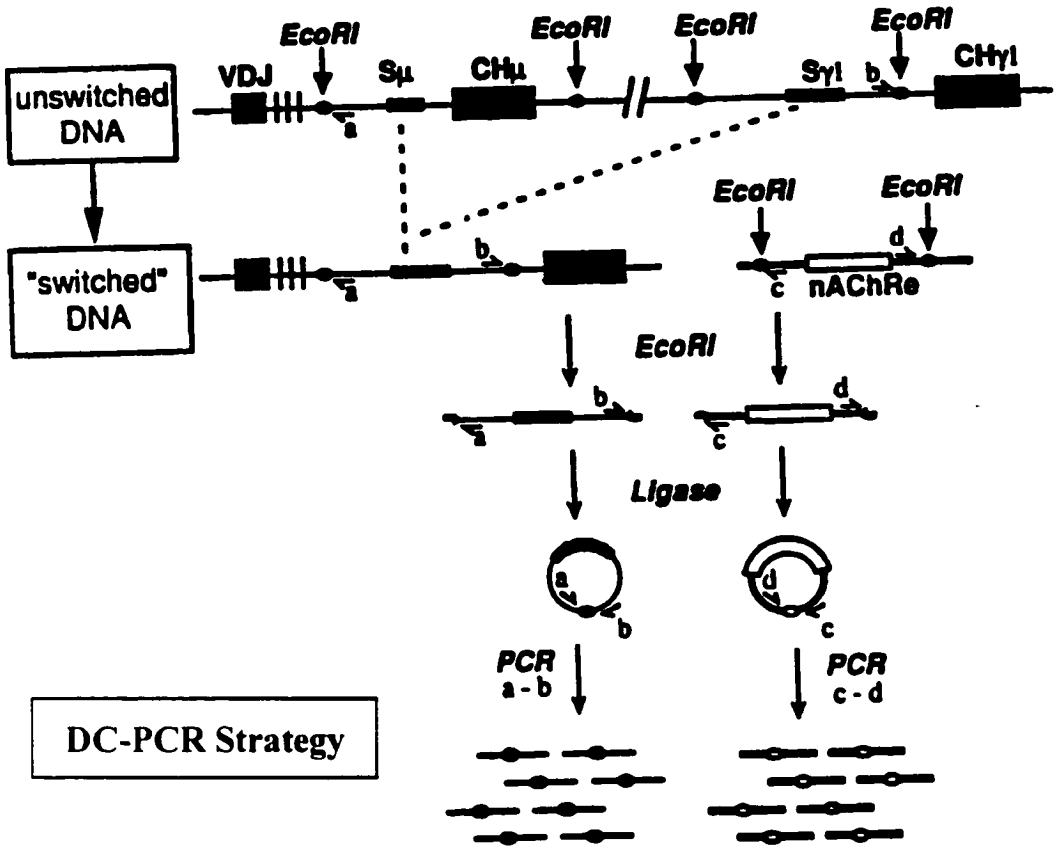


Fig 37. Schematic representaion of DC-PCR. Nonarranged chromosomal DNA is shown in the upper bar, depicting the relationships between the constant heavy chain gene of IgG1 ($C_H\gamma$ 1), the IgG1 switch region (Sγ1), $C_H\mu$, and Sμ. The sequences designated a and b, which are 5' to Sμ and 3' to Sγ1, respectively, are approximately 100 kb apart. Therefore, upon EcoR1 digestion of nonarranged DNA, a and b would be located on two separate fragments. However, if the IgG1 locus has been arranged, as shown in the lower bar, a and b would be on the same fragment. After EcoR1 digestion the fragments are circularized by T4 ligase. A new EcoR1 site is thus created, joining 3'Sγ1 to 5'Sμ. The digested, circularized DNA is then subjected to PCR, using primers complementary to a and b. If Sμ-Sγ1 recombinations are present on the genomic DNA sample, they yeild a PCR product of a distinct size (219 bp), whereas the nonrearranged IgG1 locus will not give rise to this PCR product. In a similar fashion another circularized fragment, which contains a portion of the acetyl-choline receptor (nAChRe), is created and amplified. Unlike IgG1, the nAChRe gene is not subject to rearrangements and is present equally in DNA from B cells that have or have not undergone IgG1 switch recombination. It therefore serves as a quantitative control for the efficiency of DC template generation.

