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THE UNIVERSITY OF ALBERTA

IMMUNITY TO *EIMERIA NIESCHULZI* IN RATS

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

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EDMONTON, ALBERTA

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FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled: Immunity to *Eimeria nieschulzi* in rats, submitted by Eugene McLaren Liburd, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

An *Eimeria nieschulzi*-rat model was used to study the nature and mechanism of immunity to coccidia.

The reproducible pattern of *E. nieschulzi* infections was confirmed. The reproductive potential of *E. nieschulzi* varied with dosage of oocysts; an inverse relationship between oocyst production and oocyst dosage was found. The course of infection was not altered by parenteral inoculation of oocysts or sporozoites, thymectomy, or partial resistance.

Eimeria nieschulzi was parenterally transferrable by intravenous, intramuscular, intraperitoneal, and subcutaneous injections. Parenterally induced infections were in every case milder than those elicited by the normal oral route.

Infection of rats by *E. nieschulzi* stimulated a high degree of immunity. This was demonstrable as early as 15 days after inoculation. Given optimal dosage, rats became totally resistant to challenge infection. Single inoculations of 2,500 and 3,500 oocysts stimulated total resistance in outbred and inbred rats, respectively. Following challenge inoculations of immunized rats no penetration of sporozoites or development of endogenous stages occurred.

Electrophoretic analysis of serum from infected rats showed significant increases in beta-2-globulin, and decreases in the alpha-1-globulin fractions. Slide agglutination tests demonstrated the presence of agglutinating antibody. Sporozoites agglutinated on exposure to immune serum. Despite the immobilizing and deleterious

effects of immune serum on sporozoites *in vitro*, no protective relationship between serum antibody and resistance to infection with *E. nieschulzi* could be demonstrated. Attempts to passively transfer immunity with immune serum or serum fractions were unsuccessful.

Preliminary studies using the indirect fluorescent antibody technique indicated that rats produced localized antibody in response to *E. nieschulzi* infections. The presence of tissue antibody throughout the entire small intestine supports the idea of a diffuse immune response to coccidia.

Neonatal or adult thymectomy did not inhibit or suppress development of acquired resistance to infection. The pattern and intensity of infection were the same in thymectomized and control rats, and there was no delay in the immune response.

Sporozoites when combined with thoracic duct lymphocytes from immunized rats exhibited a specific adherence. This association was deleterious to sporozoites.

Immunity was adoptively transferred to susceptible syngeneic rats. Intravenous injections of 100 million or more thoracic duct lymphocytes from immunized rats elicited varying degrees of resistance against primary infections with *E. nieschulzi* in recipients. Rats injected with 1 billion lymphocytes shed an average of 734 oocysts for each oocyst given compared to 78,000 for the controls. Three of 11 recipients of 1 billion lymphocytes were totally immune.

Sensitized thoracic duct lymphocytes adoptively transferred immunity whereas immune serum did not; thus, it is proposed that the immune response to *E. nieschulzi* is cellular.

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INTRODUCTION

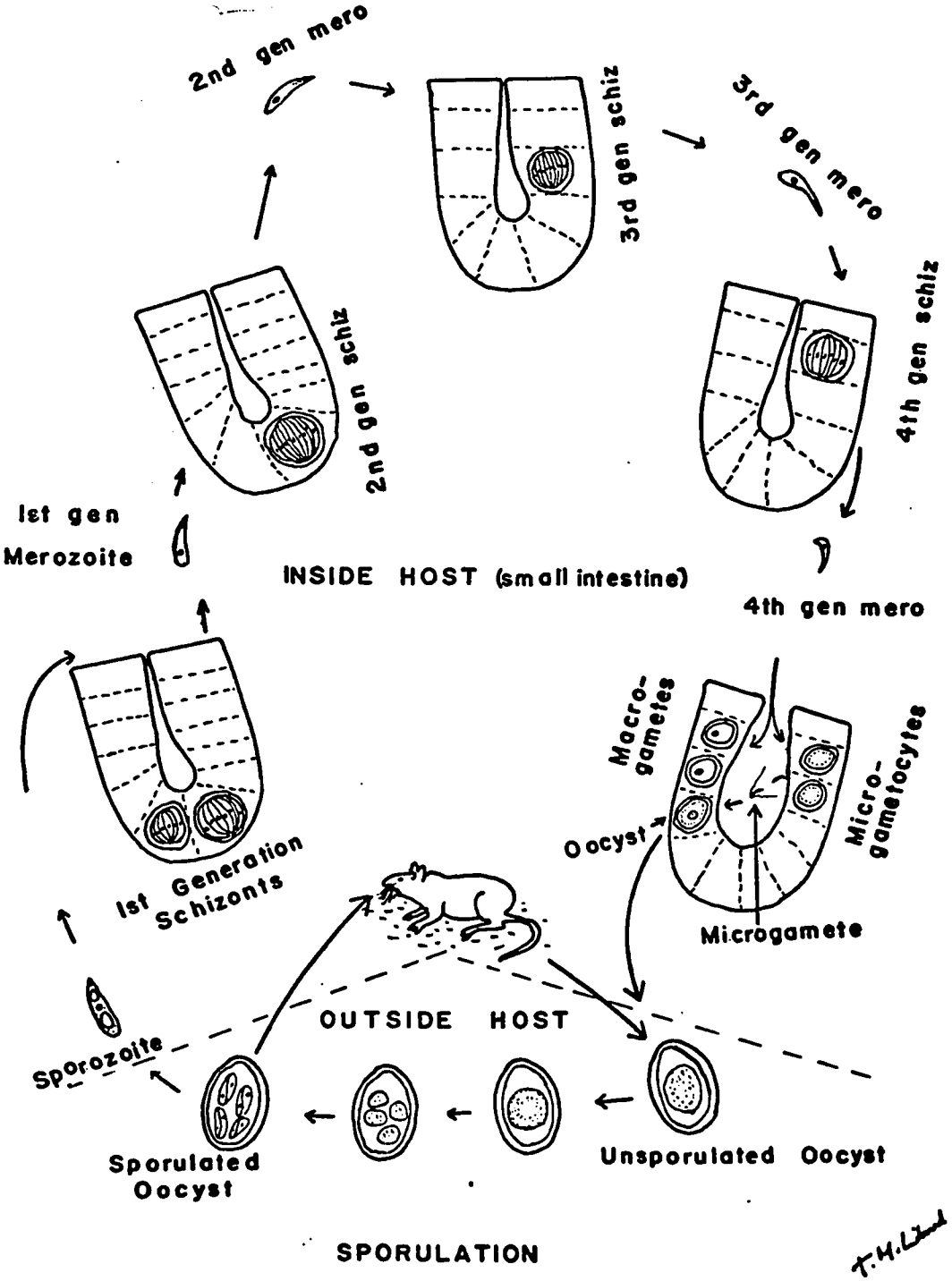
Coccidia belong to a group of one-celled organisms (Sporozoa) which are all obligatory parasites. Generally, they are intracellular parasites of the epithelia that line the alimentary tracts of vertebrates and some invertebrates. Less frequently the epithelia and/or other tissue, e.g., liver and kidney, are subject to invasion. Each species usually occupies a particular location within the host where different cells may be invaded. The location within the host cell may also vary. Some coccidial species are pathogenic, causing a disease known as coccidiosis. Due to the loss from morbidity and death in domestic animals, the disease is of major economic importance. Although coccidia are distributed widely, and with all groups of vertebrates probably being infected, most of the studies on the biology of these organisms have been confined to species of the genus *Eimeria* which are economically important, e.g., *E. tenella* in chickens, *E. bovis* in cattle and *E. stiedae* in rabbits.

Coccidia differ from other parasitic protozoa of the gut in the following ways: (i) They are obligatory intracellular parasites with one stage or numerous individuals within a single host cell. (ii) Coccidia usually show a high degree of host-specificity. (iii) They undergo a period of development outside the host (sporogony) and an alternation of asexual (schizogony) and sexual (gametogony) stages within the host. (iv) Their life cycle is self limiting, thus in the absence of reinfection the host is void of the parasite after a specific period of time.

Like other sporozoans, coccidia have a complex life cycle which has three phases: sporogony, schizogony and gametogony. A typical coccidian life cycle is that of *Eimeria nieschulzi* which infects rats (Figure 1). This life cycle was described by Roudabush (1937). In the presence of moisture and oxygen an unsporulated oocyst develops into the infective stage by a process of sporulation. This results in the formation of 8 infective sporozoites in each oocyst. When sporulated oocysts are ingested excystation occurs which liberates the sporozoites into the lumen of the small intestine. In a susceptible rat these infective organisms penetrate through cells of the villi, enter lacteals and pass down the lacteals into the crypts of Lieberkuhn (Marquardt, 1966) and begin the second phase of the life cycle, that of schizogony. When the schizont is fully mature the host cell ruptures and the released merozoites seek other host cells, penetrate and develop into another generation of schizonts. In *E. nieschulzi* there are 4 asexual generations. The fourth generation of merozoites enters new host cells and undergoes gametogony. Some of these merozoites become macrogametes whereas others develop into microgametocytes. The nucleus of each microgametocyte divides many times, thus giving rise to numerous microgametes. Following its release from the host cell a biflagellated microgamete penetrates a macrogamete and their nuclei fuse. This union results in a zygote (unsporulated oocyst) which ruptures out of its host cell and is expelled with the faeces. The endogenous development of this parasite occurs in the middle portion of the small intestine and has a prepatent period of approximately 6 and 1/2 days and a patent period of 6 days.

Figure 1 Diagramatic representation of the life cycle of *Eimeria nieschulzi* Dieben, 1924.

LIFE CYCLE OF EIMERIA NIESCHULZI DIEBEN, 1924



Acquired immunity to reinfection with the same species of *Eimeria* is well known, Tyzzer (1929); Hall (1934); Becker *et al.* (1935); Morehouse (1938); Pierce *et al.* (1962); Hammond *et al.* (1963) and others. The degree of resistance conferred varies to some extent with the species of coccidia, the size and frequency of the infective dose, and the age of the host, Tyzzer (1929); Becker (1935); Horton-Smith (1949); Hammond *et al.* (1963) and Leathem and Burns (1967, 1968). Resistance is generally determined by either the complete absence, or the presence of relatively few oocysts in the faeces of the resistant animal at the expected time in the life cycle, following challenge inoculation (Becker, 1935; Pierce *et al.*, 1962; Hammond *et al.*, 1963). This absence or marked reduction in oocyst production indicates failure of some of the parasites to complete their life cycles, Hammond *et al.* (1963). According to these latter workers, in the resistant host, the immune reaction affects the numbers, but not the timing of the various stages of the life cycle. Leathem and Burns (1968), having successfully challenged immune birds, proposed that immunity to *E. tenella*, as ordinarily understood, implies that a chicken is resistant to the clinical signs of the disease only. They further suggested that histological examination of the infected tissue be requisite to declaring a host immune if immunity is to imply resistance to invasion. Leathem and Burns found that animals which fail to display clinical signs may still harbour endogenous stages of the parasite that are capable of completing their development and producing oöcysts. They postulated that chickens immunized by multiple doses of *E. tenella* develop only

a condition of "relative immunity" which can be assessed by observing immune birds for (1) clinical signs of disease (2) amount of schizogony that occurs or (3) number of oocysts produced in response to a challenge inoculation.

The complexity of the *Eimeria* life cycle has given rise to speculation over the stage-specificity of the immune reaction. There is agreement that excystation of the sporozoites occurs normally in the immune host, but there is conflict concerning the subsequent fate of sporozoites. Morehouse (1938) observed that the sporozoites of *E. nieschulzi* failed to penetrate the epithelial cells of the intestines of resistant rats. Hammond *et al.* (1964) found that schizont stages of *E. bovis* in resistant calves were affected and they believed that this was due to failure of the sporozoites to penetrate the host cells. Pierce *et al.* (1962) failed to find intracellular stages of *E. tenella* in tissue from the caeca of immune chickens following a heavy challenge inoculation. They did, however, find oocysts in the faecal material of the immune birds which shows that some sporozoites did penetrate. Leathem and Burns (1967) observed first generation schizonts in the tissue of immune birds 48 and 72 hours after challenge inoculation. These authors also found what appeared to be second generation schizonts in sections from immune birds 96 hours subsequent to challenge. Leathem and Burns (1968) reported the presence of endogenous stages in histological sections from immune birds 72 and 96 hours after challenge inoculation.

Leathem and Burns (1967), in viability studies with the sporo-

zoites of *E. tenella* in immunized chickens, found that stages recovered from caecal epithelial cells between 12 and 24 hours after invasion, produced infections when transferred into susceptible birds whereas those obtained at 48 hours did not. This, they suggested, indicates that between 24 and 48 hours after invasion, the immune host had irreversibly affected the development of the parasite. Hammond *et al.* (1963, 1964) and Leathem and Burns (1967) found that the reaction of the immune host is not confined to any specific endogenous stage. Hammond *et al.* (1964) demonstrated that intracaecal injection of first generation merozoites of *E. bovis* will stimulate resistance which is effective against sporozoite challenge. Rose (1967) observed species differences between *E. tenella* and *E. necatrix* in their ability to immunize against homologous stages. She found that *E. tenella* infection with gametogony stages only, induced resistance to subsequent gametocyte stages but not to schizogony stages. On the other hand, caecal inoculation of second generation merozoites of *E. necatrix* which produced gametogony-only infections did not stimulate resistance since schizogony and gametogony were not suppressed in the challenge infection.

There is no clearcut evidence on the exact site of the immune reaction to *Eimeria*. The fact that the *Eimeria* are found in specific locations makes it difficult to determine whether the immune response is confined to the previous infected site, that is a local response, or is more diffuse. Burns and Challey (1959) and Horton-Smith *et al.* (1961) working with chickens, demonstrated that both caeca become re-

sistant to infection with *E. tenella* as a result of previous infections confined to one caecum. Becker, Hall and Madden (1935) in a study on the mechanisms of immunity to coccidial infections of rats indicated that immunity is not localized to only the immediate area of infection. "Immunity is capable of spreading from centers of infection over the remaining surface of the intestine. No explanation was given as to how this 'spreading' might occur. These authors considered that neither a general systemic response, nor circulating antibodies played a role in this immunity. They presumed that the epithelial cells either acquire the property of blocking the entrance of the sporozoites into their protoplasm, or, by having become sensitized, inhibit the growth of any penetrating sporozoites" (Hammond, 1964). Hammond *et al.* (1964) found that the presence of *E. bovis* in a small portion of the large intestine of calves confers immunity against *E. bovis* in the entire large intestine. Rose (1967) observed that development of *E. necatrix* (a duodenal species) in the caeca of fowls caused reduced schizogony following challenge infection in the duodenum. These observations indicate that acquired resistance to these organisms is present in areas not previously infected by them. Horton-Smith *et al.* (1961) suggested that the immunity acquired by the infected site was mediated to the previously uninfected areas through either humoral antibodies, lymphoid cells or both.

Evidence for the presence of an antibody response explaining acquired resistance to coccidia has been sought by many investigators. Bachman (1930) working with rabbits, Tyzzer *et al.* (1932) with chickens,

and Becker *et al.* (1935) with rats did not succeed in demonstrating circulating antibodies in infected animals. McDermott and Stauber (1954); Rose (1959, 1961, 1963); Rose and Long (1962); Pierce *et al.* (1962, 1963); Augustin and Ridges (1963) and Andersen *et al.* (1965) demonstrated antibodies in infected animals by use of precipitation and complement fixation tests (extracts of endogenous stages used as antigens) and by indirect fluorescent staining (on merozoites used as antigens). Heckman (1968), using blood serum of rats infected with *E. nieschulzi*, found two new bands in the gamma globulin range during days one and two following inoculation. The significance of these two new bands has not been elucidated. The role of serum antibodies in coccidial immunity is not known. Long and Pierce (1963) and Pierce and Long (1965) have suggested a minor protective role for these serum antibodies.

Efforts have been made to produce active and passive immunization of susceptible animals. Becker (1935) failed to actively immunize rats by injecting them intraperitoneally with suspensions of ground-up oocysts and infected intestine, and passively by transferring serum from resistant to susceptible animals. Augustin and Ridges (1963), Pierce *et al.* (1963), Rose (1963) and Fitzgerald (1964) did not succeed in passive transfer of immunity by inoculation of serum or serum fractions. Augustin and Ridges (1963), Long *et al.* (1963) and Burns and Challey (1965) reported that the sera of resistant animals showed an adverse and/or deleterious effect on merozoites and sporozoites by way of agglutinating, lytic, precipitating and immobili-

zing reactions. They also noted the failure of serum-treated organisms to induce infections in susceptible hosts. Andersen *et al.* (1965) observed that merozoites of *E. bovis* are adversely affected by serum from immune animals. However, they found that (a) merozoites are also affected, but to a lesser degree, by normal serum from calves, and (b) degenerative changes also occur in merozoites exposed to saline solutions. Because of the latter findings they suggested that it is difficult to determine the degree to which such changes in merozoites are caused by antibodies. Despite the *in vitro* deleterious effects of immune serum on endogenous stages of *Eimeria*, passive transfer of immunity has been unsuccessful.

The function of lymphoid organs and their influence on the immune response to coccidia have been studied. Challey (1962) reported that surgically bursectomized chickens were significantly less resistant to infection with *E. tenella*. Rouse and Burns (1971) did not substantiate Challey's observation on the effect of bursectomy. They reported that surgical bursectomy within 48 hours of hatching failed to impair the ability of chickens to survive an initial infection with *E. tenella* or to develop resistance to reinfection. Hormonal bursectomy by *in ovo* administration of testosterone also does not affect immunity. Pierce and Long (1965) and Rose (1968) found that although hormonally bursectomized chickens did not produce detectable coccidial antibodies, their ability to develop resistance was unimpaired.

Neonatal thymectomy in chickens apparently has no effect on the bird's ability to develop resistance to reinfection. Although

Pierce and Long (1965) reported slightly higher oocyst counts from thymectomized chickens than from intact controls, the thymusless birds were resistant to challenge infection. Rouse and Burns (1971) also found that neonatally thymectomized chickens were capable of developing immunity to *E. tenella*.

Becker *et al.* (1935) working with rats, Haberkorn (1970) with mice, and Rose (1968) with chickens found that splenectomy had no effect on the animal's ability to resist reinfection.

The immunological significance of lymphoid cells in resistance to coccidia has only recently been explored (Long *et al.*, 1963; Pierce and Long, 1965; Rommel, 1970; Rose, 1968a), and the possible role of thoracic duct lymphocytes (TDLs) in adoptive immunity against these parasites has had little or no attention.

It is well established that animals develop some degree of immunity to reinfection by the same species of *Eimeria*. However, the mechanism of this acquired resistance has not yet been elucidated. The literature indicates that both humoral and cellular factors may be involved, but there are no reports which have answered the basic question, what is the mechanism of immunity to coccidia?

Eimeria nieschulzi, which infects rats, was selected as a model system for this study on the nature and mechanism of immunity to coccidia. This model has many advantages. Inbred rats are readily available, easily maintained and develop complete resistance to challenge infection. *Eimeria nieschulzi* is non pathogenic, its life cycle is well known and predictable, it is host and site specific, and is

available in large numbers.

My study began with an analysis of the effects of varying dosages of *E. nieschulzi* on inbred and outbred rats. This was followed by examinations of (a) the effects of parenteral inoculations of sporozoites on both the host and the parasite, (b) serum protein changes during parasitemia, (c) the site of antibody production, (d) the effects of thymectomy on the immune response, and (e) passive and adoptive transfer of immunity. The results of these experiments are reported.

