THE IMMUNE RESPONSE OF SHEEP TO
INFECTION WITH FASCIOLA HEPATICA

by

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This thesis is my own work, except where specifically acknowledged.

R.M. Sandeman
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ABSTRACT

In vitro culture techniques were used to study the immune responses of sheep to *Fasciola hepatica* L. A precipitate which forms on the tegument of juvenile flukes when they are cultured with serum from infected sheep was found to contain sheep antibodies and parasite antigens. Two antigen-antibody interactions were found to be involved in this precipitate and the antigens were apparently secreted from the tegument.

The levels of precipitating antibodies in sera from infected sheep were measured by an in vitro assay using juvenile worms. Antibody activity was observed 1 week after a primary oral infection of sheep with metacercariae.

Assays of the liver enzyme glutamate dehydrogenase and the bile duct enzyme γ-glutamyl transferase allowed the location and extent of the damage caused by flukes to be correlated with antibody activity. The highest levels of antibody in infected sheep were found throughout the liver migratory phase of the life cycle. After the flukes became established in the bile ducts antibody and enzyme levels dropped, suggesting that the worms were isolated from host responses. However, in some sheep fluctuations in both antibody and enzyme levels occurred after patency. It is suggested that these were caused by damage to the bile ducts mediated by adult flukes, resulting in leakage of bile and fluke antigens into the liver and peritoneal cavity.
Intermittent leakage could result in antigenic stimulation and give rise to the fluctuations seen in antibody activity. Moreover, the parasite and host material could cause tissue damage by stimulating anaphylactic and autoimmune reactions resulting in fluctuations in the levels of glutamate dehydrogenase.

Assays of the antibody and enzyme responses after a challenge infection showed that although there were increased levels of damage to the liver and the bile ducts, antibody levels decreased and did not respond to the challenge antigenic stimulus. Minor fluctuations in antibody and enzyme levels were a feature during the course of the challenge infection. The suppression of the precipitating antibody responses is suggested to be due to the release from damaged bile ducts of large amounts of adult fluke antigens which, when complexed with antibody to form immune complexes, may inhibit antibody formation.

Analysis of the sheep immunoglobulin classes involved in precipitate formation was carried out by the fractionation of serum from infected and normal sheep on Sephadex columns. In vitro assays and double immunodiffusion tests revealed that both IgM and IgG1 classes were involved 3 weeks after a primary infection in sheep but that subsequently and after a challenge infection IgG1 was the only subclass detected in the precipitate.
Further analysis of the antigen-antibody reactions involved in precipitate formation confirmed that 2 reactions took place to form the juvenile fluke antigen/sheep antibody precipitate. At least 3 reactions were involved in precipitate formation around semi-mature flukes in infected sheep serum and at least 4 reactions were involved in a precipitate which formed around adult flukes in infected sheep serum. The antigens secreted by juveniles and involved in precipitate formation were also apparently secreted by the semi-mature and adult stages.

The involvement of these antigens with the glycocalyx which overlies the tegumental membranes was shown by electron microscopy. It is suggested that this glycocalyx is involved in protection of the parasite from host immune responses and that the observed secretion of antigens is related to the constant turnover of this layer.

The effects of serum and cells from infected sheep on juvenile flukes were also examined *in vitro*. Mortality of juvenile flukes was not enhanced when they were cultured with infected sheep serum. In fact the contrary was true; high antibody levels were associated with slightly lower mortalities. There were effects of infected sheep serum on the ability of cultured flukes to survive when transferred to normal rats. This effect was not observed in normal sheep. It is therefore suggested that sheep, unlike rats, are deficient in some factor which acts in concert with
antibody to destroy flukes.

Attempts to vaccinate sheep and rats with the sheep antibody/juvenile fluke antigen precipitate were not successful, although some delay in the migration of flukes in vaccinated sheep was observed.
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Despite at least 50 years of research and control efforts, parasitic diseases still present enormous human and animal health problems. Approximately half the world's population (2,000 million) live in areas where malaria, schistosomiasis, filariasis, trypanosomiasis or leishmaniasis are endemic (Busvine, 1978). Parasitic diseases of animals are widespread wherever agriculture is practiced.

For many years the main measures used in attempts to control parasitic diseases have been drug and chemical treatments. Although these have been successful in some areas for limited periods, certain diseases, notably malaria, leishmaniasis and filariasis, are now increasing in prevalence because of the resistance of the parasites to drugs and of their intermediate hosts to insecticides (Van den Bossche, 1978). The former is also true of other animal parasites, particularly gastrointestinal nematodes (Le Jambre, 1978).

A solution to some of the problems of parasitic diseases, and the potentially damaging effects of chemicals on the host and its environment, would appear to depend on the development of new control strategies. A more ecologically desirable way of controlling parasitic disease may be found by exploiting the host's capability to reject foreign material. While this has been recognised for many years, little progress has yet been made towards the development of effective vaccines against protozoan and helminth parasites. It is now acknowledged that the complexity of most parasites and
host-parasite relationships is such that the application of methods used so successfully for the production of bacterial and viral vaccines cannot be readily achieved. The complexity of host-parasite relationships is exemplified by the variety of mechanisms used by parasites to avoid the deleterious effects of host immune responses. These include antigenic variation, antigenic disguise, immune-complex suppression and tolerance, location in a privileged site (such as intracellular location), and by production of "impedins" which interfere with certain types of immune responses (Cohen, 1976). As a result studies are now being directed towards a more detailed understanding of host-parasite relationships in order to determine where, when and how manipulation of the interaction will favour the host.

1.1 *Fasciola hepatica* and fascioliasis

Fascioliasis caused by *Fasciola hepatica* Linnaeus, 1758, and *Fasciola gigantica* Cobbold, 1855, is one of the more significant animal health problems in the world. The disease is endemic in parts of Europe, Asia, Africa, North and South America and Australia. However, it is difficult to derive reliable estimates of its economic costs to communities. It causes monetary losses through the death of the host either directly or indirectly through secondary bacterial infection, condemnation of livers at the abattoir, a reduction in host fecundity and lower levels of meat and wool production. Available control measures are time consuming and costly. In Australia, over 40 million sheep
and 5 million cattle graze on pastures which are potentially endemic for *Fasciola hepatica* (Boray, 1969), and the estimated average economic loss is $20 million annually (Barger, Dash and Southcott, 1978) although when climatic conditions favour outbreaks of the disease losses may be much higher.

The control of fascioliasis is attempted at present mainly by regular chemical drenching of sheep, cattle and other hosts such as goats and pigs, and occasionally, by destruction of the snail intermediate host, *Lymnaea tomentosa*, with molluscicides (*Lymnaea columella* and *Lymnaea viridis*, recent introductions to Australia, are also suitable intermediate hosts for *F. hepatica* (Boray, 1978)). The inadequacy of these control methods is highlighted by the continuing high prevalence of fluke infected sheep and cattle at abattoirs (Barger *et al.*, 1978). Control attempts are complicated by the life cycle of *Fasciola hepatica* (Fig. 1.1). The definitive hosts, most commonly sheep or cattle, become infected by ingesting encysted metacercariae on forage. The metacercariae excyst in the duodenum, penetrate the gut wall and migrate through the mucosa into the peritoneal cavity. The juvenile flukes then enter the liver parenchyma; after about 10 weeks migrating in this tissue they enter the bile ducts. The liver migratory phase is marked by considerable damage to parenchymal tissue. In the bile ducts the flukes attain sexual maturity and produce eggs which pass into the intestine and out of the host in the faeces. The life cycle is completed after infection of the snail intermediate host.
Fig. 1.1 The Life Cycle of *Fasciola hepatica*

After Olsen (1974)

**DEFINITIVE HOST**

Adult flukes  Semi-mature flukes  Juvenile flukes

**INTERMEDIATE HOST**

*Lymnaea tomentosa*

Egg  Metacercariae  Cercariae  Redia  Sporocyst  Miracidia
by the free swimming miracidium. Within the snail the fluke undergoes asexual multiplication through sporocyst and redia stages, culminating in the development of many cercariae. These leave the snail and encyst on herbage to form metacercariae.

Until recently chemical drenches have only been able to effectively kill adult flukes resident in the bile ducts. Drenches such as rafoxanide, nitroxynil, clioxanide and the bromsalans are now available which can kill migratory flukes from 4 weeks after infection (Barger et al., 1978). However, because of the necessity for drugs to be absorbed into the host's blood stream in order for them to exert their effects on the flukes, most have relatively low safety indexes (the ratio of maximum tolerated dose to recommended dose) (Prichard, 1978). In addition, control of the infection is hampered by the difficulty of diagnosis during the 10 weeks prior to the onset of egg production, when pronounced liver damage takes place.

The application of immunological techniques would appear to be a desirable objective for the diagnosis and control of F. hepatica in its definitive hosts. The rapidly developing tissue-migrating stages presumably release many antigens which stimulate immune responses. Investigations of these responses may lead to diagnostic tests capable of detecting the early stages of fluke infection, and to immunotherapies which might kill the flukes before major tissue damage takes place.
Fascioliasis is caused by both the tissue migrating stages and adult flukes in the bile ducts. During tissue migration (the acute phase of the disease) the liver is extensively damaged with haemorrhagic tracts in evidence. These may become so numerous that haemorrhage into the peritoneal cavity occurs and death may result. More often, however, the tracts and the host's cellular reactions result in major disruption of the normal liver architecture and the deposition of large amounts of fibrotic tissue (Murray and Rushton, 1975). After the flukes become established in the bile ducts, the chronic form of the disease ensues. This is characterised by anaemia and hypoalbuminaemia which result from the flukes' feeding activities and possibly from effects on erythropoiesis (Sinclair, 1964). Mechanical obstruction and damage to the bile ducts by the adult flukes can also cause jaundice and further proliferation of fibrotic tissue around the ducts (Sinclair, 1967; Dow, Ross and Todd, 1968). The relative significance, in terms of damage to the host, of the acute tissue and the chronic bile duct phases of the disease may vary among different host species. Sheep apparently suffer severe deprivation throughout both stages of the disease.

1.2 **Immune responses to *F. hepatica***

Until recently, most studies on the immune response of the host to *F. hepatica* have been related to the development of diagnostic reagents or to vaccination against the parasite (Geyer, 1967). The general lack of success of these studies indicates that a more fundamental appraisal of
host-parasite interactions is necessary. Interest in the immune response \textit{per se} has usually been a secondary consideration. Nevertheless, previous work has demonstrated some important aspects of the host immune responses to fluke antigens, and the effects of those responses.

\textit{F. hepatica} can successfully infect a large range of hosts including man (Boray, 1969) and the principal hosts used for experimental investigations have been rabbits, mice, rats, cattle and sheep. Of these, most of the initial work was undertaken in rabbits which, in addition to being suitable laboratory animals, become infected in nature and may act as reservoir hosts when sheep or cattle are not present (Boray, 1969).

1.2.1 \textit{Immune responses of rabbits}

Evidence for the development of protective immunity in rabbits is rather equivocal. The usual assay for demonstrating the existence of protective immunity is the administration of a challenge infection after a primary infection has become patent, then comparison of the number of challenge flukes which survive to reach the bile ducts with the number of flukes in previously uninfected controls. This assay has shown evidence for protective immunity in some studies (Ross, 1966a; Kendall, Herbert, Parfitt and Pierce, 1967) but not in others (Hughes, 1962; Kendall and Sinclair, 1971). In addition, attempts to vaccinate rabbits with antigens of \textit{F. hepatica} have met with little success, beyond some retardation in the development of flukes (Kerr and Petkovich,

Rabbits have been shown to produce specific antibodies during *F. hepatica* infections (Urquhart *et al.*, 1954; Hughes, 1962; Sewell, 1964; Capron, Rose, Luffau, Biguet and Rose, 1965; Gajos, 1969; Deelder, 1973). Sinclair and Kendall (1969) studied precipitating antibody levels over the course of fluke infections. They obtained precipitin lines 3 weeks after infection and the titre increased over the remainder of the experimental period (8 weeks). They also found a correlation between the number of adult parasites recovered and the precipitin levels in individual animals.

Movsesijan and Cuperlovic (1969), using a complement fixation test and a miracidial immobilization test, found antibody against *F. hepatica* as early as 7 days after infection. Peak activities were reached 6 to 7 weeks after infection, as the flukes were leaving the liver parenchyma and entering the bile ducts, but fell soon thereafter. Gundlach (1971b) found a similar pattern of antibody response during infection using immunoelectrophoresis and passive haemagglutination as assay methods. He also demonstrated that metabolic antigens of *F. hepatica* elicited strong immune responses, especially during the prepatent period. The effects of these antibodies on flukes has not been studied, and thus their role in protecting the host is unknown.
Cell mediated reactions have been demonstrated in rabbits with fascioliasis. When an anti-spleen cell serum was injected into rabbits prior to and after infection with 200 metacercariae, the rabbits died within 4 weeks (Dodd and O'Nuallain, 1969). The livers of these animals were swollen, and haemorrhagic tracts were visible in all lobes. Control rabbits remained healthy and when autopsied showed only small numbers of haemorrhagic tracts. Despite the crude nature of the antiserum used, this experiment suggested that cell mediated processes were involved in limiting the damage caused by migrating flukes. It is unfortunate that this type of work has not been extended to determine what types of cells are involved and their roles during infection.

A recent study (Bolbol, Hammond and Sewell, 1978) obtained a reduction of about 50% in worm recovery after rabbits were challenged following 2 previous infections, each of which was terminated by chemotherapy after only 4 weeks. This suggests that a protective immune response is stimulated by the migrating stages of *F. hepatica*, which parallels findings in mice and rats (see below).

1.2.2 Immune responses of mice

Mice are not particularly satisfactory hosts for *F. hepatica* since infections with more than 2 metacercariae usually result in death of the host. Despite this, many studies have been carried out in which large numbers of mice have been infected with only 1 or 2 cysts each.
Evidence for acquired immunity in mice comes mainly from the work of Lang and his associates. Initially low numbers of mice were used (Lang, 1967, 1968), and the evidence for resistance to reinfection was equivocal (Dawes and Hughes, 1970). However, continuing studies have shown that protective immunity develops in infected mice which can be passively transferred with peritoneal exudate cells to naive mice (Lang, Larsh, Weatherly and Goulson, 1967). This response is stimulated by the liver migratory stages of the fluke (Lang and Dronen, 1972; Lang, 1974a). Studies involving transfer of flukes from donor mice to in vitro culture and then to naive recipients, have suggested a protective role for antibody and complement (Lang, 1974b). Finally, Lang (1976) and Lang and Hall (1977) successfully protected mice against infection with antigens collected from cultures of 16 day old worms and also with a crude homogenate of flukes of the same age. The antigens obtained from in vitro cultures were found to be most effective, giving an 85% reduction in worm recovery.

In other studies, Dawes (1964) could find no evidence for the development of immunity following infection with X-irradiated metacercariae (3 kr dose). However, it is possible that these metacercariae did not survive long enough to stimulate a protective response, since liver migration is apparently a prerequisite for protection to develop in mice (Lang, 1974a) and liver migration by X-irradiated metacercariae after a dose of 3 kr is limited (Movsesijan and Cuperlovic, 1969). Harness, Hughes and Doy (1976), by recovering flukes from the peritoneal cavity, found that
immunity was apparently expressed against young flukes very soon after challenge. However, their results were later explained by the more rapid migration of flukes into the liver of previously infected mice, since the number of flukes recovered 12 days after challenge was similar in both control and previously infected mice (Harness, Doy and Hughes, 1977).

Thus, there is conflicting data on whether mice are able to mount a protective immune response against *Fasciola hepatica*. In situations where protection has been demonstrated, the antigens that stimulate it are excretory or secretory products of immature worms. The amount of protection conferred is limited since significant numbers of mice die even when given a challenge of only 2 metacercariae and the response may only affect the behaviour and not the survival of the flukes. The relevance of the mouse as a model for sheep and cattle infection is perhaps questionable.

1.2.3 Immune responses of rats

Following the finding by Corba, Armour, Roberts and Urquhart (1971) that lymphoid cells could transfer protective immunity from infected to naive rats, the rat has become the host of choice for studies on immunity to *F. hepatica*. The existence of an acquired immune response in this host was first suggested by Thorpe and Broome (1962) in rats which were given a primary infection of X-irradiated metacercariae. It was not until 1972 that immunity to reinfection in rats was confirmed. Hayes, Bailer and Mitrovic (1972) found a 92% reduction in the take of challenge flukes in previously infected rats.
Subsequent studies showed that the size of the primary infection had little effect on the development of immunity (Hayes et al., 1973; Rajasekariah and Howell, 1977a), and that the adults from the primary infection could be removed with no effect on the host's resistance to reinfection (Goose and Macgregor, 1973; Hayes et al., 1974a). Moreover, adult flukes of the primary infection resident in the bile ducts were found to be unaffected by the response which killed flukes of the challenge infection (Hayes et al., 1972, 1974a); thus a state of concomitant immunity (Smithers, Terry and Hockley, 1969) exists in this host.

The apparent inability of adult flukes resident in the bile ducts to affect the persistence of immunity and the inability of the immune response to kill these adults suggested that the migratory stages were both the stimulators and targets of the protective mechanism. In support of this idea, Corba and Spaldonova (1975) found that resistance to challenge developed as soon as 4 weeks after the primary infection, and reached its maximum levels by 8 weeks when the flukes leave the liver and enter the bile ducts.

The mechanism underlying the protective response was thought at first to be cell mediated immunity, since Corba et al. (1971) were not able to passively transfer immunity to naive recipients with serum. However, Armour and Dargie (1974) and Hayes et al. (1974b) successfully transferred immunity when serum from immune rats was given to naive recipients at the
same time as the infecting dose of metacercariae. Passive transfer of immune serum was ineffective if given 2 or 4 days after the challenge dose suggesting that the response had damaging effects on the initial stages of infection (Hayes et al., 1974c). The protective effect of immune serum was abrogated by heat treatment and by absorption with live or dead flukes. Thus heat labile factors, such as complement were apparently involved.

Further studies on the time of expression of protective immunity following challenge revealed that the recovery of juvenile flukes from previously infected animals was lower than from their controls as soon as 9 h after administration of metacercariae (Rajasekariah and Howell, 1977b; Hayes and Mitrovic, 1977; Hayes, 1978). Recoveries at later times from the peritoneal cavity and liver confirmed that the gut was a major barrier to challenge flukes. It was not, as shown in studies of the mouse immune response, only a matter of a more rapid migration by challenge flukes to the liver.

Antibodies were apparently active against the flukes before they reached the liver, since after transfer of immunity with serum few lesions were observed in this organ. Following cell transfer, however, the liver was extensively damaged suggesting that flukes were not killed until after liver migration had commenced (Armour and Dargie, 1974). Thus, it was suggested that an antibody-mediated mechanism destroyed
flukes as they moved through the mucosa, while a cellular mechanism was active in the liver. In view of the current knowledge of the multiplicity and complexity of cell to cell and cell to antibody interactions in both the afferent and efferent phases of the immune response (Playfair, 1978; Mitchell, 1979), this explanation may be a simplification. It is possible that the delay in development of immunity after cell transfer relates to the lead time needed for the production of antibody by either transferred or host cells, which can then act in concert with effector cells to kill the flukes.

Investigations into the roles and characteristics of immunocompetent cells involved in the rat response to infection has progressed little beyond the initial transfer studies. Hayes et al. (1975) showed that acquired immunity still developed in splenectomised rats.

More recently, evidence for the involvement of reaginic antibodies (Capron, Dessaint, Joseph, Rousseaux, Capron and Bazin, 1977) and eosinophils (Butterworth, Sturrock, Houba, Mahmoud, Sher and Rees, 1975; Mahmoud, Warren and Peters, 1975) in immunity to schistosomes has led to similar research with Fasciola hepatica in rats. Doy, Hughes and Harness (1978a) showed that the immune response at the gut level could account for the protective effect seen in challenged rats. They also correlated immunity with the presence of large numbers of eosinophils, and some evidence for the presence of reaginic antibody, in the mucosa at the
time of challenge. In support of a role for eosinophils in immunity to *F. hepatica*, these workers have now reported that a primary infection with *Nippostrongylus brasiliensis*, which induces intestinal eosinophilia, can protect rats against a subsequent fluke infection (Doy *et al.*, 1978b).

Studies on antibody involvement in the protective response of rats to *F. hepatica* have not been extensive since it was first shown that immunity could be passively transferred with serum. Dargie, Armour and Urquhart (1973) suggested that immunoglobulin G was the antibody class involved in the passive transfer of protection. Recent work by Howell, Sandeman and Rajasekariah (1977) showed that passive transfer of protection with serum could be achieved using donor animals which had experienced a variety of immunising doses of metacercariae. In addition, *in vitro* cultures were carried out with freshly excysted metacercariae in the presence of immune rat serum. In these cultures, an antigen-antibody precipitate formed on the tegument of each juvenile fluke, and worms in immune serum had a higher mortality rate than those in normal serum over the first 48 h of incubation. When cultured juvenile flukes were washed then injected into the peritoneal cavities of rats, those that had been cultured with immune rat serum did not develop. Thus although immune serum alone can apparently kill some worms *in vitro*, its effects on cultured flukes are more fully expressed when the worms are transferred *in vivo*. Presumably, factors other than antibody can act in concert with antibody to kill flukes. Later work has also shown that
the precipitate which forms on juvenile flukes cultured in immune rat serum is able to partially protect rats against infection (Howell and Sandeman, 1979; Howell, in press). This precipitated complex was found to consist of fluke antigen (presumed to be secreted from the tegument) and rat immunoglobulin G antibody. Only one antigen-antibody reaction was apparently involved in the formation of the complex and heat labile factors were not necessary for its formation (Howell et al., 1977).

The antibody response over the course of primary and challenge infections of rats has been followed by Rajasekariah and Howell (1978a). Using several different antigen preparations and different antibody assay techniques, they found that peak antibody activity occurred while flukes were migrating in the liver parenchyma. All responses fell after the flukes became established in the bile ducts. Transfer of different developmental stages of the parasite to naive animals confirmed that all tissue migratory stages are able to elicit protective immune responses (Rajasekariah and Howell, 1978b) whereas the ability of the adult to do so is uncertain (Eriksen and Flagstad, 1974; Anderson, Hughes and Harness, 1975; Rajasekariah and Howell, 1978b).

It is now clear that the invasive and migratory stages of *F. hepatica* provide the major stimulation for protective immunity in the rat. The antigens involved are probably secreted by the worms during their migration
to and through the liver parenchyma and the roles of excretory and secretory products and the fluke tegument are now receiving greater attention in this respect (Hanna, 1978; Goose, 1978; Davies, Rickard, Smyth and Hughes, 1979). The protective response is apparently expressed as challenge flukes attempt to cross the gut wall of previously infected animals and probably involves immunoglobulin G antibodies and immunocompetent cells.

The absence of any deleterious effect of the immune response on adult flukes resident in the bile ducts raises questions as to whether the bile duct is an immunologically privileged site or whether adult flukes are able to avoid the effects of immunity. Attempts to demonstrate that adult flukes are disguised by the presence of host antigens on the tegument, as demonstrated for adult schistosomes (Clegg, Smithers and Terry, 1971; McLaren, Clegg and Smithers, 1975), have been unsuccessful (Hughes and Harness, 1973a, 1973b).

Transfer of flukes from bile ducts to ectopic sites in immune recipients has shown that adult flukes are often killed if implanted in the peritoneal cavity (Hughes, Anderson and Harness, 1976). This, together with the evidence that antibody levels fall once flukes have entered the bile ducts, suggests that adult flukes in the bile ducts are isolated from the immune system. However, adult flukes implanted subcutaneously into immune animals survive (Hughes et al., 1976) suggesting either that they lack the target antigens present in the immature stages or that immunity is
not fully expressed in the subcutaneous site. These questions have yet to be resolved.

In addition to the immunity acquired by rats during a primary infection, an increasing resistance to primary infection as rats get older has been demonstrated (Ray, 1970; Hayes *et al.*, 1974a). This resistance begins to develop at about 10 weeks of age (Rajasekariah and Howell, 1977a) but the basis for it is not known. Age resistance, like protective immunity, has been linked with the inability of young flukes to penetrate the gut (Campbell, Kelly and Dineen, 1978).

Although the rat is now the most frequently studied host of *F. hepatica*, it appears to be unique in the effectiveness and the timing of its protective immune response. In other hosts a protective response, if present, is usually only partially effective and generally acts against the liver migrating stages. Rats, however, provide a valuable model since they permit characterisation of an effective response to challenge infection which may have some relevance as far as vaccinating other hosts is concerned.

1.2.4 Immune responses of cattle

Cattle are natural hosts of *Fasciola hepatica*. However, they are apparently not as severely affected as sheep, and fascioliasis is only a major problem in calves (Boray, 1969). The resistance of cattle to fluke infection has been studied extensively. It was first noticed that after large doses of
metacercariae, many immature flukes became trapped in the liver parenchyma (Ross, 1965, 1966b, 1967a; Ross, Todd and Dow, 1966; Boray, 1969). This was attributed to the proliferation of fibrotic tissue (Ross, 1965; Boray, 1969).

Ross (1967b) also described an acquired "self cure" in cattle in which challenge flukes could apparently cause expulsion of adult flukes already resident in the bile ducts. However, Boray (1969) and Doyle (1971, 1972) found that calves would expel adult flukes from the bile ducts at about 20 weeks after infection and that a challenge infection was not necessary for this phenomenon to take place. Bile duct calcification has been suggested to cause expulsion by inhibiting the feeding of adult flukes (Boray, 1969). However, involvement of a specific immune response during the parenchymal trapping of immature flukes and the expulsion of adults has also been suggested (Dargie, Armour, Rushton and Murray, 1974). The relative importance of non-specific and specific mechanisms in the resistance of cattle to *F. hepatica* has still not been resolved.

