Analysis of the mechanisms of immune expulsion from mice of Hymenolepis diminuta and Hymenolepis nana

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A thesis submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Microbiology

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Abstract:
Normal littermates (NLM) of congenitally thymus-deficient (hude) mice expelled Hymenolepis diminuta by day 21 post-cysticercoid-inocu-Iation.

In second infections of NLM, worms were smaller, destrobilated earlier and were expelled sooner than in first infections. Nude mice failed to expel H. diminuta normally; worms were maintained by nudes for over 60 days. Nude mouse recipients of either dispersed thymus cells or thymus gland implants expelled H. diminuta in a pattern similar to NLM. Thymus competence of nude mice received thymus cells or glands was confirmed by quantitating plaque-forming cell responses to the thymus-dependent antigen sheep erythrocytes. Expulsion of H. diminuta from mice was concluded to be a thymus-dependent immune phenomenon.

NLM mice given a primary H. nana lumenal phase (cysticercoid) infection suffered, within 14-21 days post-cysticercoid-inoculation, a low level of natural reinfection involving the tissue phase; such mice, however, expelled their worms by day 35 post-cysticercoid-inoculation. NLM mice given a primary H. nana tissue phase (egg) infection did not suffer natural reinfection and expelled their worms by day 20 post-egg-inoculation. Following expulsion of an initial infection involving the tissue phase, NLM were immune to experimental reinfection with challenge eggs or cysticercoids. Nude mice infected with either eggs or cysticer-coi ds failed to expel their worms and showed no evidence of reinfection immunity; increasingly heavy worm burdens developed through progressive reinfection cycles in such mice. Nudes injected with thymus cells or implanted with thymus glands expelled both lumenal and tissue phase infections. Following contact with the tissue phase, reinfection immu-nity was generated in nude mice with thymus competence. It was con-cluded that the expulsion of H. nana and the reinfection immunity seen following contact with the tissue phase are thymus-dependent immune phenomenon.

The ability to produce humoral antibody was abrogated in Balb/c mice by treatment with rabbit anti-mouse IgM; mice so suppressed expelled H. diminuta as rapidly as did control, nonsuppressed mice. Serum from mice immune to H. diminuta did not passively transfer worm expulsion potential to nude mice. Furthermore, such immune serum, when incubated in Vitro with cysticercoids and complement, did not reduce the infectivity of H. diminuta cysticercoids. Collectively, these data suggest that specific humoral antibody is not the critical thymus-dependent component of the immune system responsible for the expulsion of H. diminuta from mice.

Balb/c mice suppressed with rabbit anti-mouse IgM and infected with H. nana eggs maintained significantly more adult worms than did control, nonsuppressed mice, suggesting that antibody may be involved in the expulsion of H. nana from mice. Because suppressed mice were immune to reinfection, immune mechanisms other than antibody must also be involved in controlling H. nana infections in mice.

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ABSTRACT

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Balb/c mice suppressed with rabbit anti-mouse IgM and infected with *H. nana* eggs maintained significantly more adult worms than did control, nonsuppressed mice, suggesting that antibody may be involved in the expulsion of *H. nana* from mice. Because suppressed mice were immune to reinfection, immune mechanisms other than antibody must also be involved in controlling *H. nana* infections in mice.

INTRODUCTION

In 1947 Stoll estimated that there existed in the world about 2000 million human nematode infections, 72 million human cestode infections, and about 148 million human trematode infections (1). Though these estimates were made in 1947, there is now good evidence that in some cases these numbers have in fact increased. Colley, for example, has indicated that currently there are an estimated 200 million cases of schistosomiasis throughout the world (2). Human parasites are found in every inhabited portion of the world, though they tend to predominate in tropical climates where environmental conditions and poor public health standards favor the completion of life cycles and the spread of infective units.

Parasitic infections produced a wide range of clinical signs and symptoms, depending upon the species of parasite, the condition of the host, the organs affected, and the intensity of infection. Clinically, parasitic infections may be asymptomatic, mildly discomforting, or severely debilitating.

