THE IMMUNOLOGY OF FASCIOLIASIS IN RABBITS

by

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PREFACE

The work described in this thesis is original and has not been submitted in any form to any other University. It was carried out by the author in the Department of Tropical Animal Health, Royal (Dick) School of Veterinary Studies, University of Edinburgh, under the supervision of Dr. M.M.H. Sewell and Dr. J.A. Hammond.

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SUMMARY

Two different aspects of the immunology of rabbits infected with Fasciola hepatica were investigated, the first being the development of host resistance to repeated infections with this parasite and the second was the in-vitro response of lymphocytes of infected animals to the presence of an extract prepared from this fluke.

Three other parameters proved to be of value in monitoring the development of the disease. The serum glutamic dehydrogenase levels and the numbers of peripheral eosinophils were both elevated while the parasites were causing parenchymal damage to the livers but fell rapidly following successful chemotherapy. The establishment of infection also resulted in the production of antibodies which could be detected in gel-precipitin tests within three to four weeks after infection.

Diamphenethide (Coriban, Burroughs, Wellcome and Company) was shown to have no apparent effect on the immature stages of F.hepatica four to five weeks after infection at an oral dose rate of 240-500mg per Kg body weight. Later studies revealed that rafoxanide (Flukanide, Merck, Sharp and Dohme) was more effective against the immature stages of the flukes than either nitroxynil (Thodax, May and Baker) or diamphenethide.

No evidence of acquired resistance was observed in

rabbits given a single challenge infection nine weeks after an initial infection with either 100 or 500 metacercariae, this initial infection having been removed by the administration of two doses of rafoxanide given four and five weeks after infection.

However, in a later study in which the challenge infection was given after two similar previous infections had been curtailed with rafoxanide, there was some reduction in the number of flukes recovered, together with changes in the gross pathology of the livers. It was not possible to decide whether this resistance was immunologically mediated or was purely or partly the result of the fibrosis and other changes in the tissues of the livers.

Both the morphological changes and the uptake of tritiated thymidine by peripheral lymphocytes of rabbits infected with F.hepatica showed evidence of an in-vitro response to the presence of an antigen prepared as a 1:5 v/v extract in Minimal Eagles Medium from adult F.hepatica somata. This antigen is also slightly mitogenic for lymphocytes from uninfected rabbits but there was a significant increase in the response within two weeks of infection.

The parameters of this technique were studied and it was found that maximum stimulation could be achieved by incubating the lymphocytes for four days and allowing labelling to occur during the final 24 hours. The

optimal number of lymphocytes in whole blood cultures of 1 ml total volume was 0.5 million and maximum labelling occurred in the presence of 0.0625 ml of the fluke antigen per culture.

Similar levels of response were shown by lymphocytes obtained from rabbits given either 100 or 500 metacercariae and there was no immediate change in the response after effective chemotherapy. There was some evidence of a slow diminution in the response in prolonged infections.

Challenge infections did not cause a further increase in the level of lymphocyte stimulation.

It was also shown that there was both an absolute and a relative increase in the numbers of large lymphocytes in the peripheral blood of rabbits infected with F.hepatica.

There was increased uptake of tritiated thymidine by unstimulated cultures from animals infected with F.hepatica compared with similar cultures from uninfected rabbits. This was probably related to the increased number of large lymphocytes into the peripheral circulation of the infected animals.

GENERAL INTRODUCTION

Fasciola hepatica (Linnaeus, 1758) is the major parasite causing liver fluke disease in domestic mammals and human beings in temperate areas while a closely related parasite, Fasciola gigantica (Cobbold, 1855), occurs widely in tropical and sub-tropical areas. Two other species of Fasciola were considered valid by Hammond (1970), these being Fasciola nyanzae (Leiper, 1910) and Fasciola tragelaphi (Pike and Condy, 1966). However, these two species have only been reported in wild animals in Africa namely the hippopotamus (Hippopotamus amphibious) and sitatunga (Tragelaphus spekei) respectively.

Both the more common species may be traced back to antiquity, F.gigantica (presumably) having been recorded in the livers of cattle represented in Egyptian tombs 4000 to 5000 years ago (Taylor, 1964).

In 1892 Neumann spoke about severe losses due to liver fluke in sheep after the annual fall of the Nile in Egypt. In recent years after the construction of the High Dam in Egypt and the development of irrigation schemes, the chance of the spread of this disease and other related trematode parasitic diseases such as schistosomiassis have increased.

Taylor (1949) also referred to a severe epidemic which occurred in the United Kingdom during the winters

of 1946-47 and 1947-48, in which the main losses were in sheep due to the acute form of the disease. Outbreaks are usually confined to the west coast of the United Kingdom and a high incidence of the disease always follows a wet summer, as shown clearly in a study of the relationship between the incidence of disease and climate carried out by Ollerenshaw and Rowlands (1959). In a recent report of a survey conducted by I.C.I. (Froyd, 1974) it has been suggested that fascioliasis is endemic throughout the United Kingdom, flukes or residual lesions occurring in some 40% of slaughtered cows, 17% of heifers and steers, 13% of ewes and 5% of sheep. It was further suggested that the loss to the farmer is at least £50 million per year.

The disease also occurs sporadically in human beings, in whom it usually runs a chronic course. Facey and Marsden (1960) reported an outbreak in Hampshire in the United Kingdom due to Fasciola hepatica infestation and the incidence appears to be higher in other parts of the world. Thus Bendezu (1969) stated that F.hepatica is a far more common zoonotic parasite than has been hitherto realized and in one village school in the high valley of Mantaro in Peru, South America, 60% of the children were found to harbour F.hepatica eggs in their stools. Few human infections with F.gigantica have been reported, but perhaps this is largely because it has not been looked for adequately (Hammond, 1974).

The life cycle within the intermediate snail host

was worked out by Leuchart (1882) in Germany and by Thomas (1883) in the United Kingdom. They showed that the life cycle of Fasciola hepatica involves a snail intermediate host of the genus Lymnaea and in Britain this is the species L.truncatula. The amphibious nature of L.truncatula, its preference for habitats which are subject to alternate flooding and drying, and also the ease with which it can be infected with miracidia of F.hepatica (Kendall and McCullough, 1951) result in it being a very efficient vector for this fluke. However, as Reinhard (1957) stated, these classical researches still left some points about the migratory route of the parasite in its final host unexamined. The first study on experimental fascioliasis was reported by Lütz (1892), using guinea-pigs and goats as hosts. The Russian worker, Sinitsin (1914), later fed metacercarial cysts to rabbits and showed that the flukes migrate directly across the abdominal cavity and actively penetrate the liver from outside. Schumacher (1938) confirmed Sinitsin's result in rabbits and guinea-pigs and showed that the young flukes reached the bile duct by the same route in sheep. The route of migration and final site in man are believed to be the same as in sheep.

The susceptibility of different mammalian hosts species to F.hepatica varies considerably. Ross (1967_b) divided the potential hosts into three groups based on their resistance to infection during the migratory

parenchymal stage and also during the bile duct stage of infection. He regarded both sheep and rabbits as showing relatively low resistance and Boray (1969) also considered sheep as a host of low resistance. In such a host the disease is likely to be relatively pathogenic and F.gigantica has been reported to be even more pathogenic to sheep than F.hepatica (Hammond 1956, Soyogen 1956). Cattle were included by Ross (1967b) in his medium resistance group, because of inhibition of the parasite's migration and the excessive fibrosis and calcification of the bile duct, while Boray (1969) considered cattle to show delayed resistance in which a reaction controls the parasites during the later phase of tissue migration. Sewell (1966a) and Hammond and Sewell (1974) considered that F.gigantica is better adapted as a parasite of cattle than F.hepatica, mainly because the former species survives longer in this host. In human beings there is a considerable tissue reaction and calcification in the bile duct similar to that in cattle, and spontaneous recovery from the infection is common.

The recent development of a commercial vaccine against the nematode parasite Dictyocaulus viviparus (Jarrett, Jennings, McIntyre, Mulligan and Urquhart, 1958) stimulated a search for such a vaccine against fascioliasis. However, Dawes and Hughes (1964) considered that there was little evidence from the literature of any significant naturally acquired resistance to

F.hepatica in sheep.

Nevertheless acquired resistance to F.hepatica infection has been shown recently in rats by Thorpe and Broome (1962) and by Armour and Dargie (1974), while Dargie, Armour, Rushton and Murray (1974) have reported some degree of protection in sheep against a challenge with normal metacercariae after they had been given six doses of 100 irradiated F.hepatica metacercariae as an initial dose.

It was pointed out by Urquhart (1954) that rabbits form a host in which the reaction appears similar to that in sheep, while the initial cost of this animal and the cost of maintaining it, is much less than that for sheep. This host has also been considered to be of epidemiological importance in maintaining the snail infection, as "The furtive habits and small size of the rabbit give it the ability to spend its time in culverts, ditches and similar out of the way places, that may only be grazed by sheep after the rest of the field becomes relatively devoid of grass; both snails and rabbits frequent these places" (Taylor, 1964). However, little work has been done on the development of resistance or the immune response in this host.

It was therefore decided to further investigate the immune response in rabbits infected with Fasciola hepatica. The investigations took two main forms. The first of these was a study of acquired resistance in this host against a challenge infection after the elimination

of a previous infection with anthelmintic drugs. Secondly there were a series of studies of the serological and cellular response of rabbits to this infection by the use of *in - vitro techniques*.

SECTION I

STUDIES ON RESISTANCE TO FASCIOLA HEPATICA

Chapter 1	...	...	Review of literature
Chapter 2	...	...	Materials and methods
Chapter 3	...	...	A pilot study on the response of rabbits to single infections with F.hepatica
Chapter 4	...	...	The activity of diamphenethide against immature F.hepatica in rabbits
Chapter 5	...	...	A comparison of the effect of three fasciolicidal drugs on the immature stages of F.hepatica
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CHAPTER ONE

REVIEW OF LITERATURE

INTRODUCTION

The available information on the resistance to F.hepatica shown by various hosts was reviewed by Ross (1967b) who noted that species related differences are expressed by resistance to infection during both the migratory parenchymal stage and the adult bile duct stage of infection. This author therefore divided the hosts into "low resistance", "medium resistance" and "high resistance" groups. He included sheep, rats and rabbits in the "low resistance" group, cattle in the "medium resistance" group and pigs and carnivores in the "high resistance" group. Boray (1969) also divided the potential hosts of F.hepatica into three main groups, these being an "early resistance" group which included pigs and carnivores and in which the infection is self limiting without harming the host as the tissues are not suitable for the parasite, a "delayed resistance" group in which the disease is self limiting, but may cause severe pathogenic lesions and mortality may occur particularly in young or debilitated animals; cattle were included in this group. In Boray's third "low resistance" group, the disease is highly pathogenic in both acute and chronic phases, rabbits, sheep and rats being included in this group.

1. Resistance to F.hepatica in cattle

(a) Previously uninfected animals

The apparent development of resistance in previously uninfected cattle was first described by Ross (1964), who observed that, in calves given a single low level oral infection of 200 - 1,300 Fasciola hepatica metacercariae, there was retardation and inhibition of immature flukes up to 23 weeks post-infection, when the studies were terminated. The host reactions were more severe at the later stages of the migratory phase. The resulting fibrosis of the liver parenchyma and cirrhosis of the bile ducts were specially marked in the ventral lobe of the liver (Ross, Todd, and Dow, 1966), in which large numbers of the immature stages were trapped. This resistance was further investigated by Ross (1965) using a single high level infection of 2,500, 5,000 or 15,000 metacercariae of F.hepatica. At 24-56 weeks post-infection, it was found that most of the migratory stages of the flukes had been blocked and trapped in the parenchyma of the liver, with the result that there were few flukes in the bile ducts. The author suggested that the resistance developed by the cattle may be a result of the fibrous reaction in the liver, which enhanced the local reaction and so produced inhibition of the parasite. This type of blocking and inhibition phenomena was considered by Urquhart, Jarrett and Mulligan (1962) to be a common feature of parasitic infections.

It was shown by Ross (1968) that there was an inter-

action between the level of infection and length of the infection in relation to the resistance manifested by the host. He infected calves with a single dose of 200 F.hepatica metacercariae and found that 75% of the infecting dose was eliminated between five to twenty-one months. Previous work by Ross (1967 a) had shown that a single high level infection of 2,500-15,000 metacercariae of F.hepatica reduced the life span of most of the flukes to around six to seven months. On the other hand, Doyle (1971) gave a single low level infection of 750 F.hepatica metacercariae to calves and found that 83% of the fluke population was eliminated between four to eight months later.

(b) Acquired resistance in a previously infected host

The role of acquired immunity in F.hepatica infection in cattle was studied by Ross (1966a) who showed that calves which had been infected with 200 metacercariae as an initial dose and then challenged with a further 300 metacercariae three or eighteen weeks later, were more resistant than a control group receiving only the challenge infection, as fewer flukes developed from the latter infection. Furthermore, an "acquired self-cure phenomenon", which resulted in the clearance of the initial infection, was observed in the group challenged in the 18th week. Doyle (1971) also indicated that an enhanced resistance to reinfection in calves challenged with 1,650 metacercariae 17 weeks after an initial infection of 750 metacercariae was demonstrated by a lower

recovery of flukes in the challenge group.

(c) Vaccination with irradiated metacercariae

Armour, Dargie, Doyle, Murray, Robinson and Rushton (1974) attempted to vaccinate calves against F.hepatica, with two immunizing doses of 1000metacercariae given at an interval of one month each which had been X-irradiated at 3.5 Kr. They challenged one group with 1,000 normal metacercariae four weeks after vaccination and the other group with 850 metacercariae eight weeks after vaccination. Control non-vaccinated animals were included with both groups. There was a reduction in fluke burdens in both groups of 30% and 70% respectively, compared with the control groups. The degree of resistance in calves using metacercariae attenuated by X-irradiation was also investigated by Nansen (1974). He used three doses of 1,500 metacercariae attenuated at a level of 3 Kr. These animals were then turned out to graze on a fluke infected pasture for "a grazing season". Twelve weeks after grazing ended, the mean burden of flukes in the vaccinated group was 24 as compared with 82 in the control group.

(d) Factors affecting host resistance

A non-specific factor, which may act as a mechanical barrier to flukes in cattle, is the heavy calcification of the bile ducts (Ross, Todd and Dow, 1966). This may limit the accommodation and food supply for the fluke. A similar suggestion was also

made by Keck and Supperer (1966).

The age of the host was considered by Boray (1969) to be a factor in resistance against F.hepatica infection. Four calves aged six to eight months were given a dose of 1,000 metacercariae and showed clinical signs of illness. In contrast, two calves given a similar infection at 16-18 months old showed high resistance as judged by the low numbers of flukes recovered from them. Different species of Fasciola may have a different effect on cattle. For example, Sewell (1966a) produced death from subacute fascioliasis in a white Fulani Zebu bullock aged two years by giving it 20,000 Fasciola gigantica metacercariae. On the other hand, Boray (1969) used Jersey x Zebu cattle and infected them with 20,000 metacercariae of F.hepatica without any deaths.

Animals infected with F.hepatica and later treated may show resistance to a challenge dose of metacercariae as compared with previously uninfected control animals. For example, Boray (1967a) treated cattle between the 14th and 19th week after infection with 1,000 metacercariae of F.hepatica. He then challenged these animals with 5,000 metacercariae three weeks after treatment, and found that they showed a high degree of resistance, which was manifested by a low recovery of flukes at autopsy. The effect of treatment on the host's resistance to challenge infection with F.hepatica was also investigated by Armour, Dargie, Doyle, Murray, Robinson and Rushton (1974). They treated cattle 11 weeks after

exposure to a primary infection of 750 metacercariae and later with 1,650 metacercariae. These cattle also showed high resistance as compared with challenge controls.

(e) Specific factors

The specific immune response against a primary infection with F.hepatica was investigated in a series of experiments using lymphoid cells and serum transfer. Armour, Čorba, Dargie and Urquhart (1971) showed that, if lymphoid cells from the hepatic lymph nodes of a previously infected donor calf were transferred to its monozygous twin, which was then also infected with F.hepatica, this latter calf showed a high degree of resistance as compared with a control calf. Dargie, Armour, Rushton and Murray (1974) similarly used lymphoid cells from the hepatic lymph nodes of infected donor calves transferred to monozygous twins, which were then challenged with 1,000 metacercariae. These calves developed a high degree of resistance of between 77% and 97% as compared with control animals. In later studies Dargie et al (1974) obtained almost complete protection against challenge with 1,000 metacercariae in cattle previously injected intraperitoneally with a large quantity of serum taken from a hyper-infected donor.

(f) The nature of host resistance

The development of this host resistance was clarified by Doyle (1973) who challenged two groups of calves seven or twelve weeks after an initial infection

with 750 metacercariae. These times were selected to cover the migratory and early bile duct stages of the parasite development respectively. He found out that the calves challenged at the later stage of infection developed a high degree of resistance as compared with calves reinfected at the earlier stage. He therefore supported the views of Boray (1967a, 1969) that the acquired resistance developed by the host at the bile duct stage was dependent on physical factors, such as fibrosis or cholangitis. However, Doyle (1973) also pointed out that immunity can also be acquired at the migratory stage of the parasite as previously reported by other workers (Čorba, Armour, Roberts and Urquhart, 1971). Dargie, Armour and Murray (1974) also observed that, although acquired immunity in cattle may be in part the result of fibrosis of the liver, other factors both humoral and cell-mediated, may be implicated in acquired immunity to Fasciola hepatica in cattle.

2. Resistance to F.hepatica in sheep

(a) Previously uninfected animals

In his studies on the disease caused by Fasciola hepatica in sheep, Sinclair (1962) indicated that there was little to show that several doses of infection induced an immunity which prevented the successful migration of the parasites. Sinclair gave a group of four sheep four successive doses of 150 metacercariae, at weekly intervals. He compared this group with another group of the same number given the same total number of metacercariae, in one single dose. No evidence of resistance was observed in the first group, and this group suffered very severely from the disease as compared with the other group. In both groups the degree of infectivity was found to be about 32%.

On the other hand, Boray (1967b) reported that when experimentally infected sheep were given single doses of 2,000, 4,000 or 6,000 metacercariae, the percentage recovery of flukes was 36.35%, 54.71% and 34.99% respectively. When the same number of metacercariae were given as four divided equal weekly doses, there were significantly lower percentage recoveries of 27.97%, 26.62% and 25.04% respectively. Furthermore, Boray indicated that overcrowding had no appreciable influence on the number of parasites established from 2,000 to 10,000 metacercariae. However, retardation of the flukes development was noticed by the same author using 2,000 metacercariae or more, and he related this effect

to the severe fibrosis of the liver.

In this respect it was shown by Dow, Ross and Tod (1968) that in the early parenchymal stages of the infection the tracts caused by the migrating parasites were numerous and hepatic cell hyperplasia was prominent. Around the seventh week the lesions consisted of open haemorrhagic spaces which do not indicate obstruction to the free movement of the parasite. They also pointed out that, although the presence of flukes in the bile duct produces fibrosis of the walls, these remained pliable and dilated and that calcification is not a prominent feature in sheep.

(b) Acquired resistance in the previously infected host

Sinclair (1971) reported evidence of acquired resistance in sheep under experimental conditions. These sheep were challenged with 600 metacercariae, after the elimination of a four weeks old primary infection of 300 metacercariae, with an intramuscular injection of emetine hydrochloride at the rate of 3mg per Kg body weight. The resistance in the challenged animals was demonstrated by a marked, but temporary, retardation of fluke development about eight weeks after infection. He related this retardation phenomenon to the early occurence of fibroblastic activity, and lymphocytic infiltration in the liver of the challenged group. These aggregations were observed in and around the tracts and triads of the challenged group as early as six weeks, but

were not seen in the control group until 12 weeks after infection. Furthermore, Sinclair suggested that early lymphocyte infiltration in the challenged group was produced in response to the initial infection. This work supported his earlier observation (Sinclair, 1970) on two sheep infected with 300 metacercariae which were killed five weeks later and showed lymphocyte infiltration in areas adjacent to portal triads, and in the fibrous capsule surrounding the degenerate remains of dead flukes.

However, previous work by Boray (1967_b) suggested that sheep did not develop any acquired resistance to F.hepatica. He reported no evidence of resistance in sheep which were infected with 500 metacercariae, and challenged with 200 or 4,000 metacercariae 12 weeks later. This group showed no significant difference in the number of flukes recovered, compared with the uninfected control groups which were also challenged with 200 or 4,000 metacercariae.

In another experiment, Boray (1967_a) infected four year old sheep on three occasions with 1,000 metacercariae and eliminated each infection after five weeks with an anthelmintic. A challenge dose of 4,000 metacercariae showed no difference in the number of flukes established in these animals compared with those in a previous uninfected control group. Furthermore, the author indicated that the previously infected animals had less severe tissue reactions than those in the

control group.

(c) Vaccination with X-irradiated metacercariae

Hughes (1963) vaccinated sheep with two doses, three weeks apart, of 500 metacercariae which had been X-irradiated at 3 Kr. These sheep were challenged with 200 normal metacercariae seven weeks later. Sixteen weeks after challenge, there was no significant difference between the number of flukes in the vaccinated and control groups. Boray (1967_a) infected a group of six adult Merino sheep, three times at six week intervals, with 1,000 metacercariae X-irradiated at 2 Kr. The animals were then challenged with 4,000 metacercariae together with six vaccinated control animals. There was again no difference between the number of flukes which developed in the vaccinated and control groups. However, Dargie, Armour, Rushton and Murray (1974) have recently shown that sheep may develop resistance when given six doses of 100 metacercariae of F.hepatica X-irradiated at 3 Kr, and then challenged with 750 normal metacercariae. These animals contained an average of 36 adult flukes, as compared with an average recovery of 105 parasites in non-vaccinated animals.

(d) Passive transfer of resistance

The transfer of immunity to F.hepatica in sheep by lymphoid cells and serum was studied by Dargie et al (1974). These authors showed that lymphoid cells obtained from the hepatic lymph nodes of donor sheep

eight weeks after infection and transferred to highly inbred animals, produced a high degree of protection against a challenge infection of 500 metacercariae, compared with an untreated control given the same dose of metacercariae. However, they also found that serum transfer may produce protection against infection with F.hepatica. They injected 400 ml of serum from hyper-infected donor sheep into recipient animals. A challenge infection of 750 metacercariae produced 80% protection as compared with controls which received the same number of metacercariae without having received the serum.

3. Resistance to F.hepatica in rats

(a) Previously uninfected hosts

It was pointed out by Lämmler (1959) that Fasciola hepatica will develop satisfactorily in rats at a dose level of ten or twenty metacercariae. Later Thorpe (1965) infected five groups of rats with doses of 5, 20, 40, 80 or 160 metacercariae per rat. He pointed out that, in the more heavily infected groups, a lower percentage of the inoculum was recovered as flukes, compared with those rats given fewer metacercariae. He also observed a degree of competitive inhibition among the flukes in the more heavily infected groups and also delayed entry into the main bile duct. Death occurred in rats infected with 80 or 160 metacercariae. Čorba (1972) infected inbred hooded Lister rats with 10, 15, 20, 30 or 50 metacercariae of F.hepatica, and found that the most extensive liver damage occurred between the fourth and sixth week after invasion. Contrary to Thorpe's earlier observation, Čorba recorded high mortality in the groups given 30-50 metacercariae and he related this to the strain of rats used in his experiment. He again noticed retardation of the flukes in the more heavily infected rats.

(b) Challenge infections with normal metacercariae

Hayes, Bailer and Mitrovic (1972) stated that rats which had been infected with five metacercariae of F.hepatica developed resistance to challenge with a

similar dose of metacercariae given seven weeks later. The resistance resulted in the survival of 95.5% fewer flukes than in similar infections in previously uninfected control rats.

The effect of an existing infection on a challenge infection was also studied by Hayes, Bailer and Mitrovic (1973) using two different levels of one or ten metacercariae 49 days later. The reduction in the number of flukes which developed in the challenge group with a pre-existing infection of one fluke was 76% as compared with previously uninfected rats. The authors concluded, that the existing infection had a marked effect on the challenge infection, and further, that non-specific liver inflammation and damage at the time of challenge may not play any role in resistance.

Goose and MacGregor (1973) showed that rats previously infected with 30 metacercariae developed resistance to a challenge with 30 metacercariae, given 63 or 98 days later. Similar resistance was also developed following an intraperitoneal transplant of adult flukes given 65 and 73 days later. Furthermore, they noted that removal of the initial infection by anthelmintic just before challenge did not interfere with the resistance of the host. This supported their belief that the resistance was not dependent on the presence of the initial immunizing infection at the time of challenge. These authors also showed that this

resistance was not directed solely against the immature stages of the parasite, as shown by the failure of adult flukes to survive after transplantation into the peritoneal cavity of resistant rats. However, they also found that resistant rats harboured an apparently normal immunizing infection in their bile ducts.

Further work on the effect of previous infection on a challenge infection was reported by Armour and Dargie (1974). They infected rats with 20 metacercariae each and treated them with an anthelmintic to eliminate this infection after eight weeks. When later challenged with a further 20 metacercariae, these rats contained a mean of 2.6 flukes as compared with 5.5 flukes from previously uninfected controls.

Evidence of resistance to challenge infection was also demonstrated in rats with long standing chronic infections by Hayes, Bailer and Mitrovic (1974). Rats which had been infected with five metacercariae and then challenged with 20 metacercariae 28 or 48 weeks later, yielded 66% or 50% less flukes respectively as compared with previously uninfected controls. These authors pointed out that a challenge infection did not significantly alter the number of flukes remaining from a pre-existing infection.

It was found by Hughes and Harness (1972) that F.hepatica implanted in the body cavities of recipient rats, survived for at least six to seven weeks inside a well

formed cyst. This was thought by the authors to be a suitable model for the study of this host parasite relationship. Based on this observation, Eriksen and Flagstad (1974) studied the protective effect of adult F.hepatica implanted subcutaneously in rats. Adult flukes from experimentally infected sheep and goats or from slaughtered cattle were used. Three weeks after the fluke transfer the implanted rats were each infected with 20 metacercariae. This resulted in a 50% decrease in infection rate compared to non-implanted control rats infected with the same number of metacercariae.

Rüther (1963) cited by Geyer (1967) demonstrated good immunity in rats after repeated administration of controlled doses of metacercariae. He pointed out that five immunizing infections caused a reduction in the number of the parasites developing to 0.75% of the number of metacercariae administered, as compared with 17.6% in single infections.

(c) Vaccination with X-irradiated metacercariae

Thorpe and Broome (1962) infected five groups of 30 rats each with 40 metacercariae per rat, irradiated at 1,000, 2,500, 5,000, 7,500, or 10,000 roentgens. A challenge dose of 20 non-irradiated metacercariae were given to two groups of ten rats from each group, seven weeks and eleven weeks respectively after vaccination. In each case, a control group of ten rats given 20 normal metacercariae were included. Seventeen to twenty-one weeks after the initial infection the animals were

killed. The author indicated that all the groups of rats given metacercariae irradiated at 1,000 or 2,500 roentgens developed immunity against the challenge.

They also noted that immunity appeared to be time dependent as the groups challenged at 11 weeks with a previous infection of 5,000 and 7,500 irradiated metacercariae showed resistance, whereas the similar groups challenged earlier at seven weeks had no detectable immunity. No immunity was observed in the groups infected with metacercariae irradiated at 10,000 roentgens. Armour and Dargie (1974) infected rats with three doses of 20 irradiated metacercariae at weekly intervals and treated them with an anthelmintic eight weeks later. These animals showed a high degree of protection against challenge infection with metacercariae, with a mean recovery of 2.4 flukes as compared with 5.4 flukes from control rats.

(d) Passive transfer of immunity in serum

Previous work by Čorba, Armour, Roberts and Urquhart (1971) observed that serum obtained from rats infected ten weeks previously and injected intraperitoneally into recipient rats, at a dose rate of 4.0 ml per rat before the animals received a challenge dose of 30 metacercariae, failed to give any significant protection. However, recent work by Armour and Dargie (1974) has shown that intraperitoneal transfer of serum

from previously infected rats, cattle or sheep gave a significant degree of protection against a challenge. They showed that the injection of 10 ml of anti-serum at the same time as the rats received an oral dose of 20 metacercariae per rat and repeated ten days later, resulted in a reduction of 67% in the fluke burden. This reduction was found to be significant when compared with the number of flukes recovered from control rats which had received a similar amount of serum from non-infected donors, and also when compared with other control rats which had received no serum. These authors stressed the significance of the volume of anti-serum necessary to attain good protection against challenge. They injected immune bovine serum in amounts of 0, 5, 10 or 20 ml and showed a decreasing number of fluke recoveries of 3.6, 2.0, 1.4 and 0.6 respectively after challenge.

(e) Cellular transfer of immunity

The resistance which follows the transfer of lymphoid cells from the hepatic and mesenteric lymph nodes and spleen of infected donor rats to other rats was studied by Čorba, Armour, Roberts and Urquhart (1971). These authors pointed out that the time factor was important, lymphoid cells obtained from donor rats eight to ten weeks after infection with F.hepatica giving the most successful transfer of cellular immunity. They also obtained good transfer of resistance using cells

obtained from rats immunized with three weekly doses of metacercariae irradiated 2.5 Kr.

The recent work by Dargie et al (1974) has also shown that syngeneic recipient rats may be protected against a primary challenge of 20 metacercariae, if cells taken from the mesenteric and hepatic lymph nodes and spleens of donor rats are transferred to these animals. They also pointed out that the transfer of immunity was dependent on the number of adult flukes in the donor rats as well as on the number of viable cells transferred.

4. Resistance to F.hepatica in rabbits

(a) Previously uninfected animals

Using single infections of 50 metacercariae per rabbit, with four groups of rabbits each receiving metacercariae from different snails, Urquhart (1954) found that metacercariae from different snails had no influence on the number of flukes recorded and that the sex and breed of rabbits had no consistent significant effect on the degree of infection developed. However, the number of flukes recovered was dependent on the technique used for infection and the natural resistance of the host.

(b) Vaccination with fluke antigen

Kerr and Petkovich (1935) immunized seven rabbits with an extract of dried powdered liver fluke extract. The injections were given intraperitoneally over a period of three weeks. The immunized group and three control rabbits were each infected with 13 metacercariae, 36 days after the last injection. These authors claimed that resistance to the infection developed in the immunized rabbits and that no eggs were detected in their faeces and also that there were signs of calcification in some of the flukes. Urquhart, Mulligan and Jennings (1954) immunized two groups, each of seven rabbits, with an alum precipitated extract of F.hepatica. The first group received a total of three injections, while the second group received a total of six

injections. Each of the immunized and control rabbits were infected with 50 metacercariae and they were autopsied 63 days after infection. The average number of flukes in the immunized group was not significantly lower than in the control group, but these authors noted retardation of the flukes recovered from the immunized groups. They reported a severe inflammatory reaction of the livers in which the most retarded flukes were found and they related this to the prolonged migratory period of the retarded flukes. Furthermore, they suggested that a state of hypersensitivity as a result of previous immunization was responsible for the exaggerated cellular reaction to the presence of the parasite. Similarly Healy (1955) immunized rabbits with either an extract of F.hepatica somata or with pooled regurgitated caecal contents from these flukes. These animals were challenged with 50 metacercariae and were autopsied after 60 days but showed no significant decrease in the number of flukes recovered as compared with controls. Finally, Shibanai, Tozawa, Takahashi and Isoda (1956) injected a dry vaccine (sic) prepared from F.hepatica into rabbits by various routes. The rabbits were then infected 70 days later and autopsied after 56 days. In the immunized rabbits, the damage was less severe than in the controls, while the development of the parasite was not affected by vaccination. However, Babadzhanov and

Tukhmanynts (1958) cited by Geyer (1967) claimed to have success in developing resistance in rabbits. They immunized 20 rabbits with six intravenous injections of an extract of F.hepatica. They stated that these extracts did not contain any protein or lipids but a considerable amount of polysaccharide. Two weeks after the last injection, the vaccinated rabbits were resistant to a challenge infection with ten metacercariae as compared with unvaccinated rabbits but did not describe what form this resistance took.

The effect of various antigens prepared from F.hepatica was investigated by Hughes (1963). Rabbits were injected intraperitoneally at weekly intervals for a period of eight weeks with antigen prepared from the adult flukes or from metacercariae and then challenged with 15 metacercariae. The number of flukes recovered 11 weeks later did not show any significant difference from the number recovered from control rabbits. Furthermore the use of subcutaneous injections of an adult fluke antigen in Freund's complete adjuvant at three weekly intervals, followed by challenge with 15 metacercariae, again gave no significant difference from the controls. Hughes also pointed out that the antigen prepared by Ershov (1959) did not protect rabbits against challenge infection, although the latter author had claimed some success in sheep. On the other hand, Hughes (1963) found that several intraperitoneal injections of

lypholised adult fluke antigen would protect rabbits against a challenge of 13 metacercariae given five weeks later. However, in a later experiment, this antigen proved less successful when used in twice or half the amount of previous experiment. The author suggested that the apparent protection obtained in his first experiment was fortuitous. Furthermore, no inhibition of the development of the flukes was observed by the author in any of his work using these different antigens. Also, Ross (1967c) vaccinated rabbits with a fluke homogenate prepared from six week old flukes. One ml of this homogenate was injected subcutaneously into three groups each of four rabbits, at two week intervals. These animals were infected with 20 metacercariae each and they were killed at six, seven and nine weeks after infection respectively. The author reported no significant difference between the recovery of flukes from these animals as compared with the control group, infected with the same number of metacercariae, but not injected with the antigen. However, the author noted a retardation of flukes from the group given the fluke homogenate at the sixth and seventh week after infection.

The possible effect of host antigen in F.hepatica infection was studied by Hughes and Harness (1973). They immunized recipient rabbits against donors' tissue and then challenged the recipient animals with adult flukes administered intraperitoneally. Unimmunized

control rabbits were similarly challenged. The authors reported no difference between the flukes in the immunized and control groups.

(c) Previously infected

Ross (1966_b) infected four rabbits with 75 metacercariae each and challenged these animals with a further 50 metacercariae 24 weeks later. Five weeks after challenge the author recorded retardation in the fluke development and a decrease in the number of flukes recovered from challenged animals, as compared with those from control rabbits which were previously uninfected. Similarly, when Kendall, Hebert, Parfitt and Peirce (1967) infected rabbits with ten metacercariae and then challenged them twelve weeks later with a further 20 metacercariae, they recovered fewer flukes from the challenged animals as compared with the number from rabbits which had received a single infection of ten or twenty metacercariae. They related this lower recovery to the overcrowding effect in the challenged animals but also to the difficulty in finding the smaller flukes at post-mortem examination.

Kendall (1967) also compared a group of rabbits with a single infection with another group in which the first infection was followed by a second infection of 20 metacercariae. He reported a 30% loss in the number of flukes recovered from the group with a double infection and related this decrease either to a true resistance or to an overcrowing effect. He also found that

varying the numbers of metacercariae given to the rabbits over a range from 10 to 160 per rabbit did not significantly affect the proportion of the infecting dose later recovered as adult flukes.

Kendall and Sinclair (1971) pointed out that rabbits may show resistance against a challenge infection when the initial infection had been eliminated by drugs. These authors suggested that the time between fluke elimination and challenge may influence the number of flukes recovered. They supported this by an experiment on two groups of rabbits, each animal of which had been infected with ten metacercariae. These two groups were treated with hexachlorophene 84 or 86 days later and then challenged with 20 metacercariae, four days or two days respectively after treatment. There was a significant reduction in the number of flukes recovered from the group which was treated two days before the challenge compared with a previously uninfected control treated group challenged with 20 metacercariae, but there was no such effect on the number from the group treated four days before challenge. These authors pointed out that a large but transient release of antigen from the dead flukes which may have stimulated lymphocyte infiltration as a result of a delayed hypersensitivity response. Another suggestion was that the destruction of the flukes of the initial infection may have produced materials which were toxic to the young flukes of the challenge

infection.

However, Rüther (1963) cited by Geyer (1967) demonstrated good immunity in rabbits after repeated infection with controlled doses of metacercariae and showed a significant decrease in the number of flukes recovered when later challenged with a further 50 metacercariae. Only 7% of the flukes developed from the challenge infection as compared with 46% in the control. This author also noted a retardation of more than 65% in the size of flukes compared with those recovered from a single infection. Also, Fortmeyer (1974) claimed to have demonstrated resistance to challenge infection in rabbits acting at the intestinal level, but possibly maintained by the adult flukes. There was no resistance after an intraperitoneal infection of metacercariae, followed by an oral infection 42 days later but there was a 65% resistance when the challenge infection was at 94 to 115 days. However, there was reduction in the growth rate of the parasites in both previously infected groups. The author also suggested that the resistance may take a long time to develop rather than that the presence of adult flukes is essential.

(d) Vaccination with X-irradiated metacercariae

The effect of X-irradiated metacercariae on a challenge infection of normal metacercariae was studied by Hughes (1963). The author vaccinated a group of rabbits with 500 metacercariae X-irradiated at 4 Kr and this was followed by a second vaccinating dose of the

same size three weeks later. These animals were challenged four weeks later with 15 normal metacercariae. When the rabbits were killed ten weeks after the challenge infection there were no significant differences between the number of flukes compared with a control group given only the challenge dose of metacercariae.

CHAPTER TWO

MATERIALS AND METHODS

The Production of Metacercariae

Before carrying out the experimental studies on rabbits it was essential to produce the infective agent, the metacercariae of Fasciola hepatica, using for this purpose snails from a stock of the intermediate host Lymnaea truncatula, kept in the Department of Tropical Animal Health, Royal (Dick) School of Veterinary Studies, University of Edinburgh. The techniques used in maintaining the snails and production of metacercariae were similar to those described by Sewell (1962).

Preparation of the algal cultures was carried out in sandwich boxes as described by Pullan (1968). The mud was mixed using a domestic food mixer (Kenwood Manufacturing Company Limited, Havant, Hampshire) which produced a mud of even consistency. Pullan (1968) stated that it was necessary to wet the mud on the first day of culture, but the use of the efficient mixer rendered this unnecessary. It was found that the maximum growth rate for the algae could be obtained by maintaining the cultures at 35 - 37°C.

The method used for maintaining the young generation of snails was that described by Hammond (1970).

Shedding of metacercariae was carried out by putting the infected snails into a small polythene bag, half filled with cold distilled water at 10°C. The

bag was supported in a small beaker, closed by a spring clip, and left for about six to seven hours. The snails were then removed from the bag and again the bag containing the shed metacercariae was left overnight. The following day the water in the bag was poured off and it was refilled with fresh distilled water, placed in a glass bottle, which was left for one week at room temperature, then wrapped in tin foil and kept at 4°C for further use.

When the metacercariae were required for use, the bag with the cysts on its wall was cut into pieces of a suitable size to lie in the centre of a watch glass, which was filled with enough water to cover the metacercariae. The number of viable cysts were counted on the polythene using a stereoscope and the required number scraped off the polythene into the watch glass. The excess water was then carefully removed with a pasteur pipette and the metacercariae were then ready for use.

The Administration of Metacercariae to Rabbits

1. The Filter Paper Technique

The required number of metacercariae in a watch glass were sucked up into a siliconized pasteur pipette, under a dissecting microscope, and transferred on to a small piece (2 x 2 cm) of Whatman No. 1 filter paper in a dish. The pasteur pipette was then checked under the microscope for any metacercariae sticking to the wall and such lost metacercariae were replaced from stock with another clean pipette. The filter paper was

folded using forceps and was ready for administration to a rabbit.

2. The Gelatine Capsule Method

The metacercariae within each watch glass were mixed with a little cellulose fibre (Whatman CF1, Whatman Biochemicals Limited, Maidstone, Kent), after most of the surrounding water had been removed. The cellulose fibre absorbs the residual water around the metacercariae forming a doughy consistency. This semi-solid mass was picked up with fine forceps and transferred to a gelatine capsule (Size No. 1, Parke, Davis and Co., Hounslow, London), which was then covered with its cap. The capsule was then ready for administration to a rabbit.

Experimental Animals

Porton cross-bred rabbits were used, which were obtained from the Centre for Laboratory Animals, University of Edinburgh, when they were five to six months of age. Equal numbers of each sex were usually used in each experiment. After being received, each rabbit was kept in its own individual cage. Food and water were offered ad lib, the food being a commercial pelleted diet Oxoid S.G. 1 (Oxoid Limited, London), with the following analysis:-

Crude protein	20.23%
Crude fibre	9.05%
Oil	4.18%
Calcium	0.89%

Phosphorous	1.05%
Chlorine	0.23%
Sodium	0.15%
Potassium	1.06%

Underneath each cage was a faecal collecting tray covered with peat moss, which was cleaned out twice a week. The rabbits were identified by individually numbered ear tags (Ketchum Manufacturing Company, Tadworth, Surrey). Each rabbit was examined at least twice before infection, including a faecal examination and a blood smear in each case.

