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THE IMMUNOMODULATION OF INTESTINAL SMOOTH MUSCLE FUNCTION

By

BRUCE A. VALLANCE, (Hons) B.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Doctor of Philosophy in Medical Science

McMaster University

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THE IMMUNOMODULATION OF INTESTINAL SMOOTH MUSCLE FUNCTION

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This thesis is dedicated to my family and friends and

to the memory of James Ronald Vallance

ABSTRACT

In response to an enteric infection, the host mobilizes inflammatory and immune cells to combat the invading pathogen. Studies suggest that physiologic tissues such as smooth muscle are also recruited to aid in host defense, and that this recruitment depends on the hosts immune response. The purpose of this thesis was to use the intestinal phase of a primary *Trichinella spiralis* infection in mice to study the accompanying changes in smooth muscle contraction, including their role in host defense and their immunological basis. Naive mice were infected with the nematode parasite *T. spiralis*. Infection caused a significant increase in intestinal longitudinal muscle contraction in response to carbachol *in vitro*. Testing several mouse strains, we found that the strains that developed the greatest increases in muscle contraction also expelled the infection the most rapidly. Two phases of increased contraction were identified, the early phase when muscle contraction first increases, and the persistent phase, when the increased muscle tension was sustained until at least day 21 post-infection. We also found that infected mice lacking T cells, and specifically CD4 +ve T cells, exhibited an attenuated increase in both phases of muscle contraction. We also tested the role of the Th2 cytokines, interleukins 4 and 5, which are produced by CD4 +ve T cells during a nematode infection. We found that during infection, IL-5 deficient mice developed a normal early phase of increased muscle contraction, but were significantly impaired in the persistent muscle response. We also examined the role of IL-4, through gene transfer. Following surgery, we infected the intestinal surface with either control adenoviral vectors, or a virus encoding the cytokine IL-4. IL-4 over-expression significantly increased intestinal muscle contraction, while the control virus had no effect. In summary, my studies show that intestinal longitudinal muscle function is subject to immunomodulation, specifically by the actions of CD4 +ve T cells, and through the cytokine mediators IL-4 and 5.

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FORMAT & ORGANIZATION OF THESIS

This thesis is organized in the "Open-faced" sandwich format, approved by McMaster University. This thesis is comprised of 7 chapters. The first chapter is a general introduction and is followed by chapters 2 and 3, which are manuscripts already published in a peer reviewed scientific journal. Chapter 4,5 and 6 are manuscripts submitted for publication in peer reviewed journals. Chapter 7 is a general discussion highlighting the overall significant contributions and ramifications for the work presented in the five manuscripts. An appendix has been included, appendix A provides key preliminary data and analysis not presented in the five manuscripts.

Contributions to Multi-Authored Papers

Chapter 2: Increased intestinal muscle contractility and worm expulsion in nematode-infected mice. 1997.

Authors: B.A. Vallance, P.A. Blennerhassett, and S.M. Collins.

Journal: American Journal of Physiology, 272: G321-327.

Comment: The experiments were conducted and data collected by the author B.A. Vallance. P.A. Blennerhassett was instrumental in the initial experimental design. Supervision for this project was provided by Dr. S.M. Collins.

Chapter 3. T lymphocyte dependent and independent intestinal smooth muscle dysfunction in the *T. spiralis* infected mouse. 1998.

Authors: B.A. Vallance, K. Croitoru, and S.M. Collins.

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Chapter 5. Interleukin-4 gene transfer to the murine intestine leads to eosinophilic inflammation and intestinal muscle hyper-responsiveness.

Authors: B.A. Vallance, C.M. Hogaboam, C.L. Addison, F.L.Graham, J. Gauldie, and S.M. Collins.

Journal: Gastroenterology, submitted.

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Chapter 6. A role for interleukin-5 and eosinophils in the intestinal muscle dysfunction following *T. spiralis* infection.

Authors: B.A. Vallance, P.A. Blennerhassett, Y. Deng, K.I. Matthaei, I.G. Young, and S.M. Collins.

Journal: American Journal of Physiology, submitted.

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Chapter 1

INTRODUCTION

INTRODUCTION

1.1 THE GASTROINTESTINAL TRACT

1.1.1 Structure

All regions of the gastrointestinal tract are composed of a similar basic structure. The mucosa is the innermost layer and lining the entire mucosa is a single layer of contiguous epithelial cells. It is these intestinal epithelial cells which come in contact with lumenal contents and are responsible for nutrient and water absorption and fluid secretion. As well, the epithelial cells play a key role in host defense, acting as a barrier to lumenal organisms and antigens, preventing their entry into the mucosa. Just beneath the epithelial cells, the mucosa contains many different cell types including parenchymal cells, immunocytes, and endothelial cells of the vascular/lymphatic networks. The major parenchymal cells are fibroblasts and smooth muscle cells which form the scaffolding supporting the villi and crypts. Also present in the mucosa are a number of immunocytes, including lymphocytes, macrophages, neutrophils, eosinophils and mast cells, which are involved in protecting the organism from any invading viruses, bacteria and parasites which might penetrate through the epithelial layer.

Beneath the mucosa lies the submucosa which is made up of connective tissue including elastic fibres and which contains the submucosal nerve plexus and the larger blood vessels and lymphatics of the intestine. Nerves of the submucosal plexus regulate the absorptive and secretory functions of the mucosa. The outermost neuromuscular layers control the motility of the gut, with the muscularis layer comprised of two layers of smooth muscle. The outer layer is composed of longitudinally oriented smooth muscle, the inner, of circular smooth muscle, with the axes of the two layers laying perpendicular to each other. The myenteric nerve plexus is located between the two layers and coordinates their movement. Fibres from the myenteric plexus project to the central nervous system, via autonomic nerves and to the submucosal nerve

plexus. Surrounding the intestine, as it does all internal organs, is a thin sheath of mesothelial cells, termed the serosa, which protects the intestine from the contents of the peritoneal cavity.

1.1.2 Function - Gastrointestinal Motility

The main functions of the gastrointestinal tract are the digestion and absorption of nutrients, and the elimination of waste products from the body. To accomplish this, motility facilitates the orderly passage of food into the gut via the oropharynx and esophagus. Following this process, food is stored and mixed in the stomach before transfer to the small intestine where contractions of the small bowel mix the food products with digestive enzymes. As well, motility promotes contact of the resulting chyme with absorptive cells over a sufficient length of bowel to optimize nutrient absorption. Finally, contractions propel the remnants of digestion into the colon, with slower passage through the large intestine to permit the formation and ultimate expulsion of solid stool. The motility patterns in the gastrointestinal tract that control these events are the result of a contiguous modulation of smooth muscle contraction; tightly controlled by complex and integrated systems involving myogenic mechanisms as well as highly organized neural circuits and hormonal influences. There is recent evidence that interstitial cells of Cajal (ICC) also play a role in the regulation of intestinal propulsive activity (for review - see Huizinga, 1998).

1.2 THE IMMUNOMODULATION OF GUT NEUROMUSCULAR FUNCTION

1.2.1 Immunophysiology

While not often immediately recognized as a participant in regulating physiology, immune cells are also part of the complex and highly integrated system that controls intestinal motility and other gut functions. As the largest immune organ in the body, with immune cells in close proximity to physiological tissues throughout its length, the gastrointestinal tract provides ample opportunity for interactions between immune and non-immune cells. It is now well established, particularly for the intestinal epithelium, that immune cells and their mediators can regulate epithelial function under both inflamed and non-inflamed conditions. This has been studied both in *in vivo* models and with the advent of epithelial cell lines, *in*

vitro. Most inflammatory mediators were found to reduce the barrier function of the epithelium, while increasing ion transport and secretion. These studies, (reviewed by Perdue & McKay, 1994) have provided clues to understanding epithelial function during enteric infections, inflammatory diseases as well as under homeostatic conditions, and has provided a basis for studying immunophysiological interactions in other areas, such as motility.

1.2.2 Immunophysiology of the Enteric Neuromuscular Layers

While the evidence for immune regulation of physiological function is strongest for epithelial cells, there is now evidence that not only nerves, muscle and ICC play roles in regulating intestinal motility, but cells of the immune system may also act in the development and maintenance of gut neuromuscular structure and function. The strongest evidence for this involvement comes from studies of gnotobiotic animals. Normally the gastrointestinal tract is maintained in a state of physiological inflammation, due to interactions between local immune cells and enteric flora. In germ free animals, this stimulation of the mucosal immune system is missing. As a result, the gastrointestinal tract develops abnormally. The caecum becomes distended and *the muscularis externa* lining the entire GI tract is very thin and unresponsive to mechanical stimuli (Iwai *et al.*, 1973, Rolfe, 1984). This results in reduced propulsive activity and slower intestinal transit in these animals (Abrams & Bishop, 1967, Riottot *et al*;, 1984). Thus the immune system and its stimulation by luminal factors are involved in the development and maintainance of normal enteric motility. It follows then, that when activated by an enteric infection or other noxious stimulus, the resulting intestinal inflammation and immune response would result in pathophysiological changes in the gut motor apparatus, altering normal motility patterns. Such interactions have been studied in several animal models of gut inflammation.

1.2.3 Altered Motility in Animal Models of Gut Inflammation

There are now a number of animal models available to study either small or large bowel inflammation. Of the experimental models of colonic inflammation which have been developed, those

using chemicals or haptens to induce inflammation are the most widely used. Because these models are easily implemented, and because they are accompanied by changes in motor activity that correlate well with the hypomotility and intestinal muscle hypocontractility observed in patients with ulcerative colitis, they have been extensively studied for changes in colonic neuromuscular function, and the data examining altered neuromuscular function in the colon has recently been reviewed (see Collins, 1996).

Among the different models of small intestinal inflammation, those of nematode infection (*Trichinella spiralis* and *Nippostrongylus brasiliensis*) have been used most extensively to study the impact of inflammation on gut function. In part this is due to the simplicity of delivery, since these parasites naturally migrate to and infect the small bowel. As well, these models are very reproducible, consistently producing an inflammatory response concurrent with changes in intestinal motility. Also, because of the immune basis of the host defense generated against these parasites, they provide a useful tool for the study of immunophysiological mechanisms in the gut.

1.2.4 Nematode Infection Models.

In 1976, pioneering studies by Castro *et al.* showed an accelerated rate of transit of ^{51}Cr through the inflamed proximal small intestine of rats infected with *T. spiralis* (Castro *et al.*, 1976) and a similar effect was subsequently documented by Farmer following *N. brasiliensis* infection in rats (Farmer, 1981). Farmer also demonstrated a substantial increase in intestinal muscle contraction in response to acetylcholine (Farmer *et al.*, 1983). Subsequent studies performed in *T. spiralis* - infected mice revealed that the increased rate of small bowel transit could be abolished by corticosteroid treatment (Suhkdeo & Croll, 1981), thus implicating the inflammatory process rather than the parasite as the cause of the altered muscle function. These studies however, did not provide an indication of which system controlling intestinal motility (ie. extrinsic or intrinsic nerves or the muscle itself) was involved. By using *in vitro* preparations of isolated intestinal segments to study propulsive activity, Alizadeh *et al.* also found that *T. spiralis* infection was accompanied by increased intestinal propulsion (Alizadeh *et al.*, 1987). Because they were using extrinsically denervated segments, their findings suggested that the altered function

resided in the intestinal smooth muscle or the intrinsic nerves of the gut wall. Subsequent *in vitro* work in nematode-parasitized rodents has provided extensive evidence of functional changes in the contractile properties of intestinal muscle (Vermillion & Collins, 1988, Vallance *et al.*, 1997a).

Specifically Vermillion showed that *T. spiralis* infection of rats is associated with a 3-fold increase in isometric muscle tension generation in the inflamed jejunum in response to different agonists, including carbachol and 5-hydroxytryptamine. The tension development increased until day 6 post-infection, where it peaked, and then returned to normal by day 23 (Vermillion & Collins, 1988). It is interesting to note that the changes in force generation were accompanied by increased thickening of the *muscularis externa* due to a combination of both hypertrophy and hyperplasia of the smooth muscle (Blennerhassett *et al.*, 1992). Since tension generation by muscle was normalized to the cross sectional area of the muscle layer, the actual increase in force generation by intestinal muscle after infection was substantially greater than 3 fold considering the significant increase in muscle mass. Even if extrapolation of *in vitro* studies to the *in vivo* situation must be considered cautiously, these findings provide a reasonable explanation for the increased small bowel propulsion described in *T. spiralis* infected animals.

Additionally, Marzio *et al.* showed in this model that longitudinal muscle contractility in the ileum was decreased which, in combination with the hyper-contractility of the jejunum, would create an aboral gradient in the force of contraction further promoting small bowel propulsion (Marzio *et al.*, 1990). One interpretation for the described motility changes is that they represent a protective mechanism mounted by the host to aid in evicting the parasite from the gut. In addition to the region specificity of inflammation induced muscle changes in different gut regions described by Marzio *et al.*, there is also evidence that the longitudinal and circular muscle layers may display diametrically different responses to the same inflammatory stimulus. In nematode infected rats, circular smooth muscle strips produced decreased responses to both contracting (carbachol) and relaxing (isoproterenol) stimuli (Crosthwaite *et al.*, 1990), whereas longitudinal jejunal strips have an increased response to both serotonin and carbachol (Vermillion & Collins, 1988). Such general agonist non-specific effects may be due to the above mentioned structural changes and to functional changes of the contractile apparatus.

Similarly in nematode infected rats, muscarinic receptor density is markedly decreased in the longitudinal smooth muscle, although contractility is increased. In that study, ouabain-sensitive ^{86}Rb uptake by longitudinal muscle was reduced by >80% compared with controls, and this was accompanied by a corresponding decrease in the activity of p-nitrophenylphosphatase, an enzyme marker of sodium-pump activity (Muller *et al.*, 1989). Considering that the sodium pump is electrogenic, its suppression during inflammation could lead to hyper-excitability of the intestinal muscle since the membrane potential would be reduced in its threshold for contraction. However it is conceivable that the increased contractility of muscle in the inflamed gut is multifactorial, since the contractile protein content of muscle is also increased in this model (Weisbrodt *et al.*, 1994).

Enteric nerve dysfunction has also been extensively studied in nematode models of intestinal inflammation. Enteric nematode infection of the rat suppresses neurotransmitter release from both cholinergic and adrenergic nerve terminals in the jejunum. Electrophysiological studies have confirmed an altered activity of the enteric nervous system, in both the submucous and myenteric plexus, however in these studies, hyper-excitability of enteric neurons was the predominant finding (Frieling *et al.*, 1994; Palmer *et al.*, 1996). No evidence has yet been provided to link the observations of impaired neurotransmitter release and nerve hyper-excitablity. Nerve dysfunction may also be related to changes in neurotransmitter receptor numbers, but few studies have examined this.

Nematode infected animals and other animal models of gut sensitization have also been used to investigate the impact of immediate or Type I hypersensitivity reactions on intestinal motility. Following the recovery of rats from the intestinal inflammation of a nematode-infection, a second exposure to the same parasite induces a new burst of motor changes that are qualitatively and quantitatively different from those observed during the primary infection. Specifically, it has been shown that in rats sensitized by prior nematode-infection, exposure of isolated intestinal segments to the same parasite (*T. spiralis*) produced changes in propulsive activity that were stimulus specific and rapid in onset (Alizadeh *et al.*, 1989). Subsequent studies *in vitro* have demonstrated the ability of *T. spiralis* to cause contraction of intestinal

muscle via the release of 5-HT from degranulating connective tissue mast cells, which were present in large numbers in muscle throughout the sensitized gut (Marzio *et al.*, 1992).

1.2.5 Altered Motility During Enteric Infection as an Adaptive Response

In the above mentioned studies, altered motility and changes in enteric neuromuscular function have been described as a consequence of inflammation, and they likely form the basis for symptom generation during enteric infections. However changes in GI motility may have originally evolved as an adaptive host response against these infections. As studied in several models, including that of nematode infection, propulsive activity in the small bowel is increased while contractions in the large bowel are reduced. These changes would promote diarrhea and accelerated transit, which in many cases would aid in evicting pathogens and other noxious stimuli from the GI tract. Infection by nematode parasites (Alizadeh *et al.*, 1987; Vermillion & Collins, 1988), protozoans (Deselliers *et al.*, 1994) and some strains of bacteria (ie. *Yersinia pseudotuberculosis*) (Scott & Tan, 1992) have all been shown to generate similar changes, increasing small bowel longitudinal muscle function. This suggests that the response by the muscle originally developed as a generalized protective response by the host, rather than as a response selectively adapted against one particular pathogen.

In parasitology literature, there have long been proponents of the "washer-sweeper" hypothesis of enteric parasite expulsion. This hypothesis suggests that increased fluid secretion, as well as increased propulsive activity by the small bowel, are the main effector mechanisms in the eviction of invading enteric parasites. In keeping with this hypothesis is the finding that the changes in muscle function during *T. spiralis* infection occur in the proximal but not the distal small bowel. This may indeed result in an aboral gradient in the degree of longitudinal muscle contraction, down the length of the gut. It is unclear if this alone would be sufficient to expel pathogens. However these changes in proximal small bowel muscle function would also alter the local environment, making it less hospitable to pathogens and providing further impetus for their movement down the length of the gut and eventually for their expulsion. This hypothesis is strengthened by the observation that when *T. spiralis* parasites are expelled,

the parasites are not killed by the expulsion process, but retain their viability (Kennedy *et al.*, 1979), suggesting they are evicted from the gut, rather than neutralized by a lethal attack by the hosts immune system. However until now, the putative role of motility in parasite expulsion has remained only a possibility, with few if any studies examining the relationship between chnages in gut motility, neuromuscular function, and host defense.

Examination of the role of altered motility in host defense has also received relatively little attention in clinical studies, however there was a recent report which found that patients who vomited during a bout of gastroenteritis, (Neal *et al.*, 1997) were less likely to develop persistent problems, ie. IBS). Whether this would hold true for other enteric infections and for increased motility in other regions of the GI tract, remains unknown, but does provide a basis for further examination.

1.2.6 Immune Basis for Altered Function

As mentioned, there is a strong association between inflammation and altered gut motility. This association implicates inflammatory mediators in the genesis of altered enteric neuromuscular function. These mediators include a variety of molecules of chemically distinct structure. They include cytokines, eicosanoids, free radical bearing molecules and many others. These molecules are produced by activated inflammatory and immune cells and by tissue resident cells, as part of the host's response to infection. Since many studies have examined acute models of inflammation, research has focused on inflammatory rather than immune mediators. In this regard, enteric nerve dysfunction appears to be caused by the inflammatory response. Corticosteroid treatment for instance, prevents sympathetic nerve dysfunction as well as preventing the increase in tissue levels of substance P seen during rat trichinosis (Swain *et al.*, 1992). Experimental evidence from a number of animal studies supports a role for inflammatory cytokines as modulators of enteric nerve function. Exogenous IL-1β, TNFα and IL-6 alter neurotransmitter release from rat jejunal neuromuscular preparations (Main *et al.*, 1993; Hurst & Collins, 1993 & 1994; Ruhl *et al.*, 1994) and more recent electrophysiological data support these findings (Xia *et al.*, 1996).

As for changes in enteric muscle during *T. spiralis* infection, an increasing body of data supports the concept that the immune system is directly involved in altering enteric muscle function. Like changes in enteric nerve function, alterations in muscle contraction are absent from nematode-infected rats in which the inflammatory response has been suppressed by corticosteroid treatment (Marzio *et al.*, 1990). However, unlike enteric nerve dysfunction, the altered muscle contractility observed during infection was absent from *T. spiralis* infected congenitally athymic rats, despite the presence of detectable mucosal inflammation (as determined by myeloperoxidase activity) in the jejunum (Vermillion *et al.*, 1991). Moreover, the changes in intestinal muscle contraction were restored through the successful reconstitution of T-lymphocyte numbers in these rats, prior to infection. There is also immunohistochemical evidence of MHC II expression within the muscle layers during *T. spiralis* infection, and both CD4 and CD8 positive T cells have been shown to infiltrate the jejunal neuromuscular layers within the first 48 hrs of infection (Dzwonkowski *et al.*, 1990). Taken together, these findings support the hypothesis that increased contraction of intestinal smooth muscle from the inflamed intestine of *T. spiralis* infected rats is T lymphocyte dependent.

1.2.7 *T. spiralis* Infection - Immune Response and Expulsion

The observation that altered muscle function during a *T. spiralis* infection depends on the presence of T cells, supports the hypothesis that gut motility can act an extension of the immune system. This also implies that during an infection, the immune response directed against the parasite probably mediates these changes in motility. Unfortunately, until now, relatively little attention has been paid to the gut motor system in host defense, so few studies have examined how the immune system can interact with gut neuromuscular tissues. Even so, there is already extensive literature examining nematode infections in mice, the interactions between the host and the parasite, and the direct actions of the immune response against the parasite. Therefore previous studies characterizing the route taken by the parasite as it infects the host, and the immune response that develops during the infection should help to identify potential mediators of altered motility during the infection.

The larval *Trichinella* is found encysted in the skeletal muscle of its host and remains dormant within its nurse cell until another animal ingests the original host's skeletal muscle. When the parasite reaches the new hosts digestive tract, the gastric acids and digestive enzymes of the stomach break down the tissue, releasing the parasite from its cyst. Following their release, the juvenile worms travel from the stomach to the duodenum/jejunum where they begin to penetrate intestinal epithelial cells and feed. Over the next 24-36 hours, the larvae undergo several molting stages until reaching adulthood when they begin to mate. Within a few days, female *Trichinella* begin to release large numbers of live larval young, which migrate from the intestine, via the lymphatics, eventually reaching the new hosts skeletal muscle. Once they arrive, they penetrate a host cell and begin their encystment. Over the next few weeks they turn the skeletal muscle cell they have infected into a nurse cell and wait to begin the infection process anew.

The gastrointestinal phase of a *T. spiralis* infection, generally lasts 2-3 weeks, at the end of which the adult worms have been expelled from the gut, damaged, but alive (Kennedy *et al.*, 1979). This phase of the infection induces a rapid inflammatory response in the jejunal mucosa, and an immune response in associated lymphoid tissues including the Peyer's patches and mesenteric lymph nodes (Svetic *et al.*, 1993; Wakelin *et al.*, 1993). There is also a strong genetic component regulating host defense to *T. spiralis* infection (Wakelin, 1988), since there are significant differences in the timing of worm expulsion, depending on the mouse or rat strain infected. These genetic differences can significantly alter the immune response, as determined by the types and levels of cytokines produced at different tissue sites during infection (Wakelin, 1988; Lammas *et al.*, 1992). Whether differences in the hosts ability to expel an infection are due solely to immunological factors, or whether other, non-immunologic responses are involved, is at present unknown.

The T cell dependence of the changes in muscle function in the *T. spiralis* infected rat provides a potential link with the expulsion of the parasite since the eventual clearance of the parasite also requires an intact T cell axis (Manson-Smith *et al.*, 1979; Ruitenberg & Steerenberg, 1974). Thus, mice lacking T cells exhibit delayed expulsion, or even develop a chronic infection. Studies by Grencis and others have shown that CD4 +ve T cells are able to adoptively transfer protection against a primary *T. spiralis*

infection (Grencis *et al.*, 1985; Korenaga *et al.*, 1989). In many infection paradigms in both humans and laboratory rodents, the CD4 +ve T cell response appears to evolve into one of two types (Mossman & Coffman 1989). One is the Th1 response in which CD4 +ve T cells secrete characteristic cytokines typified by γ-IFN and IL-2. The other is the Th2 response in which these cells secrete a characteristic profile of cytokines typified by IL-4, IL-5 and IL-10. Occasionally both responses occur simultaneously in a single host, apparently in compartmentalized form (Goyal *et al.*, 1994). Recent studies have shown that a Th2 profile is associated with protection against nematode infections (Svetic *et al.*, 1993, Urban *et al.*, 1992), consistent with a role for the eosinophilia, mastocytosis and increased levels of IgE seen during such infections. However, the exact role of these responses in host defense is unclear.

The two cytokines that have received the most attention in Th2 responses, and particularly during nematode infections, are IL-4 and IL-5. Produced predominantly by T cells, IL-5 is thought to be relatively selective in the cell types it acts on. Although it does modulate B cell function, its major activity is on eosinophils, mediating their proliferation (Sanderson, 1988). It has also been shown to be important for eosinophil chemotaxis, growth, activation and survival, by delaying eosinophil apoptosis (Simon & Blaser, 1995, Sanderson, 1988). However, despite the clear relationship between nematode infections and eosinophilia, the role of IL-5 and eosinophils in the expulsion of these infections is unclear.

In contrast, IL-4 has broader actions, modulating the activity of a number of immune and non-immune cells. Produced by both T lymphocytes and mast cells, it is an essential factor in the development of Th2 responses and its main effector function appears to be as a regulator of allergic reactions. Mice in which the IL-4 gene has been disrupted, fail to produce IgE and these mice also suffer a significant impairment in the ability of T cells to develop the polarized expression of Th2 cytokines such as IL-5 and IL-10. While IL-4 plays an important role in the development of the hosts immune response during a nematode infection, it is less clear whether this is essential for the expulsion of nematode infections. Studies using IL-4 neutralizing antibodies (Madden *et al.*, 1991) and IL-4 deficient mice (Lawrence *et al.*,

1998) have shown conflicting evidence for and against IL-4 playing a critical role in nematode parasite expulsion from the GI tract.

1.3 ALTERED MOTILITY DURING DISEASE AS A MALADAPTIVE RESPONSE

1.3.1 Introduction

Altered intestinal motility is not only a consequence of nematode infections. The susceptibility of the gut motor system to immunomodulation, allowing it to respond during an enteric infection also makes it vulnerable to immune mediated injury during non-infectious forms of inflammation. In the absence of an identifiable pathogen, any resulting alterations in function could be seen as maladaptive, particularly if they contributed to the symptomatology or pathology of the disease. Indeed, there is now extensive evidence that gastrointestinal motility is altered during a variety of disease states and that disruption of normal motility patterns contributes to several clinical symptoms of, for example, acute and chronic intestinal inflammation. These include chest pain and dysphagia in the esophagus (DiMarino *et al.*1998; Kahrilas *et al.*, 1986), while bloating, nausea and vomiting may result from perturbations of motility in the stomach and proximal small intestine (Moore *et al.*, 1986; Testoni *et al.*, 1992 & 1993; Evans *et al.*, 1997). Abdominal cramps and alterations of normal transit resulting in diarrhea or constipation are a consequence of disturbed motility in the remainder of the gut (Choi *et al.*, 1997; O'Brien *et al.*, 1996). Functional disturbances and structural changes have been described in all three components of the motor apparatus: smooth muscle, enteric nerves and endocrine cells, during inflammation. Moreover proliferation of cells in the neuromuscular layers contribute to stricture formation in the chronically inflamed gut wall (Dvorak *et al.*, 1980; Graham 1995).

While the relationship between overt inflammatory diseases of the GI tract and altered motility is clear, the role of inflammation in triggering functional gastrointestinal disorders, where mucosal inflammation is no longer present, is controversial. There is speculation that acute inflammation, of either infectious or idiopathic origin, may induce chronic changes in the neuromuscular apparatus of the gut, generating symptoms that persist long after the mucosal inflammation has resolved (Isgar *et al.*, 1983,

Collins 1996, Bergin *et al.*, 1993; Gwee *et al.*, 1996; Neal *et al.*, 1997). This persistent dysfunction could reflect the normal time course required for the inflammation induced changes in the structure and function of neuromuscular tissues to resolve. Presumably changes in mucosal function would recover more rapidly, due to the more frequent cell turnover in the mucosa. Alternatively, it could indicate the presence of subclinical mucosal inflammation, or other factors within the neuromuscular layers, continually stimulating dysfunction of these layers, long after any mucosal inflammation has resolved. Thus, post-infectious irritable bowel syndrome (IBS) and some forms of intestinal pseudo-obstruction, may reflect underlying inflammatory or immune mechanisms (Collins, 1996). Because of the prevalence of symptoms attributable to altered motility, and the associated morbidity that ensues, the mechanisms underlying the pathophysiology of enteric motor function are an important clinical concern (Collins, 1996; Gwee *et al.*, 1996).

1.3.2 Evidence for Altered Motility in Patients

The first reports of altered motility in the inflamed gut originated from studies in patients with chronic ulcerative colitis and were therefore related to the colon. In 1951, Kern *et al.*, observed a marked decrease in the motor activity of the sigmoid colon in patients with active ulcerative colitis (Kern *et al.*, 1951). Because colonic motility in these patients returned towards normal when their colitis was in remission and their colonic mucosa was healing, the authors hypothesized a causal relationship between mucosal inflammation and altered motor function. Since then, a number of investigators have reported that active ulcerative colitis is accompanied by a reduction in motor activity in the distal colon. Later studies by Garrett *et al.*, demonstrated that the colonic motor response to pharmacologic agents, including opiates, and to endoluminal stimuli, is markedly altered, producing high-amplitude, non-propagated contractions in patients with ulcerative colitis (Garrett *et al.*, 1967). More recent studies by Snape *et al.* have shown that even physiological stimulation of colonic motility following ingestion of a standard meal was abnormally decreased in patients with ulcerative colitis compared to healthy control subjects (Snape *et al.*, 1980).

Since these early studies in the colon, motor disturbances have also been reported in a variety of inflammatory diseases involving other regions of the gastrointestinal tract. Altered small bowel transit times in celiac sprue (Spiller *et al.*, 1987), disorganization of motility patterns in Crohn's disease (Annese *et al.*, 1995), antral hypomotility in gastritis (Moore *et al.*, 1986), and peristaltic dysfunction in peptic oesophagitis (Liu *et al.*, 1975) are among the prominent, although sometimes disputed findings, since at least one study failed to demonstrate a clear correlation between symptoms and motility patterns in dyspeptic patients with gastritis (Pieramico *et al.*, 1993). Abnormal motor function has also been shown to occur as a result of antigen-specific immune responses. Studies by Liu *et al.*, demonstrated accelerated small bowel transit after oral antigen challenge in patients with milk protein allergy (Liu *et al.*, 1975). Lymphoid cell infiltration of the *myenteric plexus* and *muscularis externa* has also been described in some patients with intestinal pseudo-obstruction (Chinn *et al.*, 1988, Krishnamurthy *et al.*, 1986). There are also reports of the development of acute colonic pseudo-obstruction after therapy with the cytokine interleukin-2, providing further evidence of a role for immune mediated mechanisms in the pathogenesis of some pseudo-obstruction syndromes (Post *et al.*, 1991, Schwartzenruber *et al.*, 1988).

1.3.3 Changes in Enteric Neuromuscular Structure

While demonstrations of altered motility in various diseases have been repeatedly demonstrated, these studies have not identified the mechanisms underlying the motility disturbances. In this regard, surgical specimens have been evaluated for pathologic changes in both structure and intrinsic function, as well as for the potential involvement of infiltrating inflammatory cells. Several studies have shown that intestinal neuromuscular tissues respond with changes in structure during inflammation of the GI tract, and since these types of surgical specimens are often removed from IBD patients (Farmer *et al.*, 1976), more information is known about these conditions than others.

Gut tissues removed from Crohn's patients show extensive architectural disruption of both the mucosa and the bowel wall. The *muscularis externa* and *muscularis mucosae* are often thickened in involved regions of the GI tract (Dvorak *et al.*, 1979). As well, in these regions, the neuromuscular layers

have frequently undergone extensive fibrosis and are heavily infiltrated with a variety of cells, including lymphocytes, mast cells, eosinophils, and macrophages (Dvorak *et al.*, 1979). There is also increased expression of antigen presentation molecules within the myenteric plexus, with major histocompatibility complex (MHC) class II antigens expressed by infiltrating haematopoietic cells as well as by the enteroglial sheath surrounding the myenteric nerve fibers (Geboes *et al.*, 1992). Since MHC class II glycoproteins are crucial in presenting antigen to CD4 +ve T cells (Grusby *et al.*, 1991), immunoreactions *in situ* may be triggered by these molecules, causing axonal damage to myenteric neurons and other neuromuscular tissues. (Geboes *et al.*, 1992).

These changes in structure can influence the course of Crohn's disease in several ways, but perhaps the most serious is the development of strictures. Stricture formation is one of the complications of inflammatory bowel disease, and one of the most frequent necessitating surgical intervention (Farmer *et al.*, 1976; Graham 1995). Strictures are physical obstructions of the GI tract, usually occurring in the small bowel, resulting in a partial or complete blockage of transit, and are often associated with a localized thickening of the entire muscle layer, as well as extensive deposition of collagen (Graham *et al.*, 1988). The smooth muscle cells in these areas appear to undergo a phenotypic transformation, becoming proliferative and secreting copious amounts of collagen. They have also been shown to express atypical contractile proteins (Graham *et al.*, 1988; Dvorak *et al.*, 1980). These phenotypically transformed smooth muscle cells as well as myofibroblasts are the most prominent cell types found in intestinal strictures (Graham, 1995).

Structural alterations of enteric nerves have also been reported in various chronic inflammatory bowel diseases. The main histological findings include changes in the number of ganglia, ganglion cell hypertrophy as well as axonal necrosis, and alterations in neuropeptide innervation (Strosteen *et al.*, 1953; Strobach *et al.*, 1990; Koch *et al.*, 1987). Axonal necrosis was found in both diseased areas and grossly normal resection margins of Crohn's disease resection specimens (Dvorak *et al.*, 1979). A relative increase in sympathetic nerve fibers has also been reported in the diseased gut. As well there is extensive evidence of a change in the peptidergic innervation of the gut during disease states, with substance P

receptor density on arterioles, venules and lymph nodes, significantly increased in tissues removed from Crohn's and ulcerative colitis patients (Mantyh *et al.*, 1989).

It should be noted that the inflammatory changes seen in the muscle layers do not merely reflect the responses seen in the overlying mucosa. Studies suggest that inflammatory changes in the muscle layers do not normalize as readily as do those in the mucosa, and that the activation and proliferation of T lymphocytes may be greater in the muscle layers of Crohn's disease resection tissues than in the mucosa (Fell *et al.*, 1996). Our laboratory has also recently shown that the cytokine profile within the muscle layers might be quite different from that expressed in the overlying mucosa during this disease (Lovato *et al.*, in press). Finally recent evidence suggests that the presence of inflammatory changes in enteric nerves within the myenteric plexus of resection specimens and section margins in Crohn's disease, is a predictor of post-operative recurrence. This suggests that the reoccurence might have its genesis in the myenteric plexus, although this remains to be shown. (D'Haens *et al.*, 1998)

1.3.4 Patient Studies - Changes in *In Vitro* Function.

Assessment of the contractile properties of muscle in the human inflamed gut has been investigated most extensively in inflammatory bowel disease (IBD). Studies on ulcerative colitis specimens have yielded conflicting results. Koch *et al.*, reported a weak association between the duration of symptoms and decreased frequency of spontaneous summation contractions in circular muscle from ulcerative colitis patients compared to non-inflamed controls (Koch *et al.*, 1988). However there was no discernible change in resting membrane potential or slow wave activity. In contrast, others have reported a decrease in maximal isometric tension development by bethanecol in circular muscle from ulcerative colitis patients compared to non-inflamed controls (Snape *et al.*, 1991, Mayer *et al.*, 1990). The reason for the discrepancy may reflect the use of different methodologies between laboratories and the use of intestinal specimens obtained from patients with different clinical features.

There have been very few studies of small intestinal motility in patients with IBD, probably because of the technical difficulty involved in placing a recording device in an inflamed area such as the

terminal ileum. Similarly, there have been relatively few studies evaluating small bowel muscle and nerve function, compared to those of the large bowel. However, one such *in vitro* study conducted by our laboratory demonstrated that both circular and longitudinal muscle from the inflamed ileum of Crohn's disease patients showed increased contractility when compared to muscle from patients without IBD (Vermillion *et al.*, 1993). Since stimulation of muscle with either carbachol or histamine produced similar changes in muscle contractility, these results suggested that the altered muscle response was not at the receptor level. Interestingly, no differences were observed in the passive properties, basal tone or spontaneous activity of tissues from the two groups.

1.3.5 Caveats to Patient Studies

It should be noted that examining gastrointestinal function and tissues in patients provides the most relevant data concerning the disease in question. However such studies, by the nature of the subjects, are limited. While examining motility patterns in the gastrointestinal tract of patients is quite advanced, perhaps more so than in experimental models, patient heterogeneity can provide variability in the data. This is also true with regard to the various treatment regimes that patients may be undergoing. As well, neuromuscular tissue specimens are generally only available from patients with the most severe forms of intestinal disease such as IBD, when surgery is required. It is otherwise unethical to sample neuromuscular tissues from patients, so there is little data from less severely inflamed tissues, or from functional bowel diseases such as IBS, where biopsies are limited to the mucosa. Therefore although specimens of human neuromuscular tissue are very valuable both for functional assays, and for the histological and biochemical assessment of inflammation, their relative scarcity and the inability to obtain tissues showing early or mild inflammatory events which might initiate the changes in neuromuscular function, limits the conclusions that can be obtained. Thus, there is a decided need for further study in animal models, to determine the mechanisms underlying altered enteric neuromuscular function.

1.4 THESIS - AIM & STUDY PLAN

1.4.1 Aim

Based on preliminary evidence suggesting an immune basis for altered muscle function, as well as a possible role for altered muscle function in host defence, the purpose of my studies was to investigate the immunological basis behind altered intestinal muscle function. In order to achieve this, and to exploit the available immunological tools, it was first necessary to establish and characterize altered smooth muscle function in a murine model. Since parasitology has formed the basis for exploring immunophysiological interactions in other systems, I elected to focus my studies on murine trichinosis.

1.4.2 Summary of Results

We found that infection with *T. spiralis* caused a significant increase in tension generation by muscle in response to carbachol. We found that mouse strains that rapidly expelled the infection generated a greater tension than mice which developed long lasting infections. I documented two phases, an early phase when the muscle initially changed function, and a persistent phase, where the increased muscle tension was maintained until at least day 21 post-infection. We also found that mice lacking T cells, and specifically mice lacking CD4 +ve T cells showed an attenuated increase in muscle contraction during infection. We examined the mediators known to be released by CD4 T cells during a *T. spiralis* infection and found that mice lacking in IL-5 expression and therefore lacking most of their eosinophils, were attenuated in the persistent phase of increased muscle contraction. As well, by infecting the outer layers of the small bowel using an adenoviral vector expressing interleukin-4, we found a resulting increase in muscle contraction, which was not found when a control virus was used.

Along with determining the putative cells and mediators that alter muscle function during a nematode infection, we believe these studies also provide clues to the mechanisms underlying neuromuscular pathophysiology during other forms of intestinal inflammation. While the factors that initiate infectious and idiopathic based intestinal inflammation are quite different, the similarities in the functional abnormalities that develop, suggest the underlying mechanisms are probably similar. My

studies thus support the concept that the immune system can modulate enteric muscle function. In idiopathic inflammatory diseases of the bowel, this interaction and the resulting changes in gut motility may prove maladaptive by contributing to disease symptoms. However, during the course of an enteric infection, the interaction between these two systems may prove adaptive by contributing to host defense, aiding in the eviction of pathogens through increased propulsive activity.

1.4.3 Experimental Tools for this Study

The following paragraphs outline the tools used in the experiments which make up this thesis. As in any study, interpretation of the data requires some knowledge of the strengths and limitations of the tools used. This is especially important since this thesis probably comprises the first studies utilizing gene knockout mice, and recombinant adenovirus vectors to examine the mechanisms underlying the immunomodulation of enteric muscle function.

1.4.4 Infection of Spontaneous and Gene Targeted Immunodeficient Mice

There are major limitations to the study of the functional interactions between the immune system and the enteric neuromuscular system in the rat, since there are relatively few tools and limited background literature that addresses immunology in this species. We therefore decided, before beginning the studies which make up this thesis, that there was a need to switch experimental species to the mouse. Unfortunately studies of enteric physiological mechanisms in the murine intestine have, until recently, been constrained because of the relative fragility of the mouse small bowel. However, we felt that if these difficulties could be overcome, the mouse would become a particularly useful model because of the diversity of immunologic reagents and cell markers that are available for this species, including neutralizing antibodies, and immunodeficient mouse strains suffering from either a spontaneous or a gene targeted deficiency in their immune system.

The capacity to produce specific mutations in whole animals has major implications for the analysis of pathogenesis and resistance mechanisms, as well as for the study of immunophysiology. While

gene targeted immunodeficient mice have been extensively used to study viral and bacterial infections (Malo & Skamene 1994), their use in parasitology and particularly in immunophysiological studies has lagged behind. However it is clear that this approach has the potential to clarify many *in vivo* immunobiology questions that were previously inaccessible to rigorous analysis. As well, there are a broad spectrum of immunodeficiencies available for study, from extreme cases involving the loss of both T and B cell function, to the deletion of a single cytokine gene.

Cytokine mediated effects in enteric infections are very complex and present a formidable challenge to the immunobiologist working with *in vivo* models. The development of mice with targeted disruptions of cytokine genes opens the possibility of determining whether there is an absolute requirement for a particular molecule in the host's response to a specific infection. In general, it is likely that there is considerable redundancy in the possible pathways to an effective host response, as there is often significant overlap between cytokines in their effector functions. However cytokine deficient animals do possess the advantage that they are usually totally deficient in production of the protein in question (which is a decided advantage over neutralizing antibodies). Unfortunately, they also lack the protein during embryogenesis. Thus the approach is far from straightforward, due to possible effects on the ontogeny of the immune system and the fact that these changes in the immune system can lead to substantial pathology even in the absence of a superimposed infectious process (Elson *et al.*, 1995). Thus the targeted immunodeficiency may alter development of the animal or induce compensatory mechanisms, which might not be present in unmanipulated animals. Therefore before making conclusions, it is probably advisable to show that the cytokine has some role in the disease process, as it occurs in the otherwise unmanipulated animal.

1.4.5 Adenoviral Gene Transfer to the Intestine

Studying the role of the immune system in pathology and pathophysiology has made recent advances in other systems, such as the lung, through the development of new tools that allow the localized over-expression of immunological molecules such as cytokines. For example, many laboratory groups are

examining live viral vectors that can be constructed to carry foreign genes, and once they infect cells, induce the production of the transgene products. Now there is interest in using these vectors in the intestine. Of these vectors, recombinant adenoviruses are the most efficient and extensively used vectors currently available for gene transfer *in vivo*. They are infectious across a broad range of host species and cell types, and can be grown to high titres. Following successful infection, they can direct the cellular machinery of the host cell to produce large quantities of their transgene proteins (Graham & Prevec, 1991).

These vectors have received much publicity regarding their possible medical application in the treatment of disease through the expression of therapeutic proteins, but they have also found use in the study of immune and inflammatory responses. Several recent studies have used recombinant adenoviruses, incorporating genes encoding cytokines and growth factors, to study the effects of transient elevations of these molecules on inflammation (Xing *et al.*, 1994; Sime *et al.*, 1997) and host defense (Greenberger *et al.*, 1996) in the airways. However, while there have been many advances in the field of gene transfer to the lung and other tissues, the issue of gene transfer to the intestine is more problematic, due to technical difficulties in delivering viruses to the intestine. Several studies have examined lumenal or oral delivery of these vectors, but have encountered relatively poor transfection efficiency (Sandberg *et al.*, 1994; Hamilton *et al.*, 1997). Other approaches have used the circulation to deliver the virus but these approaches lack selectivity for the intestine (Brown *et al.*, 1997), or require technically demanding surgery (Sferra *et al.*, 1997), feasible only in larger experimental animals such as the rat. Thus alternative approaches to delivering genes to the gastrointestinal tract need to be developed and tested.

Other concerns relating to transferring genes to the intestine include not only the best approach for the question being asked, but also which cell types to target and infect. Targeting the intestinal mucosa would provide additional problems because the likely target, the intestinal epithelial cells, are rapidly sloughed into the lumen (Cheng & Leblond, 1974), providing only a small window of time for transgene expression. Other possibilities involve the deeper layers of the intestinal tract, including the crypts, as well as the external muscle layers. These cell types would offer longer lasting, more stable production of

transgenes than epithelial cells, providing a way could be found to infect these cells. Once a delivery system to the intestine is developed, the overexpression of specific cytokines as well as other genes within gut tissues would allow the study of their actions in relative isolation, within an *in vivo* system. This would have implications for the study of inflammation, fibrosis, host defense and of course immunophysiology in both the intestinal mucosa and neuromuscular layers of the gut.

The following work, my first publication addresses the development of the methodology allowing measurement of murine intestinal muscle function. The paper outlines the changes in muscle function that develop in two strains of mice, their time course in relation to worm expulsion, and a correlation between the strength of the muscle response and the speed with which worms are expelled.