Despite the obvious medical importance of many, parasitic diseases, research on host-parasite systems frequently has lagged behind that done in areas involving other disease-causing agents such as bacteria and viruses. One aspect of host-parasite relationships, the mechanisms of host immunity to worm infections, in particular, has not

been studied in depth. Though a number of observations has been made on immunological phenomena involved in host-parasite systems (3, 4, 5, 6, 7), few systems have been well characterized in terms of the mechanisms of host immunity which serve to regulate helminthic infections. A number of factors including the complexity of helminthic life cycles, the multiplicity of structural and metabolic antigens, and the apparent lack of symptoms associated with many of these infections have contributed to this lack of knowledge.

Perhaps the host-parasite systems best characterized in terms of host immunity are those of *Nippostrongylus brasiliensis* in mice and rats. Evidence accumulated with these systems would indicate the involvement of both humoral antibody in initiating worm damage (8) and cell-mediated immunity in the actual expulsion of the worms (9). In addition, the eosinophilia seen in rats infected with *N. brasiliensis* (10) and the increase in the numbers of mast cells in the intestinal mucosa of infected rats (11) suggests a possible role for these cell types in the control of these infections.

In contrast to the *N. brasiliensis*-mouse and -rat systems, few other host-helminth systems have been well characterized in terms of host immunity. In particular, research on immunity to tapeworm infections (Cestoidea) has lagged behind that carried out with other groups of helminths, possibly due to the greater economic and medical importance of the latter (5). A number of review articles (5, 12, 13)

summarizing the observations made on immunological phenomena regulating tapeworm infections has been written and no attempt will be made to restate these observations in total. Two host-parasite systems involving infections of mice with the tapeworms *Hymenolepis diminuta* and *H. nana* are of particular interest because of their ease of study in the laboratory mouse and their clinical importance. Brown (14) has estimated that *H diminuta* infections in man, while rare, have been diagnosed in about 200 cases; *H. nana* infections in man, in contrast, account for over 20 million current clinical cases. In spite of the high number of human parasitic infections, including those due to these 2 tapeworms, there still exists a dearth of knowledge concerning host-parasite relationships and in particular, a lack of knowledge concerning those aspects of the host's immune system which serve to regulate the growth of parasites.

H. diminuta, a tape worm which has its normal host the rat, is a noninvasive, lumen-dwelling tapeworm of the small intestine of rats (15). In rats, following the ingestion of cysticercoids, adult worms develop and become patent about 21 days post-inoculation and continue to release eggs for many weeks. Turton (16) has provided evidence that although these infections may be of long duration, they do not go unnoticed by the immune system of the rat because antibodies of both the IgG_1 and IgE class are formed in response to the worms. These antibodies, however, are apparently incapable of causing worm expulsion.

In addition to their normal rat host, *H. diminuta* also becomes established in mice (17); in mice, however, the infections are not of long duration. Hopkins and coworkers (18) have reported that nearly all *H. diminuta* cysticercoids given to mice develop into adult *H. diminuta* which are maintained for about 10 days. Between days 10 and 17 post-inoculation in mice, in contrast to the kinetics of infection seen in the normal rat host, worms rapidly destrobilate and are expelled from the mouse host. In addition, Hopkins *et al.* provided evidence that the expulsion of *H. diminuta* from mice is the result of an immunological reaction by the host because secondary infections yielded fewer worms which were reduced in size and were expelled more rapidly than were worms in primary infections (18). Similarly, Befus (19) has reported that mice expel *H. diminuta* via an immunological response; in his studies, however, expulsion of *H. diminuta* did not occur more rapidly in mice previously infected compared with mice receiving their first infection, although stunting of worms and worm destrobilation at an earlier time did occur in mice with a second infection.

