Dark areas and the immune response to the cestode, Hymenolepis diminuta in mice and rats
by Michael Allen Larson

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Microbiology
Montana State University
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Abstract:
The gut, lumen-dwelling tapeworm, Hymenolenis diminuta, will infect mice, but after about 10 days of growth the worms are expelled. Rats do not expel their worms. During infection, areas which are opaque to transmitted light form in the worms. It has been suggested that these areas, called dark' areas (DA), are the result of an immune response to the worm.

In normal mice, DA peak in number and size 1 or 2 days before worm expulsion begins. When the intensity of infection is increased from 1 to 3 or 6 worms/mouse, worm expulsion occurs sooner but the size and number of DA are not markedly changed. Nude mice do not expel H. diminuta. In one experiment worms recovered from nude mice have few DA but in 2 subsequent experiments worms recovered did not have DA levels that differed significantly from normal littermates (NLM) or thymus-grafted (TG)-nudes. TG-nudes also expel their worms similarly to NLM controls. Mice panspecifically suppressed for antibody production by treatment from birth with heterologous anti-μ. expel worms normally, and DA levels are normal. No antibody to H. diminuta is found in serum from infected normal mice by. indirect hemagglutination. The few worms recovered from a secondary infection have few DA. DA levels are similar between worms recovered from rats and mice on the same day. Prior injection of mice with lyophilized worm homogenate (worm Ag) did not affect the number and size of DA or the expulsion kinetics even though high serum antibody titers to worm Ag are detected. When this serum is passively transferred to naive mice, it causes earlier expulsion of worms and these worms have fewer and smaller DA. Serum from rats injected with worm Ag and then infected has no detectable antibody. Worms incubated in vitro in serum or glucose-salts lose most of their DA within I5 minutes and show no damage after 4 hours. If complement is added, worms die within 30 minutes and no DA are formed. A transient delayed-type hypersensitivity response in infected mice is also seen. Electron microscopy show DA to be an edema.

DA formation is not immunologically-mediated. The mechanism that causes expulsion, which is thymus dependent, actually decreases the number and size of DA. DA are an intrinsic metabolic or maturational activity of the worm.

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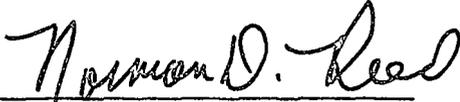of the requirements for the degree

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INTRODUCTION

"Diseases resulting from animal parasites are among the principal causes of morbidity, mortality, economic loss, and human suffering throughout the world" (16). Parasites (protozoa, helminths, and arthropods) affect man directly by infection and indirectly by reducing the food value of his domestic animals. According to Brown (12) it is estimated that 7 million people in Central and South America are infected with Chagas' disease. Malaria is present in areas of the world that include totally over a billion people. Whipworms infect about 500 million people worldwide and Ascaris lumbricoides infects 900 million, many of them in the United States. In parts of Egypt and Africa 75-95% of the residents may be infected with Schistosoma haematobium. Attention has recently been focused by the World Health Organization (WHO) on the status of parasitic infections. WHO has identified the six most prevalent infectious human diseases in tropical countries. Five of these diseases are parasitic (malaria, schistosomiasis, trypanosomiasis, filariasis, and leishmaniasis) and the sixth, leprosy, is bacterial (19).

The infection of a human host by a parasite usually

produces no symptoms or only very mild clinical symptoms
that may go unnoticed by the patient until the fulminating
stage of the infection occurs (granuloma formation in
schistosomiasis, acute lethargy in trypanosomiasis, or
muscle degeneration in trichinosis, for example). Treat-
ment at this advanced stage is ineffective. The parasites
may be in sites not accessible by significant amounts of
drugs or upon death and degeneration of the parasites
released toxins and by-products may cause an increase in
symptomology. An effective prophylactic program that
prevents or reduces initial parasitic infections could
substantially reduce morbidity and mortality.

