



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

A thesis submitted in partial fulfillment of the requirements for the degree of DOCTOR OF
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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 rapidly 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.

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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 IgG₁ (19, 22). However, IgG₂ 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, radio-sensitive (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, *Aspicularis 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 strain 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. \approx 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 5×10^3 IL) or mice (previously infected with 0.5 or 1×10^3 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, IgG₁, IgG₂, 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 ± 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.

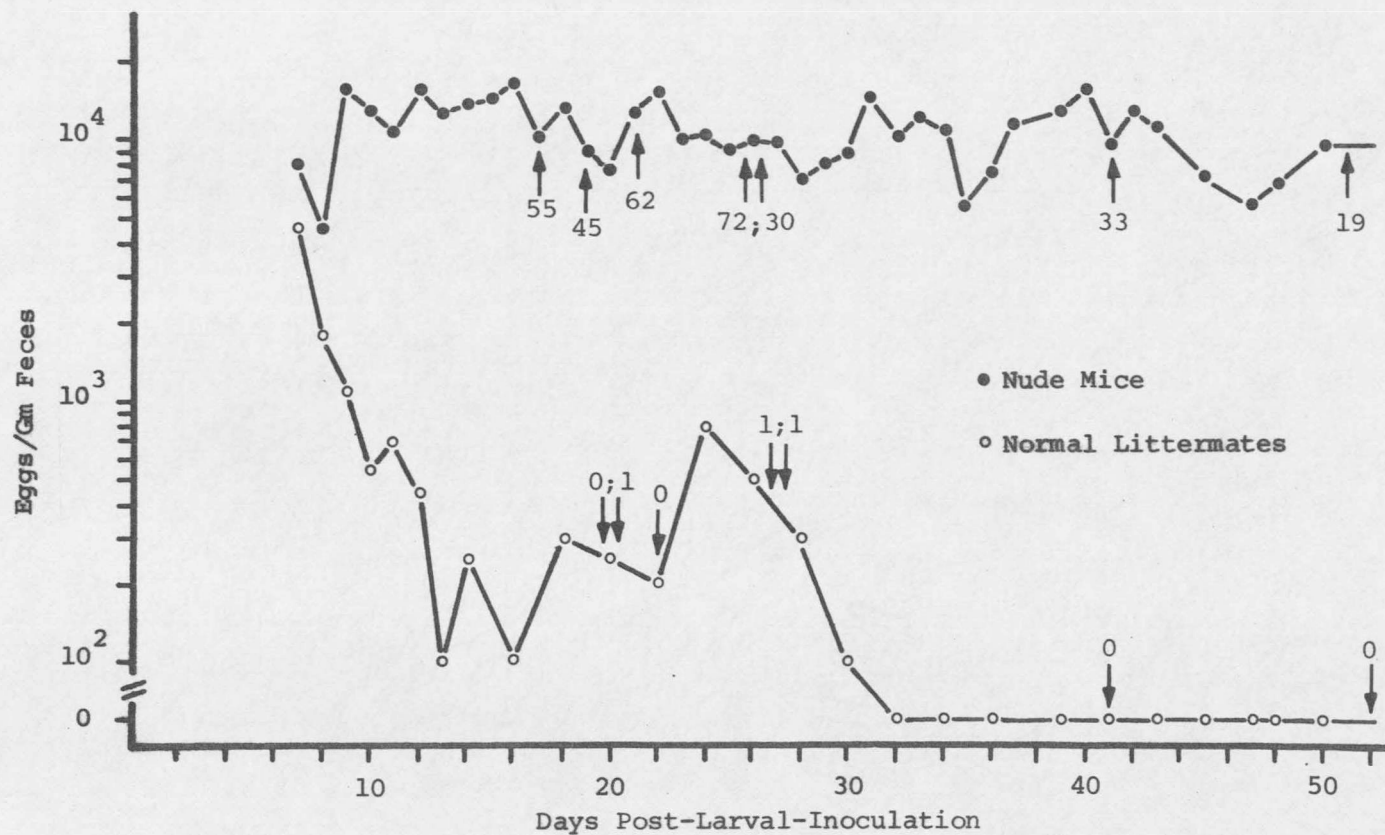


Figure 1. Comparative fecal worm egg counts of nude mice and their normal littermates inoculated with 300 infective larvae of *Nippostrongylus brasiliensis* on day 0. The arrow and accompanying number indicate the day on which individual mice died or were sacrificed and their worm burden at necropsy.

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 ± 1803 . The mean worm burden in nude mice was 61 ± 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 strain 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

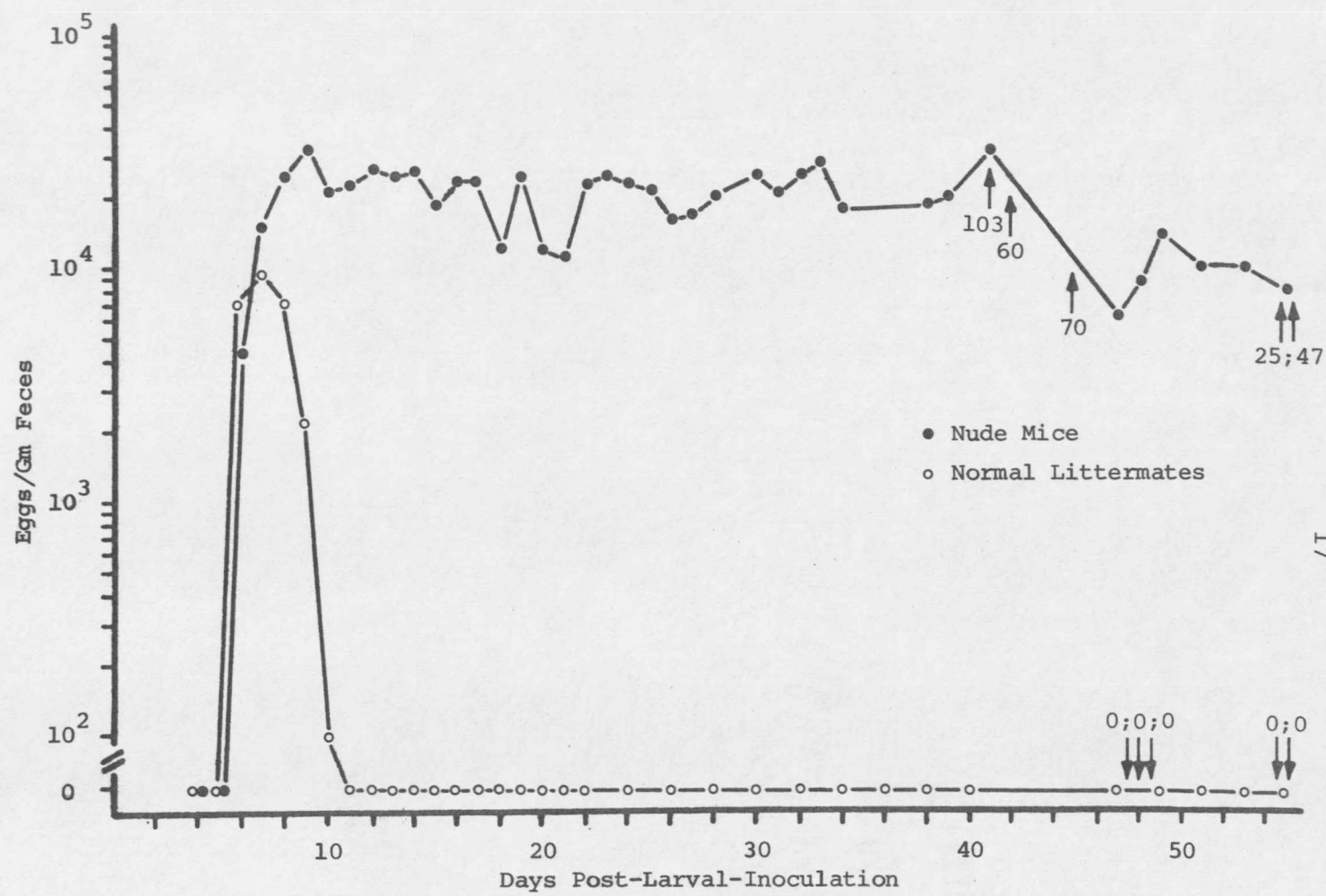


Figure 2. Comparative fecal worm egg counts of nude mice and their normal littermates inoculated with 300 infective *Nippostrongylus brasiliensis* larvae on day 0. The arrow and accompanying number indicate the day on which individual mice died or were sacrificed and their worm burden at necropsy.

burdens by about day 11 PLI. Therefore, the lack of worm expulsion capability is apparently attributable to the thymus-deficient nature of nude mice rather than the genetic background into which the nude trait is placed, at least with respect to the BALB/c and CFW strains.

Generation of Worm Expulsion Potential in Nude Mice

Nude mice have abnormalities in addition to thymus deficiency (74, 75). As a prerequisite to studies aimed at defining host immune factors required to elicit worm elimination, it was, therefore, necessary to examine *N. brasiliensis* infections in nude mice with thymus competence. In a pilot study five 6-month-old nude mice, previously given either thymus glands or thymus cells and proven thymus competent based on skin allograft rejection capability, each were inoculated with 300 IL of *N. brasiliensis*. Because no littermates of these mice were available, age matched BALB/c control mice were similarly inoculated. The worms in both groups of mice had a prepatent period of 6 days and apparently were eliminated from all mice within about 13 days PLI as determined by worm egg production (Figure 3). Because the data suggested that nude mice with thymus competence were capable of worm expulsion, three additional experiments were conducted to assess the validity of this observation.

