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THE RELATION BETWEEN TISSUE EOSINOPHILIA AND PHOSPHOLIPASE B ACTIVITY IN MICE INFECTED WITH *TRICHINELLA SPIRALIS*

THESIS

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By

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The numbers of tissue eosinophils were counted and phospholipase B activity was assayed in the intestines of nonsensitized and sensitized mice infected with Trichinella spiralis. In the nonsensitized mice the numbers of intestinal eosinphils and phospholipase B activity increased, peaked and returned to normal levels during the same time period. The numbers of intestinal eosinophils and phospholipase B activity of the sensitized mice was similar to that of the nonsensitized mice, but displayed an anamnestic response. The increase, peak and decline were parallel but within a shorter time span. The different cell population work showed that the eosinophil indeed possessed the enzyme while all other white blood cells studied were negative for it. All these findings support the hypothesis that a parasite-induced tissue eosinophililia is the source of elevated phospholipase B activity present in parasitized tissues. Finally, a methodology was modified for the extraction of phospholipase B from eosinophil rich tissues.

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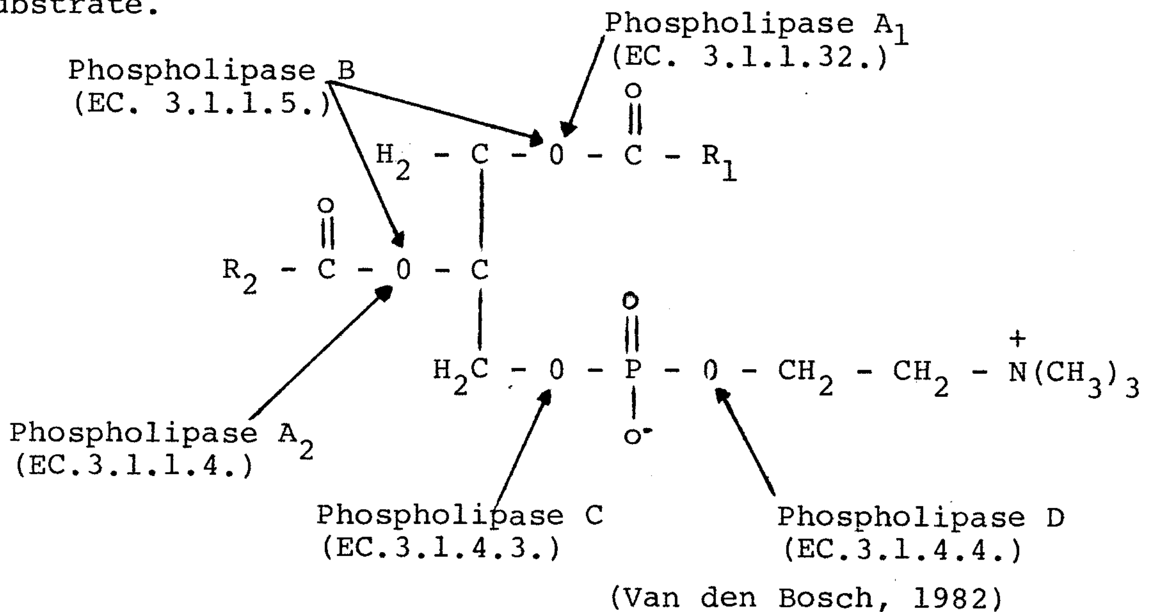
CHAPTER I

INTRODUCTION

There is considerable evidence that suggests an association between a variety of parasitic infections and elevated phospholipase B concentrations in the parasitized tissues. The literature review that follows presents what is known about this association.

Chemical properties and sources of phospholipase B. Phospholipase B (E.C.3.1.1.5), also designated lecithinase B, lysolecithinase and lysolecithin acylhydrolase, is an enzyme that catalyzes hydrolysis reactions. The action of phospholipase B and similar phospholipases is displayed below utilizing phosphatidylcholine as the enzymes substrate.



(Van den Bosch, 1982)

Thus phosphilipase B can remove both acyl chains from a diacylglycerophospholipid such as phosphatidylcholine. Such phospholipases B are also always active towards lysoglycerophosphatides. Thus they possess lysophosphilipase activity in that they can remove the fatty acid esterified in the α position from lyso-compounds such as lysolecithin (Robertson and Lands, 1962; Rossiter, 1967; Van den Bosch, 1982).

The presence of phospholipase B in rodent intestine was first reported by Epstein and Shapiro (1959). In a quantitative assessment of enzymatic activity in rodent tissue, Marples and Thompson (1960) found that intestinal (ileum), lung and splenic tissues were the most active, whereas nervous tissue and heart muscle were the least active. Comparing these findings from studies on the whole ileum (i.e., mucosa and muscle) to the findings of Epstein and Shapiro (1959), who studied only the mucosa, it appears that the greater part of the enzyme activity is associated with the mucosa.

<u>Effects of parasitic infections on host phospholipase B levels</u>. The association between intestinal parasitic infections and increased enzyme activity was demonstrated by Ottolenghi (1973a,b). In preparing phospholipase for biochemical characterization, unusually high concentrations were found in the small intestines

of mice infected with Hymenolepis nana. A tentative relation between increased phospholipase activity and the tapeworm was shown by infecting parasite-free mice with H. nana eggs (Ottolenghi, 1973b). When the course of infection (Hunninen, 1935) in the mouse host was compared with the changes in phospholipase concentrations, it became apparent that the enzyme concentrations were directly related to the migration of adult tapeworms from the upper small intestine to the lower small intestine, where the adult worms were eventually located.

Subsequently, other host-parasite models were tested which resulted in the well documented association between the presence of parasitic worms in tissue and increased concentrations of phospholipase B in the same sites. Larsh et al. (1974) demonstrated increased enzyme concentrations in the intestines of mice and rats after an initial infection with Trichinella spiralis. Mice infected with 400 larvae had elevated enzyme concentrations on the fifth day post-infection and maximum quantities of the enzyme were present on Day 14. After a gradual decline, normal amounts were reached by Day 31. Rats infected with 3,000 larvae showed increased enzyme concentrations at Day 4 that remained high through Day 13. After this, the enzyme activities decreased, indicating a return to normal.

In a subsequent paper, Larsh et al. (1975) found that sensitized mice (three stimulations with 200 larvae at three-week intervals) challenged with 400 *T. spiralis* larvae showed an anamnestic-type response as measured by enzyme concentrations in intestinal tissue. Elevated amounts were present at Day 1 and they remained high through 20 days after challenge, returning to the level of uninfected controls by 25 days post-infection.

Ottolenghi et al. (1975) found elevated phospholipase B concentrations in the lungs and intestines of nonsensitized and sensitized rats after challenge with *Nippostrongylus brasiliensis*. Rats given an initial infection exhibited increased enzyme activity in the intestinal tissue from Day 8 through Day 22, with peak amounts occurring on Day 15 after challenge. The intestinal levels of phospholipase B were greatest in the area with the largest worm population. The proximal half of the small intestine, where most of the worms are found, had an earlier and greater enzyme level than did the distal half of the intestine. After Day 15, at the time of the expulsion of worms from this area (Brambell, 1965), the enzyme concentration in the proximal half of the intestine declined faster than that in the distal half. An anamnestic-type of response was found in rats sensitized (1,000 larvae) and challenged 40 days later

with 1,000 larvae. The phospholipase B concentrations were elevated on Day 3 and reached a peak by Day 5 (Ottolenghi et al., 1975). Goven (1979a,b) showed that the phospholipase B activity of the intestines of nonsensitized and sensitized rats was directly related to the number of adult N. brasiliensis worms present in the intestine. Thus, the size of the worm burden influenced the induction and concentration of phospholipase B.

Ottolenghi et al. (1977) also found elevated enzyme concentrations in the lungs and the brains of rats infected with Angiostrongylus cantonensis during both a primary and secondary infection. Rats with an initial infection had moderately elevated amounts of phospholipase B in the lungs at eight and 15 days after infection and greatly elevated levels at 35, 43 and 49 days when compared with uninfected controls. The brain tissue contained elevated concentrations at Day 15 through 35 days post-infection. These periods of increased activity in the lungs and brain coincided with the migration patterns of the third stage larvae and the adult worms in the host. Sensitized rats challenged with an infection demonstrated an anamnestic response in both the lungs and brain. Elevated enzyme concentrations in

both organs were present on Day 1 after challenge and remained high through 35 days post challenge.

Goulson et al. (1981) showed that rats given an infection with *Strongyloides ratti* developed elevated phospholipase B levels in the lungs and small intestines during the first week of infection which remained elevated through the fourth week. Sensitized rats challenged with *S. ratti* developed an anamnestic response in both the lungs and intestines.

In addition to these helminth models Ngwenya and Capaci (1982) demonstrated elevated levels of phospholipase B in mice infected with *Plasmodium yoelii* and Laubach et al. (1982) demonstrated increased enzyme activity in rats infected with *Pasteurella pneumotropia* and *Mycoplasma pulmonis*.

The experimental findings described above demonstrate a relation between parasitic infections and increased phospholipase B concentrations. It has been suggested that this relation is due to the presence of eosinophils and inflammation found in parasitic infections (Larsh et al., 1975).

Association of phosphilipase B activity and eosinophils. There has been considerable evidence linking eosinophilic leukocytes and phospholipase B activity in animal tissues. As examples, Elsbach and Rizack (1963) have shown phospholipase B activity in homogenates of

rabbit polymorphonuclear leukocytes from peritoneal exudates. Using histochemical techniques, Ottolenghi et al. (1966) demonstrated the presence of phospholipase B in various tissues of the rat. Ottolenghi (1970) showed a close correlation between the numbers of eosinophils in histological sections and cell suspensions, and the enzymatic activity of the same preparations. In other words, the results demonstrated a relation between the concentration of phospholipase B and the number of eosinophilic leukocytes in most tissues. However, with bone marrow preparations, a lower ratio of enzyme activity to the number of eosinophils was found. This was shown to be due to the fact that bone marrow contained a fraction of immature eosinophils that lacked the enzyme.

The morphological evidence relating eosinphils to the enzyme has also been supported by results obtained using corticosteroids. Ottolenghi and Barnett (1974a,b) used dexamethasone and cyclophosphamide to vary the number of eosinophils in rat tissues. Dexamethasone produces a rapid decline in the number of peripheral cells and cyclophosphamide produces a decline in marrow eosinophil number. By doing cell counts in tissue sections and cell suspensions, along with determining phospholipase B concentrations, they found that changes in the numbers of eosinophils paralleled changes in the enzyme concentrations in the intestine and bone marrow.

Because of this direct relation between phospholipase activity and eosinophils in animal tissues, Larsh et al. (1974; 1975), Ottolenghi et al. (1975; 1977), Goven (1978a,b) and Goulson et al. (1981) found it worthwhile to measure the numbers of eosinophils in the bone marrow while at the same time monitoring enzyme concentrations in animals given parasitic infections.

Larsh et al. (1974) found in mice infected with *T. spiralis* that an increase in bone marrow eosinophils occurred a few days after the intestinal phospholipase had increased. The eosinophil numbers reached a maximum and declined concurrently with the enzyme activity. This same pattern was seen in rats infected with *T. spiralis*. Also, Larsh et al. (1975) found that in sensitized rats challenged with *T. spiralis*, the numbers of eosinophils in the bone marrow increased shortly after the elevation of enzyme concentrations in the intestinal tissue. Both responses were accelerated in comparison with the nonsensitized mice (Larsh et al., 1974). Ottolenghi et al. (1975) and Goven (1978a,b) also found a clear relation between eosinophils and enzyme activity in rats given a primary infection with *N. brasiliensis* and in sensitized rats given a challenge infection with the same parasite. In a primary infection, the activity of phospholipase B in the intestine coincided with an intense eosinophilia. Kelly and Ogilvie (1972) have

reported a great increase in eosinophils of intestinal tissue on Day 15 post-infection, which was also the period of peak phospholipase activity (Ottolenghi et al., 1975; Goven, 1978a,b). A challenge infection showed an anamnestic-type response for both parameters.

