Humoral and cellular factors in the immune eliminationn of Nippostrongylus brasiliensis from mice
by Richard Hilding Jacobson

A thesis submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Zoology
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Abstract:
Congenitally athymic (nude) mice were incapable of expelling Nippostrongylus brasiliensis while normal littermates (NLM) of nude mice eliminated their worm burdens by day 9-11 post-larval-inoculation. Nude mouse recipients of either dispersed thymus cells or thymus gland implants were, however, capable of eliminating their infections. Thymus competence of nude mice receiving thymus cells or glands was confirmed by skin allograft rejection and plaque-forming cell responses to sheep erythrocytes. Expulsion of N. brasiliensis from mice was thus determined to be a thymus dependent phenomenon.

The thymus dependency of the proposed inductive (worm damaging) and effector (worm elimination) steps in expulsion of N. brasiliensis from mice was studied. Adult worms, obtained from a 13 or 14 day infection of rats (damaged worms) and transferred via laparotomy into nude mice or NLM, were not expelled from nude mice but were rapidly eliminated from NLM. Seven-day rat worms (normal worms) were expelled from NLM 3 days later than were 14-day rat worms; thus, the data suggest the effector step in expulsion of N. brasiliensis from mice is thymus dependent.

The thymus dependency of the inductive step of worm elimination was determined by evaluating fecundity of female worms, morphological changes in the intestinal cells of the worms, and reestablishment and kinetics of infection following worm transfer into a normal host. The number of eggs per female (EPF) N. brasiliensis was significantly reduced ($P < 0.01$) in 9-day mouse worms (damaged worms) and 14-day rat worms (damaged worms) compared with 7-day rat worms (normal worms). Conversely, the mean EPF of nude mouse worms was not reduced for at least 81 days of infection and, furthermore, was not significantly different ($P > 0.05$) from that of 7-day rat worms (normal worms). In transverse sections of N. brasiliensis obtained from 14-day rat or 8-day NLM infections, the worm intestinal cells were highly vacuolated. In contrast, 15-day nude mouse worms were not vacuolated. Furthermore, although limited, the data suggest that worms obtained from 9-day infections of NLM and transferred into rats are more rapidly eliminated from rats than are 6-day NLM or 15-day nude mouse worms. Collectively, these data suggest that the inductive step of N. brasiliensis expulsion from mice is thymus dependent.

Immunosuppression of BALB/c mice by treatment from birth with heterologous anti-μ serum resulted in abrogation of antibody production potential. Anti-μ treatment did not, however, reduce the capacity of mice to expel N. brasiliensis. Passive immunization of nude mice with massive amounts (5 ml/20 gms body weight) of homologous immune serum resulted in worms which, upon transfer into normal Sprague-Dawley rats, were expelled more rapdily than were worms obtained from mice similarly treated with normal mouse serum. The passive immunization' experiments suggest that immune serum does contain a factor which is detrimental to the worms resulting in their accelerated expulsion following transfer into normal rats; however, results of the anti-μ experiment suggest that the protective factor may not be antibody.

HUMORAL AND CELLULAR FACTORS IN THE IMMUNE ELIMINATION OF *NIPPOSTRONGYLUS BRASILIENSIS* FROM MICE

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INTRODUCTION

Although many observations have been made on immunological phenomena in a variety of host-parasite systems (1-5), there are relatively few systems for which in-depth studies have been conducted on mechanisms of immunity to helminthic infections. The number and complexity of antigens of individual helminths, the lack of adequate *in vitro* correlates and the complexity of helminth life cycles all have contributed to the paucity of knowledge concerning the host immunological response to helminthic infections.

One host-parasite relationship which has been the subject of many immunological investigations is the rodent-*Nippostrongylus brasiliensis* (Travassos, 1914) system in which infected rodents develop a strong immunity (6, 7). Background information on the systematics, parasitic development, kinetics of worm egg production, worm population dynamics, physiology of the worm, and the pathologic effects of the worm on the host have been adequately reviewed (8) and will not be repeated here.

