The role of immunity in inhibited development of Obeliscoides cuniculi (Graybill), a stomach nematode of rabbits
by Joseph Carl Fox

A thesis submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Veterinary Science
Montana State University
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Abstract:
A study was designed to examine the role of immunity in the inhibited development of Obeliscoides cuniculi. Rabbits, infected per os with 1-3 doses of third stage larvae (L3), inhibited larvae in subsequent challenge infections at the 4th stage of development.

Massive infections in rabbits given 200,000-834,700 L3 resulted in almost complete worm inhibition. In rabbits infected with 100,000 L3, 50-75% of the worms were inhibited at the 4th stage. Rabbits, pre-infected with 75,000 L3, treated 10 days later with levamasole HCl and subsequently challenged with 5,000 L3 harbored infections comprised of 18.3% 4th stage larvae (L4). All 5th stage worms (L5) recovered from these infections were retarded in growth, whereas L5 recovered from controls were normal. Egg production by worms in actively immunized rabbits was complete suppressed, and in passively immunized rabbits patency did not occur until 6 days after serum transfers were terminated.

When inhibited L4 were transferred into previously unexposed rabbits, many developed to adults (28-87%). However, significant numbers failed to develop beyond the 4th stage. Also, the sex ratio of adult worms which developed from inhibited larvae following transfer shifted toward larger numbers of females, and in actively immunized recipients the differences were further accentuated. Egg production, by adult worms which developed from both inhibited and normal L4 after transfer, was suppressed in actively immunized recipients; fewer eggs were produced by worms of the inhibited type.

Two immunosuppressant drugs, 9-fluoroprednisolone (FP; corticosteroid) and cyclophosphamide (CY; alkylating agent), were given to rabbits infected with 100,000 L3 to determine if larval development would resume. When rabbits were treated with FP on days 0-28 there was an increase in the numbers of adult worms. However, development did not resume after treatments on days 0-6. Significant numbers of worms developed to adults when FP treatments were given days 9-15 or 20-26; also normal egg production occurred in these worms. Non-treated inhibition control animals (100,000 L3) passed only low number of eggs (< 100) even though 25-42% of their worms were L5. Treatment of rabbits with CY inhibited further development of larvae as only 13% of the worms progressed to the 5th stage, and none were found that contained eggs.

Treatment of rabbits with FP (total dosage 9 mg/kg) produced rapid and sustained reductions in total lymphocyte counts. There was also an early weight gain (3-5 days) and subsequent weight loss (5-12 days) following treatment. Treatments with CY (total dosage 90 mg/kg) did not significantly reduce lymphocyte counts. However, a slight lymphocytosis which occurred in the inhibition controls was not observed in the CY-treated animals. Antibody titers to sheep erythrocytes (SRBC) were highest in FP-treated rabbits and animals with low-level infections (4,000 L3); inhibition controls (100,000 L3) and CY-treated animals had comparable antibody titers. Antibody titers to O. cuniculi antigen (OCA) and sheep erythrocytes (SRBC) on day 27 p.i. showed similar relationships between treatment groups, however, anti-OCA titers were considerably lower.

Gross pathological lesions after 27 days in rabbits with high-level O. cuniculi infections included the presence of numerous petechial hemorrhages and folding and thickening of the gastric mucosa; only the cardiac and fundic areas of the stomach were affected. Histopathological lesions included massive epithelial hyperplasia, lymphocytic hyperplasia and nodule formation, lymphocyte and eosinophil infiltration into the lamina propria and cryptitis in areas near embedded larvae. Other effects on rabbits were weight loss and anorexia during the first 6-12 days p.i. Immunosuppressants did not appreciably alter the formation of lesions.

The data indicate that host immunity plays an important role in inhibited development of O. cuniculi in rabbits: (1) active immunization by repeated low-level (3,000 L3) and single high-level (100,000 L3) infections resulted in inhibition of larvae at the 4th stage, (2)corticosteroid treatments increased the number of worms that developed to the adult stage, (3) inhibition was larval dose dependent, and (4) worms were damaged during inhibition in source rabbits such that some of the L4 were unable to resume development when transferred to uninfected rabbits. Also many inhibited male L4 failed to develop into adults, and this was further accentuated in actively immunized recipients.

THE ROLE OF IMMUNITY IN INHIBITED DEVELOPMENT OF *OBELISCOIDES CUNICULI* (GRAYBILL), A STOMACH NEMATODE OF RABBITS

by

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ABSTRACT

A study was designed to examine the role of immunity in the inhibited development of Obeliscoides cuniculi. Rabbits, infected *per os* with 1-3 doses of third stage larvae (L_3), inhibited larvae in subsequent challenge infections at the 4th stage of development. Massive infections in rabbits given 200,000-834,700 L_3 resulted in almost complete worm inhibition. In rabbits infected with 100,000 L_3, 50-75% of the worms were inhibited at the 4th stage. Rabbits, pre-infected with 75,000 L_3, treated 10 days later with levamasole HCl and subsequently challenged with 5,000 L_3 harbored infections comprised of 18.3% 4th stage larvae (L_4). All 5th stage worms (L_5) recovered from these infections were retarded in growth, whereas L_5 recovered from controls were normal. Egg production by worms in actively immunized rabbits was complete suppressed, and in passively immunized rabbits patency did not occur until 6 days after serum transfers were terminated.

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INTRODUCTION

Some parasites, after being ingested by a suitable host, either fail to mature or remain for an extended period of time in an early stage of development before reaching maturity. The reasons these parasites do not develop normally are not fully understood. In most cases the mechanisms are complex and involve either extrinsic or intrinsic factors.

Dormant larval parasites are commonly encountered within the realm of parasitism. Michel (1968) reported that impaired larval development has been reported for over 30 helminth sepecies. The common gastro-intestinal nematodes of domestic cattle and sheep (Trichostrongyloidea and Strongyloidea) consistently exhibit a high degree of arrested development, especially in older animals or during the cold seasons of the year. During the early parasitic phase of their life cycle, many of these nematodes enter a histotropic stage of development (Madsen, 1962). At this stage the larvae migrate into the mucosa and undergo 2 molts (ecdyses). Larval development is usually impaired during this process.

Dormant larvae are important in the epizootiology of several nematode species. Spedding and Brown (1956) reported that a spring rise in worm egg output commonly occurred in confined sheep. Increased egg output was attributed to worms which were dormant throughout the winter and resumed development in the spring. Field, Brambell and Campbell (1960) reported increased egg production by _Haemonchus contortus_

in domestic sheep. Connan (1968a) and Gibbs (1968) stressed the importance of inhibited larvae in the spring rise phenomenon, and Crofton (1954) and Brunsdon (1966) pointed out that the spring increase in worm egg output was a primary source of infection for young animals.

Dormant larvae are generally thought to resume development after a prolonged quiescence. Mechanisms which may be responsbile for renewed development of dormant larvae are: waning of host immunity (Soulsby, 1957), changes in host diet (Connan, 1969) or seasonal hormonal changes in the host (Dunsmore, 1965; Connan, 1968b; Bliz and Gibbs, 1972b). McKenna (1974a) reported that H. contortus was expelled from sheep within 10 weeks after inhibition occurred, and thus could not contribute to spring rise. Blitz and Gibbs (1971a) produced patent infections with H. contortus by transfer of inhibited L_4 into the abomasa of parasite-free, pregnant ewes; however, development did not occur until the time of parturiton. Also, Roberts and Keith (1963) transplanted inhibited Oesophagostomum radiatum into uninfected calves, and patent infections developed. In contrast, Herlich (1974) transplanted 4th stage larvae of Ostertagia ostertagi that were inhibited in sheep into parasite-free calves and found that patent infections did not develop. However, when larvae were grown to the 4th stage (not inhibited) in calves and subsequently transferred to sheep, patent infections developed within 6 days. These data suggest that the 3rd and 4th ecdyses may be important in the inhibitory process and that this

process may be irreversible under certain circumstances.

Inhibited helminths are the etiological agents in certain disease syndromes. These were discussed in detail by Martin, Thomas and Urquhart, (1957), Anderson, Armour, Jarrett, Jennings, Ritchie and Urquhart (1965) and Armour (1970).

Factors Contributing to Inhibited Development of Nematodes

Michel (1968) reviewed some of the mechanisms of retarded larval development. These could be classified as:

A. Environmental effects
B. Diet
C. Size of infective larval dose
D. Presence of adult worms
E. Host age resistance
F. Acquired immunity

Other reviews (Taylor and Michel, 1953; Madsen, 1962; Michel, 1969; Armour, 1970; Jarrett and Urquhart, 1971, and Ogilvie and Jones, 1973) contain similar evaluations of the processes involved in inhibited larval development.

A. Environmental effects

Large numbers of dormant parasites are often observed in cattle and sheep in the autumn or early winter. Anderson et al. (1965) showed that calves grazing on contaminated pastures during late autumn acquired _Ostertagia ostertagi_ infections which were comprised primarily of 4th stage larvae, whereas few L_4 were found in animals.

which grazed the same pastures for similar periods in the spring or summer. They concluded that larval dormancy was precipitated by autumn environmental factors which induced physiological changes in either the host or the infective larvae. Similar observations have been reported for _O. ostertagi_ in calves (Armour, Jennings and Urquhart, 1969a; Reid and Armour, 1972), _Cooperia oncophora_ in calves (Herlich, 1965; Michel, Lancaster and Hong, 1970), _Haemonchus contortus_ in sheep (Connan, 1971; Brunsdon, 1973), _C. curticei_ in sheep (Sommerville, 1960), _Chabertia ovina_ in sheep (Connan, 1974), _Ostertagia spp_. in sheep (Muller, 1968; Reid and Armour, 1972, and _Haemonchus contortus_ and _O. circumcincta_ in sheep (McKenna, 1973). These reports indicate that seasonal inhibition of larvae is common with many nematode parasites of cattle and sheep.

Physiological changes in infective larvae as a result of environmental or seasonl effects are not understood. Blitz and Gibbs (1972b) observed that _H. contortus_ became dormant in sheep when the infective larvae were cultured to the 3rd stage in the laboratory and subsequently exposed to pasture conditions for several weeks during late autumn. Certain strains of _O. ostertagi_ were shown to be more prone to dormancy than others. Armour, Jenning and Urquhart (1967; 1969) demonstrated that laboratory cultures of _O. ostertagi_ which were seeded onto pastures during the fall, did not become dormant in calves, whereas larvae acquired from the naturally contaminated pastures did. Physiological

differences within the laboratory and field strains of larvae were not defined. Storage of infective larvae at 4C for 3-8 weeks also greatly enhanced the tendency for O. ostertagia to become quiescent (Bruce and Armour, 1974; Armour and Bruce, 1974; Michel, Lancaster and Hong, 1975). A similar effect was observed with H. contortus after the L_3 were stored at 5C for 60 days (McKenna, 1974a,b). Rod-like, crystalline structures have been observed within the intestinal cells of inhibited H. contorus (Blitz and Gibbs, 1971b). The significance of these crystals was not known, but they were not found in worms which had resumed development. The presence of such crystals in inhibited H. cortortus was verified by McKenna (1974a,b).

B. Diet

An animal's nutritional status is important in its ability to resist parasitism (Ackert and Beach, 1933; Ross and Gordon, 1933; Hunter, 1953; Kates and Wilson, 1955; Geiman, 1958; Brunddon, 1962a; Gibson, 1963; Bawden, 1969). Several studies have shown that resistance to helminth infections is closely linked to an animal's protein intake (Baird, Vegors, Sell and Stewart, 1956; Wells, 1962, 1963). Sheep on high protein rations exhibited greater resistance to Oesophagostomum radiatum and produced less intestinal mucin Dobson and Bawden (1974), Frick and Ackert (1948) and Dobson (1967) demonstrated that mucin was important in resistance to intestinal helminths. Vegors et al. (1956) further showed that animals on low-protein diets harbored larger numbers of both

immature and mature worms than those on a high-protein ration. A similar observation in sheep infected with O. columbianum was described by Dobson and Bawden (1974). These observations indicated that the host's diet probably does not have a direct effect on worm development. This conclusion was also expressed by Connan (1969) concerning the effects of diet on the retardation of Ostertagia spp. in lambs.

C. Size of infective larval dose

Retarded larval development associated with massive worm infections has been attributed to competition between individual parasites (Taylor and Michel, 1953), however, immunological responses may also be involved. Michel (1952a,b) observed that the course of an infection with Trichostrongylus retortaeformis in rabbits varied according to the size of the infective larval dose. In some cases, massive infections resulted in worm expulsion, while at other times, the larvae were inhibited at the late L_3 stage. The inhibited T. retortaeformis were not rejected immediately but were slowly eliminated as they matured. Martin et al. (1957) reported that development of Graphidium strigosum in rabbits was impaired in heavy infections. Infections initiated with 5,000 L_3 developed normally, whereas most of the worms were retarded in infections with Cooperia pectinata and C. oncophora in calves indicated that these species were inhibited in greater numbers as the larval dose increased. Moreover, Mapes, Coop and Angus (1973) demonstrated that the number of inhibited N. battus in lambs was directly proportional to the number of

infective larvae given. The number of Ostertagia spp. which become retarded in sheep is also proportional to larval dose. Dunsmore (1960) observed that in infections initiated with 1,000 L_3, 2% of the worms remained at the 4th larval stage as compared to almost complete inhibition of larvae in infections with 100,000 L_3. Also, the latter infections were characterized by long prepatent periods and small adult worms.

D. Presence of adult worms

Larval development may be controlled by the presence of adult worm populations. Gibson (1953) found that worm eggs (Trichonema spp.) appeared in the feces of horses following treatment with phenothiazine, even though they were not re-exposed to infective larvae. Dormant larvae were thought to resume development to replace the adult worms which were removed with anthelmintic. Dunsmore (1963) observed a similar situation in sheep that were infected with Ostertagia spp. Following treatment with phenothiazine, the numbers of dormant larvae decreased by a proportion approximately equal to the numbers of new adults. Michel (1969, 1970, 1971) reviewed and stressed the importance of adult worms in controlling the development of O. Ostertagia in calves. However, such a control mechanism was discounted by Anderson et al. (1965) and later by Michel, Lancaster and Hong (1973).

E. Host age resistance

It has been recognized for many years that mature animals are usually more resistant to parasitic infections than are the young.

Herlich (1960) studied age resistance against nematode infections in cattle. Parasite-free calves (5-8½ months old) and steers (18 months old) were pastured in contaminated paddocks. Necropsy results on these animals showed that the calves had worm burdens comparable to those of the steers, even though the steers consumed more forage. More importantly, the steers were less susceptible than the calves to the debilitating effects of parasitism. These results were thought to reflect a certain degree of resistance in the older animals.

Gibson (1959) observed that development of *Nematodirus* spp. was often retarded in mature sheep and that the capacity to inhibit worm development was acquired by lambs at approximately 2 months of age. In addition, Gibson and Everett (1963) demonstrated a reduced fecundity in adult *N. battus* harbored by older sheep. Brunsdon (1962b) stated that resistance to *Nematodirus* spp. in sheep was characterized by 1) limited establishment of larvae, 2) increased length of prepatent periods, and 3) decreased egg production by adult worms. Age resistance to *N. battus* infections in sheep was further documented by Mapes and Coop (1973) who demonstrated that the percentages of worms that were inhibited after 5 inoculations with 60,000 L_3 were smaller in 3 month-old lambs (12.4 - 22.2%) than in 8 month-old animals (47.8 - 72.3%). Gallie (1973) showed that development of *N. battus* was similarly retarded in old versus young rabbits. These reports indicate the age of the host can have a direct effect on parasite development.

Studies on H. contortus infections in sheep (Manton, Peacock, Poyter, Silverman and Terry, 1962) showed that lambs (2-4 months old) were not resistant to a challenge with 15,000 L_3. Furthermore, Urquhart, Jarrett, Jennings, McIntyre and Mulligan (1966) observed that lambs (5-week old) were not immunized against H. contortus using either single or repeated inoculations with irradiated larvae. This was verified by Lopez and Urquhart (1968) who demonstrated that sheep under 6 months of age did not develop immunity to H. contortus after vaccination with irradiated larvae, whereas older animals readily developed strong immunity using the same vaccine. Recently, Knight and Rodgers (1974) observed that sheep of different ages exhibited various degrees of resistance to primary infections with H. contortus; animals older than 12 months consistently harbored fewer worms than younger sheep.

Michel (1963) was able to inhibit the development of O. ostertagi in calves (96-272 days old) using repeated oral inoculations. In contrast, Anderson et al. (1967) were unsuccessful in inhibiting O. ostertagi in 63-day old calves by a similar method. Other workers (Armour, Jennings and Urquhart, 1969b; Smith, 1974) have stated that inhibition of O. ostertagi in calves has nothing to do with host resistance. Similar opinions were expressed concerning inhibited Ostertagia spp. and N. filicollis in sheep (Reid and Armour, 1972) and H. contortus in sheep (Brunsdon, 1973).

F. Acquired immunity

Several excellent review articles are available which discuss the role of acquired immunity in parasite development (Urquhart, Jarrett and Mulligan, 1962; Michel, 1968; Jarrett and Urquhart, 1971; Ogilvie and Jones, 1973). Because dormant parasites tend to accumulate in animals during autumn and winter, regardless of the age of the host, and because inhibition often occurs in initial infections, many people believe that larval inhibition cannot be related to host immunity. Such views were expressed in regard to inhibition of *H. contortus* in sheep by Blitz and Gibbs (1971a) and Brunsdon (1973), *O. ostertagi* in calves by Anderson et al., (1967) and *Cooperia oncophora* in calves by Michel, Lancaster and Hong (1970). Smith (1974) further suggested that host resistance had no effect on the development of *O. ostertagi*, *C. oncophora* or *N. helvetianus* in calves experiencing multiple infections.

There is substantial evidence that retarded development of nematodes in the host is enhanced by acquired immunity. *Nematodirus* spp. elicit strong immune responses in sheep. Donald, Dineen, Turner and Wagland (1964) demonstrated that *N. spathiger* infections were regulated about threshold levels in sheep according to the immune status of the host. Immune responses were characterized by 1) elimination of L_3, 2) retarded development of L_4, 3) reduced fecundity of adults or 4) expulsion of adults. Inhibition of L_4 was most pronounced after repeated infections.

Retarded development of Oesophagostomum spp. occurs rather consistently. Roberts, Elek and Keith (1962) showed that the immune response against O. radiatum in calves occurred while the larvae were in the histotropic stage. The immune response to O. radiatum was shown to be directed against 4th stage larvae by Keith and Bremner (1973), who demonstrated that the L_4 were highly effective for immunization of calves.

Keith (1967) observed abnormal spicules in male Cooperia pectinata obtained from immune calves. In addition, the prepatent periods of the C. pectinata infections were twice as long in immune animals. Sommerville (1960) demonstrated that immunity to C. curticei, a similar parasite in sheep, was directed against the L_4.

The role of immunity in inhibition of Ostertagia spp. has stimulated controversy among workers. Michel (1963, 1970) described the dynamics of O. ostertagi infections in calves which were inoculated daily with 1,500 L_3. He reported that 1) worms were constantly being eliminated, 2) inhibition of larvae seemed to be correlated with the size of the adult worm population, 3) adult worms were stunted in growth, 4) ovulation was suppressed in adult female worms or 5) the host became resistant to the establishment of new larvae. Active immunization by repeated infections, therefore, increased the numbers of inhibited larvae. Later, Michel, Lancaster and Hong (1973) verified that acquired immunity was important in the retardation of O. ostertagi in calves.

Haemonchus placei is more susceptible to retardation in calves than is *H. contortus* in sheep. Roberts (1957) showed that calves developed a strong resistance to *H. placei* during an initial infection. Resistant calves were found to harbor many more inhibited L_4 following a subsequent challenge infection than unexposed calves. In contrast, Dineen and Wagland (1966a) observed that pre-exposed sheep showed no appreciable increase in the proportions of inhibited L_4 after 1 challenge with 3,000 *H. contortus*; although after a second challenge a small increase was evident. It is possible that the pre-exposing doses of larvae used in the latter experiment (498-2,713 L_3) were not sufficient to elicit a strong resistance to challenge.

