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DIETARY PROTEIN DEFICIENCY MODIFIES SYSTEMIC AND GUT-ASSOCIATED IMMUNE RESPONSES IN MICE INFECTED WITH *HELIGMOSOMOIDES POLYGYRUS* (NEMATODA)

by

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ABSTRACT

Protein deficiency may increase susceptibility to gastrointestinal (GI) parasitic infections, possibly as a result of impaired systemic and/or intestinal effector responses induced by downregulation of Th2 cytokines and/or upregulation of Th1 cytokines. To test this hypothesis, female BALB/c mice (n=18/diet) were fed a control (24%), marginal (7%), or deficient (3%) protein diet and given a challenge infection with the GI nematode, *Heligmosomoides polygyrus*. The 3% mice had higher worm burdens at 1, 2 and 4 weeks post-challenge infection (pci), lower increases in serum IgE, reduced intestinal eosinophilia, and depressed mucosal mast cell proliferation and activation at 1 to 2 weeks pci. To determine whether these suppressed effector responses in the 3% mice were associated with altered spleen and mesenteric lymph node (MLN) cytokine profiles, cells were restimulated in vitro with parasite antigen and cytokine concentrations were measured. Deficient MLN cells secreted significantly less IL-4 and more IFN-γ at 1-2 weeks pci than did control MLN cells. Deficient spleen cells also secreted more IFN-γ at 2 weeks pci compared with control spleen cells. From RT-PCR analyses, the 3% mice also had lower IL-4 mRNA expression in spleen and MLN at 1-2 weeks pci. Our study supports the hypothesis that protein deficiency exacerbates the survival of a GI nematode parasite by decreasing IL-4 (Th2) and increasing IFN-γ (Th1) early in the infection, leading to reduced gut and systemic Th2 effector responses.

ABRÉGÉ

La carence alimentaire protidique a démontré son influence à la prédisposition aux infections parasitaires gastrointestinales chez la souris. Une relation possiblement due à une détérioration de l'action des effecteurs généraux et/ou intestinaux provoquée par une régulation à la baisse des cytokines Th2 et/ou d'une régulation à la hausse des cytokines Th1. Pour tester cette hypothèse, des souris BALB/c (n=18/diète) ont été nourri selon trois types de diètes protidiques différentes: un groupe témoin (24%), marginal (7%) et déficient (3%). De plus, ces souris étaient infestées par le nématode *Heligmosomoides polygyrus* selon un schéma de première infection suivie d'une réinfection. Le 3% groupe avait un taux d'infection plus élevé à 1, 2 et 4 semaines après la réinfection, les augmentations des taux d'IgE les plus bas dans le sérum, une éosinophilémie intestinale, ainsi qu'une prolifération et une activation réduites de mastocytes de la muqueuse à 1 et 2 semaines post-réinfection. Pour déterminer si cette diminution de l'activité des effecteurs dans le 3% groupe est le résultat d'un profile altéré de la rate et des cytokines du GLM, des cellules ont été stimulées *in vitro* avec de l'antigène de parasite. Les cellules du GLM du groupe déficient ont sécrétées beaucoup moins d'IL-4 et d'IFN-γ durant les 2 semaines suivant la réinfection que le groupe de cellules du GLM du groupe témoin. Les cellules de la rate ont elles aussi sécrétées plus d'IFN-γ durant les 2 semaines après la réinfection en comparaison aux cellules du groupe témoin. D'après des analyses au PCR, le groupe DP n'exprimait pas aussi bien IL-4 ARN messager de la rate et du GLM à 1 ou 2 semaines post-réinfection. Nos résultats soutiennent l'hypothèse que la carence protidique exacerbe la survie d'un nématode gastrointestinal en diminuant IL-4 (Th2) et en augmentant IFN-γ (Th1), qui amènent une diminution de l'activité des effecteurs Th2 intestinaux et généraux.

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Lastly but immensely, I also dedicate this thesis to J.P. Paiement. Thank you for everything that you are and more. *Elephant shoe.*

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STATEMENT OF AUTHORSHIP

All experimental work in this thesis was performed by Rebecca Y.L. Ing with the exceptions of the worm counts, histological evaluations, and RT-PCR assays. Dr. Zhong Su performed the histology and RT-PCR experiments. This thesis was written by Rebecca Y.L. Ing. Dr. M.E. Scott and Dr. K.G. Koski served as research/thesis supervisors, critiqued data presentation and analyses, and pre-edited all written material. The authors gratefully acknowledge Farzaneh Jalili for her work in counting the worm burdens.

LIST OF ABBREVIATIONS

ADCC	antibody-dependent cellular cytotoxicity
ANOVA	analysis of variance
APC	antigen presenting cell
BSA	bovine serum albumin
BUN	blood urea nitrogen
CMI	cell-mediated immunity
ConA	concanavalin A
DNA	deoxyribonucleic acid
DTH	delayed type hypersensitivity
GALT	gut-associated lymphoid tissue
GM-CSF	granulocyte-macrophage colony stimulating factor
HBSS	Hank's Balanced Salt Solution
HEV	high endothelial venule
HPRT	hypoxanthine phosphoribosyltransferase
IE	intraepithelium
IEL	intraepithelial lymphocytes
IL	interleukin
IFN-γ	interferon-gamma
L_3	third-stage larvae
L_4	fourth-stage larvae
LP	lamina propria
LPS	lipopolysaccharide
mAB	monoclonal antibody
mRNA	messenger ribonucleic acid
MLN	mesenteric lymph nodes
MHC	major histocompatibility complex
MMC	mucosal mast cell

MMCP-1	mucosal mast cell protease-1
NK	natural killer
PBS	phosphate buffered saline
pci	post-challenge infection
PHA	phytohemagglutinin
pi	post-infection
ppi	post-primary infection
PWM	pokeweed mitogen
RNA	ribonucleic acid
RT-PCR	reverse transcriptase - polymerase chain reaction
SRBC	sheep red blood cell
Th	T helper
Th1	T helper type 1
Th2	T helper type 2
TCR	T cell receptor
TNF-α	tumour necrosis factor - alpha

CHAPTER I
INTRODUCTION

Protein malnutrition and helminth infections of the human gastrointestinal tract are among the most common causes of morbidity and mortality worldwide (WHO, 1987). The coexistence of enteric helminth infections with dietary deficiencies, particularly in populations of developing countries, has been well-documented in epidemiological and clinical studies (Crompton, 1986). Although the prevailing socioeconomic and environmental conditions (e.g. poor sanitation, inadequate preventive health care) may predispose the individual to both malnutrition and parasitic infection, the complex bidirectional interactions between the two conditions may influence disease patterns and the efficacy of nutritional repletion or anti-parasitic interventions (Bundy & Golden, 1987). Malnutrition is known to depress immunocompetence in humans and experimental animals (Good & Lorenz, 1992; Kuvibidila et al., 1993), leading to increased susceptibility to parasitic infections. Alternatively, intestinal parasites may influence host defenses (e.g. villous atrophy and damage to the mucosal epithelial integrity) which in turn disrupt the uptake and utilization of dietary nutrients (Upadhyay *et al.*, 1985; Solomons, 1993). Parasitic infections are also implicated in human wasting and nutrient loss associated with protein deficiency due to expenditure of resources for immunological processes rather than for tissue synthesis, to excessive intestinal blood loss and to the leakage of plasma proteins across the damaged gut epithelial barrier (Lunn & Northrop-Clewes, 1993). Thus, host immune function may be one mechanism underlying the mutually aggravating interactions of malnutrition and helminth infections. Given the possible relationships in the malnutrition-infection-immunity complex, this project was aimed at determining the effect of protein malnutrition on host immune response to intestinal nematode parasites.

Protein deficiency, in conjunction with inadequate energy intake, has been associated with profound growth retardation (stunting), metabolic dysfunctions, increased child mortality, and greater prevalence of intestinal parasitic infections (WHO, 1987; Latham, 1990; Kuvibidila et al., 1993; Nesheim, 1993). Epidemiological studies have suggested a strong association between protein malnutrition and immune function

(Chandra, 1983), however, the evaluation of causal relationships in the human setting is complicated by the presence of co-existing confounding variables which make it difficult to establish whether the impaired immune function is a direct result of protein deficiency or whether it is secondary to the presence of concomitant infection, unrelated diseases, other nutritional deficiencies, or environmental factors. Despite the intrinsic interpretative problems in epidemiologic data, the conclusions from human studies have been supported by experiments showing that protein deficiency depressed various indices of immune function in uninfected animals (e.g. Conzen & Janeway, 1988; Lamont *et al.*, 1988; McGee & McMurrary, 1988; Woodward *et al.*, 1995) and increased the transmission and/or survival of *Nippostrongylus brasiliensis*, *Trichuris muris* , and *Heligmosomoides polygyrus* in rodents (Duncombe *et al.*, 1981; Slater, 1988; Keymer & Tarlton, 1991; Michael & Bundy, 1992a). However, the majority of these studies either did not involve parasitized hosts when investigating the role of protein nutrition in host immunocompetence or did not examine immunological mechanisms relevant to the control of helminthiasis. Therefore, much is unknown about the effect of protein malnutrition in host-parasite interactions.

Functional immunity to nematode parasites depends on specific cytokine production by type 2, T helper cells (Th2) which induce and mediate antibody-dependent effector responses involving IgE, IgG1, mucosal mast cells (MMC) and eosinophils (Monroy & Enriquez, 1992; Needham & Lillywhite, 1994). Yet, only a few studies have investigated the effect of dietary protein deficiency on Th2-type host immune responses to intestinal nematode parasites (Slater & Keymer, 1988; Boulay *et al.*, 1998) and therefore it is currently unclear whether protein deficiency potentiates intestinal parasitic infections by impairing the development and maintenance of specific T cell-dependent humoral immune responses. In addition, the severe levels of protein restriction (e.g. 0.5%) used in studies on protein malnutrition and immune function (e.g. Woodward *et al.*, 1992a & 1992b) limit the generalizability of the results to human populations in which mild to moderate malnutrition is much more prevalent than profound nutrient deficiencies (Kuvibidila *et al.*, 1993). Few researchers have evaluated comprehensively the relative impact of mild to moderate protein deficiency on protective immunity to nematode parasites in a dynamic host-parasite system.

Also, there is little information regarding the interactions of host immunity and protein malnutrition during secondary parasitic infections despite accumulated evidence that the magnitude and efficacy of immune responses are influenced by the frequency of antigen exposure. In experiments using repeated or challenge infection protocols that stimulate protective immunity in normally immunocompetent hosts, protein deficiency (2-3%) has been shown to prolong *H. polygyrus* survival and to impair the production of secondary total IgG1 and eosinophil responses (Slater & Keymer, 1986; Keymer & Tarlton, 1991; Boulay *et al.*, 1998). To date, the immunological parameters examined in these studies and in most previous research on host immunity to nematode parasites has been confined to the effector mechanisms in systemic compartments such as the peripheral circulation and spleen. As a result, we know less about the nutritional determinants of gut mucosal immunity despite reports that the gut mucosa has one of the highest protein turnover rates of all tissues in the body (Nakshabendi *et al.*, 1995) and is potentially very sensitive to changes in host nutritional status (Wykes *et al.*, 1996). Research on gut mucosal immunity is essential to the understanding of host malnutrition-parasite interactions because the gut and its associated lymphoid tissues comprise the first site of contact between host immune defenses and invasive intestinal nematodes. Recent studies have shown that the expression of Th2 immunity in intestinal lymphoid tissues may determine the survival of *H. polygyrus* during primary infection in mice (Behnke *et al.*, 1993; Finkleman *et al.*, 1997; Wahid *et al.*, 1994). The putative importance of gut mucosal immunity in the resolution of nematode infection and its potential for nutritional modulation are persuasive reasons for evaluating intestinal as well as systemic immune processes in order to fully ascertain the mechanisms underlying the relationships between protein deficiency and intestinal parasitism.

The overall objective of this research project was to determine the systemic and intestinal Th2 immune responses by which protein deficiency may influence the duration and severity of an intestinal nematode infection. To better understand the complex interrelationships of protein deficiency, host immunity and intestinal parasitism, this study integrated all three conditions using an experimental *H. polygyrus*-mouse system as a model for gastrointestinal helminthiasis and expression of Th2-type immune responses. Mice were

fed varying levels of dietary protein and subsequently subjected to a challenge infection protocol that has been shown to stimulate acquired resistance to secondary infections with *H. polygyrus* in well-nourished hosts (Behnke & Robinson, 1985). We hypothesized that protein deficiency would reduce the magnitude of intestinal and/or systemic cytokine and effector responses during challenge infection with *H. polygyrus*. Also, we expected that the level of diet-induced impairment of host protective immunity would be proportional to the severity of protein restriction such that a low protein diet (e.g. 3%) would exacerbate parasite survival by inhibiting acquired immunity to a greater extent than would a marginally deficient diet (e.g. 7%). We had anticipated that a certain degree of physiological adaptation to maintain homeostasis and functionality might occur with mild to moderate malnutrition but that there would be a threshold of nutritional deficiency beyond which immunocompetence would be notably impaired. Assuming that gut mucosal immunity is important for the rapid termination of a nematode infection and for priming systemic immunity, we postulated that protein deficiency would cause a more profound impairment of acquired immunity at the intestinal level than that at the systemic level. Finally, we speculated that protein deficiency would impact cytokine-mediated effector mechanisms to varying intensities at different phases of a nematode infection. To determine this possible effect of protein deficiency on the kinetics of Th2 immune responses, we conducted a time course study of various effector and cytokine outcomes at multiple time points after primary and challenge infection with *H. polygyrus*. Addressing these hypotheses would advance our understanding of the mechanisms of parasite survival in protein malnourished populations and thereby help define immunological or nutritional targets for therapeutic interventions.

CHAPTER II
LITERATURE REVIEW

1. HOST IMMUNITY TO NEMATODE PARASITES

In general, immune responses in mice to foreign antigens involve two categories of T helper (Th) cells: type 1 Th (Th1) cells are responsible for cell-mediated immunity (CMI) and type 2 Th (Th2) cells mediate antibody-dependent immunity. Each Th cell response phenotype is characterized by distinct patterns of cytokine and immune effector production that correlate well with their immunologic specificity (see Figure 1). Presentation of parasite-derived antigens by antigen-presenting cells (APC) to $CD4^+$ T cells induces the differentiation of Th cells to the Th2 functional phenotype. Th2 cells secrete interleukin (IL)-4, IL-5, IL-9 and IL-10 which promote the proliferation and activation of Th2-associated effectors such as IgE- or IgG1-secreting plasma cells, eosinophils, and mucosal mast cells (MMC). These effectors are features of IgE-mediated host defense mechanisms against many helminth parasites (Cox & Liew, 1992). Alternatively, Th1 cells produce IL-2 and IFN-γ and interact with APCs to synthesize IL-12. These Th1-associated cytokines are important in macrophage activity and isotype selection for IgG2a, IgG2b and IgG3, which mediate antibody-dependent cellular cytotoxicity (ADCC) and complement activity against bacterial or viral infections (Locksley, 1994). Both Th cell types secrete IL-3, TNF-α, and GM-CSF (Mosmann & Sad, 1996). The polarized patterns of cytokine and immune cell production correspond to activated effector Th phenotypes generated during immune response to a particular antigen but are not observed in naive $CD4^+$ T cells or even long-term, quiescent memory cells (Mosmann & Sad, 1996; Bunce & Bell, 1997).

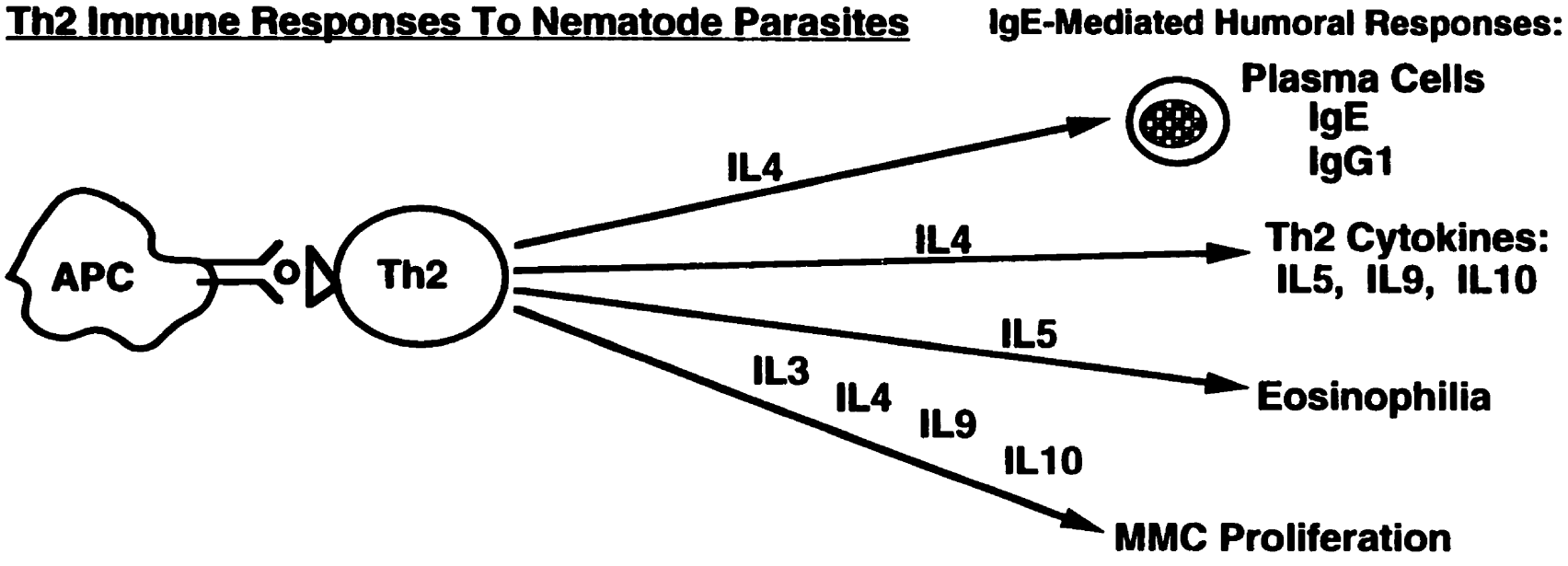

Figure 1: Th2 and Th1 Immune Response Phenotypes

1.1 Th1 Versus Th2 Response Phenotype as Mediated by Il-4 and IFN-γ

The characteristic cytokine products of Th1 and Th2 cells are mutually antagonistic for the differentiation and activity of effectors belonging to the reciprocal phenotype (see Figure 2). Hence, IL-4 and in some circumstances IL-10 inhibit cytokine synthesis by Th1 cells and macrophage function (Kopf et al., 1993; Lagoo et al., 1994; Mosmann & Sad, 1996) whereas IFN-γ suppresses the production of Th2-type effectors and cytokines (King et al., 1993; Urban et al., 1993). This cross-regulation of Th cell polarization and immune responses may explain the strong bias towards Th2 responses during nematode infections and why the alteration of Th2 response patterns by IFN-γ or other Th1 cytokines (e.g. IL-12) reverses host resistance to nematode infection (see Urban *et al.*, 1993; Finkleman *et al.*, 1994). In humans infected with helminth parasites, serum IgE and IgG1 levels are directly proportional to parasite-induced IL-4 production and inversely related to IFN-γ synthesis (King et al., 1993; King & Nutman, 1993) whereas IgE-mediated eosinophil and mast cell activities are associated with effective resolution of helminth infections (Allen & Maizels, 1996). Experimental evidence to support the view that development of a Th2 cytokine response stimulates strong resistance to nematode parasites has been derived from studies that measured the cytokine profile of cells isolated from tissues of genetically resistant animals (Wahid *et al.*, 1994), conducted adoptive transfer of protection with immune cells known to secrete Th2 cytokines *in vitro* (Korenaga *et al.*, 1989; Ramaswamy *et al.*, 1994a), analyzed knockout animals (Kopf *et al.*, 1993), used neutralizing antibodies to Th2 cytokines (Coffman *et al.*, 1989; Urban *et al.*, 1991b), and analyzed the effects of different cytokines on the *in vitro* development of Th1 and Th2 cell lines (e.g. Maggi *et al.*, 1992). Collectively, observations from these studies suggest that functional immunity to helminths involves Th2-type cytokines and effectors and that the reciprocal amounts of parasite-elicited IL-4 and IFN-γ produced determine the magnitude of Th2 immunity raised against the nematode parasites.

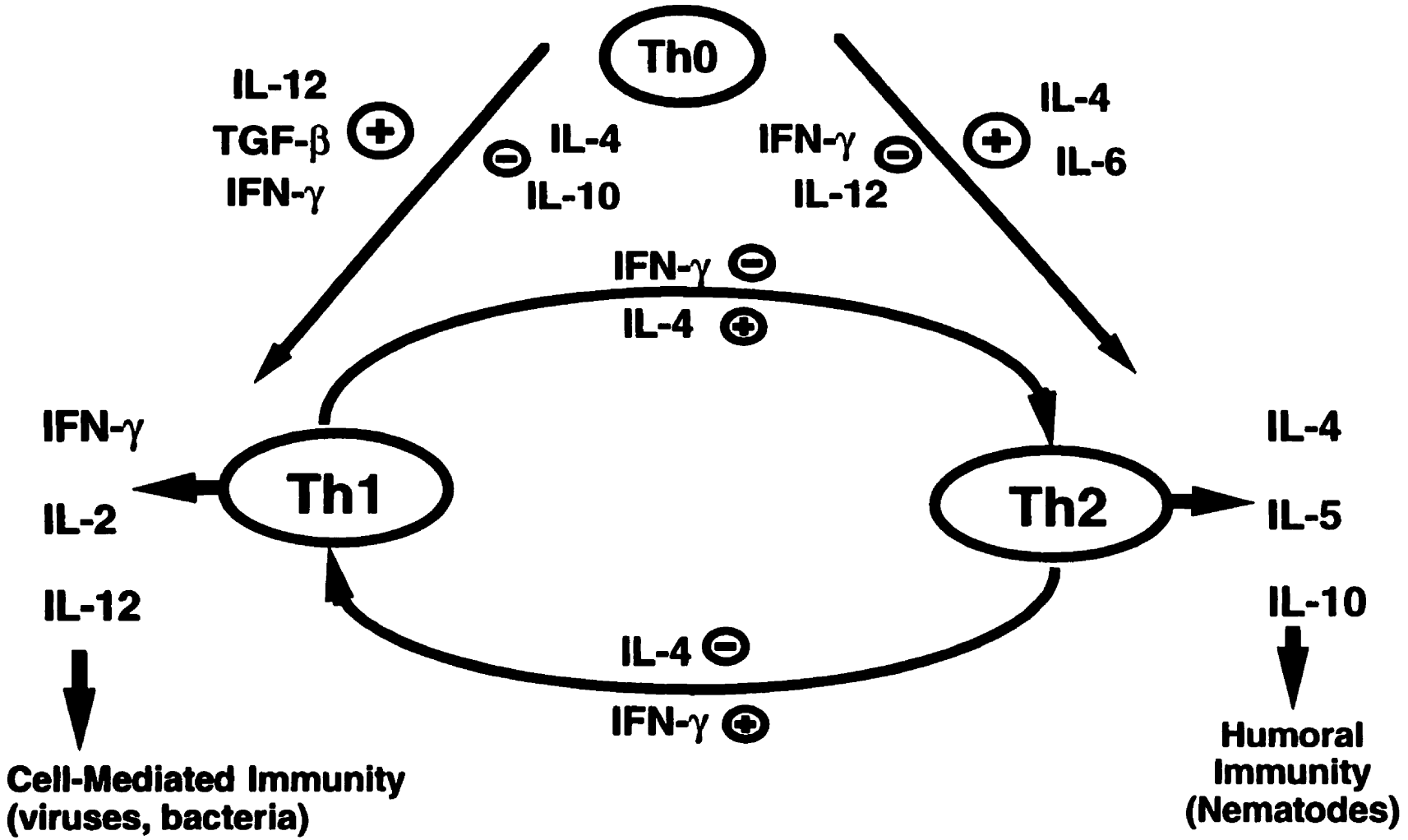

Figure 2: Differentiation of CD4$^+$ T helper cells into Th1 or Th2 type: cross-regulation by IL-4 and IFN-γ. Adapated from Mosmann & Sad, 1996

1.2 Th1 Versus Th2 Cell Differentiation

The source and principal early events affecting Th1/Th2 cell differentiation ultimately influence the type, speed and magnitude of the host immune response to nematode parasites. Naive $CD4^+$ T cells and even some subsets of memory Th cells are uncommitted and have the potential to secrete cytokines belonging to both phenotypes. When stimulated by antigen presented on APCs, these Th0 cells secrete primarily IL-2 as well as small amounts of other cytokines, including IL-12, IFN-γ and IL-4. There is agreement that IL-12 and IFN-γ direct differentiation to a Th1 phenotype (Seder *et al.*, 1991; Bradley *et al.*, 1996) and IL-4 drives differentiation to a Th2 phenotype (Le Gros *et al.*, 1990; Swain *et al.*, 1990). Several studies suggest that IL-12 is derived from macrophages and IFN-γ is secreted by NK cells in response to IL-12 or by naive $CD4^+$ T cells independently of APC or IL-12 (Swain, 1988). The initial source of IL-4 and factors required for the generation of IL-4-producing precursors are less well-defined. Classical APCs (e.g. macrophages, dendritic cells) are not known to produce IL-4 while the proportion of IL-4-producing T cells among naive T cell populations is very small (Swain *et al.*, 1988; Le Gros *et al.*, 1990). In fact, naive $CD4^+$ T cells freshly isolated from murine spleen do not secrete detectable quantities of IL-4 until after the cells are primed and restimulated with ConA or antibodies to the TCR (Swain *et al.*, 1988) or if exogenous IL-4 is added to the priming culture (Le Gros *et al.*, 1990; Seder *et al.*, 1992). Mast cells and eosinophils can produce IL-4 (Seder *et al.*, 1991; Moqbel *et al.*, 1995; Nakajima *et al.*, 1996) and provide cell contact signals required for IgE production by B cells (Gauchat *et al.*, 1993), but these cells are not abundant in lymphoid tissues where T cell priming and differentiation occur upon first exposure to the antigen. IL-4 is also produced by a minor subpopulation of T cells, $CD3^+CD4^+NK1.1^+$ T cells (Yoshimoto & Paul, 1994) but how these cells become activated to secrete IL-4 is not known.

Similarly, the driving force for initial Th2 priming during nematode infection has not been clearly elucidated. IL-4 mRNA expression during primary infection in mice with *Heligmosomoides polygyrus* is restricted to $CD4^+$, $TCR\alpha/\beta^+$ cells, occurs relatively late compared to other Th2 cytokines, and is not preceded by elevations in IL-2 mRNA (Svetic *et al.*, 1993) as observed in some Th cell transitions from Th0 to Th2 expression. Also, NK T cells with the markers, CD6L-low, CD44-high, CD45RB-low, from mice genetically deficient in IL-4 receptor retain their capacity to produce IL-4 during *Nippostrongylus brasiliensis* infection (Noben-Trauth *et al.*, 1997), suggesting that the induction of Th2-type responses to nematode parasites does not require early IL-4 or signaling through the IL-4 receptor. Considering that production of IL-4 by mast cells, eosinophils or $CD4^+$ T cells in response to nematode parasites appears to be a late event without an initial transient Th0 phase dominated by IL-2 synthesis, other cytokines or cell types may be involved in initial Th2 differentiation. A recent study has shown that IL-6 produced by APCs can trigger the Th2 pathway by inducing naive $CD4^+$ T cells to produce IL-4 which in turn would act as an autocrine factor to potentiate the upregulation of IL-4 gene expression while inhibiting IFN-γ production (Rincon *et al.*, 1997). The $CD4^+$ T cells are then polarized to a Th2 phenotype using their own source of IL-4 or IL-4 secreted by simultaneously activated basophils and eosinophils. It is possible that these IL-6-dependent events leading to IL-4-producing Th2 cells occur during responses to nematode parasites because Svetic *et al.* (1993) have reported that non-T cells ($CD4^-/CD8^-$) are likely the early source for elevated expression of IL-3, IL-5 and IL-9 mRNA and $CD4^+$ T cells become the later source of these cytokines including IL-4. Although IL-6 was not analyzed by Svetic *et al.* (1993) or other related studies on helminth immunology, it may be a potential initial factor influencing the choice between a Th1 or Th2 response to nematode parasites.

