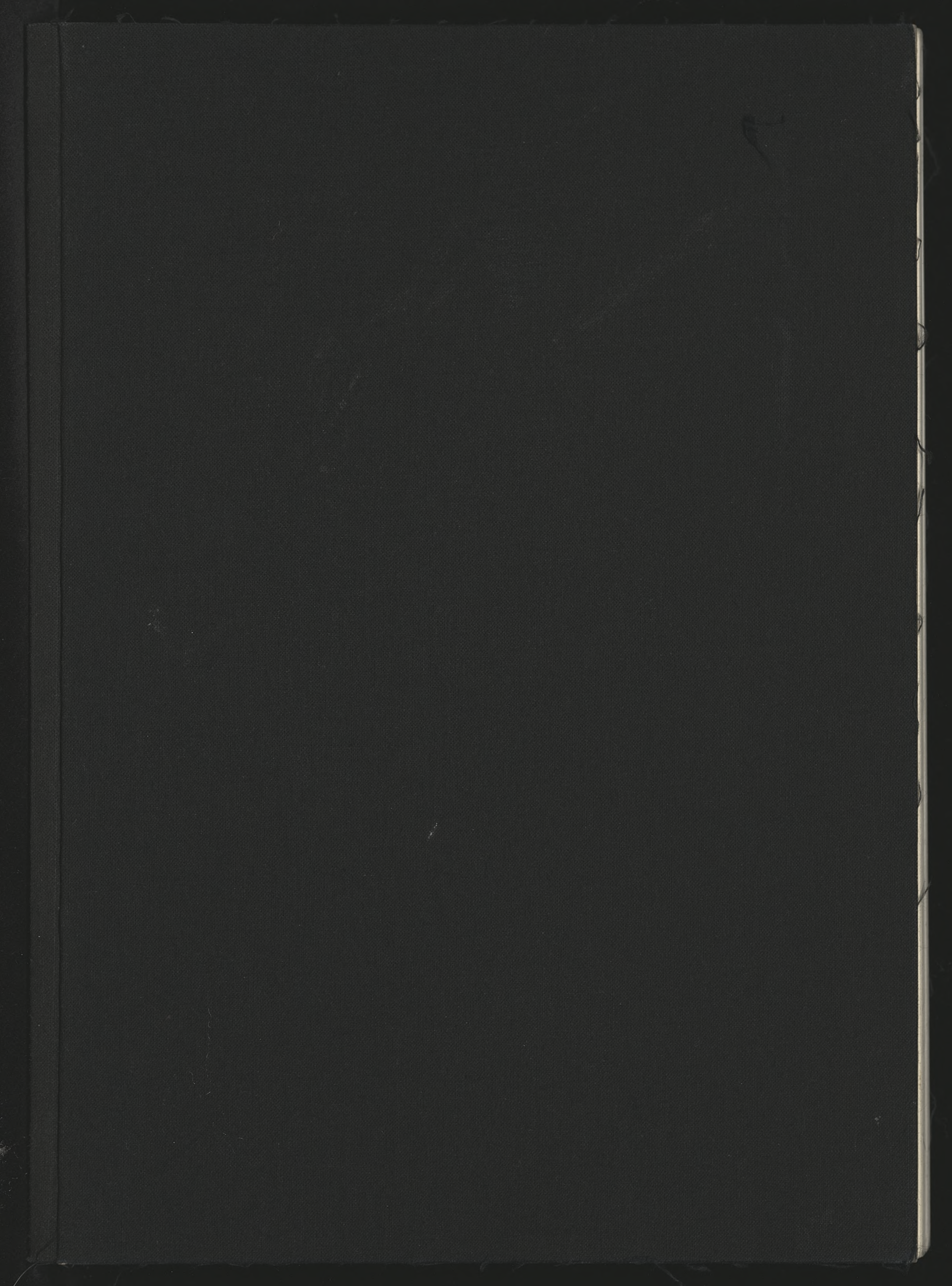

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AN INVESTIGATION INTO THE USE OF MICE TO MONITOR THE PROTECTIVE EFFECT OF *FASCIOLA* IMMUNE SERUM

BY

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ABSTRACT

A review of the literature of immunity to fascioliasis is presented. Following a description of the materials and methods used, an account of attempts to demonstrate immunity to *Fasciola hepatica* in mice by passive transfer of immune serum from rats is given. No immunity to *F. hepatica* was demonstrated. The possible reasons for this are discussed and suggestions are made for further studies.

INTRODUCTION

Fasciola hepatica (Linnaeus 1758) is a digenetic trematode commonly found in its adult form in the bile ducts of its mammalian host. It causes great economic losses in livestock resulting from the pathogenic effects produced during the course of the infection. These effects were summarized by Dargie (1975) as (a) antigenic stimulation leading to elevated serum globulins and eosinophilia, (b) liver damage resulting in elevated serum enzymes, anaemia and hypoalbuminaemia, (c) inappetance with a consequent loss of weight or poor weight gain, (d) haemorrhage via the bile due to the blood sucking activities of adult parasites. The severity of these effects is influenced by many factors including the number of flukes in the host.

The economic importance of the parasite to the livestock industry has stimulated research on host-parasite relationships and comprehensive data has become available on experimental infections of small laboratory animals. Mice, rats, rabbits and guinea-pigs were found to be very useful experimental animals (Boray, 1969), particularly the mouse which Lang (1967) suggested was suitable for immunological work.

Although some mammalian species can acquire a considerable ability to resist reinfection with *F. hepatica* (Doyle, 1971; Goose and MacGregor, 1973a) it has not yet been possible to produce a vaccine.

Attempts to transfer immunity with the use of *Fasciola* immune serum has shown significant results in some species of laboratory

animals (Dargie, Armour and Urquhart, 1973; Hayes, Bailer and Mitrovic, 1974a and 1974b). The present study was designed to demonstrate the possibility of using mice to monitor the protective effect of such immune serum.

A REVIEW OF THE LITERATURE

I. IMMUNITY TO FASCIOLIASIS

There are two generally recognised species of Fasciola but there are others of disputed validity. Fasciola hepatica (Linnaeus, 1758) and F. gigantica (Cobbold, 1855). The former parasite is distributed in the temperate areas of the world, Australia and the Americas. It is also found in the tropics at high altitudes (Dinnik and Dinnik, 1959; Kendall, 1965). F. gigantica is widespread in the tropics and subtropics (Hammond, 1970).

The production of a vaccine against these parasites would greatly help in control programmes and hence reduce the losses in livestock, particularly in countries where snail infested water is the only source and the use of fasciolicides is very expensive.

A. Ruminants

1. Cattle It is generally recognized that cattle are more resistant to both F. hepatica and F. gigantica than sheep (Taylor, 1949). After a primary infection they are resistant to challenge even when the primary infections are drug terminated (Ross, 1966; Boray, 1969).

Kotlan (1953) stated that mainly young cattle suffer from clinical fascioliasis and adult animals exposed to repeated infection showed a high degree of resistance to reinfection. Davtyan (1956) found that in cattle, F. hepatica was less infective but more pathogenic than F. gigantica.

Previous investigations in cattle (Ross, 1965) showed that with high level single infections of 2,500 to 15,000 metacercariae (m/c) of *F. hepatica*, a severe parenchymal reaction develops around the immature flukes. This inhibited the development of the migrating young flukes and only small numbers developed to adult flukes in the bile ducts. Following challenge infections it was observed that migrating parasites in the parenchyma were more prone to inhibition than in single infections of comparable levels.

Acquired immunity to *F. hepatica* in calves was studied by Ross (1967). Extracts of immature parasites were injected into calves and a retarded growth of the challenge infection was observed. He also found that with low level challenge infection (200 m/c) given 18 weeks after a similar initial infection an "acquired self cure" was observed whereby the existing adult infection was expelled. This phenomenon did not exist in the case of *F. gigantica* infections and this would support the suggestion that resistance to *F. gigantica* infection in cattle may be merely related to the fibrosity of the liver parenchyma (Hammond, 1970).

Doyle (1971) found that calves previously infected with *F. hepatica* were highly resistant to reinfection as only 16 percent of the fluke burden of the controls was recovered from the reinfected calves.

Doyle (1973) found that resistance to reinfection could not be demonstrated in calves with seven-week-old infections. However, significant resistance was demonstrated in calves with twelve-week-old infections; only 27.5% of the fluke burden found in the previously uninfected control calves was recovered.

Corba, Armour, Roberts and Urquhart (1971) protected a calf against reinfection by the transfer of lymphoid cells from its identical twin which had been infected 14 weeks previously. The transfer of 2.4 l serum failed to protect.

The pathogenesis of the disease caused by *F. gigantica* in cattle is not dissimilar from that caused by *F. hepatica* (Sewell and Hammond, 1974). The chronic disease in cattle is self-limiting with both parasites in that the numbers of adult flukes in the bile ducts begin to fall soon after patency.

Infectivity of *F. gigantica* in cattle tends to be greater than that of *F. hepatica* and the infection is more prolonged compared with infections of similar magnitude with *F. hepatica*. It was therefore concluded that *F. gigantica* is better adapted to cattle than *F. hepatica* (Hammond and Sewell, 1975).

2. Sheep There is no evidence that sheep become immune to reinfection with *F. hepatica* (Boray, 1969; Taylor, 1980). Boray (1969) found that repeated infections terminated with anthelmintics did not produce any resistance to reinfection, though the hepatitis was less severe after a challenge dose than in sheep with primary infections.

The development and pathogenicity of *F. hepatica* were compared in two groups of sheep, one previously uninfected and the other which had its infection terminated four weeks post-infection (Boray, 1969). There was evidence of acquired resistance in the challenged group as judged by a marked but temporary retardation of fluke development about eight weeks after infection. Recoveries

of flukes were similar but there was some evidence of delayed entry to the bile ducts in the challenged group.

Serum from sheep with 12- to 14-week-old infections conferred protection on rats. As little as 2-5 ml given intraperitoneally on the day of challenge and repeated two days later gave a reduction of 34% in total worm burden. Doses of 5 ml and 10 ml gave much better results (Dargie, Armour and Urquhart, 1973; Armour and Dargie, 1974).

