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
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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

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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.

When inhibited L_4 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 L_4 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 L_3 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 L_3) passed only low number of eggs ($\leq$ 100) even though 25-42% of their worms were L_5. 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 L_3); inhibition controls (100,000 L_3)

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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 L_3) and single high-level (100,000 L_3) 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 L_4 were unable to resume development when transferred to uninfected rabbits. Also many inhibited male L_4 failed to develop into adults, and this was further accentuated in actively immunized recipients.

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 gastrointestinal 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).