The presence of an appropriate pattern of cytokine production is essential for the development of beneficial immune responses to intestinal helminths. Even though the initial sources of IL-4 remain uncertain, it is well recognized that IL-4 is a crucial regulator of the commitment of $CD4^+$ T cells to the production of IL-4. The inhibition of early IL-4 production using antibodies to IL-4 and the IL-4 receptor is sufficient to promote the

differentiation of Th1 cells by increasing IL-2 and IFN-γ production by naive $CD4^+$ T cells (Seder *et al.*, 1992; Tanaka *et al.*, 1993). Consistent with these data, mice deficient in Stat6 gene (the signal transducer and transcriptional activator of IL-4) and infected with *N. brasiliensis* secreted lower amounts of Th2 cytokines (Takeda *et al.*, 1996) and showed decreased production of IgE but increased levels of IgG2a, IgG2b and IgG3 which are dependent on IFN-γ (Shimoda *et al.*, 1996). Moreover, blocking of IL-4 induced the development of memory effector Th1 cells (Bradley *et al.*, 1995). Unlike IL-4-producing T cells which require priming and restimulation, naive Th cells secrete IL-2 and IFN-γ upon first antigenic stimulation even in absence of IL-12 (Bradley *et al.*, 1996). Taken together, these findings imply that the pattern of Th1 cytokines appears to be the default phenotype in naive mice as well as in nematode-infected mice when IL-4 synthesis or activity is restricted. Also, it is clear that IL-4 is an absolute requirement for Th2 cell differentiation and resultant Th2 immune responses because it selectively promotes the expansion of Th2-committed cells via positive feedback and/or suppresses IFN-γ synthesis. Multiple sources of IL-4 including $CD4^+$ T cells, APC, mast cells, eosinophils and their costimulatory molecules (e.g. signaling between B7.1 or B7.2 on B cells and CD28 or CTLA-4 on T cells; expression of CD40 ligand on activated Th cells) are involved in inducing the differentiation of Th2 cells and subsequent production of IL-4 (Lu *et al.*, 1994; Lu *et al.*, 1996; Greenwald *et al.*, 1997). A selective impairment of these cells and/or of their contact signals may down-regulate Th2 response and/or concomitantly up-regulate Th1 responses, resulting in an inappropriate or inadequate host immune response to nematode parasites. Because immune effectors are controlled by T cell-derived cytokines, the following paragraphs will review briefly the immunologic roles of the major Th2 and Th1 cytokines. It is apparent that each cytokine has pleiotropic effects on multiple types of lymphoid cells and that there is much duplication of function amongst different cytokines.

1.3 Role of Th2 Cytokines

IL-4 is involved in the activation and maintenance of most Th2 effector responses. IL-4 produced by Th2 cells early after antigenic stimulation drives all subsequent immune responses toward the Th2 phenotype by acting as an autocrine growth factor for differentiated Th2 cells (Mosmann & Sad, 1996), enhancing the production of other Th2 cytokines (Kopf *et al.*, 1993), and inhibiting IFN-γ production by Th1 cells (Tanaka *et al.*, 1993). Mice deficient in IL-4 receptor displayed markedly diminished IgE, IgG1, IL-5, and IL-10 responses to *N. brasiliensis* infection (Noben-Trauth *et al.*, 1997), indicating that induction of Th2 responses to helminths is dependent upon signaling through the IL-4 receptor. IL-4 promotes isotype switching of membrane IgA-positive and IgM-positive B cells to IgE- and IgG1-expressing cells (Finkelman *et al.*, 1988b; Zhang *et al.*, 1991). As IgM^+ B cells are frequently found in peripheral blood, the IgM to IgE switch may account for much of circulating IgE after nematode infection (Zhang *et al.*, 1991). Also, IL-4-mediated switch of IgA to IgE-expressing B cells may be important for protective mucosal immunity given the abundance of IgA^+ B cells in gut lymphoid tissues such as Peyer's patches (Cebra & Shroff, 1994) and the association of IgE with anthelminthic resistance. Katona *et al.* (1991) demonstrated that antibodies to the IL-4 protein and receptor completely inhibited secondary IgE responses to *H. polygyrus* but did not affect the ability of IgE-expressing B cells to differentiate into IgE-secreting cells. Taken together, these data suggest two important aspects of IL-4-induced immunoglobulin responses: 1) IL-4 is required by virgin B cells to express the IgE and IgG1 isotype but may not be necessary for the terminal differentiation of these cells into IgE- and IgG1-secreting plasma cells; and 2) IgE and IgG1 responses generated in mice challenged with *H. polygyrus* are not derived from memory B cells that were primed to express IgE and IgG1 during the initial exposure but from IgA^+ or IgM^+ B cells that were induced by IL-4 to switch to IgE and IgG1 expression during secondary exposure. IgE and IgG1 in turn, when crossed-linked by parasite-derived antigen, trigger the degranulation of mast cells and eosinophils (Gounni *et al.*, 1994; Chen & Enerback, 1994 & 1996). Also, IL-4 has been shown to enhance IL-5-

mediated chemotaxis of eosinophils and to induce MMC hyperplasia along with IL-3 and stem cell factor (Madden *et al.*, 1991; Rennick *et al.*, 1995). IL-4 up-regulates the expression of endothelial adhesion molecules (e.g.VCAM-1) on intestinal endothelial cells and therefore may be important in the regulation of effector cell trafficking and homing of lymphocytes, eosinophils and basophils to intestinal mucosal sites (Chin *et al.*, 1991; Haradlsen *et al.*, 1996; Schleimer *et al.*, 1992). Similarly, IL-4 treatment *in vivo* has been shown to enhance the uptake of injected IgE into the gut mucosa and lumen following infection with *Trichinella spiralis*, possibly by upregulating the affinity of IgE receptors on MMC and eosinophils present in the infected intestinal tissues (Ramaswamy *et al.*, 1994). In addition to promoting the expansion and recruitment of Th2 effectors, IL-4 may be crucial for effector cell activation and function. IL-4 stimulates and promotes IgE binding to mast cells and IgE-induced degranulation of mast cells when sensitized by antigen, possibly via control of mast cell FcεRI expression (Banks & Coleman, 1996; Toru *et al.*, 1996). Lastly, preliminary experiments by Urban *et al.* (1996) suggest that IL-4 amplifies smooth muscle contraction in the small intestine through mast cell and leukotriene D4-dependent mechanisms which could in turn expedite worm expulsion from the intestine.

The main activities of IL-5 are to promote the growth and terminal differentiation of eosinophils, to attract eosinophils to sites of inflammation, and to enhance IgE- or IgG1-induced eosinophil degranulation (Coffman *et al.*, 1989; Kita *et al.*, 1992). These activities are complemented by IL-4 (e.g. via IgE or IgG switching and eosinophil recruitment) and by IL-10 (e.g. via IgG1 switching). IL-5 is secreted by Th2 cells, basophils and eosinophils after IgE- and IgG1-induced activation, thus providing an autocrine pathway for local eosinophil activation (Dubucquoi *et al.*, 1994). The production of IL-5 in T and NK cells is augmented by IL-4 and inhibited by IL-12 (Warren *et al.*, 1995).

Preliminary data suggest that IL-9 potentiates IL-4-induced synthesis of IgE and IgG1 by LPS-primed or antigen-stimulated B lymphocytes (Dugas *et al.*, 1993; Petit-Frere *et al.*, 1993), optimizes mucosal mastocytosis synergistically with IL-3 and IL-4 (Behnke *et al.*, 1993; Hultner *et al.*, 1990), and stimulates the transcription of genes coding for mast cell granules such as mouse mast cell protease-I (MMCP-1) which is secreted specifically

by intestinal MMC (Eklund *et al.*, 1993). The role of IL-9 in mediating protective immunity to nematode parasites has not been studied as extensively as that of other Th2 cytokines, however, a recent paper has demonstrated that IL-9 mRNA is expressed early during *Trichuris muris* infection and its elevation *in vivo* is associated with enhanced intestinal mastocytosis, production of IgE and IgG1, and loss of the parasite from the intestine (Faulkner *et al.*, 1998). Collectively, these observations indicate that IL-9 contributes to host resistance to helminths by promoting IgE-dependent MMC responses.

A recognized role of IL-10 in mediating Th2 responses is its inhibition of Th1 cell differentiation via suppression of IFN-γ production by Th1-committed cells. IL-10 does not act directly on Th1 cells but impairs the capacity of splenic and peritoneal macrophages to stimulate Th1 cytokine synthesis by Th1 cells (Fiorentino *et al.*, 1991). Ding *et al.* (1993) showed that this effect is partly due to the ability of IL-10 to suppress the activity of B7.1 (CD80) costimulatory molecule on macrophages while de Waal *et al.* (1991) found that IL-10 down-regulated the expression of class II MHC on APCs that interact with antigen-activated Th1 cells to induce cytokine synthesis. IL-10 contributes to mucosal mastocytosis by promoting the growth of mast cell progenitors and expansion of later colonies in the presence of IL-3 and IL-4 (Behnke *et al.*, 1993; Thompson-Snipes *et al.*, 1991). Similar to the effect of IL-9, IL-10 induces the transcription of genes for MMCP-1 and other serine proteases secreted by MMCs of nematode-infected mice (Ghildyal *et al.*, 1992 & 1993). IL-10, along with IL-4 and stem cell factor, induces the synthesis of histamine in MMC (Bissonette *et al.*, 1995). Also, IL-10 may promote isotype switching to IgG4 in humans (Lagoo *et al.*, 1994); although human IgG4 is homologous to murine IgG1, this particular function of IL-10 has not been shown with murine B lymphocytes.

IL-3 is a cytokine secreted by Th0, Th1 and Th2 cells (Mosmann & Sad, 1996). It is a multi-colony stimulating factor for several haematopoietic cells and their progenitors *in vitro*. IL-3 is an essential cofactor for the growth and maturation of MMC along with IL-4 and stem cell factor (Madden *et al.*, 1991; Rennick *et al.*, 1995) and its administration into nematode-infected mice induces strong mucosal mastocytosis and successful expulsion of the nematode *Strongyloides ratti* (Abe & Nawa, 1988; Abe *et al.*, 1993).

1.4 Role of Th1 Cytokines

IFN-γ is an important Th1 cytokine that serves primarily to activate natural killer cells and mononuclear phagocytes (e.g. macrophages and neutrophils) which are responsible for cytotoxic activity against tumor cells and microbes (Mosmann & Sad, 1996). Research suggests that IFN-γ inhibits or delays protective immunity to nematodes by suppressing the growth and expansion of Th2 cells via a down-regulation of IL-4 (Maggi *et al.*, 1992; Urban *et al.*, 1992), inhibiting production of IgG1 (Finkelman *et al.*, 1988a), suppressing the ability of IL-4 to elicit IgE switching and differentiation (King *et al.*, 1993; Uchikawa *et al.*, 1994), blocking eosinophilia (Urban *et al.*, 1993), decreasing the TNF-α-dependent cytotoxicity of MMC (Bissonette *et al.*, 1995), and delaying IgE response and MMC hyperplasia in *N. brasiliensis*-infected rats (Urban *et al.*, 1993). These inhibitory effects of IFN-γ on Th2 effector responses have been associated with prolonged survival of intestinal nematode parasites such as *N. brasiliensis* and *T. muris* in rodents (Urban *et al.*, 1993; Else *et al.*, 1994).

IL-12 is secreted by macrophages and B lymphocytes and potentiates Th1 phenotypic expression by enhancing priming for IFN-γ production by naive $CD4^+$ T cells (Seder *et al.*, 1993) and natural killer cells (Chan *et al.*, 1991). IL-12 also induces Th1 differentiation after antigenic stimulation by enhancing IFN-γ synthesis and inhibiting the development of IL-4-producing Th cells (Manetti *et al.*, 1993; Windhagen *et al.*, 1996), even in the absence of the costimulatory receptor CD28 on T cells (Chu *et al.*, 1997). The presence of IL-12 *in vitro* has been shown to reduce the suppressing effects of IL-4 on IFN-γ production by naive $CD4^+$ T cells (Seder *et al.*, 1993). These anti-Th2 effects of IL-12 have also been demonstrated *in vivo*: IL-12 inhibits IgE synthesis and corresponding IL-4 production by lymphocytes isolated from helminth-infected patients (King *et al.*, 1995) while administration of IL-12 to *N. brasiliensis*-infected mice prolonged worm survival, stimulated IFN-γ production, inhibited IL-4, IL-5 and IL-9 mRNA expression, and prevented increases in nematode-induced IgE, MMC and eosinophil responses (Finkelman *et al.*, 1997).

1.5 Overview of Gut-Associated Mucosal Immune Responses

Much of our knowledge about specific immune responses to nematode infection is based on analyses of antibody concentrations from blood and cytokine production by splenocytes. The underlying assumptions in these studies are that peripheral levels of effectors or cytokines reflect intestinal levels and that protective T cells exert their principal function by releasing Th2 cytokines in gut mucosa where the parasite resides and reproduces. The first assumption was deemed invalid by findings of a study showing that serum IgE concentration in *T. spiralis*-infected mice represented only 0.2 to 1.0% of total amount of IgE produced at the peak of intestinal antibody response and that the vast majority of IgE produced *in vivo* is directed to the small intestine and does not enter the bloodstream (Negarao-Correa *et al.*, 1996). Therefore, to understand the full capacity of host resistance to intestinal parasites, we need to examine immune responses occurring in gut mucosal tissues in addition to those in systemic compartments.

The small intestine contains specialized lymphoid tissues that are categorized into two anatomically and functionally distinct compartments: 1) the afferent limb consists of aggregated lymphoid cells (Peyer's patches), isolated lymphatic follicles and mesenteric lymph nodes (MLN), where APCs present antigen to naive T lymphocytes (Bogen *et al.*, 1991; Laissue *et al.*, 1993; Regoli *et al.*, 1994) and 2) the efferent limb contains the intraepithelium (IE) and lamina propria (LP) of the intestinal microvilli, where antigen-specific effectors such as IgE, IgG1, MMC and eosinophils mediate their anthelminthic reactions (Beagley & Elson, 1992). Recently, Saito *et al.* (1998) described a new intestinal lymphoid compartment (cryptopatches) located under the layer of epithelial cells lining the gut which consisted of immature and differentiated intraepithelial lymphocytes (IEL). Although progenitors of all immune cells originate from bone marrow, Saito *et al.* (1998) found that T cell precursors in cryptopatches were capable of maturing in the gut without passing through the thymus and thus provide evidence that the intestinal mucosa contains a population of thymus-independent mature T cells that may interact directly with enteric antigens. Collectively, these immunological compartments are known as gut-associated

lymphoid tissues (GALT) which help ensure mucosal integrity and comprise the first line of host protection against ingested antigens and intestinal pathogens. The function of the gut mucosa includes nonspecific defenses provided by the epithelial barrier and mucus as well as specific immune responses mediated by gut-associated cytokines and effectors.

The continuous, nonrandom migration of lymphocytes between mucosal lymphoid tissues and the bloodstream is an essential process in the development of immune responses at the appropriate sites of infection anywhere in the body. Naive lymphocytes first interact with antigens in the Peyer's patches and isolated lymphoid follicles and then further differentiate and mature in the germinal centers of the lymphoid follicles (Guy-Grand *et al.*, 1978). Thereafter, the antigen-primed cells leave these inductive sites of GALT via mucosal lymphatics, collect in the thoracic duct and enter the peripheral circulation (see Figure 3). From the blood, the lymphocytes migrate to systemic lymphoid organs such as the spleen and peripheral lymph nodes where the lymphocytes continue to mature and proliferate to become effector cells (e.g. secrete cytokines) and/or memory cells (Salmi & Jalkanen, 1991). Early studies on lymphocyte recirculation proposed a common mucosal immune system in which lymphocytes migrate freely among the gut, peripheral lymph nodes, genital tissues and the lung (McDermott & Bienestock, 1979). However, recent papers suggest that lymphocytes of lung and gut origin have distinct recirculation routes and express different homing adhesion receptors that bind to high endothelial venules (HEV) lining the mucosal lymphoid tissues (Joel & Chanana, 1985; Abitorabi *et al.*, 1996). For example, activated and memory gut-derived lymphocytes preferentially extravasate from the blood to intestinal effector sites by expressing integrin $\alpha 4\beta 7$ and L-selection, adhesion receptors which are much less expressed by lymphocytes recirculating through the lung (Schweighoffer *et al.*, 1993; Abitorabi *et al.*, 1996). These homing molecules are receptors for mucosal vascular addressin cell adhesion molecule (MAdCAM)-1 which is a vascular addressin found selectively on endothelia of the gut and associated lymphoid tissues (Nakache *et al.*, 1989; Berlin *et al.*, 1993; Roberts & Kilshaw, 1993). Thus, most of the differentiated lymphocytes that enter the effector sites of the GALT (LP, IE) are likely to have had prior contact and specific activation with antigens located in the gut mucosa.

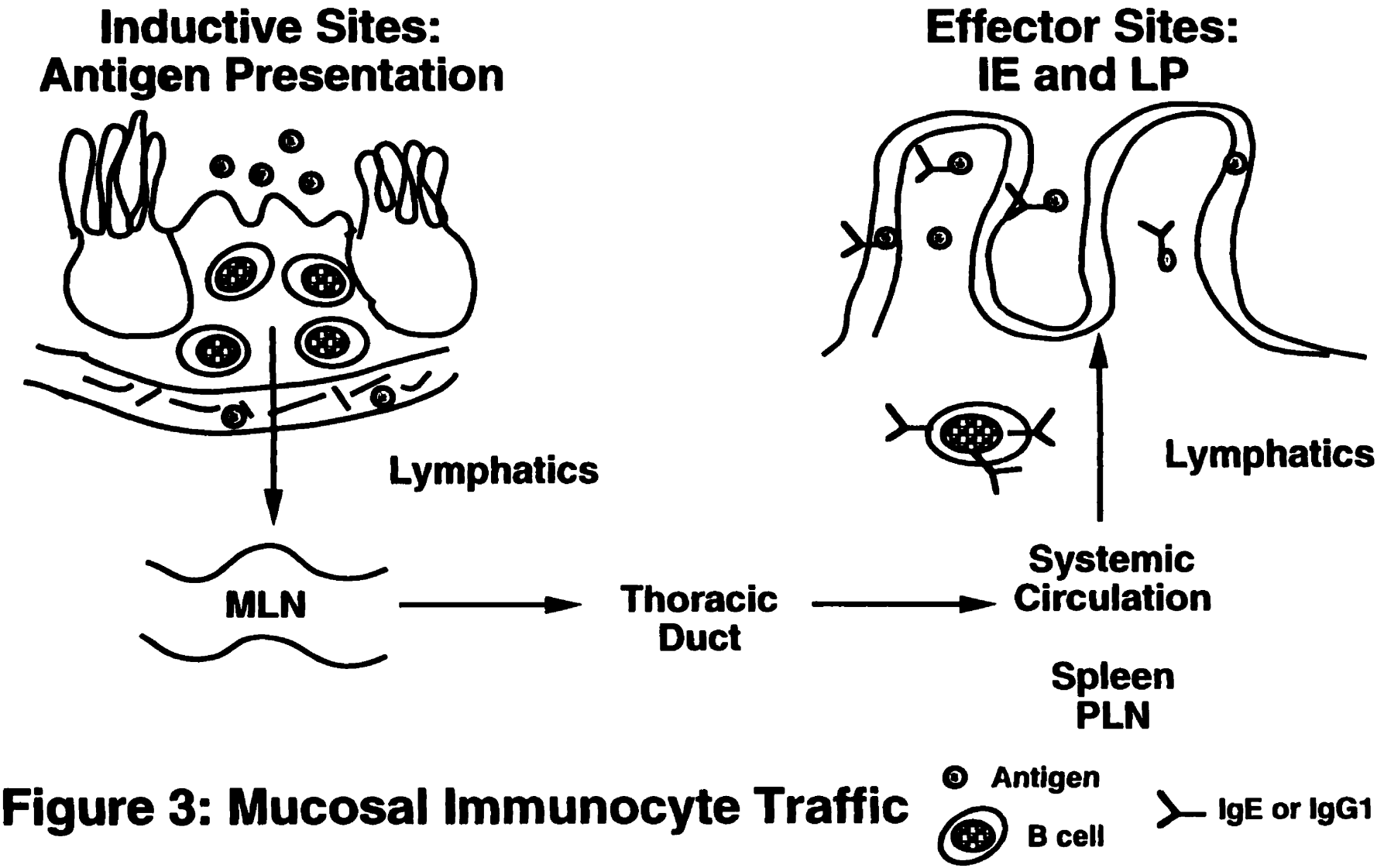

Figure 3: Mucosal Immunocyte Traffic

In addition to the intestinal migration of lymphocytes, MMC progenitors, basophils, and circulating eosinophils are attracted by chemotaxis induced by gut-associated cytokines to the IE and LP where they mature and degranulate after cross-linking of their FcεRI receptors by IgE and parasite antigens (Pearce *et al.*, 1982; Smith & Weis, 1996). MMC in the gut mucosa or MLN are histochemically and functionally different from mast cells of the peritoneal cavity and connective tissues (Shanahan *et al.*, 1986; Befus *et al.*, 1987) and are the predominant type of mast cells that proliferates in response to parasitic infections of the gastrointestinal tract (Arizono *et al.*, 1993 & 1994). Infection with *N. brasiliensis* induces migration of MMC precursors from peripheral blood to the intestine (Kasugai *et al.*, 1995), development of MMC cells located *in situ* (Miller & Jarrett, 1971), and release of serine proteases that may alter intestinal mucosal permeability (King & Miller, 1984; Wastling *et al.*, 1997). Mucosal mastocytosis is dependent on T cell-derived IL-3, IL-4 and IL-10 (Haig *et al.*, 1982; Madden *et al.*, 1991; Rennick *et al.*, 1995). In turn, MMC produce Th2 cytokines (e.g. IL-4) and provide contact signals for IgE synthesis which help augment local Th2 reactions (Plaut *et al.*, 1989; Seder *et al.*, 1991; Gauchat *et al.*, 1993).

Cytokines such as IL-4, IFN-γ and TNF-α may enhance the adhesiveness of endothelial ligands on HEV cells for lymphocytes (Thornhill *et al.*, 1990 & 1991; Chin *et al.*, 1991). Previous studies have documented the synthesis of Th2 cytokines by lymphocytes derived from Peyer's patches and MLN (Svetic *et al.*, 1993; Wahid *et al.*, 1994) as well as the GALT-specific production and/or uptake of Th2 effectors early after nematode infection (Ramaswamy *et al.*, 1994; Uchikawa *et al.*, 1994; Wahid *et al.*, 1994). Cytokine gene expression in GALT and transport of IgE into intestinal lumen occur within 24 hours after primary *T. spiralis* infection (Ramaswamy *et al.*, 1994; Svetic *et al.*, 1993) while cytokine secretion by MLN cells, mucosal mastocytosis, and IgE-mediated MMC degranulation occur by 5 to 10 days of *N. brasiliensis* and *H. polygyrus* infection (Arizono *et al.*, 1994; Wahid *et al.*, 1994; Chen & Enerback, 1996, 1996). Th2 cytokine levels in the intestinal lymph of *T. spiralis*-infected mice were found to accurately reflect the responses in the gut mucosa, leading to the conclusion that the most important source of local cytokines during early infection is the mucosal cells residing in the gut and not the draining

lymph nodes (Ramaswamy *et al.*, 1996). Similarly, others have observed that the majority of cytokines produced in MLN, which receive both activated $CD4^+$ T cells that were primed in Peyer's patches and naive lymphocytes circulating from systemic compartments, are produced by cells derived from intestinal lymphoid tissues (Flo & Massouh, 1997). Moreover, lymphocytes in the MLN were found to be the most potent source of growth-stimulating activity for MMC hyperplasia during *N. brasiliensis* infection compared to those in spleen or peripheral lymph nodes (Haig *et al.*, 1984). These results imply that cytokine production is initiated locally at intestinal sites of infection and that these gut-associated cytokines induce chemotactic recruitment of lymphocyte and effector progenitors to IE or LP where they mature and differentiate into antigen-specific cell populations. Furthermore, the migrational pattern of mucosal lymphocytes from the gut to systemic sites suggest that immunological mediators produced in GALT are important in priming and perpetuating host responses in systemic compartments.

A further indication that immune responses in the mucosa are unique is the large percentage of T cells with the γδ receptor. This γδ population, which constitutes only 5% of circulating T cells (the majority carry the αβ receptor), makes up approximately 50% of the T lymphocytes in the gut mucosa of mice (Kaufmann, 1996). The role of γδ T cells in Th2- type responses to nematode parasites has not been clearly elucidated. Most γδ cells are not $CD4^+$ T cells which have been shown to be important for protective immunity to nematode parasites (Urban *et al.*, 1991a; Koyama *et al.*, 1994) and γδ cells do not recognize MHC-associated peptides. (Raulet, 1989). However, there is evidence that γδ T cells mediate Th2 immune responses by providing a contact signal for B cell isotype switching to IgE production in the presence of IL-4 (Gascan *et al.*, 1992; Wen *et al.*, 1996) and by preferentially secreting Th2 cytokines spontaneously (Hiroi *et al.*, 1995) or during infection with *N. brasiliensis* in mice (Ferrick *et al.*, 1995). $CD4^+$ γδ T cells are rare but may proliferate during infection in the gut and help drive the development of Th2 clones *in vitro* (Wen *et al.*, 1998). The selective expression of Th2 cytokines by γδ T cells and their substantial accumulation in the gut mucosa of mice suggest that γδ T cells play an important role in the induction of Th2 response to intestinal nematode parasites.

2. THE *HELIGMOSOMOIDES POLYGYRUS*-MOUSE MODEL

Heligmosomoides polygyrus (= *Nematospiroides dubius*) is a trichostrongyloid parasite of murine rodents and has been proposed as a experimental model for human hookworm infection (Bartlett & Ball, 1972) and other nematode infections of veterinary importance (Monroy & Enriquez, 1992). The chronicity of primary infection, the capacity for acquired immunity to secondary infections, and the similarity of epidemiological patterns between laboratory-maintained and free-living populations (Scott & Tanguay, 1994) make the *H. polygyrus*-mouse system a suitable model for studying chronic gastrointestinal helminthiasis in humans.

The life cycle of *H. polygyrus* involves both free-living and parasitic stages and occurs predominately in the small intestine of its murine host. As described by Bryant (1973), parasite eggs are passed in the feces of the infected host, hatch within approximately 36 hours to become the first larval stage (L_1), which then undergoes two moults to form the ensheathed non-feeding infective third larval stage (L_3). Ingested L_3 exsheath in the lumen of the stomach and migrate to the small intestine where they penetrate the serosal musculature and mature to form the fourth larval stage (L_4). Within 7-8 days post-infection (pi), the pre-adults migrate out of intestinal mucosa and emerge into the intestinal lumen. Adult worms migrate preferentially to the anterior duodenum where they mature, mate and produce eggs. Significant levels of egg output are observed within 12-14 days pi (Bryant, 1973) and an average of 1200-1500 eggs per female worm per day are released in the feces of mice infected once with 100 infective L_3 (Kerboeuf, 1982).

2.1 Host Immunity to Primary Infection with *H. polygyrus*

Primary infections are chronically maintained for 4 to 10 months depending on the mouse strain (Lawrence & Pritchard, 1994; Wahid *et al.*, 1989). Apart from nutritional factors, parasite establishment, survival and fecundity during primary infection are influenced by multiple external variables such as host population density, level of exposure, genetic background, and host immune response phenotype (Behnke & Wahid, 1991;

Kerboeuf, 1985; Keymer & Tarlton, 1991; Scott, 1990). Even in mouse strains which are able to reject primary infection with *H. polygyrus*, the process of worm expulsion is protracted over several weeks with considerable variation among individual animals (Behnke & Robinson, 1985). Primary *H. polygyrus* infections in most murine hosts are long-lived despite the ability for gene expression and production of Th2 cytokines (Svetic *et al.*, 1993) and the presence of parasitic-specific effector responses such as IgG1 (Wahid & Behnke, 1993). This chronic survival of *H. polygyrus* during primary infection is believed to be related to the immunosuppressing excretory-secretory proteases of adult worms (Monroy *et al.*, 1989). These substances released by the adult worm have been shown to suppress the activity of host generated immune cells, to restrict intestinal peristalsis and mucus secretion, and to neutralize cytotoxic substances such as free oxygen radicals produced by mucosal mast cells (MMC) or eosinophils (Dehlawi *et al.*, 1987; Dehlawi & Wakelin, 1988; Monroy & Enriquez, 1992; Pritchard *et al.*, 1994; Reed *et al.*, 1988; Smith & Bryant, 1986). Although these proteolytic and antioxidant proteases may modulate host immune responses, the overall contribution of these immunosuppressing activities of the parasite to the chronicity of primary infection has not been definitively resolved.

Primary infection with *H. polygyrus* induces marked enlargement and increased cellularity of the spleen and MLN (Parker & Inchley, 1990a). Preliminary work by Ali and Behnke (1985) suggested that the intensity and rapidity of the proliferative response in secondary lymphoid organs are related to the inherent capacity of the mouse strain to expel the worms. In support of these findings, Parker and Inchley (1990b) reported that an increase in the proportion of B lymphocytes in MLN (taken as a crude measure of active humoral immunity) was greatest and most prolonged in moderate to high responder strains (BALB/c and NIH mice) compared to slow responders (CBA mice). T cell proliferation was delayed in comparison with changes in B cell frequency, while total T cell numbers and the ratio of T helper to T suppressor cells were not different among the three strains. Maximal hyperplasia of lymphoid organs and peak B lymphocyte cellularity occurred within 7-12 days post-infection and coincided with the appearance of L_4 in the gut mucosa (Parker & Inchley, 1990a & 1990b). These results support other observations that L_4 stimulate a

strong antibody-dependent inflammatory and granulomatous response in intestinal tissues of responder strains (Penttila & Jenkin, 1983; Monroy & Enriquez,1992).