Meek and Morris (1979) were unable to demonstrate the development of immunity in sheep given different levels of infection with F. hepatica m/c. No significant differences in the mean number, prepatent periods or fecundity of the flukes, established as a result of the challenge dose of m/c, were detected between the control and experimental groups. It was concluded that previous infection with F. hepatica conferred no significant resistance to a future challenge.

(a) Heterologous immunity in ruminants

Cysticercus tenuicollis has a migratory pattern similar to F. hepatica, is non-pathogenic for sheep and stimulates a strong immunity to homologous challenge. The possibility of cross-immunity between it and the liver fluke was investigated by Campbell, Kelly, Townsend and Dineen (1977); Dineen, Kelly and Campbell (1978). Campbell et al. (1977) found that infection of sheep with C. tenuicollis for 12 weeks generated a high level of protection (79.5%) against intra-ruminal challenge with m/c of F. hepatica as measured by recovery of flukes from liver and bile ducts and counts of fluke

eggs in faeces. The animals were resistant whether challenge was superimposed upon the cestode infection or after removal with mebendazole. However, Dineen, et al. (1978) found that sheep in which the initial C. tenuicollis infections were terminated by a cesticidal drug at 12 weeks post-infection, were resistent to the primary infection with F. hepatica but became fully susceptible after nine months. These results suggest that maintenance of resistance depends on persistence of the cestode infection.

Hughes, Harness and Doy (1978) were unable to replicate these results in sheep, cattle or goats. However, there were some differences in the two trials, only one of which was considered important by Hughes et al. (1978). This was the use by Campbell et al. (1977) of levamisole in the sheep before the trial started. Levamisole is known to be an immunostimulent and may have accounted for the results obtained.

(b) Attempts to vaccinate ruminants

Early attempts using irradiated m/c did not give good results in both cattle and sheep (Boray, 1969).

Later attempts used higher doses of irradiation (Armour, Dargie, Doyle, Murray, Robinson and Rushton, 1974). Two groups of seven calves were given two doses, one month apart, of 1,000 3.5 KR-irradiated m/c. One group was challenged at four weeks, the other at eight. Reduction in worm burdens were 30% and 70% respectively. However considerable liver damage occurred in the vaccinated animals.

Details of a field trial of a similar vaccine were given by Nansen (1975). He obtained a significant resistance to natural

infection in the field in calves infected with m/c attenuated at a gamma-irradiated level of 3 K rad. The mean fluke burden of vaccinated calves was 29% of the controls. The gamma-ray dose of 3 K rad did not prevent a small proportion of the irradiated m/c reaching and maturing in the bile ducts.

Culture incubate antigens and sonicated antigens prepared from 16-day-old flukes were used by Hall and Lang (1978) in trials involving 36 cattle. The animals were challenged with 200 m/c 38 or 100 or 200 days after vaccination and slaughtered 65-115 days later. Multiple doses of culture incubate antigens or culture incubate antigen plus sonicated antigens resulted in hypersensitivity and liver damage. Protection from multiple doses of the vaccine (culture incubate and sonicated antigens combined) was not as good as a single dose of sonicated antigen only. A reduction in worm counts of 98% was obtained after a subcutaneous injection of the sonicated antigen. Although this trial was very successful the problem of producing the vaccine economically still exists.

B. Laboratory Animals

It is well known that certain species of laboratory animals can develop a considerable resistance to *F. hepatica* following a primary infection with that parasite. Host-parasite relationships have been intensively studied (Lang, 1967, 1968 and 1974a; Boray, 1969; Smithers, 1975).

On the other hand, little is known about pathogenicity and immunity of *F. gigantica* (Cobbold, 1855) infection in laboratory animals.

(a) Fasciola gigantica

A number of authors have worked with F. gigantica in laboratory animals. Mango, Mango and Esamal (1972) studied the susceptibility of small laboratory animals to infection with F. gigantica. Rabbits and guinea pigs were given 25 m/c and mice, rats and hamsters were given 20 in one experiment. In another experiment each animal received only 5 m/c. They found that guinea pigs and hamsters were highly susceptible to infection, mice and rabbits were less susceptible to infection, but rats were completely refractory. None of these laboratory animals excrete F. gigantica eggs in the faeces. This seemed to indicate that F. gigantica is not capable of developing to maturity in these animals. They concluded that these laboratory animals are not suitable hosts for F. gigantica.

These findings were confirmed later in the same year by Srivastava and Singh (1972). They used guinea pigs only after failing to produce infection in other laboratory animals. Sixteen guinea pigs were given either 200, 500 or 1,000 F. gigantica m/c orally. Only a total of 15 flukes were recovered from abdominal cavities and liver parenchyma of three guinea pigs. These flukes had failed to enter the bile ducts of guinea pigs and hence did not reach maturity. The authors concluded that guinea pigs are not very susceptible to infection.

Rabbits were also infected with 500 and 1,000 m/c (Srivastava and Singh, 1972). No pathological changes were seen in livers of rabbits infected with 500 m/c. Those receiving 1,000 m/c showed some pathological changes which were described by the author as

subacute as compared with infection in sheep.

Due to the above limitations most workers have used F. hepatica instead of F. gigantica in laboratory animals.

(b) Fasciola hepatica

1. Mice Wikerhauser (1961) noted a reduction in the pathogenicity of metacercariae of F. hepatica for mice following x-irradiation with 3,000 roentgens. Similar results were obtained by Hughes (1962a).

Dawes (1964) failed to demonstrate immunity to reinfection 22 days after dosing mice with 40 cysts irradiated with 3 KR. He suggested shortening the interval between immunizing and challenge doses.

Lang (1967a) immunized mice with two stimulating infections of two F. hepatica m/c given 60 days apart. Forty days later a challenge infection of the same size was given to immunized and control mice. In the experimental mice killed 20 days later there was an earlier migration of the flukes from the liver parenchyma to the common bile duct. Fluke recoveries were 2.25 adults and 1.2 immature flukes from the second infection. The controls were found to harbour 1.6 immature flukes. Forty days after initial infection these mice had significantly fewer worms (2.1 adults and 0.5 immatures each) than the controls (1.5 immature flukes). Lang suggested that various host measurements (body weight, total and differential leukocyte count, spleen weight, haemagglutinating antibody titre and liver histopathology) indicated a much more rapid response by the experimental animals on the basis of the

nature and timing of lymphocyte infiltration in, and the histopathology of, the liver. He also suggested that delayed (cellular) hypersensitivity may play a prominant role in the earlier migration of the flukes to the common bile ducts. Lang (1967b) demonstrated even more rapid response and a lower fluke burden in the recipient mice following the transfer of peritoneal exudate cells from isologous donors, than in the controls.

Lang (1968, 1972) studied factor proper for the release of the specific antigens responsible for the induction of acquired immunity. He transferred 8- and 16-day-old flukes into mice by the peritoneal routes. He found that the transferred 8-day-old flukes were present in the liver parenchyma of recipient mice for 21 to 22 days while the 16-day-old flukes were in the parenchyma for only 13 to 14 days. These flukes were used as immunizing infections in two groups of mice to control the duration of the liver migration. When transferred flukes had a total age of 40 days, immunized mice were given a challenge infection of two m/c per mouse. At 25 days after challenge fewer flukes were recovered from mice immunized with 8- and 16-day-old flukes compared to the controls which had been infected with metacercariae _per os_. He suggested that young flukes are capable of inducing acquired immunity during the entire liver migration. The duration of the liver migration, at least 10 to 11 days, and not the specific age of the young fluke is responsible for the stimulation of acquired immunity (Lang, 1974a).

The migratory behaviour of young flukes in immunized and control mice has been studied by Harness, Hughes and Doy (1976a,

1976b, 1977a, 1977b). They immunized two groups of mice with either one oral dose of 20 irradiated m/c per mouse or two such doses given seven days apart. Three weeks later both the immunized and the control groups were dosed orally with 100 normal m/c. Two days later the immature flukes were recovered from the peritoneal cavities of the mice as described by Harness et al. (1973). The number of flukes recovered from both immunized groups of mice was significantly lower than that obtained from the control groups. This was thought to indicate that a protective mechanism operated at the intestinal wall. However after flukes were recovered from liver parenchyma two days after infection the author suggested that this apparent immunity was probably due to an accelerated migration from the peritoneal cavity into the liver. However when the immunizing infection was allowed to mature there was a significant reduction in recoveries from the liver of 14-day-old flukes of the challenge infection.

The reason for this effect on the migration of the challenge infection, in mice carrying an immature infection, is not fully understood but a severe inflammatory cellular reaction occurred in the intestinal wall of the sensitized mice. The inflammation started at six hours post-infection in sensitized mice and reached a peak at two days. In the controls this severe inflammation was not observed. Six or twelve hours after challenge the same number of migrating flukes in each group were recovered from the peritoneal cavities, while at two days three times the number of flukes reached the liver in the sensitized mice.

Lang (1974a) studied the role of early migration in the immune response of the mouse. He surgically transferred juvenile flukes, 12-, 14-, 18- and 24-day-old, into the peritoneal cavities of normal recipient mice. These flukes were found to enter the common bile duct when their total age was 30 to 32 days; however he found that 12-day-old flukes spent 16 to 17 days migrating in the liver parenchyma before reaching the common bile duct whereas 20-day-old flukes spent only 3 days in migration. These mice were each challenged with 2 m/c 40 days after the infection of the donor mice and he found that the mean recoveries of the challenge infection from mice receiving 12-, 14- or 18-day-old flukes were 0.26 to 0.47 whereas in mice receiving the old flukes 1.1 to 1.5 flukes were recovered from each mouse. These flukes were then incubated in immune and normal serum before transfer to test the factors governing the induction of immunity (Lang, 1974b). This resulted in low recoveries when compared with incubation in normal or heat inactivated immune serum. This effect did not depend on the age of the fluke and this indicates that the duration of the liver migration is the prime factor governing the development of immunity to reinfection by F. hepatica in the mouse.

Attempts to immunize mice by administration of "fluke incubates" (excretory/secretory product) were made by Lang (1976). Lang incubated the flukes for four hours in a suitable medium and these "fluke incubates" were injected intraperitoneally 30 days before challenge. Mortality was significantly reduced in the vaccinated mice compared to controls but fluke recoveries were similar.