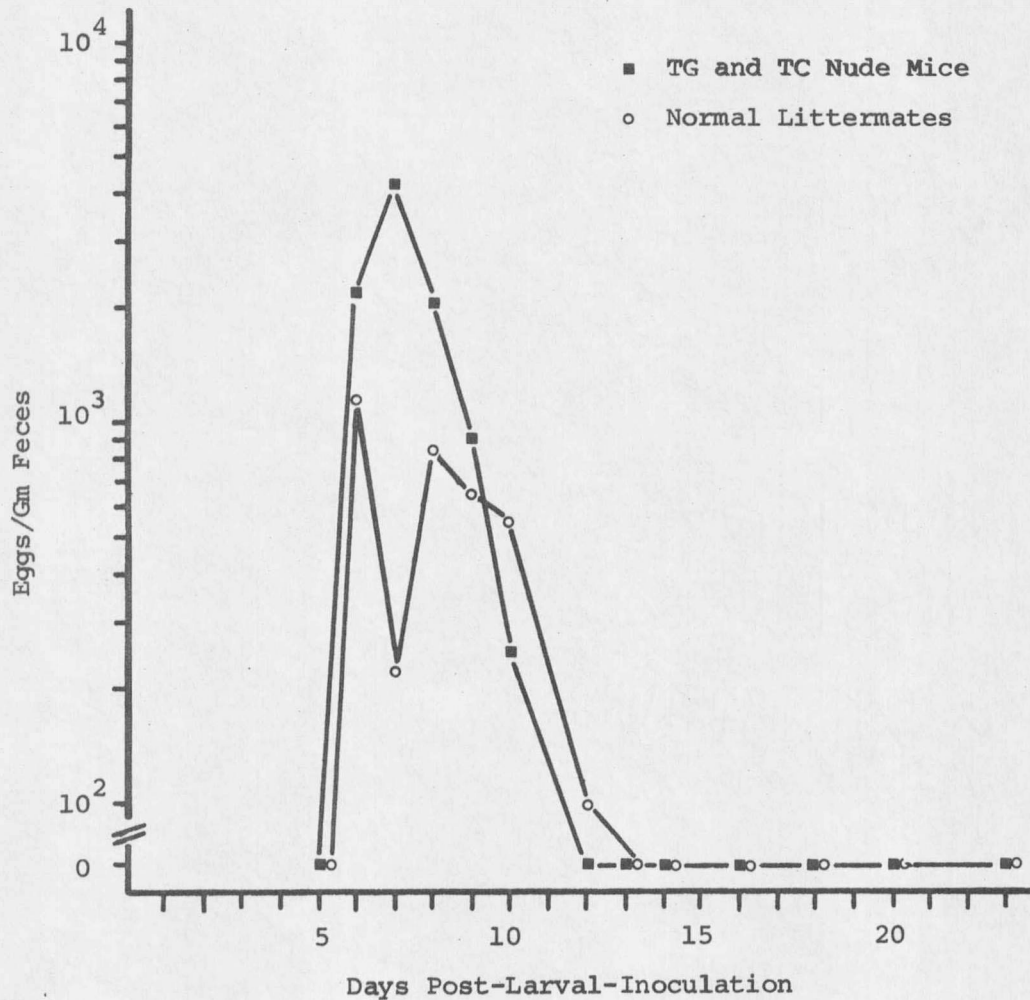


Figure 3. The effect of thymus gland (TG) implantation or thymus cell (TC) inoculation on *Nippostrongylus brasiliensis* fecal worm egg counts in nude mice. All mice were inoculated with 300 infective larvae on day 0.

In the first of these experiments, mice 4-6 weeks of age were randomized and placed into 3 experimental groups. Six nude mice each were given an intravenous (i.v.) inoculation of 1.59×10^8 thymus cells (TC) obtained from 2-week-old BALB/c donor mice. Approximately six hours later, these mice, 7 nude littermates and 8 NLM each were inoculated with 300 infective larvae of *N. brasiliensis*. Mice in all groups became patent on day 6 PLI. The NLM rapidly eliminated their worms whereas the untreated nude mouse group remained infected for the duration of the experiment (Figure 4). The precipitous fall in EPG counts which generally appears between 7 and 10 days of an NLM infection did not occur in TC nude mice until between days 19 and 24 PLI. Thereafter, the TC nude group egg counts fluctuated between 500 and 2250 EPG until day 47 PLI when they became negative. This fluctuation in EPG counts was subsequently clarified upon necropsy of TC nude No. 4 which died on day 47 of the experiment. This mouse experienced the wasting syndrome characteristic of untreated nude mice and at necropsy, 37 adult *N. brasiliensis* were recovered. Furthermore the negative EPG count of the TC nude group on day 47 PLI was based on feces from mice exclusive of TC nude No. 4 feces. Collectively, these data suggest that this mouse was probably responsible for the long duration of positive EPG counts in the TC nude group and because it wasted, was probably thymus incompetent.

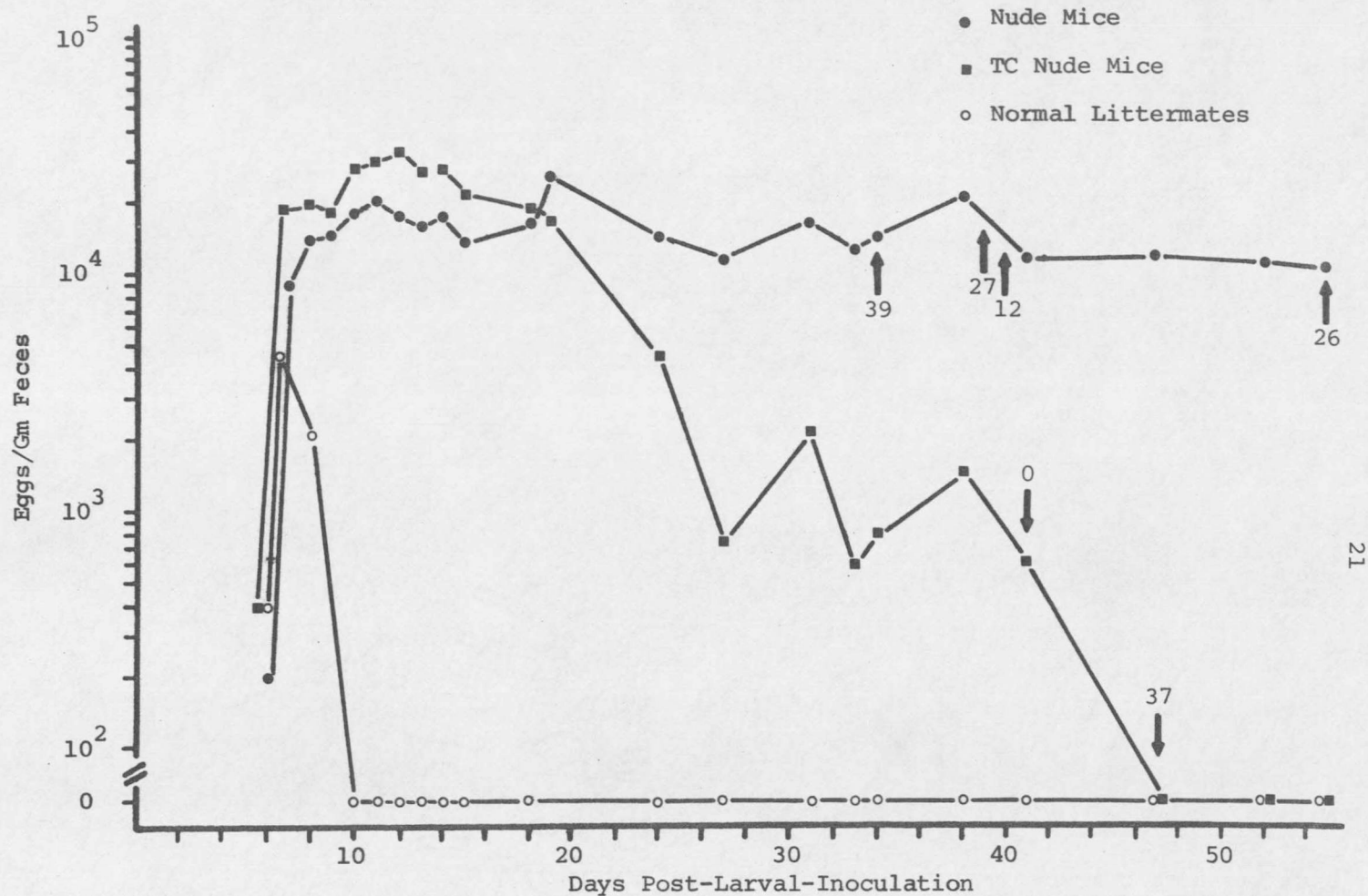


Figure 4. The effect of thymus cell (TC) inoculation on *Nippostrongylus brasiliensis* fecal egg counts in nude mice.

TC nude mice were inoculated i.v. with 1.59×10^8 viable thymus cells 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.

TC nude mouse No. 6 was accidentally killed by an anesthetic overdose during skin grafting (see below) on day 41 PLI. In contrast to TC nude No. 4, this mouse was negative for *N. brasiliensis* at necropsy.

In order to assess thymic function in TC nude mice, the response to allografts of CBA skin was determined for 4 TC nude mice (TC nude mice Nos. 4 and 6 had died - see above), the 4 surviving nude mice and 3 of the NLM. Allograft rejection time averaged 14 and 13 days for all TC nude and NLM mice, respectively. None of the untreated nude mice rejected their skin grafts.

In addition to the 2 TC nude mice previously described, necropsy data are available for only 5 of 6 nude mice. These mice which died between days 34 and 81 of the experiment had an average of 31 ± 4.3 worms. Although the NLM and TC nude mice were not necropsied, the egg count data for these groups on days 52 and 55 suggested that these mice were negative for *N. brasiliensis* upon termination of the experiment.

Because a lag period occurs between inoculation of T cells and establishment of T cell populations in peripheral lymphatic tissues of nude mice, we hypothesized that the interval between larval inoculation and worm elimination would be reduced if T cells were administered to nude mice prior to inoculation with *N. brasiliensis*. Thus, the experiment was repeated. In addition to nude mice which received T cells on day 0, another group of nude mice received T cells 3 weeks prior to larval inoculation. The design of this experiment is

outlined in Table I. The results indicated that indeed the interval between larval inoculation and worm elimination was reduced in nude mice given T cells 3 weeks prior to infection (Figure 5).

It is interesting to note that nude mouse No. 8 was experiencing the wasting syndrome as early as day 11 PLI even though it had received T cells about 4½ weeks prior to that time. This mouse died on day 15 PLI and was heavily infected with *N. brasiliensis* (Table I). Likewise, mice Nos. 13 and 15 from group II had not eliminated their worms at the time they died (24 and 50 days, respectively, after T cell administration). The lack of worm expulsion capability may indicate a concurrent lack of thymus competence. In marked contrast, all TC nude mice which survived until termination of the experiment had greatly reduced worm counts and clearly had developed thymus competence as determined by their skin allograft rejection capability and plaque forming cell responses to SE (Table I). Collectively, data from these two experiments indicated that generation of worm expulsion potential may be accomplished in nude mice which have been inoculated intravenously with dispersed thymus cells.