Although primary *H. polygyrus* infection in mice induces infiltration of inflammatory mediators (e.g. neutrophils and eosinophils) and parallel increases in serum IgE and IgG1, mucosal mastocytosis is limited early after infection and noticeably absent once adults have established in the anterior duodenum (Dehlawi & Wakelin, 1988; Reed *et al.*, 1988). The suppression of mucosal mastocytosis by adult *H. polygyrus* is not due to intrinsic defects of mast cells because mastocytosis can be stimulated by concomitant *T. spiralis* infection (Dehlawi & Wakelin, 1988) but is associated with the selective down-regulation of IL-9 and IL-10 production in MLN (Behnke *et al.*, 1993). Although IL-3 and IL-4 are necessary for helminth-induced mucosal mastocytosis (Madden *et al.*, 1991), both IL-9 and IL-10 have been shown to be important for optimal MMC proliferation and production of MMC-derived granule proteases (Ghildyal *et al.*, 1992 & 1993; Hultner *et al.*, 1990; Rennick *et al.*, 1995). In contrast, other researchers have found high numbers of intestinal MMC in mice infected with *H. polygyrus* compared with that of naive mice (Urban *et al.*, 1995) but this nematode-induced mucosal mastocytosis appears only after, and not before, pronounced worm expulsion (Wahid *et al.*, 1995). It has been suggested that impaired IgE- or IgG1-induced activation of mast cells, and thus the reduced degranulation and release of granule proteins, may be a more important reason for the prolonged worm survival during primary infection than the insufficient enlargement of MMC in GALT (Wahid *et al.*, 1994; Woodbury *et al.*, 1984). Thus, the chronicity of primary infection may result from not only immunosuppressive secretions from the parasite but also, and perhaps more importantly, from parasite-induced dysregulation of Th2-type cytokine and effector responses.

It is controversial as to whether IgG1, IgE, and eosinophil responses are beneficial to the host infected once with *H. polygyrus*. Pritchard *et al.* (1983) reported that purified IgG1 from immune serum of mice infected with *H. polygyrus* caused severe stunting of worms and promoted adherence of peritoneal exudate cells to the surface of L_4 and adult worms *in vitro*. However, a later *in vivo* study by Wahid *et al.* (1993) showed that parasite-specific IgG1 responses did not correlate with the rate of worm expulsion and

were marginally elevated in mice with heavier worm burdens. Moreover, the inhibition of IL-4 and its receptor by neutralizing antibodies increased worm burdens in primary *H. polygyrus* infection without concurrent alterations in IgG1 responses (Finkelman *et al.*, 1988b; Katona *et al.*, 1991). Later studies showed that adult worms induced mass production of nonspecific IgG1 (Robinson & Gustad, 1995) that could be bound with proteins on the surface of *H. polygyrus* (Robinson *et al.*, 1997). This was interpreted by these authors as evidence of an attempt by the parasite to mask specific antigens from recognition and interaction with host immune cells. Other researchers have also questioned the functional role of Th2 effectors during primary *H. polygyrus* infection. The neutralization of secreted IgE by an anti-IgE antibody failed to promote worm survival although injection of anti-IL-4 antibody, which suppressed serum IgE concentrations, resulted in increased worm burdens and fecundity (Urban *et al.*, 1991b). IL-4 treatment eliminated an established primary *H. polygyrus* infection in immunocompetent mice but was not associated with changes in eosinophil or serum IgE levels (Urban *et al.*, 1995). Furthermore, injection of an anti-IL-5 antibody suppressed the development of eosinophilia but had no effect on worm numbers or egg production (Urban *et al.*, 1991b). In contrast to these results, Wahid *et al.* (1994) found that the ability of mice to expel adult *H. polygyrus* during primary infection was directly related to their potential to secrete Th2 cytokines and to produce prominent and sustained IgG1, IgE, and MMC responses. Thus, an increase in parasite-induced production of Th2-type effectors reflects a capacity for specific immunological responsiveness but the precise functions of these Th2 effectors in mediating host immunity to *H. polygyrus* infection remain poorly defined. The inhibition of a single parameter of Th2 defense mechanism without a subsequent detectable effect on worm expulsion or fecundity does not necessarily prove that these effectors are not functionally active or important in the host immune response to nematode parasites, but suggests that there may be some redundancy of host defenses which has evolved to prevent the selection of parasites that are resistant to any one of these defenses (as reviewed by Finkelman *et al.*, 1997). To this end, the host may have developed a complex set of defense mechanisms that collectively, through their interactions and compensatory responses, act to effectively resist

infections with nematode parasites. However, for reasons that remain poorly understood, this putative network of cooperative host effector responses fails to provide adequate protection against primary infections with *H. polygyrus*. In summary, low immunological responsiveness and chronic primary infection with *H. polygyrus* appear to be related to genetically determined host susceptibility to parasite-induced immunomodulation, although the critical protective mechanisms that are suppressed are not clearly defined.

Despite the equivocal evidence for the benefit of Th2-type effector mechanisms, the production of Th2-type cytokines is thought to be crucial for the resolution of *H. polygyrus* infection. Injection of anti-IL-4 or anti-IL-4 receptor antibodies into mice infected with *H. polygyrus* increased worm survival and fecundity in mice (Urban *et al.*, 1991b). The importance of IL-4 to protection against primary infection was confirmed by a later study which found that treatment of *H. polygyrus*-inoculated mice with exogenous purified IL-4 significantly limited parasite survival and completely suppressed fecundity (Urban *et al.*, 1995). Interestingly, the effector mechanisms by which IL-4 terminated the primary infection were unclear because levels of MMC, IgE, and eosinophils did not differ between IL-4-treated and untreated mice. The independent ability of increased IL-4 concentrations *in vivo* to selectively enhance these Th2 effector responses in the absence of altered concentrations of other Th2 cytokines is not known. The observation that IL-4 treatment inhibited adult worm survival to a greater extent than larval establishment led Urban *et al.* (1995) to speculate that IL-4 may activate nonspecific immune defenses such as intestinal smooth muscle contraction, mucus secretion, or peristalsis which in turn may help facilitate worm expulsion from the small intestine. Recently, preliminary data suggest that IL-4 may increase gut spasticity, small intestinal permeability, and decrease fluid absorption in the small intestine (Urban *et al.*, 1996). These changes in smooth muscle contractility and intestinal fluid dynamics may facilitate expulsion of *H. polygyrus* during primary infection by decreasing parasite attachment to the intestinal villi (thus limiting parasite establishment) and/or by restricting access of worms to their food source within mucosal tissues. Alternatively, IL-4 treatment may have initiated immune-mediated responses that damaged the larvae or young adult worms and thereby reduced their potential for chronic survival.

2.2 Systemic Versus Gut Mucosal Immunity to Primary Infection with *H. polygyrus*

Aside from the analysis of mucosal mastocytosis, much of our knowledge on immune responses to *H. polygyrus* has been based on serum levels of Th2-associated antibodies (e.g. IgE and IgG1) or cytokine production (IL-4, IL-5, IL-9 or IL-10) in the spleen. Studies examining the specific advantages of Th2 effector mechanisms such as IgE-mediated immune reactions have produced inconsistent results perhaps because they have not examined these Th2 immune responses at the usual site of infection in GALT. It appears logical that induction of Th2 cytokine and effector cell production also occurs in intestinal mucosal tissues where the nematode parasite first interacts with the host and spends the majority of its life cycle. However, only recently has there been concentrated research on the role and relative importance of gut mucosal immunity in the resolution of intestinal nematode infections.

Parker & Inchley (1990a) reported that the peak cellular proliferative response to *H. polygyrus* infection occurred earlier in MLN than in spleen (Parker & Inchley 1990a), thus suggesting that the first lymphocytic response to an intestinal nematode is restricted to infected enteric regions. Unfortunately, the authors did not determine whether this earlier cellular proliferative response in MLN resulted in a more rapid onset of functional immunity at the gut level. To assess time-dependent compartmentalization of Th2 immune responses to *H. polygyrus*, Svetic *et al.* (1993) studied Th2 cytokine gene expression in spleen and GALT (MLN and Peyer's patch) within 12 days after primary infection. The authors found that IL-3, IL-4, IL-5, and IL-9 mRNA expression as measured by a RT-PCR assay was markedly elevated in the Peyer's patch and MLN cells within 2 days pi and remained above uninfected levels for up to 12 days after infection. In contrast, the gene expression for these Th2 cytokines remained unchanged in splenic cells throughout the experimental period. These results suggested that the early functional immune response to *H. polygyrus* as measured by mRNA activity was localized at intestinal sites of infection. Currently, no study has confirmed whether the higher Th2 cytokine mRNA expression in the gut during *H. polygyrus* infection is accompanied by equivalent increases in protein secretion although

gene expression usually correlates well with protein synthesis. Table 1 shows a compilation of data on the kinetics and site-specificity of Th2 cytokine and effector responses following primary infection with *H. polygyrus*. The principal conclusion to note from this table and the above studies is that Th2 cytokine gene expression and secretion in GALT usually precede the production of Th2 cytokines in the spleen as well as the onset and peak of Th2 effector responses in the peripheral circulation.

Because worm outcomes were not assessed by Parker & Inchley (1990a) or by Svetic *et al.* (1993), it is unclear whether this earlier onset of cellular and cytokine proliferative responses in GALT is critical for protective immunity to the nematode parasite. In one study that examined the correlation between Th2 immune responses in GALT and worm burden of a primary response, Wahid *et al.* (1994) determined whether Th2 cytokine secretion in MLN as well as MMC responses differed between fast and slow responder mouse strains. The fast responder strain (SAR mice) expelled over 96% of the worms by 8 weeks pi compared to the slow responder strain (CBA mice) which experienced no significant worm loss throughout the experimental period. The MLN of SAR mice secreted consistently higher amounts of IL-3, IL-4, and IL-9 over the course of infection compared to MLN of CBA mice. Moreover, significantly higher MMC counts and serum concentrations of MMCP-I (used as a systemic marker of mucosal activation and/or mastocytosis) were found in SAR mice versus the CBA mice. Thus, survival of the parasite and ultimately the chronicity of infection may depend on the intensity and persistence of Th2 cytokines and effector responses at the intestinal level. The relative importance of gut mucosal versus systemic immunity to infection outcomes such as worm survival has not been studied in our experimental *H. polygyrus*-mouse model.

Table 1: Kinetics of Th2 Cytokine and Effector Production Post-primary (PPI) and Post-challenge (PCI) Infection with *H. polygyrus* in BALB/c mice (unless otherwise indicated).

Time Post-infection PPI	PCI	Cytokine/Effector Response	Tissue Location
6-12 hrs		Onset of IL-3, IL-5, IL-9 gene expression	PP[1]
		Marginal increase in IL-5 gene expression	MLN[1]
1-2 days		Sustained IL-3, IL-9 gene expression	PP & MLN[1]
2-4 days		Onset of IL-4 gene expression	PP & MLN[1]
		Onset of IL-3 & IL-4 secretion (SAR mice)	MLN[2]
	3-5 days	Onset of eosinophilia (NIH mice)	Whole Blood[3]
4-6 days		Peak IL-5 gene expression	PP[1]
		Peak IL-3 gene expression	PP & MLN[1]
	4-6 days	Onset of total IgE response	Serum[4, 7]
		Onset of total IgG response (NIH mice)	Serum[3]
	5-7 days	Peak eosinophilia	Whole Blood[3, 6]
		Onset of parasite-specific IgG1 (NIH mice)	Serum[9]
6-8 days		Sustained IL-5 gene expression	MLN[1]
		Peak IL-9 secretion (SAR mice)	MLN[2]
		Marginal increase in IL-10 secretion	MLN[2]
		Onset of MMCP-1 increase	Serum[2]
		Onset of total IgG1 response (NIH mice)	Serum[3]
	7 days	Peak total IgG1 response	Serum[6]
	8-9 days	Peak total IgE response	Serum[4, 7]
		Decline of eosinophilia (NIH mice)	Whole Blood[3]
7-14 days		Onset of total IgE and total IgG1 response	Serum[4, 6]

continued

PP = Peyer's patches
MLN = Mesenteric lymph nodes

Table 1: continued

Time Post-infection PP	PC	Cytokine/Effector Response	Tissue Location
12 days		Peak MMC numbers (SAR, CBA mice)	Small Intestine[2]
		Peak IL-5 gene expression	MLN[1]
10-15 days		Peak parasite-specific IgG1 response	Serum[5]
14-15 days		Peak eosinophilia	Whole Blood[6]
		Peak total & specific IgG1 response	Serum[6]
	14-21 days	Peak total & specific IgG1 response	Serum[6, 8]
		Peak serum IgE response	Serum[6]3
16 days		Peak MMCP-1 levels (SAR, CBA mice)	Serum[2]
20-21 days		Second peak of IL-9 secretion	MLN[2]
		Decline of IL-4 secretion (SAR/CBA mice)	MLN[2]
		Peak total IgE response	Serum[4]
24-30 days		Decline of MMC numbers (SAR mice)	Small Intestine[2]
		Decline of MMCP-1 levels (CBA mice)	Serum[2]

MLN = Mesenteric lymph nodes

[1] Svetic *et al.*, 1993
[2] Wahid *et al.*, 1994
[3] Slater & Keymer, 1988
[4] Urban *et al.*, 1991a
[5] Wahid & Behnke, 1993
[6] Boulay *et al.*, 1998
[7] Urban *et al.*, 1991b
[8] Katona *et al.*, 1991
[9] Wahid & Behnke, 1992

2.3 Host Immunity to Challenge Infection with *H. polygyrus*

The host is capable of developing effective resistance against secondary exposures to *H. polygyrus* following the removal of adult worms from a primary infection by an anthelmintic-abbreviated regime (Behnke & Wakelin, 1977; Behnke & Robinson, 1985), oral inoculation with irradiated larvae (Slater & Keymer, 1988), or other immunizing methods (Urban *et al.*, 1991a). The rapidity and intensity of acquired immunity achieved upon challenge infection depend on the dose of the immunizing inoculum, the genetic susceptibility of the host, and the infectivity of the larvae (Jacobson *et al.*, 1982; Ali & Behnke, 1985; Behnke & Robinson, 1985; Dobson *et al.*, 1985; Enriquez *et al.*, 1988). Protective immunity to challenge infection with *H. polygyrus* is demonstrated by reduced worm size, depressed fecundity, and more rapid expulsion of adult worms (as reviewed by Monroy & Enriquez, 1992). The L_3 and L_4 larvae of *H. polygyrus* are believed to be potent inducers of protective immunity in responder strains of mice (Monroy & Enriquez, 1992; Wahid & Behnke, 1992). In most murine strains, primary *H. polygyrus* infections, which are terminated before the parasites mature to adulthood by drug treatment or irradiation, induce high levels of acquired resistance to larval challenge infections (Behnke *et al.*, 1983; Behnke & Robinson, 1985; Wahid & Behnke, 1992). The strong and rapid acquired immunity to secondary larval exposures following an immunizing regime is thought to overcome any inherent immunomodulatory strategies employed by the nematode parasites (Monroy & Enriquez, 1992). However, development of this protective immunity depends on the stage of the parasite used in the immunizing procedures because no protection was evident in mice when adult worms were used for the initial sensitization, when the primary infection was terminated after the appearance of adults worms in the intestinal lumen, and when the mice were challenged with adult worms (Behnke *et al.*, 1983; Enriquez *et al.*, 1988; Jacobson *et al.*, 1982; Pleass & Bianco, 1994). The appearance of increased effector responses upon larval challenge of previously immunized hosts suggests that immunological processes primed during primary exposure are important in the effective resolution of secondary infections (as reviewed by Monroy & Enriquez, 1992).

Immunological memory is characterized by accelerated speed, increased size and persistence, all of which are observed during secondary immune responses to challenge or repeated infections with nematode parasites. Immunocompetent hosts are able to develop strong resistance to challenge infection with *H. polygyrus* as evidenced by the dramatic reduction in worm survival, worm size, and fecundity shortly after secondary exposure (Behnke & Wakelin, 1977; Behnke & Robinson, 1985). This rapid and intense termination of secondary *H. polygyrus* infections is thought to result from the mobilization of protective Th2 immune responses that had been primed during previous antigenic exposure. Acquired immunity to challenge infection following an anthelminthic-abbreviated primary infection is $CD4^+$ T cell dependent and involves Th2 effector responses (Urban *et al.*, 1991a). Indeed, serum IgG1 rose ten-fold from resting levels in mice repeatedly immunized with immune mouse serum (Williams & Behnke, 1983) and three-fold in mice challenged with the parasite compared to mice given only a single inoculation (Urban *et al.*, 1991a). Passive transfer of primary infection serum containing parasite-specific IgG1 did not protect unprimed mice from *H. polygyrus* (Williams & Behnke, 1982) whereas multiple infection sera were capable of transferring immunity to naive recipients (Behnke & Parish, 1979). Also, concentrations of serum IgE and numbers of MMC were significantly higher in challenged mice relative to primary infection controls (Dehlawi & Wakelin, 1988; Urban *et al.*, 1991a). Consistent with the concept that secondary exposure to *H. polygyrus* in previously immunized mice stimulates rapid activation of memory Th2 immunity, Table 1 shows that the onset and peak of IgE, IgG1 and eosinophil responses in the peripheral circulation of challenged mice generally occurs 2 days to a week earlier than that following primary infection. Unfortunately, there are no studies evaluating specific cytokine profiles during challenge infection and therefore it is unconfirmed as to whether an elevated secretion of Th2 cytokines is responsible for the apparent potent activation of Th2-type effector responses in mice challenged with nematode parasites. However, this cytokine and effector interaction in the gut is highly probable considering that cytokines are the primary factors that induce and regulate effector responses. A cytokine inhibition study by Urban *et al.* (1991b) provided some indirect evidence that Th2 cytokines are important for the

resolution of secondary infections. Injection of an anti-IL-4 antibody suppressed secondary IgE responses and was associated with increased worm counts and fecundity on day 48 pci. Interestingly, the role of IL-5 and associated eosinophil response in acquired immunity to *H. polygyrus* is not clear because neutralization of IL-5 blocked nematode-induced eosinophilia but did not affect worm burden or fecundity in challenged mice.

The relative contribution of naive and memory effector cells to immune responses observed during challenge infection is not clear. Memory Th cells are not as stringent in their requirements for IL-4 or costimulatory signals (CD28/CTLA4 and B7.1/B7.2 interactions) during activation and effector activity compared to naive Th cells (Gause *et al.*, 1995; Huang *et al.*, 1997). It is possible that the priming of Th2 cytokines during primary infection may be a more critical immunological event than secondary responses for the development of protective immunity to nematode parasites. However, some studies have suggested that memory $CD4^+$ T cells are unstable, depend on the persistence of antigen, and may revert to a resting, quiescent form that is phenotypically indistinguishable from naive $CD4^+$ T cells (Bunce & Bell, 1997; Bell *et al.*, 1998). IL-4 was found to be crucial for maximal capacity for Th2 cytokine secretion by memory Th effector cells that came from a heterogeneous pool of resting memory $CD4^+$ T cells (Bradley *et al.*, 1995) and therefore may be important in potentiating previously committed active T cells as well as in reactivating memory revertant T cells. In addition, Katona *et al.* (1991) and Finkelman *et al.* (1997) reported that IgE responses in secondary infections with *H. polygyrus* were derived mostly from naive or uncommitted B cells rather than from IgE-committed memory B cells produced during initial nematode infection. These preliminary results must be verified by experiments on other Th2 effectors to provide precise description of the immune populations and regulatory factors that are activated during challenge infection.

In summary, functional immunity to *H. polygyrus* appears to be a Th2-dependent process because it involves specific cytokines such as IL-4 and effectors such as IgE-dependent eosinophils and MMC. The magnitude and efficacy of Th2-mediated immune mechanisms may differ according to the infection protocol used with the greatest level of protection seen after secondary exposure. Preliminary data point to the importance of Th2 immunity in GALT which may play a role in promoting the observed increases in serum Th2 effector responses. Given that immunocompetent hosts are able to mount a strong and effective acquired immunity upon subsequent infections, it would be important to determine the magnitude, kinetics and compartmentalization of Th2 immune responses during challenge infection and to assess whether these characteristics are altered by nutritional deficiencies such as protein malnutrition.

3. EFFECT OF PROTEIN DEFICIENCY ON HELMINTH INFECTIONS

Protein deficiency is generally synergistic with both natural and experimental helminth infections and thereby results in an interaction that is more seriously harmful than would be expected from the occurrence of either condition alone (Scrimshaw *et al.*, 1968). Protein malnutrition and intestinal parasitism share similar geographical distribution patterns with the same individuals experiencing both disease states simultaneously throughout their lifetime. For example, a study of rural Nigerian children found that protein intakes were inversely proportional to the number of co-existing intestinal parasitic infections (Rosenberg & Bowman, 1984). Additionally, *Trichuris* egg counts in children living in an urban slum region of Indonesia were correlated with stunting, a physiological indicator of chronic protein calorie malnutrition (Hadju *et al.*, 1995. These data supports the concept that malnourished children living in helminth endemic areas are more heavily parasitized than their well-nourished counterparts. However, given the multi-dimensional nature of malnutrition-parasite interactions and the interpretative problems of epidemiological data, it is difficult to ascertain which condition was the preceding or causative factor.

3.1 Effect of Protein Deficiency on Primary Infection with *H. polygyrus*

Controlled experiments are more conducive to studying the nature of putative casual relationships between protein malnutrition and intestinal parasitic infection. These systems attempt to reconstruct the natural transmission of nematode parasites occurring in human settings by establishing a cohort of susceptible animals inhabiting an area of endemic helminth infection. Slater (1988) housed mice in large, unrestricted cages which provided free access to a 2% or 16% protein diet. An initial cohort of mice was infected with *H. polygyrus*, and at 5 week intervals, subsequent cohorts of young uninfected mice were introduced into the enclosures while previous cohorts were removed. The rate at which the uninfected mice acquired the parasites was higher in the low protein colony, thus indicating that protein deficiency may enhance the rate of parasite transmission within a mobile population of hosts. The prevalence of infection among protein-deficient mice was higher

compared with that of the well-nourished mice and may be related to the observed increase in survival of adult worms in these deficient animals over the experimental period. These results show that, under natural infection conditions, protein deficiency may potentiate the prevalence and intensity of a nematode parasitic infection.

Evidence to support these findings using controlled primary infection protocols is inconclusive. An early study by Bawden (1969) showed that a marginal protein diet (white bread with 12.3% crude protein) produced greater establishment of *H. polygyrus* in mice, significant stunting of female adult worms, and more widespread distribution of nematodes along the small intestine relative to the normal protein diet (pelleted concentrate with 25.2% protein). Interestingly, protein restriction had no effect on the long-term expulsion rate of nematodes but was associated with decreased fecundity at 28 days post-infection. Bawden suggested that the retarded growth and altered distribution of female worms recovered in the malnourished hosts adversely affected their reproductive capacity. Given that *H. polygyrus* worms tend to mate and produce eggs in the anterior duodenum, the longer migration route induced by protein restriction may have subsequently impeded their usual reproductive processes. It is difficult to interpret these results due to the intermediate level of protein restriction and the use of diets with different macronutrient constituents. These uncontrolled dietary factors may have been responsible for the absence of nutritional impact on chronic worm survival. Using purified synthetic diets, Brailsford and Mapes (1987) reported that a more severe restriction of dietary protein (2% casein) did not significantly increase worm establishment or survival despite inhibiting body weight gain and producing overt hypoalbuminaemia during primary infection. These authors found that worm burdens in control (20% protein) and protein-deficient (2% protein) animals remained equivalent at 3-4 weeks post infection. Previous studies in our laboratory found that marginal (7%) and low (3%) protein diets resulted in greater worm burdens at day 29 post-primary infection (Boulay *et al.*, 1998). These results differ from those of Brailsford & Mapes (1987) perhaps because Boulay *et al.* (1998) found unusually low parasite numbers in the control diet group (rather than larger worm burdens in the deficient mice) which may have lead to significant differences in worm survival between dietary groups.

It is possible that a primary infection protocol in which laboratory animals are exposed only once to the nematode parasite may not be a sensitive experimental system to evaluate the influence of dietary protein on outcomes of *H. polygyrus* infection. Primary infections with *H. polygyrus* are usually chronic in otherwise immunocompetent murine hosts, suggesting that the parasite has the inherent capacity to down-regulate or prevent protective host immune responses (Behnke *et al.*, 1992; Dehlawi & Wakelin, 1988; Smith & Bryant, 1986). Assuming that secondary infections normally stimulate development of protective immunity in well-nourished immunocompetent hosts (Monroy & Enriquez, 1992) and that protein deficiency impairs immunological processes, a repeated or challenge infection protocol may help to maximize the chances of detecting any possible effects of dietary protein deficiency on intestinal helminth population dynamics.

3.2 Effect of Protein Deficiency on Challenge Infection with *H. polygyrus*

Experimental infection studies show that the level of dietary protein influences the extent to which the host can develop protective immunity to repeated or secondary infections. Slater and Keymer (1986) fed mice 2% or 8% protein diets and repeatedly infected animals with varying doses of infective larvae of *H. polygyrus* every 2 weeks for a period of 12 weeks. Mice fed the 2% protein diet accumulated worms in direct proportion to the intensity of exposure throughout the experiment whereas mice fed the 8% protein diet showed partial resistance to repeated infection at higher doses. Also, net egg production was increased in mice fed the low protein diet due to both higher per capita egg production of female worms and heavier worm burdens. The authors attributed the increased survival and fecundity of worms in the malnourished mice to either a diet-induced inhibition of acquired immunity to reinfection or to increased sensitivity to parasite-induced immunosuppression. Both explanations rely on the assumption that protein deficiency impairs optimal immunocompetence. Consistent with the results obtained by Slater and Keymer (1986), Keymer and Tarlton (1991) observed higher worm burdens in repeatedly infected mice fed 3% protein diet compared with those fed 16% protein diet. These authors also used a challenge infection protocol in which the primary infection was

abbreviated by an anthelmintic drug and the mice were then reinfected once with 100 infective larvae. At two weeks post-challenge infection, mice fed the low protein diet had more worms than their well-nourished counterparts, suggesting that protein deficiency impairs development of immunological memory normally primed by previous exposure to infectious pathogens. Using a challenge infection protocol, Slater & Keymer (1988) recovered significantly more worms in female NIH mice fed diets containing 2% protein compared with 16% protein at 21 days post-challenge infection. In contrast to results of their previous study (Slater & Keymer, 1986) which had employed a repeated infection protocol, Slater & Keymer (1988) found that *per capita* fecundity of a female worm in mice challenged with larval *H. polygyrus* was not altered significantly by the low protein diet. It is unknown whether repeated infection protocols elicit different immunological responses than challenge infection protocols and whether this may be responsible for these conflicting effects of protein deficiency on worm fecundity. Boulay *et al.*, (1998) reported that a 3% protein diet increased total egg output compared to a 24% protein diet throughout four weeks of challenge infection with *H. polygyrus*. Taken together, protein restriction impaired the capacity for acquired host resistance to secondary infection as indicated by the increased worm burdens in previously immunized protein-deficient mice.

In summary, findings from controlled laboratory and experimental epidemiology studies demonstrate that the overall protein nutriture of the host affects the population dynamics of a nematode parasite. Although many researchers have attributed the increased survival and fecundity of nematodes in protein-deficient hosts to impaired host immunity, only a few studies directly examined Th2 immune responses after both nematode infection and protein deprivation (e.g. Boulay *et al.*, 1998). Inasmuch as host immune defenses are important in mediating susceptibility to parasitic infection, the immunologic mechanisms by which protein deficiency may exacerbate intestinal parasitism deserve in-depth investigation. To this end, the following sections discuss in detail some of the literature on protein malnutrition and host immunity.

4. EFFECT OF PROTEIN DEFICIENCY ON SYSTEMIC CELL-MEDIATED AND HUMORAL IMMUNITY

The predominant approach of previous literature on interactions of malnutrition and immunity has been to examine diet-induced structural and functional changes in systemic lymphoid tissues and their associated immune cell populations. The thymus and bone marrow are essential tissues for the production of lymphocyte, monocyte and mast cell progenitors, however, they are not specifically designed to facilitate immune responses upon antigenic stimulation. Initiation of most primary immune responses to blood-borne pathogens including antigen presentation, activation of B and T lymphocytes, and the production of antibodies and T cell-derived cytokines occurs mainly in the spleen. Antigen-specific immune effectors (e.g. immunoglobulins and differentiated T cells) produced in the spleen enter the systemic circulation to migrate to sites of infection and are detectable in the peripheral blood. Thus, the term systemic immunity used in this paper will apply to nonspecific and specific immune responses in peripheral sites such as the bloodstream, thymus, or spleen.

