MONTANA STATE UNIVERSITY

The immunology of spontaneous cure in the Nippostrongylus brasiliensis-mouse system
by William Hiram Benjamin

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Microbiology
Montana State University

Abstract:
Either cells or serum from immune animals has been found to be able to abrogate the patency of a larval N. bra-siliensis infection when given to naive mice. All pools of cells that could cause abrogation of a larval patency were mesenteric lymph node cells collected from mice on days 9-12 after a primary N. brasiliensis infection. This correlated with a lymphadenopathy that was shown to occur at about days 6-12 after larval infection. No cell pools collected from secondary infections demonstrated the ability to protect a naive mouse to this extent nor did cells collected before or after this period. Pools of serum from mice 13 days after a second infection were commonly found to be able to transfer as high a level of protection as protective cells. A lot of variability in the degree of protectivity was found in both the serum and cell pools, even in pools that were apparently prepared in the same manner.

The serum factor was found to precipitate in 50% saturated NH4(SO4)2, was resistant to 56 C for 30 minutes, was dialyzable, was freeze-thaw resistant and apparently didn't bind to larvae in a way that adaptively transferred immune cells could act on the larvae. Protective serum was more effective when given on day 3 of a larval infection than when given on day 0 only. Protective serum injected intraduodenally on day 4 of an infection had no effect on the patency of the worm infection.

Expulsion of a N. brasiliensis larval infection by mice is very radio-resistant as shown by normal expulsion after 350 rads on day -1 only slightly delayed expulsion after 450. rads, The lesion created by 450-550 rads given before a larval infection can be repaired by 1 x 10^8 immune mesenteric lymph node cells and worms are expelled nearly at the normal time in mice given 550 rads and normal thymocytes on day -1.

Attempts were made to transfer immunity to nude mice with protective immune serum or immune mesenteric lymph node cells or a combination of the two, none of which transferred any apparent protection.

Evidence is presented that suggests that the expulsion of N. brasiliensis from mice isn't as prostaglandin dependent as the N. brasiliensis rat system. Neither aspirin nor in-domethacin delayed expulsion times. Experiments in which prostaglandins were injected intraduodenally also had little effect on an ongoing infection which is different than had been shown in the rat system.

The role of macrophages in the expulsion of a N. brasiliensis infection in mice was approached by using a macrophage lysozyme inhibitor, trypan blue. Larval brasiliensis infections in mice pretreated with trypan blue, then maintained on a chronic treatment schedule maintained their worms longer than controls. Mice given normal adult worms intra-duodenally after pretreatment and continued chronic treatment with trypan blue expelled their worm burden about the same time as non-trypan blue treated mice. These results suggest that trypan blue interfered in an afferent vs. effector immune function.

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Date 5 Oct 1979

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by

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A thesis submitted in partial fulfillment
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MASTER OF SCIENCE

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Microbiology

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June, 1980

ACKNOWLEDGMENTS

I gratefully acknowledge the support and guidance given by Dr. N. D. Reed, my advisor. Thanks is given for his taking time when there was no time in his busy schedule and especially for his confidence in me.

Special thanks go to fellow graduate students, Dennis Bier and Brad Brooks, for many hours of discussion, technical help and friendship.

I would also like to thank my wife, Sarah, for her encouragement, love, patience and typing ability displayed during this work. I also appreciate the training in the art of mouse catching received from my daughter, Heather.

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INTRODUCTION

There are several reasons to study the immune response of mammalian hosts to intestinal nematodes. The most obvious is the large number of humans and their economically important domestic animals that are infected with nematodes (18,32). Some of the more common human intestinal nematodes are; Ascaris, which infects approximately one in every four people in the world; hookworms, which infect approximately 400 million people; and Trichuris, which infects approximately 350 million people worldwide (53). Intestinal nematodes aren't just a third world problem as shown by Warren's estimates of human infection in the United States (64). Other studies show half or more of children in some low-income rural communities in the Southeast U.S. are infected with Ascaris or Trichuris (1). A surprising amount of disease--both chronic disease, such as anemia and malnutrition, and the more acute medical emergencies, which are usually caused by Ascaris--occurs in the U.S. as a result of intestinal nematode infection (1).

The immunological response of humans to these nematodes isn't well studied. The apparent lack of expulsion of the adult worms and even the apparent lack of developed resistance to subsequent larval infection is well known (47) (although normal adults in Ascaris endemic areas seem to be

more resistant to challenge than their counterparts from nonendemic areas (37)). The lack of protection is evident even in apparently normal adult individuals, even though the individuals have antibodies of several classes to several antigens released by or present on the parasites (37,38). Some researchers have demonstrated cell-mediated immunity to be active in intestinal nematode infections in humans without evidence of protective immunity (59).

Currently, the best way to control most of these intestinal infections, at least in humans, is education and higher living standards. However, considering the poor response to this approach, vaccines seem to be a useful, possible adjunct. Because of the parasite-related problems incurred in livestock management, nematode vaccines will always be in demand (47). Much effort has been made in trying to prepare vaccines in nematode-host systems with very few usable results (5).

The first vaccines tested for nematode diseases were patterned after the bacterial vaccines. Attenuated (irradiated) worms and various preparations of killed worms have been tried. These have multiple deficiencies such as: lack of effective protection, being hard to prepare and store, and requiring adjuvants and large volumes.

The next step in preparing vaccines against intestinal nematodes seems to be finding out how these parasites can be acted upon by the host, the host response which is responsible for protection, and the antigen that stimulates this protective response (5). The best system in which to study this problem would be a parasite that stimulates long-lasting and complete immunity in its host. Several such systems with laboratory animals as the host are known (62), but even in the best characterized of these systems, neither the mechanism of expulsion nor the protective antigen(s) is known.

The Nippostrongylus brasiliensis rat system is probably the most completely studied of all of the intestinal nematode-host systems. I will now confine most of my remarks to this system because the system I am using is the analogous system of N. brasiliensis in mice. The rat system has been extensively reviewed (10,11,18,27,42,43,44) in the fairly recent literature and I won't attempt to duplicate this. The biology, host, geographical distribution, systematics and life cycle have been adequately reviewed by Haley (10,11). I will briefly give the life cycle here.

N. brasiliensis was first described in 1914 by Travassos and has been recovered in the wild from Rattus norvegi-

cus, Rattus rattus and Mus muscularis. Typical of all nematodes, there are four moults during the life cycle of N. brasiliensis. The immature eggs are passed in the feces of the host and, in proper conditions, embryonate and hatch in about 1 day. The first moult occurs on about day 1, with the second shortly thereafter. These first two larval stages, called rhabditoid larvae, feed on live bacteria in soil-feces or feces-charcoal mixtures. The third larval stage, which is the filariform or infective stage, crawl up to a high point where they wait until a prospective host passes by. The larvae, which are actively thermotactic, penetrate normal rat skin and feed their way to the lungs via the bloodstream and/or lymphatic channels in 11-15 hours. The larvae feed on blood in the lungs and moult at about 36 hours after infection. They are then coughed up and swallowed and the fourth stage larvae reach the small intestine where they take up residence and begin the fourth moult in about 48 hours (42). Fertile eggs are found in the feces of the host at 6 days post infection. The adult worms live in the duodenum and jejunum where they feed on host tissue but cause little in the way of specific pathologic lesions in light infections.

The infection in rats is essentially terminated after

12 days (42). The worms are eliminated by spontaneous cure which may leave a small residual worm burden but which confers complete immunity to subsequent infections. This phenomenon is probably the best studied expression of host immunity to a gut nematode and has been reviewed extensively (18,42,43,44).

Most of the work conducted to determine the active components and mechanisms of action in the expulsion of the worms during spontaneous cure has been done in rats. Studies have shown the immune expulsion of larval infection in mice closely parallels or is identical to that seen in rats, except it occurs a little earlier in mice (day 9 or 10) than in rats (day 12)(28,29). This work was done with a rat strain of N. brasiliensis. It was found that mice reject both the rat strain and the mouse adapted strain of N. brasiliensis on the same day but a greater percentage of the mouse adapted strain reach the adult stage (66).

One approach to understanding the mechanisms of expulsion and to affirm the immunological nature of spontaneous cure is to try to inhibit expulsion of worm burdens by immunosuppression. Some tools that delay expulsion are neonatal thymectomy, cortisone derivative treatment, lethal x-irradiation, thoracic duct drainage, and antithymocyte

serum (27). Recently, it has been shown that athymic mice (nude) don't expel a worm burden unless they are thymus grafted or injected with thymocytes (15,16,34). Taken together, these results suggest a thymus dependent step is necessary in the development of immune expulsion of N. brasiliensis.

Rats were observed to retain their worm burden longer than normal when infected during two special periods of the normal rat life cycle. One was during the neonatal period before 7-9 weeks of age, depending on the strain (40), and the other was during lactation (4). When the worms remaining after day 14 from either of these cases are examined carefully, it is evident that they are abnormal; these worms are given the label "damaged". Damaged worms are rapidly eliminated from normal rats if transferred directly into the duodenum. Normal worms, when transferred, are eliminated in 8-10 days, but damaged worms are eliminated in 5-6 days. The damaged worms have altered gut structure, the cells are vacuolated and the gut is distended. There are changes in isoenzyme patterns, reduced feeding on host tissue as measured by P^{32} uptake from the host, and the female worms have a reduced number of eggs in their uteri. During an infection in a normal rat, the worms are found to

be damaged by day 11 also, but are then rapidly eliminated from the host (44). In mice, the damage occurs earlier and is evident by day 6 (28).

A second method used to determine the mechanism of self-cure is to attempt to passively immunize naive animals with components from immune animals. Several different investigators have found that some degree of protection can be achieved with certain serum pools (41,52). Large amounts of serum are required and the passive protection is generally not nearly as effective as active immunity in eliminating worms. In a large group (48 pools) of sera tested, 1 out of 3 pools was somewhat protective (41). Hyperimmunization of donors with repeated larval infections didn't seem to increase the probability of a pool being protective or increase the titer of protection (41). A few cell preparations from immune rats and mice have been found which will protect nonimmune animals when transferred alone into them. However, protection approaching that seen in immune animals challenged with a larval infection has been achieved reproducably only by giving a rat or mouse both immune cells and immune serum before challenge (28,29).

Combining the two tools of passive and adoptive immunization and the neonatal and lactating rat models which are

defective in worm expulsion, a hypothesis of two step requirement for expulsion has been put forth (42). Both neonatal rats and lactating rats can cause the phenotypic changes in worms which is characteristic of damage, but in both of these systems the adult worms are expelled very slowly. Immune cells transferred into either of these systems from adult mice will cause expulsion in normal animals. Serum transferred from neonatal and lactating rats has been shown to cause increased protection of recipients against larval challenge. From this work it is felt that there is a humoral factor (antibody) that damages the worms which must precede the step requiring lymphocytes which causes the rapid expulsion (42,44).

Several groups of researchers then attempted to define the damaging factor(s) and the expulsive factor(s). To study the expulsive factor, a truncated infection was used. Worms were left in one rat until damaged, then transferred to the experimental host to determine what cells were required for the second rat to eliminate the worms (42,44).

Using this approach, damaged worms were transferred into rats irradiated at sublethal (440 rads) or lethal (750 rads) levels. These rats were then reconstituted to find out the cellular requirment for expulsion. It was found

that the lethally-irradiated animals required both an immune lymph node cell, presumably lymphocytes, and a bone marrow component to effect expulsion within 4 days. The sublethally irradiated animal needed only the immune lymph node cell (6,25). Several cell types were postulated as being the one supplied by the bone marrow. One of the more favored possibilities was the monocyte or macrophage (44).

It recently has been shown that rats lethally irradiated with 650-750 rads, then given damaged worms intraduodenally, can be reconstituted to expel the worms in 3 days if given Ig^- thoracic duct lymphocytes collected from rats infected 7-8 days or 13 days before collection. One of two pools of MLNC also caused expulsion from lethally irradiated animals (45).

Another interesting thing shown by this work is that even though there are in thoracic duct lymph on days 7 and 8 cells capable of causing expulsion, the worms normally wouldn't even be damaged until day 10 or 11. These results suggest that in a primary infection with N. brasiliensis the damaging step actually occurs after there are cells capable of effecting the expulsion in the thoracic duct lymph, making the damaging step the rate limiting step. It is well documented that pools of cells from like infections

have different activity when transferred (23,41).

The mechanism of action of the cell causing the expulsion is unknown. It is felt that the worms aren't killed in the small intestine but die in the adverse environment of the colon (42). Therefore, the cell that causes expulsion would only have to cause the worm to lose its hold for awhile. Because no cell has been seen to attach to the worm in the lumen of the intestine, it is thought to be a substance released by cells or an environmental change in the gut that causes expulsion (46).

Workers in this system have assumed that the humoral damaging factor is antibody and have tried to find the class that was produced at the right time in an infection to cause damage. One interesting thing about parasitic infections has been the high IgE levels, both specific for worm antigen and specific for unrelated antigens the animal comes in contact with (43). The role of IgE in passive protection and its active amine-releasing role in active infections has largely been discounted in damage or expulsion of N. brasiliensis (43,44). Recently, worm-specific IgA has been found in the gut of infected rats at the time that damage is occuring. The importance of this class in the host-parasite relationship hasn't been defined (49,54).

One study (20) demonstrated that the *in vivo* protective fraction of protective sera fractionated on one type of an ion exchange column contained mostly antibody of the IgG_1 subclass and protective fractions were found that contained little IgE, IgM or IgA.

One real problem with studying the damaging antibody is that it can only be demonstrated to damage worms *in vivo*. Worms live just as long in immune protective serum as they do in normal rat serum *in vitro* (21,31). Another reason that the effect of antibody can't be monitored *in vitro* is because, even in the optimum cultural conditions, after the worm is kept *in vitro* for 3 days, it looks exactly like *in vivo* damaged worms in all respects (31).

Recently, non-steroid anti-inflamatory agents such as aspirin and indomethacin have been demonstrated to delay expulsion of worms in a rat larval infection. Because a known activity of these agents is to inhibit prostaglandin synthesis, workers in Australia (9) injected purified prostaglandins directly into the gut on day 6 of an infection and found that this increased expulsion. This work interpreted with some even more recent work suggests that prostaglandins by themselves cause damage to the worm (50).

This inconclusive evidence, coupled with the fact that

a serum protective capacity doesn't correlate with any known antibody activity and the fact that sera from multiply infected rats don't protect more often nor at higher dilutions than sera from animals infected only once, suggests a factor other than antibody being the "damaging antibody". This is also suggested by the inconsistency of analogous pools of antiserum to transfer passive protection (41). Some work done in the mouse model also suggests that the damaging factor may be other than immunoglobulin. Mice panspecifically immunosuppressed with anti-μ expelled their worms at the same time as the normal control suggesting that specific antibody isn't necessary for expulsion (17).