There is now fairly strong evidence that cattle can acquire resistance to challenge infections after a primary infection. Ross (1967c) found reduced recoveries of challenge flukes, although the numbers of animals used in the experiment made statistical analysis difficult. However, reduced recoveries were confirmed by Boray (1969) and Doyle (1971). Doyle (1973a) later showed that resistance did not develop
until 12 weeks after primary infection, when: the liver migratory period had been completed. Nansen (1975) found that resistance developed after the administration of γ-ray attenuated metacercariae. The level of γ irradiation used did not prevent some irradiated flukes from reaching the bile ducts, which in view of Doyle's (1973a) results, may be a requirement for resistance to develop.

Acquired resistance was ascribed by Boray (1969) to the development of liver fibrosis caused by migrating worms of the primary infection. Doyle (1973a), however, suggested that a specific anti-\textit{F. hepatica} immune response was responsible for resistance. Support for the latter view was provided by Corba \textit{et al.} (1971), who successfully transferred protection between a pair of monozygous twin calves with lymphoid cells. Passive transfer of immunity was also accomplished by Dargie \textit{et al.} (1974) with both cells and serum. However, both these studies involved very few experimental animals. Further work is needed on the passive transfer of resistance to confirm these findings and to determine the relative effectiveness of antibody and immunocompetent cells, and the contribution to resistance of liver fibrosis.

The antibody response of cattle to \textit{Fasciola hepatica} has been studied by a number of workers, but the relationship of the findings to resistance to reinfection has not been clarified. Nansen (1969) observed increased synthesis of total immunoglobulin G during infection, but the levels of specific anti-fluke antibodies were not established.
Flagstad and Eriksen (1974) showed increases in the number of immunoglobulin secreting cells in the liver after infection. These cells were mainly secreting IgA but some IgM cells were also present. Following a challenge infection IgG₄ secreting cells were the predominant type. Again, the secretion of specific antibodies was not investigated.

Homocytotropic or reaginic antibodies to antigens of *F. hepatica* have been detected by passive cutaneous anaphylaxis during *F. hepatica* infections in cattle (Doyle, 1973b). The highest levels were reached at that period during which expulsion of adult worms occurred. There were similarities between these antibodies and the IgE class of human antibodies.

Specific anti-*F. hepatica* antibodies in cattle have been found by a number of methods. Doyle (1973c) detected precipitating antibody 2-4 weeks after infection by double immunodiffusion. Van Tiggele and Over (1976) used an indirect haemagglutination assay (IHA), counter-immunoelectrophoresis and double immunodiffusion to determine antibody levels during infection. IHA was found to be the most sensitive technique; titres rose 2 weeks after infection and, following a temporary drop after 5-6 weeks, rose to a maximum at about 13 weeks after infection, when flukes enter the bile ducts and commence egg production. Subsequently, antibody levels declined slowly. The temporary fall in IHA titre at around 5 weeks after infection has also been observed in precipitating antibody
titre (Kendall, Sinclair, Everett and Parfitt, 1978). These workers suggested that this might be due to the changing site of immunological stimulation from the gut and peritoneal cavity to the liver. After a challenge infection, precipitating antibody titres increased, but to levels below the highest detected during the primary infection (Kendall et al., 1978). The apparent lack of any anamnestic response was considered to be due to the killing of juvenile flukes of the challenge infection by the acquired immune response and thus a loss in the antigenic stimulus needed for a secondary response to take place. Recoveries of adult flukes after challenge were 80% lower than controls.

In the serological assays described above, only adult fluke antigen extracts were utilised, which may explain the consistent finding of peak antibody activities at about the time flukes are entering the bile ducts and attaining sexual maturity. In a study of antibodies directed against antigens of freshly excysted metacercariae of the closely related fluke *F. gigantica*, Hanna and Jura (1977), using a fluorescent labelling technique, found peak antibody levels at about 6 weeks after infection, perhaps indicating that juveniles express different antigens to adults.

In summary, cattle are able to acquire resistance to challenge infections of *F. hepatica*. This resistance is stimulated by the liver migratory stages of a primary infection and a complete liver migration is apparently necessary for its
development. The resistance probably has an immunological basis, but non-specific factors such as fibrosis may also effect fluke migration. The expulsion of adult flukes from the bile ducts may also involve immunological factors and non-specific factors such as bile duct calcification. It is not known at what stage challenge flukes are killed in cattle. There is also very little known about the age resistance which develops after about a year and which may be due to increased amounts of liver connective tissue seen in older cattle (Boray, 1969).

1.2.5 Immune responses of sheep

The main economic impact caused by *F. hepatica* in Australia is due to infections in sheep. This is probably a direct result of the apparent lack of resistance to infection in this host. In the field situation, sheep grazing on infested pastures continually ingest metacercariae. These migrate through the liver and they may build up in numbers in the bile ducts and cause the death of the host. If sheep are removed from the source of infection, adult flukes will remain alive inside the bile ducts for many years (Durbin, 1952) causing chronic disease. Thus it appears that, at least, in the field situation, there is little resistance, in any form, to infection or reinfection. Studies in the laboratory have tended to confirm this.

In attempts to understand the particular susceptibility of sheep many studies and reviews have examined
the pathology of the disease in this host (Dawes and Hughes, 1964; Sinclair 1964, 1967, 1973, 1975; Boray, 1967, 1969; Dow et al., 1968; Thorpe and Ford, 1969; Dargie, Holmes, Maclean and Mulligan, 1969; Murray and Rushton, 1975; Rushton and Murray, 1977; Berry and Dargie, 1978; Dargie, Berry and Parkins, 1979). Briefly, these studies have shown that damage caused by migrating flukes in the liver stimulates a massive cellular infiltration consisting mainly of eosinophils, but also large numbers of neutrophils, macrophages and giant cells. At later stages of the liver migration collagen production occurs and increases rapidly in amount, causing post necrotic scarring. In addition, pericellular and monolobular fibrosis affects large parts of the liver, even at sites remote from fluke tracts (Rushton and Murray, 1977). Pathological changes in the bile ducts are most marked after the infection is patent although hyperplasia of the bile duct wall is evident well before flukes enter the ducts (Dow et al., 1968; Iseroff, Girard and Leve, 1977). The epithelial lining of the duct becomes hyperplastic and surrounded by eosinophils and mononuclear cells. Eventually large areas of the epithelium are removed and the ducts become fibrosed. Lesions in other organs including the spleen, kidneys, brain and adrenal glands are suggested to be caused by toxic substances released by the flukes (Locatelli and Simonic, 1974).

The infiltration of immunocompetent cells into the liver and bile ducts indicates that immune mechanisms are stimulated. However, Boray (1967) could find no evidence for
acquired resistance against *F. hepatica* in sheep. He did however, describe some retardation in the growth of flukes after large doses of metacercariae and suggested that this was due to severe fibrosis in the liver. Sinclair (1971a) also observed growth retardation in flukes of a challenge infection. This effect was correlated not only with the earlier appearance of fibrotic tissue, but also with earlier lymphocyte infiltration. Sinclair suggested that these cells mediated an anti-*F. hepatica* response. Further studies of the cellular infiltration suggested that hypersensitivity-type reactions took place (Sinclair, 1973), but the relative importance of this response compared with fibrosis in retarding the development of flukes was not clarified.

As in cattle, attempts to determine if the sheep immune response can effect *F. hepatica* have involved passive transfer experiments. Sinclair (1971b) transferred spleen and lymph node homogenates from infected to naive sheep then infected the sheep with *F. hepatica* metacercariae. A retardation of fluke development was obtained, but it occurred in both the experimental and control animals suggesting that the delay was a non-specific effect caused by a homograft reaction against allogeneic cells. Dargie *et al.* (1974) transferred cells or serum from infected to naive animals and obtained a specific and significantly lower recovery of worms from the animals that received cells from infected sheep. Serum had a marginal effect on fluke recovery. They concluded that resistance was related to immune effects and not to hepatic
fibrosis. However, many details of these experiments were not given and it is not possible to compare the study with other work. More recent studies (Sinclair, 1975; Rushton, 1977; Campbell, Gregg, Kelly and Dineen, 1978) have found no evidence of resistance as indicated by adult fluke recoveries following challenge infections, although in line with earlier work referred to above some retardation of the growth of challenge flukes was evident. The role of fibrosis is still in doubt; Murray and Rushton (1975) have suggested that this response may be under immunological control and the lack of hepatic fibrosis during _F. hepatica_ infections in splenectomised sheep (Sinclair, 1970) may support this suggestion.

Apart from Dargie _et al._ (1974), the only other study to claim evidence for resistance in sheep is Ross (1967c), who transplanted adult flukes intramuscularly into sheep and then challenged the hosts with metacercariae. Fewer adult flukes were recovered from the bile ducts of sheep with intramuscular implants. However, only 4 lambs were used and the results were significant at the 10% level. Thus, this experiment provides little support for the development of resistance to _F. hepatica_ in sheep.

Recently, it has been claimed that infection of sheep with the larval cestode _Cysticercus tenuicollis_ for 12 weeks almost totally protects them from a subsequent infection with _F. hepatica_ (Campbell, Kelly, Townsend and Dineen, 1977; Dineen, Kelly and Campbell, 1978). Moreover, it was reported
that prior *F. hepatica* infection could reduce the number of *C. tenuicollis* that developed following challenge (Campbell, Dineen and Kelly, 1979). Attempts to repeat this work in goats, sheep and cattle have failed (Hughes, Harness and Doy, 1978). In addition, antigens from *C. tenuicollis* were unable to protect rats or mice against infection with *F. hepatica* (Rajasekariäh, Rickard, Montague and Mitchell, 1979). If the work of Campbell *et al.* (1977) could be substantiated it would indicate that sheep have the capacity to develop resistance to *F. hepatica*, and experimental approaches different to those previously used are necessary for resistance to be generated.

There have been few studies on the antibody responses of sheep to fluke infection. Movsesijan, Jovanovic, Aalund and Nansen (1975) incubated slices of adult *F. hepatica* with sera from infected sheep, then labelled bound immunoglobulin with a fluorescent tag (fluorescein isothiocyanate). This test was found to detect antigen mainly in the gut epithelium. The highest antibody titres were detected about 6 weeks after infection and the levels fell once the infection became patent. This suggests that the liver migration period causes greatest antigenic stimulation and that adult flukes resident in the bile ducts are isolated from the immune system. The declining antibody levels cannot be readily explained by a change of antigens as flukes become adult, since Movsesijan *et al.* used an adult fluke antigen preparation. However, Van Tiggele and Over (1976), who also used adult fluke antigens in indirect haemagglutination, counter-immunoelectrophoresis and double
immunodiffusion tests, did not observe peak activities of antibody until up to 18 weeks after infection. Levels fell only marginally after 18 weeks suggesting continued antigenic stimulation of the host. This suggests that the bile duct is not isolated from the immune system. An explanation for these diverse results might relate to the selective absorption of different antigens from the host gut, a factor which has been largely ignored in discussions on bile duct isolation, but which has been shown to be important in adult cestode infections (Williams, 1979).

Sheep are not apparently able to mount a protective immune response against *Fasciola hepatica*. However, cellular reactions and antibody production take place indicating the presence of responses which may be responsible for the delays observed in the growth and migration of flukes of challenge infections. Moreover, serum from infected sheep can apparently protect rats against infection when it is passively transferred (Armour and Dargie, 1974), suggesting that protective antibodies are present in infected sheep.

To date most studies on the immune response of sheep to *F. hepatica* have utilised adult worm antigens. Several factors now suggest that the invasive juvenile stages and the liver migrating semi-mature stages may be more important in stimulating immune responses. These include the findings that the juvenile and immature stages can stimulate protective immunity in rats, mice and cattle; that the immune response in
rats acts mainly on juvenile flukes as they cross the gut wall; and that a juvenile fluke antigen/rat antibody complex is able to partially protect rats against infection. Studies of the antigens released by these stages may, therefore, lead to a better understanding of the development of the host immune response to *F. hepatica* and may enable the characterisation of antigens which are functional (Soulsby, 1963), that is, antigens which stimulate protective immunity.

1.3 Proposed work on the immune response of sheep

Studies involving the use of helminth antigens are hampered by the antigenic complexity of the organisms and the difficulty in obtaining large amounts of antigen. *In vitro* culture, which offers a possible solution to these problems, has rarely been successful with helminths and this is one of the reasons for the limited nature of many immunological studies on helminths. During studies on *Fasciola hepatica* in rats (Howell *et al*., 1977) a simple method for short term culture of excysted metacercariae *in vitro* was developed. This allowed examination of antigens and antibodies involved in the host-parasite relationship, in isolation from the complexities of the *in vivo* situation, for periods of up to 2 weeks. The adaptation of this method to studies with sheep serum and cells has permitted work on particular components of the sheep's response to infection. It has also enabled comparisons of the sheep's response with some of the protective responses of the rat which have been characterised both *in vivo* and *in vitro*. 
The major aims of this study were to:

1. Evaluate the effects of serum from infected sheep on juvenile flukes in vitro.

2. Use in vitro techniques to investigate the sheep antibody response to juvenile fluke antigens over the course of both primary and challenge infections.

3. Fractionate serum from infected sheep to identify those components responsible for in vitro effects on the flukes.

4. Analyse antigen-antibody complexes that develop in vitro and compare them with in vivo reactions.

5. Determine the nature and the origin of antigens isolated in vitro.

6. Study the mechanisms by which flukes avoid destruction by the sheep immune response.

7. Examine the value of antigens and antibodies isolated in vitro for diagnostic and vaccination studies.

It was hoped that these studies would lead to a better understanding of the susceptibility of sheep to F. hepatica and the identification of factors important to parasite or sheep survival during the course of infection.

Chapters 2 to 8 are accounts of experimental work. In Chapter 9 the results are discussed within the framework of previous studies and with regard to their wider implications.
IN VITRO ASSAYS OF THE ANTIBODY RESPONSE OF SHEEP TO INFECTION WITH *FASCIOLA HEPATICA*

2.1 Introduction

The formation of precipitate around helminths cultured in serum from infected hosts is a well documented phenomenon. Their formation on the cuticle and around the orifices of nematodes (Sarles, 1937; Otto, 1939; Jackson, 1959, 1960; Douvres, 1962a, 1962b; Michel, 1969; Leventhal and Soulsby, 1975) and on the tegument of cestodes (Heyneman and Welsh, 1959; Kowalski and Thorson, 1972) suggests that secretory and excretory antigens of the parasites and host antibodies are involved in these reactions. A precipitin reaction which develops around cercariae of *Schistosoma mansoni* in infected host serum (Papirmeister and Bang, 1948) has been utilised as a serological test for schistosomiasis - the "cercarienhüllen reaktion".

The significance of these precipitation reactions in the host-parasite relationship has not usually been investigated. However, other studies with nematodes (Thorson, 1970), cestodes (Williams, 1979) and schistosomes (Cox, 1979), have shown that excretory-secretory antigens often stimulate immune responses harmful to the parasite.

Formation of precipitates around juvenile *Fasciola hepatica* in serum from infected hosts was first observed by Wikerhauser (1961). No difference was found between the
survival of worms cultured in infected and normal serum, but impaired viability was demonstrated when flukes incubated in serum from *F. hepatica* infected animals failed to develop when injected into guinea pigs. However, the results were equivocal because of bacterial contamination of cultures and the low numbers of worms and guinea pigs used. Ruther (1963, cited in Geyer, 1967) observed precipitate formation around immature flukes obtained from mice when they were incubated in serum from immune rabbits. These flukes died soon after incubation, whereas flukes in normal rabbit serum did not show precipitates and did not die. These interesting results have not been confirmed. Lang (1974b) also found precipitate formation around immature *F. hepatica* when they were incubated in the serum of infected mice. After incubation, the worms were transferred to previously uninfected mice and a significant loss in worm viability was observed. The ability of excretory-secretory antigens collected from flukes cultured in normal serum to elicit protective immunity in mice was later confirmed (Lang, 1975).

Precipitates form on the teguments of *F. hepatica* metacercariae in the presence of immune rat serum (Howell, *et al.*, 1977). The precipitate is a complex of a rat antibody (probably IgG) and an excretory-secretory antigen of the parasite; it can be used as a vaccine to partially protect rats against infection (Howell and Sandeman, 1979; Howell, in press), and therefore contains a functional antigen of the parasite.
In this chapter some preliminary findings concerning the effects of sheep serum, taken throughout the course of fluke infection, on juvenile *F. hepatica* are described. Similar methods to those developed for examining the effects of immune rat serum on juvenile flukes (Howell *et al.*, 1977; Howell and Sandeman, 1979) were used. Thus, attempts were made to determine the nature of any precipitate which formed on juvenile worms in the presence of infected sheep serum and to quantify the amount of precipitate at various intervals after infection. The migratory activity of flukes in the liver parenchyma, followed by their emergence into the bile ducts, was monitored by the assay of serum levels of the liver enzyme, glutamate dehydrogenase (GLDH) (Boyd, 1962; Thorpe and Ford, 1969; Bundesen and Janssens, 1971; Anderson, Berrett, Brush, Herbert, Parfitt and Patterson, 1977) and the bile duct epithelial enzyme, γ-glutamyl transferase (γ-GT) (Anderson *et al.*, 1977; Rew, Colglazier and Enzie, 1978). These enzymes have been described as the most sensitive for the detection of liver and bile duct damage respectively during *F. hepatica* infections (Anderson *et al.*, 1977; Rowlands and Clampitt, 1979).

2.2 Materials and Methods

2.2.1 General

Metacercariae of *Fasciola hepatica* were obtained from experimentally infected snails maintained essentially as described by Boray (1969). Following removal of the outer cyst wall, they were considered viable if they had a translucent
appearance and the refractile excretory granules were conspicuous.

The levels of the liver mitochondrial enzyme GLDH and the bile duct enzyme \( \gamma \)-GT were assayed in the weekly collections of serum using commercially available kits (Boehringer-Mannheim, GLDH activated test kit; C system 10 \( \gamma \)-GT kit).

2.2.2 Sheep

Six merino-cross lambs were reared worm free from birth. Food and water were provided \textit{ad libitum}. Four weeks after weaning the sheep were weighed, a blood sample was taken and they were separated into 3 groups of 2 animals. Lambs in group 1 received 100 metacercariae by oral intubation, group 2 received 200 metacercariae by the same route; group 3 lambs acted as uninfected controls. Each week after infection, blood samples were collected. Beginning 6 weeks after infection, faecal samples were taken weekly to determine the onset of patency (see below). All blood samples were kept at 4\(^\circ\)C for approximately 4 h before centrifugation at 4000 g for 30 min. The sera from lambs in each group was pooled and stored at -20\(^\circ\)C until required.

Twenty weeks after infection the animals in groups 1 and 2 and 1 lamb from group 3 were killed, and flukes were recovered from the bile ducts.
2.2.3 Faecal egg counts

Faeces was collected from the rectum of sheep. Two grams of the sample was broken up in approximately 200 ml of tap water in a plastic cup. The solution was allowed to sediment for 10 to 15 min before the supernatant was poured off. Water was again added and the sedimentation and decantation repeated a total of 3 times. The sediment was then washed through a 44 mesh then an 85 mesh sieve (Endecott Ltd, London) and the filtrate was collected. After two further sedimentation and decantation steps, 0.1 ml of 0.1% methylene blue was added and the volume was brought to 20 ml. This was bubbled continuously while a 2 ml sample was taken and placed in a McMaster slide. *F. hepatica* eggs were counted and the total was multiplied by 5 to give the number of eggs per gram of faeces.

2.2.4 *In vitro* cultures

Metacercariae were excysted in an artificial digestion medium following the method of Wikerhauser (1961). Two solutions were used:

1. Solution 1 contained 0.25% pepsin (3 x Crystalline, porcine stomach mucosa, Calbiochem) in 0.85% NaCl. The pH was adjusted to about 2.0 with 1 M HCl.

2. Solution 2 contained 0.4% trypsin (2 x Crystalline), salt free, Nutritional Biochemicals Corporation), 1% NaHCO₃ and 20% centrifuged and filtered sheep bile in 0.85% NaCl. The pH was adjusted to 7.0 with 1 M HCl. Care was taken to collect bile from sheep which showed no evidence of current or prior infection with *F. hepatica*. 
Metacercariae were initially incubated in 5 ml of Solution 1 in a shaking water bath at 37°C for about 2 h. They were then washed several times with 0.85% NaCl to raise the pH to about 7.0. Solution 2 was then added and incubation continued for 3 to 4 h. Periodic shaking of the culture vessel by hand appeared to promote excystment. Between 50 and 80% of the metacercariae excysted after 3 to 4 h in the bile/trypsin solution.

The juvenile flukes were then transferred aseptically into cultures in a laminar flow cabinet (Clemco). The transfer procedure was as follows:

1. The excysted metacercariae were washed 4 x by sedimentation, with sterile medium 199 (Commonwealth Serum Laboratories) containing 100 units/ml penicillin G (Crystapen, Glaxo), 100 μg/ml streptomycin (Streptomycin sulphate, Glaxo), and 2.5 μg/ml amphotericin B (Fungizone, E.R.A. Squibb).

2. They were then transferred to a sterile Leighton tube and again washed 4 x. Finally, they were transferred to a fresh sterile Leighton tube and washed once prior to the addition of the culture medium. Throughout the washing process sterile glassware was used and aseptic procedures were followed. Pasteur pipettes were siliconized with Ajax Silicone Glass Treatment Solution to prevent flukes from adhering to them.
Approximately 100 excysted metacercariae were placed in a sterile Leighton tube with 5 ml of a sample of normal or infected sheep serum. All serum samples had previously been sterilised by Millipore filtration and contained 100 units/ml penicillin G, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B. The tubes were incubated in a shaking water bath at 37°C for 160 h and regular observations made using an inverted microscope. Any precipitate which formed on the flukes or in the medium (see Results) was separated from the flukes by agitation and decantation. The young flukes were then washed several times with phosphate buffered saline (PBS) until no further precipitate was recovered, and the washings were pooled with the medium. The precipitate was recovered by centrifugation at 4000 g for 30 min, the supernatant was discarded and the pellet washed 3 x in PBS. The precipitate was finally resuspended in 1 ml of PBS and frozen until assayed for protein content by the method of Lowry, Rosebrough, Farr and Randall (1951). Because of variations in the final numbers of parasites in culture (± 20) protein levels were corrected to µg/ml serum/100 juvenile flukes.

To test the involvement of heat labile factors in precipitate formation, cultures were set up in which juvenile flukes were incubated with heat inactivated sheep serum. Metacercariae were excysted and washed as above and 100 were then transferred to sterile Leighton tubes to which was added 5 ml of 20% heat inactivated (56°C for 30 min) serum from sheep.
infected 8 weeks previously with 100 metacercariae or with heat inactivated normal sheep serum of the same age, in 199. Cultures were incubated at 37°C for 160 h as described above.

2.2.5 **Fluorescent antibody test (FAB)**

A direct FAB test was carried out on juvenile flukes cultured in normal serum and serum from infected sheep using fluorescein isothiocyanate (FITC) labelled rabbit anti-sheep immunoglobulin G (Burroughs-Wellcome). This antiserum is not heavy chain specific and thus could be expected to react with other sheep immunoglobulin classes in addition to IgG. Juvenile flukes were gently washed 3 x in PBS (with care not to dislodge any attached precipitate), then exposed for 60 min to the labelled anti-sheep immunoglobulin diluted 1:16, and finally 3 x with PBS. Examination was carried out under a Leitz u.v. microscope equipped with a BG 12 exciter filter and blue absorbent barrier filters.

2.2.6 **Micro-Ouchterlony tests**

These were carried out in 1% agar (Ionagar No. 2) in PBS with 0.1% NaN₃ and 10% NaCl added. The addition of NaCl has been found to enhance the development of precipitin lines with sera from cattle and sheep (Soulsby, 1967). This was confirmed in the present study for micro-Ouchterlony tests, but not standard Ouchterlony tests. Immunoelectrophoresis templates were used to pour the gels on microscope slides. An LKB gel punch was adapted to punch 3 mm holes, 3 mm apart as shown in Fig. 2.6. Juvenile worm excretory-secretory antigen
(JWS) was collected by incubating approximately 500 excysted metacercariae in 5 ml of 20% normal sheep serum in medium 199 with antibiotics as described above. The medium was removed after 160 h and concentrated to approximately 0.2 ml on a Minicon B15 concentrator (Amicon). JWS were tested against infected and normal sheep serum and against absorbed and unabsorbed infected sheep serum. Absorbed serum was raised by incubating excysted metacercariae in 20% infected sheep serum (taken 9 weeks after infection) in 199 until precipitate formation had ceased. The medium was then concentrated 5 x back to the original serum volume. Unabsorbed serum was raised by incubating a second batch of infected sheep serum for the same period but in the absence of juvenile flukes.

2.2.7 Scanning electron microscopy

Following incubation in cultures containing infected and normal sheep sera, some juvenile flukes were prepared for scanning electron microscopy. The flukes were removed from culture, washed gently 3 x in phosphate buffered saline (PBS) and fixed in 3% glutaraldehyde in 0.01 M phosphate buffer, pH 7.3. After further washes with phosphate buffer they were post fixed in 1% osmic acid in 0.01 M phosphate buffer and then washed several times in distilled water. Fixed flukes were placed on double sided tape, on a stub, in liquid nitrogen and freeze dried. They were then rotary coated with gold in a Dynavac rotary coating unit and viewed under a Cambridge Stereoscan electron microscope.
2.3 Results

When excysted metacercariae were cultured in serum from infected sheep a precipitate developed on their teguments and in the surrounding medium (Fig. 2.1). In sheep serum with a high level of antibody, precipitate formation could be seen on the flukes after only 15 min in culture. Development first occurred on the anterior half of each worm and especially on the tegument surrounding the oral sucker. Later, it also developed on the posterior tegument. Initially it appeared as granulated blebs of material on the tegument (Fig. 2.2). Larger aggregates gradually formed and the worm became progressively covered. Although precipitate first formed on the tegument it was also seen in the medium after 12 h in culture. After 160 h large amounts of precipitate attached to each worm and in the surrounding medium made the flukes difficult to distinguish (Fig. 2.3). Occasionally precipitate could be seen sloughing off the tegument of active worms. Precipitate did not develop on excysted metacercariae cultured in normal sheep serum (Fig. 2.4).