Many attempts have been made to confer to rats protective immunity against infections of *N. brasiliensis* by passive transfer of immune serum (9 - 20). Protection conferred has been extremely variable and has seldom been comparable to that developed in rats experiencing an active infection. Fractions of antiserum pools which most frequently afforded protection against *N. brasiliensis* contained predominately IgG_1 (19, 22). However, IgG_2 and "occasionally other immunoglobulin classes" may be protective (19). Very high levels of reaginic antibody

(detected by a 72-hr. passive cutaneous anaphylaxis reaction) invariably accompany infections of *N. brasiliensis* in rats (23, 24), but its role, if any, in protective immunity to helminthic infections remains obscure. It has been suggested, however, that adult *N. brasiliensis* are damaged by protective antibodies (25) resulting in severe gut cell degeneration (26, 27) and changes in the isoenzyme patterns of acetylcholinesterase and acid phosphatase of the worms (28). The mechanism of this proposed action of antibody remains undefined.

Other studies have been conducted to determine the role of cellular components in worm expulsion. Immunity conferred by adoptive transfer of lymphoid cells (20) was recently confirmed (29) but limited to activity of only mesenteric lymph node cells (MLNC). Subsequently, it was shown that MLNC obtained only from immune donors caused worm expulsion in both irradiated (400 rads) and non-irradiated syngeneic recipient rats (30). Recently, it was observed that a third, radiosensitive (750 rads), bone-marrow derived component is required for worm expulsion (31, 32). Although this third cellular component remains unidentified, it is thought to be of myeloid origin (32).

In addition to lymphocytes, other specific cell types have either been implicated or tentatively considered nonfunctional in worm regulation. Although the data are limited, it has been suggested that macrophages are excluded from a role in the worm expulsion mechanism

(33). Further investigations are necessary to test the validity of this suggestion. Eosinophilia invariably accompanies many parasitic infections (34). The functional role, if any, of eosinophilia which develops during infections of *N. brasiliensis* (35, 36) has not been assessed. Although the induction of the eosinophilic response apparently has antigenic specificity (37), the immunologic function of eosinophilia remains obscure as does its role in immunopathological processes. A highly significant increase in the numbers of mast cells occurs in the intestinal mucosa of rats infected with *N. brasiliensis* (38 - 40). The kinetics of the mast cell response and functional role of this cell type in worm expulsion is not defined and remains a controversial subject (8, 22, 38).

Two diametrically opposed hypotheses emerge in the literature, each of which attempts to explain the expulsion of *N. brasiliensis* from the rat host. In the first of these hypotheses (see review 38), it is suggested that reaginic antibody developed in response to the worms reacts with mast cells and worm antigen resulting in amine release (41, 42) which is responsible for an increase in gut mucosal permeability (43) allowing for translocation of circulating antiworm antibody which in turn leads to worm expulsion (38, 44, 45). Based on a variety of observations, others have indicated that the reagin-mast cell interaction may not be required for effecting worm elimination (23, 35, 46).

In the second hypothesis of worm expulsion, antibodies and cells act cooperatively in a sequence of events which, ultimately, results in worm expulsion (see review 47). Briefly, expulsion of *N. brasiliensis* from the rat host is thought to require at least two separate and sequential steps (8, 25). In step 1, worms are damaged by protective antibody (25) as described above. Step 2 is lymphocyte dependent (30, 33) and, according to the hypothesis, occurs only after the worms have been damaged by antibody (25, 48). The means whereby the sensitized lymphocytes cause expulsion of worms is, however, unknown. Furthermore, the role of a proposed radiosensitive myeloid component (described above) remains undefined.

Although the immune response of rodents to *N. brasiliensis* has probably been studied as intensively as that of any host-helminth system, knowledge of the immune mechanism is both fragmentary and incomplete. The system has been plagued with variability, often making interpretation of results controversial. This variability has been observed in: 1) passive or adoptive immunization studies (19, 20, 29), 2) determination of the class(es) of protective antibody (19), 3) effects of neonatal thymectomy, antilymphocyte serum (ALS) and antithymocyte serum (ATS) treatments (49, 50, 51), 4) the kinetics of mast cell proliferation and amine release in infected rats (review 38 vs. reviews 8 and 22), and 5) the degree and significance of reaginic antibody production by intact rats (23). In addition, due

to the rapid onset of active immunity, it has been difficult to separate the effects of passively administered immune serum and cells from active immunity generated by recipient rats in response to a challenge infection (20). Finally, it has been necessary to employ both physical and chemical immunosuppressant treatments as aids in understanding the worm expulsion mechanism. Such immunosuppressants often have effects on organs and tissues other than those toward which the treatment is directed (2), thus possibly creating an unfavorable habitat for worm development and maintenance. Furthermore, the effects of immunosuppressant treatments often are incomplete, as in neonatal thymectomy (49 - 51).