Attempts to control parasitic infections are aggra-
vated by low standards of health in much of the world.
In many tropical underdeveloped countries, people have
neither the money to buy shoes nor the desire to wear
shoes and are, therefore, constantly exposing themselves
to the infective larvae of hookworms and other nematodes.
The dense population of insect vectors is virtually im-
possible to control, even in urban areas, leading to a
continuous exposure of humans to malaria and sleeping
sickness. A lack of adequate plumbing and sewage treat-
ment yields water supplies teeming with the cercariae of

schistosomes and amebas. Cultural habits or a lack of fuel to adequately cook foods leads to trichinosis and cestode infections. The primary problems in controlling parasites, therefore, seem to be economic and social. Rather than trying to change lifestyles and customs in order to control parasitic infections, it seems more feasible to induce acquired immunity via vaccination.

In order to utilize the potentials of the human immune system in combatting parasitic infections, several major areas need to be investigated further:

1) The life cycle of the parasite must be understood in relation to the host immune response. For example, it is the eggs of schistosomes that cause much of the pathology in schistosomiasis; the adult form evokes little response.

2) Which component of the immune system, humoral or cell-mediated immunity or both, is responsible for the destruction and/or expulsion of the parasite? The expulsion of the nematode Nippostrongylus brasiliensis from rodents is proposed to be both antibody-mediated and cell-mediated, the antibody producing initial damage (Step 1) to the worm and cell-mediated functions being

mainly responsible for final expulsion of the worm
(Step 2) (58). IgE and IgG$_1$ specific for N.
brasiliensis are produced (59); IgA may also play
a role in local immunity to the worm in the rat
(64). Marked eosinophilia is seen in rats shortly
after worm expulsion (36). Both antibody-med-
iated damage and expulsion have been found to be
thymus-dependent. Nude mice do not expel their
worm burdens up to 40 days post-infection whereas
in normal mice worms are usually gone by day 14.
However, if nudes are given a thymus gland graft
or thymus cells, expulsion occurs similarly to that
seen in normal mice (31). However, mice treated with
heterologous anti-μ (which severely reduced their
ability to produce antibodies) expelled their worms
at a rate similar to normal mice (32) suggesting
that initial antibody-induced worm damage is not
essential for expulsion.

3) Knowing how the host responds to a natural
infection, how then is it possible to prime the
immune system to resist penetration or development
of the infective forms of a given parasite?
Immunization with larval homogenates of the cestode

<u>Mesocestoides</u> <u>corti</u> substantially reduces the
number of tetrathrydia developing upon challenge
infection (40), but it does not prevent infection
entirely. Further studies are necessary to determine
which antigens provide protection against subsequent
specific challenge.

Progress has been slow in applying immunological
techniques to parasitology. Many recent studies have
been directed toward developing methods for stimulating
immunity artificially before an understanding of the
underlying immunological mechanisms involved in the host-
parasite relationship is gained. Clearly, basic research
on the immune response of humans and animals to parasitic
infection needs to be undertaken as a prelude to any
vaccination programs.

One model host-parasite system that may elucidate
the host response to helminthic infections is the mouse
infected with the cestode <u>Hymenolepis</u> <u>diminuta</u>. This
tapeworm is a lumen-dwelling, noninvasive cestode of the
small intestine which produces no known inflammatory
response or lesions. Unlike most tapeworms, the rostellum
of <u>H.</u> <u>diminuta</u> is not armed with a circlet of hooks.
The rat is the natural host for the worm and will retain

the adult form for many months or years. Worms in rat
hosts will often attain lengths greater than 50 cm. The
life cycle for the worm begins when mature proglottids
containing eggs are shed in the feces and are consumed
by an intermediate host, usually a beetle. Unlike H.
nana eggs, the eggs of H. diminuta are not directly
infective to mammals. The tapeworm eggs hatch upon en-
tering the intermediate host and release hexacanth embryos.
The embryos penetrate into the insect haemocel and develop
into cysticercoids in about 10 days. A cysticercoid is
a mature, inverted scolex with a short, immature neck
region attached. Upon ingestion of the infected insect
by the rodent, the scolex is released and attaches to
the duodenum. Strobilation progresses and mature eggs
are found in terminal proglottids by day 14 post-cysti-
cercoid infection thus completing the cycle. This tape-
worm can infect humans, but only about 200 cases of
infection have been reported (73).