Because some of the nude mice given thymus cells i.v. in the preceding two experiments apparently failed to develop thymus function, we postulated that thymus gland implantation may be a more efficient means of generating thymus competence in nude mice. Accordingly, 6 nude mice were given thymus gland implants (TG nude mice) 30 days prior

Table I. Generation of worm expulsion potential in congenitally athymic (nude) mice injected with thymus cells.

Group	Mouse ^c No.	Day PLI of death or sacrifice	No. <i>N.</i> <i>brasiliensis</i> recovered at necropsy	Graft rejection time (days) ^d	PFC/e 10 ⁶ ^e
I Nude mice; T-cells given day -21 or -22 ^a	1	61	0	16	193
	2	61	0	16	22
	3	61	0	16	5
	4	61	1	14	138
	6	61	0	15	138
	7	61	0	14	84
	8	15	129	M.D.	M.D. ^g
	9	20	0	M.D.	M.D.
	II Nude mice; T-cells given day 0 ^b	11	61	2	15
12		61	0	15	83
13		24	130	M.D.	M.D.
14		60	2	15	338
15		50	103	M.D.	M.D.
III Nude mice	16	51	103	N.R. ^f	M.D.
	17	59	3	N.R.	M.D.
	18	44	98	M.D.	M.D.
	19	58	47	N.R.	M.D.
	20	34	118	M.D.	M.D.
IV NLM	21	61	0	14	335
	22	61	0	14	237
	23	61	0	14	504
	24	61	0	14	150

^a 1.25 x 10⁸ viable T-cells inoculated into each mouse i.v.

^b 1.34 x 10⁸ viable T-cells inoculated into each mouse i.v.

^c All mice inoculated with 300 infective larvae of *N. brasiliensis* on day 0.

^d All surviving mice given CBA skin allografts on day 41 PLI.

^e All surviving mice inoculated with sheep erythrocytes (SE) on day 56

PLI and assayed for anti-SE responses on day 61 PLI.

^f N.R. - skin allograft not rejected.

^g M.D. - mouse died prior to assay.

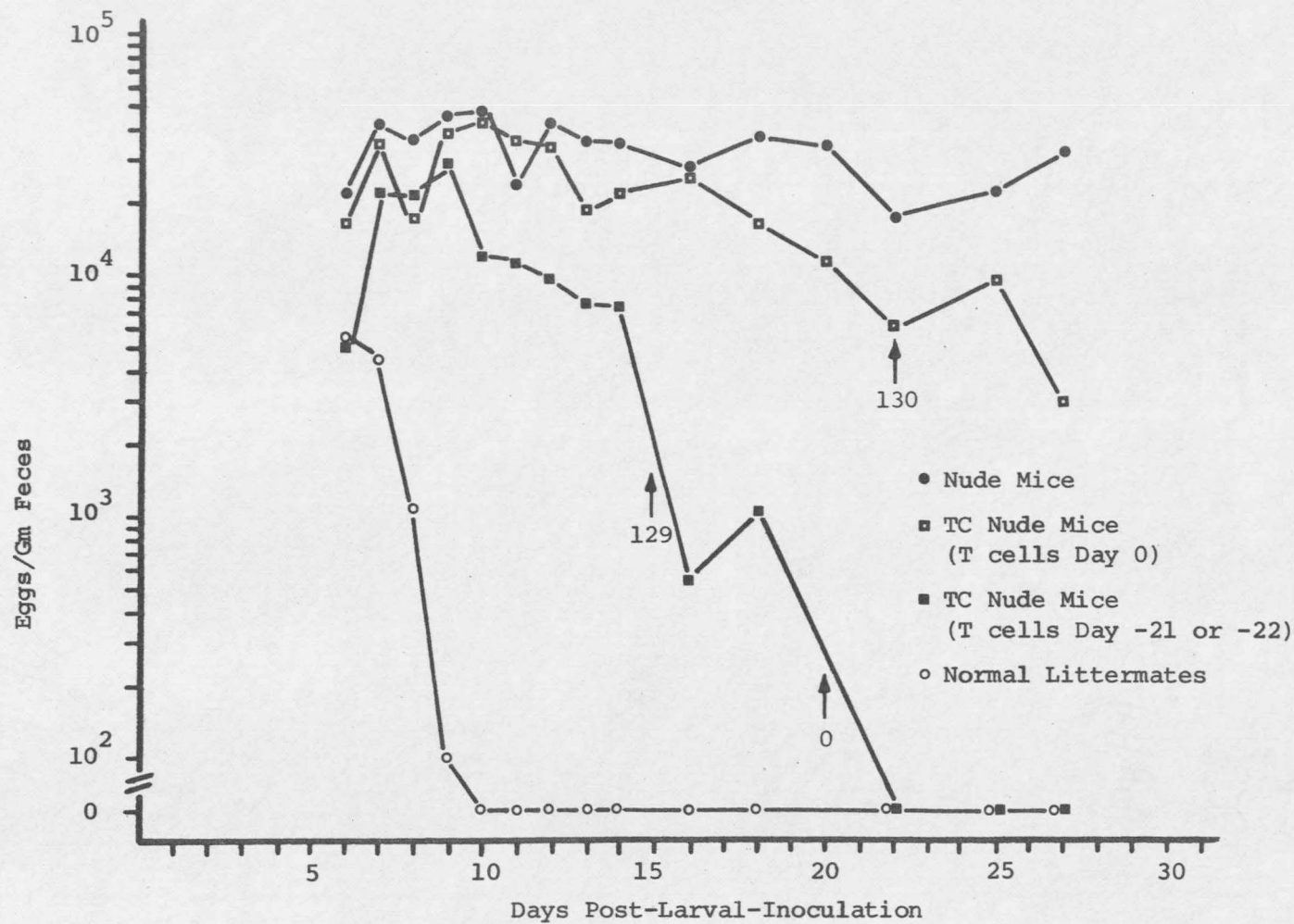


Figure 5. The effect of thymus cells (TC), as a function of time of administration, on fecal worm egg counts of *Nippostrongylus brasiliensis* in nude mice. TC nude mice were inoculated with 1.25×10^8 viable thymus (T) cells on days -21, -22, or 0. Arrow and number indicate day on which individual mice died and their worm burden at necropsy.

to larval inoculation (Table II). These mice, 5 nude mice and 5 NLM all were inoculated with 300 infective larvae of *N. brasiliensis* on day 0. The results of daily EPG counts (Figure 6) indicated that NLM rejected their worm burdens by about day 9 PLI and infections in nude mice persisted until termination of the experiment. The TG nude mice did show a precipitous fall in EPG counts similar to that of NLM with the exception that the phenomenon was delayed by about 5-6 days.

At necropsy, an average of 1.7 ± 1.2 worms was recovered from TG nude mice as compared with a burden of 69 ± 24 worms from nude mice. No worms were found in any NLM. To confirm generation of thymic function in TG nudes, skin allografts were made on all mice on day 15. Five days after grafting, healing in was accomplished in all mice. The grafts of NLM and TG nudes had become indurated and inflamed by day 10 post-grafting and by days 12-13 rejection was complete. At no time did grafts on nude mice show signs of rejection and the grafts remained healthy for the lifetime of the recipient. On day 28 PLI, all of the mice were injected with SE. On day 33, the plaque forming cell (PFC) response of nude mice and NLM was 34 ± 12 and 517 ± 139 PFC/ 10^6 spleen cells, respectively. Thymus-implanted nude mice responded with 280 ± 157 PFC/ 10^6 spleen cells.

These data, coupled with those of the TC nude mouse experiments, present direct evidence that 1) nude mice are incapable of expelling

Table II. Generation of worm expulsion potential in congenitally athymic (nude) mice given thymus gland implants

Group	Mouse No. ^b	No. worms recovered at necropsy ^c	Graft rejection time ^d	PFC/10 ⁶ ^e
I T-gland ^a nude mice	1	0	12	213
	2	1	12	401
	3	0	12	225
	4	1	13	138
	5	0	T.F. ^f	163
	6	0	13	538
II Nude mice	7	83	N.R. ^g	66
	8	157	N.R.	0
	9	49	N.R.	38
	10	29	N.R.	48
	11	28	N.R.	16
III LM	12	0	12	383
	13	0	13	910
	14	0	12	16 ^h
	15	0	13	498
	16	0	12	276

^a Given thymus gland implants subcutaneously 30 days prior to larval inoculation.

^b All mice inoculated with 300 infective larvae of *N. brasiliensis* on day 0.

^c Mice necropsied on day 33 PLI.

^d All mice skin grafted on day 15 PLI.

^e All mice given 0.2 ml of a 20% suspension of SE on day 28 PLI.

^f Technical failure.

^g N.R. - skin graft not rejected.

^h Not included in determination of mean PFC response. Probably a low response because of improper inoculation of SE antigen.