Few attempts have been made to characterize the nature of the immune response involved in expelling *H. diminuta* from mice. Hopkins *et al.* (20) have reported that mice immunosuppressed with cortisone acetate, sodium methotrexate or antilymphocyte serum were unable to expel *H. diminuta* as rapidly as were control, nonsuppressed mice. The effects of such drugs, however, are numerous and immunosuppression is

often incomplete. In addition, they frequently affect both humoral and cellular components of the immune system and therefore fail to distinguish between the role of antibody and cell-mediated immunity in the expulsion of worms. To date, no further attempts have been made to elucidate the nature of the immune mechanism or mechanisms responsible for expelling *H. diminuta* from mice. Because of the lack of knowledge regarding the nature of expulsion and the immune mechanism(s) responsible for expulsion, studies reported here were undertaken to clarify our understanding of immunity in mice to *H. diminuta*.

H. nana, a tapeworm which has as its normal host the mouse, has been studied by a number of investigators and is somewhat unique because of the dual life cycle pattern exhibited by this parasite. In the direct life cycle (21), eggs ingested by the definitive mouse host hatch in the small intestine, release hexacanth larvae (oncospheres) which invade the intestinal villi and develop, via the tissue phase, to the cysticercoid stage in about 5-6 days (22). Cysticercoids within the intestinal villi then emerge, loose their protective membranes and develop into adult tapeworms which become patent 13-24 days post-egg-ingestion. Alternatively, *H. nana* may develop via an indirect cycle following the ingestion of cysticercoids (23) which have developed in an intermediate insect host such as the flour beetle, *Tribolium confusum* (24).

A number of investigators have studied the immunity to reinfection

present in mice previously exposed to *H. nana* (25, 26, 27, 28, 29, 30). Heyneman (31) reported that immunity following primary infections involving the tissue phase (i.e. following the ingestion of eggs) was more complete than that following primary infection involving the lumenal phase (i.e. following the ingestion of cysticercoids). These observations, however, were somewhat clouded by the observation that mice receiving primary infections with the lumenal phase frequently suffered natural reinfection involving the tissue phase, either by internal autoinfection (32) or by copraphagia, and thus were rendered immune to experimental reinfection to an extent comparable with animals receiving initial infections involving only the tissue phase. The immunity observed by Heyneman was more effective at inhibiting subsequent infections involving the tissue phase than the lumenal phase.

Attempts to characterize the mechanism(s) responsible for immunity to reinfection in mice previously exposed to *H. nana* have led to varying conclusions. Weinmann (33) and Larsh (34), for example, have reported that splenectomy of mice has no apparent effect on acquired immunity to *H. nana*. Because the spleen is a major site of antibody production in mice (35), these investigators suggested that antibody may be of limited importance in immunity to *H. nana* in mice. Friedberg *et al.* (36), however, reported that immunity to reinfection could be transferred to irradiated recipients by the injection of spleen cells from immune mice but not by the injection of spleen cells from nonimmune mice. In

addition, Coleman and coworkers (37) have provided indirect evidence for the involvement of antibody in acquired immunity to *H. nana*. In their studies X-irradiated mice produced less antibody and maintained greater worm burdens than did nonirradiated mice, suggesting that antibody may play a role in immunity to *H. nana* in mice.

More direct evidence for the involvement of humoral antibody in acquired resistance to reinfection with *H. nana* in mice has been reported by several investigators. Using passive transfer techniques, Hearin (27) observed that serum from immune mice could confer a significant level of immunity to *H. nana* in mice not previously exposed. In these experiments, the transfer of serum from nonimmune mice failed to confer immunity to infection in recipient mice.

The lack of cell-mediated immunity in acquired resistance to *H. nana* is suggested by observations on the immunity established in mice which had been neonatally thymectomized. Wienmann (33) found that neonatal thymectomy did not abolish or significantly reduce the capacity of mice to develop resistance to reinfection with *H. nana* following primary infections. Early work with neonatally thymectomized mice (38) clearly established the importance of the thymus glands for the development of cell-mediated immune responses in later life.