Rats are considered the normal host of *N. brasiliensis*. However, because of the extensive background information available on mouse immunobiology, there are many advantages in using the mouse-helminth model for studies on host immune responses to parasitic infections. It has been shown that the rat strain of *N. brasiliensis* will infect laboratory mice but at a reduced rate compared with infections in rats (52, 53) and may result in unpredictable numbers of adult worms (54). A more recent study, however, has confirmed that the response of mice to rat strain *N. brasiliensis* parallels that of rats with only minor differences in the kinetics of the responses (55). Furthermore, these objections have been overcome through adaptation of a rat strain of *N. brasiliensis* to mice by serial passage of the parasite through over

400 worm generations in mice (56, 57, & R. B. Wescott, personal communications). Mice develop a strong active immunity to the mouse-adapted strain and the immune response is very similar to that of rats (55, 58, 59).

Because humoral responses to thymus-dependent antigens and cell-mediated responses are severely impaired in congenitally athymic (nude) mice (60, 61), we concluded that the nude may have potential as a useful tool in experiments designed to clarify the role of cellular and humoral factors required for regulation of helminthic infections of rodents. Previous work with *Trichinella spiralis* and the mouse pinworms, *Aspiculuris tetraptera* and *Syphacia obvelata*, in nude mice (62, 63) have confirmed this prediction.

In an attempt to define the mechanism of immune elimination of *N. brasiliensis* from rodents, it has been my approach to characterize the host response to *N. brasiliensis* through use of nude mice and their phenotypically normal thymus-bearing littermates (NLM). The effects of various humoral and cellular factors were analyzed in experiments designed either to reconstitute the worm expulsion capability of nude mice or to eliminate a specific factor required for the worm expulsion mechanism which occurs in thymus-bearing mice.

MATERIALS AND METHODS

Animals

Mice used in most experiments were either BALB/c or congenitally thymus-deficient (nude; nu/nu) mice and their normal thymus-bearing littermates (NLM). Nude mice crossed on a BALB/c genetic background were obtained from breeding stock heterozygous for the nude trait. Such breeders were not congenic and their progeny were used in most of the experiments. In cell transfer experiments, however, nude mice which served as cell recipients were congenic with BALB/c donor mice (crossed-intercrossed; generation 9).

BALB/c, normal littermates of nude mice and CFW mice were used as maintenance hosts for the mouse adapted stain of *N. brasiliensis*. In worm transfer studies, rats of the Lewis strain, nude mice and their normal littermates served as donors of adult worms while rats of the Lewis or Sprague Dawley strains, nude mice and their normal littermates were used as recipients of worms.

All animals were maintained on autoclaved 5010C Purina Mouse Chow and acidified-chlorinated water. Bedding was routinely sterilized before use. No medication was administered to experimental mice except in some worm transfer procedures as outlined below.

Parasites

The mouse-adapted strain of *N. brasiliensis* used in these studies was obtained from Dr. R.B. Wescott, Washington State University, Pullman, WA. This strain was originally derived from rats and has been passaged serially through over 400 worm generations in mice (56, 57 and R.B. Wescott, personal communications). In our laboratory, this strain has been maintained by subcutaneous inoculation of normal littermates of nude mice, BALB/c, or CFW source mice with infective larvae approximately on a weekly schedule according to techniques previously described (56).

Fecal material was obtained from source mice on days 6 and 7 post-larval-inoculation (PLI). The feces containing worm eggs was mixed with moist granular animal bone charcoal (VWR Scientific, San Francisco, CA.) at a v/v ratio of about 1 to 4 and incubated at room temperature (25 - 27 C) for 5 days or more before use of the resultant infective larvae (IL).

Fecal Examinations

The modified McMaster technique of Whitlock (65) was further altered for use in estimating the number of worm eggs per gram of feces (EPG). One gram of fecal material was comminuted in 30 cc of saturated NaCl solution (sp. gr. $\simeq$ 1.20). An aliquot 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.