This thesis will address the following areas of the N. brasiliensis system:

1) The macrophage has been suggested as a possible active participant in the expulsion of N. brasiliensis from rats. I will investigate the role of the macrophage in the self-cure phenomenon in mice by using the macrophage lysosome inhibitor, trypan blue.

2) The role of prostaglandins in the expulsion of N. brasiliensis will be investigated using prostaglandin inhibitors and synthetic prostaglandins.

3) I believe that the humoral damaging factor has been

incompletely studied. Other groups have just assumed that it is antibody and tried to find out what class of immunoglobulin is active or the antigenic specificity of the protective antibody. I propose that the humoral protective factor may not be immunoglobulin and will attempt to find protective sera pools from immune mice and characterize the factor.

MATERIALS AND METHODS

Parasites

A mouse-adapted strain of *N. brasiliensis* was used in all experiments. This strain was derived from a rat strain by Dr. R. B. Wescott, Washington State University, Pullman, WA. (56,66) and has been passaged serially through at least 400 generations in mice.

N. brasiliensis was maintained in our laboratory by injecting 900 IL_3 in 0.1 ml saline subcutaneously on the dorsal aspect of the mouse. Feces were then collected on days 6, 7 and 8 post-infection by keeping mice in cages with 0.8 cm mesh wire bottoms over moist paper towels. The feces were then comminuted with water and mixed with moist granular animal bone charcoal (VWR Scientific, San Francisco) in a feces to charcoal ratio of 1:4 V:V. The mixture was divided between petri dishes and incubated at room temperature. Moisture was maintained throughout the incubation period. Larvae were used between days 5 and 20 after the culture was initiated.

The larvae were harvested from charcoal cultures by placing 40 C 0.85% NaCl in a small funnel with a screen (30 mesh/cm) which was covered by 2 layers of Kimwipes (Kimberly Clark Co., Neenan, Wisconsin). The larvae

migrated through the Kimwipes and were recovered after 1 hour by opening a clamp on the end of the funnel. The larvae were then washed twice in 0.85% NaCl by centrifuging at 200 g for 1.5 minutes. A suspension of 3,000 or 9,000 IL_3/ml was prepared by counting worms in 0.1 ml of suspension under a stereoscope, then resuspended in the correct volume of saline. Mice were then infected with 300 or 900 IL_3 as above.

Fecal Examinations

The modified McMaster technique of Whitlock (67) was further altered for use in estimating the number of worm eggs per gram of feces (EPG). Fecal material was comminuted with saturated NaCl (sp. gr. 1.20) in a ratio of 1 gm feces to 30 ml saline. Commonly, 0.25 gm feces was used with 7.5 ml saline. A portion of this suspension was rapidly transferred to a fecal egg counting chamber (Cutler-Haver-Lockhart Laboratories, Shawnee Mission, KS.). Counts of eggs from both grids of the chamber were averaged and corrected by a dilution factor of 200 to arrive at the EPG count.

Animals

All mice used in this work are either BALB/c raised in

this institution or a BALB/c Bom. line carrying the nude (nu) gene. The latter line was bought from Bomholtgoard, Ltd., Ry, Denmark and maintained in this laboratory by mating heterozygotes (+/nu) to obtain nude (nu/nu) congenitally thumus-deficient mice and their littermates (hereafter designated LM) which are one-third wild type (+/+) and two-thirds heterozygotes (+/nu). All mice were maintained on Wayne Lab blox and acidified water ad libitum. All animals were between 8- and 12-weeks old at the beginning of an experiment unless otherwise noted. The rats used to donate worms were Lewis strain females.

Necropsy and Worm Recovery

Mice were killed by cervical dislocation and the entire small intestine was quickly removed. The entire length of the small intestine was opened with scissors and placed in 37 C 0.8% NaCl on 1 thickness of gauze suspended in a plastic 250 ml specimen cup. The temperature was maintained in a water bath for 1-2 hours. Intermittent mixing was used to allow the adult worms to migrate to the bottom of the cup. The gut was then examined for residual worms. The worms that had migrated to the bottom of the cup were then counted under a stereoscope at about 10X as they were

picked up with a Pasteur pipette on a 10 ml syringe.

EP♀

The number of eggs in individual female worms (EP♀) was counted under a microscope at 63X on lined microscope slides. EP♀ from 20 to 50 female worms were counted from each mouse and the mean was calculated. EP♀ from mice which had less than 20 female worms weren't recorded.

Cell Transfer

Cells for transfer were prepared by dissecting out the organ of interest--either the mesenteric lymph node or thymus--and gently screening through a 100 mesh stainless steel screen to prepare single cell suspensions. The cells were then counted in a hemocytometer, washed once in balanced salt solution and resuspended to the correct concentration and, unless otherwise indicated, 1×10^8 cells were injected IV in a 0.5 ml volume.

Trypan Blue

Trypan blue was purchased from Kallestad Labs, Inc., Minneapolis, Minnesota and prepared according to Hibbs (13). The trypan blue was dissolved in double-distilled water and dialized against water for 72 hours at 4 C.

The dialyzed material was then lyophilized and stored at room temperature until used. Trypan blue was resuspended in 0.85% NaCl to a concentration of 10 mg/ml. Mice in the trypan blue treated groups received 3 mg IP 24 hours or 48 hours before the beginning of the experiment and 0.5 mg or 1 mg IP 3 hours or 1 day before the experiment. Mice in all treated groups were then given 0.5, 1 or 2 mg every third day subcutaneously until the experiment was terminated. Dosage protocols are indicated in footnotes to tables and figure legends.

Aspirin

Aspirin was acquired in tablet form from Bayer Company, New York, N.Y. The percentage active ingredient (acetylsalicilic acid) was determined. Two hundred mg of the active ingredient were dissolved in 2 ml of 95% ethyl alcohol then diluted to 50 ml with a 0.5% Tween 80 in water solution. Thus, the final concentration of acetylsalicilic acid was 4 mg/ml and 20 gm mice were given 0.25 ml, which is approximately 50 mg/kg, twice daily. Control groups were given the diluent without aspirin (3,24).

Indomethacin

Capsules of Indocin were obtained from Merck, Sharp &

Dohme. It was found that the active ingredient (indomethacin) was 10% of the capsule content's weight. The drug was dissolved in 0.125% Tween 80 to an active ingredient concentration of 0.04 mg/0.2 ml which was given by stomach tube twice daily beginning on day 0 (2).

Per Os Treatment Procedure

Mice were given drugs per os using intramedic polyethylene tubing (Clay Adams, Parsippany, N.J.) with outside diameter of 0.048 inches. A 1 ml tuberculin syringe with a 21 G needle inserted into the tubing was used to inject the drug. A tongue depressor was cut to 7 mm in width on one end which would fit crosswise in the mouse's mouth. A hole was drilled in the tongue depressor just large enough to allow the tube to pass through. It was positioned so the tube was guided down the esophagus when the awake mouse was held to maintain a straight neck. The tubing was inserted with a gentle twisting motion until it reached the stomach and the contents of the syringe were then emptied into the mouse's stomach.

Ammonium Sulfate Precipitation of Serum

Saturated ammonium sulfate was added dropwise to serum that had been diluted 1:1 with 0.85% NaCl until a volume

equal to the diluted serum had been added. Precipitation was then allowed to proceed at 4 C for 30 minutes. Then, the precipitate was separated by centrifuging 800 g for 30 minutes at 4 C. The precipitate was resuspended in phosphate buffered saline pH 7.2 (PBS) and both the precipitate (globulin-rich) and the supernatent (albumin-rich) fractions were dialized against 200 volumes PBS for 12 hours. Each fraction was reconstituted to the original serum volume and given to test mice, 0.5 ml IV and 0.5 ml IP (65).

Irradiation of Mice

Mice were irradiated using a ^{60}CO source while individually immobilized in a slowly-revolving plastic container. Source to skin distance was always 70 cm; the final dose received is listed in the appropriate places in the text.

SRBC Plaque Assay

The antibody response of mice was quantitated by a slide modification (33) of Jerne's (19) localized hemolysis-in-gel technique. Mice were plaqued on day 4 after being immunized IV with 0.25 ml of a 10% SRBC suspension.

Laporotomy for Inserting Adult Worms Intraduodenally

Normal rat worms (day 7 post-infection) were transferred to the duodenum of mice using the method of Jacobson & Reed (16). Briefly, the mouse's stomach and proximal duodenum were exteriorized through a 3-5 mm ventral incision. A purse string suture was placed in the greater curvature of the stomach and a Pasteur pipette containing 150 worms was inserted into a puncture wound in the area circumscribed by the purse string. The 150 worms were deposited in the duodenum approximately 1-2 cm from the pyloric sphincter. The pipette was removed and the purse string suture was tightened.

Intraduodenal Injections

The duodenum was exteriorized as in worm transfer and the drug or serum was then injected intralumenally into the duodenum within 1 cm of the pyloric sphincter with a 30 G needle.

Serum

Mice were bled retroorbitally while under ether anesthesia. The blood was then allowed to clot at room temperature for 2 hours, then it was refrigerated for 24 hours.

The serum was then separated from the clot and centrifuged to separate cells from the serum. The serum was then passed through a 0.45 μm millipore filter and stored at -20 C until it was used, when it was thawed at 37 C.

Freezing, Thawing and Heating Serum

Serum was frozen in a methanol bath at -50 C and thawed on ice. This was repeated 5 times. Serum was then used in the appropriate experiments. A second sample of serum was frozen, thawed and then maintained in a 56 C water bath for 60 minutes, then used in the appropriate experiments.

Cyclophosphamide

Cyclophosphamide (Mead, Johnson & Co., Evansville, Indiana) was dissolved in PBS to a concentration of 4 mg/ml. One-half ml was given to mice on day 2 of a larval infection (34).

PCA

IgE responses were measured in Lewis female rats using heterologus passive cutaneous anaphylaxis (PCA) assay (48). Mouse serum specimens were diluted serially 2-fold and 0.1 ml of these dilutions were injected intradermally into the shaved back of a rat. Four serum specimens could be titrat-

ed to 2^8 or 2^{10} in one rat. Forty-eight hours later the rats were given 1 ml IV of a solution containing 2,000 worm equivalents of N. brasiliensis antigen and 5 mg of Evans blue. The rats were killed after 30 minutes and the results were read as positive where a blue circle could be seen on the underside of the skin. The antigen used in this test was prepared by collecting worms from rats infected with 4,500 IL_3 8 days before collection. These worms were counted and macerated thoroughly in a glass tissue homoginizer. The preparation was then centrifuged at about 700 g for 10 minutes and the supernatent was frozen and used as antigen in the PCA test.

Prostaglandins

Synthetic prostaglandins used in this study were kindly donated by the UpJohn Company, Kalamazoo, Michigan. The prostaglandins PGE_1, PGE_2 and $PGF_{2\alpha}$ were received as crystalline solids and were dissolved in 95% ethyl alcohol, 10 mg in 1 ml. This was further diluted 1:10 with 0.2 M sodium phosphate buffer, pH 7.4. One-tenth ml of this was injected intraduodenally for a dosage of 100 µg/mouse (24).

Statistical Treatment

Worm recovery data are usually given as arithmatic mean

± standard deviation (SD). Because of the variance seen in this system, the number of worms recovered from individual mice is also listed most of the time. The method used to get EPG data precluded any statistical treatment as feces from all mice in a group were collected on the same tray. The Student T test was used to assess the significance of differences noted between means and a probability (p) value of less than 0.05 indicated significance.

RESULTS

Chronic Trypan Blue Treatment of Mice and the Elimination of *N. brasiliensis*

The role of macrophages in elimination of an infection of *N. brasiliensis* was approached by the method of inhibiting macrophage lysosomal enzymes with trypan blue (TB). This chemical inhibition was chosen because of its apparent lack of marked effect on the afferent and efferent limbs of the acquired immune response, except for the killing by macrophages of tumor cells (13,26).

The experimental design of the first TB experiment is shown below:

	Day -3	Day -1	Day 0	Day 3 and Every 3rd Day Thereafter
Group 1	-	-	$300IL_3$	-
Group 2	3mg TB IP	0.5mg TB IP	$300IL_3$	0.5mg TB SQ

Four of the mice in the experimental group died before monitoring of egg count began. Egg counts per gram (EPG) feces were monitored daily starting on day 6 (Figure 1).

The egg counts in the TB group were sustained at a high level for about 6 days longer than those in the control group. There was a definite high plateau in the EPG counts which suggests that the worm damage phase was delayed.

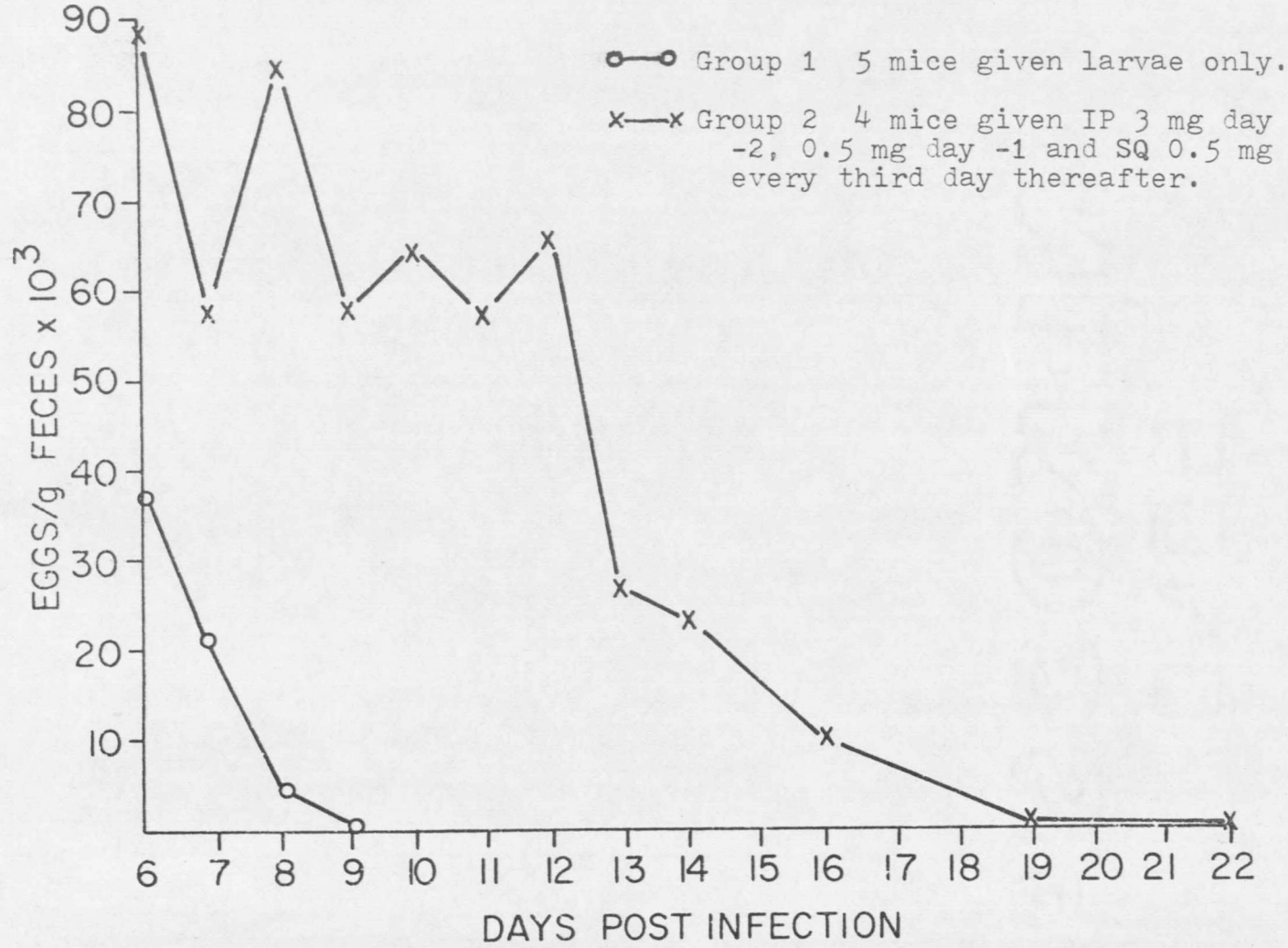

Figure 1. The effect of chronic trypan blue treatment on the EPG production of a N. brasiliensis larval infection.

