STUDIES ON THE PASSIVE TRANSFER OF RESISTANCE TO *FASCIOLA HEPATICA*

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

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ABSTRACT

The main aims of this study were to determine the times after infection or reinfection that immune serum from bullocks infected with Fasciola hepatica would transfer protection to rats and to devise means of sensitising bullocks by which the animals could be stimulated to produce a strongly protective immune serum.

The serological response by enzyme-linked immunosorbent assay (ELISA), using either a somatic or a metabolic antigen, the serum glutamic dehydrogenase (GD) activities, and the peripheral eosinophil counts were monitored from both bullocks and rats during the infections.

Firstly, two bullocks of about 18 months of age were given an initial infection of 1,000 metacercariae of F. hepatica and immune sera (IBS) collected at three-weekly intervals and passively transferred to rats, to monitor their protective effect. Serum obtained during the prepatent period, 6-9 weeks after initial infection, gave partial protection. In an attempt to obtain a more strongly protective serum that could transfer a more effective resistance, the bullocks were given two more infections (22 weeks apart) of 1,000 metacercariae to each animal on each occasion. However, the IBS from previously infected bullocks gave only relatively weak protection. Indeed IBS from one of the bullocks (94), collected after repeated infections was not protective at all.

From this study it is clear that juvenile flukes are immunogenic, giving rise to humoral protective agents, since IBS collected at weeks 6-9 after initial infection was protective. Conversely, it is also clear that repeated oral infection is not a suitable method for enhancing the humoral protective response, probably because the

challenge flukes are soon killed by the cellular response in previously sensitised animals.

It was thought that age might influence the ability of immune serum from bullocks to transfer resistance. Accordingly three adult mature bullocks (4-5 years old) were infected with 1,000 metacercariae of F. hepatica and 30 weeks later another 1,000 metacercariae were administered. However the immune serum collected at week $9_{(1)}$ from one of the bullocks was not protective while that from the other two was rather weak. After secondary infection, the immune sera collected from 204 appeared to give some protection but not that from 203 or M199.

The opposite hypothesis was then examined, namely that older, immunologically mature animals rely more on a cellular response and consequently have a weaker humoral protective response. Accordingly four immature bullocks (6 months old) were infected with 1,000 metacercariae once, IBS collected six weeks later and again when the animals were killed nine weeks after infection. However these sera were not strongly protective to naive rats.

Following initial infection the animals tended to show a biphasic reaction in all the monitored parameters. However, because of great variations between animals, the pattern for some of the parameters was not always clear in individual bullocks.

The first ELISA peak occurred before patency and the second after patency. This phenomenon of a biphasic serological response was observed by Gundlach (1971) in rabbits, using metabolic or somatic antigen in complement fixation tests. It is suggested that the first ELISA peak coincides with liver migration and the active feeding by

the juvenile flukes on the parenchymal cells, while the second peak, which is usually lower than the first peak, coincides with an immune response to products released from flukes in the bile duct.

Following repeated infections, the ELISA values tended to be higher than those following primary infection. There was, therefore, no evidence of any direct relationship between the ELISA value and the protective effect of the sera. At the same time the serum GD activities and the peripheral eosinophil counts tended to be lower and faecal egg counts were very low after repeated infections, showing that the animals had resisted the challenge infections.

The GD and eosinophil levels rose to an initial peak at week $6_{(1)}$, with the maximum values being reached at weeks $12\text{-}16_{(1)}$ in bullocks. Again it is suggested that the first peak results from the direct effect of the parenchymal liver migration by the juvenile flukes, while the second peak probably results from the combined effect of both the fibrotic healing process in damaged liver and the presence of antigen-antibody complex in the liver.

It therefore appears that, although the bullocks previously sensitised by oral infection had acquired strong resistance against challenge infections, this was not related to the increased concentrations of antibodies in their immune serum but was more likely to be a cellular effect. Thus it was thought that other means than simply challenging previously infected animals would be necessary to produce more strongly protective antisera. Implantation of encapsulated flukes, which might be protected from the cellular protective mechanisms in the hosts and so be able to release the protective-inducing immunogen for a more extended period, was therefore adopted.

A preliminary study on the effect of implanting flukes in diffusion chambers on the immune response of animals was carried out in rats. The ELISA results suggested that there was an elevated ELISA response in the rats following implantation but this was short-lived. In the definitive experiment in bullocks, for logistic reasons, the serum was collected eight weeks after implantation, when a shorter period might have been optimal. Nevertheless, in the associated passive transfer study it was found that immune serum from the implanted bullocks tended to give better protection, except in one animal, than that from orally challenged bullocks. However, these results cannot be considered conclusive because of the limited numbers of experimental animals used.

The relatively short elevation in the ELISA value after implantation was attributed to encapsulation of the diffusion chambers containing the flukes by the host's cellular reaction against diffusing metabolic antigens.

CHAPTER ONE

GENERAL INTRODUCTION

Fascioliasis is a parasitic disease that affects all types of domestic ruminants. It is caused by the trematodes Fasciola hepatica and Fasciola gigantica, of which the former is the most common in temperate areas and the latter in the tropics.

Fasciola has an indirect life history. It requires a molluscan intermediate host in which it undergoes asexual reproduction.

From the infected mammals, the Fasciola eggs are voided with the faeces. The eggs embryonate in shallow water in about 17 days at 22°C and hatch on exposure to light, releasing a ciliated miracidium. The life span of a hatched miracidium is only about 24 hours (Smyth, 1962) in which time it must penetrate a snail host. The most common snail host for F. hepatica in Europe is Lymnea truncatula, an amphibious snail, whereas in the tropics F. gigantica commonly develops in Lymnea auricularis or one of its sub-species.

Inside the snail the miracidia develops into a sporocyst. Each germinal cell in the sporocyst, by growth and repeated divisions, becomes a germinal ball, which develops into a redia. Like the sporocysts the redia becomes packed with germinal balls, these being progenitors of a second generation redia. The germinal cells of these second generation rediae develop into a third kind of larvae, the cercariae. The cercariae are released from the snail and, after a brief swim, migrate a short distance above the water level on the surface of plants and encyst losing their tails in the process, to become a metacercaria. This is the form that is infective for cattle, sheep and other grazing animals.

In the mammalian host the metacercarial cyst is digested away, partly by the host's enzymes in the small intestine but partly by the activities of the young fluke. The young fluke then penetrates the wall of the intestine and crosses the peritoneal cavity to the liver, which it penetrates. After several weeks of migration through the hepatic parenchyma, the young flukes enter the bile ducts, mature into adult flukes and begin laying eggs (Gorgi and Theodorides, 1980). In rats infected with F. hepatica this takes about six weeks (Kendall, 1967), whereas in sheep it takes about eight weeks (Sinclair, 1962; Boray, 1967b; Kendall, 1967), and in cattle about nine weeks (Kendall, 1967). F. gigantica has a longer prepatent period of about 12 weeks (Soulsby, 1982).

The development of the disease can be divided into acute and chronic phases. The former phase occurs while immature flukes are migrating through the liver parenchyma. Disease seldom develops in cattle during the acute phase but deaths occur in sheep. On the other hand, the chronic phase, which commences when the flukes enter the bile duct, is responsible for heavy economic losses and its occurrence in cattle is very common.

Chick (1979) divided the economic losses due to chronic fascioliasis into direct and indirect losses. The direct loss is brought about by the condemnation of the livers of affected animals at slaughter (Argana and Sandili-Argana, 1971; Leimbacher, 1978), while indirect loss is caused by the reduced growth rate which arises from the increased protein catabolism coupled with reduced food intake (Sinclair, 1964; Sewell, 1976; Dargie, Berry and Parkins, 1979) and lower feed conversion efficiency (Armstrong and Miller, 1980; Chick, Coverdale and Jackson,

1980). These losses are aggravated when a lower plane of nutrition is available to the animals, especially during autumn (Roseby, 1970; Reid, Doyle, Armour and Jennings, 1972; Dargie and Berry, 1978) or a tropical dry season (Webster and Wilson, 1980).

Other indirect losses during the chronic phase of fascioliasis include a reduction in the yield and quality of the milk (Castagnetti, Losi and Morini, 1977; Froyd, 1978), a decrease in the number and activity of spermatozoites (Ramaniuk, 1978) and reduced wool growth in sheep (Hawkins and Morris, 1978).

To date the control of fascioliasis has involved managemental procedures and the use of anthelmintics or molluscicides. These are expensive and not always available. For example, the pasture management or controlled grazing procedures, which may be used as control measures against Fasciola infection, require additional labour and fencing (Sewell, 1976), while repeated use of anthelmintic is also expensive. Moreover some types of anthelmintic, especially the cheaper ones such as carbon tetrachloride, hexachlorophene and hexachloroethane, are less effective against the immature stages of the parasite (Boray, 1963; Kendall, 1973) and so cannot prevent much of the damage caused by the parasites. On the other hand, the disadvantages of using molluscicides lie in the host, the rapid repopulation by snails from outwith the treated area, the sophisticated understanding of the bionomics of the molluscs needed if the procedure is to succeed and the possibility of poisoning of animals exposed to the agent or of disturbing the ecosystem (Coyle, 1956; Berg, 1964).

The use of an effective non-living vaccine in the control of fascioliasis might be economical and practical if it could be produced

in a large quantity in the laboratory, administered with ease and was without harmful effects. However, despite much effort, such a vaccine has yet to be developed.

Rats (Thorpe, 1965; Hayes, Bailer and Mitrovic, 1972) and cattle (Ross, 1967b; Doyle, 1971) are known to be able to develop resistance to challenge infection with F. hepatica and the studies to be described are a contribution to the understanding of this phenomenon intended to facilitate the development of such a vaccine.

CHAPTER TWO

REVIEW OF THE LITERATURE

Immunity to Fasciola infection is the ability of animals to resist the pathogenic effects of the parasite and the toxic components of its secretions or excretions. This ability may be expressed in a variety of ways including a reduction in the number of flukes reaching the liver or bile duct, retardation of development of the flukes in the liver, elimination of the parasites from the liver parenchyma during the immature phase or reduction of the life span of adult trematodes in the bile ducts (Ross, 1967f; Armour and Dargie, 1973).

Immunity to fascioliasis may be divided into innate and acquired immunity. Innate immunity is the capacity to resist the pathogenic effect of the parasite directly associated with inherent qualities such as age, sex, species, breed or strain. This does not require previous contact with the parasite and is often non-specific. On the other hand, acquired immunity is an enhanced capacity of an animal to resist the infection brought about by the stimulation of its immune defence mechanisms. This therefore requires previous contact with the trematode. There is of course a sense in which the ability to acquire such resistance is itself an innate quality.

2.1 Innate Immunity

The difference in the response of animals within the same group to infection of Fasciola is termed individual idiosyncrasy. This phenomenon is brought about by the balance of the inherent qualities of the animals i.e. histological structure of the liver, age, sex and

strain of animals. This would to some degree be inseparable from the ability of the host to acquire resistance during the initial infection and effective against the parasites involved in this initial infection.

However, there are also consistent differences in innate immunity between the various potential host species. Thus it has been held that cattle and pigs are more resistant to Fasciola hepatica infection than other animals because of the relatively large amount of fibroblastic tissue in their livers (Ross, 1967f).

Age has been shown to influence resistance by animals to Fasciola infection. In rats, Ray (1970), Armour and Dargie (1973), Dargie (1973) and Hayes, Bailer and Mitrovic (1974a) showed that younger animals are more susceptible than older ones. In cattle, although an age effect has not been clearly described, Ross (1968) noted that 14-17 month-old steers were more resistant to initial heavy infections than younger ones.

Kendall, Herbert, Parfitt and Pierce (1967) reported a lack of any age resistance in rabbits. Although they recovered lower proportions of flukes from larger doses of metacercariae, they suggested that this might be a manifestation of competitive inhibition similar to that reported in rats by Thorpe (1965).

In sheep there has been no report on the influence of age on acquired resistance, however, Taylor (1949) noted no evidence of natural resistance to F. hepatica, and Durbin (1952) observed that infection in sheep can persist for at least 11 years.

Sex and strain of animals are other inherent factors that contribute to resistance to fascioliasis (Goose and MacGregor, 1974; Hughes, Harness and Doy, 1976), though these factors may well operate through the acquired resistance mechanism.

2.2 Acquired Immunity

This develops following contact of animals with the parasite. It may be demonstrated in cattle, mice or rats and possibly in sheep, goats or rabbits after oral or parenteral exposure to the trematode or after passive transfer of immune serum and/or lymphoid cells and leucocytes. Acquired immunity can thus either be active or passive.

2.2.1 Active acquired immunity

Contact with the parasite or its antigenic components is an essential factor for the induction of active acquired immunity. This contact sensitises the animals which are thus stimulated to produce antibodies and immune lymphoid cells against the parasites.

There are many ways of sensitising animals against Fasciola infection. These include oral infection with either normal or irradiated metacercariae, parenteral implantation of living flukes and active immunisation using either somatic or metabolic antigens.

2.2.1.1 Sensitisation by oral infection

Following oral infection the excysted flukes undergo a series of developments of their tegument that relates to the formation of different types of tegumental antigens. Bennett and Threadgold (1973, 1975) and Hanna (1980c) studied the development of tegumental 0 (T0), tegumental 1 (T1) and tegumental 2 (T2) antigens. T0 antigens originated from T0 granules which are formed from the time the fluke excysts and migrates to the peritoneal cavity and liver parenchyma. By the time the young flukes penetrate the liver, T1 granules have replaced T0. When flukes enter the bile duct the T1 bodies are replaced by T2. Therefore, during the whole course of Fasciola infection,

tegumental (T0, T1 and T2) and gut-released antigens are elaborated (Hanna and Trudgett, 1983; Demaree and Hillyer, 1982).

(a) Oral sensitisation by normal metacercariae

After an oral infection with normal metacercariae the animals are stimulated to produce several kinds of immune responses. These immune responses may be manifested by the reduction of the existing or challenge infection, serologically, or by changes in various biochemical or haematological parameters.

(i) Resistance to primary infection: Resistance to primary infection may be expressed as a reduction of eggs in the faeces or a reduction of fluke burden. It is influenced by a number of factors such as duration of infection, dose of infection, species, strain or age of the host.

In cattle exposed to infection with 200 metacercariae of F. hepatica, Ross (1968) observed a fall in faecal egg counts with time, corresponding to a reduction in the number of flukes in the bile ducts. From 10-18 months after infection, 50% of the faecal egg counts were negative, and from 18-26 months 70% were negative. At necropsy, a mean of only 13 flukes was recovered from animals slaughtered 21 months after infection, compared to a mean of 57 flukes recovered from calves slaughtered 2-5 months after infection. He suggested that extreme fibrosis and calcification of the duct wall, which occurs in cattle, was responsible for this reduction in the fluke burden.

Doyle (1971) observed elimination of 85% of the population derived from the single infection of 750 metacercariae of F. hepatica between 16 and 30 weeks after primary infection in calves.

In another experiment, Doyle (1972) recovered an average of 65 (13%) flukes from a single experimental infection of 500 metacercariae in calves killed 12, 16 and 20 weeks after infection. In contrast, only an average of 24 (4.8%) flukes were recovered from calves killed 24 weeks after infection. He suggested that given a single infection of 500 to 750 metacercariae, cattle start to eliminate their fluke burden between 20 and 24 weeks after infection. This phenomenon is preceded by a period of seven to eight weeks of reduced biological activity by the flukes that results in reduced faecal egg counts.

The dose of infection has been shown by some workers to influence the numbers of Fasciola that develop from a single infection (Ross, 1964, 1967e). Ross (1965a) observed a reduction in the fluke burden in cattle given heavy infections of between 1,300 and 15,000 metacercariae. In these infections many of the immature flukes reached the parenchymal migratory stage but were trapped in the liver parenchyma. It was also noted that fibrosis in the bile duct was not severe, indicating the presence of fewer flukes. In contrast in cattle given lighter infections of 200 to 300 metacercariae, more Fasciola reached the bile ducts resulting in severe fibrosis of the wall. However, liver cirrhosis was not as severe as in heavier infections. A similar observation of reduced fluke burden in rabbits given a high level infection when compared with those given a low level was made by Kendall and Sinclair (1971).

Ross, Todd and Dow (1966) also observed retardation of fluke development in calves given heavy infections of 1,300 metacercariae as against 200 or 300 metacercariae. The mean length of the flukes in the high level infections was 4.5 mm at week 23, while in the lighter

infections the fluke length was already 8.6 mm by week 14-19 after infection. They suggested that overcrowding was responsible for the reduction of fluke development in the heavier infections.

Boray (1967a) observed sexually immature F. hepatica in calves given a single infection with 10,000 metacercariae 10-14 weeks after infection, while in calves infected with 1,000 metacercariae the flukes became patent. There was spontaneous recovery in three out of four calves that were given infections with 1,000 metacercariae.

In sheep, Boray (1967b) studied the number of flukes that could be recovered following the use of varying sizes of infection. He observed that with infections of 200-1,000 metacercariae the mean recovery rate was 47%, but only 33% from infections with 2,000-10,000 metacercariae. Following infection with 2,000 or more metacercariae the development of the flukes was retarded by approximately 3 mm at 8-10 weeks and by 7 mm at 12 weeks after infection. The maturity of the flukes was also affected by the level of infection. Infection with 200 metacercariae resulted in patency at nine weeks after infection, with 500 metacercariae patency was reached by week 13-15, while following infection with 1,000 or more metacercariae F. hepatica did not reach patency before the death of the sheep in nine weeks.

After observing that there is variation in the rate at which individual flukes migrate to the liver, Boray (1969) suggested that the liver lesions induced by the flukes that reached the liver first rendered the environment unsuitable for the flukes that arrived later, thereby reducing the fluke burden in single infections.

In animals given multiple doses of metacercariae, Sinclair (1962) failed to demonstrate protection in sheep given four doses of 150

metacercariae each. However, he observed delayed migration of the parasite, reduction of egg production and later maturation of the adults. Likewise, Knight (1980a) failed to demonstrate protection in sheep given single or repeated infections, but noted a significant difference in the number of globule leucocytes in the liver and caecal tissues of infected sheep as compared to those in uninfected controls. These globule leucocytes did not appear to confer resistance to re-infection of the lambs with F. hepatica.

Sokolic, Sofrenovic, Sibalic, Cuperlovic, Dordevic and Lepojev (1968) studied the effect of repeated doses of metacercariae on the subsequent development of flukes in rats. They observed 10%, 2% and 3.5% recovery in rats given respectively one, two and three doses of 25 metacercariae each.

Over (1974) inoculated steers with primary, secondary and tertiary infections of F. hepatica at six week intervals. He did not find any reduction in worm burden from the second and third infections, but observed a "self-cure" phenomenon in all primary infections.

Factors such as species or strains, age or sex of the hosts have been shown by some workers to influence resistance to single infection.

Cattle are not likely to die during the migratory phase of the parasite in field infection (Ross, 1966b,c, 1968; Ross and Dow, 1966; Ross and Todd, 1968), whereas sheep often die of acute fascioliasis (Sewell, 1966; Ross, 1965b; 1967c,d,f; Reid, Armour, Urquhart and Jennings (1970).

Hughes, Harness and Doy (1976) studied the susceptibility of two strains of rats and noted that Piebald Virol Glaxo was more susceptible than Sprague Dawleys.

Studying the influence of sex and age, Dargie (1973) observed that male rats were more susceptible to F. hepatica than female rats, and also that young animals were more susceptible than old ones. The reason for this, he suggested, was the immature development of the immune response and poor development of hepatic fibrous tissue in young animals.

The susceptibility of Wistar rats to F. hepatica infection increases with age from weaning to reach a peak at puberty. After puberty they become more resistant. At puberty male rats are more susceptible than the female rats. Both sexes are resistant to challenge following an initial immunising infection but this resistance is stronger in female rats. This phenomenon was studied by Goose and MacGregor (1974).

Rajasekariah and Howell (1977b) studied the effects of the age of the host on the establishment of F. hepatica infection in male Wistar rats. They observed that within any one age group of rats (5, 10 or 25 weeks old), the proportion of metacercariae which developed to maturity was the same irrespective of dose. They suggested that neither a crowding effect nor competitive inhibition occurred. Five-week-old rats were generally susceptible while 10-week-old rats were resistant. In another study Rajasekariah and Howell (1981) showed that older rats were more resistant to both the oral and intraperitoneal routes of infection than younger rats and suggested that the resistance in older rats was related to the maturation of their haemapoietic system.

Urquhart (1954) also studied the influence of sex, age and the source of metacercariae on acquired resistance in rabbits or sheep

to Fasciola infection. He concluded that the factors that most influenced the number of flukes recovered from rabbits were the techniques used to infect the hosts or recover the parasites and the natural resistance of the animals. He further noted that the natural resistance of sheep to infection with F. hepatica was about the same level and within the same limits as that of rabbits.

Prior sensitisation of the hosts with Bacillus Calmette-Guerin (BCG) vaccine and its effect on subsequent infection was studied by Thompson and Howell (1979). They observed no significant difference in fluke burden between male Wistar rats vaccinated with BCG and non-vaccinated groups. However, rats which received BCG had mounted a cell-mediated immune response as indicated by marked hyperplasia of T-cells dependent areas of the spleen.

(ii) Resistance to challenge infection: Animals sensitised by previous oral infection with F. hepatica have been shown to acquire resistance to homologous challenge. This results in a reduction in the number of flukes establishing after challenge and was reported by Ross (1966a) and Kendall, Herbert, Parfitt and Pierce (1967) in rabbits; by Lang (1967) in mice; by Boray (1967a) and Ross (1967a,b) in cattle and by Hayes, Bailer and Mitrovic (1972, 1973), Campbell, Kelly and Martin (1979), Kelly and Campbell (1979) and Doy, Hughes and Harness (1978, 1981b) in rats.

Goose and MacGregor (1973a) described resistance to intraperitoneal challenge, while Hughes, Anderson and Harness (1976) described a resistance to subcutaneous challenge in rats sensitised by oral infection of F. hepatica. Likewise, Doyle (1971) observed a significant

reduction in the number of challenge flukes present in a group of cattle that was infected 17 weeks previously with 750 metacercariae of F. hepatica and killed 13 weeks after reinfection with 1,650 metacercariae of F. hepatica, as compared with cattle receiving only the challenge infection. This resistance to reinfection was also reflected in higher weight gains, relatively elevated serum albumin and lower serum globulin levels in the reinfected calves.

Workers who failed to confer such resistance to experimental hosts include Doyle (1973a) and Sinclair (1973, 1975a,b).

Doyle (1973a) failed to confer resistance to reinfection by calves when a challenge infection was given seven weeks after the initial infection of 750 metacercariae. However, he demonstrated resistance when calves were reinfected 12 weeks after the initial infection.

Likewise, Sinclair (1973) did not confer an ability to reduce the number of flukes that became established following a challenge infection in sheep previously infected with 500 metacercariae of F. hepatica and challenged 13 weeks later with the same number of metacercariae but did observe a temporary reduction in the development rate of the flukes, with delayed entry to the bile duct and later onset of clinical signs, and also an earlier rise in the number of peripheral eosinophils. Likewise Sandeman and Howell (1981) failed to demonstrate resistance to challenge in sheep previously infected with 100 metacercariae of F. hepatica and challenged 16 weeks later with a further 100 metacercariae, as compared with previously uninfected controls. They suggested that the presence of adult flukes in the bile ducts of the previously infected animals suppressed the antibody response following challenge.

Using the intraruminal, subcutaneous or intramuscular route of infection, Kelly and Campbell (1979) failed to confer resistance in sheep to intraruminal challenge infection with 400 metacercariae given 14 weeks after the previous sensitising infection of 400-500 metacercariae of F. hepatica.

The time at which the second infection is administered in previously infected animals had been shown by many workers to influence acquired resistance to challenge.

Ross (1966d, 1967b) observed a dramatic fall in the number of adult flukes originating from a challenge infection when this was given to calves 18 weeks after the previous infection. However, when the challenge was given only three weeks after the initial infection, there was no significant reduction in the number of flukes recovered. He concluded that the resistance to challenge was the result of a combination of the immune mechanism, the efficacy of which was related to the age of the existing infection, and cirrhosis of the liver.

Doyle (1973a) conferred resistance to challenge in calves given 1,300 metacercariae 12 weeks after the initial infection of F. hepatica, but failed to confer resistance when the challenge was given seven weeks after the previous infection.

In rats, many workers have investigated the influence of the time at which a challenge infection is administered on acquired resistance to the challenge. Campbell, Kelly and Martin (1979) noted a significant resistance to challenge of 400 metacercariae of F. hepatica given nine weeks after the initial infection with the same number of cysts; Goose and MacGregor (1973a,b), a similar high degree of resistance to challenge infection with 30 metacercariae when this was administered 63 and

98 days after an initial infection of 30 metacercariae; Hayes, Bailer and Mitrovic (1974a), a 66% or 50% reduction in the number of flukes developing after challenge with 20 metacercariae when this was given 28 and 48 weeks respectively after previous infection with five metacercariae of F. hepatica; Hayes, Bailer and Mitrovic (1972), a 92.5% reduction in the number of flukes developing after challenge with five metacercariae of F. hepatica when given 49 days after the initial infection of the same amount of metacercariae, compared with only 61.5% reduction in previously uninfected control; Doy, Hughes and Harness (1978) also found a high degree of resistance to challenge with 200 metacercariae of F. hepatica when this was given three weeks after the primary infection with 30 F. hepatica cysts.

Hayes and Mitrovic (1977) after successfully demonstrating resistance by necropsy 24 or 48 hours after challenge in rats given 30 metacercariae seven or 10 weeks after the previous infection with 10 or 15 cysts of F. hepatica, concluded that resistance to F. hepatica in rats is expressed within the first 24 hours after challenge. The earliest stage of the parasite would therefore seem to be the most likely source of an antigen with which to protect the host.

Lang (1966, 1968a) observed a significant reduction in the number of flukes developing after challenge in mice which received one or two immunising infections of two metacercariae each of F. hepatica as compared with previously uninfected controls. He further noted that the immune factors responsible for the observed immunity were effective between 20 and 25 days after challenge. He suggested that the specific antigen responsible for the induction of acquired immunity was released from the flukes between eight and 17 days before they migrated into the common bile duct.

The influence of dose of challenge on acquired resistance to challenge by previously sensitised rats was investigated by Rajasekariah and Howell (1978a,b). They observed that acquired resistance could be consistently demonstrated against a challenge of 30 metacercariae but not against five metacercariae in rats previously sensitised seven weeks beforehand by oral infection of five metacercariae.

The mechanisms involved in acquired resistance to challenge by previously sensitised animals have been suggested by Rajasekariah and Howell (1977a), Campbell, Kelly and Dineen (1978), Kelly, Campbell and Dineen (1980) and Hughes, Hanna and Symonds (1981) to operate in the gut region. This was supported by experiments using either oral or intraperitoneal route to challenge in previously sensitised rats. They observed fewer flukes recovered following orally challenged than after intraperitoneally challenged rats.

Doy and Hughes (1982) suggested that two mechanisms were involved in acquired resistance to challenge in previously infected rats. The first is a T-cell dependent system effective at the gut wall, while the second is effective after penetration of the gut wall but also requires a T-cell dependent response for full expression.

Studying the effect of an existing heterologous infection on F. hepatica challenge, Sirag, Christensen, Nansen, Monrad and Frandsen (1981) observed resistance against F. hepatica challenge in calves harbouring a patent 10-week-old Schistosoma bovis infection. The numbers of flukes establishing was reduced and resistance was also expressed in less severe liver damage as indicated by lower gamma-glutamyltranspeptidase levels, as compared with the challenge control group.

Christensen, Nansen and Frandsen (1978) observed that primary infection with Schistosoma mansoni, 2-28 days old, did not stimulate resistance to heterologous challenge with F. hepatica in mice. However, in older infections (54-60 days old) of S. mansoni, mice were protected from challenge with F. hepatica. Older infections with either male or female (mono-infection) S. mansoni did not protect against heterologous F. hepatica challenge. Conversely there was no heterologous resistance to S. mansoni in mice harbouring 7 to 23-day-old F. hepatica infection, but primary infections with 28, 32, 42 or 50-day old F. hepatica conferred significant resistance to heterologous challenge with S. mansoni. The existing F. hepatica infection was not affected by the challenge S. mansoni infection.

Campbell, Kelly, Townsend and Dineen (1977) found that there was a high level of protection in sheep infected with metacestodes of Taenia hydatigena (Cysticercus tenuicollis) against an intraruminal challenge with F. hepatica as measured by the number of flukes recovered from the liver and bile ducts and the counts of Fasciola eggs in the faeces. This resistance was demonstrated whether the existing 12-week-old metacestode infection was terminated by anthelmintics or not. In another study, Campbell, Dineen and Kelly (1978) again observed a significant protection against an intraruminal challenge with F. hepatica, as measured by recovery of flukes from liver and bile ducts and counts of fluke eggs in the faeces from sheep that had been harbouring a 12-week-old infection of C. tenuicollis. These sheep were protected from the pathologic effect of a challenge infection with 500 metacercariae of F. hepatica. When half of the sheep that had been challenged with F. hepatica were treated with rafoxanide to remove their fluke

infection, there was a high level of protection against a further F. hepatica challenge. They therefore suggested that sheep, suitably stimulated, can acquire significant protection against F. hepatica infection.

Campbell, Kelly and Martin (1979) observed a significant resistance against challenge with Taenia taeniaeformis in rats infected with F. hepatica. Infection with T. taeniaeformis protected rats against a homologous challenge given six weeks later. However, a primary infection with T. taeniaeformis did not protect rats against heterologous F. hepatica challenge.

(iii) Serological response: Many workers have studied the serological response of animals after an infection with normal metacercariae using different antigens in different serological diagnostic tests.

Movsesijan, Jovanovic, Aalund and Nansen (1975) monitored the humoral response of lambs infected with 500 metacercariae of F. hepatica, using two-month-old flukes derived from rabbits as antigen in an indirect fluorescent antibody technique (IFAT). They observed a marked increase of fluorescent antibody titres during the first six weeks, reaching a peak at week 6 after infection. They suggested that the peak at week 6 coincided with the time when tissue migration of the parasite was nearly completed and the parasite could be found in a localised phase before entry into the bile duct. After the infection became patent they still observed high titres, but from week 16 onwards these gradually decreased. They further observed increased levels of IgG-1 at weeks 6 and 16 after infection but there was no increase in IgG-2.

Using a somatic extract as the antigen to monitor the antibody response of sheep with fascioliasis by immunoelectrophoresis and a double diffusion test, Gundlach and Sadzikowski (1980) first detected antibodies two to three weeks after infection, and then these remained detectable up to week 19. The precipitins were higher in animals with heavier infections and during patency than in animals which had naturally acquired a light infection. In experimental fascioliasis they found immunoelectrophoresis more useful and sensitive than the double diffusion. However both the tests were less useful in natural infections.

In calves, Hanna and Jura (1977) studied the applicability of IFAT to the early diagnosis of fascioliasis using in vitro excysted flukes as antigen, and monitored the antibody response to Fasciola gigantica throughout a 30-week period of infection. They observed positive titres from week 2 until week 18 after infection. The maximum titres of 1/128 were recorded in serum samples collected between three and six weeks after infection.

In rats, Sinclair and Wassal (1981) monitored the serological response of animals given single or double infections of F. hepatica using the enzyme-linked immunosorbent assay (ELISA) and the Ouchterlony double diffusion precipitation test. Antibodies were demonstrated in all rats receiving double infections by the two tests. However, following single infection, antibodies were detected only by ELISA two weeks after infection. Using a peroxidase antiperoxidase (PAP) staining technique to demonstrate IgG in the liver sections of infected rats, they showed an increased concentration of IgG in the liver three to nine days after the second infection. They suggested that IgG

played a role in the immune mechanism of the rat.

Finally, in rabbits, Gundlach (1971) monitored the serological response of these hosts when infected with single or double doses of metacercariae using whole somatic, metabolic or acid polysaccharide fraction (C) as antigens in the complement fixation test (CFT), passive haemagglutination, gel precipitation and immunoelectrophoresis. He observed the first appearance of antibodies on the 14th day, with the highest level on the 5th to 9th week after infection, and suggested that the appearance and level of antibodies in the serum was dependent on the intensity and duration of infection. The suitability of the metabolic or somatic antigens for diagnostic purposes using CFT and the antigenic fraction C in the haemagglutination test was noted.

Other workers have demonstrated that the different serological diagnostic tests are mainly detecting different types of antibodies.

Hanna (1979, 1980b) tested the specificity of sera from infected sheep collected throughout a 30-week period of infection by indirect fluorescent antibody labelling using as antigen JB4-plastic embedded sections of flukes of various ages up to 30 weeks old obtained from rats. He demonstrated that sera from early infections (before six or seven weeks) gave strong labelling over the tegument of juvenile fluke tissues but that the reaction became progressively weaker with older fluke tissues. This was associated with the decline in the number of T1 bodies in the tegument as revealed by electron microscopy. T1 bodies contain glycocalyx precursor substance and, as mentioned earlier (Section 2.2.1.1), during development they replace the antigenically similar T0 secretory bodies, which are characteristic of early juvenile flukes.

Hanna (1980d) and Hughes, Hanna and Symonds (1981) estimated the concentrations of IgG and IgA specific for fluke tegumental and gut antigens in the sera and bile of infected calves using an indirect fluorescent antibody labelling and plastic-embedded sections of juvenile and adult flukes as antigens. Antibodies against juvenile (T1) tegument and gut antigens reached peak concentrations four to six weeks after infection and declined slowly thereafter as the flukes became established in the bile duct. IgG against adult tegument (T2) antigens appeared in the serum six weeks after infection, but no IgA against T2 was detected. In the bile, both IgG and IgA titres against T1 and gut antigens rose to peak values at weeks 4-6 after infection, but there was no activity against T2 antigens.

Doyle (1973b) first detected precipitating antibodies to Fasciola somatic antigens two to four weeks after infection in calves using double diffusion in agar in a two or in a single dimension in a glass tube. The precipitating antibodies were detected throughout the course of infection and were still present up to 28 weeks after infection. In another study Doyle (1973c) demonstrated haemocytotropic antibodies in the sera of calves experimentally infected with F. hepatica using a lipid-free extract of F. hepatica in a passive cutaneous anaphylaxis (PCA) test. The highest level of haemocytotropic antibodies were noted between 20 and 25 weeks after infection, coinciding with the period of expulsion of the parasite from the bile duct of the animals.

Flagstad and Eriksen (1974a,b) demonstrated IgA in the livers of calves experiencing a single infection of 200 metacercariae of F. hepatica, using the fluorescent antibody technique. IgG-1 was demonstrated in calves receiving repeated infections. In the hepatic lymph nodes

IgG-1 was demonstrated in calves receiving either single or repeated infections.

Furmaga and Gundlach (1980) showed elevated levels of serum β-globulins in calves from week 10 after infection with F. hepatica as compared with the uninfected control. The levels of γ-globulins increased from eight to nine weeks after infection until the end of the experiment, first appearing as early as the 4th week with two peaks at six and 12 weeks after infection. The levels of α-globulins did not differ greatly from the uninfected control.

Using the PCA test, Ershov and Poluektova (1978) demonstrated reagin-like antibodies in rabbits 28-58 days after a single infection. Repeated infections stimulated the formation of high titres of reaginic antibodies until day 120, with a peak at day 42 to 47 after infection. Similar reaginic antibodies were demonstrated by Doy, Hughes and Harness (1981a) in the serum of rats three weeks after infection.

Finally, using either the passive haemagglutination test or CFT, Sokolic (1968) was able to demonstrate complement-fixing antibodies in mice infected with either normal or irradiated metacercariae, but failed to show haemagglutinating antibodies. Following infection with normal metacercariae, complement-fixing antibodies disappeared four weeks after initial infection but increased to high levels after repeated infections.

(iv) Biochemical response: Boyd (1962) observed glutamic dehydrogenase (GD) to be an enzyme specific for liver damage in cattle and also high levels of glutamate pyruvate transaminase (GPT) in rat liver. Sewell (1967) utilised serum GD activity to monitor liver damage in ovine fascioliasis. Anderson, Berrett, Brush, Herbert, Parfitt

and Patterson (1977) and Burden, Hughes, Hammet and Collis (1978) used gamma-glutamyltranspeptidase (GGT), while Pullan, Sewell and Hammond(1970) and Sykes, Coop and Robinson (1980) utilised aspartate aminotransferase (Aspt) in a similar way.

The activities of these enzymes have since been used by many workers to monitor resistance to Fasciola infection, as indicated by lower levels at certain stages of infection in the plasma of resistant animals because fewer flukes cause less parenchymal damage.

Nansen (1975) observed a reduced level of plasma gamma-glutamyl-transpeptidase in calves infected with irradiated metacercariae before challenge as compared with the challenge controls. Likewise, Rajasekariah and Howell (1980) observed a lower level of serum GD activities in rats previously infected with 30 metacercariae and later challenged as compared with previously uninfected controls. They suggested that the presence of these enzymes in the serum could be used as an indicator of resistance to reinfection.

In contrast Anderson, Matthews, Berrett, Brush and Patterson (1981) showed an increase in plasma GD and GGT activities in calves given 10, 50 or 100 metacercariae weekly for 12 consecutive weeks. The animals that received higher doses had the greater increase in GD. The maximum increase of individual animals occurred between the 13th and 15th week after the first infection or between one and three weeks after the end of the dosing schedule. Likewise, Sykes et al. (1980) observed a significant increase in plasma GD activity, which correlated with the levels of infection after 9, 22 and 23 weeks, and in GGT activity after 12, 24 and 32 weeks for groups of sheep receiving weekly doses of 14, eight and three metacercariae respectively over a period of 22 weeks. Changes in Aspt were not so clearly related to dose level. They concluded that GD and GGT activities were more

sensitive indicators of liver damage in chronic sub-clinical fascioliasis than Aspt activity.

(v) Haematological response: Eosinophilia is another manifestation of the immune response found in animals following Fasciola infection. It was considered by Furmaga and Gundlach (1978) to relate to the "cell-participation-defence process" against the parasites. In calves infected and reinfected with F. hepatica they showed a many-fold increase in the eosinophil counts as compared with the uninfected control.

In rabbits infected with 500 or 1,000 metacercariae, Bolbol (1975) observed eosinophilia up to seven weeks after infection, and Furmaga, Gundlach and Sobieszewski (1974) observed a similar response in rabbits and sheep during the acute phase of invasion (3-10 weeks) by the fluke. Thereafter the counts dropped to approximately normal levels by week 11.

Akahane (1975) studied the response of heavily and slightly infected rabbits and concluded that the eosinophilia appeared earlier in heavily infected than in slightly infected animals.

Doy, Hughes and Harness (1981b) observed eosinophilia in rats after primary infection, reaching a peak between four and six weeks, and falling to control levels by week 14. After reinfection the increase was more pronounced than in primary infection.

(vi) Other response: Foster (1981) after infecting rats with 25 metacercariae of F. hepatica was able to demonstrate an increase in the number of epithelial cells in the common bile duct. This type of hyperplasia occurred without the mechanical presence of an adult fluke. Although he did not suggest this phenomenon was an immune response, Foster hypothesized that the enlargement of the bile duct would render the environment unsuitable for flukes.

(b) Oral sensitisation by irradiated metacercariae

It has been shown that irradiation with gamma rays or x-rays reduces the pathogenicity of metacercariae (Hughes, 1962a). He and other workers noted that attenuation of metacercariae depended on the dose of radiation imposed on the cysts. Accordingly by using irradiated metacercariae as against normal metacercariae for immunising animals, larger doses can be tolerated by the host without inducing disease (Sokolic, 1968).

Hughes (1962a) monitored the development of irradiated metacercariae in mice. He observed that the minimum level of x-irradiation which would reduce the pathogenicity of metacercariae, as indicated by the ability of animals to survive after an oral infection with irradiated metacercariae, was between 2 and 4 Kiloroentgens (Kr). Following 4 Kr irradiation, living immature flukes could be found in the liver up to 32 days after infection but most of them died in the liver 6-28 days after infection.

Using metacercariae of F. gigantica, Wikerhauser (1961a) observed 3 to 20 Kr was sufficient to reduce pathogenicity of the cysts; while Jarrett, Jennings, MacIntyre, Mulligan, Sharp and Urquhart (1959), using the same species of fluke, noted 5 Kr was sufficient to inactivate metacercariae.

By electron microscopic examination of irradiated metacercariae, Hughes, Doy, Burden and Oldham (1982), Burden, Bland and Hughes (1982) and Burden, Bland, Hughes and Hammet (1983) showed that after 3 Kr irradiation there was sequential appearance of T0, T1 and T2 granules in the flukes, while at 4 Kr most flukes failed to develop beyond the T0 granules stage.

(i) Resistance to challenge infection: The effect of varying the dose of radiation imposed on metacercariae on acquired resistance to challenge in rats was studied by Thorpe and Broome (1962). They found that the administration of 40 metacercariae that had been irradiated at 1-7.5 Kr led to rats showing resistance to a subsequent challenge with normal metacercariae. The resistance was stronger and developed more quickly following immunisation with metacercariae that had received 1-2.5 Kr than with those receiving higher levels. Metacercariae irradiated at 10 Kr did not elicit resistance.

Using multiple doses of metacercariae irradiated at varying levels of radiation, many workers have conferred resistance to challenge with normal metacercariae of F. hepatica in different hosts.

Corba, Armour, Roberts and Urquhart (1971) and Armour and Dargie (1974) observed a significant reduction in the number of flukes developing after challenge in rats immunised with two or three doses of 20 metacercariae each at weekly intervals. The metacercariae had been irradiated at 2-3 Kr. Likewise, Dargie (1973) recovered fewer flukes from rats previously exposed to three doses of 20 irradiated metacercariae (3 Kr) at weekly intervals than from non-vaccinated controls exposed to the same challenge of 20 metacercariae.

Harness, Hughes and Doy (1976) observed a significant reduction in the number of flukes recovered from the peritoneal cavity two days after challenge with 100 normal metacercariae in mice vaccinated with two doses, seven days apart, of metacercariae irradiated at 3.8 Kr. However in another study, using a similar vaccine, Harness, Doy and Hughes (1977b) did not observe any difference in the numbers of flukes recovered after challenge with 100 normal metacercariae of F. hepatica

between the vaccinated and non-vaccinated groups necropsied 14 days after challenge. They therefore suggested that the lower number of challenge flukes recovered from the peritoneal cavity of the vaccinated mice killed two days after reinfection was due to early migration of the flukes to the liver in immunised mice.

In natural infections Nansen (1974, 1975) observed significant protection in calves which had each been vaccinated with three doses of 1,500 metacercariae attenuated with gamma-irradiation at 3 Kr as compared with non-vaccinated controls, while in an experimental infection using metacercariae of F. gigantica irradiated at 3 Kr, Bitakamire (1973) observed a significant reduction in the fluke burden after challenge with 1,000 non-irradiated metacercariae of F. gigantica in calves vaccinated with 1,000 metacercariae of F. gigantica irradiated at 3 Kr as compared with non-vaccinated controls.

In sheep given six doses of 100 metacercariae irradiated at 3 Kr and then challenged with 750 normal metacercariae, Dargie, Armour, Rushton and Murray (1974) observed a mean of 36 flukes in the vaccinated group and 105 in non-vaccinated sheep. However, there was much variation and the difference in mean fluke recoveries was not statistically significant.

Other workers who failed to confer resistance to challenge infection with normal metacercariae by animals immunised with irradiated metacercariae of F. hepatica include Hughes (1962b) in mice and rabbits; Hughes (1963) in rabbits and sheep; Dawes (1964) and Sokolic (1967) in mice; and Chiroboga, Leon, Liard, Velarde and Gomez (1976) in rats.

Although Boray (1967a) did not observe any difference in the number of flukes recovered after challenge between groups of calves

vaccinated with metacercariae irradiated at 20 Kr and a non-vaccinated group, he observed fewer pathological changes in the vaccinated group. Likewise, Campbell, Gregg, Kelly and Dineen (1978) failed to demonstrate resistance against an intraruminal challenge in sheep vaccinated twice, six weeks apart, with 100 or 1,000 metacercariae of F. hepatica irradiated at 2.5 Kr. However they did observe a significant increase in the proportion of retarded flukes in the vaccinated sheep.

An influence of time of challenge in animals vaccinated with irradiated metacercariae was observed by Armour, Dargie, Doyle, Murray, Robinson and Rushton (1974). They infected rats one month apart with two doses of 1,000 metacercariae irradiated at 3.5 Kr. They noted a 30% reduction in the number of flukes after challenge when the animals were reinfected with 1,000 normal metacercariae four weeks after vaccination and a 70% reduction in those given the challenge infection eight weeks after vaccination. However, there was no clear difference in the liver lesions between the vaccinated and non-vaccinated groups.

(ii) Serological response: Hughes, Hanna and Doy (1982) monitored the antibody response of cattle, sheep and rats infected per os with metacercariae of F. hepatica irradiated at 4 Kr, to metacercarial tegumental (T0), juvenile tegumental (T1), adult tegumental (T2) and gut antigens using an indirect fluorescent antibody labelling technique. They failed to show antibodies against T0, T1 or gut antigens but they demonstrated the late appearance of antibodies against T2. They suggested that the appearance of antibodies against T2 at a titre lower than that following infection with normal metacercariae, was caused by the flukes which "broke through" the developmental barrier imposed by irradiation.

Similarly, Chiroboga et al. (1976) failed to show precipitins in the sera of rats infected with metacercariae of F. hepatica irradiated with cobalt-60 gamma-rays. Precipitating antibodies were observed in the sera of rats infected with normal metacercariae.

(iii) Biochemical response: Nansen (1975) utilised plasma GGT activity to show the protective effect of an initial infection with irradiated larvae of F. hepatica to subsequent natural infection in calves. He showed an increase of about five-fold in plasma GGT activity in non-vaccinated calves, compared with only a two-fold increase in vaccinated animals.

2.2.1.2 Sensitisation by primary infection terminated by anthelmintic

The elimination of an existing primary infection has been employed to exclude the influence of liver damage induced by the primary infection on acquired resistance to challenge infection, and to assess the persistence of the resistance afforded by the primary infection after it had been removed (Sinclair, 1971).

(i) Resistance to challenge infection: Workers who have observed resistance to challenge infection after elimination of primary infection by anthelmintic in various hosts include Boray (1967a), Armour, Dargie, Doyle, Murray, Robinson and Rushton (1974) and Kendall, Sinclair, Everett and Parfitt (1978) in cattle; Sinclair (1975a) in sheep; Goose and MacGregor (1973a), Armour, Dargie et al. (1974) and Haroun, Hammond and Sewell (1980) in rats; and Bolbol (1975) in rabbits given two infections before administration of the anthelmintic.

On the other hand, workers who did not observe any significant resistance to challenge after elimination of a previous infection by

anthelmintic include Boray (1967a), Sinclair (1971) and Meek and Morris (1979) in sheep; Harness et al. (1977a,b) in mice; Kendall and Sinclair (1971) and Haroun et al. (1980) in rabbits; and Bolbol (1975) in rabbits given a single pre-challenge infection.

Although Rushton, Murray, Armour and Dargie (1974) did not observe resistance to challenge in terms of the numbers of flukes which developed, they noted a prolonged phase of parenchymal migration, as reflected in an increased SGOT in the previously infected sheep as compared to the challenge controls. This prolonged phase of parenchymal migration was thought to be brought about by the enhanced accumulation of local lymphoid cells surrounding the challenge flukes.

Boray (1967a) found no significant resistance in sheep repeatedly infected with metacercariae, treated with anthelmintic and challenged, as compared to untreated controls. However the treated sheep lived longer, there was a delayed onset in the anaemia and fewer liver lesions as compared to untreated controls. Likewise, Knight (1980b) did not observe any reduction in the number of flukes developing after challenge in sheep previously given repeated infections that were eliminated by anthelmintic as compared to previously uninfected challenge controls. However there were more small-sized flukes in the previously infected sheep than in the controls. He considered the retardation of development of the flukes to be evidence of acquired resistance by the sheep.

Corba and Spaldonova (1975) observed a reduction in the number of sexually developed flukes following challenge in rats when an immunising infection had been eliminated at week 8-10. Kendall and Sinclair (1971) observed a reduction in the number of flukes derived from a

challenge infection in rabbits whose primary infection had been terminated by hexachlorophene two days before challenge.

(ii) Serological response: Haroun (1979) observed increasing antibody titres by ELISA from the 2nd week until the 14th to 18th week after reinfection in rats whose mature or immature primary infection was terminated by diamphenethide before challenge. In another study using rabbits he observed a steady rise in ELISA values despite treatment and reinfection, to reach a constant plateau by the 10th week. The highest ELISA value was reached 20 weeks after the initial infection.

Using IFAT on serial sections of plastic-embedded fluke tissue as the test antigen, Hanna, Hughes and Taylor (1982) failed to demonstrate antibodies against T1 antigen in sheep in which the primary infection had been terminated with anthelmintic three weeks after infection. Treatment of the primary infection resulted in a decline in antibody titre against the T2 and gut antigens. In contrast, in the untreated controls the antibody titres against T1, T2 and gut antigens appeared normally.

(iii) Biochemical response: Haroun (1979) observed a significantly reduced peak in GD activity at week 4 or 6 after challenge in the serum of rats or rabbits whose primary infection was eliminated by anthelmintic as compared with untreated controls.

Anderson, Berrett and Patterson (1978) found that there was no increase in plasma GD or GGT activities in calves reinfected 35 or 54 weeks after a previous 31-week-old infection with 1,000 metacercariae had been terminated by anthelmintic. In contrast, there was

a large increase in plasma GGT activity in calves receiving the primary infection alone.

(iv) Haematological response: In a series of experiments on acquired resistance to challenge infections by rats after immature or mature infections with F. hepatica were eliminated by anthelmintic, Haroun (1979) observed increasing eosinophil counts with a lower peak at week 4 or 6 after challenge in treated rats as compared to untreated controls. In contrast, rabbits whose mature or immature infections were also terminated by rafoxanide, the peak eosinophil counts at weeks 4 or 6 after challenge were not significantly different from those in the untreated control.

2.2.1.3 Sensitisation by parenteral implantation of adult flukes

Parenteral implantation of adult flukes by-passes the hosts' gut region, thereby eliminating any influence of the intestinal wall on acquired resistance to challenge (Hughes, Harness and Doy, 1981). If the implanted flukes are also encapsulated the technique may also be employed to expose the animals solely to the metabolic products excreted or secreted by the flukes.

(a) Sensitisation by free flukes

The level of protection against an oral challenge afforded by implantation of living adult flukes, as indicated by the reduction in the number of flukes developing after challenge in implanted animals, may be influenced by the site of implantation or the age of the implanted flukes.

(i) Resistance to challenge: Ross (1967a) implanted 6-week-old flukes into the intercostal muscles of lambs. Following oral challenge with 1,000 metacercariae there was a reduction in the pooled mean fluke

numbers in animals receiving single or double implants as compared to the challenge controls. However this was only significant at the 10% level.

Anderson, Hughes and Harness (1975) observed a significant reduction in the fluke burden following challenge in rats implanted subcutaneously with living adult flukes. Similarly although Haroun (1979) failed to confer resistance to challenge in rabbits implanted subcutaneously with adult F. hepatica, he observed a significant reduction in the number of flukes developing after challenge in rats similarly implanted with adult flukes.

The influence of the age of implanted flukes on acquired resistance to F. hepatica in mice was studied by Lang (1968b, 1974a) and Lang and Dronen (1972).

Lang (1974a) implanted 12, 14, 18, 20 and 21 day old juvenile F. hepatica into the intraperitoneal cavity of normal mice in an attempt to relate the duration of liver migration and fluke egg to the stimulation of acquired immunity. He observed that 12-18 day old flukes produced a significant reduction in the numbers of flukes developing after challenge as compared with an unimplanted control, while 22-24 day old flukes did not stimulate a significant resistance.

Eriksen and Flagstad (1974a,b) studied the effect of the source of subcutaneously implanted flukes on acquired resistance of F. hepatica in rats. Adult flukes taken from sheep, goats or cattle and transferred at week 3 or week 4 before challenge reduced by 50% the fluke burden which developed following challenge. They suggested that the production of protective antibodies was stimulated by undamaged living flukes but did not identify the antigen involved.

(ii) Biochemical and haematological responses: To date the only work which monitors the biochemical and haematological responses of rats or rabbits implanted with free mature flukes and challenged is that of Haroun (1979).

Haroun (1979) observed after infection increasing GD levels in rats implanted subcutaneously with one or two mature F. hepatica and challenged two weeks later with 20 metacercariae of F. hepatica. However the maximum level at week 4 was significantly lower in the implanted group than in the non-implanted control. In contrast, in rabbits implanted subcutaneously with two, three or five mature flukes and challenged two weeks later with 100 metacercariae of F. hepatica, Haroun (1979) observed no statistically significant difference in GD levels at week 4 between the implanted and non-implanted groups.

In the same studies, Haroun (1979) observed a significantly lower peak of eosinophil counts at week 4 in implanted rats, but not in rabbits, than in the appropriate non-implanted controls.

(iii) Other responses: Lang (1968b) observed the death of mice ten days after they had been implanted intraperitoneally with two adult flukes. He did not attribute this death to liver damage because by that time the flukes had not reached the livers of the mice. He suggested that young flukes, 20 days old or slightly older, produce toxic excretions or secretions that were responsible for the deaths.

Isseroff, Girard and Leve (1977) observed enlargement of the bile duct of rats implanted with five or more flukes. This enlargement resembled that in rats having a 20- to 40-day old oral infection. They suggested that the enlargement was brought about by chemical factors, as there was no direct contact between the flukes, which had been encapsulated by the host, and the liver.

(b) Sensitisation by encapsulated flukes

Implantation of living adult flukes encapsulated in diffusion chambers allows stimulation only by the metabolic antigens which are released through the membrane.

(i) Resistance to challenge: Haroun (1979) studied the immune response of rats implanted subcutaneously or intraperitoneally with one or two adult flukes in a diffusion chamber and observed a significant reduction in the numbers developing from the challenge infection as compared with those in rats implanted with empty chambers or in non-implanted controls. There was no difference between groups of rats implanted with flukes in diffusion chambers and those implanted with free flukes. After studying the effect of surgically removing the subcutaneously implanted encapsulated flukes on acquired resistance to challenge by rats, he concluded that the resistance was due to the immunogenic effect of the metabolic products from the flukes. By carrying out repeated intraperitoneal implantations he further concluded that resistance could be increased by multiple implantations.

(ii) Serological response: Using a semi-quantitative immunodiffusion technique, Haroun (1979) first detected precipitating antibodies to metabolic antigen five days after intraperitoneal implantation of rats with adult flukes in diffusion chambers. These precipitating antibodies continued to be detected up to week 11 and to week 12 in one rat. However, one week after the second implantation antibodies were again detected.

(iii) Biochemical response: In rats implanted with adult flukes, whether the implants were given subcutaneously or intraperitoneally, Haroun (1979) observed significantly lower peaks of plasma GD at week 6 after challenge than in non-implanted controls. Again no similar effect was observed in rabbits. These observations clearly correlated with the number of flukes developing in these hosts.

(iv) Haematological response: Haroun (1979) observed a significantly lower peak of eosinophil counts at week 4 or 6 after challenge in rats implanted subcutaneously with adult flukes than in challenge controls. However there was no significant difference in the eosinophil counts in rabbits at week 4 or 6 after challenge as between the implanted and non-implanted controls. Again these results were related to the numbers of flukes developing in the hosts.