Chapter 2

Increased intestinal muscle contractility and worm expulsion in nematode-infected mice.

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ABSTRACT

Intestinal nematode infections are accompanied by mucosal inflammation and an increase in propulsive motor activity that may contribute to parasite eviction from the gut. To examine whether differences in worm expulsion correspond to the increased intestinal muscle contractility that accompanies nematode infection, we studied mice with genetically-determined differences in their ability to expel the nematode *parasite Trichinella spiralis*. Specifically, we examined isometric contraction of longitudinal muscle, worm counts and inflammation, as measured by myeloperoxidase (MPO) activity, in two strains of mice infected with *T. spiralis*. The strong responder strain, NIH Swiss, expelled the parasites by day 16 P.I., while the poorer responding B10.BR strain was still heavily infected by day 21 P.I. However, both strains developed similar increases in jejunal MPO activity. Both strains demonstrated increased isometric tension development following infection, but peak tension occurred earlier in NIH Swiss mice (day 8 vs day 12 P.I.) and was of significantly greater magnitude than in the B10.BR mice. We conclude that the ability to expel *T.spiralis* from the small bowel is not related to the degree of granulocyte dependent mucosal inflammation, but is reflected in the magnitude of the accompanying increase in force generation by intestinal smooth muscle.

2.1 INTRODUCTION

The gastrointestinal tract represents one of the body's largest interfaces with the environment and possesses a specialized immune system to assist in its defence against a variety of environmental threats that include infection by viruses, bacteria and parasites. The mucosal immune system may also recruit other tissues in defence of the gut against infection. The observation that many enteric pathogens, expelled in the feces, remain viable and virulent, suggests that a process of eviction occurs in addition to immunologically mediated neutralization (18) of pathogens. This process may involve changes in the secretion of mucus and water, in addition to alterations in gastrointestinal motility.

Previous studies have shown that changes in intestinal motility accompany primary infection of rats or mice with the nematode parasite *Trichinella spiralis*. There is an increase in motor activity (3) resulting in a decrease in transit time (22). Since this is also observed in extrinsically denervated segments of bowel (1), the

underlying mechanisms may involve previously documented changes in muscle contractility (16,28). Populations of T lymphocytes, among other cells, localize to the gut during nematode infections (6,31). Studies performed with athymic rats have shown that infection induced changes in muscle function require T lymphocytes (26). Interestingly, the ability of animals to expel *T.spiralis* has also been shown to be T lymphocyte-dependent (20). Thus, it appears likely that the ability to expel the nematode and accompanying changes in smooth muscle contraction share a common immunological basis, and possibly the two processes are causally linked.

To investigate the hypothesis that worm expulsion and altered intestinal muscle contractility are linked, we examined the consequences of *T.spiralis* infection on mucosal inflammation, worm distribution and muscle contractility in two strains of mice that exhibit quite different patterns of worm expulsion following *T.spiralis* infection; NIH Swiss mice are strong responders to *T.spiralis* infection, evicting the parasite quickly, whereas B10.BR mice are weak responders and expel the parasite slowly from the gastrointestinal tract (2,29). To enable us to exploit this difference, it was necessary to first adapt experimental approaches, used previously in the rat, to the murine intestine. Our results show that it is possible to obtain reproducible measurements of isometric contraction of intestinal muscle in the mouse, that primary infection by *T.spiralis* results in a significant increase in muscle contraction, and that this increase is significantly greater in magnitude and occurs more rapidly, in the NIH Swiss mouse compared to the B10.BR strain, even though both strains undergo similar degrees of jejunal inflammation during infection.

2.2 MATERIALS AND METHODS

2.2.1 Mice

Studies were performed on male NIH Swiss mice (obtained from the National Cancer Institute, Frederick, Maryland), and on male B10.BR mice (obtained from the Jackson Laboratory, Bar Harbor, Maine) between 6 and 10 weeks of age. Mice were kept in filter-isolated cages in groups of 4-5 in positive pressure rooms with a constant ambient temperature and photoperiod (14:10 h light-dark cycle). All experiments were

approved by the Animal Care Committee at McMaster University and were conducted in accordance with the guidelines of the Canadian Council on Animal Care.

2.2.2 *Trichinella* infection

Mice were infected by administration of 0.1 ml of phosphate-buffered saline (PBS) containing 350-400 *Trichinella spiralis* larvae by gavage. To minimize differences between infections, when possible both strains were infected from the same larval preparation. The larvae were obtained from infected rodents 30 - 90 days post-infection using a modification (28) of the technique described by Castro and Fairbairn.(4) The *T. spiralis* culture originated in the Department of Zoology at the University of Toronto and the colony was maintained through serial infections alternating between male Sprague-Dawley rats and male CD1 mice.

2.2.3 Worm counts

The entire length of the small intestine was removed and divided into four equal sections for ease of counting. All the adult worms within each section were counted using a modification of the method described by Castro and Fairbairn (4). Briefly, the mucosa was separated from the underlying muscularis by scraping with a glass microscope slide and mixed in 1 ml of PBS. The worms were then counted using a scored petri dish and an inverted microscope. The worm counts in the four sections were then added and expressed as the total number of worms recovered per mouse. In accordance with established practices, worm rejection was considered complete when at least 98% of the infective dose had been expelled from the gut (29).

2.2.4 Myeloperoxidase Assay

The degree of jejunal inflammation was monitored by assay of myeloperoxidase activity (MPO). Animals were killed by cervical dislocation and samples of jejunum (50-100mg), approximately 2 cm distal to the ligament of Treitz were removed for MPO measurement. The samples were snap frozen in liquid nitrogen and stored at -70?C. MPO is an enzyme contained in the azurophilic granules of neutrophils as well as other myeloid cells and is commonly used as an index of neutrophil infiltration and inflammation (21). MPO was

measured using a modified version of an assay as previously described (30). MPO activity is reported as units of MPO per mg of wet tissue. One unit of MPO is defined as the quantity of enzyme able to convert 1 μmole of hydrogen peroxide to water in 1 minute at room temperature.

2.2.5 Measurement of muscle contraction

Tissue Preparation. The jejunum was removed and placed in oxygenated (95% O_2-5% CO_2) Kreb's solution containing (in mM) 120.9 NaCl, 1.2 NaH_2PO_4, 15.5 $NaHCO_3$, 5.9 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 11.1 glucose; pH 7.4. Four 1cm sections of whole gut were removed from the jejunum, beginning at the ligament of Treitz and proceeding distally. The lumen of each segment was flushed gently with Kreb's buffer prior to the insertion of short (2-3mm) lengths of Silastic tubing (0.065 inches outside diameter, 0.030 inches internal diameter (Dow Corning, Midland, MI) into the open ends of the gut segments. The tubing was then tied in position with surgical silk. The insertion of the tubing was found to prevent the tissue dilatation and eventual loss of responsiveness that was found to typically occur over the course of an experiment, when both proximal and distal ends of the gut segments were tied off. Segments were then hung in the longitudinal axis and attached at one end to a Grass FT03C force transducer (Grass, Quincy, MA) (see Figure 1) and responses were recorded on a Grass 7D polygraph. Tissues were equilibrated for 30 minutes at 37°C in Kreb's solution, oxygenated with 95% O_2 - 5% CO_2 before starting the experiment.

Analysis of Length-Tension Relationships. Experiments were then conducted to examine the length-tension characteristics of the muscle before and after infection. Segments were stretched by applying tension equivalent to 0-1250 mg of weight, and contraction was assessed following stimulation with 1μM carbachol (Sigma Chemical, St.Louis, MO) which gives a submaximal response, according to carbachol dose response studies. Initial experiments indicated that this tension range was sufficient to determine the maximum responsiveness of both control and inflamed tissues. After each application of increasing tension, the length of the tissue and the contractile response were recorded. Tissues were then rinsed twice and equilibrated at the next tension level in fresh Kreb's for 15 minutes, prior to the next addition of carbachol. At the end of each

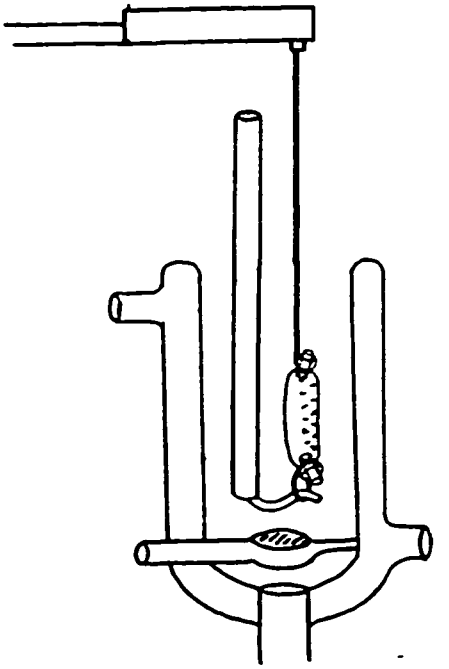

Figure 2.1: Position and setup of a full thickness small bowel section in a tissue bath. Silastic tubing is inserted into both ends of the lumen of the gut, and the proximal end of the tissue is attached to the force transducer above, while the distal end is attached to the tissue hook below.

experiment, tissue segments were removed, blotted and weighed. The degree of applied tension producing the maximum response to carbachol was identified as the optimal tension (T_O) and the tissue length at the optimal tension was then used to calculate the cross sectional area of the tissue.

Carbachol-induced Contraction. The previously identified T_O for a tissue was then applied in carbachol dose response experiments, prior to the addition of the first dose of carbachol. Gut segments were then exposed to noncumulative final bath concentrations of carbachol ranging from 1nM to 1mM by adding microliter aliquots to the 20 ml baths. After the maximal response to each dose was obtained, tissues were rinsed twice and equilibrated in fresh Krebs for 15 minutes before adding the next agonist dose.

2.2.6 Data Presentation and Statistical Analysis.

In both length-tension and dose response studies, the maximum force generated by the tissue was expressed in milligrams and normalized for cross sectional area. Cross sectional area was determined using the following equation:

$$\text{CSA } (mm^2) = \text{tissue wet weight (mg)} / (\text{tissue length (mm)} \times \text{density } (mg/mm^3))$$

where the density of smooth muscle was assumed to be 1.05 mg/mm^3. The ED_{50} concentration (the concentration of an agonist required to induce a 50% maximal response) was determined by the probit analysis of Litchfield and Wilcoxon (13) for each tissue, and a mean ED_{50} calculated for each mouse. The reported isometric tension is expressed in milligrams per square millimetre of cross sectional area of the tissue. For each mouse, the mean tension value was calculated from at least three segments. All results are expressed as the means ± SEM, n refers to the number of mice used. Statistical significance was calculated using the Student's t test for comparison of two means or a one way analysis of variance for the comparison of three or more means. Multiple comparisons were performed using the Newman-Keuls multiple comparison test. A p value of < 0.05 was considered significant.

2.3 RESULTS

2.3.1 Worm Counts

Evaluation of the parasite load in the small intestine indicated that between 65 and 90% of the gavaged larvae attained adulthood by day 4 P.I. This percentage is similar to earlier published studies (2,29). As shown in Figure 2, the number of worms found in the B10.BR mice was significantly elevated over the number recovered from the NIH Swiss mice, at all time points after day 6 P.I.

Worm expulsion is determined to have occurred when 98% of the infective dose has been eliminated from the gut (29). As shown in Figure 2, worm rejection occurred by day 14 to 16 P.I. in NIH Swiss mice, whereas expulsion remained incomplete by as late as day 21 P.I. in the B10.BR strain, and significant numbers of worms (10 - 20) were still present in the small intestine by as late as day 35 P.I. These results confirm previous reports that mice derived from the NIH Swiss strain respond strongly and effectively to *T. spiralis* infection (2,8,29) whereas the B10.BR strain respond poorly and are prone to develop chronic infections (2,29).

2.3.2 Myeloperoxidase Activity

As illustrated in Figure 3, infection with *T.spiralis* larvae caused a significant and time-dependent increase in MPO activity in the jejunum of both NIH Swiss and B10.BR mice. In both strains, MPO activity reached a maximum on day 10 P.I. and declined thereafter. In NIH Swiss mice, MPO activity returned to levels not significantly elevated over control, by day 12 to 16 P.I., corresponding to the time course of worm expulsion in that strain (see Figure 2). In B10.BR mice, the maximum MPO activity was also observed on day 10 P.I., and the magnitude of the increase was similar to that observed in the NIH Swiss strain. However, the increase in MPO activity remained significantly elevated over control at day 16 P.I. in B10.BR mice (6.4±1.2 units), whereas MPO activity had returned to approximately normal levels in the NIH Swiss strain (2.9 ± 0.8 units). The persistent elevation in MPO activity in B10.BR mice corresponded to the prolonged presence of worms in the small intestine of this strain at day 16 P.I. and beyond (see Figure 2).

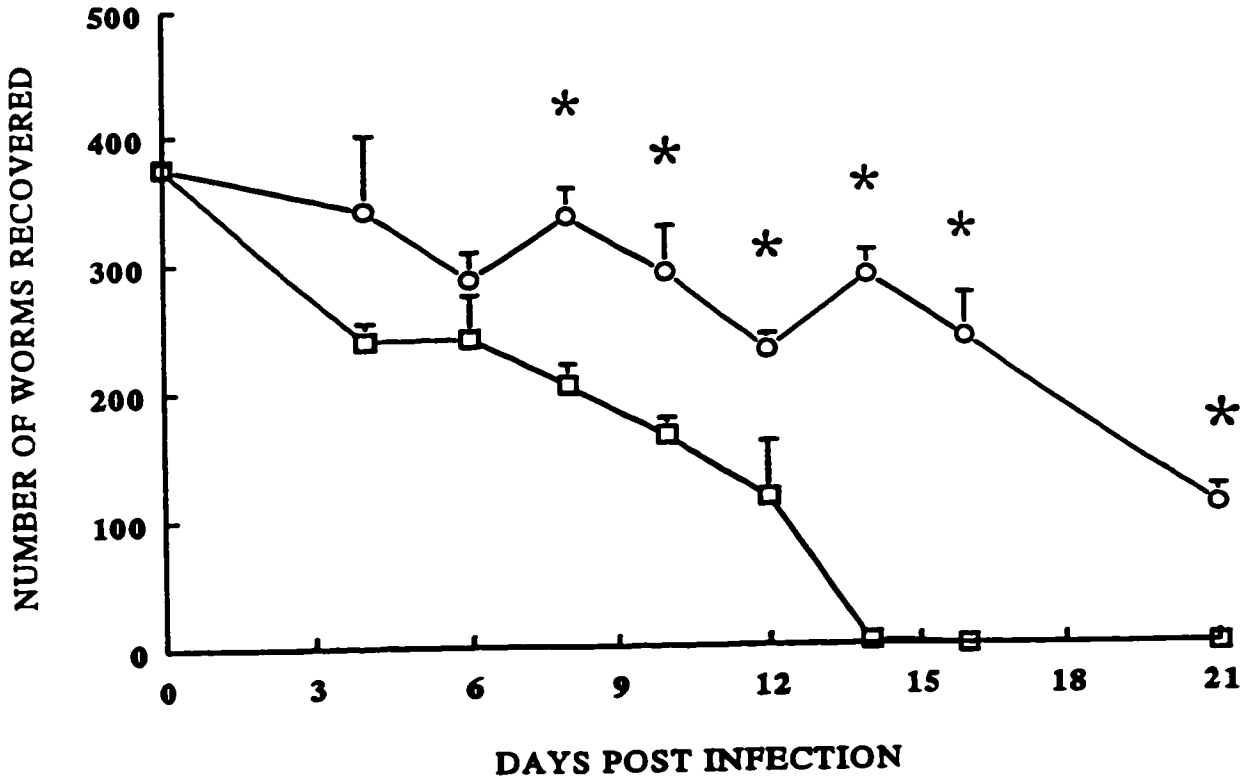

Figure 2.2: The number of adult *T. spiralis* worms recovered from the small intestine of infected B10.BR mice (open circles) and NIH Swiss mice (open squares) over the course of three weeks post-infection. Results shown are the mean ± 1 SE of groups of 4-6 animals. The asterisk denotes a significant difference ($p < 0.05$) compared to NIH Swiss at the same time point.

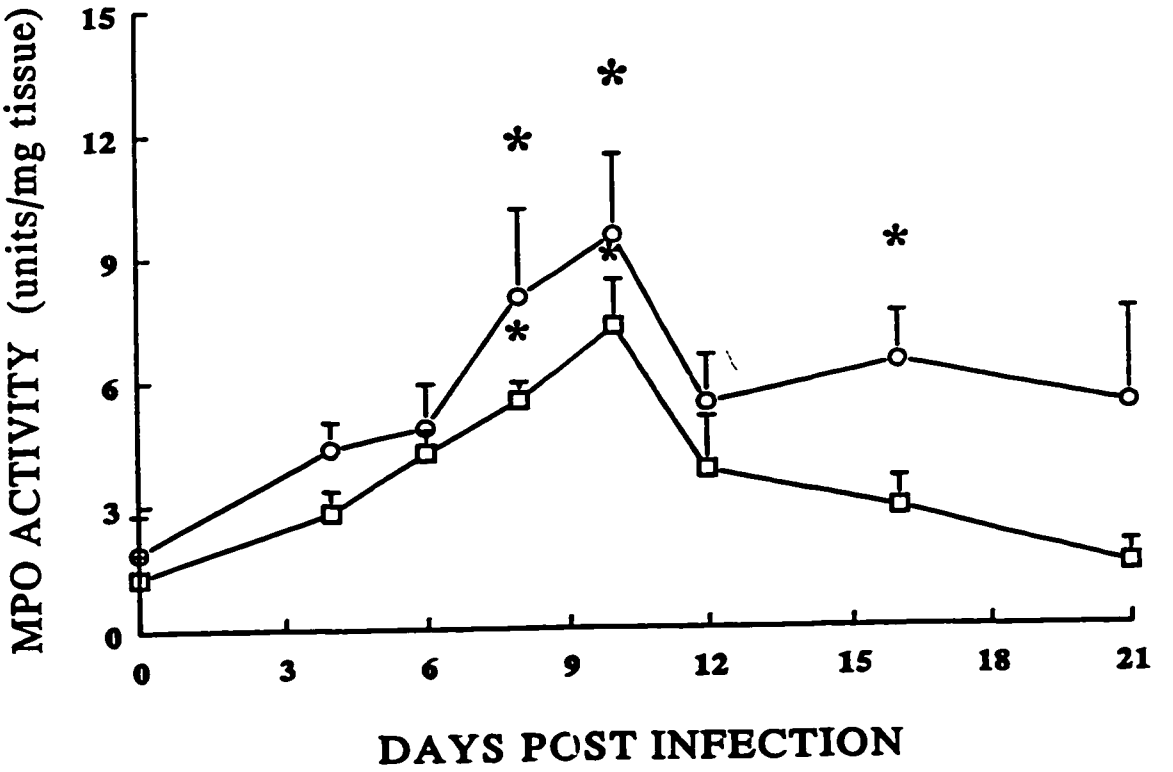

Figure 2.3: Myeloperoxidase activity (MPO) from the small intestine of B10.BR mice (open circles) and NIH Swiss mice (open squares) over the indicated time post-infection. Results are means ± SE of groups of 4-6 animals. The asterisk denotes a significant difference ($p < 0.05$) compared to the control MPO activity for the same mouse strain.

2.3.3 Muscle Contraction

2.3.3.1 Optimal Tension.

As shown in Figure 4, there was no significant difference between the two strains of mice under control conditions in terms of the amount of applied tension required to generate a maximum contraction to carbachol (ie. optimal tension (T_O)). After infection, both strains developed a biphasic pattern of significantly increased T_O, where the first rise in T_O was evident on days 4-6 P.I. By day 10 P.I., the T_O had dropped to approximately control levels, followed by a second smaller but still significant elevation as of day 12 P.I. in both strains. The rise and fall pattern of optimal tension seen during the infection was remarkably similar in the two strains, except between days 4 and 8 P.I., at which time the NIH Swiss mice reached their maximum T_O as the culmination of their first peak in the biphasic pattern of T_O increase. In contrast, by day 8 P.I., the T_O of B10.BR mice was already past its first rise in T_O and had dropped significantly lower than the T_O of the NIH Swiss on day 8 P.I.

2.3.3.2 Responses to Carbachol

As shown in Figure 5, the response to 1μM carbachol in both mice strains, increases in a time-dependent manner following *T.spiralis* infection with tension generation significantly elevated over control at all time points after day 4 P.I. In NIH Swiss mice, the maximum increase was observed at day 8 P.I., 4 days before the maximum increase was seen in B10.BR mice. In addition, the maximum increase in carbachol-induced contraction was substantially (50%) greater in NIH Swiss mice compared to that observed in the B10.BR mice. Interestingly, the response to carbachol remained significantly elevated above control by day 28 and 35 P.I. in the NIH Swiss but not the B10.BR strain (data not shown).

The dose response relationships for carbachol induced contraction for NIH Swiss and B10.BR mice are shown in Figures 6 and 7 respectively. For the NIH Swiss on day 8 P.I., the applied tension was 1100 mg whereas 800 mg was applied to day 12 P.I. B10.BR tissues. In both non-infected and day 8 P.I. NIH Swiss mice, the response to carbachol was maximal between 10 and 100 uM carbachol. There was no significant difference

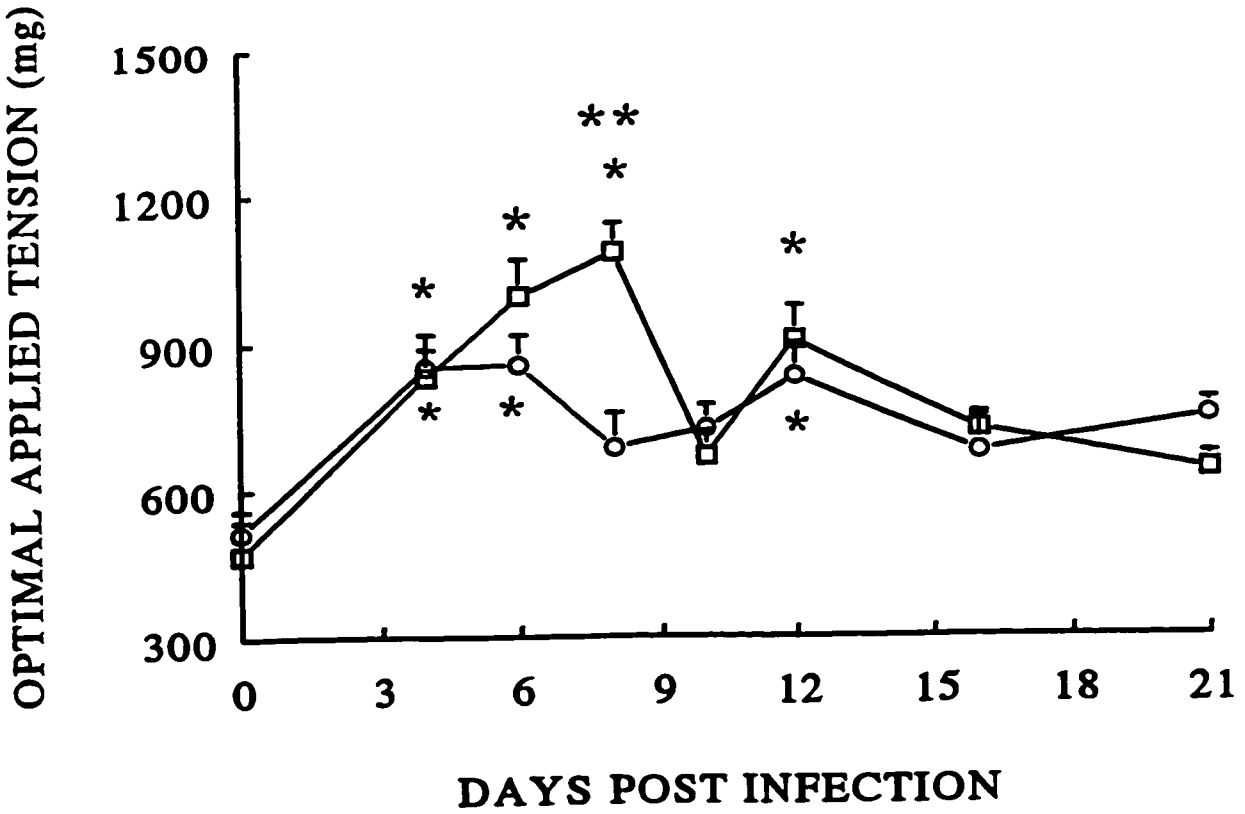

Figure 2.4: Optimal tension (in mg) applied to muscle strips in order to generate a maximal contractile response to 1uM carbachol in B10.BR mice (open circles) and NIH Swiss mice (open squares). Results are the means ± 1 SE of groups of 4-6 animals. The single asterisk (*) denotes a significant difference ($p < 0.05$) from the control value in the same mouse strain. The double asterisk (**) indicates a significant difference ($p < 0.05$) between NIH Swiss and B10.BR mice at the same time point.

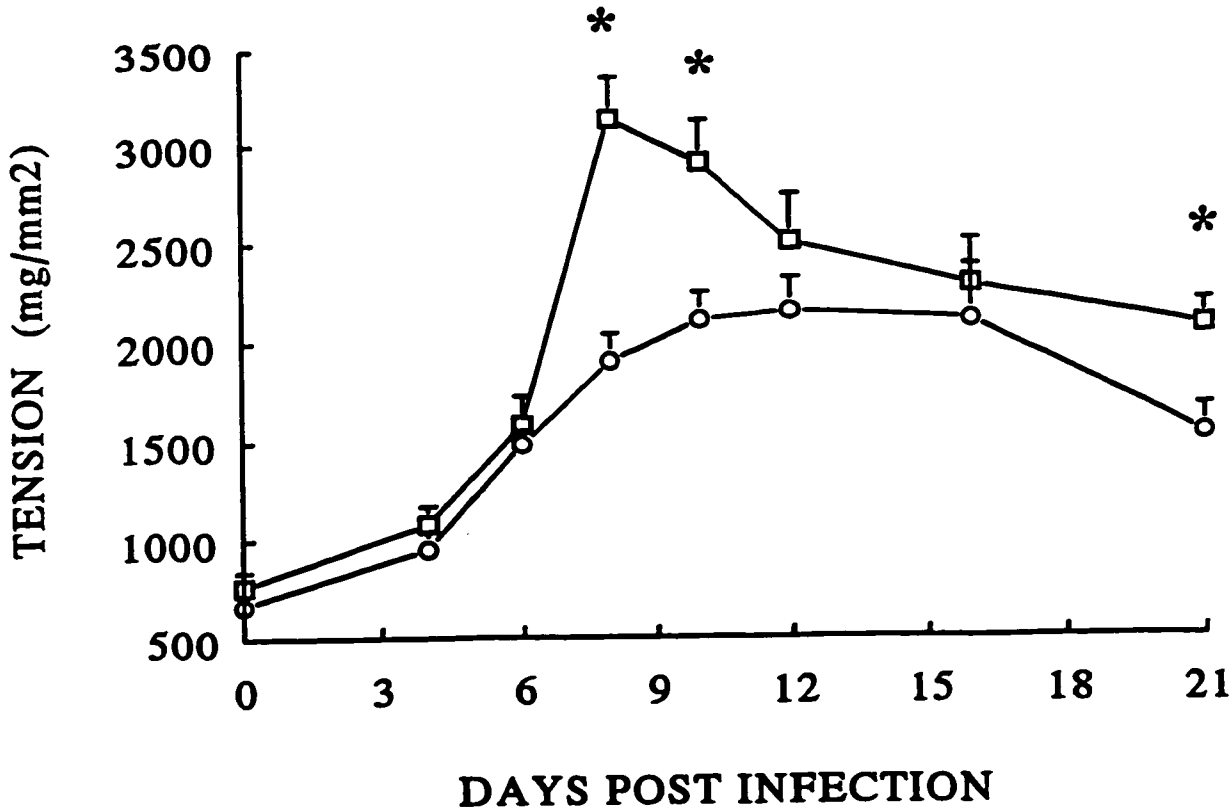

Figure 2.5: Maximum tension generated by muscle in response to 1uM carbachol over the indicated time course of the infection in B10.BR mice (open circles) and NIH Swiss mice (open squares). Results are the means ± 1 SE of groups of 4-6 animals. The asterisk indicates a significant ($p < 0.05$) difference between the responses obtained in the two strains for a given time point.

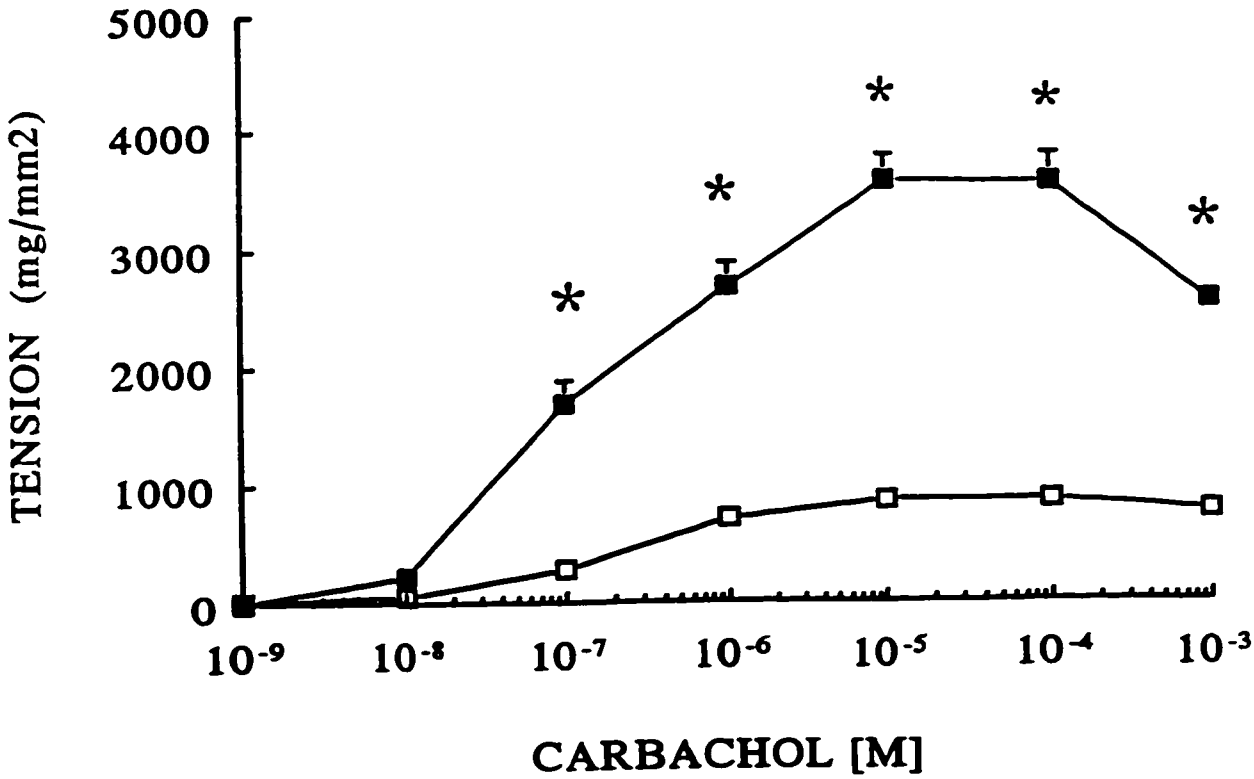

Figure 2.6: The dose response relationships for carbachol-induced contraction of muscle from control NIH Swiss mice (open squares) and mice of the same strain infected 8 days previously with *T.spiralis* (closed squares). Results are the means ± 1 SE of groups of 4 animals. The asterisk indicates a significant ($p < 0.05$) difference between control and infected mice at a given carbachol concentration.

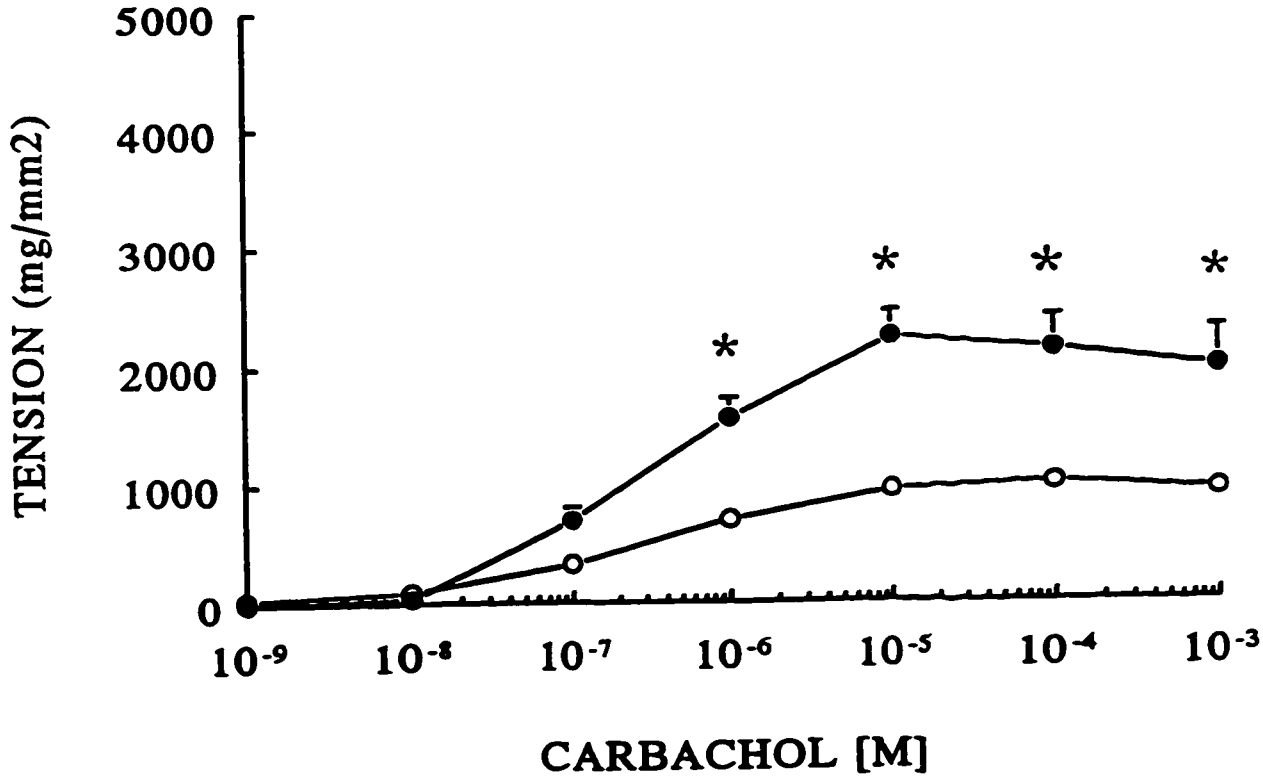

Figure 2.7: The dose response relationships for carbachol-induced contraction of muscle from control B10.BR mice (open circles) and mice of the same strain infected 12 days previously with *T.spiralis* (closed circles). Results are the means ± 1 SE of groups of 4 animals. The asterisk indicates a significant ($p < 0.05$) difference between control and infected mice at a given carbachol concentration.

in the ED_{50} value for carbachol between control NIH Swiss mice and that observed on day 8 P.I. ($1.34 \pm 0.84 \times 10^{-7}$ M vs $6.3 \pm 0.9 \times 10^{-8}$ M respectively). A similar profile was observed in the B10.BR mice where the ED_{50} values were $2.14 \pm 0.99 \times 10^{-7}$ M in controls and $1.50 \pm 0.83 \times 10^{-7}$ M on day 12 P.I.. While neither strain showed a significant change in ED_{50} for carbachol during the infection, a comparison of Figures 6 and 7 further underscores the observation that the magnitude of contraction generated by carbachol in infected tissues was greater in NIH Swiss than B10.BR and this occurred over a broad range of concentrations.

2.4 DISCUSSION

This study was prompted not only by the availability of mouse strains that exhibit differences in their ability to expel nematode parasites from the gut, but also by a general need to switch species from the rat to the mouse, in order to facilitate our work on the immunomodulation of intestinal smooth muscle function. The mouse is a particularly useful model because of the diversity of immunological reagents and cell markers that are available for this species. Unfortunately, studies of physiological mechanisms in the murine intestine have been constrained due to the relative fragility and difficulty in handling small amounts of delicate tissue. Once these technical difficulties are overcome, however, the advantages of using mouse models to examine immunophysiological mechanisms should in most circumstances, outweigh the benefits of other model species. It was therefore reassuring to observe similarities between this study and those previously conducted in the *T.spiralis*-infected rat (28) which show that infection-induced inflammation of the small intestine is accompanied by a time-dependent increase in tension development by smooth muscle.

Our results support previous studies which demonstrate that certain mouse strains exhibit diverse abilities to expel *T. spiralis* infection (2). Several studies have shown that NIH Swiss derived strains, such as the NIH, NFR/N and NFS/N mice expel *T. spiralis* infections extremely rapidly during a primary infection (2,29). In contrast, the same studies have demonstrated that B10.BR mice expel the parasite very slowly and often develop a chronic infection (2,29). While previous studies have addressed the kinetics of worm expulsion (2,29) and have

explored underlying immunological mechanisms (29,35), no previous study has addressed pathophysiological changes that may contribute to the ability of the host to expel the parasite from the gut.

The main finding in the present study was the difference in the profile of altered muscle contractility between the NIH Swiss and B10.BR mice following infection. The NIH Swiss strain developed significantly greater tension than the weaker responder B10.BR mice during the course of infection. In addition, there were significant differences in the length-tension characteristics between the two strains post-infection; more stretch (applied tension) was required to demonstrate maximal responses to carbachol in NIH Swiss compared to B10.BR and this was most evident days 4 through 8 P.I. The increase in optimal applied tension may reflect tissue edema and changes in the extracellular matrix during inflammation, rendering the full-thickness tissue sections less compliant or alternatively, since inflammation is known to increase the actin content of intestinal muscle in the rat (32), the difference between strains could reflect more pronounced changes in the contractile protein content of smooth muscle in the NIH Swiss mouse, compared to the B10.BR strain. It is, however, unclear whether the differences in altered contractile capabilities are based upon dissimilar tissue responses to the nematode-induced inflammation, since in both strains, a similar degree of intestinal muscle thickening was observed. The peak in increased tension developed earlier in the NIH Swiss mouse compared to the B10.BR (day 8 vs day 12 P.I.). Interestingly, the increased contractility observed in the NIH Swiss mice persisted after worm expulsion was complete and the inflammatory reaction had subsided. While the genetic determinants influencing the degree and persistence of the altered muscle function seen in these two strains are unclear, the observations made in this study suggest that the development and degree of intestinal muscle dysfunction following intestinal inflammation in Man (27) may be dependent upon genetic background, as well as other factors.

We believe that differences in the magnitude and kinetics of the contractility changes in muscle between NIH Swiss and B10.BR mice do not reflect differences in worm distribution or the severity and duration of the mucosal inflammatory response. Indeed, the increase in MPO activity, which is a reliable marker of the acute inflammatory reaction in the gut (21), was not significantly different between the two strains. Moreover, the time course of altered muscle contractility was not congruous with that of MPO activity. Myeloperoxidase is formed by granulated cell types, such as neutrophils and eosinophils, which constitute a major component of the

acute inflammatory response to nematodes (17,33,34) and have been considered important, along with mucosal mast cells, in worm expulsion (15,34). However, blocking eosinophilia and even mucosal mastocytosis has failed to prevent worm expulsion (5,14). These findings, as well as infection studies using athymic rodents, suggest that other components of the inflammatory response, such as T lymphocytes, (which do not contain myeloperoxidase) play an important role in worm expulsion.

The role of T lymphocytes in the expulsion of nematode parasites has been investigated (20), using a variety of mouse strains, including NIH and B10.BR mice. Although there is no unanimity, it is generally believed that nematode expulsion following a primary infection is mediated by a Th2 cytokine-producing T cell population (8,35). Studies using *Nippostrongylus brasiliensis* infections suggest that Th2 cytokines are predominant at the site of intestinal infection and that the cytokine interleukin-4 (IL-4) may be critical in the immune response to helminth infections (23,24). Recent studies with SCID mice infected with *N. brasiliensis* and *Heligosomoides polygyrus* suggest that during infection, IL-4 may act directly on non-lymphoid intestinal cells to create an environment that is unfavorable to the parasite (24). Taken together, these findings suggest that T cells play a major role in the eviction of nematode and other parasites from the gut and that Th2 cytokines may mediate expulsion, with a particular emphasis on IL-4. It should be noted that both strong responders, such as NIH, as well as poor responders such as B10.BR have been shown to develop Th2 responses following *T. spiralis* infection, as evidenced by IL-4 secretion from stimulated mesenteric lymph node cells (8,20), however NIH cells appear to respond more readily to antigen stimulation than B10.BR cells.

Studies in the rat have also shown that the increased contractility of smooth muscle that accompanies *T.spiralis* infection in the rat is dependent upon T lymphocytes, which accumulate in the muscle layers of the inflamed gut during nematode infection (6); the increase in contractility was absent in muscle from infected athymic rats but was present in infected T cell reconstituted athymic rats (26). Preliminary data suggest a similar mechanism in T.spiralis infected Balb/c mice (25). It is known that mouse strains, such as NIH mice, that are strong in their ability to expel nematodes show a greater proliferative T lymphocyte response to *T. spiralis* infection than poorer responders, such as B10.BR (35). Taken together, these findings raise the possibility that the more prominent changes in muscle contractility seen in the NIH Swiss mouse reflect an enhanced T

lymphocyte response to the infection. If indeed T cell mediated changes in muscle contractility in nematode infected mice contribute to the eviction of the parasite from the gut, IL-4 is a putative mediator. This notion is supported by preliminary data showing that IL-4 is able to increase intestinal smooth muscle contraction (7). Studies are in progress to formally evaluate the role of IL-4 in the development of increased contractility of intestinal muscle in the nematode-infected mouse.

This study correlates increases in intestinal muscle contractility with the ability of mice to expel *T.spiralis* from the gut. The process of expulsion contains components of eviction as well as neutralization of the parasite (17), which can retain its fecundity upon recovery following expulsion (11). It is thus tempting to speculate that changes in muscle function and increased propulsive forces in the gut participate in the eviction of the parasite from the host and this eviction may be aided by other enteric physiologic changes such as increased fluid secretion into the lumen of the bowel. In the *T.spiralis*-infected rat, increased muscle contractility is evident in the jejunum but not in the distal ileum (28), thus creating an aboral gradient in force generation by muscle. In vivo studies have shown rapid intestinal transit during *T.spiralis* infection (3,22). Furthermore, evidence of increased propulsive activity was found in extrinsically denervated intestinal segments taken from *T.spiralis* infected guinea pigs (1), indicating that tissues intrinsic to the gut wall, such as smooth muscle, are responsible for generating this aboral force. More recent work on helminth infection has utilized WW^v and bone marrow reconstituted WW^v mice. *N. brasiliensis* infected WW^v mice exhibit a delayed worm rejection (12) which was only partially restored by bone marrow reconstitution (10). While the reconstitution normalized the host response to the migratory or lung phase of the infection, the higher susceptibility and slower expulsion of the parasite seen in the intestinal phase in the WW^v mice remained unchanged after reconstitution (10). This supports the concept that myeloid cells, such as mast cells, are not pivotal in the process of worm eviction in the intestinal phase of the infection. Interestingly, the WW^v mouse exhibits highly abnormal intestinal motility (9), adding further to the notion that motor function is an important determinant of successful expulsion of parasites from the nematode infected gut.

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The following work was published in the American Journal of Physiology and continues from the previous publication. The experiments use the same model - examining jejunal longitudinal muscle function in *T. spiralis* infected mice, however in this study we examined the role of T lymphocytes in the changes in muscle function. We show that mice that possess T cells develop an early increase in muscle contraction which is sustained until at least day 21 PI. In contrast, mice which lack T cells are delayed in the onset of muscle hyper-contractility and this response is not sustained. We were able to restore both the early and the sustained responses in T cell deficient mice given T cells. This is the first demonstration that there are both T cell dependent and independent phases of enteric muscle hyper-contractility during *T. spiralis* infection.