MATERIALS AND METHODS

Eimeria nieschulzi Dieben, 1924, Landers isolate was used for the study. The coccidium was maintained in our laboratory by serial passage in albino laboratory rats. Faeces were collected from the animals during the patent period of infection. Oocysts were sporulated in 2.5 per cent potassium dichromate solution at 30 C and then stored at 4 C. Fresh inoculum containing over 97 per cent sporulated oocysts was used. In preparing the inoculum, the oocysts were washed free of potassium dichromate by repeated centrifugation in water, and the number estimated by the McMaster method (Whitlock, 1948). The volume of the inoculum was one ml. Inoculation was accomplished by injecting the oocysts into the stomach of the lightly anaesthetized rat through a stomach tube attached to a syringe. Prior to inoculation the rats were starved for approximately 18 hours.

During the patent period (7-12th day post inoculation) the total faecal output of each rat was collected daily on moist paper and a randomly chosen 10 gram sample examined in order to calculate the total oocyst output (Whitlock, 1948) of the individual rat. Faecal examinations were also made by the Lane Direct Coverslip Flotation (DCF) method (Levine, 1961) in order to detect very low numbers of oocysts in the early and late periods of patency. The data are based upon daily counts of the total number of oocysts discharged by individual rats during each 24 hour period. The intensity of infection established and the degree of acquired resistance developed by the host (against challenge infections) were assessed by the number of oocysts discharged

during the patent periods.

Rats of 2 strains, CD (outbred) and CD-F (inbred), age 4-15 weeks were supplied by Charles River Breeding Laboratories, Wilmington, Mass. According to the suppliers the inbred rats were derived from 68th generation Fischer 344 inbred rats obtained from Dr. Wilhelmina F. Dunning in 1960. These are presently in the 92nd generation of their inbred nucleus. The breeders do their own skin and bone grafts annually. None of the inbred animals died following injections of serum, serum globulins, lymph or thoracic duct lymphocytes from syngeneic donors.

Prior to inoculation daily faecal examinations (for 3-5 days) were made to determine if the rats had natural infections. No natural infections were detected. Each rat was kept in a separate wire-floored cage. There was no contact between animals. Except where otherwise indicated all experimental animals were inoculated with sporulated oocysts via stomach tube attached to a syringe.

PART 1 ACQUIRED RESISTANCE TO *EIMERIA NIESCHULZI* INFECTIONS

This series of experiments was designed to study some of the host-parasite relationships that exist between our strain of *E. nieschulzi*, and the rat. I began the programme by (a) timing the parasite's life cycle, prepatent and patent periods, in inbred and outbred rats, and (b) studying the effects of various sized infective doses on both parasite and host. From these, the optimal dose size, degree of immunity conferred, and a dose response curve were established. Next, experiments were set up to: induce infection by parenteral routes with sporozoites of *E. nieschulzi*; compare the differences in the intensities if infection was established; and study the efficiency of parenteral and oral inoculations in inducing infection. Finally I tried to determine if there was sporozoite penetration and subsequent intracellular development of endogenous stages of *E. nieschulzi* in immunized rats.

MATERIALS AND METHODS

Correlations between primary dose size and resistance conferred against secondary infections

In pilot experiments 10 rats were each inoculated with 500 oocysts.

Experiment 1. Each of 20 male rats (outbred) was inoculated with 1,000 oocysts; each of 20 with 2,500; each of 20 with 5,000. Fifteen days after the primary inoculation the above animals, plus

4 uninoculated ones, were each given 50,000 oocysts.

Experiment 2. Ten male rats (inbred) were each inoculated with 1,500 oocysts, 10 with 2,500; and 10 with 3,500. Each of the above animals, plus 4 uninoculated controls, was given 50,000 and 100,000 oocysts at 15 and 30 days respectively after original inoculation.

Experiment 3. Ten male and 10 female rats (outbred) and 10 male and 10 females (inbred) were each inoculated with 3,500 oocysts. Fifteen days post sensitization each animal was challenged with 50,000 oocysts.

Five rats from each group inoculated with 1,000; 1,500; 2,500; 3,500; 5,000 and 50,000 sporulated oocysts were examined to determine the number, if any, passed in the first 24 hours after inoculation. Total faeces were examined and the number of oocysts estimated by the DCF method (Levine, 1961).

Parenteral infections with sporozoites

Three experiments with a total of 38 male rats (outbred), 10 weeks of age, were conducted. To determine whether the infections came from the injected inoculum, control animals were kept in close proximity to, but not in the same cages as the inoculated animals. In all cases the controls remained uninfected.

Preparation of inoculum. Faeces from infected rats were collected in pans containing 2.5% potassium dichromate solution. Oocysts were sporulated in this solution at 29 C, with constant stirring for 48 hours. Purification and recovery of sporulated oocysts were done

by a continuous-flow differential density flotation method (Vetterling, 1969). Microscopic examinations of 500 oocysts showed that 96% were sporulated. These were free of bacteria and virtually debris-free (Figure 15). Suspensions of purified and unpurified oocysts were incubated on nutrient agar. No bacterial growth occurred on the plates with the purified suspensions, only on those with the unpurified oocysts. The purified oocysts were stored in sterile distilled water at 4 C.

Sporozoites were obtained by *in vitro* excystation. They were released from oocysts by grinding for 3-4 minutes in a glass tissue grinder and then treating with 0.5% trypsin and 5% bile at pH 7.5 (Hibbert and Hammond, 1968). Sigma Type III purified Bovine Pancreas Trypsin and Difco Bacto Bile Salts were used. This suspension was incubated at 39-40 C for 1 hour. Excystation of the sporozoites was usually complete in 91-94% of the sporocysts in 5-20 minutes. Following removal of the excystation fluid by repeated centrifugation in Ringer's solution, the sporozoites were purified by passage through a glass bead column (Wagenbach, 1969). The number of free sporozoites was counted in a haemocytometer. Some sporozoites excysted when the oocysts were broken by grinding, without any other stimulus.

Parenteral inoculations. Prior to inoculation, the viability of the sporozoites was assessed by mixing 0.5 ml of the sporozoite suspension with 5 drops of 0.05% trypan blue solution. Over 95% of the sporozoites were alive as judged by their exclusion of the dye and motility under phase-contrast microscopy at room temperature.

Rats were lightly anaesthetized by ether inhalation and injected in either the tail vein (IV), the muscle of the thigh (IM), subcutaneously (SC) or intraperitoneally (IP). Oocysts produced by these inoculations were examined microscopically to study their morphology (no differences were observed), sporulated in 2.5% potassium dichromate solution at 29 C and used successfully in other experiments.

Five outbred rats were used in a pilot experiment. Three were injected intraperitoneally with intact sporulated oocysts, one was inoculated with oocysts via stomach tube and the other was uninoculated. Seven days after inoculation 2 parenterally inoculated and the uninoculated control rats were killed. Segments of tissue, 1 cm long, were taken at 10 cm intervals from the small intestine and from the large intestine. These were prepared histologically and examined microscopically for endogenous stages of the parasite.

Experiment 1. In this experiment 28 rats were divided into seven groups of 4 each. The animals in Groups I-V were each inoculated with 9,600 sporozoites while those of Group VI were given 1,200 sporulated oocysts each. Rats in Group VII served as inoculated controls. Group I animals were inoculated intravenously (IV), Group II intraperitoneally (IP), Group III intramuscularly (IM), Group IV subcutaneously (SC) and Groups V and VI by stomach tube (stomach). Fifteen days post inoculation each rat in Groups I-VI was inoculated with 50,000 sporulated oocysts.

Experiment 2. Five rats were each inoculated intraperitoneally with 24,000 sporozoites and challenged 15 days later by intraperitoneal

injection of 40,000 sporozoites.

Experiment 3. Five rats were each orally inoculated with 3,000 sporulated oocysts. Fifteen days later each animal was injected intraperitoneally with 40,000 sporozoites.

Influence of the site of infection against secondary infections

Sixteen male outbred rats, 8-10 weeks of age, were each inoculated with 75,000 oocysts. Fifteen days later each rat was challenged with 50,000 oocysts. Twelve hours after the challenge inoculation one rat was killed. Excysted sporozoites found in the intestinal lumen were recovered by drawing up the intestinal fluid in an eyedropper and immediately placing it in Ringer's solution. To test the viability of these sporozoites they were injected into the stomach of 2 non-immunized rats. Two of the immunized rats were killed at intervals of 24 hours. As controls 2 susceptible rats were each inoculated with 75,000 oocysts and killed at 24 and 38 hours after inoculation. Portions of the middle third and other areas of the small intestine and colon from each rat were fixed in Helly's fluid, sectioned at 7 microns, stained in haematoxylin and eosin, and PAS-A-O for the detection of early endogenous stages. The sections were examined microscopically for the endogenous stages of the parasite. One of the 16 immunized rats was kept until the end of the known patent period. No oocysts were shed by this animal.

RESULTS

Correlations between primary dose size and resistance conferred against secondary infections

Following inoculation, a portion of the inoculum was voided with the faeces. For the first 24 hour period post inoculation the percentile loss ranged from 3.6 for a dosage of 1,000 to 29.7 for a dosage of 50,000 oocysts (Table I).

The basic pattern of *E. nieschulzi* infection for outbred and inbred rats as determined by oocyst output during the patent period is shown in Figures 2 and 3. Oocysts were present in the faeces 7 days after inoculation, reached a peak on day 9, then rapidly decreased until they were absent from the faeces by day 13. The average patent period was 6 days. Rats showed great variability in potential for the production of oocysts during experimental infections with *E. nieschulzi* (Figures 2 and 3). The larger the infective dose of oocysts inoculated, the greater the total number of oocysts shed; but the ratio of oocysts shed per oocyst inoculated during patency was not constant (Figure 4). In Figure 4 is recorded the mean yields for different sized infective doses. The reproductive potential is clearly inversely proportional to the size of the infective dose.

E. nieschulzi appears to be only mildly pathogenic in rats since no clinical signs of disease were observed in animals infected with up to 50,000 oocysts. However, in infections resulting from inoculations of 50,000 oocysts, the rats showed a loss of appetite and had a marked

reduction in faecal output during days 6-9 after inoculation. There was no mortality of infected animals.

Rats infected with *E. nieschulzi* developed resistance to re-infection. The level of immunity was dependent on the size of the infective dose (Figures 2 and 3). In outbred rats an infective dose of 1,000 oocysts stimulated only partial resistance while doses of 2,500 and 5,000 conferred complete resistance (Figure 2). Inbred rats required higher sensitizing doses before total immunity was produced (Figure 3). Animals inoculated with 1,500 and 2,500 oocysts respectively became partially resistant and developed mild infections when challenged 15 days after primary inoculations. Immunizing doses of 3,500 oocysts rendered the inbred rats completely resistant to secondary, challenge infections (Figure 3).

There was no significant difference between the sexes in either the intensity of infection established or the development of resistance.

Parenteral infections with sporozoites

In the pilot experiment the rats inoculated intraperitoneally with oocysts became infected. The course of infection paralleled that obtained by the oral inoculation (Figures 2 and 3). Tissue (endogenous) stages of the parasite were found in the middle third of the small intestine only. No infection occurred in the uninoculated control.

All rats were successfully infected by sporozoites of *E. nieschulzi* parenterally inoculated at 4 different sites. The pattern of infection was similar to that obtained by the normal oral route of

inoculation. Oocysts were found in the faeces from day 7 through day 12. The intensity of infection was mildest following intramuscular and intravenous inoculations. The infections caused by intraperitoneal injections of sporozoites were more successful than the other parenteral routes (Table II). Oral administration of intact sporulated oocysts resulted in greater intensities of infection than those elicited by the parenteral inoculations of sporozoites.

The oocyst output of rats inoculated intraperitoneally with 24,000 sporozoites is shown in Table V. These animals were totally resistant to challenge inoculations with sporozoites given intraperitoneally.

All rats in experiment 3 which were orally inoculated with oocysts were completely resistant to challenge infections with sporozoites injected intraperitoneally.

Influence of the site of infection against secondary infections

A total of 48 sections from each rat was examined. No penetrated sporozoites or developing endogenous stages were found in the intestinal tissues of the immunized rats. In the controls, no penetrated sporozoites were seen in the tissues, but all other endogenous stages were found.

Susceptible rats inoculated with sporozoites obtained from the intestinal lumen of challenged immunized rats became infected and exhibited the normal pattern of infection for this parasite.

TABLE I

Mean percentage of sporulated oocysts recovered from animals over the first 24 hour period after inoculation (5 rats per group).

Dosage (oocysts)	Percent recovered in 24 hours
1,000	3.6
1,500	3.4
2,500	4.9
3,500	5.4
5,000	5.8
50,000	29.7

TABLE II

Oocysts produced by inoculating rats parenterally with sporozoites of *Eimeria nieschulzi*.

Site of inoculation	Size of inoculum	Mean total No. of oocysts shed x 10^6 (4 rats/ group)	Range x 10^6	Mean No. of oocysts shed/ sporozoite or oocyst given	Oocysts shed after challenge with 50,000 oocysts (x 10^6)
IV	9,600	0.37	0.12-0.65	39	0.00015
IP	9,600	7.44	1.10-9.85	775	None
IM	9,600	0.27	0.10-0.72	28	0.00011
SC	9,600	2.37	0.62-4.53	247	None
Stomach	9,600	22.99	6.00-28.3	2,395	None
*Stomach (oocysts)	1,200	38.86	10.20-43.0	32,383	None
**Stomach (oocysts)	50,000				367.38

*Oocysts were used as control inoculum.

**This group was inoculated at the time when the other rats were challenged.

Figure 2 Average oocyst production in relation to immunization and challenge with *Eimeria nieschulzi* oocysts in outbred rats.

Vertical lines represent the range in oocyst output observed.

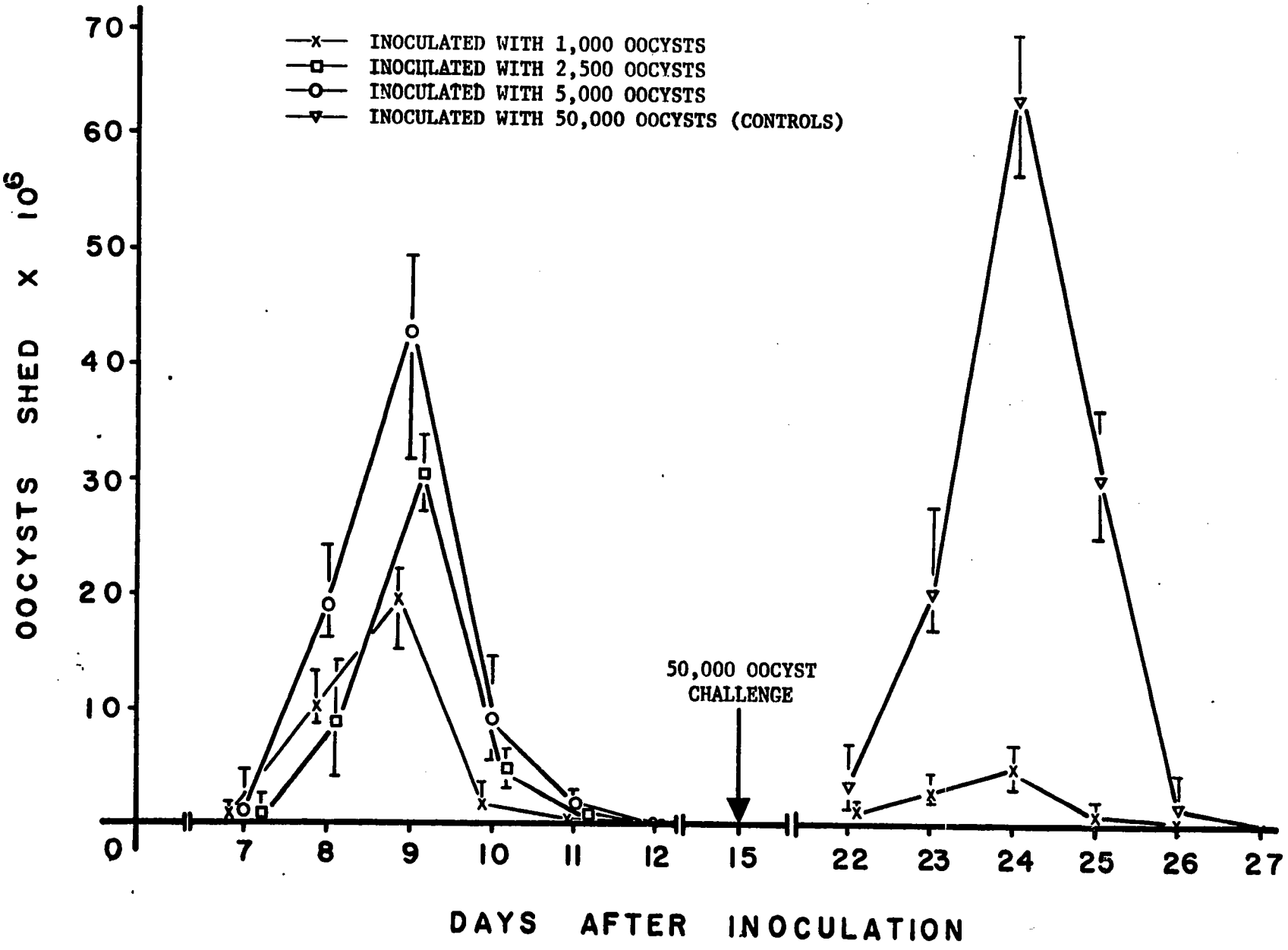

INOCULATED WITH 1,000 OOCYSTS
INOCULATED WITH 2,500 OOCYSTS
INOCULATED WITH 5,000 OOCYSTS
INOCULATED WITH 50,000 OOCYSTS (CONTROLS)
OOCYSTS SHED X 10^6
70
60
50
40
30
20
10
0
50,000 OOCYST CHALLENGE
7
8
9
10
11
12
15
22
23
24
25
26
27
DAYS AFTER INOCULATION

Figure 3 Average oocyst production in relation to immunization and challenge with *Eimeria nieschulzi* oocysts in inbred rats.

Vertical lines represent the range in oocyst output observed.

