



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, *Hymenolepis 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 15 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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TABLE OF CONTENTS

	Page
VITA	ii
ACKNOWLEDGEMENTS	iii
TABLE OF CONTENTS	iv
LIST OF TABLES	vii
LIST OF FIGURES	ix
ABSTRACT	xi
INTRODUCTION	1
MATERIALS AND METHODS	10
Maintenance of Parasites	10
Mice	11
Worm Recovery and Dark Area Measurements	13
Immunosuppression with Heterologus Anti- μ Immunoglobulin	15
Ouchterlony Gel Diffusion for Measuring Residual Anti- μ and Serum IgM	16
Localized Hemolysis-in-Gel Technique	17
Thymus-Gland Grafting of Nude Mice	18
Preparation of Worm Antigen and Metabolite Antigen	19
Indirect Hemagglutination	19
Determining Delayed-Type Hypersensitivity (DTH) to <u>H. diminuta</u> by Foot Pad Swelling	21

	Page
Electron Microscopy Techniques	21
Staining and Photography for Light Microscopy	22
<u>In Vitro</u> Incubation of Worms in HBSS or Serum	23
Statistical Treatment of Data	24
RESULTS	25
Worm Expulsion and Dark Areas in Normal Mice Infected with <u>H. diminuta</u>	25
Worm Expulsion and DA Kinetics in Mice Given a Secondary Infection with <u>H. diminuta</u>	35
Comparison of Dark Areas in Mice and Rats Infected with <u>H. diminuta</u>	37
The Course of Worm Expulsion and DA Formation in Nude Mice	42
Effect of Pan-Specific Antibody Suppression with Heterologous Anti- μ on Expulsion and DA Formation	47
The Effect of Injecting Mice with Worm Antigen on Expulsion, DA Formation, and Circulating Antibody Levels	51
Passive Transfer of Serum from Mice Previously Injected with Worm Antigen or Infected with <u>H. diminuta</u>	59
Injection of Rats with Worm Antigen	63
The Effect of Feeding Worm Antigen on Mean DA and Expulsion of <u>H. diminuta</u> from Mice	65

	Page
Incubation of Worms <u>In Vitro</u> in Glucose-Salts or Serum	69
Delayed-Type Hypersensitivity to <u>H. diminuta</u> Infection in Mice	72
Morphology of Cellular Infiltrate in a Foot Pad Undergoing a DTH Response to Worm Antigen	73
Morphological Comparison Between Dark Areas and Normal Tissue	76
Electron Microscopy of DA and Normal Tissue of <u>H. diminuta</u> from Mice	79
Morphology of Worms Incubated in Concentrated or Diluted Hank's Balanced Salts Solution	85
Scanning Electron Microscopy of <u>H. diminuta</u>	89
DISCUSSION	91
LITERATURE CITED	104

LIST OF TABLES

Table	Page
1. Comparison of mean DA _c peak and starting expulsion date in the 3 separate experiments with mice given a 3c infection	29
2. Course of DA formation, worm growth, and expulsion in nude and NLM mice	43
3. DA formation, worm growth, and expulsion in nude, TG-nude, and NLM mice infected with <u>H. diminuta</u>	45
4. Effect of anti-μ suppression on a 3 cysticeroid <u>H. diminuta</u> infection in mice	48
5. The effect of heterologous anti-μ suppression in <u>H. diminuta</u> -infected mice in a second experiment	51
6. Serum antibody titers from mice given combinations of <u>H. diminuta</u> infections and/or antigenation	53
7. Comparison of nudes and NLM given Ag or PBS and infected with 3 <u>H. diminuta</u> cysticeroids	58
8. The effect of passively transferred serum on <u>H. diminuta</u> in mice	61
9. Expulsion and mean DA _c of worms from antigenized rats ^c	64
10. The effect of oral antigenation on the mean DA _c and expulsion of <u>H. diminuta</u> from mice	67
11. The effect of a single, oral antigenation of mice with 20 mg worm Ag on the course of DA formation and worm expulsion	68

Table

Page

12. Delayed-type hypersensitivity response to worm Ag in a 6c infection in mice	74
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LIST OF FIGURES

Figure		Page
1.	Development of adult <u>H. diminuta</u> in mice given cysticercoïds of varying age	12
2.	Elimination of <u>H. diminuta</u> from mice given a 3c infection in 3 separate experiments	26
3.	Change in mean DA _c of <u>H. diminuta</u> during the course ^c of a 3c infection in the 3 separate experiments	28
4.	Mean DA _c and % Worm Recovery for a 1c infection	31
5.	Comparison of expulsion kinetics in mice given 1c, 3c, or 6c infections with <u>H. diminuta</u>	33
6.	Mean DA _c of worms from 1c, 3c, or 6c <u>H. diminuta</u> infections in mice	34
7.	Expulsion of <u>H. diminuta</u> in primary and secondary infections	36
8.	Dark area formation in worms from primary and secondary infections with <u>H. diminuta</u>	38
9.	Dark area formation in <u>H. diminuta</u> from rats	39
10.	Comparison of worm expulsion and mean DA _c in mice and rats given 3 <u>H. diminuta</u> cysticercoïds	41
11.	Time of worm expulsion in mice given Ag injections over a 2-week period	55
12.	Mean DA _c of <u>H. diminuta</u> from antigenized and control mice	56

Figure		Page
13.	Control mouse foot pad morphology	75
14.	Cellular infiltrate (CI) in worm Ag- injected foot pad of <u>H. diminuta</u> - infected mouse at 48 h post-injection	75
15.	Wet mount of <u>H. diminuta</u> showing +1 and +2 scoring DA	77
16.	DA of <u>H. diminuta</u> with a score of +4	77
17.	DA with a total score of +8	77
18.	Toluidine Blue stained section of normal <u>H. diminuta</u> tissue	78
19.	Toluidine Blue stained section of <u>H. diminuta</u> DA	78
20.	Normal <u>H. diminuta</u> tissue	80
21.	Dark area tissue in <u>H. diminuta</u>	82
22.	Neighboring strobila from a single worm ...	83
23.	Pyknotic cuticle in normal <u>H.</u> <u>diminuta</u> tissue	84
24.	Vacuole in pyknotic cuticle in DA of <u>H. diminuta</u>	84
25.	Normal tissue from <u>H. diminuta</u> incubated in 0.2X HBSS	87
26.	DA from <u>H. diminuta</u> incubated in 0.2X HBSS	87
27.	Normal tissue from a single <u>H.</u> <u>diminuta</u> incubated in 5X HBSS	88
28.	Normal <u>H. diminuta</u> strobila under SEM	90
29.	DA of <u>H. diminuta</u> under SEM	90

ABSTRACT

The gut, lumen-dwelling tapeworm, Hymenolepis 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.

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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). Treatment 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 aggravated 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 impossible 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 treatment 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 IgG₁ 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-mediated 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

Mesocestoides corti substantially reduces the number of tetrathyridia 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 Hymenolepis diminuta. 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 H. diminuta 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 entering the intermediate host and release hexacanth embryos. The embryos penetrate into the insect haemocoel 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-cysticercoid infection thus completing the cycle. This tapeworm can infect humans, but only about 200 cases of infection have been reported (73).

In mice, H. diminuta infections are of short duration, seldom lasting more than 14 days, with destrobilation 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, IgG₁, IgG₂, 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 cysticeroid (1c) infection, expulsion did not begin until day 16-20 (7).

2) A 6c infection leads to a quicker and earlier 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 laparotomy, 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 droplets 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 surgically-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 correspondingly 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 cysticeroids 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 cysticeroids 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 cysticeroids) 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.

Cysticeroids 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 cysticeroids. The drop in infectivity for cysticeroids 35 days old (Figure 1) is probably nonspecific and not related to the age of the cysticeroids.

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

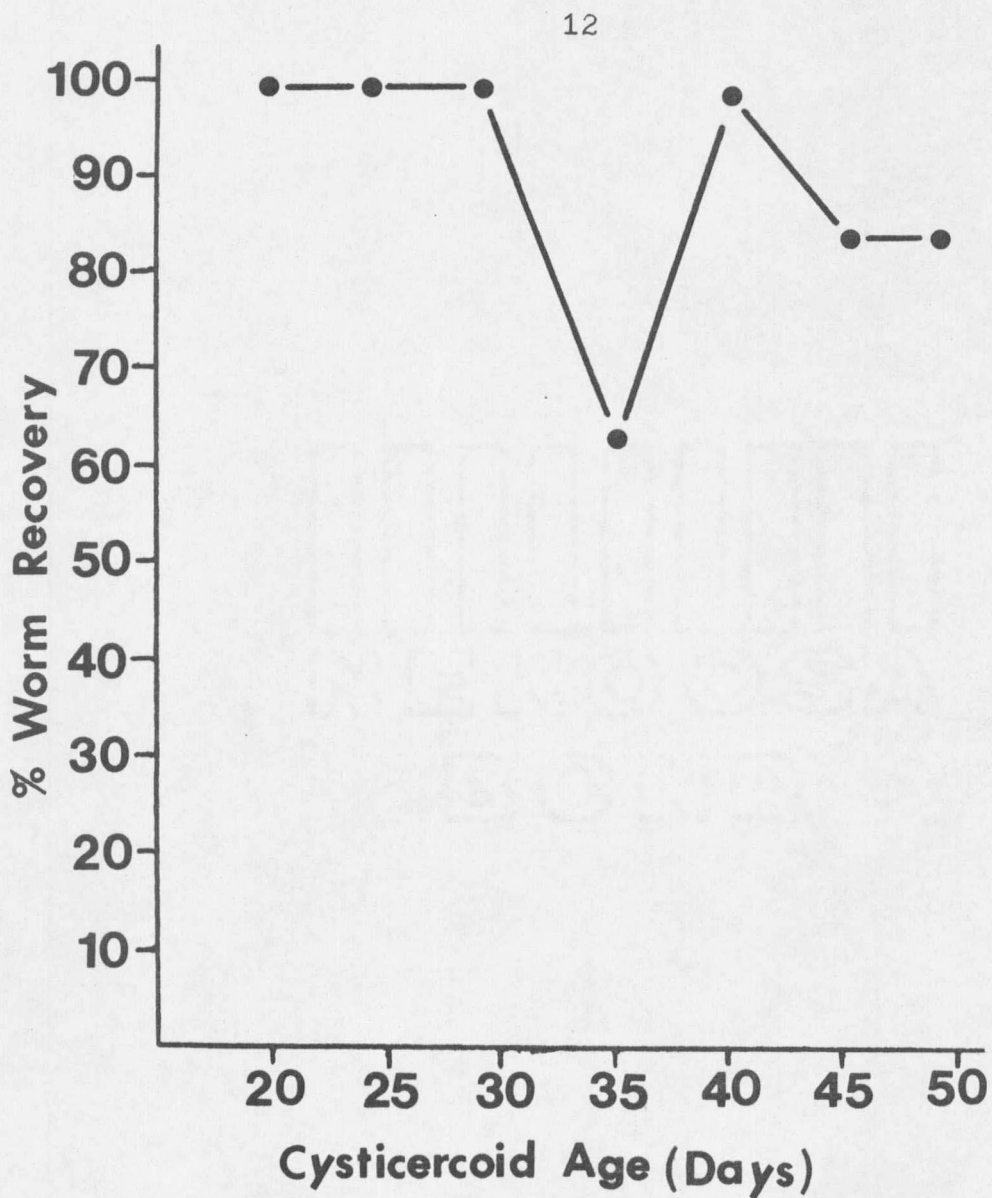


Figure 1. Development of adult *H. diminuta* in mice given cysticercoids of varying age. The four mice/group were each given three cysticercoids. Seven days later the mice were killed and examined for adult worms.

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 cysticercoids, 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 posteriorly as expulsion begins. The small intestine was flushed with saline from a wash bottle into a tray containing 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 1½ 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 pentobarbital) 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°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, inactivated 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 μ l of 1% NRS diluent were added to each well. Twenty-five μ l of serum were added to the first well and 2-fold serially diluted to the penultimate well. Then 25 μ l of sensitized or control cells were added, and the plate was covered and incubated at room temperature 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°C. The fixative was 5% glutaraldehyde in Millonigs buffer containing 5% sucrose 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, Stockholm, 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 consisted of fixing 7-day old worms whole in cold glutaraldehyde 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 μ m 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

serum was tested with 2 different worms. Complement was added to one well of each serum after 80 minutes. The other well received nothing. All worms were examined at intervals of 20 minutes for any change in their DA patterns.

Statistical Treatment of Data

The mean DA_c for a given day p.i. was compared between groups of animals in the same experiment using the Student's t test. The mean DA_c was considered significantly different when probability (p) values were less than 0.05.

RESULTS

Worm Expulsion and Dark Areas in Normal Mice Infected with *H. diminuta*

To determine if dark areas (DA) vary in number and size during the course of a 3 cysticeroid (3c) infection with *H. diminuta* in normal Balb/c mice 5 weeks old, three separate but similar experiments were done. In the first experiment, 49 mice were each given 3c. On days 7-11 post-infection (p.i.), 9 or 10 mice/day were killed and the small intestine examined for worms. In the second experiment, 55 mice were infected with 3c and on days 5, 6, 12, and 13 p.i., 10 mice/day were killed; on days 7, 9, and 11 p.i., 5 mice/day were killed. In the third experiment, 80 mice were infected with 3c and 10 mice/day on days 5-12 p.i. were killed.

Expulsion was considered to be occurring on the first day p.i. that per cent worm recovery was 60% or less. Using a similar system, Befus set the dividing line at $\leq 50\%$ (7). Both of these limits were set arbitrarily. Stunted worm growth (as measured by length) is also used as an indicator of expulsion in some cases.

Expulsion began between days 8-10 p.i. (Figure 2) with all or most worms gone by day 12. On day 5 p.i.,

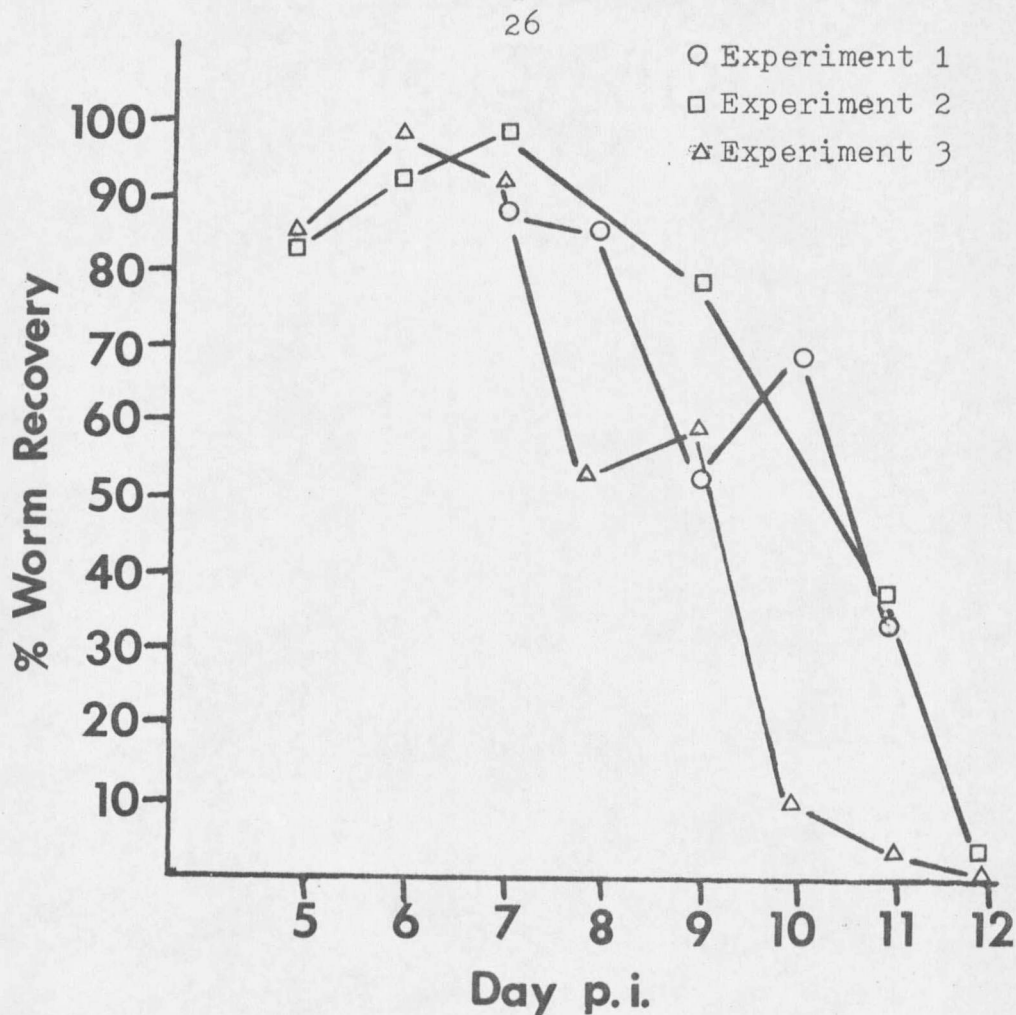


Figure 2. Elimination of *H. diminuta* from mice given a 3c infection in 3 separate experiments. In experiment 1, 49 mice were each given 3c and on days 7-11 post-infection (p.i.), 9 or 10 mice/day were killed and examined for worms. In experiment 2, 55 mice were each given a 3c infections and on days 5, 6, 12, and 13 p.i., 10 mice/day were killed. On days 7, 9, and 11 p.i., 5 mice/day were killed. In experiment 3, 80 mice were each given 3c and on days 5-12 p.i., 10 mice/day were killed.

some smaller worms (< 5 mm long) could not be found with the methods used and this led to a lower per cent worm recovery on that day. Worms had few DA on day 5 but gained more DA until just before expulsion began (Figure 3). The first known mean DA_c peak occurred on or before the day expulsion began (Table 1). There seems to be a downward trend of mean DA_c after expulsion begins in experiment 3 but not in the other 2 experiments. In experiment 1, the mean DA_c declines before expulsion begins and levels out after expulsion begins. There was no consistent pattern of how the mean DA_c varies in magnitude before or after expulsion began, making the relationship between expulsion and DA formation unclear.