Chapter 3

T lymphocyte dependent and independent intestinal smooth muscle dysfunction in the *T. spiralis* infected mouse

B.A. Vallance, K. Croitoru, S.M. Collins

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ABSTRACT

We examined the profile of increased intestinal muscle contractility following primary infection with *Trichinella spiralis* in the mouse, correlating it with parasite expulsion. We also examined the extent to which the changes in muscle contraction were T lymphocytes dependent, by infecting athymic and SCID mice. Infection was accompanied by increased tension development by intestinal muscle. Two components of this response were identified, a rapid peak increase in tension generation observed on day 6 post-infection, and a smaller but sustained increase in tension evident thereafter in euthymic BALB/c mice. The peak muscle response was significantly delayed in infected athymic and SCID mice, along with a corresponding reduction in the magnitude of the sustained component. These changes were accompanied by reduced parasite expulsion in athymic and SCID mice, compared to euthymic mice. Reconstitution of T cell function in athymic mice restored both the acute and sustained profile of muscle contraction seen in euthymic mice, and this was accompanied by faster expulsion of the worms. These results identify T cell dependent and independent components of the muscle response to nematode infection in the mouse, and suggest that the onset of the peak contractile response, as well as the magnitude of the sustained muscle response, contribute to parasite eviction from the gut.

3.1 INTRODUCTION

The enteric phase of a primary nematode infection is associated with structural and functional changes in the intestinal mucosa (25). In addition, studies from this and other laboratories have shown that *Trichinella spiralis* infection also induces changes in the deeper neuromuscular layers of the intestine, accompanied by accelerated intestinal transit (4,6,38,41,45). Since the increased propulsion is observed in extrinsically denervated gut segments (1), the changes in propulsive activity must reflect changes in those components of the motility apparatus that are intrinsic to the bowel wall, thus implicating enteric nerves and muscle.

Previous studies have demonstrated that these changes in intestinal physiology are influenced by the hosts immune response to the invading parasite (9,13,46). We have postulated that the increased propulsive activity is due to a recruitment of the intestinal motor system by the host immune response, enabling it to act as an extension of the immune system in evicting the parasite from the gut. In the rat, both worm expulsion and increased tension generation by intestinal muscle are attenuated in the absence of functioning T lymphocytes (46), suggesting a common immunologic mechanism. We have continued to pursue this investigation in mouse in order to exploit a broader range of investigative tools. In our first study in the mouse, we showed that *T. spiralis* infection was accompanied by increased tension development by intestinal muscle. Interestingly, we showed that the magnitude of this increase was greater in mouse strains that evicted the parasite rapidly (eg. NIH Swiss) (2,41) compared to that seen in mice that evicted the parasite slowly and incompletely, such as the B10.BR strain (41). These observations strengthened the association between worm expulsion and changes in muscle contractility, and prompted a closer evaluation of this relationship and underlying immunological mechanisms.

The present study was undertaken to investigate the time course of changes in muscle contraction in relation to worm expulsion in the mouse, and to determine the extent to which contractility changes are mediated by T cells. The previous study in rats examined only one time point post-infection (PI), and left open the possibility that other immunological or inflammatory mechanisms may contribute to enhanced muscle contractility within the overall time frame of primary infection by this nematode parasite.

The results from this study identify two distinct components of increased contractility in the mouse intestine following *T. spiralis* infection. There is an early phase that occurs rapidly within 6 days of infection, followed by a smaller sustained phase that persists for up to 21 days. In athymic and SCID mice, the early phase is delayed several days and the sustained phase is reduced in magnitude. This attenuated profile of muscle contractility is accompanied by a delay in worm expulsion. Reconstitution of T cell function in athymic mice is accompanied by restoration of the profile of muscle contraction seen in euthymic mice, and by faster worm expulsion. These results suggest that both the timing and magnitude of muscle changes are important in worm expulsion, and that there are both T cell dependent and

independent mechanisms that underlie the enhanced contractility of intestinal smooth muscle in this model.

3.2 MATERIALS AND METHODS

3.2.1 Mice

Athymic (nu/nu) BALB/c mice as well as their heterozygous (nu/+) euthymic littermates were purchased from the National Cancer Institute (Frederick, MD), while CB.17 SCID/beige mice, which are congenic with BALB/c mice, were bred and kept in the animal facilities of McMaster University, Hamilton, Ontario, Canada. For these experiments, male mice, aged 4-6 months (in order to be age-matched to T lymphocyte reconstituted mice), were used. Mice were kept in sterilized and filter-isolated cages, under specific pathogen free (SPF) conditions, in groups of four to five in positive pressure rooms with a constant ambient temperature and photoperiod (14:10 h light-dark cycle). All experiments were approved by the Animal Care Committee at McMaster University and were conducted in accordance with the guidelines of the Canadian Council on Animal Care.

3.2.2 *Trichinella* Infection

Mice were infected by administration of 0.1 ml of PBS containing approximately 375 *Trichinella spiralis* larvae by gavage. Athymic, euthymic and reconstituted mice were infected together, with the same larval preparation to minimize differences. SCID/beige mice were infected in separate experiments along with euthymic BALB/c mice, tested as controls to ensure that the infections were successful. The larvae were obtained from rodents 30-90 days post-infection using a modification of the technique described by Castro and Fairbairn (5). The *T. spiralis* parasites used in this study originated in the Department of Zoology at the University of Toronto and the colony was maintained through serial infections alternating between male Sprague-Dawley rats and male CD1 mice.

3.2.3 Worm Counts

Mice were sacrificed by cervical dislocation and the entire length of the small intestine was removed. All the adult worms within the small bowel were then counted using a modification of the method described by Castro and Fairbairn (5). Briefly, the intestine was opened longitudinally, the mucosa was separated from the underlying muscularis by scraping with a glass microscope slide and mixed in 1 ml of PBS. The worms were then counted using a scored petri dish and an inverted microscope and expressed as the number of adult worms recovered per mouse. In accordance with established practices, worm rejection was considered complete when at least 98% of the infective dose had been expelled from the gut (47).

3.2.4 Myeloperoxidase Assay

MPO is an enzyme contained in the azurophilic granules of neutrophils as well as other myeloid cells and is commonly used as an index of neutrophil infiltration and inflammation (32). MPO was measured using a modified version of an assay as previously described (49). After sacrifice, samples of the jejunum (50-100mg), approximately 4 cm distal to the ligament of Treitz were removed for MPO measurement. The samples were snap frozen in liquid nitrogen and stored at -70°C. The activity of MPO is reported as units of MPO per mg of wet tissue where a unit of MPO is defined as the quantity of enzyme able to convert 1 mmole of hydrogen peroxide to water in 1 minute at room temperature.

3.2.5 T Lymphocyte Reconstitution

Euthymic BALB/c mice, 6-10 weeks old, were sacrificed by cervical dislocation, their spleens removed aseptically, placed in RPMI media with 10% fetal bovine serum, cut into small pieces and crushed between two sterile glass slides. Once a single cell suspension was generated, the cells were placed in covered culture dishes in a 37°C incubator and incubated for 2 hours. Then non-adherent cells were removed and spun down over Ficoll-Hypaque (Pharmacia, Sweden), for 20 minutes at 1500 rpm. The buffy coat of mononuclear cells was collected, washed twice with RPMI media and then pelleted

through centrifugation and resuspended in a small volume of phosphate buffered saline (PBS). The cells were then counted, analyzed for viability using Trypan blue exclusion, and 30 million viable cells were injected intravenously (iv) into the tail vein of recipient athymic mice (6-10 weeks of age). The reconstituted athymic mice were then left for 2-3 months, allowing sufficient time for the re-population of their T lymphocyte complement.

3.2.6 Determination of Lymphocyte Reconstitution by FACS Analysis

To confirm the immunodeficiency of the SCID/beige and athymic mice, and to assess the success of T lymphocyte reconstitution, SCID/beige mice, athymic and euthymic BALB/c mice, as well as T cell reconstituted athymic mice (2-3 months after reconstitution) were euthanized, their spleens removed and prepared as for reconstitution experiments. The isolated mononuclear cells were then stained and analyzed by FACS (FACScan flow cytometer, Becton Dickinson, Mountain View, CA) with a specific biotinylated mAb to CD3 (2C11), followed by streptavidin-phycoerythrin (PE) (Becton Dickinson). Cells were then fixed in 0.1% paraformaldehyde and analyzed on a FACScan. Data analysis was performed using the PClysis software (Becton Dickinson).

3.2.7 Measurement of Muscle Contraction

The preparation of sections of jejunal longitudinal muscle for muscle contractility analysis and the analysis of the length-tension relationships have been described previously (41). In brief, the jejunum was removed and placed in oxygenated (95% O_2 - 5% CO_2) Kreb's solution and 1 cm sections of whole gut were cut from the jejunum, beginning at the ligament of Treitz and proceeding distally. The lumen of each segment was flushed with Kreb's buffer, prior to the insertion of short (2-3 mm) lengths of Silastic tubing (0.065 OD, 0.030 ID) (Dow Corning, Midland, MI) into the open ends of the gut segments. Tubing was then tied in place with surgical silk. The insertion of the tubing was found to maintain patency of the gut segments over the course of the experiments. Segments were then hung in the longitudinal axis and attached at one end to a grass FT03C force transducer (Grass, Quincy, MA) and responses were recorded

on a Grass 7D polygraph. Tissues were equilibrated for 30 minutes at 37°C in Kreb's solution, oxygenated with 95%O_2 - 5%CO_2 before starting the experiment. Experiments were then conducted to examine the length-tension characteristics of the muscle before and after infection. Segments were stretched by applying tension equivalent to 0 - 1250 mg of weight, and contraction was assessed following stimulation with 1 mM carbachol (Sigma Chemical, St. Louis, MO). Initial experiments indicated that this tension range was sufficient to determine the maximal responsiveness of both control and inflamed tissues at this carbachol concentration. Tissues were then rinsed twice and equilibrated at the next tension level in fresh Kreb's for 15 minutes, prior to the next addition of carbachol. At the end of each experiment, tissue segments were removed, blotted and weighed. The degree of applied tension or stretch producing the maximum response to carbachol was identified as the optimal tension (T_O) and the tissue length at this tension was then used to calculate the cross sectional area of the tissue.

3.2.8 Data Presentation and Statistical Analysis

Responses to carbachol were recorded from tracings, followed by the calculation of contractile activity, which were expressed as milligrams of tension per cross sectional area as described previously (37). For each mouse, the mean tension was calculated from at least three segments. The results comparing euthymic, athymic and reconstituted mice were pooled from three separate infections, all of which gave similar results, while the SCID mouse data are from an additional two infections, which were similar and were also pooled. Results are expressed as the means ± 1 SEM; n refers to the number of mice tested. Statistical significance was calculated using the Student's t test for comparison of two means or a one way of analysis of variance (ANOVA) for the comparison of three or more means. Multiple comparisons were performed using the Neuman-Keuls multiple comparison test. $P < 0.05$ was considered significant.

3.3 RESULTS

3.3.1 Euthymic BALB/c Mice: Muscle Contraction.

During the course of a *T. spiralis* infection, euthymic BALB/c mice developed a significant increase in isometric tension development by jejunal longitudinal smooth muscle. The data shown represent the contractile response to a sub-maximally effective concentration of carbachol (1mM) at each time point post-infection. As shown in Figure 1A, in BALB/c mice there are two components of this contractile response to carbachol. There is an early phase which is rapid in onset, occurs by day 6 post-infection (PI), which is also the time point of maximum contraction. Subsequently, the magnitude of the contractile response decreases.

The second component is the sustained phase of increased contraction which persists until at least day 21 PI. This biphasic contractile profile is seen in other immuno-competent *T. spiralis* infected mice such as the NIH Swiss strain (41).

3.3.2 T Cell Deficient Athymic BALB/c Mice: Muscle Contraction.

In contrast to the pattern seen with euthymic mice, the rapid phase of contraction was delayed in onset until day 8 PI in athymic BALB/c mice, and did not peak until day 12 PI (see Figure 1B). In addition, the sustained phase of contraction was significantly smaller in magnitude than that seen in euthymic BALB/c mice, with the tension generated at day 14 PI and thereafter, not significantly different from control.

3.3.3 T Cell Deficient CB.17 SCID/beige Mice: Muscle Contraction.

The pattern of increased isometric tension development by longitudinal muscle from infected SCID/beige mice is also shown in Figure 1B and is similar to that seen in infected athymic mice. The peak component of increased contraction was delayed until day 12 PI. The increased contraction was not well sustained, such that by day 21 PI, values were not different from those of uninfected SCID/beige mice.

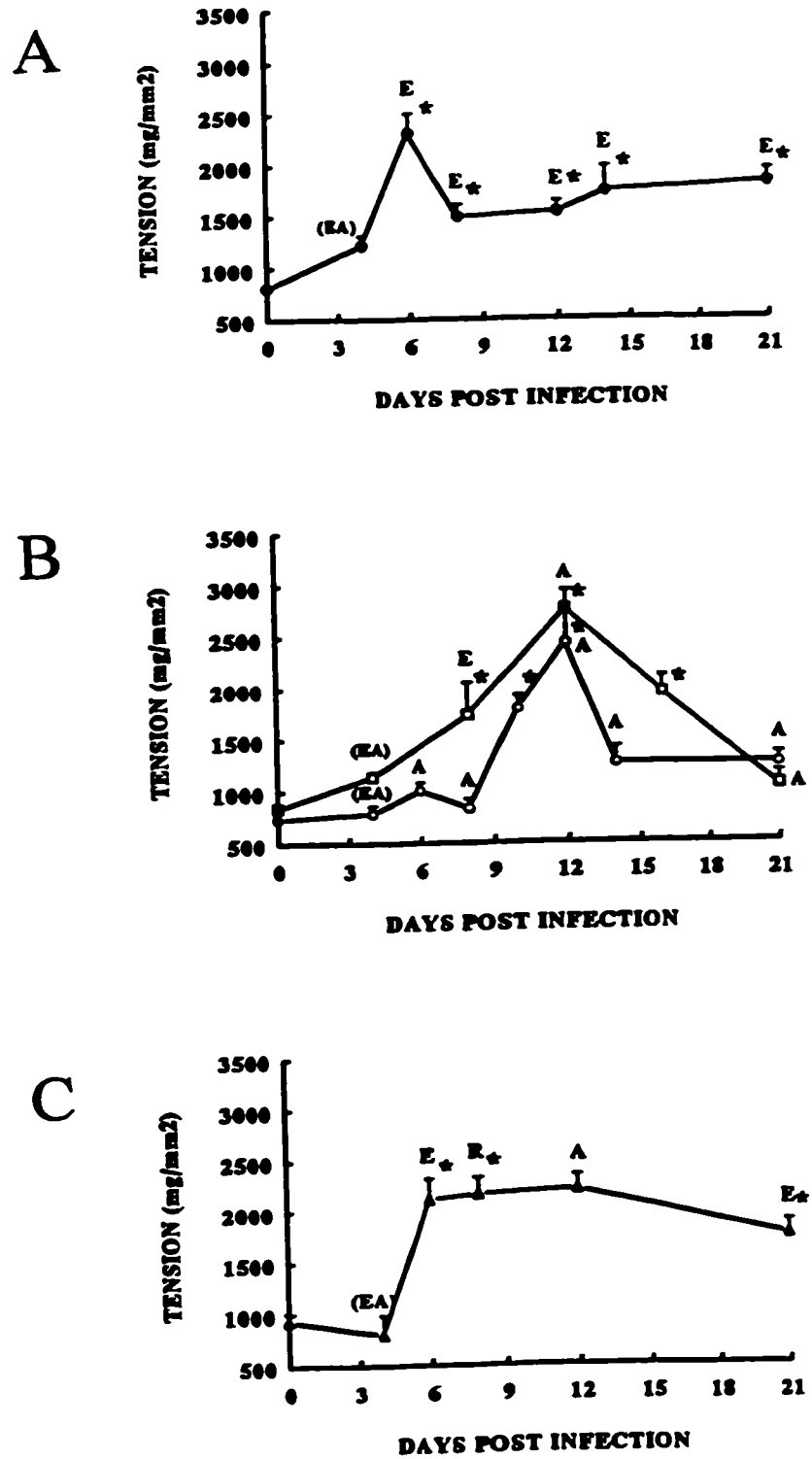
Figure 3.1: Panel A: Maximum tension generated by muscle in response to 1 mM carbachol over the indicated time course of the infection in euthymic BALB/c mice (closed circles), the thymus bearing littermates of athymic BALB/c mice. Results are the mean ± 1 SEM of groups of 4-6 animals, The asterisk indicates tension generation significantly greater than that generated by uninfected mice, as determined by a Students t test (P <.05).

Panel B: Maximum tension generated by muscle in response to 1 mM carbachol over the indicated time course of the infection in athymic BALB/c (open circles) and CB.17 SCID/beige mice (open squares). Results are the mean ± 1 SEM of groups of 4-6 animals. The asterisk denotes a significant increase in tension generation when compared to uninfected mice of the same strain, as determined by a Students t test (P <.05).

Panel C: Maximum tension generated by muscle in response to 1 mM carbachol over the indicated time course of the infection in T cell reconstituted athymic BALB/c mice (closed triangles). Results are the mean ± 1 SEM of groups of 4 animals. The asterisk indicates tension generation significantly greater than that generated by athymic mice at the same time point post-infection, as indicated by a Students t test (P <.05)

All data are ranked with analysis performed by ANOVA (P <.05), with comparisons made by the Neuman- Keuls multiple comparison test. E indicates no significant difference from euthymic mice at the same time point, but a significant difference from athymic mice. A indicates no significant difference from athymic mice, but a significant difference from euthymic mice at the same time point. (EA) indicates no significant difference from either euthymic or athymic mice, while R indicates a significant difference from both euthymic and athymic mice - again at the same time point. No significant difference was seen between any of the strains at day 0.

Figure 3.1


A
TENSION (mg/mm2)
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3000
2500
2000
1500
1000
500
0
3
6
9
12
15
18
21
DAYS POST INFECTION
(EA)
E
B
A
C
R

3.3.4 T Cell Reconstituted Athymic BALB/c Mice: Muscle Contraction.

T cell reconstitution of athymic mice resulted in restoration of a profile of contraction similar to that seen in euthymic BALB/c mice. The onset of the early phase of contraction occurred by day 6 PI. compared to day 10 PI in athymic mice, and the sustained phase of contraction persisted from day 8 until at least day 21 PI when its magnitude was significantly greater than that seen in athymic mice on day 21 (compare Figures 1B and 1C). Similar to infected euthymic mice, tension generated by reconstituted mice on day 21 PI was also significantly greater than that generated by uninfected mice.

3.3.5 Evaluation of T cell Deficiency and T Cell Reconstitution

As illustrated in Figure 2, less than 1.0 % of the splenic cells recovered from SCID/beige mice and only 3.0 % of the mononuclear population recovered from the spleen of athymic BALB/c mice were CD3 +ve, consistent with their deficiency in the generation of T lymphocytes (20,29). The small number of positive cells seen in SCID mice likely represents only background, since SCID mice are considered to be almost completely deficient in T cell function. In contrast, euthymic BALB/c mice possessed a greater number of CD3 positive splenic cells (12.2 %) and reconstituted mice were found to possess an even greater portion of CD3 positive splenic cells (17.1 %) than the euthymic mice, reaching as high as 25% of the cell population (not shown), confirming the successful reconstitution of T lymphocytes in the mononuclear cell injected athymic mice.

3.3.6 MPO Activity In SCID, Euthymic, Athymic And Reconstituted Mice.

In contrast to the differences observed in muscle contraction between euthymic and athymic mice during infection, maximum MPO activity levels were not significantly different between infected euthymic and athymic mice (7.9 ± 1.5 vs 8.9 ± 2.6 units/mg tissue respectively). However, the onset of maximum MPO activity was delayed by 2 days in the athymic mice (day 8 PI) compared to the euthymic mice (day 10 PI), as shown in Figure 3. MPO was significantly elevated over control values on days 8-12 in

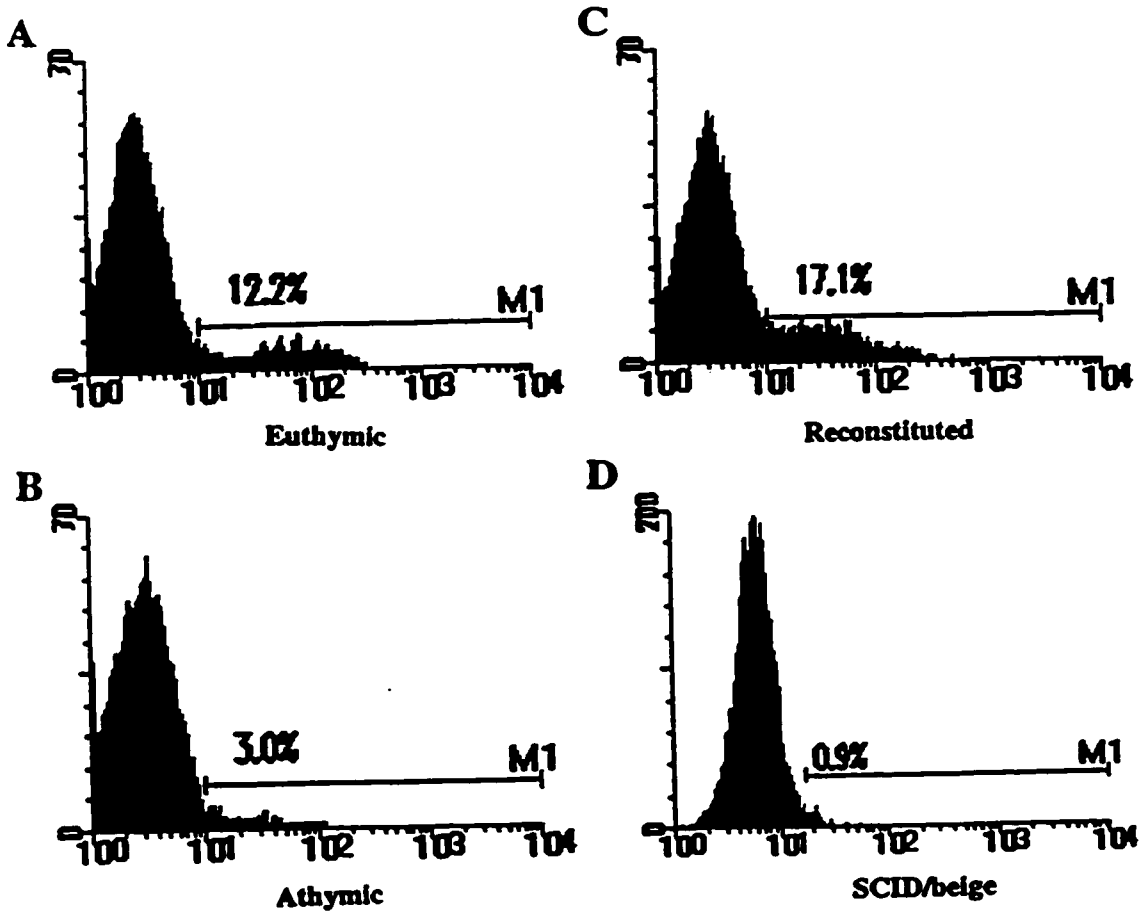
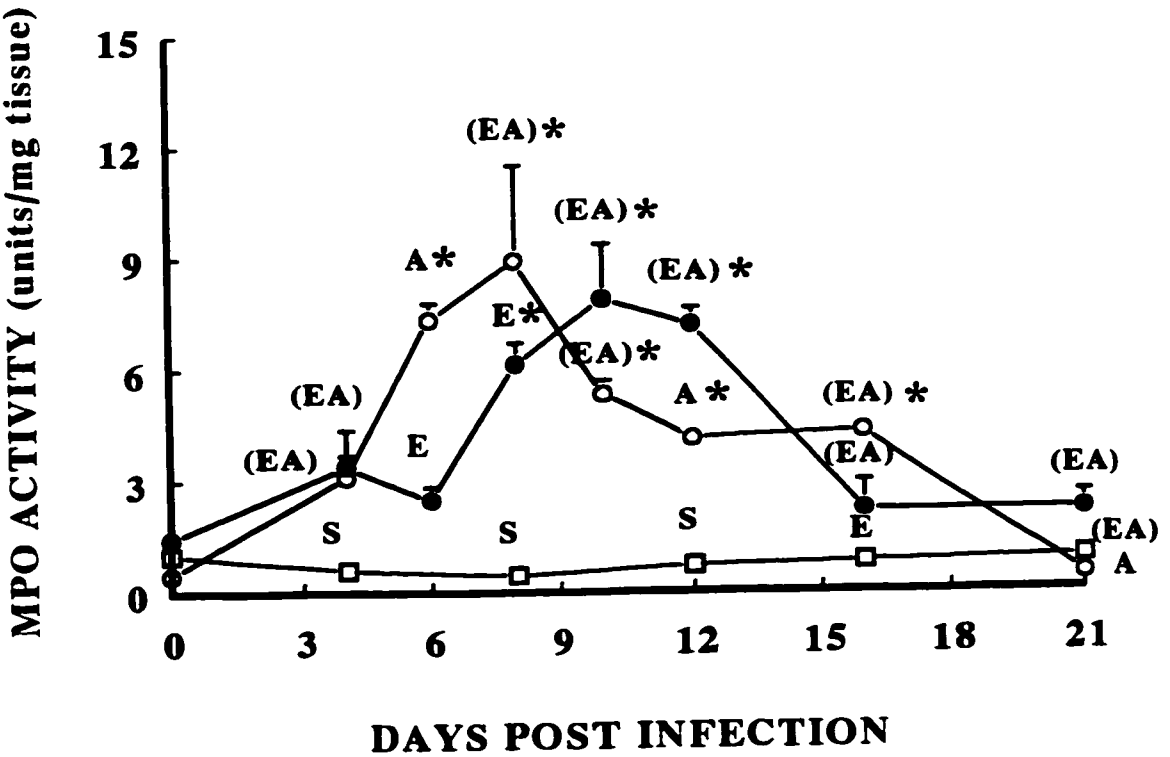


Figure 3.2: FACS analysis of mononuclear cells isolated from the spleens of euthymic (Panel A), athymic (Panel B) and T cell reconstituted athymic BALB/c (Panel C), as well as SCID/beige mice (Panel D). Cells were stained with a biotinylated anti-CD3 mAb followed by streptavidin-conjugated PE. The profiles of CD3 staining are displayed as histograms, after gating on small lymphoid cells. Numbers in histograms represent percentage of small lymphoid cells which were CD3 +ve.

Figure 3.3:Myeloperoxidase activity (MPO) from the small intestine of CB.17 SCID/beige mice (open squares) as well as athymic BALB/c mice (open circles) and their euthymic littermates (closed circles), over the indicated time course post-infection. Results are the mean ± 1 SEM of groups of 4-6 mice. The asterisk denotes a significant increase compared to uninfected mice of the same type, as determined by Students t test ($P < .05$). All data are ranked with analysis performed by ANOVA ($P < .05$), with comparisons made by the Neuman- Keuls multiple comparison test. E indicates no significant difference from euthymic mice at the same time point, but a significant difference from athymic mice. A indicates no significant difference from athymic mice, but a significant difference from euthymic mice at the same time point. (EA) indicates no significant difference from either euthymic or athymic mice, while S indicates a significant difference from both euthymic and athymic mice - again at the same time point. No significant difference was seen between any of the strains at day 0.


MPO ACTIVITY (units/mg tissue)
15
12
9
6
3
0
0
3
6
9
12
15
18
21
DAYS POST INFECTION
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(EA)*
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euthymic BALB/c mice and on days 6-16 in athymic BALB/c mice. MPO activity returned to normal levels by day 16 in euthymic mice and on day 21 in infected athymic animals. Reconstituted athymic infected mice also developed an increase in MPO activity with levels of 7.1 ± 1.3 units/mg tissue found on day 10 PI. Interestingly, no increase in MPO activity was found during *T. spiralis* infection in SCID/beige mice, indicating either a requirement for T and B cells in the recruitment of neutrophils to the infected gut, or possible effects of the beige mutation.

3.3.7 Recovery of parasites from the gut in euthymic, athymic and T cell reconstituted mice.

During infection of BALB/c mice, there appeared to be an early component that determined the survival of *T. spiralis* larvae to adulthood. Only 50% of the larvae gavaged into euthymic mice survived to be counted on day 4 PI, compared to 90-100% in athymic mice (see Figure 4). The larvae which survived to adulthood in the euthymic mice were then expelled between days 10 and 21 PI, with complete expulsion obtained by day 21 PI. In contrast, worm expulsion was slower and occurred in 2 phases in athymic mice, with the first phase of eviction occurring between days 8 and 10 PI, when approximately one-third of the worm burden was expelled. This first phase of expulsion corresponded with the transient increase in muscle contraction seen in this strain (see Figure 1B). Following this phase, worm numbers remained stable until day 16 PI, after which the athymic mice started to expel the remaining parasites. In contrast to euthymic mice, expulsion was incomplete in athymic mice on day 21 PI, with a small but significant number of worms (43 ± 11) remaining in the intestine on day 21 PI. This represented 11.5% of the total original infective load.

Intestinal worm numbers were also determined 21 days after infection in SCID/beige mice. Similar to athymic mice, worm expulsion was incomplete in these animals with 32 ± 13 adult *T. spiralis* worms recovered on day 21 PI. This again shows a role for T lymphocytes in the successful expulsion of a *T. spiralis* infection.

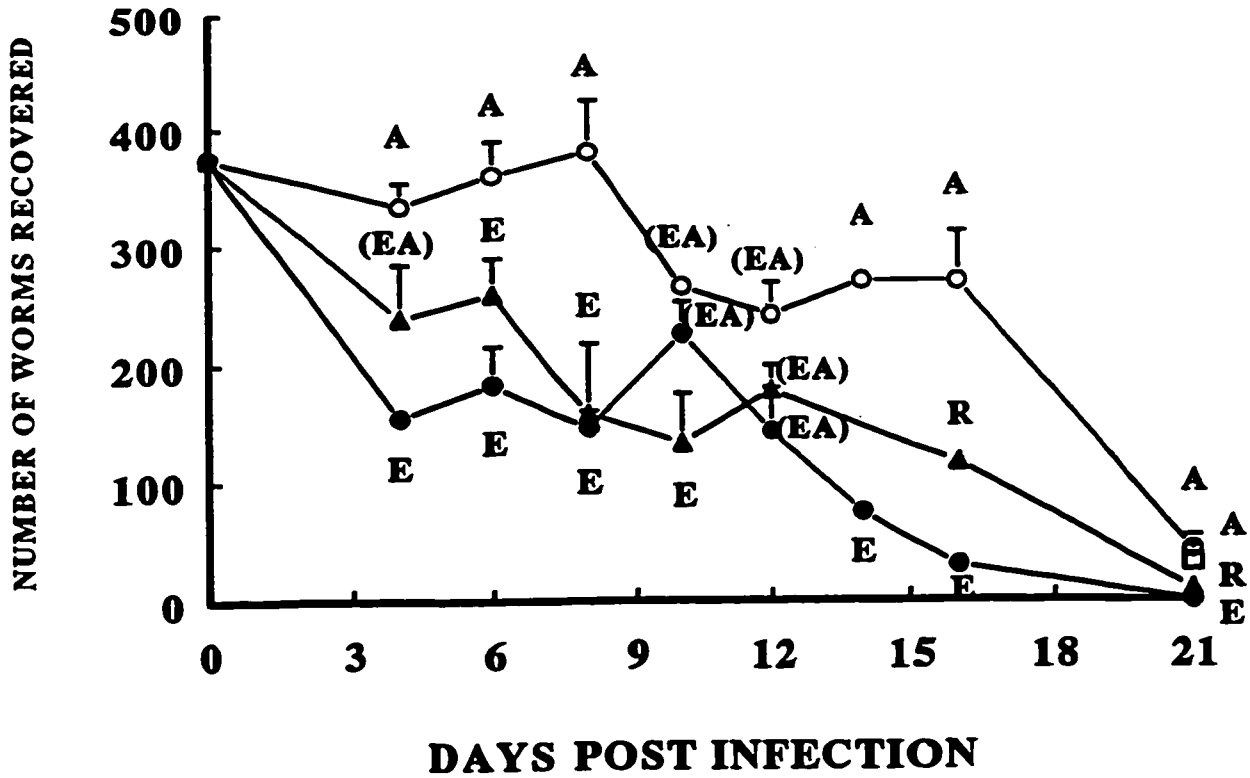

Figure 3.4: The number of adult *T. spiralis* worms recovered from the small intestine of infected athymic BALB/c (open circles), their infected thymus bearing littermates (closed circles) and T cell reconstituted athymic BALB/c mice (closed triangles) over the course of three weeks post-infection. SCID/beige data for day 21 PI are in open squares. Results shown are the mean ± 1 SEM of groups of 4-6 animals. Data are ranked with analysis performed by ANOVA ($P < .05$), with comparisons made by the Neuman- Keuls multiple comparison test. E indicates no significant difference from euthymic mice at the same time point, but a significant difference from athymic mice. A indicates no significant difference from athymic mice, but a significant difference from euthymic mice at the same time point. (EA) indicates no significant difference from either euthymic or athymic mice, while R indicates a significant difference from both euthymic and athymic mice - again at the same time point.

T cell reconstitution of athymic mice changed the survival curve of gavaged larvae to one that was intermediate (70%) between euthymic and athymic mice. The pattern of worm expulsion in reconstituted mice was also changed, becoming significantly more rapid than that found in unreconstituted athymic mice. Significantly fewer worms were recovered from reconstituted mice than unreconstituted athymic mice on days 6, 8, 10, 16 and 21 PI. Expulsion continued to completion by day 21 PI in the reconstituted mice, compared to the small but significant parasite load which remained in the gut of athymic mice on day 21 PI. However, worm expulsion from reconstituted mice did not quite reach that seen in euthymic mice, since reconstituted mice retained significantly more worms than euthymic mice on day 16 PI.

3.4 DISCUSSION

This study builds upon previous work in the rat showing that in that species, the increased tension development by intestinal muscle on day 6 post-*T.spiralis* infection was T cell dependent. The present study not only confirms that finding in mouse, but also identifies T cell independent components of contraction and, importantly, addresses the temporal relationship between worm expulsion and both T cell dependent and independent components of intestinal muscle contraction.

The hypothesis that T cells play a role in altering muscle function was based upon and supported by several studies, including the identification that the phenomenon of worm expulsion following primary infection by *T.spiralis* is, at least in part, dependent on T lymphocytes (21,30). Second, we have shown that activation of T-cells *in vivo*, using an anti-CD3 antibody, leads to accelerated intestinal transit as well as increased tension generation by intestinal smooth muscle, demonstrable *in vitro* (27). Third, we have previously demonstrated that T cells infiltrate the *muscularis externa* during *T.spiralis* infection (11) and that intestinal muscle cells increase T cell survival by reducing apoptosis (19). In addition, muscle cells have been shown to interact directly with T cells in the context of MHC II linked antigen presentation (14,15) and via other co-stimulatory molecules such as B7-2 and ICAM-1 (14,15). One outcome of this interaction is the production of large amounts of interleukin (IL)-4 by lymphocytes exposed to muscle cells

(15). IL-4 expression is elevated within the intestine during nematode infection (39), and we have recently shown an increase in tension generation by murine intestinal muscle following transfer of an IL-4 encoding adenovirus to the gut (43). Others have demonstrated increased intestinal muscle contraction via a neural mechanism following administration of IL-4 to mice (12). Taken together, these findings suggest that the interactions between muscle and T cells are bi-directional and complex, but may in fact, result in the augmentation of the contractile properties of muscle. With respect to the changes seen in the inflamed intestine, it is of potential relevance to recall that T lymphocytes have also been implicated in the genesis of airways muscle hyper-responsiveness in a murine models of asthma (7).

However, we also chose to examine the role of T cells in the modulation of murine intestinal muscle in this study, based upon the discovery that SCID and athymic mice, which lack functional T lymphocytes (20,29), developed increased tension generation by intestinal muscle during infection, implying that the T cell dependency found previously in rat could not be confidently extrapolated to the mouse. This may reflect differences in the pathological regulation of murine versus rat intestinal smooth muscle or reflect the limited number of time points tested in the *T. spiralis* infected rat, since others have shown that limited changes in intestinal epithelial function can occur in nematode infected athymic rats (24). In either case, the presence of altered muscle function in the T cell deficient mice indicated that the pathological regulation of intestinal smooth muscle function may be more complex than simple dependence upon a single cell type. Our results show that in the infected athymic and SCID/beige mouse, the peak increase of muscle contraction was delayed in onset and that the subsequent sustained component of contraction was lower in magnitude than that seen in euthymic mice, suggesting a partial dependence on T lymphocytes. Following reconstitution of T cell populations, as verified by flow cytometry, most of the differences in the profile of contraction between non-reconstituted athymics and euthymics were abrogated. In particular, the onset of peak contraction was accelerated and the magnitude of the sustained level of contraction was increased. These changes cannot be ascribed to differences in the magnitude of the acute inflammatory response to the infection as maximum MPO activity was similar in the three groups of BALB/c mice post-infection.

Interestingly, SCID/beige mice showed no increase in MPO activity during the course of *T. spiralis* infection. This may indicate that their extreme immunodeficiency inhibited the infiltration of neutrophils into the gut (confirmed by histology), however this may also involve the beige mutation which results in an abnormal structure and function in some granulated cell types, including neutrophils and natural killer (NK) cells (35). No studies to date have characterized whether myeloperoxidase activity is affected by this mutation. While it is possible that this mutation may play a role in the neutrophilic response to *T. spiralis* infection, it likely plays no role in worm expulsion, since several studies have shown that the beige mutation had no impact upon the expulsion of nematode infections (36), or the anti-parasite activity of inflammatory cells *in vitro* (18). Nor is it likely that the beige gene or a deficiency in NK cells plays a role in the changes in muscle function during infection, since athymic mice develop a similar timing and magnitude of increased muscle contrraction to that seen in the SCID/beige mouse, yet athymic mice are known to have a heightened NK cell activity (28). However, the development of muscle dysfunction in the SCID/beige mice, in the absence of an increase in MPO does confirm previous speculation (6,46), that neutrophils play no role in the pathological regulation of intestinal muscle function.

While the elaboration of IL-4, or other mediators, by T cells may reflect at least part of the mechanism underlying increased tension development by muscle, our results indicate that T cells are not the sole determinants of increased tension development in this model, as significant contractile changes were observed in non-reconstituted infected athymic and SCID mice. Therefore other possibilities also need to be considered. Parasitic infection can induce an eosinophilia in athymic mice (37), and mucosal mast cells, which are present in T cell deficient mice, have been shown to release Th2 cytokines including IL-4 (26). Thus the cellular locus of action of this or other mediators on muscle contractility requires further elucidation. Studies in nematode infected rats have revealed several loci of change in the intestinal muscle cell including suppression of Na-K ATPase, altered calcium handling (45) and an increased actin content (50). Since corticosteroid treatment completely abolished the increased tension development in the *T.spiralis* infected rat (22) and mouse (Collins *et al.*, unpublished data), the changes that persist in the

absence of T cells presumably still reflect host factors rather than a direct effect of the parasite. Currently, our investigation is focusing on the roles of macrophages, eosinophils and mast cells (42,44), in the context of other mechanisms that might underlie muscle changes. An intriguing observation is the persistent increase in tension generation evident at day 21 PI in BALB/c mice, when worms have already been expelled and the inflammatory response resolved. In the NIH Swiss strain, this persistent increase in tension development was evident 6 weeks after infection (2). It is highly unlikely that inflammatory cells sustain this persistent change as the tissue was histologically normal. We have postulated a smooth muscle origin for the factors that sustain this persistently increased tension generation, as previous studies have shown that muscle cells can elaborate arachidonic acid metabolites as well as cytokines (6). Autocrine regulation of such factors is a putative mechanism to explain altered muscle function in the absence of lymphocytes or other immune or inflammatory cells in the muscle layer at these late time points PI (6).

A major goal of this study was also to evaluate the changes in muscle contraction and worm expulsion at various time points in our experimental paradigms. Our results suggest that the two parameters are related. In euthymic mice, the peak increase in tension generation by muscle occurred within 6 days PI, however tension generation was sustained above control levels over the following 15 days, during which time, the infection was completely expelled. In contrast, in athymic mice, the peak increase in tension development occurred at days 10-12 PI when about one third of the parasites were evicted. Tension generation then fell to a lower level than observed in euthymics and was accompanied by a slow eviction of the remaining two thirds of the worm burden, with over 11% of the original worm burden remaining by day 21 PI. In successfully reconstituted athymic mice, the peak increase in tension generation occurred by day 6 PI and the worms were completely evicted between days 10-21 PI when tension generation was sustained at a level equal to or higher than that seen in euthymic mice. Thus, the initial peak increase in tension development may be required to initiate worm expulsion and the sustained component may assist in the expulsion of the remaining parasites over a longer time frame. The higher level of sustained tension development seen in reconstituted mice may have been necessary to eliminate the larger initial worm burden seen on day 4 PI in reconstituted mice (70% of the initial dose) compared

to that seen in euthymic mice (50% of the initial dose), or may be the result of the increased number of T cells recovered from reconstituted mice, compared to normal euthymic mice (see Figure 2).

Our results suggest a relationship between the timing and the magnitude of the increased tension development by muscle in this model, and the eviction of the parasite from the gut. This is supported by other studies, including a recent publication where we found a positive correlation between the ability of certain mouse strains to expel the parasite and the tension generated by intestinal muscle during infection (41). In that study, NIH Swiss mice, which are efficient in expelling the parasite, also developed the greatest increase in tension generation whereas the B10.BR mouse, which is very inefficient in its ability to evict the parasite such that they can develop chronic infections (3), had a smaller and delayed increase in tension development. Further evidence for a role for motor activity in worm expulsion comes from studies utilizing the WW^V mouse strain which lacks both the c-kit receptor and mast cells. In the WW^V mouse, expulsion of the nematode parasite *Nippostrongylus brasiliensis* was markedly delayed following primary infection, but this delay could not be corrected by restoring mast cell populations following bone marrow reconstitution (17). Since the WW^V mouse also lacks the interstitial cells of Cajal (ICC), which control slow wave and propulsive activity in the gut (16), the delayed expulsion of the parasite most likely reflects altered motility. In addition, an earlier study demonstrated that opiate-induced inhibition of gut motility resulted in delayed expulsion of *T.spiralis* from mice (38). Together, these results invoke intestinal motility as an important determinant of successful worm expulsion from the gut. Since increased propulsive activity was demonstrated in extrinsically denervated gut segments during nematode infection (1), cells, like smooth muscle, which are intrinsic to the gut wall are important in determining propulsive motor activity. Taken together, these studies also provide indirect support for a major conclusion of the present study, that increased tension generation by intestinal smooth muscle and the efficient expulsion of the parasite from the gut contraction are linked.

A role for increased propulsive motor activity in the expulsion of enteric infections has also been suggested by Urban *et al.*(40) and this is supported indirectly by the demonstrations of altered motility in a number of enteric infections including bacteria (31), protozoa (8) and nematode parasites (1,6,38,41,45).

Changes in motility likely combine with other mechanisms, including increased fluid secretion (48), to evict parasites from the gut (6), rendering the intestine an inhospitable environment for the parasite, leading to both decreased fecundity and the parasites expulsion (40). The involvement of lymphocytes in the generation of altered muscle contraction also has bearing on non-infective GI diseases associated with altered motility such as IBD (34) and pseudoobstruction (23,33). In Crohn's disease, lymphocytes have been demonstrated in the muscle layers (10) and massive lymphoid infiltration of the neuromuscular layers has been found in patients with certain types of acquired pseudoobstruction (23,33). These observations thus extend the relevance of our study beyond that of enteric infections. As well, these results indicate a more complex pathogenesis for altered intestinal muscle function than was previously suspected.

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The following work, which has been submitted to the Journal of Immunology continues to address the imunological dependence of the increased muscle contraction and worm expulsion seen *during T. spiralis* infection. Specifically the paper identifies a dependence on CD4 +ve T cells and class II antigen presentation in the development of increased muscle function, and in the expulsion of the infection, while identifying no crucial role for CD8 +ve T cells in either response.

Chapter 4

CD4 T cells and MHC class II expression regulate worm expulsion and increased intestinal muscle contraction during *T. spiralis* infection.