(Chu et al 1993; Mandler et al 1993)

amplicon size for nAChRe, was detected at uniform levels with the same amount of template and 35 amplification cycles (non-saturating conditions). Similar results were obtained with TSI-18. The identity of the amplicon was confirmed by both sequence analysis and EcoRI restriction. The identification of the amplicon by sequence analysis was based on the 100 percent match of the 117 nucleotide bases obtained from the sequencing gel autoradiograph which was queried to Genbank for analysis. Their report matched this partial sequence of 117 nucleotides to the mouse nicotinic acetylcholine receptor beta subunit gene. This observation was critical since it demonstrated the establishment of this very important technique and the specificity of DC-PCR. Furthermore, the detection of two fragments following restriction of the amplicon with EcoRI, confirming the presence of a single EcoRI restriction site, provided additional confirmation for the identity of the amplicon and the adequacy of the technique to assess immunoglobulin DNA recombination.

As with nAChRe, the IgG1 switch DNA recombination product was amplified from DNA isolated from the IgG1 hybridoma TSI-18. An amplicon of 219 bp, similar to the published amplicon for IgG1 was obtained (Chu et al 1992; Chu et al 1993; Mandler et al 1993a; Nakanishi et al 1995; Zelazowski et al 1997). I was unable to sequence the amplicon. Amplicon digestion (although not quite visible on the scanned image of the gel) confirmed the presence of an EcoRI site within this amplicon. Mapping of the nucleotide sequence through Genbank

predicted the presence of only the one EcoRI site generated from the ligation of the two ends. The Sμ-Sγ1 amplification in TSI-18 is specific, as a control hybridoma (IgG2a-producing hybridoma IB4) was negative for the product. In all DC-PCR, controls lacking template were always negative for either nAChRe or Sμ-Sγ1 amplification. Therefore contamination with exogenous template DNA did not complicate the interpretation of results. The DC-PCR technique has been optimized, in these experiments, as a reliable quantitative assay for the level of IgG1 class switch.

Using this system, we examined DNA isolated from spleen cells of naive, *Nb*, FIA, and AWH treated mice for the presence and extent of switched DNA. The IgG1 switched DNA amplicon (Sμ-Sγ1; 219 bp) was detected at significant levels in DNA preparations of spleen cells from both *Nb* and AWH treated mice at three weeks post treatment. The level of switch was higher in cells from *Nb* infected mice than from cells obtained from mice injected with AWH, corresponding to the amount of IgG1 induced in the serum. At week one, no switch was detected in any group of mice, but at week two, IgG1 switch recombination was detected in the *Nb* and AWH treated mice with the level increasing further at three weeks post treatment. The lack of detectable switch at week 1 might be due to the decrease in IgG1 levels observed in the serum of *Nb* infected mice (Zakroff et al 1989) indicating some form of purge of IgG1 plasma cells. Alternatively, it may simply be due to the fact that such responses take time.

as evidenced by the fact that significant humoral immune responses to *Nb* infection are usually detected only after seven days (Urban et al 1984; Finkelman et al 1988; Zakroff et al 1989; Lee and McGibbon 1993).

A low level Sμ-Sγ1 switch was detected in control animals as the experiment progressed (week three). This low level. switch was not detected in DNA isolated at week one or two. The detection of low level IgG1 switch in control. untreated mice at week three, is indicative of the slight increase in serum IgG1 levels that is associated with mice as they get older. The observation of substantial IgG1 switch recombination in splenic B cells from *Nb* infected mice. is supported by an earlier report by Yoshida and colleagues (Yoshida et al 1990). Their study. using Southern blotting, indicated that switched DNA excision products of immunoglobulin heavy-chain constant region genes could be found in DNA isolated from the mesenteric lymph nodes (MLN) of *Nb* infected mice. This provides supportive evidence to our more specific and sensitive DC-PCR demonstration of a marked increase in IgG1 switched DNA in the spleens of *Nb* infected mice. The demonstration of specific class switch in the spleen. along with the presence of type-2 cytokines. suggests that the response in the spleen is an extension of that in the MLN. It also demonstrates the magnitude and widespread nature of *Nb* generated immune responses.

