IN VITRO CULTURE OF FASCIOLA HEPATICA AND THE IMMUNOLOGY ASSOCIATED WITH THE METABOLIC PRODUCTS OF THE TREMATODE.

by

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PREFACE

The work described in this thesis is original and has not been submitted in any form to any other University. It was carried out by the author in the Centre for Tropical Veterinary Medicine, University of Edinburgh, under the supervision of Dr M.M.H. Sewell.

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SUMMARY

This work is presented in three Sections, concerned respectively with the *in vitro* maintenance of *Fasciola hepatica*, production and properties of the metabolic antigens of *F. hepatica* and immunisation of the mammalian host with these antigens.

In Section One a number of studies were made on the *in vitro* maintenance of adult *F. hepatica*, with a view to establishing a routine method for maintaining large numbers of flukes together, to facilitate subsequent studies on the antigenic properties of their metabolic products. A detailed description is given of a continuous-flow culture apparatus for the axenic *in vitro* maintenance of groups of adult flukes. The suitability of various media and culture conditions was assessed by measuring the rate of production of ammonia, an end-product of trematode protein metabolism.

It was shown that when the medium consisted of a simple balanced salts solution (modified Earle's salts) flukes showed 100% survival over a five day culture period, but the rate of ammonia production declined rapidly during the initial three days. However, when the medium was supplemented with increasing amounts of serum, the rate of ammonia production was maintained at levels more closely approaching the initial level. Medium 199 (a multi-component tissue culture medium) was shown to be slightly more favourable than the simple balanced salts solution, but less so than those media which contained high proportions of serum. It was shown that inclusion of clotted blood in the medium had no effect on ammonia production, but an increased

flow-rate of the culture medium was associated with the output of significantly greater amounts of ammonia, presumably due to the more rapid dispersal of toxic metabolic waste products.

A series of experiments was undertaken to investigate the possibility of maintaining juvenile flukes *in vitro*, from the metacercarial stage. It was shown that the survival period could be increased from two days, in a balanced salts solution, to 11 days by the inclusion of more complex nutrients such as serum, blood or liver extract in the medium. However, little or no growth occurred under these conditions. Survival and growth were maximal when the young flukes were able to feed on monolayers of living cells, but even under the most favourable conditions growth was limited and compared very unfavourably with that expected *in vivo*.

In Section Two the continuous-flow culture apparatus was modified to form a recirculating system, in which large groups of adult flukes could be maintained for reasonably prolonged periods. Following concentration, the medium in which flukes had thus been maintained showed strong antigenic properties against sera from fluke-infected animals. When flukes were maintained in Medium 199, it was shown that they continued to produce antigenic metabolites throughout a nine day culture period, although the amounts produced decreased with time.

Subsequent studies indicated that the concentrated culture medium contained a variety of antigenic components, including both heat-stable and heat-labile substances, which reacted to differing degrees

with serum from fluke-infected rabbits, sheep, rats and bovines. These reactions were investigated using double diffusion immunoprecipitation in agar and the Enzyme Linked Immunosorbent Assay (ELISA), a serological technique which had not previously been applied to fascioliasis. It was shown that metabolic antigen could be used to follow the serological response developing in experimentally infected animals, but there was no evidence to suggest that it had any particular advantage over more readily obtainable somatic fluke antigen. ELISA was found to be a useful technique, which in some cases enabled the serological response to be detected at an earlier stage than by immunodiffusion.

In view of the apparently complex nature of the metabolic antigen, it was concluded that in its present form it was not likely to be suitable for application to the serodiagnosis of fascioliasis in the field. However, since it is probably less complex than somatic fluke antigen, it may provide a more suitable starting material from which to attempt to isolate purified and species-specific antigens for this purpose.

In Section Three a series of experiments was undertaken to establish whether laboratory animals immunised with metabolic antigens derived from F. hepatica maintained in vitro would respond with the production of antibodies and, if so, whether these were associated with any degree of protection against a subsequent challenge infection.

Rabbits immunised with metabolic antigen derived from adult flukes produced antibodies, which showed certain features in common with those

present in the serum of rabbits carrying a patent fluke infection. There was some evidence that these antibodies had an effect on the course of the challenge infection, since the mean size of the flukes recovered from the immunised animals was significantly smaller than that of the flukes recovered from non-immunised control rabbits. However, there was no significant difference between the two groups of rabbits in terms of the numbers of flukes recovered. The host response to the challenge infection, as indicated by the eosinophilia and increase in plasma glutamate dehydrogenase, was consistently less in the immunised rabbits, but there were marked within-group variations in these parameters and the differences between the two groups were not of statistical significance.

A series of experiments was then carried out, in which rats, of a strain known to be capable of developing acquired resistance to F. hepatica, were immunised with metabolic antigen prepared in various ways from either adult or immature flukes. In all cases the immunised animals produced antibodies to the immunising antigen, which again showed some features in common with those present in the serum of infected animals. However, it was also apparent that there were significant differences between the antibody components of the two types of sera. In particular, it was evident that the immunised rats were not responding well to the non-protein component of the metabolic antigen, in contrast to the infected animals, which showed a strong reaction with this component of the antigen.

There was no evidence that the antibodies resulting from the

immunisation of rats with metabolic antigen of F. hepatica had any effect on the course of a subsequent challenge infection. In no case was there any significant difference between the immunised and control rats in terms of the numbers or sizes of flukes recovered from the challenge infection, or the host response to challenge, as indicated by the eosinophilia and plasma glutamate dehydrogenase assays.

An attempt was made to verify the claim of Lang (1976) that mice could be successfully protected against F. hepatica by immunisation with the medium in which 16-day-old flukes had been incubated for 24 hours. The design of this experiment was similar, but not identical, to that of Lang, but the results were in marked contrast, there being no differences between the immunised and control mice in terms of the infection rate, mortality rate or numbers of flukes recovered from the challenge infection.

It was therefore concluded that metabolic antigen derived from adult or immature F. hepatica maintained in vitro as described was incapable of stimulating resistance to a challenge infection, even in a strain of animals known to be capable of demonstrating acquired immunity to this parasite. This indicated that either the metabolic antigens obtained in vitro were significantly different from those produced in vivo, to the extent that they had lost the ability to stimulate protective immunity in the host animal, or that there is a need for some alternative or additional stimulus to the host before such immunity can be initiated.

GENERAL INTRODUCTION

The common liver fluke, Fasciola hepatica (Linnaeus 1758) is a digenetic trematode, which has long been recognised as a parasite of major economic importance. F. hepatica is a highly successful organism, found in widely distributed temperate areas of the world and capable of infecting an extensive range of mammalian species. A closely related species, Fasciola gigantica (Cobbold 1856) predominates in tropical areas.

The life cycle of F. hepatica, as of all digenetic trematodes, is indirect and involves a period of development and asexual reproduction in a molluscan intermediate host, which in Great Britain is the amphibious snail Lymnaea truncatula. Reinhard (1957) in a review article on the discovery of the life cycle of F. hepatica gave an extensive account of the historical aspects of fascioliasis, noting that the economic importance of the disease had been recognised from the Middle Ages. However, the details of the life cycle and in particular the role of the intermediate host were only elucidated by Thomas and Leuckart who worked independently between 1881 and 1883. Dawes and Hughes (1964) gave a detailed account of the early investigations into the development of the parasite within the mammalian host, which demonstrated that there is a period of migration through the hepatic parenchyma before the sexually mature flukes become established within the bile ducts.

The economic importance of F. hepatica in Great Britain lies in its ability to infect sheep and cattle. In these species fascioliasis

is generally described as occurring as either an acute condition, resulting from massive invasion of the hepatic parenchyma by immature flukes, or a chronic condition, associated with the presence of adult parasites within the biliary system. The acute form of the disease may result in the early death of the host animal, whilst the chronic form is associated with more insidious losses, such as poor weight gains and lowered reproductive function. Further economic losses result from the rejection of parasitised livers at the slaughter house and finally, F. hepatica is associated with the bacterium Clostridium novyi Type B in the pathogenesis of the acute toxaemic condition of sheep and cattle known as infectious necrotic hepatitis ('black disease').

It is extremely difficult to accurately quantify the losses resulting from fascioliasis, particularly those associated with the chronic form of the disease. The most recent survey in Great Britain (Froyd, 1975), which was based on the examination of slaughter house material indicated that 40% of cows, 17% of heifers and steers, 13% of adult sheep and 5% of lambs had been exposed to infection at some time. It was suggested that fascioliasis was more widespread than had been previously thought, particularly in cattle.

Outbreaks of fascioliasis in Great Britain are confined to those areas which are inhabited by Lymnaea truncatula and studies on the epidemiology of the disease and the ecology of the snail have revealed a relationship between the incidence of acute fascioliasis and the prevailing climatic conditions. This has facilitated the development of a method of forecasting outbreaks of fascioliasis

with some reliability (Ollerenshaw and Rowlands, 1959; Ollerenshaw, 1959; Michel and Ollerenshaw, 1963).

To date, attempts have been made to control fascioliasis in livestock either by treating infected animals with appropriate anthelmintics or by attempting to prevent the mammalian host from coming into contact with the intermediate host. Although both these techniques are theoretically of value in controlling the disease, in practice they have not always proved to be satisfactory. Although there are now a number of safe and effective drugs which will kill F. hepatica, even at an early stage in its development, it is only possible to achieve optimum results with them if they are used in conjunction with a complete understanding of the epidemiology of the disease. In other circumstances the use of such drugs is likely to prove expensive and of limited value in the control of endemic fascioliasis.

Measures which may be taken to try and prevent the mammalian host from coming into contact with the intermediate host include fencing or drainage of land, or the application of molluscicides to areas known to harbour snails. Such measures are also likely to be expensive and may not entirely preclude the possibility of further infection.

There can be little doubt that an effective vaccine would be of great potential value in the control of fascioliasis. The successful introduction of the commercial vaccine against bovine lungworm (Jarrett, Jennings, Martin, MacIntyre, Mulligan, Sharp and Urquhart,

1958) has stimulated much research into attempts to induce artificial immunity to many other helminth parasites. Such efforts are only likely to succeed in those cases where the host-parasite relationship favours the development of immunological resistance to reinfection under natural field conditions. It is only within relatively recent years that it has been conclusively shown that certain host species can develop an effective resistance to F. hepatica. This phenomenon has been shown to occur in mice (Lang, 1967), rats (Hayes, Bailer and Mitrovic, 1972, 1973; Goose and MacGregor, 1973a,b; Armour and Dargie, 1974; Čorba and Špaldonová, 1975) and cattle (Boray, 1967a; Ross, 1966a, 1967c; Doyle, 1971, 1972, 1973). Subsequent experiments involving the transfer of immune serum or lymphoid tissues have indicated that such acquired resistance to reinfection is a result, at least in part, of immunological factors. In some other species, notably rabbits and sheep, there is still only slight evidence of an ability to develop resistance to reinfection with F. hepatica.

It has become apparent that even in those cases where the host-parasite relationship favours the development of resistance, attempts to immunise the host species by injection of antigens derived from helminth tissues (somatic antigens) generally produce little or no resistance to a subsequent challenge infection. The apparent need for the host animal to be exposed to the living parasite before protective immunity can be initiated has therefore stimulated much interest in antigenic substances which are produced by the live parasite and excreted or secreted into its environment (metabolic antigens). There is now substantial evidence that such antigens play an important role in many host-parasite relationships and there

have been a number of reports that immunisation with helminth metabolic antigens can stimulate protective immunity to a greater or lesser degree.

There have been a number of attempts to induce protective immunity to F. hepatica by injection of somatic fluke antigens, but these have resulted in little or no protection to subsequent challenge. The only systematic attempt to use metabolic antigens to immunise a host species against F. hepatica was made by Lang (1976), who claimed that injection of mice with medium in which 16-day-old flukes had been incubated for 24 hours stimulated significant resistance to a challenge infection. It would therefore appear that further studies on the effects of immunising animals with fluke metabolic antigens are justified.

Another major topic of interest within the field of the immunology of helminth infections has been that of serodiagnosis. A great deal of research effort has been devoted to this subject, often with conflicting and, from the practical point of view, disappointing results. Major problems have arisen as a result of non-specific reactions and cross-reactions between different helminth species. These problems have been most apparent when serodiagnostic methods have been applied to field studies, rather than to experimentally infected animals maintained under controlled conditions. It has become apparent that more reliable results can be obtained if use is made of purified helminth antigens in serodiagnostic studies and numerous attempts have been made to identify species-specific helminth

antigens. The majority of investigations into the serodiagnosis of helminth diseases have utilised somatic antigens, whilst comparatively few workers have made use of metabolic antigens. Metabolic antigens would only be of value if they were shown to be more species-specific than are somatic antigens. There is little evidence to suggest this, but the matter has yet to be fully investigated.

The major problem associated with attempts to study helminth metabolic antigens is that of obtaining them in adequate quantities. In most cases it is necessary to maintain the parasite *in vitro* for this purpose and consequently most of the studies which have been made to date have made use of those species for which adequate *in vitro* culture methods have been developed. In order to be more certain that metabolic products produced *in vitro* reflect those normally produced by that species *in vivo*, it is necessary to establish the most favourable *in vitro* conditions, which will allow metabolic processes to continue at an optimum rate for the longest possible period of time.

The purpose of the present work was therefore to undertake studies on the *in vitro* maintenance of *F. hepatica*, with a view to developing methods which would allow for the prolonged maintenance of large numbers of flukes in an active metabolic state. It was then intended to make a study of the properties of the antigenic substances produced by flukes *in vitro*, in particular to attempt to assess their potential value in the serodiagnosis of fascioliasis and to determine whether their administration to laboratory animals would stimulate protective immunity to a subsequent challenge infection.

SECTION ONE

IN VITRO CULTURE OF FASCIOLA HEPATICA

CHAPTER ONE

REVIEW OF THE LITERATURE

Introduction

It has long been recognised that the successful maintenance of a parasitic organism *in vitro* can facilitate detailed studies of many aspects of that organism's biology, which are not possible under the *in vivo* situation.

To date, *in vitro* studies have been most widely applied within the fields of virology, bacteriology and mycology, where the comparatively simple organisms can often be successfully maintained outwith the host animal and in many instances will even continue to reproduce in the artificial environment. Although there have been numerous attempts to culture helminth parasites *in vitro*, there have been far fewer successes, largely due to the complex nutritional and environmental requirements of many such species. General discussions and reviews of the subject of *in vitro* maintenance of helminths include those of Hobson (1948), Weinstein (1958), Dougherty (1959), Smyth (1966), Silverman (1963, 1965), Clegg and Smyth (1967) and Taylor and Baker (1968).

In the past, the major reasons for wishing to culture helminths *in vitro* were to study aspects of their biochemistry and physiology and to evaluate anthelmintic drugs. In recent years, an interest has developed in the immunological properties of the excretory and

secretory products of helminth parasites, which can most conveniently be obtained from cultured individuals.

One of the major pitfalls of *in vitro* studies is that the organism may be undergoing an altered form of metabolism *in vitro* from that which normally occurs *in vivo*. There is thus a need to develop *in vitro* systems which adequately satisfy the major needs of the organism, so that *in vitro* findings may be accepted with more confidence as reflecting the true natural activities of that species. This necessitates the ability to assess the performance of the cultured parasite under various conditions, so as to determine those which are the most suitable for it. Since many helminth parasites contain endogenous food reserves, they may be able to survive in relatively simple media for prolonged periods of time, although their metabolic processes will be continuing in a manner far removed from normality. It is therefore essential to distinguish between those conditions and media which merely allow survival of the parasite and those which permit growth, development, maturation and ultimately reproduction of the individual *in vitro*.

A wide variety of criteria have been used to evaluate success of *in vitro* culture systems for helminth parasites. According to Silverman (1965) these have included worm motility and reaction to stimulus, increase in size, changes in external morphology, physiological criteria such as utilisation of substrates, organogeny, gametogenesis and oviposition, cytological evidence for increased mitotic activity and completion of the life cycle. Bell and Smyth (1968), discussing criteria for evaluating the development of

trematodes and pseudophyllidean cestodes *in vitro*, pointed out that such criteria should always be related to the normal pattern of development in the natural host and this should be adequately investigated before meaningful results can be obtained from *in vitro* studies. Unfortunately, in many cases, whilst the broader aspects of the life cycle of the parasite may have been known for some time, there is a lack of detailed knowledge of the normal processes of maturation and general metabolism. Bell and Smyth suggested that useful criteria for assessing development *in vitro* should be precisely defineable, readily recognisable and cover a whole range of maturation processes. They stated that the more precise is the criterion, the more rapidly can changes from normality *in vitro* be recognised, so that prolonged culture would not be necessary before the suitability of any particular medium could be assessed.

Dougherty (1959) classified culture media for helminths as being either oligidic (where the medium contains crude organic materials), meridic (where some components of the medium are of unknown chemical composition) or holidic (where the entire medium has been precisely chemically defined). Obviously the aim in any experimental work on *in vitro* cultivation of a helminth parasite would be to establish an holidic medium. However, in most cases this is not yet likely to be possible, due to the biological complexity of the nutritional requirements of many species.

The term 'axenic culture' was introduced by Baker and Ferguson (1942) to describe the cultivation of an organism in the absence of

any other organisms. This is obviously a desirable feature of any helminth culture system, since contamination of the medium by organisms such as bacteria or fungi might influence the performance of the helminth and invalidate results. However in many cases it is difficult to obtain the parasite free from such contaminants and special measures such as repeated washing in antibiotics may be necessary prior to cultivation.

Numerous nematode, cestode and trematode species have been used in axenic in vitro studies since Glaser (1940) first succeeded in cultivating the nematode parasite Neoaplectana glaseri through successive generations without return to its normal host, the Japanese beetle grub, Popillia japonica. Unfortunately, it has seldom proved possible to emulate this success with parasites of mammals. Nippostrongylus muris was the first nematode parasite of a mammal to be grown to the adult stage in vitro (Weinstein, 1954; Weinstein and Jones, 1956, 1957, 1959). Amongst the trematodes, there have been attempts to culture members of the strigeidae, plagiorchidae, troglotrematidae and schistosomatidae as well as the fasciolidae. Smyth (1966) noted that the cultivation of trematodes in vitro presents a number of special problems. These may be summarised as:

1) Trematodes live in biological habitats, which possess complex chemico-physical characteristics. The exact nature of these habitats is not fully understood in many instances, thus replicating them in vitro may be impossible. In order to define the environment of the parasite completely, it would be necessary to have detailed knowledge of such factors as pH, pO_2, pCO_2,

redox potential, osmotic pressure, temperature, amino acid and sugar levels and concentration of physiological ions.

2) Trematodes feed on complex biological materials, which may be impossible to provide under *in vitro* conditions. Furthermore, in some cases the exact source and nature of the nutrients *in vivo* is unknown.

3) Many species live in non-sterile habitats, thus special measures may be necessary to achieve axenic culture conditions.

4) Allowance must be made for the removal of toxic waste products of metabolism from the locality of the cultured parasite.

5) The complex life cycles of many trematodes may involve a number of different host species. Thus different stages of the life cycle will require different physico-chemical conditions. Furthermore, different stages within one particular host may be exposed to markedly different environments and 'trigger stimuli' may be necessary before one stage can progress to the next.

Thus any attempt to culture a trematode species *in vitro* must take account of these various factors if there is to be any hope of success.

A relatively large amount of work has been undertaken on the culture of schistosomes, reflecting the economic importance of members of this family and a number of useful and interesting aspects of experimental *in vitro* culture work have been investigated. These have included the successful development of axenic culture (Lee and Chu, 1935), demonstration of the benefits of flow cultures (Newsome

and Robinson, 1954; Robinson, 1960; Clegg, 1961), the development of chemically defined media (Senft and Senft, 1962; Senft, 1963, 1965) and the culture of juvenile stages of the parasite (Senft and Weller, 1956; Robinson, 1957; Cheever and Weller, 1958; Clegg, 1959, 1965).

Attempts to maintain adult F. hepatica in vitro

The earliest systematic attempts to maintain adult F. hepatica in vitro were made by Stephenson (1947a). He collected flukes from slaughtered animals, one to six hours after slaughter and transported them to the laboratory in cold bile. Flukes were maintained in 500 ml jars in a variety of media, with motility being used as the measure of survival. The culture medium was changed every 24 hours and the mean batch survival time calculated under various physical and chemical conditions. Borate was used to control bacterial growth. It was found that a pH range of 8.2-8.6 and a temperature of 36°C gave the best results. Glucose was shown to be beneficial to fluke survival, aerobic conditions were preferable to anaerobic, whilst the osmotic potential of the medium was apparently of little significance. By ligating the oral sucker, it was demonstrated that glucose could be taken up through the cuticle. Stephenson's most satisfactory medium contained 150 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 6 mM sodium borate and 30 mM glucose, in which flukes survived for 60 hours.

Dawes (1954) maintained adult flukes for a maximum of 12 days in sterile Hedon-Fleig solution. To achieve this, flukes were extracted

in a sterile manner from warm livers and individual flukes were placed in 25 ml volumes of the medium, contained in 3x1 inch sterile tubes, plugged with cotton wool. Dawes considered that certain airborne bacteria were lethal to cultured flukes, but also considered that borate as used by Stephenson was harmful. Dawes judged survival of flukes on the maintenance of the natural red colour and fleshy appearance and found Hedon-Fleig solution to be superior to Stephenson's medium.

Rohrbacher (1957) repeated Stephenson's work. He removed flukes (without aseptic precautions) from the livers of recently slaughtered animals and transferred them to the laboratory in sterile saline containing antibiotics at 37°C. He found that survival time was increased if the interval between slaughter and removal of the flukes was minimised and also noted that excessive crowding of flukes during transport and culture led to decreased viability. Vigorous and visually uncontaminated specimens were cultured individually in four ounce oil sample bottles, sealed with a rubber stopper. Different physical and chemical environments were investigated and again viability was judged on the appearance of the flukes. Rohrbacher showed that flukes could be maintained for up to 21 days in Tyrode's solution, although numbers diminished from seven days onwards. He confirmed that glucose was beneficial to survival and that it could be absorbed through the tegument as well as through the oral sucker. Liver extract was found to be beneficial, but an amino acid mixture, chicken plasma, horse serum or beef embryo extract were not. Cholesterol appeared to be of some benefit. Rohrbacher confirmed

that fluke survival was better under aerobic rather than anaerobic conditions. Although Sobotka (1937) had shown that the oxygen tension in bile is very low (0.0-0.2 volumes per cent), Rohrbacher suggested that since adult flukes live in very close proximity to the biliary mucosa, they might be exposed to higher levels of oxygen, by diffusion from adjacent blood vessels.

Clegg (1957) adopted a new approach to the assessment of the suitability of various environments for the maintenance of flukes. He quantified the rate of deterioration of various reproductive processes in the cultured flukes and was able to show that physical abnormalities in spermatogenesis or oogenesis were initiated as soon as three hours after the start of in vitro culture. Clegg made use of experimentally infected rabbits as his source of flukes and described a method of aseptically removing flukes from this host. Flukes were maintained within cellulose tubing, which provided a surface for attachment and survived for up to 17 days in Hedon-Fleig solution. In the absence of glucose, survival was reduced to 38 hours. Clegg examined a range of physical and biochemical parameters which influence fluke metabolism in vitro. His major findings were that an osmotic pressure greater than the physiological normal was desirable, inclusion of serum was beneficial and provision of a supporting surface led to better survival. Cooling of the flukes prior to culture was shown to be harmful, whilst the inclusion of whole, clotted or haemolysed blood was apparently of no significance.

Nymark (1961) confirmed that flukes survived longer in Hedon-Fleig

solution than in Stephenson's saline, whilst Bénex (1966) reported a survival time of 40-50 days in a medium consisting of Hank's balanced salt solution, 3% glucose, 20% foal serum and 0.05% sheep erythrocytes.

Sewell (1968) reported that flukes could be maintained in relatively large groups within a continuous-flow culture system and was able to achieve a 50% survival rate over a 30 day period. The medium consisted of 10% sheep serum in modified Earle's salts solution. Sewell monitored the production of ammonia (an end-product of protein metabolism) during the course of the culture and used this as an assessment of fluke metabolism.

Martinetto and Capuccinelli (1968) also used a continuous-flow system, which they found to be superior to a stationary flask-type system. Flukes survived for up to 13 days in the flow system, compared to only 8 days in a flask.

Ratcliffe, Guevara-Pozo and Lopez-Roman (1969) used egg production <u>in vitro</u> as the criterion to assess the suitability of different culture media. They found that 30% calf serum and 10% calf blood were favourable, whereas cholesterol and penicillin were not.

Locatelli and Paoletti (1969) also examined egg production <u>in vitro</u>. It was found that egg production declined rapidly over the first few days and by six days had reached zero. The number of abnormal eggs also increased with time. The medium consisted of Tyrode's solution supplemented with 20% bovine serum.

Wikerhauser and Cvetnić (1967) attempted to maintain adult fluke on monolayers of cultured cells, but found that survival time was longer in a cell-free medium.

Cornish and Bryant (1976) investigated the levels of intermediate metabolites and end-products of carbohydrate metabolism in adult flukes after 24 and 48 hours in Hedon-Fleig solution with added glucose and compared them with the levels immediately after removal from the host. It was concluded that flukes equilibrated with the medium in which they were placed and that energy metabolism was not adversely affected by 48 hours maintenance in vitro.

A number of useful conclusions may be drawn from these studies. It would seem that experimentally infected animals are preferable to animals slaughtered in an abbatoir as the source of flukes for culture. The main advantages of experimentally infected animals being that they allow for more rapid transfer of flukes from the animal to the culture, thus avoiding the deleterious effects of cooling. It should also be easier to obtain uncontaminated flukes under such conditions.

Although a number of workers have demonstrated that flukes can survive adequately in static culture systems, there would seem to be several reasons why a continuous-flow system is more appropriate to the maintenance of adult flukes. A flow system allows for the continual provision of fresh nutrients to the flukes, together with removal of potentially toxic waste products. Thus the flukes may be

kept in sizeable groups, which is of value in certain studies. Furthermore, adult flukes contained within a tubular structure through which medium is flowing, will be in an environment which approximates more closely to their natural site within the bile ducts and this is likely to be beneficial. Finally, provided the flow system incorporates an adequate reservoir of medium, there will be no necessity to add to the medium during the course of the culture, so there will thus be less risk of contamination, as is likely with a static culture, where fresh medium must be added at intervals.

Of the various techniques used to assess fluke performance under in vitro conditions, that of Sewell (1968) would seem to offer a number of advantages. Ammonia assay is relatively simple and accurate, it offers a precise index of fluke metabolic activity and, as it does not require the destruction of the parasite for the measurement to be made, the same individual flukes may be monitored over a period of time. Ammonia is an end-product of protein metabolism in many simple animals (von Brand, 1966) and various workers have shown that it is produced by F. hepatica (Ehrlich, Rijavec and Kurelec, 1963; Moss, 1970). The exact biochemical pathway for its production is unclear, as there are several alternatives. Ammonia can be formed by different enzymes, such as L- amino oxidases, glutamic acid dehydrogenase and urease. Although other metabolic end-products could be used to monitor fluke metabolism in vitro, it would seem to be logical to make use of an end-product of protein metabolism, since F. hepatica must have a very high rate of protein metabolism in vivo to allow for its high

reproductive capacity. Thus one might expect that if protein metabolism could be adequately maintained *in vitro*, then the organism as a whole would be in an active metabolic state.

Urea is another end-product of fluke protein metabolism which could be used for the same purpose, but there has been some controversy over the relative importance of ammonia and urea as end-products of protein metabolism in *F. hepatica*. Ehrlich, Rijavec and Kurelec (1963) reported that *F. hepatica* excreted a number of nitrogenous waste products into its environment, of which urea was the most abundant. Flukes were incubated axenically in Ringer's solution, fortified with glucose and it was found that both ammonia and urea were released at a fairly constant rate. Urea output was found to be lower in Ringer's solution than in serum, whereas ammonia output was higher in Ringer's solution than in serum.

These workers also identified all the essential constituent amino acids of the ornithine cycle in fluke tissue homogenates and showed that the addition of ornithine to the medium led to an increased output of urea and diminished excretion of ammonia. They concluded that the synthesis of urea was following the pattern of the well documented Krebs-Hensleit ornithine cycle.

Moss (1970) measured the output of various end-products of protein metabolism from flukes maintained in either static or flow cultures. He showed that the production of ammonia declined rapidly after 30 minutes incubation in Hedon-Fleig solution, under both

aerobic and anaerobic conditions and this was not due to the toxic effects of the accumulating ammonia. Moss showed that urea formed 21-30% of the excreted nitrogen and that the inclusion of glucose in the medium led to a reduction in the ammonia production, but not in urea output. He also showed that small flukes produced more ammonia per unit of body weight than did larger flukes. Goil (1958) investigated the end-products of metabolism of a number of trematodes, including F. gigantica. He was unable to identify urea, but showed that ammonia and uric acid were produced.

Aspects of the physiology of adult F. hepatica which are of significance to in vitro studies

In order to make a rational attempt to maintain a helminth parasite in vitro it is necessary to first make a study of the known facts relating to its normal in vivo physiology. Wherever possible, the in vitro culture system should then seek to replicate the situation to which the organism has become adapted in nature.

The physiology of F. hepatica has been reviewed in detail by Smyth (1966) and Pantelouris (1965, 1967). Adult flukes may be found at various locations within the biliary tract of the parasitised mammalian host, which may be any one of a wide range of species. The major contents of mammalian bile are the bile salts and pigments, whilst minor constituents include cholesterol lecithin, inorganic salts, enzymes, and a number of diffusible plasma constituents such as amino acids, glucose and urea (Hill, 1970). None of these constituents would appear to be of sufficient

nutritive value to maintain an organism with the high level of metabolic activity shown by adult liver flukes. Sobotka (1937) made a study of the physiological chemistry of the bile of a number of mammalian species. He noted that there is a wide variation in many of the physiological characteristics of bile, depending on the species. This is of some interest since it implies that F. hepatica, which can parasitise a wide range of hosts, must be adaptable to various conditions. Sobotka found that the pH of the bile varied according to the site from which it was taken, ranging from 6.0 to 8.2, with gall bladder bile tending to be more acidic than hepatic bile. Again, since flukes may be found at different sites within the biliary tract and also within the gall bladder, it may be assumed that pH values are not critical. It was also found that the osmotic potential of the bile varied according to its location, as a result of the concentration of the hepatic secretions within the gall bladder.

Sobotka also noted that the oxygen content of bile was very low (0.0-0.2 volumes per cent). This was in accordance with the findings of Moss (1970). However the question as to whether F. hepatica has an aerobic or anaerobic form of metabolism has caused some controversy. The characteristic end-products of anaerobic carbohydrate metabolism are the fatty acids and the abundance of these substances in the excretory products of F. hepatica (Stephenson, 1947c; van Grembergen, 1949; Mansour, 1959a,b; Lahoud, Prichard, McManus and Schofield, 1971) indicates that this form of metabolism is occurring. Several authors have quantified the uptake of oxygen by flukes in vitro (van Grembergen, 1949; Mansour, 1959b).