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ABSTRACT

The expulsion of intestinal nematode parasites and the associated increased tension of intestinal muscle are T cell dependent, since both are attenuated in athymic rodents. However, it is unclear what role(s) CD4 or CD8 T cell subsets play in either process. We infected C57BL/6 mice, athymic, CD4 deficient, CD8α deficient, and MHC class II deficient (C2d) mice with *T. spiralis* larvae. Thereafter, we examined intestinal worm numbers, isometric longitudinal muscle contraction, and MHC II expression. Numerous MHC II positive cells were identified within the jejunal *muscularis externa* of infected but not uninfected C57BL/6 mice. C57BL/6 and CD8α deficient mice developed a large increase in muscle contraction, expelling the parasite by day 21 post infection. Athymic and C2d mice exhibited much smaller increases in muscle contraction and had delayed parasite expulsion. CD4 deficient mice exhibited intermediate levels of muscle contraction and delayed parasite expulsion. To further examine the role of MHC II and CD4 T cells, we irradiated C2d mice and reconstituted them with C57BL/6 bone marrow alone or together with C57BL/6 CD4 T cells. C57BL/6 bone marrow alone had no effect upon muscle function, or worm expulsion in recipient C2d mice. Bone marrow plus CD4 T cells restored increased muscle contraction, but worm expulsion remained delayed. Thus, both hematopoietic MHC II expression and mature CD4 T cells are required for the pathophysiology of intestinal muscle. Both are likely required but not sufficient for worm expulsion. However, MHC II expression on non-hematopoietic cells may be required for complete expulsion of this parasite.

4.1 INTRODUCTION

The gastrointestinal (GI) tract is continuously exposed to environmental antigens, which may include potential pathogens and other noxious stimuli (1). Usually the mucosal immune system can differentiate between useful and harmful antigens encountered in the lumen (2), and initiate the appropriate immune response. In this regard, most studies of enteric infections have focused on the direct

effector actions against invading pathogens by the immune system. However there is growing evidence that during such infections, the host develops a complex and integrated response involving the coordinated actions of all the tissues in the gastrointestinal tract. Normally passive physiological tissues are recruited by the immune system, altering their function to actively aid in host defense (1,3). This immunophysiological interaction, such as occurs following infection with the gastrointestinal nematode parasite *T. spiralis*, results in increased fluid secretion into the lumen of the small bowel (3,4) as well as increased intestinal propulsive activity and more rapid intestinal transit (1,5,6,7). These changes occur in association with a hyper-responsiveness of jejunal longitudinal muscle (8,9,10). Physiological responses of intestinal muscle have been hypothesized to play a role in the expulsion of the infection (1,11) and to involve T lymphocytes, as supported by the observation that both processes are attenuated during infection of athymic rats (12,13,14).

While T lymphocytes are undoubtedly major contributors to these responses, the respective roles of CD4 and CD8 +ve T cells in worm expulsion and enteric muscle function during a primary *T. spiralis* infection remain unclear. Both CD4 and CD8 +ve lymphocytes infiltrate the jejunal muscle layers during the early stages of *T. spiralis* infection in rats (unpublished observation), and both T cell subsets are capable of mediating tissue damage, either directly or indirectly (15,16). While several studies have shown that CD4 T cells from infected rodents can adoptively transfer protection against infection to naive animals (17,18,19), this is potentially quite different from the roles that newly recruited CD4 and CD8 T cells could play during a primary infection. CD4 T cell activation and differentiation requires their interaction with antigen presenting cells (APC), and the role and identity of various APC within the intestine during nematode infections is not clearly understood. MHC II expression within the non-inflamed intestine is generally limited to professional APC found within the organized lymphoid aggregates, the Peyer's patches, or scattered within the lamina propria (20,21). Some limited expression by intestinal epithelial cells also occurs (22,23). However, during nematode infection the pattern of MHC II expression within the intestine is altered (24). More professional APC are recruited to the gut, and non-immune cells such as smooth muscle (25,26) and glial cells (27) may be stimulated by inflammatory

cytokines to express MHC II and possibly play an active role in antigen presentation (25,26,27).

Our previous work primarily examined muscle function and worm expulsion in the infected rat. (8,13). However, to examine the questions concerning T cell subsets, we switched experimental species to the mouse, in order to take advantage of the gene targeted immunodeficient mouse strains currently available. CD8α chain deficient (28), CD4 deficient (29), and MHC II deficient (C2d) mice that lack CD4 T cells and class II antigen presentation (30) were infected with *T. spiralis* as a first approach. This allowed us to identify the critical role of CD4 T cells and MHC II expression, in the physiologic responses of increased muscle tension and in parasite expulsion. These results may have implications not only for immunophysiological interactions during enteric infections, but also for the maladaptive changes in physiologic function found in disease states involving the GI tract.

4.2 MATERIALS AND METHODS

4.2.1 Mice

Euthymic and athymic (nu/nu) C57BL/6 mice were purchased from Taconic (Germantown, New York), as were mice lacking MHC class II (class II-/-), which were originally produced by targeted gene mutation as described by Grusby *et al* (30). These mice were later back-crossed onto the C57BL/6 background five times (and thereafter designated as C2d mice). A colony of C2d mice has been continuously bred in the central animal facility at McMaster University. CD4 (29) and CD8α (28) deficient mice (also back-crossed onto a C57BL/6 background) were obtained from the Jackson Laboratories and bred at McMaster University. Only male mice (aged 6 to 10 weeks) were used in this study, and all animals were kept under specific pathogen free (SPF) conditions. As the original mixed background MHC class II deficient mice were described to spontaneously developing colitis by 4-6 months of age (31), we examined our C2d mice for any signs of intestinal inflammation or other pathologies. Colitis was not observed in these mice until at least 6 months of age, and only in a small percentage of the animals, indicating that spontaneous intestinal inflammation should not be a factor in our studies.

4.2.2 CD4 T Lymphocyte Purification

C57BL/6 mice were euthanized, their mesenteric, brachial and inguinal lymph nodes removed, placed in RPMI media containing 10% fetal bovine serum (RPMI-10), and crushed between two sterile glass slides. The resulting single cell suspension was placed in covered culture dishes in a 37°C incubator for 2 hours. Non-adherent cells were then removed and spun down over Ficoll Hypaque (Pharmacia, Sweden), for 20 minutes at 1500 rpm. The mononuclear cells were then collected from the interface, washed twice with media and resuspended in a small volume of HBSS. Cells were then counted, and then incubated with magnetic beads (Miltenyi Biotec Inc. Sunnyvale CA.) labeled with rat anti-mouse CD4 mAb (clone GK1.5) for 20 minutes at 4°C. The cells were then passed through a VS type, "Minimacs" column (Miltenyi) previously washed with column buffer (phosphate buffered saline, 2mM EDTA, 0.5% BSA at pH 7.2) and chilled to 4°C. The unbound cells were allowed to pass through the column that was then washed 3 times with 3 mls of column buffer. The column was then removed from the magnet and the purified CD4 positive cells eluted with 5 mls of column buffer. The isolated cells were then counted and analyzed for viability, which was found to be >95%, using Trypan blue exclusion. Flow cytometric analysis confirmed previous studies (32) showing >90% purity of CD4 T cells following this procedure.

4.2.3 Production of Bone Marrow Chimeras

The protocol was adapted from that previously published (33). In brief, recipient MHC II deficient mice received 9.50 Gy of gamma irradiation delivered from a ^{137}Cs source. Donor C57BL/6 mice were euthanized by cervical dislocation, their femurs removed and flushed with HBSS. The bone marrow cells were then pooled, washed and counted, using Trypan blue exclusion. 2-5 hours after irradiation, recipient mice were reconstituted by tail vein injection of 20 million viable bone marrow cells in sterile PBS and then given Septra in their drinking water ad libidum, for two weeks, until the bone marrow graft had taken. There was a 90% survival rate for mice undergoing this procedure. Mice given both CD4 T cells plus bone marrow underwent the same procedure, except that 3-5 million positively selected CD4 T

cells were mixed with the bone marrow cells, and given to recipient mice in one injection.

4.2.4 Flow Cytometry Analysis

Flow cytometry staining for MHC II expression and CD4 T cells was performed on the peripheral blood of uninfected C2d, C57BL/6 or chimaeric mice (4-6 weeks after reconstitution), as well as on a mixed population of spleen and mesenteric lymph node (MLN) cells isolated from various mice infected 8 days previous with *T. spiralis*. Blood lymphocytes were isolated from 200 μl of EDTA treated blood centrifuged on Ficoll. Spleen and MLN cells were isolated as described above for CD4 T lymphocyte isolation. The isolated blood cells were incubated with FITC-labeled anti-IA^b mAb (clone M5) prepared in our laboratory and PE-anti-CD4 (clone GK1.5, Pharmingen) or biotin-anti-CD4 (Pharmingen) plus R-PE-streptavidin (Molecular Probes, Eugene, OR). Isolated spleen plus MLN cells were stained with FITC-anti-CD3 (clone 145-2C11, Pharmingen), PE-anti-CD4, and biotin-anti-IA^b (our laboratory) plus Cychrome-streptavidin (Pharmingen). Cells were then fixed in 1.0% paraformaldehyde and analyzed by flow cytometry using a FACScan™ instrument (Becton Dickinson, San Jose, CA). Data analysis for CD4 +ve T cells and MHC II expressing cells was performed using the PClysis software (Becton Dickinson), by gating on lymphocytes using forward and side scatter parameters. A minimum of 10,000 events were collected within the lymphocyte scatter gate.

4.2.5 Parasites and Infection of Mice

The *T. spiralis* nematode parasites used in this study originated in the laboratory of Dr. S. Desser, Department of Zoology, at the University of Toronto, and the colony was maintained through serial infections alternating between male Sprague-Dawley rats and male CD1 mice at McMaster University. Muscle larvae were isolated 30-90 days post-infection using a modification of the technique described by Castro and Fairbairn (34). Mice were infected by administration of 0.1 ml of phosphate-buffered saline (PBS) containing 350-400 *Trichinella spiralis* larvae by gastric gavage. To minimize differences between infection, control and gene deficient or reconstituted mice were infected from the same parasite

preparation whenever possible.

4.2.6 Worm Counts

The entire length of the small intestine was removed and for ease of counting, divided into four equal sections. All the adult worms within the given section were then counted using a modification of the method described (34). Briefly, the mucosa was separated from the underlying muscularis by scraping with a glass microscope slide and mixed in 1 ml of PBS. The worms were then counted using a scored petri dish and an inverted microscope. The worm counts in the four sections were then added and expressed as the total number of worms per mouse. In accordance with published norms (35), worm rejection was considered complete when at least 98% of the maximum worm burden had been expelled from the gut.

4.2.7 Immunohistochemistry

Tissues were rinsed with ice-cold phosphate buffered saline (PBS), then embedded in OCT compound, frozen using isopentane and liquid nitrogen and stored at -70^{0}C. Serial sections were then cut at a thickness of 6 μm and fixed in ice-cold acetone for 10 minutes. To detect MHC II expression, the biotinylated monoclonal rat anti-mouse MHC II antibody (clone 25-9-17) (30) (Gibco) was used. Immunohistochemical staining was performed according to the streptavidin-biotin-peroxidase complex method and the antibody was diluted in 1% BSA solution, in Tris-buffered saline, pH 7.2 (TBS). In brief, immunostaining was performed in the following manner: tissue sections were washed twice in TBS and endogenous peroxidases were blocked by submerging the slides in 1% H_2O_2 (in TBS) for 30 minutes. Sections were again washed twice with TBS and blocked with 1% BSA in TBS for 30 minutes. BSA was then washed off with another 2 TBS washes. Biotinylated anti-MHC II was then added to the slides at a 1:300 dilution and left over-night at room temperature. The next morning the antibody was washed off and streptavidin-horseradish peroxidase conjugate (1:300 dilution), also purchased from Gibco was added to the slides for 30 minutes. Again, after 2 washes, visualization of the antibodies was performed using 3-

amino-9-ethylcarbazole (AEC). Red color development was quenched by immersion of the slides in tap water, followed by counterstaining with haematoxylin. Photomicrographs were taken using a Zeiss camera.

4.2.8 Measurement of Muscle Contraction

The preparation of the jejunal longitudinal muscle sections for muscle contractility analysis and the analysis of the length-tension relationships have been described previously (9). In brief, the jejunum was removed and placed in oxygenated (95% O_2 -5% CO_2) Kreb's solution and 1 cm sections of whole gut were cut from the jejunum, beginning at the ligament of Treitz and proceeding distally. The lumen of each segment was flushed with Kreb's buffer, prior to the insertion of short (2-3 mm) lengths of Silastic tubing (0.065 OD, 0.030 ID) (Dow Corning, Midland, MI) into the open ends of the gut segments. Tubing was then tied in place with surgical silk. The insertion of the tubing was found to maintain patency of the gut segments, over the course of the experiments. Segments were then hung in the longitudinal axis and attached at one end to a grass FT03C force transducer (Grass, Quincy, MA) and responses were recorded on a Grass 7D polygraph. Tissues were equilibrated for 30 minutes at 37°C in Kreb's solution, oxygenated with 95%O_2 - 5%CO_2 before starting the experiment. Experiments were then conducted to examine the length-tension characteristics of the muscle before and after infection. Gut segments were stretched, by applying a tension equivalent to 0-1250 mg of weight. Contraction was then assessed following stimulation with 1 μM carbachol (Sigma Chemical, St. Louis, MO). Initial experiments indicated that this tension range was sufficient to determine the maximal responsiveness of both control and inflamed tissues. After each application of tension, the length of the tissue and the contractile response were recorded. Tissues were then rinsed twice and equilibrated at the next tension levels in fresh Kreb's for 15 minutes, prior to the next addition of carbachol. At the end of each experiment, tissue segments were removed, blotted and weighed, and the optimal tension (T_O) and the tissue length giving the maximum contractile response was used to calculate the cross sectional area of the tissue.

4.2.9 Data Presentation and Statistical Analysis

Responses to carbachol were recorded from tracings, followed by the calculation of contractile activity, expressed as milligrams of tension per cross sectional area as described previously (9). For each mouse, the mean tension was calculated from at least three segments. All the results are expressed as the means ± 1 SEM. Statistical significance was calculated using the Student's t test for comparison of two means or a one way of analysis (ANOVA) for the comparison of three or more means. Multiple comparisons were performed using the Neuman-Keuls multiple comparison test. $P < 0.05$ was considered significant.

4.3 RESULTS

4.3.1 Euthymic C57BL/6 Mice Develop Increased Muscle Contraction During *T. spiralis* Infection.

We infected euthymic C57BL/6 mice and studied the kinetics of jejunal longitudinal muscle contraction in response to 1 μM carbachol. The muscle response significantly increased by day 6 post-infection (PI) and peaed by day 8 PI, at levels three times higher than those of tissues from uninfected control mice. A significant increase in muscle contraction was maintained until at least day 21 PI (Figure 1).

4.3.2 Athymic, CD4 Deficient, and C2d Mice have Attenuated Muscle Function *During T. spiralis* Infection.

Euthymic, athymic, CD4 deficient, CD8α deficient mice, and C2d mice were infected with *T. spiralis* to determine the impact of their respective immunodeficiencies on muscle contractility. Intestinal tissues were tested on day 8 PI, the time of maximum increase in contraction by intestinal muscle in euthymic C57BL/6 mice during *T. spiralis* infection. All 5 strains of immunodeficient mice showed similar carbachol-induced muscle contraction (mean range, 800-1000 mg/mm^2), prior to infection (Figure 2A). However, while tissues from normal C57BL/6 mice again demonstrated a large increase in

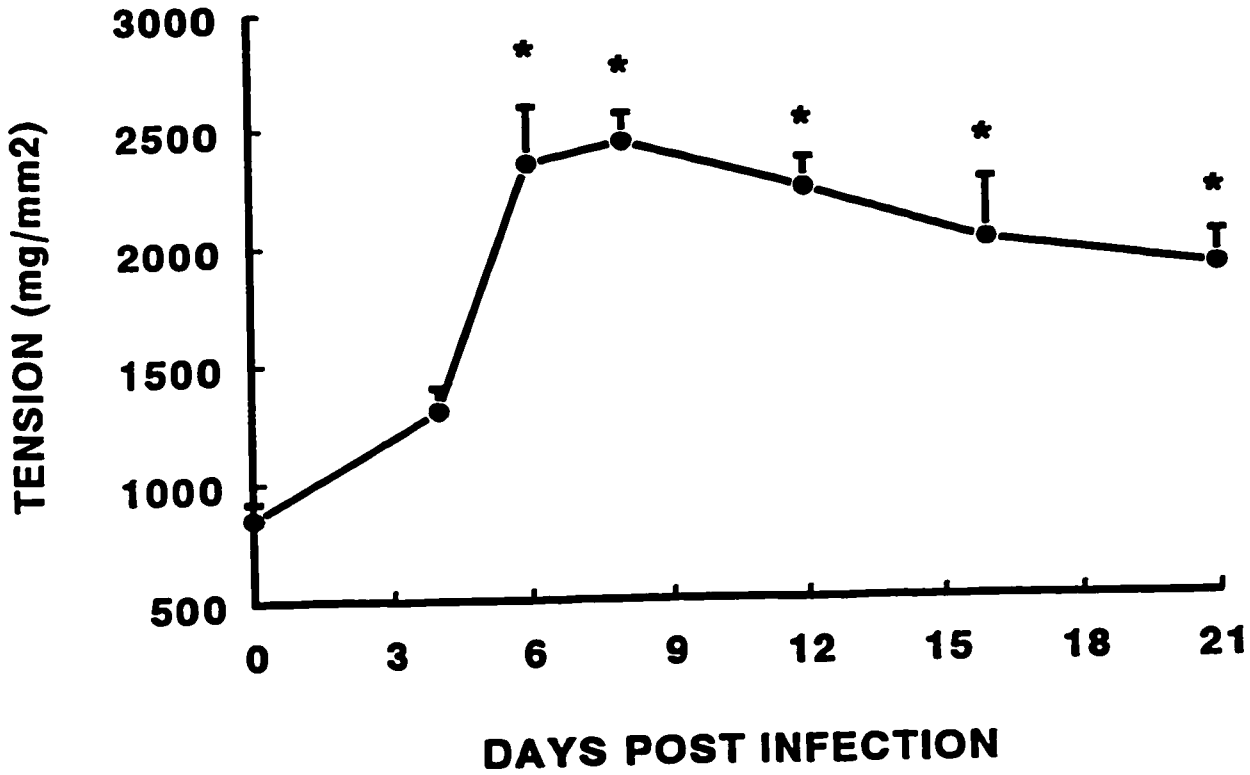


Figure 4.1:Time course of contractile response to 1μM carbachol (y axis = maximum tension generated) by intestinal muscle taken from infected C57BL/6 mice (solid circles). Data shown are the mean ± 1 SEM of groups of 4-6 animals. The asterisk denotes a significant increase in tension generation when compared to uninfected mice (time =0).

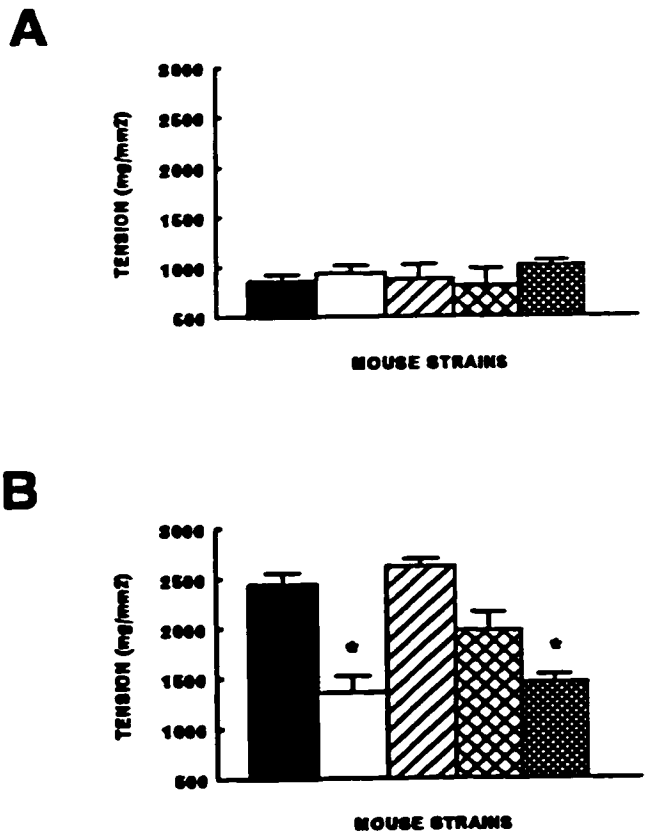


Figure 4.2:Maximum tension generated by muscle taken from uninfected mice (A) or day 8 PI mice (B), in response to 1μM carbachol. Mouse strains shown are euthymic C57BL/6 (solid bar), athymic C57BL/6 (open bar), CD8α deficient (hatched bar), CD4 deficient (cross hatched bar), and C2d (gray bar) mice. Data shown are the mean ± 1 SEM from groups of 4-6 animals. The asterisk denotes significantly lower tension generation by muscle compared to the infected C57BL/6 mice.

contraction at day 8 PI, tissues from athymic mice had very little increase in muscle contraction (Figure 2B) which was not significantly greater than that generated by uninfected mice. This result clearly indicated a major role for T cells in the increased intestinal muscle contraction, and confirms the previous findings in the athymic rat (13). CD8 deficient mice developed a high level of muscle contraction similar to that seen in the C57BL/6 mice. CD4 deficient mice showed an intermediate increase in muscle function, that on average was somewhat less than C57BL/6 muscle but there was no statistically significant difference between the muscle responses of the two strains of mice.

In contrast to intestinal tissue from CD4 and CD8α deficient mice, tissues from C2d mice had very low muscle responses on day 8, equivalent to those of athymic mice (Figure 2B). The tension generated by tissues from infected C2d mice were not significantly higher than that generated by tissues from control uninfected mice. Thus, only C2d and athymic mice had significantly attenuated muscle response, indicating a clear requirement for MHC class II expression and CD4 T cells to generate the increased muscle response. Absence of CD4 expression had little effect, presumably because the CD4 deficient mice still express MHC II and have been shown to retain a significant amount of MHC II-restricted T cell function (30,36).

4.3.3 Athymic, CD4 Deficient, and C2d Mice, but Not CD8α Deficient Mice Fail to Expel *T. spiralis*.

To confirm previous findings (13,14) of a major contribution by T lymphocytes in *T. spiralis* expulsion, euthymic and athymic C57BL/6 mice were infected with *T. spiralis* larvae and sacrificed 21 days later. The time course of their worm expulsion indicated that normal C57BL/6 mice expelled greater than 90% of their parasites by day 16 and were worm free by day 21, whereas athymic mice had a chronic infection with a substantial load of worms up to day 21 (Table 4.1). Mice with CD4 or CD8α deficiencies were also infected, to determine if their immunodeficiencies could affect worm expulsion. CD8α deficient mice, were devoid of adult worms by day 21, similar to normal euthymic C57BL/6 mice, but CD4 deficient mice also had substantial numbers of worms at day 21 (Table 4.2).

Table 4.1. Time-course of intestinal worm burden during *T. spiralis* Infection

Strain	C57BL/6	Athymic B6	C2d
Day 4 PI	252 ± 45	204 ± 3	200 ± 38
Day 12 PI	100 ± 7	121 ± 21	205 ± 36[b]
Day 16 PI	14 ± 8	83 ± 17[a]	88 ± 7[a]
Day 21 PI	0 ± 0	25 ± 8[a]	28 ± 3[a]

NOTE. Values are the mean number of worms recovered per mouse ± 1 SEM for groups of 4-6 mice [a] Significant difference from C57BL/6 mice at the same time point ($P < 0.05$). [b] Significant difference from both euthymic and athymic mice at the same time point ($P < 0.05$).

Table 4.2. Chronic worm burden in athymic, CD4 deficient, but not CD8α deficient mice

Strain	C57BL/6	Athymic B6	CD4 -/-	CD8α -/-
Day 21 PI	0 ± 0	40 ± 9[a]	28 ± 15[a]	0 ± 0

NOTE. Values are the mean number of worms recovered per mouse ± 1 SEM, obtained from groups of 4-6 mice. [a] Statistically difference from C57BL/6 worm numbers ($P < 0.01$).

The time course of worm expulsion was followed in C2d mice, along side that of euthymic and athymic mice (Table 4.1). Similar high numbers of adult *T. spiralis* worms were recovered from the intestines of athymic, euthymic, and C2d mice, as of day 4 PI. Over the next 2 weeks of infection, all 3 mouse strains showed a time dependent loss of adult *T. spiralis* worms. However the early period of worm loss (between 4 and 12 days) was significantly slower in mice lacking MHC II expression (Table 4.1). Worm expulsion continued to completion in euthymic mice between days 16 and 21 PI. Over the same

time, C2d mice (like athymic mice) continued to retain a significant number of worms, with approximately 15% of their peak load still present on day 21.

4.3.4 Bone Marrow and Peripheral CD4 T Cell Reconstitution of C2d Mice

Questions of the role of CD4 T cells and MHC II expression could best be answered by reconstitution of C2d mice, by bone marrow and CD4 T cell transfer from congenic C57BL/6 mice. We generated these congenic bone marrow chimaeras and validated the success of our reconstitutions by flow cytometric analysis of recipient peripheral blood and lymphoid tissues. As expected, C57BL/6 mice had many circulating MHC II positive cells, and approximately 27% of their lymphocytes were CD4 positive (Table 4.3). In contrast, C2d mice had no detectable MHC II expression, and were lacking almost all CD4 T cells (only 1.5% of all blood lymphocytes). Reconstitution of C2d mice with C57BL/6 bone marrow led to the presence of a large population of MHC II +ve cells within the blood, however there was no significant increase in the proportion of CD4 +ve cells in their peripheral blood. This was predictable because the C2d thymus cannot generate CD4 T cells due to lack of MHC II expression on the thymic epithelium, and consequent lack of positive selection (30,33,37). Following the injection of C57BL/6 bone marrow, along with purified CD4 +ve T, there was a partial restoration of the CD4 T cell population within the blood, with their frequency reaching almost 4% of all lymphocytes. However, bone marrow reconstitution of C57BL/6 mice with syngeneic C57BL/6 bone marrow alone, regenerated MHC II +ve cells in blood, and restored normal numbers of CD4 +ve T cells (25.1%).

Table 4.3. MHC II expression and CD4 T cells in blood of normal or bone marrow chimera mice.

Strain	C57BL/6	C2d	C2d +BM	C2d +BM+CD4	C57BL/6 + BM
MHC II expression	56.7 ± 1.9	0.2 ± 0.0	70.9 ± 5.1	65.9 ± 1.3	62.5 ± 0.8
CD4 T cells	26.5 ± 1.1	1.5 ± 0.1	1.7 ± 0.0	3.7 ± 0.1	25.1 ± 0.6

Flow cytometric analysis of peripheral blood cells from uninfected euthymic C57BL/6 mice, C2d mice, C2d mice given C57BL/6 bone marrow (BM) or BM plus CD4 T cells, and C57BL/6 mice given syngeneic bone marrow. Cells were isolated 6 weeks after transplant prior to *T. spiralis* infection, and stained as described in Methods. MHC II expression refers to the percentage of MHC II positively staining cells within the blood (detection limit <0.1%). The proportion of blood cells expressing CD4 are also shown as percentage data. Values shown are the mean data calculated from 3 mice per group.

CD4 T cells were also assessed within the spleen and mesenteric lymph nodes of reconstituted C2d mice, during the height of *T. spiralis* infection, day 8 PI. As shown in Figure 3, the percentage of CD4 positive cells was very low in MHC II deficient mice whether they had undergone bone marrow reconstitution or not (1.3% vs 0.1%). In the mice that had been given both purified CD4 +ve T cells and MHC II +ve bone marrow, there was a partial restoration of CD4 T cells representing 11% of lymphoid cells compared to in infected age matched C57BL/6 mice that had 33% at the same time point.

4.3.5 Immunohistochemistry of Intestinal MHC II Expression

Staining for MHC II on frozen sections of the small intestine of uninfected C57BL/6 mice revealed MHC II staining predominantly on cells within lymphoid follicles and by intestinal epithelial cells (Figure 4A). No staining could be detected within the muscle layers. Athymic mice also showed positive staining within lymphoid follicles (not shown) but staining on epithelial cells was decreased or absent, compared to euthymic mice. As expected, no staining was observed on tissues removed from C2d mice (Figure 4B). We also examined tissues from C57BL/6 mice during the height of infection (day 8 PI) and found reduced MHC II expression on epithelial cells, but increased expression in the crypt regions and within the muscle layers (Figure 4C). Stained cells were also seen within the crypt and muscle regions of infected athymic mice, however fewer positive cells were seen (Figure 5D). As expected, no positive cells were seen in infected C2d mice (Figure 4E). However, following bone marrow reconstitution of C2d

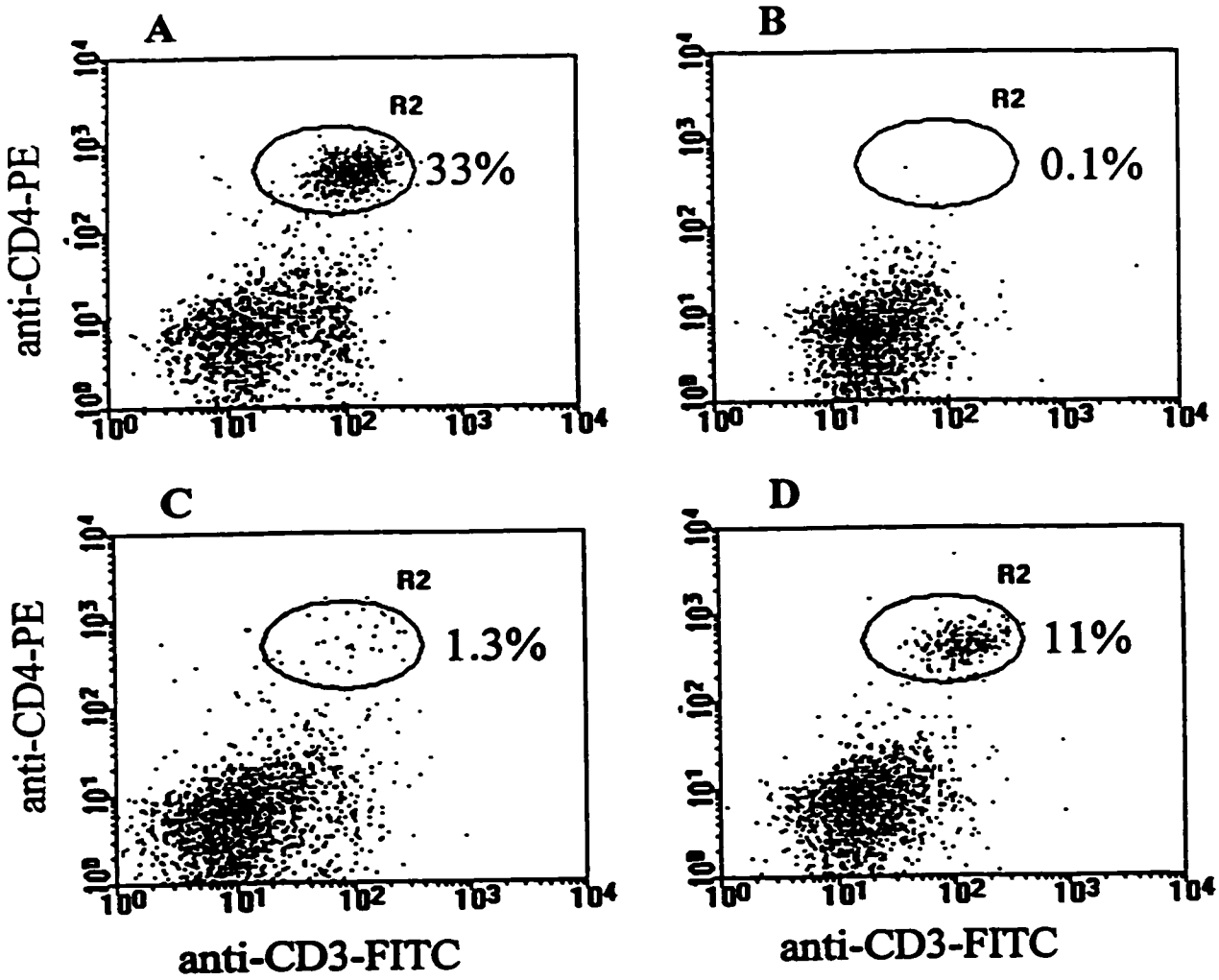


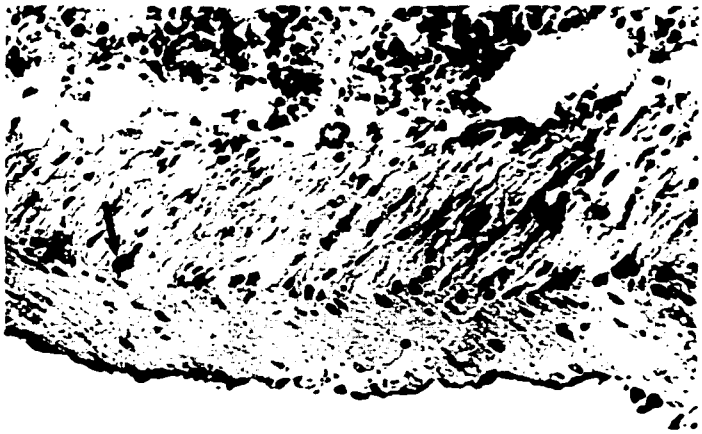
Figure 4.3:Flow cytometric analysis of mixtures of mononuclear cells isolated from the spleen and mesenteric lymph nodes of (A) euthymic C57BL/6 mice, (B) unreconstituted C2d mice, as well as (C) C2d mice reconstituted with just C57BL/6 bone marrow, or (D) bone marrow plus CD4 T cells. The mice were infected eight days previous, with *T. spiralis*. Cells were stained and analyzed as described in Methods. The CD4 stained T cells are displayed as double stained (anti-CD4 and anti-CD3) populations. Percentages of double stained cells in the total gated lymphocyte population are shown for cells within the oval, R2 region.

Figure 4.4: Immunohistochemistry for detection of cells expressing MHC II within the small intestinal tissues of normal uninfected mice (A-B) or mice at 8 days PI (C-F). A. Jejunal cross-section from an uninfected euthymic C57BL/6 mouse (x40). Dark staining is localized to cells within a lymphoid follicle (arrow, bottom right) as well more diffuse staining of some epithelial cells (upper arrow). B. Jejunal cross section of an uninfected C2d mouse, revealing absence of staining, even in a lymphoid follicle (x40). C. Numerous stained cells localized (arrows) to the external muscle layers and crypt region of jejunal tissue from a euthymic C57BL/6 mouse, at eight days PI (x400). Panel D. Some stained cells were found in the external muscle layers and crypts of an athymic mouse, at day 8 PI - see arrow (x400). E. Absence of staining in the external muscle layers and crypts of a C2d mouse at day 8 PI (x400). F. Many cells stain for MHC II within the muscle layers, myenteric plexus and crypts of a C2d mouse - see arrows, reconstituted with MHC II +ve bone marrow, at 8 days PI with *T. spiralis*.

A

B

C



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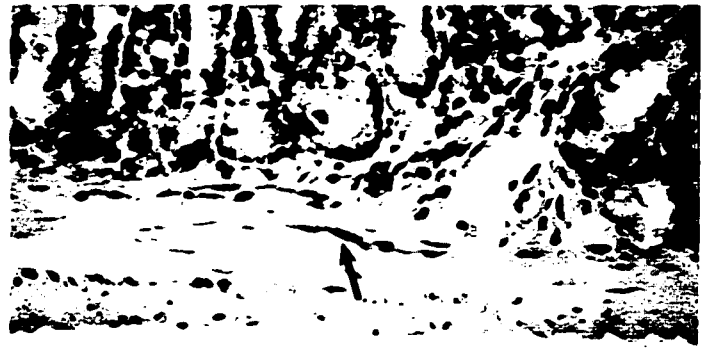
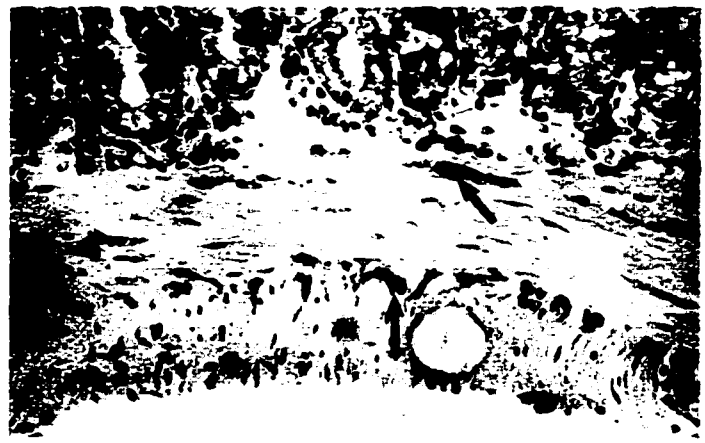


E

F



mice using C57BL/6 mice as donors, infection was accompanied by the presence of numerous MHC II +ve staining cells, again predominantly within the crypt regions and muscle layers (see Figure 4F).

4.3.6 Muscle Contraction and Worm Expulsion in Bone Marrow and CD4 T Cell Reconstituted C2d Mice

To examine the effects of the reconstitution protocols on muscle function, experiments were conducted on day 8 PI. As shown in Figure 5, bone marrow reconstitution of C2d mice had no effect upon muscle function, with a tension generation similar to that seen in non-reconstituted C2D mice. In both cases, tension generation by C2d mice was significantly lower than that generated by infected C57BL/6 mice. The combined reconstitution of MHC II +ve bone marrow and CD4 +ve T cells to C2d mice resulted in a significant increase in muscle tension, greater than both bone marrow reconstituted as well as unmanipulated C2d mice, and not significantly different from infected C57BL/6 mice. C57BL/6 mice which had undergone syngeneic bone marrow grafting also developed a similar level of muscle contraction during infection to that generated by tissues from infected unmanipulated C57BL/6 mice (data not shown).

C2d Mice were similarly reconstituted to examine the dependence of worm expulsion on MHC II expression and the presence of CD4 T cells. Neither bone marrow reconstitution alone nor the addition of mature CD4 T cells from C57BL/6 mice had any significant effect upon worm expulsion that was still incomplete as of day 21 PI and was therefore similar to untreated C2d mice and athymic mice (Table 4.4). In a control group, we determined if bone marrow reconstitution procedure was itself capable of restoring normal worm expulsion in syngeneic C57BL/6. These mice when infected with *T. spiralis* parasites exhibited complete expulsion of worms by day 21 (Table 4.4), indicating that the bone marrow reconstitution procedure was sufficient within the C57BL/6 recipient.

Table 4.4. Effects of bone marrow and CD4 T cell reconstitution on duration of *T. spiralis* infection in C2d mice.

Strain	C57BL/6	C2d	C2d +BM	C2d +BM + CD4	C57BL/6 + BM
Day 21	0 ± 0	30 ± 5[a]	25 ± 5[a]	24 ± 6[a]	0 ± 0

Data are mean number of worms recovered per mouse ± 1 SEM, from groups 4-6 mice.

[a] Significant difference from C57BL/6 mice ($P < 0.01$).

4.4 DISCUSSION

Our results clearly point to a dynamic interaction between T cell functions and intestinal muscle hyper-responsiveness, during *T. spiralis* infection. The increased muscle tension we observed in C57BL/6 mice had a similar time course to that seen in other immunocompetent mouse strains (9). Muscle tension generation increased early, peaking on day 8 PI and remained significantly elevated over control until at least day 21 PI. In contrast, the muscle response was significantly reduced in athymic mice, indicating a pivotal role for T cells in the development of increased muscle contraction. The CD8α knockout mice exhibited the same pattern of altered muscle function as normal C57BL/6 mice, indicating that CD8α expression and the altered immune function of CD8 T cells in these mice (28) were not essential to the muscle hyper-responsiveness. However, a prominent role for CD4 T cells as well as MHC class II expression, was identified following infection of C2d mice, because tissues from these mice demonstrated attenuated muscle contraction during infection, to a similar degree as athymic mice. In contrast, infected, CD4 deficient mice gave intermediate muscle function, not much different than normal C57BL/6 mice. Thus, CD4 expression per se is not essential. However, because these mice are known to retain some class II restricted T helper cell function, it is the expression of that function which must be most important to the role of CD4 T cell subsets in the muscle hyper-responsiveness.

The reconstitution studies of the C2d mice showed that even a small population of CD4 T cells, in the presence of MHC II positive myeloid cells, could restore increased muscle contraction during

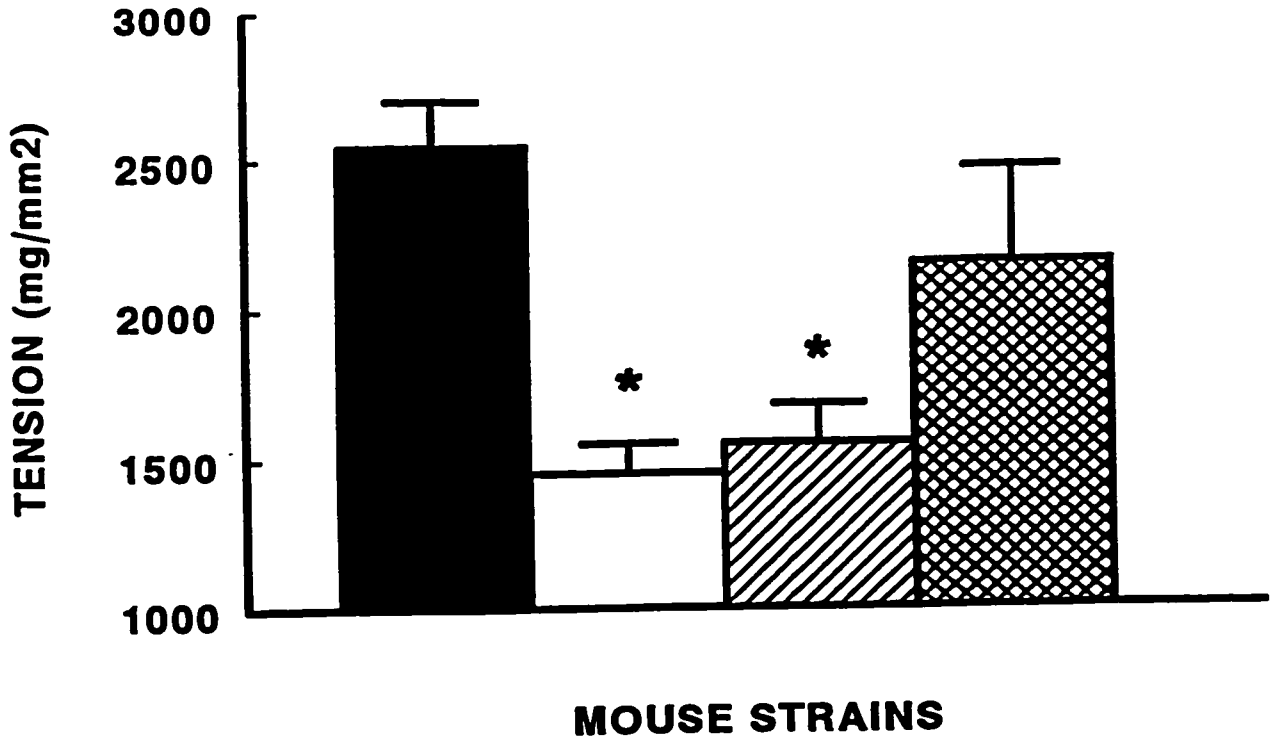


Figure 4.5:Maximum tension generated in response to 1μM carbachol, by intestinal muscle from infected euthymic C57BL/6 mice (solid bar), C2d mice without manipulation (open bar), or reconstituted with bone marrow from C57BL/6 mice (hatched bar), or with bone marrow plus CD4 +ve T cells (cross hatched bar) 8 days PI. Results shown are the mean ± 1 SEM of groups of 4 animals. The asterisk denotes a significantly lower tension generation by muscle compared to the infected C57BL/6 mice.

infection. Those results demonstrate clearly that class II restricted CD4 T cells are the major T cell subset involved, and are essential for the increased intestinal muscle contractility. The minimal increase in muscle function seen in unreconstituted C2d mice could also indicate a minor or compensatory role for CD8 positive T cells. Their ability to produce cytokines and mimic some of the functions of CD4 T cells under certain conditions could be at play during nematode infections as in other pathologies or infections (38). Similarly, the minimal response seen in athymic mice could also reflect the actions of the small population of γδ-TcR T cells that are extrathymically derived and frequently localize to the gut mucosa (39).

Similarly, the worm expulsion that occurred by day 21 PI in the immunocompetent C57BL/6 mice was found to be mediated, at least in part by CD4 +ve T cells. CD4 deficient mice, as well as athymic and C2d mice all had significant numbers of worms remaining in their small bowels as of day 21 PI, while at the same time, expulsion was complete in CD8 knockout mice. In addition, the numbers recovered from athymic mice were similar to those recovered from CD4 and C2d mice. Taken together, these results suggest that CD8 T cells play no significant role in worm expulsion, but that CD4 T cells may have a significant contribution. The inability of CD4 T cell plus MHC II positive bone marrow reconstitution to restore normal worm expulsion to C2d mice was surprising. However this likely reflects a requirement for greater numbers of available CD4 T cells for clonal development of antigen specific immune response relevant to worm expulsion. This would agree with several previous studies demonstrating that strain differences in the degree of T cell proliferation during *T. spiralis* infection correlated with the speed of expulsion (35).