Examination of Faecal Samples

Faecal samples were examined using the Sellotape technique using clear cellulose self-adhesive tape (Sellotape Products Limited, Boreham Wood, Hertfordshire). This technique was described by Sewell and Hammond (1972) for detection of Fasciola hepatica eggs. Coccidial oocysts were occasionally seen in a few rabbits and these were treated with sulphamezathine for at least three successive days. No other parasites or helminth eggs were ever seen.

Collection of blood and serum

Blood was taken from the peripheral vein of the rabbit's ear. The marginal area of the ear was shaved with a scalpel, and cleaned with 70% alcohol. The tip of the ear was swabbed with xylol. Pressure was applied on the vein close to the head and a small incision made into the engorged vein with a 20g needle (Becton,

Dickinson U.K. Limited, Wembley, Middlesex). Blood flowed easily and was collected into a 2.5 ml plastic vial containing sequestrine (E.D.T.A.) as anti-coagulant (Becton, Dickinson, U.K. Limited, Wembley, Middlesex). About one ml of blood was collected and the container was inverted several times to mix the blood with the anti-coagulant.

For serum collection blood was taken into universal bottles, about five ml of blood from each rabbit being collected into each bottle. The tops were screwed on and the bottles were left at room temperature for one to two hours and then at 37°C for a further two hours, until a clot was formed and serum separated. The bottles were further left at 4°C for two hours to allow for retraction of the clot. The separated serum was poured into centrifuge tubes and centrifuged at 2500 r.p.m. for 30 minutes. The clear supernatant serum was then transferred into small plastic tubes which were labelled and stored at -20°C for biochemical and serological studies.

Haematological Techniques

The blood in the E.D.T.A. bottles was mixed for at least two minutes in a rotary mixer (Matburn Limited, London) before carrying out any haematological techniques.

All haematological estimations were carried out on an electronic cell counter (Model F.N., Coulter Electronics, South Dunstable, Bedfordshire) and its

associated equipment, using the techniques and materials recommended by this manufacturer.

Blood smears were prepared on glass slides (76 x 25mm, 0.8mm thick, Chance Proper Limited, Smethwick, Warley, Warwickshire). The slides were first cleaned by dipping them in acid alcohol and then in 70% alcohol and air drying. The blood smears were made using a blood spreader (Midland Industrial Company Limited, Birmingham) at an acute angle. The smears were left to dry for 24 hours on a clean surface, away from any dust. They were then stained with the Undritz modification of the peroxidase staining method (Undritz, 1952) to facilitate differentiation of the heterophils from the eosinophils, as these are difficult to differentiate in rabbits by the usual Romanowski stains. The use of this stain was discussed by Purvis (1971). Differential white cell counts were made by examining 100 cells per slide and expressing the cell types as a percentage. Absolute eosinophil counts could then be calculated from the total white cell count using the percentage figure.

Glutamic dehydrogenase assay

The assay was carried out as described by Ford and Boyd (1962) and Sewell (1967) using a cuvette of one cm light path in an SP 1800 spectrophotometer (Pye Unicam Limited, Cambridge) at a wavelength of 340mm and a slit width of 0.26mm. The drop in the optical density was determined on a recording instrument (Unicam AR2 Linear Recorder). A reduction in the optical density of 0.001

is equivalent to the oxidation of 4.83×10^{-4} moles of the diphosphopyridine nucleotide (DPNH) (Sigma Chemicals Company, Baltimore, U.S.A.) so that the activity of the sera in μ moles per litre per minute (I.U.S.) can thus be estimated.

Preparation of Fasciola hepatica antigens for serological use

Standard fluke antigen (SFA)

Adult flukes were collected from the bile ducts of infected livers and washed in saline; they were processed immediately or stored at -20°C. For preparation of the antigen, the flukes were chopped up with a pair of scissors and their wet volume estimated in a measuring cylinder. They were then mixed with veronal buffer saline, pH 7.4 (100 ml of 0.04 M sod. diethyl barbiturate plus 15.3 ml of 0.2 N HCl at a concentration of 1:5 V/V and homogenised in universal bottles attached to a laboratory homogeniser (M.E.S. Limited, Manor Royal, Crawley, Sussex). The resultant homogenate was incubated at 4°C overnight and then centrifuged at 2,500 r.p.m. for 20 minutes, the supernatant being stored at -20°C for serological studies.

Immuno precipitin

The technique for immuno precipitation test was carried out as described by Sewell (1966_b), who described a simple apparatus involving the study of precipitating system.

Fasciolicidal Drugs

The drugs used were:-

Diamphenethide "Coriban" (Burroughs, Wellcome and Company, Berkhamsted, Hertfordshire), available in bottles containing two litres of a suspension containing 18% W/W diamphenethide. The dose in rabbits is 240mg to 500mg per Kg body weight.

Nitroxynil "Trodax" (May and Baker Limited, Dagenham, Essex), available as a 20% solution in multi-dose bottles containing 250 ml each. The dose is about 20mg per Kg body weight.

Rafoxanide "Flukanide" (Merck, Sharp and Dohme Limited, Hoddesdon, Hertfordshire), available as 4.2 litre and 1,050 ml bottles containing 2.7% W/V of rafoxanide. The dose is 26mg per Kg body weight.

Diamphenethide and Rafoxanide were given orally, while Nitroxynil was given by subcutaneous injection. The weight of each rabbit was estimated separately before dosing.

For oral dosing the animal was wrapped in a towel and held by an assistant. A stomach tube consisting of a narrow p.v.c. tube, 20cm x 3mm o.d. was connected at one end to a polypropylene luer-to-record adaptor which fitted the luer nozzle of a syringe. The stomach tube was introduced by hand into one side of the mouth until it touched the base of the tongue, then pushed gently into the oesophagus. The other hand was holding the head and, by finger, pressure was gently applied to

the jaws to help keep the mouth open. The required dose was then injected using a 10ml plastic syringe (Becton, Dickinson U.K. Limited, Wembley, Middlesex) fitted into the stomach tube. A few ml of tap water was further injected into the stomach tube to wash out all the drug and the stomach tube was quickly withdrawn from the mouth. The subcutaneous injections of Trodax were given using a one ml syringe, after the site of injection had been cleared of hair and cleaned with 70% alcohol.

Autopsy

At the end of each experiment surviving rabbits were killed by an intravenous injection of Euthatal (Abbot Laboratories, Queensborough, Kent), while animals which died during the experiments were examined as quickly as possible after death. A longitudinal incision was made through the abdominal muscle until the viscera were exposed. Another incision was made across the abdominal muscle near the liver. Any fluid in the abdomen was collected into a separate container. The abdominal cavity was then washed with saline and the washings were collected in the same container. The liver was very carefully freed from the surrounding viscera, and then excised at the junction of the bile duct with the intestine. It was then transferred into a separate container and examined externally. The larger bile ducts and the gall bladder were opened with

scissors and any flukes present in them removed and counted.

The liver was then cut with scissors into smaller pieces about two cm in length and 0.5 cm in width and any flukes were removed with forceps. All the pieces of liver were then placed in a beaker of saline and kept at 37°C for three to four hours, after which the saline and pieces of liver were poured into a sieve of 500 μ aperture, the fluid passing through being retained. The pieces of liver were then transferred into another beaker containing warm saline for a further hour and any flukes found on the sieve were placed in petri-dishes for further counting and examination. The process of sieving was again repeated on the pieces of liver and also on the fluid collected after the first sieving and on the abdominal fluid and washing, so as to ensure that any flukes contained in the fluids were recovered. The numbers of Fasciola hepatica from each liver was recorded.

CHAPTER THREE

A PILOT STUDY ON THE RESPONSE OF RABBITS TO SINGLE INFECTION

Fasciola hepatica spends part of its life cycle migrating through the parenchymal tissue of the liver of its host and Dawes (1961) has pointed out that the young flukes appear to feed primarily on hepatic cells. The cellular and humoral responses of the host are a reaction to the presence of the parasite and the damaged tissue (Dargie, 1973). An experiment was designed to:-

(1) Follow the peripheral eosinophilia as a measure of cellular response of the host

(2) Follow the serum glutamic dehydrogenase levels as an indication of the severity of the damage

(3) Study the serological response as shown by the immuno-gel precipitation technique

in rabbits infected with different numbers of metacercariae.

Experimental Design

Seven rabbits were divided into two groups, these being a control group of three rabbits (Group 1) and one group of four rabbits (Group 2) in which each rabbit was infected with 500 metacercariae. The metacercariae used were three weeks old. They were administered by the filter paper technique. At a later date two further rabbits were infected with 1,000 metacercariae each by the gelatine capsule technique. These latter rabbits

form Group 3 of this experiment. Blood was taken from each animal at weekly intervals for haematology and for serum.

The serum was stored at -20°C and used as soon as possible for measuring the glutamic dehydrogenase level and the precipitin antibodies.

The rabbits were killed 12 weeks after the start of the experiment to give enough time for adult flukes to be recovered and to allow examination of these parameters for a longer period after infection.

Results

The results are shown in Table 3.1, Plates 3.1 and 3.2, Figs. 3.1 - 3.3 and Appendix Tables 3.1 and 3.2.

Haematological Data

The total and differential leucocyte counts of each group were estimated. There was a slight tendency for the eosinophil level in the normal rabbits to fall with time, but regression analysis showed that this trend was not significant ($t_b < 1$).

All the infected rabbits showed elevated eosinophil counts (Fig. 3.1). There were consistently low counts in the uninfected rabbits (Appendix Table 3.1). Group 2 showed gradually increasing level of eosinophilia after infection and this increase was maximal seven weeks later. In Group 3, there was a marked increase by two weeks after infection with a maximum increase three weeks after infection, after which there was a terminal fall in

the eosinophilia, both the animals in this group dying from acute fascioliasis.

The rabbits in Group 3 also showed significant increases in their heterophil counts (Fig. 3.2), these being markedly increased by one week after the infection and remaining so thereafter. No significant differences were observed in the heterophil counts from Groups 1 and 2.

No significant differences were observed in lymphocyte counts or in the packed cell volumes of any of the groups, except perhaps for a terminal fall in one of the two rabbits in Group 3.

Serum Glutamic Dehydrogenase (G.D.)

There was a slight tendency for the G.D. levels in the normal rabbits to fall with time, but regression analysis showed that this trend was not significant. ($t_b < 1$ - where t_b is the value of student's t for the difference between the regression coefficient and zero). The results of G.D. assays on the sera are shown in Appendix Table 3.2 and Fig. 3.2. The highest levels were found in Group 3 at three weeks after infection (Fig. 3.2) although there was a slight elevation in the levels in Group 2 at six to seven weeks after infection.

Precipitins

No precipitin lines were seen at any time in the sera from the control rabbits. The rabbits infected with 500 metacercariae first showed precipitins by five

weeks after infection and those infected with 1,000 metacercariae first showed precipitins by three weeks after infection. All these rabbits continued to show precipitins for the remainder of the experiment.

Post-mortem examination

Detailed post-mortem examinations were carried out on all rabbits 12 weeks after infection except for Group 3 in which two rabbits died during the experiment.

There were no signs of flukes in any of the rabbits in Group 1. In Group 2, there were signs of bile duct hyperplasia and the gall bladders were slightly swollen and engorged. There was some fibrosis in the liver parenchyma. In Group 3, RB 14 died at the fourth week after infection and RB 13 died at the fifth week after infection and, in both rabbits, relatively large numbers of flukes were recorded (Table 3.1). The livers of these two rabbits were severely damaged and deformed with multiple areas of necrosis where the young flukes were embedded (Plate 3.2) and this was shown by cutting the liver tissue into small pieces and counting the young flukes under stereoscopic microscope.

The capsules were severely inflamed and the peritoneal cavity contained dark reddish fluid.

Discussion

It was clear from the small numbers of flukes recovered from the rabbits in Group 2 (Table 3.1), that

the filter paper technique, although convenient with ruminant hosts such as sheep, is not suitable for use in rabbits, and the gelatine capsule method used later on in RB 13 and RB 14 was much superior to the filter paper technique. These two rabbits died from acute fascioliasis at four to five weeks after infection. This suggested that the infectivity rate was satisfactory, and this was confirmed at post-mortem examination.

The parameters measured were closely related to the severity of infection; all the uninfected rabbits in Group 1 remained around normal levels. In Group 3 the first indication of precipitin at three weeks coincided with the peaks of the glutamic dehydrogenase levels and eosinophil counts.

TABLE 3.1

Results from rabbits infected with Fasciola hepatica

Group	Infective dose of Metacercariae	Rabbit number	Fluke burden	Mean burden of percentage of dose
1	0	RB 1	0	-
		RB 8	0	
		RB 15	0	
2	500	RB 3	8	2
		RB 7	10	
		RB 10	9	
		RB 12	7	
3	1000	RB 13	189	29
		RB 14	400	

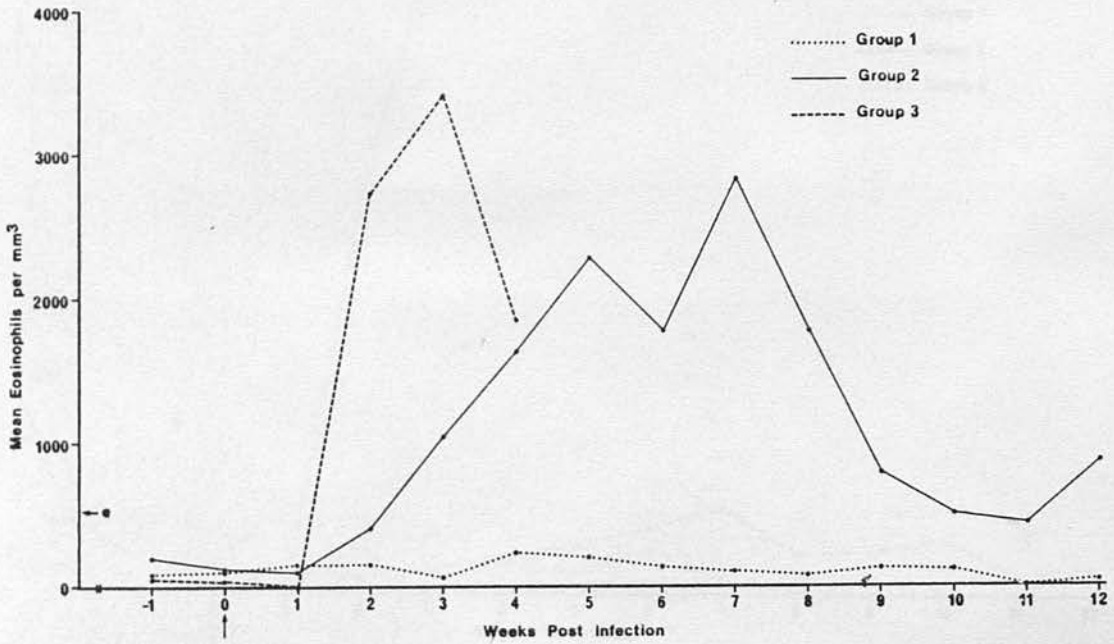


Fig. 3.1. Mean peripheral eosinophil counts. The uninfected control rabbits (Group 1), the rabbits infected with 500 metacercariae (Group 2), and those infected with 1,000 metacercariae (Group 3).
-e Mean ± 3 s.d.

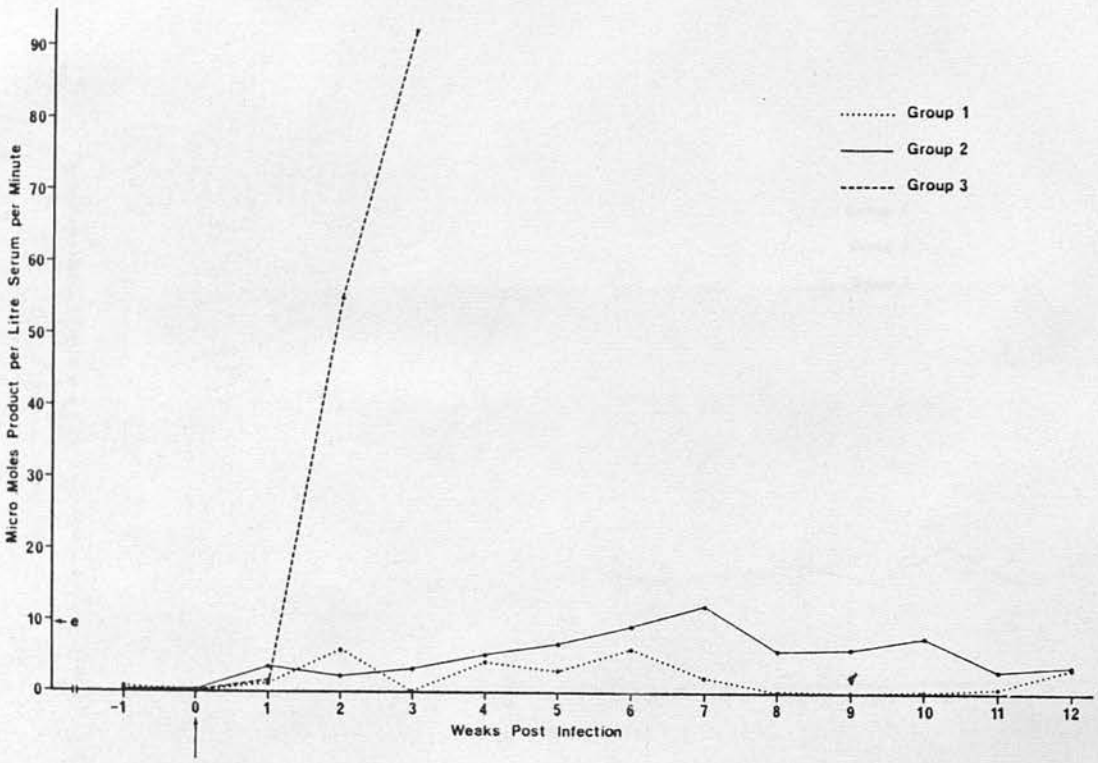


Fig. 3.2. Mean serum glutamic dehydrogenase levels. The uninfected control rabbits (Group 1), the rabbits infected with 500 metacercariae (Group 2), and those infected with 1,000 metacercariae (Group 3).

-e Mean $\pm$ 3 s.d.

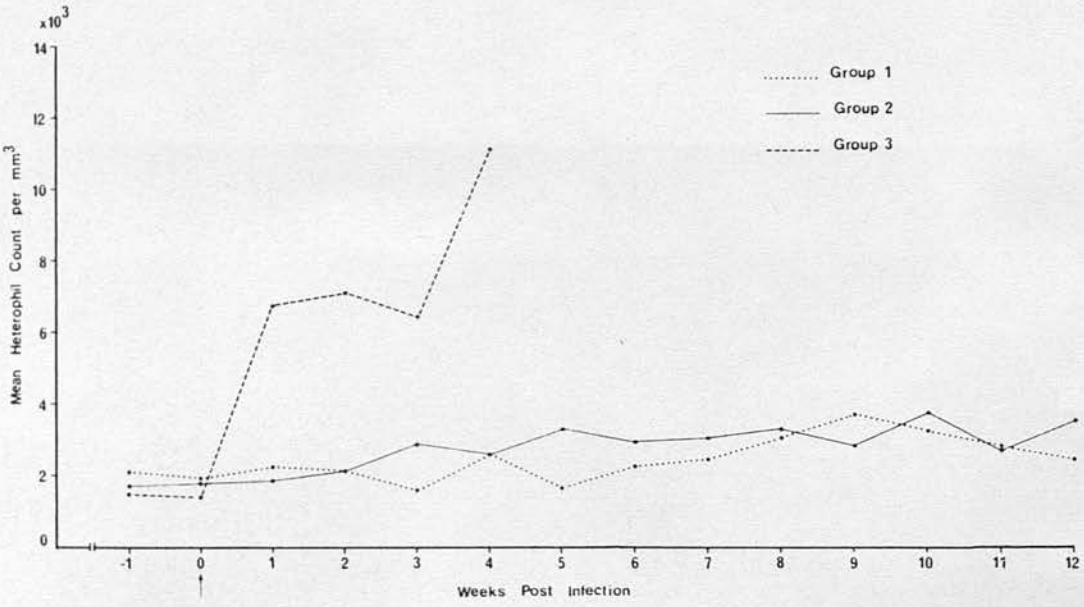
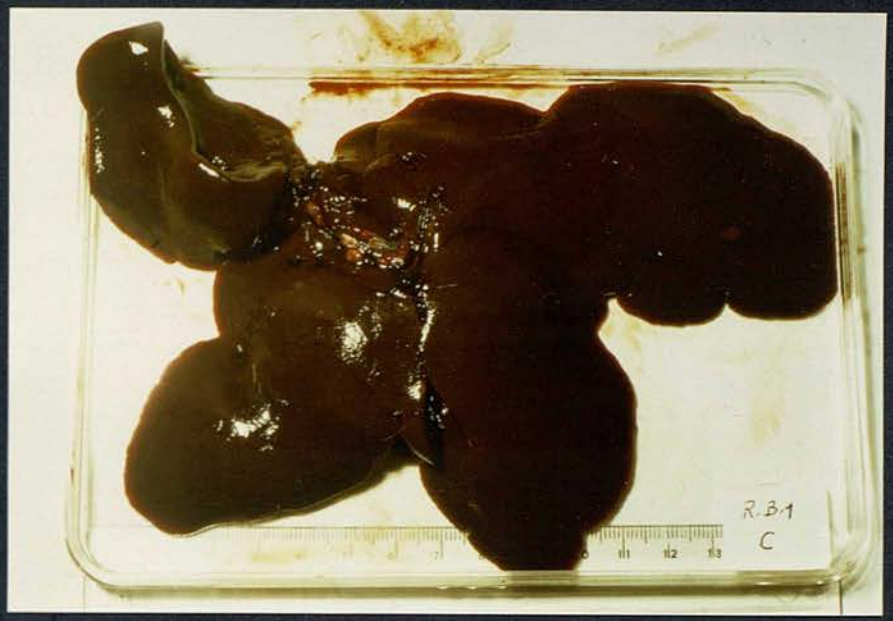


Fig. 3.3. Mean heterophil counts.
The uninfected control rabbits (Group 1), the rabbits infected with 500 metacercariae (Group 2), and those infected with 1,000 metacercariae (Group 3).

Plate 3.1. Liver from a control uninfected rabbit.

Plate 3.2. Liver from a rabbit which died following infection with 1,000 metacercariae of F.hepatica orally.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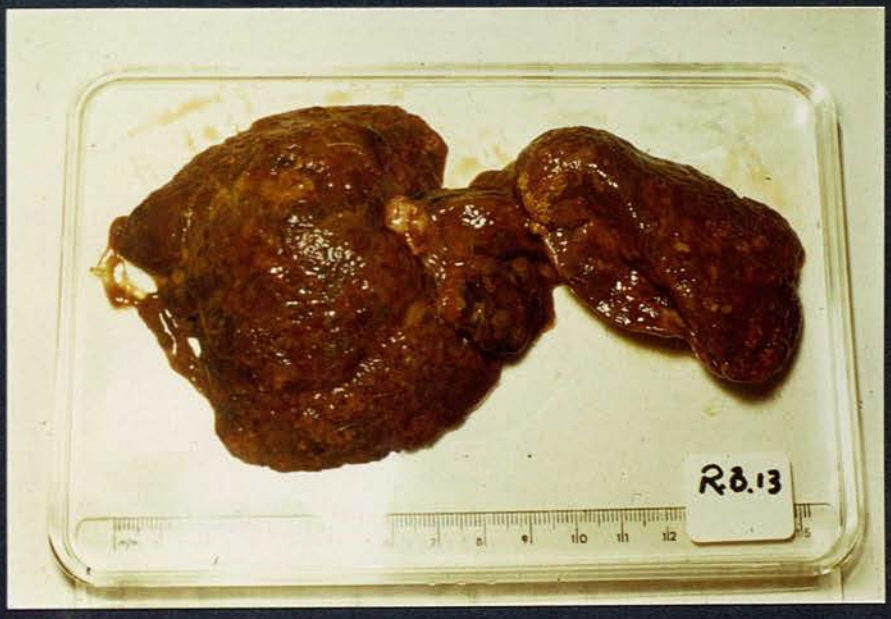
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CHAPTER FOUR

THE ACTIVITY OF DIAMPHENETHIDE AGAINST IMMATURE FASCIOLA HEPATICA IN RABBITS

General Introduction

The object of this experiment was to investigate the effect of diamphenethide on the immature stages of F.hepatica in rabbits experimentally infected with different doses of metacercariae. It was desired to be able to eliminate an initial infection in order to study the immunological response of animals challenged with metacercariae in the absence of flukes from the initial infection.

Dickerson, Hareenist and Kingsbury (1971) used mice with immature infections of F.gigantica and rabbits with adult infections of F.hepatica for screening different compounds for fasciclicidal activity. They confirmed their results in sheep infected with F.hepatica. The most characteristic effect of diamphenethide was its effect on the immature stages of the parasite. In sheep, an oral dose of 80-100mg per Kg body weight was 95-100% effective against three days to six weeks old F.hepatica. They reported also that a dose of 80-120mg per Kg body weight was 85-95% effective against the adult flukes. It therefore appeared probable that diamphenethide would be a suitable anthelmintic for the desired use, although there were no reports of its use against immature flukes in rabbits.

Experimental Design

Animals. Sixteen four month old rabbits were divided into four equal groups. Group 1 consisted of uninfected controls while Groups 2, 3 and 4 were infected with 50, 500 and 1,000 metacercariae respectively by the gelatine capsule method. The metacercariae used in this experiment were eight weeks old. Each group was then subdivided into two subgroups, one of which was dosed orally with 240mg per Kg body weight at the fourth week and again at the fifth week after infection, while the other subgroup was left as the undosed control. All the rabbits were killed and subjected to post-mortem examination eight weeks after infection.

Results

All the rabbits survived until eight weeks after infection. The number of flukes then recovered from the rabbits are shown in Table 4.1. The treated rabbits did not contain significantly fewer flukes than the untreated rabbits. The individual infection rates ranged from 3-56% with a mean of 15%, but the mean figure for the rabbits which had received 50 flukes was 34.5% compared with 5.9% for the more heavily infected rabbits.

Haematological data

The leucocyte series. Eosinophil and heterophil counts are shown in Appendix Tables 4.1 and 4.2. There were no significant changes in either the eosinophil or the heterophil counts in the uninfected rabbits, with or without treatment. There was a slight tendency

for the eosinophil and heterophil levels in the normal rabbits to fall with time, but regression analysis showed that this trend was not significant ($t_b < 1$) for either parameter.

All the infected rabbits showed significant increases in the eosinophil and heterophil counts by two weeks after infection and the latter tended to rise earlier in the more heavily infected animals. There was however no clear difference between the treated and untreated groups. Overall means for these data are shown in Figs. 4.1, 4.2, 4.3, and 4.4.

The erythrocyte series. No consistent differences were observed between the treated and untreated animals within the same group, although there were considerable individual variations within the groups. Some of the more heavily infected rabbits were developing anaemia by the end of the experiment.

Serum Glutamic Dehydrogenase

The results are shown in Appendix Table 4.3.

There were no significant changes in the glutamic dehydrogenase levels in the uninfected rabbits, whether treated or untreated. As in the previous experiment there was a tendency for the G.D. level to fall with time, but this was again not significant ($t_b = 1.8$, $N = 38$, $0.1 \succ p \succ 0.05$).

All the infected rabbits showed significant increases in the enzyme levels by three to four weeks

after infection and, although there were no significant differences as between the treated and untreated animals (Fig. 4.5), there was a tendency for the enzyme concentration in the more heavily infected rabbits to rise more steeply and to higher overall levels (Fig. 4.6).

Precipitins

No precipitin lines were observed with sera from rabbits in Group 1, while precipitin lines were detected with sera from all the rabbits in the other groups between three to four weeks after infection and continued to occur until the end of the experiment. There was some tendency for lines to be seen earlier when using sera from the more heavily infected rabbits.

Post-mortem examination

The liver of each animal was examined separately and the pathological lesions were recorded.

Group 1. No pathological lesions were observed.

Group 2. Most of the rabbits showed only a few scattered areas of necrosis, but the damage was much pronounced in the rabbit with 28 flukes: its bile duct was hyperplastic and its gall bladder was engorged.

Group 3. The livers were damaged and the whole surface was scattered with whitish necrotic areas. These lesions were particularly pronounced in the two more heavily infected animals.

Group 4. The lesions were distinctly clearer than in other groups. The livers were covered with fibrinous areas and there were adhesions between the liver and other abdominal organs. The livers were very inflamed and friable, with many areas of necrosis. Peritonitis was observed and the abdominal cavities of all the animals in the group were filled with dark reddish fluid.

There were no differences to be seen in the liver lesions between the treated and untreated animals within each group. The flukes recovered from all the animals were mature and living. In particular, no dead flukes were recovered from any of the treated animals.

Discussion and Conclusions

The similarity of the eosinophil counts and of the serum glutamic dehydrogenase levels in infected rabbits, whether they had been treated or untreated, suggested that the cellular reaction of the host and the damage caused by the parasite were still occurring at similar levels in both groups, and the post-mortem findings confirmed this deduction.

It therefore appeared that diamphenethide has no effect against immature stages of flukes in rabbits. In view of the high level of activity of this drug in other hosts, this was considered to be a surprising result, so it was decided to directly compare the activity of diamphenethide and other fasciolicidal drugs in a further study (Chapter Five).

The high infection rate in Group 2 (50 metacercariae) confirmed the suitability of the gelatine capsule infection technique.

The lower proportion of the infective dose of metacercariae recovered from the heavily infected rabbits may have been caused by competition between the flukes, although such an effect had not been seen previously (Chapter 3).

TABLE 4.1

The effect of diamphenethide on survival of *Fasciola hepatica* in rabbits

Group	1		2		3		4	
Number of Metacercariae given	Nil		50		500		1000	
Treatment with diamphenethide 4 and 5 weeks post-infection	-	+	-	+	-	+	-	+
Number of flukes present in each rabbit 8 weeks post-infection	0	0	10	28	30	17	70	60
	0	0	15	16	20	40	80	49

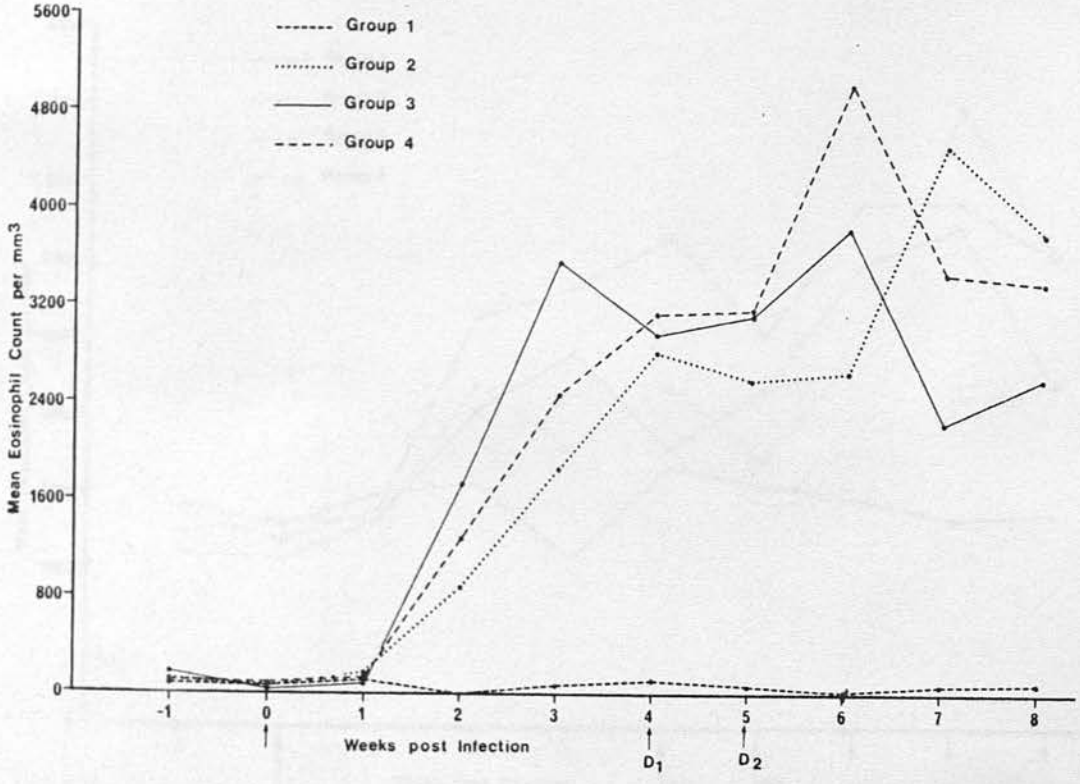


Fig. 4.1. Mean peripheral eosinophil counts.
The uninfected control rabbits (Group 1), the rabbits infected with 50 metacercariae (Group 2), those infected with 500 metacercariae (Group 3), and those infected with 1,000 metacercariae (Group 4).
At D_1 half the animals in each group were dosed with diamphenethide at 240mg/Kg and at D_2 this dose of diamphenethide was repeated.

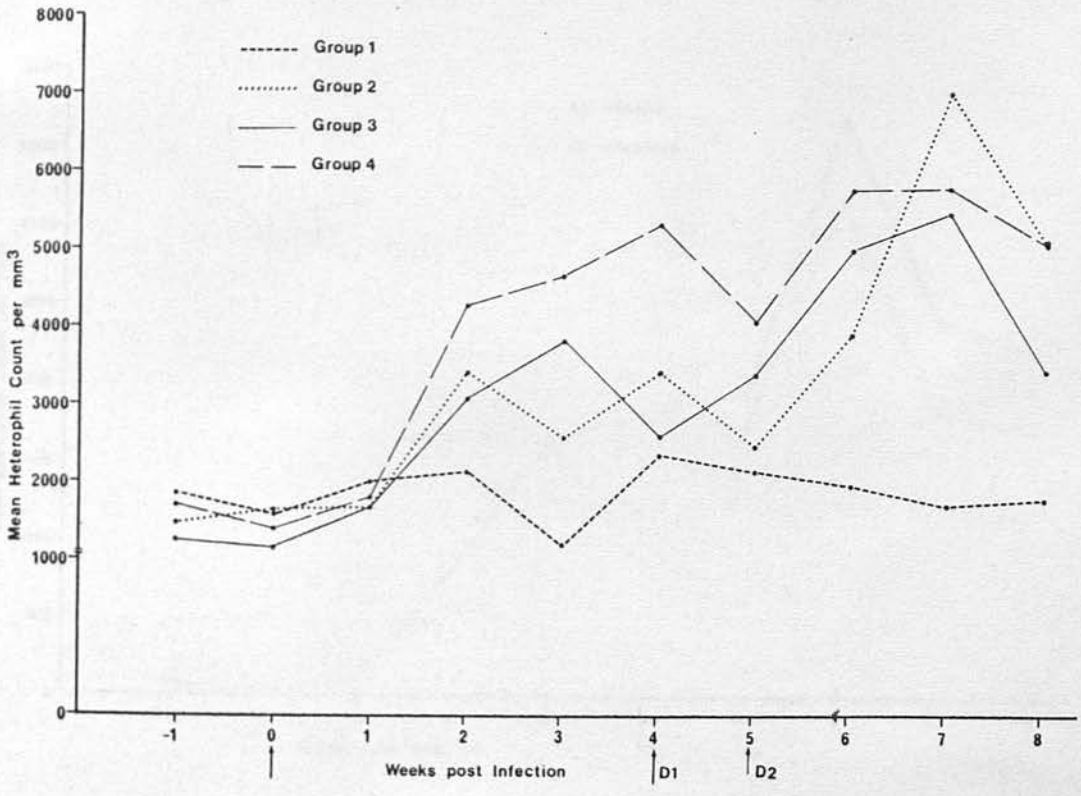


Fig. 4.2. Mean heterophil counts.

The uninfected control rabbits (Group 1), the rabbits infected with 50 metacercariae (Group 2), those infected with 500 metacercariae (Group 3) and those infected with 1,000 metacercariae (Group 4).

At D_1 half the animals in each group were dosed with diamphenethide at 240mg/Kg and at D_2 this dose of diamphenethide was repeated.

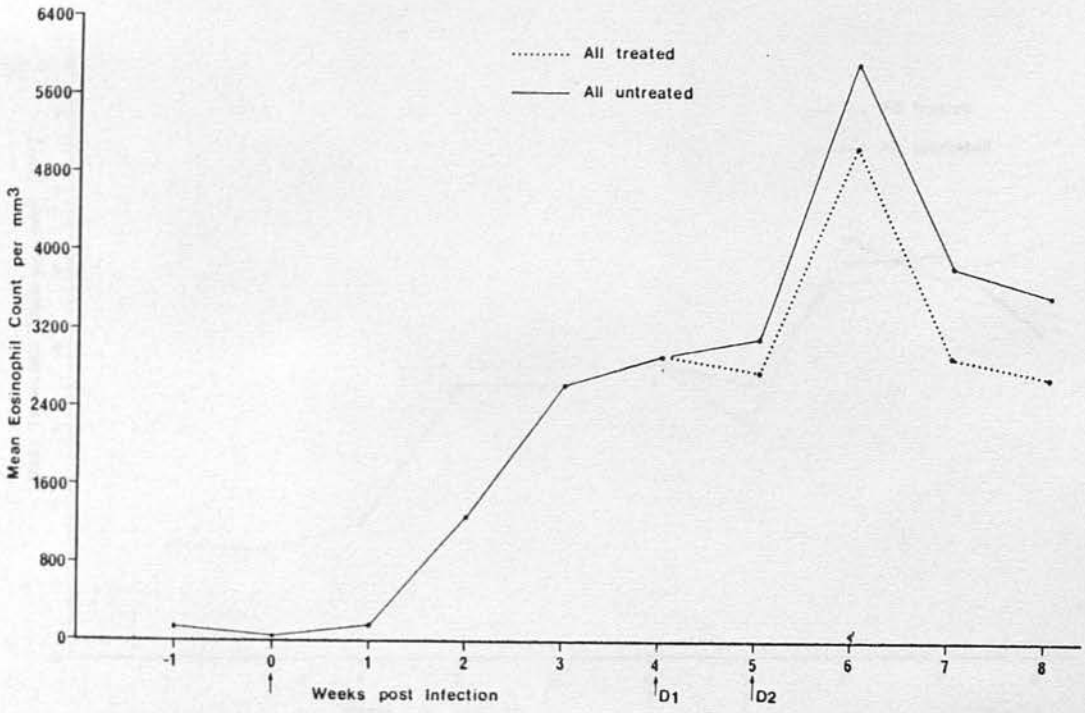


Fig. 4.3. Mean peripheral eosinophil count in rabbits infected with different numbers of metacercariae. The data are pooled together until four weeks after infection.

At D_1 half the animals in each group were dosed with diamphenethide at 240mg/Kg and at D_2 this dose of diamphenethide was repeated.

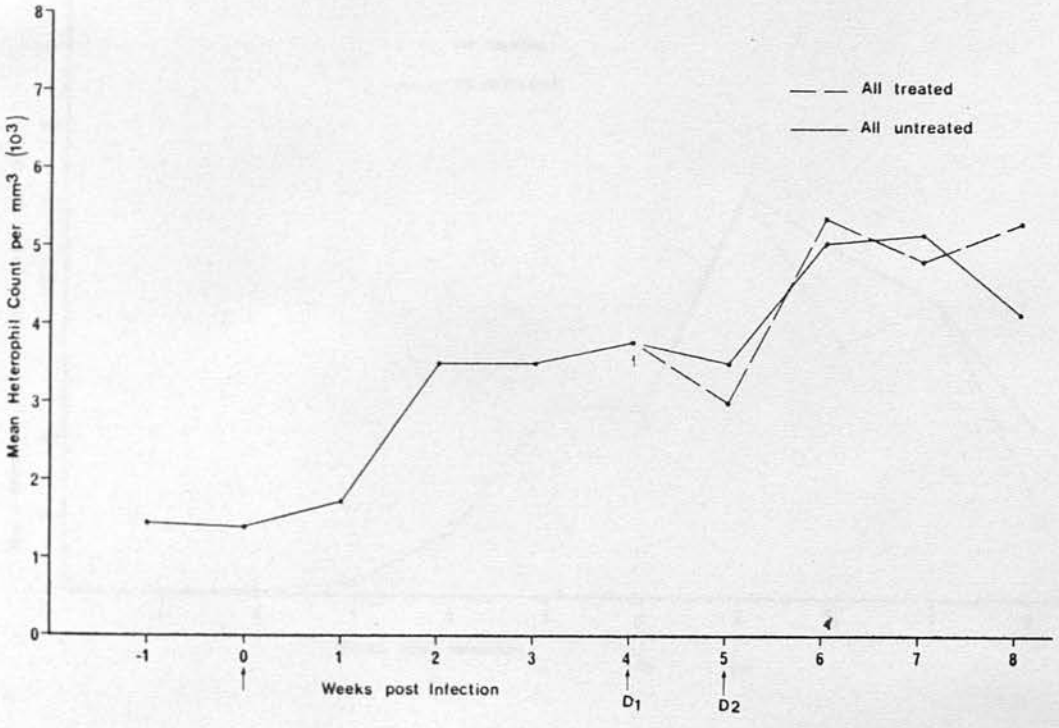


Fig. 4.4. Mean heterophil count in rabbits infected with different numbers of metacercariae. The data are pooled together until four weeks after infection.