2.2.1.4 Sensitisation by non-living antigens

The most commonly used materials for active immunisation are somatic and metabolic antigens. Somatic antigens are derived from the body tissues of the fluke, while metabolic antigens are derived from the excretory/secretory products of living flukes.

(a) Somatic antigen

To date the only work that has indicated that an acquired resistance by animals to challenge infection may follow vaccination with fluke somatic antigen is that of Oldham and Hughes (1982) and Oldham (1983a). They observed a reduction in the numbers of flukes developing following challenge infection with 30 metacercariae of F. hepatica in rats. They further obtained an enhancement of resistance by increasing the amount of antigen (Oldham, 1983a) or by the addition of Bordetella pertussi to the adjuvenated antigen (Oldham et al., 1982).

Workers who failed to confer resistance to challenge infection by vaccinating animals include Hughes (1962b), using homogenised whole fluke antigen in rabbits, Ross (1967a) using an adjuvenated somatic antigen injected subcutaneously in calves, rabbits or lambs

which had been simultaneously intramuscularly implanted with adult fluke antigen before challenge, Hughes et al. (1981a), using adult fluke antigen injected intramuscularly on two occasions, three weeks apart, in rats and Burden, Harness and Hammet (1982), using somatic antigen derived from 10- to 16-day-old flukes injected subcutaneously in rats or mice.

Urquhart, Mulligan and Jennings (1954) failed to confer resistance to challenge infection by rabbits immunised with protein antigens of F. hepatica but observed inhibition of development of the parasite in the sensitised animals as compared with unvaccinated rabbits. Likewise, Healy (1955) failed to demonstrate resistance to challenge in rabbits previously immunised with either somatic material or pooled regurgitated caecal contents from flukes, although he observed small numbers of abnormal fluke eggs in the faeces of vaccinated animals.

In rabbits immunised subcutaneously with a fluke homogenate, Ross (1967a) observed no difference in the fluke burden following challenge with 50 metacercariae of F. hepatica in vaccinated or non-vaccinated groups. However, he observed a significant difference in the mean fluke lengths between the two groups at weeks 6 and 7 after challenge, those in the vaccinated animals being smaller. However by week 9 after challenge the difference in lengths was no longer significant.

A similar result to those obtained above, using adult somatic antigen, was found by Kozar (1974), who studied the effect of antigen derived from tissues of Galba truncatula and Lymnea tomentosa (i.e. in effect a larval somatic antigen) on acquired resistance to challenge infection in rats. He observed a reduction in intensity of infection, pathological changes in the liver and inhibited development

of flukes in the vaccinated animals.

Overall, it appears that the use of somatic antigen to stimulate acquired resistance to challenge infection is not promising. However, it was observed by Urquhart et al. (1954) and Oldham (1983b) that following vaccination of animals with somatic antigens an immune response was stimulated as indicated by the appearance of antibodies in the serum.

(b) Metabolic antigen

The use of metabolic antigen of F. hepatica to transfer acquired resistance appears more promising since Lang and Hall (1977) and some other workers have successfully protected mice against challenge infection using culture fluid in which liver flukes had been incubated.

(i) Resistance to challenge infection: Lang (1976) observed significant protection against challenge infection with two F. hepatica in mice vaccinated with metabolic products from 16-day-old flukes elaborated over a 24-hour period, but failed to confer protection with metabolic products from 16-day-old F. hepatica elaborated over four hours. Likewise, Rajasekariah, Mitchell, Chapman and Montague (1979) conferred significant resistance against challenge to mice immunised with excretory/secretory products of 4-week-old flukes. However immunisation of mice with metabolic products of 8-week-old flukes did not confer resistance to challenge as compared with challenge controls. They concluded that "host protective antigen" for rats was present in the excretory products of immature F. hepatica.

On the other hand, other workers such as Davies, Rickard, Smyth and Hughes (1979), Burden and Hammet (1980), Lehner and Sewell (1980) and Burden, Harness and Hammet (1982) have failed to confer protection against F. hepatica in rats, mice or rabbits immunised with metabolic antigens of the homologous parasite.

The use of antigen-antibody complex obtained from in vitro culture of newly excysted metacercariae to immunise animals appeared to afford some resistance in rats (Howell, 1979), but gave no success in sheep (Sandeman, Howell and Campbell, 1980).

(ii) Serological, haematological and biochemical responses: Lehner (1977) detected specific anti-Fasciola antibody by ELISA in the serum of rats or rabbits six weeks after immunisation with metabolic products of F. hepatica, collected in medium 199 during seven weeks of in vitro culture. However, he did not find any evidence of an anamnestic response to subcutaneous booster injections.

In the same rats and rabbits, Lehner (1977) observed lower eosinophil counts at four, six and eights weeks after challenge with 20 or 100 metacercariae of F. hepatica than in similarly infected but unvaccinated controls. However this difference was not statistically significant. In addition, he observed a reduced mean GD level at weeks 4 and 6 after challenge, but again the differences were not statistically significant when compared with the GD levels in unvaccinated challenge controls.

2.2.2 Passive acquired immunity

The induction of passive acquired immunity does not involve the active stimulation of the immune system of the animals. Either antibodies (humoral) or lymphoid cells and granulocytes (cellular) may be used to transfer resistance.

2.2.2.1 Humoral

The role of immune serum in acquired resistance to Fasciola infection has not been fully elucidated. However both IgG and IgE have been implicated (Doyle, 1973b; Sinclair and Wassal, 1981).

(i) Effect of immune serum on parasites: When parasites are incubated in immune serum several morphological changes may be observed. Thus Thorson (1954) observed a loss of activity of larvae Nippostrongylus muris incubated in the sera of rats infected with the homologous parasite, demonstrable when the larvae are injected intraperitoneally into rats. This loss of activity was arrested if the larvae were washed with distilled water before they were transferred to the rats. Jamuar and Lewert (1967) noted the absence of movement and alteration to the surface of miracidia of Schistosoma japonicum incubated in immune serum.

The presence of precipitates around the body openings of parasites incubated in immune serum was observed by Sarles (1937, 1938) with larvae of N. muris; Oliver-Gonzales (1940) with adult Trichinella spiralis; Standen (1952) with Schistosoma mansoni; Wikerhauser (1961b), Howell, Sandeman and Rajasekariah (1977) and Sandeman and Howell (1980) with newly excysted F. hepatica and by Howell and Sandeman (1979) on the surface of metacercariae of F. hepatica. Sher (1976) observed "rosette" formation by purified mast cells attached to schistosomula incubated in immune serum.

The chemical composition of the precipitates formed around the tegument of metacercariae of F. hepatica incubated in immune serum was studied by Howell and Sandeman (1979). They found that they were antigen-antibody complex. When the precipitates were injected into rats they observed a 50% reduction in the number of flukes developing following a challenge infection as compared to the challenge controls.

Hanna (1980a) noted a continuous layer of IgG on the tegument of flukes incubated in immune serum. There was active replacement of this layer with a new, antigenically similar layer when the fluke was transferred into a medium free of immune serum. Likewise, Duffus and Franks (1980) observed layers of antigen-antibody complex on the surface of flukes brought about by the build-up of antibodies present in the serum. They suggested, after observing the eventual shedding of adherent granulocytes brought about by the turnover of the outer glycocalyx of juvenile flukes, that the intimate attachment of these granulocytes to the flukes could be a prerequisite for cell-mediated damage to the flukes.

The action of these precipitates, as suggested by Sarles (1939), may be to deprive the parasites of nutrients thereby affecting their biological activities. Eckblad, Woodard and Lang (1981), using scanning electron microscopy (SEM), demonstrated damage to the tegumental surface of immature F. hepatica after in vitro incubation for four hours in serum of a calf infected with F. hepatica. They further observed that after incubation for 8-12 hours, there was some decomposition of the fluke and after incubation for 16 hours there was complete destruction. Using latex particles as immunological markers for SEM studies, they were able to show the involvement of both IgG1 and IgG2.

(ii) Resistance to challenge: Resistance to challenge in animals protected with immune serum was observed by Mitchell, Armour, Ross and Halliday (1981). They noted a significant reduction of 64% in the numbers of flukes developing following challenge with 20 metacercariae of F. hepatica in rats immunised with immune serum from sheep collected ten weeks after infection as compared to naive control group. They further observed that the transfer factor prepared from lysed leucocytes of rats harbouring a 10-week-old infection could reduce the fluke burden in rats by 55.6% as compared to unimmunised controls, while the transfer factor prepared from the leucocytes of infected sheep or calves did not protect rats.

Dargie, Armour and Urquhart (1973) and Armour and Dargie (1974) conferred protection against F. hepatica challenge (20 metacercariae) to rats immunised intraperitoneally with homologous immune serum, given simultaneously with the challenge and Dargie, Armour, Rushton and Murray (1974) conferred a high degree of protection against primary challenge with 20, 500 or 1,000 metacercariae of F. hepatica to rats, sheep or cattle by transfer of immune serum from infected donors.

Hayes, Bailer and Mitrovic (1974b,c) observed significant protection against challenge infection with 10 or 20 metacercariae of F. hepatica in rats given serum from infected rats collected 49 to 56 days after infection. However, serum from rats infected for 25 weeks was not protective. They further observed that immune serum given at the same time as the oral challenge gave protection, while immune serum given three or four days after challenge had only a slight effect and immune serum given six or eight days after challenge had no protective effect. The protective effect of immune serum was eliminated by heat treatment or by absorption with live or dead F. hepatica.

Similarly, Howell et al. (1977) conferred significant resistance against challenge with 10 or 30 metacercariae of F. hepatica to rats immunised with serum taken from rats given primary and secondary infections of F. hepatica. Likewise, Haroun, Hammond and Sewell (1981) conferred resistance to challenge with 20 metacercariae of F. hepatica in rats passively immunised with immune serum obtained from rats, rabbits or cattle eight, nine and ten weeks after infection with F. hepatica. They further observed that serum obtained from infected rats or cattle conferred some protection to rabbits against challenge with 100 metacercariae of F. hepatica but sera from infected rabbits did not protect rabbits.

Lang (1974b, 1976) studied the development of 12-, 16-, 18-, 20- and 24-day-old F. hepatica incubated for four hours in immune sera of mice before intraperitoneal transplantation into normal mice. He observed a significant reduction in the resulting fluke burden as compared to those obtained using flukes incubated in heat-inactivated immune sera, normal sera, medium M199 or buffered saline before transfer.

Studying other trematodes, Baalawy (1975) observed a 25% to 48% protection against F. gigantica in rabbits immunised with homologous immune serum given on the day of challenge with 20 metacercariae of F. gigantica and two days later, and 78% when they were immunised with serum from goats infected nine weeks earlier, while in contrast Stirewalt and Evans (1953) failed to confer protection against Schistosoma mansoni challenge to mice immunised with rat immune serum.

(iii) Haematological and biochemical responses: Haroun (1979) observed a significantly lower peak in the peripheral eosinophil counts at week 6 after challenge with 20 metacercariae of F. hepatica in rats

immunised with homologous immune serum as compared with controls receiving homologous normal serum. However in neither rats nor rabbits immunised with immune serum of rabbits was there any significant difference at week 6 after challenge as compared with controls receiving normal serum.

In the same experiments on passive transfer of resistance to rats or rabbits by immune serum, Haroun (1979) observed a significantly lower serum GD at week 4 or 6 after similar challenge in rats immunised with immune serum or gamma globulins from rats, rabbits or cattle infected with F. hepatica, or from rats implanted with mature flukes in diffusion chambers than in rats which had received normal serum or gamma globulins from normal serum. In contrast, serum from infected sheep did not protect rats from the challenge as indicated by similar serum GD activities in the supposedly immunised animals as compared with those which had received normal serum.

2.2.2.2 Cellular

The first in vitro action of granulocytes and lymphoid cells against worms to be demonstrated was their attachment onto the parasite. This was observed by Sher (1976) using mast cells, Perez and Smithers (1977) using peritoneal cells, Capron, Capron, Torpier, Bazin, Bout and Joseph (1978a) and Capron, Capron, Bazin, Torpier and Joseph (1978b) using eosinophils, and Caulfield, Korman, Butterworth, Hogan and David (1980) using eosinophils and neutrophils on schistosomulae. This adherence occurred with or without the presence of complement (Caulfield et al., 1980) but was dependent on the presence in immune serum of anti-schistosome IgE antibodies (Capron, Dessaint and Capron, 1975) or IgG2a (Capron et al., 1978a,b).

With F. hepatica, Doy, Hughes and Harness (1980) observed adherence of eosinophils derived from either normal or infected rats on the tegument of F. hepatica in the presence of immune serum. This adherence occurred independently of complement, was not affected by the age of the sensitising infection and could not be induced by the administration of non-living fluke antigens.

Following adherence to schistosomulae, degranulation of the eosinophils further followed by release of enzymes was observed by Butterworth (1977). Phagocytosis of the antigen-antibody complex was observed by Sabesin (1963). Damage to the flukes is also caused by the human major basic protein of eosinophils as noted by Butterworth, Wassom, Gleich, Loegering and David (1979) and Duffus, Thorne and Oliver (1980).

The use of a labelling technique to demonstrate the release of ^{51}Cr from damaged flukes was introduced by Butterworth, Sturrock, Houba and Taylor (1976). Other workers who have demonstrated antibody-dependent damage to schistosomulae as shown by ^{51}Cr release from labelled parasites include Butterworth, Coombs, Gurner and Wilson (1976), Butterworth, David, Franks, Mahmoud, David, Sturrock and Houba (1977), Glauert and Butterworth (1977) and Vadas, David, Butterworth, Pisani and Siongok (1979).

(i) Resistance to challenge: Corba et al. (1971) and Dargie et al. (1973, 1974) observed a high degree of resistance against F. hepatica challenge by rats (30 metacercariae) or cattle (1,000 metacercariae) immunised with lymphoid cells from infected donors.

Corba et al. (1971) also observed that lymphoid cells taken 8-10 weeks after infection and transferred to naive rats protected the

animals against F. hepatica challenge. However, when lymphoid cells were taken four weeks after infection of donor and similarly transferred, they afforded no protection against challenge to the naive rats.

Armour and Dargie (1974) also observed resistance to F. hepatica challenge (20 metacercariae) in rats given lymphoid cells from a homologous donor infected with F. hepatica. They suggested that the extent of protection obtained with the cells appeared to be related to the quantity and persistence of the antigenic stimulus in the donor.

On the other hand Lang, Larsh, Weatherly and Goulson (1967) observed a significant reduction in the numbers of flukes developed from challenge (two metacercariae of F. hepatica) in mice given an intraperitoneal injection of 2.75×10^6 peritoneal exudate cells derived from isologous donors and concluded that delayed hypersensitivity played a role in the resistance.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Preparation of Metacercariae

3.1.1 Culture of green algae (Oscillatoria spp.)

The method for preparing the algal cultures was essentially that described by Pullan (1968).

Local top soil containing the minimum number of stones or grass was collected in a bucket. It was passed through a sieve with a 4 mm mesh to remove stones and other debris before being sterilised by autoclaving at 1 kg/cm^2 for 30 minutes. After the soil had cooled, it was mixed with a mineral solution (Lehner, 1977), using a spatula, to produce mud of about the consistency of dough. The mud was smoothed into the bottom of a plastic box (104 mm x 178 mm, Stewart Plastic Products), to a depth of approximately 15 mm, to give a flat surface on which the algae could be spread.

A small amount of alga from an old mixed culture (mainly Oscillatoria spp.) was then placed in the middle of the mud, and the whole surface very gently wetted with distilled water. The prepared boxes were placed in a warm room at 23°C, 60 mm beneath four 30-watt warm white 1200 mm fluorescent strip lights (Crompton). The algae were watered daily with distilled water or mineral solution if the culture turned brownish or pale.

3.1.2 Culture of snails

The culture method used with young snails was developed from those described by Sewell (1961) and Hammond (1970).

Algal cultures containing egg masses of adult Lymnea truncatula from the stock colony maintained at the Centre for Tropical Veterinary Medicine were placed in a window at room temperature during warm weather or in an incubator at 23°C during cold weather. When the egg masses hatched, young snails were harvested, using a blunt scalpel and a fine brush, and transferred onto a fresh algal culture. As many as 50 young snails could be placed in each culture box. When they reached a length of 2-5 mm some of the snails were infected and others were transferred into a cold room (4°C) as stock, for a period of aestivation not exceeding three months.

As needed, the snails were taken out from the cold room and dead ones were removed. Live snails can easily be distinguished from the dead ones by placing them in a petri dish containing distilled water at room temperature, when the live ones become active.

3.1.3 Obtaining miracidia

3.1.3.1 Collection of Fasciola eggs

Eggs were collected from the gall bladder or faeces of animals known to be infected with Fasciola hepatica.

The gall bladders, taken from infected animals taken at a local abattoir, were opened with pointed scissors and the contents emptied into a glass beaker. The bladder was washed out with tap water and the washings added to the beaker. The contents of the beaker were then washed through a 150 μm aperture sieve (Endecotts) and into a 38 μm aperture sieve. The eggs were retained in the second sieve and washed into a glass bottle using a fine jet of water. The collected eggs were then washed several times by sedimentation in distilled water before they were incubated or stored in distilled water at 4°C.

On occasions when no infected livers were available at the abattoir, collection of Fasciola eggs from the faeces of infected sheep kept at the Centre for Tropical Veterinary Medicine was resorted to. The faeces were collected by gloved fingers direct from the rectum. They were broken up in a clean plastic bowl containing a small amount of tap water. The suspension was then washed through a nylon coffee strainer having a pore size of 1 mm and then through a sieve of 250 μm aperture and into one of 38 μm aperture. The eggs retained in the 38 μm aperture sieve were placed in universal bottles, washed many times with distilled water and stored at 4°C.

This suspension of eggs still contained a little fine debris. However, when the eggs hatched, the miracidia could easily be obtained free of debris by sucking them up with a pasteur pipette, swimming about in the water.

Clean Fasciola eggs that have been kept for about a month in the cold (4°C) in distilled water will still develop in the incubator.

3.1.3.2 Culture of Fasciola eggs

The procedure used to hatch Fasciola eggs was that described by Sewell (1961).

The washed eggs were kept in an amber bottle in just enough distilled water to submerge them in the dark at 23°C. The incubated eggs were washed daily for seven days with distilled water. Thereafter the eggs were kept continuously in the dark until the miracidia were required. Miracidia would hatch on exposure to daylight by 14 days after setting up the culture and were used to infect the snails as soon as possible thereafter, as they only survive for a few hours after hatching.

3.1.4 Infection of snails with miracidia

Infection of Lymnea truncatula with miracidia was carried out in a similar way to that described by Haroun (1979).

The snails were infected when they were 2 to 5 mm long. They were placed individually in the wells of a polystyrene flat-bottomed microtitre tray (Flow Laboratories). Each well also contained a little distilled water into which had been placed 5-7 miracidia. The plate was covered and left overnight at room temperature for infection to occur.

The infected snails were maintained at 23°C in the incubator during cold weather or at room temperature during warm weather on fresh algae which were changed regularly. At four weeks after infection the snails were examined under a stereoscope for rediae and any uninfected snails discarded. By five weeks after infection the cercariae could be shed.

3.1.5 Shedding and storage of cercariae

The technique of Pullan (1968) was used for shedding snails for cercariae.

All available snails with mature infections were placed in a small polythene bag (120 x 75 mm) half-filled with cold (5°C) distilled water, closed with a spring-clip and left in the light at room temperature (25°C). The cercariae were shed as the water warmed up to room temperature. After five to six hours the snails were removed and placed onto a fresh algal culture. The bag was reclosed and left at room temperature overnight. The following day the snail debris in the bag was gently washed off and autoclaved. The bag was then half-filled with distilled water and left in a 100 ml wide-mouthed

bottle at room temperature for a week before it was transferred to a cold room at 4°C for prolonged storage.

3.2 Infection of Animals

3.2.1 Sheep and cattle

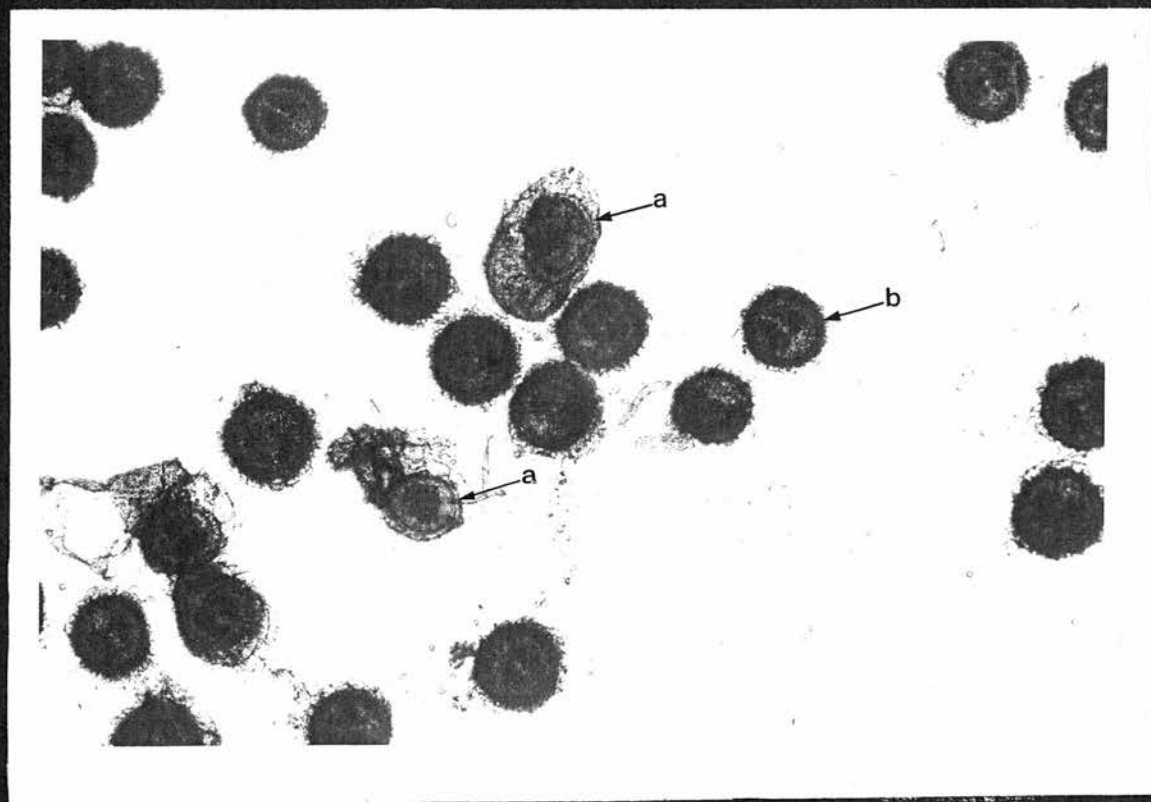
The water in a polythene bag containing metacercariae was poured into a beaker and later boiled so that it could be safely disposed of. The bag was then removed from the bottle with forceps and placed in a plastic petri dish being handled thereafter with forceps while wearing gloves. The plastic bag was opened with scissors and cut into manageable pieces (about 10-15 mm^3). The metacercariae were examined for viability under a stereoscopic microscope at 50x (40M, Vickers Instruments). Viable metacercariae have a characteristic appearance (Plate 3.1).

After being scraped off the polythene with pointed needles, the required number of viable metacercariae was sucked up using siliconised pasteur pipette and placed in a sheet of Whatman No. 4 filter paper (W & R Balston) in a glass funnel. After the paper had drained and the part which did not bear any metacercariae had been cut off, the filter paper was folded, with the metacercariae inside, and administered orally to the animals using a balling gun.

3.2.2 Rats

The required number of viable metacercariae was dispensed into sufficient wells of a haemagglutination plate (Flow Laboratories) containing 1.5 ml of 1% suspension of gum tragacanth (Boots). The metacercariae were sucked up into a siliconised pasteur pipette at the tip of which was attached a 100 x 2 mm i.d. transparent silicone rubber tubing.

Plate 3.1 Three-week-old metacercariae of Fasciola hepatica showing a non-viable cyst without the characteristic granular appearance (a) and a viable one with the characteristic granular appearance (b).


a
b
a

Each rat was anaesthetized with ether, held by its neck so that it hung vertically, the tubing on the pipette inserted into its pharynx and the dose administered.

To ensure that all the metacercariae had been administered, the pipette and the tubing were then examined under the stereoscopic microscope at x50 and washed out three times in distilled water. Any metacercariae observed were again administered.

3.2.3 Rabbits

The required number of viable metacercariae was dispensed into sufficient wells of a haemagglutination plate as above (Section 3.2.2) and transferred into an empty 20 mm gelatin capsule (Parke Davies) using a siliconised pasteur pipette. The gelatin capsule was then administered orally using forceps.

3.3 Bleeding the Animals

3.3.1 Cattle

To collect bulk samples the blood was extracted from the jugular vein with a 32 mm, 18 g disposable hypodermic needle (Sherwood) to which was attached a 1200 mm of 4 mm i.d. siliconised tubing (ESCO Rubber). The blood that passed through the tubing was collected into 500 ml Kilner jars, allowing it to flow along the wall of the jar, to avoid haemolysis. As much as 500 ml blood was collected from each bullock.

Smaller amounts of blood were collected from the jugular vein with a 32 mm, 18 g needle attached to a sterile 10 ml disposable syringe (Becton Dickinson).

3.3.2 Sheep

Only 20 ml was collected at any one time, from the jugular vein with a 32 mm, 18 g needle into a sterile disposable syringe (Becton Dickinson).

3.3.3 Rats

The rat was anaesthetized with ether and placed on its back in a box provided with an opening for the tail. The ventral side of the tail was shaved to avoid the hair becoming matted with blood and rubbed with cotton wool previously damped with xylene. The engorged vein was incised with a sharp scalpel blade and the blood collected by sucking it into a plastic 0.3 ml capillary vessel (Sarstedt).

3.3.4 Rabbits

The tip of the ear was rubbed with xylene to engorge the veins. The blood was then obtained by puncturing the vessel with a 21 g disposable needle. Up to 5 ml was collected.

3.4 Treatment of Body Fluids

3.4.1 Serum

The following procedure was used with bulk blood samples.

The blood was allowed to clot for one hour at 37°C. The clot was then cut into small pieces to allow better retraction, using a sharp sterile 200 mm knife, and kept at 4°C overnight. The serum was then transferred to a sterile plastic centrifuge bottle (MSE) and centrifuged at 2500 g for 30 minutes at 4°C. The serum was then stored in aliquots of 20 ml in disposable plastic universal bottles (Sterilin) or in aliquots of 0.2 ml in 1.5 ml plastic microcapped centrifuge tubes (Hughes and Hughes) and kept at -20°C.

When the bullocks were slaughtered the animal was raised on a hoist by one hind leg, the jugular veins cut and the blood collected in a clean bucket while it was being stirred with a clean piece of wood made for the purpose. When no fibrin could be trapped on the wood, the defibrinated blood was transferred into sterile plastic centrifuge bottles (MSE) and centrifuged at 2500 g for 30 minutes at 4°C. The serum was then stored in aliquots of 20 ml at -20°C.

For small blood samples of up to 20 ml, the blood was put in a 10 ml centrifuge test tube, kept at 37°C for one hour and then overnight at 4°C, and centrifuged at 2500 g for 30 minutes at 4°C. The serum was stored in aliquots of 5 ml in plastic vials (Luckham) and stored at -20°C.

Rat serum was collected by centrifugation of the blood collected in 0.3 ml plain capillary blood collection tubes (Sarstedt) at 2500 g for 30 minutes at 4°C. The serum was stored in aliquots of 0.05 ml in 1.5 ml microcapped centrifuge tubes (Hughes and Hughes) at -20°C.

3.4.2 Plasma

Blood collected from rats in heparinised capillary plastic vessels (NH_4-heparin, Sarstedt) was centrifuged at 2500 g for 30 minutes and the plasma dispensed in aliquots of 0.1 ml in plastic vials and stored at -20°C.

3.5 Eosinophil Counts

The technique described by Archer (1965) was used, the diluting fluid being:-

Eosin Y 1% solution	5 ml
Acetone	5 ml
Distilled water	90 ml

This fluid is stable for some weeks on the bench.

Eosinophils are stained by the eosin, while the other leucocytes and erythrocytes are lysed by the acetone and distilled water.

Blood was diluted 1:10 in the fluid in a clean test tube, mixed and allowed to stain for at least five minutes. A Neubauer Improved Haemocytometer was filled with the resuspended mixture and the cells falling within the whole ruled area (9 mm^2 on each side) were counted. The cell depth is 0.1 mm.

The total eosinophil count was calculated as follows:-

$$\frac{\text{No. of cells x Dilution factor}}{\text{Volume counted in } mm^3} = \frac{\text{No. of cells x 10}}{1.8}$$

3.6 Serum Glutamic Dehydrogenase (GD) Assay

This was used as an indicator of liver damage (Sewell, 1967). The principle of the test is:-

$$\alpha\text{-oxoglutarate} + \text{NADH} + NH_4 \xrightleftharpoons{\text{GLDH}} \text{glutamate} + NAD^+ + H_2O$$

The assays were done in a SP 1800 spectrophotometer (Pye Unicam). Glutamic dehydrogenase (Type IV, Sigma Chemical) prepared from bovine liver with an activity of 36 units mg^{-1} protein and containing 10.4 mg ml^{-1} protein, as measured by biuret (Sigma Chemical), was diluted 1:300 in 0.1M phosphate buffered saline, pH 7.2, and used as the positive control for the assays. Stock diluent (Boehringer) was used as the negative control.

A preliminary study to compare the activated (Boehringer) and inactivated (Ford and Boyd, 1962) techniques was done. The purpose of this study was to compare the two techniques as to their ease and rapidity of assay. This was necessary because of the large number of samples to be assayed, especially from rats. The assay of test samples had to be done at regular intervals, as keeping the serum for two or

three days changes the GD concentration (Boehringer, 1982). It was therefore necessary to use a technique that would facilitate the assay of many samples at one time.

It was observed that the two techniques gave similar results with sera from both rats and bullocks, but that the activated technique was much more convenient as it took only six hours to assay more than 80 samples using this technique as opposed to 48 hours using the inactivated technique.

3.6.1 Inactivated technique

The technique was described by Ford and Boyd (1962). The following reaction mixture was placed in a 4.5 ml plastic cuvette (Sarstedt) with a 10 mm light path:-

Solution	Amount (ml)
Reduced coenzyme (DPNH, 1 mg/ml) (Sigma Chemical)	0.2
0.1M phosphate buffer, pH 7.5	2.2
33% ammonium sulphate	0.2
Plasma sample	0.2

After 15 minutes pre-incubation, the test was started by the addition of 0.2 ml α-ketoglutaric acid (Sigma Chemical) previously adjusted to pH 7.5. The decrease in optical density at 340 nm was recorded graphically on an "AR2" linear recorder (Pye Unicam) and plotted against time. The number of μmoles utilised per hour was calculated from the initial rate of reaction and the molecular extinction coefficient of reduced enzyme (DPNH), whereby a reduction in optical density of 0.001 is equivalent to the oxidation at 4.81×10^{-4} μmoles of DPNH.

3.6.2 Activated technique

The technique used was that described by Boehringer (1982). In this technique the test sample was diluted with GLDH stock diluent (Boehringer). This stock diluent contains adenosine diphosphate (ADP) which serves as an activator for the GD in the test sample. The activation of GD in the test sample results in a "creep" reaction in the absence of the substrate, which has to be allowed for in the technique. This also has the effect of allowing for the non-specific reaction in the test sample (Walker, 1982, personal communication*).

For assaying the serum glutamic dehydrogenase activity of cattle, 0.5 ml of test serum, 2.5 ml of GLDH stock diluent (Boehringer) and 0.1 ml α-oxoglutarate were used. While in rats, because of the limited amount (0.1 ml) of blood plasma that could be obtained, which is less than the 0.5 ml required by the Boehringer test kit, the amount of GLDH stock diluent and test starter (α-oxoglutarate) was initially scaled down to 1.5 ml and 0.1 ml respectively. This resulted in high values for the "creep" reaction which, according to Walker (1982, personal communication), were due to the large amount of ADP in the test relative to the volume of the test sample. Hence in study 2 (Section 5.2) and in subsequent studies the volume of GLDH stock diluent and α-oxoglutarate was reduced to 1 ml and 40 µl respectively (Campbell, 1982, personal communication*).

The assay was done in 10 mm light-path plastic cuvettes (Sarstedt) for cattle serum and in 4 mm light-path plastic cuvettes (Sarstedt) for rat plasma.

After three minutes incubation in water bath (25°C) of the test sample diluted with GLDH stock diluent, the absorbance (A_1) was read

*Customer Services, Boehringer Corporation

at wavelength 340 nm. After five minutes further incubation the absorbance (A_2) was again read. The difference between A_1 and A_2 constituted the "creep" reaction for the test.

The test proper was then started by the addition of the α-oxoglutarate. Immediately after adding this, the absorbance was again read (A_3). After incubation for another five minutes, the final absorbance (A_4) was read. The difference between A_3 and A_4 constituted the absorbance in the actual test. This absorbance and the "creep" effect could then be used to calculate the absorbance due to the enzyme in the test sample (A_t) from the following formula:-

$$A_t = \frac{(A_3 - A_4) - (A_1 - A_2)}{5}$$

To convert this absorbance into international units, factors of 197 for the 0.5 ml test sample or 361.9 for the 0.1 ml test sample were used. These factors were derived from the formula (Boehringer, 1982) below:-

$$\text{Enzyme activity (in } \mu\text{m/ml)} = \frac{A_t \times 1000 \times V}{\varepsilon \times d \times v}$$

where

A_t is change in O.D. per minute caused by the enzyme activity of the sample

V is the total reaction volume

v is sample volume

d is the light path

ε is extinction coefficient of NADH/NADPH at 340 nm i.e. 6.3 cm^3/μmole.

3.7 Preparation of Somatic Fasciola Antigen

Somatic Fasciola antigen was prepared as described by Farrel, Sheer, Wescott and Lang (1981).

Live adult flukes taken from recently killed hosts were placed

in a disposable petri dish containing 0.1M carbonate buffer (pH 9.6) with 0.02% sodium azide at room temperature for 30 minutes or so. The flukes were then weighed and placed in a mortar. One ml of the same buffer per gram (wet weight) of the worms was added gradually while the parasite was ground in a mortar at 4°C. When no visible tissue particles remained, the resultant mixture was further macerated with a tissue homogeniser at 4°C, until a uniform suspension was obtained. The material was held at 4°C for 24 hours and then centrifuged at 12,000 g for 30 minutes at 4°C. The supernatant was pipetted off, its protein concentration estimated (see Section 3.9) and the antigen stored in aliquots of 0.05 ml in microcapped centrifuge tubes (Hughes and Hughes) at -20°C.

3.8 Production of Metabolic Product Antigen

A modified version of the continuous-flow culture system described by Lehner (1977) was used to produce metabolic antigen from adult *Fasciola hepatica*, the apparatus being set up and sterilised as described by Lehner.

The flukes used for the culture were obtained from rabbits which had a *F. hepatica* infection of not less than 12 weeks old. The removal of flukes from the liver was carried out in a sterile airflow hood (Microflow). The bile ducts were opened aseptically, the flukes removed with sterile forceps and placed in sterile modified Earle's medium, which had been kept for 48 hours at 37°C before they were transferred into the 300 mm by 40 mm nylon sleeving (Portex) containing the culture medium.

The culture medium used for the maintenance of the flukes was made by dissolving the constituents of the original formula of Earle's

balanced salt solution (Earle, 1943) in 9L instead of 10L of distilled water.

The medium was made in a steel pressure vessel (Millipore) for sterilisation by pressure filtration. The culture medium was then passed through a 0.22 μm pore-size 142 mm diameter membrane filter (Millipore) into a sterile 10L "pyrex" aspirator (Jobling Laboratory), which served as the reservoir, using the technique described in the Millipore operating manual.

Before use the sterile culture medium was placed in a warm room at 37°C for at least 48 hours. Penicillin G (Crystopen, Glaxo) at 0.6 mg^{ml-1} (1000 $units^{ml-1}$), streptomycin sulphate BP (Glaxo) at 0.6 mg^{ml-1} (100 $units^{ml-1}$) and amphotericin B (Fungizone, E.R. Squibb and Sons) at 2 mg^{ml-1} were added to the medium.

The medium was passed into a 300 mm by 40 mm diameter nylon sleeving (Portex), in which the flukes were placed, through silicone rubber tubing (MacFarlane/Robson) of appropriate sizes and the flow rate was controlled by means of a peristaltic pump (Desaga PLG-multipurpose pump 13 21000, Camlab).

The flow of the used medium containing the metabolic antigen into a conical flask, which was placed in a refrigerator, was maintained at 1 ml per fluke per hour. Eighteen adult flukes taken from rabbits with 12-week-old infections were used in the culture.

The flukes were still alive after five days when the medium ran out and the culture was terminated.

The medium containing the metabolic antigen was collected every 24 hours. It was filtered through Whatman No. 4 filter paper to remove eggs and other debris and centrifuged at 2500 g for 30 minutes at 4°C.

It was then concentrated to one third of the original volume by dialysis against 50% (w/v) polyethylene glycol 6000 (BDH Chemical), and further dialysed against several changes of phosphate buffered saline (PBS) (pH 7.3) (Oxoid Ltd.). Finally it was ultracentrifuged at 12,000 g for 30 minutes at 4°C so as to remove any residual small molecules from the culture which were found to interfere with subsequent protein estimation, and the supernatant, which constituted the "metabolic antigen", was pipetted off and its protein content estimated. The metabolic antigen was dispensed in aliquots of 0.05 ml in microcapped centrifuge tubes (Hughes and Hughes) and stored at -20°C.

3.9 Estimation of Protein Content

The protein concentration of the antigens was estimated by the method of Warburgh and Christian (1941) as described by Dawson, Elliott, Elliott and Jones (1969).

This technique depends mainly on the phenylalanine, tryptophan and tyrosin content in the sample and so does not necessarily measure the total protein accurately. It is however particularly useful because of its ease and rapidity and the fact that it is non-destructive.

The extinction of an appropriately diluted solution of antigen was measured at both 260 nm and 280 nm in 0.2 mm light-path quartz cells (Pye Unicam) in a SP 1800 spectrophotometer (Pye Unicam). The E260:E280 ratio was calculated. Using this ratio the proportion of nucleic acids in the protein sample and a factor for calculating the protein content was read off from a standard table (Warburgh and Christian, 1941). The protein concentration was then estimated using the formula: protein concentration (mg^{ml-1}) = extinction at 280 x factor x 1/d, where d is length of light path in cm.

3.10 Faecal Examination

Two techniques were used to examine faeces for Fasciola eggs. A modification of the Sellotape technique (Sewell and Hammond, 1972) was used for faeces from bullocks and a flotation technique modified from Spedding (1952) for faecal samples from rats.

The bulkiness and fibrous nature of the faeces from cattle demanded the use of the Sellotape technique as the concentration and trapping of the eggs on the sellotape allows them to be detected at very low concentrations.

3.10.1 Sellotape technique

A suspension of 1 gram of faeces in about 12 ml of water in a round-bottom, lipped, plastic centrifuge tube of 15 mm diameter was centrifuged, the sediment resuspended in saturated salt solution and again centrifuged. The sediment was then resuspended in zinc sulphate solution of specific gravity 1.3, so that the convex meniscus was slightly above the top of a lipped plastic centrifuge tube. A short length (c. 45 mm x 25 mm) of sellotape (Sellotape Products) was then placed flat over the top of the tube and gently pressed onto the rim. After further centrifugation at 250 g for five minutes the sellotape was examined under the microscope at x 100.

3.10.2 Flotation technique

Approximately 1 gram of faeces (1 ml by water displacement) was placed in a 10 ml calibrated centrifuge tube containing 9 ml tap water. The faeces were then broken by means of a clean stirring rod and the suspension passed through a double layer of butter muslin (Turnball and Wilson) into a clean test tube. The filtered suspension

was transferred into a glass centrifuge tube and centrifuged at 250 g for one minute. The sediment was then resuspended in a zinc sulphate solution of specific gravity 1.3 and mixed well. The tube was topped up with zinc sulphate solution, so that the meniscus was slightly above the top of the tube. A clean coverslip was placed carefully over the top of the tube, which was then centrifuged at 250 g for one minute. The coverslip was removed vertically, placed on a clean glass slide and examined under the microscope at x100.

3.11 Fasciolicidal Treatment of Rats

Hexachlorophene was used for deworming rats which had an 18-week-old infection with F. hepatica. Corba (1973) found that the drug was 85.5% effective and without side effects against an 8-week-old infection in rats, this being better than any other of the fasciolicides he tested.

3.11.1 Preparation of anthelmintic

The drug was prepared as described by Todd and Wade (1979) in the eleventh edition of the British Pharmaceutical Codex.

Hexachlorophene (Sigma Chemical)	50 g
Liquid paraffin (Evans Medical Ltd.)	150 ml
Pure vegetable oil (Boots the Chemist)	1 L

The hexachlorophene was dissolved in 750 ml vegetable oil by gentle heating. Upon cooling the liquid paraffin was added together with the vegetable oil to 1 L and the solution mixed. The preparation, which contained 50 mg hexachlorophene in 1 ml, was stored in an air-tight container protected from light.

A pilot study to determine the efficacy and margin of safety of the preparation was conducted on two rats which had high Fasciola

egg counts (281 and 594 e.p.g.) in their faeces by 14 weeks after infection. One week after treatment neither of the rats showed any eggs in the faeces. However, when necropsied two weeks later, one rat showed a partly decomposed fluke in the bile duct.

3.11.2 Treatment of experimental rats

All the rats received two treatments at 50 mg^{kg-1} body weight one week apart. Following the first treatment, three of the rats still showed eggs in the faeces (Appendix Table 3.1). However, following the second treatment only one rat (No. 15) had any eggs in its faeces (Appendix Table 3.1). This rat was again treated one day later for the third time. Following the third treatment this rat also no longer had any eggs in its faeces.

The drug was administered orally using a 1 ml syringe fitted with a 14 mm, 18 g needle (Arnold), to which was attached a 113 mm x 2 mm translucent p.v.c. (MacFarlane Robson). The tubing was inserted into the oesophagus of ether-anaesthetised rats and the preparation administered.

3.12 Necropsy and Recovery of Worms

3.12.1 Rats

The animals were killed by over-exposure to ether (Anaesthetic ether B.P., May and Baker).

The abdominal cavity was opened by making a longitudinal incision in the skin along the linea alba, from the promontory of the pelvis to the thoracic inlet. A second incision was made horizontally along the long axis of the last rib and another along the brim of the pelvis, so that skin flaps were formed. The fascia, muscle

and peritoneum were then cut to expose the viscera. By deflecting the liver anteriorly the congested bile duct was exposed. It was incised along its long axis and adult flukes were picked out using forceps.

The liver was removed from the viscera, cut into approximately 10 mm^3 pieces and placed in a labelled plastic petri dish (Sterilin) containing citrated saline (0.05 mg^{ml-1} sodium citrate dihydrate plus 0.08 mg^{ml-1} NaCl) and held in a warm room at 37°C for 5-6 hours. The pieces of liver were then squeezed into 1000 ml plastic beaker containing 500 ml tap water and disposed of. The citrated saline and flukes were poured into the same beaker. The washing fluid was changed twice using tap water so that the supernatant became clear. The sediment was transferred to a plastic petri dish and the flukes in it were counted.

3.12.2 Rabbits

Most of the animals were killed by intravenous injection of pentobarbitone sodium (Euthatal, May and Baker Veterinary Products) at a dose of 143 mg^{kg-1} body weight.

The rabbits used as the source of flukes for the culture of adult Fasciola for the production of metabolic antigen were killed with a humane killer.

The necropsy procedure and recovery of adult flukes were similar to the procedure for the rats, except that the livers of rabbits were not cut into small pieces since the purpose of using rabbits was only to obtain adult flukes for somatic antigen. It was not therefore necessary to obtain an estimate of the number of flukes in the rabbits.

3.12.3 Cattle

These were killed with a humane killer.

The animal was evicerated and the liver, with the intact bile duct was placed in a clean white 370 mm x 300 mm enamel pan. The bile duct was cut open and the flukes in them collected using forceps. The liver was then cut into small (45 mm x 45 mm) pieces, put in a 320 mm x 250 mm x 137 mm plastic basin uncovered (Woolworth, F.W.) containing warm tap water and placed in a warm room at 37°C. After 5-6 hours incubation the pieces of liver were removed and the supernatant passed through a clean kitchen sieve of 2 mm pore-size. The flukes were collected from the sieve and counted.

In the case of cut flukes, only the half bearing the anterior sucker was counted.

3.13 Implantation Procedures

3.13.1 Preparation of maintenance medium for flukes

The maintenance medium used was RPMI-1640 grade IX (Gibco Europe). To 500 ml of this medium was added 100 ml sodium pyruvate (Gibco Europe) containing 100 $i.u.^{ml-1}$ benzyl penicillin sodium (Crystapen, Glaxo) and 100 mg^{ml-1} streptomycin (Diamycin, Glaxo). The medium was held overnight in a warm room at 37°C before use.

3.13.2 Preparation of adult flukes

The flukes were taken from rats which had had a long-standing (at least 12 weeks old) infection. To avoid cooling the flukes, after the animal had been killed by over-dosage with ether, it was held in a warm room at 37°C. As soon as possible thereafter and

always within 20 minutes, the flukes were removed from the bile duct under a laminar airflow hood (Microflow) using sterile forceps and immediately placed into warm sterile medium (2 ml per fluke) in a sterile plastic dish (Sterilin).

3.13.3 Preparation of diffusion chamber

The diffusion chambers were constructed under the laminer airflow hood (Microflow).

Two sterile plexiglass rings (Millipore) of 0.14 mm internal diameter and 2 mm thickness were stuck together with "MF Cement" (Millipore). Two sterile millipore membranes ("MF Filter", Millipore) of pore-size 0.45 μm were moistened with sterile distilled water. This caused the membrane to swell slightly and allowed them to form a taut cover when dry. One of the membranes was glued with "MF Cement" (Millipore) to one side of the double ring.

A small amount of sterile medium was placed inside the chamber together with one or two adult flukes. The other membrane was then glued to the other side of the ring to make the complete chamber.

3.13.4 Surgical procedure for rats

This was done in the Department of Veterinary Surgery.

Anaesthesia of the rats was carried out using a Boyle's Circle Carbon-dioxide Absorber (BOC). The rat was restrained on its back on a small wooden block placed on top of the operating table. Two litres of oxygen and two litres of nitrogen oxide were allowed to flow through a mask for about two minutes and then halothane (May and Baker Veterinary Products) was added at 4% for 2-3 minutes. Once the animal was anaesthetised the halothane was reduced to 1-2%.

During the recovery phase the halothane and nitrogen-oxide were turned off and the oxygen increased to 4 litres per minute for about two minutes.

The incision site was shaved and hibitane (ICI) at a concentration of 1:200 was applied. A longitudinal incision about 25 mm long was made in the ventral abdominal region. The peritoneal cavity was opened and the diffusion chamber was inserted. The incision was closed using catgut No. 2 (Dexon).

3.13.5 Surgical procedure for cattle

The operation was done while the animals were standing either held in a pen or in a crush in the case of the large animal. The site of incision in the right flank region, extending from the last thoracic rib to the anterior part of the pelvic zone, was shaved at least 24 hours before surgery and the animals were fasted overnight.

Paravertebral anaesthesia was induced with 2% lignocain HCl (Astra Pharmaceuticals) in the ventral and dorsal branches of the third thoracic and the first and second lumbar spinal nerves.

A 1:200 concentration of hibitane (ICI) was applied to the shaved area and a small longitudinal incision (about 13 mm long, 75 mm below the thoracic transverse processes) was made through the skin, muscle and peritoneum. The diffusion chambers were inserted into the peritoneal cavity. At least 10 diffusion chambers were implanted intraperitoneally in each animal. The chambers containing Fasciola had one or two parasites in them, depending on the size of the flukes.

The peritoneum and muscle were closed with two layers of Dexon No. 2 (Cyanamid) using a simple continuous suture and the skin with Dexon No. 2 using interrupted mattress sutures.

3.14 Enzyme-linked Immunosorbent Assay (ELISA)

The microplate system described by Lehner (1977) was used with some modifications.

The antigen optimally diluted in 0.1M sodium carbonate buffer (pH 9.6) with 0.02% sodium azide was added (150 µl) to each well of a microtitre plate (Dynatech Laboratories). The plate was incubated at 37°C for three hours and then overnight at 4°C or until needed, but not for more than three days. The plates were then washed three times for five minutes each with excess washing fluid (9 g^{L-1} NaCl in distilled water containing 0.5g^{L-1} Tween 20 (Sigma Chemical). Then 150 µl of optimally diluted serum in 0.1M phosphate buffered saline (PBS) (Oxoid) containing Tween 20 was added to each well and the plates incubated for one hour at 37°C.

The plates were washed again three times for five minutes each with excess washing fluid and 150 µl of optimally diluted horseradish peroxidase conjugate (Nordic Immunological Laboratories) in PBS containing 0.5 g^{L-1} Tween 20 added to each well. The plates were further incubated for one hour at 37°C. After another wash by the same procedure, 150 µl of the substrate O-phenylenediamine (OPD) was added. This had been prepared as follows:- 35 mg of OPD (Sigma Chemical) in 100 ml of 0.1M citric/phosphate buffer (pH 6.0) containing 167 µl hydrogen peroxide. The plates were incubated at 37°C for no less than 30 minutes and no more than 40 minutes, depending on a visual assessment of the reaction.

The reaction was stopped with 150 µl of 1M H_2SO_4 (BDH, Aristar) and the absorbance read in a flow-through colorimeter using a 490 nm filter (MSE Scientific Instruments).

A preliminary study using ELISA was conducted with Fasciola somatic antigen derived from cattle or rats, cattle or rat antiserum and the respective rabbit anti-host immunoglobulins. It was found that the backgrounds (normal and positive sera) were rather high with either antigen (Appendix Table 3.2). It was thought that this was due to host material that had been absorbed onto the surface of the flukes and was therefore present in the antigens and so reacting with the rabbit anti-immunoglobulin.

In order to avoid this spurious reaction, somatic antigen prepared from Fasciola obtained from rabbits was used in later studies and gave much lower background results (Appendix Table 3.2).

Preliminary studies using bullock sera to determine the optimum dilutions of somatic antigen, serum and enzyme-labelled anti-immunoglobulin were also first carried out. The results are given in Appendix Tables 3.3.2, 3.3.3 and 3.3.4 and the conclusion was that this antigen should be used at the rate of 10 μg^{ml-1} protein, with a serum dilution of 1:1000, and a conjugate dilution of 1:2000.

Results of similar studies using rat or bullock sera and the metabolic antigen are given in Appendix Tables 3.4.2, 3.4.3, 3.4.4, 3.5.2, 3.5.3 and 3.5.4. The conclusions are given in Table 3.1.

Table 3.1 Optimum concentration when using serum from:

	Bullocks	Rats
Antigen ($\mu g\ ml^{-1}$)	20	5
Serum (1:)	1000	500
Conjugate (1:)	2000	2000

3.15 Statistical Analyses

The comparisons of ELISA values between more than two animals or groups of animals were carried out by Single Classification Analysis of Variance, while those between weeks within animals or between two animals were carried out by paired "t" tests using the differences between the replicate assays of the sera being compared.

The correlations between the fluke burden and the serum glutamic dehydrogenase levels were assessed by calculating the Pearson Correlation Coefficients and their probabilities.

Because of the over-dispersion, which is characteristic of such data, the comparisons between the numbers of flukes recovered from the rats were carried out by the non-parametric Mann-Whitney test.

CHAPTER FOUR

RATIONALE FOR EXPERIMENTAL STUDIES

It has been shown by Haroun (1979) that immune serum from cattle can passively transfer significant protection to rats. However, from these studies it is not known whether or how the serological response of cattle relates to the protection afforded by their immune sera nor how this protection varies with the age of the cattle or with their experience of infection. If these factors could be elucidated and strongly protective sera could be reliably obtained from cattle, it should be feasible to use such sera in absorption studies and by immuno-affinity chromatography to identify and isolate those immunogen/s from the flukes which cause the production of the protective antibodies. Once such functional antigens could be isolated and characterised it is possible that they might be produced from bacteria by means of genetic engineering or by using _in vitro_ culture techniques with _Fasciola_ material. Hence studies were undertaken in which bullocks were infected with single or repeated doses of _Fasciola hepatica_ in an attempt to produce such a strongly protective serum.

In the first study the immune response of cattle of about 18 months old, which had been given primary, secondary and tertiary infections with 1,000 metacercariae of _F. hepatica_, was monitored. The results of this study led to a trial in which the immune response of older animals (4-5 years old) was studied, after they had received similar initial and secondary infections, in the expectation that they might give a stronger immune response. However it transpired that the immune response in the older animals was in fact rather lower than that in the first study and that reinfection did not enhance this.

Hence the use of a single infection in younger animals (six months old) was examined. Again these relatively immature cattle did not give a consistently strong immune response and it was considered that other methods of sensitising the bullocks to induce a stronger immune response would be needed. Hence studies on implantation of bullocks or rats with adult flukes in diffusion chambers were undertaken.

CHAPTER FIVE

STUDIES USING YOUNG ADULT CATTLE

Introduction

Most previous studies in cattle given single or repeated infections with Fasciola hepatica have monitored the serological and other responses but the protective activity of the sera was not considered. Thus Doyle (1971) studied the serological, biochemical and haematological responses and faecal egg counts in 3-4 month old calves given primary and secondary infections with 750 and 1,650 metacercariae of F. hepatica. In other studies Doyle (1972; 1973a) monitored the immune response, in groups of 3-4 month old calves given primary infection with 750 metacercariae of F. hepatica and in another group given the same initial infection and challenged with 1,300 metacercariae, by monitoring the biochemical and haematological effects and faecal egg counts in the faeces. On the other hand, Hanna and Jura (1977) studied the immune response of cattle infected with 500 metacercariae of F. hepatica by monitoring the antibody titres by IFAT only.

In the following chapters a study is described in which the serological responses of cattle were monitored and the protection afforded by their immune sera following single and repeated infections with F. hepatica was assessed.

5.1 The Response of 18-month-old Cattle to Infection with Fasciola hepatica

Experimental design

Two 18-month-old Ayrshire bullocks (Nos. 89 and 94) were each given an initial infection with 1,000 metacercariae of F. hepatica

and 22 weeks later the animals were each given another 1,000 metacercariae. A third infection with 1,000 metacercariae of F. hepatica was again administered to each bullock 22 weeks after the second infection.

Small (10-15 ml) blood and faecal samples were collected from each bullock at two-weekly intervals throughout the studies to monitor peripheral eosinophil counts, serum glutamic dehydrogenase activities (using the activated technique), serology (using either rabbit-derived somatic or metabolic antigen by ELISA) and faecal egg counts.

Larger blood samples (c. 500 ml) were taken from these animals before initial infection and at three-weekly intervals after infection.

For the assay of serum glutamic dehydrogenase activity and eosinophil counts the samples were duplicated, while in the ELISA test the samples were replicated four times.

Results

Serology

(i) Somatic antigen: The corrected units of absorbance in ELISA using serum from bullocks 89 and 94, using somatic antigen in the test, are shown in Figure 5.1.1 and the mean values are shown in Appendix Table 5.1.1.

Both bullocks showed elevated ELISA values within two weeks of infection. In bullock 94, the peak occurred relatively early at week 2 and thereafter the ELISA values tended to decline. In bullock 89, the peak occurred at week 6 and again the values tended to decline thereafter.