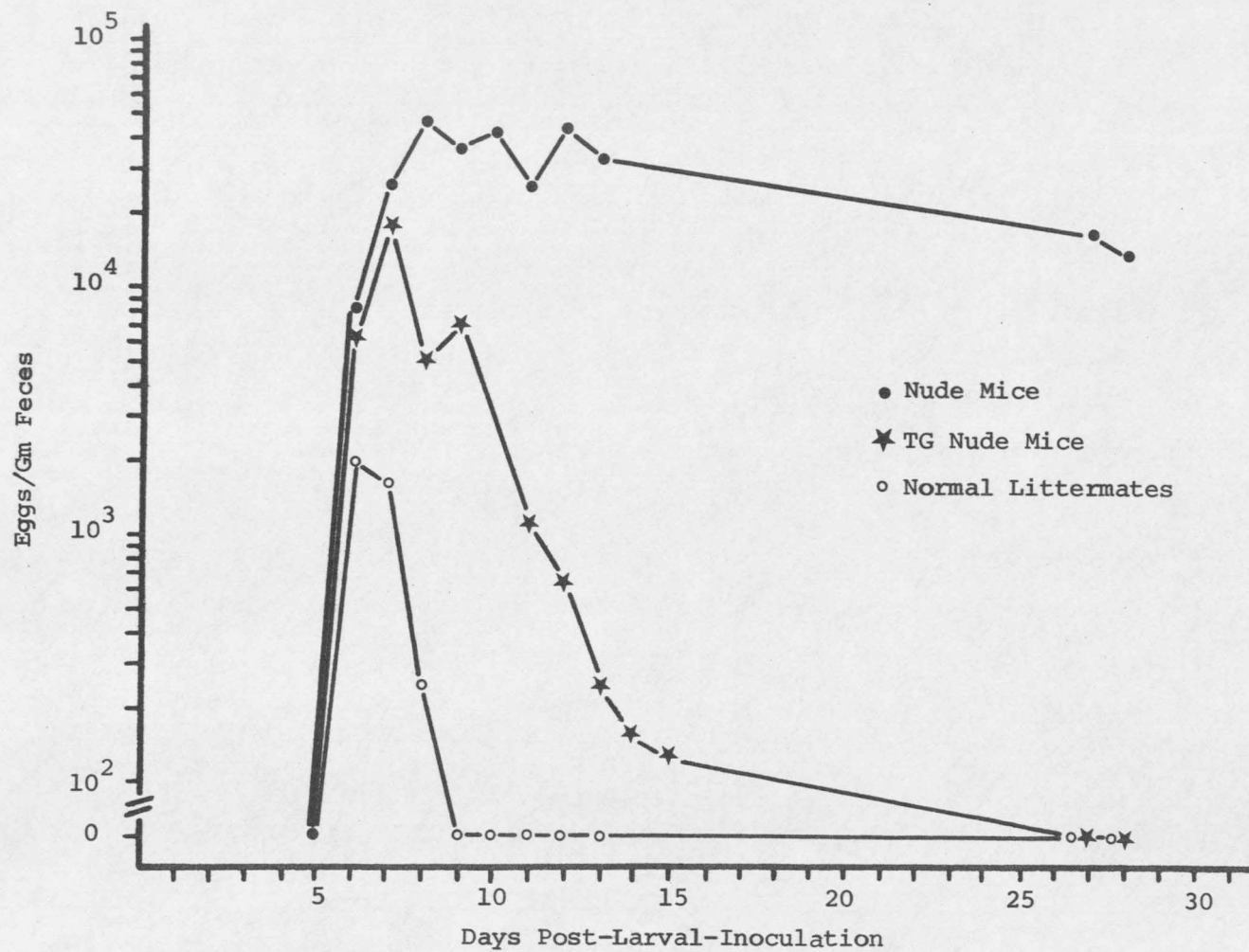


Figure 6. The effect of thymus gland (TG) implantation in nude mice on *Nippostrongylus brasiliensis* fecal egg counts.

N. brasiliensis, at least for more than 50 days PLI, and 2) worm expulsion is a thymus dependent phenomenon.

Thymus Dependence of the Effector Step in
Expulsion of *N. brasiliensis*

With establishment of the thymus dependence of *N. brasiliensis* expulsion from mice, we then proceeded to determine whether the proposed inductive step (antibody-mediated worm damage), the effector step (worm elimination) or both require thymus function for their elicitation in mice. It has been shown by others (26) that adult worms in rats are not damaged either histologically or functionally by 6-9 days PLI; such worms are called "normal". By days 10-14 PLI, however, both structural and functional worm damage is apparent (26). Functional damage has been detected by measuring comparative worm expulsion times following transfer of adult normal or damaged worms via laparotomy into normal recipient rats. Damaged adult worms are eliminated by about day 5-6 while normal worms are not expelled until day 9-11 after transfer (47). The technique of adult worm transfer via laparotomy was thus used to determine the thymus dependence of the effector step in worm elimination. Adult *N. brasiliensis* were harvested from Lewis rats which had been inoculated with 3×10^3 or 5×10^3 IL 14 or 15 days previously. Three hundred of these worms were transferred into the duodenum of each of 8 nude and 8 NLM

recipient mice. Beginning on the day after worm transfer, daily EPG counts were made for each group.

Results indicated (Figure 7) that NLM rapidly eliminated their worm burdens while nude mice were incapable of expelling their worms over either a 30 or 42 day observation period. Four NLM were sacrificed on days 15 and 42 post-worm-transfer. All but 1 NLM were negative for *N. brasiliensis* at necropsy. The positive NLM, from the group sacrificed on day 15, harbored 5 worms. In contrast, nude mice which died at various intervals following worm transfer (Figure 7) had an average worm burden of 190 ± 29.6 . These results appear to suggest that the effector of worm expulsion is thymus dependent. Such a conclusion rests on the assumption that 13 or 14-day rat worms were damaged prior to transfer into NLM and that the NLM expulsion of normal worms (both inductive and effector steps required) is distinguishable from NLM expulsion of damaged worms (effector step only required). If expulsion of damaged and normal worms from NLM controls is not distinguishable, then one cannot clearly ascertain which step (inductive or effector) is thymus dependent in the previous experiment (Figure 7). Therefore, 7-day (normal) and 13 or 14-day (damaged) rat worms were transferred into NLM and their expulsion times determined.

Based on fecal egg counts (Figure 8), 13 or 14-day rat worms were rapidly expelled from NLM while expulsion of 7-day rat worms from NLM

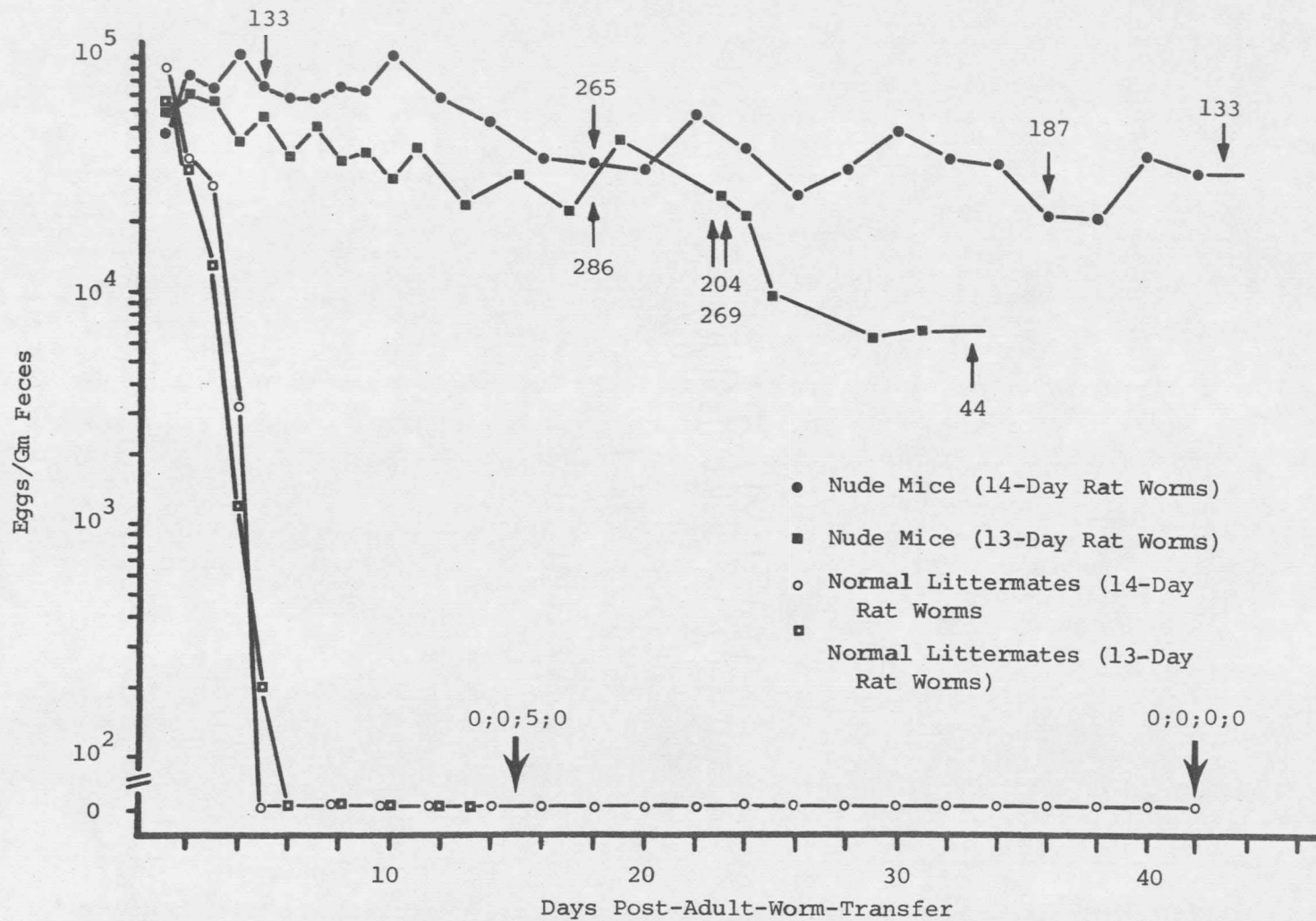


Figure 7. Thymus dependence of the effector step in worm elimination. 13 or 14-day adult *Nippostrongylus brasiliensis* transferred from rats into nude and normal littermate mice on day 0. The arrow and accompanying number indicate the day on which individual mice died or were sacrificed and their worm burden at necropsy.