The standard deviation (s) for the mean DA_c was usually high on all days in each experiment often equalling or exceeding the mean DA_c value (Figure 3). The vertical bars represent the positive standard deviation only (for clarity).

Worm length increased steadily during the course of the infection. On day 5 worm length averaged slightly less than 1 cm and worms grew to about 11 cm by day 10.

To test the effect of a lower intensity infection on DA formation and worm expulsion, 80 mice each were given

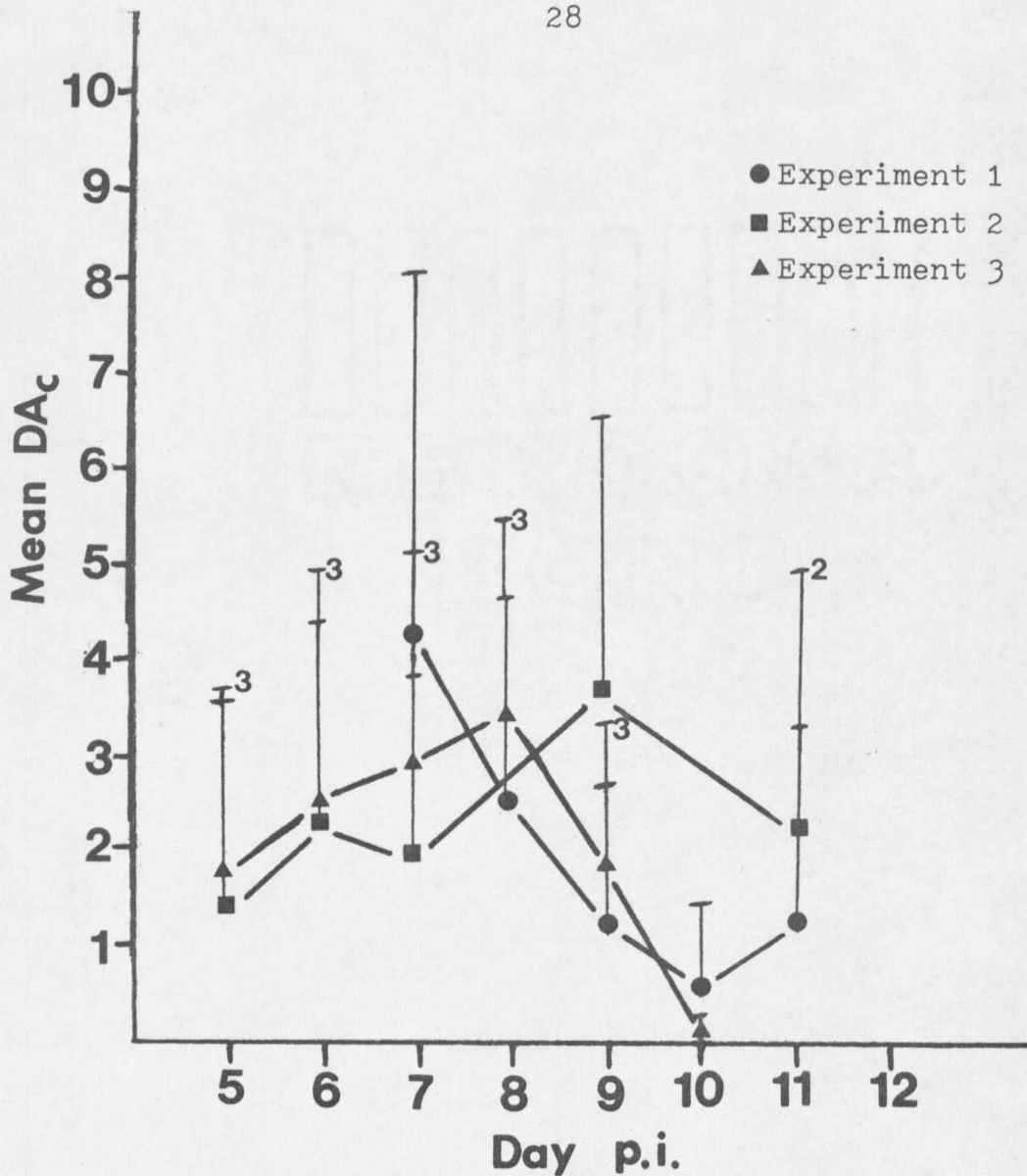


Figure 3. Change in mean DA_c of *H. diminuta* during the course of a 3c infection in the 3 separate experiments. See legend on Figure 2 for details of experiments. Vertical bars represent the positive standard deviation only (for clarity). Numbers next to the vertical bars represent the experiment number point from which the standard deviation bar originates.

Table 1. Comparison of mean DA_c peak and starting expulsion date in the^c 3 separate experiments with mice given a 3c infection.^a

Experiment Number	Day Expulsion Begins ^b	DA _c Peak
1	9	7 ^c
2	10	9
3	8	8

^a See legend for Figure 2 for experimental details.

^b First day when % worm recovery was $50 \pm 10\%$.

^c First known mean DA_c peak observed in that experiment. No mice^c were examined earlier than day 7 p.i.

1 H. diminuta cysticeroid and 10 mice/day on days 5-12 p.i. were killed and examined for worms. Expulsion did not begin until day 11 but only 20% of the worm burden remained at day 12 (Figure 4). The beginning of expulsion was delayed 1-3 days as compared to a 3c infection. The mean DA_c peaked on day 6, earlier and higher than in a 3c infection, and again on day 11. The mean DA_c peak was elevated on both days 6 and 11 because several worms with very high DA_c (>10) were found on each of these days. This was reflected in the grossly elevated standard deviation seen on both days. The mean DA_c graph pattern was more erratic than those for 3c infections but what this difference means is unknown.

Worm length increased in a 1c infection similarly to that in a 3c infection.

To directly compare the differences in DA and worm expulsion using different levels of infection, 192 mice were first divided into three groups of 64 mice each. Mice in one group were given 1c, those of a second group 3c, and a third 6c; all cysticeroids were from the same pool. On days 5-12 p.i., 8 mice/day/group were killed and examined for worms. In the 1c infection expulsion did not begin until day 11, whereas 3c and 6c infection

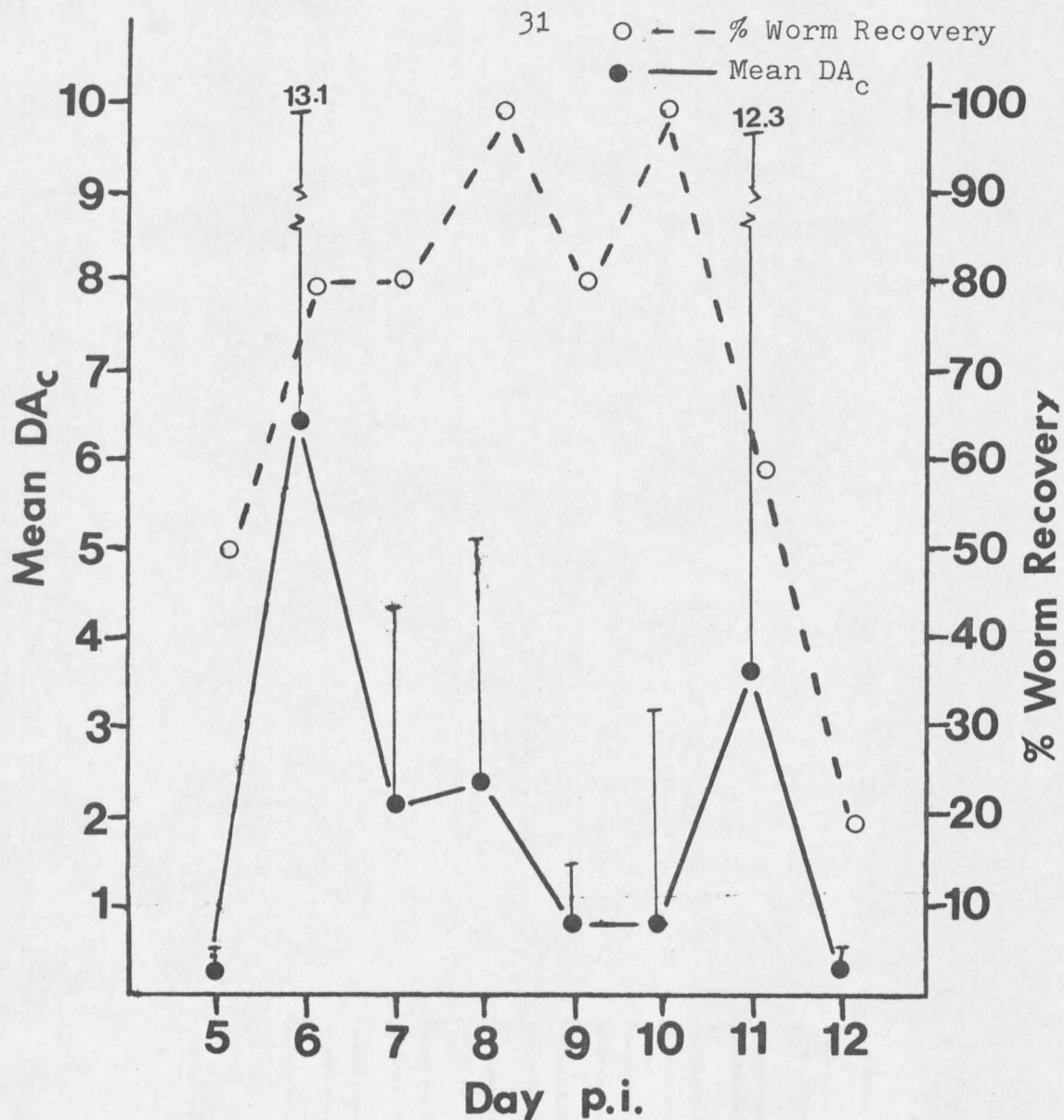


Figure 4. Mean DA_c and % Worm Recovery for a 1c infection. Each of 80 mice was given 1 *H. diminuta* cysticercoid. On days 5-12 p.i., 10 mice/day were killed and examined for worms. Numbers above the vertical bars represent the value the standard deviation would reach if the graph were extended.

expulsion both began on day 10 (Figure 5). The time when expulsion began and the rate thereafter were similar for the 3c and 6c infections. In a 1c infection the mean DA_c peaked on day 8 (two days later than in the previous 1c experiment) at 9.0 and then dropped sharply by day 9. The 3c infection mean DA_c also peaked on day 8 at 2.4. In the 6c infection, two mean DA_c peaks were found: a peak of 3.4 on day 7 and another on day 10 at 3.0 (Figure 6). Standard deviation varied between 2 and 4 for most days tested. Note the very high standard deviation on day 8 p.i. for a 1c infection. This is again due to several worms having a grossly elevated DA_c . Expulsion kinetics were similar between 3c and 6c infections as opposed to a 1c infection. Mean DA_c graphs were similar in all infection groups according to the Student's t test. Only the 1c mean DA_c point on day 8 differed significantly from the other groups on that day. This difference is probably due to 2 worms (out of 7 recovered) with a $DA_c > 15$. The level of infection generally does not appear to affect DA formation though the time when expulsion began was affected.

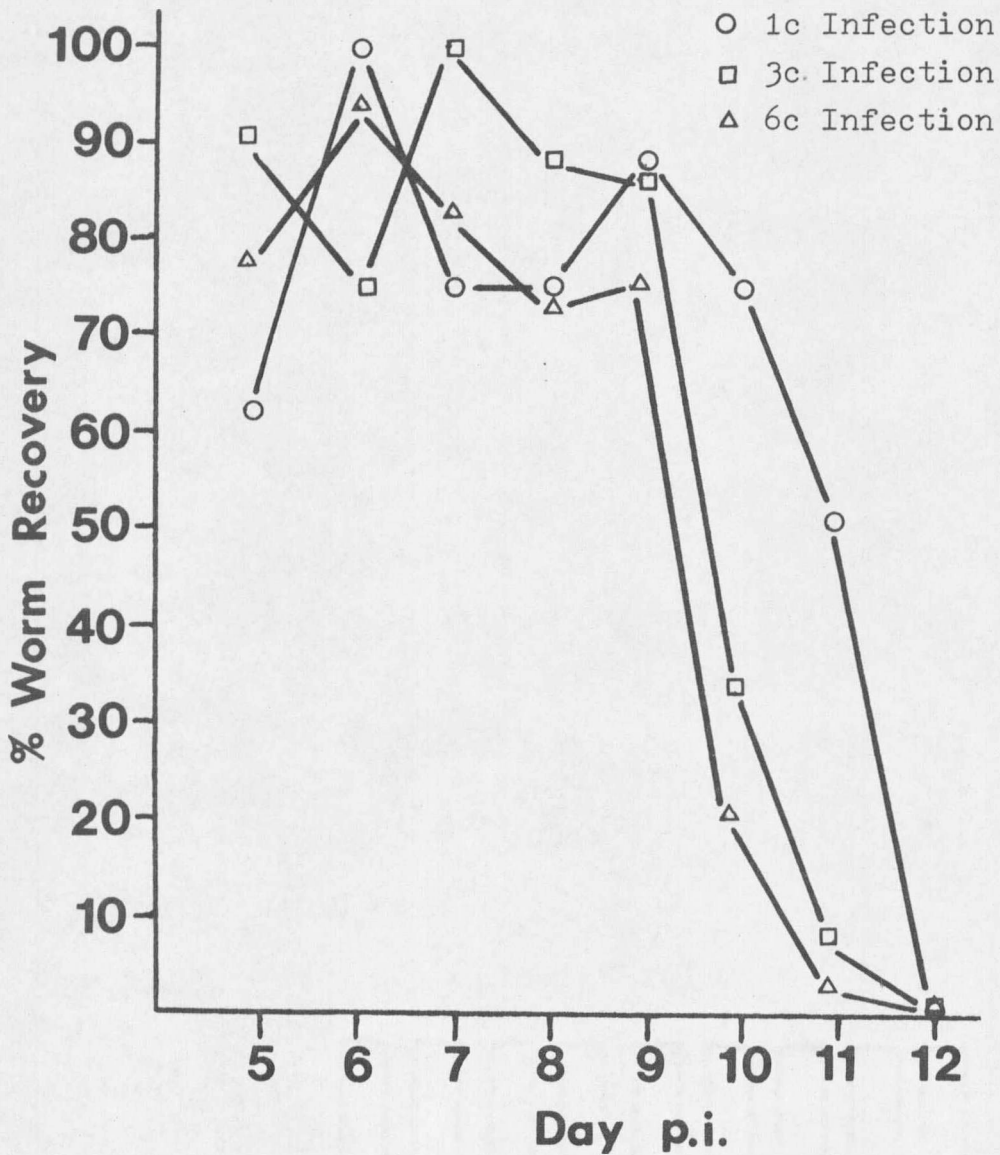


Figure 5. Comparison of expulsion kinetics in mice given 1c, 3c, or 6c infections with *H. diminuta*. Three groups of 64 mice each were infected with either 1, 3, or 6 *H. diminuta* cysticercoids. On days 5-12 p.i., 8 mice/day/group were killed and examined for worms.

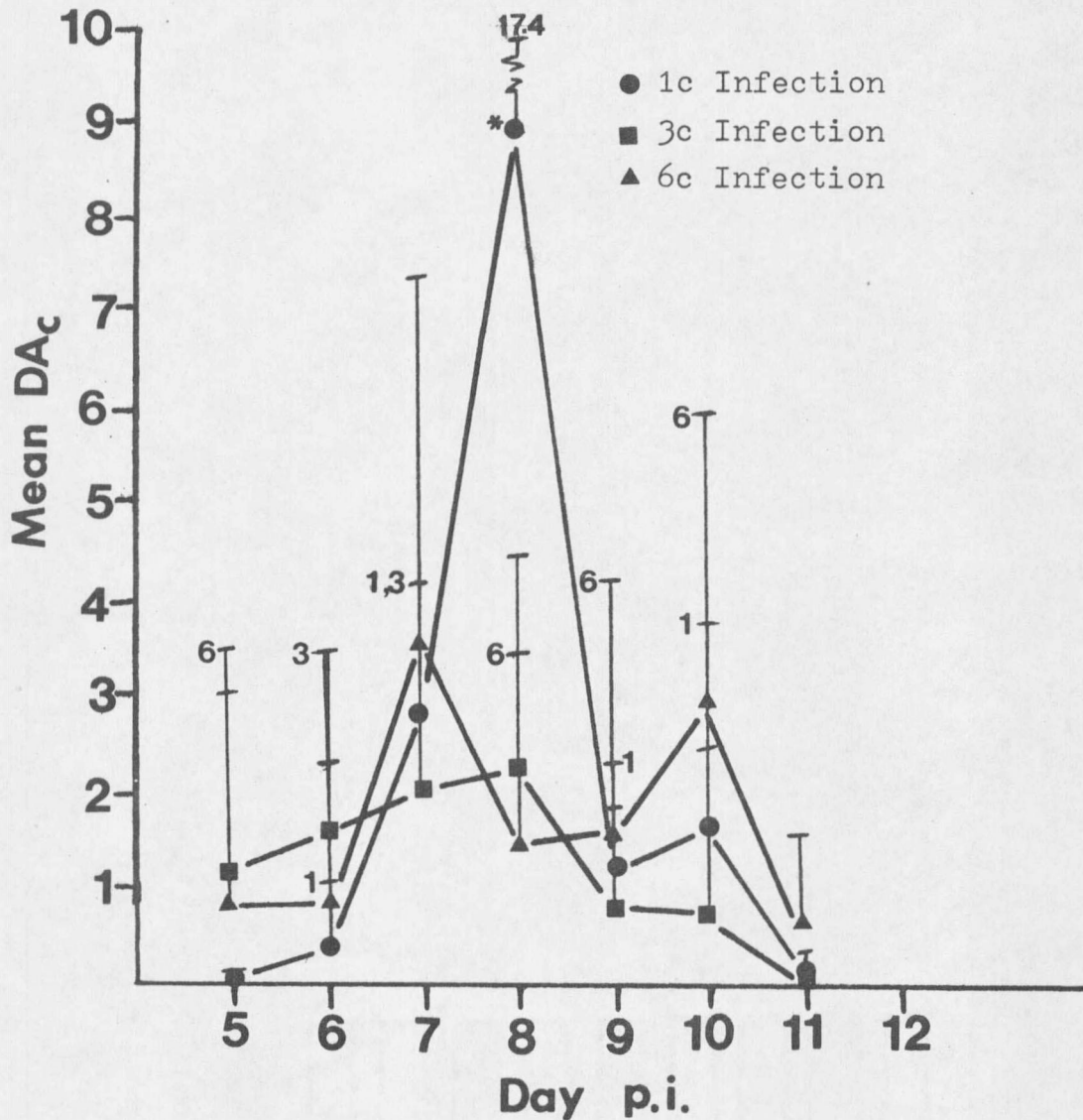


Figure 6. Mean DA of worms from 1c, 3c, or 6c *H. diminuta* infections in mice. See legend on Figure 5 for experimental details. Numbers to the side of the vertical bars represent the number cysticeroid infection point that the standard deviation bar originated from. Asterisk indicates $p < 0.05$ for that point.