Following secondary infection, the ELISA values from bullock 94

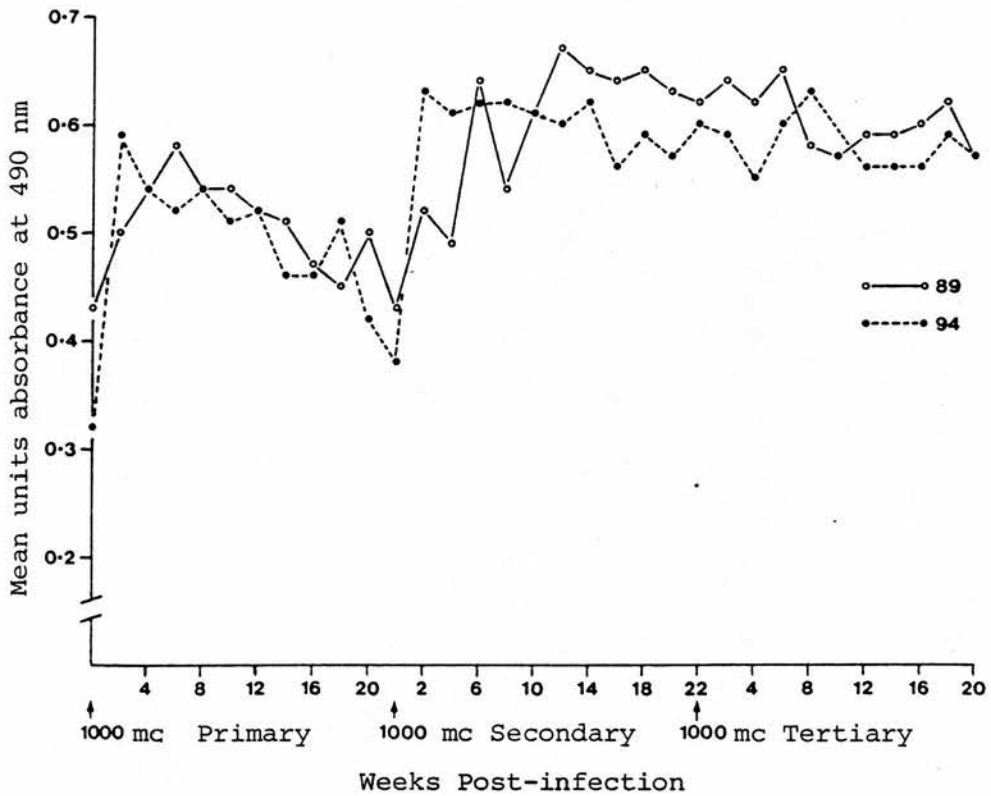


Figure 5.1.1 ELISA values from 18 month-old bullocks infected with Fasciola hepatica using somatic antigen

again rose rapidly and thereafter the titres remained constantly at relatively high levels. In bullock 89, there was a slower somewhat irregular rise in the ELISA values after secondary infection, to a maximum value at 12 weeks, after which the values remain constantly high. There was little change in the ELISA values from either animal after tertiary infection and they were consistently higher than those after primary infection.

Statistical analysis of ELISA values in both bullocks is shown in Tables 5.1 and 5.2.

Table 5.1 Comparisons of the ELISA values in the same bullocks

Bullock No.	Weeks compared	t-value	Probability
94	0 v 2(1)	10.8	<.01
	2(2)	12.8	<.01
	2(3)	21.0	<.001
	4(3)	17.5	<.01
89	0 v 2(1)	7.2	<.01
	6(1)	4.1	<.05
	6(2)	4.5	<.05
	2(3)	17.4	<.05

Means and standard deviations are given in Appendix Table 5.1.1

Moreover there were highly statistically significant differences in ELISA values between the two bullocks.

Table 5.2 Comparisons of the ELISA values between bullocks 89 and 94

Week	t-value	Probability
2(1)	13.18	<.001
2(2)	13.20	<.001
2(3)	2.60	<.10
6(1)	2.20	<.10
6(2)	4.7	<.02
6(3)	4.6	<.02

(ii) Metabolic antigen: The results are shown in Figure 5.1.2 and in Appendix Table 5.1.2.

The values obtained using pre-infection sera were lower with this antigen than those with the somatic antigen.

The values remained consistently above the values given by the pre-infection sera following primary infection in both animals although with bullock 94, as with the somatic antigen, the value rose more rapidly by weeks 2 and 10 to a low peak at week 14. The values from bullock 89 rose more slowly to a lower peak from weeks 10-14 and the ELISA value from this animal was barely above the normal level at week 22.

After secondary infection the values rose steadily with both bullocks, although in bullock 94 the maximum value was reached at week 8, while in bullock 89, this was at week 6. The ELISA values then temporarily declined, most markedly with bullock 94, but rose consistently from about week 12 to week 22. After the third infection the values remained relatively high in both animals, but were rather variable with bullock 89.

Statistical analysis of ELISA results from both bullocks is shown in Tables 5.3 and 5.4.

Table 5.3 Comparisons of the ELISA values in the same bullocks

Bullock No.	Weeks compared	t-value	Probability
94	0 v 2 (1)	6.4	<.01
	10 (1)	9.0	<.01
	2 (2)	3.9	<.05
	4 (3)	3.3	<.05
	16 (3)	6.7	<.01
89	0 v 2 (1)	2.8	<.10
	2 (1)	6.0	<.01
	2 (2)	21.5	<.001
	12 (3)	31.1	<.001
	(3)		

Means and standard deviations are given in Appendix Table 5.1.2.

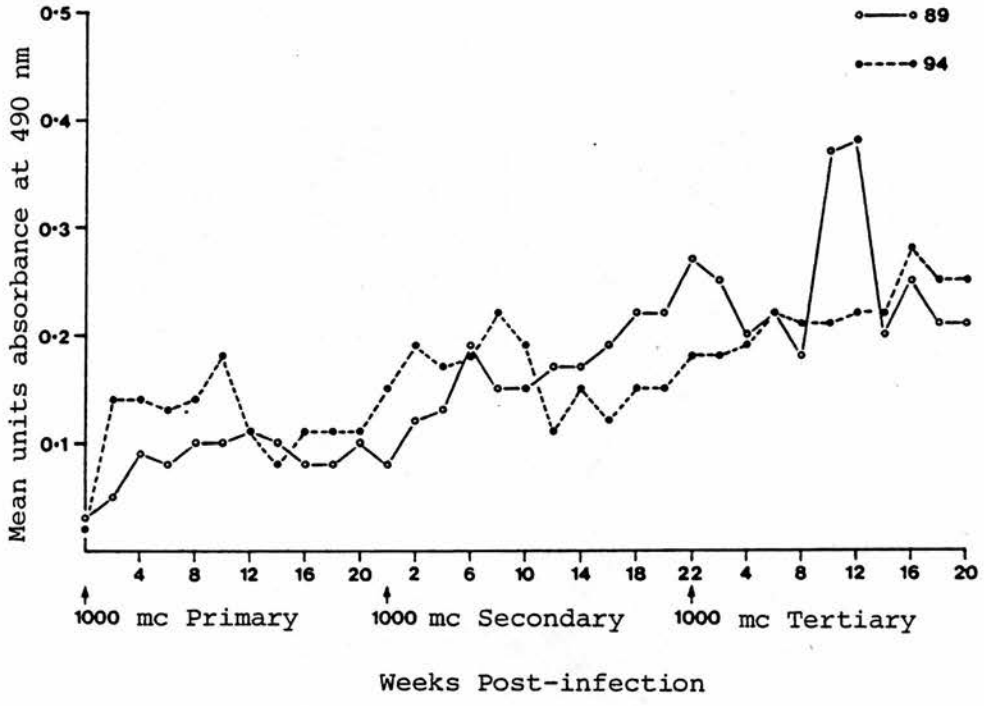


Figure 5.1.2 ELISA values from 18-month-old bullocks infected with Fasciola hepatica, using metabolic antigen.

Table 5.4 Comparisons of the ELISA values between bullocks 89 and 94

Week	t-value	Probability
$2_{(1)}$	15.7	<.001
$2_{(2)}$	3.3	<.05
$2_{(3)}$	15.1	<.001
$6_{(1)}$	4.5	<.05
$6_{(2)}$	4.9	<.02

Serum glutamic dehydrogenase assay

The results are shown in Figure 5.1.3.

After primary infection the GD values progressively increased in both bullocks, reaching a maximum at week 6, thereafter dropping temporarily but rising to a much higher peak at weeks 12 and 16 in bullock 94 and at weeks 14 and 16 in bullock 89. The values then dropped again to reach approximately normal values by weeks 18-22 in both bullocks.

After secondary infection the value rose at first only slightly and remained relatively low in bullock 94, although bullock 89 had a higher peak at week 10 than it had after primary infection. Moreover in bullock 89, the GD value rose at weeks $20_{(2)}$ and $22_{(2)}$. After tertiary infection the values in bullock 94 fluctuated but tended to be fairly low, apart from the isolated result at week $12_{(3)}$, and dropped to approximately normal levels at week $14_{(3)}$. In bullock 89, the values peaked at weeks 2 and 6 and thereafter the GD values fluctuated but also dropped to approximately the normal level at week 16.

Peripheral eosinophil counts

The results are shown in Figure 5.1.4.

The peripheral eosinophil counts in the two bullocks gave a

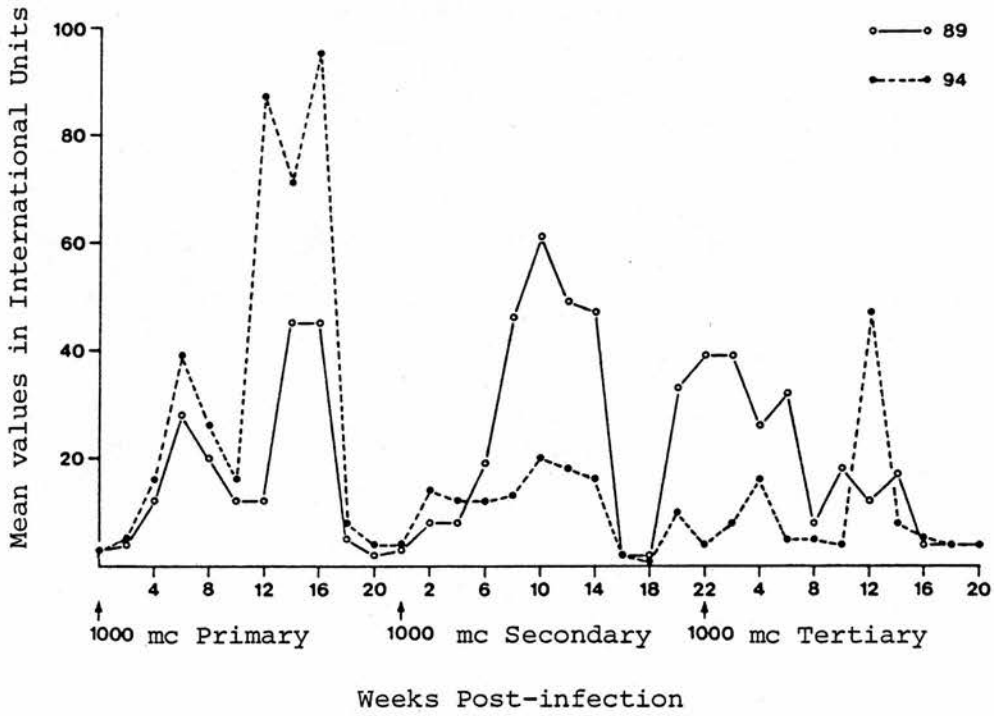


Figure 5.1.3 Serum glutamic dehydrogenase activities from 18-month-old bullocks infected with Fasciola hepatica.

biphasic response after primary infection with peaks at week 4-6 and again at about weeks 14-16, although the second peak was much less evident in bullock 89. Thereafter the counts decreased, by returning to approximately normal values in both bullocks by week 22. Following secondary infection there was a moderate increase in bullock 89, but a higher one in bullock 94 by week $2_{(2)}$, maintained in both cases until weeks $10\text{-}16_{(2)}$. However, these increases were lower than the peaks after primary infection. In both bullocks the counts dropped near to normal levels by week $18_{(2)}$ and then briefly rose again at week $2_{(3)}$. This increase was lower than that following the increase in primary or secondary infection. Thereafter the values fluctuated but had fallen near to normal levels by the end of the experiment.

Faecal egg counts

The individual faecal egg counts in bullocks 89 and 94 are shown in Figure 5.1.5.

Patency for the flukes in the two bullocks occurred at week $12_{(1)}$ when egg counts of 2-3 per gram of faeces were observed. The egg counts reached a peak of 42 e.p.g. at week $14_{(1)}$ in bullock 94 and of 32 e.p.g. at week $16_{(1)}$ in bullock 89. Thereafter the counts in each bullock fell below 10 e.p.g. with some fluctuations. There may have been some increase about weeks $10\text{-}16_{(2)}$ but the eggs completely disappeared from the faeces by weeks $18\text{-}20_{(2)}$. The egg counts were temporarily discontinued at weeks $22_{(2)}$ and weeks $4\text{-}8_{(3)}$ but following tertiary infection, eggs had reappeared by week $12_{(3)}$ and low peaks occurred at week $14_{(3)}$ (No. 94) and week $18_{(3)}$ (No. 89).

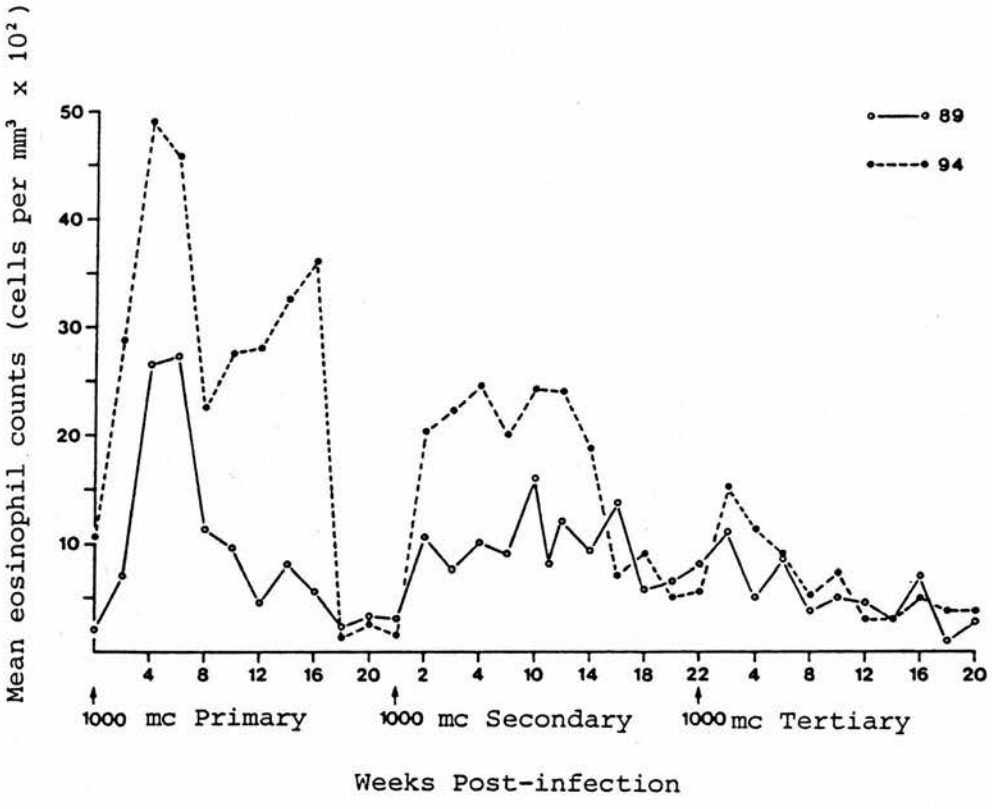


Figure 5.1.4 Peripheral eosinophil counts from 18-month-old bullocks infected with Fasciola hepatica.

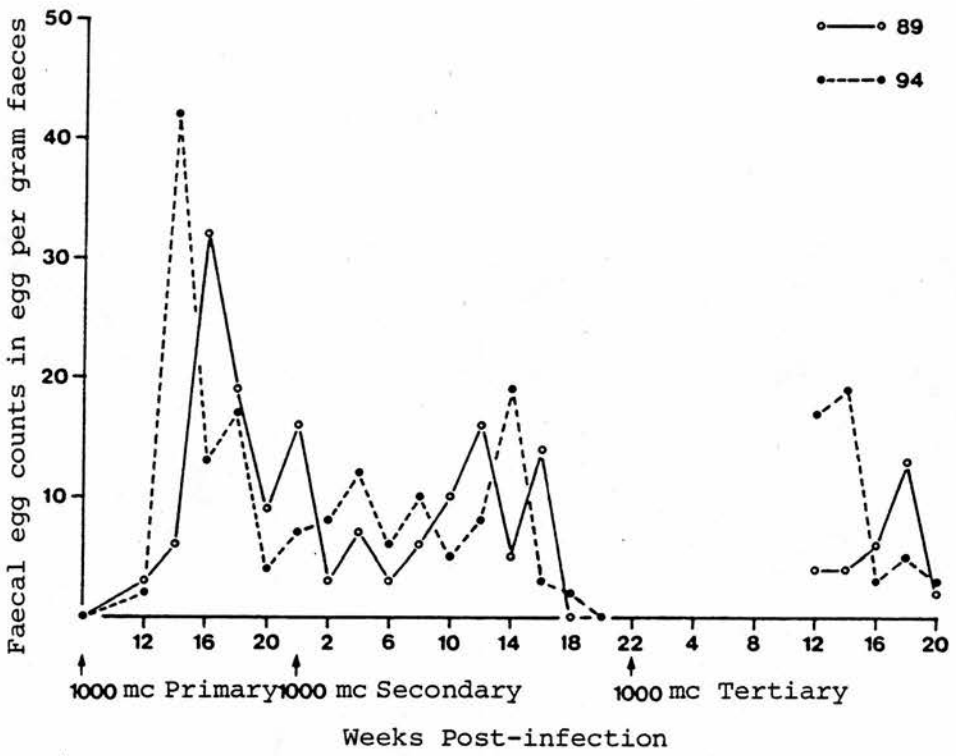


Figure 5.1.5 Faecal egg counts from 18 month-old bullocks infected with Fasciola hepatica.

Discussion

The results clearly show that the bullocks are mounting an immune response. This is indicated by the increased ELISA values and by the elevated eosinophil counts within two weeks after initial infection.

The ELISA levels, using either somatic or metabolic antigen tended to increase following repeated infections, although the two bullocks varied in their responses. This individual difference in serological response was statistically significant at certain weeks following infection or reinfection.

The different values in antibody titres as measured by ELISA, using either somatic or metabolic antigen, indicate that the two antigens were reacting with different kinds of antibodies. Inasmuch as the values with normal serum, measured by metabolic antigen, were much lower than the ones measured by somatic antigen, it appears that the metabolic antigen reacts with more specific antibodies. The ratio of the reaction of immune sera to normal sera using the metabolic antigen was about 2-3, much greater than that with the somatic antigen of about 1.5.

The eosinophil counts and serum glutamic dehydrogenase levels were greatly increased after primary infection, reaching peak values at weeks 6, 12 and 18 respectively. A similar increase followed both the secondary and tertiary infections. Indeed in 89 the peak GD value was higher after the secondary infection than after the primary infection, although all the eosinophil peaks and the other GD peaks were lower than after the initial infection, as were the numbers of eggs (e.p.g.) recovered from the faeces after repeated infections. This probably reflects the animal's increasing resistance to challenge.

This observation on GD is in agreement with Doyle (1971), who observed a rise in SGOT two weeks after infection with 750 metacercariae of F. hepatica in 17-week-old dairy calves. However, reinfection with 1,650 metacercariae 13 weeks later did not alter the mean SGOT level as compared to a group of calves which were not reinfected. He concluded that cattle which had been previously exposed to adult stage of F. hepatica develop an immunity to subsequent infection.

The increase in egg counts after patency is also in agreement with Doyle (1971), who observed maximum egg counts 13-16 weeks after infection in 17-week-old dairy calves and with Kendall, Sinclair, Everett and Parfitt (1978), who observed a maximum egg count at about 19 weeks after infection in 9-16-week-old Friesian calves, although the primary infections became patent at 11 weeks and 9-10 weeks respectively. Kendall et al. (1978) further observed that the peak in egg counts after repeated infection was two times less than that after the primary infection and they suggested that repeated infections enhanced resistance to challenge as indicated by the lower number of flukes recovered from repeatedly infected calves than from those given single infections.

For financial reasons it was not possible to recover flukes from these bullocks and, in any case, the nature of the study did not permit necropsy after primary or secondary infection. However, the biochemical and haematological results and faecal egg counts following repeated infections, suggest that the bullocks had developed resistance to subsequent challenge.

5.2 Passive Transfer of Resistance to Rats by Immune Serum from 18-month-old Bullocks Collected at Three-weekly Intervals after Primary Infection

Introduction

Previous work on passive transfer of resistance to F. hepatica has utilised sera from infected donors collected at one time after infection or reinfection. Thus Armour and Dargie (1974) have shown that sera from rats infected ten weeks previously with 30 metacercariae of F. hepatica or sera from cattle infected with 1,000 metacercariae and challenged 14 weeks later can transfer resistance to recipient rats. Likewise, Howell, Sandeman and Rajasekariah (1977) have shown that sera from rats given a primary infection with five metacercariae of F. hepatica and challenged seven weeks later with 30 metacercariae can transfer significant protection against challenge in a homologous recipient. Similarly, Haroun (1979) showed that sera collected from cattle 8-10 weeks after a single infection with 1,000 metacercariae of F. hepatica can transfer resistance against challenge in rats.

From these studies it can be concluded that sera from infected donors can protect against challenge. However it is not known the degree of protection afforded by the serum varies during the course of the infection in the donor animal.

Experimental design

Two experiments were undertaken consecutively. The first consisted of separate groups of rats immunised with serum (IBS) from bullocks 89 or 94 collected at three, six and nine weeks after infection.

The second consisted of groups of rats receiving pooled IBS from the same bullocks collected at weeks 12, 15 and 18 after primary infection. Pooling of sera was done by mixing equal volumes of serum from each bullock in sterile universal bottles before immunisation. It was decided to pool the sera from the two bullocks in this second experiment in order to economise in the use of rats. Moreover it has been shown in previous studies by Haroun (1979) that pooling the sera from infected cattle did not appear to have any significant effects on their capacity to transfer resistance against challenge to the rats.

In the first experiment 80 six-week-old male Wistar rats (approximately 180-200 grams at the time of infection) were randomly assigned into groups (A and B) of 40 rats. Group A was immunised with normal or immune sera from bullock 89, while group B received normal or immune sera from bullock 94.

All the rats in each group were further assigned randomly into four groups of 10 rats. Groups A1 and B1, which served as control groups, were immunised with normal serum from bullocks 89 and 94 respectively; groups A2 and B2 received IBS collected at week $3_{(1)}$; groups A3 and B3 received IBS collected at week $6_{(1)}$ and groups A4 and B4 received IBS from the respective donors collected at week $9_{(1)}$.

In the second experiment 40 six-week-old male Wistar rats were randomly assigned into four groups (1-4) of ten rats each. Group 1, which served as the control, was immunised with pooled normal serum; group 2 received IBS collected at week $12_{(1)}$; group 3 received IBS collected at week $15_{(1)}$ and group 4 received IBS collected at week $18_{(1)}$.

All the rats in each group were infected orally with 20 metacercariae each of F. hepatica and immediately after infection each rat was immunised intraperitoneally with 10 ml of normal or immune serum from the appropriate bullock or pool. Immunisation was repeated two days later.

Serum glutamic dehydrogenase activities were monitored fortnightly, using the activated technique, and necropsy of rats and recovery of adult flukes was carried out eight weeks after infection.

Results

Three rats in the first experiment and one in the second died within two weeks after infection and immunisation. The cause of death was not determined but it was suspected, based on the presence of bloody exudate in the peritoneal cavity, that the animals died of internal haemorrhage which was probably associated with the parenchymal migratory phase of the fluke development.

Fluke recovery

The results are shown in Table 5.2.1 and the statistical analysis using the Mann-Whitney U-test, is shown in Appendix Table 5.2.1.1.

Study 1: There were significantly fewer flukes recovered from groups A3 and A4 ($p < .001$) as compared to the control group (A1). Moreover there was a statistically significant difference in groups A3 and A4 ($p < .001$) as compared to group A2. There was no significant difference between groups A1 and A2 and between groups A3 and A4. Likewise there was a statistically significant difference in the numbers of flukes developing after challenge in groups B3

($p < .10$) and B4 ($p < .025$) as compared to the control group B1 immunised with normal serum from bullock 94, but there was no significant difference when group B2 was compared with group B1 ($p > .10$). Comparison between groups B2 and B3 ($p < .025$) and B2 and B4 ($p < .05$) also showed a statistically significant difference in the number of flukes recovered.

Study 2: There was near significant differences between the control group 1 and group 2 ($p < .10$) and between groups 2 and 4 ($p < .10$). In contrast there was no statistically significant difference in flukes developed from challenge in groups 3 and 4 when compared to the control group 1.

Serum glutamic dehydrogenase assay

The data are shown in Appendix Tables 5.2.1 and 5.2.2 for studies 1 and 2 respectively.

Study 1: Owing to significant activation of the GD in the plasma samples during the assay brought about by an excess amount of ADP in the test, which resulted in a high absorbance in the "creep reaction", the data presented in Appendix Table 5.2.1 consisted of the uncorrected absorbance values observed in the actual test only. Figure 5.2.1 shows the mean GD levels.

There were increasing levels of GD from week 2, reaching peaks at week 4. Thereafter the levels declined and dropped to nearly normal levels by week 8.

The GD values at weeks 4 and 6 in the treated groups (A2,A3 and A4) were less than those from the control group (A1), but this difference was not statistically significant.

Table 5.2.1

Passive transfer of resistance to 20 metacercariae of F. hepatica in rats by serum from infected bullocks. Two 10 ml volumes of serum given by intraperitoneal injection on the day of challenge and two days later.

Donor Bullock	Serum		Nos. of flukes recovered at P.M. Individual	Mean ± SD
89 (Group A)	Normal serum	(A1)	6, 6, 2, 5, 4, 4, 8, 7, 3, -	5.0 ± 1.9
	Immune serum $3_{(1)}$	(A2)	6, 5, 5, 4, 6, 2, 6, 4, 6, 4	4.8 ± 1.3
	Immune serum $6_{(1)}$	(A3)	0, 2, 1, 2, 3, 0, 1, 0, 2, 2	1.3 ± 1.1
	Immune serum $9_{(1)}$	(A4)	0, 0, 0, 1, 1, 0, 4, 3, 3, -	1.3 ± 1.6
94 (Group B)	Normal serum	(B1)	8, 2, 2, 5, 3, 7, 3, 1, 3, -	3.8 ± 2.4
	Immune serum $3_{(1)}$	(B2)	6, 4, 3, 2, 3, 2, 6, 4, 2, 5	3.7 ± 1.6
	Immune serum $6_{(1)}$	(B3)	2, 2, 3, 2, 1, 3, 2, 3, 2, 2	2.2 ± 0.6
	Immune serum $9_{(1)}$	(B4)	1, 1, 2, 3, 1, 2, 0, 4, 0, 1	1.5 ± 1.3
89/94 pooled	Normal serum	(1)	3, 5, 3, 7, 5, 6, 2, 3, 7, -	4.6 ± 1.9
	Immune serum $12_{(1)}$	(2)	3, 4, 5, 1, 4, 5, 4, 3, 2, 0	3.1 ± 1.7
	Immune serum $15_{(1)}$	(3)	3, 3, 5, 2, 5, 2, 4, 4, 8, 4	4.0 ± 1.8
	Immune serum $18_{(1)}$	(4)	3, 3, 7, 5, 6, 8, 4, 5, 2, 3	4.6 ± 2.0

- Animal died

No significant differences occurred between the groups using the sera from bullock 94.

Study 2: The mean group GD levels are shown in Figure 5.2.2. There was increased GD levels within two weeks after infection, to reach a maximum peak at week 4. Thereafter the levels declined.

The peak values in treated groups (2, 3 and 4) at week 4 were lower than the control group, even for group 2 which had the lowest GD level, this difference was not statistically significant ($t = 1.21$, $p > .10$). Likewise, at week 6, there was no significant difference between the treated groups and the control group.

Discussion

The groups of rats immunised with immune serum collected at weeks 6 or 9 (pre-patency) after primary infection from either bullock had significantly fewer flukes than the corresponding controls. However this resistance is not absolute because not all the flukes developed from challenge were killed. On the other hand, the immune serum collected at week 12 (patency) gave less than either pre-patency IBS. Whether this relatively weak protection was influenced by the pooling of sera is not known. Immune serum from bullock 89 collected at week $9_{(1)}$ gives better protection (58%) than the corresponding serum from bullock 94 (39%).

Rajasekariah and Howell (1980) utilised GD as an indicator of resistance to challenge infection in previously infected rats and observed that fewer flukes cause less parenchymal damage, and Haroun (1979) observed a significantly less serum GD peak at weeks 4 or 6, using the unactivated technique, in rats immunised with sera from rats or cattle infected with *F. hepatica*.

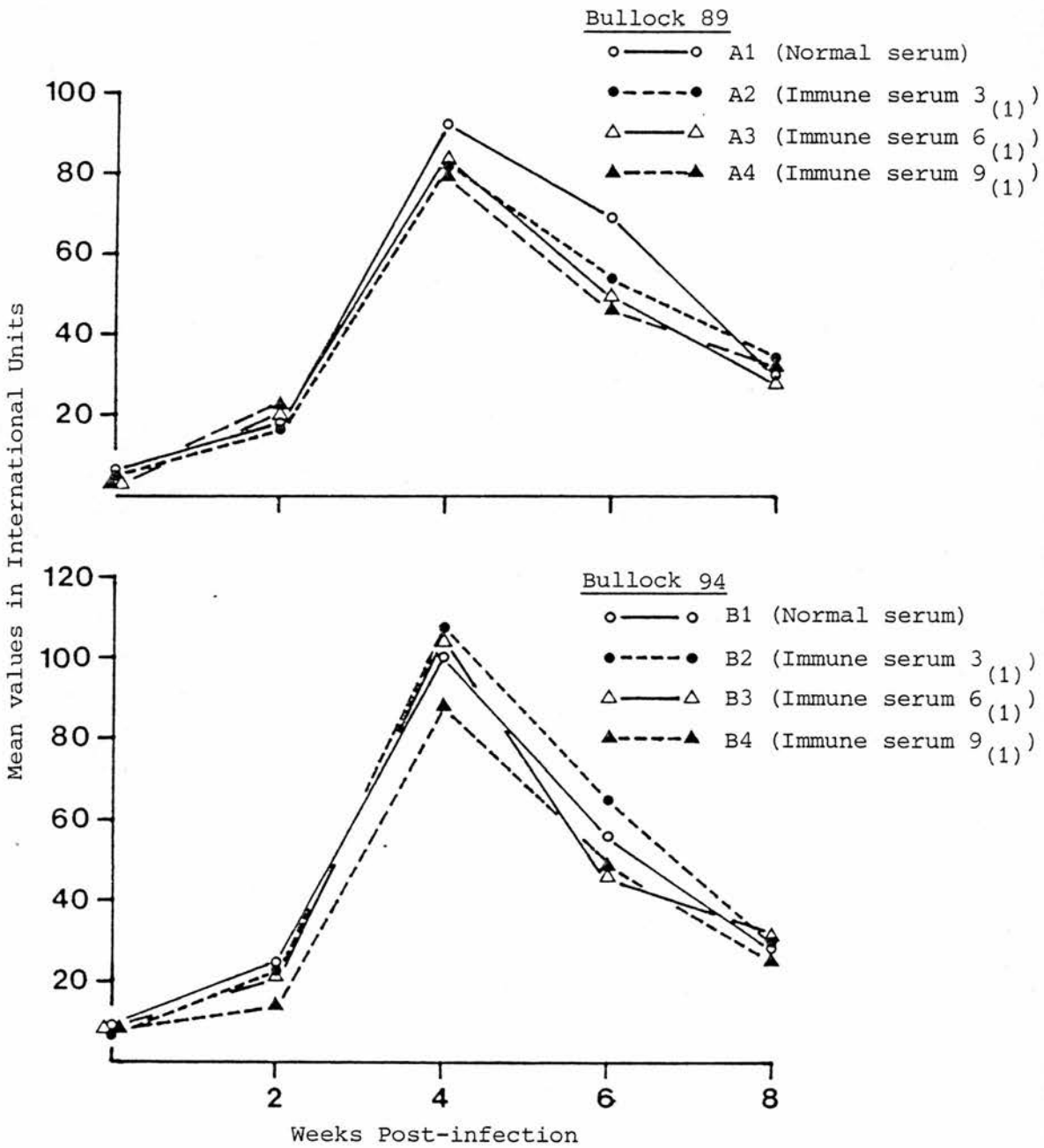


Figure 5.2.1 Serum glutamic dehydrogenase activities from rats immunised with immune serum.

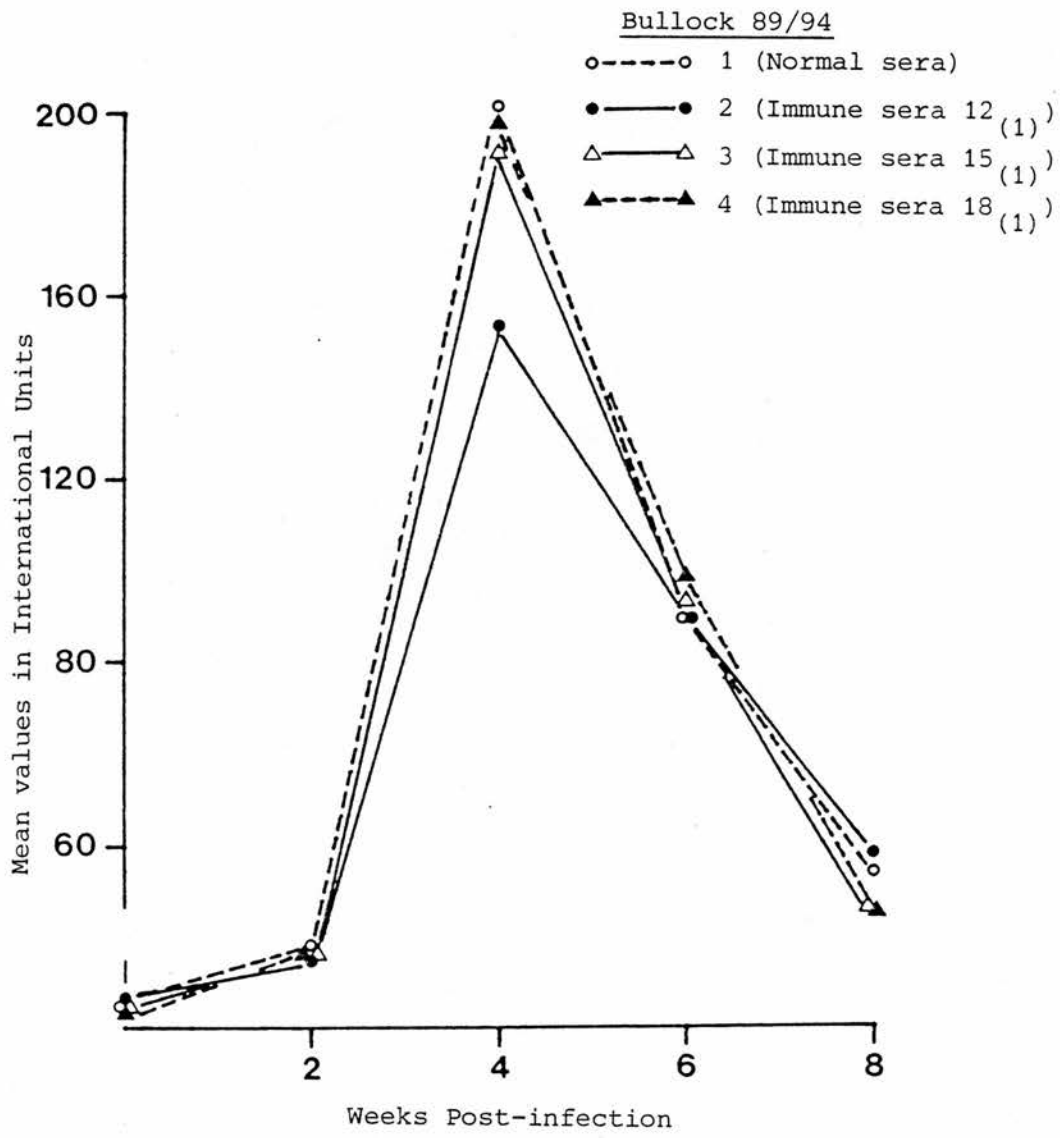


Figure 5.2.2 Serum glutamic dehydrogenase activities from rats immunised with pooled immune sera.

Moreover the GD levels at weeks 4 and 6 showed no statistically significant difference between groups treated with IBS from either bullock and control groups treated with normal serum, although the mean group GD values showed a lesser liver damage in the former group than in the latter groups.

Neither is there any significant relationship between the liver damage, as indicated by the concentration of GD in the plasma, and the numbers of flukes developed from the challenge in individual rats. In study 1, this lack of relationship may have been due to the hyperactivation of GD in the plasma during the creep reaction, brought about by the excess amount of ADP used (see Section 3.6.2), resulting in high absorbance in the creep effect. However in study 2, although the GD values in group 2 at week 4 were lower than in the control group 1, statistical analysis again showed no relationship with the fluke burden. Thus in the present study, although the numbers of flukes developed from challenge did not relate directly to the liver damage, statistical analysis on the pooled data showed a significant correlation (study 1, $r_{(1,75)} = 0.52$, $p < .05$) (study 2, $r_{(1,37)} = 0.84$, $p < .05$) between the GD activities at week 4 and the numbers of flukes recovered at necropsy.

5.3 Passive Transfer of Resistance in the Rats by Immune Serum from 18-month-old Bullocks Collected after Secondary Infection Introduction

Section 5.2 shows that IBS collected at weeks 6, 9 or 12 after primary infection gave statistically significant protection. However this resistance was not absolute.

In an attempt to obtain a more strongly protective immune serum, the two bullocks (Nos. 89 and 94), which had had a previous infection of 1,000 metacercariae, were reinfected with the same number of metacercariae 22 weeks after the initial infection and immune sera collected at three-weekly intervals.

For reasons mentioned in Section 5.2, the immune sera were once again pooled as in Section 5.2, study 2.

Experimental design

Seventy six-week-old male Wistar rats were randomly assigned into seven groups of ten rats each. Group A, which served as the control, was treated with normal bovine serum; Group B, with IBS collected at week $3_{(2)}$; Group C, with IBS collected at week $6_{(2)}$; Group D, with IBS collected at week $9_{(2)}$; Group E, with IBS collected at week $12_{(2)}$ and Group F, with IBS collected at week $15_{(2)}$. Group G, which served as the immunised primary control, was immunised with IBS collected at week $21_{(1)}$.

All the rats in each group were infected orally with 20 metacercariae each of F. hepatica and immediately after infection they were immunised intraperitoneally with 10 ml pooled normal or IBS as the case may be. Immunisation was repeated two days later.

Serum glutamic dehydrogenase activity was monitored fortnightly, using the activated technique, and necropsy of rats was carried out eight weeks after infection.

Results

All the rats in each group were alive at the end of the experiment.

Fluke recovery

The numbers of flukes recovered at post-mortem are shown in Table 5.3.1 and the statistical analysis of the results, using Mann-Whitney U-test, is shown in Appendix Table 5.3.1.1.

The numbers of flukes recovered from the treated groups were consistently fewer than in the controls (A and G). However, only in groups C and E did this approach significance when compared with the control group A, immunised with normal serum.

There were more flukes in group G than in group A, but this difference was not significant.

A comparison of flukes developed from challenge in group C or E with the other treated groups is shown in Appendix Table 5.3.1.1.

Serum glutamic dehydrogenase assay

The mean group GD and the results in individual rats in each group are shown in Figure 5.3.1 and in Appendix Table 5.3.1 respectively.

The GD levels in all groups rose slightly within two weeks of infection and reached maximum levels at week 4. Thereafter the levels declined and dropped to nearly normal values at week 8. In each case the mean peak GD levels in the groups of rats receiving IBS obtained following the secondary infection were lower than those in either control, but none of these differences were significant. The group in which the peak was most nearly significantly lower than that for the control group A was group C (serum $6_{(2)}$, $t = 2.09$, $p < .10$).

Table 5.3.1

Passive transfer of resistance to 20 metacercariae of *F. hepatica* in rats by serum from infected bullocks 89/94. Two 10 ml volumes of pooled serum given by intraperitoneal injection on the day of challenge and two days later.

Donor Bullock	Serum		Nos. of flukes recovered at P.M. Individual	Mean ± SD
89/94	Normal serum	(A)	5, 1, 5, 2, 8, 4, 5, 2, 4, 2	3.8 ± 2.1
	Immune serum $3_{(2)}$	(B)	6, 6, 4, 3, 3, 2, 3, 4, 1, 2	3.4 ± 1.6
	Immune serum $6_{(2)}$	(C)	1, 3, 0, 3, 3, 2, 4, 2, 1, 3	2.2 ± 1.2
	Immune serum $9_{(2)}$	(D)	2, 5, 0, 1, 6, 6, 4, 3, 3, 3	3.3 ± 2.0
	Immune serum $12_{(2)}$	(E)	4, 4, 3, 2, 2, 2, 2, 3, 2, 3	2.7 ± 0.8
	Immune serum $15_{(2)}$	(F)	1, 3, 5, 2, 2, 3, 1, 4, 1, 5	2.7 ± 1.6
	Immune serum $21_{(1)}$	(G)	3, 4, 2, 6, 4, 2, 6, 0, 7, 7	4.1 ± 2.4

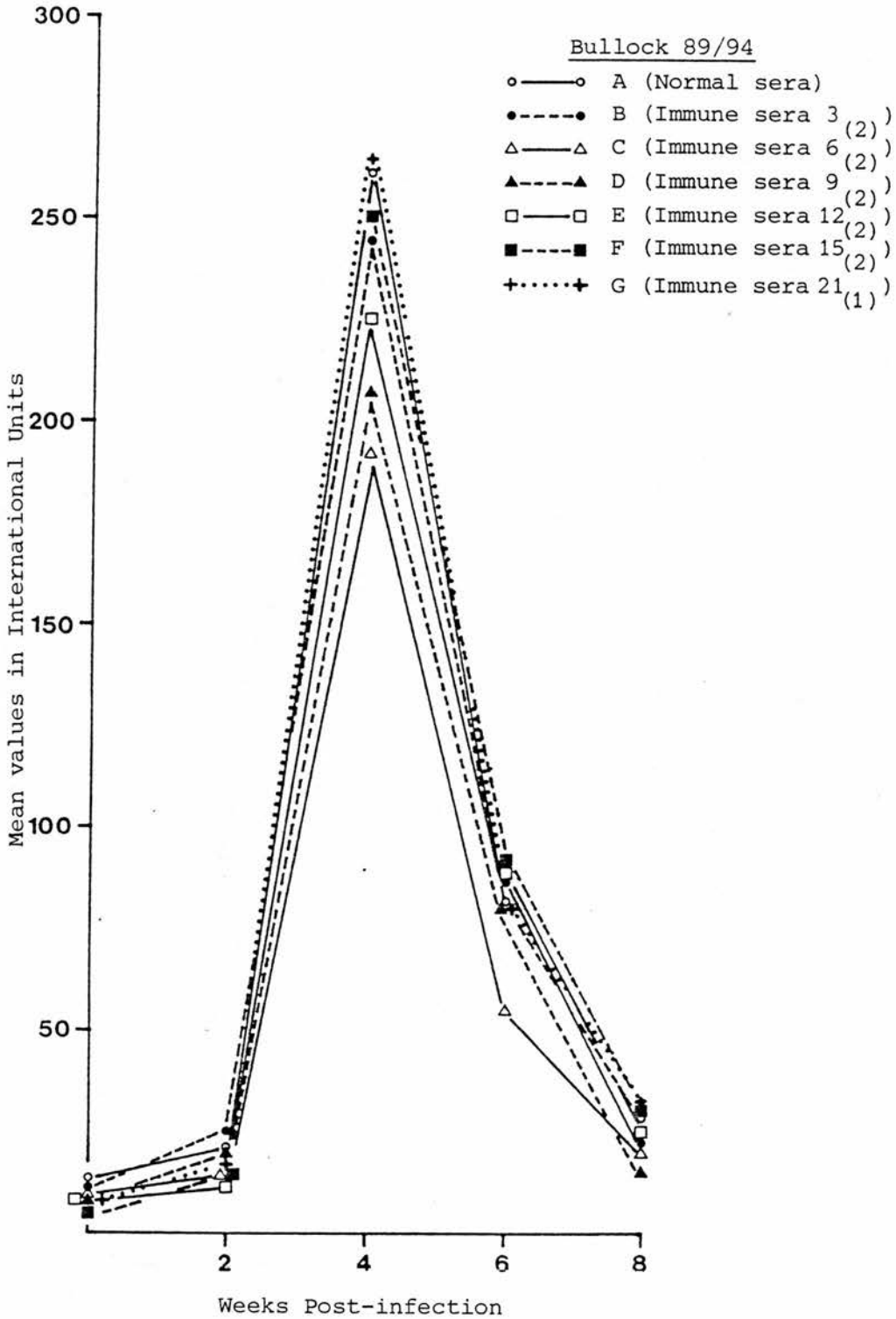


Figure 5.3.1 Serum glutamic dehydrogenase activities from rats immunised with pooled immune sera.

Discussion

The results of this study showed that IBS collected at weeks 6 and 12 were protective, as indicated by significantly fewer flukes developed from challenge. Nevertheless overall the protection afforded by these sera was not strong and was weaker than that given by IBS collected after primary infection.

It was thought that the inconsistency, wherein more flukes developed from challenge in rats immunised with IBS collected at week $9_{(2)}$ (group D) than in rats immunised with IBS collected at week $12_{(2)}$ (group E) or week $15_{(2)}$ (group F), may have been associated with the pooling of the sera or it may have been associated with the mounting of a rapid and more effective cellular response of the bullock at this stage of infection, which depressed the humoral protective response by reducing the exposure to the appropriate antigen.

Again there was no relationship between the GD activities at week 4 and the numbers of flukes recovered in groups C or E. However, on the pooled data there was a significant correlation ($r_{(1,68)} = 0.67$, $p < .05$) between the GD activities at week 4 and the numbers of flukes recovered at necropsy.

5.4 Passive Transfer of Resistance in Rats by Immune Serum from 18-month-old Bullocks Collected after Tertiary Infection

Introduction

Immune serum from bullocks given primary and secondary infections with F. hepatica had been shown to confer protection in rats, although this protection was not very strong. Furthermore

it had been shown in Section 5.2, Study 1, that IBS (week $9_{(1)}$) from bullock 89 gave better protection than the immune sera from bullock 94 collected at the same week. It is possible that the protective effect of immune sera from bullock 89 was diluted. Therefore the two bullocks were given a third infection of 1,000 metacercariae, 22 weeks after the second infection, and the immune sera collected at $3_{(3)}$ weekly intervals were used separately.

Experimental design

Eighty-four six-week-old male Wistar rats were assigned into two main groups (A and B) of 42 rats each. Group A was immunised with IBS from bullock 89 and group B, with IBS from bullock 94.

The rats in each main group were further allocated randomly into seven groups of six rats each. Groups A1 and B1, which served as controls, were immunised with normal serum; groups A2 and B2 and groups A3 and B3 were given IBS collected at weeks $6_{(2)}$ and $9_{(2)}$ respectively; while groups A4 and B4 were immunised with IBS collected at week $3_{(3)}$; groups A5 and B5, with IBS collected at week $6_{(3)}$; groups A6 and B6, with IBS collected at week $9_{(3)}$; and groups A7 and B7, with IBS from respective donors collected at week $12_{(3)}$.

As usual all the rats in each group were infected orally with 20 metacercariae each of F. hepatica and immediately after infection 10 ml of normal or immune sera was administered intraperitoneally. Immunisation was repeated two days later.

Serum glutamic dehydrogenase activities were monitored at weeks 0, 4 and 6 only after infection. This was so because previous studies, using activated technique, showed that GD levels peaked at week 4 and thereafter declined.

Necropsy of rats and recovery of adult flukes was carried out eight weeks after infection.

Results

One rat in each of groups A3 and B5 died, within four weeks and one week respectively after infection, of internal haemorrhage which was probably associated with the parenchymal migratory phase of the fluke development.

Fluke recovery

The data are shown in Table 5.4.1 and statistical analysis is given in Appendix Table 5.4.1.1.

The numbers of flukes recovered in groups of rats immunised with IBS from bullock 89 were consistently fewer than those from the corresponding group immunised with normal serum from the same bullock. This difference was not significant when using sera $^{6}_{(2)}$.

In groups of rats immunised with IBS from bullock 94, the mean numbers of flukes developed from challenge were fewer than in the control group immunised with normal serum, except for groups B4 and B5 which had received sera $^{3}_{(3)}$ and $^{6}_{(3)}$ respectively. However, none of these differences were statistically significant. With bullock 89, the group of rats receiving sera $^{9}_{(2)}$ contained more flukes than those receiving sera $^{6}_{(2)}$.

Serum glutamic dehydrogenase assay

The mean group GD levels are shown in Figure 5.4.1 and the individual data in Appendix Table 5.4.1.

In groups immunised with IBS from bullock 89, the mean GD level at week 4 was lesser than in the control (A1). Nevertheless

Table 5.4.1

Passive transfer of resistance to 20 metacercariae of F. hepatica in rats by serum from repeatedly infected bullocks. Two 10 ml volumes of serum given by intraperitoneal injection on the day of challenge and two days later.

Donor Bullock	Serum		Nos. of flukes recovered at P.M. Individual	Mean ± SD
89 (Group A)	Normal serum	(A1)	6, 5, 5, 6, 6, 4	5.3 ± 0.8
	Immune serum $6_{(2)}$	(A2)	2, 5, 7, 2, 3, 2	3.5 ± 2.1
	Immune serum $9_{(2)}$	(A3)	4, 4, 6, 4, 8, -	5.2 ± 1.8
	Immune serum $3_{(3)}$	(A4)	3, 1, 5, 6, 7, 7	4.8 ± 2.4
	Immune serum $6_{(3)}$	(A5)	3, 2, 4, 3, 6, 4	3.7 ± 1.4
	Immune serum $9_{(3)}$	(A6)	1, 2, 1, 3, 9, 3	3.2 ± 3.0
	Immune serum $12_{(3)}$	(A7)	6, 2, 3, 7, 2, 4	4.0 ± 2.1
94 (Group B)	Normal serum	(B1)	5, 6, 3, 7, 3, 6	5.0 ± 1.7
	Immune serum $6_{(2)}$	(B2)	5, 3, 3, 5, 8, 5	4.8 ± 1.8
	Immune serum $9_{(2)}$	(B3)	4, 6, 2, 6, 3, 7	4.7 ± 2.0
	Immune serum $3_{(3)}$	(B4)	5, 5, 6, 3, 6, 5	5.0 ± 1.1
	Immune serum $6_{(3)}$	(B5)	7, 8, 5, 5, 5, -	6.0 ± 1.4
	Immune serum $9_{(3)}$	(B6)	5, 6, 5, 1, 5, 4	4.3 ± 1.7
	Immune serum $12_{(3)}$	(B7)	4, 2, 3, 6, 8, 5	4.7 ± 2.2

- Animal died

these differences were not statistically significant.

In groups immunised with IBS from bullock 94, the GD levels at week 4 in all experimental groups, with the exception of group B5 (serum $6_{(3)}$), were greater than in the normal control (B1). However, and again, these differences were not statistically significant.

Discussion

As in the study using sera from the primary infection the prepatent (6-9 weeks) IBS from bullock 89 gave better protection than the immune serum from bullock 94 collected at the same time. The highly elevated ELISA values after tertiary infection of bullock 89, using either somatic (Figure 5.1.1) or metabolic (Figure 5.1.2) antigen, decreased GD levels at weeks 10-$20_{(3)}$ (Figure 5.1.3) and low eosinophil counts (Figure 5.1.4) after secondary and tertiary infections, all of which suggest the immune response in this animal was greater than in bullock 94.

Thus pooling the serum in the previous studies probably did have some adverse effect on the capacity of IBS from bullock 89 to transfer resistance, although as before, the IBS collected at week $9_{(2)}$ (Table 5.3.1) from either bullock was not protective. There is no explanation for this other than the mounting of a more rapid and effective cellular response of the animals at this stage of infection, which depressed the humoral protective response by reducing the exposure to the appropriate antigen.

Overall there exists great variations in immune response between bullocks and secondary and tertiary infections did not increase the protective effect of their immune serum.

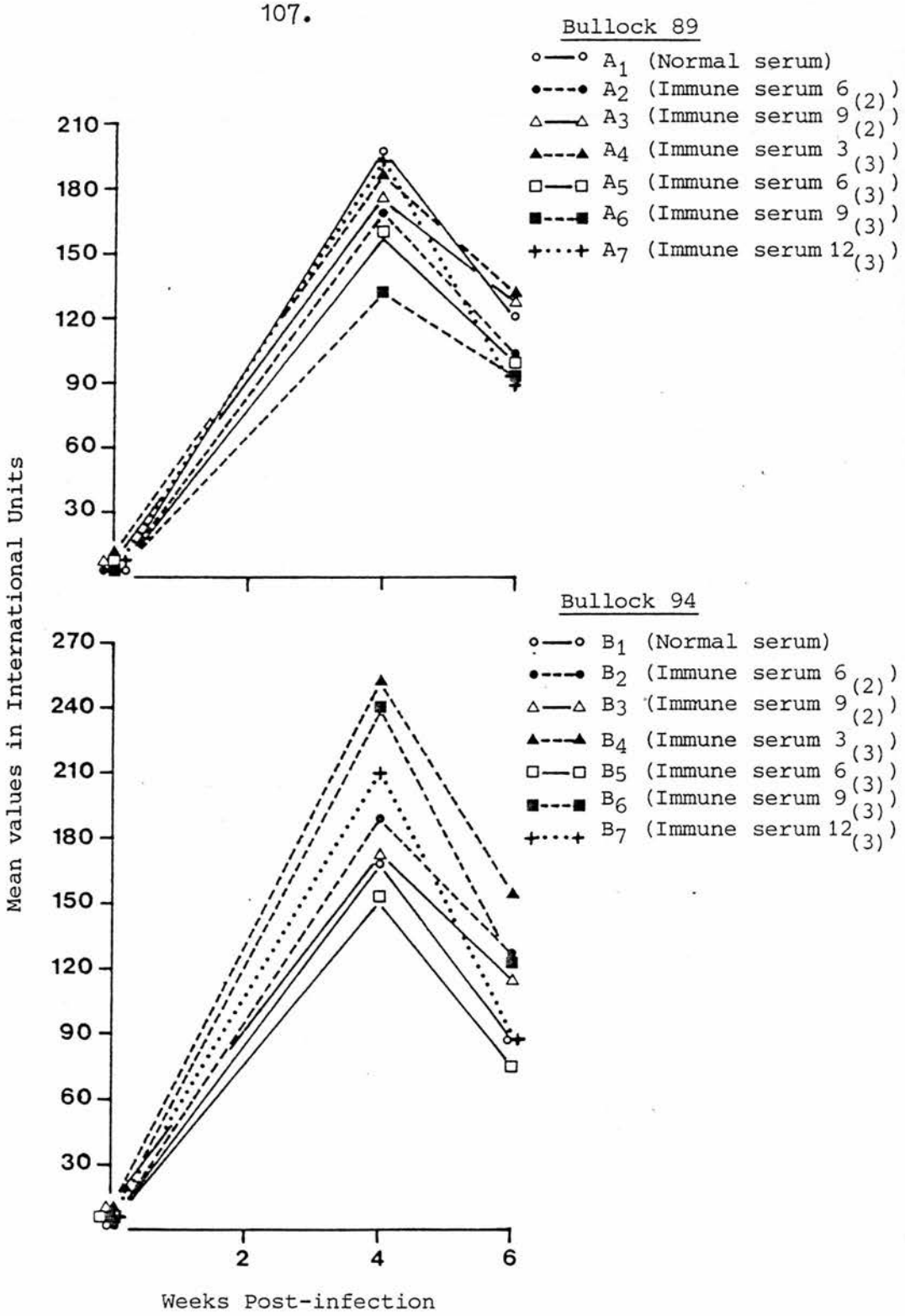


Figure 5.4.1 Serum glutamic dehydrogenase activities from rats immunised with immune sera.