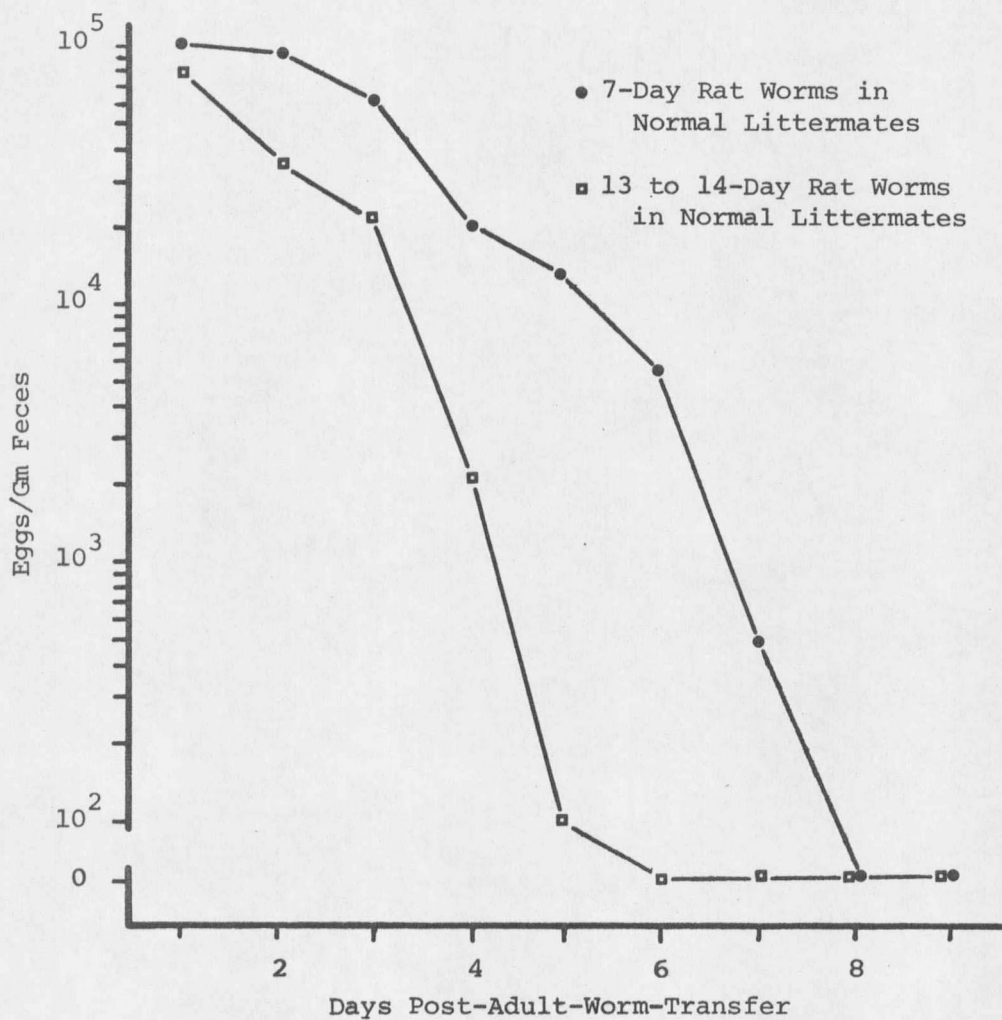


Figure 8. The temporal relationship in elimination by mice of normal (7-day) and damaged (13 to 14-day) *Nippostrongylus brasiliensis*. Worms were obtained from 7 and 13 or 14-day infections of rats and transferred via laparotomy into normal littermates of nude mice on day 0.

was delayed for about 3 days. Furthermore, histological sections of 13 or 14-day rat worms confirmed the conclusion of others (26) that such worms have vacuolated intestinal cells and are definitely damaged. Collectively, these data support the conclusion of others that the effector step in the *N. brasiliensis* expulsion process is a thymus dependent phenomenon.

Thymus Dependence of the Inductive
Step in Expulsion of *N. brasiliensis*

Included in the criteria used to define a damaged worm population are: a) reduction in reproductive capacity, b) reestablishment and kinetics of elimination when transferred into a normal host (8), and c) structural changes in the cellular morphology of individual worms (26). The thymus dependence of the worm damaging process (inductive step) has not been established and, therefore, was the basis for the following series of experiments.

a) Reduction in Reproductive Capacity. Figures 1, 2, 4-8 present evidence that *N. brasiliensis* in nude mice maintain a high level of egg production virtually for the lifetime of the host. A more direct measure of worm reproductive capacity is a determination of the number of eggs in the uteri of individual female worms (eggs/female; EPF). Thus, the EPF was determined for a total of 597 worms derived from nude mice, their NLM or Lewis rats which had been infected for varying

lengths of time with *N. brasiliensis*. The data (Table III) indicate that worms derived from nude mice infected for 17 to 81 days had an average EPF of 34.45 ± 1.13 which was not significantly different ($P > 0.01$) than that of nude mice infected for 7 to 12 days (EPF of 31.54 ± 0.99). Furthermore, worms, obtained from nudes, regardless of duration of infection, had an average EPF which was not significantly different ($P > 0.05$) from that of "normal" worms derived from a 7 or 8-day infection of rats (EPF of 31.93 ± 0.43).

Conversely, female worms obtained from a 14-day infection of rats (EPF of 7.34 ± 0.48) or a 9-day infection of NLM (EPF of 14.93 ± 0.56) had a highly significant reduction in EPF compared with worms derived from nude mice ($P < 0.01$). These data clearly indicate that nude mice are incapable of causing worm damage as determined by the criterion of worm egg production capacity.

b) Worm Reestablishment and Kinetics of Expulsion Following Transfer into a Normal Host. Differential rates of expulsion of adult worm infections have been used to determine if worms are normal or damaged. Rats which are recipients of normal adult worms expel them by about day 10-11 after transfer. Conversely, rats given damaged adult worms expel their worm burdens within about 6 days of worm transfer (47). In a series of 8 experiments the technique of adult worm transfer was used to determine whether the inductive step in worm expulsion is thymus dependent. In all but one of these experiments worms were derived from

Table III. An analysis of the number of eggs per female *N. brasiliensis* as a function of duration of infection.

Group	Duration of infection (days)	No. worms examined	Avg. EPF	S.E. ^e	Max.-Min.	T-Statistic
Nude mice	17-81	97	34.45 ^a	1.13	68-15	30.46
Nude mice	7-12	50	31.54 ^a	0.99	47-22	31.86
Normal litter-mates	6	100	23.21 ^b	0.73	51-6	15.95
Normal litter-mates	8	100	22.91 ^b	0.55	53-13	41.97
Normal litter-mates	9	100	14.93 ^c	0.56	32-1	26.70
Lewis rats	7-8	150	31.93 ^a	0.43	46-21	73.97
Lewis rats	14	100	7.43 ^d	0.48	33-0	15.31

^{a,b,c,d} Duncan's multiple range test (Reference 21). Any two means not having the same letter superscript are significantly different ($P < 0.01$).

^e Standard error of the mean.

normal donor mice on day 6 or day 8 PLI according to a protocol established using rat strain *N. brasiliensis* in mice (55). In all cases, worms transferred from normal to nude mice, regardless of duration of infection in donor mice, were not expelled from recipient nude mice.

In order to establish more firmly that nude mice are unable to generate the inductive step, it was necessary to show the inductive step does not take place by day 6 PLI in donor mice, thus rendering the worms damaged prior to transfer. Although the experiment was attempted repeatedly, our data indicate that no difference was detectable in time of worm expulsion following transfer of either 6 or 8-day worms into NLM or rats. Therefore, we elected to transfer 9-day mouse worms into rats to compare their time of expulsion with 6-day normal mouse worms or 15-day nude mouse worms. Based on fecal egg counts, (Figure 9) there was a 1-3 day reduction in expulsion time in recipients of 9-day versus 6-day worms derived from normal mice. Although the rats receiving nude mouse worms did not expel their worms for an extended period (Figure 9) their EPG counts are not strictly comparable with those of the other groups of mice due to differences in the number of worms transferred. At best, the data are merely suggestive that the inductive step can be differentiated from the effector step by the technique of adult mouse worm transfer into rats. Clearly, further studies are needed to clarify this point.

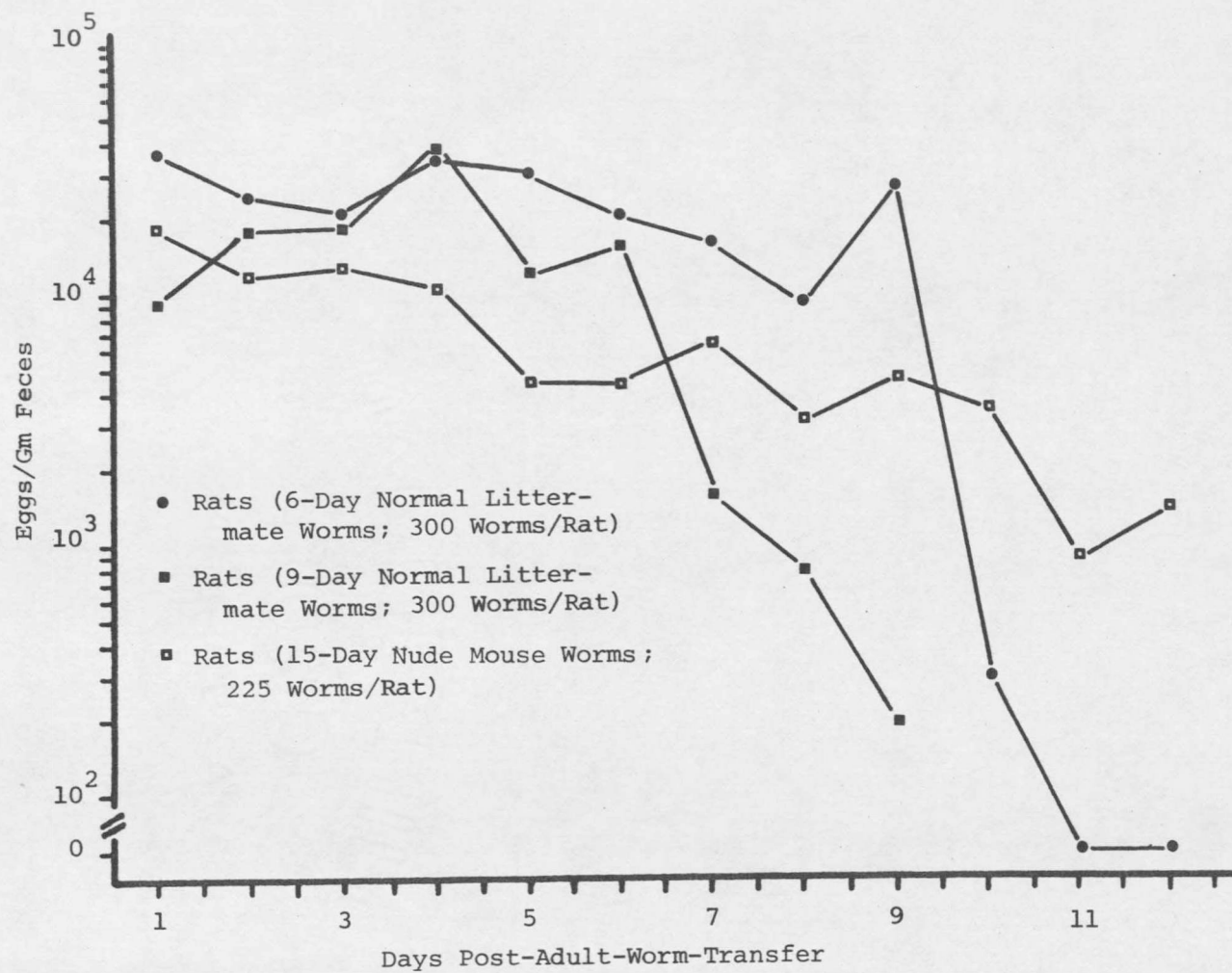


Figure 9. Elimination of normal (6-day normal littermate or 15-day nude mouse) and damaged (9-day normal littermate mouse) worms from Sprague Dawley rats. In all cases, mouse worms were transferred via laparotomy into rats on day-0.

c. Structural Changes in Cellular Morphology of Worms. In transverse sections of *N. brasiliensis*, a varying morphology of the worm intestinal cells has been correlated with the different steps in the worm expulsion process (26). Briefly, in light microscope sections, intestinal cells of normal worms derived from 7-day rat infections stain densely with hematoxylin. Conversely, the intestinal cells of damaged worms obtained from 14-day infections of rats are markedly vacuolated. Studies using the electron microscope have revealed that densely staining ribosomes of normal worms are replaced by large vacuoles containing material of low electron density (26).