Repeated inoculations with infective larvae have been shown to immunize sheep effectively against *H. contortus*. Dineen, Donald, Wagland and Offner (1965) observed that 3,000 *H. contortus*, administered at the rate of 100 L_3/day for 30 days, yielded infections comprised of 350-387 inhibited L_4, whereas 3,000 L_3 in a single inoculation resulted in only 17-63 retarded larvae. In addition when large numbers of infective larvae are superimposed upon existing populations of *H. contortus*, sheep became immunologically exhausted or "tolerant" (Dineen and Wagland, 1966b). This is an important concept to consider when animals are repeatedly infected or when natural pasture infections are employed to evaluate the dynamics of worm populations. Wagland and Dineen (1967) further observed that animals became tolerant after only

6 inoculations with 3,000 L_3, however, resistance returned 4-8 weeks later. As previously discussed, the histotropic larval stages of many nematodes are highly immunogenic, and such is also the case with H. contortus in sheep (Christie, Brambell and Charleston, 1965; Bitakaramire, 1966; and Wagland and Dineen, 1967).

Immunosuppression has been used to study the role of immunity in the inhibited development of helminths. Dunsmore (1961) combined X-irradiation and cortisone treatments in sheep infected with Ostertagia spp. and found that fewer larvae were inhibited in treated animals (0.7-31.0%) than in untreated controls (28.2-71.4%). Michel and Sinclair (1969) tested the effects of the corticosteriods B-methazone and prednisolone on inhibition of O. ostertagi in calves but did not find a reduction in the number of inhibited larvae. They did show, however, that worm egg production was greatly enhanced following treatment. Similarly, Pritchard, Donald and Hennessy (1974) failed to induce development of inhibited O. ostertagi using the corticosteroid dexamethazone trimethylacetate. Soulsby (1966) observed increased worm egg counts in sheep following treatment with chlorambucil, an alkylating agent, and suggested that the increased worm egg production occurred after inhibited larvae had matured (also Soulsby and Owen, 1965). Other studies regarding the effects of immunosuppression on worm populations were reviewed by Gibbs (1968). Such studies should be interpreted carefully, however, as it has been demonstrated that the

time at which a drug is administered (relative to the time of antigenic stimulation), dosage rate, and the type of immunosuppressant used are extremely important in the degree and type of suppression produced by a drug (Gabrielson and Good, 1967: Lagrange, Mackaness and Miller, 1974; Kerckhaert, 1974; and Kerckhaert, van den Berg, and Hofhius, 1974).

Work, heretofore reported, indicates that many factors may be involved in the inhibition phenomenon. In the past some discrepancies have existed among workers using the same or similar host-parasite systems. It seems reasonable that nematode species should vary in their susceptibility to or proclivity for retarded development. Variation may also occur because immune responses in animals vary according to the species and/or the degree of immunocompetence which they possess. These factors may account for much of the confusion regarding the role of immunity in the inhibitory process.

Inhibited Development of Obeliscoides cuniculi

The bionomics and development of O. cuniculi were described by Alicata (1932) from infections in guinea-pigs. The 3rd ecdysis was found to occur between 3 and 5 days post-larval-inoculation. Fourth stage females measured 2.3 mm by day 5 and the genital structures were beginning to take form. Fourth stage males measured 4.3-4.8 mm and possessed a prominant tail spike. By day 12 many 5th stage larvae and adults were present. Sollod, Hayes and Soulsby (1968) described the

development of _O. cuniculi_ in rabbits. They reported that 3rd ecdysis occurred at approximately 72 hr post-larval-inoculation. By day 10 some larvae were in the 5th larval stage, but 93% were still in the 4th stage. The asynchronous development of the larvae could not be explained, but it was postulated that many of the L_4 were retarded. Samuel (1970) observed retarded _O. cuniculi_ within the gastric mucosa of rabbits up to 225 days post-larval-inoculation.

Retarded development of _O. cuniculi_ may result from several factors. Russell, Baker and Raizes (1966) showed that the number of inhibited L_4 depended on the size of the larval dose. In rabbits infected with 2,500 and 25,000 L_3/kg of body weight, 6.0% and 70.9% of the worms, respectively, were inhibited. Also, prepatent periods were lengthened, and worm egg production was reduced in worms in the high-level infections.

Obeliscoides cuniculi also becomes dormant in rabbits if the infective larvae are stored under cold conditions for a prolonged period (Fernando, Stockdale and Ashton, 1971; Stockdale, Fernando and Lee, 1970). When L_3 were stored at 15 C for 28 days and then cooled to 5 C and left for an additional 5 days, 42% of the worms became retarded at the 4th stage (hutchinson, Lee and Fernando, 1972). The physiological changes which occurred in these larvae were not defined, nor was any reason given for all the larvae not becoming dormant.

Studies have not been done to determine whether development of _O. cuniculi_ is altered by an immunological process. This study was

designed to determine whether host immunity is a factor in the inhibition of development of _O. cuniculi_ in domestic rabbits. Such parameters as single repeated infections, high-level infections, passive immunizations and immunosuppression of the host were evaluated as to their effects on worm development. The results of the present study show that the immunological status and the degree of antigenic stimulation in rabbits directly influenced the development of _O. cuniculi_ such that large numbers of worms became inhibited at the 4th larval stage. It is therefore concluded that host immunity plays a significant role in imparing the development of _O. cuniculi_ in rabbits.

MATERIALS AND METHODS

Rabbits

Domestic rabbits (*Oryctolagus cuniculus* L.) were obtained from 3 local rabbit producers. Several breeds were used depending on their availability. Californian, New Zealand White and Palomino breeds were used in controlled tests, while Champagne d'Argent and some cross-bred rabbits were used as source animals. Antibiotics were occasionally administered to rabbits with diarrhea or respiratory infections. Test animals were usually allowed to acclimate for at least 2 weeks and were fed medicated Peavey pelleted ration (Peavey Co., Minneapolis, Minnesota) *ad libitum*.

Helminth Cultures

The *Obeliscoides cuniculi* strain used in this study was originally isolated from a cottontail rabbit (*Sylvilagus floridanus mearnsi*) collected in Ohio in 1959 and was maintained in laboratory rabbits that were routinely infected with 3,000-5,000 third stage larvae (L_3). The strain was usually passaged every 3-5 months. Infective larvae were cultured from the feces of infected source rabbits. To prevent desiccation, feces were collected in trays containing damp wood shavings covered with paper towels. Fecal pellets were first softened by soaking in tap water for 2-3 hr, after which peat moss was added (approx. 2/3 peat moss and 1/3 feces) and mixed with an electric mixer.

This material was then placed in 10-inch plastic piettes (Polly-flex Products, Chicago, Ill.) and incubated at ambient temperature (approx. 25 C) for 7-10 days. Larvae were recovered from the cultures by the standard Baermann technique (Baermann, 1917). Baermannization was done through 1 layer of cellulose tissue (Kimwipe, Kimberly-Clark) supported by a 60-mesh screen in an 8-inch polyethylene funnel. The larvae were washed several times and either used immediately in tests or stored at 4 C until sufficient larvae were available for specific experiments. Some larvae were stored at -20 C to be used in the preparation of worm antigen.

Inoculations with Infective Larvae

The inocula were prepared from an aqueous suspension of L_3 by counting the number of active larvae in an aliquot. Volumes of the suspension containing the appropriate number of larvae were then inoculated into rabbits by gavage using a size 6 Bard catheter (Bard woven venous cannula, C. R. Bard Inc., Murray Hill, N. J.) which was covered with a tight-fitting piece of Tygon tubing (U. S. Stoneware, Akron, Ohio) as a stomach tube.

Fecal Examinations

Fecal egg counts were determined using either a modified McMaster technique or a Lane flotation procedure. In the McMaster technique,

5 gm of rabbit feces and 150 ml water were blended for 2 min. Thirty ml of the mixture were collected in a graduated test tube and sedimented in a centrifuge at 1,500 g for 3 min. The supernate was poured off, and the sediment was resuspended in saturated NaCl solution to a volume of 15 ml. After mixing, a portion of the suspension was examined in a McMaster chamber. Both grids of the chamber were scanned at 60 X magnification on a compound microscope. The worm eggs were counted and the totals were multiplied by a correction factor (50) to obtain the number of eggs per gram of feces (EPG).

For Lane flotations, 5 gm of feces were macerated in 150 ml of water as described above. Duplicate 15 ml aliquots were collected in 15 ml calibrated test tubes and sedimented at 1,500 g for 3 min. The supernate was removed and replaced with saturated NaCl solution. The sedimented material was resuspended and addition NaCl solution was added until a meniscus formed at the top of the tube. A cover slip was placed on the top of each test tube after which they were centrifuged at 700 g for 2 min. A cover slip was removed from each sample, placed on a microscope slide and examined under low-magnification using a compound microscope. All eggs were counted, and a correction factor (2) was used to obtain the EPG count.

Necropsies

All rabbits were euthanatized with Beuthanasia Special (H. C. Burns Pharmaceuticals, Oakland, Calif.) administered via an ear vein.

Each rabbit was immediately opened and the stomach removed. The stomach contents were rinsed into a container and formalin was added (5-10% final concentration) to fix the worms. In some cases a 1 cm^2 piece of stomach tissue was removed from the dorsal aspect of the fundus in the area directly across from the cardiac sphincter. These tissue specimens were stored in 10% phosphate buffered formalin for subsequent histological examinations.

The remaining stomach tissue was digested for 8 hr at 37 C with agitation in 200 ml of a solution containing 1 gm of pepsin (1:10,000, Difco)/300 ml of 0.1 normal HCl. Both the stomach contents and the digested material were rinsed on a 200-mesh sieve, and the remaining material was stored in 10% formalin for later worm counts.

The numbers of 4th stage larvae and 5th stage worms were counted in aliquots of both the stomach contents and the tissue digests. The size of the aliquots depended on the number of worms in the sample but usually ranged from 1/20 to 1/2 the total material. Occasionally it was necessary to examine all the material to obtain an accurate worm count. Male L_4 were distinguished by the presence of a tail spike instead of a bursa and females L_4 by their size and the presence of rudimentary reproductive organs (Fig. 21).

Helminth Transfers

Larval O. cuniculi for use in transfer experiments were collected from infected source rabbits by placing stomach tissue into an "8"

diameter glass funnel filled with warm (37-40 C) physiological saline solution (PSS). When sufficient L_4 had migrated from the tissue (1/2-1 hr) they were counted and administered *per os* to recipient rabbits.

Helminth Measurements

Worms were mounted on microscope slides and images of the worms were projected onto a white background using a Bausch and Lomb Microprojector. Measurements were made by bending a piece of small-diameter catheter tubing along the conformation of the projected image. The actual length was calculated using a conversion factor obtained from the projected image of a 1 mm stage micrometer.

Anthelmintics

Two anthelmintics, thiabendazole (TBZ) and levamisole HCl (LHC), were tested for their efficacy against the immature larvae of *O. cuniculi* in rabbits. The Omnizole formulation of TBZ (Merck, Sharp, and Dohme, Rahway, N. J.) was administered at a dosage rate of 500 mg/kg. The LHC (American Cyanamid, Princeton, N. J.) was dissolved in water and administered to rabbits at the rate of 70 mg/kg. Both drugs were administered via gavage.

Immunosuppressant Drugs

Two drugs were used for immunosuppression of rabbits. These included the corticosteroid 9-fluoroprednisolene (Predef 2X; Upjohn Co., Kalamazoo, Mich.) and the alkylating agent cyclophosphamide (Cytoxin; Mead-Johnson, Evansville, Ind.). Both drugs were administered intramuscularly at the dosage described in protocols of the respective experiments.

Histopathology

Tissues were fixed in 10% phosphate buffered formalin, paraffin embedded, thin sectioned, and stained with a hematoxylin-eosin (H & E). The tissue sections were examined using a compound microscope for the presence of lesions or embedded parasites.

Hematology

Packed red blood cell volumes, total leukocyte counts and white blood cell differentials were measured using standard hematological techniques. The number of lymphocytes/mm^3 of blood was calculated from concurrent total WBC counts and WBC differential counts.

Antigens

Obeliscoides cuniculi antigen (OCA) was prepared from a pool of third stage larvae using a lipid-extraction tecnhique. Following 4

washings in tap water the larvae were rinsed 4 times in cold absolute ethyl alcohol and extracted in the same solution for 1 1/2 hr at 4 C. The latter step was then repeated using diethyl ether. The larvae were removed from the ether, placed on a watch glass and allowed to desiccate at 4 C for 12 hr. The dry preparation was weighed (168 mg), placed in a mortar with glass fragments, and ground into a powder. The powdered preparation was rehydrated with 10 ml or PBS (ph 7.4) and sonicated 3 times (2 min at an intensity of 73.5 W/cm^2) using a Biosonik III Sonicator (Bronwill Scientific, Rochester, N. Y.). The antigen was then eluted in PBS at 4 C for 22 hr. After subsequent centrifugation the supernate containing the soluble antigen was stored in ampoules at -20 C.

Sheep red blood cells (SRBC) for use as antigen were collected in Alsever's solution and stored at 4 C. A 5% suspension of washed SRBC in PSS was prepared just prior to use. Each rabbit received 5 ml via an ear vein.

Antisera

Antisera against _O. cuniculi_ were obtained from rabbits which previously had been inoculated with 100,000-200,000 L_3. Serum was collected 5-10 weeks post-larval-inoculation (p.i.). Sera that reacted with OCA in gel diffusion tests were incorporated in a pooled anti-OCA anti-serum which was used for passive immunization of rabbits and as a control serum in serological tests.

Sera from test rabbits were similarly processed and examined separately for antibody titers against OCA or SRBC. Normal rabbit serum (NRS) was collected from non-infected rabbits for use in serological tests and passive immunization experiments.

Serological Tests

Antibody titers against OCA and SRBC were determined by immunodiffusion, indirect hemagglutination, and hemolysin tests.

A. Immunodiffusion test

The immunodiffusion test was patterned after a method described by Campbell (1970). Agar gel was prepared by mixing 1 gm of agarose (SeaKem, Marine Colloids, Inc., Springfield, N. J.) with 94 ml of PBS (pH 7.4) and 5 ml of borate buffer. After heating the mixture to dissolve the agarose, 1 ml of 1% merthiolate in PSS was added as a preservative. The agar was then stored in 10 ml aliquots at 4 C. Just prior to use the agar was melted by heating and poured into formvar (1% formvar in carbon tetrachloride) coated glass petri dishes to a depth of 3 mm. After the agar solidified, wells (3 mm in diameter and 1-2 mm apart) were cut in the gel. Antisera were added to the outer wells, and antigen was placed in the center well. The agar plates were then placed in a humidity chamber for 12-18 hr, after which they were examined for the presence of precipitin lines.

B. Indirect hemagglutination (IHA) test

The IHA test, used to determine OCA antibody titers, was patterned after a microtiter method described by Herbert (1973). Antigen coated horse red blood cells (HRBC) were prepared by mixing 2 ml of 0.1% chromic chloride in PSS and 5 ml of OCA solution (0.37 ml OCA diluted to 5 ml in PSS) with 1 ml of a 50% suspension of washed HRBC in PSS. Following absorption for 1 hr at 25 C the erythrocytes were washed 6 times in PSS.

The IHA tests were done in V-well Microtiter Plates (Cooke Laboratory Products, Alexandria, Va.). Serial 2-fold dilutions of 0.025 ml of heat-inactivated serum were prepared in 0.025 ml of PSS after which 0.025 ml of a 1% suspension of the OCA-coated HRBC was added to each well. Each test also included HRBC, NRS, PSS and anti-OCA controls. The plates were covered with cellophane tape, agitated and left at 25 C for 2-3 hr. Anti-OCA titers were determined by examination of the plates for agglutination of cells.

C. Hemolysin (HL) test

The HL test was used to determine antibody titers to SRBC. Serial 2-fold dilutions of heat-inactivated serum were made as described above using PBS in place of PSS. Afterwards, 0.025 ml of a 0.5% suspension of SRBS in PSS and 0.01 ml of guinea-pig complement (1:10 dilution in PSS) were added to each well. The plates were then covered with cellophane tape, agitated and incubated at 37 C for 1 hr. Antibody titers were

determined by examination of the plates for hemolysis of the erythrocytes. These tests also included PBS, NRS and complement controls.

RESULTS

Repeated Infections with *Obeliscoides cuniculi*

The objective of this experiment was to determine whether repeated exposures to low-level infections with *O. cuniculi* would enable rabbits to resist development of subsequent challenge infections. For this purpose 5 groups of rabbits (3.4 kg mean wt), each consisting of 1 Californian, 1 New Zealand White, and 2 animals of unknown breed, were pre-infected by inoculating them with 3,000 L_3 at 2-week intervals according to the schedule in Table 1. Following pre-infections, all 20 rabbits were treated twice (days 42 and 49) with TBZ at a dosage rate of 500 mg/kg, and subsequently challenged (day 68) with 3,000 L_3. Packed red blood cell volumes (hematocrits), white blood cell (WBC) differential counts, and weight changes were determined at weekly or biweekly intervals. On day 94, all animals were necropsied, and feces were collected and examined for helminth eggs using the Lane flotation method.

One rabbit in group 5 died before the end of the experiment. Necropsy data and mean eggs per gram (EPG) at day 26 post-challenge (day 94) for the remaining animals are listed in Table 1. Group I animals (controls) harbored the largest number of worms ($\bar{x}$ 587) of which 12.8% were fourth stage larvae (L_4). In contrast, the animals in the 4 pre-infected groups (II-V) contained fewer worms ($\bar{x}$ 245-411) of which 19.3%-56.5% were inhibited at the 4th larval stage.

TABLE 1. DEVELOPMENT OF *OBELISCOIDES CUNICULI* IN PREVIOUSLY INFECTED (PRE-INFECTED) RABBITS FOLLOWING A CHALLENGE[A] WITH 3,000 INFECTED LARVAE.

GROUP	NUMBER OF RABBITS	DAY OF PRE-INFECTION	MEAN WORM COUNT (S.E.M.)[C]	PER CENT L_4	MEAN EPG COUNT[D]
I	4	CONTROL	587 (± 49.6)	12.8	908
II	4	0	258 (± 59.9)	46.4	498
III	4	0, 14, 28	341 (±161.3)	19.3	525
IV	4	14, 28	411 (±173.8)	52.3	403
V	3	28	245 (± 45.7)	56.5	353

[A]RABBITS CHALLENGED DAY 68.

[B]RABBITS PRE-INFECTED WITH 3,000 L_3 PER DOSE.

[C]STANDARD ERROR OF THE MEAN.

[D]EGG COUNTS DAY 26 POST-CHALLENGE (DAY 94 OF EXPERIMENT).