Adult Worm Transfers

Although transfer of adult worms from donor to recipient rats via laparotomy has been accomplished repeatedly by others (16), the technique required modification for use in mice. Adult worms were harvested from donor rats (previously infected with 3 or 5 x 10^3 IL) or mice (previously infected with 0.5 or 1 x 10^3 IL) according to the technique described by Ogilvie and Hockley (26). They were counted under a dissecting microscope and allotted to aliquots of 300 worms each. A 3 mm ventro-medial incision just caudal to the xiphoid process of the sternum was made in mice anesthetized with sodium pentobarbital (66). The stomach and proximal portion of the duodenum were exteriorized and a purse-string suture of 6-0 silk was placed in the serosa of the greater curvature of the stomach about 3 mm from the pylorus. A puncture was made into the lumen of the stomach within the 4 mm^2 area circumscribed by the purse-string suture. A Pasteur pipette containing approximately 300 adult worms was introduced through the opening in the stomach wall, on through the pylorus and for a distance of 1-2 cm into the duodenum where the worms were deposited. After withdrawal of the pipette, the purse-string suture was drawn

and tied, thus closing the opening into the stomach. Attempts to introduce the worms directly into the lumen of the intestine generally resulted in peritonitis and death of the mouse due to failure of the suturing procedure in the fragile duodenal mucosa. In some experiments laparotomized mice were given 5000 units of penicillin and 5000 µg streptomycin intraperitoneally (Grand Island Biological Co., Grand Island, N.Y.) on each of 3 consecutive days beginning on the day of surgery.

Immunosuppressive Treatment

In order to produce mice deficient in humoral immunoglobulin production potential, a portion of each of several litters of BALB/c mice was injected intraperitoneally with rabbit anti-mouse IgM heavy chain (anti-µ) serum. The remaining littermates were allotted to groups similarly injected either with normal rabbit serum (NRS) or phosphate buffered saline. Mice received injections on the day of birth (0.05 ml/mouse) and every second day thereafter (to a maximum individual dose of 0.15 ml at any given injection) until termination of the experiment. The anti-µ serum was produced and provided by Dr. D.D. Manning, University of Wisconsin Medical School, Madison, WI. The technique for anti-µ serum production has been described elsewhere (67,68) and its effects reviewed (69).

Thymus Gland and Thymus Cell Transfer

In experiments designed to impart thymus function capability to nude mice, thymus glands or dispersed thymus cells were given to such mice. Neonatal BALB/c mice served as donors of thymus glands which were implanted surgically in the subcutaneous tissues of the axillary region of congenic 4-week-old nude mouse recipients. Dispersed thymus cells were obtained from thymus glands of 2-week-old donor mice by mincing the glands over 80-mesh screens in chilled phosphate buffered saline in 1% normal mouse serum. The cells were enumerated and assayed for viability by a trypan blue exclusion test (70); subsequently, 1.25×10^8 to 1.34×10^8 viable cells were inoculated intravenously into each recipient mouse.

Immunization

Mice subjected to immunosuppressive (anti-μ) treatments and infected with *N. brasiliensis*, or infected nude mice given thymus cells or thymus glands were immunized by intraperitoneal injections of sheep erythrocytes (SE). From 5×10^8 to 7.2×10^8 SE were given either to nude mice and their NLM controls as a single injection 5 days prior to necropsy, or to anti-μ suppressed mice and their controls on days 16, 10 and 6 prior to necropsy.

Antibody Assays

Mice were tested for their antibody response to SE by a slide modification of the hemolysis-in-gel (Jerne Plaque) technique (71). Assays for both direct (IgM) and indirect (IgG) plaque formation were conducted in the immunosuppressive treatment experiments, whereas only direct plaque formation was determined in all other experiments. Rabbit anti-mouse immunoglobulin was used to facilitate indirect plaque formation. Counts of indirect plaques were recorded as the difference between the number of direct and facilitated plaques.

Serum from anti-μ-treated mice and their controls was tested for the presence of class-specific IgM, IgG_1, IgG_2, and IgA using monospecific antisera. In addition, antibody titers against NRS and residual anti-μ levels were determined. All assays were done semi-quantitatively by the serial dilution Ouchterlony gel diffusion technique of Arnason et al. (72). These tests were conducted, as part of a collaborative study, by Dr. D.D. Manning at the University of Wisconsin Medical School, Madison, WI.