At D_1 half the animals in each group were dosed with diamphenethide at 240mg/Kg and at D_2 this dose of diamphenethide was repeated.

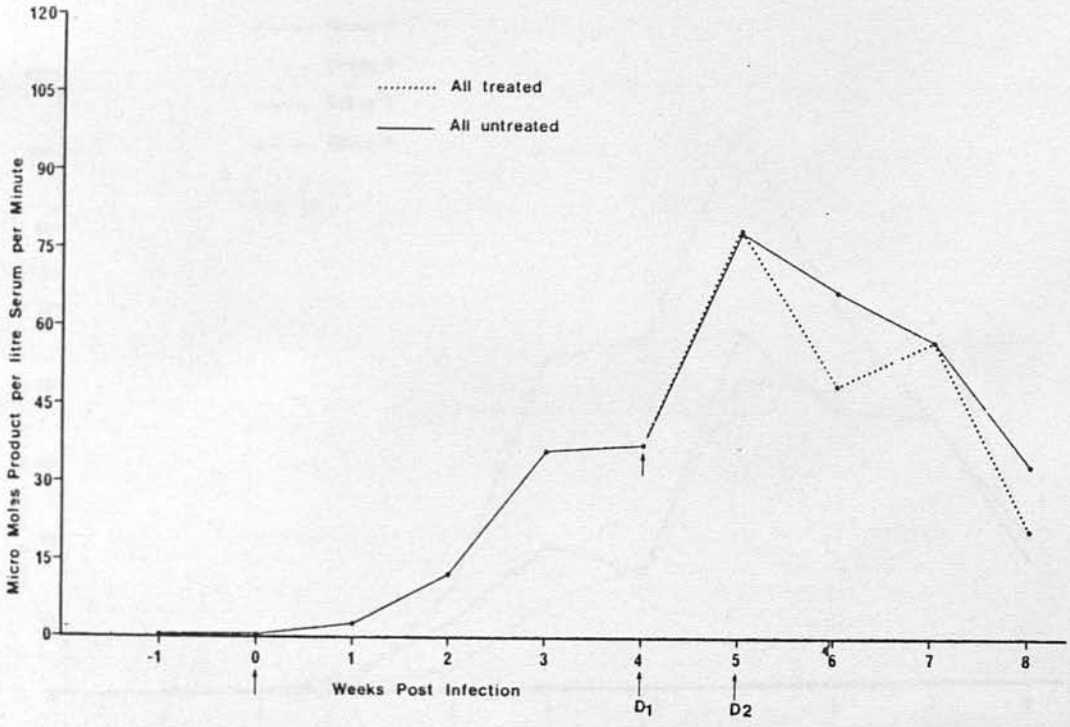


Fig. 4.5. Mean serum glutamic dehydrogenase levels in rabbits infected with different numbers of metacercariae. The data are pooled together until four weeks after infection. At D_1 half the animals in each group were dosed with diamphenethide at 240mg/Kg and at D_2 this dose of diamphenethide was repeated.

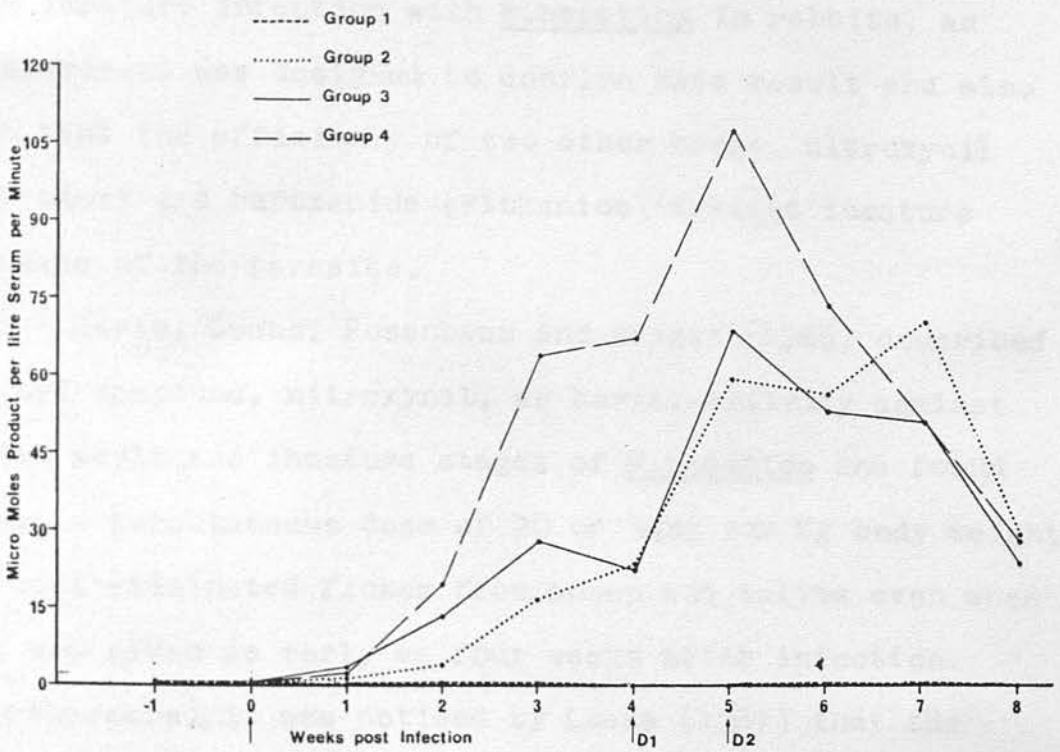


Fig. 4.6. Mean serum glutamic dehydrogenase levels. The uninfected control rabbits (Group 1), the rabbits infected with 50 metacercariae (Group 2), those infected with 500 metacercariae (Group 3) and those infected with 1,000 metacercariae (Group 4).

At D_1 half the animals in each group were dosed with diamphenethide at 240mg/Kg and at D_2 this dose of diamphenethide was repeated.

CHAPTER FIVE

A COMPARISON OF THE EFFECT OF THREE FASCIOLICIDAL DRUGS ON THE IMMATURE STAGES OF FASCIOLA HEPATICA

Introduction

Following the failure of diamphenethide to eliminate an immature infection with F.hepatica in rabbits, an experiment was designed to confirm this result and also to test the efficiency of two other drugs, nitroxynil (Trodax) and rafoxanide (Flukanide) against immature stages of the parasite.

Davis, Lucas, Rosenbaum and Wright (1966) described a new compound, nitroxynil, as having activity against both adult and immature stages of F.hepatica and found that a subcutaneous dose of 20 or 34mg per Kg body weight almost eliminated flukes from sheep and calves even when it was given as early as four weeks after infection. Furthermore, it was noticed by Lucas (1967) that the drug was equally potent against the mature stages of this parasite in rabbits.

An earlier observation on the use of rafoxanide was made by Mrozik, Jones, Friedman, Schwartzkope, Schardt, Patchett, Hoff, Yokstis, Riek, Ostlind, Plishker, Butler, Cuckler and Campbell (1969), who reported a wide therapeutic index for this drug. A more recent investigation by Armour and Čorba (1970) has shown the anthelmintic activity of rafoxanide against the immature stages of F.hepatica infection in sheep and they also observed

rapid regression of F.hepatica lesions in lambs treated at four to six weeks after infection.

Experimental Design

Sixteen rabbits were divided into four groups each of four rabbits, these comprising a control group, and three experimental groups, the rafoxanide treated group, the diamphenethide treated group and a third group to be treated with nitroxynil. Each group was again equally sub-divided (Table 5.1) and the rabbits in each subgroup infected with 100 or 500 metacercariae respectively. Uninfected controls were not included in this experiment as it was primarily designed to demonstrate the efficacy, if any, of the anthelmintic used.

Packed cell volumes, erythrocyte counts, haemoglobin estimations, eosinophil and total leucocyte counts were determined weekly.

Serum was collected for determination of serum glutamic dehydrogenase levels and immuno-precipitation tests.

Sterile whole blood was also obtained weekly from these rabbits for in-vitro lymphocyte stimulation studies, the results of which will be discussed separately in a later chapter in Section II (page 149).

After it had been found from the last experiment that diamphenethide at a dose rate of 240mg per Kg body weight appeared to have no effect on the immature stages of F.hepatica in rabbits, it was decided to use a higher

dose of 500mg per Kg body weight in this further trial. Rafoxanide was administered orally at a dose rate of 26mg per Kg body weight and nitroxynil was given subcutaneously at a dose rate of 20mg per Kg body weight. The drugs were first given four weeks after infection and this dose was repeated again at the fifth week in an attempt to ensure the destruction of immature flukes.

All the rabbits were killed and subjected to post-mortem examination eight weeks after infection as in the previous experiment (Chapter 4) to maintain a similar pattern and to allow sufficient time for any flukes which may have escaped the effects of dosing to establish in the bile ducts.

Results

In the group treated with diamphenethide the two rabbits in the more heavily infected subgroup both died two weeks after the second dosing, while in the nitroxynil treated group, one of the heavily infected animals also died at that time. In the control group one animal died accidentally, three weeks after the beginning of the experiment.

Otherwise no consistent differences were seen in any of the parameters as between the rabbits receiving 100 metacercariae and those receiving 500 metacercariae within any group and the results have therefore been pooled together.

No side effects were observed after treatment, apart

from slight ulceration around the site of injection in two of the rabbits injected with nitroxynil.

The numbers of flukes recovered are recorded in Table 5.1. The rafoxanide treated group and the nitroxynil treated group contained significantly lower numbers of flukes as compared with the control group and the group treated with diamphenethide. Of the two former groups, the percentage reduction in fluke numbers was significantly greater with rafoxanide. Diamphenethide again caused no reduction in fluke numbers.

Eosinophils

The eosinophil counts are shown in Appendix Table 5.1. In all groups there was an increase in the eosinophil counts (Fig. 5.1) by two weeks after infection. There was no significant difference between the eosinophil counts from the untreated rabbits and those treated with diamphenethide, except at week eight, by which time both groups were reduced in size. In view of this and of the other results suggesting that diamphenethide is not effective against immature flukes in rabbits, the results from these two groups were therefore pooled for further statistical analysis in this experiment. However, the rabbits in the rafoxanide treated group all showed a sharp increase in the level of eosinophilia after the first treatment with the drug but the count then decreased quickly after the second treatment and remained low until the end of the experiment. These results were significantly lower than those from the rabbits in the control

and diamphenethide groups at weeks seven and eight ($N = 10$, $t = 2.44$ and 4.24, $p < 0.02$ and 0.01 respectively). However, the apparent peak in the eosinophil count from the rafoxanide group at five weeks post infection just fails to be significant ($N = 9$, $t = 2.25$, $p < 0.1 > 0.05$). The eosinophil counts in the rabbits in the nitroxynil treated group did not show this short-lived increase after the first dose of the drug, but fell after dosing, though not to the low levels observed in the rabbits treated with rafoxanide, before again rising to levels similar to those in the control group by the end of the experiment. These results were only significantly different from the rabbits in the control and diamphenethide groups at week five ($N = 11$, $t = 2.48$, $p < 0.05$).

Erythrocytes

The erythrocyte values tended to fall in all the groups for five to six weeks after infection but tended to rise thereafter, especially in the rabbits treated with rafoxanide (Fig. 5.3). In the group treated with rafoxanide, the p.c.v. level was not significantly lower than the other groups except at week eight ($N = 10$, $t = 5.4$ and $p < 0.001$).

Precipitins

Precipitin lines were observed with sera from all animals in the group by three weeks after infection and continued to occur for the rest of the experiment, even after treatment.

Glutamic Dehydrogenase Assay

The results are shown in Appendix Table 5.2 and Fig. 5.3. There was a distinct increase in the enzyme level by the second week after infection. All rabbits then showed considerably elevated enzyme levels prior to anthelmintic dosing. There was no significant difference between the enzyme levels in the control group and the diamphenethide treated group although the levels in both groups fell towards the end of the experiment. These results were therefore pooled for further statistical analysis. The results from the group treated with nitroxynil were significantly different from those for the untreated controls, pooled with the diamphenethide group, at weeks six and eight ($N = 9$, $t = 2.27$, $p < 0.05$, and $N = 6$, $t = 4.1$, $p < 0.01$ respectively). In the rafoxanide treated group there was a marked fall in the enzyme level after the fifth week which was then progressive until the end of the experiment. These results were significantly lower than those from the other rabbits by six weeks after infection ($N = 10$, $t = 4.2$ and $p < 0.005$) and remained so thereafter. The rabbits in the nitroxynil treated group showed a slight fall after the second dose of the drug but this fall was less than seen in the rafoxanide treated group (Fig. 5.2).

Post-mortem examination

This was carried out as soon as possible on the rabbits which died during the experiment and the rest of

the rabbits were autopsied eight weeks after infection.

Damage was pronounced in the livers of all the rabbits in the control group, with necrotic areas scattered throughout the tissue, which was relatively firm when cut by a knife. At post-mortem examination of RB 122, which died three weeks after infection, there were signs of damage and areas of whitish-yellow necrosis were seen in the liver tissue. The bile ducts were normal and the young flukes were recovered from the tissue of this animal. In the remainder of the animals in this group the bile ducts were hyperplastic and inflamed, this being especially noticeable in the subgroup infected with 500 metacercariae. Adult flukes were recovered from the hyperplastic bile ducts.

The colour of the livers in the rabbits treated with rafoxanide were normal and, on cutting into the tissues, they appeared to be of normal consistency. The bile ducts were normal and there were no signs of inflammation or necrosis detected on the liver surface apart from a small necrotic area which measured 1 x 1 cm seen on the edge of the liver tissue in RB 125, in which part of a dead fluke was seen. The small numbers of immature flukes found in two of the livers were not seen in the bile duct but only during examination of the parenchyma.

The livers of the animals treated with diamphenethide were similar to those of the control rabbits and

the bile ducts of RB 129 and RB 117 were particularly severely inflamed and hyperplastic. Furthermore, the gall bladders of these two animals were engorged and relatively large numbers of adult flukes were recovered from the bile ducts.

The livers of the animals treated with nitroxynil were still showing signs of damage and the cut surface was tough and fibrous. There was no sign of adult flukes in the bile duct, only immature flukes being found in the liver parenchyma, even in the three rabbits that survived for eight weeks. In the single rabbit which died earlier, there was no sign of flukes in the bile duct, and the parenchyma was too badly degenerated to permit detailed examination, although 15 immature flukes were recovered.

Discussion

The reduction in peripheral eosinophilia and serum enzyme levels after the second dose of rafoxanide suggested that both the cellular reaction of the host and the damage caused by the flukes were regressing and post-mortem examination supported this view, although a complete cure was not obtained in all the rabbits. Nitroxynil had a considerable effect in reducing the number of flukes in the rabbits but the drug was less effective than rafoxanide and failed to prevent the death of one heavily infected animal. The sharp increase in the eosinophil counts in the rabbits in this

group immediately after the first dose of rafoxanide may be an indication of an increased host response to the relatively sudden presence of dead fluke material. If so, it would suggest that nitroxynil perhaps fails to kill the flukes so quickly, thus allowing a continuing pathogenic effect.

Higher doses of diamphenethide still appeared to have no effect on the immature stages of F.hepatica in rabbits.

It thus appeared that rafoxanide had both a greater lethal effect against the immature stages of flukes than the other drugs and also resulted in a greater regression in the effects of the F.hepatica infection in the rabbits, so supporting the earlier work of Armour and Čorba (1970) on lambs.

TABLE 5.1

Infection level and the number of fluke recovery at P.M.

Groups	Control Group		Rafoxanide Treated		Diamphenethide Treated		Nitroxynil Treated	
Rabbit Numbers	118 122+	130 131	124 125	126 116	127 128	129++ 117++	119 121	120++ 123
Infective dose of metacercariae per animal	100	500	100	500	100	500	100	500
Number of flukes recovered at P.M.	20 18*	45 50	1* -	2* -	45 35	95 80	4* 7*	15* 3*
Percentage reduction in burden	-		98		-		78	

+ Died by four weeks after infection. ++ Died by seven weeks after infection. * Immature.

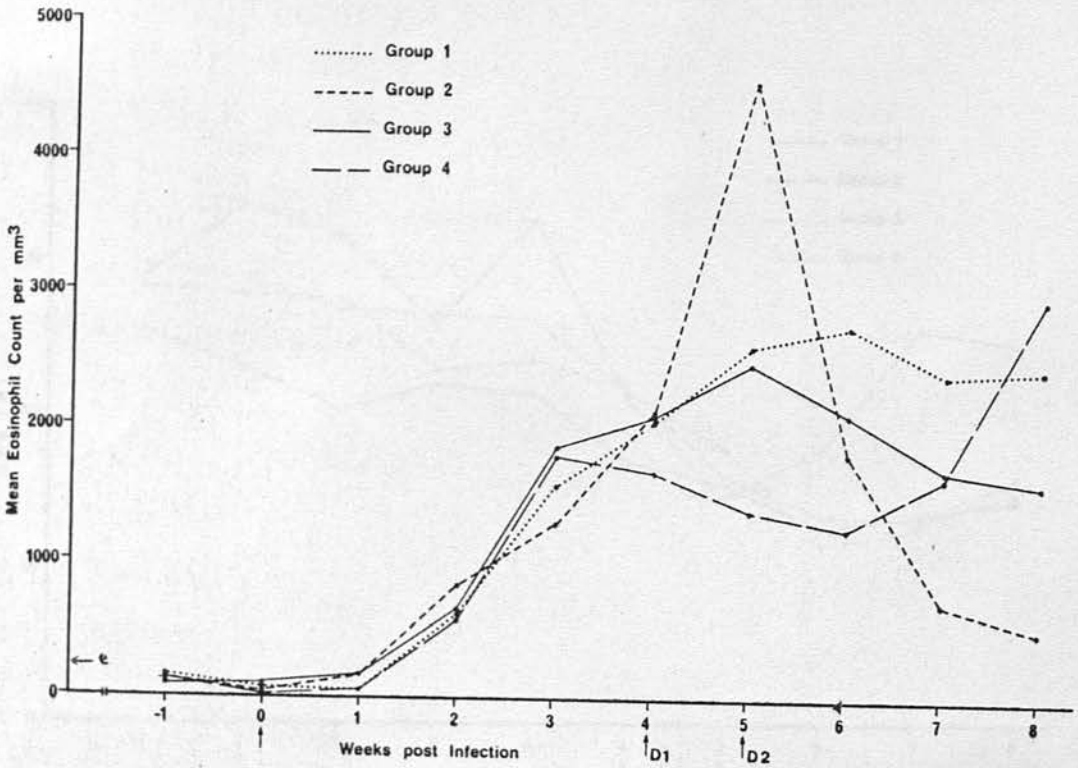


Fig. 5.1. Mean peripheral eosinophil count, in rabbits infected with different levels of metacercariae and treated with different fasciolicidal drugs.

Group 1 = the control untreated group.

Group 2 = rafoxanide treated group (26mg/Kg).

Group 3 = diamphenethide treated group (500mg/Kg).

Group 4 = nitroxynil treated group (20mg/Kg).

— e - Mean $\pm$ 3 s.d.

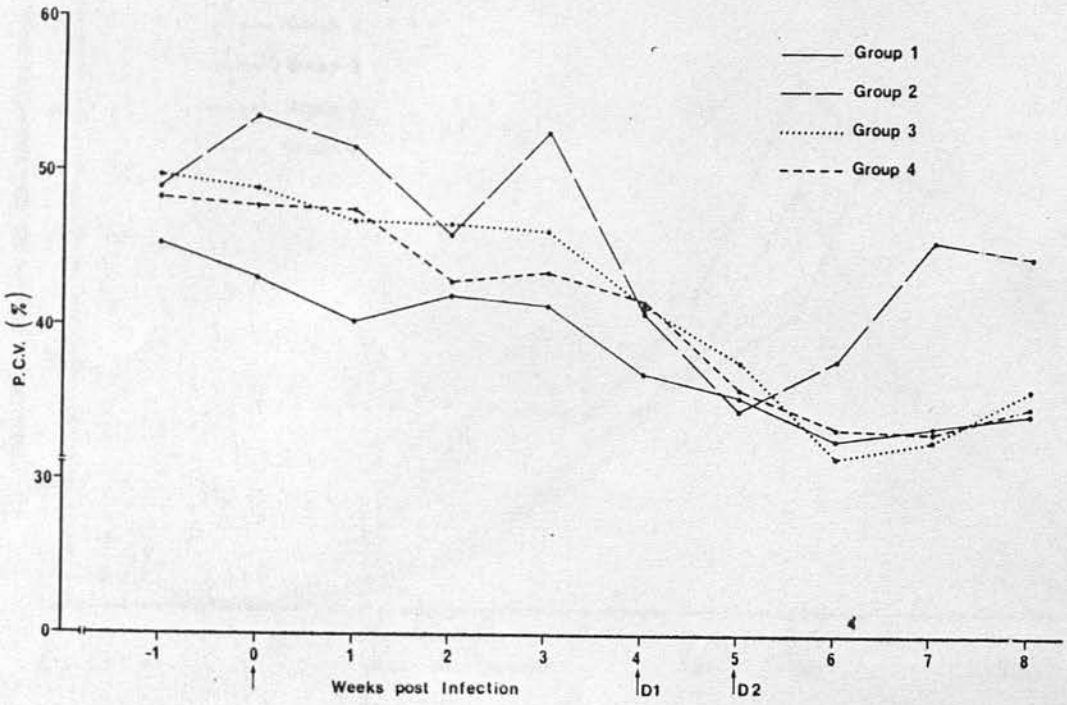


Fig. 5.2. Mean packed cell volume (p.c.v.) in rabbits infected with different levels of metacercariae and treated with different fasciolicidal drugs.

Group 1 = the control untreated group.

Group 2 = rafoxanide treated group (26mg/Kg).

Group 3 = diamphenethide treated group (500mg/Kg).

Group 4 = nitroxynil treated group (20mg/Kg).

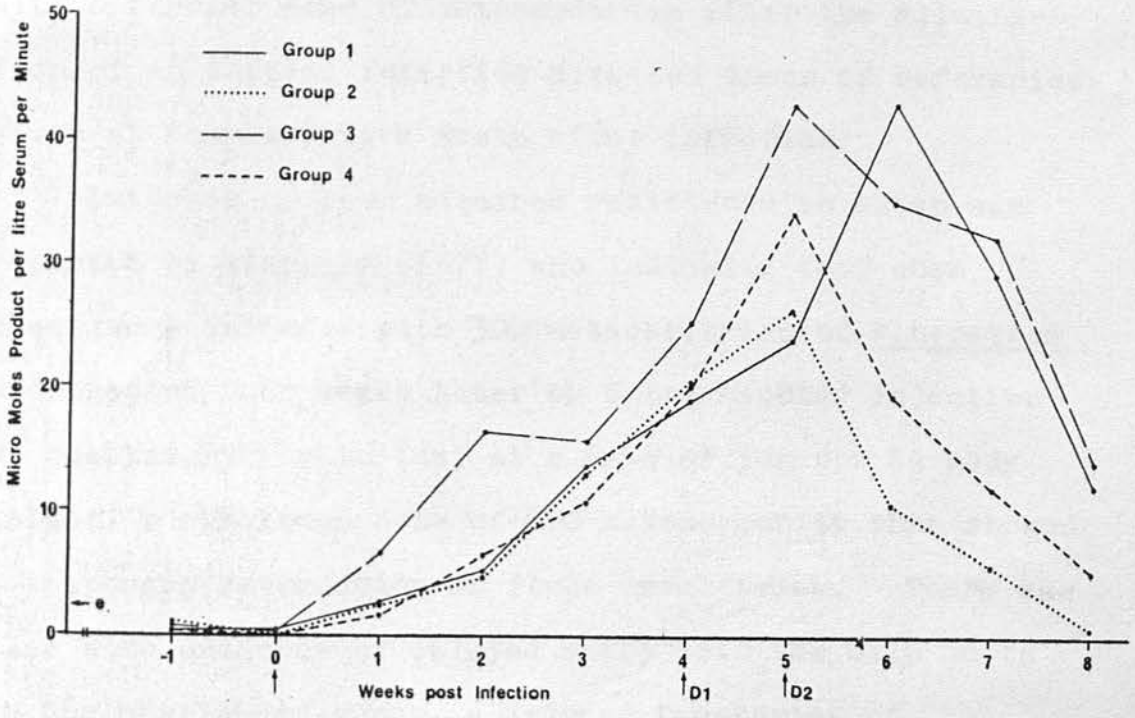


Fig. 5.3. Mean serum glutamic dehydrogenase levels in rabbits infected with different levels of metacercariae and treated with different fasciolicidal drugs.

Group 1 = the control untreated group.

Group 2 = rafoxanide treated group (26mg/Kg).

Group 3 = diamphenethide treated group (500mg/Kg).

Group 4 = nitroxynil treated group (20mg/Kg).

— e - Mean $\pm$ 3 s.d.

CHAPTER SIX

RESISTANCE TO RE-INFECTION WITH FASCIOLA HEPATICA IN RABBITS AFTER ONE PREVIOUS INFECTION

Introduction

This experiment was designed to demonstrate if there was any acquired resistance in rabbits challenged with a further dose of metacercariae after the elimination of an initial infection with two doses of rafoxanide given at four and five weeks after infection.

Evidence of some acquired resistance in sheep was reported by Sinclair (1971) who indicated that when sheep were infected with 300 metacercariae of F.hepatica and treated four weeks later by intramuscular injection of emetine hydrochloride, at a rate of 3mg per Kg body weight, a challenge dose of 600 metacercariae then showed a temporary retardation of fluke development. There was also some evidence of delayed entry into the bile ducts in the challenged group. Reduced recoveries of F.hepatica from challenge infections in rabbits were observed by Kendall, Hebert, Parfitt and Peirce (1967), who accounted for this reduction as arising from inhibition of growth and the technical difficulty of recovering very small flukes. Recent work by Kendall and Sinclair (1971) has shown that significantly fewer flukes were recovered from a challenge infection of F.hepatica in rabbits when this was given two days after an initial infection had been terminated by hexachlorophene.

Experimental Design

Sixteen rabbits were divided into four groups each of four rabbits (Table 6.1). Each animal in Group A was infected with 100 metacercariae, and in Group B with 500 metacercariae. Both these groups were treated with rafoxanide at 26mg per Kg body weight at four weeks and again at five weeks after infection and then all rabbits in both groups were each challenged with a further 100 metacercariae four weeks after the last treatment. The four rabbits in Group C, the previously uninfected controls, were each challenged with 100 metacercariae four weeks after being treated with rafoxanide. Group D, the treated control group, were divided into two subgroups in which the rabbits were infected with 100 and 500 metacercariae respectively. All the rabbits in this group received the same doses of rafoxanide as those in Groups A, B and C but did not receive the challenge infection.

The metacercariae used for the initial infection were three weeks old, while those for the challenge infection were six weeks old.

All the rabbits were killed 13 weeks after the initial infection, and four weeks from the challenge infection. This gave enough time for the challenge infection to be examined at post-mortem examination, while at the same time allowing the young flukes of the challenge infection to be distinguished from any flukes remaining from the initial infection.

The data obtained from rabbit RB 138, one of the previously uninfected control group (Group C), has been excluded from all the means calculated for this group as the evidence from all the parameters studied suggested that this animal was never infected.

Results

The results are shown in Appendix Tables 6.1, 6.2 and 6.3.

No consistent differences were seen between any of the parameters studied as between the rabbits receiving 100 metacercariae and those receiving 500 metacercariae. Accordingly the results for all the rabbits in Group D have been pooled together and also those for Groups A and B.

The number of flukes recovered from all the rabbits are recorded in Table 6.1. There was clearly no significant difference in the mean numbers of flukes recovered as between Groups A and B. The mean number of flukes recovered from Group C, the challenge control group, was higher than that from either Group A or B, which were previously infected and challenged, but this difference is not significant. The results from Group D, the unchallenged controls show that the rafoxanide had almost eliminated the previously existing infection with F.hepatica.

Leucocytes

The rabbits in Groups A, B and D all showed an increase in the eosinophil counts (Fig. 6.1) by two

weeks after infection and there were no significant differences between the levels of eosinophils in these groups. A sharp increase was observed after the first treatment in most of the rabbits in these groups but the counts then tended to decrease rapidly and remained low in the unchallenged rabbits in Group D (Fig. 6.1), apart from a slight rise after the ninth week. However, the counts again rose rapidly after the challenge infection in Groups A and B. The rabbits in Group C did not show any increase in the number of eosinophils until two weeks after they received the challenge infection. There was a significant ($N = 21$, $t = 2.8$, $p < 0.05$) tendency for the eosinophil count to be higher four weeks after second infection than four weeks after initial infection.

The heterophil counts also showed similar patterns in Groups A and B (Fig. 6.2) with a rise by three to five weeks after initial infection and again after the challenge infection and in this case also the count was significantly higher ($N = 21$, $t = 3.9$, $p < 0.001$) four weeks after the second infection than four weeks after the initial infection.

The rabbits in Group D showed the rise after the first infection but tended to decrease later, while the rabbits in Group C showed a rise in the number of heterophils only after the challenge infection (Fig. 6.2).

Serum Glutamic Dehydrogenase

There was a marked increase in the glutamic dehydrogenase levels in the serum by two weeks after the first infection in all the rabbits except those in Group C (Fig. 6.3). These levels reached a peak by five weeks after infection. The levels then fell sharply and remained low in the unchallenged rabbits in Group D, although they rose sharply after the challenge infection in Groups A, B and C.

There was a slight tendency for the G.D. level to be lower four weeks after the second infection than four weeks after initial infection, but this tendency is not significant ($N = 21$, $t = 1.18$, $0.4 > p > 0.2$).

Post-mortem examination

The livers of all rabbits were examined at the end of the experiment, 13 weeks after the initial infection. There was evidence of damage in all the livers in the Groups A, B, C and D except in rabbit No. 138 but there was much individual variation within the groups and also some differences between the groups. Areas of whitish irregular necrotic lines were seen in the liver parenchyma underneath the capsule. Adhesions between the lobes were especially noticeable in RB 75. In all cases the bile ducts appeared normal and were not hyperplastic (Plates 6.1, 6.2, 6.3 and 6.4).

There tended to be more liver damage in Group B than in Group A (Plates 6.1 and 6.2). The adhesions were more marked and the capsules were severely torn and haemorrhagic, especially in RB 48 and RB 47. The lesions in the infected livers in Group C (Plate 6.3), were similar but generally less severe than those in Groups A and B. The necrotic lines were more distinct, while the haemorrhagic lesions were not clear as compared with Groups A and B. The liver of RB 138 showed no lesions and the liver appeared to be normal.

The livers of the animals in Group B (Plate 6.4) were normal in appearance and colouration but the cut surface was soft and friable. RB 73 had a small encapsulated area of necrotic tissue measuring 1.5 x 1 cm on the edge of the medium lobe. This contained necrotic tissue, probably dead flukes. RB 60 had small necrotic spots located at one side of the lateral lobe.

The flukes recovered from these livers were obtained from the parenchyma by cutting the livers into small pieces which were then examined under a stereoscopic microscope.

Discussion

There was no significant difference between the number of flukes recovered from Groups A and B, which suggested that the difference in number of metacercariae given at the initial infection had no significant effect

on the challenge infection. The rabbits in Group C, the challenge controls, showed a slight increase in the mean numbers of fluke recovery as compared with the previously infected and challenged rabbits in Groups A and B, although the increase in the fluke recovery from the rabbits in Group C was not consistent. The exclusion of RB 138 from Group C appears to be justified in view of lack of any evidence of infection but this adds an additional difficulty in evaluating the result from this group, especially as it is clearly impossible to entirely rule out the possibility that RB 72 may have accidentally received a double infection.

The increase in both the glutamic dehydrogenase level and the peripheral eosinophilia after both the initial and the challenged infections, taken together with the low levels after treatment and between the infections, indicates that rafoxanide both kills the flukes and allows a rapid recovery of the liver. However, the initial infection clearly does not prevent the flukes from the challenge infection causing damage to the host.

The difference in gross pathology between the challenge controls and the previously infected rabbits, suggests that further doses of metacercariae may even be more deleterious to the host.

Table 6.1

The effect of challenge infection on the number of fluke recovery

Group	Rabbit Number	Initial Infection of Metacercariae	Number of flukes recovered at P.M. examination four weeks after challenge			Mean number of flukes from challenge only recovered from each group
			Mature adult	Immature*	Young	
A	RB 74	100	0	0	6	14
	RB 71	100	0	0	16	
	RB 75	100	0	1	17	
	RB 53	100	0	1	15	
B	RB 48	500	0	0	22	16
	RB 47	500	0	0	11	
	RB 58	500	0	0	15	
	RB 57	500	0	0	16	
C	RB 72	Nil	0	0	32	22
	RB 51	Nil	0	0	25	
	RB 138	Nil	0	0	0	
	RB 78	Nil	0	0	10	
D	RB 70	100	1	0	0	—
	RB 73	100	0	0	0	
	RB 45	500	0	0	0	
	RB 60	500	0	2	0	

All rabbits treated with Rafoxanide four and five weeks after the initial infection and, with the exception of those in Group D, challenged with 100 metacercariae four weeks after last treatment.

*Immature flukes are those which were clearly bigger than the young flukes recovered from the challenge infection, but did not contain any eggs.

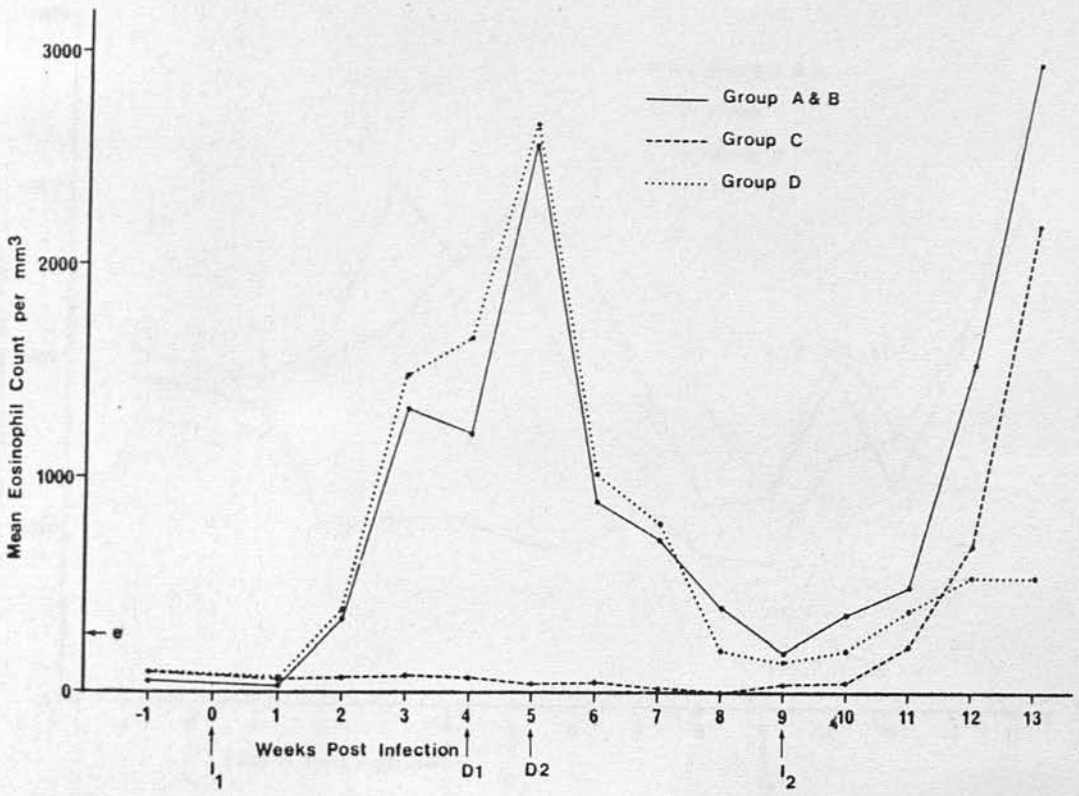


Fig. 6.1. Mean peripheral eosinophil counts.

Group A + B = rabbits infected with 100 and 500 metacercariae.

Group C = previously uninfected control.

Group D = treated control.

I_1 ..... initial infection of metacercariae.

I_2 ..... challenge infection.

D_1 ..... first dose of rafoxanide (26mg/Kg).

D_2 ..... repeated dose of rafoxanide (26mg/Kg).

— e ..... Mean $\pm$ 3 s.d.

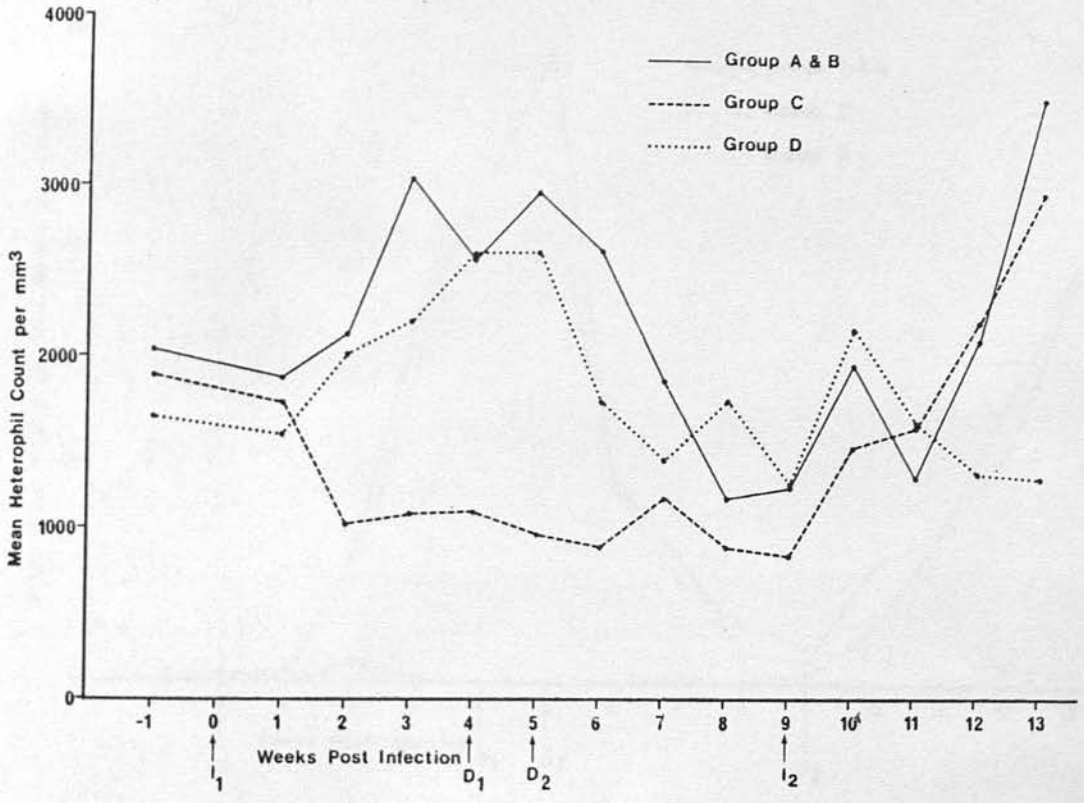


Fig. 6.2. Mean heterophil counts.

Group A + B = rabbits infected with 100 and 500 metacercariae.

Group C = previously uninfected control.

Group D = treated control.

I_1 ..... initial infection.

I_2 ..... challenge infection of 100 metacercariae.

D_1 ..... first dose of rafoxanide (26mg/Kg).

D_2 ..... repeated dose of rafoxanide (26mg/Kg).