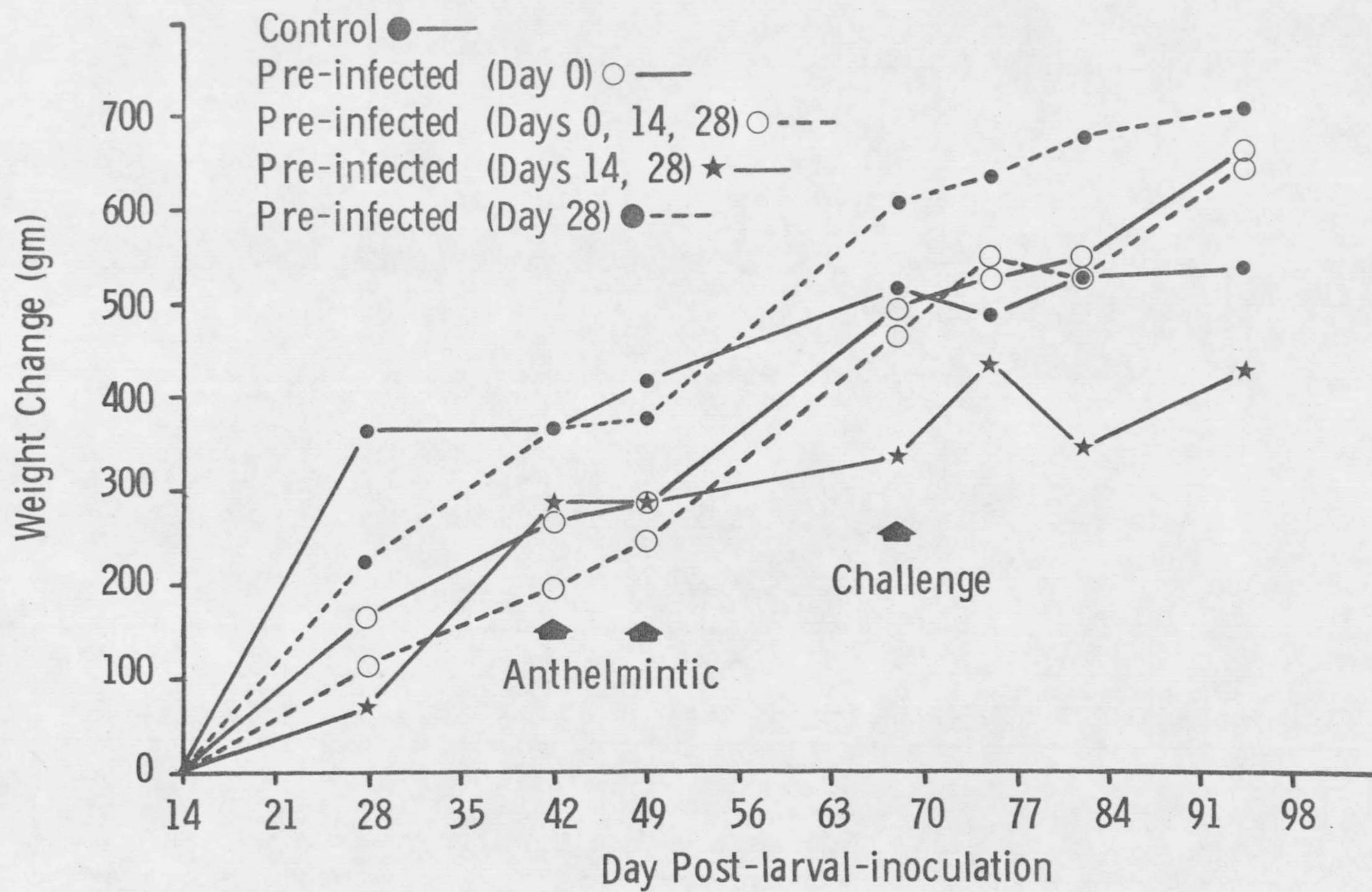

Figure 1. Mean weight changes associated with repeated low-level (3,000 L_3) infections with <u>Obeliscoides cuniculi</u> in rabbits.

Pre-infections, therefore, produced conditions in these rabbits under which both establishment and development of worms in challenge infections were impaired. Although an F-test showed that the variation between treatment means was not significant, the data suggest that inhibition of larval development was enhanced in the previously infected animals. Mean EPG counts 26 days after challenge were almost twice as high in control rabbits. This further indicates that the worms in pre-infected animals did not develop normally. It is also possible that immune responses initiated by previous infections in rabbits may have reduced the fecundity of worms in the challenge infections.

Pathological changes in rabbits with repeated low-level infections were only minor. Mean weight changes (Fig. 1) indicated that the pre-infected rabbits gained weight at a rate comparable to the controls. Hematological values for rabbits with single or repeated low-level infections are provided in the Appendix. Changes were not significant in either the packed red cell volumes or WBC differential counts.

High-level Infections with *Obeliscoides cuniculi*

The purpose of this experiment was to determine the effect of massive infections with *O. cuniculi* in rabbits. Eight New Zealand White (12-14 weeks old) were allocated into 4 groups consisting of 2 animals each. Group I rabbits served as uninfected controls, while those in groups II, III and IV were inoculated orally with 200,000, 400,000 and 834,700 L_3 respectively (Table 2). Sera were collected at biweekly

intervals and tested for antibody against O. cuniculi antigen (OCA) using a gel diffusion test. Fecal samples were collected daily (day 14-80) and examined by the Lane flotation procedure. Pretreatment fecal examinations were negative for helminth ova. Animals in groups II and III were necropsied on day 80, while those in groups I and IV were used in other tests.

One rabbit (IV-a) showed periodic symptoms of diarrhea and anorexia throughout the test. Anorexia was observed in all the infected animals from approximately day 3 to day 9 p.i. Only 1 rabbit in each of the infected groups became patent. Rabbit IV-a became patent day 47, whereas rabbits II-a and III-a began to pass worm eggs on days 61 and 63, respectively. In all cases the worm egg counts were low and egg output ceased after 3-10 days. The prolonged prepatent periods (47-63 days) indicate that the worms did not develop normally.

The mean weight changes in rabbits with high-level O. cuniculi infections (Fig. 2) showed a weight loss up to day 10, but thereafter weight increased at a rate comparable to the uninfected controls. At day 59, the infected animals had not gained as much weight as the controls; groups III, II and IV weighed an average of 200, 420 and 630 gm less, respectively.

At necropsy, group II rabbits harbored a mean of 38,850 worms and group III, 22,286. These were predominantly 4th stage larvae (L_4), with only a few 5th stage worms (L_5) present. These data

TABLE 2. DEVELOPMENT OF *OBELISCOIDES CUNICULI* IN RABBITS WITH HIGH-LEVEL INFECTIONS.

GROUP[A]	NUMBER LARVAE ADMINISTERED	DAY PATENT	MEAN WORM COUNT[B] (S.E.M.)[C]
I	NONE	-	ND[D]
II	200,000	63	38,850 (± 3,250)
III	400,000	61	22,286 (±21,413
IV	834,700	47	ND

[A]TWO AMINALS PER GROUP.

[B]RABBITS IN GROUPS II AND III WERE NECROPSIED ON DAY 80 P.I.

[C]STANDARD ERROR OF THE MEAN.

[D]NOT DONE.

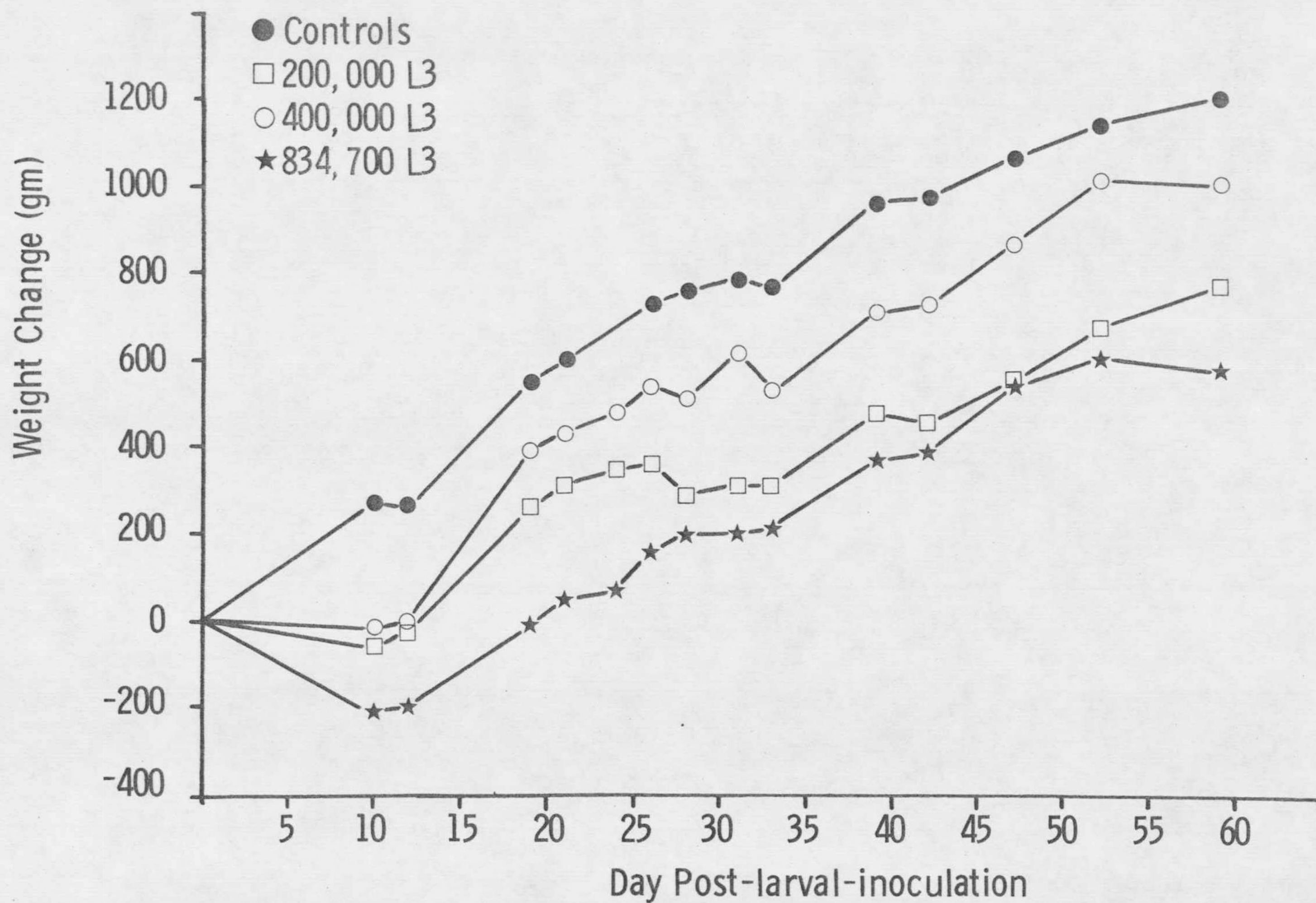

Figure 2. Mean weight changes associated with high-level _Obeliscoides cuniculi_ infections in rabbits

indicate that massive infections with O. cuniculi resulted in almost total retardation of the larvae at the 4th stage of development.

Gross pathological lesions found in the gastric mucosa included numerous petechial hemorrhages and a thickened, rough, whitish epithelium. Only the fundic and cardiac regions of the stomach were affected.

All rabbits in groups II and III developed sufficient antibody titers by day 80 p.i. to give a positive gel diffusion test. Serum from rabbit IV-b produced the most prominent precipitin lines, whereas rabbit IV-a did not develop a measurable antibody response. This may explain the early patency (day 47) observed in the latter animal.

Elimination of Immature *Obeliscoides cuniculi* with Anthelmintics

In order to use high-level infections of O. cuniculi as a means of actively immunizing rabbits, a method of expelling large numbers of worms had to be devised. An initial attempt to expel inhibited O. cuniculi from rabbits in group IV of the previous experiment failed. These animals were given 834,700 L_3 and after approximately 5 months p.i. were treated with TBZ at a dosage rate of 500 mg/kg. At necropsy there were still 30,000-50,000 L_4 present. A study was then designed to test the efficacy of LHC against immature O. cuniculi larvae. For this test, 8 Californian rabbits (10-12 weeks old) were allocated to 4 groups each consisting of 2 animals, which were inoculated *per os* with 100,000 L_3. Beginning day 8 p.i., rabbits in

TABLE 3. EFFICACY OF LEVAMISOLE HCl (LHC) AGAINST IMMATURE *OBELISCOIDES CUNICULI* IN RABBITS.[A]

GROUP[B]	TREATMENT[C] (70 MG/KG LHC)	DAY NECROPSIED (P.I.)	MEAN NUMBER L_4	PER CENT REDUCTION
I	CONTROL	16	10,614	---
II	1 DOSE	10	3	99.9
III	2 DOSES	14	15	99.8
IV	3 DOSES	16	8	99.9

[A]RABBITS WERE INOCULATED WITH 100,000 INFECTIVE LARVAE 8 DAYS BEFORE TREATMENT.

[B]TWO ANIMALS PER GROUP.

[C]ANTHELMINTIC WAS ADMINISTERED BY GAVAGE AT 2-DAY INTERVALS.

groups II, III and IV were given either 1, 2 or 3 doses of LHC at a rate of 70 mg/kg, respectively. A 2-day interval was allowed between doses when more than 1 treatment was given. Group I rabbits served as untreated controls. All treated rabbits were necropsied 2 days after they received their last dose of anthelmintic, and the controls were necropsied on day 16.

At necropsy the control group harbored a mean of 10,614 L_4 as compared to 3-15 L_4 in the treated groups (Table 3). Therefore, LHC was $>$ 99.8% effective in removing the immature worms in all 3 groups. Toxic effects were not noticeable in the treated animals, even after repeated treatments.

Transfer of Inhibited *Obeliscoides cuniculi* to Normal Rabbits

Experiment A:

Two experiments were designed to determine whether inhibited L_4 could resume development when transferred to previously uninfected (normal) rabbits. In experiment A, 5 rabbits were inoculated with 4,000 L_3 and served as sources of normal L_4. Normal 4th stage larvae were removed from the rabbits on day 5 p.i. Inhibited L_4 were obtained from a rabbit which had been infected with 5,000 L_3 and subsequently challenged (5 months later) with 200,000 L_3; the inhibited L_4 were harvested day 14 p.i. Four Californian rabbits (14-16 weeks old) were inoculated *per os* with 500 inhibited L_4, while 4 others received 500 normal L_4. Fecal samples were examined by the Lane flotation procedure

on day 18 p.i. All rabbits were necropsied on day 33 post-larval-transfer. One hundred adult male and 100 adult female worms from each group were measured, and the results analyzed using the Student's T-test. The remaining data were analyzed using an F-test.

Table 4 contains the necropsy results (day 33 post-larval-transfer) for experiment A as well as EPG counts for feces collected on day 18 post-larval-transfer. Approximately 50% of both the inhibited and normal 4th stage larvae that were transferred to the recipient rabbits were recovered at necropsy. Of these 87.6% of the inhibited and 98.2% of the normal larvae developed to adulthood. However, the 12.4% of the inhibited L_4 transfers which remained in the 4th developmental stage represented a statistically significant number ($P \leqslant .005$). These data suggest that some of the inhibited larvae were damaged during inhibition and were not capable of further development.

An analysis of the adult worm sex ratios (F-test) showed a significant shift in the inhibited L_4 recipients ($P \leqslant .025$) to higher numbers of female worms. Evidently male worms were more adversely affected during inhibitory process than females. Fecal examinations showed that worms which developed from normal L_4 produced slightly higher EPG counts. Measurements of 100 adult males and 100 adult females from each group showed that the worms were not significantly different in total length. Inhibited males measured 9.70($\pm$0.5; S.E.M.), normal males 9.58($\pm$0.6) mm, inhibited females 13.47($\pm$1.2) mm, and normal females 13.86($\pm$0.9) mm.

TABLE 4. DEVELOPMENT OF INHIBITED AND NORMAL 4TH STAGE LARVAE (L_4)[A] OF *Obeliscoides cuniculi* AFTER TRANSFER TO NORMAL RABBITS.

Larvae transferred	Mean worm count		Worm sex ratio (male:female)	Mean EPG count day 18
	Per cent recovered	Per cent adults		
Inhibited[B] (500 L_4)	45.3	87.6	1:1.61	600
Normal[C] (500 L_4)	55.8	98.2 ($P < .005$)[D]	1:1.01 ($P < .025$)	900

[A]Four rabbits per group.

[B]Obtained from L_4-day infection with 200,000 L_3.

[C]Obtained from 5-day infection with 4,000 L_3.

[D]F-test for analysis of variance.

Experiment B:

Experiment B differed from A in that recipient animals were included which were pre-infected with O. cuniculi prior to transfer of the inhibited and normal L_4. Seventeen rabbits (14-16 weeks old) were allocated to 3 experimental groups. Groups I and II contained subgroups of 4 pre-infected (A) and 3 normal (B) rabbits (Table 5); each subgroup was comprised of 2 New Zealand Whites and either 1 or 2 Palomino rabbits. Group III was made up of 3 New Zealand Whites which served as pre-infection controls. Rabbits in Group III and subgroups I-A and II-A were pre-infected using oral inoculations with 75,000 L_3. All rabbits (groups I-III) were treated 7 days after pre-infection with 1 dose of LHC at a rate of 70 mg/kg. Ten days later, the 4th stage larvae were transferred to all recipient rabbits. The L_4 for transfer were obtained as in experiment A except that the inhibited L_4 were obtained from a rabbit only 8 days after inoculation with 200,000 L_3. The L_3 used to produce both the inhibited and normal L_4 came from the same larval pool. Group I rabbits were inoculated with 400 inhibited L_4 each, and group II animals received 325 normal L_4 each. Group III rabbits did not receive a transfer of larvae. Necropsies were done 26 days after larval transfer. Fecal samples, collected days 14-26 post-larval-transfer, were examined by a modified McMaster technique. Data were compared using an F-test for analysis of variance.

The combined data of subgroups A & B (Table 5) showed significant differences in the numbers of inhibited (28.5%) versus normal (64.6%) L_4 which became established in recipient rabbits following transfer.

A significantly smaller percentage of the inhibited larvae developed to adults, of which females were more numerous than males. These data indicate the larvae involved in a high-level infection for only 8 days were substantially altered in their ability to develop properly, and it appears that the males were more affected than females.

Worm populations were further altered in pre-infected recipients. Approximately 5% more worms were present in the pre-infected rabbits of both transfer groups. This increase cannot be attributed to residual worms from pre-infections, as only 17 worms were found in the pre-invection controls. Larger numbers of inhibited larvae developed to the adult stage in pre-infected recipients (34.3%) than in normal recipients (18.2%). In contrast, all of the normal L_4 developed to maturity in normal recipients. The male-to-female ratio of adult worms which developed from inhibited L_4 in pre-infected recipients was 1:8.3 as compared to 1:3.3 in the normal recipient rabbits. A shift in sex ratios was not observed in animals which received normal L_4. Therefore, active immunization of rabbits altered worm development with respect to 1) the percentages of worms which were recovered at necropsy, 2) the percentage of worms which remained in the L_4 stage and 3) the number of male worms which developed to maturity. Although some of the retarded

TABLE 5. DEVELOPMENT OF INHIBITED $(I\text{-}L_4)^A$ AND NORMAL $(N\text{-}L_4)^B$ 4TH STAGE LARVAE OF <u>OBELISCOIDES CUNICULI</u> AFTER TRANSFER TO PREVIOUSLY INFECTED (PRE-INFECTED)[C] OR UNEXPOSED (NORMAL) RABBITS.

RECIPIENT RABBITS	PER CENT RECOVERED		PER CENT ADULTS		ADULT WORM SEX RATIO (MALE:FEMALE)	
	$I\text{-}L_4$	$N\text{-}L_4$	$I\text{-}L_4$	$N\text{-}L_4$	$I\text{-}L_4$	$N\text{-}L_4$
PRE-INFECTED (4 RABBITS)	30.6	65.5	34.3	91.2	1:8.3	1:1.2
NORMAL (3 RABBITS)	25.7	60.5	18.2	100.0	1:3.3	1:1.2
COMBINED DATA (7 RABBITS)	28.5*	64.6*	28.1*	98.4*	1:7.4*	1:1.2*
STATISTICALLY SIGNIFICANT*	($P \leqslant .001$)		($P \leqslant .001$)		($P \leqslant .01$)	

[A] $I\text{-}L_4$ OBTAINED FROM 8-DAY INFECTIONS WITH 200,000 L_3; 400 LARVAE TRANSFERRED.

[B] $N\text{-}L_4$ OBTAINED FROM 5-DAY INFECTIONS WITH 4,000 L_3; 325 LARVAE TRANSFERRED.

[C] RABBITS PRE-INFECTED WITH 75,000 L_3 AND DEWORMED WITH LEVAMISOL HC1.