When flukes were incubated in serum from these vaccinated mice they showed poor survival compared with those incubated in normal serum.

On the other hand Rajasekariah, Mitchell, Chapman and Montague (1979) used *in vitro* excretory/secretory products of four-week immature and eight-week mature flukes. These products were administered intraperitoneally to mice followed by a 100 viable m/c *per os* -vaccination with excretory/secretory products derived from immature *F. hepatica* did not confer any significant protection.

2. *Rats* Hayes, Bailer and Mitrovic (1972) studied the effect of superinfection with *F. hepatica* in the rat in relation to immunity. They infected rats with five metacercariae (m/c) and repeated the infection 49 days later. Control rats received a similar infective dose on day 49. Flukes were recovered on day 70 after the first infection whereby the first and second infections were distinguishable. Controls given the initial infection only had a mean of 2.7 flukes in their bile ducts, while rats given only the second infection had 3.1 flukes in the liver parenchyma. Rats given both infections had 2.9 flukes in their bile ducts from the first infection and 0.23 flukes in the liver parenchyma from the second infection. It was therefore suggested that rats are highly immune to super-infection (92.5% reduction in flukes recovery). Age or weight of the rats at the time of infection did not alter fluke recoveries and the first infection was not detectably affected by the second. To test whether this constituted a specific immune response or a non-specific effect related to liver damage, Hayes

et al (1973) compared the response of rats receiving an initial dose of either one or ten m/c. Rats receiving ten metacercariae strongly resisted reinfection and those given one metacercaria showed a reduction of 76% as compared with the controls. There was only slight liver damage from the challenge infection in rats receiving one m/c and it was concluded that specific immunity was involved.

Goose and MacGregor (1973a, 1973b) initially infected rats with 30 m/c and found that they developed a marked degree of resistance to challenge. Reductions of 80 to 88.8% were obtained. Removal of immunizing infection by anthelmintic treatment at 2 to 13 weeks did not interfere with resistance to a challenge infection 10 to 14 weeks after the initial infection. The rats were also found to be resistant to infection with adult worms transferred intraperitoneally.

It was confirmed by Dargie (1973) that the removal of initial infection does not affect the development of immunity. Irradiated m/c were also believed to confer protection; however if infection was by subcutaneous or intraperitoneal injection the level of immunity was lower than that obtained from oral infection (Armour, Dargie, Doyle, Murray, Robinson and Rushton, 1974).

Eriksen and Flagstad (1974) surgically transferred adult F. hepatica from sheep, goats and cattle into the subcutaneous muscles of rats four weeks before challenge. They also found a decrease in infection rate as compared with controls.

Subcutaneous implantation of F. hepatica was also used by Anderson, Hughes and Harness (1975). They implanted one fluke in

the flank muscles. Histological examination showed changes believed to be characteristic of cell mediated immunity and antibody production was also induced in the prefemoral lymph node draining the site of implanatation. There was a positive intradermal hypersensitivity to fluke antigen and precipitating antibodies were found. When these rats were challenged orally with 20 metacercariae, a reduction of 34% in the number of flukes in the bile duct was obtained. However the histopathological changes in the liver in both groups were equally severe. These results provided further evidence for the role of acquired immunity in resistance to F. hepatica.

Studies on the influence of splenectomy on acquired immunity were carried out by Hayes, Bailer and Mitrovic (1975). They obtained a significant resistance to a second infection of F. hepatica. Thus splenectomy appeared to have no influence on the development of immunity.

Peritoneal recoveries of one- to two-day-old flukes were also reduced in immune rats (Hayes and Mitrovic, 1977). Rats given an initial infection of three or five m/c 7 or 11 weeks previously showed reductions of 83.9% to 99.0% when challenged.

A marked eosinophilic infiltration was observed in sections of small intestine two days after challenge, in rats with three-week-old infections (Doy, Hughes and Harness, 1978). This was in contrast to the findings in mice (Harness et al., 1977b). Subsequent liver recoveries confirmed that this was a real immunity and not an effect of fluke migration.

The possibility that the gut is the site of immunity to reinfection in the rat was investigated by Rajasekariah and Howell (1977). Although rats infected orally with five m/c seven weeks earlier showed no immunity when challenged intraperitoneally they were immune when challenged orally.

Hughes, Harness and Doy (1977) suggested that rats in which flukes have reached the bile ducts progressively lose their ability to stop the development of challenge infections. They also suggested that the flukes living in the bile ducts either do not produce the right antigenic stimulus or do not present it in the right situation for processing.

The early migratory stages of F. hepatica were found to be responsible for the activation of immunity in the rat (Rajasekariah and Howell, 1978). The authors sensitized the rats by subcutaneous implantation of either m/c, four-week-old immature flukes, adult flukes or eggs. These rats were challenged two weeks later and killed after eight weeks. There was a reduction in the worm burden in all groups except that implanted with adult flukes which failed to confer any significant resistance even in prolonged sensitization.

Immunity to reinfection in rats following termination of the initial infection at an early stage was studied by Haroun, Hammond and Sewell (1980a). Rats were given 20 m/c and treated with diamphenthide at four and five weeks post-infection. The dosing was repeated one week later. Another group of rats was similarly treated at 10 and 11 weeks post-infection. These two groups and a control group were challenged at the twelfth week. Mean fluke

recovery per rat was 0.9 for the rats with four-week-old terminated infection, 0.5 for the ten-week-old and 2.9 for the controls. It was concluded that immature infections confer better resistance to reinfection.

Rats were also found to be resistant to reinfection by implanting mature flukes subcutaneously or intraperitoneally in diffusion chambers (Haroun, Hammond and Sewell, 1980b). The resistance was not affected when the implants were removed two weeks before challenge. The authors concluded that the continuing presence of the sensitizing flukes was not necessary for the maintenance of resistance.

The effect of age of the host in resistance to reinfection was found to be influenced by the gut (Campbell, Kelly and Dineen, 1978). Rats were orally or intraperitoneally infected with m/c at 2-, 6-, 15- and 35-weeks-old. The number of flukes recovered decreased as host age increased. In two- and six-week-old rats equal numbers of flukes were recovered at four and ten weeks after infection. In 15-week-old rats fluke burdens four weeks after infection were significantly greater following intraperitoneal than oral infection. No effect on route or age of infection was found in 35-week-old rats.

Rats sensitized on day 0 and 12 by subcutaneous injection of ova and/or excretion/secretion products of adult flukes, failed to resist challenged infections. However, the flukes recovered from the rats sensitized by excretion/secretion products were found to be smaller in size than those recovered from control groups (Burden

and Hammet, 1980).

Immature and adult flukes were transferred by Hughes and Harness (1973a, 1973b) from rabbits or goats into rats, and from rats or goats into rabbits immunized against the donor with lymphoid tissues. The flukes were unaffected by the host antigen.

3. Rabbits Boray (1969) had successfully used rabbits as experimental hosts but failed to demonstrate significant immunity.

Rabbits were infected orally after one mature or two immature infections had been terminated by an anthelmintic. No resistance was observed on challenge (Haroun et al., 1980a, 1980b). They also attempted, without success, to demonstrate immunity in rabbits after intra-peritoneal implantation of flukes in diffusion chambers.

4. Experimental infection in other animals Hörchner, Dalchow, Berliner and Münchener (1972) infected 39 pigs at 3 to 4½ months of age with 90-2,500 F. hepatica m/c. No clinical signs were observed although mature flukes were recovered from 29 of these pigs.

Further work was carried out by Nansen, Anderson, Harmer and Rüsing (1972) to study the effect of age on development of resistance to infection in the pig. Pigs were infected with F. hepatica m/c shortly after birth and comparable numbers of flukes to those from very susceptible host species were recovered; whereas a considerable resistance was observed in pigs infected at eight weeks of age. The increasing resistance with age coincided with the development of a marked fibroblastic reaction in response to the infection. It was suggested that the fibrous tissues may act as a mechanical barrier to the migration of the parasite into the liver

parenchyma.

Monkeys, on the other hand, were found to be as susceptible as mice, rabbits or sheep to infection with F. hepatica (Tomimura, Kotani, Takemoto, Yokota, Yamagami and Yoshida, 1975). Eleven monkeys were infected experimentally with 20 to 800 m/c of the "Japanese species" of the liver fluke, Fasciola species and all were found to be highly susceptible to the infection.

Experimental data showed that the horse exhibits a pronounced resistance to the establishment of a liver fluke infection (Nansen, Anderson and Hesselholt, 1975). With oral doses of up to 800 m/c of F. hepatica a patent infection was established in only one out of ten horses. Histological, haematological and immunological results provided evidence to suggest that the majority of parasites were eliminated or immobolized at an early stage of the infection, presumably before reaching the liver. This hypothesis was supported by the finding that about 15% of the excysted flukes implanted intraperitoneally in two horses, succeeded in reaching maturity in the bile ducts.

(a) Heterologous immunity in laboratory animals

In an investigation of heterologous immunity rats and mice were infected with Taenia hydatigena and challenged with F. hepatica m/c. However no such immunity was demonstrated (Rajasekariah, Rickard, Montague and Mitchell, 1979).

Cross-immunity between T. taeniaeformis metacestodes and F. hepatica was studied in rats by Campbell, Kelly and Martin (1979). Resistance to homologous infection was present in rats challenged

with *T. taeniaeformis* at six weeks, and with *F. hepatica* at nine weeks post-infection. *F. hepatica* protected against *T. taeniaeformis* at four, eight or nine weeks but the reciprocal cross-immunity did not occur.

Cross-resistance in *Schistosoma mansoni* and *F. hepatica* was investigated in mice by Christensen, Nansen, Frandsen, Bjørneboe and Monrad (1978). A primary infection of 7 to 28-day-old *S. mansoni* did not stimulate a significant level of resistance to challenge with *F. hepatica*. In contrast, in older *S. mansoni* infections (54 to 65-day-old) there was a significant level of resistance to the challenge with *F. hepatica*.