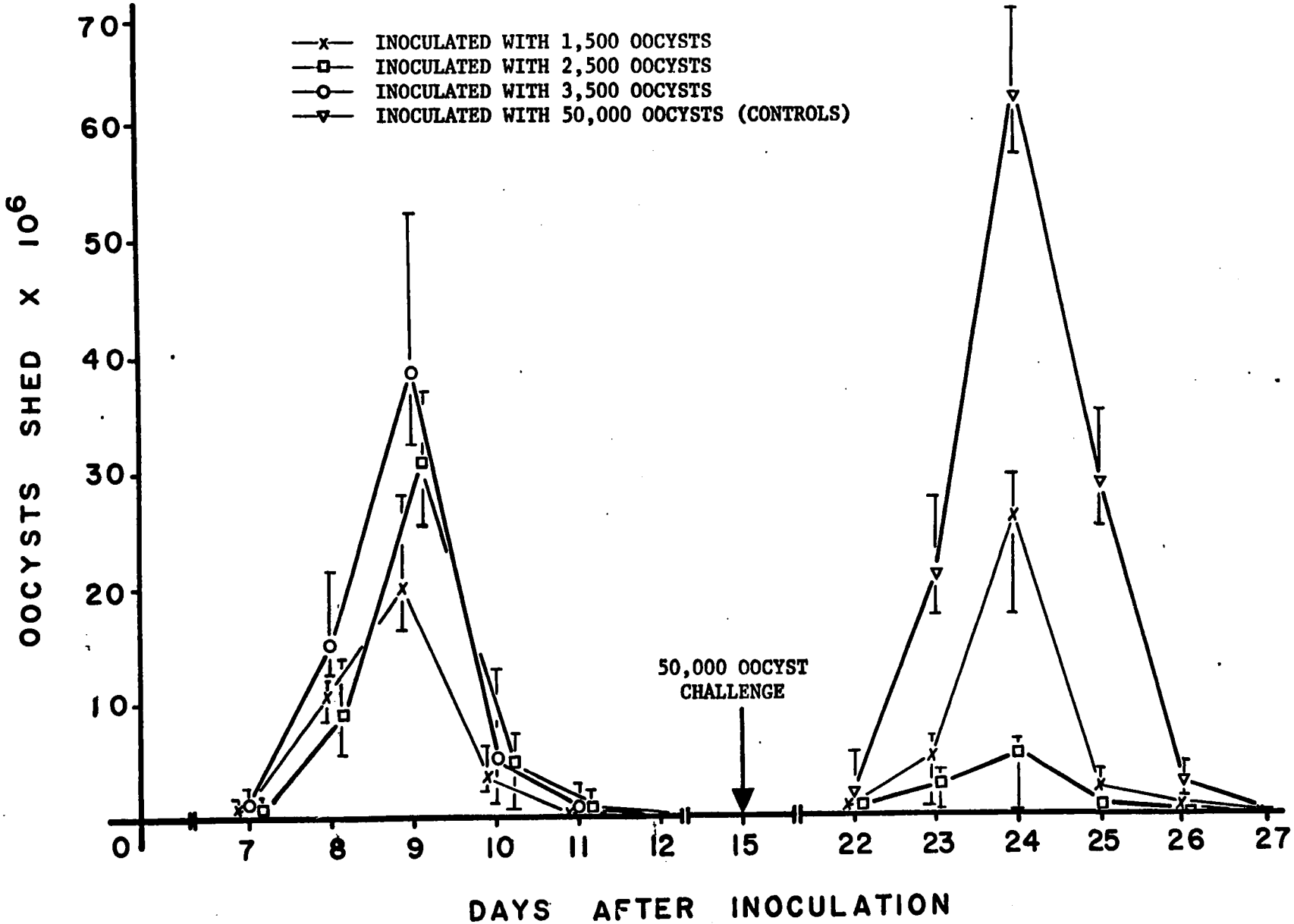
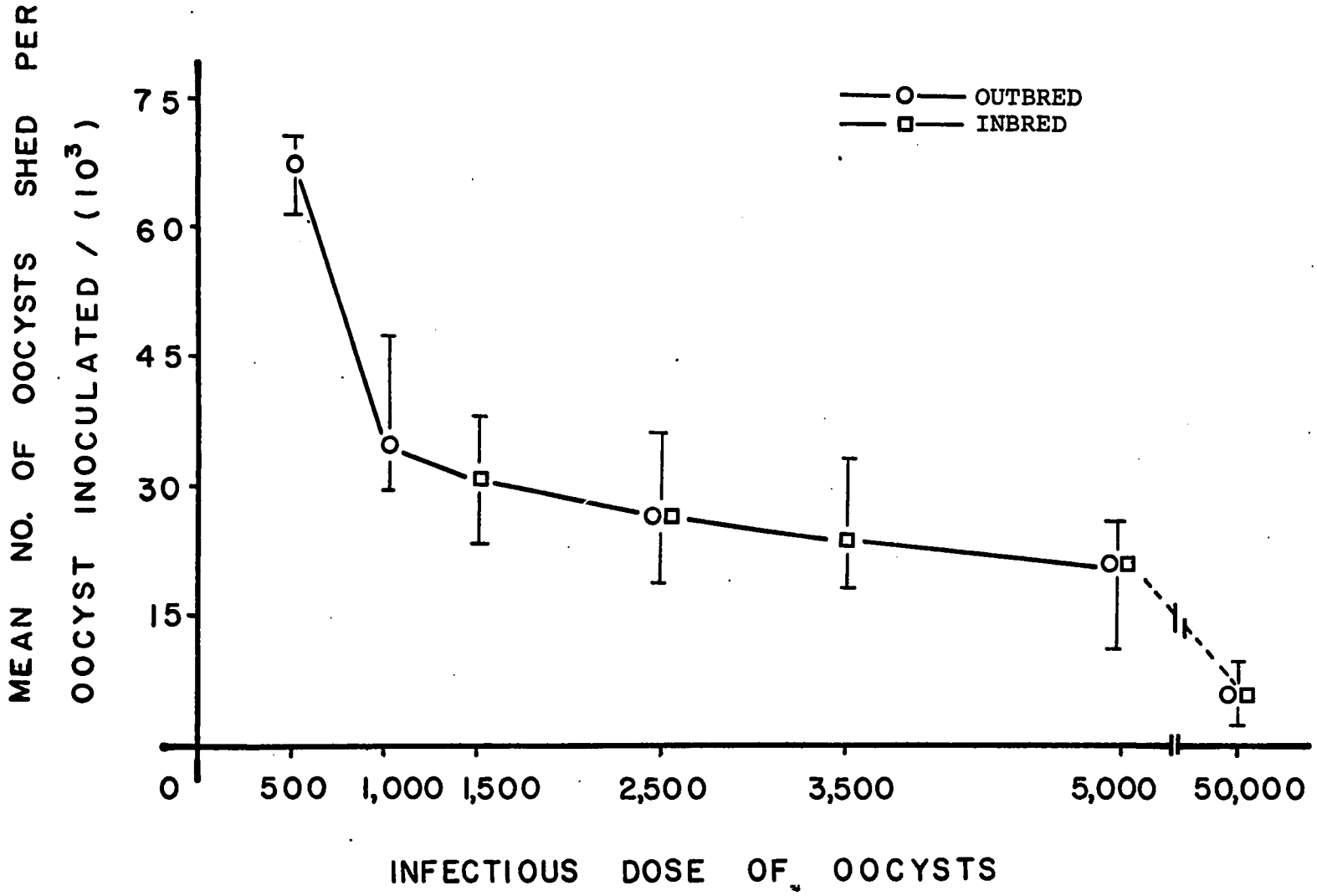

INOCULATED WITH 1,500 OOCYSTS
INOCULATED WITH 2,500 OOCYSTS
INOCULATED WITH 3,500 OOCYSTS
INOCULATED WITH 50,000 OOCYSTS (CONTROLS)
OOCYSTS SHED X 10^6
70
60
50
40
30
20
10
0
50,000 OOCYST CHALLENGE
7
8
9
10
11
12
15
22
23
24
25
26
27
DAYS AFTER INOCULATION

Figure 4 Oocyst response curve for each infectious dose of *Eimeria nieschulzi* oocysts inoculated into outbred and inbred rats.
Vertical lines represent the range in oocyst output observed.


MEAN NO. OF OOCYSTS SHED PER OOCYST INOCULATED / (10^3)
75
60
45
30
15
0
500
1,000
1,500
2,500
3,500
5,000
50,000
OUTBRED
INBRED
INFECTIOUS DOSE OF OOCYSTS

PART 2 ACQUIRED ANTIBODIES DURING PRIMARY INFECTIONS

These experiments were designed to study (a) changes in serum proteins of rats infected with *E. nieschulzi*, (b) the effects of serum on sporozoites, *in vitro*, (c) the site of antibody production, and (d) attempts at passive transfer of immunity against *E. nieschulzi*. It was hoped that these experiments would help in elucidating a role for acquired antibodies to *Eimeria*, and also find a correlation between the presence of serum antibodies and immunity that would aid in understanding the immune response to coccidial infections.

MATERIALS AND METHODS

Electrophoretic analysis of serum proteins

Experimental infections. Each of 20 rats was inoculated with 5,000 oocysts. Fifteen days later half of the animals (5 inbred and 5 outbred) was each given 50,000 oocysts to determine if there was a detectable secondary immune response. Ten rats (5 inbred and 5 outbred) served as uninfected controls. Oocysts were collected and counted as in Part 1.

Collection of blood samples. Blood samples were collected from all rats on day 0 prior to inoculation, and on day 1, 4, 7, 10, 13, 16, 19, 22, 36, 50 and 64 after inoculation. The uninfected control rats were bled on these same days. After the patent period of the immunizing infection, the animals were transferred to clean cages and faecal samples examined for oocysts each time blood samples were taken. No oocysts were found after the initial patent period.

Bleeding was done by snipping off the tip of the tail from the lightly anaesthetized rat and aseptically drawing off the blood under vacuum (Figure 16) in a 5 ml sampling tube. Approximately 1 ml of blood was collected from each animal at the time of sampling. After the blood had clotted 3-4 hours at room temperature, it was centrifuged for 20 minutes and the serum harvested with a sterile pipette and placed in sterile 1-dram phials. All sera were stored at -20 C until time of use.

Electrophoresis. Part of each serum sample was transferred to cellulose-acetate strips (Sepraphore III) of a Gelman Deluxe Electrophoresis Chamber buffered at a pH of 8.8 and an ionic strength of 0.05M. The samples were run for 60 minutes, stained in Ponceau S, cleared in 5% acetic acid and dried at 105 C. Differential protein analysis was carried out with the aid of a Beckman Analytrol densitometer. The amount represented by each fraction was calculated and expressed in gram per cent. The data from the electrophoretic separation of the serum proteins were tabulated and computer analysed using Duncan's Multiple-Range and Multiple-F tests (Duncan, 1955).

Slide agglutination of sporozoites

Sporozoites of *E. nieschulzi* were used as the antigen in these experiments. They were obtained by *in vitro* excystation as described in Part 1. Sporozoite suspensions were stored for a maximum of 48 hours, at 4 C. Prior to use the suspensions were allowed to reach room temperature. A small drop of the suspension was placed in a

depression slide, then 2 drops of undiluted serum, or serum diluted 1:20, 1:40, 1:80, 1:160, 1:320, or 1:640 with PBS pH 7.1 were added to each slide. Serum samples were the same as those used for electrophoretic analysis. The mixture was allowed to react for 7-9 minutes at room temperature after which time it was examined under phase contrast microscopy at 100X to determine the relative number of sporozoites which had agglutinated. The results were recorded as negative if no sporozoites had clumped (Figure 5), 1+ if 1/4 were clumped, 2+ if 1/2 were clumped, 3+ if 3/4 were clumped and 4+ if all had clumped (Figure 6). The last serial dilution which gave a definite agglutination (1+) was taken as the titer. Physiological saline and nonimmune serum were used as controls. At the start of each test, the serum to be tested was allowed to thaw at room temperature. All serum from an individual rat was tested at the same time. Sporozoites were also tested in (a) heat inactivated immune serum (56 C) at full strength and diluted 1:20 with PBS and (b) in inactivated immune serum plus an equal amount of normal immune serum at 37 C for 60 minutes.

Site of antibody production

Two experiments using a total of 36 inbred rats were conducted. Twelve non-infected rats served as controls while each of 24 was inoculated with 5,000 oocysts. On day 1, 4, 7, 10, 13 and 16 after inoculation 2 non-infected and 4 inoculated animals were killed. Segments of tissue (0.5 cm long) were taken from the upper, middle and lower third of the small intestine and from the large intestine of each rat. The tissue was immediately snap frozen in an acetone-dry ice

mixture, placed in an air tight phial and stored at -20 C. Frozen tissue was kept for a maximum of 14 days before being sectioned. Sections of 7 microns were made in a microtome-cryostat at -25 C, placed on gelatinized slides, fixed in formaldehyde vapours for 10 minutes and stored overnight at -4 C.

Fresh immune serum of high agglutinating titers from infected rats, and serum from non-immunized rats were used. The indirect method of fluorescent staining was used (Nain, 1969). One drop of serum diluted 1:10 with phosphate buffered saline (PBS) at pH 7.1 was placed on each tissue section and allowed to react for 25-30 minutes. The uncombined serum was rinsed away by gently squirting PBS over the section, and the slide blotted with filter paper. A drop of fluorescent-tagged goat anti-rat globulin (obtained from Hyland Laboratories Inc.) was layered over the moist tissue and allowed to react for 25-30 minutes at room temperature in a covered Petri dish containing filter paper moistened with PBS. The slide was then rinsed twice with PBS, 5 minutes each, air dried and mounted in glycerol-saline (90:10) at pH 9. Finally, the tissue was examined under a Zeiss microscope equipped with a BG 12 exciter filter, No. 53 barrier filter and phase-contrast-fluorescence. The presence of antibody was shown by bright fluorescence at the site of antibody. This was assessed qualitatively and rated as negative, 1+, 2+, 3+ and 4+ depending on the assessed brightness. Slides tested with (a) known uninfected intestinal tissue, (b) without a layer of rat serum, (c)

with PBS and (d) with only a thin layer of gelatin, were used as controls.

Passive immunity studies

Thirty male inbred rats, 9-10 weeks of age were immunized by inoculating each with 5,000 oocysts. Fifteen days later each animal was challenged with 50,000 oocysts to test its resistance. (All were totally resistant). To obtain a pool of serum from these donor rats they were terminally bled on days 24-25 post inoculation. Non-immune serum was collected from 10 control (uninfected) rats.

Globulin fractions were prepared by precipitating sera in 100% saturated ammonium sulfate solution (Nain, 1969). With constant stirring, a volume of saturated ammonium sulfate solution to give a final concentration of 50% saturation, was added dropwise to the serum. (This final concentration of 50% saturation precipitates alpha, beta, and gamma globulin, and not albumin, Nain, 1969). The mixture was then centrifuged for 15 minutes at 5,000 rpm. The globulin pellet was then redissolved in PBS (pH 7.1) to give a total volume equal to 1/2 the original serum volume. These procedures were conducted at 4 C. The redissolved globulin precipitate was then dialysed against PBS (pH 7.1) at room temperature for 16-20 hours. Serum protein concentrations were determined by the Folin phenol reagent method of Lowry *et al.* (1951), on duplicate samples. All sera and globulin fractions were kept at 4 C and were injected into recipients within 96 hours of bleeding the donor rats. Sera were examined for agglutinating antibodies by reacting undiluted, and portions diluted 1:20,

1:40, 1:80, or 1:160 with PBS, against sporozoites.

Thirty-five inbred male rats 7-9 weeks of age were divided into seven groups of five rats.

Group I: Five rats were each given a single IV or IP injection of 3 ml of normal serum 24 hours before inoculation.

Group II: Five rats each received one IV or IP injection of 6 ml of immune serum 24 hours before inoculation.

Group III: Five rats each received three IV or IP injections of immune serum; 3 ml at 48 and 24 hours prior to and at inoculation.

Group IV: Five rats each received IV or IP a total of 0.75 g globulins/kg body wt. 24 hours before inoculation.

Group V: Five rats each received a total of 0.6 g globulins/kg body wt. IV or IP divided equally among 3 injections; one at 48 hours and 24 hours prior to and one at inoculation.

Group VI: Five rats each received a total of 0.75 g globulins/kg body wt. IV or IP, divided equally into 4 injections; one at 72, 48 and 24 hours prior to inoculation, and one 24 hours after.

Group VII: Five uninjected rats served as controls.

Following IV or IP injections of whole serum or globulins, each rat was inoculated with 3,500 oocysts. Fifteen days later each animal was challenged with 50,000 oocysts. Control animals in Group VII were each given the same doses of oocysts.

RESULTS

Changes in serum proteins

The electrophoretic separation and characterization of serum proteins from one infected and one normal rat are illustrated in Figure 17. This figure was included to show how the various fractions were identified. Curves illustrating the gross changes of the serum proteins from day 0 to day 64 after inoculation are represented by Figure 18. Day to day variations are shown in Table VI. When analysed against day 0 values only variations of the alpha-1 and beta-2 protein fractions were significantly different (Figures 9 and 10). There were no significant variations in day to day values of serum proteins of non-infected rats (Table VII).

The average alpha-1-globulin values of inoculated rats from both strains are shown in Figure 9. Levels of this fraction were highest on day 0. All infected animals demonstrated a gradual decline in the alpha-1-globulin levels from day 1 to day 16 after primary inoculation. In 2 animals the peak representing this fraction all but disappeared. On or about day 19 there was a gradual increase in the levels of this protein fraction. By day 64 the level of alpha-1-globulin was almost back to day 0 values. Figure 10 illustrates the data for the beta-2-globulin in inoculated rats. From day 1 there was a gradual increase until a peak was reached on day 13 after inoculation. At this time the values obtained were significantly higher than on day 0. After day 13 there was a gradual decline in levels of this fraction until at day 64 they were close to the levels noted on day 0.

Figure 9 Pattern of fluctuations in serum alpha-1-globulin in 20 rats inoculated with *Eimeria nieschulzi* oocysts. Vertical lines represent the range in values observed. Duncan's Multiple-Range tests indicated that the fluctuations of this protein fraction were significant. Mean 6.233 ± 2.048 (± one standard deviation).