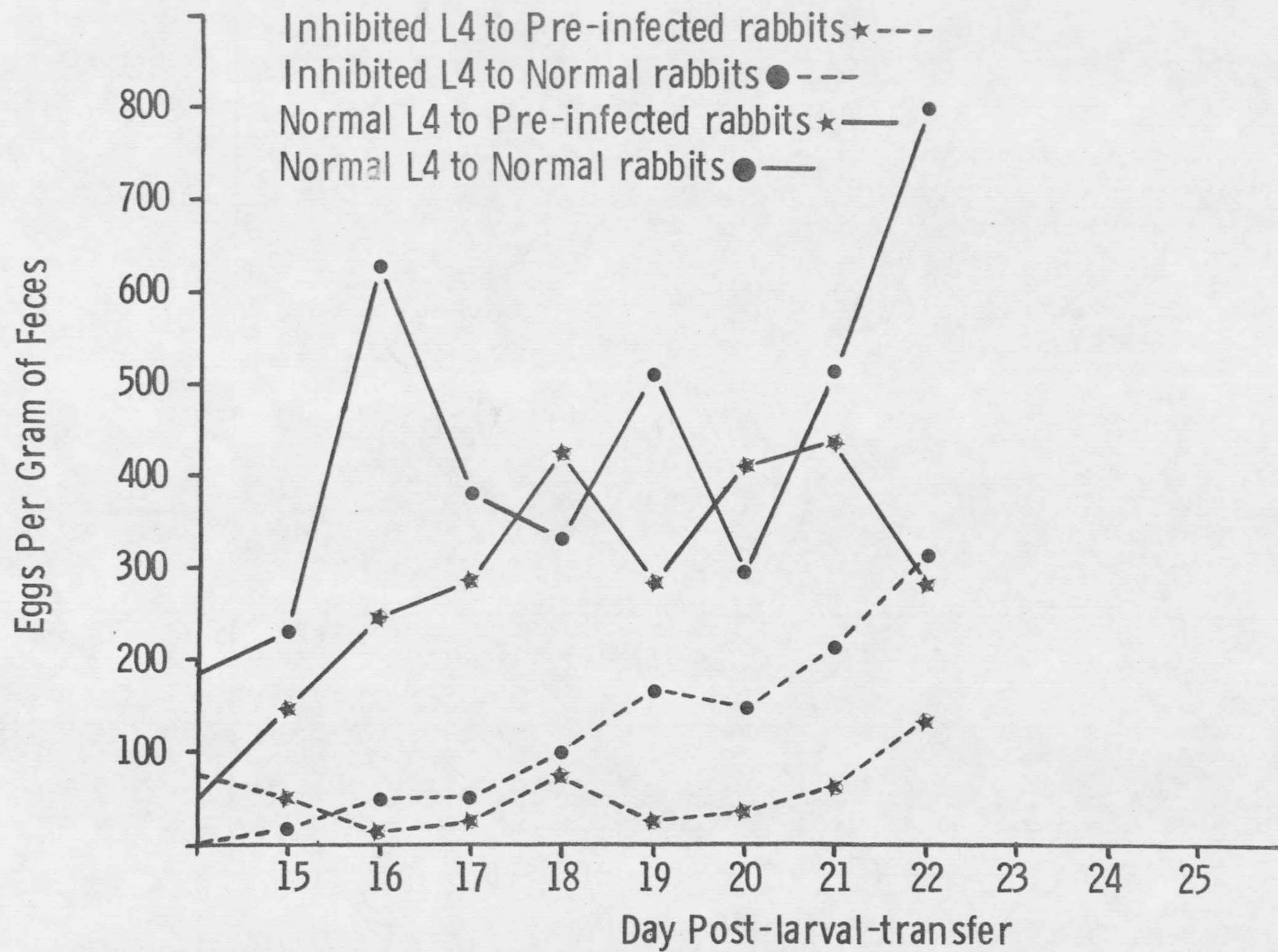

Figure 3. Mean egg production by <u>Obeliscoides cuniculi</u> following transfer of inhibited and normal 4th stage larvae (L_4) to previously infected or normal rabbits.

larvae were impaired in their ability to resume development following transfer to normal rabbits, many L_4 did develop to maturity indicating that short term inhibition was not completely irreversible.

Differences also were found in worm egg production following transfer of inhibited and normal L_4 (Fig. 3). Most recipient animals developed patent infections before day 14 post-larval-transfer. Patency normally occurs about day 18 when infections are initiated with 3rd stage larvae. In general, EPG counts were lower in rabbits which received the inhibited L_4. Also egg counts were lowest in the pre-infected recipients within both groups. These data suggest that adult worms which developed from the inhibited L_4 were not as prolific as those from the normal L_4, and that actively immunized animals further suppressed egg production by adult worms.

Effects of Pre-infections and Passive Immunizations in Rabbits on Development of *Obeliscoides cuniculi*

The purpose of this experiment was to ascertain whether active immunization via exposure to pre-infections or passive-transfers of immune serum in rabbits would alter the development of *O. cuniculi*. Fifteen New Zealand White rabbits (12-16 weeks old) were allocated to 5 groups of 3 animals. Rabbits in groups I and II were pre-infected with 75,000 L_3 *per os*. Ten days later, rabbits in all groups were treated with LHC at a rate of 70 mg/kg. Fifteen days after anthelmintic treatment, rabbits in groups I, III, IV and V were challenged with

5,000 L_3, whereas animals in group II were not challenged and served as pre-infected controls. At challenge, animals in group III were injected (intraperitoneally) with 12 ml of pooled rabbit anti-OCA serum. Similar injections were continued at 2-day intervals for a total of 9 injections (108 ml). Group IV rabbits received a similar regimen of NRS and served as normal serum controls, whereas animals in group V did not receive treatments after challenge and served as patency controls. Fecal samples were collected days 14-24 and were examined by the modified McMaster method. All animals were necropsied 26 days post-challenge.

Although the pre-infected animals (group I) harbored the largest number of worms, there was no evidence that this was due to residual infection persisting other treatment (Table 6). Immune serum recipients (group III) did not have increased worm counts. Because worm counts in both of the immunized groups were highly variable it is possible that individual rabbits did not respond to the infections to the same degree. Pre-infected animals (group I) harbored the highest percentages of inhibited L_4. In addition, the 5th stage worms in the pre-infected animals were either stunted in growth or infertile. Pre-infections in rabbits therefore resulted in abnormal development of the young adult worms (L_5) and enhanced inhibition of the 4th stage larvae, whereas anti-OCA serum did neither.

The pre-infection controls (not challenged) did not pass helminth eggs during the test. Patent infections developed in the NRS controls and the patency controls by days 17 and 18, respectively. In both cases

TABLE 6. DEVELOPMENT OF *OBELISCOIDES CUNICULI* IN PRE-INFECTED[A] AND PASSIVELY IMMUNIZED RABBITS.

GROUP[B]	TREATMENT	MEAN WORM COUNT[C] (S.E.M.)[D]	PER CENT	CONDITION OF ADULT WORMS
I	PRE-INFECTED	701 (±248.2)	18.3	STUNTED OR INFERTILE
II	ANTHELMINTIC CONTROL	6 (± 2.8)	----	---------
III	IMMUNE SERUM	505 (±318.2)	0.4	NORMAL
IV	NORMAL SERUM	459 (± 27.2)	0.0	NORMAL
V	PATENCY CONTROL	594 (±112.0)	0.1	NORMAL

[A]RABBITS PRE-INFECTED WITH 75,000 L_3 AND DEWORMED WITH LEVAMISOLE HCl PRIOR TO CHALLENGE WITH 5,000 L_3.

[B]THREE RABBIT GROUPS.

[C]RABBITS NECROPSIED 26 DAYS POST-CHALLENGE.

[D]STANDARD ERROR OF THE MEAN.

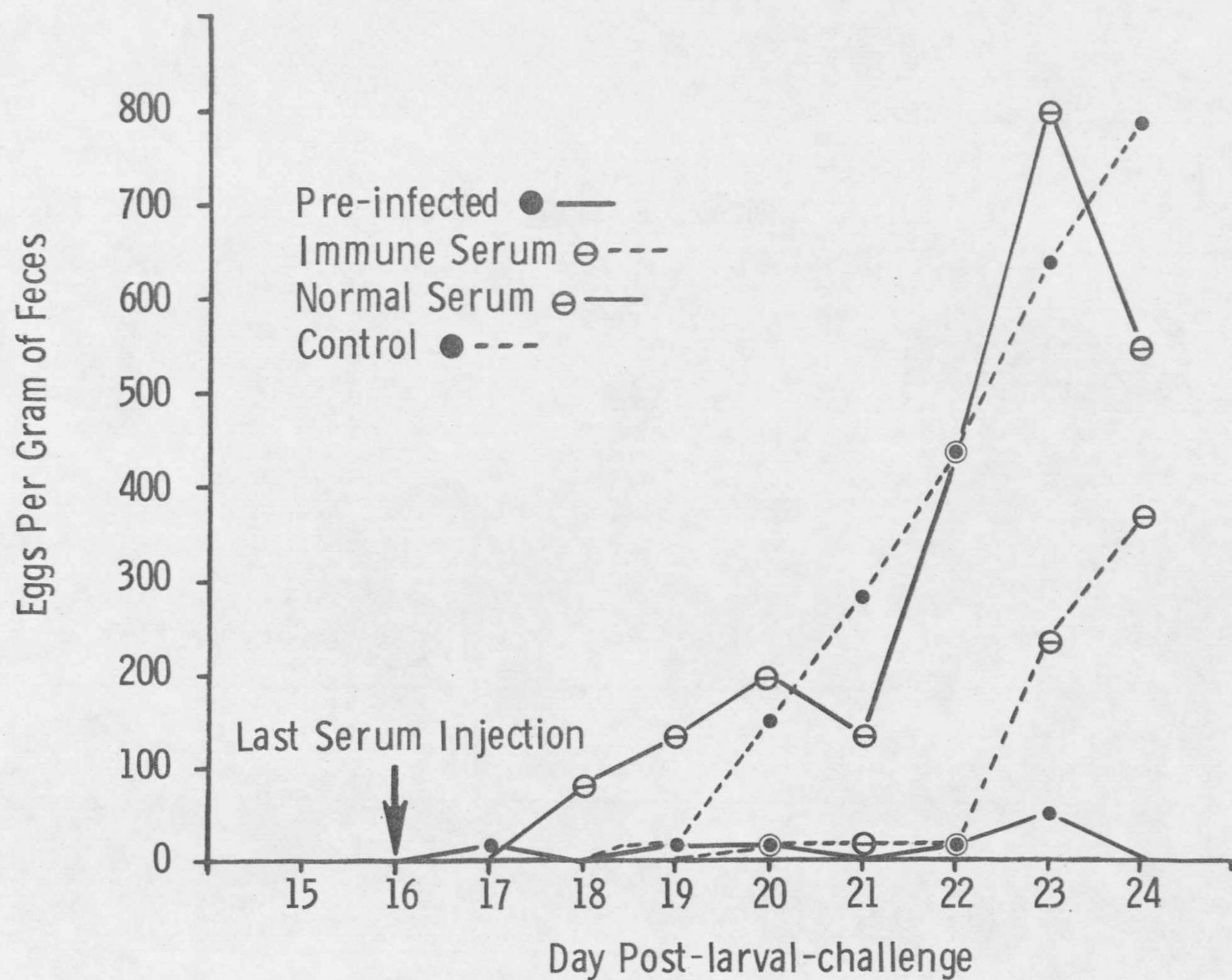

Figure 4. Mean egg production by Obeliscoides cuniculi in previously infected or passively immunized rabbits.

worm egg production increased normally (Fig. 4). The pre-infected animals (group I) passed only a few worm eggs after a slightly prolonged patency. This further supports the observation that many of these worms were either stunted or infertile. Worm egg production was suppressed in the passively immunized rabbits until day 23 after which EPG counts increased normally. Immune serum evidently obviated worm egg production until the amount of passively transferred antibody fell below critical levels. Therefore, rabbits possibly produce 2 types of response to *O. cuniculi*: one which may or may not be antibody dependent and results in larval inhibition, and another which is antibody dependent and causes suppression of worm egg production.

The Development of *Obeliscoides cuniculi* in Rabbits Treated with the Corticosteroid 9-fluoroprednisolone

Experiment A:

Two experiments were designed to examine the effects of 9-fluoroprednisolene (FP) treatments in rabbits on the development of concurrent infections with *O. cuniculi*. In experiment A, 8 rabbits (12-14 weeks old) were separated into 2 groups. Each group consisted of 2 New Zealand Whites and 2 Californians. On day 0, all rabbits were inoculated *per os* with 100,000 L_3. Animals in the treatment group were given injections (intra-muscular) with FP at dosage rated of 2 mg/kg/day (days 0-6) and 0.5 mg/kg at 2-day intervals thereafter (days 8-28). Fecal egg counts were determined days 17-40 using the Lane flotation technique. Total

WBC counts were determined on days 19 and 42. All rabbits were necropsied on day 42.

Two of the FP-treated rabbits died during the initial phase of the test, and 1 control animal was sacrificed for a worm burden comparison. The necropsy data for the remaining rabbits are listed in Table 7. The FP-treated rabbits harbored slightly larger number of worms than controls. In the control animals, 49.5% of the O. cuniculi present at necropsy had developed to the L_5 stage, but only 3.5% were late L_5 (fully mature). Comparable data for the FP-treated group were 57.5% and 20.9%, respectively. Therefore, substantially more worms attained maturity in the FP-treated animals. However, the egg count data revealed that worm egg production was impaired in animals of both groups. None of the controls became patent, and the FP-treated animals passed only small numbers of eggs (8-72 mean EPG) for a short period (days 30-36). The reason egg production was suppressed even though large numbers of adult worms were present is not understood.

Mean WBC counts in the FP-treated rabbits were considerably lower than in the controls; counts on days 19 and 42 were 5,138 and 5,075 cells/mm^3 of blood, respectively, in FP-treated animals and 9,979 and 8,100 cells/mm^3 in controls. In addition, FP produced early weight losses (Fig. 5) in the treated rabbits (days 4-12) after which they began to gain weight slowly. By day 29 the FP-treated rabbits weighed an average of 892 gm less than the controls.

Table 7. Development of *Obeliscoides cuniculi* in rabbits with high-level[A] infections and treated with the corticosteroid 9-fluoroprednisolone (FP).

Group	Number of rabbits	Mean worm count[B] (S.E.M.)[C]	Per cent L_5[D]	Per cent late L_5
FP-treated (Days 0-28)	2	7,382 (±2013)	57.5	20.9
Controls	3	6,911 (±2110)	49.5	3.5

[A]Infections initiated with an oral inoculation of 100,000 L_3.

[B]Necropsies performed 42 days post-larval-inoculation.

[C]Standard error of the mean.

[D]Early and late 5th stage worms (late - fully mature).

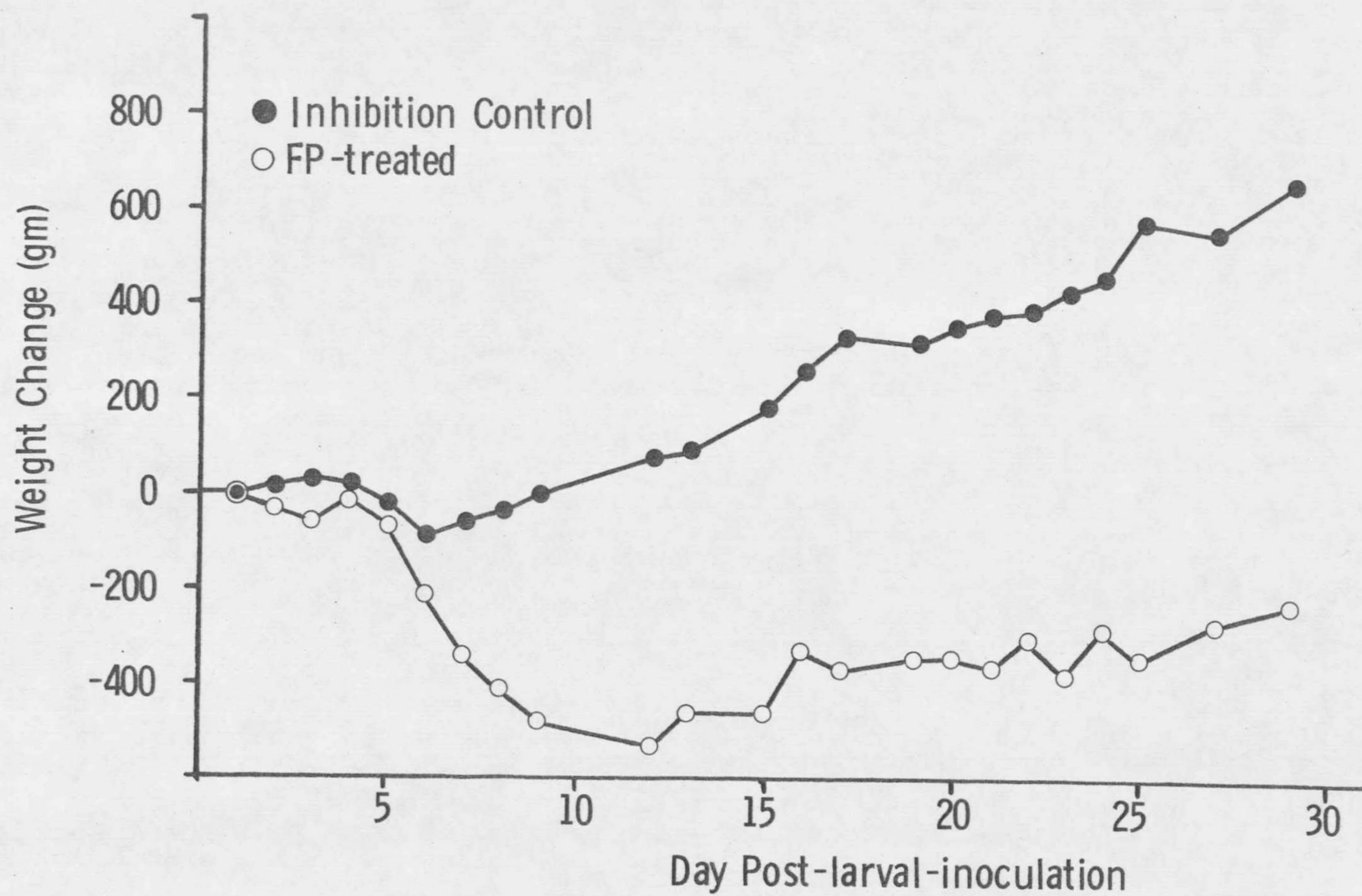

Figure 5. Mean weight changes in rabbits given high-level infections with <u>Obeliscoides cuniculi</u> and treated with 9-fluoroprednisolone (FP).

Necropsy examinations on the FP-treated rabbits that died on days 5 and 9 revealed numerous petechial hemorrhages within the mucosa of the fundic and cardiac regions of the stomachs, and 10,848 and 14,170 4th stage larvae were recovered from these 2 animals. The control rabbit sacrificed on day 9 had similar petechial hemorrhages and harbored 8,364 L_4.

Experiment B:

Because FP treatment in rabbits resulted in increased numbers of mature O. cuniculi, a second test was designed to determine the effects of FP on worm development when administered to rabbits at 2 different time intervals. In experiment B, 15 rabbits (12-14 weeks old) were allocated to 3 groups (Table 8). Each group consisted of 3 New Zealand Whites and 2 Californians. An additional group consisting of 2 Californians (6 months old) were used as patency controls. On day 0, animals in groups I, II and III were inoculated per os with 100,000 L_3 and those in group IV, with 3,000 L_3. The rabbits in group I (FP-0) were given FP injections (i.m.) on days 0-6 at the dosage rates of 2 mg/kg/day for 2 days and 1 mg/kg/day for an additional 5 days. A similar regimen of FP was administered to animals in group II (FP-20) on days 20-26. Rabbits in groups III (inhibition controls) and IV (Patency controls) did not receive FP injections. Weight changes and WBC counts were determined regularly throughout the test for animals in groups I, II and III. Fecal samples were collected on days 14-38 and

examined using a modified McMaster technique. Serum samples were collected at 6-day intervals and examined for antibodies against OCA using the gel diffusion test. The animals in groups I, II and III were necropsied on day 42.