Smyth (1966) noted that the egg shells of flukes are hardened by the process of quinone tanning, which requires oxygen. Both Stephenson (1947a) and Rohrbacher (1957) found that *in vitro* survival was better under aerobic rather than anaerobic conditions, whilst Prichard and Schofield (1971) recorded that the uptake of oxygen by flukes was proportional to the oxygen tension to which they were exposed. Thus the opinion of Vernberg (1963) that digenetic trematodes may utilise both aerobic and anaerobic forms of metabolism would seem to be applicable to *F. hepatica*. Rogers (1949) demonstrated that whilst the lumen of the mammalian intestine has a very low oxygen tension, the microenvironment adjacent to the mucosa may in fact be rich in oxygen, as a result of diffusion from adjacent blood vessels. A similar situation may apply within the biliary tract, thus allowing flukes which are in close proximity to the mucosa to utilise oxygen for their metabolic functions.

Knowledge of the details of the nutrition of adult flukes *in vivo* would obviously be of great value to *in vitro* studies. Unfortunately, this subject is another in which there has been much controversial reporting. Stephenson (1947b) cited a number of early workers who thought that flukes fed on bile, bile duct epithelium or blood. Müller (1923) concluded that flukes were not feeding on blood, but rather on the viscous proteinaceous contents of the bile ducts, desquamated bile duct epithelium and leucocytes which had infiltrated the biliary mucosa. A number of workers have reported finding blood cells or haemoglobin derivitives in the caecal contents of adult flukes (Hsü, 1939; Stephenson, 1947b; Urquhart, 1955; Todd and Ross,

1966) although it should be noted that there is evidence that haemoglobin-like substances may normally occur in trematodes. Stephenson (1947b) further showed that flukes would ingest clotted blood *in vitro* and convert it to acid haematin.

Anaemia and hypoalbuminaemia are well documented features of chronic fascioliasis, but their precise cause has been the subject of much controversy in the past. Some authors have stated that the anaemia is caused by toxins liberated by the parasites (Lapage, 1956; Smith, Jones and Hunt, 1972), whilst Sinclair (1964, 1965) considered that anaemia arose secondary to a disorder of the reticulo-endothelial sytem which led to decreased erythrocyte production and increased erythrocyte destruction. However, in recent years, evidence from experiments in which erythrocytes or plasma constituents have been labelled with radioactive markers has clearly indicated that the presence of adult flukes in the biliary tract is associated with a marked loss of blood cells and serum proteins through the bile ducts into the intestine (e.g. Sinclair, 1967b, 1970a, 1972; Sewell, 1967a; Holmes, McLean, Dargie, Jennings and Mulligan, 1967; Sewell, Hammond and Dinning, 1968; Symons and Boray, 1967a,b, 1968; Dargie, Holmes, McLean and Mulligan, 1968a,b, 1970; Dargie and Mulligan, 1970).

Jennings, Mulligan and Urquhart (1955, 1956), Pearson (1963) and Symons and Boray (1967a,b, 1968) have shown further that such radioactive labels can be found in higher concentration in flukes or their caecal contents than in surrounding tissues, thus indicating a selective uptake of blood by the parasites.

Dawes and Hughes (1964) in a review of the nutrition of adult flukes noted that whilst a number of workers had supported the hypothesis that adult flukes feed on blood, none had indicated how flukes within the bile ducts might have access to a source of blood. Dawes (1963b) made a detailed study of the pathological changes occurring in the bile ducts of fluke infected animals and stated that there was little evidence of any vascular tissue within the immediate vicinity of the adult flukes. He concluded that the parasites were feeding on a 'pasture' of the hyperplastic biliary epithelium rather than on blood. However, in contrast to this, Symons and Boray (1967a,b, 1968) have reported finding histological evidence of blood clots on the biliary mucosa at the sites where flukes were located and were of the opinion that this proved that adult flukes within the bile ducts could feed on blood. Sinclair (1967b) speculated that the anaemia of fascioliasis may result from vascular leakage and haemolysis associated with the cellular reaction of the host to toxic substances produced by the parasite, rather than from the direct feeding activities of the flukes.

Overall, the evidence seems to be strongly in favour of blood as the main source of nutrients for adult flukes, although it is possible that the parasites also obtain part of their nutritive requirements from the damaged biliary mucosa itself, as suggested by Dawes (1963b).

Attempts to maintain juvenile F. hepatica in vitro

There have been few reports of attempts to culture juvenile

flukes from the metacercarial stage, presumably because this stage of the parasite develops in an exceedingly complex environment, which is virtually impossible to simulate in vitro.

Before juvenile flukes can be cultured, they must be caused to excyst from the metacercaria. A variety of different systems have been reported to cause excystment. Susuki (1931) used an artificial digestive juice containing 0.2% HCl and 1% pepsin, which was followed by 0.2% $NaHCO_3$, 1% pancreatin and 5-7% bile. Wikerhauser (1960) reported a rapid method of assessing the viability of metacercariae using a system similar to that of Susuki. An 80% success rate was achieved using 20% bile, whilst a lower rate was seen if the bile was reduced to 10% and little excystment occurred in the absence of bile. Wikerhauser found that flukes excysted in this manner would survive for up to 42 hours in Tyrode's solution, after removal of the excysting solutions. Dixon (1964, 1966) showed that excystment was an active two stage process. The first stage, activation, was stimulated by carbon dioxide, reducing conditions and a temperature of 39°C, whilst the second stage, emergence from the cyst, was triggered by bile. Sewell and Purvis (1969) further showed that excystment was not dependent on the presence of the enzymes pepsin and trypsin, but was improved by use of bile. 0.04 M sodium dithionite as a reducing agent was found to be an alternative to bile.

Wikerhauser and Cvetnić (1967) obtained excysted flukes by the method of Wikerhauser (1960) and following repeated washing in sterile saline containing antibiotics, were able to maintain them

under axenic conditions for some time. Survival was judged on spontaneous contractions and normal appearance, but was limited to two days in Hedon-Fleig solution and only three days in more complex media incorporating horse serum, calf serum or lactalbumin hydrolysate. The survival period was extended up to 14 days if the flukes were maintained on monolayers of cultured cells. A variety of cell lines were investigated, including calf testis, bovine embryo kidney, pig kidney, pig embryo kidney and monkey heart. It was found that the abundance of the cells in the culture was of more significance to fluke survival than the cell type. Wikerhauser, Cvetnić and Brudnjak (1970) in a continuation of this work found that replacement of cells and medium would prolong the survival of young flukes up to a maximum of 29 days. There was no evidence of any growth or development of the young flukes in any of these studies.

Osuna Carillo de Albornoz and Guevara Pozo (1973, 1974) cultured excysted flukes in normal inactivated horse serum, together with sheep red cells washed in Alsever's solution, under an atmosphere of 10% carbon dioxide and 90% nitrogen. Flukes survived for a maximum of 54 days and increased in length to 700 µm from the initial 150-200 µm. Some development of the genital and digestive systems was also recorded.

Hanna, Baalawy and Jura (1975) excysted metacercariae of F. gigantica, using a modification of the technique of Sewell and Purvis (1969). The juvenile flukes thus obtained were maintained for up to 60 days on monolayers of foetal calf spleen cell cultures, using

various media and a gas phase of 5% carbon dioxide in air. A fourfold increase in size was recorded after 25 days in the culture, when growth ceased. Hanna and Jura (1976) showed that juvenile F. gigantica which had been maintained on cell monolayers in a medium consisting of 10% foetal calf serum in Eagle's Minimum Essential Medium, retained the ability to develop normally after intraperitoneal injection into mice. Even after 60 days in such a culture system the flukes were able to continue their normal development within the mammalian host, having apparently existed in a state of 'suspended animation' within the in vitro culture.

Aspects of the physiology of immature F. hepatica which are of significance to in vitro studies

As previously indicated, successful in vitro cultivation of a parasitic helminth is more likely to occur if the known facts relating to the physiology of the species in vivo are applied to the in vitro situation.

Although the basic life cycle of F. hepatica had been elucidated by the end of the last century, the precise details of the migratory route of the young parasite within the mammalian host remained in doubt for many years. Dawes and Hughes (1964) gave an account of early investigations into this problem.

Dawes (1961a,b,c; 1962a,b,c; 1963a,b,c,d) gave a detailed account of the development and nutrition of F. hepatica from the metacercaria to the adult stage in mice. His findings confirmed

those of earlier workers in that excystment occurred in the small intestine of the host and the young flukes moved freely over the intestinal mucosa immediately after excysting. Feeding started at this early stage and Dawes recorded that the young flukes were ingesting host tissues whilst penetrating the intestinal wall. Thus during the first 24 hours, the flukes had been exposed to a variety of different tissues and had fed on a number of them. There was no evidence for blood feeding at this stage. By 24 hours after excysting, the flukes had reached the peritoneal cavity, where they were observed 'creeping over the viscera'. By injecting the host with trypan blue dye, Dawes was able to show that flukes were feeding off the peritoneal surface of the abdominal viscera at this stage.

Entry into the liver was seen by two to three days after excystment, when young flukes were seen to bury themselves into the structure of that organ and start the migratory phase which would eventually take them to the bile ducts. During this phase of the life cycle, the young flukes were seen to be actively tunnelling through the hepatic tissues, using the oral sucker to burst and break down liver cells to form a cytoplasmic homogenate, which was then ingested. Dawes stated that the mouth of the fluke was almost always found pressing into the hepatic tissue, indicating that feeding is more or less a continuous process. Although they must be exposed to blood whilst migrating through an organ as vascular as the liver, Dawes was of the opinion that the young flukes were not primarily feeding off blood, but rather off liver cells. At a later stage in the hepatic phase, when host liver damage was maximal, flukes were

seen to be lying within blood-filled cavities, but when this occurred, there was a tendency for the parasites either to leave the liver and return to the abdomimal cavity or to proceed to the bile ducts and the growth rate diminished under these circumstances. The precise mode of entry into the bile ducts was not established, but Dawes suggested that it occurred at the time when the risk to the host was maximal and might be the only alternative left to the fluke in order to maintain a satisfactory host-parasite relationship.

Dawes made a study of the growth rate of juvenile flukes and also followed the development of the various organs, particularly the gut and genital system. Wide individual variation in growth rate was noted, but overall the growth rate was very rapid throughout the intra-hepatic phase. By the third day after excystment the mean length had increased from 0.17mm to 0.29mm, indicating a degree of growth during the stage of leaving the intestine and reaching the peritoneal cavity. Thereafter the flukes doubled in length every three to four days. Dawes (1962a) gave details of the growth, including measurements of length, breadth, diameter of oral and ventral suckers as well as development of the gut and genitalia, which may form a useful reference for in vitro studies.

These reports indicate that the in vitro culture of juvenile F. hepatica is likely to present formidable problems. It is apparent that the flukes at this stage are voracious feeders, and grow remarkably rapidly. The hepatic tissues are able to provide a suitably rich and varied source of nutrients to allow for this, which

would be difficult in emulate *in vitro*. Furthermore, the hepatic parenchyma will be providing a highly complex physical environment for the parasite, which again would be difficult to simulate *in vitro*.

CHAPTER TWO

MATERIALS AND METHODS

Production of metacercariae

Throughout the course of this work it was necessary to continually produce metacercariae of F. hepatica.

A stock of Lymnaea truncatula was maintained in the laboratory to serve as the intermediate host for the parasite. Snails were kept in plastic sandwich boxes on a shallow layer of mud, on which was grown green alga to serve as a food supply. The preparation of the algal cultures was as described by Pullan (1968) except that it was found desirable to use a specially formulated mineral solution in the preparation of the mud, since the use of tap water alone with the local soil would support only limited algal growth. The composition of this solution (Table 2.1) was determined by comparative analysis of the local soil with soil supplied by the Ministry of Agriculture, Weybridge, which readily supported algal growth (Sewell, M.M.H., personal communication).

The collection of fluke eggs from bile, the production of miracidia and the infection of snails was as described by Sewell (1961). Infected snails were maintained at 23°C, their management and the subsequent shedding of cercariae into polythene bags being undertaken as described by Pullan (1968). The metacercariae thus obtained were kept at room temperature for seven days, before being stored at 4°C in a 125 ml glass bottle containing distilled water.

Solution A. (g/l).		Solution B. (mg/100 ml)		Solution C. (g/l)	
Potassium nitrate	8.00	Ammonium meta -vanadate	2.0	Calcium chloride	55.0
Potassium dihydrogen ortho phosphate	10.32	Boric acid	286.0		
Sodium chloride	0.16	Cobalt nitrate	5.0		
Sodium carbonate	6.00	Copper sulphate ($5\ H_2O$)	8.0		
Trisodium citrate	0.08	Magnesium sulphate ($7\ H_2O$)	25,000.0		
Ferrous sulphate ($7\ H_2O$)	0.08	Manganese Chloride ($4\ H_2O$)	180.0		
		Sodium molybdate	20.0		
		Zinc sulphate ($7\ H_2O$)	2.0		

Final solution: 250.0 mls Solution A; 1.0 ml Solution B; 1.0 ml Solution C; distilled water to 1000.0 mls.

TABLE 2.1 Mineral solution for use in algal culture.

Infection of animals with metacercariae

In order to achieve high infection rates, the freshest available metacercariae were used, although metacercariae were never used in the 48 hours immediately after their production.

A small piece of the polythene bag, on which encystment of the cercariae had occurred, was cut out and transferred to a solid watch glass containing a small volume of distilled water. The required number of cysts were then scraped off the polythene using a blunt mounted needle, making efforts to reject those which did not show the characteristic granular appearance denoting viability.

In order to infect rats and mice, the metacercariae were taken up in a pasteur pipette, to the end of which was attached a short length of 1.0 mm internal diameter plastic tubing. In order to prevent the metacercariae from sticking to the glass, the pipette was pretreated with silicone water repellent ('Repelcote', Hopkin and Williams Ltd., Chadwell Heath, Essex).

The animals were lightly anaesthetised with ether and the tube inserted into the pharynx. The cysts were expelled and the pipette and tube examined under a steroscopic microscope to ensure that no cysts remained.

In order to infect rabbits, sheep and cattle, a small quantity of cellulose powder ('Whatman CF1', Whatman Biochemicals Ltd., Maidstone, Kent) was added to the watch glass containing the

metacercariae. The powder adsorbed the water and the cysts adhered to the resulting wet mass, which was then transferred to a gelatine capsule (Parke Davies and Co., Hounslow, London). The capsules were then administered to the animals, by force feeding in the case of rabbits and by means of a balling-gun in the case of sheep and cattle.

Detection of fluke eggs in faeces

Since it was necessary to establish when mature flukes were present within the bile ducts of experimentally infected animals, routine faecal examinations were carried out. The method used was the sellotape modification of the standard zinc sulphate flotation technique, as described by Sewell and Hammond (1972).

Sterilisation procedures

Metal surgical instruments were sterilised by dry heat in an oven for two hours at 160°C.

Glassware, drapes and other materials were sterilised by autoclaving at a pressure of 103 kPa for 20 minutes.

Small volumes of medium were sterilised by filtration through a 25 mm 0.22 μm membrane filter (Millipore U.K. Ltd., London) contained within a 25 mm Swinnex filter holder (Millipore U.K. Ltd., London). Prior to use, the holders and filters were assembled according to the manufacturer's instructions, wrapped in aluminium foil and autoclaved at 103 kPa for 20 minutes. The medium was taken up in a suitable

syringe and injected through the sterile filter into a sterile container.

Large volumes of medium were sterilised by filtration through a 142 mm 0.22 μm membrane filter (Millipore U.K. Ltd., London) contained within a 142 mm filter holder (Millipore U.K. Ltd., London). Again the filters and holders were assembled according to the manufacturer's instructions, the outlet was plugged with non-absorbent cotton wool and the whole unit sterilised by autoclaving. The medium was placed in an 11.4 l stainless steel pressure vessel (Millipore U.K. Ltd., London) which was then filled with nitrogen or compressed air to a pressure of 345 kPa. The pressure vessel was then connected to a 142 mm filter holder containing a depth filter (Type AP15, Millipore, U.K., London) and a series of membrane filters of pore sizes 1.2 μm, 0.8 μm and 0.45 μm respectively, the purpose of which were to remove larger contaminating particles. This filter holder was in turn connected to the pre-sterilised 142 mm filter holder containing the 0.22 μm membrane filter. The medium, having been forced through the system by the pressurised gas, was collected into a sterile container attached to the outlet of the sterile filter holder.

Removal of adult flukes from infected animals

Adult flukes to be used in in vitro studies were removed from the bile ducts of experimentally infected animals once they had reached maturity, as indicated by positive faecal egg examination.

Rabbits and sheep were used as hosts in these studies. In order to establish axenic cultures, it was necessary to remove flukes from the bile ducts using aseptic techniques.

Rabbits were killed by cervical fracture rather than by chemical methods, which it was thought might affect the flukes. The skin over the ventral thorax and abdomen was shaved and then thoroughly cleaned with chlorhexidine ('Hibiscrub', Imperial Chemical Industries, Macclesfield, Cheshire) followed by 70% ethyl alcohol. The animal was then placed in dorsal recumbency in a laminar air-flow cabinet (Microflow Ltd., Fleet, Hants.).

All subsequent procedures were undertaken using aseptic techniques and sterile equipment. Sterile drapes were arranged around the shaved area, so as to cover the remainder of the body. A midline skin incision was made over the xiphoid process, extended anteriorly to the mid thorax and posteriorly to the umbilicus. The abdominal wall was then incised along the linea alba from the umbilicus to the xiphoid process. The ribs on each side were incised along a line approximately 2 cm from the midline, the incision being extended to the mid thorax. The posterior section of the sternum was then pulled forward so as to expose the liver. The liver was grasped and by carefully cutting the diaphragm, it could be extracted from the abdominal cavity. The exteriorised liver was then pulled anteriorly to expose the visceral surface and the common bile duct. The common bile duct was clamped with a fine pair of curved artery forceps close to its point of entry into the duodenum, so as to

prevent the possible entry of intestinal contents into the biliary tract during subsequent handling. A short longitudinal incision was then made into the common bile duct. Flukes were expressed through this incision, carefully picked up with smooth forceps and rapidly transferred to a beaker containing sterile medium at 37°C. The beaker was immediately returned to a water bath, so as to maintain the contents at this temperature. It was found convenient to prepare several such beakers, which were used in sequence as flukes were removed. The beakers were covered with sterile aluminium foil, to prevent contamination of the contents. If necessary, additional incisions were made in the bile ducts at different sites, until all the flukes had been removed.

With practice, it was possible to remove flukes aseptically within a very short time of the death of the host animal. The rapid transfer of flukes to the medium and the maintenance of the medium at 37°C ensured that the deleterious effects of cooling were minimised.

The removal of flukes from sheep followed essentially the same principles as for rabbits. The animals were killed with a captive bolt pistol and the incision site prepared as previously described. Since the liver of the sheep is relatively small and inaccessible, the midline abdominal incision was extended along the right costal margin to provide better exposure of the organ. It was necessary to perform the initial stages of the process in the post-mortem room, where the animal had been killed. Having exposed the liver, the

common bile duct was clamped with two pairs of artery forceps at a point near to the duodenum. An incision was made between the forceps and the whole liver was removed from the abdominal cavity by carefully dissecting it free of its various attachments.

The liver was then rapidly transferred to the laminar air-flow cabinet, where flukes were removed as previously described. This process of extracting the whole liver from the sheep meant that there was likely to be a greater degree of cooling of the flukes, so rabbits were used as the source of flukes in most cases.

Flukes which had been thus collected were washed by transferring them two or three times to beakers containing fresh medium at 37°C. They were then ready to be transferred to a culture.

Continuous-flow culture system for the maintenance of adult Fasciola hepatica

The continuous-flow system which was routinely used in studies on the *in vitro* maintenance of adult *F. hepatica* is illustrated in Plate 2.1 and represented diagramatically in Fig.2.1.

A 2 l 'Pyrex' aspirator (A) (Jobling Laboratory Division, Stone, Staffordshire) served as the reservoir for the culture medium. A silicone bung (B) was inserted into the top inlet of the aspirator. The medium was introduced into the aspirator through silicone tubing (C), which was attached to a length of 5 mm diameter stainless steel tubing, which pierced the bung. A similar piece of silicone tubing

(D) was attached to the steel tubing within the aspirator and reached to the bottom of the vessel in order to prevent excessive foaming of the medium as it entered the aspirator. The bung (B) was also pierced by three wide-bore hypodermic needles. Two of these needles had hubs of the standard Luer type, which allowed Swinnex 25mm filter holders (E,F) (Millipore U.K. Ltd., London) to be attached as shown. The filter holders contained 0.22 μm membrane filters (Millipore U.K. Ltd., London) and allowed for the equilibration of air pressure between the aspirator and the exterior, whilst maintaining sterility of the culture. The third needle, which was taken from an intravenous 'giving set' (Avon Medicals Ltd., Birmingham) had a smooth male hub, to which was attached an inverted Swinnex 25 mm filter holder (G), containing a 0.22 μm membrane filter. A length of silicone tubing (H) was attached to the end of this needle within the aspirator and reached to the floor of the vessel. The purpose of this inverted filter holder was to allow for the removal of samples of the medium from the aspirator in an aseptic manner. A short length of silicone tubing (I) was attached to the outlet nozzle of the holder. By connecting this tubing to the nozzle of a 20 ml syringe, medium could be withdrawn from the aspirator by suction.

A silicone bung was inserted into the side-outlet of the aspirator and held in place by an aluminium yoke (J). The bung was pierced by a short length of 5 mm diameter stainless steel tubing, to which was attached a length of silicone tubing (K). This tubing was in turn connected to finer bore tubing by means of a male hubbed hypodermic needle (L). The fine bore tubing then passed

through a peristaltic pump (M), (Desaga 'PLG-multipurpose peristaltic pump 13 21 00', Camlab, Cambridge). The use of this multi-channel pump enabled a number of cultures to be run simultaneously. The flow rate through the system could be varied, either by altering the speed of the pump, or by changing the diameter of the tubing passing through the pump. Thus 1.0 mm internal diameter tubing was used for high flow rates and 0.5 mm internal diameter tubing for slower rates.

Having passed through the pump, the silicone tubing was connected to a 'bubble-trap' (N), the purpose of which was to collect gas bubbles which tended to accumulate in the preceding tubing and prevent them being carried further down the apparatus. The 'bubble-trap' consisted of a 100x10 mm test tube with rubber bung ('Vacutainer', Becton, Dickinson U.K. Ltd., Wembley, Middlesex). The silicone tubing from the pump was attached to the shaft of a wide bore hypodermic needle (O) which pierced the bung. A similar needle shaft (P), to which was attached a length of silicone tubing (Q) sufficient to reach the bottom of the test tube, served as the exit for the medium from the 'bubble-trap'. A male hubbed hypodermic needle, to which was attached a short length of silicone tubing (R) plugged with non-absorbent cotton wool, allowed gas to escape from within the test tube.

The medium then passed along a length of narrow bore silicone tubing to a 30 cm length of 8 mm diameter nylon sleeving (S), (Portex Ltd., Hythe, Kent) in which were placed the flukes. Each end of the nylon sleeving was stretched slightly with forceps and slipped over the end of a 4 cm length of 10 mm diameter stainless steel tubing

(T,T'). The sleeving was held tightly in place over the tubing by cuffs of silicone tubing (U,U'). Rubber bungs (V,V'), taken from 100x10 mm test tubes ('Vacutainer', Becton, Dickinson U.K. Ltd., Wembley, Middlesex) and each pierced by the shaft of a wide-bore hypodermic needle, were then attached to each of the steel tubes. The tubes were supported by clips on a wooden frame as shown.

A length of narrow-bore silicone tubing (W) connected the outlet from the sleeving to a wide-bore needle shaft, which pierced a silicone bung (X) inserted into the neck of a 250 ml conical flask, in which the medium was collected. A hypodermic needle (Y), plugged with non-absorbent cotton wool, allowed for equilibration of air pressure between the flask and the exterior.

All materials used in the construction of the continuous-flow apparatus were heat stable, so that the whole apparatus could be sterilised by autoclaving prior to use, to enable the setting up of axenic cultures. Prior to autoclaving all joints in the system were sealed with liquid silicone sealing compound ('Aquaseal', Dow Corning, Windsor) which prevented subsequent leakage of the medium. The apparatus was then prepared for autoclaving as shown in Plate 2.2.

Having assembled the culture apparatus, the medium-inlet tube (C) was plugged with non-absorbent cotton wool and clamps were applied to tubes I and K. A length of 10 cm diameter nylon sleeving (Portex Ltd., Hythe, Kent) was then placed over the top of the aspirator and taped to the glass with indicating autoclave tape

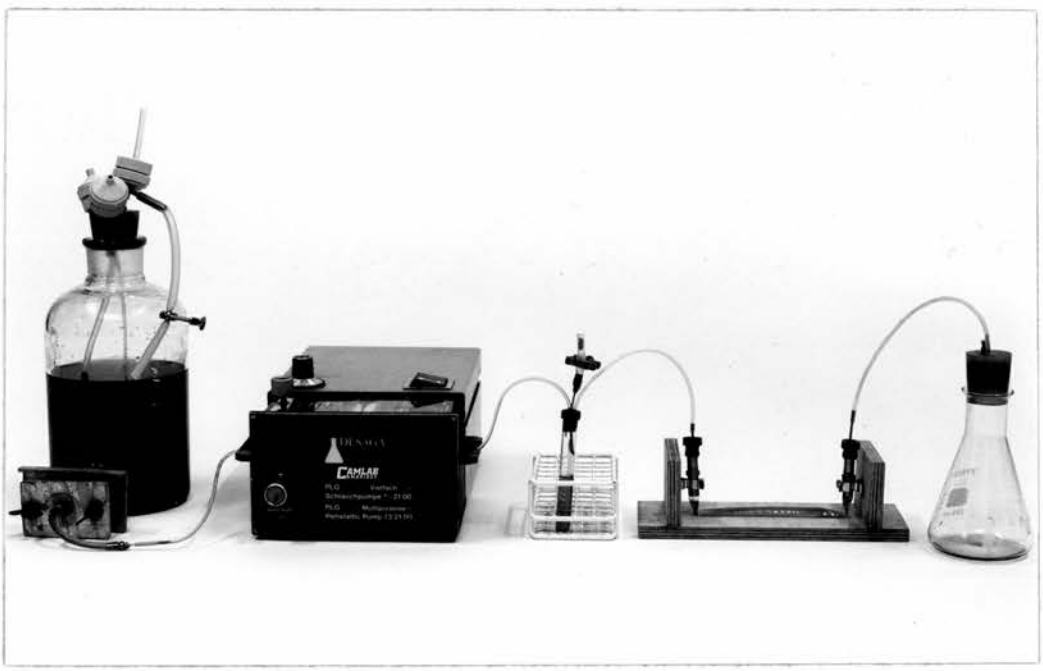

Plate 2.1. Continuous-flow culture apparatus for the in vitro maintenance of adult F. hepatica.

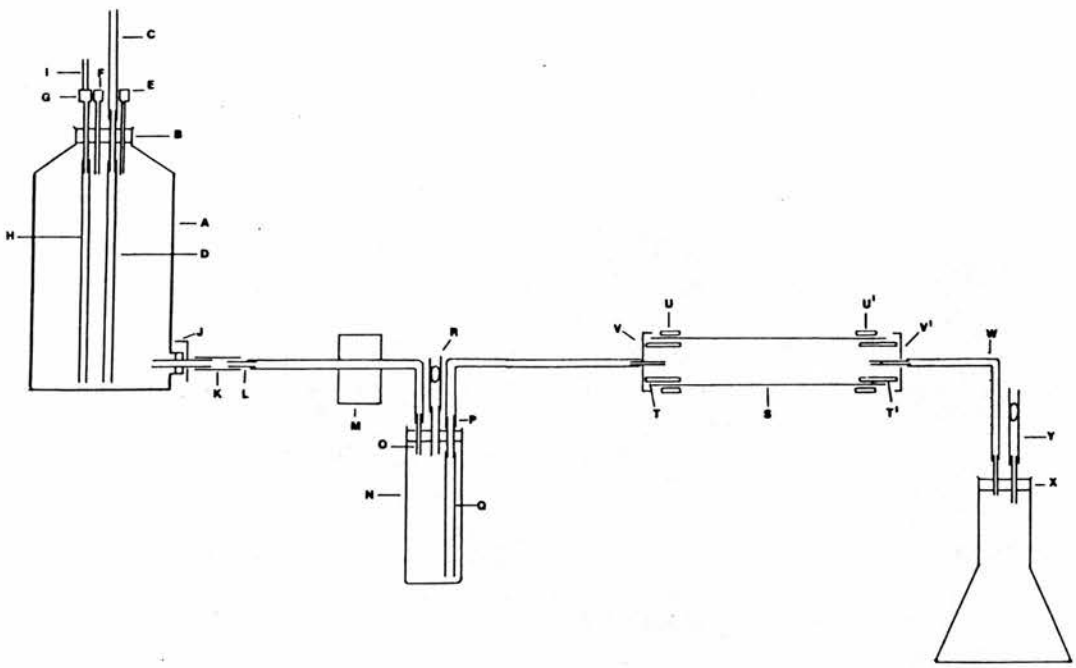

Fig. 2.1. Diagramatic representation of the continuous-flow culture system for the in vitro maintenance of adult F. hepatica (not to scale).
Refer to text for key.

(Medical Products Company, Loughborough, Leicestershire). Non-absorbent cotton wool was wrapped about the inlet tube (C) and the nylon sleeving tied tightly with string. The bung (B) was loosened so as to allow steam to enter the aspirator. A bag was formed from a length of similar nylon sleeving, by taping shut one end. The entire length of the culture apparatus was then placed in this bag. Non-absorbent cotton wool was wrapped around the neck of the side-outlet of the aspirator and the nylon bag tied tightly around the neck with string.

The culture apparatus, having been thus prepared, was autoclaved for 15 minutes at a pressure of 103 kPa. On removal from the autoclave, the bung (B) was reinserted into the neck of the aspirator, which was allowed to cool.

The medium was then introduced into the aspirator. Using aseptic techniques, the cotton wool was removed from the inlet tubing (C), which was then connected to the outlet of a presterilised 142 mm filter holder (Millipore U.K. Ltd., London) containing a 0.22 μm membrane filter. The medium was forced into the aspirator by gas pressure, having been sterilised by passage through a series of membrane filters as previously described. When the required volume of medium had entered the aspirator, the inlet tubing was clamped. The apparatus was then removed from the nylon sleeving in which it had been sterilised, attached to the peristaltic pump, tube K unclamped and medium pumped along the system. When the 'bubble-trap' test tube was approximately half filled with medium, the air outlet tube (R) was clamped. The whole apparatus was then allowed to stand

Plate 2.2. Continuous-flow apparatus prepared for sterilisation.