Moreover, although there was no significant difference in GD activities in groups of rats whose numbers of flukes recovered were significantly reduced (see Appendix Table 5.2.1) when compared with the control groups, it appears that there is a high correlation ($r_{(1,266)} = 0.53$, $P < .05$) on the pooled data between the GD activities and the numbers of flukes recovered at necropsy. Thus the GD activity is a relatively good indicator for liver damage induced by the juvenile flukes as shown by Rajasekariah and Howell (1980).

CHAPTER SIX

STUDIES USING MATURE ADULT CATTLE

Introduction

The previous study in young mature cattle (about 18 months old) showed that, although the animals had acquired resistance to challenge infections as indicated by low GD levels, low eosinophil and faecal egg counts after repeated infections, their sera tended to be less protective than that obtained following the initial infection.

Haroun (1979) utilised 8-10 month old cattle as a source of immune serum for passive transfer of resistance in rats. Boray (1969) did not study the use of immune sera from F. hepatica infected cattle for passive transfer of resistance, but concluded that the age of the host was a more important factor in the resistance. In his study, although the 6-8 month old calves infected with 1,000 metacercariae developed clinical chronic fascioliasis with anaemia and high egg counts, the animals recovered spontaneously. When these animals were challenged with 1,000 metacercariae together with controls at 16-18 months of age, both groups showed high resistance and low recoveries.

It was considered that the use of immune sera from older cattle (i.e. 4-5 year old) for passive transfer of resistance against Fasciola hepatica infection might afford stronger protection. Such cattle have not previously been used for this purpose.

It seemed possible that more mature animals, whose haemopoietic system might be further developed, as shown by Rajasekariah and Howell (1977b) in rats, could be better stimulated than younger cattle to produce a strongly protective serum.

6.1 Studies in Infected Cattle

Experimental design

Three 4-5 year-old Ayrshire bullocks (Nos. 203, 204 and M199) were each infected with 1,000 metacercariae of F. hepatica and 30 weeks later each animal was given another 1,000 metacercariae.

Small (10-15 ml) blood and faecal samples were collected from each bullock at two-weekly intervals up to 12 weeks after primary infection, following which the samples were collected at three-weekly intervals until they recovered from the second infection and then again at two-weekly intervals, to monitor peripheral eosinophil counts, serum glutamic dehydrogenase (GD) activities, serology and faecal egg counts.

Larger blood samples (c. 500 ml) were taken from these animals before initial infection and at weeks $9_{(1)}$, $12_{(1)}$, $6_{(2)}$ and $9_{(2)}$.

For the assay of GD activities (using the activated technique) and eosinophil counts the samples were duplicated, while four replicate samples were assayed by ELISA, using either rabbit-derived somatic or metabolic antigen.

Results

Serology

(i) Somatic antigen: The corrected ELISA values from bullocks 203, 204 and M199 are shown in Figure 6.1.1 and the detailed results are in Appendix 6.1.1.

As in Chapter 5, Section 5.1, the increase in ELISA values in the three bullocks after primary infection was not dramatic, especially as the normal sera from 204 and M199 gave high absorbance values.

However there was a steady increase following infection to a level which was maintained, with some variations, from about week 15 in M199 and week 24 in 204, the second infection having little effect. In 203, on the other hand, there was an increase in titre by week 4 to a peak at week 8. The values then fluctuated but remained above normal for that animal until week $6_{(2)}$. They then fell to normal levels for two weeks.

The statistical analysis of the ELISA values in these three bullocks is shown in Table 6.1 and 6.2.

Table 6.1 Comparisons of the ELISA values in the same bullocks

Bullock No.	Weeks compared	t-value	Probability
203	0 v $2_{(1)}$	5.8	< .02
	$6_{(1)}$	9.3	< .01
	$12_{(1)}$	6.2	< .01
	$2_{(2)}$	20.3	< .001
	$4_{(2)}$	9.0	< .01
	$6_{(2)}$	8.0	< .01
	$12_{(2)}$	3.1	< .10
204	0 v $2_{(1)}$	1.2	> .10
	$6_{(1)}$	3.4	< .05
	$15_{(1)}$	2.6	< .10
	$2_{(2)}$	3.8	< .05
	$4_{(2)}$	3.2	< .10
M199	0 v $2_{(1)}$	2.3	> .10
	$6_{(1)}$	2.6	< .10
	$15_{(1)}$	7.5	< .01
	$2_{(2)}$	4.7	< .02
	$4_{(2)}$	2.7	< .10

Means and standard deviations are given in Appendix Table 6.1.1.

Statistical analysis of ELISA values among the three bullocks, using a single classification ANOVA, showed that there were significant differences both before and after infection.

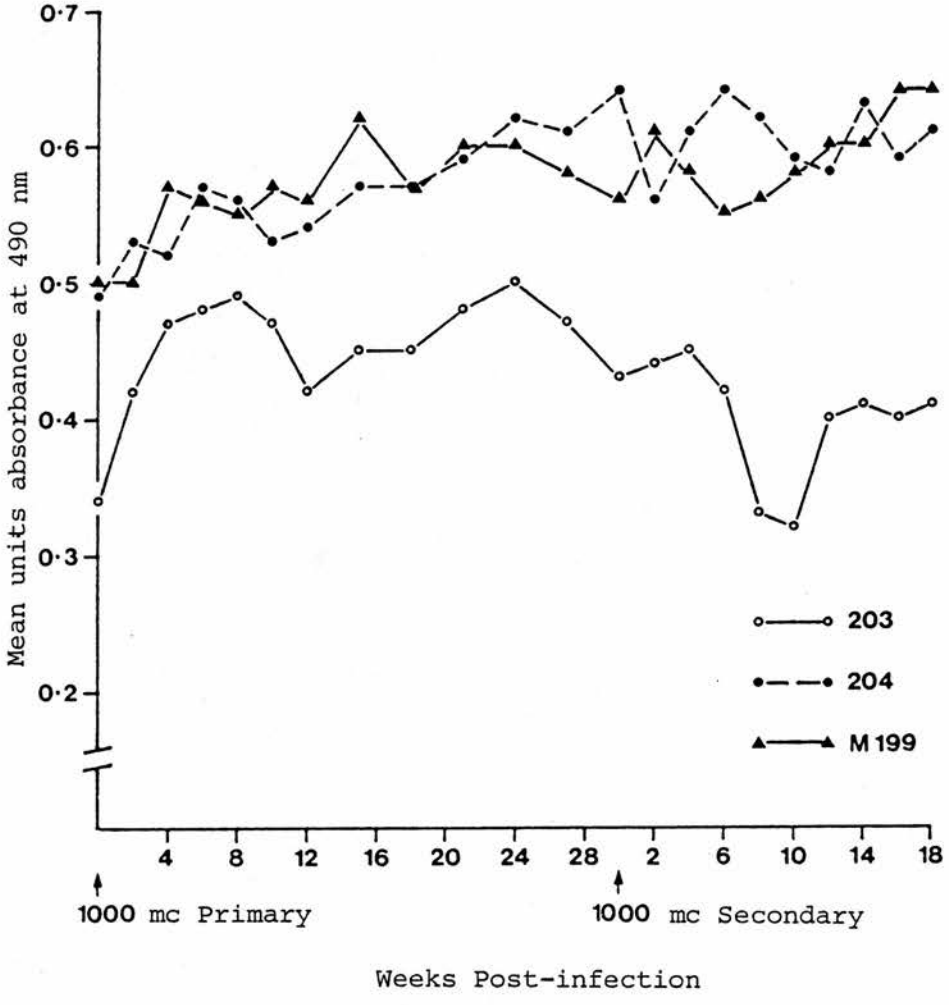


Figure 6.1.1 ELISA values from 4-5-year-old bullocks infected with Fasciola hepatica, using somatic antigen.

Table 6.2 Comparisons of the ELISA values among bullocks 203, 204 and M199

Week	F-value	Probability
0	22.7	< .05
$2_{(1)}$	37.5	< .05
$12_{(1)}$	120.0	< .05
$18_{(1)}$	68.9	< .05
$2_{(2)}$	65.9	< .05
$6_{(2)}$	190.0	< .05
$10_{(2)}$	87.5	< .05
$16_{(2)}$	645.0	< .05

(ii) Metabolic antigen: The corrected ELISA values are shown in Figure 6.1.2 and the detailed results are in Appendix Table 6.1.2.

Bullocks 204 and M199 again had higher normal values than 203 and also than the animals in Chapter 5 (Section 5.1). After infection two of the animals (203 and M199) gave similar response patterns, with temporarily elevated levels from weeks $2_{(1)}$ to $4\text{-}10_{(1)}$ followed by a return to approximately the level seen in the normal sera. The levels in these animals were tending to rise again before the second infection and were maintained above normal thereafter.

With the antigen it was 204 which showed a different pattern, with the initial peak being somewhat delayed but the second rise occurring much sooner, so that the level only fell to a near normal level at week $12_{(1)}$.

The statistical analysis of these ELISA results is shown in Tables 6.3 and 6.4.

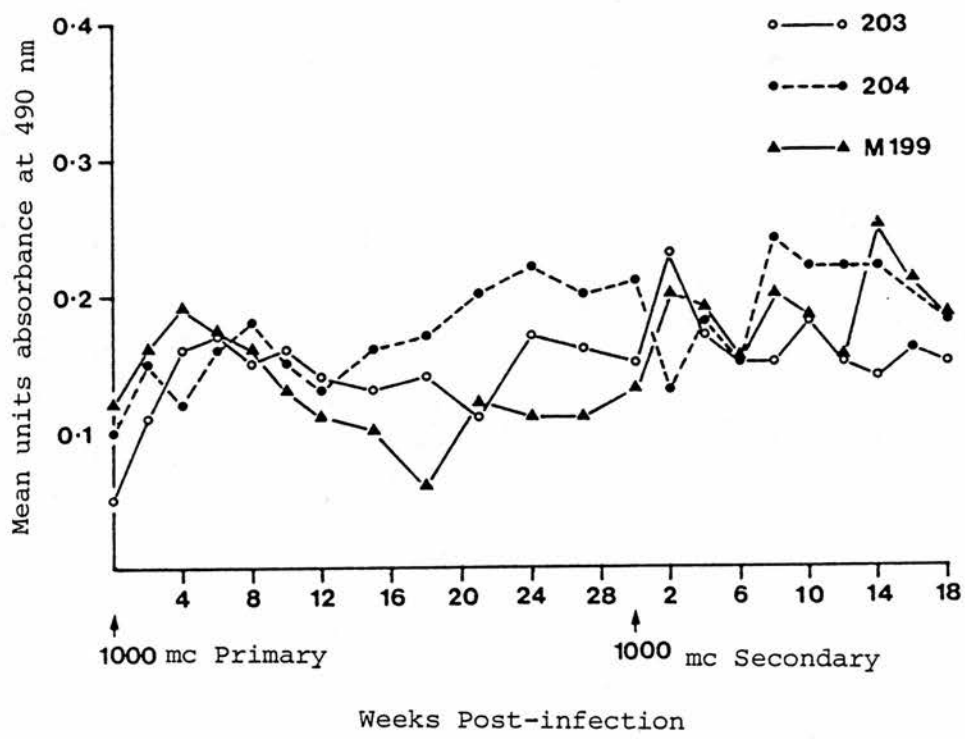


Figure 6.1.2 ELISA values from 4-5-year-old bullocks infected with Fasciola hepatica, using metabolic antigen.

Table 6.3 Comparisons of the ELISA values in the same bullocks

Bullock No.	Weeks compared	t-values	Probability
203	0 v $6_{(1)}$	4.7	< .02
	$10_{(1)}$	11.0	< .01
	$12_{(1)}$	6.8	< .01
	$15_{(1)}$	11.3	< .01
	$21_{(1)}$	5.0	< .02
	$2_{(2)}$	16.1	< .001
	$14_{(2)}$	9.2	< .01
	$16_{(2)}$	10.8	< .01
204	0 v $2_{(1)}$	6.9	< .01
	$8_{(1)}$	5.2	< .02
	$12_{(1)}$	1.8	> .10
	$15_{(1)}$	4.0	< .05
	$8_{(2)}$	17.7	< .001
	$14_{(2)}$	11.7	< .01
M199	0 v $4_{(1)}$	4.1	< .05
	$12_{(1)}$	0.6	> .10
	$18_{(1)}$	5.0	< .02
	$2_{(2)}$	2.2	> .10
	$8_{(2)}$	8.1	< .01

Means and standard deviations are shown in Appendix Table 6.1.2.

Table 6.4 Comparisons of the ELISA values among bullocks 203, 204 and M199

Week	F-value	Probability
0	22.1	< .05
$21_{(1)}$	40.9	< .05
$30_{(1)}$	47.6	< .05
$12_{(2)}$	14.7	< .05

Serum glutamic dehydrogenase assay

The results are shown in Figure 6.1.3.

In all three bullocks the GD values rose above the pre-infection values by four weeks after infection and remained so until after the second infection. From week $10_{(1)}$ to $20_{(1)}$ the values were consistently high apart from two isolated results from 204 and M199

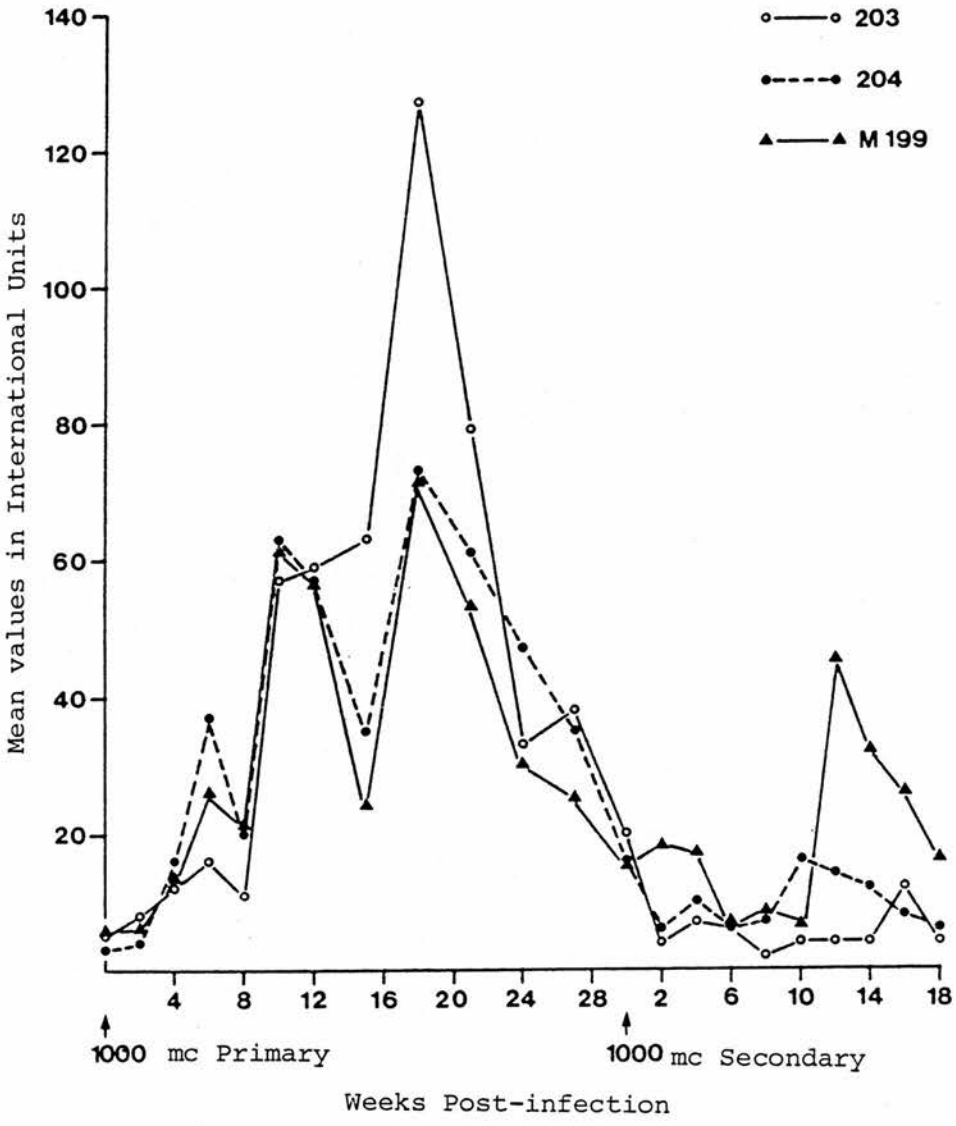


Figure 6.1.3 Serum glutamic dehydrogenase activities from 4-5-year-old bullocks infected with Fasciola hepatica.

at week $15_{(1)}$. There was little response after the second infection. The values remaining low except for a brief period in weeks $12_{(2)}$-$16_{(2)}$ with M199.

Peripheral eosinophil counts

The data are shown in Figure 6.1.4.

The eosinophil counts in all three bullocks rose by two weeks after infection. In bullocks 204 and M199, the counts may have shown three peaks one at week $6_{(1)}$, which was the maximum, a second at week $12_{(1)}$ and the third, which was the smallest, at weeks $18_{(1)}$ in 204 and $21_{(1)}$ in M199. On the other hand, in 203, the counts peaked only once at week $6_{(1)}$ and thereafter fell relatively steadily to nearly normal levels from weeks $21_{(1)}$-$30_{(1)}$.

Following secondary infection the eosinophil response was biphasic in all three bullocks. In M199, the first peak occurred at week $2_{(2)}$, while in 203 and 204, it was not reached until week $6_{(2)}$. The second peak was at week $10_{(2)}$ in all three bullocks. Again as in bullocks 89 and 94, the maximum counts after secondary infection in three bullocks were far lower than the ones after primary infection.

Faecal egg counts

The results are shown in Figure 6.1.5.

The patency period for 203 and M199 was 12 weeks, while with 204 eggs were first detected at 11 weeks after infection.

Following patency there was a steady increase in egg counts to reach a peak at week $18_{(1)}$ in 203 and M199 and at week $15_{(1)}$ in 204. Thereafter the counts fell to low levels which, with some variations, were maintained throughout in M199 and until week $10_{(2)}$ in 203 and 204.

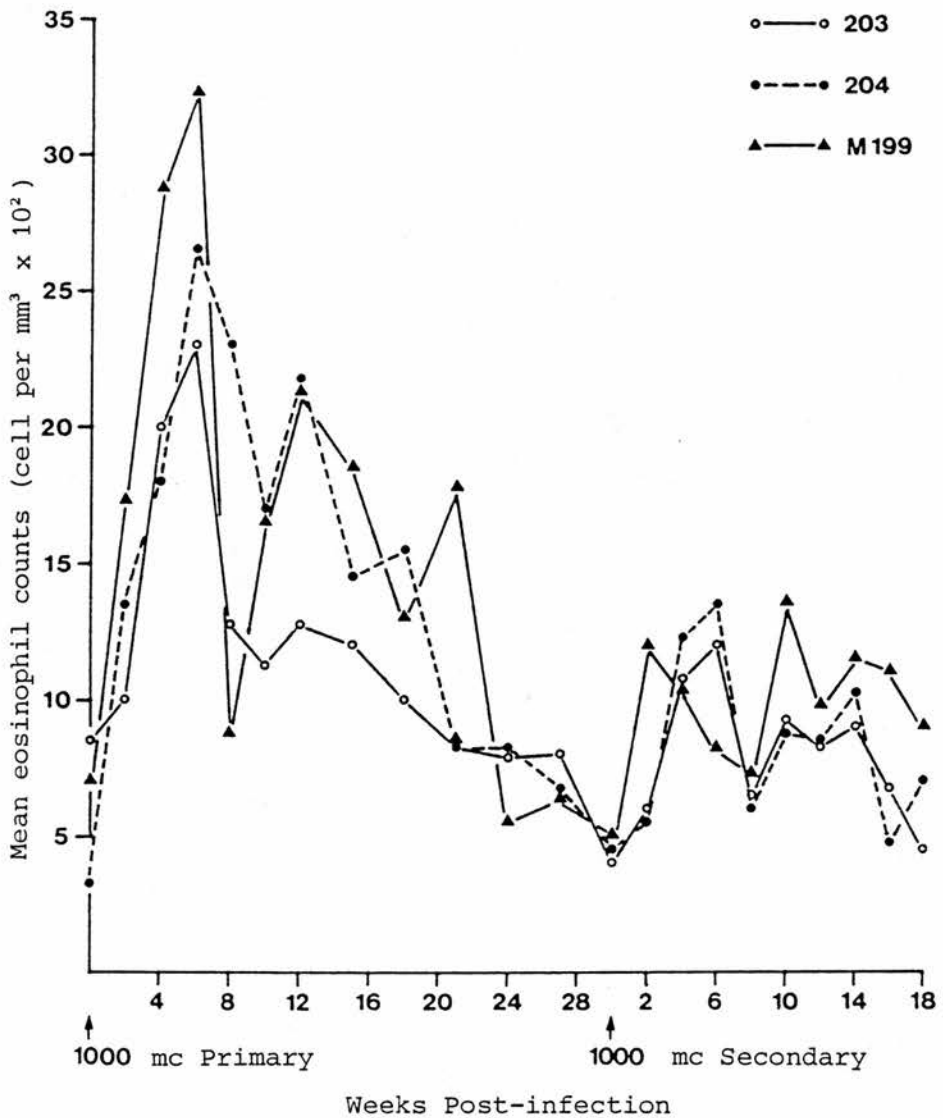


Figure 6.1.4 Peripheral eosinophil counts from 4-5-year-old bullocks infected with Fasciola hepatica.

Except for a slight increase at weeks $14_{(2)}$ and $16_{(2)}$ in M199, there was no evidence of any mature flukes developing following the second infection.

Discussion

As in Chapter 5, Section 5.1, necropsy could not be undertaken in these bullocks and the worm burdens were not determined. One of the animals (No. 203) was later used in the implantation study (see Chapter 8). However the lower serum glutamic dehydrogenase levels and very low eosinophil and faecal egg counts after secondary infection suggest that the bullocks were resisting the challenge infection. This observation is in agreement with Doyle (1971, 1972) and Boray (1969). Doyle (1971) observed that significantly reduced numbers of flukes developed after challenge even in 3-4 month old calves previously infected with 750 metacercariae and challenged 17 weeks later with 1,000 metacercariae.

Again as in bullocks 89 and 94, there were considerable variations in the serological responses between the bullocks.

The ELISA levels by the two antigens in 204 and M199 following secondary infection were similar to those observed with 89 and 94; in that the levels remained relatively high, as indeed they did after the tertiary infection. On the other hand, with the somatic antigen, the ELISA level in bullock 203 decreased after secondary infection, but with the metabolic antigen the level increased. This difference in ELISA results with the two antigens further indicates that the metabolic antigen was probably reacting with a different spectrum of antibodies.

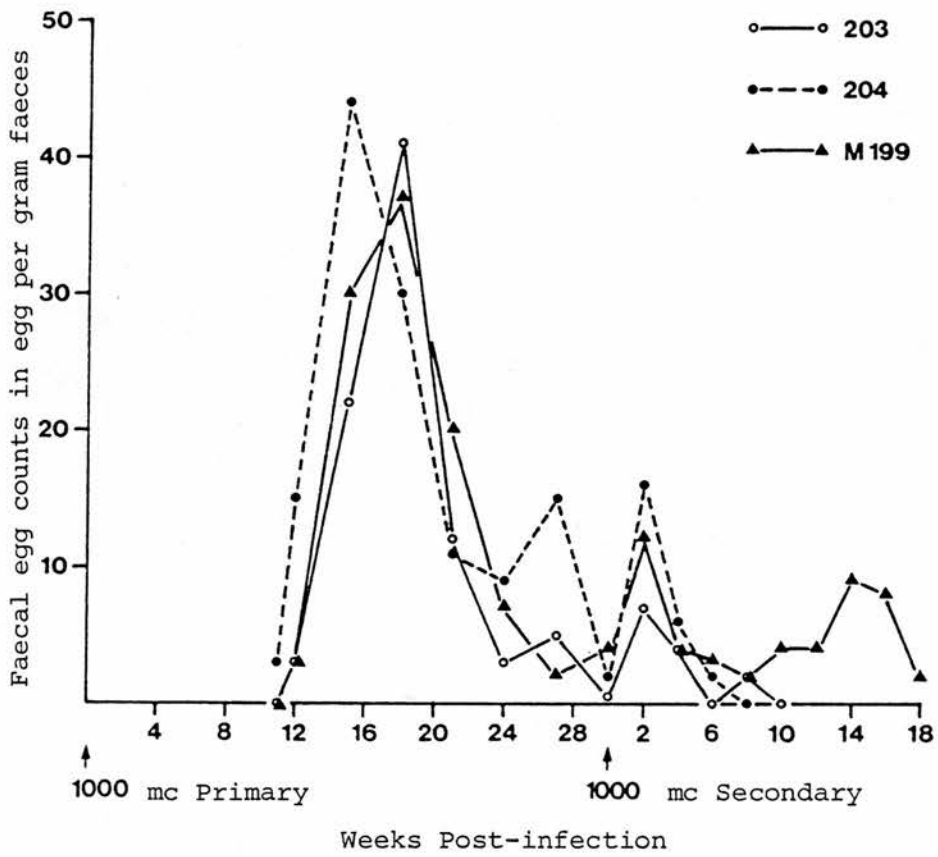


Figure 6.1.5 Faecal egg counts from 4-5-year-old bullocks infected with Fasciola hepatica.

Overall the pattern of the GD values for these three bullocks was similar to that for bullocks 89 and 94. The maximum levels occurred after primary infection and after secondary infection the values were lower. However, in 89 and 94, the values after the second infection were higher than with these older animals, a similar response to the latter occurring after the tertiary infection.

6.2 Passive Transfer of Resistance in the Rats by Immune Serum from 4-5 year-old Bullocks Collected after Primary Infection

Introduction

The ability of immune serum from 18-month-old bullocks infected with 1,000 metacercariae of F. hepatica and challenged twice with the same number of metacercariae to protect rats against this parasite had proved to be rather limited. In Section 5.2 it was shown that immune sera from bullock 89 collected at weeks $6\text{-}9_{(1)}$, 6 and $12_{(2)}$ or $6\text{-}9_{(3)}$ gave significant protection in rats against challenge. However such protection was not very strong, was not increased by repeated infection and was even less clear using sera from bullock 94.

In an attempt to obtain a strongly protective immune bovine serum (IBS), it was hoped that older bullocks might be more easily stimulated and produce a more effective serum. This section describes the work done to show if this was correct.

Experimental design

Sources of IBS were the three 4-5 year-old Ayrshire bullocks (Nos. 203, 204 and M199) which were each given 1,000 metacercariae of F. hepatica and bled at day 0 and at weeks 9 and 12 only after infection.

Fifty-four six-week-old male Wistar rats were randomly assigned into three main groups (A, B and C) of 18 rats. Group A was immunised with normal or immune serum as the case may be from bullock 203; group B, with normal or immune serum from bullock 204; and group C, with normal or immune serum from bullock M199.

The rats in each group were further assigned randomly into three groups of six rats. Groups A1, B1 and C1, which served as controls, were immunised with normal serum from the respective bullocks; groups A2, B2 and C2, with IBS collected at week $9_{(1)}$ from the respective donors and groups A3, B3 and C3, with IBS collected at week $12_{(1)}$ from the respective bullocks.

All the rats in each group were each infected orally with 20 metacercariae of F. hepatica and immediately after infection 10 ml of normal or IBS was administered intraperitoneally. Immunisation was repeated two days later.

Serum glutamic dehydrogenase activities were monitored fortnightly, using the activated technique, and the necropsy of rats and recovery of adult flukes was carried out eight weeks after infection.

Results

One rat in group A2 died four weeks before the end of the experiment. The animal died of internal haemorrhage which was probably associated with parenchymal migratory phase of the flukes development.

Fluke recovery

The data are shown in Table 6.2.1 and the statistical analysis is given in Appendix Table 6.2.1.1.

Except in group C2, the mean number of flukes developed from the challenge in all the treated groups were fewer than in their respective controls. However, with the marginal exception of A2, immunised with IBS (week $9_{(1)}$) from bullock 203 ($U = 24$, $P < .10$), the numbers recovered from the treated groups were not significantly reduced as compared with the respective controls.

Serum glutamic dehydrogenase assay

The group mean GD activities are shown in Figure 6.2.1 and the detailed results are shown in Appendix Table 6.2.1.

The serum GD concentrations at week 4 in all treated groups, with the exception in group C2 immunised with IBS (week $9_{(1)}$) from bullock M199, were lower than in the corresponding control group, but again these differences were not statistically significant.

Discussion

In contrast to the hypothesis on which the study was based, the IBS from these mature adult cattle collected after primary infection was less able to transfer protection than the sera from the younger cattle in the previous study. The immune serum collected at week $9_{(1)}$ from bullock M199 apparently gave no protection. The same observation was noted in IBS from bullock 94 collected at week $6_{(3)}$ (see Table 5.4.1). There is no explanation for this discrepancy other than the individual variations in the immune response of bullocks. It is however possible that these older animals were mounting a more rapid and effective cellular response, which depressed the humoral protective response by reducing the exposure to the appropriate immunogen.

Table 6.2.1

Passive transfer of resistance to 20 metacercariae of *F. hepatica* in rats by serum from infected bullocks. Two 10 ml volumes of serum given by intraperitoneal injection on the day of challenge and two days later.

Donor Bullock	Serum		Nos. of flukes recovered at P.M. Individual	Mean ± SD
203 (Group A)	Normal serum	(A1)	5, 6, 2, 5, 5, 5	4.7 ± 1.4
	Immune serum $9_{(1)}$	(A2)	3, 1, 5, 4, 3, -	3.2 ± 1.5
	Immune serum $12_{(1)}$	(A3)	4, 2, 2, 8, 3, 2	3.5 ± 2.3
204 (Group B)	Normal serum	(B1)	3, 6, 4, 3, 2, 2	3.3 ± 1.5
	Immune serum $9_{(1)}$	(B2)	1, 1, 0, 2, 7, 3	2.3 ± 2.5
	Immune serum $12_{(1)}$	(B3)	4, 0, 2, 2, 3, 1	2.0 ± 1.4
M199 (Group C)	Normal serum	(C1)	4, 3, 5, 2, 1, 4	3.2 ± 1.5
	Immune serum $9_{(1)}$	(C2)	5, 7, 5, 9, 5, 1	5.3 ± 2.7
	Immune serum $12_{(1)}$	(C3)	2, 4, 1, 0, 2, 2	1.8 ± 1.3

- Animal died

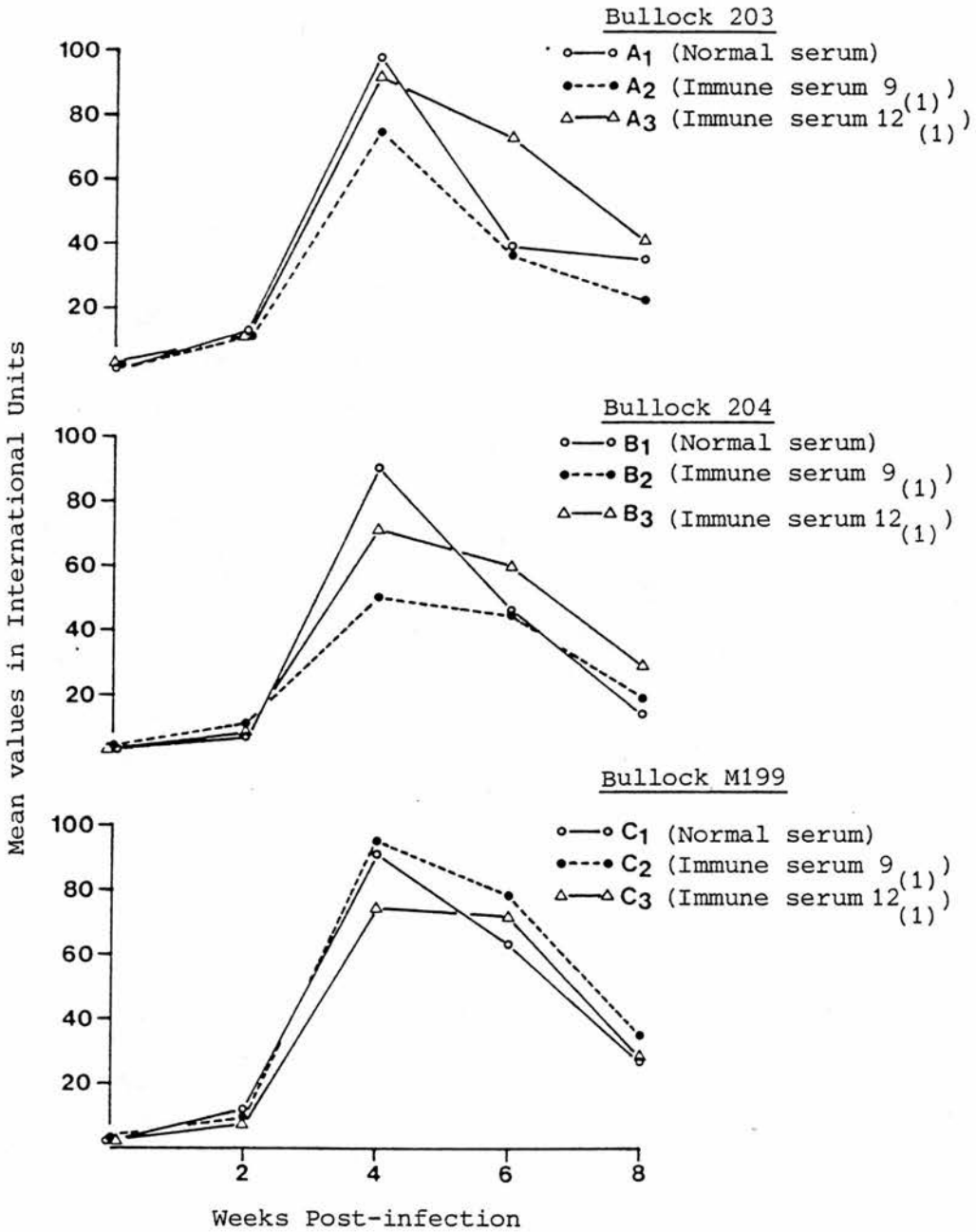


Figure 6.2.1 Serum glutamic dehydrogenase activities from rats.

6.3 Passive Transfer of Resistance in the Rats by Immune Serum from 4-5 year-old Bullocks Collected after Secondary Infection

Introduction

The protection afforded by the serum obtained from mature adult bullocks given only a primary infection was less effective than that given by IBS from younger adult cattle. This was indicated from the numbers of flukes which developed from challenge. However because of the time involved in carrying out the protection experiment this could not be known before commencing the second part of this study, using sera obtained after the secondary infection. It was therefore decided to complete the study using the sera obtained after the second infection, even though such sera had not given enhanced protection when obtained from the younger animals in the first study.

Inasmuch as the IBS collected at weeks $9_{(1)}$ or $12_{(1)}$ did not give significant protection and since it had been noted in the previous study (Section 5.2) that IBS collected at weeks $6_{(2)}$ or $9_{(3)}$ from the young adult cattle (No. 89) was significantly protective, it was decided to restrain this part of the study on passive transfer to IBS collected at weeks $6_{(2)}$ and $9_{(2)}$ only.

Experimental design

The donors of the IBS were bullocks 203, 204 and M199, which had already experienced a 30-week-old infection, and were reinfected with 1,000 metacercariae each.

Fifty-four six-week-old male Wistar rats were randomly assigned into three groups (A, B and C) of 18 rats each. Group A was injected with normal or immune serum from bullock 203; group B, with normal

or immune sera from bullock 204; and group C, with normal or immune serum from bullock M199.

The rats in each group were further assigned into three groups of six rats. Groups A1, B1 and C1, which served as controls, were immunised with normal serum from the appropriate bullocks. Groups A2, B2 and C2 were each immunised with IBS collected from the respective bullocks at week $6_{(2)}$ and groups A3, B3 and C3 were immunised with IBS from the same animals collected at week $9_{(2)}$.

All the rats in each group were infected orally with 20 metacercariae of F. hepatica each and immediately after infection they were immunised intraperitoneally with 10 ml normal or immune serum as appropriate. Immunisation was repeated two days later.

Serum glutamic dehyrogenase activity was monitored, using the activated technique, at weeks 0, 4 and 6 only and necropsy and recovery of the adult flukes was carried out eight weeks after infection.

Results

One of the rats in group C1 died within two weeks after infection. The cause of death was the same as in Section 6.2, which was probably associated with the parenchymal migratory phase of the fluke's development.

Fluke recovery

The data are shown in Table 6.3.1.

The numbers of flukes which developed from the challenge in all treated groups were fewer than in their respective control groups, with the exception of group A3. However, once again, with the

exception of group B2 ($P < .05$) the differences were not significant. This is shown in detail in Appendix Table 6.3.1.1.

Serum glutamic dehydrogenase assay

The mean group GD levels are shown in Figure 6.3.1 and the detailed results are shown in Appendix Table 6.3.1.

With the exception in group A3, whose GD values at week 4 were greater than the control group A1, the GD values in all treated groups were lower than in their respective control at week 4. Again these differences were not statistically significant.

At week 6, the GD levels dropped in all groups.

Discussion

Overall the IBS from these mature adult cattle collected after secondary infection was less protective than the IBS from the younger adult bullock (No. 89).

On the other hand, the consistency with which the groups receiving IBS harbouring more flukes than those receiving normal serum does suggest that some protection was being conferred. By the sign test, the probability of this occurring by chance is $< .01$.

This relative lack of protective effect by the IBS from these three bullocks may be attributed to the age of the animals, since it is possible that these older bullocks were more capable of mounting cellular response.

So far there have been no observations made on the influence of age of mature adult cattle on the ability of their immune sera to transfer resistance. However Boray (1969) observed the influence of age of cattle on the resistance of the animals and subsequently

Table 6.3.1

Passive transfer of resistance to 20 metacercariae of *F. hepatica* in rats by serum from infected bullocks. Two 10 ml volumes of serum given by intraperitoneal injection on the day of challenge and two days later.

Donor Bullock	Serum		Nos. of flukes recovered at P.M. Individual	Mean ± SD
203 (Group A)	Normal serum	(A1)	7, 4, 6, 1, 5, 5	4.7 ± 2.1
	Immune serum $6_{(2)}$	(A2)	1, 3, 7, 3, 5, 5	4.0 ± 2.1
	Immune serum $9_{(2)}$	(A3)	8, 5, 8, 2, 2, 6	5.2 ± 2.7
204 (Group B)	Normal serum	(B1)	7, 5, 8, 7, 6, 13	7.7 ± 2.8
	Immune serum $6_{(2)}$	(B2)	2, 5, 4, 5, 6, 7	4.8 ± 1.7
	Immune serum $9_{(2)}$	(B3)	4, 4, 6, 8, 7, 3	5.3 ± 2.0
M199 (Group C)	Normal serum	(C1)	1, 5, 3, 7, 6, -	4.4 ± 2.4
	Immune serum $6_{(2)}$	(C2)	4, 1, 6, 7, 2, 6	4.3 ± 2.4
	Immune serum $9_{(2)}$	(C3)	3, 7, 5, 4, 4, 2	4.2 ± 1.7

- Animal died

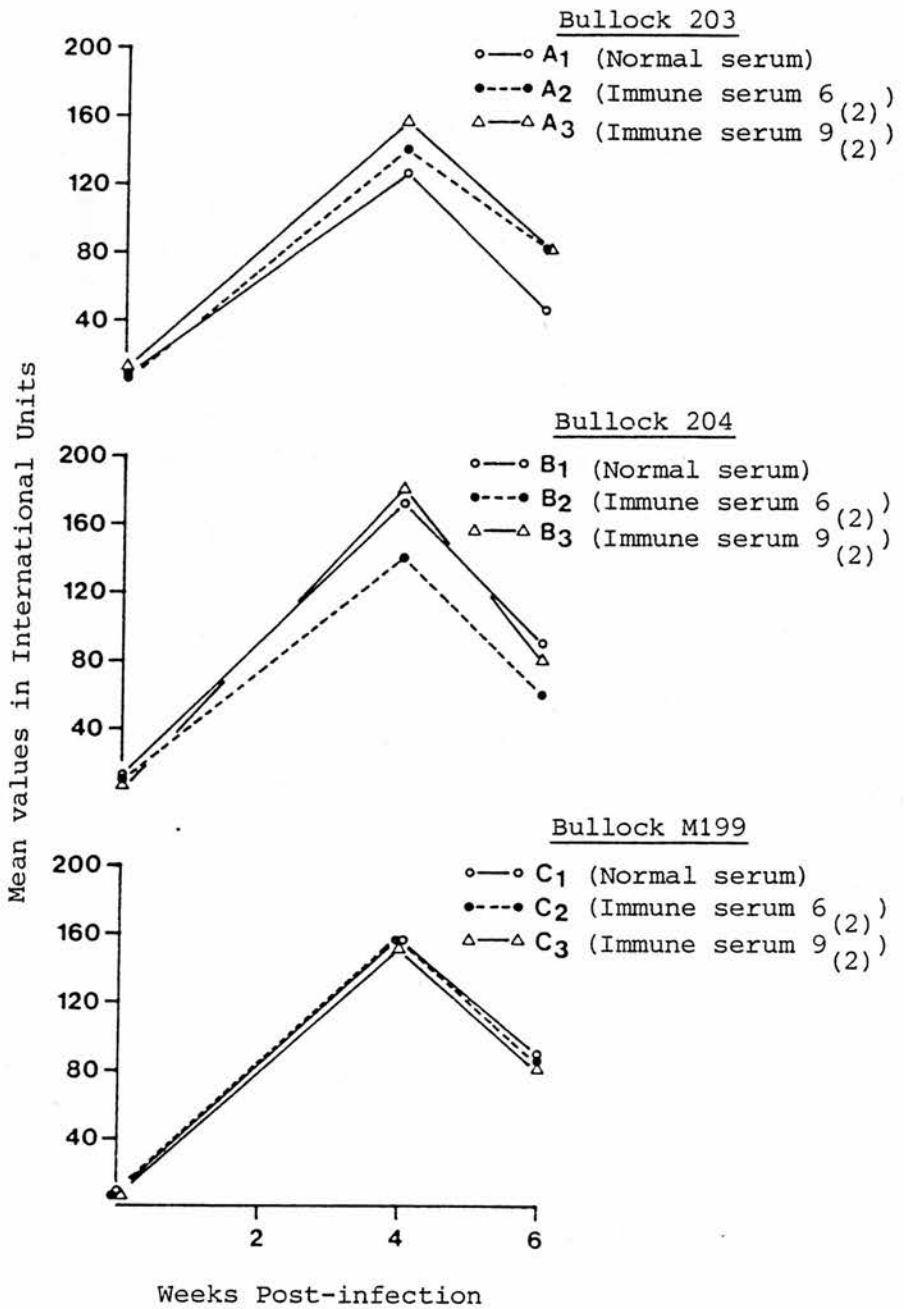


Figure 6.3.1 Serum glutamic dehydrogenase activities from rats.

in host's reaction against F. hepatica. He further observed a great variation of host reaction to Fasciola infection brought about by the innate resistance of cattle to F. hepatica.

CHAPTER SEVEN

STUDIES USING IMMATURE CATTLE

Introduction

It has been shown in Chapters 5 and 6 that immune sera from mature cattle did not confer absolute protection against Fasciola hepatica challenge to rats, and that there was considerable individual variation, but immune sera from young mature bullocks appeared to give better protection, although even this was not very strong. In view of the fact that Haroun (1979) had obtained rather better protection using animals of 8-10 months of age, it was thought that younger animals might depend more on the humoral component to acquire resistance, if so would develop a stronger resistance and hence the immune sera from these bullocks challenged with 1,000 metacercariae and passively transferred might confer stronger protection to rats.

The cattle were infected once and their immune sera collected at weeks $6_{(1)}$ and $9_{(1)}$ only were used to passively transfer to rats. This was so because the previous studies (Section 5.2) had shown that immune bovine serum (IBS) collected at weeks 6 and 9 after a single infection gave as much protection as any other.

7.1 Studies in Infected Cattle

Experimental design

Four six-month-old Ayrshire bullocks (Nos. 176, 177, 178 and 186) were each infected orally with 1,000 metacercariae of F. hepatica and nine weeks later they were killed, immune sera ($9_{(1)}$) collected in bulk from each animal and the flukes recovered from the bile ducts.

Larger blood samples (c. 500 ml) had also been collected from these animals prior to infection and at week $6_{(1)}$.

Small (10-15 ml) blood samples were collected from each bullock at two-weekly intervals to monitor peripheral eosinophil counts, serum glutamic dehydrogenase activities (using the activated technique) and anti-Fasciola antibody production, using either rabbit-derived somatic or metabolic antigen by ELISA.

For the assay of GD activities and eosinophil counts the samples were duplicated, while four replicate samples were assayed by ELISA.

Results

Serology

(i) Somatic antigen: The mean ELISA values, using the somatic antigen, are shown in Figure 7.1.1 and the individual data are shown in Appendix Table 7.1.1.

As in the previous studies normal serum gave a considerable non-specific reaction with this antigen but there was also a steady rise in titres in all four bullocks after infection, reaching peak values at week 4 in bullock 176 and at week 6 in 177, 178 and 186. The values from 186 were consistently lower than those from the other three animals.

Statistical analysis of ELISA values from bullocks is shown in Tables 7.1 and 7.2.

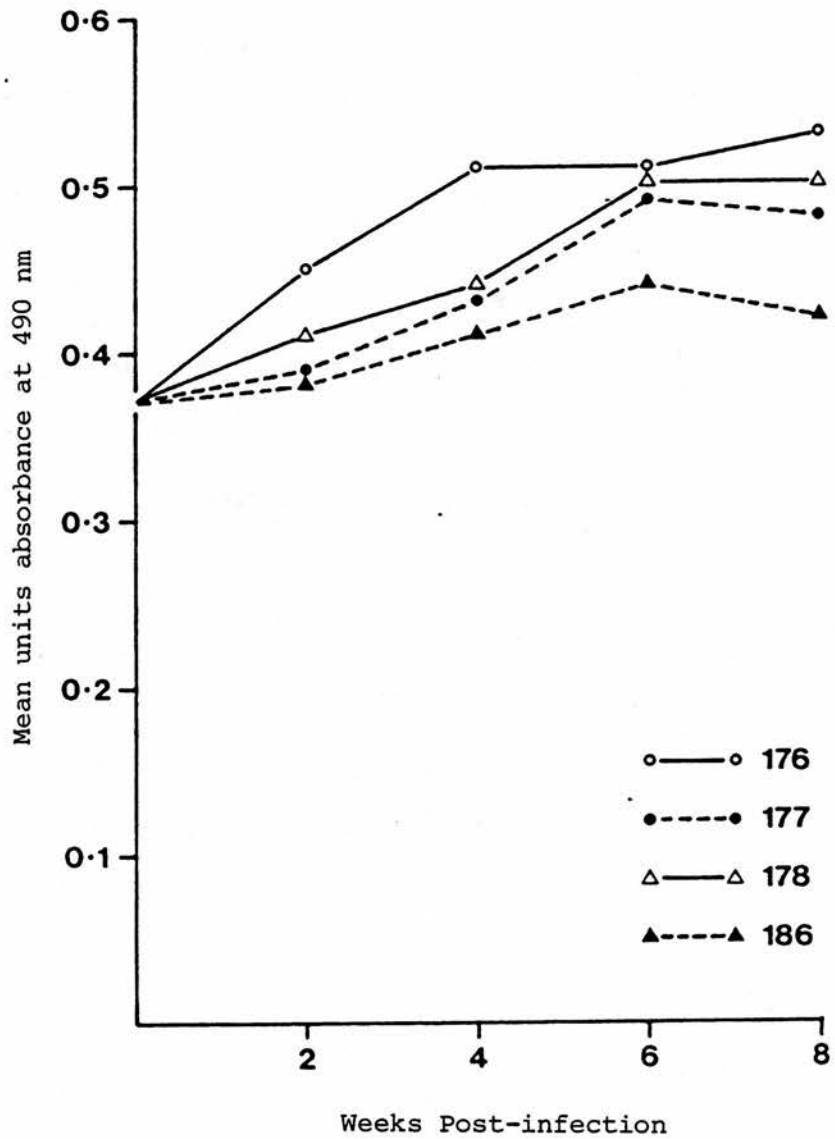


Figure 7.1.1 ELISA values from six-month-old bullocks infected with Fasciola hepatica, using somatic antigen.

Table 7.1 Comparisons of the ELISA values from the same bullocks

Bullock No.	Weeks compared	t-values	Probability
176	0 v $4_{(1)}$	29.7	< .001
	$6_{(1)}$	11.3	< .001
177	0 v $4_{(1)}$	6.5	< .01
	$6_{(1)}$	25.6	< .001
178	0 v $4_{(1)}$	26.0	< .001
186	0 v $4_{(1)}$	3.6	< .05
	$6_{(1)}$	12.3	< .01

Means and standard deviations are given in Appendix Table 7.1.1.

Table 7.2 Comparisons of the ELISA values among bullocks

Week	F-value	Probability
$2_{(1)}$	18.33	< .05
$4_{(1)}$	85.94	< .05
$6_{(1)}$	16.89	< .05

(ii) Metabolic antigen: The mean data are shown in Figure 7.1.2 and the details in Appendix Table 7.1.2.

The ELISA values rose slightly to reach maximum levels in all four animals at week 6. Although the increase was not very dramatic. The proportionate difference as compared with the results obtained with the normal serum was again greater than that using the somatic antigen. With this antigen, 186 gave stronger results than the other three animals.

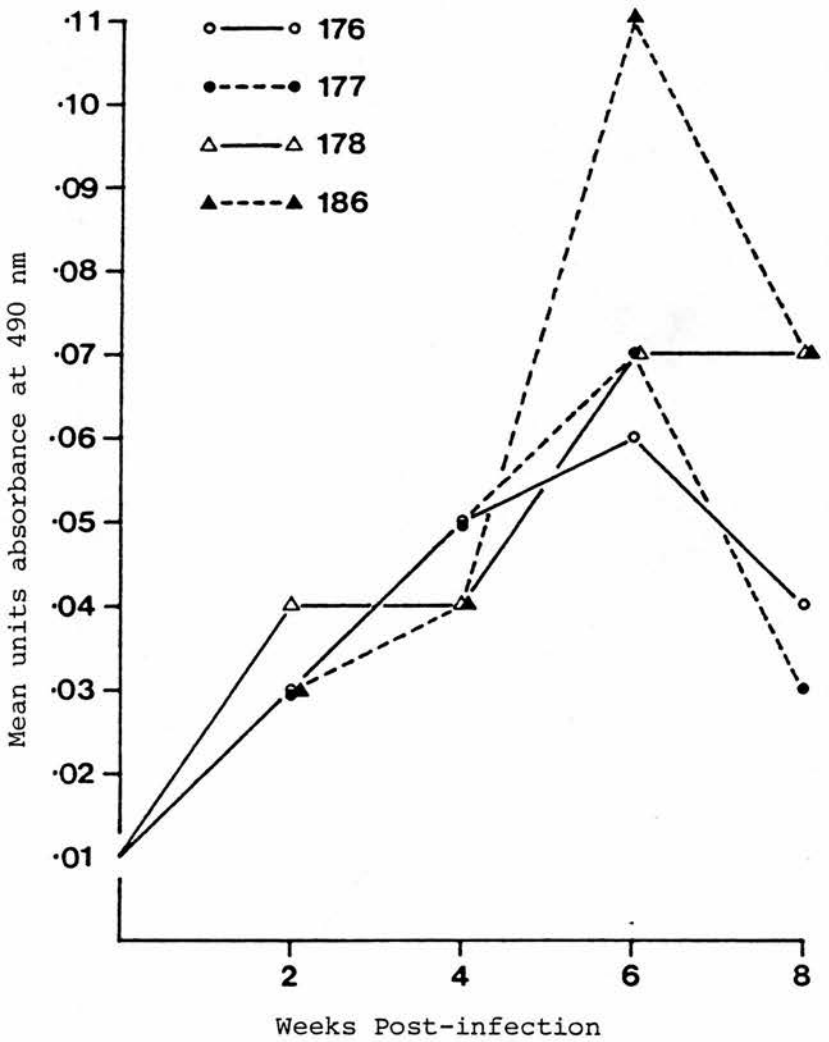


Figure 7.1.2 ELISA values from 6-month-old bullocks infected with Fasciola hepatica, using metabolic antigen.

Table 7.3 Statistical analysis of ELISA values from the same bullocks

Bullock No.	Weeks compared	t-value	Probability
176	0 v $4_{(1)}$	4.5	< .05
	$6_{(1)}$	8.7	< .01
177	0 v $4_{(1)}$	9.0	< .01
	$6_{(1)}$	14.7	< .001
178	0 v $4_{(1)}$	12.1	< .01
	$6_{(1)}$	4.2	< .05
186	0 v $4_{(1)}$	5.4	< .02
	$6_{(1)}$	20.4	< .001

Means and standard deviations are given in Appendix Table 7.1.2.

Table 7.4 Comparisons of ELISA values among bullocks

Week	F-value	Probability
$2_{(1)}$	10.97	< .05
$4_{(1)}$	0.64	> .05
$6_{(1)}$	5.37	< .05

Serum glutamic dehydrogenase assay

The data are shown in Figure 7.1.3.

There was increasing levels of GD in the four bullocks following infection to reach maximum values at week $4_{(1)}$ in bullock 176 and at week $6_{(1)}$ in 177, 178 and 186. Thereafter the values dropped.

Peripheral eosinophil counts

These are shown in Figure 7.1.4.