In mice, H. diminuta infections are of short dura-
tion, seldom lasting more than 14 days, with destrobila-
tion occurring on about day 10 and final expulsion by
day 12. This destrobilation and expulsion has been found
to be immunologically mediated. Worms from a secondary

infection of CFLP or Porton mice were stunted and the percent recovery of worms was lower by 20% than in primary infections (23). Cortisone- and antilymphocyte serum (ALS)-treated mice did not expel their worms on schedule but retained them up to 18 days. Worm growth was also enhanced in cortisone-treated hosts (24). Similar results were found in cortisone-treated mice infected with the bile duct cestode H. microstoma (37, 54). Nude (thymus-deficient) mice did not expel their worm burdens up to day 33 (10) supporting the idea that expulsion is a thymus-dependent phenomenon. Nudes reconstituted with viable thymus cells or a thymus gland expelled their worms on schedule (28). However, Andreassen (1,2) found that nude mice began to expel their worms after day 13 with all worms gone by day 20, expulsion being dependent on the level of infection. No measurable IgE was produced in infections in normal mice and no specific circulating antibodies have been detected in serum from infected mice, though IgA, IgG_1, IgG_2, IgM, and C3 have been found on the integument of the worm (4). IgA was detected earliest and in greatest abundance on the integument.

Further support for an immunological basis for worm expulsion is:

1) In young mice (2-4 weeks old) given a 1
cysticercoid (1c) infection, expulsion did not
begin until day 16-20 (7).

2) A 6c infection leads to a quicker and ear-
lier expulsion than is found in a 1c infection (5).
Worms are also smaller in 6c infections.

3) Worms surgically transplanted into the
duodenum of immune mice were rejected in 4 days.
If immune mice were given 550 rads before lapar-
otomy, expulsion was delayed until day 6 but still
occurred appreciably sooner than expulsion in
naive untreated mice (25).

If animals are infected with a large number of
cestodes, some nonspecific loss of worms is evident.
This is thought to be due to inter-worm competition for
carbohydrates (34) or oxygen (67) and is known as the
crowding effect.

An investigation of immunologically-mediated worm
damage could be very revealing of the immune mechanisms
involved in expulsion (57). Befus and Threadgold (8)
found opaque or dark areas (DA) on the integument of
H. diminuta taken from mice. They noted that DA increased
in number until expulsion began and that worms from a

6c infection had more DA than those from a 1c infection.
Morphological differences from normal tissue in DA as
shown by electron micrographs included more lipid drop-
lets in the parenchymal cells, a reduced amount of rough
endoplasmic reticulum, and a general increase in the
electron density of the tegument. These changes are
similar to those caused by dehydration (6). Worms damaged
mechanically in vitro also formed DA. Befus postulated
that DA on worms from mice may represent immunological
damage (8). These lesions have also been found in sur-
gically-mutilated worms from mice (20) and on worms from
rats (15).

The questions this thesis will attempt to answer are:

1) Do DA have an immunological etiology?

2) If the immune status of the mouse host is
altered, are the number and size of DA correspond-
ingly changed?

3) Are morphological changes other than those
previously observed evident in DA?

4) Can antibody or delayed-type hypersensitivity
responses to worm infection be detected? Are
these responses related to DA?

MATERIALS AND METHODS

Maintenance of Parasites

Hymenolepis diminuta cysticeroids were dissected in saline from infected beetles (see below). Five cysticercoids were drawn into small guage tubing (Intramedic PE 60, Clay-Adams, Parsippany, NJ) connected to a 22 guage needle on a 1 ml syringe. Adult rats were lightly anaesthetized with ether. The tubing was inserted into the esophagus of the rat and the cysticercoids expelled with the syringe.

The beetle, Tribolium confusum, was used as the intermediate host. The beetles were maintained in the dark at room temperature in jars containing stone-ground flour and oats. Beetles were separated from the flour into a glass container with no flour and starved for 5-7 days. Donor rats were killed (at least 14 days after inoculation with cysticercoids) and the small intestines were removed and flushed out with physiological saline. Terminal proglottids containing infective eggs were removed from the tapeworms and mashed onto a small piece of moist filter paper and fed to starved beetles in a humid chamber overnight. The beetles were removed the next day and put in a flour-oats jar.

Cysticercoids used for infecting mice and rats were
dissected from beetles 3-6 weeks after feeding worm eggs
to the beetles. Figure 1 shows this to be the time of
optimal infectivity of cysticercoids. The drop in
infectivity for cysticercoids 35 days old (Figure 1) is
probably nonspecific and not related to the age of the
cysticercoids.

