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Strongylus vulgaris: Protection, pathology and humoral immune responses in the immunized pony

Monahan, Clifton Michael, Ph.D.

The Louisiana State University and Agricultural and Mechanical Col., 1993

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Ann Arbor, MI 48106

STRONGYLUS VULGARIS: PROTECTION, PATHOLOGY AND HUMORAL IMMUNE RESPONSES IN THE IMMUNIZED PONY

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Interdepartmental Program in
Veterinary Medical Sciences through
the Department of Veterinary Microbiology and Parasitology

by
Clifton Michael Monahan
B.S., Colorado State University, 1982
D.V.M., Colorado State University, 1985
December 1993

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I would like to begin by acknowledging Dr. Johannes Storz for extending to me the offer of a stipend for graduate studies which enticed me to Louisiana. Without the offer, none of this would have occurred. Four years and four months ago I arrived at the L.S.U School of Veterinary Medicine. I waited by the elevator for my first trip to the third floor, and when the doors opened, out stepped Joe Bertone. I hadn't seen Joe since Colorado State where he and Alicia had done their residencies in equine medicine and surgery respectively. Joe asked what I was doing here, and when I told him that I was going to do graduate work in parasitology he told me without hesitation, "Tom Klei, work with Tom Klei. He's the best there is." Joe also told me to eat at the Silver Moon Café because that's Alicia's favorite restaurant. Joe gave me good advice on both accounts.

Dr. Klei earns the respect and admiration of all those who work around him. There were many moments during these four years and four months when I considered giving up in frustration and trying instead a career in the food service sector, but my sincere respect for Dr. Klei became the motivation to finish when I had exhausted all other reasons. I am also deeply indebted to the help and friendship of the people he has gathered around him, who have worked with Dr. Klei for many years, and I would like to acknowledge Sharon Coleman and Melanie Chapman for their tolerance, support, and help during these four years. Dr Klei would be the first to admit that his esteemed position as Boyd Professor of Parasitology would have been difficult if not impossible to attain if not for the loyalty and profficient support he has received from Melanie and Sharon for so many years. More recently, Chau Nguyen has joined Dr. Klei's laboratory group, and I have greatly

appreciated her contribution to the lab ambiance. People of this caliber are a credit and testament to the respect and loyalty Dr. Klei merits.

I have many other friends who rallied around me during the final months of my work, and I will thank them all personally rather than list them here, but I would like to say that their cards, letters, phone calls and care packages worked magic. I would also like to thank the Thomas family, Seabelle, Sonia, Andrew and Ken, at the Silver Moon for the nourishment I received from their care and their cooking.

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I would also like to acknowledge the contributions of Paravax, Inc., which provided funding for the project, and Ribi Immunochem Research, Inc., which graciously donated the Ribi adjuvant used in our immunizations. I extend my thanks and gratitude to both organizations for their cooperation.

PREFACE

This study may be criticized by readers who feel that many important questions were left unanswered, and this is so, but not because these questions were ignored or not examined. I ask the readers, if any, to bear in mind the limitations of the equine system for a graduate study of such a complex host-parasite system. Parasite-free ponies are a prohibitively expensive model and commercially available reagents to clarify their responses did not exist during the time that this study was performed and written. Due to these limitations, many of the most interesting questions could only be pondered but not performed. My personal inexperience in the laboratory likewise limited many of the studies which were executed. I was sufficiently naïve at the onset that I didn't understand the limitations of the system nor whether to ask if a single person could perform certain tasks alone. I plunged into areas which, due to the limitations of the system, could not be repeated once experience was gained. I do not offer these explanations as excuses to those who find this document fairly light in content, just as explanations that many studies were left inchoate because the system did not allow for a second chance. The system would be better for an experience laboratory technician than a neophyte. I do appreciate the insight it has given me, and would relish the opportunity to have done this in a model where I could have repeated valuable phases of the immunization.

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ABSTRACT

Protective resistance to *Strongylus vulgaris* infection was examined by immunization of ponies with radiation-attenuated larvae or soluble parasite homogenates. Twelve yearling ponies raised and maintained under parasite-free conditions were divided into four groups which received either radiation-attenuated L_3; aqueous-soluble adult somatic antigens plus adjuvant; aqueous-soluble larval somatic antigens with excretory/secretory products and adjuvant; or media with adjuvant. Ponies were immunized twice; radiation-attenuated L_3 were administered orally and soluble antigens or control injections were given intramuscularly. Six weeks following the second immunization, ponies were challenged *per os* with virulent *S. vulgaris* L_3. Ponies were monitored twice daily for rectal temperatures and signs of discomfort. Hematologic exams were performed weekly. Six weeks following challenge ponies were euthanized and necropsy performed. Challenge larvae were recovered from dissections of the cranial mesenteric artery (CMA) and major branches.

Ponies immunized with radiation-attenuated L_3 had fewest post-challenge febrile episodes, developed anamnestic eosinophilias and were greater than 91% protected from challenge determined by larval recoveries from arterial dissections. Ponies immunized with either Adult or Larval antigens or Controls had similar larval burdens recovered from arterial dissections, thus were equally susceptible to homologous challenge. Ponies which received Adult and Larval immunizations, however, had more severe clinical signs and necropsy lesions, suggesting that prior sensitization to these antigens exacerbated their post-challenge inflammatory response.

Histological differences included the number and staining characteristics of eosinophils. Tissues from radiation-attenuated recipients demonstrated severe eosinophilic infiltrates with dark-staining eosinophils which lacked distinct cytoplasmic granules. Sections of the CMA showed that these recipients maintained arterial architectural integrity while ponies which received either Adult or Larval immunizations and Controls all suffered severe arterial destruction secondary to larval migration.

Analysis by enzyme-linked immunosorbent assay (ELISA), Western blot and indirect fluorescent antibody (IFA) assay demonstrated differences which further characterized these groups. Radiation-attenuated larvae recipients developed higher antibody recognition of larval surface antigens determined by IFA than their counterparts, but recognized fewer somatic antigens on Western blots. Protection was associated with recognition of antigens on larval surfaces.

INTRODUCTION

Strongylus vulgaris is an ubiquitous intestinal nematode parasite of equids and, presently, still the single most important equine parasite due to the severity of lesions caused by the arterial migration of infective larvae. Horses can develop a resistance to subsequent infections following a primary exposure, such as that gained during a grazing season, but this primary exposure will lead to damaging lesions in the arteries which supply the large intestine (Amborski *et al.*, 1974; Duncan, 1973, 1975; Klei *et al.*, 1986). Such resistance is not complete, and appears to prevent further larval migration of recently-acquired infective larvae rather than elimination of those arterial-dwelling larvae already established. This resistance is not long-lasting, thus must be reestablished during subsequent grazing seasons. Studies examining CMA lesions at necropsy have shown little age-related correlation with the presence of active larval lesions in the CMA, but that a seasonal correlation exists (Lyons *et al.*, 1981; Ogbourne and Duncan, 1985; Morgan *et al.*, 1989). Annual vaccination would be a preferable method for achieving this reexposure considering the severity of the consequences from arterial lesions which follow natural exposure.

Anthelmintic control of *S. vulgaris* infections is possible on well-managed farms, but foals or horses from these farms would not develop resistance to infection without a primary exposure, thus would be susceptible to infection when they encounter *S. vulgaris* larvae at other locations, such as shows, events, trail rides or breeding farms where anthelmintic programs may not be as rigorous, or equine traffic too frequent for control. Previous studies in our laboratory have shown that: 1) *S. vulgaris* can be eliminated from a closed herd by judicious use of anthelmintics over a two-year period; 2) daily treatment of foals with

pyrantel tartrate feed preparations can prevent infection with *S. vulgaris*, but that foals reared in this manner are as susceptible to the effects of challenge as parasite-naïve foals.

Radiographic evidence of lesions secondary to larval migration within the CMA have been demonstrated 21 days following administration of as few as 50 infective larvae (Slocombe *et al.*, 1977; Holmes *et al.*, 1990), and experimental infection with 200 larvae has resulted in death within 5 weeks (Klei *et al.*, 1990). These findings demonstrated that significant arterial lesions can develop during the usual interval between anthelmintic treatments, and suggest that protection with an effective vaccine would be a useful adjunct to effective anthelmintic control programs (Klei, 1986).

Immunization of ponies with radiation-attenuated larvae can be effective in generating protection from experimental challenge without producing arterial lesions (Klei *et al.*, 1982; 1989). Live larval vaccines have inherent difficulties in production, distribution, storage, and approval from regulatory agencies (Miller, 1978). Radiation-attenuated larval vaccines have been developed, but the vaccine for *Dictyocaulus viviparous* is the only live larval product currently available (Jarrett *et al.*, 1957). Miller's experience developing an effective vaccine for hookworm in dogs leads one to believe that no product derived of a fecal culture will reach licensing in an injectable form due to the bacterial counts inherent in a product of fecal culture. The *D. viviparous* vaccine has an oral route of administration which reduces the potential impact of such bacterial counts. Again, an effective subunit vaccine would be a preferable alternative, based on acceptance by consumers and regulatory agencies alike.

Immunization of animals with soluble somatic nematode extracts has been performed by several groups, followed by studies defining the antigens recognized preferentially by those animals protected from challenge (Adams, 1989; Adams *et al.*, 1982; East *et al.*, 1989, 1993; Gasbarre and Canals, 1989; Jasmer *et al.*, 1991; Munn *et al.*, 1993). For these reasons, previous studies on *S. vulgaris* radiation-attenuated immunizations were expanded in the present study by comparing the post-challenge responses of parasite-free ponies immunized with soluble worm extracts to the responses of ponies immunized with radiation-attenuated larvae, including the protection from larval migration, pathological lesions associated with immunization or challenge, and the humoral immune responses to various *S. vulgaris* antigen preparations.

G.F. Mitchell advised that "Macerating a worm must yield a soup of which little can be expected by way of immunobiological activity." and that "Because immunopathology can be a major aspect of parasitic disease, immunization with crude antigen mixtures may have entirely undesirable consequences." (Mitchell, 1989). While prophetic in a sense, such immunization with the entire worm would mimic the exposure received when an animal's immune system deals with the decay of a parasite killed within the host's tissues. This approach utilizing the crude mixture risks development of nonprotective immunopathology, but that is often the case in natural infections prior to the establishment of protective immunity. One must compare these responses in order to evaluate which antigens are important in protective recognition, as well as the repercussions of an inappropriate response.

Since parasites are complex infectious agents, often with intermediate hosts and somatic migrations, it makes sense that development of a method for protective immunization would be a more complex task than for viruses or bacteria. Vaccine development is the culmination of a series of studies examining immunization with a variety of antigens, routes of immunization, and adjuvants. Preliminary studies, such as the present study, which use crude extracts are a necessary first step.

REVIEW OF LITERATURE

Strongylus vulgaris has been studied and reviewed in depth (Georgi, 1973; Ogbourne and Duncan, 1985). Georgi, in his translation of Karl Enigk's works, cited that Enigk credited Kikuchi with the postulation that *S. vulgaris* infective larvae, larval stage 3 (L_3), cross the intestinal wall and migrate through the capillaries and arteries of the intestine. The popular conception held at the time was that infectious larvae followed a typical nematode migration pattern which involves penetration across the intestinal wall, travel through the liver and lung before returning to the lumen of the intestine where the adults reside.

Enigk elaborated on Kikuchi's earlier studies by infecting parasite-free foals and performing necropsies at sequential timepoints, thus elucidating the lifecycle, proposed by Georgi as the Kikuchi - Enigk model for the migration of *S. vulgaris* in the horse. Further studies have confirmed the migration and the range of lesions formed as a result of larval migration, as well as the development of natural resistance to infection (Amborski *et al.*, 1974; Duncan, 1973, 1975; Drudge and Lyons, 1983).

Briefly, infective *S. vulgaris* L_3 ingested by horses while grazing on contaminated pastures penetrate the mucosa of the intestine, principally the cecum and ventral colon, and migrate as far as the submucosa. Infective L_3 then become quiescent for several days, during which time they molt to the fourth stage (L_4). Subsequent to this molt, the L_4 somatic migration follows arterioles and arteries from the submucosa upstream to the root of the CMA at its junction with the caudal aorta. Aref (1982) postulated that migrating larvae sense the curvature of the vessel and follow this stimulus to the root of the CMA. Rather than the

CMA being a prediliction site where some biological stimulus is provided for further development, he proposed that it is a mathematical prediliction site. Further larval maturation occurs at at the root of the CMA before the immature adults (L_5) return to the intestine, again via the arteries, to complete their lifecycle.

Adult *S. vulgaris* reside in the lumen of the cecum and ventral colon where they attach to the mucosa and feed on blood. It is not, however, the bloodfeeding habits of the adults which are responsible for the association of *S. vulgaris* with colic, but the arterial lesions which form secondary to *S. vulgaris* larval migration. These lesions make *S. vulgaris* the most important equine parasite because damage to the intestinal arteries can compromise perfusion of distal vascular beds even after the larvae have been removed by effective anthelmintic treatment. Occlusion of a major branch of the CMA can produce ischemia of the bowel, pain and colic, a condition known as verminous arteritis or thromboembolic colic. The ultrastructure of these arterial lesions has received considerable attention (White *et al.*, 1983; Slocombe *et al.*, 1987; Drudge and Lyons, 1989; Morgan and Van Houten, 1989; Morgan *et al.*, 1991). The precise mechanisms behind the perfusion compromise remain unclear, but include mechanical and physiological factors, such as the mechanical occlusion of a parasitized vessel, as well as the physiological occlusion following vasospasm of the vascular smooth muscle of vessels in otherwise healthy capillary beds.

Arterial architecture can be destroyed by migrating larvae which lift the endothelial lining from the inner elastic lamina by their movements as well as through the actions of secreted proteases. Tortuous subintimal tracts can be visualized on the lumen surface of an opened artery where these migratory tunnels have been formed and subsequently filled with

granulation tissue. Disruption of the endothelial lining exposes subendothelial collagen to platelets, inflammatory cells, and humoral clotting factors which recognize and respond to the exposed collagen, initiating thrombus formation and a progression of the inflammatory response. Destruction of the intima leads to changes within the tunica media which can be visualized histologically as a disruption of the normal pattern of smooth muscle cells, increased extracellular collagen deposition, and infiltration of inflammatory cells. The tunica adventitia also responds to this inflammation with increased fibrosis. Further destruction, whether from mechanical damage by larvae or through inflammatory insult, can lead to complete loss of the endothelial lining and the inner elastic lamina. Inflammation progresses into the media of the artery, followed by a weakening and remodeling of the wall which leads to formation of diverticuli known as verminous aneurysms. Architectural changes such as these disrupt normal laminar blood flow. Turbulent flow over the roughened surface further encourages continued formation of diverticuli. Alteration in the collagen and elastin ratio within the extracellular matrix reduces the flexibility of the arterial wall, and it is this flexibility and contractility which is responsible for smooth flow of blood during the systole/diastole cycle of the heart. Reduction of this flexibility reduces the overall flow of blood through the dependent vascular beds.

Thrombus formation begins on the exposed collagen when the endothelium and inner elastic lamina are destroyed. Thrombus formation compromises the lumen of the artery, which in turn decreases the effective blood flow. Thrombi can detach from their sites of formation and be carried in the bloodstream towards distal capillary beds until reaching a lumen diameter small enough to prevent further passage. Blockage of a major vessel in this manner may halt perfusion of large areas of the intestinal mucosa before collateral circulation can

establish. If the ensuing ischemia leads to necrosis of sufficient areas of the bowel mucosa, a potentially fatal endotoxemia can result.

Mechanical occlusion of an artery by thromboemboli is one explanation of the pathogenesis of verminous arteritis and thromboembolic colic, but Morgan (1989) decribed other architectural changes which could contribute to ischemia of the intestine without formation of thromboemboli. Complete destruction of the endothelial lining and inner elastic lamina as described above was characteristic of active lesions in parasitized vessels. Morgan also noted severe subendothelial hyperplasia in arteries possessing intact endothelial linings and inner elastic laminae. This hyperplasia in otherwise healthy arteries or arterioles was characterized by an increase in both the number of smooth muscle cells and the extracellular collagen deposition. Such hyperplasia was noted to reduce the effective lumen diameter (Morgan *et al.*, 1991). Throughout Morgan's histological and morphometric study, a wide variation in the effective lumen diameters of arteries with either acute or chronic arteritis was documented. The variation in lumen diameter was dependent on the relative effects of thrombus formation, subintimal hyperplasia, and changes of the tunica media. Any combination was possible. Heavily-parasitized arteries could be found with an increased lumen diameter despite the thrombi present where sufficient thinning and diverticulation of the arterial wall led to an overall lumen diameter increase. In non-parasitized vessels where there was no destruction of the intima nor changes within the tunica media, however, the resulting subintimal hyperplasia always caused a significant decrease of lumen diameter. Vessels compromised in this fashion may serve as mechanical barriers to thromboemboli, or, in the event of physiological vascular constriction, could reduce circulation to negligible levels (Ward *et al.*, 1986; Bottoms *et al.*, 1986). If numerous arteries and arterioles

supplying large areas of bowel are affected with these lumen reductions, collateral circulation might be difficult to establish in the event of a mechanical blockage by thromboemboli.

The cause of this subintimal hyperplasia is undocumented, but may involve a host response to inflammatory cytokines generated against the migrating larvae. Angiogenesis is a necessary step in healing and is stimulated by a choreography of cytokines, particularly platelet-derived growth factor(PDGF), transforming growth factor beta (TGF-beta) interleukin-1 (IL-1), and tumor necrosis factor (TNF) (Leibovich *et al.*, 1987; Fràter-Schröder *et al.*, 1987; Jaatela, 1991; Kovacs, 1991; Nathan and Sporn, 1991). It is possible that healthy tissue would respond to this same stimulus with hyperplasia, since there is no post-inflammatory remodeling necessary. Such hyperplasia has been demonstrated in the subendothelial changes of non-parasitized vessels (Morgan *et al.*, 1989). Inflammatory cytokines produced in response to arterial-dwelling larvae would be in highest concentration in the vessels immediately distal to their production, which further supports this possible mechanism behind the subendothelial changes documented by Morgan.

Resistance to *Strongylus vulgaris* infection can be generated following natural exposure (Amborski *et al*, 1974; Duncan, 1973, 1975; Klei *et al.*, 1986), or exposure to radiation-attenuated larvae (Klei *et al*, 1982; 1989). Natural infection, of course, generates CMA lesions before resistance to further infection develops, and this resistance is neither complete nor long-lasting. Several studies have demonstrated the lack of a clear age-related association of CMA lesions at necropsy (Lyons *et al*, 1981; Ogbourne and Duncan, 1985; Morgan *et al*, 1989). These studies suggest that resistance to *S. vulgaris* infection is seasonal

rather than age-related, based on a high prevalence of active lesions in older horses at necropsy. This implies that resistance must be reestablished with each transmission season, and this resistance serves to reduce the severity of acute lesions but does not eliminate their occurrence (Ogbourne and Duncan, 1985; Morgan *et al*, 1989).

The role of humoral factors, antibodies in particular, in resistance or immunity to *Strongylus vulgaris* infection has been examined by several authors but remains enigmatic (reviewed by Bailey, in Soulsby, 1987). Animals may develop similar changes in serum globulins during the course of an *S. vulgaris* infection, but vary greatly in their resistance, or development of lesions. One common change seen during *S. vulgaris* infection is a marked elevation of serum globulins, particularly IgG(T) in the beta globulin fraction, but no demonstrable role for this IgG(T) in the course of infection or disease has been made. Polyclonal mitogenic effects of *S. vulgaris* antigen fractions were demonstrated for lymphocytes of equids, and other species as well, and the increase in IgG(T) was proposed to be a result of this non-specific stimulation (Adefeya *et al.*, 1985; Bailey *et al.*, 1989). A positive precipitin reaction using IgG(T) and this mitogenic fraction indicated that some of the IgG(T) did recognize *S. vulgaris* antigens (Adeyefa, 1992), but no other *S. vulgaris*-specific role for IgG(T) has been demonstrated.