If the inductive step of worm expulsion (worm damaging process) is thymus dependent, it follows that nude mice should be incapable of evoking worm damage. Accordingly, *N. brasiliensis* adults were collected from the intestines of NLM which had been infected for 6 or 8 days, from nude mice infected 15 days, and from Lewis rats infected either 7 or 14 days. We observed that intestinal cells of worms obtained from 7-day infections of rats were not vacuolated and these were considered normal (Figure 10). In contrast, the dense staining intestinal cell cytoplasm of the 7-day rat worms was replaced by large vacuoles in 14-day rat worms. In contrast, worms derived from 15-day infections of nude mice appeared as normal worms.

Slides of either 6 or 8-day NLM worms had some sections of worms which appeared nearly normal with only slight cell vacuolation, and

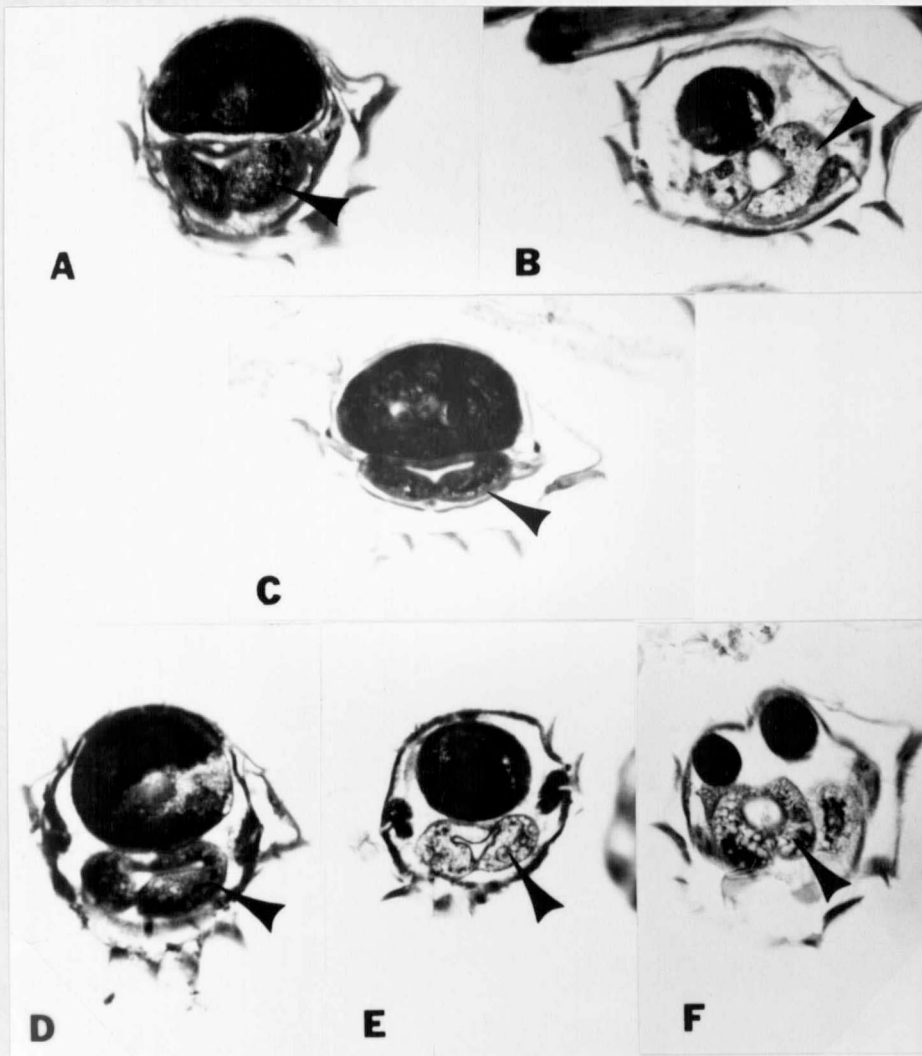


Figure 10. Intestinal cell morphology of *Nippostrongylus brasiliensis* obtained from infections of varying duration in mice or rats. A) Normal Rat Worms (7-day infection); B) Damaged Rat Worms (14-day infection); C) Nude Mouse Worms (15-day infection); D) 6-day normal littermate mouse (NLM) worms (normal); E) 6-day NLM worms (damaged); F) 8-day NLM worms (damaged). Arrow indicates worm intestinal cell. All sections are 400X magnification.

other sections in which gut cells were severely vacuolated, regardless of worm age. No clear pattern of damage emerged in 6 or 8 day NLM worms.

Although the temporal sequence of the worm damaging process in NLM remains unclear, these data support the conclusion that nude mice are incapable of inducing worm damage, at least through 15 days of an infection.

Effect of Immunosuppression by Heterologous Anti- μ
Serum on *N. brasiliensis* Expulsion from Mice

It has been concluded by others (see review 47) that antibody is required to elicit worm damage and additionally, worm damage is the first and necessary prerequisite in the sequence of steps leading to worm expulsion. Without worm damage, no expulsion will occur (47). We postulated, therefore, that abrogation of antibody production potential would curtail *N. brasiliensis* expulsion from mice. Accordingly, individual BALB/c mice from 5 litters were randomized among 3 experimental groups. Each of 7 mice in the first group received intraperitoneal injections of rabbit anti-mouse μ chain antiserum (anti- μ) on their day of birth and every second day thereafter according to the following schedule: days 0 (day of birth) and 2, 0.05 ml; day 4, 0.7 ml; day 6, 0.8 ml; days 10 through 20, 0.10 ml; days 22 through 2 days prior to necropsy, 0.15 ml. Seven mice each received normal rabbit serum (NRS)

and 8 mice each received phosphate buffered saline (PBS) in equal amounts and on the same schedule as outlined for anti- μ treated mice. Mice in these groups, in addition to 6 untreated BALB/c and 6 nude mice, each were inoculated with 300 IL of *N. brasiliensis* on day 33 of the experiment (because of varying birth dates, mice were 27-33 days old on day 33 of the experiment).

Based on daily group EPG counts, mice treated with NRS or PBS and their untreated BALB/c controls eliminated their worm burdens as expected (Figure 11; egg counts of PBS-treated mice not plotted). Surprisingly, mice treated with anti- μ also expelled *N. brasiliensis* virtually within the same time period as the controls. Nude mice, however, remained infected throughout the experiment.

To determine the anamnestic responsiveness of anti- μ -treated mice, all but the nude mice were given a challenge of 300 infective *N. brasiliensis* larvae 17 days after the initial infection (Figure 11). Nude mice had either died or were wasting and thus were not challenged. Two additional control groups, one consisting of 6 nude and the other comprised of 6 NLM, were infected with *N. brasiliensis* on the day challenge infections were given to NRS, PBS and anti- μ mice. The lower EPG counts following a second infection suggest that mice treated with anti- μ were able to muster a secondary response to *N. brasiliensis*.

The immunoglobulin production potential of mice treated with anti- μ , NRS or PBS and untreated BALB/c controls was determined by PFC

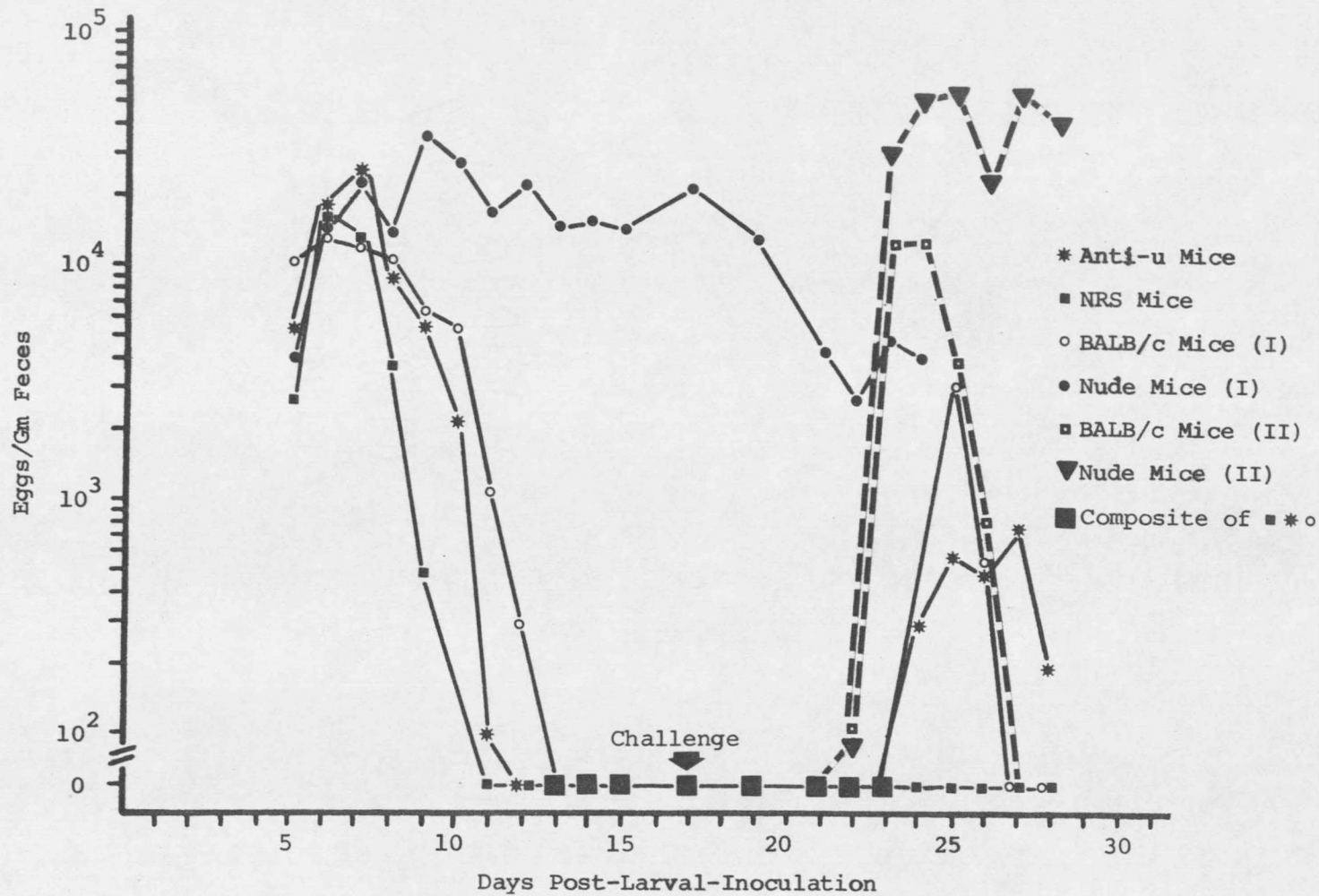


Figure 11. The effect of anti- μ serum on expulsion of *Nippostrongylus brasiliensis* from normal mice. Anti- μ Mice; treated with anti- μ at birth and every second day thereafter. All mice inoculated with 300 IL *N. brasiliensis* on day 0 (exclusive of group II Mice) and challenged with 300 homologous IL on day 17 (all but Nude I mice).