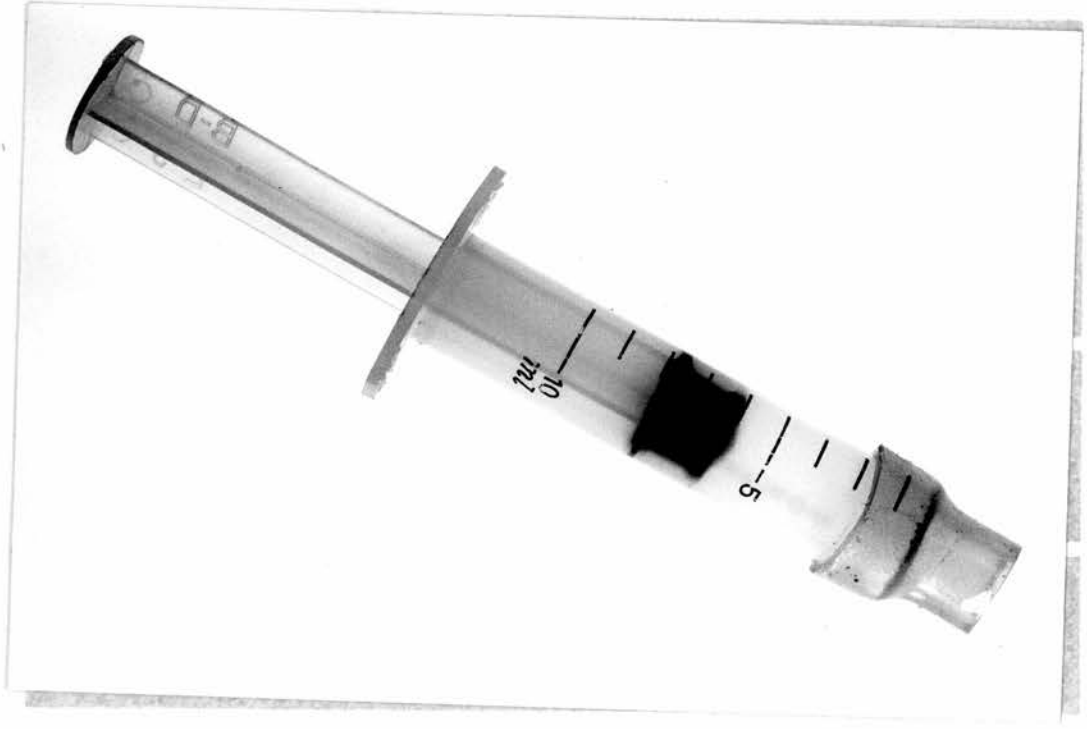

Plate 2.3. Modified syringe for inserting adult flukes into continuous-flow culture.

overnight in a warm-room at 37°C, before the flukes were introduced.

Introduction of flukes into the continuous-flow apparatus

Adult flukes, which had been removed from the host animal as previously described, were introduced into the continuous-flow apparatus by means of a specially constructed device shown in Plate 2.3. The end of the barrel of a 20 ml plastic syringe (Becton, Dickinson U.K. Ltd., Wembley, Middlesex) was cut off and a cuff of 8 mm internal diameter silicone tubing fitted to the cut end. Prior to use, the modified syringe was wrapped in aluminium foil and sterilised by autoclaving. Using aseptic techniques, the plunger of the syringe was drawn back and the flukes, together with a small volume of medium, placed in the barrel of the syringe using blunt forceps. The bung (V') was detached from the culture apparatus and the silicone cuff on the syringe fitted carefully over the end of the stainless steel tubing (T'). The flukes were then 'injected' into the length of nylon sleeving (S), the syringe detached and the bung (V') replaced. Flukes could thus be inserted into the culture in a rapid and aseptic manner, with a minimum of physical handling.

Cleaning the culture apparatus

Between successive cultures, those parts of the apparatus which were to be reused were first washed well with tap water to remove gross debris. They were then soaked overnight in detergent (2% 'Decon 90', Decon Laboratories Ltd., Brighton). The apparatus was then washed with hot running tap water to remove all traces of the detergent, rinsed twice in distilled water and dried in an oven.

Media used in experiments on the in vitro culture of adult flukes

Modified Earle's balanced salts solution for use in the culture of adult flukes was made by dissolving the constituents of the original formula (Earle, 1943) in 900 ml of distilled water rather than in 1000 ml. The purpose of this was to increase the osmotic potential of the solution, which was shown by Clegg (1957) to be of value in the culture of adult F. hepatica.

Bovine serum for use in the flow cultures was required in relatively large volumes and special measures were necessary to produce a sterile product. Blood was collected from cattle at the time of slaughter at the local abbatoir. The livers of the animals from which blood had been collected were examined for evidence of fascioliasis and only blood from apparently uninfected animals was retained. The blood was placed in a 40 l plastic tub and allowed to clot at room temperature. The clot was cut up with a knife and, after standing overnight at 4°C to allow the clots to retract, the liquid fraction was poured off. This was found to contain large amounts of cellular debris and further treatment was necessary before sterile serum could be obtained by filtration. The crude serum was passed through a small cream separator ('Model 100AE', Alfa Laval Co.Ltd., Cumbran, Gwent), which rapidly removed most of the cellular debris. The serum was then centrifuged for one hour at 1,500 g in 1 l plastic flasks on a 'Mistral 4 L' centrifuge (M.S.E. Ltd., London).

The resulting supernatant could then be sterilised by filtration

through a 142 mm 0.22 μm membrane filter (Millipore U.K. Ltd., London) as previously described.

By this technique, large volumes of sterile serum could be prepared. Serum was stored at -20°C until required.

Medium 199 was obtained commercially (Gibco Bio-Cult, Paisley, Scotland). Clotted sheep blood was obtained by collecting blood from the jugular vein into a sterile evacuated test tube ('Vacutainer', Becton, Dickinson U.K. Ltd., Wembley, Middlesex). The blood was allowed to clot at room temperature. The clot was then removed from the tube using aseptic techniques and cut into suitable pieces for insertion into the culture.

All media were supplemented with 100 i.u. benzyl penicillin per ml ('Crystapen', Glaxo Labs Ltd., Greenford, England), 100 μg per ml streptomycin/dihydrostreptomycin ('Dimycin', Glaxo Labs Ltd., Greenford, England) and 2 μg per ml amphotericin B ('Fungizone', E.R.Squibb and Sons Inc., New York).

Assay of ammonia in culture medium

The ammonia in the culture medium was quantified using an ammonia probe ('Laboratory Model 8002 - 2', Electronic Instruments Ltd., Chertsey, Surrey). This instrument responds to free ammonia in solution and is unaffected by ions. Therefore, for the detection of total ammonia, or of ammonium ion in solution, the pH must be raised to a value greater than 12, so as to convert all ammonium to free

ammonia. When the probe is in contact with free ammonia, the internal solution between the pH electrode membrane and the gas permeable membrane gains or loses ammonia gas through the latter, until the partial pressure of ammonia is the same on each side. The pH of the internal filling solution next to the membrane is therefore proportional to the free ammonia concentration of the sample. Changes in pH were measured on the expanded millivolt scale of a 'Model 291 Mk 2' pH meter (Pye Unicam, Cambridge).

To make a measurement, 9 ml of the sample was quickly mixed with 1 ml 1M sodium hydroxide in a small beaker. The probe end was then dipped into the mixture and the reading noted. The probe was calibrated with a number of standard ammonium chloride solutions and a calibration graph prepared by plotting the potentials developed by the standards on the linear axis, against the solution concentrations on the log axis of semi-logarithmic graph paper. The ammonia concentration of the unknown sample could then be read from the graph. The probe end was well rinsed with distilled water between successive readings.

It was found that it was essential that all solutions to be assayed should be at the same temperature, as the probe was sensitive to small changes in temperature. Hence when samples from a fluke culture at 37°C were to be assayed, they were first allowed to cool at room temperature.

Production of juvenile flukes for use in in vitro studies

Juvenile flukes to be used in in vitro studies were caused to excyst from the metacercariae by the method of Wikerhauser (1960), special precautions being taken to allow for the establishment of axenic cultures.

Metacercariae were produced as previously described and the polythene bag bearing the cysts which were to be used was washed repeatedly in distilled water to remove gross debris. The metacercariae were stored at 4°C in a sterile 125 ml bottle, containing distilled water, to which was added 100 i.u. per ml benzyl penicillin ('Crystapen', Glaxo Labs Ltd., Greenford, England), 100 µg per ml streptomycin/dihydrostreptomycin ('Dimycin', Glaxo Labs Ltd., Greenford, England) and 2 µg per ml amphotericin B ('Fungizone', E.R.Squibb and Sons Inc., New York).

Aseptic techniques and sterile instruments and solutions were used in all subsequent procedures. A piece of polythene bearing a suitable number of metacercariae was cut out and placed in a 35 x 10 mm sterile plastic petri dish (Becton, Dickinson U.K. Ltd., Wembley, Middlesex). Approximately 2 ml of a solution containing 0.8 g per cent NaCl and 0.5 g per cent pepsin in 0.05N HCl was then added to the petri dish. The metacercariae were gently scraped off the polythene using a mounted needle. After incubation at 37°C for two hours, an equal volume of a solution containing 1.0 g per cent $NaHCO_3$, 0.8 g per cent NaCl and 0.4 g per cent trypsin in distilled water, with 20% v/v ox bile was added. Incubation at 37°C was continued for

a further three hours, after which time the flukes which had excysted were washed three times, by withdrawing the solution in the petri dish with a finely drawn pasteur pipette and replacing it with an equal volume of the medium to be used in the subsequent experiment. Every effort was made to ensure that the flukes were maintained at 37°C during these procedures.

The newly excysted flukes were examined under a stereoscopic microscope and actively moving, intact specimens were taken up in a finely drawn pasteur pipette and transferred to 25 cm^2 plastic tissue culture flasks (Becton, Dickinson U.K. Ltd., Wembley, Middlesex), containing 5 ml of the medium in which they were to be cultured.

The assessment of the suitability of different media for the maintenance of juvenile flukes

A series of tissue culture flasks containing the medium under consideration were set up, into each of which were placed 40 viable young flukes, as described above. The flasks were then incubated at 37°C.

At intervals (generally two to three days), the flukes within one flask were removed and placed in a petri dish. Individuals which were still showing spontaneous movements were taken up in a finely drawn pasteur pipette and transferred with a small volume of the medium to a microscope slide. An equal volume of methyl green acetic stain was then added and the slide examined under a microscope. On exposure to the stain the flukes were killed and contracted to a uniform oval shape, the length and breadth of which was measured with

a calibrated eyepiece. The stain also highlighted details of the internal morphology of the flukes, allowing for an assessment of organ development. Twenty such flukes were measured, the process being continued until insufficient live flukes remained in the cultures. Thus an indication of the 50% survival time, as well as growth rate, was obtained for each medium under consideration.

Media used in experiments on the in vitro maintenance of juvenile flukes

Earle's salts solution was made according to the original formula (Earle, 1943). Sterile calf serum was obtained commercially (Gibco Bio-Cult Ltd., Paisley, Scotland).

Whole sheep blood was prepared by aseptically collecting blood from the jugular vein into a sterile 10 ml syringe (Becton, Dickinson U.K.Ltd., Wembley, Middlesex). The blood was then immediately transferred to a sterile universal bottle containing 50 units of heparin (Evans Medical Ltd., Liverpool). The required quantities of whole blood were then transferred to the culture flasks.

A strain of human liver cells ('Chang Liver') was obtained commercially (Gibco Bio-Cult Ltd., Paisley, Scotland) and maintained according to the manufacturer's instructions. The maintenance medium was Minimum Essential Medium (MEM) with Hank's salts (Gibco Bio-Cult Ltd., Paisley, Scotland) supplemented with 10% calf serum. The cells were grown on 25 cm^2 plastic tissue culture flasks (Becton, Dickinson U.K.Ltd., Wembley, Middlesex).

To prepare a liver extract, 60 g of fresh rabbit liver was finely chopped in 50 ml of MEM, and then homogenised for 3 minutes with a mechanical laboratory homogeniser. The homogenate was allowed to stand overnight at 4°C, centrifuged at 2,500 g for 30 minutes and the resulting supernatant recentrifuged at 25,000 g for 30 minutes on a Model L265B Ultracentrifuge (Beckmann Instruments Ltd., Glenrothes, Scotland). The supernatant liver extract was sterilised by filtration through a 0.22 µm membrane filter (Millipore U.K.Ltd., London) and stored at -20°C.

All media were supplemented with antibiotics and amphotericin B as previously described.

Gas phase for the maintenance of juvenile flukes

The influence of various gas phases on the maintenance of juvenile flukes was investigated by placing the tissue culture flasks, with loosened caps, in a McIntosh and Fyldes anaerobic jar (Baird and Tatlock Ltd., Chadwell Heath, Essex), which was then filled with the appropriate gas mixture.

CHAPTER THREE

A PRELIMINARY STUDY ON THE MAINTENANCE OF ADULT F. HEPATICA IN A CONTINUOUS-FLOW CULTURE SYSTEM

Introduction

As previously indicated, a continuous-flow culture system appears to offer a number of advantages over a stationary culture for the *in vitro* maintenance of adult *F. hepatica*. The purpose of this preliminary study was to establish whether it was possible to maintain adult flukes under axenic conditions in a continuous-flow apparatus and whether measurement of the production of ammonia by such flukes was likely to provide a useful means of assessing fluke viability *in vitro*.

Experimental design

Two sets of the continuous-flow apparatus were assembled, sterilised and filled with modified Earle's salts solution as previously described. Five adult flukes were placed in the first culture immediately after removal from a rabbit. Five similar flukes, which had been killed by keeping them at 4°C for 16 hours, were placed in the second culture to serve as controls. The two cultures were then run side-by-side, with a medium flow-rate of approximately 5 ml per hour.

At 24 hour intervals, samples were withdrawn from the reservoir of each culture for ammonia assay. At the same time, medium which had passed over the flukes and been collected in the conical flask was removed, the volume measured and the ammonia content determined.

Samples of media which had passed over the flukes were submitted for bacteriological examination. The cultures were terminated after five days.

Results

The flukes in the first culture were all alive and active at the end of the culture period. It was noticeable that during the course of the culture flukes migrated along the nylon sleeving in which they were contained, against the direction of flow of the medium, until they were all congregated at the end of the sleeving nearest to the pump.

Ammonia assays for both cultures are recorded in Appendix Table 3.1 and the rate of ammonia output represented graphically in Fig.3.1. No ammonia was detected at any time in the samples taken from the reservoirs of medium, nor from the medium collected after passing over the dead flukes. However, medium which had passed over the live flukes did contain ammonia. It was evident that the rate of production of ammonia by the live flukes declined steadily during the course of the culture, with the most rapid decline occurring in the first three days.

Bacteriological examination of medium which had passed over dead or living flukes failed to reveal the presence of any organisms.

Discussion

This experiment showed that it was possible to maintain flukes under axenic conditions in a continuous-flow culture system. Even in

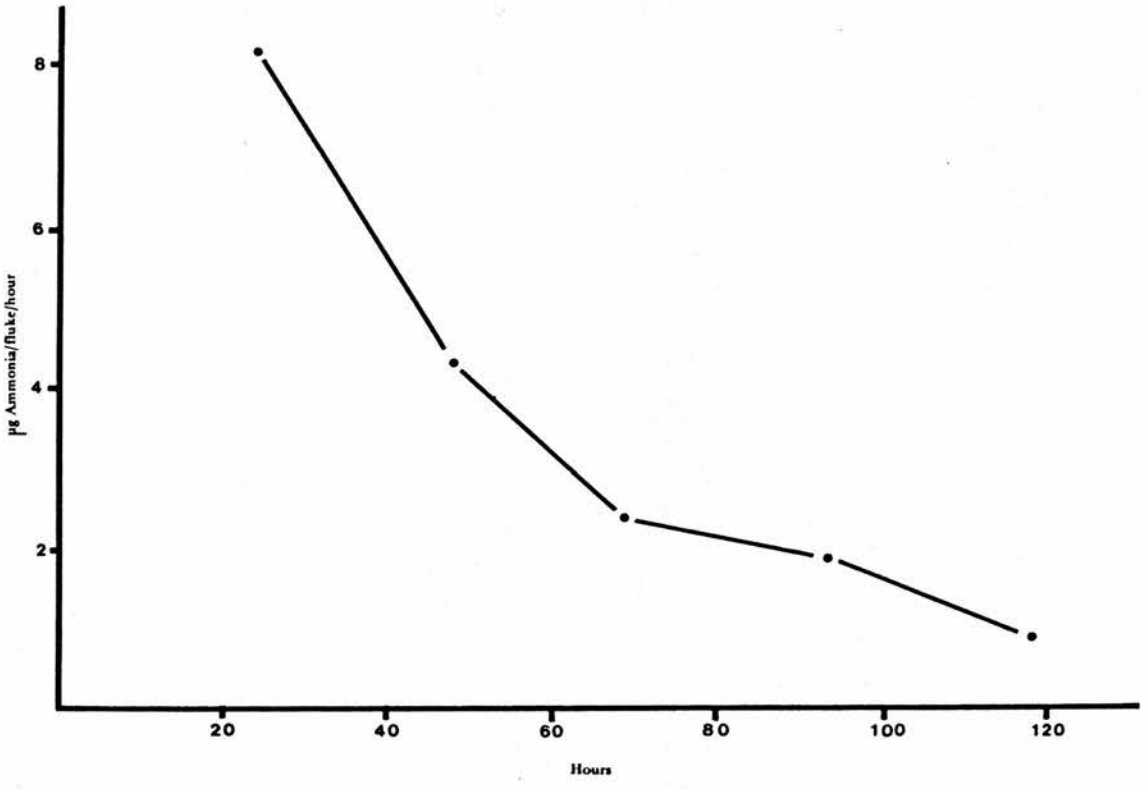

Fig. 3.1. Production of ammonia by adult F. hepatica maintained in modified Earle's salts solution.

a basic saline medium flukes showed 100% survival over a five day period. The results confirmed the findings of earlier workers that adult F. hepatica produces ammonia in measurable quantities. It was evident that the ammonia was being produced by some active metabolic process and not by either bacterial activity or mere autolytic processes, since no ammonia was detected in the fluid from the culture containing dead flukes. The reason for killing the flukes by cooling, rather than by any other method such as exposure to toxic chemicals, was to preserve any bacteria which might be present and to allow subsequent degenerative processes to take place in the normal manner.

The rapid decline in ammonia production during the initial three days of the culture would seem to indicate that the flukes were utilising their endogenous food reserves during this period. When these had been depleted, it appeared that the flukes were only able to continue a low level of metabolic activity in this medium, which is of very low nutritive potential.

The tendency for the flukes to migrate against the flow of the medium may be a mechanism designed to prevent them being swept out of their natural environment by the flow of bile, or to take them away from areas contaminated by their metabolic waste products.

In view of these findings further experiments were undertaken in order to determine whether ammonia production could be increased, or the rate of decline of ammonia production diminished, by the use of potentially more nutritious media and also to study other parameters which might influence fluke metabolism in vitro.

The fact that in this initial experiment the greatest decline in ammonia output occurred in the initial three days of the culture period indicated that future studies should concentrate on attempts to improve metabolism during this period. Thus, prolonged cultures would not be necessary before it was possible to determine whether any modifications to the system were of potential value. In this respect, measurement of ammonia production fulfills one of the major criteria laid down by Bell and Smyth (1958) for methods of assessing the performance of trematodes *in vitro*.

CHAPTER FOUR

THE INFLUENCE OF INCREASING THE SERUM CONTENT OF THE MEDIUM ON THE METABOLISM OF ADULT F. HEPATICA IN VITRO

Introduction

In view of the strong evidence that adult flukes *in vivo* feed on blood, an experiment was conducted to investigate the effect of adding serum to the culture medium used to maintain adult flukes *in vitro*.

The previous experiment had indicated that fluke protein metabolism *in vitro* declined rapidly in a simple balanced salt solution. It was of interest to know whether the quantity of ammonia produced, or the rate of decline of ammonia output could be altered by use of a protein-rich medium such as serum.

Experimental design

Four sets of the continuous-flow apparatus were assembled, sterilised and filled with medium. The media consisted of 90%, 60%, 30% and 10% bovine serum respectively in modified Earle's salts solution. Five adult flukes removed from a rabbit were placed in each culture. The cultures were then run side-by-side at a flow-rate of approximately 5 ml per hour. At intervals, samples of medium were withdrawn from each of the reservoirs for ammonia assay and at the same time the medium which had passed over the flukes and been collected in the conical flasks was removed, the volumes measured and ammonia assayed. Samples were again submitted for bacteriological examination. The cultures were terminated after five days.

Results

The flukes again showed a marked tendency to migrate against the flow of the medium. All the flukes were alive and active at the end of the experiment and no bacterial contamination of the medium was detected.

The ammonia assays for each culture are recorded in Appendix Table 4.1. It was evident that the serum used in this experiment contained appreciable amounts of ammonia, since samples taken from the reservoirs throughout the course of the experiment consistently contained ammonia, in amounts proportional to the quantity of serum in the medium. It was also apparent that the level of ammonia in the reservoirs was increasing with time, presumably as a result of the breakdown of nitrogenous serum constituents.

Ammonia production by flukes maintained in a medium consisting of 10% serum/90% modified Earle's salts solution declined rapidly during the initial three days of the culture, in a similar manner to that observed in the previous experiment. However, it was evident that as the level of serum in the medium increased, whilst the initial output of ammonia was relatively unchanged, the rate of decline of ammonia output diminished, apparently in proportion to the quantity of serum in the medium. The calculated least squares regression lines ($\log_{10}$ rate of ammonia production per flukes, against time) for the cultures containing 90% and 10% serum respectively are shown in Fig.4.1. The regression lines for the cultures containing 60% and 30% serum lie between these two, but are not shown for the sake of clarity. Analysis of variance indicated that there were

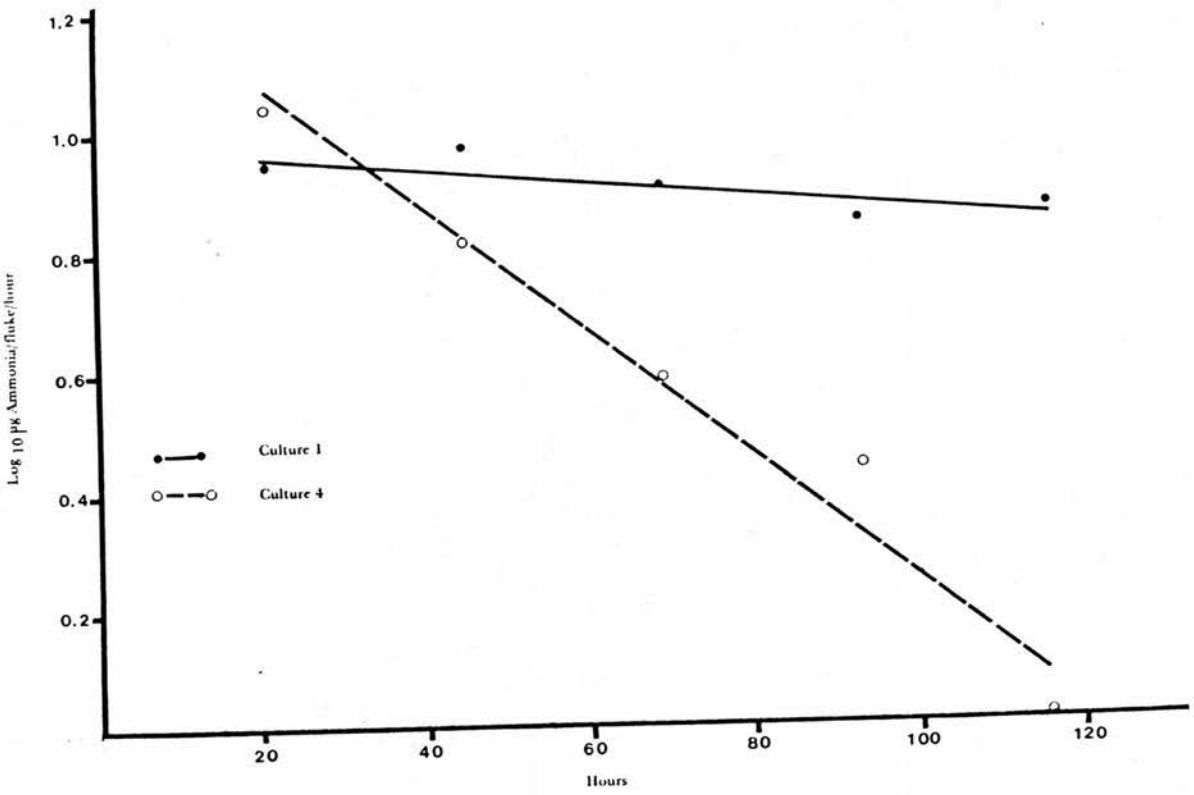

Fig. 4.1. Calculated least squares regression lines ($\log_{10}$ rate of ammonia production per fluke, against time) for cultures containing 90% serum (Culture 1) and 10% serum (Culture 4).

Culture 1 $y = -0.001x + 0.980$, $r = -0.84$.

Culture 4 $y = -0.010x + 1.280$, $r = -0.98$.

c.f. Culture 2 (60% serum) $y = -0.004x + 1.052$, $r = -0.84$.

Culture 3 (30% serum) $y = -0.005x + 1.044$, $r = -0.95$.

significant differences between the four regression coefficients ($F = 9.25$; degrees of freedom = 3, 12; $p < 0.005$). The relationship between the regression coefficients and the serum contents of the four cultures approached significance at the 5% level ($r = -0.94$; $0.1 > p > 0.05$).

Discussion

The results of this experiment showed clearly that the rate of decline of ammonia production by adult flukes maintained *in vitro* could be diminished by the addition of increasing amounts of serum to the medium. When the medium was supplemented with 90% serum, the output of ammonia over the five day culture period was maintained at a level approaching that occurring at the time of removal of the flukes from the host animal. A medium composed solely of serum might have proved even more favourable. The results provide additional evidence that the production of ammonia *in vitro* is the result of active metabolism rather than mere degenerative processes, since if the latter were the case, one would not expect the addition of increasing amounts of potential nutrients to the medium to influence the output of ammonia.

These results were not unexpected, in view of the substantial evidence that adult flukes *in vivo* feed on blood. Although ammonia production is only reflecting one part of the overall metabolism of the organism, it is not unreasonable to assume that those *in vitro* conditions which favour optimal ammonia output will also favour other metabolic processes. It may therefore be concluded that the inclusion of a high percentage of serum in the medium has a favourable effect

on fluke metabolism *in vitro*, a conclusion which is in accord with the findings of other workers (Clegg, 1957; Bénex, 1966; Ratcliffe, Guevara-Pozo and Lopez-Roman, 1969).

CHAPTER FIVE

A COMPARISON OF MODIFIED EARLE'S SALTS SOLUTION WITH MEDIUM 199 FOR THE MAINTENANCE OF ADULT F. HEPATICA IN VITRO

Introduction

The previous experiment indicated that increasing amounts of serum in the culture medium were associated with a less rapid decline in the production of ammonia by the flukes. However, it was envisaged that in certain subsequent experiments it would be necessary to maintain flukes in a protein-free environment. It was therefore of interest to establish whether the use of a protein-free multi-component tissue culture medium was more favourable than basal saline, in which ammonia production had been found to decline rapidly over the initial period of the culture.

Experimental design

Two sets of the continuous-flow apparatus were assembled, sterilised and filled with medium. The media consisted of modified Earle's salts solution and Medium 199 respectively.

Five flukes removed from a rabbit were placed in each culture. The cultures were then run side-by-side at a flow-rate of approximately 8 ml per hour and ammonia assays were made at intervals on samples of media withdrawn from the aspirators and also on media which had passed over the flukes.

The cultures were terminated after five days.

Results

All the flukes in both cultures were alive and active at the end of the experiment. Individual ammonia assays are recorded in Appendix Table 5.1. The calculated least squares regression lines ($\log_{10}$ rate of ammonia production per fluke, against time) for the two cultures are shown in Fig.5.1. The flukes maintained in modified Earle's salts solution again showed a rapid decrease in ammonia output during the initial three days *in vitro*. The initial rate of production of ammonia by the flukes maintained in Medium 199 was similar to that of the flukes maintained in the basal saline, but, although there was a subsequent decline in the rate, the later ammonia levels were consistently higher than those of the flukes maintained in modified Earle's salts solution. However, analysis of variance showed that the difference between the slopes of the two regression lines was only significant at the 10% level ($F = 4.66$; degrees of freedom = 1,6; $p < 0.1$).

Discussion

The results of this experiment suggested that Medium 199 was a slightly more favourable medium than modified Earle's salts solution for the *in vitro* maintenance of adult F. hepatica, since the rate of decline of ammonia production was slower in the former medium. Although the differences in the slopes in this experiment were not conventionally significant, a comparison of the results from a number of other experiments in which one or other of the media were used supported the conclusion that the difference was real.

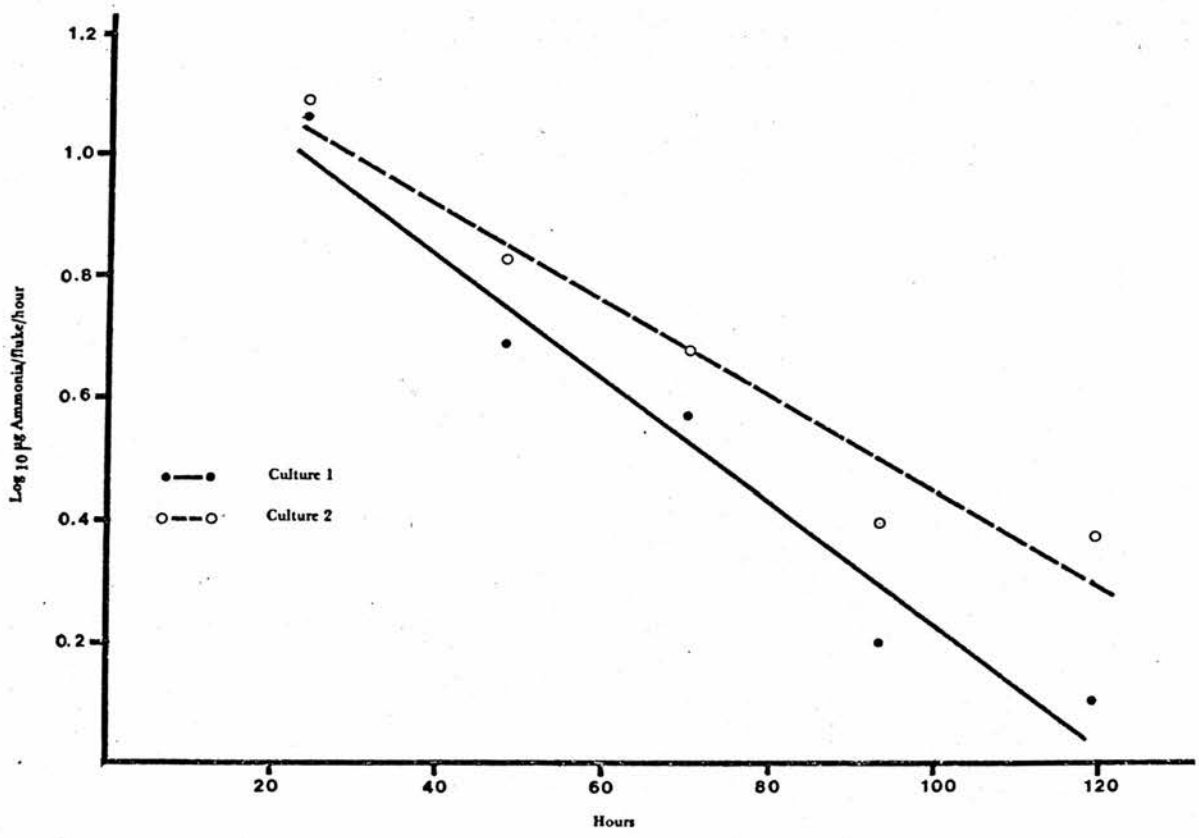

Fig. 5.1. Calculated least squares regression lines ($\log_{10}$ rate of ammonia production per fluke, against time) for cultures containing modified Earle's salts solution (Culture 1) and Medium 199 (Culture 2).