Gamma globulins produced in response to *S. vulgaris* infection have been shown to recognize *S. vulgaris* larval and adult antigens, some in a stage-specific or species-specific fashion (Klei *et al.*, 1983; Nichol *et al.*, 1987; Dennis *et al.*, manuscript submitted). Rabbit anti-*S. vulgaris* antibodies have likewise demonstrated stage- and species-specific antigens in both somatic and excretory/secretory products when analysed using agar gel

immunoprecipitation (Wynne *et al.*, 1981). These authors examined antibodies which developed to *S. vulgaris* antigens following natural or experimental infections. The present study, however, was designed to ask whether *S. vulgaris*-specific antibodies in circulation at the time of challenge could protect parasite-naïve ponies from challenge, and if so, which would be the most important antigens for a subunit vaccine. If this immunization could not provide that protection, then the recognition profiles could be compared with that developed against radiation-attenuated *S. vulgaris* L_3 which have been demonstrated as effective in generating protective immunity. By such a comparison, non-protective epitopes could be eliminated.

Another possibility is that the manner in which the antigen is recognized confers the immunity. Protection or pathology may prove to be a difference of antibody isotype and cellular responses rather than a difference in which antigen is recognized. The difference in resistance to *Schistosoma mansoni* imparted by IgE or IgG_4 is one example of isotype-related differences in susceptibility (Hagan, 1991). The differential development of T cell subsets and their effects on the outcome of disease has been demonstrated in parasitic infections (reviewed by Cox and Liew, 1992; Finkleman *et al.*, 1991; Locksley and Scott, 1991). Antigen can be presented with different adjuvants to elicit responses from these different T cell populations (Grun and Maurer, 1989; East *et al.*, 1993; Bomford, 1980; Mallon *et al.*, 1991).

Antibodies to helminths often result in immunopathology rather than protection, and immunizations may promote disease rather than protection (Mitchell, 1989). The possibility of simultaneous protection and pathology has been demonstrated (Gasbarre and Canals,

1989). Cattle immunized with crude *Oesophagostomum radiatum* antigens had fewer worms present at necropsy than did non-immunized controls, but the immunized cattle suffered more weight loss during the larval stage of the challenge infection than controls. Using HPLC, these authors then refined the crude extract to five major immunogens recognized by IgG_{2a} which imparted equal immunity to challenge and equal clinical response as well. Similar protection was demonstrated with high molecular weight *O. radiatum* antigens prepared by gel filtration (East *et al.*, 1989, 1993).

Radiation-attenuated larval immunization provides protection in a number of host-parasite relationships and some of the proposed mechanisms for this protection are reviewed by Wales and Kusel (1992). These authors mention impaired protein synthesis as one of the most strongly supported mechanisms behind attenuation. Examining the *S. vulgaris* attenuated larvae model, this could translate into delayed maturation of the L_3 to the L_4. Such a delay would prolong the period of exposure to this larval stage. Impaired protein synthesis could also be manifest in the elaboration of ineffective excretory/secretory products by the L_4. Migration of the L_4 through tissue requires the aid of proteases. Proteases have been identified in extracts of *S. vulgaris* larvae and adults (Klei, unpublished data). Alterations resulting in defective protein synthesis could reduce the efficacy of an enzyme and slow or halt migration. Changes in glycosylation of proteins could lead to more rapid degradation. Either way, this may translate into impaired migration of the L_4 which would also prolong the exposure and allow development of immune recognition.

The major questions posed by the present study are: can protective immunity equal to that of radiation-attenuated L_3 be induced by sensitization with soluble somatic homogenates; is

there a difference in the clinical response to challenge following immunization; if protection is not provided by immunization with soluble homogenates, can a comparison of antigen recognition profiles elucidate differences in the antigens recognized; and, if the difference does not lie with the antigens, can the difference in the response be identified?

MATERIALS AND METHODS

Experimental Animals - Twelve yearling ponies were raised from birth and maintained under parasite-free conditions as previously described (Bello *et al*, 1972). During the immunization experiment, these ponies were housed in pairs in concrete stalls, fed a pelleted ration (Ralston Purina Co., St. Louis, MO) and water ad libitum.

Experimental Design - Ponies were assigned to one of four immunization groups to provide a sex ratio of two males and one female per group. All ponies were yearlings, and body size was not considered in the assignments to immunization groups. The four immunization groups were: radiation-attenuated *S. vulgaris* L_3 (Irradiated); aqueous-soluble *S. vulgaris* adult somatic antigen homogenate (Adult) plus Ribi adjuvant (Ribi Immunochem Research Inc., Hamilton, MT); aqueous-soluble *S. vulgaris* L_3 and L_4 somatic antigens plus excretory/secretory products (E/S) from *in vitro* culture (Larval) plus Ribi adjuvant; or serum-free culture media (Control) plus Ribi adjuvant.

Each pony was immunized twice with its appropriate preparation. Irradiated *S. vulgaris* L_3 were prepared from fecal cultures of *S. vulgaris* monospecifically-infected donor ponies. Following harvest from the Baermann apparatus, larvae were washed in tap water and stored at 4° C for less than a month. Each dose consisted of 500 *S. vulgaris* L_3 counted manually with the aid of a dissecting microscope, put into individual syringes with tap water, and attenuated with 90 kRads ^{60}Co on the day of inoculation. These larvae were given orally by syringe behind the tongue, then the syringe was rinsed and the rinsate administered orally as

well. Irradiated immunizations were separated by a three-week period following the protocol previously described (Klei *et al.*, 1982).

Adult, Larval and Control immunizations consisted of 1 ml of antigen preparation at a concentration of 1 mg ml^{-1} (Adult or Larval), or 1 ml serum-free culture media (Control). Injections were made intramuscularly in the left side of the neck, and were separated by a four-week interim according to the manufacturer's recommendations (Dr. T. Ulrich, Ribi Immunochem Research, Inc., personal communication).

The two immunization protocols were synchronized such that the second immunizations coincided. Six weeks following the second immunization, all ponies were challenged *per os* with 750 virulent *S. vulgaris* L_3. Challenge larvae were prepared from fecal culture of the same *S. vulgaris* monospecifically-infected donor ponies. Larvae were counted manually with the aid of a dissecting microscope and aliquoted to individual syringes. Challenge larvae were administered to all ponies in the same fashion as previously mentioned for the Irradiated immunizations.

Following challenge, ponies were monitored twice daily for signs of colic, depression, anorexia, and rectal temperatures. Blood was drawn weekly for hematological exams, and serum or plasma from these collections was stored at -70° C. Six weeks following oral challenge, all ponies were euthanized and necropsy examination performed as described below.

Rectal Temperatures - Ten days prior to the experiment, ponies were moved from a parasite-free facility into cleaned concrete stalls and allowed to acclimate to their new surroundings. Parasite-free conditions were maintained in this facility. During this ten day period, rectal temperatures were recorded for all ponies and a ten-day mean rectal temperature was calculated. Post-challenge febrile episodes were recorded as those days which the morning rectal temperature of a pony exceeded the ten-day mean rectal temperature by two standard deviations. Mean febrile days post-challenge were calculated from the number of febrile episodes experienced by the individual ponies in an immunization group. A daily group mean temperature was also calculated and plotted graphically to demonstrate the temporal pattern of the group febrile episodes.

Antigen Preparation - *Strongylus vulgaris* L_3 for use in the live Irradiated immunizations, larval antigen immunizations, surface extract preparations, or fluorescent antibody staining were recovered from Baermann sedimentations of fecal cultures prepared from *S. vulgaris* monospecifically-infected donor ponies. For any experiments or preparations which used *S. vulgaris* larvae, L_3 were exsheathed with a mild sodium hypochlorite solution and washed to remove the cuticles. At this stage of preparation, larvae were described as 'freshly-exsheathed' and used immediately. Those L_3 intended for experiments requiring *in vitro* culture were exsheathed and incubated in NCTC-135 media (Gibco, Grand Island, NY) supplemented with 2.8 g L^{-1} Bacto-Peptone (Difco Laboratories, Detroit, MI) 2.25 g L^{-1} yeast extract (Difco), 2.8 g L^{-1} dextrose (Difco), antibiotics (sodium penicillin G: 400 U ml^{-1}; streptomycin sulfate: 400 μg ml^{-1}; amphotericin B: 1 μg ml^{-1}; Gibco, Grand Island, NY) and 50% fetal calf serum, as a modification of Douvres (1970). A gas mixture of 7.5%

CO_2, 5% O_2, and nitrogen was bubbled through the media and larvae prior to incubation at 37° C in 5% CO_2 and 95% RH.

Preparation of the Larval immunizations utilized L_3, L_4, and the E/S products or the L_4. The L_3 were cultured to the L_4 stage *in vitro*. Media was changed after seven days. On Day 10, at which time most larvae had molted to the L_4 stage, larvae were washed to remove bovine serum, cast cuticles and debris, recultured overnight in supplemented, serum-free NCTC-135 with aprotinin added as a protease inhibitor. Following overnight culture, L_4 and media containing E/S products were frozen at -70° C until processed with an equal number of freshly exsheathed L_3 as follows. Frozen L_4 in their culture media were thawed, combined with the freshly-exsheathed L_3, and pelleted by centrifugation. The pelleted L_3 and L_4 were subjected to repeated cycles of grinding in a glass tissue homogenizer followed by freezing in an alcohol bath maintained at -70° C, then further grinding as the frozen pellet thawed. Following five cycles, this homogenate was combined with the L_4 supernatant and rocked overnight at 4° C. Further steps for preparation of the immunizations doses follow the description of the Adult antigen preparation.

Adult antigen preparation utilized adult male and female *S. vulgaris* which were recovered from horse intestines at a commercial abbatoir, washed repeatedly in PBS and stored at -70° C until used. Male and female adults were washed in PBS and homogenized through five freeze/thaw cycles in supplemented, serum-free NCTC cuture media as described above for the larval preparation, then rocked overnight at 4° C. Following overnight solubilization, adult and larval antigen preparations were centrifuged at 20,200 x g, for 45 minutes at 4° C. Supernatants were filtered through a 0.2 μm nylon filter (Gelman, Ann Arbor, MI).

Protein content of these soluble homogenates was determined using a commercial kit (Pierce Chemical Co., Rockford, IL). These preparations were then aliquoted and stored at -70° C until used.

Immunization doses were prepared by diluting the appropriate antigen preparations to a concentration of 1 mg ml^{-1} using serum-free NCTC media. Adult, Larval and Control immunizations consisted of 1.0 ml of the appropriate antigen or media preparation plus 50 μl Ribi adjuvant. Antigen preparations and adjuvant were mixed by sonication immediately prior to injection.

Western blot assays utilizing the enhanced chemiluminescence technique were performed with *S. vulgaris* L_3 surface antigens extracted from freshly-exsheathed L_3, or from L_3 cultured *in vitro* for four different timepoints: 24 hours; 48 hours; 72 hours; or 96 hours. Freshly-exsheathed L_3 were placed into media composed of PBS (pH 7.2), 2.4 g L^{-1} dextrose (Difco), antibiotics (sodium penicillin G: 400 U ml^{-1}; streptomycin sulfate: 400 μg ml^{-1}; amphotericin B: 1 μg ml^{-1}; gentamicin sulfate: 50 μg ml^{-1} (Gibco, Grand Island, NY), 10 mM HEPES, with 4 μg ml^{-1} aprotinin (Sigma Chemical Co., St. Louis, MO) and 1 U ml^{-1} $alpha_2$ macroglobulin (Sigma) as protease inhibitors. Larvae were cultured in this media for eight hours at 37° C to collect E/S, after which the surface antigens were extracted.

Surface antigens were extracted using a modification of a protocol by Pritchard (Pritchard *et al.*, 1986) with a detergent solution composed of 50 mM tris (pH 8.0), 0.25% cetyl trimethylammonium bromide (CTAB, Boehringer Mannheim Biochemicals, Indianapolis IN), with 1 mM phenylmethylsulfonyl fluoride (PMSF, Boehringer Mannheim) and 5 mM

ethylenediamine tetraacetic acid (EDTA, Sigma), as protease inhibitors, and 0.02% sodium azide. Larvae were incubated at 37° C for four hours with occasional agitation, then centrifuged, and the detergent extract removed. Both pellet and extract were mixed 1:1 with 2X SDS sample buffer with 2-mercaptoethanol, boiled for 6 minutes and frozen until use. Larvae for extraction of surface proteins at specified *in vitro* timepoints were removed from culture and washed 4X with sterile E/S collection media to remove bovine serum. These larvae were cultured for eight hours in E/S collection buffer as described above, centrifuged and E/S products were removed and frozen. The surface antigens of these larvae were solubilized as described above.

Hematology - Blood drawn weekly for hematology was analysed in a routine fashion by the Clinical Pathology Laboratory at the L.S.U. School of Veterinary Medicine. Total white blood cell counts (WBC) were analyzed using a Baker 9000 analyzer (Serono-Baker, Allentown, PA). Total protein (TP) and heat precipitated protein (HPP) were determined manually with a refractometer. Differentials on blood smears were determined by counts of 100 cells examined on oil immersion.

Gross Lesion Evaluation - Necropsy examinations were performed on all immunized ponies. Any lesions were recorded and tissue samples fixed in buffered 10% formalin for histological examination. The CMA and its major branches were dissected intact from the cecum and large colon for evaluation in the laboratory.

The peritoneal cavity was opened and the quantity and characteristics of fluid present were noted. The serosal surfaces of the organs were examined for lesions. The pleural cavity was

opened and the heart and lungs were removed and examined. The aorta was severed near the heart and stripped from its attachment to the body wall. The diaphragm was cut and the visceral organs removed intact from the peritoneal cavity. The aorta was removed from its attachment to the body wall and severed near the caudal mesenteric artery. The aorta and arteries remained with their associated organs.

The liver, kidneys and spleen were removed and examined for lesions. The pyloris and duodenum were ligated with string, the duodenum severed, and the stomach was opened and examined for lesions. The small intestine was stripped from the mesentery and ligated near the ileo-cecal-colic valve. The small intestine was examined for serosal lesions such as hemomalasmae ilei, which were recorded, and the small intestine was opened. Lesions or representative tissue samples of the small intestine were taken at this time.

The arteries of the cecum and colon were examined *in situ*, then removed intact with the caudal aorta and CMA for evaluation in the laboratory as well as recovery of challenge larvae. The cecum and colon were examined for serosal lesions, then opened. Representative tissue samples were taken as well as any lesions found.

Arterial Dissections - The caudal aorta, the CMA and its branches were opened in the laboratory and evaluated for changes secondary to challenge larval migration. The range of lesion scores was from parasite-free (0) to severe (4). The lesion categories were: subintimal tracts from larval migration; thrombus formation in the arterial lumen; thickening of the arterial wall; and aneurysm formation, or vessel dilation.

The scoring of tracts due to larval migration was 0 if the endothelial surface was smooth and glistening; 1 if a few, distinct tracts were visible; 2 if the tracts coalesced in some areas; 3 if tracts were diffuse and coalesced, but did not cover the entire lumen surface; and 4 if the tracts coalesced and covered the entire surface of the lumen.

Thrombosis of the vessel was scored as 0 if the endothelial surface was free of lesions and was smooth and glistening; 1 if there were a few, focal thrombi present but were small; 2 if the thrombi present were multifocal but small; 3 if the thrombi were diffuse and some were large; and 4 if the thrombi were large enough to threaten the vessel with occlusion.

Thickening of the arterial wall was scored as 0 if the vessel wall was flexible and free of lesions as in a parasite-free animal; 1 if there was some focal thickening but this was easily cut with scissors; 2 if the thickening was diffuse or for the entire length of the vessel but was still easily cut with scissors; 3 if the thickening had become more fibrous, causing the vessel wall to lose its pliability and become difficult to cut with scissors; and 4 if the vessel wall had become thickened and rigid and calcified such that it would crunch when cut with scissors.

Aneurysm formation or vessel dilation was scored as 0 if the lumen of the artery was continuous as in a parasite-free animal; 1 if there were small areas of dilation; 2 if these areas had developed into distinct pockets or diverticuli; 3 if there were numerous pockets and overall lumen dilation due to the diverticuli; 4 if these diverticuli had coalesced into a large cavernous aneurysm.

The criteria used in this evaluation are identical to those previously described for evaluation of gross lesions, scoring of the CMA changes, and recovery of larvae from dissections of the CMA and major branches (Klei *et al.*, 1980), but the numerical description has been dropped in this study in favor of the verbal descriptions of parasite-free, mild, moderate or severe due to the author's preference.

Larval Recoveries - Following dissection and evaluation of lesions, the lumen of the CMA and its branches were scraped to remove the intima and any thrombi present. Larvae were recovered from these scrapings in saline under a dissecting microscope. Percent protection was calculated by the following formula: [1-(the total number of larvae recovered from arteries of an immunization group/the total number of larvae recovered from arteries of Controls) X 100].

Histological Evaluation - Tissue samples were taken routinely from the ICC, cecal tip, ventral colon, liver, as well as any lesion found. Tissues were fixed in buffered 10% formalin, embedded in parafin, processed and stained with hematoxylin and eosin, by the Histology Laboratory in the Department of Pathology, L.S.U. School of Veterinary Medicine. Tissue sections were examined with conventional light microscopy.

Enzyme-Linked Immunosorbent Assay - The circulating antibody response to larval and adult *S. vulgaris* antigens was monitored by enzyme-linked immunosorbent assay (ELISA) using serum collected weekly during the immunization and challenge periods. Soluble antigens which were prepared for the immunizations were diluted in 0.01 M carbonate buffer (pH 9.6) to a protein concentration of 10 μg ml^{-1}. These preparations, in 50 μl

volumes, were added to wells of 96-well, flat-bottom, polystyrene microtiter plates (Dynatech Industries, Alexandria, Virginia) and incubated overnight at 37° C. Eight wells were incubated with carbonate buffer alone for use as negative controls. Following overnight fixation, plates were washed with PBS, and non-specific protein binding sites were blocked using 250 μl well^{-1} PBS - 0.05% Tween 20 for 4 hours at room temperature. After blocking, plates were dried, sealed, and stored at -20° C until used.

Weekly serum samples from individual ponies were analyzed in triplicate. A high-responder serum sample served as a standard positive control for each plate. Sera were diluted 1:100 in PBS - 0.05% Tween - 3% bovine serum albumin (BSA). Serum dilutions were added to appropriate wells in volumes of 50 μl well^{-1}, and plates were incubated at room temperature at least 2 hours. Following incubation, plates were washed 3X with PBS - 0.05% Tween.

Affinity-purified, alkaline phosphatase-labeled goat anti-horse IgG (heavy and light chain-specific, Kirkegaard and Perry Laboratories, Gaithersburg, Maryland) was diluted 1:200 in PBS - 0.05% T - 3% BSA. Quantities of 50 μl well^{-1} were added to appropriate wells and incubated at least 2 hours. Following incubation, plates were washed 3X with PBS - 0.05% T. As substrate, *p*-nitrophenylphosphate (*p*-NPP) in diethanolamine buffer (Kirkegaard and Perry Laboratories) was used at 25 μl well^{-1}. Following development, the reaction was stopped with 100 mM EDTA. Optical density (OD) was recorded using a Dynatech MR 700 automated microtiter plate reader (Dynatech Industries) with absorbance set at 410 nm.