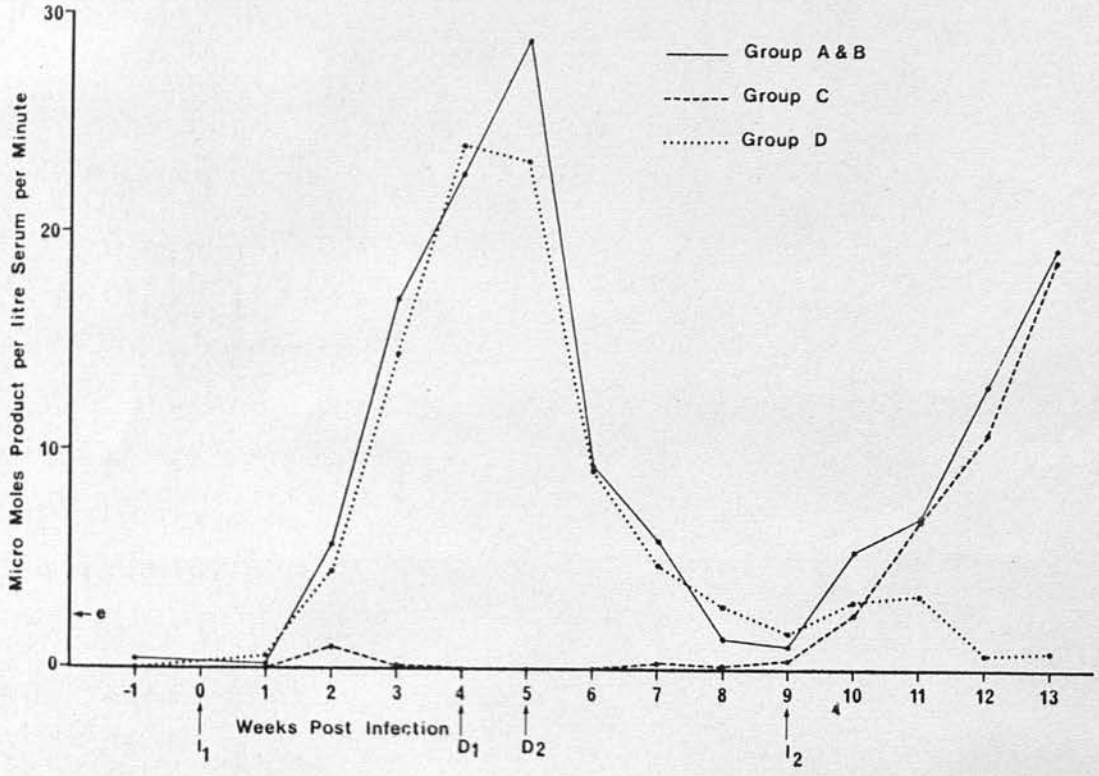


Fig. 6.3. Mean glutamic dehydrogenase levels.

Group A + B = rabbits infected with 100 and 500 metacercariae.

Group C = previously uninfected control.

Group D = treated control.

I_1 ..... initial infection.

I_2 ..... challenge infection.

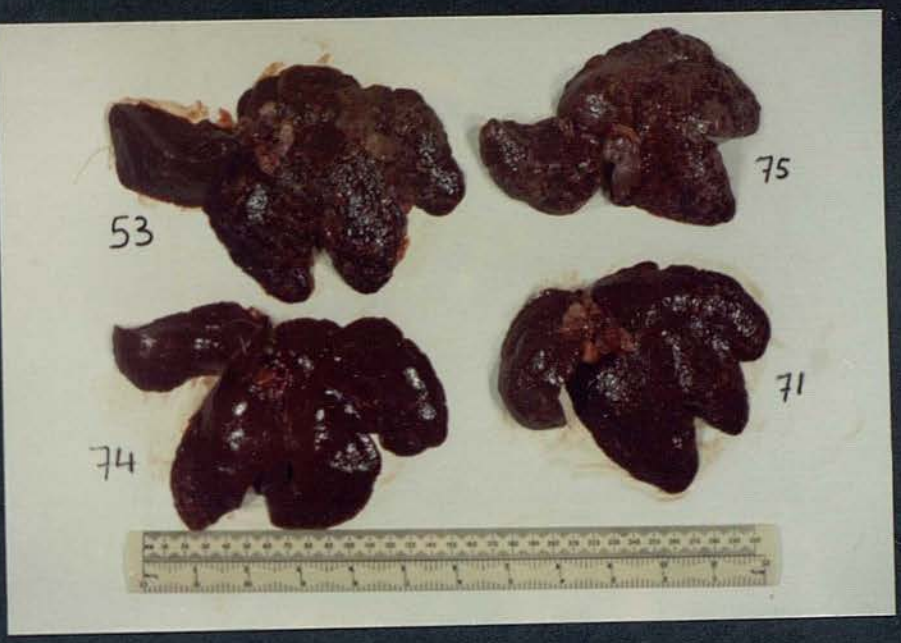
D_1 ..... first dose of rafoxanide (26mg/Kg).

D_2 ..... repeated dose of rafoxanide (26mg/Kg).

— e ..... Mean $\pm$ 3 s.d.

Plate 6.1. Livers from rabbits (Group A) infected with 100 metacercariae of F.hepatica each orally, treated with rafoxanide at 26mg/Kg at four and five weeks after infection and challenged with 100 metacercariae each, nine weeks later.

Plate 6.2. Livers from rabbits (Group B) infected with 500 metacercariae of F.hepatica each orally, treated with rafoxanide at 26mg/Kg at four and five weeks after infection and challenged with 100 metacercariae each, nine weeks later.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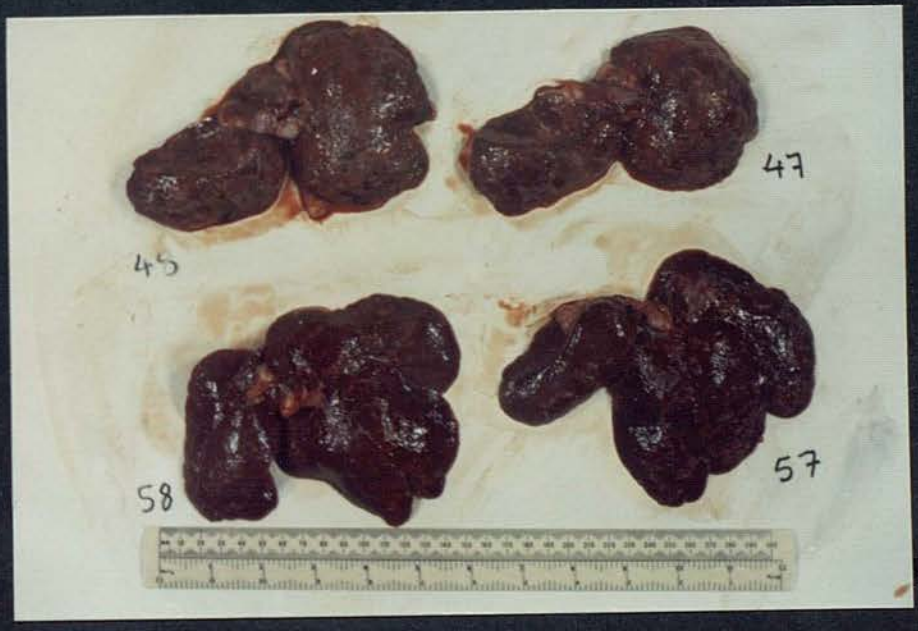
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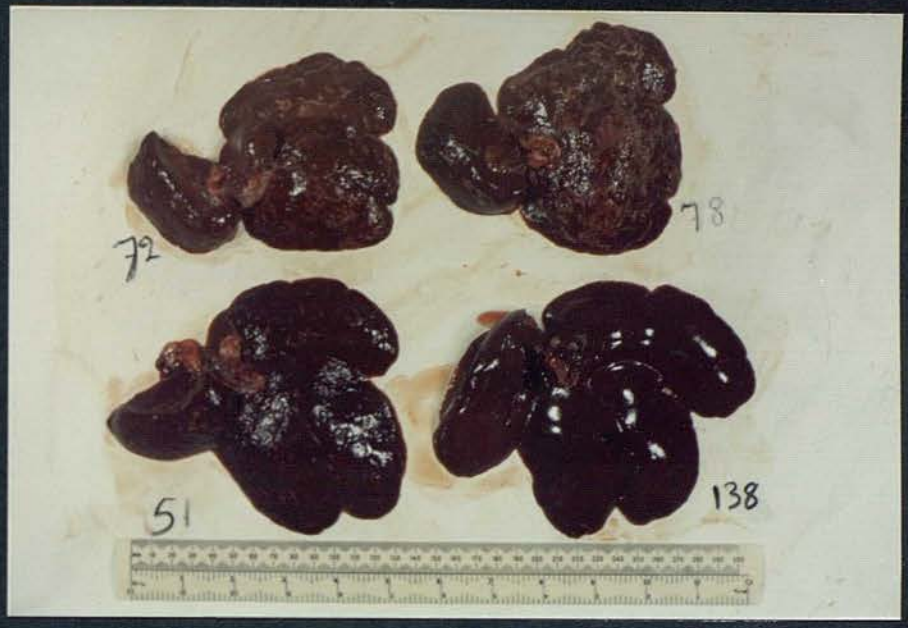
Plate 6.3. Livers from rabbits (Group C) previously uninfected, but challenged with 100 metacercariae of F.hepatica each orally.

Plate 6.4. Livers from rabbits (Group D) treated control group, which had received 100 or 500 metacercariae, and been treated with rafoxanide four and five weeks at 26mg/Kg and weeks prior to slaughter.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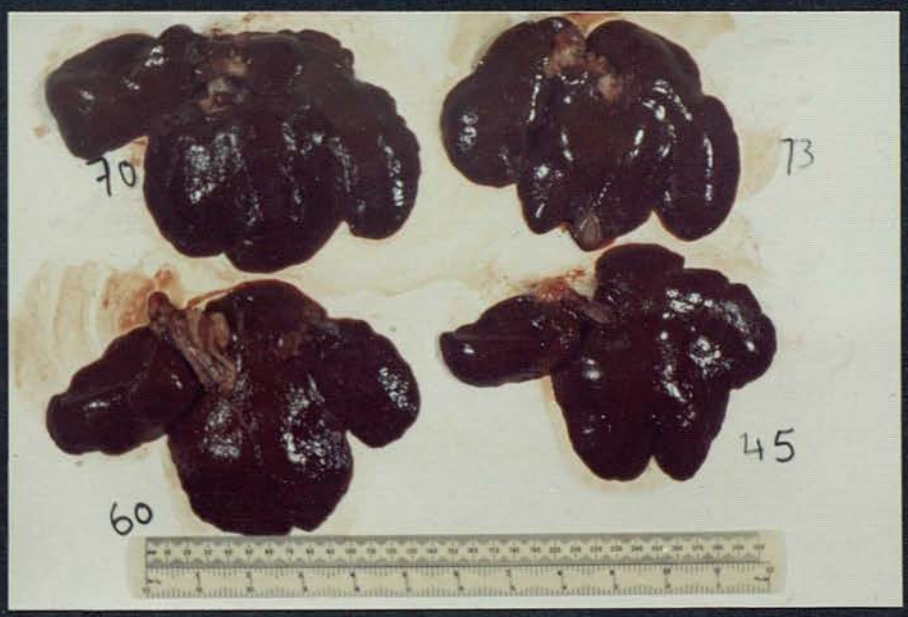
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CHAPTER SEVEN

RESISTANCE TO RE-INFECTION IN RABBITS FOLLOWING TWO PREVIOUS INFECTIONS OF FASCIOLA HEPATICA

Introduction

Following the experiment in which previously infected and treated rabbits received a further challenge infection with a single dose of metacercariae (Chapter 6) and in which resistance was not clearly demonstrated, it was decided to design an experiment to study the effect of giving one or two infections of metacercariae to a group of rabbits, after their previous infections had been terminated by anthelmintic treatment prior to the challenge infection.

Experimental Design

Sixteen rabbits were divided into three groups of four rabbits and the fourth group was sub-divided into two subgroups of two. Details of the experimental design are shown in Table 7.1. All the rabbits were treated with rafoxanide at a rate of 26mg per Kg body weight at four weeks and again at five weeks after either infection. Group 3 acted as the challenge control group, while the two subgroups in Group 4 formed the treatment control group.

The metacercariae used in each infection were about four weeks old.

Eosinophil and total leucocyte counts were determined every second week and, at the same time, serum was

collected for determination of glutamic dehydrogenase levels.

Sterile whole blood was also obtained every second week for *in-vitro* lymphocyte stimulation studies, the results of which will be discussed separately in Chapter 14 (page 169).

All the rabbits were killed and subjected to *post-mortem* examination 22 weeks after the start of the experiment and four weeks from the last infection, thus permitting the young flukes recovered from the last infection to be distinguished from any remaining flukes from the previous infections.

Results

The results are shown in Appendix Tables 7.1 and 7.2, and in Figs. 7.1, 7.2, 7.3 and 7.4. The numbers of flukes recovered from all rabbits are recorded in Table 7.1. There was no significant difference in the mean number of flukes recovered as between Groups 2 and 3 but the mean number of flukes recovered from Group 1 was significantly less than from Groups 2 or 3 ($N = 12$, $t = 3.8$, $p < 0.01$). The results from Group 4, the treated control, indicated that the drug had almost eliminated the previous infections.

Eosinophils

All the rabbits showed an increase in eosinophil counts (Figs. 7.1 and 7.2) by two weeks after either the initial infection or the successive infections and in

each case the counts fell after treatment.

There was no consistent or significant difference in the response following initial or subsequent infections. In particular, the tendency for the eosinophil count to be greater following second infection seen in the previous experiment was not repeated, and there were no significant differences between the eosinophil counts following one, two or three infections.

Serum Glutamic Dehydrogenase

In all groups there was a marked increase in the serum concentrations of this enzyme (Figs. 7.3 and 7.4) by two weeks after initial infection. After treatment with the drug, the level of the enzyme fell sharply but rose again after challenge infection. In both subgroups of Group 4 the level of the enzyme fell after the last treatment and then remained low till the end of the experiment.

Post-mortem examination

The livers of all the rabbits were examined at the end of the experiment, and are shown in Plates 7.1 - 7.4.

There were a number of differences between the livers of the different groups, although these could only be subjectively assessed. Thus, as compared with Group 3 (Plate 7.3) the migration tracts were less clearly demarcated in the livers from Group 1 (Plate 7.1) and there were also more adhesions between liver and other abdominal organs and the liver was harder to cut in the

latter group. In rabbit RB 156 (Group 1, Plate 7.1) the stomach was firmly adherent to the liver. The abnormalities in the livers from Group 2 were intermediate between Groups 1 and 3.

The livers of the animals in Group 4 (Plate 7.4) were relatively normal in appearance and colouration, although there was evidence of fibrotic contractions and areas of hard tissue were observed while cutting the tissue, especially in RB 167.

Discussion

There was no significant difference between the number of flukes recovered from Groups 2 and 3 which suggested that the two doses of infective metacercariae in Group 2 had not produced any effective resistance in these animals. The significantly lower mean number of flukes recovered from the rabbits in Group 1 suggests that repeated doses of metacercariae may cause a reduction in the survival of the flukes.

The low glutamic dehydrogenase level in Group 1 correlates well with the lower number of recovered flukes in some of the animals in this group, although the differences in eosinophilia between the different groups did not show such a correlation. There was also a tendency for the G.D. levels four to five weeks post infection tofall successively from the first to the third infection. On the other hand, the equivalent G.D. levels from first infection in Groups 1, 2 or 3 and following

second infections in Groups 1 and 2 were not significantly different and when these were pooled together, it could be shown that the differences between the results following the successive infections were significant at $p < 0.05$, that between the first and third infection being significant at $p < 0.01$.

The additional evidence provided by the gross pathology of the liver tissue, and in particular the tough nature of the livers in Group 1, may suggest that the reduced number of flukes recovered from Group 1 could be explained on the purely mechanical basis, that this type of modified liver parenchyma may have been a relatively unsuitable habitat for the developing flukes. However, further investigation and, in particular, histological studies, would be needed to decide this matter.

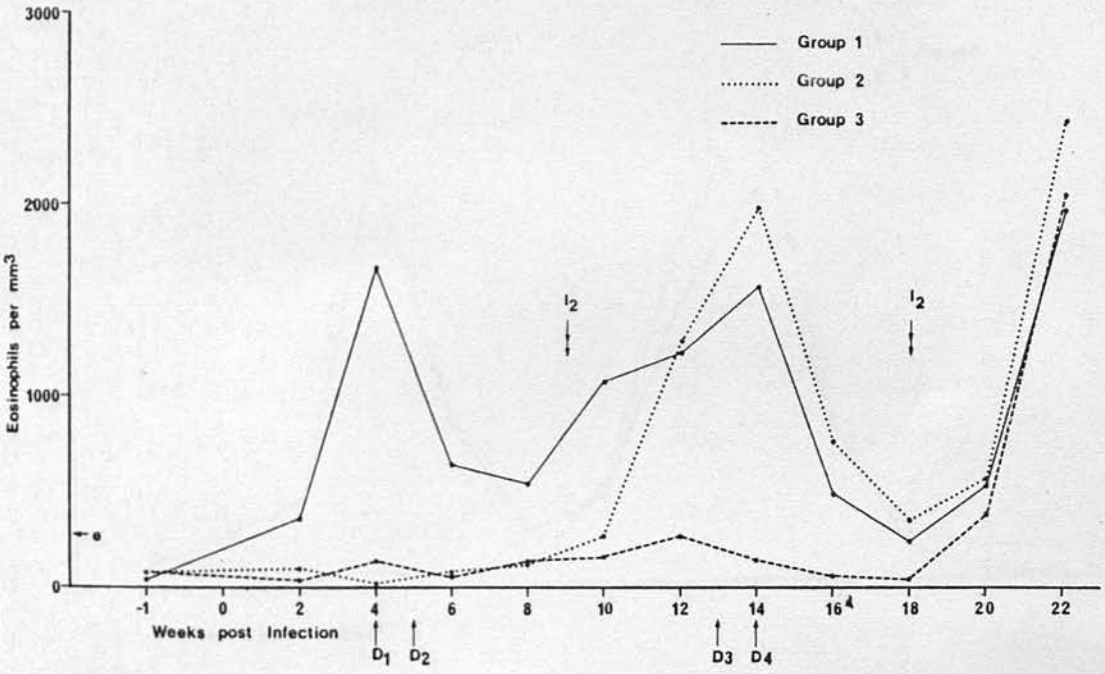


Fig. 7.1. Mean peripheral eosinophil counts.

Group 1 = challenged with three doses of 100 metacercariae at 0, 9 and 18 weeks.

Group 2 = challenged with two doses of 100 metacercariae at 9 and 18 weeks.

Group 3 = previously uninfected but challenged with 100 metacercariae at 18 weeks.

I_2 = challenge infection of 100 metacercariae.

$D_1 - D_4$ = dosing with rafoxanide at a rate of 26 mg/Kg.

— e = Mean $\pm$ 3 s.d.

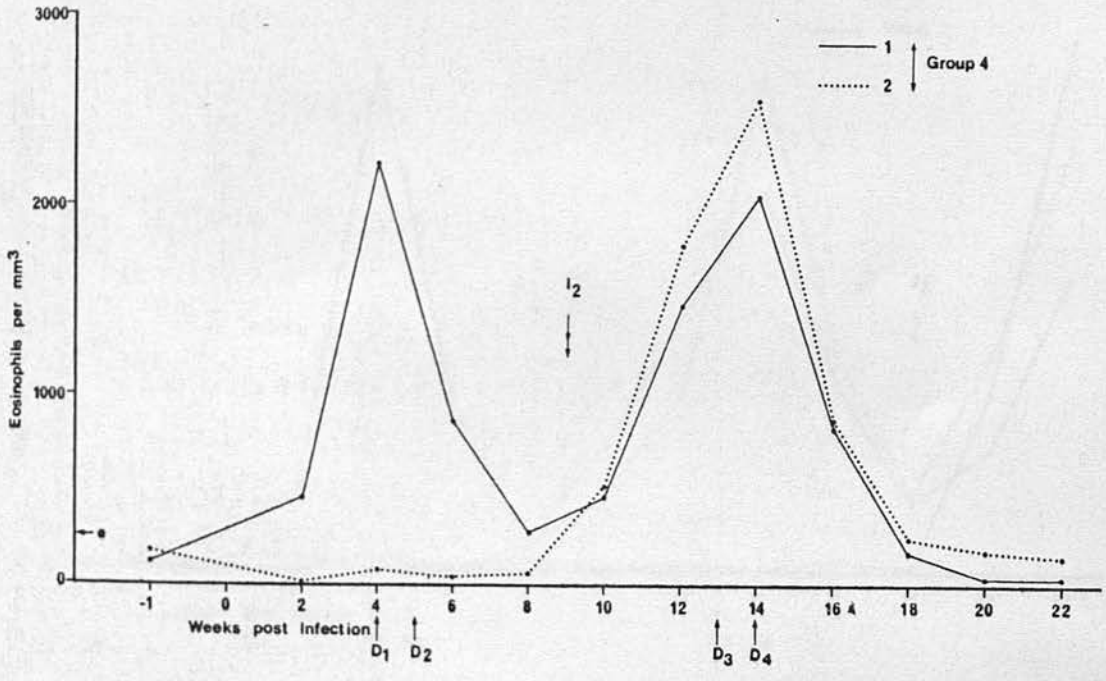


Fig. 7.2. Mean peripheral eosinophil counts.

Group 4 = treatment control group.

I_2 = challenge infection of 100 metacercariae.

$D_1 - D_4$ = dosing with rafoxanide at a rate of 26mg/Kg.

— e = Mean $\pm$ 3 s.d.

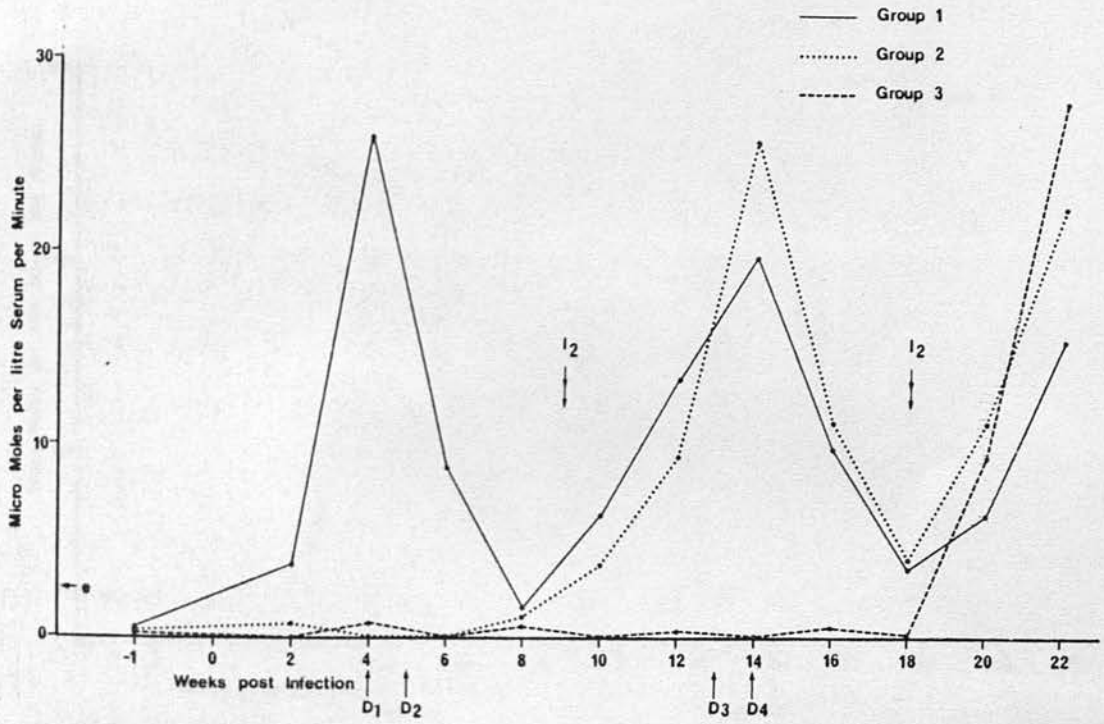


Fig. 7.3. Mean serum glutamic dehydrogenase levels.

Group 1 = challenged with three doses of 100 metacercariae at 0, 9 and 18 weeks.

Group 2 = challenged with two doses of 100 metacercariae at 9 and 18 weeks.

Group 3 = previously uninfected but challenged with 100 metacercariae at 18 weeks.

I_2 = challenge infection of 100 metacercariae.

$D_1 - D_4$ = dosing with rafoxanide at a rate of 26mg/Kg.

— e = Mean $\pm$ 3 s.d.

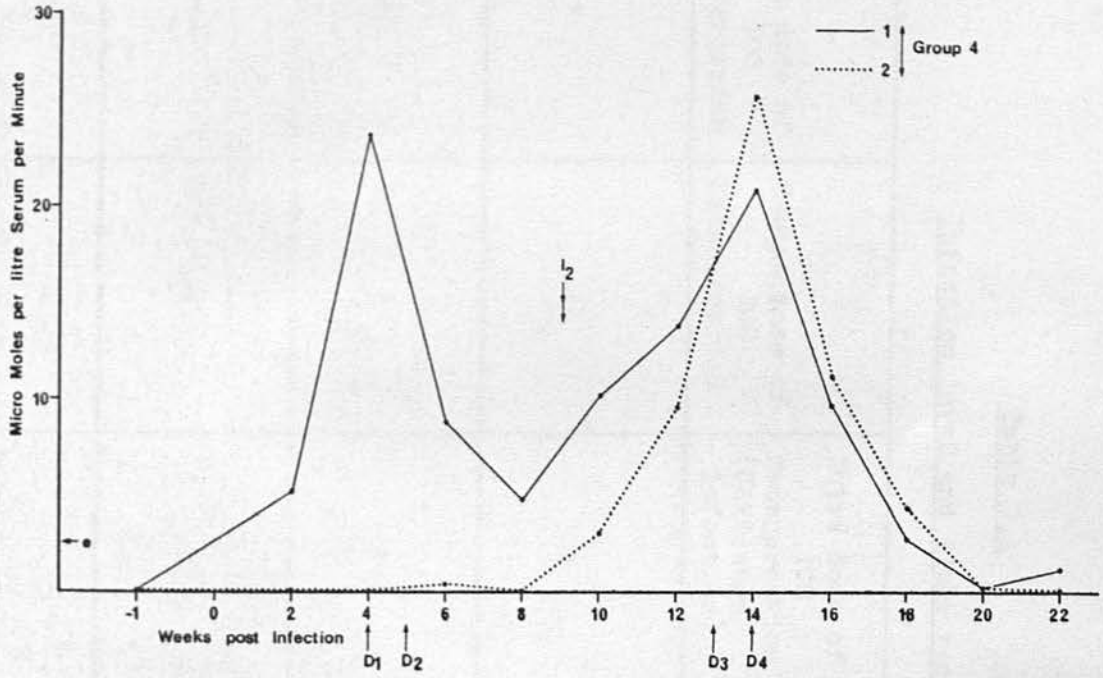


Fig. 7.4. Mean serum glutamic dehydrogenase levels.

Group 4 = treatment control group.

I_2 = challenge infection of 100 metacercariae.

$D_1 - D_4$ = dosing with rafoxanide at a rate of 26mg/Kg.

— e = Mean $\pm$ 3 s.d.

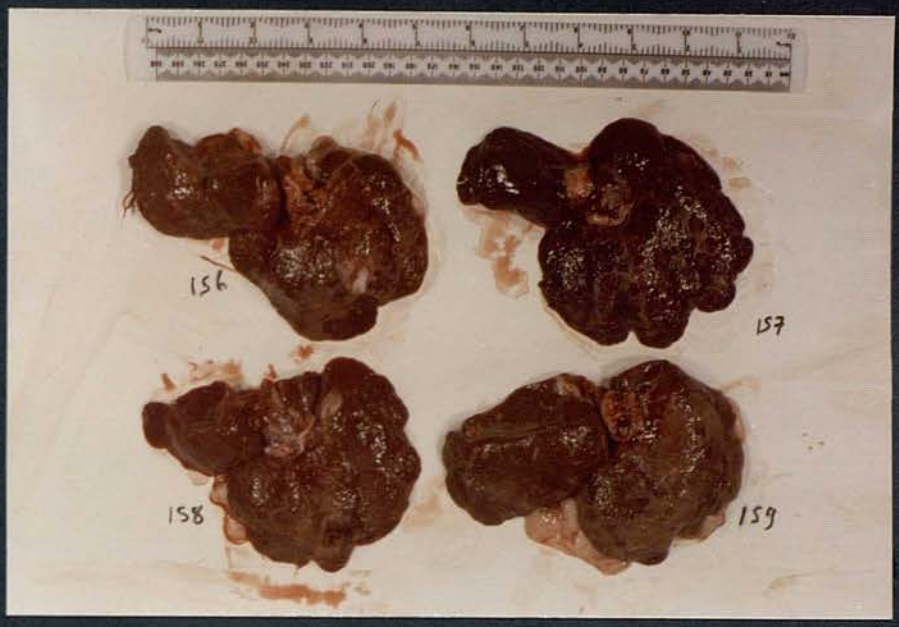
Table 7.1

Infection level and fluke recovery

Group	Rabbit Number	First dose of 100 Metacercariae	Second dose of 100 Metacercariae	Third dose of 100 Metacercariae (Challenge infection)	Number of flukes recovered at P.M.		Mean number of flukes recovered from the challenge infection
					Immature	Young	
1	RB 156	+	+	+	-	14	8.75
	RB 157				-	7	
	RB 158				-	4	
	RB 159				-	10	
2	RB 168	-	+	+	-	15	16.25
	RB 169				-	18	
	RB 170				-	18	
	RB 171				-	14	
3	RB 162	-	-	+	-	12	16.00
	RB 163				-	20	
	RB 165				-	14	
	RB 174				-	18	
4	RB 160	+	+	-	1	-	-
	RB 161				-	-	
	RB 166	-	+	-	-	-	
	RB 167				1	-	

Plate 7.1. Livers from rabbits (Group 1) infected with 100 metacercariae of F.hepatica each orally, treated with 26mg/Kg at four and five weeks after infection and chellanged with 100 metacercariae each at nine and eighteen weeks later.

Plate 7.2. Livers from rabbits (Group 2) infected with 100 metacercariae of F.hepatica each orally, treated with rafoxanide at 26mg/Kg at four and five weeks after infection and challenged with 100 metacercariae each at nine weeks later.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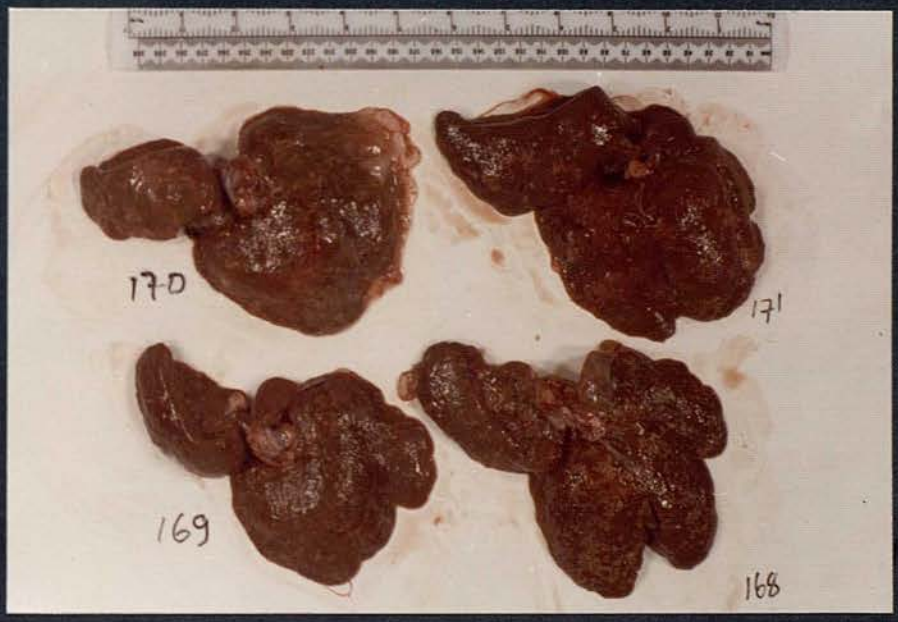
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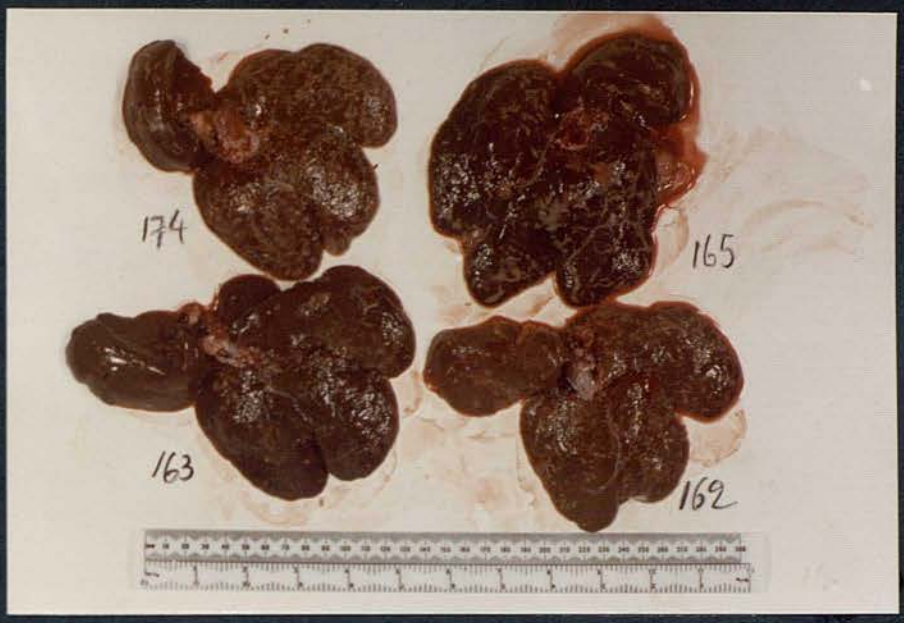
Plate 7.3. Livers from rabbits (Group 3) previously uninfected, but challenged with 100 metacercariae of F.hepatica each orally.

Plate 7.4. Livers from rabbits (Group 4), treated control group, which had received 100 metacercariae and been treated with rafoxanide four and five weeks after each infection at 26mg/Kg.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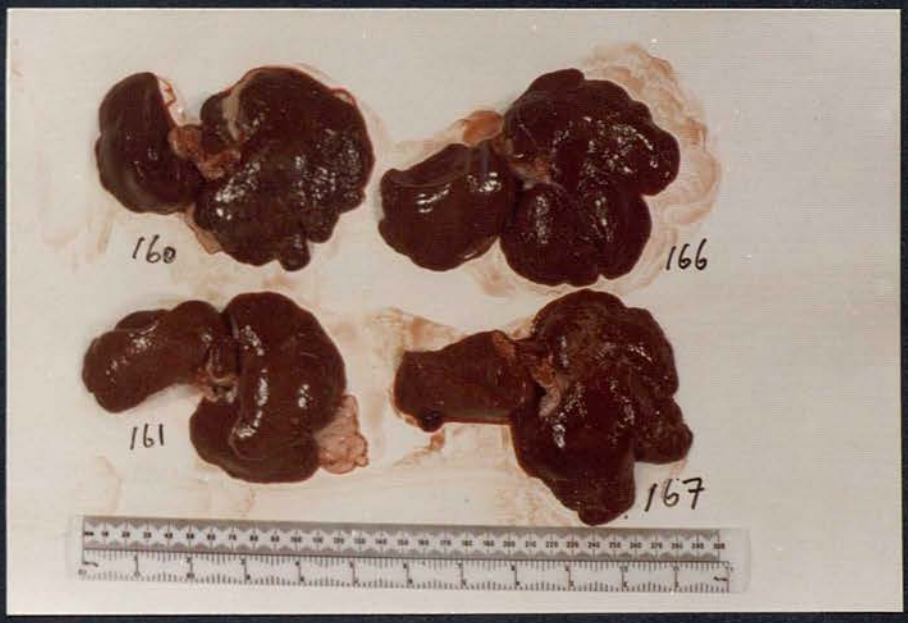
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CHAPTER EIGHT

DISCUSSION

The considerable degree of individual variation between the rabbits, taken together with the logistic difficulties which limited the number of animals used, limited the information which could be obtained from all the experiments. Nevertheless, the results have demonstrated some interesting features concerning this host's resistance to Fasciola hepatica.

Administration of Metacercariae

It was clear from the results described in Chapter 3 that the filter paper technique is a much less effective method for infecting rabbits than the gelatine capsule technique. Thus rabbits numbers (RB3, RB7, RB10 and RB12; Table 3.1) all showed a much lower recovery of flukes than RB 13 and RB 14, although the filter paper technique had previously been found to be effective in sheep (Pullan, Sewell and Hammond, 1970). It is probable that this method's inefficiency in rabbits arises from the difference in the digestive system of the two hosts, as only the ruminant is able to digest away the filter paper, so releasing the metacercariae for excystation in the small intestine.

Single infections in untreated animals

(a) Glutamic dehydrogenase assay

There are no reports on the use of the G.D. assay in rabbits infected with F.hepatica but, because of the

similarity of the effects of this parasite in rabbits and sheep (Ross, 1967b), it was decided to use this assay technique on sera from the rabbits as a means of estimating the liver damage during the course of infections with F.hepatica. The results from the preliminary study suggested that this approach was justified (Chapter 3). Thus in RB 13 and RB 14, which were infected with 1,000 metacercariae, there was a sharp rise in the serum G.D. concentration two weeks after infection (Fig. 3.2), which could be related to the high numbers of flukes feeding on the liver tissue (Table 3.1). In contrast, the low G.D. level in the group infected with 500 metacercariae by the inefficient filter paper technique (Fig. 3.2) was associated with the low take of metacercariae. A significant correlation between the numbers of flukes present in the rabbits and the serum G.D. levels was confirmed in the later studies described in Chapters 4 and 5 ($N = 14$, $r = 0.755$, $p < 0.01$).

In untreated rabbits (Group 1, Chapter 5) the greatest increase in the serum G.D. level (Fig. 5.3) was found at six weeks after infection and this probably correlates with maximum damage caused by the flukes to the liver tissue. This was followed by a decrease in the level during the following weeks, by which time the flukes were entering the bile duct. This observation agrees with that reported by Sewell (1967) who indicated that an elevated G.D. level in the serum is associated with the liver damage caused by acute fascioliasis and it was

suggested by this author that the enzyme test might be of value as an aid to prognosis during the acute stages of fascioliasis in sheep. Thus assay of the serum G.D. level in rabbits infected with F.hepatica appears to afford a means of estimating the severity of infection in this host. Sewell (1966a) also indicated that, as soon as the flukes have entered the bile duct in sheep, parenchymal repair is rapid and dysfunction is soon no longer detectable by liver function tests. In these rabbits the level of G.D. also returned to low levels soon after the flukes entered the bile duct.

(b) Eosinophil counts

In the preliminary experiment the eosinophilic response was higher in the groups infected with 1,000 metacercariae (Fig. 3.1) than in the lightly infected group, which suggests that it is also to some extent dependent on the numbers of flukes present. An increase in the numbers of circulating blood eosinophils has been shown to be a manifestation of F.hepatica infection in both sheep (Sinclair, 1962; Pullan, Sewell and Hammond, 1970) and in man (Ashton, Boardman, D'SA, Everall and Houghton, 1970; Hardman, Jones and Davies, 1970). However, in rabbits infected with different doses of metacercariae (Table 4.1), the eosinophilic response (Fig. 4.1) was not consistently related to the numbers of flukes recovered from each group. For example, the animals infected with 500 metacercariae had a mean higher count than the group infected with 1,000 metacercariae at three

weeks after infection, while at seven weeks the mean count from the group infected with 50 metacercariae was higher than that from the group infected with 500 metacercariae. Correlation analysis between the number of flukes and peak eosinophilia showed no significant differences. It therefore appears that, provided the level of infection attains a threshold value, the eosinophil response is similar regardless of the number of flukes in the host or the severity of the liver damage.

(c) Pathology

During the second study on rabbits infected with a single high level of F.hepatica metacercariae (Table 4.1) a reduced take of flukes was noticed from these animals at post-mortem examination compared with animals infected with low levels of metacercariae. This may have been due to competition between flukes in the heavily infected animals, although such competition was not observed in the earlier work in which two rabbits (RB 13 and RB 14, Chapter 3) were infected with 1,000 metacercariae each and in which both animals died from acute fascioliasis at three and four weeks after infection. Such a decrease in fluke survival in rabbits receiving increasing doses of metacercariae was not observed by Kendall (1967). These latter two rabbits appear to have died from acute fascioliasis, arising from the relatively large numbers of immature flukes in their livers and confirmed by the gross pathology which indicated severe damage to the liver (Plate 3.2).

Although it is not possible to quantify the comparison, further observations were made on the effect of the level of infection on the pathological picture of the livers from the untreated animals discussed in Chapter 4. In these animals clearer lesions were associated with the groups which had received the higher doses of metacercariae, although the pathological picture in the more heavily infected group (Chapter 4) was not comparable with the two previously heavily infected animals (RB 13 and RB 14, Chapter 3), this difference being explicable as a result of the lower numbers of flukes in the rabbits discussed in Chapter 4.