Dietary protein deficiency has been shown to adversely affect the morphology of systemic lymphoid organs, particularly in the immediate postweaning period which represents a crucial stage of development and maturation of the lymphomyeloid system (Spear & Edelman, 1974). Thymic and splenic atrophy is characteristic of protein-energy malnutrition in children and young laboratory animals (Chandra, 1983). Bell *et al.* (1976) found significant organ weight loss, decreased cell yields, and structurally disorganized cells in the thymus and spleen of weanling mice fed a 4% protein diet for four weeks compared to mice fed a 28% protein diet. Given the importance of the thymus and spleen in the production and maturation of T lymphocytes, respectively, a likely consequence of lymphoid organ involution is decreased numbers or altered proportions of T lymphocyte subpopulations. Protein-deficient rats and patients with kwashiorkor have lower counts of total circulating or splenic T lymphocytes (Abbott *et al.*, 1986; Bises *et al.*, 1987; Slobodianik *et al.*, 1984) while protein-malnourished adults also had lower percentages of $CD3^+$, $CD4^+$, and $CD8^+$ T cells in blood (Abbott *et al.*, 1986). Contradicting these findings,

Woodward and Miller (1991) and Woodward *et al.* (1995) reported that a very low protein diet (0.5% protein) increased the percentages of $CD4^+$ and $CD8^+$ cells relative to total cell numbers in spleens of weanling mice. The biological significance of these proportional increases is unclear considering that the total cell population was drastically reduced (96-99%) by the severe protein deficiency. Some researchers found greater proportion of cytotoxic/suppressor ($CD8^+$) T cells relative to that of T helper/inducer ($CD4^+$) cells in protein-deficient animals (Nimmanwudipong *et al.*, 1992) while others reported that the ratio of $CD4^+$ to $CD8^+$ cells did not vary from control values in protein malnourished animals or humans (Abbott *et al.*, 1986; Bises *et al.*, 1987; Woodward & Miller, 1991). Therefore, it is equivocal as to whether an altered ratio of helper to suppressor T cells contributes to the Th cell-dependent immunodepression observed in protein malnourished animals (Woodward & Miller, 1991). A recent paper examining the effects of protein deficiency in experimental pulmonary tuberculosis in guinea pigs reported that protein deprivation (10% ovalbumin versus 30%) did not affect the proportions of $\alpha\beta$ T cells ($CD4^+$ and $CD8^+$) in the spleens of naive animals (Mainali & McMurray, 1998). When mycobacterial infection was superimposed on the protein deficiency, there was a dramatic loss of $\alpha\beta$ T cells from the spleen and a large decrease in the $CD4^+/CD8^+$ ratio in lymph nodes due to a disproportionate increase of the $CD8^+$ subset at the expense of $CD4^+$ T cells. These results imply that analysis of immune cells in uninfected animals may not necessarily reflect induced responses to antigenic stimulation. Lower numbers of $CD4^+$ cells would have obvious implications for the capacity of the host to respond effectively to nematode parasites because $CD4^+$ T cells control the development and activation of B cells and effectors via secretion of cytokines or direct cellular interactions. Interestingly, Woodward *et al.* (1995) observed higher numbers of $CD4^+$ and $CD8^+$ cells expressing the $CD45RA^+$ surface marker of quiescent or hyporesponsive T cells relative to cells with the $CD45RA^-$ marker of memory-phenotype T cells. These results provide the first indication that protein deficiency decreases the number of responsive T cell subsets in the absence of exposure to an external antigen. The exact mechanisms by which protein deficiency contributes to depressed activation and maturation of T cells remain to be elucidated.

Recent data point to the involution and dysfunction of specific thymic compartments, namely the thymic epithelium which promotes functional differentiation of T lymphocytes through the release of thymulin and other hormonal factors, as contributing factors in the decrease in T lymphocyte numbers seen in protein depleted subjects (Wade *et al.*, 1988; Woodward *et al.*, 1992a). Administration of thymulin by Parent *et al.* (1994) to malnourished children with marasmus and/or kwashiorkor increased the percentages of $CD3^+$, $CD4^+$ and $CD8^+$ and decreased the proportion of immature T lymphocytes (CD1a) in their peripheral blood. Also, thymulin is reputed to exert a positive influence on proliferative capacity of peripheral T cells. Thus, the thymic and splenic atrophy induced by protein malnutrition in weanlings may result in depressed maturation and proliferative capacity of specific T lymphocytes. Unfortunately, none of these studies examined the functional implications of decreased or altered T cell subpopulations during infection in malnourished animals or humans.

In addition to altered numbers of specific types of T lymphocyte, protein deficiency may have detrimental effects on the efficacy of CMI responses to mitogenic or antigenic stimulation. Deitch *et al.* (1992) found that splenic lymphocytes from mice fed a nearly protein-free diet (0.03% protein) for 7, 14 or 21 days had progressively lower blastogenic responses to two T cell mitogens (PHA and ConA) compared to baseline levels. The lower mitogen responsiveness of T lymphocytes from mice fed a low protein diet is a crude indicator of depressed nonspecific CMI but does not necessarily reflect altered T cell responses to specific antigens. Lamont *et al.* (1988) showed that the transfer of ovalbumin-primed lymphocytes from donor mice fed a 24% protein diet to the footpads of naive mice fed 4% protein diet resulted in a depressed cutaneous delayed type hypersensitivity (DTH) response (a characteristic feature of CMI) whereas a positive DTH response was elicited in footpads of naive mice fed 24% protein. Using a graft rejection assay, Lamont *et al.* (1988) found that splenic T cells derived from mice fed 4% protein induced significant splenomegaly in well-nourished recipients. The authors interpreted these results as evidence that protein deficiency did not impair the functional capacity of T cells *per se* but rather it altered other cell-mediated immune responses (e.g. infiltration of leukocytes to site of

antigen challenge or T cell-dependent activation of macrophages) which are necessary for a positive DTH response. Thus, protein deficiency may decrease not only the proliferative capacity of T lymphocytes but also impaired T cell-dependent immune processes and interfered with cellular interactions required for effective CMI responses.

Although thymic involution and subsequent alterations in T lymphocyte numbers are frequent manifestations of protein deficiency, evidence is inconclusive as to whether CMI is more seriously impaired by malnutrition than is thymus-dependent humoral immunity. Severe protein restriction (0.5% protein) induced a greater reduction in splenic B lymphocyte numbers than in T lymphocyte numbers (Woodward & Miller, 1991). However, changes in lymphocyte numbers do not necessarily correlate with altered functional lymphocyte responsiveness. Woodward *et al.* (1992b) found that protein deficiency impaired DTH to sheep red blood cells (SRBC) but had no effect on the serum titres of anti-SRBC antibodies. Human studies consistently report depressed DTH responses, lower mitogen-stimulated proliferative capacity of T lymphocytes, and lower numbers of circulating T lymphocytes but preserved B cell function and hyperimmunoglobulinemia in children with protein malnutrition (Chandra, 1983; Ozkan *et al.*, 1993; Purtilo *et al.*, 1976). In support of these findings, Deitch *et al.* (1992) reported that severe protein deficiency (0.03% protein) decreased blastogenesis of splenic lymphocytes to T cell mitogens (PHA and ConA) to a greater extent than that to a B cell mitogen (PWM). Although these mitogens do not elicit exclusive proliferation of either T or B lymphocytes, these results do imply that competence of CMI rather than of humoral immunity is more sensitive to changes in dietary protein level. In summary, protein deficiency causes structural and functional defects in systemic lymphoid organs and peripheral lymphocytes which are required for the induction and propagation of CMI activities and T cell-dependent humoral responses.

5. EFFECT OF PROTEIN DEFICIENCY ON GUT MUCOSAL IMMUNITY

The functional relevance of systemic immune responses to intestinal helminths is not clear because infections with these parasites are predominantly localized in enteric tissues. Given that the gut contains specialized secondary lymphoid tissues that can react directly with antigens present in intestinal mucosa or lumen, it is of crucial importance to examine the consequences of protein deficiency on gut mucosal immunity to nematode parasites. The impact of protein deficiency on gut mucosal immunity can be first observed by changes in the gross morphological characteristics of GALT. Bell *et al.* (1976) reported that dietary protein restriction (4% protein) induced atrophy of MLN of weanling mice as seen by the reduction in organ size and loss of mature B cells over the four week feeding period. However, the authors found that the tissue loss and structural defects in the protein deficient mice were less pronounced in MLN than those in the thymus and spleen. Similarly, Lochmiller *et al.* (1992) reported no significant differences in the number and average total weight of Peyer's patches in the small intestine among weanling rats fed 4% and 16% protein diets for 6 weeks. When expressed relative to body weight, Peyer's patches were 84 to 95% larger in the protein-deficient rats compared to the well-nourished rats, indicating that the low protein diet caused a greater decrease in total body weight than in the weight of Peyer's patches. In contrast, Woodward and Miller (1991) and Deitch *et al.* (1992) found that severe protein malnutrition equally depressed splenic and MLN organ weight and lymphocyte pools. The CD4/CD8 ratio and percentage of $CD4^+$ or $CD8^+$ cells in MLN were not affected by feeding a very low protein for 3 weeks (Lee & Woodward, 1996). Taken together, these studies indicate that gut lymphoid tissues may be more resistant to gross organ stunting induced by moderate protein restriction than peripheral lymphoid organs or body mass in growing animals. However, the very severe levels of protein restriction used in both studies (0.5% and 0.03% protein, respectively) and the use of naive animals preclude any applicable conclusions to be drawn from these findings toward nematode infections in protein-depleted humans. Regardless of the relative extent of gross structural changes to intestinal lymphoid tissues, there is considerable evidence that

protein deficiency induces morphological abnormalities in the intestinal villi (e.g. crypt hypoplasia) and increases permeability of the gut mucosal barrier (Cummins *et al.*, 1987a & 1987b; Deitch *et al.*, 1992). Diet-induced villus atrophy and a disrupted mucosal barrier integrity may enable parasites to penetrate and establish in gut mucosal tissues and thus could be more detrimental to host resistance than gross changes in lymphoid organs and lymphocyte numbers. Moreover, an increasingly permeable gut barrier may allow leakage of plasma proteins and nutrients which may further compromise gut-associated immunity.

In addition to the observed morphological defects, research has documented impaired functional capacity of gut-associated immunity in protein-restricted animals. Blastogenic responsiveness of lymphocytes from MLN and Peyer's patches to T cell mitogens (PHA and ConA) was depressed over time in mice fed 0.03% protein diet (Deitch *et al.*, 1992). Although B cell responsiveness to mitogen is relatively retained in protein-deficient mice (Deitch *et al.*, 1992), protein restricted diets (2 to 4% protein) have been associated with reduced intestinal IgA concentrations (Lim *et al.*, 1981; McGee & McMurray, 1988; Sullivan *et al.*, 1993), impaired differentiation of IgA^+ precursors in Peyer's patches to a mature IgA-expressing B cell phenotype (Lopez & Roux , 1989), and diminished ability of gut lymphocytes to mediate antibody-dependent cell-mediated cytotoxicity against SRBC (Lim *et al.*, 1981). Recent reports show that the reduced intestinal IgA concentrations during protein-energy malnutrition may not result from impaired IgA synthesis but more likely from decreased transport of IgA precursors to the intestinal epithelium (Ha *et al.*, 1996; Ha & Woodward, 1997). The relevance of these defects in gut-associated IgA and mitogen-stimulated proliferative responses to helminth infection is not clear, although decreases in the intestinal IgA^+ B cell pool may lower the potential for nematode-elicited production of IgE- and IgG1-expressing B cells in GALT.

Another mechanism by which protein deficiency may contribute to impaired host immunity to intestinal parasites is alteration of the homing patterns of mucosal lymphocytes. McDermott *et al.* (1982) transferred radiolabelled MLN lymphoblasts prepared from well-nourished donor rats to recipients with or without protein-calorie malnutrition and found that radioactivity in small and large intestines did not differ among normal or malnourished rats. In contrast, radiolabelled MLN lymphoblasts from malnourished donors injected into well-fed recipients resulted in decreased radioactivity recovered in small and large intestines. The authors concluded that protein-calorie malnutrition did not alter the capacity of the gut to take up mucosal lymphocytes but did impair the selective intestinal localization of GALT-derived lymphoblasts. Although these preliminary data do not provide any information regarding molecular or cellular mechanisms, the authors speculated that protein deficiency may have modified the function and/or structure of lymphocyte homing receptors that mediate lymphocyte binding to the endothelium of HEV in gut mucosal tissues. Composition of the diets and the degree of deprivation induced in the recipient rats were not specified and therefore these results are difficult to interpret and to generalize to systems involving both malnutrition and intestinal parasitic infection.

6. EFFECT OF PROTEIN DEFICIENCY ON IMMUNE RESPONSES TO INTESTINAL NEMATODES INCLUDING *H. POLYGYRUS*

Given that T helper lymphocytes are crucial for antibody responses, depressed T lymphocyte numbers, impaired functional capacity of CMI responses, and defects in homing of gut-derived T lymphocytes have important implications for Th2-mediated humoral immunity to intestinal nematode parasites. It is premature, however, to conclude that these changes observed in blood and lymphoid organs of naive subjects interfere with host immunological responsiveness to helminths, especially considering that the impact of protein deficiency on host protection depend on the proliferative and functional capacity of Th2 cells. As discussed previously, changes in humoral responses are not characteristic of protein malnutrition in uninfected humans. Malnourished children have been reported to have elevated antibody titres in blood (Ozkan *et al.*, 1993) and elevated total IgE levels (Abbassy *et al.*, 1974) while other studies found no relationship between malnutrition and IgE levels (Purtilo *et al.*, 1976). However, these results on total antibody levels do not reflect antigen-specific responses during protein malnutrition. Hagel *et al.* (1995) reported that *Ascaris*-infected children had increased total serum IgE but also lower anti-*Ascaris* IgE concentrations. This study showed that malnutrition may enhance polyclonal IgE synthesis induced by the parasite and in turn diminish the ability to mount specific anti-parasite IgE responses that are important in host resistance to helminthic infections.

In another study that is relevant to anthelminthic immunity, Rickard and Lagunoff (1989) found that a 0.5% protein diet reduced the number of IgE receptors on peritoneal mast cells of naive rats but did not affect the number of mast cells nor the histamine content per cell. The authors suggested two possibilities for these results: 1) protein deficiency is more detrimental to IgE receptor synthesis than to synthesis of proteins necessary for mast cell or histamine production and/or 2) decreased levels of circulating IgE down-regulated the expression of mast cell receptors for IgE. The first explanation requires that protein malnutrition does not cause generalized hypoimmunoglobulinemia, however, the latter is equally plausible in individuals whose parasite-specific IgE synthesis is impaired by protein restriction (Hagel *et al.*, 1995). Interestingly, Chen & Enerback (1994 & 1996) found that

the density of FcεRI receptors and binding of IgE to FcεRI on peritoneal mast cells of naive or *N. brasiliensis*-infected rats were independent of T lymphocytes as demonstrated by the lack of differences in these parameters between athymic and euthymic rats. In athymic rats which lack T lymphocytes, IgE synthesis may be induced by Th2 cytokines and cell contact signals released from mast cells and basophils (Gauchat *et al.*, 1993; Huels *et al.*, 1995). However, the IgE-mediated histamine release by mast cells in athymic rats was significantly lower compared with that of the normal rats despite similar levels of serum IgE and IgE receptors. Thus, interaction with T cells and their mediators may be necessary for activation and degranulation of mast cells. The potential for protein deficiency to impair the synthesis of IgE receptor and the secretory capacity of MMC by depressing Th2 proliferation and cytokine production remains to be investigated.

In one study that examined gut-associated immunological outcomes of protein deficiency during nematode infection, Cummins *et al.* (1987a & 1987b) found that a 6% protein diet versus a 20% protein diet impaired worm expulsion and decreased the number of intestinal MMC but did not affect serum levels of rat mucosal mast cell protease II (released upon MMC degranulation) in rats infected once with *N. brasiliensis*. Thus, increased worm survival in the protein-deficient rats may be attributed to depressed MMC proliferation rather than to defective mast cell activation. It is possible that the relatively mild level of protein deficiency (6% protein) was not conducive to determining the impact of protein malnutrition on histamine releasibility of mast cells. Also, protease concentrations are an indirect measure of MMC activation and may be influenced by the rate of protease turnover in blood. Given that cytokine production was not measured in these studies, it is unknown whether protein deficiency down-regulates the growth and maturation of MMC by decreasing the production and release of cytokines from Th2 and other immune cells. These findings of diet-related impairment of intestinal immunity have obvious implications for the capacity for resistance to nematodes in malnourished hosts.

Surprisingly, there are few studies that have examined whether protein deficiency potentiates chronicity of infection with nematode parasites by impairing Th2 immune responses. A low protein diet (4%) increased the establishment, survival and egg output of *T. muris* in mice (Michael & Bundy, 1991) compared with a 16% protein diet, but this was not associated with defects in humoral immunity as indicated by the normal to elevated serum titres of parasite-specific IgG1 and IgA in the protein deficient group (Michael & Bundy, 1992a & 1992b). These findings were interpreted by the researchers as evidence that protein deficiency is detrimental to immunological processes such as CMI or intestinal barrier function but has little impact on systemic antibody-mediated responses. Studies using the mouse-*H. polygyrus* system have produced more conclusive data showing the detrimental effect of protein malnutrition on Th2 immunity during nematode infection. Slater and Keymer (1988) fed mice a 2% or 16% protein diet and then challenged the animals with *H. polygyrus*. The worm burdens at day 21 post-challenge infection were significantly higher in mice fed the low protein diet compared to those recovered in the protein sufficient mice. The blood eosinophil response was significantly delayed and reduced in the protein deficient mice over the challenge infection period. The rate of increase in parasite-specific IgG response was significantly reduced in the protein deficient mice while absolute concentrations in IgG did not differ between the diet groups. It is unclear whether this slower onset of IgG response may account for the lower level of acquired protection observed in these animals because changes in total IgG response may not necessarily correspond with that of subset IgG1 response which is known to be mediate protective immunity against *H. polygyrus*. Consistent with these findings by Slater and Keymer (1988), past experiments in our laboratory showed that mice fed a low (3%) protein diet had significantly higher worm burdens and higher net egg production at 4 weeks post-challenge infection compared to mice fed 24% protein (Boulay *et al.*, 1998). The lack of functional resistance to challenge infection in these protein-deficient mice was related to their lower peripheral eosinophilia and lower serum total IgG1 concentrations observed throughout the challenge infection process. Considering that Slater and Keymer (1988) and Boulay *et al.* (1998) did not measure production of other Th2 immune effectors

such as IgE, intestinal eosinophils, MMC and specific Th2 cytokines, there are still major gaps in our understanding of the specific immunological mechanisms underlying the interrelationship between protein deficiency and challenge infection with *H. polygyrus*. In particular, we know very little about how protein deficiency affects the development of protective immunity to secondary exposures to *H. polygyrus* in GALT. It is obvious from the limited scope of current literature that much more research on protein deficiency and specific Th2 immunity to helminth infections is needed. Our study integrated all three conditions (i.e. protein deficiency, intestinal nematode infection, and host immunity) in a single experimental host-parasite system with particular focus on them systemic and intestinal immunological mechanisms mediating the interactions between protein malnutrition and intestinal parasitism.

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CHAPTER III
STUDY OBJECTIVES

Previous studies have reported independently that protein deficiency increases the prevalence and intensity of experimental nematode infections and impairs systemic and gut mucosal immune function in naive animals. However, very few studies have examined the interactions between all three conditions (i.e. protein deficiency, nematode infection and host immunity) in the same host-parasite system. Inasmuch as host resistance to challenge *H. polygyrus* infection depends on intense, rapid and sustained Th2 immune responses, it is important to examine whether the capacity for acquired immunity to intestinal nematodes is adversely modified by protein malnutrition. The overall purpose of this study was to determine whether dietary protein deficiency potentiates a challenge infection in mice with the gastrointestinal nematode, *H. polygyrus*, by impairing the magnitude, site-specificity and kinetics of secondary cytokine and effector responses. A time course study of serum IgE, parasitic-specific IgG1, peripheral and intestinal eosinophils, MMC, MMCP-1, and cytokine production in spleen and MLN was performed from days 3 to 28 pci. These immune responses were also measured in naive mice and/or after primary infection to compare the level of protective immunity acquired during challenge infection.

The first specific objective of this study was to determine the effects of dietary protein deficiency on systemic and intestinal Th2 immune responses to *H. polygyrus* infection as indicated by the level of serum effectors (eosinophils, IgE, parasite-specific IgG1), the production of spleen cytokines (IL-4, IL-5, IL-10, IFN-γ), the proliferation of gut mucosal effectors (MMC, intestinal eosinophils, MMCP-1), and the synthesis of gut-associated cytokines in MLN. Secondly, we wanted to ascertain whether the severity of dietary protein restriction (7% versus 3%) would affect the extent of impaired Th2 immunity and parasite survival. The third objective was to compare the impact of protein deficiency on systemic versus intestinal Th2 immune responses to validate our assumption that gut mucosal immunity may be more susceptible to protein malnutrition. Finally, we intended to characterize and contrast the kinetics of systemic and local Th2 immune responses during graded levels of protein deficiency in mice challenged with *H. polygyrus*.

CHAPTER IV

PROTEIN DEFICIENCY PROLONGS SURVIVAL OF A GASTROINTESTINAL PARASITE IN MICE BY SUPPRESSING TH2 IMMUNITY

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ABSTRACT

Protein deficiency has been shown to increase susceptibility to gastrointestinal (GI) parasitic infections in mice, possibly as a result of impaired systemic and intestinal effector responses induced by the down-regulation of Th2 cytokine and/or up-regulation of Th1 cytokine. To test this hypothesis, BALB/c mice (n=18/diet/time point) were fed a control (24%), marginal (7%), or deficient (3%) protein diet and given a challenge infection with the nematode, *Heligmosomoides polygyrus*. The protein deficient (PD) mice had higher food intake per gram of body weight, lower body weight gain, reduced BUN concentration, and depressed relative spleen and mesenteric lymph node (MLN) weights compared to the control and marginal mice. The PD mice had higher worm burdens at 1, 2 and 4 weeks post-challenge infection (pci), suggesting chronic absence of a functional immune response. Indeed, the PD mice had lower increases in serum IgE, reduced peripheral and intestinal eosinophilia, and depressed MMC proliferation and activation at 1 to 2 weeks pci. Parasitic-specific IgG1 was not affected by protein deficiency. To determine whether these suppressed effector responses in the PD mice may have resulted in part from altered spleen and MLN cytokine profiles, cells were stimulated *in vitro* with parasite antigen and cytokine concentration in the culture supernatants was measured by sandwich ELISA. The deficient MLN cells secreted significantly less IL-4 and more IFN-γ within 2 weeks pci than did control MLN cells. The deficient spleen cells also secreted more IFN-γ at 2 weeks pci compared with control spleen cells. There were no significant differences in IL-5 or IL-10 secretion by MLN or spleen cells among the dietary groups. From RT-PCR analyses, the PD mice also had lower IL-4 mRNA expression in spleen and MLN at 1-2 weeks pci. In summary, protein deficiency has detrimental type- and site-specific effects on cytokine and effector responses at early pci time points critical for expulsion of *H. polygyrus*. Our data support the hypothesis that protein deficiency exacerbates the survival of a GI nematode parasite by decreasing IL-4 (Th2) and increasing IFN-γ (Th1), leading to reduced gut and systemic Th2 effector responses.

INTRODUCTION

Protein malnutrition and gastrointestinal nematode infections are chronic diseases that frequently co-exist in human populations of developing countries (Crompton, 1986). Acquired resistance to intestinal parasitism depends on the magnitude and rapidity of Th2 cytokine (e.g. IL-4, IL-5, IL-10) and effector responses (eosinophilia, IgE, mastocytosis) as well as the concomitant absence of Th1 responses such as IFN-γ and macrophage activity (Urban *et al.*, 1993; Monroy & Enriquez, 1992; Urban *et al.*, 1991). Although protein deficiency increases susceptibility to nematode parasites during repeated and challenge infections (Slater & Keymer, 1988; Keymer & Tarlton, 1991), it remains unclear whether protein deficiency potentiates intestinal parasitic infections by impairing the development and maintenance of specific Th2-dependent immune responses in peripheral and/or intestinal tissues. Earlier studies showed that higher worm burdens in mice fed low protein diets were associated with depressed blood eosinophilia and lower titres of circulating parasite-specific IgG (Slater & Keymer, 1988; Boulay *et al.*, 1998). However, it is unknown whether milder protein restriction has the same effect on Th2 immunity and whether the impaired effector responses resulted from diet-induced perturbations in cytokine synthesis. Additionally, little information exists on the consequences of protein deficiency on host protective immunity in gut-associated lymphoid tissues (GALT) even though immune responses in GALT comprise the first line of defense against intestinal parasites and may influence the appropriateness, proliferative magnitude and trafficking of systemic responses. The rate of protein turnover in gut mucosal tissues is 25-30 times greater than that observed for muscle (Nakshabendi *et al.*, 1995) and is potentially very sensitive to changes in host protein status (Wykes *et al.*, 1996).

Our laboratory has been involved in studies on the interactions between acquired immunity, parasitic infection, and nutritional deficiencies in mice infected with a gastrointestinal mouse nematode, *Heligmosomoides polygyrus*. Primary infections with *H. polygyrus* are usually chronic but immunocompetent hosts are capable of developing resistance to challenge infections. The capacity to resist secondary infections and the intestinal site of infection make the *H. polygyrus*-mouse system a suitable model for

studying the influence of nutritional factors on acquired immunity and gut mucosal immune responses during gastrointestinal helminthiasis. Previous experiments in our laboratory have shown that protein deficiency increased worm burdens, decreased serum IgG1 and lowered eosinophilia in mice challenged with *H. polygyrus* (Boulay *et al.*, 1998). Gut-associated Th2 effector responses and cytokine production in a protein deficient host challenged with *H. polygyrus* have not been characterized. The overall objective of this study was to determine the effects of graded levels of protein deficiency on the magnitude, site-specificity and time course of Th2 immune responses during challenge infection with *H. polygyrus*. We postulated that protein deficiency prolongs parasite survival by impairing host cytokine responses in gut-associated and/or systemic lymphoid tissues that normally help to resolve a secondary *H. polygyrus* infection. The reduced Th2 cytokine responses and/or increased Th1 cytokine synthesis may subsequently lead to decreased concentrations of Th2 effectors in intestinal and systemic sites. Assuming that gut mucosal immunity is more susceptible than systemic immunity to protein malnutrition and given its putative role in priming and directing host resistance, we expected protein deficiency to cause a more profound impairment of intestinal immunity than of systemic responses. Also, we expected that a low protein diet (3%) would inhibit acquired immunity, and in turn increase parasitism, to a greater extent than would a marginally deficient diet (7%). Finally, we speculated that these effects would differ in intensity at different phases of infection given that the most intense stimulus for acquired immunity is elicited by tissue-resident larvae at 1-2 weeks post-challenge infection (Wahid & Behnke, 1992).

Our results show that protein deficiency promoted survival of *H. polygyrus* by altering cytokine and effector responses in a selective manner depending on the tissue compartment, the level of restriction and the time point of infection. In particular, the host responses that normally help to resolve challenge infections with *H. polygyrus* (increased IL-4, decreased IFN-γ, elevated IgE, mucosal mastocytosis, MMC activation) were profoundly impaired by protein deficiency. These data support the hypothesis that protein deficiency prolongs survival of a nematode parasite by decreasing gut-associated IL-4 production and Th2 effector responses in the gut and peripheral circulation.

MATERIALS & METHODS

Experimental Design

Mice were fed a diet that was sufficient (24%), marginal (7%), or deficient (3%) in protein and given primary or challenge infections with *H. polygyrus*. Peripheral eosinophilia was measured in tail vein blood using the Unopette Test (#5877 Fisher Scientific, Montreal, PQ). Worms in small intestines of infected mice were counted at 6 and 28 days post-primary infection (ppi) and at 6, 14, 21 and 28 days post-challenge infection (pci). Cardiac blood was separated into serum and plasma samples and stored at -80^{0}C in Microtainer tubes (Becton Dickinson, Rutherford, NJ, USA) for later antibody and mucosal mast cell protease-1 (MMCP-1) analyses. Intestinal tissues were obtained for histological counting of mucosal mast cells (MMC) and intestinal eosinophils. At 3, 6, 14 and 28 days pci, spleen and mesenteric lymph nodes (MLN) cell suspensions were prepared, and their cytokine production and gene expression were determined *in vitro*.