Optical density values for samples were equilibrated by standardizing the OD values for the identical positive control serum used on all plates. This was performed to compensate for

minor variations in timing of the assays and temperature variations of incubation which were experienced by individual plates. The highest OD value recorded for the standard positive control of all plates was divided by OD value of the standard positive control OD for each individual plate. Test serum ODs were then multiplied by the equilibration factor calculated for that plate. Mean OD values and standard error were then calculated from these adjusted values. As an example, if the highest OD value for standard positives of the twelve plates was 1.600 and the standard positive value for a second plate was 1.459, then all OD values for this second plate were elevated by a factor of 1.600/1.459, or by 1.096.

Protein Electrophoresis - *Strongylus vulgaris* antigens were separated on preparative 8 - 20% polyacrylamide gradient gels using a discontinuous sodium dodecylsulfate polyacrylamide gel (SDS-PAGE) system. The acrylamide concentration in the stacking gel was 4%. The ratio of acrylamide to bisacrylamide was 30:1 for all gels. High and low molecular weight prestained markers (Bethesda Research Laboratories, Gaithersburg, Maryland) were used for molecular weight estimation. Samples to be separated were mixed 1:1 with sample buffer for a final concentration of 1% SDS, 10% glycerol, 60 mM tris (pH 6.8), 5% 2-mercaptoethanol, and 0.005% bromphenol blue.

Following separation, proteins were transferred to nitrocellulose paper (Bio-Blot, Costar, Cambridge, MA), .45 μm pore size), in a Tris-Glycine-Methanol buffer (25 mM Tris, 190 mM Glycine, 20% methanol), or Tris-Glycine without methanol. Following transfer, nitrocellulose papers were rinsed in distilled water to remove the transfer buffer, dried in a vacuum chamber overnight at 37° C, sealed in plastic wrap and stored at 4° C until used.

Western Blot - Western blots were performed using *S. vulgaris* antigens on nitrocellulose sheets using either pooled or individual sera collected at several timepoints during the course of immunization and challenge. Western blots were performed on *S. vulgaris* adult or larval somatic antigens using alkaline phosphatase-labeled secondary antibodies. For the ECL Western blots of detergent extracts of larval surfaces, horseradish peroxidase-labeled secondary antibodies were used. Western blotting using the ECL method has theoretical sensitivity equal to radioiodination without the complications of radioisotopes. The fluorescent reaction occurs when peroxidase catalyzes the oxidation of a cyclic diacylhydrazide which emits light when decaying to the ground state. This technique has the added advantage that the antibody recognition is recorded on film rather than as permanent color stain of conventional Western blots. This permits the primary and secondary antibodies to be stripped from the nitrocellulose and the sheet can be used again.

Nitrocellulose sheets for Western blots were processed in two ways depending on the secondary antibody enzyme/substrate system. For the alkaline phosphotase reactions, nitrocellulose sheets with transferred antigens were cut into 5 mm strips which were then placed into individual troughs of a plexiglass incubation tray. For the ECL Western blots using horseradish peroxidase luminescent reactions, entire sheets were placed into a multichannel plexiglass miniblotter (Immunetics, Cambridge, MA).

Solutions for blocking nonspecific binding sites on the nitrocellulose, dilution of antibodies, and wash steps were prepared with consideration of the enzyme/substrate of the secondary antibodies. Alkaline phosphatase-labeled antibody assays were performed using Tris-buffered saline (TBS; 25 mM Tris, 140 mM sodium chloride, 2.7 mM potassium chloride, pH 8.0),

with 0.02% sodium azide as preservative. Blocking and antibody dilutions were performed using TBS and 20% fetal calf serum. Washes were performed with TBS. Western blots using HRP-labelled antibodies were performed using phosphate-buffered saline (PBS) and thymersol as preservative in all steps. Blocking and antibody dilutions were performed with PBS, 5% dried milk powder, and 0.2% Tween 20. Washes were performed with PBS - 0.2% Tween 20. Initial washes were performed with 1X PBS, and the final two washes following secondary HRP-labeled antibody incubations were performed with 2X PBS - Tween 20.

Frozen aliquots of sera collected weekly during the course of the experiment were thawed and diluted 1:100 using PBS, 1% BSA, and either sodium azide or thymersol as preservative. Nitrocellulose strips or sheets were incubated with serum dilutions for at least one hour on a rocking platform at room temperature. Following incubation, nitrocellulose strips or sheets were washed four times with the appropriate wash buffer for 5 minutes per wash.

Secondary antibodies were then applied to the nitrocellulose and incubated for one hour at room temperature on a rocking platform. Affinity-purified, Fc specific, alkaline phosphatase-labeled goat anti-horse IgG secondary antibodies were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). These antibodies were diluted 1:2000 with TBS, 1% BSA and sodium azide as preservative. Affinity-purified, horseradish peroxidase-labeled goat anti-horse were obtained from two sources: anti-IgG Heavy and Light chain-specific antibodies were obtained from Kirkegaard and Perry Laboratories; anti-IgG Fc fragment-specific antibodies were obtained from Jackson Immunoresearch Laboratories,

West Grove, PA. HRP-labelled antibodies were diluted 1:2000 with azide-free PBS, 1% BSA, 0.2% Tween 20, and thymersol as preservative. Following incubation with secondary antibodies, the nitrocellulose was washed five times for five minutes per wash, the first two washes with either 2X PBS or TBS containing 0.2% Tween 20. The final three washes were performed with 2X PBS or TBS.

Alkaline phosphatase substrate was 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitroblue tetrazolium (NBT) in 0.1 M Tris buffer (Kirkegaard and Perry Laboratories). LumiGLO™ (Kirkegaard and Perry Laboratories) was used as the substrate for ECL Western blots as directed. Due to high background and very rapid overdevelopment, this solution was diluted 1:1 with distilled water to slow and lower the background of the reaction. Luminescence reactions were recorded with XAR-5 film (Eastman Kodak Co., Rochester, NY).

Nitrocellulose sheets were stripped of primary and secondary antibodies after development of the ECL Western blots by immersing the sheets in 60 mM Tris buffer (pH 8.0) with 100 mM 2-mercaptoethanol for 30 minutes at 50° C. Following the stripping, sheets were washed twice with 2X PBS buffer and either dried as previously described or placed immediately into blocking solution overnight for a repeat of the ECL procedure.

Indirect Fluorescent Antibody Test - Antibody recognition of the *S. vulgaris* larval surface by the four immunization groups was examined using an indirect fluorescent antibody test (IFAT) using serum collected at different timepoints during the immunization experiment: post-immunization/pre-challenge; two weeks post-challenge; four weeks post-challenge, and

at necropsy. *Strongylus vulgaris* larvae were tested as freshly exsheathed L_3, L_3 cultured *in vitro* overnight, or for two days, and as L_4. Larvae were exsheathed and cultured using methods described above. *Strongylus edentatus* larvae were tested as freshly exsheathed L_3 or as L_4 from *in vitro* culture as described above.

Preliminary assays were performed to ascertain the dilutions and techniques best suited for the assay. The dilutions for the assay were chosen to provide a transition between a clear positive and negative, and be performed in one plate. Initial trials using tenfold and fivefold serum dilutions gave endpoints which were too abrupt and overlapped. Twofold dilutions were not adequate for a single plate since the range of endpoints carried beyond the range of seven wells in a column. Threefold dilutions were used as a compromise. The end point was decided at the serum dilution for which 50% of the larvae were negative for fluorescence.

A 3-fold serial dilution of pooled sera was generated in flat-bottom polystyrene microtiter plates (Dynatech Industries). A larval suspension in PBS-1% BSA was made at an approximate concentration of 5 L_3 μl^{-1} and was added to all wells in 3 μl volumes such that each well contained between 10 - 20 L_3. Larvae were incubated one hour in serum dilutions at room temperature on an orbital shaker. Wells were then washed 3X with PBS, allowing the L_3 to settle for 5 minutes between washes. PBS wash was gently aspirated using a multichannel pipetor leaving approximately 50 μl residual volume in each well with the larvae.

Affinity-purified, fluorescein-labeled goat anti-horse IgG (H+L chain specific, Kirkegaard and Perry Laboratories) as a 1:15 dilution with PBS - 1% BSA was added to appropriate

wells in 25 μl volumes and plates were incubated one hour on the orbital shaker. Wells with larvae and serum alone were used as a negative control. Larvae were then washed 4X in PBS as above, and examined on a Nikon DIAPHOT-TMD inverted microscope equiped with epi-fluorescence (Nippon Kogaku, Tokyo, Japan). Positive wells were recorded as those with more than 50% of the intact larvae fluorescing above background seen in negative control wells. The condition of larvae could be verified by switching from epifluorescence to conventional phase contrast microscopy, and was essential for this verification. Degenerating larvae exhibit mild autofluorescence which is difficult to differentiate from weakly positive staining, and shed cuticles trap labeled antibody. These differences were noted and such artifacts not included in the count of stained larvae.

Surface-Associated Antibody - *Strongylus vulgaris* and *S. edentatus* freshly exsheathed L_3 and L_4 from *in vitro* culture were incubated in flat-bottom 96-well Immulon microtiter plates (Dynatech) in a 1:100 dilution of immune serum and stained with fluoroscein-labeled antibody as described above. Larvae were cultured in media at either 37° C, 20° C, or 4° C and examined at various timepoints during a 24-hour period to determine the loss of fluorescent label. One culture at 37° C contained media with sodium azide to test the dependence of antibody loss on larval viability.

RESULTS

Clinical Signs - Following immunization and challenge, Adult recipients experienced the most febrile days while the Irradiated recipients experienced the least. Larval and Control recipients experienced similar numbers of post-challenge febrile episodes (Table 1). An initial febrile episode was recorded for all experimental ponies within 48 hours of challenge. The post-challenge febrile response experienced by Adult, Larval and Control ponies was biphasic (Figure 1), and coincided temporally with the penetration, molt and migration of infective *S. vulgaris* larvae. The timing of the second febrile peak by the Adult, Larval and Control groups coincided with the molt and migration of L_4 within arterioles of the intestinal submucosa, migrating craniad towards the root of the CMA. The Irradiated immunization group did not experience this second febrile peak, instead, maintained a group mean rectal temperature within the normal, pre-experimental range for the duration of the challenge period.

Adult and Larval recipients were often anorexic and visibly lost body condition compared to Irradiated recipients or Control ponies, all of which were in good condition at the end of the challenge period. Weights were not taken during this study because of the potential complications of moving experimental animals from the parasite-free environment to the scales. Loss of body condition, while a vague term, was assessed as such based on the visible and palpable loss of skeletal muscle mass which became notable due to the prominence of the spinous processes of the vertebrae as well as the protrusion of the spines of the scapulae. These landmarks became palpable and subsequently visible in ponies which

Table 1. Post-challenge febrile days.

	Immunization Group			
Temperature Range (°C)	Irradiated	Adult	Larval	Control
Non-Febrile Days (≤38.8°)	40.3 ± 0.6[1]	20.0 ± 10.7	33.0 ± 3.0	33.6 ± 2.5
Total Febrile Days (>38.8° C)	1.7 ± 0.6[2]	22.0 ± 13.0[3]	9.0 ± 3.0	8.3 ± 2.5
Febrile Days> 40.0°	0.3 ± 0.6	1.7 ± 1.5	1.0 ± 1.0	0.3 ± 0.6

[1] Mean number of days ± standard error.
[2] ($p < 0.05$) using Scheffe's test of multiple pairwise comparisons to Adult, Larval and Control means.
[3] ($p < 0.05$) using Scheffe's test of multiple pairwise comparisons to Irradiated, Larval and Control means.

For 10 days prior to immunization, rectal temperatures for all experimental ponies were recorded. The mean rectal temperature for this period was 38.2° C ± 0.3° C. A febrile day was recorded if a pony had a morning rectal temperature above the pre-immunization mean plus two times the standard error. Statistical analysis was performed using a general linear model utilizing Scheffe's test of multiple pair-wise comparisons (SAS Institute Inc., Carey, North Carolina).

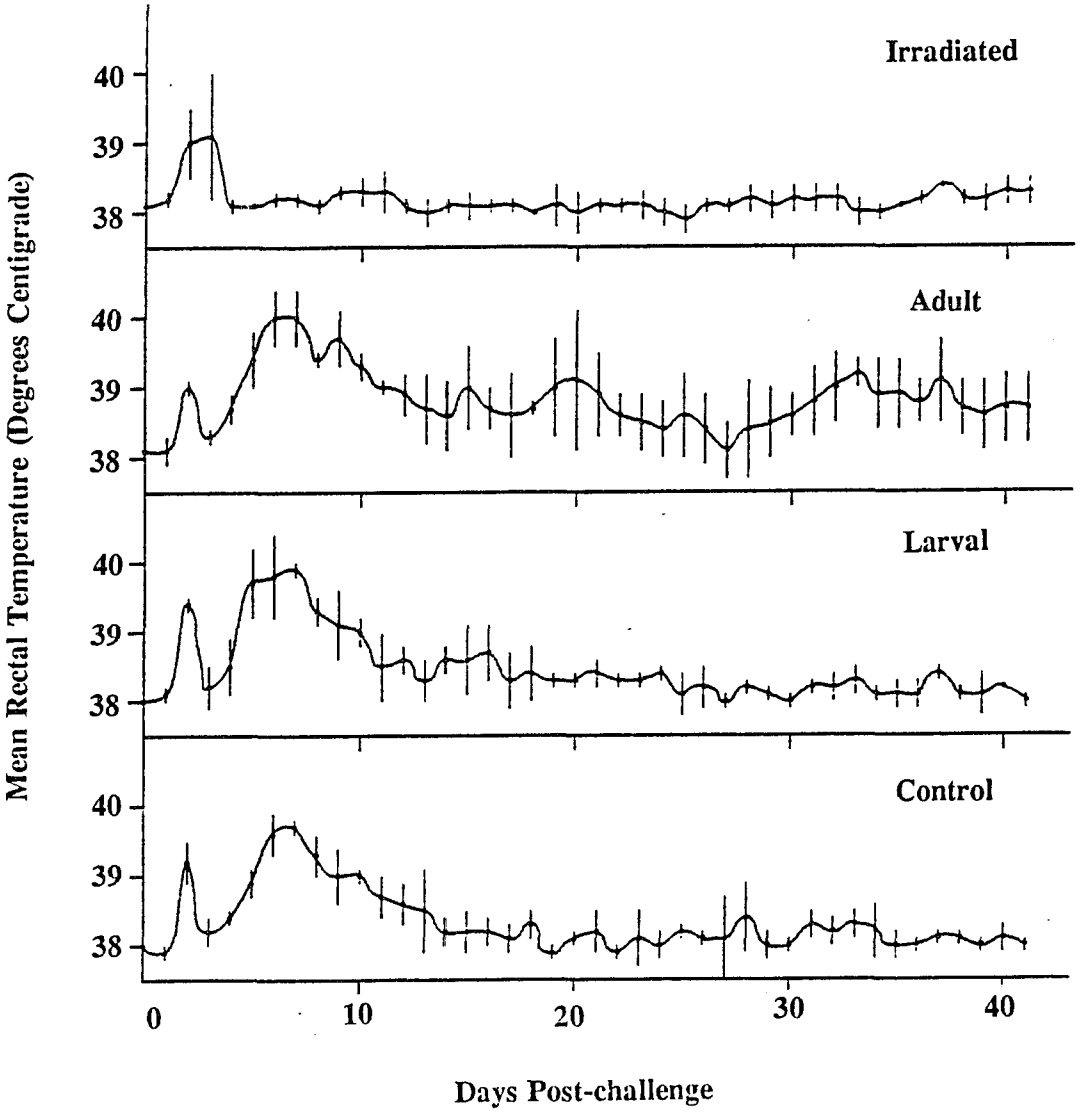


Figure 1. Post-challenge febrile episodes experienced by immunization groups. Temperatures represent group mean rectal temperatures taken during morning observations. Irradiated recipients experience a single febrile period post-challenge while Adult and Larval immunized ponies and Controls experienced a biphasic response. The initial febrile episode follows penetration of the intestinal mucosa by the L_3 and the second febrile episode corresponds temporally with the onset of migration by the L_4.

received the soluble homogenate immunizations but not so in any of the Controls nor the Irradiated recipients.

White Blood Cell Counts - Following challenge, the Irradiated, Adult and Larval groups developed elevated WBC counts ($>12{,}000$ cells μl^{-1}), peaking between 2 - 3 weeks post-challenge (Figure 2). Differentials on weekly blood smears revealed that an anamnestic eosinophilia accounted for a high percentage of the white count elevation seen in the Irradiated recipients during this period, up to 34% of circulating WBC were eosinophils (Figure 3).

Plasma Proteins - Plasma protein determinations from weekly hematological exams were performed for heat precipitated protein (HPP) (Table 2), and total protein (TP) (Table 3). Values considered normal for HPP in the Louisiana State University School of Veterinary Medicine Clinical Pathology Laboratory are less than 500 mg dl^{-1}; for TP from plasma, 5.2-7.8 g dl^{-1}. Rises in HPP and TP post-challenge are non-specific indicators of inflammation. Irradiated recipients maintained the lowest values for HPP throughout the challenge period during which time their total WBC counts rose and fell. Adult recipients maintained high HPP values and elevated WBC counts, indicative of a prolonged inflammatory response.

Gross Necropsy Findings - Irradiated recipients appeared parasite-free on gross evaluation of viscera at necropsy. Intact livers appeared normal, however, on cut section there were severe fibrotic cuffs which surrounded and accentuated the bile ducts and vessels. These fibrotic tracts appeared as irregular, ropy, white cords of increasing diameter which could be traced from the periphery to the hilus of the liver (Figure 4 A). Gross examination

Table 2. Total protein values pre- and post-challenge.

Group	Pre-Challenge Value[1]	High Value Post-Challenge	Low Value Post-Challenge	Number of Weeks with Elevated Values[2]
Irradiated	6.8 ± 0.2	7.1 ± 0.7	6.2 ± 0.0	0
Adult	5.9 ± 0.3	8.2 ± 0.5	5.9 ± 0.3	2
Larval	6.9 ± 0.1	8.1 ± 0.2	6.7 ± 0.1	2
Control	6.4 ± 0.1	7.5 ± 0.1	6.3 ± 0.1	0

[1] Recorded as g dl^{-1}.
[2] Above high normal value of 7.8 g dl^{-1} for the Clinical Pathology Laboratory in the Louisiana State University School of Veterinary Medicine.

Table 3. Heat precipitated protein values pre- and post-challenge.

Group	Pre-Challenge Value[1]	High Value Post-Challenge	Low Value Post-Challenge	Number of Weeks with Elevated Values[2]
Irradiated	367 ± 170	433 ± 125.0	300 ± 39.5	0
Adult	300 ± 81.6	766 ± 47.5	300 ± 81.6	4
Larval	367 ± 47.0	633 ± 47.5	367 ± 47.0	2
Control	200 ± 0.0	600 ± 81.6	200 ± 0.0	1

[1] Recorded as mg dl^{-1}. Mean value for all experimental ponies prior to challenge = 366.6 mg dl^{-1}; Standard error = 110.5 mg dl^{-1}.
[2] Values greater than the mean + 2X standard error.

Total plasma protein and heat precipitated protein values were determined manually by refractometer in the Clinical Pathology Laboratory at the Louisiana State University School of Veterinary Medicine.