The eosinophil counts were significantly increased in all four bullocks by two weeks after infection to reach maximum values at

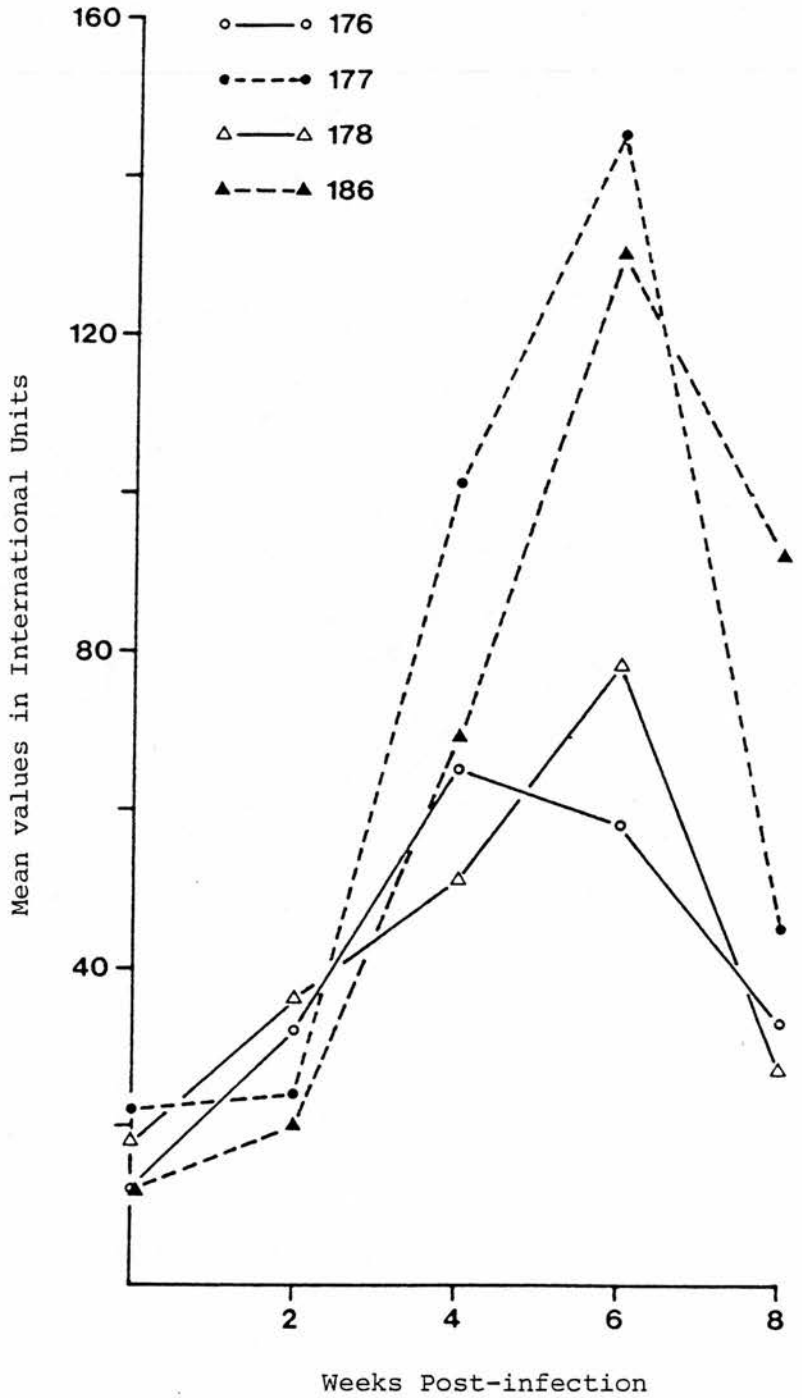


Figure 7.1.3 Serum glutamic dehydrogenase activities from six-month-old bullocks infected with Fasciola hepatica.

week 6. The peak in bullock 176 was higher than in the other three bullocks. Thereafter the counts fell.

Fluke recovery

The numbers of flukes recovered from each bullock are shown in Table 7.5.

Table 7.5 Flukes recovered from bullocks infected with 1,000 metacercariae of F. hepatica.

Bullock No.	No. of flukes	Percentage
176	108	10.8
177	143	14.3
178	33	3.3
186	111	11.1

Discussion

As in the previous study, using mature bullocks, the ELISA values were increased by two weeks after infection, although there were individual differences in the peak values for the two antigens. Using somatic antigen, the ELISA values in 176 peaked at week $4_{(1)}$ and in the other bullocks at week $6_{(1)}$. On the other hand, by metabolic antigen, ELISA values in all four bullocks peaked at week $6_{(1)}$.

Likewise, as in the earlier bullocks, the two antigens gave different results in these bullocks. Thus bullock 186 had the highest ELISA value at week $6_{(1)}$ using metabolic antigen whilst with the somatic antigen, the ELISA value was highest in bullock 176. These differences further indicate the great variation in individual responses to F. hepatica infection and tend to confirm that the two antigens are detecting a different spectrum of antibodies.

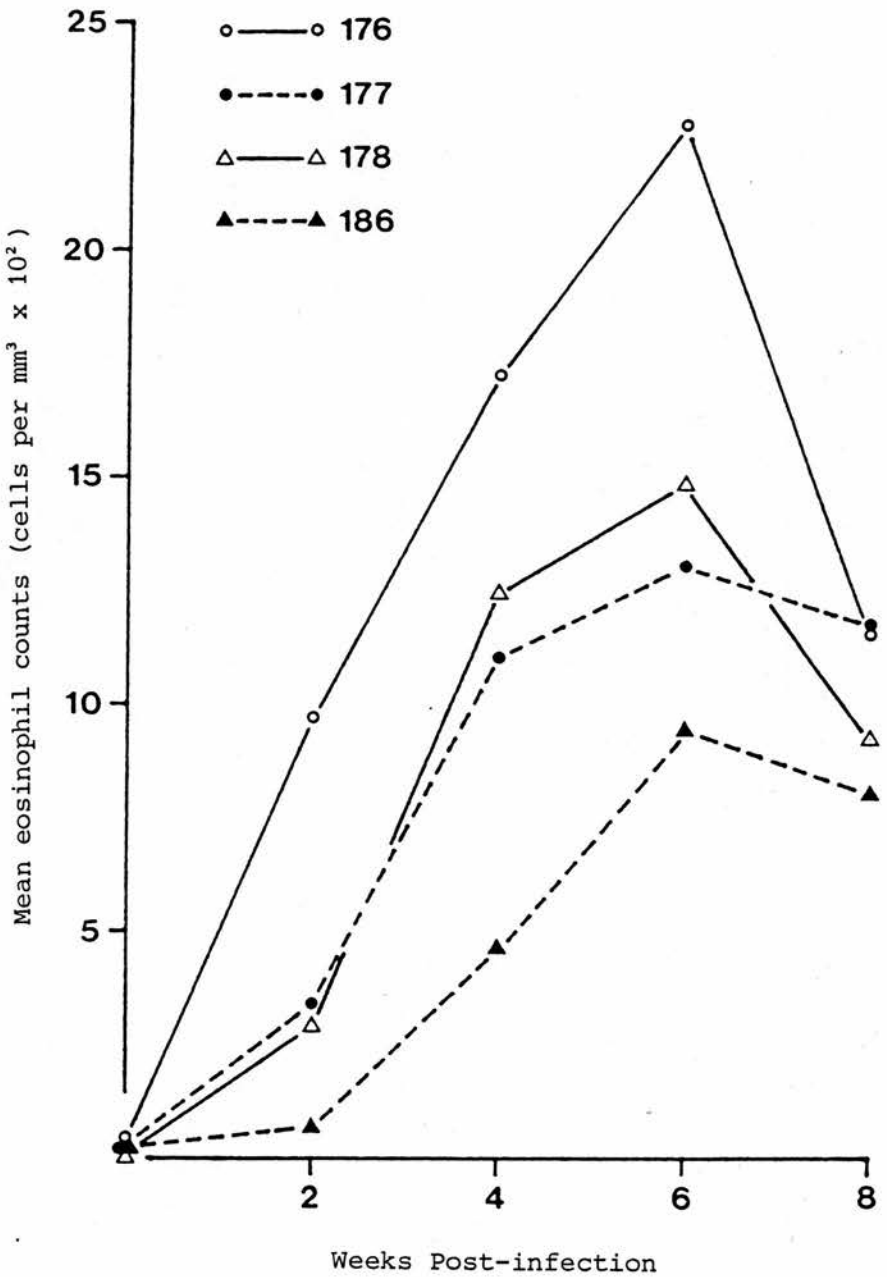


Figure 7.1.4 Peripheral eosinophil counts from 6-month-old bullocks infected with Fasciola hepatica.

The GD values, as in young and adult mature bullocks, corresponded with the migration of juvenile flukes in the liver. The values reached maximum level at week $6_{(1)}$, except in 176 whose GD values peaked early at week $4_{(1)}$.

The peak in eosinophil counts at week $6_{(1)}$ in all four bullocks followed that of the GD values. However, in 176, the eosinophil count at week $6_{(1)}$ was higher than in the other three bullocks, whereas its GD value was the lowest at week $6_{(1)}$. The early peaking in the GD values from this animal at week $4_{(1)}$, may indicate earlier migration of some of the flukes in the liver of this animal. The liver damage and immune complexes formed as a result of this earlier migration may have been responsible for the further eosinophilia stimulation in this bullock. Such an explanation may also account for the higher eosinophil count and numbers of flukes recovered at necropsy when compared with 177 or 186. A similar phenomenon may also be responsible for the relatively high eosinophil count in 176 despite the small numbers of flukes recovered at necropsy.

There was no correlation between the serological response of the animals and the numbers of juvenile flukes recovered from the liver and, although the GD values in those bullocks having less flukes were also having less GD values, statistical analysis showed no correlation ($r_{(1,2)} = 0.60$, $P > .05$) between the GD levels and the number of flukes recovered. This lack of correlation could be due to the limited numbers of experimental bullocks.

7.2 Passive Transfer of Resistance to Rats by Immune Serum from Six-month-old Bullocks Collected at Weeks 6 and 9 after Single Infection

Experimental design

Seventy-two six-week-old male Wistar rats were randomly assigned into four main groups (A, B, C and D) of 18 rats each. Each main group was further assigned into three groups of six rats. Groups A1, B1, C1 and D1, which served as controls, were immunised with normal serum from bullocks 176, 177, 178 and 186 respectively. Groups A2, B2, C2 and D2 were immunised with immune bovine serum (IBS) collected at week $6_{(1)}$ from the respective bullocks and groups A3, B3, C3 and D3 were immunised with IBS collected at week $9_{(1)}$ from the respective donor bullock.

All the rats in each group were infected orally with 20 metacercariae of F. hepatica and immediately after infection each rat was immunised intraperitoneally with 10 ml normal or immune serum as relevant. Immunisation was repeated two days later.

Serum glutamic dehydrogenase activities were monitored at weeks 0, 4 and 6 only, using the activated technique, and necropsy of rats and recovery of adult flukes was carried out eight weeks after infection.

Results

One rat in group A2, immunised with IBS from bullock 176 collected at week $6_{(1)}$ died one week after infection of internal haemorrhage which was probably associated with the parenchymal migratory phase of the fluke's development.

Fluke recovery

The results are shown in Table 7.2.1 and the statistical analysis using the Mann-Whitney U-test, is in Appendix Table 7.2.1.1.

The numbers of flukes recovered in all treated groups were fewer than in the appropriate control except for group B2. However only in groups A3 ($P < .025$) and D3 ($P < .05$) were the differences significant as compared to their appropriate controls.

Serum glutamic dehydrogenase

The data are shown in Figure 7.2.1 and in Appendix Table 7.2.1.

The GD values reached maximum levels at week 4 and thereafter the levels fell. However, in groups A2, A3 and B3, the peak GD at week 4 was higher than in the respective control, while in the other treated groups the maximum value at week 4 was lower than in the appropriate control. Again these differences were not statistically significant.

Discussion

The IBS from these relatively immature bullocks collected at weeks $6_{(1)}$ and $9_{(1)}$ did not give any better protection in rats than that from the young mature bullock 89, as indicated by the numbers of flukes developed after challenge. The resistance afforded by the IBS from these immature bullocks was indeed similar to that given by IBS from the adult mature cattle.

Moreover it appears that regardless of whether the animals were infected singly or repeatedly its immune sera collected at weeks 6 or 9 (prepatency) gave better protection. However, such capacity of IBS to transfer resistance may vary among bullocks.

Table 7.2.1

Passive transfer of resistance to 20 metacercariae of *F. hepatica* in rats by serum from infected bullocks. Two 10 ml volumes of serum given by intraperitoneal injection on the day of challenge and two days later.

Donor Bullock	Serum		Nos. of flukes recovered at P.M. Individual	Mean ± SD
176 (Group A)	Normal serum	(A1)	5, 8, 5, 4, 11, 12	7.5 ± 3.4
	Immune serum $6_{(1)}$	(A2)	3, 8, 8, 6, 4, -	5.8 ± 2.3
	Immune serum $9_{(1)}$	(A3)	3, 3, 5, 5, 2, 4	3.7 ± 1.2
177 (Group B)	Normal serum	(B1)	4, 7, 6, 2, 4, 4	4.5 ± 1.8
	Immune serum $6_{(1)}$	(B2)	1, 3, 3, 8, 7, 7	4.8 ± 2.9
	Immune serum $9_{(1)}$	(B3)	7, 2, 7, 1, 2, 4	3.8 ± 2.6
178 (Group C)	Normal serum	(C1)	1, 6, 7, 8, 5, 6	5.5 ± 2.4
	Immune serum $6_{(1)}$	(C2)	1, 11, 3, 7, 3, 5	5.0 ± 3.6
	Immune serum $9_{(1)}$	(C3)	4, 2, 5, 7, 5, 4	4.5 ± 1.6
186 (Group D)	Normal serum	(D1)	5, 8, 7, 5, 7, 8	6.7 ± 1.4
	Immune serum $6_{(1)}$	(D2)	8, 8, 5, 4, 3, 6	5.7 ± 2.1
	Immune serum $9_{(1)}$	(D3)	7, 4, 6, 4, 2, 4	4.5 ± 1.8

- Animal died

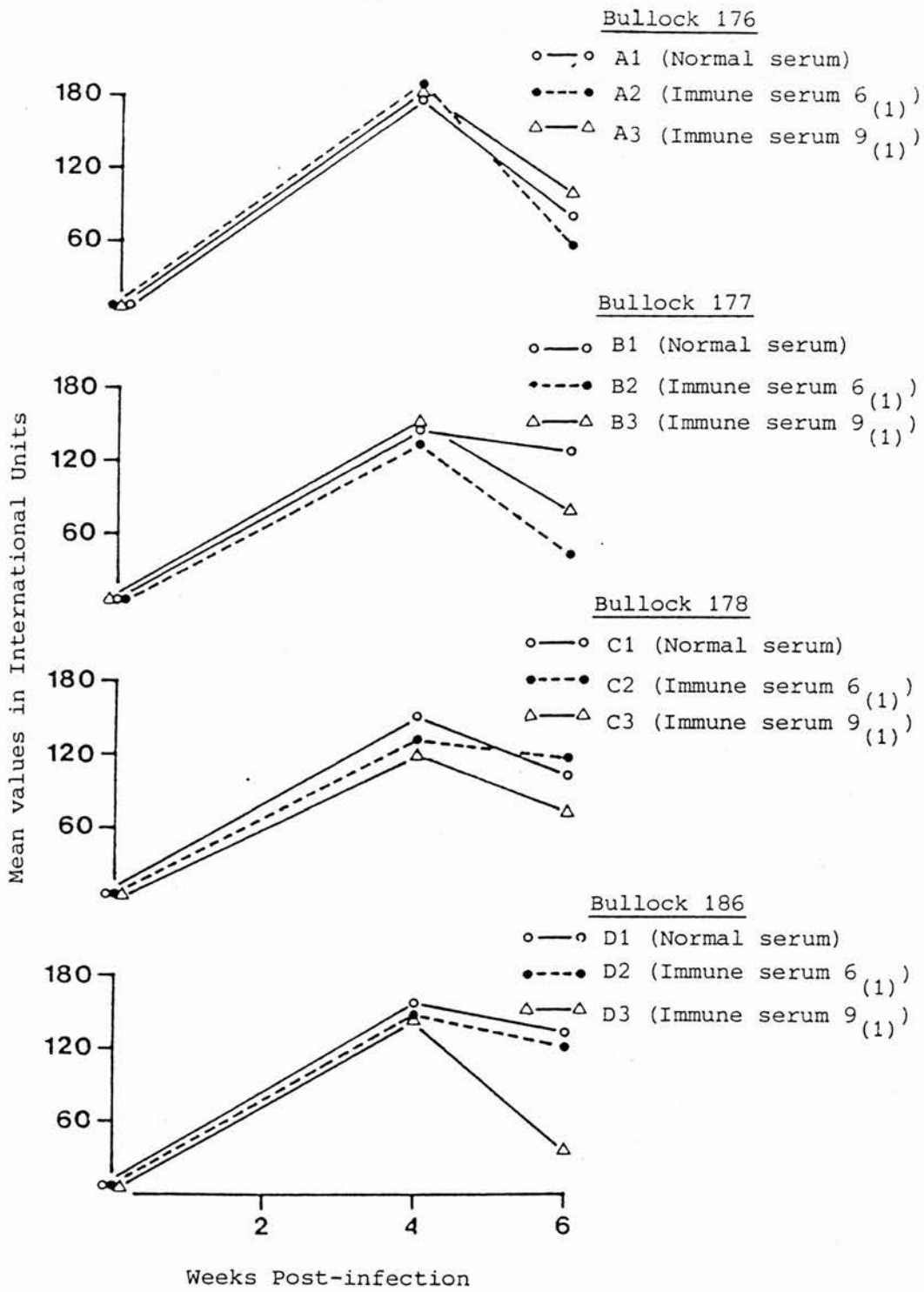


Figure 7.2.1 Serum glutamic dehydrogenase activities from rats immunised with immune sera.

Hayes, Bailer and Mitrovic (1974b) observed that immune serum from rat donors infected for 7-8 weeks (subacute) was effective, while serum from rats infected for 25 weeks (chronic) was not protective to challenge infection in a homologous recipient. On the other hand, Haroun, Hammond and Sewell (1981) observed significantly fewer flukes in rats immunised with immune sera collected at 8-10 weeks from bullocks given single and repeated infections 22 weeks apart.

CHAPTER EIGHT

STUDIES ON IMPLANTED RATS AND BULLOCKS

Introduction

A previous study by Haroun (1979) showed that immune serum from rats implanted intraperitoneally with adult flukes in diffusion chambers was able to confer protection against challenge infection when transferred to naive rats.

In the work described in Chapter 5, 6 and 7, it was found that, although previously infected bullocks developed strong resistance against challenge, their immune sera could only confer a relatively weak resistance against challenge to rats.

Furthermore the age of the infected donor made little difference to the protective ability of its serum, the activity of the serum waned from about 12 weeks after initial infection and did not increase following challenge. It appeared possible that this failure to induce increased activity by challenge occurred because the flukes derived from the challenge infection did not survive long enough in the hosts. Therefore it was postulated that intraperitoneal implantation with adult Fasciola in diffusion chambers, whereby there is a continuous release of metabolic antigen from the adult flukes, might induce a stronger immune response in previously infected bullocks.

As a preliminary part of this study, the effect of implanting flukes into previously infected rats on their serological response as indicated by ELISA, was assessed.

8.1 Serology Response in Implanted Rats

Experimental design

Thirty-four six-week-old male Wistar rats were first assigned

randomly into two groups (A and B). Group A, which consisted of five rats, served as a non-infected control and the 29 rats in group B were infected orally with 20 metacercariae of Fasciola hepatica each.

Prior to infection and at two-weekly intervals after infection, all the rats, including the non-infected control, were bled to monitor their eosinophil counts, serum glutamic dehydrogenase activity, using the activated technique, and serology by ELISA, using a rabbit-derived metabolic antigen.

At weeks 6 and 7 after infection and at two-weekly intervals thereafter the faeces of the infected rats were examined for fluke eggs.

Eighteen weeks after infection all rats in group B were treated with hexachlorophene at the rate of 50 mg/kg body weight on two successive weeks (see Appendix Table 3.1). However, prior to this treatment regimen, two of the rats, which had high faecal egg counts, were treated to determine the margin of safety of the anthelmintic preparation and one week later they were killed (see Section 3.11.1). After the second treatment at week 20, when eggs could no longer be demonstrated in the faeces, another two rats were randomly selected and necropsied to see if the treatment was effective. All the surviving rats, except those in group A, were randomly grouped into three groups (C, D and E). The rats in group A were infected orally with 20 metacercariae of F. hepatica. Group C, which consisted of nine rats, was left uninfected. The eight rats in group D were implanted intraperitoneally with one or two (depending on the size of the flukes) rat-derived flukes in diffusion chambers and the eight rats in group E were reinfected with 20 metacercariae of F. hepatica.

Monitoring of the eosinophil counts, serum glutamic dehydrogenase activity, serology and faecal egg counts continued at two-weekly intervals. Twenty weeks later all the rats were killed and the flukes recovered.

Results

None of the four rats killed as anthelmintic controls contained any flukes.

Two rats in group D died two days after the surgery and one rat in group E died 15 weeks after reinfection, but the cause of death could not be assessed because of severe injury in the abdominal and tail regions caused by cannibalism.

Serology

The mean corrected ELISA values are shown in Figure 8.1.1 and the detailed results are shown in Appendix Tables 8.1.1A and 8.1.1B.

There was an increased mean ELISA value within two weeks in the rats receiving the first infection and maximum value was reached at week $6_{(1)}$. Thereafter the level was variable but tended to fall until week $14_{(1)}$. In contrast, in non-infected rats (A), the ELISA values were consistently low.

After treatment of the rats in group B with anthelmintic, at weeks 18 and 19, the mean ELISA value was significantly increased at week 20. Thereafter the values varied with the different treatments. In the implanted group (D), there was a brief sustained rise, which lasted until four weeks after implantation, after which the ELISA values fell away at a similar rate to those in group C. In group C, the level declined after the initial rise following treatment.

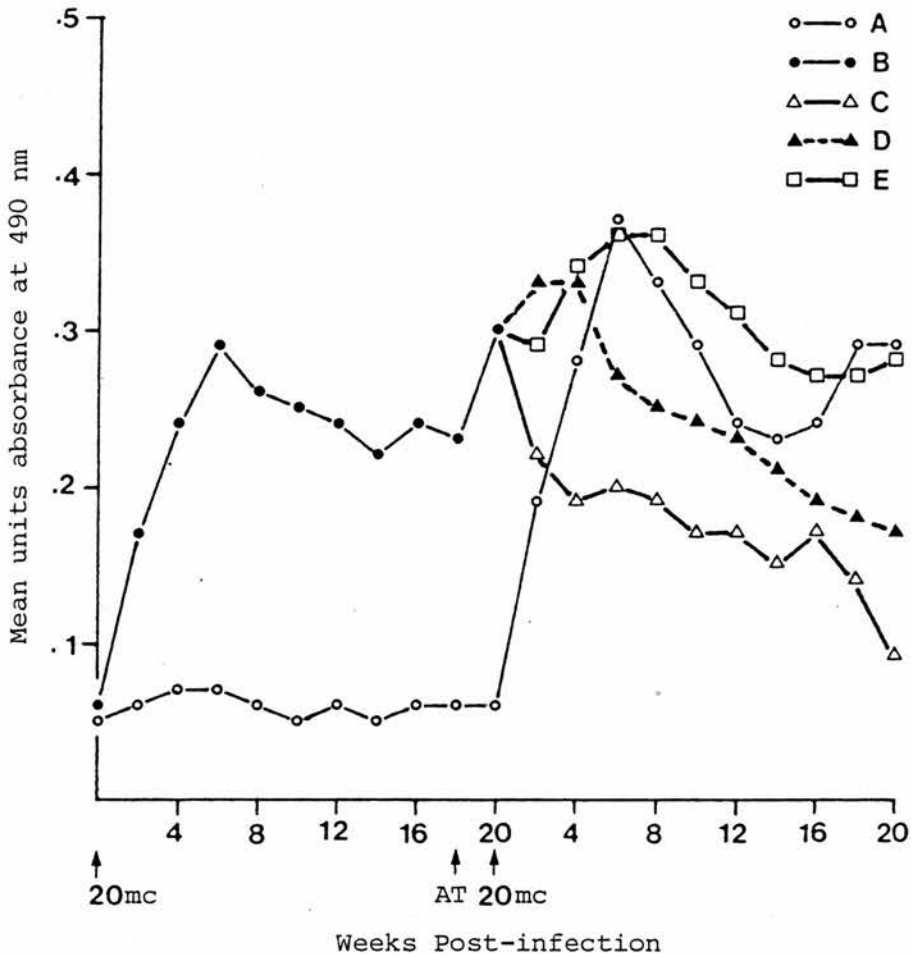


Figure 8.1.1 ELISA values from infected control rats (A), infected rats and treated with anthelmintic (B) and then left uninfected (C), implanted with adult flukes in diffusion chamber (D) or re-infected with 20 metacercariae of F. hepatica (E).

On the other hand, in the reinfected group (E), the level fell briefly at week $2_{(2)}$ and thereafter rose to reach a brief plateau at weeks $6_{(2)}$ and $8_{(2)}$ before falling away at much the same rate as in the rats in the infected control group (A).

Statistical analysis of ELISA values from the same groups and within groups, using the independent t-test and single ANOVA is shown in Tables 8.1.1 and 8.1.2 respectively.

Table 8.1.1 Comparisons of ELISA values from the same group

Group No.	Weeks compared	t-value	Probability
A	0 v $2_{(1)}$	4.9	< .01
B	0 v $2_{(1)}$	54.1	< .001
	$18_{(1)}$ v $20_{(1)}$	3.7	< .01

Table 8.1.2 Comparison of ELISA values between groups

Groups compared	Week	t-value	Probability
E v D	$2_{(2)}$	1.5	> .10
	$6_{(2)}$	3.2	< .01
(E+D) v C	$2_{(2)}$	1.0	> .10
	$4_{(2)}$	1.8	< .10

Serum glutamic dehydrogenase assay

The mean group GD activities are shown in Figure 8.1.2 and in Appendix Tables 8.1.2A and 8.1.2B.

The only dramatic change in GD in infected group (B) occurred at weeks $4_{(1)}$, when the level rose to maximum value, and at week $6_{(1)}$ when the GD fell.

Following treatment, in groups C and D, the GD levels were consistently low, while in group E, the level rose sharply to a peak

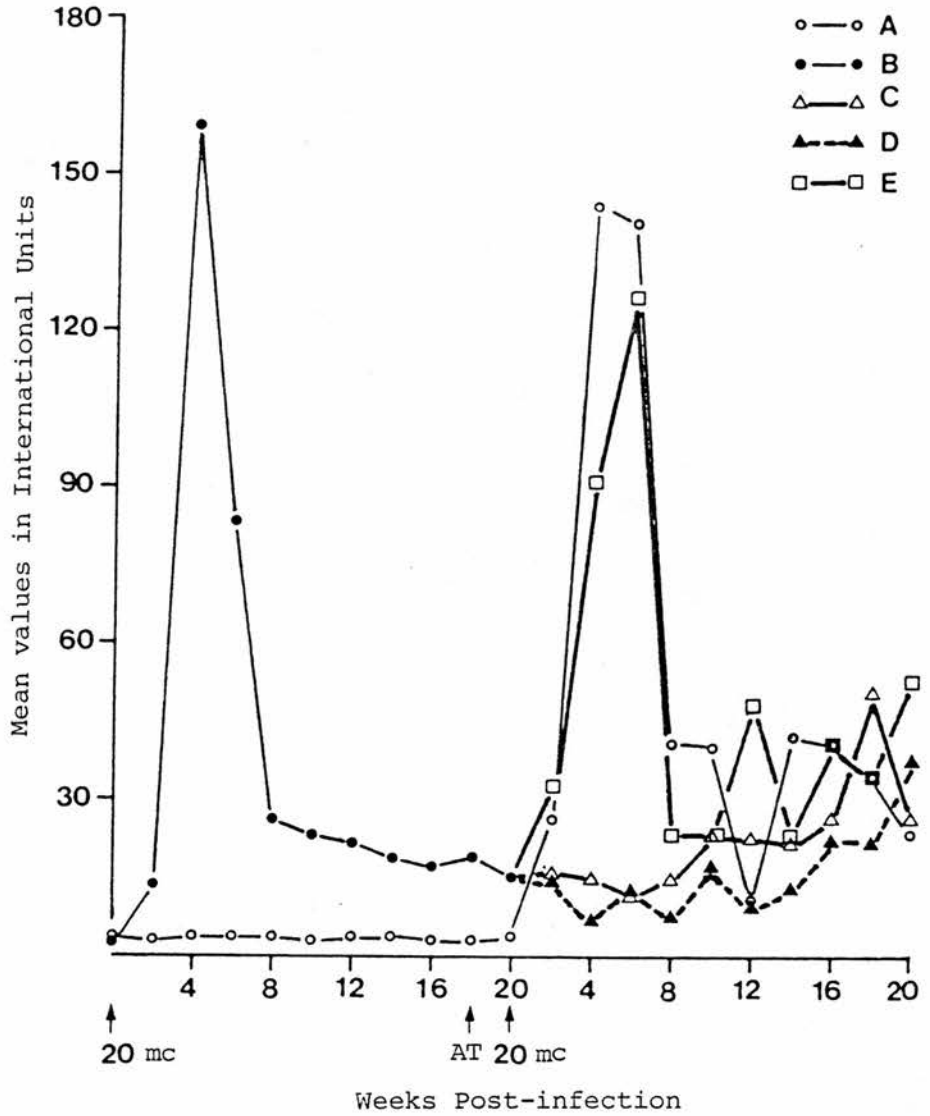


Figure 8.1.2 Serum glutamic dehydrogenase from infected control rats (A), infected rats and treated with anthelmintic (B) and then left uninfected (C), implanted with adult flukes in diffusion chamber (D) or reinfected with 20 metacercariae of F. hepatica (E).

at week $6_{(2)}$ although this was lower than the levels in the infected control group (A).

Peripheral eosinophil counts

The data are shown in Figure 8.1.3 and in Appendix Tables 8.1.3A and 8.1.3B.

As with the GD, the eosinophil counts rose to peak values at week $6_{(1)}$ after infection in all infected rats. Following treatment the eosinophil counts at week 6 in the groups that were left uninfected (C) and implanted (D) were lower than the reinfected group (E) and challenge control (A).

Statistical analysis of eosinophil counts within groups is shown in Table 8.1.3.

Table 8.1.3 Comparison of eosinophil counts between groups

Groups compared	Week	t-value	Probability
D v C	$2_{(2)}$	2.3	< .05
	$4_{(2)}$	6.4	< .001

Faecal egg counts

The results are shown in Figure 8.1.4 and in Appendix Tables 8.1.4A and 8.1.4B.

Patency occurred at week 7 after infection, when less than 10 eggs per gram faeces (e.p.g.) were recovered. The maximum egg counts were noted at week $16_{(1)}$. Again there were great variations in the faecal egg counts both from week to week and between individual infected rats.

After the second treatment, faecal eggs were demonstrable in the faeces in only one of the rats (Appendix Table 3.1). This rat

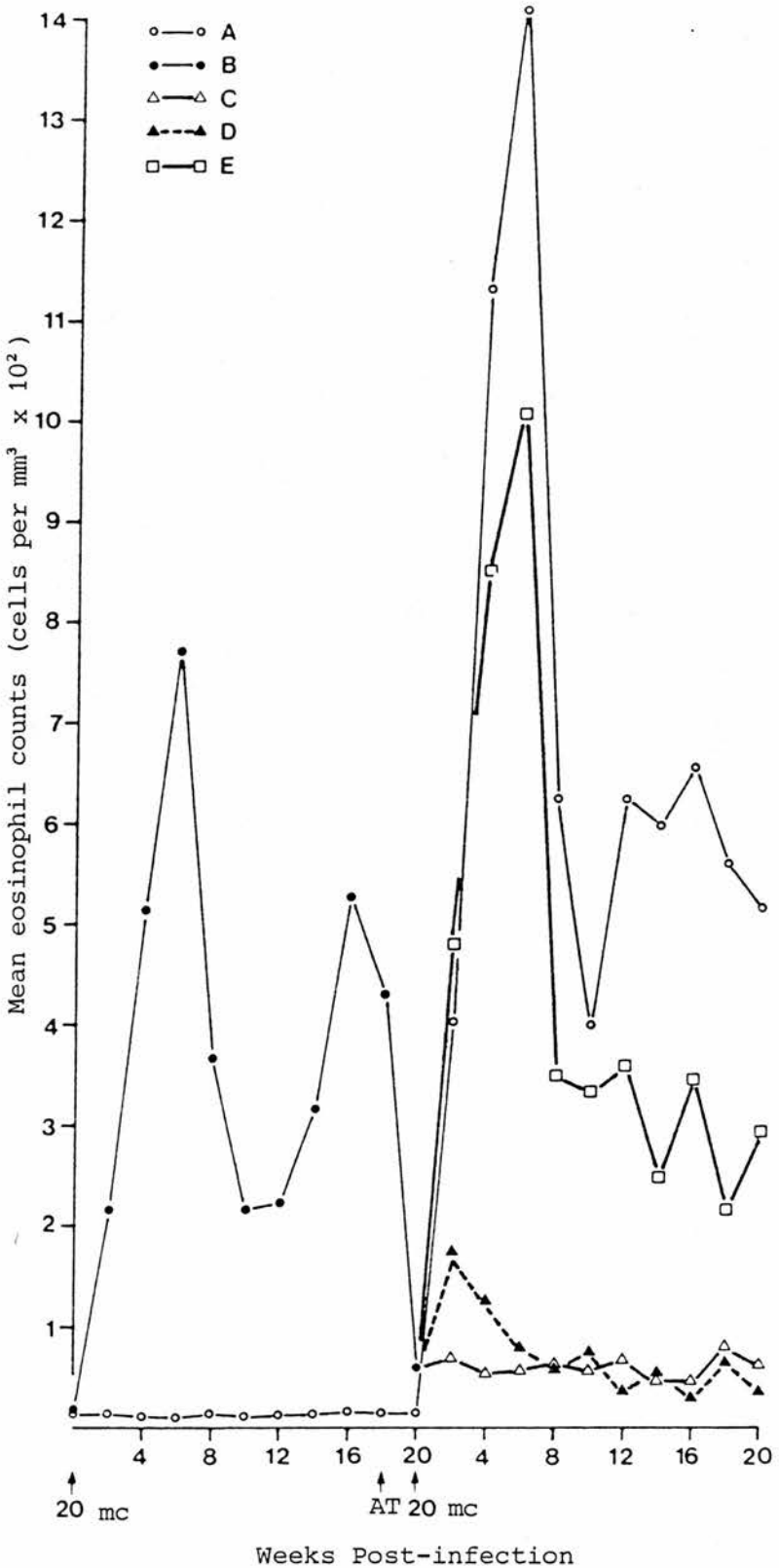


Figure 8.1.3 Peripheral eosinophil counts from infected control rats (A), infected rats and treated with anthelmintic (B) and then left uninfected (C), implanted with adult flukes in diffusion chamber (D) or reinfected with 20 metacercariae of F. hepatica (E).

was given a third dose of hexachlorophene and a week later eggs were no longer demonstrable in the faeces.

In the reinfected group (E), the eggs were again recovered from one of the rats quite early at week $6_{(2)}$ and by week $7_{(2)}$ two-thirds of the rats had eggs in their faeces of more than 10 e.p.g. In con-contrast to the pre-treatment egg counts at week $7_{(1)}$ only one-third of the rats showed eggs in their faeces and the mean count was less than 5 e.p.g. However, this was probably merely a reflection of the variability in the two batches of metacercariae used, since most of the rats in group A also had high egg counts by seven weeks after infection and the peak counts were higher than in any of the groups after the first infection.

The faecal egg counts in reinfected group (E) reached its maximum level at week $18_{(2)}$, while in challenge infection control (A), the peak was at week 12 after infection. This difference may also be fortuitous but could be a reflection of a degree of resistance in the former animals.

Fluke recovery

The data are shown in Table 8.1.4.

There were no flukes recovered from groups C and D. The numbers of flukes in the rats in group E were fewer than those in the challenge control (A) and this difference was highly significant ($P < .005$) - again probably the result of some resistance in the former group.

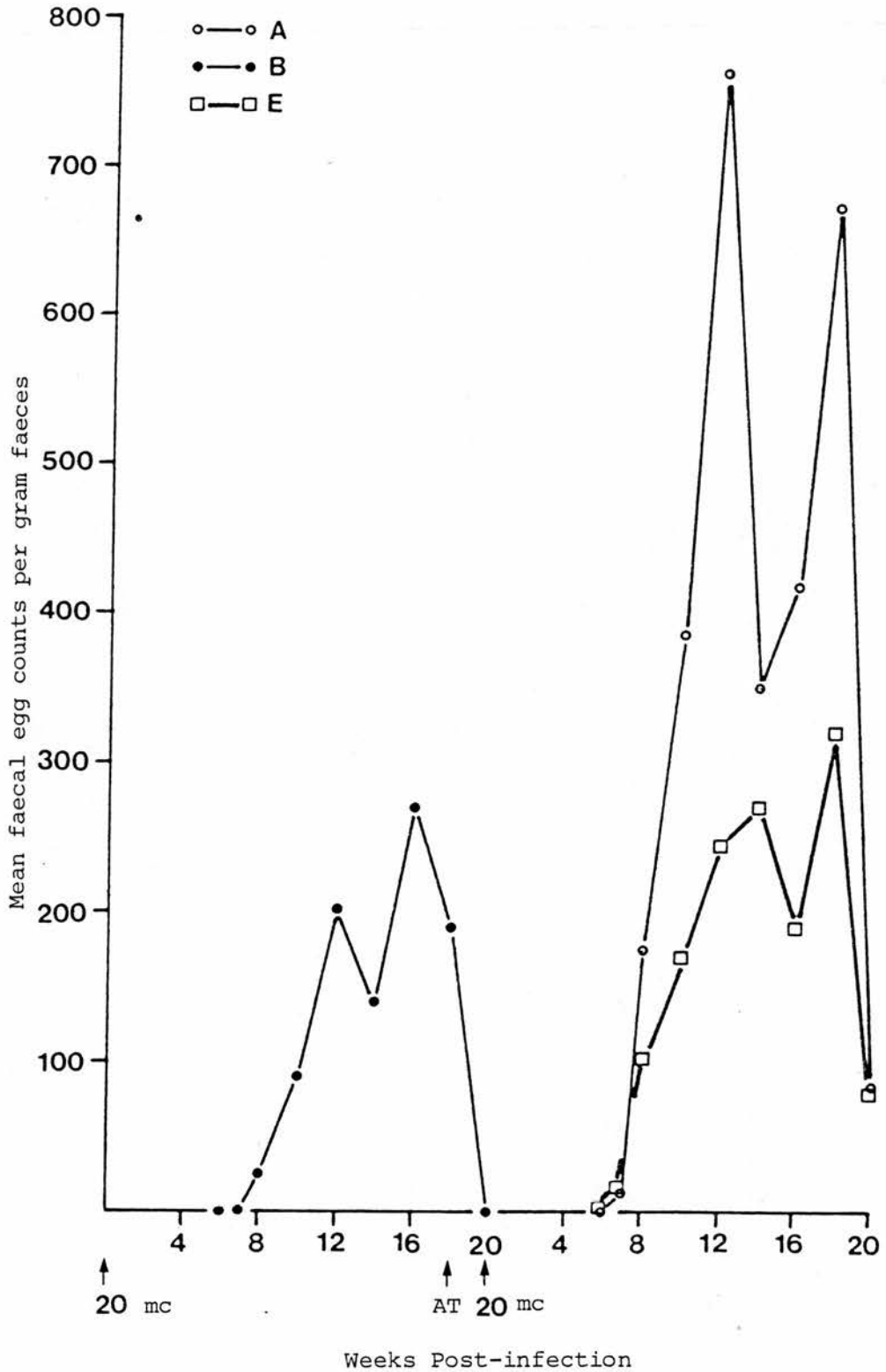


Figure 8.1.4 Faecal egg counts from infected control rats (A), infected rats and treated with anthelmintic (B) and then reinfected with 20 metacercariae of F. hepatica (E).

Table 8.1.4

Fluke recovery from groups of rats given 20 metacercariae of F. hepatica (A), given 20 metacercariae, treated and remaining uninfected (C), given 20 metacercariae, treated and implanted with adult flukes in diffusion chambers (D) and given 20 metacercariae, treated and reinfected with 20 metacercariae of F. hepatica.(E).

Group	Rat No.									Mean ± SD
	1	2	3	4	5	6	7	8	9	
A	7	10	7	8	9					8.2 ± 1.3
C	0	0	0	0	0	0	0	0	0	
D	0	0	0	0	0	0				
E	3	5	0	2	6	5	1			3.1 ± 2.3+

+A v D ($U.005_{(5,7)} = 34$, $U = 35$)

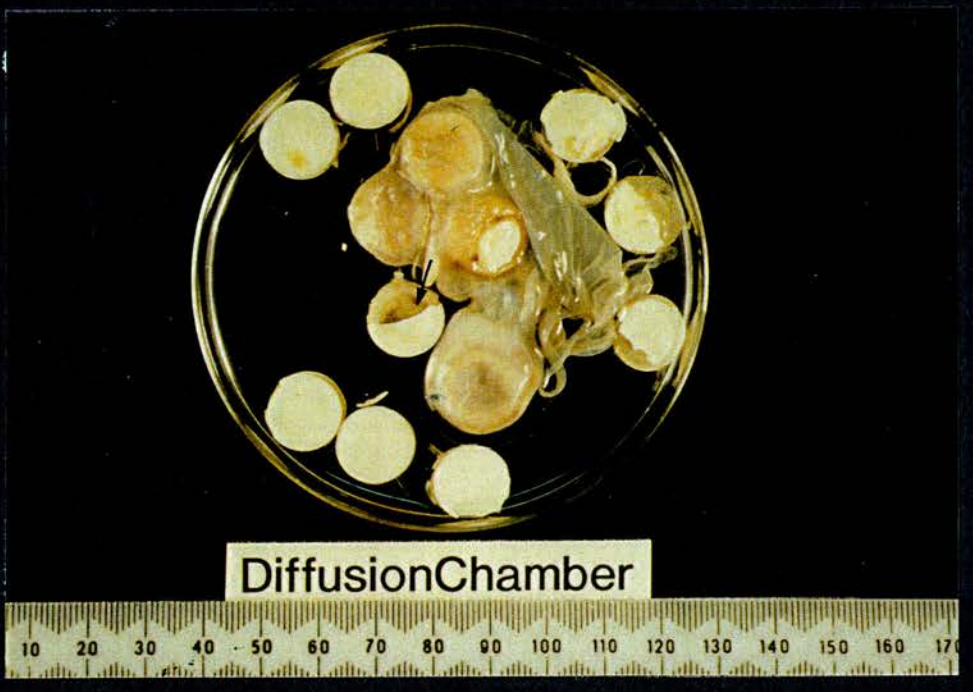
Discussion

The ELISA values in rats infected with 20 metacercariae rose to maximum values at week 6 after infection, indeed the response in rats was stronger than in bullocks. The increase observed two weeks after the elimination of flukes by anthelmintic may have been caused by a release of antigens from dying flukes in the bile duct.

The relatively short-lived increase in ELISA levels at weeks 2-4 after implantation in group D as compared with group E suggests that the release of metabolic antigen by the flukes in diffusion chambers was rather brief. It was probable that after about four weeks the flukes were dead or there was encapsulation of the diffusion chamber by the host's cellular tissues, as happened in those flukes implanted in the bullocks (see Plate 8.1), which may have blocked further release of the metabolic antigen.

Rajasekariah and Howell (1978a) studied the acquired immunity to F. hepatica in rats and first detected precipitating antibodies in rats two weeks after receiving infection. The appearance of these antibodies corresponded to the time when juvenile flukes began to invade the liver. About 4-5 weeks after infection, the antibodies reached a peak which corresponded to the time when active migration of the young flukes in the liver parenchyma was taking place. Thereafter the antibodies fell and this corresponded to the emigration of flukes from the liver to enter the bile ducts. There are two possibilities for this fall; one is that the adult stage of F. hepatica, after reaching the bile duct, may undergo some changes as a result of which the host does not recognise its presence (mimicry) and antibody production against it ceases or, perhaps more likely, because the adult worm is located in the bile

Plate 8.1 Diffusion chambers containing adult Fasciola hepatica recovered 20 weeks after they had been implanted in rats two weeks after the rats had been treated with anthelmintic to remove a previous infection of 20 metacercariae. Shows the host's tissue membrane encapsulating the chambers and dead fluke in an opened chamber (arrow).


DiffusionChamber
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160

duct, antigenic materials released by it are effectively isolated from the immune system of the host.

From the numbers of flukes recovered, it is clear that group E was partially resistant to the challenge infection. Indeed, in terms of the serum GD levels there was almost as much liver damage as in the naive group A. It is possible that this is a reflection of a more severe reaction by the liver parenchyma in the previously infected animals, resulting in the early death of a higher proportion of the invading flukes.

The enhanced serological response obtained following the administration of the encapsulated flukes, was disappointingly short-lived but was considered sufficient to merit using the same approach in an attempt to enhance the protective humoral response in bullocks.

8.2 Immune Response of Implanted Bullocks

Experimental design

Six six-month-old Ayrshire bullocks (Nos. L2, L6, L3, L4, L7 and L17) were each infected with 1,000 metacercariae of F. hepatica. Six weeks later two of the animals (L2 and L6) were killed, sera collected in bulk and the flukes recovered. At the same time, one bullock (L3) was reinfected with 1,000 metacercariae, killed six weeks later, sera collected in bulk and flukes recovered. The last three young bullocks (L4, L7 and L17) and a seventh animal (No. 203) which had been given two previous infections (see Section 6.1), were implanted intraperitoneally with adult flukes in 10 diffusion chambers (one or two flukes in each chamber, depending on the size of the parasite), killed eight weeks later, sera collected in bulk and the flukes recovered.

A day before the administration of the initial infection each animal (except No. 203) was bled (c. 500 ml) to collect normal serum for pooling. Further small amounts of blood (10 ml) were taken at day 0 and at weeks $6_{(1)}$ (except in bullock 203), $2_{(2)}$ and $6_{(2)}$ from all these animals to monitor the eosinophil counts, serum glutamic dehydrogenase activities, using the activated technique, and serology, using rabbit-derived somatic or metabolic antigen by ELISA.

The eosinophil counts and assays for GD activity were duplicated, while in the ELISA three replicates were undertaken. The pre-infection sera from the six bullocks were pooled in the assay of ELISA.

Results

Serology

The mean ELISA values, using somatic and metabolic antigens, are shown in Tables 8.2.1 and 8.2.2 respectively.

Table 8.2.1 Mean ELISA values (units of absorbance) from bullocks given single (L2 and L6) and repeated (L3) infections, implanted after given single infection (L4, L7 and L17) and implanted after given repeated infections (203), using somatic antigen in the test.

Bullock No.	Weeks after Infection			
	0 (Pool)	$6_{(1)}$	$2_{(2)}$	$6_{(2)}$
L2	0.11 ± 0.03	0.39 ± 0.03		
L6	0.11 ± 0.03	0.40 ± 0.02		
L3	0.11 ± 0.03	0.36 ± 0.02	0.48 ± 0.03	0.54 ± 0.04
L4	0.11 ± 0.03	0.28 ± 0.01	0.36 ± 0.03	0.34 ± 0.04
L7	0.11 ± 0.03	0.38 ± 0.01	0.39 ± 0.02	0.25 ± 0.02
L17	0.11 ± 0.03	0.32 ± 0.05	0.41 ± 0.03	0.39 ± 0.02
203*	-	0.48 ± 0.04	0.50 ± 0.03	0.52 ± 0.01

*Weeks $18_{(2)}$, $2_{(3)}$, $6_{(3)}$ for this bullock

Table 8.2.2 Mean ELISA values (units of absorbance) from bullocks given single (L2 and L6) and repeated (L3) infections, implanted after given single infection (L4, L7 and L17) and implanted after given repeated infections (203), using metabolic antigen in the test.

Bullock No.	Weeks after Infection			
	0 (Pool)	$^{6}_{(1)}$	$^{2}_{(2)}$	$^{6}_{(2)}$
L2	0.05 ± 0.02	0.17 ± 0.02		
L6	0.05 ± 0.02	0.19 ± 0.01		
L3	0.05 ± 0.02	0.18 ± 0.03	0.22 ± 0.03	0.29 ± 0.05
L4	0.05 ± 0.02	0.17 ± 0.02	0.23 ± 0.03	0.19 ± 0.04
L7	0.05 ± 0.02	0.16 ± 0.02	0.28 ± 0.01	0.23 ± 0.04
L17	0.05 ± 0.02	0.15 ± 0.02	0.30 ± 0.04	0.29 ± 0.02
203*		0.35 ± 0.03	0.39 ± 0.03	0.31 ± 0.03

*Weeks $^{18}_{(2)}$, $^{2}_{(3)}$ and $^{6}_{(3)}$ for this bullock.

As in the previous studies, using young and adult mature bullocks, the ELISA value in pre-infection serum was greater with the somatic than with the metabolic antigen. Likewise, with both antigens, the ELISA values increased after infection and were further raised at week $^{2}_{(2)}$. In both the reinfected and implanted bullocks, they then remained fairly stable with the somatic antigen, except in L7, when there was a fall in the ELISA activity. With the metabolic antigen between weeks $^{2}_{(2)}$ and $^{6}_{(2)}$ the ELISA activity increased in L3 fell in L4 and was relatively stable in L17 and 203.

All the ELISA values following infection were significantly greater than the pre-infection values in the same animal ($P < .005$).

Serum glutamic dehydrogenase assay

The results are shown in Appendix Table 8.2.1.

As in the previous studies in bullocks, the GD levels increased in all animals after infection. However, in contrast to previous

studies, the GD values in bullock L3 increased greatly at week $6_{(2)}$. In implanted bullocks, there were high GD levels in bullocks L7, L4 and 203 at week $2_{(2)}$, although in the last two animals the values fell at week $6_{(2)}$. However, in bullock L17, the GD level was very low two weeks after implantation but rose by week $6_{(2)}$.

Eosinophil counts

The data are shown in Appendix Table 8.2.2.

After infection, at week $6_{(1)}$, there was an increase in eosinophil counts in all animals, although the counts were surprisingly low in L6 and L17. In L3, the eosinophil counts continued to rise by two weeks after reinfection and the counts fell at week $6_{(2)}$.

In the implanted bullocks, the counts were relatively high in L17 and L4 at two weeks after implantation and six weeks later the counts fell sharply except in L7 and 203, whose eosinophil counts showed a very slight increase. These changes were within the range of responses seen in unimplanted bullocks in the previous study (Section 7.1).

Fluke recovery

The number of flukes recovered from each bullock are shown in Table 8.2.3.

Table 8.2.3 Numbers of flukes recovered from bullocks infected and/or implanted with F. hepatica

Bullock No.	Number of	
	Mature flukes	Immature flukes
L2	-	172
L6	-	137
L3	132	36
L7	176	-
L17	104	-
L4	33	-
203	4	-

As shown in Plate 8.2, the flukes at week $6_{(1)}$ from L6 were clearly immature, having been retrieved from the liver parenchyma. Those recovered from L2 were similarly immature. In contrast, those recovered from the implanted bullocks and most of those from L3 were very mature. There were also few immature flukes recovered in liver parenchyma in L3 at week $6_{(2)}$.

Discussion

The increased ELISA values, after infection at week $6_{(1)}$, showed that these animals, as in the previous studies, were mounting a serological response. In implanted bullocks, there was an increased ELISA value at week $2_{(2)}$. However, by week $6_{(2)}$, the values had fallen. As in the rats, this may have been due to death of the flukes in the capsule or the encapsulation of the diffusion chamber containing the fluke by the host's cellular tissue (see Plate 8.3), obstructing further release of the metabolic product antigen.

Again, as in the previous studies, the ELISA values using the metabolic antigen are lower than with the somatic antigen.

The increased GD at week $6_{(1)}$ confirmed that the animals had been infected. In bullock L3 the increased GD after secondary infection (week $6_{(2)}$) suggests that some flukes from the challenge infection were causing liver damage and this was confirmed by the 36 juvenile flukes recovered. Nevertheless this does not necessarily indicate that the animal had not resisted the challenge infection, because this is a smaller proportion of the challenge dose than developed after the initial infection and, if the animal had not been killed at week $6_{(2)}$ and the infection had been allowed to continue,

Plate 8.2 Adult Fasciola hepatica recovered from three bullocks.

Left to right:-

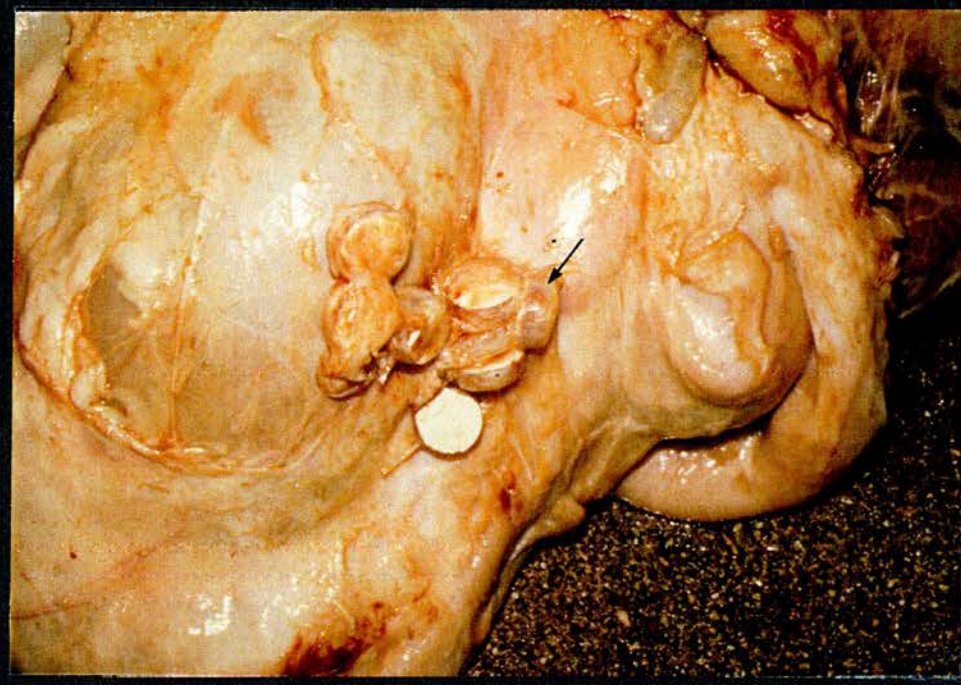
L7 - Eight weeks after implantation from bullock infected per os with 1,000 metacercariae and six weeks later implanted with adult flukes in diffusion chamber.

L3 - Six weeks after secondary infection from bullock infected per os twice, six weeks apart with 1,000 metacercariae of F. hepatica

L6 - Six weeks after infection from bullock infected per os with 1,000 metacercariae of F. hepatica.


Calf No. L 7
Calf No. L 3
Calf No. L 6

Plate 8.3 Diffusion chambers containing adult Fasciola hepatica recovered eight weeks after they had been implanted intraperitoneally in a bullock six weeks after previous infection with 1,000 metacercariae. Shows the host's tissue membrane encapsulating the chambers (arrow) and one chamber not encapsulated. It seems possible that the fluke in the latter died soon after implantation.



these young flukes might well have been eliminated by the immune reaction of the host in the liver, before they could establish themselves in the bile duct.