Necropsies were not performed on 2 FP-0 rabbits that died (1 on day 6 and one on day 7), and the rabbits in group IV were not sacrificed at the end of the test. Necropsy data for the remaining animals are found in Table 8. Rabbits in both FP-treated groups harbored an average of approximately 3,000 more worms than the inhibition controls. The percentages of worms which did not develop beyond the 4th stage were 68.0% (FP-0), 28.5% (FP-20) and 58.1% (inhibition controls). Thus, FP treatments administered days 20-26 produced a significant reduction in inhibited larvae, whereas treatments on days 0-6 did not. In addition, only 12.2% of the FP-0 worms and 29.6% of the inhibition control worms progressed to the late 5th stage as compared to 50.1% of those from the FP-20 group. These data indicate that the FP treatments given days 20-26 allowed inhibited L_4 to resume development. It was not clear why FP treatments administered days 0-6 resulted in an enhancement of worm inhibition. However, it is known that immunosuppressants have varied effects on immune responses in animals depending on the time the drug is administered relative to an antigenic stimulus.

Rabbits in the patency control group began to pass helminth eggs on day 19, followed by a normal rise in mean EPG counts to a peak of 3,300 (Fig. 6). In contrast, the other groups exhibited prolonged

prepatent periods. The FP-0 and inhibition control groups started passing eggs on days 35 and 37, respectively, and the mean EPG counts remained below 100 in both cases. Yet, eggs first appeared in the feces of rabbits in the FP-20 group only 6 days after FP treatments were begun (day 26), and the mean EPG counts rose to a peak of 780. The egg count data correlate well with the necropsy results which showed that many adult worms were present in the FP-20 groups.

Treatment of rabbits with FP resulted in significant changes in WBC counts (Fig. 7). The FP-0 group showed an immediate drop, and counts remained at low levels throughout the test. Both the FP-20 and inhibition controls exhibited markedly depressed counts on day 6 followed by an immediate rise to an elevated level. The WBC drop may reflect a period in which lymphocytes were migrating to the gastric mucosa, while the rise probably coincided with mobilization of cells from lymphoid tissues. The WBC counts in the FP-20 animals also dropped rapidly after treatment began (day 20) but did not remain as low as in the FP-0 group.

Treatments with FP produced a specific weight change pattern (Fig. 8) which was characterized by an immediate and extensive increase in weight followed by a precipitous drop. During the period of initial weight increase, the rabbits engorged themselves with food, after which they stopped eating for a period of 6-9 days. Once the rabbits again started eating they gained weight at a somewhat normal rate but never achieved the weight of the untreated animals. The latter observations

TABLE 8. DEVELOPMENT OF *OBELISCOIDES CUNICULI* IN RABBITS WITH HIGH-LEVEL[A] INFECTIONS AND TREATED WITH THE CORTICOSTEROID 9-FLUOROPREDNISOLONE (FP).

GROUP	NUMBER OF RABBITS	TREATMENT	MEAN WORM COUNT[B] (S.E.M.)[C]	PER CENT L_4	PER CENT ADULTS
I	3	FP-TREATED[D] (DAY 0-6)	8,969 (±2067)	68.0	12.2
II	5	FP-TREATED (DAY 20-26)	8,326 (±1612)	28.5	50.1
III	5	INHIBITION CONTROL	5,513 (± 751)	58.1	29.6

[A]INFECTIONS INITIATED WITH 100,000 L_3.

[B]NECROPSIES 42 DAYS POST-LARVAL-INOCULATION.

[C]STANDARD ERROR OF THE MEAN.

[D]TWO INJECTIONS (I.M.) AT A RATE OF 2 MG/KG/DAY FOLLOWED BY 5 INJECTIONS AT 1 MG/KG/DAY.

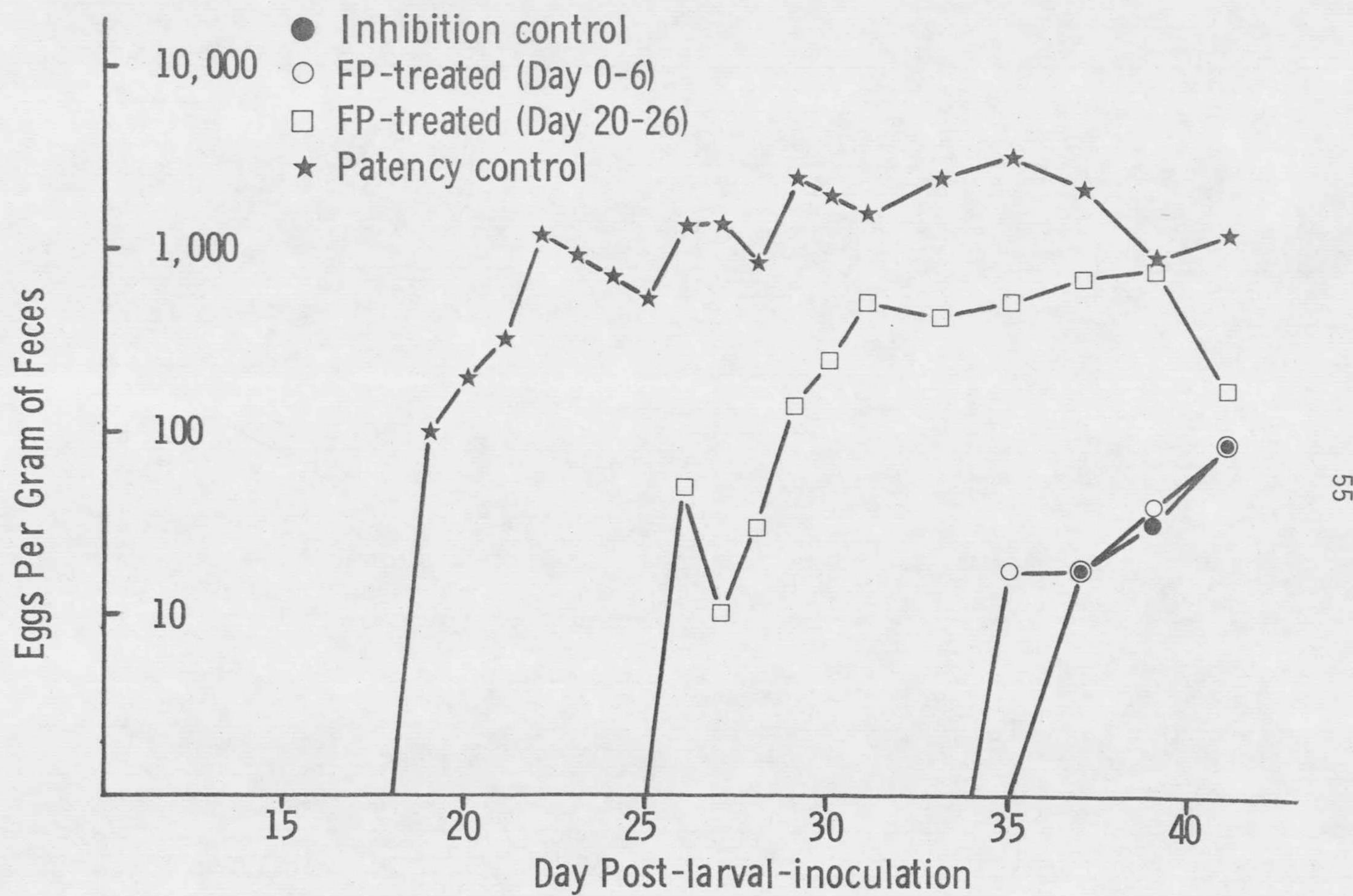

Figure 6. Mean egg production by Obeliscoides cuniculi in rabbits treated with 9-fluoroprednisolone (FP).

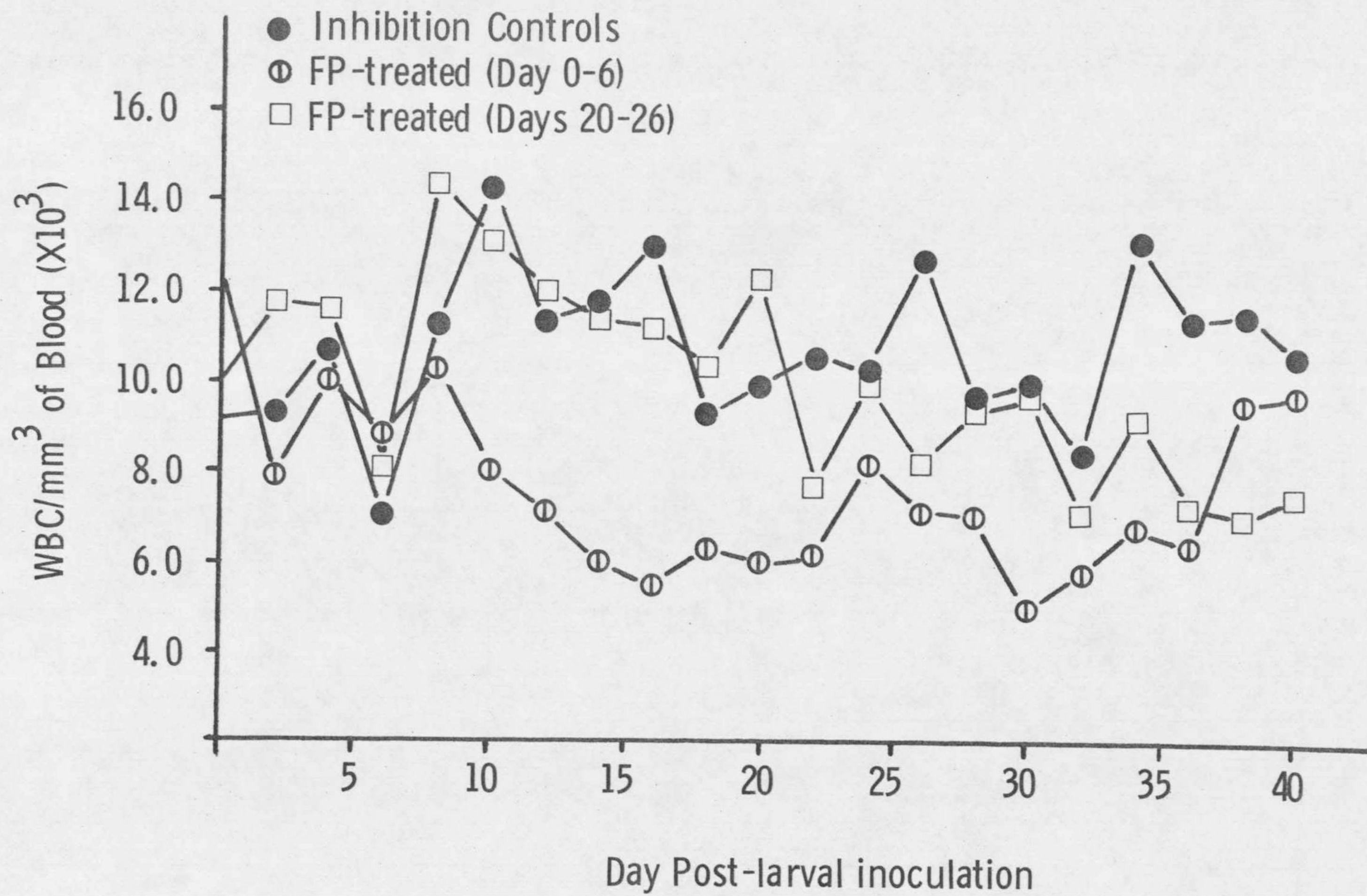

Figure 7. Mean white blood cell (WBC) counts in rabbits given high-level <u>Obeliscoides cuniculi</u> infections and treated with 9-fluoroprednisolone (FP).

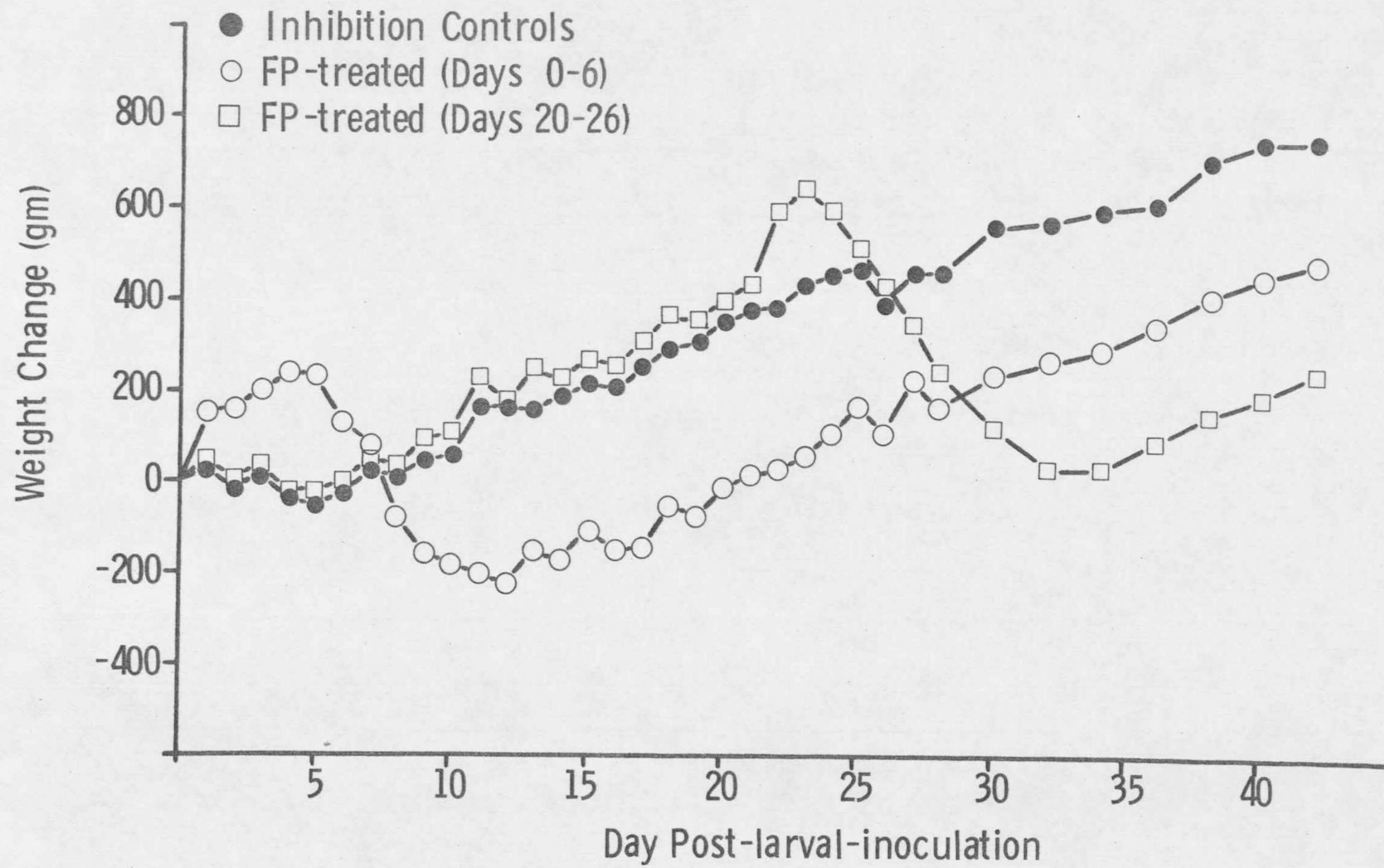

Figure 8. Mean weight changes in rabbits given high-level *Obeliscoides cuniculi* infections and treated with 9-fluoroprednisolone (FP).

are probably indicative of the hormonal effects of the corticorsteroid on glucose metabolism.

Serum antibodies against OCA were detected in only 2 rabbits during the 42-day observation period. One rabbit in the FP-20 group was positive on days 11 and 17 but antibody was not detected after FP treatments began. Also, 1 rabbit in the inhibition control group was positive on day 17. The gel diffusion test probably was not sensitive enough to detect low concentrations of antibody.

Development of *Obeliscoides cuniculi* in Rabbits Treated With Cyclophosphamide or 9-fluoroprednisolone

This test was designed to study the development of *O. cuniculi* in rabbits given immunosuppressive drugs with different biological activities. Three Palomino and 2 New Zealand White rabbits (approximately 6 months old) were allocated to each of 4 experimental groups (Table 9). On day 0, all rabbits in groups I, II and III were inoculated with 100,000 L_3, and rabbits in group IV with 4,000 L_3. Total WBC counts were done at 2-day intervals. Serum (3-5 ml blood) was collected and WBC differential counts were determined every 6 days. On day 9, all rabbits were injected with 5 ml of a 5% suspension of SRBC as a test antigen. Rabbits in group I were given a regimen of FP (2 days at 2 mg/kg and 5 days at 1 mg/kg) on days 9-15. A regimen of CY (2 days at 20 mg/kg and 5 days at 10 mg/kg) and was given days 9-15 to animals in group II. Group III rabbits served as inhibition controls and Group IV as patency controls. All rabbits were weighed daily during the time of drug treatment and on alternate

days thereafter. Fecal egg counts were measured on days 14-26 using the modified McMaster technique. All rabbits were necropsied on day 27. Serum antibody titers against SRBC were measured via a hemolysin test, and antibody against OCA was detected by indirect hemagglutination. Stomach tissues were examined histologically for lesions.

One rabbit in each group died early in the test. Necropsy data for the remaining 16 animals are listed in Table 9. The FP-treated rabbits harbored a mean of 14,562 worms, of which 61.7% were inhibited L_4. One FP-treated rabbit did not have a sustained reduction in numbers of peripheral blood lymphocytes after treatment as did the other animals in the group, nor was its worm burden comparable (16,340 worms; 83.2% inhibited). Comparable data for groups II, III and IV were 10,785, 85.2%; 8,978, 75.6%, and 416, 47.8% respectively. A Duncan's multiple range analysis showed that the percentage of inhibited larvae in FP-treated rabbits and patency control animals were statistically similar. Likewise, the percentages of retarded worms in the CY-treated group were similar to those of the inhibition controls. These data indicate that FP treatments given days 9-15 allowed a large number of L_4 larvae to resume development. Conversely, CY treatments days 9-15 prevented development of L_4 to mature adults. The data suggest that CY may have enhanced the inhibitory process. This conclusion is substantiated by the small numbers of L_5 stages found in the CY-treated animals. Therefore, FP and CY appeared to have opposite effects on the ability of rabbits to retard development of _O. cuniculi_. Furthermore, the effects of CY

administered days 9-15 were similar to the effects of FP given days 0-6 in the previous test.

Egg production by O. cuniculi in immunosuppressed rabbits is shown in Fig. 9. Egg production was not observed in the CY-treated rabbits (Fig. 9). Also very few helminth eggs were passed by the inhibition controls. Patency controls developed patent infections on day 18 and EPG counts increased normally. Egg output in the FP-treated group also showed a normal rapid increase, but patency was delayed until day 22 (7 days after FP treatments began). These data indicate that FP treatments in rabbits allowed development of retarded larvae to resume, whereas an opposite effect occurred in the CY-treated animals.

Changes in the numbers of rabbit peripheral blood lymphocytes (PBL) are shown in Fig. 10. Treatment with FP produced the greatest reduction in the numbers of PBL. The reduction occurred within 6 days after treatment began and persisted to the end of the test. Treatment with CY did not significantly depress PBL counts, however, it did prevent the lymphocytosis that was observed in the inhibition controls at approximately day 16. These results show that FP had a significantly greater effect on lymphocyte populations in the peripheral blood than did CY. These data also suggest that some lymphoid cell type may mediate the suppression of worm development.