As with IgG1 levels in the serum. the observed increase in γ1 switched DNA in B cells from mice injected with AWH. was due to specific activation of

the IgG1 response by AWH, rather than it being a consequence of non-specific stimulation of all immunoglobulin isotypes. Examination of DNA isolated from the spleen cells of mice injected with killed Mycobacteria showed no detectable increase in the level of γ1 switched DNA. The level of γ1 switched DNA in mice injected with killed Mycobacteria was at the same level as mice treated with the vehicle alone. This is consistent with the expectation that, being an inducer of type-1 associated immune responses, Mycobacteria will not induce an increase in γ1 switched DNA but rather γ2a switched DNA.

Numerous attempts were made to adapt the DC-PCR technique, as modified by Purkerson and Isakson (1994) and Zelazowski et al (1997), for the detection of IgE (ε) switch. None of these attempts were successful. I was able however, to amplify the control (globin) gene for this system confirming that the system parameters were correct. Amplification of DNA templates from the different treatment groups for the globin gene, yielded the expected amplicon size of 589 bp. Different optimization approaches for the detection of IgE class switch recombination events, using primers designed by Purkerson and Isakson (1994) were unsuccessful. Because of the fact that both IgG1 and IgE are upregulated by *Nb* infection and other Th2 activating treatments (Lebrun and Spiegelberg 1987; Finkelman et al 1988; Beck and Spiegelberg 1989; Sudowe et al 1997), the evidence of sequential switch to IgG1 then to IgE (Yoshida et al 1990; Mills et al 1992; Mandler et al 1993b), and the resulting similarities in the regulation of these

immunoglobulins. I chose to concentrate on IgG1 as a marker for induction of immunoglobulin class switch by *Nb*.

The DC-PCR data strongly support the hypothesis that the induction of increased IgG1 and IgE levels in *Nb* infected and AWH treated mice is primarily due to stimulation of *de novo* class switch to these type-2 associated immunoglobulins. This premise is supported by the extent of switched DNA recombination for IgG1 detected in the spleen of mice treated with AWH compared to controls. This high level of switch, which is induced specifically by AWH, and the consequent high IgG1 levels in the serum, suggests a strong connection between *de novo* immunoglobulin heavy chain γ1 DNA recombination and the level of γ1 immunoglobulin in the serum (Rothman et al 1988; Stavnezer et al 1988). Further evidence to support this hypothesis comes from the observation that AWH alone does not induce an increase in IgG1 levels *in vitro*, except in the presence of a B cell activator, thus confirming that increased antibody production from previously switched γ1 B cells is not occurring. (There will have to be a number of switched γ1 B cells in a spleen B cell population). Moreover, AWH alone does not have the ability to expand γ1 B cells, confirming that γ1 B memory cell expansion is not the cause of the increased IgG1 levels in these mice. Addition of cytokines involved in B cell activation, such as IL-1, IL-2, IL-4, IL-5, and IL-6 (Clark et al 1996; Takatsu 1997; Rieckman et al 1997) to *in*

vitro cultures did not affect the inability of AWH alone to expand B cells or to induce production of IgG1 from B cells.

5.2 Characterization of the modulatory activity of AWH on B cell proliferative response

The data discussed in the preceding section showed, among other things, that AWH alone did not expand B cell populations *in vitro*. In fact, the observation was made that AWH inhibited the proliferative activity of B cells. This observation is very interesting. It demonstrates the diversity in the modulatory activity of nematodes, and suggests a tentative link between the two phenomenon (discussed below).

Inhibition of B cell function by *Nb* has not been widely reported. The emphasis has been on the stimulatory activities (Zakroff et al 1989). However, suppression of mitogen responses in both B and T cells has been reported in filarial nematodes. In this case, alterations in lymphocyte regulatory mechanisms have been postulated (Lammie and Katz 1983; Leiva and Lammie 1988; Lal et al 1990; Allen et al 1996). Alterations in lymphocyte reactivity have also been reported in other helminths such as *Fasciola hepatica* (Cervi and Masih 1997) and *Acantholinema viteae* (Harnett and Harnett 1993). However, no in depth study of the mechanism by which these modulatory effects are regulated, or their relationship to type-2 immunoglobulin production, has been attempted.