responses to SE and by determination of class-specific Ig levels in their serum. The data (Table IV) indicate that anti- μ -treated mice were virtually incapable of responding to SE. The low response of mice treated with NRS was probably due to antigenic competition between the massive amount of NRS and SE.

Analysis of Ig levels revealed that Ig production potential was virtually abrogated in anti- μ -treated mice (Table V). IgM was not detectable in serum of anti- μ -treated mice and IgG₁, IgG₂ and IgA levels were in every case below control group levels. It is significant that circulating anti- μ was detectable in anti- μ -treated mice, indicating that sufficient quantities of the immunosuppressant were administered.

Because our supply of anti- μ was exhausted, the experiment was terminated prior to complete elimination of the second infection of *N. brasiliensis*. Necropsy data indicated that nude mouse control group I (control on initial infection) and nude mouse control group II (control on challenge infection) both remained infected during the experiment (average worm burdens = 77 ± 14 and 100 ± 24 worms, respectively, for the two groups). The anti- μ mice, in contrast, harbored only 8.4 ± 2.6 worms. An average of 0.4 ± 0.3 and 1.8 ± 0.8 worms were recovered from PBS and BALB/c control mice, respectively. No worms were found in NRS controls. Except for those recovered from nude mice, all worms including those recovered from anti- μ treated

Table IV. Effect of anti- μ treatment on plaque forming cell (PFC) responses of mice.*

Group	No. Mice	Direct PFC		Indirect PFC	
		PFC/Spleen	PFC/10 ⁶	PFC/Spleen	PFC/10 ⁶
Anti- μ **	6	50	0.6	14	0.1
NRS	7	813	4.6	1,281	8
PBS	7	5,469	22	170,469	651
BALB/c	8	5,417	22	221,875	937

* All mice infected with 300 IL of *N. brasiliensis* at 4 weeks of age and injected with 0.2 ml of a 10% suspension of sheep erythrocytes on days 16, 10 and 6 prior to necropsy.

** Anti- μ treatment initiated on day of birth and thereafter, every second day until termination of the experiment.

Table V. Effect of anti- μ antiserum on serum immunoglobulin levels of BALB/c mice.

	Anti- μ treated mice	Mean Serum Level*		
		Normal Rabbit Serum Control	Phosphate buffered saline control	Untreated BALB/c control
Immunoglobulin Detected				
IgM	0	32	40	43
IgG ₁	139	1664	480	469
IgG ₂	28	192	128	192
IgA	6	16	12	16
Normal Rabbit Serum	4	272	0	0
Free anti- μ	5	-	-	-

* Numerical average of highest individual reciprocal serum dilutions producing precipitin bands in Ouchterlony gel diffusion.

animals, were stunted and malformed; only one of 24 female worms recovered from anti- μ -treated mice had eggs in its uterus.

Effect of Passive Immunization of Nude Mice on Worms
Subsequently Transferred Into Rats

Because it appeared that abrogation of antibody production potential via anti- μ treatment did not impair the ability of BALB/c mice to expel *N. brasiliensis* (Figure 11), we questioned whether immune serum would have an effect on this parasite in mice. Thus, BALB/c or normal littermates of nude mice each were inoculated with 900 IL and exsanguinated on days 16, 17 or 18 PLI; the serum (hereafter designated immune serum) was collected and stored at -20 C until use. After thawing and prior to inoculation into recipient mice, the serum was centrifuged at 7500 G for 25 minutes and subsequently passed through a millipore filter of pore size 0.45 microns (Millipore Corp., Bedford, MA.). Normal mouse serum (NMS) was obtained from BALB/c donors and treated as outlined for immune serum.

On day -1 of the experiment, 12 nude mice each were inoculated intraperitoneally (i.p.) with 2 ml immune serum; likewise, 11 additional nude mice each received 2 ml NMS. On day 0, all serum-injected nudes in addition to 5 untreated nude and 8 NLM control mice each were inoculated with 300 IL of *N. brasiliensis*. On day 2 PLI, a second injection of immune or normal mouse serum was given i.p. to mice which

on day -1 had received the same type of serum. The volume of this second serum dose was adjusted so that individual mice received, in the two injections, a total of 5 ml of serum/20 gm body weight.

The results of daily fecal worm egg counts indicated that worms became established in treated and control mice; all three groups of nude mice maintained their infections and the NLM controls expelled their worms as expected. On day 14 PLI, nude mice from the immune serum and NMS groups were sacrificed and their worms transferred via laparotomy into 11-week-old Sprague Dawley female rats. Each of 3 rats received 250 adult worms from the immune-serum-treated nude mouse group; likewise, an additional 3 rats were given 250 worms derived from NMS-treated nude mice. EPG counts of these two groups of rats were made for 6 consecutive days following worm transfer. Based on the criteria of others (20), the antiserum was considered protective only if there was a significant reduction in worm burden 6 days after worms had been transferred into rats. Thus, all rats were sacrificed late on day 6 post-worm-transfer.

The group EPG and individual rat worm counts (Table VI) indicated that, indeed, worms derived from immune-serum-treated nude mice were eliminated more rapidly from their rat host than were worms derived from NMS-treated nude mice.

Table VI. Effect of passive immunization of nude mice on *N. brasiliensis*.*

Days post-adult-worm-transfer from donor mice to rats	Recipient of worms from NMS-treated-nude mice	Recipients of worms from immune-serum-treated nude mice
Daily EPG Counts of Recipient Rats		
1	10,100	10,025
2	15,800	12,950
3	27,300	14,050
4	15,300	9,500
5	22,100	1,300
6	14,300	200
Average Worm Burden		
6	84 ± 42.7**	25 ± 9.3

* Worms subjected to antiserum in nude mice were transferred on day 0 via laparotomy into 11-week-old Sprague Dawley rats.

** One of 3 rats was negative for *N. brasiliensis*; remaining 2 rats had worm burdens of 112 and 140, respectively.

DISCUSSION

Our data present direct evidence for the thymus dependency of *N. brasiliensis* expulsion from mice (Figures 1-6; Tables I and II). Others (50) have observed that neonatal thymectomy of rats delays worm expulsion but only by several days. Furthermore, the results of numerous experiments (51) indicated that the response of individual neonatally thymectomized rats was extremely variable. Kelly (49) found that *N. brasiliensis* in neonatally thymectomized rats maintained a significantly higher level of egg production than did sham-operated controls during a 26-day infection period. Significantly, however, the EPG counts of thymectomized animals dropped about 33 fold and worm burdens were reduced during the 20 days of patency. These studies indicated that worm expulsion was at least in part a thymus dependent phenomenon. In contrast, the inability of nude mice in our studies to eliminate *N. brasiliensis* for at least 81 days and the generation of worm expulsion potential in thymus cell (Figures 4 and 5; Table I) or thymus gland (Figure 6; Table II) treated nude mice not only proved the thymus dependency of the worm expulsion process but also suggested the potential usefulness of the nude mouse for immunohelminthological studies.

Based on the high degree of host specificity attributable to a majority of parasites, it is probable that the physiological

requirements for establishment and maintenance of a parasite species in a host are very specific. Therefore, the use of chemical or physical immunosuppressant treatments, such as drugs or irradiation, may alter host physiology to the extent that the normal habitation of the parasite becomes untenable. For studies on mechanisms of immunity to helminthic infections, we have shown that nude mice may offer an excellent alternative for use in experimental designs requiring immunosuppressed hosts.

It has been shown (26) that worm senescence is not responsible for changes in worms obtained from rats at the end of a primary infection (26). In the nude mouse system, we observed that worms maintained in healthy nude mice for periods in excess of 3 times the duration of NLM infections were still apparently normal based on morphological and functional parameters (Table III; Figure 2). The changes that occur in worms during an infection of mice are, therefore, dependent upon thymus competence for their elicitation and are not attributable to worm senescence.

A phenomenon common to immunodeficient animals is a wasting process which ultimately results in their death. Nude mice, not held under germ free or specific pathogen free conditions may exhibit this phenomenon as they age. Although no quantitative data are available for assessment of which mice wasted in our experiments, it was observed that periodically some individuals infected with *N. brasiliensis*

had symptoms of wasting for extended periods (2-4 weeks) prior to death. Invariably these mice had very low worm burdens compared with other healthy nude mice in the same group. This observation suggests that possibly, due to an altered physiology or a change in intestinal flora associated with the nude mouse wasting syndrome, the gut habitat becomes unsuitable for worm maintenance. In support of this suggestion it has been observed by others (53) that fewer adult worms were recovered from germ free rats compared with conventional controls.

The thymus is apparently necessary for development of immunity to all helminths studied so far (reviewed in 22). It has been demonstrated that the effector step in expulsion of *N. brasiliensis* from rats is thymus dependent (33). Our experiments confirm and extend this observation; in mice the effector of *N. brasiliensis* elimination is also thymus dependent (Figures 7, and 8). There is no information linking thymus dependency with the inductive (antibody mediated) step of worm expulsion in the rat-*N. brasiliensis* system. Our work with nude mice, however, suggests that the inductive (worm damaging) step is indeed thymus dependent (Table III, Figures 9 & 10) but concludes nothing about the requirement for antibody in this step.