The involvement of cell types other than the thymus-dependent lymphocytes responsible for cell-mediated immune responses can not be ruled out however. Baily (39) reported that *H. nana* cysticercoids

developing within the intestinal villi of infected mice stimulated the accumulation of large numbers of eosinophils in the lamina propria of the gut. Also,mice given a second infection accumulated greater numbers of eosinophils at an earlier time than did mice given their first infection.

Although a number of investigators have provided evidence for the immunologically mediated expulsion of *H. diminuta* and *H. nana* from mice; there still exists a void of knowledge concerning the mechanisms of expulsion and the subsequent immunity established following initial infection with these parasites. It has been suggested that congenitally thymus-deficient (nude) mice may prove useful as a model system for studying the cellular and humoral components involved in immunity to parasites because they have a number of immunological deficiencies including: 1) decreased antibody production in response to thymus-dependent antigens (40, 41, 42); 2) the inability to reject allografts (40,42) and xenografts (43); 3) the lack of delayed type hypersensitivity responses (44); 4) the failure to produce eosinophilia (45), and 5) the inability to produce reaginic antibody (46). The ability to correct these immunological defects with grafted thymus glands or injected thymus cells extends the usefulness of the nude mouse-parasite system. A number of nude mouse-parasite systems have been studied to date (47, 48, 49, 50) and work with these systems has confirmed the usefulness of nude mice in immunoparasitology.

Because of the incompleteness and the frequent discrepancies present in previous work on immunity to *H. diminuta* and *H. nana* in mice, studies reported here were initiated in an attempt to clarify the nature of the immunity generated in mice as a consequence of infection with either of these 2 parasites. In an attempt to do so, nude mice and their phenotypically normal, thymus-bearing littermates (NLM) were used first to determine the thymus-dependency of tapeworm expulsion from mice. In subsequent experiments, both humoral and cellular aspects of the mouse's immune system were analyzed for their role in worm expulsion either by selective immunological reconstitution of nude mice or by selective elimination of factors required for worm expulsion in thymus-bearing mice.

MATERIALS AND METHODS

Animals

The principle experimental animals used throughout this study were congenitally thymus-deficient (nude; nu/nu) mice and their phenotypically normal, thymus-bearing littermates (NLM; nu/+ or +/+). The majority of such animals were derived from heterozygus breeding stock initially crossed (crossed-intercrossed, generation 2-4) on a Balb/c genetic background and then maintained as a clean, barrier isolated colony bred unit. Experiments involving Balb/c thymus cell injection into nude mouse recipients were done using generation 9 nudes. Balb/c mice were also used as thymus gland donors and as experimental animals in experiments involving suppression of antibody synthesizing ability with rabbit anti-mouse IgM antisera. Animal colonies from which the animals used in these studies were obtained had no history of natural tapeworm infections, as determined by periodic random fecal examination.

Nude and NLM mice were also used as the definitive maintenance hosts for *H. nana* while Lewis strain rats were used as the definitive maintenance hosts for *H. diminuta*.

All animals were maintained on autoclaved 5010C Purina Mouse Chow and acidified-chlorinated water as previously described (51).

Parasites

H. diminuta, obtained initially from Dr. Austin MacInnis at UCLA,

was maintained in rat definitive hosts and flour beetle (*Tribolium confusum*) intermediate hosts. Rats anesthetized with ether were inoculated per os with 6 *H. diminuta* cysticercoids obtained by dissecting infected flour beetles in tap water. In addition, eggs obtained from mature terminal proglottids macerated in a Thomas tissue homogenizer were used to infect flour beetles. Beetle cultures were maintained and infected as previously described (52). Briefly, uninfected beetles were sifted from their stone ground flour culturing medium and starved at least 5 days in advance of egg feeding. Egg suspensions obtained from macerated proglottids were pipetted onto filter paper discs placed on absorbant pads. Eggs trapped on the filter paper as the water was drawn into the pad were placed in petri dishes with starved beetles. Beetles were allowed to feed on the egg preparation in humid chambers for 24 hours before culture medium was added.