Worm Expulsion and DA Kinetics in Mice Given a Secondary Infection with *H. diminuta*

To further clarify the relationship between host immunity and DA formation kinetics, an experiment was done to determine if any acquired immunity to *H. diminuta* was seen after a primary infection and, if so, whether or not it affects the magnitude of the mean DA_c . First 154 mice were divided into two groups. On day -14, ninety mice were given a 6c primary infection and 64 mice were sham-infected with saline. On day -7, ten mice in the infected group were killed to ascertain the success of the primary infection. All worms from the primary infection should be gone by day 0 (See Figure 5). On day 0 all mice were given 3c and 8 primary-infected mice/day and 10 secondary-infected mice/day were killed on days 5-12 p.i. Worm recovery on day -7 was 91.7% indicating a normal, active 6c primary infection. Control mice (sham-infected and later given 3c) showed normal expulsion kinetics with expulsion starting by day 10 and most worms were gone by day 11. Worms were expelled faster from secondary-infected mice with only 30% worm recovery on day 6 and less than 5% worm recovery through day 10 (Figure 7). Mean DA_c peaked on day 7 at 4.0 in primary-

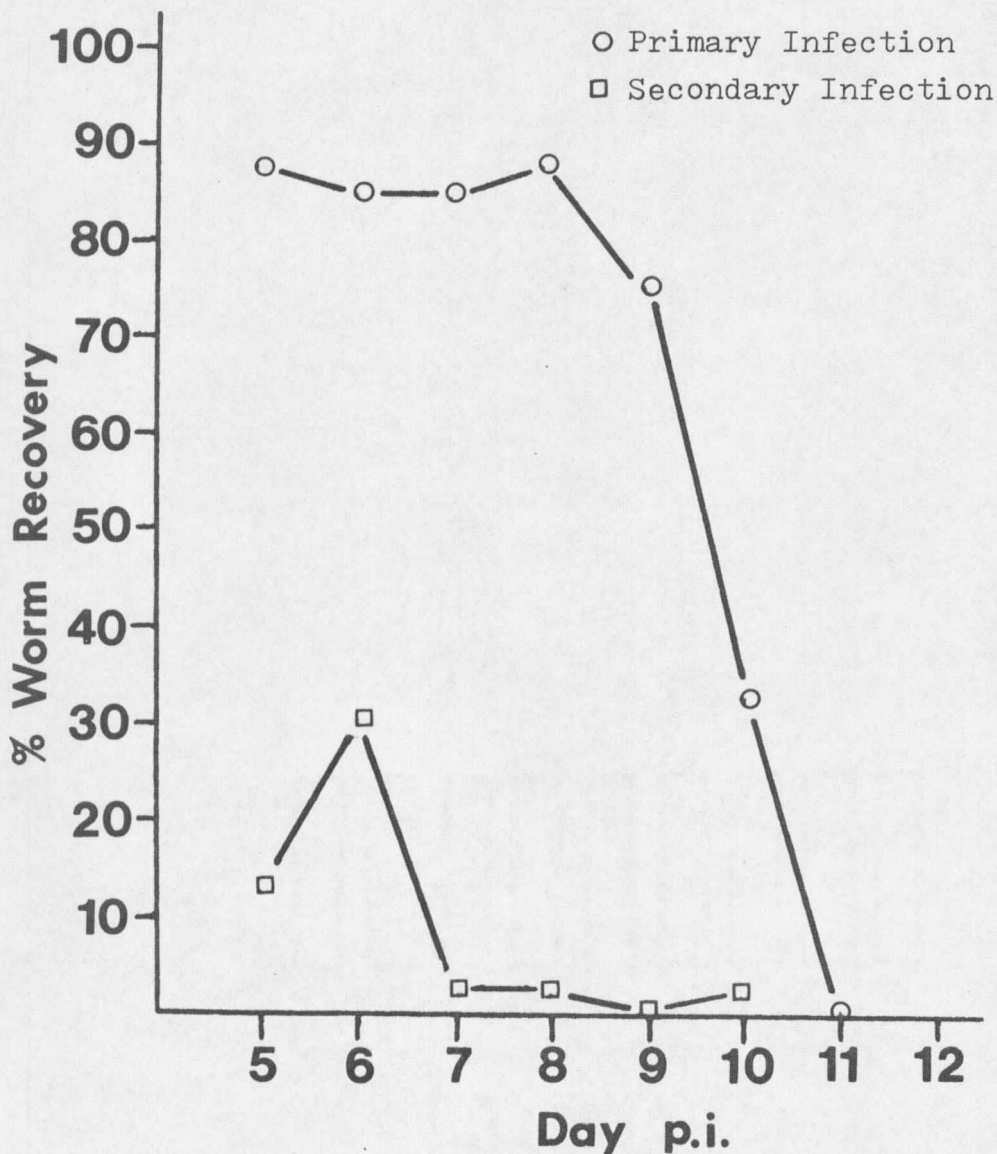


Figure 7. Expulsion of *H. diminuta* in primary and secondary infections. Ninety mice were given 6 cysticeroids on day -14 and 64 mice were sham infected on the same day. Two weeks later (day 0) all mice in both groups were given 3c. On days 5-12 p.i., 8 or 10 mice/day/group were killed and examined for worms.

infected mice but reached only 0.4 on day 6 in secondary-infected mice (Figure 8). However, insufficient worms were recovered on successive days from secondary-infected mice to permit evaluation of DA kinetics.

Worm length was not significantly different between primary- and secondary-infected mice on day 6 (1.0 and 0.9 cm, respectively).

Comparison of Dark Areas in Mice and Rats Infected with *H. diminuta*

Since rats maintain their worm burdens over six months and are not thought to immunologically expel their worms, worms from rats were examined for DA. In the first experiment, 16 adult Holtzman rats were given a 4c infection. Four rats/day were killed on days 7, 14, 28, and 56 p.i. and the worms removed. Only the upper one-third of the worm was examined for DA because in rats maturing proglottids as well as DA are opaque to transmitted light. A mean DA_c similar in magnitude to that found in mice on day 7 p.i., was found in rats on day 7 p.i. By day 14 most worms recovered from rats had few and small DA. On days 28 and 56 p.i., worms had many DA but a low mean DA_c was found because of the long worm length (Figure 9). Per cent worm recovery was high on

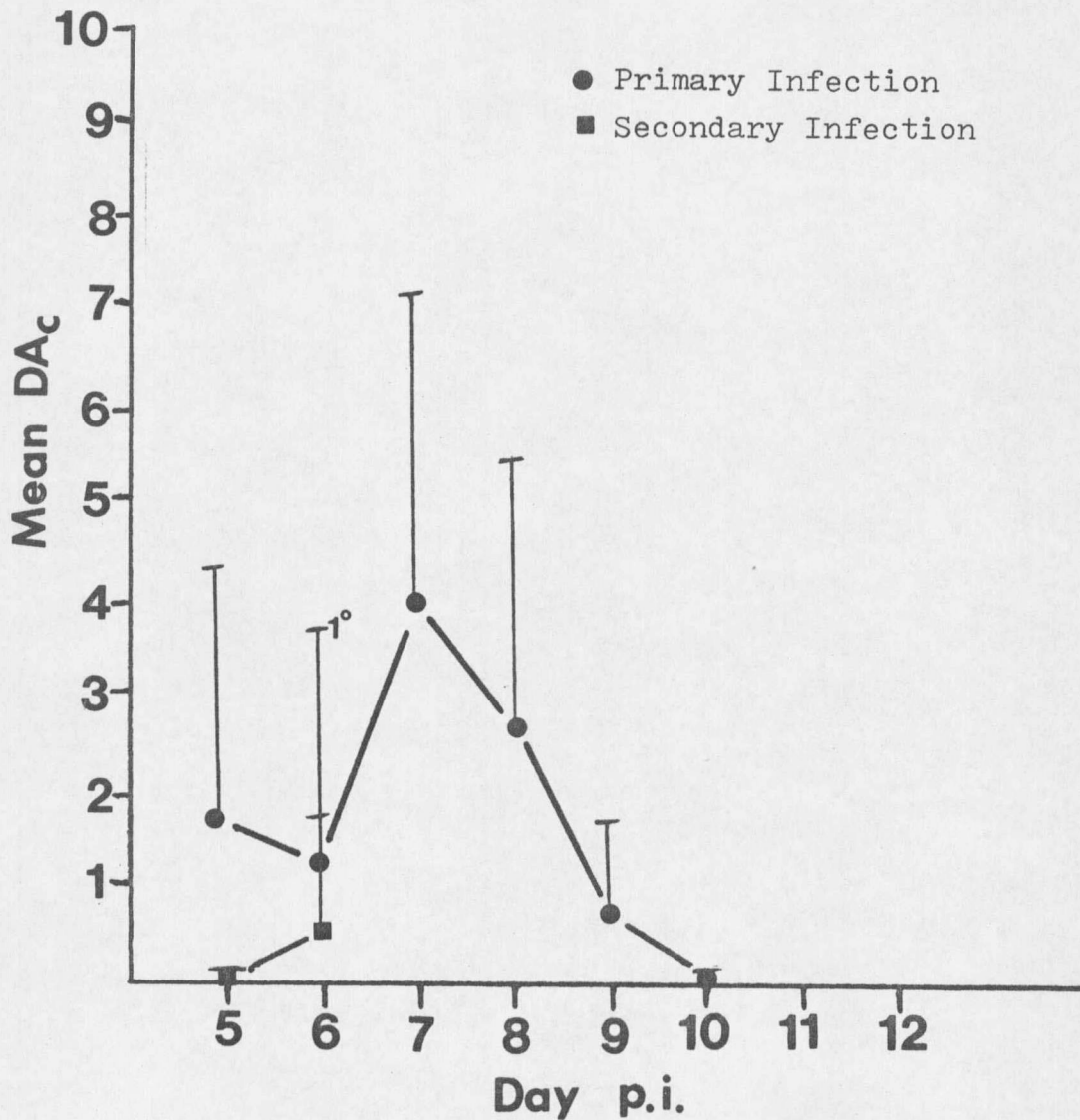


Figure 8. Dark area formation in worms from primary and secondary infections with *H. diminuta*. See legend for Figure 7 for experimental details. The vertical bars represent the standard deviation.

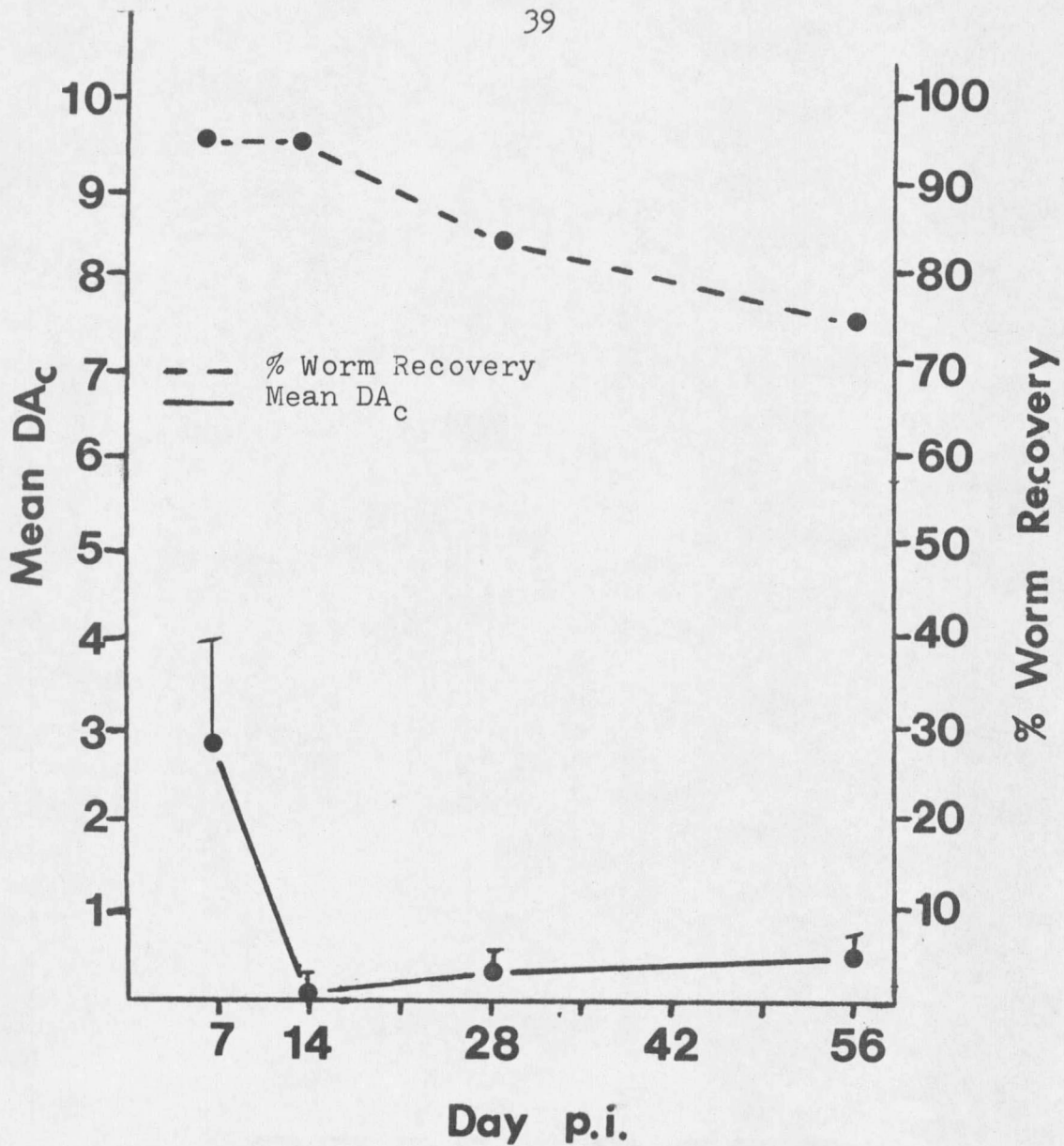


Figure 9. Dark area formation in *H. diminuta* from rats. Sixteen rats 10 weeks old each were given 4c. On days 7, 14, 28, and 56 p.i., 4 rats/day were killed and examined for worms and the worms were examined for DA.

all days tested. A lowered standard deviation was found for the mean DA_c on days 14, 28, and 56 p.i. Worms recovered from rats also had DA but this is probably not related to a host expulsion mechanism since the rats did not expel their worms.

Worms had attained a length of almost 50 cm by day 56 p.i.