Culture 1 $y = -0.010x + 1.239$, $r = -0.98$.

Culture 2 $y = -0.008x + 1.230$, $r = -0.97$.

This again was not unexpected, in view of the fact that Medium 199 contains a wide range of potentially valuable nutritive substances, including amino acids, glucose, vitamins and coenzymes, as compared to modified Earle's salts solution, which contains only glucose. It would seem probable that the flukes maintained in both media were initially utilising endogenous food supplies, but that when these were exhausted, the flukes maintained in Medium 199 were able to maintain a somewhat higher metabolic rate, by utilising components in the medium which were not present in the basal saline solution. None the less, it was evident that Medium 199 was much less favourable for the maintenance of adult flukes than were the media in the previous experiment which were supplemented with large proportions of serum.

CHAPTER SIX

THE INFLUENCE OF INCLUDING WHOLE BLOOD IN THE MEDIUM ON THE METABOLISM OF ADULT F. HEPATICA MAINTAINED IN VITRO

Introduction

Flukes feeding on blood *in vivo* will be exposed to the cellular elements of blood as well as to serum and, as previously described, a number of workers have identified blood cells in the caecal contents of adult flukes.

A previous experiment had indicated that serum was favourable to fluke metabolism *in vitro* and the present experiment was designed to see whether the presence of whole blood in the medium had any effect on the quantity of ammonia produced, or the rate of decline of ammonia output.

Experimental design

Three sets of the continuous-flow apparatus were set up, sterilised and filled with a medium consisting of 80% bovine serum/ 20% modified Earle's salts solution. Small pieces of clotted sheep blood were then introduced into two of the cultures, so that the clots lay approximately half way along the nylon sleeving. Nine adult flukes were placed into one of the cultures containing clotted blood and nine similar flukes were placed in the culture containing no blood clot. No flukes were placed in the remaining culture, which served as a control to indicate whether any ammonia was produced as a result of the presence of the blood clot alone.

The three cultures were run side-by-side at a flow-rate of approximately 7 ml per hour. Ammonia assays were made at intervals on the medium in the culture reservoirs and on the medium after it had passed over the flukes. The cultures were terminated after four days.

Results

As before, the flukes tended to migrate along the nylon sleeving, against the flow of the medium. The flukes in the culture which contained the blood clots thus encountered the clots as they migrated, but seven of the nine flukes passed over the blood and continued along the sleeving. The remaining two flukes buried themselves in the blood clots and remained there for the rest of the culture period.

Individual ammonia assays are recorded in Appendix Table 6.1. The calculated least squares regression lines ($\log_{10}$ rate of ammonia production per fluke, against time) for the two cultures are shown in Fig.6.1. It was again evident that the medium in the reservoirs contained appreciable quantities of ammonia, which increased during the course of the experiment. The presence of the blood clot was associated with a small additional increase in ammonia concentration in the control culture.

It was clear that the presence of the clotted blood had no significant effect on the initial quantities of ammonia produced by the flukes and analysis of variance indicated that there was no significant difference between the slopes of the two regression lines ($F = 0.2$; degrees of freedom = 1,4; $p > 0.1$).

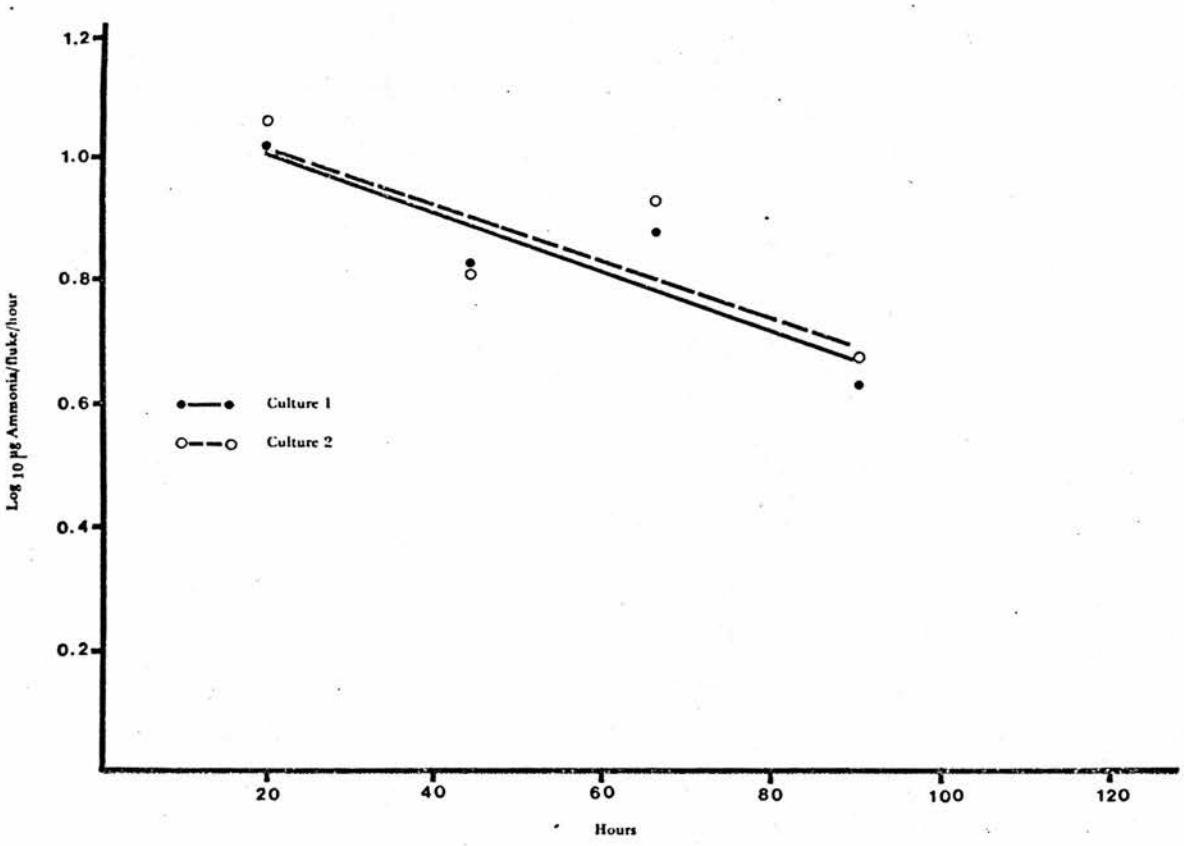

Fig. 6.1. Calculated least squares regression lines ($\log_{10}$ rate of ammonia production per fluke, against time) for culture lacking blood (Culture 1) and culture containing blood (Culture 2).

Culture 1 $y = -0.004x + 1.104$, $r = -0.90$.

Culture 2 $y = -0.005x + 1.121$, $r = -0.82$.

Discussion

The results of this experiment indicated that for the majority of the flukes the blood clot was a lesser attraction than the stimulus which made them migrate against the flow of the medium. The two flukes which remained in association with the blood clot were probably ingesting cellular material, but this had no apparent effect on either the overall amount of ammonia produced, or the rate of decline of ammonia output of the group, as compared to flukes maintained in a culture which lacked clotted blood.

CHAPTER SEVEN

THE INFLUENCE OF THE FLOW-RATE OF THE MEDIUM ON THE METABOLISM OF ADULT F. HEPATICA MAINTAINED IN VITRO

Introduction

Adult flukes within the bile ducts of the infected animal will be subjected to the natural flow of the bile and it was evident from all the previous experiments that the flow of the medium had an effect on fluke behaviour *in vitro*.

It is probable that the flow of medium over the flukes is of benefit to them, in that it provides a means of dispersing potentially toxic waste products of metabolism. It was therefore of interest to see whether the output of ammonia was influenced in any way by alterating the flow-rate of the medium.

Experimental design

Two sets of the continuous-flow apparatus were assembled. In order to achieve different flow-rates through the cultures, the tubing which passed over the pump was of diameter 0.5 mm in the case of the first culture and diameter 1.0 mm in the case of the second culture. The two sets of apparatus were sterilised and filled with medium consisting of 80% bovine serum/20% modified Earle's salts solution. Six flukes removed from a rabbit were placed in each culture, which were then run side-by-side. Ammonia assays were made at intervals on samples of the media withdrawn from the reservoirs and having passed over the flukes. The cultures were terminated after four days.

Results

The use of tubing of different diameters in the two cultures resulted in a flow-rate of approximately 5 ml per hour in the case of the first culture and approximately 12 ml per hour for the second culture.

All the flukes were alive and active at the end of the culture period. Individual ammonia assays are recorded in Appendix Table 7.1 and the calculated least squares regression lines ($\log_{10}$ rate of ammonia production per fluke, against time) for the two cultures are shown in Fig.7.1. It was evident that the quantity of ammonia produced per fluke was considerably higher in the case of the high flow-rate culture than in the slow flow-rate culture. Analysis of variance indicated that there was a significant difference between the intercepts of the two regression lines ($F = 42.7$; degrees of freedom = 1, 4; $p < 0.005$). It was also evident that the rate of decline of ammonia output for the two cultures was very similar, there being no significant difference between the slopes of the two lines ($F = 0.07$; degrees of freedom = 1, 4; $p > 0.1$).

Discussion

The results of this experiment indicated that adult flukes excrete greater amounts of ammonia when the medium in which they are contained is flowing at a higher rate.

It would seem probable that ammonia is toxic to F. hepatica, as it is to many other organisms. Organisms living in an aqueous environment are able to excrete nitrogenous wastes as ammonia without

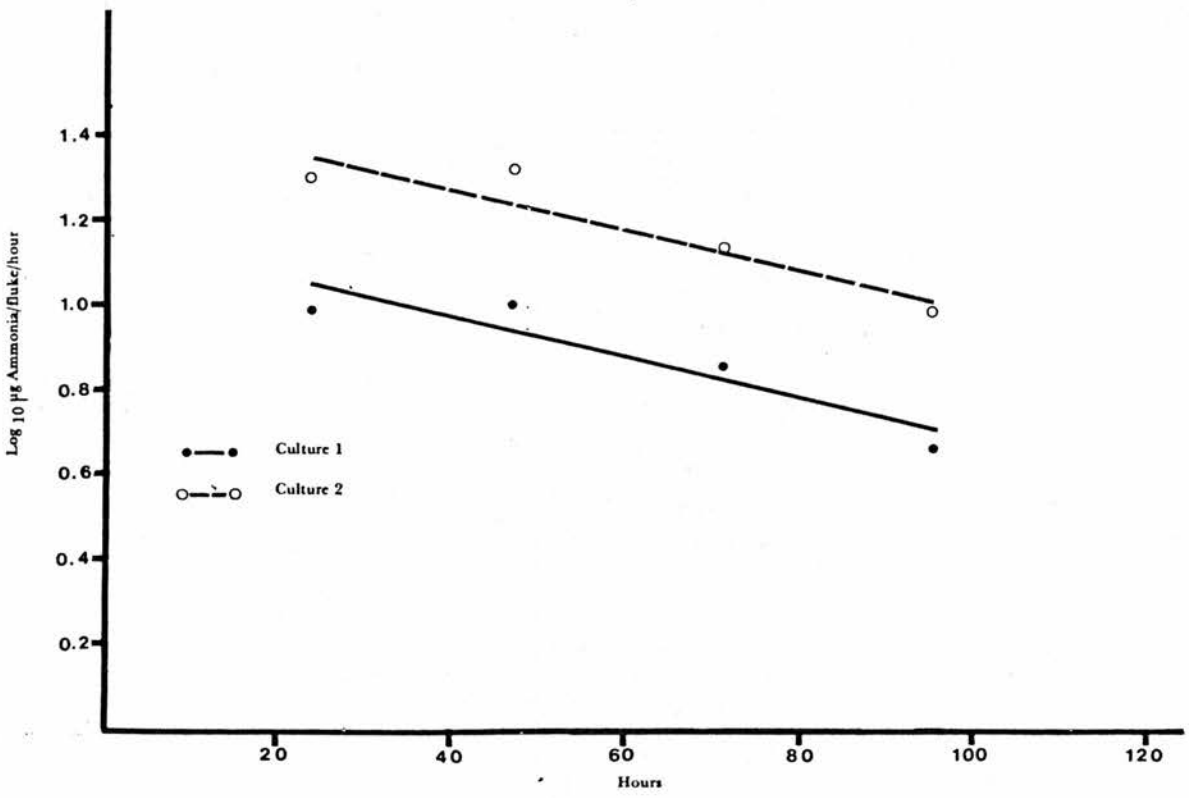

Fig. 7.1. Calculated least squares regression lines ($\log_{10}$ rate of ammonia production per fluke, against time) for slow flow-rate culture (Culture 1) and fast flow-rate culture (Culture 2).

Culture 1 $y = -0.005x + 1.159$, $r = -0.92$.

Culture 2 $y = -0.005x + 1.473$, $r = -0.94$.

suffering from toxic effects, since the ammonia is able to rapidly diffuse away from the immediate proximity of the organism. Undoubtedly, this dispersion of ammonia and any other potentially toxic waste products would be aided by the flow of medium in which the organism was located.

It might therefore be suggested that as the ammonia being produced by the flukes in the high flow-rate culture was being dispersed more rapidly, this allowed the preferential excretion of nitrogenous wastes as ammonia, rather than in other forms such as urea or uric acid. In order to verify this it would be necessary to make comparative studies on the proportions of the various end-products of protein metabolism produced by flukes maintained under different flow-rates.

Cavier and Savel (1954) made rather similar observations to the above, noting that Ascaris lumbricoides was able to alter the proportions of nitrogenous waste excreted as ammonia or urea, depending upon the nature of its environment. Thus, when maintained fully immersed in a relatively large volume of liquid, the organism excreted greater amounts of ammonia than urea, whereas when maintained in narrow bore tubing, surrounded by only a thin film of liquid, the position was reversed and urea formed the greater proportion of the excreted nitrogen. The authors concluded that this effect was due to the inability of ammonia to be adequately dispersed from the environment of the worm when it was contained within the narrow tubing, with the result that there was a subsequent preferential excretion of urea, which is less toxic than ammonia.

It would therefore seem that the use of a high flow-rate is a desirable feature of a system for the *in vitro* maintenance of adult *F. hepatica*, particularly to ensure the rapid dispersal of toxic waste products when large numbers of worms are confined within a relatively small volume of medium.

CHAPTER EIGHT

THE INFLUENCE OF CROWDING ON THE METABOLISM OF ADULT F. HEPATICA MAINTAINED IN VITRO

Introduction

As noted in the previous experiment, the dispersion of potentially toxic waste products from the vicinity of flukes maintained *in vitro* would be aided by the flow of the culture medium. However, it would seem possible that even in a flow culture, the effects of such toxic materials might be accentuated if a larger number of flukes were maintained in close proximity. The present experiment was designed to investigate the influence of crowding on the output of ammonia by adult flukes *in vitro*.

Experimental design

Two sets of the continuous-flow apparatus were assembled, sterilised and filled with medium consisting of 80% bovine serum/20% modified Earle's salts solution. Five flukes taken from a rabbit were placed in the first culture, whilst 23 similar flukes were placed in the second.

The two cultures were then run side-by-side at a flow-rate of approximately 8.0 ml per hour and ammonia assays were made at intervals on samples of media removed from the reservoirs and having passed over the flukes. The cultures were terminated after four days.

Results

All the flukes were alive and active at the end of the culture period. The individual ammonia assays are recorded in Appendix

Table 8.1 and the calculated least squares regression lines ($\log_{10}$ rate of ammonia production per fluke, against time) for the two cultures are shown in Fig.8.1.

It was clear that the initial output of ammonia was very similar in each culture, as was the subsequent decline in the rate of ammonia production, although the quantities of ammonia produced in the culture containing the smaller number of flukes were consistently higher than those produced in the other culture. Analysis of variance indicated that there was no significant differences between the intercepts or slopes of the two regression lines ($F = 5.56$; degrees of freedom = 1, 4; $p > 0.1$ for intercepts; $F = 1.44$; degrees of freedom = 1, 4; $p > 0.1$ for slopes).

Discussion

There was little evidence to suggest that the degree of crowding used in the present experiment had any influence on the rate of production of ammonia. However, it was anticipated that in later studies there would be a need to maintain very much larger numbers of flukes within the same culture and it is possible that under such circumstances adverse effects due to crowding would become more apparent. In the light of the evidence of the previous experiment, it would seem to be advisable to use a high flow-rate of medium so as to avoid such potential problems.

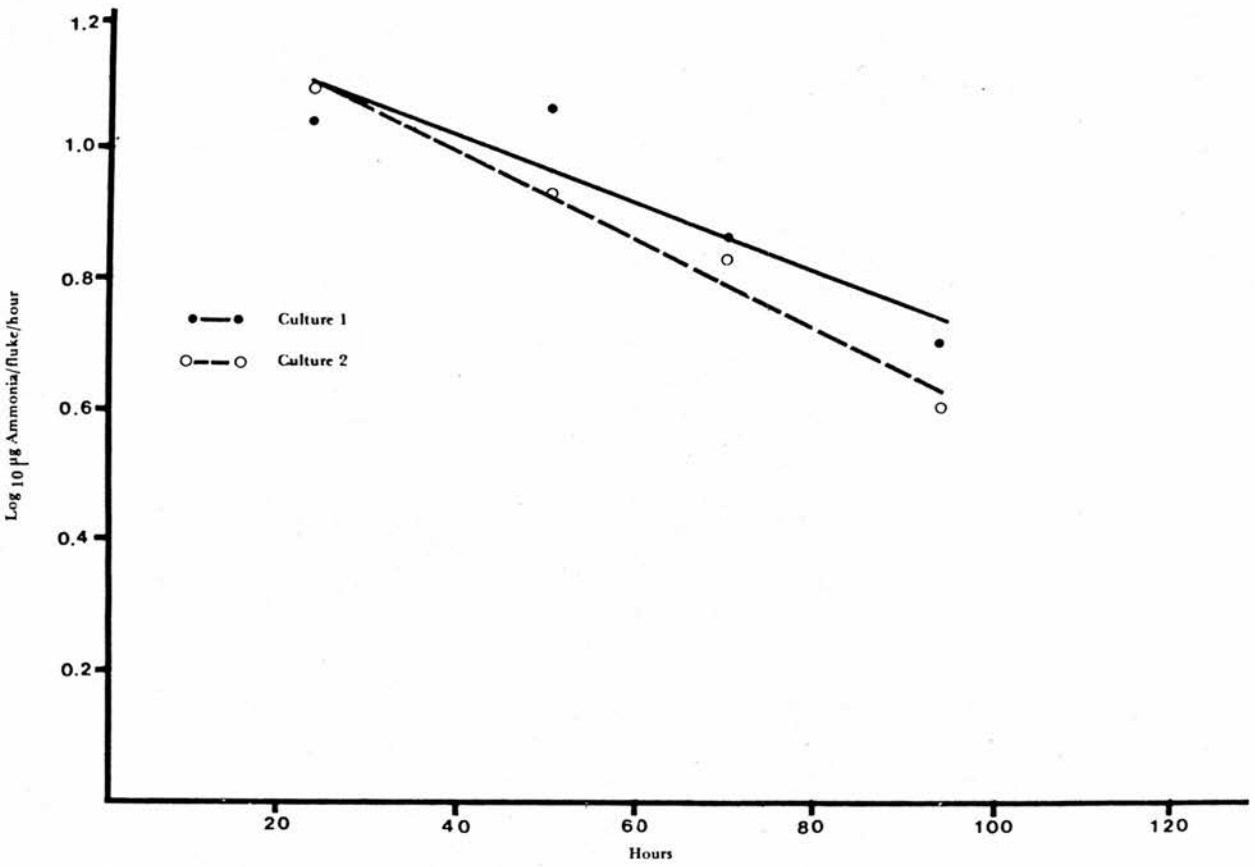

Fig. 8.1. Calculated least squares regression lines ($\log_{10}$ rate of ammonia production per fluke, against time) for culture containing 5 flukes (Culture 1) and culture containing 23 flukes (Culture 2).

Culture 1 $y = -0.005x + 1.222$, $r = -0.91$.

Culture 2 $y = -0.007x + 1.271$, $r = -0.99$.

CHAPTER NINE

EXPERIMENTS ON THE MAINTENANCE IN VITRO OF JUVENILE F. HEPATICA

Introduction

A series of experiments was undertaken to investigate the survival and growth of newly excysted F. hepatica under a variety of conditions.

Experimental design

In each of the experiments 40 newly excysted flukes were placed in each of a series of tissue culture flasks containing 5 ml of the medium under investigation. In order to avoid possible differences between different batches of metacercariae, the flukes used in each experiment were all derived from the same snail-shedding.

The flukes were incubated at 37°C under the appropriate gas phase. The media and gas phases studied are recorded in Table 9.1. In those cultures which lasted more than a few days, most of the medium was aseptically drawn off at four or five day intervals and replaced with a similar volume of fresh medium. Any flasks which showed evidence of bacterial or fungal contamination were immediately discarded.

Flukes were removed from individual flasks at intervals and the lengths and breadths of viable specimens measured, so as to establish the 50% survival period and extent of any growth under the prevailing conditions.

Experiment no.	Medium
1.	Earle's salts solution
2.	Whole sheep blood
3.	Calf serum
4.	Minimum Essential Medium (MEM) + 10% calf serum
5.	MEM + 50% rabbit liver extract + 10% calf serum
6.	MEM + 10% calf serum + Chang liver cell monolayer
7.	MEM + Chang liver cell monolayer
8.	MEM + 10% calf serum + Chang liver cell monolayer (under N_2)
9.	MEM + 10% calf serum + Chang liver cell monolayer (under 97.6% N_2/1.9% O_2/0.5% CO_2)

Table 9.1. Media used in experiments on the *in vitro* maintenance of juvenile *F. hepatica*.

In certain cases, flukes which had been maintained *in vitro* were injected intraperitoneally into mice, in order to see whether they were still capable of continuing their development *in vivo*. Newly excysted flukes were similarly injected into mice for comparative purposes.

Results

The measurements and survival periods of individual flukes in these experiments are recorded in Appendix Tables 9.1 - 9.8. The 50% survival times and mean fluke sizes (lengths x breadths) at the start and end of each experiment are shown in Table 9.2.

It was clear that the 50% survival period was limited to only two days when the flukes were maintained in Earle's salts solution and there was only a small increase in the mean size of the flukes during this time. The survival time could be greatly increased by the addition of blood, serum or liver extract to the medium. Flukes incubated in whole sheep blood showed clear evidence of ingestion of erythrocytes (Plate 9.1), but there was no significant increase in size during the seven day 50% survival period. However, small but statistically significant increases in size were noted for flukes maintained in calf serum, Minimum Essential Medium (MEM) supplemented with 10% calf serum or MEM supplemented with 50% liver extract and 10% calf serum.

The most prolonged survival period (in excess of 21 days) occurred when the flukes were maintained in a medium consisting of 90% MEM/10% calf serum over a monolayer of Chang liver cells. Under

Expt. no.	50% survival period (days)	Mean size ± S.D. ($\mu m^2 \times 10^3$):		t	Significance
		at start of culture	at end of culture		
1	2	24.36±4.39	22.43±4.13	2.79	$p > 0.1$
2	7	22.09±4.59	22.74±3.97	0.48	$p > 0.1$
3	10	22.13±4.37	26.80±3.79	3.62	$p < 0.002$
4	11	19.50±3.83	27.86±5.27	5.73	$p < 0.001$
5	8	21.66±4.96	27.47±2.32	4.76	$p < 0.001$
6	> 21	23.36±3.52	67.03±15.16	12.55	$p < 0.001$
7	5	21.71±2.91	20.36±3.52	1.32	$p > 0.1$
8	> 14	22.34±3.26	27.87±5.97	3.64	$p < 0.002$
9	> 20	18.66±3.40	71.25±18.86	12.29	$p < 0.001$

Table 9.2. 50% survival times and mean sizes of flukes at beginning and end of each experiment.

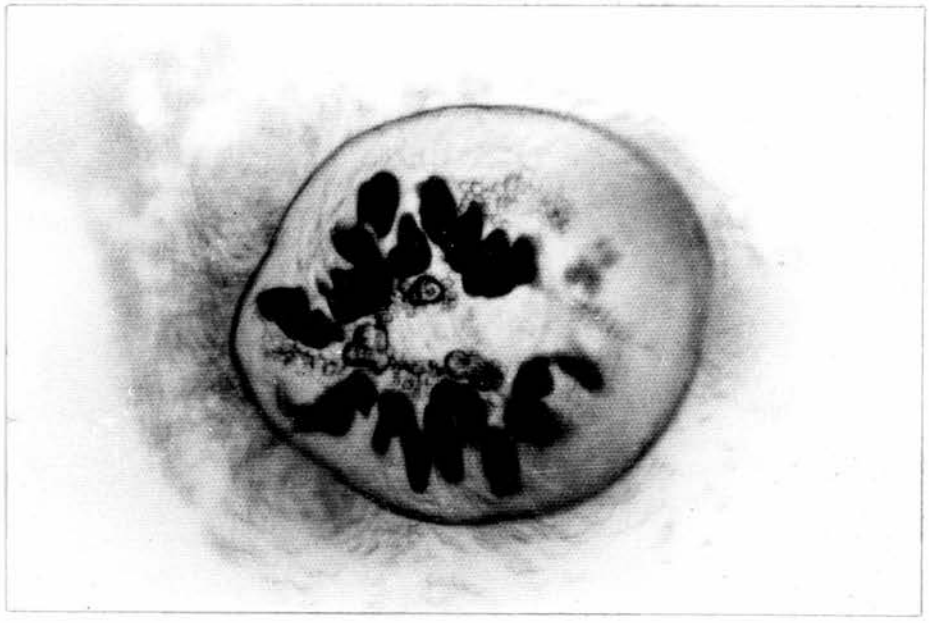

Plate 9.1. Juvenile F. hepatica maintained for seven days in whole sheep blood, demonstrating ingestion of erythrocytes. (x 70).
(c.f. Plate 14.12).

these conditions a much greater degree of growth was recorded, although examination of stained specimens revealed little evidence of organ development. Although fluke viability was still high at 21 days it appeared that the growth rate was declining (Fig.9.1). A gas phase of air or 97.6% nitrogen/1.9% oxygen/0.5% carbon dioxide (made by mixing nitrogen and 5% carbon dioxide in air at proportions of 9:1) gave virtually identical results when the flukes were maintained in 90% MEM/10% calf serum over a cell monolayer. However, an atmosphere of pure nitrogen appeared to inhibit growth, but not survival. The importance of serum in the medium was emphasised by the short survival period (five days) and absence of growth when flukes were maintained in MEM alone over a cell monolayer.

Flukes which had been incubated for 14 days in a medium consisting of 90% MEM/10% calf serum over a liver cell monolayer were able to continue their normal course of development in vivo following intraperitoneal injection into mice. Five such flukes were injected each of four mice, whilst four similar mice each received five newly excysted flukes. All the mice died between 18 and 23 days after the administration of the infection and a similar degree of liver damage was noted in each case. Live flukes of approximately the same size were recovered from all the mice.

Despite the care taken to set up axenic cultures, there was a fairly high failure rate in this respect. Evidence of bacterial or fungal contamination was generally apparent within 48 hours of setting up the cultures and it was noticeable that such contamination had a lethal effect on the young flukes.

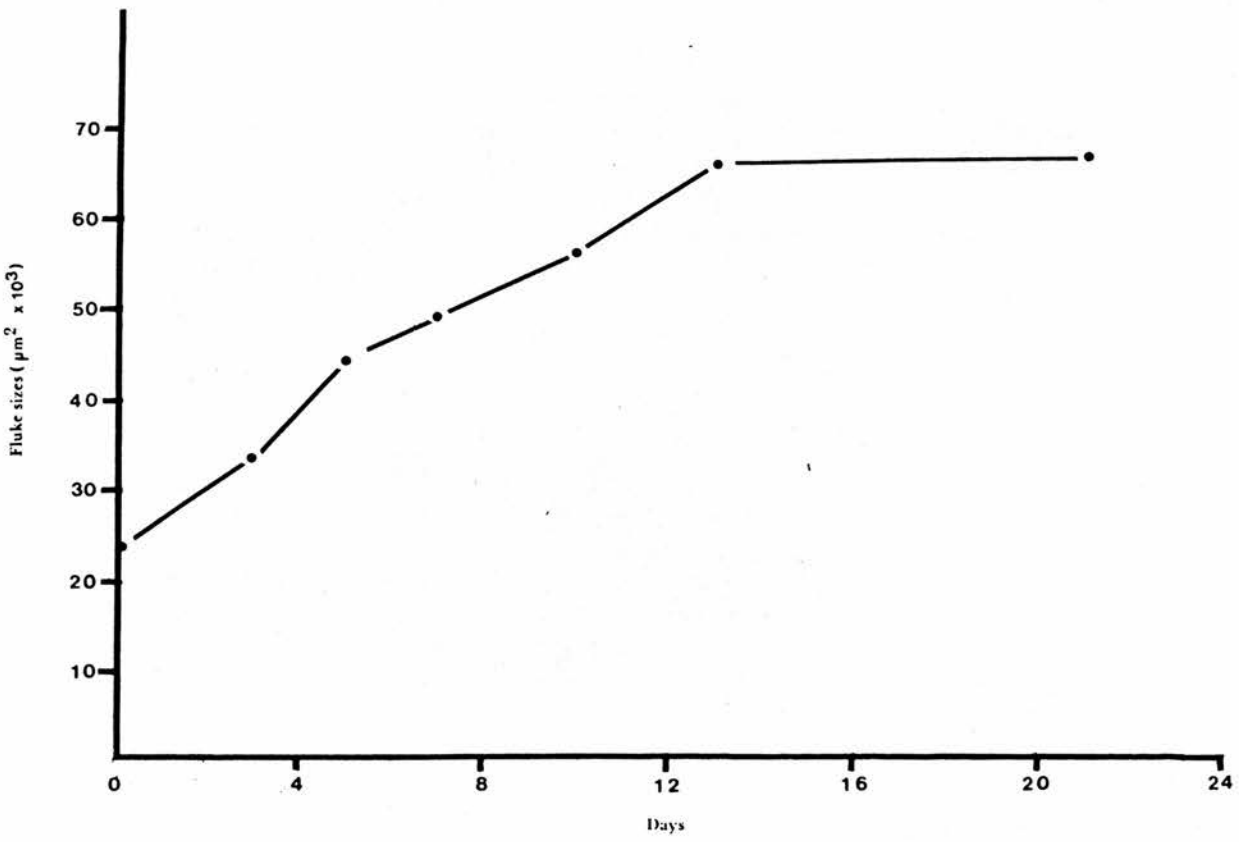

Fig. 9.1. Growth of immature F. hepatica maintained in 90% MEM / 10% calf serum over Chang liver cell monolayer.

Discussion

In general, the results of these experiments were similar to those of other workers who have studied the *in vitro* maintenance of juvenile *F. hepatica*, although there were some points of difference.