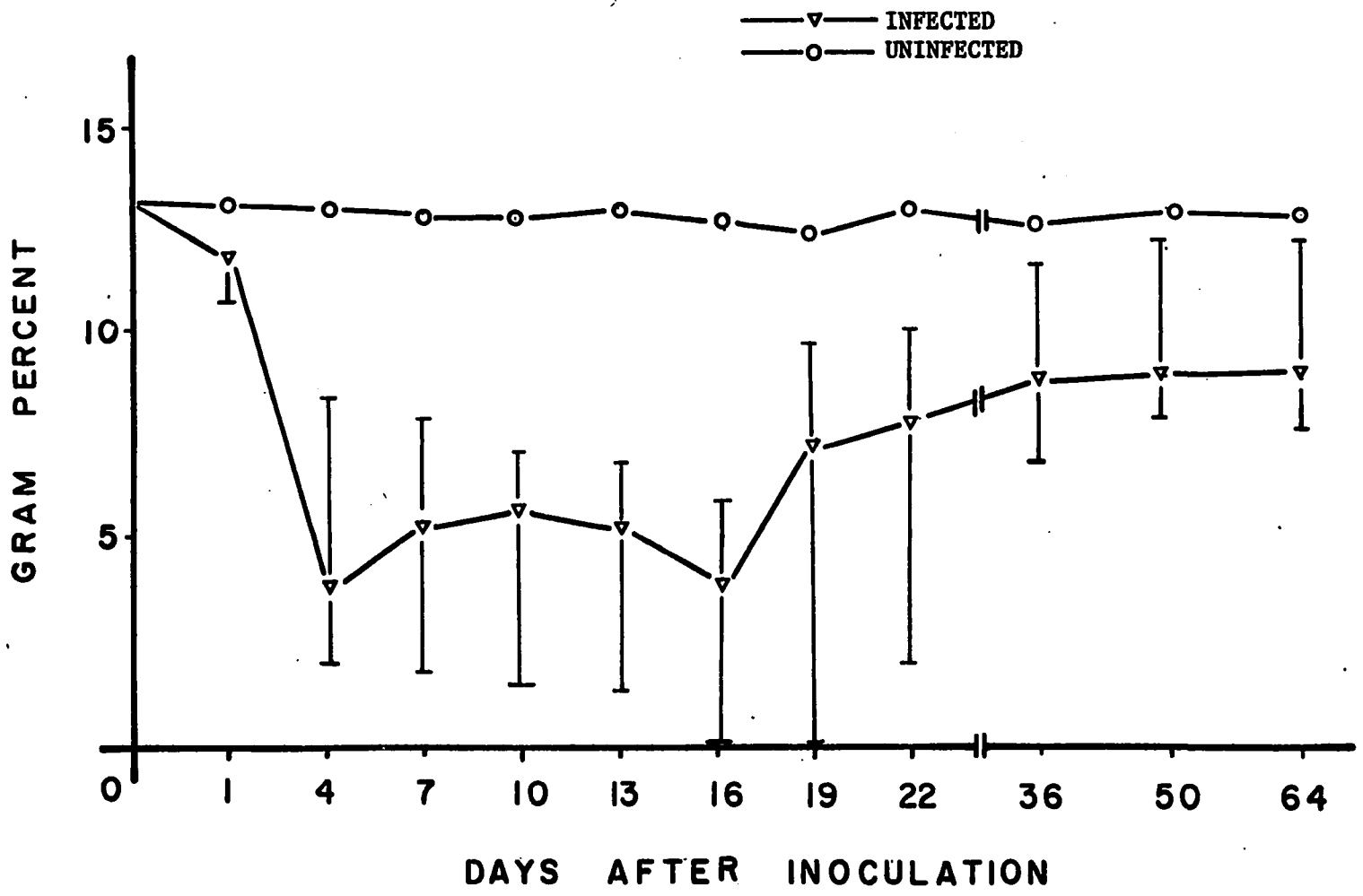
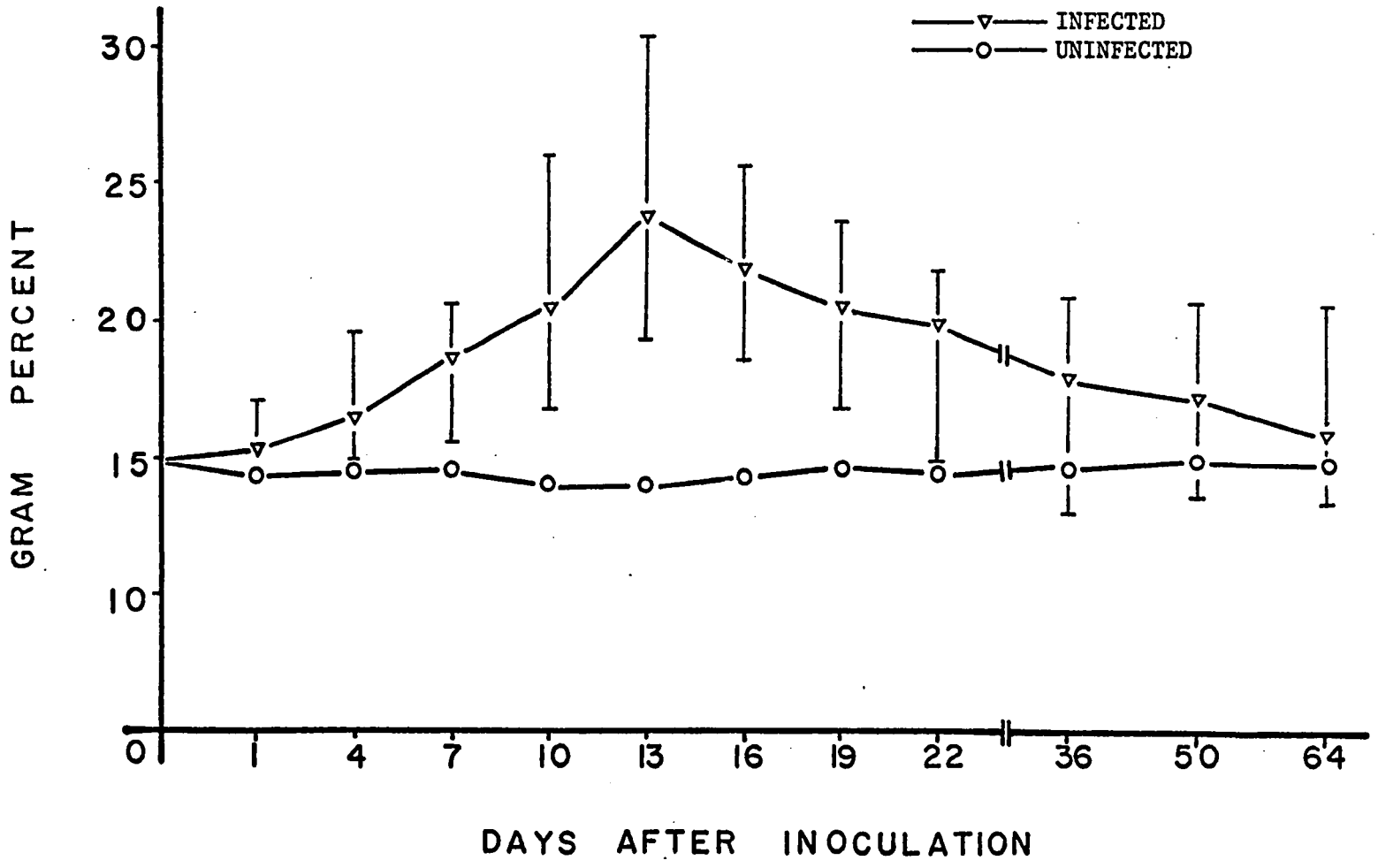

INFECTED
UNINFECTED
GRAM PERCENT
15
10
5
0
1
4
7
10
13
16
19
22
36
50
64
DAYS AFTER INOCULATION

Figure 10 Pattern of fluctuations in serum beta-2-globulin in 20 rats inoculated with *Eimeria nieschulzi* oocysts. Vertical lines represent the range in values observed. Duncan's Multiple-Range tests indicated that the fluctuations of this protein fraction were significant. Mean 19.187 ± 3.324 (± one standard deviation).


INFECTED
UNINFECTED
GRAM PERCENT
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36
50
64
DAYS AFTER INOCULATION

Similar observations were noted in all groups of rats.

The infections that resulted from the inoculation of oocysts were similar in pattern to those observed in Part 1 of this study (Figures 2 and 3).

Slide agglutination of sporozoites

Specific agglutinins were present in the serum of parasitized rats and also in the serum of those which had recovered from *E. nieschulzi* infections. No detectable agglutinating antibody was found in the non-immunized control rats. Agglutinating antibody was first detected between day 4 and day 7 after primary inoculation (Table VIII). Serum diluted 1:20 with buffered PBS gave reactions of at least 1+ at the first indication of antibody. The degree of agglutination increased with subsequent serum samples until maximum agglutination was observed between day 10 and day 16 after inoculation in rats which received a single inoculation, and between day 22 and day 36 after inoculation in animals which received a second inoculation 15 days after the first (Table VIII). Rats which received a primary inoculation of 5,000 oocysts followed by a second with 50,000 oocysts 15 days later showed agglutinating titers which were higher than those receiving one inoculation (Figure 21). The level of the titers also remained high for longer periods. In the doubly inoculated animals there was a larger interval between first detection of antibody and occurrence of maximum titers (Table VIII). After occurrence of maximum titers, the average agglutinating antibody level of each rat declined; however, antibody was still demonstrable 64 days after primary inocu-

lations (Figure 21). No significant difference in titers occurred between the inbred and outbred rats.

Locomotion of sporozoites consisted of gliding and occasional flexing or bending movements. Those sporozoites incubated in saline, nonimmune or heat inactivated immune serum did not agglutinate (Figure 5) whereas those incubated in immune serum or heat inactivated plus normal immune serum became agglutinated (Figure 6). On being exposed to immune serum the sporozoites moved in a random circular manner until two became stuck together (Figure 7). Groups of two (Figure 8) then randomly clumped until a conglomerate was formed (Figure 6). In sporozoites which were exposed to immune serum a granular discharge (Figure 7) was observed at the posterior end of the organism. This phenomenon did not occur in those sporozoites which were incubated in saline, heated immune serum or nonimmune serum. The significance of this discharge and its relationship to agglutination is not known. Deleterious effects of immune serum occurred whether or not the sporozoites were suspended in distilled water or PBS.

Site of antibody production

Antibody was first detected on day 7 after inoculation. The degree of fluorescence was highest at the site of infection. This was rated as 2+ for day 7 and 4+ for days 10, 13 and 16. The upper and lower thirds of the small intestine were rated as 1+ on day 7, 2+ on days 10 and 16 and 3+ on day 13 (Figure 22). Tissue from the large intestine and from the non-infected rats did not fluoresce.

Maximum fluorescence, 4+, occurred in the area of the crypts

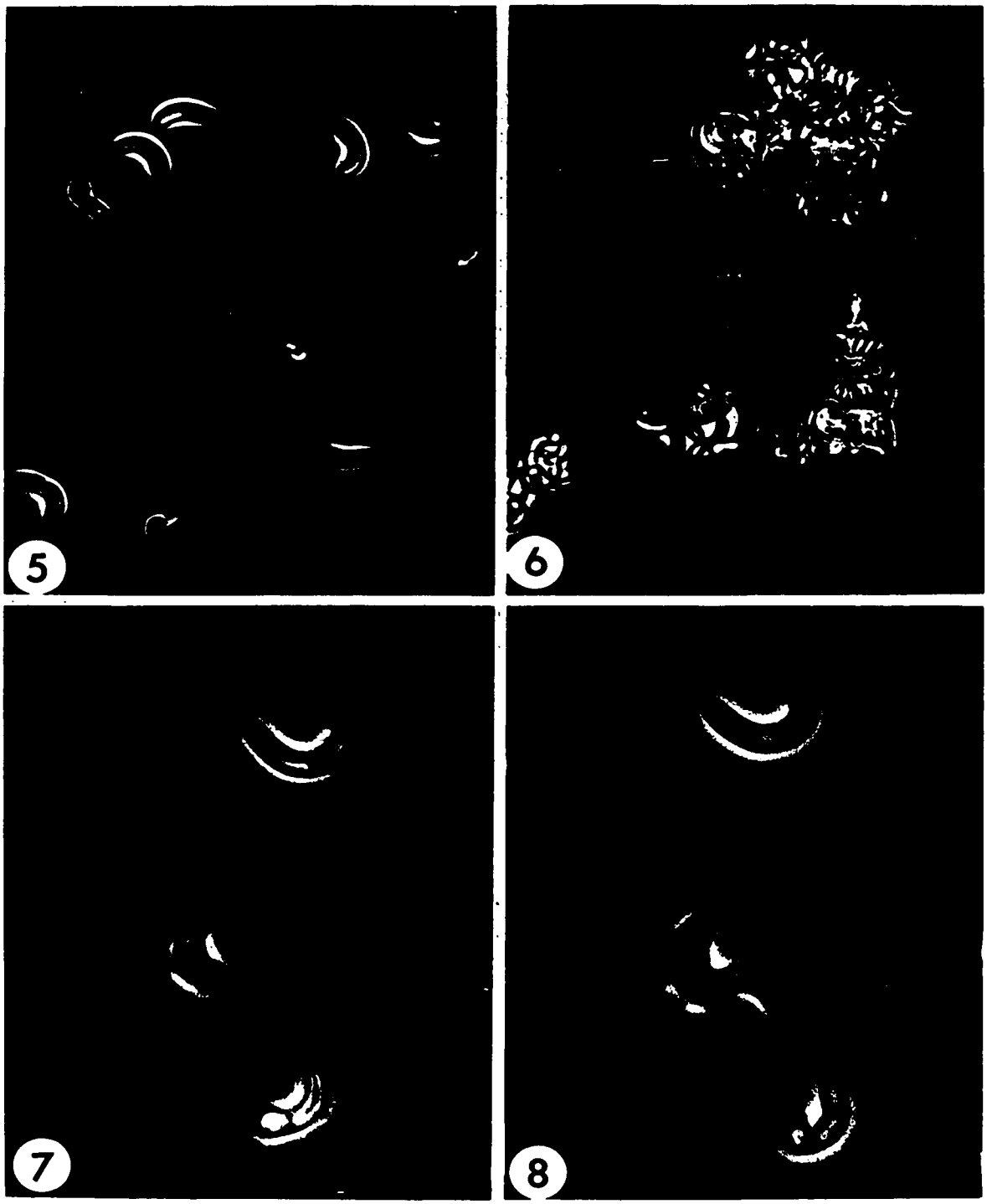
Figure 5 Fresh sporozoites in Ringer's solution (negative agglutination reaction). Phase-contrast microscopy; X 650.

Figure 6 Fresh sporozoites in immune serum diluted 1:20 with Ringer's solution (positive agglutination reaction). Phase-contrast microscopy; X 650.

Figure 7 Sporozoites incubated in immune serum showing two joined together by a granular discharge at the posterior end. Phase-contrast microscopy; X 1300.

A: granular discharge

Figure 8 Sporozoites incubated in immune serum showing groups of two clumped together. Phase-contrast microscopy; X 1300.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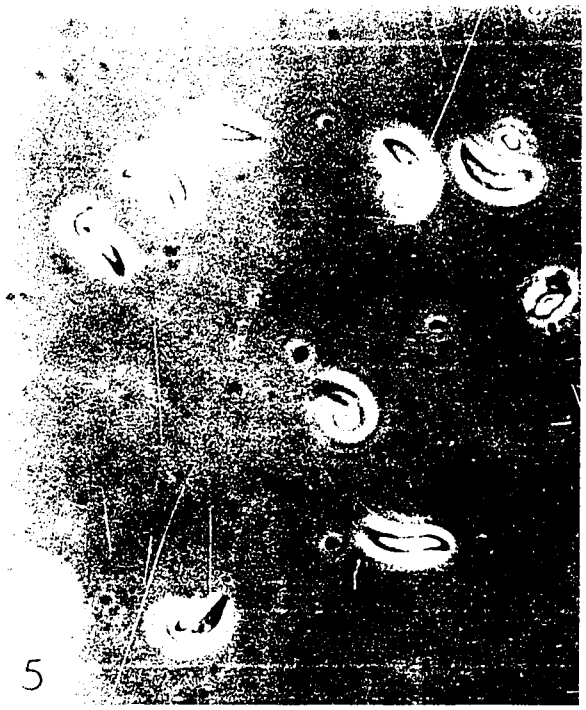

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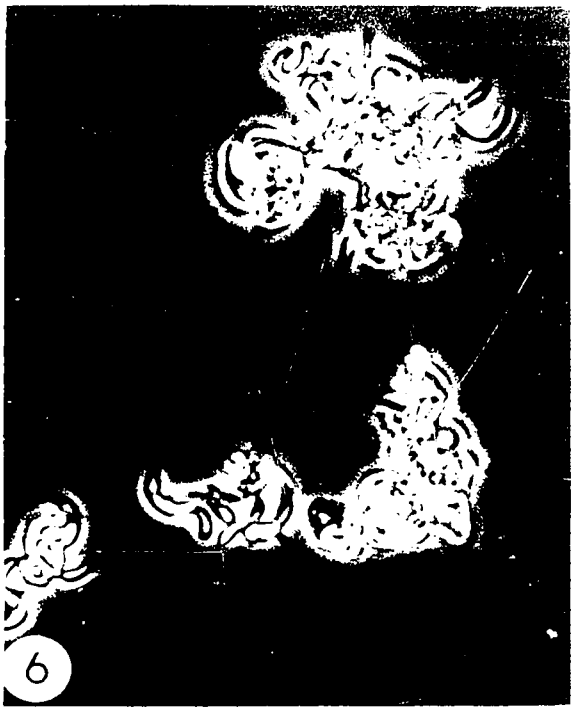

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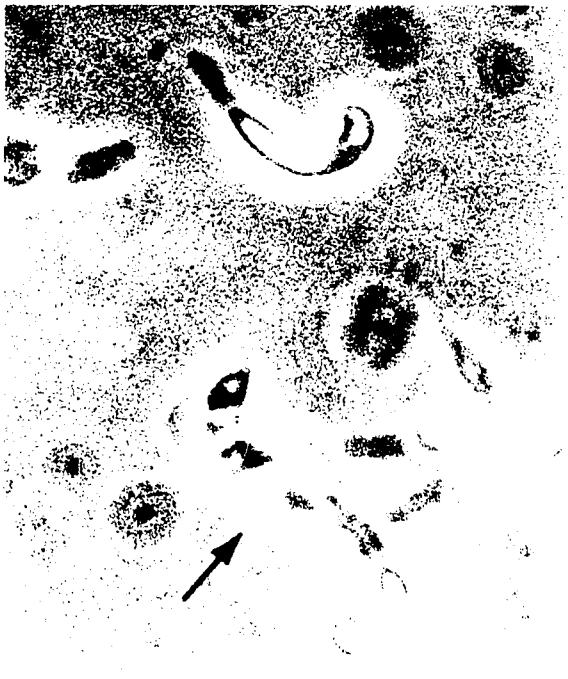


in the middle third of the small intestine. This is the site of infection where the endogenous stages of *E. nieschulzi* usually develop. There was also some fluorescence, rated 2+, in the lamina propria of the villi and in the submucosal area of the site of infection. Fluorescence in the upper and lower thirds of the small intestine occurred mainly at the base of the crypts. Neither the muscular layers nor the columnar epithelial cells of the villi fluoresced.

Passive immunity studies

No passive transfer of resistance was detected even though relatively large amounts of immune serum or serum globulins were injected into susceptible rats and subsequently mild doses of inoculum given (Table III). Experimental infections in the injected rats were similar in intensity and pattern to those in the uninjected ones. Oocyst production was the criterion for determining if passive transfer of serum or serum fractions had any effect on the course of the coccidial infection. There were no significant differences between the groups in the number of oocysts shed during infection. The routes of injecting serum or serum fractions had no effect on the intensity of infection established.

TABLE III

Oocyst production in rats injected with immune serum or immunoglobulins, and inoculated with 3,500 *Eimeria nieschulzi* oocysts.

	Group I	Group II	Group III	Group IV	Group V	Group VI	*Group VII
SERUM INJECTED	Normal	Immune	Immune				
Route	IP IV	IP IV	IP IV				
No. of rats	3 2	3 2	4 1				5
No. of injections	1	1	3				
Volume/rat (ml)	3	6	9				
IMMUNOGLOBULINS							
Route				IP IV	IP IV	IP IV	
No. of rats				3 2	4 1	4 1	
No. of injections				1	3	4	
Globulins/kg body wt.(g)				0.75	0.6	0.75	
Oocysts shed/oocyst given	20,128	19,659	20,034	20,785	20,318	19,998	20,127

*Uninjected controls.

PART 3 CELLULAR ASPECTS OF IMMUNITY

MATERIALS AND METHODS

Thymectomy

This experiment was designed to investigate the effect of neonatal and adult thymectomy on the immune response of rats against *E. nieschulzi*. Male inbred rats were thymectomized by the suppliers within 24 hours of birth; they were weaned at 21 days and then shipped. Adult rats were thymectomized when 8 weeks old and then shipped. None of the thymectomized rats died or showed signs of "wasting." Five rats were kept for 4 months after termination of the experiment.

Experiment 1. Five neo-natally thymectomized and 5 non-operated rats, 26 days old, were each inoculated with 1,500 oocysts. Fifteen days later each animal was challenged with 50,000 oocysts. Two weeks later they were again inoculated with 75,000 oocysts each.

The size of the sensitizing inoculum was deliberately kept small because the effects of *E. nieschulzi* on neonatally thymectomized rats were not known.

Experiment 2. Five neo-natally thymectomized, 8 thymectomized at 8 weeks of age and 5 non-operated rats were each inoculated with 3,500 oocysts when 9 weeks old. At 15 and 30 days post sensitization they were each challenged with 50,000 and 75,000 oocysts respectively. All thymectomized plus 3 non-operated control rats were autopsied at the end of both experiments and examined grossly and histologically for thoracic thymic tissue. No thymic tissue was ob-

served grossly or histologically in any of the thymectomized rats.

Immuno-cyto-adherence

This experiment was designed to determine if thoracic duct lymphocytes (TDLs) from rats actively immunized against *E. nieschulzi* contained cell associated antibody. Sporozoites obtained by *in vitro* excystation were used as antigenic material (Part 1). Thoracic duct lymphocytes collected during the first 24 hours of thoracic duct drainage of immunized rats (see below) were washed 3 times, 5 minutes each, in Dulbecco's medium at 5 C. Two drops of TDL suspension were placed in a culture chamber (1 cm diameter x 3 mm deep). One drop of sporozoite suspension was then added. This mixture was layered with several drops of paraffin oil, covered with a coverslip and observed for up to 60 minutes. The culture chamber with its contents was maintained at 37 C by a Sage air-curtain incubator. During incubation the reaction between TDLs and sporozoites was observed with a Zeiss inverted microscope at a magnification of 400 x. Photomicrographs were made with a Nikon 35 mm camera attached to the system.