The increased GD at week $6_{(2)}$ in bullocks L7 and L17 and the smaller drop in the relatively high eosinophil counts in all the implanted animals are surprising and no firm explanation for this can be given. They may however be the result of the host's reaction to the products released from the capsule. In contrast, in bullocks L4 and 203, the GD level dropped to nearly normal levels at week $6_{(2)}$ although there was a slight increase in eosinophil counts in the latter animal at this time. However, since there was little correlation after implantation between the GD and the eosinophilia, it is clearly unlikely that either was caused by flukes escaping from the ruptured capsules.

The relatively low eosinophil counts in L6 and L17 at week $6_{(1)}$ may be an indication that the levels had started to fall by this time and that there had been an earlier rise in eosinophil counts at week $4_{(1)}$. The GD activities and eosinophil counts were monitored at week 6 after infection in bullocks because in previous bullocks (young and adult mature and immature bullocks) these parameters reached peak values at that time.

Based on the numbers of flukes recovered, it appears that L4 and 203 and perhaps L17 may have rejected some of their fluke burden. Whether such rejection was due to the implanted flukes is not known. However, Ross (1968) observed spontaneous recovery in cattle 16-18 months after infection based on negative faecal egg counts and Doyle (1971) observed spontaneous recovery of fluke burdens in cattle given

750 metacercariae of F. hepatica between 16 and 30 weeks after primary infection.

8.3 Passive Transfer of Resistance to Rats by Immune Serum from Implanted Bullocks

Experimental design

Fifty-four six-week-old male Wistar rats were randomly assigned into nine groups of six rats each. Group A, which served as the normal control, was inoculated with pooled normal sera from the six bullocks. Three groups (B1-B3) of six rats each were assigned as primary controls and given IBS collected at week $6_{(1)}$. Group C was given IBS collected at week $6_{(2)}$ from bullock L3. Three groups (D1-D3) of six rats were given IBS collected at week 8 after implantation. Group E was immunised with IBS collected at week 8 after implantation from bullock 203.

All the rats were infected orally with 20 metacercariae each and immediately after infection they were immunised with normal or the appropriate immune serum. Immunisation was repeated two days later.

Serum glutamic dehydrogenase activity was monitored at weeks 0, 4 and 6, while necropsy and fluke recovery was carried out eight weeks after infection.

Statistical analysis on the numbers of flukes recovered and GD activities for groups B1-B3 and D1-D3 are given in Appendix Tables 8.3.1, 8.3.2, 8.3.3 and 8.3.4, using single Independent Analysis of Variance (Sokal and Rohlf, 1981). They showed no significant differences. Therefore, the data from the three groups B1-B3 and also from

D1-D3 were pooled when these were analysed, using the Mann-Whitney U-test for fluke recovery, and the t-test for GD activities.

Results

One rat in group B2, immunised with IBS from L2 died within two weeks after infection. The death was due to internal haemorrhage probably related to the parenchymal migratory phase of the juvenile flukes.

Fluke recovery

The data are shown in Table 8.3.1 and the statistical analysis is given in Appendix Table 8.3.1.1.

The mean number of flukes in all the treated groups were fewer than in the control group, immunised with normal serum. However these differences were only significant in group E, when compared with the normal serum control.

Serum glutamic dehydrogenase assay

The results are shown in Figure 8.3.1 and in Appendix Table 8.3.5. Except in group D3 (L4), the GD levels at week 4 in all treated groups were lower than in normal control. However the pooled GD in group D's was less than in the control group. Statistical analysis showed that the GD levels in group E were significantly lower ($t_{(10)} = 6.04$, $P < .01$) than those from normal serum control (group A).

Discussion

As in Chapter 7, the IBS for these bullocks collected at week $6_{(1)}$ only gave slight protection. Likewise the IBS (week $6_{(2)}$) from

Table 8.3.1

Passive transfer of resistance to 20 metacercariae of F. hepatica in rats by serum from infected or infected and implanted bullocks. Two 10 ml volumes of serum given by intraperitoneal injection on the day of challenge and two days later.

Donor Bullock	Serum		Nos. of flukes recovered at P.M. Individual	Mean ± SD	
	Pooled Normal Serum	(A)	2, 5, 4, 1, 7, 10	4.8 ± 3.3	
176	Immune serum $6_{(1)}$	(B_1)	3, 3, 2, 4, 4, 3,	3.2 ± 0.7	
L2	Immune serum $6_{(1)}$	(B_2)	5, 4, 5, 3, 3, -	4.0 ± 1.0	3.6 ± 1.5
L6	Immune serum $6_{(1)}$	(B_3)	7, 1, 6, 4, 2, 3	3.8 ± 2.3	
L3	Immune serum $6_{(2)}$	(C)	5, 6, 6, 6, 2, 2	4.5 ± 2.0	
L7	Immune serum $8_{(2)}$	(D_1)	5, 1, 2, 1, 1, 3	2.2 ± 1.6	
L17	Immune serum $8_{(2)}$	(D_2)	1, 1, 6, 4, 3, 1	2.7 ± 2.1	3.2 ± 2.0
L4	Immune serum $8_{(2)}$	(D_3)	8, 3, 4, 4, 2, 6	4.5 ± 2.2	
203	Immune serum $8_{(2)}$	(E)	4, 2, 0, 3, 2, 0	1.8 ± 1.6	

- Animal died

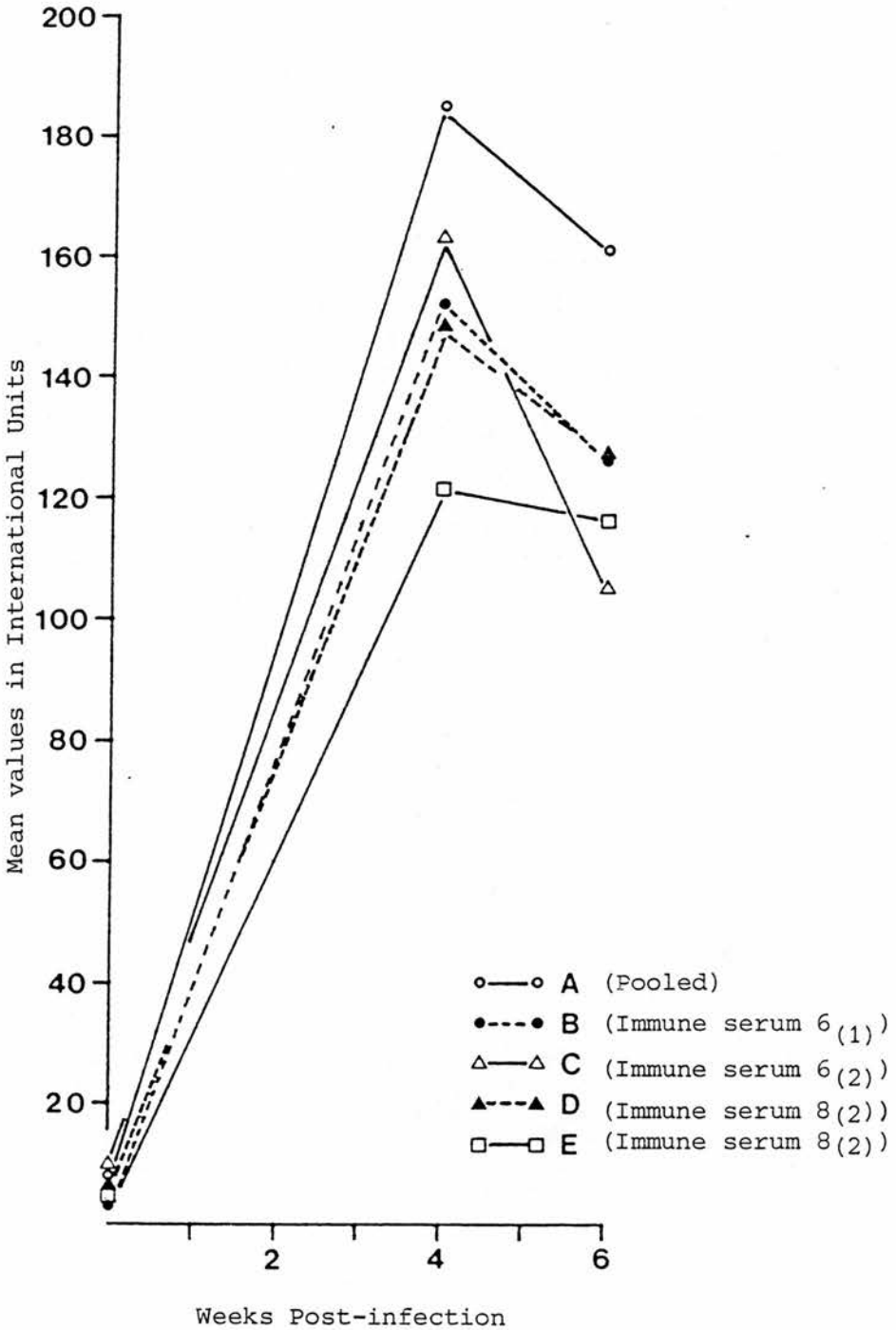


Figure 8.3.1 Serum glutamic dehydrogenase activities from rats immunised with pooled normal serum (A), immune serum from bullocks 176, L-2 and L-6 (B), immune serum from bullock L-3 (C), immune serum from bullocks L-4, L-7 and L-17 (D) and immune serum from bullock 203 (E).

bullock L3, which had received repeated infections, also gave little protection. On the other hand, the IBS from two of the young implanted bullocks (L7 and L17) appeared to give somewhat better protection although serum from the other immature implanted bullock L4 failed to do so. The IBS from the implanted adult mature bullock given repeated infections gave stronger and significant protection, although, as in bullock 89, this protection was not absolute. Further experimentation would be needed to show if this relatively strong protection obtained by administering encapsulated flukes to mature, previously infected bullocks can be reliably repeated. On reflection, however, in view of the short-lived enhancement of the serological response obtained following the administration of encapsulated flukes to rats, it might have been better to have used sera from week $2_{(2)}$ in the attempts to transfer protection to rats.

Again, as in previous passive transfer studies, there is no relationship between the GD activities and the numbers of flukes recovered from individual immunised groups of rats. However, on the pooled data there was a significant correlation ($r_{(1,51)} = 0.66$, $P < .05$) between the GD activities at week 4 and the numbers of flukes recovered at necropsy.

CHAPTER NINE

GENERAL DISCUSSION

The prime objective in this series of studies was to devise a procedure by which cattle could be reliably induced to develop antibodies which would render their serum more protective to naive rats challenged with Fasciola hepatica than is the case following a single infection. This objective was not fully achieved, although there may be some indication in the last experiment that exposing animals to repeated infections and repeated implantation with encapsulated flukes might be effective (Table 8.2.1). It might also be better to use juvenile flukes rather than adult flukes in the implanted chambers.

An important feature of this study is that all the experiments have involved a comparison between numbers of flukes recovered from rats receiving the immune serum and the numbers recovered from rats receiving a normal pre-infective serum from the same animal(s) as provided the immune serum. This is essential since it is known (Haroun, Hammond and Sewell, 1981) that the administration of normal serum will consistently reduce the numbers of flukes recovered from the rats.

The results from this study amply confirm that serum from infected bullocks will afford some protection to naive rats. This occurred in 48 of the 55 immune transfer studies with an overall protection of 21 ± 22% (Table 9.1) and statistical analysis, using "sign test" (Sokal and Rohlf, 1981), shows a significant difference ($P < 0.1$).

Following an initial challenge by oral infection with 1,000 metacercariae of F. hepatica, the immune serum from the two young mature bullocks (about 18 months old) collected between 6 and 12 weeks after

infection appeared to give better protection to rats than that of the other age group or from sera obtained at different times (Tables 9.1 and 5.1.1) and statistical analysis between $sera_{(6-12)}$ and $sera_{(>12)}$ ($U_{(22,3)} = 57$, $t_\infty = 2.09$, $P < .05$) or between $sera_{(6-12)}$ and $sera_{(all\ multiple)}$ ($U_{(22,21)} = 329.5$, $t_\infty = 2.39$, $P < .02$) shows a highly significant difference. The fact that IBS collected before patency was relatively protective indicates that antigens derived from juvenile flukes were effective in this respect. This view is in agreement with Haroun (1979). However after repeated infections the protection afforded by IBS was relatively weak (Tables 9.1, 5.3.1 and 5.3.4), which might be due to the fact that the juvenile flukes from challenge infections were rapidly killed in the sensitised animals, possibly by a cellular mechanism, so that there was no enhancement of the humoral immune response. If so, it appears that stimulation of an immune response, which would induce production of strongly protective serum, would require the persistence of juvenile flukes. In this respect Corba and Spaldonova (1975) have shown a direct dependence between the time of persistence of immunising infections of F. hepatica and the intensity of the immune response by the host.

The use of irradiated metacercariae, wherein the developed flukes do not survive beyond the juvenile stage and so might be expected to induce a strong immune response, has been tried by Wikerhauser (1961a), Corba, Armour, Roberts and Urquhart (1971), Sokolic (1971) and Dargie (1973). Although by this technique there is persistence of juvenile flukes, it has been shown by Hughes, Hanna and Doy (1982) that some of these irradiated metacercariae "break through" the development barrier imposed by irradiation. The dose of radiation, which would

allow the parasite only to persist within the immunogenic stage, had not been established. In any case this method does not appear to induce good protection.

The method of implanting naked or encapsulated adult flukes had also been tried (Haroun, 1979). Again the persistence of antigenic stimulus would be the critical factor since the host forms a cyst around the flukes (Burden and Hammet, 1980) or the chamber, as seen in the present study. However, the fact that there was a brief and sustained ELISA level after implantation in rats offers promise with this technique.

In retrospect and in view of the ELISA results obtained with implanted rats it would perhaps have been better to have used IBS obtained two weeks after implantation in the passive transfer study. The reason this was not done was because the experiment in rats took rather a long time and could not be analysed in detail before the same experiment had to be started in bullocks. However, as was observed by Haroun (1979) and amply confirmed in the present study, a high antibody titre in the serum is not necessarily associated with its ability to transfer resistance, so it is by no means certain the earlier serum would have given better results. Despite this possibility that the serum was not obtained at the optimal time, IBS from implanted bullocks previously receiving oral infections was able to transfer a relatively good resistance (Tables 9.1 and 8.2.1). However, because of the limited numbers of bullocks that could be used, this finding cannot be regarded as conclusive, although the level of protection afforded by sera from the implanted animals is significantly greater than that from those that had received multiple infection

(Tables 9.1, 5.3.1, 5.4.1 and 6.3.1, Mann-Whitney $U_{(22,4)} = 74.5$, $t_{\infty} = 2.17$, $P < .05$).

Serologically there was a humoral antibody response demonstrable by ELISA in both bullocks and rats, using either a somatic or a metabolic antigen. The results, using the somatic antigen, appeared somewhat stronger than those using the metabolic antigen but further analysis showed (Figures 9.1 and 9.2) that this could mostly be accounted for by the difference in the reaction between these antigens and normal bovine serum (Table 9.2). This latter reaction was much stronger with the somatic antigen, especially with sera from mature animals.

With both antigens, the first ELISA peak occurred before patency and there was usually the second smaller peak after patency (Figures 5.1.1 and 5.1.2; Figures 6.1 and 6.1.2; Figure 8.1.1). However there was considerable variation in ELISA values with individual animals and the second ELISA peak occurred at varying weeks in different animals. This variation may be associated with the biological variation in both host and parasite (Meek and Morris, 1979).

The appearance of a further ELISA peak after patency and the fluctuation of levels thereafter suggests that even when the flukes were localised in the bile duct there was release of antigenic materials from the flukes and this view is in agreement with Sandeman and Howell (1981).

The ELISA levels after repeated infections in either bullocks or rats were higher than the levels after primary infection which suggests that the juvenile flukes from challenge infections were releasing sufficient antigenic materials to cause the increased

Table 9.1

Apparent protection afforded to rats by various types of immune bovine sera (pooled results)

Serum	No. of sera	% protection* ± SD
$^{3}_{(1)}$	2	3
$^{6}_{(1)}$	8	24 ± 24
$^{9}_{(1)}$	9	31 ± 31
$^{12}_{(1)}$	4	35 ± 8
6 month $^{6-12}_{(1)}$	9	18 ± 17
18 month $^{6-12}_{(1)}$	5	57 ± 19
mature $^{6-12}_{(1)}$	6 (5)	23 ± 28
$^{6}_{(2)}$	6	16.5 ± 16
$^{9}_{(2)}$	5	4 ± 10
All primary (6-12)	21	29 ± 25
All primary (15-21)	3	2 ± 11
All secondary	14	14 ± 15
All tertiary	8	13 ± 19
All implants	4	42 ± 25
All multiple	22	13 ± 16
All	55	21 ± 22

$$*\%\ \text{protection} = \frac{\text{No. of flukes in control} - \text{No. of flukes in experiment}}{\text{No. of flukes in control}} \times 100$$

Table 9.2

Raw ELISA reactions with normal bovine sera and *Fasciola* antigens

Serum from bullocks aged:	Mean ± SD ELISA values with:	
	Somatic antigen	Metabolic antigen
6 months	0.33 ± 0.18	0.18 ± 0.01
18 months	0.49 ± 0.03	0.16 ± 0.01
4-5 years	0.53 ± 0.09	0.23 ± 0.04

antibody production. This in turn implies that the juveniles from the challenge infections were able to localise in the liver parenchyma before they were being killed by the immune reaction and were not being killed in the gut region as suggested by Rajasekariah and Howell (1978a), and is therefore in agreement with Kelly, Campbell and Dineen (1980), who concluded after infecting rats orally and intraperitoneally with F. hepatica that the passage of juvenile flukes through the gut was not essential for the acquisition or expression of acquired resistance to F. hepatica in the rat.

On the other hand, the increased level of ELISA values after repeated infections is in contrast with Sandeman and Howell (1981) who observed decreased antibody levels after challenge infection in sheep by Ouchterlony double diffusion in gels. Whether this contrasting observation is due to the host used, in that sheep show poor resistance to F. hepatica challenge, or to the immunodiagnostic test, is not known. However it has been shown that different serological tests show different peak titres of precipitating antibodies (Doyle, 1973b; Hillyer and Diaz, 1976). There was no evidence of any direct relationship between the ELISA values, using these rather crude and complex antigens, and the protective effect of the sera.

Even after offsetting the differing reactions with the normal sera the ELISA values tended to be somewhat higher with the somatic antigen than with the metabolic antigen (Figures 9.1 and 9.2). However these antigens were clearly reacting with different antibody spectra since the variation in activities of successive sera from the various animals did not consistently follow each other, notably with 203 and 94.

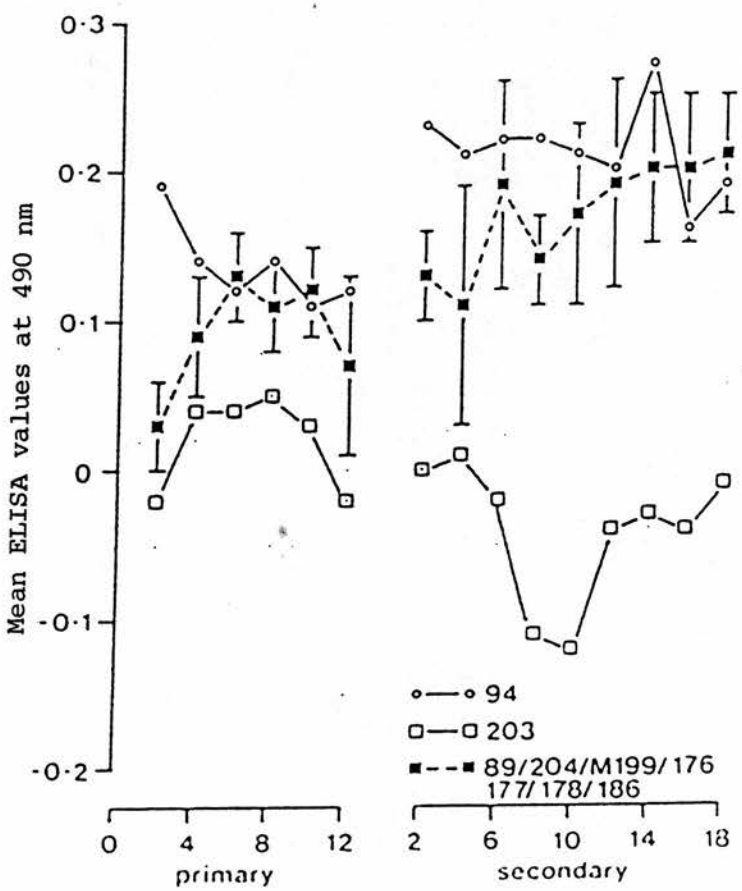


Figure 9.1 Increased over pooled normal ELISA values from bullocks, using somatic antigen.

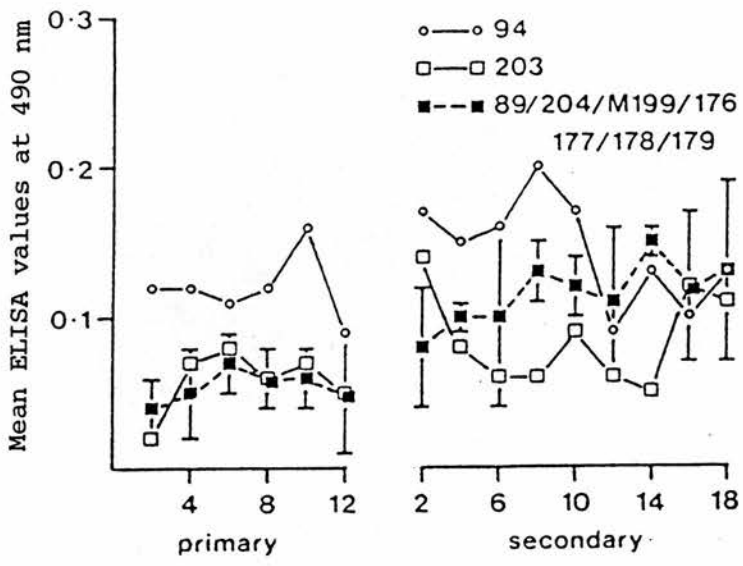


Figure 9.2 Increased over pooled normal ELISA values from bullocks, using metabolic antigen.

With the exception of 203, with somatic antigen, and 94, with metabolic antigen, the ELISA values after primary infection from the rest of the animals were similar, indicating that age has little influence in the serological response of bullocks. Following secondary infection the ELISA values tended to be somewhat higher with both antigens but there was also considerable fluctuation. Again this fluctuation is probably influenced by the variation in the biology of both the animal host and the parasites (Meek and Morris, 1979).

Figures 9.1 and 9.2 further show that, although the specific ELISA values with metabolic antigen tended to be lower than the values with somatic antigen, the markedly different overall ELISA values for the different antigens, as shown in the earlier figures, were largely a reflection of the relatively high reaction between normal bovine serum and the somatic antigen. This further indicates the complex nature of this antigen (Sewell, 1964; Hillyer, 1980) as compared with the metabolic antigen (Lehner and Sewell, 1980).

The serological response of rats (group B) two weeks after treatment with anthelmintic was markedly increased (Figure 8.1.1) and this was probably brought about by the dead flukes in the bile duct. This hypothesis further confirms the view of Sandeman and Howell (1981) that through the physical obstruction of the bile duct there is passage of antigenic materials into the circulation.

The serological response in bullocks or rats after implantation was short-lived. The encapsulation of the diffusion chamber, probably resulting from the host's cellular reaction against diffusing metabolic antigen, appears to have caused the death of the flukes, so preventing further release of the antigen and consequent reduction

in antibody stimulation (Burden and Hammet, 1980).

The GD and eosinophil reactions in bullocks and rats after primary infection were also biphasic. However, in the case of GD in bullocks, the second peak was higher (week $6_{(1)}$ V week $10_{(1)}$, $t_{(16)} = 2.33$, $P < .05$) than the first, as shown in Figure 9.1.3 where the values from closely responding animals are pooled. In contrast, in rats (Figure 8.1.2), the second peak at week $18_{(1)}$ was relatively inconspicuous, probably because in these animals there was less severe liver damage than in cattle. On the other hand, the second peak in the eosinophil counts in both bullocks (Figure 9.1.4) and rats (Figure 8.1.3) was lower than the first peak. The fact that this biphasic reaction is common to both parameters may indicate that both were related more to the parenchymal liver damage than directly to the presence of the flukes in the infected animals. Moreover the second peak in ELISA after patency is probably related to the release of metabolic antigen by the flukes in the bile duct.

The first peak in GD and eosinophil counts occurred before patency (week $6_{(1)}$ in bullocks or week $4_{(1)}$ in rats) and this was probably the result of a non-specific phenomenon involving liver damage followed by repair after the flukes localised in the bile duct (Sewell, 1966). On the other hand, the second peak which occurred after patency may well be the result of a specific reaction involving immunocompetent cell infiltration in the liver (Rajasekariah and Howell, 1981).

It has been shown that severe tissue destruction can result from the presence of immune complexes in bacterial and viral diseases (WHO Technical Report, 1977) and Coombs (1976) showed that an auto-

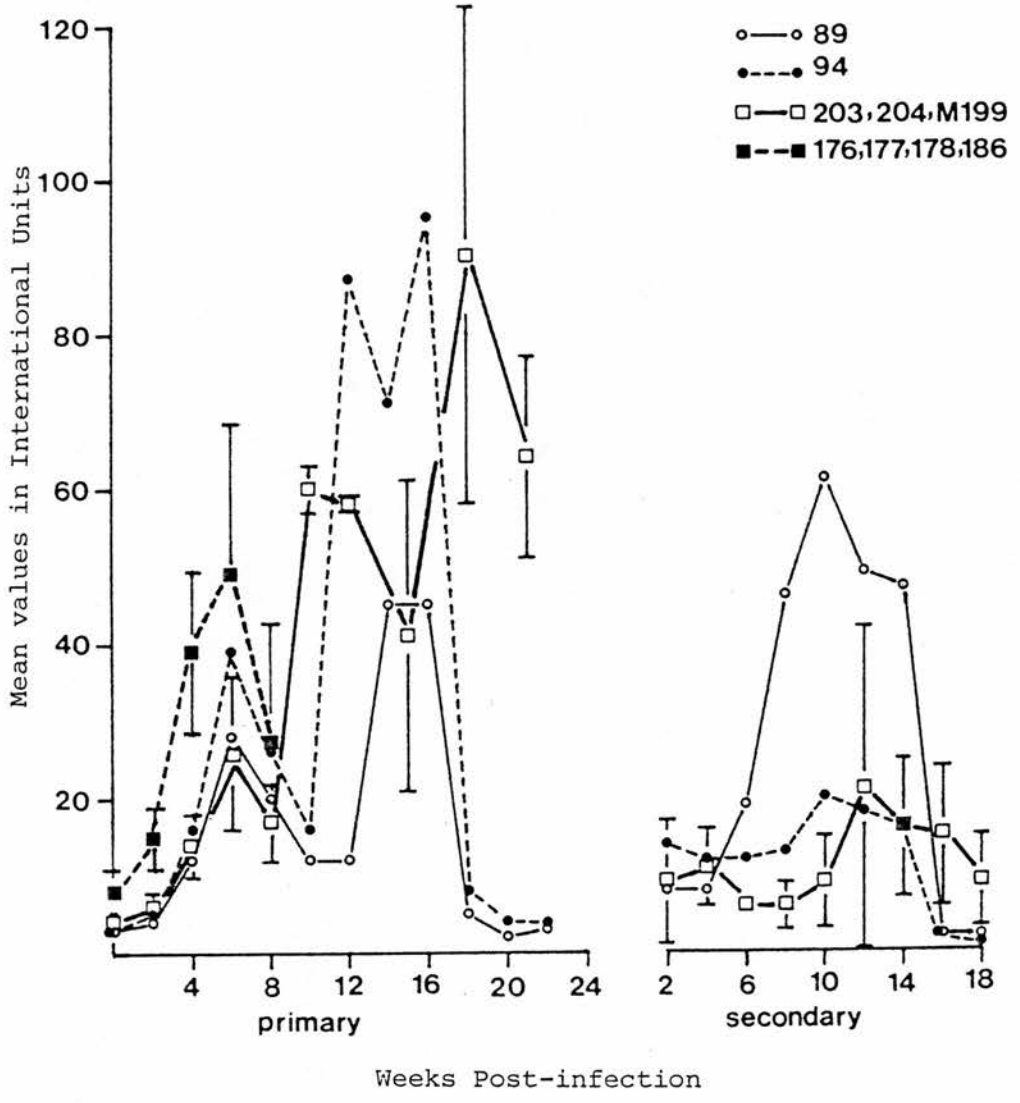


Figure 9.1.3 Serum glutamic dehydrogenase activities from bullocks infected with Fasciola hepatica.

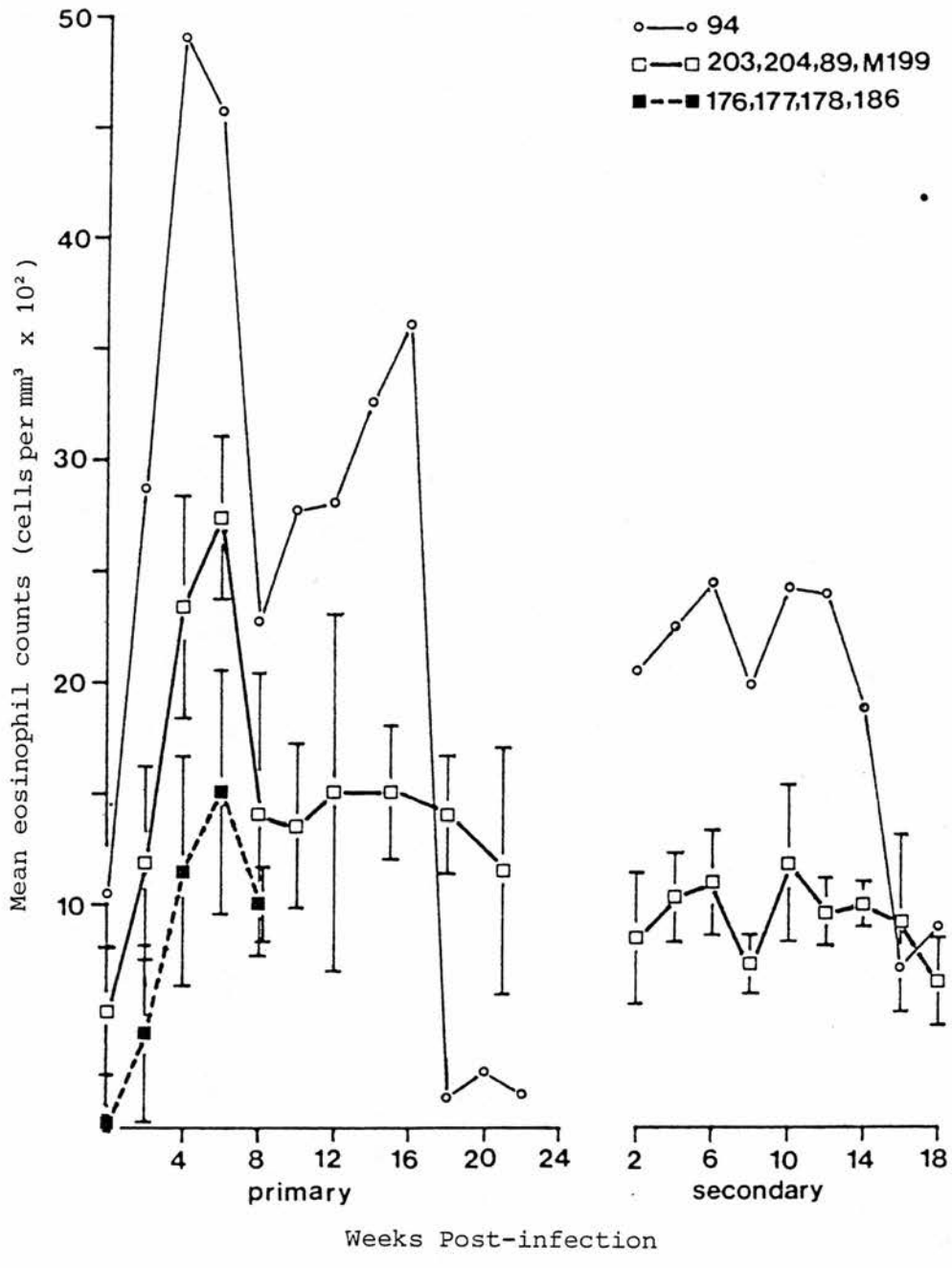


Figure 9.1.4 Peripheral eosinophil counts from bullocks infected with Fasciola hepatica.

immune reaction may cause tissue damage. On the other hand, Sandeman and Howell (1980, 1981) showed that precipitated fluke antigen-antibody complex will cause release of GD when injected intramuscularly into sheep and suggested that such material released from the bile duct, which will include not only fluke antigen but also host antigens, might induce a number of reactions leading to destruction in the surrounding liver cells and thereby releasing GD into the serum.

On the other hand, Sabesin (1963) found that peritoneal eosinophilia can be elevated in the guinea-pig by transfer of antigen-antibody complex and Flagstad, Andersen and Nielsen (1972) showed that eosinophils are attracted by immune complexes.

Following secondary or tertiary infections there was a rise in GD and eosinophils in bullocks but these increases were relatively low. Taken together with the lower faecal egg counts after repeated infections these lower peaks are probably an indication that the animals had acquired resistance against challenge so that these lower peaks in GD or eosinophil may have been induced partly by a smaller number of flukes migrating through the liver parenchyma and also by a hypersensitivity reaction in the liver (Sinclair, 1973).

The increased GD and eosinophil counts in reinfected rats whose primary infection had been eliminated by anthelmintic is not an indication of any lack of acquired resistance, because these animals had significantly fewer flukes than the challenge controls, but is rather the result of chance variation in the infectivity of the different batches of metacercariae used.

In rats implanted with adult flukes in diffusion chambers, the GD or eosinophil counts remained low with only a small peak, possibly

induced by an auto-immune reaction or hypersensitivity of the liver. In contrast, in implanted bullocks, the GD and eosinophil counts were very inconsistent. There was no clear explanation for this but it is presumably related to the different responses in the liver parenchyma to the products diffusing from the chambers, since there was no evidence to suggest that the flukes had escaped from the diffusion chamber to penetrate the liver in any of the animals.

From the individual passive transfer studies, it appeared that there was no direct relationship between the GD level in the rats at week 4 after infection and the numbers of flukes recovered at necropsy and the reason for this is probably the limited numbers of animals used in each study. However, statistical analysis of the pooled data from all these studies, comparing the GD values at week 4 and the numbers of flukes recovered showed a highly significant correlation ($r_{(1,495)} = 0.49$, $P < .01$).

The results of the passive transfer studies confirmed that IBS from bullocks collected before patency and IBS collected from bullocks previously infected and implanted with adult flukes in diffusion chambers are able to transfer resistance to rats. However this ability of the serum to transfer resistance shows considerable individual variation between bullocks and although the IBS from young mature bullock (No. 89) gave relatively strong protection, the age of the animals seems to have no consistent influence.

On the other hand, the lack of ability of IBS to transfer absolute resistance to rats suggests that oral sensitisation, especially by repeated infections, does not induce a strong humoral immune response in cattle capable of producing a serum that can transfer

absolute resistance to naive rats. The implantation procedure possibly using juvenile flukes, rather than adult flukes in diffusion chambers, given after single or repeated oral infections, merits further investigation.

REFERENCES

Akahane, H. (1975). The characteristic changes of haematological findings and immunological response of the rabbits infected with Fasciola sp. by the migration of flukes in the liver. 1. The characteristic changes of haematological findings. Japanese Journal of Parasitology, 24: 347-352.

Anderson, J.C., Hughes, D.L. and Harness, E. (1975). The immune response of rats to subcutaneous implantation with Fasciola hepatica. British Veterinary Journal, 131: 509-517.

Anderson, P.H., Berrett, S., Brush, P.J., Herbert, C.N., Parfitt, J.W. and Patterson, D.S.P. (1977). Biochemical indicators of liver injury in calves with experimental fascioliasis. Veterinary Record, 100: 43-45.

Anderson, P.H., Berrett, S. and Patterson, D.S.P. (1978). Resistance to Fasciola hepatica in cattle. II. Biochemical and morphological observations. Journal of Comparative Pathology, 88: 245-251.

Anderson, P.H., Matthews, J.G., Berrett, S., Brush, P.J. and Patterson, D.S.P. (1981). Changes in plasma enzyme activities and other blood components in response to acute and chronic liver damage. Research in Veterinary Science, 31: 1-4.

Archer, R.K. (1965). Haematological techniques for use in animals. Blackwell Scientific Publications, Oxford.

Argana, E.C. and Sandili-Argana, A.M. (1971). An economic survey of liver fluke infection at Zamboanga City abattoir. BAI Recorder, 21(1-3): 13.

Armour, J. and Dargie, J.D. (1973). Studies on immunity to Fasciola hepatica. Annali della Facolta di Medicina Veterinaria di Torino, 30 (Supplement): 135-147.

Armour, J. and Dargie, J.D. (1974). Immunity to Fasciola hepatica in the rat. Successful transfer of immunity by lymphoid cells and by serum. Experimental Parasitology, 35(3): 381-388.

Armour, J., Dargie, J.D., Doyle, J.J., Murray, M., Robinson, P. and Rushton, B. (1974). Immunisation against fascioliasis. In Proceedings of the Third International Congress of Parasitology, 25-31 August, 1974, Munich, 1: 494.

Armstrong, D.A. and Miller, C.R. (1980). Liver fluke in cattle: life cycle, pathology, economic impact. Norden News, 55 (Part 3): 30-33.

Baalawy, S.S. (1975). Studies on immune mechanism of Fasciola gigantica infection in rabbits. Bulletin of Animal Health and Production in Africa, 23(1): 99-102.

Bennett, C.E. and Threadgold, L.T. (1973). Electron microscope studies of Fasciola hepatica. XIII. Fine structure of newly excysted juvenile. Experimental Parasitology, 34: 85-99.

Bennett, C.E. and Threadgold, L.T. (1975). Fasciola hepatica: Development of tegument during migration in mouse. Experimental Parasitology, 38: 38-55.

Berg, C.O. (1964). Snail control in trematode diseases: the possible value of Sciomyzid larvae snail-killing diptera. In Advances in Parasitology, 2: 259-309. Academic Press, London and New York.

Bitakamire, P.K. (1973). Preliminary studies on the immunization of cattle against fascioliasis using gamma-irradiated metacercariae of Fasciola gigantica. In Isotopes and Radiation in Parasitology, III: 23-32. International Atomic Energy Agency, Vienna.

Boehringer (1982). BCL, Mannheim, Boehringer, Catalogue 1982.

Bolbol, A.S. (1975). The immunology of fascioliasis in rabbits. Ph.D. Thesis, University of Edinburgh.

Boray, J.C. (1963). Standardisation of techniques for pathological and anthelmintic studies of Fasciola spp. In Proceedings of the First International Conference of the World Association for the Advancement of Veterinary Parasitology, The Evaluation of Anthelmintics, Hanover, 34-45. Edited by E.J.L. Soulsby, Merck, Sharp and Dohme International, New York.

Boray, J.C. (1967a). The effect of host reaction to experimental Fasciola hepatica infections in sheep and cattle. Proceedings of the Third International Conference of the World Association for the Advancement of Veterinary Parasitology, Lyons, France. Veterinary Medical Review, 84-95. Edited by E.J.L. Soulsby.

Boray, J.C. (1967b). Studies on experimental infections with Fasciola hepatica, with particular reference to acute fascioliasis in sheep. Annals of Tropical Medicine and Parasitology, 61(4): 439-450.

Boray, J.C. (1969). Experimental fascioliasis in Australia. In Advances in Parasitology, 7: 95-210.

Boyd, J.W. (1962). The comparative activity of some enzymes in sheep, cattle and rats-normal serum and tissue levels and changes during experimental liver necrosis. Research in Veterinary Science, 3(3): 256-268.

Burden, D.J., Bland, A.P. and Hughes, D.L. (1982). Development of the tegument of Fasciola hepatica after γ-irradiation. An electron microscope study. Parasitology, 84: lxii

Burden, D.J., Bland, A.P., Hughes, D.L. and Hammet, N.C. (1983). Fasciola hepatica: development of the tegument of normal and γ-irradiated flukes during infection in rats and mice. Parasitology, 86: 137-145.

Burden, D.J. and Hammet, N.C. (1980). Fasciola hepatica: attempts to immunise rats using fluke eggs and in vitro culture products. Veterinary Parasitology, 7: 51-57.

Burden, D.J., Harness, E. and Hammet, N.C. (1982). Fasciola hepatica: attempts to immunise rats and mice with metabolic and somatic antigens derived from juvenile flukes. Veterinary Parasitology, 9(3/4): 261-266.

Burden, D.J., Hughes, D.L., Hammet, N.C. and Collis, K.A. (1978). Concurrent daily infection of calves with Fasciola hepatica and Ostertagia ostertagi. Research in Veterinary Science, 25: 302-306.

Butterworth, A.E. (1977). The eosinophil and its role in immunity to helminth infection. Current topics in Microbiology and Immunology, 77: 127-168.

Butterworth, A.E., Coombs, R.A.A., Gurner, B.W. and Wilson, A. (1976). Receptors for antibody-opsonic adherence on the eosinophils of guinea pig. Int. Archs. of Allergy and Applied Immunology, 151: 368-377.

Butterworth, A.E., David, J.R., Franks, D., Mahmoud, A.A.F., David, P.H., Sturrock, R.F. and Houba, V. (1977). Antibody-dependent eosinophil-mediated damage to ^{51}Cr-labelled schistosomula of Schistosoma mansoni: damage by purified eosinophils. The Journal of Experimental Medicine, 145: 136-150.

Butterworth, A.E., Sturrock, R.F., Houba, V. and Taylor, R. (1976). Schistosoma mansoni in baboons. Antibody-dependent cell-mediated damage to ^{51}Cr-labelled schistosomula. Clinical and Experimental Immunology, 25:95-102.

Butterworth, A.E., Wassom, D.L., Gleich, G.J., Loegering, D.A. and David, J.R. (1979). Damage to schistosomula of Schistosoma mansoni induced directly by eosinophil major basic protein. The Journal of Immunology, 122(1): 221-229.

Campbell, N.J., Dineen, J.K. and Kelly, J.D. (1978). Resistance in sheep to Fasciola hepatica generated by infection with Cysticercus tenuicollis. In Fourth International Congress of Parasitology, 19-26 August, 1978, Warsaw, Poland, Short communications, Section C, 78-79.

Campbell, N.J., Gregg, P., Kelly, J.D. and Dineen, J.K. (1978). Failure to induce homologous immunity to Fasciola hepatica in sheep vaccinated with irradiated metacercariae. Veterinary Parasitology, 4: 143-152.

Campbell, N.J., Kelly, J.D. and Dineen, J.K. (1978). The effect of age of host in resistance expressed at the gut level in rats infected with Fasciola hepatica. Veterinary Parasitology, 4: 317-325.

Campbell, N.J., Kelly, J.D. and Martin, I.C.A. (1979). Stimulation of resistance to Taenia taeniaeformis in rats by infection with Fasciola hepatica. International Journal for Parasitology, 9(5): 469-474.

Campbell, N.J., Kelly, J.D., Townsend, R.B. and Dineen, J.K. (1977). The stimulation of resistance in sheep to Fasciola hepatica by infection with Cysticercus tenuicollis. International Journal for Parasitology, 7: 347-351.

Capron, M., Capron, A., Torpier, G., Bazin, H., Bout, G. and Joseph, M. (1978a). Eosinophil-dependent cytotoxicity in rat schistosomiasis. Involvement of IgG2a antibody and role of mast cells. European Journal of Immunology, 8: 127-133.

Capron, M., Capron, A., Bazin, H., Torpier, G. and Joseph, M. (1978b). Eosinophil-dependent cytotoxicity in rat schistosomiasis. In Fourth International Congress of Parasitology, 19-26 August, 1978, Warsaw, Poland, Short Communications, Section E, 30-31.

Capron, A., Dessaint, J.P. and Capron, M. (1975). Specific IgE antibodies in immune adherence of normal macrophages and Schistosoma mansoni schistosomula. Nature, 253(5491): 474-475.

Castagnetti, G.D., Losi, G. and Morini, D. (1977). Effect of the infection by Fasciola hepatica on chemical composition and technicological characters of milk. Scienze E Tecnica Lattiero Casearia, 28(3): 187-202.

Caulfield, J.P., Korman, G., Butterworth, A.E., Hogan, M. and David, J.R. (1980). Partial and complete detachment of neutrophils and eosinophils from schistosomula: evidence for the establishment of continuity between a fused and normal parasite membrane. The Journal of Cell Biology, 86:64-76.

Chick, B.F. (1979). Economic significance of Fasciola hepatica infestation of beef cattle - a definitive study based on field trial and grazier questionnaire. In Proceedings of the Second International Symposium on Veterinary Epidemiology and Economics, 7-11 May, 1979, Canberra, Australia, 377-382. Edited by W.A. Goernig, R.T. Roe and L.A. Chapman.

Chick, B.F., Coverdale, D.R. and Jackson, A.R.B. (1980). Production effects of liver fluke (Fasciola hepatica) infection in beef cattle. Australian Veterinary Journal, 56: 588-592.

Chiriboga, J., Leon, D.C., Liard, F., Velarde, A. and Gomez, D. (1976). Immunity and serum precipitins to Fasciola hepatica induced by irradiated metacercariae in rats. IRCS Medical Science Literary Compendium, 4(4): 145.

Christensen, N.Ø., Nansen, P. and Frandsen, F. (1978). Studies on heterologous resistance between Fasciola hepatica and Schistosoma mansoni in the mouse. In Fourth International Congress of Parasitology, 19-26 August, 1978, Warsaw, Poland, Short communications, Section C, 108.

Coombs, R.R.A. (1976). Immunology of Parasitic Infections. Edited by S. Cohen and E.A. Sadun. Blackwell Scientific Publications, Oxford.

Corba, J. (1973). The effect of some recent anthelmintics on Fasciola hepatica in rats. Helminthologia, 14/15 (1/4): 459-466.

Corba, J., Armour, J., Roberts, R.J. and Urquhart, G.M. (1971). Transfer of immunity to Fasciola hepatica infection by lymphoid cells. Research in Veterinary Science, 12:292-295.

Corba, J. and Spaldonova, R. (1975). Development of host resistance to Fasciola hepatica after the elimination of primary infection with diamphenethide. Folia Parasitologia (PRAHA), 22: 133-139.

Coyle, T.J. (1956). Liver fluke in Uganda. Bulletin of Epizootic Diseases of Africa, 4: 47-55.

Dargie, J.D. (1973). Fascioliasis: Immunity. In Helminth Diseases of Cattle, Sheep and Horses in Europe. Proceedings of a Symposium held at the University of Glasgow Veterinary School, Scotland (1973), 109-114. Edited by G.M. Urquhart and J. Armour.

Dargie, J.D., Armour, J., Rushton, B. and Murray, M. (1974). Immune mechanisms and hepatic fibrosis in fascioliasis. Proceedings of the Sixth International Conference for the Advancement of Veterinary Parasitology, Vienna, 249-272. Edited by E.J.L. Soulsby in Parasitic Zoonoses. Academic Press Inc., New York and London.

Dargie, J.D., Armour, J. and Urquhart, G.M. (1973). Studies on immunity to Fasciola hepatica. Parasitology, 67: xxv.

Dargie, J.D. and Berry, C.I. (1978). The influence of host nutrition on the development of fascioliasis in sheep. In Fourth International Congress of Parasitology, 19-26 August, 1978, Warsaw, Poland, Short communications, Section C, 88.

Dargie, J.D., Berry, C.I. and Parkins, J.J. (1979). The pathophysiology of ovine fascioliasis: studies on the feed intake and digestibility, body weight and nitrogen balance of sheep given rations of hay or hay plus a pelleted supplement. Research in Veterinary Science, 76: 289-295.

Davies, C., Rickard, M.D., Smyth, J.D. and Hughes, D.L. (1979). Attempts to immunise rats against infection with Fasciola hepatica using in vitro culture antigens from newly excysted metacercariae. Research in Veterinary Science, 26(2): 259-260.

Dawes, B. (1964). A preliminary study of the prospect of inducing immunity in fascioliasis by means of infections with X-irradiated metacercarial cysts and subsequent challenge with normal cysts of Fasciola hepatica L. Parasitology, 54: 369-389.

Dawson, R.M.C., Elliott, D.C., Elliott, W.H. and Jones, K.M. (1969). Data for Biochemical Research. Oxford University Press, London.

Demaree, R.S. and Hillyer, G.V. (1982). Immunoperoxidase localization of Fasciola hepatica worm tegument antigens by electron microscopy. International Journal for Parasitology, 12(2/3): 179-183.

Doy, T.G. and Hughes, D.L. (1982). Evidence for two distinct mechanisms of resistance in the rat to reinfection with Fasciola hepatica. International Journal for Parasitology, 12(4): 357-361.

Doy, T.G., Hughes, D.L. and Harness, E. (1978). Resistance of the rat to reinfection with Fasciola hepatica and the possible involvement of intestinal eosinophil leucocytes. Research in Veterinary Science, 25(1): 41-44.

Doy, T.G., Hughes, D.L. and Harness, E. (1980). The relative adherence of rat eosinophils to newly excysted Fasciola hepatica in vitro. Research in Veterinary Science, 29: 98-101.

Doy, T.G., Hughes, D.L. and Harness, E. (1981a). Hypersensitivity in rats infected with Fasciola hepatica: lack of correlation between serum reaginic antibody levels and rejection of flukes. Research in Veterinary Science, 30: 357-359.

Doy, T.G., Hughes, D.L. and Harness, E. (1981b). Hypersensitivity in rats infected with Fasciola hepatica: possible role in protection against a challenge infection. Research in Veterinary Science, 30: 360-363.

Doyle, J.J. (1971). Acquired immunity to experimental infection with Fasciola hepatica in cattle. Research in Veterinary Science, 12: 527-534.

Doyle, J.J. (1972). Evidence of an acquired resistance in calves to a single experimental infection with Fasciola hepatica. Research in Veterinary Science, 13(5): 456-459.

Doyle, J.J. (1973a). The relationship between the duration of a primary infection and the subsequent development of an acquired resistance to experimental infections with Fasciola hepatica in calves. Research in Veterinary Science, 14(1): 97-103.

Doyle, J.J. (1973b). The precipitin response induced in calves by a single experimental infection with Fasciola hepatica. Research in Veterinary Science, 15: 119-120.

Doyle, J.J. (1973c). Homocytotropic antibodies induced in calves by infection with Fasciola hepatica. Int. Arch. Allergy, 45: 744-751.

Duffus, W.P.H. and Franks, D. (1980). In vitro effect of immune serum and bovine granulocytes on juvenile Fasciola hepatica. Clinical and Experimental Immunology, 41: 430-440.

Duffus, W.P.H., Thorne, K. and Oliver, R. (1980). Killing of juvenile Fasciola hepatica by purified bovine eosinophil proteins. Clinical and Experimental Immunology, 40: 336-344.

Durbin, C.G. (1952). Longevity of the liver fluke, Fasciola sp. in sheep. Proceedings of the Helminthological Society of Washington, 19(2): 120.

Earle, W.R. (1943). Production of malignancy in vitro. IV. The mouse fibroblast cultures and changes seen in the living cell. Journal of the National Cancer Institute, 4: 165-212.

Eckblad, W.P., Woodard, L.F. and Lang, B.Z. (1981). Scanning electron microscopy of in vitro serum-mediated destruction of juvenile Fasciola hepatica. Journal of Parasitology, 67(6): 784-789.

Eriksen, L. and Flagstad, T. (1974a). Studies of immunity to Fasciola hepatica infection in vaccinated rats. In Proceedings of the Third International Congress of Parasitology, 25-31 August, 1974, Munich, 1: 491.

Eriksen, L. and Flagstad, T. (1974b). Fasciola hepatica: influence of extrahepatic adult flukes on infections and immunity in rats. Experimental Parasitology, 35: 411-417.

Ershov, S. and Poluektova, P.F. (1978). Reagin-like antibodies in rabbits infected with Fasciola hepatica. Trudy Vsesoyuznogo Instituta Gel'mintologii im, K.I. Skryabina, 24: 47-52.

Farrel, C.J., Sheer, D.T., Wescott, R.B. and Lang, B.Z. (1981). An enzyme-linked immunosorbent assay for diagnosis of Fasciola hepatica infection in cattle. American Journal of Veterinary Research, 42(2): 237-240.

Flagstad, T., Andersen, S. and Nielsen, K. (1972). The course of experimental Fasciola hepatica infection in calves with a deficient cellular immunity. Research in Veterinary Science, 13: 468-475.

Flagstad, T. and Eriksen, L. (1974a). Hepatic immunoglobulin synthesis in Fasciola hepatica infected calves. Research in Veterinary Science, 17: 59-63.

Flagstad, T. and Eriksen, L. (1974b). Local immunoglobulin synthesis in fascioliasis. In Proceedings of the Third International Congress of Parasitology, 25-31 August, 1974, Munich, 1: 490-491.

Ford, E.J.H. and Boyd, J.W. (1962). Cellular damage and changes in biliary excretion in a liver lesion of cattle. Journal of Pathology and Bacteriology, 83: 39-48.

Foster, J.R. (1981). A study of the initiation of biliary hyperplasia in rats with Fasciola hepatica. Parasitology, 83; 253-258.

Froyd, G. (1978). Fascioliasis in cattle and sheep. In Fourth International Congress of Parasitology, 19-26 August, 1978, Warsaw, Poland, Short communications, Section C, 81-82.

Furmaga, S. and Gundlach, J.L. (1978). Behaviour of morphotic blood elements and levels of iron and transferin in the blood serum of calves experimentally infected with Fasciola hepatica (Trematoda). Acta Parasitologica Polonica, 25: 179-189.

Furmaga, S. and Gundlach, J.L. (1980). Changes in the serum protein in the course of experimental fascioliasis of calves. Acta Parasitologica, Polonica, 26: 219-230.

Furmaga, S., Gundlach, J.L. and Sobieszewski, K. (1974). Studies on the behaviour of white blood cell systems in experimental fascioliasis in sheep and rabbits. In Proceedings of the Third International Congress of Parasitology, 25-31 August, 1974, Munich, 1: 497.

Glauert, A.M. and Butterworth, A.E. (1977). Morphological evidence for the ability of eosinophils to damage antibody-coated schistosomula. Transactions of the Royal Society Tropical Medicine and Hygiene, 71(5): 392-395.

Goose, J. and MacGregor, M. (1973a). Naturally acquired immunity to Fasciola hepatica in the rat . British Veterinary Journal, 129: xlix-lii.

Goose, J. and MacGregor, M. (1973b). Naturally acquired immunity to Fasciola hepatica in the rat. Parasitology, 67, xxv.

Goose, J. and MacGregor, M. (1974). Influence of age and sex on rat susceptibility to Fasciola hepatica infection. Parasitology, 69, xvii.

Gorgi, J.R. and Theodorides, V.J. (1980). Parasitology for Veterinarians. 3rd ed., 58-60. W.B. Saunders Company, Philadelphia, London and Toronto.

Gundlach, J.L. (1971). A study on the phenomena of immunity in the course of experimental fascioliasis in rabbits. Acta Parasitologica Polonica, XIX: 285-307.

Gundlach, J.L. and Sadzikowski, A. (1980). Precipitin dynamics and use of serodiagnostic reactions of immunoelectroprecipitation and double diffusion in fascioliasis in sheep. Medycyna Weterynaryjna, 36(1): 21-26.

Hammond, J.A. (1970). Studies on fascioliasis with special reference to Fasciola gigantica in East Africa. Ph.D. Thesis, University of Edinburgh.