The Effect of Anthelmintics

(a) Rafoxanide

Rafoxanide clearly prevented the death of heavily infected rabbits (RB 126 and RB 116 in Chapter 5) and almost eliminated the flukes in the animals treated with the drug at four and five weeks after infection (Table 5.1), the percentage reduction in the treated group being 98% as compared with the control untreated group. There was also a rapid regression of the four week old lesions in the livers of animals treated with rafoxanide (RB 124, RB 125, RB 126 and RB 116, Chapter 5), shown at post-mortem examination by the return of the livers to their normal shape, with cut surfaces of normal consistency and normal bile ducts.

The rapid reduction in the serum enzyme levels and to a lesser extent in the eosinophilia after the

administration of rafoxanide (Figs. 5.1 and 5.3) suggested that the drug acts rapidly to destroy the parasite. In rabbits treated with rafoxanide, the occasional rise in eosinophils after the first dose (Fig. 5.1) may have been caused by the death and disintegration of the immature flukes, while the later decrease in eosinophil numbers after the second dose would arise from the fact that the regression process had already started.

This efficacy of rafoxanide was clearly confirmed in the later work concerned mainly with the demonstration of resistance of rabbits to challenge infection of metacercariae (Plates 6.4 and 7.4). This observation was in agreement with those reported by Kimura (1973) who studied the efficacy of rafoxanide in 21 rabbits given a single oral dose of 50, 75 or 100mg per Kg body weight after experimental infection with 20 Fasciola sp metacercariae each. Autopsy 24 to 77 days after infection failed to reveal any surviving worms, whereas autopsy of three similarly infected controls 39 to 62 days after infection yielded three to nine immature worms and one mature worm. Rafoxanide appears to have been even more effective when used as two doses of 26mg per Kg at four and five weeks after infection (Table 5.1) as autopsy at 65 days after infection then revealed only one surviving fluke.

The return of the livers in these animals to their normal gross appearance agrees well with the findings of Armour and Čorba (1970) on the pathology of the livers in

rafoxanide treated lambs previously infected with F.hepatica. These authors reported a rapid regression of the liver lesions in this host and showed that the drug has a good anthelmintic activity against both four and six week old F.hepatica in sheep. They therefore suggested that rafoxanide makes both the control and treatment of acute fascioliasis a distinct possibility.

The use of rafoxanide as an anthelmintic against the immature stages of F.hepatica in the comparative anthelmintic trial (Chapter 5) and in the experiments in Chapters 6 and 7 also confirmed the value of the G.D. assay as a monitor of the damage caused by these flukes. This agrees well with the observations reported by Campbell and Barry (1970) who found that another enzyme, serum glutamic transaminase (S.G.O.T.) was indicative of the liver damage caused by immature flukes in rats, being greatly elevated three weeks after infection but falling to near normal levels within one week of dosing with a known fasciolicidal drug. The fall in the enzyme S.G.O.T. values in the treated rats was considered by Campbell and Barry to indicate both the drug's efficacy and its lack of hepato-toxity, as the post-treatment values were similar to those in control uninfected rats. It thus appears to have been possible to effectively monitor the efficacy of rafoxanide on the young stages of F.hepatica in rabbits using the serum G.D. level.

(b) Nitroxynil

When nitroxynil was used against the immature stages of F.hepatica at a dose rate of 20mg per Kg body weight given at four weeks and again at five weeks after infection to rabbits infected with either 100 or 500 metacercariae (Group 4, Chapter 6), the drug reduced the numbers of flukes later recovered from the rabbits but did not prevent deaths among the animals infected with 500 metacercariae. This suggests that the drug does not have adequate efficiency against immature flukes in the severely damaged livers of rabbits infected with heavy doses of metacercariae. Even in the surviving rabbits, the action of nitroxynil against immature stages of the parasite only resulted in a reduction of 78% in the burden, as judged by the number of flukes recovered at post-mortem examination eight weeks after infection (Table 5.1).

The interpretation of the observations on the serum G.D. levels and eosinophil counts is complicated by the fact that all the young flukes in the rabbits were not killed after dosing with nitroxynil. There was an earlier reduction in the serum G.D. level (Fig. 5.3) in the group treated with nitroxynil as compared with the groups treated with diamphenethide or untreated but not to the extent of the steep fall seen in the group treated with rafoxanide, which correlates well with the relatively greater efficiency of rafoxanide as a fasciolicidal drug. This relatively slow fall in the serum G.D. level in

rabbits is consistent with the observation by Hughes, Treacher and Harness (1973) who recorded a rapid fall in the plasma sorbitol dehydrogenase level in goats after treatment with nitroxynil at 15mg per Kg body weight subcutaneously but not in the plasma G.D. level.

The results of the serum G.D. assay, taken together with the post-mortem examination of the livers of rabbits treated with nitroxynil, suggested that the use of the drug resulted in little or no recovery of the structure and function of these livers. However, there were no adult flukes in the bile ducts of these animals, only immature flukes being found in the liver parenchyma, and it would seem probable that their development had been retarded by the action of the drug. An alternative possibility is that this drug only killed those flukes which were most mature at four and five weeks after infection, sparing a small number of relatively immature and innately slowly developing individuals. However, such immature flukes were never found in the liver parenchyma of untreated animals killed at 65 days after infection. It is also probable that it was these remaining immature flukes which had continued to cause the pathological effects on the livers of these animals. The presence of these immature flukes in the liver may also have led to the irregularity in the level of peripheral eosinophils seen after treatment as compared with the group treated with rafoxanide. The eosinophil counts from the group treated with nitroxynil were only

significantly different from those from the untreated controls (pooled with the diamphenethide group) at week five ($N = 11$, $t = 2.48$, $p < 0.05$).

The death of RB 120 soon after treatment with nitroxynil added further evidence on the inefficiency of the drug in curing heavily infected rabbits. Earlier work by Lucas (1967) who tested the activity of nitroxynil in rabbits, sheep and cattle, has shown that the drug was very potent against the mature flukes in rabbits, sheep and cattle by injection but that immature flukes were less susceptible. However, Lucas indicated that the drug is useful in controlling the chronic disease and in preventing some of the deaths due to the acute disease by reducing the number of live parasites and by delaying the development of those parasites that survive the treatment, so limiting the damage caused by the developing infection to within the capacities of the host. The present observations in rabbits would tend to support these latter suggestions.

(c) Diamphenethide

The decision to use diamphenethide against the immature stages of F.hepatica was based on previous observations by other authors (Kingsbury and Rowland, 1972), who indicated that diamphenethide was 100% efficient against all liver flukes up to six weeks of age when administered orally to sheep at 100mg per Kg body weight. These authors found that the majority of adult flukes were also removed at this dose level and they noticed that the

younger immature flukes and the flukes big enough to be in the bile ducts quickly disappeared after treatment but that older immature flukes still in the liver tissue, die and become an opaque white colour. Annen, Boray and Eckert (1973) have also shown this drug to have an efficiency of between 99.9% and 100% against liver flukes aged one day to six weeks old at a dose rate of 100mg per Kg body weight. Kendall and Parfitt (1973), using the same dose rate as Annen et al (1973) found the drug to be 100% efficient against one week old infection, 99% against three, five and seven weeks old, 83% against nine weeks old and 85% against eleven week old flukes.

However, the similarity in the eosinophil counts in the serum glutamic dehydrogenase levels and in the numbers of flukes ultimately recovered from infected rabbits (Figs. 4.3 and 4.5), whether or not they have been treated with diamphenethide at 240mg per Kg body weight, suggested that the drug has no effect on the immature stages of flukes at four to five weeks after infection in this host. Furthermore the pathological picture of the treated rabbits was not distinguishable from that in the untreated rabbits. The inefficiency of diamphenethide in rabbits was confirmed using a higher dose of 500mg per Kg body weight (Group 3, Chapter 5). Even at this high dose level, the drug did not prevent deaths among heavily infected rabbits (RB 129 and RB 117, Chapter 5) and the numbers of flukes recovered from the group treated with

diamphenethide (Table 5.1) was as high as those from the control untreated rabbits at a comparable level of infection.

Multiple infections

A previous infection with F.hepatica may influence the development or survival of later infections in several ways. For example, the presence of flukes already in the rabbit may compete with the developing young flukes and result in them being retarded or destroyed. The influence of such an existing population of F.hepatica on a subsequent infection in rabbits was considered by Kendall, Hebert, Parfitt and Peirce (1967) who showed that there was an apparent decrease in the number of flukes developing from the challenge infection but considered that this had probably resulted from an "inhibition of growth and the technical difficulty of recovering very small flukes". Firstly, in the present study a significant degree of competition from flukes remaining from the previous infection in the challenged groups seems unlikely, since the existing infection had been almost entirely removed by the use of rafoxanide.

Secondly, non-specific factors, such as the mechanical effect of the fibrosis in the liver tissue caused by the successive challenge or a combination of the action of the drug on the immature stages of the parasite and cellular infiltration from the host, may be

involved. This latter suggestion would be in agreement with that made by Kendall and Sinclair (1971) who associated the destruction of the parasite in rabbits by chemotherapy with a "large but transient outpouring of antigen which evokes a delayed (cellular) hypersensitivity throughout the liver by direct combination with the reticuloendothelial cells of the liver, or by reactions with circulating antibody within the liver leading to lymphocytic infiltration." These two phenomena might then cause a non-specific allergic inflammation which is inimical to the challenge infection. Alternatively, it was suggested by these authors that the destruction of the parasites within the liver temporarily produces materials, as a result of the breakdown of fluke tissue, which are toxic to the young flukes of the second infection, although whether these would still be present some four weeks later is perhaps doubtful.

Thirdly, there may be a specific immune response by the host which is harmful to the invading young flukes or, finally, the earlier infections may have little or no effect on the flukes developing from the most recent infection.

In the present study there was no indication that a reduced number of flukes developed following the second infection in the groups of rabbits which received a single challenge dose of 100 metacercariae after an initial dose of 100 or 500 metacercariae (Table 6.1) as compared with a challenge control group. A reduced number

of flukes was recovered from the rabbits challenged after two previous infections with F.hepatica (Table 7.1) but this may well have been more a result of the mechanical barrier of fibrosis resulting from the previous infection than of an immunological host-response.

The gross post-mortem findings suggested that a second dose of metacercariae may even be more deleterious to the host than a similar initial dose (Plates 6.1 and 6.2) and the livers from the rabbits which had been infected three times (Plate 7.1) looked smaller in size than those from the other rabbits. This may have been due to fibrous tissue contraction, as indicated from the tough nature of the livers and the hard texture of their cut surfaces. The marked pathological changes in these livers as compared with those from the treatment and single infection controls is itself evidence that these rabbits reacted more strongly to the invading flukes than those which had experienced only one or no previous infections. Thus the question as to whether it was a specific immune response or the cellular changes in the liver which had resulted in the reduction in fluke numbers is to some extent unreal.

In rabbits which had received a single previous dose of metacercariae, the serum G.D. levels were also similar after challenge to those observed following an initial infection (Fig. 6.3). The reduction in the G.D. level in these rabbits four weeks after the third infection with 100 metacercariae, as compared with a similar period

after earlier infections probably occurred because of the smaller numbers of flukes developing in these animals.

The rabbits which received one, two or three doses of metacercariae all reacted on each occasion with eosinophil responses which were indistinguishable in magnitude from each other (Figs. 6.1 and 7.1). Otherwise the changes in the eosinophil counts followed much the same pattern as the serum G.D. levels, this observation being in agreement with that reported by Sinclair (1973) working in sheep. Sinclair explained these changes on the basis of a hypersensitivity reaction in the liver of the challenged sheep and referred to the eosinophilia as affording support for his hypothesis. It was also considered by Barbaro (1972) that eosinophils play a secondary role in the immediate-type hypersensitivity response and that their appearance is the consequence of an antigen-antibody reaction. Sinclair (1973) also considered delayed (cellular) hypersensitivity to be of importance because of the probable involvement of sensitized lymphocytes in the reaction of sheep to challenge infection with F.hepatica. Lymphocytic involvement in the reaction to the presence of the flukes within the liver parenchyma is rendered more probable in view of the findings referred to in the next section of this study. The possible deleterious effects of such hypersensitivity should be borne in mind in attempts to control parasitic diseases by means of worm antigen (Urquhart, Mulligan and Jennings, 1954).

Nevertheless it is clear that some constraint to the survival and development of the flukes can be elicited without the rabbits having been exposed to a biliary infection and with a period of four weeks elapsing between the removal of the previous infection and challenge, although this is less marked than has been observed by other authors working with other hosts and with other treatment and infection schedules (Armour and Dargie, 1974; Čorba and Špaldonová, 1975). These findings may be associated with the relatively high susceptibility of the rabbit to infection with F.hepatica.

Suggestions for further investigation:-

It is suggested that in future studies concerning the resistance to F.hepatica in rabbits, histological examination of the livers would be of great interest. Such a study would allow observations on the developing fibrous and cellular infiltration in the liver tissue as possibly indicating the significance of these in relation to the development of resistance to F.hepatica in rabbits.

It would also be desirable to study whether there is a similar time-related response in the development of resistance to F.hepatica in rabbits to that shown in rats by Čorba, Armour, Roberts and Urquhart, 1971; Čorba and Špaldonová, 1975.

SECTION II

STUDIES ON THE IN-VITRO RESPONSE OF LYMPHOCYTES FROM RABBITS INFECTED WITH F.HEPATICA

Chapter 9	... ...	Review of literature
Chapter 10	... ...	Materials and methods
Chapter 11	... ...	The ability of extracts of F.hepatica to stimulate rabbit lymphocytes in-vitro
Chapter 12	... ...	The in-vitro activity of the peripheral lymphocytes of rabbits following experimental infection with F.hepatica at two different levels and later treatment with fasciolocidal drugs
Chapter 13	... ...	Studies on the parameters of the in-vitro stimulation of rabbit lymphocytes by an extract of F.hepatica
Chapter 14	... ...	The in-vitro activity of the peripheral lymphocytes of rabbits following multiple infections with F.hepatica
Chapter 15	... ...	Discussion

CHAPTER NINE

REVIEW OF LITERATURE

Timofejewsky and Benewolenskaja (1928) cited by Lamvik (1966) first drew attention to the fact that mitotic activity is sometimes to be seen in cultures of human leucocytes, while modern leucocyte culture techniques began with the demonstration by Hungerford, Donnelly, Nowell and Beck (1959) and by Nowell (1960) of the *in-vitro* mitogenic effect of phytohaemagglutinin.

Carstairs (1961) was the first to show that it is the small lymphocytes that are the source of the dividing cells in cultures of human lymphocyte. Cultures of mononuclear cells, comprising 84% small lymphocytes, 13% medium and large lymphocytes, 3% granulocytes and less than 0.1% monocytes, in the presence of a mitosis stimulating agent, contained large cells of more primitive appearance than any of the original cells after two days. About 40% of the cell population showed mitosis between the fourth and fifth day, which suggested that most of the small lymphocytes have the power to divide.

Lymphocytes of several species survive comparatively well in simple media and are easily obtained. They remain in a quiescent non-dividing state unless a stimulant is added (Ling, 1968). However, it was evident from the work of Ling and Husband (1964) that a fairly large group of agents, both specific and

non-specific are capable of stimulating human lymphocytes. These include purified protein derivatives of tuberculin (P.P.D.), tetanus toxoid, Staphylococcal aureus filtrate, Escherichia coli filtrate, phytohaemagglutinin and highly purified Shigella shigae endotoxin and Salmonella enteritidis endotoxin.

Stimulation results in the morphological enlargement of small lymphocytes to form larger lymphoblasts. The term "blast-like" cell was used by MacKinny, Stohlman and Brecher (1962) to describe the transformed human lymphocyte, which has the ability to undergo mitosis and to synthesise deoxyribonucleic acid (DNA). Robbin (1964) termed this transformation process "blastogenesis".

Nowell (1960) showed that non-specific stimulants such as phytohaemagglutinin will act on lymphocytes of human origin without prior sensitization of the donor to that substance. Another non-specific stimulant "Staphylococcal filtrate" (Ling, Spicer, James and Williamson, 1965) also acts in this way.

On the other hand, Shrek (1963) and Pearmain, Lycette and Fitzgerald (1963), showed that tuberculin acts as a specific stimulant for lymphocytes from a person with a positive tuberculin reaction but not from a non-reacting individual.

Initial attempts in animals by Lycette and Pearmain (1963) using guinea-pig lymphocyte cultures failed to

provide convincing evidence that phytohaemagglutinin can induce blastoid transformation, because of clot formation in the culture bottle and difficulties in leucocyte separation. The same difficulties were reported by Marshall and Roberts (1963), who failed to stimulate leucocytes from guinea-pigs, rabbits, mice or rats with phytohaemagglutinin and who also found that phytohaemagglutinin agglutinated the red cells of all these animals. However Knight, Ling, Sell and Oxnard (1965) using non-specific stimulants, mainly phytohaemagglutinin and staphylococcal filtrate, in cultures of peripheral lymphocytes of monkeys, rabbits, guinea-pigs, hamsters and rats, showed that lymphocytes of these different animals were transformed, although, in mice, occasional transformed cells were regularly found amongst the cell debris after 24 and 36 hours in cultures, but not after longer periods of culture, since it was observed that the erythrocyte of mouse culture underwent lysis after short periods. Large blast-like cells with prominent nucleoli and basophilic cytoplasm were seen in stimulated cultures of rabbit lymphocytes.

All lymphocytes, whether they were derived from blood, lymph nodes or spleen were affected by the non-specific stimulants and this was considered to be a useful mitogen for the study of cell transformation and activation.

The separation of lymphocytes from other blood

constituents presented many difficulties but Jago (1956) described a method which involves a combination of sedimentation and centrifugation. This method required the addition of no chemical reagents other than heparin for separating living lymphocytes from granulocytes in normal human blood with a minimum of erythrocyte contamination. However Knight et al (1965) found that a defibrination-gelatin technique, based on that of Coulson and Chalmers (1963), was satisfactory for the separation of viable lymphocytes from rabbit blood. They considered that the heparin-dextran procedure used by Ling et al (1965) for human blood was not satisfactory for rabbit or guinea-pig blood since the heparinised plasma of these animals clotted after a short period of incubation with culture media.

This lymphocyte activation process was considered by Oppenheim (1964) to be an essential part of the delayed hypersensitivity response. In his study on the in-vitro response of lymphocytes from guinea-pigs following immunization with various antigens, he used cell suspensions of lymph nodes draining the site of injection, distant lymph nodes, spleen and peripheral blood. He found that lymphocytes from the lymph nodes draining the site responded in-vitro to the antigens, as determined by their uptake of tritiated thymidine, before the other lymphocytes and before the skin test became positive. He therefore suggested that the

lymphocyte transformation test may be a more sensitive technique for the detection of delayed hypersensitivity than the skin test.

The lymphocyte transformation technique has been used by many authors as a parameter for cell-mediated immune response in different infections. For instance, Roberts (1973) studied the cell-mediated immune response to Mycoplasma hypopneumoniae in pigs, using the in-vitro transformation of sensitized lymphocytes by a specific antigen prepared from this organism. He showed a statistically significant increase in activity in the presence of this antigen as indicated by the uptake of tritiated thymidine. The technique was also used for the study of cellular immune responses to vaccinia virus and Herpes simplex virus (Rosenberg, Farber and Notkins, 1972). Lymphocytes from rabbits immunized with these viruses showed increased activity as compared with cells from unimmunized animals when incubated with an antigen prepared from the virus.

When rabbits were immunized against purified protein, tissue extracts or sheep erythrocytes, with or without Freund's adjuvant, skin reactions of delayed type only developed in animals given antigen together with the adjuvant although all rabbits developed serum antibodies and most of them developed Arthus type reactions (Benezra, Gery and Davies, 1969). These authors also showed that lymphocytes of all the immunized animals

showed similar transformation activity when cultured with the immunizing antigen, whether they showed delayed type hypersensitivity or not. In this work the cultured cells were obtained by heart puncture, three to six weeks after immunization and were stimulated by 100mg of the immunizing protein or tissue antigens or by 0.1 ml of 2% sheep red blood cells. The degree of response was determined after five days incubation by quantifying both the percentage of transformed lymphocytes and the uptake of tritiated thymidine.

These authors therefore concluded that the blast transformation phenomenon correlated with both cellular and humoral immune response and not with delayed type hypersensitivity alone. Similar results had been obtained by Loewi, Temple and Vischer (1968), who showed that lymphocytes taken either from a guinea-pig which had been immunized with sheep erythrocytes in Freund's adjuvant and which showed strong delayed hypersensitivity or from animals immunized intravenously with sheep erythrocytes, which failed to show delayed hypersensitivity reactions, both responded in-vitro in the presence of sheep erythrocytes, as indicated by significant incorporation of tritiated thymidine.

Krahenbuhl, Gaines and Remington (1972) who studied infection in man with Toxoplasma gondii, using an antigen prepared from trophozoites of the RH strain of Toxoplasma. They showed that cells from persons with

serological evidence of the disease transform more frequently than lymphocytes from serologically negative individuals.

There have been relatively few reports of this technique being used with antigens derived from metazoan parasites, although the test has recently been used by Dobson and Soulsby (1974), who studied the kinetics of the lymphocyte transformation reaction in guinea-pigs infected with Trichostrongylus colubriformis. The uptake of tritiated thymidine in-vitro was used as an index of lymphoid cell activity. They used blood from infected and normal guinea-pigs and the antigen was prepared as an extract of the adult worms in 0.1M Tris-HCL, pH 8.2. This extract was brought to a final protein concentration of 0.6 mg/ml with sterile Eagle's minimal essential medium (MEM), plus 20% faetal calf serum. Cultures were made using phytohaemagglutinin, worm antigen and a negative control with MEM. The rate of transformation was determined after three days in cultures containing phytohaemagglutinin and after five days in cultures containing worm antigen or the negative control cultures. It was found that the greatest response to the worm antigen occurred on the 15th and 25th day after infection. They also reported some spontaneous transformation in unstimulated cultures from both normal and infected guinea-pigs.

Lymphocyte transformation and production of the

migration inhibitory factor (MIF) have been used by Warren (1974) as an in-vitro correlate of delayed hypersensitivity in Schistosomiasis mansoni. It was suggested that the onset of egg granulomas of S.mansoni in the guinea-pigs coincided with delayed skin reactivity, lymphocyte transformation and MIF.

Furthermore, it was found by Chen and Dean (1974) that peripheral blood lymphocytes and oil induced peritoneal mononuclear cells from guinea-pigs infected with S.mansoni, respond very strongly to antigens prepared from different stages of the parasite. They used blastogenesis and the production of MIF and chemotacic factor in response to these antigens as parameters indicating the response. They showed that the responses due to antigens reached a peak between four to eight weeks after infection.

The lymphocyte transformation test was used by Dobson (1974) as a parameter for measuring cell-mediated immunity in rodents infected with ascaroid nematodes. Heparinized whole blood cultures were used with antigens prepared from adult Toxocari canis and Ascaris suum by homogenising whole worms in saline. The first response by the lymphocytes was observed after five days and a peak was reached by the tenth day after infection in both cases.

In-vitro assessment of blastogenesis and rosette formation techniques were used by Khoury and Soulsby

(1974) for the demonstration of the local immune response in groups of normal and previously immunized guinea-pigs infected with Ascaris suum. Each group was infected with different stages of the parasite by different routes. They cultured lymphocytes from different lymph nodes for four days with antigen prepared from Ascaris suum, and studied their blastogenic activity after pulsed with tritiated thymidine and processed for liquid scintillator spectrometry. They showed a peak response at the time when the parasite was migrating through the tissue and the response was much higher in the previously infected immune animals.

The sensitivity of lymphocytes in experimentally immunized rabbits after single and repeated infections with Strongyloides papillosus has been investigated after Geyer, Manteuffel, Schmidt and Havemann (1974). They used the macrophage migration inhibition assay for this purpose. The antigens used were prepared from third stage larvae and adult female worms, also antigen prepared from intestinal/faecal bacteria of rabbits. The peritoneal exudate cells were obtained from these animals as a source of macrophages, also macrophages from control rabbits were used. In the control rabbits, maximum migration was obtained in the presence of third stage larval antigen, adult worm antigen and control saline medium. On the contrary, macrophages

from immunized animals showed a significant inhibition in the presence of adult worms and intestinal/faecal bacterial antigen, while in the presence of larval antigen, little inhibition or absence of inhibition was demonstrated. Furthermore, it was suggested by these authors that the adult parasite played an important role in the immune response.

In liver fluke disease an in-vitro test based on the inhibition of leucocyte migration in the presence of liver fluke antigen has been used by Aalund, Nielsen and Ericksen (1972). They used this to study the delayed hypersensitivity response of leucocytes obtained from peripheral blood and from the hepatic and prescapular lymph nodes of sheep and goats infected with F.hepatica. The technique used in their study was based on the leucocyte migration test (Aalund, Hoerlein and Adler, 1970). The antigen used was an extract of adult fluke with phosphate buffered saline. Four different antigen levels were tested by applying one, two, three or four drops respectively to one ml of diluent into the migration chamber and positive results were indicated by the inhibition of migration of leucocytes.

Furthermore, an indirect macrophage migration inhibition test was used by Renz (1972) to test the cellular response of 19 rabbits infected with Fasciola hepatica. The macrophages were derived from guinea-pigs, while the antigen used was a supernatant or

sediment of homogenised parasites. He indicated that lymphocytes of 15 rabbits produced migration inhibition factor (MIF), in the presence of supernatant but not with the sediment. Leucocyte migration inhibition was also used by Genchi, Locatelli and Sartorelli (1973) to detect the immune response in cattle infected with F.hepatica. They considered the test as positive in 71 out of 80 infected cattle, while 40 uninfected cattle proved negative.

CHAPTER TEN

MATERIALS AND METHODS

Removal of relatively large volumes of blood from rabbits

1. Cardiac puncture

The rabbit was gently manually controlled. A sterile solution of pheno-barbitone (Nembutal, Abbot Laboratories Limited, Queensborough, Kent) was injected intravenously into the ear vein at a dose rate of 0.4 ml per Kg body weight. Cardiac puncture was then carried out on the anaesthesized rabbit using an 18g, 37mm long needle (Yale Microlance, Plastipak, Becton, Dickinson U.K. Limited, Wembley, Middlesex) attached to a sterile 50 ml polypropylene syringe obtained from the same company. The site of the injection was cleaned with 70% alcohol, the needle being inserted through the third intercostal space directly into the heart and 30 - 40 ml of blood slowly withdrawn.

2. Syringe bleeding

By this method about 10 - 20 ml of blood could easily be obtained from the peripheral vein. The rabbit was controlled in a wooden box 240 mm long, 215 mm high, with a 55 mm diameter hole for the rabbit's head. The ear was prepared as before and the vein entered with a 20g needle, (Yale Microlance), attached to a 10 or 20 ml sterile polypropylene disposable syringe. It was much easier to collect the blood if the needle was inserted against the venous flow and the blood

flowed more easily if a slight pressure was applied to the vein proximally to the insertion site.

3. Needle puncture

It was ultimately found however that the needle puncture method described in the previous section was easier and the blood obtained appeared to be no less sterile than when the above two techniques were used, even when 10 - 20 ml of blood was required. This technique also appeared to be generally less upsetting to the rabbits.

Defibrination

1. Manual

Sterile universal bottles containing about six glass beads of 5mm diameter were used. About five to eight ml of blood was placed in each bottle and the bottles were tightly capped and gently shaken up and down for about five minutes until a tight clot had formed around the glass beads. This also served to remove most of the granulocytes, while the lymphocytes remained free in the blood.

2. Mechanical

A machine (Plate 10.1.) which could slowly rotate universal bottles about their short axis was made in the Field Station work shop. When using this machine about eight glass beads were put in each universal bottle with 10 - 15 ml of blood and the bottle rotated at about 60 r.p.m. The major advantage of this machine

over the manual method is that one person can then carry out the bleeding and defibrination procedure without assistance.

Methods for separating lymphocytes

1. The Gelatine Method

This was carried out in the same way as described by Knight, Ling, Sell and Oxnard (1965). Defibrinated blood from each animal was placed into separate sterile universal bottles. To each volume of blood an equal volume of molten, sterile 3% gelatine (Sigma Chemical Company, Baltimore, U.S.A.) was added. This was prepared by dissolving 3g of gelatine in 100 ml of saline in a water bath at 45°C. The solution was then filtered through a 25mm Millipore GS (0.22μ pore size) filter in a Swinnex filter holder (Millipore (U.K.) Limited, London) into a sterile universal bottle. The gelatine solution was poured slowly into the bottle containing the blood, with gentle mixing. The blood-gelatine mixture was then transferred into another sterile universal bottle and kept at 37°C for 40 minutes to one hour, by which time the mixture had separated into a supernatant clear layer, which contained many lymphocytes and a lower layer which contained mainly red cells. The supernatant clear layer was then aspirated with a sterile pasteur pipette into another sterile universal bottle and centrifuged at 500 R.C.F. for ten minutes with the temperature of the centrifuge adjusted to 20°C. After centrifugation,

most of the supernatant was pipetted off and the sediment containing the lymphocytes was resuspended in one ml of the supernatant. The number of lymphocytes per mm^3 of the mixture was determined using an electronic cell counter (Model F.N., Coulter Electronics, South Dunstable, Bedfordshire).

2. Ficoll-Triosil Method

This was carried out in the same way as described by Harris and Ukoejiofo (1970). The technique was tried when a relatively pure suspension of lymphocytes was needed during the early work of establishing the in-vitro lymphocyte culture technique. For this purpose defibrinated blood was obtained as described above. One volume of this blood was mixed with three volumes of phosphate buffered saline pH 7.3 in a sterile test tube. One ml of the diluted blood was carefully layered down the side of a sterile test tube (100 x 10mm) containing two ml of a mixture of Ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden) and Triosil (Glaxo Laboratories, Greenford, Essex) using a one ml sterile plastic syringe (Becton, Dickinson U.K. Limited, Wembley, Middlesex). This Ficoll-Triosil mixture was composed of 24 parts of 9% Ficoll plus ten parts of 34% Triosil. The tubes containing the layered blood were then centrifuged at 500 R.C.F. for 20 minutes.

Four distinct layers were easily recognized after centrifugation. There was an upper clear plasma layer,

underneath which was a white layer composed mainly of lymphocytes and just below this layer was a third layer which was formed of Ficoll Triosil mixture. At the bottom of the test tube there was a thin layer of red cells. The lymphocytes could be aspirated off using a sterile pasteur pipette into another sterile tube. This was mixed with two ml of phosphate buffered saline and then recentrifuged at 1,000 R.C.F. for ten minutes. The supernatant was then pipetted off and the bottom cell layer was resuspended in one ml of culture medium (TC - 199). The concentration of lymphocytes per mm^3 was then determined using the electronic cell counter.

Culture Media

1. TC 199 medium (Wellcome Reagents Limited, Beckenham, Kent) was used during the early work of developing the technique, enriched with either 10% calf serum (Gibco Biocult, Glasgow), or with 10% autologous plasma.

2. Minimal Eagle's Medium (MEM). This medium, without L-glutamine, was obtained from Gibco Biocult and, just before setting up the cultures, L-glutamine (Gibco Biocult) was added to a final concentration of 2mM and penicillin-streptomycin (Gibco Biocult) at a concentration of 5,000 units and 5,000 gm per 100 ml respectively was added to the culture media.

Culture Stimulants

1. Staphylococcal filtrate (SF)

Staphylococci of canine origin (Strain M711/73)

were obtained from the Bacteriology Department, Royal (Dick) School of Veterinary Studies, University of Edinburgh, and staphylococci of human origin (Strain UMB) were obtained from the Medical School, University of Edinburgh. Staphylococcal extracts were prepared by the method described by Ling (1968). The extracts were filtered using an 0.45 μ pore size Millipore membrane.

The efficiency of staphylococci of canine origin as a stimulant for lymphocytes was compared with staphylococci of human origin (UMB). The latter gave clearly higher counts, the mean ($\pm$ standard deviation) for six replicates being 6883 $\pm$ 2581, compared with 198 $\pm$ 75, these differences being very highly significant ($F = 1172.75$, $t = 8.75$, $p < 0.001$).

2. Fasciola hepatica antigen (FA) for lymphocyte stimulation studies

Fasciola hepatica were collected from naturally infected sheep at Glasgow abattoir. The flukes were briefly washed in tap water to remove any mucous and, after a further wash in physiological saline, they were placed in a dry, clean petri-dish. They were then cut into very small pieces with a scalpel and placed in a 100 ml volumetric cylinder to estimate the wet volume. The macerated flukes were then poured into a kilner jar and five volumes of culture media (MEM) were added. The mixture was homogenised using a heavy duty laboratory

emulsifier (Silverson Machines Limited, Chesham, Buckinghamshire) and extracted overnight at 4°C. The extracted homogenate was then centrifuged at 2,500 - 3,000 r.p.m. for one hour. The supernatant was collected and stored in 25 ml universal bottles at -20°C until required. The homogenate was filtered through a 0.45μ pore size Millipore membrane.

Cleaning and sterilization of equipment

All the glassware was first rinsed in tap water to get rid of any gross contamination. It was then soaked in 2% Decon 90 (Dikon Laboratories Limited, Brighton) for three to four days. The culture tubes were then washed with hot running water about six times to get rid of any traces of the detergent and finally rinsed twice with distilled water. They were then dried in a hot oven.

The aluminium caps (Oxoid Limited, London) which were used to cover the culture tubes were washed by being soaked in hot water, washed in distilled water and dried in a hot oven. Millipore 25mm Swinnex filters (Millipore (U.K.) Limited, London) were dismantled and the filter membrane removed and discarded. The filters were then washed in the same way as the glassware.

Sterilization procedure

All solutions and glassware, except for the culture

tubes, were sterilized by autoclaving at 1 Kg/cm^2 for 20 minutes in a portable autoclave (Griffin and George Limited, East Kilbride, Glasgow) and Millipore filters were similarly sterilized after being assembled together with a GS membrane, wrapped in aluminium foil and sealed with indicating autoclave tape (Medical Products Company, Loughborough, Leicestershire). Culture tubes and aluminium caps were sterilized using dry heat at 160°C for two hours.

Methods for culturing lymphocytes

1. Culture of separated lymphocytes in bijou bottles

The tissue culture medium (MEM) was enriched with 10% of the autologous plasma-gelatine mixture and added to aliquots of the lymphocyte suspension in universal bottles so as to give the desired final concentration of lymphocytes. The stimulants were added as required. The lymphocytes were carefully suspended in the culture fluid and two ml aliquots of this suspension were transferred into a bijou bottle using five ml sterile disposable syringes (Becton, Dickinson U.K. Limited, Wembley, Middlesex). The bottles were then gassed in a 30 cm diameter dessicator (Gallenkamp Limited, East Kilbride, Glasgow) with 5% CO_2 in air (British Oxygen Company Limited, Edinburgh) for about two minutes. The lid was sealed with silicone grease (Edward, High Vacuum Limited, Royal Crawley, Sussex) and incubated at 37°C

for two days, after which 1/$^{\mu c}$ tritiated thymidine (Radiochemical Centre, Amersham, Buckinghamshire) was added to each bijou bottle. The gassing was repeated and the dessicator was kept at 37^{o}C for a further 24 hours. The contents of each bijou bottle were suspended and poured into a separate sterile culture tube (75mm x 12mm) for further processing.

However, it was found that this technique was time consuming, the amount of culture media used was relatively large and the space available in the dessicator was too limited when large numbers of cultures were needed. Accordingly a more economical and convenient technique was developed.

2. Culture of separate lymphocytes in test tubes

This technique was similar to that described above using bijou bottles with the following modifications.

A McIntosh and Fyldes anaerobic jar (Baird and Tatlock Limited, Chadwell Heath, Essex) was used instead of the dessicator. This was modified to hold 60 culture tubes (75mm x 12mm o.d.) in cylindrical racks which would fit inside these jars. These racks were made from aluminium in the Field Station workshop. Each rack (Plates 10.2 and 10.3) holds 30 culture tubes and two racks will fit inside each jar. The modifications to the jars (Plate 10.4) were as follows:-

(a) The chemical indicator which is normally present in the capsule on the side-arm was replaced by an

aliquot of the culture medium containing phenol red indicator to assist in assessing the adequacy of the gassing.

(b) The catalyst provided inside the jar was removed.

(c) A piece of polythene tube, which would pass down to the bottom of the jar was fitted to the inside of the inlet valve, to ensure that the gas would flush out the air inside the whole jar.

(d) 5% CO_2 in air was passed into the jar through the inlet valve for two minutes, while the outlet valve was kept open. If the lid of the jar was not tight enough on the rubber sealing or the inside polythene tube was blocked, this could be detected as gas stopped issuing throughout the outlet valve.

This technique was found to be much easier and quicker during the preparation of the cultures and the amount of lymphocyte suspension in culture media used per culture tube was reduced to one ml per culture as compared with two ml in the bijou bottle technique. During the culturing procedure the culture tubes were covered with different coloured aluminium caps (Oxoid Limited, London) so that a simple colour code could be used to show the treatments of the lymphocytes. This cap can also be easily removed, to allow the insertion of the stimulant or thymidine using syringes as described for the bijou bottle technique. Furthermore it was

found that chance contamination was reduced as compared with the former method.

The efficiency of bijou bottle and culture tube methods for culturing the lymphocytes was compared when these cells had been stimulated by SF. The latter gave clearly higher counts, the mean ($\pm$ standard deviation) for six replicates being 13,177 $\pm$ 2,971 compared with 4,110 $\pm$ 1,103, these differences being very highly significant ($F = 7.25$, $t = 7.71$, $p < 0.01$).

3. Cultures of lymphocytes in whole blood

Defibrinated blood was collected as described earlier. The number of leucocytes per mm^3 was directly estimated using the electronic cell counter. In defibrinated blood almost all the leucocytes are lymphocytes (Ling, 1968). The desired amount of blood was then transferred into another universal bottle containing culture media. Different universal bottles were used as required in the experiment, each with a known number of lymphocytes, with different mitogens or without any mitogens. The lymphocyte-culture mixtures were then transferred into culture tubes in one ml aliquots and cultured in the anaerobic jar as described above. It was found that this technique is preferable to either of the techniques using separated lymphocytes, as it is more economical in terms of both material and time and also tends to give somewhat higher counts. The volume of blood that was needed from each rabbit was also much less than when using separated lymphocytes.

In a comparison of the gelatine separation method and the whole blood technique using six replicates in each case, the latter gave higher mean counts. The mean ($\pm$ standard deviation) being 13,177 $\pm$ 2,971 and 17,289 $\pm$ 3,865 respectively. However, this difference was not quite significant ($F = 1.7$, $t = 2.1$, $p<0.1$).

4. Spleen cell culture

The spleen was removed aseptically and freed from its surrounding fat. About a quarter of the organ was then macerated in sterile culture medium (MEM). The macerate was sieved through a fine sterile stainless steel mesh (100/^{4} pores) to remove the large particles and the material passing through the mesh, which contained the cells, was transferred into a sterile universal bottle. The number of splenic cells per mm^3 was determined using the electronic cell counter. The cells were cultured in bijou bottles as described above, except that the lymphocyte culture media was enriched with 10% autologous serum.

Staining with Giemsa

Air-dried smears of the cultured cells were fixed in methanol for one minute and then stained with Giemsa stain (Gurr, B.D.H. Chemicals Limited, Poole, Dorset) in buffered saline at pH 6.8 at a dilution of 1:20 for 20 minutes. The smears were then washed in distilled water for a few seconds and examined under the microscope for differentiation and rewashed if necessary. Plate 10.7 showed stimulated lymphocytes from an uninfected rabbit, obtained using the SF (UMB) as a stimulant, with unstimulated cultured lymphocytes in Plate 10.8 for comparison.

Preparation of cultured lymphocytes for counting

The tubes containing lymphocytes were centrifuged at 500 R.C.F. for ten minutes. Most of the supernatant was then decanted from each tube and the sedimented cells resuspended in the residual supernatant (about two drops). The erythrocytes were haemolysed by adding 1.5 - 2 ml one per cent acetic acid in saline to each culture tube when using separated lymphocytes or spleen derived cells, while three to four ml were added when using whole blood cultures and the tubes again centrifuged at 500 R.C.F. for ten minutes. The supernatant containing acetic acid was again decanted off and the sediment resuspended in two ml of physiological saline. About 0.5 ml of 5% trichloroacetic acid was added to each tube and the tubes were left in a refrigerator for at least two hours to allow the precipitate to form.

The lymphocytes which had been thus treated with trichloroacetic acid were trapped by pouring the resuspended contents of each tube on to a separate glass fibre disc (Type GF/A, 21mm diameter, Whatman Biochemicals Limited, Maidstone, Kent).