Figure 11 shows that FP was more effective in suppressing total leukocyte counts than was CY. A drop in WBC counts occurred in the FP group 2 days after treatment began. Reductions in total leukocyte

counts were not as great as the reduction in numbers of lymphocytes. This suggests that other cell-types were mobilized as the circulating lymphocytes numbers were depleted. The WBC differentials indicated that the greatest increase in leukocyte numbers occurred in the neutrophil populations; eosinophilia was not evident in the peripheral blood.

Both drugs produced weight losses in rabbits (Fig. 12). Treatment with FP caused an initial weight increase followed by a rapid drop. This pattern was also described in the previous experiment. Treatments with CY caused a rapid weight loss without an initial increase. Thus, the effects of CY on rabbits are different than those of FP. Animals in the 2 control groups gained at a fairly constant rate throughout the test. Even though CY did not reduce the numbers circulating lymphocytes, the depressed weight gains in the recipient rabbits indicate that the drug produced an adverse physiological effect.

The results of the hemolysin tests to detect antibodies against SRBC are shown in Fig. 13. Six days after SRBC were administered (day 15), all rabbits which were infected with 100,000 L_3 had similar mean anti-SRBC antibody titers. However, those rabbits which were inoculated with only 4,000 L_3 had considerably higher antibody titers against SRBC. Thus, the massive worm infections may have suppressed the antibody response to SRBC. These findings again suggest that FP and CY had different effects on the immune systems in these rabbits.

A comparison of the antibody titers against OCA and SRBC on day 27 is shown in Fig. 14. In both cases the CY-treated animals had the lowest titers and the FP-treated animals, the highest, while the inhibition controls had suppressed titers to both antigens. Even though the differences in anti-OCA titers were not statistically significant (F-test) and differences in the anti-SRBC titers were significant at only the $P \leq .10$ level, the relative similarities in titers to both antigens suggest that CY and FP exerted opposite effects on both antibody levels and worm development.

The gross pathological lesions in the stomachs of these animals were similar to those previously described. The histological lesions (Figs. 14-20) within the fundic and cardiac regions of the gastric mucosa were characterized by focal lymphoid hyperplasia in the base of the mucosa and lymphocytic infiltration into the lamina propria. There was mild eosinophilic infiltration throughout the mucosa but especially in areas associated with parasitic larvae or lymphoid follicles. Some lymphoid cells infiltrated through the muscularis mucosa into the submucosa. The mucosa was thrown into folds as a result of epithelial hyperplasia. Occasionally a mild cryptitis was associated with the presence of nematode larvae. Lesions in the FP and CY treated animals were similar to those in the inhibition controls but in general there was less lymphocytic proliferation in the treated rabbits. However, considerable variation occurred within the immunosuppressed groups, and

in some animals the lesions were almost as severe as in the inhibition controls. Lesions associated with low-level infections (patency controls) were not severe and consisted of mild lymphocytic hyperplasia and only limited epithelial hyperplasia.

TABLE 9. DEVELOPMENT OF <u>OBELISCOIDES CUNICULI</u> IN RABBITS TREATED[A] WITH 9-FLUOROPREDNISOLONE (FP) OR CYCLOPHOSPHAMIDE (CY).

GROUP[B]	NUMBER OF INFECTIVE LARVAE	MEAN WORM COUNT[C] (S.E.M.)[D]	PER CENT L_4
FP-TREATED (DAYS 9-15)	100,000	14,562 (±1996)	61.7[Y]
CY-TREATED (DAYS 9-15)	100,000	10,785 (±1565)	87.2[X]
INHIBITION CONTROL	100,000	8,978 (±1254)	75.6[X]
PATENCY CONTROL	4,000	416 (± 33)	47.8[Y]

[A]RABBITS GIVEN A TOTAL OF 9 MG/KG OF FP AND CY-TREATED 90 MG/KG CY.

[B]FOUR RABBIT GROUPS.

[C]NECROPSIES DAY 27 POST-LARVAL-INOCULATION.

[D]STANDARD ERROR OF THE MEAN.

[XY]MULTIPLE RANGE DESIGNATIONS TO DENOTE DIFFERENCES IN TREATMENT MEANS.

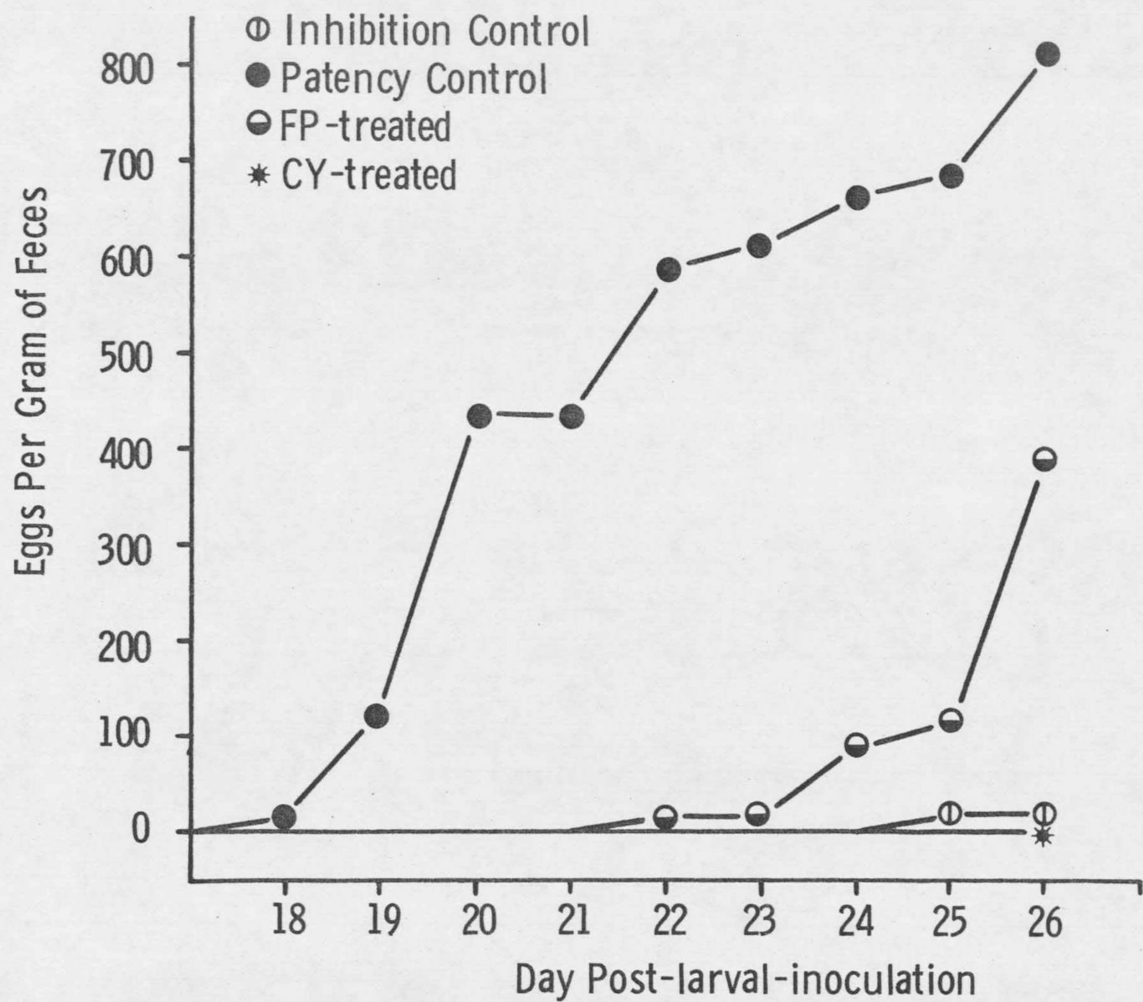

Figure 9. Mean egg production by _Obeliscoides cuniculi_ in rabbits treated with 9-fluoroprednisolone (FP) or cyclophosphamide (CY).

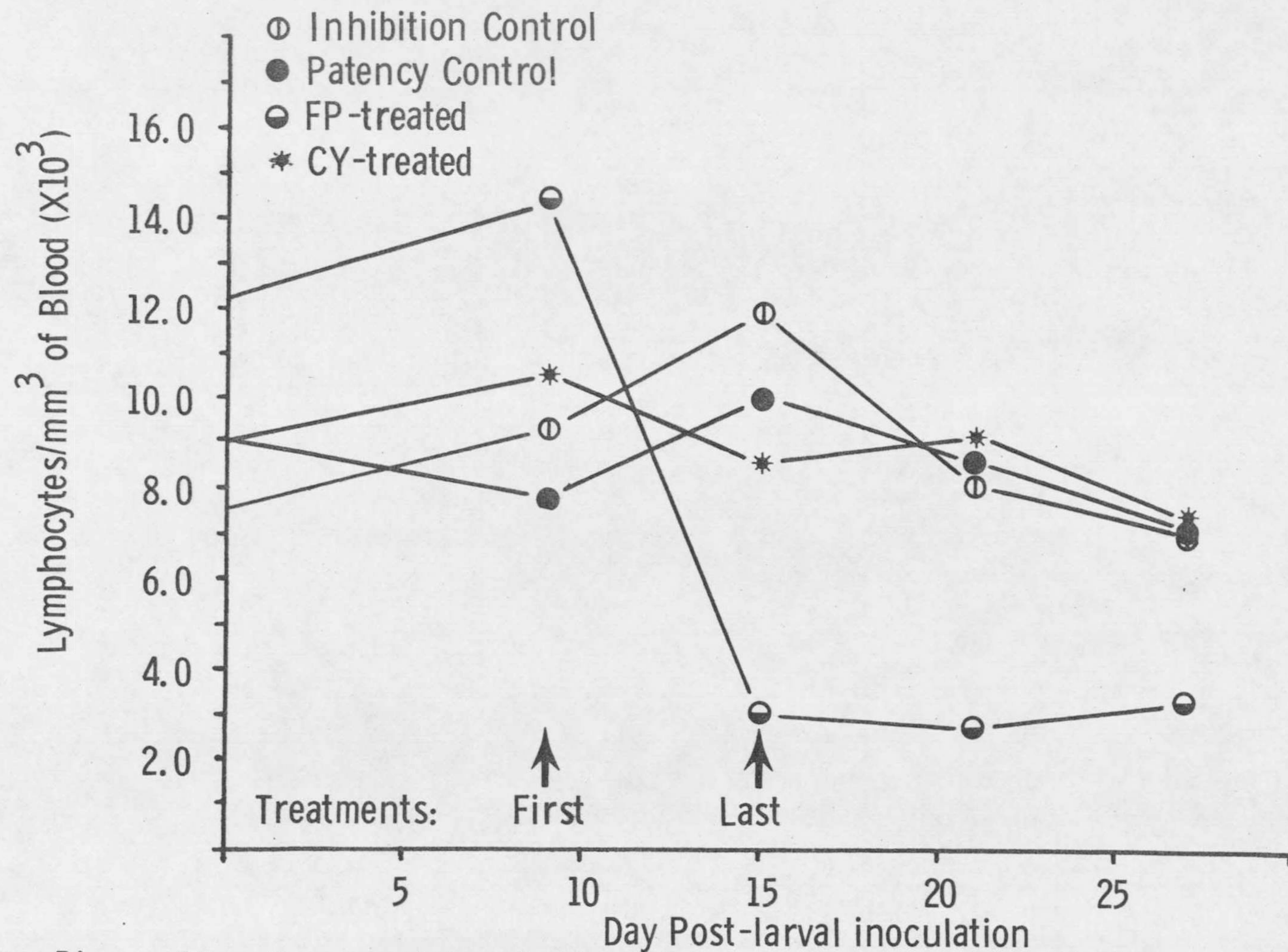

Figure 10. Mean peripheral blood lymphocyte counts in rabbits infected with <u>Obeliscoides cuniculi</u> and treated with 9-fluoroprednisolone (FP) or cyclophosphamide (CY).

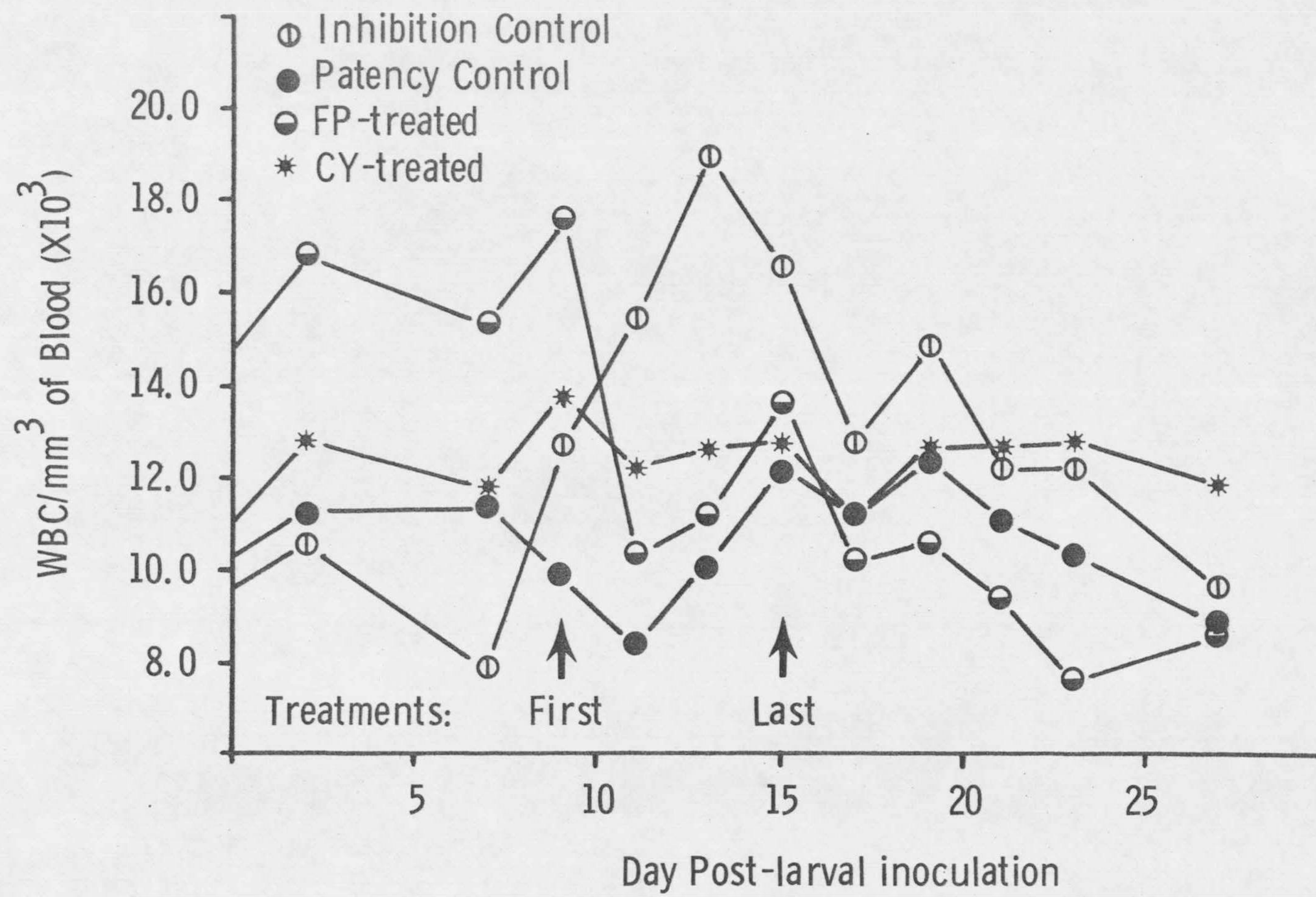

Figure 11. Mean white blood cell (WBC) counts in peripheral blood of rabbits infected with <u>Obeliscoides cuniculi</u> and treated with 9-fluoroprednisolone (FP) or cyslophosphamide (CY).

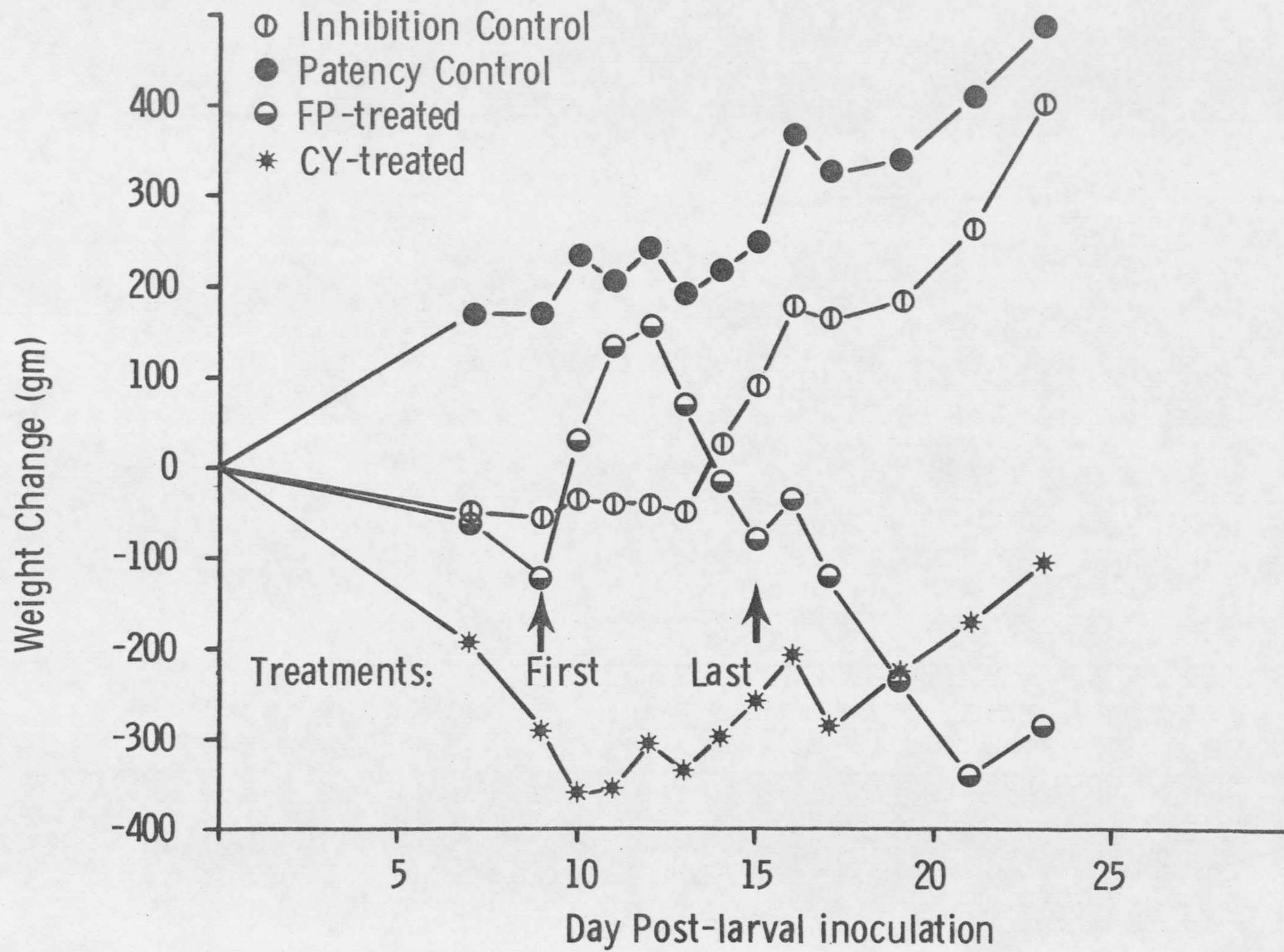

Figure 12. Mean weight changes in rabbits infected with Obeliscoides cuniculi and treated with 9-fluoroprednisolone (FP) or cyclophosphamide (CY).