Expulsion occured, but may have been delayed a little. The worm specific IgE production in these mice was measured by passive cutaneous anaphylaxis (PCA) and was found to be rising from day 18 to day 25 in the TB-treated group, which might be expected if the worms were maintained longer. The PCA titers achieved in the TB-treated mice shows that the IgE response was fairly normal (Table I).

Because the first TB experiment (Figure 1) suggested that TB prolongs the time of egg production in a larval infection, the following experiment was designed to determine if TB inhibits the damaging step or the expulsion step.

	Day -2	Day 1	Day 0	Day 2 and Every 3rd Day Thereafter
Group 1	-	-	$300IL_3$	-
Group 2	3mg TB, IP	1mg TB, SQ	$300IL_3$	1mg TB, SQ

Some mice from each group were killed on days 6, 8, 10 and 12 and the number of worms recovered was recorded. As a measure of damage, the number of eggs per female (EP♀) was also determined (Table II). The TB-treated group apparently allowed more worms to reach maturity by day 6 ($P<.05$) and also retained more worms on day 10 ($P<.05$) than did the control group. The EP♀ data were inconclusive. By day 12,

Table I. Specific N. brasiliensis PCA titer of serum of normal and trypan blue-treated mice.

		Day 18[a]	Day 25[a]
Group 1	Control mice	128	64
Group 2	Trypan blue-treated mice[b]	64	256

[a] Day post infection.
[b] See Figure 1, treatment of group 2.

Table II. Effects of trypan blue treatment of mice on a N. brasiliensis larval infection.

	Control				Trypan blue[a]			
	EPG[c]	No. Mice Killed	Worms[b] Rec.	EP♀[d]	EPG[c]	No. Mice Killed	Worms[b] Rec.	EP♀[d]
Day 6	9,000	4	73 (±327)	8.3	31,200	5	135.4 (±33.6)	12.5
Day 7	4,900				44,000			
Day 8	900	4	98 (±112)	13.7	18,300	4	157 (±47.7)	19.3
Day 9	400				16,300			
Day 10	0	4	.5 (±1)	-	6,600	5	145 (±53.2)	13.4
Day 11	0				4,600			
Day 12	0	3	.33 (±1)	-	1,400	4	15 (0-61)	-

[a] Each mouse given 3 mg TB IP day -2, 1 mg SQ day -1 and 1 mg TB SQ every third day thereafter.
[b] Worms Rec. = average number of worms recovered/infected mouse.
[c] EPG = average worm eggs/gram of infected mouse feces.
[d] EP♀ = average eggs/female worm.

Worms recovered
Day 6 Control vs. Test $P<.05$
Day 8 Control vs. Test NS
Day 10 Control vs. Test $P<.050$

the worms had virtually all been expelled from all but one mouse in the experimental group. The EPG were higher in the TB-treated group and remained higher about 5 days (Table II).

I believed that if adult worms were inoculated into the gut of TB-treated mice, there would be less stimulation of the immune response and a greater effect of TB might be realized. Therefore, worms were recovered from 7-day infections of Lewis rats and 150 worms were placed by laporotomy in the duodenum of mice of 2 groups. The control group was made up of normal 10-week-old littermates. The test group was 10-week-old littermates which had been given 2 primary doses (3 mg day -2, 1 mg day -1, IP) of TB, and then were maintained on 1 mg TB subcutaneously every third day. EPG feces counts, monitored beginning on the day after laporotomy, were similar in all groups (Figure 2).

It was possible that the worms were damaged during the transfer from rats to immunologically competent mice and that by transferring worms into nude mice, the status of the transferred worms could be deduced. To test this, 3 groups of mice were used. Two groups consisted of 10-week-old littermates. The mice in Group 2 were pretreated with TB then maintained on 2 mg every third day, whereas, the mice

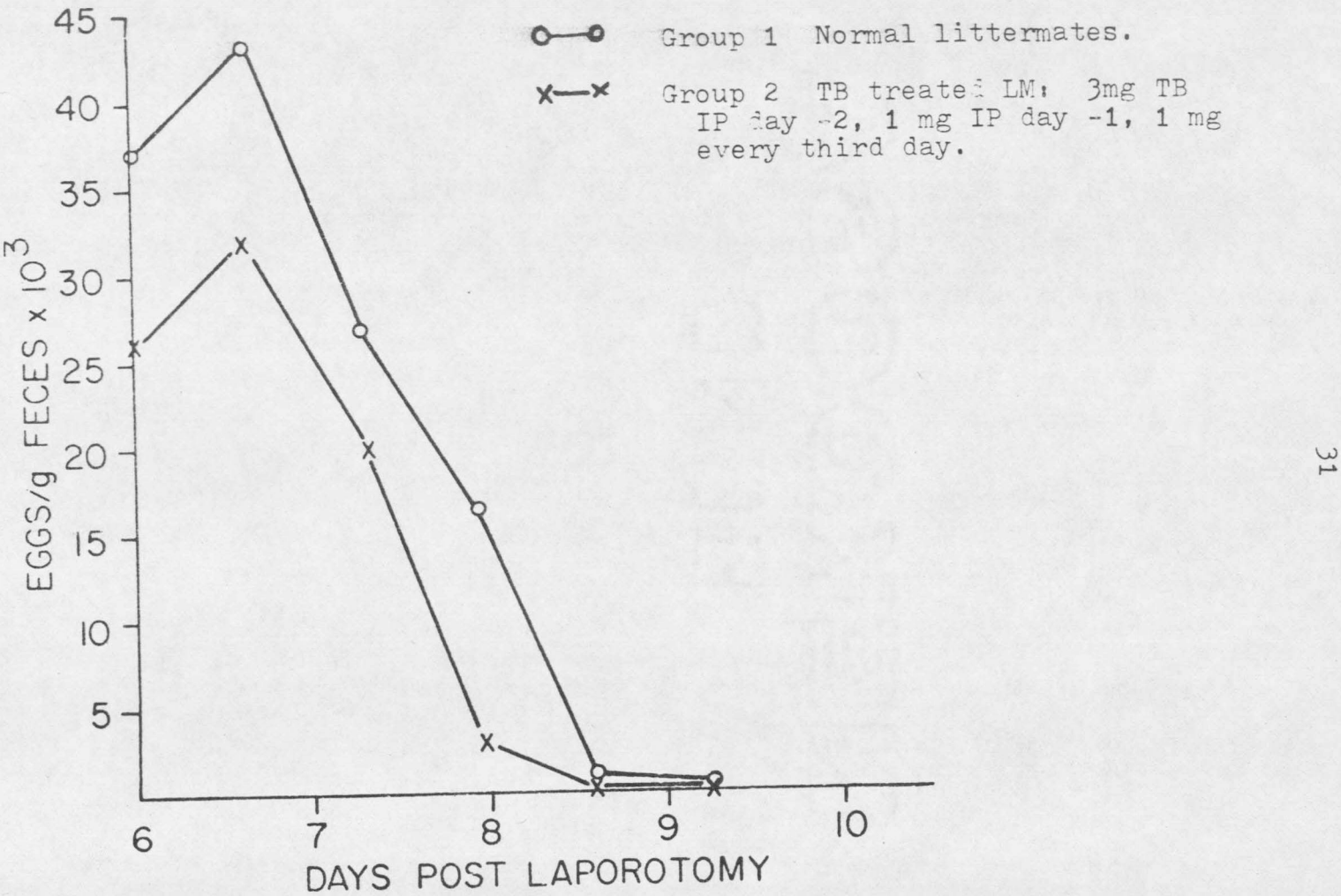

Figure 2. The effect of trypan blue treatment of mice on the elimination of normal rat worms given by laporotomy to mice.

in Group 1 were normal controls. The third group was 6 nude mice. One hundred fifty normal rat worms, which contained 28.6 EP♀, were transferred by laporotomy on day 0 into all mice and egg counts were monitored beginning on day 1 post transfer (Figure 3 and Table III).

The egg counts didn't appear to be different. Worms were recovered from the nudes when they died (Table III). The surgical procedure apparently was very stressful to the nudes, causing all but one to die within 9 days. The worms recovered from some nudes appeared to be damaged as measured by EP♀, though it isn't known whether this was caused by the poor condition of the nudes or whether the worms were damaged between the time they were removed from the rat and the time they were placed in the nudes. The high and variable numbers of worms recovered from groups 1 and 2 as late as days 8 and 9 raised the question of the surgical procedure interfering with expulsion more than the TB (Table III).

Collectively, these data show that chronic TB treatment of mice before and during a larval infection with N. brasiliensis allows more worms to reach the adult stage and the worms are retained in the host longer (Table II). This results in higher EPG counts that are maintained longer

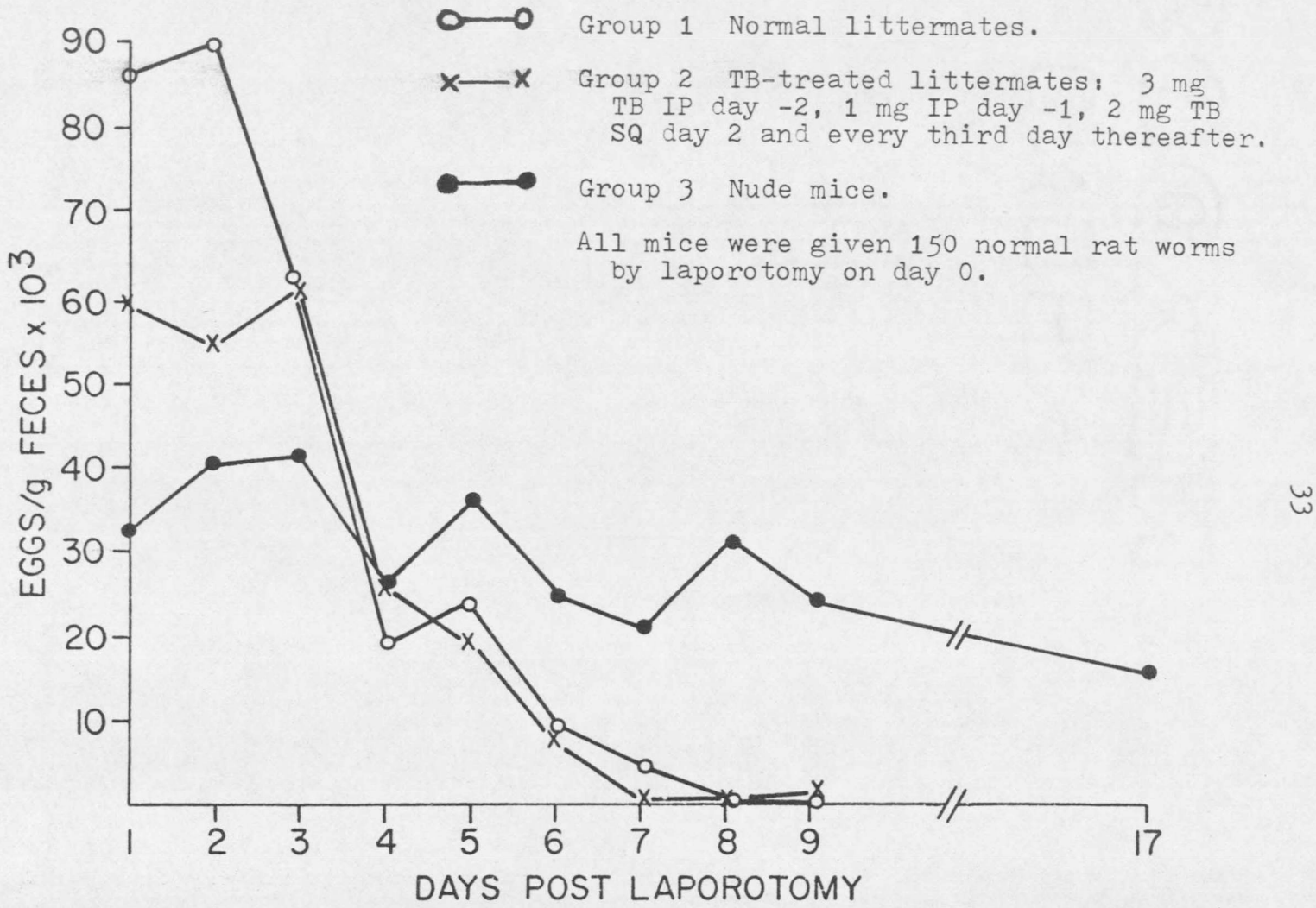

Figure 3. The effect of trypan blue treatment of mice on the elimination of normal rat worms given by laporotomy to mice.

Table III. Worms recovered after transfer of normal rat worms into normal, trypan blue-treated and nude mice.