Cellular Immunological Assay

To determine if cell-mediated immune competence had been generated in nude mice given BALB/c thymus glands or thymus cells, nude mice and their BALB/c controls were grafted with CBA skin allografts. The technique used was that of Billingham (73). Graft rejection time was

calculated as the number of days post-grafting at which the skin graft was lost.

Necropsy Procedures

For recovery of *N. brasiliensis* from mice, the entire small intestine was excised from euthanatized animals. The ingesta were flushed from the intact intestine with tap water under pressure. The resultant material containing the worms was then washed over a 200-mesh screen to remove soluble and fine particulate debris. The worms, retained on the screen, were backwashed into petri dishes and counted under a dissecting microscope. The intestinal tissue was pressed between glass plates and examined microscopically for the presence of adherent worms not recovered by the flushing procedure. In most experiments, male and female worms were enumerated separately.

Histopathology

Adult *N. brasiliensis* obtained from rats and mice on various days post-larval-inoculation were examined histologically for alteration of worm intestinal cell morphology. Worms were harvested from the excised small intestine of rats or mice by baermannization in warm PSS. The worms were promptly transferred to Carnoy's fixative and stored in 70% alcohol. To facilitate manipulation of the worms during preparation for sectioning, they were placed into a 1% agar matrix. The worms were sectioned at 6 microns and stained in hematoxylin-eosin.

RESULTS

N. brasiliensis Infection in Congenitally Athymic (nude) and Normal Mice

The initial experiments were designed to determine the kinetics of infection in the nude mouse-*N. brasiliensis* host-parasite system. In the first experiment, 7 seven-week-old nude mice on a BALB/c genetic background and 7 NLM each were inoculated subcutaneously with 300 infective larvae of *N. brasiliensis*. Group fecal worm egg counts were initiated on day 7 (161 hours) post-larval-inoculation (PLI) and thereafter were continued on virtually a daily schedule until the last nude mouse died on day 51 PLI. The results (Figure 1) revealed that fecal worm egg counts for the nude mouse group remained at high levels throughout the experiment and averaged 10,760 $\pm$ 488 (standard error) eggs per gram of feces (EPG). The NLM group, however, showed a peak EPG count at the first fecal examination on day 7 PLI. Thereafter, NLM egg counts dropped rapidly and fluctuated below 1000 EPG until day 31 of the experiment when they became negative.

Individual nude mice died and equal numbers of NLM were sacrificed at intervals between days 17 and 52 PLI (Figure 1). At necropsy, the mean number of adult *N. brasiliensis* recovered from nude mice was 45 $\pm$ 7. In marked contrast, only 3 of 7 NLM were positive at necropsy for *N. brasiliensis* and only one worm was recovered from each of the positive NLM mice.