Wikerhauser and Cvetnić (1967) and Wikerhauser, Cvetnić and Brudnjak (1970) also found that the survival time of juvenile flukes was most prolonged when they were maintained over living cells. However, these workers were not able to demonstrate any increase in fluke size, whereas in the present experiments there were statistically significant increases in size, which were most marked when the flukes were maintained over cell monolayers. On the other hand, Osuna Carillo de Albornoz and Guevara Pozo (1973, 1974) recorded growth and development of flukes maintained in a medium containing serum and sheep erythrocytes, under an atmosphere of 10% carbon dioxide and 90% nitrogen. The present experiments clearly demonstrated that young flukes were ingesting erythrocytes, but there was no evidence of growth when the flukes were maintained in whole sheep blood, under an atmosphere of air. It is possible that these differences were the result of the use of different gas phases, since it was shown in the present series of experiments that fluke development could be influenced by the gas phase over the medium. In particular it appeared that an atmosphere of pure nitrogen was not favourable for fluke growth.

The present results were similar in most respects to those of Hanna, Baalawy and Jura (1975) and Hanna and Jura (1976), who found that juvenile *F. gigantica* could be maintained for prolonged periods

in media containing serum and cell monolayers and also reported growth under these conditions. In their experiments, as in the present ones, the growth rate *in vitro* declined after a period. These workers also noted that flukes which had been maintained *in vitro* for prolonged periods were still capable of continuing their normal development *in vivo*, following intraperitoneal injection into mice.

Although a degree of growth was recorded in some of the present experiments under the most favourable conditions, it should be noted that this compared very unfavourably with the growth rate expected *in vivo*. The data of Dawes (1962a) relating to the growth of juvenile flukes during normal development in the liver of the mouse indicated that after 21 days the mean size of the fluke had increased by a factor of 345, as compared to a factor of less than three in the present studies.

It would have been possible to have continued these studies, investigating numerous other media and culture conditions. However, in view of the unpromising results and the time-consuming nature of such experiments, further work was not undertaken.

It was apparent that the *in vitro* maintenance of juvenile flukes was most successful when the flukes were able to ingest living cells, as they would *in vivo*. Further studies within this field might therefore be concerned with methods of supplying the flukes with an adequately rich source of living cells, possibly by techniques such as three dimensional tissue culture or organ culture.

CHAPTER TEN

CONCLUSIONS AND DISCUSSION

The series of experiments on the *in vitro* maintenance of adult *F. hepatica* revealed a number of facts which were of general interest and some which were of particular significance to the intended application of this work to studies on the metabolic antigens of the parasite.

It was evident that it was possible to extract adult flukes from the bile ducts of the host animal and establish axenic cultures, even when the medium was of a type well suited to the growth of bacterial or fungal contaminants. On certain occasions, notably in the early stages of this work, cultures did become contaminated and although the results obtained from such cultures were always disregarded, it was noticeable that the contamination appeared to have little effect on fluke viability.

The continous-flow culture system provided a successful means of maintaining groups of adult flukes, high survival rates being noted in all experiments. Although long-term cultures were not attempted, the studies of Sewell (1968) had indicated that adult flukes could be successfully maintained in large groups for prolonged periods of time using a similar culture system. Although at first sight the continuous-flow apparatus appears to be complex and cumbersome, with practice it proved to be easy to assemble and reliable in operation. The use of the silicone sealing compound was of particular value in preventing leakages of medium during cultures.

Flukes showed 100% survival over a five day period when maintained in a basal saline solution (modified Earle's salts solution). However, it was evident that under these conditions there was a rapid decline in the production of ammonia by the flukes during the initial three days *in vitro*. It is probable that the flukes were utilising endogenous food reserves during this period and when these had been depleted the flukes were only able to maintain a low level of metabolic activity in this medium of strictly limited nutritive potential.

It was apparent that the ammonia was being produced as the result of active metabolic processes, since no ammonia was detected in a control culture containing dead flukes. Furthermore, there was no evidence of bacterial or fungal contamination of the medium, which might possibly have contributed to the accumulation of ammonia.

In marked contrast, when the medium was supplemented with increasing amounts of serum, the rate of production of ammonia by the flukes was maintained at a level more closely approaching that which was occurring in the initial period after their removal from the host. It was clear that the beneficial effects of the serum were directly related to the percentage of serum in the medium. A major conclusion of this work must therefore be that for optimal maintenance of adult flukes *in vitro*, the medium should be supplemented with a high proportion of serum.

It was envisaged that in later studies on the metabolic antigens of adult *F. hepatica* there would be a need to maintain the flukes in

a protein-free medium. The present studies provided some evidence that the multi-component tissue culture medium, Medium 199, was more favourable for this purpose than modified Earle's salts, although Medium 199 was clearly less suitable than those media which had been supplemented with high proportions of serum.

In contrast to the findings of Stephenson (1947b) who reported that flukes in a stationary culture were actively attracted to clotted blood, in the present studies it appeared that the stimulus causing the flukes to migrate against the flow of the medium was stronger than the attraction of the blood.

It was shown that a high flow-rate of medium over the flukes was associated with the production of significantly greater amounts of ammonia, although the subsequent decline in the rate of output was not altered. It was nevertheless concluded that a high flow-rate of the medium is a desirable feature of a system for the in vitro maintenance of adult F. hepatica, to ensure the rapid dispersal of potentially toxic waste products of metabolism. However, an experiment designed to study the effects of crowding of flukes in vitro failed to demonstrate any adverse effects, although the numbers of flukes used were not very great. It was anticipated that in later studies on the metabolic antigens of adult flukes there would be a need to maintain very much larger groups of flukes together, under which circumstances adverse effects resulting from crowding might become more apparent unless high flow-rates were used. The use of high flow-rates with the type of culture apparatus used in the present studies would introduce problems if the cultures were prolonged,

since large volumes of medium would be required. For this reason, in later studies use was made of a modified form of the apparatus, in which the medium was recirculated over the flukes.

The use of the measurement of ammonia production as an indicator of the suitability of various media and culture conditions for the maintenance of adult flukes proved to be successful and fulfilled a number of criteria laid down by Bell and Smyth (1958) for methods of assessing the performance of trematodes in vitro. In particular, ammonia assay was able to indicate the potential value of different media and conditions within a relatively short period, thus avoiding the necessity of undertaking prolonged experiments. In addition, ammonia assay is precise, repeatable, easy to perform and does not require the death of the flukes or their removal from the culture for the measurement to be made. However, it should be noted that ammonia production is only reflecting one aspect of the overall metabolism of the organism and there can be little doubt that for a more complete indication of fluke metabolism in vitro it would be necessary to measure the output of other end-products of both carbohydrate and protein metabolism.

It would have been possible to have continued these studies, investigating numerous other media and culture conditions. However, it was not the primary aim of this work to undertake a detailed investigation of fluke metabolism in vitro but rather to establish a successful routine method of maintaining large numbers of adult flukes in vitro, so as to facilitate subsequent studies on their metabolic products. In this respect, this series of experiments can be

considered to have been successful.

The series of experiments on the *in vitro* maintenance of juvenile flukes from the metacercarial stage revealed little that was not already known, although the results differed in certain details from those of other workers who have studied the same subject.

It was clear that the survival period and growth were maximal when the young flukes were able to feed on monolayers of living cells. However, even under the most favourable conditions, growth was strictly limited and compared very unfavourably with that expected *in vivo*. It was also evident that, as showed by Hanna and Jura (1976) using *F. gigantica*, the young flukes maintained for prolonged periods *in vitro* were not fundamentally damaged by the culture procedure, but were only retarded, since they were able to continue their normal course of development following intraperitoneal injection into mice. This suggests that the *in vitro* environment was not deleterious *per se*, but was lacking some factor or factors necessary for the continued maturation of the organism.

Again the primary objective of these studies on the maintenance of juvenile flukes *in vitro* was to try and establish a routine method for maintaining large numbers of the young parasites in an actively metabolising and, hopefully, growing state. In view of the limited success achieved, the experiments must be considered a failure in this respect. There is no doubt that the highly complex physical environment and nutritive requirements of juvenile *F. hepatica* *in vivo*

are extremely difficult to simulate *in vitro* and the present results merely serve to underline this observation.

SECTION TWO

PRODUCTION AND PROPERTIES OF THE METABOLIC ANTIGENS OF FASCIOLA HEPATICA

CHAPTER ELEVEN

REVIEW OF THE LITERATURE

Introduction

It is now well established that in many instances an animal which has suffered an infection with a helminth parasite may develop a degree of resistance to reinfection with that same species of helminth at a later date. Whilst this resistance may be the result of a variety of factors, there is ample evidence that a specific immunological response by the host animal is often of major significance. It is apparent that, as in other infectious diseases, the animal is able to respond to the primary infection by production of appropriate antibodies or cellular responses which may protect it to a greater or lesser degree against a subsequent challenge infection.

It has also become apparent that immunisation of the host animal with antigens derived from the tissues of the helminth (somatic antigens) will provoke an antibody response, but this response does not generally provide significant protection against a challenge infection. Soulsby (1958) stated that this phenomenon of failure of somatic antigens to provoke protective immunity is one of the outstanding aspects of helminth immunology, in that the conventional bacteriological techniques for producing artificial immunity are not applicable.

In an organism as biologically complex as a helminth parasite there are likely to be many substances with antigenic potential. Oliver-Gonzáles (1946) described helminth antigens as being either

'functional' or 'non-functional' with regard to their ability to stimulate protective immunity in the host animal. Since there is apparently a need for the host to be exposed to the living parasite before protective immunity can be initiated, it has been suggested that substances produced by the living parasite and liberated into its environment may play an important role in the immunological aspects of the host-parasite relationship. The terms 'metabolic antigens' or 'excretory-secretory antigens' have come into use to describe such immunologically active substances and the theories as to their significance can now be supported by substantial experimental evidence.

Very little work has been undertaken on metabolic antigens as compared to that relating to somatic antigens. This is of course a direct consequence of the difficulty in obtaining sufficient quantities of the metabolic antigens to allow for extensive experimental investigations. Due to the inaccessibility of most parasitic helminths *in vivo*, most of the studies have made use of metabolic antigens derived from helminths maintained *in vitro*. It is therefore apparent that successful *in vitro* maintenance of a parasite would greatly facilitate studies on its metabolic antigens. Weinstein (1958) pointed out the desirability of maintaining parasites under conditions which would allow them to maintain their normal metabolic activities, so that metabolic antigens obtained *in vitro* would accurately reflect those produced *in vivo*. Consequently, most of the investigations into the nature of helminth metabolic antigens have been undertaken using those nematode species for which adequate *in vitro* systems have been developed.

A number of workers have demonstrated antigenic activity in the 'exsheathing fluid' of larval nematodes (e.g. Soulsby, Sommerville and Stewart, 1959; Soulsby and Stewart, 1960), which may also be considered to fall within the definition of metabolic antigens.

Evidence for the existence of metabolic antigens

The first evidence for the antigenic nature of substances liberated by living helminths arose from the observations of Sarles (1937, 1938) that when Nippostrongylus muris was incubated in serum taken from an animal which had been infected with that species, there was precipitate formation about the parasite, particularly at the natural body orifices. Oliver-Gonzáles (1940) using Trichinella spiralis, Otto (1940) using Ancylostoma caninum, Otto, Schugam and Groover (1942) using Necator americanus and subsequent workers have confirmed these findings. Sarles and Taliaferro (1936) and Taliaferro and Sarles (1939) also noted the presence of precipitates around Nippostrongylus muris in vivo. These early workers assumed that such precipitates were the result of reaction between antigens produced by the helminth and antibodies in the serum. This theory was confirmed by Jackson and Lewert (1957), who showed by fluorescent antibody staining that the precipitates around Trichinella spiralis resulted from a specific antigen-antibody complex.

Otto (1940) noted that incubation of larvae of Ancylostoma caninum in immune serum reduced their subsequent infectivity. Mauss (1940) confirmed this using Trichinella spiralis, whilst Thorson (1954) showed that the inhibition of larvae of Nippostrongylus muris incubated in immune serum could be removed by washing the

larvae.

Culbertson (1941) suggested that the precipitates acted by mechanically blocking the orifices of the parasite, thus interfering with normal metabolism, but Soulsby (1957a) thought that the reactions were more complex and that the antibodies were penetrating the helminth to interfere with metabolic processes. This theory was supported by the finding of Schwabe (1957), who showed that the oxygen consumption of Nippostrongylus muris fell rapidly on incubation in immune serum, even before precipitates had formed. He suggested that the worm enzyme systems were being antagonised by the antibodies, which must be actually entering the parasite to reach the site of enzyme activity.

Chandler (1953) suggested that anti-parasite immunity is anti-enzymic in nature, which would explain why immunisation with somatic antigens generally fails to induce protective immunity, as there would be few residual enzymes in a somatic extract. Thorson (1953) demonstrated a lipolytic factor in the metabolic products of Nippostrongylus muris, which could be inhibited by immune serum. Thorson (1956a) also showed that the secretions from the oesophageal glands of hookworms contained proteolytic enzymes, whose activity could also be inhibited by immune serum. Thorson (1956b) further showed that an extract of these glands could provoke a degree of protective immunity in the canine host. Tran Van Ky, Vaucelle, Capron and Vernes (1967) used immunoelectrophoresis together with enzyme characterisation to demonstrate 12 different antigen-antibody

complexes with enzymic properties in Fasciola hepatica.

There are few reports on studies on the metabolic antigens of F. hepatica. Wikerhauser (1961a) demonstrated precipitate formation about newly excysted flukes incubated in immune bovine and rabbit sera. The precipitates were most marked around the oral cavity and the excretory pore, but were not seen around empty cysts. Wikerhauser demonstrated that such precipitates did not affect the in vitro survival of the flukes, to any marked extent. Precipitates did not form if the infected serum was first incubated with a metabolic antigen derived from adult flukes. It was shown that exposure of newly excysted flukes to immune bovine serum prior to intraperitoneal injection into guinea pigs resulted in a lighter infection than did exposure to normal serum. A similar effect was seen if young flukes were injected intraperitoneally together with immune serum.

Ruther (1963), cited by Geyer (1967) saw precipitates around young flukes taken from mice 22 days after infection and then incubated in immune rabbit serum. The flukes died after a short period of incubation in immune serum, but did not die so soon in normal serum. The same author noted that if metacercariae were incubated in immune serum prior to infection, there was a significant suppression of infectivity. However, Ruther was unable to detect precipitates in young flukes migrating in animals which were strongly immune to infection. It was suggested that the relatively rapid rate of migration might prevent the formation of stable precipitates.

Lang (1974b) showed that incubation of 12, 16, 18, 20 and 24-day -old flukes in immune serum for four hours followed by intraperitoneal transfer to normal mice resulted in a significantly reduced worm burden as compared to similar incubation in normal or heat-treated immune serum. Lang (1976) further demonstrated that incubation of 16-day-old flukes in serum from a 25-day-old infection, or in serum from mice immunised with metabolic antigens would reduce their infectivity, whereas incubation in serum from a 100-day-old infection, or in serum raised against a somatic fluke antigen would not.

In recent years, the technique of labelling antibodies with fluorescent markers has made it possible to identify the physical site of antigens within the tissues of a helminth. Thus, if anti-fluke antibodies are labelled and then exposed to histological sections of flukes, they will bind to their homologous antigen and indicate its location. Such studies have been undertaken by a number of workers and have indicated that the antigens which are active during the course of the infection are associated with tissues which have excretory or secretory functions. This provides strong support for the theory that metabolic antigens are of major importance in the immunology of fascioliasis.

Thus, Thorpe (1965) described the distribution of antigen in F. hepatica at different stages of its development. He reported that the sites of specific staining in both immature and mature flukes were those from which effete cells and metabolic products were likely to be lost into the environment. Positively stained material was also noted in fluke tracts in the liver, indicating that antigens had

been shed by the migrating flukes. Two and four week old flukes showed fluorescence in the caecal lining, cuticle and excretory ducts. Adult flukes showed strongest staining in the caecal lining and excretory ducts and also in the lining of the uterus and the Mehlis gland.

Movsesijan, Sokolić and Sibalić (1967) used the fluorescent antibody technique to diagnose fascioliasis and Movsesijan and Čuperlović (1970) demonstrated antigenic activity in the surface of the epithelial cell lining of the caecae, the surface of the uterus, excretory ducts, spermatogenic cells and also in the caecal contents. The most specific fluorescence was located in the caecal lining, which they suggested indicated that some specific excretory substances had antigenic activity. Movsesijan, Jovanović, Aalund and Nansen (1975) used an indirect fluorescence test to follow the serological response in experimentally infected lambs. They stated that the antigens active in the infection were those associated with the gut epithelium and the reproductive tract.

Čuperlović, Movsesijan and Lalić (1974) reported that in young flukes antigen was located on the inner surface of the digestive tract, the caecal epithelium and on the cuticle. In adults, the antigens were located in the caecal epithelium, caecal contents and the reproductive and excretory systems. The specific fluorescence of the caecal epithelium and caecal contents disappeared or diminished after absorption of the serum with a metabolic antigen derived from cultured flukes.

Thus there is substantial evidence that antigenic substances produced by the living fluke and excreted or secreted into its environment are of significance in the immunology of fascioliasis. However, as yet, there have been few investigations into the nature of such substances.

Sewell (1968) studied the metabolic products obtained by the incubation of adult flukes in vitro. The culture medium was greatly concentrated and the serologically active components were then studied by immunodiffusion techniques. It was noted that different protein components of the metabolic products reacted with sera from different species, whilst the non-protein components would react with infected sheep or bovine sera, but not with infected rabbit serum. These non-protein elements were found to be irreversibly absorbed by polysaccharide anion exchangers. None of the serologically active components would absorb out the antibodies concerned in the formation of precipitates around newly excysted flukes incubated in infected serum.

Čuperlović (1972) and Čuperlović and Lalić (1972) described a method for fractionating and purifying a metabolic antigen derived from adult flukes. They collected the antigen liberated by flukes which were maintained for six hours in saline and compared them with somatic antigen (extract of whole fluke) and with bile from infected animals. They found immunological identity between these three types of antigen on the basis of gel precipitation. The three antigenic groups were fractionated on Sephadex G-100 and the active fractions identified. The flukes used were taken from infected sheep and

sheep serum proteins were identified in both the metabolic and somatic antigens. Rabbits were immunised with the metabolic antigen and responded with specific antibodies. These workers also used the metabolic antigen to follow the immune response which developed in experimentally infected and immunised rabbits and sheep. The complement fixation test was found to give good results. It was found that immunisation of rabbits with a saline fluke extract led to the production of antibodies, which would react with the metabolic antigen in the agar gel precipitin test and that lines of identity were shown by infected and immunised sera against the metabolic antigen.

Čuperlović and Movsesijan (1972) demonstrated that the antigen fraction which showed greatest activity with infected rabbit serum was protein in nature.

Čuperlović (1975) incubated adult flukes in saline, which contained radioactively labelled methionine and lysine. He showed that the flukes took up these amino acids and incorporated them into tissue proteins. In addition, it was shown that antigenic metabolites excreted by the flukes into the medium during a relatively short incubation period were also labelled. This finding is of great significance, since it provides evidence that the antigens produced by flukes <u>in vitro</u> are originating from active metabolic processes, rather than from mere degeneration of existing tissues.

<u>The use of metabolic antigens as immunogens</u>

There is now substantial evidence that helminth metabolic

antigens, when injected into the host animal, may stimulate a degree of protective immunity to subsequent challenge by that same species of parasite.

Most of the evidence has arisen from experiments which have utilised metabolic antigens derived from nematodes, for which adequate in vitro culture methods have been developed. There is also some evidence that metabolic antigens derived from cultured cestodes and trematodes can provoke a protective immune response.

A more detailed discussion of this topic is included in the Review of the Literature in Section 3.

The use of metabolic antigens in the immunodiagnosis of helminth infections.

To date, many attempts to diagnose helminth infections in the field by immunological methods have proved to be unsatisfactory due to the prevalence of false results, non-specific reactions and cross-reactions between different helminth species. It has long been recognised that a complex organism such as a helminth parasite will contain a large number of antigenic components, only few of which are likely to be species-specific. A great deal of the work within the field of the immunodiagnosis of helminth infections has been directed towards the search for such species-specific antigens, in the hope that they will facilitate the development of more accurate and reliable methods of diagnosis. Most of these studies have involved the use of somatic antigens, whilst comparatively few have involved metabolic antigens. The main indication for the use of metabolic antigens would

be if they were shown to be more species-specific than somatic antigens, but there has been little experimental evidence of this as yet.

Once again, most use has been made of metabolic antigens derived from helminths for which adequate *in vitro* methods have been developed. Thus Sprent (1951) used metabolic antigens derived from *Ascaris* to demonstrate anaphylaxis in guinea pigs infected with that genus. Sadun and Norman (1957) used metabolic antigens of *Trichinella spiralis* in a flocculation test, concluding that they conferred greater sensitivity in the test than did somatic antigens.

Several groups of workers have studied the role of metabolic antigens of schistosomes in immunodiagnostic tests. Kagan and Oliver-Gonzáles (1958) obtained promising results with metabolic antigens from adults and cercariae of *Schistosoma mansoni* in the haemagglutination test, whilst Minning, Newsome and Robinson (1958) used the complement fixation test. Sadun, Lin and Walton (1959) found no particular advantage of metabolic antigen over somatic antigen in the complement fixation or intradermal tests. On the other hand, both Anderson, Sadun, Rosen, Weinstein and Sawyer (1962) and Oliver-Gonzáles and Levine (1962) claimed that metabolic antigens were superior to somatic antigens for the diagnosis of *Angiostrongylus cantonensis* and *Trichinella spiralis* infections respectively.

The vast majority of reports on the immunodiagnosis of fascioliasis have been concerned with the use of somatic rather than metabolic antigens, and the literature on the subject is extremely

extensive. It is not intended to undertake a full discussion on the use of somatic antigens here, since reviews by Pantelouris (1965), Geyer (1967), Sinclair (1967a) and Platzer (1970) are available for more detailed information on the topic.

Briefly, an examination of the literature on the use of somatic antigens reveals that a great variety of different antigens have been applied to a wide range of immunodiagnostic techniques. Many of the results have been contradictory and few have been claimed to be wholly satisfactory. Problems associated with non-specific effects, false results and cross-reactions between different species of helminths have been noted in many instances. The fact that no single type of antigen or immunodiagnostic test has found universal favour may be taken as an indication that further investigations are justifiable.

To date, the few reports on the use of metabolic antigens for the immunodiagnosis of fascioliasis have not indicated any marked benefit over the use of somatic antigens. Minning, Newsome and Robinson (1958) collected antigens from adult flukes maintained in Stephenson's medium and found that they would react in the complement fixation test with sera from patients infected with F. hepatica, with no cross-reaction with Schistosoma mansoni. Masu (1955) and Wikerhauser and Bartulić (1961) successfully used metabolic antigens in the intradermal test. Paris and Capron (1962) found no cross-reaction in gel diffusion tests between sera from patients suffering from infection with F. hepatica, Schistosoma, Taenia,

Ascaris, Onchocerca or Dracuncula species using a metabolic antigen derived from adult F. hepatica.

Gundlach (1971) compared metabolic with somatic fluke antigens for the immunodiagnosis of experimental fascioliasis in rabbits, using the complement fixation test, haemagglutination, gel precipitation and immunoelectrophoresis. Metabolic antigen was found to give the better results during the prepatent period of the infection, whereas during the patent phase, somatic antigen was more effective.

Čuperlović (1972) and Čuperlović and Lalić (1972) made more detailed studies on metabolic antigens derived from adult flukes. The antigens were purified and fractionated by gel filtration and used to follow the serological response in experimentally infected rabbits and sheep and in rabbits immunised with fluke extract. It was shown that the metabolic antigen had no anti-complementary effect and, after linking to the appropriate antibody, had a high potency for fixing complement. A response was first detected by 7 days after infection of rabbits, and by 14 days for sheep.

CHAPTER TWELVE

MATERIALS AND METHODS

Recirculating continuous-flow apparatus for the production of metabolic antigens

This apparatus, which is shown in Plate 12.1 was a modification of the continuous-flow system described in Section 1.

Since greater numbers of flukes were to be used in these experiments, the length of the nylon sleeving, in which the flukes were contained, was increased to 90 cm from the original 30 cm. The medium, having passed over the flukes, was returned to the aspirator by way of a length of silicone tubing (W), rather than being collected in a flask as in the previous work.

The sterilisation of the apparatus and the introduction of the medium and flukes were performed as previously described.

Preparation of metabolic antigen

Flukes were generally maintained for approximately seven days in a recirculating continuous-flow culture system containing 2 l of medium. At the end of this period, the medium was removed and filtered through a 142 mm 0.22 μm membrane filter (Millipore U.K. Ltd., London) to remove particulate debris such as fluke eggs. The medium was then concentrated by ultrafiltration through a 'Pellicon' membrane (Millipore U.K. Ltd., London), which was contained within a 142 mm 'Hi-Flux U-F Cell' system (Millipore U.K. Ltd., London). A

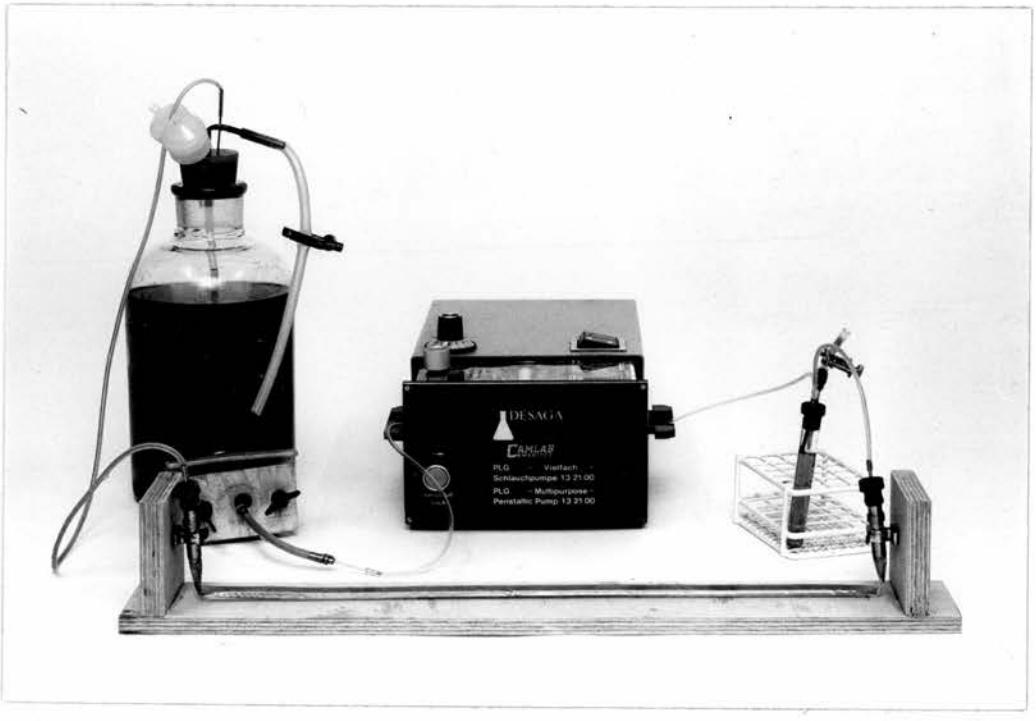

Plate 12.1. Recirculating continuous-flow culture system.

type PSAC membrane, with molecular weight 'cut-off' of 5,000 was used. The apparatus was assembled according to the manufacturer's instructions and the medium placed in the cell, which was then connected to a source of nitrogen at a pressure of approximately 345 kPa. The gas pressure forced molecules with molecular weights less than 5,000 through the membrane, whilst larger molecules were retained within the cell. The volume of the medium was thus reduced to approximately 50 ml. The volume was reduced further by dialysis against polyethylene glycol 6000 at a concentration of approximately 50% in distilled water.

The concentrated medium was then dialysed extensively against phosphate buffered saline pH 7.4 (Oxoid Ltd., London) so as to remove any residual small molecules from the culture medium, as these were found to interfere with subsequent protein estimations. The dialysed concentrate was designated 'whole metabolic antigen' and was stored at -20°C.

Preparation of the non-protein component of the metabolic antigen

Protein was removed from the crude antigen by boiling for 10 minutes or by precipitation with trichloracetic acid.

Trichloracetic acid was added to the antigen solution to a final concentration of 0.25 M. After standing for 30 minutes at 4°C the solution was centrifuged at 2,500 g for 10 minutes. The supernatant was removed and the precipitation procedure repeated. The pH of the final supernatant was restored to approximately 7.0 by

titration with 2N sodium hydroxide. It was found that a certain amount of the non-protein antigen was carried down with the precipitate on each occasion and this was partially recovered by dissolving the precipitates in saline and reprecipitating with trichloracetic acid on two occasions.

Collection of blood and serum

Blood was collected from the peripheral ear vein of rabbits and from the jugular vein in the case of sheep and cattle, using the standard techniques as described by Archer (1965).

Rats were bled from the tail veins. The animal was lightly anaesthetised with ether and the tail smeared with petroleum jelly. A small transverse scalpel incision was then made across the ventral aspect of the tail and the animal placed in a large filter funnel, with the tail extending down the neck of the funnel. The funnel was then supported in a large conical flask. A short length of silicone tubing was attached to the end of the neck of the funnel and the blood which dripped from the tail wound was collected into a suitable vial, which was inserted into the free end of the silicone tubing.

Whole blood for haematological studies was collected into 2.5 ml plastic vials containing E.D.T.A. as anti-coagulant (Becton, Dickinson U.K. Ltd., Wembley, Middlesex). Plasma was obtained by centrifugation of the whole blood sample at 2,500 g for 15 minutes.

Serum was prepared by similar centrifugation of clotted blood,

which had been collected into plain plastic vials.

Both serum and plasma were stored in plastic vials at -20°C until required.

Protein estimation

Protein estimation was routinely performed using the Folin-Ciocalteau method, as described by Dawson, Elliott, Elliott and Jones (1969). 'Lab Trol' (Dade Division American Hospital Supply Corporation, Miami, Florida) was used as the protein standard.

Somatic fluke antigen

Adult flukes were collected from the bile ducts of experimentally infected animals. After washing in normal saline to remove excess bile, the flukes were placed in a petri dish and cut into small pieces with a scalpel. A universal bottle was then filled to a depth of 1 cm with the macerated flukes, to which was added approximately 5 volumes of phosphate buffered saline (Oxoid Ltd., London). The mixture was then homogenised for three minutes with a mechanical laboratory homogeniser and allowed to stand overnight at 4°C.

After centrifugation at 2,500 g for 30 minutes, the supernatant fluid constituted the somatic fluke antigen. The antigen solution was passed through a 0.22 μm filter to remove particulate material and was then stored at -20°C until required.

Immunodiffusion techniques

The agar used in all the immunodiffusion techniques was made up as a 1% solution in barbiturate buffer. Concentrated buffer was made by dissolving 5.75 g diethyl barbituric acid and 3.75 g sodium barbitone in approximately 500 ml of hot distilled water, the solution then being made up to 2 l with distilled water. Five grams of agar ('Oxoid no.2 Ionagar', Oxoid Ltd., London), 0.5 g sodium azide and 50 g sodium chloride were placed in a dry 500 ml bottle, to which was added 500 ml of a mixture comprised of 100 ml of the concentrated buffer and 400 ml distilled water. The bottle was placed in a boiling water bath, in order to dissolve the agar and the molten agar was then dispensed into universal bottles.