Despite this effect of infected sheep serum on juvenile flukes their mortality was no different than that of juvenile flukes in serum (see Chapter 7).

Precipitate development occurred in cultures containing serum from infected sheep from each of the weekly samples. However, it was less extensive in serum collected 1 week after infection than subsequently.
Fig. 2.1. Precipitate on the tegument of juvenile fluke cultured with infected sheep serum for 160 h.

a) Light microscopy; b) Scanning electron microscopy
Fig. 2.2 Blebs of precipitate on the tegument of a juvenile fluke cultured for 48 h with infected sheep serum.

Fig. 2.3 Precipitate in a culture of juvenile flukes and infected sheep serum at 160 h.
Fig. 2.4 Juvenile flukes cultured with normal serum for 160 h.

a) Light microscopy; b) Scanning electron microscopy.
The presence of sheep antibody(ies) in the precipitate was confirmed by the fluorescent antibody test. The precipitate on the tegument and in the medium fluoresced brightly following treatment with fluorescein labelled rabbit anti-sheep IgG (Fig. 2.5). No fluorescence was observed on excysted metacercariae cultured in normal sheep serum.

Precipitate development occurred in the presence of heat inactivated serum from infected sheep demonstrating that heat labile factors were not involved in its formation.

Results of a micro-Ouchterlony test used to assay for the presence of juvenile fluke antigens in the precipitate are shown in Fig. 2.6. Two precipitin lines were seen to form between unabsorbed serum from infected sheep and JWS. After infected sheep serum was cultured with excysted metacercariae neither line developed. No precipitin lines formed between normal serum and JWS.

Protein assays on precipitates isolated from cultures gave a relative measure of the amount of antibody in the serum each week after infection (Fig. 2.7). Serum taken from both infected groups (groups 1 and 2) contained detectable antibody 1 week after infection. Antibody levels then rose and reached a peak at 10 weeks in group 1, which coincided with the first appearance of fluke eggs in the faeces. After this time antibody levels dropped. About 13 weeks after infection a plateau was reached in group 1 which persisted until 1 week before the experiment was terminated when there was a further drop. In group 2 antibody levels rose to high levels at 6 weeks and remained at about this level until 9 weeks.
Fig. 2.5 Fluorescence associated with the precipitate after a fluorescent antibody test on juvenile flukes cultured for 160 h in infected sheep serum.

Fig. 2.6 Micro-Ouchterlony Test. Juvenile excretory-secretory antigen (JWS) run against infected sheep serum (InSS), serum absorbed with juvenile worms (JAS) and normal sheep serum (NSS).
Fig. 2.7 Antibody activity (µg protein/ml serum) measured in weekly collections of sheep serum by isolation of precipitate from *in vitro* cultures.

- Dotted line: 100 metacercariae
- Solid line: 200 metacercariae
- Star: Control
Antibody levels then declined until 13 weeks after which fluctuations occurred through to autopsy. The group which received 100 metacercariae (group 1) showed higher levels of antibody in their sera (a peak of 75 μg/ml serum) than the group which received 200 metacercariae (group 2) (41 μg/ml serum). The amount of precipitated or insoluble protein isolated from control cultures remained at very low levels throughout the course of the experiment.

The levels of the enzymes GLDH and γ-GT in pooled serum samples from group 1 are shown in Fig. 2.8. The antibody levels of this group are also shown for comparison. GLDH activity in the serum rose 2 or 3 weeks after infection, reached a peak at 9 weeks, and decreased to negligible levels after about 14 weeks. At 19 and 20 weeks GLDH again increased in activity. In contrast γ-GT showed increased activity only after 7 weeks; it remained at high levels from 9 to 12 weeks then declined to normal values by 15 weeks.

The enzyme and antibody levels of group 2 are shown in Fig. 2.9. GLDH and γ-GT activities in serum were similar to those of group 1 until 9 weeks after infection. Subsequently, marked fluctuations were observed in the levels of both enzymes. Analysis of serum from individual lambs in this group showed that these fluctuations occurred in 1 animal. The other lamb showed enzyme levels very similar to those of group 1.

The number of flukes recovered from the bile ducts of the sheep that were autopsied is shown in Table 2.1. Approximately 30% of the initial dose of metacercariae was recovered as adult worms from both infected groups.
Fig. 2.8 Enzyme levels (units/litre serum) and antibody activities (µg protein/ml serum) in weekly collections of serum from sheep infected with 100 metacercariae.

- Antibody activity
- γ-GT levels
- GLDH levels
Fig. 2.9 Enzyme levels (units/litre serum) and antibody activities (µg protein/ml serum) in weekly collections of serum from sheep infected with 200 metacercariae.

- Antibody activity
- γ-GT levels
- GLDH levels
Table 2.1  Adult fluke recovered from the bile ducts of sheep at necropsy.

<table>
<thead>
<tr>
<th>Sheep number</th>
<th>Number recovered</th>
<th>% of dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td>(100 mc)</td>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td>Group 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>71</td>
<td>35.5</td>
</tr>
<tr>
<td>(200 mc)</td>
<td>2</td>
<td>25</td>
</tr>
<tr>
<td>Group 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>(Control)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.4 Discussion

The precipitate which forms on juvenile *F. hepatica* when they are cultured in serum from infected sheep is similar to the precipitate which forms when juvenile flukes are cultured in immune rat serum (Howell *et al.*, 1977). That is, it forms on the teguments of the worms and it contains host antibodies and parasite antigens. Comparable phenomena described by Wikerhauser (1961) and Ruther (1963, cited in Geyer, 1967) with infected serum from rabbits and cattle and by Lang (1974b) with mice indicates that this is a common reaction between *F. hepatica* and serum from its infected hosts. It would seem probable that similar or the same parasite antigens are involved in its formation in each case.

The involvement of at least two antigen-antibody reactions in precipitate formation and the excretory or secretory nature of the antigens was shown by the micro-Ouchterlony test performed with JWS. Evidence for the active secretion of the antigens from the tegument is provided by the dynamics of precipitate formation. Thus precipitate apparently forms at the surface of the fluke but is then forced away by subsequent secretion. This question is considered further in later chapters.

Heat labile factors such as complement are apparently not necessary for precipitate formation to occur. However, this does not preclude the binding of complement to antibodies involved in precipitate formation. This may be important if the antibodies are to have any effects on the parasite.
Precipitating antibody, as measured by the culture technique, was detected 1 week after incubation. Thus the sensitivity of this method compares favourably with indirect haemagglutination and counterelectrophoresis (Van Tiggele and Over, 1976) and indirect fluorescent antibody (Movsesijan et al., 1975) assays.

Antibody activity increased rapidly while flukes were migrating through the liver (as determined by GLDH assays). Maximum antibody levels at this time coincided with greatest liver damage and the time at which flukes were leaving the liver parenchyma and entering the bile ducts. The movements of flukes through the wall of the intrahepatic bile ducts is shown by the appearance of γ-GT in the serum; eggs also first appear in the faeces at about this time. When flukes had become established in the bile ducts the antibody and enzyme levels fell. Thus, the antibody response is linked closely with the liver migratory phase of the fluke's life cycle. This relationship between the sheep antibody response and fluke migration has also been demonstrated by Movsesijan et al. (1975).

Once the flukes leave the liver parenchyma and enter the bile ducts antigenic stimulation of the host appears to be markedly reduced since antibody levels fell. As discussed in Chapter 1, this may be a result of either the relative isolation of adult worms from the host immune system and/or a change in the antigenic nature of the flukes as they become sexually mature. That the bile duct is an immunologically privileged
site within the host is also supported by the work of Hughes et al. (1976) who found that if flukes are removed from the ducts and implanted in other sites they are often killed. However, Van Tiggele and Over (1976) showed that high antibody levels persisted well after patency, suggesting that the bile duct is not a privileged site. This difference might be explained in terms of the immunological test used and selective absorption of fluke antigens from the gut of the host, a factor shown to be important in adult cestode infections (Williams, 1979).

The fluctuations seen in the antibody and enzyme levels of 1 lamb in group 2, 10 weeks after infection and subsequently, might suggest that successive waves of fluke migration occurred in this animal. Boray (1967) found delayed fluke migration in sheep although only after much heavier infections than used here. A criticism of this explanation is that continued liver migration by fluke has not been recorded in sheep up to 20 weeks after infection. Boray (1967) found patency delayed up to 15 weeks after infection but only after administering 2000 metacercariae. The prepatent period after giving 200 metacercariae was 9 weeks and this was also the case in this study.

An alternative mechanism might result from the bile duct damage caused by the adult flukes. Bile duct blockage and bile stasis which occur in patent F. hepatica infections (Murray and Rushton, 1975) might lead to the continuing release
of γ-GT into the serum. The fluctuations in GLDH, which is indicative of liver cell damage, and in antibody levels, might then result from the sporadic release of bile and fluke antigens from damaged bile ducts into the liver parenchyma. The timing and magnitude of these effects may depend on the individual sheep and on the fluke burden. The increase in GLDH activity after 18 weeks in group 1 might also result from bile duct damage by adult flukes, although the lack of a corresponding increase in γ-GT suggests that a different phenomenon may be responsible.

It is of interest that the antibody response in the sheep given 100 metacercariae was greater than in sheep given 200 metacercariae, yet there were only about half as many flukes recovered from the animals given the lower dose. This lowered antibody response, despite a presumed higher degree of antigenic stimulation, may have been due to a number of factors including variation in the responses of individual sheep, or immunosuppression caused by the greater number of parasites.

A similar precipitate to that described in this chapter develops when excysted metacercariae are cultured in immune rat serum. Juvenile flukes are also affected by immune rat serum in vitro in a manner which impairs their viability when they are transferred to previously uninfected hosts (Howell et al., 1977). When rats are injected with precipitate in Freund's complete adjuvant some protection is conferred against a subsequent infection (Howell and Sandeman, 1979;
Howell, in press). It is therefore considered that damage to juvenile flukes by immune rat serum *in vitro* is associated, at least in part, with precipitate formation. The results of experiments aimed at determining whether juvenile flukes are similarly damaged by serum from infected sheep are presented in later chapters.
3.1 Introduction

Hosts of *Fasciola hepatica* such as rats, cattle and mice mount an immune response to a primary infection, and exhibit resistance to a challenge infection. Resistance may be conferred by the levels of immune effectors stimulated by the primary infection. Alternatively, it might depend on an anamnestic response to the challenge infection resulting in the invading flukes encountering higher levels of immune effectors soon after reinfection than would be encountered following the primary infection. Studies of the immune response of hosts following challenge infections may determine which of these mechanisms is applicable. If flukes of a challenge infection are destroyed by existing antibodies and activated cells then an anamnestic response may not be evident. If, however, a secondary immune response to challenge must be stimulated before protective immunity can be effected then a classic anamnestic response might be expected. To date, few studies have investigated the immune response of hosts to a challenge infection with *F. hepatica*. These have obtained results which favour the hypothesis that the invading challenge flukes are killed by existing antibodies and activated cells and a secondary response is not an essential component of protective immunity.

*Kendall et al.* (1978) used an adult fluke antigen to assay precipitating antibody titres during primary and
challenge infections in cattle. They found that after a challenge, antibody levels rose more slowly and did not reach the levels seen after primary infection. The apparent lack of an anamnestic response was thought to be due to the rapid killing of challenge worms with the result that there was a negligible secondary antigenic stimulus. Rajasekariah and Howell (1978a), studying antibody responses of rats to primary and challenge infection, also found that the secondary precipitating antibody response was slower to develop, and of lower magnitude, than the primary response. Antibody titres assayed by a miracidial immobilisation test showed a similar trend. However, antibody titres, as measured by an indirect fluorescent antibody technique on frozen sections of adult flukes, showed a slight increase following challenge to levels greater than seen following the primary infection. Rajasekariah and Howell (1978a) suggested that the absence of an anamnestic response following challenge infection might be due to destruction of invading flukes in the intestine (either in the lumen or mucosa) resulting in a relatively low level of secondary antigenic stimulation. As suggested above, the absence of an anamnestic response in hosts which kill challenge worms may indicate that antibodies and cells activated by the primary infection are responsible for protective immunity. Gundlach (1971b) studied secondary infections in rabbits, a host which shows little resistance to challenge. Immunoelectrophoresis, passive haemagglutination and complement fixation tests with somatic antigens all showed there was a slight increase in antibody titre after a secondary infection,
especially at low levels of infection. However, complement fixation with excretory-secretory antigens showed falling titres after the primary infection which were not affected by the challenge. This was most noticeable at higher levels of infection. It suggests that the antigenic stimulation provided by the invading flukes, was modified by other factors so that antibody levels remained depressed.

Clearly then, the situation among hosts whose responses to challenge have been examined to date, is to some extent paradoxical. There is no distinct correlation between their responses and whether they show resistance to reinfection.

The antibody response of sheep to challenge infection with *F. hepatica* has not previously been investigated, possibly because of the apparent lack of any resistance to challenge in this host. However, comparisons between sheep and other hosts that exhibit a protective immunity may provide some insights into the susceptibility of sheep to reinfection.

In this chapter, levels of precipitating antibodies to *F. hepatica* in sheep during primary and challenge infections, as assayed by the *in vitro* culture method, are presented. The course of the infections was followed by monitoring the activities of the liver and bile duct enzymes, GLDH and γ-GT, in the serum.
3.2 Materials and Methods

Methods for the assay of antibody, GLDH and \( \gamma \)-GT levels in serum samples and for *F. hepatica* eggs in the faeces were as described in Chapter 2.

Two sheep, raised worm free, were infected with 100 metacercariae *per os*, and reinfeated 16 weeks later with a further 100 metacercariae. A control animal, uninfected until 16 weeks, was also infected with 100 metacercariae at this time. Serum and faecal samples were taken from these animals each week for 36 weeks after the initial infection (ie. until the time of autopsy). At autopsy, the livers were removed and adult flukes recovered. In the control lamb, antibody levels were measured at 4 week intervals.

3.3 Results

The precipitate which formed on excysted metacercariae in cultures containing serum from sheep given primary and challenge infections was indistinguishable in appearance from that which formed in the presence of serum from sheep given only a primary *F. hepatica* infection (Chapter 2).

The pattern of the precipitating antibody response during the primary infection of this experiment (Fig. 3.1) was similar to that described in the previous chapter. Thus, the rise and fall of antibody levels mirrored the liver migratory phase of the flukes life cycle; antibody levels rose when
Fig. 3.1 Antibody activity (μg protein/ml serum) and enzyme levels (units/litre serum) in weekly collections of serum from sheep infected with 100 metacercariae then challenged with 100 metacercariae.

- Antibody activity;
- GLDH levels
- γ-GT levels
contact with the liver parenchyma was established and fell after adult flukes had reached the bile ducts. The same pattern was observed in the control lamb in the present experiment (Fig. 3.2). However, the antibody activity measured during the primary infection in the experimental lambs reached much higher levels (120 μg protein/ml serum) than were measured previously (Chapter 2) or in the control animal in this experiment.

Following challenge, the antibody response differed from that seen following the primary infection. Two weeks after reinfection antibody levels had risen to their maximum post-challenge value, but this was only 39% of the maximum value recorded during the primary infection. Subsequently, the mean antibody level dropped, but marked fluctuations were observed and these continued until autopsy.

The activities of both GLDH and γ-GT in the serum during the primary infection (Fig. 3.1), were similar to those seen previously (Chapter 2). Thus the rise in GLDH at 3 weeks, a peak at 10 weeks and fall at 11 weeks was indicative of liver damage as the flukes migrated through the parenchyma. The peak in γ-GT activity at 10 weeks coincided with the first appearance of *F. hepatica* eggs in the faeces and indicated the movement of flukes into the bile ducts and their attainment of sexual maturity. After this time, when flukes could be assumed to have established in the bile ducts, fluctuations in the levels of both enzymes were observed.
Fig. 3.2 Antibody activity (μg protein/ml serum) and enzyme levels (units/litre serum) in serum from a challenge control lamb infected with 100 metacercariae.

★ Antibody activity

GLDH levels

γ-GT levels
Following administration of the challenge infection, GLDH activity rose rapidly to high levels, although marked fluctuations continued. The highest peak was recorded 12 weeks after challenge, which coincided with a sharp rise in faecal egg counts. GLDH activity then fell marginally, although fluctuations continued until autopsy.

Fluctuations in the γ-GT response during the primary infection and which continued after challenge (Fig. 3.1), made it difficult to determine when worms of the challenge infection began to reach the bile ducts. However, a steep rise occurred about 8 weeks after challenge, which coincided with the increase in γ-GT levels recorded in the control animal (Fig. 3.2). Peak activities were reached at 10 weeks, and were sustained at this level until 13 weeks after challenge; this rise coincided with the rise in faecal egg counts at about 10 weeks. Levels of γ-GT then dropped, although at autopsy they had not regained normal values. Analysis of the GLDH and γ-GT levels of the individual lambs of the challenged group revealed that although both lambs showed fluctuations in the levels of these enzymes after the primary infection had become patent, the variations were more marked, and the level of activity was higher, in one lamb than in the other.

In the challenge control lamb, γ-GT activity showed a peak 9 weeks after infection which coincided with the appearance of *F. hepatica* eggs in the faeces. Levels then dropped as flukes established in the bile ducts. However, after
16 weeks, a second rise in the level of \( \gamma \)-GT occurred. GLDH activity in this animal showed no elevation until 6 weeks after infection; levels then increased with a peak at 8 weeks. Marked fluctuations in activity then continued with a substantial increase occurring 16 weeks after infection.

Fluke recoveries at autopsy are shown in Table 3.1. About 40% of the combined dose of 200 metacercariae was recovered as adult flukes from the challenged group, while 50% was recovered from the control lamb given a single infection of 100 metacercariae.

3.4 Discussion

The recoveries of adult flukes from the sheep reinfected with \( F. \) hepatica were not different from those of sheep which received a single infection of 200 metacercariae (Chapter 2, Table 2.1). This result supports previous work on \( F. \) hepatica infections in sheep (Boray, 1967; Sinclair, 1975; Rushton, 1977) and indicates that these sheep showed no evidence of resistance to challenge infection.

Antibody and enzyme responses during the primary \( F. \) hepatica infection were similar to those discussed in Chapter 2. Thus, these responses were tied to the migratory activities of the flukes, coinciding with the entry of the flukes into the liver parenchyma and their exit into the bile ducts. They also suggest that the adult flukes are isolated, at least temporarily, from the host immune system and liver.
tissue after they enter the bile ducts (Chapter 2).

The maximum levels of precipitating antibody measured after primary infection in the experimental group were greater than those observed in the previous experiment, and in the control lamb in this experiment. Clearly, there is considerable variation among sheep in their precipitating antibody response to *F. hepatica* infection.

After flukes are presumed to have become established in the bile ducts, as judged by the appearance of eggs in the faeces and the rise and fall in $\gamma$-GT levels, fluctuations in enzyme and antibody levels occurred. These fluctuations, which were seen in only 1 lamb in the previous experiment (Chapter 2), were observed in the 3 infected animals in this experiment. As suggested previously, the fluctuations in $\gamma$-GT levels might result from damage caused to the bile ducts by adult flukes. In addition, the periodic release of bile and fluke antigens from bile duct lesions could cause destruction of adjacent hepatocytes, thereby explaining the variations in the levels of GLDH. Precipitating antibody levels could vary in response to the intermittent release of fluke antigens.

Following the challenge infection, both GLDH and $\gamma$-GT activities reached higher levels than observed during the primary infection. When juvenile flukes of the challenge infection migrate through the liver and into the bile ducts the damage they cause, and thus the release of GLDH and $\gamma$-GT,
would be superimposed on that caused by the activities of adult flukes of the primary infection which has been referred to above.

Precipitating antibody levels, despite their fluctuations, remained at relatively low levels following challenge. There was no apparent anamnestic response to the challenge with 100 metacercariae, many of which would have successfully established tissue contact and provided a significant antigenic stimulus. This suggests that the antibody response during this period was in some way suppressed. Similar observations in cattle (Kendall et al. 1978) and rats (Rajasekariah and Howell, 1978a) have been attributed to acquired immune mechanisms killing young flukes soon after challenge, thus resulting in a low level of secondary antigenic stimulation. Clearly this mechanism cannot be operating in sheep since this host shows no resistance to challenge; secondary exposure to antigen might be expected to elevate the antibody level back to and above that observed during the primary infection (Bach, 1978).

The apparent suppression of the antibody response following the challenge infection could be the result of a number of factors. Adult flukes may release an immunosuppressive agent. Alternatively, the release of antigenic material by adult flukes through damaged bile ducts, with the formation of immune complexes (as postulated in Chapter 2), could alone account for the observed fall in the level of free antibody.
The suppression of T and B cell responses by immune complexes is well documented (Sinclair, Lees, Abrahams, Chan, Fagan and Stiller, 1974; Wright, Hargreaves, Bernstein and Hellstrom, 1974; Morgan and Tempelis, 1977; WHO Technical Report, 1977, Morgan, Rodrick and Tempelis, 1978). Not only are responses to antigens associated with the complex suppressed, but also responses to unrelated antigens (Gorzynski, Kilburn, Knight, Norbury, Parker and Smith, 1975; WHO Technical Report, 1977; Morgan et al., 1978). Thus, the release of adult antigens may result in the suppression of antibody responses against juvenile fluke antigens that are perhaps unrelated to those of the adult. Immune complexes may also contribute to the tissue damage which apparently occurs after adult flukes are established in the bile ducts. Such complexes are known to cause tissue destruction in other diseases (Alberti, Realdi, Tremolada and Spina, 1976; Allison and Houba, 1976; WHO Technical Report, 1977; Andres, 1979).

Gundlach (1971b) also found there was no anamnestic response to a challenge infection in rabbits. This was most noticeable at higher levels of infection and in the titres of antibody directed against excretory-secretory antigens. Current views on immunosuppression by antigen-antibody complexes suggest that it occurs in a situation of antigen excess (Morgan et al., 1978). Higher levels of infection are more likely to create this condition thus these results may also support the idea of immunosuppression by antigen-antibody complexes.
The low levels of antibody observed in this experiment from the time of patency and after challenge may be important in the survival of juvenile flukes in sheep with existing adult fluke burdens. However, if the persistence of adult flukes is necessary for an immunosuppressed state to be maintained, an explanation is needed for the survival of juvenile flukes of a challenge infection after adult flukes from the primary infection have been removed (Sinclair, 1975; Rushton, 1977). It is possible that once suppression is established, a state of tolerance to certain antigens is maintained despite removal of the adults. Alternatively, sheep, by comparison with other hosts that exhibit resistance to challenge, may be lacking or deficient in some component of the afferent arc of their immune response to the parasite, so that juvenile flukes can survive irrespective of antibody levels. Finally, the antibodies assayed in this and previous tests (Kendall et al., 1978; Rajasekariah and Howell, 1978a) may have no effect on flukes, although similar precipitating antibodies affect the survival of juvenile *F. hepatica* in rats (Howell and Sandeman, 1979; Howell, in press).

Further studies of the apparent suppression of the antibody response are required. A repetition of this experiment, with the addition of a group treated with anthelmintic to remove the adults of the primary infection before the challenge was administered, would be desirable. Lack of time has prevented this important experiment from being carried out during the course of this study. Evidence which
provides indirect support for the existence of suppressed antibody levels is further discussed in Chapter 7 in conjunction with studies of the effects of infected sheep serum on juvenile flukes in culture. In the next chapter, an analysis of the immunoglobulins involved in precipitate formation on juvenile flukes in vitro is presented.
Table 3.1. Adult fluke recoveries from the bile ducts of sheep at necropsy.

<table>
<thead>
<tr>
<th>Lamb</th>
<th>Dose of metacercariae</th>
<th>Number of fluke recovered</th>
<th>% of dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100 + 100</td>
<td>73</td>
<td>36.5</td>
</tr>
<tr>
<td>2</td>
<td>100 + 100</td>
<td>82</td>
<td>41.5</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>51</td>
<td>51</td>
</tr>
</tbody>
</table>
4.1 Introduction

Ruminant immunoglobulins have been characterised into at least 3 classes; immunoglobulin M (IgM); immunoglobulin G (IgG) and immunoglobulin A (IgA) (Aalund, Osebold and Murphy, 1965; Aalund, Blakeslee, Butler, Duncan, Freeman, Jenness, Kehoe, Mach, Rapacz, Vaerman and Winter, 1971). The immunoglobulin G class can be further divided into IgG₁ and IgG₂ (Silverstein, Thorbecke, Kraner and Lukes, 1963) and possibly a third class IgG₁A (Curtain, 1969). These classes have been characterised by their electrophoretic mobilities, sedimentation coefficients, molecular weights and carbohydrate contents (Harrison and Mage, 1967; Pan, Kaplan, Morter and Freeman, 1968; Heimer, Clark and Maurer, 1969) and by their antigenic cross reactivity with comparative human classes (Heimer et al., 1969; Aalund, 1972). IgM, IgA and IgG₁ from goats, cattle and sheep show cross reactions of partial identity, probably reflecting the common evolutionary origin of these animals (Curtain and Fudenberg, 1973). The existence of IgG₁A and an IgD-like molecule in ruminants is possible (Heimer et al., 1969), but this has not been confirmed because of the difficulties of isolating these antibodies from complex mixtures. To date, immunoglobulin-secreting cytomas, which would aid the characterisation of ruminant immunoglobulins, are not available.