Elevated numbers of eosinophils in the bone marrow were also found to be related to increased enzyme levels in the lungs and brains of rats during a primary infection with A. cantonensis and in sensitized rats given a challenge infection (Ottolenghi et al., 1977). This relation was best shown when the time pattern of bone marrow eosinophilia was compared to the increases in lung phospholipase B levels during a primary infection. Ottolenghi et al. (1977) found a biphasic enzyme response in the lungs. They concluded that the first response was due to the larvae migrating through the lungs to the brain, and that the second response was due to the worms reinvading the lungs to become established in the branches of the pulmonary artery. A close temporal relation was found between this biphasic enzyme response and a similar biphasic bone marrow eosinophilic response. An anamnestic response for both parameters was noted after a challenge infection of sensitized animals.

Bone marrow eosinophils were also found to be elevated throughout the course of infection of rats with S. ratti (Goulson et al., 1981). These eosinophils were

increased at Days 5 through 21 during the primary infection and demonstrated an anamnestic response during a challenge infection.

Association of inflammation and phospholipase B activity. Larsh et al. (1974; 1975) found that the increase in intestinal phospholipase B concentrations in nonsensitized and sensitized mice challenged with *T. spiralis* corresponded closely in time and degree with the inflammatory response reported in previous work (Larsh and Race, 1975).

Larsh and Race (1954) reported that after an initial infection with *T. spiralis* an acute inflammatory response developed in about four days, and by Day 8 the panmucosal inflammations reached their peaks. This acute response diminished gradually so that by 14 days after infection a chronic inflammation was present. In sensitized mice challenged with *T. spiralis*, the response was similar except for the more rapid appearance of acute inflammation, within seven to 12 hours after infection, which peaked at four days.

In nonsensitized mice, Larsh et al. (1974) found the first increased enzyme concentration on Day 5, which followed the inflammatory response present on Day 4. The enzyme levels, which peaked at Day 14, were high throughout the known inflammatory time span (14 days) and remained elevated until 29 days after infection.

The increased enzyme concentration within 24 hours after the *T. spiralis* challenge in sensitized mice followed the inflammatory response present 12 hours after challenge (Larsh et al., 1975). The enzyme level peaked at Day 11 and remained elevated for at least 20 days.

The enzyme concentrations in sensitized mice increased earlier, but did not reach as high a peak or last for as long as those in nonsensitized mice. This can be explained by conclusions drawn by Larsh and Race (1975) from studies on the inflammatory pattern found in mice infected with *T. spiralis*. They found that not only does direct association exist between acute intestinal inflammation and the expulsion of *T. spiralis* adults but, also, that a similar association exists between the degree of sensitivity of the host at challenge and the timing and intensity of inflammation, and loss of worms. In sensitized animals, the inflammatory response developed much sooner than in nonsensitized animals (Larsh et al., 1975). It is logical to assume, because of the temporal pattern described above, that this early inflammatory response was linked to the earlier rise in phospholipase B activity. Also, since a significant number of adult worms which initiate the inflammatory response, was expelled a week earlier in sensitized animals, it is logical to assume that the inflammation would subside earlier in these animals (Larsh et al.,

1975). This would account for the lower concentrations and shorter durations of phospholipase B activity in sensitized animals.

The association between inflammation and enzyme activity can be found in the lungs and brain of rats infected with *A. cantonensis* (Ottolenghi et al., 1977), the lungs and intestine of rats infected with *N. brasiliensis* (Ottolenghi et al., 1975; Goven 1979a,b), and the intestines of rats infected with *S. ratti* (Goulson et al., 1981).

Because of the above mentioned results, Larsh et al. (1974; 1975) and Ottolenghi et al. (1975) postulated that the elevated intestinal enzyme activities found in parasitic infections were due to the presence of inflammation. This inflammation stimulated the bone marrow to increase production of eosinophils, the source of the enzyme. Larsh and Weatherly (1974) have reported that the immunity against the adult worms of *T. spiralis* in mice is cell-mediated, and Ogilvie and Jones (1973) and Kelly and Dineen (1972) have acknowledged that cell-mediated immunity plays a role in the Nippostrongylus/rat model. Larsh et al. (1974; 1975) and Ottolenghi et al. (1975) believe that T-lymphocytes present in the damaged tissues provided this stimulus. Basten and Beeson (1970) have shown that a diffusible product from sensitized lymphocytes implanted intraperitoneally in diffusion chambers

produced eosinophilia in normal rats. These eosinophils migrated to areas where tissue injury had produced an inflammatory response. Colley (1973) has shown that sensitized lymph node cells, after interaction with an antigen, will cause the migration of eosinophils. Once in the inflammed areas, the eosinophils will release their contents, thus increasing the amounts of phospholipase B (Larsh et al., 1974). This would continue until the worms were expelled and the inflammation subsided, thereby lessening the stimulus for bone marrow eosinophil production (Larsh et al., 1974).

In testing the hypothesis, Goven and Moore (1980) have shown that nude mice, which lack a T-lymphocyte population, do not develop either an eosinophilia or increased phospholipase B activity after *T. spiralis* infection. This demonstrates that both responses are probably T-lymphocyte-dependent. In addition, Goven (1983) has shown that the suppression of the eosinophil response in *T. spiralis* infected mice with antieosinophil serum also results in a diminished phospholipase B activity, thus indirectly demonstrating the eosinophil as the source of the enzyme.

The present study demonstrates a direct relation between the accumulation of tissue eosinophilia, caused by a *T. spiralis* infection, and an elevated phospholipase B activity in the same site in primary or challenge

infected mice given *T. spiralis*. Thus, this study gives direct support for the hypothesis that elevated phospholipase B levels present in both a primary or challenge infection with *T. spiralis* are the result of chemotaxis of eosinophils to sites of parasitized tissue. The present study also demonstrates the eosinophil as the only inflammatory cell that contains phosphilipase B. Finally, a crude extraction of phospholipase B from the small intestines of mice infected with *T. spiralis* was accomplished.

CHAPTER II

MATERIAL AND METHODS

Mice

Male mice, BALB/c, were used in all experimental procedures. All mice used were adults, 10 weeks old. The mice were housed in a temperature controlled animal room in plastic cages with five mice in each cage. They were supplied with commercially prepared feed and water *ad libitium*.

To obtain the supply of mice for experimental studies a breeder colony was established. The original mice from which this colony was started were obtained from Charles River Laboratories, Wilmington, Massachusetts. The males and females were mated over a two week period (two females and one male). Pregnant females were then individually housed until giving birth. Approximately four weeks after birth the young were weaned and separated according to sex.

Parasite

The strain of *Trichinella spiralis* was originally obtained from Dr. N.F. Weatherly, Department of Parasitology and Laboratory Practice, University of

North Carolina, Chapel Hill. It has subsequently been maintained in the above mentioned mice. The techniques of Larsh and Kent (1949) and Weatherly (1970) were used for isolation, collection, standardization of the inocula, and infection of experimental animals.

Isolation of Larvae

The larvae were isolated from a mouse that had been infected with *Trichinella spiralis* for at least 45 days. The mouse was killed by cervical dislocation, the carcass skinned and eviscerated and rinsed in cool tap water. The carcass was coarsely minced with scissors and homogenized in a blender with digestion medium (1% HCl, 0.7% Merck granular pepsin, 1000 ml H_2O). The resultant solution was incubated at 37°C for two hours with constant agitation. After two hours the mixture was strained through two layers of cheese cloth into a one liter graduated cylinder. This was then incubated at 37°C for one hour to allow settling of the larvae. Following the incubation all but 200 ml of the solution was aspirated off and the remaining 200 ml poured into four 50-ml centrifuge tubes. These were incubated at 37°C for 10-15 minutes to again facilitate settling of the larvae. The larvae were present as a small pellet in the bottom of the tubes. With a Pasteur pipette they were removed and transferred to a 50-ml centrifuge tube filled with 0.85%

saline prewarmed to 37°C. This tube was incubated for 15 minutes at 37°C. The saline was aspirated down to 10 ml, being careful not to disturb the larvae pellet at the bottom of the tube.

Standardization of the Inocula

The collected larvae were standardized in a 5% gelatin nutrient broth suspension. The gelatin-nutrient broth suspension was heated to boiling and then cooled to 37°C before addition of the larvae. The approximate amount of broth needed was calculated on the basis of a 0.2 ml infecting dose per mouse and the number of mice to be infected. The broth was added to a 15-ml centrifuge tube and the larvae added drop by drop until the correct infecting dose was reached. The infecting dose was determined by counting the number of larvae present in 0.05 ml of the larvae-broth mixture using a stereoscopic microscope. The larvae-broth suspension was mixed and streaked on a glass plate with the use of a 1-ml tuberculin syringe fitted with a blunted 18 gauge needle and a length of plastic tubing. Larvae were added or removed from the broth-gelatin mixture until 25 percent of the desired number of live larvae were present in 0.05 ml of sample. This would render the desired number of larvae in the infecting dose, 0.2 ml.

Infection of the Animals

After reaching the proper concentration of larvae in the broth-gelatin mixture the mice were then infected. It was important that the mixture be maintained at 37°C to keep the larvae alive. Each mouse was intubated with 0.2 ml of larvae-broth suspension by using the 1-ml tuberculin syringe fitted with 18 gauge needle and a length of plastic tubing.

Collection and Preparation of Tissues for Eosinophil Enumeration and Phospholipase B Assay

The mice were sacrificed on the days post infection indicated in the tables and figures. The mice were killed by cervical dislocation. The entire small intestine was promptly removed and placed on ice. The small intestine was then freed of adhering tissues, the contents extruded from its entirety and cut into either two (anterior, posterior) or four (anterior, mid-anterior, mid-posterior, posterior) equal segments. The first centimeter of tissue from each segment was removed and embedded in Tissue-Tek II (Miles Laboratories, Naperville, Illinois) for tissue-eosinophil enumeration. These embedded samples were then frozen using Cryo-Quik (International Equipment Company, Nedham, Massachusetts) and maintained frozen (-10°C) until the sections could be cut. The remaining

small intestine was used for phosphilipase B determination. This technique allowed the numbers of tissue eosinophils and phosphilipase B to be compared in the same tissue segment.

The phosphilipase B activity was determined on each tissue segment. The method used to prepare and assay intestinal tissue samples for enzyme activity were exactly as described by Larsh et al. (1974).

Source and Preparation of Lysolecithin

Lysolecithin (Sigma Chemical Company, St. Louis, Missouri) was dissolved in distilled water to give a concentration of 2×10^{-2} M and stored in aliquots of 5 ml at -10°C. Immediately before use, the frozen samples were thawed in a water bath at 37°C.

Determination of Phospholipase B Activity

Each segment of tissue was weighed separately and minced with scissors. Segments were then individually homogenized in 19 volumes of ice cold 12.5% glycerol buffer medium (12.5% glycerol, 0.1 M potassium phosphate pH 6.6, 5×10^{-3} M $MgCl_2$, 2×10^{-3} M ethylenediaminetetracetic acid) in a motor driven, chilled Teflon glass homogenizer (Arthur H. Thomas, Philadelphia, Pennsylvania). After homogenization the brei was poured into 15-ml centrifuge tubes and placed on ice.