	Group 1 Control[a]		Group 2 Trypan blue[a,b]		Group 3 Nudes[a]	
	Worms Rec.	EP♀	Worms Rec.	EP♀	Worms Rec.	EP♀
Day 5	177	4	183	3.3	154	7.7
Day 6					137	28
Day 7					160	11.9
					132	5.5
Day 8	74	6.25	193	6.0		
	0	-	4	-		
Day 9	53	3.9	170	6.6	115	22.5
	17		1			
	9		1			
	0		0			
	0		0			
			0			

[a] All mice given 150 normal rat worms by laporotomy on day 0.
[b] See legend Figure 2 for TB administration schedule.

(Figure 1, Table II). TB has much less effect on an infection in mice obtained by transferring adult normal rat worms into TB-treated mice (Figure 2, Table III). At this time, it is unknown whether the effects of TB are caused by a specific depression of macrophage effector function or whether it is just a depression of the general health of the mouse.

Prostaglandins and the Response of Mice to a Larval Infection of N. brasiliensis

Expulsion of a N. brasiliensis infection in the rat can be delayed by prostaglandin inhibitors (8,9). The following series of experiments was done to examine the possibility that prostaglandins are important in the expulsion of worms from mice also.

The first experiment was designed to determine if aspirin (acetylsalicilic acid), a prostaglandin inhibitor, would inhibit worm expulsion. Eight-week-old Baylor mice were divided into 5 groups. One group, the control, got only larvae. Mice in 2 other groups each got only aspirin diluent, 1 group orally and the other group intraperitoneally. Mice in 1 test group each received 50 mg/kg aspirin twice a day orally via gastric tube and mice in the second test group each received 50 mg/kg intraperitoneally. This

dose was adopted from work done in mice using aspirin as a prostaglandin synthesis inhibitor (3) and was used only after the dosage (3 mg/kg, twice daily) given by Dineen, et al (9) to rats was found not to delay expulsion of a larval infection (data not shown).

Two mice in the oral aspirin and 1 in the intraperitoneal aspirin group died of intestinal bleeding, and stomach ulcers were grossly visible in 2 of the 3 mice. The egg counts in all groups dropped between day 7 and 8 and all groups were virtually negative by day 9. Because egg counts were similar in all control groups, they were combined for presentation in Table IV. Likewise, the 2 different aspirin-treated groups were combined in Table IV. As in the previous experiment (data not shown), aspirin did not reproducably increase the length of patency or delay expulsion of the worms.

A second drug which is a known prostaglandins inhibitor, indomethacin, was also used. Indomethacin previously had been used in a nematode (Trichuris muris)-mouse system and the interpretation of the experimental results was that indomethacin delayed expulsion by inhibiting prostaglandin synthesis (2).

Three groups of 7-week-old BALB/c males were given 300

Table IV. EPG and worms recovered from N. brasiliensis infected mice treated with aspirin[a].

	Control[b]	Aspirin[b,c] Treated Mice	
EPG Day 6	21,600	31,000	
EPG Day 7	20,600	36,300	
EPG Day 8	3,000	5,700	
EPG Day 9	200	1,300	
Worms Recovered Day 10	Control 0,0,0, 0,0,0	Oral ASA[cc] 117,45,0, 0,0	IP ASA[cd] 33,12,1, 0,0

[a] All mice given 300 IL_3 on day 0 and were killed and worms counted on day 10.

[b] The EPG counts for all control groups were similar and were combined. Likewise, the EPG counts of the 2 different aspirin treatment groups were similar and were combined.

[c] 50 mg/kg aspirin given twice daily from day 0, either orally (cc) or intraperitoneally (cd).

IL_3 and used in this experiment. Group 1 received no further treatment. Mice in group 2 received 0.2 ml 0.125% Tween 80 (indomethacin diluent) *per os* twice daily. Mice in group 3 were given 0.04 mg indomethacin twice daily in 0.2 ml volume as was used by Bruce & Wakelin (2), beginning 6 hours before larval infection and continuing until the mice were killed. EPG were determined for each group from day 6 until day 10.5 when the mice were killed and the worms were recovered (Table V). It appears the mice were killed a day too early to determine if expulsion was delayed by indomethacin. The great variability in the number of worms recovered in each group suggests no difference between the dilution control and indomethacin groups and that both groups were expelling their worm burden.

The ultimate test of whether prostaglandins would cause expulsion is to inject preparations of purified prostaglandins into the gut and see if a worm burden is expelled earlier than normal. This was tried next. Prostaglandins, PGE_1 and PGE_2, when injected intraduodenally into rats on day 6 of a *N. brasiliensis* infection caused the expulsion of the worms by day 10 (24). Therefore, two experiments were designed to test the effect of prostaglandins on mouse infections. The first trial consisted of 5 groups of litter-

Table V. EPG and worms recovered from *N. brasiliensis*[a] infected mice treated with indomethacin.

	Group 1 Untreated Control	Group 2 Diluent[b] Control	Group 3 Indomethacin[c]
EPG Day 6	47,300	45,700	44,300
EPG Day 7	47,500	42,500	30,800
EPG Day 8	81,200	50,700	31,700
EPG Day 9	47,000	27,200	6,700
EPG Day 10	0	400	200
Worms Recovered on Day 10:5		262,110,68, 56,3,1	156,133,102,86, 67,12,0
Average (P=NS)		83±97	92±72.2

[a] All mice given 300 IL_3 on day 0.
[b] 0.2 0.125% Tween 80 *per os* twice daily from day 0 to day 10.
[c] 0.04 mg/mouse indomethacin *per os* twice daily from day 0 to day 10.

mates infected on day 0 with 300 IL_3. One group received no further treatment (Group 1). On day 5 post-infection, a second group (Group 2) was injected with 0.1 ml prostaglandin diluent intraduodenally. Mice in 3 groups each were given 100 µgm of either PGE_1 (Group 3), PGE_2 (Group 4) or $PGF_{2\alpha}$ (Group 5). EPG feces were monitored until the egg counts dropped. No increased expulsion was shown by the prostaglandin-treated mice as judged by the egg counts and, as this experiment had an unusually long patency period, I conclude that prostaglandins didn't damage the worms (Table VI).

The same experiment was done with nudes. Because nudes don't damage the worms (16,34), prostaglandin-induced damage should be easier to detect in nudes. Five groups of 6-week-old nudes were infected on day 0 with 300 IL_3. Group 1 was used as infection control and wasn't manipulated further. One group (Group 2) was given 0.1 ml of prostaglandin diluent intraduodenally on day 5. Mice in 3 other groups each were given 100 µgm of either PGE_1 (Group 3), PGE_2 (Group 4) or $PGF_{2\alpha}$ (Group 5) intraduodenally. As before, egg counts were monitored through day 16 (Table VII).

Table VI. The effect of prostaglandin (PGE_1, PGE_2, $PGF_{2\alpha}$) injected intraduodenally on day 5 of a larval infection in normal mice.[a]

	Group 1 Unmanipulated	Group 2 Buffer Control[b]	Group 3 PGE_1[c]	Group 4 PGE_2[d]	Group 5 $PGF_{2\alpha}$[e]
No. Mice	7	7	6	6	6
Day 6	32,300	41,400	16,800	33,300	36,600
Day 7	82,300	64,600	58,900	38,700	71,700
Day 8	51,600	34,000	18,000	27,600	34,700
Day 9	40,200	13,300	9,900	13,800	24,600
Day 10	30,600	7,200	8,300	3,900	15,500
Day 11	5,400	3,400	5,500	7,900	8,700
Day 12	3,400	3,400	600	3,500	10,100
Day 14	1,700	100	4,400	4,300	4,200
Day 16	200	100	500	0	300

[a] 300 IL_3 given to all mice on day 0; all mice were 6-week-old LM.
[b] 0.1 ml 10% ethyl alcohol in phosphate buffer injected intraduodenally on day 5. 100µgm of PGE_1[c], PGE_2[d] and $PGF_{2\alpha}$[e] injected intraduodenally on day 5.

Table VII. The effect of prostaglandin (PGE_1, PGE_2, $PGF_{2\alpha}$) injected intraduodenally on day 5 of a larval infection in nude mice.[a]

	Group 1 Unmanipulated	Group 2 Buffer Control[b]	Group 3 PGE_1[c]	Group 4 PGE_2[d]	Group 5 $PGF_{2\alpha}$[e]
Day 6	36,700	41,400	60,500	39,000	30,600
Day 7	189,800	176,600	100,300	146,600	154,200
Day 8	188,400	143,000	169,600	185,400	78,800
Day 9	86,000	125,600	103,200	76,800	52,400
Day 10	137,800	124,000	104,200	93,800	76,000
Day 12	119,400	125,000	88,600	70,000	49,600
Day 16	90,400	56,400	74,000	38,000	29,000

[a] 300 IL_3 given to all mice on day 0; all mice were 6-week-old nude mice.
[b-e] See Table VI.

The data don't suggest any damage to the worm in any group. Anorexia, caused by the surgery, decreases the volume of feces, which in turn increases the EPG. Although none of these experiments (Tables IV, V, VI, VII) is definitive, I believe that all three taken together give reason to believe that the mouse N. brasiliensis system may not have as much of a dependence on prostaglandin to cause expulsion as the N. brasiliensis rat system.

Immune Serum Given with IMLNC to Normal Mice

a) Classification of Immune Serums as Mouse Protective or Worm Protective. Attempts to either damage worms earlier than normal or prevent their damage for an appreciable amount of time in vivo having failed, I decided to try to passively transfer from immune mice to naive mice the ability to damage the worm. Immune serum and immune cells are apparently necessary to get protection that approximates natural immunity in both the rat (28) and mouse (29,44) systems. The next set of experiments was designed to get a constant type of immune cells and find protective pools of serum by giving immune mesenteric lymph node cells (IMLNC) that would synergize with protective pools of serum to cause expulsion similar to that observed in a second infection.

For the first experiment, I chose IMLNC from mice which had been given 300 IL_3 on days -56 and -32 and 900 IL_3 on day -13. These IMLNC were given IV in a dose of 1×10^8 nucleated cells on the same day (0) that the recipient mice were infected with 300 IL_3. Twelve pools of serum collected at various times after one or more N. brasiliensis larval infections were used as the immune serum. Each of the 12 pools was tested in a group of 3 mice injected with 1 ml each, 0.5 ml IV and 0.5 ml IP. All mice also received IMLNC on day 0.

Mice in one control group received normal mesenteric lymph node cells (NMLNC) only, and mice in a second control group received IMLNC only. EPG feces were monitored beginning on day 6. The egg count curves resulting from treatment with these serum pools divide the pools into three different groups: 1) Seven of 12 pools tested weren't unlike the control groups (normal or IMLNC only). These pools were collected from mice 13, 15, 20, 21, 23, 24 and 25 days after a primary infection. 2) In 3 of the pools, the EPG counts were lower than the controls on day 6, but eventually went very high and had a prolonged duration of patency. These were called worm protective sera. These serum pools were taken from mice 19 or 24 days after a primary infection

and from mice infected on both day 10 and day 32 before harvesting serum (Figure 4). 3) Serum from 2 of the 12 pools was classified as mouse protective serum because the EPG feces were very low and dropped to 0 before the controls did (Figure 5). One of these sera was from a day 17 primary infection. The second was from the IMLNC donor mice which were bled 13 days after the third infection. This last pool hadn't been frozen and was injected into mice within 24 hours after collection (Figure 5).

The ratio of mouse protective pools to nonprotective pools is less than the one of three reported in the literature, but is comparable (41). I didn't find any correlation between the time after infection when a serum was harvested and the protective titer of the serum. Multiple infections may be an important factor. One of the pools that was mouse protective hadn't been frozen which suggested the possibility of a freeze-thaw labile factor.

b) Ammonium Sulfate Fractionation of Mouse Protective Serum. Another series of experiments was designed to explore some of the parameters of this mouse protective factor. All test animals were given 1 x 10^8 IMLNC from 3-month-old BALB/c of either sex which had been infected with 300 IL_3 48 and 15 days before the cells were collected.

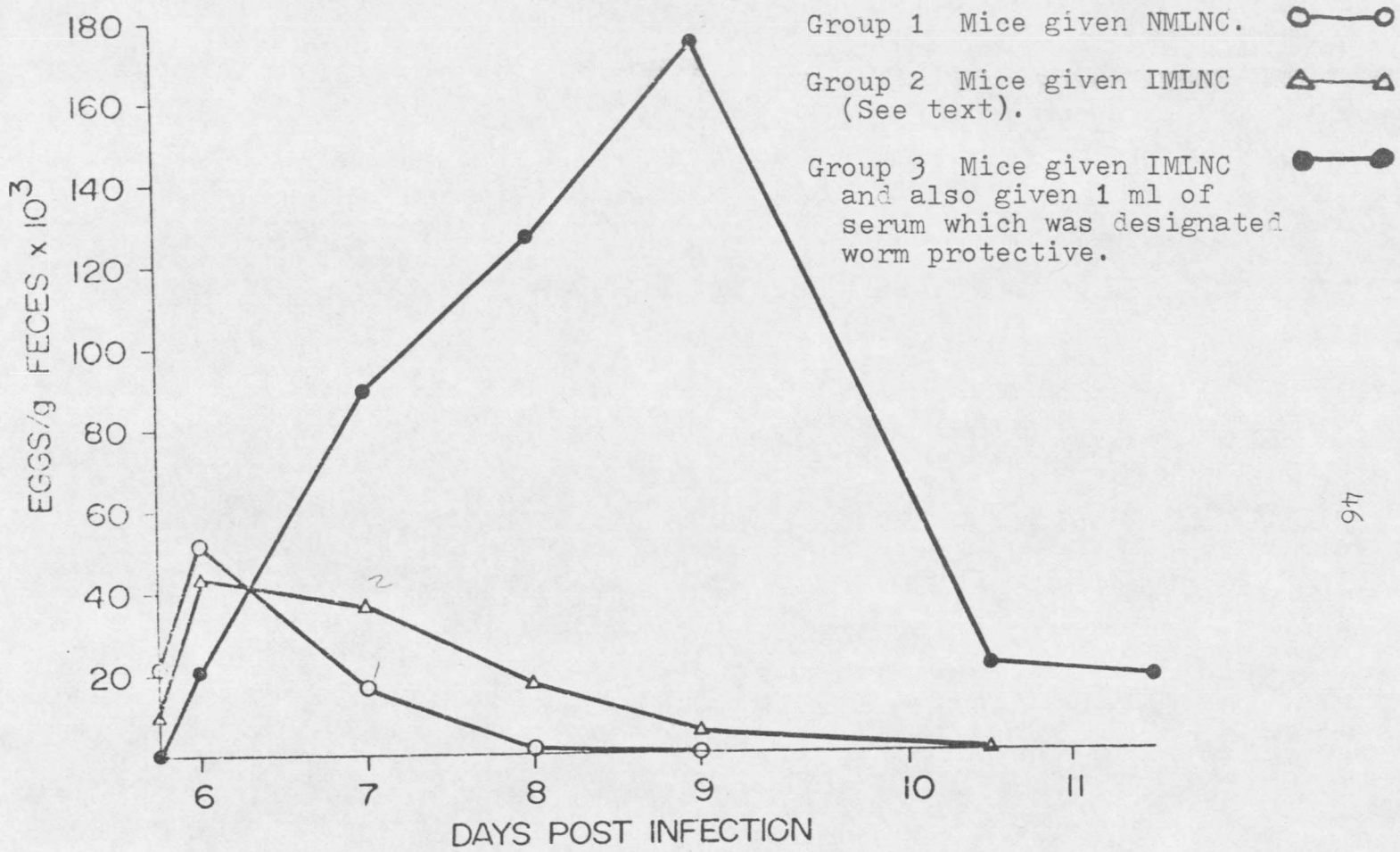

Figure 4. EPG feces from mice given IMLNC and pools of immune serum which were found to be "worm protective".