Our studies not only indicate an important role for CD4 T cells but also for MHC class II expression during *T. spiralis* infection. Previous studies using panels of congenic mouse strains found that MHC as well as non-MHC genes influence the susceptibility or resistance to *T. spiralis* infection (35), however the important cell types expressing MHC II were not identified. Under uninfected, specific pathogen free conditions, we found that MHC II expression within the mouse was localized only to cells within lymphoid follicles, as well as some intestinal epithelial cells. There was little or no constitutive

expression within the muscle layers. However, during infection MHC II expression on epithelial cells was reduced and numerous MHC II positive cells appeared within the *muscularis externa* and crypt regions of both euthymic and athymic mice. The mechanisms for MHC II expression at these sites that are relatively distant from where the actual pathogen resides, are unclear. The identity of these MHC II positive cells was also unclear from initial immunohistochemical studies. They could represent infiltrating inflammatory cells, or resident cells such as smooth muscle and glial cells responding to proinflammatory cytokines within their microenvironment. These resident cells express MHC II ectopically in culture (25,26,27) and in human disease (23). However, our results from the bone marrow transplantation experiments with recipient C2d mice showed clearly that cells expressing MHC II (and therefore most definitely of myeloid origin) do localize in the jejunal *muscularis externa* layers and crypt regions, during *T. spiralis* infection of those chimaeric mice. This is good suggestive evidence that the infiltrating cells likely include macrophages and/or dendritic cells.

One alternate explanation for the lack of expulsion of the parasite worms by C2d mice given bone marrow and CD4 T cells remains an intriguing possibility. There may be a necessary dependence on the cooperation of class II restricted CD4 T cells with non-myeloid cells that express MHC class II under inflammatory conditions during *T. spiralis* infection. Epithelial, smooth muscle, and glial cells all could potentially participate in such activity. Even if sufficient CD4 T cells were present in the transplanted C2d mice, MHC II would only be expressed on myeloid cells, so MHC II-restricted interactions with non-myeloid cells would be impossible. By contrast the syngeneic transplant mice would have full MHC II expression and hence allow worm expulsion.

As shown in this and other studies, the host responds to enteric nematode infections with changes in intestinal physiology (1,3,4) that are moderated by the immune system. Although this study and others indicate a prominent role for T lymphocytes in altering the function of gut muscle, it is still not clear whether T cells interact directly with muscle cells, whether they act through an intermediary cell type, or at a relative distance by paracrine cytokine production. Few studies have examined the direct effects of T cells on enteric muscle, but preliminary studies in culture and *in vivo* suggest that they can occur, with IL-

4 as primary candidate effector cytokine (unpublished observations). Indirect interactions are also possible, for example through the recruitment and hyperplasia of both eosinophils (40) and mast cells (41) which are both dependent upon CD4 T cells of the Th2 class. We have preliminary data that IL-5 and eosinophils as well as c-kit dependent cells, which include mast cells and interstitial cells of Cajal (ICC) are involved in the increased muscle function following a *Trichinella* infection (unpublished observations). However, the role of these cell types in the expulsion of a *T. spiralis* infection, and whether they are a necessary requirement for host defense is still controversial (42,43).

These results not only have implications for host defense against nematodes, but may have broader implications for clinical gastroenterology. CD4 +ve T cells have already been implicated in the pathogenesis of inflammatory bowel diseases, and are thought to initiate much of the tissue damage seen in these diseases (44,45). As well, both MHC II expression and T lymphocytes have been demonstrated within the external muscle layers (46,47) of Crohn's disease, and recent studies indicate that these infiltrating T cells are both activated and dividing, suggesting that they are responding to antigen, and antigen presentation within the muscle layers (47). There are also cases of intestinal pseudo-obstruction where immune cells have been identified within the neuromuscular layers of the gut (48,49). This study thus raises the possibility that CD4 +ve T cells are a putative cause of the motility disturbances associated with these diseases, as well as the functional bowel disorders which can arise during enteric infections and persist long after the resolution of the mucosal inflammation and immune activation.

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The following work, my fourth publication, diverges from my earlier studies. Instead of using *T. spiralis* infection to study the interactions between the immune and intestinal neuromuscular systems, we examined the ability of an adenoviral vector expressing interleukin-4 to alter intestinal muscle function. To accomplish this, we first had to develop and characterize adenoviral gene transfer to the jejunal serosal surface using a pluronic gel. In this study we not only show that gene transfer to the intestine is feasible, but that localized over-expression of IL-4 increases jejunal longitudinal muscle contraction.

Chapter 5

Interleukin-4 gene transfer to the murine intestine leads to eosinophilic inflammation and intestinal muscle hyper-responsiveness.

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Gastroenterology, 1998 (submitted)

ABSTRACT

Introduction: Intestinal gene transfer is a potentially attractive experimental and therapeutic tool, however attempts through the lumen have met with little success. This study explored an alternative approach, by applying recombinant human type 5 adenoviruses to the outer layers of the gut, and examined the effects of a virus over-expressing interleukin (IL)-4 upon enteric neuromuscular function. **Methods**: Following surgery, adenoviruses were mixed in pluronic gel and painted onto the surface of murine small bowel IL-4 protein expression was analyzed as was the resulting inflammatory response and muscle function. **Results**: Virus application infected numerous cells within the intestine and in the surrounding mesentery. Transgene derived IL-4 protein was synthesized for almost 2 weeks. Viral infection led to an inflammatory response involving predominantly macrophages, lymphocytes and neutrophils, however IL-4 transgene expression also led to the recruitment of eosinophils, peritoneal mast cells and plasma cells. IL-4 expression was associated with significant intestinal longitudinal muscle hyper-contractility, evident by day 7 P.I. in the IL-4 virus infected mice, but not in the control virus infected animals. **Conclusion**: This study thus identifies a novel strategy for gene transfer to the intestine and provides evidence of a role for IL-4 in the pathological regulation of enteric smooth muscle function.

5.1 INTRODUCTION

Gene transfer and the ability to direct the site of transgene expression, has potential both for studying the physiological and pathophysiological roles of these genes within specific tissues, as well as eventual medical application in the treatment of disease through the ectopic expression of therapeutic proteins (1). Recombinant adenoviruses are the most efficient and extensively used vectors currently available for gene transfer *in vivo*. Infectious across a broad range of host species and cell types, adenovirus vectors can be grown to high titres and following infection, direct the cellular machinery of

host cells to produce large quantities of transgene proteins (2). Beyond their potential therapeutic application in the correction of genetic defects and autosomal recessive diseases such as cystic fibrosis (1), recombinant adenoviruses have also found use in the study of immune and inflammatory responses. Several recent studies have used recombinant adenoviruses, incorporating genes encoding cytokines and growth factors, to study the effects of transient elevations of these immunomodulatory molecules upon inflammation (3), fibrosis (4) and host defense (5) within the airways.

Since many of the same immunomodulatory factors are thought to be involved in enteric immune responses, transferring these genes to intestinal tissues could provide valuable information concerning their role in gastrointestinal (G.I.) pathology and pathophysiology. However, while many advances have been made in the field of gene transfer to the lung and other tissue sites, intestinal gene transfer is still in its infancy, due in part to the numerous technical difficulties found in delivering viral vectors to intestinal tissues (6). *In vivo* infection of intestinal epithelial cells, through lumenal or oral introduction of viral vectors, has been attempted by several groups but all have encountered similarly poor transduction efficiency *in vivo* (6,7). Other approaches, using the circulation to deliver viruses to the intestine have proven more successful, but either lack selectivity for the intestine (8), or require technically demanding surgery, feasible only in larger experimental animals such as the rat (9). Thus, alternative approaches to intestinal gene transfer need to be developed and tested.

We have previously shown that inflammatory events in the intestinal mucosa lead to altered function of the underlying smooth muscle; which becomes hyper-reactive to most contractile stimuli (10-12). Changes in intestinal muscle contractility have been demonstrated in inflammatory bowel diseases (IBD) (13,14) and during many forms of enteric infection (11,15,16), and are thought to contribute to the pain, nausea and diarrhea associated with G.I. inflammation. Because of the complex nature of these inflammatory conditions, and because direct experimental evidence identifying the principle pathogenic cells and mediators is lacking, the underlying mechanisms remain unclear. However, the ability to locally overexpress specific immunomodulatory molecules in proximity to the enteric neuromuscular apparatus could facilitate our understanding of the mediators involved in the generation of smooth muscle hyper-

reactivity, and help define potential targets for therapeutic intervention. Based upon our studies of the intestinal inflammation caused by nematode infections, and the association between the Th2 immune response to these parasites and the onset of smooth muscle hyper-responsiveness (11,17), we hypothesized a role for the cytokine interleukin (IL)-4 in the regulation of enteric smooth muscle function during inflammation.

Thus the aim of our study was to develop a means to overexpress IL-4 within the intestine and specifically in the *muscularis externa*. Because of the previously mentioned difficulties, we examined an alternative route, which involved targeting the *muscularis externa* through the application of adenoviral vectors, diluted in a pluronic gel, and painted onto the serosal surface of the mouse small intestine. Biocompatible pluronic gels have been previously shown to improve the efficiency of adenoviral gene transfer to endothelial and vascular smooth muscle cells both *in vitro* (18) and *in vivo* (19). In the present study, this approach resulted in the infection of a large population of cells on the surface of the intestine, as well as in the intestinal mesentery, as assessed by histochemistry for the marker virus encoded enzyme (LACZ). Infection with the LACZ virus resulted in a mild inflammatory response involving neutrophils, macrophages and lymphocytes. However when an IL-4 encoding virus was used (AdCAIL-4), the magnitude of the inflammatory response was significantly increased and its cellular composition was shifted to one typical of allergic inflammation, including numerous eosinophils, plasma cells and mast cells. This was accompanied by the development of altered intestinal muscle function, with jejunal longitudinal muscle taken from IL-4 virus infected mice exhibiting a strikingly increased contractile response to carbachol, which was not seen in LACZ infected mice. This study not only identifies a novel approach to intestinal gene transfer, but also demonstrates a putative role for IL-4 in the pathological regulation of intestinal smooth muscle function.

5.2 MATERIALS AND METHODS

5.2.1 Mice

Specific pathogen-free, male C57BL/6 mice (6 to 10 weeks old) were purchased from Taconic (Germantown, New York) and kept in filter topped cages and given *ad libitum* access to autoclaved food and water prior to and during the study. The protocols employed were in direct accordance with the guidelines of the McMaster University Animal Care Committee and the Canadian Council on the Use of Laboratory Animals.

5.2.2 Recombinant Adenovirus Vectors

The recombinant human type 5 adenovirus with mouse IL-4 cDNA incorporated in the E3 region of the viral genome (Ad5CAIL-4) has been previously constructed and well characterized *in vitro* (20). This recombinant virus possesses the human cytomegalovirus (hCMV) promotor and polyadenylation signal sequences, upstream and downstream of the inserted IL-4 cDNA, respectively. The adenovirus encorporating the *Escherichia coli*- beta-galactosidase cDNA (Ad5LacZ) in the E3 region was constructed in a similar fashion (21). High titers of viral constructs were generated as previously described (21). Virus was then purified by CsCl gradient centrifugation, and dialyzed thoroughly as previously described (20,21), aliquoted, and stored at -70^{0}C until use.

5.2.3 Surgery - Study Protocol and Establishment of Viral Infection

Mice were anaesthetized by intra-peritoneal injection of ketamine and xylazine(70ng and 6ng/gram body weight respectively). Following a midline abdominal incision, the small bowel was gently exposed and covered with saline soaked gauze. A filter sterilized gel solution containing 20% (w/v) F127 pluronic gel (BASF, Wyandotte Corp., Wyandotte, MI) in phosphate buffered saline (PBS) pH 7.4 was mixed with 2 x 10^{8} pfu of adenovirus, to give a final gel concentration of 15% (w/v) in a total volume of 80 ml. The virus-gel mixture was then painted onto a 6-8 cm region of jejunum, begining distal to the ligament of Trietz. The bowel was then covered with gauze, and left for 5 minutes to allow the gel to set. Intestinal peristalsis was still present in all animals following the procedure. The small bowel was then

returned to the abdominal cavity and the incision closed with discontinuous sutures. Following surgery, the animals were allowed to recover, and then euthanized over the next three weeks for tissue collection.

5.2.4 Histology and Histochemical Localization of Beta-Galactosidase (LACZ) Product

Intestinal tissues were fixed in 10% neutral buffered formalin overnight, followed by storage in 70% ethanol. Formalin fixed tissues were then embedded in paraffin, sectioned at 3 μm and stained with either haematoxylin and eosin or haematoxylin and congo red, using standard staining procedures. Staining for LacZ expression was as described by Mastrangeli *et al.* (22) with minor modifications. Briefly, over the time course of the infection, mice were euthanized, their small bowel removed and flushed of lumenal contents and then fixed with 2% formaldehyde in PBS at 4^0C for 1 hour. The intestine was then rinsed twice with PBS and immersed in staining solution containing 5mM $K_4Fe(CN)_6$, 5mM $K_3Fe_3(CN)_6$, 2 mM $MgCl_2$, and 0.5 mg/ml of the X-gal stain (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside: (Boehringer Mannheim Corp., Indianapolis, IN) at 37^0C over-night. The stained intestinal tissues were then paraffin-embedded, sectioned at 6 μm, and counterstained with nuclear fast red.

5.2.5 Detection of Interleukin-4 by RT-PCR

For analysis of IL-4 mRNA expression, LACZ and IL-4 virus infected mice were euthanized three days after infection, and the LMMP, including serosa was stripped from the infected jejunum. For *in vitro* analysis, murine smooth muscle cultures were prepared as previously described (23), infected at a concentration of 10 pfu of adenovirus/cell. Cells were harvested 3 days later. Total cellular RNA was isolated using a previously described guanidium isothiocyanate method (24). The RNA concentration was determined by measuring absorbance at 260 nm and its purity confirmed using the ratio of absorbency at 260 nm to that at 280 nm. mRNA was then used for reverse transcription-polymerase chain reaction (RT-PCR) as previously described (24) to yield cDNA. Two μl aliquots of cDNA (3 μg) were then mixed with

ACT ACG AGT AAT CCA-3') primers for murine IL-4 (30). The house-keeping gene was hypoxanthine phosphoribosyl transferase (HPRT), and 20 pmol of each sense primer (5'-GTT GGA TAC AGG CCA GAC TTT GTT G-3') and anti-sense primer (5'-GAT TCA ACT TGC GCT CAT CTT AGG C-3') were used to detect it (25). IL-4 and HPRT were co-amplified for 33 cycles and PCR products visualized as previously described (26). The 383 bp product corresponds to IL-4 and the 164 bp product corresponds to HPRT.

5.2.6 Peritoneal Lavage

Following euthanization, mice underwent peritoneal lavage which involved the injection of 4 mls of cold PBS into the peritoneal cavity of each mouse, and after 30 seconds of abdominal "massage", the abdominal cavity was opened and the fluid recovered and placed on ice. 1 ml of the fluid was centrifuged at 1000 rpm for 8 minutes. The supernatant was then saved and stored at -70^{0}C for assay of interleukin-4. Another 1 ml was used for total and differential cell counting. Differential cell counts were performed using Diff Quik (Dade Diagnostics, Aquada, USA) on aliquots from PLF samples, counting and identifying a minimum of 350 to 450 cells per sample.

5.2.7 Detection of IL-4 Protein by ELISA

Levels of murine IL-4 within the peritoneal lavage were determined using a standardized sandwich ELISA protocol, with antibodies puchased from PharMingen (San Diego, CA), as previously described (26). In brief, ELISA plates were coated with the rat anti-mouse IL-4 capture antibody (BVD4-1D11) 10 mg/ml) and blocked with bovine serum albumin. Following addition of experimental samples, the biotinylated rat anti-mouse IL-4 detection antibody (BVD6-24G2) was added for 3 h, and streptavidin-alkaline phosphatase (1:20,000 dilution) was used to visualize the IL-4. An ELISA amplification system (Gibco BRL, Burlington, ON) was used to increase the colour intensity generated and recombinant murine IL-4 (Intermedico, Markham, Canada) was used to generate the standard curve.

5.2.8 Muscle Function and Carbachol dose response curve

The preparation of the gut sections for muscle contractility analysis has been described previously (10). In brief, the jejunum was removed and placed in oxygenated (95% O_2 - 5% CO_2) Kreb's solution and 1 cm sections of whole gut were cut from the jejunum, beginning at the ligament of Treitz and proceeding distally, or from the distal ileum, beginning 1 cm from the ileo-caecal junction and proceeding proximally. The lumen of each segment was flushed with Kreb's buffer, prior to the insertion of 2-3 mm lengths of Silastic tubing (0.065 OD, 0.030 ID) (Dow Corning, Midland, MI) into the open ends of the gut segments, a process which was found to maintain their patency. Tubing was then tied in place with surgical silk. Segments were then hung in the longitudinal axis and attached at one end to a grass FT03C force transducer (Grass, Quincy, MA) and responses were recorded on a Grass 7D polygraph. Tissues were equilibrated for 30 minutes at 37^{0}C in oxygenated Kreb's solution, before starting the experiment. Experiments were then conducted to examine the length-tension characteristics of the muscle before and after infection. Segments were stretched by applying tension equivalent to 0-1250 mg of weight, (a range previously found sufficient to determine the maximal responsiveness of both control and inflamed tissues) and contraction was assessed following stimulation with 1 mM carbachol (Sigma Chemical, St. Louis, MO). After each application of tension, the length of the tissue, and the contractile response were recorded. To examine neural contributions to contraction, tissues were exposed to the neurotoxin, tetrodotoxin (TTX: 1mg/ml) for 15 minutes to inhibit neural activity. At the end of each experiment, tissues were removed, blotted and weighed, and the optimal tension (T_O) and the tissue length which gave the maximum contractile response were used to calculate the cross sectional area of the tissue. For dose response studies, the previously identified T_O for a tissue was applied first, followed by exposure of the gut segments to noncumulative final bath concentrations of 1nM-1 mM carbachol by addition of microliter aliquots to 20 ml baths. After the maximal response to each dose was obtained, tissues were rinsed twice and equilibrated in fresh Krebs solution for 15 minutes before adding the next agonist dose.

5.2.9 Data Presentation and Statistical Analysis

Responses to carbachol were expressed as mgs of tension per cross sectional area as previously described (10). Mean tension was calculated from at least three segments per mouse. All results are expressed as the means ± 1 SEM, n refers to the number of mice tested. Statistical significance was calculated using the Student's t test for comparison of two means or an ANOVA for the comparison of three or more means. Multiple comparisons were performed using the Neuman-Keuls multiple comparison test. $P < 0.05$ was considered significant.

5.3 RESULTS

5.3.1 Preliminary Experiments (Intra-Lumenal and Intra-Peritoneal Application.)

Initial gene transfer experiments to the lumen of the murine G.I. tract were attempted by injecting the marker virus LACZ, diluted in PBS, or in 15% pluronic gel, directly into the small bowel following surgery, or through intrarectal instillation into the colon. However, few infected cells were detectable by LACZ histochemistry. We also examined i.p. administration of the virus, and as others have found (27), the majority of the transgene expression occurred within the liver, with few infected cells detected within the G.I. tract.

5.3.2 Serosal Application of the Virus and Time Course of Infection

Since lumenal application of the virus was unsuccessful, we adopted an approach of applying the virus to the external surface of the small bowel, following its dilution in a pluronic gel solution, which has been previously shown to increase gene transfer efficiency as well as anti-sense oligonucleotide delivery to vascular smooth muscle and endothelial cells through intra-arterial infusion (19,28). As with the earlier studies, we found that application of the gel alone had no deleterious effects upon animal survival, as mice undergoing surgery with or without the subsequent application of the gel had similar survival rates (95%).

Mice recovered from surgery quickly and upon sacrifice of sham treated mice, the small bowel appeared normal, except for occasional signs of mild edema. Following staining for beta-galactosidase, numerous blue, positively stained cells were seen on the surface of the gut, (Figure 1A), most being in the region of the original gel application. Under higher magnification, the majority of the infected cells were identified as serosal cells (Figure 1B), and this identification was confirmed when cross sections were examined (Figure 1C). Occasionally other cell types, just beneath the serosa and with the appearance of smooth muscle cells, also stained blue (not shown). As well, many cells in the intestinal mesentery were also infected (Figure 1D). LACZ expression lasted at least 1 week, with the number of infected cells seen after 1 day of infection remaining constant until day 7 PI. By day 12 PI however, the number of positively stained cells had decreased by approximately 50%, and by day 21 PI, only a few scattered blue stained cells could still be detected.

5.3.3 IL-4 Gene Expression (PCR & mRNA)

To examine IL-4 transgene mRNA within the small bowel, we semi-quantitatively measured IL-4 mRNA expression, using RT-PCR, with total RNA extracted from the longitudinal muscle - myenteric plexus (LMMP) as well as the serosa of the infected jejunum. As our positive and negative controls, we extracted mRNA from intestinal smooth muscle cultures infected with either the Ad5CA-IL-4 or the Ad5E1 viruses. As can be seen in Figure 2, the predicted 383 bp sized PCR product for IL-4 was detected in the gut tissue of mice receiving Ad5CA-IL4 and in the cells infected with the IL-4 virus, but not in the tissues or cells infected with the control vector.

5.3.4 IL-4 Protein Levels in Peritoneal Lavage Fluid (PLF).

To assess the production of IL-4 transgene protein, we assayed peritoneal lavage fluids from infected mice by ELISA. Peak IL-4 protein levels within the peritoneal cavity were detected on day 1 PI, reaching almost 70 ng of IL-4 protein (approximately 17ng/ml of PLF). Consistent with the observed time course of LACZ transgene expression, high levels of IL-4 protein remained detectable in the PLF for at

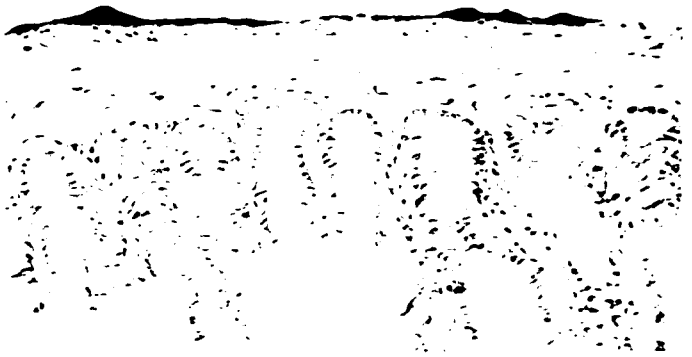
Figure 5.1:Histological demonstration of beta-gactosidase expression.

Panel A. Macroscopic appearance of the small bowel removed from a mouse infected 3 days previously with LACZ virus and subsequently stained for beta-galactosidase activity. Note the blue green stained cells clustered on the surface of the intestine (arrow). Panel B. Higher magnification of the same tissue. Note the numerous positively stained cells on and occasionally below the surface of the intestine (x10). Panels C & D. Cross sections of LACZ infected small bowel. Note the serosal cells as well as cells in the intestinal mesentery staining positive for the beta-galactosidase enzyme (x200).

A

B

C



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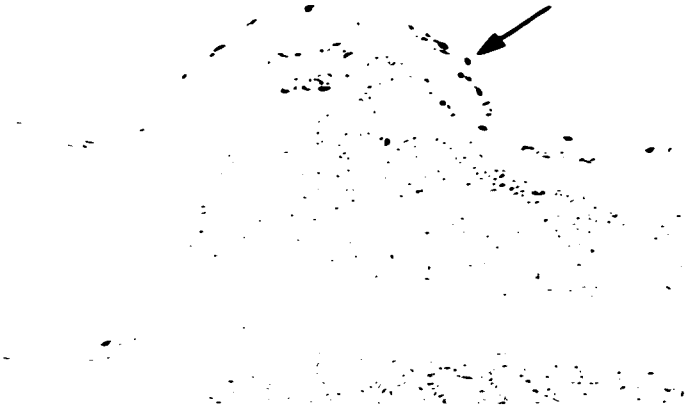
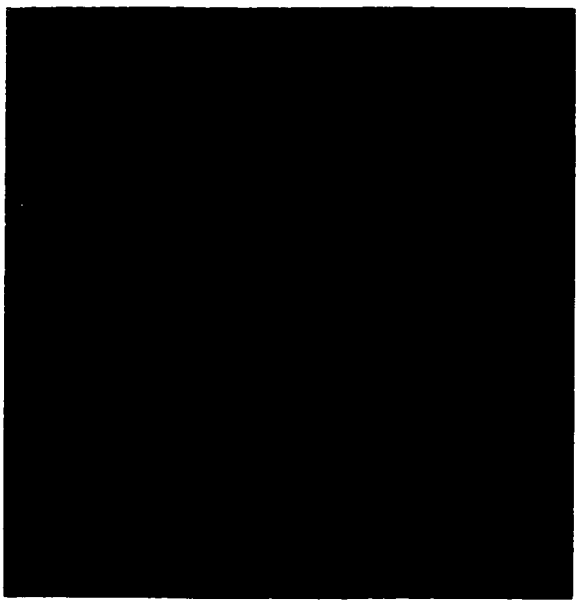


Figure 5.2: IL-4 gene transcription

IL-4 gene transcription within the serosa and external muscle layers of the small bowel of mice infected 3 days before, with either the Ad5CA-IL4 or Ad5E1 virus, or intestinal smooth muscle cells in culture, infected with the same viruses, also 3 days previous. Lane 1: Ad5CA-IL4 virus infected mouse Lane 2: Ad5E1 infected mouse Lane 3: Ad5CA-IL4 infected intestinal smooth muscle cells in culture. Lane 4: Ad5E1 infected intestinal smooth muscle cells in culture. HPRT is used as a the internal housekeeping control in each lane.



IL-4

HPRT

1 2 3 4

least 12 days following IL-4 gene transfer. However, by day 21 PI, levels of IL-4 had become almost undetectable (Figure 3). In contrast, the endogenous levels of IL-4 detected in the PLF from mice receiving the control vector were extremely low, never reaching total levels greater than 2 ng.

5.3.5 IL-4 Protein Levels in Serum.

To determine whether IL-4 transgene expression was detectable within the circulation, sera was taken at various timepoints and assayed for IL-4 by ELISA. Levels of detectable IL-4 within the sera of mice receiving the IL-4 virus were very low (< 1 ng) and athough the levels were higher than those found in mice receiving the Ad5E1 virus, they did not go above background levels (not shown). However other routes of adenoviral infection were capable of elevating IL-4 serum levels, since mice given 2 x 10^8 pfu of AdCAIL-4 virus by intra-muscular injection were found to have over 10 ng of IL-4 per ml of serum, as of day 3 PI. (not shown).

5.3.6 Changes in the Cellular Components in PLF Post-IL-4 Gene Transfer.

To quantify the inflammatory and immune response, we performed total and differential cell counts within the PLF. Uninfected mice had fewer than 1 million cells in the peritoneal cavity, most being peritoneal macrophages (75%) and mast cells (15%). Following infection with either adenoviral vector, there was a rapid increase in cell numbers. Peritoneal macrophages, lymphocytes and neutrophils were increased in number by day 1 PI, with either virus, however the expression of IL-4 also recruited eosinophils and mast cells. This trend continued over the following days, with the number of inflammatory cells continuing to increase in both groups of animals, though to a greater extent in the Ad5CA-IL-4 infected mice (see Figure 4A), peaking on day 12 P.I. (> 180 million cells) compared to day 7 in the AdLACZ infected mice (45 million). While the cellular response to LACZ infection consisted of macrophages, lymphocytes, neutrophils and a small number of peritoneal mast cells (Figure 4B), the addition of IL-4 not only increased the number of macrophages and lymphocytes, over that of the

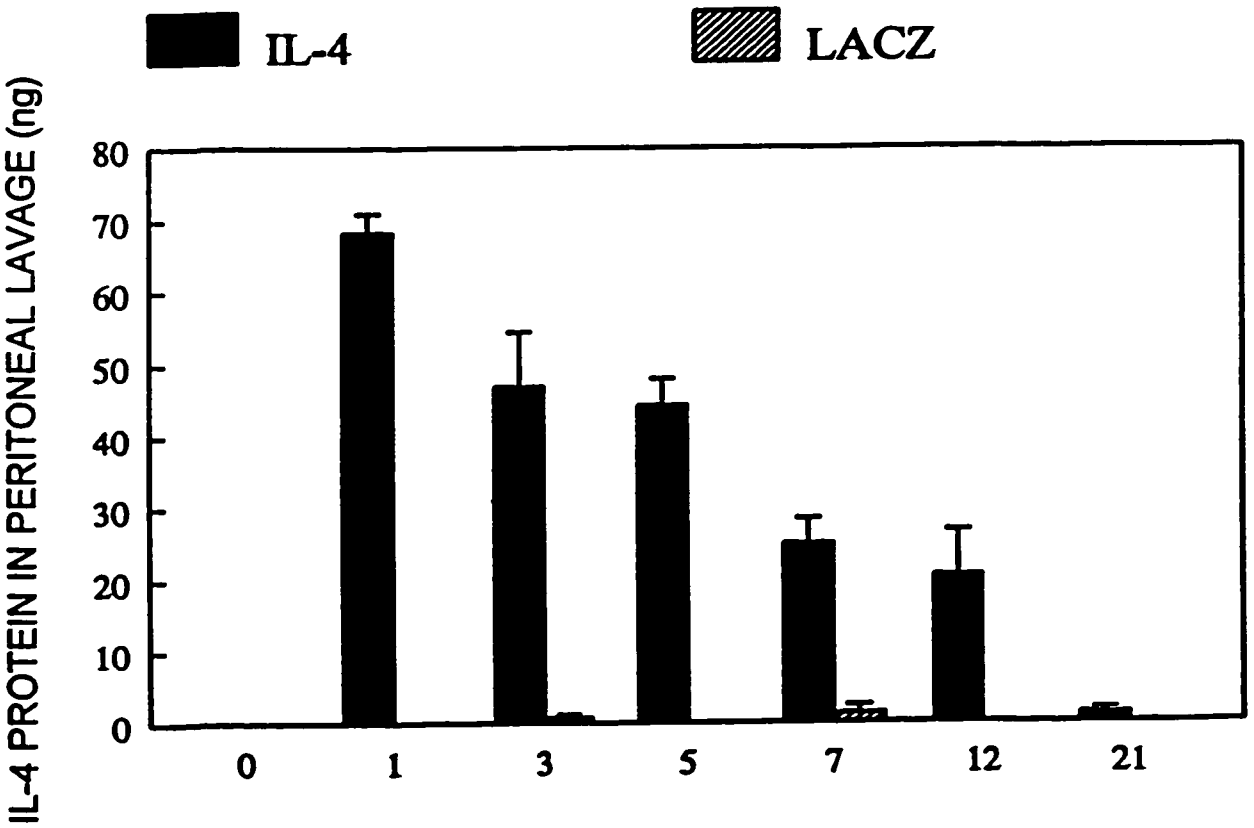


Figure 5.3:IL-4 protein levels in peritoneal lavage fluid

The amount of IL-4 protein detected by ELISA per ml of peritoneal lavage fluid taken over the course of the infection from both control and IL-4 virus infected mice. The levels in the IL-4 virus infected mice are represented by the closed bar while the LACZ virus infected levels are shown by the open bar, over the course of three weeks post-infection. Results shown are the mean ± 1 SEM of groups of 4-6 animals.

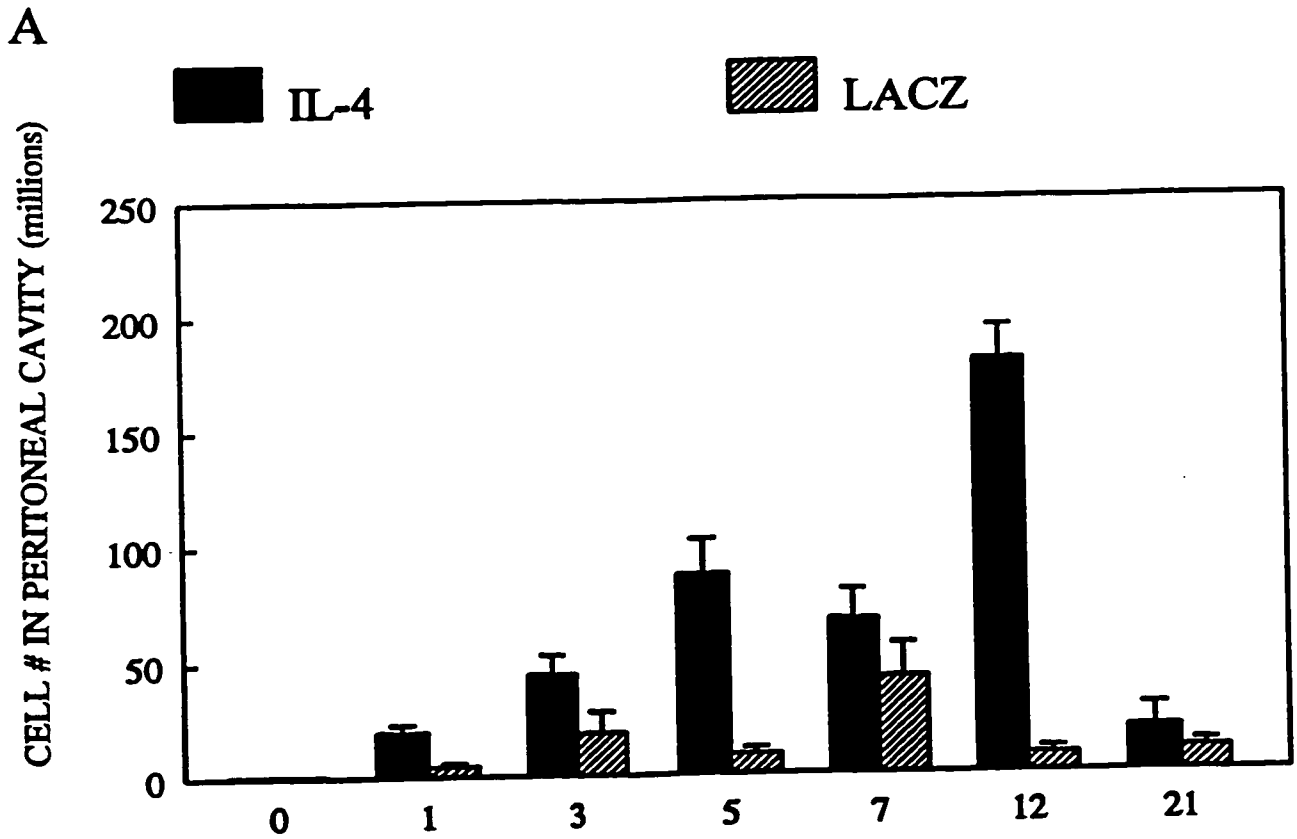
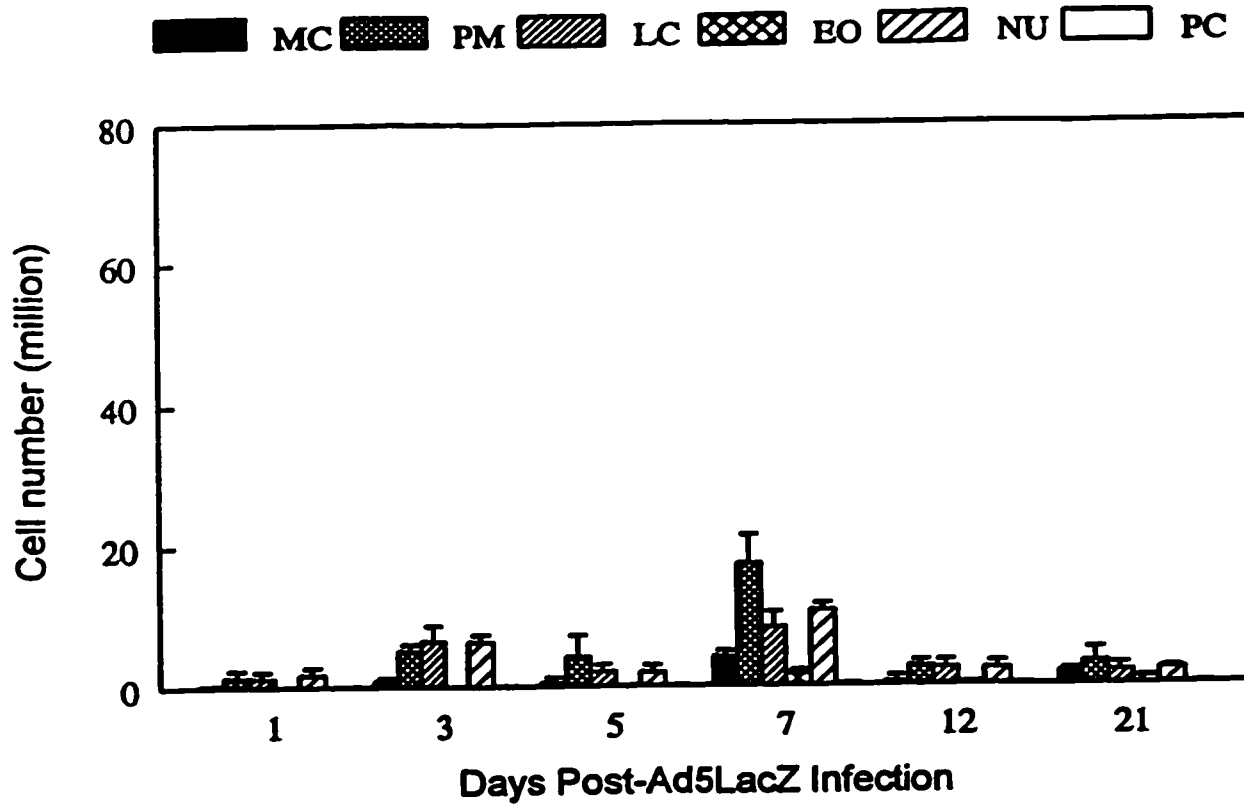


Figure 5.4:Inflammatory response in peritoneal lavage fluid

Panel A. The total number of cells recovered from the peritoneal lavage fluid taken from both control and IL-4 virus infected mice over three weeks post-infection. The number of cells recovered from the IL-4 virus infected mice are represented by the closed bar while the LACZ virus infected numbers are shown by the open bar. Results shown are the mean ± 1 SEM of groups of 4-6 animals. Panel B. Kinetics of mast cells (MC), peritoneal macrophages (PM), lymphocytes (LC), eosinophils (EO), neutrophils (NU) and plasma cells (PC), recovered and identified within PLF of Ad5LACZ infected and (Panel C.) Ad5CA-IL-4 infected mice. Results shown are the means ± 1 SEM of groups of 4 animals.

B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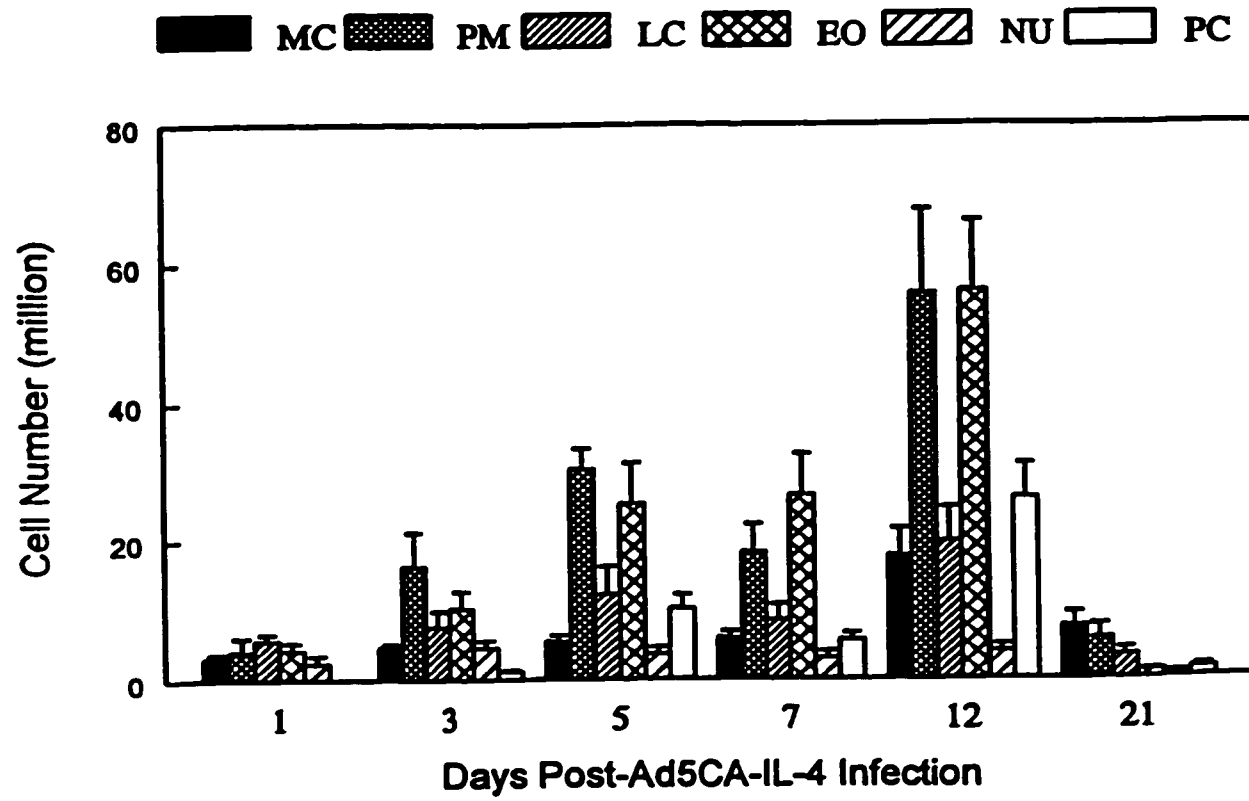
Days Post-Ad5LacZ Infection

C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Days Post-Ad5CA-IL-4 Infection

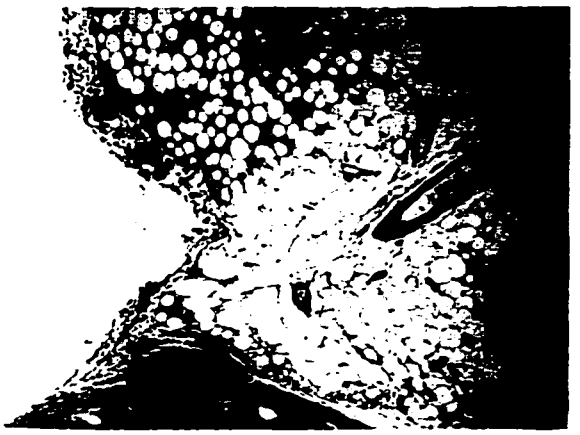
control virus by approximately 200%, but also resulted in a substantial eosinophilia and plasmacytosis (Figure 4C). Peritoneal mast cell numbers were also dramatically increased in IL-4 virus infected mice. By day 21, as the infections were cleared, the number of inflammatory cells decreased, although they were still elevated 15 and 30 fold over that of control mice, with similar phenotypic cell populations in LACZ and IL-4 virus infected mice, however mast cells and plasma cells were more numerous within the IL-4 virus infected mice.

5.3.7 Tissue eosinophilia and mastocytosis

The tissue inflammatory response was also examined, in order to assess the intestinal involvement and to determine what effect IL-4 over-expression had upon the cellular phenotype of the cells infiltrating the intestine. Consistent with the PLF findings, the control vector elicited a mild inflammatory response comprising a mix of mononuclear cells with neutrophils, localized to the serosa and external muscle layers of the jejunum, with little involvement deeper within the intestinal wall (Figure 5A). Eosinophils were rarely seen in the Ad5LACZ infected mice. In contrast, significant tissue eosinophilia was observed in the muscle layers of mice overexpressing IL-4, along with the infiltrating macrophages, lymphocytes and neutrophils seen with the control virus. The response to the IL-4 virus was of a greater magnitude and penetrated deeper into the intestinal wall than with the LACZ virus, even reaching the intestinal crypts (Figure 5B). Congo red staining revealed that eosinophil accumulation began as early as 1 day after IL-4 gene transfer and was marked between days 2 and 12 in the region where the virus was applied. The tissue eosinophilia was patchy however, since some regions showed far more infiltrating eosinophils (Figure 5C) than others (Figure 5D). However the eosinophil numbers were always greater in the IL-4 virus infected mice, than with the control virus (Figure 5E). Despite the eosinophilia seen in IL-4 infected mice, no obvious increase in mucosal mast cell numbers was seen, as assessed by alcian blue staining. While a minor increase in connective tissue mast cell numbers was seen in IL-4 virus infected mice some 30 days PI (using toluidine blue staining), this was also seen in tissues infected with the control virus (not shown).