Following antigenic stimulation of mammals the first immunoglobulin to appear in the serum is generally IgM.
However, this rise is transient, and IgG quickly takes over as the predominant class (Aalund, 1972; Spiegelberg, 1974; Bach, 1978). The relative amounts of the IgG subclasses which are present after antigenic stimulation, is usually related to their initial serum levels (Spiegelberg, 1974). In most animals the subclass of highest concentration is IgG\(_1\) and it generally predominates, although this is dependent on the antigen and its method of introduction into the animals (Spiegelberg, 1974). English, Adams and Morris (1976) have also pointed out that the method used for assaying antibody levels can select particular immunoglobulins and so bias the results.

IgA is found at a low level in the serum of ruminants and its level does not usually increase markedly during the immune response. However, it is locally produced in many organs and may be important in reactions at these sites (Curtain and Anderson, 1971). Its local production in the hepatic parenchyma of cattle during *F. hepatica* infections may point to a role against this parasite (Flagstad and Eriksen, 1974).

Little work has been carried out on the immunoglobulin responses of sheep to helminth infections, particularly in regard to the identity of antibodies to specific parasites. In other hosts, immunoglobulin responses which have been extensively studied include those of rats and mice to *Taenia taeniaeformis, Schistosoma mansoni, Nippostrongylus*
brasilensis, Ascaris suum and recently Mesocestoides corti. Mice mainly form IgG\textsubscript{1} antibodies following infection with T. taeniaeformis and S. mansoni, and this subclass usually contains antibodies which can protect naive mice against these parasites (Musoke and Williams, 1975a; Sher, Smithers, Mackenzie and Broomfield, 1977). Antibodies of other classes are apparently formed, but at lower levels and later in the infection (Sher et al., 1977). During Mesocestoides corti infections mice produce very large amounts of IgG\textsubscript{1} and low levels of IgG\textsubscript{2} (Mitchell, Marchalonis, Smith, Nicholas and Warner, 1977). However, it is not known whether most of the IgG\textsubscript{1} represents antibody directed against the parasite (Chapman, Knopf, Anders and Mitchell, 1979). The stimulation of large numbers of immunoglobulin-secreting B cells is also a feature of other parasitic infections in mice (Crandall, Crandall and Franco, 1974; Rosenberg, 1978; Prowse, Mitchell, Ey and Jenkin, 1978; Chapman, Knopf, Hicks and Mitchell, 1979). Mitchell and his associates suggest that this may be due to the chronicity of antigen exposure.

In rats infected with T. taeniaeformis or N. brasiliensis, IgG\textsubscript{1} again seems to be the predominant subclass. However, IgG\textsubscript{2}\textsubscript{a} is often formed, and it can confer protection in passive transfer experiments especially if recovered from serum after secondary infection (Jones, Edwards and Ogilvie, 1970; Ogilvie and Jones, 1973; Musoke and Williams, 1975b). IgG\textsubscript{2}\textsubscript{a} has also been found to be important in the transplacental transfer of resistance to T. taeniaeformis in the rat (Musoke, Williams, Leid and Williams, 1975).
Ascaris infections are known to stimulate the production of reaginic or homocytotropic antibodies in many hosts (Ogilvie, 1970). These are usually IgE or IgG₁ antibodies which elicit the release of soluble mediators of hypersensitivity reactions, for example, vasoactive amines, from certain cell types such as mast cells (Zvaifler, 1976). Recently IgE has been shown to activate cells which can then kill S. mansoni schistosomula in vitro (Capron et al., 1977). The class with homocytotropic activity in sheep is apparently IgG₁a, since this subclass has been associated with passive cutaneous anaphylaxis reactions during Ostertagia circumcincta infection (Hogarth-Scott, 1969; Curtain and Anderson, 1971). In addition, an IgE-like molecule has been described in cattle (Doyle, 1973b) and its existence cannot be ruled out in sheep.

The immunoglobulin response of the host during F. hepatica infection is essentially similar to that seen in other helminth infections. IgG antibodies are produced by rats in response to Fasciola hepatica infection (Howell et al., 1977). In ruminants, a rise in IgG₁ is the most significant effect following infection (Movsesijan et al., 1975), with only marginal increases in the levels of other immunoglobulins. The involvement of mercaptoethanol-sensitive antibodies (probably IgM) in the anti-parasite response was observed. Apart from this study, there have been no examinations of anti-F. hepatica immunoglobulin responses of sheep during the course of infection. The in vitro culture technique described in Chapter 2, using precipitate formation on the teguments of juvenile flukes as an
assay of antigen/antibody interactions, enabled the immunoglobulins involved in precipitate formation to be identified and the sequential appearance of some of the immunoglobulin classes and subclasses to be followed.

4.2 Materials and Methods

4.2.1 Immunoglobulin separation

Serum was separated by two descending gel filtration steps to obtain enriched fractions of IgM, IgG₁ and IgG₂. The methods used were basically those of Aalund et al. (1965), Mackenzie (1968) and Watson, Brandon and Lascelles (1972). The first separation was carried out on a 2.6 x 100 cm glass column (Pharmacia) packed with 17 g of Sephadex G200 (Pharmacia). The gel was swollen in 0.01 M hydroxymethyl-methylamine (Tris) buffer, pH 8.0 in 1 M NaCl and heated in a water bath for 2 h to remove air bubbles. It was then cooled and the column was poured. After equilibration with the buffer, 10 ml of sheep serum was placed on the column and allowed to move into the gel. Buffer was then placed on top and 5 ml fractions were collected on a LKB Radirac fraction collector. The protein content of the eluate was assayed by an LKB Uvicord at 280 nm. The whole apparatus was kept at 4°C to minimise protein degradation. A representative trace is shown in Fig. 4.1. Fractions were pooled from the ascending and descending sides of each peak, concentrated against 20% polyethylene glycol (PEG, Aquacide III, Calbiochem) in 0.85% NaCl to 5 ml, then dialysed against
Fig. 4.1 Protein content (OD 280 nm) of the eluate from a G200 Sephadex column during separation of 10 ml of sheep serum. Fractions were pooled from the ascending and descending sides of each peak.
0.85% NaCl for at least 48 h with at least 2 changes of saline. Identification of the fractions was carried out by immunoelectrophoresis (see below).

The second separation of IgG into its IgG₁ and IgG₂ components was carried out on a 1.6 x 100 cm column (Pharmacia) packed with 7 g of Sephadex DEAE A-50 gel. The gel was swollen in 0.05 M phosphate buffer, pH 8.0, heated as described above, and the column was poured. The fractions containing predominantly IgG from the G200 separation were pooled and concentrated to 5 ml before addition to the top of the column. A gradient mixing apparatus was used with a starting buffer of 0.05 M phosphate, pH 8.0, and a final buffer of 0.3 M phosphate, pH 8.0. The high molarity final buffer meant that this column had to be run at 20°C to maintain the phosphates in solution. Five ml fractions were collected on a Gilson mini MTDC fraction collector and assayed for protein content on an LKB Uvicord. A representative trace from this separation is shown in Fig. 4.2. The whole of peak 1 was pooled. This was usually also the case with peak 2, although secondary peaks on the descending side of peak 2 occasionally forced its division into ascending and descending fractions. The pooled fractions were returned to the original volume applied to the column by concentration against 20% PEG then dialysed against 0.85% NaCl before immunoelectrophoresis.
Fig. 4.2 Protein content (OD 280 nm) of the eluate from an A-50 ion-exchange column during separation of 5 ml of a pooled IgG fraction of sheep serum. Fractions were pooled from peaks 1 and 2.
4.2.2 Immunoelectrophoresis

This was carried out according to the procedures given by McInnes and Voge (1970), using 1% agar (Ionagar, No. 2, Oxoid) in 0.01 M veronal buffer, pH 7.2, containing 0.01% NaN₃ to prevent microbial growth. An LBK gel punch was used to cut wells and troughs. Gels were run on an LKB electrophoresis apparatus at 4°C with 0.01 M veronal buffer as the electrolyte solution. After equilibration for 1/₄ h at 10 mA the wells were filled with the test fractions obtained from the G200 and A-50 separations (see above). The gel was then run for 1½ to 2 h using 1% trypan blue as a marker. The troughs were cut out and filled with rabbit anti-whole sheep serum which diffused into the gel in a humidified container at room temperature (about 20°C). Rabbit anti-whole sheep serum was raised by injection of 1 ml of sheep serum subcutaneously into a New Zealand white rabbit. Four booster injections were given at 4 week intervals and blood was collected from the ear vein 1 week after the final injection. The serum was collected by centrifugation and stored at -20°C until required.

When precipitin lines had developed the gels were desalted for 24 h in 2% NaCl, washed for 24 h in distilled water containing 0.01% NaN₃ and 1% glycerol which prevented the gel cracking when it was dried. After drying the gels were stained with 0.1% amido black for 5-10 min, washed in 2% acetic acid and allowed to dry. Representative slides from the G200 and A-50 separations are shown in Fig. 4.3. Lines were identified according to Heimer et al. (1969) and Brandon (pers.
Fig. 4.3 Immunoelectrophoresis of samples 1 to 6 of sheep serum fractionated on a G200 column and of samples 1 and 2 from the IgG fraction of sheep serum, separated on an A-50 column.
comm.). Appropriate fractions were selected as enriched for IgM, IgG₁, IgG₂ and albumin.

4.2.3 Sheep serum

Eight samples of sheep sera were separated by the above methods. Four were derived from infected animals; the sera were taken 3, 9 and 20 weeks after a primary infection with 100 metacercariae of *F. hepatica*, and 3 weeks after a challenge infection of 100 metacercariae (19 weeks after the primary infection of 100 metacercariae). The other serum samples came from uninfected sheep of the same age as the test animals. Each fraction was tested for antibody activity against juvenile flukes *in vitro* and by double immunodiffusion tests (see below).

4.2.4 *In vitro* cultures

The procedures for excystment of metacercariae and their preparation for culture were as described in Chapter 2. Culture media, which consisted of 20% of the test fraction, 20% normal sheep serum and 60% 199 were sterilised by Millipore filtration (0.45 μ filter), and 5 ml of each was added to 50 or 100 metacercariae in a Leighton tube. Cultures were incubated in a shaking water bath for 160 h and the development of precipitate was observed. On completion of the culture period, a fluorescent antibody test was performed on the cultured flukes as described in Chapter 2, using FITC labelled rabbit anti-sheep immunoglobulin.
4.2.5 Micro-Ouchterlony tests

These were carried out as described in Chapter 2. Antibody activity in the immunoglobulin fractions was determined by running it against a juvenile worm excretory-secretory antigen (JWS) which was prepared as described in Chapter 2. Approximately 10 μl of JWS was placed in the centre well, and samples of the immunoglobulin fractions and a sample of the original whole serum were placed in the surrounding wells.

4.2.6 Ouchterlony tests

These were carried out according to McInnes and Voge (1970). A 1% solution of agar (Ionagar, No. 2) in PBS containing 0.1% NaN₃, was melted and 5 ml were placed in 5.5 cm petri dishes (Falcon plastics). Wells were punched with an Ouchterlony gel punch (8 mm holes, 6 mm apart) and then sealed with a small amount of molten agar. JWS antigen was placed in the centre well and immunoglobulin fractions and whole serum taken 3 weeks after infection with 100 metacercariae, were placed in the surrounding wells. Diffusion was allowed to proceed at room temperature until precipitin line development had ceased (96 h). The plate was washed in 2% NaCl, then in distilled water containing 0.01% NaN₃ and 1% glycerol. The gels were dried and stained as described for micro-Ouchterlony tests in Chapter 2.
4.3 Results

4.3.1 In vitro cultures

The addition of 20% normal sheep serum to immunoglobulin fraction cultures was found to be necessary to overcome the high worm mortality observed in cultures of test fractions and 199 alone. The high mortality in its absence was non-specific; it occurred in serum fractions from both infected and uninfected sheep. The effect was considered to be due to the depletion of nutrient factors in the medium, brought about by the separation procedures.

Extensive dialysis of the fractions before culture was also found to be most important. If this was not carried out, fine precipitates occasionally formed in cultures or the worms died. Even after extensive dialysis, worms sometimes appeared to suffer damage to the tegument which could be confused with attached precipitate, although the fluorescent antibody test usually overcame this problem. These effects were non-specific since they occurred in the presence of normal and infected sheep serum fractions.

The results of culturing juvenile flukes in various serum fractions from infected sheep are shown in Table 4.1. A precipitate developed on the teguments of juvenile flukes and in the culture medium in the presence of the IgM and IgG1 fractions of serum taken from sheep 3 weeks after infection. More precipitate appeared to develop in the presence of the IgM fraction (Fig. 4.4). The fluorescent antibody test showed
Fig. 4.4 Precipitate on a juvenile fluke cultured with the IgM fraction of serum from sheep infected with 100 metacercariae for 3 weeks.

Fig. 4.5 Fluorescence associated with precipitate on the surface of a juvenile fluke cultured with the IgM fraction of sheep serum taken 3 weeks after infection.
Table 4.1 Precipitate formation on the tegument of juvenile flukes cultured in serum fractions from infected sheep.

<table>
<thead>
<tr>
<th>Time after infection that serum was taken</th>
<th>Serum fraction tested</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgM</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>3*</td>
<td>-</td>
</tr>
</tbody>
</table>

*3 weeks after challenge infection

+ precipitate present

- precipitate absent
bright fluorescence of the IgM precipitate (Fig. 4.5) but worms from the IgG₁ culture had little precipitate attached and only occasional worms showed bright fluorescence.

Precipitate development was detected only in the presence of the IgG₁ fraction of serum taken from sheep 9 and 20 weeks after infection (Fig. 4.6). More precipitate was observed to form in the presence of IgG₁ from 9 weeks after infection than at 20 weeks. In both cases, the presence of sheep antibody in the precipitate was confirmed by the fluorescent antibody test.

Three weeks after challenge infection, precipitate development was only obtained in the presence of the IgG₁ fraction. The presence of antibody in the precipitate was confirmed by the fluorescent antibody test.

No precipitate was observed to form on juvenile flukes or in the medium when normal sheep serum or its IgM, IgG₁, IgG₂ and albumin fractions were tested.

4.3.2 Micro-Ouchterlony tests

Results of these tests are shown in Fig. 4.7. A single precipitin line formed between JWS and serum taken 3 weeks after infection and its IgG₁ fraction. No lines developed between JWS and the IgM or IgG₂ fractions. Two precipitin lines formed between JWS and serum taken 9 weeks after infection and its IgG₁ fraction. No lines formed between JWS
Fig. 4.6 Precipitate on the surface of a juvenile fluke cultured with the IgG1 fraction of serum from sheep infected for 9 weeks with 100 metacercariae.
Fig. 4.7 Micro-Ouchterlony Test. Sheep serum samples and their immunoglobulin fractions run against juvenile fluke excretory-secretory antigen (J).

Fig. 4.8 Ouchterlony test. Sheep serum taken 3 weeks after infection and its immunoglobulin fractions against juvenile fluke excretory-secretory antigen (J).
and the other fractions. A faint precipitin line developed against serum taken 20 weeks after infection and its IgG₁ fraction.

Two lines formed between JWS and serum taken 3 weeks after a challenge infection and its IgG₁ fraction.

No lines developed between JWS and serum from uninfected sheep or any of its immunoglobulin fractions.

4.3.3 Ouchterlony test

This test was performed to re-examine the results obtained with fractions of serum taken 3 weeks after infection. When JWS was run against this serum 1 line formed. (Fig. 4.8). One line also formed against its IgG₁ fraction and 2 against its IgM fraction. No other lines developed.

4.4 Discussion

The precipitate which formed when juvenile fluke were cultured in immunoglobulin fractions of infected sheep serum was very similar to that which forms in whole infected serum. However, although it appeared at first on the tegument, it apparently sloughed off the tegument more readily than that in whole serum and was seen free in the culture medium at an earlier stage of incubation. As a result of this, precipitate did not form the extensive covering on worms as observed in whole serum cultures (Fig. 2.1). These observations suggest that other serum factors may be required for extensive precipitate formation or to stabilize the precipitate.
Two immunoglobulin classes were found to contribute to the antigen-antibody precipitate. Three weeks after infection both IgM and IgG₁ antibodies were detected in the precipitate; only the latter were detected at later stages of infection and after a challenge infection.

The two assay methods initially gave different results with serum taken 3 weeks after infection. The in vitro culture-fluorescent antibody method suggested that IgM antibodies predominated, while the micro-Ouchterlony tests showed a line only against IgG₁. The differences probably derived from the nature of the techniques used. It was found that washing juvenile flukes prior to exposure to the fluorescent labelled antibody dislodged some precipitate from the tegument and that this may have been discarded if insufficient time was allowed for its sedimentation prior to the subsequent washing procedures. The low levels of fluorescence observed on worms incubated in the 3 week IgG₁ fraction might be accounted for in this way. Tests carried out at later time intervals involved centrifugation of juvenile flukes at low speed for 5 min between washings to overcome the problem. The strong fluorescence of flukes cultured in the presence of IgM prepared 3 weeks after infection suggests either that this precipitate was more tightly bound to the tegument, that heavier aggregates formed, or that larger amounts were present initially. The absence of a precipitin line against this same IgM fraction on the micro-Ouchterlony test may have related to the large size of IgM molecules (1 x 10⁶ daltons). Since IgM
molecules would move relatively slowly through the gel, a precipitin line, if it formed, would be nearer the antibody well. Alternatively the high salt concentration, used to enhance the development of precipitin lines, may have inhibited the formation of IgM-antigen complexes (Crowle, 1973). To determine if the salt concentration was responsible for the failure to obtain a precipitin line with the IgM fraction, an Ouchterlony test in which NaCl was omitted was set up with the 3 week fraction. Lines formed against whole serum, its IgG1 fraction and its IgM fraction confirming that both IgM and IgG1 antibodies 3 weeks after infection form precipitating complexes with juvenile fluke antigens. This test also suggests that high salt concentration inhibits precipitin formation with IgM antibodies.

The failure to detect IgG2 precipitating antibodies at any time during infection suggests either that they are not produced, or that the levels are beyond detection by the methods used. Previous studies have detected increases in total IgG2 levels in sheep serum after *F. hepatica* infection (Nansen, 1969) and after stimulation with defined antigens (Heimer *et al*., 1969; English *et al*., 1976). However, anti-*F. hepatica* IgG2 antibodies have not been demonstrated (Movsesijan *et al*., 1975). The level of IgG2 in sheep serum is considerably lower than IgG1 or IgM, and thus it is possible that antibodies of this subclass with anti-*F. hepatica* activity are beyond detection by the methods used.
It is clear that the formation of IgG₁ antibodies is elicited by *F. hepatica* in sheep, a finding in agreement with previous work (Nansen, 1969; Movsesijan *et al.*, 1975) using different assay systems. Other helminth and bacterial infections also elicit IgG₁ responses (Aalund, 1972). Although IgG₁ remains the major antibody class throughout primary and challenge infections, the appearance during this time of new antibodies of the same class is not precluded. In fact, the changing number of precipitin lines which formed against JWS in the double diffusion tests suggests that this occurs. Further tests to determine in more detail the nature of these changes are described in Chapter 5.

The involvement of IgA and IgG₁A was not investigated in this study, but the occurrence of large numbers of IgA secreting cells in the liver of cattle (Flagstad and Eriksen, 1974) and the existence of homocytotropic antibodies in other sheep infections (Hogarth-Scott, 1969), together with their involvement in the killing of schistosomes (Capron *et al.*, 1977), suggests that studies of these immunoglobulin classes would be worthwhile.
Chapter 5

PRECIPITATE FORMATION AND ANTIGEN-ANTIBODY
REACTIONS IN SERA FROM SHEEP INFECTED WITH

FASCIOLA HEPATICA

5.1 Introduction

The majority of studies on antigens of *Fasciola hepatica* have been directed towards the development of diagnostic reagents and vaccines (Geyer, 1967; Clegg and Smith, 1978). There has been little attempt to analyse and define those antigens expressed by *F. hepatica* during normal infections. Most vaccination and diagnostic trials have utilised adult worm homogenates or fractions thereof; many of the antigens present in such a complex may never come in sufficiently close contact with the host's immune system during the normal course of infection, to stimulate an immune response.

Capron, Biguet, Vernes and Afchain (1968) found that adult *F. hepatica* homogenates gave at least 25 distinct precipitin lines against a rabbit anti-homogenate serum. However, only 4 to 6 precipitin lines were seen using a similar antigen against sera collected from infected rabbits (Capron *et al.*, 1965). Thus, only a proportion of the potential antigens present in flukes are actually expressed during infection in a manner that elicits a detectable antibody response.

Studies on immune responses to liver fluke in cattle, rats and mice have indicated that the main developmental stages of the parasite which elicit protective
immunity are the juvenile and semi-mature migratory worms rather than adult flukes (Doyle, 1973a; Lang, 1976; Rajasekariah and Howell, 1977b; Hayes and Mitrovic, 1977). Hence the functional antigens of the parasite, namely those that stimulate protective immunity, appear to be expressed by immature worms rather than adult flukes. If this is the case, there would be little prospect of obtaining antigens capable of stimulating protective immunity from preparations of adult *F. hepatica*. The lack of success in the use of such preparations as vaccines provides some support for this conclusion (Geyer, 1967; Clegg and Smith, 1978).

The isolation of a precipitate with antigen and antibody components from cultures of juvenile flukes in infected sheep serum overcomes some of the problems associated with previous studies. Firstly, the antigens are expressed during normal infections since they react with antibodies in infected sheep serum. Secondly, the antigens are expressed by juvenile flukes, the developmental stage which first establishes contact with the host's immune system when it penetrates the gut wall and which in other hosts is a major target of protective immunity.

In this chapter the complexity of the antigen-antibody reactions which form this precipitate are examined over the course of primary and challenge infections of *F. hepatica* in sheep. In addition, specificity of the antigens involved in precipitate formation for various developmental
stages of the parasite are determined. Finally, precipitating antigen-antibody reactions between semi-mature and adult flukes and infected sheep serum are investigated in order to follow their development and complexity during the immune response.

5.2 Materials and Methods

Double diffusion in gel was performed by both Ouchterlony and micro-Ouchterlony techniques (McInnes and Voge, 1970), which were as described in Chapters 4 and 2 respectively. The scoring of line development in these gels was carried out before and after the desalting and washing process. Photography was carried out after washing, before drying and, if necessary, after staining.

5.2.1 Antigens

Excretory-secretory antigens of juvenile flukes (JWS) were prepared as described in Chapter 2.

Semi-mature worm excretory-secretory antigen (SMWS) was obtained by culturing flukes obtained from mice infected 3 weeks previously with 5 metacercariae each. The flukes were dissected from the livers and placed in tissue culture medium 199 at 37°C and incubated for several hours to allow regurgitation of their caecal contents. They were then washed several times in sterile 199 containing antibiotics as described for juvenile flukes in Chapter 2. Ten worms were placed in a sterile Leighton tube with 5 ml of 20% normal sheep serum in 199. Cultures were incubated at 37°C in a shaking water bath for 160 h before the medium was
centrifuged to remove debris; then concentrated to 0.5 ml on a Minicon B15 concentrator.

Adult worm excretory-secretory antigen (AWS) was obtained by culturing adult flukes recovered from the bile ducts of sheep slaughtered at Goulburn Abattoir, NSW. The methods used were as described for semi-mature worms, but only 2 adult flukes were placed in each culture tube. It was found that the condition of adult worms appeared to deteriorate more rapidly than that of semi-mature worms so the medium was collected after 96 h. The medium was centrifuged and concentrated to 0.5 ml. Falls in pH were especially noticeable with adult flukes and this was corrected by the addition of sterile 2.8% NaHCO$_3$.

5.2.2 Sheep sera

Sera collected from sheep after primary and challenge infections with *F. hepatica* (Chapters 2 and 3) were tested against the various antigen preparations.

5.2.3 Sheep serum absorptions

Serum absorbed with juvenile worms was prepared as described in Chapter 2.

Serum absorbed with semi-mature worms was prepared by incubating 5, 3 week old flukes from mice in 5 ml of 20% high antibody titre sheep serum, collected 8 weeks after infection, in medium 199. When cultures were terminated at
160 h, the medium was centrifuged to remove precipitate and concentrated to the original serum volume of 1 ml on a Minicon B15 concentrator.

Serum absorbed with adult worms was obtained by incubating 2 adult *F. hepatica* in 5 ml of 20% sheep serum collected 8 weeks after infection, in medium 199. When cultures were terminated at 48 h the medium was collected, centrifuged to remove precipitate and concentrated to 1 ml as described for semi-mature worm absorbed serum.

Unabsorbed serum was prepared by incubating serum collected 8 weeks after infection for the same culture period as used to prepare each of the absorbed sera.