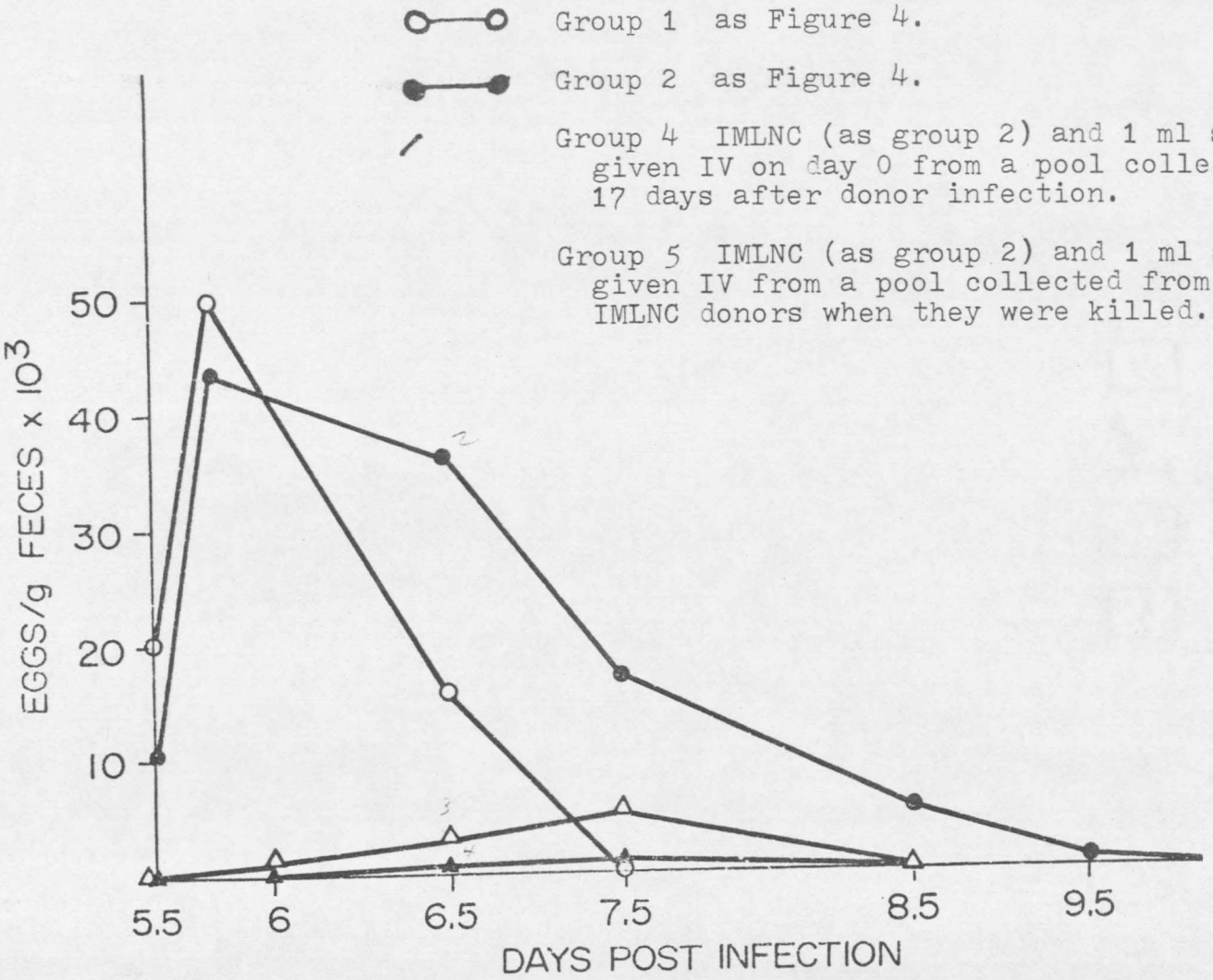

Figure 5. EPG feces from mice given IMLNC and pools of immune serum which were found to be "mouse protective".

A new pool of mouse protective serum was used. It was from the same mice from which the IMLNC were taken. The serum was precipitated once with 50% ammonium sulfate and the albumin-rich supernatent and the globulin-rich precipitate were dialized against phosphate buffered saline and resuspended to the original volume. One group of mice was given 1 ml of the immune serum. A second group was given 1 ml of the globulin-rich and a third group of 5 was given 1 ml of the albumin-rich supernatent (Table VIII).

The protective activity was found only in the globulin-rich fraction. This experiment also shows the mouse protective factor is dializable and isn't irreversibly denatured by ammonium sulfate precipitation.

c) Mouse Protective Serum Adsorbed on IL_3 and Absorbed with IL_3. The next experiments were designed to find out: a) if the protective factor in serum could be adsorbed onto larvae in vitro and allow immune cells to act on the larvae when they were injected subcutaneously into mice, and b) if the activity of an immune serum can be absorbed out using a large number of infectious larvae. The experimental design was:

Group 1 - 300 normal IL_3

Group 2 - IMLNC donors infected with 300 IL_3 28 and 14

Table VIII. Activity of ammonium sulfate fractions of a mouse protective serum.

	Group 1 Control	Group 2 IMLNC[a]	Group 3 IMLNC[a] Immune Serum[b]	Group 4 IMLNC[a] Albumin-Rich[c] Fraction of Immune Serum	Group 5 IMLNC[a] Globulin-Rich[c] Fraction of Immune Serum
Day 6	5,100	19,200	0	8,700	100
Day 7	15,000	31,000	100	35,100	1,600
Day 8	2,500	17,600	0	15,200	1,300
Day 9	100	4,600	0	10,500	0
Day 10	0	600	0	0	0

[a] Mice received 1 x 10^8 IMLNC from mice given 300 IL_3 48 and 15 days before the IMLNC were collected.

[b] 1 ml of serum given IV to each mouse from a pool made from serum collected from mice infected with 300 IL_3 48 and 15 days before bleeding.

[c] The serum (b) was precipitated with 50% ammonium sulfate and both the precipitate (globulin-rich) and the supernatent (albumin-rich) were dialyzed and reconstituted to the starting serum volume and the equivalent of 1 ml serum was given to each mouse in groups 4 and 5 from either the albumin-rich fraction or the globulin-rich fraction.

days before MLNC were collected. 300 normal IL_3.

Group 3 - IMLNC (as group 2) and 1 ml of mouse protective serum given 6 hours before infection with 300 normal IL_3.

Group 4 - IMLNC (as group 2) and 1 ml of the pool of serum used in group 3 after absorption with 46,200 IL_3.

Group 5 - IMLNC (as group 2) and 300 IL_3 which had been adsorbed in serum from the same pool as was given group 3.

Group 6 - IMLNC (as group 2) and 300 IL_3 which had been adsorbed in NMS.

The EPG data (Table IX) suggest that larvae incubated in immune serum were able to initiate a normal infection, even in mice given IMLNC from day 14 after a second infection. Furthermore, the serum wasn't depleted of its protective factor using even large numbers of larvae. This is very inconclusive because the serum wasn't titrated and the 1 ml of serum given may have been more than necessary. Such a small volume of larvae may have resulted in incomplete absorption and might not have been adequate to show a difference in the assay used.

I believe these data (Table IX) show that the mouse protective factor won't adsorb onto larvae in a way that adoptively transferred immunity can act on the larvae. The second part of the experiment proved hard to do correctly as

Table IX. The effect of *in vitro* incubation in protective serum on IL_3 and the ability of *in vitro* incubation of IL_3 to deplete serum of protective factor.

	Group 1[a]	Group 2[a] IMLNC[b] NMS	Group 3[a] IMLNC[b] IMS[c]	Group 4[a] IMLNC[b] Absorbed IMS[d]	Group 5 IMLNC[b] IL_3-Ads IMS[e]	Group 6 IMLNC[b] IL_3-Ads NMS[f]
EPG Day 6	29,300	36,200	0	0	35,000	30,000
EPG Day 7	72,800	46,700	0	0	49,500	42,800
EPG Day 7.5	38,200	14,200	100	0	11,100	10,000
Worms Recovered On Day 7.5	261,192, 139,40	216,194, 149	5 1	0 3	-	-
Average	158±93	186±34	3±2.8	1.5±2.1		

[a] 300 normal IL_3.
[b] 1×10^8 IMLNC collected from donors which had been infected with 300 IL_3.
[c] Protective pool of immune serum from mice infected 48 and 15 days before bleeding. 0.5 ml IV and 0.5 IP given 6 hours before infection.
[d] Each mouse was given 1 ml of serum from the pool given to group 3 which had been absorbed with 46,200 IL_3 for 2 hours.
[e] Each mouse received 300 IL_3 which had been incubated for 30 minutes in serum from the pool given to group 3.
[f] 300 IL_3 which had been incubated in NMS.

Group 1 vs. 2 = NS Group 4 vs. 5 = NS Group 1+3 vs. 4+5 = $P<.05$

it is technically difficult to get the large number of larvae that would be necessary to adequately absorb enough serum to be used in this assay.

Adoptive Transfer with IMLNC to Mice of Immunity to *N. brasiliensis* Larval Infection Without Immune Serum

The immune cells used in the above experiments had been collected about day 14 after a second larval infection of donors. Having a pool of known protective serum available, I wanted to find out if 2 infections were necessary to produce IMLNC capable of cooperating with protective serum to cause abrogation of the patency of an infection.

Two pools of cells were prepared to test the ability of immune cells to co-operate with immune serum. One pool was from mice which had been given a 300 IL_3 primary infection 9 days before cell collection. The second pool was prepared by giving 2 infections of 300 IL_3 to the donor mice on days 32 and 9 before the cells were collected. Both pools of cells were given to mice with and without known mouse protective serum as shown below:

Group 1 - Control, 300 IL_3 day 0 only.

Group 2 - Each mouse received 1 x 10^8 IMLNC from primary infection donors.

Group 3 - Each mouse received 1 x 10^8 IMLNC as group 2 and 1 ml of a known protective pool of serum.

Group 4 - Each mouse received 1 x 10^8 IMLNC from donor mice given 2 larval infections.

Group 5 - Each mouse received IMLNC as group 4 and 1 ml of a known mouse protective pool of serum.

All recipient groups were then given 300 IL_3 the same day they were given IMLNC.

The results of this experiment (Table X) were surprising because the cells from day 9 of the primary infection seemed to be very effective by themselves at eliminating worms, whereas (as had been found in previous work (Figures 3 & 4, Table VIII)) the cells from the secondary infection weren't. The next step suggested by these results was to transfer cells at different days after an infection and see on which days the cells are active by themselves and on which days they need protective serum to effect expulsion of a worm burden.

Groups of 8-week-old BALB/c female mice were infected with 300 IL_3 at 2 day intervals so that at day 0 cells could be collected from infections of 4, 6, 8, 10, 12 and 14 days duration. From these donor mice, IMLNC were transferred into recipient mice and all recipients were infected with 300 IL_3 on that same day. EPG were counted on days 6 and 7 post-infection. The mice were killed on day 8.5 to

Table X. The ability of IMLNC collected after a primary or secondary infection of N. brasiliensis given with or without protective serum to effect adoptive transfer of protection from a 300 IL_3 challenge to naive mice.

EPG	Group 1[a] Control	Group 2[a] Primary[c] IMLNC	Group 3[a] Serum[b] Primary[c] IMLNC	Group 4[a] Secondary[d] IMLNC	Group 5[a] Serum[b] Secondary[d] IMLNC
Day 6	69,900	1,700	0	14,700	2,300
Day 7	86,100	3,300	0	55,600	4,200
Day 8	60,400	2,575	100	62,800	6,000
Day 9	25,700	900	100	30,400	2,000
Day 10	19,900	0	100	14,900	0
Worms Recovered On Day 10	235,212, 209,205, 171,170, 95,61	20 10 0 0	1 - 0	205 149 57	10 5 4
Average	169.8± 61.2	7.5±9.6	.5±1	136.7±74	6.3±3.2

[a] All mice received 300 IL_3 on day 0.
[b] 1 ml IV of known protective pool of serum on day 0.
[c] 1 x 10^8 IMLNC from mice infected with 300 IL_3 9 days before transfer.
[d] 1 x 10^8 IMLNC from mice infected with 300 IL_3 on days 32 and 9 before transfer.

Group 1 vs. 4 = NS
Group 1 vs. 2 = $P<.05$
Group 2 vs. 3 = NS
Group 4 vs. 5 = $P<.05$

recover worms (Table XI).

The egg count and worms recovered data suggest that if the mice had been killed a day earlier there would have been a more noticable difference in the worm counts. The worm recovery data are consistent with all immune cells increasing expulsion by day 8.5 but the EPG feces suggest that day 8 and 10 cells were active earlier than any of the other cell preparations.

The previous experiment was repeated but this time day 10 cells were titrated (Table XII & Table XIII). The worms recovered in this experiment have better separation among groups, possibly because the mice were killed a day earlier. The day 8 cells show very little activity in this experiment, which demonstrates the variability often observed in this system. The day 10 IMLNC group is the only group in which the worms recovered data is significantly different from the control at the $P<.05$ level, but the day 12 cell group is significantly different at the $P<.06$ level, suggesting that the time that the cells from the MLN were able to cause expulsion shifted from before day 10 to after day 10 in this experiment.