This disc was mounted above a sintered polythene disc in a filter block (Plate 10.9) which was attached to a water-vacuum pump, so as to allow the removal of the fluid portion of the suspension, while the particulate matter remained on the glass fibre disc. Further washing with about one ml of methyl alcohol was carried

out using a pasteur pipette so as to remove the last traces of the culture fluid and also to help in removing any colouration from the blood sticking to the disc and in fixing the solid particles to the glass fibre disc. Each disc was removed carefully with a fine pair of forceps and placed on to filter paper to allow the alcohol to evaporate off. The discs were finally dried at 40-60°C for about half an hour and each disc was transferred into a separate scintillator vial, containing 10 ml of scintillation fluid, for counting.

Counting Procedure

The amount of tritiated thymidine incorporated in the cultured lymphocytes was estimated using a scintillation counter. (The Corumatic-200 System, I.C.N. Pharmaceutical (U.K.) Limited, Tracer Labs Instrument Division, Horsham, Surrey).

A toluene based scintillator fluid was used with two standard phosphors, the primary phosphor being 2,4 - diphenyloxazole (PPO) (Nuclear Enterprises Limited, Sighthill, Edinburgh) at 0.5% and the secondary phosphor 1,4 - bis - (5 - phenoxyazole - 2 - yl) benzene (POPOP) (Nuclear Enterprises Limited) at 0.03%. The scintillator fluid was added in 10 ml aliquots to the scintillator vials (I.C.N. Pharmaceutical (U.K.) Limited). The glass fibre discs bearing the washed cell debris were then placed in separate vials. Known blanks and

positive controls were included on each occasion. The activity in the vials was then counted using the settings shown in Tables 10.1 and 10.2.

TABLE 10.1

(Sample Changer)

Sample Changer	External Standard	Programme	Cycle per Sample
Continuous	off	one	one

TABLE 10.2

(Sample counting)

Mode I	Back-ground	Coarse Gain	Fine Gain	Window	Threshold
Contin-uous	0	16	1.2	in	25

Plate 10.1. A rotatory machine for defibrinating small volumes of whole blood.

Plate 10.2. The separated aluminium racks, tubes and caps used for lymphocyte cultures in anaerobic jars, showing the pattern of the holes and culture tubes.

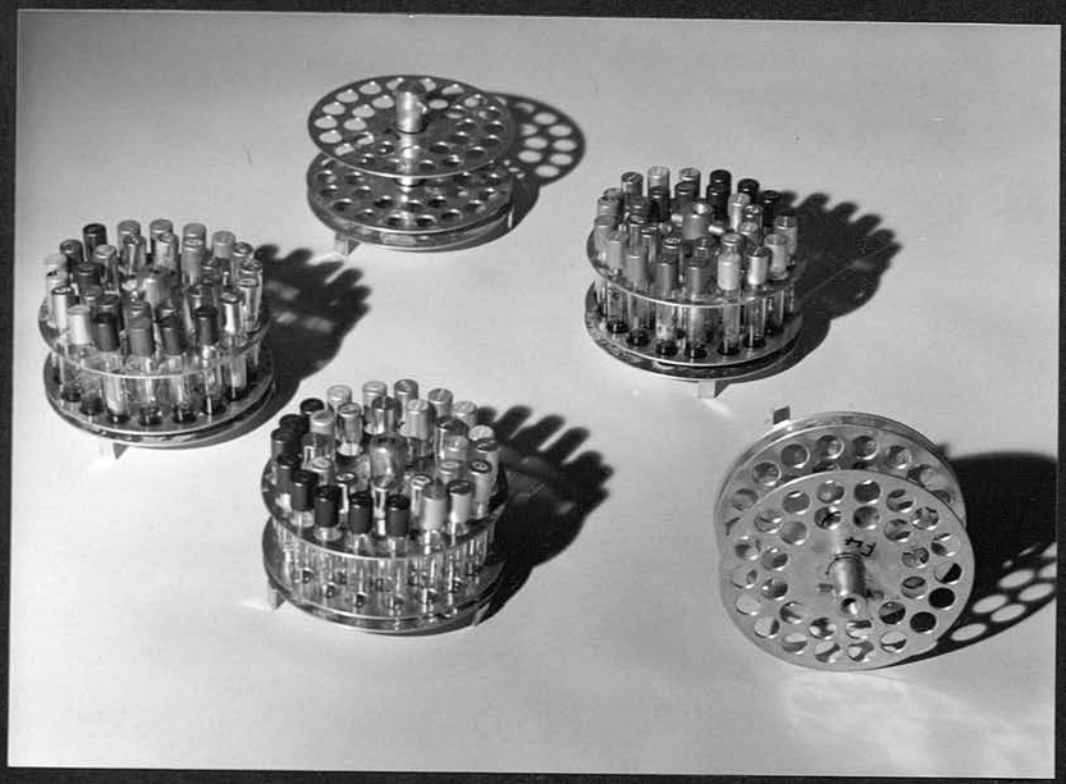
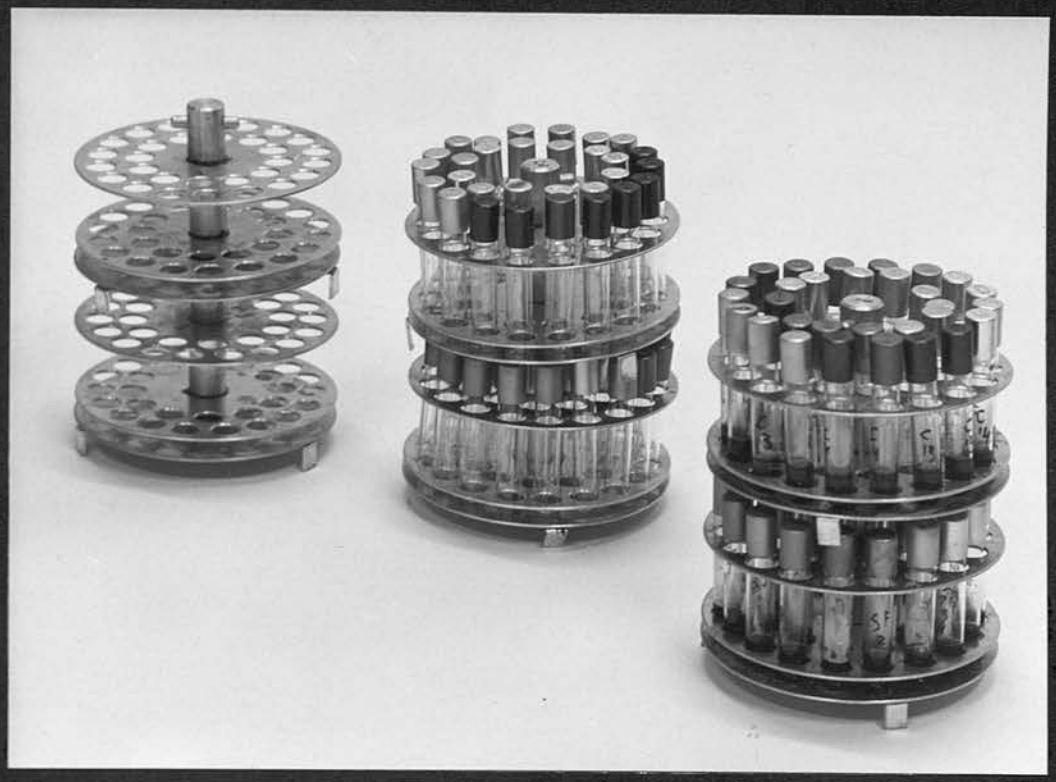


Plate 10.3. The two parts of aluminium racks attached to each other, with (from left to right) no culture tubes, empty culture tubes and culture tubes containing 1 ml with MEM.

Plate 10.4. McIntosh and Fyldes jar, showing the length of tubing which was attached to the inlet valve, and the absence of the catalyst. Also a closed jar, showing the inlet and outlet valve.

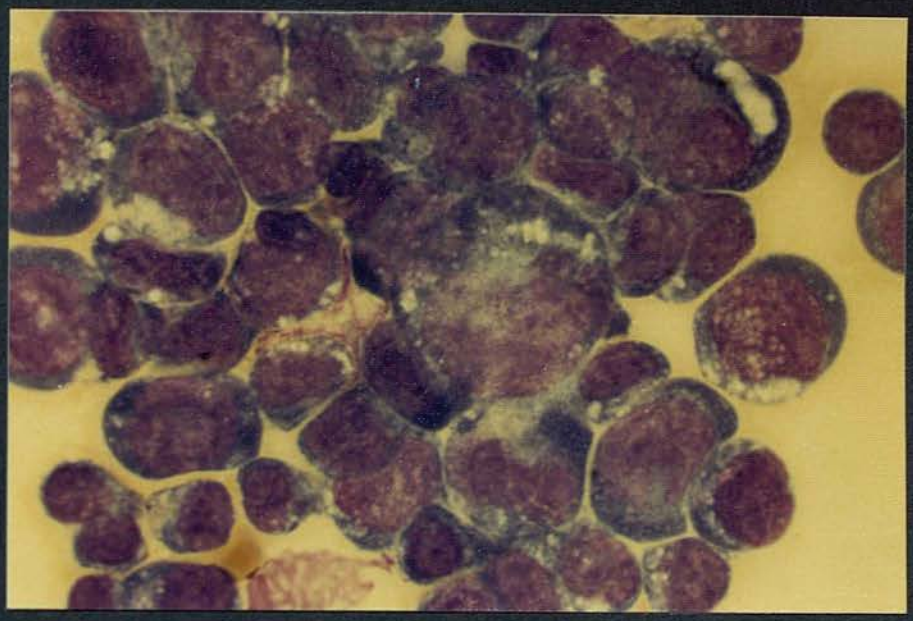


Plates 10.5 and 10.6.

Stimulated lymphocytes from infected rabbits

Smears from in-vitro whole blood culture. Numerous large cells are present. The cytoplasm stains bluish and contains an increased number of granules and also vacuoles of different sizes.

The nuclei are enlarged and some of them are already dividing. Small forms are also seen in the smear stained Undritz (approx. x 1094).



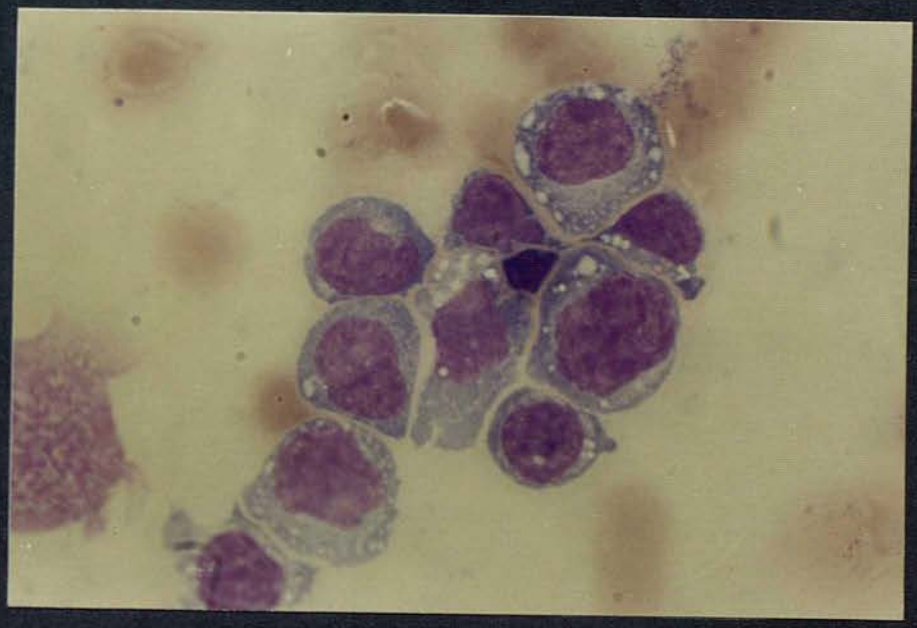
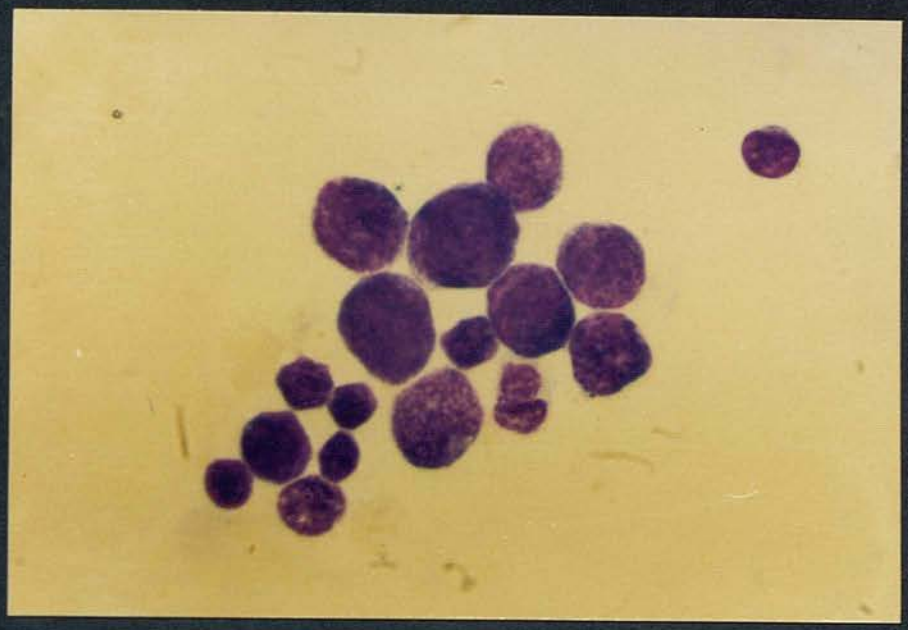


Plate 10.7. Smear from in-vitro lymphocyte culture stimulated with SF (UMB). The separated lymphocytes were obtained from an uninfected rabbit. The cells are enlarged, spread out and not clumped together.
Giemsa (approx. x 1094).

Plate 10.8. Smears from in-vitro lymphocyte culture without any stimulant. The lymphocytes were obtained from an uninfected rabbit. The cells are not enlarged.
Giemsa (approx. x 1094).



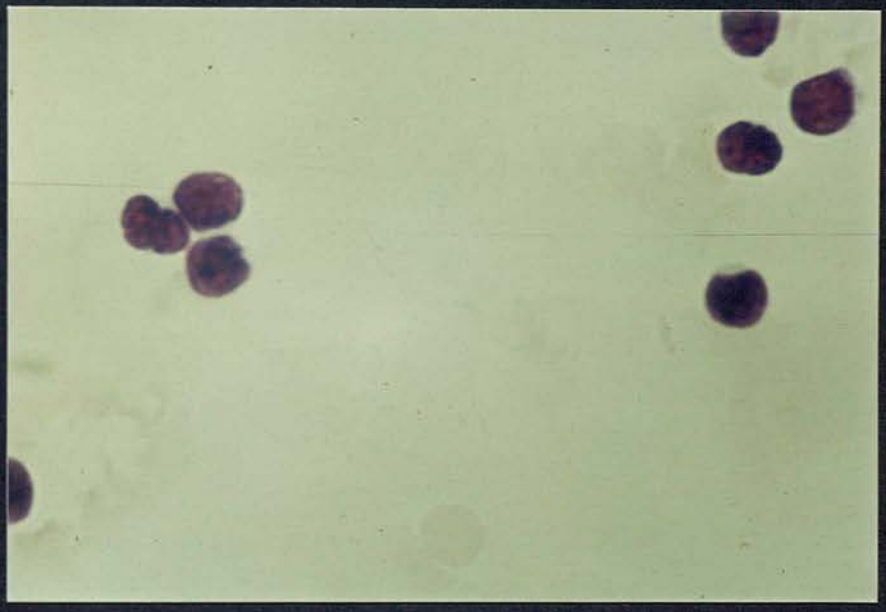
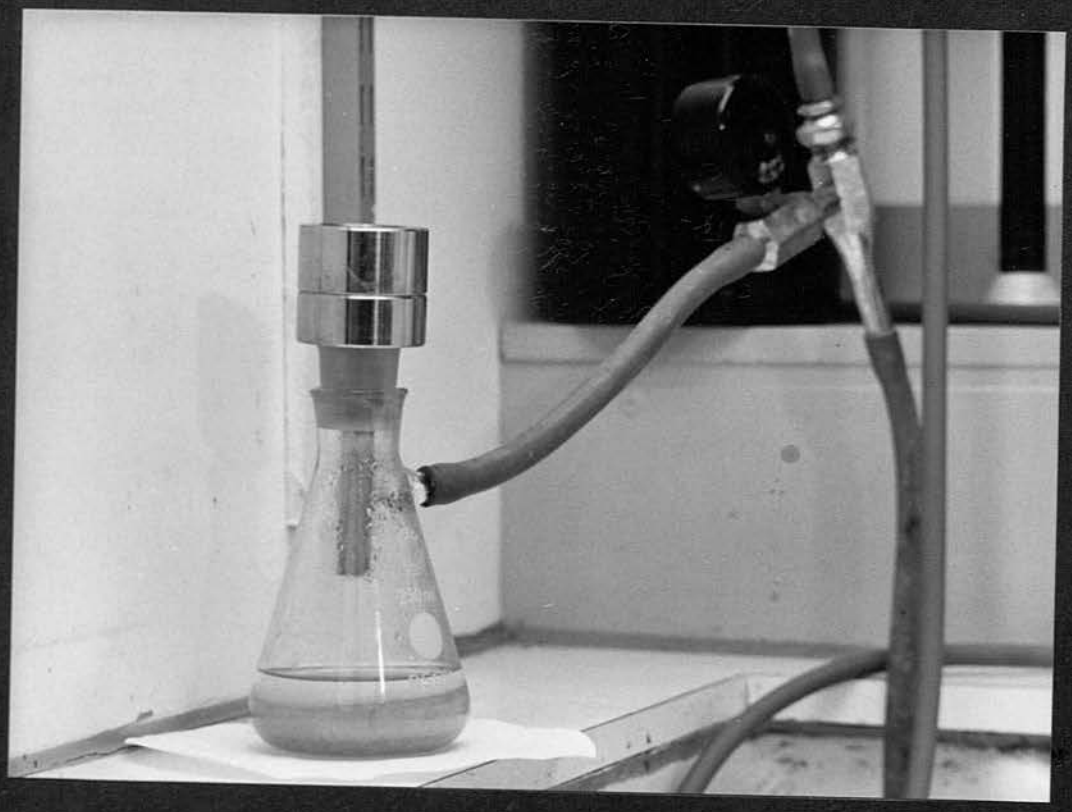


Plate 10.9. Filter block attached to a water-vacuum pump for removal of fluid portion of suspensions of cultural lymphocytes.



CHAPTER ELEVEN

THE ABILITY OF EXTRACTS OF FASCIOLA HEPATICA TO STIMULATE RABBIT LYMPHOCYTES IN-VITRO

Introduction

The aim of this work was to ascertain whether an antigen prepared from Fasciola hepatica would trigger the transformation of peripheral blood lymphocytes from rabbits infected with F.hepatica.

Experimental Design

Two groups of rabbits were chosen, the first group consisting of six rabbits which had been infected with varying numbers of metacercariae of F.hepatica for varying periods (Table 11.1), while the second group consisted of 16 uninfected rabbits.

Lymphocytes were separated by the gelatine technique and cultures were incubated for two days before the addition of tritiated thymidine. All cultures were carried out in duplicate. Smears were stained with Giemsa.

In an extension of this study a comparison was made between the activity of peripheral and splenic lymphocytes.

Results

(i) The radioactive uptake of tritiated thymidine by peripheral blood lymphocytes

The results are shown in Table 11.1. All the infected animals showed a clear increase in the count

when cultured in the presence of fluke antigen as compared with those without any stimulant. However, there was some indication that the counts may be reduced when the lymphocytes were obtained from rabbits in more prolonged infection as shown by RB 7 and RB 8 (Table 11.1).

In most cases there was also a small increase in the uptake of radioactive thymidine by lymphocytes from uninfected animals in the presence of fluke antigen. The mean count ($\pm$ standard deviation) was 86 $\pm$ 44 without FA and 136 $\pm$ 67 with FA. This difference is significant ($t = 2.56$, $p < 0.02$) but the size of the increase is much less than that which occurred with lymphocytes from the infected rabbits.

(ii) Morphological examination

The results are shown in Plates 11.1, 11.2 and 11.3. No difference was observed between the morphological characters of lymphocytes from the infected rabbits (Plate 11.1) cultured without the addition of FA and those obtained from uninfected rabbits (Plate 11.2) cultured with or without the addition of FA. However, when FA was included with the cultured lymphocytes from infected rabbits, there was considerable enlargement of the cells and their nuclei (Plate 11.3), the cytoplasmic area increased, and there was a basophilic granulation not seen in unstimulated cells.

(iii) The radioactive uptake of tritiated thymidine by splenic lymphocytes as compared with that by peripheral blood lymphocytes

The results are shown in Table 11.2 in which the results of counts on peripheral blood lymphocytes are compared with those on lymphocytes obtained from the spleen of the same rabbit (RB 8) at the same time. Both FA and SF stimulated both types of lymphocytes and the total count after stimulation with SF was higher than with FA, although this was much less marked with the splenic lymphocytes.

Discussion

The peripheral and splenic lymphocytes obtained from rabbits infected with F.hepatica clearly responded to the presence of a soluble extract of adult flukes, although these rabbits were only giving a relatively small response to this antigen at the time (See Table 11.1).

However there was no indication that the use of splenic lymphocytes would offer any advantage sufficient to offset the technical difficulties which this would prevent.

The difference in the degree of uptake of tritiated thymidine by lymphocytes from uninfected rabbits between the cultures containing no stimulants and those containing FA suggests that FA has some non-specific mitogenic activity.

It was concluded that this technique, using

peripheral lymphocytes, offered a potentially useful means for monitoring the cellular response of rabbits to infection with F.hepatica.

Table 11.1

Uptake of tritiated-thymidine by peripheral lymphocyte from rabbits infected with F.hepatica

Infection Data			Mean C.p.m. per million cells (less background) in the presence of	
Rabbit Number	Days after Infection	Number of Metacercariae	No Stimulant	Fluke Antigen
RB 10	30	100	388	2241
RB 15	35	100	166	1390
RB 9	60	50	137	6174
RB 1	95	500	79	1280
RB 7	130	100	147	396
RB 8	130	50	35	566

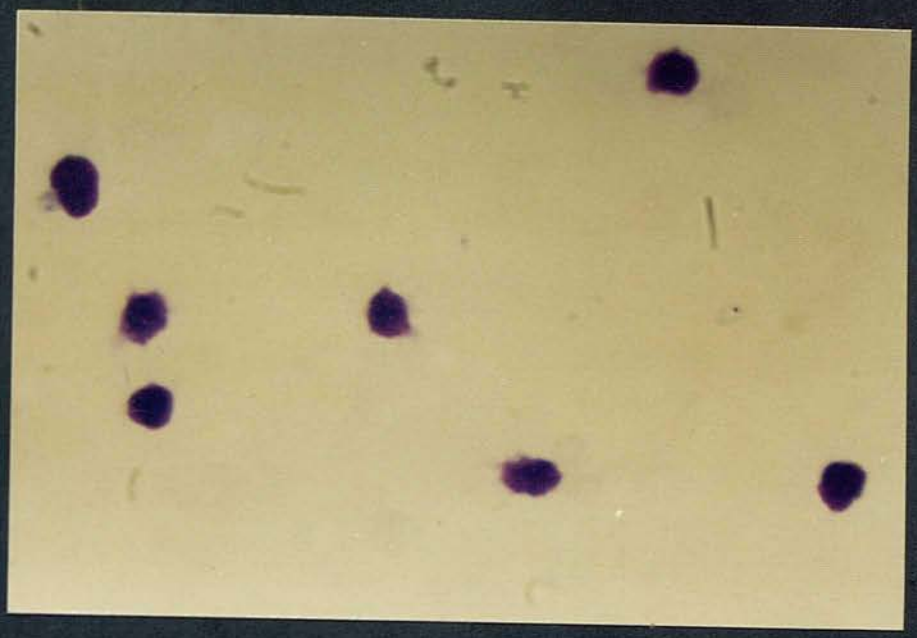
Table 11.2

Comparison of average stimulation between peripheral and splenic lymphocytes

Peripheral Lymphocytes			Splenic Lymphocytes		
Average C.p.m.			Average C.p.m.		
C	FA	SF	C	FA	SF
80	747	9999	295	1086	2327

Plate 11.1. Smear of cultured lymphocytes obtained from an infected rabbit (RB 9) without the addition of any stimulant. The lymphocytes are of normal size and are undifferentiated cells, staining deep blue. Giemsa (approx. x 1094).

Plate 11.2. Smear of cultured lymphocytes obtained from an uninfected rabbit (RB 11) in the presence of FA. These are normal unstimulated cells stained deep blue. Giemsa (approx. x 1094).



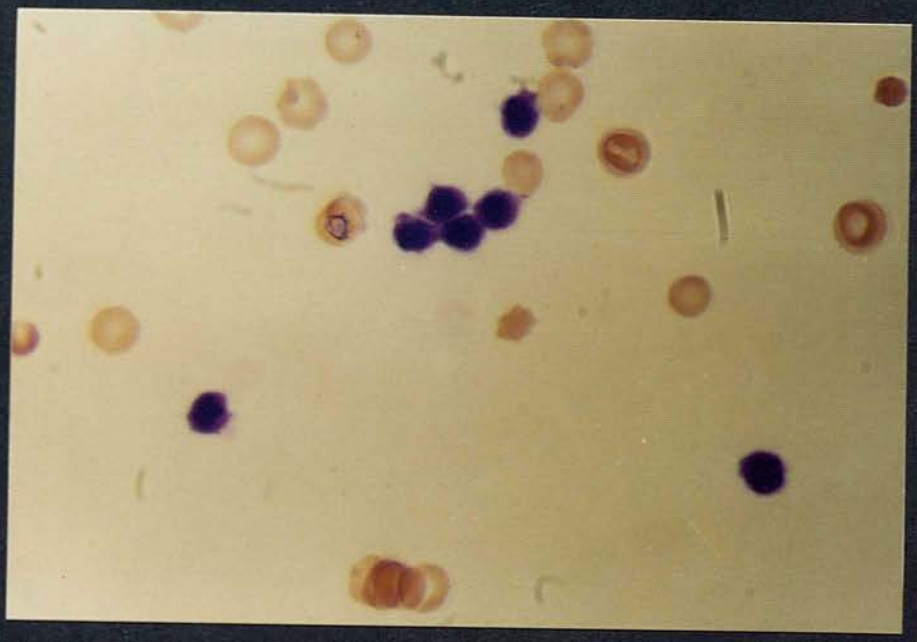
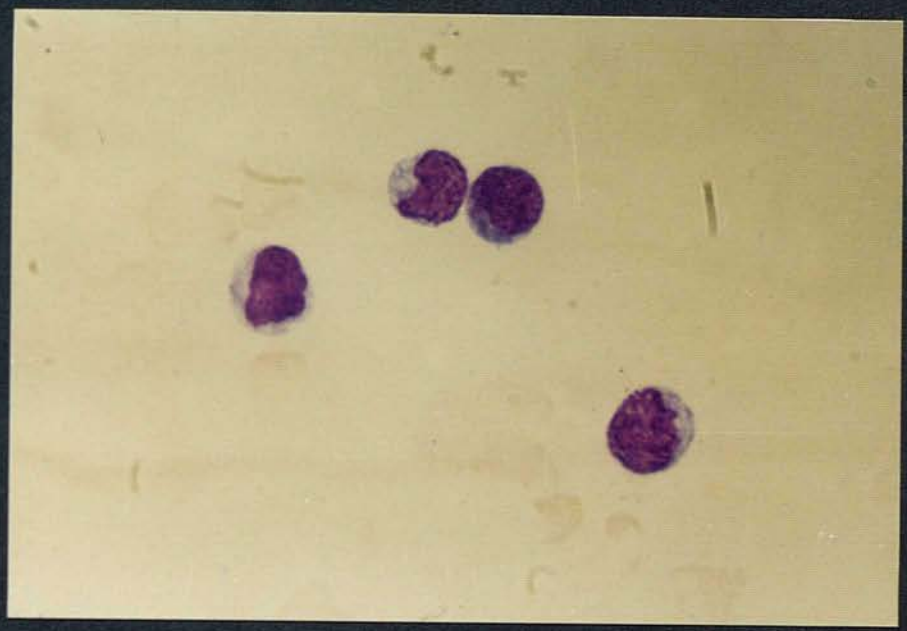


Plate 11.3. Smear of cultured lymphocytes obtained from an infected rabbit (RB 9) in the presence of FA. The cells and nuclei are enlarged, with basophilic granular cytoplasm. Giemsa (approx. x 1094).



CHAPTER TWELVE

THE IN-VITRO ACTIVITY OF THE PERIPHERAL LYMPHOCYTES OF RABBITS FOLLOWING EXPERIMENTAL INFECTION WITH F.HEPATICA AT TWO DIFFERENT LEVELS AND LATER TREATMENT WITH FASCIOLICIDAL DRUGS

Introduction

Following the results described in the previous chapter, in which in-vitro antigenic stimulation of lymphocytes from rabbits infected with F.hepatica was demonstrated, it was decided to use this technique in a pilot experiment to study the development of this lymphocyte activity in experimentally infected rabbits. The rabbits were to be infected with different numbers of F.hepatica and some of them were to be treated with different fasciolicidal drugs, to see if either of these treatments caused any consistent difference in this activity.

Experimental Design

The details of this experiment are given in Chapter 5, where they are discussed in relation to the efficacy of the anthelmintics used. However a summary of the details is again shown in Table 12.1.

TABLE 12.1

Group	Number of rabbits receiving 100 metacercariae	Number of rabbits receiving 500 metacercariae	Anthelmintic used at 4 and 5 weeks after infection
1	2	2	-
2	2	2	Rafoxanide
3	2	2	Diamphenethide
4	2	2	Nitroxynil

Materials and Methods

Sterile blood was collected by the needle puncture method at weekly intervals. Cultures were prepared in tubes using separated lymphocytes obtained by the gelatine technique and were incubated in modified McIntosh and Fyldes jars.

On each occasion seven cultures were made from each animal, three controls without any stimulant, three with FA and a positive control culture with SF. The cultures were incubated for two days before the addition of tritiated thymidine and then further incubated for another 24 hours before harvesting. As mentioned previously, in Chapter 5, haematological examination, including total and differential leucocyte counts were carried out at weekly intervals on the blood from these rabbits.

The absolute numbers of large and small lymphocytes

in these samples could thus be calculated.

Results

The activities of the cultures of the lymphocytes stimulated with either FA or SF and of the control cultures are shown in Appendix Tables 12.1, 12.2, 12.3 and 12.4. The deaths which occurred during the course of this experiment caused some difficulty in the interpretation of the results. However a Wilcoxson's signed ranks test ($N = 26$, $T = 157$, $Z = 0.13$, n.s.) indicated that there were no overall differences in the activities of their lymphocytes between rabbits infected with different doses of metacercariae, and accordingly the results for each group were pooled. Most of the rabbits showed some increase in lymphocyte activity by one week after infection and, at this time, there was a tendency for the mean counts from the group infected with 500 metacercariae (Mean $\pm$ s.d. = 314 $\pm$ 212) to rise faster than those from the group infected with 100 metacercariae (178 $\pm$ 93). The increase in the counts over this week was significant by the Mann-Whitney test (Mean increase $\pm$ s.d. = 264 $\pm$ 156) for the more heavily infected group and 107 $\pm$ 129 for the group infected with 100 metacercariae ($T = 49$, $N_1 = N_2 = 8$, $p < 0.05$). There was a further marked increase in all cases by two weeks after infection. Thereafter the counts fluctuated somewhat and there was a slow overall tendency for the counts to fall in the untreated rabbits ($y = 2900 - 199\ x$ for x from 2-8 weeks) but there was

also marked fluctuation so that this tendency was not significant (for the difference of the regression coefficient from zero, N = 39, t = 1.1).

There was no evidence that treatment with any of the drugs caused a clear or consistent change in the activity of the lymphocytes (Fig. 12.1). Thus analysis of variance showed no significant differences between the regression of the counts on time for four to eight weeks post infection in any of the treatment groups (Table 12.2).

TABLE 12.2

Source of Variation	d.f.	s.s.	MS	F	
Among regressions	3	3167748	1055916	1.18	-n.s.
Within regressions	198	177254788	895226		

The numbers of lymphocytes present in the blood from these rabbits are shown in Appendix Table 12.5, large lymphocytes were always present in smaller numbers than small lymphocytes but after infection there was a gradual increase in the absolute and relative numbers of large lymphocytes over the period 0 - 6 weeks post infection. Regression analysis showed that this trend was highly significant ($y = 289x + 177$ for the absolute data, with $N = 79$, $t = 8.3$, $p < 0.01$ for the difference of the regression coefficient from zero). Furthermore, in this and later experiments, activated lymphocytes were frequently seen in blood smears in various stages of division (Plates 12.1 and 12.2).

Discussion

The tendency for an increased in-vitro response after infection was clear and it appears that this technique gives the earliest immunological evidence of infection with F.hepatica.

Despite the marked variation in the uptake of tritiated thymidine, it could be discerned that treatment with the different fasciolicidal drugs failed to have any effect on the transformation response in any of the rabbits.

The numbers of large lymphocytes in the peripheral circulation was clearly increased after infection, (Appendix Table 12.5), and this change was highly significant. It would seem probable that this was related to the animals response to the infection or to the damaged

liver tissue.

The greater response to *in-vitro* stimulation by the first week after infection in the heavily infected animals as compared with those that had received the lower infection was probably related to the greater stimulus from the larger numbers of young migrating flukes in the former group by that time. However this difference was not maintained, suggesting that the rabbits were reacting maximally to both levels of infection by two weeks after infection.

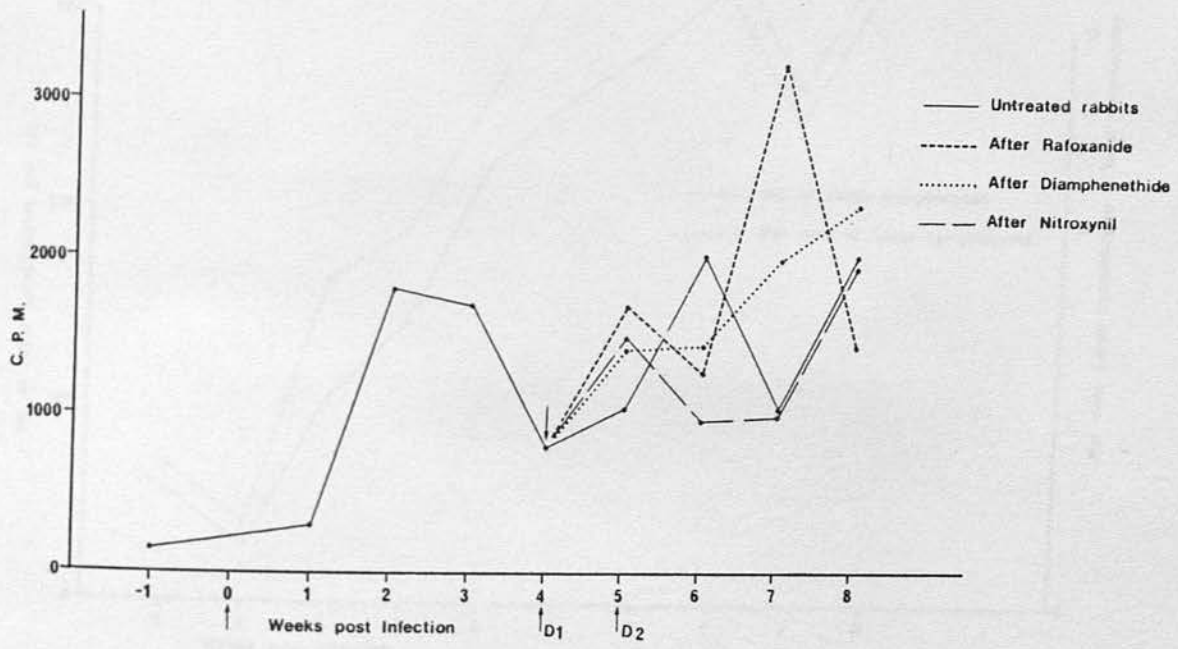


Fig. 12.1. Mean counts on triplicate culture of lymphocytes from four groups of rabbits.

——— Untreated control rabbits.

----- Rabbits dosed with rafoxanide (26mg/Kg).

..... Rabbits dosed with diamphenethide (500mg/Kg).

— — Rabbits dosed with nitroxynil (20mg/Kg).

D_1 first dosing.

D_2 second dosing.

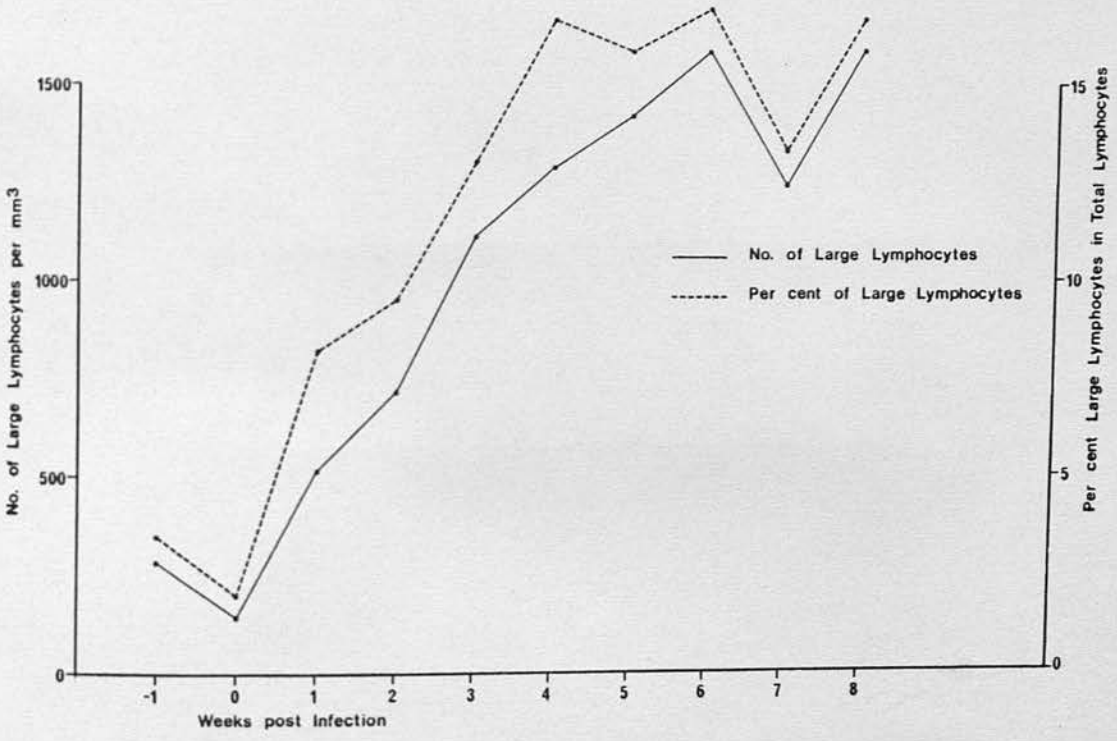
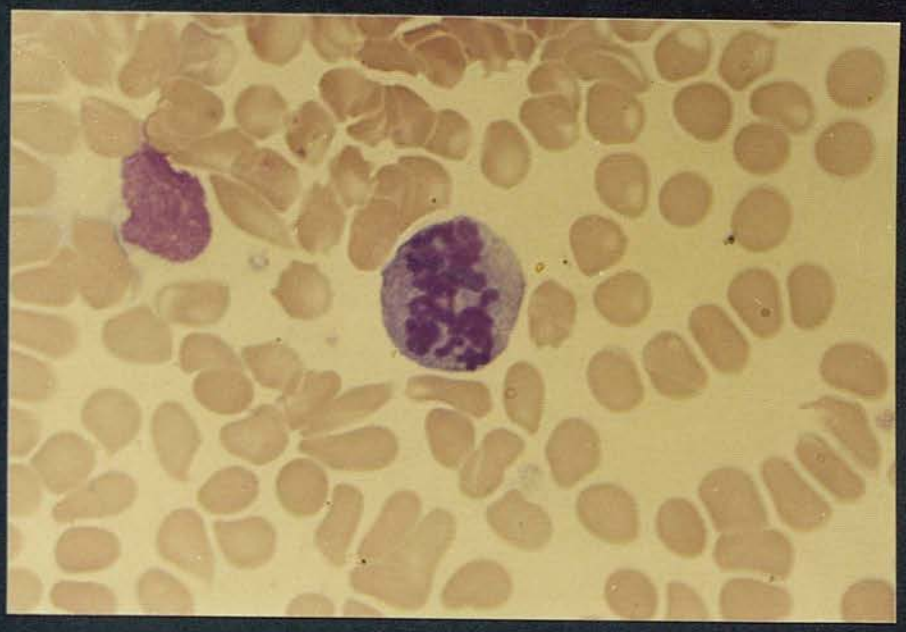
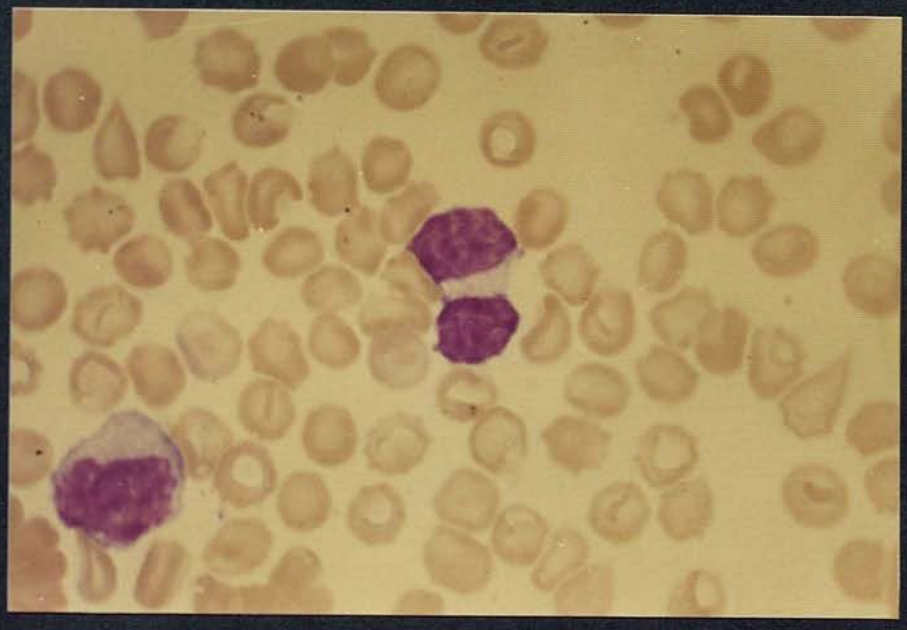


Fig. 12.2. Comparison of numbers of large lymphocytes, observed at weekly intervals in smears and counts from rabbits, following infection with F.hepatica.