Experimental Protocol

The experiment was conducted using an anthelmintic-abbreviated immunizing protocol that has been shown to stimulate protective immunity against a challenge infection with *H. polygyrus* (Behnke & Robinson, 1985). Three weeks after feeding with experimental diets, mice (6/diet group/time point) were intubated orally with 100 third-stage larvae (L_3) of *H. polygyrus* (day 0 ppi); 12 mice per diet group remained uninfected (Figure 1). Six days after primary infection, 6 mice per diet group were killed to confirm the infectivity of L_3 (P6 mice). On days 9 and 14 ppi, the remaining infected mice were treated orally with pyrantel pamoate (Pfizer Canada Inc., Kirkland, Quebec, Canada, dose of 172 mg/kg body weight) to remove the adult parasites from the intestine. Six mice per dietary group were killed to ensure efficacy of the drug treatment which required an absence of worms in the intestine. One week following the second anthelmintic treatment (day 21 ppi), mice (6/diet group/time point) were reinfected with 100 L_3 of *H. polygyrus* (challenge cohort).

Challenge Cohort

-21 D	D 0	D 6	D 9	D 14	D 21 → Post-primary Infection (ppi) D 0	D 3	D 6	D 9	D 14	D 21	D 28 → Post-challenge Infection (pci)
Feeding different diets	100 L_3 Primary Infection	P6	Drug	Drug	100 L_3 Challenge Infection	C3	C6	C9	C14	C21	C28

Primary Cohort

-21 D	D 0	D 28 → Post-primary Infection (ppi)
Feeding different diets	100 L_3 Primary Infection	P28

Naive Cohort

-21 D	D 28
Feeding different diets	Naive controls

Figure 1: Schematic representation of the experimental protocol and various infection groups. The numbers on top of each line represent time course (in days) of the infection protocol. The values below the line represent the procedures performed at the corresponding day and infection groups killed at each time point.

Two groups of control mice were included in the experiment. One group of previously uninfected mice (6 per diet group) were given a primary infection with 100 L_3 at the time of challenge infection and killed at 28 days ppi (P28 mice). These primary infection mice were used to compare the degree of protection acquired during the challenge infection protocol. A second cohort of mice (6 per diet group) remained uninfected throughout the experimental period to serve as naive infection controls and were killed on day 28 pci (naive controls). Mice given the challenge infection were killed at 3, 6, 9, 14, 21 and 28 days pci and are referred to as C3, C6, C9, C14, C21 and C28 mice, respectively.

Mice

Three week old female BALB/c mice (Charles River, St. Constant, PQ, Canada) were acclimatized to a 14h light-10h dark cycle in a temperature-controlled (22-25°C) animal room for 3 days while fed the protein-sufficient diet. Mice were then weighed and randomly allocated to one of the 3 dietary groups. All experimental diets were offered *ad libitum* in plastic Mouse Powder Feeders (Lab Products Inc., Montreal, PQ) specifically designed to minimize food spillage. Distilled water was available to all animals *ad libitum*. Mice were housed individually in Nalgene cages (Fisher Scientific, Montreal, PQ) with stainless steel covers, filter tops, and heat-treated hardwood bedding. All procedures were approved by the McGill Animal Care Committee according to the Canadian Council on Animal Care (1984).

Diets

Mice were fed a semi-purified, biotin-fortified diet with spray-dried egg-white solids as the sole source of protein (Boulay *et al.*, 1998). The 24% (control) protein level is considered adequate and not excessive for the laboratory mouse (NRC, 1995). The protein-restricted diets contained either 7% (marginal) or 3% (deficient) protein and were made isoenergetic to the 24% protein diet by substitution of protein with equivalent amounts of cornstarch on a weight basis (Table 1). All other nutrients were identical among the three diets and were included at levels sufficiently above the NRC (1995) mouse requirements to ensure that a 30% reduction in food intake as a result of protein restriction or infection would not generate any other nutrient deficiencies in the marginal or deficient protein groups. Biotin was added at 18 times the NRC recommended level to counteract the biotin-chelating effects of avidin in the egg-white solids.

Parasite and parasite antigen

Infective third-stage larvae (L_3) of *H. polygyrus* were obtained by culturing the feces of stock CD1 mice (Charles River) on moist filter paper for 7 days. The cultured larvae were suspended in distilled water (approximately 100 L_3/20 µl) and administered by oral gavage to mice using a Gilson pipetman (Mandel Scientific, Guelph, Ontario). The accuracy of the dose and viability of the larvae was estimated by counting the number of live larvae per 20 µl after every 15-20 intubations.

Parasite antigen was prepared using fourth stage larvae (L_4) of *H. polygyrus* obtained from small intestines of mice infected with L_3 6-7 days previously. The collected L_4 were washed with PBS and sonicated on ice for 10-15 min. The homogenate was centrifuged at 1500 *g* at 4°C for 1 hr. The recovered supernatant was sterilized using a 0.22 µm Acrodisc (Gelman Sciences, Ann Arbor, MI, USA) and stored at -20°C. Protein concentration of the antigen homogenate was determined using a dye-binding protein assay (Bio-Rad, Mississauga, Ontario, Canada) with BSA as the standard.

Table 1: Nutrient composition of experimental diets.[1]

Ingredient[2]	Protein sufficient (24%)	Protein marginal (7%)	Protein deficient (3%)
Egg white	240	70	30
Corn oil	80	80	80
Corn starch	296	466	506
Glucose	296	296	296
Alphacel	30	30	30
Vitamin mix[3]	12	12	12
Mineral mix[4]	46	46	46

[1] Ingredients are g/kg diet

[2] ICN Biochemicals, Division of ICN Biochemicals, Inc.

[3] Vitamin mix (mg/kg diet) as formulated with ICN Biochemicals & Anachemia Science (Anachemia Canada, Inc.); niacin 50.0; d-calcium panthothenate 32.0; riboflavin 24.0; pyridoxine hydrochloride 24; thiamin hydrochloride 16.0; folic acid 2.0; d-biotin 3.6; cyanocobalamin 0.05; α-tocopheryl acetate (1.0 IU/mg) 96.0; menaquinone 9.0; vitamin D_3 (400,000 IU/g) 7.5; vitamin A acetate (500,000 IU/g) 8.0; butylated hydroxytoluene 100.0; choline chloride 4000.0; alphacel 3748.5.

[4] Mineral mix (g/kg diet) as formulated with ICN Biochemicals, Anachemia Science, Sigma Chemicals (Sigma Canada), & Fisher Chemicals (Fisher Scientific Canada); dicalcium phosphate 27.16; potassium bicarbonate 10.25; sodium chloride 2.54; magnesium sulphate 4.95; chromium potassium sulphate 0.0384; cupric carbonate 0.0157; potassium iodate 0.001; ferrous sulphate 0.2489; manganous carbonate 0.1883; zinc carbonate 0.115; sodium selenite 0.0004; sodium molybdate 0.00045; potassium fluoride 0.0099; citric acid 0.48;

Parasite worm burdens

The numbers of L_4 parasites were counted at 6 days after primary or challenge infection. The numbers of adult worms in challenged mice were determined at days 14, 21 and 28 pci and in the primary group at day 28 post-infection. L_4 parasites embedded in the intestinal mucosa were counted from the small intestine that was pressed between two glass slides. Parasite survival was indicated by the number of worms recovered from the entire small intestine at necropsy.

Nutritional measures

Mouse body weight and food intake were measured every 3-4 days. Body weight gain was calculated by subtracting the initial body weight taken prior to randomization to the experimental dietary groups from the final body weight obtained at necropsy. The weights of spleen, thymus and MLN are expressed relative to the final body weight. The concentration of albumin in plasma samples was assayed by dye-binding of bromcresol green to the protein (No. 631, Sigma, St-Louis, MO) as measured with the Abbott VP Sumper System Discrete Analyzer (Abbott Diagnostic, Mississauga, Ontario). Blood urea nitrogen (BUN) concentration was determined by coupled enzyme reactions (No. 67-uv, Sigma) as measured with the Abbott Analyzer.

ELISAs for IgE, parasite-specific IgG1 and MMC protease-I

Total serum IgE and MMCP-1 were measured by two-site ELISA. Immulon II plates (Dynatech Laboratories Inc., Chantilly, VA) were coated with purified rat anti-mouse IgE mAb (02111D clone R35-72, PharMingen, San Diego, CA) at 2 μg/ml and incubated overnight at 4°C. Plates were blocked with 1% BSA in PBS for 2 hrs at room temperature to minimize nonspecific binding in wells. Serial dilutions of purified mouse IgE standard mAb (03121D, PharMingen) and diluted sera were added to the wells and incubated overnight at 4°C. Biotinylated, streptavidin-horseradish peroxidase conjugated rat anti-IgE mAb (02122D clone R35-92, PharMingen) at 2 μg/ml was used as a detecting antibody. Avidin-peroxidase conjugate (Cedarlane Laboratories Limited, Hornby, ON) was used as

the secondary layer and the reaction was visualized by addition of 2,2'-azino-(3-ethyl-benzthiazoline-6-sulphonic acid) (ABTS; Sigma) and read at 405 nm after 25 min with an ELISA microplate reader (Dynatech MR5000, Dynatech Laboratories). IgE concentrations were determined from standard curves and are expressed as mean μg/ml ± SE.

For measurement of serum MMCP-1, Immulon II plates (Dynatech Laboratories) were coated with sheep anti-MMCP-1 capture antibody (Moredun Scientific Ltd., Peniciuk, Scotland) at 2 μg/ml and incubated for 24 hr at 4°C. Purified mouse MMCP-1 isolated from mouse small intestine was used as the standard (Moredun Scientific). After overnight incubation at 4°C with standards and diluted serum samples, rabbit anti-MMCP-1 mAb (Moredun Scientific) conjugated to streptavidin-horseradish peroxidase was added as the detecting antibody. The reaction was visualized by the substrate, orthophenylene diamine (OPD, Sigma) and read at 450 nm with ELISA reader after 25 min incubation. MMCP-1 concentrations are derived from MMCP-1 standard curves and expressed as ng/ml ± SE.

Parasite-specific IgG1 in sera was determined by direct ELISA. Immulon II plates (Dynatech Laboratories) were coated with L_4 antigen homogenate (2.5 μg/ml), prepared as above, in PBS and incubated overnight at 4°C. Plates were blocked with 1% BSA in PBS for 2-3 hr at room temperature. Serum samples and internal controls from uninfected and infected mice diluted 1:100 in PBS with 1% BSA were incubated in wells at 4°C overnight. Biotinylated, streptavidin-horseradish peroxidase conjugated rat anti-mouse IgG1 mAb (PharMingen) was used as the detecting antibody. Avidin-peroxidase conjugate (Cedarlane Limited) was added and plates were developed with ABTS substrate solution for 12 min. The reaction was read at 405 nm with an ELISA reader. The results are reported as mean optical density (OD) ± SE.

Intestinal mast cell and eosinophil histology

Segments of small intestine (approximately 0.5 cm long, 10 cm from pylorus) were fixed in Carnoy's fixative (for MMC) or in 4% paraformaldehyde at 4°C overnight (for intestinal eosinophils). The fixed tissue was dehydrated and embedded in paraffin following standard histological procedure. To count mast cells, sections (5-6 μm thick) were stained with Alcian Blue, counterstained with Safranine O, and the number is expressed as mean per 20 villus crypt units (v.c.u.). Eosinophils in intestinal mucosal sections (5-6 μm thick) were stained with 0.5% Chromotrope 2R and are expressed as mean counts per v.c.u.

Spleen cell and mesenteric lymph node cell preparation

Spleens and MLN were weighed, teased apart with needles, and then passed aseptically through a 60 mesh stainless steel screen (Sigma). Red blood cells were lysed by osmotic shock with cold NH_4Cl lysing buffer solution. Membrane debris was removed through repeated washings with RPMI 1640 supplemented with 10% FBS and centrifugation at 469 *g* for 10 min at 4°C. Cell viability was determined by trypan blue exclusion (0.1% Trypan Blue in PBS; Gibco) and was always greater than 90%. The cells were resuspended in complete medium consisting of RPMI 1640 (Gibco, Burlington, Ontario), 10% heat-inactivated FBS (Gibco), 20mM HEPES buffer (Sigma), 200 U/ml penicillin (Sigma), 200 μg/ml streptomycin sulfate (Sigma), 25 μg/ml gentamicin (Gibco), and 50 μM 2-mercaptoethanol diluted in Hank's balanced salt solution (Gibco). The stock concentration was adjusted to 10×10^6 cells/ml.

Production of Th2 (IL-4, IL-5 and IL-10) and Th1 (IFN-γ) cytokines

Spleen and MLN cell suspensions were plated in complete medium at a final concentration of 5×10^6 cells/ml. Aliquots of 1.0 ml were incubated in 24-well flat-bottom cell culture plates (Costar, Charlotte, NC) with L_4 antigen (7.5 μg protein/ml) prepared as above. This concentration of parasite antigen was determined in a separate experiment to be the lowest concentration required to stimulate maximal cytokine secretion by infected spleen cells. Cell cultures were incubated at 37°C in 5% CO_2 atmosphere with 90%

humidity. Supernatants were recovered after 48 hr incubation by centrifugation of the suspensions at 300 x *g* for 10 min. The supernatants were stored at -80°C until they were assayed for IL-4, IL-5, IL-10 and IFN-γ. A subset of the cultured cells (3 per diet, second block) were lysed with Trizol (Gibco) for mRNA analysis.

Cytokine concentrations in the supernatants were measured by two-site sandwich ELISA that involved the following paired antibodies: purified rat anti-mouse IL-4 mAb 11B11 (18191D, PharMingen, San Diego, CA) and biotinylated rat anti-mouse IL-4 mAb BVD6-24G2 (18042D, PharMingen); purified rat anti-mouse IL-5 mAb TRFK-5 (18051D, PharMingen) and biotinylated rat anti-mouse IL-5 mAb TRFK-4 (18062D, PharMingen); purified rat anti-mouse IL-10 mAb JES5-2A5 (18141D, PharMingen) and biotinylated rat anti-mouse IL-10 mAb SxC-1 (18152D, PharMingen); and purified rat anti-mouse IFN-γ mAb R4-6A2 (18181D, PharMingen) and biotinylated rat anti-mouse IFN-γ mAb XMG1.2 (18112D, PharMingen). Immulon II plates (Dynatech Laboratories) were coated with the capture antibody at 2 μg/ml in 0.1 M Na_2HPO_4 (pH 9.0) and incubated overnight at 4°C. Serial dilutions of cytokine standards and supernatants were added and incubated overnight at 4°C. Biotinylated anti-cytokine detection antibodies were added at 1 μg/ml and streptavidin-horseradish peroxidase conjugate (Cedarlane Laboratories Limited, Hornby, ON, Canada) was used as the secondary layer. The reaction was visualized with the ABTS (Sigma). The concentration of cytokine was calculated from standard curves generated from known concentrations of recombinant (r) murine IL-4 (19231V, PharMingen), rIL-5 (19241V, PharMingen), rIL-10 (19281V, PharMingen), and rIFN-γ (19301T, PharMingen).

Cytokine mRNA expression by semi-quantitative RT-PCR

Cytokine mRNA levels in cultured spleen and MLN cells were quantitated by RT-PCR assay. RNase free plastics and water were used throughout the assay. Concentration of the extracted RNA was determined spectrophotometrically. The RNA was suspended in water, heated to 65°C for 10 min and reverse transcribed in a 65 µl final volume containing 6.5 µl of a 10 mM mix of four deoxynucleotide triphosphates (dNTP), 13 µl of 5X RT buffer, 2.6 µl of 0.1M dithiothreital, 0.26 µl of RNase inhibitor (40 U/µl), 0.52 µl Oligo dT (0.5 µg/µl), 0.65 µl of Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (200 U/µl) (Gibco), 36.27 µl distilled water, and 5.2 µl total RNA (0.3 µg/µl). The mix was incubated at 37°C for 1 hr and then heated to 95°C for 5 min. A 10-µl aliquot of the RT reaction was used in a 100 µl PCR reaction which contained 0.5 µl of Taq DNA polymerase (Gibco), 10 µl of 10x PCR buffer, 0.2 µl of 10 mM dNTP, 4 µl of 50 mM MgCl, 1 µl of each appropriate forward and reverse primers (50 pm/µl). The sequence primers for IL-4, IL-5, IL-10 and IFN-γ and the reference gene, hypoxanthine phosphoribosyltransferase (HPRT) were selected as described by Svetic *et al.* (1993). The samples were subjected to 29 cycles as follows: 1) 94°C for 0.5 min; 2) 56°C for 1 min; and 3) 72°C for 2 min. A final extension step was performed by heating to 72°C for 8 min. The number of PCR cycles and the total RNA input in the RT reaction were determined in a preliminary experiment to ensure that the amplification was well below the saturation under this assay condition. After PCR amplification, a 10 µl aliquot was mixed with 2 µl loading buffer and electrophoresed on a 1.2% agrose gel containing ethidium bromide (0.25 µg/ml). The resulting DNA bands in the gel were photographed using a Polaroid camera. Quantitation of cytokine mRNA was accomplished by densitometric scanning of the photograph using Macintosh Photoshop and NIH Image 160 software. HPRT mRNA levels did not differ significantly among dietary groups ($F_{1,42}$=0.47; p=0.49), infection times ($F_{3,42}$=1.77; p=0.17), or tissues ($F_{1,42}$=0.06; p=0.81). The mRNA level for each cytokine was normalized to HPRT and expressed as ratio to HPRT mRNA for each sample.

Statistical analyses

The experimental protocol was repeated 3 times over a period of 8 months. The results for nutritional, parasite, antibody and histological outcomes were obtained from 3 blocks. The cytokine concentration data were from the second and third blocks while the mRNA expression data were obtained from a subset (n=3/diet/time point) in the second block. Results are reported as means ± SE. Bartlett's test was used to determine homogeneity of variance among groups to be compared. When the variances of the comparison groups were non-homogenous ($P<0.05$), analyses of eosinophils, IgE, MMCP-1, histological counts, cytokine were performed with logarithmic transformed data.

To assess the overall effects of diet and time, data on worm burdens, antibody titres, histological counts, MMCP-1 concentration, cytokine production and cytokine mRNA expression were pooled by infection protocol (primary or challenge) and each was analyzed for the effects of diet, time and diet by time using a two-way analysis of variance (ANOVA). Interactions between diet and time were reported only if significant. Repeated measures ANOVA was used to assess effects of diet and time on peripheral eosinophils. One-way ANOVA was used to detect the effects of diet on nutritional parameters of challenged mice killed on day 28 and on parasite and immunological data of naive mice.

Although our study was designed to examine the overall effects of diet and time during the entire course of the primary or challenge infection (as assessed using two-way ANOVA), we also studied the effect of diet at each post-infection time point because we expected dietary modulation of host immune response to vary in magnitude at different stages of the infection. To this end, the worm burdens, antibody titres, histological counts, MMCP-1 concentration, and cytokine data were analyzed for the effect of diet at each post-infection time point with a one-way ANOVA followed by *post hoc* pairwise comparisons (Tukey) when diet was significant. Only results from *post-hoc* comparisons are reported. The analyses were performed using SAS (SAS Institute Inc., Cary, NC) and considered significant at $P < 0.05$.

RESULTS

Nutritional Outcomes

Table 2 summarizes the nutritional outcomes of a representative group, challenged mice killed on day 28 pci. Although challenged mice fed the 3% protein diet ate the same amount of food cumulatively, and hence calories, as mice fed the 24% protein diet, they gained significantly less body weight. Thus, the 3% protein diet induced protein but not energy deficiency that frequently accompanies dietary protein restriction. In fact, the 3% protein group ate significantly more food per gram body weight compared to the other 2 dietary groups. However, this increased relative energy intake did not support normal growth as shown by the 71% lower body weight gain of protein deficient mice compared to the control mice. In contrast to the 3% group, the marginal protein (7%) group ate significantly more food overall and as a result gained comparable weight as the 24% group.

Protein restriction at both the 3% and 7% level significantly decreased relative weight of mesenteric lymph nodes (MLN). The 3% protein diet also significantly lowered relative spleen weight. Thymus weight was not affected by protein restriction. Dietary protein deficiency modified biochemical indices of protein status: plasma albumin concentration was decreased by the 3% protein diet only when compared with the 7% group, but neither groups differed from the 24% mice. However, blood urea nitrogen (BUN) concentration showed a dose-dependent response to protein level in the diet.

Table 2: The effect of protein deficiency on nutritional parameters of C28 mice[1]

Nutritional Parameter[2]	24%	7%	3%	Diet Effect (P)[4]
Total Food Intake (g)	173.2 ± 2.2^{a}	193.5 ± 1.9^{b}	176.3 ± 3.4^{a}	0.0001
Relative Food Intake (g/g body weight/day)	149.5 ± 1.8^{a}	156.9 ± 1.7^{a}	192.9 ± 2.8^{b}	0.0001
Body Weight Gain (g)	8.7 ± 0.4^{a}	8.5 ± 0.4^{a}	2.5 ± 0.4^{b}	0.0001
MLN Relative Weight[3]	5.1 ± 0.2^{a}	4.1 ± 0.2^{b}	3.7 ± 0.2^{b}	0.0007
Spleen Relative Weight[3]	5.7 ± 0.2^{a}	5.3 ± 0.2^{a}	4.6 ± 0.2^{b}	0.0018
Thymus Relative Weight[3]	1.9 ± 0.9	1.8 ± 0.8	1.8 ± 1.7	0.8125
Plasma Albumin (g/dL)	$2.45 \pm 0.11^{a,b}$	2.77 ± 0.11^{a}	2.26 ± 0.16^{b}	0.0234
Blood Urea Nitrogen (mg/dL)	24.7 ± 1.3^{a}	10.5 ± 0.4^{b}	4.8 ± 0.2^{c}	0.0001

[1] Values are means ± SE.
[2] Values within each parameter with different letter superscripts are statistically different ($P<0.05$) based on post-hoc comparisons (Tukey).
[3] ($\times 10^{-3}$)
[4] Significance of main effect of diet from ANOVA.

Parasite Outcomes

The functional significance of protein deficiency on immunity is shown in Figure 2 which presents the worm burdens of mice fed the experimental diets and given either a primary or challenge infection with *H. polygyrus*. Previously immunized mice fed the 24% and 7% protein diets were capable of expelling over 95% of their parasites by day 28 pci compared to their primary infection counterparts who eliminated only 56-60% of the infective dose. In contrast, worm burdens were significantly higher in mice fed the 3% protein diet throughout the primary and challenge infections, thus supporting the premise that protein deficiency impairs the development of protective immunity to *H. polygyrus*. During challenge infection, protein deficiency increased both the establishment of *H. polygyrus* at day 6 pci and the survival of adult worms at days 14-28 pci. The worm burdens of the 3% mice were also significantly higher compared to the 24% and 7% mice during primary infection, however, these were not as dramatic as that observed during challenge infection.

Serum Effector Responses

To assess the effects of protein deficiency on serum IgE responses, we measured IgE concentration in naive mice, during primary infection and throughout the challenge infection (Figure 3A). Serum levels of IgE were nearly undetectable in uninfected mice and low in primary mice but increased dramatically after challenge infection. Serum IgE levels in all dietary groups changed over the course of challenge infection (time effect, $P<0.05$): mice fed 24% and 7% protein diets had noticeable peaks in serum IgE at days 6 to 9 pci while this elevation was blunted in mice fed 3% protein. In all challenged mice, serum IgE levels gradually declined over time and by 28 days pci were similar to levels observed during primary infection. Mice fed 3% protein had significantly lower serum IgE levels compared to mice fed 24% protein throughout the challenge infection and on day 28 ppi. Serum IgE levels in the 7% mice were similar to the 24% mice on days 3, 6 and 14 pci, were intermediate between the 24% and 3% groups on days 9 and 21 pci, and were as low as the 3% group by day 28 pci, thus suggesting a possible differential response over time to graded levels of dietary protein.

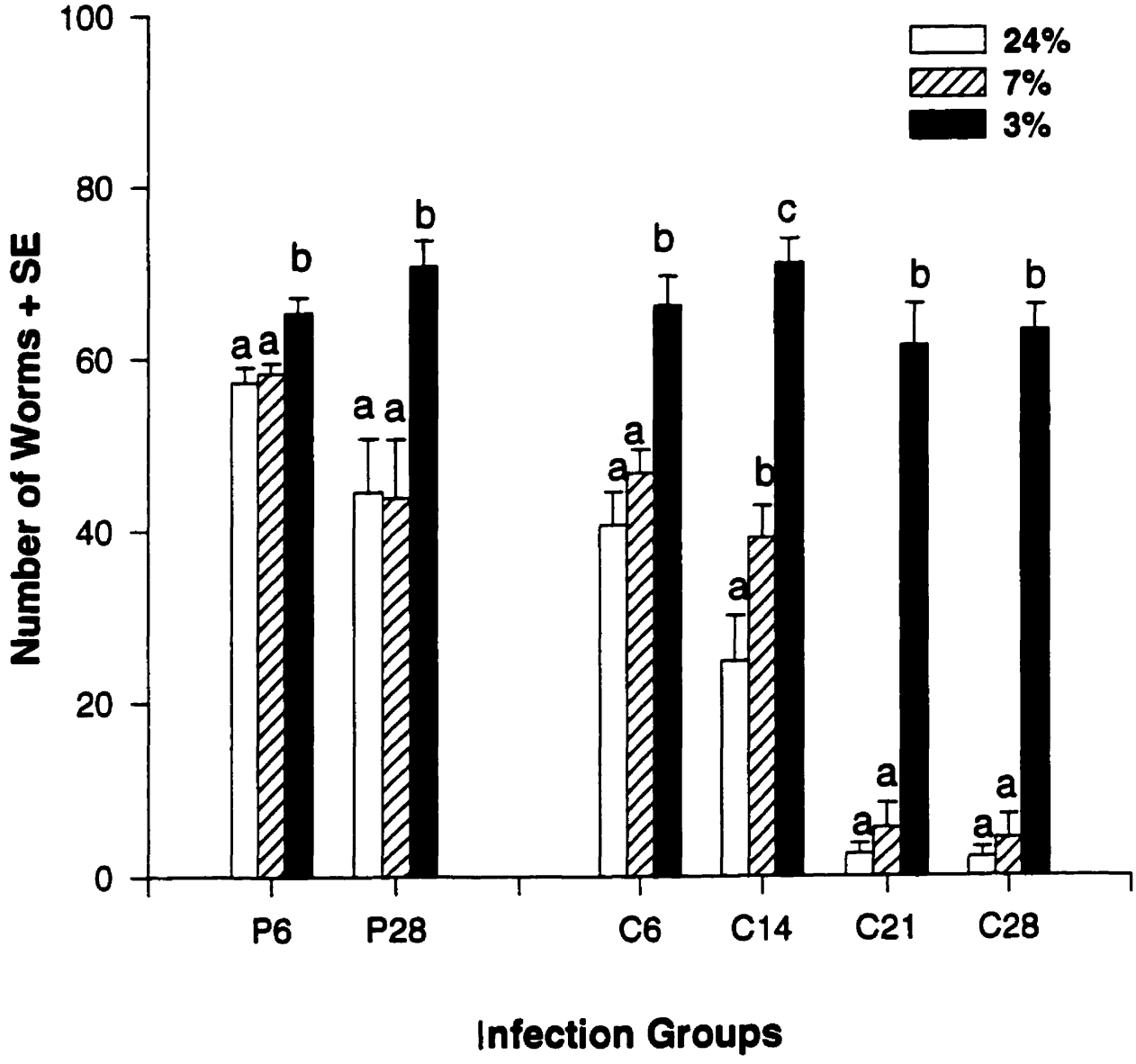
100
80
60
40
20
0
Number of Worms + SE
24%
7%
3%
a a b
a a b
a a b
a b c
a b b
a a b
P6
P28
C6
C14
C21
C28
Infection Groups

Figure 2

Figure 2. Effects of dietary protein deficiency and time on worm burdens of mice given a primary or challenge infection with *H. polygyrus*. Values are mean + SE. During primary infection, the number of worms was significantly higher in 3% mice compared to 24% and 7% mice ($F_{2,92}$=13.35, P=0.0001) and was significantly higher on day 6 ppi compared to day 28 ppi ($F_{1,92}$=5.29, P=0.024). During challenge infection, the worm burdens were significantly higher in 3% mice compared to 24% and 7% mice ($F_{2,153}$=163.37, P=0.0001) and were significantly affected by time ($F_{3,153}$=47.63, P=0.0001). There were significant diet and time interactions in primary infection ($F_{2,92}$=4.22, P=0.018) and in challenge infection ($F_{6,153}$=9.15, P=0.0001), indicating that 24% and 7% mice but not 3% mice eliminated progressively more worms over time. Different letters at each time point represent significant difference between dietary groups (P<0.05) based on *post-hoc* pairwise comparisons (Tukey).