I believe that a cell population was either migrating through the MLN on days 8-12 or was being produced in the

Table XI. Experiment A. The ability of MLNC collected on different days after a primary infection of mice to adoptively protect naive mice from a challenge with 300 IL_3.[a]

	Grp 1	Grp 2[b] Day 4 MLNC	Grp 3[c] Day 6 MLNC	Grp 4[d] Day 8 MLNC	Grp 5[e] Day 10 MLNC	Grp 6[f] Day 12 MLNC	Grp 7[g] Day 14 MLNC
EPG Day 6	22,200	11,300	11,900	600	1,100	9,500	15,300
EPG Day 7	28,400	6,100	3,400	200	500	8,900	7,400
EPG Day 8	6,900	1,300	1,100	0	0	200	1,600
Worms Recovered	220,163, 128,89, 73,57	59,19, 30,0	10,126, 46,0	38,0, 0,0,0	1,0,0, 0,0,0	121,50, 31,1,0	116,43 23,1,0
Average	121.6± 61.7	27±24.7	48±55.5	7.6±17	.2±.41	40.6± 57.3	36.6± 47.8

[a] All recipient mice were given 300 IL_3 on day 0.
All mice received 1 x 10^8 MLNC from donor mice given 300 IL_3 4[b], 6[c], 8[d], 10[e], 12[f] or 14[g] days before the cells were transferred.

Table XII. Experiment B. The ability of MLNC collected on different days after a primary infection of mice to adoptively protect naive mice from a challenge with 300 IL_3.[a]

	Grp 1	Grp 2[b] Day 4 MLNC	Grp 3[c] Day 6 MLNC	Grp 4[d] Day 8 MLNC	Grp 5[e] Day 10 MLNC	Grp 6[f] Day 12 MLNC	Grp 7[g] Day 14 MLNC
EPG Day 6	29,400	25,300	2,900	5,700	0	14,100	17,400
EPG Day 7	23,400	31,300	9,700	7,400	0	7,200	12,100
Worms Recovered On Day 7	253	167	157	223	1	150	226
	207	165	143	142	1	144	170
	181	148	111	123	1	112	157
	150	144	106	118	1	88	112
	139	100	21	41	0	49	82
	64	65		9	78	4	
Average	165.7± 64.6	131.2± 40.4	107.5± 52.9	109± 76.1	13.7± 31.6	91± 56.7	148± 55.3

[a] All recipient mice were given 300 IL_3 on day 0.
All mice received 1 x 10^8 MLNC from donor mice given 300 IL_3 4[b], 6[c], 8[d], 10[e], 12[f] or 14[g] days before the cells were transferred.

Worms recovered
1 vs. 2,3,4,7 = NS
1 vs. 5 = $P<.05$
1 vs. 6 = $P<.06$

Table XIII. The ability of MLNC collected from donor mice on different days after a secondary infection with 300 IL_3 to protect naive recipient mice from a challenge with larvae.[a]

	Group 1	Group 2[b] Day 3 MLNC	Group 3[c] Day 6 MLNC	Group 4[d] Day 9 MLNC	Group 5[e] Day 11 MLNC	Group 6[f] Day 13 MLNC
EPG Day 6	10,300	14,900	2,200	8,000	9,100	4,500
EPG Day 7	15,300	18,100	4,600	6,000	9,000	6,300
EPG Day 8	9,700	8,700	900	800	5,900	1,200
Worms Recovered	134,103, 95,85,78	116,109, 85,85,71	91,84,54 43,34	109,81,65 58,49	108,88,78, 70,2	77,69,50 49.28
Average	99±21.8	92.2± 19.4	61.2±25.1	72.4±23.5	69.2±40.2	53.6± 18.3

[a] All recipient mice were given 300 IL_3 on day 0.
All donor mice were infected on day 29 before transfer and a second infection was given on days 3[b], 6[c], 9[d], 11[e] or 13[f]. All mice also received 1×10^8 IMLNC on day 0.

Worms recovered
1 vs. 2,4,5 = NS
1 vs. 3,6 = $P<.05$

MLN by division and that this population might be the cell that is responsible for producing the serum factor which is the limiting factor in primary worm infection rejection as shown by worm transfer experiments (28,29,44). To find out if there is an analogous collection of cells in the MLN during the second infection, an experiment was designed so that cells would be transferred on different days after the second infection. Donor mice were infected with 300 IL_3 29 days before cells were transferred. Groups of these mice were then reinfected with 300 IL_3 on 13, 11, 9, 6 or 3 days before they were used as cell donors. Mice in all experimental groups received 1 x 10^8 IMLNC from the appropriate donors on day 0. EPG feces were monitored 6, 7 and 8 days after IMLNC transfer and 300 IL_3 infection. Worms were recovered 8 days after the transfer and infection (Table XIII). Compared with cells from a primary infection with N. brasiliensis, there was a lot less protection with cells from a second infection. Cells transferred day 6 and day 13 after the second infection caused significant ($P<.05$) reduction of worm recovery by day 8 which is about what has been seen by others with immune cells alone (28,29).

In these adoptive transfer experiments, I was looking for a more significant drop in worm counts on day 7 or 8

because this borderline level of expulsion is commonly found in the rat system when immune cells are transferred (28,29). The interesting observation in these experiments was that pools of cells from both primary infections caused the challenge infection to be nonpatent and to greatly reduce the worm burden on days 7 and 8 (Tables XI, XII). These are the results that would be expected in a mouse immunized by a normal infection. None of the pools of cells collected after the second infection approached these criteria for adoptive transfer of immunity. These results demonstrate that there is in a primary infection a high percentage of cells that, when transferred to naive mice, can cause a challenge infection to become nonpatent and that the percentage drops after day 10 or 12 and apparently doesn't reach as high a percentage in a secondary infection.

Mesenteric Lymphadenopathy Observed During *N. brasiliensis* Larval Infection

An interesting observation made in these experiments (Tables XI, XII, XIII) was the lymphadenopathy that occurred during the infection in the IMLNC donors. The total number of cells recovered from the mesenteric lymph nodes from primary infections of varying lengths is presented in Table XIV. The number of cells recovered was lowest on day 4 and

Table XIV. The number of nucleated cells recovered from the MLN of mice at various times after a primary or secondary N. brasiliensis infection.

Day After Last Infection[a]	No. of Cells Recovered Primary Infection[b]	No. of Cells Recovered Secondary Infection[c]
3		2.37×10^8
4	1.57×10^8	
6	3.25×10^8	4.06×10^8
8	3.15×10^8	
9		3.56×10^8
10	3.75×10^8	
11		2.4×10^8
12	3.58×10^8	
13		1.2×10^8
14	3.45×10^8	

[b] The number of cells recovered from mesenteric lymph nodes per mouse on days[a] after a primary infection.

[c] The number of cells recovered from mesenteric lymph nodes per mouse on days[a] after a secondary infection when the first infection was given 29 days before.

increased until day 10, then decreased some before day 14; most of the activity was found in the cells collected on days 8-12 (Tables XI, XII).

MLN collected on different days after a second infection (Table XIII) also showed lymphadenopathy which peaked earlier than the primary infection (day 6 vs. day 10) (Table XIV) and by day 13 had returned to near normal size (unreported observation). The protective capacity of cells transferred during infection with N. brasiliensis appears to correlate somewhat with the lymphadenopathy (Tables XI, XII, XIII). The cells recovered from the larger nodes are more protective than equal numbers of cells from smaller nodes, except day 13 after the second infection when the cells were significantly protective even after involution had occurred (Tables XIII, XIV).

Various Doses of γ-Irradiation of Mice on Subsequent IL_3 Challenge and the Restoration of Normal Expulsion with IMLNC in Irradiated Mice

To further characterize the factor-producing cell, the radio resistance of worm expulsion was determined. This was done by irradiating mice at different doses and then infecting them with 300 IL_3 and monitoring egg counts to determine when expulsion occurred. Groups of 5 mice were given

either 100, 250, 350, 450 or 550 rads of γ-irradiation 24 hours before being infected with 300 IL_3. EPG were monitored beginning on day 6 (Table XV).

These data show that 450 rads were required to cause any delay in expulsion of the worm burden, and 550 rads were required to get any appreciable delay of expulsion. This suggests that the cell(s) involved in factor production are very radio resistant and can function normally after mice receive 350 rads of irradiation.

As a second part of this experiment, groups of 3 animals from each irradiation dose were given 1 x 10^8 IMLNC from a day 10 primary infection to attempt to repair the expulsive mechanism. In a previous experiment, immune cells prepared in the same manner transferred the ability of immune expulsion to normal mice by themselves. In this experiment, as shown in Table XVI, Group 2, those results weren't reproducable, however these cells were able to repair the expulsive mechanism in the 450 rad group and cause expulsion to be effected 6 or 7 days earlier in the 550 rad group (Table XVI). The expulsive mechanism in mice functioned normally in mice receiving 350 rads or less of γ-irradiation, was delayed very little in mice receiving 450 rads and was severely delayed in 550 rad-irradiated mice (Table XVI).

Table XV. The effect on the expulsion of worms of different doses of γ-irradiation given before a N. brasiliensis larval infection.

	Group 1 Control	Group 2[a] 100 rad	Group 3 250 rad	Group 4 350 rad	Group 5 450 rad	Group 6 550 rad
No. of Mice	6	6	6	6	6	6
EPG Day 6[b]	20,300	33,900	61,700	52,700	59,000	58,400
EPG Day 7	27,900	50,000	82,200	72,200	92,400	118,800
EPG Day 8	11,700	8,200	25,300	28,800	80,000	109,800
EPG Day 10	0	0	2,500	0	16,100	110,000
EPG Day 14					0	64,000
EPG Day 18						37,600
EPG Day 21						2,700

[a] Mice were given indicated doses of irradiation 24 hours before infection.
[b] Day post 300 IL_3 infection.

Table XVI. The ability of day 10 IMLNC to repair the expulsive mechanism of mice given 450 or 550 rads γ-irradiation before a larval challenge.

	Group 1 Control	Group 2 IMLNC[b]	Group 3 450 Rads[a] IMLNC	Group 4 450 Rads No Cells	Group 5 550 Rads IMLNC	Group 6 550 Rads No Cells
No. of Mice	6	3	3	6	3	6
EPG Day 6[c]	20,300	11,800	5,400	52,700	20,300	58,400
EPG Day 7	27,900	15,000	6,300	72,200	32,300	118,800
EPG Day 8	11,700	3,100	8,400	28,800	15,100	109,800
EPG Day 10	0	0	100	0	11,600	110,000
EPG Day 14					3,500	64,000

[a] Rads γ-irradiation given to mice 24 hours before infection.
[b] IMLNC--1 x 10^8 from donor mice given a primary infection 10 days earlier.
[c] Day post 300 IL_3 infection.

This defect in expulsion could be repaired to normal in mice given 450 rads and partially repaired in mice given 550 rads by giving IMLNC from donor mice given a primary 300 IL_3 infection 10 days before cell transfer (Table XVI).

An experiment was done to see if normal thymocytes could repair the worm expulsive capacity of 550 rad-irradiated mice. Five groups made up of 8-week-old BALB/c mice were used: Group 1 received only 300 IL_3 on day 0, as did all other groups; Group 2 received 1 x 10^8 IMLNC from donor mice given a primary larval infection 9 days before transfer of cells; Group 3 received 550 rads γ-irradiation; Group 4 received 550 rads γ-irradiation and also 1 x 10^8 thymocytes from 3-week-old syngeneic donors; Group 5 received 550 rads γ-irradiation and 1 x 10^8 IMLNC as Group 2 did. EPG were monitored beginning on day 7. Again the day 9 IMLNC didn't cause expulsion by themselves in normal mice (Table XVII, Group 2), but they were able to reconstitute 550 rad-irradiated mice to give a near normal expulsion time (Group 5). The interesting group in this experiment was Group 4 which was given thymocytes (Table XVII, Group 4). Mice in this group expelled their worms by day 14 although when plaqued against sheep red blood cells (SRBC's) which were given on day 18, both of these mice showed a greatly reduced

Table XVII. The ability of day 9 IMLNC or normal thymocytes to reconstitute the ability of mice given 550 rads of γ-irradiation before a larval challenge to expel the infection.

	Group 1 Control	Group 2 IMLNC[b]	Group 3 550 Rads[a]	Group 4 550 Rads[a] Thymocytes[c]	Group 5 550 Rads[a] IMLNC
No. of Mice	5	5	5	5	5
EPG Day 7[d]	23,300	45,900	73,900	93,200	26,800
EPG Day 8	4,800	22,200	56,100	116,200	21,600
EPG Day 10	0	0	62,100	55,900	700
EPG Day 12			107,800	30,400	600
EPG Day 14			85,400	600	0
EPG Day 16			44,300		0
EPG Day 18			33,500		
EPG Day 20			30,800		
EPG Day 22			42,200		

[a] γ-irradiation given 24 hours before cell transfer and larval challenge.
[b] 1×10^8 IMLNC from donor mice infected 9 days before transfer.
[c] 1×10^8 thymocytes from 3-week-old syngeneic donors.
[d] Days after 300 IL_3 infection.

Table XVIII. Direct plaque-forming cells (PFC) against SRBC on day 4 after antigen challenge in mice given 550 rads and 1 x 10^8 thymocytes 18 days before.

	PFC/Spleen	PFC/10^6
Normal Control	211,250±17,628	660±85.0
550 Rads and Thymocyte Reconstituted[a]	7,688±265	42.0±24.0

[a] Two mice from Group 4 in Table XVII.

ability to produce direct plaques to this thymus-dependent antigen (Table XVIII).

This experiment shows that mice which are given thymocytes after 550 rads γ-irradiation can expel a larval infection of N. brasiliensis (Table XVII, Group 4) before their antibody-producing potential is recovered (Table XVIII). IMLNC, even though not causing abrogation of patency in normal mice (Table XVII, Group 2), were able to repair 550 rad-irradiated mice to a near normal expulsion time (Table XVII, Group 5).

Immune Serum Tested For Its Mouse-Protective Activity When Given Alone

The immune cells I had been using to collaborate with the immune serum didn't cause expulsion when used alone (Figures 3,4 & Table VIII), so I decided to test the protective serum for activity by itself. This should have been a control in earlier experiments (Figures 3,4, & Tables VII, IX), but because of the limited amount of active serum available, serum was given alone only to small groups after it was known to work with cells.

In one of the first experiments, normal littermates (NLM) were used and serum given with IMLNC from a day 16 infection was no more effective than serum given alone (Table

Table XIX. The effect of mouse protective serum on a 300 IL_3 infection when given alone or with IMLNC.

	Group 1 Control		Group 2 IMLNC[a] + 1ml Serum[b]		Group 3 1ml Serum[b]		Group 4 .5ml Serum[b]	
EPG Day 6[c]	12,000		0		100		100	
EPG Day 7	8,000		0		0		200	
EPG Day 8	800		100		0		600	
Worms Recovered On Day 8	Worms	EP♀[d]	Worms	EP♀	Worms	EP♀	Worms	EP♀
	141	13.3	113	2.1	118	12.7	74	1.6
	96	9.2	74	0	20	-	36	3.7
	91	4.9	27	-	3	-		
	72	8.6	0	-	0	-		
	9	-	0	-	0	-		
	1	-						

[a] 1×10^8 IMLNC from syngeneic donor mice given 300 IL_3 16 days before cells were transferred.
[b] Serum from a known protective pool given IV on the same day as a 300 IL_3 infection.
[c] Post infection.
[d] EP♀ = Eggs recovered per female worm. Eggs in 20-50 female worms were counted and the mean taken.