To directly compare the mean DA_c of worms from mice and rats, 20 rats and 32 mice each were given 3c. On days 6, 8, 9, and 10 p.i., 5 rats and 8 mice/day were killed. Expulsion began on day 10 in mice but not in rats (Figure 10). The mean DA_c peak for worms from rats is 1 or 2 days earlier than for mouse worms and the mean DA_c peaks for worms from both rats and mice were of similar magnitude (Figure 10). Worms from both rats and mice have few DA by day 10 though worms from rats grew faster and matured earlier. In comparing the mean DA_c of the same day p.i. between worms from mice and rats using the Student's t test, it was found that they differed only on day 6 p.i. Worms from rats form more and larger DA earlier than in mice but thereafter DA formation kinetics are similar in mice and rats. In two vastly different host, one which expels worms and one which does not, it

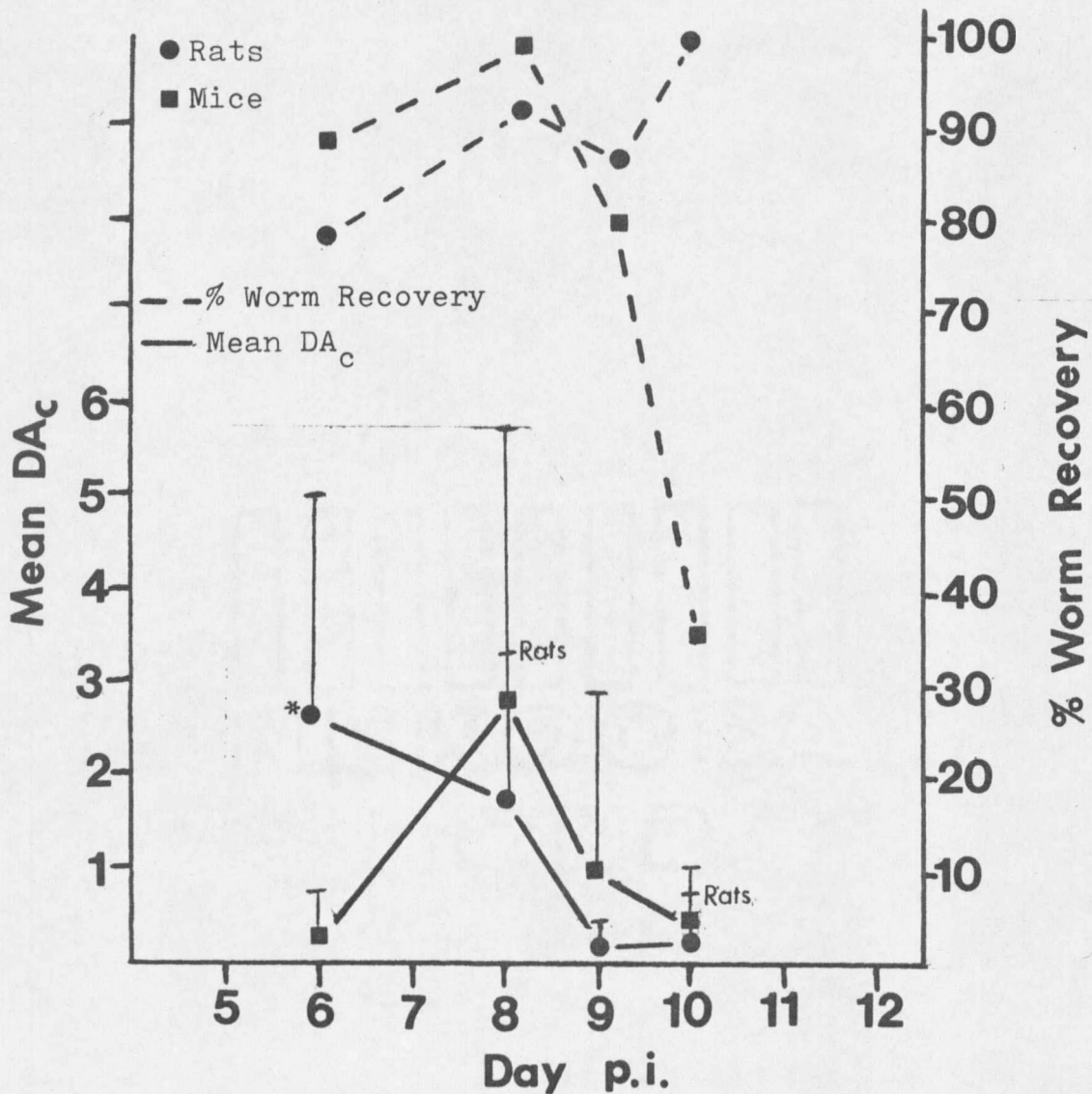


Figure 10. Comparison of worm expulsion and mean DA_c in mice and rats given 3 *H. diminuta* cysticercoids. Nineteen rats and 32 mice were infected. On days 6, 8, and 9 p.i., 5 rats and 8 mice/day were killed and examined for worms. On day 10 p.i., 4 rats and 8 mice were killed. Asterisk indicates $p < 0.05$.

would be expected that DA formation kinetics would vary markedly. Therefore, DA formation may not be totally host-dependent or influenced by the host immune response but rather may be intrinsic to the worm.

The Course of Worm Expulsion and DA Formation in Nude Mice

To see if DA formation is thymus dependent 10 nudes (8-9 weeks old) and 5 NLM (8 weeks old) each were given a 3c infection. About one-half each group was killed on days 7 and 14 p.i. Worms from nude mice were shorter on day 7 and had significantly fewer DA ($p < 0.05$) than worms from NLM controls (Table 2). By day 14 all NLM had expelled their worms but 100% of the worms were recovered from nudes and these worms had DA. This could mean that thymic competency is necessary for the formation of higher levels of DA or that DA are related to the growth rate of the worms. In the earlier experiment involving infected mice and rats, worms from rats grew faster than those from mice and their mean DA_c peaked sooner. So the shorter worms found in nude mice could have fewer DA because they are growing slower than worms in NLM.

To distinguish whether DA formation was related to

Table 2. Course of DA formation, worm growth, and expulsion in nude and NLM mice.^a

Mice	Day p.i.	% Worm Recovery	Mean DA _c	Mean Worm Length (cm)
Nude	7	87	0.4*	0.8
	14	100	1.2	5.4
NLM	7	80 ^b	1.6	2.0
	14	0 ^b	---	---

^aTen nude mice and 5 NLM, 8-9 weeks old, each were given 3 H. diminuta cysticercoids. On days 7 and 14 p.i., about one-half of each group was killed and examined for worms.

^bNo worms were recovered.

* $p < 0.05$ for mean DA_c on day 7 p.i. between NLM and nude mouse worms.

worm growth rate or thymus competency, 14 thymus-grafted (TG) nudes, 11 nude mice and 15 NLM each were given a 3c infection. On days 7, 11, and 14 p.i., 3-5 mice/group were killed and examined for worms. Worms from NLM had a significantly higher ($p < 0.05$) mean DA_c on day 7 than both TG-nude worms and nude worms (Experiment 1, Table 3). Again worms from nude mice were shorter on day 7 than worms from TG-nudes or NLM. By day 11, no worms were found in NLM and the few worms found in TG-nudes had no DA. On day 11 the mean DA_c for nudes had not changed significantly from day 7. Nude mice retained all their worms through day 14 and the mean DA_c for worms from nudes remained relatively constant. At least one kidney had a viable thymus gland under the renal capsule in all TG-nudes killed. Isaak (27) showed that TG-nude mice, 6 weeks after receiving a thymus-gland graft, had a PFC response to SRBC similar to the PFC response of NLM and substantially higher than nude mice. These TG-nudes also expelled H. diminuta on schedule. So a restoration of thymus-dependent functions (seen by an increased PFC response) in TG-nudes also led to a restoration of the worm expulsion mechanism. In my experiments the restoration of immune competency was not tested in TG-nudes by

Table 3. DA formation, worm growth, and expulsion in nude, TG-nude, and NLM mice infected with H. diminuta.

	Mice							
	NLM		TG-Nude			Nude		
			Day p.i.					
	7	11	7	11	14	7	11	14
Experiment 1 ^a								
Number of Mice	5	7	5	4	5	5	3	3
% Worm Recovery	100	0	93	33	13	93	100	100
Mean DA _c	5.5*	--	2.3	0	0	1.6	1.1	0.6
Mean Worm Length (cm)	1.9	--	1.8	7.5	5.0	1.0	6.9	10.9
* * * * * Experiment 2 ^b * * * * *								
Number of Mice	7	7	4	3	NT	5	5	NT
% Worm Recovery	81	0	75	11	NT	100	100	NT
Mean DA _c	2.2	--	1.6	--	NT	3.0	0.7	NT
Mean Worm Length (cm)	2.9	--	1.4	--	NT	1.0	4.5	NT

^aFifteen NLM, 13 nudes, and 14 thymus grafted nudes (TG-nudes) were each given 3 H. diminuta cysticercoids. All mice were 17 weeks old. On days 7, 11, and 14 p.i., 3-5 mice/group were killed and examined for worms. *p 0.05.

^bFourteen NLM, 7 TG-nudes, and 10 nudes were each given a 3c infection. On days 7 and 11 p.i., one-half of each group was killed. NT = Not Tested.

determining a PFC response. Based on Isaak's work, if TG-nudes expel their worms, then the thymus-graft is viable and did restore thymus-dependent functions.

To confirm and clarify the results of the first experiment using TG-nudes a second experiment involving TG-nudes was done. Eight nudes (5 or 10 weeks old), 7 TG-nudes (14 weeks old) and 12 NLM (8 or 14 weeks old) were each given 3c. On days 7 and 11 p.i., one-half of each group was killed and examined for worms. On day 7 p.i., per cent worm recovery for all 3 groups was normal. All TG-nudes had a viable thymus gland under each renal capsule. Also on day 7, there was no significant difference in mean DA_c between the 3 groups (Experiment 2, Table 3) according to the Student's t test. Again worms from nude mice were shorter on day 7 than worms from TG-nudes or NLM. By day 11 all worms were gone from NLM, one worm was recovered for the TG-nude group, and 100% of the worms were recovered from the nudes. A low mean DA_c was found in worms from nudes on day 11. In the experiment involving only nudes and NLM (Table 2), a significant difference was found in the mean DA_c between worms from nudes and NLM. In experiment 1 (Table 3) involving TG-nudes, this is also true but the mean DA_c

was different between worms from TG-nudes and NLM. In experiment 2 (Table 3), no difference in mean DA_c is seen in worms from NLM, TG-nudes, and nudes. Due to this conflicting data, no firm conclusion can be drawn about the thymus competency of DA formation.

Effect of Pan-Specific Antibody Suppression with Heterologous Anti- μ on Expulsion and DA Formation

In the first experiment, a total of 10 Balb/c mice (Cumberland View Farms, Clinton, TN) treated from birth with either heterologous anti- μ , PBS, or normal rabbit serum (NRS) (See Materials and Methods for injection schedule) each were given a 3c infection at 5 weeks of age. On day 7 all 10 mice were killed. Table 4 shows that the mean DA_c did not vary significantly between groups. Both the PBS and NRS groups had good PFC responses to SRBC whereas the anti- μ mice had almost no plaque-forming ability and thus antibody production to SRBC was effectively suppressed by anti- μ (Table 4).

In the second experiment, 23 Balb/c mice were treated from birth with heterologous anti- μ , PBS, or normal goat serum (NGS). Each mouse was infected with 3c at 4-5 weeks of age. On day 7 p.i., four anti- μ -, 4 NGS-, and 4 PBS-treated mice were bled, killed, and

Table 4. Effect of anti- μ suppression on a 3 cysticer-coid H. diminuta infection in mice.^a

	Mice Treated with		
	PBS	NRS	Anti- μ
	7	Day p.i. 7	7
Number of Mice	4	3	3
% Worm Recovery	88.7	88.7	88.7
Mean DA _c	2.2	2.1	2.9
Mean Worm Length (cm)	2.5	1.1	2.7
Mean PFC/10 ⁶ Spleen cells	60.1	27.7	1.4

^aTen mice treated from birth with heterologous anti- μ , NRS, or PBS were infected at 5 weeks of age with 3 H. diminuta cysticercoids. On day 2 p.i. all mice were given 0.2 ml 10% SRBC i.p. On day 7 all mice were bled, killed, examined for worms, and the spleens were used in the hemolysis-in-gel assay to measure the number of PFC to SRBC.

^bNo significant difference among groups was found.

examined for worms. The remaining mice were injected with SRBC on day 11, and bled, killed, examined for worms, and PFC on day 16 p.i. On day 7, per cent worm recovery was high for all groups. By day 16 only one anti- μ -treated mouse had any worms. No worms were found in any NGS- or PBS-treated mice or in any other anti- μ -treated mouse. On day 7 the mean DA_c was similar between the two control groups and between the worms from NGS- and anti- μ -treated mice. However, the mean DA_c was significantly different between worms from the PBS- and anti- μ -treated mice. Why the mean DA_c of the PBS control group is statistically different from the anti- μ group but the NGS control group is not different from the anti- μ group is unknown. There was almost no difference in worm length between groups on day 7.

A normal PFC response to SRBC was seen in PBS-treated mice but low responses were seen in NGS- and anti- μ -treated mice (Table 5). The low response in NGS-treated mice could have been due to incomplete adsorption of any anti-mouse activity from the serum or because of anti-SRBC present in the NGS. The serum IgM levels indicate that the anti- μ treated mice were effectively suppressed in regard to IgM production whereas PBS- and NGS-treated

Table 5. The effect of heterologous anti- μ suppression in H. diminuta-infected mice in a second experiment.^a

	Mice Treated with					
	PBS		NGS		Anti- μ	
	Day p.i.					
	7	16	7	16	7	16
Number of Mice	3	4	4	4	4	4
% Worm Recovery	88.9	0	100	0	100	25
Mean DA _c	2.4 ^b	--	4.0	--	4.6	--
Mean Worm Length (cm)	3.4	--	3.7	--	3.4	--
Mean PFC/10 ⁶						
Spleen cells	NT	25	NT	2	NT	0
Residual Circulating Anti- μ Titer	--	--	--	--	3	1
Serum IgM Levels Titer	5	10	7	11	0	0

^aTwenty-four mice were each given injections of PBS, NGS, or anti- μ from birth. At 4 weeks of age all mice were given a 3c infection. On day 7 p.i. one-half of the mice in each treated group was bled, killed and examined for worms. On day 11 p.i. the remaining mice were injected i.p. with 0.2 ml 10% SRBC. On day 16 p.i. the remaining mice were bled, killed, examined for worms, and the spleens used to determine the PFC response to SRBC. Serum was tested for residual anti- μ and circulating IgM with Ouchterlony plates.

^bMean DA_c differs significantly only between worms from PBS-treated mice and those from anti- μ -treated mice.

mice were producing detectable antibody. Anti- μ treatment does not prevent worm expulsion and probably does not affect DA formation kinetics.

The Effect of Injecting Mice with Worm Antigen on Expulsion, DA Formation, and Circulating Antibody Levels

Theoretically, if immunodeficient animals have worms with fewer DA, then immuno-stimulated animals should have worms with more DA. To test this 14 mice were each given worm Ag (2 mg/ml in PBS) injections according to the following schedule:

Amount	Route	Day
0.25 ml	s.c.	0
0.10 ml	s.c.	11
0.15 ml	i.p.	60

A total of 1.0 mg of worm Ag was administered over the 2-month period. Mice were each infected with 3c on day 67 and killed on day 7 or 9 p.i. Both on days 7 and 9 p.i., only 14% of the worms were recovered. Two rats infected from the same cysticeroid pool showed 87.5% recovery on day 14 p.i. indicating normal and viable cysticeroids. Serum obtained from worm Ag-injected mice was pooled for each day and tested for antibody by

indirect hemagglutination (IHA). Serum from this experiment (labelled 7Ag and 9Ag, corresponding to serum from antigenized mice taken on days 7 or 9 p.i.) had titers of 10, 240 (Table 6). Tentatively, it appeared that worm Ag-injected mice expelled their worms faster. However, a lack of a PBS-injection control group makes the results equivocal. Other serums from mice given various infection regimens were tested and their titers are also shown in Table 6. Serum from mice naturally infected had no detectable antibody even if the mice had been given 3 infections over a 4 week period. Serum from infected rats or nude mice also showed no antibody to worm antigens.

To see if worm Ag-injection does speed expulsion, a second experiment with PBS controls was done. Sixty-four mice were given worm Ag according to the following schedule:

Amount	Route	Day
0.20 ml	s.c.	0
0.20 ml	s.c.	4
0.10 ml	s.c.	7
0.10 ml	i.p.	11
0.15 ml	i.p.	14

A total of 1.5 mg worm Ag was administered over a 2 week

Table 6. Serum antibody titers from mice given combinations of H. diminuta infections and/or antigenation^a.

Serum	Titer	
	Infected or Antigenized	Controls ^b
NMS--Normal Mouse Serum	0	0
IS--Immune Serum. These mice had received 3c, 3 times over a 4-week period and were bled at 5 weeks.	0	0
Ag--These mice had received 3 injections with worm Ag over a 5-week period and were bled at 6 weeks.	256	0
7Ag--Mice that received 3 Ag injections over a 2 month period, infected with 3c, and then bled on day 7 p.i.	10,240	0
9Ag--As above except bled on day 9 p.i.	>1024	0
7 _{3k} --Serum from mice used in the third 3c experiment. The larger number indicates the day p.i. when mice were bled.	0	0
8 _{3k} --As above except bled on day 8 p.i. (See Figure 3).	0	0
9 _{3k} --Bled on day 9 p.i.	0	0
10 _{3k} --Bled on day 10 p.i.	0	0
11 _{3k} --Bled on day 11 p.i.	0	0
12 _{3k} --Bled on day 12 p.i.	0	0
50 _{3k} rat--Serum from a rat with a 50 day infection.	0	0
60 rat--60-day infection in a rat.	0	0
7 nude--Serum from nude mice taken on day 7 p.i.	0	0
14 nude--Serum from nude mice taken on day 14 p.i.	0	0

^aAll serums were adsorbed with SRBC and heated to 56°C for 30 minutes. Antibody was tested for by IHA.

^bControls were tanned cells not coated with worm Ag.

period. Sixteen mice were also given PBS according to the same schedule. Mice were infected with 3c on day 21 and 8 worm Ag-injected mice/day were killed on days 5-12 p.i. One-half of the control mice were killed on days 7 and 9 p.i. Figure 11 shows that expulsion was occurring on day 9 in both control and worm Ag-injected mice. Two mean DA_c peaks were seen for worms from worm Ag-injected mice: one on day 6 and one on day 9 (Figure 12). These were due to several worms with a high DA_c . Control mean DA_c did not differ significantly from the mean DA_c for worms from worm Ag-injected mice according to the Student's t test. Worm lengths were similar between worm Ag-injected and control groups.