After a few minutes of settling, each intestinal homogenate was tested for phospholipase B activity. Samples of each homogenate (20-50 μl) were transferred to 15-ml conical centrifuge tubes and brought to a final volume of 0.6 ml with glycerol buffer medium. The tubes were incubated in a 37°C water bath for four minutes before starting the reaction to allow them to equilibrate. The reaction was started by the addition of 0.3 ml of $2x10^{-2}$ M lysolecithin prewarmed to 37°C. The contents of each tube were then gently mixed. For each tissue sample tested, an identical sample was incubated without lysolecithin, which served as a reaction blank. The incubation was continued until the reaction was terminated either at a fixed time (30 or 60 minutes) or upon formation of a cloudy precipitate. If a precipitate formed before five minutes had elapsed the original sample was diluted and run again. The reaction was stopped by the addition of 0.1 ml of 2N sulfuric acid and 1.0 ml of isopropyl alcohol. The tubes were mixed after each addition. After stopping the reaction, 0.4 ml of distilled water was added and the fatty acids extracted by the addition of 2.0 ml of heptane. To aid in the extraction of the fatty acids, the tubes were mixed on a Vortex-Genie (Scientific Industries Incorporated, Bohemia, New York) for two minutes. The tubes were then centrifuged at 500 xg for 10 minutes to

facilitate the separation of the heptane containing the extracted fatty acids from the aqueous part of the mixture. Precisely 1.0 ml of the heptane layer containing the fatty acids was then transferred by micropipette to a clean 15-ml glass centrifuge tube for titration. Tubes serving as reaction blanks were treated in the same manner as their corresponding experimental tubes.

Fatty acids were titrated according to the techniques described by Dole (1956). One ml of thymol blue indicator (Difco Laboratories, Detroit, Michigan) was added to each experimental tube and blank tube. The indicator was made fresh (1.0 ml stock, 40.0 ml isopropyl alcohol) before each use from stock solution (500 mg thymol blue, 500.0 ml absolute ethyl alcohol). The extracted fatty acids were then titrated with 0.01 N sodium hydroxide using a syringe microburet (Micro Metric Instrument Company, Cleveland, Ohio) fitted with a volume displacement syringe that delivered 1.0 microliter per division (Micro Metric Instrument Company, Cleveland, Ohio). The 0.01 N sodium hydroxide was prepared fresh each day from a stock solution and calibrated by titration against 1.0 ml of a reference standard of palmitic acid in heptane (2 μM/ml) (Eastman Kodak Company, Rochester, New York).

Throughout the titrations a stream of nitrogen gas was bubbled through the solution using a glass capillary tube. This served to expel carbon dioxide and kept the solution mixed during titration. The end point of the titration was reached when the color changed from pink to blue. The titration values were recorded for both the experimental and blank tubes along with the length of time the reaction ran and any additional dilutions that may have been necessary. The phospholipase B activity was expressed as millimoles of lysolecithin hydrolzyed per gram of wet tissue per hour.

Determination of Eosinophilia in Bone Marrow, Blood and Tissue

The percentage of bone marrow eosinophils was determined according to the methods of Larsh et al. (1974). The femur of the mouse was removed and freed of adhering muscle. The extremities were clipped with scissors and one end of the bone was inserted into a short section of plastic tubing mounted on a syringe. The femur was flushed with five ml of ice-cold isotonic saline. The extruded marrow was collected in a Teflon-glass homogenizer (Arthur H. Thomas, Philadelphia, Pennsylvania) and the marrow dispersed with 15-20 gentle strokes. The marrow cell suspension was then transferred to a 15-ml glass centrifuge tube. The cells were

separated from the fluid by centrifugation at 600 xg for six minutes. The fluid was drawn off and the cells resuspended in 0.2 ml of saline.

Eosinophil and total white cell counts were then made by transferring 0.05 ml of the bone marrow suspension to separate tubes containing either 0.5 ml of Discombe's dilution fluid (0.1 gm aqueous eosin, 10.0 ml acetone, 90.0 ml water) or 0.5 ml of white blood cell diluting fluid (3.0 ml acetic acid, 97 ml water, 1 drop gentian violet). The numbers of eosinophils and numbers of total white cells were counted using a hemacytometer. The percentage was then calculated by dividing the number of eosinophils by the number of total white cells and multiplying by 100.

The percentage of peripheral blood eosinophils was determined using established techniques (Kirk et al., 1975). The tail of each mouse was clipped and a blood smear was prepared. After drying, the smear was stained with Camco Quik Stain (Cambridge Chemical Products, Ft. Lauderdale, Florida) for 15 seconds and rinsed for 1 minute in distilled water. The smear was then gently flooded with distilled water to remove any stain precipitate. One hundred cells were counted which yielded the peripheral blood percentage of eosinophils.

For tissue eosinophil enumeration, frozen tissue samples were retrieved and six to 10 micron thick sections

were cut at -20°C using a Cryo-Cut Microtome (American Optical Corporation, Buffalo, New York). The sections were transferred to microscope slides and maintained frozen until they could be fixed and stained. The sections were fixed in chilled calcium-formal fixative for 10 minutes and then rinsed briefly in two changes of 200 ml of 0.1 M Tris buffer at room temperature (Ottolenghi et al., 1966). Sections were stained in Eosinophil Stain (Banco Laboratories, Ft. Worth, Texas) for two minutes, rinsed twice in distilled water, destained for 15 seconds in methanol, dehydrated in ethanol-xylene and mounted using Permount (Fisher, Fairlawn, New Jersey). Five sections were cut from each segment of the small intestine. The number of eosinophils was obtained by averaging the number of eosinophils present in five microscopic fields (x630) per tissue section. The fields were chosen at random with the one stipulation being that the outer muscular wall of the intestine be present in all fields. Where the tissue eosinophil response was reported for the entire small intestine, the number of eosinophils from all segments was averaged.

Collection of Cells for Histochemical Determination of Phospholipase B

Collection of Neutrophils

Peritoneal exudates were stimulated and cells collected as described by Mahmoud et al. (1973). The mouse's abdomen was alcohol swabbed and then 1.5 ml of sterile 10% proteose peptone (Difco, Detroit, Michigan) was injected intraperitoneally. The proteose peptone was also filtered through an in-line filter (Gelman, Ann Arbor, Michigan) to insure its sterility. After 18 hours the mice were sacrificed by etherization and five ml of Hanks Balanced Salt Solution (HBSS) was injected intraperitoneally. The abdomen of the mouse was gently massaged to bring all the cells free into the solution. The skin was then excised being careful to leave the peritoneum intact. The cell-containing medium was extracted from the peritoneal cavity with a 5-ml syringe. The cell suspension was transferred to a 15-ml centrifuge tube and the cells washed twice in a volume of 15 ml of HBSS. The cells were spun down at 100 xg for 10 minutes at 4°C. After the final wash the medium was drawn off with a pasteur pipette being careful not to disturb the pellet. The cells were resuspended in a volume of 1.0 ml of HBSS. The collected, concentrated

cells were then fixed to microscope slides using 30% albumin.

Collection of Lymphocytes

Lymphocytes were collected by orbital bleeding several mice and pooling the blood in heparinized containers. The blood was then layered on a Histopaque density gradient (Sigma Chemical Company, St. Louis, Missour) in 15-ml centrifuge tubes. The tubes were centrifuged at 400 xg for exactly 30 minutes at 4°C to facilitate the separation of the lymphocytes from granulocytes and red blood cells. Following separation the lymphocytes, present as an opaque band in the medium, were removed using a pasteur pipette and washed twice in 15-ml volumes of HBSS by centrifugation at 100 xg for 10 minutes at 4°C. The final wash was carefully removed and the cells resuspended in 1.0 ml of HBSS. The collected cells were fixed to microscope slides using 30% albumin.

Collection of Macrophages

Macrophages were collected as described by Unanue (1968). The mouse's abdomen was alcohol swabbed and 1.5 ml of sterile 10% proteose peptone was injected intra-peritoneally to stimulate peritoneal exudates. The proteose peptone was also filtered through an in-line filter to insure its sterility. After 3-4 days the cells

were collected. The mice were sacrificed by etherization and five ml of HBSS was injected intraperitoneally. The abdomen of the mouse was gently massaged to bring all the cells free into the solution. The skin was then excised being careful to leave the peritoneum intact. The cell-containing medium was extracted from the peritoneal cavity with a 5-ml syringe. The cell suspension was transferred to a 15-ml centrifuge tube and the cells were washed twice in a volume of 15 ml of HBSS. The cells were spun down at 100 xg for 10 minutes at 4°C. After the final wash the medium was drawn off with a pasteur pipette being careful not to disturb the pellet. The cells were resuspended in a volume of 1.0 ml of HBSS. The collected, concentrated cells were then fixed to microscope slides using a 30% albumin solution.

Collection of Eosinophils

Eosinophils were collected as described by Mahmoud et al. (1973). The mice were intubated with 250 *Trichinella spiralis* larvae as described earlier for isolation, collection, standardization of inocula and infection of experimental animals. Fourteen days after the initial larvae intubation an antigen challenge was made with *Trichinella spiralis*. The antigen was injected intraperitoneally at a concentration of approximately 1 mg/ml. This was done to boost the eosinophil response.

Subsequent antigen injections were made every seven days to keep the eosinophil level elevated.

The antigen was prepared from *Trichinella spiralis* larvae. The larvae were collected from a mouse that had been infected with *Trichinella spiralis* for at least 45 days as described above. Approximately 0.3 ml of washed, packed larvae and 5.7 ml of phosphate buffered saline were placed in a motor driven Ten Broeck Tissue Grinder (Corning Glass Works, Corning, New York). The grinder was submerged in shaved ice and the larvae ground at 300 rpm until no large fragments of larvae were found upon low power microscopic observation of the mixture. The grinder was then rinsed with 4.0 ml of phosphate buffered saline while operating at 300 rpm for 5 minutes. The wash was added to the centrifuge tube. The tube was placed at 4°C and left overnight to allow settling of the larger fragments and extraction of the protein. The next day the solution was centrifuged in a Sorval Superspeed RC2-B refrigerated centrifuge (4°C) at 1000 xg for 20 min. The supernatant was pulled off and the amount of protein present was determined by the Waddell technique (Waddell, 1956). The protein was diluted with 0.9% saline. With a saline standard the absorbance was read at A215 and A225. To calculate the protein concentration the absorbance at A225 was subtracted from the absorbance at A215, the difference was multiplied by 144 and then by

the dilution. This gave the protein concentration in micrograms per ml which when divided by 1000 gave the concentration in milligrams per ml. All protein determinations were determined using a Coleman Model 124 Hitachi Double Beam Grating Spectrophotometer. The antigen sample was then administered intraperitoneally with a 1-ml tuberculin syringe fitted with a 27 gauge needle. The *T. spiralis* antigen was administered in a volume containing approximately 0.5 mg of protein.

Four days after the fourth antigen injection the cells were collected. The mice were sacrificed by etherization and five ml of HBSS was injected intraperitoneally. The abdomen of the mouse was gently massaged to bring all the cells free into the solution. The skin was then excised being careful to leave the peritoneaum intact. The cell containing medium was extracted from the peritoneal cavity with a 5 ml syringe. The cell suspension was transferred to a 15-ml centrifuge tube and the cells were washed twice in a volume of 15 ml of Ottolenghi's media. The cells were spun down at 100 xg for 10 minutes at 4°C. After the final wash the medium was drawn off with a pasteur pipette being careful not to disturb the pellet. The cells were resuspended in a volume of 1.0 ml of HBSS. The collected, concentrated cells were then fixed to microscope slides using a 30% albumin solution.