Plate 12.1. Fresh blood smear from rabbit infected with 100 metacercariae for 28 days, showing an activated lymphocyte in early anaphase. Undritz (approx. x 1094).

Plate 12.2. Fresh blood smear from rabbit infected with 100 metacercariae for 28 days, showing a lymphocyte apparently in late telophase (Top) and an enlarged lymphocyte (Bottom). Undritz (approx. x 1094).





CHAPTER THIRTEEN

STUDIES ON THE PARAMETERS OF THE IN-VITRO STIMULATION OF RABBIT LYMPHOCYTES BY AN EXTRACT OF FASCIOLA HEPATICA

Introduction

Following the demonstration of stimulation of rabbit peripheral lymphocytes by FA, using separated lymphocytes obtained by the gelatine technique, it was considered that it would be worthwhile to study the parameters of this response in some detail. However, it was apparent that this would require the development of a technique which would allow large numbers of lymphocyte cultures to be processed easily and quickly. Following the preliminary study discussed in Chapter 10, it was therefore decided to use cultures prepared from whole defibrinated blood instead of separated lymphocytes, as the latter is considerably more laborious and requires more blood to be taken from each animal.

Lymphocyte transformation experiments using whole blood rather than lymphocyte enriched preparations have been previously reported by Jung, Hoekstra, Wolfe and Deinhardt (1970) and Firket, Leclercq-Foucart and Geubelle (1971).

Experimental Design

Six rabbits were infected with 100 metacercariae each and these, together with four uninfected rabbits, were used as a source of lymphocytes. Blood was obtained by the needle puncture method and the lymphocytes

cultured by the whole defibrinated blood technique.

The following parameters were studied:-

1. Length of incubation.
2. Number of cells.
3. Concentration of FA.

Blood was collected from different groups of rabbits at intervals of not less than 14 days. The activity of the lymphocytes was studied by both the morphological and the radio-tracer techniques.

Results

Lymphocyte stimulating activity varied from animal to animal but was always greater when using lymphocytes from infected animals. The cells from the uninfected rabbits showed only the small increase in activity caused by the non-specific mitogenic activity of the FA, previously discussed in Chapter 11. Furthermore, there were no marked changes in the activity of the unstimulated cells from the infected rabbits apart from a tendency for their activity to rise with longer incubation in the presence of tritiated thymidine.

(i) The effect of varying the length of incubation

The results of these experiments are shown in Figs. 13.1, 13.2 and 13.3. In the first experiment, cultures were made from blood taken from a rabbit ten days after infection with F.hepatica. These were incubated for one to four days and were then labelled with tritiated thymidine for 3, 18, 24 or 42 hours. A slight increase in the

activity in the cells was seen in all cultures by three hours after the addition of thymidine, and the maximum increase was usually seen by 24 hours. This increase was most marked in the cultures incubated for four days (Fig. 13.1).

In a second experiment, cultures were prepared using blood from a rabbit with a 20 day old infection and incubated for three to five days. The counts followed the same pattern as in the previous experiment and again the maximum count occurred after incubation for four days (Fig. 13.2) and usually after exposure to tritiated thymidine for 24 hours.

In a final experiment of this type, blood was obtained 35 days post infection from two rabbits at the same time and cultures were incubated for two to seven days (Fig. 13.3). In all cases the cells were then labelled with tritiated thymidine for 24 hours before harvesting. Once again the maximum count was seen in cultures maintained for four days prior to the addition of the thymidine.

(ii) <u>The effect of varying the number of cells</u>

The results are shown in Fig. 13.4. The counts increased over the range from one to five x 10^5 cells per culture but then decreased until the maximum number of lymphocytes used (2.5 x 10^6 per culture) gave a low count similar to that obtained when the smallest number of cells were used. The maximum count per 10^5 cells used

was obtained at four x 10^5 cells but was not greatly different from that at five x 10^5 cells.

(iii) The effect of varying the concentration of FA

The results are shown in Fig. 13.5. The maximum count was seen when using the FA at a dilution of 1:4, which is the equivalent of adding 0.0625 ml of the undiluted antigen to each culture. No consistent change was seen in the non-specific mitogenic activity of the FA with changes in concentration.

In each case analysis of variance showed the differences arising from the various treatments in these three experiments were significant (See Tables 13.1 - 13.5).

Analysis of Variance

Table 13.1

Source	ss	d.f.	MS	F	P
Days	950647	3	316883	30	0.01
Hours	2066666	3	688899	65	0.01
Inter-actions	1172884	9	130320	12*	0.01
Residual	486998	46	10587		
Total	4677195	61			

* Although the interaction appeared to be significant, it was considered on a priori grounds that this was unlikely to be a real effect and breakdown analysis confirmed the high significance of the main effects.

Table 13.2

Source	ss	d.f.	MS	F	P
Days	5380698	2	2690349	6.9	< 0.05
Hours	20451897	3	6817299	17	< 0.01
Inter-actions	5267912	6	877985	2.2	n.s
Residual	4683038	12	390253		
Total	35783545	23			

Table 13.3

Source	ss	d.f.	MS	F	P
Days	229310991	5	45862198	12	< 0.01
Hours	74795140	1	74795140	20	< 0.01
Inter-actions	4505180	5	1036	< 1	n.s
Residual	134617101	36	3739364		
Total	443228412	47			

Table 13.4

	d.f.	MS	F	P
Between ss = 61500198	8	7687525	14	< 0.01
Within ss = 14735914	27	545775		
Total ss = 76236112	35			

Table 13.5

	d.f.	MS	F	P
Between ss = 3534814	6	589136	3.1	< 0.05
Within ss = 2660571	14	190041		
Total ss = 6195385	20			

Discussion

It was concluded from this work that the optimum number of lymphocytes per culture using the whole blood technique was 0.5 million and that these lymphocytes should be incubated in the presence of 0.0625 ml of F.hepatica antigen (FA), for four days prior to the addition of tritiated thymidine for 24 hours. In this way the maximum stimulation was obtained as indicated by the higher uptake of tritiated thymidine. These parameters were thereafter used consistently.

The number of lymphocytes per ml of culture is similar to that suggested by other workers (Valentine, 1971) and although the period of culture is rather

longer than has sometimes been recommended (Knight and Ling, 1969; Mansfield and Wallace, 1973), most of these earlier studies have been concerned with the reaction between lymphocytes and non-specific mitogens.

The use of whole blood cultures instead of the separated lymphocytes was earlier recommended by Paul and Sokal (1972), who referred to the possibility of the maintenance of a more stable PH and oxygen tension, when cultures contain relatively large numbers of erythrocytes.

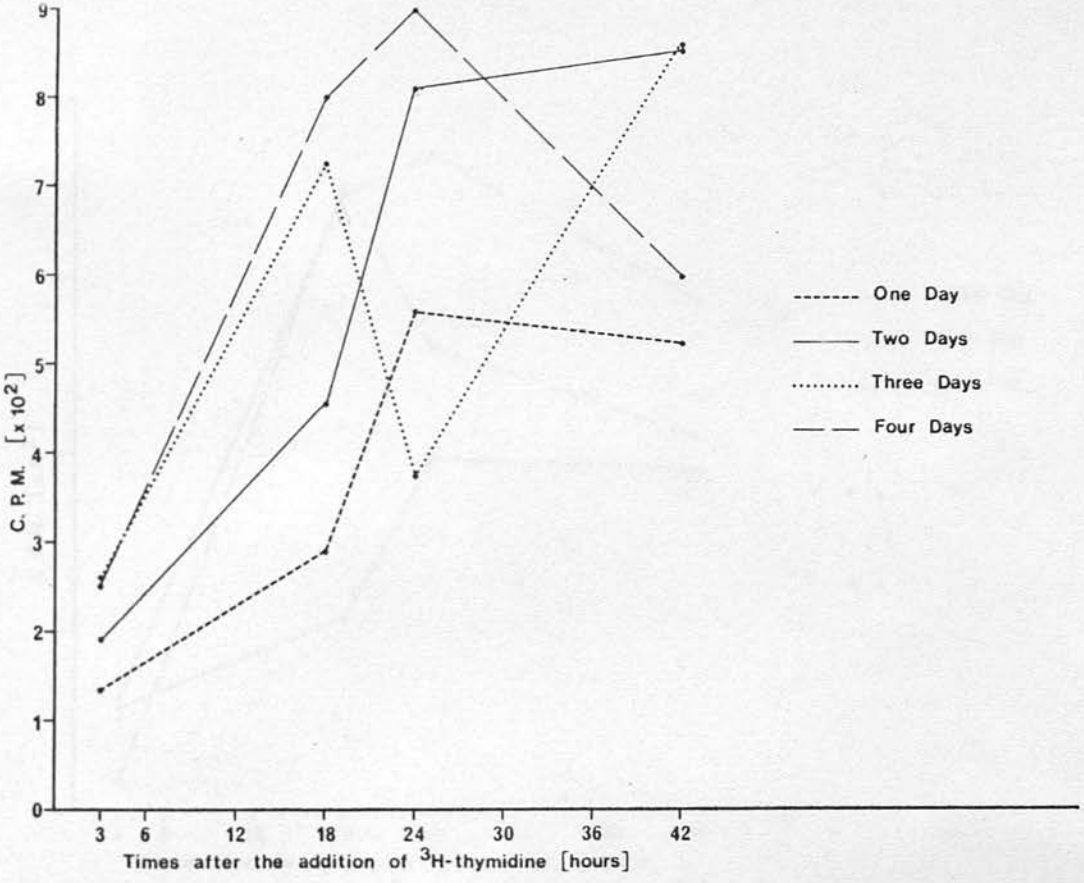


Fig. 13.1. Mean counts on quadruplicate whole blood cultures in tubes of lymphocytes from a rabbit with a ten day old infection with 100 metacercariae.

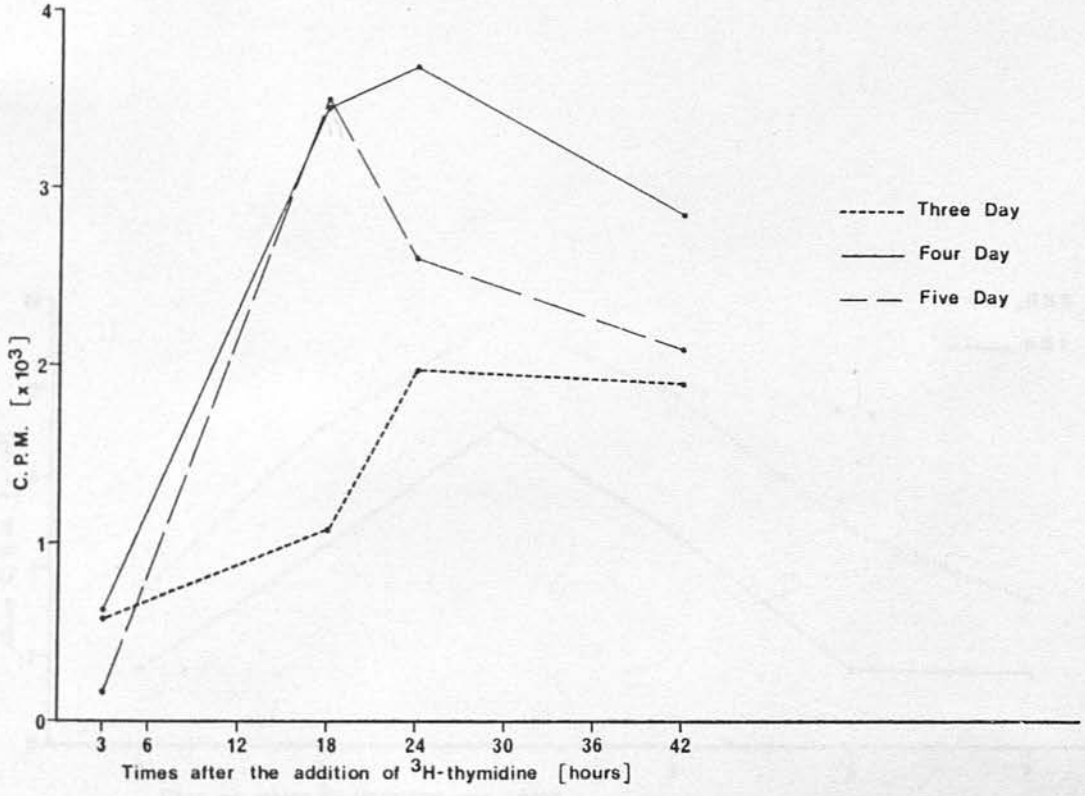


Fig. 13.2. Mean counts on duplicate whole blood cultures in tubes of lymphocytes from a rabbit with a 20 day old infection of 100 metacercariae.

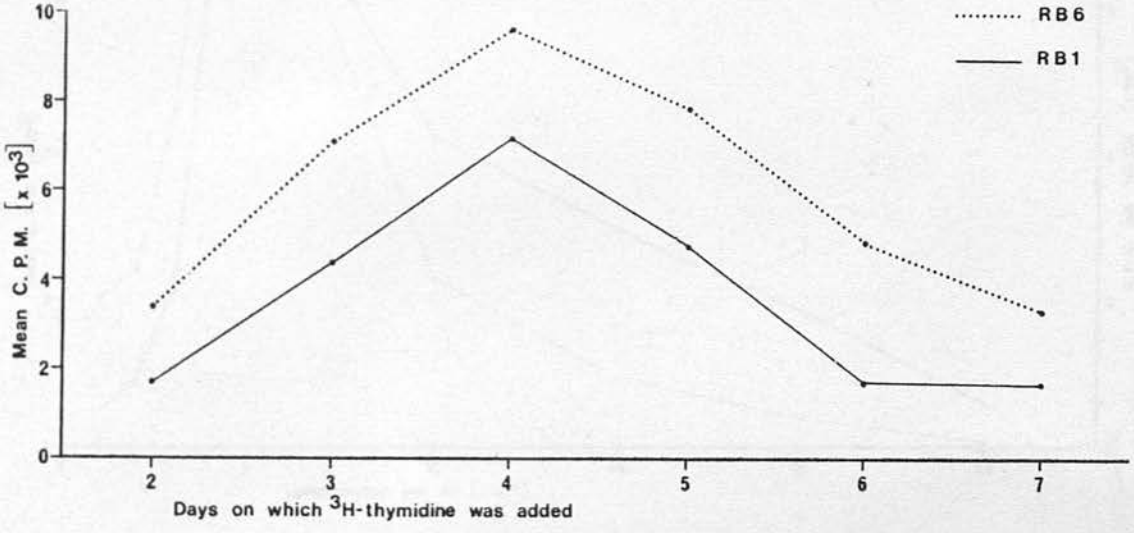


Fig. 13.3. Comparison of the counts on quadruplicate whole blood cultures in tubes of lymphocytes from two rabbits, 35 days after infection with 100 metacercariae.

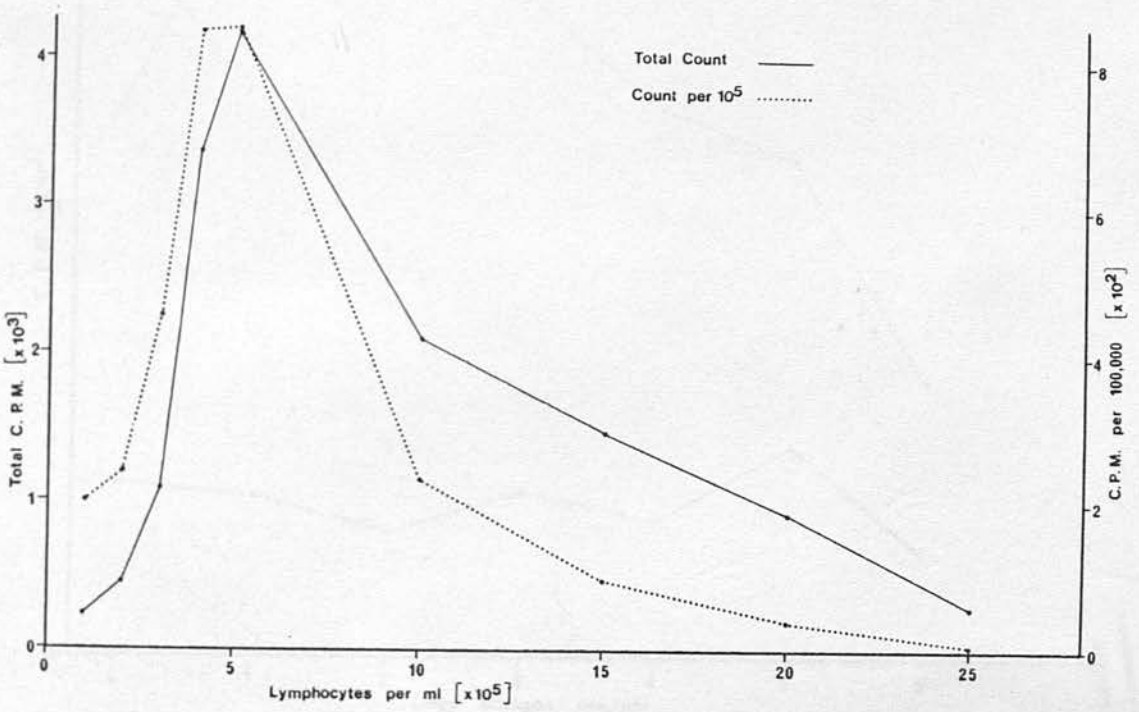


Fig. 13.4. Mean counts on quadruplicate whole blood cultures in tubes containing different numbers of lymphocytes.

The solid line represents the mean of the total counts.

The dotted line represents the count per 10^5 lymphocytes.

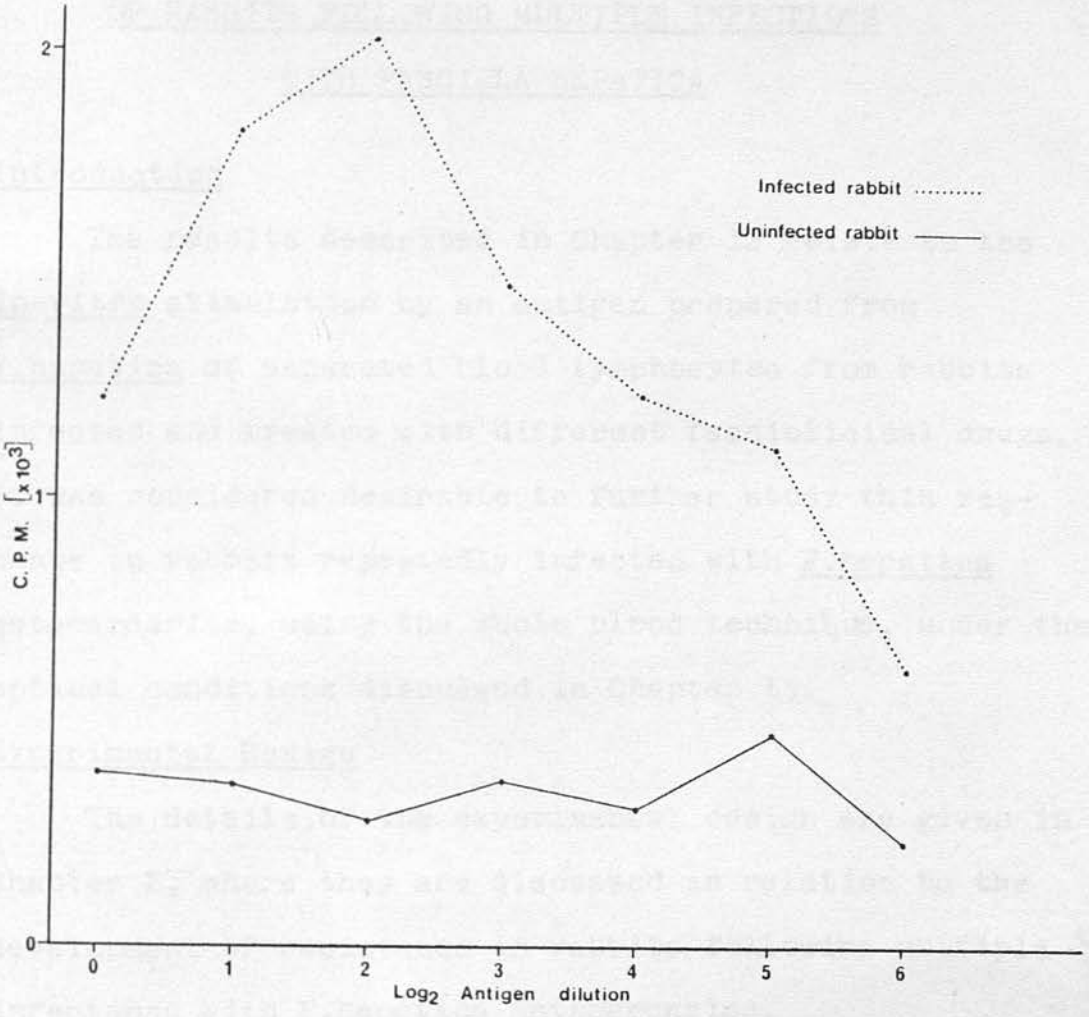


Fig. 13.5. Mean counts on triplicate whole blood cultures in tubes of lymphocytes.

0 represents 0.25 ml of neat fluke antigen (FA) prepared as a 1:5 V/V extract of adult F.hepatica homogenised in Minimal Eagles Medium.

1-6 represents the Log 2 antigen dilution.

CHAPTER FOURTEEN

THE IN-VITRO ACTIVITY OF THE PERIPHERAL LYMPHOCYTES OF RABBITS FOLLOWING MULTIPLE INFECTIONS WITH FASCIOLA HEPATICA

Introduction

The results described in Chapter 12 relate to the in-vitro stimulation by an antigen prepared from F.hepatica of separated blood lymphocytes from rabbits infected and treated with different fasciolicidal drugs. It was considered desirable to further study this response in rabbits repeatedly infected with F.hepatica metacercariae, using the whole blood technique, under the optimal conditions discussed in Chapter 13.

Experimental Design

The details of the experimental design are given in Chapter 7, where they are discussed in relation to the development of resistance in rabbits following multiple infections with F.hepatica metacercariae.

Sterile blood was collected by the needle puncture method at two-weekly intervals. The cultures were prepared using the whole blood technique and incubated in modified McIntosh and Fyldes jars.

On each occasion five cultures were made from each animal, two controls without any stimulant, two with FA and a positive control culture with SF. The cultures were incubated for four days before the addition of the tritiated thymidine and then incubated for a further 24

hours.

Results

The results are shown in Figs. 14.1 and 14.2 and in Appendix Tables 14.1.a and 14.1.b. In all the groups there was a marked increase in the activity by one to two weeks after initial infection, the mean (± standard deviation) increase in the count over this time being 3629 ± 2531. However, following first and second challenge infections, the mean counts (± standard deviation) fell over the next one to two weeks by 1854 ± 1286 and 1751 ± 1001 respectively. At first sight this would seem a very significant and consistent finding but the animals in Group 4 which received the initial infection and the first challenge only also showed a fall in the count at the time of the second challenge of a similar size (1863 ± 600).

Treatment had no consistent effect on lymphocyte transformation as the counts with one to two weeks of treatment varied in an apparently random manner, the mean change in count (± standard deviation) being 753 ± 2382, which was not significant by Wilcoxons paired ranks test ($Z = 1.27$, $P > 0.2$).

The peak counts of lymphocyte transformation activity on the six infected and ten uninfected rabbits during the first eight weeks, using cultures without any stimulants, showed a significant increase in the activity in the count in the infected group ($t = 5.6$, $p < 0.01$) as compared with the uninfected animals.

Discussion

This work supported the previous observations in Chapter 12, in that there was a marked increase in the uptake of tritiated thymidine by the cultured lymphocytes within one to two weeks after the initial infection. However challenge infections did not cause a further increase in the level of lymphocyte stimulation and may have caused a decrease, although this latter observation was rendered rather doubtful because of the variation in the counts and needs confirmation.

Treatment had no apparent effect over the time scale of the experiment but some effect will have been seen if the observations had continued for a longer period.

The increased uptake of tritiated thymidine by unstimulated cultures from animals infected with F.hepatica is probably related to the increased numbers of large lymphocytes occurring in the peripheral blood of such rabbits, as is discussed in Chapter 12. It may be postulated that a number of these cells were already stimulated - perhaps by fluke antigens or by tissue degradation products in the host - and continued to transform and multiply in culture, consequently taking up the tritiated thymidine.

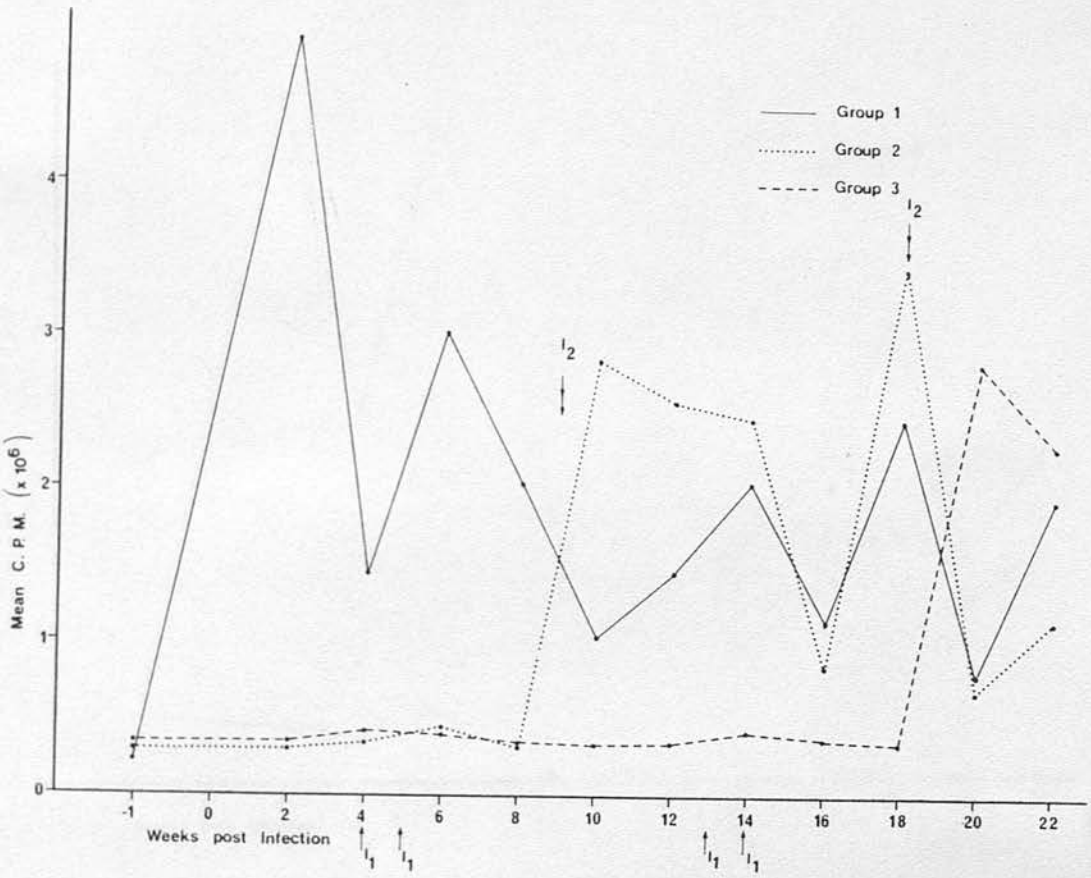


Fig. 14.1. Mean counts on duplicate whole blood cultures in tubes of lymphocytes from rabbits.

Group 1 = challenged with three further doses of 100 metacercariae at 0, 9 and 18 weeks.

Group 2 = challenged with two further doses of 100 metacercariae at 9 and 18 weeks.

Group 3 = previously uninfected, but challenged with 100 metacercariae at 18 weeks.

I_1 All animals treated with 26mg/Kg rafoxanide.

I_2 Re-infection dates.

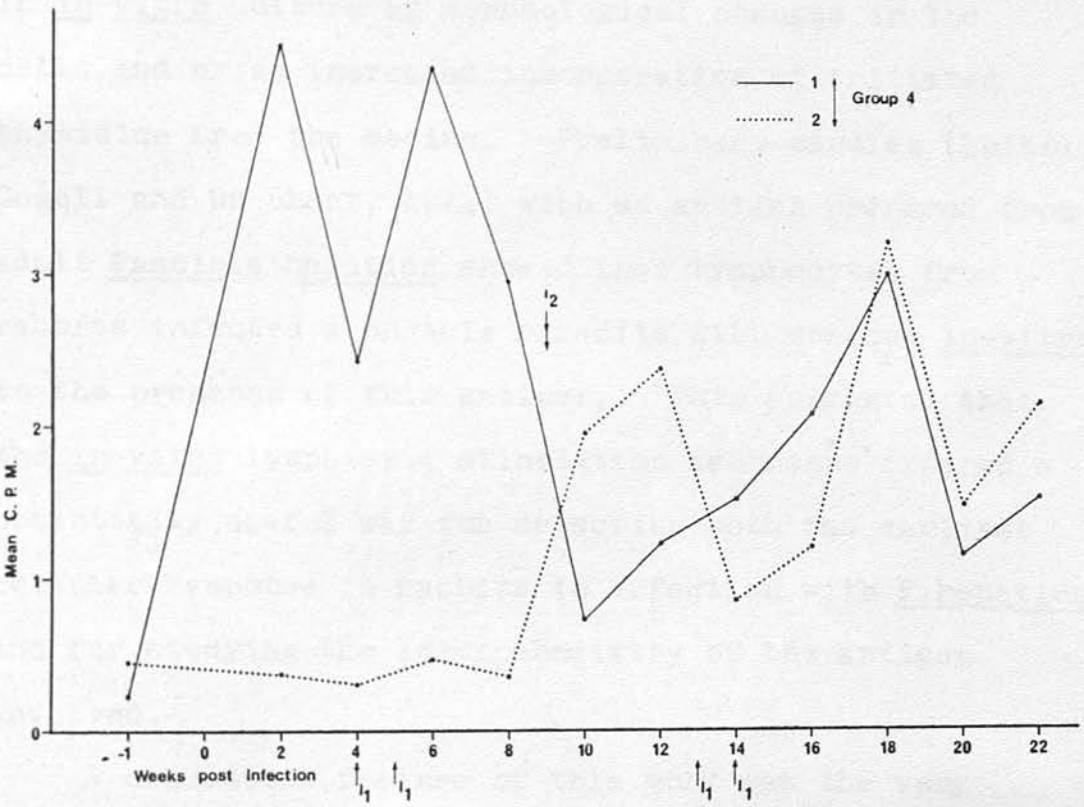


Fig. 14.2. Mean counts on duplicate whole blood cultures in tubes of lymphocytes from rabbits.

Group 4 = treatment control groups.

I_1 All animals treated with 26mg/Kg rafoxanide.

I_2 Re-infection dates.

CHAPTER FIFTEEN

DISCUSSION

One of the most fundamental immunological responses is that shown by lymphocytes when they are stimulated by a specific antigen and this reaction can be demonstrated in in-vitro culture by morphological changes in the cells and by an increased incorporation of tritiated thymidine from the medium. Preliminary studies (Bolbol, Sewell and Urquhart, 1974) with an antigen prepared from adult Fasciola hepatica showed that lymphocytes from rabbits infected with this parasite will respond in-vitro to the presence of this antigen. This suggested that the in-vitro lymphocyte stimulation technique offered a potentially useful way for detecting both the earliest cellular response in rabbits to infection with F.hepatica and for studying the immunochemistry of the antigen involved.

A consistent feature of this work was the very marked variation in lymphocyte stimulation activity seen from day to day, animal to animal, and even within the replicates of the same animal on the same day. It seems most probable that this must be due to minor variations in technique, such as differences in the time taken to bleed the animals or to set up the cultures. However, despite this variation, a number of consistent and significant deductions can be made.

Thus antigen-induced transformation of lymphocytes was usually detected as early as the first week after infection in rabbits infected with different numbers of

metacercariae and, although lymphocytes from uninfected rabbits did respond to the extract of F.hepatica, this non-specific response was small, being much less than that given by lymphocytes from infected rabbits. Nevertheless it was shown that the extract acts as a low grade non-specific mitogen.

The in-vitro lymphocyte response could be detected by both the uptake of tritiated thymidine and by morphological changes. The maximum response was usually seen by 14 days after infection with F.hepatica. On the other hand, Dobson and Soulsby (1974), working with Trichostrongylus colubriformis in guinea pigs, reported maximum in-vitro lymphocyte transformation 25 days after infection. Dobson and Soulsby suggested that the maximum stimulation obtained at that time was probably due to the release of a large pool of sensitized lymphocytes into the circulation and considered this to be an early indication of the induction of an immune response. The increased uptake of tritiated thymidine by unstimulated cultures from animals infected with F.hepatica and the increased numbers of large lymphocytes present in the peripheral blood shown in the studies described in Chapters 12 and 14 possibly arise from a similar phenomenon to that suggested by Dobson and Soulsby (1974). It would seem probable that it is these large lymphocytes which continue to multiply and so to take up thymidine in-vitro, having been previously stimulated while in-vivo.

It is always advisable to include a positive control in this type of study, and it was found that a filtrate prepared from staphylococci of human origin (UMB) was the most useful for this purpose. Cells which had been stimulated by the mitogen were quite discrete in smears (Plate 11.7) and not clumped together as happened in the presence of phytohaemagglutinin and as had been earlier reported by Ling, Spicer, James and Williamson (1965). Thus UMB facilitates the morphological differentiation of stimulated cells. A filtrate prepared from staphylococci of canine origin however failed to stimulate rabbit lymphocytes.

Although incorporation of labelled thymidine is a convenient quantitative technique for measuring the response of lymphocytes, the less elaborate morphological examination is also considered a good measure of this response. A morphological comparison of the stimulated cells using both fluke antigen (FA) and staphylococci filtrate (UMB) stimulants showed that UMB stimulated lymphocytes from both normal and infected rabbits, while FA seemed to stimulate lymphocytes from infected rabbits only (Plates 10.5, 10.6 and 11.3). This suggests that morphological examination of the cells offers a useful preliminary guide as to the efficacy of any particular stimulant or antigen.

There was also evidence that, 28 days after infection with F.hepatica, a similar response by lymphocytes

was occurring in-vivo (Plates 12.1 and 12.2). This in-vivo activity may represent migration of sensitized cells from the lymph nodes to the circulation, where they may react with the parasitic antigen. The appearance of these transformed cells 28 days after infection may also correlate with the maximum damage to the liver tissue which occurs at about that time, in which case these cells form a part of the large numbers of lymphocytes migrating to the damaged area.

The magnitude of the in-vitro response is rather stereotyped, showing little or no difference with varying doses of metacercariae, so that groups of animals infected with 100 or 500 metacercariae (Chapter 12) both showed a similar response, although the onset of the response tended to be faster in the latter group. There would probably be some relationship between infection rate and response at lower levels of infection.

There seemed to be a slow decline in the activity of lymphocytes in prolonged chronic infection, thus RB 7 and RB 8 (Chapter 11), which were infected for a longer period, responded poorly. However the maintenance of this response was apparently unaffected by re-infection (Fig. 14.2) or by the removal of flukes with rafoxanide. Challenge infections (Chapter 14) certainly did not cause a further increase in the level of lymphocyte stimulation and, although they may have caused a decrease in this level, the variability of the counts and the

similar change which occurred in unchallenged animals at the same time casts considerable doubt on the validity of this effect.

Rabbits infected with a larger number of metacercariae also tend to produce precipitating antibodies earlier than those infected with lower doses of metacercariae (Chapters 3 and 4). It therefore seems possible that the ability of the lymphocytes to respond in-vitro and the production of precipitins were associated with each other.

As regards the technique used for culturing lymphocytes, the whole blood culture has considerable advantages such as the ease with which different sets of cultures could be set up at one time and the avoidance of the prolonged procedure used for separating the lymphocytes. Furthermore the whole blood technique seemed to result in rather more stimulation of the lymphocytes (Chapter 10), possibly because of the reduced number of manipulations required before the cultures are set up. However, it must not be concluded that these two techniques are necessarily measuring an identical response, because of the possibility that the presence of other leucocytes and erythrocytes will modify the response of the lymphocytes.

The maximum response was obtained using 500,000 cells (Fig. 13.4) per ml of culture media. Valentine (1971) also suggested that the cell concentration should be kept under 1×10^6 cells per ml in order to facilitate

maintenance of PH and cell nutrition. He pointed out that with human blood leucocyte cultures, 250,000 - 500,000 lymphocytes per ml will usually give a vigorous response. This could be explained on the basis that higher numbers of cells may cause considerable acidity in the medium, which may reduce the transforming capacity of the cells. The last explanation received some support from Knight, Ling, Sell and Oxnard (1965), who also indicated that acid production was relatively low in granulocyte-poor leucocyte preparation cultured in the presence of SF and PHA, which further suggests that the presence of other leucocytes tends to modify the response of the lymphocyte. Spontaneous transformation of control cultures was not seen in this study and this correlates well with the observation by Knight, Ling, Sell and Oxnard (1965) who did not observe such spontaneous transformation in their control cultures. Sabesin (1965) reported a high value for spontaneous transformation in cultures of rabbit lymphocytes but it was suggested by Sabesin that this was related to the lymphocytic aggregation that always occurred in cells which were cultured in the absence of phytohaemagglutinin. The most probable explanation for these variations is that they are due to differences in culture techniques, as was also suggested by Benezra, Gery and Davies (1967).

GENERAL CONCLUSIONS

The following conclusions were drawn from the results obtained in this work:-

(1) There was considerable individual variation among the rabbits for all the parameters studied.

(2) For infecting of rabbits with metacercariae of F.hepatica, the gelatine capsule technique used is much more effective than the filter paper technique.

(3) The reduced recovery of flukes in some of the heavily infected animals may be suggestive of competitive inhibition among the flukes, or may be related to the host response, which was reflected by the hepatic fibrosis.

(4) Diamphenethide is ineffective as an anthelmintic against the immature stages of F.hepatica in rabbits.

(5) Rafoxanide is an effective anthelmintic against the immature stages of F.hepatica in rabbits as shown by the very low recovery of young flukes and by the rapid recovery of the livers in animals treated with this drug.

(6) Gel-precipitin tests using serum from infected rabbits and an antigen prepared from adult F.hepatica affords a method of early diagnosis of the infection during the period in which the parasite is still migrating through the liver tissue and the infection is not yet patent.