Obviously, the thymus dependence of immunity to helminths does not clarify the role of cell mediated or humoral factors in mechanisms of helminth immunity. In the rat-*N. brasiliensis* system, a number of studies (reviewed in 22) have indicated that rats can be passively

immunized with antisera from immune donors. There has, however, been a considerable amount of variation in the capacity of antiserum pools to evoke worm expulsion (20). In one study using 48 antiserum pools, only 1 in 3 was protective (20). Furthermore, extremely large volumes of antiserum are required to effect a response in the recipient rat. Although worm damage (26, 27) observed in the inductive step of rat worm expulsion has been attributed to antibody, an array of at least 3 antibody classes, which were predominant in various antiserum pools, have been identified as protective (19). The variability in potency of antiserum pools, the large amounts of antiserum required, the variety of predominant antibody classes in various protective antiserum pools and the lack of correlation between hyperimmunization of donors and protectivity of their antisera all defy satisfactory explanations relative to the role of antibody as the sole effector in the worm damaging process. An equally perplexing observation indicated that via an immunofluorescence technique, humoral antibody of the classes IgG, IgM or IgA were not detectable in sections of adult *N. brasiliensis* until days 10, 11 and 12 of mouse infections (21). Worm rejection occurred during days 10-12 but IgG was not detectable in peripheral circulation until day 15 PLI. Finally, protective antibody to *N. brasiliensis* in rats can not be detected in the serum until day 17 PLI (19) which is after the worms have been expelled. The data generally have been interpreted, however, that antibody is the factor

in immune serum which evokes worm damage, a process which must precede worm expulsion (reviewed in 47).

We postulated that possibly factors in antiserum pools other than antibody were responsible for the inductive step (worm damaging step) in *N. brasiliensis* expulsion. Consequently, we employed the powerful immunosuppressant of mouse immunoglobulin production (69) heterologous anti-heavy chain antiserum (anti- μ) in an attempt to determine if worm expulsion could be precluded by abrogating antibody production. In anti- μ -treated mice, although direct and indirect plaque forming cell responses to sheep erythrocytes were virtually abolished and immunoglobulin levels were well below control levels (Tables IV and V), surprisingly, worms were expelled from these mice in a manner similar to control infections (Figure 11). One might argue that antibody production was only depressed in anti- μ -treated mice, thus allowing antibody-mediated worm damage to occur in spite of anti- μ treatment. If true, this argument would be difficult to reconcile with the variability intrinsic in passive transfer studies, especially with regard to 1) the amounts of antiserum required, 2) the lack of potency of many antiserum pools, and 3) the lack of correlation between hyperimmunization of serum donors and protectiveness of their serum.

Because the results obtained in the anti- μ experiment suggest that mice eliminate *N. brasiliensis* by a mechanism which may not require antibody, we examined the effect of passive immunization of nude mice on the worms. Massive amounts of homologous antiserum were injected into nude mice one day preceding and 2 days after a larval infection of *N. brasiliensis*. On day 14 PLI, adult worms were transferred into recipient rats. According to criteria of others (20) daily EPG counts and worm burdens in rats on day 6 post-worm-transfer (Table VI) indicated the antiserum was protective. Although limited, our data suggest that a factor was present in antiserum which caused worm damage resulting in an accelerated expulsion of damaged worms from rats. There is nothing in these data to suggest whether antibody or other serum factors constitute the worm damaging factor. However, if interpreted in conjunction with the anti- μ experiment, the data suggest that the protective factor is present in antiserum but may not be antibody.

The two-step mechanism of worm expulsion described for rats has been difficult to define in mice infected with rat strain *N. brasiliensis*. Ogilvie (54) concluded, by transplant of damaged rat worms into mice, that the second step of expulsion is not generated in mice. In subsequent studies, Love (55) indicated that cells and antiserum do collaborate in two sequential steps in mice resulting in worm expulsion. Using the mouse-adapted strain of *N. brasiliensis*,

we have repeatedly attempted, without success, to detect a differential expulsion time in either mouse or rat recipients of 8-day or 6-day mouse worms (normal and damaged worms, respectively, by criteria of Love; 55). This apparent similarity in 6 and 8-day mouse worms was also reflected in the reproductive capacity and morphological characteristics of these worms. No significant difference was observed in the number of eggs/female worm (EPF) when comparisons were made between worms derived from 6 and 8-day infections of NLM mice (Table III). Interestingly, however, the EPF of worms derived from either 6 or 8-day NLM infections was significantly lower than the EPF of worms obtained from nude mice. This observation suggests that thymus dependent host factors have a detrimental effect on reproductive capacity of worms, as early as the first day of patency (day 6). In support of this concept, morphological damage to intestinal cells of *N. brasiliensis* was commonly observed in transverse sections of worms derived from 6-day mouse infections (Figure 10). Collectively, these data suggest that initiation of the inductive step occurs several days earlier in mice than in rats and thus, may be responsible for the more rapid expulsion of *N. brasiliensis* from mice.

The identity of thymus dependent cell type(s) or cell products required in the expulsion of *N. brasiliensis* was not within the scope of the present study and will be the subject of future investigations. Immunity has been conferred to heavily irradiated (750 rads) rats by

adoptive transfer of sensitized mesenteric lymph node cells and a bone marrow-derived cellular component thought to be of myeloid origin (31, 47). With current knowledge, however, one can only speculate about which thymus dependent cell types are required for generation of worm expulsion. It is possible that helper T cells are required for induction of protective antibody production. However, if antibody is precluded as a requirement for effecting worm damage, possibly one of the products elaborated from sensitized T cells is the inducer of worm damage or the effector of expulsion of previously damaged worms.

Eosinophilia is a hallmark of most parasitic infections; however, its immunologic function remains obscure as does its role in immunopathologic processes. With the development of the technique for production of anti-eosinophil serum (76), studies may now be conducted on this cell type. Furthermore, with the mounting evidence (77, 78) of the thymus dependence of eosinophilia, the role of this enigmatic cell type which accompanies *N. brasiliensis* infections (35) warrants further investigation.

There are several recent studies on the temporal relationship of mast cell infiltration into the lamina propria of the intestine and worm expulsion (reviewed in 38), and on reaginic antibody production in response to *N. brasiliensis* infections (51, 79). An attractive hypothesis states that reaginic antibody, produced in response to infections of *N. brasiliensis*, is recruited to the surface of an

increasing number of mast cells, and react with worm allergen resulting in degranulation of mast cells and subsequent release of amines into the environment of the worm. The amines, according to the hypothesis, are either directly antagonistic to the worms or make the environment of the worms unsuitable, resulting in worm expulsion. Because greatly increased IgE production and mast cell proliferation almost invariably occur in response to *N. brasiliensis* (8), the possible role of each in worm expulsion needs clarification. It has been shown that anti-serum devoid of passive cutaneous anaphylaxis activity can evoke worm damage in recipient rats (19). Therefore, an exclusive role of IgE as a worm-damaging immunoglobulin in the inductive step of the expulsion mechanism seems precluded. However, its possible role as a mediator of mast cell degranulation, under the previously stated hypothesis, has not been excluded. Experimentation has been hampered by the non-availability of anti-IgE serum for use in eliminating IgE responses *in vivo*. In preliminary studies, Manning and Reed (unpublished) have observed that anti- μ treatment of mice suppresses their IgE production capability. If confirmed, this observation coupled with our studies on anti- μ suppression, would suggest that IgE is precluded as a requirement for expulsion of *N. brasiliensis* from mice.

In conclusion, the data gathered in this study have indicated that both the inductive and effector step of worm expulsion in mice are thymus dependent. We have also seen that interpretation of

data is made less difficult when using the nude mouse host because of its complete inability to muster a protective immune response against the parasites. Our studies with anti- μ serum may have raised more questions than they have answered; however, they do provide the basis for a plausible hypothesis which may assist in clarifying the actual role of antibody in the worm expulsion process.

Because of its defect in immune capacity, the nude mouse has provided a valuable tool for use in the pursuit of an understanding of the host immune response (or lack of it) to parasitic infections. Possibly only through use of such unique model systems will we be able to determine the basis of immunoprophylaxis against parasitic infections.

LITERATURE CITED

1. Soulsby, E. J. L., (editor), 1972. *Immunity to Animal Parasites*. Academic Press, New York.
2. Targett, G. A. T., 1973. Thymus dependency and chronic antigenic stimulation: Immunity to parasitic protozoans and helminths. In *Contemporary Topics in Immunobiology*, Edited by A. J. Davies and R. L. Carter, Vol. 2, p. 217, Plenum Press, New York-London.
3. Jackson, G. J., R. Herman, and I. Singer, (editors), 1970. *Immunity to Parasitic Animals*, Vol. 2, Appleton-Century-Crofts, New York.
4. World Health Organization Expert Committee Report. Immunology and Parasitic Diseases. W. H. O. Techn. Rep. Ser. No. 315, 1965.
5. Soulsby, E. J. L., (editor), 1968. *The Reaction of the Host to Parasitism*. Elwert, Marburg/Lahn, Germany.
6. Africa, C. M., 1931. Studies on the host relations of *Nippostrongylus muris*, with special reference to age resistance and acquired immunity. *J. Parasit.*, 18:1.
7. Schwartz, B., J. E. Alicata, and J. T. Lucker. 1931. Resistance of rats to superinfections with a nematode, *Nippostrongylus muris* (Yokogawa, 1920), and an apparently similar resistance of horses to superinfection with nematodes. *J. Wash. Acad. Sci.*, 21:259.
8. Ogilvie, B. M., and V. E. Jones. 1971. *Nippostrongylus brasiliensis*: A review of immunity and the host-parasite relationship in the rat. *Exp. Parasit.*, 29:138.
9. Graham, G. L., 1934. Resistance studies with the nematode, *Nippostrongylus muris*, in laboratory rats. *Amer. J. Hyg.*, 20:352.
10. Porter, D. A., 1935. A comparative study of *Nippostrongylus muris* in rats and mice. *Amer. J. Hyg.*, 22:444.
11. Sarles, M. P., and W. H. Taliaferro. 1936. The local points of defense and the passive transfer of immunity to *Nippostrongylus muris* in rats. *J. Infect. Dis.*, 59:207.