Hanna, R.E.B. (1979). Surface antigenicity of Fasciola hepatica during development. Parasitology, 79(3): xxxi.

Hanna, R.E.B. (1980a). Fasciola hepatica: glycocalyx replacement in the juvenile as a possible mechanism for protection against host immunity. Experimental Parasitology, 50: 103-114.

Hanna, R.E.B. (1980b). Fasciola hepatica: an immunofluorescent study of antigenic changes in the tegument during development in the rat and sheep. Experimental Parasitology, 50: 155-170.

Hanna, R.E.B. (1980c). Fasciola hepatica: autoradiography of protein synthesis, transport and secretion by the tegument. Experimental Parasitology, 50; 297-304.

Hanna, R.E.B. (1980d). Further immunofluorescent studies on fascioliasis using plastic embedded antigens: antibodies in blood and bile of infected calves. Parasitology, 81: xxxv-xxxvi.

Hanna, R.E.B., Hughes, D.L. and Taylor, S.M. (1982). Fasciola hepatica: antibody levels in sheep serum before and after treatment with anthelmintic. Research in Veterinary Science, 33: 328-332.

Hanna, R.E.B. and Jura, W. (1977). Antibody response of calves to a single infection of Fasciola gigantica determined by an indirect fluorescent antibody technique. Research in Veterinary Science, 22: 339-342.

Hanna, R.E.B. and Trudgett, A.G. (1983). Fasciola hepatica: development of monoclonal antibodies and their use to characterize a glycocalyx antigen in migrating flukes. Parasite Immunology, 5: 409-425.

Harness, E., Doy, T.G. and Hughes, D.L. (1977a). Further observations on host-parasite relationships of Fasciola hepatica in the mouse. Parasitology, 75: v.

Harness, E., Doy, T.G. and Hughes, D.L. (1977b). The early migratory behaviour of young Fasciola hepatica in sensitised mice. International Journal for Parasitology, 7(1): 51-54.

Harness, E., Hughes, D.L. and Doy, T.G. (1976). The demonstration of prehepatic immune response to Fasciola hepatica in the mouse. International Journal for Parasitology, 6: 15-17.

Haroun, E.M. (1979). Studies on resistance to Fasciola hepatica in rats and rabbits. Ph.D. Thesis, University of Edinburgh.

Haroun, E.M., Hammond, J.A. and Sewell, M.M.H. (1980). Resistance to Fasciola hepatica in rats and rabbits following sensitising infection and treatment. Research in Veterinary Science, 28(3): 377-379.

Haroun, E.M., Hammond, J.A. and Sewell, M.M.H. (1981). Passive transfer of humoral resistance to Fasciola hepatica in rats and rabbits. Research in Veterinary Science, 30: 309-311.

Hawkins, C.D. and Morris, R.S. (1978). Depression of productivity in sheep infected with Fasciola hepatica. Veterinary Parasitology, 4(4): 341-352.

Hayes, T.J., Bailer, J. and Mitrovic, M. (1972). Immunity in rats to superinfection with Fasciola hepatica. The Journal of Parasitology, 58(6): 1103-1105.

Hayes, T.J., Bailer, J. and Mitrovic, M. (1973). Immunity to Fasciola hepatica in rats: the effect of two different levels of primary infection on superinfection. The Journal of Parasitology, 59(5): 810-812.

Hayes, T.J., Bailer, J. and Mitrovic, M. (1974a). Acquired immunity and age resistance in rats with chronic fascioliasis. Journal of Parasitology, 60(2): 247-250.

Hayes, T.J., Bailer, J. and Mitrovic, M. (1974b). Serum transfer of immunity to Fasciola hepatica in rats. The Journal of Parasitology, 60(4): 722-723.

Hayes, T.J., Bailer, J. and Mitrovic, M. (1974c). Studies on the serum transfer of immunity to Fasciola hepatica in rats. The Journal of Parasitology, 60(1): 930-934.

Hayes, T.J. and Mitrovic, M. (1977). The early expression of protective immunity to Fasciola hepatica in rats. The Journal of Parasitology, 63(3): 584-587.

Healy, G.R. (1955). Studies on immunity to Fasciola hepatica in rabbits. The Journal of Parasitology, 41 (Abstract): 25.

Hillyer, G.V. (1980). Isolation of Fasciola hepatica tegument antigens. Journal of Clinical Microbiology, 12(5): 695-699.

Hillyer, G.V. and Diaz, de A.L. (1976). Use of immunologic techniques from latest chemotherapeutic success in infections with Fasciola hepatica. I. Rabbit Infections. The American Journal of Tropical Medicine and Hygiene, 25(2): 307-311.

Howell, M.J. (1979). Vaccination of rats against Fasciola hepatica. The Journal of Parasitology, 65(5): 817-819.

Howell, M.J. and Sandeman, R.M. (1979). Fasciola hepatica: some properties of a precipitate which forms when metacercariae are cultured in immune rat serum. International Journal for Parasitology, 9: 41-45.

Howell, M.J., Sandeman, R.M. and Rajasekariah, G.R. (1977). In vivo and in vitro studies of the effects of immune rat serum on Fasciola hepatica. International Journal for Parasitology, 7: 367-377.

Hughes, D.L. (1962a). Reduction of the pathogenicity of Fasciola hepatica in mice by X-irradiation. Nature, 193: 1093-1094.

Hughes, D.L. (1962b). Observations on the immunology of Fasciola hepatica infections in mice and rabbits. Parasitology, 52: 4p.

Hughes, D.L. (1963). Some studies on the host-parasite relations of Fasciola hepatica. Ph.D. Thesis, University of London.

Hughes, D.L., Doy, T.G., Burden, D.J. and Oldham, G. (1982). Stage-specific immunity in the rat as demonstrated by γ-irradiated Fasciola hepatica. Parasitology, 84, xiii.

Hughes, D.L., Hanna, R.E.B. and Doy, T.G. (1982). Antibody response in cattle, sheep and rats to infection with γ-irradiated metacercariae of Fasciola hepatica. Research in Veterinary Science, 32: 354-358.

Hughes, D.L., Hanna, R.E.B. and Symonds, H.W. (1981). Fasciola hepatica: IgG and IgA levels in the serum and bile of infected cattle. Experimental Parasitology, 52, 271-279.

Hughes, D.L., Harness, E. and Doy, T.G. (1976). The establishment and duration of Fasciola hepatica infections in two strains of rats and the development of acquired resistance. Research in Veterinary Science, 20: 207-211.

Hughes, D.L., Harness, E. and Doy, T.G. (1981a). The different stages of Fasciola hepatica capable of inducing immunity and the susceptibility of various stages to immunological attack in the sensitised rat. Research in Veterinary Science, 30, 93-98.

Hughes, D.L., Anderson, J.C. and Harness, E. (1976). Fasciola hepatica: The effect of challenge by the subcutaneous or intraperitoneal route with living adult flukes in sensitised rats. Experimental Parasitology, 40(3): 355-362.

Isseroff, H., Girard, P.R. and Leve, M.D. (1977). Fasciola hepatica: bile duct enlargement induced in rats after intraperitoneal transplantation. Experimental Parasitology, 41(2): 405-409.

Jamuar, M.P. and Lewert, R.M. (1967). Effect of immune serum on the miracidial surface of Schistosoma japonicum. The Journal of Parasitology, 53(1): 220.

Jarrett, W.F.H., Jennings, F.W., McIntyre, W.I.M., Mulligan, W., Sharp, N.C.C. and Urquhart, G.M. (1959). Practical vaccination against helminth diseases. Reports of the 16th International Veterinary Congress, Madrid, II: 567-569.

Kelly, J.D. and Campbell, N.J. (1979). The effect of route of infection on acquired resistance to Fasciola hepatica in the rat and sheep. Research in Veterinary Science, 27(2): 205-209.

Kelly, J.D., Campbell, N.J. and Dineen, J.K. (1980). The role of the gut in acquired resistance to Fasciola hepatica in the rat. Veterinary Parasitology, 6(4): 359-367.

Kendall, S.B. (1967). Host specificity as evidenced by species of Fasciola. Helminthologia, 8: 223-233.

Kendall, S.B. (1973). The chemotherapy of fascioliasis. Helminthologia, 14/15(1/4): 467-473.

Kendall, S.B., Herbert, N., Parfitt, J.W. and Pierce, M.A. (1967). Resistance to reinfection with Fasciola hepatica in rabbits. Experimental Parasitology, 20: 242-247.

Kendall, S.B. and Sinclair, I.J. (1971). Barriers to reinfection with Fasciola hepatica in the rabbit. Research in Veterinary Science, 12: 74-79.

Kendall, S.B., Sinclair, I.J., Everett, G. and Parfitt, J.W. (1978). Resistance to Fasciola hepatica in cattle. I. Parasitological and serological observations. Journal of Comparative Pathology, 88: 115-122.

Knight, R.A. (1980a). Relationship of globule leukocytes to infections with Fasciola hepatica in sheep. Journal of Parasitology, 66(5): 844-845.

Knight, R.A. (1980b). Response of lambs to challenge infections after repeated inoculations with Fasciola hepatica cysts. Proc. Helminth. Society Washington, 47(2): 186-191.

Kozar, M. (1974). The effect of immunization of the final host with intermediate host antigens on the development of Fasciola hepatica and the course of fascioliasis in rats. Acta Parasitologica Polonica, XXII (fasc. 44), 473-483.

Lang, B.Z. (1966). Host-parasite relationships of Fasciola hepatica in the white mouse. I. Response to a primary infection. The Journal of the Mitchell Science Society, 82: 195-203.

Lang, B.Z. (1967). Host-parasite relationships of Fasciola hepatica in the white mouse. II. Studies on acquired immunity. The Journal of Parasitology, 53(1): 21-30.

Lang, B.Z. (1968a). Acquired immunity to Fasciola hepatica in the laboratory white mouse. The American Journal of Tropical Medicine and Hygiene, 17(4): 561-567.

Lang, B.Z. (1968b). Host-parasite relationships of Fasciola hepatica in the white mouse. III. Worm transfer. Proc. Oklahoma Acad. Sciences, 47: 81-84.

Lang, B.Z. (1974a). Host-parasite relationships of Fasciola hepatica in the white mouse. V. Age of fluke responsible for the induction of acquired immunity. The Journal of Parasitology, 60(1): 90-92.

Lang, B.Z. (1974b). Host-parasite relationship of Fasciola hepatica. VI. Studies on the effects of immune and normal sera on the viability of young worms transferred to normal recipients. The Journal of Parasitology, 60: 925-929.

Lang, B.Z. (1976). Host-parasite relationships of Fasciola hepatica in the white mouse. VII. Effects of anti-worm incubate sera on transferred worms and successful vaccination with a crude incubate antigen. The Journal of Parasitology, 62(2): 232-236.

Lang, B.Z. and Dronen, N.O. (1972). Host-parasite relationships of Fasciola hepatica in the white mouse. IV. Studies on worm transfer and the induction of acquired immunity by worms of different ages. The Journal of Parasitology, 58(1): 84-87.

Lang, B.Z. and Hall, R.F. (1977). Host-parasite relationships of Fasciola hepatica in the white mouse. VIII. Successful vaccination with culture incubate antigens and antigens from sonic disruption of immature worms. The Journal of Parasitology, 63(6): 1046-1049.

Lang, B.Z., Larsh, J.E., Weatherly, N.F. and Goulson, H.T. (1967). Demonstration of immunity to Fasciola hepatica in recipient mice given peritoneal exudate cells. The Journal of Parasitology, 53(1): 208-209.

Lehner, R.P. (1977). In vitro culture of Fasciola hepatica and the immunology associated with the metabolic products of the trematode. Ph.D. Thesis, University of Edinburgh.

Lehner, R.P. and Sewell, M.M.H. (1980). A study of the antigens produced by adult Fasciola hepatica maintained in vitro. Parasite Immunology, 2(2): 99-109.

Leimbacher, F. (1978). Experience with the 'MT' system of forecasting fascioliasis in France. In World Meteorological Organization. Technical Note No. 159, Weather and Parasitic Disease, 6-13. Edited by T.E. Gibson.

Meek, A.H. and Morris, R.S. (1979). The effect of prior infection with Fasciola hepatica on the resistance of sheep to the same parasite. Australian Veterinary Journal, 55(2): 61-64.

Mitchell, G.B.B., Armour, J., Ross, J.G. and Halliday, W.G. (1981). Successful passive transfer of resistance to Fasciola infection in rats by immune serum and transfer factor. Research in Veterinary Science, 30(2): 246-247.

Movsesijan, M., Jovanovic, B., Aalund, O. and Nansen, P. (1975). Immune response of sheep to Fasciola hepatica infection. Research in Veterinary Science, 18: 171-174.

Nansen, P. (1974). Studies on the resistance to Fasciola hepatica in calves vaccinated with attenuated metacercariae. In Proceedings of the Third International Congress of Parasitology, 25-31 August, 1974, Munich, 1: 496.

Nansen, P. (1975). Resistance in cattle to Fasciola hepatica induced by γ-ray attenuated larvae: results from a controlled field trial. Research in Veterinary Science 19(3): 278-283.

Oldham, G. (1983a). Protection against Fasciola hepatica in rats with adult fluke antigen in Freund's adjuvant: influence of antigen batch, antigen dose and number of sensitising injections. Research in Veterinary Science, 34(2): 240-244.

Oldham, G. (1983b). Antibodies to Fasciola hepatica antigens during experimental infections in cattle measured by ELISA. Veterinary Parasitology, 13: 151-158.

Oldham, G. and Hughes, D.L. (1982). Fasciola hepatica: immunisation of rats by intraperitoneal injection of adult fluke antigen in Freund's adjuvant. Experimental Parasitology, 54: 7-11.

Oliver-Gonzales, J. (1940). The in vitro action of immune serum on the larvae and adults of Trichinella spiralis. Journal of Infectious Diseases, 67: 292-300.

Over, H.J. (1974). Reinfections with F. hepatica in cattle. In Proceedings of the Third International Congress of Parasitology, 25-31 August, 1974, Munich, 1: 499-500.

Perez, H.A. and Smithers, S.R. (1977). Schistosoma mansoni in the rat: the adherence of macrophages to schistosomula in vitro after sensitization with immune serum. International Journal for Parasitology, 7: 315-320.

Pullan, N.B. (1968). Acute fascioliasis in sheep. M.Sc. Thesis, University of Edinburgh.

Pullan, N.B., Sewell, M.M.H. and Hammond, J.A. (1970). Studies on the pathogenicity of massive infections of Fasciola hepatica L. in lambs. British Veterinary Journal, 126: 543-547.

Rajasekariah, G.R. and Howell, M.J. (1977a). The fate of Fasciola hepatica metacercariae following challenge infection of immune rats. Journal of Helminthology, 51(4): 289-294.

Rajasekariah, G.R. and Howell, M.J. (1977b). Fasciola hepatica in rats. Effects of age and infective dose. International Journal for Parasitology, 7: 119-121.

Rajasekariah, G.R. and Howell, M.J. (1978a). Acquired immunity to the trematode Fasciola hepatica in rats. Australian Journal of Experimental Biology and Medical Sciences, 56(6): 747-756.

Rajasekariah, G.R. and Howell, M.J. (1978b). Immunity to Fasciola hepatica in rats. In Fourth International Congress of Parasitology, 19-26 August, 1978, Warsaw, Poland, Short communications, Section E, 125.

Rajasekariah, G.R. and Howell, M.J. (1980). Enzyme assay for detecting resistance to reinfection with the liver fluke Fasciola hepatica in rats. Research in Veterinary Science, 29: 124-125.

Rajasekariah, G.R. and Howell, M.J. (1981). Age-associated responses in susceptible and resistant rats to infection with Fasciola hepatica. International Journal for Parasitology, 11: 59-65.

Rajasekariah, G.R., Mitchell, G.F., Chapman, C.B. and Montague, P.E. (1979). Fasciola hepatica: attempts to induce protection against infection in rats and mice by injection of excretory/secretory products of immature worms. Parasitology, 79: 393-400.

Ramaniuk, K. (1978). Influence of Fasciola hepatica invasion on the reproduction of its final host. In Fourth International Congress of Parasitology, 19-26 August, 1978, Warsaw, Poland, Short Communications, Section F, 22-23.

Ray, R.J. (1970). Studies on the effect of various anthelmintics upon Fasciola hepatica in albino rats. Research in Veterinary Science, 11: 1-6.

Reid, J.F.S., Armour, J., Urquhart, G.M. and Jennings, F.W. (1970). Studies on ovine fascioliasis. I. Observations on the sequential development of the naturally-acquired disease. The Veterinary Record, 86: 242-249.

Reid, J.F.S., Doyle, J.J., Armour, J. and Jennings, F.W. (1972). Fasciola hepatica infection in cattle. Veterinary Record, 90: 486-487.

Roseby, F.B. (1970). The effect of fascioliasis on the wool production of merino sheep. Australian Veterinary Journal, 46: 361-365.

Ross, J.G. (1964). Experimental infection of cattle with Fasciola hepatica. In Proceedings of the First International Congress of Parasitology, 21-26 September, 1964, Rome, 2: 885-886. Edited by Augusto Corradetti.

Ross, J.G. (1965a). Experimental infections of cattle with Fasciola hepatica: a comparison of low and high infection rates. Nature (London), 208(5013): 907.

Ross, J.G. (1965b). Acute fascioliasis in sheep. The Veterinary Record, 77(10): 289-290.

Ross, J.G. (1966a). Studies of immunity to Fasciola hepatica. Naturally acquired immunity in rabbits. British Veterinary Journal, 122: 209-211.

Ross, J.G. (1966b). Fascioliasis in Northern Ireland cattle. Irish Veterinary Journal, 20: 131-134.

Ross, J.G. (1966c). An abattoir survey of cattle liver infections with Fasciola hepatica. British Veterinary Journal, 122: 489-494.

Ross, J.G. (1966d). Experimental infections of cattle with Fasciola hepatica: challenge infections. Nature, 212(5069): 1464-1465.

Ross, J.G. (1967a). Studies of immunity to Fasciola hepatica: acquired immunity in cattle, sheep and rabbits following natural infection and vaccine procedures. Journal of Helminthology, XLI(4): 393-399.

Ross, J.G. (1967b). Experimental infections of cattle with Fasciola hepatica: the production of an acquired self cure by challenge infection. Journal of Helminthology, XLI(2/3): 223-228.

Ross, J.G. (1967c). An epidemiological study of fascioliasis in sheep. The Veterinary Record, 80(6): 214-217.

Ross, J.G. (1967d). A further season of epidemiological studies of Fasciola hepatica infections in sheep. The Veterinary Record, 80(12): 368-371.

Ross, J.G. (1967e). Experimental infections of cattle with Fasciola hepatica: high level single infections in calves. Journal of Helminthology, XLI(2/3): 217-222.

Ross, J.G. (1967f). A comparison of the resistance status of hosts to infection with Fasciola hepatica. Proceedings of the Third International Conference of the World Association for the Advancement of Veterinary Parasitology, Lyon, France. Veterinary Medical Review, 96-105. Edited by E.J.L. Soulsby.

Ross, J.G. (1968). The life span of Fasciola hepatica in cattle. The Veterinary Record, 82: 587-589.

Ross, J.G. and Dow, C. (1966). The problem of acute fascioliasis in cattle. The Veterinary Record, 78: 670.

Ross, J.G. and Todd, J.R. (1968). Epidemiological studies of fascioliasis. A third season of comparative status with cattle. The Veterinary Record, 82: 695-699.

Ross, J.G., Todd, J.R. and Dow, C. (1966). Single experimental infections of calves with the liver fluke Fasciola hepatica (Linnaeus, 1758). Journal of Comparative Pathology, 76: 67-81.

Rushton, B., Murray, M., Armour, J. and Dargie, J.D. (1974). The pathology of primary and reinfection lesions of fascioliasis in the ovine liver. In Proceedings of the Third International Congress of Parasitology, 25-31 August, 1974, Munich, 1:498-499.

Sabesin, S.M. (1963). A function of the eosinophil: phagocytosis of antigen-antibody complexes. Proc. Society of Experimental Biology and Medicine, 112: 667-670.

Sandeman, R.M. and Howell, M.J. (1980). In vitro studies of the response of sheep to infection with Fasciola hepatica. Veterinary Parasitology, 6: 347-357.

Sandeman, R.M. and Howell, M.J. (1981). Response of sheep to challenge infection with Fasciola hepatica. Research in Veterinary Science, 30: 294-297.

Sandeman, R.M., Howell, M.J. and Campbell, N.J. (1980). An attempt to vaccinate sheep against Fasciola hepatica using a juvenile fluke antigen sheep antibody complex. Research in Veterinary Science, 29(2): 255-259.

Sarles, M.P. (1937). The in vitro action of immune rat serum on Nippostrongylus muris (Nematoda). The Journal of Parasitology, 23 (Abstract), 560-561.

Sarles, M.P. (1938). The in vitro action of immune rat serum on the nematode, Nippostrongylus muris. Journal of Infectious Diseases, 62: 337-348.

Sarles, M.P. (1939). Protective and curative action of immune serum against Nippostrongylus muris in the rat. Journal of Infectious Diseases, 65: 183-195.

Sewell, M.M.H. (1961). The immunology of fascioliasis with special reference to its relationship to the disease process. Ph.D. Thesis, University of Cambridge.

Sewell, M.M.H. (1964). The immunology of fascioliasis. II. Qualitative studies on the precipitin reaction. Immunology, 7: 671-680.

Sewell, M.M.H. (1966). The pathogenesis of fascioliasis. The Veterinary Record, 78(3): 98-105.

Sewell, M.M.H. (1967). Serum enzyme activities in acute ovine fascioliasis. The Veterinary Record, 80: 577-578.

Sewell, M.M.H. (1976). The role of management in the control of helminth disease. In Beef Cattle Production in Developing Countries, 138-149. Edited by A.J. Smith. Lewis Reprints Ltd., Tonbridge.

Sewell, M.M.H. and Hammond, J.A. (1972). The detection of Fasciola eggs in faeces. Veterinary Record, 90: 510-511.

Sher, A. (1976). Complement-dependent adherence of mast cells to schistosomula. Nature (London), 263(5575): 334-336.

Sinclair, I.J. and Wassal, D.A. (1981). The peroxidase anti-peroxidase technique for the demonstration of immunoglobulin G in rat livers infected with Fasciola hepatica. Journal of Comparative Pathology, 91: 573-579.

Sinclair, K.B. (1962). Observations on the clinical pathology of ovine fascioliasis. British Veterinary Journal, 118: 37-53.

Sinclair, K.B. (1964). Some observations on ovine fascioliasis. In Proceedings of the First International Congress of Parasitology, 21-26 September, 1964, Rome, 2: 884-885. Edited by Augusto Corradetti.

Sinclair, K.B. (1971). Acquired resistance to Fasciola hepatica in sheep. British Veterinary Journal, 127: 125-136.

Sinclair, K.B. (1973). The resistance of sheep to Fasciola hepatica: studies on the development and pathogenicity of challenge infections. British Veterinary Journal, 129: 236-249.

Sinclair, K.B. (1975a). Pathophysiological studies on sheep exposed to challenge infections with Fasciola hepatica. In Facts and Reflections. II. Workshop on Fascioliasis, Parasit. Dept. Cert. Diergeneeskundig Inst., Lelystad, Netherlands, 23-24 May, 1975, Rotterdam, The Netherlands, 55-57. Edited by H.J. Over and J. Armour.

Sinclair, K.B. (1975b). The resistance of sheep to Fasciola hepatica: studies on the pathophysiology of challenge infections. Research in Veterinary Science, 19: 296-303.

Sirag, S.B., Christensen, N.Ø., Nansen, P., Monrad, J. and Frandsen, F. (1981). Resistance to Fasciola hepatica in calves harbouring primary patent Schistosoma bovis infections. Journal of Helminthology, 55: 63-70.

Smyth, J.D. (1962). Introduction to Animal Parasitology, 1st ed. The English University Press Ltd., London.

Spedding, C.R.W. (1952). Variation in the egg content of sheep faeces within one day. Journal of Helminthology, XXVI(2/3): 71-86.

Sokal, R.R. and Rohlf, F.J. (1981). Biometry. The Principles and Practice of Statistics in Biological Research. 2nd ed. W.H. Freeman and Company, San Francisco, U.S.A.

Sokolic, A. (1967). Preliminary studies of irradiated and normal metacercariae of Fasciola hepatica in mice. In Proceedings of the Third International Conference of the World Association for the Advancement of Veterinary Parasitology, Lyons, France. Veterinary Medical Review, 258-263. Edited by E.J.L. Soulsby.

Sokolic, A. (1968). Immunological studies of Fasciola hepatica. Clinical and immunological response in mice to infection with irradiated and normal fluke cysts. In Isotope and Radiation in Parasitology. Proceedings of Panel Meeting 31 July-4 August, 1967, 93-103. International Atomic Energy, Vienna.

Sokolic, A. (1971). Note on host resistance stimulated by irradiated parasitic forms. Comptes Rendus 1er Multicolloque Europeen de Parasitologie, France, 99-103. Edited by J.M. Doby.

Sokolic, A., Sofrenovic, D., Sibalic, S., Cuperlovic, K., Dordevic, M. and Lepojev, O. (1968). The response of albino rats to infection with irradiated and non-irradiated metacercariae of Fasciola hepatica. Acta Veterinaria Beograd, 18: 187-201.

Soulsby, E.J.L. (1982). Helminths, Arthropods and Protozoa of Domesticated Animals. 7th edition. Bailliere, Tindall and Cassel, London.

Standen, O.D. (1952). The in vitro effect of normal and immune serum upon the cercariae of Schistosoma mansoni. Journal of Helminthology, XXXI(3): 25-42.

Stirewalt, M.A. and Evans, A.S. (1953). An unsuccessful attempt to protect mice against Schistosoma mansoni by transfer of immune rat serum. Proceedings of the Helminthological Society of Washington, 20(1): 15-19.

Sykes, A.R., Coop, R.L. and Robinson, M.G. (1980). Chronic subclinical ovine fascioliasis: plasma glutamate dehydrogenase, gamma-glutamyltranspeptidase and aspartate aminotransferase activities and their significance as diagnostic aids. Research in Veterinary Science, 28: 71-75.

Taylor, E.L. (1949). The epidemiology of fascioliasis in Britain. Report of the 14th International Veterinary Congress, London, 2: 81-87.

Thompson, R.C.A. and Howell, M.J. (1979). Effect of BCG on the resistance of rats to infection with Fasciola hepatica. Zeitschrift fur Parasitenkunde, 61: 93-98.

Thorpe, E. (1965). Liver damage and the host-parasite relationship in experimental fascioliasis in the albino rat. Research in Veterinary Science, 6: 498-509.

Thorpe, E. and Broome, A.W.J. (1962). Immunity to Fasciola hepatica infection in albino rats vaccinated with irradiated metacercariae. The Veterinary Record, 74: 755-756.

Thorson, R.E. (1954). Effect of immune serum from rats on infective larvae of Nippostrongylus muris. Experimental Parasitology, 3: 9-15.

Todd, R.G. and Wade, A. (1979). The Pharmaceutical Codex. 11th edition, 409-411. The Pharmaceutical Press, London.

Urquhart, G.M. (1954). The rabbit as host in experimental fascioliasis. Experimental Parasitology, 3: 38-44.

Urquhart, G.M., Mulligan, W. and Jennings, F.W. (1954). Artificial immunity to Fasciola hepatica in rabbits. 1. Some studies with protein antigens of F. hepatica. The Journal of Infectious Diseases, 94: 126-133.

Vadas, M.A., David, J.R., Butterworth, A., Pisani, N.T. and Siongok, T.A. (1979). A new method for the purification of the human eosinophils and neutrophils, and a comparison of the ability of these cells to damage schistosomula of Schistosoma mansoni. The Journal of Immunology, 122(4): 1228-1236.

Warburgh, O. and Christian, B.Z. (1941). Protein estimation by extinction at 260 nm and 280 nm. In Data for Biochemical Research, 625-626. Edited by R.M.C. Dawson, D.C. Elliott, W.H. Elliott and K.M. Jones. Clarendon Press, Oxford.

Webster, C.C. and Wilson, P.N. (1980). Agriculture in the Tropics. In Tropical Agricultural Series, 2nd edition. Edited by D. Rhind. William Clowes and Sons Ltd., London and Beccles.

WHO Technical Report (1977). The role of immune complexes in disease. World Health Organization Technical Report Series 606: 5-58.

Wikerhauser, T. (1961a). On the effect of roentgen radiation upon metacercariae of Fasciolagigantica. Veterinarski Arhiv, 31: 229-236.

Wikerhauser, T. (1961b). Immunobiologic diagnosis of fascioliasis. II. The in vitro action of immune serum on the young parasitic stage of F. hepatica - a new precipitin test for fascioliasis. Veterinarski Arhiv, 31: 71-79.

Appendix Table 3.1

Faecal egg counts (e.p.g.) from rats infected with 20 metacercariae of F. hepatica

Rat No.	Weeks after infection									
	6	7	8	10	12	14	16	18+	19	20
1	0	0	36	-	62	356	369	483	0	0
2	0	0	35	123	137	94	294	216	5	0
3	0	3	58	90	D					
4	0	3	69	349	120	56	554	357	0	0
6	0	0	20	94	194	199	D			
7	0	0	11	59	10	409	409	109	1	0
8	0	-	-	42	475	194	381	308	0	0
9	0	-	-	16	22	122	56	90	0	0
10	0	0	0	75	160	43	190	237	0	0
11	0	0	21	27	0	0	0	-	0	0
13	0	0	4	56	477	25	73	64	0	0
14	0	2	41	95	66	0	170	113	0	0
15	0	-	-	5	414	208	204	379	39	24
16	0	0	1	0	0	0	6	2	0	0
17	0	9	89	140	-	136	132	3	0	0
18	0	0	0	35	338	239	248	611	0	0
19	0	1	40	-	255	51	445	163	0	0
20	0	0	0	24	53	22	85	0	0	0
21	0	2	46	228	287	158	335	238	0	0
22	0	2	41	84	23	116	78	37	0	0
23	0	0	9	36	225	202	353	251	0	0
24	0	0	14	2	302	206	390	171	0	0
25	0	0	24	453	841	186	408	349	0	0
26	0	0	3	63	179	241	300	94	0	0
28	0	0	0	10	142	28	190	168	0	0
29	0	0	-	43	104	78	596	27	0	0
39	0	3	52	117	192	315	532	90	0	0

+treatment with hexachlorophene

- no sample

D Animal died

Appendix Table 3.2

ELISA values (units of absorbance) for bullock 89, infected twice with 1000 metacercariae of Fasciola hepatica, using somatic antigen derived from different animal hosts. Serum dilution 1:1000, conjugate dilution 1:2000, antigen concentration of $5\mu g^{ml-1}$ and OPD in the substrate.

Source antigen	Bullock serum	Replicates 1	2	3	4	5	6	Mean ± SD
Cattle	Normal	0.49	0.62	0.77	0.70	0.75		0.67 ± 0.11
	Positive+	1.45	1.47	1.20	1.38	1.41		1.38 ± 0.11
	Saline	0.22	0.25	0.37	0.26	0.27		0.27 ± 0.06
Rat	Normal	0.30	0.27	0.28	0.29	0.30	0.28	0.29 ± 0.01
	Positive+	1.10	1.11	1.11	1.10	1.10	1.11	1.10 ± 0.01
	Saline	0.17	0.14	0.15	0.16	0.22	0.21	0.17 ± 0.03
Rabbit	Normal	0.07	0.07	0.07	0.07			0.07 ± 0.00
	Positive+	0.31	0.37	0.30	0.33			0.33 ± 0.03
	Saline	0.05	0.04	0.04	0.04			0.04 ± 0.01

+obtained 10 weeks after second infection

Appendix Table 3.3.1

Titres of all available sera from bullock 89 using somatic antigen at 10 μg^{ml-1}, serum dilution 1:1000, conjugate dilution 1:1000.

Week p.i. Bullock (primary)	Absorbance	Week p.i. Bullock (secondary)	Absorbance
0	0.15	2	0.30
2	0.27	4	0.44
4	0.33	6	0.43
6	0.33	8	0.43
8	0.34	10	0.37
10	0.36	12	0.36
12	0.31	14	0.38
14	0.32	16	0.35
16	0.36	18	0.36
18	0.35	20	0.35
20	0.33	saline	0.10
21	0.21		

Appendix Table 3.3.2

The effect of varying the concentration of somatic antigen in the assay of bullock serum with conjugate dilution 1:1000, serum dilution 1:1000

Ag conc. (μg^{ml-1})	Absorbance			
	P1	P2	Normal serum	Carbonate buffer
2.5	0.34	0.24	0.13	0.05
5	0.34	0.26	0.14	0.06
10	0.36	0.27	0.15	0.07
20	0.37	0.29	0.17	0.08
50	0.37	0.29	0.19	0.10
100	0.39	0.31	0.20	0.11
carbonate buffer	0.04	0.04	0.04	0.02

P1 (high titre) - week 10 (secondary)
P2 (low titre) - week 21 (primary)

Appendix Table 3.3.3

The effect of varying the serum dilution in the assay of bullock serum with conjugate dilution 1:1000, somatic antigen concentration 10 μg^{ml-1}.

Serum dilution	Absorbance		
	P1	P2	Normal serum
1:250	0.61	0.50	0.36
1:500	0.52	0.42	0.31
1:1000	0.45	0.37	0.24
1:1500	0.38	0.30	0.20
1:2000	0.37	0.27	0.18
saline	0.06	0.07	0.04

Appendix Table 3.3.4

The effect of varying the dilution of enzyme labelled anti-immunoglobulin in the assay of bullock serum using somatic antigen at 10 μg^{ml-1} and serum dilution 1:1000.

Conjugate dilution	Absorbance			
	P1	P2	Normal serum	PBS/Tween
1:500	0.50	0.37	0.26	0.15
1:1000	0.42	0.33	0.23	0.12
1:1500	0.41	0.29	0.21	0.11
1:2000	0.37	0.25	0.14	0.09
1:3000	0.33	0.23	0.14	0.06
PBS/Tween	0.02	0.03	0.04	0.03

Appendix Table 3.4.1

Titres of all available sera of a group of rats previously infected and treated with hexachlorophene and reinfected. Metabolic antigen 5 μg^{ml-1}, serum dilution 1:1000, conjugate dilution 1:1000.

Week p.i. rat (primary)	Absorbance	Week p.i. rat (secondary)	Absorbance
0	0.12	2	0.46
2	0.24	4	0.49
4	0.32	6	0.44
6	0.38	8	0.40
8	0.40	10	0.38
10	0.36	12	0.37
12	0.39	14	0.32
14	0.38	16	0.29
16	0.36	18	0.28
18	0.38	20	0.29
saline	0.09		

Appendix Table 3.4.2

The effect of varying the concentration of metabolic antigen in the assay of rat serum with conjugate dilution 1:1000, serum dilution 1:1000

Ag conc. μg^{ml-1}	Absorbance			
	P1	P2	Normal serum	Carbonate buffer
1	0.06	0.04	0.03	0.01
2.5	0.10	0.08	0.07	0.01
5	0.27	0.12	0.07	0.01
10	0.28	0.14	0.10	0.02
20	0.33	0.19	0.15	0.08
carbonate buffer	0.02	0.02	0.02	0.01

P1 (high titre) - week 12 (secondary)
P2 (low titre) - week 2 (primary)

Appendix Table 3.4.3

The effect of varying the serum dilution in the assay of rat serum with a conjugate dilution 1:1000, metabolic antigen concentration 5 μg^{ml-1}.

Serum dilution	Absorbance		
	P1	P2	Normal serum
1:250	0.51	0.35	0.19
1:500	0.44	0.31	0.17
1:1000	0.37	0.29	0.15
1:1500	0.33	0.27	0.14
1:2000	0.30	0.28	0.16
saline	0.14	0.14	0.09

Appendix Table 3.4.4

The effect of varying the dilution of enzyme-labelled anti-immunoglobulin in the assay of rat serum using metabolic antigen at 5 μg^{ml-1} and serum dilution 1:500.

Conjugate dilution	Absorbance			
	P1	P2	Normal serum	PBS/Tween
1:250	0.41	0.35	0.24	0.12
1:500	0.35	0.25	0.17	0.07
1:1000	0.26	0.18	0.13	0.05
1:1500	0.26	0.18	0.13	0.06
1:2000	0.21	0.16	0.08	0.04
1:3000	0.17	0.10	0.07	0.05
PBS/Tween	0.01	0.01	0.01	0.01

Appendix Table 3.5.1

The effect of varying the concentration of metabolic antigen in the assay of bullock (89) serum with conjugate dilution 1:2000, serum dilution 1:1000.

Ag conc. $\mu g ml^{-1}$	Absorbance			
	P1	P2	Normal serum	Carbonate buffer
0.5	0.27	0.24	0.25	0.11
1	0.31	0.24	0.25	0.11
1.5	0.35	0.25	0.25	0.12
2.5	0.39	0.29	0.27	0.13
5	0.41	0.29	0.26	0.13
10	0.47	0.37	0.31	0.16
20	0.56	0.43	0.31	0.17
carbonate buffer	0.21	0.22	0.23	0.10

Appendix Table 3.5.2

The effect of varying the serum dilution in the assay of bullock serum with conjugate dilution 1:2000, metabolic antigen concentration 20 μg^{ml-1}.

Serum dilution	Absorbance		
	P1	P2	Normal serum
1:250	0.36	0.36	0.24
1:500	0.28	0.25	0.19
1:1000	0.21	0.24	0.14
1:1500	0.25	0.22	0.15
1:2000	0.20	0.19	0.11
saline	0.13	0.13	0.07

Appendix Table 3.5.3

The effect of varying the dilution of enzyme-labelled anti-immunoglobulin in the assay of bullock serum using metabolic antigen 20 μg^{ml-1} and serum dilution 1:1000.

Conjugate dilution	Absorbance			
	P1	P2	Normal serum	PBS/Tween
1:250	0.59	0.54	0.53	0.50
1:500	0.50	0.44	0.37	0.36
1:1000	0.40	0.30	0.28	0.25
1:1500	0.33	0.28	0.22	0.16
1:2000	0.35	0.25	0.21	0.16
1:3000	0.28	0.21	0.15	0.09
PBS/Tween	0.05	0.06	0.06	0.06

P1 (high titre) - week 10 (secondary)
P2 (low titre) - week 21 (primary)

Appendix Table 5.1.1

Mean ELISA values (units of absorbance) from bullocks repeatedly infected with 1,000 metacercariae of F. hepatica, using somatic antigen in the test

Bullock No.	Weeks after primary infection											
	0*	2	4	6	8	10	12	14	16	18	20	22
89	0.52±0.03	0.59±0.01	0.63±0.02	0.67±0.06	0.62±0.02	0.63±0.01	0.61±0.05	0.60±0.02	0.57±0.07	0.54±0.01	0.58±0.01	0.51±0.02
94	0.47±0.04	0.68±0.01	0.63±0.02	0.61±0.02	0.63±0.04	0.60±0.04	0.61±0.01	0.55±0.02	0.55±0.03	0.60±0.02	0.51±0.04	0.47±0.02

	Weeks after secondary infection										
	2	4	6	8	10	12	14	16	18	20	22
89	0.61±0.02	0.51±0.02	0.75±0.01	0.63±0.01	0.70±0.01	0.76±0.03	0.74±0.03	0.73±0.03	0.74±0.01	0.72±0.01	0.70±0.01
94	0.72±0.02	0.70±0.01	0.71±0.02	0.71±0.01	0.70±0.04	0.69±0.02	0.76±0.03	0.65±0.01	0.68±0.02	0.66±0.01	0.69±0.04

	Weeks after tertiary infection									
	2	4	6	8	10	12	14	16	18	20
89	0.74±0.02	0.71±0.02	0.74±0.02	0.67±0.01	0.66±0.02	0.68±0.01	0.68±0.01	0.69±0.01	0.71±0.01	0.66±0.01
94	0.68±0.03	0.64±0.02	0.69±0.02	0.72±0.01	0.66±0.02	0.65±0.01	0.65±0.01	0.65±0.02	0.68±0.02	0.66±0.01

*Administration of infection

Appendix Table 5.1.2

Mean ELISA values (units of absorbance) from bullocks repeatedly infected with 1,000 metacercariae of F. hepatica, using metabolic antigen in the test

Bullock No.	Weeks after primary infection											
	0*	2	4	6	8	10	12	14	16	18	20	22
89	0.17±0.02	0.19±0.01	0.23±0.01	0.22±0.02	0.24±0.03	0.24±0.01	0.25±0.01	0.24±0.01	0.22±0.02	0.22±0.02	0.24±0.01	0.22±0.01
94	0.16±0.03	0.28±0.01	0.28±0.01	0.27±0.02	0.28±0.01	0.32±0.02	0.25±0.01	0.22±0.02	0.25±0.01	0.25±0.02	0.25±0.01	0.29±0.04

	Weeks after secondary infection										
	2	4	6	8	10	12	14	16	18	20	22
89	0.26±0.02	0.27±0.01	0.33±0.02	0.29±0.03	0.29±0.02	0.31±0.01	0.31±0.01	0.33±0.02	0.36±0.01	0.36±0.01	0.41±0.02
94	0.33±0.04	0.31±0.01	0.32±0.03	0.36±0.04	0.33±0.01	0.25±0.01	0.29±0.01	0.26±0.02	0.29±0.03	0.29±0.02	0.32±0.01

	Weeks after tertiary infection									
	2	4	6	8	10	12	14	16	18	20
89	0.39±0.01	0.34±0.03	0.36±0.02	0.32±0.02	0.51±0.01	0.52±0.01	0.34±0.01	0.39±0.01	0.35±0.01	0.35±0.02
94	0.32±0.01	0.33±0.01	0.36±0.01	0.35±0.03	0.35±0.01	0.36±0.05	0.36±0.02	0.42±0.01	0.39±0.05	0.39±0.01

*Administration of infection

Appendix Table 5.2.1.1

Mann-Whitney U-test analysis of flukes recovered at post-mortem as shown in Table 5.2.1.

Group comparison	U-value	Probability
A1 v A2	47.5	>.10
A1 v A3	86.5	<.001
A1 v A4	75.0	<.001
A2 v A3	97.0	<.001
A2 v A4	85.5	<.001
A3 v A4	47.0	>.10
B1 v B2	47.5	>.10
B1 v B3	64.0	<.10
B1 v B4	73.5	<.025
B2 v B3	79.0	<.025
B2 v B4	87.0	<.05
B3 v B4	64.5	>.10
A1 v B1	51.0	>.10
A1 v B2	63.0	<.10
A1 v B3	82.5	<.001
A1 v B4	83.5	<.001
A2 v B1	61.0	>.10
A2 v B2	70.5	<.10
A2 v B3	94.0	<.001
A2 v B4	95.5	<.001
A3 v B1	76.5	<.01
A3 v B2	86.0	<.05
A3 v B3	74.5	<.05
A3 v B4	52.5	>.10
A4 v B1	73.5	<.025
A4 v B2	76.5	<.01
A4 v B3	62.0	>.10
A4 v B4	48.5	>.10
1 v 2	62.5	<.10
1 v 3	53.0	>.10
1 v 4	45.5	>.10
2 v 3	60.5	>.10
2 v 4	69.0	<.10
3 v 4	60.5	>.10

Appendix Table 5.2.1

Serum glutamic dehydrogenase activities from rats infected with 20 metacercariae each and immunised intraperitoneally with normal bovine serum from bullocks 89 (A1) and 94 (B1), immune bovine serum collected at week $3_{(1)}$ (A2 and B2), week $6_{(1)}$ (A3 and B3) and week $9_{(1)}$ (A4 and B4) from bullocks 89 and 94 respectively.

Group No.	Bullock No.	Weeks p.i.	Rat Number 1	2	3	4	5	6	7	8	9	10	Mean ± SD
		0*	4	2	8	0	6	12	16	12	0	4	6.4 ± 5.5
		2	16	20	24	16	12	16	28	20	12	D	18.2 ± 5.3
A1	89	4	110	87	47	87	75	95	126	154	51		92.4 ± 34.2
		6	91	69	24	91	51	106	63	104	20		68.8 ± 32.3
		8	32	28	24	28	16	8	43	4	91		30.4 ± 25.7
Fluke Numbers			6	6	2	5	4	4	8	7	3	D	5.0 ± 1.9
		0*	8	4	4	6	4	0	6	12	4	8	5.6 ± 3.2
		2	12	16	8	18	16	20	28	12	24	16	17.0 ± 5.9
A2	89	4	91	106	95	51	95	47	87	102	67	83	82.4 ± 20.6
		6	71	47	114	16	63	12	95	47	32	43	54.0 ± 32.6
		8	16	20	25	47	43	32	0	87	16	59	34.5 ± 25.3
Fluke Numbers			6	5	5	4	6	2	6	4	6	4	4.8 ± 1.3
		0*	2	8	4	2	6	8	4	2	0	4	4.0 ± 2.7
		2	12	16	20	18	35	24	12	4	39	20	20.0 ± 10.6
A3	89	4	16	67	83	134	95	83	146	8	87	114	83.3 ± 44.8
		6	8	83	35	102	4	87	43	16	47	63	48.8 ± 34.4
		8	4	47	20	0	43	71	0	55	12	28	28.0 ± 25.0
Fluke Numbers			0	2	1	2	3	0	1	0	2	2	1.3 ± 1.1
		0*	8	2	4	0	6	4	12	2	2	4	4.4 ± 3.5
		2	4	8	16	24	32	8	43	24	47	D	22.9 ± 15.5
A4	89	4	12	24	16	154	95	20	114	146	134		79.4 ± 60.8
		6	4	0	8	67	83	16	87	67	83		46.1 ± 38.0
		8	0	2	4	43	67	12	51	87	24		32.2 ± 31.4
Fluke Numbers			0	0	0	1	1	0	4	3	3	D	1.3 ± 1.6
		0*	12	4	4	10	12	12	D	16	4	2	8.4 ± 5.0
		2	24	16	28	32	32	24		32	12	20	24.4 ± 7.3
B1	94	4	150	91	63	114	59	122		165	55	87	100.7 ± 39.9
		6	51	110	87	16	39	43		35	106	20	56.3 ± 35.7
		8	4	12	20	8	63	24		75	4	47	28.6 ± 26.6
Fluke Numbers			8	2	2	5	3	7	D	3	1	3	3.8 ± 2.4
		0*	2	0	2	16	4	8	12	14	0	16	7.4 ± 6.6
		2	12	24	39	12	12	16	43	24	12	28	22.2 ± 11.6
B2	94	4	173	118	130	91	110	55	154	71	24	150	107.6 ± 47.4
		6	59	35	83	87	75	102	28	122	8	51	65.0 ± 35.4
		8	47	24	16	43	55	32	0	67	0	16	30.0 ± 22.8
Fluke Numbers			6	4	3	2	3	2	6	4	2	5	3.7 ± 1.6
		0*	12	8	4	10	4	12	0	12	8	2	7.2 ± 4.4
		2	8	39	24	24	28	35	12	24	12	12	21.8 ± 10.5
B3	94	4	126	102	154	99	35	83	134	146	95	76	105.0 ± 36.1
		6	12	20	16	87	63	32	59	8	75	87	45.9 ± 31.7
		8	4	47	12	43	24	12	47	67	16	39	31.1 ± 20.4
Fluke Numbers			2	2	3	2	1	3	2	3	2	2	2.2 ± 0.6
		0*	4	6	12	2	6	16	4	0	8	12	7.0 ± 5.0
		2	20	12	12	8	24	4	20	4	8	28	14.0 ± 8.5
B4	94	4	95	118	79	173	75	87	12	141	20	79	87.9 ± 49.1
		6	51	42	91	67	24	71	4	126	0	16	49.2 ± 40.4
		8	35	24	55	24	16	32	0	63	4	2	25.5 ± 21.5
Fluke Numbers			1	2	1	3	1	2	0	4	0	1	1.5 ± 1.3

*Administration of infection; D Animal died

Appendix Table 5.2.2

Serum glutamic dehydrogenase activities from rats infected with 20 metacercariae each and immunised intraperitoneally with pooled normal bovine serum (1) from bullocks 89 and 94, pooled immune bovine serum (89 and 94) collected at week $12_{(1)}$ (2), week $15_{(1)}$ (3) and week $18_{(1)}$ (4).

Group No.	Weeks p.i.	Rat Number 1	2	3	4	5	6	7	8	9	10	Mean ± SD
	0*	4	8	6	8	6	2	0	4	8	D	5.1 ± 2.8
	2	12	35	12	18	18	16	26	12	14		18.1 ± 7.7
1	4	118	355	158	177	197	177	256	217	158		201.4 ± 69.6
	6	69	128	49	81	49	106	118	99	99		88.7 ± 28.5
	8	39	39	20	12	39	28	30	43	61		34.6 ± 14.2
Fluke Numbers		3	7	2	3	5	5	7	6	3		4.6 ± 1.9
	0*	12	6	0	8	4	4	6	8	10	6	6.4 ± 3.4
	2	13	8	35	8	14	8	12	18	18	8	14.7 ± 8.4
2	4	197	99	374	39	158	39	138	197	197	99	153.7 ± 98.2
	6	30	104	73	55	122	108	100	99	95	102	88.8 ± 27.9
	8	20	79	39	2	55	47	0	49	59	30	38.0 ± 25.3
Fluke Numbers		4	3	5	1	4	0	3	5	4	2	3.1 ± 1.7
	0*	4	6	8	6	4	6	2	6	2	6	5.0 ± 1.9
	2	24	14	12	24	16	12	24	16	12	18	17.2 ± 5.1
3	4	236	177	158	236	177	138	276	158	138	217	191.1 ± 47.3
	6	99	69	108	118	0	47	146	89	148	108	93.2 ± 45.1
	8	20	55	4	30	24	45	28	49	2	0	25.7 ± 19.8
Fluke Numbers		5	4	2	5	4	2	8	3	3	4	4.0 ± 1.8
	0*	8	4	2	6	6	4	4	6	6	0	4.6 ± 2.3
	2	12	8	16	26	16	10	20	22	32	12	17.4 ± 7.6
4	4	138	118	177	296	177	138	217	236	315	158	197.0 ± 67.6
	6	73	49	87	148	114	108	118	87	99	99	98.2 ± 26.9
	8	28	0	12	53	28	39	0	4	30	59	25.3 ± 21.2
Fluke Numbers		2	3	5	6	4	3	5	7	8	3	4.6 ± 2.0

*Administration of infection; D Animal died

Appendix Table 5.3.1.1

Mann-Whitney U-test analysis of flukes recovered at post-mortem as shown in Table 5.3.1.

Group comparison	U-value	Probability
A v B	54.5	>.10
A v C	73.0	<.10
A v D	54.4	>.10
A v E	71.5	<.10
A v F	67.5	>.10
G v A	55.0	>.10
G v B	60.5	>.10
G v C	74.5	<.05
G v D	61.0	>.10
G v E	69.5	<.10
G v F	69.0	<.10
C v B	70.0	<.10
C v D	67.0	>.10
C v E	60.0	>.10
C v F	57.5	>.10
E v B	62.5	>.10
E v D	61.0	>.10
E v F	53.0	>.10
F v B	69.5	<.10
F v D	60.0	>.10

Appendix Table 5.3.1

Serum glutamic dehydrogenase activities from rats infected with 20 metacercariae each and immunised intraperitoneally with pooled normal sera (A) and pooled immune bovine sera from bullocks 89 and 94 collected at week $3_{(2)}$ (B), week $6_{(2)}$ (C), week $9_{(2)}$ (D), week $12_{(2)}$ (E), week $15_{(2)}$ (F) and week $21_{(1)}$ (G).

Group No.	Weeks p.i.	Rat Number 1	2	3	4	5	6	7	8	9	10	Mean ± SD
	0*	20	10	10	10	10	0	10	20	20	20	13.0 ± 6.7
	2	20	20	10	20	30	0	39	20	30	30	21.9 ± 11.2
A	4	177	276	355	256	315	266	236	158	404	167	261.0 ± 81.4
	6	110	28	32	146	95	16	134	32	138	87	81.8 ± 50.8
	8	24	12	75	12	55	8	43	0	47	12	28.8 ± 24.7
Fluke Numbers		1	5	5	2	8	4	4	2	5	2	3.8 ± 2.1
	0*	20	0	10	0	20	10	20	10	10	0	10.0 ± 8.2
	2	20	39	30	20	20	30	10	20	20	39	24.8 ± 9.4
B	4	286	394	266	256	236	177	148	197	256	227	244.3 ± 67.7
	6	114	102	47	177	71	39	24	43	150	91	85.8 ± 50.6
	8	51	12	4	12	28	24	12	16	8	63	23.0 ± 19.5
Fluke Numbers		6	6	4	3	3	2	2	1	4	3	3.4 ± 1.6
	0*	0	0	10	20	0	20	0	10	10	20	9.0 ± 8.8
	2	0	0	20	20	20	10	20	10	20	20	14.0 ± 8.4
C	4	187	246	197	335	187	128	236	128	118	158	192.0 ± 66.8
	6	99	4	8	59	63	8	71	75	71	95	55.3 ± 35.8
	8	83	4	8	12	24	8	8	4	0	47	19.8 ± 26.1
Fluke Numbers		1	3	0	3	3	1	4	2	2	3	2.2 ± 1.2
	0*	0	20	10	10	10	0	10	0	10	10	8.0 ± 6.3
	2	39	30	20	39	10	0	39	0	10	10	19.7 ± 15.9
D	4	207	207	30	177	266	217	276	197	207	286	207.0 ± 72.2
	6	35	39	12	95	95	55	146	134	165	35	81.1 ± 53.7
	8	8	12	4	4	32	43	33	6	4	6	15.2 ± 14.8
Fluke Numbers		2	5	0	3	6	1	6	4	3	3	3.3 ± 2.0
	0*	10	0	0	10	10	0	10	10	20	10	8.0 ± 6.3
	2	20	0	0	10	20	0	10	20	10	30	12.0 ± 10.3
E	4	217	276	197	315	236	197	197	256	227	138	225.6 ± 49.2
	6	138	20	87	35	67	150	130	16	91	146	88.0 ± 52.2
	8	4	12	39	55	4	20	6	16	67	14	23.7 ± 22.3
Fluke Numbers		4	2	2	3	4	2	2	3	3	2	2.7 ± 0.8
	0*	0	10	10	10	0	10	0	10	0	10	6.0 ± 5.2
	2	0	10	39	20	0	30	0	20	0	20	13.9 ± 14.1
F	4	246	256	286	187	158	345	246	296	148	335	250.3 ± 68.8
	6	130	0	146	87	185	185	51	12	83	39	91.8 ± 67.6
	8	91	4	20	0	12	12	49	8	63	47	30.6 ± 30.3
Fluke Numbers		3	1	5	2	1	5	3	2	1	4	2.7 ± 1.6
	0*	10	0	0	10	10	10	10	0	10	20	8.0 ± 6.3
	2	20	0	0	20	20	10	30	20	20	20	16.0 ± 9.7
G	4	276	266	355	256	266	187	315	217	246	256	264.0 ± 46.7
	6	24	59	55	268	16	16	130	0	20	209	79.7 ± 92.4
	8	51	67	8	59	12	8	20	0	28	71	32.4 ± 27.0
Fluke Numbers		4	4	6	7	3	0	7	2	2	6	4.1 ± 2.4

*Administration of infection

Appendix Table 5.4.1.1

Mann-Whitney U-test analysis of flukes recovered at post-mortem as shown in Table 5.4.1.