Figure 5.5:Tissue inflammatory response. Panel A. Inflammatory response within the mesentery and on the surface of the intestine of a LACZ infected mouse, day 7 P.I. Panel B. Inflammatory response within the mesentery and on the surface of the intestine of an IL-4 virus infected mouse, day 7 P.I. Panel C. Congo red staining showing jejunal muscle layers of an IL-4 infected mouse demonstrating significant eosinophilia (x200) with an arrow designating an eosinophil. Panel D shows a milder tissue eosinophilia (x200), also from an IL-4 virus infected mouse, again with an arrow designating an eosinophil (x400). Both C and D are day 7 P.I. At day 7 post-LACZ infection - no eosinophils are seen in the jejunal *muscularis externa* (Panel E) (x400).

A



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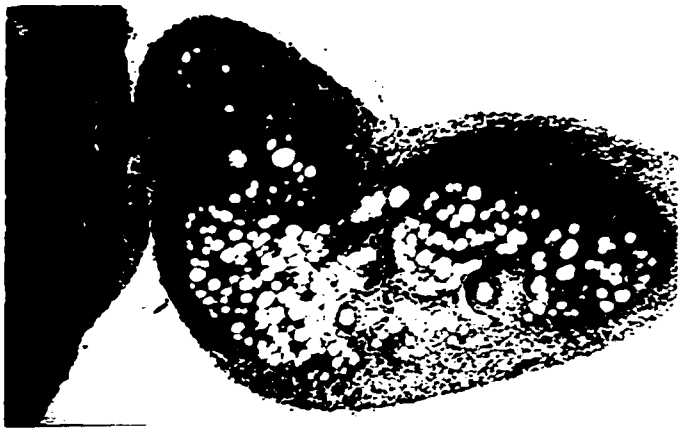

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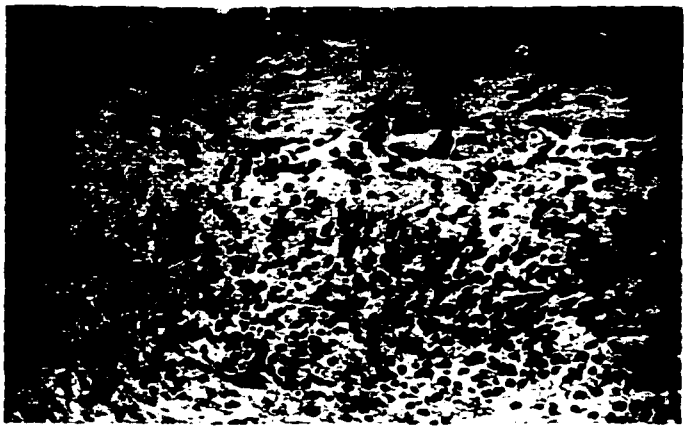

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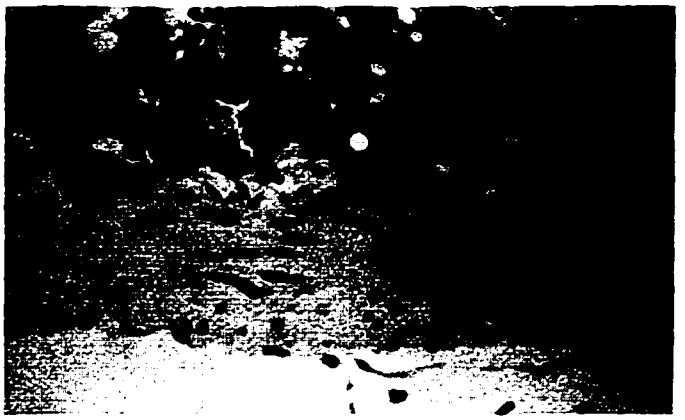


5.3.8 Muscle Function

Increased tension generation by jejunal longitudinal muscle from IL-4 virus infected mice was observed by day 5 PI (not shown), but by day 7 PI, the contractile response to 10^{-6} M carbachol was significantly elevated, compared to the tension generated by both control and day 7 P.I. LACZ infected tissue (see Figure 6). The neurotoxin TTX had no effect on the muscle hyper-responsiveness, indicating that the changes in function were myogenic and not neurally based. In contrast, no change in the maximum tension generation by longitudinal muscle was observed during the course of LACZ infection. We also examined the contractile response of ileal smooth muscle, following IL-4 gene transfer to the jejunum, to determine if alterations in muscle function affected the entire small bowel, or were selective for the jejunum. As seen in Figure 7A, while the IL-4 treatment led to jejunal smooth muscle hyper-responsiveness, significantly increased over control and LACZ infected tissues over a broad dose range, no significant change was seen in ileal muscle function (Figure 7B).

5.4 DISCUSSION

This is the first demonstration of successful, site directed gene transfer to the murine small bowel. Other groups have tried to target transgene expression to the intestine, however unlike other tissues, the simplest route of delivery, the lumen, is not the most effective, often requiring an initial insult before achieving any appreciable transgene expression (6,29). While targeting genes to the mucosa might better mimic pathological conditions and as a therapy, result in transgene expression in closer proximity to the site of disease, the difficulties encountered targeting gene transfer through the lumen may be insurmountable. Beyond the protective epithelial and mucus barriers that line the lumen, preventing viral contact and infection of host cells, the desired targets of infection, the intestinal epithelial cells; have only a small window of potential transgene expression, since they are rapidly sloughed from the tips of the villi into the intestinal lumen (30). Additional routes for intestinal gene transfer include the blood circulation and the surrounding peritoneal cavity. Tail vein injection of adenoviral vectors can yield transgene expression within the murine colon, however infection of the intestine appears incidental, with the

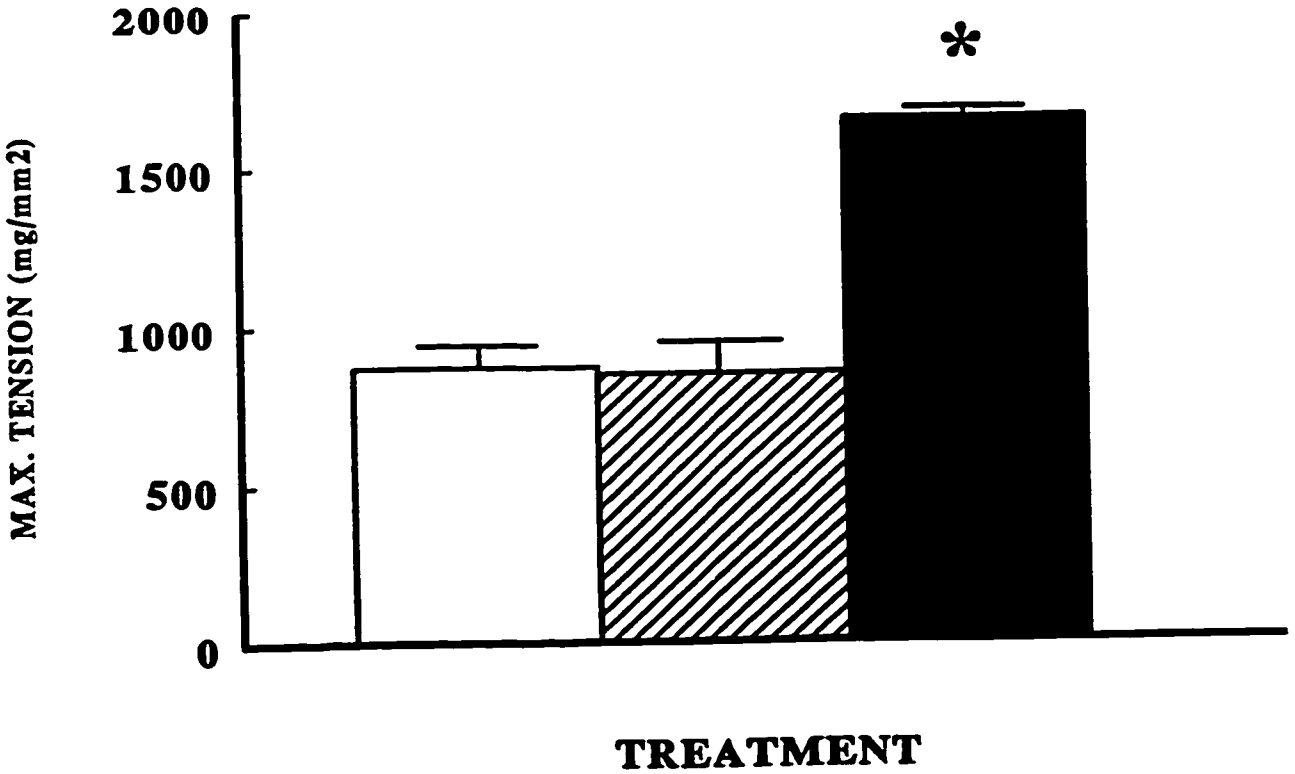
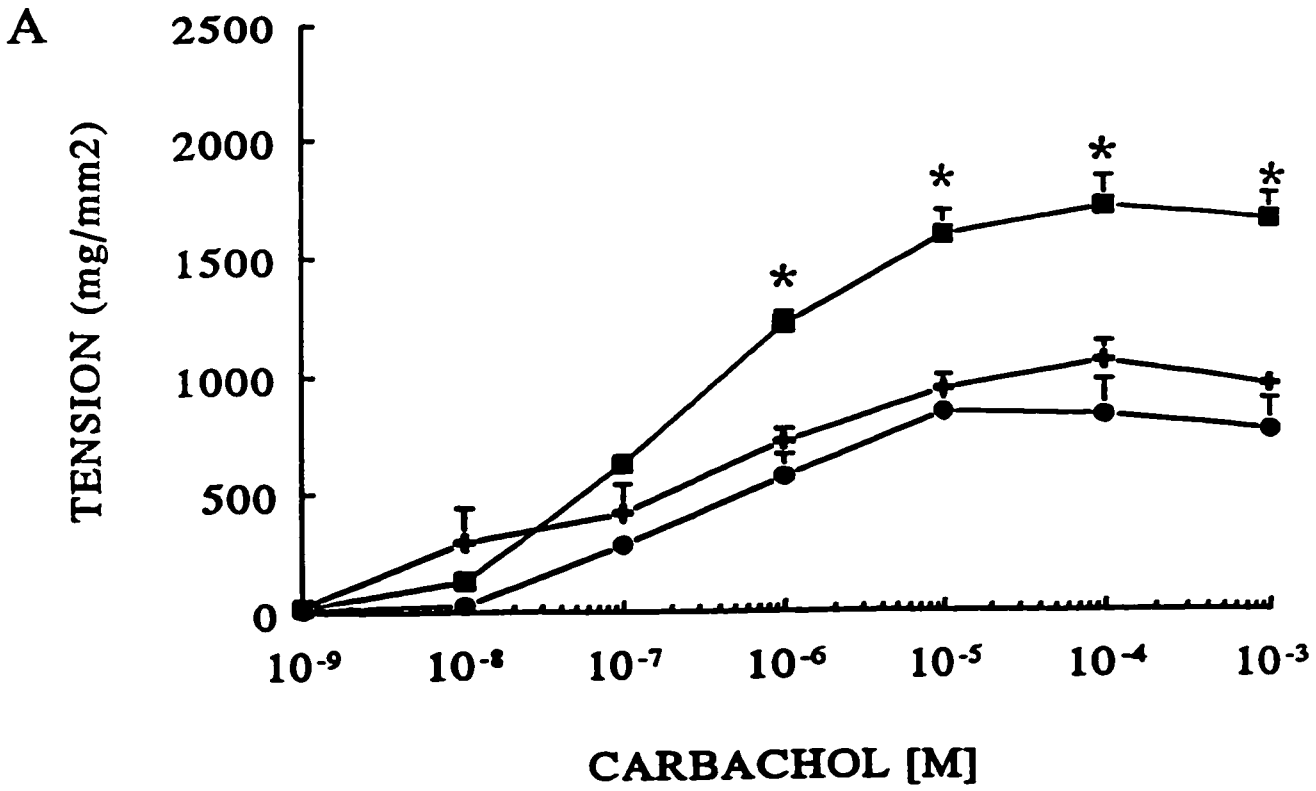


Figure 5.6. Muscle contractility - changes due to infection

Maximum tension generated by muscle in response to 1mM carbachol on day 7 P.I. from C57BL/6 mice given the marker virus (hatched bar), the IL-4 virus (closed bar) or nothing at all (open bar). Results are the mean ± 1 SEM of groups of 4-6 animals. The asterisk denotes a significant increase in tension generation when compared to the other two groups.

Figure 5.7. Muscle contractility - jejunal and ileal smooth muscle dose response curves

Dose response relationships for carbachol induced contraction of jejunal (Panel A) longitudinal smooth muscle taken from either control C57BL/6 mice (crosses), or mice infected 7 days earlier with the AdLACZ (closed circles) or the AdCA-IL4 virus (closed squares) painted on the jejunal surface. Panel B shows baseline ileal tissue function (crosses) as well as ileal smooth muscle function when the jejunum has been infected by the LACZ virus (open circles), or the IL-4 virus (open squares). Results shown represent the mean tension ± 1 SEM of groups of 4-6 animals. The asterisk denotes a significant increase in tension generation when compared to control and to LACZ infected mice.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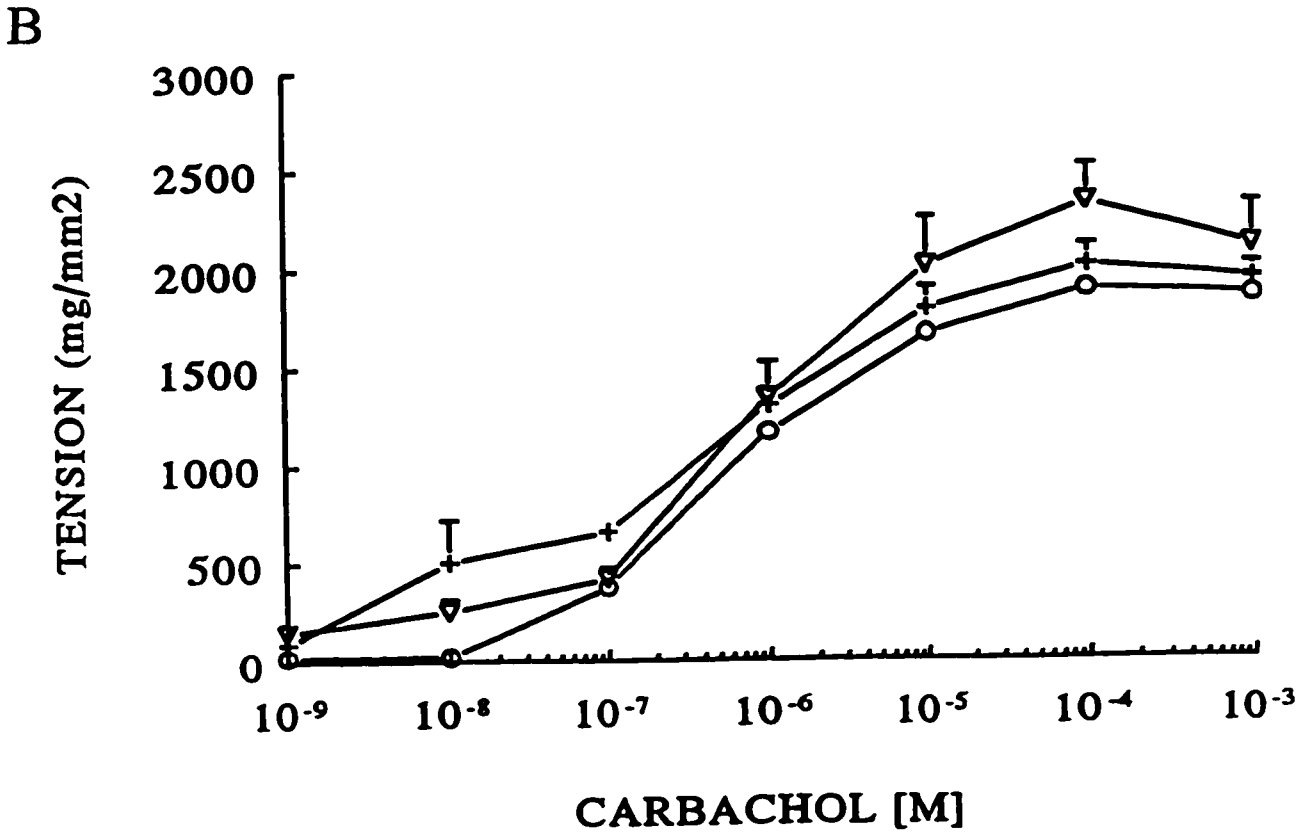
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CARBACHOL [M]

majority of the transgene expression occurring within the liver (8). Others have tried more selective approaches, including injecting adenovirus into the arteries feeding the small bowel. While this can result in transgene expression within the intestinal mucosa, the surgical procedure is difficult and the identity of the cells transfected by this method remain unclear (9).

We have circumvented these problems by bypassing the intestinal lumen and targeting the outer layers of the gut, through the surface application of adenoviral vectors diluted in the biocompatible pluronic gel, F127. Thermally reversible gels have been used in a number of studies, including gene transfer (19), and because of their low toxicity and lack of irritancy, they have also been investigated as drug delivery systems for opthalmic, rectal and subcutaneous use (31,32). Gel application, in this study, allowed for the targeting of specific regions of the intestine, and increased the duration of the viruses bioavailability, enabling adenoviral infection of the intestine.

The potential advantages of targeting the outer layers of the gut can be seen in this study, with the expression of transgenes having dramatic effects upon the function of the enteric neuromuscular apparatus. Not only can this approach be used to study interactions between the immune and intestinal neuromuscular systems, but also potentially in the study of both systems in enteric host defense (11). As well, serosal and muscle cells are more stable populations of cells than intestinal epithelial cells, and as this study shows, are among the most accessible intestinal cell types to target, however not all approaches give the same degree of transfection. Another group recently attempted direct injection of plasmid DNA into the submucosa of the rat stomach, however although the injections did result in gene transfer to the intestinal muscle layers, the extent of transfer was very limited, with only a few muscle cells expressing the transgene at the site of injection (33).

The approach adopted in this study led to the infection of large numbers of serosal cells on the surface of the intestine as well as cells within the adjacent mesentery. LACZ expression persisted for at least 2 weeks following infection and we found IL-4 protein release into the peritoneal cavity over the same time period. Infection with either virus led to an inflammatory and immune response within the peritoneal cavity involving predominantly macrophages, lymphocytes and neutrophils within the

peritoneal lavage fluid of AdLACZ infected mice. However, the number of inflammatory cells was significantly increased following IL-4 virus infection, with an additional accumulation of eosinophils, mast cells and plasma cells. The response by mast cells and plasma cells to the IL-4 virus was unexpected, since previous studies using this vector have only shown an eosinophil recruitment to the site of infection (20). The mast cell and plasma cell recruitment may have been due to the actions of IL-4 as a growth factor for these cell types, or have been a result of IL-4 inhibiting the anti-viral response. The intestine was also affected, with macrophages and lymphocytes infiltrating the jejunal serosa and external muscle layers of LACZ infected mice. These cells also infiltrated the intestine following IL-4 virus infection, however tissue eosinophilia was also prominent. Within seven days of the IL-4 virus infection, we also observed a significant increase in tension generation by intestinal longitudinal smooth muscle in response to carbachol. This response appeared to be more effective on jejunal muscle than ileal, since ileal muscle was only affected when the virus was applied directly to the ileal surface, while jejunal muscle was altered in its function no matter where on the small bowel the virus was applied. Neither jejunal nor ileal smooth muscle showed a change in function following LACZ infection.

The choice to deliver the Ad5CAIL-4 virus in this study was based upon the critical role IL-4 plays in the initiation of Th2 immune responses as well as previous studies demonstrating an association between allergic inflammation and altered smooth muscle function, in both the G.I. and respiratory tracts (11,34,35). In inflammatory diseases such as asthma and IBD, altered muscle physiology is thought to contribute to the disease process as well as the symptomatology, bronchoconstriction in asthma (35), and pain, nausea and diarrhea in IBD (11). IL-4 has also been implicated in several other diseases including rheumatoid athritis (36), in association with the deposition of matrix proteins and tissue dysfunction. Recently, over-expression of IL-4 using the Ad5CA-IL-4 virus was found to increase the fibrotic response in a model of granuloma formation (37), providing further evidence of a role for IL-4 in both tissue fibrosis, and in the pathophysiology of non-immune cells.

Increased contraction by small bowel longitudinal muscle has also been shown to occur during enteric infection by nematode parasites (10-12), bacteria (15) and protozoa (16). We and others have

postulated that the changes in muscle function and motility are an adaptive host response, the immune system recruiting the gut neuromuscular apparatus to aid in the expulsion of pathogens through increased propulsive (10,11,17). In the present study, over-expression of IL-4 affected jejunal smooth muscle but not ileal smooth muscle. This may relate to the IL-4 only having local effects, or perhaps the potential role of these changes in host defense, with increased motility of the proximal small bowel potentially propelling pathogens distally, as one step in their eventual expulsion (11). Others have shown that high doses of systemically administered IL-4 accelerates the expulsion of gastointestinal nematodes, and this treatment also led to an increase in electrical field stimulated acetylcholine release by enteric nerves concurrent with a mucosal mastocytosis (17). In contrast, the altered function in the present study occurred at the level of the smooth muscle, since TTX had no effect.

While the the AdCAIL-4 virus produced dramatic changes in intestinal smooth muscle function, whether a direct interaction occurred between IL-4 and muscle is unclear. The altered function may have occurred through the actions of the cell types recruited, since both eosinophils and mast cells produce mediators which can contract smooth muscle *in vitro*, and are frequently associated with smooth muscle hyper-responsiveness. However the mere presence of eosinophils and mast cells within tissues appears insufficient to alter muscle function based upon previous studies from this lab (11,38) and others (39). Since relatively little is known about how changes in smooth muscle function occur and what cell types are responsible, IL-4 may act through an as yet unknown mechanism.

While this study was not designed to model IBD, since both ulcerative colitis and Crohn's disease predominantly affect the mucosa, the findings in this study may prove relevant to the transmural involvement (40) seen in some cases of IBD. There are also cases of eosinophilic gastroenteritis which predominantly affect the serosa and external muscle layers of the bowel (41). This study also demonstrates the potential to express other genes within the intestine, as putative therapies, possibly by replacing the defective expression of mutated genes involved in recessive genetic diseases, or delivering vaccines to the G.I. tract. The recent demonstration that i.p. administration of adenoviral vectors encoding the cytokines IL-4 and IL-10 ameliorated experimental colitis (26,42) is also encouraging. Eventually, directing viral

vectors encoding immunosuppressive transgenes to the G.I. tracts of patients with IBD may provide an effective therapy, with fewer systemic side effects than are found with current treatments.

We have thus developed an effective approach to intestinal gene transfer, through the surface application of adenoviral vectors diluted in a pluronic gel, and shown IL-4 mRNA expression in the gut and the release of significant amounts of IL-4 protein within the peritoneal cavity following use of an IL-4 encoding virus. Infection led to the generation of an inflammatory response, with IL-4 also recruiting cell types characteristic of allergic or eosinophilic inflammation. Concurrent with this response was the onset of a hyper-responsive state in intestinal longitudinal smooth muscle function. Similar alterations in muscle function have been found during enteric infections, indicating that IL-4 may be the mediator responsible. This novel approach to gene delivery to the intestine should help to clarify how alterations in intestinal motility are influenced by the immune system and demonstrates that viral based gene therapy for intestinal diseases may one day be possible.

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The following work returns to the *T. spiralis* infection model. Based upon our work with *Trichinella* and with the IL-4 expressing adenovirus, we hypothesized a role for eosinophils in the muscle hyper-contractility seen in this model. We infected IL-5 deficient mice, which are known to be impaired in developing eosinophilic responses, and found that while they did not develop an intestinal eosinophilia during infection, there was no effect upon the onset of intestinal muscle function. However the persistence of the muscle contraction in the IL-5 deficient mice was reduced compared to wildtype mice, as was their ability to expel a *T. spiralis* infection.

Chapter 6

A role for interleukin-5 and eosinophils in the intestinal muscle dysfunction following *T. spiralis* infection.

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ABSTRACT

Enteric nematode infections lead to increased interleukin (IL)-5 expression, eosinophilic inflammation and intestinal smooth muscle hyper-contractility. While eosinophils are known to release inflammatory mediators that cause smooth muscle contraction, the role of IL-5 and eosinophils in enteric smooth muscle hyper-contractility is unclear. IL-5 deficient mice and their wild-type controls were infected with the nematode *Trichinella spiralis*. Intestinal parasites and eosinophils were counted and jejunal longitudinal muscle contractility assessed. During infection, IL-5 gene expression significantly increased in wild-type mice, and this was accompanied by a significant intestinal eosinophilia in wild-type but not IL-5 deficient mice. While both strains developed increases in enteric muscle contractility during infection, this was better sustained by day 21 post-infection in the wild-type than the IL-5 deficient mice. Adult parasite expulsion was also delayed in IL-5 deficient mice. Thus, in the nematode infected mouse, IL-5 appears essential for intestinal eosinophilia, is involved in the persistence of muscle hyper-contractility, and contributes to the expulsion of *T. spiralis* from the gut.

6.1 INTRODUCTION

Blood and tissue eosinophilia are characteristic of the mammalian host response to nematode infections (42). The cytokine interleukin (IL)-5 is thought to mediate the proliferation of eosinophils during infection (51). While there is a clear relationship between nematode infections and eosinophilia, it is uncertain whether eosinophils are actually responsible for the expulsion of nematodes from the intestine of infected hosts. Eosinophilic inflammation in general, and nematode infections in particular, are also associated with local tissue dysfunction (32,34). We, and others, have previously described the development of increased intestinal smooth muscle contractility during the course of enteric nematode infections (6,15,44,46). We have also recently shown that the changes in the intestinal neuromuscular apparatus can persist long after a *T. spiralis* infection has been cleared (3).

The changes in muscle function have been shown to be, at least in part, immune mediated and involve T lymphocytes (9). Since worm expulsion is also T cell dependent, we have hypothesized that the increases in intestinal muscle contractility and the expulsion of the parasites may be linked. In this regard, attention has focused on the role of Th2 cytokines (9), including both IL-4 and IL-5. We recently demonstrated that local over-expression of IL-4 by infecting the serosal surface of mouse small intestine with an adenoviral vector encoding the IL-4 gene, led not only to a localized eosinophilic inflammation, but to an increase in the muscle contraction generated by jejunal longitudinal muscle (45). The prominence of eosinophils in this infection, as well as during nematode infections led us to consider a causal role for eosinophils in the altered enteric muscle function and in worm expulsion. Supporting this hypothesis are experiments showing that eosinophil derived mediators can cause smooth muscle contraction in tissue baths (1,18) as well as kill *T. spiralis* larvae *in vitro* (30). Therefore worm expulsion and increased muscle function could have a common basis in the eosinophil, with eosinophil derived products maintaining host defense not only by damaging the parasite but also by causing increased gut propulsion through their effects on muscle.

Unfortunately, little direct experimental evidence exists identifying a causal role for eosinophils in nematode expulsion or in the pathophysiology of enteric smooth muscle, however IL-5 and eosinophils have been implicated in bronchial muscle hyper-reactivity, although results appear to depend on the system used (10,16,23). Several studies using neutralizing monoclonal antibodies to IL-5 to block the eosinophilia during nematode and helminth infections found little role for eosinophils in controlling these infections at either the adult or larval stage (8,22,35). However, these results conflict with *in vitro* data and may reflect an inability to completely neutralize the target IL-5 protein and its function. Added to the inherent limits of neutralizing antibodies, are the often prohibitive costs associated with their chronic administration.

To overcome these difficulties, recent advances in recombinant DNA technology have permitted the development of mice that specifically lack a functional IL-5 gene (28). Using these mice, as well as their IL-5 expressing controls, we examined the role of IL-5 in intestinal muscle pathophysiology as well as host defense during *T. spiralis* infection. Our results demonstrate a critical role for IL-5 in the recruitment of eosinophils to the intestine throughout a primary *T. spiralis* infection. IL-5 deficient mice were also significantly impaired in

expelling worms from the intestine at the late stages of the infection. However IL-5 expression had no effect upon the number of muscle stage larvae recovered. Finally, although IL-5 was not critical for the initiation of intestinal smooth muscle hyper-contractility, it appeared necessary to sustain increased tension development after the infection was resolved. These studies therefore indicate that IL-5 and eosinophils play a dual role, both in controlling the intestinal phase of a *T. spiralis* infection and in maintaining the chronicity of enteric smooth muscle hyper-contractility.

6.2 MATERIALS AND METHODS

6.2.1 Mice

C57BL/6 mice lacking interleukin 5 (IL-5 -/-) were originally produced by targeted gene mutation as described by Kopf *et al* (28). Breeding pairs of IL-5 -/- and +/+ mice were obtained from the John Curtin School of Medical Research, ANU, Canberra, Australia and were kept and bred under specific pathogen free (SPF) conditions at the animal facilities of McMaster University, Hamilton, Ontario, Canada. Animals were kept in sterilized, filter topped cages, handled in tissue culture hoods and fed autoclaved food. As well, sentinel animals were routinely tested for common pathogens. The protocols employed were in direct accordance with guidelines drafted by the McMaster University Animal Care Committee and the Canadian Council on the Use of Laboratory Animals.

6.2.2 *Trichinella* Infection

The *T. spiralis* parasites used in this study originated in the Department of Zoology at the University of Toronto and the colony was maintained through serial infections alternating between male Sprague-Dawley rats and male CD1 mice. The larvae were obtained from infected rodents 60-90 days post-infection, using a modification of the technique described by Castro and Fairbairn (7). Mice were infected by administration of 0.1 ml of phosphate-buffered saline (PBS) containing 375 *T. spiralis* by gavage. To minimize differences between infections, both IL-5 -/- and +/+ mice were infected with the same *T. spiralis* preparations.

6.2.3 Detection of Interleukin-5 by RT-PCR

For analysis of IL-5 mRNA expression within the external muscle layers, control uninfected IL-5 +/+ C57BL/6 mice and mice infected 6 days previously with *T. spiralis* were euthanized. Following removal of the small bowel, the longitudinal muscle-myenteric plexus, including serosa was stripped from the jejunum, beginning at the ligament of Trietz and proceeding 4 cm distally. Total cellular RNA was isolated using a previously described guanidium isothiocyanate method (26). The concentration of RNA was determined by measuring absorbance at 260 nm and its purity was confirmed using the ratio of absorbency at 260 nm to that at 280 nm. RNA was stored at –70°C until used for reverse transcription-polymerase chain reacton (RT-PCR). mRNA was then reverse transcribed as previously described to yield cDNA. Two μl aliquots of cDNA (0.1 μg) were then mixed with 20 pmol of sense (5'-ATG AGA AGG ATG CTT CTG CAC TTG-3') and anti-sense (5'-GGA ACT CTT GCA GGT AAT CCA GGA-3') primers for IL-5 (5). The positive control used in the PCR reaction was the housekeeping gene, hypoxanthine phosphoribosyl transferase (HPRT), and 20 pmol of each sense primer (5'-GTT GGA TAC AGG CCA GAC TTT GTT G-3') and anti-sense primer (5'-GAT TCA ACT TGC GCT CAT CTT AGG C-3') were used to detect it (38). PCR was performed in 50 μl volumes containing dNTP (200 μM), Mg^{2+} (1.5 mM), 2.5 U Taq polymerase (Gibco BRL, Burlington, ON) with corresponding buffer and distilled water. IL-5 and HPRT were co-amplified for 45 cycles using the following cycle parameters: denaturation 94°C for 30 seconds, annealing 55°C for 30 seconds, and extension at 72°C for 60 seconds. PCR products were loaded onto a 2.5% agarose gel and then visualized under ultraviolet light after ethidium bromide staining. The 366 bp product corresponds to IL-5 and the 164 bp product corresponds to HPRT. The IL-5 and HPRT band densities were determined for each sample (each lane represents a different mouse) and the ratios of IL-5 : HPRT were calculated. The mean of the ratios was then calculated for uninfected and infected conditions.

6.2.4 Histology and Eosinophil Counts

Mice were sacrificed and jejunal tissue, skeletal muscle from the hind legs and tissue from the diaphragm were fixed in 10% neutral buffered formalin (NBF). 3 μm sections were cut and sections were stained with haematoxylin-eosin, or stained with congo red and lightly counterstained with haematoxylin. Intestinal tissue eosinophil counts were performed by counting the number of polymorphonuclear leukocytes that stained intensely with congo red, found within 20 villus crypt units (vcu), including the underlying muscle, per mouse (53). The number of eosinophils was then referred to as eosinophils per vcu. Photomicrographs were taken using a Zeiss camera.

6.2.5 Adult Worm Counts

The entire length of the small intestine was removed, opened longitudinally, and all adult worms within the small intestine were then counted using a modification of the method described by Castro and Fairbairn (7). Briefly, the mucosa was separated from the underlying muscularis by scraping with a glass microscope slide and mixed with 1 ml of PBS. The worms were then counted using a scored petri dish and an inverted microscope. In accordance with established practices, worm rejection was considered complete when at least 98% of the infective dose had been expelled from the gut (47).

6.2.6 Skeletal Muscle Larvae Counts

Skeletal muscle larvae numbers were determined by homogenizing skinned, eviscerated mice, using a mixture of phosphate buffered saline (PBS), hydrochloric acid and pepsin in a kitchen blender, again using a modification of the method described by Castro and Fairbairn (7). The homogenate was transferred to 1000-ml flasks and bubbled with 95%O_2 - 5%CO_2 for 2 hours. The digestate was then strained through several layers of gauze and the worms allowed to settle at unit gravity. Following tissue digestion, the supernatant was removed and the worms resuspended in PBS, pH 7.4. The larvae were then washed, collected and counted under an inverted dissecting microscope.

6.2.7 Muscle Function

The preparation of the sections of jejunal longitudinal muscle for muscle contractility analysis and the analysis of the length-tension relationships have been described previously (3,44). In brief, the jejunum was removed and placed in oxygenated (95% O_2 - 5% CO_2) Kreb's solution and 1 cm sections of whole gut were cut from the jejunum, beginning at the ligament of Treitz and proceeding distally. The lumen of each segment was flushed with Kreb's buffer, prior to the insertion of short (2-3 mm) lengths of Silastic tubing (0.065 in. OD, 0.030 in. ID) (Dow Corning, Midland, MI) into the open ends of the gut segments. Tubing was then tied in place with surgical silk. The insertion of the tubing was found to maintain patency of the gut segments, over the course of the experiments. Segments were then hung in the longitudinal axis and attached at one end to a grass FT03C force transducer (Grass, Quincy, MA) and responses were recorded on a Grass 7D polygraph. tissues were equilibrated for 30 minutes at 37^{0}C in Kreb's solution, oxygenated with 95%O_2 - 5%CO_2 before starting the experiment. Experiments were then conducted to examine the length-tension characteristics of the muscle before and after infection. Segments were stretched by applying tension equivalent to 0-1250 mg of weight, and contraction was assessed following stimulation with 1 μM carbachol (Sigma Chemical, St. Louis, MO). Initial experiments indicated that this tension range was sufficient to determine the maximal responsiveness of both control and inflamed tissues. After each application of tension, the length of the tissue, and the contractile response were recorded. At the end of the experiment, the tissue was removed, blotted and weighed, and the optimal tension (T_O), and the tissue length which gave the maximum contractile response was used to calculate the cross sectional area of the tissue.

6.2.8 Carbachol dose response curve

The previously identified T_O for a tissue was applied in carbachol dose-response experiments before the addition of the first dose of carbachol. Gut segments were then exposed to noncumulative final

bath concentrations of 1nM - 1mM carbachol by addition of microliter aliquots to 20 ml tissue baths. After the maximal response to each dose was obtained, tissues were rinsed twice and equilibrated in fresh Krebs solution for 15 min before addition of the next agonist dose.

6.2.9 Data Presentation and Statistical Analysis

Responses to carbachol were recorded from tracings, followed by the calculation of contractile activity which were expressed as milligrams of tension per cross sectional area as described previously (44). For each mouse, the mean tension was calculated from at least three segments. All the results are expressed as the means ± 1 SEM, n refers to the number of mice tested. Statistical significance was calculated using the Student's t test for comparison of two means or a one way of analysis (ANOVA) for the comparison of three or more means. Multiple comparisons were performed using the Neuman-Keuls multiple comparison test. $P < 0.05$ was considered significant.

6.3 RESULTS

6.3.1 *T. spiralis* Infection - Macroscopic

Both strains behaved similarly following infection, during the course of which they developed the normal changes in behaviour associated with *T. spiralis* infection, including the adoption of a hunched appearance, reduced movement and piloerection by day 4-6 of infection. Both strains lost similar amounts of body weight (15-20%) by day 12 PI, recovering the weight over the following days (not shown). Upon sacrifice however, it was consistently noted that tissue edema, as well as the redness of the intestinal tissue, appeared to be reduced in the IL-5 -/- mice, compared to the IL-5 +/+ mice.

6.3.2 IL-5 Gene Expression

Since IL-5 can act as an eosinophil chemoattractant, and eosinophils are seen infiltrating the muscle layers, as well as the mucosa during *T. spiralis* infection, we wished to determine whether IL-5 was expressed within the neuromuscular layers, and whether it increased during infection. To examine IL-5 expression within

the enteric longitudinal muscle layer, we semi-quantitatively measured IL-5 mRNA expression, using RT-PCR on total RNA extracted from the longitudinal muscle - myenteric plexus (LMMP) of the jejunum from uninfected mice and IL-5 +/+ mice infected 6 days previous with *T. spiralis*. As can be seen in Figure 1A, the predicted 366 bp sized PCR product for IL-5 was detectable in all 3 uninfected mice, but was significantly increased in tissues from the three infected mice. Note that the housekeeping gene HPRT did not significantly change expression during infection. Figure 1B quantifies the bands seen in Figure 1A, and shows that the ratio of IL-5 gene expression compared to HPRT expression, significantly tripled at day 6 PI. As expected IL-5 mRNA was not detected in IL-5 deficient mice in the absence or presence of infection (data not shown).

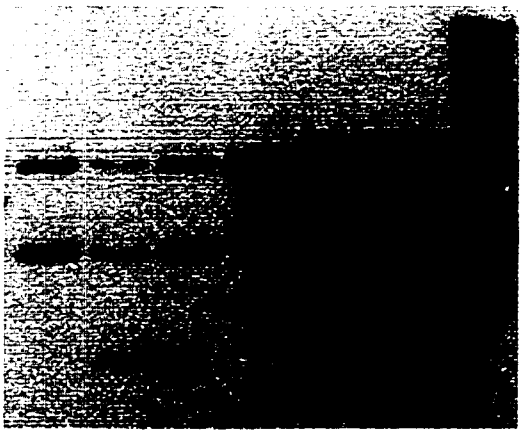
6.3.3 Worm Counts

Evaluation of the parasite load in the small intestine of *T. spiralis* infected IL-5 deficient and IL-5 sufficient mice, revealed a time dependent decrease in worm numbers from the small bowel of both mouse genotypes over the course of a primary infection (Figure 2). While a larger number of worms were recovered from the small bowel of IL-5 -/- mice at both day 12 and 16 PI, the difference between IL-5 +/+ and IL-5 -/- mice was only significant on day 16 PI. By day 21 PI, both IL-5 -/- and +/+ mice had expelled their parasite load from the small bowel. However a few (5-10) adult *T. spiralis* worms were also found in the caecum and colon of IL-5 -/- mice, but not in IL-5 +/+ mice at day 21 PI. During the infection, eosinophils identified in histological sections from IL-5 +/+ mice were often seen in close proximity to *T. spiralis* parasites (Figure 3A). However parasites found in IL-5 -/- mice had few inflammatory cells in their vicinity (Figure 3B), with the eosinophils replaced by neutrophils, macrophages and lymphocytes.

6.3.4 Eosinophil Counts

The number of eosinophils identified microscopically, in the jejunum of IL-5 -/- and +/+ mice, were evaluated per villous crypt unit both prior to and during a primary *T. spiralis* infection (see Figure 4). Prior to infection, the number of intestinal eosinophils was similar in IL-5 +/+ (0.8 eosinophils/vcu) and IL-5 -/- (0.5 eosinophils/vcu) mice. Following infection, eosinophil numbers rose significantly by day 8 PI (14.0

Figure 6.1. Panel A. IL-5 gene expression within the jejunal LMMP of uninfected IL-5 +/+ C57BL/6 mice (lanes 1-3) and IL-5 +/+ mice 6 days after *T. spiralis* infection (lanes 4-6). Size markers are in lane 7. Note that the IL-5 bands are increased in the infected mice, while the housekeeping gene HPRT is unchanged. Panel B. Demonstrates the mean ratio ± 1 SEM of the IL-5 band densities compared to the HPRT band densities, in uninfected (open bar) and infected (shaded bar) mice. Note that *T. spiralis* infection results in a significant tripling of the IL-5 : HPRT ratio ($P < 0.01$).