Histochemical Determination of Phospholipase B at the Cellular Level

The methods of Ottolenghi et al. (1966) were used to demonstrate the presence or absence of phospholipase B in neutrophils, macrophages, lymphocytes or eosinophils. Briefly, populations of these cells were bound to microscope slides using a 30% albumin solution. The cells were fixed in chilled calcium - formal fixative for 10 minutes and then briefly rinsed in two changes of 200 ml of 0.1 M Tris buffer at room temperature. The microscope slides were placed in Coplin jars and incubated in 20 ml of reaction medium consisting of 20 ml Tris buffered medium at pH 6.6 containing 2 ml of 2.2×10^{-2} M lysolecithin and 1% cobalt acetate. The incubations were carried out in a water bath at 37°C for a time span ranging from 1 to 3 hours. After incubation the microscope slides were rinsed in a beaker containing approximately 200 ml of 0.9% sodium chloride, and placed in a Coplin jar containing dilute ammonium sulfide (8-10 drops of 22% ammonium sulfide in 35 ml of cold isotonic saline) and left for 30 seconds. While still wet the cells were counter stained with nuclear fast red stain (Allied Chemical, New York, New York) and rinsed in distilled water. The cells were then allowed to dry, mounted using Permount (Fisher, Fairlawn, New Jersey),

examined and photographed (x630) using Kodachrome 64 film and a Zeiss Standard 18 Microscope and Zeiss MC63 automatic/semiautomatic camera system (Carl Zeiss, Oberkochen, West Germany).

Extraction of Phospholipase B From Eosinophil Rich Intestinal Tissue

Phospholipase B was extracted from the small intestines of mice two weeks after infection with 400 *T. spiralis* larvae using a six-step procedure. For ease, the procedure will be presented in a step by step manner.

1. *Homogenization.* The small intestines were removed from eight mice and the contents extruded. The tissue was weighed, minced and homogenized in 19 volumes ice cold medium (8% sucrose, $1x10^{-2}$ M Tris pH 7.4, $1x10^{-3}$ $MgCl_2$, $1x10^{-3}$ M dithiothreitol). The homogenates were pooled and centrifuged (8,000 xg, 10 min.)

2. *Protamine precipitation.* The supernatant was added to 0.25 volumes of protamine sulfate (1% in H_20) at room temperature, incubated at 4°C for 20 minutes and centrifuged (25,000 xg, 15 min) in 50-ml tubes. The pellet contains the enzyme activity.

3. *Extraction with Tris-Triton X-100.* Five ml of 0.3 M Tris (ph 7.4, with $1x10^{-3}$ M dithiothreitol) and 0.5 ml Triton X-100 (1% in H_20) was added to each tube, the

pellet dispersed, the suspension diluted with 15 ml H_2O (with $1x10^{-3}$ M dithiothreitol) and transferred to an ice cold beaker. Centrifugation (25,000 xg, 15 min) yielded a supernatant containing 50% of the total activity.

4. Fractionation with ammonium sulfate. The supernatant (approx. 140 ml) was mixed with 1 volume of cold, 80% saturated $(NH_4)_2SO_4$ and stored at 4°C for 20 minutes. The light precipitate was centrifuged (25,000 xg, 15 min) out and the supernatant added to 0.5 volume cold saturated $(NH_4)_2SO_4$. After 30 min at 4°C a well defined precipitate was separated by centrifugation (25,000 xg, 15 min).

5. Fractionation on calcium phosphate gel. The precipitate was disolved in 40 ml cold 0.1 M Tris (pH 7.4, with $1x10^{-3}$ M dithiothreitol) and 12 ml calcium phosphate gel suspension was added. After mixing, the gel was separated by centrifugation (2000 xg, 10 min), washed two times with 60 ml NaCl (1%, $1x10^{-3}$ M dithiothreitol) and the enzyme was extracted with 20 ml 0.4 M K_2HPO_4 buffer (pH 7.4).

6. Dialysis and storage. The gel eluate was dialyzed for 24 hr against two changes of distilled H_2O (8L), and the clear solution stored at -15°C.

CHAPTER III

RESULTS

Studies were designed to determine if the tissue eosinophilia present during a parasitic infection was responsible for increased phospholipase B activity during both primary and challenge infections. Histochemical studies were also designed to determine if the eosinophil was the only infiltrating cell that carried enzyme activity. Finally, a procedure was modified to enable crude phospholipase B to be extracted from mouse intestinal tissue.

Intestinal phospholipase B activity and eosinophilia in mice given a primary infection with *Trichinella spiralis*. This experiment was done to determine the relation between increases in phospholipase B activity and increases in the number of tissue eosinophils in the small intestine of mice during a primary infection with *Trichinella spiralis*.

Animals were divided into two groups: 21 infected experimental BALB/c mice which received 200 *T. spiralis* larvae; and four noninfected control mice. Assays for phospholipase B and eosinophilia (bone marrow, peripheral, tissue) were done on three experimental mice on the days

indicated in the tables and figures. These results are expressed as the mean ± SE of three animals. A single control mouse was killed on days 7, 14, 21, and 30. These results are expressed as the mean ± SE of all four animals.

The data presented in Table 1 and Figure 1 demonstrate the bone marrow, peripheral blood and intestinal tissue eosinophil response, along its entire length, in animals infected with 200 *T. spiralis* larvae. All three parameters were in the range of those of the control animals on day 4 after infection. Control animals averaged 3.5% ± 0.2 eosinophils in the bone marrow, 2% ± 0.5 in the peripheral blood and 15.3 ± 0.1 per tissue section of the small intestine. Beginning on day 7 the experimental animals demonstrated a bone marrow and peripheral blood eosinophilia along with a tissue eosinophilia over the entire length of the small intestine. The bone marrow eosinophil was the most rapid in developing, reaching a peak response on day 10 (14.6% ± 1.2). This was followed by a paralleled increase in peripheral blood and tissue eosinophilia, both responses peaked on day 14 of the infection (peripheral blood 19.3% ± 1.4; tissue 35.9 ± 0.3 eosinophils per tissue section). All the parameters returned to the levels of the control animals by day 21.

TABLE 1 - Average* Bone Marrow, Peripheral Blood and Tissue** Eosinophilia in Mice Infected with 200 _Trichinella Spiralis_ Larvae (± Standard Error of the Mean).

Days Post Infection	Average Percentage Of Bone Marrow Eosinophils	Average Percentage Of Peripheral Blood Eosinophils	Average Number Of Tissue Eosinophils
4	5.3 ± 0.2	3.3 ± 0.3	14.9 ± 0.3
7	9.3 ± 1.5	7.6 ± 0.3	25.2 ± 0.5
10	14.6 ± 1.2	9.6 ± 0.3	27.6 ± 0.4
14	13.5 ± 2.1	10.3 ± 1.4	35.9 ± 0.3
18	3.9 ± 1.0	4.0 ± 0.5	27.8 ± 0.4
21	2.5 ± 0.3	1.5 ± 0.4	18.3 ± 0.3
30	3.8 ± 1.7	3.5 ± 0.4	18.2 ± 0.4
Controls***	3.5 ± 0.2	2.0 ± 0.5	15.3 ± 0.1

* = Average of 3 animals.
** = Average of all 4 segments of the small intestine for all animals.
*** = Average of 4 noninfected control mice killed on days 7, 14, 21, and 30.

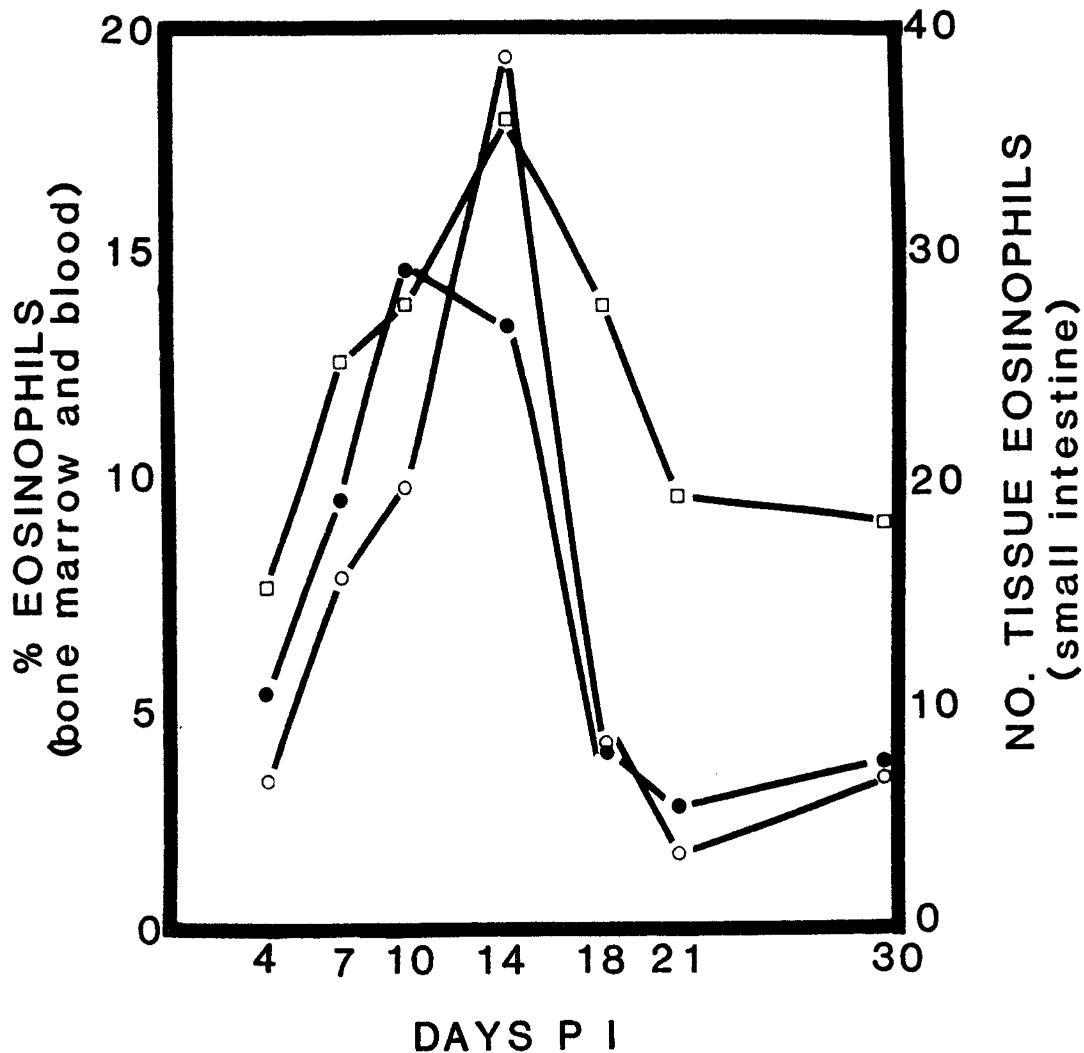


FIGURE 1 - Percentage of bone marrow (●—●) and peripheral blood (○—○) eosinophils and numbers of tissue eosinophils along the entire length of the small intestine (□—□) in mice infected with 200 *Trichinella spiralis* larvae. Each point represents the mean of 3 animals ± SE. SE bars that fell within the symbols were omitted for clarity.

The data shown in Tables 2 and 3, and Figure 2, A through D, describes the relation between tissue phospholipase B activity and numbers of eosinophils in the anterior, mid-anterior, mid-posterior and posterior segments of the small intestine. The results demonstrate that both tissue enzyme activity and numbers of tissue eosinophils increased (day 7 through 14), peaked (day 14) and returned to levels of the control animals (after day 21) over the same time period in each of the segments of the small intestine. Control animals averaged the following number of eosinophils and phospholipase B activity (μM lysolecithin hydrolyzed/gm. wet tissue/hr) in the segments of the small intestine with standard errors of about 20 to 50%: anterior, 15.0 and 206; mid-anterior, 17.0 and 489; mid-posterior, 15.2 and 968; posterior, 11.7 and 1,559.