To see if injecting worm Ag affected the course of worm expulsion or DA formation in nude mice as well as to determine if nude mice will respond to worm Ag with antibody formation, 12 nudes and 12 NLM were divided into 4 groups of six mice each:

Group Number	Mouse	Treatment
1	NLM	PBS
2	NLM	Worm Ag
3	Nude	PBS
4	Nude	Worm Ag

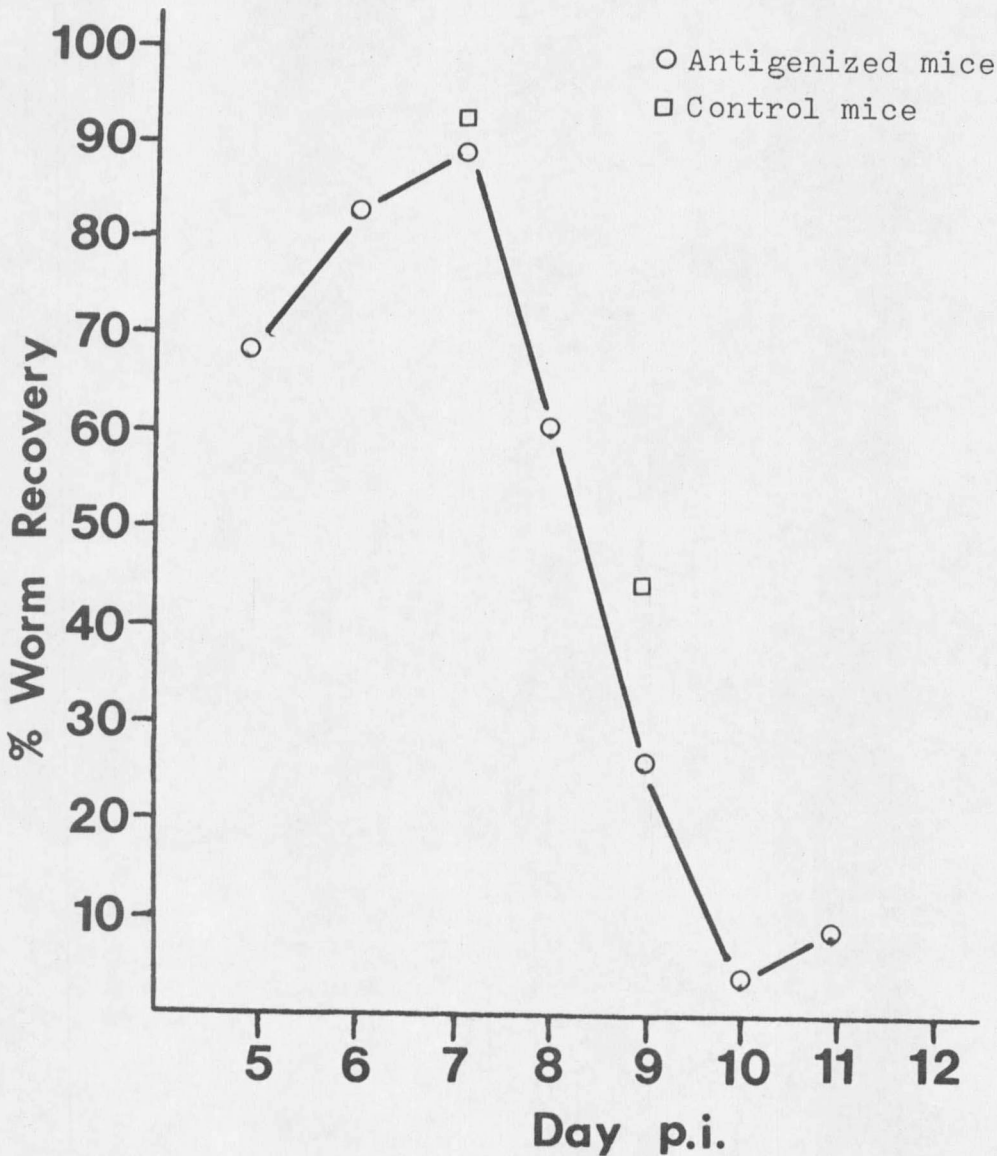


Figure 11. Time of worm expulsion in mice given Ag injections over a 2-week period. Sixty-four mice were given Ag i.p. or s.c., 5 times over a 2-week period. Sixteen mice were given PBS. All mice were given 3c and on days 5-12 p.i., 8 antigenized mice/day were bled, killed, and examined for worms. On days 7 and 9 p.i., 8 PBS-treated mice/day were killed and examined for worms.

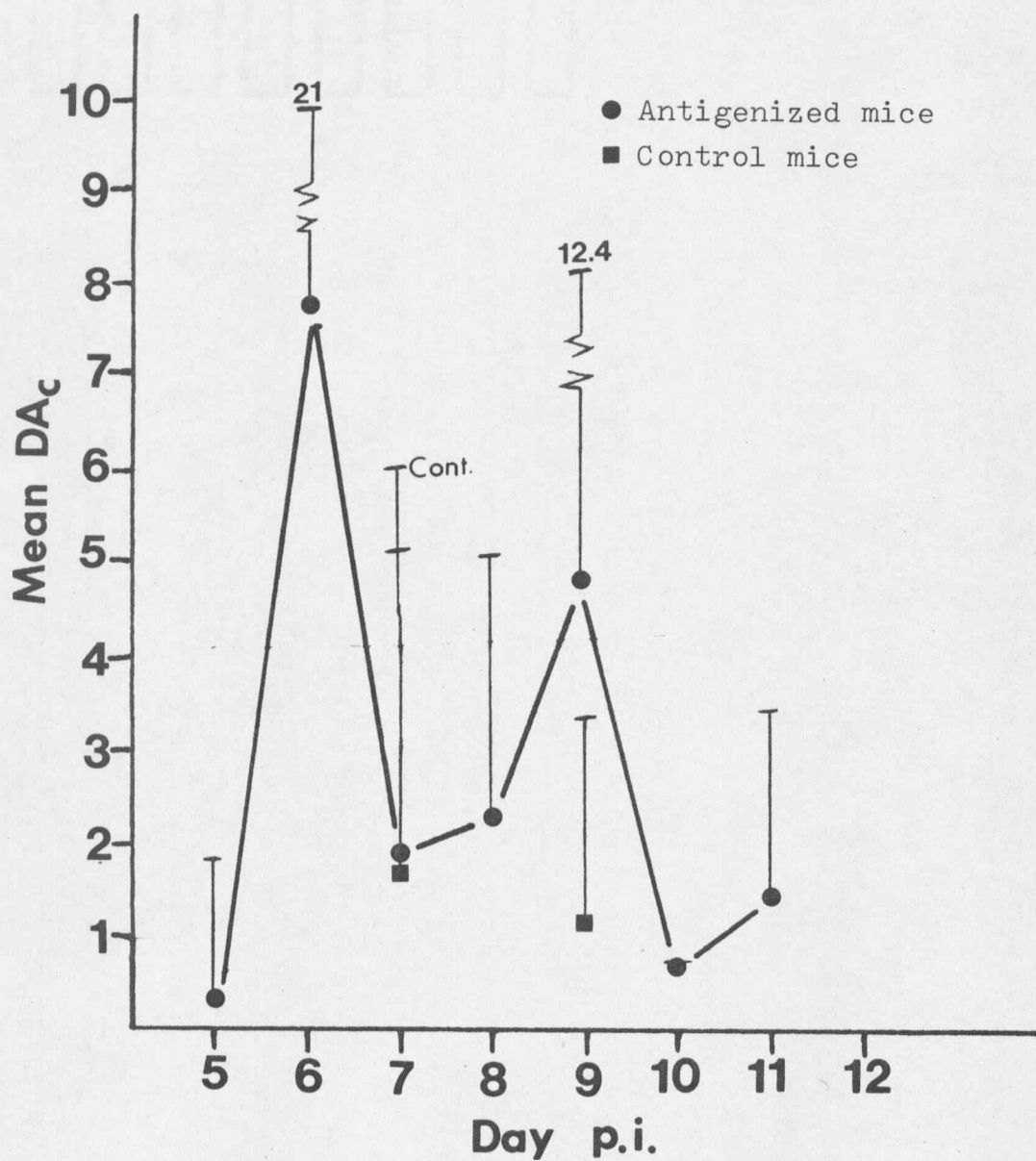


Figure 12. Mean DA_c of *H. diminuta* from antigenized and control mice. See legend for Figure 11 for experimental details.

Mice were given injections of PBS or worm Ag (2 mg/ml in PBS) according to the following schedule:

Amount	Route	Day
0.20 ml	s.c.	0
0.10 ml	s.c.	7
0.15 ml	i.p.	14

A total of 0.90 mg worm Ag was given to each mouse receiving worm Ag. Mice were each infected with 3c on day 21. One-half of each group were killed on day 7 p.i. and the rest on day 14 p.i. One worm Ag-injected nude and 1 worm Ag-injected NLM were bled for serum immediately before infection. Serum was recovered from the killed mice on days 7 and 14 p.i. to test for antibody. On day 7 low per cent worm recovery was found in both groups of nude mice but worms from NLM mice were recovered at normal levels (Table 7). The low recovery from nude mice was probably due to shorter worms (and hence nondetectable by the recovery techniques used) in nude mice (See Table 2) rather than some expulsion mechanism or a lack of cysticercoid infectivity. Worms from both groups of nude mice were about the same length (1.2 and 1.0 cm) and worms from PBS-injected NLM were almost 1 cm longer than worms from worm Ag-injected NLM (2.3

Table 7. Comparison of nudes and NLM given Ag or PBS and infected with 3 H. diminuta cysticercoids.^a

Group	Day p.i.	% Worm Recovery	Mean DA _c	Mean Worm Length (cm)	Titer
NLM-PBS	7	77.8	5.2 ^b	2.3	0
	14	0	---	---	0
NLM-Ag	7	88.9	1.5	1.2	256
	14	0	---	---	64
Nude-PBS	7	55.6	0	1.0	0
	14	100.0	0.2	13.8	0
Nude-Ag	7	66.7	2.5	1.2	0
	14	88.9	0.4	9.4	0

^aSix nudes and 6 NLM were injected with worm Ag and 6 other nudes plus 6 other NLM were given PBS according to the same 2 week schedule. All mice were given a 3c infection. Three mice/group were bled, killed, and examined for worms on days 7 and 14 p.i.

^bNo significant difference between groups was found on day 7 p.i. for mean DA_c.

and 1.2 cm, respectively). Injection with worm Ag appeared to stunt worm growth in NLM in this experiment. No significant difference was found between the mean DA_c in any group on day 7 p.i. according to the Student's t test.

By day 14 p.i. both NLM groups had expelled their worms but both nude groups had a high per cent worm recovery. The level of DA was low in both nude groups, though worms from PBS-injected nudes were somewhat longer than those from worm Ag-injected nudes. No antibody was found in the serum of any nude mouse. Worm Ag-injected NLM had a mean serum titer of 256 on day 7 p.i. and 64 on day 14. PBS-injected NLM had no measurable levels of circulating antibody. Worm Ag injection did not appear to affect DA formation in worms from nudes or NLM even though antibody is formed in NLM in response to worm Ag injections. Also, antigenation did not affect worm expulsion.

Passive Transfer of Serum from Mice Previously
Injected with Worm Ag or Infected with *H. diminuta*

To test the effect of serum from infected and/or worm Ag injected mice on worm expulsion and DA formation, five groups of mice (5-6 mice/group) 9 weeks old were

given 0.5 ml of the following serum i.p. on day -1:

- | | |
|---------|---|
| Group 1 | Normal mouse serum-NMS |
| 2 | Serum from mice with a secondary infection-2 ^o |
| 3 | Serum from worm Ag-injected mice (titer of 16)-IV |
| 4 | Serum from worm Ag-injected then infected mice (titer of 32)-1 st Ag K |
| 5 | Serum from worm Ag-injected mice (titer of 64)-Ag 1 |

All serums were filtered to remove any cells. On day 0, mice were each given 3c. On day +1, all mice were again given 0.5 ml of the appropriate serum i.p. On day 7 p.i., all mice were killed and examined for worms. Two mice from each group were bled to determine residual antibody titer, if any. Worm recovery from NMS- and 2^o-treated mice was normal (Table 8) and the per cent worm recovery for worms from Ag 1-treated mice was low. The low per cent worm recovery for mice given IV or 1st Ag K serum indicates that expulsion was occurring by day 7 p.i. or that many of the worms did not grow. Worms from mice given serum with detectable antibody were less than 1 cm long whereas worms from NMS- or 2^o-treated mice were 1.6 and 1.3 cm respectively. Mean DA_c was at normal levels for NLM- and 2^o-treated worms, but was dramatically re-

Table 8. The effect of passively transferred serum on H. diminuta in mice.^a

Group	Serum	Passive Antibody Titer	% Worm Recovery	Mean DA _c	Residual Antibody Titer	Mean Worm Length (cm)
1	NMS ^b	0	83.3	4.3 ^c	0	1.6
2	2 ^o	0	86.7	3.1	0	1.3
3	IV _T	16	60.0	0.3	2	0.9
4	1 st Ag K	32	53.3	0.8	4	0.7
5	Ag 1	64	73.3	0.2	0	0.7

^a Five groups of mice (5-6 mice/group) 9 weeks old were given 0.5 ml serum i.p. on day -1. On day 0, all mice were given a 3c infection. On day 1 p.i., all mice were again given 0.5 ml serum i.p. On day 7 p.i., all mice were killed and examined for worms. Two mice from each group were bled and the serum tested for residual antibody.

^b Abbreviations used in this experiment are as follows:

NMS--Normal mouse serum.

2^o--Serum from mice with a secondary infection.

IV_T--Serum from antigenized mice.

1st Ag K--Serum from antigenized, then infected mice.

Ag 1--Serum from antigenized mice.

^c The mean DA_c for groups 1 and 2 was statistically equivalent as was the mean DA_c of groups 3, 4, and 5. However, the mean DA_c for groups 1 and 2 was statistically different from the mean DA_c for groups 3, 4, and 5 with the exception that the mean DA_c for group 2 was equivalent to the mean DA_c for group 4.

duced in worms from mice given IV, 1st Ag K, or Ag 1 serum. The mean DA_c for worms from NMS- and 2^o-treated mice was statistically equivalent as was the mean DA_c for worms from IV-, 1st Ag K-, and Ag 1-treated mice. The mean DA_c for groups 3, 4, and 5 was different from the mean DA_c for groups 1 and 2 (Table 8), with the exception that the mean DA_c for group 2 was equivalent to the mean DA_c for group 4. Residual serum antibody to worm Ag in serum from passively immunized mice was 0 for Ag 1-treated mice and 4 and 2 for 1st Ag K- and IV-treated mice, respectively. The data would seem to indicate that serum from mice injected with worm Ag when passively transferred into naive mice causes expulsion to occur earlier. However, as seen in previous experiments, antigenized mice with measurable circulating antibody to worm Ag did not expel their worms earlier than normal mice (Figure 11). It is unknown why serum from worm Ag-injected mice, when passively transferred to naive hosts, does cause earlier expulsion but does not cause earlier expulsion in the original worm Ag-injected hosts. Also the mean DA_c was much lower in worms from all groups of mice receiving passively transferred serum from worm Ag-injected mice. This seems to indicate that there are

fewer and smaller DA formed in the face of an immune response. Therefore, DA actually decrease in the presence of an immune response.

Injection of Rats with Worm Ag

If mice respond to worm Ag injection with antibody formation and this serum is protective when passively transferred, then what effect does worm Ag injection have on rats? To test this 8 rats and 16 mice were given worm Ag injections and infected with 3c according to the same schedule used for the nude mouse worm Ag injection experiment (page 54). Four other rats were given injections with PBS. On days 9 and 11 p.i., one-half of the mice and one-half of the worm Ag-injected rats were killed. PBS-injected rats were all killed on day 11 p.i. Most worms were gone from the mice by day 9 and mice were only bled. There was no difference in mean DA_c in worms from worm Ag-injected and PBS-injected rats or between days 9 and 11 in worm Ag-injected rats (Table 9). Worms from worm Ag-injected rats are somewhat longer than those from PBS-injected rats. Antibody titers of 128 and 256 were found in serum from worm Ag-injected mice on days 9 and 11 p.i. respectively. However, no antibody was

Table 9. Expulsion and mean DA_c of worms from antigenized rats.¹

Group	Number of Animals	Day p.i.	% Worm Recovery	Mean DA _c	Mean Worm Length (cm)	Antibody Titer
Rats-Ag	4	9	100	1.1 ²	10.1	0
	3	11	100	0.5	25.4	0
Rats-PBS	4	11	100	0.37	18.6	0
Mice-Ag	8	9	0	---	---	128
	8	11	0	---	---	256

¹Eight rats and 16 mice were given worm Ag injections over a 2-week period. Four other rats were given PBS. All animals received 3 *H. diminuta* cysticercoids. On day 9 p.i., one-half of the antigenized rats and mice were bled, killed, and examined for worms. The remaining animals were killed on day 11 p.i.

²No significant difference was found between groups for the mean DA_c on any day.

detected in serum from any of the worm Ag-injected rats or PBS-injected rats. Rats evidently do not recognize worm Ag as foreign or, alternatively, low dose tolerance was induced by the injection schedule. A lack of responsiveness of rats to worm Ag probably is related to the inability of rats to expel their worm burdens.

The Effect of Feeding Worm Ag on Mean DA and
Expulsion of *H. diminuta* from Mice

Feeding proteins orally has been shown to induce tolerance (17, 78). Will prefeeding mice worm Ag delay worm expulsion or affect DA formation? To test this, 12 mice, 8 weeks old, were given worm Ag (2 mg/ml worm Ag in PBS) orally by stomach tube according to the following schedule:

Amount	Day
0.20 ml	0
0.20 ml	4
0.10 ml	7
0.10 ml	11
0.15 ml	14

Ten mice were given the same amount of PBS. Mice were each given a 3c infection one week after the last day of feeding worm Ag. A total of 1.5 mg worm Ag was administered over a two week period. One-half of each

group was bled and killed on days 7 and 11 p.i. On day 7 p.i., worm recovery in both worm Ag-fed and control groups was similar and by day 11 worms were gone from both groups (Table 10). The mean DA_c on day 7 p.i. was significantly different between mice given worm Ag orally and those not. Serum from mice given worm Ag orally also had measurable antibody titers to worm Ag.

To see if a different dosage would affect expulsion or DA formation, a second experiment with feeding worm Ag was done. A single large dose of 20 mg worm Ag was given orally to 8 mice and 8 other mice received PBS. Fourteen days later all mice were given 3c and all mice were killed on day 9 p.i. Per cent worm recovery for worm Ag-fed mice and normal controls was almost equal (Table 11). According to the Student's t test there was no significant difference between the mean DA_c of worms from worm Ag-fed and control mice. Worm lengths were also equivalent between groups. No serum was collected to test for antibody. In summary, feeding worm Ag can stimulate antibody formation but does not affect the time when worms are expelled. The reason why the mean DA_c is different between PBS- and worm Ag-fed mice in one experiment but not the other is unknown.

Table 10. The effect of oral antigenation on the mean DA_c and expulsion of H. diminuta from mice.^{a c}

Mice	Day p.i.	% Worm Recovery	Mean DA_c	Mean Worm Length (cm)	Antibody Titer
Oral	7	77.8	5.5 ^c	1.4	--- ^b
	11	0	---	---	128
PBS	7	85.7	1.3	1.7	0
	11	0	---	---	0

^a Twelve mice 8 weeks old were fed worm Ag and 10 controls were given PBS over the same 2 weeks. All mice were given 3c and on days 7 and 11 p.i., one-half of each group was bled, killed, and examined for worms.