Mice

Balb/c mice 6 weeks old and of both sexes (Charles
River Breeding Labs, Wilmington, MA) were used in all
experiments unless otherwise stated. They were maintained
on Wayne Lab Blox (Allied Mills, Chicago, IL) mouse feed
and given acidified-chlorinated water ad libitum.

Congenitally thymus-deficient (nude; nu/nu) mice and
their phenotypically normal littermates (NLM; nu/+ or
+/+) were bred in restricted access rooms using homozygous
(nu/nu) males and heterozygous (nu/+) females of Balb/c
background. Some nudes were obtained from homozygous
males and heterozygous females bred in mono-contaminated
gnotobiotic units. These nude mice were removed from the
gnotobiotic unit and housed in a laboratory for at least
one week before they were used in experiments. Male and
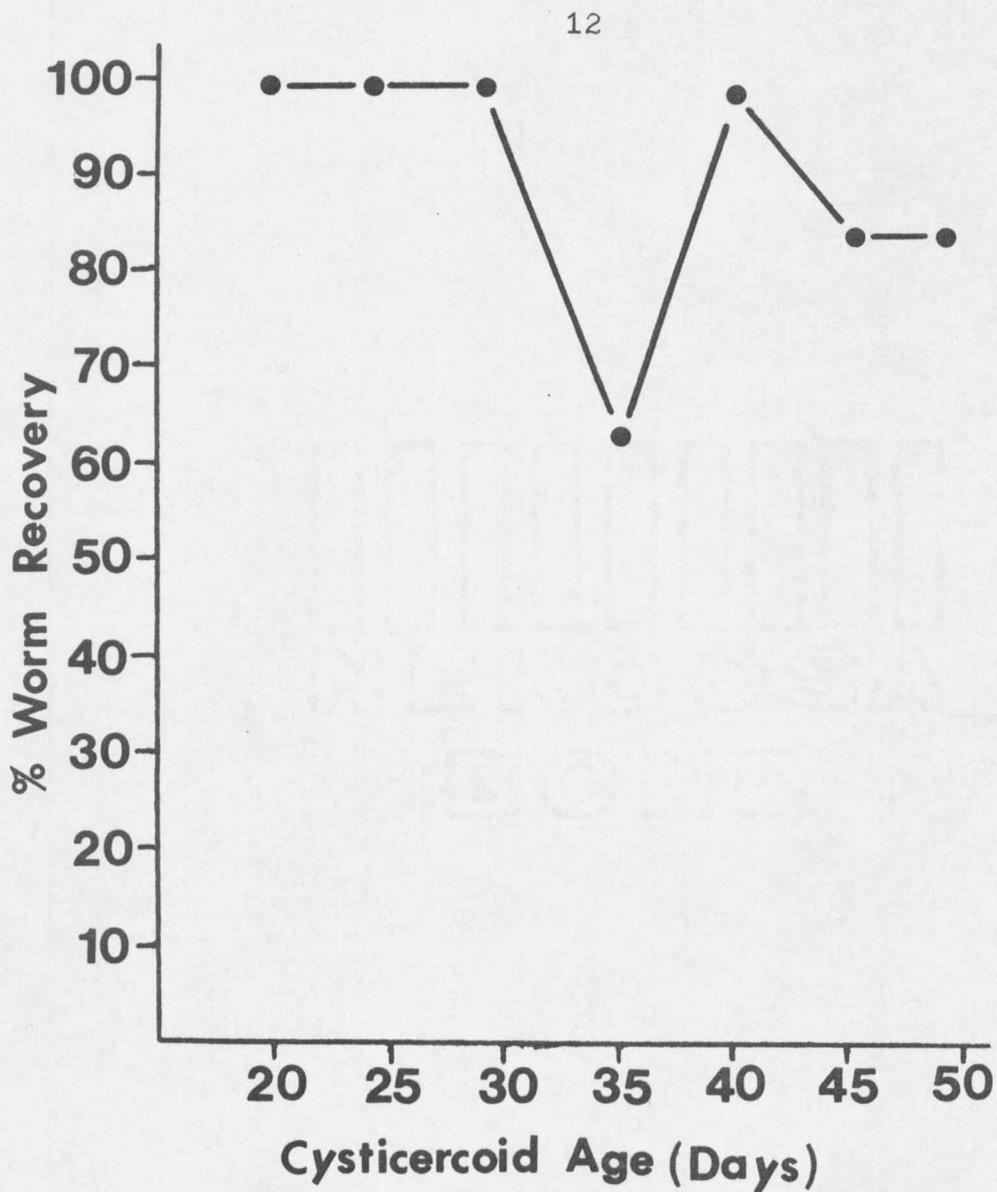
Figure 1. Development of adult <u>H. diminuta</u> in mice given cysticercoids of varying age. The four mice/group were each given three cysticercoids. Seven days later the mice were killed and examined for adult worms.