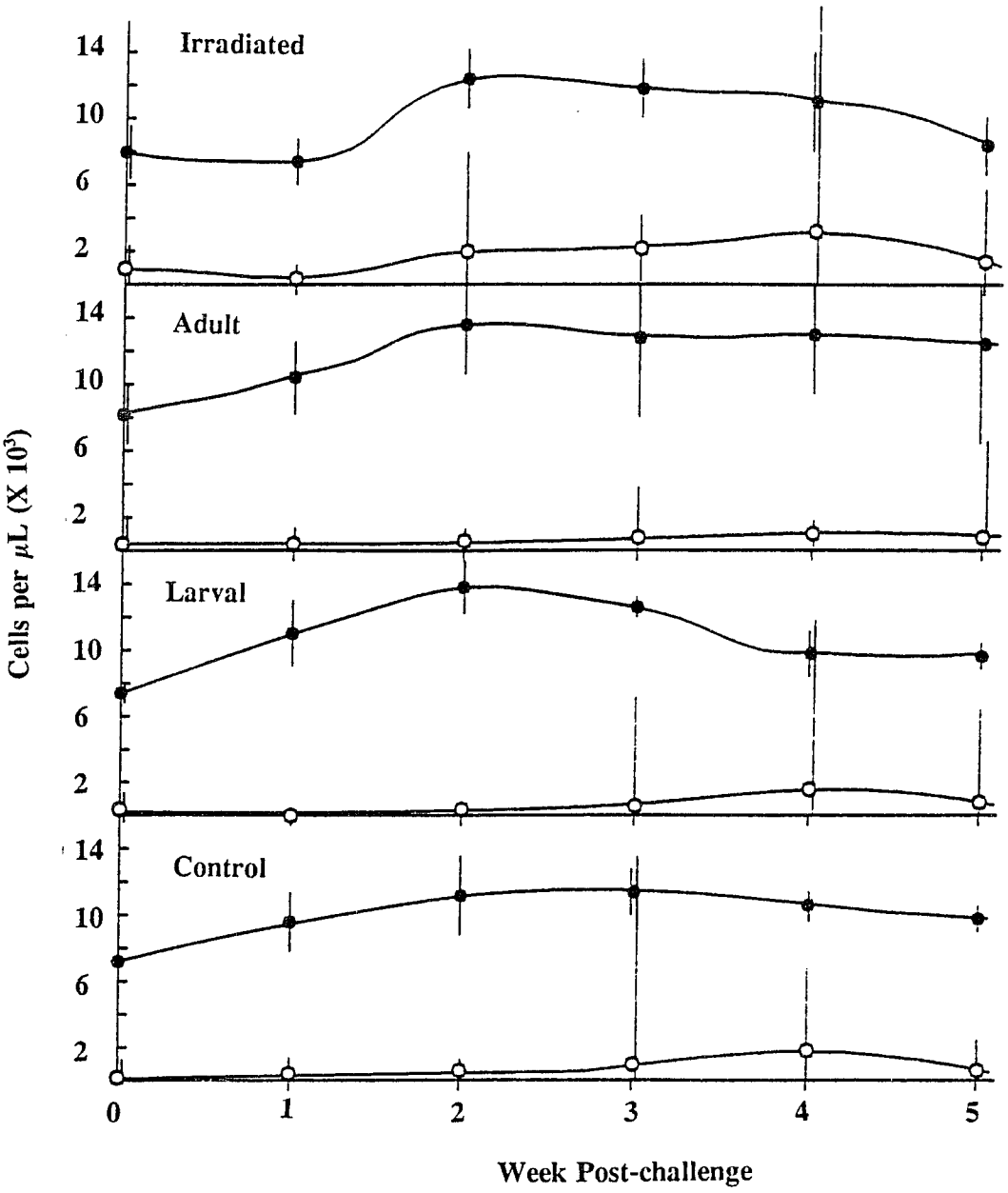


Figure 2. Post-challenge white blood cell and eosinophil counts. White blood cell values represent means of individual counts analyzed by a Baker 9000 automated counter. Eosinophil values were calculated by multiplying the total WBC count per microliter by the percent eosinophilia determined by manual count of 100 cells on blood smears examined by light microscopy on oil immersion. Cell counts and differentials were performed in the Louisiana State University School of Veterinary Medicine Clinical Pathology Laboratory. Closed circles represent WBC counts; open circles represent eosinophil counts.

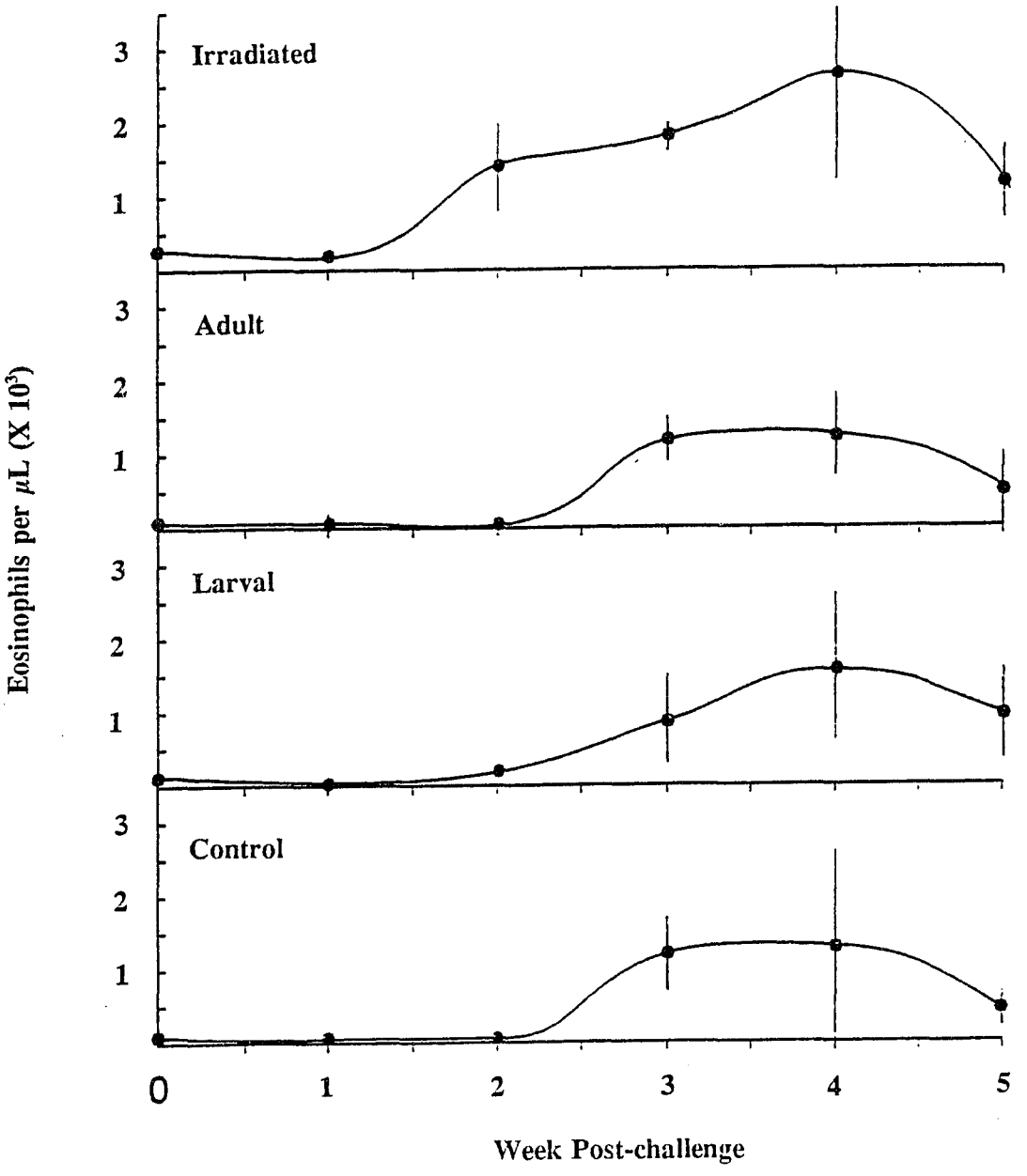
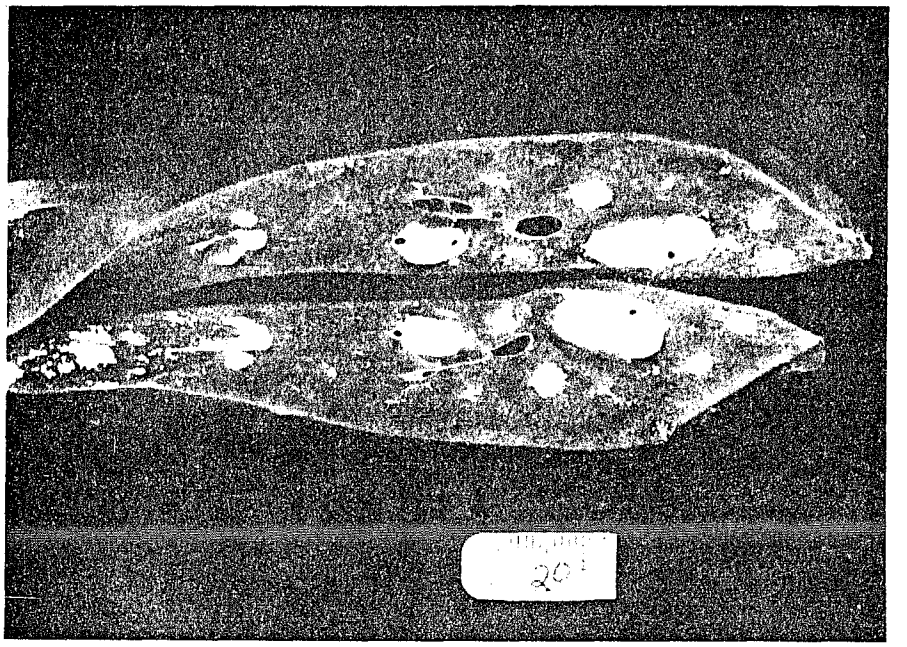


Figure 3. Post-challenge eosinophil counts. Eosinophil numbers represent means of individual eosinophils per microliter calculated by multiplying the total WBC count by the percent eosinophilia determined from a manual count of 100 cells on blood smears examined by light microscopy on oil immersion. Cell counts and differentials were performed in the Louisiana State University School of Veterinary Medicine Clinical Pathology Laboratory.

(A)



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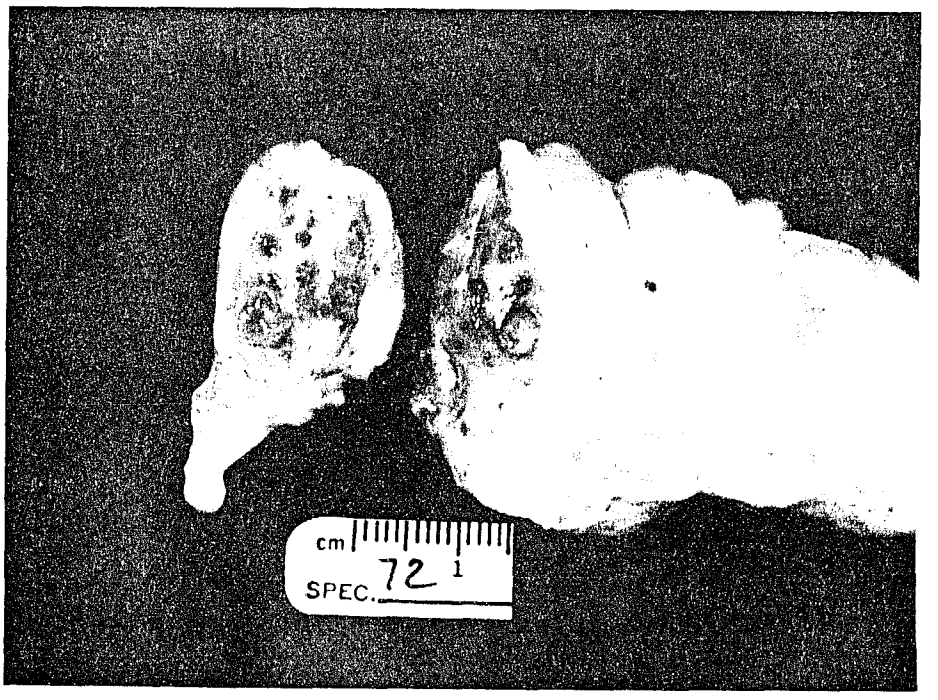


Figure 4. Gross lesions from necropsy exams. (A) Periportal fibrosis seen only in the livers of ponies immunized with Irradiated L_3. (B) Cross-section of the ventral colic artery demonstrating the perivascular fibrosis and edema of cecal and colic arteries seen in ponies immunized with Adult and Larval antigen preparations.

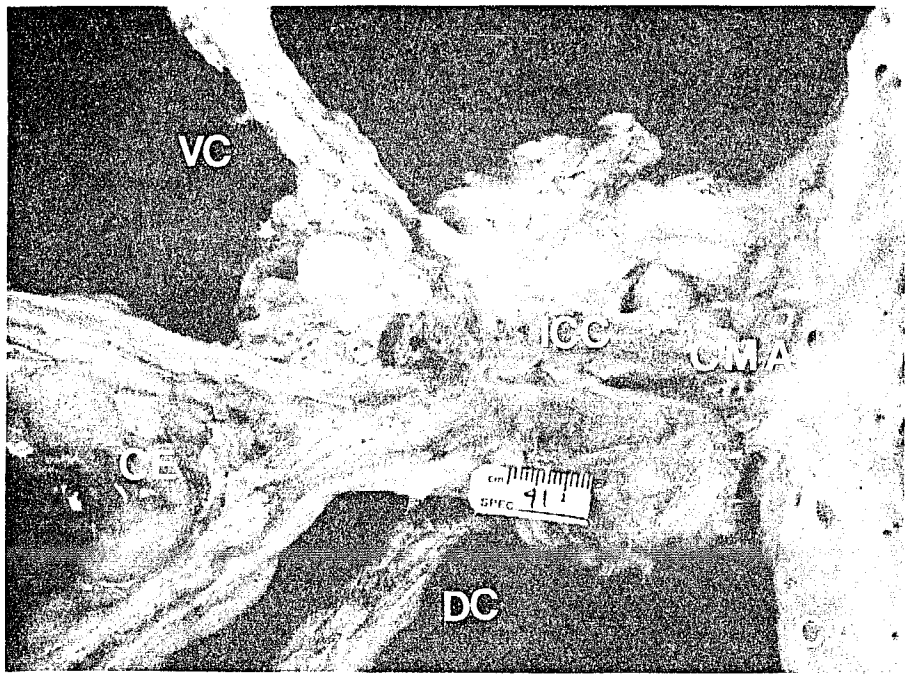
showed that the cecal and colic branches of the CMA taken from Irradiated recipients were free from visible lesions. The CMA was not thickened, and associated lymph nodes from the CMA and its branches were normal.

At necropsy, Adult and Larval recipients were notably thinner than Irradiated recipients or Controls, in both the loss of skeletal muscle mass mentioned above, and loss of peritoneal fat. The ventral colic and cecal arteries from Adult and Larval recipients were grossly thickened and edematous for the majority of their lengths (Figure 4 B). This thickening was diffuse and perivascular, and not limited to foci of larval development within the arterial wall. Lymph nodes associated with these arteries and with the CMA were greatly enlarged. The livers and other visceral organs, however, appeared normal.

Control ponies had few notable gross lesions. The cecal and colic arteries had several discreet nodules associated with developing larvae, but did not show the extensive perivascular edema seen in the Adult and Larval recipients. Lymph nodes associated with these arteries were slightly enlarged. The CMA was palpably thickened and firm, as were many of the associated CMA lymph nodes. Livers of Control ponies appeared normal.

Arterial Dissections - When opened, the CMA from Irradiated recipients had few arterial changes and appeared parasite-free (Figure 5 A). The most striking feature of these arteries was the abundance of larval tracts visible in the intima of the ileo-cecal-colic branch and which extended into the caudal aorta. These tracts were severe, but did not compromise the lumen of the arteries in any appreciable manner. There were minor, focal erosions and occasional tiny thrombi adhered to the intimal surface, which displayed mild hyperemia. The

(A)



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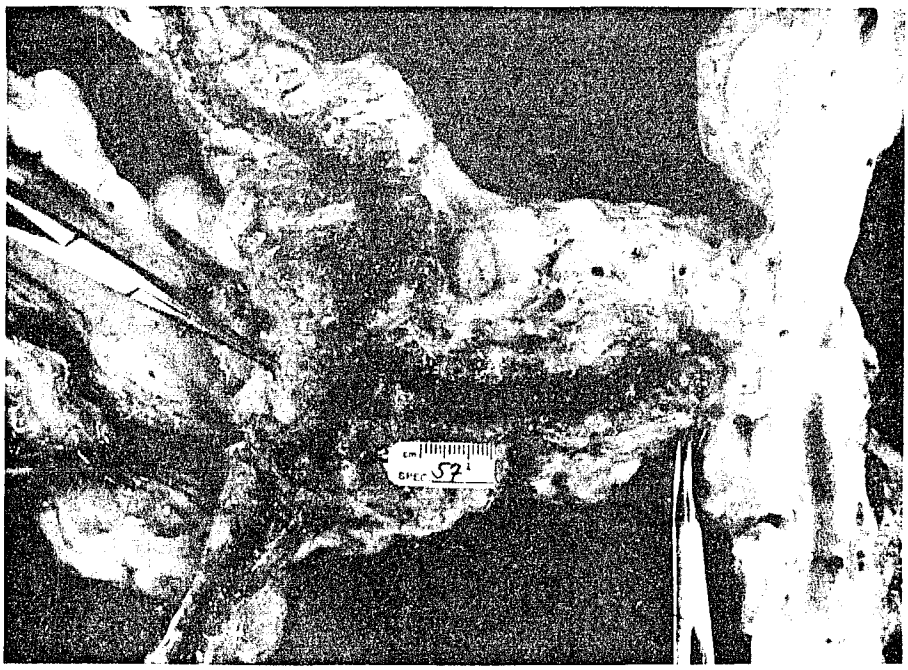


Figure 5. Comparison of cranial mesenteric artery dissections. (A) Dissection of an artery typical of ponies immunized with Irradiated L_3. There is mild hyperemia within the intima of the artery, but no visible thrombosis nor changes in the architecture of the arterial wall. (B) Artery dissection typical of ponies immunized with Adult or Larval antigen preparations and Control ponies. There is severe destruction of the intima with thrombus formation within the lumen of the artery, and destruction of the arterial wall with formation of diverticuli. CA - caudal aorta; CMA - cranial mesenteric artery; ICC - ileo-cecal-colic branch; VC - ventral colic branch; DC - dorsal colic branch; CE - cecal branches.

normal arterial architecture was intact, there were no diverticulations or verminous aneurysms present. No compromise of the lumen was apparent.

Arterial dissections from the Adult and Larval recipients and Controls displayed lesions typical of acute verminous arteritis. These changes included erosion of the intimal surfaces, thrombosis of many areas within the lumen of the CMA and its branches, diverticulation and fibrosis of the arterial walls, and numerous foci of abcessation (Figure 5 B). The prominent larval tracts so clearly visible in arteries of the Irradiated recipients could not be seen in any of these dissections due to complete destruction of the intima where such tracts would be located. Distal arteries from Adult and Larval recipients were different from Controls due to the severe, perivascular fibrosis and edema which extended the majority of the length of these arteries. Dissection revealed that the lumen and intima were intact through the majority of their length, except for discreet areas containing larvae. Changes typical of verminous arteritis were found in the intima and wall in these areas.

Larval Recoveries - Irradiated recipients demonstrated a 91.8% reduction of migrating larvae compared with Controls (Table 4). Adult and Larval recipients had larval recoveries similar to those of Controls. Larval recoveries had no statistical significance, however, due to the large standard error and small population number in each group.

Histological Lesions (Cranial Mesenteric Artery) - Two major differences were seen in histological sections of the CMA taken from each pony. Utilizing these histological differences, ponies could be characterized as either protected from arterial damage secondary to challenge (Irradiated recipients) or unprotected (Adult, Larval and Control

Table 4. Larval recoveries from cranial mesenteric artery dissections.