Further trials on cross-resistance to *F. hepatica* were attempted using single-sex schistosome infections (Christensen, Monrad, Nansen and Frandsen, 1980). Single-sex *S. mansoni* infection in mice did not stimulate any detectable level of heterologous resistance to challenge with *F. hepatica* after 22 to 76 days. However statistically significant resistance to challenge with *F. hepatica* was demonstrated in the presence of patent mixed-sex *S. mansoni* infection.

Finch (1980) studied the cross-immunity between *S. mansoni* and *F. hepatica* by transferring serum from mice infected with the heterologous parasite species. There was some indication of immunity to *F. hepatica* but this was not statistically significant.

C. Passive transfer of immunity

1. *Mice* Lang, Larsh, Weatherly and Goulson (1967) found that mice given 2.75 million peritoneal exudate cells from donors with

35-week-old infection were partially immune to challenge three weeks later. Fluke recoveries were 40% in the challenge group compared with 75% in the controls.

It was shown by Lang (1976) that migrating flukes were debilitated after incubation in serum from mice with 25-day-old F. hepatica infections as measured by worm recovery and host mortality. However serum from mice with 100-day-old infections produced no such effect.

2. Rats A series of experiments showed that lymphoid cells transferred from rats infected with F. hepatica to isogenic recipients conferred a high degree of protection against challenge (70% reduction). Similar results were obtained with twin calves. On the other hand four ml of serum from ten-week-old infections failed to confer any significant protection in recipient rats (Corba, Armour, Roberts and Urquhart, 1971).

The degree of protection in rats by transferred serum was found to be directly related to the volume of serum. Fractionation of the immune serum indicated that the presence of IgG is essential for successful protection (Dargie, Armour and Urquhart, 1973).

Lymphoid cells from rats vaccinated with three doses of 20 irradiated m/c gave complete protection to recipient rats. Donors with three flukes failed to confer any protection whereas those harbouring six to seven flukes gave up to 80% reduction in the number of flukes recovered (Armour and Dargie, 1974).

Armour and Dargie (1974) also demonstrated successful transfer of immunity by using larger doses of serum than used by Corba et al.

(1971). Two intraperitoneal injections of 10 ml immune serum given two days apart conferred significant protection against infection. It was also observed that liver damage did not occur in animals receiving serum, whereas it occurred in lymphoid cell recipient rats where cellular infiltration was observed around the dead flukes. The authors suggested that the role of lymphocytes may be in a hypersensitivity reaction rather than a source of antibody.

A single intraperitoneal injection of 5 ml immune serum also resulted in a highly significant degree of resistance in rats exposed to _F. hepatica_ infection at the time of transfer (Hayes, Bailer and Mitrovic, 1974a). However, serum given 14 days after infection did not significantly affect the challenge infection. Thus the resistance is apparently expressed against flukes younger than 14 days.

Although serum from donors with seven to eight-week-old infections was effective in producing resistance, serum from rats infected for 25 weeks was not protective (Hayes, _et al._, 1974b). While immune serum given on the day of infection resulted in significantly fewer flukes that given two or four days after infection had only a slight effect, and when given six or eight days after infection had no effect. No effect was also observed when the serum was heated or incubated with dead or living flukes.

Rajasekariah and Howell (1979) found that serum from rats with a primary infection was not protective. This may have been due to the low fluke burdens in the donors (0.9 fluke/rat) (Armour and Dargie, 1974). Serum from rats which had two immunizing

infections however protected recipients from reinfection and liver damage.

Immune rat serum or gammaglobulins precipitated from such serum, transferred at the time of challenge and again two days later resulted in a significant level of resistance in terms of both the number of flukes recovered from the challenge infection and the serum glutamate dehydrogenase levels (Haroun, 1979).

In vitro studies showed that incubation of freshly excysted flukes in rat immune serum did not kill them but their development was impaired when injected intraperitoneally into rats (Howell, Sandeman and Rajasekariah, 1977). The authors suggested that the precipitate which formed round the flukes during incubation caused this impairment.

Several experiments failed to demonstrate resistance to infection with *F. hepatica* in rats following vaccination with metabolic products obtained from flukes maintained *in vitro* (Lehner and Sewell, 1979; Davies, Rickard, Smyth and Hughes, 1979). However such resistance was demonstrated in mice (Lang, 1976; Lang and Hall, 1977). The authors suggested this difference may be due to a different response to infection in mice and rats or it may reflect the differences in the developmental stages of the flukes used to prepare the culture incubate antigens.

No evidence has been found in the published literature that mice have been used to study the protective effect of *Fasciola* immune serum. Experiments on the use of mice to monitor such effect are therefore described.

II. SERUM ENZYME CHANGES DUE TO LIVER DAMAGE

Serum enzyme changes have been investigated in many diseases in man (Henley, Sorensen and Pollard, 1959) and have been found to be useful aids to diagnosis and prognosis.

Boyd (1962) showed in rats with experimental hepatic necrosis that there was an increase in certain serum enzyme levels and he suggested that this was due to the leakage of these enzymes from damaged liver cells.

Ford and Boyd (1962) experimentally produced liver cellular damage in cattle, using dimidium bromide, to study the serum enzymme changes. They found a marked increase in the serum levels of glutamic oxaloacetic transaminase, lactic dehydrogenase, isocitric dehydrogenase and glutamic dehydrogenase. They concluded that serum enzyme levels are sensitive indicators of early and mild hepatocellular damage.

An increase in serum glutamic dehydrogenase activity gave little indication of the severity of the infection but it could be used as an aid to prognosis during the acute stages of fascioliasis (Sewell, 1967). This hypothesis was used in the present study to measure the degree of damage cuased by the migrating young flukes in the liver.

MATERIALS AND METHODS

A. Production of infective material

Metacercariae (m/c) were produced in the laboratory using the method described by Sewell (1961). Eggs of *Fasciola hepatica* were recovered from the faeces of experimentally infected sheep at the Centre for Tropical Veterinary Medicine, University of Edinburgh. After shedding m/c were suspended in distilled water and kept at $4^{o}C$.

B. Experimental animals

1. Rats Twenty five-week-old Porton-Wistar males were used weighing approximately 280 to 325 g each.

2. Mice These were T.O. or C.F.1(random bred albino) females (20-30 g) according to availability.

Both species were kept in groups of five in separate cages. Food pellets (Oxoid modified diet 41B, Wade Road, Basingstoke, Hampshire) and water were provided *ad lib*.

C. Infection procedures

1. Oral route

(a) Rats (immune serum donors) Metacercariae were examined microscopically before use and only those with typical morphology of viability (Hayes, Bailer and Mitrovic, 1972) were selected. Twenty viable cysts were counted and transferred in distilled water to a WHO haemagglutination plate well. Each rat was then anaesthetized by ether and the cysts were administered by oesophageal intubation using a rubber tube fitted to an 18-gauge blunt needle and a 2 ml syringe. The syringe, needle, rubber tubing and WHO

plates were coated with 'Repelcote' (Hopkins and Williams, Chadwellheath, Essex) to prevent cysts sticking. The syringe was washed out in distilled water in a WHO plate well and the washings were examined for cysts to check accurate inoculum delivery. If any were present they were administered to the rat.

(b) Mice The same technique was also used but the mice were not anaesthetized.

2. Intraperitoneal route (I/P)

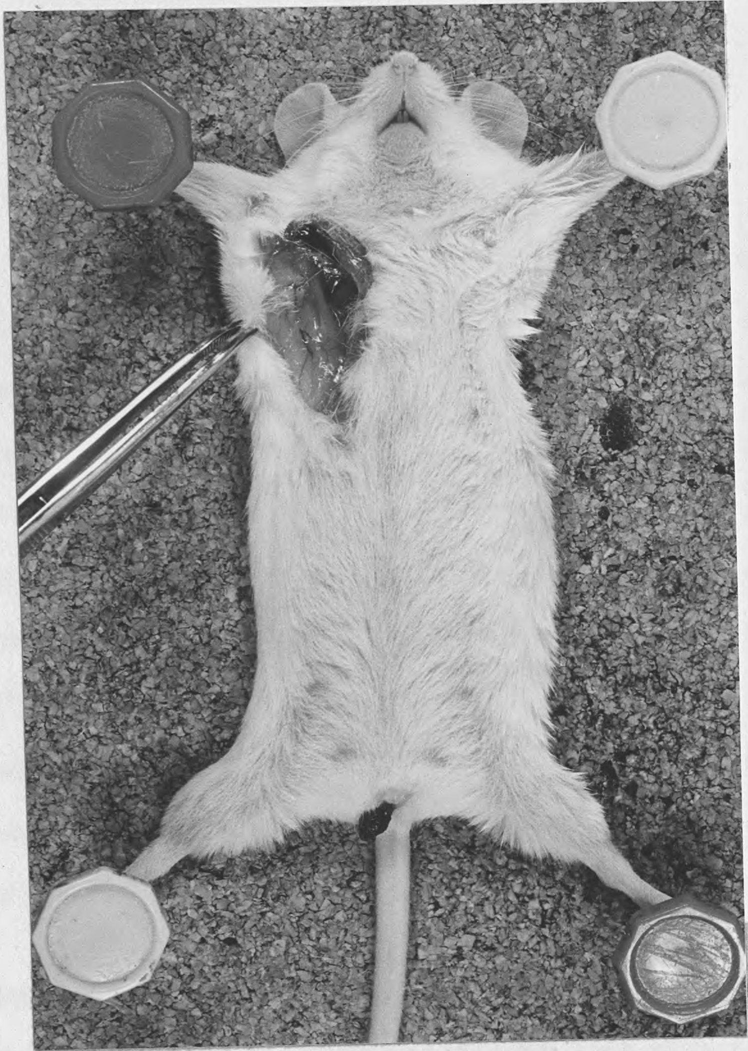
Mice Four-day-old m/c were used. Excystation procedure used was as described by Wikerhauser (1960). Young excysted flukes were placed in fresh sterile 0.8% saline in WHO haemagglutination plate wells and washed in two changes of 0.8% saline to get rid of the artificial digestive juice used for excystation.

Intraperitoneal infection procedure was modified from that used by Lang and Dronen (1972); an 18-gauge needle was used to inject the newly excysted flukes instead of 16-gauge because of the possible leakage of fluid when using 16-gauge needle. Syringes and needles were also coated with 'Repelcote'. The young excysted flukes readily passed through the 18-gauge needle without cooling to 13°C as described by Lang and Dronen (1972). Mice were starved overnight before injection.