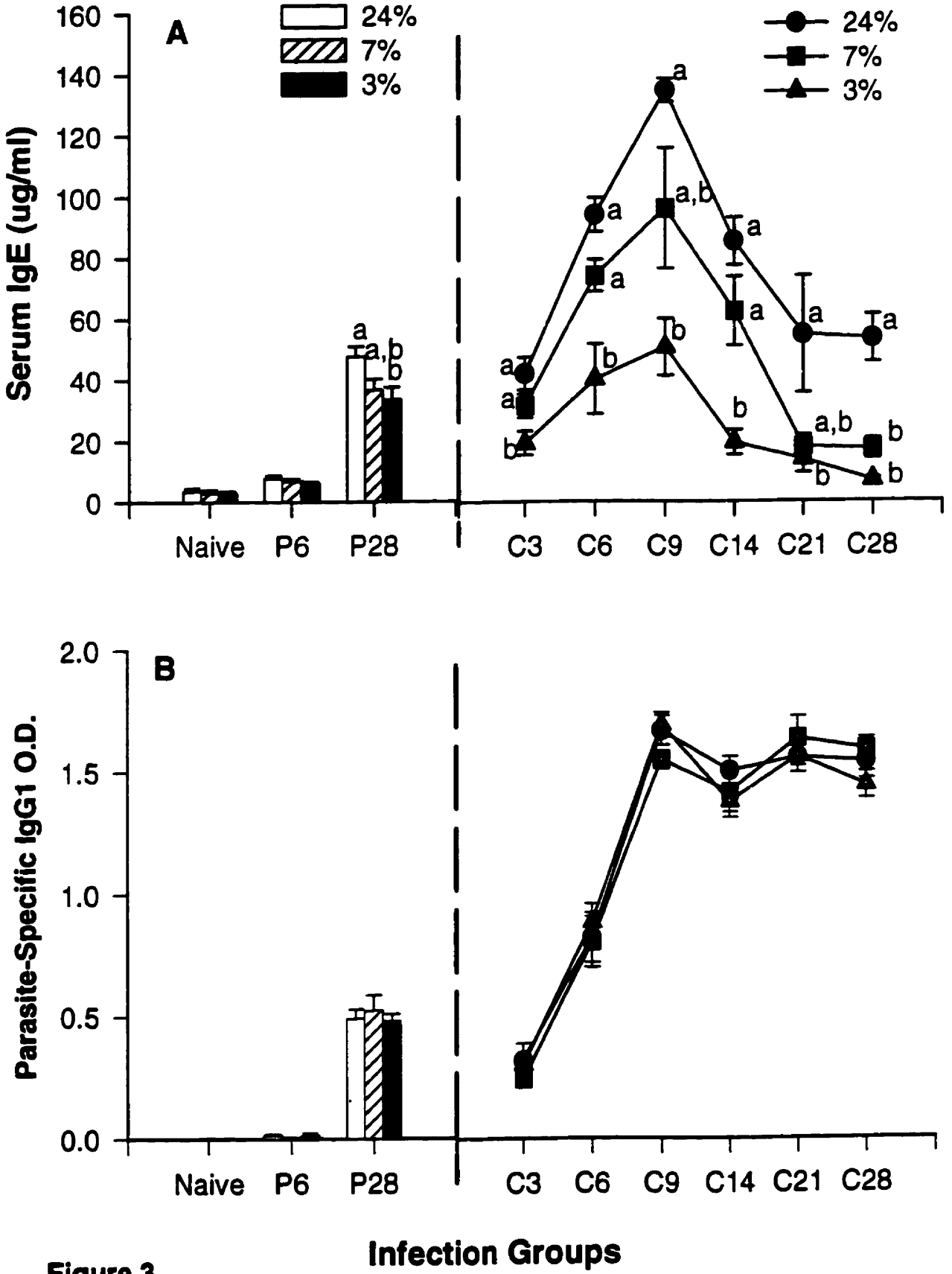
A
24%
7%
3%
Serum IgE (ug/ml)
160
140
120
100
80
60
40
20
0
Naive
P6
P28
C3
C6
C9
C14
C21
C28
B
Parasite-Specific IgG1 O.D.
2.0
1.5
1.0
0.5
0.0
Infection Groups

Figure 3

Figure 3. Effects of dietary protein deficiency and time on serum antibody levels. Values are ± SE. Total serum IgE (A) and parasite-specific IgG1 (B) were determined in naive mice, primary infection (days 6 and 28 ppi), and challenge infection (days 3, 6, 9, 14, 21, and 28 pci). A: In naive mice, there were no significant differences in serum IgE detected between the dietary groups ($F_{2,19}$=0.89, P=0.43). During primary infection, serum IgE was not affected by diet overall ($F_{2,90}$=2.43, P=0.094) but was significantly higher on day 28 ppi compared to day 6 ppi ($F_{1,90}$=254.5, P=0.0001). During challenge infection, serum IgE was significantly different among all three diets ($F_{2,213}$=44.3, P=0.0001) and significantly altered over time ($F_{5,213}$=17.67, P=0.0001). There were no significant interactions of diet and time in serum IgE during primary or challenge infection. B: Parasite-specific IgG1 concentration was not significantly affected by diet overall in naive mice, primary groups or challenge groups. Time significantly affected parasite-specific IgG1 levels among dietary groups during primary infection ($F_{1,81}$=583.18, P=0.0001) and during challenge infection ($F_{5,214}$=150.62, P=0.0001). For IgE, different letters represent significant difference between dietary groups (P<0.05) based on *post-hoc* pairwise comparisons (Tukey) performed following significant effect of diet in one-way ANOVAs at each time point.

Parasite-specific IgG1 responses were also measured in the different infection and dietary groups (Figure 3B). Similar to IgE responses, parasite-specific IgG1 was not detected in naive mice or early during primary infection but was significantly elevated after challenge infection. However, protein deficiency did not impair the synthesis of parasite-specific IgG1 at any time during the primary or challenge infection period. In all dietary groups, parasite-specific IgG1 rose rapidly early after challenge infection and peaked by day 9 pci (time effect, $P<0.05$) to levels greater than that observed during primary infection. Higher parasite-specific IgG1 levels persisted in all dietary groups after 4 weeks pci despite the near absence of worms in the mice fed 24% or 7% diets.

The patterns of peripheral eosinophil responses were determined during primary and challenge infection with *H. polygyrus* (Figure 4). Protein deficiency significantly lowered the numbers of peripheral eosinophils throughout the primary infection (Figure 4A). Although eosinophil counts appeared to be higher in the 24% mice compared to the 3% mice on day 5 pci, the overall effect of diet on peripheral eosinophilia throughout challenge infection was not significant (Figure 4B). At day 0 pci (on which the challenge mice received their second infection and the primary mice received their first infection), blood eosinophil counts in previously immunized mice of the challenge group were higher than in mice of the primary group, indicating the more intense secondary response characteristic of acquired immunity. In the first 2 days after challenge infection, circulating eosinophil numbers in tail vein blood decreased in all dietary groups, suggesting a preferential recruitment of blood eosinophils to the parasite-infected intestine. This was followed, in mice fed 24% or 7% protein, with a sharp increase in blood eosinophilia during the first week of infection which was double the eosinophilia observed in primary mice fed either diets. Blood eosinophil responses declined rapidly after day 5 pci in the challenged mice fed 24% or 7% protein possibly because of the decreasing number of worms in the gut. In contrast, elevated blood eosinophilia persisted in the protein sufficient mice throughout the primary infection.

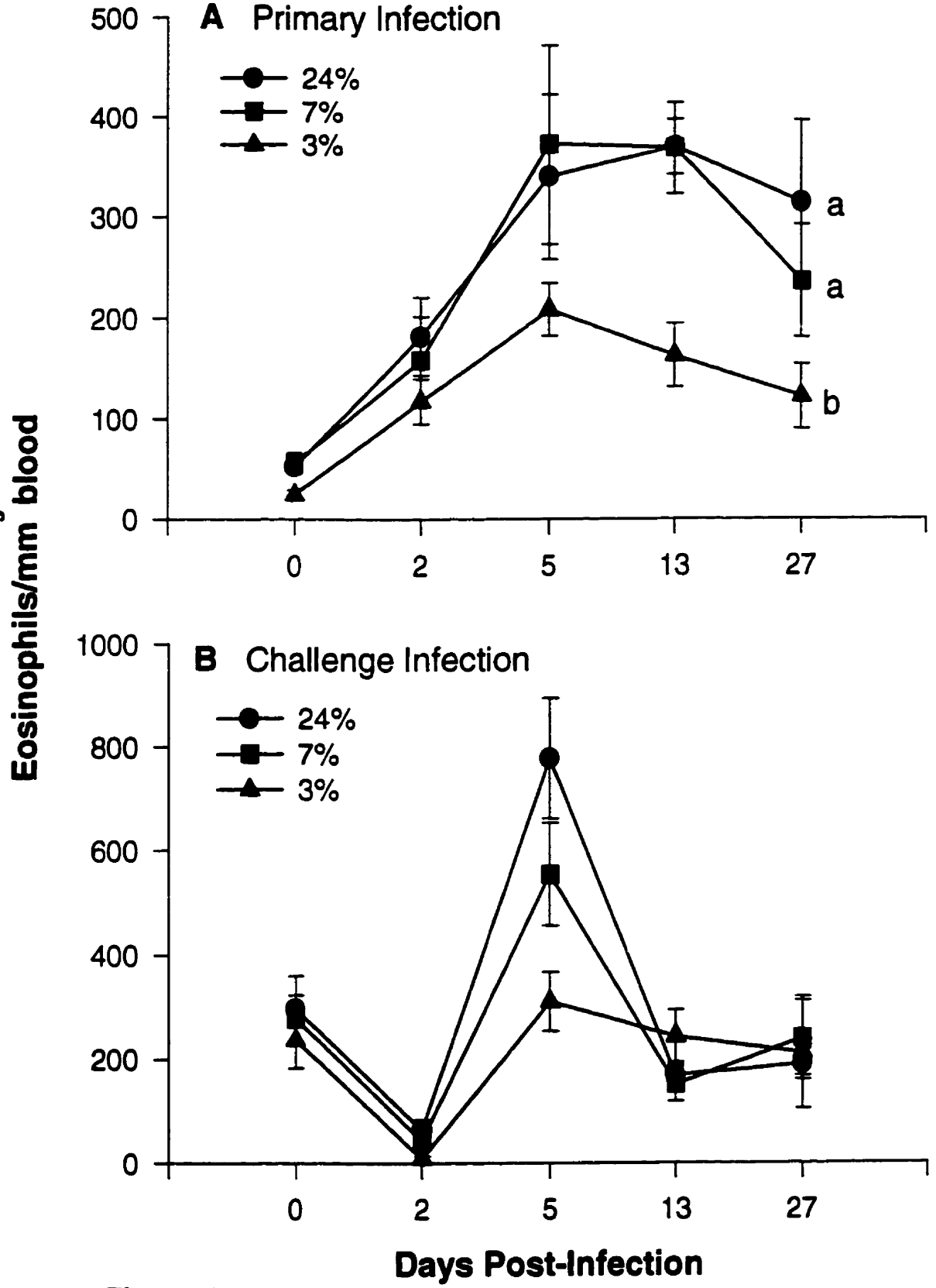
A Primary Infection
24%
7%
3%
500
400
300
200
100
0
a
a
b
0
2
5
13
27
B Challenge Infection
24%
7%
3%
1000
800
600
400
200
0
0
2
5
13
27
Eosinophils/mm 3 blood
Days Post-Infection

Figure 4

Figure 4. Effects of dietary protein deficiency on eosinophilia in tail vein whole blood during both primary (A) and challenge infection (B) with *H. polygyrus*. Values are ± SE. A: During primary infection, peripheral eosinophilia was significantly affected by diet ($F_{2,29}$=8.71, P=0.001) and time ($F_{6,174}$=47.99, P=0.0001). B: During challenge infection, peripheral eosinophilia was significantly affected by time only ($F_{6,180}$=6.87, P=0.0001). There were no significant interactions of diet and time during primary or challenge infection.

Intestinal Effector Responses

We counted the number of MMC and intestinal eosinophils in sections of intestinal tissues obtained from naive mice, primary mice, and challenged mice (Figure 5). In all challenged mice, the numbers of intestinal MMC increased 5-fold by day 3 pci compared with the uninfected levels and were consistently higher at all pci time points compared with early primary mice (Figure 5A). Overall, the 3% protein diet significantly impaired MMC proliferation during challenge infection but not during primary infection. High numbers of MMC in challenged mice fed 24% or 7% protein diets persisted throughout the infection period (time effect, $P>0.05$) but dropped in the 3% group after day 9 pci (time effect, $P<0.05$). Therefore, significant differences in MMC numbers between 24% and 3% groups were detected on day 9 pci and successively thereafter. Numbers of intestinal MMC were lower in the 7% protein diet compared with the 24% group only on day 9 pci, and at other pci time points were either similar to 24% mice or intermediate between the 24% and 3% groups. Hence, the marginal protein mice maintained intermediate but adequate levels of MMC during challenge infection. MMC counts were not statistically different among the dietary groups in uninfected mice or during primary infection.

Protein deficiency also significantly prevented the increase of intestinal eosinophils during challenge infection but did not affect numbers in naive or primary mice (Figure 5B). In spite of the fluctuation in the numbers of intestinal eosinophils after challenge infection, they showed an overall dose-response to the dietary protein level. Compared to the uninfected level, a doubling of intestinal eosinophil numbers was apparent within 2 weeks pci only in mice fed 24% protein. Marked increases in intestinal eosinophilia were not observed in the challenged mice fed 7% or 3% protein compared to their naive counterparts, and therefore, gut eosinophils failed to proliferate after challenge infection in these protein restricted groups. Due to the considerable variability in counts obtained at each pci time point (e.g. days 3 and 9 pci), intestinal eosinophil levels did not change significantly over time in any of the dietary groups (time effect, $P>0.05$). It is important to note that high numbers of gut eosinophils were found in 24% mice on day 3 pci despite low numbers of circulating eosinophils observed very early after challenge infection (Figure 4).

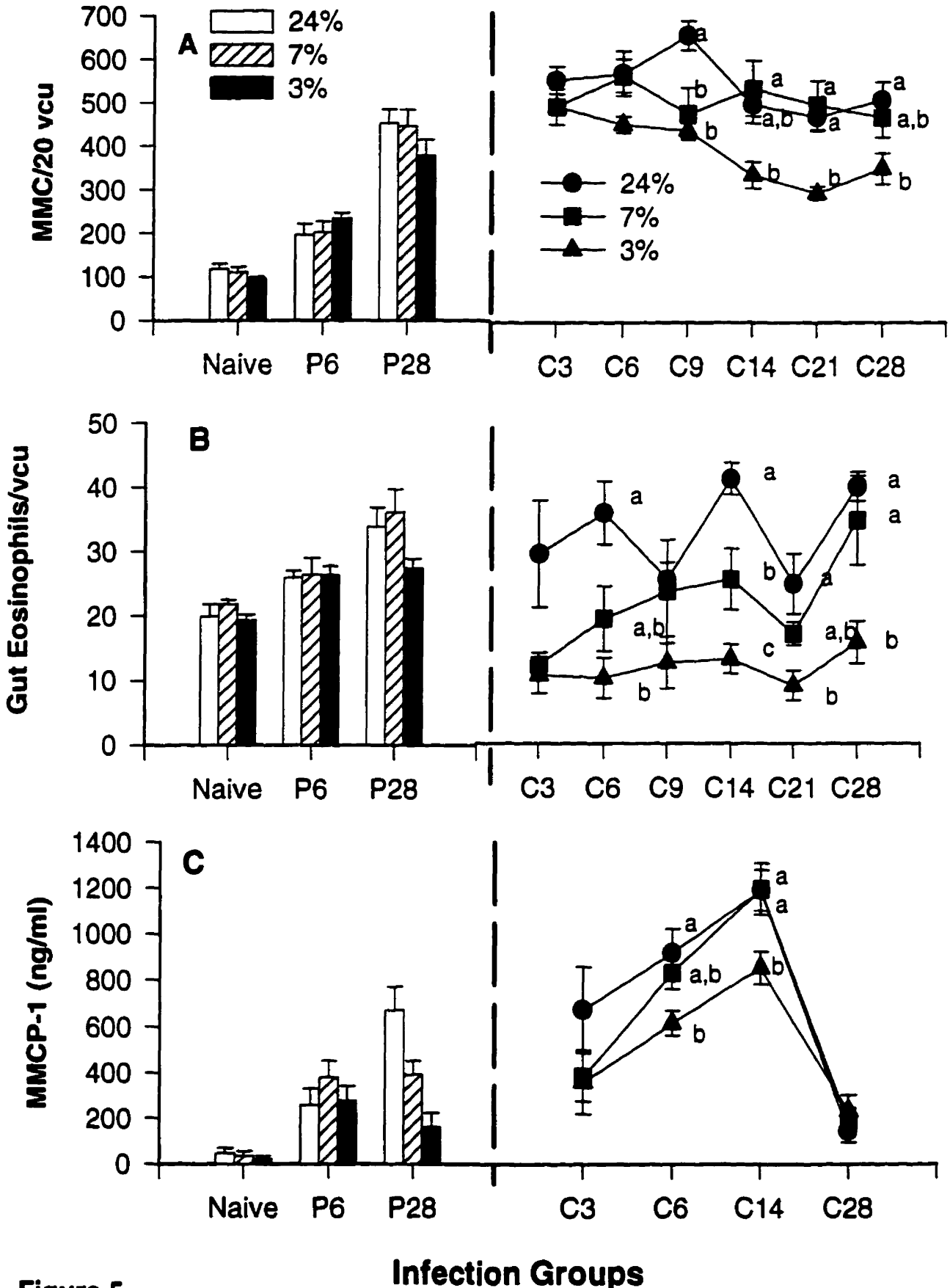
A
24%
7%
3%
MMC/20 vcu
700
600
500
400
300
200
100
0
Naive
P6
P28
C3
C6
C9
C14
C21
C28
B
Gut Eosinophils/vcu
50
40
30
20
10
0
C
MMCP-1 (ng/ml)
1400
1200
1000
800
600
400
200
0
Infection Groups

Figure 5

Figure 5. Effects of dietary protein deficiency and time on the numbers of MMC per 20 vcu (A), numbers of intestinal eosinophils per vcu (B), and the secretory capacity of MMC as measured by MMCP-1 concentration (C) in naive mice, primary mice (6 and 28 days ppi), and challenge mice (3, 6, 9, 14, 21 and 28 days pci). Values are ± SE. A: In naive mice, the numbers of MMC were not significantly affected by diet. During primary infection, the numbers of MMC were not significantly different among dietary groups but were significantly altered over time ($F_{1,27}$=70.19, P=0.0001). During challenge infection, the numbers of MMC were significantly lower in 3% mice compared to 24% and 7% mice ($F_{2,59}$=21.69, P=0.0001) and significantly affected by time ($F_{5,59}$=4.09, P=0.003). B: Numbers of intestinal eosinophils were not significantly affected by diet overall in naive or primary mice. During primary infection, intestinal eosinophilia was significantly higher on day 28 ppi compared to day 6 ppi ($F_{1,24}$=7.02, P=0.014). During challenge infection, the numbers of intestinal eosinophils were significantly different among all dietary groups ($F_{2,58}$=29.61, P=0.0001) and significantly different over time ($F_{5,58}$=3.67, P=0.0059). C: Serum MMCP-1 concentration was not significantly affected by diet in naive mice. During primary infection, serum MMCP-1 levels were not significantly affected by diet or time but there was a significant diet and time interaction ($F_{2,50}$=3.84, P=0.028). During challenge infection, MMCP-1 concentration was not significantly affected by diet overall but was significantly affected by time ($F_{3,185}$=44.59, P=0.0001). For MMC, gut eosinophils and MMCP-1, the different letters represent significant difference between dietary groups (P<0.05) based on *post-hoc* pairwise comparisons (Tukey) following significant effect of diet in one-way ANOVAs at each time point.

To determine the secretory capacity of MMC during nematode infection in mice with varying degrees of protein deficiency, we measured the concentration of serum MMCP-1, a protease expressed predominantly in intestinal MMC (Figure 5C). Protein deficiency did not alter MMCP-1 levels in uninfected mice, primary mice or in challenged mice. Although there were no overall diet effects during challenge infection, we found that protein deficiency significantly inhibited the increase of MMCP-1 secretion at days 6 and 14 pci ($P<0.05$). The time course of MMCP-1 response parallels that of serum IgE such that MMCP-1 concentration in the protein sufficient mice were higher after challenge infection compared with uninfected or primary mice. In all dietary groups, peak MMCP-1 concentration occurred on day 14 pci (time effect, $P<0.05$) which occurred after peak MMC numbers in the gut mucosa (in 24% mice on day 9 pci) and peak levels of serum IgE and parasite-specific IgG1 (day 9 pci).

Systemic and Local Cytokine Responses to Parasite Antigen in vitro

To determine whether the impaired effector responses observed in the protein deficient mice were related to diet-induced perturbations in cytokine production in local and/or systemic lymphoid tissues, we measured post-challenge Th2 cytokine (IL-4, IL-5, IL-10) and Th1 cytokine (IFN-γ) production by spleen and MLN cells restimulated with parasite antigen *in vitro* (Figure 6). In general, Th2 cytokine secretion was higher in the first week of primary infection compared with the challenge infection (with the exception of IL-5 production in spleen), indicating a strong priming of $CD4^+$ cells to secrete Th2-type cytokines. Also, the Th2 cytokine profile during challenge infection was similar among the 3 dietary groups in that the response was greater in MLN cells than in spleen cells, peaked by one week pci, and declined rapidly thereafter. Effects of protein deficiency on Th2 cytokine production were detected only in IL-4 secretion by MLN cells early after challenge infection (Figure 6A & 6B). The MLN cells of the 7% protein group secreted significantly less IL-4 at day 3 pci compared to their 24% protein counterparts. Similarly at day 6 pci, IL-4 production was significantly lower in MLN of mice fed 3% protein compared to mice fed 24% protein. We also observed differences in mucosal IL-4

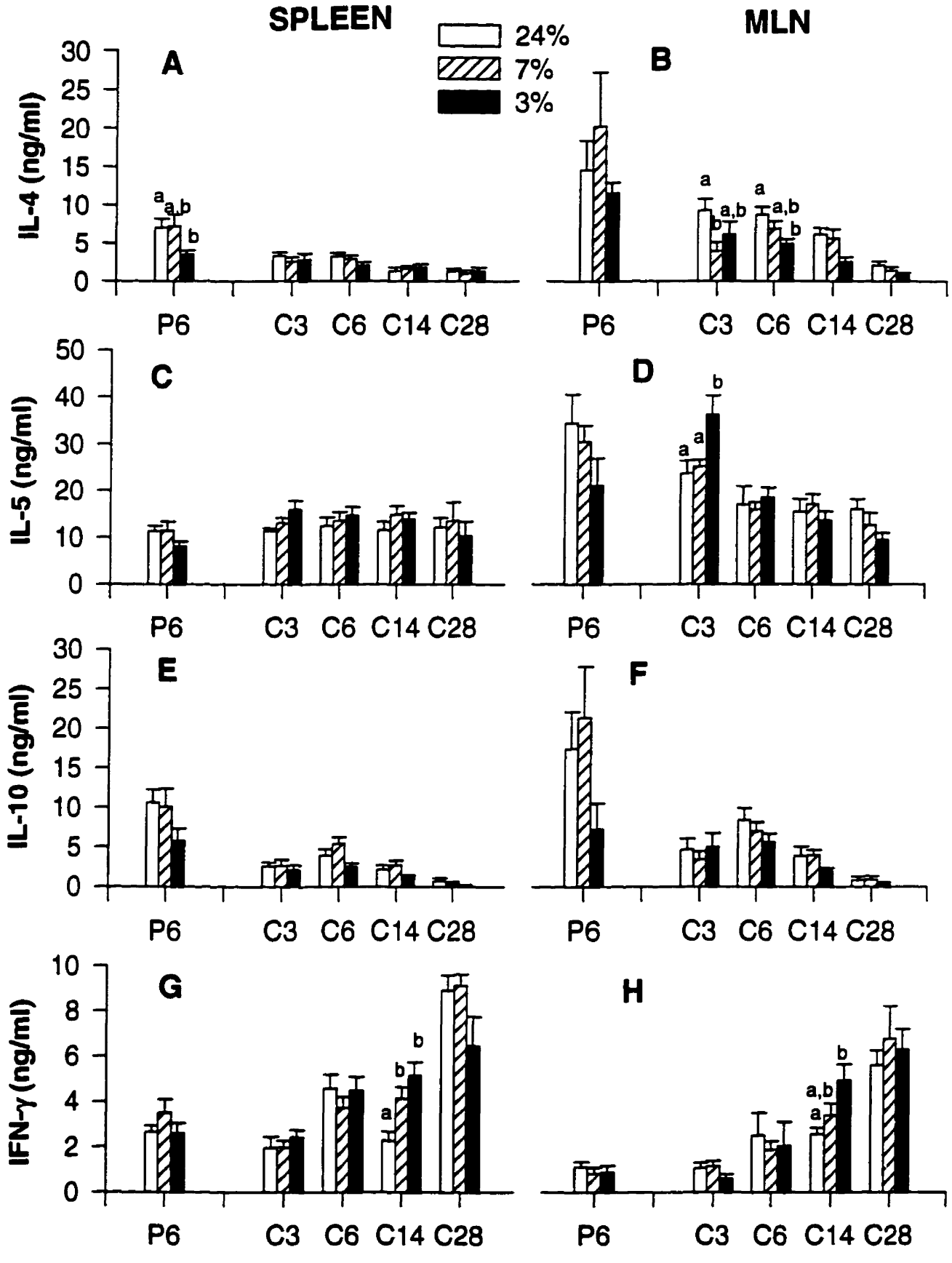
SPLEEN
MLN
24%
7%
3%
A
B
C
D
E
F
G
H
IL-4 (ng/ml)
IL-5 (ng/ml)
IL-10 (ng/ml)
IFN-γ (ng/ml)
P6
C3
C6
C14
C28
Infection Groups

Figure 6

Figure 6. Effects of dietary protein deficiency on Th2 cytokine (IL-4, IL-5, IL-10) and Th1 cytokine (IFN-γ) production in spleen (A, C, E, G) or MLN (B, D, F, H) cells in mice with either primary or challenge infection of *H. polgyrus*. Values are ± SE. Spleen and MLN cells from P6, C3, C6, C14 and C28 mice were cultured, restimulated with parasite antigen *in vitro*, and assayed for cytokine secretion using sandwich ELISA. Values are mean ng/ml ± SE. A and B: At day 6 ppi, IL-4 production was significantly lower in spleen of 3% mice compared to 24% mice ($F_{2,33}$=3.87, P=0.031) but not in MLN. During challenge infection, IL-4 production in spleen was significantly affected by time only ($F_{3,102}$=6.55, P=0.0004) and in MLN was significantly affected by both diet overall ($F_{2,93}$=6.08, P=0.0033) and time ($F_{3,93}$=14.06, P=0.0001). C and D: IL-5 secretion by spleen or MLN cells was not significantly different among dietary groups at day 6 ppi and during challenge infection. Time significantly affected IL-5 production only in MLN cells during challenge infection ($F_{3,101}$=16.74, P=0.0001). E and F: IL-10 production by spleen or MLN cells was not significantly affected by diet overall at day 6 ppi and throughout the challenge infection. During challenge infection, time significantly affected IL-10 production in spleen ($F_{3,82}$=13.41, P=0.0001) and in MLN ($F_{3,87}$=9.97, P=0.0001). G and H: IFN-γ production was not significantly affected by diet overall at day 6 ppi or throughout challenge infection. During challenge infection, time significantly affected IFN-γ production in spleen ($F_{3,102}$=35.06, P=0.0001) and in MLN ($F_{3,92}$=30.89, P=0.0001). For all cytokines produced in spleen or MLN, different letters represent significant differences between dietary groups (P<0.05) based on *post-hoc* pairwise comparison (Tukey) following significant effect of diet in one-way ANOVAs at each post-challenge time point.

production between the dietary groups at a time immediately prior to challenge infection (basal challenge levels). In these primed mice, the MLN cells of the 24% group produced more IL-4 (8.66 ng/ml ± 0.71, n=2) compared to 3% group (3.98 ng/ml ± 0.96, n=2) while the MLN of mice fed 7% protein secreted an intermediate level (5.16 ng/ml ± 2.43). The 7% mice were capable of increasing IL-4 production by 6 days pci, suggesting a capacity to up-regulate IL-4 production at a post-infection time critical for maximal stimulation of effector responses. By comparison, the decreased production of IL-4 in MLN cells of mice fed 3% protein preceded the impairment of intestinal and systemic Th2 effector responses. As shown on Figure 6C to 6F, the profiles of IL-5 and IL-10 production in spleen and MLN were similar to IL-4 responses but, unlike IL-4, were not significantly affected by protein deficiency overall during challenge infection. Despite an overall nonsignificant effect of diet, we found that IL-5 secretion in MLN was higher in mice fed 3% protein compared with either 24% or 7% groups at day 3 pci but this was not associated with marked elevation of gut eosinophils.