An antiserum against precipitate obtained from cultures of juvenile flukes in infected sheep serum was raised in sheep. 1.4 mg of precipitate and 1 ml PBS was emulsified with an equal volume of Freund's complete adjuvant and injected intramuscularly into worm-free sheep (see Chapter 8). The methods of precipitate isolation were as described in Chapter 2. Two injections separated by 4 weeks were administered. The serum collected 1 week after the second injection was designated anti-precipitate serum (APS). Control serum was obtained after the injection of PBS emulsified with Freund's complete adjuvant (AFS).
5.3 Results

5.3.1 Juvenile fluke antigens and precipitate formation

In Chapter 2 it was found that 2 precipitin lines formed when JWS were run against infected sheep serum (collected 9 weeks after infection) in a micro-Ouchterlony double diffusion test. It was also shown that these precipitin lines were not obtained if the infected serum was first absorbed by culturing juvenile flukes in its presence (Fig. 2.6). The relationship between these precipitin lines and the formation of precipitate around juvenile flukes when cultured in infected sheep serum was confirmed as follows: JWS were tested against APS raised in sheep and 2 precipitin lines were observed (Fig. 5.1). Both of these lines formed reactions of identity with the lines that formed against infected sheep serum referred to above. No lines formed against AFS.

In order to determine whether 2 antigen-antibody reactions of a comparable nature were also detectable at other times after infection, JWS were tested against samples taken at 3 week intervals during the course of infection (Fig. 5.2 and Table 5.1). Three weeks after infection 1 precipitin line developed; at 6, 9 and 12 weeks, 2 lines formed close to each other in the gel. At 15 weeks after infection, 2 lines again formed, but further apart than previously. The challenge dose of metacercariae given 16 weeks after the primary infection did not alter the 2 line pattern observed at 15 weeks. From 27 weeks after infection
Fig. 5.1 Micro-Ouchterlony test. Developmental stage antigens run against anti-precipitate serum (APS), anti-PBS/Freund's control serum (AFS) and serum from infected sheep (InSS).

- J = juvenile excretory-secretory antigen
- S = semi-mature excretory-secretory antigen
- A = adult excretory-secretory antigen.
Fig. 5.2 Micro-Ouchterlony test. Juvenile fluke excretory-secretory antigen (J) run against serum samples taken over the course of infection with *F. hepatica* in sheep.
Table 5.1 Precipitin line development over the course of *F. hepatica* infections in sheep.

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*The schematic representation is intended to show the relative position of the various precipitin lines with antigen well on left of each column and antibody well to the right.
until autopsy a single broad band replaced the 2 line pattern.

5.3.2 Precipitins detected with semi-mature fluke antigens

The precipitate which formed in cultures of semi-mature worms and infected sheep serum was very similar in appearance to the juvenile fluke-infected sheep serum precipitate. It appeared at first on the tegument and was also later observed in the medium. By comparison with the juvenile fluke precipitate, it was more copious and large amounts had formed within 48 h of culture. The number of antigen-antibody interactions which were involved in this semi-mature worm precipitate was determined by testing SMWS against absorbed and unabsorbed sera by micro-Ouchterlony double diffusion (Fig. 5.3). Serum taken 8 weeks after a primary infection gave 3 lines against SMWS and none developed if the serum was absorbed by prior incubation with semi-mature worms.

Ouchterlony tests using SMWS as antigen were used to characterise the number of precipitin lines at various times after primary and challenge infection of sheep with *F. hepatica* (Fig. 5.4 and Table 5.1). Precipitin lines first formed at 3 weeks after infection when a faint line was observed near the antibody well; at 4 weeks 3 lines developed. Three lines also developed against serum taken 6 weeks after infection, but not all the lines at 4 and 6 weeks were identical. Two of the precipitin lines detected at 4 weeks appeared to have
Fig. 5.3 Micro-Ouchterlony test. Semi-mature fluke excretory-secretory antigen (S) run against semi-mature fluke absorbed (SAS) and unabsorbed (US) infected sheep serum.
Fig. 5.4 Ouchterlony tests. Semi-mature fluke excretory-secretory antigen (S) run against serum collected over the course of infection with *F. hepatica* in sheep.
coalesced to form a single line at 6 weeks. The 3 line pattern was maintained, however, because a new line appeared close to the antibody well. After 6 weeks the situation remained stable until 14 weeks when 1 of the 3 precipitin lines formed closer to the antibody well than it had previously. At 16 weeks only 2 lines were detected. At 18 weeks, 2 weeks after challenge infection, 3 lines were again detected, with a new line appearing near the antibody well. A further precipitin line appeared at 20 weeks resulting in 4 lines. However, at 22 weeks 2 of the precipitin lines seen at 20 weeks had coalesced and 3 lines were present. At 24 weeks 1 broad band apparently replaced 2 of the lines seen at 22 weeks. This pattern continued until 30 weeks after the primary infection (14 weeks after challenge) when 1 of the bands appeared to have split giving rise to a single diffuse band and 2 distinct lines. At 32 weeks the line which was nearest the antibody well at 30 weeks did not develop, the remaining lines were not distinct, and 1 broad band formed. A single line was also obtained at 34 and 36 weeks.

5.3.3 Precipitins detected with adult worm antigens

The precipitate which formed when adult worms were cultured in serum collected from sheep 8 weeks after infection was similar to that which formed when juvenile and semi-mature worms were cultured in infected sheep serum. Thus, it formed on the teguments of the worms but was also found in the culture medium shortly after cultures were initiated. The amounts that formed were judged to be more copious than with either juvenile or semi-mature flukes.
Analysis of the number of antigen-antibody interactions involved in the formation of this adult precipitate revealed that 3 precipitin lines formed in diffusion tests of AWS against serum collected 8 weeks after infection. These lines did not form if the serum was absorbed with adult flukes prior to the test (Fig. 5.5).

Ouchterlony tests were used to characterise the number of antigen-antibody interactions that formed against AWS over the course of primary and challenge infections of sheep with *F. hepatica* (Fig. 5.6 and Table 5.1). The patterns which developed in these tests were complex and difficult to interpret because the bands were diffuse and often lacked sharp boundaries. In addition, a greater number of bands were usually present in these gels than in former tests. However, at 3 weeks after infection a broad faint line formed near the antibody well; at least 2 interactions appeared to take place 4 weeks after infection; one was in the form of a broad precipitin band near the antibody well and the other a sharp precipitin line nearer the antigen well. At 8 weeks a third diffuse band was detected and another line formed at 12 weeks after infection suggesting 4 major interactions. This 4 line pattern was subsequently observed throughout the primary infection. After the challenge infection at 16 weeks there was little change in the precipitin line pattern until 26 weeks when a fifth line was detected. At 28 weeks after the primary infection and until autopsy, 4 lines were again observed.
Fig. 5.5 Ouchterlony test. Adult fluke excretory-secretory antigen (A) run against adult fluke absorbed (AAS) and unabsorbed (US) infected sheep serum.
Fig. 5.6 Ouchterlony test. Adult fluke excretory-secretory antigen (A) run against serum taken over the course of infection with *F. hepatica* in sheep.
5.3.4 *Stage specificity of antigens*

In addition to the studies of reactions to each kind of antigen preparation described above, several comparative tests were performed to analyse the cross reacting and non-cross reacting components of the 3 different excretory-secretory antigen mixtures.

In the first test JWS, SMWS and AWS were each run against the 3 lots of sera absorbed with either juvenile or semi-mature or adult flukes and unabsorbed sera (Fig. 5.7 and Table 5.2). In all cases the antigen preparations gave no precipitin lines with sera absorbed with the homologous stage of development from which the antigen preparations were derived. In addition, no precipitin lines formed between JWS and serum absorbed with semi-mature worms. However, 1 line formed between JWS and serum absorbed with adult worms. A single precipitin line formed between SMWS and serum absorbed with juvenile flukes, and 1 line formed between SMWS and serum absorbed with adult flukes. AWS gave 2 lines against serum absorbed with juvenile flukes and 1 faint line against serum absorbed with semi-mature worms.

The 3 antigen preparations were also each tested with sera raised in sheep against the juvenile fluke antigen-sheep antibody precipitate (APS) and its control serum (AFS) (Fig. 5.1 and Table 5.3). Two precipitin lines formed against all 3 antigen preparations.
Fig. 5.7 Micro-Ouchterlony test. Antigen preparations of various stages of development of *Fasciola hepatica* run against serum absorbed with these different stages of development.  
J = juvenile fluke excretory-secretory antigen  
S = semi-mature fluke excretory-secretory antigen  
A = adult fluke excretory-secretory antigen  
JAS = juvenile fluke absorbed serum  
SAS = semi-mature fluke absorbed serum  
AAS = adult fluke absorbed serum.
Table 5.2 Precipitin lines between antigen preparations of various stages of development of *Fasciola hepatica* and serum absorbed with these different stages of development.

<table>
<thead>
<tr>
<th>Antigen preparation</th>
<th>Precipitin lines against:</th>
<th>Serum* absorbed with:</th>
<th>Unabsorbed serum</th>
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<tr>
<td>SMWS</td>
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<td>1</td>
</tr>
<tr>
<td>AWS</td>
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<td>1 faint</td>
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</tbody>
</table>

*This serum was taken 8 weeks after infection, before the full complement of adult antigens were expressed (see text).*
Table 5.3  Precipitin line development between antigen preparations of various stages of development of *Fasciola hepatica* and anti-juvenile fluke precipitate serum

<table>
<thead>
<tr>
<th>Serum</th>
<th>Antigen preparation</th>
<th>JWS</th>
<th>SMWS</th>
<th>AWS</th>
</tr>
</thead>
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<td>APS</td>
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<td>2</td>
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<tr>
<td>AFS</td>
<td>0</td>
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5.4 Discussion

Interpretation of the patterns obtained in these double diffusion tests was based on Crowle (1973). A single precipitin line in the gel was considered to reflect 1 antigen-antibody reaction. However, this approach may only denote the minimum number of antigen-antibody reactions. This is especially the case when testing complex mixtures of antigens (Crowle, 1973). The excretory-secretory products of helminths would fall into this category. Some antigens in the antigen preparations used are probably at low concentrations, and they may not be detected by the relatively insensitive double diffusion technique. It should also be noted that any complex molecules which may be secreted by *F. hepatica* could have a number of different antigenic determinants and thus a number of antibodies directed against them. This may further complicate the formation of precipitin lines.

It has already been shown that two antigen-antibody reactions are involved in the precipitate which develops on the teguments of juvenile flukes cultured in infected sheep serum (Chapter 2). Confirmation of this result was obtained in this chapter by raising an antiserum in sheep against this precipitate; when tested against juvenile worm excretory-secretory antigens (JWS), the antiserum gave 2 precipitin lines. That the *in vitro* culture precipitate was homologous with the precipitin lines which formed in double diffusion tests was confirmed by prior absorption of infected sheep
serum with juvenile flukes. When this was done, the precipitin lines did not form.

A maximum of two antigen-antibody precipitation reactions also occurred between JWS and sera collected from sheep throughout the course of both primary and challenge infections of *F. hepatica* (Table 5.1). At first, 3 weeks after infection, a single line was detected which probably reflects either low antibody concentration in the serum, causing a lack of definition in line formation, or the absence of antibody to the second antigen at this time. From 6 weeks after infection two lines formed and these persisted until well after the challenge infection when they coalesced to form a single wide band. This may again have resulted from low antibody concentrations (Crowle, 1973). There was no apparent effect of the challenge infection on precipitin line formation. This may have been due to the fact that there was no apparent anamnestic response to juvenile fluke antigens after a challenge (Chapter 3). The only notable change in the double line pattern occurred between 12 and 15 weeks after the primary infection, when the previously close precipitin lines apparently moved further apart. This might be explained by a fall in concentration of one of the antibodies resulting in a shift of one of the lines towards the antibody well. Measurement of the position of the lines does in fact show movement of one towards the antibody well by comparison with their respective positions at 12 weeks.
The presence in semi-mature and adult flukes of juvenile fluke antigens involved in precipitate formation was analysed by testing JWS against sera absorbed with homologous and heterologous fluke developmental stages, and by testing the 3 different excretory-secretory antigen preparations against APS (Table 5.2 and 5.3). These tests showed that both juvenile antigens detected in the precipitate are also present in semi-mature and adult worms.

Analysis of the antigen-antibody reactions which formed the precipitate in cultures of semi-mature flukes and infected sheep serum showed that at least 3 semi-mature worm antigens were involved. This was confirmed by the finding that prior absorption of infected sheep serum with semi-mature flukes resulted in the loss of all 3 precipitin lines.

Ouchterlony double diffusion analysis of reactions between SMWS and sera from sheep undergoing *F. hepatica* infections revealed a complex and changing pattern of precipitin line development. A faint precipitin line first developed at 3 weeks after infection and this may represent IgM and IgG₁ antibody-antigen reactions. Four weeks after infection 3 lines formed but at 6 weeks 2 previously separate lines appeared to have coalesced and a new line appeared near the antibody well. These changes probably represent the production of new antibodies to antigens already experienced by the host. However, the loss of a line at 16 weeks, which was preceded by the movement of that line towards the antibody well at 14 weeks, suggests a fall in antibody concentration which may
reflect the isolation of flukes as they enter the bile ducts and attain sexual maturity at about 10 weeks. Two weeks after the challenge infection a third line appeared near the antibody well, possibly reflecting the reintroduction of the host to an antigen characteristic of juvenile flukes. The brief appearance of 4 lines 4 weeks after the challenge infection may once again be due to the production of new antibodies to existing antigens, this time stimulated by the challenge infection.

Studies of SMWS run against each sera absorbed with one of the 3 developmental stages, revealed that 2 of the 3 antigen-antibody reactions seen with semi-mature worms were shared with juvenile flukes (Table 5.2). This confirmed the results with JWS discussed above. In addition, 2 reactions were found to be shared with adults, one of which was also common to juvenile flukes.

The precipitin line pattern that developed between adult worm excretory-secretory antigen (AWS) and adult fluke absorbed and unabsorbed sera showed that at least 3 antigen-antibody interactions were involved in the precipitate which formed in association with adult flukes and infected sheep serum in vitro. The wide bands of precipitate seen in these double diffusion tests and those with SMWS antigen may result from the high concentrations of antigens in the preparations of semi-mature and adult flukes. An excess of antigen over antibody in the gel can result in shifts in the position of precipitin lines; wide bands of precipitate
may result and also Liesegang lines (multiple lines) may form within the band (Crowle, 1973). This may explain the complex nature of the broad precipitin band which forms near the antibody well in these tests.

The antibody-antigen reactions which formed between AWS and serum collected over the course of *F. hepatica* infections in sheep showed more variation than tests involving JWS or SMWS. Three weeks after infection a single line formed close to the antibody well probably representing IgM and IgG1 antibody-antigen interactions (see Chapter 4). Four weeks after infection 2 IgG-like precipitin lines were detected; however, 3 appeared 8 weeks after infection and 4 at 12 weeks. These changes probably reflect the release of new antigens by the developing flukes and thus the appearance of new antibodies in the serum. Four precipitin lines were detected 12 weeks after infection and this pattern persisted subsequently. Thus, the tests of AWS against adult fluke absorbed and unabsorbed sera taken 8 weeks after infection, which showed only 3 major reactions (see Table 5.1 and 5.2), did not reveal the full range of adult antigens that might become involved in precipitate formation at later stages of infection. A challenge infection administered 16 weeks after the primary infection once again had little effect on the precipitin line pattern. A brief appearance of 5 lines, 10 weeks after the challenge infection, may reflect a change in antibody concentration or the production of a new type of antibody to an existing antigen.
When AWS was tested against the different lots of absorbed sera, 2 lines formed against juvenile fluke absorbed serum and 1 against semi-mature fluke absorbed serum (Table 5.2). As mentioned above, 3 lines formed against unabsorbed serum which was taken 8 weeks after infection. Thus these results suggest that the adult stage shares 1 antigen with juvenile flukes and 2 with semi-mature worms (Table 5.2). In contrast, the anti precipitate serum test suggested that the adult worms secreted both of the juvenile antigens. Because the absorbed sera assays are only indirect measures of shared antigens, it is probable that the result obtained with APS is correct. This also suggests that the antigen-antibody reactions at each stage may be more complex than these assays indicate.

The results obtained with AWS against serum collected 12 weeks and subsequently after infection suggest that 4 major precipitating antigen-antibody reactions take place. It appears that 2 of these are specific to the adult stage since they do not appear until late in the infection. However, the APS result suggests that adult worms express at least 5 antigens, in which case one of the broad bands seen against AWS may represent 2 antigen-antibody reactions.

Fig. 5.8 schematically summarises the antigens that have been found to precipitate with antibody and which seem to be present at each stage of development of *F. hepatica* in sheep, and the time after infection at which they appear to
Fig. 5.8 A schematic summary of the antigens produced by each developmental stage of *F. hepatica*.

*Developmental stages of F. hepatica*

<table>
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<th>Weeks after infection (sheep)</th>
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<th>Adults</th>
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The time of appearance of these antigens is based on the appearance of new precipitin lines in the analysis of JWS, SMWS and AWS versus sera from infected sheep. As a result of the time taken for detectable levels of antibody to be produced, the actual time of an antigen appearance may be 1 or 2 weeks before that given.
be first expressed. In summary, the results of these tests suggest that the juvenile fluke secretes 2 antigens which stimulate the formation of precipitins and that these are also secreted by semi-mature (3 weeks old) and probably by adult flukes. Semi-mature flukes secrete an additional antigen which is not shared with juveniles but is shared with adults. Adult flukes were found to secrete 4 or 5 antigens which precipitate with antibodies in infected sheep serum; 2 are unique to the adult, 1 is shared with semi-mature flukes but not juveniles and 2 are probably shared with both semi-mature and juvenile flukes. Thus, 2 of the antigens which stimulate the production of precipitating antibody are apparently produced by all developmental stages of *F. hepatica*.

Comparisons between this study and others on precipitins during *F. hepatica* infections are difficult because most other studies have used adult fluke homogenate-derived antigens and because studies of this type on sheep have not been previously reported. Gundlach (1971a, 1971b) studied precipitin line development between an adult fluke metabolic antigen and sera collected over the course of *F. hepatica* infections in rabbits. He observed 2 precipitin lines to develop 3 weeks after infection but this increased to 3 at 4 weeks, 4 at 5 weeks and to 5 or 6 by 16 weeks after infection. Thus, there is a general correlation between these results and those obtained in this study with AWS. Most other studies have observed the formation of larger numbers of precipitin lines with adult homogenate antigens against
sera from rabbits, although there is still a sequential appearance of these antigens as the flukes develop (Sewell, 1964; Capron et al., 1965; Sinclair and Kendall, 1969; Gundlach, 1971b; Deelder, 1973). No other studies have previously attempted to analyse other developmental stages of *F. hepatica* to determine the stage specificity of antigen production.

The apparent tegument origin and secretory nature of the antigens involved in the formation of precipitate in cultures of juvenile flukes with infected sheep serum was discussed in Chapter 2 and is further investigated in Chapter 6. The similarities in appearance and mechanism of formation of the semi-mature and adult precipitates to the juvenile precipitate, and the occurrence of common antigens in these precipitates, suggests that the semi-mature and adult precipitating antigens are also secreted from the tegument. A comparison with the ultrastructure of the tegument of developmental stages of *F. hepatica* is warranted.

Bennett and Threadgold (1975) have described the development of the tegument in flukes recovered from experimentally infected mice. They found that different types of secretory granules are produced in the tegument at different stages of development. In freshly excysted juveniles, Type 0 tegumental cells produce Type 0 (To) granules. When the flukes enter the mouse liver the cells start producing Type 1 (T₁) granules and after 3 weeks of infection T₁ granules
predominate; the tegumental cells are then reclassified as Type 1. Type 2 cells differentiate 2-3 days after infection and begin to produce Type 2 (T₂) granules as the flukes move through the liver. Large numbers of these granules are released into the tegument when the flukes enter the bile ducts. The contents of these different granules are apparently released onto the surface of the flukes. Comparison of these granule types with the appearance of the different antigens of developing flukes (Fig. 5.8) suggests some parallels. However, it should be noted that fluke development to sexual maturity in mice is 4-5 weeks, but 9-10 weeks in sheep. The timing of the appearance of granule types is, therefore, probably faster in mice, especially during the liver migratory period.

The timing of the appearance of these granules in terms of the development of *F. hepatica* agrees with the apparent times of appearance of the different antigens discussed above (Table 5.1 and Fig. 5.8). The production of the juvenile T₀ granules correlates with the appearance of the juvenile antigens X and/or Y. The T₁ granules produced by the semi-mature and adult flukes correlates with production of antigen A and the T₂ granules produced by the adults and possibly also by late migratory stages correlates with antigen B and/or antigen C. Antigen X and/or Y could be present in both T₀ and T₁ granules, which are produced by the same cell type. This would explain their production by all developmental stages.
Bennett and Threadgold (1975) have suggested that the 3 types of secretory granules produced by the tegumental cells may be involved in the formation of the glycocalyx which covers the plasma membrane of *F. hepatica*. The different types and sequential appearance of secretory granules would be concerned with the changing functions of the glycocalyx as the fluke moves into and through different host tissues. The gradual turnover of the glycocalyx which is assumed to occur in *F. hepatica* (Bennett and Threadgold, 1975), but known to occur in *Schistosoma mansoni* (Wilson and Barnes, 1977) with a half life of 2-3 h (Wilson and Barnes, 1979), would also explain the dynamics of formation of precipitate on the tegument as described in Chapter 2. Attempts to determine whether the antigens analysed in these experiments are involved with the glycocalyx are discussed in the next chapter.
6.1 Introduction

The outermost surface layer of trematodes, the tegument, is a metabolically active structure being involved in nutrient uptake, excretion and protection of the parasite (Lee, 1966, 1972). The protective function is achieved by a number of mechanisms. For example, in schistosomes host antigens are absorbed on to the tegument and may disguise parasite antigens (Clegg et al., 1971; McLaren et al., 1975), and a glycocalyx is secreted which may be able to mask surface membrane antigens (Wilson and Barnes, 1977). These mechanisms are considered to protect adult flukes from host immunity. The early invasive stages of the parasite, the schistosomula, which lack a fully developed glycocalyx and host antigens on their surfaces, are susceptible to the effects of host immunity (McLaren et al., 1975; McLaren and Hockley, 1976). Thus a state of concomitant immunity exists; the presence of adult worms in a host resistant to reinfection (Smithers et al., 1969).

Absorbed host antigens are not apparently present on the tegument of Fasciola hepatica (Hughes and Harness, 1973a, 1973b). However, the liver fluke does possess a glycocalyx covering the outer membrane of the tegument (Threadgold, 1968), and a state of concomitant immunity also exists with respect to some of its hosts. The glycocalyx may, therefore, serve as a major defence against the host's immune responses.
The presence of parasite antigens in the precipitate which forms on the teguments of juvenile *F. hepatica* cultured in vitro with infected sheep serum, suggested that these antigens might be derived from the tegument and become associated with the surface glycocalyx. In an attempt to study this question several experiments were carried out including analysis of the secretion of antigens from the tegument of juvenile flukes, tests of the chemical nature of antigens involved in precipitate formation, and analysis of the origin of these antigens both on the surface and within the tegument of the flukes. In addition, a comparison was made of juvenile, semi-mature and adult fluke antigen/sheep antibody precipitates by polyacrylamide gel electrophoresis.

6.2 *Materials and Methods*

6.2.1 *Active secretion of antigens*

The secretion of antigens involved in precipitate formation from the teguments of juvenile flukes was analysed by culturing juvenile flukes in the presence of serum taken from sheep 15 weeks after infection with 100 metacercariae. After 160 h the flukes were removed, washed vigorously with PBS to remove loosely attached precipitate, then placed in cultures containing 5 ml of 20% normal sheep serum in medium 199. Methods for excystment and culture of flukes were as described in Chapter 2.
6.2.2 Chemical nature of antigen

Tests for the presence of lipid and polysaccharide moieties in the antigens involved in precipitate formation were carried out after juvenile flukes had been cultured with 5 ml of full strength serum taken from sheep infected 14 weeks previously with 100 metacercariae, or from uninfected sheep of the same age. Methods for excystment and culture of flukes were as described in Chapter 2. Tests for the presence of lipid were carried out by staining juvenile flukes, fixed in 10% formalin, with a saturated solution of Sudan IV in propylene glycol. Carbohydrate was detected by the periodic acid Schiff (PAS) reaction (McManus and Mowry, 1964).

6.2.3 Electron microscopy

Attempts to determine the origin of the antigens involved in precipitate formation within the tegument was carried out by immunochemical labelling techniques and electron microscopy. Methods were adapted from Kraehenbuhl and Jamieson (1974), McLaren et al. (1975) and Threadgold and Befus (1977). The first technique was designed to label the fluke antigen/sheep antibody precipitate attached to the surface of juvenile flukes cultured in serum from sheep 10 weeks after infection with 200 metacercariae, or in the IgG₁ fraction of that serum. Juvenile flukes cultured in normal sheep serum and its IgG₁ fraction were included as controls. Cultures were set up containing 20% of the test serum in 199 or its IgG₁ fraction as described (Chapters 2 and 4). Worms
were removed after 90 h and washed gently several times in cold (4°C) PBS. They were fixed for 1 hour in 0.5% glutaraldehyde (Ladd Laboratories) in 0.01 M phosphate buffer, pH 7.3, containing 0.15 M sucrose and 0.5 mM CaCl₂ at 4°C. Following overnight washing with 0.01 M phosphate buffer they were incubated with a 1:10 dilution of rabbit anti-whole sheep serum (see Chapter 4) in PBS for 2 h at room temperature. After a wash with the 0.01 M phosphate buffer they were incubated with a 1:10 dilution of horseradish peroxidase conjugated goat anti-rabbit IgG (Miles-Yeda) in PBS. Following overnight washing in phosphate buffer, the enzyme was localised with 5 mg of 3-3'diaminobenzidine tetrahydrochloride (DAB, BDH) in 10 ml of 50 mM Tris-HCl buffer, pH 7.6, containing 0.01% hydrogen peroxide, for 1 h at 37°C. The worms were washed 3 times in Tris-HCl buffer before post-fixing in 1% osmic acid (BDH) in 0.1 M phosphate for 1 h at 4°C. The worms were then washed 3 times in distilled water before dehydration in an alcohol series and embedding in Spurr A (Glauert, 1975).