Group comparison	U-value	Probability
A1 v A2	28.0	<.10
A1 v A3	18.0	>.10
A1 v A4	18.5	>.10
A1 v A5	30.5	<.05
A1 v A6	30.0	<.05
A1 v A7	24.5	>.10
B1 v B2	20.5	>.10
B1 v B3	19.5	>.10
B1 v B2	26.5	>.10
B1 v B7	20.5	>.10
A2 v A3	23.0	>.10
A2 v A4	24.0	>.10
A2 v A5	20.5	>.10
A2 v A6	22.5	>.10
A2 v A7	17.0	>.10
A3 v A4	14.5	>.10
A3 v A7	21.0	>.10
A5 v A3	23.5	>.10
A5 v A4	24.5	>.10
A5 v A6	25.5	>.10
A5 v A7	17.5	>.10
A6 v A3	25.0	<.10
A6 v A4	24.5	>.10
A6 v A7	24.0	>.10
A2 v B1	27.0	>.10
A5 v B1	26.0	>.10
A6 v B1	28.0	<.10

Appendix Table 5.4.1

Serum glutamic dehydrogenase activities from rats infected with 20 metacercariae each and immunised intraperitoneally with normal bovine serum from bullocks 89 (A1) and 94 (B1), immune bovine serum collected at week $6_{(2)}$ (A2 and B2), week $9_{(2)}$ (A3 and B3), week $3_{(3)}$ (A4 and B4), week $6_{(3)}$ (A5 and B5), week $9_{(3)}$ (A6 and B6) and week $12_{(3)}$ (A7 and B7) from bullocks 89 and 94 respectively.

Group No.	Bullock No.	Weeks p.i.	Rat Number 1	2	3	4	5	6	Mean ± SD
		0*	0	8	8	0	8	8	5.3 ± 4.1
A1	89	4	134	150	173	244	173	292	194.3 ± 60.9
		6	130	43	51	188	116	195	120.5 ± 64.9
Fluke Numbers			6	5	5	6	4	6	5.3 ± 0.8
		0*	8	0	0	8	8	0	4.0 ± 4.4
A2	89	4	134	173	181	221	158	134	166.8 ± 32.9
		6	123	-	166	14	174	29	101.2 ± 75.5
Fluke Numbers			2	5	7	2	3	2	3.5 ± 2.1
		0*	8	16	0	0	8	8	6.7 ± 6.0
A3	89	4	197	110	189	142	189	213	173.3 ± 39.0
		6	188	72	D	14	181	181	127.2 ± 79.6
Fluke Numbers			6	4	D	4	4	8	5.2 ± 1.8
		0*	8	12	0	8	12	8	8.0 ± 4.4
A4	89	4	165	95	197	213	292	150	185.3 ± 66.5
		6	14	87	109	203	268	94	129.2 ± 91.0
Fluke Numbers			3	1	6	7	7	5	4.8 ± 2.4
		0*	4	8	0	8	0	16	6.0 ± 6.1
A5	89	4	71	189	236	102	229	142	161.5 ± 67.8
		6	29	123	0	94	210	134	98.3 ± 76.0
Fluke Numbers			2	3	4	3	6	4	3.7 ± 1.4
		0*	0	8	0	0	12	4	4.0 ± 5.1
A6	89	4	118	63	79	118	240	173	131.8 ± 65.3
		6	7	87	72	0	224	163	92.2 ± 87.8
Fluke Numbers			2	1	1	3	9	3	3.2 ± 3.0
		0*	8	4	8	8	16	0	7.3 ± 5.3
A7	89	4	102	134	158	323	315	123	192.5 ± 99.7
		6	43	130	98	109	14	126	86.7 ± 47.4
Fluke Numbers			2	3	2	7	6	4	4.0 ± 2.1
		0*	8	0	8	0	0	0	2.7 ± 4.1
B1	94	4	150	213	118	299	95	134	168.2 ± 75.5
		6	7	80	22	203	87	123	87.0 ± 71.3
Fluke Numbers			3	6	5	7	3	6	5.0 ± 1.7
		0*	0	0	0	8	0	4	2.0 ± 3.3
B2	94	4	221	189	142	134	221	224	188.5 ± 41.2
		6	210	65	0	123	210	145	125.5 ± 82.6
Fluke Numbers			8	5	3	3	5	5	4.8 ± 1.8
		0*	12	4	8	0	8	8	6.7 ± 4.1
B3	94	4	175	236	173	102	126	236	174.7 ± 55.1
		6	166	217	94	7	14	181	113.2 ± 89.0
Fluke Numbers			4	6	6	2	3	7	4.7 ± 2.0
		0*	8	4	8	16	0	4	6.7 ± 5.5
B4	94	4	236	205	323	158	284	299	250.8 ± 62.6
		6	174	174	253	152	109	65	154.5 ± 64.1
Fluke Numbers			5	5	6	3	6	5	5.0 ± 1.1
		0*	8	0	8	8	8	0	5.3 ± 4.1
B5	94	4	158	173	D	221	118	95	153.0 ± 49.1
		6	51	130		72	109	14	75.2 ± 46.1
Fluke Numbers			5	7	D	8	5	5	6.0 ± 1.4
		0*	8	0	0	8	12	0	4.7 ± 5.3
B6	94	4	268	315	165	189	252	244	238.8 ± 54.4
		6	29	101	51	181	195	181	123.0 ± 72.7
Fluke Numbers			5	6	1	5	5	4	4.3 ± 1.7
		0*	8	4	8	8	0	8	6.0 ± 3.3
B7	94	4	219	165	236	284	197	158	209.8 ± 47.2
		6	63	101	65	-	-	123	88.0 ± 29.1
Fluke Numbers			5	3	6	8	4	2	4.7 ± 2.2

*Administration of infection; D Animal died; - No sample

Appendix Table 6.1.1

Mean ELISA values (units of absorbance) from bullocks repeatedly infected with 1,000 metacercariae of F. hepatica, using somatic antigen in the test.

Bullock No.	Weeks after primary infection												
	0*	2	4	6	8	10	12	15	18	21	24	27	30
203	0.43±0.02	0.51±0.01	0.57±0.01	0.57±0.01	0.58±0.01	0.56±0.01	0.51±0.02	0.54±0.04	0.54±0.02	0.57±0.03	0.59±0.01	0.56±0.01	0.56±0.01
204	0.58±0.05	0.62±0.03	0.61±0.01	0.66±0.01	0.64±0.01	0.62±0.02	0.63±0.01	0.65±0.01	0.65±0.01	0.68±0.02	0.71±0.01	0.70±0.03	0.73±0.02
M199	0.59±0.04	0.60±0.02	0.66±0.02	0.65±0.03	0.64±0.01	0.66±0.02	0.65±0.01	0.71±0.01	0.66±0.01	0.69±0.01	0.69±0.01	0.67±0.01	0.69±0.01

	Weeks after secondary infection								
	2	4	6	8	10	12	14	16	18
203	0.53±0.02	0.54±0.02	0.51±0.01	0.42±0.01	0.41±0.04	0.49±0.02	0.50±0.01	0.49±0.03	0.50±0.01
204	0.64±0.02	0.70±0.01	0.73±0.03	0.71±0.02	0.68±0.01	0.67±0.01	0.72±0.02	0.68±0.01	0.70±0.02
M199	0.70±0.01	0.67±0.01	0.64±0.01	0.64±0.01	0.66±0.01	0.68±0.02	0.69±0.01	0.73±0.01	0.73±0.01

*Administration of infection

Appendix Table 6.1.2

Mean ELISA values (units of absorbance) from bullocks repeatedly infected with 1,000 metacercariae of F. hepatica, using metabolic antigen in the test.

Bullock No.	Weeks after primary infection												
	0*	2	4	6	8	10	12	15	18	21	24	27	30
203	0.19±0.01	0.25±0.01	0.30±0.01	0.31±0.01	0.29±0.02	0.30±0.03	0.28±0.02	0.27±0.02	0.28±0.01	0.25±0.01	0.31±0.03	0.30±0.01	0.29±0.01
204	0.24±0.02	0.29±0.01	0.26±0.01	0.30±0.03	0.32±0.01	0.29±0.03	0.27±0.02	0.30±0.02	0.31±0.01	0.34±0.02	0.36±0.02	0.34±0.03	0.35±0.02
M199	0.26±0.02	0.30±0.02	0.33±0.02	0.31±0.01	0.30±0.03	0.27±0.01	0.25±0.01	0.24±0.01	0.20±0.03	0.26±0.01	0.25±0.01	0.25±0.01	0.27±0.02

	Weeks after secondary infection								
	2	4	6	8	10	12	14	16	18
203	0.37±0.01	0.31±0.01	0.29±0.03	0.29±0.01	0.32±0.04	0.29±0.03	0.28±0.01	0.35±0.03	0.34±0.01
204	0.27±0.02	0.32±0.03	0.29±0.01	0.38±0.01	0.36±0.01	0.36±0.02	0.36±0.01	0.30±0.02	0.32±0.02
M199	0.34±0.08	0.33±0.02	0.29±0.01	0.34±0.01	0.32±0.01	0.29±0.01	0.39±0.01	0.35±0.01	0.32±0.03

*Administration of infection

Appendix Table 6.2.1.1

Mann-Whitney U-test analysis of flukes recovered at post-mortem as shown in Table 6.2.1.

Group comparison	U-value	Probability
A1 v A2	24.0	<.10
A1 v A3	26.5	>.10
B1 v B2	26.0	>.10
B1 v B3	26.5	>.10
C1 v C3	27.0	>.10

Appendix Table 6.2.1

Serum glutamic dehydrogenase activities from rats infected with 20 metacercariae each and immunised intraperitoneally with normal bovine serum from bullocks 203 (A1), 204 (B1) and M199 (C1), immune bovine serum collected at week 9 (1) (A2, B2 and C2) and week 12 (1) (A3, B3 and C3) from the respective bullocks.

Group No.	Bullock No.	Weeks p.i.	Rat Number 1	2	3	4	5	6	Mean ± SD
		0*	0	4	0	4	0	0	1.3 ± 2.1
		2	20	8	4	24	8	12	12.7 ± 7.8
A1	203	4	47	114	47	102	146	126	97.0 ± 41.4
		6	35	24	12	114	8	43	39.3 ± 38.9
		8	29	24	29	94	8	29	35.5 ± 29.8
Fluke Numbers			5	6	2	5	5	5	4.7 ± 1.4
		0*	4	4	2	0	0	4	2.3 ± 2.0
		2	8	12	12	8	12	12	10.7 ± 2.1
A2	203	4	89	47	91	75	55	87	74.0 ± 18.8
		6	4	26	75	65	D	8	35.6 ± 32.7
		8	4	22	58	14		12	22.0 ± 21.1
Fluke Numbers			3	1	5	4	D	3	3.2 ± 1.5
		0*	4	0	4	4	0	0	2.0 ± 2.2
		2	6	8	-	24	8	8	10.8 ± 7.4
A3	203	4	63	59	122	138	79	87	91.3 ± 32.0
		6	31	59	106	138	69	31	72.3 ± 42.5
		8	43	14	94	29	58	0	39.7 ± 33.6
Fluke Numbers			2	2	4	8	3	2	3.5 ± 2.3
		0*	4	4	4	0	4	-	3.2 ± 1.8
		2	8	-	8	4	8	8	7.2 ± 1.8
B1	204	4	98	126	142	-	47	35	89.6 ± 47.3
		6	35	83	35	59	0	67	46.5 ± 29.4
		8	29	22	29	0	0	4	14.0 ± 14.2
Fluke Numbers			3	4	6	3	2	2	3.3 ± 1.5
		0*	4	4	4	6	8	0	4.3 ± 2.7
		2	16	8	4	12	18	8	11.0 ± 5.3
B2	204	4	39	31	8	71	83	69	50.2 ± 28.8
		6	33	25	8	55	83	65	44.8 ± 27.8
		8	7	7	0	7	72	20	18.8 ± 26.8
Fluke Numbers			1	1	0	2	7	3	2.3 ± 2.5
		0*	4	0	0	4	4	4	2.7 ± 2.1
		2	12	4	4	8	6	16	8.3 ± 4.8
B3	204	4	106	47	71	31	95	79	71.5 ± 28.4
		6	78	47	47	12	104	69	59.5 ± 31.6
		8	57	40	7	7	43	22	29.3 ± 20.6
Fluke Numbers			4	0	2	2	3	1	2.0 ± 1.4
		0*	0	0	4	4	4	0	2.0 ± 2.2
		2	24	8	16	10	4	10	12.0 ± 7.0
C1	M199	4	91	138	71	100	102	43	90.8 ± 32.0
		6	87	104	35	91	59	4	63.3 ± 38.3
		8	39	14	22	14	65	7	26.8 ± 21.7
Fluke Numbers			4	5	3	2	4	1	3.2 ± 1.5
		0*	0	4	4	4	4	2	3.0 ± 1.7
		2	12	6	-	16	-	4	9.5 ± 5.5
C2	M199	4	110	91	91	91	110	75	94.7 ± 13.4
		6	87	87	57	91	69	-	78.2 ± 14.6
		8	14	36	14	110	0	-	34.8 ± 44.0
Fluke Numbers			7	5	5	5	9	1	5.3 ± 2.7
		0*	0	4	0	4	4	0	2.0 ± 2.2
		2	6	14	8	6	0	6	6.7 ± 4.5
C3	M199	4	20	91	95	43	83	114	74.3 ± 35.5
		6	55	16	173	35	91	55	70.8 ± 55.9
		8	0	29	87	14	14	22	27.7 ± 30.6
Fluke Numbers			0	2	2	1	2	4	1.8 ± 1.3

*Administration of infection; D Animal died; - No sample

Appendix Table 6.3.1.1

Mann-Whitney U-test analysis of flukes recovered at post-mortem as shown in Table 6.3.1.

Group comparison	U-value	Probability
A1 v A2	22.5	>.10
B1 v B2	31.0	<.05
B1 v B3	27.0	>.10
A2 v B1	32.0	<.025
A2 v B2	20.0	>.10
A2 v B3	24.0	>.10
A2 v C1	17.0	>.10

Appendix Table 6.3.1

Serum glutamic dehydrogenase activities from rats infected with 20 metacercariae each and immunised intraperitoneally with normal bovine serum from bullocks 203 (A1), 204 (B1) and M199 (C1), immune bovine serum collected at week $6_{(2)}$ (A2, B2 and C2) and week $9_{(2)}$ (A3, B3 and C3) from the respective bullocks

Group No.	Bullock No.	Weeks p.i.	Rat Number 1	2	3	4	5	6	Mean ± SD
		0*	14	7	7	0	14	7	8.2 ± 5.3
A1	203	4	166	195	137	22	145	94	126.5 ± 61.1
		6	22	36	14	137	65	7	46.8 ± 48.7
Fluke Numbers			4	7	5	1	6	5	4.7 ± 2.1
		0*	0	14	0	7	7	14	7.0 ± 6.3
A2	203	4	116	188	166	116	109	145	140.0 ± 32.0
		6	22	130	159	-	51	51	82.6 ± 58.6
Fluke Numbers			5	7	3	3	1	5	4.0 ± 2.1
		0*	0	22	22	14	0	7	10.8 ± 10.1
A3	203	4	195	159	145	123	137	188	157.8 ± 28.6
		6	210	43	130	7	0	101	81.8 ± 81.1
Fluke Numbers			8	5	6	2	2	8	5.2 ± 2.7
		0*	14	21	7	29	7	7	14.2 ±9.2
B1	204	4	230	194	137	130	174	166	171.8 ± 37.1
		6	130	43	51	58	138	123	90.5 ± 44.1
Fluke Numbers			13	7	5	6	7	8	7.7 ± 2.8
		0*	14	7	14	14	0	7	9.3 ± 5.7
B2	204	4	123	109	145	116	174	181	141.3 ± 30.6
		6	0	58	138	58	43	65	60.3 ± 44.7
Fluke Numbers			2	5	5	4	6	7	4.8 ± 1.7
		0*	7	0	0	14	0	22	7.2 ± 9.2
B3	204	4	253	188	116	207	224	94	180.3 ± 62.5
		6	130	58	29	174	51	43	80.8 ± 57.7
Fluke Numbers			7	4	4	6	8	3	5.3 ± 2.0
		0*	7	0	0	14	22	0	7.2 ± 9.2
C1	M199	4	D	158	148	151	123	195	155.0 ± 26.0
		6		22	14	123	108	174	88.2 ± 68.6
Fluke Numbers			D	5	3	6	1	7	4.4 ± 2.4
		0*	7	0	7	7	14	0	5.8 ± 5.3
C2	M199	4	174	65	174	174	181	159	154.5 ± 44.4
		6	43	159	0	36	203	80	86.8 ± 78.5
Fluke Numbers			6	1	6	2	7	4	4.3 ± 2.4
		0*	7	14	7	0	7	0	5.8 ± 5.3
C3	M199	4	123	144	152	109	188	195	151.8 ± 34.3
		6	-	14	29	181	0	174	79.6 ± 90.0
Fluke Numbers			3	4	4	2	5	7	4.2 ± 1.7

*Administration of infection: D Animal died; - No sample

Appendix Table 7.1.1

Mean ELISA values (units of absorbance) from bullocks infected with 1,000 metacercariae of F. hepatica, using somatic antigen in the test.

Bullock No.	Weeks after primary infection				
	0*	2	4	6	8
176	0.46 ± 0.01	0.54 ± 0.01	0.60 ± 0.01	0.60 ± 0.02	0.62 ± 0.01
177	0.46 ± 0.01	0.48 ± 0.01	0.52 ± 0.01	0.58 ± 0.02	0.57 ± 0.01
178	0.46 ± 0.01	0.50 ± 0.01	0.53 ± 0.01	0.59 ± 0.01	0.59 ± 0.02
186	0.46 ± 0.01	0.47 ± 0.02	0.50 ± 0.01	0.53 ± 0.01	0.51 ± 0.01

*Administration of infection

Appendix Table 7.1.2

Mean ELISA values (units of absorbance) from bullocks infected with 1,000 metacercaria of F. hepatica, using metabolic antigen in the test.

Bullock No.	Weeks after primary infection				
	0*	2	4	6	8
176	0.15 ± 0.01	0.17 ± 0.02	0.19 ± 0.01	0.20 ± 0.02	0.18 ± 0.01
177	0.15 ± 0.01	0.17 ± 0.01	0.19 ± 0.02	0.21 ± 0.01	0.17 ± 0.01
178	0.15 ± 0.01	0.18 ± 0.01	0.18 ± 0.01	0.21 ± 0.02	0.21 ± 0.02
186	0.15 ± 0.01	0.17 ± 0.01	0.18 ± 0.01	0.25 ± 0.01	0.21 ± 0.01

*Administration of infection

Appendix Table 7.2.1.1

Mann-Whitney U-test analysis of flukes recovered at post-mortem as shown in Table 7.2.1.

Group comparison	U-value	Probability
A1 v A2	19.5	>.10
A1 v A3	31.5	<.025
B1 v B3	19.5	>.10
C1 v C3	25.5	>.10
D1 v D2	23.0	>.10
D1 v D3	31.0	<.05

Appendix Table 7.2.1

Serum glutamic dehydrogenase activities from rats infected with 20 metacercariae each and immunised intraperitoneally with normal bovine serum from bullocks 176 (A1), 177 (B1), 178 (C1) and 186 (D1), immune bovine serum collected at week $6_{(1)}$ (A2, B2, C2 and D2) and week $9_{(1)}$ (A3, B3, C3 and D3) from the respective bullocks.

Group No.	Bullock No.	Weeks p.i.	Rat Number 1	2	3	4	5	6	Mean ± SD
		0*	7	14	7	0	7	4	6.5 ± 4.6
A1	176	4	195	203	65	181	181	210	172.5 ± 53.9
		6	7	36	72	7	116	80	53.0 ± 43.8
Fluke Numbers			8	11	5	4	5	12	7.5 ± 3.4
		0*	7	7	0	0	4	14	5.3 ± 5.3
A2	176	4	181	166	D	188	188	210	186.6 ± 15.9
		6	33	101		22	65	25	49.2 ± 33.6
Fluke Numbers			4	3	D	6	8	8	5.8 ± 2.3
		0*	7	7	0	4	14	7	6.5 ± 4.6
A3	176	4	188	152	195	195	181	181	182.0 ± 16.0
		6	90	43	4	174	29	145	80.8 ± 67.7
Fluke Numbers			5	2	3	5	3	4	3.7 ± 1.2
		0*	7	0	11	7	0	7	5.3 ± 4.4
B1	177	4	123	195	195	80	130	159	147.0 ± 45.0
		6	101	159	94	51	232	112	124.8 ± 62.9
Fluke Numbers			7	6	4	2	4	4	4.5 ± 1.8
		0*	0	14	11	7	7	4	7.2 ± 5.0
B2	177	4	51	36	159	195	170	166	129.5 ± 67.8
		6	58	29	40	29	65	40	43.5 ± 14.9
Fluke Numbers			1	3	3	8	7	7	4.8 ± 2.9
		0*	0	7	14	7	7	0	5.8 ± 5.3
B3	177	4	210	188	203	72	94	152	153.2 ± 58.3
		6	65	29	29	192	36	116	77.8 ± 65.1
Fluke Numbers			7	2	7	1	2	4	3.8 ± 2.6
		0*	11	7	0	7	0	4	4.8 ± 4.4
C1	178	4	58	188	166	210	116	159	149.5 ± 54.8
		6	29	7	188	152	33	203	102.0 ± 88.6
Fluke Numbers			1	6	7	8	5	6	5.5 ± 2.4
		0*	4	7	14	14	0	7	7.7 ± 5.5
C2	178	4	4	188	152	159	159	130	132.0 ± 65.4
		6	14	192	159	127	166	36	115.7 ± 73.6
Fluke Numbers			1	11	3	7	5	3	5.0 ± 3.6
		0*	7	7	0	4	14	0	5.3 ± 5.3
C3	178	4	80	65	185	145	101	152	121.3 ± 46.6
		6	7	188	87	0	98	54	72.3 ± 69.4
Fluke Numbers			2	4	7	5	5	4	4.5 ± 1.6
		0*	0	7	7	14	7	14	8.2 ± 5.3
D1	186	4	130	109	116	181	224	181	156.8 ± 45.5
		6	134	112	217	138	130	51	130.3 ± 53.3
Fluke Numbers			5	5	7	8	8	7	6.7 ± 1.4
		0*	7	11	4	7	0	7	6.0 ± 3.7
D2	186	4	116	166	210	123	58	210	147.2 ± 59.6
		6	181	268	7	51	116	109	122.0 ± 93.0
Fluke Numbers			5	6	8	4	3	8	5.7 ± 2.1
		0*	7	0	7	0	11	7	5.3 ± 4.4
D3	186	4	79	145	166	188	137	159	145.7 ± 37.2
		6	43	43	0	14	7	109	36.0 ± 40.1
Fluke Numbers			2	4	4	7	4	6	4.5 ± 1.8

*Administration of infection; D Animal died

Appendix Table 8.1.1A

Corrected ELISA values from uninfected rats (A) and rats infected with 20 metacercariae each (B), using metabolic antigen in the test.

Group No.	No. of Rats	Weeks after Infection										
		0	2	4	6	8	10	12	14	16	18	20
		0.06	0.07	0.06	0.05	0.08	0.04	0.05	0.08	0.09	0.09	0.10
		0.06	0.02	0.04	0.03	0.03	0.04	0.04	0.04	0.05	0.06	0.04
A	5	0.06	0.09	0.09	0.09	0.08	0.08	0.08	0.04	0.07	0.07	0.08
		0.07	0.07	0.07	0.07	0.06	0.07	0.06	0.04	0.04	0.04	0.06
		0.02	0.06	0.07	0.09	0.04	0.04	0.05	0.05	0.05	0.05	0.04
Mean ± SD		0.05±0.02	0.06±0.03	0.07±0.02	0.07±0.03	0.06±0.02	0.05±0.02	0.06±0.01	0.05±0.02	0.06±0.02	0.06±0.02	0.06±0.03
		0.06	0.10	0.11	0.11	0.10	0.09	0.08	0.09	0.19	0.16	0.09
		0.09	0.20	0.27	0.21	0.21	0.17	0.17	0.16	0.22	0.17	0.25
		0.07	0.15	0.26	0.35	0.38	0.38	0.30	0.30	0.30	0.29	0.50
		0.01	0.14	0.23	0.27	0.24	0.25	0.17	0.11	0.14	0.10	0.44
		0.01	0.18	0.26	0.31	0.26	0.21	0.18	0.19	0.21	0.15	0.13
		0.08	0.13	0.13	0.18	0.16	0.15	0.14	0.14	0.14	0.13	0.13
		0.03	0.21	0.33	0.47	0.48	0.41	0.32	0.33	0.35	0.37	0.49
		0.07	0.34	0.44	0.53	0.40	0.36	0.40	0.34	0.40	0.37	0.43
		0.01	0.13	0.17	0.15	0.13	0.10	0.11	0.14	0.15	0.27	0.17
		0.05	0.17	0.24	0.28	0.21	0.22	0.24	0.24	0.29	0.26	0.27
		0.10	0.25	0.28	0.36	0.31	0.26	0.29	0.28	0.29	0.31	0.40
		0.03	0.16	0.25	0.31	0.22	0.25	0.18	0.20	0.22	0.20	0.35
B	25	0.04	0.14	0.18	0.23	0.23	0.13	0.18	0.14	0.19	0.19	0.22
		0.08	0.19	0.24	0.25	0.21	0.20	0.22	0.19	0.22	0.23	0.24
		0.08	0.14	0.24	0.31	0.29	0.22	0.26	0.29	0.24	0.22	0.23
		0.06	0.15	0.26	0.29	0.27	0.24	0.25	0.27	0.25	0.23	0.24
		0.07	0.17	0.22	0.26	0.25	0.22	0.23	0.24	0.25	0.22	0.28
		0.04	0.09	0.11	0.17	0.13	0.21	0.16	0.16	0.16	0.13	0.18
		0.07	0.12	0.17	0.28	0.25	0.24	0.23	0.19	0.20	0.18	0.28
		0.01	0.11	0.19	0.25	0.27	0.24	0.26	0.25	0.24	0.24	0.37
		0.01	0.10	0.23	0.28	0.27	0.29	0.29	0.23	0.22	0.19	0.30
		0.12	0.21	0.32	0.40	0.35	0.34	0.33	0.36	0.45	0.40	0.43
		0.14	0.21	0.30	0.36	0.35	0.36	0.36	0.31	0.27	0.30	0.36
		0.14	0.24	0.35	0.39	0.39	0.32	0.43	0.31	0.31	0.30	0.38
		0.01	0.14	0.19	0.28	0.27	0.31	0.15	0.16	0.22	0.25	0.33
Mean ± SD		0.06±0.04	0.17±0.06	0.24±0.08	0.29±0.10	0.26±0.09	0.25±0.08	0.24±0.09	0.22±0.08	0.24±0.08	0.23±0.08	0.30±0.11

Appendix Table 8.1.1B

Corrected ELISA values from rats infected with 20 metacercariae each of F. hepatica (A), infected (20 metacercariae), treated with anthelmintic and left uninfected (C), infected (20 metacercariae), treated with anthelmintic and implanted with adult fluke in diffusion chamber (D), infected (20 metacercariae), treated with anthelmintic and reinfected with 20 metacercariae, each of F. hepatica (E), using metabolic antigen in the test.

Group No.	Rat No.	Weeks after Infection									
		2	4	6	8	10	12	14	16	18	20
A	1	0.23	0.27	0.37	0.31	0.24	0.23	0.23	0.25	0.24	0.23
	2	0.28	0.41	0.45	0.42	0.44	0.30	0.32	0.32	0.33	0.33
	3	0.15	0.21	0.37	0.27	0.26	0.24	0.23	0.21	0.28	0.29
	4	0.19	0.34	0.39	0.32	0.27	0.23	0.20	0.19	0.31	0.31
	5	0.09	0.16	0.28	0.33	0.26	0.20	0.18	0.23	0.30	0.27
Mean ± SD		0.19±0.07	0.28±0.10	0.37±0.06	0.33±0.05	0.29±0.08	0.24±0.04	0.23±0.05	0.24±0.05	0.29±0.03	0.29±0.04
C	20	0.09	0.08	0.09	0.08	0.07	0.11	0.10	0.11	0.11	0.09
	21	0.08	0.08	0.12	0.09	0.09	0.11	0.09	0.09	0.09	0.10
	14	0.41	0.46	0.32	0.36	0.32	0.17	0.15	0.15	0.13	0.04
	13	0.33	0.22	0.26	0.25	0.23	0.20	0.19	0.18	0.16	0.04
	15	0.11	0.09	0.09	0.08	0.08	0.09	0.09	0.15	0.13	0.11
	16	0.13	0.12	0.14	0.13	0.13	0.20	0.21	0.22	0.23	0.14
	18	0.44	0.37	0.39	0.34	0.31	0.33	0.21	0.25	0.04	0.03
	19	0.42	0.22	0.36	0.33	0.29	0.32	0.30	0.31	0.29	0.24
	11	0.01	0.04	0.03	0.02	0.01	0.02	0.02	0.03	0.07	0.04
Mean ± SD		0.22±0.17	0.19±0.14	0.20±0.13	0.19±0.13	0.17±0.12	0.17±0.10	0.15±0.08	0.17±0.09	0.14±0.08	0.09±0.07
D	1	0.30	0.36	0.25	0.26	0.22	0.22	0.22	0.20	0.21	0.17
	2	0.37	0.33	0.31	0.25	0.24	0.22	0.21	0.19	0.17	0.16
	4	0.41	0.37	0.29	0.23	0.20	0.21	0.14	0.18	0.15	0.15
	7	0.34	0.34	0.27	0.28	0.26	0.24	0.20	0.21	0.21	0.24
	8	0.25	0.32	0.23	0.20	0.20	0.21	0.23	0.19	0.20	0.19
	9	0.31	0.24	0.29	0.31	0.30	0.27	0.27	0.19	0.15	0.14
Mean ± SD		0.33±0.06	0.33±0.05	0.27±0.03	0.25±0.04	0.24±0.04	0.23±0.02	0.21±0.04	0.19±0.01	0.18±0.03	0.17±0.04
E	22	0.19	0.19	0.25	0.23	0.22	0.19	0.17	0.22	0.15	0.15
	23	0.25	0.32	0.35	0.34	0.34	0.31	0.27	0.19	0.21	0.20
	24	0.33	0.36	0.32	0.27	0.25	0.24	0.19	0.16	0.16	0.17
	25	0.24	0.26	0.34	0.37	0.22	0.28	0.21†			
	26	0.42	0.45	0.52	0.49	0.55	0.47	0.44	0.44	0.43	0.42
	28	0.36	0.39	0.41	0.44	0.41	0.31	0.33	0.28	0.30	0.34
	29	0.20	0.39	0.36	0.36	0.30	0.30	0.30	0.30	0.34	0.34
	30	0.34	0.35	0.36	0.37	0.36	0.35	0.33	0.32	0.33	0.34
Mean ± SD		0.29±0.08	0.34±0.08	0.36±0.08	0.36±0.08	0.33±0.11	0.31±0.08	0.28±0.09	0.27±0.09	0.27±0.10	0.28±0.10

†Animal died fifteen weeks after reinfection

Appendix Table 8.1.2A

Serum glutamic dehydrogenase activity from uninfected rats (A) and rats infected with 20 metacercariae of F. hepatica (B)

Group No.	No. of Rats	Weeks after Infection										
		0	2	4	6	8	10	12	14	16	18	20
		8	6	10	8	7	8	6	8	8	6	8
		0	2	0	0	1	0	2	0	0	0	0
A	5	0	0	1	0	1	0	0	0	1	2	0
		0	0	1	1	1	1	0	2	0	0	2
		10	9	8	9	10	8	8	8	7	8	10
Mean ± SD		4±5	3±4	4±5	4±4	4±4	3±4	3±4	4±4	3±4	3±4	4±5
		0	4	0	8	0	7	14	7	0	14	7
		0	32	378	55	7	0	14	0	0	0	0
		16	20	197	16	7	14	14	14	7	14	14
		0	24	95	95	58	7	14	51	43	33	29
		8	24	307	221	29	18	22	14	22	14	43
		0	16	8	4	7	-	14	7	7	14	0
		0	16	189	95	54	0	29	7	7	22	14
		0	8	95	79	29	36	43	51	51	101	14
		4	4	8	8	0	7	7	14	7	14	18
		0	24	205	166	14	0	14	4	7	14	0
		0	0	228	63	18	7	72	29	65	29	0
		8	31	165	116	80	87	14	22	7	7	14
B	25	0	24	236	181	55	14	36	43	36	14	14
		0	0	307	174	0	0	14	0	7	0	30
		0	0	95	65	58	87	14	7	-	7	14
		16	8	150	101	14	7	22	43	43	7	7
		8	16	276	71	14	24	0	39	0	22	36
		0	8	39	16	14	18	14	7	0	0	0
		8	8	79	51	36	18	22	18	22	22	22
		8	31	252	189	29	-	22	43	72	94	29
		0	31	197	110	87	87	39	7	14	0	-
		0	0	71	43	22	65	14	7	0	22	10
		8	8	126	8	4	7	14	22	14	14	7
		0	16	126	80	14	-	22	14	0	0	14
		4	8	205	55	7	7	11	14	7	14	14
Mean ± SD		3±5	14±11	161±101	83±63	26±25	23±30	21±14	19±16	18±22	20±25	15±12

- No sample

Appendix Table 8.1.2B

Serum glutamic dehydrogenase activity from rats infected with 20 metacercariae each (A), infected (20 metacercariae), treated with anthelmintic and left uninfected (C), infected (20 metacercariae) treated with anthelmintic and implanted intraperitoneally with adult fluke in diffusion chamber (D) and treated with anthelmintic and infected with 20 metacercariae each of F. hepatica (E).

Group No.	Rat No.	Weeks after Infection									
		2	4	6	8	10	12	14	16	18	20
A	1	14	166	155	22	65	7	130	72	58	22
	2	36	152	163	29	22	22	22	22	36	29
	3	22	130	109	109	87	7	14	87	29	22
	4	43	130	109	29	14	14	22	14	29	29
	5	14	137	166	14	14	7	29	14	22	14
Mean ± SD		26±13	143±16	140±29	41±39	40±34	11±7	43±49	42±35	35±14	23±6
C	20	22	29	14	14	-	51	43	36	65	29
	21	7	7	7	22	18	14	22	22	-	36
	14	0	22	36	14	29	7	22	22	65	7
	15	7	0	0	0	7	22	14	14	14	36
	16	0	7	0	0	0	11	0	14	14	22
	18	28	22	14	14	43	43	36	36	65	18
	19	36	22	0	22	29	22	29	43	51	36
	11	11	0	0	7	14	14	0	0	87	36
Mean ± SD		14±13	14±11	9±13	12±9	20±15	23±16	21±16	23±14	52±28	27±11
D	1	7	7	25	14	14	14	14	14	14	51
	2	21	7	7	7	36	7	14	36	-	18
	4	11	0	0	7	14	7	14	29	29	51
	7	29	14	29	14	29	22	22	22	51	87
	8	7	7	7	0	7	7	7	0	0	22
	9	7	7	7	7	0	7	7	29	22	7
Mean ± SD		14±9	7±4	12±12	8±5	17±13	11±6	13±6	22±13	23±19	39±29
E	22	11	137	203	7	36	22	14	7	36	36
	23	43	80	72	58	7	65	14	36	14	22
	24	43	29	29	36	-	116	36	22	14	130
	25	7	94	239	0	0	14	14+			
	26	29	65	94	65	36	22	58	58	51	94
	28	43	-	232	14	43	58	25	87	94	58
	29	87	181	101	7	22	7	22	58	22	14
	30	0	51	36	0	14	7	0	11	14	7
Mean ± SD		33±28	91±52	126±86	23±26	23±16	39±38	23±18	40±29	35±29	52±46

- No sample
+ Animal died 15 weeks after infection

Appendix Table 8.1.3A

Peripheral eosinophil counts from uninfected rats (A) and rats infected with 20 metacercariae of F. hepatica (B).

Group No.	No. of Rats	Weeks after infection										
		0	2	4	6	8	10	12	14	16	18	20
A	5	6	10	6	11	14	9	10	6	11	17	11
		10	9	8	6	10	11	22	14	12	6	17
		11	17	22	6	16	11	6	22	28	11	11
		16	17	11	17	22	22	11	17	17	17	22
		28	22	17	17	11	10	16	14	12	17	16
Mean ± SD		14±8	15±5	13±7	11±5	15±5	13±5	13±6	15±6	16±7	14±5	15±5
B	25	17	89	89	106	94	57	39	39	44	72	61
		17	133	606	689	328	139	194	156	528	300	44
		22	411	683	700	61	244	322	244	306	411	39
		0	250	961	722	239	283	144	311	606	544	28
		6	322	833	894	272	267	328	833	1067	667	267
		33	67	83	133	50	28	28	39	83	128	61
		11	189	556	1011	461	211	122	278	633	550	89
		0	206	750	745	378	89	228	417	378	83	28
		50	89	89	133	94	56	28	72	89	33	33
		11	361	861	878	239	194	500	333	406	611	56
		0	456	683	806	533	200	389	617	728	450	17
		12	128	1122	1150	489	322	406	489	750	917	128
		33	189	333	844	717	311	90	350	556	433	83
		28	150	739	1022	311	206	261	572	511	833	72
		6	150	572	800	589	350	50	161	167	361	67
		61	250	761	1033	283	183	294	389	450	444	39
		28	467	900	1178	683	161	594	428	1106	589	22
		22	250	806	844	200	272	83	156	233	128	106
		6	150	511	617	411	44	217	389	683	483	33
		22	367	339	572	239	161	261	57	528	461	28
		22	194	883	850	856	933	144	450	811	744	33
		0	144	672	911	156	233	239	411	522	294	39
		17	139	472	833	706	28	178	156	567	450	33
		11	183	756	733	583	222	172	394	589	433	79
		0	78	294	1050	122	200	289	172	811	383	28
Mean ± SD		17±16	216±118	614±281	770±287	364±230	216±176	224±146	316±197	526±278	432±231	60±51

Appendix Table 8.1.3B

Peripheral eosinophil counts from rats infected with 20 metacercariae each (A), infected (20 metacercariae), treated with anthelmintic and left uninfected (C), infected (20 metacercariae), treated with anthelminctic and implanted intraperitoneally with adult fluke in diffusion chamber (D) and infected (20 metacercariae), treated with anthelmintic and reinfected with 20 metacercariae each of F. hepatica (E).

Group No.	Rat No.	Weeks after Infection									
		2	4	6	8	10	12	14	16	18	20
A	1	650	1344	1944	467	472	994	428	628	400	450
	2	411	1178	1378	1089	328	400	561	644	472	106
	3	472	1150	1289	828	539	522	506	589	689	328
	4	350	861	1278	522	344	339	583	667	622	1161
	5	133	1122	1156	217	311	872	906	750	628	539
Mean ± SD		403±188	1131±174	1409±309	625±339	399±101	625±292	597±182	656±60	562±121	517±395
C	20	94	44	89	78	89	22	33	50	72	56
	21	33	44	28	61	67	83	17	67	44	50
	14	122	61	78	22	56	78	56	39	72	50
	13	50	44	33	28	22	56	39	22	178	94
	15	44	44	28	67	56	28	94	117	61	50
	16	50	61	28	78	44	78	56	28	28	83
	18	67	73	94	128	72	128	78	56	94	61
	19	72	83	61	33	56	72	78	39	94	33
	11	89	33	72	78	56	50	28	61	72	89
Mean ± SD		69±28	54±16	57±28	64±33	58±18	66±32	53±26	53±28	79±43	63±21
D	1	111	133	150	83	122	44	111	78	33	39
	2	100	72	44	78	89	11	94	11	122	28
	4	450	139	72	44	94	28	83	78	106	33
	7	94	122	67	39	44	0	33	17	50	50
	8	156	144	56	50	44	50	56	0	11	17
	9	133	139	100	67	78	78	78	100	56	44
Mean ± SD		174±137	125±27	81±38	60±18	78±30	35±28	76±28	47±43	63±43	35±12
E	22	344	1261	956	250	344	244	289	250	278	372
	23	1044	1033	1094	761	767	983	194	267	433	156
	24	94	211	56	17	89	106	117	89	72	94
	25	372	722	1533	611	150	400	33+			
	26	372	950	672	444	456	172	167	444	294	283
	28	583	1117	1833	328	322	394	339	550	228	300
	29	883	1000	1006	239	367	306	572	628	106	733
	30	133	467	900	139	167	256	278	194	94	133
Mean ± SD		478±339	845±354	1006±534	349±246	333±216	358±272	249±164	346±198	215±132	296±217

+Animal died 15 weeks after reinfection.

Appendix Table 8.1.4A

Faecal egg counts (e.p.g.) from rats infected with 20 metacercariae each of F. hepatica (B).

Group No.	No. of Rats	Weeks after infection									
		6	7	8	10	12	14	16	18	19	20
		0	0	0	24	53	22	85	0	0	0
		0	2	46	228	287	158	335	238	0	0
		0	2	41	95	66	0	170	113	0	0
		0	0	4	56	477	25	73	64	0	0
		0	-	-	5	414	208	204	379	39	24
		0	0	1	0	0	0	6	2	0	0
		0	0	0	35	338	239	248	611	0	0
		0	1	40	-	255	51	445	163	0	0
		0	0	21	27	0	0	0	-	0	0
		0	0	36	-	62	356	369	483	0	0
		0	0	35	123	137	94	294	216	5	0
		0	3	69	349	120	56	554	357	0	0
B	25	0	0	11	59	10	409	409	109	1	0
		0	-	-	42	475	194	381	308	0	0
		0	-	-	16	22	122	56	90	0	0
		0	0	0	75	160	43	190	237	0	0
		0	9	89	140	-	136	132	3	0	0
		0	1	41	84	23	116	78	37	0	0
		0	0	9	36	225	202	353	251	0	0
		0	0	14	2	302	206	390	171	0	0
		0	0	24	453	841	186	408	349	0	0
		0	0	3	63	179	241	300	94	0	0
		0	0	0	10	142	28	190	168	0	0
		0	0	-	43	104	78	596	27	0	0
		0	3	52	117	192	315	532	90	0	0
Mean ± SD			1±2	25±25	90±113	203±199	139±115	272±173	190±160		

- No sample

Appendix Table 8.1.4.B

Faecal egg counts (e.p.g) from rats infected with 20 metacercariae each (A), infected (20 metacercariae), treated with anthelmintic and left uninfected (C), infected (20 metacercariae), treated with anthelmintic and implanted intraperitoneally with adult fluke in diffusion chamber (D) and infected with 20 metacercariae, treated with anthelmintic and reinfected with 20 metacercariae of F. hepatica (E).

Group No.	Rat No.	Weeks after Infection									
		4	6	7	8	10	12	14	16	18	20
A	1	0	0	26	365	424	162	316	341	650	46
	2	0	0	7	70	492	594	298	456	848	125
	3	0	0	26	203	294	375	553	528	446	114
	4	0	0	0	108	467	1454	262	569	668	43
	5	0	0	3	128	254	1217	323	189	740	103
Mean ± SD		0	0	12±13	175±117	386±106	760±553	350±116	417±154	670±148	86±39
C	20	0	0	0	0	0	0				
	21	0	0	0	0	0	0				
	14	0	0	0	0	0	0				
	13	0	0	0	0	0	0				
	15	0	0	0	0	0	0				
	16	0	0	0	0	0	0				
	18	0	0	0	0	0	0				
	19	0	0	0	0	0	0				
	11	0	0	0	0	0	0				
D	1	0	0	0	0	0	0				
	2	0	0	0	0	0	0				
	4	0	0	0	0	0	0				
	7	0	0	0	0	0	0				
	8	0	0	0	0	0	0				
	9	0	0	0	0	0	0				
E	22	0	3	54	-	-	96	-	128	335	43
	23	0	0	-	25	257	151	177	189	680	267
	24	0	0	-	0	0	0	0	0	0	0
	25	0	0	23	135	247	111	111+			
	26	0	0	0	17	72	34	312	43	116	60
	28	0	0	14	478	430	477	559	317	474	116
	29	0	0	14	22	110	881	566	464	-	78
	30	0	0	0	35	69	215	162	-	-	17
Mean ± SD		0	0.4±1	17±20	102±172	169±149	246±296	270±220	190±175	321±273	83±90

- No sample

+Animal died 15 weeks after reinfection

Appendix Table 8.2.1

Serum glutamic dehydrogenase activities from bullocks given single (L2 and L6) and repeated (L3) infections, implanted after given single infection (L7, L4 and L17) and implanted after given repeated infections (203).

Bullock No.	Weeks after Infection			
	0 (Pool)	$6_{(1)}$	$2_{(2)}$	$6_{(2)}$
L2	11 ± 2.1	89 ± 2.1		
L6	3 ± 2.8	18 ± 2.1		
L3	9 ± 0.4	164 ± 2.8	112 ± 1.8	337 ± 3.8
L4	10 ± 2.8	49 ± 2.5	85 ± 2.1	22 ± 1.4
L7	16 ± 4.2	85 ± 1.8	136 ± 2.8	217 ± 2.8
L17	14 ± 2.8	89 ± 2.8	6 ± 1.4	181 ± 1.4
203*			124 ± 1.4	11 ± 0.7

*Weeks $2_{(3)}$ and $6_{(3)}$ for this bullock

Appendix Table 8.2.2

Eosinophil counts from bullocks given single infection (L2 and L6) and repeated infections (L3), implanted after given single infection (L7, L4 and L17) and implanted after given repeated infections (203).

Bullock No.	Weeks after Infection			
	0 (Pool)	$6_{(1)}$	$2_{(2)}$	$6_{(2)}$
L2	50 ± 14	972 ± 59		
L6	61 ± 27	81 ± 18		
L3	22 ± 10	1456 ± 82	2444 ± 291	1542 ± 34
L4	42 ± 14	1261 ± 71	2400 ± 156	242 ± 42
L7	75 ± 7	1242 ± 20	258 ± 42	778 ± 90
L17	47 ± 17	103 ± 7	1775 ± 47	700 ± 25
203*			519 ± 30	764 ± 65

*Weeks $2_{(3)}$ and $6_{(3)}$ for this bullock

Appendix Table 8.3.1

ANOVA table of flukes in groups of rats immunised with IBS from bullocks 176, L2 and L6

Source of variance	df	SS	MS	F	
Between treatment	2	2.2157	1.1078	0.46	N.S.
Within treatment	14	33.6667	2.4048		
Total	16				

$F.05^{(2,14)} = 3.74$

Appendix Table 8.3.2

ANOVA table of flukes in groups of rats immunised with IBS from bullocks L7, L17 and L4

Source of variance	df	SS	MS	F	
Between treatment	2	21.0	10.5	2.56	N.S.
Within treatment	15	61.5	4.1		
Total	17				

$F.05^{(2,15)} = 3.68$

Appendix Table 8.3.3

ANOVA table of GD activities in groups of rats immunised with IBS from bullocks 176, L2 and L6

Source of variance	df	SS	MS	F	
Between treatment	2	643.0745	321.537	0.18	N.S.
Within treatment	14	25269.8669	1804.99		
Total	16				

$F.05^{(2,14)} = 3.74$

Appendix Table 8.3.4

ANOVA table of GD activities in groups of rats immunised with IBS from bullocks L7, L17 and L4

Source of variance	df	SS	MS	F	
Between treatment	2	2902.11	1451.055	0.56	N.S.
Within treatment	15	38886.833	2592.4555		
Total	17				

$F.05^{(2,15)} = 3.68$

Appendix Table 8.3.1.1

Mann-Whitney U-test analysis of flukes recovered at post-mortem as shown in Table 8.3.1.

Group comparison	U-value	Probability
A v B	60.5	> .10
A v C	18.5	> .10
A v D	71.5	> .10
A v E	28.5	< .10
E v B	81.0	< .025
E v C	30.0	< .05
E v D	72.0	> .10
E v F	27.0	< .05
A v F	15.5	> .10
B v F	56.0	> .10
D v F	65.0	< .10

Appendix Table 8.3.5

Serum glutamic dehydrogenase activities from rats infected with 20 metacercariae each and immunised with pooled normal sera from bullocks L2, L3, L4, L6, L7 and L17 (A), immune bovine sera from bullocks 176, L2 and L6 collected at week 6(1) (B), immune bovine serum from bullock L3 collected at week 6(2) (C), immune sera from bullocks L7, L17 and L4 collected at week 8 after implantation (D) and immune bovine serum collected at week 8 after implantation from bullock 203 previously given two infections (E).

Group No.	Bullock No.	Weeks p.i.	Rat No. 1	2	3	4	5	6	Mean ± SD
A	Pooled	0*	7	22	0	0	7	14	8.3 ± 8.5
		4	210	174	188	188	195	159	185.7 ± 17.5
		6	268	80	203	22	210	188	161.8 ± 92.0
		Fluke Numbers	5	4	2	7	10	1	4.8 ± 3.3
B	176	0*	0	7	0	0	0	0	1.2 ± 2.9
		4	224	123	80	173	177	181	159.7 ± 50.5
		6	246	116	174	123	159	51	144.8 ± 65.4
		Fluke Numbers	4	3	2	3	4	3	3.2 ± 0.7
B	L2	0*	14	7	0	0	7	7	5.8 ± 5.3
		4	159	159	188	116	D	101	144.6 ± 35.4
		6	210	101	94	109		203	143.4 ± 57.9
		Fluke Numbers	4	5	5	3	-	3	4.0 ± 1.0
B	L6	0*	0	0	14	7	0	0	3.5 ± 5.9
		4	166	80	181	152	138	185	150.3 ± 38.7
		6	145	0	43	166	152	51	92.8 ± 69.9
		Fluke Numbers	3	1	6	4	2	7	3.8 ± 2.3
C	L3	0*	7	14	14	7	7	7	9.3 ± 3.6
		4	195	145	181	188	166	101	162.7 ± 35.1
		6	72	181	195	174	7	0	104.8 ± 89.8
		Fluke Numbers	5	6	6	6	2	2	4.5 ± 2.0
D	L7	0*	7	14	14	0	0	0	5.8 ± 6.9
		4	188	138	116	203	87	159	148.5 ± 43.8
		6	72	58	58	181	275	116	126.7 ± 86.6
		Fluke Numbers	2	1	1	5	1	3	2.2 ± 1.6
D	L17	0*	0	7	7	7	0	7	4.7 ± 3.6
		4	174	166	138	138	195	87	149.7 ± 37.7
		6	80	203	130	210	109	7	123.2 ± 76.8
		Fluke Numbers	3	4	1	1	6	1	2.7 ± 2.1
D	L4	0*	7	7	0	7	7	0	4.7 ± 3.6
		4	181	188	185	203	185	174	186.0 ± 9.6
		6	268	58	261	145	211	159	183.7 ± 79.7
		Fluke Numbers	4	6	4	8	3	2	4.5 ± 2.2
E	203	0*	7	7	0	0	11	7	5.3 ± 4.4
		4	116	130	123	152	112	94	121.2 ± 19.4
		6	174	101	7	181	87	145	115.8 ± 65.4
		Fluke Numbers	2	3	0	4	2	0	1.8 ± 1.6

*Administration of infection

D Animal died

Names and Addresses of Commercial Companies

Arnold, Richfield Avenue, Reading, Berkshire, England

Astra Pharmaceuticals Ltd., Watford, Hertfordshire, England

Becton Dickinson & Co. Ltd., N. Ireland

BDH Chemical Ltd., Poole, Dorset, England

BOC Ltd., London, England

Boehringer Corp. (London) Ltd., Sussex, England

Boots the Chemist, Beeston, Nottingham, England

Camlab, Cambridge, England

Crompton, England

Cyanamid, Gosport, Hampshire, England

Dynatech Laboratories Ltd., Billingshurst, Sussex, England

Endecotts Ltd., London, England

ESCO Rubber Co. Ltd., Teddington, Middlesex, England

Evans Medical Ltd., Greenford, England

Flow Laboratories Ltd., Irvine, Ayrshire, Scotland

Gibco Europe Ltd., Uxbridge, Middlesex, England

Glaxo Laboratories Ltd., Greenford, Middlesex, England

Hughes & Hughes Ltd., Romford, Essex, England

ICI, Macclesfield, Cheshire, England

Jobling Laboratory Division, Stone, Staffordshire, England

Luckham Ltd., Burgess Hill, Sussex

MacFarlane Robson Ltd., Thornliebank, Glasgow, Scotland

May & Baker Veterinary Products Ltd., Dagenham, Essex, England

Microflow Ltd., Fleet, Hants., England

Millipore Corp., Bedford, Massachusetts, U.S.A.

MSE Ltd., Manor Royal, Crawley, Sussex, England

Nordic Immunological Laboratories, Maidenhead, Berkshire, England

Oxoid Ltd., London, England

Parke Davies & Co., London, England

Portex Ltd., Hythe, Kent, England

Pye Unicam Ltd., York Street, Cambridge, England

Sarstedt Ltd., Beaumont Leys, Leicester, England

Sellotape Products Ltd., Boreham Wood, Hertfordshire, England

Sherwood Ltd., Medical Industries, Deland, Florida, U.S.A.

Sigma Chemical Co., St. Louis, Missouri, U.S.A.

E.R. Squibb & Sons Inc., Princeton, New Jersey, U.S.A.

Sterilin Ltd., Teddington, Middlesex, England

Stewart Plastic Products, Edinburgh, Scotland

Turnball & Wilson, South Clerk Street, Edinburgh, Scotland

W & R Balston Ltd., England

Woolworth F.W. & Co. Ltd., Edinburgh, Scotland

Vickers Instrument Ltd., York, England

List of Abbreviations and Symbols (excluding standard abbreviations for SI units)

ADP	Adenosine 5'-diphosphate
ANOVA	In statistics: Analysis of variance
AT	In figure: Anthelmintic treatment
At	In GD activated technique: change in O.D. at 340 nm ($25^{o}C$) per minute
c.	with
D	In Appendix Table: animal died
d	In GD activated technique: light path
DPNH	Diphosphonucleotide, reduced form
ELISA	Enzyme-linked immunosorbent assay
e.p.g.	Eggs per gram faeces
g	centrifugal force
GD	Glutamic dehydrogenase
H_2O	Water
IBS	Immune bovine serum
ip	Intraperitoneal
Kr	Kilorentgen
mc	metacercariae
NAD	Nicotinamide-adenine dinucleotide, oxidised form (Boehringer)
NADH	Nicotinamide-adenine dinucleotide, reduced form (Boehringer)
NH_4	Ammonium
NS	In statistics: not significant, probability more than 0.10.
O.D.	Optical density (absorbance)
OPD	Orthophenylene diamine (Sigma)
P_1	In Appendix Table: high titre serum
P_2	In Appendix Table: low titre serum

PBS	Phosphate buffered saline (Dulbecco "A" - Oxoid)
p.i.	In Appendix Table: post-infection
p.v.c.	polyvinyl chloride
RPMI	Rosewell Park Memorial Institute cell culture medium - 1640 (Gibco)
t	In statistics: Student t-test
V	In GD activated technique: total reaction volume
v	In GD activated technique: sample volume
1°	Primary
2°	Secondary
-	In Tables: animal died; in Appendix Tables: no sample and not done
ε	In GD activated technique: extinction coefficient of NADH/ NADPH at 340 nm

To describe the sera the following notation has been used:-

$T_{(i)}$, where T = number of weeks since last infection

i = number of successive infections received by the animals