Double immunodiffusion tests were performed either on a macro- or micro-scale. In the macro-system approximately 5 ml of molten agar was pipetted into a 60 mm plastic petri dish (Becton, Dickinson U.K. Ltd., Wembley, Middlesex). 5 mm diameter wells were cut with a metal tube and the plugs of agar removed with a needle.

The micro-test was performed on microscope slides, using the modified L.K.B. apparatus (L.K.B. Instruments Ltd., South Croydon, Surrey) as described by Sewell (1966).

Semi-quantitative immunodiffusion techniques

The serological response of infected and immunised animals was routinely monitored by double immunodiffusion. Doubling dilutions of the serum in normal saline were prepared and placed in the outer wells

of a hexagonal pattern cut in agar, using the micro-system described by Sewell (1966). Antigen was placed in the central well and the titre was taken as being the highest serum dilution which gave a visible precipitin line after 24 hours incubation.

For immunoadsorbtion studies and to quantify the production of metabolic antigen the semi-quantitative technique described by Sewell (1967b) was used, as this allows more precise determination of the dilution of antigen which will completely react with an equal volume of antibody (or vice versa).

Two parallel rows of wells were cut in the agar, using the micro-immunodiffusion system. Into each well of the upper row was placed a mixture consisting of equal volumes of different dilutions of antigen and serum (see Fig.12.1). In the lower row of wells were placed alternately plain antigen and serum. On incubation, precipitin lines develop as shown in Fig.12.2. If either antigen or antibody is in excess in the upper wells, the line is deflected accordingly by the reagents in the lower wells. Where the antigen-serum mixture in the upper wells is such that reaction is complete and neither is in excess, the precipitin line is not deflected (Well no.3, Fig.12.2). This is the end point of the assay.

Immunoadsorption studies

The semi-quantitative immunodiffusion technique of Sewell (1967b), as described above was used in immunoadsorption studies to compare the antibody constituents of different sera, each capable of reacting with a particular antigen.

	Tube number				
	1	2	3	4	5
Antigen diln.	$\frac{1}{4}$	$\frac{1}{2}$	1	1	1
Antigen vol.	1	1	1	1	1
Serum diln.	1	1	1	$\frac{1}{2}$	$\frac{1}{4}$
Serum vol.	1	1	1	1	1
Total vol. of antigen/serum mix.	2	2	2	2	2

Fig.12.1 Preparation of dilutions of antigen and antiserum, which are then mixed in equal volumes for use in the semi-quantitative immunodiffusion technique.

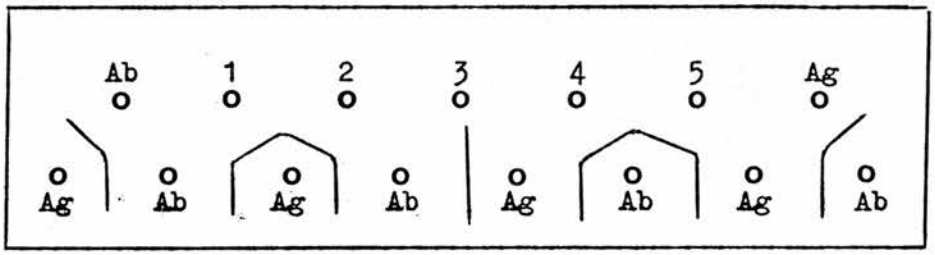

Fig.12.2. Well pattern for semi-quantitative immunodiffusion technique. Ag = plain antigen; Ab = plain antiserum; Antigen/serum mixtures from Tubes 1-5 (Fig.12.1) are placed in wells 1-5 of the upper row. The undeflected precipitin line at well 3 indicates that the mixture in that well is at optimal proportions for complete reaction, with neither reagent in excess.

For example, to compare the antibodies present in two sera A and B, the concentration was determined at which each would react completely with an equal volume of the antigen, so as to leave no residual antigen activity against the same serum.

Such a mixture of serum A with the antigen was then tested by immunodiffusion against serum B. If the two sera contain the same range of antibodies, no reaction will be seen between the adsorbed antigen and serum B. However if serum B contains a different range of antibodies to serum A, a reaction will occur. Similarly, the mixture of serum B with the antigen was tested against serum A. Controls incorporating saline in place of the serum in each of the mixtures were included in each case, to ensure that any differences noted were not merely the result of dilution of the reagents.

The Enzyme-Linked Immunosorbent Assay (ELISA).

The test was performed in 10.7 x 54.8 mm flat bottomed plastic tubes (Luckham Ltd., Burgess Hill, Sussex), following a similar procedure to that described by Engvall and Perlmann (1972).

The antigen was diluted in 0.1 M sodium carbonate buffer (pH 9.6) with 0.02% sodium azide. Unless otherwise indicated all buffers used in the assay and the preparation of reagents were made according to the formulae described by Dawson, Elliott, Elliott and Jones (1969).

One millilitre of the diluted antigen was then added to each plastic tube and the tubes were incubated in a water bath at 37°C

for three hours, during which time the antigen was adsorbed onto the wall of the tube. Antigen-coated tubes were stored at 4°C until required. Before assay, the tubes were washed three times by repeatedly filling them with a solution containing 0.9% sodium chloride and 0.05% Tween 20 and emptying by suction. The serum under investigation was diluted in phosphate buffered saline (Oxoid Ltd., London), containing 0.05% Tween 20 and 0.02% sodium azide and 1 ml was added to each antigen-coated tube. After incubation at room temperature for six hours the tubes were again washed three times and 1 ml of the peroxidase-conjugated anti-immunoglobulin diluted in phosphate buffered saline with 0.05% Tween 20 was added to each tube. The tubes were allowed to incubate overnight (16 hours) at room temperature and again washed three times. The amount of peroxidase bound to the tubes was then determined by addition of a substrate for the enzyme, as described by Ruitenberg, Steerenberg and Brosi (1975). The substrate was made by dissolving 80 mg of 5 amino salicylic acid in 100 ml of hot distilled water. The pH was adjusted to 6.0 with 1 N sodium hydroxide. To 9 ml of this solution was added 1 ml of 0.05% hydrogen peroxide. One millilitre of the substrate was added to each tube and on incubation at room temperature the reaction between the enzyme and the substrate caused the production of a brown colour. The intensity of this colour was proportional to the amount of peroxidase-conjugated antiglobulin which had bound to the tube and thus indirectly to the amount of globulin in the serum which had specifically bound to the antigen adsorbed onto the tube. After a suitable incubation period (generally 30 minutes) the enzyme reaction was terminated by the addition of 0.1 ml of 2 M sodium hydroxide to each tube. The intensity of the colour change was then measured on

a spectrophotometer at 449 nm, the reaction of the unknown serum samples being directly compared with that for a known negative serum and the results being expressed in units of absorbance.

Peroxidase-conjugated antiglobulins

Initially a peroxidase-conjugated rabbit-anti-sheep globulin was prepared for use in ELISA. During the course of this work it became possible to purchase such conjugates for the various species from a commercial source (Nordic Immunological Laboratories, Maidenhead, Berks.) and further conjugates were not prepared in the laboratory, since it was found to be an extremely time-consuming procedure.

Sheep IgG was prepared by ammonium sulphate precipitation as described by Campbell, Garvey, Cremer and Sussdorf (1964), followed by anion exchange chromatography. 500 ml of sheep serum was placed in a conical flask and saturated ammonium sulphate added dropwise from a burette with continual stirring, until the final concentration of the ammonium sulphate was 33%. After stirring for two hours the crude globulin and albumin precipitate was recovered by centrifugation at 2,500 g for 30 minutes. The precipitate was made up to the original volume with normal saline and the proteins reprecipitated with ammonium sulphate. After further centrifugation, the precipitate was made up to half the original volume in 0.01 M phosphate buffer (pH 6.8) and excess ammonium sulphate removed by extensive dialysis against 0.01 M phosphate buffer (pH 6.8). The desalted preparation was then purified by fractionation on a column of DE 52 Cellulose (Whatman Biochemicals, Maidstone, Kent), which was prepared according

to the manufacturer's instructions. The column was equilibrated with 0.01 M phosphate buffer (pH 6.8).

Under the prevailing conditions of molarity and pH the IgG_2 fraction of the globulin did not bind to the anion exchanger but passed straight through the column, being collected in a flask. The IgG_1 fraction was then liberated from the exchanger by altering the buffer to 0.035 M phosphate (pH 7.6). The two IgG fractions thus purified were pooled. The purity of the two fractions was confirmed by immunoelectrophoresis.

A 1% solution of agar ('Oxoid no.2 Ionagar', Oxoid Ltd., London) was prepared in barbital buffer pH 8.2 (made by dissolving 15.85 g sodium barbital in a solution containing 230 mls 0.1 N hydrochloric acid and 770 ml distilled water). Ten millilitres of the molten agar was poured onto a 5 x 7 cm glass plate and the wells and trough cut using a specially made perspex template. The wells were filled with the IgG samples or whole sheep serum and electrophoresis carried out for 90 minutes at 150 Volts in a tank containing the same buffer as that used to prepare the agar. The trough in the agar was then filled with a rabbit-anti-sheep serum prepared by immunising rabbits with whole sheep serum in a similar manner as that described below for the preparation of rabbit-anti-sheep IgG. The plate was allowed to stand overnight at $4^{\circ}C$, when the precipitin lines were clearly visible.

Rabbit-anti-sheep IgG was prepared by injection of rabbits with

the pooled sheep IgG. A solution containing 10 mg IgG per ml was emulsified in an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan). A series of three intramuscular injections of 1.0 ml of the emulsion were given at fortnightly intervals. By two weeks after the final injection the titres (as measured by immunodiffusion) were found to be sufficiently high and blood was taken for the preparation of serum.

Purified rabbit-anti-sheep IgG was then obtained from the serum by immunoadsorption with insolubilised sheep IgG, along similar lines to those described by Avrameas and Ternynck (1969).

Two hundred and fifty milligrams of the pure sheep IgG, prepared as described, were made up in 5.0 ml of 0.1 M phosphate buffer, pH 7.0. One millilitre of a 2.5% solution of glutaraldehyde ('Grade iv', Sigma Chemical Company Ltd., London) was then added to the stirred IgG solution. The resulting gel was allowed to stand at room temperature for three hours. Five hundred milligrams of the insolubilised IgG were homogenised by small portions in 200 ml of 0.2 M phosphate buffer (pH 7.4) using a loose fitting glass homogeniser. The resulting suspension was centrifuged for 15 minutes at 2,500 g at 4°C, the precipitate being rehomogenised and centrifuged twice more. The insolubilised IgG was then suspended in 200 ml of 0.1 M glycine - HCl buffer (pH 2.8) and centrifuged at 2,500 g for 15 minutes at 4°C. This operation was repeated. The IgG was then washed by centrifugation in 0.2 M phosphate buffer until the supernatant had an optical density of 0 at 280 nm.

In order to adsorb rabbit-anti-sheep IgG from the immune rabbit serum, appropriate volumes of the serum were mixed with the insolubilised sheep IgG, stirred at room temperature for 30 minutes and centrifuged at 2,500 g for 15 minutes. The supernatant fluid was retained and tested against sheep IgG by immunodiffusion to indicate the effectiveness of the immunoadsorbtion. The precipitate was suspended in phosphate buffered saline and washed repeatedly by centrifugation, until the supernatant had an optical density of less than 0.04 at 280 nm. The rabbit-anti-sheep IgG, which had been adsorbed into the insolubilised sheep IgG, was then eluted with 0.1 M glycine - HCl buffer (pH 2.8). The insolubilised material was suspended in approximately 4.0 ml of the buffer, stirred at room temperature for five minutes and centrifuged at 2,500 g for 15 minutes at 4°C. The supernatant was retained and the process repeated twice more. The supernatants were pooled, passed through a 0.45 µm membrane filter contained within a Swinnex 25 mm filter holder (Millipore U.K. Ltd., London) and dialysed against phosphate buffered saline at 4°C. The pure rabbit-anti-sheep IgG thus obtained was then conjugated to horse radish peroxidase according to the method of Nakane (1975).

Five milligrams of horse radish peroxidase (Sigma Chemical Company Ltd., London) were dissolved in 1 ml of freshly prepared 0.3 M sodium bicarbonate (pH 8.1) and treated with 0.1 ml fluorodinitrobenzene at a concentration of 1% in absolute ethanol for one hour at room temperature. One millilitre of 0.05 M sodium metaperiodate was then added, the reaction being stopped after 30 minutes by the addition of 1 ml of 0.16 M ethylene glycol. After stirring for one

hour, the solution was dialysed extensively against 0.01 M sodium carbonate buffer (pH 9.5). The oxidised peroxidase was then mixed with 5 mg of the purified rabbit-anti-sheep IgG and after three hours 5 mg sodium borohydride was added. The mixture was left at 4°C overnight and then dialysed extensively against 0.01 M phosphate buffered saline. The conjugated immunoglobulin was then isolated from the mixture by fractionation on a column of Sephadex G- 200 (Pharmacia Fine Chemicals, Uppsala, Sweden) in a manner similar to that described below for the fractionation of the metabolic antigen.

The purified rabbit-anti-sheep IgG conjugate was stored at 4°C as a solution containing 250 µg of protein per ml.

The peroxidase-labelled anti-immunoglobulin obtained commercially was supplied as a freeze-dried formulation, which was dissolved in 1 ml of distilled water in accordance with the manufacturer's instructions. The solution was then diluted as required immediately prior to use.

Fractionation of metabolic antigen on Sephadex G-200

Metabolic antigen was fractionated by gel filtration on a column of Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden). A 2.5 cm diameter column was filled to a depth of 78 cm with the gel, which had been prepared in 0.1 M tris-HCl buffer (pH 8) in accordance with the manufacturer's instructions. Antigens were applied to the column in a volume of 1 ml and eluted with the same buffer. The optical density of the eluant was measured at 220 nm

using a 'Spectrochrome F' analyser (Gilson, Villies-le-bel, France) and recorded on an 'L.K.B. 6520-4' recording device (L.K.B. Instruments Ltd., South Croydon, Surrey). Fractions of the eluant were collected by means of an 'L.K.B. 7000A UltroRac' fraction collector (L.K.B. Instruments Ltd., South Croydon, Surrey).

The required fractions were restored to the initial antigen volume (1 ml) by concentration against polyethylene glycol 6000 at a concentration of 50% in distilled water.

The molecular weights of the various antigen fractions were calculated by the method of Andrews (1964, 1965), the void volume of the column being determined by application of a solution of blue dextran, molecular weight 2 million (Sigma Chemical Company Ltd., London) at a concentration of 2.0 mg/ml.

CHAPTER THIRTEEN

A PRELIMINARY STUDY ON THE PRODUCTION OF METABOLIC ANTIGEN BY ADULT F. HEPATICA MAINTAINED IN VITRO

Introduction

The purpose of these preliminary studies was to establish whether adult F. hepatica maintained in vitro were producing metabolites with antigenic properties and, if so, to investigate some of the parameters which might influence their production.

Studies in Section 1 indicated that a high proportion of serum in the culture medium was beneficial to fluke metabolism in vitro. However, the use of high levels of serum in cultures intended for the production of metabolic antigen introduces a number of problems. It was envisaged that in order to obtain metabolic antigen in a potentially useful form, the culture medium in which the flukes had been maintained would need to be very greatly concentrated. A high percentage of serum in the medium would preclude this, unless the serum proteins were first removed. Such removal of serum proteins would also result in the loss of the protein components of the metabolic antigen and it was anticipated that these might form an important part of the antigen.

The earlier studies also indicated that a high flow-rate through the culture was of benefit, especially when large numbers of flukes were to be maintained together. In order to conserve culture medium during the relatively prolonged culture periods, which it was anticipated would be necessary to obtain useful quantities of antigen,

it was apparent that a system whereby the medium could be recirculated would offer some advantages. Such a system would also avoid the necessity of having to replenish the medium reservoir during the course of the culture, with the attendant risks of introducing contamination.

For these reasons, attention was focussed mainly on the development of a continuous-flow recirculating system, incorporating a protein-free medium.

Experimental design

Initially a single continuous-flow recirculating culture was set up, in which were placed approximately 100 adult flukes recovered from an experimentally infected sheep. The culture contained 2 l of Medium 199 with antibiotics as previously described and was maintained for seven days, at a flow-rate of approximately 20 ml per hour. At the end of this period, antigen was prepared by concentrating the medium to a volume of 10 ml.

In order to study the rate of production of the antigen during such a culture, a similar culture was set up, containing approximately 80 flukes recovered from a sheep. After three days of culture, the aspirator containing the reservoir of medium was disconnected from the culture, using aseptic techniques and replaced by a similar aspirator containing 2 l of fresh Medium 199. After a further three days this process was repeated, the second aspirator again being replaced by a fresh one. After a further three days the culture was terminated, the contents of the third aspirator being retained.

Antigen was recovered from the medium in each of the three aspirators by concentration, the final volume being 10 ml in each case. The quantity of antigen in each sample was measured against a standard infected rabbit serum using the semi-quantitative immunodiffusion method.

A similar technique was used to investigate whether the presence of 10% bovine serum in the medium resulted in the production of appreciably more non-protein metabolic antigen than when the culture contained Medium 199 alone. Two similar continuous-flow recirculating cultures were set up, into each of which were placed 80 flukes recovered from a sheep. One culture contained 2 l of Medium 199 supplemented with 10% bovine serum, whilst the second contained 2 l of Medium 199 alone. The cultures were run side-by-side, the flow rate in each being the same.

After three days, the aspirators were disconnected from each culture and replaced with fresh ones, each containing 2 l of the appropriate medium. After another three days this was repeated and the cultures were terminated after a final three days. The non-protein component of the antigen was recovered from each of the samples of medium by precipitation with trichloracetic acid, followed by concentration to a final volume of 10 ml. The antigen in each sample was then quantified against a standard infected sheep serum using the semi-quantitative immunodiffusion technique.

Results

Examination of the flukes maintained in recirculating cultures

as described indicated 100% survival during the course of all the experiments.

The concentrated culture medium obtained from the initial recirculating culture showed strong antigenic activity when tested by immunodiffusion against infected rabbit and sheep sera. The protein concentration was 1800 μg per ml. After removal of the protein with trichloracetic acid or boiling, no immunodiffusion reaction was obtained with infected rabbit serum but activity was maintained with infected sheep serum.

The results of the experiment to investigate the rate of production of metabolic antigen during the nine day culture in Medium 199 are shown in Table 13.1. It was apparent that the dilution of antigen which would react completely with an equal volume of the standard rabbit serum diminished during the course of the culture. The amount of antigen produced during the initial three days of the culture was twice the amount produced during the second three days and eight times the amount produced during the final three days.

Apparently the rate of production of antigen capable of reacting with infected sheep serum did not fall so rapidly during the course of the culture. The quantity of this component of the antigen produced during the first three days was twice the amount produced in the second three days, which was equal to the amount produced in the final three days.

The results of the experiment to investigate whether the inclusion of 10% bovine serum in the medium led to the production of more of the non-protein antigen are shown in Table 13.2. It was apparent that the inclusion of the serum did not affect the quantity of non-protein antigen produced.

Discussion

The initial experiment in this series demonstrated that adult flukes maintained in a recirculating continuous-flow apparatus containing a protein-free medium were liberating appreciable quantities of metabolites with antigenic properties into their environment. These metabolites apparently included both protein and non-protein components, which reacted to a different degree with the sera of rabbits and sheep, the non-protein antigens giving no precipitin reaction with infected rabbit serum.

Although there was 100% survival of flukes under these culture conditions, as anticipated, the quantity of antigen produced declined during the course of a nine day culture. The fall in production of the protein component of the metabolic antigen was apparently more rapid than that of the non-protein component. Moreover, the fall in production of the protein component was greater between six and nine days than in the first six days of culture. On the basis of this finding, future cultures were generally maintained for approximately seven days.

The inclusion of 10% bovine serum apparently did not stimulate the production of increased amounts of the non-protein component of

Days after start of culture	Dilution of antigen for complete reaction with an equal volume of:	
	Infected rabbit serum	Infected sheep serum
0-3	1/8	1/8
3-6	1/4	1/4
6-9	1	1/4

TABLE 13.1. Rate of production of metabolic antigen during a nine day culture period.

Days after start of culture	Dilution of antigen for complete reaction with an equal volume of infected sheep serum	
	Medium 199 alone	Medium 199/10% serum
0-3	1/8	1/8
3-6	1/4	1/4
6-9	1/4	1/4

TABLE 13.2. Comparison of the rate of production of non-protein metabolic antigen in cultures containing protein-free medium or 10% bovine serum.

the metabolic antigen. It is possible that the protein component of the antigen might be stimulated by the inclusion of serum in the medium but, as explained previously, it was not possible to investigate this.

CHAPTER FOURTEEN

FURTHER STUDIES ON THE PROPERTIES OF METABOLIC ANTIGEN OBTAINED FROM ADULT F. HEPATICA MAINTAINED IN VITRO

Introduction

Having established that adult flukes maintained in a protein-free medium in a continuous-flow recirculating system were producing antigenic metabolites, further studies were undertaken on the properties of these substances.

Experimental design

Metabolic antigen used in these studies was produced from adult flukes which were recovered from experimentally infected rabbits or sheep. In either case, the flukes were maintained for seven days in a continuous-flow recirculating culture apparatus containing 2 l of Medium 199. Between 80 and 120 flukes were included in each culture and the final volume of the concentrate was 10 ml.

Immunodiffusion reactions between infected rabbit serum and the metabolic antigen, bile from a rabbit carrying a patent fluke infection and somatic fluke antigen were compared, so as to indicate the extent of any immunological identity between these three antigens.

The immunodiffusion reactions of whole and protein-free metabolic antigen with serum from rabbits, rats, sheep and cattle carrying patent fluke infections were studied and compared. Immunoadsorption studies were undertaken, in which metabolic antigen was adsorbed with infected rabbit, rat or sheep sera and then tested against sera from

alternative species, in order to demonstrate the differing antigen-antibody reactions occurring in the various species.

The immunodiffusion reaction between metabolic antigen, produced from flukes originating from rabbits and sheep, and antisera raised against the host species' serum were studied, in order to assess the extent to which the antigen was contaminated with materials originating from the host.

Metabolic antigen derived from flukes taken from rabbits was compared by immunodiffusion with antigen derived from flukes taken from sheep, in order to see whether there was any difference between the antigens obtained from flukes originating from different host species.

Newly excysted flukes were incubated in normal and infected rabbit serum and in infected rabbit serum, which had been completely adsorbed with metabolic antigen, and examined for the formation of immune precipitates.

Results

The immunodiffusion reactions of metabolic antigen, infected bile and somatic fluke antigen with infected rabbit serum are shown in Plate 14.1. Metabolic antigen and infected bile showed immunological identity, whilst metabolic antigen and somatic antigen showed only partial identity.

The metabolic antigen gave a positive immunodiffusion reaction

with infected sheep, rabbit, rat and bovine sera. Three precipitin lines could be distinguished in the case of the reaction between sheep serum and the antigen (Plate 14.2), although sheep sera tended to give rather blurred lines on immunodiffusion, making it difficult to accurately identify individual lines. A single precipitin line was seen between infected rabbit serum and metabolic antigen (Plate 14.3), two lines in the case of infected rat serum (Plate 14.4) and a single blurred line in the case of infected bovine serum (Plate 14.5).

Infected rabbit serum gave no immunodiffusion reaction with the non-protein component of the metabolic antigen, but reactions were seen between infected sheep, rat and bovine sera and the non-protein antigen. Two sharp lines were seen with sheep serum, (Plate 14.6) whilst a single line was obtained in the case of rat or bovine sera (Plates 14.7, 14.8).

The results of the immunoadsorption studies are shown in Table 14.1. It was evident that metabolic antigen which had been completely adsorbed with infected serum from any one of the three species involved would still give a precipitin reaction with serum from the other two species.

Metabolic antigen derived from flukes originating from rabbits gave a strong precipitin reaction when tested against a serum prepared by immunising goats with whole rabbit serum (kindly supplied by L.J.S.Harrison), (Plate 14.9). Similarly metabolic antigen derived from flukes originating from sheep gave a strong

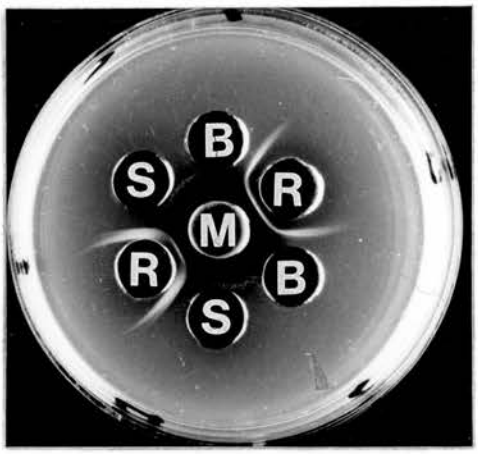

Plate 14.1. Immunodiffusion reactions between infected rabbit serum (R) and metabolic antigen (M), somatic antigen (S) and infected bile (B).

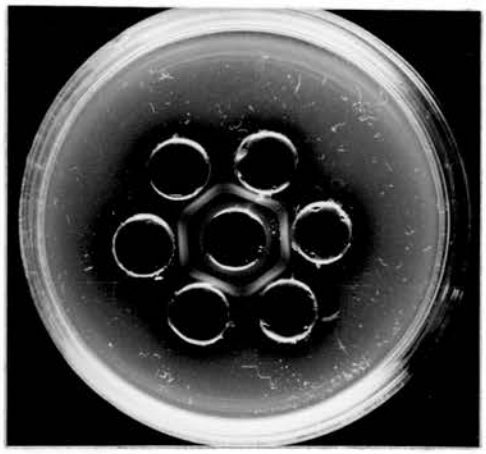

Plate 14.2. Immunodiffusion reaction between whole metabolic antigen (centre well) and infected sheep serum (outer wells).

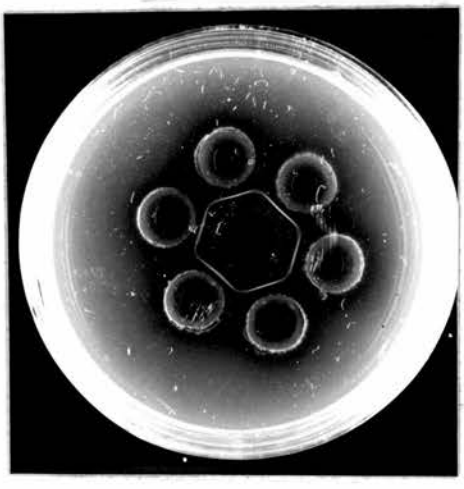

Plate 14.3. Immunodiffusion reaction between whole metabolic antigen (centre well) and infected rabbit serum (outer wells).

Plate 14.4. Immunodiffusion reaction between whole metabolic antigen (centre well) and infected rat serum (outer wells).

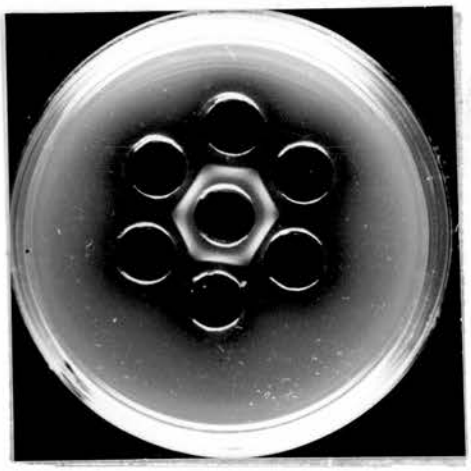

Plate 14.5. Immunodiffusion reaction between whole metabolic antigen (centre well) and infected bovine serum (outer wells).

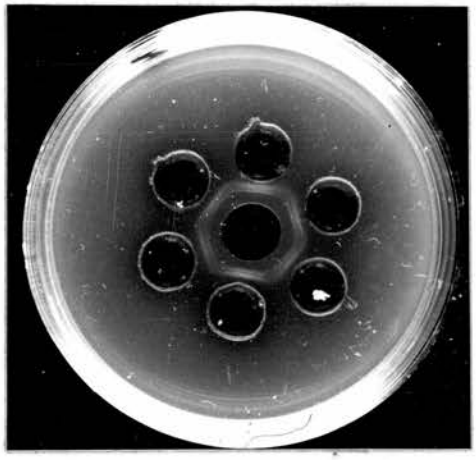

Plate 14.6. Immunodiffusion reaction between non -protein component of the metabolic antigen (centre well) and infected sheep serum (outer wells).

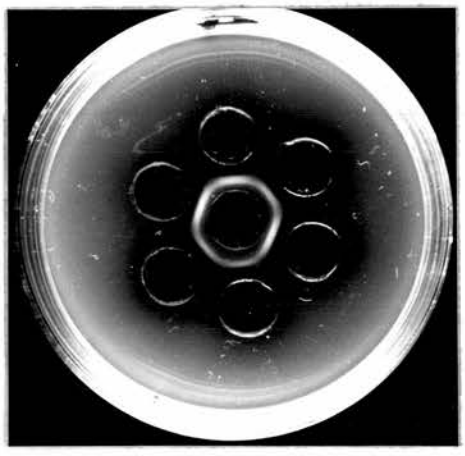

Plate 14.7. Immunodiffusion reaction between non -protein component of the metabolic antigen (centre well) and infected rat serum (outer wells).

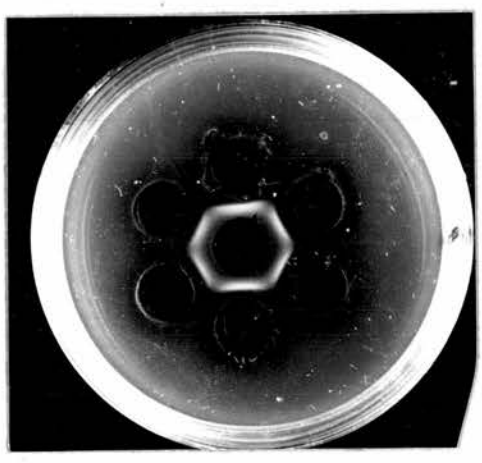

Plate 14.8. Immunodiffusion reaction between non -protein component of the metabolic antigen (centre well) and infected bovine serum (outer wells).

Metabolic antigen adsorbed with:	Adsorption mixture tested against:	Result
Infected rabbit serum	Infected rabbit serum	-
	Infected sheep serum	+
	Infected rat serum	+
Infected sheep serum	Infected sheep serum	-
	Infected rabbit serum	+
	Infected rat serum	+
Infected rat serum	Infected rat serum	-
	Infected rabbit serum	+
	Infected sheep serum	+

TABLE 14.1 Immunodiffusion reactions of metabolic antigen after immunoadsorption with infected sera.
+ = positive immunodiffusion reaction
- = negative immunodiffusion reaction

precipitin reaction with a serum prepared by immunising rabbits with whole sheep serum (Plate 14.10). Otherwise, antigen derived from flukes taken from sheep was apparently identical to antigen derived from flukes taken from rabbits (Plate 14.11).