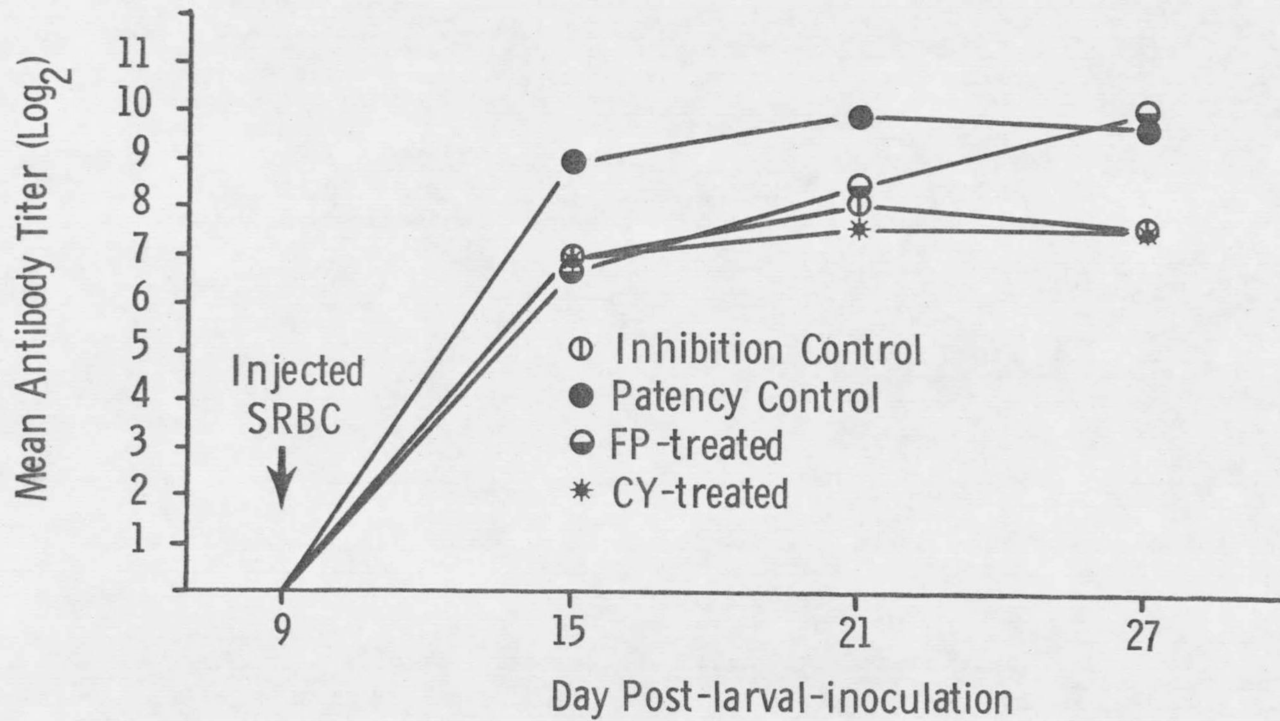

Figure 13. Mean antibody titers to sheep red blood cells (SRBC) in rabbits infected with *Obeliscoides cuniculi* and treated with 9-fluoroprednosolone (FP) or cyclophosphamide (CY).

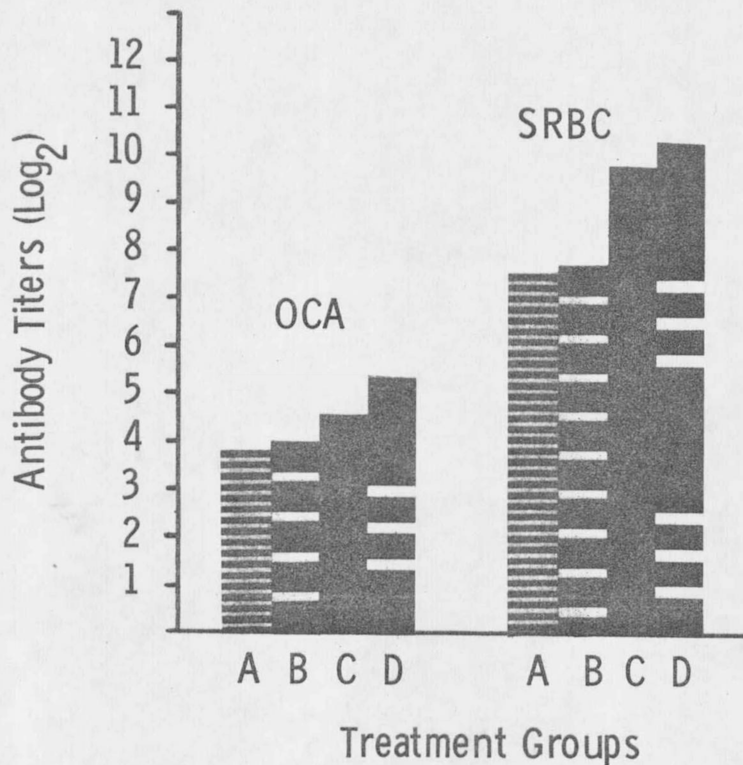

Figure 14. A comparison antibody titers against *Obeliscoides cuniculi* antigen (OCA) and sheep red blood cells (SRBC) in immunosuppressed rabbits. Sera collected day 27 post-larval-inoculation (18 days after SRBC injections): (A) infected with 100,000 *O.c.* and treated with cyclophosphamide; (B) infected with 100,000 *O.c*; (C) infected with 4,000 *O.c.*; and (D) infected with 100,000 *O.c.* and treated with 9-fluoroprednisolone.

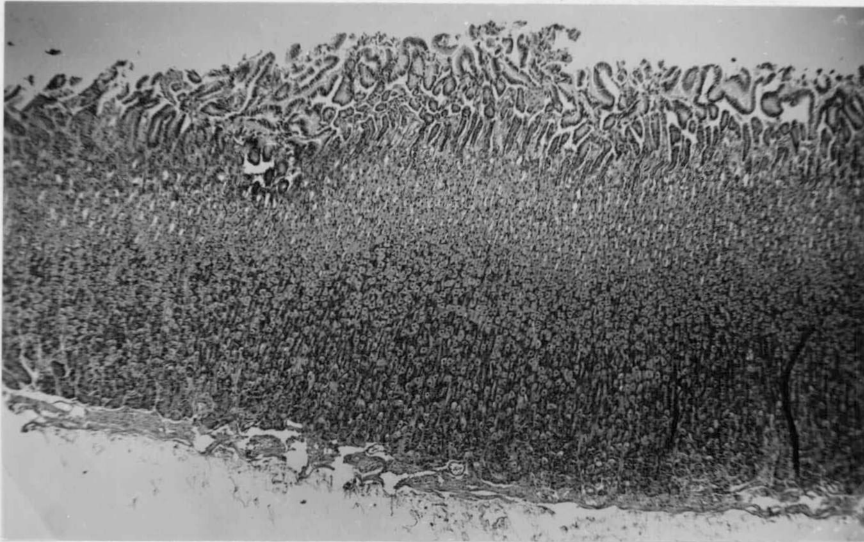

Figure 15. Gastric mucosa from uninfected (normal) rabbit. H & E stain, x 40.

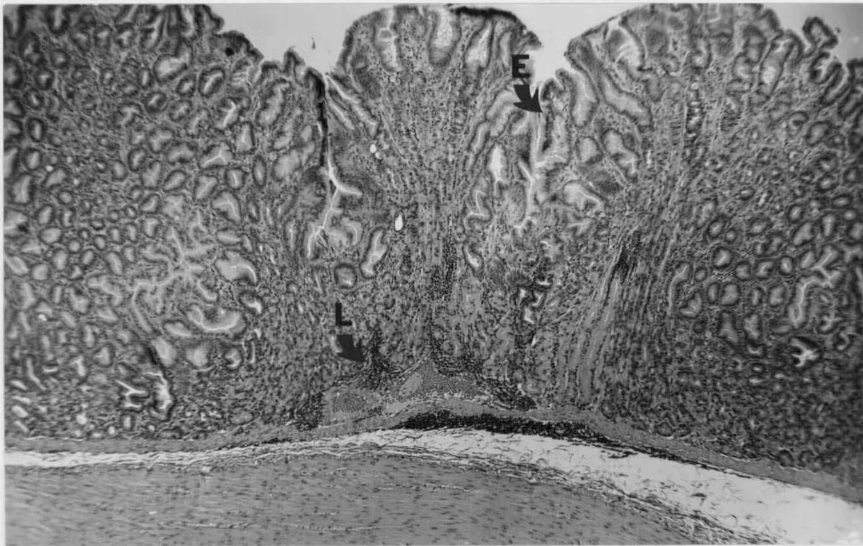

Figure 16. Epithelial (E) lymphocytic (L) hyperplasia in the gastric mucosa of a rabbit after a 27-day infection with 100,000 Obeliscoides cuniculi. H & E stain, x 40.

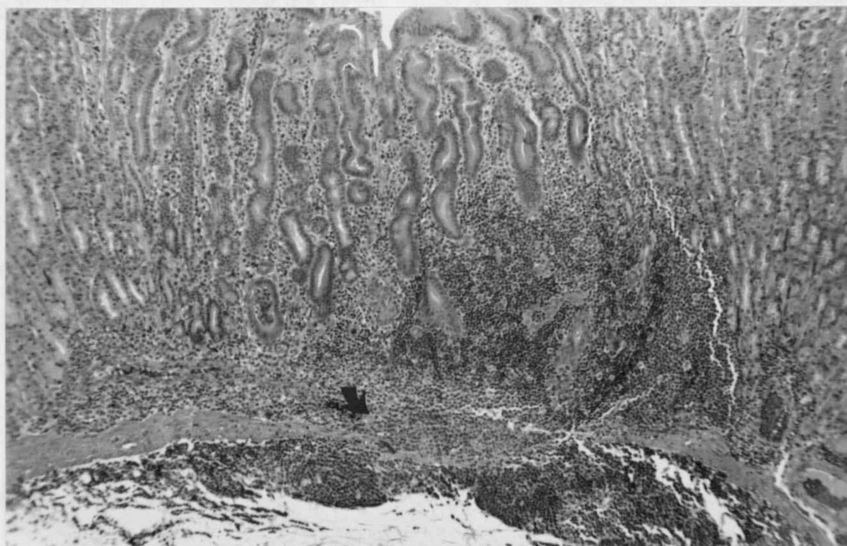

Figure 17. Area of lymphocytic hyperplasia in the gastric mucosa where lymphocytes have infiltrated the muscularis mucosa and into the submucosa of a rabbit infected with 100,000 *Obeliscoides cuniculi*. H & E stain, x 64.

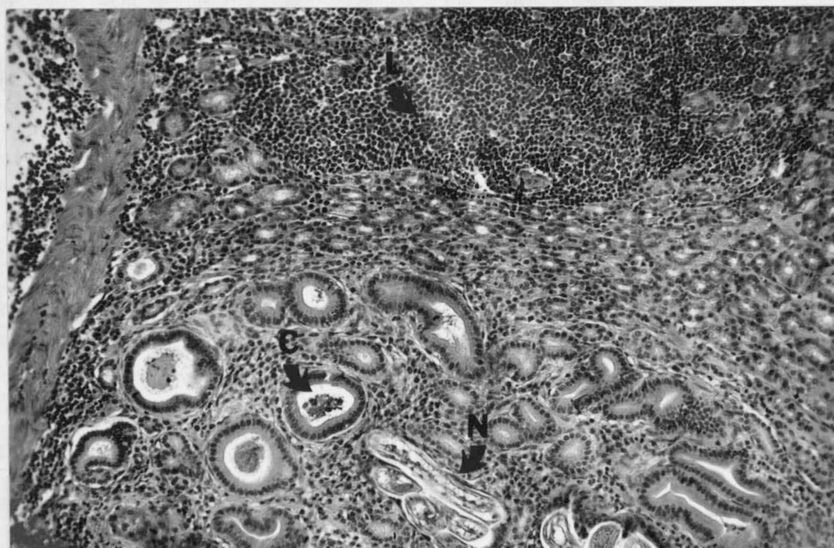

Figure 18. Lymphocytic hyperplasia (L), cryptitis (C) embedded nematode larvae (N), and lymphocytic infiltration in the gastric mucosa of a rabbit infected with 100,000 *Obeliscoides cuniculi*. H & E stain, x 100.

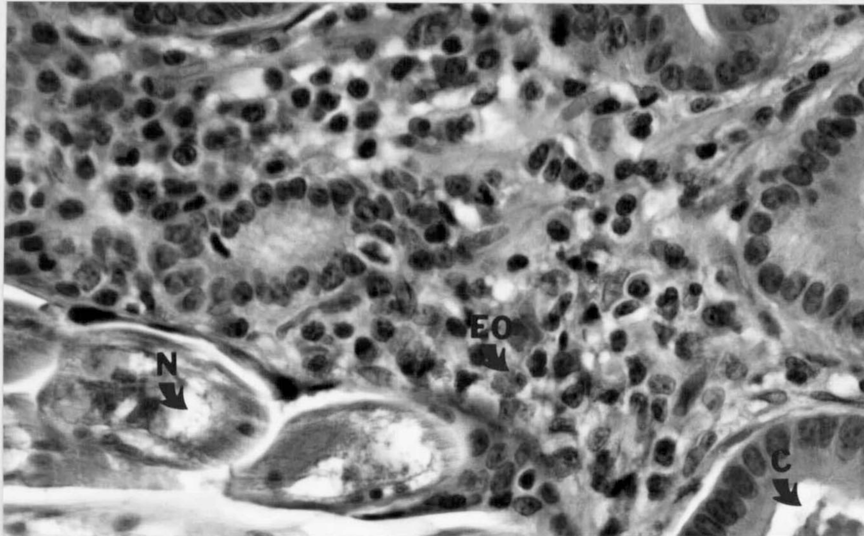

Figure 19. Embedded nematode larvae (N), cryptitis (C) and eosinophils (EO) in the gastric mucosa of a rabbit infected with 100,000 Obeliscoides cuniculi. H&E stain, x 375.

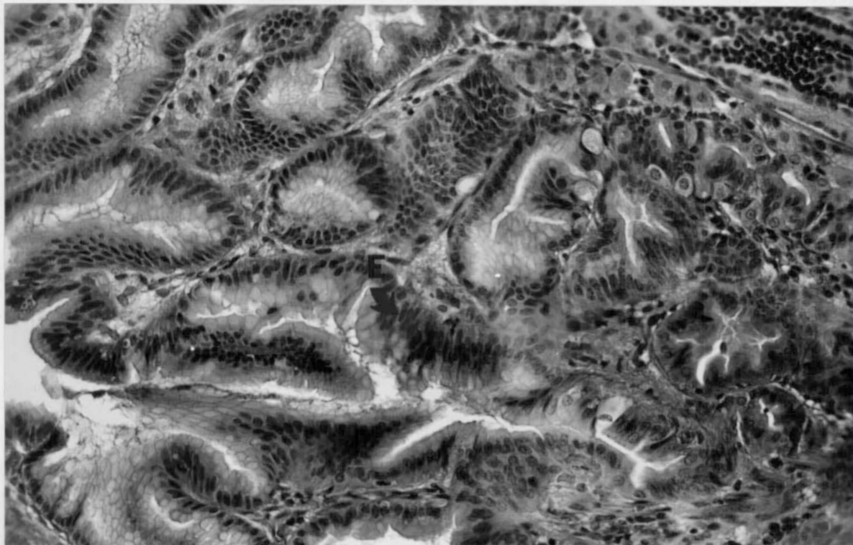

Figure 20. Epithelial hyperplasia (E) of the gastric mucosa in a rabbit during an infection with 100,000 Obeliscoides cuniculi. H&E stain, x 100.

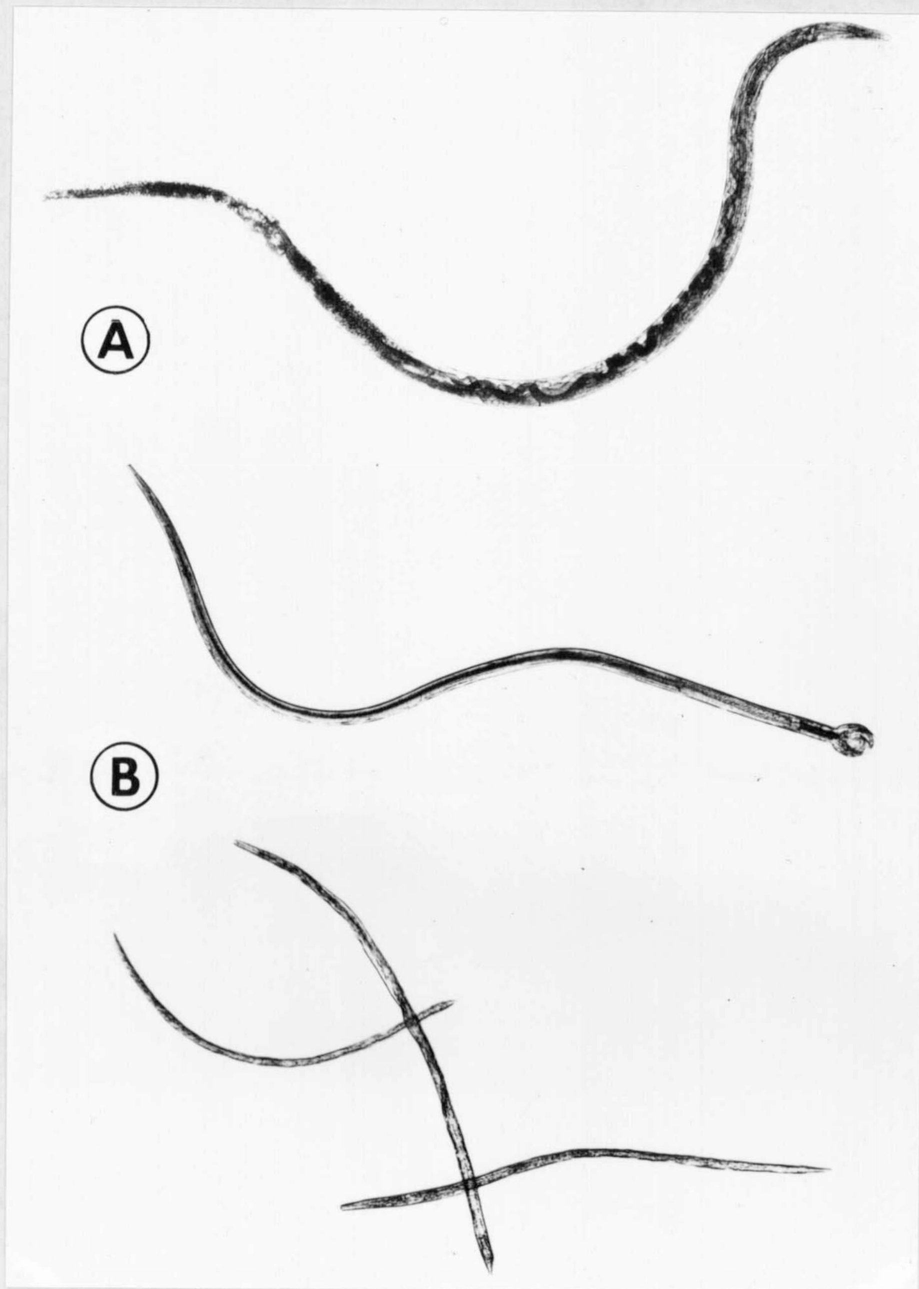

Figure 21. Normal adults (A; x 10) and inhibited 4th stage larvae (B; x 40) of _Obeliscoides cuniculi_ from rabbits 27 days after inoculation with 4,000 L_3 and 100,000 L_3, respectively.