XIX). The EPG feces counts seemed low for the number of worms recovered at necropsy but the EP♀ was very low in the serum-treated mice, suggesting that the worms were damaged earlier than normal.

A second experiment was done using 10-week-old BALB/c females. Three groups were randomly chosen, the first of which consisted of mice which got only a 300 IL_3 infection on day 0. The second group consisting of 3 mice, was given 1 ml of a known protective pool of serum 6 hours before the larvae. The third group consisting of 4 mice was given 0.5 ml of the same pool also 6 hours before challenge.

The results of this experiment (Table XX) show a marked reduction in the EPG counts of mice given the immune serum, but because of the great variability, there is no statistical difference in the worm recovery data. The EP♀ are statistically lower ($P<.05$) in both of the immune serum-treated groups. The EPG counts are increasing in each group of animals given immune serum, suggesting that the serum factor is being depleted, possibly because of a short half-life, and the worms are recovering.

The experiment suggested by the above results was to give immune serum on both day 0 and day 3 to see if a high enough titer of serum factor could be maintained to cause

Table XX. The effect on EPG and worm recovery of mouse protective serum alone.

	Group 1 Control		Group 2 1ml Serum[a]		Group 3 .5ml Serum[a]	
EPG Day 6 PI[b]	21,100		300		700	
EPG Day 7 PI	24,850		3,400		2,000	
Worms Recovered On Day 7	Worms	EP♀	Worms	EP♀	Worms	EP♀
	220	14.2	211	-	204	8
	208	13.2	158	1	157	6
	182	12.6	3	4.4	137	6.3
	179	11.3			2	-
	162	10.2				
	142	9.8				
	48	6.2				
Average	163± 57	11.1± 2.7	124± 108	1.7± 2.1	125± 86.7	5±3.5

[a] Known protective pool of serum given IV on day 0.
[b] Post infection--300 IL_3 had been given on day 0.

Worms recovered
1 vs. 2,3 = NS
1 vs. 3 = $P<.05$

expulsion. Three different protective serum pools were used in this experiment. One ml of each pool was given to each mouse in groups of 3 or 4 mice on the day of larval infection and other groups of 3 or 4 mice were given 0.5 ml from each pool on both days 0 and 3. EPG were determined on days 6 and 7 and all mice were killed on day 7. The worms recovered were counted. All groups given the same treatment with serum were pooled because results of all pools were statistically alike (Table XXI). The worm counts from the control infection had a high standard deviation, but were significantly ($P<.05$) different from the group that received serum on both days 0 and 3. This experiment showed that 1 ml of protective serum given on day 0 only wasn't any more active than 1 ml split between day 0 and day 3.

I wanted to know at what stage in the life cycle of the worm the serum was effective. Sarles & Taliaferro (52) suggested that serum may cause delayed migration or immune precipitate-mediated killing in host tissue, especially lung. Later work hasn't supported this idea and suggests that worms are expelled from immune rats only after they reach the gut (42). To solve this problem, I gave 0.5 ml of a protective pool of serum 6 hours before the infection to the mice in one group and in a second group, each mouse got 0.5

Table XXI. The effect of one or two injections of mouse protective serum on a larval infection.

	Group 1[a] Control	Group 2 Immune Serum[a,b] Day 0	Group 3 Immune Serum[a,c] Days 0 and 3
EPG Day 6	19,500	200	0
EPG Day 7	8,800	600	0
Worms Recovered On Day 7	186	158	20
	127	71	4
	94	31	2
	79	30	1
	22	5	1
	0	5	1
		2	0
		0	0
		0	0
		0	0
Average	85±68	30±50	2.9±6

[a] 300 IL_3 given to all mice on day 0.
[b] 1 ml of a known mouse protective pool of serum given IV on day 0.
[c] 0.5 ml of serum from b given IV on day 0 and 0.5 ml more given IV on day 3.

Worms recovered
1 vs. 2 = NS
1 vs. 3 = $P<.05$

Table XXII. Effect of mouse protective serum given on day 0 or day 3 of a larval infection.

	Control	Day 0[a]	Day 3[b]
EPG Day 6[c]	13,200	0	0
EPG Day 7	13,900	0	0
Worms Recovered On Day 7	242	50	1
	161	22	1
	126	8	0
	112	2	0
	79	2	0
	16	1	
	6		
Average	106±82	14.2±19.25	.4±.55

[a] 0.5 ml of mouse protective serum given to each mouse on day 0.
[b] 0.5 ml serum (from pool used above[b]) given to each mouse on day 3.
[c] Days after a 300 IL_3 challenge on day 0.

Worms Recovered
1 vs. 2 = $P<.05$
1 vs. 3 = $P<.05$

ml on day 3 of the infection (Table XXII).

Protective serum given IV on day 3 appears to be more effective in causing expulsion than when given on day 0. This would suggest that the elimination of a larval infection might be more efficient after the worms reach the gut than while they are in the skin or lungs.

The protective factor has been reported to be heat labile (38). To test this in my system, the serum was heated 56 C for 30 minutes then given to mice--0.5 ml on day 3. I believed that I had evidence that the factor was freeze-thaw labile, so one group was given on day 3 0.5 ml of serum which had been frozen and thawed 5 times (Table XXIII). More worms were found in the mice receiving the heated serum, but there was still a lot of activity in the serum, suggesting that either 56 C for 30 minutes was inadequate to inactivate all of the protective factor or the protective factor isn't as heat labile as was thought.

The leak lesion theory of antibody damage proposes that the damaging step is to get IgE-mediated anaphylaxis of the gut, thus allowing protective antibodies of other classes to get into the gut and cause expulsion of the worms. I designed an experiment to test this idea by injecting the protective serum into the gut on day 4 when all of the worms

Table XXIII. Effect of heat or freeze-thaw on the activity of mouse protective serum.

	Group 1 Control	Group 2 Immune Serum[a]	Group 3 Immune Serum Heated[b]	Group 4 Immune Serum Frozen & Thawed[c]
EPG Day 6[d]	13,200	0	1,400	0
EPG Day 7	13,900	0	0	0
Worms Recovered On Day 7	242	0	100	1
	161	0	83	0
	126	0	11	0
	112	1	5	0
	79	1	0	0
	16			0
	6			
Average	106±82	.4±.55	39.8±47.7	.17±.41

[a] Each mouse received 0.5 ml IV of a known mouse protective pool of serum on day 3.

[b] Serum from the pool used in Group 2 was heated 30 minutes at 56 C; each mouse received 0.5 ml of the heated serum IV on day 3.

[c] Serum from the pool used in Group 2 was freeze-thawed 5 times; each mouse received 0.5 ml of the freeze-thawed serum IV on day 3.

[d] All mice received 300 IL_3 on day 0.

would be in the gut, but not all moulted to the adult stage yet. One half ml of the protective serum was injected into the duodenum via laporotomy. Another group was given 0.5 ml of normal mouse serum intraduodenally (Table XXIV). The serum was protective when given IV but not when given intraduodenally. Either the serum wasn't in contact with the worms for a long enough time or serum isn't active against the worms in the gut lumen. It would be difficult to inject serum into the duodenum more than a few times because of surgical trauma.

Cyclophosphamide has been reported to delay expulsion of a worm infection for about 6 days (34). In that study, cyclophosphamide given on day -2 relative to infection did not have an effect, but if given on day +2 it delayed expulsion. Because I had the serum system working, I believed I could determine whether this cyclophosphamide-induced delay was in the humoral damaging step or a later expulsion step. I gave the mice 2 mg cyclophosphamide IV, which is about half the dose given subcutaneously by Mitchell's group (34). The mice were divided into 6 groups and the mice in each group were given:

Group 1 - 300 IL_3 on day 0, as were all other groups.

Group 2 - Immune serum on day 0.

Table XXIV. The effect on a larval infection of mice of protective serum injected into the duodenum.

	Group 1 Control	Group 2 Immune Serum IV[b]	Group 3 Immune Serum ID[c]	Group 4 NMS[d] ID
No. of Mice	6	6	6	6
EPG Day 6[a]	14,000	0	21,200	9,400
EPG Day 7	14,800	0	85,600	9,400
EPG Day 8	1,000		6,800	4,800
EPG Day 8.5	0		7,600	2,900

[a] All mice were infected with 300 IL_3 on day 0.
[b] Mice were given 0.5 ml IV of a known protective pool of serum on day 0.
[c] On day 4 post infection, mice were injected intraduodenally with 0.5 ml of the pool used IV in Group 2.
[d] On day 4 post infection, mice were injected intraduodenally with 0.5 ml of NMS.

Group 3 - Immune serum on day 3.

Group 4 - Cyclophosphamide on day 2.

Group 5 - Immune serum day 0, cyclophosphamide day 2.

Group 6 - Cyclophosphamide day 2, immune serum day 3.

The results (Table XXV) of this experiment were a surprise and more experiments need to be done before they can be interpreted. The EPG counts were depressed on day 6 in both groups receiving serum and cyclophosphamide (Groups 5, 6) but had reached normal levels by day 7, after which the worms were apparently expelled normally. One possible explanation of these results is that a threshhold dose of both immune serum and cyclophosphamide was used. This is shown in the group which received cyclophosphamide only, as worm expulsion was near normal. Also, if the serum dose was the minimum needed, the cyclophosphamide may have decreased the production of the factor in the mice which was necessary to supplement the immune serum given to get the full benefit of the serum.

Transferring Passive Immunity Into Nudes Using IMLNC and Protective Serum

Two methods of transferring the worm damaging step to naive normal mice have been examined; passive transfer of protection with immune serum (Table XXII) and adoptive

Table XXV. Effect of cyclophosphamide or cyclophosphamide and protective serum on a larval infection of mice.

	Group 1 Control	Group 2 Serum[b] Day 0	Group 3 Serum[b] Day 3	Group 4 Cyclophos-phamide[c]	Group 5 Cyclophos-phamide[c] Serum[b] Day 0	Group 6 Cyclophos-phamide[c] Serum[b] Day 3
EPG Day 6[a]	13,200	0	0	27,400	1,100	7,700
EPG Day 7	13,900	0	0	39,500	24,000	20,000
EPG Day 8	-	-	-	10,400	2,400	500
EPG Day 8.5	-	-	-	5,800	400	200
Worms Recovered				107 18 0 0 0	1 1 1 0 0 0	10 4 2 1 0 0
Average	106±82[d]	14.2[d] Range 1-50	.4[d] Range 0-1	25±46.5[e]	.5±.55[e]	2.8±3.8[e]

[a] All mice given 300 IL_3 on day 0.
[b] Mice given 0.5 ml of one pool of serum IV on days indicated.
[c] Mice given 2 mg cyclophosphamide IV on day 2.
[d] Worms recovered on day 7.
[e] Worms recovered on day 8.5

transfer of immunity with IMLNC (Tables XI, XII). Next, the requirement for transfer of some amount of passive immunity to nude mice was studied. When given thymus grafts or when given thymocytes 21 days before challenge, nudes expel their worms in a near normal pattern (16,34). However, it takes approximately 14 days to get expulsion after giving either normal spleen cells or thymocytes to the nude at the same time they are infected. No one has reported giving nudes protective serum or cells that are protective by themselves.

An experiment was designed to test the ability of known protective serum and immune cells to collaborate in a nude to cause expulsion before day 14 when nudes given normal splenocytes and thymocytes would expel a worm burden. The groups used in this experiment are:

Group 1 - LM control, 300 IL_3 on day 0 as all other groups.

Group 2 - LM given 1 x 10^8 IMLNC IV from donors infected 16 days before transfer.

Group 3 - LM given 1 ml IV of a known protective pool of serum on day 0.

Group 4 - Nudes given larval infection only.

Group 5 - Nudes given immune serum as Group 3.

Group 6 - Nudes given IMLNC as Group 2 and immune serum as Group 3.

Nudes given mouse protective serum alone (Table XXVI,

Table XXVI. The effect of protective serum or protective serum and IMLNC on a larval infection of nude mice.

EPG	Group 1 Control NLM	Group 2 NLM IMLNC[b]	Group 3 NLM Immune[c] Serum	Group 4 Nude	Group 5 Nude Immune[c] Serum	Group 6 Nude IMLNC[b] Immune[c] Serum
Day 6[a]	12,000	13,000	0	18,300	51,800	1,000
Day 7	8,000	9,400	0	32,800	69,700	34,600
Day 8	800	1,500	0	74,500	160,200	91,400
Day 12				38,900	89,200	33,900
Day 15				95,400	101,800	11,800
Day 17				69,500		1,700
Day 19				55,200		6,800
Day 21				111,400		3 mice, 300 2 mice, 58,800
Day 23				67,300		

[a] All mice given 300 IL_3 on day 0.
[b] Mice given 1 x 10^8 IMLNC from donor mice infected 16 days before cell transfer on day 0.
[c] Mice given 1 ml of a known protective pool of serum IV on day 1

Group 5) didn't expel their worms and died earlier than nudes not given mouse protective serum (Group 4). Nudes given mouse protective serum and day 16 IMLNC (Group 6) showed a delay in egg production by one day. EPG counts seemed to drop between days 15 and 17, but it is difficult to tell because 3 of the nudes were apparently reconstituted and expelled their worms, while 2 others weren't reconstituted and died with high EPG counts. This shows that each nude mouse needs to have EPG counted separately so the data from those that were reconstituted could be separated from those which weren't.

The lack of a pool of undifferentiated T cells was felt to be a possible explanation for the failure of protective serum to have an effect on an infection in nudes, so I selected 3 groups of 2 nudes and on day 9 of a N. brasiliensis infection I gave them different combinations of cells and mouse protective serum as follows:

Group 1 - 1×10^8 thymocytes IP, 0.5 ml immune serum IP.

Group 2 - 1×10^8 thymocytes IP, 0.5 ml protective serum and 1×10^8 IMLNC from donor LM mice given a primary infection 9 days before.

Group 3 - 1×10^8 thymocytes, IMLNC as Group2.

EPG were monitored (Table XXVII).

Table XXVII. The effect of various combinations of protective serum, IMLNC and thymocytes on a larval infection of nude mice.