In this thesis, AWH, added at the onset of culture, showed a dose-dependent inhibition of LPS stimulated B cell proliferation. At the highest concentration of AWH, the proliferative response of B cells was inhibited by greater than 90% demonstrating a very potent inhibitory activity.

Although AWH strongly inhibits B cell proliferation in response to LPS stimulation, it does not have any effect on the proliferation of T cells to potent activators. This confirms that the lymphocyte inhibitory effect is restricted to B lymphocytes. This observation is surprising. In most reports, both B and T cells are subject to the effects of inhibitory factors, whether from nematodes, platyhelminths or from other origins. For example, a phosphorylcholine-containing preparation of the nematode *B. malayi* (Lal et al 1990), an extract of *A. suum* (Soares et al 1987; Ferreira et al 1995) and the immunosuppressive drug rapamycin (Kay et al 1991), have been demonstrated to inhibit the functional activities of both B and T cells. Interestingly, an earlier report by Price and Turner (1986) indicated that extracts from *H. polygyrus* and *N. brasiliensis* inhibited proliferative responses of spleen cells to Con A (T cell mitogen) and LPS (B cell mitogen). The inhibition was observed optimally when the extract was added to the cells 24 hours prior to the addition of mitogens. In contrast, the experimentation in this thesis revealed that the inhibitory effect of *N. brasiliensis* AWH is specific to B cells, with no effect on T cells and that AWH is able to inhibit LPS-induced B cell proliferation long after the inductive phase, as indicated

by the inhibition observed when the extract was added as late as 48 hours following exposure to LPS. The reasons for this discrepancy are unclear, but may relate to the manner in which the *N. brasiliensis* extract was prepared by Price and Turner.

The inhibitory activity of AWH appears to be consistent over time in culture. Evidence of AWH inhibition of LPS-mediated B cell proliferation was seen as early as 24 h. However, the inhibition was greater in cultures incubated for 72 hours prior to the addition of thymidine. This data argues that the inhibitory effect of AWH is due to the production and subsequent accumulation of factors *in vitro* and not due to non-specific cellular cytotoxicity of the extract.

Indeed, the data presented in this thesis show that AWH is not toxic to cells. This was immediately evident in the T cell culture experiments where AWH had no effect on T cell proliferation. The data also show that AWH is not selectively toxic to B cells. The viability of B cells after 24 hours in culture was similar between cells stimulated with LPS alone and those stimulated with both LPS and AWH. Further, the exposure of B cells to 50 μg/ml of AWH for 4 to 6 hours, followed by washing in fresh media, did not have an effect on their proliferative response upon re-stimulation with LPS. Similar short exposure of B cells to agents with cytotoxic potential induces cell death. Additional evidence that AWH was not mediating its inhibitory activity by inducing cell death is provided by the

demonstration of a similar level of apoptosis (measured by JAM assay) observed in LPS-stimulated B cells cultured with or without AWH.

Others have shown that other helminths' immunomodulatory factors do not mediate their effects by cytotoxicity. For example, Cervi and Masih (1997) showed that the suppressive effect of the trematode *F. hepatica* glycoprotein was not attributable to cytotoxic action. They found that the viabilities of cells cultured in the presence of the glycoprotein were higher than those of control cells that were not exposed to the parasite extract. Studies with nematode extracts (Price and Turner 1986, Leiva and Lammie 1989; Lal et al. 1990, and Harnett and Harnett 1993), have shown similar findings. Indeed, the findings in this thesis are most akin to those reported by Peçanha and co-workers (Peçanha et al 1993) and Marcelletti (1996) with respect to cytokine mediated modulation of B cell proliferation. They demonstrated that the inhibitory activity of IL-10 on LPS-induced B cell proliferation is not a result of cytotoxicity since IL-10 treatment failed to increase apoptosis, and did not alter cell viability.