H. nana, obtained initially from an isolated, naturally infected animal colony maintained at the Veterinary Research Laboratory at Montana State University, was maintained in nude and NLM definitive hosts and flour beetle intermediate hosts. Source mice were inoculated with 5 cysticercoids obtained again by dissecting infected flour beetles. Procedures used to infect beetles with *H. nana* were as previously described for *H. diminuta*.

Thymus Gland Grafting

Thymus glands obtained from neonatal Balb/c mice were held in

phosphate buffered saline (PBS) on ice until recipient nude mice were anesthetized. Nude mice 4-6 weeks old were anesthetized with sodium pentabarbitol (53) and thymus grafted using the technique established by Dukor *et al.* (54). Briefly, a 1 cm incision was made lateral to the dorsal midline directly over the right kidney. Using a pair of forceps, the kidney was manipulated onto the surface of the recipient and a small incision was made through the renal capsule. One thymus gland was placed under the capsule, the kidney was returned to its normal position and the incision was closed by suturing. This process was then repeated for the opposite kidney so each thymus-grafted nude (TG-Nu) received two thymus glands. At least 42 days were allowed to pass after grafting before TG-Nu were used in experiments.

Thymus Cell Transfers

Thymus glands obtained from Balb/c mice 2-3 weeks old were converted to single cell suspensions in cold saline plus 1% fetal calf serum by teasing over 60 mesh stainless steel screens. These suspensions were quantitated by trypan blue exclusion (55) to determine the percentage of viable cells and adjusted to contain 3×10^8 viable thymocytes per ml. Each recipient nude was injected intraperitoneally (I. P.) with 0.5 ml so each thymus cell-injected nude (TC-Nu) received 1.5×10^8 viable thymocytes. At least 21 days were allowed to pass after injection before TC-Nu were used in experiments.

Necropsy Procedures

The intensity of infection was determined by counting the number of lumen-dwelling *H. diminuta* or *H. nana* present in the small intestine and by counting the number of cysticercoids present in the villi of the small intestine of *H. nana* infected mice. To count the number of lumen-dwelling worms, the small intestine was severed from the stomach at the pyloric sphincter and from the cecum at the ileocecal valve, freed of adhering mesenteric tissue, and placed in tap water. Gut contents were then flushed with tap water under pressure and, subsequently, the small intestine was split longitudinally and washed, along with the flushed contents, over a 200 mesh screen. Washed material was observed under 20X power of a dissecting scope for the number of worms. In cases where worm burdens were heavy, counts were made on representative aliquots of washed gut material.

The number of *H. nana* cysticercoids within the villi of infected animals was quantitated using the technique described by Hunninen (56). Briefly, the isolated small intestines were freed of adhering mesenteric tissue, split longitudinally, scraped free of mucus, and allowed to autolyze at 4^{o} C in 0.5% saline overnight. The partially autolyzed gut was then pressed between glass plates and the number of cysticercoids was determined by examination under a dissecting scope (30X). Cysticercoids were most easily observed by finding the circular row of

hooklets on the rostellum or by noting the swollen base of an infected villus.

Fecal Examinations

Worm egg production in mice and rats infected with *H. nana* and *H. diminuta* respectively was monitored qualitatively by examining fecal material comminuted in saturated NaCl (sp. gr. = 1.20) as previously described (57).