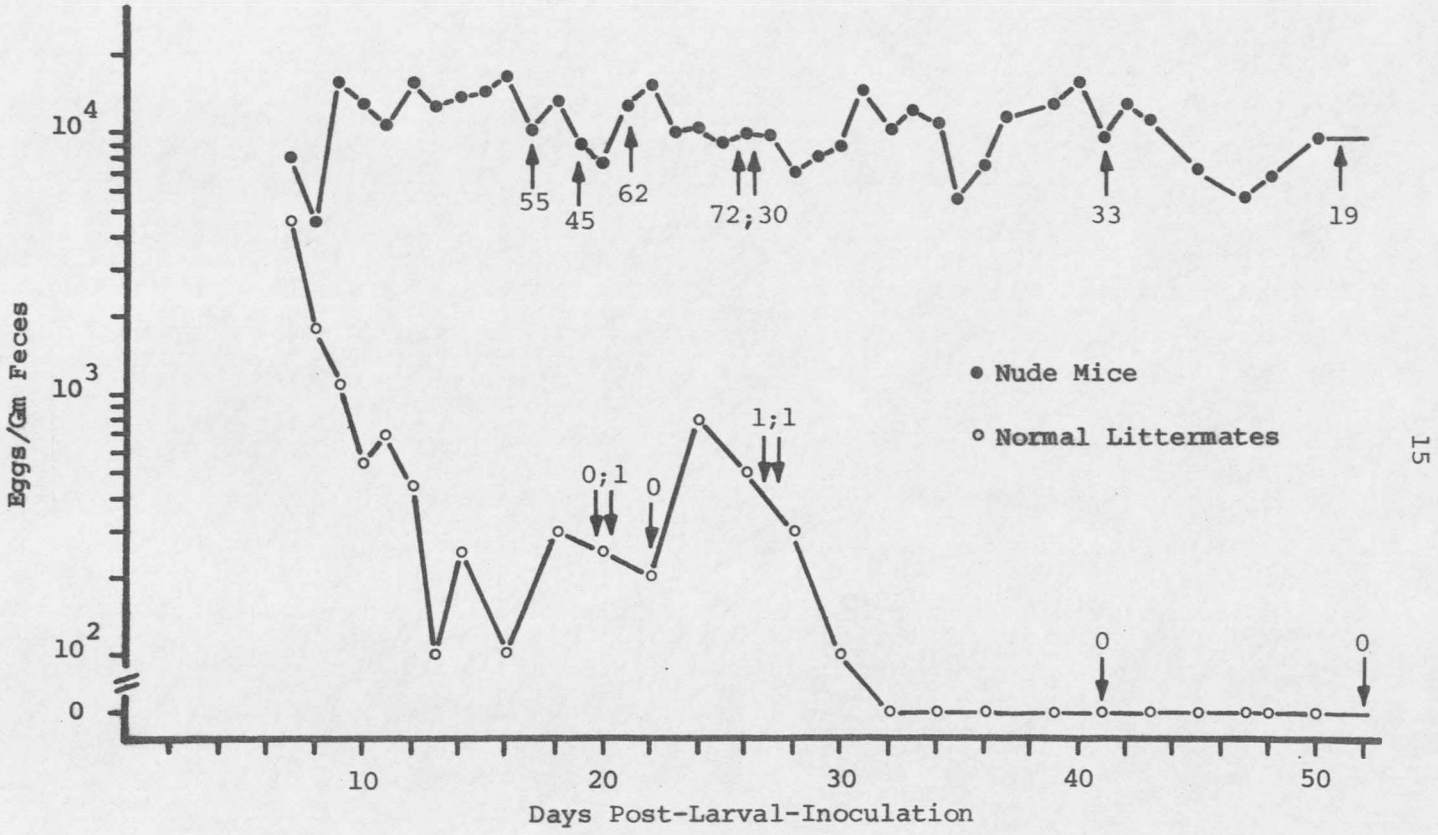

Figure 1. Comparative fecal worm egg counts of nude mice and their normal littermates inoculated with 300 infective larvae of *Nippostrongylus brasiliensis* on day 0. The arrow and accompanying number indicate the day on which individual mice died or were sacrificed and their worm burden at necropsy.

Because the prepatent period was not determined in the first experiment, the work was repeated and fecal examinations were initiated on day 4 (100 hours) PLI instead of day 7. Five nude and 5 NLM mice each were inoculated with 300 infective larvae (IL) of *N. brasiliensis*. Worm eggs were first detected in the feces of both nude and NLM on day 6 PLI (Figure 2). As in the first experiment, worms in the nude mouse group maintained a high level of egg production for the duration of the experiment. The average of all positive EPG counts for the nude mouse group during a 49 day observation period was 19,930 $\pm$ 1150. In contrast, EPG counts for NLM reached a maximum of 9800 on day 7 PLI and dropped to 0 on day 11. All subsequent NLM fecal examinations remained negative for *N. brasiliensis* ova. The average EPG count during patency of NLM was 5270 $\pm$ 1803. The mean worm burden in nude mice was 61 $\pm$ 12.9. No worms were found in any of the NLM mice.

A similar experiment was then conducted to determine whether nude mice derived from a line crossed onto the outbred CFW stain would respond to *N. brasiliensis* in a manner similar to nude mice on a BALB/c genetic background. Thus, 5 six-week-old CFW-nude mice and 5 of their NLM were inoculated with 300 IL and fecal worm egg counts were made according to the protocol outlined for the preceding experiments. Over a 50 day observation period, the profile of daily EPG counts paralleled that observed in the two experiments described above, i.e., CFW nude mice remained infected while their NLM eliminated their worm