Immunization Group	Larvae Recovered[1]	Percent Protection[2]
Irradiated	7.0 ± 2.8	91.8
Adult	88.3 ± 30.1	-3.5
Larval	74.7 ± 13.9	12.5
Control	85.3 ± 53.3	0.0

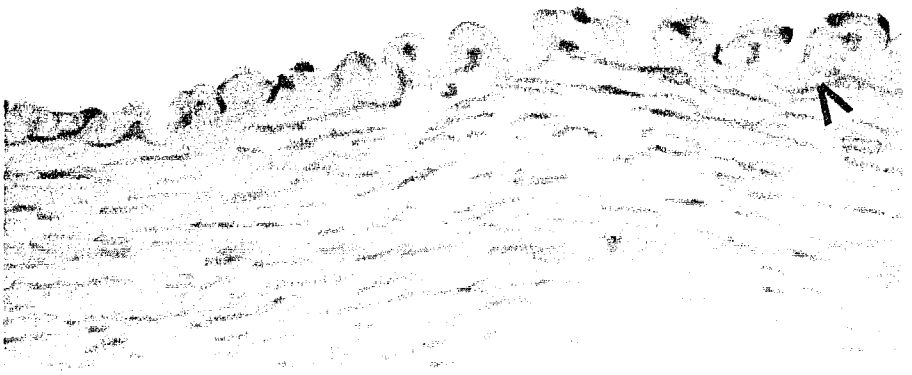
[1] Mean ± standard error.

[2] Percent protection was calculated by the formula [1-(total larvae recovered from arteries of immunization group/total larvae recovered from arteries of Controls) x 100].

ponies). Protected ponies retained normal arterial architecture with an intact intimal lining and inner elastic lamina. The smooth muscle of the media was organized and not infiltrated by an abundance of inflammatory cells nor prominent deposition of extracellular collagen (Figure 6 A). Those cells present were predominantly eosinophils. The adventitia was not fibrotic and thickened, as judged by the relative displacement of the associated nerve trunks, but there was a profound infiltration of eosinophils in the adventitia (Figure 7. A). Typically, eosinophils seen in any tissue of the protected ponies stained a dark grayish green with a faint or indistinct outline of the cytoplasmic granules (Figure 8 A). This staining was an identifying characteristic of histological sections taken from the Irradiated recipients.

Histological sections of the CMA from ponies characterized as unprotected (Adult, Larval and Control ponies) displayed severe destruction of normal arterial architecture. This included the loss of a demonstrable intimal lining and inner elastic lamina. These were replaced by granulation tissue occupying the lumen, and a lymphoplasmacytic infiltrate was seen within this granulation tissue as well as within the disorganized smooth muscle of the media (Figure 6 B). The adventitia was greatly fibrotic and thickened, and the associated nerve trunks were surrounded by granulation tissue. There was a mild cellular infiltrate in the adventitia, but few eosinophils (Figure 7 B). Few eosinophils were visible in the cellular infiltrate of the CMA, whether the intima, media or adventitia. Those present in these tissues demonstrated the characteristic eosinophil cytoplasmic granules with distinct outlines and which stained bright orange from the uptake of eosin dye (Figure 8 B). The cellular infiltrate in the granulation tissue of the intima and the smooth muscle of the media was characteristically lymphoplasmacytic (Figure 9 A).

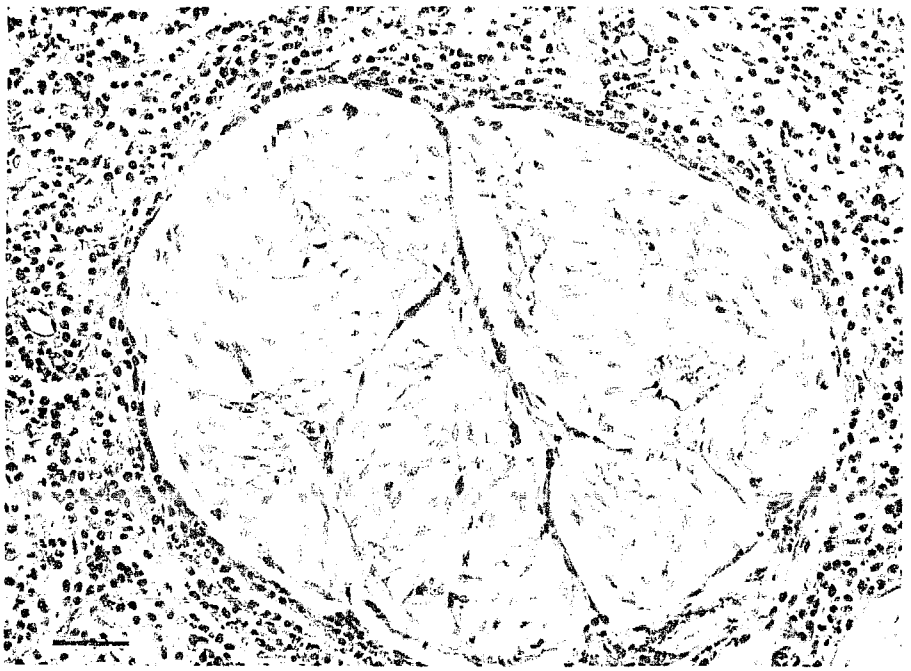
(A)



(B)

Figure 6. Histological sections of cranial mesenteric arteries. (A) Histological section representative of ponies immunized with Irradiated L_3. The smooth endothelial lining and the undulating, inner elastic lamina (open arrow) remain intact, as well as the organized smooth muscle cells of arterial wall. (B) Low magnification of a section of the cranial mesenteric artery representative of ponies immunized with Adult or Larval antigen preparations, or Control ponies. The endothelium and inner elastic lamina have been destroyed and replaced by granulation tissue. The organization of the smooth muscle of the arterial wall has been destroyed by an inflammatory cell infiltrate.

(A)



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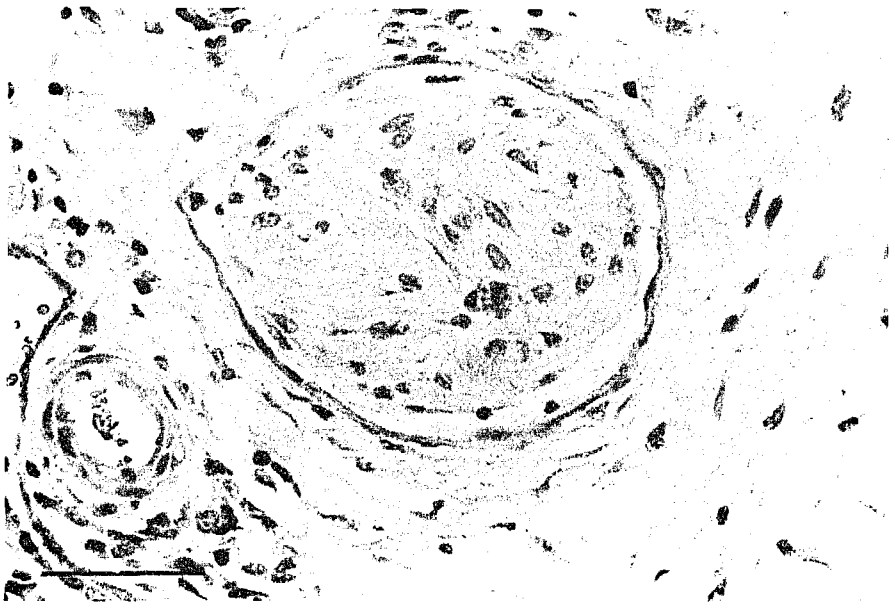


Figure 7. Comparison of the adventitia of the cranial mesenteric arteries. (A) Histological section typical of the adventitia of ponies immunized with Irradiated $_3$. There is a loose network of fibrous connective tissue with a severe eosinophil infiltrate. (B) Histological section typical of the adventitia of ponies immunized with Adult or Larval antigens, or Control ponies. The fibrous response surrounded the nerve bundles associated with the tunica adventitia and there was a minor cellular infiltrate seen focally. Scale bar represents 50 μm.

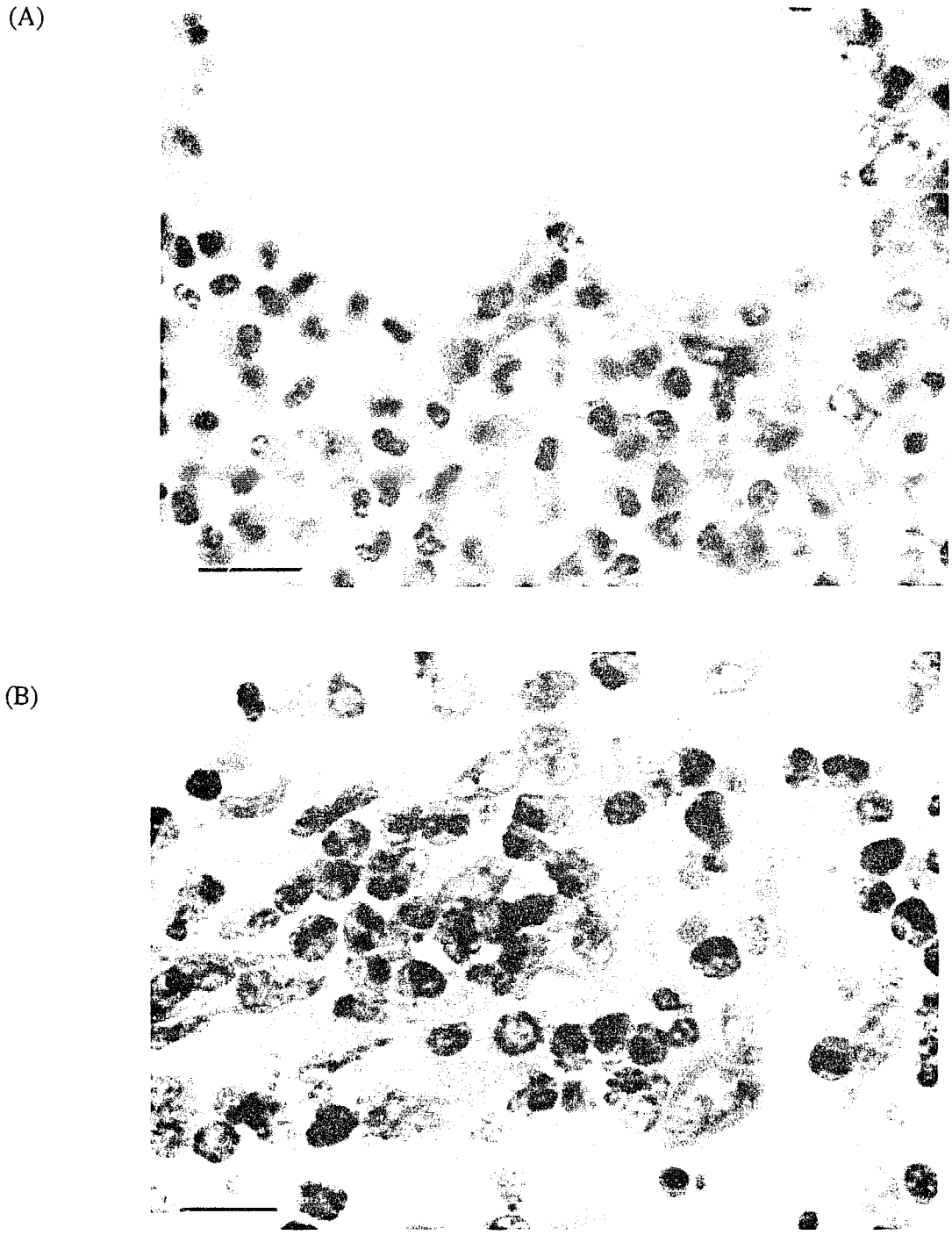
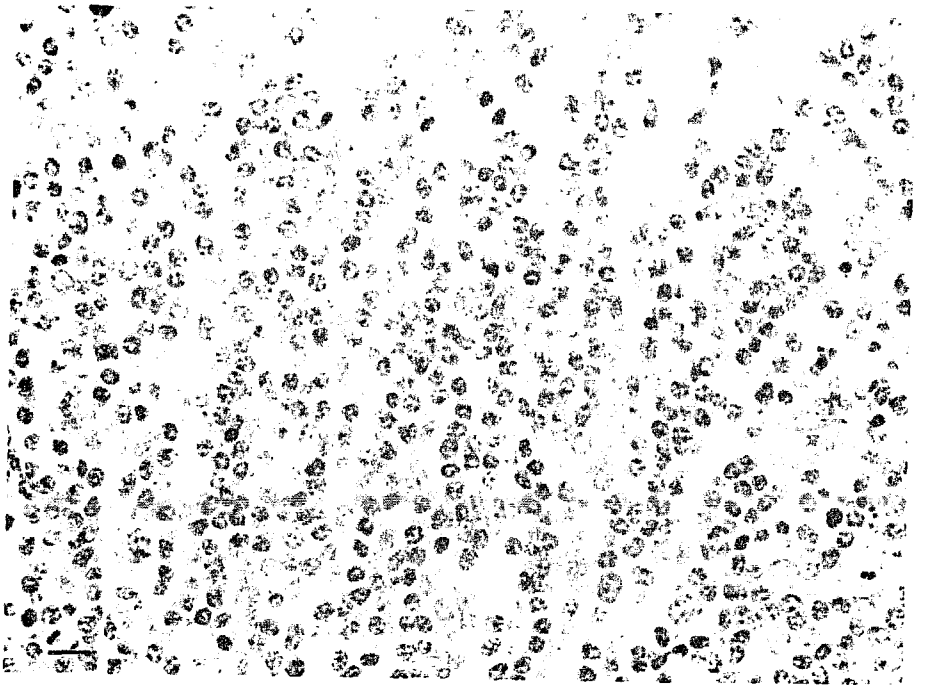


Figure 8. Comparison of eosinophil infiltrates. (A) Eosinophils in the adventitia from ponies immunized with Irradiated L_3. Cytoplasmic granules are indistinct and do not stain brightly with H&E stain. (B) Eosinophils within the granulation tissue of the adventitia from ponies immunized with Adult or Larval antigen preparations, or Control ponies. Cytoplasmic granules are distinct and stain brightly with H&E stain. Scale bar represents 15 μm.

(A)



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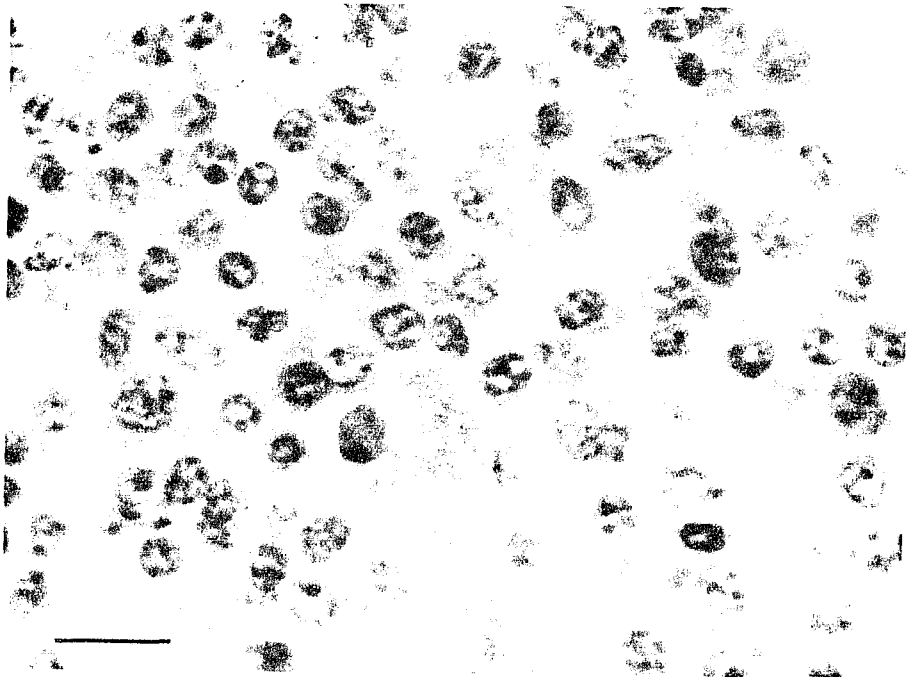


Figure 9. Lymphoplasmacytic infiltrate. Cellular infiltrate in the media of the cranial mesenteric artery charateristic of ponies immunized with Adult or Larval antigen preparations. Scale bar represents 15 μm.

Histological Lesions (Intestine) - The degree of eosinophilic infiltration seen in histological sections of the intestine was similar for all immunization groups, but the staining of those eosinophils was a differentiating characteristic. Sections examined from Irradiated recipients were distinguishable from sections from Adult, Larval and Control ponies based on the appearance of eosinophil cytoplasmic granules (Figure 10 A and B).

Histological Lesions (Liver) - The fibrous lesions visible grossly at necropsy in two of three Irradiated recipients consisted of severe fibrosis of portal areas with regions of lymphocytic infiltration, pronounced biliary hyperplasia and focal necrosis (Figure 11 A). These lesions were clearly delimited and did not invade adjacent parenchyma. Liver sections from Adult, Larval and Control ponies also displayed periportal changes which ranged from mild lymphocytic cuffing, to lymphocytic infiltration with fibrosis and biliary hyperplasia, but never exceeding the severity seen in the Irradiated group (Figure 11 B).

Enzyme-Linked Immunosorbent Assay - Values of ELISA ODs demonstrated that Adult and Larval recipients developed high concentrations of antibody to their respective antigen preparations following immunization (Figures 12 and 13). Larval recipients developed lower ELISA ODs to adult antigens than did Adult recipients to larval antigens. Irradiated recipients produced ELISA OD values to larval antigens which were intermediate to the levels produced by the Larval and Adult recipients but Irradiated recipients produced a weak response against adult antigens. Following challenge, ELISA OD values for all groups against larval antigens rose and converged. Against the adult antigen preparations, the OD values of the Adult and Larval recipients rose and converged while OD values of the Irradiated recipients and Controls remained low.