To assess the mode of action of AWH on B cells, it was important to investigate whether this effect seen with LPS was mitogen specific (restricted), or whether AWH would influence the effects of B cells to a variety of activating stimuli. The data in this thesis clearly demonstrates that AWH also inhibits the proliferation of B cells stimulated with other potent activators. B cells stimulated with mitogens (LPS and PWM) were more susceptible to the inhibitory effect of

AWH, but cells activated by cross-linking either CD40 (by anti-CD40 mAb) or surface Ig (by SAC) also showed decreased proliferation in the presence of AWH. This observation suggests that either there is a difference in the sensitivity level of the different signaling pathways activated in the cells to AWH or AWH is affecting the co-stimulatory signaling pathways required, probably to a greater extent, by mitogen activated B cells. With the other activators, another pathway that is probably resistant to AWH or partially blocked by AWH may explain the difference in inhibition.

The fact that AWH inhibitory activity is not restricted to LPS-mediated B cell proliferation argues for the involvement of downstream signaling pathway in the inhibitory effect of AWH. This is supported by the finding that, AWH also inhibited B cell proliferation stimulated by the combination of PMA and Ionomycin. The effect of AWH does not appear to be mediated by interfering with the binding of LPS to its receptor, since the inhibitory effect was observed when the extract was added to the cultures as late as 48 hours following LPS stimulation. Stimulation of B cells with LPS for 24 hours is adequate for the cells to maintain their proliferative capacity when subsequently incubated in fresh media, in the absence of mitogen, although at a somewhat reduced level (data not shown). The fact that AWH suppressed the proliferation of B cells, even after LPS would have bound to its receptor, confirms that the inhibition of proliferation could not be due to blockade of LPS binding to its receptor, or down-regulation of other early stages

of B cell activation, but rather is due to activation events downstream to receptor ligation, perhaps including intracellular signaling events such as those mediated by late acting tyrosine kinases. This observation is consistent with that of Harnett and Harnett (1993) and Deehan et al (1997) which demonstrated that the inhibition of anti-Ig induced B cell proliferation by an extract of *A. viteae* was mediated by downregulation of protein kinase C expression and activity. A report by Deehan et al (1998) also demonstrated that a nematode extract inhibited B cell proliferation by modulating sIg coupling to Ras/MAP kinase and the PI-3-K signaling pathway.

AWH does not affect B cell proliferation by binding directly to (mopping up) LPS, preventing its action. Stimulation of B cells with very high doses of LPS (100 μg/ml) did not change AWH activity, neither did they enhance proliferation beyond the level observed with lesser doses of LPS (5 μg/ml), used throughout the study. This observation is consistent with those of Price and Turner (1986), Lal et al (1990), Harnett and Harnett (1993) and Marcelletti (1996) who showed that addition of high levels of the respective B cell activator did not rescue the cells from the inhibition of their proliferative responses.

Macrophages, as a source of co-stimulatory signals, are essential for the generation of robust proliferation of B and T cells (Corbel and Melchers 1983). So, it is therefore not surprising that they appear to be primary target for factors modulating lymphocyte function. A number of reports have demonstrated effects of parasite factors on macrophage activities (Kadian et al 1994; Allen et al 1996;

Motran et al 1996; Rakha et al 1996; Cervi and Masih 1997). The data in this thesis show that macrophages are involved in the inhibitory activity of AWH on LPS-stimulated B cells. This is demonstrated by the observation that a B cell population, which had been purged of macrophages (by plastic adherence for 24 hours) was resistant to the inhibitory effect of AWH. These experiments were controlled for the late addition of AWH to these cells (after 24 hour incubation to remove macrophages) by using a group of B cells which were treated, 24 hour post culture, with AWH. These control cell populations, which still contain macrophages, showed similar suppression of proliferation by AWH as cells treated with AWH at the time of initiation of culture. Thus, it was the lack of macrophages, not the late addition of AWH that rendered AWH incapable of inducing the suppression in these experiments.