The second method attempted to label antigens within the tegument of juvenile *F. hepatica*. Metacercariae were excysted and washed as described in Chapter 2 but they were then fixed in 5% paraformaldehyde in 0.01 M phosphate, pH 7.3, containing 4% sucrose and 0.4 mM CaCl₂, for 4 h at 4°C. The worms were washed overnight in 0.01 M phosphate buffer at 4°C, then centrifuged to form a pellet. This was mounted in O.C.T compound (Lab-Tek) on a cryostat (International Equipment).
and frozen sections of about 40 μm cut. The embedding medium was removed by washing at 4°C with 0.01 M phosphate buffer. The thick sections were then exposed at room temperature for 4 h with 1:10 dilutions in PBS of one of the following: sheep serum collected 9 weeks after infection with 100 metacercariae, normal sheep serum, the infected sheep serum IgG₁ fraction, or the normal sheep serum IgG₁ fraction. After an overnight wash with 0.1 M phosphate buffer they were incubated with 1:10 rabbit anti-sheep serum for 4 h, washed overnight and finally incubated with 1:10 horseradish peroxidase conjugated goat anti-rabbit IgG for 4 h. Following overnight washing in phosphate buffer the sections were refixed in 2% glutaraldehyde in 0.1 M phosphate with sucrose and CaCl₂ for 1 h at 4°C. They were then washed overnight, the enzyme was localised with DAB, and embedded as described above.

Silver sections were cut on a Reichert OMU 2 microtome, floated on to 200 mesh LKB grids and viewed in a Hitachi HU 12 electron microscope.

6.2.4 *Sodium dodecyl sulphate - Polyacrylamide gel electrophoresis (SDS-PAGE)*

This method was used in an attempt to resolve host and parasite components of the precipitate. The SDS-polyacrylamide system is a powerful technique for the separation of protein mixtures on the basis of molecular weight (Ryden and Walker, 1978). The effects of the charge and shape of
the molecules during electrophoresis are apparently negated by the binding of dodecyl sulphate ions to the molecules, which not only results in most proteins carrying a net negative charge but also packages them into uniform "rod like" structures (Ryden and Walker, 1978). The addition of a reducing agent such as dithiothreitol to the protein-SDS mixture also dissociates disulphide bonds and thus antibody molecules are broken down to their constituent light and heavy chains (Stanworth and Turner, 1978).

Methods used in these assays were essentially those of Laemmli (1970). Gels contained 5% (stacking gel) or 8% acrylamide (separation gel; Eastman) and 0.8% of N,N'-bis-methylene acrylamide (Eastman). The final concentrations in the separation gels were 0.375 M Tris-HCl, pH 8.8, and 0.1% sodium dodecyl sulphate (SDS; BDH). The gels were polymerised by the addition of tetramethylethylene diamine (TEMED; Fluka) to a final concentration of 0.44%. Slab gels (14 x 17 x 0.15 cm) were prepared between glass plates. After polymerization 200 μl samples (see below) were added to the stacking gel and the gel was run vertically on a Raven electrophoresis apparatus at 100 V for 6 h. Samples were prepared in a solution containing 0.0625 M Tris-HCl, 10% glycine and 2% SDS at pH 6.8 with 40 mM DL-dithiothreitol (Sigma). The samples run on the gel included, juvenile fluke antigen/sheep antibody precipitate, semi-mature fluke antigen/sheep antibody precipitate and adult fluke antigen/sheep antibody precipitate (see Chapter 5). In addition, several
standard proteins of known molecular weight (Weber and Osborn, 1969) were run to aid in the identification of molecular weights of the sample proteins. These include haemoglobin (MW 15,000, Sigma), pepsin (MW 35,000, Sigma), bovine serum albumin (BSA, MW 68,000, Sigma), transferrin (MW 80,000, Sigma) and glutamate dehydrogenase (GLDH, MW 53,000, Boehringer Mannheim). Serum fractions enriched for IgG₁ (see Chapter 4) were also run to show the positions of the heavy and light chains of the immunoglobulin.

Fixing and staining methods were essentially those of Fairbanks, Steck and Wallach (1971). The proteins were fixed in the gel with 25% isopropyl alcohol and 10% acetic acid overnight then stained for 8 h in the same solution but with 0.5% Coomassie blue added. The gels were then destained in 10% isopropyl alcohol, 10% acetic acid overnight and for several hours in 10% acetic acid. The last solution was changed once and the gels remained in this until a clear background was achieved.

Gels were also stained for carbohydrate using the PAS procedure. The samples were fixed as outlined above but without Coomassie blue in the second solution. The gels were stained by immersion in 0.5% periodic acid for 2 h, 0.5% sodium arsenite in 5% acetic acid for 30 to 60 min, 0.1% sodium arsenite in 5% acetic acid for 20 min (3 times) and acetic acid for 10 to 20 min. Schiff's reagent was then
added and left overnight. The gel was treated with several changes of 0.1% sodium metabisulphite in 0.01 M HCl for several hours until the solution no longer turned pink upon addition of formaldehyde. Schiff's reagent turns red in the presence of free aldehydes and this test indicates when all of the Schiff's reagent has been removed from the gel.

6.3 Results

Juvenile flukes cultured for 160 h in infected sheep serum became enveloped by large amounts of precipitate. After vigorous washing most of this precipitate was removed and that which remained appeared to be firmly attached to the tegument. Twelve hours after transferring the flukes to cultures containing normal sheep serum precipitate could be seen free in the medium and sloughing off the teguments of some worms. By 72 h the more active flukes had lost their covering of precipitate and some was present in the medium.

Staining tests carried out on the precipitate attached to the surface of juvenile flukes after culture in infected sheep serum were strongly positive for the presence of polysaccharides (Fig. 6.1) but not lipid.

Attempts to specifically label the antigen involved in precipitate formation with horseradish peroxidase were not successful. There were no marked differences in the staining pattern of flukes cultured with infected or normal
Fig. 6.1 A periodic acid Schiff reaction carried out on juvenile flukes after culture with infected sheep serum. The attached precipitate stained strongly positive.
sheep serum. The major site of staining was the glycocalyx which overlies the tegumental membranes (Fig. 6.2) (Threadgold, 1976). There was also staining of the precipitate attached to juvenile flukes cultured with infected sheep serum (Fig. 6.3). Application of the staining procedure to frozen sections of juvenile flukes did not stain tegumental cell organelles but stained the glycocalyx and precipitate as described for worms which were processed intact. Similar results were obtained using the IgG1 fractions of infected or normal sheep serum.

The results of SDS-PAGE analysis of the precipitate are shown in Fig. 6.4 (protein) and 6.5 (carbohydrate). Ten protein bands were detected when a juvenile fluke antigen/sheep antibody sample was run, 8 developed with semi-mature fluke antigen/sheep antibody and 5 with adult fluke antigen/sheep antibody precipitate. These have been numbered in Fig. 6.4, from 1 to 12 in order of decreasing molecular weight. Bands with the same number in the different precipitate samples are assumed to represent the same or very similar proteins. Using this system, bands 1, 2, 3 and 4 are apparently unique to the juvenile fluke precipitate; bands 8 and 9 are absent but these appear in the semi-mature fluke precipitate. The adult precipitate only has bands 5, 6, 10, 11 and 12. The fraction enriched for IgG1 shows several bands and obviously contains a number of proteins other than IgG1. A major contaminant is the line (C) at a position corresponding with a molecular weight of
Fig. 6.2 Electron microscopy of horseradish peroxidase labelled juvenile flukes after culture with normal sheep serum a) low magnification, $\times 19\,000$

b) high magnification. $\times 68\,000$
Fig. 6.3 Electron microscopy of horseradish peroxidase labelled juvenile flukes after culture with infected sheep serum a) low magnification, ×13000
b) high magnification. × 50000
**Fig. 6.4** Sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Coomassie blue stained gel.

- **JP** = juvenile fluke antigen/sheep antibody precipitate
- **SP** = semi-mature fluke antigen/sheep antibody precipitate
- **AP** = adult fluke antigen/sheep antibody precipitate.

- **Hb** = Haemoglobin; **P** = Pepsin; **BSA** = Bovine serum albumin; **T** = transferrin; **GLDH** = glutamate dehydrogenase; **IgG₁** = IgG₁ enriched fraction of sheep serum.
Fig. 6.5 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Periodic acid Schiff stained gel. Abbreviations as for Fig. 6.4.
approximately 100,000. The heavy chain line (H) is seen at just over 50,000 and the light chain (L) at about 25,000. The approximate molecular weights of unknown bands was determined from the position of the standard proteins (Fig. 6.4).

Following PAS staining at least 2 bands were seen to develop from the sample of juvenile fluke precipitate; at least 4 from the semi-mature fluke precipitate and at least 5 from the adult fluke precipitate. These have been identified with the number of the protein band they corresponded to when the gel was stained with Coomassie blue. Thus the 2 juvenile precipitate PAS positive lines corresponded with bands 1 and 11 in Fig. 6.4. The semi-mature precipitate PAS bands corresponded with bands 5, 6, 11 and 12 and the adult precipitate PAS bands also corresponded with bands 5, 6, 11 and 12. In addition, there is a very high molecular weight PAS-positive band in the adult precipitate (O) which has no corresponding protein band. The fraction enriched for IgG\textsubscript{1} shows several PAS positive bands including the contaminating protein (C), the heavy chain (H) and the light chain (L) of the immunoglobulin, although both H and L are faint bands.

6.4 Discussion

The antigens of *Fasciola hepatica* involved in precipitate formation *in vitro* are apparently secreted continuously from the tegument of juvenile flukes since the
precipitate forms initially on the tegument and is later found in the medium. The transfer of worms with precipitate attached to them from cultures containing infected sheep serum to cultures containing normal sheep serum results in dislodgement of the precipitate. The juvenile flukes thereby assume the appearance of those cultured continuously in normal serum. This observation supports the notion of a continuous turnover of material from the tegument. In addition, about 12 h is taken for precipitate to become completely dislodged from the tegument following transfer to normal serum; this is similar to the time taken for precipitate to first appear in the medium of cultures containing infected sheep serum.

The strongly positive PAS reaction given by the precipitate bound to juvenile flukes indicates the presence of carbohydrate. This is probably of parasite origin although IgG₁, the antibody mainly involved in precipitate formation (see Chapter 4), is known to have small amounts of carbohydrate (Aalund, 1972). However, the apparent secretory nature of the antigens involved also suggests that carbohydrate might be present and that the antigens may therefore be glycoprotein molecules. Alternatively, the carbohydrate detected in the precipitate may not be an integral part of any of the parasite's antigens; it may be released from the tegument and become loosely associated with the precipitate.
Further evidence for the existence of glycoprotein in the complex is suggested by the results of electron microscopy, which show that the precipitate is bound to the glycocalyx layer. Indeed, it appears almost as an extension of the glycocalyx (Fig. 6.3). Since the glycocalyx contains carbohydrate (Threadgold, 1976) and assuming turnover of the glycocalyx the precipitate carbohydrate presumably derives from the glycocalyx. Previous studies have also found that excretory-secretory products derived by incubating adult flukes in vitro contain glycoprotein antigens which form precipitin lines in gel diffusion tests against serum from infected rabbits (Sewell, 1964; Gundlach, 1971a), as shown for the antigens involved in precipitate formation in vitro (Chapter 5). Thus it appears likely that the antigens involved in formation of the juvenile fluke antigen/sheep antibody precipitate form part of the glycocalyx and that they may be glycoproteins. As discussed in Chapter 5, the glycocalyx may be important in protection of the fluke from the effects of the host immune response. Thus the glycocalyx may mask surface membrane antigens and the binding of host antibodies may enhance this masking (Clegg, 1974). In addition, the constant turnover of the glycocalyx may remove antibodies and other effectors from the surface before damage can be inflicted on young worms. In this connection the rate of turnover of the antigens seems slow in juvenile flukes (12 h) by comparison with the turnover of the glycocalyx in adult schistosomes (2 to 3 h) (Wilson and Barnes, 1977, 1979). However, when semi-mature and adult F. hepatica were cultured with serum from infected sheep (Chapter 3), the
appearance of precipitate in the medium occurred only a short time after it was first noticed on the fluke's surface. On active flukes precipitate formed initially on the tegument and appeared shortly thereafter in the medium, giving a turnover time of 2 to 3 hours or less. The apparent slower turnover in juvenile flukes might be an important factor in the ability of the protective immune response of rats to kill this stage (Rajasekariah and Howell, 1977b; Hayes, 1978) since the rat's immune effectors may be able to bind and damage the worms more rapidly than they can be removed.

A slower rate of antigen secretion may, paradoxically, facilitate survival in naive hosts by reducing the degree of antigenic stimulation of the immune system, thereby retarding the development of protective immune effectors. Thus the juvenile flukes may "sneak through" as has been suggested to occur during the growth of certain tumour cell lines (Klein, 1972).

Although the juvenile fluke antigens appeared by the electron microscopy to be associated with the glycocalyx, the failure of the procedure to label structures within the tegument precluded an assessment of the relationship between the secretory granules in the tegumental cells and the antigens which reach the surface of the tegument. In addition, the non-specific staining which occurred on juvenile flukes which had prior contact with normal sheep serum made a satisfactory interpretation of the micrographs
difficult. Non-specific staining may have been due either to the binding of 3-3' diaminobenzidine (the reagent used to localise horseradish peroxidase) to charged molecules in the glycocalyx (Seligman, Shannon, Hoshino and Plapingen, 1973; Threadgold, 1976), or possibly to the non-specific adsorption of immunoglobulins in normal serum onto the glycocalyx with the binding of peroxidase to this immunoglobulin. In future studies of this type, freshly excysted flukes which have not been exposed to normal sheep serum and controls localised with DAB in the absence of H$_2$O$_2$ (Seligman et al., 1973) should be processed.

The results obtained when samples of juvenile, semi-mature and adult fluke precipitates were run on SDS-PAGE gels seemed to conflict with immunodiffusion data presented in Chapter 5. The juvenile fluke precipitate gave more protein bands than the semi-mature precipitate and this also gave more bands than the adult precipitate. This is in contrast to the increasing antigenic complexity with development seen in Chapter 5. Bearing in mind that SDS and dithiothreitol cause some disruption of protein molecules, an additional explanation may be that the proteolytic activity known to occur in the caecal excretions of *F. hepatica* (Stephenson, 1947; Thorsell and Björkman, 1965; Howell, 1973) caused a breakdown of antigens and antibodies in *in vitro* cultures, especially in those containing semi-mature and adult flukes.
Of the protein bands that were detected on the gels, only the juvenile fluke precipitate shows a line which corresponds in position to an IgG heavy chain (2 and/or 3) whereas all precipitates show bands corresponding to the IgG light chain (9 and/or 10). This anomaly might again be explained by the release of proteolytic enzymes by the flukes. These enzymes are known to include hydrolases (Stephenson, 1947; Thorsell and Björkman, 1965; Locatelli and Simonie, 1974) which could cleave the antibody molecule into (Fab)\(_2\) and Fc fragments (Speigelberg, 1974). If this were the case, precipitation could still take place via the (Fab)\(_2\) fragment. However, when the precipitate was reduced by SDS and dithiothreitol prior to electrophoresis, only chains with molecular weights around 25,000 would be obtained, that is, 2 light chains and 2 Fd fragments. An alternative mechanism might operate through hydrolysis and removal of the Fc region after precipitation has taken place. Following cross linkage of antigen by the Fab ends of the antibody molecule and precipitation Fc ends may be left exposed to proteolytic activity and thereby be selectively removed. In cultures both these processes may take place to remove most of the Fc ends in the precipitate. The reasons for the finding of some intact heavy chains in the juvenile precipitate is presumably because this stage of development produces much less proteolytic enzyme than semi-mature and adult flukes.

It should be noted that not only immunoglobulins but also fluke antigens are probably susceptible to degradation.
by hydrolytic enzymes. Hence, the number of bands appearing in the gels may not necessarily reflect the number of antigen molecules released by the flukes but instead the cleavage products of the antigens (resulting from the action of hydrolytic enzymes secreted by the flukes) which persist in the precipitate; the 10 bands which developed from the juvenile fluke precipitate sample could be derived from 2 or 3 antigens. The high molecular weight bands (1 and/or 2) could represent intact antigenic molecules. In contrast the low molecular weight diffuse band (12) may be antigenic fragments which persist in the precipitate. In the semi-mature and adult fluke precipitates this band is more diffuse and apparently contains more protein. There are also bands corresponding to a molecular weight of about 35,000, suggesting that in these samples most of the larger molecules are cleaved. In addition to the above explanation, the dissociation of samples in dithiothreitol and SDS prior to electrophoresis may bring about fragmentation of antigen molecules, especially large branched molecules (Atassi, 1977; Tokács, 1979). These considerations make it difficult to compare the 3 precipitates for differences in their antigenic composition.

Interpretation of gels stained by the PAS technique is also difficult for the same reasons as discussed above. The number of PAS positive bands did, however, correlate with the number of antigens considered to be released by each developmental stage in Chapter 5. Thus, the juvenile precipitate samples showed 2 bands, the semi-mature precipitate
showed 4 and the adult precipitate 5 bands. Of the 2 juvenile precipitate bands, the high molecular weight one corresponded with the position of protein band 1 and thus, as stated above, this may represent an intact glycoprotein antigen. The low molecular weight band which corresponded in position to protein band 10 or 11 may be either the light chain or Fd chain fragments of immunoglobulin or both. This band increased in intensity of PAS staining in the semi-mature and adult precipitates, which may support this view. In addition the heavy and light chain bands in the IgG enriched fraction showed faint positive PAS staining, confirming the small amount of carbohydrate bound to these molecules. The PAS positive bands in the semi-mature and adult precipitate samples, which corresponded to protein bands 5, 6 and 12, provide some support for the suggestion of antigen breakdown by proteolytic enzymes. Finally, in the adult precipitate sample a very high molecular weight carbohydrate band was detected which has no counterpart on the protein stained gel. This large carbohydrate may be a molecule released by the adults for some specific formation allied to the bile duct environment.

Further analysis of the antigen-antibody precipitates may require them to be formed under conditions where proteolysis is limited, such as in double diffusion gels. Studies on precipitates eluted from such gels, by SDS-PAGE and by some other method such as isoelectric focussing, may facilitate determination of the number of antigen molecules
and their production by different developmental stages. It may also be worthwhile to attempt to dissociate the precipitate without disrupting the immunoglobulin and antigen components. This might be achieved by treating the precipitate with a dissociating agent such as urea. Such a procedure would probably have little effect on the integrity of the antigens and antibodies present. Unfortunately, the limited availability of material has not enabled these tests to be attempted to date.

Proteolytic enzymes secreted by *F. hepatica* may have a protective effect for flukes *in vivo*. Such enzymes may cleave antibodies and complement proteins and inhibit the activities of other immune effectors in the vicinity of the flukes. In support of this view, it has been shown that excretory-secretory products, including caecal contents of adult flukes, can inhibit cell binding to juvenile flukes (Goose, 1978). Moreover, the breakdown of large molecules with multiple antigenic sites such as may be released by the flukes, could facilitate suppression of antibody responses through the production of many small, soluble, immune complexes (Howard and Mitchison, 1975). These phenomena and others whereby the liver fluke may avoid the consequences of host immune attack, are discussed further in Chapter 9.
THE EFFECTS OF SERUM AND CELLS FROM INFECTED SHEEP ON JUVENILE FASCIOLA HEPATICA IN VITRO

7.1 Introduction

The importance of juvenile and semi-mature *F. hepatica* rather than adult flukes in eliciting immunity to challenge infection in some hosts has been shown by two types of study. Firstly, implantation of developmental stages into naive rats followed by an oral challenge has shown that metacercariae, 4 week old flukes and the eggs of *F. hepatica* can all stimulate a significant degree of protection (Rajasekariah and Howell, 1978b). In contrast, the results obtained with adult flukes have been rather equivocal and the immunity which has developed may be due to the release of eggs by implanted adults (Eriksen and Flagstad, 1974; Anderson, Hughes and Harness, 1975; Rajasekariah and Howell, 1978b). Secondly, there is direct evidence that juvenile flukes are a target of protective immunity in rats, particularly as they move through the gut wall (Rajasekariah and Howell, 1977b; Hayes and Mitrovic, 1977; Hayes, 1978). Moreover, *in vitro* studies in rats have shown that juvenile flukes cultured in immune rat serum have a higher mortality rate than those cultured in normal rat serum and that after a period in culture their ability to survive in a naive host is impaired (Howell *et al.*, 1977).

In sheep the juvenile and semi-mature stages stimulate the production of high levels of antibody (Chapter 2). In contrast, adult flukes appear to cause some
suppression of antibody production, since there is no anamnestic response following a challenge infection (Chapter 3). However, it is not clear whether juvenile flukes are themselves affected by the immune response of sheep. In vivo studies suggest that there is little or no protective immunity elicited against challenge infections (Boray, 1967; Sinclair, 1971a, 1971b, 1975; Rushton, 1977; Campbell, Gregg, Kelly and Dineen, 1978). Many factors may contribute to this state. The flukes may be able to evade immune attack, other factors in the host may antagonise or inhibit immune effector mechanisms which have the potential to damage the parasites, or the host may lack some factor which is essential for immune damage to be inflicted on the parasite.

The in vitro culture method outlined in Chapter 2 allows analysis of the effects of different immune factors in isolation from other intrinsic properties of the host. The value of this kind of approach is illustrated by studies on host immune responses to schistosomes. Several effective and novel anti-schistosome mechanisms, which are apparently also active in vivo, have been demonstrated (Mahmoud et al., 1975; Rousseaux-Prevost, Capron, Bazin and Capron, 1978; McLaren and Ramalho-Pinto, 1979).

In this chapter, attempts to determine whether various components of the immune response of sheep to F. hepatica have damaging effects on juvenile flukes in culture are described. This approach involved monitoring the
rates of juvenile fluke mortality in cultures containing serum from infected sheep and the viability of cultured flukes when transferred to previously uninfected hosts.

7.2 Materials and Methods

7.2.1 Mortality assays

Cultures monitored for mortality of juvenile flukes in the presence of full strength infected sheep serum included those set up to measure precipitating antibody levels in serum after primary infections of *F. hepatica* in sheep (Chapter 2). Methods for excystment of metacercariae and transfer to culture are described in Chapter 2. Mortality of juvenile flukes was assessed by a combination of a lack of movement, opacity, disruption of the tegument and/or disintegration of the worms. Using these criteria, it was found that dead worms could be easily distinguished from living, but perhaps moribund, worms.

7.2.2 Cultures containing spleen cells

Mortality assays were also carried out on juvenile flukes cultured in the presence of sheep serum and spleen cells. Spleen cells were obtained from a sheep infected 20 weeks previously with 200 metacercariae of *F. hepatica* and from a sheep autopsied 20 weeks after a challenge infection and 36 weeks after primary infection, with each infection consisting of 100 metacercariae. Normal spleen cells were obtained from a sheep of the same age as those that had undergone only the primary infection.
A small sample of the spleen was obtained immediately after the sheep was killed. Using aseptic procedures the sample was cut into small pieces and rubbed gently through a mesh spoon into sterile phosphate buffered saline (PBS) containing 2% foetal calf serum (Flow Laboratories). The cell suspension was centrifuged at 600 g for 7 min, resuspended in 6 ml of Dulbecco's modified Eagles medium (DMM) (Flow Laboratories) and allowed to stand on ice for 5 min. Clumps of cells settled out and were discarded. The suspended cells were removed, centrifuged as described and resuspended in 6 ml of DMM. Cell viability was determined using 0.01% trypan blue in PBS.

Metacercariae were excysted and washed as described in Chapter 2. Approximately 100 juvenile flukes were then placed in sterile Leighton tubes containing 5 ml of medium which consisted of 20% of the test serum plus 1 ml of the cell suspension in culture medium 199. Two trials were carried out. In the first, $10^4$ spleen cells were added to each culture to give $2 \times 10^3$ cells/ml of culture medium. In the second, $10^6$ spleen cells were added to give $2 \times 10^5$ cells/ml of culture medium. In the first trial serum taken 10 weeks after infection with 200 metacercariae and normal serum from sheep of the same age were tested for their effects on juvenile fluke mortality in the presence of spleen cells from normal and infected sheep. Four different cultures were set up as follows:
ducts and by mincing and incubating the liver in PBS (Rajasekariah and Howell, 1977a).

A viability experiment similar to those described above was also performed with 6 sheep that had been raised worm free and given food and water *ad libitum*. Three sheep were each injected intraperitoneally with 200 juvenile flukes which had been cultured in the presence of serum from a sheep infected with 100 metacercariae 10 weeks previously (Chapter 2). The other 3 sheep were injected with juvenile flukes cultured in normal sheep serum. In order to avoid injecting juvenile flukes into the rumen, the syringe was pulled back slightly before injection to check for rumen contents. A freshly excysted control group was not included in this experiment.

7.3 Results
7.3.1 Mortality of juvenile flukes in vitro

The number of juvenile flukes which died over the culture period varied markedly between cultures. Overall there appeared to be few differences between the mortality rates of flukes cultured in normal and infected sheep serum. However, comparisons between flukes cultured in serum with a high precipitating antibody titre (>50 μg/ml) and those in normal sheep serum showed a greater mortality in the latter (Fig. 7.1). In both types of culture the mortality rate was low for the first 64 h. After this time the rate increased and was greater in cultures containing normal sheep serum. After 160 h
Fig. 7.1 Mortality of juvenile fluke over time in culture with infected (antibody titre >50 μg/ml serum) or normal sheep serum.