Newly excysted flukes incubated in infected rabbit serum developed marked precipitates, which were clearly visible by 24 hours, when they virtually encapsulated the organism (Plate 14.12). Flukes incubated in infected rabbit serum which had been completely adsorbed with metabolic antigen developed similar precipitates. Incubation of newly excysted flukes in normal rabbit serum did not result in the formation of such precipitates.

Discussion

These studies indicated that the crude metabolic antigen obtained from adult flukes, maintained as described, contained a variety of different antigen components, which reacted to varying degrees with infected sera from different host species.

The immunological identity seen between the metabolic antigen and infected bile was noteworthy, since it suggested that the antigens produced *in vitro* had not undergone radical changes from those produced by flukes *in vivo*. The partial identity between metabolic and somatic fluke antigens was to be expected, since the flukes used to prepare the somatic antigen would undoubtedly contain quantities of those metabolites which, when liberated into the culture medium, constitute the metabolic antigen. In addition, the somatic antigen would be expected to contain a wide range of antigenic components not

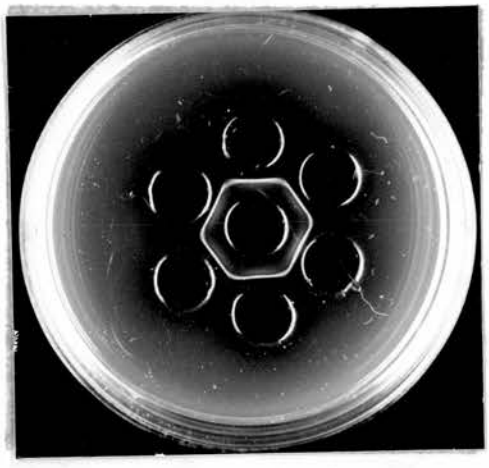

Plate 14.9. Immunodiffusion reaction between metabolic antigen derived from flukes taken from rabbits (centre well) and goat-anti-rabbit serum (outer wells).

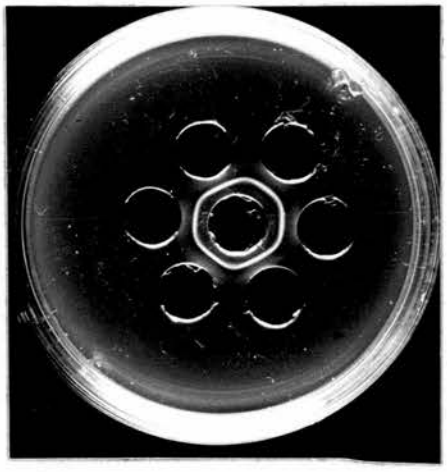

Plate 14.10. Immunodiffusion reaction between metabolic antigen derived from flukes taken from sheep (centre well) and rabbit-anti-sheep serum (outer wells).

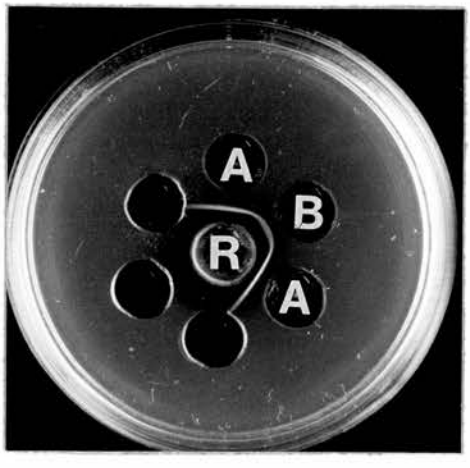

Plate 14.11. Immunodiffusion reactions between infected rabbit serum (R) and metabolic antigens derived from flukes taken from rabbits (A) and sheep (B).

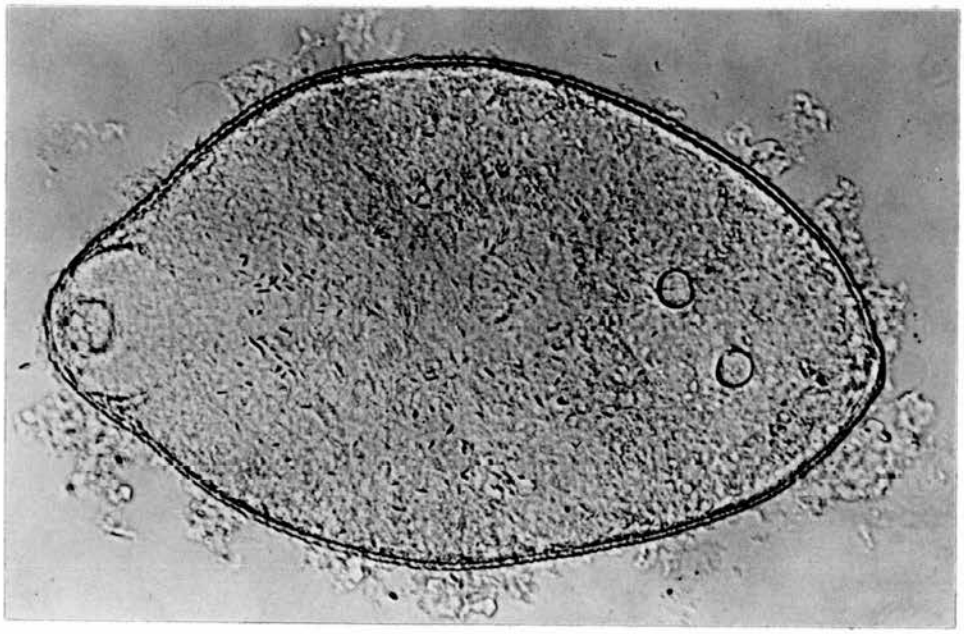

Plate 14.12. Juvenile fluke incubated for 24 hours in infected rabbit serum, demonstrating formation of immune precipitates. (x 200).

present in the metabolic antigen.

The immunodiffusion studies on infected sera from rabbits, sheep, rats and cattle indicated that the metabolic antigen contained a range of antigenic components. This was confirmed by the immuno-adsorption studies, which showed that each of the species studied was producing antibodies against different components of the metabolic antigen.

It was apparent that metabolic antigen prepared as above was contaminated with materials originating from the host species from which the flukes had been taken. Although flukes tended to regurgitate their caecal contents during the washing processes prior to being placed in the culture, appreciable quantities of the ingesta were apparently retained within the digestive tract and liberated during the course of the culture. As an extension of this study, the antigen obtained between six and nine days of culture in the experiment described previously was tested against rabbit-anti-sheep serum and again a positive reaction was obtained. This indicated that the liberation of materials from the digestive tract was prolonged and that it would not be possible to obtain metabolic antigen free from such contaminants merely by discarding the medium collected during the initial period in vitro.

An alternative explanation for this prolonged excretion of materials related to host serum proteins is that certain of the fluke metabolic antigens might be closely related to the host antigens, thus providing the parasite with a measure of protection against the host's defence

system, as is known to occur with schistosomes. However, it is probably more likely that the antigenic substances were in fact merely residual food products from the flukes' digestive tracts, since Hughes and Harness (1973a,b) were unable to demonstrate a 'host-antigen' effect with F. hepatica.

Although contamination of the metabolic antigen with host materials is of little significance in many studies, it is of concern where the antigen is to be used in the immunisation of host species. Thus, administration to rabbits of antigen derived from flukes taken from a sheep would stimulate the production of antibodies to the sheep contaminants in the antigen, possibly resulting in a less effective response to the purely fluke components of the whole antigen. This problem could be most readily overcome by administering antigen derived from flukes taken from the same species of host as that to be immunised.

The finding that adsorption of infected rabbit serum with metabolic antigen did not prevent the development of immune precipitates about newly excysted flukes subsequently incubated in the serum was in agreement with that of Sewell (1968), but differed from that of Wikerhauser (1961a), who reported that precipitates were not formed about newly excysted flukes if the infected serum was first adsorbed with metabolic antigen derived from adult flukes.

The fact that the metabolic antigen used in the present study was not able to adsorb those antibodies in infected rabbit serum

which are involved in the formation of immune precipitates about newly excysted flukes is of some interest, in that it implies that the antigens being produced by the young flukes differ from those produced by adult flukes. If this is so, it is possible that attempts to immunise the host animal with metabolic antigen derived from adult flukes will not be successful and more emphasis should be placed on the role of antigens derived from immature flukes.

CHAPTER FIFTEEN

PRELIMINARY STUDIES ON THE ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Introduction

As previously noted, studies on the serodiagnosis of fascioliasis have been undertaken by numerous workers, often with conflicting results.

As with many other helminth diseases, the major problems have been those associated with false results, non-specific effects and cross-reactions with other species. A wide variety of antigens have been used in such studies, in the hope of identifying those which are specific to F. hepatica and thus the most reliable for serodiagnosis of that species. Similarly, many different serological tests have been used, in the hope of identifying those which are most sensitive and reliable.

The fact that no single antigen preparation or serodiagnostic technique has found universal acceptance indicates that further studies in this field are justified.

The Enzyme-Linked Immunosorbent Assay was first described by Engvall and Perlmann (1971), who used the technique to quantify antigen (rabbit IgG). The antigen was labelled with enzyme (alkaline phosphatase) and reacted with excess insolubilised antibody (sheep-anti-rabbit IgG-serum). After suitable washing, the extent to which the antigen-antibody reaction had occurred was indicated by exposing the conjugate to a suitable enzyme substrate

(p-nitrophenylphosphate).

The reaction between enzyme and substrate resulted in a change in the colour of the solution, which was directly proportional to the initial amount of labelled antigen. The test was found to be as sensitive and precise as the radioimmunosorbent technique (RIST), allowing for the determination of antigen at a concentration of a few ng per ml. ELISA however had the advantage that enzyme-labelled reagents can be stabilised, so that one preparation could be used for a prolonged period. Moreover, the measurement of the enzyme activity requires simpler and more readily available apparatus than does the measurement of radioactivity.

Engvall and Perlmann (1972) extended ELISA to the quantitative determination of antibodies. Antigen was adsorbed onto disposable polystyrene tubes, which were then incubated with antiserum, followed by enzyme-labelled anti-immunoglobulin. The amount of enzyme remaining in the tube after washing was then indicated by the addition of a suitable substrate, the reaction causing a change in the optical density of the solution proportional to the amount of specific antibody present in the serum. The extreme sensitivity of the technique was confirmed, antigen being used at a concentration of a few μg per ml and antibody being detected at concentrations of less than 1 ng per ml.

Ruitenberg, Steerenberg and Brosi (1975) described a modification of ELISA which made use of microplates rather than tubes, with a resulting decrease in the quantities of reagents required.

ELISA has since been widely used in the serodiagnosis of a variety of conditions. The application of ELISA to parasitic diseases was reviewed by Voller, Bartlett and Bidwell (1976). Within the field of helminthology, ELISA was first used for the immunodiagnosis of Trichinella spiralis infections of pigs (Ljungström, Engvall and Ruitenberg, 1974; Ruitenberg, Steerenberg, Brosi and Buys, 1974; Ruitenberg, Steerenberg, Brosi, Buys, Ljungström and Engvall, 1974; Ruitenberg, Steerenberg, Brosi and Buys, 1975), where it was shown to be highly sensitive and capable of detecting antibodies at a very early stage of the infection. ELISA has also been applied to the serodiagnosis of schistosomiasis (Bout, Dugimont, Farag and Capron, 1975; Huldt, Lagerquist, Phillips, Draper and Voller, 1975; Voller, Bartlett and Bidwell, 1976), hydatid disease (Farag, Bout and Capron, 1975) and onchocercosis (Bartlett, Bidwell and Voller, 1975). Most of these studies have emphasised the sensitivity of the technique, but it was also apparent that the problems of cross-reactions and non-specificity remained when crude helminth antigens were used in the test, particularly when studying field cases rather than experimental infections.

ELISA has not previously been applied to the serodiagnosis of fascioliasis. The extreme sensitivity of the technique suggested that it might be of value in the present study, where the metabolic antigen was available in relatively limited quantities, so that alternative serodiagnostic tests which are less economical on reagents were unsuitable.

The purpose of these preliminary studies was to establish whether metabolic antigen was suitable for use in ELISA and, if so, to establish the optimal concentrations of antigen, serum and enzyme-labelled anti-immunoglobulin for the test, according to the method of Engvall and Perlmann (1972).

Experimental design

The metabolic antigen used in all the following studies was obtained from adult flukes maintained for seven days in a recirculating continuous-flow system containing Medium 199. In order to avoid any possible reaction between the enzyme-labelled anti-immunoglobulin and the host serum contaminants in the metabolic antigen, the antigen used was generally derived from flukes taken from a species different from that whose serum was to be assayed.

The first series of experiments was designed to study the influence of varying the concentrations of antigen, serum and enzyme-labelled anti-immunoglobulin when using ELISA to assay rabbit serum. The metabolic antigen was derived from flukes taken from a sheep. Negative and positive serum samples were obtained from normal rabbits and rabbits infected 70 days previously with 100 metacercariae. Horseradish peroxidase-conjugated goat-anti-rabbit IgG was obtained commercially (Nordic Immunological Laboratories, Maidenhead, Berkshire).

In the initial experiment, antigen was used at concentrations of 60.0, 6.0, 0.6 and 0.12 µg of protein per ml, positive and negative sera at a dilution of 1/50, and the enzyme conjugate at a dilution of 1/500.

The effect of varying the dilution of the serum was then investigated. Antigen was used at a concentration of 5.0 μg of protein per ml, positive and negative sera at dilutions of 1/25, 1/50, 1/100 and 1/500, and the enzyme conjugate at a dilution of 1/500.

Finally the effect of varying the dilution of the anti-immunoglobulin was studied. Antigen was again used at a concentration of 5.0 μg of protein per ml, sera at a dilution of 1/50 and the enzyme conjugate at dilutions of 1/200, 1/300, 1/500, 1/600 and 1/1000.

Similar studies were then undertaken on the effect of varying the concentrations of serum and anti-immunoglobulin when using ELISA to assay sheep serum. In this case the antigen used was derived from flukes taken from rabbits. Negative and positive serum samples were obtained from normal sheep and sheep infected 70 days previously with 500 metacercariae. Horseradish peroxidase-labelled rabbit-anti-sheep IgG was either prepared in the laboratory, or obtained commercially (Nordic Immunological Laboratories, Maidenhead, Berkshire).

The antigen was used at a concentration of 5.0 μg of protein per ml in all the experiments. In the first experiment, sera were used at dilutions of 1/25, 1/50 and 1/100, with the commercial enzyme preparation at a dilution of 1/500. The effect of varying the dilution of the conjugated anti-immunoglobulin was then studied. With sera at a dilution of 1/50, the commercial preparation was used at dilutions of 1/100, 1/300, 1/500, 1/700 and 1/1000, and the laboratory prepared conjugate at concentrations of 5.0, 6.2, 8.3 and

12.5 µg of protein per ml.

For the assay of rat serum, the antigen used was derived from flukes taken from rabbits. Negative and positive serum samples were obtained from normal rats and rats infected 56 days previously with 20 metacercariae. The antigen was used at a dilution of 5.0 µg of protein per ml, and sera at a dilution of 1/25. Horseradish peroxidase-labelled rabbit-anti-rat IgG was obtained commercially (Nordic Immunological Laboratories, Maidenhead, Berkshire) and used at dilutions of 1/25, 1/50, 1/100 and 1/200.

Antigen derived from flukes taken from a sheep was used in the assay of bovine sera by ELISA. Negative and positive sera were obtained from normal calves and calves infected 28 days previously with 800 metacercariae. The antigen was used at a dilution of 5.0 µg of protein per ml and sera at a dilution of 1/25. Horseradish peroxidase-labelled rabbit-anti-bovine IgG (Nordic Immunological Laboratories, Maidenhead, Berkshire) was used at dilutions of 1/100, 1/200, 1/300 and 1/500.

Experiments were also undertaken to see whether the non-protein components of the metabolic antigen would react in ELISA. The antigen was obtained by boiling metabolic antigen derived from flukes taken from sheep, initially at a concentration of 5.0 µg of protein per ml. The boiled antigen was then used at that same dilution, with rabbit and sheep sera at a dilution of 1/50, and commercial goat-anti-rabbit and rabbit-anti-sheep enzyme conjugates at a dilution of 1/500 and 1/700 respectively.

Results

The effect of varying the concentration of the antigen in the assay of rabbit serum is shown in Table 15.1. It was apparent that as the concentration of antigen increased, so did the difference in optical density between the positive and negative sera. In future experiments antigen was used at a concentration of 5.0 μg of protein per ml, as this appeared to offer a reasonable combination of economy of materials and differentiation of positive from negative sera. The effect of varying the dilution of the serum is shown in Table 15.2. Again the difference between positive and negative sera was greatest when the serum was diluted least. In future work serum was used either at a dilution of 1/25 or 1/50. The influence of differing dilutions of the peroxidase-labelled goat-anti-rabbit IgG is shown in Table 15.3. The difference between positive and negative sera was greatest at lower dilutions of the conjugate, but it was apparent that the use of lower dilutions also increased the non-specific reaction of the negative serum. In future studies this enzyme preparation was used at a dilution of 1/500, as this allowed for adequate differentiation of positive and negative sera and largely avoided the non-specific effects with the negative serum.

The results of the study of the effects of varying the dilution of sheep serum, using antigen at a dilution of 5.0 μg of protein per ml and commercial rabbit-anti-sheep enzyme conjugate at a dilution of 1/500 are shown in Table 15.4. As with rabbit sera, the greatest difference between positive and negative sera was seen when the serum was diluted least. A dilution of 1/25 was used in future studies. A dilution of 1/700 was found to be satisfactory for the commercial

Antigen concentration (μg protein/ml)	Absorbance	
	Positive serum	Negative serum
60.0	0.56	0.00
6.0	0.36	0.00
0.6	0.16	0.00
0.12	0.09	0.00
0.00	0.00	0.00

TABLE 15.1. The influence of varying the concentration of the antigen in the assay of rabbit serum by ELISA

Serum dilution	Absorbance	
	Positive serum	Negative serum
1/25	0.41	0.02
1/50	0.32	0.00
1/100	0.26	0.00
1/500	0.13	0.00

TABLE 15.2. The influence of varying the dilution of the serum in the assay of rabbit serum by ELISA

Anti-immunoglobulin dilution	Absorbance	
	Positive serum	Negative serum
1/200	0.63	0.12
1/300	0.54	0.08
1/500	0.33	0.02
1/600	0.28	0.00
1/1000	0.20	0.00

TABLE 15.3 The influence of varying the dilution of the enzyme-labelled anti-immunoglobulin in the assay of rabbit serum by ELISA.

peroxidase-labelled rabbit-anti-sheep preparation (Table 15.5). Again at lower dilutions of the conjugate there was a marked non-specific effect with the normal serum. The laboratory-prepared anti-immunoglobulin gave a satisfactory result at a concentration of 8.3 µg of protein per ml (Table 15.6).

The effect of varying the dilution of the peroxidase-labelled rabbit-anti-rat IgG is shown in Table 15.7. This preparation was apparently much weaker than the previous ones and was only effective at dilutions of 1/50 or less.

Similarly the rabbit-anti-bovine preparation was rather weak, with differentiation of positive and negative sera only occurring at dilutions of less than 1/200 (Table 15.8).

The results of the experiments using the non-protein component of the antigen are shown in Table 15.9. It was apparent that in the case of both rabbits and sheep, the non-protein antigen was reacting to a similar extent to the whole metabolic antigen.

Discussion

These preliminary studies indicated that ELISA was potentially a useful technique for quantifying the antibodies to F. hepatica present in the serum of infected animals. The technique proved to be highly sensitive and was particularly economical in the use of antigen, which was of benefit in this work, where the metabolic antigen was available in relatively limited amounts.

Serum dilution	Absorbance	
	Positive serum	Negative serum
1/25	0.48	0.21
1/50	0.40	0.20
1/100	0.35	0.19

TABLE 15.4. The influence of varying the dilution of the serum in the assay of sheep serum by ELISA.

Anti-immunoglobulin dilution	Absorbance	
	Positive serum	Negative serum
1/100	0.42	0.25
1/300	0.40	0.22
1/500	0.38	0.19
1/700	0.19	0.08
1/1000	0.12	0.04

TABLE 15.5. The influence of varying the dilution of the enzyme-labelled anti-immunoglobulin (Commercial preparation) in the assay of sheep serum by ELISA

Anti- immunoglobulin concentration (µg protein/ml)	Absorbance	
	Positive serum	Negative serum
12.5	0.46	0.28
8.3	0.22	0.04
6.2	0.09	0.00
5.0	0.05	0.00

TABLE 15.6. The influence of varying the concentration of the enzyme-labelled anti-immunoglobulin (laboratory preparation) in the assay of sheep serum by ELISA.

Anti-immunoglobulin dilution	Absorbance	
	Positive serum	Negative serum
1/25	0.40	0.23
1/50	0.15	0.00
1/100	0.00	0.00
1/200	0.00	0.00

TABLE 15.7. The influence of varying the dilution of the enzyme-labelled anti-immunoglobulin in the assay of rat serum by ELISA

Anti-immunoglobulin dilution	Absorbance	
	Positive serum	Negative serum
1/100	0.35	0.28
1/200	0.11	0.04
1/300	0.00	0.00
1/500	0.00	0.00

TABLE 15.8. The influence of varying the dilution of the enzyme-labelled anti-immunoglobulin in the assay of bovine serum by ELISA.

Serum	Absorbance	
	Positive serum	Negative serum
Rabbit	0.31	0.00
Sheep	0.17	0.06

TABLE 15.9. The use of non-protein metabolic antigen in the assay of rabbit and sheep sera by ELISA.

It appeared that the concentration and activity of the enzyme-labelled anti-immunoglobulin were the factors of greatest practical importance in these studies. If the enzyme preparation was too weak, positive and negative sera were not clearly differentiated, whilst if it was too strong, there was a marked non-specific reaction associated with the negative samples. It was apparent that the rabbit-anti-rat and rabbit-anti-bovine enzyme preparations contained very much less activity than did the other preparations and so had to be used at relatively low dilutions in order to differentiate positive from negative sera. At low dilutions of conjugate, the cost of performing each assay became significant and, particularly in the case of the assay of rat serum, the necessity of using the conjugate at a dilution of only 1/50 would make it uneconomical to extend the method to the assay of large numbers of serum samples. Attempts to obtain alternative and more active preparations of these two conjugates from commercial sources were not successful. Alternatively, these preparations could have been prepared in the laboratory but the experience with the laboratory-prepared rabbit-anti-sheep enzyme preparation indicated that this was an extremely time-consuming procedure.

The fact that the non-protein component of the metabolic antigen reacted in ELISA with both infected rabbit and sheep sera, to a similar extent as the whole metabolic antigen was of some interest. Earlier studies had indicated that infected rabbit serum showed no immunodiffusion reaction with the non-protein component of the metabolic antigen, whilst the present findings indicated that the infected rabbit serum did contain antibodies to the non-protein antigen. Thus it would appear that the immunodiffusion technique and ELISA are not measuring the same antigen-antibody reactions.

CHAPTER SIXTEEN

THE USE OF METABOLIC ANTIGEN TO MEASURE THE SEROLOGICAL RESPONSE OF ANIMALS EXPERIMENTALLY INFECTED WITH F. HEPATICA

Introduction

Having demonstrated that metabolic antigen obtained by the *in vitro* maintenance of adult flukes was able to react with serum from infected animals in the immunodiffusion test and ELISA, these two techniques were then used to follow the development of the serological response during the course of experimental fluke infections in rabbits, sheep, rats and cattle.

It was of interest to discover how soon after infection a serological response could be detected and to compare the efficiency and sensitivity of the two techniques. It was also of interest to compare metabolic antigen with somatic antigen in these serodiagnostic tests.

Experimental design

Four adult New Zealand White rabbits were each infected with 100 metacercariae. Serum samples were obtained fortnightly until eight weeks after infection. The metabolic antigen used in the immunodiffusion test was derived from flukes taken from a sheep and used at a concentration of 600 µg of protein per ml. The same antigen was used in ELISA, at a concentration of 5 µg of protein per ml, with serum diluted 1/50 and the commercial peroxidase-labelled goat anti-rabbit IgG at a dilution of 1/500. Similar tests were carried out using the non-protein component of the antigen at the same dilutions.

Somatic antigen, derived from flukes taken from a sheep, was used at a concentration of 600 μg of protein per ml in the immunodiffusion test and at a concentration of 5 μg of protein per ml in ELISA, with sera and anti-immunoglobulin diluted as above.

Four adult female sheep of mixed breeding were each infected with 500 metacercariae and the serological response to the infection measured in a similar manner. The metabolic antigen used in the immunodiffusion test was derived from flukes taken from a sheep, at concentration of 600 μg of protein per ml. The antigen used in ELISA was derived from flukes taken from rabbits, at a concentration of 5.0 μg of protein per ml. Serum was used at a dilution of 1/50 in ELISA and the commercial peroxidase-labelled rabbit-anti-sheep IgG at a dilution of 1/700. Similar tests were carried out using the non-protein component of the metabolic antigen and somatic antigen, derived from flukes taken from a rabbit, with all reagents diluted as above.

Four adult male Porton Wistar rats were each infected with 20 metacercariae. The serological response to metabolic antigen was measured by immunodiffusion and ELISA using the same antigen as described above for the study of the immune response of infected rabbits. In ELISA, serum was used at a dilution of 1/50 and the commercial peroxidase-labelled rabbit-anti-rat IgG at a dilution of 1/50.

Two male Ayrshire calves were each infected with 800 metacercariae.

The serological response to metabolic antigen was measured by immunodiffusion and ELISA, using antigen derived from flukes taken from a sheep at a concentration of 800 μg of protein per ml in the case of immunodiffusion and 20 μg of protein per ml in ELISA. Serum was used at a dilution of 1/10 and the commercial peroxidase-labelled rabbit-anti-bovine IgG at a dilution of 1/200 in ELISA.

Results

The individual immunodiffusion titres of the infected rabbits to metabolic antigen are shown in Appendix Table 16.1 and group mean titres in Fig.16.1. It was evident that a response was detectable by two to four weeks after infection, after which time the titres continued to rise. The individual ELISA values to the metabolic antigen are recorded in Appendix Table 16.2 and group mean values in Fig.16.1. In all four animals there was a clearly detectable response by two weeks after infection.

Using the non-protein component of the metabolic antigen, none of the infected rabbits showed a precipitin response at any time during the course of the infection. However, the non-protein component of the metabolic antigen gave results which were virtually identical to those obtained with the whole antigen in ELISA (Appendix Table 16.3 and Fig.16.2).

The somatic antigen gave results which were very similar to those obtained with the whole metabolic antigen in both the immunodiffusion test and ELISA (Appendix Tables 16.4, 16.5 and Fig.16.1).

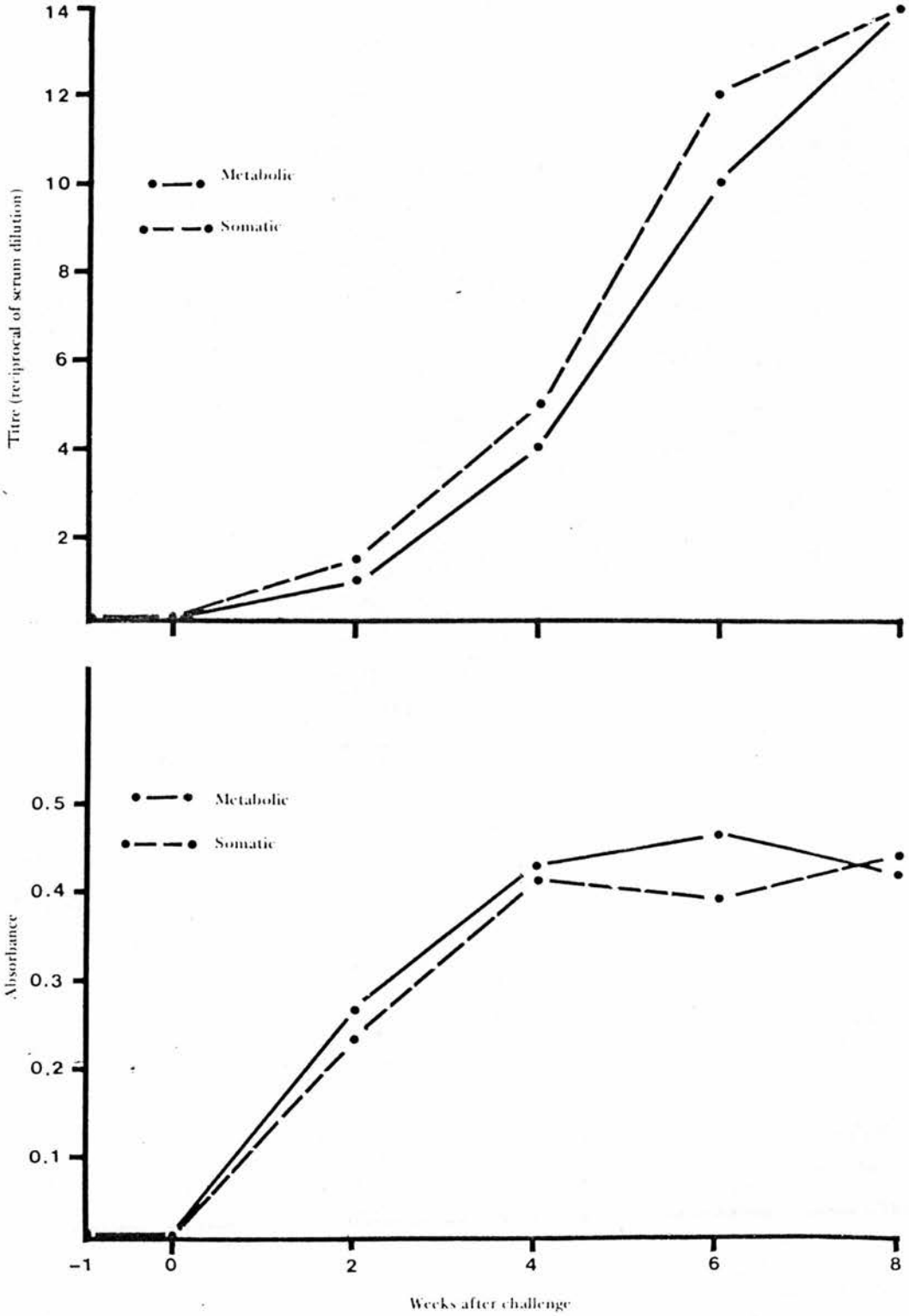

Fig. 16.1. Serological response of rabbits infected with F. hepatica to whole metabolic antigen and somatic antigen. Group mean immunodiffusion titres (upper figure) and group mean ELISA values (lower figure).