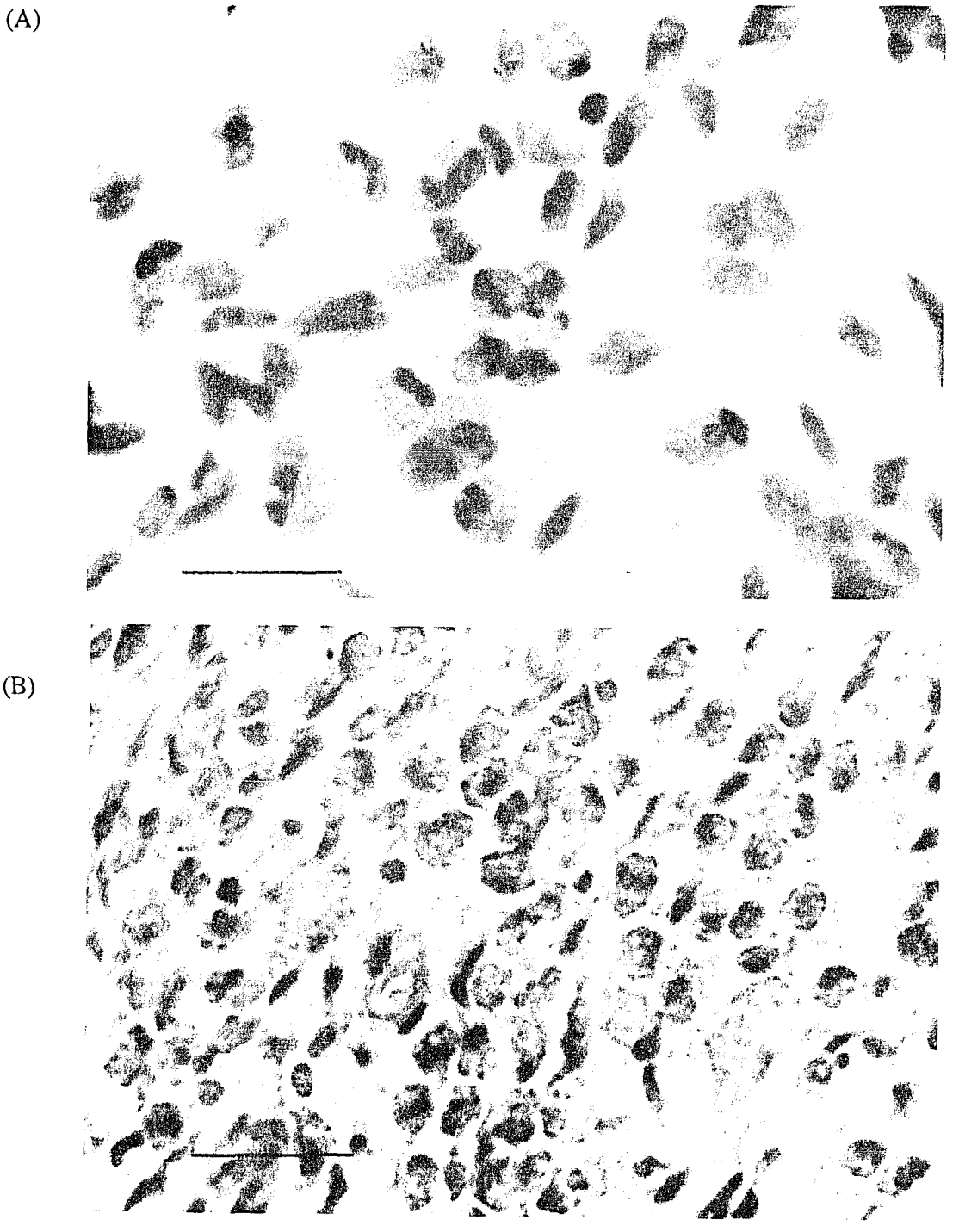
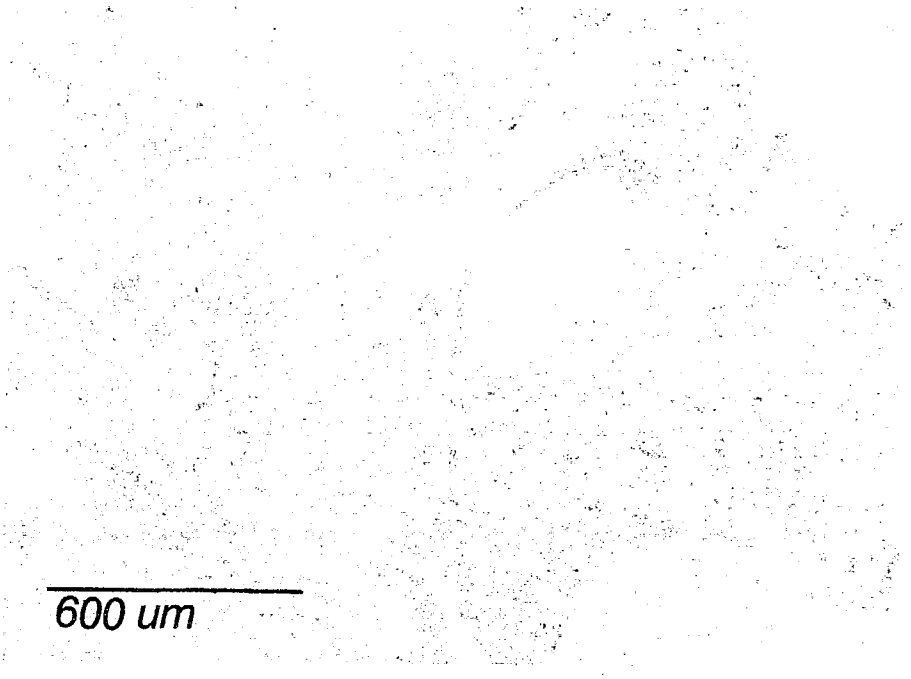


Figure 10. Comparison of eosinophils in the intestinal epithelia and submucosa. The relative cellularity of the infiltrates was not identifiably different, but the staining and morphology of the cytoplasmic granules were differentiating characteristics. (A) The eosinophils of Irradiated L_3 recipients characteristically demonstrated indistinct cytoplasmic granules. (B) Eosinophils typical of ponies immunized with Adult or Larval antigens, or Control ponies, demonstrated cytoplasmic granules which stained brightly and had distinct outlines. Scale bar represents 15 μm.

(A)



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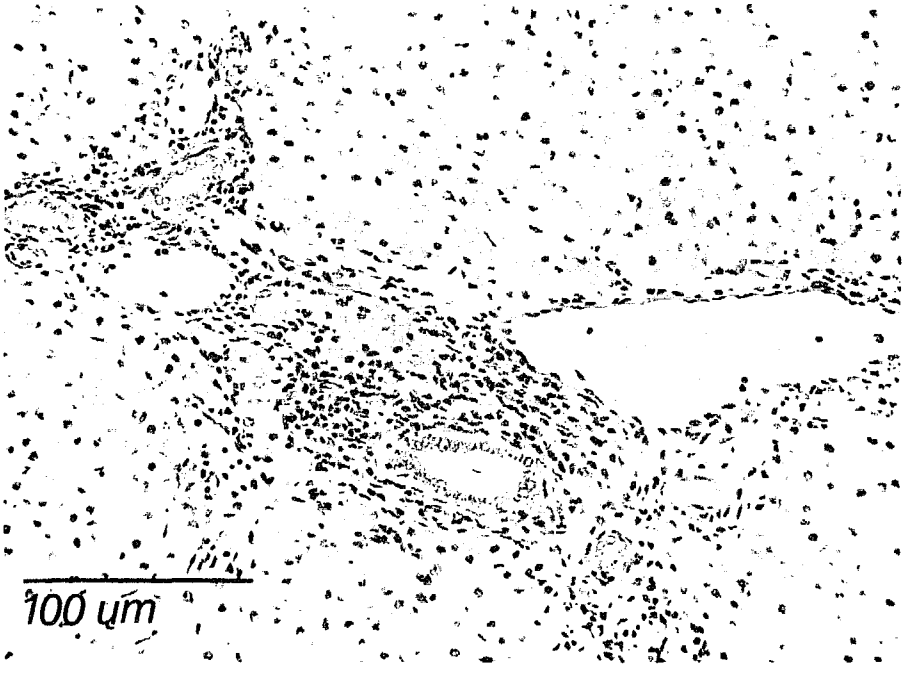

Figure 11. Histological sections of hepatic lesions. (A) Severe periportal fibrosis seen in livers of ponies immunized with radiation-attenuated larvae. (B) Periportal cuffing of lymphocytes typically seen in livers of ponies immunized with Adult or Larval antigen preparations, or Control ponies.

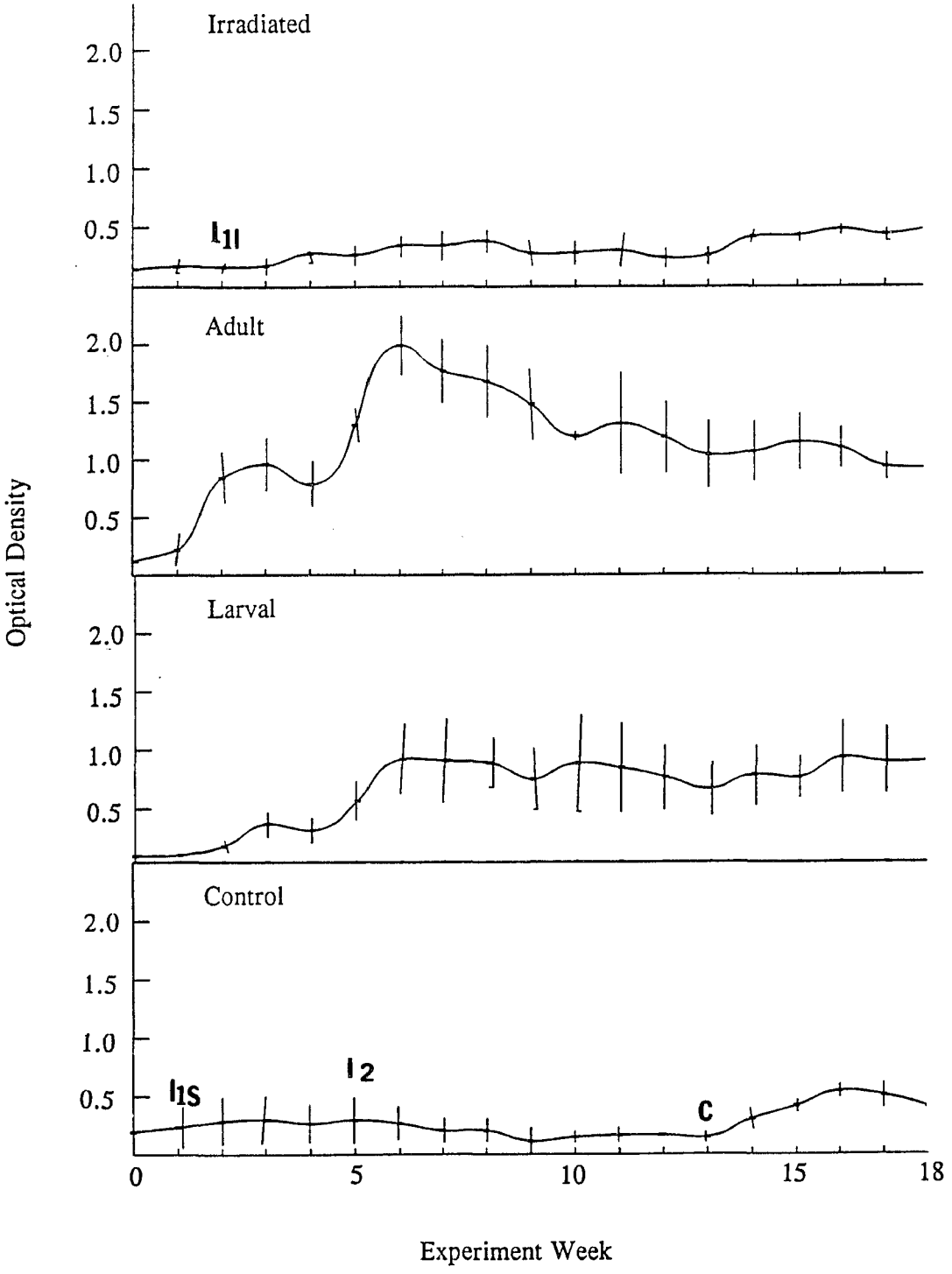

Figure 12. ELISA optical density values: Adult antigens. Values represent group means of individual serum samples analyzed in triplicate. Adult antigen used in these assays were identical to the antigen preparation used for immunizations and Western blots. I_{1S} - first immunization for soluble antigen recipients and Controls; I_{II} - first immunization for Irradiated recipients;I_2 - second immunization for all immunization groups; C - challenge.

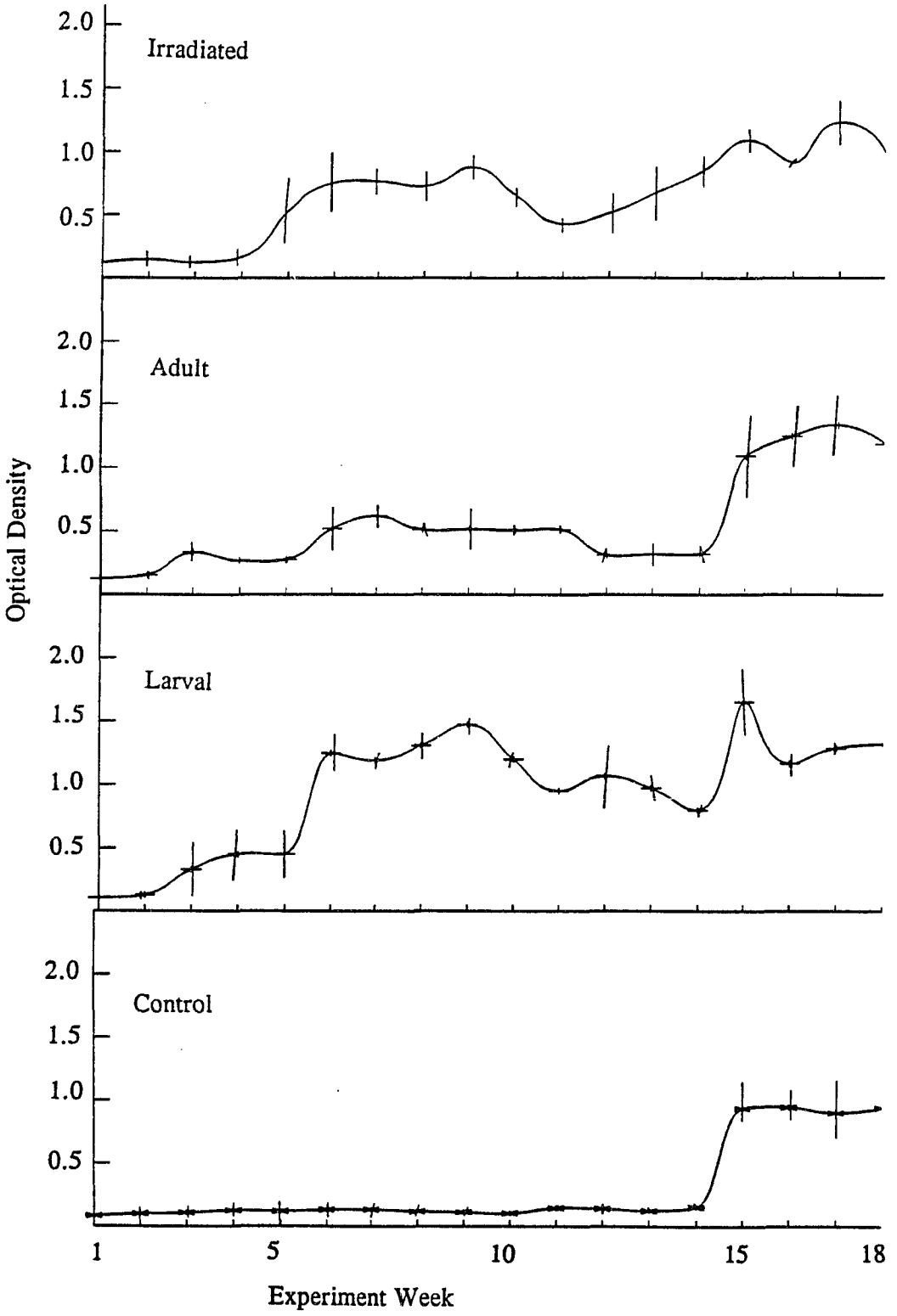

Figure 13. ELISA optical density values: Larval antigens. Values represent group means of individual serum samples analyzed in triplicate. Larval antigens used in these assays were identical to the antigen preparation used for immunizations. I_1 - first immunization; I_2 - second immunization; C - challenge.

Western Blot - The most striking finding of Western blot analysis using either adult or larval *S. vulgaris* antigen preparations and sera collected at several pre- and post-challenge timepoints was the paucity of antigen bands demonstrable with sera from the Irradiated recipients (Figures 14 and 15). There were few bands recognized by these sera at the pre-challenge timepoint, and a slight increase in the number and intensity of the bands recognized at post-challenge timepoints, but this was weak in comparison to the changes demonstrable with sera from ponies immunized with the Adult and Larval antigens, and sera from controls. Following immunization, at the pre-challenge timepoint, sera from Adult and Larval recipients recognized numerous bands in both the Adult and Larval antigen preparations. Following challenge, the number and intensity of band recognition increased greatly for both groups. Sera from Controls recognized bands in a pattern similar to the Adult and Larval recipients rather than that of Irradiated recipients.

Interestingly, recognition of a larval protein with an approximate weight of 43 kDa by Irradiated recipients increased following challenge but recognition of this band decreased in the three other groups following challenge. The response of these groups to a band of approximate molecular weight of 95 kDa was the reverse, decreasing in recognition by the Irradiated group but increasing in Adult and Larval recipients and Controls. Also of interest was the recognition of multiple bands between 43 and 95 kDa by pre- and post-challenge sera of Adult and Larval recipients and, post-challenge, by the Controls. Most of these bands were at best faintly demonstrable or not seen at all in post-challenge sera of the Irradiated recipients.

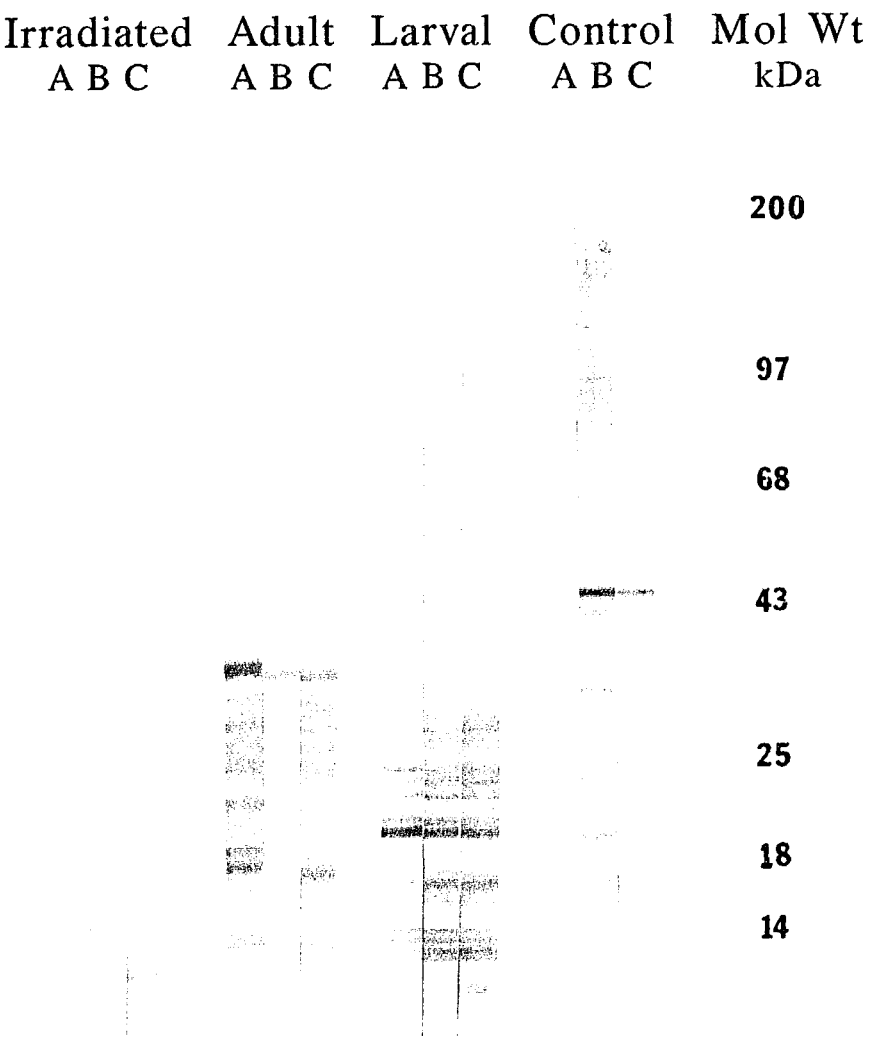

Figure 14. Western blot of *Strongylus vulgaris* adult antigen preparation. Pooled sera from three timepoints were used: A - Post-immunization/Pre-challenge; B - Two weeks post-challenge; C - Four weeks post-challenge. Proteins were separated on a 8-20% acrylamide gradient SDS-PAGE gel and transfered to nitrocellulose. The secondary antibody was alkaline phosphatase-labeled goat anti-equine IgG Fc fragment-specific, and the substrate was BCIP/NBT.