- Infected sheep serum cultures (n = 13)
- Normal sheep serum cultures (n = 10)
in culture, 30.6 ± 17.8% of the flukes in these cultures were dead but only 10.6 ± 9.2% were dead in the infected sheep serum cultures.

In Fig. 7.2 the number of juvenile flukes dead after 160 h incubation in all infected sheep serum cultures is compared with the amount of antibody isolated from these cultures. There is an apparent negative correlation between the mortality after 160 h of culture and the level of antibody in the serum. The difference between juvenile fluke mortalities in cultures with antibody levels greater than 50 µg/ml serum and in cultures with antibody levels below this is significant (Students T-test, p<0.05).

7.3.2 Cultures containing spleen cells

When juvenile flukes were cultured with spleen cells and serum from infected sheep, a precipitate was observed to form on the teguments of the worms and cells were seen attached to this precipitate (Fig. 7.3). As precipitate formation continued large aggregates of cells and precipitate were observed attached to the worms and free in the medium. In these cultures the precipitate appeared more granular than in cultures of juvenile flukes and infected sheep serum (Fig. 7.4). Cells were rarely observed attached directly to the tegument of the flukes. Spleen cells from both normal and infected sheep attached to the precipitate which formed on juvenile flukes in infected sheep serum cultures; larger numbers appeared to attach when cells from infected rather
Fig. 7.2 The number of juvenile fluke dead after 160 h incubation in vitro compared with the amount of antibody isolated from these cultures. The sample size (n) for each 10 µg range is printed within each bar.
Fig. 7.3 Precipitate and cell aggregates attached to juvenile flukes cultured for 160 h in the presence of serum and spleen cells from infected sheep. 

a) Light microscopy, b) Scanning electron microscopy.
Fig. 7.4 Detail of precipitate on the surface of juvenile flukes cultured for 160 h in a) infected sheep serum, b) serum and cells from infected sheep. 

S = surface of the fluke
C = cells attached to the precipitate.
than normal sheep were present. In cultures containing normal sheep serum, spleen cells from infected and normal animals did not attach to active flukes (Fig. 7.5).

Spleen cells adhered to dead flukes and metacercarial cysts in cultures containing both normal and infected sheep serum (Fig. 7.6). The numbers of spleen cells which attached to dead worms in cultures containing infected sheep serum was such that it was often difficult to distinguish dead flukes from precipitate-cell aggregates free in the medium, especially when the higher spleen cell inoculum was used. In addition, dead worms apparently disintegrated more rapidly in cell cultures than in cultures of serum alone. It was found that mortality data could be reliably collected only from the first trial involving cells, since fewer cells were present in each culture and dead flukes could be distinguished. In all cultures containing spleen cells, juvenile flukes ingested red blood cells which were present in the spleen cell suspensions, since the caeca became prominent because of the presence of a dark brown pigment, presumably haematin (Fig. 7.7).

The mortality rates of the worms in the cultures containing $2 \times 10^3$ spleen cells/ml are shown in Fig. 7.8. Flukes cultured with serum and spleen cells from infected sheep showed a slightly higher mortality rate than those in other cultures. No other treatment appeared to have an appreciable effect on the survival of juvenile flukes.
Fig. 7.5 Juvenile flukes cultured with serum and cells from normal sheep.

a) Light microscopy, b) Scanning electron microscopy.
Fig. 7.6 Spleen cells from infected sheep attached to metacercarial cysts in vitro.

Fig. 7.7. A juvenile fluke cultured for 160 h with spleen cells showing dark brown pigment in the caeca.
Fig. 7.8 Mortality of juvenile flukes over time in cultures containing sheep spleen cells and serum.

- Normal serum and cells
- Infected serum and normal cells
- Normal serum and sensitised cells
- Infected serum and sensitised cells
7.3.3 Viability experiments

The recoveries of adult flukes from rats injected intraperitoneally with juvenile flukes that had been cultured in sheep serum or with freshly excysted metacercariae are shown in Table 7.1. In the first experiment, significantly fewer flukes (Mann-Whitney U test; p<0.05) were recovered from rats which had been injected with juvenile flukes cultured in primary infection serum with a relatively high level of precipitating antibody than from rats which received flukes cultured in normal sheep serum. Also, fewer flukes were recovered from the latter group than from the group which received freshly excysted metacercariae.

In the second experiment, 4 rats which were injected with juvenile flukes cultured in normal sheep serum died and they were not autopsied. Recoveries from the remaining rats in this group are included since they do not apparently differ from the other groups. No significant differences were found among the worm recoveries from the other groups in this experiment.

The third experiment was designed to repeat and extend the observations made in the first two experiments. Results are shown in Table 7.2. There were no significant differences between the worm recoveries of group 1, which received flukes cultured in serum collected 8 weeks after a primary infection, and group 2 which received flukes cultured in serum taken 2 weeks after a challenge infection. However,
Table 7.1 Fluke recoveries from rats infected with cultured or freshly excysted metacercariae.

Experiment 1. Mean recovery ± S.E. 9 rats/group.

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluke cultured in serum collected 10 weeks after primary infection</td>
<td>Fluke cultured in normal sheep serum</td>
<td>Freshly excysted metacercariae</td>
</tr>
<tr>
<td>0.22 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.33 ± 0.47&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.0 ± 0.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

a<b<c; p<<0.05 Mann-Whitney U Test.

Experiment 2. 6 rats/group

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluke cultured in serum collected 12 weeks after challenge infection</td>
<td>Fluke cultured in normal sheep serum</td>
<td>Freshly excysted metacercariae</td>
</tr>
<tr>
<td>5.67 ± 1.0</td>
<td>4.0*</td>
<td>4.5 ± 0.34</td>
</tr>
</tbody>
</table>

No significant differences p>0.05 Mann-Whitney U Test.

*Mean of only 2 rats (see text).
Table 7.2  Fluke recoveries from rats infected with juvenile flukes cultured in sheep serum or with freshly excysted metacercariae.

Experiment 3.  Mean ± S.E.  6 rats/group.

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Group 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluke cultured in serum taken</td>
<td>Fluke cultured in serum taken</td>
<td>Fluke cultured in serum taken</td>
<td>Fluke cultured in normal sheep</td>
<td>Freshly excysted metacercariae</td>
</tr>
<tr>
<td>8 weeks after primary infection</td>
<td>2 weeks after challenge infection</td>
<td>8 weeks after challenge infection</td>
<td>serum</td>
<td></td>
</tr>
<tr>
<td>5.5 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.3 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.3 ± 0.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.33 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.4 ± 0.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Groups with the same superscript not significantly different.

a<b<c;  p<0.05 Mann-Whitney U Test.
Table 7.3 Fluke recoveries from sheep infected with juvenile flukes cultured in sheep serum.

<table>
<thead>
<tr>
<th>Sheep number</th>
<th>Group 1: Fluke cultured in sheep serum taken 10 weeks after primary infection</th>
<th>Group 2: Fluke cultured in normal sheep serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>143</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>91</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>126</td>
<td>103</td>
</tr>
</tbody>
</table>
seems contrary to what might be expected. However, in such cultures each fluke became encapsulated by the precipitate (antigen-antibody complex) which formed on its tegument (Fig. 2.2); this layer of precipitate may present a barrier to other factors in serum which have a damaging effect on the flukes. The failure of these precipitating antibodies to significantly damage worms may reflect their inability to reach critical sites on the tegument, possibly because of the constant secretion of antigen from the tegument (Chapter 6; Clegg, 1974). Alternatively, these precipitating antibodies may not be protective and in fact may act to enhance fluke survival by blocking attachment to the tegument of other protective antibodies or cells, as postulated in other parasitic infections (Jerusalem, Weiss and Poels, 1971; Rickard, 1974) and in immune responses to tumours (Klein, 1972). Another possibility for the enhanced survival of juvenile worms when cultured in serum with a high antibody titre might be that the antigen-antibody precipitate is ingested by the worms thus providing an additional source of nutrients.

The enhanced survival of flukes cultured in infected sheep serum is in contrast to results obtained with rat serum (Howell et al., 1977). A higher mortality rate occurred in immune rat serum than in normal rat serum in the first 36 h of culture. This may indicate that rat antibodies have the ability to cause damage to flukes but sheep antibodies do not. Support for this view comes from studies which show that rats develop immunity to reinfection (Hayes et al., 1972;

The relevance of enhanced fluke survival in cultures containing relatively high precipitating antibody levels to the \textit{in vivo} situation in sheep is unknown. However, since sheep do not develop resistance to reinfection, the events in challenged animals and in culture may be comparable; the \textit{in vitro} phenomenon may thus be worthy of further investigation.

The addition of spleen cells to cultures containing sheep serum had no marked effect on the survival of worms. In fact, the addition of cells (including red blood cells) in the spleen cell mixture may have enhanced survival by providing an additional nutrient source. There was indication that the survival of worms was more markedly impaired in the presence of both spleen cells and serum from infected sheep than by the other treatments. However, these experiments could only be regarded as preliminary and further work is necessary for the effects of cells on juvenile flukes to be fully explored.

The patterns of attachment of spleen cells to flukes in the cultures containing sheep serum indicated that immunocompetent cells are unable to bind directly to the tegument of active parasites. In cultures containing infected sheep serum with either normal or infected spleen cells, the antigen-antibody precipitate was the major site of cell
attachment. Cells were rarely seen attached directly to the tegument of the fluke and in these cases the presence of precipitated complex between the attached cell and the fluke could not be ruled out. This observation, together with the lack of cell attachment to active parasites in cultures containing normal sheep serum, suggests that the constant secretion of antigens from the tegument and into the culture medium precludes the adherence of cells to the surface membranes of the parasites. When secretion of antigen stops cells readily become attached to the flukes. This may explain the rapid disintegration of dead worms in these cultures by comparison with cultures containing sheep serum alone.

A previous in vitro study of the binding of rat peritoneal cells to juvenile F. hepatica is in contrast to the findings outlined above. Goose (1978) found that cells adhered to excysted metacercariae in the presence of immune rat serum. However, his culture methods were markedly different from those used in this study: 2,000 juvenile flukes were cultured with 0.1 ml of immune rat serum and 6 x 10^6 peritoneal cells in a total volume of 0.3 ml for 2 h. Precipitate formation was not reported under these conditions, but it is possible that the cells became bound to such a complex on the fluke's tegument. If the culture period had been extended beyond 2 h the adherent cells may have been forced from the tegument by continued secretion of antigen. However, this study indicates that at high cell densities adhesion to the flukes' surface is able to take place, even if only for a limited period, and that this might be important
in the protective immune response of a host such as the rat.

The viability experiments carried out in rats revealed that culture in infected sheep serum could adversely affect the ability of juvenile flukes to continue development when transferred to normal hosts. The magnitude of this effect was dependent on the time after the sheep were infected that the test serum was collected. In the first experiment serum collected 10 weeks after a primary infection, which had a high precipitating antibody titre (75 μg/ml), resulted in an almost complete loss of fluke viability. However, there was no effect in the second experiment with serum taken 12 weeks after challenge infection (24 μg/ml). In the third experiment, serum collected 8 weeks after a primary infection (118 μg/ml) and 2 weeks after challenge infection (47 μg/ml) were effective, whereas serum taken 8 weeks after a challenge infection (15 μg/ml) was not. Thus, there seems to be a threshold level of precipitating antibody in serum before it can impair fluke viability. This seems in conflict with the mortality experiments where it was found that higher mortality in vitro was associated with low antibody levels. This suggests that the effect on fluke viability is not necessarily dependent on antibody damage to the flukes in vitro but that rats are able to provide some component which acts in concert with sheep antibody to damage and kill flukes in vivo. Rats apparently have some humoral factor other than antibody, which can participate in the killing of juvenile flukes when they
are cultured with immune rat serum (Howell et al., 1977). This factor may or may not be the same as that which acts in vivo with sheep antibody to impair viability.

Non-specific effects on fluke viability were also observed. Thus, fewer adult flukes were generally recovered from hosts that received juvenile flukes cultured in normal sheep serum than freshly excysted metacercariae. A similar phenomenon was observed with rat serum (Howell et al., 1977).

Although an association between precipitating antibody titres in sheep serum and juvenile fluke viability has been demonstrated, there is no proof that the damaging effects are caused by the antibodies involved in precipitate formation. In rats, however, there is some evidence that an analogous antibody is involved in the destruction of juvenile worms (Howell and Sandeman, 1979; Howell, in press). That serum from infected sheep contains protective antibodies is strongly suggested by its ability to impair fluke viability in vitro, and by the observation that passive transfer of infected sheep serum into naïve rats can protect them against fluke infection (Armour and Dargie, 1974). Thus the apparent differences between sheep and rats in relation to their ability to resist challenge infections must presumably lie in some component of their resistance mechanisms other than in their respective abilities to produce protective antibodies.
The experiments in this Chapter would appear to provide some support for the apparent suppression of antibody responses by adult worms discussed in Chapter 3. Those sera which did not effect juvenile fluke viability were taken after adult worms were established in the bile ducts, when suppression is suggested to occur. Only 1 batch of serum from sheep with a patent infection effected viability and this had the highest post-challenge antibody activity measured.

The viability experiment performed in sheep was marred by the injection of flukes into the rumen rather than the peritoneal cavity in two animals. The worm recovery obtained from the remaining animal in the group which received flukes cultured in normal sheep serum, was within the range of values obtained from the group which received flukes cultured in infected sheep serum. This indicates that in contrast to rats, sheep are not able to impair development of pre-cultured juvenile flukes. However, as discussed earlier, sheep apparently produce protective antibodies and so, compared with rats and possibly other hosts, sheep are apparently deficient in some factor, other than antibody, which is essential for juvenile flukes to be killed in vivo. The identity of this factor might be discovered by a comparison of the immune responses of rats and sheep to antigens other than those of *F. hepatica*, since so little is known of the immune response to *F. hepatica* antigens. This idea will be discussed further in Chapters 8 and 9.
ATTEMPTS TO VACCINATE SHEEP AND RATS AGAINST FASCIOLA HEPATICA USING THE JUVENILE FLUKE ANTIGEN/SHEEP ANTIBODY PRECIPITATE

8.1 Introduction

Attempts to vaccinate sheep against *F. hepatica* have consistently failed to bring about a reduction in the numbers of challenge flukes reaching the bile ducts (Geyer, 1967; Clegg and Smith, 1978). However, most of these attempts have used adult fluke antigens which, as pointed out in Chapter 5, may not stimulate protective immune responses. Recently, rats and mice have been vaccinated against infection by using antigens derived from juvenile and semi-mature worms. Lang (1976) and Lang and Hall (1977) have shown that excretory-secretory antigens of 16 day old flukes can protect mice. Howell and Sandeman (1979) and Howell (in press) obtained partial protection of rats after injections of a precipitated complex of juvenile worm antigen and rat antibody which has been the subject of study in other Chapters of this thesis. These findings provide support not only for the view that the migratory stages are particularly important for the stimulation of protective immune responses but also that metabolic antigens released by living flukes elicit such responses.

The studies referred to above prompted the vaccination experiments described in this chapter. The effects of vaccinating sheep and rats with fluke antigen/sheep antibody precipitate, on an oral infection with metacercariae,
was examined. The degree of protection was assessed by adult fluke recovery from the bile ducts of vaccinated hosts at autopsy. In sheep the level of precipitating antibodies against *F. hepatica* and the activities of glutamate dehydrogenase (GLDH) and γ-glutamyl transferase (γ-GT) in weekly serum samples were assayed. Ouchterlony tests were performed on serum from vaccinated rats collected one week prior to infection, in order to analyse their response to the injection of the fluke antigen/sheep antibody precipitate.

8.2 *Materials and Methods*

8.2.1 *Animals and experimental procedures*

8.2.1.1 *Rats*

Seventeen, 5 week old male Wistar rats were divided into 3 groups. Each of the 6 animals in group 1 received fluke antigen/sheep antibody precipitated complex (see below), suspended in 0.1 ml of PBS and emulsified with 0.1 ml Freund's complete adjuvant (FCA, Difco), into each hind footpad. Group 2 animals each received 0.2 ml of an emulsified PBS/FCA mixture into each hind footpad. The 5 animals in group 3 were not injected. Three weeks after the first injection, groups 1 and 2 received booster injections of the same amount of precipitate/FCA or PBS/FCA. One week after the booster injection all rats were bled via the tail vein for the collection of rat anti-precipitate serum \((APS_R)\) from group 1, rat anti-PBS/FCA control serum \((AFSR)\) from group 2 and normal rat serum from group 3. Two weeks after the booster injections each rat in all groups was infected with 20
metacercariae by oral intubation. Rats were autopsied 7 weeks after infection; flukes were removed from the bile duct as well as from the liver after it had been minced and incubated at 37°C in PBS for 2 h (Rajasekariah and Howell, 1977a).

8.2.1.2 Sheep

Fifteen Merino ewe lambs, raised worm-free on concrete at the Veterinary Research Station, Glenfield, NSW, were divided into 3 groups of 5 animals. The lambs were 5 months old with a mean body weight of 21 kg at the start of the experiment. Each lamb in group 1, the vaccinated group, received an intramuscular injection of precipitated complex (see below), suspended in 1 ml of PBS and emulsified with 1 ml of FCA, into the posterior aspect of the thigh. Each lamb in group 2 received 1 ml of PBS emulsified with 1 ml of FCA intramuscularly. Group 3 animals were not injected.

Groups 1 and 2 received 2 injections, 4 weeks apart. One week after the second injection each animal in these groups and in group 3 was infected with 200 metacercariae of F. hepatica intraruminally. Metacercariae of F. hepatica were obtained by artificial infection of field collected Lymnaea tomentosa maintained in shallow aquaria (Whitlock, Campbell, Chow, Rolfe, Portier and Kelly, 1977).

Blood was collected weekly from 8 weeks before infection until 16 weeks after infection. Serum was prepared
as described in Chapter 2 and stored at -20°C until required. Faecal egg counts were monitored from 12 weeks after infection using the method described in Chapter 2.

All sheep were killed 16 weeks after infection. The livers were removed and the flukes were recovered and counted. The sites of injection in groups 1 and 2 were inspected and samples of tissue were fixed in Bouin's fluid and embedded in paraffin for histological examination. Sections were stained in either Masson's trichrome, haematoxylin and eosin or Van Gieson's stain (McManus and Mowry, 1964).

8.2.2 Precipitated complex

Precipitated complex (sheep antibodies-parasite antigens) was obtained from cultures of juvenile flukes in infected sheep serum as described in Chapter 2. The complex was stored in PBS at -20°C until required; its protein content was determined by the method of Lowry et al. (1951).

Precipitate for the rat vaccination experiment was pooled, suspended in 1.6 ml of PBS and emulsified with 1.6 ml of FCA prior to injection. Each rat in group 1 received approximately 100 μg of precipitated protein at each injection.

In the sheep vaccination experiment each animal in group 1 received the equivalent of 1.4 mg of precipitated protein at each injection.
8.2.3 Antibody and enzyme assays of sheep sera

Precipitating antibody levels were assayed by the *in vitro* culture method described in Chapter 2. However, in order to reduce the number of excysted metacercariae required, only 50 were placed in each culture with 2.5 ml of sterile, pooled serum. Serum was tested at 3 week intervals and additionally at weeks considered important in terms of the vaccination and infection protocols. Because of variations in the final numbers of parasites in culture (± 10) antibody protein levels were corrected to μg/ml serum/50 juvenile flukes.

Serum samples from the animals in each group were pooled and assayed for the presence of the liver mitochondrial enzyme GLDH and the bile duct epithelial enzyme γ-GT. These enzymes were used as indicators of the levels of tissue damage in the liver and bile ducts respectively (see Chapter 2).

8.2.4 Ouchterlony tests

Ouchterlony tests were carried out on serum collected from vaccinated rats 1 week prior to challenge, that is, 1 week after the booster injection of precipitate. The methods were as outlined in Chapter 4. Semi-mature fluke excretory-secretory antigen (SMWS\textsubscript{R}) was prepared as described in Chapter 5. In order to avoid any cross reactions between the serum from rats injected with precipitated complex containing sheep antibodies (APS\textsubscript{R}) and sheep serum, SMWS\textsubscript{R} was
prepared in 5 ml of 20% normal rat serum in 199 rather than in normal sheep serum. The SMWSR was tested against serum from vaccinated rats (as above), serum taken from sheep 8 weeks after infection with 100 metacercariae, immune Wistar rat serum (IRS) prepared 1 week after a challenge infection with 10 metacercariae (9 weeks after a primary infection with 10 metacercariae), rat anti-FCA serum (AFSR) and normal rat serum (NRS).

8.3 Results
8.3.1 Rats

In the first vaccination experiment, no significant differences were found between the numbers of flukes recovered from the 3 groups of rats (Table 8.1, p>0.05). One rat in group 3 died after infection but it was not possible to autopsy this animal. However, the worm recoveries from the remaining animals showed no departure from those of the other groups.

When APSR collected from group 1 rats (vaccinated) was run against SMWSR a complex series of lines formed (Fig. 8.1). However, only the faint line approximately half way between the wells probably represents a reaction between APSR and SMWSR. The 2 lines near the SMWS well, which cross to meet the empty well, are probably reactions between APSR and the infected sheep serum well. It is thought that these lines are unlikely to be reactions between APSR and SMWSR because they do not
Fig. 8.1 Ouchterlony test. Semi-mature fluke excretory-secretory antigen prepared in rat serum run against rat anti-juvenile fluke antigen/sheep antibody precipitate serum ($APSR$), infected sheep serum (InSS), immune rat serum (IRS), rat anti-FCA control serum ($AFSR$) and normal rat serum (NRS).
Table 8.1  Rat vaccination experiment.
Adult fluke recoveries (Mean ± S.E.)

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precipitate/FCA injected</td>
<td>PBS/FCA injected</td>
<td>Control</td>
</tr>
<tr>
<td>4.33 ± 0.9</td>
<td>4.0 ± 0.6</td>
<td>5.5 ± 0.95</td>
</tr>
</tbody>
</table>

No significant differences. Mann-Whitney U Test
extend to the AFS well. They are more likely to be reactions between APSR and infected sheep serum because the line occurs about equidistant from these wells and curves around the SMWSR well, probably as a result of sheep serum passing around or through this well. Moreover, other Ouchterlony plates in which infected sheep serum was diffused directly against APSR showed two very prominent lines and other less intense lines, suggesting that rat anti-sheep antibodies are at very high concentrations in the APSR.

Apart from these reactions, 2 lines formed between SMWSR and infected sheep serum and they formed lines of identity with 2 lines between SMWSR and IRS.

8.3.2 Sheep

The numbers of flukes recovered from the 3 groups of sheep are shown in Table 8.2. There were no significant differences between the groups (Mann-Whitney U test, p>0.05). Faecal egg counts were lower in group 1 (vaccinated) than in group 3 at 12 weeks and group 2 at 15 weeks (Table 8.3). There were no other differences between the groups.

The levels of the liver enzyme GLDH in the weekly collections of serum are shown in Fig. 8.2. The activity in the 3 groups over the course of the infection was similar to that seen after primary infections (Chapters 2 and 3). Thus there was a sharp increase in activity as the flukes entered and migrated through the liver, followed by a decrease after
Fig. 8.2 Glutamate dehydrogenase activity (units/litre) measured in weekly collections of sheep serum.

- Group 1 (complex injected)
- Group 2 (injected control PBS/FCA)
- Group 3 (control)
Table 8.2 Sheep vaccination experiment.

Fluke recovery at autopsy (Mean ± S.E.)

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precipitate/FCA injected</td>
<td>PBS/FCA injected</td>
<td>Control</td>
</tr>
<tr>
<td>119.6 ± 7.1</td>
<td>120.2 ± 12.8</td>
<td>115.4 ± 9.4</td>
</tr>
</tbody>
</table>

No significant differences,  
p > 0.05, Mann-Whitney U test.
Table 8.3  Faecal egg counts (Mean Eggs per gram and S.E.)

<table>
<thead>
<tr>
<th>Weeks after infection</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>84 ± 30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>512 ± 182</td>
<td>284 ± 62</td>
<td>412 ± 146&lt;sup&gt;a&lt;/sup&gt;</td>
<td>658 ± 296</td>
</tr>
<tr>
<td>Group 2</td>
<td>462 ± 229</td>
<td>632 ± 128</td>
<td>632 ± 157</td>
<td>1080 ± 333&lt;sup&gt;b&lt;/sup&gt;</td>
<td>717 ± 153</td>
</tr>
<tr>
<td>Group 3</td>
<td>530 ± 150&lt;sup&gt;b&lt;/sup&gt;</td>
<td>832 ± 268</td>
<td>472 ± 108</td>
<td>480 ± 100</td>
<td>600 ± 76</td>
</tr>
</tbody>
</table>

<sup>a</sup><b>, p<0.05, Mann-Whitney U test</b>
they had entered the bile ducts. There were, however, some differences between the vaccinated and control groups. Firstly, sheep in group 1, unlike controls, showed a rise in serum GLDH activity 2 weeks after the first injection with precipitated complex (3 weeks prior to infection). One week after infection, GLDH levels in these animals had returned to their control values. Secondly, a marked elevation of GLDH levels after infection became apparent a week earlier in group 1 than in the other groups.

The levels of the bile duct epithelial enzyme, γ-GT, (Fig. 8.3) in the 3 groups of sheep showed a transitory peak of activity between 9 and 12 weeks, probably due to the movement of flukes from the liver parenchyma into the bile ducts (see Chapter 2). This rise began at 8 weeks in group 3, and peak activity was reached at 9 weeks. In group 2, the rise began at 8 weeks, but peak activity was not reached until 10 weeks. In group 1, the rise began at 9 weeks and activity reached a peak at 11 and 12 weeks. There was no marked elevation of the level of γ-GT in the serum of group 1 sheep soon after vaccination as observed with GLDH. However, there was some erratic activity in all groups prior to infection.