D. Serum collection

1. Rats Rats were anaesthetized by ether and an incision was made along the ventral right side (Plate 1) to expose the brachial vein and artery which were then excised. More blood was obtained than using the heart puncture method. Blood was collected in

Plate I: Bleeding of a mouse



universal bottles, left on the bench for one hour and then kept in the refrigerator at 4°C overnight. It was then centrifuged at 2500 r.p.m. for twenty minutes. Serum was then separated, pooled and stored at -20°C.

2. Mice The procedure described above was also used. Blood was collected in individually labelled bijou bottles. Sera were assayed on the same day of collection.

E. Post-mortem procedure and recovery of flukes from mice

Mice were anaesthetized by ether and bled out. The livers were then removed and placed in individually labelled Petri dishes containing enough warm 0.8 %. saline to cover them and incubated at 37°C for one hour. Gross pathological changes were recorded. The livers were sliced and incubated to recover the flukes, as described by Harness, Doy and Hughes (1977a). Flukes that had migrated out from the liver tissues into the saline were counted and removed. The livers were then gently squeezed using the back of curved forceps to remove any remaining flukes. The entire suspension was then examined under a dissecting microscope and the total number of flukes recovered from each liver was recorded.

F. Serum glutamate dehydrogenase assay (GLDH)

The method used was described in Boehringer test kits (Mannheim GmbH, Mannheim, West Germany). Chemicals used and preparations are listed in Appendix II. The test was performed in one ml cuvettes. Serum (0.1 ml) was added to one ml buffer prewarmed to 25°C, mixed well and left for three minutes at 25°C in a water bath. Absorbance (A_1) was then measured using the sp 1800 spectro-

photometer (Pye Unicam Ltd., York Street, Cambridge) at wave length 340 nm. The machine was zeroed against air. Absorbance 2 (A_2) was measured five minutes after reading A_1 and α-ketogluterate (0.1 ml) was added to the above mixture. Absorbance 3 (A_3) was then measured immediately and absorbance (A_4) after five minutes.

Change of absorbance (ΔA) was then calculated. This is the activity of GLDH in international units per litre of serum.

$$\Delta A = \frac{(A_3 - A_4) - (A_1 - A_2)}{6.3} \times \frac{1.2}{0.1} \times \frac{1}{5} \times 1000 \text{ units/litre}$$

i.e.

$$\Delta A = \frac{(A_3 - A_4) - (A_1 - A_2)}{6.3} \times \frac{\text{Volume of cuvette}}{\text{Volume of sample}} \times \text{Dilution factor} \times 1000$$

EXPERIMENTAL DESIGNS AND RESULTS

A. Experiment I: Pilot study to determine the optimum dose of metacercariae of F. hepatica using the oral route

1. Experimental design Two batches of 20 mice each were used. Each batch was further divided into four groups of five mice each (A_1-A_4 and B_1-B_4). The mice of Groups A_1 and B_1 each received one metacercaria; Groups A_2 and B_2 each received two; Groups A_3 and B_3 each received four, and Groups A_4 and B_4 were not infected. The mice were examined every other day and the general health was recorded. Mice within each group were not individually labelled. Dead mice were necropsied. The mice of Groups A_1-A_4 were killed after two weeks from infection and Groups B_1-B_4 after three. Mice were bled for serum, carcases and livers were weighed, flukes recovered were counted and measured and any pathological changes in the livers were recorded.

2. Results One mouse in Group B_2 died two days after infection and showed no pathological changes when necropsied. Mice in both groups showed no abnormal clinical signs during the course of infection.

The mice in Groups A_1-A_4 were killed two weeks after infection. One mouse in Group A_1 showed haemorrhagic lesions in the liver and one fluke 3 mm long was recovered from it. Two other mice showed slightly engorged gall bladder but there was no evidence of fluke infection. The other two mice showed no pathological lesions. The livers and carcases of Groups A_1-A_4 were not weighed. The mean serum glutamate dehydrogenase level in Group A_1 was higher than

in the controls.

Only one mouse in Group B_1 was found to harbour one fluke. Although there was evidence of infection no flukes were recovered from the other three mice.

Three mice in Groups A_2 and B_2 showed evidence of infection with serous fluid in the peritoneal cavity. Three flukes were recovered from each of Groups A_3 and B_3. All the mice in both groups showed haemorrhagic tracks in the livers. There was a slight difference in the liver weights, carcase weights and serum enzyme levels between any of the groups (Table I and Appendix Table I).

3. Discussion The infection rates were low in this experiment. This is in accord with the concept of the "wastage" of potential flukes developed by Dawes and Hughes (1964) who reported recovery rates of between 15 and 55%. They also quoted that the technique of administration is acknowledged to be a factor in the infection rate obtained.

As the carcase weights were only slightly affected by these infections they could not be used as an aid to diagnosis. Similarly only very slight changes were found in the weights of the infected livers.

Normal glutamate dehydrogenase levels in mice were not found in the literature and therefore it was difficult to compare the results obtained from the control groups in this experiment. The great difference between the serum enzyme levels in the controls of both groups can not be explained.

Table I Results of pilot study to determine the optimum dose of metacercariae of **F.** hepatica using the oral route

Group[a]	Number of mice	Mean carcase weight (gm) $\pm$ SD[b]	Mean liver weight (gm) $\pm$ SD	Flukes/mouse $\pm$ SD	Infection rate (%)	Fluke length (mm) $\pm$ SD	SGD[c] $\pm$ SD U/L
A_1	5	-	-	0.2 ± 0.4	20	3.0	19.05 ± 3.81
A_2	5	-	-	0.2 ± 0.4	20	4.0	21.33 ± 20.01
A_3	5	-	-	0.6 ± 0.8	60	2.7 ± 0.47	19.81 ± 24.38
A_4	5	-	-	0	0	-	0
B_1	5	30.8 ± 0.98	2.6 ± 0.2	0.2 ± 0.4	20	5.0	15.24 ± 9.64
B_2	5 (4)	32.25 ± 1.79	2.75 ± 0.75	0.2 ± 0.4	20	4.0	38.08 ± 27.10
B_3	5	32.8 ± 2.92	2.8 ± 0.24	0.6 ± 0.8	60	3.7 ± 0.47	25.14 ± 16.26
B_4	5	31.4 ± 0.8	2.7 ± 0.24	0	0	-	12.19 ± 3.7

[a] A_1 received one m/c; A_2 received two; A_3 received four; A_4 controls
B_1 received one m/c; B_2 received two; B_3 received four; B_4 controls
Group A killed after 2 weeks; Group B at three

[b] SD = standard deviation

[c] SGD = Serum glutamate dehydrogenase. Units/litre of serum.

Although the mice in Groups A_2 and B_2 had lower fluke burdens (0.2 ± 0.4) than the mice in Groups A_3 and B_3 (0.6 ± 0.8) the serum enzyme levels were higher. However this enzyme is not liver specific (Boyd, 1962) so the levels found in Groups A_2 and B_2 could have been due to leakage from other tissues. However no lesions were found at post-mortem examination which might have caused such change. Although glutamate dehydrogenase has this limitation to its use as an indication of liver damage it was decided to use it in the next experiment in order to obtain further information.

The optimum infective dose was found to be four m/c as this resulted in infections in a much higher proportion of the mice than with the lower dose levels. An infection time of three weeks was found to be better than two weeks because flukes recovered at this time were relatively longer and were easier to find under stereo-microscope.

B. <u>Experiment II</u>: <u>Pilot study to determine the optimum number of juvenile F. hepatica using the intraperitoneal route</u>

1. <u>Experimental design</u> Two batches of 20 mice each were used. Each batch was further divided into four groups of five mice each (A_1-A_4 and B_1-B_4). The mice of Groups A_1 and B_1 were uninfected controls; the mice of Groups A_2 and B_2 each received one juvenile fluke intraperitoneally contained in one ml 0.85% sterile saline; the mice of Groups A_3 and B_3 each received two flukes contained in one ml 0.85% sterile saline and those of Groups A_4 and B_4 each

received four flukes in 0.85% sterile saline. The mice were clinically examined every other day. Those within each group were not individually labelled. The mice of Groups A_1-A_4 were killed at two weeks after infection and those in Groups B_1-B_4 at three weeks. Serum was collected, carcases and livers were weighed and flukes recovered from each liver were counted and measured.

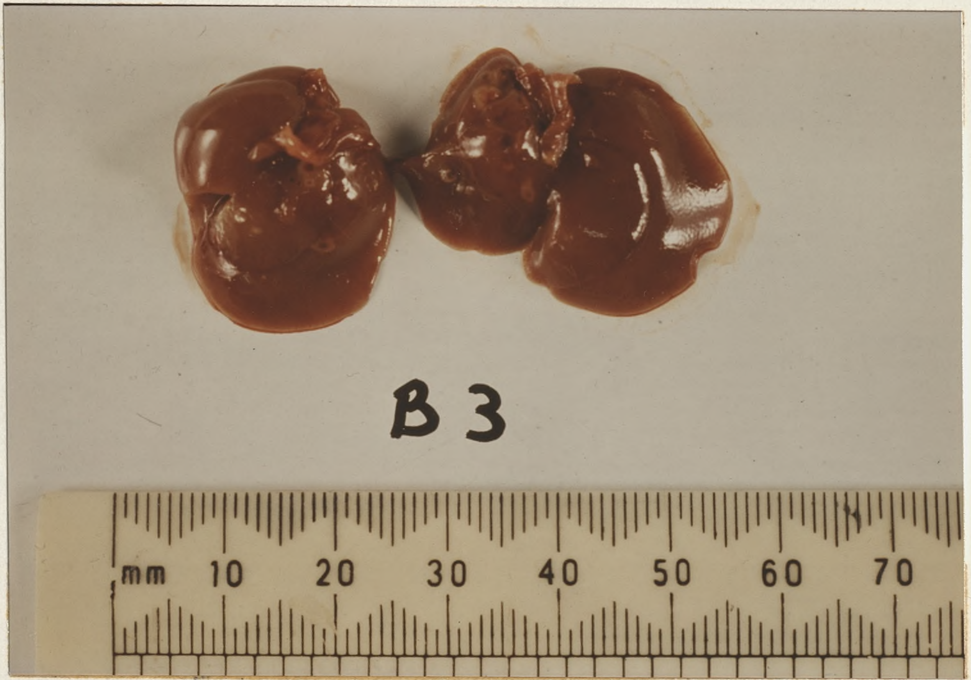
2. Results These are given in Table II and Appendix Table II. No deaths occurred in Groups A_1-A_4. Only one mouse in Group B_4 died 20 days after infection, and the post-mortem examination revealed haemorrhagic tracks in the liver. The peritoneal cavity contained bloody exudate. In general liver lesions in the B groups were more severe than in the A groups.