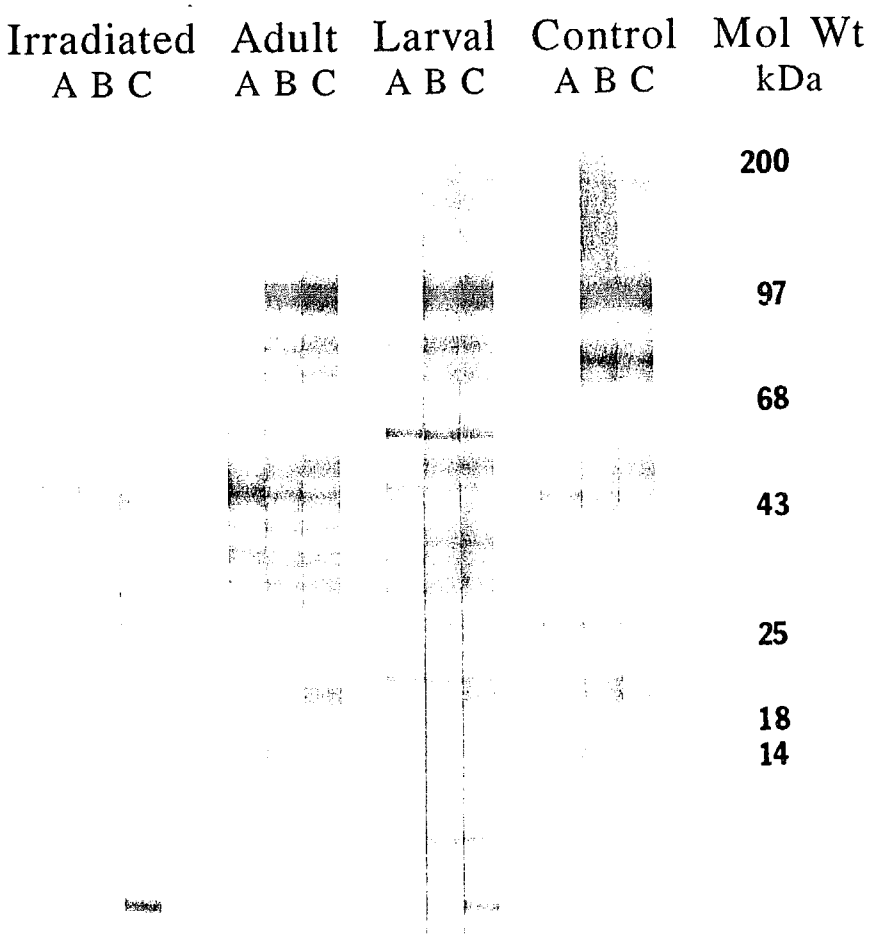

Figure 15. Western blot of *Strongylus vulgaris* larval antigen preparation using pooled serum from three timepoints: A - Post-immunization/Pre-challenge; B - Two weeks post-challenge; C - Four weeks post-challenge. Proteins were separated on a 8-20% acrylamide gradient SDS-PAGE gel and transfered to nitrocellulose. The secondary antibody was alkaline phosphatase-labeled goat anti-equine IgG Fc fragment-specific, and the substrate was BCIP/NBT.

Western Blots of *S. vulgaris* adult antigens showed similar patterns to those of the L_5 antigens, particularly the paucity of demonstrable bands using the Irradiated recipient sera. Adult and Larval recipients recognized numerous small bands in doublets, triplets or quadruplets with molecular weights ranging from approximately 14 to 43 kDa. Some of these bands were recognized faintly by Control sera as well, but most of these bands were not seen by sera from Irradiated recipients.

Enhanced Chemiluminescence Western Blot - Western blots using the ECL procedure proved difficult to develop and capture on film. Due to a dearth of larvae for extraction of surface antigens, insufficient reagents were available to permit refinement of the technique, and sheets of nitrocellulose with surface antigens adhered to them had to be stripped of primary and secondary antibodies and reused. This is, in principle, a technique which allows nitrocellulose sheets to be blocked and blotted a second time, but in practice with the surface antigens of *S. vulgaris* L_3, this reduced the clarity of recognized bands to a degree that the blots were tantamount to an ELISA on nitrocellulose sheets. Important information could still be discerned from these assays, but this information merely confirmed the IFAT studies which demonstrated that the Irradiated immunization group strongly recognized surface antigens of the freshly-exsheathed L_3 and of L_3 from *in vitro* culture when sera from pre-challenge and post-challenge timepoints were examined (Figure 16). Sera from the Adult, Larval and Control groups did not recognize these surface antigens.

Indirect Fluorescent Antibody Test - Irradiated recipients had higher antibody titers to the surface of freshly-exsheathed infective *S. vulgaris* L_3 when sera drawn on the day of

Figure 16. Enhanced chemiluminescence Western blot of *Strongylus vulgaris* L_3 surface antigens. Surface antigens of *S. vulgaris* L_3 cultured *in vitro* for 48 hours were solubilized in a detergent solution containing 0.25% cetyltrimethylammonium bromide. Proteins were separated on an 8 - 20% acrylamide gradient gel and transferred to nitrocellulose. Pooled sera from immunization groups were: I - Irradiated; A - Adult; L - Larval; C - Control. Timepoints analyzed were: Pre - sera taken immediately prior to challenge; 1 - one week post-challenge; 2 - two weeks post-challenge.

challenge were tested by IFAT (Table 5). This recognition of the L_3 was also higher at two weeks post-challenge.

When *S. vulgaris* L_4 from *in vitro* culture were tested using IFAT, Irradiated recipients again had higher pre-challenge titers than did Adult, Larval and Control ponies, but at two weeks post-challenge this was not the case, and Adult and Larval recipients, as well as Controls, had higher IFAT titers (Table 6).

When freshly-exsheathed *S. vulgaris* L_3 were cultured *in vitro* overnight or for three days, the IFAT differences were even more pronounced, and at these timepoints of larval culture the Irradiated recipients recognized the larval surface much more strongly than the other groups (Table 7).

Using *Strongylus vulgaris*-positive serum to assess the species specificity of larval recognition, positive serum recognized *S. vulgaris* L_3 and L_4, as demonstrated above, and recognized *S. edentatus* L_4, but did not recognize freshly exsheathed *S. edentatus* L_3. This indicates that the *S. edentatus* L_4 expresses cross-reacting epitopes on its surface which are not seen on the L_3.

When sequential *in vitro* cultures of *S. edentatus* were examined IFAT results revealed that during development of the L_3, the first and only structures recognized by anti-*S. vulgaris* serum were the three large lips which appeared at two or three days in culture. When the developing L_4 retracted within the L_3 sheath, the lips continued to bind anti-*S. vulgaris*

Table 5. Indirect fluorescent antibody titer[1] to *Strongylus vulgaris* L_3[2] surface antigen.

Immunization Group	Pre-Challenge[3]	2 Weeks Post-Challenge	6 Weeks Post-Challenge
Irradiated	960	7680	1920
Adult	240	1920	1920
Larval	120	960	960
Control	neg	960	960

[1] Numeric value represents the reciprocal of the dilution of pooled serum.
[2] Freshly-exsheathed L_3.
[3] Serum samples were collected immediately prior to challenge.

Strongylus vulgaris L_3 were freshly-exsheathed and then prepared in a media dilution to deliver at least 10 - 20 larvae in 5 μl volumes into serum dilutions previously prepared in 96 well flat-bottom microtiter plates. End-points were determined at the serum dilution where half the larvae in that well did not exhibit fluorescence above the negative control larvae which had been cultured without pony serum but cultured with affinity-purified, polyclonal, FITC-labeled, goat anti-horse IgG secondary antibody.

Table 6. Indirect fluorescent antibody titer[1] to *Strongylus vulgaris* L_4 surface antigen.

Immunization Group	Pre-Challenge[2]	2 Weeks Post-Challenge	6 Weeks Post-Challenge
Irradiated	960	1920	1920
Larval	480	3840	1920
Adult	480	3840	1920
Control	neg	3840	1920

[1] Numeric value represents the reciprocal of the dilution of pooled serum.
[2] Serum samples were collected immediately prior to challenge.

Strongylus vulgaris L_3 were cultured *in vitro* until the L_4 stage and then prepared in a media dilution to deliver at least 10 - 20 larvae in 5 μl volumes into serum dilutions previously prepared in 96 well flat-bottom microtiter plates. End-points were determined at the serum dilution where half the larvae in that well did not exhibit fluorescence above the negative control larvae which had been cultured without pony serum but cultured with affinity-purified, polyclonal, FITC-labeled, goat anti-horse IgG secondary antibody.

Table 7. Indirect fluorescent antibody titer[1] to *in vitro* culture *S.vulgaris* L_3.

	Pre-challenge		2 Weeks Post-challenge	
Immunization Group	Overnight[2]	3-Day[2]	Overnight	3-Day
Irradiated	1350	4050	12150	12150
Adult	150	450	4050	4050
Larval	450	450	4050	1350
Control	-	150	4050	450

[1] Numeric value represents the reciprocal of the dilution of pooled serum.
[2] *In vitro* culture period.

Strongylus vulgaris L_3 were exsheathed and cultured *in vitro* either overnight or for 3 days and then prepared in a media dilution to deliver at least 10 - 20 larvae in 5 μl volumes into serum dilutions previously prepared in 96 well flat-bottom microtiter plates. End-points were determined at the serum dilution where half the larvae did not exhibit fluorescence above the negative control larvae which had been cultured without pony serum but cultured with affinity-purified, polyclonal, FITC-labeled, goat anti-horse IgG secondary antibody.

Table 7. Indirect fluorescent antibody titer[1] to *in vitro* culture *S.vulgaris* L_3.

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Irradiated	1350	4050	12150	12150
Adult	150	450	4050	4050
Larval	450	450	4050	1350
Control	-	150	4050	450

[1] Numeric value represents the reciprocal of the dilution of pooled serum.
[2] *In vitro* culture period.

Strongylus vulgaris L_3 were exsheathed and cultured *in vitro* either overnight or for 3 days and then prepared in a media dilution to deliver at least 10 - 20 larvae in 5 μl volumes into serum dilutions previously prepared in 96 well flat-bottom microtiter plates. End-points were determined at the serum dilution where half the larvae did not exhibit fluorescence above the negative control larvae which had been cultured without pony serum but cultured with affinity-purified, polyclonal, FITC-labeled, goat anti-horse IgG secondary antibody.

antibodies. Following the molt, the entire surface of the emerging L_4 stained brightly using *S. vulgaris*-positive serum.

Surface-Associated Antibody - Larvae cultured at different temperatures following IFA staining were examined for a loss of fluorescence indicative of a sloughing of surface antigens recognized by immune serum. The L_4 cultured at 37° C lost fluorescence in patches at the anterior end, often extending caudally past the excretory pore. These larvae maintained the fluorescence caudal to these areas, demonstrating that the fluorescent label did not simply quench nor decay during the culture period. Those larvae cultured at 4°, or in media containing sodium azide did not lose the surface-associated fluorescent staining, indicating that this change was larvae-induced. The freshly-exsheated L_3 did not lose the labeled antibody during these timepoints, indicating that the larvae-induced shedding of surface-associated antibody is an L_4 phenomenon.

DISCUSSION

Experimental Design - The number of challenge larvae used in the present study was reduced, from 5,000 L_3 established previously, to 750 because severe reactions were seen in non-protected animals which received the higher number (Klei *et al.*, 1982a, 1982b, 1989). Previous studies examined whether protection could or could not be provided by radiation-attenuated larvae, and the prevention of severe reactions through immunization proved advantageous for this demonstration. The purpose of the present study was a comparison of the protection afforded by attenuated larvae or homogenate antigen immunizations as well as an analysis of the immune responses generated by these different forms of exposure. Examination of non-protective responses was regarded as equally important for an evaluation of the mechanisms behind both protection and pathogenesis of *S. vulgaris* infections, and loss of any experimental animals through euthanasia due to severe reactions prior to the termination of the experiment was undesirable.

The 21-day post-challenge period established in the previous protocol had been chosen so that challenge larvae could be distinguished from viable immunization larvae based on substantial differences in their sizes and degrees of development. In the present study, only the Irradiated immunization group could fall into this problematic area, and refinement of the radiation-attenuation permitted us the confidence that attenuated immunization larvae would not be confused with those of challenge. The post-challenge period in the present study was extended for several reasons: to allow time for larval development in the event that the response generated by homogenate immunization was not immediate but could work

through the removal of L_4 in the CMA; and, to allow differences in the cellular and humoral responses between immunization groups to mature.

Clinical Signs - Differences in post-challenge clinical signs, necropsy lesions, larval recoveries and histological changes all indicated that ponies immunized with the Irradiated larvae generated a protective response to challenge while ponies immunized with the Adult and Larval antigen preparations did not. The similarity in numbers of challenge larvae recovered from the arterial dissections of Adult and Larval recipients and Control ponies further demonstrated that the exacerbated clinical signs and lesions of Adult and Larval recipients were not due to differences in total worm burdens following challenge. This strongly suggested that the antigens of immunization sensitized these recipients and precipitated higher and more prolonged fevers, greater loss of body condition, and elevated plasma protein profiles indicative of greater inflammation following challenge.

Rectal Temperatures - The biphasic febrile response in Adult and Larval recipients and Control ponies following challenge coincided temporally with the migration and maturation of *S. vulgaris*. The initial febrile period followed penetration of the mucosa by infective larvae, and this episode was experienced by all ponies. The subsequent decline in fever coincided with the quiescent developmental period of the L_3 prior to the molt and migration of the L_4, The second febrile episode was experienced by Adult and Larval recipients and Control ponies, and corresponded temporally to the L_4 migration within the arterioles and arteries. The pattern of febrile episodes declined towards 20 days post-challenge, a time when most larvae would have completed migration. The inflammation and fever associated

with L_4 migration may be in response to mechanical damage, or from the proteases secreted by the migrating larvae.

Similar biphasic temperature rises in *S. vulgaris* infections have been documented post-challenge (Klei *et al*, 1982, Lester, *et. al.*, 1989), while other studies have shown that fevers post-challenge remained elevated (Patton and Drudge, 1977). Differences in the number of challenge larvae may explain these observations. A similar biphasic pattern in the myoelectrical activity of the cecum and colon following experimental infection of parasite-naïve ponies with *S. vulgaris* L_3 was correlated with larval migration and was not seen in the previously-exposed animals (Lester, *et. al.*, 1989).

Irradiated Immunization Changes - Ponies in the Irradiated group developed a slightly more prolonged febrile period following the initial penetration of the mucosa by L_3 and did not experience a second febrile peak. They also did not develop the serum protein changes indicative of inflammation, nor did they develop arterial lesions subsequent to larval migration. The lack of arterial lesions, in particular, strongly suggests that the site of their protective response was within the intestinal submucosa. Had migration occurred, vessels craniad to this site would have been affected with lesions. Grossly-apparent periportal fibrosis unique to the livers from ponies in the Irradiated group could lend support to this hypothesis if such lesions serve as evidence of portal clearance of inflammatory cytokines generated within the intestinal submucosa during such a response. These periportal lesions had not been seen during prior *S. vulgaris* experiments due to the shorter post-challenge period previously employed. The extended post-challenge period of the present study allowed for such fibrotic lesions to develop and mature.

Inflammatory Cytokines - Inflammatory cytokines, such as interleukin 1 (IL-1), tumor necrosis factor alpha (TNF-alpha), interleukin 6 (IL-6), platelet activating factor (PAF), platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-beta), and fibroblast growth factor (FGF) have pleuripotent activities, including fibrosis and angiogenesis which are important and necessary components of tissue remodeling following inflammation (Leibovich *et al.*, 1987; Fràter-Schröder *et al.*, 1987; Jaatela, 1991; Kovacs, 1991; Nathan and Sporn, 1991). The periportal fibrosis seen in the Irradiated recipients could be a manifestation of the fibrogenic effects of cytokines produced or released in the intestinal submucosa and subsequently cleared via the portal circulation to the liver. These lesions could be indicative of a high concentration of such cytokines. Although grossly visible, such periportal lesions would be less severe than the counterpart arterial lesions seen in the other experimental groups, and in naturally-infected animals as well because the portal system would transport a complement of cytokines within a network of vessels of increasing lumen diameter, thus mechanistically less susceptible to procoagulant effects and thrombus trapping. The immediate, life-threatening severity of such portal lesions would be far less than the thrombosis of a major branch of the CMA supplying the cecum or colon.

An identical complement of such inflammatory cytokines produced in response to *S. vulgaris* larvae migrating within branches of the CMA would be produced and transported within a network of vessels of decreasing lumen diameters, from arteries towards capillary beds. The procoagulant effects of many of these cytokines would favor activation of endothelial surfaces as well as circulating platelets and inflammatory cells, possibly potentiating thrombus formation and trapping (Bevilacqua *et al*, 1986; Cotran, 1987; Cybulsky *et al*, 1988a, 1988b). The subintimal hyperplasia demonstrated in otherwise healthy branches of

the CMA (Morgan *et al.*, 1989) would further reduce the lumen diameter and potentiate the thrombus-trapping by dependent vascular beds.

The periarterial fibrosis and edema of the cecal and colic arteries seen in the Adult and Larval immunization groups may be indicative that prior sensitization produced an anamnestic response which led to production of higher levels of cytokines in response to identical challenge. Circulating *S. vulgaris*-specific opsonizing antibodies could potentiate the release of many cytokines by responding cells. This periarterial fibrosis found in the branches of the CMA of Adult and Larval immunization groups would be the corollary to the periportal fibrosis seen in the Irradiated group.

The severity of a thromboembolic lesion is dictated by the size of vessel affected and the extent of its dependent vascular bed, as well as the ability of the compromised circulatory system to reestablish collateral circulation before ischemic necrosis occurs. Taking into account the subintimal hyperplasia of arteries and vasa vasora of the CMA of naturally-infected horses (Morgan *et al.*, 1989), and seen in the arteries of ponies in this study as well, smaller thrombi could be trapped higher in the arterial tree, thus increasing the scope of the affected area downstream of such a blockage. Blood flow through non-thrombosed vessels would be decreased further due to the reduction of lumen diameters secondary to subintimal hyperplasia. Mechanical obstruction would be potentiated by the release of vasoactive substances from platelets, endothelial cells and leucocytes, such as thromboxane and leucotrienes, which stimulate contraction of the endothelium and vascular smooth muscle and are found in circulation of many horses suffering colic (Ward *et al.*, 1986). The combination of thromboembolism and vasoactive substances would hamper the establishment

of colateral circulation. Éndotoxemia following ischemia of small areas of the intestinal mucosa would further exacerbate the development of hemostasis and life-threatening shock (Bottoms *et al.*, 1986).

Histological Differences - Histological differences between the groups were pronounced. Sections of tissues from Irradiated recipients demonstrated severe infiltrates of eosinophils which stained in a fashion unique to this group. The eosinophil cytoplasm lacked the normal, refractile, brightly eosinophilic granules, staining instead as a dark greenish-brown (Figure 10 A). The uptake of eosin, an acidic dye, is related to the presence of basic proteins in the eosinophil granule, principally major basic protein (MBP) and eosinophil cationic protein (ECP) (Gleich *et al*, 1986), suggesting that this decrease of eosin staining represents a loss of MBP and ECP from the cytoplasmic granules. Faint outlines of granules could be visualized in some tissue eosinophils, but the majority had degranulated or selectively discharged enough basic proteins to alter their dye uptake.