There are a number of ways AWH could modulate macrophage activity. The most widely investigated mechanisms of action involve i) the suppression of cytokines (IL-6, TNF-α) that produce positive signals or ii) the upregulation of production of cytokines (IL-10, TGF-β) and other non-specific suppressive factors (nitric oxide, prostaglandins, hydrogen peroxide and superoxide anion) which produce negative signals (Schebesch et al 1997). Several reports have described a prominent role for macrophages in immune modulation mediated by helminth parasites (Kadian et al 1994; Allen et al 1996; Motran et al 1996; Rakha et al 1996; Cervi and Masih 1997). Suggested mechanisms of immune modulation by

macrophages involve the induction of both such positive and negative factors (Albina et al 1991, Allen et al 1996, Motran et al 1996, Cervi and Mashi 1997). For example, Cervi and Mashi (1997) reported that *F. hepatica* induced the production of hydrogen peroxide and superoxide anion, by macrophages, which inhibited T cell proliferation in response to Con A. Products of other helminths have been reported to also induce the development of suppressive peritoneal exudate cells (PEC), by modifying macrophage accessory functions and production of non-specific inhibitory factors, which inhibit both B and T cell proliferation *in vitro* (Rakha et al 1991; Kadian et al 1994; Rakha et al 1996; Allen et al 1996; Allen and MacDonald 1998; MacDonald et al 1998). These studies demonstrate that interference with the normal accessory role of macrophages, by parasites, will have profound effects on lymphocyte responses. However, these studies do not account for the B cell specific effects seen with AWH. Such an effect on B cells, sparing T cells, argues for a specific cytokine mediated effect, rather than a more non-specific effect mediated by reactive oxygen intermediates.

In order to determine the effect AWH was having on cytokine production by macrophages, cytokine levels in culture supernatants of macrophage stimulated with LPS alone or in combination with AWH were assayed by ELISA. Production of IL-6, IL-10, IL-12 and TNF-α was all decreased by 30-40%, in cultures of macrophages stimulated with both LPS and AWH. These results indicate a general down-regulation of macrophage cytokine potential, which could result in

decreased proliferation of B cells. However, addition of exogenous IL-6 and TNF-α (both cytokines required for optimal B cell proliferation: Rieckmann et al 1997; Takatsu 1997) to cultures of B cells stimulated with LPS and AWH, either alone or in combination, did not restore proliferation to the level of B cells stimulated with LPS alone. IL-6 did have a partial reversal effect on the inhibitory effect of AWH on B cell proliferation stimulated by LPS. Although the effect might be slightly significant, the level of proliferation was still markedly lower than LPS stimulated B cell proliferation. The addition of other cytokines (IL-1, IL-2, IL-4 and IL-5) which have been reported to affect B cell function (Rieckmann et al 1997; Takatsu 1997), also, did not restore B cell proliferation. These results show that the reduction in cytokine level does not appear to be the mechanism by which AWH mediated the inhibitory effect on LPS-stimulated B cell proliferation. All cytokines added to the cultures were bioactive, as assessed in bio-assays.

In addition to cytokine modulation, parasites also modulate macrophage function by inducing increased production of suppressive factors such as prostaglandins, nitric oxide, hydrogen peroxide and arginase (Albina et al 1991). These factors, as stated previously, are known to inhibit proliferation of B and T cells to various activators (Rakha et al 1991; Kadian et al 1994; Rakha et al 1996; Allen et al 1996; Allen and Macdonald 1998). The influence of these, non-specific suppressive factors in AWH mediated inhibition was also assessed. In these experiments, it was demonstrated that the inhibitory effect induced by AWH was

independent of any of these factors, because addition of their inhibitors failed to restore the proliferative response of B cells. They did, however, restore B cell proliferation slightly, but not significantly (data not shown).

Taken together, these data provide evidence that the downregulation in proliferation of B cells in the presence of AWH is not mediated by a lack of well described cytokine enhancers or by the production of non-specific cell suppressors such as reactive oxygen intermediates. The data argue for a more specific, perhaps cytokine mediated inhibition of B cell function. One such potential candidate cytokine is IL-10. A number of studies have demonstrated the inhibition of B cell proliferation by IL-10 (Itoh and Hirohata 1995; Marcelletti 1996). However, AWH did not induce an increase in IL-10 production in the cultures, rather it suppressed IL-10 production by about 30-40%. There may be a role for IL-10 if B cells are inhibited because they are more sensitive to effects of IL-10 in the presence of AWH. Increased cellular responsiveness to cytokines has, in fact, been demonstrated to result from parasitic infection (R. Liwski *J. Immunol.*; in press).