female Balb/c heterozygotes (nu/+) (GL. Bomholtgaard, Ry, Denmark) were bred as another source of nudes.

Worm Recovery and Dark Area Measurements

On varying days post-infection (p.i.) with cysti-cercoids, mice or rats were killed by cervical dislocation and the small intestine removed from each animal. In mice on days 5, 6, and 7 p.i., the small intestine was severed about 2 cm below the pyloric valve and 3 cm above the ileo-cecal junction. On later days p.i., the cuts were made 2 cm below the pyloric valve and at the ileo-cecal junction because the worms tend to migrate poster-iorly as expulsion begins. The small intestine was flushed with saline from a wash bottle into a tray con-taining saline. The intestinal contents were examined grossly for worms. If all worms were not found, the small intestine was opened longitudinally with scissors and agitated; any worms still inside the intestine were readily evident and could be removed with forceps.

Worm length was measured to the nearest cm while worms were still in the tray. To determine the number and size of dark areas (DA), worms were placed on a slide and examined under 100X of a microscope. Dark areas were

scored using the following criteria:

+1--DA covered less than one proglottid.

+2--DA covered at least one full proglottid
or parts of 2 adjacent proglottids.

+3--DA covered 2 proglottids.

+4--DA covered 3 or more proglottids. If
a DA covered over 7 proglottids, two +4's were
scored.

The dark areas of each worm were evaluated and a dark
area coefficient (DA_c) was calculated for each worm
using the following formula:

$$DA_c = \frac{\text{Sum of DA Scores}}{\text{Worm Length (cm)}}$$

DA Scores = Score for each particular DA on
one worm.

Worm length (cm) = Measured worm length to
the nearest centimeter.

On a given day p.i., the DA_c/ worm for all worms recovered
was averaged as a mean DA_c for that day. A standard
deviation (s) for each mean DA_c was also calculated.
Per cent worm recovery was calculated by:

$$\frac{\text{Number of worms recovered}}{\text{Total possible worms recoverable}} \times 100$$

The mean worm length was also calculated for all worms recovered on a given day.

Immunosuppression with Heterologous Anti-μ Immunoglobulin

In order to assess the requirement of humoral antibody for DA formation and worm expulsion, mice were treated with heterologous anti-μ. Anti-μ has been shown to pan-specifically suppress production of antibody of the IgG, IgM, IgA, and IgE classes (47, 49). Briefly, rabbits were immunized with purified mouse IgM in incomplete Freund's adjuvant. Serum was collected from the rabbits and activity against other mouse Ig's was removed through column chromatography. Purity was determined using Ouchterlony gel diffusion (48). In the first experiment anti-μ (minimum titer of 32 and generously supplied by Bradford Brooks, M.S.) was administered to neonatal mice according to the following schedule:

Day	Amount of Anti-μ (ml)
0(birth), 1,2,4,6	0.05
8,10	0.10
13,16,18,20,25	0.15
28,30,32	0.20
35,37,39	0.25
42,44,46	0.30

Injections were given intraperitoneally (i.p.) on

three or four alternate days during each week. Phosphate buffered saline (PBS) and normal rabbit serum (NRS) were administered as controls to different mice in the same litters as the anti-µ-treated mice according to the same schedule. Injection schedules were maintained during the course of worm infection.