Besides the histological differences in the CMA arterial architecture, the profound infiltration of eosinophils and alteration of their staining was the single most identifying characteristic which differentiated tissues of the Irradiated group from Adult, Larval or Control groups. Repeated blind trials based on the eosinophil staining characteristics, regardless of the tissue, demonstrated that unbiased pathologists placed histological slides from the experiment in groups which directly correlated with protection from challenge. These eosinophil changes suggest that exposure to attenuated larvae established a T cell population which produced eosinopoetic, chemotactic and activating cytokines as well as promoted the production of *S. vulgaris*-specific cytophilic antibodies capable of inducing

degranulation following antigen binding. This concept is consistent with previous findings in our lab which demonstrated the production of an eosinophil chemotactic factor by *S. vulgaris*-sensitized peripheral blood mononuclear cells following *in vitro* stimulation with *S. vulgaris* antigens but not when stimulated with *S. edentatus* antigens (Dennis *et al*, 1988), and the adherence of eosinophils to *S. vulgaris* L_3 when these larvae were incubated with *S. vulgaris*-immune serum but not when incubated with naïve serum (Klei *et al*, 1992).

Eosinophils - Other host-nematode systems have demonstrated that adherence of eosinophils to infective larvae, or deposition of MBP, was a parasite-specific antibody-dependent phenomenon (Badley *et al*, 1987; Deardorff *et al*, 1991; Kazura, 1981). The precise role of the eosinophil in host protection is under considerable debate, and may be as multifaceted as it is contentious. Eosinophils contain numerous toxic factors and enzymes, and the orchestration of their release has been shown to be related to the activation and signals received by the cell through different inflammatory mediators, including cytokines (Walls and Beeson, 1972; Veith *et al.*, 1985; Silberstein and David, 1987; Popper *et al.*, 1989; Fujisawa *et al.*, 1990; Spry *et al.*, 1992; Weller, 1992). These findings support the concept that eosinophils will exhibit either inflammatory/cytotoxic, or antiinflammatory properties depending on the cytokine stimulus, as well as the antibody/receptor combination on the cell surface.

The release of eosinophil products is also related to the surface receptor/ligand interactions (Moqbel *et al.*, 1990). These researchers have identified receptors for IgA on the surface of eosinophils as well as for IgE. Plasma cell precursors for the production of IgA for secretion into the gut lumen have been characterized and discussed (Bienenstock and Befus, 1984;

Cebra *et al.*, 1980). What remains to be identified are the precise roles of the factors which draw eosinophils into the tissue via chemotaxis and activate them on arrival. Interleukin-8 has been identified as one eosinophil accumulator (Collins *et al.*, 1993) and IL-9 has been shown to potentiate the IL-4-induced release of IgE and IgG_1 from B lymphocytes (Petit-frere *et al.*, 1993). As studies such as these document the choreography of such responses, elucidation of the antigens necessary for this induction and the adjuvants and delivery systems needed to potentiate these responses will progress rapidly.

T Helper-1 Versus T Helper-2 Subsets - The $CD4^+$ lymphocyte subsets in the mouse have been defined according to the cytokine profiles the $CD4^+$ T cells produce following stimulation. The T helper-1 subset (TH1) is characterized by the production of interferon-gamma (IFN-gamma) and interleukin-2 (IL-2), while the T helper-2 (TH2) subset produces interleukin-4 (IL-4) and interleukin-5 (IL-5) (Mosmann *et al.*, 1986; Mosmann and Coffman, 1989; Locksley and Scott, 1991; Cox and Liew, 1992). This dichotomy of T helper responses provides a conceptual basis for the development of protection or susceptibility to certain infections. Each helper subset does generate both cellular and humoral components which fit within their cytokine profiles, but described simplistically in terms of parasitism, these subsets favor the development of either cellular responses, which protect against intracellular parasites (TH1), or humoral responses which are typically generated against metazoan helminths (TH2). These two subsets orchestrate susceptibility or resistance to parasitic infections through the recruitment or activation of other cells via their cytokine products (Mosmann and Coffman, 1989; Locksley and Scott, 1991; Cox and Liew, 1992). These helper subsets are also associated with delayed-type hypersensitivity (DTH) reactions (TH1), and with allergic responses (TH2).

Mouse strains which develop a TH1 response to *Leishmania major*, an intracellular protozoan parasite, resolve lesions and survive, while susceptible strains of mice which develop a TH2 response die (Sadick *et al.*, 1986; Locksley and Scott, 1991). Susceptible mice can be cured of the infection with a monoclonal antibody to IL-4 (Sadick *et al.*, 1990). Ablation of this IL-4 response in susceptible mice results in a switch to a TH1 response with higher levels of IFN-gamma produced. Mouse strains infected with *Trichinella spiralis*, a gastrointestinal nematode with an intracellular larval stage, show a similar pattern of resistance (Pond *et al.*, 1989). Those strains which are more resistant produce more IFN-gamma than susceptible strains which produce higher levels of IL-4.

Helminth infections are associated characteristically with eosinophilia and high levels of IgE, traits of a TH2 response. Production of IgE is under the control of IL-4, which also stimulates production of IgG_1 while antagonizing the production of IgG_{2a} and IgG_3. Interferon-gamma most often displays a mutually antagonistic relationship with IL-4 in its inhibition of IgE synthesis and promotion of IgG_{2a} synthesis (Mosmann and Coffman, 1989; Mosmann and Moore, 1991). This mutual antagonism is seen as well with the role of IL-10 in the downregulation of TH1 cell proliferation (Mosmann and Moore, 1991). Immunological inertia can be established through the early development of one T cell subset which inhibits the the other, either through inhibition of cytokine production or cell proliferation. As shown with the Leishmania model in mice, early establishment of the appropriate T cell subset results in cytokines and responses which lead to protection. Protective immunizations must exploit these characteristics.

Eosinophilia has been shown to be a T cell dependent phenomenon regulated by cytokines, principally under the control of IL-5 (Coffman *et al.*, 1989; Weller, 1992). The importance of IL-5 in the generation of an eosinophilia was demonstrated in mice infected with *Nippostrongylus brasiliensis* and treated with either a monoclonal antibody to IL-4 or IL-5, or control antibodies (Coffman *et. al.*, 1989). Anti-IL-5 monoclonal antibodies ablated the production of eosinophils, while anti-IL-4 antibodies ablated the production of IgE. Removal of the eosinophil response with anti-IL-5 monoclonals did not alter susceptibility of mice to infection with *Trichinella spiralis* (Herndon and Kayes, 1993), while treatment of guinea pigs with anti-eosinophil serum did increase their susceptibility to infection or reinfection with *Trichostrongylus colubriformis* (Gleich *et al*, 1979). In a mouse-schistosome model, treatment of mice with anti-IL-5 monoclonal antibodies did not abrogate the immune response in animals vaccinated with irradiated cercaria (Sher *et al*, 1990). These differences in resistance or susceptibility following eosinophil abrogation may be in part due to the sequence of exposure to a parasite and subsequent removal of the eosinophil response. Removal of an anamnestic eosinophilia may be more detrimental to a host than preventing the development of an eosinophilia. These studies do clearly demonstrate that an eosinophil response is not the sole means of resistance to parasitic infections.

Other studies have correlated resistance to helminth infections by certain lines or breeds with differences in IL-5 production (Else and Grencis, 1991), or the generation of a peripheral eosinophilia (Dawkins *et al*, 1989), and increased production by helminth-infected human patients (Limaye *et al.*, 1990). The role of the eosinophil remains enigmatic, since many studies have shown that resistance can occur in the absence of eosinophils. Still, the

characteristic of helminth infections is the development of a TH2 type response with production of IL-4 and IL-5, resulting in high levels of IgE and circulating eosinophils.

Protection seen during the present study and others using radiation-attenuated *S. vulgaris* L_3 in our laboratory has been associated with an anamnestic eosinophilia. The present study further demonstrates that differences in the numbers and staining of tissue eosinophils is pronounced when histological sections from immunization groups are compared. Our findings strongly suggest that generation of a cellular response as manifest in the anamnestic eosinophilia must be coupled with other factors which provide a mechanism for these eosinophils to become activated and discharge granule contents. Our findings and those of others further suggest that this mechanism entails the generation of a cytophilic antibody response. This fits the development of a TH2 response in the protected ponies and suggests development of an unprotective TH1 response in those which received soluble antigens, or in the Controls.

Lymphocyte Migration - Perhaps the generation of an anamnestic response *in situ*, where penetrating larvae pass several days in their molt to the L_4 stage, may be the single most effective aspect of the oral immunization. Clearly, Adult and Larval recipients were sensitized to *S. vulgaris* antigens and had high levels of circulating antibodies at the time of challenge, but intramuscular injection in the neck did not generate an effective, protective response in the submucosa of the bowel as did the radiation-attenuated larvae. By the time challenge larvae induced immune recognition, or generated a *de novo* recognition in the intestinal submucosa of Adult and Larval vaccinates, those challenge larvae had already molted and migrated to the arterioles and arteries of the CMA. Sensitization through

exposure to homogenate antigens was associated with the augmented perivascular lesions seen in Adult and Larval recipients compared with the lesions seen in Controls.

The establishment of an immune response begins with antigen presentation to the T cells. Subsequently, naïve and memory T cells show distinct pathways of lymphocyte recirculation (Mackay *et al.*, 1990; Dunkley and Husband, 1989). This organ specificity of lymphocyte migration is dictated by highly selective lymphocyte recognition of organ-specific cell-surface determinants on the high endothelial venules (Butcher *et al.*, 1980; Chin *et al.*, 1984). The generation of TH1 or TH2 responses is also enhanced or established by antigen presentation by distinct antigen presenting cell (APC) populations (Fitch *et al.*, 1993). Adherent cell populations, such as macrophages, present antigen effectively to cells of the TH1 subset while B cells present antigen effectively to the TH2 subset. Macrophages can further dictate the inertia of the immune response because the production of IL-10 by TH2 cells suppresses macrophage signals which encourage the development of the TH1 response. Macrophages also produce IL-1 which is required for some but not all TH2 clones to proliferate in response to IL-2 or IL-4.

With these concepts in mind, it is possible to visualize the establishment of a TH2 response generated in the gut following oral immunization with radiation-attenuated larvae, or the establishment of a TH1 response following intramuscular injection of soluble antigens. The protective response from oral immunization resulted in the production of an anamnestic eosinophilia, and the staining characteristics of the eosinophils in this immunization group strongly suggests production of a cytophilic antibody. These findings are consistent with this scenario of TH2 responses generated through Peyer's patches and subsequent production of

IgA and IgE by resident B cells. The soluble antigen immunization groups were not primed in this fashion by parenteral immunization and responded with the same level of protection as naïve controls. These immunizations furthermore established circulating antibodies which appeared to exacerbate post-challenge clinical signs and promote perivascular lesions. Whether or not parenteral immunization can establish an effective immune response in gut will depend not on the antigens chosen as effective targets but moreso on the adjuvants which facilitate the establishment of a TH2 type response.

Antibody Responses - Circulating antibodies alone may promote opsonization of infective larval antigens but do little to impede larval migration, or, may serve to promote an inapproriate inflammatory response. The ELISA ODs which developed following immunization support this idea because the highest OD values were recorded from sera of the Adult and Larval immunization groups when tested against their respective antigens. The ELISA in this study was limited by the lack of commercially-available secondary reagents to different equine antibody isotypes. Anti-equine IgG heavy and light chain antibodies were used to compensate as best one could, with the hope that anti-light chain molecules would cross-react between isotypes, however, isotype-specific secondary antibodies would be essential for the demonstration of isotype differences generated by the different immunization protocols and their relationship to resistance or perivascular lesions. The low ELISA ODs seen in the Irradiated recipients cannot be assumed to represent low levels of *S. vulgaris*-specific antibody but may be due to production of IgA and IgE isotypes which may not circulate in high concentration and would not be well recognized by the commercially-available reagents used in this study. Since eosinophils from other animal models have been documented to express surface receptors for both of these isotypes and release of granule

proteins follows antigen binding, it seems logical to suggest that the low ELISA ODs and the histological changes of tissue eosinophils in the Irradiated immunization group point towards the localized generation of a parasite-specific antibody response of an isotype unrecognized by our reagents, or which circulate in low concentration due to the adsorbance to surface receptors on the responding eosinophils.

An appropriate cellular response *in situ* and in conjunction with circulating antibody, or cytophilic antibody, appears to be needed in protection from migration of *S. vulgaris* larvae through the vasculature. Since the group which was protected from larval migration had ELISA ODs which were intermediate to those of the Adult and Larval immunization groups, and recognized fewer bands on Western blots of somatic extracts, but did have much higher titers to surface epitopes as demonstrated by the IFAT titers, as well as anamnestic eosinophilias which stained profoundly different from their counterparts in other immunization groups and Controls, antibody production of a cytophilic nature and directed towards the larval surface appears to be the key to protection from *S. vulgaris* larval migration.

Radiation-attenuated *S. vulgaris* larvae could induce this antibody development by allowing the immune system a prolonged antigenic view of the L_3 surface through interference with protein synthesis. Disfunctional protein synthesis may slow the speed at which L_3 develop. This hypothesis is supported by the greater recognition of the L_3 surface than that of the L_4 by the Irradiated group. Coupled with the ability of the L_4 to shed some of the antibody bound to the anterior end, shedding which may be enhanced during migration through tissue, L_3 surface antigens appear to be the targets of choice for future immunizations.

Adjuvant - Freund's complete adjuvant (FCA) would be unacceptable in any commercial vaccine due to the adverse tissue reactions (Amyx, 1987). Ribi adjuvant has been shown to be effective in generating both humoral and cellular responses without the severe inflammatory side effects of FCA (Ribi, 1986; Mallon *et al.*, 1991). The selection of Ribi as an adjuvant, in retrospect, was made before the mechanisms of protection (or lack thereof in the soluble antigen recipients), was seen. Ribi adjuvant was chosen for its ability to stimulate both the cellular and humoral responses to antigen. Since the Adult and Larval immunization groups did have high circulating antibodies to *S. vulgaris* antigens, Ribi adjuvant must be accepted as capable of helping promote a humoral response. Since the eosinophil response to challenge was not different in the Adult, Larval and Control groups, one could speculate that Ribi adjuvant did not stimulate a T cell response which produced appreciable amounts of IL-5 when challenged. One must consider as well the site of inoculation, but for the time being, it appears that Ribi adjuvant used with soluble nematode antigens injected intramuscularly in the neck does not promote a strong TH2 response.

The field of adjuvant research has grown tremendously as the understanding of mechanisms of immune responses has unfolded (reviewed by Knussler and Thomson, 1992). Generation of mucosal responses, as appears necessary for protection against *Strongylus vulgaris* larval migration, may be possible utilizing ISCOMs first developed by Morein (Lovgren and Morein, 1991; Mowat and Donachie, 1991), or biodegradable microspheres (Eldridge *et al.*, 1991) which would allow surface antigens of *S. vulgaris* infective larvae to be delivered by an oral route.

SUMMARY

Immunization with radiation-attenuated *Strongylus vulgaris* L_3 provided protection from homologous challenge while immunization with soluble extracts of *S. vulgaris* did not. Immunization with soluble antigens sensitized recipients and exacerbated the inflammatory response to challenge, resulting in more severe clinical signs and lesions at necropsy. Ponies immunized with Irradiated L_3 had fewer febrile episodes post-challenge, developed anamnestic eosinophilias, had few arterial lesions, and were greater than 91% protected from challenge. Hepatic lesions characterized by severe periportal fibrosis were found in Irradiated larvae recipients at necropsy. Histology showed than normal cranial mesenteric arterial architecture was maintained in the Irradiated group alone, and that there was a prominent infiltration by eosinophils which were characteristically dark-staining with indistinct cytoplasmic granules.

Ponies immunized with soluble antigens had more febrile episodes post-challenge, more severe lesions at necropsy, and larval recoveries equal to those of Controls, thus unprotected from challenge. Soluble antigen recipients had enlarged cecal and colic arteries which suggested that sensitization to *S. vulgaris* antigens promoted the development of perivascular edema and fibrosis following challenge. Histology of tissues from these groups demonstrated severe destruction of normal arterial architecture with a lymphoplasmacytic infiltrate. Eosinophils of these groups stained brightly and had distinct cytoplasmic granules.

ELISA demonstrated that recipients of soluble antigen immunizations developed antibodies to their respective antigen preparations prior to challenge; radiation-attenuated recipients had

higher titers to larval extracts than to adult extracts, but titers were lower than those induced by soluble antigen immunizations.

Western blots showed that recipients of soluble extracts recognized numerous somatic proteins while attenuated larvae recipients did not. Indirect fluorescent antibody assays and Western blots using detergent extracts of larval surface proteins showed that immunization with Irradiated larvae generated greater antibody recognition of the larval surface prior to challenge. This recognition was greatest for L_3 larvae from *in vitro* culture either overnight or 3 days than for freshly-exsheathed L_3 or L_4 from *in vitro* culture.

CONCLUSIONS

Protection from *S. vulgaris* infection by prior immunization with radiation-attenuated larvae is strongly associated with the development of an anamnestic eosinophilia. Changes in the histological staining of eosinophils from protected ponies were consistent with cell activation and degranulation, which further suggests that a cytophilic antibody response specific for parasite antigens was also induced. These two parameters are characteristically associated with the development of a TH2 response.

Ponies immunized with soluble antigens by peripheral injection developed high levels of circulating antibodies following immunization. These ponies did not develop an anamnestic eosinophilia, and those eosinophils seen in histological sections from these animals did not appear degranulated. The development of high levels of circulating antibody of a non-cytophilic nature and the lack of an anamnestic eosinophilia strongly suggest that this form of immunization promoted the development of a TH1 type response. Parasite-naïve control ponies developed a response like that of the soluble antigen immunizations.

Recognition of surface antigens of the infective larval stage was highly associated with protection, and was a characteristic of the radiation-attenuated immunization alone. Development of a subunit vaccine for protection against *Strongylus vulgaris* infection should be targetted at these surface antigens, possibly in conjunction with proteases produced by the migrating L_4 stage. Adjuvants which stimulate a TH2 response to these antigens will be beneficial. Adjuvants or delivery mechanisms such as ISCOMs or lipid microspheres which can be administered orally could favor the establishment of an effective immune response

within the intestinal submucosa. An effective response in the submucosa will prevent the development of the arterial lesions secondary to *S. vulgaris* larval migration.

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VITA

Clifton Michael Monahan was born in Pontiac, Michigan, on February 12th, 1954. He dutifully attended parochial schools until 1972 at which time he entered the University of Michigan, at Ann Arbor, Michigan. His brief tenure at the U of M was colorful but not academically or professionally productive, so he transferred to the University of Jackson Hole, Jackson, Wyoming, beginning in the fall of 1974 as a snow science major. Following his years at the U of JH, he moved to Aspen, Colorado, for further studies in the field. He returned to academia in the fall of 1978, at Colorado State University in Fort Collins, Colorado, where he completed his D.V.M. in 1985. Adventure with a tinge of altruism took him to the high volcanic region in Kivu province of eastern Zaire where he worked as an extension veterinarian for a cattle cooperative. Here he saw gorillas in the jungle, volcanic eruptions, trapped okapi in the Ituri Forest with the Mputi (Pygmies) while developing an interest in, and personal experience with, tropical medicine of man and beast. This interest led him to pursue further graduate training in parasitology at the Louisiana State University School of Veterinary Medicine in Baton Rouge, Louisiana. His work there was guided by Dr. Thomas R. Klei, Boyd Professor of Parasitology. Dr Monahan presently resides somewhere, living life like a tourist.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

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Major Field: Veterinary Medical Sciences

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Approved:

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