^b Nonspecific agglutination in control wells.

^c $p < 0.05$ when comparing mean DA_c on day 7 p.i. between worms from orally antigenized and control mice.

Table 11. The effect of a single, oral antigenation of mice with 20 mg worm Ag on the course of DA formation and worm expulsion.^a

Group	Day p.i.	% Worm Recovery	Mean DA _c	Mean Worm Length (cm)
PBS	9	33.0	0.6	3.8
Oral	9	37.5	6.3 ^b	3.1

^aSixteen Balb/c mice were divided equally into 2 groups: one was given 20 mg Ag orally via stomach tube and the other was given PBS. After 14 days, both groups were given a 3c infection and all mice were killed on day 9 p.i.

^bNo significant difference between groups was found.

Incubation of Worms In Vitro in
Glucose-Salts or Serum

To see how DA on worms responded once the worm was removed from its host, H. diminuta (harvested on day 7 p.i.) were placed in 5 ml of Hank's Balanced Salts Solution (HBSS) at room temperature for up to 11 h and worms were observed for changes in DA about every 30 min. Worm 1 had no DA originally and was cut in half; a DA formed on the half containing the scolex at the cut but the rear section did not form a DA at the cut. The DA on the half with the scolex remained throughout the entire 11 h. Worm 2 began with no DA but was gouged and DA formed on both sides of the gouge. These DA remained throughout the 11 h. One other DA appeared elsewhere on the worm 9 h later but was gone within an hour. Worm 3 was missing both the scolex and the most rear strobila. DA appeared in great numbers at 3 h and disappeared within an hour to be replaced by a second, different set of DA 2 h later. No DA were found at 11 h. Worm 4 had DA before incubation but these were gone within 20 min. of the worm being placed in HBSS; DA increased from 9 h on. Worm 6 began with many DA but all DA were gone after 90 min. in HBSS. DA appear to be very transient and

factors other than gross mechanical damage can mediate their formation.

DA may be the result of dehydration (8). In an attempt to mimic possible DA formation in vivo, the concentration of salts in HBSS was varied in vitro. Seven-day old H. diminuta were placed in 5 ml of 0.2X, 1X (normal), and 5X HBSS for 1 h at room temperature. Two worms/HBSS concentration were tested. DA disappeared from worms placed in 1X (isotonic) HBSS within 10 min. of incubation and several DA appeared and disappeared before the hour was up. Worms remained active throughout the hour. In hypotonic HBSS (0.2X), worms relaxed and became elongated. Worms became inactive by 20 min. After 30 min., the scolex was retracted and the strobila developed several "pinched" areas. After 45 min., some extrusion of internal worm material was noted and DA formed around the "pinched" areas. Worms held in hypertonic (5X) HBSS became inactive by 5 minutes and most of the strobila turned dark. Integument disruption was noted by the presence of debris around the worm. Hypotonicity or hypertonicity appear to be able to mimic DA in vitro.

To test the role of serum antibody and/or complement

in DA formation, H. diminuta were washed in HBSS and placed into a spot plate well containing 0.5 ml serum + 0.5 ml HBSS at room temperature. Each serum was done in duplicate and the following sera were used:

Serum	Titer
NMS	0
Immune Serum-Thrice infected mice	0
Serum from worm Ag-injected mice	10,240
Serum from worm Ag-injected mice	16
HBSS	--

Observations were made every 10 min. during the first hour. After 1 h, 0.3 ml of 1:10 guinea pig complement was added to alternate wells. Most worms that began with DA lost them 15 min. after being placed in serum regardless of the titer. Some worms that began with no or few DA showed more DA within 15 min. but by 30 min. all DA were gone. A third group of worms started out with DA, repaired them by 15 min. but shortly thereafter developed a different set of DA which persisted for 90 minutes or the entire 3 h. Only 1 worm died. No extrusion of worm contents or precipitate was seen.

Complement had no effect on DA nor on worm viability.

In a second experiment worms were incubated in either NMS (not heat inactivated) or in serum from worm Ag-

injected mice (titer $>10^4$, heat inactivated). The worms were examined at +40 min., complement was added, and the plate was incubated at a higher temperature of 37°C for 30 min. Worms were then examined again. At +40 min., worms in NMS were dead but no extrusion of worm contents was evident. Worms in antisera were active. At +70 min., all worms were dead, and copious amounts of internal worm contents were extruded. No DA were seen at +40 or +70 min. on any worm. Complement alone or with antibody will kill the worms but worms do not form DA as a result of the action of complement.

Delayed-Type Hypersensitivity to *H. diminuta*
Infection in Mice

Mice given a 6c infection were challenged with worm Ag in one hind foot pad and with PBS in the other hind foot pad on days 7, 9, 11, and 14 p.i. and swelling was measured with dial-guage calipers at times 0 (before injection), 3, 24, and 48 h. Uninfected controls were challenged in the same manner. The infection level was high (91.7% worm recovery) in 4 infected mice killed on day 7 p.i. In noninfected mice, a transient, mild inflammation was seen at 3 h post-challenge by a slight swelling in the foot receiving worm Ag. The foot returned to

normal by 24 h. This same nonspecific 3 h swelling was also seen in infected mice. No DTH response (swelling at 48 h) was evident on days 7, 11, and 14 p.i. However, on day 9 p.i., a very definite significantly different response was present at 48 h (Table 12). The DTH response was transient and roughly correlated with when expulsion began in previous experiments. The transient nature of the DTH reaction could possibly be due to a lack of certain antigens in the worm Ag preparation.

Morphology of Cellular Infiltrate in a Foot Pad
Undergoing a DTH Response to Worm Ag

Swollen foot pads were removed from infected mice which were challenged with worm Ag on day 9 p.i. Control foot pads were removed from mice which were not infected but were challenged with worm Ag. The foot pads were fixed, embedded, sectioned and stained as stated elsewhere. Control foot pad morphology is seen in Figure 13. A very definite cellular infiltrate (CI) is seen between the epidermis and underlying muscle tissue (Figure 14). On higher power most of the cells in the CI have the indented nucleus that is characteristic of monocytes. Other cell types seen include many small to medium lymphocytes, some eosinophils, and a few polymorphonuclear

Table 12. Delayed-type hypersensitivity response to worm Ag in a 6c infection in mice.^a

Number of Mice	Day p.i.	Infection ^b	Challenge Solution ^c	Time			
				0	3	24	48
6	7	+	Ag	1.9 ^d	2.3	2.2	2.3
			PBS	2.0	2.1	2.1	2.0
4	7	-	Ag	1.8	2.4	1.9	1.9
			PBS	1.9	2.2	1.9	1.9
6	9	+	Ag	1.8	2.1	2.1	3.1 ^e
			PBS	1.8	2.0	2.0	1.9
4	9	-	Ag	1.8	2.1	1.9	2.0
			PBS	1.8	2.0	1.9	2.0
5	11	+	Ag	1.8	2.3	2.0	2.1
			PBS	1.9	2.1	2.0	1.9
6	14	+	Ag	2.0	2.4	2.2	2.2
			PBS	2.0	2.0	1.9	2.0

^aTwenty-seven 6c infected mice and 8 control uninfected mice were tested for DTH responses by foot pad swelling on day 7, 9, 11, and 14 p.i. with dial-guage calipers after challenge with Ag or PBS.

^bMice infected with 6c-- +
Uninfected mice ----- -

^cOne hind foot was injected with 0.03 ml Ag and the other with 0.03 ml PBS.

^dMean foot pad thickness in mm.

^e $p < 0.05$ when compared with the PBS control and with uninfected mice.

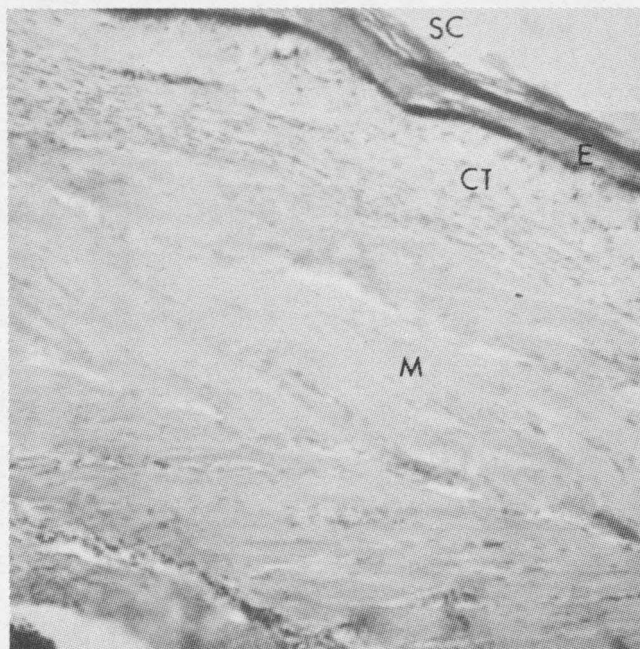


Figure 13. Control mouse foot pad morphology. The stratum corneum (SC) is clearly seen overlaying the epidermal layer (E). Beneath the epidermis is a layer of connective tissue (CT) containing fibroblasts followed by muscle (M) bundles.

Magnification = 100X.

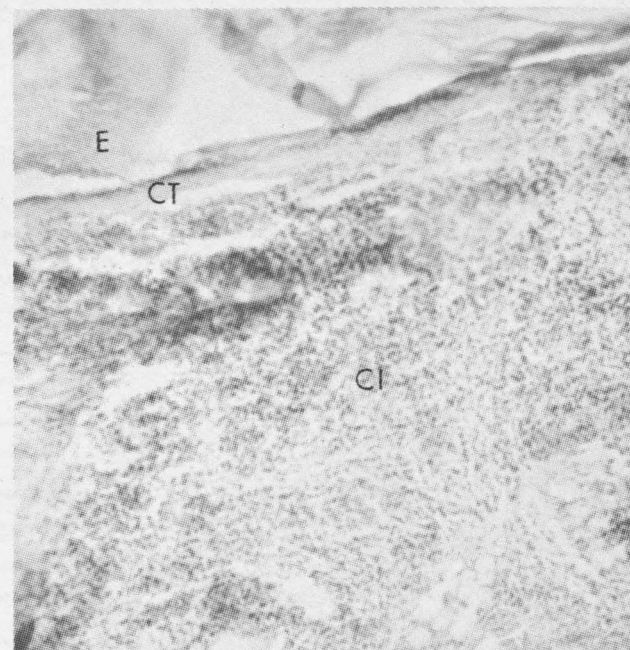


Figure 14. Cellular infiltrate (CI) in worm Ag-injected foot pad of *H. diminuta*-infected mouse at 48 h post-injection. The swelling is so marked that muscle tissue is not seen and the layer of connective tissue (CT) is compressed.

Magnification = 100X.

neutrophils. The predominance of these cell types and time when swelling occurs (48 h) indicates that the foot pad swelling can be classified as due to delayed-type hypersensitivity.

Morphological Comparison Between Dark Areas and Normal Tissue

DA from fresh, unstained 7-day old worms are seen in Figures 15-17. A very marked difference in the ability of the tissue to transmit light is clearly seen. The DA shown in Figures 16 and 17 are +4 and +8, respectively. Scores of +1 and +2 are seen in Figure 15. No difference between normal tissue and DA could be seen using H & E staining probably due to thick sections. However, epoxy-embedded tissue stained with basic (pH 9) Toluidine Blue O showed distinct differences between normal areas and DA. Normal tissue had more cells per unit area and the cells had more cytoplasm and cytoplasmic extensions than DA (Figure 18). In DA sections a pink-staining fluid filled the interstitial spaces between cells and the cells were smaller and fewer in number. Cells in DA had fewer vacuoles than cells in normal tissue (Figure 19). DA appear to be characterized by an internal accumulation of fluid which disturbs the normally densely

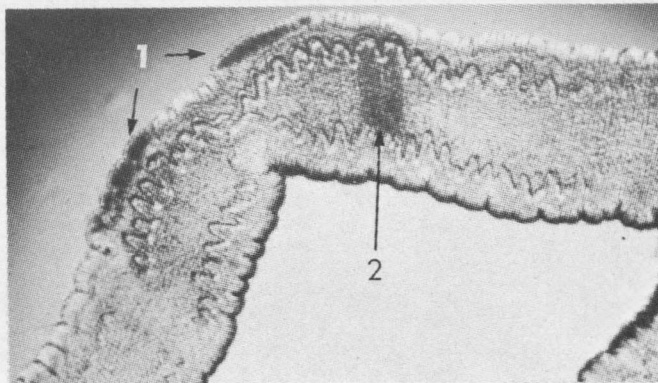


Figure 15. Wet mount of H. diminuta showing +1 and +2 scoring DA. Magnification = 40X.

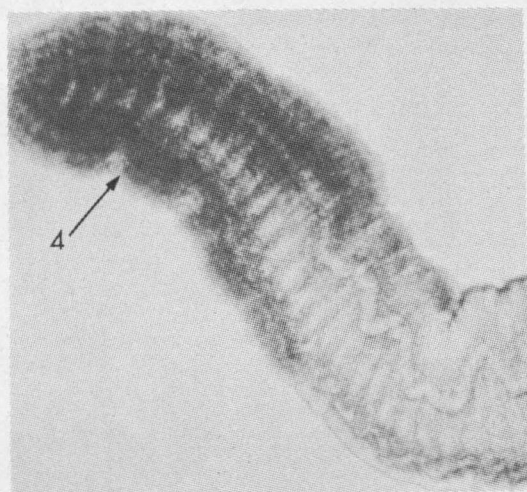


Figure 16. DA of H. diminuta with a score of +4. Magnification = 50X.

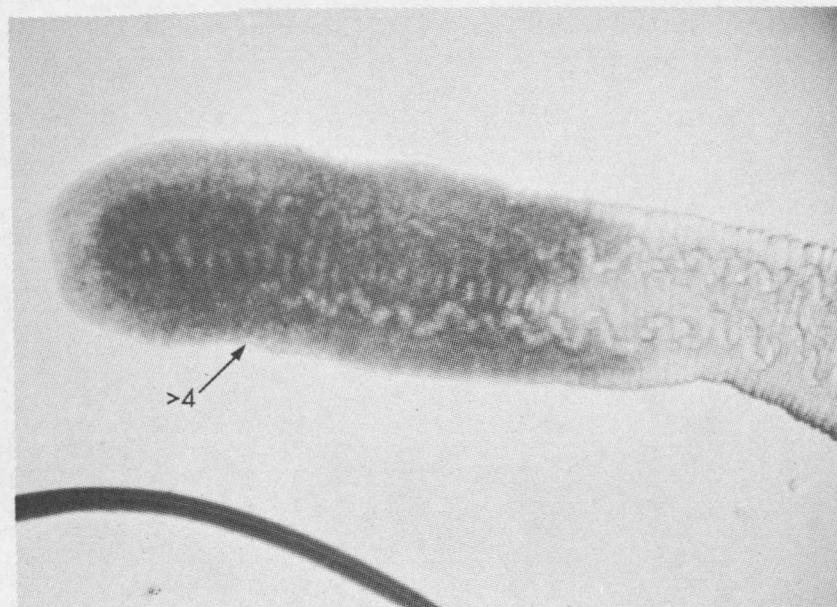


Figure 17. DA with a total score of +8. Magnification = 50X.

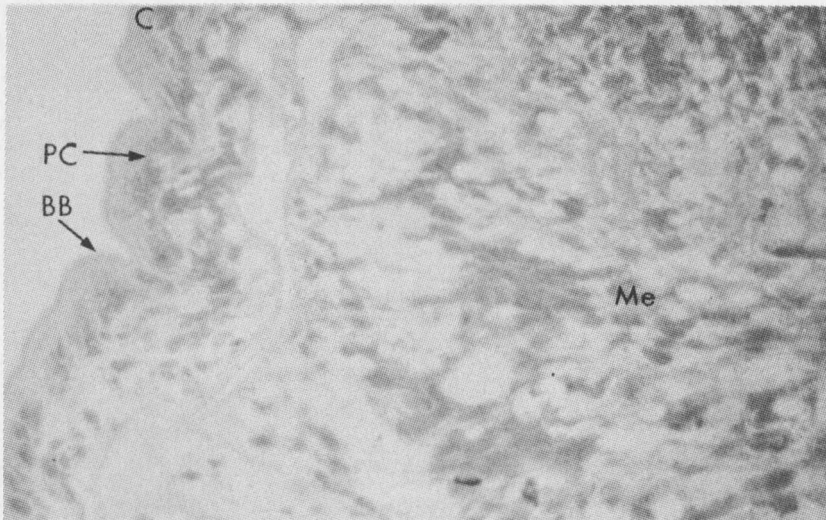


Figure 18. Toluidine Blue stained section of normal H. diminuta tissue. Brush border (BB), cuticle (C), parenchymal cells (PC), and medullary region (Me). Magnification = 256X.



Figure 19. Toluidine Blue stained section of H. diminuta DA. The cells are smaller and less densely packed than in normal tissue. Legend and magnification are the same as above.

packed parenchymal cells. The origin or content of the fluid or why it accumulates is unknown. The fluid is slightly basic as seen by it's pink staining reaction.