Intestinal phospholipase B activity and eosinophilia in sensitized mice given a challenge infection with Trichinella spiralis. This experiment was designed to demonstrate the existence of an association between the anamnestic response of phospholipase B in the small intestine of sensitized mice, upon challenge with *T. spiralis*, with an anamnestic response of numbers of eosinophils in the same parasitized tissues.

TABLE 2 - Average* Phosphilipase B Activity** in the Anterior, Mid-Anterior, Mid-Posterior and Posterior Segments of the Small Intestines of Mice Infected with 200 _Trichinella spiralis_ Larvae. (± Standard Error of the Mean).

	Phospholipase B Activity			
Days Post Infection	Anterior Small Intestine	Mid-Anterior Small Intestine	Mid-Posterior Small Intestine	Posterior Small Intestine
4	5,584±530	5,328±490	999±42	2,314±982
7	13,140±5,108	26,226±7,411	20,178±492	18,264±7,011
10	39,040±4,241	60,840±9,290	51,440±7,711	48,000±1,558
14	75,680±3,139	80,240±4,764	86,720±9,846	89,600±6,879
18	19,747±1,743	29,415±1,845	31,610±5,529	3,612±3,977
21	7,249±3,536	13,159±237	8,532±1,340	9,738±2,944
30	157±38	24±9	130±41	528±22.6
Controls***	206±60	489±224	968±322	1,599±341

* = Average of 3 animals.
** = Phospholipase B measured as micromoles of lysolecithin hydrolyzed per gram of wet tissue per hour.
*** = Average of 4 noninfected control mice killed on days 7, 14, 21 and 30.

TABLE 3 - Average* Number of Tissue Eosinophils** in the Anterior, Mid-Anterior, Mid-Posterior and Posterior Segments of the Small Intestines of Mice Infected with 200 _Trichinella spiralis_ Larvae. (± Standard Error of the Mean).

Days After Infection	Number of Tissue Eosinophils			
	Anterior Small Intestine	Mid-Anterior Small Intestine	Mid-Posterior Small Intestine	Posterior Small Intestine
4	14.6±0.2	24.0±0.6	11.9±0.4	9.0±0.2
7	23.7±0.7	32.0±0.7	29.9±0.5	15.0±0.2
10	28.2±0.3	35.0±0.4	27.2±0.7	20.0±0.3
14	31.2±0.3	36.0±0.3	41.8±0.4	34.8±0.2
18	26.0±0.3	26.0±0.4	30.1±0.6	29.0±0.4
21	19.0±0.3	19.0±0.4	18.9±0.5	16.0±0.4
30	17.8±0.2	26.6±0.9	20.4±0.1	14.0±0.3
Controls***	15.0±0.3	17.0±0.5	15.2±0.2	11.7±0.3

* = Average of 3 animals.

** = The number of eosinophils was obtained by averaging the number of eosinophils from 5 microscope fields (X630) per tissue section from 5 tissue sections per segment.

*** = Average of 4 noninfected control mice killed on days 7, 14, 21 and 30.

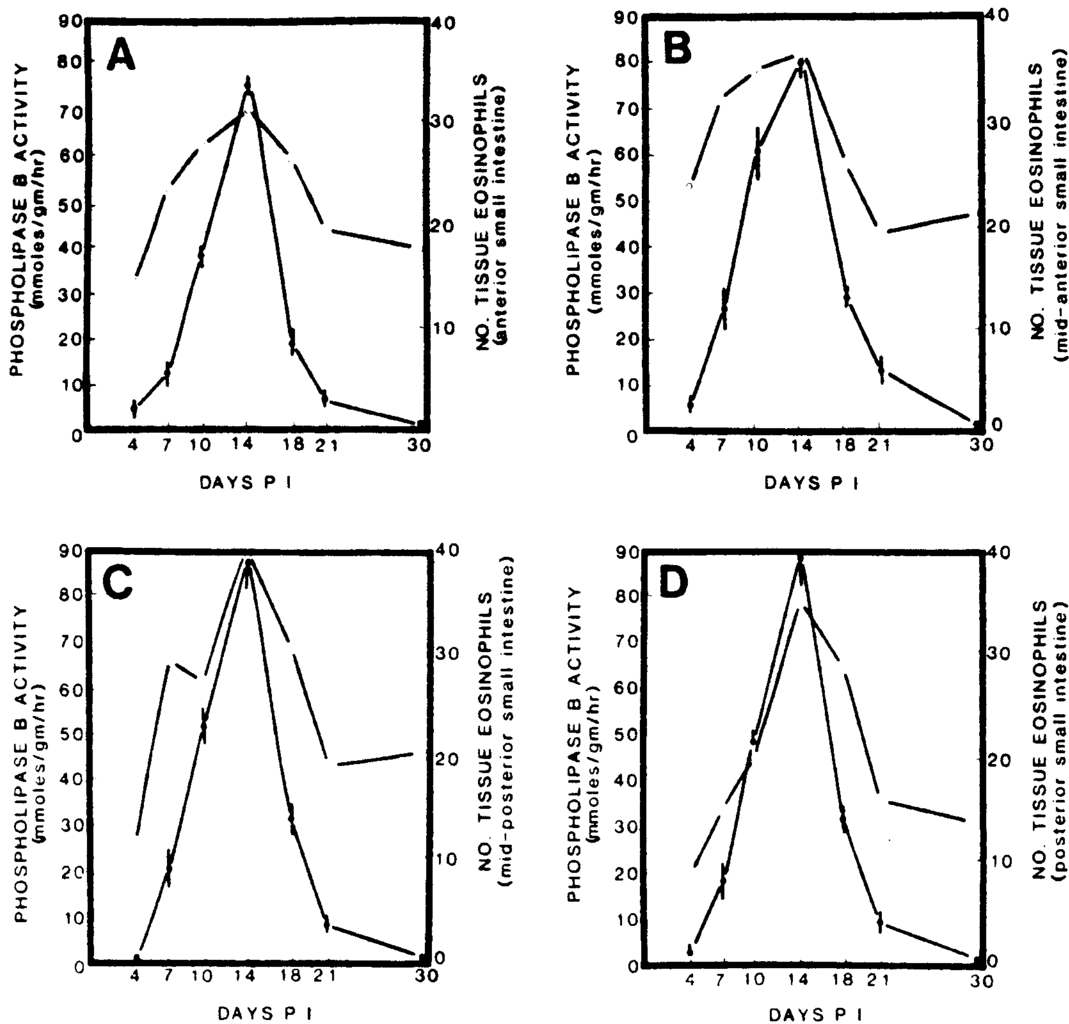


FIGURE 2 - Number of tissue eosinophils (o—o) and phospholipase B activity (●—●) in the anterior (A), mid-anterior (B), mid-posterior (C) and posterior (D) segments of the small intestine of mice infected with 200 Trichinella spiralis larvae. Each point represents the mean of 3 animals ± SE. SE bars that fell within the symbols were omitted for clarity.

Twenty-one mice were each sensitized twice with 200 *T. spiralis* larvae; the sensitizations were given 21 days apart. After the second sensitization 30 days were allowed to elapse before challenge to insure that the tissue inflammation had subsided and that the enzyme activity in the gut had returned to normal. After challenge with 200 *T. spiralis* larvae three mice were killed on each of the days indicated in Tables 4, 5, 6 and 7 and Figures 3, 4 and 5.

The percentage of bone marrow and peripheral blood eosinophils was found and the number of tissue eosinophils along the entire length of the small intestine was determined (Table 4, Figure 3B). The results were compared with the results found in the primary experiment reported above (Table 5, Figure 3A).

The number of eosinophils in the anterior, mid-anterior, mid-posterior and posterior part of the small intestine was determined along with the phospholipase B activity on the same tissue. The data from the anterior and mid-anterior was combined as was the data from the mid-posterior and posterior. This was done to facilitate the reporting of the data. These results are reported in Table 6 and Figure 4. These results were compared with the results found in the primary experiment reported above (Table 7 and Figure 5). To facilitate the

TABLE 4 - Average* Bone Marrow, Peripheral Blood and Tissue** Eosinophilia in Mice Sensitized Twice with 200 _Trichinella Spiralis_ Larvae and Challenged with 200 _Trichinella Spiralis_ Larvae (± Standard Error of the Mean).

Days Post Infection	Average Percentage Of Bone Marrow Eosinophils	Average Percentage Of Peripheral Blood Eosinophils	Average Number Of Tissue Eosinophils
2	10.2 ± 0.4	6.3 ± 0.6	13.1 ± 0.6
3	11.7 ± 0.3	12.3 ± 1.3	23.0 ± 0.1
4	15.8 ± 2.5	12.3 ± 0.9	27.7 ± 0.1
7	18.8 ± 0.5	24.3 ± 3.1	29.5 ± 0.1
10	25.7 ± 1.2	24.3 ± 3.1	30.4 ± 0.1
14	12.3 ± 2.2	17.7 ± 1.4	21.4 ± 0.1
24	6.5 ± 0.8	7.7 ± 0.7	17.2 ± 0.1
Controls***	7.3 ± 0.7	2.3 ± 0.2	13.9 ± 0.3

* = Average of 3 animals.
** = Average of all 4 segments of the small intestine for all animals.
*** = Average of 4 noninfected control mice killed on days 7, 10, 14 and 24.

TABLE 5 - Average* Bone Marrow, Peripheral Blood and Tissue** Eosinophilia in Mice Infected with 200 *Trichinella spiralis* Larvae (± Standard Error of the Mean).

Days Post Infection	Average Percentage Of Bone Marrow Eosinophils	Average Percentage Of Peripheral Blood Eosinophils	Average Number Of Tissue Eosinophils
4	5.3 ± 0.2	3.3 ± 0.3	14.9 ± 0.3
7	9.3 ± 1.5	7.6 ± 0.3	25.2 ± 0.5
10	14.6 ± 1.2	9.6 ± 0.3	27.6 ± 0.4
14	13.5 ± 2.1	19.3 ± 1.4	35.9 ± 0.3
18	3.9 ± 1.0	4.0 ± 0.5	27.8 ± 0.3
21	2.5 ± 0.3	1.5 ± 0.4	18.3 ± 0.3
30	3.8 ± 1.7	3.5 ± 0.4	18.2 ± 0.4
Controls***	3.5 ± 0.2	2.0 ± 0.5	15.3 ± 0.1

* = Average of 3 animals.
** = Average of all 4 segments of the small intestine for all animals.
*** = Average of 4 noninfected control mice killed on days 7, 14, 21 and 30.