The liver and carcase weight changes in this experiment were not consistent. Two mice from Group A_3 showed characteristic migratory tracks in their livers, however, only one fluke measuring one mm was recovered from one of them. All the mice in Groups A_4 and B_4 and 40% of those in Groups A_3, B_2 and B_3 were infected. The other groups were not infected. Infection with two flukes caused only slight liver lesions (Plate II) whereas infection with four flukes caused more severe lesions (Plate III). The serum enzyme levels in Groups A_4 and B_4 were much higher than in the other groups.

3. Discussion It was clear that an infective dose of one or two juvenile *F. hepatica* caused little damage to the liver

Plate II: Macroscopic lesions in the livers of mice intraperitoneally infected with two juvenile *F. hepatica* for three weeks.

Plate III: Macroscopic lesions in the livers of mice intraperitoneally infected with four juvenile *F. hepatica* for three weeks.


B 3
mm 10 20 30 40 50 60 70

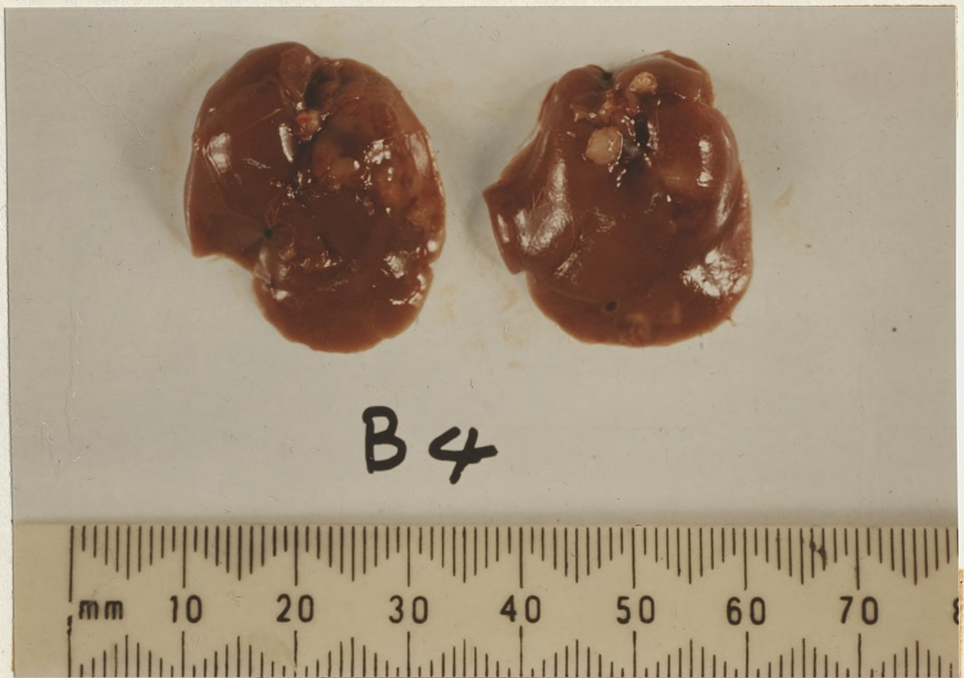

B 4
mm 10 20 30 40 50 60 70

Table II Results of pilot study to determine the optimum number of juvenile *Fasciola hepatica* using the intraperitoneal route

Group[a]	Number of mice	Mean carcase weight (gm) ± SD	Mean liver weight (gm) ± SD	Flukes/mouse ± SD	Infection rate (%)	Fluke length (mm) ± SD	SGD[c] U/L ± SD
A_1	5	24.7 ± 1.83	1.98 ± 0.04	0	0	0	0.76 ± 1.52
A_2	5	23.1 ± 1.56	1.92 ± 0.21	0	0	0	15.24 ± 3.41
A_3	5	23.3 ± 1.6	2.06 ± 0.23	0.2 ± 0.4	40	1.0	14.48 ± 12.14
A_4	5	23.9 ± 0.49	1.88 ± 0.19	1.4 ± 0.49	100	2.4 ± 0.44	41.14 ± 16.59
B_1	5	26.2 ± 1.75	1.74 ± 0.17	0	0	0	23.62 ± 15.69
B_2	5	24.6 ± 1.24	1.94 ± 0.08	0	40	0	29.72 ± 32.89
B_3	5	26.1 ± 1.66	1.96 ± 0.08	0.4 ± 0.8	40	4.5 ± 0.5	26.67 ± 22.86
B_4	5 (4)	26.25 ± 2.02	2.38 ± 0.41	1.25 ± 0.83	100	4.6 ± 0.49	57.14 ± 45.47

[a] A_1 and B_1 controls; A_2 and B_2 received one fluke intraperitoneally in 0.85% saline; A_3 and B_3 received two; A_4 and B_4 received four. Group A killed after 2 weeks and Group B after 3

[b] SD = standard deviation

[c] SGD = Serum glutamate dehydrogenase. Units/litre of serum.

parenchyma, as judged by the low levels of serum glutamate dehydrogenase. However in Experiment I the change in serum enzyme levels did not follow a consistent pattern and the possibility of enzyme leakage from other damaged tissues was a prime suspect. The increased serum enzyme levels which resulted from infection with four juvenile flukes indicated more liver damage as was supported by the necropsy findings.

The presence of tracks in the liver of one mouse from Group A_3 without the recovery of flukes was regarded as evidence of infection, the migrating flukes having died after entering the liver as was suggested by Eriksen and Flagstad (1974).

The higher infection rates following the intraperitoneal routes as compared with the oral routes would suggest that the oral infective doses had been "wasted" in the gut (Dawes and Hughes, 1964) or the metacercariae used were not able to excyst.

The optimum infective dose was found to be four juvenile flukes as this resulted in infections of all the mice. An infection time of three weeks was found to be better than two weeks because flukes recovered at this time were longer and were easier to find.

C. Experiment III: Serum donor rats

1. Experimental design Two groups of ten five-week-old rats each were used. One group received an oral dose of 20 metacercariae of *Fasciola hepatica* and was killed for immune serum at eight weeks. The other group was not infected and was killed for normal serum

at eight weeks.

2. Results These are given in Table III. Only one rat in the infected group died, 53 days after infection, and the post-mortem examination revealed extensive jaundice. Six flukes were recovered from the bile ducts of this rat. The liver showed severe lesions characteristic of fascioliasis.

The other infected rats also had liver lesions with enlarged bile ducts. Thirty nine flukes were recovered from the nine surviving infected rats.

3. Discussion Five-week-old rats are generally more susceptible to infection of *F. hepatica* than other age groups (Rajasekariah and Howell, 1977).

Armour and Dargie (1974) reported that at least six flukes in a rat induces better immunity than lower numbers. The infective dose of 20 m/c was therefore chosen with this in mind.

D. Experiment IV: An attempt to protect mice against oral infection with Fasciola hepatica by injection of serum from rats infected with F. hepatica

1. Experimental design Three groups of ten mice were used (A, B and C). Each mouse was infected with four metacercariae of *F. hepatica*. The mice in Group A were left as the infection controls. Immediately after infection the mice in Group B were each injected intraperitoneally with one ml normal rat serum. This was repeated two days later. Group C was similarly injected twice

Table III Results of necropsies of *F. hepatica* antiserum donors

Number of rats	Infection dose (m/c)	Number of flukes recovered at necropsy
10 (9)	20	3, 6, 6, 5, 5, 4, 3, 6, 1
		mean $\pm$ SD = 4.33 $\pm$ 1.63

All rats were killed after eight weeks

One rat died at 53 days after infection

with immune serum from rats which had been infected with 20 metacercariae eight weeks previously.

All the mice were killed three weeks after infection, serum was collected, carcases and livers were weighed and the flukes recovered from each liver were counted and measured.

2. Results These are given in Table IV and Appendix Table IV. No deaths occurred. Eight mice in Group A were found to be uninfected; the other two showed haemorrhagic tracks in their livers and bloody exudate was recovered from their peritoneal cavities. Only three flukes were recovered from these mice. Therefore the infection rate of this group was 20%.

The infection rate of the mice in Group B was 30%. Four flukes were recovered. All the infected mice in Group B showed severe lesions characteristic of fascioliasis.

Three of the mice in Group C showed evidence of infection. One fluke was recovered from each of them. The infection rate was similar to Group B(30%). There was a depression in the carcase weight in the mice of Group C compared with the other two groups but this was not significant. Similarly no significant difference occurred in the liver weights of the mice in all groups. The serum glutamate dehydrogenase level in the mice of Group C was higher than in the other two groups but, again, these differences were not significant.

3. Discussion The results obtained were not consistent. The mice receiving immune serum showed higher infection rates and

Table IV Results of an attempt to protect mice against infection with _Fasciola hepatica_ metacercariae by injection of serum from rats previously infected with _F. hepatica_

Group	Number of mice	Mean[a] carcase weight (g) $\pm$ SD	Mean[a] liver weight (g) $\pm$ SD	Mean fluke/mouse $\pm$ SD	Infection rate (%)	Mean fluke length (mm) $\pm$ SD	Mean[a] serum glutamate dehydrogenase (U/L) $\pm$ SD
A	10	27.6 $\pm$ 2.70	2.8 $\pm$ 0.24	0.3 $\pm$ 0.64	20	5.3 $\pm$ 0.47	3.42 $\pm$ 4.33
B	10	29.85 $\pm$ 2.88	3.05 $\pm$ 0.35	0.4 $\pm$ 0.66	30	5.2 $\pm$ 0.43	7.24 $\pm$ 9.09
C	10	25.8 $\pm$ 3.51	2.6 $\pm$ 0.3	0.3 $\pm$ 0.46	30	5.3 $\pm$ 0.47	16.76 $\pm$ 32.69

SD = standard deviation

[a]No significant difference using Analysis of variance

Group A received 4 flukes each; Group B received 4 flukes and normal rat serum; Group C received 4 flukes and immune rat serum

increased serum glutamate dehydrogenase levels than the infection control group. This difference cannot be explained satisfactorily other than to suggest that the metacercariae used were not all viable or may have failed to excyst. The technique of infection, as stated before, is acknowledged to be a factor in the low infection rate achieved (Dawes and Hughes, 1964), however, great attention was given to ensure accurate infective dose delivery.