Electron Microscopy of DA and Normal Tissue of *H. diminuta* from Mice

Tissue from *H. diminuta* 7 days old was first fixed in glutaraldehyde, dehydrated, embedded in epoxy resin, sectioned and post-section stained with uranyl acetate and Reynolds lead citrate. Normal tissue is shown in Figure 20. The finger-like projections on the surface are microtriches (Mt) (46) and they form the brush border (BB). They are conical and enclosed by a unit membrane (arrow). Below the microtrichal border is a noncellular syncytium called the cuticle (C) composed mostly of dumbbell-shaped discoid secretory bodies (DSB), microtubular complexes (MTC), and mitochondria (M). The microtubular complexes are thought to function in ion or water transport (76) or in sensory reception (69). Separating the cuticle from the parenchymal cells is the fibrous basement layer (F). This layer is composed of tightly packed fibers consisting of a protein similar to collagen(44). Longitudinal and circular muscle bundles (MB) are below this in the parenchyma. The parenchymal

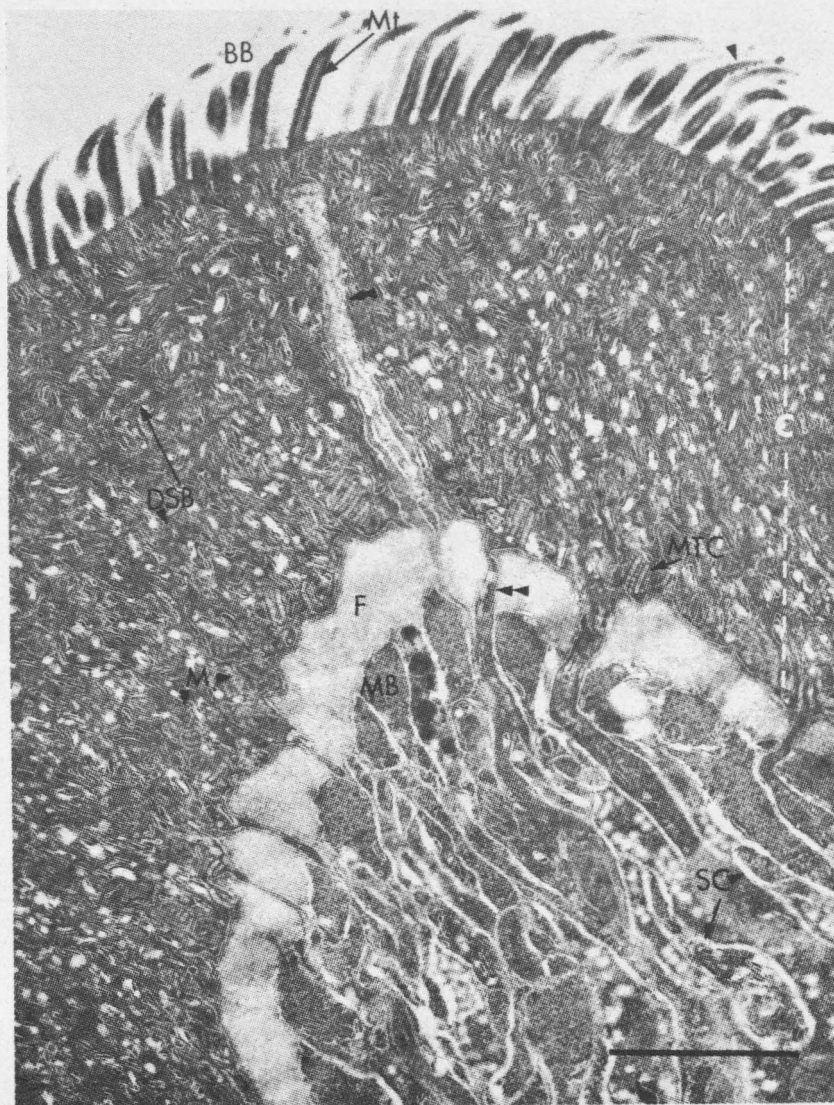


Figure 20. Normal *H. diminuta* tissue. Line bar = 1 μ m. The single lone arrow denotes the unit membrane covering the entire worm. The double arrows indicate cell processes connecting the cuticle with the parenchymal cells. All other abbreviations in this and following micrographs are as follows:

- BB: Brush border
- C: Cuticle
- DSB: Discoid secretory body
- F: Fibrous basement layer
- M: Mitochondria
- MB: Muscle bundle
- Mt: Microtriche
- MTC: Microtubular complex
- SC: Subcuticular canal
- V: Vacuole

cells have many processes which are interspersed with many winding subcuticular canals (SC). The main organelles visible in the parenchymal cells are the mitochondria and nuclei. Endoplasmic reticulum and the Golgi apparatus are generally not found. Oftentimes cellular processes extended from the parenchyma up into the syncytium (double arrows, Figure 20) and vacuoles were quite often seen in muscle bundles and cell processes. Tissue fixed in both glutaraldehyde and osmium tetroxide had fewer vacuoles; the osmium evidently stained the contents of the vacuoles. Vacuoles also occasionally occurred in the cuticle. The cuticle was sometimes pyknotic in normal tissue.(Figure 23).

DA exhibited some structural changes as compared to normal tissue. The most striking change seen was the disarray of the parenchymal cell processes and muscle bundles. The fibrous basement layer was also less loosely packed with individual fibers easily seen (Figure 21). DA from 10-15 worms examined, all exhibited this disorientation of the parenchymal stroma and the loosely packed fibrous layer. The contrast is clearest in Figure 22 where two neighboring strobila show both normal and DA morphology. The microtrichal border is



Figure 21. Dark area tissue in H. diminuta. Note the disarrayed fibrous layer and parenchyma. Some DSB appear to have swollen and expelled their contents. Line bar = 1 μ m.

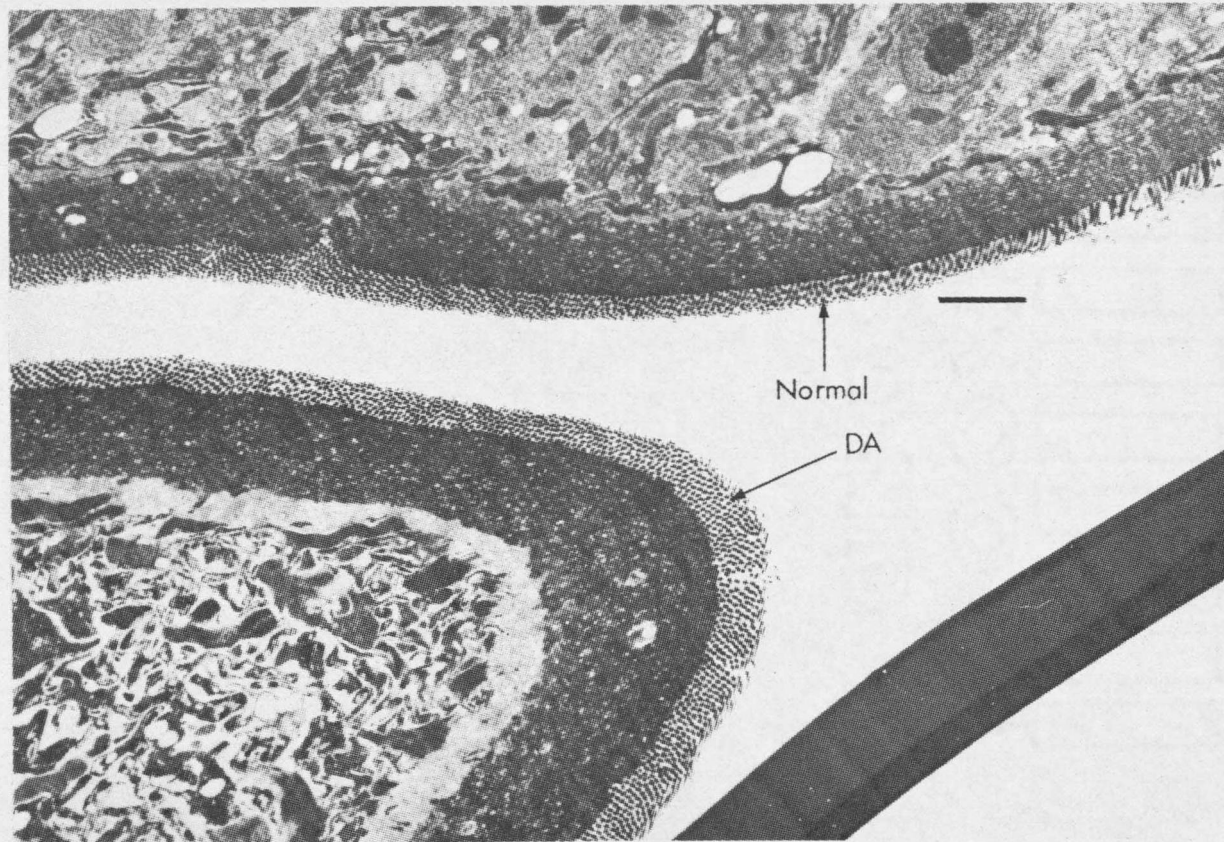


Figure 22. Neighboring strobila from a single worm. One proglottid shows morphology typical of normal tissue and the adjacent proglottid shows a DA being formed. Line bar = 2 μ m.

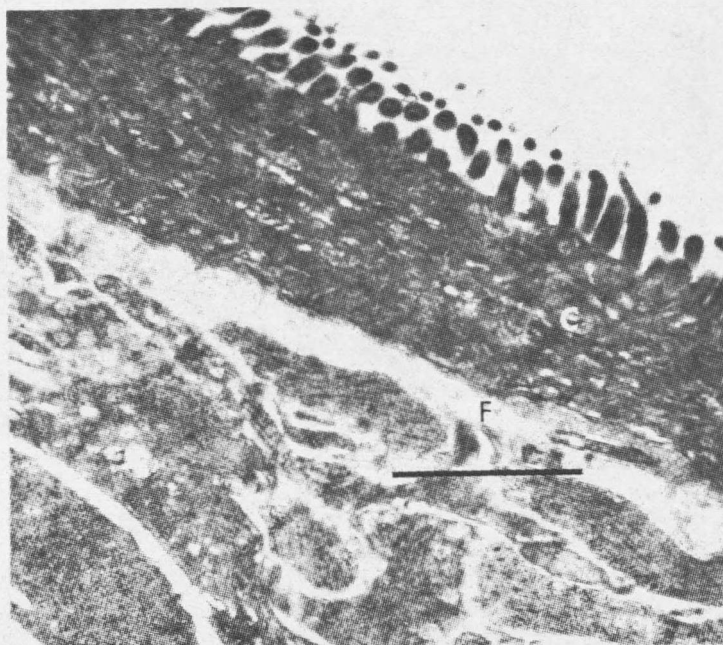


Figure 23. Pyknotic cuticle in normal H. diminuta tissue. Line bar = 1 μ m.

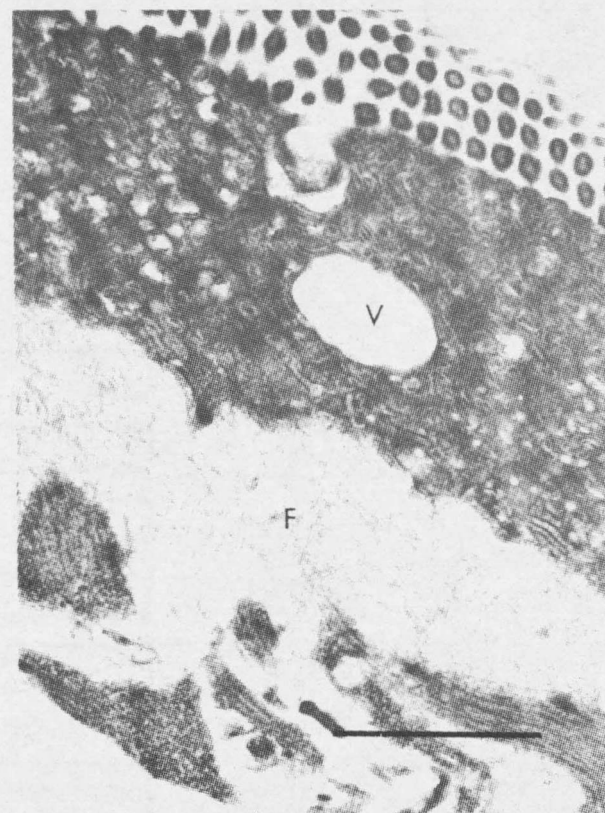


Figure 24. Vacuole in pyknotic cuticle in DA of H. diminuta. Again the DSB are swollen. Line bar = 1 μ m.

intact. More occasions of a pyknotic cuticle were found, though it was still rare (Figure 24). Mitochondria and nuclei were apparently unaffected. The discoid secretory bodies nearer the fibrous layer appeared to be empty and expanded as if they had emptied their contents internally (Figure 21). In DA some vacuoles were found in the cuticle but this also was rare (Figure 24).

DA and normal tissue taken from worms from mice treated with anti- μ , nude mice, or worm Ag-injected mice (nude or normal) exhibited no morphological differences from that described above. This seems to indicate that the presence or absence of an immune response to the worm does not affect the basic tissue morphology. DA and normal tissue morphology did not change with immune status of the mouse host.

Morphology of Worms Incubated in Concentrated or Diluted Hank's Balanced Salts Solution

DA and normal tissue were excised from H. diminuta recovered on day 7 p.i. which had been previously incubated in 0.2X, 1X, or 5X HBSS in an earlier experiment (page 70). DA and normal tissue from worms from 1X HBSS were no different than DA or normal tissue morphology as shown in previous photos. Normal-appearing tissue

taken from worms incubated in hypotonic (0.2X) HBSS appeared vacuolate and the muscle bundles were very enlarged (Figure 25). The basement fibrous layer was not tightly packed, and individual fibers were easily seen. The cuticle appeared intact. DA tissue from these worms was similar except the lack of parenchymal cells and muscle bundles was more evident. The DSB also appear to be swollen and a vacuole appeared in the cuticle (Figure 26).

Normal tissue from worms incubated in hypertonic (5X) HBSS showed a disrupted brush borders (arrows) and disintegrating mitochondria in the cuticle (Figure 27). DA showed similar disruptions but again the parenchymal elements were more sparse. Normal tissue incubated in hypotonic HBSS most closely mimiced the morphology of DA. This points to the idea that DA represent an area of the worm that is having difficulty maintaining a correct fluid-salt balance with it's environment. Other factors, however, are also involved as the normal tissue incubated in hypotonic HBSS did not become opaque to transmitted light.

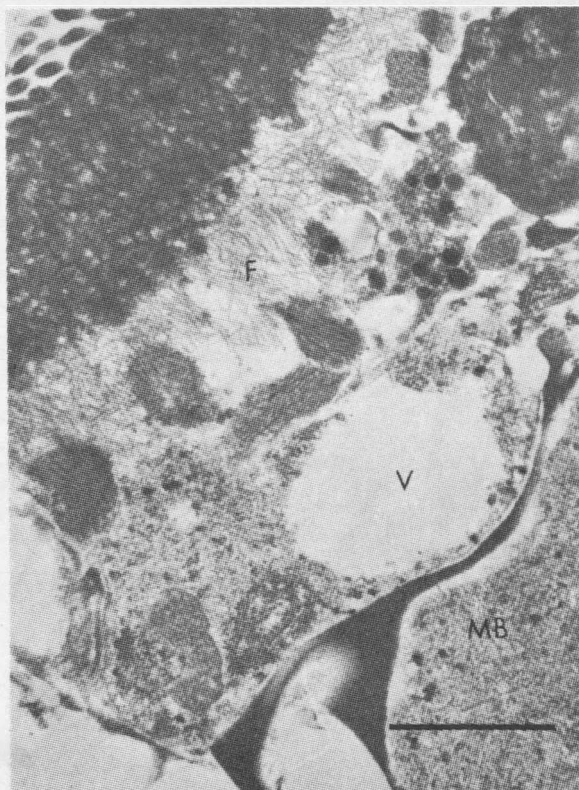


Figure 25. Normal tissue from *H. diminuta* incubated in 0.2X HBSS. Note the large vacuole, loosely packed fibrous layer and the enlarged muscle bundle. Line bar = 1 μ m.

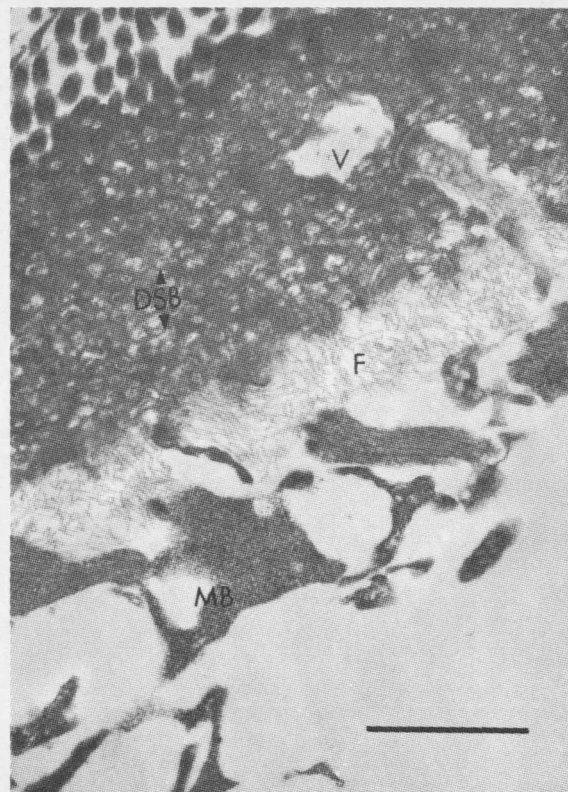


Figure 26. DA from *H. diminuta* incubated in 0.2X HBSS. The parenchymal tissue is particularly sparse and DSB appear swollen. Line bar = 1 μ m.