As shown in Figure 6G & 6H, IFN-γ secretion by both spleen and MLN cells restimulated with parasite antigen *in vitro* increased significantly throughout the challenge infection in all dietary groups (time effect, $P<0.05$), except in spleens of mice fed 3% protein (time effect, $P>0.05$) This higher IFN-γ response in spleen and MLN over time coincided with decreasing levels of IL-4 and IL-10 in MLN and increasing worm loss in the protein sufficient mice. We did not detect an overall significant effect of diet on IFN-γ production during challenge infection period. However, at day 14 pci, mice fed the 3% protein secreted significantly more IFN-γ compared to mice fed 24% protein in spleen ($P=0.0003$) and in MLN ($P=0.023$). Taken together, protein deficiency did not alter the inherent capacity of spleen or MLN cells to synthesize IFN-γ but increased IFN-γ production at the onset of adult worm expulsion (day 14 pci) in a challenge infection.

Systemic and Local Cytokine mRNA Expression

To address the question of whether altered IL-4 and IFN-γ profiles in protein deficient mice resulted from diet-induced changes in cytokine mRNA expression, we analyzed levels of cytokine mRNA using semi-quantitative RT-PCR (Figure 7). The spleen and MLN cells of mice fed either 24% or 3% protein (n=3) were restimulated with parasite antigen *in vitro* and then assayed for IL-4, IL-5, IL-10 and IFN-γ mRNA. In mice fed 3% protein, IL-4 mRNA expression in spleen and MLN cells failed to increase and remained low throughout the challenge infection (Figure 7A & 7B). The differences between dietary groups were statistically significant on day 14 pci in spleen cells and on days 6 and 14 pci in MLN cells. Also, IL-10 mRNA expression in spleen and MLN was significantly lower in mice fed 3% protein compared with their 24% protein counterparts at day 14 pci (Figure 7E & 7F). The levels of IL-5 (Figure 7C & 7D) and IFN-γ (Figure 7G & 7H) mRNA expression were not significantly affected by protein deficiency at any time point during challenge infection.

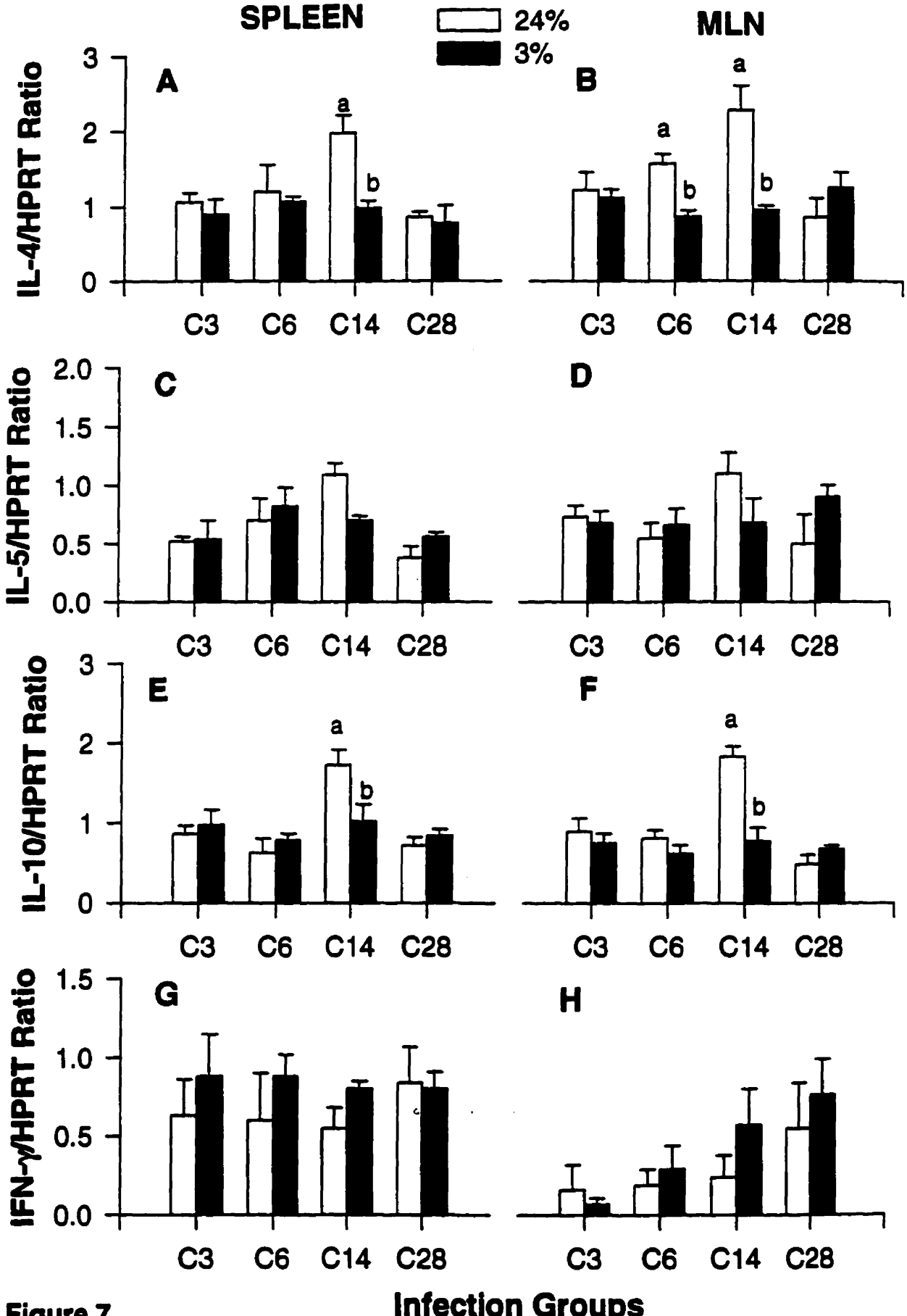
SPLEEN
24%
3%
MLN
A
B
C
D
E
F
G
H
IL-4/HPRT Ratio
IL-5/HPRT Ratio
IL-10/HPRT Ratio
IFN-γ/HPRT Ratio
C3
C6
C14
C28
Infection Groups

Figure 7

Figure 7 Effects of dietary protein deficiency and time on Th2 cytokine mRNA (IL-4, IL-5, IL-10) and Th1 cytokine mRNA (IFN-γ) expression in spleen (A,C, E, G) or MLN (B, D, F, H) cells in mice with challenge infection of *H. polygyrus*. Spleen and MLN cells from C3, C6, C14 and C28 mice were cultured and restimulated with parasite antigen *in vitro*. Cells from the supernatants were lysed for total mRNA and mRNA expression of each cytokine was determined with semi-quantitative RT-PCR. Values are expressed as the ratio of cytokine mRNA to HPRT mRNA for individual samples (mean ± SE, n=3). A and B: IL-4 mRNA levels in spleen and MLN were significantly affected by diet ($F_{1,39}$=12.28, P=0.0015) and time ($F_{3,30}$=6.43, P=0.0017). C and D: No significant differences in IL-5 mRNA expression in spleen and MLN were detected between the dietary groups or *time points*. E and F: IL-10 mRNA levels in spleen and MLN were significantly affected by diet ($F_{1,33}$=6.64, P=0.015) and time ($F_{3,33}$=17.18, P=0.0001). G and H: IFN-γ mRNA expression in spleen and MLN was not significantly affected by diet or time. For all cytokine mRNA levels in spleen or MLN, different letters represent significant differences between dietary groups (P<0.05) based on *post-hoc* pairwise comparisons (Tukey) following significant effect of diet in one-way ANOVAs at each time point.

DISCUSSION

This study is the first comprehensive investigation on the effects of dietary protein deficiency on the magnitude and kinetics of Th2 immune responses in mice to a challenge infection with a gastrointestinal nematode parasite, *Heligmosomoides polygyrus*. Contrary to conventional belief, dietary protein deficiency did not suppress all immune processes but instead exerted differential site- and type-specific effects on cytokine and effector responses to *H. polygyrus* infection. To illustrate this point, four important findings are noted: 1) Protein deficiency impaired the proliferation of gut eosinophils and mucosal mast cells (MMC) but only during the early phase of infection; 2) Protein deficiency decreased the levels of circulating IgE but not of parasite-specific IgG1; 3) Protein deficiency down-regulated IL-4 production only in a gut lymphoid tissue and did not affect secondary responses of other Th2 cytokines; and 4) Protein deficiency up-regulated levels of IFN-γ at 2 weeks pci which is a critical period for expression of acquired immunity. Additionally, the level of dietary protein influenced the degree of protective immunity and parasite survival during a challenge infection with *H. polygyrus*. Our results support the hypothesis that protein deficiency prolongs survival of a gastrointestinal nematode parasite by decreasing gut-associated IL-4 (Th2) and increasing IFN-γ (Th1) early in the infection, leading to reduced intestinal and systemic Th2 effector responses.

Acquired immunity to secondary infections with *H. polygyrus* is associated with strong and persistent Th2 effector responses (Monroy & Enriquez, 1992), although the precise mechanisms by which these Th2 effectors exert their protective effect have not been clearly elucidated. Eosinophilia is a typical Th2 inflammatory response observed during nematode parasitic infections but some researchers have suggested that host resistance to *H. polygyrus* or *Trichinella spiralis* infections in mice is not dependant on eosinophilia. Prevention of eosinophilia induced by the neutralization of IL-5 antibody or deletion of the IL-5 gene did not affect worm survival or fecundity (Urban *et al.*, 1991; Herndon & Kayes, 1992; Takamoto *et al.*, 1997). However, these authors measured eosinophilia in circulating blood, an indicator that does not necessarily reflect eosinophil responses in intestinal sites of

infection which are more relevant to host immunity against enteric parasites (e.g. Svetic *et al.*, 1993; Negrao-Correa *et al.*, 1996). An example of the strong bias towards gut mucosal localization of eosinophils during nematode infection is our finding of a transient drop in blood eosinophils at day 2 pci that coincided with elevated numbers of intestinal eosinophils in mice fed 24% protein. We interpret this pattern as evidence that eosinophils are transported preferentially to the infected intestine immediately following challenge infection until gut-associated lymphoid tissues are capable of producing high quantities of local eosinophils. Similar to this reputed migrational pattern of eosinophils during *H. polygyrus* challenge infection, others have observed that the uptake of IgE by intestinal cells is greatly increased within 24 hours after *T. spiralis* infection (Ramaswamy *et al.*, 1994) and that the vast majority of intestinal IgE (>99%) detected by 4 days post-infection is produced locally in the gut (Negrao-Correa *et al.*, 1996). These results suggest that high levels of immune effectors in the gut are functionally more important than peripheral quantities during intestinal nematodiasis. Thus, our finding that intestinal but not peripheral eosinophilia was inhibited by protein deficiency during challenge infection may be one factor contributing to the prolonged worm survival in the protein deficient mice. However, the role of eosinophils in protective immunity to helminth infections has been challenged by studies showing that murine eosinophils, unlike human eosinophils, lack IgE receptors (the high-affinity FcεRI and the low-affinity FcεRII/CD23) but do express IgG receptors (de Andres *et al.*, 1997). Accordingly, the cross-linkage of IgG receptors by parasite antigen may be the main activation pathway of eosinophil degranulation and oxidative burst. Thus, the functional significance of reduced gut eosinophilia in mice fed 3% protein to parasite expulsion remains uncertain because the synthesis of parasite-specific IgG1 (the IgG isotype associated with Th2 responses to nematodes) after challenge infection with *H. polygyrus* was not impaired by protein deficiency.

This lack of responsiveness of parasite-specific IgG1 to the detrimental effects of protein deficiency during primary or challenge infection with *H. polygyrus* corresponds with previous results from our laboratory (Boulay *et al.*, 1998) and may be explained by literature suggesting that the regulation of IgG1 response does not require IL-4. Early studies demonstrated that the synthesis and expansion of IgG1-secreting B cells is dependent on $CD4^+$ T cells and that IL-4 promotes isotype switching of IgM^+ B cells to membrane expression and antibody secretion of IgG1 (Vitetta *et al.*, 1985; Coffman *et al.*, 1986). However, later animal studies showed that IgG1 responses can be induced during *H. polygyrus* or *Nippostrongylus brasiliensis* infections even in the absence of IL-4 activity *in vivo* and the disruption of the murine IL-4 gene (Finkelman *et al.*, 1988; Katona *et al.*, 1991; Kopf *et al.*, 1993). Furthermore, treatment with exogenous IL-4 increased the transport and uptake of IgE but not of IgG1 into the intestines of *T. spiralis*-infected rats (Ramaswany *et al.*, 1994). Therefore, any disturbances in IL-4 production caused by protein malnutrition as observed in our study may not necessarily lead to subsequent changes in IgG1 responses during *H. polygyrus* infection. Also, the observation that protein deficiency significantly prolonged parasite survival without impairing concomitant IgG1 responses indicate that we should reappraise the immunologic role of IgG1 in protective immunity to nematode parasites. Pritchard *et al.* (1983) reported that purified IgG1 from immune sera of mice infected with *H. polygyrus* caused severe stunting of worms and promoted adherence of peritoneal exudate cells to the surface of immature and adult worms *in vitro*. However, Wahid *et al.* (1993) later showed that parasite-specific IgG1 responses did not correlate with the rate of worm expulsion and were marginally elevated in mice with heavier worm burdens. Moreover, the inhibition of IL-4 and its receptor by neutralizing antibodies prolonged worm survival in primary *H. polygyrus* infection without concurrent impairment of IgG1 responses (Finkelman *et al.*, 1988; Katona *et al.*, 1991). The unaltered parasite-specific IgG1 levels observed in the heavily parasitized protein deficient mice further support the contention that IgG1 may contribute less to host protection against intestinal parasites than other Th2 effectors in a challenge infection.

MMC hyperplasia is another prominent feature of Th2 effector responses to intestinal helminths including *T. spiralis* and *N. brasiliensis* (Woodbury *et al.*, 1984; Dehlawi *et al.*, 1987). The presence of adult *H. polygyrus* in the intestinal lumen is associated with poor mastocytosis, chronic parasite survival, and inhibition of MMC responses stimulated by concomitant *T. spiralis* infections, indicating that adult *H. polygyrus* exerts an immunosuppressive effect on MMC development and proliferation (Dehlawi & Wakelin, 1988; Behnke *et al.*, 1993). Moreover, spontaneous expulsion of *H. polygyrus* in some resistant strains of mice and elimination of *N. brasiliensis* in rats were not accompanied by concurrent mucosal mastocytosis which had peaked after maximal worm loss (Woodbury *et al.*, 1985; Dehlawi *et al.*, 1988; Wahid *et al.*, 1994). However, increased size and accelerated development of MMC responses have been observed during challenge infections with *H. polygyrus* in which there was also a reduction in the number of penetrating larvae and expedited elimination of adult worms (Dehlawi *et al.*, 1987), thus implying that MMC may contribute to acquired resistance by arresting larval development or damaging young adult worms following lumenal emergence. Indeed, we found that a challenge infection with *H. polygyrus* induced prominent and persistent MMC responses that peaked (day 9 pci) prior to marked elimination of adult parasites (beginning on day 14 pci). The concept that secondary MMC responses are a critical mechanism of acquired resistance is supported by our finding that protein deficiency impaired the intestinal proliferation of MMC within 2 weeks pci but did not affect MMC numbers during primary infection in which more worms were recovered than after challenge infection. Furthermore, the onset of depressed mucosal mastocytosis immediately preceded the loss of adult worms in the protein sufficient mice, thereby implying that MMC responses were elicited by larvae resident in the gut mucosa and that local MMC induced early after challenge infection directly participated in parasite expulsion in conjunction with IgE or IgG1.

IgE response is an essential component of acquired immunity to nematode parasites and any increases in this antibody represent an induced response in helminth infections. We found that serum IgE levels were low in uninfected mice and during primary infection but increased substantially after challenge infection. However, this nematode-induced elevation in IgE was absent in the protein deficient mice which also had lower MMC levels in the gut mucosa. The importance of IgE in mediating protective immunity to helminth parasites is inferred from studies showing that the neutralization of IL-4 *in vivo* or inhibition of IL-4 receptor ameliorated serum IgE responses and increased worm numbers (Finkleman *et al.*, 1990; Urban *et al.*, 1991). Moreover, mice with a mutation of the IgE gene have increased burdens of *Schistosoma mansoni* despite prominent elevations of antigen-specific IL-4-secreting cells (King *et al.*, 1997). Binding of IgE to high affinity receptors on mast cells and cross-linking by parasite antigen cause the degranulation of MMC and release of inflammatory mediators. Accordingly, an effective IgE-mediated immune response requires not only sufficient levels of MMC and associated IgE receptors but also abundant IgE antibody at the appropriate sites of infection in order to enhance mast cell FcεRI expression and secretory capacity (Chen *et al.*, 1994; Yamaguchi *et al.*, 1997). In our study, the time courses of serum IgE response, MMC proliferation and parasite expulsion were tightly correlated such that the onset of parasite expulsion in the protein sufficient mice (day 14 pci) occurred immediately following peak serum IgE and intestinal MMC numbers (day 9 pci). Furthermore, the protein deficient mice had higher worm burdens throughout 4 weeks of challenge infection as well as consistently decreased serum IgE titres and lower numbers of MMC in the gut mucosa. Taken together, these results provide additional evidence that protective immunity to nematode parasites requires maximal induction of IgE production and concurrent proliferation of MMC *in situ* or intestinal trafficking of mast cell precursors.

Although serum IgE concentration was found to correlate poorly with intestinal IgE levels (Negrao-Correa *et al.*, 1996) and with IgE occupancy or density of IgE receptors on mast cells (Chen & Enerback, 1994 & 1996), our results on the secretory capacity of MMC likely implicate a functional consequence of lower serum IgE levels detected in the protein deficient mice. Inasmuch as MMC activation and degranulation depend on expression of IgE receptors and acquisition of IgE by the mast cell, the lower MMCP-1 concentrations in protein deficient mice at days 6 and 14 pci may be due to both decreased MMC numbers and reduced levels of IgE in gut-associated lymphoid tissues. Lower serum MMCP-1 levels likely represent the true extent of the immunosuppressive effects of protein deficiency on MMC secretory capacity given that the intestine is the major source of systemically secreted MMCP-1 and that serum levels correlate positively with concentrations in the intestinal lumen of nematode-infected mice (Wastling *et al.*, 1997). There are also preliminary indications that MMCP-1 has a physiological role in parasite expulsion by affecting epithelial permeability and translocation of antibodies from blood to the gut lumen (Wastling *et al.*, 1997). Lower IgE responses, depressed MMC numbers and impaired MMC secretory activity were apparent by day 9 pci in mice fed 3% protein at a time point that preceded the onset of marked parasite expulsion in immunocompetent mice fed 24% protein. Although we did not measure MMCP-1 on day 9 pci, other studies have shown that MMC protease concentration in blood peaks slightly later than maximal intestinal mastocytosis in nematode-parasitized mice and rats (Woodbury *et al.*, 1984; Wastling *et al.*, 1997). Thus, prolonged worm survival in mice fed 3% protein may be attributed in part to the impaired IgE-dependent MMC proliferative and secretory responses observed early after challenge infection with *H. polygyrus*.

We investigated whether these impaired effector responses in protein deficient mice were related to perturbations in Th2 cytokine production. It is currently accepted that IL-4 is the critical cytokine directing Th2 differentiation and response phenotype during nematode infections (Mosmann & Sad, 1996). IL-4 promotes the differentiation of Th cells to the Th2 phenotype (Rincon *et al.*, 1997), the generation of memory Th2 and B cells (Bradley *et al.*, 1995), isotype switching of B cells from IgA^+ and IgM^+ to IgE^+ (Zhang *et*

al., 1991), the proliferation of IgE-committed cells and differentiated MMC (Finkleman *et al.*, 1988; Katona *et al.*, 1991), mast cell FcεRI expression (Toru *et al.*, 1996), binding of plasma IgE to intestinal cells of *T. spiralis*-infected mice (Ranaswany *et al.*, 1994), and the expulsion of established *H. polygyrus* in mice (Urban *et al.*, 1991). Our findings that protein deficiency decreased the capacity of MLN cells of *H. polygyrus*-infected mice to secrete IL-4 and to express IL-4 mRNA, depressed Th2 effector responses, and prolonged parasite survival support previous reports (e.g. Urban *et al.*, 1991 & 1995) that IL-4 is a crucial cytokine required for strong and persistent Th2 effector responses and rapid elimination of nematode parasites. In contrast to IL-4, protein deficiency did not impair IL-5 and IL-10 production even though the 3% protein diet reduced IL-10 mRNA levels. (Yamaguchi *et al.*, 1988; Coffman *et al.*, 1989; Behnke *et al.*, 1993). This indicates that relatively small amounts of IL-4 may be sufficient for production of other Th2 cytokines and that protein deficiency may affect gene expression without impairing translation of mRNA to protein. In fact, IL-5 secretion by MLN was relatively higher in mice fed 3% protein at day 3 pci, but the functional significance of this event is unclear because the up-regulated IL-5 response at this early time point was not associated with concomitant elevation in gut eosinophilia. It is possible that IL-5 and IL-10 are constitutively expressed during nematode infection and their regulation is less responsive to exogenous factors. Other researchers have questioned the functional impact of IL-5 and IL-10 during nematode infection. The expression of IL-10 mRNA does not correlate with the mRNA levels of other Th2 cytokines in *H. polygyrus* infection (Svetic *et al.*, 1993) and IL-12 can enhance the production of IL-10 by human T cells (Windhagen *et al.*, 1996), thus implying that IL-10 may not be a strictly Th2-restricted cytokine. Also, IL-10 gene expression is stimulated by IL-12 treatment in mice during *N. brasiliensis* infection in which there was also prolonged worm survival and reduced IgE, MMC and eosinophil responses (Finkelman *et al.*, 1994). Similarly, Th2-type mechanisms of resistance to nematode parasites may be independent of IL-5 as shown in experiments in which the neutralization of endogenous IL-5 failed to affect parasite loads in *T. spiralis*, *H. polygyrus* or *N. brasiliensis* infections even though anti-IL-5 antibody prevented eosinophilia in blood and lung mucosa (Herndon &

Kayes, 1992; Finkleman *et al.*, 1997). This selective impact of dietary protein deficiency on host immune function has also been reported in studies on cytokine production in murine tuberculosis (Chan *et al.*, 1996) and on neutrophil mobilization during staphylococcal infection (Nwankwo *et al.*, 1985). Taken together, the differential effects on Th2 cytokines suggest protein deficiency impairs specific components of Th2 cytokine response (IL-4 production in intestinal lymphoid tissues) that are important for development of acquired immunity to *H. polygyrus.*

The significant effect of time on Th2 cytokine response profiles suggests that kinetics of cytokine production during challenge infection are important to the regulation of host protection. Cytokines are likely most effective in promoting effector responses during the first week of infection when *H. polygyrus* is in its tissue-dwelling larval stage which is known to elicit the most intense expression of acquired immunity in mice (Wahid & Behnke, 1992). Indeed, we found that Th2 cytokine concentrations in MLN were higher than in spleen and that levels gradually decline after 14 days pci. Consistent with these time-dependent effects of protein deficiency, Chan *et al.*, (1996) detected depressed cytokine mRNA expression in lungs of protein deficient mice only at early times (<14 days) after tuberculosis infection. In our study, the early intense response in local lymphoid tissue reflects the strong antigenic stimuli provided by the invasive and tissue-resident larvae. Once the worm matures and enters the gut lumen, there is much less antigenic stimulation to gut-associated lymphoid cells for cytokine synthesis. Also, the presence of adult worms in the anterior duodenum has been associated with suppression of mucosal mastocytosis (Dehlawi & Wakelin, 1988) and selective down-regulation of IL-9 and IL-10 production (Behnke *et al.*, 1993), leading to the conclusion that adult parasites are capable of compromising host immune responses. Taken together, these results suggest that acquired resistance to *H. polygyrus* requires elevated Th2 cytokine production in intestinal lymphoid tissues within one week pci before adult parasites emerge into the gut lumen. During challenge infection with *H. polygyrus*, protein deficiency modified IL-4 production by MLN only during the first week pci in a selective manner depending on the severity of restriction. The MLN cells of marginal protein mice were able to rebound from initial impaired IL-4

synthesis (day 3 pci) whereas the MLN cells of the protein deficient mice were unable to secrete sufficient quantities of IL-4 at a slightly later time point (day 6 pci). This later time point may have been critical for stimulation of subsequent Th2 effector responses because peak serum IgE and MMC responses occurred on day 9 pci. Moreover, we found that basal IL-4 production by MLN of mice fed 24% protein was high prior to challenge infection and remained elevated during the first week pci but that IL-4 secretion in MLN of mice fed 3% protein was low at baseline and had failed to increase after challenge infection. Therefore, sustained elevations in IL-4 production in the early phase of challenge infection may be equally or more important for priming of effector mechanisms than transient increases at any specific pci time point.

It is important to note that Th2 cytokine production by spleen and MLN was prominent early after primary infection as similarly reported by Svetic *et al.* (1993) and Shi *et al.* (1997). However, this intense primary cytokine response was not associated with subsequent marked increases in IgE, eosinophilia or mucosal mastocytosis which were significantly higher after challenge infection. Secondary IL-4 and IL-10 cytokine responses to *H. polygyrus* were relatively low compared to primary levels and elevated levels were limited to the first week of challenge infection. It is possible that the priming or initiation of Th2 cytokine synthesis may be a more important immunological event than secondary responses for the development of B cell or Th2 memory and phenotypic expression. However, IL-4 produced during challenge but not primary infection with *H. polygyrus* is required to generate and sustain secondary IgE responses which derive mostly from naive or uncommitted B cells rather than from IgE-committed memory B cells produced during primary infection (Finkelman *et al.*, 1990; Katona *et al.*, 1991). Therefore, the impaired production of gut-associated IL-4 in protein deficient mice observed early in challenge infection may be the determining factor responsible for the subsequent impaired IgE, MMC and intestinal eosinophil responses, and in turn for the prolonged worm survival.

The reciprocal amounts of IL-4 and IFN-γ produced during challenge infection may be an additional factor affecting the magnitude of Th2 immunity raised against nematode parasites. IL-4 and IFN-γ are mutually antagonistic for Th cell differentiation and effector activity belonging to reciprocal Th cell phenotypes. IL-4 inhibits cytokine synthesis by Th1 cells and impairs macrophage function (Kopf *et al.*, 1993; Tanaka *et al.*, 1993; Lagoo *et al.*, 1994) whereas IFN-γ suppresses the production of Th2 effectors and cytokine and reverses host resistance to nematode parasites (Maggi *et al.*, 1992; King *et al.*, 1993; Urban *et al.*, 1993). Collectively, these data indicate that a high level of IL-4 and the concomitant low quantity of IFN-γ ultimately influence the appropriateness, speed and magnitude of Th2 immune responses raised against nematode parasites. We found that protein deficiency decreased gut-associated IL-4 response (protein level and mRNA expression) and increased both gut-associated and systemic IFN-γ production (protein level on day 14 pci). Although we did not detect effects of diet on IFN-γ gene expression, it is possible that protein deficiency altered other steps in the IFN-γ synthesis pathway such as mRNA translation or post-translational modification. The up-regulated IFN-γ production during challenge infection coincided with decreasing IL-4 levels in MLN and thus may be a by-product of decreasing antigenic stimulation of Th2-associated responses within gut mucosal tissues. Despite an overall nonsignificant effect of diet on IFN-γ throughout challenge infection, we detected significantly higher IFN-γ production in spleen and MLN of mice fed 3% protein compared to controls at day 14 pci. During the initial 2 weeks pci, a critical time for development of appropriate and sufficient Th2 responses, the increased IFN-γ coupled with decreased IL-4 may have contributed to the lower Th2 effector responses in the protein deficient mice. The mechanisms by which protein deficiency could up-regulate nonprotective IFN-γ and simultaneously down-regulate protective IL-4 are unknown but these results suggest that malnutrition alters the balance of Th2/Th1 cytokine responses that regulate IgE-dependent effector mechanisms during challenge infection with *H. polygyrus*.

The selective impact of protein malnutrition on host immunity was further evidenced in the graded responses to varying levels of dietary protein. We found that 3% protein deficiency significantly decreased body weight gain, relative spleen and MLN weights and BUN concentration but had no effect on plasma albumin concentration despite a feeding period of 10 weeks. The absence of hypoalbuminaemia in our mice fed 7% or 3% protein is consistent with previous experiments in our laboratory using similar diet and infection protocols (Boulay *et al.*, 1998) and with protein restriction in rabbits (Weidel *et al.*, 1994). Although biochemical indicators of protein deficiency typically have included levels of transport proteins, urea nitrogen and hematocrit (Golden, 1982), plasma albumin concentration has been regarded classically as an index of long-term protein status (McFarlane *et al.*, 1969). However, recent studies on protein deficiencies in pigs and rabbits show that plasma albumin concentration does not reflect albumin turnover nor provide information on the mechanisms of how the plasma albumin pool is maintained (Jahoor *et al.*, 1996). Several studies have reported that protein deficiency significantly reduces the fractional rates of albumin synthesis and catabolism (Kirsch *et al.*, 1968; Jahoor *et al.*, 1996), but the rate of albumin turnover was similar between control and protein deficient animals (Weidel *et al.*, 1994). These metabolic adaptations in albumin turnover rate may explain the unchanged plasma albumin concentration during protein deficiency despite obvious impairment of growth and other physiological functions. Immune indices are a sensitive parameter of nutritional status and help define the functional consequences of malnutrition by predicting morbidity and disease outcomes (Chandra, 1991). To this end, our results show that impaired immune function occurs prior to detectable changes in plasma albumin concentration. Also, we can suggest that the smaller body weight in the protein deficient mice may have enabled these mice to maintain plasma albumin homeostasis at the expense of protein synthesis for other functional capacities such as host immunocompetence. Indeed, the suppressed Th2 immunity observed in *H. polygyrus*-infected mice fed 3% protein confirmed that we had created a functional protein deficiency without concurrent reductions in plasma albumin concentration.