Adoptive immunity with thoracic duct lymphocytes

The aim of the experiment was to determine (a) if immunity could be transferred by TDLs from immunized to normal rats, and (b) cell dose required to produce adoptive immunity.

All experiments were performed on inbred (syngeneic) male rats 11-15 weeks of age and weighing 200-250 grams. Donor rats were immunized by inoculating them with 5,000 oocysts. Cannulation and

lymph collection were performed on day 14, the period of maximum agglutinating titers (Part 2). Donor rats used as controls were uninfected.

Thoracic duct drainage. Ninety minutes before the induction of anaesthesia the animal was given 1.5 ml of corn oil, via stomach tube. After an intraperitoneal injection of Nembutal the thoracic duct was cannulated at its intra-abdominal region, using the method of Bollman *et al.* (1948). The cannula (PE 20 or PE 50 Intramedic tubing) was bent by heating in boiling water, soaked in merthiolate 1:1000, flushed with a heparin-saline solution and fixed in position in the thoracic duct by a stitch (Figure 19) and the surgical adhesive Isobutyl 2-cyanoacrylate (Ethicon Sutures, Peterborough, Ontario). The adhesive prevented lymph leakage. The cannula was brought out through the skin (held by a stitch) of the back of the animal. On closing the wound 100 units of heparin were injected. Cannulated rats were placed on modified Bollman type cages (Figure 20) which allowed them to exercise. Thoracic duct drainage was carried out for 5 days. Lymph was collected in iced flasks which contained 2 ml of Dulbecco's T.C. medium to which 5 drops of heparin (without preservative) were added. Every 12 hours the total volume of lymph was measured and the number of cells drained were counted in a haemocytometer. During the period of restraint the animals drank 5% sucrose in saline and were fed rat pellets. Control rats were subjected to sham operation and restraint to evaluate the effect of stress on the operated rats.

Transfusion of thoracic duct lymphocytes. Lymph from several donor rats, which was drained within the first 24 hours after cannulation, was pooled and then centrifuged at 1,000 rpm for 10 minutes at 5 C. The pellet of cells was resuspended in cold Dulbecco's medium and then injected intravenously into the tail of the recipient. In pilot experiments TDLs were injected into recipients 12 hours before inoculation with *E. nieschulzi* oocysts. However, in subsequent experiments they were injected 4 hours prior to inoculation.

The viability (93%) of the TDLs was judged by their motility under phase contrast microscopy and by their ability to exclude a 0.05% trypan blue solution (0.5 ml of cell suspension + 5 drops 0.05% trypan blue).

The number of TDLs injected ranged from 1 million to 1 billion. Control rats were either uninjected prior to inoculation with 5,000 *E. nieschulzi* oocysts, or injected with TDLs obtained from non-immunized rats. The effect of the transfused TDLs on the recipients was assessed by counting the total number of oocysts shed per oocyst given.

In these experiments a different technique for inoculating the rats was used. The animals were not starved prior to inoculation and the inoculum was given in 0.5 ml of water. During the patent period the faeces were crushed with a Sorvall Omni-mix mechanical blender. These procedures gave higher, and more consistent oocyst counts.

RESULTS

Thymectomy

The pattern of infection was not altered by neonatal or adult

thymectomy (Figures 23 and 24). Statistical analysis of the oocyst output showed no significant differences between thymectomized rats and intact control rats.

Thymectomy did not impair the ability of rats to develop resistance to reinfection. In Experiment 1 where the primary inoculation was only 1,500 oocysts, thymectomized and control rats were not totally resistant to the first challenge inoculation of 50,000 oocysts (Figure 23). This substantiates earlier results where it was found that inbred rats required a larger sensitizing dose (Figure 3). In Experiment 2 there was no significant difference in oocyst production between the neonatally and adult thymectomized rats. Both groups of animals were totally resistant to challenge inoculations (Figure 24).

Immuno-cyto-adherence

Thoracic duct lymphocytes from uninfected rats mixed with *E. nieschulzi* sporozoites did not adhere (Figure 11) whereas sporozoites adhered to TDLs from immunized animals. Many of the lymphocytes which took part in adhesion possessed pseudopodia-like projections (Figure 12) in contrast to the other lymphocytic cells. One sensitized TDL may interact with several sporozoites (Figures 12 through 14). Sporozoites which came into contact with certain lymphocytes lost their motility, became granular and were completely immobilized. Some sporozoites were able to pull themselves free but soon became reattached. The entire adhesion process took place in a very short period of time, within 10 seconds. However, lysis of the sporozoites took a much longer time. After being adhered for approximately 30 minutes the sporozoites were

immobile and granular. Not all lymphocytes took part in the sporozoite-lymphocyte adhesion reaction. TDLs from non-immunized rats that were exposed to sporozoites, and observed for up to 60 minutes, were ineffective. After 60 minutes of observation the sporozoites were still motile.

Adoptive immunity with thoracic duct lymphocytes

In pilot experiments cannulated rats were restrained, and drained for various periods of time up to a maximum of 8 days. Animals which survived for this period became emaciated, listless and eventually died. No attempts were made to determine the cause of death. Sham-operated rats survived restraint without mortality.

Figure 25 shows the volume of lymph and the number of lymphocytes drained daily for 5 consecutive days. The greatest number of lymphocytes was always collected within the first 24 hour period after cannulation. This was followed by a marked decline in cell output. The volume of lymph drained increased sharply on the second day, then gradually levelled off. The increase in lymph output of the second day was perhaps due to the large intake of 5% sucrose in saline that operated rats drank during restraint. In most cases the volume of lymph in ml drained over a 5 day period exceeded the weight of the rat in grams.

Thoracic duct lymphocytes from donor rats immunized against *E. nieschulzi*, were effective in adoptive transfer of immunity to susceptible syngeneic recipients. Adoptive immunity of recipient rats was demonstrated by a high degree of resistance to the establishment of primary infection of *E. nieschulzi* after injection of massive doses

of TDLs. The degree of resistance to primary infection in the recipient was directly correlated with the number of transferred TDLs (Table IV). Relative intensities of infection measured by oocyst production in the recipients, injected with 125 x 10^6 to 1 x 10^9 TDLs were much reduced by comparison with the controls. Few oocysts were produced by those rats which received 1 x 10^9 cells. Three animals which received 1 x 10^9 cells were totally resistant to primary infections while 8 others had very low oocyst output (mean number of oocysts shed per oocyst given, 734). All control animals had high oocyst counts (mean number of oocysts shed per oocyst given, 78,355). TDLs from non-immunized donors did not confer resistance to the recipients. The number of oocysts shed by controls and those rats injected with 10 million or less TDLs (immune) were remarkably uniform and no significant quantitative differences were found (Table IV).

It is evident from these results that large dosages of thoracic duct lymphocytes from immunized donors were effective in adoptive transfer of immunity against *E. nieschulzi* when injected into susceptible syngeneic rats.

TABLE IV

Adoptive transfer of immunity against *Eimeria nieschulzi* by thoracic duct lymphocytes.

No. of animals	No. of TDLs injected IV (x 10^6)	**Mean No. of oocysts shed/oocyst given
10 (Controls)	Uninjected	78,036
8 (Controls)	*100	78,347
8 (Controls)	*500	78,375
6 (Controls)	*1,000	78,662
4	1	75,557
4	10	73,789
4	100	60,457
4	125	35,924
4	150	27,822
4	250	13,954
4	350	9,810
8	500	3,111
***11	1,000	734
		****47,275 ± 31,229

*Control animals injected with TDLs from non-immunized rats.

**Each rat inoculated with 5,000 sporulated *E. nieschulzi* oocysts 4 hours after intravenous injection of TDLs.

***Three rats were totally resistant to infection.

****± one standard deviation.

Figure 11 Fresh sporozoites in thoracic duct lymphocyte suspension from non-immunized rats (negative adherence reaction). Phase-contrast microscopy; X 650

A: sporozoite

Figure 12 Fresh sporozoites in thoracic duct lymphocyte suspension from immunized rats (positive adherence reaction). Phase-contrast microscopy; X 500

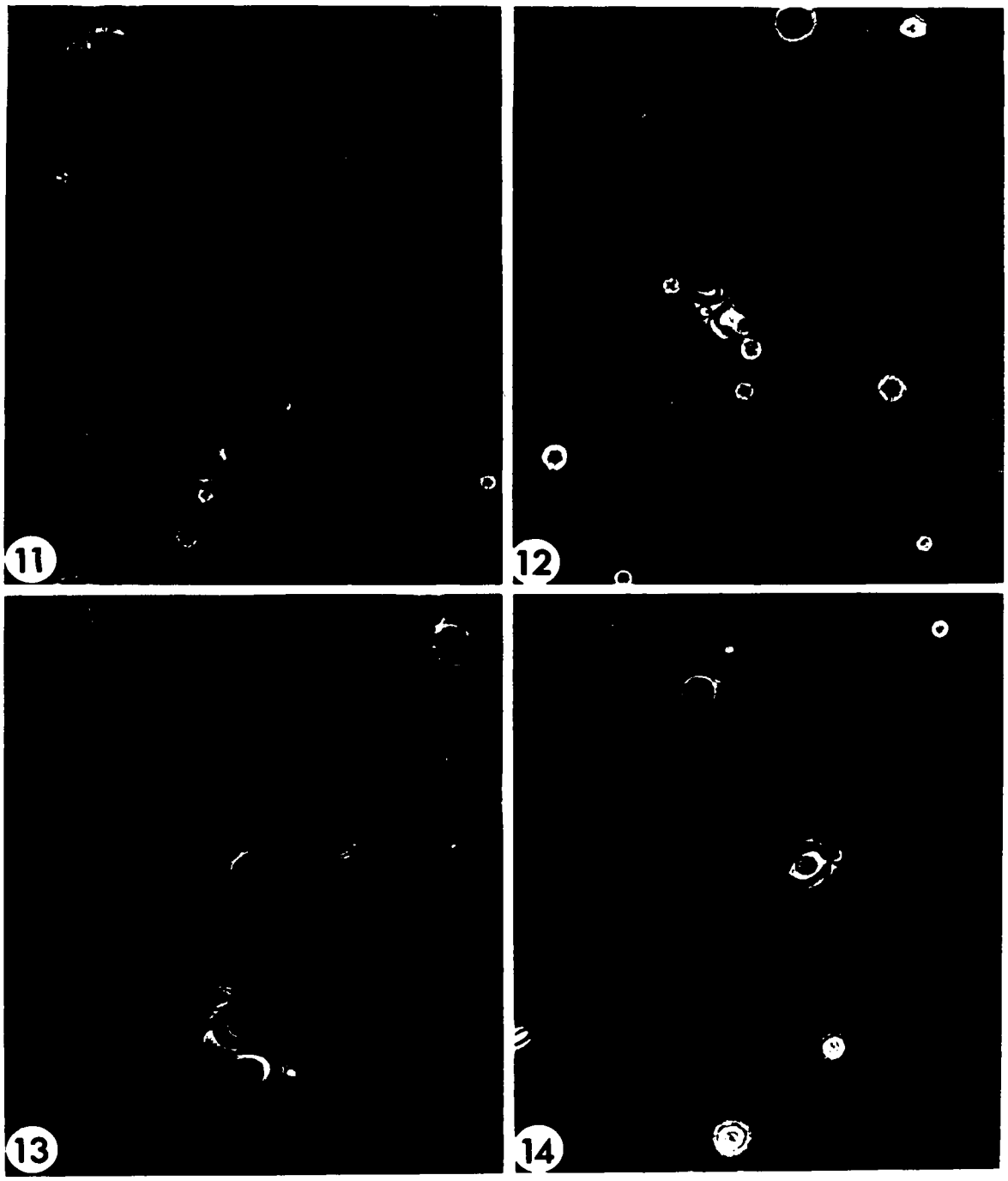
B: sporozoites adhered to lymphocyte with pseudopodia-like projections.

Figure 13 Sporozoites incubated in thoracic duct lymphocyte suspension from immunized rats. Phase-contrast microscopy; X 650.

D: 3 sporozoites adhered to 1 lymphocyte.

Figure 14 Sporozoites incubated in thoracic duct lymphocyte suspension from immunized rats. Phase-contrast microscopy; X 650.

C: 3 sporozoites adhered to 1 lymphocyte.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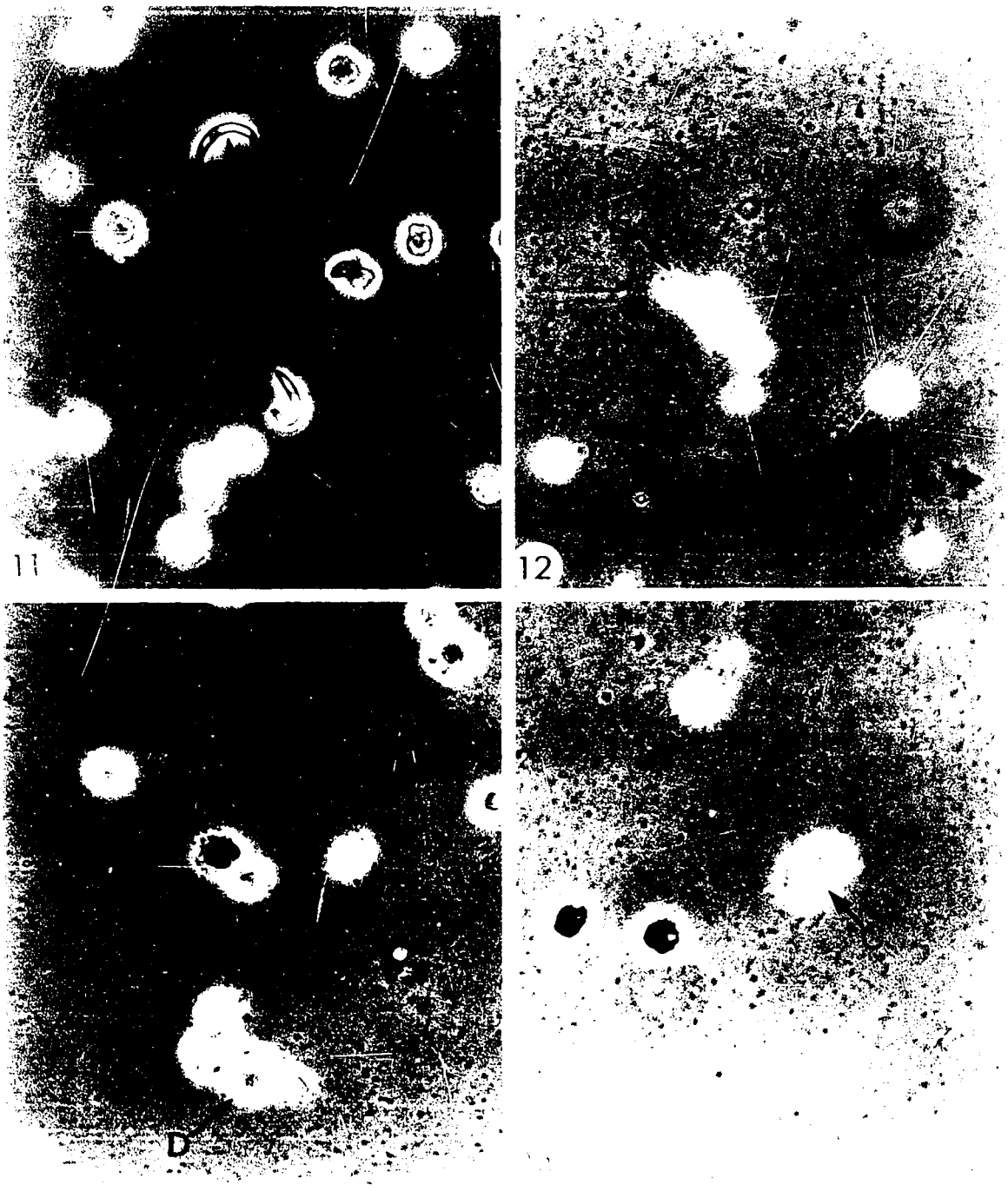
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DISCUSSION

In this study an *Eimeria nieschulzi*-rat model was used to study the immune response of the host to this parasite. The approaches chosen were straightforward. First, I examined the pattern of infection, minimum number of sporulated oocysts needed to produce total immunity in rats, and the effects of parenteral inoculations on intensity of infection. Second, I studied the gross changes that occurred in the serum proteins of parasitized rats and attempted to find out whether the response was only at the local site of infection, or systemic. Third, I examined the effect of thymectomy on the immune response. Fourth, I determined the effects of transferring serum and serum fractions, and thoracic duct lymphocytes from immunized rats to susceptible syngeneic recipients. The major question was: can immunity to *E. nieschulzi* be either passively or adoptively transferred from immunized to normal syngeneic rats?

The pattern of *E. nieschulzi* infection in the strain of rats used was similar to that observed by Hall (1934), Becker *et al.* (1935) and Roudabush (1937). Oocyst discharge started on day 7, reached a peak on day 9 and then rapidly declined until cessation on or before day 12 after inoculation. However, the results obtained on the reproductive potential for our strain of *E. nieschulzi* differ markedly from those reported by Hall, and Becker *et al.* for their strain of the parasite. They found that an infective dose of 1,500 oocysts yielded 192,200 oocysts for each oocyst fed. In my experiments similar dosages produced approximately 30,000 oocysts for each oocyst inoculated.

This disparity in numbers of oocysts produced is not uncommon, and is perhaps due to some or all of the following: differences in techniques; the reproductive potential of each individual parasite in the host; physiological variability within experimental animals; strain differences of the host in susceptibility to parasites (Brackett and Bliznick, 1952).

Throughout this study an inverse relationship between oocyst production and oocyst dosage was found. Larger infective doses produced fewer oocysts per oocyst fed. This corroborates the findings of Hall (1934), and Becker *et al.* (1935) for *E. nieschulzi*, Brackett and Bliznick (1952) for chicken coccidia, and Haberkorn (1970) for *E. falciformis* in mice. The phenomenon of an inverse relationship between parasite input and output is a common one and in this case might reflect a "crowding" effect. Much variation was noted in the number of oocysts produced between individual rats which were inoculated with the same number of oocysts. This finding is similar to that reported by Becker *et al.* (1935) and perhaps reflects the biologic variation of the host since inoculation and other techniques were kept standard and as consistent as possible.

After infections caused by an optimal dosage of *E. nieschulzi* oocysts, rats became totally resistant to subsequent challenge infection with the same species. Hall (1934) found that dosages of 1,500 oocysts conferred about 99.9% resistance while 15,000 oocysts caused total resistance. She also observed that an infective dose of 15,000 oocysts caused mortality in over 50% of the rats. My work showed

that dosages of 2,500 and 3,500 oocysts conferred total resistance in outbred and inbred rats respectively. Smaller infective doses caused only partial resistance. Infective doses of up to 75,000 oocysts did not cause mortality in rats. Rats developed complete immunity to reinfection with no penetration of host tissue and subsequent development of endogenous stages. This concurs with the findings of Morehouse (1938) and Hammond *et al.* (1964), but differs from the observations that in chickens immunized against coccidia, sporozoites were able to penetrate the host cell although further development was prevented (Leathem and Burns, 1967, 1968; and Pierce *et al.*, 1962).