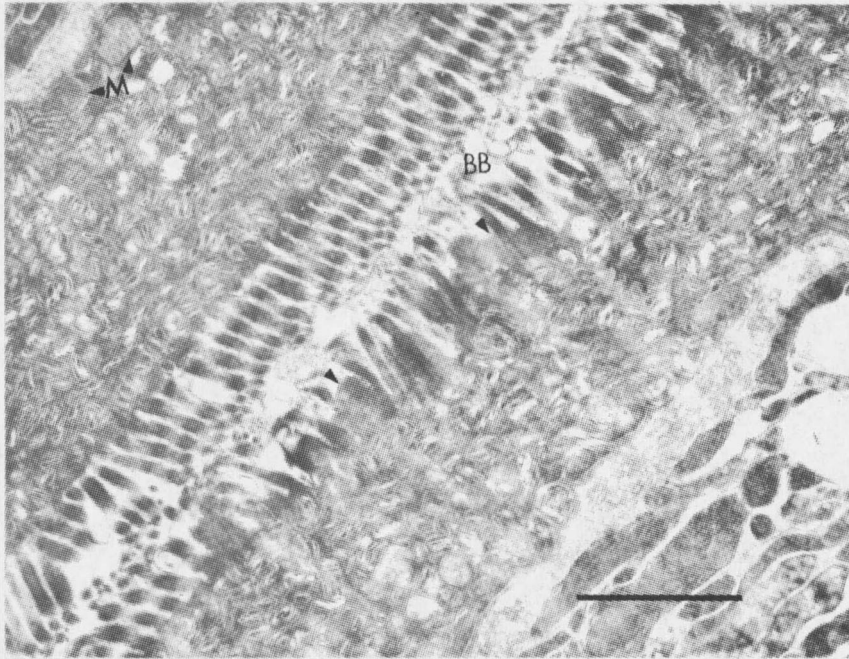


Figure 27. Normal tissue from a single *H. diminuta* incubated in 5X HBSS. Note the disintegrating brush border (arrows) and the loss of structure in cuticular mitochondria. Line = 1 μ m.

Scanning Electron Microscopy of *H. diminuta*

H. diminuta were viewed by light microscopy to note the location of DA. The worms were then fixed whole in glutaraldehyde, dehydrated, and critical point dried. By scanning electron microscopy, Figure 28 shows a normal worm section and Figure 29 shows an area where DA were seen by light microscopy. Note the swollen areas at the arrows; this may be due to an internal accumulation of fluid. This swelling was also seen in another worm in the area of a large DA. High magnification showed little detail of the surface of the worm due to poor resolution. However microtriches have been clearly seen by other workers (77).

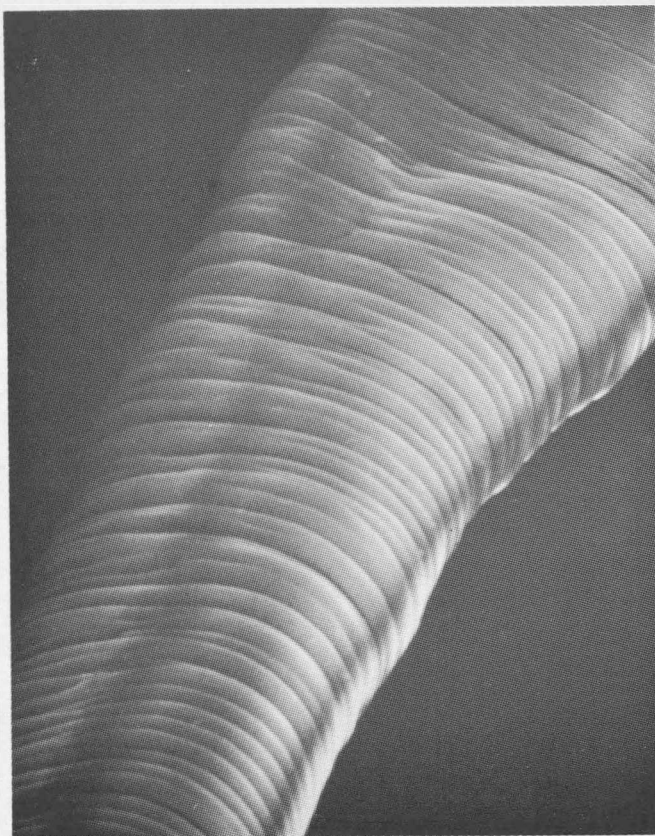


Figure 28. Normal H. diminuta strobila under SEM. Magnification = 525X.

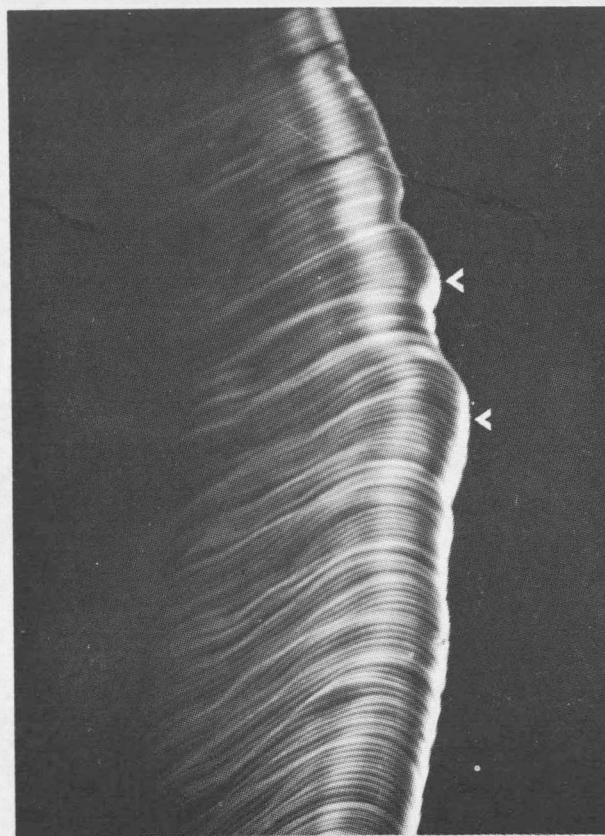
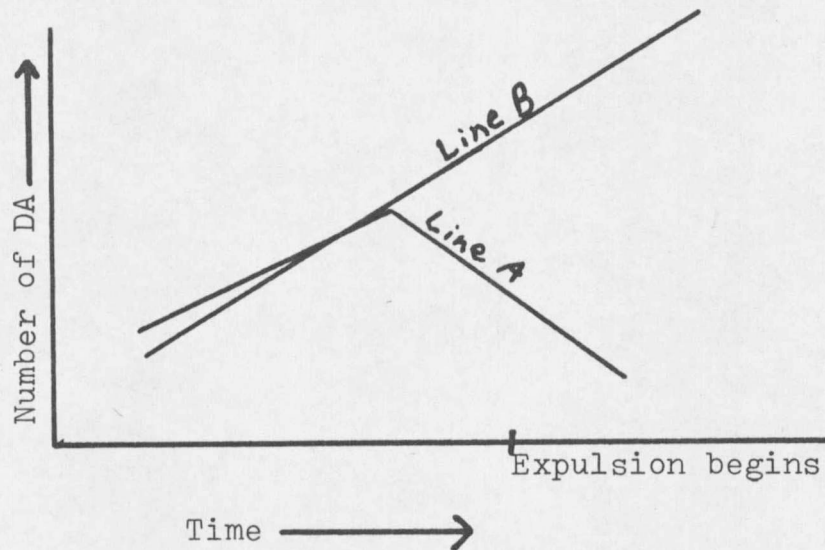


Figure 29. DA of H. diminuta under SEM. Two swollen areas (arrows) in the center represent two +3 DA. Magnification = 175X.

DISCUSSION

The pattern of DA formation seen in the experiments described herein could be generally represented by line A in the graph below:



DA increased in number and size until 1 or 2 days before expulsion began and then decreased, the decline continuing as expulsion began. However, Befus and Threadgold (8) found the pattern of DA formation in their system generally followed line B in the graph above. They noted that DA increased in number up to and beyond the point where the expulsion of worms began. On the basis of their data, they hypothesized that the formation of DA is immunologically mediated, that DA are the result of the host immune response to the worm and represent worm

damage. Line A and line B are obviously different. Does this mean, then, that my data will still support the idea that DA formation is immunologically mediated?

If DA formation is immunologically mediated, then the pattern of DA formation should vary with the immune status of the host. In normal mice, increasing the immunogenic dose by changing the intensity of the worm infection resulted in earlier expulsion, which is immunologically mediated, but the mean DA_c graph was not significantly changed. Also, the few worms recovered from secondary infections had few and small DA and they should have had more DA if DA were the result of an immune response. These data support the idea that DA may not be the result of the host immune response.

When mice and rats were infected from the same cysticeroid pool and killed on the same day p.i. for worm removal, it was found that the mean DA_c differed only on 1 day out of the 4 tested. Rats do not expel their worms but do form IgE in response to an infection (21); mice expel their worms but do not form any circulating IgE or other classes of antibody specific for the worm (4). Also mice respond to worm Ag injection with antibody formation but rats do not (Table 9). It is

quite clear that the host immune response to the worms differ. This suggest that DA may not even be the result of the host immune response but rather could be a phenomenon intrinsic to worm metabolism or maturation.

Worms from an immunologically compromised animal should have few or no DA. The nude (athymic) mouse provided the ideal model to test this. Nude mice lack the ability to reject allografts (60,80) or xenografts (72), and have a drastically reduced antibody response to T-dependent antigens (38,60,80). They cannot mount a delayed-type hypersensitivity response (65) and fail to produce eosinophilia (56) or reagenic antibody (50). Nude mice cannot expel many parasites unless given a thymus-gland graft (31,68,70) and in some cases the parasite burden overwhelms nude mice (11,30,66). Isaak (28) found that nude mice do not expel H. diminuta and the experiments described herein confirmed this (Table 3). However, nude mice given thymus-gland grafts did expel their worms. This indicates that the expulsion mechanism is thymus dependent.

In one experiment, the mean DA_c for worms from nudes was indeed significantly lower than worms from NLM (Table 2). However, in the two subsequent experiments using

nudes, TG-nudes, and NLM no difference was found between the mean DA_c for nudes and TG-nudes on day 7 p.i. (Table 3). In one (but not both) of these experiments, worms from NLM had a significantly higher mean DA_c than nudes or TG-nudes. This seems to indicate that some other factor besides the thymus gland is causing the worms from nudes and TG-nudes to have fewer DA, if, in fact, they do (Table 3, Experiment 2). Nude mice have other anomalous conditions, besides being athymic, which include an altered adrenal cortex (the absence of the zona reticularis and zona glomerulosa which secrete mineraloactive corticosteroids)(45), decreased fertility (60), and they tend to survive poorly in normal surrounding, often wasting after 3 months of age. Perhaps one of these other deficiencies has a more direct effect on DA. The restoration of thymus competency to nudes clearly resulted in worm expulsion; however, no firm conclusion can be made about the thymus dependency of DA formation.

Worms from nude mice were generally smaller than those recovered from NLM on the same day p.i. (Tables 2 and 3). However, the mean worm lengths differed between experiments for worms from TG-nudes. In one experiment the mean worm length of worms from TG-nudes was similar

to that of worms from NLM and in another case was similar to that of worms from nudes (Table 3). No conclusion can be drawn about the effect of thymus competency on worm length until more experiments using H. diminuta infections in nude and TG-nude mice are done. It is of interest to note that nude mice are more resistant to the intra-erythrocytic protozoan Babesia rhodaini than NLM. Mitchell (51) postulates that some T-cell dependent phenomenon may accelerate the development of parasitemia. Could it be that some thymus dependent phenomenon stimulates worm growth?

Nude mice are not always more susceptible to parasite infections. Nudes and NLM do not differ in their susceptibility to an oral dose of *Ascaris suum* eggs (53). This is also true of nudes and NLM infected with Schistosoma mansoni (61), Fasciola hepatica (Mitchell, et al., unpublished data), and Plasmodium berghei (52). It is quite obvious that the immune responses to the parasite by the host differ in type and intensity depending on the parasite studied.

Another immunodeficient mouse model used is mice treated with heterologous anti- μ . The expulsion kinetics of several parasites was not affected by anti- μ treatment

(27,29,32). However, antibody is important in the immunological control of other parasites. Mice injected with heterologous anti- μ die when infected with Plasmodium yoelii whereas normal controls survive the infection (79).

H. diminuta from mice treated with heterologous anti- μ from birth did not have a mean DA_c that differed significantly from control groups treated with normal serum or PBS on day 7 p.i. However, in the second experiment, a higher mean DA_c for worms from anti- μ -treated mice as compared to PBS-treated mice was found. The mean DA_c between worms from NGS- and anti- μ -treated mice or PBS- and NGS-treated mice was equivalent. If antibody to worms antigens directly or indirectly affect DA formation, it would be expected that worms from anti- μ -treated mice would have a lower instead of a higher mean DA_c than control groups. This is not found experimentally. Therefore, it seems likely that the higher mean DA_c in the second experiment is anomalous. A lack of antibody-producing potential in mice does not affect the mean DA_c . DA are probably not caused by specific antibody interacting with the living worm.

Immunization with larval or whole worm homogenates does confer specific protection against challenge in

some systems. This has been found in infections with Mesocestoides corti (40), Ascaris suum (74), and Taenia taeniaeformis (42).

H. diminuta from immunopotentiated (antigenized) mice were not expelled any faster than controls and showed a DA formation response similar to normal controls. Mice were known to be immunopotentiated because circulating antibody to worm Ag was found and when this serum was passively transferred, an increased expulsion rate was found. The mice were responding immunologically to worm Ag but this response did not affect DA formation kinetics. Again no relationship between mean DA_c and the immune status of the host could be detected.

Rats vaccinated orally with excretory-secretory antigen from adult N. brasiliensis had a high level of immunity upon challenge (63). With H. diminuta, worm Ag fed orally to mice gave no protection (Tables 10 & 11). However, circulating antibody to worm Ag was found in serum from mice fed worm Ag orally. In one case worms from orally fed mice had a higher mean DA_c than those from controls and in one case they did not. No firm conclusion can be drawn about the effect of prefeeding worm Ag has on DA formation.

Passively transferred antibody can enhance parasite expulsion. Antibody passively transferred will reduce the reproductive rate of Mesocostoides corti (39), interfere with the development and reorganization of H. nana oncospheres (18) and, in some cases, cause earlier expulsion of N. brasiliensis (58).

Serum passively transferred from mice antigenized with worm Ag caused worm expulsion to occur earlier and significantly reduced the mean DA_c as compared to controls (Table 8). So there is a correlation between expulsion and the mean DA_c . DA formation is reduced in the presence of an immune interaction between serum and the worm. However, the expulsion of H. diminuta induced by passively transferred serum could be by a different mechanism than the expulsion seen in a normal infection. No antibody was found in serum from naturally infected mice and expulsion probably occurs by a mechanism other than antibody.

Complement is cestocidal. When Echinococcus granulosus protoscoleces or adult worms are placed in any serum containing complement, they immediately lyse and die (22). The adult worms contract and darken. Then they become swollen, distorted, and the tegument

ruptures. Also E. multilocularis protoscoleces incubated for 30 minutes in fresh rat serum showed tegumental disruption and damage to underlying tissue. This complement-mediated damage is probably activated by antibody on the surface of the protoscoleces (35). The incubation of T. taeniaeformis larvae in rat antisera in vitro for 6 h led to a marked uptake of ^{14}C -glucose when compared to controls. This was found to be complement dependent (55). Complement and/or immune serum damage other parasites also (14,33).

Complement does not appear to be part of the mechanism causing DA. H. diminuta incubated in complement in vitro do not darken before or after death. In electron micrographs of DA, the brush border and external plasma membrane are intact and no holes are visible. This implies that DA formation is probably not due to complement disrupting the integrity of the external plasma membrane.

Collectively, the data discussed above suggest that DA are not immunologically mediated. Then, how can the decline in DA formation (Line A, p.91) be explained? I would suggest that DA actually disappear in the face of an immune response. The decline in mean DA_c before

expulsion begins could mean that the host immune response is affecting the parasite and this is manifested by a loss of DA. Worms found between days 9 and 11 p.i. in normal infections represent worms that are being acted on immunologically (and hence have a reduction in DA size and number). Then the worms are expelled.

DA evidently are not a measure of the host immune response. However, other parameters were measured which indicate that mice do immunologically respond to H. diminuta. A transient DTH response was seen at 48 h post-challenge in H. diminuta-infected mice challenged with worm Ag in a rear foot pad on day 9 p.i. Challenging on earlier or later days p.i. yielded no DTH responses. The lack of a DTH response later than day 9 p.i. could be due to a lack of certain antigens in the challenge solution or it could mean that the responding cells are only primed in the presence of an active infection. The absence of certain antigens in the challenge solution is probably the most likely explanation of why no DTH responses are seen after day 9 p.i.

Cell-mediated responses are seen in infections involving other cestodes and nematodes. A cell-mediated and immediate-type response to T. taeniaeformis in rats

(41,42) and to T. spiralis (43) has been shown. Also the spleen cells of infected animals exposed to proto-scolex or hydatid fluid of E. granulosus showed an increased thymidine uptake (3).

DA are probably the result of an edema caused by an accumulation of worm fluids internally. The fluid probably does not come from the external environment into the worm because worms incubated in hypotonic HBSS or in complement do not turn dark. DA do not appear to be due to an increase in cuticular permeability. The probably scenario of how DA are formed is as follows: The excess fluid of unknown origin swells the discoid secretory bodies (DSB) of the cuticle (Figure 22). The DSB eventually burst, releasing their contents internally. The fluid level inside the worm rises and eventually the parenchymal cell processes and fibers in the fibrous layer are forced apart by the pressure of the fluid. DSB are thought to be packages of worm secretory or excretory products passing between the parenchymal cells and the external worm environment and/or enzyme products and solutes being brought into the worm (13). So the local fluid accumulation might be caused by a breakdown in the transport system of materials via the DSB.

Some possible areas of investigation for future experiments could include:

1) More experiments to clarify the nature of the DTH reaction to worm Ag. Is it under thymic control? Is it transferrable by cells? Is it related to worm expulsion? Why does it appear to be so transient?

2) Why do worm Ag injections not confer resistance on injected mice yet serum from antigenized mice which is passively transferred does? Will nude mice given passively transferred serum from antigenized mice expel their worms? Are cellular controls involved somehow?

3) Do DA have higher levels of Ig's on their surfaces as opposed to normal tissue? What are the classes and when are they seen on the integument during the course of an infection?

4) Does DA formation have a physiological basis? Will "poisoning" the ion transport system cause DA to form?

5) Why don't rats respond to worm Ag injection with antibody formation? Is this related to the rats' inability to expel their worm burdens? Is

H. diminuta in the rat truly an endocommensal (26)?

6) Are immunoglobulins present on the integument of worms from anti- μ -treated mice?

7) Is specific antibody found in the intestinal contents of mice infected with H. diminuta?

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