Finally, this study is important because it suggests that a certain degree of physiological adaptation occurs with marginal malnutrition but there is a threshold of nutritional deficiency below which immune function is significantly compromised. Mice fed a marginal protein diet (7%) were capable of eliminating most of their parasites by day 28 pci and showed intermediate but adequate levels of IgE, MMC numbers, and eosinophilia. In contrast, a further decrease in dietary protein level caused obvious defects in host protective immunity at both intestinal and peripheral levels which lead to increased parasite survival. The7% protein group adapted to their marginal protein intake by eating more food which resulted in these mice ingesting more energy than the control and deficient groups. This was confirmed by the higher plasma albumin concentration in the 7% mice compared with the 3% group. Increased energy intake may spare endogenous protein for utilization in protein synthesis and allow nitrogen balance to be achieved during limited protein intake (Waterlow, 1986). Indeed, many of the nutritional and immunological outcomes of the 7% mice were not statistically different from those of the 24% mice, suggesting that the higher energy consumption compensated for the marginal protein intake and in turn help protect these 7% mice against reinfection with *H. polygyrus*.

A comparison of immunological responses between the 7% and 3% protein groups may help to clarify the significance of Th2 effector responses in protective immunity against nematode parasites. Previous studies in which a single parameter of Th2 defense mechanisms was inhibited by neutralizing antibodies (e.g. Urban *et al.*, 1991) or deletion of a cytokine-specific gene (e.g. Takamoto *et al.*, 1997) were inconclusive as to whether Th2 effectors such as IgE, MMC and eosinophils were functionally important in host resistance to nematode parasites, but raised the possibility that there are some redundancies in host immune function. Our study examining multiple parameters and tissue compartments of host immunity suggests that Th2 effector and cytokine defenses act collectively and/or synergistically to promote acquired resistance to *H. polygyrus*. The marginally restricted mice were capable of eliciting intermediate levels of Th2 effector responses that were apparently effective for rapid parasite expulsion. In contrast, the 3% mice had lower gut-associated IL-4, higher gut-associated and splenic IFN-γ, and consistently decreased IgE,

MMC and intestinal eosinophil responses during challenge infection which cumulatively may have resulted in their higher parasite burdens.

In summary, our study demonstrated that protein deficiency promotes parasite survival by altering cytokine and effector responses in a selective manner depending on the specific tissue compartment, the level of protein restriction and the time point of infection. We found that protein deficiency was more detrimental to IL-4 production in MLN than in spleen during the early phase of a challenge infection which may have been responsible for the subsequent decreased IgE, MMC and gut eosinophil responses. Based on our results, we suggest that protein deficiency exerts its adverse effects at the following steps along the cascade of immunological responses to a Th2-inducing parasite. Adequate intake of protein is necessary for mRNA expression and protein synthesis of IL-4 in GALT but does not affect IL-5 or IL-10 protein production which appeared to be normal even in mice with limited capacity to produce IL-4. This decreased IL-4 combined with increased IFN-γ led to reduced levels of IgE, MMC and gut eosinophils and in turn to prolonged parasite survival in the protein deficient mice. That intestinal MMC and eosinophil responses were impaired in the 3% mice despite adequate production of IL-5 and IL-10 suggests that dietary protein affects cytokine-independent pathways in the cellular proliferation or trafficking of immune effectors. Finally, the integration of protein deficiency into the *Hp*-mouse system provided evidence that, among the many cytokines and effectors elicited during an *Hp* infection, the set of IL-4-dependent, IgE-mediated MMC and eosinophil responses in the gut mucosa is more critical to host protection than IL-5, IL-10, peripheral eosinophils or IgG1. Broadly, we conclude that dietary protein deficiency predisposes individuals to acquire higher levels of nematode infection by suppressing specific Th2 immunity at the intestinal level. By contributing to the understanding of the immunological mechanisms of nematode parasite survival in protein-depleted populations, our results reveal immunological targets and nutritional strategies that may help improve the natural abilities of the host immune system to protect against gastrointestinal nematode parasites.

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CHAPTER IV
GENERAL DISCUSSION

Protein malnutrition and gastrointestinal nematode infections are chronic diseases that are frequently endemic in populations of the developing world. Protein deficiency is associated with impaired host immunocompetence; however, the specific mechanisms by which protein deficiency may influence the duration and severity of an intestinal nematode infection are complex and poorly understood. Inasmuch as host resistance to intestinal nematode infections depends on the development and maintenance of Th2-type cytokine and effector responses, we investigated whether protein deficiency exacerbates survival of a murine nematode parasite, *Heligmosomoides polygyrus*, by impairing parasite-induced Th2 immunity in systemic and/or intestinal compartments. Previous studies on the interactions of protein deficiency, nematode infection and host immunity showed that increased parasite survival was associated with depressed antibody (IgG, total IgG1) and eosinophil responses. The effects of protein deficiency on secondary effector responses in the GALT (e.g. MMC, intestinal eosinophilia) and on gut-associated or splenic cytokine production have not been characterized. Our study was designed to address these current gaps in knowledge as well as to assess the temporal and compartmentalization aspects of protein malnutrition and host immunity to nematode parasites. The major finding of this study was that protein deficiency at the 3% level promoted parasite survival in previously immunized mice in association with altered cytokine responses to *H. polygyrus* (i.e. up-regulated Th1 and down-regulated Th2) and impaired proliferation and activation of Th2 effectors within 2 weeks pci. Specifically, protein deficiency decreased gut-associated IL-4 production, increased gut-associated and systemic IFN-γ synthesis, and inhibited increases in intestinal and serum Th2 effector responses that were observed in well-nourished hosts during challenge infection. Interestingly, protein deficiency appeared to exert differential site- and type-specific effects on cytokine and effector responses to *H. polygyrus* challenge infection. For example, protein deficiency inhibited the parasite-elicited responses of intestinal eosinophils, IgE, MMC and IL-4 but did not significantly depress peripheral eosinophilia, parasite-specific IgG1 synthesis, or production of other

Th2 cytokines (IL-5 and IL-10). Also, the intestinal compartment (gut eosinophils, IL-4 production in MLN) was more adversely affected by protein restriction than systemic responses in peripheral circulation or spleen. The responses that were profoundly impaired during protein malnutrition (IL-4/IFN-γ balance, elevated IgE titres, mucosal mastocytosis) are those known to be the most important markers associated with host protective immunity to *H. polygyrus*. These impaired responses observed in the protein deficient mice were detected by 3 days pci (e.g. IgE) until 2 weeks pci (e.g. up-regulated IFN-γ), suggesting that functional Th2 immunity is most effective at terminating the infection when activated at the early stage of challenge infection before the parasite matures and migrates to the intestinal lumen. Once a worm reaches the gut lumen it is less in contact with immune effectors in the gut mucosa and thus is capable of avoiding the potentially damaging mediators of host immunity. Also, the level of protein restriction influenced the magnitude of host protection and parasite survival such that mice fed a marginal protein diet were able to mobilize adaptive mechanisms (e.g. increased total food intake) which may have enabled them to mount an effective immune response and in turn eliminate a challenge infection. However, a further decrease in dietary protein level from 7% to 3% caused profound defects in secondary Th2 immune responses as well as significantly prolonged parasite survival.

This study is the first comprehensive investigation of the interactions between protein deficiency, Th2 immunity and nematode parasitic infection, and the results reveal several points for further investigation. Cytokine production in MLN likely reflects the activities of lymphocytes in the Peyer's patches and cryptopatches of the gut mucosa that are in more direct contact with parasite antigens. Considering that MLN receives immunological components from both intestinal tissues and the peripheral bloodstream, a definitive study of malnutrition-associated changes in cytokine production would require measurement of cytokine production by lymphocytes in Peyer's patches or other epithelial lymphoid tissues. Also, it would be important to determine the kinetics of cytokine production during primary infection in order to ascertain whether primary cytokine responses direct the magnitude and direction of secondary cytokine responses and resultant effector mechanisms. Moreover, the potential for immunomodulation by protein

malnutrition early after challenge infection (i.e. 2 weeks pci) suggests that nutritional interventions (e.g. refeeding) or immunological therapy (e.g. IL-4 treatment) may be most successful when implemented before or immediately following reinfection with nematode parasites. Lastly, the differential outcomes of the marginal protein group (7%) and the protein deficient group (3%) indicate that total energy or caloric intake may have an important impact on host immunocompetence irrespective of, or in conjunction with, the level of dietary protein. The greater food intake by the marginal protein group which had successfully terminated a challenge infection suggests that one reasonably cost-effective and feasible strategy to improve host resistance to nematode parasites is to increase the amount of energy consumption without a concomitant supplementation of dietary protein.

Broadly, our study supports the premise that protein deficiency exacerbates the survival of a gastrointestinal nematode parasite by decreasing gut-associated IL-4 (Th2) and increasing systemic and gut-associated IFN-γ (Th1) early in the challenge infection, leading to reduced gut and systemic Th2 effector responses. These data contribute to our understanding of the immunological mechanisms of parasite survival in protein-depleted populations. Also, the results reveal immunological targets and tissue compartments for therapeutic modulation in order to improve the natural abilities of the host immune system to respond to intestinal nematode parasites. Clearly, a consideration of the nutritional status in the population is essential to the success of vaccination programs aimed at enabling rapid and strong acquired immunity to secondary parasitic infections.

APPENDICES

APPENDIX A: RESULTS FROM TWO-WAY ANOVA ON EFFECTS OF DIET, TIME , & DIET BY TIME INTERACTION.

APPENDIX B: RESULTS FROM ONE-WAY ANOVA ON MAIN EFFECT OF DIET.

APPENDIX C: RESULTS FROM ONE-WAY ANOVA ON MAIN EFFECT OF TIME.

APPENDIX A: Results from two-way ANOVA analyses of diet, time and diet*time interaction main effects on parameters measured postprimary infection (PPI) or postchallenge infection (PCI). Numbers in parentheses are degrees of freedom.

Variable	Diet	Time	Diet x Time
Worms - PPI	F (2,92) = 13.35 P=0.0001	F (1, 92) = 5.29 P=0.0237	F (2, 92) = 4.22 P=0.0177
Worms - PCI	F (2,153) = 163.37 P=0.0001	F (3, 153) = 47.63 P=0.0001	F (6,153) = 9.15 P=0.0001
Blood Eosinophils - PPI	F=8.71 P=0.0011	F=47.99 P=0.0001	F=1.75 P=0.0623
Blood Eosinophils - PCI	F=0.18 P=0.6858	F=6.87 P=0.0001	F=1.07 P=0.3963
Serum IgE - PPI	F (2, 90) = 2.43 P=0.0940	F (1, 90) = 254.47 P=0.0001	F (2, 90) = 1.28 P=0.2843
Serum IgE - PCI	F (2, 213) = 44.3 P=0.0001	F (5, 213) = 17.67 P=0.0001	F (10, 213) =1.27 P=0.2515
Serum IgG1 - PPI	F (2, 81) =0.74 P=0.4817	F (1, 81) = 583.18 P=0.0001	F (2, 91) =0.81 P=0.4490
Serum IgG1 - PCI	F (2, 214) = 0.31 P=0.7303	F (5, 214) = 150.62 P=0.0001	F (10, 214) = 0.52 P=0.8751
MMC - PPI	F (2, 27) =0.00 P=0.9998	F (1, 27) =70.19 P=0.0001	F (2, 27) =2.18 P=0.1330
MMC - PCI	F (2, 59) = 21.69 P=0.0001	F (5, 59) = 4.09 P=0.0029	F (10, 59) = 1.50 P=0.1614
Gut Eosinophils - PPI	F (2, 24) = 1.06 P=0.3615	F (1, 24) = 7.02 P=0.0140	F (2, 24) = 1.23 P=0.3089
Gut Eosinophils - PCI	F (2, 58) = 29.61 P=0.0001	F (5, 58) = 3.67 P=0.0059	F (10, 58) = 0.59 P=0.8143

APPENDIX A continued.

Variable	Diet	Time	Diet x Time
MMCP1 - PPI	F (2, 50) = 0.11 P=0.8947	F (1, 50) = 3.34 P=0.0734	F (2, 50) = 3.84 P=0.0281
MMCP1 - PCI	F (2, 185) = 2.30 P=0.1030	F (3, 185) = 44.59 P=0.0001	F (6, 185) = 0.79 P=0.5794
IL-4 Spleen - PCI	F (2, 102) = 0.00 P=0.9985	F (3, 102) = 6.55 P=0.0004	F (6, 102) = 1.75 P=0.1160
IL-5 Spleen - PCI	F (2, 109) = 2.41 P=0.0948	F (3, 109) = 0.40 P=0.7563	F (6, 109) = 0.30 P=0.9338
IL-10 Spleen - PCI	F (2, 92) = 2.40 P=0.0968	F (3, 92) =13.41 P=0.0001	F (6, 92) = 1.41 P=0.2185
IFN-γ Spleen - PCI	F (2, 102) = 1.39 P=0.2531	F (3, 102) = 35.06 P=0.0001	F (6, 102) = 4.21 P=0.0008
IL-4 MLN - PCI	F (2, 93) = 6.08 P=0.0033	F (3, 93) = 14.06 P=0.0001	F (6, 93) = 2.02 P=0.0702
IL-5 MLN - PCI	F (2, 101) = 0.02 P=0.9839	F (3, 101) = 16.74 P=0.0001	F (6, 101) = 2.06 P=0.0640
IL-10 MLN - PCI	F (2, 87) = 1.70 P=0.1879	F (3, 87) = 9.97 P=0.0001	F (6, 87) = 1.55 P=0.1721
IFN-γ MLN - PCI	F (2, 92) = 0.35 P=0.7031	F (3, 92) = 30.89 P=0.0001	F (6, 92) = 1.24 P=0.2950

APPENDIX B. Results from one-way ANOVA analyses on the main effect of diet on parameters measured at each postprimary (PPI) and postchallenge (PCI) time point and in naive mice.

Variable	Diet	Posthoc Comparisons
Worm Burdens - P6	F (2, 48) = 7.11 P=0.0020	3% > (24% = 7%)
Worm Burdens - P28	F (2, 44) = 8.33 P=0.0009	3% > (24% = 7%)
Worm Burdens - C6	F (2, 48) = 15.75 P=0.0001	3% > (24% = 7%)
Worm Burdens - C14	F (2, 46) = 36.05 P=0.0001	3% > 7% > 24%
Worm Burdens - C21	F (2, 12) = 100.98 P=0.0001	3% > (24% = 7%)
Worm Burdens - C28	F (2, 47) = 195.14 P=0.0001	3% > (24% = 7%)
Serum IgE - Naive	F (2, 19) = 0.89 P=0.4259	
Serum IgE - P6	F (2, 46) = 0.26 P=0.7693	
Serum IgE - P28	F (2, 44) = 3.67 P=0.0336	3% < 24%; 7% = 3% & 24%
Serum IgE - C3	F(2, 48) = 10.40 P=0.0002	3% < (24% = 7%)
Serum IgE - C6	F (2, 47) = 18.96 P=0.0001	3% < (24% = 7%)
Serum IgE - C9	F (2, 12) = 8.45 P=0.0051	3% < 24%; 7% = 3% & 24%
Serum IgE - C14	F(2, 47) = 15.06 P=0.0001	3% < (24% = 7%)
Serum IgE - C21	F (2, 12) = 5.06 P=0.0255	3% < 24%; 7% = 3% & 24%
Serum IgE - C28	F (2, 47) = 18.12 P=0.0001	(3% = 7%) < 24%
Parasite-specific IgG1 Naive	F (2, 4) = 1.87 P=0.2671	
Parasite-specific IgG1 - P6	F (2, 37) = 0.73 P=0.4873	
Parasite-specific IgG1 - P28	F (2, 44) = 0.03 P=0.9708	
Parasite-specific IgG1 - C3	F (2, 48) = 0.78 P=0.4619	
Parasite-specific IgG1 - C6	F (2, 48) = 0.52 P=0.6006	
Parasite-specific IgG1 - C9	F (2, 12) = 2.45 P=0.1280	

APPENDIX B continued.

Variable	Diet	Posthoc Comparisons
Parasite-specific IgG1 - C14	F (2, 47) = 0.84 P=0.4399	
Parasite-specific IgG1 - C21	F (2, 12) = 0.33 P=0.7233	
Parasite-specific IgG1 - C28	F (2, 47) = 1.47 P=0.2400	
MMC - Naive	F (2, 15) = 1.32 P=0.2973	
MMC - P6	F (2, 9) = 0.80 P=0.4774	
MMC - P28	F (2, 18) = 1.54 P=0.2406	
MMC - C3	F (2, 7) = 1.70 P=0.2496	
MMC - C6	F (2, 8) = 2.54 P=0.1370	
MMC - C9	F (2, 9) = 6.88 P=0.0154	(3% = 7%) < 24%
MMC - C14	F (2, 9) = 5.92 P=0.0228	3% < 7%; 24% = 3% & 7%
MMC - C21	F (2, 8) = 14.81 P=0.0020	3% < (24% = 7%)
MMC - C28	F (2, 18) = 4.95 P=0.0193	3% < 24%; 7% = 3% & 24%
Gut Eosinophils - Naive	F (2, 15) = 0.98 P=0.3984	
Gut Eosinophils - P6	F (2, 9) = 0.01 P=0.9915	
Gut Eosinophils - P28	F (2, 15) = 2.18 P=0.1477	
Gut Eosinophils - C3	F (2, 6) = 3.92 P=0.0814	
Gut Eosinophils - C6	F (2, 8) = 10.40 P=0.0059	3% < 24%; 7% = 3% & 24%
Gut Eosinophils - C9	F (2, 11) = 1.54 P=0.2565	
Gut Eosinophils - C14	F (2, 10) = 19.96 P=0.0003	3% < 7% < 24%
Gut Eosinophils - C21	F (2, 7) = 4.83 P=0.0480	3% < 24%; 7% = 3% & 24%
Gut Eosinophils - C28	F (2, 16) = 8.84 P=0.0026	3% < (24% = 7%)

APPENDIX B continued.

Variable	Diet	Posthoc comparisons
MMCP-1 - Naive	F (2, 6) = 1.64 P=0.2705	
MMCP-1 - P6	F (2, 30) = 1.94 P=0.1616	
MMCP-1 - P28	F (2, 20) = 1.85 P=0.1835	
MMCP-1 - C3	F (2, 45) = 0.75 P=0.4769	
MMCP-1 - C6	F (2, 47) = 4.37 P=0.0182	3% < 24%; 7% = 3% & 24%
MMCP-1 - C14	F (2, 47) = 5.41 P=0.0077	3% < (24% = 7%)
MMCP-1 - C28	F (2, 46) = 0.47 P=0.6287	
IL-4 Spleen - P6	F (2, 33) = 3.87 P=0.0309	3% < 24%; 7% = 3% & 24%
IL-4 Spleen - C3	F (2, 28) = 1.14 P=0.3336	
IL-4 Spleen - C6	F (2, 29) = 1.91 P=0.1665	
IL-4 Spleen - C14	F (2, 25) = 2.39 P=0.1126	
IL-4 Spleen - C28	F (2, 20) = 0.18 P=0.8334	
IL-5 Spleen - P6	F (2, 32) = 1.23 P=0.3048	
IL-5 Spleen - C3	F (2, 30) = 1.78 P=0.1852	
IL-5 Spleen - C6	F (2, 30) = 0.39 P=0.6820	
IL-5 Spleen - C14	F (2, 27) = 1.59 P=0.2229	
IL-5 Spleen - C28	F (2, 22) = 0.30 P=0.7456	
IL-10 Spleen - P6	F (2, 32) = 2.62 P=0.0880	
IL-10 Spleen - C3	F (2, 25) = 0.12 P=0.8856	
IL-10 Spleen - C6	F (2, 30) = 3.14 P=0.0576	
IL-10 Spleen - C14	F (2, 28) = 0.76 P=0.4765	
IL-10 Spleen - C28	F (2, 9) = 1.63 P=0.2484	

APPENDIX B continued.

Variable	Diet	Posthoc comparisons
IFN-γ Spleen - P6	F (2, 32) = 1.29 P=0.2897	
IFN-γ Spleen - C3	F (2, 25) = 1.15 P=0.3319	
IFN-γ Spleen - C6	F (2, 28) = 0.57 P=0.5728	
IFN-γ Spleen - C14	F (2, 25) = 11.19 P=0.0003	(24% = 7%) < 3%
IFN-γ Spleen - C28	F (2, 24) = 3.10 P=0.0633	
IL-4 MLN - P6	F (2, 14) = 0.09 P=0.9114	
IL-4 MLN - C3	F (2, 23) = 4.69 P=0.0195	7% < 24%; 3% = 24% & 7%
IL-4 MLN - C6	F (2, 26) = 5.21 P=0.0125	3% < 24%; 7% = 3% & 24%
IL-4 MLN - C14	F (2, 26) = 2.12 P=0.1402	
IL-4 MLN - C28	F (2, 18) = 0.38 P=0.6895	
IL-5 MLN - P6	F (2, 14) = 1.57 P=0.2427	
IL-5 MLN - C3	F (2, 24) = 4.44 P=0.0229	3% < 24%; 7% = 3% & 24%
IL-5 MLN - C6	F (2, 26) = 0.48 P=0.6253	
IL-5 MLN - C14	F (2, 29) = 0.72 P=0.4933	
IL-5 MLN - C28	F (2, 22) = 2.11 P=0.1452	
IL-10 MLN - P6	F (2, 12) = 2.44 P=0.1289	
IL-10 MLN - C3	F (2, 22) = 0.70 P=0.5075	
IL-10 MLN - C6	F (2, 26) = 1.07 P=0.3564	
IL-10 MLN - C14	F (2, 29) = 1.85 P=0.1756	
IL-10 MLN - C28	F (2, 10) = 3.22 P=0.0835	

APPENDIX B continued.

Variable	Diet	Posthoc comparisons
IFN-γ MLN - P6	F (2, 11) = 0.67 P=0.5330	
IFN-γ MLN - C3	F (2, 21) = 1.48 P=0.2496	
IFN-γ MLN - C6	F (2, 22) = 0.29 P=0.7506	
IFN-γ MLN - C14	F (2, 29) = 4.31 P=0.0230	24% < 3%; 7% = 3% & 24%
IFN-γ MLN - C28	F (2, 20) = 0.06 P=0.9408	
Body Weight Gain - C28	F (2, 47) = 85.35 P=0.0001	
Total Food Intake - C28	F (2, 47) = 16.63 P=0.0001	
Relative Food Intake - C28	F (2, 47) = 77.03 P=0.0001	
Relative Spleen Weight - C28	F (2, 39) = 7.27 P=0.0018	
Relative MLN Weight - C28	F (2, 32) = 8.57 P=0.0007	
Relative Thymus Weight C28	F (2, 32) = 0.21 P=0.8125	
Plasma Albumin Concentration - C28	F (2, 37) = 4.16 P=0.2340	
Blood Urea Nitrogen - C28	F (2, 38) = 158.20 P=0.0001	

APPENDIX C. Results from one-way ANOVA analyses of time on parameters measured within each dietary group (24%, 7%, 3%) postprimary infection (PPI) or postchallenge infection (PCI).

Variable	Time	
Worm Burdens - 24% PPI	F (1, 28) = 3.93	P=0.0573
Worm Burdens - 7% PPI	F (1, 31) = 5.25	P=0.0289
Worm Burdens - 3% PPI	F (1, 33) = 2.37	P=0.1333
Worm Burdens - 24% PCI	F (3, 49) = 22.29	P=0.0001
Worm Burdens - 7% PCI	F (3, 49) = 44.43	P=0.0001
Worm Burdens - 3% PCI	F (3, 55) = 1.31	P=0.2815
Serum IgE - 24% PPI	F (1, 30) = 95.91	P=0.0001
Serum IgE - 7% PPI	F (1, 29) = 80.34	P=0.0001
Serum IgE - 3% PPI	F (1, 31) = 78.53	P=0.0001
Serum IgE - 24% PCI	F (5, 69) = 7.28	P=0.0001
Serum IgE - 7% PCI	F (5, 69) = 9.46	P=0.0001
Serum IgE - 3% PCI	F (5, 75) = 4.43	P=0.0014
Parasite-specific IgG1-24% PPI	F (1, 27) = 163.41	P=0.0001
Parasite-specific IgG1 - 7% PPI	F (1, 26) = 199.08	P=0.0001
Parasite-specific IgG1 - 3% PPI	F (1, 28) = 227.11	P=0.0001
Parasite-specific IgG1-24% PCI	F (5, 69) = 43.98	P=0.0001
Parasite-specific IgG1 - 7% PCI	F (5, 69) = 54.22	P=0.0001
Parasite-specific IgG1 - 3% PCI	F (5, 76) = 53.09	P=0.0001

APPENDIX C continued.

Variable	Time	
MMC - 24% PPI	F (1, 9) = 5.09	P=0.0504
MMC - 7% PPI	F (1, 10) = 1.59	P=0.2392
MMC - 3% PPI	F (1, 9) = 0.51	P=0.4935
MMC - 24% PCI	F (5, 21) = 2.41	P=0.0711
MMC - 7% PCI	F (5, 18) = 0.54	P=0.7459
MMC - 3% PCI	F (5, 20) = 6.02	P=0.0015
Gut Eosinophils - 24% PPI	F (1, 7) = 0.00	P=0.9645
Gut Eosinophils - 7% PPI	F (1, 8) = 0.07	P=0.7942
Gut Eosinophils - 3% PPI	F (1, 8) = 0.35	P=0.5686
Gut Eosinophils - 24% PCI	F (5, 21) = 3.45	P=0.1960
Gut Eosinophils - 7% PCI	F (5, 18) = 1.40	P=0.2708
Gut Eosinophils - 3% PCI	F (5, 19) = 0.54	P=0.7471
MMCP1 - 24% PPI	F (1, 19) = 13.47	P=0.0016
MMCP1 - 7% PPI	F (1, 18) = 0.14	P=0.7136
MMCP1 - 3% PPI	F (1, 13) = 0.24	P=0.6307
MMCP1 - 24% PCI	F (3, 61) = 18.19	P=0.0001
MMCP1 - 7% PCI	F (3, 58) = 19.85	P=0.0001
MMCP1 - 3% PCI	F (3, 66) = 10.23	P=0.0001

APPENDIX C continued.

Variable	Time	
IL-4 Spleen - 24% PCI	F (3, 35) = 6.85	P=0.0009
IL-4 Spleen - 7% PCI	F (3, 33) = 2.33	P=0.0922
IL-4 Spleen - 3% PCI	F (3, 34) = 1.35	P=0.2747
IL-5 Spleen - 24% PCI	F (3, 37) = 0.18	P=0.9073
IL-5 Spleen - 7% PCI	F (3, 35) = 0.67	P=0.5761
IL-5 Spleen - 3% PCI	F (3, 37) = 0.15	P=0.9308
IL-10 Spleen - 24% PCI	F (3, 32) = 2.09	P=0.1205
IL-10 Spleen - 7% PCI	F (3, 29) = 7.30	P=0.0009
IL-10 Spleen - 3% PCI	F (3, 31) = 4.56	P=0.0093
IFN-γ Spleen - 24% PCI	F (3, 35) = 22.97	P=0.0001
IFN-γ Spleen - 7% PCI	F (3, 37) = 31.52	P=0.0001
IFN-γ Spleen - 3% PCI	F (3, 30) = 2.62	P=0.0690
IL-4 MLN - 24% PCI	F (3, 33) = 19.32	P=0.0001
IL-4 MLN - 7% PCI	F (3, 35) = 4.56	P=0.0085
IL-4 MLN - 3% PCI	F (3, 25) = 5.58	P=0.0045
IL-5 MLN - 24% PCI	F (3, 34) = 2.11	P=0.1166
IL-5 MLN - 7% PCI	F (3, 36) = 6.03	P=0.0019
IL-5 MLN - 3% PCI	F (3, 31) = 12.48	P=0.0001
IL-10 MLN - 24% PCI	F (3, 31) = 5.50	P=0.0038
IL-10 MLN - 7% PCI	F (3, 29) = 1.76	P=0.1760
IL-10 MLN - 3% PCI	F (3, 27) = 5.25	P=0.0055
IFN-γ MLN - 24% PCI	F (3, 31) = 10.18	P=0.0001
IFN-γ MLN - 7% PCI	F (3, 32) = 8.15	P=0.0004
IFN-γ MLN - 3% PCI	F (3, 29) = 14.20	P=0.0081