Parenteral inoculations with oocysts and sporozoites of *E. nieschulzi* caused infections in susceptible rats. These infections were less severe than those elicited by a corresponding number of parasites given orally. This is in accordance with the findings of Davies and Joyner (1962), Landers (1960), and Sharma (1964). However, Haberkorn (1970) found that oral, subcutaneous, intramuscular, intravenous and intraperitoneal inoculation of *E. falciformis* oocysts in mice, caused identical infections. In my study both the pattern, and the duration of infection of the parenterally infected rats paralleled that observed in control rats inoculated orally with intact oocysts. This indicated that some of the organisms reached the site of infection shortly after inoculation and had a normal life cycle. The prepatent period for this species was not prolonged as was observed for coccidia of fowls (Davies and Joyner, 1962). Infections caused by parenteral inoculations were largest when the intraperitoneal route was used, and smallest following

intravenous and intramuscular injections. Haberkorn found that the infections caused by parenterally inoculating oocysts into mice were identical. Davies and Joyner found that *E. tenella* infections were heaviest following intravenous injection of sporozoites than by other parenteral routes. Neither the mechanisms for the transportation of the parenterally inoculated parasite to the infective site, nor the causes for the different intensities of infection established by various routes have been elucidated.

Intraperitoneal inoculation of intact oocysts caused heavier infections than a corresponding number of sporozoites inoculated by the same route. Sporozoites may be more susceptible to host internal influences than are intact oocysts.

Coccidial infections often cause serum protein changes which are detectable by electrophoretic analysis (Dunlap *et al.*, 1959; Schlueter, 1959; Fitzgerald, 1964a; Heckman, 1968). Dunlap *et al.* found significant increases in both beta and gamma globulin fractions, but no changes in serum albumin or total proteins in rabbits infected with *E. stiedae*. Some of their results were similar to mine. In my experiments a significant increase was found in the beta-2-globulin fraction, a decrease in the alpha-1-globulin fraction and no changes in either gamma - globulin components. The importance of the serum protein changes during coccidial infections in relation to acquired immunity is not known. All reports indicate that serum protein changes were not influenced by challenge infections since demonstrable secondary response was not made evident by electrophoretic analysis. It seems

therefore, that serum protein changes do not have a direct correlation with the immune status of the host during coccidial infections.

Direct evidence that an antibody response is elicited during coccidial infections has been shown by *in vitro* tests (McDermott and Stauber, 1954; Rose and Long, 1962; Andersen *et al.*, 1965). McDermott and Stauber observed that sera from chickens infected with *E. tenella* caused agglutination of second generation merozoites. Andersen *et al.* found that merozoites of *E. bovis* agglutinated in immune serum. In my experiments sporozoites agglutinated in immune rat serum. The slide agglutination technique was useful in following the course of antibody production during and after coccidial infection.

Agglutinating antibody was first detected between day 4 and day 7 after inoculation. The sampling period of every 3 days might have exaggerated the variability in first appearance of antibody in rats. Maximum agglutinating titers were highest in rats which received two inoculations. Titers reached 1:160, between 10 and 16 days after inoculation in rats which received one inoculation, and 1:640 between 22 and 36 days in rats receiving two inoculations. Higher maximum titers in rats which received two inoculations suggest a secondary response. Although a secondary response was not evident by electrophoretic analysis of serum, the higher agglutinating titers indicate that such a response occurred. Andersen *et al.* (1965) also reported higher maximum titers in calves which received two inoculations of *E. bovis*.

The effects of immune serum on sporozoites were similar to

those reported by Andersen *et al.*, (1965) for first generation merozoites of *E. bovis*. Sporozoites became granular, discharged material at the posterior end, and became clumped and immobile. These observations demonstrate that humoral antibodies produced in animals infected with coccidia have deleterious and immobilizing effects against various endogenous stages of the parasite, *in vitro*.

Attempts to demonstrate a protective role by passively transferring immune serum or serum fractions from immunized to normal recipients have been unsuccessful (Becker *et al.*, 1935; Augustin and Ridges, 1963; Pierce *et al.*, 1963; Rose, 1963; Fitzgerald, 1964). The results of the present study corroborate their findings. My attempts at passive immunization with immune serum or serum globulins were ineffective in demonstrating any protective function against *E. nieschulzi* in susceptible rats. The transfer of high-titer serum did not moderate the infection (Table III), despite the immobilizing effects observed against sporozoites *in vitro*. Long and Rose (1965) were able to protect susceptible chickens from *E. tenella* infections with sporozoites injected intravenously, by injecting immune serum globulins at a time when the sporozoites were circulating in the blood. They found that globulins were ineffective once the sporozoites had penetrated. Recently, Rose (unpublished data quoted by Ogilvie, 1970) reported a definite protective role for immunoglobulins (antisera) against *E. maxima* in fowls. Complete details were not available, but she found a reduction in oocyst output in challenged,

passively immunized birds. Apparently the resistance transferred was only partial.

Any attempts at assessing the efficacy of passive transfer of immunity by humoral antibodies, or when correlating this efficacy with volume of serum transferred, should consider the dilution factor in the circulating and extravascular fluids of the recipients. The availability of specific antiparasite immunoglobulins to the site of infection at the precise time to inhibit penetration of sporozoites or stop their development must also be taken into account (Long and Rose, 1965).

Serum antibodies are thought to have a minor protective role in coccidial immunity (Long and Pierce, 1963; Pierce and Long, 1965; Long and Rose, 1965; Rouse and Burns, 1971). Long and Pierce, and Pierce and Long found no suppression of immunity in birds bursectomized *in ovo* by the administration of testosterone. Neither lytic antibodies to sporozoites, nor gamma-globulins were detectable in these birds. It should be pointed out that the effects of testosterone as a hormonal suppressant are too general to allow precise interpretations of their findings. They also omitted the sex of the fowls used - this is an important omission (Ruth, personal communication). Recently, Rouse and Burns substantiated the results of Long and Pierce, and Pierce and Long. They found that surgical bursectomy of chickens did not affect their ability to develop resistance to *E. tenella* infections.

The production of humoral antibodies during coccidial infections is well documented. Several workers have used serological methods to demonstrate acquired antibodies against coccidia (McDermott and Stauber,

1954; Rose, 1959, 1961, 1963; Rose and Long, 1962). Recently, a more sensitive serological technique, the indirect fluorescent antibody staining method (developed by Coons and Kaplan, 1950) was used by Andersen *et al.* (1965) in a study of humoral antibody production to *E. bovis*. These workers used first generation merozoites as antigenic material to detect the presence of acquired antibodies in the serum of calves infected with *E. bovis*. They were the first to use immunofluorescence in demonstrating humoral antibody to coccidia. My experiments were designed to use the indirect fluorescent antibody technique in demonstrating localization of antibodies in tissues during *E. nieschulzi* infections in rats. Although this study was very preliminary, it was apparent that rats produced localized antibody in response to *E. nieschulzi* infection. This was demonstrated by varying amounts of fluorescence throughout the small intestine. The greater degrees of fluorescence in the lamina propria of the villi and crypts at the site of infection indicated the presence of more antibody or antibody forming cells at these locations. Fluorescence could not be seen in individual cells, but was rather confined to the areas mentioned. As noted earlier, these tests were preliminary and the results obtained can serve merely as guidelines. Nonetheless, demonstrating tissue antibody throughout the entire small intestine supports the idea of a diffuse response that was suggested by Burns and Challey (1959), Horton-Smith *et al.* (1961) and Hammond *et al.* (1964).

The elucidation of the immune response

The elucidation of the mechanism of immune response to coccidia has proved to be a challenging problem for fundamental immunology. As discussed earlier, attempts to passively transfer coccidial immunity with serum or serum fractions have been entirely unsuccessful, except for the recent report by Rose (quoted in Ogilvie, 1970). The investigations on cellular immunity to coccidial infections have been equally fruitless (Becker *et al.*, 1935; Burns and Challey, 1968; Long and Pierce, 1963; Pierce and Long, 1965; Rose, 1968a; Rose and Orlans, 1968; Rommel, 1970). However, the use of syngeneic hosts in the present study offered the possibility of a more critical examination into cellular immunity to coccidia.

The importance of the thymus in the development of immunologic competence in vertebrates is generally accepted. The thymus as a separate lymphoid organ is of importance in development of complete immunological competence (Miller, 1964). Removal, neonatally or when adult, often depresses immunological responses in some species (Brown, *et al.*, 1968; Metcalf, 1966; Miller, 1961; Waksman *et al.*, 1962). However, the concept that immunological competence is inhibited or suppressed by thymectomy does not seem to apply in certain forms of parasitism of the intestines (Pierce and Long, 1965; Rose, 1968b; Rouse and Burns, 1971; Wilson *et al.*, 1967). It appears that in these associations the animal's immunological competence is not thymus-directed. Evidence for this view was obtained by Good *et al.* (1966) who proposed that peripheral lymphoid tissue e.g. Peyer's patches of

the intestines, may function in a capacity comparable to the thymus, against intestinal parasites. Waksman *et al.* (1962) supported this view by suggesting the possibility of a second population of small lymphocytes in the rat, the function of which is unrelated to the thymus lymphocytes.

My thymectomy experiments were aimed primarily at altering the rat's cellular immune mechanism. The results demonstrated that (a) rats thymectomized at birth and infected within 3 weeks of thymectomy, (b) rats neonatally thymectomized and infected when adult (8-9 weeks), and (c) rats thymectomized when adult and then infected, have the ability to develop acquired resistance to *E. nieschulzi*. Thymectomized rats responded similarly to control animals when infected with *E. nieschulzi*. The pattern of infection was the same and there was no delay or suppression of the immune response.

These observations suggest that a thymus-dependent mechanism is not involved in the control of *E. nieschulzi* infections in rats. The results of other studies also favour this interpretation for *Eimeria* infections (Pierce and Long, 1965; Rose, 1968b; Rouse and Burns, 1971) in that thymectomized chickens became as resistant to coccidial infections as the controls. On the other hand, my results conflict with the observation of Pierce and Long that thymectomized animals tended to produce more oocysts in the immunizing infections. This disparity may be due to the differences in both host and parasite species studied. No evidence of increased oocyst production in thymectomized rats was observed in my experiments.

Several explanations are possible which may be responsible for the seeming lack of effect of thymectomy on suppression of resistance to coccidia. Complete neonatal thymectomy is difficult to achieve, however, histological examination of my thymectomized rats did not reveal the presence of any residual thymic tissue. Waksman *et al.* (1962) suggested that (a) there are two types of small lymphocytes, thus removal of the thymus could affect the type of cells not responsible for development of immunity to coccidia; (b) thymectomy is not performed sufficiently early in the life of the rat, since the organ is large and mature in appearance at birth, hence seeding from the thymus might have already occurred; (c) there is the possibility that thymectomized rats had small amounts of residual or ectopic thymus. It is not known which, if any, of these factors might have influenced the results obtained.

In my experiments neither neonatal nor adult thymectomy produced detectable suppression of the rat's ability to develop acquired resistance to *E. nieschulzi* infections.

The last area to be examined was the cellular components (thoracic duct lymphocytes) of the immune response and their ability to adoptively transfer resistance to *Eimeria*. Several workers have demonstrated adoptive immunity to other parasitic protozoa (Frenkel, 1967; Roberts and Tracey-Patte, 1969). There are no reports which have unequivocally linked cellular components to the immune response against coccidia. Several investigators have suggested that lymphoid cells have a role in cellular immunity to coccidia (Pierce and Long,

1963, 1965; Rose, 1968b). Rose (1968b) found equivocal results in her attempts to adoptively transfer immunity against *E. tenella* by way of peripheral leukocytes, spleen, caecal tonsil and peripheral exudate cells. Her attempts to modify immunity to *Eimeria* spp. with antilymphocyte serum were also unsuccessful. Rommel (1970) was unsuccessful in trying to adoptively transfer immunity to susceptible pigs against *E. scabra* with lymphocytes from regional lymph nodes of immunized pigs.

My studies on thoracic duct drainage demonstrated several characteristics which were described by Gowans (1959). One of the most outstanding was the fall in lymphocyte output which followed extended drainage of lymph from the thoracic duct. Another feature was that most cells drained for any 24 hour period were obtained within the first 24 hours of cannulation. Gowans (1962) demonstrated that lymphocytes collected after prolonged drainage may be functionally different from those which emerge in the lymph of a freshly cannulated rat. He also pointed out that thoracic duct drainage diminishes the serum globulin levels by removal of the cells or their precursors responsible for synthesis. He further showed that in lymph collected during the first day after cannulation about 95% of the cells are typically small lymphocytes, and prolonged drainage caused a progressive fall in the output of the percentage of these small lymphocytes. Gowans and Uhr (1966) postulated that the small lymphocytes are responsible for immunocompetence and immunological memory. In view of the above and in order to avoid bacterial contamination and mortality

of the TDLs during drainage, only cells collected during the first 24 hours of cannulation were used in immuno-cyto-adherence and adoptive immunity experiments.

In experiments involving thoracic duct lymphocyte-sporozoite interactions it was observed that when brought together in culture the lymphocytes were seemingly able to adhere to and eventually cause destruction of the sporozoites. No phagocytosis of sporozoites was observed. Although the studies cannot be compared directly, it is noteworthy that the lymphocyte-sporozoite adherence was similar to the formation of "rosettes" between lymphoid cells and target cells described by Storb and Weiser (1967), and Denham *et al.* (1969). These workers showed that when incubated together, sensitized lymphoid cells were capable of adherence on target cells. With time, the sensitized cells were capable of destroying the target cells in culture. In my experiments only sensitiized cells were capable of adherence. One lymphocyte often adhered to two or three sporozoites and vice versa.

The cause of adherence and eventual destruction of the sporozoites is not known. Pearsall and Weiser (1970), proposed that lymphocytes can carry specific recognition factors in the form of cytophilic antibodies which promote adherence and destruction of antigen bearing particles. The deleterious effects of sensitized lymphocytes on the sporozoites were not unlike those noted by Denham *et al.* (1969), who reported the specific cytotoxic action that antigenically stimulated sheep lymph cells had on lymphoma cells when cultured together. The mechanism of cell death of the target cell is not known, but may be due

to a change in permeability of surface membrane which often follows cellular interactions (Pearsall and Weiser, 1970).

Thoracic duct lymphocytes are responsible for immunocompetence and immunological memory (Gowans and Uhr, 1966) and are able to adoptively transfer antibody producing to syngeneic recipients (Gowans and McGregor, 1965). When injected into recipients, lymphocytes are able to localize in various organs including the small intestines (Gowans and Knight, 1964; Armstrong, personal communication) within 1 hour after injection (Hall and Smith, 1970). These latter workers observed that approximately 20% of the injected lymphocytes are found in the small intestine between 1-5 hours after injection. There is some relevance between the observations of these workers and the lowering of intensities of infection in susceptible rats, after receiving large doses of thoracic duct lymphocytes from immunized rats, that I observed. It is proposed that protection conferred by the injected cells occurred at the site of sporozoite penetration which was populated by the sensitized lymphocytes. Lymphocyte-sporozoite adherence tests indicated that the sensitized lymphocytes were immunocompetent cells.

Immunity has been adoptively transferred to other intracellular protozoan parasites. Frenkel (1967) found that direct transfer of spleen and lymph node cells were effective in inducing immunity to *Besnoitia jellisoni* and *Toxoplasma gondii*. These species of obligatory parasites are closely related to coccidia. Roberts and Tracey-Patte (1969) adoptively transferred immunity to *Plasmodium berghei*

by transferring sensitized spleen cells to susceptible rats. The lowering of intensity of infections in the susceptible recipients that was reported by these workers was also found in my study. That protection against penetration of sporozoites occurred was made evident by the massive reduction in parasite load that rats receiving doses of 125 million or more lymphocytes showed.

In my experiments, acquired immunity to *E. nieschulzi* has been adoptively transferred to susceptible syngeneic rats with sensitized thoracic duct lymphocytes. The lymph cells used were a mixed population and no attempt was made to identify the cell types involved in the various responses. Cell sensitization is rather important as cells from non-immunized rats were ineffective in their ability to adhere to sporozoites or transfer immunity. Successful transfer of cells was accomplished by using syngeneic rats, thus no prior conditioning was necessary and there was no mortality following massive injections of lymphocytes. The ability of the lymphocytes to transfer immunity on being taken after 14 days of infection, is consistent with the appearance of clinical immunity. Rats inoculated with *E. nieschulzi* develop resistance to challenge infections, with the same species, given 14-15 days after primary inoculation (Becker *et al.*, 1935; Morehouse, 1938; personal observation).

This first demonstration of the ability of thoracic duct lymphocytes to transfer immunity against *E. nieschulzi* offers a new approach for studying the immune response to coccidia. Although the precise immune mechanism to coccidia was not elucidated in this study,

transferred TDLs probably act either directly on the parasite, or indirectly in setting up an unfavourable environment for the parasite in the host. That immunity was transferred to recipient rats by sensitized thoracic duct lymphocytes and not by immune serum would indicate that the immune response to *E. nieschulzi* is primarily cellular. The results of the study not only point to a cellular immune response, but also supply an affirmative answer to my major question: can acquired resistance to *E. nieschulzi* be passively or adoptively transferred? The study offers new tools for elucidating the immune mechanism to coccidia. This feature makes the *Eimeria nieschulzi*-rat model a valid one for studying intracellular parasitism and immunity.

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APPENDIX A

TABLE V

*Oocyst production in 5 outbred rats intraperitoneally inoculated on day 0 with 24,000 *E. nieschulzi* sporozoites and with 50,000 sporozoites 15 days later.

Rat No.	Days after inoculation						Total	Oocyst shed/ oocyst given
	7	8	9	10	11	12		
224	**1,468	4,320	46,300	8,200	80	16.5	60,385	20.1
225	866	9,720	52,100	4,358	56	9.7	67,110	22.3
226	988	8,600	30,200	3,600	164	12.7	43,565	14.5
227	2,528	5,310	75,000	4,750	250	5.2	87,843	29.3
228	1,370	4,150	64,000	3,900	75	20	73,515	24.5
Total	7,220	32,100	267,600	24,808	625.2	64.2	332,417	
Average	1,444	6,420	53,520	4,961	125.04	12.8	66,483	22.2

*No oocysts were produced following challenge inoculations.

**All numbers / (x 10^3).

TABLE VI

Average serum protein values for rats inoculated with *Eimeria nieschulzi* oocysts (10 rats per group).

Day of sample	Albumin %	Globulin %				
		Alpha-1	Alpha-2	Beta-1	Beta-2	Gamma
*Group A						
0	50.5	12.8	2.8	4.4	13.5	16
1	55.5	8.1	2.6	2.9	15.7	15.2
4	55.1	4.9	3.2	4.4	16.3	16.1
7	56.8	4.9	2.4	2.7	19.1	14.1
10	50.9	6.7	3.4	2.5	21.4	15.1
13	50.6	4.4	3.1	2.2	25.3	14.4
16	52.4	4.8	3.5	2.7	21.8	14.8
19	52.7	7	2.9	2.3	21.6	13.5
22	52.1	7.6	4.7	3	19.5	13.1
36	55.7	7.5	3.1	2.3	16	15.4
50	52.2	5.8	3.6	3.5	17.6	17.3
64	52	5.5	3.5	3.2	15.7	20.1
**Group B						
0	48.1	10	3.5	3.1	15.1	20.2
1	48.3	7.7	3.2	4.4	15.9	20.5
4	47.9	5.4	1.4	6.1	18.6	20.6
7	47.2	6.7	1.3	3.7	21.5	19.6
10	48	7	2.1	3	24.5	15.4
13	50.9	4.2	1.8	2.7	26.3	14.1
16	53.4	3.4	2.5	1.1	21.7	17.9
19	50.2	5	2.3	2	21.6	18.9
22	51.7	4.1	2.4	3.7	18.1	20
36	51	4.4	3.5	3	18.6	19.5
50	50.5	5.4	3.4	3.4	18.4	18.9
64	51.9	6.3	3.6	3.6	16.7	17.9

*Group A, each rat inoculated with 5,000 oocysts.