As a result of this experiment it was decided to conduct another serum protection trial using the intraperitoneal route for infection instead of the oral.

E. <u>Experiment V</u>: <u>An attempt to protect mice against intraperitoneal infection with Fasciola hepatica by injection of serum from rats infected with F. hepatica</u>

1. <u>Experimental design</u> Three groups of ten mice were used (A, B and C). The mice were each infected intraperitoneally with four juvenile flukes. In Group B this was immediately followed by intraperitoneal injection of each mouse with one ml of normal rat serum and this was repeated two days later. Group C was similarly injected with immune serum obtained from rats which had been infected with 20 metacercariae eight weeks previously. Group A was left as the infection control.

All the mice were killed three weeks after infection, serum was collected, carcases and livers were weighed, and the flukes recovered from each liver were counted and measured.

2. <u>Results</u> These are given in Table V and Appendix Table V. No deaths occurred. Only one mouse, in Group C, was observed to have lost condition during the third week after infection. Ninety percent of the mice in Group A and 50% of those in Groups B and C were infected, however the differences in the infection rate were not significant ($X_1^2 = 2.6$, $p > 0.05$). The infected mice in all groups showed migratory lesions in the livers characteristic of fascioliasis, however, the lesions were less severe in the mice of Group C. The differences between the liver and carcase weights of all groups were not significant ($p > 0.05$). Similarly the difference between the number of flukes recovered from each group was not significant. The infection was followed by insignificant changes in the serum glutamate dehydrogenase levels in all groups.

3. <u>Discussion</u> Rats are recognized as being highly resistant to reinfection with <u>F. hepatica</u> (Hayes <u>et al</u>., 1972; 1973) and this resistance can be transferred with serum and is dose dependent (Dargie <u>et al</u>., 1973).

It was clear from the results obtained from this experiment that while homologous immune serum can confer a significant resistance against <u>F. hepatica</u> infection in the rat (Dargie <u>et al</u>., 1973; Haroun, 1979) it was unable to protect mice. This difference may reflect that the serum was not protective.

It appeared from the reduction in the fluke recoveries in mice receiving normal serum that a "flushing effect" of the serum on the juvenile flukes in the peritoneal cavity contributed to this

Table V Results of an attempt to protect mice against intraperitoneal infection with *F. hepatica* by injection of serum from rats infected with *F. hepatica*

Group	Number of mice	Mean[a] carcase weight (g) ± SD	Mean[a] liver weight (g) ± SD	Mean fluke/mouse ± SD	Infection rate (%)[b]	Mean fluke length (mm) ± SD	Mean[a] serum glutamate dehydrogenase (U/L) ± SD
A	10	24.9 ± 2.37	1.95 ± 0.17	1.3 ± 0.64	90	3.9 ± 0.93	96.33 ± 94.51
B	10	25.8 ± 2.92	2.0 ± 0.25	0.8 ± 0.87	50	3.8 ± 1.27	83.81 ± 86.95
C	10	23.95 ± 3.47	1.95 ± 0.15	0.7 ± 0.78	50	3.3 ± 1.03	87.24 ± 88.56

[a]No significant difference using Analysis of variance

[b]No significant difference using chi-squared

Group A received 4 flukes each; Group B received 4 flukes each and normal serum

Group C received 4 flukes and immune serum

reduction (Haroun, 1979). This suggested that the reduction in the fluke recoveries in mice receiving immune serum was due to a similar effect.

Haroun (1979) obtained a significant reduction in fluke recoveries using homologous immune serum in rats.

The difference between the group mean serum glutamate dehydrogenase levels was not significant and the range within groups was large. Therefore it is unlikely that these can be used as an indication of infection with _F. hepatica_.

The present results are surprising in view of the results of Armour and Dargie (1974), Hayes, Bailer and Mitrovic (1974c) and Haroun (1979) in the rat; it was expected that two injections of one ml immune serum each, two days apart, would be sufficient to protect mice against the infection with _F. hepatica_.

GENERAL DISCUSSION AND CONCLUSIONS

Despite the evidence that immune rat serum confers protection against challenge infections with *F. hepatica* in homologous recipients (Dargie *et al*., 1973; Haroun, 1979) the passive transfer to mice of this immunity was not demonstrated in this study. Haroun (1979) found that immune rat serum protected rabbits against infection with *F. hepatica* therefore its protective effect could be transferred and is not only confined to rats. It is suggested that the serum used in this study might have had no protective effect against *F. hepatica* infections even in the homologous host. Therefore it is suggested that in further work rats should be used as homologous controls to confirm that the serum is actually protective. It is also possible that the method of processing and handling of the serum affected the protective antibodies present. Furthermore some of the immune serum donors were found to harbour less than six flukes, which is the lowest number required to induce a satisfactory immunity (Armour and Dargie, 1974). Consequently the protective antibodies present were diluted when the serum was pooled.

It is also suggested that all further work is carried out using pooled serum only from rats harbouring at least six flukes. This could be achieved by infecting the serum donor rats with 20 m/c of *F. hepatica* and allowing them to carry the infection for eight weeks (Haroun, 1979).

The intraperitoneal route of infection using excysted flukes was found to be better than the oral route, in terms of fluke

recoveries and consistent results, although the oral route has been used with success by many authors (Lang, 1967).

Armour and Dargie (1974) and Haroun (1979) reported a direct relationship between the volume of serum transferred and the degree of resistance obtained. From their findings it was expected that a dose of one ml immune serum given immediately after infection and repeated two days later would be sufficient to protect mice against the infection with *F. hepatica*.

Serum glutamate dehydrogenase was found to be unsatisfactory as an indicator of infection with *F. hepatica* in the mouse. In particular there were large variations in serum levels in individual mice in all groups, including the uninfected controls. However Haroun (1979) obtained satisfactory results when he used this enzyme in studies on *F. hepatica* infections in rats and rabbits.

The practical significance of the present study lies in the potential use of mice instead of rats in passive transfer studies using serum from cattle or rats infected with *Fasciola hepatica*.

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APPENDIX I

Appendix Table 1 Experiment I - Results of pilot study to determine the optimum dose of m/c of *F. hepatica* using the oral route.

Groups A_1 - A_4

Group	Mouse	Carcase weight (g)	Liver weight (g)	Evidence[a] of infection	Flukes recovered	Fluke length (mm)	Serum enzyme level U/L
A_1	1	-	-	No	1	3	15.24
	2	-	-	No	0	-	15.24
	3	-	-	No	0	-	22.86
	4	-	-	No	0	-	22.80
	5	-	-	No	0	-	-
A_2	1	-	-	Yes	1	4	7.62
	2	-	-	No	0	-	45.71
	3	-	-	No	0	-	45.71
	4	-	-	No	0	-	0
	5	-	-	No	0	-	7.62
A_3	1	-	-	Yes	2	2,3	0
	2	-	-	Yes	1	3	0
	3	-	-	Yes	0	-	0
	4	-	-	No	0	-	45.71
	5	-	-	No	0	-	53.33
A_4	1	-	-	No	0	-	0
	2	-	-	No	0	-	0
	3	-	-	No	0	-	0
	4	-	-	No	0	-	0
	5	-	-	No	0	-	0

Mice in Group A_1 received one m/c each; Group A_2 received two; Group A_3 received four; Group A_4 were uninfected controls.
All mice were killed at 2 weeks after infection
a = evidence of infection = haemorrhagic tracks in liver

Appendix Table I Experiment I - Results of pilot study to determine the optimum dose of m/c of *F. hepatica* using the oral route. Groups B_1 - B_4

Group	Mouse	Carcase weight (g)	Liver weight (g)	Evidence of infection	Flukes recovered	Fluke length (mm)	Serum enzyme level U/L
B_1	1	29	2.5	Yes	0	-	22.86
	2	31	2.5	No	0	-	7.62
	3	31	3.0	No	0	-	7.62
	4	31	2.5	No	0	-	7.62
	5	32	2.5	Yes	1	5	30.48
B_2	1	34	2.5	Yes	1	4	53.3
	2	31	2.5	No	0	-	60.95
	3	34	4.0	No	0	-	-
	4	30	2.0	No	0	-	0
Dead	5	-	-	-	-	-	-
B_3	1	30	2.5	Yes	2	4,3	15.24
	2	29	2.5	No	0	-	26.67
	3	36	3.0	Yes	0	-	38.09
	4	33	3.0	Yes	1	4	0
	5	36	3.0	No	0	-	45.71
B_4	1	32	3.0	No	0	-	7.62
	2	32	2.5	No	0	-	15.24
	3	31	2.5	No	0	-	15.24
	4	32	3.0	No	0	-	15.24
	5	30	2.5	No	0	-	7.62

Mice in Group B_1 received one m/c; Group B_2 received two; Group B_3 received four; Group B_4 uninfected controls. All mice were killed at 3 weeks after infection.