DISCUSSION

Rabbits which were repeatedly inoculated with 3,000 L_3 developed the capacity to retard the development of *O. cuniculi*. This was demonstrated by the presence of large numbers of inhibited 4th stage larvae in rabbits which were pre-infected and dewormed prior to receiving a challenge dose of infective third stage larvae (Table 1). Enhanced inhibition associated with repeated infections was also reported for *Nematodirus spathiger* in sheep (Donald et al., 1964), *Haemonchus contortus* in sheep (Dineen et al., 1965), *Oesophagostomum radiatum* in sheep (Roberts et al., 1962), *Ostertagia ostertagi* in calves (Michel, 1963; Michel et al., 1973) and *H. placei* in calves (Roberts, 1957). In each case the larvae were inhibited at the 4th larval stage. There is much evidence that the 4th stage larvae of nematodes are highly immunogenic (Sommerville, 1960; Christie et al., 1964; Bitakarmire, 1966; Wagland and Dineen, 1967; Keith, 1967; Keith and Bremner, 1973; Michel et al., 1973). Soulsby, Sommerville and Steward (1959) and Silverman, Poynter and Podger (1962) demonstrated that immunogenicity is related to excretion of exsheathing fluid by worms while molting. Sollod et al. (1966) showed that *O.* cuniculi larvae molt twice while embedded in the gastric mucosa; once at 72 hr and again at 5-10 days post-inoculation. This molting process likely elicits an immune response in rabbits which inhibits development of worms.

Development of Obeliscoides cuniculi was almost completely inhibited during initial infections with 200,000-834,700 infective larvae (Table 2). These results fit a pattern demonstrated by Russell et al. (1966) in which the percentages of inhibited O. cuniculi in rabbits increased with the size of the infective larval doses. In the present study infections initiated with 100,000 L_3 were comprised of 50-75% inhibited larvae (Tables 7, 8 and 9). Therefore, dose dependent inhibition in the O. cuniculi-rabbit system is rather consistent. Trichostrongylus retortaeformis (Michel, 1952a) and Graphidium strigosum (Martin et al., 1957) are also inhibited in rabbits in proportion to the size of the infective larval dose. The degree of inhibition possibly is related to the amount of antigenic stimulus the rabbit receives. Dose response curves (Davis, et al. 1973) indicate that an optimal amount of antigenic stimulus is necessary to elicit maximum antibody production. It would be difficult to measure the amount of antigenic stimulus a rabbit receives from nematode larvae which are embedded in the mucosal lining of the gastrointestinal tract. However, small numbers of larvae probably do not elicit a sufficient immune response to inhibit worm development, unless the animal is repeatedly infected. In contrast, massive infections produce a high degree of antigenic stimulus, thus the animal would develop a strong immune response. Such a relationship could explain why almost complete inhibition occurs in high-level infections with O. cuniculi.

Previous reports (Gibson, 1953; Reid, Armour, Jennings, Kirkpatrick, and Urquhart, 1968; Armour, 1970) have indicated that dormant larvae are not susceptible to certain anthelmintics. It was found in this study that immature _O. cuniculi_ were not eliminated from rabbits by 2 treatments with thiabendazole (500 mg/kg). _Obeliscoides cuniculi_ may be atypical in this respect as Armour, Bairden and Reid (1975) reported that thiabendazole (55-75 mg/kg) was effective against dormant _Ostertagia_ spp. in sheep. Levamisole HCl was tested against inhibited _O. cuniculi_ in the present study and was found to be highly efficacious at the rate of 70 mg/kg (Table 3). McKenna (1974b) demonstrated that levamisole was effective against inhibited _Haemonchus contortus_ in sheep at a rate of only 8 mg/kg. Further investigation is therefore necessary to determine if lower dosage rates of levamisole HCl would be effective against _O. cuniculi_. These reports indicate that inhibited larvae of nematodes are not invulnerable to anthelmintics as previously suggested (Armour, 1970).

The time of the 4th ecdysis and the emergence of _O. cuniculi_ from the gastric mucosa have not been definitely established in rabbits. Sollod et al. (1968) thought that the molt to the 5th stage occurred 8-10 days p.i., however, 41% of the worms were still in the 4th larval stage on day 14. These data indicate that the infective larvae used in their tests were inhibited, thus these figures do not represent normal molting periods. A subsequent report (Sollod and Allen, 1971)

indicated that most worms in similar infections (1,000 L_3) had developed to the 5th stage and had migrated to the surface of the mucosa by day 8. In the present study normal 4th stage larvae for use in transfer experiments could not be obtained from source rabbits after 8 days p.i. but were easily recovered on day 5. Therefore, the molt to the 5th stage and emergence from the gastric mucosa must occur between days 5 and 8 of infection. When the larvae remain in the tissues for more than 8 days they probably do so as a result of inhibitory factors whether intrinsic or extrinsic in nature.

The question as to whether larvae resume development following retardation has not been answered for most nematode species. In this study, inhibited 4th stage larvae were obtained from source animals with high-level infections (200,000 L_3) and transferred to either normal or pre-infected (actively immunized) rabbits. In experiment A, 87.6% (Table 4) of the inhibited larvae developed to maturity following transfer to normal rabbits as compared to only 18.2% (Table 5) in experiment B. The differences in results suggest that the extent to which 4th stage larvae are affected during the inhibitory process varies from rabbit to rabbit. Thus, failure to mature was a result of damage incurred under inhibiting conditions in the source rabbits.

Under the conditions of this study, large numbers of _O. cuniculi_ were able to resume development after transfer to other rabbits. Similar observations have been reported for inhibited _Oesophagostomum_

radiatum in calves (Roberts et al., 1963) and inhibited Haemonchus contorus in ewes (Blitz and Gibbs, 1971a). In contrast, Ostertagia ostertagi which were inhibited in sheep and transferred to calves did not resume development (Herlich, 1974).

Following transfer to normal rabbits, inhibited male L_4 did not develop to maturity as readily as normal male L_4 (Tables 4 and 5). In experiment B, the male to female ration was 1:3.3 for inhibited L_4 and 1:1.2 for normal L_4 transfers (Table 5). Possible explanations for the reduced numbers of males are: 1) fewer males were transferred to the recipient animals or 2) some of the inhibited males failed to resume development after transfer. The latter explanation is most likely as further reductions in the numbers of males occurred in the pre-infected recipients (1:8.3). The inhibited L_4, transferred to both the pre-infected and the normal rabbits, came from the same larval pool and should have produced similar adult worm ratios. Changes in sex ratios were not evident in pre-infected rabbits which received normal L_4. Thus, the damage to male worms occurred during the initial inhibitory process in the source rabbit and was potentiated by some factor in the actively immunized recipient. Why male worms failed to resume development after transfer is not understood. Either they remained dormant following transfer or were eliminated from the recipient animals via unknown mechanisms. The latter results were unique in that no such immunologically medicated effect has been described in the development of larvae of other nematode species.

In high-level infections (200,000-834,000 L_3), almost all worms became inhibited, and they remained within the mucosa for periods of 3 months and longer. Normally about 50-75% of the worms in infections initiated with 100,000 L_3 were retarded at the 4th larval stage (Tables 7, 8, 9). Russell et al. (1966) showed that graded doses of larvae (2,500 L_3/kg-25,000 L_3/kg) resulted in corresponding increases in the percentages of worms which became inhibited. Samuel (1970) reported that O. cuniculi were found in rabbits up to 225 days post-larval-inoculation in a low-level infection (3,000 L_3). Thus, the size of the infective larval dose does not determine the length of time larvae remain inhibited, but only the number which become inhibited. The latter observation further indicated that crowding of worms (Taylor and Michel, 1952) does not account for all larval inhibition. In one experiment in the present study (Table 9), 47.8% of the worms in the patency control group (4,000 L_3) were inhibited. In this case, larval inhibition may have been related to the age of the rabbits (6 months). Compare these data with those in Table 6 where patency control animals (5,000 L_3) which were 12-16 weeks old did not contain inhibited L_4.

Active immunization of rabbits resulted in 2 changes in the developmental pattern of O. cuniculi. First, inhibition of L_4 was augmented (Table 6). Almost all adult worms recovered from the pre-infected animals were stunted in growth, and adult females contained

few if any eggs. These data indicated that a host immune response directly inhibited worm development. Second, egg production was slightly reduced in adult worms which developed from inhibited and normal L4 in actively immunized rabbits (Fig. 4). Similar observations have been described in Nematodirus spathiger infections in sheep (Donald et al., 1964) and O. ostertagi in calves (Michel, 1963; 1970). Also, abnormal spicule development occurs in male Cooperia pectinata in immune calves (Keith, 1967).

Worm egg counts were influenced through reduction in the total number of mature worms and by suppression of egg production in adult worms. Passive immunizations of rabbits with immune serum did not inhibit development of worms (Table 6) but did suppress egg production (Fig. 4). These data indicate that antibodies suppressed egg production in mature worms. Antibody-mediated suppression of egg production has not been reported for O. cuniculi. However, reduced egg production in second infections have been demonstrated many times with other nematode species (Michel, 1969). Worley (1963) demonstrated that O. cuniculi produced higher worm egg counts in rabbits infected with 1,000 L_3 than with 10,000 L_3. The latter observation could represent either antibody-suppressed egg production or restricted development of worms.

In summarizing the results of active and passive immunizations, it is evident that inhibition of worm development and suppression of egg production in adult worms are under separate control mechanisms.

Egg production was suppressed with hyperimmune serum and is therefore antibody dependent. Also, following transfer of inhibited and normal L_4, egg counts were lower in actively immunized rabbits than in normal animals (Fig. 3), even though comparable numbers of adult worms were present in both types of recipients (Table 5). Egg production was lowest in worms which were transferred to the pre-infected rabbits, and in both cases these rabbits harbored large numbers of adult worms (Table 5). Larval inhibition may or may not involve humoral antibody because worm inhibition was not augmented with immune serum.

Cyclophosphamide treatments in rabbits did not promote worm development. Instead it resulted in an increase in the percentages of inhibited L_4, and few of the 5th stage worms were normal (3.5%). Thus, CY seemed to enhance the inhibitory process. Immunosuppressive agents have not altered inhibitory processes in some host-parasite systems (Michel, 1969; Blitz and Gibbs, 1972a; Pritchard et al., 1974). However, Dunsmore (1961) was successful in causing Ostertagia spp. in sheep to develop following combined corticosteroid and X-irradiation treatments. Also, Sollod and Allen (1971) reported that cyclophosphamide treatments in rabbits suppressed antibody response to O. cuniculi antigen and conalbumen, and produced a concomitant increase in worm burdens and maturation of O. cuniculi.

It has been demonstrated that cyclophosphamide is effective in inhibiting antibody production to SRBC in mice at dosage rates of

200-300 mg/kg. When the drug was given to mice before sensitization with sheep erythrocytes the result was an enhanced DTH or cell-mediated immune response (Lagrange Mackaness and Miller, 1974; Kerckhaert, 1974; Kerckhaert et al., 1974). The dosage rate used in the present study was considerably lower (90 mg/kg total), and antibody production was not significantly suppressed. However, Askenase, Hayden and Gershon (1975) demonstrated that CY treatments of 20 mg/kg enhanced DTH responses to SRBC in mice, even though it did not abrogate antibody production. If CY has similar effects in rabbits, inhibition of O. cuniculi may have been controlled by cell-mediated immune mechanisms.

Immunosuppression of rabbits with FP provided strong evidence that development of O. cuniculi was under immunological control. In 3 separate experiments (Table 7, 8 and 9), FP treatment of rabbits with high-level infections (100,000 L_3) with O. cuniculi resulted in an increased number of adult worms. The effects produced by FP varied according to the time the drug was administered. Treatments given to rabbits on days 9-15 and 20-26 p.i. resulted in significantly larger numbers of adult worms; patency occurred on days 22 (Fig. 9) and 26 (Fig. 6), respectively. Also worm egg counts increased at a steady rate. Treatments given days 0-6 did not alter worm development (Table 8), whereas prolonged administration of corticosteroid for 28 days (Table 7) resulted in development of slightly larger numbers of worms.

The pathology of O. cuniculi infections, initiated with 100,000 L_3,

has been described (Russell et al., 1970). Gross lesions included petechial hemorrhages and folding and thickening of the gastric mucosa. Russell et al. suggested that this process was caused by mucosal edema. In the present study, histological examination of stomach tissue at 27 days p.i. revealed that the folding of the mucosa was caused by epithelial hyperplasia (Figs.16, 20) and only mild mucosal edema was present. Also, by day 27 p.i., mucous-cell hyperplasia was not evident. Russell found that the latter condition began early (24 hr) and subsided by day 22. Other histological lesions seen in rabbits in the present study included lymphocytic hyperplasia, lymphocytic and eosinophilic infiltration into the lamina propria and in the vicinity of embedded parasites (Figs. 17-20). In some areas lymphocytic cells were observed to infiltrate through the muscularis mucosa into submucosa. Similar lesions also occurred to a limited extent in rabbits with low-level _O. cuniculi_ infections (Samuel, 1970).

The effect of FP in rabbits points to the possibility that larval inhibition is related to cell-mediated immunity. Results shown in Fig. 10 indicated that FP significantly reduced the numbers of circulating lymphocytes in rabbits. _In vitro_ transformation of rabbit peripheral blood lymphocytes with T-cell mitogens (PHA and Con A) indicate that most peripheral blood lymphocytes in rabbits are T-cells (Sell and Sheppard, 1973). If so, FP may have selectively reduced the T-cell populations (the lymphocytes involved in DTH reactions) in these rabbits.

Many studies have shown that T-cells are susceptible to corticosteroids (Davis et al., 1973; Fauci, 1975; Fauci and Dale, 1974; Yu, Clements, Paulus, Peter, Levy and Barnett, 1974).

Animals differ in their sensitivity to corticosteroids. The hamster, mouse, rat and rabbit are corticosteroid-sensitive species, whereas ferret, monkey, guinea-pig and man are resistant. Subpopulations of lymphocytes in sensitive species vary in susceptibility to corticosteroids. Claman (1972) reported that bone marrow derived lymphocytes (B-cells) are most susceptible during the precursor stage or prior to being activated by antigen when in lymphoid compartments other than the bone marrow. Thymus drived lymphocytes (T-cells) in the thymus cortex are susceptible but not those in the medulla. Also, T-cells are sensitive during blast transformation by mitogens but not when activated by antigen. Therefore, antibody producing cells, B-lymphocytes or plasma cells, are not effected by cortiocosteroids. Also, antigen activated T-cells are not destroyed by corticosteroids, however, in some cases, their effector mechanisms may be sensitive.

The fact that activated B-cells are not effected by corticosteroids would explain why antibody titers against OCA and SRBC were not suppressed (Figs. 13 and 14). Yet the mass destruction of T-cells could have produced a deficit in T-cells necessary for the regulation of the worm populations. The enhanced antibody titers against sheep erythrocytes (Fig. 13) also suggest a selective effect by FP on the

T-cell populations in rabbits. The FP treated animals had higher antibody levels to SRBC than the inhibition controls. Taniguchi and Tada (1974) showed that selective depletion of T-cll compartments in rabbits using antithymocyte serum or adult thymectomy resulted in elevated anti-DNP antibody titers.

The results of this study only infer an involvement of T-cells in the regulation of development of O. cuniculi in rabbits. It has been shown that T-lymphocytes are involved in the regulation of several other helminth parasites (Targett, 1973; Kelly, 1973; Ogilvie and Jones, 1973). Recent studies using congenitally athymic mice showed that resistance to the mouse pinworms Aspicularis tetraptera and Syphacia obvelata (Jacobson and Reed, 1974a) and the rat heligmosomid Nippostrongylus brasiliensis (Jacobson and Reed, 1974b) was thymus-cell dependent. These reports provide evidence that T-cells function in the regulation of several unrelated helminth systems. Therefore, T-cells could be involved in the regulation of other parasites as well.

The conclusion from this study is that host immunity plays an important role in inhibited development of O. cuniculi. The data show that (1) active immunization of rabbits by single or repeated low-level (3,000 L3) or single high-level (75,000 L3) infections resulted in inhibition of larvae at the 4th stage, (2) 9-fluoroprednisolone treatment increased the numbers of worms which developed to the adult stage, (3) the amount of inhibition varied with larval-dose, and

(4) worms were damaged during inhibition (especially males) such that not all inhibited L4 were able to resume development when transferred into uninfected rabbits.

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APPENDIX

Mean percent packed red blood cell volumes in rabbits given single and repeated infections[a] with *Obeliscoides cuniculi*.

Day of test	Group				
	I (1)[b]	II (2)	III (4)	IV (3)	V (2)
14	43.3	46.3	42.8	42.5	41.3
28	40.0	44.0	42.5	43.3	45.5
42	41.5	46.0	42.5	41.8	43.0
49	40.8	45.8	42.5	44.3	43.3
68	42.0	44.8	43.5	43.5	43.3
75	41.8	44.8	42.0	42.3	42.7
82	40.5	42.3	40.5	41.3	41.3
94	40.3	41.3	37.8	38.0	41.7

[a]Infections with 3,000 infective larvae.

[b]Number of inoculations.

Mean percent lymphocytes from white blood cell differential counts on rabbits given single and reported infections[a] with Obeliscoides cuniculi.

Day of test	Group				
	I (1)	II (2)	III (4)	IV (3)	V (2)
28	61.8	68.5	59.3	67.3	71.5
42	69.8	74.0	77.3	80.0	82.7
49	67.3	83.0	73.0	79.8	83.7
68	78.3	78.8	73.5	78.0	79.0
75	74.5	66.8	80.8	73.5	74.7
82	73.0	73.3	72.5	82.0	73.7
94	68.3	86.0	83.3	88.3	89.7

[a]Infections with 3,000 infective larvae.

[b]Number of inoculations.

Mean percent monocytes from white blood cell differential counts on rabbits given single and repeated infections[a] with *Obeliscoides cuniculi*.

Day of test	Group				
	I (1)[b]	II (2)	III (4)	IV (3)	V (2)
28	3.8	6.0	6.0	5.0	5.5
42	1.8	3.0	1.8	2.0	0.3
49	2.3	1.5	1.0	1.5	2.0
68	1.5	2.0	1.3	1.0	1.0
75	0.5	0.8	0.0	1.0	0.0
82	1.5	2.8	2.5	0.8	2.0
94	1.3	1.8	1.5	1.3	3.0

[a]Infections with 3,000 infective larvae.

[b]Number of inoculations.

Mean percent neutrophils from white blood cell differential counts on rabbits given single and repeated infections[a] with Obeliscoides cuniculi.

Day of Test	Group				
	I (1)[b]	II (2)	III (4)	IV (3)	V (2)
28	30.8	23.0	30.5	16.8	19.0
42	26.3	19.0	16.3	13.0	7.0
49	26.5	14.3	21.5	12.0	12.0
68	17.3	16.8	21.3	15.0	18.3
75	22.3	18.8	13.8	20.0	21.0
82	22.3	19.8	19.3	12.5	19.3
94	28.5	10.3	12.8	11.0	5.0

[a]Infections with 3,000 infective larvae.

[b]Number of inoculations.

Mean percent eosinophils from white blood cell differential counts on rabbits given single and repeated infections[a] with *Obeliscoides cuniculi*.

Day of test	Group				
	I (1)[b]	II (2)	III (4)	IV (3)	V (2)
28	1.3	0.5	1.3	4.3	1.0
42	0.0	2.0	1.3	1.0	3.0
49	1.1	0.5	0.5	1.5	1.0
68	0.3	1.5	0.5	1.5	0.0
75	1.3	2.0	3.0	2.0	3.0
82	1.3	1.0	1.0	2.8	3.0
94	0.8	0.8	1.0	1.0	0.7

[a]Infections with 3,000 infective larvae.

[b]Number of inoculations.

Mean percent basophils from white blood cell differential counts on rabbits given single and repeated infections[a] with *Obeliscoides cuniculi*.

Day of test	Group				
	I (1)[b]	II (2)	III (4)	IV (3)	V (2)
28	2.5	2.0	3.0	6.8	3.3
42	2.3	2.0	3.5	4.3	3.0
49	3.0	0.8	4.0	5.0	1.3
68	2.8	0.3	3.5	4.5	3.7
75	1.8	2.3	2.5	3.3	1.7
82	2.3	3.3	4.8	2.0	2.3
94	1.3	1.5	1.5	1.0	1.0

[a]Infections with 3,000 infective larvae.

[b]Number of inoculations.

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