**Group B, each rat inoculated with 5,000 oocysts and challenged 15 days later with 50,000 oocysts.

TABLE VII

Average serum protein values for 10 non-immunized rats.

Day of sample	Albumin %	Globulin %				
		Alpha-1	Alpha-2	Beta-1	Beta-2	Gamma
0	49.1	13	3.5	4.4	15.7	14.3
1	49.5	12.7	3.5	3.5	14.9	15.9
4	49.1	10.8	3.5	3	15.6	18
7	47.5	13.5	3.6	4.5	15.8	15.1
10	47.8	12.2	3.2	3.7	14.4	18.7
13	50.2	12.4	3.6	3.1	14.7	16
16	50.2	11.9	3	3.5	15.2	16.2
19	48.2	13.2	3.5	3.5	15.4	16.2
22	49.6	11.4	3.6	3.2	14.9	17.3
36	49.8	12.7	3.9	3.9	15.3	14.4
50	50.7	13.2	3.5	3.9	16.3	12.4
64	48	13.6	3.8	4.7	15.2	14.7

TABLE VIII

Occurrence of agglutinating antibodies against *Eimeria nieschulzi* as demonstrated by agglutination tests.

Rat No.	Earliest	Maximum		Latest	
	Day	Day	Titer	Day	Titer
Group I					
501	7	16	1:160	64	1:20
502	4	13	1:160	64	1:20
503	7	13	1:80	50	1:40
504	7	13	1:160	64	1:20
505	7	16	1:160	64	1:40
Group III					
601	7	13	1:80	64	1:20
602	7	10	1:160	64	1:20
603	4	16	1:160	64	1:20
604	7	16	1:80	64	1:40
605	7	16	1:160	50	1:20
Means	6.4	14.2	1:136	61.2	1:26
Group II					
506	4	22	1:320	64	1:40
507	7	22	1:320	64	1:80
508	7	22	1:320	64	1:80
509	7	22	1:640	64	1:20
510	7	22	1:320	50	1:40
Group IV					
606	4	22	1:320	64	1:40
607	7	22	1:320	64	1:40
608	7	22	1:320	64	1:20
609	4	36	1:320	64	1:40
610	7	22	1:320	64	1:80
Means	6.1	23.4	1:352	62.6	1:48
Level of significance (Grp. I, III, vs. II, IV)	NS	0.001	0.001	NS	0.05

Groups I and III, each rat inoculated with 5,000 oocysts.

Groups II and IV, each rat inoculated with 5,000 oocysts and challenged 15 days later with 50,000 oocysts.

APPENDIX B

FIGURES AND EXPLANATION OF FIGURES

(15 THROUGH 25)

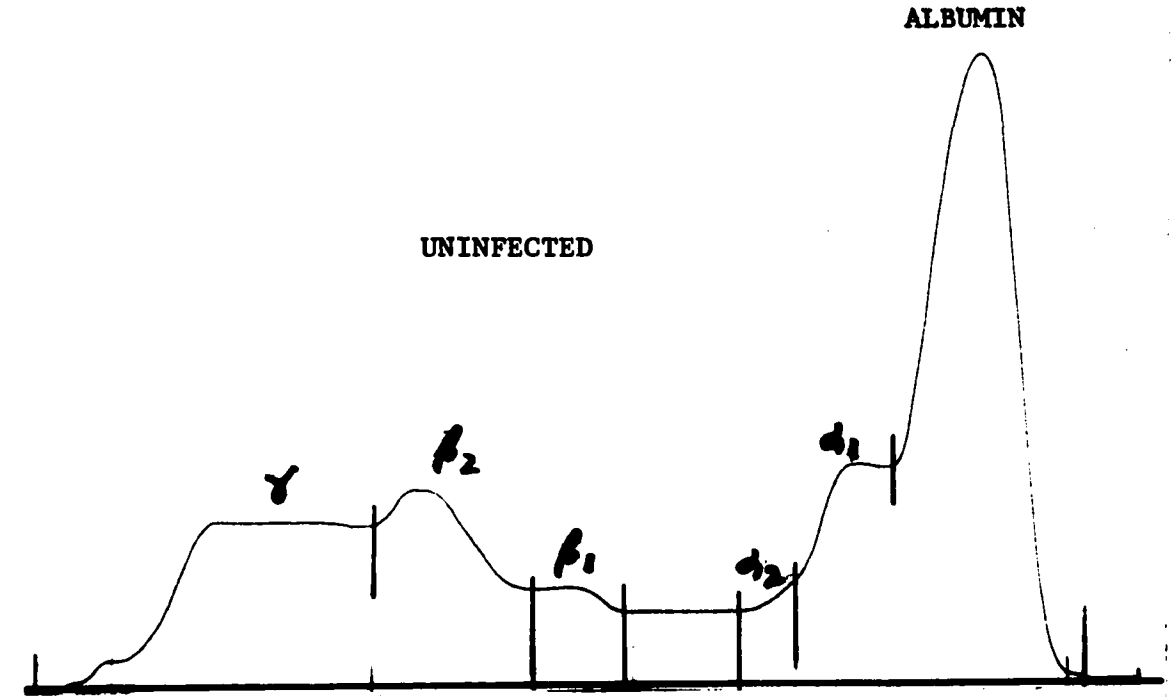
Figure 15 Fresh, cleaned oocysts in Ringer's solution.
X 1500

Figure 15 Fresh, cleaned oocysts in Ringer's solution.

X 1500

Figure 16 Apparatus and technique used in collecting blood samples from rats.

Figure 17 Electrophoretic patterns of the serum of uninfected and infected rats.


ALBUMIN
UNINFECTED
γ
β2
β1
α2
α1

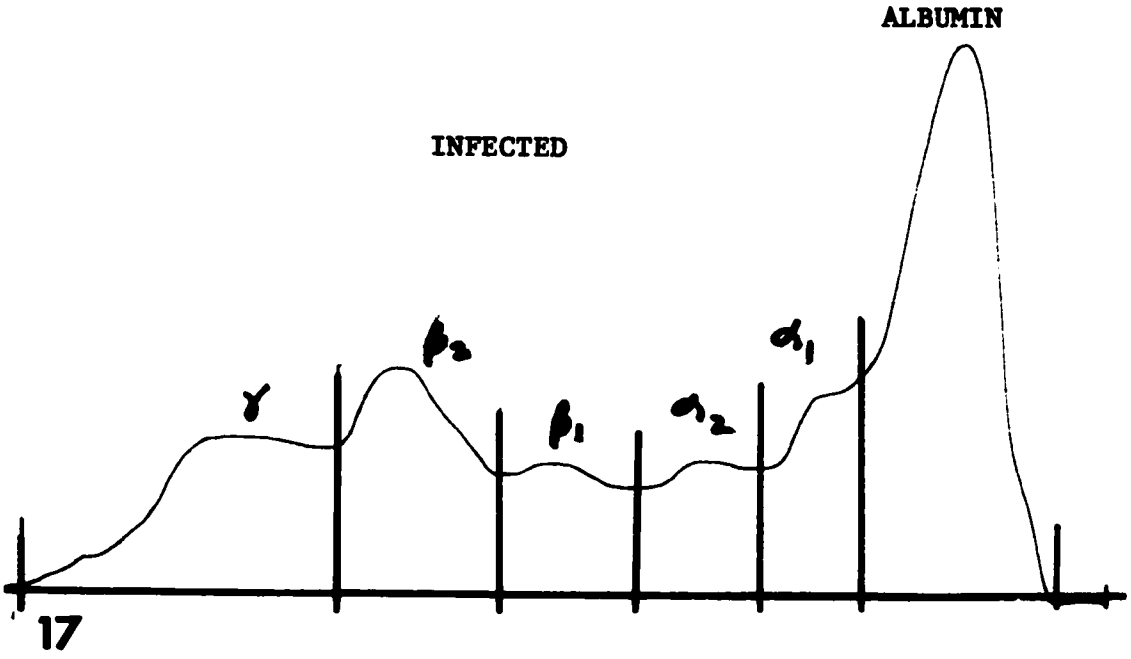

ALBUMIN
INFECTED
γ
β2
β1
α2
α1

Figure 18 Electrophoretic changes occurring in the blood serum protein of a rat following inoculation with *Eimeria nieschulzi* oocysts.

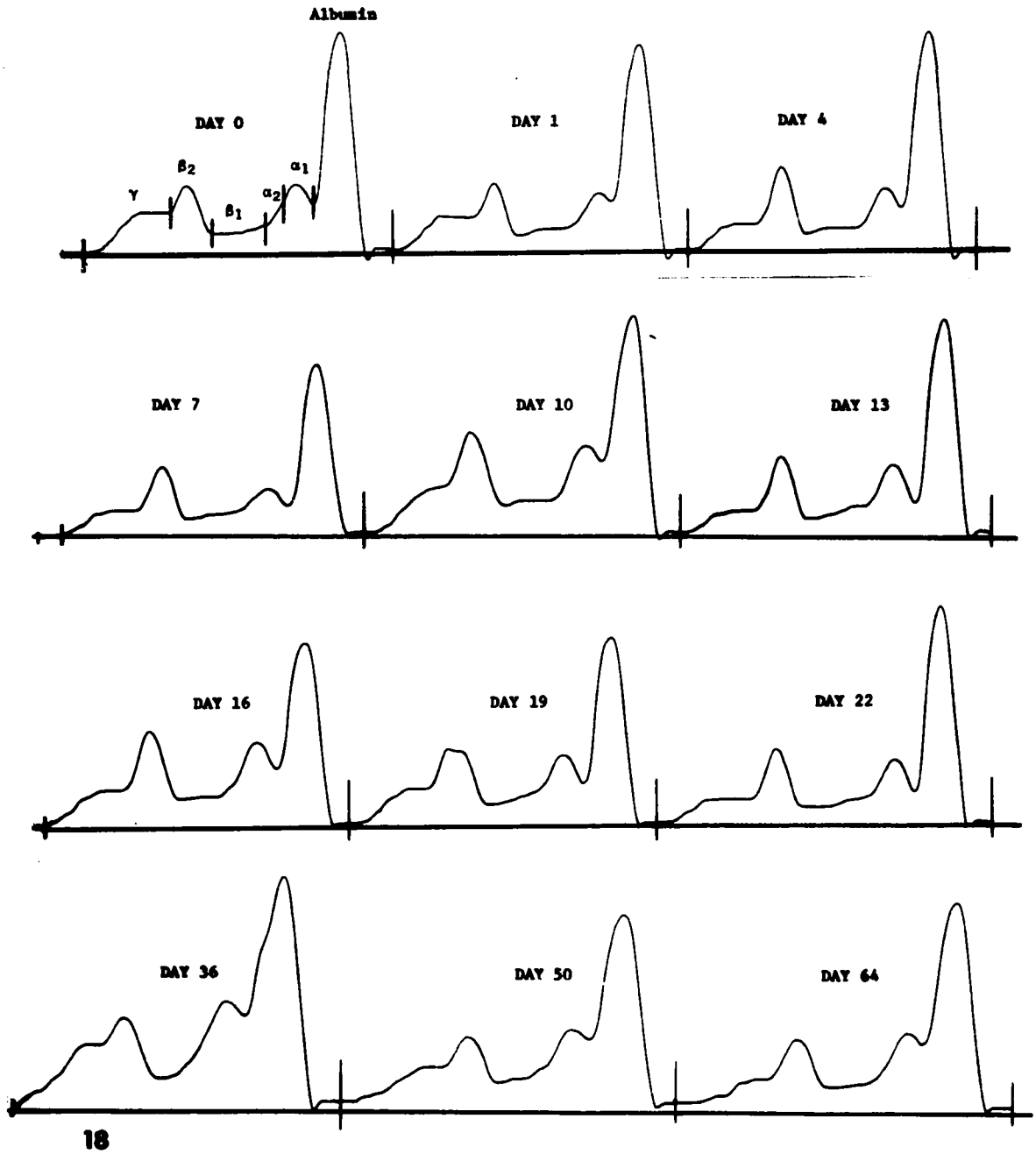
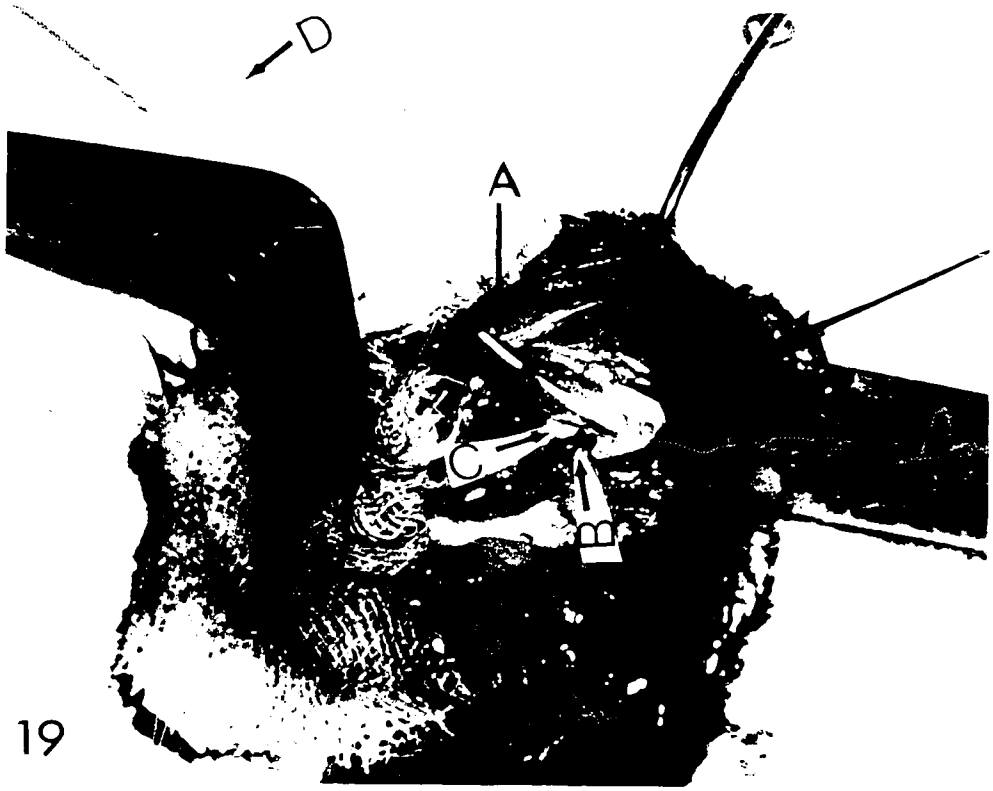

Albumin
DAY 0
DAY 1
DAY 4
γ
β2
β1
α2
α1
DAY 7
DAY 10
DAY 13
DAY 16
DAY 19
DAY 22
DAY 36
DAY 50
DAY 64

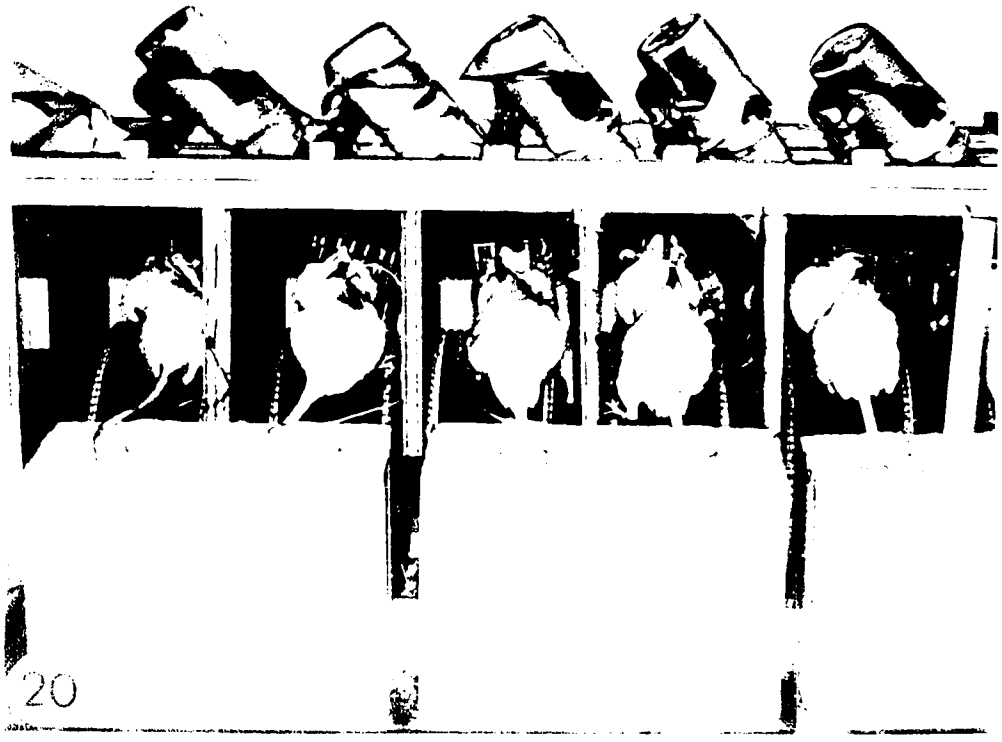
Figure 19 The thoracic duct cannula in place.

A, cannula with lymph; B, stitch holding cannula; C, thoracic duct; D, cannula leaving through the back of the rat.

Figure 20 Restrained rats on a Bollman-type cage.



19



20

Figure 21 Average course of antibody production in 10 rats (- x -) inoculated on day 0 with 5,000 *Eimeria nieschulzi* oocysts, and 10 rats (- o -) inoculated on day 0 with 5,000 and on day 15 with 50,000 oocysts, as demonstrated by agglutination.