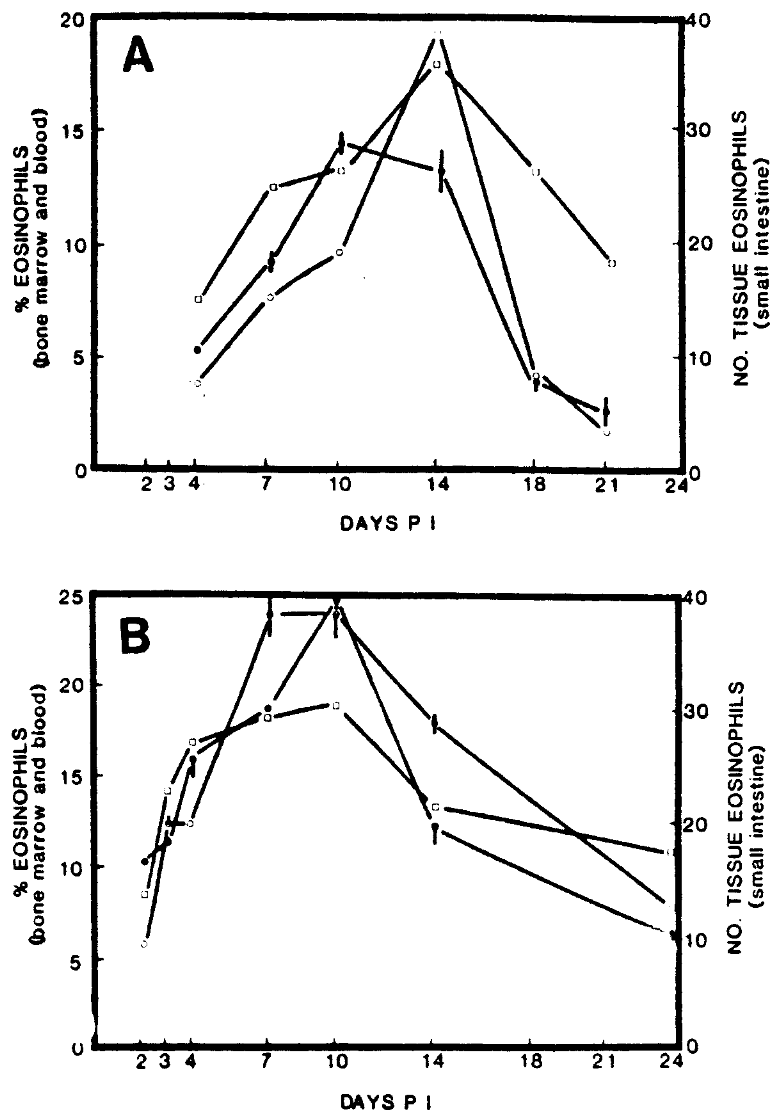


FIGURE 3 - Percentage of bone marrow (●—●) and peripheral blood (○—○) eosinophils and numbers of tissue eosinophils along the entire length of the small intestine (□ □) in mice given a primary infection of 200 *Trichinella spiralis* larvae (A) and in sensitized mice given a challenge infection of 200 *Trichinella spiralis* larvae (B). Each point represents the mean of 3 animals ± SE. SE bars that fell within the symbols were omitted for clarity.

comparison of these results with those of the previous experiment the four segments assayed in the primary infection were converted to two segments by combining the data from the anterior and mid-anterior segments and combining the data from the mid-posterior and posterior segments. This is reported in Table 7 and Figure 5. Normal eosinophil and phospholipase B data were obtained from an additional 4 uninfected mice which were killed on days 7, 10, 14 and 24.

The data in Table 4 and 5 and Figure 3 demonstrates the bone marrow, peripheral blood and intestinal tissue eosinophil response, along its entire length, in nonsensitized mice infected with 200 *T. spiralis* larvae and in sensitized mice challenged with 200 *T. spiralis* larvae.

The challenge infection (Table 4, Figure 3B) resulted in an anamnestic response, with all three parameters demonstrating an eosinophilia by day 3 versus day 7 for the primary infection (Table 5, Figure 3A). Unlike the primary infection the peripheral blood eosinophilia reached a peak response (day 7 and 10; 24.3 ± 3.1) before the bone marrow compartment (day 10; 25.7 ± 1.2). This suggests that there is a stimulation for the early release of eosinophils that precedes or occurs simultaneously with their accelerated production as found by Spry (1971a,b)

in rats challenged with intravenous *Trichinella* larvae. The number of eosinophils present in the tissues closely paralleled the peripheral blood response with a peak eosinophilia present on days 7 (29.5 ± 0.1) and 10 (30.5 ± 0.1).

Table 6 and 7 and Figure 4 and 5 demonstrate that one of the results of the accumulation of eosinophils in parasitized tissues in both the nonsensitized and sensitized mice infected with *T. spiralis* is an increase in phospholipase B activity.

The elevation in phospholipase B activity closely paralleled the increase in the number of tissue eosinophils in both the anterior and posterior segments of the small intestines in the animals given a primary infection (Table 7, Figure 6). When compared to the primary infection both the eosinophil number and enzyme activity in the mice given a challenge infection (Table 6, Figure 5) demonstrated an anamnestic response.

Both the tissue eosinophil and phospholipase B responses showed an increase by day 3 post infection. The peak enzyme activity occurred in both segments on day 4 (anterior 70,183 ± 2,983; posterior 65,613 ± 3,984) and was well on its way to control levels (anterior 348 ± 142 enzyme, 16 ± 0.4 eosinophil; posterior 1,263 ± 331 enzyme, 13.5 ± 0.2 eosinophil) in both segments by day 21 post infection. The tissue eosinophilia found in the anterior

TABLE 6 - Average* Phospholipase B Activity** and Tissue Eosinophilia*** in the Anterior and Posterior Segments of the Small Intestines of Mice Sensitized Twice with 200 Larvae and Challenged with 200 *Trichinella spiralis* Larvae (± Standard Error of the Mean).

Days Post Infection	Anterior Segment Phospholipase B/Tissue Eosinophilia	Posterior Segment Phospholipase B/Tissue Eosinophilia
2	12,539±2,259/15.2±0.2	10,773±1,547/10.9±0.2
3	53,733±1,365/27.7±0.4	51,866±5,095/18.4±0.2
4	70,183±2,983/31.9±0.2	65,613±3,984/23.5±0.2
7	49,910±3,004/32.2±0.3	42,805±6,215/30.6±0.2
10	26,875±3,958/31.2±0.4	38,050±1,294/26.4±0.3
14	6,923±2,938/22.6±0.3	15,093±3,955/22.9±0.3
21	686±425/17.6±0.2	2,001±363/17.4±0.2
Controls****	348±142/16.0±0.4	1,263±331/13.5±0.2

* = Average of 3 animals.
** = Phospholipase B measured as micromoles of lysolecithin hydrolyzed per gram of wet tissue per hour.
*** = The number of eosinophils was obtained by averaging the number of eosinophils from 5 microscopic fields (X630) per tissue section from 5 tissue sections per segment.
**** = Average of 4 noninfected control mice killed on days 7, 14, 21 and 30.

TABLE 7 - Average* Phospholipase B Activity** and Tissue Eosinophilia*** in the Anterior and Posterior Segments of the Small Intestines of Mice Infected with 200 _Trichinella spiralis_ Larvae (± Standard Error of the Mean).

Days Post Infection	Anterior Segment Phospholipase B/Tissue Eosinophilia	Posterior Segment Phospholipase B/Tissue Eosinophilia
4	5,456±341/19.3±0.1	1,206±234/10.5±0.2
7	19,683±4,142/27.8±0.4	19,221±6,411/22.5±0.2
10	49,940±4,381/31.6±0.1	49,720±3,641/26.5±0.3
14	77,960±1,555/33.6±0.1	88,160±6,553/38.3±0.1
18	24,581±1,001/26.0±0.1	31,611±2,389/29.5±0.2
21	10,204±1,938/19.0±0.1	9,135±1,346/17.5±0.1
Controls****	348±39/16±0.2	1,263±156/13.5±0.1

* = Average of 3 animals.
** = Phospholipase B measured as micromoles of lysolecithin hydrolyzed per gram of wet tissue per hour.
*** = The number of eosinophils was obtained by averaging the number of eosinophils from 5 microscope fields (630X) per tissue section from 5 tissue sections per segment.
**** = Average of 4 noninfected control mice killed on days 7, 14, 21 and 30.

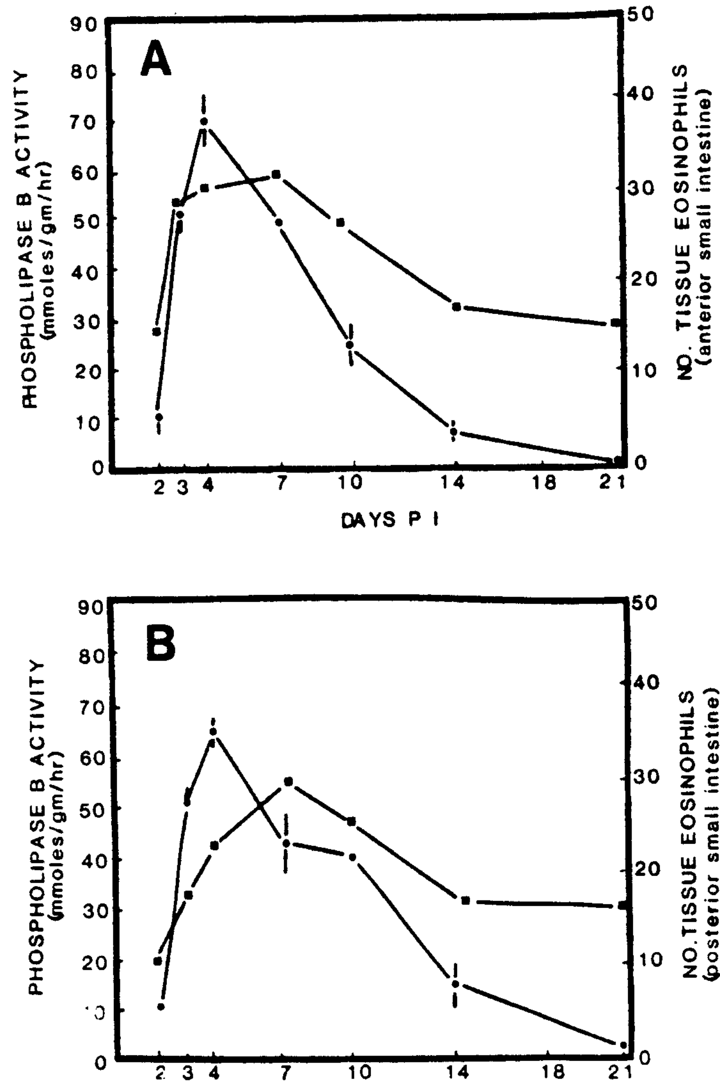


FIGURE 4 - Number of tissue (eosinophils (■—■) and phospholipase B activity (●—●) in the anterior (A) and posterior (B) segments of the small intestine of sensitized mice challenged with 200 Trichinella spiralis larvae. Each point represents the mean of 3 animals ± SE. SE bars that fell within the symbols were omitted for clarity.