(7) The serum glutamic dehydrogenase level is a useful parameter in estimating the degree of liver damage caused by the migrating flukes.

(8) The peripheral eosinophilia may be reflected by the cellular response of the host to the infection, such an eosinophil response being commonly associated with tissue invasive parasites.

(9) There was no significant difference in the numbers of recoverable flukes or in the other parameters as between rabbits challenged with a further dose of infective metacercariae after removal of a previous infection and controls which had received only the challenge infection.

(10) In animals challenged after two previously curtailed infections there was some reduction in the numbers of flukes recovered, but this may have arisen from the more fibrous nature of the livers in these animals.

(11) There was no clear difference in the gross pathology of the livers as between animals infected with 500 or 100 metacercariae and it appeared that 100 metacercariae of F.hepatica is a suitable higher dose for infecting rabbits.

(12) Peripheral lymphocytes obtained from infected rabbits respond in-vitro to the presence of an antigen prepared as a 1:5 v/v extract of adult F.hepatica in Minimal Eagles Medium, as shown by both their morphological changes and their uptake of tritiated thymidine.

(13) The fluke extract is also a mild non-specific mitogen for rabbit lymphocytes but the response of lymphocytes from infected rabbits is significantly greater than that from uninfected rabbits by two weeks after infection.

(14) There is both a relative and an absolute increase in the numbers of large lymphocytes in the peripheral blood of rabbits infected with F.hepatica.

(15) Filtrates prepared from staphylococci of animal origin cannot be relied upon as non-specific mitogens.

(16) Staphylococcal filtrate using organisms of human origin facilitated the morphological examination of the stimulated lymphocytes as they do not clump together as they do with phytohaemagglutinin.

(17) The whole defibrinated blood technique was preferable for the studies on the in-vitro response of the lymphocytes to those using separated lymphocytes on grounds of facility, economy and efficiency.

(18) The optimal number of cells per culture of a total volume of 1 ml was found to be 0.5 million and maximum responses were obtained in the presence of 0.0625 ml antigen in cultures incubated for four days prior to labelling with tritiated thymidine for 24 hours.

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APPENDIX TABLE 3·1.

Haematological results - Eosinophils (cells per mm^3)

Rabbit Number	Pre-infection		Weeks after infection												Infective Dose of Metacercariae
	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	
RB 1	152	95	184	352	92	0	420	186	96	0	92	282	0	0	Control
RB 8	116	224	0	89	0	595	204	0	0	212	0	0	0	113	
RB 15	0	0	249	0	89	194	0	212	208	0	276	73	0	0	
RB 3	115	0	87	261	1905	5160	4642	3650	6739	1290	666	801	188	0	500
RB 7	206	91	276	468	1128	828	655	1270	1368	2952	328	348	588	2178	
RB 10	204	94	0	492	485	420	2295	8345	1521	1661	1254	328	556	1024	
RB 12	84	170	55	445	624	1034	1484	1827	1628	1161	885	595	432	315	
RB 13	91	0	0	1560	1683	536	426								1000
RB 14	84	121	0	2720	3400	1855	—								

APPENDIX TABLE 3·2.

Glutamic Dehydrogenase level (micro moles product per litre serum per minute)

Rabbit Number	Pre-infection		Weeks after infection											Infective Dose of Metacercariae
	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	
RB 1	0	0	4·8	5·8	0·5	0·5	0	4·8	0	0	0	0	0	7·2	Control
RB 8	0	0	0	4·8	0	7·2	1·4	4·3	0	0	0	0	0	2·4	
RB 15	2·4	0	0	5·3	0	6·3	4·8	9·6	6·0	0	0	1·0	2·4	1·0	
RB 3	0	0	4·8	7·7	5·8	4·3	9·7	11·7	6·8	0	5·3	4·8	7·2	3·4	500
RB 7	0	0	5·3	6·3	2·9	2·4	7·2	7·2	7·7	7·2	4·8	4·8	2·9	2·9	
RB 10	0	0	0·5	1·5	3·9	7·2	6·8	4·8	12·1	8·7	2·4	13·4	0	0	
RB 12	0·5	1·4	3·4	9·7	1·0	7·2	2·9	3·4	21·7	0	12·1	8·7	2·4	8·7	
RB 13	0	1·0	3·0	31·0	50·7	70·1	82·1								1000
RB 14	0	0	0	108·7	132·8	—	—								

APPENDIX TABLE 4·1.

Eosinophils (cells per mm^3)

Rabbit Number	Dose of Metacercariae	Pre-infection		Untreated Animals: Weeks after infection							
		-1	0	1	2	3	4	5	6	7	8
RB 3	C	91	80	100	0	85	110	0	0	90	100
RB 4		97	0	92	0	98	100	143	0	83	91
RB 8	50	103	100	75	550	981	1664	1110	2208	5859	1300
RB 15		0	0	200	876	1232	1264	2415	2880	2080	7298
RB 13	500	206	99	105	1550	2880	1448	3120	7412	3090	3842
RB 16		0	0	142	712	2156	4312	3717	4590	4071	1690
RB 9	1000	274	103	0	1804	3800	3140	4664	8550	3830	950
RB 11		103	0	255	1950	3660	5292	3760	10,186	1410	6490

Rabbit Number	Dose of Metacercariae	Pre-infection		Treated Animals: Weeks after infection							
		-1	0	1	2	3	4	5	6	7	8
RB 1	C	0	90	75	0	0	109	0	0	98	0
RB 2		110	85	186	0	87	120	115	98	0	103
RB 5	50	113	0	148	1430	2380	5100	4299	10,120	3723	2607
RB 6		250	103	198	650	2907	3200	2470	5544	6450	4040
RB 7	500	268	91	116	3060	5600	2304	2964	1350	280	264
RB 10		228	0	0	1560	3528	3336	2709	2120	1602	4572
RB 12	1000	96	100	102	399	640	660	830	1730	1780	3040
RB 14		0	81	172	960	1727	3540	3519	9800	3948	2132

APPENDIX TABLE 4·2.

Glutamic Dehydrogenase level (micro moles per litre serum per minute)

Rabbit Number	Dose of Metacercariae	Pre-infection		Untreated Animals: Weeks after infection							
		-1	0	1	2	3	4	5	6	7	8
RB 3	C	3·3	0	0	1·4	1·4	0	0	0	0	0
RB 4		0	0	0	1·4	0·9	0	0	2·4	0	0
RB 8	50	1·4	0	3·3	7·2	19·3	28·9	96·6	14·4	12·1	21·7
RB 15		0	1·2	0	3·3	14·4	21·7	48·8	70·0	48·3	12·6
RB 13	500	0·4	0	0	19·3	48·3	30·4	83·5	103·8	111·1	41·1
RB 16		0·2	1·4	2·4	14·4	33·8	24·5	33·8	108·7	60·4	39·6
RB 9	1000	0	1·4	0	16·9	45·8	42·5	103·8	56·5	36·2	45·8
RB 11		0·4	0	2·4	24·1	74·8	67·6	101·4	50·7	79·7	41·8

Rabbit Number	Dose of Metacercariae	Pre-infection		Treated Animals: Weeks after infection							
		-1	0	1	2	3	4	5	6	7	8
RB 1	C	·5	0	0	0	1·2	0	0	0·9	0	0
RB 2		0·5	4·8	0	1·4	0·5	0	0	0	0·5	0
RB 5	50	1·4	0	2·4	2·4	9·1	12·5	18·5	36·7	132·8	55·5
RB 6		0	0	1·4	4·8	21·7	31·4	74·3	103·8	89·3	28·5
RB 7	500	0	2·4	11·1	12·1	9·2	16·7	40·3	21·7	12·3	7·7
RB 10		0	0	0	6·7	21·7	14·5	115·4	43·4	21·7	9·6
RB 12	1000	2·4	0	0	9·6	60·4	74·8	108·6	95·6	84·5	16·9
RB 14		0	·9	7·2	28·5	74·8	86·9	118·3	91·7	4·8	9·6

APPENDIX TABLE 4·3.

Heterophils (cells per mm^3)

Rabbit Number	Dose of Metacercariae	Pre-infection		Untreated Animals: Weeks after infection							
		-1	0	1	2	3	4	5	6	7	8
RB 3	C	1092	1200	2200	2478	1020	2090	1974	1350	2076	1300
RB 4	C	1940	1953	2208	2120	1568	2500	2100	1819	1494	1729
RB 8	50	1030	1800	1425	3080	1210	2080	888	1950	9548	1950
RB 15	50	1280	1539	2500	3504	2618	3318	3220	5600	5280	6302
RB 13	500	1030	1188	1995	3410	4224	3080	6960	6322	5150	4972
RB 16	500	1200	1440	994	4736	4158	3432	3363	3060	2124	1950
RB 9	1000	1918	1030	1940	3116	4560	3744	3180	6555	5250	4750
RB 11	1000	1648	1634	1530	3900	5856	5488	3525	6984	3798	5015

Rabbit Number	Dose of Metacercariae	Pre-infection		Treated Animals: Weeks after infection							
		-1	0	1	2	3	4	5	6	7	8
RB 1	C	1485	1890	1275	2071	994	2180	2037	1290	1960	1863
RB 2	C	2860	1275	2325	1792	1218	2640	2530	3528	1296	2369
RB 5	50	2599	1700	1702	3900	3042	4250	3405	4301	7446	9243
RB 6	50	1000	1545	1089	3120	3420	4075	2324	3927	5934	2915
RB 7	500	972	819	1856	1270	3800	1584	1036	2250	1400	1584
RB 10	500	1828	1260	1911	2860	3190	2363	2322	8480	13,350	7366
RB 12	1000	768	1100	2346	4389	3840	4884	3276	6574	6408	4940
RB 14	1000	2490	1863	1462	5760	4368	7224	6417	6860	7896	5952

APPENDIX TABLE 5·1.

Eosinophils (cells per mm^3)

Rabbit Number	Initial Infection "Metacercariae"	Drug	Pre-infection		Weeks after infection							
			-1	0	1	2	3	4	5	6	7	8
RB 118	100	Control	103	0	0	1170	3486	3400	4140	4902	2100	2170
RB 122	100		278	103	152	332	1848	—	—	—	—	—
RB 130	500		224	119	140	420	334	636	1622	1520	1296	2670
RB 131	500		0	94	0	630	670	2093	3060	1880	3781	2450
RB 124	100	Rafoxanide	234	98	158	714	1152	3320	4600	1370	550	824
RB 125	100		107	87	97	565	936	846	2304	882	605	440
RB 126	500		113	98	198	1064	1980	3859	5880	2844	1170	226
RB 116	500		118	0	222	948	1098	1503	4644	2189	576	576
RB 127	100	Diamphen-ethide	126	166	78	1150	2898	1742	2896	1840	1440	1644
RB 128	100		116	105	186	345	1972	3078	3240	3006	2004	1683
RB 129	500		0	80	0	904	1834	2010	2814	3066	—	—
RB 117	500		214	174	376	178	756	1620	1080	492	—	—
RB 119	100	Nitroxynil	123	0	0	544	1812	1989	1120	1002	1128	1780
RB 121	100		91	0	0	702	1296	732	1072	1216	1616	3080
RB 120	500		246	0	110	399	1404	1836	1400	664	—	—
RB 123	500		113	103	140	752	2682	2192	2040	2236	2170	4114

APPENDIX TABLE 5·2.

Glutamic Dehydrogenase level (micro moles per litre serum per minute)

Rabbit Number	Infective Dose of Metacercariae	Drug	Pre-infection		Weeks after infection							
			-1	0	1	2	3	4	5	6	7	8
RB 118	100	0	0·48	0	2·8	2·4	14·4	20·8	15·9	33·8	10·2	9·2
RB 122	100		0	0	0	4·8	9·1	—	—	—	—	—
RB 130	500		0	1·4	5·3	6·2	12·1	10·6	19·2	34·4	21·7	13·5
RB 131	500		0	0	3·3	7·2	19·6	26·1	37·1	62·6	57·9	14·1
RB 124	100	Rafoxanide	0	0	0	0	3·8	18·3	16·4	13·9	3·8	0·5
RB 125	100		2·89	0	1·5	4·8	14·3	16·9	17·4	6·3	1·0	0
RB 126	500		0	0	6·3	9·6	14·7	24·9	48·3	14·5	13·5	2·4
RB 116	500		1·45	0	2·3	4·8	19·6	20·2	24·5	8·7	4·8	0
RB 127	100	Diamphen-ethide	1·25	1·21	4·8	4·3	12·1	14·4	25·1	20·3	21·5	11·1
RB 128	100		0	0	3·9	7·2	17·8	34·7	30·9	19·8	42·5	17·4
RB 129	500		0	0	9·7	15·8	19·3	28·8	73·0	49·7	—	—
RB 117	500		0	0	8·8	14·5	14·5	24·5	44·5	41·1	—	—
RB 119	100	Nitroxynil	0·48	0	2·9	4·5	6·8	19·8	37·2	17·3	9·5	4·3
RB 121	100		0	0	0	4·8	10·1	15·9	28·5	12·3	14·5	4·9
RB 120	500		0	0	3·9	5·9	12·3	21·7	30·9	27·5	—	—
RB 123	500		0	0	0	10·6	14·5	24·2	41·5	21·7	12·5	7·1

APPENDIX TABLE 5·3.

Packed cell volume (%)

Rabbit Number	Infective Dose of Metacercariae	Drug	Pre-infection		Weeks after infection							
			-1	0	1	2	3	4	5	6	7	8
RB 118	100	0	42·3	41·8	42·9	43·6	39·3	33·7	34·5	34·0	36·4	36·7
RB 122	100		41·0	39·7	37·3	42·8	41·1	—	—	—	—	—
RB 130	500		48·2	45·2	41·8	39·7	44·2	41·5	41·3	31·9	31·9	35·4
RB 131	500		49·3	45·3	40·2	41·1	39·7	35·5	30·0	32·2	32·2	32·0
RB 124	100	Rafoxanide	46·2	54·5	47·7	40·8	46·8	41·2	33·3	35·4	35·1	43·0
RB 125	100		46·3	50·0	49·6	50·5	53·8	45·6	38·2	38·2	55·4	45·1
RB 126	500		47·6	54·5	51·1	42·9	50·7	37·4	32·0	32·1	55·5	51·1
RB 116	500		55·5	54·1	56·8	49·2	58·5	39·5	34·1	36·4	36·1	41·2
RB 127	100	Diamphen-ethide	52·1	51·0	45·2	42·7	45·4	38·3	39·1	37·4	31·9	35·1
RB 128	100		53·5	51·0	47·3	43·7	43·3	39·9	38·2	32·1	33·3	36·9
RB 129	500		50·6	49·7	47·1	45·3	47·3	40·6	39·2	23·8	—	—
RB 117	500		42·2	43·2	40·5	42·5	49·8	45·6	34·1	33·0	—	—
RB 119	100	Nitroxynil	45·9	42·3	43·5	41·6	41·2	45·2	36·2	36·1	31·7	35·4
RB 121	100		48·7	45·9	58·6	36·3	44·0	38·5	36·5	34·1	31·2	32·0
RB 120	500		49·3	57·0	44·8	41·2	42·9	41·2	35·4	29·3	—	—
RB 123	500		48·3	45·3	41·8	42·2	45·5	39·7	35·6	33·4	36·9	37·5

APPENDIX TABLE 6·1.

Eosinophils (cells per mm^3)

Group	Rabbit Number	Initial Infective Dose of Metacercariae	Pre-infection	Weeks after Initial infection									Weeks after challenge			
			-1	1	2	3	4	5	6	7	8	9	10	11	12	13
A	RB 74	100	0	180	255	570	1430	2256	156	630	216	0	231	305	460	1612
	RB 71	100	0	115	103	1143	1875	2888	111	226	208	85	218	0	348	2070
	RB 75	100	0	0	602	889	2718	2880	1078	644	595	420	935	1648	1921	3620
	RB 53	100	0	123	232	1104	1332	2885	488	558	444	0	122	384	1920	2379
B	RB 48	500	279	109	264	1250	1716	1777	1528	1136	206	213	91	280	1016	2560
	RB 47	500	109	0	388	1440	1560	2580	1768	1260	279	76	288	270	464	1848
	RB 58	500	0	0	720	2369	1320	1545	1639	872	400	210	425	525	4020	4862
	RB 57	500	0	0	288	1944	1845	3768	500	452	475	525	648	940	2486	3675
C	RB 72	Nil	0	0	61	0	60	0	70	60	0	55	60	126	300	1152
	RB 51	Nil	182	182	126	170	91	0	0	0	0	0	105	380	1296	3672
	RB 138	Nil	78	75	78	184	65	65	0	0	0	0	0	218	368	365
	RB 78	Nil	82	0	60	80	55	138	80	0	0	75	0	166	508	1980
D	RB 70	100	192	91	388	1610	1624	1904	1320	752	321	475	552	440	297	496
	RB 73	100	0	0	340	1495	2451	3358	630	623	0	88	305	425	954	615
	RB 45	500	70	0	210	1350	1365	3680	1004	968	130	70	300	360	410	288
	RB 60	500	95	190	243	1500	1236	1810	1170	896	360	0	232	327	444	726

APPENDIX TABLE 6·2.

Heterophils (cells per mm^3)

Group	Rabbit Number	Initial Infection "Metacercariae"	Pre-infection	Weeks after Initial infection									Weeks after challenge			
			-1	1	2	3	4	5	6	7	8	9	10	11	12	13
A	RB 74	100	1440	1890	1785	2610	2420	3102	2340	990	1944	858	924	1525	1840	3100
	RB 71	100	2560	2415	2987	3032	2250	2584	3330	1808	1560	765	2725	1840	2784	3588
	RB 75	100	1881	1840	2580	4191	2718	3600	980	1104	2890	1995	2125	3193	2260	3620
	RB 53	100	1512	2706	2204	2622	2331	2790	1586	2046	2220	1805	1220	2176	2720	4392
B	RB 48	500	1581	1635	2200	3375	2244	2553	3583	1420	1236	1704	1729	1710	2286	3520
	RB 47	500	2398	1582	2037	4320	4056	4644	3404	1890	1209	532	2016	1530	1276	2926
	RB 58	500	2030	1500	1584	2781	2310	1957	2023	2616	1200	1120	2125	1725	2144	3740
	RB 57	500	2919	1500	2016	2481	2460	2752	3875	3277	1330	1275	2916	846	1695	3675
C	RB 72	Nil	2139	1495	854	1045	1200	858	560	900	540	770	1500	1512	1950	2880
	RB 51	Nil	1911	1365	1008	850	1001	710	960	1248	720	900	735	1330	1620	2448
	RB 138	Nil	1326	1425	1326	2116	1300	845	1155	1022	1440	1050	2392	2332	1564	1695
	RB 78	Nil	1640	1908	1200	1360	1100	1343	1200	1408	1440	900	2240	1992	3175	3696
D	RB 70	100	1824	2457	3104	2415	2784	2856	1320	1316	2354	2375	2668	1650	1386	1488
	RB 73	100	1221	1116	2125	1725	2838	1898	1710	1424	1110	880	1365	1445	1378	1476
	RB 45	500	1470	1120	1750	1950	2850	3496	2460	1232	1170	700	900	2040	1066	960
	RB 60	500	2090	1520	1134	2750	1957	2250	1521	1405	2430	1100	3828	1417	1554	1331

APPENDIX TABLE 6·3.

Glutamic Dehydrogenase level (micro moles per litre serum per minute)

Group	Rabbit Number	Initial Infection "Metacercariae"	Pre-infection	Weeks after Initial infection									Weeks after challenge infection			
			-1	1	2	3	4	5	6	7	8	9	10	11	12	13
A	RB 74	100	0·4	1·4	7·2	9·6	28·8	29·8	10·4	4·8	0	0	4·8	0	6·8	16·9
	RB 71	100	0	0	1·4	15·8	21·8	30·8	13·2	10·3	0	0	2·4	7·2	4·8	18·2
	RB 75	100	0	0	3·8	21·8	19·8	26·2	7·2	5·8	2·4	3·3	4·8	4·8	9·6	20·3
	RB 53	100	0	0	0	12·8	16·4	21·2	7·7	7·2	0	0·9	12·2	13·2	14·2	18·3
B	RB 48	500	0	0	6·3	24·1	24·5	48·3	12·1	9·2	4·4	0	2·8	13·2	19·8	29·8
	RB 47	500	0	0	12·4	19·7	31·6	26·2	12·4	0	0	0	0	4·8	17·2	21·2
	RB 58	500	2·4	0	14·3	18·5	20·8	30·4	9·6	0	4·8	4·8	7·3	8·6	18·6	15·7
	RB 57	500	0	0	0	13·5	17·2	17·2	2·4	9·6	0	0	9·7	8·3	13·2	17·2
C	RB 72	Nil	0	0	0	0	0	0	0	0	1·4	0	0	9·6	11·1	14·8
	RB 51	Nil	0	0	0	0	0	0	0·9	0·5	0	0	3·8	4·8	12·1	24·3
	RB 138	Nil	0	0	2·4	1·4	0	2·4	0	0	0	0	0	0	3·8	5·3
	RB 78	Nil	0	0	0·8	0	0	0	0	0	0	0	4·8	6·3	9·2	17·2
D	RB 70	100	0	0	2·9	0	19·8	11·1	12·1	6·3	7·2	4·4	7·2	4·8	2·9	0
	RB 73	100	0	0	0	12·2	24·5	26·6	5·3	5·8	0	0	2·4	0	0	0
	RB 45	500	0·1	2·4	9·6	19·5	21·5	33·3	11·8	4·8	4·4	2·4	2·8	4·8	0	0
	RB 60	500	0	0	5·3	25·8	30·2	21·7	7·2	2·4	0	0	0	3·8	0	3·3

APPENDIX TABLE 7·1.

Eosinophils (cells per mm^3)

Rabbit Number	Pre-infection	Weeks after infection										
	-1	2	4	6	8	10	12	14	16	18	20	22
RB 156	0	270	1197	565	240	1400	1476	2850	570	327	578	3432
RB 157	0	480	2054	380	412	280	584	450	308	154	332	720
RB 158	113	575	1860	600	570	445	1596	1250	399	840	721	1599
RB 159	80	195	1580	928	904	2105	1190	1677	625	180	364	2176
RB 168	170	78	0	89	178	246	1353	1596	721	300	492	1287
RB 169	95	92	0	0	0	190	1017	1378	620	195	351	4032
RB 170	0	166	0	272	186	306	1872	3690	936	568	624	2110
RB 171	0	81	0	0	80	210	837	1209	712	240	705	2288
RB 162	103	80	158	0	162	99	90	91	93	73	393	2115
RB 163	0	0	162	95	162	206	202	78	81	0	224	1467
RB 165	0	96	180	156	70	242	339	206	72	103	492	1836
RB 174	83	0	67	65	60	0	246	202	100	95	369	2715
RB 160	65	760	2505	828	240	480	1125	1474	798	204	80	81
RB 161	170	160	1896	903	320	450	1768	2574	854	154	0	0
RB 166	0	0	83	80	0	392	1974	2586	876	231	186	225
RB 167	90	0	90	0	60	618	1612	2431	810	288	182	69

APPENDIX TABLE 7·2.

Glutamic Dehydrogenase level (micro moles product per litre serum per minute)

Rabbit Number	Pre-infection	Weeks after infection										
	-1	2	4	6	8	10	12	14	16	18	20	22
RB 156	0	3·3	32·5	9·2	2·2	6·2	13·1	17·2	7·2	2·3	6·2	20·4
RB 157	1·5	5·3	26·8	10·9	2·5	3·8	9·1	18·3	8·6	3·2	5·3	12·8
RB 158	0·5	2·4	18·2	6·2	3·4	6·4	9·2	23·3	9·2	4·0	4·2	9·4
RB 159	0	6·4	25·9	8·9	4·2	7·3	8·6	20·3	10·3	5·2	9·8	19·2
RB 168	0	0	0	0	0	0	6·8	24·0	9·2	5·2	9·2	21·8
RB 169	0·3	0	0	0	0	2·8	12·1	32·0	12·3	3·4	13·2	26·2
RB 170	0	1·1	0	0	0·5	2·4	11·2	29·0	17·3	3·2	6·4	22·2
RB 171	0	1·2	0	0·5	0	0	10·3	17·0	6·2	4·3	15·4	18·2
RB 162	0·4	0	0	0	0	0	0	0	0	0	7·4	17·4
RB 163	0	0	0	0	0	0	0	0	2·2	0·4	12·2	30·0
RB 165	0	0	1·5	0	0	0	0·4	0	0·5	0	8·6	28·2
RB 174	0	0	1·2	0	0	0	0	0	0	0	9·3	35·9
RB 160	0	4·3	19·6	9·6	5·3	10·7	16·4	18·2	7·2	2·4	0·5	0
RB 161	0	6·2	20·2	7·2	3·6	9·6	11·2	23·2	12·3	3·4	0	2·3
RB 166	0	0	0	0	0	2·8	9·1	21·3	8·2	5·3	0	0
RB 167	0	0	0	0	0	3·4	10·1	30·1	14·2	3·3	0·4	0

APPENDIX TABLE 12·1.

Weekly uptake of tritiated thymidine by lymphocytes "control group"

Weeks after infection	Infected with 100 Metacercariae						Infected with 500 Metacercariae					
	Rabbit Number RB 118			Rabbit Number RB 122			Rabbit Number RB 130			Rabbit Number RB 131		
	C	FA	SF	C	FA	SF	C	FA	SF	C	FA	SF
-1	100 99 100	204 196 190	3550	128 247 144	227 267 300	4410	128 126 130	204 190 185	6933	165 192 138	163 162 160	6446
1	140 215 208	381 287 212	1180	97 72 116	254 238 313	3638	147 128 176	385 806 625	1170	106 136 130	256 364 307	3007
2	122 162 264	1066 1129 1394	10,374	121 148 95	2167 2060 3389	11,043	206 154 211	2709 3663 2041	10,151	200 139 142	2509 3090 2031	8981
3	180 135 188	827 728 1268	11,278	134 140 143	726 1123 2638	12,619	319 101 173	805 629 1193	6969	-	-	-
4	127 129 131	1546 2780 1154	2549	-	-	-	123 83 110	269 680 543	2101	91 95 94	290 633 361	435
5	105 115 72	418 716 629	1776	-	-	-	284 318 247	1750 1727 719	4314	165 210 153	1415 1460 1424	9219
6	111 146 153	1724 4855 4089	1960				210 217 199	2054 913 1397	4298	134 140 143	726 1123 2638	2916
7	127 178 125	1052 422 1211	5492	-	-	-	245 216 230	567 403 1481	8590	107 136 139	607 423 525	6857
8	96 111 104	1714 1980 1571	2128				234 260 198	2174 3293 3245		180 137 169	1525 1967 1025	1318

APPENDIX TABLE 12·2.

Weekly uptake of tritiated thymidine by lymphocytes "Rafoxanide treated group"

Weeks after infection	Infected with 100 Metacercariae						Infected with 500 Metacercariae					
	Rabbit Number RB 124			Rabbit Number RB 125			Rabbit Number RB 126			Rabbit Number RB 116		
	C	FA	SF	C	FA	SF	C	FA	SF	C	FA	SF
-1	89 49 39	131 128 185	12,348	59 81 104	136 133 111	2895	105 174 64	127 100 150	4650	142 272 136	215 206 159	6600
1	86 76 153	202 110 170	1703	68 60 96	201 341 296	2771	76 193 76	225 233 321	3086	178 184 100	593 395 482	3769
2	151 105 115	2406 2428 2642	16,331	95 75 90	1775 2200 1883	15,572	204 137 115	1403 1233 1014	10,078	203 170 159	2571 2250 2240	14,228
3	128 120 109	8249 8206 6211	24,815	69 60 48	2493 1617 1382	19,451	86 115 135	2480 1991 1781	12,726	123 105 103	1453 1250 1458	1990
4	117 64 61	364 512 438	2549	112 61 86	482 502 480	2700	129 88 62	1432 1182 1307	3505	107 108 71	298 289 295	2492
5	111 131 299	1587 1834	3387	94 105 101	3334 2485 3786	994	112 102 101	1012 516 657	1287	95 113 75	1573 1089 1347	3849
6	125 182 181	557 576 532	894	69 87 85	667 559 770	4876	127 145 369	3227 2957 4863	8880	88 109 91	452 259 444	1531
7	173 159 119	4192 3125 3426	12,639	72 116 90	6237 1750 2908	7891	75 94 86	3476 4100 3025	10,675	94 112 72	2004 1500 1465	2844
8	164 125 188	1423 1409 2115	2543	63 91 108	1056 1291 1142	1967	80 132 44	1893 1835 1716	3082	106 77 84	1421 1194 1981	4537

APPENDIX TABLE 12·3.

Weekly uptake of tritiated thymidine by lymphocytes "Diamphenethide treated group"

Weeks after infection	Infected with 100 Metacercariae						Infected with 500 Metacercariae					
	Rabbit Number RB 127			Rabbit Number RB 128			Rabbit Number RB 129			Rabbit Number RB 117		
	C	FA	SF	C	FA	SF	C	FA	SF	C	FA	SF
-1	171 59 182	270 212 250	7213	47 64 40	59 65 59	3927	124 96 113	231 145 88	2645	119 291 334	190 139 329	7336
1	166 86 93	249 305 165	2244	43 73 50	241 382 246	2822	219 136 56	364 381 277	1899	118 108 73	333 308 371	4773
2	140 128 143	1849 2836 1891	15,297	117 135 203	821 944 1051	11,835	115 80 139	1953 2413 2330	10,822	131 115 110	1795 2101 2273	11,634
3	89 104 87	3510 3513 3511	19,990	112 116 101	1857 2090 2077	15,114	95 72 88	2905 3197 1992	15,114	112 116 101	1857 2090 2077	15,114
4	90 83 103	803 793 1211	2912	71 119 89	617 503 309	2223	72 59 82	693 523 637	1985	76 58 61	820 731 442	9145
5	121 156 132	801 1233 608	2854	171 127 111	1124 915 618	4128	224 163 189	1630 1620 1347	3901	195 182 178	1695 1262 1024	4147
6	101 68 94	1239 1101 2434	4789	115 78 91	1260 893 1267	5613	151 222 201	2080 2396 2023	8701	83 81 57	1916 1474 2041	7811
7	132 122 79	2943 2657 1758	4951	94 98 111	1645 1400 2013	3567	-	-		-	-	
8	104 146 153	3755 4019 1724	3166	91 89 103	1546 1312 2111	3656	-	-		-	-	

APPENDIX TABLE 12·4.

Weekly uptake of tritiated thymidine by lymphocytes "Nitroxynil treated group"

Weeks after infection	Infected with 100 Metacercariae						Infected with 500 Metacercariae					
	Rabbit Number RB 119			Rabbit Number RB 121			Rabbit Number RB 120			Rabbit Number RB 123		
	C	FA	SF	C	FA	SF	C	FA	SF	C	FA	SF
-1	82 99 92	107 82 103	4410	167 182 152	219 289 291	2770	122 97 94	172 152 112	2254	140 108 122	208 257 256	4362
1	121 213 258	471 397 842	1980	189 154 151	239 365 389	4100	109 137 174	318 282 342	1465	156 130 136	1195 636 911	3778
2	150 175 146	1972 1407 1535	11,043	142 99 117	852 1227 1083	8981	107 97 115	695 525 719	7331	111 109 123	1387 2090 1403	10,151
3	127 89 130	2226 1956 2309	18,503	145 80 113	1056 1336 1186	12,009	108 109 123	2198 954 1208	12,619	349 153 157	2624 1935 3382	12,456
4	110 92 114	1154 1205 1185	2614	95 80 90	930 633 589	1898	68 74 86	361 463 613	4192	94 110 91	314 432 729	1806
5	137 177 172	2599 2943 2178	6122	185 175 176	1595 2263 1014	3146	128 172 191	711 1024 718	4208	199 182 169	1688 749 2364	2177
6	126 137 136	2177 1070 789	3292	151 281 143	987 699 794	1496	161 126 128	423 808 551	1960	132 195 193	1199 1424 1261	2449
7	161 190 126	868 973 500	3136	176 185 155	1013 946 767	2562	-	-		224 104 139	1827 1781 1704	6857
8	128 108 51	4368 3992 2842	2128	63 173 83	1124 1189 587	1865	-	-		94 122 107	1269 1575 1369	1318

APPENDIX TABLE 12·5.

Comparison of the number of small and large lymphocytes before and after infection

Rabbit Number	Infective Dose of Metacercariae	Drug	Pre-infection				Weeks post-infection															
			-1		0		1		2		3		4		5		6		7		8	
			LL	SL	LL	SL	LL	SL	LL	SL	LL	SL	LL	SL	LL	SL	LL	SL	LL	SL	LL	SL
RB 118	100	Nil	123	9594	89	7476	166	5478	1002	7488	664	7968	1190	5440	621	8280	3096	10,578	1670	8016	1550	9300
RB 122	100		182	6916	282	7708	532	4104	581	4814	616	6314	-	-	-	-	-	-	-	-	-	-
RB 130	500		123	8610	200	7600	140	4060	756	4956	364	5332	1166	5194	952	5848	912	6840	972	8424	1780	8188
RB 131	500		226	7810	327	7416	1560	10,764	945	6720	804	9380	644	7889	1530	7140	1650	8250	796	9552	1750	8925
RB 124	100	Flukanide	206	7622	106	7420	729	3318	833	5831	1008	7344	664	5644	2990	7820	1370	6028	220	6380	1545	5871
RB 125	100		317	9147	206	6695	629	4753	565	7119	780	8268	1269	7300	792	9900	822	8694	484	7865	660	8910
RB 126	500		224	7728	0	8062	495	6138	766	9880	1320	8415	2497	7037	2205	12,005	474	9480	580	7722	1243	7458
RB 116	500		357	7140	94	6768	999	5772	1264	9164	1098	8601	1503	8183	2064	9030	2786	6965	1584	7776	1296	8784
RB 127	100	Diamphen-ethide	234	8775	98	6174	546	3822	805	5960	2576	6279	670	5092	2210	6800	1560	8424	2016	6048	2055	7124
RB 128	100		428	7918	261	5964	0	5487	345	8050	1404	7800	1296	7614	1782	5994	1503	8350	2340	6847	2295	6885
RB 129	500		339	8375	196	7350	560	5680	791	6328	786	6943	804	6968	1742	5092	2336	5986	-	-	-	-
RB 117	500		282	6674	0	8475	470	5546	801	5429	864	5292	972	3996	972	4800	1845	3936	-	-	-	-
RB 119	100	Nitroxynil	378	8694	83	6059	200	7700	216	8568	755	7701	1377	7497	980	7000	2672	9018	2444	8084	1958	9790
RB 121	100		580	8584	105	7875	142	5254	546	3510	756	5508	1586	5490	938	7102	608	7600	606	8686	1078	6930
RB 120	500		105	6615	240	6800	110	8580	399	9177	1092	8736	1683	8415	2625	8225	996	8300	-	-	-	-
RB 123	500		535	7490	87	5394	910	4060	752	4700	2831	8493	2045	4795	816	7752	1132	8256	1086	10,850	1870	6358

LL = large lymphocytes.

SL = small lymphocytes.

APPENDIX TABLE 14·1(a).

Weekly uptake of tritiated thymidine by lymphocytes

Weeks after infection	Group 1								Group 2							
	RB 156		RB 157		RB 158		RB 159		RB 168		RB 169		RB 170		RB 171	
	C	FA	C	FA	C	FA	C	FA	C	FA	C	FA	C	FA	C	FA
-1	190	213	142	212	283	270	231	280	136	226	172	232	258	309	209	297
	201	290	159	211	189	233	179	220	250	362	153	212	164	254	184	240
2	105	3073	411	4717	490	1323	271	6864	280	326	223	274	240	329	157	230
	326	7387	318	6320	404	681	305	8475	292	432	227	157	251	379	272	428
4	155	2103	255	1543	159	531	595	2558	200	423	271	340	274	302	235	304
	182	1892	416	1163	92	538	561	1323	295	322	210	230	426	394	183	247
6	294	1230	574	1040	103	3357	430	2642	211	386	281	207	187	258	259	465
	449	2385	304	2101	195	5328	330	4961	386	534	293	371	151	217	283	548
8	370	1150	306	1267	214	1883	292	3472	209	294	238	305	291	345	351	336
	328	3893	253	1004	456	1009	192	2603	313	269	217	273	176	373	297	268
10	265	684	186	1034	248	1221	189	1798	154	2482	142	3690	332	2322	281	3001
	306	742	262	813	416	908	114	1169	300	2220	128	2706	256	2603	125	2591
12	334	1584	297	2033	256	1003	192	2906	164	3142	241	2500	363	3121	379	2014
	307	923	252	1013	341	2135	214	1096	210	2110	228	3617	129	1512	290	2691
14	511	2111	393	866	275	1732	547	2097	324	1857	199	2458	269	3623	274	2448
	317	1530	228	2804	341	1967	209	3169	304	2261	279	1261	299	4528	280	1741
16	373	717	266	603	197	869	263	2249	345	964	231	809	239	704	220	706
	411	702	327	1886	261	777	285	1428	273	892	311	1601	258	599	213	540
18	252	1733	199	2474	217	1360	195	4437	154	1826	286	2736	113	4309	185	2868
	395	2927	200	2020	152	1496	124	3374	295	3591	164	4101	195	5165	181	2380
20	524	520	188	908	194	770	185	893	132	639	252	591	193	581	207	987
	292	612	235	597	162	739	213	776	229	746	213	830	211	525	234	690
22	214	5022	242	1673	80	1842	220	1236	144	1001	217	1309	129	1800	134	909
	125	1572	156	1324	135	932	187	1092	123	604	190	903	181	1733	167	1031

APPENDIX TABLE 14·1(b).

Weekly uptake of tritiated thymidine by lymphocytes

Weeks after infection	Group 3								Group 4							
	RB 162		RB 163		RB 165		RB 174		RB 160		RB 161		RB 166		RB 167	
	C	FA	C	FA	C	FA	C	FA	C	FA	C	FA	C	FA	C	FA
-1	188	288	176	495	183	320	295	282	180	215	175	225	235	354	202	403
	222	331	295	305	194	289	208	349	222	249	191	231	212	343	182	392
2	172	346	213	274	152	289	139	396	265	9336	326	9436	224	401	210	418
	165	273	144	250	273	294	235	279	259	6434	323	8628	308	344	462	383
4	290	529	229	409	311	501	343	477	275	2736	223	2411	301	255	250	290
	276	304	411	433	145	314	275	396	524	2729	158	1983	167	314	271	333
6	226	371	297	273	416	488	260	591	228	9510	385	3154	306	515	298	563
	343	400	305	265	230	438	288	339	359	6912	304	2692	271	313	326	390
8	249	210	301	432	267	349	416	252	421	2740	227	1963	312	608	215	193
	185	245	253	364	314	291	270	292	378	1874	246	4015	447	235	289	371
10	188	297	125	320	233	304	287	441	302	931	126	409	279	1564	267	1453
	194	233	261	319	296	340	231	392	259	798	257	678	238	2534	237	2321
12	199	267	136	310	318	398	139	376	212	1311	125	697	291	1009	256	2351
	293	330	271	339	213	347	287	410	239	1890	239	888	316	936	235	4216
14	252	353	198	264	273	372	251	365	463	1592	185	951	289	1036	232	616
	270	461	180	392	382	471	321	347	312	2162	262	1362	324	1020	179	642
16	266	349	129	260	253	365	340	436	281	2150	206	1950	582	1498	229	646
	371	459	317	379	311	379	200	363	436	1516	165	2670	372	1592	379	957
18	231	293	290	304	307	391	261	397	367	3250	120	3196	253	4771	126	2230
	167	250	211	285	263	296	283	377	225	3530	236	2025	386	789	197	4290
20	351	3264	268	3378	361	2250	500	3487	301	1142	205	1032	475	1490	363	766
	344	2080	405	3068	293	2630	390	2138	426	1074	330	1164	362	1993	252	1320
22	220	2087	332	3892	319	2103	269	2395	323	1788	234	1216	174	1333	123	2325
	234	2692	224	2496	276	1035	218	1804	216	1683	291	1001	236	1546	215	2103

LABORATORY MEETING OF THE SCOTTISH BRANCH OF THE SOCIETY

Held in the Appleton Tower, University of Edinburgh, 7 May, 1974

DEMONSTRATIONS

Antigen induced *in vitro* transformation by the peripheral lymphocytes of rabbits infected with *Fasciola hepatica*

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Morphological and radio-tracer studies using ^{3}H thymidine have both shown that the peripheral lymphocytes of rabbits infected with *Fasciola hepatica* will transform in response to an antigen present in a supernatant extract prepared from homogenized, packed adult flukes, 1 : 5 v/v in MEM. The presence of the antigen caused the cells to incorporate at least 6 times the amount of the label as compared with other cells taken from the same pool but cultured in the absence of antigen. The extract also possesses a much smaller, non-specific effect.