Appendix Table II Experiment II - Results of pilot study to determine the optimum number of juvenile *F. hepatica* using the intraperitoneal route Groups A_1 - A_4

Group	Mouse	Carcase weight (g)	Liver weight (g)	Evidence[a] of infection	Flukes recovered	Fluke length (mm)	Serum enzyme level U/L
A_1	1	28.0	2.0	No	0	-	0
	2	25.0	2.0	No	0	-	3.81
	3	24.0	1.9	No	0	-	0
	4	24.0	2.0	No	0	-	0
	5	22.5	2.0	No	0	-	0
A_2	1	25.0	2.1	No	0	-	19.05
	2	24.0	2.0	No	0	-	11.43
	3	24.0	2.0	No	0	-	19.05
	4	21.0	1.5	No	0	-	11.43
	5	21.5	2.0	No	0	-	15.25
A_3	1	25.0	2.5	No	0	-	0
	2	24.5	2.0	No	0	-	0
	3	24.0	2.0	Yes	0	-	26.67
	4	20.5	1.8	No	0	-	19.05
	5	22.5	2.0	Yes	1	1	26.67
A_4	1	24.5	2.0	Yes	1	3	15.24
	2	24.0	1.9	Yes	2	3,2	57.14
	3	23.0	2.0	Yes	1	2	38.09
	4	24.0	2.0	Yes	2	2.5,2	60.95
	5	24.0	1.5	Yes	1	2	34.29

Mice in Group A_1 received no flukes; Group A_2 received one; Group A_3 received two; Group A_4 received four.

All mice were killed after 2 weeks.

a Evidence of infection = haemorrhagic tracks

Appendix Table II Experiment II - Results of pilot study to determine the optimum number of juvenile *F. hepatica* using the intraperitoneal route Groups B_1 - B_4

Group	Mouse	Carcase weight (g)	Liver weight (g)	Evidence of infection	Flukes recovered	Fluke length (mm)	Serum enzyme level U/L
B_1	1	28.0	1.8	No	0	-	19.05
	2	26.5	1.8	No	0	-	15.24
	3	25.0	1.6	No	0	-	22.86
	4	28.0	2.0	No	0	-	53.33
	5	23.5	1.5	No	0	-	7.62
B_2	1	24.5	1.9	No	0	-	15.24
	2	27.0	2.0	No	0	-	15.24
	3	23.5	2.0	Yes	0	-	95.24
	4	24.0	1.8	No	0	-	15.24
	5	24.0	2.0	Yes	0	-	7.62
B_3	1	23.5	1.8	No	0	-	15.24
	2	26.0	2.0	No	0	-	11.43
	3	25.5	2.0	Yes	0	-	45.71
	4	27.0	2.0	Yes	2	5,4	60.95
	5	28.5	2.0	No	0	-	0
B_4	1	27.0	2.5	Yes	2	5,5	83.81
	2	23.5	2.0	Yes	1	4	15.24
	3	25.5	3.0	Yes	2	4,5	118.09
	4	29.5	2.0	Yes	0	-	11.43
Dead	5	-	-	-	-	-	-

Mice in Group B_1 received no flukes; Group B_2 received one; Group B_3 received two; Group B_4 received four.
All mice were killed 3 weeks after infection.

Appendix Table IV Experiment IV - Results of serum transfer (infection via oral route) Group A and B

Group	Mouse	Carcase weight (g)	Liver weight (g)	Evidence of infection[a]	Flukes recovered	Fluke length (mm)	Serum enzyme level U/C
A	1	24.0	2.5	No	0	-	0
	2	32.0	3.0	No	0	-	0
	3	26.5	3.0	No	0	-	3.81
	4	28.0	3.0	Yes	2	5,6	3.81
	5	26.0	2.5	No	0	-	3.81
	6	31.0	3.0	No	0	-	0
	7	25.0	2.5	No	0	-	0
	8	27.0	3.0	No	0	-	0
	9	31.0	3.0	Yes	1	5	11.43
	10	25.0	2.5	No	0	-	11.43
B	1	33.0	3.0	No	0	-	3.81
	2	32.0	3.0	No	0	-	3.81
	3	32.0	3.0	No	0	-	0
	4	30.0	4.0	Yes	1	6	15.24
	5	24.0	3.0	Yes	1	5	11.42
	6	29.0	3.0	Yes	2	5,5	30.48
	7	32.0	3.0	No	0	-	3.81
	8	32.0	2.5	No	0	-	0
	9	25.5	3.0	No	0	-	0
	10	29.0	3.0	No	0	-	3.81

Mice in Group A received four m/c orally;
Group B received four m/c, and normal rat serum.
All mice were killed at three weeks after infection.
a Evidence of infection = haemorrhagic tracks in liver

Appendix Table IV Experiment IV - Results of serum transfer (Infection via oral route) Group C

Group	Mouse	Carcase weight (g)	Liver weight (g)	Evidence of infection[a]	Flukes recovered	Fluke length (mm)	Serum enzyme level U/L
C	1	24.0	2.5	No	0	-	3.81
	2	25.0	3.0	Yes	1	5	34.29
	3	21.0	2.0	No	0	-	0
	4	23.0	2.5	No	0	-	3.81
	5	26.0	2.5	No	0	-	0
	6	25.0	2.5	No	0	-	3.81
	7	35.0	3.0	Yes	1	6	0
	8	27.0	3.0	Yes	1	5	110.48
	9	27.0	2.5	No	0	-	3.81
	10	25.0	2.5	No	0	-	7.62

Mice in Group C each received four m/c orally, and immune rat serum.

Appendix Table V Experiment V - Results of serum transfer (Infection via intraperitoneal route) Group A and B

Group	Mouse	Carcase weight (g)	Liver weight (g)	Evidence[a] of infection	Flukes recovered	Fluke length (mm)	Serum enzyme level U/L
A	1	23.0	2.0	Yes	1	5	285.71
	2	26.5	2.0	Yes	1	5	3.81
	3	26.5	2.0	Yes	1	4	26.67
	4	24.0	2.0	Yes	2	5,4	118.09
	5	27.0	2.2	Yes	2	3,3	87.62
	6	20.5	1.5	No	0	-	7.62
	7	28.0	2.2	Yes	1	4	11.43
	8	24.0	1.9	Yes	2	4,3	53.33
	9	27.0	2.0	Yes	1	3	243.81
	10	23.0	1.9	Yes	2	2.5,5	125.71
B	1	23.0	1.9	No	0	-	19.05
	2	27.5	2.1	Yes	1	6	95.24
	3	22.0	1.8	No	0	-	15.24
	4	21.5	1.9	No	0	-	19.05
	5	30.0	2.0	No	0	-	3.81
	6	25.0	1.5	No	0	-	3.81
	7	29.0	2.5	Yes	2	5,5	213.33
	8	28.5	2.2	Yes	2	3,4	213.33
	9	26.5	2.0	Yes	1	3	49.52
	10	24.5	2.1	Yes	2	2,3	205.71

Mice in Group A each received four juvenile flukes intraperitoneally
Group B received four juvenile flukes, and normal rat serum.
All mice were killed at three weeks after infection.
a Evidence of infection = haemorrhagic tracks in liver

Appendix Table V Experiment V - Results of serum transfer (Infection via intraperitoneal route) Group C

Group	Mouse	Carcase weight (g)	Liver weight (g)	Evidence of infection	Flukes recovered	Fluke length (mm)	Serum enzyme level U/L
C	1	26.0	2.1	Yes	2	3,4	148.57
	2	23.5	1.8	No	0	-	30.48
	3	26.5	1.9	No	0	-	22.86
	4	26.0	2.3	Yes	1	4	34.29
	5	25.0	1.9	No	0	-	175.24
	6	26.5	2.0	Yes	1	3	95.24
	7	28.0	1.8	No	0	-	30.48
	8	21.5	1.9	Yes	1	5	297.14
	9	20.5	1.8	No	0	-	11.43
	10	16.0	2.2	Yes	2	2,2	26.67

Mice in Group C each received four juvenile flukes intraperitoneally and immune rat serum.

All mice were killed at three weeks after infection.

APPENDIX II

Chemicals

Solution 1. It is stable for a few months when stored at room temperature.

To make one litre of buffer (pH 8.0):

(a) Triethanolamine (64.5 m molar)

$$\frac{\text{Molecular weight x m molar}}{1000}$$

$$= \frac{185.6 \times 64.5}{1000} = 11.97 \text{ g/L}$$

(b) Ammonium acetate (129 m molar)

$$\frac{77.08 \times 129}{1000} = 9.94 \text{ g/L}$$

(c) Ethylene Diamine Tetra-acetic Acid (3.22 m molar)

(Dipotassium Salt, 2_2HO)

$$\frac{404.47 \times 3.22}{1000} = 1.30 \text{ g/L}$$

Mix (a) + (b) + (c) + 1000 ml distilled water

Solution 2. Stable for one week at 4°C.

To make one ml of the solution:

(a) Adenosine Diphosphate (32.2 m molar)
(ADP monosodium salt)

$$\frac{429.4 \times 32.2}{1000} = 13.8 \text{ mg/ml}$$

(b) Nicotinamide Adenine Dinucleotide (Reduced, 6.45 m molar)
(NADH)

$$\frac{709.4 \times 6.45}{1000} = 4.6 \text{ mg/ml}$$

(c) Lactic dehydrogenase (65 units/ml) (LDH)

Mix (a) + (b) + 0.1 ml solution (c) + 0.9 ml distilled water

Solution 3. Stable for a few months at room temperature.

To make 10.0 ml of the solution:

(a) α-ketogluterate (217.0 molar)

(Sodium salt)

$$\frac{168.1 \times 217}{1000} = 36.48 \text{ mg/ml} = 364.8 \text{ mg/10 ml}$$

Mix (a) + 10 ml distilled water

For one serum glutamate dehydrogenase assay:

Mix 2.5 ml from Solution 1 and 0.1 ml from Solution 2.

The mixture is stable for 24 hours at 4°C and for eight hours at 15-25°C.

APPENDIX III

Explanation of Units of Radiation

kR Kiloroentgen. 1kR = 1000 R

One roentgen measures the number of ion pairs produced within 1 ml of air under standard temperature and pressure by gamma rays or X-rays. Its value in SI units is:

1 R = 0.000258 C/kg (coulombs per kilogram)

Krad Kilorad. 1 krad = 1000 rad

One rad (radiation absorbed dose) represents the deposition of 100 ergs per gramme in any absorbing medium by any type of ionizing radiation. Its value in SI units is:

1 rad = 0.01 Gy (gray)

The conversion of exposure (measured in roentgens) to absorption (measured in rads) depends on the nature of the material exposed.

References: Cited by Finch (1980)

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