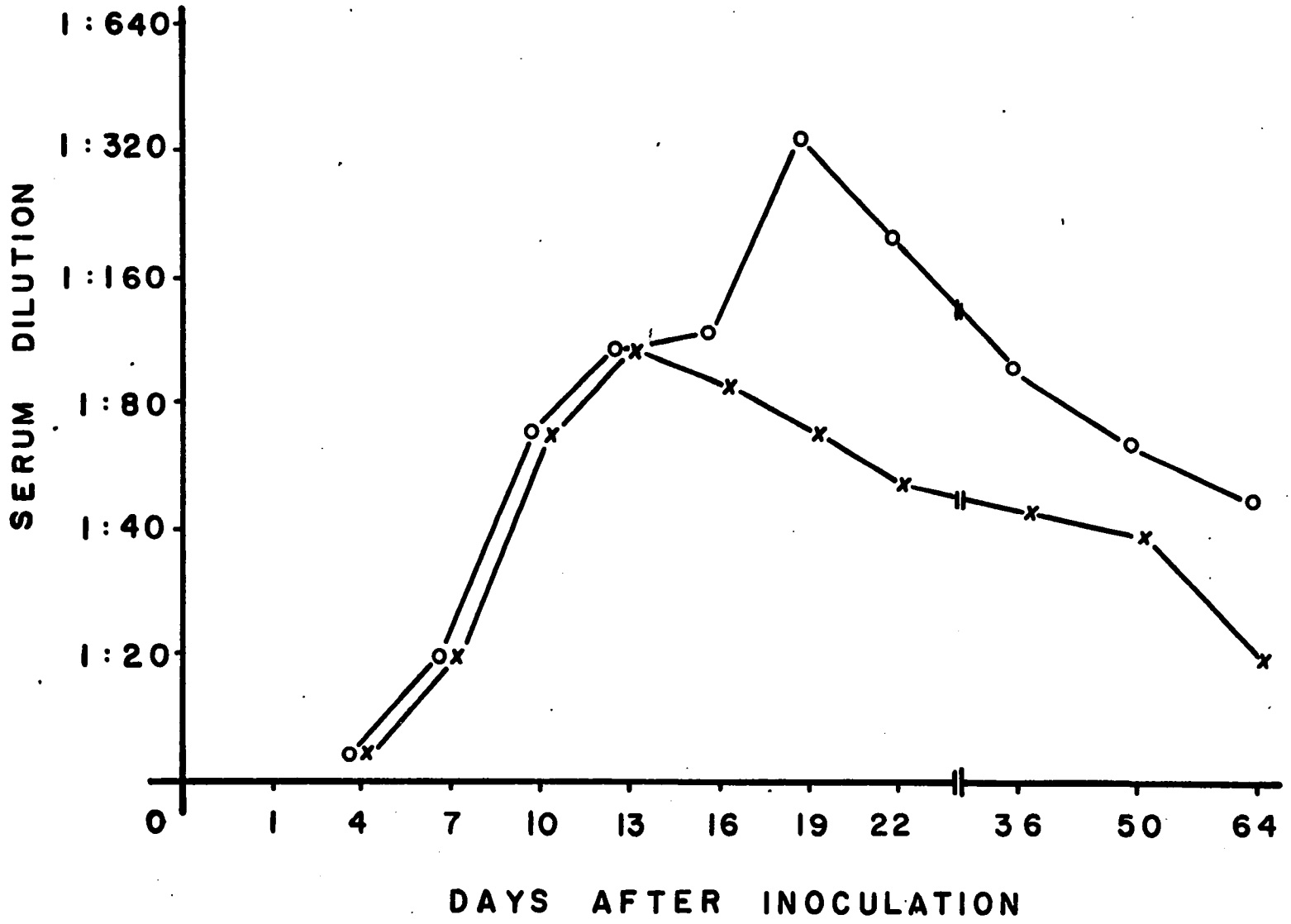

1 : 640
1 : 320
1 : 160
1 : 80
1 : 40
1 : 20
SERUM DILUTION
0
1
4
7
10
13
16
19
22
36
50
64
DAYS AFTER INOCULATION

Figure 22 Site of antibody reaction, and day of maximum fluorescence in intestinal tissue of rats inoculated with 5,000 *E. nieschulzi* oocysts, as demonstrated by immunofluorescence.

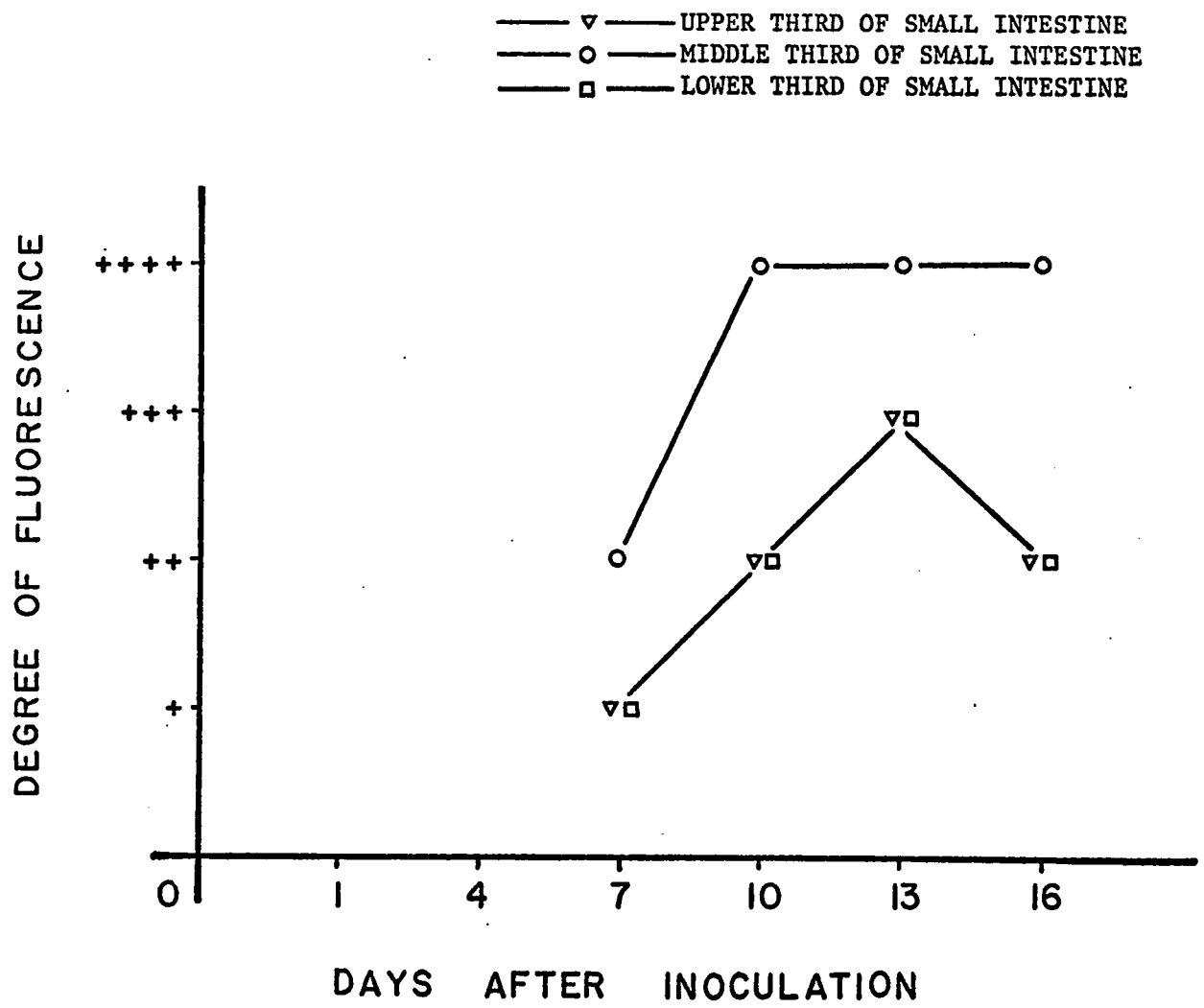

UPPER THIRD OF SMALL INTESTINE
MIDDLE THIRD OF SMALL INTESTINE
LOWER THIRD OF SMALL INTESTINE
DEGREE OF FLUORESCENCE
++++
+++
++
+
0
1
4
7
10
13
16
DAYS AFTER INOCULATION

Figure 23 Average oocyst production in neonatally thymectomized rats inoculated with 1,500 and challenged 15 days later with 50,000 oocysts of *Eimeria nieschulzi*.

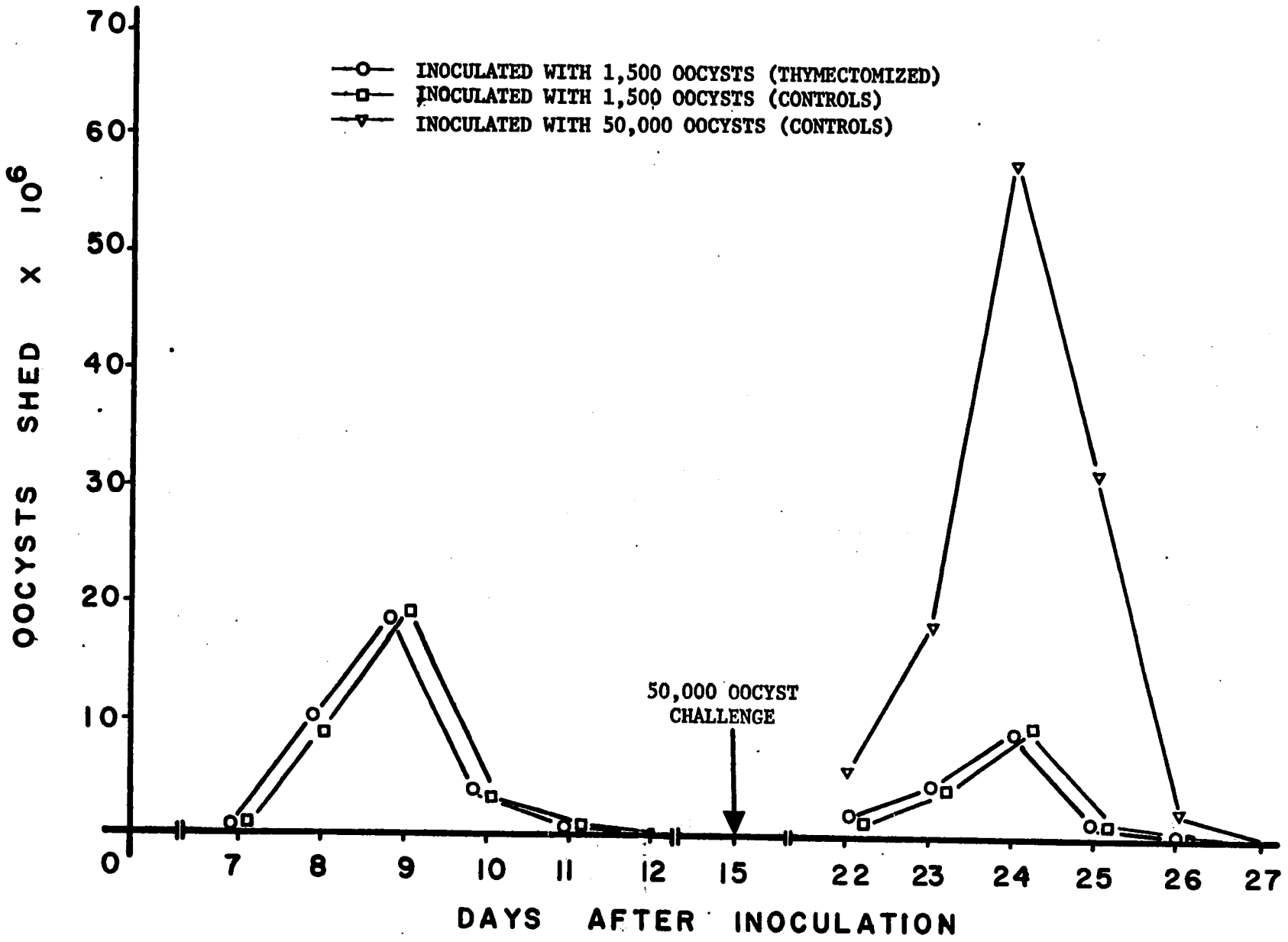

INOCULATED WITH 1,500 OOCYSTS (THYMECTOMIZED)
INOCULATED WITH 1,500 OOCYSTS (CONTROLS)
INOCULATED WITH 50,000 OOCYSTS (CONTROLS)
OOCYSTS SHED X 10^6
70
60
50
40
30
20
10
0
50,000 OOCYST CHALLENGE
7
8
9
10
11
12
15
22
23
24
25
26
27
DAYS AFTER INOCULATION

Figure 24 Average oocyst production in thymectomized (neonatal and adult) and normal rats inoculated with 3,500 and challenged with 50,000 oocysts of *Eimeria nieschulzi*.

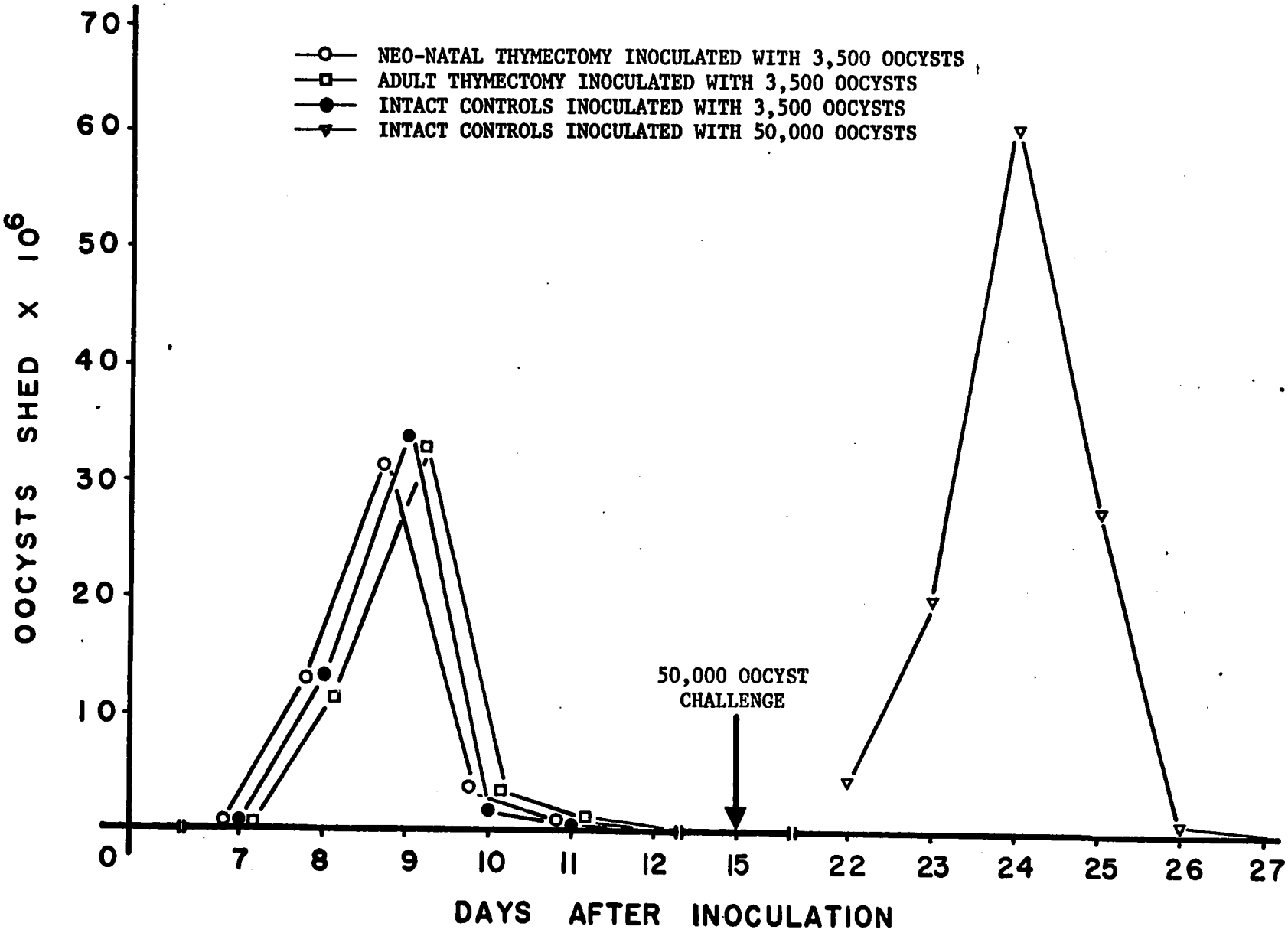
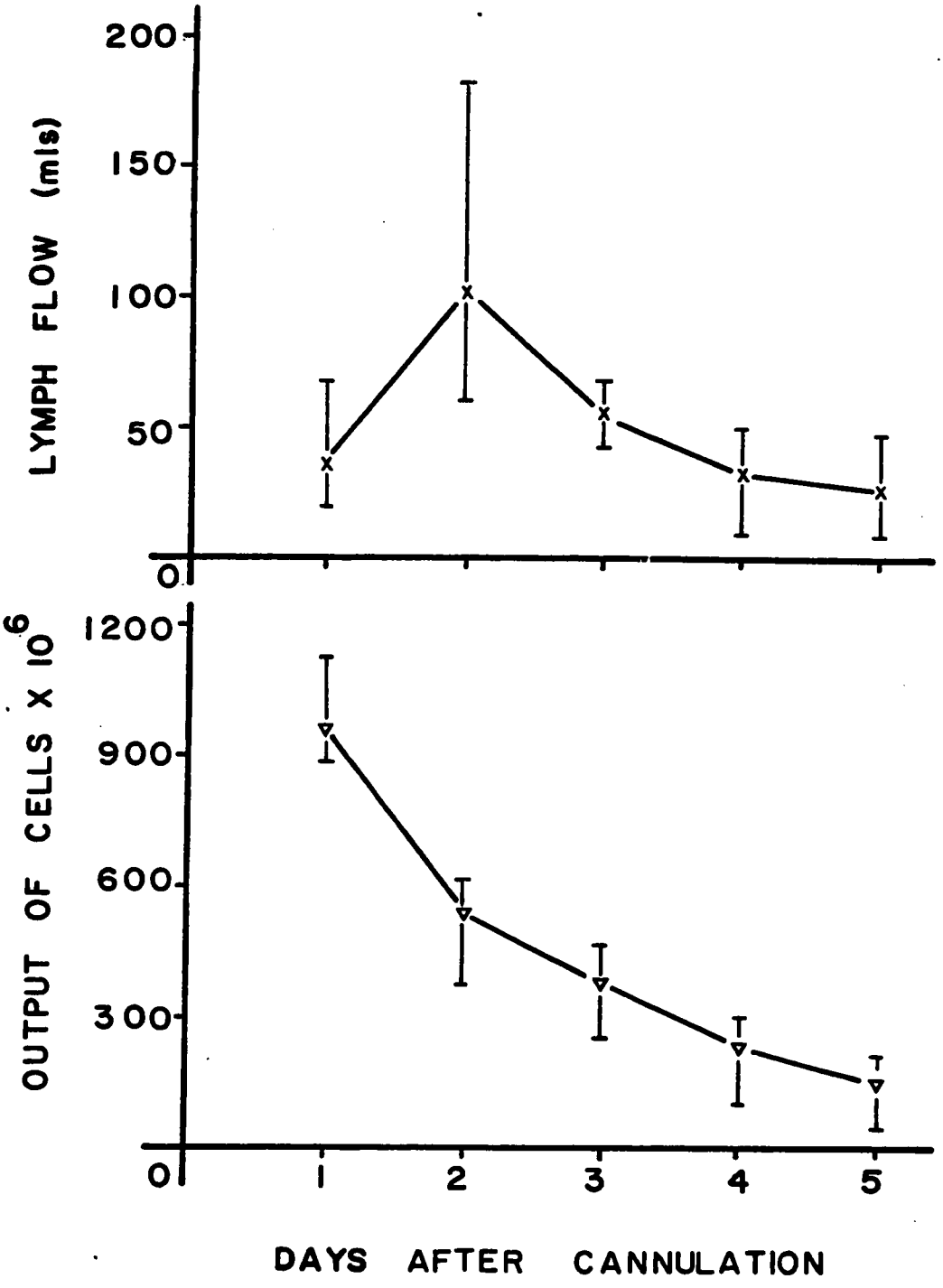

NEO-NATAL THYMECTOMY INOCULATED WITH 3,500 OOCYSTS
ADULT THYMECTOMY INOCULATED WITH 3,500 OOCYSTS
INTACT CONTROLS INOCULATED WITH 3,500 OOCYSTS
INTACT CONTROLS INOCULATED WITH 50,000 OOCYSTS
OOCYSTS SHED X 10^6
70
60
50
40
30
20
10
0
50,000 OOCYST CHALLENGE
7
8
9
10
11
12
15
22
23
24
25
26
27
DAYS AFTER INOCULATION

Figure 25 Volume of lymph and number of lymphocytes drained from the thoracic duct of 12 cannulated rats.


200
150
100
50
0
LYMPH FLOW (mls)
1200
900
600
300
0
OUTPUT OF CELLS X 10^6
1
2
3
4
5
DAYS AFTER CANNULATION