Another candidate cytokine is TGF-β. TGF-β is a cytokine with potent inhibitory activity on lymphocyte proliferation (Fox et al 1992; Bouchard et al 1994). In this study, the addition of an anti-TGF-β antibody to cultures of B cells stimulated with LPS and AWH increased the proliferation of the cells. However, it also increased the proliferative response of B cells stimulated with LPS alone.

This suggests that TGF-β production in the cultures is not related to AWH but results from LPS activation. This does not support a hypothesis that TGF-β mediates the inhibition seen here.

Although the exact mechanisms through which AWH induces the inhibition of LPS-mediated B cell proliferation remain unclear, the data presented in this thesis confirms that macrophages are the source of the activity that impairs the proliferative response of B cells. B cells require macrophages to respond appropriately to LPS (Corbel and Melchers 1983). Thus it was thought that if AWH was added at the initiation of culture, the initial stimulatory signals given to the B cell by the macrophage could be interrupted by AWH. This possibility was eliminated, by stimulating the B cells with LPS, in the presence of macrophages, for 24 hours in the absence of AWH. This allows activation of the B cells to occur. The subsequent addition (at 24 hours) of AWH was still able to inhibit proliferation but only in the presence of macrophages. This confirms that AWH is not blocking proliferation by inhibiting the delivery of a late accessory signal from macrophages but is somehow mediating active suppression of the response. The partial reversal of the inhibitory effect of AWH on LPS-mediated B cell proliferation by inhibition of macrophage activity with IL-4 (data not shown), further confirms the active involvement of macrophages in AWH mediated inhibition of B cell proliferation. This partial reversal of AWH inhibitory activity could be explained by the ability of IL-4 to suppress macrophage activity (Hart et

al 1991; Maizels et al 1993; Doherty et al 1993; MacDonald et al 1998; Nasarre et al 1998).

It was of significant interest, in this context, that ligation of CD40 molecules on B cells, with anti-CD40 antibody, restored LPS-induced proliferation in AWH-containing B cell cultures. Others (Liu et al 1988; Marcelletti 1996) have found that addition of anti-CD40 rescues B cells from inhibition. Also, it has been demonstrated in a number of studies that ligation of the CD40 molecule on B cells rescues the cells from undergoing apoptosis (Wang et al 1995; Schauer et al 1996, 1998). Marcelletti (1996) has suggested that anti-CD40 rescue of B cell inhibition by IL-10 results from rendering these cells resistant to IL-10 mediated inhibition (rather than the activation of a B cell sub-population that is IL-10 resistant). Data from this study would support the same conclusion here. First, since the proliferation of B cells in the presence of LPS and anti-CD40 antibodies without AWH is additive, it suggests that two activation pathways, with different sensitivities to AWH, are operational. If the anti-CD40 is not having an effect on the AWH sensitive LPS-stimulated B cells (90% of the population), then it would have to induce a massive proliferation in the remaining 10% of the B cells. Second, the level of proliferation induced by anti-CD40 antibodies alone is significantly lower than both combined and is inhibited by 30-40% with AWH. As such this cannot account for the high level of proliferation. Third, the addition of anti-CD40 antibodies, 24 hours after initiation of B cell culture with LPS and

AWH. also restored B cell proliferation (data no shown). Since unstimulated B cells die within 24 hours. the B cells responding to anti-CD40 antibodies. when the antibody is added 24 hr later. are most likely the LPS-responsive population. and not unstimulated cells. As have been reported in other B cell systems. the activity of anti-CD40 in the rescue of AWH-mediated inhibition of B cell proliferation may be related to the activation of the anti-apoptotic cascade of gene products by CD40 ligation. For example Wang and colleagues have shown the involvement of the anti-apoptotic genes *bcl-x* and *bcl-2* in the rescue of murine B cells from sIg-induced apoptosis. mediated by CD40 engagement on the cells (Wang et al 1995). It will be interesting to investigate whether this is a possible mechanism of anti-CD40 mediated rescue of AWH inhibited B cell proliferation stimulated by LPS.