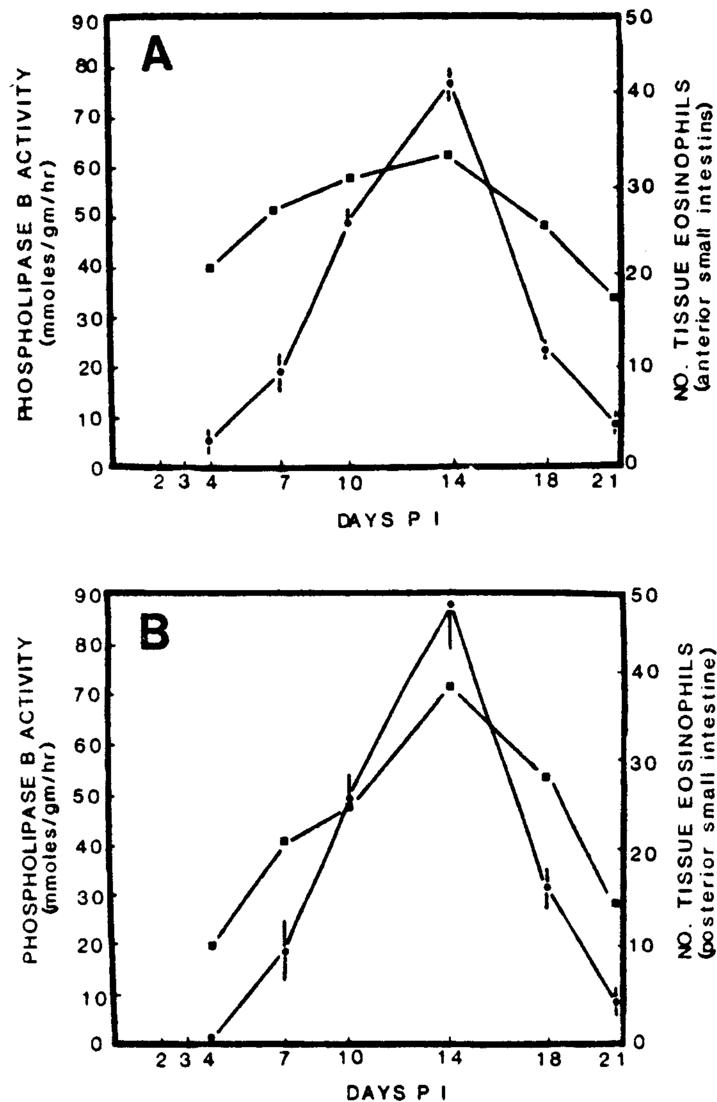


FIGURE 5 - Number of tissue eosinophils (■—■) and phospholipase B activity (●—●) in the anterior (A) and posterior (B) segments of the small intestine of mice given a primary infection with 200 *Trichinella spiralis* larvae. Each point represents the mean of 3 animals ± SE. SE bars that fell within the symbols were omitted for clarity.

segment of the intestine formed a plateau on days 4 (31.9 ± 0.2), 7 (32.2 ± 0.3) and 10 (31.2 ± 0.4), the same time period of peak enzyme activity in this tissue. The posterior segment of the intestine demonstrated peak tissue eosinophilia on day 7 (30.6 ± 0.2), somewhat after the peak enzyme activity of this tissue, but during the time of very elevated enzyme activity (days 4 through 10).

Histochemical staining of leukocytes for determination of phospholipase B content. For identification purposes cells that were harvested from peritoneal exudates were stained (Camco Quik Stain, Cambridge Chemical Products, Ft. Lauderdale, Florida) to determine their morphology. Figure 6, A through D, shows the individual cells as they were stained, identified and photographed. All cells were described and photographed at the same magnification (x630). Cells were counterstained with nuclear fast red stain.

For the demonstration of phospholipase B the cells were incubated in Tris-cobalt solution containing lysolecithin for 60, 90, 120 or 180 minutes. The cells that reacted positively appeared dark because of the reaction of phospholipase B, which they contained, with the specific substrate, lysolecithin. This reaction results in the liberation of fatty acids by enzyme hydrolysis, which are trapped as cobalt precipitates.

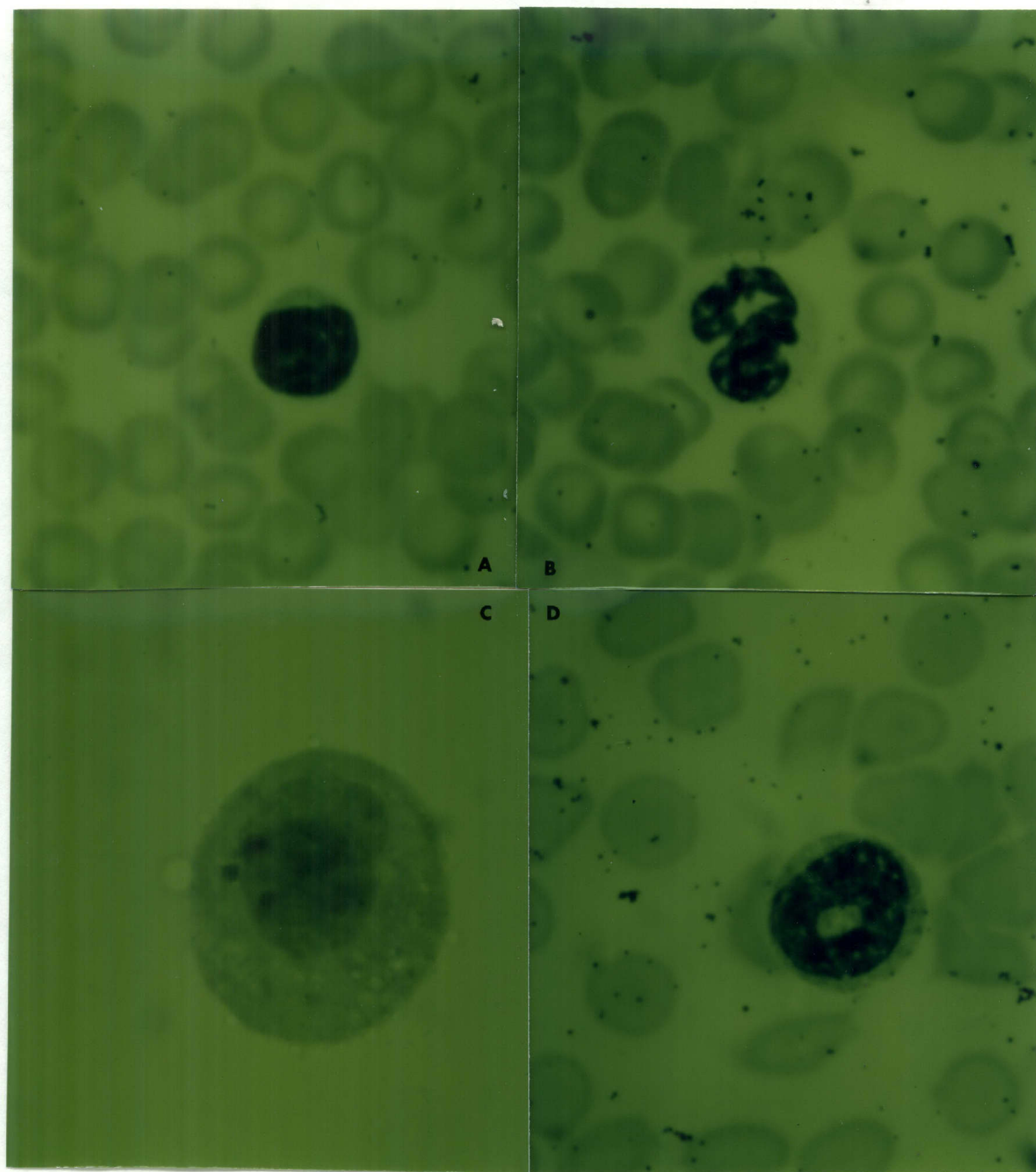


FIGURE 6 - Peritoneal exudate cells: A. Lymphocyte; B. Neutrophil; C. Macrophage; D. Eosinophil. All cells stained with Wrights-Giemsa. (X630)

When treated with ammonium sulfide the cobalt precipitates are converted to a dark color. The observation of these localized dark areas was the basis for the positive presence of phosphilipase B.

Figure 7, A through D, shows the different cell populations isolated after the reaction had been carried out. The best results seemed to be at 120 minutes incubation, for this reason all photographs are of cells after two hours incubation. Eosinophils Figure 7D, incubated in the Tris-cobalt medium with lysolecithin and treated with ammonium sulfide were the only cells to give a positive reaction. Small darkly stained areas were observed within and around the doughnut shaped nucleus. All other cells studied i.e., lymphocytes, neutrophils and macrophages were negative for any of this before mentioned staining.

Extraction of phospholipase B from eosinophil rich intestinal tissues. A crude extraction of phospholipase B was prepared from the small intestines of mice two weeks after an infection with 400 *T. spiralis* larvae. The purpose was to determine if the procedure could be performed in our laboratory and to prepare a supply of enzyme for later purification.

The product extracted from the intestine did contain enzyme activity. It hydrolyzed 5,108 micromoles of

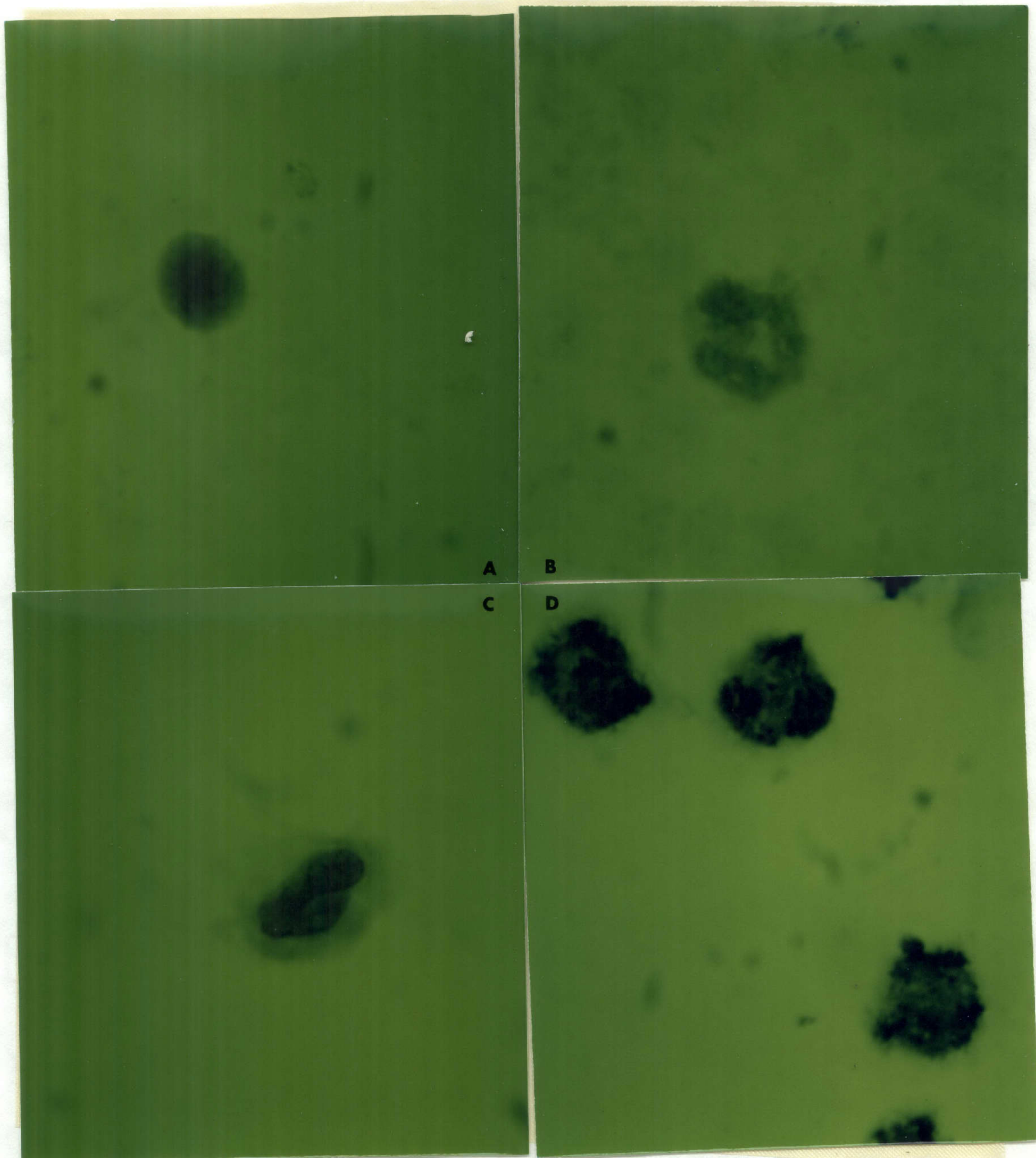


FIGURE 7 - Peritoneal exudate cells incubated with Tris cobalt-acetate medium and lysolecithin for 120 minutes: A. Lymphocyte; B. Neutrophil; C. Macrophage; D. Eosinophils. (X630)

lysolecithin per 100 microliter sample. This product was stored at -20°C for further purification.