The untreated control group, group 3 (Fig. 8.4), showed an antibody response very similar to that previously observed in sheep following a primary infection (Chapter 2 and 3). This was characterised by a rise in antibody levels to a peak 6 weeks after infection, then a subsequent fall to a
Fig. 8.3 γ-glutamyl transferase activity (units/litre) measured in weekly collections of sheep serum.

- ■■■■■ Group 1 (complex injected)
- Group 2 (injected control PBS/FCA)
- Group 3 (control)
Fig. 8.4 Antibody activity (µg protein/ml serum) measured in collections of serum by isolation of precipitate from *in vitro* cultures.

- Group 1 (complex injected)
- Group 2 (injected control PBS/FCA)
- Group 3 (control)
lower level after flukes became established in the bile ducts. The antibody level in group 2 (injected with adjuvant) rose rapidly to a peak 3 weeks after infection, then fell over the ensuing 3 weeks; a further rise and fall occurred over the following 9 weeks with the second peak 12 weeks after infection. The group 1 antibody level showed a marked rise 3 weeks after the first injection of complex in FCA, which was sustained or temporarily elevated at all times after the second injection and the challenge infection until autopsy. The challenge infection had no marked effect on the antibody level in this group, although there was a distinct elevation 12 weeks after infection. Antibody levels reached their highest values, about 53 μg protein/ml serum, in group 1 12 weeks after infection. There was a small amount of insoluble protein isolated from cultures with preinfection and preinjection sera in the various groups (Fig. 8.4). This never accounted for more than about 18 μg protein/ml serum.

At autopsy, lesions which had developed at the injection sites in groups 1 and 2 were similar in size and gross appearance. The histopathological appearance of the lesions in both groups was also similar. There was massive infiltration by mononuclear cells, although some multinucleate cells were seen in more or less direct contact with remnants of the injected material. Granulomata formation around islands of injected material was evident; each was set off by well defined concentric bands of collagen. Often, the outer collagen bands of adjacent granulomata coalesced.
Many empty vacuoles were enclosed by the granulomata. These probably represented spaces occupied by oil components of the adjuvant which were leached out during histological processing. There were no marked differences between the groups other than a more extensive deposition of collagen in the vaccinated group.

8.4 Discussion

The vaccination of rats and sheep with precipitated complex isolated from cultures of juvenile flukes and infected sheep serum had no effect on the number of adult flukes recovered following a challenge infection. Although this result was not altogether unexpected with sheep, since there is very little evidence that sheep can develop effective immunity against *F. hepatica* (Sinclair, 1975; Rushton, 1977), the result in rats was somewhat surprising. Previous studies had shown that a similar precipitating complex isolated from cultures of juvenile flukes and immune rat serum could protect rats against infection (Howell and Sandeman, 1979; Howell, in press).

The failure of the sheep antibody/fluke antigen precipitate to stimulate protective immunity suggests that this precipitate contains different fluke antigens to those present in the rat antibody/fluke antigen precipitate. However, preliminary evidence supporting the contention that the same fluke antigens are common to both precipitates was obtained when immune rat serum and infected sheep serum were run against
SMWS$_R$ (Fig. 8.1). Two precipitin lines formed between SMWS$_R$ and IRS and these formed lines of identity with those which formed between infected sheep serum and SMWS$_R$. These results suggest that the fluke antigens involved in the formation of precipitate with infected sheep serum are also those involved in precipitate formation with immune rat serum. However, further tests are required with immune rat serum absorbed with juvenile flukes to confirm that the reactions which occur between IRS and SMWS$_R$ are also those involved in rat precipitate formation in vitro.

If it is assumed that the same fluke antigens are common to both sheep and rat precipitates then the injection of heterologous sheep antibodies, which are present in the sheep precipitate, may have directly or indirectly interfered with the rat anti-fluke antigen response so as to abrogate the development or action of a protective response. The single line between APS$_R$ and SMWS$_R$ (Fig. 8.1) is fairly weak when compared to that which forms between infected sheep serum and SMWS prepared in sheep serum (Fig. 5.3). This may support the idea of interference by the heterologous antibody with the rat's response to fluke antigens in the precipitate. However, Ouchterlony tests are obviously required in the absence of infected sheep serum to avoid confusing reactions. It is unfortunate that an anti-rat precipitate serum raised in rats was not available to confirm the presence or absence of protective antibodies in the rats given sheep precipitate.
Although vaccination failed to reduce the numbers of flukes which established in sheep several other effects were observed. Injection of precipitated complex influenced the levels of enzymes and antibody in serum collected during the experiment. The most noticeable effect was the release of GLDH into the serum shortly following vaccination. Since this was not observed in group 2 it is suggested that the complex was responsible for tissue damage, most probably at the site of injection. GLDH is known to occur in muscle as well as in liver (Wilkinson, 1970) and would be released if cell damage took place. A preliminary attempt to determine whether this GLDH was of muscle or liver origin by polyacrylamide disc gel electrophoresis, using the staining method of Shaw and Prasad (1970), was unsuccessful. False positive results, that is, positive results in the absence of substrate were obtained, possibly due to the presence of "nothing" dehydrogenase (Shaw and Koen, 1965) which prevented a distinction between GLDH from muscle and liver being made.

The finding that complexed antigen causes tissue damage raises questions as to its role during a natural infection. It seems possible that antigen-antibody complexes could aggravate the mechanical damage caused by the fluke's migration and feeding. Such damage is known to occur in other parasitic infections, notably schistosomiasis (WHO Technical Report, 1977). In fascioliasis, this type of reaction might explain some of the less understood pathological changes such as portal canal fibrosis seen in hepatic and
portal veins, even in sites remote from fluke tracts (Murray and Rushton, 1975).

Histological examination of the injection sites in the sheep of groups 1 and 2 showed few differences apart from more extensive collagen deposition in group 1. This suggests that greater amounts of damage occurred when complex was injected. However, since autopsy took place 17 weeks after the second injection little can be concluded about the relative intensity of the tissue reactions in the 2 groups.

The activity of GLDH in group 1 after infection appeared to rise a week earlier, and to reach higher levels initially than in the other groups. This could result either from an earlier entry of flukes into the liver or from rapid complex formation between fluke antigens and the antibodies resulting from vaccination leading to greater liver damage.

γ-GT activities showed some differences between groups. Most noticeably, the onset of release of γ-GT and the peak levels occurred later in group 1 than group 2 and later in group 2 than group 3. The delay was about a week in group 2 compared with the control and about 2 weeks in the vaccinated group. Delays in fluke migration have previously been observed during secondary infections (Sinclair, 1971a; Rushton, 1977) and ascribed variously to liver damage and
fibrosis or the immune response. The results presented here seem to support the view that the immune response is the cause of these delays, since the flukes would not encounter fibrosed tissue in the absence of a prior infection. The delay in reaching the bile ducts by flukes in group 1 was mirrored by faecal egg counts which did not approach those of the other groups until 13 weeks after infection. The erratic activity of γ-GT in serum from all groups prior to infection cannot be adequately accounted for.

The levels of precipitating antibody measured in serum showed marked variation between groups. The group 3 (untreated) response was as expected after a primary infection (Chapter 2 and 3). Group 2, which received FCA only, showed a similar response to group 1 but the initial peak in antibody level was reached 3 weeks earlier. Furthermore, antibody levels were slightly higher in group 2 than group 3. Clearly, the adjuvant enhanced the response of the sheep immune system to fluke antigens.

In group 1, injections of the precipitate complex in FCA stimulated the production of antibody prior to infection. After the first injection there was a delay of about 2 weeks before a rapid rise in antibody level. This may have been due to the slow release of antigen from the intramuscular site. There was little apparent effect of either the second injection or infection on antibody levels apart from a peak at 12 weeks. The rise in antibody level at this time, which
was also seen in group 2, may have been due to the persistence of migrating flukes in the liver.

Despite the high level of antibody in group 1 throughout the entire period of infection, there was no apparent effect on the ability of flukes to survive to patency. This lack of a protective effect suggests that the antigens present in the complex used for vaccination are not functional in sheep, even though the antigens in a similar antigen-antibody complex confer partial protection in rats (Howell and Sandeman, 1979; Howell, in press) and Ouchterlony tests suggest that the same antigens are involved in both of these precipitates (Fig. 8.1). Alternatively, sheep, unlike rats, may be deficient in some component of the afferent arc of their response which, by acting in concert with antibody, is essential for flukes to be killed in this host. If this is the case then comparisons of the functional aspects of the rat's immune response to *F. hepatica* with the sheep's immune response may enable the deficiency in sheep to be identified. However, the lack of knowledge surrounding the effective anti-*F. hepatica* mechanisms in rats and of immune responses in sheep precludes any conclusions at this stage. Further studies should therefore be undertaken on the differences between rat and sheep immune responses, especially with regard to helminth infections.
GENERAL DISCUSSION

9.1 The precipitate reaction

A precipitate forms on the teguments of *Fasciola hepatica* (juvenile, semi-mature or adult) cultured *in vitro* in the presence of serum from infected rabbits, cattle, mice and rats (Wikerhauser, 1961; Ruther, 1963 cited in Geyer, 1967; Lang, 1974b; Howell *et al.*., 1977). The results of this study indicate that the same phenomenon also occurs in the presence of serum from infected sheep. Precipitates which form in the presence of sera from infected rats and sheep have been shown to contain host antibodies and fluke antigens. Thus, it is probable that these reactions which take place *in vitro* also occur *in vivo*. However, Ruther (1963, cited in Geyer, 1967) could find no evidence for precipitate formation *in vivo*. A possible explanation for this is that the continual secretion of the antigens involved and the movement of migratory flukes would prevent a build up of precipitate around the worms. Precipitated complex deposited in fluke tracts would probably attract host cells (Chapter 7) and be partly responsible for the massive cellular infiltration seen following fluke migration (Rushton and Murray, 1977). Tissue damage, which may be due to anaphylactic-type reactions induced by immune complexes (Allison and Houba, 1976), might also follow from the presence of precipitated complex in the liver. The finding that this complex can cause tissue damage when it is injected intramuscularly (Chapter 8) and that host cells are attached to it (Chapter 7) supports these suggestions.
The apparent similarities in appearance and formation between the precipitates which form on and around flukes in different host sera suggests that the same antigens are involved. If this is the case then this reaction, which appears to be a fundamental property of all relationships between *F. hepatica* and its hosts so far examined, may have potential for the diagnosis of fascioliasis. The specificity of the reaction for *F. hepatica* alone clearly warrants further investigation.

9.2 *Antibody responses to fluke infection*

The levels of precipitating antibodies were measured by the *in vitro* culture method in the sera of sheep infected with *F. hepatica*. Over the prepatent period these levels showed a pattern similar to that observed in previous studies of sheep and other hosts, which used a variety of immunological tests (Capron *et al.*, 1965; Gundlach, 1971b; Deelder, 1973; Movsesijan *et al.*, 1975; Van Tiggele and Over, 1976; Rajasekariah and Howell, 1978a). The later liver migratory period is consistently the stage of infection which stimulates the highest levels of antibody production by the host. This also coincides with the period of maximum liver damage as measured by enzyme assays. Following patency, that is, after flukes had become established in the bile ducts, a less consistent pattern of antibody response has been observed. Thus, antibody levels to some antigens remained high after patency (Gundlach, 1971b; Van Tiggele and Over, 1976) while others fell (Movsesijan *et al.*, 1975; Rajasekariah and Howell, 1978a). The latter can be explained
if adult flukes within the bile ducts are isolated from the host's immune system. Support for this view derives from the observation that adult flukes survive for long periods in the bile ducts of immune animals but are killed if transferred to the peritoneal cavity (Hughes et al., 1976). That some antibodies remain at high levels after patency could be due to the selective absorption of antigens through the gut of the host, a phenomenon shown to occur during cestode infections (Williams, 1979).

The antibody response after patency is complicated by the finding in this study that antibody levels first fell then showed large fluctuations (Chapters 2 and 3). Similar results have not been observed by previous workers. These fluctuations were associated with similar variations in the levels of the bile duct epithelial enzyme, γ-GT, and the liver parenchyma enzyme, GLDH. Thus it appears that bile duct damage caused by the adult flukes may result in the periodic release of fluke antigens into the liver and probably also into the peritoneal cavity. This release of fluke antigen apparently effects damage in the surrounding liver tissue, probably through anaphylactic and autoimmune reactions (Allison and Houba, 1976; Coombs, 1976; WHO Technical Report, 1977). Although damage to the bile ducts could allow the escape of antigenic material and intermittent antigenic stimulation, the continuing survival of adult flukes suggests that immune effectors are still not able to attack them within the bile ducts.
The release of large amounts of antigen and the resulting formation of antigen-antibody complexes may also lead to tissue damage of sites remote from the bile ducts. Lesions recorded in the spleen, kidneys, brain and adrenal glands during F. hepatica infections in sheep and cattle have been ascribed to toxic substances released by the flukes (Locatelli and Simonic, 1974). However, antigen-antibody complexes cause damage in these sites during other diseases and parasitic infections (Allison and Houba, 1976; Alberti et al., 1976; WHO Technical Report, 1977; Andres, 1979), suggesting that the lesions seen during fascioliasis may also be caused by antigen-antibody complexes. If this is the case then the pathology of the disease in sheep might be reduced by the use of strategies which either suppress the production of antibodies that are associated with the damaging complexes or increase the efficiency of the cellular mechanisms which process these complexes. The second alternative would appear to be preferable since this would remove the antigen from circulation and prevent other complications such as tissue damage through cell mediated hypersensitivity reactions.

It should be noted that damaged bile ducts would release not only fluke antigens but also host antigenic material. Autoimmune reactions to this material might result in tissue damage in the liver and elsewhere. Autoimmune reactions may also explain the high levels of anaemia after patency. Sinclair (1964) suggests that the
anaemia in fascioliasis is due not only to the feeding activities of adult flukes but also to the effects of toxic factors, released by adult flukes, on erythropoiesis. A further possibility is that regurgitated gut contents may contain modified red cell antigens that are auto-antigenic. Auto antibodies to liver cell antigens in ovine fascioliasis have been demonstrated by Rushton (1976) and autoimmune responses have been shown to cause anaemia and tissue damage in malaria (Rosenberg, 1978) and trypanosomiasis (Thoongsuwan and Cox, 1978).

9.3 Suppression of antibody responses

The apparent suppression of the precipitating antibody response to F. hepatica in sheep following patency and after a challenge infection, which has been reported in this study, may be due to the release of large amounts of fluke antigen from the bile ducts and the consequent formation of soluble immune complexes of antigen and antibody. These hypotheses are derived from a consideration of the literature reviewed below.

Immune complexes are thought to play major role in the regulation of immune responses (Diener and Feldman, 1972; Diener, 1974; Wright et al., 1974; Fitch, 1975; Kontainen and Mitchison, 1975; Howard and Mitchison, 1975; Morgan and Tempelis, 1977; Taylor, Tite and Manzo, 1979). Complexes can either enhance or suppress the immune responses depending on the relative concentration of antigen and antibody; suppression usually occurs in antigen excess (Diener and
Franks, David and Sturrock, 1977). However, they have not as yet been shown to directly cause suppression of anti-parasite responses, a phenomenon which has been observed during schistosomiasis (Dessaint, Camus and Capron, 1976; Pelley, Ruffier and Warren, 1976; Mota-Santos, Tavares, Gazzinelli and Pellegrino, 1977; Nash, Ottesen and Cheever, 1978; Ottesen, 1979) and other parasite infections including trypanosomiasis (Ramus, Lamoyi, Feoli, Rodriguez, Perez and Ortiz-Ortiz, 1978; Rowland and Kuhn, 1978; Pearson, Roelants, Pinder, Lundin and Mayor-Withey, 1979), malaria (Playfair, 1978), filariasis (Ottesen, Weller and Heck, 1977), Trichinella spiralis (Barriga, 1978), Nematospiroides dubius (Behnke, Wakelin and Wilson, 1978), Ascaris suum (Komatsu, Nishimura, Sano and Shinka, 1979) and Haemonchus contortus (Adams, 1978).

The immunosuppression observed during many parasitic infections has similarities with that induced by tumours. Tumours provide a continual source of antigenic stimulation to the host (Klein, 1972) and there is a progressive loss of immunocompetence associated with their growth (Schumm and Bilmire, 1976). The effect of tumours on the immune system has been mimicked by continual injection of antigen; at first the antigen induces an immune response but then causes specific and non-specific suppression (Gras, 1970; Miller, 1971). While immunosuppression may be a direct effect of continual antigenic exposure, it may also be induced in tumour-bearing mice by immune complexes (Gorczynski et al., 1979). Thus it is possible that the continual antigenic stimulation provided
by many long term parasitic infections such as *F. hepatica* in sheep together with the presence of immune complexes, may induce a similar type of immunosuppression to that induced by tumours.

The existence of immunosuppressive effects caused by *F. hepatica* in hosts other than sheep, such as rabbits, rats and cattle, is perhaps suggested by the absence of anamnestic antibody responses to some antigens following challenge infections (Gundlach, 1971b; Rajasekariah and Howell, 1978a; Kendall *et al.*, 1978). However, the effect has not been as marked as that observed in sheep in this study. The importance of immunosuppression in the survival of challenge flukes will depend on whether those antibodies that are suppressed are involved in protective immune responses.

9.4 **Immunoglobulins in precipitate formation**

IgG₁ was the principal immunoglobulin in infected sheep serum associated with precipitate formation on juvenile *F. hepatica* in vitro. Studies involving tests of total immunoglobulin levels (Nansen, 1969), assays of specific anti-*F. hepatica* antibodies (Movsesijan *et al.*, 1975) and assays of antibodies during other infections (Aalund, 1972) have shown that IgG₁ is the main immunoglobulin found following antigenic stimulation of this host. IgG₁ antibodies have been shown to have the major complement binding capacity of sheep immunoglobulins (Feinstein and Hobart, 1969) and, although complement is not necessary for precipitate formation on
juvenile flukes (Chapter 2), it may be an important factor in reactions which damage flukes. Studies with bovine IgG₁ have shown that this immunoglobulin can mediate passive cutaneous anaphylaxis and phagocytosis reactions (McGuire, Musoke and Kurtti, 1979). It remains to be determined whether the IgG₁ antibody found in the precipitated complex has any of these properties.

Although IgM and IgG₁ precipitating antibodies were the only classes detected during *F. hepatica* infections by the assays used in this study, the existence of anti-*F. hepatica* antibodies belonging to other classes is suggested by previous work. Nansen (1969) and Movsesijan et al. (1975) have detected increased levels of IgG₂ during *F. hepatica* infections while Flagstad and Eriksen (1974) have detected large numbers of IgA secreting cells in the livers of infected cattle. IgG₂ antibodies are able to take part in precipitation reactions (McGuire *et al*., 1979) but they were not detected by the assays used in this study. The role of reaginic and IgA antibodies also requires investigation, especially since both classes are found at high concentration in the gut wall during infection with gastrointestinal parasites in sheep (Curtain and Anderson, 1971) and IgA has been shown to passively protect mice against *Taenia taeniaeformis* infection (Lloyd and Soulsby, 1978). IgG₁A also appears to be the class with the major homocytotropic antibody activity in sheep (Curtain and Anderson, 1972).
9.5 Antigen-antibody reactions in precipitate formation

The number of antigen-antibody reactions that take place to form the precipitate which was observed around *F. hepatica* in vitro, apparently increases as the parasite undergoes its development (Chapter 5). This increasing antigenic complexity probably reflects the increasing functional complexity of the growing fluke and the different host environments encountered during migration. However, certain antigens are apparently common to all stages. The 2 antigens involved in formation of precipitate around juvenile flukes are also involved in precipitate formation around semi-mature and adult flukes. These antigen-antibody reactions may be useful for diagnostic tests since they should detect all stages of the parasite. Moreover, these antigens may be more readily isolated from adult flukes since they are easier to obtain in large quantities than juvenile stages.

The existence of these common antigens also suggests that adult flukes are not protected from the rat immune response (Hayes *et al.*, 1972, 1974a) because they express different antigens to those expressed by juveniles, but through some other mechanism such as isolation from the immune system by their location in the bile ducts.

9.6 Mechanisms of evasion of the host immune response

Several mechanisms whereby flukes can avoid the consequences of the host immune response are suggested by the studies reported in this thesis.
Juvenile flukes may avoid destruction by eliciting only weak or delayed antibody responses until such time as they become established in the host and bring other evasion mechanisms into play. This strategy is apparently employed by certain tumour cell lines during their establishment in naïve animals (Klein, 1972). In *F. hepatica* infections, some support for this type of mechanism is provided by the finding that antibody levels do not show marked increases until 3 weeks after infection (Chapter 2; Capron *et al.*, 1965; Van Tiggele and Over, 1976; Rajasekariah and Howell, 1978a; Kendall *et al.*, 1978).

The major mechanisms by which invasive and migratory fluke are protected from host immune effectors are probably functions of the glycocalyx, which covers the tegument (Chapter 6; Threadgold, 1976). The glycocalyx may protect the surface membranes by acting as a shield that masks surface antigens and through its constant turnover, which would remove antigen-bound immune effectors from the vicinity of the flukes. The masking function of the glycocalyx may be aided by the apparent low immunogenicity of such structures and by their ability to alter the immunogenicity of associated molecules (Apffel and Peters, 1970). As a result, antibodies and cells may be prevented from binding to antigens associated with the glycocalyx until it has been dislodged from the surface and the antigens have become separated from it. In this way, binding of antibodies and cells to potentially functional antigens may occur at sites remote from the flukes.
The turnover of the glycocalyx on *F. hepatica* has not previously been demonstrated but it is known to occur on schistosomes (Wilson and Barnes, 1977). In this study precipitate formation on the surface of juvenile flukes *in vitro* was found to be associated with the glycocalyx layer (Chapter 6). Thus, it appears probable that the antigens involved in precipitate formation are components of the glycocalyx and that their secretion (Chapter 6) is a manifestation of the turnover of this layer. The role of this turnover in removing host immune effectors is suggested by the sloughing of precipitated antigen-antibody complexes from the tegument and the apparent inability of cells to attach to the surface of flukes (Chapter 6). In addition, the formation of antigen-antibody complexes, which can bind complement, and their secretion from the surface may reduce the levels of complement proteins in the vicinity of the parasite. These types of mechanisms have also been suggested to be functions of the glycocalyx which is present on the surface of schistosomes (Clegg, 1974; Wilson and Barnes, 1977).

The release by flukes of proteolytic enzymes, although they may be mainly concerned with fluke nutrition (Howell, 1973), may indirectly have a parasite protective function. Proteolytic enzymes may have damaging effects on host antibodies, complement proteins and cells. These enzymes could be responsible for the cytotoxicity of excretory-secretory products of adult flukes to immunocompetent cells (Goose, 1978) and for the anti-complementary action of the
same products on sheep serum (Howell, pers. comm.). Similar depletion of complement in host sera has been shown to protect certain cestodes against immune attack (Williams, 1979). In addition, proteolytic enzymes may attack and cleave parasite antigens released from the tegument. This could also enhance parasite survival. Cleavage of large molecules with multiple antigenic sites could lead to an increase in the kinds and amounts of small soluble immune complexes, with a consequent increase in the potential for immunosuppression (Howard and Mitchison, 1975).

Other parasite evasion mechanisms such as antigenic disguise as observed in schistosomes (McLaren et al., 1975) and antigenic variation as observed in trypanosomes (Vickerman, 1978) do not seem to be of importance in F. hepatica infections (Hughes and Harness, 1973b; Chapter 5). The existence of blocking antibody, that is, antibody which is not able to damage flukes and which blocks the binding of antibodies and other immune effectors that can, is possible. In fact, the existence of this type of antibody might be suggested by the finding of enhanced fluke survival in vitro in the presence of high antibody levels (Chapter 7). It is not known whether the antibodies involved in precipitate formation have a blocking effect. However, it seems unlikely since precipitate, when injected into sheep, caused some delay in the migration of a challenge infection (Chapter 8) and an analogous precipitate can partially protect rats against infection (Howell and Sandeman, 1979; Howell, in press).
9.7 **Immune responses of rats and sheep to *F. hepatica***

The antigens involved in precipitate formation on juvenile flukes cultured *in vitro* with serum from infected sheep appear to stimulate the formation of antibodies which, though not able to mediate killing of flukes, can cause a delay in fluke migration through the liver (Chapter 8). Since delayed migration is the only manifestation of resistance to *F. hepatica* recorded in sheep (Sinclair, 1971a; Rushton, 1977), these antigens would seem worthy of further study. In rats similar antigens elicit responses that kill a proportion of the flukes of a challenge infection (Howell and Sandeman, 1979; Howell, in press). Thus it would appear that these antigens are functional in the sense defined by Soulsby (1963).

Juvenile flukes were killed when incubated with serum from infected sheep and then transferred to normal rats, but not when transferred to normal sheep (Chapter 7). Moreover, serum from infected sheep has been reported to passively protect rats, but not sheep, against infection (Armour and Dargie, 1974). These results suggest that sheep produce protective antibodies, which are possibly associated with the precipitate, although other non-precipitating antibodies may also be involved. However, these antibodies are not effective in killing worms in the homologous host but are effective in the heterologous host. Thus it would appear that a factor(s) is present in rats but not sheep which acts in concert with antibody to irreparably damage liver flukes. At present the identity of this factor(s) can only be speculated upon. One
resistance to reinfection. However, sufficient information is not available as yet for any firm conclusions to be drawn. It will probably be necessary to identify these differences if satisfactory vaccination procedures for protecting sheep against *F. hepatica* are to be devised.
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