Immunosuppressive Treatment

Using techniques modified from those established by Manning and Jutila (58), rabbit anti-mouse IgM antiserum (anti-IgM) was prepared by immunizing rabbits with mouse IgM obtained from mice bearing the IgM producing plasmocytoma MOPC 104E. IgM in serum harvested from such mice was concentrated and purified by a variety of techniques including NH_4SO_4 precipitation, distilled water precipitation, and column chromatography using Sephadex G-200 (Pharmacia Fine Chemicals), Ultrogel AcA 22 (Industric Biologique Fracaise) and Watman DE 52 (W. and R. Balston Ltd.). The resulting antigenic preparation was 95-98% IgM with the remaining material consisting largely of IgG and IgA. This antigen was prepared and generously provided by Kennith Lee at Montana State University. Rabbits were immunized initially with 2-10 mg doses (subcutaneously) in complete Freunds adjuvant 10 days apart. Booster injections every 20 days consisted of 5 mg antigen in incomplete Freunds.

The resulting anti-IgM was absorbed 2 times with mouse red blood cells (2%) and titered against the IgM antigen used for immunization. Titers varied from 1/32 to 1/128, depending on the time interval between immunization and harvesting of the antiserum.

Newborn Balb/c mice were injected I. P. with either the anti-IgM, normal rabbit serum (NRS), phosphate buffered saline (PBS) or were left untreated. Animals were treated on alternate days, from day 1 through day 19 with 0.1 ml and from day 21 through day 31 with 0.15 ml. At 31 days of age the animals were infected with either *H. diminuta* cysticercoids or *H. nana* eggs. From day 31 until the day of necropsy, the animals were treated with 0.25 ml of the appropriate material on alternate days.

Antibody Assays

Assays for specific antibodies against sheep erythrocytes (SE) consisted of the localized hemolysis in gel assay (59) to detect anti-SE-specific plaque-forming cells (PFC), hemagglutination tests (60) to detect anti-SE-specific hemagglutinating antibodies and hemolytic tests (61) to detect anti-SE-specific hemolytic antibodies.

Sera from anti-IgM treated mice and their controls were tested for the presence of class-specific IgM, IgG_1, IgG_2 and IgA using monospecific antisera (Meloy Laboratories) in the serial dilution Ouchterlony gel diffusion technique described by Arnason *et al.* (62).

Cellular Immunity Assay

In some experiments the ability to mount cell-mediated immune responses in thymus gland-implanted nudes was assessed using the skin grafting technique established by Billingham and Silvers (63). TG-Nu, nude, and NLM mice were grafted with skin from CBA mice. Rejection was considered to be the number of days between the time of grafting and the time of 100% graft destruction, as evidenced by total sloughing of the graft.

Histology

At necropsy all gland grafted nudes were examined for the presence of thymus tissue under their renal capsules. Representative glands were sectioned, stained with hematoxylin-eosin, and observed for normal thymic architecture.

RESULTS

H. diminuta Infections in Rats

The initial experiments with *H. diminuta* were designed to determine the kinetics of infection in the normal rat host. Three month old Lewis strain rats were inoculated per os with 5 or 6 *H. diminuta* cysticercoids. Fecal examinations revealed that such infections usually became patent about 21 days post-inoculation. Animals were necropsied at monthly intervals. Throughout the first 5 months of observation the number of worms recovered from rats was 80-100% of the number of cysticercoids given (Figure 1). Subsequently, the percentage of cysticercoids recovered as adult worms decreased to 60% at 6 months. Worms recovered were usually 20-40 cm long and there was no apparent difference in worm lengths on the different necropsy days.

H. diminuta Infections in NLM Mice

Because the kinetics of infection with a given parasite frequently differs in abnormal hosts as compared to that seen in the normal host, NLM mice were inoculated with *H. diminuta* cysticercoids. NLM mice 6-10 weeks old were infected per os with 3 cysticercoids. On alternate days beginning on day 6, representative animals were killed and examined for the presence of adult worms. Results shown in Figure 2 indicate that all cysticercoids given could be recovered as adult worms on day 6. After day 6, however, the percentage of cysticercoids recovered as adult