Days After[a] Cell Transfer	Group 1 Thymocytes[b] Immune Serum[c]	Group 2 Thymocytes[b] IMLNC[d] Immune Serum[c]	Group 3 Thymocytes[b] IMLNC[d]
(No. of Mice)	(2)	(2)	(2)
-2	28,900	75,100	82,000
1	72,100	71,000	127,000
3	33,300	45,400	54,300
5	53,200	71,600	143,000
7	57,800	58,400	71,500
9	44,600	35,100	46,150
11	59,500	31,400	32,000
13	31,800	20,800	3,100
	-1 died		
14	41,000	88,900	14,350
16	47,000	63,600	7,900
19	31,400	42,300	2,100

[a] All mice were given 300 IL_3 9 days before cell transfer.
[b] All mice were given 1 x 10^8 thymocytes IP from 3-week-old littermates.
[c] Mice given 0.5 ml of a known protective pool of serum on the day of cell transfer, IP.
[d] Mice given 1 x 10^8 IMLNC from donor littermates given a primary infection of 300 IL_3 9 days before cell transfer.

This is a very preliminary experiment, but none of the nude's counts were depressed until about day 14 after the cells were given. The thymocytes apparently didn't reconstitute the nudes as the group that didn't get IMLNC wasted, one dying on day 12 after transfer. The activity of these day 9 IMLNC in a littermate infection is unknown because the larvae used in this experiment didn't cause an infection in littermates, but did give a uniform EPG distribution in nudes.

Looking at Groups 2 and 3 in this experiment, it appears as though giving serum on the day IMLNC's were given caused the worms to be retained longer than in the nudes that received IMLNC, but no serum. The serum could have caused the worms to become adapted or it may have acted as a blocking serum, not allowing the host cells to react to the antigens. At this point, the requirements to passively immunize a nude are unknown.

DISCUSSION

The most popular theory for *N. brasiliensis* elimination is the two step theory put forth by Ogilvie and co-workers (42,43,44). In the first step, "antibodies" damage the worms thereby making them in some unknown way susceptible to the action of a cell-mediated reaction (second step) which results in their elimination from the host by an unknown mechanism. Some observations which support this proposed mechanism are: worms recovered in the later stages of a normal infection in rats (days 10-14) (42) or mice (day 8) (29) when inoculated into normal rats show an accelerated elimination pattern (<day 4 vs. >day 8) and are called "damaged worms"; neonatal and lactating rats don't expel their worms even though the worms appear damaged nearly as soon as in a normal infection (the damage has been assessed cytologically and biochemically and by earlier expulsion after transfer); expulsion can be facilitated in neonatal and lactating animals by giving them normal lymphocytes (22); serum from immunoincompetent neonates can accelerate expulsion in normal animals given a larval infection. From these observations, it has been deduced that antibodies found in the serum of lactating and neonatal rats cause damage to the worm, but lymphocytes from an immunocompetent adult animal are needed to cause the expulsion of the worm

from immunoincompetent animals.

Attempts to transfer to naive animals the level of immunity to larval N. brasiliensis infection that is seen in previously infected animals has very rarely been accomplished either passively with immune serum or adoptively with immune spleen or IMLNC. Love (28,29) found that to get mice or rats to expel their worms as previously infected animals would, both hyperimmune serum and immune cells were required. There are only two reports in the literature of IMLNC preparations causing expulsion of a larval N. brasiliensis infection before the infection became patent. Both of these were the exceptional case in a series of experiments (23,29). Two other gut-dwelling nematode-rodent systems (Trichuris muris-mouse (60) and Trichinella spiralis-mouse (62)) that have been extensively studied have been reported to require both immune cells and immune serum to transfer to naive hosts a high degree of protection to challenge with infective larvae.

There has been little work on the damaging factor, either its real identity or its origin. The damaging factor found in serum has been assumed by most workers in this field to be worm-specific antibody. Because the factor is thought to be antibody, little work has been done to iden-

tify the cell producing it. The lack of effect of immune serum on the worm *in vitro* (14,51) has left the mechanism of action of the "antibody" unknown. The recent work of Jacobson, *et al* (17) suggesting that the factor in mice isn't antibody has not been followed up in the literature yet. In experiments reported here, 5 pools of cells given at a dose of 1×10^8 cells per mouse on day 0 of an infection (Tables X, XI, XII) caused total ablation of EPG feces and very low worm counts on days 8 and 10. All of these cell pools were prepared from mice that had been infected for 8-12 days after a primary infection. Several pools of cells collected from mice 4, 6 and 14 days (Tables X, XI, XII) after a primary infection or 9-14 days (Figures 4,5 and Tables VII, IX) after a second infection didn't cause any difference in the infection as monitored by egg counts when transferred to naive recipients. The kinetic study of cells taken after a second infection showed some decrease in EPG and depressed worm counts on day 8 (Table XIII), but did not show the level of immunity seen in other cell preparations (Tables X, XI, XII).

Because of the effect on the challenge infection in the recipient mice, I believe that the cells producing the worm damaging factor must have been transferred or that the cells

transferred were committed helper cells which caused the effector cell in the recipient mouse to produce large quantities of the factor. I always transferred a constant number of MLNC in the experiments and it was always the cell preparation from donors 9-12 days after a primary infection that caused ablation of patency suggesting a high percentage of factor-producing or helper cells in the cells that were transferred. The activity of the cell preparations correlated with the peak lymphadenopathy (Table XIV) seen in the MLN which implies that the cell type responsible for the increase in cellularity of the MLN is the cell responsible for acceleration of worm damage in the recipient animal.

Work published recently demonstrated the same short time of high activity against a larval infection in recipients (34) of thoracic duct lymphocytes collected from rats during a primary N. brasiliensis infection (cells from days 10 and 15 post infection were active, cells from days 5 and 20 were not). The active cell in this work was an Ig^- population which caused the authors to postulate that damage and expulsion are caused by the same cell population and that the total number of cells is most important (36). The alternative possibility they offered is that the Ig^- cells that they found active in accelerating expulsion of a larval

infection was a helper cell that collaborated with the B cells of the recipient animal to produce the accelerated antibody response needed to get early expulsion. They never considered the possibility of a T cell product causing the damage.

Two experiments reported here support the fact that mice with limited antibody forming potential, both γ-irradiated (350 or 450 rads) animals not given any cells (Table XV) and the 550 rad γ-irradiated mice given thymocytes (Tables XVII, XVIII), can still expel their worm burden at about the normal time. These mice, though having a low number of specific antibody forming cells (as shown by SRBC-specific PFC seen (Table XVIII) and personal communication with Brad Brooks), expelled their worms by day 14 which shows that the damaging factor was produced at almost the normal rate even though the B cell competence of these animals is very low.

One major problem with my kinetic experiments (Tables XI, XII, XIII) was that each day cell donors were immunized with a different larval batch which introduced a lot of variance because each of the immunizing infections behaved a little differently. Ideally, mice infected with one larval preparation should be killed on different days and

cells transferred to groups of mice with controls infected the same time as each group that received cells. The mesenteric lymphadenopathy correlated very well with the ability of cells to adoptively transfer immunity to N. brasiliensis. Both the peak of lymphadenopathy and the activity of the cells was just at or just after the time that the worms are being damaged and expelled from the donor mice. A constant number of cells (1×10^8 nucleated cells) was transferred so the relative numbers of the damaging factor-producing cells must have increased during the lymphadenopathy which would suggest a local increase in the cell capable of transferring the ability to cause damage to a larval infection to a naive animal. The proliferation of the specific cells may have been in the MLN or they may have proliferated elsewhere and their migration pattern carried them to the MLN. Blast cells from the MLN and thoracic duct lymph preferentially localize in the small intestine of rats when injected into a recipient animal. Inflammation such as that caused by a N. brasiliensis infection causes an even larger percentage of these blast cells to localize in the small intestine (30) which would suggest that the MLN may be in the normal traffic pattern of blast cells specific for antigens in the gut.

After I found that some preparations of cells could adoptively transfer a high level of protective immunity to a larval challenge in naive mice, I tried giving serum alone to mice before a larval infection (Table XIX). The results were unexpected because Love (29) had shown previously that to get the same high level of protection that I found, both immune cells and immune serum had to be given. I found a lot of variability in the number of worms recovered after giving immune serum alone to mice. With good pools of mouse protective serum the egg counts were always very depressed on day 6, then possibly the worm escaped and produced a few eggs by day 7 or 8 and the mouse developed an immune response and caused the worms to be expelled. I believe that this may be a phenomenon of the passive serum factor being depleted before the mouse makes enough of an immune response --either damaging factor or expelling mechanism--to prevent egg production and/or cause expulsion. This phenomenon of egg production being delayed 1 day was seen several times in low serum doses and twice in immunocompromised animals--nude and cyclophosphamide-treated normal mice (Tables XXV, XXVI). The data suggesting that nudes given both immune cells and mouse protective serum can delay patency by one day suggests that something from the cells must synergize with the serum

To get damage or delayed patency in a larval infection in nudes (Table XXVI).

The observation that immune serum is more efficient at causing expulsion when given on day 3 than when given on day 0 could be explained in several ways. One possibility is that the immune serum present when the larvae are migrating causes the worms to become "adapted" (44) and survive longer even in an immunocompetent host. A second possibility is that the worm antigens are blocked by the antibodies in the passively transferred immune serum and it takes a longer time for the mouse to react actively to the worm infection and delays the worm expulsion. Either of these possibilities could account for the worm protective serums seen in Figure 4. I don't know if the worm protective factor is the same as the mouse protective factor or if the time of administration or the dose is the deciding factor as to whether the end result will be prolonged or ablated patency. If the 2 factors are different, the relative concentration could be important and total dose of each may alter the end outcome. I have some unreported data that would suggest that one serum specimen can be both worm protective and mouse protective at different doses but, because of the shortage of worm protective serum, all of the proper controls weren't

run at one time. Therefore, no definitive statement can be made.

How the serum factor acts on the worm is unknown. IL_3 incubated in immune serum *in vitro* live as long as and are indistinguishable from normal worms (21). It has been found that adult worms maintained under the best known conditions are damaged in the same way that "antibody damaged" worms are damaged *in vivo*, which suggests that the factor may just cause the worm to move to a less optimum environment in the small intestine (31).

I found that incubating IL_3 in active mouse protective serum didn't alter their infectivity even when they were used to infect a mouse given IMLNC (Table IX). I also injected mouse protective serum into the duodenum on day 4 when the worms were there and the serum didn't seem to have any detrimental affect on the worms (Table IX). More doses of serum given intraduodenally would be needed to definitively show that serum given in the gut lumen wouldn't cause damage to the worm, but this would be technically difficult. It has been shown that worms in the gut are labeled with p^{32} when given to the host animal IV better than when the p^{32} is given in the gut. This observation suggests that the worms may need to ingest the serum factor or the serum factor may

act on an unknown cell or tissue in the mouse when given IV vs. ID which could cause the worm to move from its preferred site in the gut.

I found the factor responsible for worm damage *in vivo* to be relatively stable. I found the mouse protective factor to be much less heat labile (56 C) than Ogilvie (38) reported (Table XXIII). The previous report of the heat lability of the protective factor (38) also demonstrated that the PCA activity was destroyed, as would be expected, but the report also stated that worm specific precipitin in gel was diminished, which is unexplained. I also found the factor to be resistant to freeze-thawing (Table XXIII) which suggests that it is different than Jackson's larval lethal factor found in serum (14). The activity in immune serum was found in the globulin-rich fraction of ammonium sulfate precipitation fractions and wasn't irreversibly denatured by the precipitation procedure or lost during dialysis (Table VIII).

My attempts to adsorb the factor onto IL_3 suggest that the factor didn't adhere to the larvae, in that the larvae weren't noticeably damaged even after injection into a host that had been given IMLNC (Table IX). It has been reported that IL_3 injected in as little as 0.1 ml of immune serum

could cause decreased numbers of adult worms (59). This same worker also found that larval culture fluid--excretion and secretion antigens--could neutralize the protective action of the serum, while larval extract wouldn't (59). None of the chemical characterization parameters I have defined have suggested that the protective factor couldn't be an immunoglobulin. I believe that I have the system sufficiently worked out so that by using affinity chromatography and anti-immunoglobulin serum, it could be determined if the factor is immunoglobulin and if it is, the class responsible for passive protection.

There is only one characterized chemical that has been reported to accelerate damage in the N. brasiliensis rat system and that is prostaglandin E (PGE_1 & PGE_2). The data that support the claim that PGE is important in vivo are: fractions of semen containing prostaglandins injected intraduodenally during an infection increased expulsion of worms (54); prostaglandin inhibitors (acetylsalicilic acid and d-propoxyphene) delayed expulsion; the level of prostaglandins increased in the small intestine during expulsion (7); synthetic PGE_1 and PGE_2 injected intraduodenally during an infection caused earlier expulsion.

More recently adult worms have been incubated in vitro

with prostaglandins. After incubation as short as 18 hours, adult worms incubated in PGE_1 were expelled by recipient rats as if they were damaged, while control worms were expelled later (50). While far from being definitive, experiments reported here failed to demonstrate any difference in expulsion times in mice given either prostaglandin inhibitors--aspirin (Table IV), indomethacin (Table V)--or synthetic prostaglandins (Tables VI, VII). I used doses and treatment schedules comparable to those used in the rat work for aspirin (3,24) and prostaglandins (24). Indomethacin was given as it has been reported to delay expulsion in the Trichuris muris mouse system (2).

Some low-dose immunosuppressive agents used in this work caused higher EPG counts than controls early in the infection even though the mice weren't anorexic and expelled their worms on time. This suggests that more worms reached the gut or damage occurred later. The agents are low doses of Y-irradiation, 100-350 rads (Table XV), and cyclophosphamide, as shown in Table XXV. The EPG data in these studies resemble somewhat the larval infections in trypan blue-treated mice in which the increased EPG appear to be caused by more larvae reaching the adult stage (Table II, day 6). The problem is to decide whether trypan blue

inhibits the host response in more ways than just the macrophage effector function or whether the other agents used (cyclophosphamide and γ-irradiation) also inhibit macrophage effector function. Strause (57) recently published work showing depression of specific plaque forming response in mice given doses of trypan blue much lower than the ones I was using. I can't make a final decision on the importance of macrophages as effector cells in N. brasiliensis infections. All I can report is that high doses of trypan blue allows increased numbers of larvae to develope into adults and delays their expulsion a little (Table II, Figure 1). Trypan blue apparently had little or no effect on the expulsion of normal adult worms transferred into trypan blue-treated mice (Figures 2 & 3).

In conclusion, I feel that I have shown an assay system for characterizing the humoral worm damaging factor and also to rapidly evaluate serum pools for their worm damaging ability. I also have found a source of sensitized cells which, when given to a normal animal, can produce or stimulate the animal to produce damage in a larval infection earlier than normal. Either or both of these methods of damaging worms should be applied to immunodeficient animals (γ-irradiated and/or nude) to identify the components

necessary to co-operate with the factor in causing damage, if any is necessary.

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