In a second experiment, anti-µ (titer of 256) obtained from goats injected with purified mouse IgM and prepared as above was generously supplied by Dr. Dean Manning. The injection schedule was as follows:

Day	Amount of Anti-µ (ml)
0(birth),1,2	0.06
4	0.08
6,8,10	0.10
12,14	0.15
16,19	0.20
21,23	0.25
26,28,30	0.30
33,35,37	0.35
40,42,44	0.40

Controls were littermates given either PBS or normal goat serum (NGS) according to the same schedule.

Ouchterlony Gel Diffusion for Measuring Residual Anti-µ and Serum IgM

Ouchterlony double diffusion plates were used to

test for serum levels of anti-μ and IgM from mice used
in the second anti-μ experiment. Four ml of 1%
agarose in 0.05M barbital buffer (pH 8.4) with 0.1%
sodium azide was dispensed into each 15 X 60 mm plastic
petri dish. Punch wells were 4 mm in diameter and 10 mm
apart.

To test for serum IgM, the center well was filled
with anti-μ (titer of 256) and 2-fold serial dilutions
of the appropriate serum were placed in the outer wells.
A pool of normal mouse serum was used as a standard to
test for residual anti-μ. Plates were incubated at room
temperature until the precipitin bands were clearly
visible.

Localized Hemolysis-in-Gel Technique

To determine the antibody response of mice to sheep
red blood cells (SRBC), the animal was given 0.2 ml of
10% SRBC i.p. or intravenously (i.v.). Five days later
the spleen was removed and a single cell suspension was
made by rubbing the spleen over a fine mesh screen in
5 ml of Hank's Balanced Salt Solution (HBSS). Cell
clumps were filtered out through glass wool. Dilutions
of 1:5 and 1:25 were then made of spleen cell suspensions.

To a tube containing 0.4 ml of 0.8% agarose at 47°C, 100 µl of the appropriately diluted spleen cell suspension and 50 µl of 8% SRBC were added. The mixture was vortexed and poured onto an agarose-coated slide and incubated in a humid chamber at 37°C for 2 h. Duplicate slides were done for each spleen cell dilution. A 1:10 dilution of guinea pig complement (Colorado Serum Co., Denver, CO) was added and after another $1\frac{1}{2}$ h at 37°C, plaques were counted on slides with between 30 and 300 total plaques. The number of plaque-forming cells (PFC) per spleen was determined.

Thymus-Gland Grafting of Nude Mice

Nude mice were anesthetized with Nembutal (sodium pentabarbital) i.p., the dosage being dependent upon body weight (62). A 1 cm incision lateral to the dorsal midline was made through the skin and peritoneum over one kidney. The kidney was gently worked up onto the surface of the skin. A small tear was made in the renal capsule and one lobe of a neonatal Balb/c thymus gland was inserted under the capsule with forceps. The kidney was placed back into the mouse and the incision in the peritoneum was sutured. The skin incision was closed with

wound clips. The same procedure was repeated for the other kidney. To allow time for thymocytes to mature and be distributed throughout the mouse, mice were held at least 6 weeks after grafting before being used.

Preparation of Worm Antigen and Metabolite Antigen

Mature worms were taken from rats and frozen and thawed 4X. The worms were homogenized in a Tenbroeck homogenizer on ice and the homogenate was centrifuged at 2500 rpm for 15 minutes. The supernatant was then spun at 20,000g for 1 h at $4^{\circ}C$ in an ultracentrifuge. The supernatant was dialyzed against deionized water, lyophilized, and used as antigen (worm Ag) in the indirect hemagglutination (IHA) test and for antigenization.

To obtain metabolite antigen (Met. Ag) freshly harvested living worms 7-11 days old were washed once in saline and once in HBSS. Then 10-50 of these worms were incubated in 10 ml of HBSS for 4 h at room temperature. The worms were filtered out through glass wool after 4 h and the HBSS containing any worm secretions or excretions constituted Met. Ag.

Indirect Hemagglutination

Serum to be tested for antibody to worms was col-

lected from the retro-orbital sinus or tail vein, in-activated at 56°C for 30 minutes, and absorbed once with SRBC for 10 minutes.

Fresh SRBC were washed 3X in PBS and suspended to 2% in PBS. An equal volume of fresh tannic acid (a 1:40 dilution of a 0.1% solution in PBS) was added and the mixture incubated at 37°C for 10 minutes. These cells were washed 2X in PBS and resuspended to 2%. A 5 ml sample was removed for control. The remaining suspension was combined with an equal volume of 1.5 mg/ml worm Ag in PBS at 37°C for 10 minutes. The cells were washed twice to remove excess worm Ag and diluted to 2%.