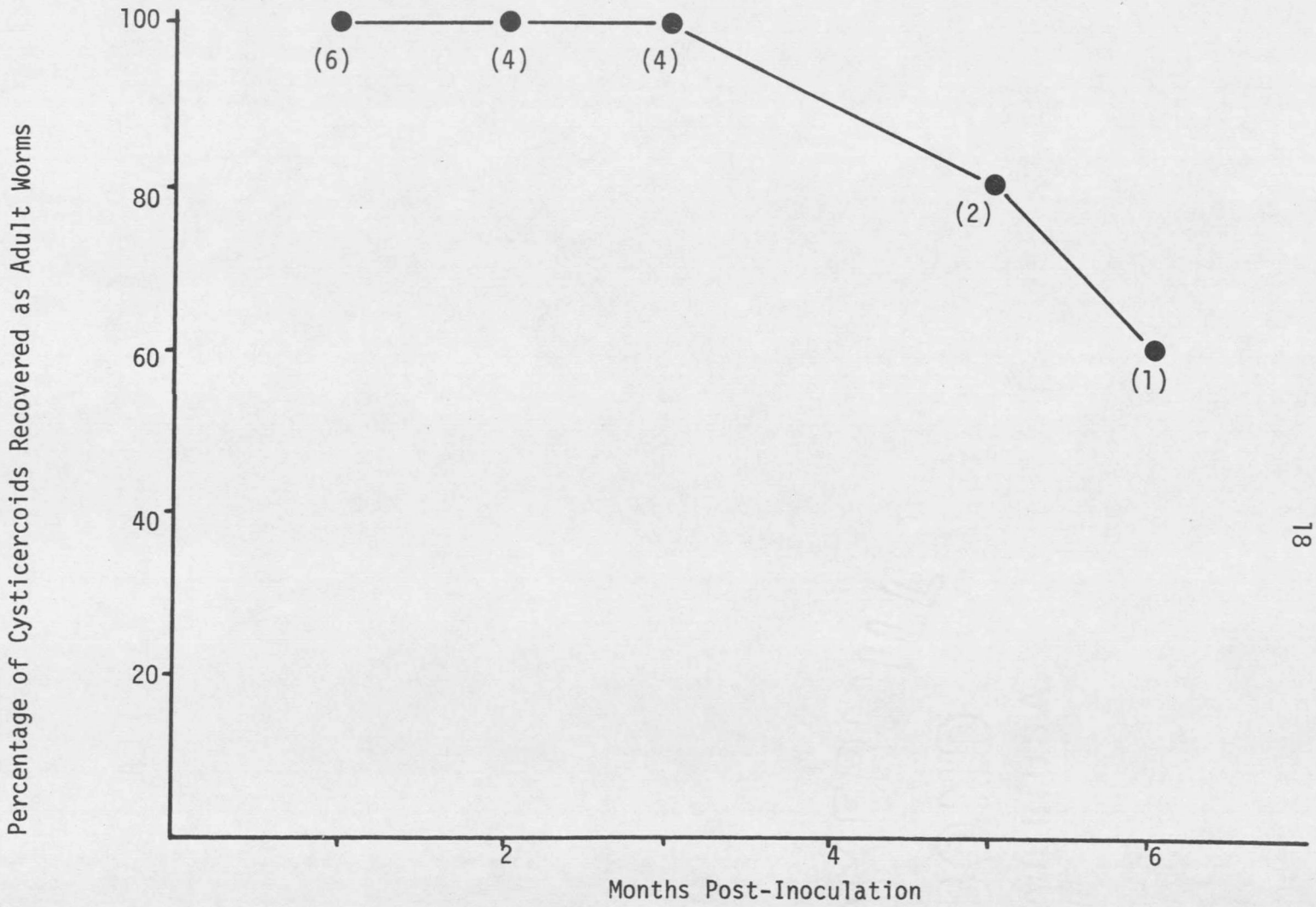

Figure 1. Long Term Survival of *H. diminuta* in Rats.
Rats given 5 or 6 cysticercoids per os were killed at monthly intervals post-inoculation and examined for the number of adult worms present in the small intestine. Numbers in parenthesis indicate the number of animals examined.