CHAPTER IV

DISCUSSION

Previous studies have shown an association between elevated phospholipase B activity and bone marrow and peripheral blood eosinophilia in parasitic infections (Ottolenghi, 1973; Ottolenghi et al., 1975, 1977; Larsh et al., 1974, 1975; Goven 1979a,b, 1983; Goulson et al., 1981; Ngwenya and Capaci, 1982; Laubach, 1982). However, no investigators have attempted to demonstrate a direct association between tissue phospholipase B activity and the number of eosinophils in the same parasitized tissue. In the present study the association between bone marrow eosinophilia, peripheral blood eosinophilia and the number of eosinophils in parasitized tissues were compared in mice infected with *Trichinella spiralis*. The relationship of the number of tissue eosinophils in the small intestine to the phosphilipase B activity in this same tissue was of particular significance.

The initial study in this series of investigations examined the relation between eosinophils and phospholipase B activity in mice given a primary infection with *Trichinella spiralis* larvae. The results indicate that increased enzyme activity was directly associated with

eosinophilia; most importantly, the tissue enzyme activity was directly related to the numbers of tissue eosinophils present in the parasitized tissue over the course of infection.

Larsh et al. (1974, 1975) and Ottolenghi et al. (1975) have proposed a hypothesis to explain the association between the presence of a T. spiralis induced eosinophilia and elevated phospholipase B activity in the parasitized tissues. They suggested that delayed hypersensitivity, a T-lymphocyte dependent inflammatory response, was responsible for the increased production of eosinophils and their migration to sites of inflammation. The temporal relation between the development of bone marrow and peripheral blood eosinophilia with increased tissue phospholipase B activity during helminth infection led to the suggestion that the eosinophil was the source of the enzyme. It was suggested that the bone marrow eosinophilia and migration of the cells to sites of infection was due to a lymphokine (Colley's "eosinophil stimulation promotor") produced by T-lymphocytes after interaction with worm antigens, and that once in parasitized tissues the eosinophils released their stores of phosphilipase B.

Considerable evidence has been collected using the T. spiralis - rodent model to support the role of the T-lymphocyte in the induction and augmentation of eosinophilia (Weller and Goetzl, 1979). It has been shown

that specifically sensitized T-lymphocytes produced in helminth infections were critical for the stimulation of eosinopoiesis as well as eosinophil chemotaxis (Basten and Beeson, 1970; Colley, 1973). It has also been shown that thymus-derived cells play a central role in the eosinophil - phospholipase B relation (Goven and Moore, 1980) and the eosinophil has been indirectly shown to be the source of the enzyme (Goven, 1983).

The bone marrow and blood eosinophilia of the present experiment reported in Table 1 and Figure 1 paralleled those of Larsh et al. (1974, 1975) and Goven (1983). These results describe an almost immediate release of bone marrow eosinophils into the blood, indicative of the shortened eosinophil cycle found in animals given a _T. spiralis_ infection (Spry, 1971a,b). The tissue eosinophilia in the first experiment (Table 1 and Figure 1) demonstrated a persisting accumulation of peripheral eosinophils, produced in the bone marrow, into the site of inflammation, the intestine. The factors that are involved in directing this circulating eosinophil pool into tissues have not been proven, however, as noted above, it has been suggested that several T-lymphocyte dependent systems are involved.

The data presented in Table 2 and 3 and Figure 2, A through D, demonstrate that the accumulation of eosinophils in the small intestines is directly associated with an

increased phosphilipase B activity. These data, along with previous findings, provide evidence that the infiltration of eosinophils into sites of parasite induced inflammation results in an increase in the tissue phospholipase B activity of the parasitized tissue. The temporal association between the parasite induced tissue injury and parasite expulsion, previously described by Larsh & Race (1954), with the tissue eosinophilia - phospholipase B response reported here suggest that the enzyme, as an eosinophil product, is part of the inflammatory mechanism of the host to *T. spiralis* infection in the small intestine. Any effect that the enzyme has upon the parasite has yet to be answered; however, it is possible that the enzyme may act directly on the worm itself as proposed by Goetzl & Austin (1977).

The second study in this series examined the relation between eosinophils and phospholipase B activity in sensitized mice given a challenge infection with *T. spiralis* larvae. The results indicate that an anamnestic enzyme response was directly associated with an anamnestic eosinophil response where compared to mice given a primary infection.

The results for the bone marrow, peripheral blood and intestinal tissue eosinophil response in nonsensitized mice infected with 200 *T. spiralis* larvae and sensitized

mice challenged with 200 *T. spiralis* larvae can be found in Table 4 and 5 and Figure 3.

The challenge infection (Table 4, Figure 3B) resulted in an anamnestic response, with all three parameters demonstrating an eosinophilia by day 3 versus day 7 for the primary infection (Table 5, Figure 3A). Unlike the primary infection, the peripheral blood eosinophilia reach a peak response (day 7 and 10; 24.30 ± 3.10) before the bone marrow compartment (day 10; 25.7 ± 1.2). This is unlike the expected results as depicted by the primary response and suggests that there is a stimulation for the early release of eosinophils that precedes or occurs simultaneously with their accelerated production as found by Spry (1971a,b) in rats challenged with intravenous *Trichinella* larvae. The results did show a close correlation between the tissue and peripheral blood eosinophil levels. Both had a parallel increase, peak and decline in percentage and number as would be expected for this type of parasite infection response. The number of eosinophils present in the tissues closely paralleled the peripheral blood response with a peak eosinophilia present on days 7 (29.5 ± 0.1) and 10 (30.4 ± 0.1).

The close temporal association between the bone marrow, peripheral blood and tissue eosinophilia, in both the nonsensitized and sensitized animals, demonstrates

a parasite induced bone marrow eosinophilia, the release of these cells into the peripheral blood and the chemotaxis of these cells from the general circulation into sites of inflammation caused by the parasites, the small intestine.

The factors that are involved in the stimulation of eosinopoiesis and those involved in directing the circulating eosinophil pool into parasitized tissue, resulting in a persisting accumulation, have not been identified; however, considerable evidence has been collected using the *T. spiralis* - rodent model to support the role of the T-lymphocyte in influencing the eosinophil (Weller & Goetzel, 1979). It has been shown that specifically sensitized T-lymphocytes produced in helminth infections are critical for the stimulation, chemotaxis and augmentation of eosinophilia (Basten and Beeson, 1970; Rand and Colley, 1982; Spry, C.J.F., 1971). One of the results of the accumulation of eosinophils in parasitized tissues in both the nonsensitized and sensitized mice infected with *T. spiralis* is a direct increase in phospholipase B activity.

The elevation in phospholipase B activity closely paralleled the increase in the number of tissue eosinophils in all segments of the small intestine assayed in the animals given a primary infection (Table 7, Figure 5). Both of these responses occur during the same time period

as the parasite induced inflammatory response and the well documented worm expulsion (Larsh et al., 1974; Larsh and Weatherly, 1974).

The results for the challenged animals (Table 6, Figure 4) demonstrate an anamnestic response for both numbers of tissue eosinophils and enzyme activity. Both responses showed an increase by day 3 post infection; however, the peak tissue eosinophilia occurred after the peak tissue phosphilipase B activity in both segments of the small intestine. At first glance this goes against the hypothesis that suggests the eosinophil as the source of the enzyme. An explanation for these findings is that the tissue eosinophilia represents a persisting accumulation of eosinophils from the bone marrow compartment. Figure 3B shows that the bone marrow and peripheral blood eosinophil response is elevated during the same time period; however, due to the early immune expulsion of the adult worms, the eosinophils may not be programmed to degranulate or release their enzymes in response to antigen sensitized T-lymphocytes. Precedence for this explanation has been shown by Rand and Colley (1982) who have demonstrated that eosinophil stimulation promotor (ESP), a T-lymphocyte lymphokine produced upon exposure to helminth antigens, causes selective and time-dependent changes in the biochemical effector function of murine eosinophils. Another explanation may be that the eosinophils do not just

release their stores of phospholipase B into the tissues, but synthesize the enzyme in response to the presence of the worm antigen and/or T-lymphocyte stimulus. Thus, the number of eosinophils continue to increase slightly after the peak enzyme levels, but these eosinophils do not synthesize the enzyme due to the lack of action by the antigen or T-lymphocyte, because of the early worm expulsion.

As noted in the introduction and above Larsh et al. (1974, 1975) and Ottolenghi (1975) have proposed a hypothesis to explain the association between the presence of a parasite induced eosinophilia and elevated tissue phospholipase B activity. The present study demonstrates a direct relation between the accumulation of tissue eosinophilia, caused by a *T. spiralis* infection, and an elevated phospholipase B activity in the same site in primary or challenge infected mice given *T. spiralis*. Thus, this study gives direct support for the hypothesis that elevated phospholipase B levels present in both a primary or challenge infection with *T. spiralis* are the result of chemotaxis of eosinophils to sites of parasitized tissue.

The next study in this series of experiments was concerned with the demonstration of the eosinophil as the sole source of phospholipase B enzyme among the inflammatory cells. The results, Figure 7, demonstrate using

histochemical techniques that only the eosinophil possesses stores of phospholipase B. All other infiltrative cells, by view of their negative staining for the enzyme can be eliminated. These results lend support to the theory that the eosinophil is the only source of phospholipase B among the inflammatory cells in a parasitic induced inflammatory response (Larsh et al., 1974, 1975; Ottolenghi et al., 1975, 1977).

Finally, the extraction and partial purification of phospholipase B from the intestine of mice infected with *T. spiralis* was accomplished. This preparation will be used in the future for the development of a further purification scheme. A pure enzyme could be used to make an antibody that could be used to further study the role of the enzyme in worm expulsion.

The temporal relation between increased enzyme concentration, the inflammatory process and subsequent reduction in worm burden raises the question of the role of phospholipase B in the inflammatory process and its contribution to the host defense against parasites. The consistent finding of elevated levels of this enzyme in a variety of parasite infections involving different tissues indicates that phospholipase B may prove to be part of the common mechanism of the host for controlling the extent and direction of the infection process (Ottolenghi et al., 1977).

Ottolenghi et al. (1975; 1977) speculated that increased amounts of phospholipase B resulting from parasitic infections are probably indirectly involved in worm expulsion. It is possible that the enzyme is involved in the synthesis of prostaglandins, which have been shown to have a direct effect in parasite expulsion. The reaction of the enzyme with cell membrane phospholipids results in the production of free fatty acids. These fatty acids, such as arachidonic acid, could be converted to primary prostaglandins of the PGE and PGF series by prostaglandin synthase (Kelly and Dineen, 1976).

It has been shown by Dineen et al. (1974) that an infection of *N. brasiliensis* in rats could be expelled by prostaglandin-like factors recovered from rams' semen. Later, Kelly et al. (1974) demonstrated that prostaglandin E was highly effective in causing the expulsion of *N. brasiliensis* from the small intestine. Finally, Dineen and Kelly (1976) demonstrated that the concentrations of prostaglandin in the small intestine was elevated during a primary infection and showed an anamnestic type response in a secondary infection.

Therefore, although direct evidence is lacking, it can be speculated that the worm burden is eliminated in part by the indirect action of phospholipase B, through its production of prostaglandin precursors. Through this

action, the enzyme would play an important role in the complex mechanism of parasite elimination from the host.

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