It is important to note that only ligation of CD40 reversed the anti-proliferative effects of AWH. Crosslinking of surface Igs with either goat-anti-mouse IgM or goat-anti-mouse IgG1 antibodies did not reverse the anti-proliferative effects of AWH on LPS-induced B cell proliferation (data not shown). This finding suggests a high level of complexity of the response to AWH by B cells. Clearly. simple activation of the B cell receptor (BCR) is not sufficient to ablate the effects of AWH.

AWH is a complex mixture of proteins (Dorzok et al 1989) with an inhibitory effect on LPS-induced B cell proliferation as reported in this thesis. Analysis of the extract by SDS-PAGE. size exclusion and ion exchange

chromatography confirms the complex nature of the AWH preparation used in this study. A preliminary characterization of the nature and size of the factor(s) responsible for the inhibitory activity of AWH, revealed that they are sensitive to denaturation by boiling and proteolytic enzyme treatments. This suggests that the active factor is proteinaceous in nature. This result was not unexpected since other extracts with inhibitory effects on lymphocyte proliferation have been suggested (and in some cases, shown) to be protein dependent (Price and Turner 1986; Lal et al 1990; Soares et al 1992; Harnett and Harnett 1993; Pearlman et al 1995; Cervi and Masih 1997). Nematodes have been shown to undergo changes in protein composition as they develop from larvae to adults, thereby possessing proteins that are unique to specific stages of the life cycle (Phillip et al 1980; Maizels et al 1983). Others (Pery et al 1979; Petit et al 1980; Dorzok et al 1989) have analyzed the protein profile of extracts from larvae and adult *Nb* and showed the presence of a number of stage specific proteins. Therefore, to obtain preliminary data regarding the nature of the factor, it was important to determine whether the active factor in AWH was restricted to any one stage of the life cycle. To address this, B cells stimulated with LPS were exposed to the same concentration of either extract from adult worms (AWH) or extract from larval worms (LWH), and their effects on B cell responsiveness to LPS assessed. Results from this experiment showed that unlike AWH, which inhibited LPS-induced B cell proliferation, LWH had no inhibitory effect on the proliferative response of B cells, even at the highest

concentration of 20 μg/ml. This observation is consistent with the factor responsible for the inhibitory activity of AWH being specific to the adult stage in the developmental life cycle. One major protein in the adult worms and absent in the larvae is the haemoglobin which gives the adult its characteristic red colour (Sharpe and Lee 1981). Preliminary fractionation experiments to determine the molecular size of the factor indicated that the activity co-purified with the fraction containing the haemoglobin. This does not nessarily indicates that the haemoglobin is the active factor but it does confirm that the size of the factor is generally similar to the molecular size of the haemoglobin protein, which is between 65-75 kDa (Sharpe and Lee 1981). The molecular weight of the factor responsible for the inhibitory activity of AWH was determined, by fractionation analysis, to be greater than 50 kDa but less than 100 kDa. Only this fraction inhibited the proliferative response of B cells stimulated with LPS, as did whole, (unfractionated) AWH. Obviously there are many protein components of the AWH extract that fall in this range (Dorzok et al 1989) and further conclusions cannot be drawn about the nature of the factor without further experimentation.

Although the data reported here are from *in vitro* experimentation, it is likely that this B cell inhibitory activity has significant *in vivo* relevance. The role of antibody in the clearance of nematode infection is still controversial, but clearly any dysfunction of B cell expansion will have profound effects on the progress of the immune response elicited by the infection. In addition to anti-parasite

immunity. this modulation could also have profound effects on the development of immune responses to other infectious agents in nematode infected individuals.

It is thus very likely that the modulation of Ig class switch and the dysregulation in B cell expansion will dramatically effect immune responses in infected individuals. Whether both of these aspects of the modulation of the immune response are mediated as an "escape mechanism" is unclear. The whole concept of "immune evasion" as it relates to nematode infection is still hotly debated. However. the potential for abnormal immune responses in infected individuals is obviously high. as is the potential for the development of novel immunotherapies by studying these interesting biological systems.

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