In the assay (71), U-bottom microtiter plates (Linbro Chemical Co. Inc., New Haven, CN) were used with the Microtiter System (Cooke Engineering Co., Alexandria, VA). Twenty-five µl of 1% NRS diluent were added to each well. Twenty-five µl of serum were added to the first well and 2-fold serially diluted to the penultimate well. Then 25 µl of senstitzed or control cells were added, and the plate was covered and incubated at room tempera-ture for 1-3 h. Agglutination reactions were scored from 0 (no agglutination) to +4 (total agglutination). Wells with agglutination indicated the presence of antibody to

the worm and/or worm Ag.

Determining Delayed-Type Hypersensitivity (DTH) to H. diminuta by Foot Pad Swelling

Foot pad thickness on both feet was measured to the nearest 0.1 mm with dial guage calipers (Schnelltaster, H.C. Kroeplin GMBH, Hessen, Germany). Mice were then lightly anesthetized with ether. With a 30g needle, 0.03 ml of 2 mg/ml worm Ag in PBS was injected into the left hind foot pad and an equal amount of PBS was given in the right hind foot pad. At 3, 24, and 48 h after injection, foot pad thickness of each foot was measured with the calipers. Swelling at 48 h was indicative of a DTH reaction to worm Ag.

Electron Microscopy Techniques

Worms were harvested from mice on day 7 p.i. and normal or dark areas were cut out with a razor blade and placed in fixative for 24 h at $4^{\circ}C$. The fixative was 5% glutaraldehyde in Millonigs buffer containing 5% surcrose and 0.5 mM $CaCl_2$. The specimens were then washed 3X in Millonigs buffer containing 3% sucrose, dehydrated through an ethanol gradient, and embedded in Spurr's epoxy resin (Polysciences, Inc., Warrington, PA). Silver

sections were cut on a Reichert OM-U2 ultramicrotome
using glass knives (LKB 7800A Knifemaker, Stockhom,
Sweden). The sections were post-sectioned stained in
Reynolds lead citrate and uranyl acetate. They were
viewed and photographed in a Zeiss EM 9S-2 transmission
electron microscope.

Preparation for scanning electron microscopy con-
sisted of fixing 7-day old worms whole in cold glutar-
aldehyde in Millonigs buffer + 5% sucrose for 4 h at
room temperature, dehydrating through an ethanol gradient
and an amyl acetate gradient, and then critical-point
drying (Sorvall Critical Point Drying System). An ISI
Model Super II SEM was used for viewing and photography.

Staining and Photography for Light Microscopy

To photograph DA in whole mounts, 7-day old worms
were taken from mice and dipped in 1% glutaraldehyde
briefly. They were then placed on a slide with saline
and covered with a cover glass. Photography was done
immediately on any visible DA.

Tissue to be stained with Hematoxylin and Eosin
(H&E) was fixed in 10% buffered formalin, embedded in
paraffin and 6 μm sections were cut. Sections were

placed on a coated slide, deparaffinized, rehydrated, stained in H&E, dehydrated to xylene, and mounted.

Sections to be stained with Toluidine Blue were cut on the ultramicrotome from the same specimens as those used for transmission electron microscopy. The sections were placed on a drop of water on a gelatin-coated slide and dried at 250°F. Toluidine Blue in borate buffer (pH 9) was added to the slide and heating continued for another 20 minutes; any moisture evaporating was replaced with distilled water. The slide was rinsed under running tap water and mounted wet using nail polish to seal the cover slip and the slide was stored in a humid environment.

In Vitro Incubation of Worms in HBSS or Serum

Living 7-day old worms were examined under 100X of a light microscope for DA and then were washed in sterile PBS and HBSS and were placed in 10 ml HBSS in a small petri dish at room temperature. At specified intervals, the worms were examined under 100X and any change in their DA pattern was noted.

For incubation in serum, 7-day old worms were placed in 0.5 ml of heat inactivated serum + 0.5 ml of HBSS in spot plate wells and incubated at room temperature. Each