IL-5

HPRT

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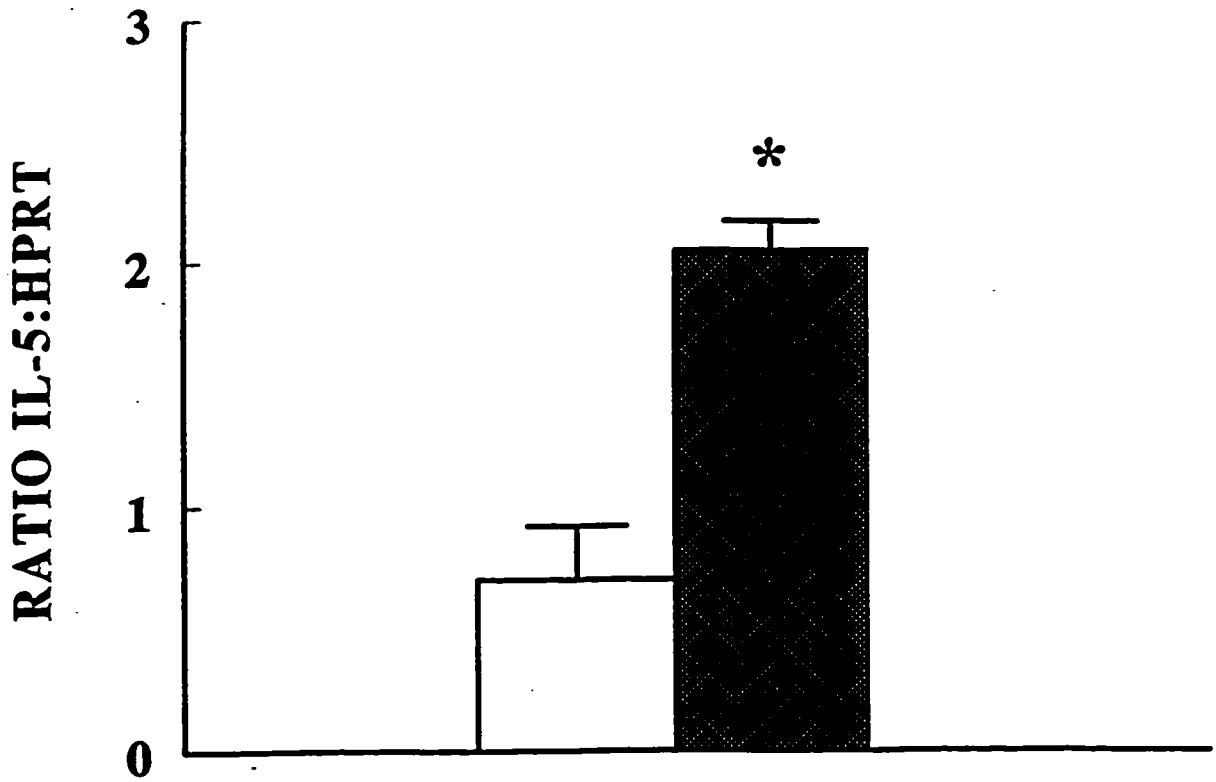
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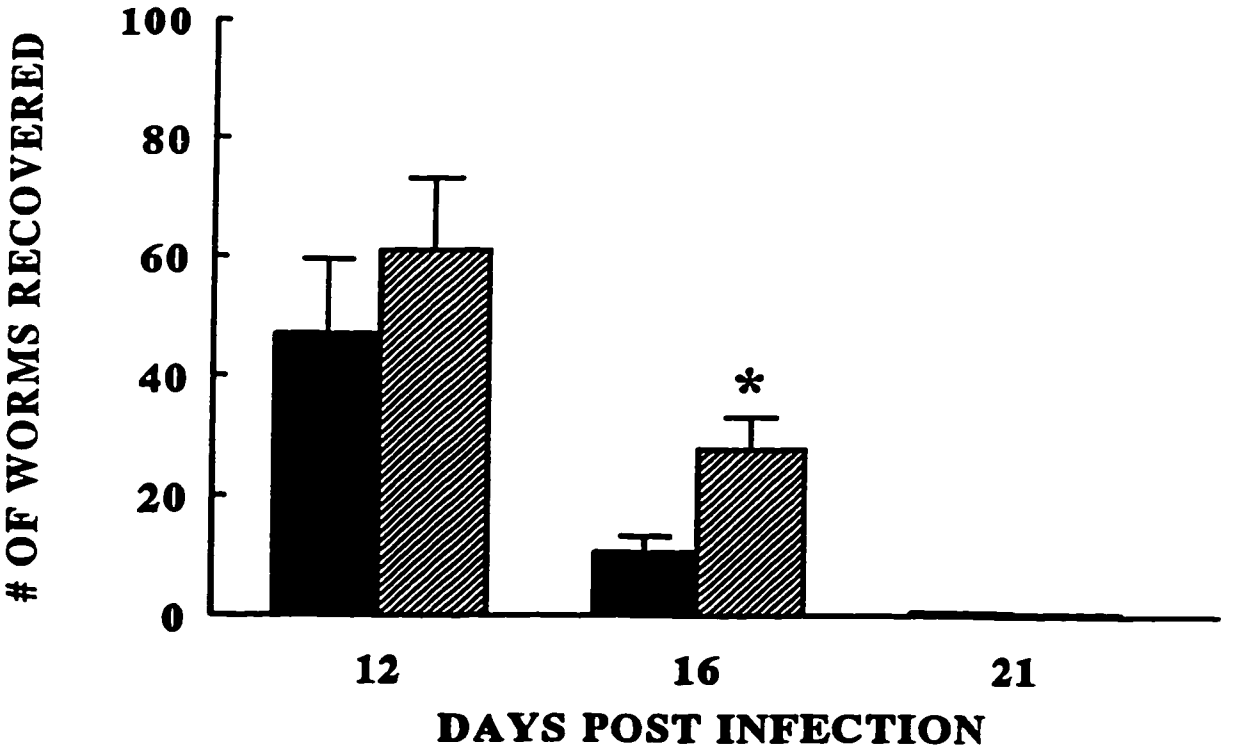
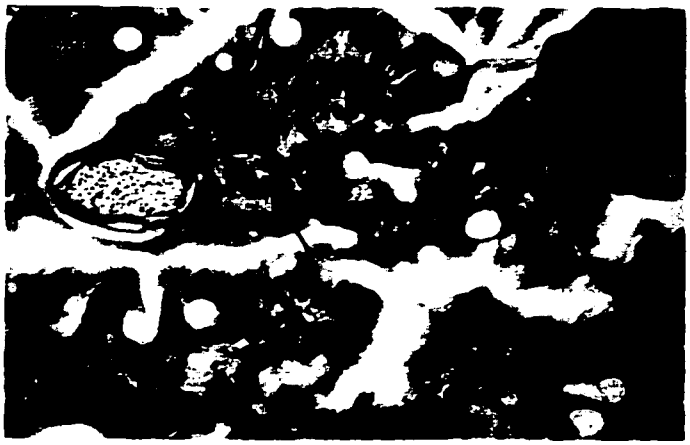


Figure 6.2. The number of adult *T. spiralis* worms recovered from the small bowel of infected IL-5 +/+ (solid bar) and -/- mice (hatched bar) on days 12, 16 and 21, following a primary infection with 375 *T. spiralis* larvae by gavage. Results shown represent the mean ± 1 SEM of groups of 5 mice. The asterisk denotes a significant difference compared to IL-5 +/+ mice at the same time point. Note that the *T. spiralis* worms had been completely expelled from the small bowel of both mouse strains by day 21 PI.

Figure 6.3. Panel A. Histologic appearance of an adult *T. spiralis* worm and the nearby inflammatory cells in the mucosa of an infected IL-5 +/+ mouse, eight days following primary infection. Note the numerous eosinophils (arrows) in close proximity to the parasite. Panel B. *T. spiralis* parasite in the mucosa of an IL-5 -/- mouse, eight days following infection. Note the absence of eosinophils and the relative paucity of inflammatory cells in the vicinity. Panel C. Histology showing inflammatory cell infiltration into the submucosa and muscle layers on day 8 PI in a IL-5 +/+ mouse and (Panel D), in a IL-5 -/- mouse at the same time point. Tissue sections were stained with a combination of congo red and haematoxylin. Note the numerous eosinophils in the IL-5 +/+ mouse, and their relative paucity in the IL-5 -/- mouse. (Original magnification for all was 400x).

A



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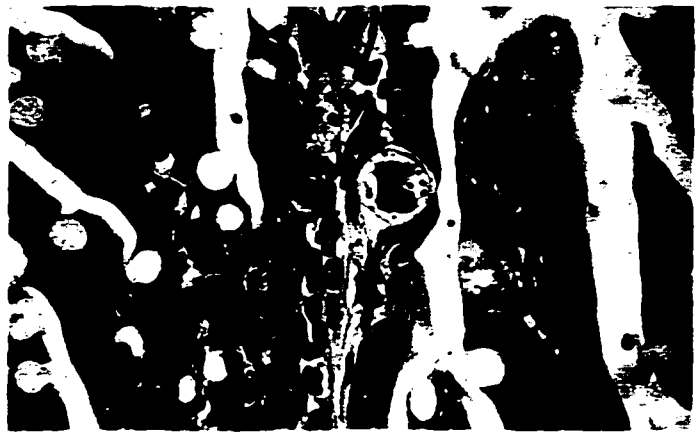

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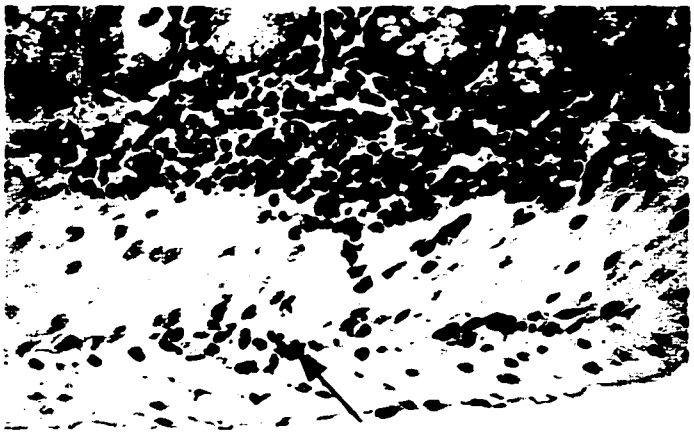

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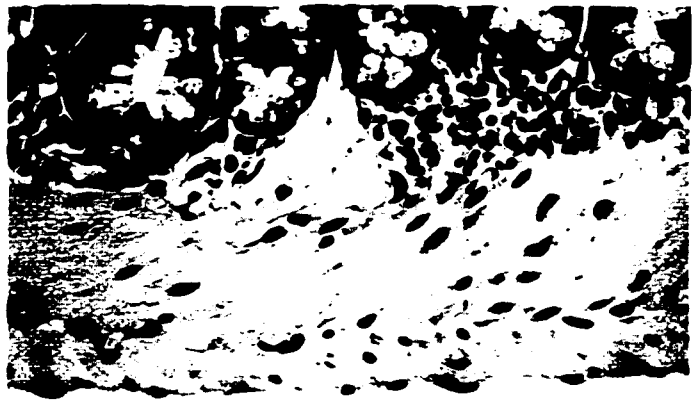

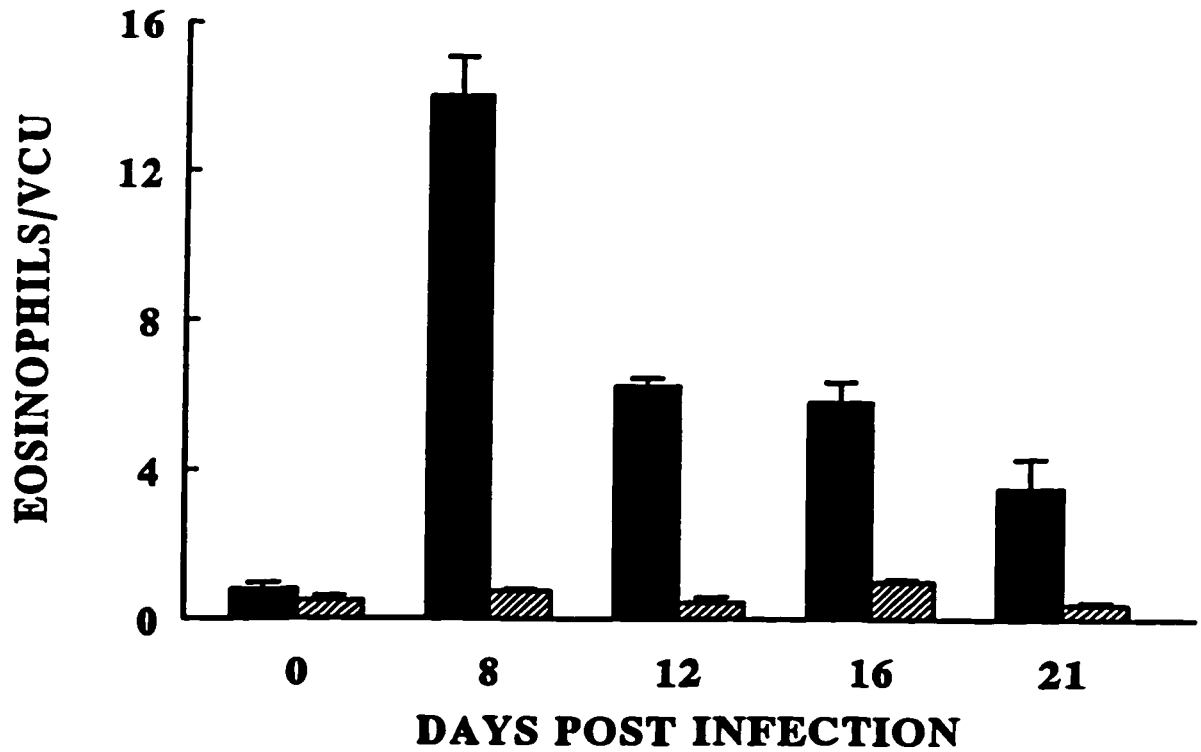

Figure 6.4. The number of eosinophils/villus crypt unit identified in jejunal cross sections from uninfected IL-5 +/+ (solid bar) and -/- mice (hatched bar) as well as on days 8, 12, 16 and 21 following a primary infection with 375 *T. spiralis* larvae by gavage. Results shown represent the mean ± 1 SEM of groups of 4 mice. The asterisk denotes a significant difference compared to IL-5 -/- mice at the same time point.

eosinophils/vcu) in the IL-5 +/+ mice. After this peak, eosinophil numbers dropped as of day 12 (6.3.eosinophils/vcu) and day 16 PI (5.8 eosinophils/vcu), and continued to decrease until reaching 3.5 eosinophils/vcu at day 21 PI. In contrast, no significant increase in the number of intestinal eosinophils was seen in the IL-5 -/- mice over the course of the infection, with numbers ranging from 0.4 to 1.0 eosinophils/vcu.

6.3.5 Eosinophil Distribution

During infection of IL-5 +/+ mice, eosinophils made up a significant fraction of the inflammatory cells infiltrating the intestine, with eosinophils seen both in the tips of the jejunal villi as well as at the base of the villi and in the crypt regions. Eosinophils were also seen infiltrating the external muscle layers and myenteric plexus (see Figure 3C). In contrast, the cellular infiltrate was reduced in the IL-5 deficient mice, and was composed predominantly of mononuclear cells, rather than granulocytes. Again, this was found both in the mucosa and in the external jejunal muscle layers (see Figure 3D). While an occasional eosinophil was seen infiltrating the intestine of infected IL-5 -/- mice, they were extremely uncommon.

6.3.6 Skeletal Muscle Larvae Counts

During infection, *T. spiralis* larvae migrated from the intestine to the skeletal muscle and 40-60 days following infection, were completely encysted within their nurse cells in the skeletal muscle. Digestion of the bodies of previously infected mice recovered approximately 25,000 muscle larvae per body with no significant difference found between IL-5 +/+ and IL-5 -/- mice (Figure 5). A mixed inflammatory cell infiltrate was seen surrounding the encysted muscle larvae, containing numerous eosinophils (Fig. 6A) in IL-5 +/+ mice. The inflammatory response against the encysted larvae was smaller in IL-5 deficient mice, with eosinophils again replaced by mononuclear cells (Figure 6B).

6.3.7 Muscle Response to Carbachol

The contractile response by jejunal longitudinal muscle to 10^{-6} M carbachol was similar between IL-5 deficient and wild-type mice prior to infection. As can be seen in Figure 7, eight days following *T. spiralis*

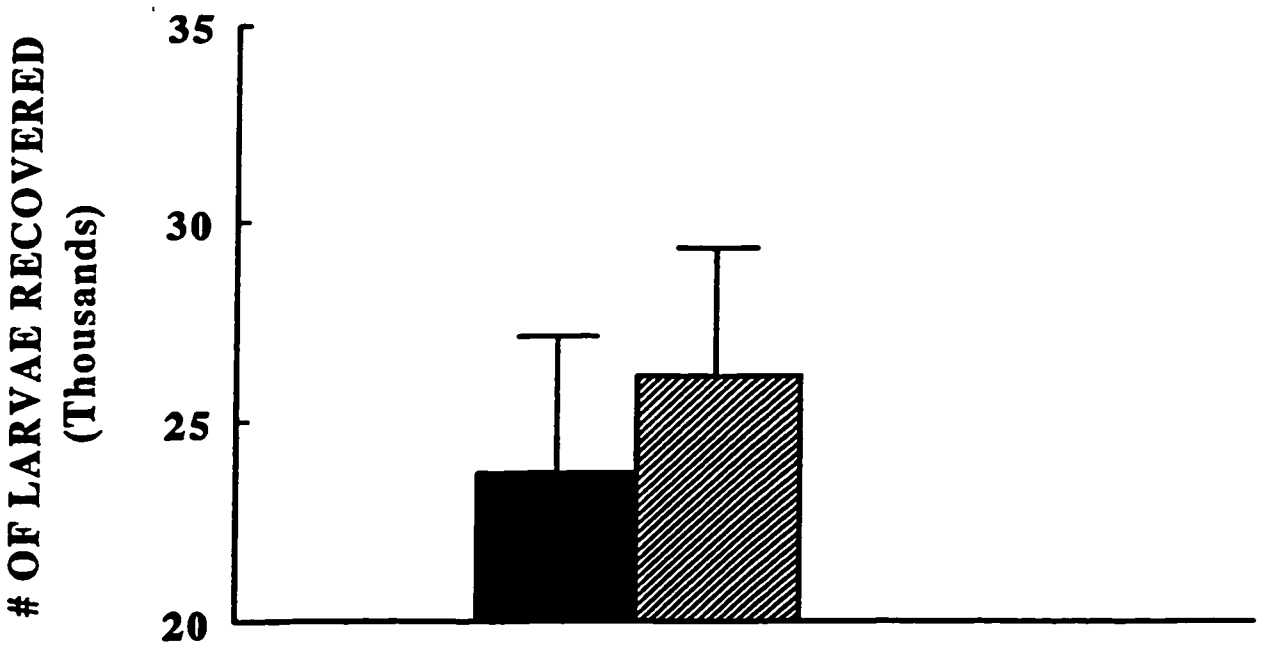

Figure 6.5. The number of *T. spiralis* skeletal muscle larvae recovered following digestion of the body of IL-5 +/+ (solid bar) and IL-5 -/- mice (hatched bar) infected 40-80 days previously with 375 *T. spiralis* worms. Results shown are the mean number of larvae recovered per body ± 1 SEM for groups of 5 animals.

Figure 6.6. Histologic appearance of the cysts of *T. spiralis* larvae located in the hind leg skeletal muscle of mice infected 40 days previously with 375 *T. spiralis* parasites. Tissue sections were stained with a combination of congo red and haematoxylin. Panal A: Cyst located in the skeletal muscle of an IL-5 +/+ mouse. Note the numerous eosinophils surrounding the cyst. Panel B: Cysts located in the skeletal muscle of an IL-5 -/- mouse. Note the presence of mononuclear cells rather than eosinophils surrounding the encysted larvae. (Original magnification for all was 640x).

A

B

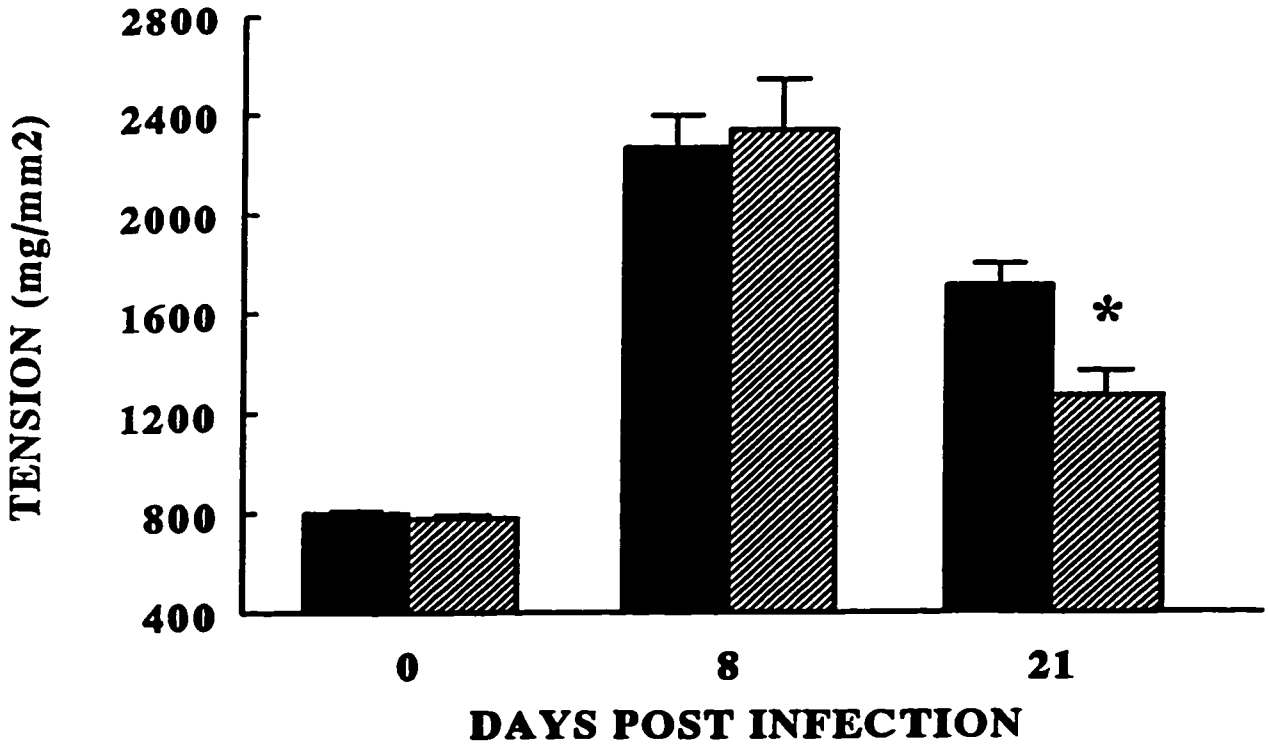

Figure 6.7. The tension generated in response to 10^{-5} M carbachol, by jejunal longitudinal muscle from uninfected IL-5 +/+ (solid bar) and IL-5 -/- mice (hatched bar) as well as mice infected 8 and 21 days previously. Results shown represent the mean tension ± 1 SEM of groups of 4 mice. The asterisk denotes a significant difference compared to IL-5 +/+ mice at the same time point.

infection, there was a substantial and significant ($p < 0.01$) increase in the tension generated by longitudinal muscle from both IL-5 competent and deficient mice. Generated tension increased approximately 3 fold over that of controls, with no significant difference between the 2 mouse strains. The contractile response generated by longitudinal muscle from IL-5 +/+ mice was sustained over control data by day 21 PI, but the magnitude had decreased from 2300 to 1700 mg/mm^2. In contrast, tension had dropped from 2350 mg/mm^2 on day 8 PI to 1200 mg/mm^2 on day 21 PI in the IL-5 deficient mice. This was significantly less tension than that generated by muscle from IL-5 +/+ mice at the same time point. However, muscle contraction had still not returned completely to normal in the IL-5 deficient mice on day 21 PI, remaining significantly elevated over that of uninfected IL-5 -/- mice.

The dose-response relationships for carbachol induced contraction in IL-5 +/+ and -/- mice are shown in Figure 8. In non-infected mice, the response to carbachol was maximal between 10^{-5} and 10^{-4} M and there was no significant difference between control IL-5 +/+ and IL-5 -/- mice in their dose response to carbachol. However by day 21 PI, the contraction generated by muscle from IL-5 +/+ mice was significantly greater than that generated by IL-5 deficient mice, over a dose range of 10^{-6} M to 10^{-3} M carbachol. The maximum response to carbachol remained at a dose range of 10^{-5} and 10^{-4} M for both mouse strains on day 21 PI.

6.4 DISCUSSION

Gastrointestinal roundworm infections result in both tissue and blood eosinophilia, which develop as one arm of a tripartite host response to infection, in concert with elevated serum levels of IgE (47,48,50) and mucosal mast cell hyperplasia (31,48). IL-5 is thought to be particularly important for the proliferation of eosinophils *in vivo* (49), as well as their growth (33), activation (33) and survival (36,37). IL-5 is one of the first cytokines expressed in the intestine during nematode or helminth infections. Svetic *et. al.* described elevated IL-5 mRNA expression in the Peyer's patches of mice during *H. polygyrus* infection (39) and high levels of IL-5 protein are produced by cells isolated from the mesenteric lymph nodes within a few days of a *T. spiralis* infection (20). The presence of infiltrating eosinophils within the jejunal neuromuscular levels raised the question of whether IL-5 expression also increased in these layers.

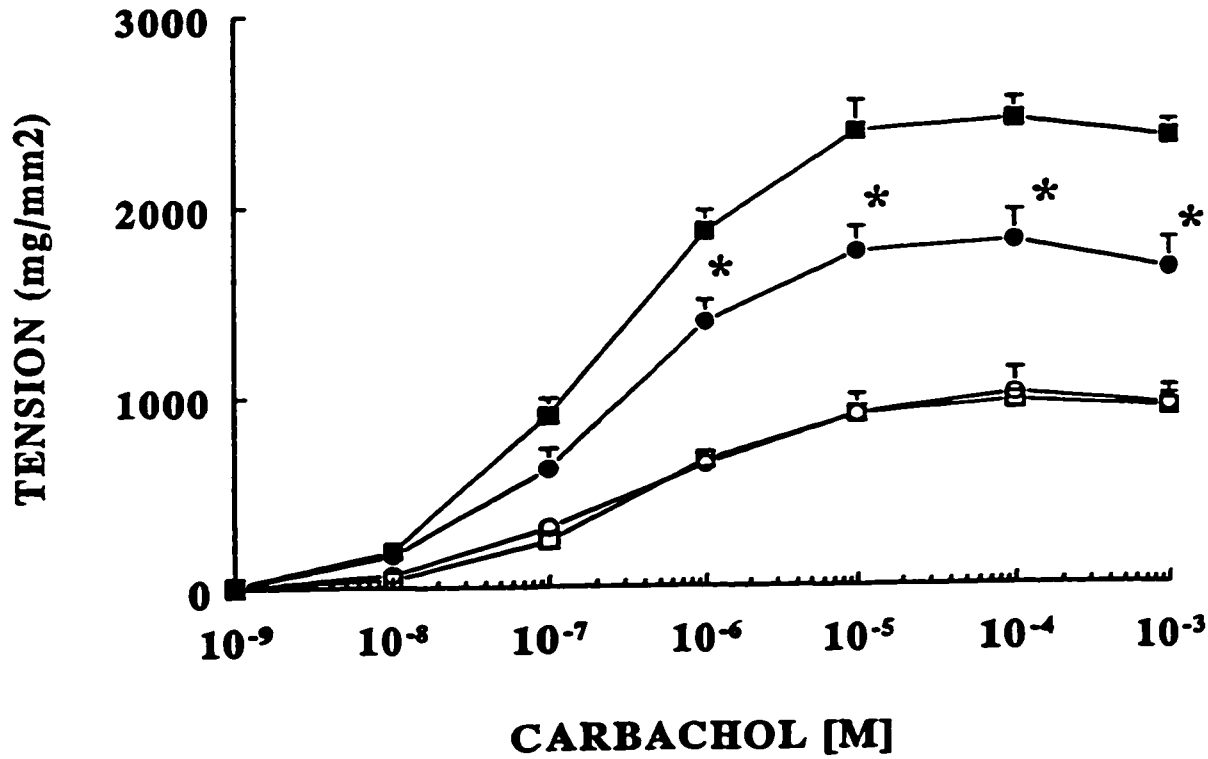

Figure 6.8. Dose-response relationships for carbachol-induced contraction of muscle from IL-5 +/+ control (open circles) and IL-5 -/- (open squares) mice and mice of the same strains (closed circle and square respectively) infected 21 days previously with *T. spiralis*. Values are means ± 1 SEM of groups of 6 animals. The asterisk denotes a significantly decreased tension compared to day 21 PI IL-5 +/+ mice at the same concentration of carbachol.

When compared to uninfected mice, we found a significant tripling of IL-5 expression within the LMMP of wild-type C57BL/6 mice on day 6 PI. This was accompanied by a strong intestinal eosinophilia affecting both the muscle layers and mucosa. The chemotactic actions of IL-5 may thus be responsible, in part for the accumulation of eosinophils within the muscle layers. While the cellular source of the IL-5 within the muscle layers of infected mice is unclear, IL-5 can be expressed by several cell types including eosinophils, and T lymphocytes which have been previously shown to quickly infiltrate the jejunal muscle layers during a nematode infection (14).

We also discovered that the intestinal eosinophilia that developed in IL-5 +/+ mice during *T. spiralis* infection, was completely abolished in the absence of IL-5. Only a small population of eosinophils was found within the jejunum of IL-5 -/- mice throughout the infection, similar in number to that seen in uninfected mice. Thus, although IL-5 production is not a requirement for the recruitment of eosinophils to the small bowel under physiological conditions, IL-5 does appears essential for the increased production and recruitment of eosinophils to the gut during infection. The other eosinophil growth and chemotactic factors that are presumably active during the infection are thus unable to compensate in the absence of IL-5. We did not characterize the numbers of circulating eosinophils in this study since the presence of a peripheral blood eosinophilia during nematode infections has been well documented, as has its abrogation in the face of IL-5 neutralization (8,22) or deficiency (28).

In addition, we found that worm burdens were greater in the small bowel of IL-5 deficient mice than in wild-type mice, but the difference was only significant at the late stages of infection (day 16 PI). These results suggest that IL-5 and eosinophils do play a protective role in the infected host. However there was a significant delay between the time that eosinophils infiltrated the infected bowel and the expulsion of the parasite. A significant intestinal eosinophilia was seen within the infected gut by day 8 PI, while the absence of IL-5 and eosinophils had no effect upon worm expulsion until eight days later. Since adult Trichinella possess a thick tegument, the delay may indicate the difficulty that even eosinophils have in damaging the parasites enough to induce their expulsion. Alternatively, it may indicate a delay between the arrival of eosinophils to the infected intestine and their activation to an anti-

parasitic role. Eosinophils have previously been shown to require additional activation signals such as IgE, to effectively damage parasites *in vitro* (30), and significant elevations in IgE levels during *T. spiralis* infection take approximately 2 weeks to develop (29). Curiously, no difference was found in the number of skeletal muscle larvae recovered from IL-5 deficient and expressing mice. This was surprising considering the number of eosinophils found in the inflammatory infiltrate surrounding the encysted *T. spiralis* larvae in the IL-5 +/+ mice. However, unlike the adult worms that are continually exposed to the host's immune response, the larvae are only exposed for a few hours during their migration from the intestine, after which the larvae are protected by the nurse cells they invade and eventually by the cysts that form around them.

Intestinal neuromuscular dysfunction has already been described in rodents following infection by several gastrointestinal parasites including *N. brasiliensis* (15), *H. polygyrus* (19,43) and *T. spiralis* (44,46), and studies from our laboratory have characterized longitudinal smooth muscle hyper-contractility during *T. spiralis* infection in both rats (46) and mice (3,44). Just as IL-5 and eosinophils appeared to have no significant effect upon adult worm survival in the early stages of infection, their absence also had no impact on the induction of intestinal smooth muscle hyper-contractility. Both mouse strains developed a similar 3 fold increase in muscle contraction on day 8 PI, similar in magnitude to the levels seen in other immunocompetent mouse strains during infection (44).

However, we also recently described that *T. spiralis* infection can lead to sustained changes in intestinal muscle function, that may persist at least one month following the resolution of the infection, in certain strains of mice (3). The persistence of these changes appears to be genetically regulated and corresponds to the strength of the immune response against the parasite. C57BL/6 mice have already been well defined in terms of their immune responsiveness to *T. spiralis* infection, being intermediate responders (47). Thus we suspected that the increased muscle function seen in the IL-5 +/+ mice would not be as sustained as in other, stronger responding mouse strains (3,44). We therefore elected to study the persistence of the muscle dysfunction following 21 days of infection, rather than as previously done, at day 28 or 42 PI (3). Interestingly, although the early phase of muscle contraction was similar in IL-5 -/- and

+/+ mice, we found that the changes in muscle function that persist in the wild-type mice, even after the infection has been resolved, were significantly reduced in the IL-5 deficient mice. Recent studies examining the mechanisms of the persistent muscle dysfunction in the strong responding NIH Swiss mouse strain found that the increased muscle function could be partially attenuated by post-infectious treatment with corticosteroids (2). This suggests that the state of muscle hyper-contractility is being actively maintained through a steroid sensitive and presumably inflammatory process. Our data suggests that eosinophils and eosinophil derived products could contribute to this process.

It is also interesting that significant roles for IL-5 and eosinophils in both worm expulsion and in the changes in muscle function were only seen at the late stages of infection. While this may simply indicate that both functions rely upon the same eosinophil derived mediators, it may also reflect a closer and possibly causal relationship between the two. The increased longitudinal muscle contractility is thought to contribute to increased intestinal propulsive activity during infection. Although immunologically mediated damage to the parasite may be an initial requirement, the increased propulsion in concert with water and mucus secretion into the lumen should act to dislodge the damaged parasite and flush it from the bowel. This theory is supported by the observation that nematode parasites are often damaged but alive (48) when they are expelled from their hosts. An eviction rather than a neutralization of the parasite is also supported by the finding that by day 21 PI, a small population of *T. spiralis* worms were recoverable from the caecum and colon of some infected IL-5 deficient mice.

In this study we have exclusively attributed the differences in worm expulsion and muscle function to the effects of IL-5 on eosinophils. IL-5 does have other functions including acting as a B cell growth (27) and IgA enhancing factor (52). However IL-5 deficient mice have already been shown to have normal antibody responses as well as cytotoxic T cell development during helminthic infections (28). Thus the primary activity of IL-5 during *T. spiralis* infections is most likely on eosinophils. Not only can it act as an eosinophil growth and differentiation factor (33,34,49), but it can also attract them to a site of inflammation or infection and once there, delay their apoptosis and induce them into a more active, hypodense state (34,36,37,49). However, while the data in this study implicates eosinophils in both worm

expulsion and the maintenance of muscle hyper-contractility, the mechanisms by which eosinophils act are unclear. Mediators such as major basic protein (MBP) and eosinophil cationic protein (ECP), when released by eosinophils, have been shown to cause significant tissue damage (49). These plus other mediators, including leukotrienes (34,49) and other products of the arachidonic pathway, are released by activated eosinophils and individually, or in combination, have been shown to contract muscle strips (11,24) and kill *T. spiralis* larvae *in vitro* (1,21,30). Determining the specific role of these mediators in both host defense and tissue dysfunction *in vivo* remains a considerable challenge for the future.

Thus IL-5 appears not only to be necessary for the development of the intestinal eosinophil response to *T. spiralis* infection, but mice deficient in IL-5 production and the resulting eosinophil response were less protected against *T. spiralis* infection than their wild type controls. As well, although IL-5 and eosinophils appear to not play any crucial role in the initiation of intestinal smooth muscle hyper-contractility, they do appear to be involved in sustaining the changes in this model. These findings thus have bearing on the frequent clinical finding that functional motility disorders often follow a bout of enteric infection, persisting long after the infection has been resolved (9). This is termed post-infectious irritable bowel syndrome (9). As well, the demonstration of the crucial role IL-5 plays in the recruitment of eosinophils to the gut as well as the role of eosinophils in gastrointestinal damage and dysfunction also has implications for non-infective inflammatory diseases of the GI tract. Celiac disease (12,41), eosinophilic gastroenteritis (41) and certain types of food allergies (4) are all characterized by the presence of increased numbers of activated eosinophils, in association with evident tissue damage and gut dysfunction. Moreover ulcerative colitis (17) and the early lesions of Crohn's disease (13) are associated with increased IL-5 expression, suggesting that eosinophils may be involved in the pathogenesis of IBD. Taken together, these results suggest that although the eosinophil is an important component of host defense within the GI tract, attention should also be focused on the potential role of IL-5 and eosinophils in the pathophysiology of several GI diseases.

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Chapter 7

DISCUSSION

DISCUSSION

7.1 SUMMARY

While alterations in organ and tissue function commonly accompany inflammatory diseases, understanding the immunological basis for these changes is still at its earliest stages. The purpose of this study was first to determine if *T. spiralis* infection in the mouse resulted in increased jejunal muscle contraction, as had been shown in the rat. The second purpose was to examine the immunological basis for this response by identifying the cell types and mechanisms involved in the enhanced intestinal muscle contraction. We did find that *T. spiralis* infection resulted in increased jejunal longitudinal muscle contraction in various strains of mice (Vallance *et al.*, 1997a), and that the increase in muscle function was typically evident by day 6 PI, and persisted until at least day 21 PI. However in some strains of mice, the increased muscle contraction was sustained until at least 42 days after infection (Vallance *et al.*, 1997a; Barbara *et al.*, 1997). We confirmed previous results in the rat, showing that the early phase of muscle contraction (day 6 PI) was absent during infection in athymic rodents. However by examining later time points in several strains of immunodeficient mice, we discovered that there was a delayed and transient T cell independent muscle response, peaking at day 12 PI, and returning to normal levels by day 21 PI (Vallance *et al.*, 1998b). Reconstitution of splenic mononuclear cells to athymic mice restored not only T cell numbers in these animals but also the early and persistent phases of increased muscle contraction seen during *T. spiralis* infection (Vallance *et al.*, 1998b).

Based on the prominent role indicated for T lymphocytes in the development of increased muscle contraction, we next examined whether it was the CD4 or CD8 T cell subsets that were involved in the changes in muscle function, by infecting gene targeted mice, deficient in these particular T cell subsets (Fung-Leung *et al.*, 1991; Rahemtulla *et al.*, 1991). We found that the loss of CD8 +ve T cells had no effect on the increased muscle function during infection, and the loss of CD4 expressing T cells only partially attenuated the response (Vallance *et al.*, 1998d). However the combined loss of CD4 T cells and MHC II expression, as found in MHC II deficient mice, significantly attenuated the response during infection. Furthermore, bone marrow grafting of MHC II expressing cells to MHC II deficient mice, along

with the injection of CD4 +ve T cells, restored the increased muscle contraction normally seen during infection (Vallance *et al.*, 1998d).

CD4 +ve T cells normally play a directoral role in immune responses, activating and interacting with other cell types through the release of cytokines (Sher & Coffman, 1992). Since nematode infections are associated with a Th2 profile of cytokine generation (Svetic *et al.*, 1993; Urban *et al.*, 1992), we examined the Th2 cytokines interleukin-4 and 5 as possible mediators of the enteric muscle hyper-responsiveness. Infecting IL-5 deficient mice, as well as their wild type controls, we found that although IL-5 deficient mice were almost completely attenuated in the development of intestinal eosinophilia, they still developed a normal early increase in muscle contraction. However, the degree of persistently increased muscle contraction seen at day 21 PI was significantly attenuated in the IL-5 deficient mice (Vallance *et al.*, 1998a). These results indicate that IL-5 and eosinophils play no crucial role in the initial change in muscle function. However they do appear to contribute in some fashion to the persistence of the altered muscle function, enabling it to maintain a high degree of hyper-responsiveness even after the infection has resolved.

We also examined the role of IL-4 in the development of muscle pathophysiology by adopting a gene transfer approach. By painting adenoviral vectors onto the serosal surface of the small bowel, following a midline laparotomy, we demonstrated that gene transfer of an IL-4 encoding virus, but not control viruses, led to longitudinal muscle hyper-contractility by day 7 PI (Vallance *et al.*, 1996). This approach also led to a localized inflammatory response, involving both the intestine and the peritoneal cavity. While the control viruses brought about an inflammatory response involving neutrophils, macrophages and lymphocytes, the IL-4 virus created a quantitatively stronger response also involving eosinophils and mast cells. While this increased response by muscle may have been mediated, indirectly, through the actions of an intermediate cell type such as eosinophils or mast cells, there is also the possibility that the actions of the IL-4 produced by the virus were directly on intestinal smooth muscle. Determining the basis for this interaction remains a challenge for the future.

7.2 Motility and Phases of Increased Muscle Function

7.2.1 Relationship Between Changes in Muscle Function and Motility

As mentioned previously, the control of intestinal motility is a complex regulatory process involving both myogenic and neurogenic components, as well as hormonal influences and pacemaking activity by the interstitial cells of Cajal (Huizinga *et al.*, 1995). The studies in this thesis examined only the changes that occur in the longitudinal muscle component of small bowel motility during inflammation. Thus, we cannot directly correlate our observations to the increase in motility described during nematode infection in several other studies. Theoretically, since motility is regulated by so many components, each factor would have to be examined in isolation and compared to the changes in motility as a whole to determine their relative contributions to the altered motility seen during inflammation. However it has recently been shown that ICC function is disrupted during nematode infections, at least in the early stages of infection (Der-Silaphet *et al.*, 1996). Circular smooth muscle function during infection has also been shown to be reduced (Crosthwaite *et al.*, 1990), and nerve function, as assessed by neurotransmitter release (Vermillion & Collins 1988; Haapala *et al.*, 1998) and by electrical field stimulation in tissue baths (Barbara *et al.*, 1997) is decreased. Therefore, with the reduced function of these other components, longitudinal muscle may be the dominant factor controlling and contributing to the increased jejunal motility that occurs during *T. spiralis* infection.

7.2.2 Phases of Increased Muscle Contraction

Unlike our earlier studies on *Trichinella spiralis* infected rats, we examined jejunal longitudinal muscle function over the full time course of infection in the mouse. This led to the discovery that the increase in jejunal longitudinal muscle contraction was not a brief response by the host, but frequently lasted until day 21 PI or beyond (Barbara *et al.*, 1997). This finding was of interest because examining the relationship between acute inflammation and the onset of muscle dysfunction was assumed to be relevant only to motility disturbances associated with overt idiopathic or infectious based intestinal inflammation

(Garrett *et al.*, 1967; Kern *et al.*, 1951). However a persistent change in muscle function following the resolution of an enteric infection and the associated inflammation might have a similar basis, or at least provide clues towards the understanding of functional bowel diseases, such as irritable bowel syndrome, as seen in gastroenterology clinics (Bergin *et al.*, 1993; Gwee *et al.*, 1996, Neal *et al.*, 1997).

Since, as seen in the rat, not all intestinal muscle dysfunction persists (Vermillion *et al.*, 1988), we decided to examine the factors controlling both the onset of muscle dysfunction as well as the factors involved in its maintenance, since these two stages of altered function could well have different effector mechanisms. We therefore decided to separately examine both the early stage of muscle function, when the contraction first increases (day 6-8 PI), as well as the contraction at day 21 PI, when the infection and the inflammation had resolved in most mouse strains. While the distinction between the early and the sustained muscle contraction may seem somewhat artificial, it enabled us to simplify the analysis of the muscle responses by the various mouse strains tested.

7.3 Immunological Basis for Altered Muscle Function

7.3.1 T Cell Dependent Increase in Muscle Function

The major focus of this thesis was the determination of the immunological basis for the increased muscle contraction seen during *T. spiralis* infection. Previous studies in the rat had already examined the changes which occur within the smooth muscle itself during infection, some of which may contribute to the increased muscle contraction (Weisbrodt *et al.*, 1994; Fox-Robichaud & Collins 1986; Khan & Collins 1993; Muller *et al.*, 1989). However identifying the role of the immune system in these changes was limited to the demonstration that athymic rats (Vermillion *et al.*, 1991) as well as rats treated with corticosteroids (Marzio *et al.*, 1990) developed little or no increase in muscle contraction during infection, as assessed on day 6 PI. Working from this basis, we confirmed that T cell deficient mice were attenuated in the development of muscle hyper-contractility. This finding was strengthened by a recent study showing that an anti-CD3 antibody which directly activates T cells in mice resulted in, among other changes in small bowel function, a hyper-contractility of longitudinal muscle (Radojevic *et al.*, 1996).

7.3.2 T Cell Independent Increase in Muscle Contraction

Interestingly, examination of later time points during *T. spiralis* infection in athymic mice determined that a delayed and transient increase in muscle function occurred in these immunodeficient mice (Vallance *et al.*, 1998b). A similar delayed response was also seen in SCID/beige mice which lack functional T and B cells, and are also attenuated in natural killer cell function. With the discovery that the muscle response to infection was not completely T cell dependent, questions were raised not only about the source of the transient response, but also about the directness of the interaction between T cells and muscle in this model. The transient T cell independent muscle response may represent a compensatory mechanism, acting through a totally different effector pathway than occurs during infection of an immunocompetent host. More interestingly, it may instead indicate a common final effector mechanism in both T cell deficient and competent mice. In this scenario, the changes in muscle function would be mediated through intermediate cell types which are strongly and persistently activated to alter muscle function in the presence of T cells, but also in the absence of T lymphocytes, are delayed in their activation and are only transiently or suboptimally stimulated. Such cells could include macrophages, mast cells, eosinophils, and neutrophils among others. However, with respect to neutrophils, several studies have already found no correlation between neutrophilic MPO activity and increased muscle contraction. In fact similar MPO levels were found in athymic and euthymic mice during infection, suggesting that neutrophils do not play a role in the muscle pathophysiology.

7.3.3 Macrophages

Another effector cell type which could potentially be involved in altering muscle function is the macrophage, which would be activated, even in the absence of a functioning immune response to *T. spiralis* infection. We examined macrophages, partially because of the mediators they are known to release, such as prostaglandins, IL-1β and TGF-β, which have been shown to alter smooth muscle

function in other systems (Martin *et al.*, 1996; Nilsson 1993, De *et al.*, 1995). As well there are other studies which indicate that macrophages and macrophage-like cells exist in close proximity to enteric muscle and within the myenteric plexus, during the course of infection (Galeazzi *et al.*, 1998) and to a lesser degree, under constitutive conditions (Mikkelson *et al.*, 1988, Galeazzi *et al.*, 1998).

These recent immunohistochemical studies revealed the presence of numerous F4/80 +ve cells within the muscle layers of infected mice, beginning by day 3 PI, with the numbers peaking between days 6 and 10 PI (Galeazzi *et al.*, 1998). In order to determine the role of macrophages in intestinal smooth muscle pathophysiology, liposomes encapsulating the drug clodronate, were injected intravenously into infected mice. Once in the circulation, these liposomes are engulfed by macrophages and other phagocytic cells. The intake of clodronate eventually causes the cells death, resulting in significant depletion of these cell populations. Liposome treatment was found to reduce macrophage numbers within the infected gut, however preliminary results indicated no effect on muscle function, but did cause a reversal of the attenuated nerve function normally seen during infection. The involvement of macrophages in altered nerve but not muscle function during *T. spiralis* infection, has recently been confirmed in this laboratory by infecting mice lacking macrophage - colony stimulating factor (M-CSF) (Yoshida *et al.*, 1990), and as a result, most of their mature macrophages (F. Galeazzi personal communication).

7.3.4 CD4 T Cell Involvement

Further investigation of T cell involvement determined that CD4 but not CD8 T cells were involved. This finding reduced the possible mechanism of T cell action since CD4 T cells typically act through the release of soluble mediators termed cytokines, while CD8 T cells are typically cytotoxic, directly causing tissue damage. However, while CD4 T cells appear to play a significant role in altering intestinal motility, the T cell mediators active in this response still needed to be defined. Nematode infections are associated with a Th2 immune response, and in other model systems, particularly in the lung, there appears to be a correlation between Th2 responses and physiological dysfunction. We therefore decided to examine several of these cytokines in depth by testing the role of these cytokines themselves

during infection, as well as the cell types dependent on their expression. Since we were dealing with nematode infections, we examined the roles of IL-4 and IL-5 as well as the classical Th2 effector cells, eosinophils and mast cells.

7.3.5 IL-5 and Eosinophils

We examined eosinophils since they have long been known to release mediators associated with bronchial hyper-reactivity, as found in asthmatic disease. The cytokine interleukin (IL)-5 has been shown to be the primary growth factor for eosinophils (Sanderson 1988; Simon & Blaser 1995), and appears to be crucial for the development of peripheral eosinophilia during parasite infections. However whether eosinophils contribute to the expulsion of intestinal parasitic nematodes is still unclear, although they are known to release numerous mediators which can cause tissue damage as well as induce smooth muscle to contract. In IL-5 deficient mice (Kopf *et al.*, 1996) infected with *T. spiralis*, there was an almost complete abrogation of the intestinal eosinophilia normally seen during this infection. Even so, IL-5 deficient and control mice developed similar levels of increased muscle tension, by day 8 PI, although the functional changes were significantly less by day 21 PI. Expulsion of both primary and secondary (manuscript in preparation) *T .spiralis* infections were delayed in IL-5 deficient mice.

The finding that eosinophils were not required for the onset of the muscle dysfunction was surprising considering the evidence for their involvement in smooth muscle pathophysiology in other systems, but may reflect that eosinophils require activation before they can contribute to changes in muscle contraction. Our finding of a role for IL-5, in the late, but not the early stages of the infection, may suggest that the eosinophils only become activated in the late stages of infection. While there are many ways in which eosinophils can be activated, one form is through the binding of IgE to the eosinophils Fc receptors. Interestingly, it is already known that IgE levels increase during *Trichinella* infection after about 2 weeks (Lammas *et al.*, 1992), at roughly the same time that differences in worm expulsion and muscle contraction are noted between the IL-5 deficient and expressing mice. Thus eosinophils, perhaps activated by IgE, may contribute to the changes in muscle function found late during the infection.

7.3.6 Mast Cells

In a similar fashion, mast cells are known to release a number of mediators, such as histamine and serotonin that can cause smooth muscle to contract. While no mediators have yet been shown to alter muscle function, as seen in this model, mast cells were putative candidates to be involved in these changes. To test their role, we infected WW^V mice, a strain that is almost completely deficient in the expression of the c-kit receptor and in the production of mast cells. Several studies have clearly demonstrated that this mouse strain expels nematode infections poorly (Ishikawa *et al.*, 1994; Kojima *et al.*, 1980). Initially this delay was thought to be due to the absence of mast cells, however further examination found that bone marrow reconstitution, which restores mast cells to these animals, was not able to normalize the delayed expulsion of *Nippostrongylus brasiliensis* from the gut. The reason may lie in recent findings indicating that these mice also lack a portion of their ICC, cells that are responsible for intestinal slow wave propagation and the coordinated propulsive activity of the small bowel (Huizinga *et al.*, 1995). Studies are currently underway in our laboratory to examine the relative roles of mast cells and ICC in the increased muscle function and in the expulsion of a *Trichinella* infection (Vallance *et al.*, 1997b).

7.3.7 Interleukin-4

We also examined a cytokine involved in both eosinophil and mast cell recruitment and activation, namely interleukin-4. We transferred an adenovirus to the serosal surface of the small bowel and found that it caused increased smooth muscle contraction as well a proliferation of mast cells and an influx of eosinophils. A similar study using an IL-5 encoding virus recruited eosinophils to infiltrate the intestine, but did not bring about a change in muscle function (Radojevic *et al*, 1998). While the actions of IL-4 could be directly on the smooth muscle, it is also possible that it acts through intermediaries such as eosinophils or mast cells, and that IL-4 is necessary to properly activate these cells to participate in

altering muscle function. I will return to the role of IL-4 in regulating muscle function later in the discussion.

7.4 Proximity of Muscle and Putative Effector Cells

A question that arises from these studies is how does inflammation, ostensibly localized to the mucosa, bring about an increase in longitudinal muscle function? In one respect the inflammation does impinge on these tissues directly, since inflammatory and immune cells do infiltrate the external muscle layers during infection (Dzwonkowsi *et al*, 1990; Vallance *et al.*, 1998d). However as assessed by light microscopy, far fewer infiltrating cells are seen in the neuromuscular layers than in the mucosa and submucosa. Of the cells that do infiltrate these tissues, the predominant sites of infiltration are the circular muscle and myenteric plexus, while the longitudinal muscle remains relatively uninvolved. However it is unclear that the cells responsible for the changes in muscle function have to be in direct contact with the muscle cells. Instead, it possible that the responsible cells are in the mucosa, or more likely, the submucosa, and are able to act on muscle cells through soluble mediators. Alternatively, the immune and inflammatory cells may act indirectly, through local nerve networks. However since the changes in muscle function are tetrodotoxin insensitive (Vermillion *et al.*, 1988; Vallance *et al.*, 1997a), any nerve involvement must be transient and lead to changes in function that become intrinsic to the muscle.

Along these lines, it is possible that the muscle may require only limited stimulation before it can begin releasing its own mediators (Kao & Zipser 1988). Several studies have shown that intestinal smooth muscle cells in culture can release the pro-inflammatory cytokines IL-1β and IL-6 following stimulation by IL-1β. As both a target for and a source of these cytokines, potentially auto-regulation and self-stimulation of these cells could act to augment or maintain the heightened muscle function seen in this model (Khan *et al.*, 1995, Collins 1996).

7.5 Persistence of Muscle Hyper-Contractility

The discovery that in some strains of mice, muscle contraction remained persistently increased over control values until at least day 42 PI (Vallance *et al.*, 1997a, Barbara *et al.*, 1997), was in contrast to earlier studies in the rat, which showed a normalization of muscle function by day 23 PI (Vermillion *et al.*, 1988). Comparing the muscle response by different mouse strains during infection, the persistence of the muscle dysfunction appeared to be genetically determined (Vallance *et al.*, 1997a), and correlated with the ability to expel the infection. However it was unclear, based on these early studies, whether the persistence simply reflected the normal time course required for the resolution of the events initiated by the acute infection, or some active and potentially reversible inflammatory process in the neuromuscular tissues.

In more recent studies, our laboratory has carried this examination further, determining that there is a role for inflammatory mediators in the persistent muscle dysfunction. In several studies we have shown that the persistence is T cell dependent, and to a lesser degree eosinophil dependent. In a recent study by our laboratory, Barbara *et al.*, treated NIH Swiss mice with corticosteroids for 3 consecutive days following the resolution of both the infection and the mucosal inflammation (days 23-25 PI). Dexamethasone treatment abrogated >85% of the muscle hyper-responsiveness seen at day 28 PI, suggesting an active (steroid sensitive) process. The role of arachidonic acid metabolites was also examined with the *in vitro* addition of indomethacin or the 5'lipoxygenase inhibitor MK-886 to muscle strips taken from nice infected 28 days previous with *T. spiralis*. Both drugs were found to attenuate the increased muscle contraction (Barbara *et al.*, 1998).

These results strongly indicate an ongoing steroid sensitive stimulation of the muscle, involving both leukotrienes and prostaglandins, however the cellular source of this stimulation remains unclear. In the absence of a discernible inflammatory cell infiltrate, these results may suggest that resident cells of the neuromuscular layers elaborate mediators that sustain the altered physiology. However, since this assessment was based on routine histology, one cannot confidently rule out the involvement of small numbers of inflammatory cells such as eosinophils, or their granules (Persson & Erjefalt, 1997), or cells poorly visible under routine histology, such as mast cells.

7.6 Role of Increased Intestinal Motility and Muscle Function in Host Defense

Another aim of this thesis was to investigate the concept that enteric muscle alters its function and structure in response to mucosal inflammation, as an adaptive host response to infection. The studies outlined in this thesis identify a correlation between the speed with which worms are expelled from the small bowel, and the degree and persistence of the increase in jejunal longitudinal muscle function (see Appendix A.3). Obviously the concept that changes in motility occur in response to intestinal inflammation is not new, as evidenced by the number of clinical studies on the subject (Choi *et al.*, 1997, O'Brien *et al.*, 1996, Moore *et al.*, 1986). However these changes in the neuromuscular apparatus have been typically examined in the context of a maladaptive event, contributing to disease progression and symptoms. The concept that these pathophysiological changes may play a protective role during enteric infection may provide a better understanding of their genesis during inflammation and their role as part of the integrated response by the host tissues.

In terms of host defense, Holmgren and Lyke have already distinguished between two levels of defense against enteric pathogens (bacteria, viruses and parasites) (Holmgren *et al.*, 1992). The first line of defense consists of nonimmunologic (gastric acidity, proteolytic enzymes, peristalsis) and immunologic factors that comprise a barrier to invasive infections. The second line of defense contains almost the full set of systemic and mucosal immune factors (serum derived antibodies operating in conjunction with the complement system and phagocytic cells, and cells with the capacity for cell mediated immune reactions).

These and other studies suggest that during at least some forms of enteric infection, these two lines of defense can interact, with the immune system altering the generally passive defenses of smooth muscle function and peristalsis into a more active form of host response/defense. This may have developed as a generalized defense against pathogens which could bypass the mucosal barriers, or against which the mucosal immune system response has little effect. *T. spiralis* parasites for example, possess thick teguments which protect them from most inflammatory mediators, and they penetrate and dwell within intestinal epithelial cells, which are a relatively protected site. However they are eventually expelled from

the host, and this process does not irreversibly damage them, during the process of expulsion, suggesting they are evicted, ie. through augmented propulsive activity, rather than neutralization (Kennedy *et al.*, 1979).

Unfortunately few studies have even indirectly examined a possible role for intestinal motility in nematode worm expulsion. An obvious approach would be to give compounds, such as opioids, which are known to reduce motility and see if there is an effect on worm expulsion. However a major impediment to such studies is that while such drugs would decrease motility, they are usually not selective for the GI tract. Among their systemic actions are anti-inflammatory effects. As mentioned earlier, the intestinal phase of nematode infections are significantly delayed in WW^V mice, when compared to their controls, even following bone marrow reconstitution. We have carried out similar studies with *Trichinella spiralis* infection in WW^V mice, and found that expulsion of this parasite was delayed by up to 2 weeks compared to their +/+ controls (Vallance *et al.*, 1997b). In addition, changes in muscle function were attenuated in WW^V mice, compared with their controls (Appendix A 1.1). This attenuated response by muscle may be one factor, along with the aberrant propulsive activity in these mice, which contribute to their poor ability to expel. Bone marrow reconstitution experiments, currently underway, should help determine the relative roles of mast cells and ICC in the changes in muscle function and in the expulsion of this parasite. Unfortunately these studies will not be able to identify the precise role of ICC in worm expulsion as the ICC function cannot be restored. However these experiments will aid in determining whether a myeloid or non-myeloid cell type is involved.

7.7 IL-4 Effects on the Enteric Neuromuscular System.

As mentioned earlier, based on the critical role that IL-4 plays in the development of a Th2 response, we hypothesized a role for it in the genesis of altered muscle function. We investigated this by infecting IL-4 deficient mice with *T. spiralis* and by overexpressing IL-4 near the neuromuscular layers, using an adenoviral vector. As mentioned, IL-4 overexpression, in the context of a viral infection, did increase jejunal longitudinal muscle function. It is unclear if the altered muscle function seen in this gene

transfer model exactly mimicks that seen in *T. spiralis* infection, since although IL-4 does appear to be involved in increased muscle tension seen during *Trichinella* infection, it was not a requirement for its development. Preliminary studies infecting IL-4 deficient mice found a slight delay in onset and a moderate attenuation of the increased muscle function, however significant increases in muscle function still occurred (Appendix A 1.2). This suggests that although IL-4, in the context of an adenoviral infection proved sufficient to increase intestinal muscle function, its expression was not necessary for such muscle function to occur.

Recently several other studies have examined IL-4 in the context of its role during nematode infections, and its possible interactions with non-immune cells. Finkelman and colleagues found that mice injected with large doses of a long-lasting IL-4 protein complexed to neutralizing anti-IL-4 antibodies, caused a massive increase in mucosal mast cell numbers within the small bowel. In related studies, Goldhill *et al.*, found this treatment was associated with a significant increase in the electrical field stimulated response of longitudinal smooth muscle, and this was found to be mediated by mast cells and leukotrienes, as assessed by the use of WW^V and 5-lipoxygenase deficient mice (Goldhill *et al.*, 1997). Based on further studies using various immunodeficient mice, the IL-4 treatment was found to increase enteric parasite expulsion, apparently through direct actions on a non-lymphoid cell type, such as smooth muscle. This effect was hypothesized to be the basis of a generalized form of intestinal host defense.

More recently, Lawrence *et al.* were able to separate the intestinal pathology normally seen during *T. spiralis* infection, from the actual expulsion of the parasites (Lawrence *et al.*, 1998) They showed using IL-4 deficient mice, that worm expulsion and intestinal pathology were IL-4 dependent. However the pathology was also dependent on the TNF receptor type 1, since they found that expulsion still occured with minimal pathology in TNFR1-deficient mice. In related studies, they showed no relationship between mast cell numbers and worm expulsion and postulated that IL-4 may act in a direct fashion on non-lymphoid intestinal cells, such as smooth muscle, to create an inhospitable environment for the parasite.

At present however, there is no proof that enteric smooth muscle cells possess a receptor for IL-4, however other cells of similar lineage (fibroblasts) do, and recent studies by Hawker *et al.*, found that IL-4 significantly inhibited proliferation of human lung smooth muscle cells in culture (Hawker *et al.*, 1998). Preliminary studies from our laboratory have identified strong mRNA expression of the α chain of the IL-4 receptor in preparations of murine jejunal longitudinal muscle-myenteric plexus. However further studies are required to determine if enteric smooth muscle cells are the expressing cell types, as well as whether there is a difference in expression between longitudinal and circular muscle tissues.

7.8 Clinical Significance and Conclusions

7.8.1 Immunomodulation of Enteric Muscle Function

Our studies have provided evidence that the gut motor system is subject to modulation by the immune system. While these data have implications for the involvement of smooth muscle and motility in host defense in the context of parasitic infection, we believe that these findings have broader implications. First they identify mucosal inflammation and immune activation as putative mechanism leading to motility disturbances. While this is not necessarily new in the context of enteric infections (Castro *et al.*, 1976, Alizadeh *et al.*, 1987), it provides insights into symptom generation in intestinal infections (Collins, 1992), inflammatory bowel diseases (Kern *et al.*, 1951, Snape *et al.*, 1980, Isgar *et al.*, 1983, Koch *et al.*, 1988), as well as in cases of intestinal pseudo-obstruction in which immune cells have been identified in the neuromuscular layers of the gut (McDonald *et al.*, 1985, Smout *et al.*, 1985). Second, these data illustrate that the motility apparatus is altered in the presence of inflammation, ostensibly limited to the mucosa. Thus, clinical conditions such as gastritis, characterized by low grade superficial inflammation, may be a cause of altered motility, and thus may be relevant to the development of dyspepsia in the absence of ulceration. Third, it is apparent that transient enteric infections and inflammation may produce changes in nerves and muscle function that persist after mucosal integrity is restored. These findings

provide mechanistic insights into the development of irritable bowel syndrome that is precipitated following brief enteric infection (Bergin *et al.*, 1993, Gwee *et al.*, 1996, Neal *et al.*, 1997).

7.8.2 T cell and MHC II interactions in the neuromuscular layers

Taken together with studies from our laboratory and others, it appears that immune cell activation and antigen presentation may be occurring within the external muscle layers of the intestine during enteric infections and immune responses. Our finding that much of the MHC II expression seen during *Trichinella* infection occurred in the muscle layers fits with studies showing numerous MHC II positive cells within the muscle layers of Crohn's resected tissues (Geboes *et al.*, 1992), as well as other studies of IBD tissues showing significantly more actively dividing T lymphocytes in the external muscle layers than in the lamina propria (Fell *et al.*, 1996). In studies conducted in our laboratory, mRNA for interleukin-15, a well recognized mediator of T cell survival, was found to be expressed to a significantly greater degree in the muscle layers than in the intestinal mucosa of both involved and uninvolved Crohn's resection tissues, but not in control tissues (Lovato *et al.*, 1998). Considering the large number of T lymphocytes found in the muscle layers of both macroscopically involved and uninvolved Crohn's tissues, these studies imply that the muscle layers protect and harbor T lymphocytes (Main *et al.*, 1996) which may be involved in the pathogenesis and perhaps in the relapsing nature of Crohn's disease

7.8.3 Role for IL-5 and eosinophils in nematode worm expulsion

There has long been a controversy over the role and importance of IL-5 and eosinophils in the expulsion of nematode infections. Since an estimated 1 billion people world wide are infected with gastrointestinal roundworm parasites, and these infections are associated with high morbidity and even mortality, this is of obvious clinical importance. Several studies have examined the role of IL-5 in experimental models of parasitic infection and have generally found little or no role for IL-5 or eosinophils. However these studies used neutralizing antibodies, and validated the effectiveness of treatment through peripheral eosinophilia. These studies probably overestimated the effectiveness of their

treatment, and thereby underestimated the role of eosinophils by not examining local numbers of eosinophils at the sites of infection. Our studies confirm more recent findings with IL-5 deficient mice, that IL-5 and eosinophils do play at least some host protective role in a primary nematode infection, and play a much greater role during subsequent infections (manuscript in preparation). Whether this information can be used clinically to control gastrointestinal nematode infections by boosting the activity of eosinophils is at present unclear, however understanding what aspects of the host defense are at play can help in the understanding of the pathology during parasitic infections and allergic disease.

7.8.4 Gene Transfer to the Intestine

A great deal of interest has recently been expressed in the potential use of viral vectors for the immunotherapy of disease. In this regard, the G.I. tract, as the largest immune organ in the body, seems well suited for this potential treatment since immune regulation in the GI tract mediates oral tolerance, while dysregulation likely mediates the idiopathic inflammatory bowel diseases. The GI tract also has great potential as a site for DNA vaccination and the production of therapeutic proteins, not only for intestinal diseases, but also for diseases that involve a deficiency in circulating proteins such as clotting factors. Establishing the intestine as a solid organ reservoir for the production of such proteins, following gene delivery would be an attractive therapeutic option.

Unfortunately, there are a number of key problems with viral gene therapy - among these the magnitude and duration of the gene therapy. These problems are magnified in the gastrointestinal tract, particularly with luminal based gene transfer approaches, since the intestine is well adapted to prevent viral infection from the lumen, thereby limiting the potential magnitude of transfection. As well, the target cells, the intestinal epithelium, are continually being replaced, and thus have a relatively short half life, thereby limiting the duration of any transgene expression.

In contrast, in the studies presented in this thesis, we infected serosal cells on the outer surface of the gut. While this approach necessitated surgery, as well as the use of a pluronic gel, we were able to efficiently target the intestine and demonstrate a functional effect of the gene transfer. It is this

demonstration, that gene transfer to the GI tract is feasible and effective, that is of potential clinical importance. We have continued to examine the interactions between the neuromuscular and immune systems, using this approach and recently demonstrated that the effects of the transfer of the IL-4 gene on enteric muscle function is selective, since transfer of an adenovirus encoding IL-5 had no effect on intestinal muscle function (manuscript in preparation). As for clinical relevance, in cases involving severe motility disturbances such as pseudo-obstruction, targeting the muscle layers through laparoscopic surgery or via deep endoscopic injection, using viral vectors encoding proteins that would promote motility, might prove useful. Of course while this approach worked well for our studies, it would also be interesting to test the effect of the same cytokines delivered via the lumen, since that in some respects would better mimic the way that the immune response develops during enteric infections.

Several recent papers have approached the problem of intestinal gene transfer and found that they were able to transduce cells within the intestinal mucosa, by delivering viral vectors through the circulation (Brown *et al.*, 1997) and perorally (During *et al.*, 1998). However the mechanisms by which the infection occurs, as well as the true efficacy of these approaches remains to be proven. We have also recently developed an approach to infect the colonic epithelium, through intra-rectal administration of adenoviral vectors, following a mild inflammatory stimulus to bypass the mucus and epithelial barriers (Vallance *et al.*, 1998c). Overexpression of immunomodulatory molecules within the colon, and their effect on colonic motility are planned. What is clear from these and other studies is that intestinal gene transfer and genetic therapies targeting the gut, need to be optimized and improved upon, and that the field will see extensive growth in the next few years.

7.9 Future Directions:

7.9.1 Immunomodulation of smooth muscle function

While the studies described in this thesis have advanced the understanding of the potential interactions between the immune and intestinal motor systems seen in this model and in others, further elucidation of the mechanisms involved is required. The observation that IL-4 gene transfer can increase

longitudinal muscle contraction is intriguing and requires further exploration. If the effects of IL-4 are directly on the smooth muscle, then it needs to be determined if smooth muscle cells express a functional IL-4 receptor. This could have significant implications in the pathogenesis of smooth muscle dysfunction not only for diseases of the GI tract, but also in the airways. If the interaction between IL-4 and smooth muscle is indirect, then IL-4 gene transfer to the small bowel of IL-5 deficient mice as well as mast cell deficient WW^V mice may identify whether there is a role for either eosinophils or mast cells.

While the gene transfer data indicate that IL-4 is involved in the development of increased muscle contraction, it is unclear if IL-4 plays such an important role in altering smooth muscle function during *Trichinella* infection. In fact, studies using IL-4 deficient mice on a C57BL/6 background did not support such a simplistic etiology, since these mice still developed an increase, albeit reduced compared to their controls, in muscle contraction during infection (see Appendix A 1.2). The role of IL-4 in the development of increased muscle function may be more complex, involving interactions with the hosts genetic background, therefore examining IL-4 deficient mice on other genetic backgrounds such as BALB/c may prove useful. As well, the cytokine IL-13 has been shown to mimic many of the actions of IL-4 and may act to compensate in its absence (Zurawski & de Vries 1994). Therefore testing IL-13 knockout mice, or even mice deficient in the production of both cytokines would provide information on their relative contributions to the process. Both cytokines interact with receptors containing the alpha chain of the IL-4 receptor and are thought to signal through a pathway involving STAT6. Since it was recently demonstrated that the STAT6 pathway, as well as the IL-4 receptor are necessary for nematode worm expulsion (Urban *et al.*, 1998), examining the role of these molecules during *T. spiralis* infection would determine whether they are as critical in the development of increased muscle contraction.

Carrying the results found with muscle function to an *ex vivo* measurement of motility would also prove interesting. Determining whether the immunodeficiencies that negate so much of the change in muscle function associated with infection, correlate with a reduction in intestinal transit, would of course validate these *in vitro* studies immensely. As well, in order to examine the role of motility in expulsion we have already begun to infect WW^V mice, and compare them to WW^V mice having undergone bone

marrow reconstitution. WW^V mice have the most aberrant small bowel motility of any mouse model tested so far, and following the restoration of their mast cells through bone marrow grafting, likely offer the most potential for demonstrating the contribution of intestinal motility to enteric nematode expulsion.

Finally, it would be of great interest to test motility changes in other models of GI infection, particular involving bacteria such as *Salmonella typhi* and *Escherichia coli*. In North America and Europe, such bacterial infections are a far greater clinical concern than nematode infections and are the types of infection most commonly associated with the development of post-infectious IBS. If the host enteric neuromusculature responds to these forms of enteric infection through similar changes and pathways to that seen with nematode infections, this would strongly implicate these changes in the pathogenesis of post-infectious IBS. As well, with the growing numbers of antibiotic resistant bacterial infections appearing, a better understanding of how the gut responds to these and other enteric infections is needed. This will help, not only in understanding the pathogenesis of these infections but perhaps in manipulating and augmenting the hosts ability to defend itself, when current therapies, such as antibiotics, become ineffective.

APPENDIX A

A.1 TESTING OTHER IMMUNODEFICIENT MOUSE STRAINS

Aside from the immunodeficient mouse strains tested in the preceding chapters, I also performed experiments on other mouse strains in order to test the role of other cell types (such as mast cells) and immune factors (such as IL-4) in the generation of increased muscle contraction during *T. spiralis* infection.

A.1.1 WW^V MICE

Aside from eosinophils, the other inflammatory cell type most closely associated with nematode infections, and with smooth muscle hyper-reactivity, is the mast cell (Goldhill *et al.*, 1997). For years the standard mouse strain to test the role of mast cells was the WW^V mouse. However recently it was shown that these mice, which almost totally lack expression of the c-kit receptor protein are also lacking in a population of interstitial cells of Cajal (Huizinga *et al.*, 1995), regulators of slow wave mediated propulsive activity. As shown in Figure A.1.1, these mice are also impaired in the development of increased muscle contraction at day 8 PI when compared to their +/+ genetic controls. However because of the multiple deficiencies suffered by these mice, it is unclear which cell population is responsible. Bone marrow reconstitution, as mentioned previously, will aid in this determination.

A.1.2 IL-4 DEFICIENT MICE

The purpose of this experiment was to examine the effects of a loss of IL-4 expression on the magnitude and time course of increased muscle contraction seen during *T. spiralis* infection. The IL-4 deficient mice were compared to C57BL/6 mice as the genetic background strain. The C57BL/6 data in Figure A.1.2 is taken from chapter 4, Figure 4.1. While the data for the C57BL/6 and the IL-4 deficient mice shown in this figure are from separate infections, I did infect and test the muscle function at day 6, 8, 12 and 16 PI, of several C57BL/6 mice infected at the same time as the IL-4 deficient mice. This infection induced a similar increase in muscle contraction by C57BL/6 mice, as shown in the figure.

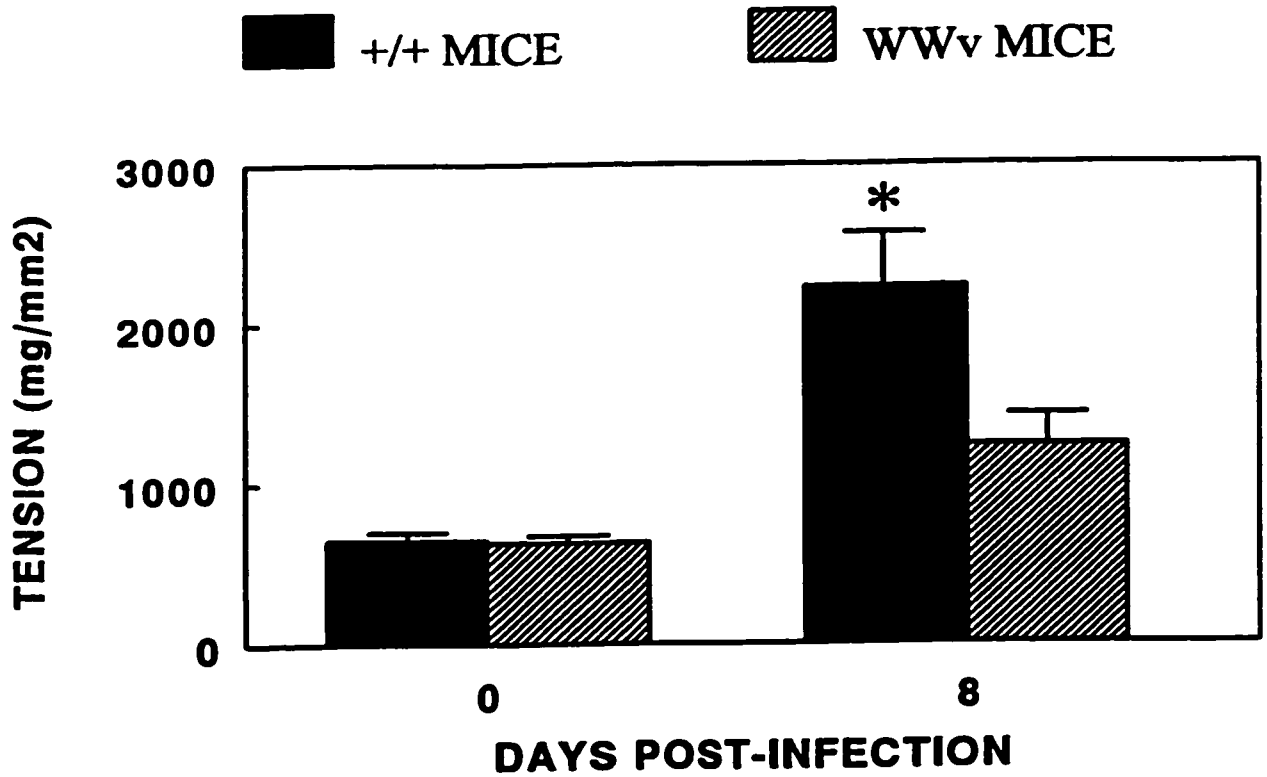

Figure A.1.1: Maximum tension generated by muscle taken from uninfected mice (day 0) or day 8 PI mice in response to 1μM carbachol. Mouse strains shown are +/+ littermates (solid bar) of WW^v (hatched bar) mice. Data shown are the mean ± 1 SEM from groups of 4-6 animals. The asterisk denotes significantly greater tension generation by muscle compared to the infected WW^v mice.

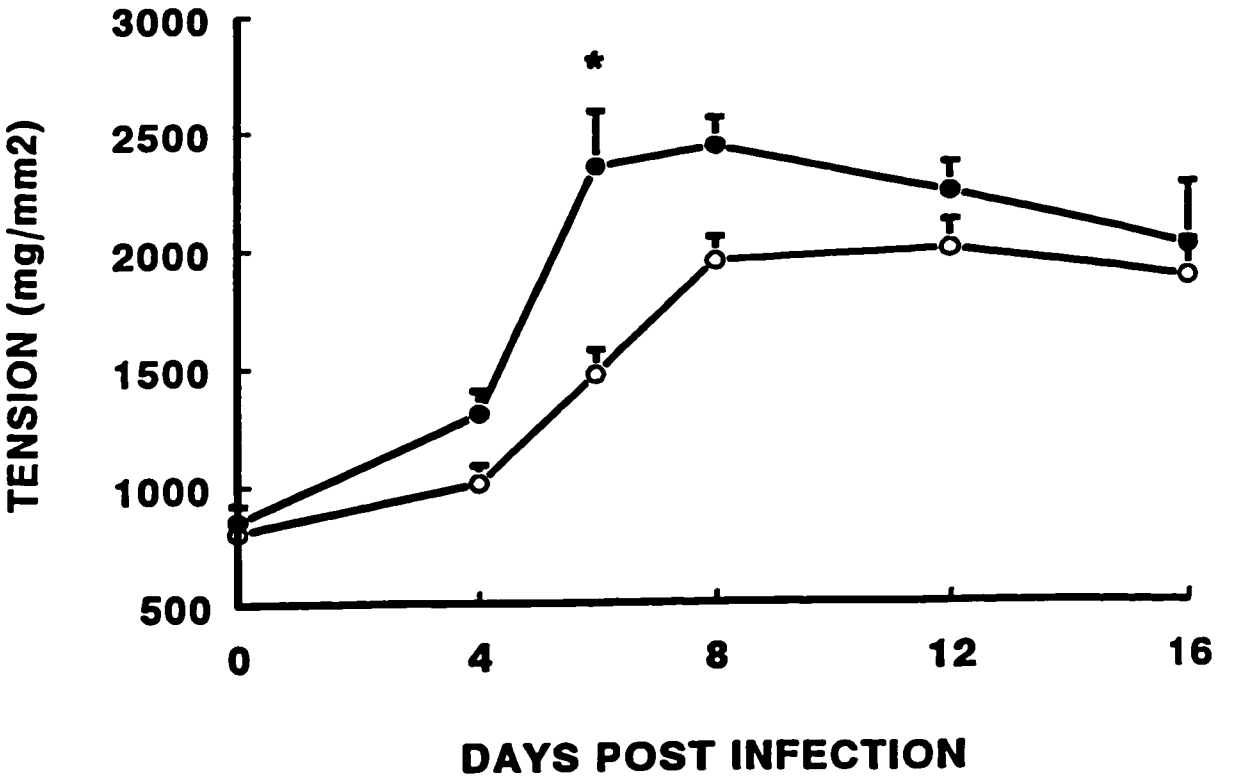

Figure A.1.2: Time course of contractile response to 1μM carbachol (y axis = maximum tension generated) by intestinal muscle taken from infected C57BL/6 mice (solid circles) and IL-4 deficient mice (open circles). Data shown are the mean ± 1 SEM of groups of 4-6 animals. The asterisk denotes a significant increase in tension generation when compared to IL-4 deficient mice at the same time point.

The early increase in contraction in the IL-4 deficient mice was delayed until day 8 PI compared to day 6 in C57BL/6 mice, and the peak muscle contraction was reduced, although not significantly. This indicates that IL-4 is probably not a necessary requirement for the onset of increased muscle contraction during a *T. spiralis* infection, even if it may be sufficient to induce it (see Figure 5.6).

A.2 SUMMARY: - IMMUNE CONTRIBUTIONS TO INCREASED MUSCLE CONTRACTION

The purpose of all these studies was of course to determine which cells and mediators play a role in the development as well as in the maintenance of the increased muscle contraction. The following Tables outline the findings of these studies.

Table A.2.1 Role of T Lymphocytes in Increased Muscle Contraction

Cell Type/Mediator	**Experiment/ Mice Infected**	**Muscle Function Early Phase**	**Muscle Function Persistent Phase**
T Lymphocytes	Athymic BALB/c	Reduced/Delayed	Reduced
	Athymic C57BL/6	Reduced/Delayed	Reduced
	SCID/beige	Reduced/Delayed	Reduced
	T Cell Reconstituted Athymic Mice	Restored	Restored

Conclusion T cells are involved in both the early and persistent phases of increased muscle contraction, during *T. spiralis* infection.

Table A.2.2 Role of T Cell Subsets in Increased Muscle Contraction

Cell Type/Mediator	**Experiment/ Mice Infected**	**Muscle Function Early Phase**	**Muscle Function Persistent Phase**
T Cell Subsets	CD8α Deficient Mice	No Change	Not Tested

	CD4 Deficient Mice	Reduced	Not Tested
	MHC II Deficient Mice	Reduced	Reduced
	MHC II Deficient Mice Given MHC II +ve Bone Marrow and CD4 +ve T Cells	Partially Restored	Not Tested

Conclusion CD4 +ve but not CD8 +ve T cells are involved in both the early and persistent phases of increased muscle contraction, during *T. spiralis* infection. MHC II expression may be required for the actions of the CD4 +ve T cells.

Table A.2.3 Role of Th2 Mediators and Cell Types in Increased Muscle Contraction

Cell Type/Mediator	**Experiment/ Mice Infected**	**Muscle Function Early Phase**	**Muscle Function Persistent Phase**
Th2 Cytokines, Eosinophils and C-kit Receptor	IL-4 Gene Transfer	Increased	Not Tested
	IL-4 Deficient Mice	Delayed	Not Tested
	IL-5 Deficient Mice	No Change	Reduced
	WW^V Mice	Reduced	Not Tested

Conclusion The Th2 cytokine IL-4 can induce increased muscle contraction, but is not essential for the alteration of muscle function during a *T. spiralis* infection. IL-5 and eosinophils are not required for the early phase of increased muscle contraction, but in their absence, the change in muscle function is less persistent. Some deficiency in the c-kit receptor deficient WW^V mouse strain is important in the development of increased muscle contraction during infection. It is unclear if it is mast cells or ICC.

A.3 CORRELATION BETWEEN INCREASED MUSCLE CONTRACTION AND THE SPEED OF WORM EXPULSION.

As mentioned earlier - there appeared to be a correlation between the degree of increased muscle contraction generated during a *T. spiralis* infection, and the speed with which the parasite was expelled from the host. While this could mean many thing - a possible scenario is that the greater the contraction, the greater the propulsive activity acting to dislodge and evict the parasite from the gut. In these last two figures, I have separated all the mouse strains tested (both immunocompetent and immunodeficient) into 3 groups. Strains which expelled the parasite in less than 16 days, strains which took between 16 and 21 days, and strains which took longer than 21 days to evict the parasite. This has been compared to the maximum jejunal longitudinal muscle contraction generated by these strains, in both the early phase (days 6-8) (Figure A.3.1), and in the persistent phase (day 21 PI) (Figure A.3.2). While there are not enough data points in all three groups to do statisitics, the trend is obvious and provides evidence for a link between worm expulsion and altered intestinal muscle function.

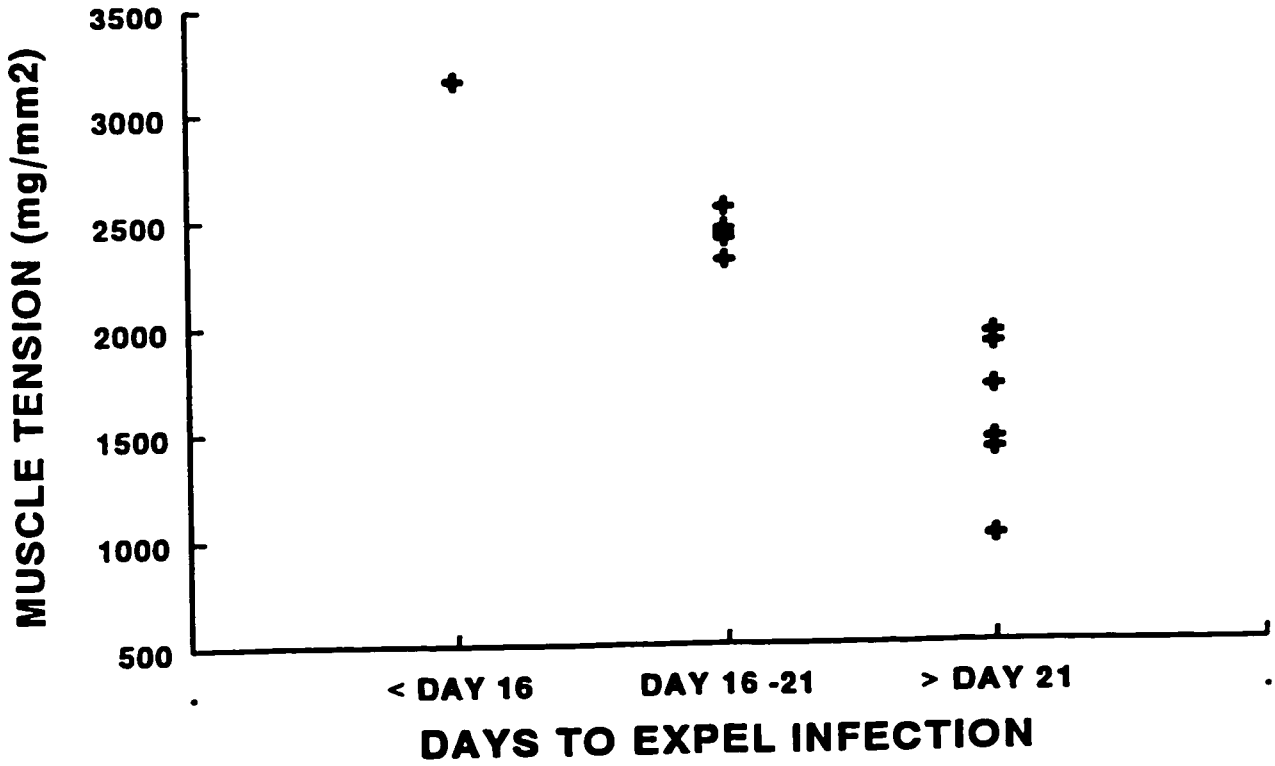

Figure A.3.1: This scatterplot examines the increased muscle contraction generated by various mouse strains in the early phase (day 6-8 post-infection) when compared to the speed with which they expelled a primary *T. spiralis* infection. The mouse strains described in this thesis were divided into 3 groups, those expelling the infection in less than 16 days, between day 16 and day 21, and those that took longer than 21 days to expel the infection. Each cross represents the maximum contraction generated by one mouse strain. The <day 16 group are NIH Swiss, the day 16 -21 group encompass almost all the immunocompetent strains, while the >day 21 group comprises the B10.BR strain, any strains lacking CD4 +ve T cells, as well as WW^V mice. There appears to be a relationship between muscle tension generated, and the speed of expulsion, but there are insufficient numbers of mice that rapid expel an infection, to do the proper analysis.

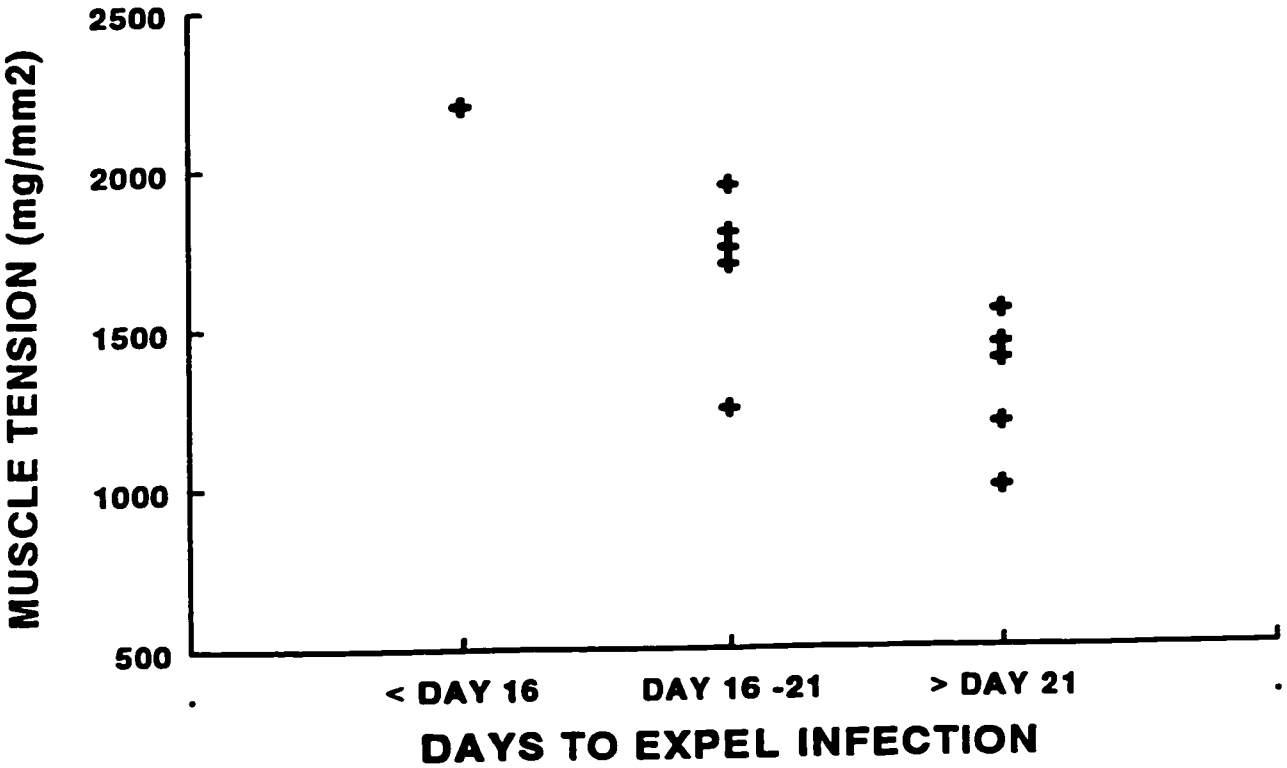

Figure A.3.2: This scatterplot examines the increased muscle contraction generated by various mouse strains in the early phase (day 21 post-infection) when compared to the speed with which they expelled a primary *T. spiralis* infection. The mouse strains described in this thesis were divided into 3 groups, those expelling the infection in less than 16 days, between day 16 and day 21, and those that took longer than 21 days to expel the infection. Each cross represents the maximum contraction generated by one mouse strain. The <day 16 group are NIH Swiss, the day 16 -21 group encompass almost all the immunocompetent strains, while the >day 21 group comprises the B10.BR strain, and any strains lacking CD4 +ve T cells. There again appears to be a relationship between muscle tension generated, and the speed of expulsion, although the relationship is less clear than that seen in Figure A.3.1. Again there are insufficient numbers of mice that rapid expel an infection, to do the proper analysis.

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