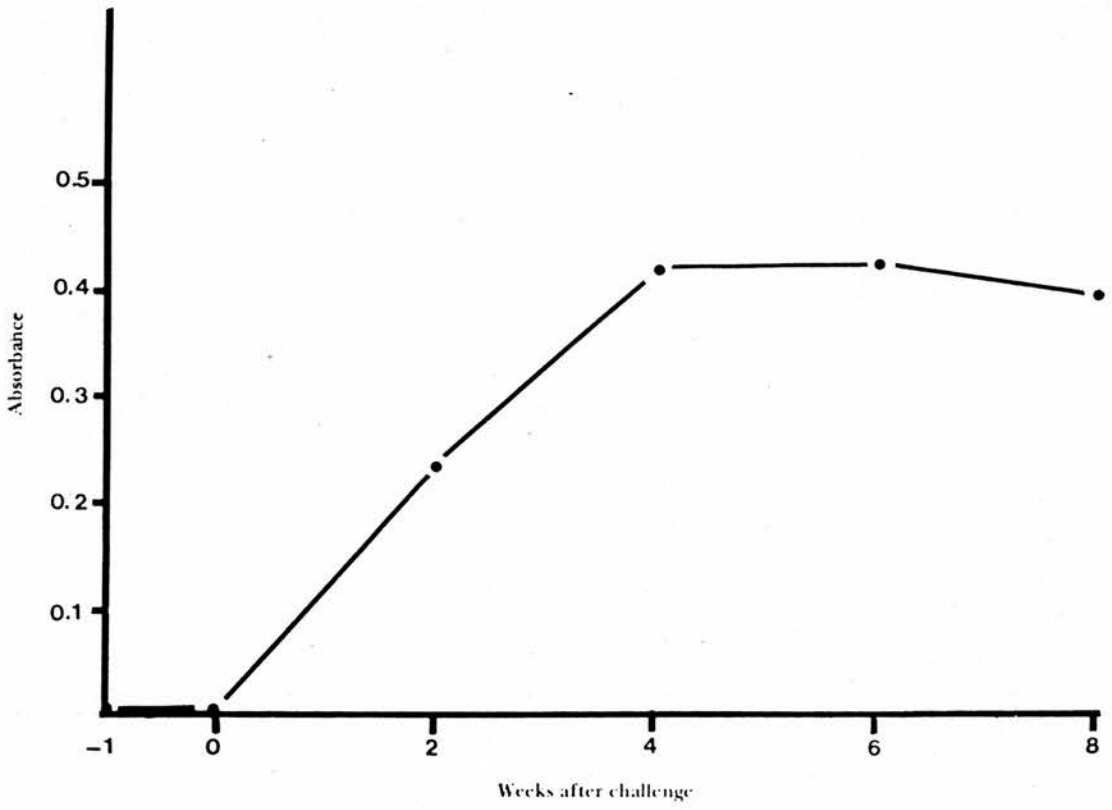

Fig. 16.2. Serological response of rabbits infected with F. hepatica to the non-protein component of the metabolic antigen. Group mean ELISA values.

The serological responses of the infected sheep to the whole metabolic antigen were similar to those of rabbits. The individual immunodiffusion titres are shown in Appendix Table 16.6 and group mean titres in Fig.16.3. Again a response was detectable by two to four weeks after infection. Using ELISA, a response was observed in all the animals by two weeks after infection. Individual ELISA values are recorded in Appendix Table 16.7 and group mean values in Fig.16.3.

The use of the non-protein component of the metabolic antigen gave virtually identical results to those obtained with the whole antigen in both the immunodiffusion test and ELISA (Appendix Tables 16.8, 16.9 and Fig.16.4), as did the somatic antigen (Appendix Tables 16.10, 16.11 and Fig.16.3).

In the case of the infected rats, all four animals showed a positive response to the metabolic antigen by two weeks after infection, in both the immunodiffusion test and ELISA (Appendix Tables 16.12, 16.13 and Fig.16.5).

The two infected calves showed only weak serological responses, despite the fact that the reagents were used at greater concentrations than with the previous species. The individual immunodiffusion titres are recorded in Appendix Table 16.14. A response was detectable by four weeks after infection in both animals, but there was little subsequent increase in titres. The ELISA responses were small and transient, (Appendix Table 16.15). In each animal a small increase in absorbance was seen at two weeks after infection, which increased

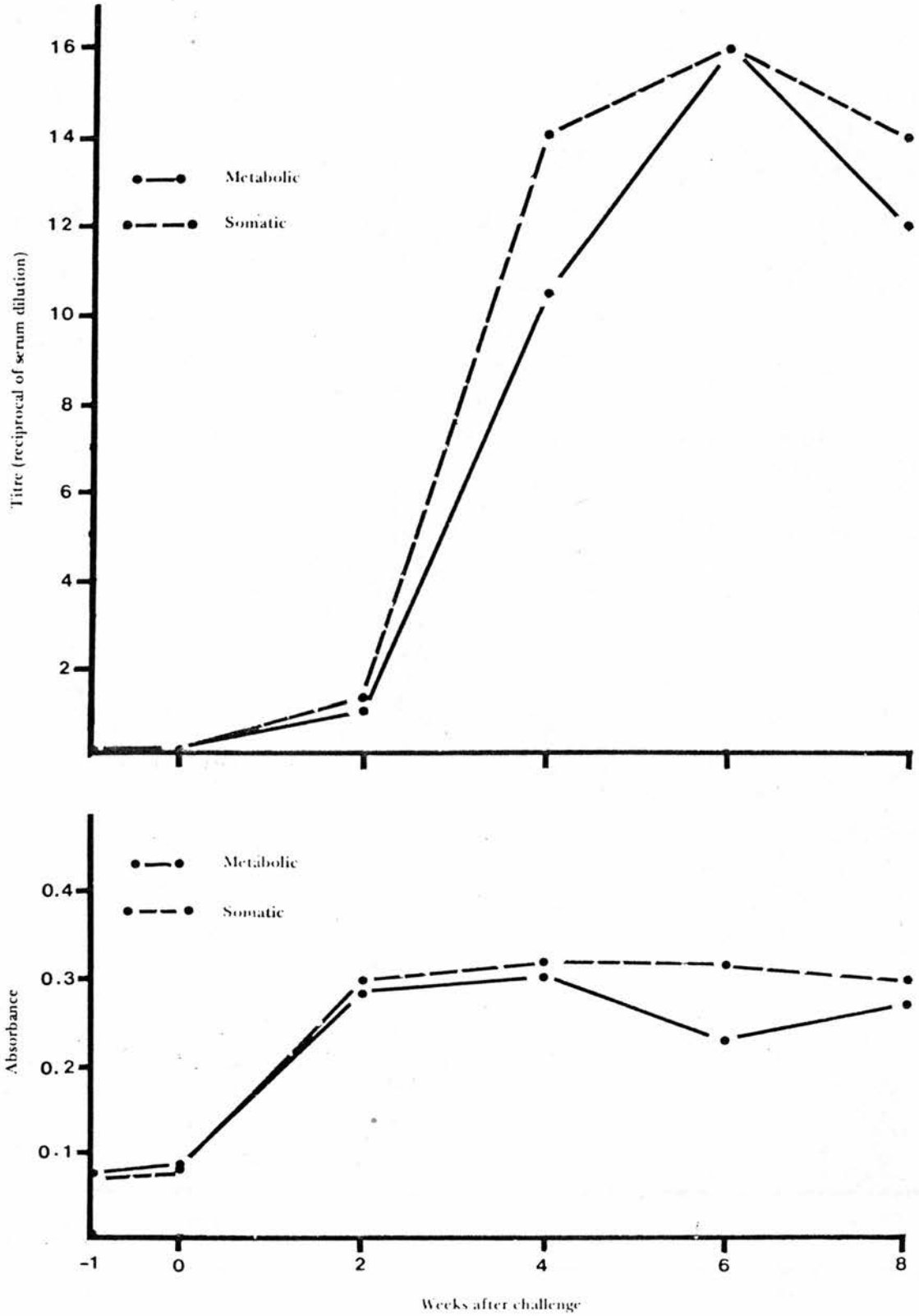

Fig. 16.3. Serological response of sheep infected with F. hepatica to whole metabolic antigen and somatic antigen. Group mean immunodiffusion titres (upper figure) and group mean ELISA values (lower figure).

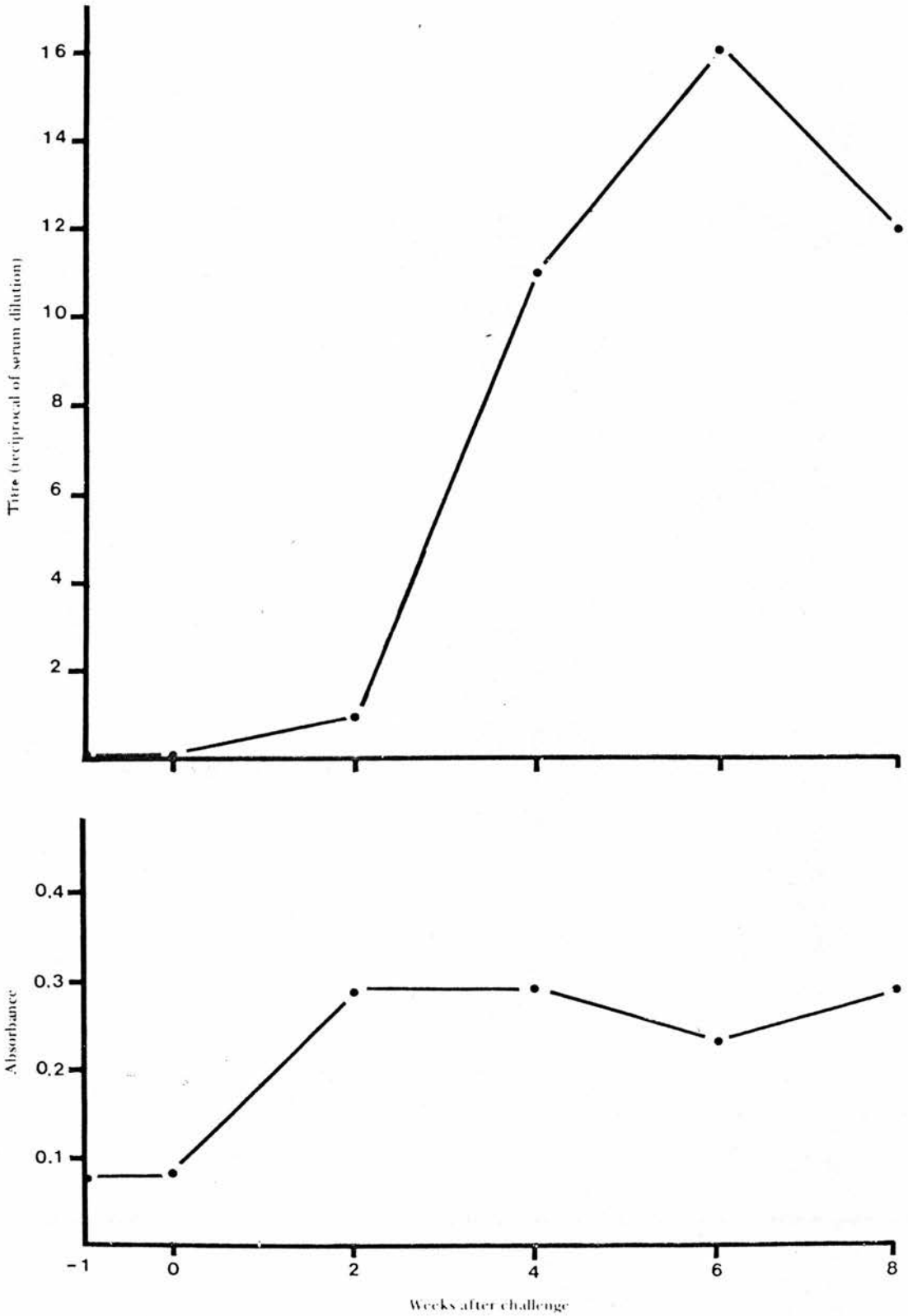

Fig. 16.4. Serological response of sheep infected with F. hepatica to non-protein component of the metabolic antigen. Group mean immunodiffusion titres (upper figure) and group mean ELISA values (lower figure).

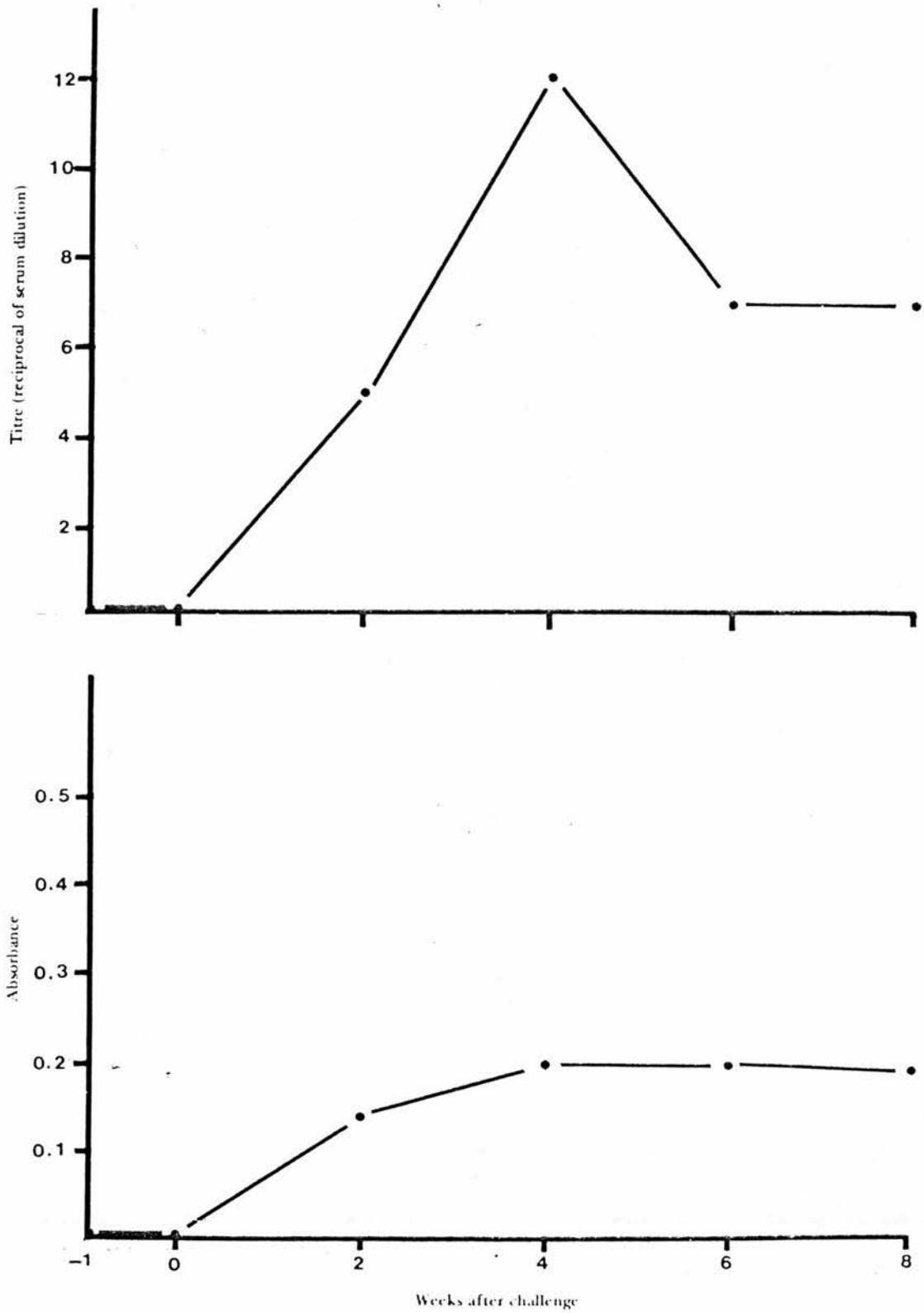

Fig. 16.5. Serological response of rats infected with F. hepatica to whole metabolic antigen. Group mean immunodiffusion titres (upper figure) and group mean ELISA values (lower figure).

slightly for three or four weeks before declining to normal levels. The group mean immunodiffusion titres and ELISA values are shown in Fig.16.6.

Discussion

In the case of the infected rabbits and sheep it appeared that ELISA was able to detect the serological response somewhat earlier than immunodiffusion, although the differences were not great. The infected rats however showed strong immunodiffusion reactions as well as increased ELISA values by two weeks after infection.

The two calves showed only weak responses, as measured by either technique. However, immunodiffusion appeared to give the more satisfactory results, the responses being detected for a longer period than by ELISA. Other workers have commented on the relatively poor serological response of cattle to F. hepatica as compared to other host species (Van Tiggele and Over, 1976). The two calves used in the present experiments were given only relatively small doses of metacercariae and it is possible that these were not highly infective. It would therefore appear that further studies involving the administration of higher infective doses would be necessary to establish the real value of ELISA as a method of diagnosing bovine fascioliasis.

The present results confirmed the earlier findings that the non-protein component of the metabolic antigen gave no immunodiffusion reaction with infected rabbit serum, but did react in ELISA with that species. In contrast, the non-protein antigen was active in both

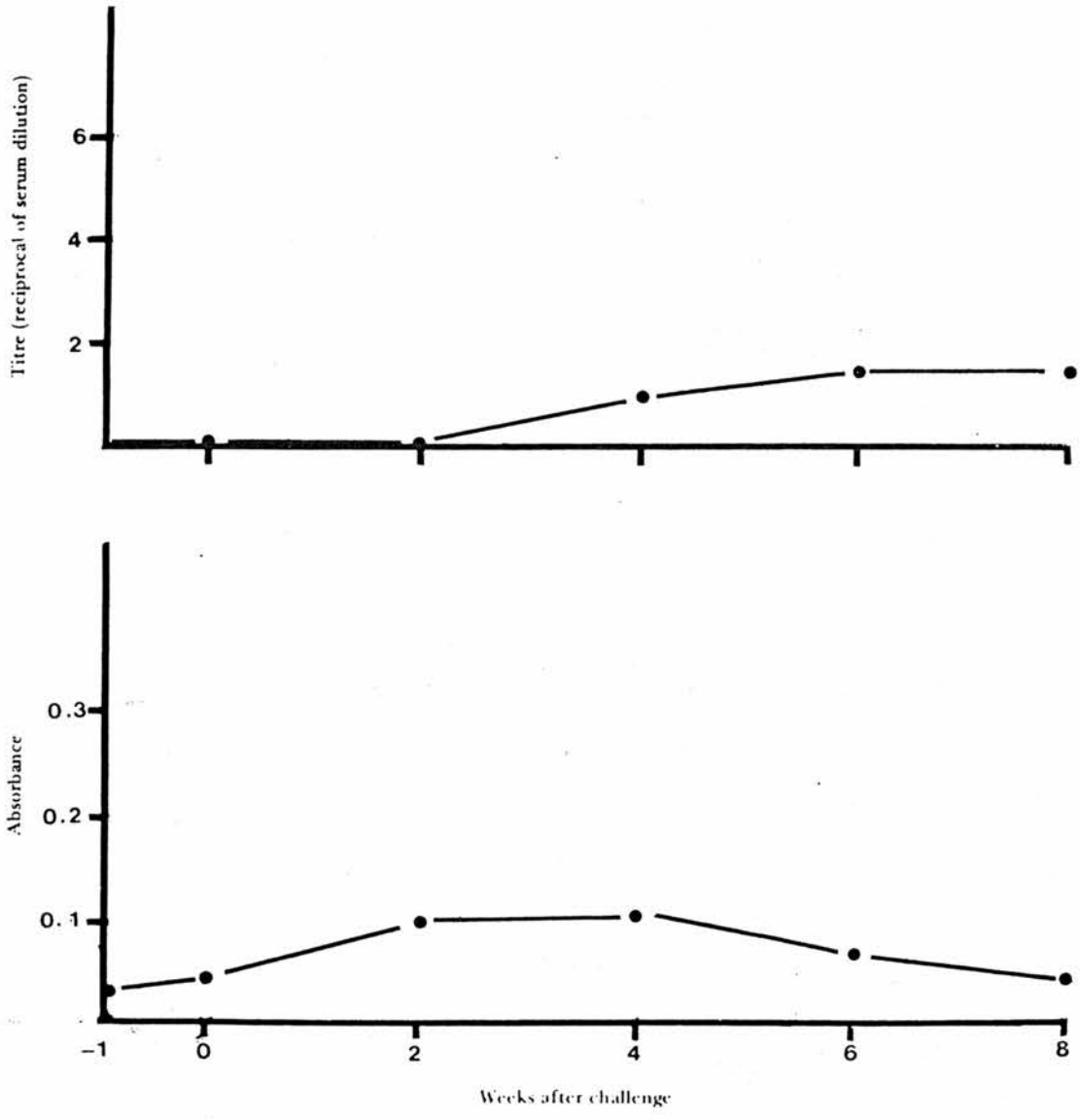

Fig. 16.6. Serological response of calves infected with F. hepatica to whole metabolic antigen. Group mean immunodiffusion titres (upper figure) and group mean ELISA values (lower figure).

immunodiffusion and ELISA with infected sheep serum.

There was no evidence to suggest that metabolic antigen was more favourable than somatic antigen, or vice versa, in either of the tests.

On the basis of these studies, ELISA appeared to have little advantage over the simpler technique of immunodiffusion, apart from allowing for the detection of the immune response slightly earlier in the case of infected rabbits and sheep. It would perhaps have been interesting to have extended these studies so as to discover whether the immune response was detectable sooner than two weeks after infection when using ELISA and also to compare the two methods in more long standing infections. However, it should be borne in mind that the major need for immunological methods of diagnosing fascioliasis is during the prepatent period of the infection, since coprological methods are generally adequate at later stages.

ELISA does provide a more accurate quantification of the immune response and it would be interesting to use the technique to study the relative responses of animals infected with increasing numbers of metacercariae, in order to see whether the degree of response was related to the severity of the infection.

As indicated earlier, there is little reason to suppose that ELISA would allow greater specificity and accuracy than alternative immunodiagnostic methods in the diagnosis of field cases of fascioliasis, unless a purified and species-specific antigen was used in the test. The apparently complex nature of the metabolic antigen

as used in these studies would suggest that it would not be particularly suitable for use in the serodiagnosis of fascioliasis in the field without additional purification. However, should such specific antigens become available, there can be little doubt that ELISA would be a most useful diagnostic method, if only for the fact that it utilises such small quantities of antigen.

Preparation of enzyme-labelled anti-immunoglobulin is a time-consuming procedure, which in certain circumstances might preclude the use of ELISA as a serodiagnostic method. The availability of commercial preparations at a reasonable price would overcome this problem. In the present study, only two of the four commercial preparations had sufficient activity to make their use on a large scale economically viable. However if commercial enzyme preparations with adequate activity were available for all species, ELISA would offer a useful means of assaying large numbers of sera, since the actual test is extremely easy to perform and lends itself to automation.

CHAPTER SEVENTEEN

FRACTIONATION OF THE METABOLIC ANTIGEN ON SEPHADEX G-200

Introduction

A number of the earlier studies had indicated that the crude metabolic antigen obtained by the *in vitro* maintenance of adult *F. hepatica* contained a range of active components, which reacted to different degrees with infected serum from different host species.

Fractionation of the antigen by the process of gel filtration through Sephadex G-200 was undertaken in an attempt to further identify the active components. Passage of a solution containing a mixture of ingredients through a column of Sephadex G-200 results in the fractionation of the constituents in accordance with their molecular size, larger molecules passing through the column more rapidly than small molecules, which become trapped within the gel particles.

Experimental design

A 1 ml sample of metabolic antigen derived from adult flukes taken from a sheep, containing 800 µg of protein per ml, was applied to the column of Sephadex G-200. Fifteen fractions of the eluant were obtained and each was concentrated to the original volume of 1 ml. The fractions were tested for antigenic activity by immunodiffusion and ELISA against infected rabbit, sheep and rat sera. The fractions were also tested by immunodiffusion against a serum produced by immunising rabbits with whole sheep serum, in order to identify the sheep serum contaminants in the antigen.

A 1 ml sample of the same antigen, which had been boiled for 10 minutes to destroy the proteins, was similarly fractionated and tested against infected sheep serum.

The approximate molecular weights of the various fractions were calculated according to the method of Andrews (1964, 1965).

Results

The elution profile of the whole antigen and the location of the 15 fractions used in subsequent studies are shown in Fig.17.1.

The immunodiffusion titres of these fractions against serum from infected rabbits, sheep and rats are shown in Table 17.1. It was evident that the infected rabbit serum was reacting only with components of the antigen within fractions 10-14, whilst sheep and rat sera showed additional activity with fractions 4-7 and 5-6 respectively. Fractions 10-14 were calculated to contain molecules of molecular weight 10,000 - 25,000, whilst fractions 4-7 contained larger molecules within the molecular weight range 100,000 - 500,000.

The reactions of the antigen fractions with the infected sera in ELISA are shown in Table 17.2. In this case the antigenic activity was found to lie mainly in the higher molecular weight fractions for all three species. Rabbits and sheep showed major ELISA activity in fractions 4-7, with lesser activity in fractions 11-13 and 10-11 respectively. The infected rat serum showed ELISA activity with fractions 4-6 only.

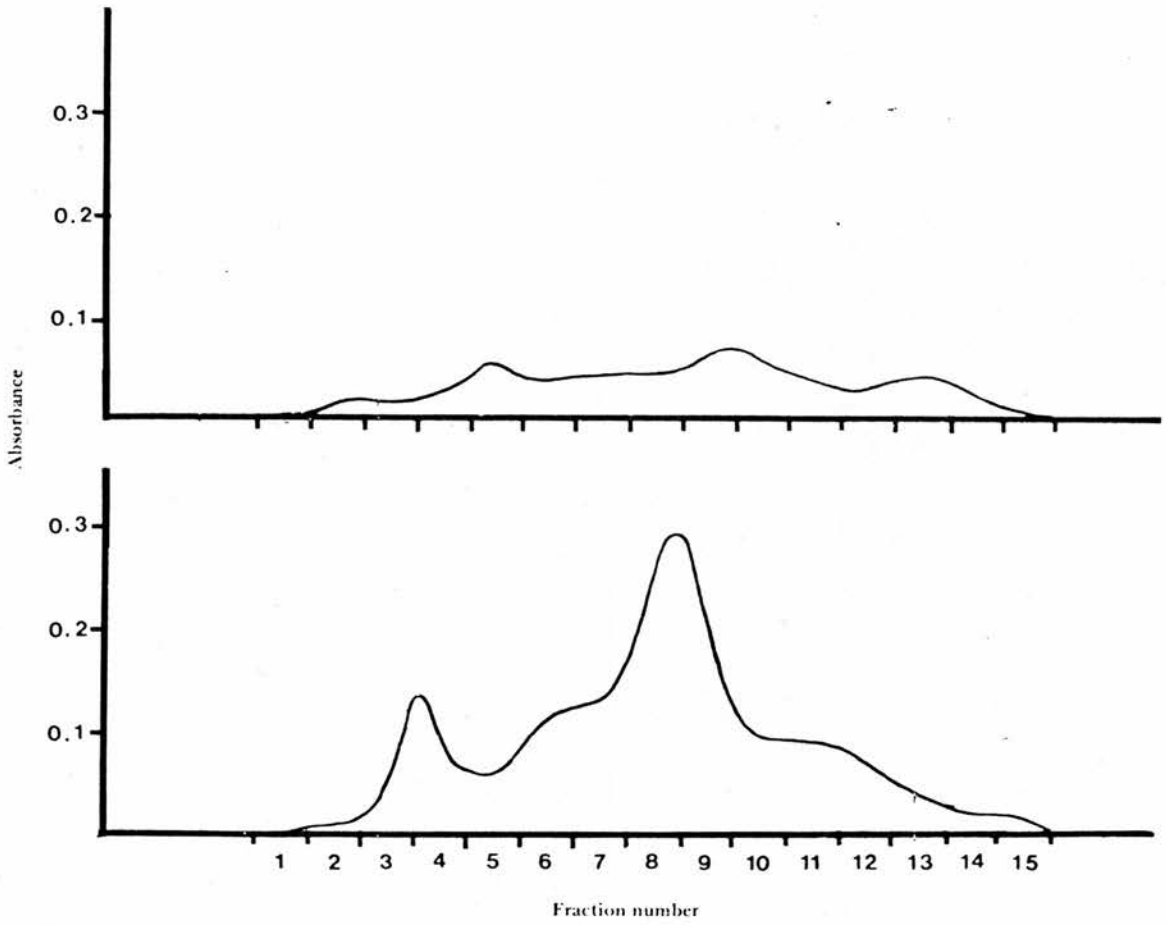

Fig. 17.1. Fractionation of metabolic antigen on Sephadex G-200. Elution profiles of heat-treated antigen (upper figure) and whole antigen (lower figure).

Fraction no.	Immunodiffusion titres			
	Rabbit serum	Sheep serum	Rat serum	Rabbit-anti-sheep serum
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	0	1	0	1
5	0	1	2	1
6	0	1	1	2
7	0	1	0	4
8	0	0	0	2
9	0	0	0	0
10	1	2	1	0
11	1	4	1	0
12	2	4	2	0
13	1	2	2	0
14	1	0	0	0
15	0	0	0	0

TABLE 17.1. Immunodiffusion titres (reciprocals of serum dilutions) of fractions of metabolic antigen obtained by gel filtration on Sephadex G-200 with infected rabbit, sheep and rat sera and with rabbit-anti-sheep serum.

Fraction no.	ELISA values		
	Rabbit serum	Sheep Serum	Rat Serum
1	0.00	0.08	0.00
2	0.00	0.07	0.00
3	0.00	0.08	0.00
4	0.06	0.11	0.08
5	0.19	0.15	0.10
6	0.10	0.12	0.06
7	0.15	0.14	0.00
8	0.01	0.07	0.00
9	0.01	0.07	0.00
10	0.00	0.11	0.00
11	0.03	0.10	0.00
12	0.09	0.07	0.00
13	0.04	0.08	0.00
14	0.00	0.07	0.00
15	0.00	0.07	0.00

TABLE 17.2. ELISA values (units of absorbance) of fractions of metabolic antigen obtained by gel filtration on Sephadex G-200 with infected rabbit, sheep and rat sera.

The immunodiffusion reactions of the antigen fractions with the rabbit-anti-sheep serum are shown in Table 17.1. These confirmed that the metabolic antigen was heavily contaminated with host materials.

The elution profile of the heat-treated antigen is shown in Fig.17.1. It was evident that a large proportion of the components of the whole antigen must have been protein in nature and had been destroyed by boiling.

The immunodiffusion and ELISA reactions of the fractions of the heat-treated antigen with infected sheep serum are shown in Table 17.3. It was apparent that the antigenic activity which remained was located within fractions 4-6 in each case, corresponding to the high molecular weight components of the antigen.

Discussion

These experiments confirmed the earlier findings that the metabolic antigen contains a range of antigenically active components which react to differing degrees with the serum from different host species infected with F. hepatica.

It was shown that the whole metabolic antigen contained high molecular weight substances which would react with infected sheep and rat sera in the immunodiffusion test, but not with infected rabbit serum. However, the high molecular weight components reacted with sera from all three species in ELISA and it was also shown that the antigenically active non-protein components of the antigen were

Fraction no.	Immunodiffusion titre	ELISA value
1	0	0.08
2	0	0.07
3	0	0.07
4	1	0.11
5	1	0.14
6	1	0.10
7	0	0.07
8	0	0.07
9	0	0.08
10	0	0.07
11	0	0.07
12	0	0.07
13	0	0.08
14	0	0.08
15	0	0.07

TABLE 17.3. The immunodiffusion titres (reciprocals of serum dilutions) and ELISA values (units of absorbance) of fractions of non-protein metabolic antigen obtained by gel filtration on Sephadex G-200, with infected sheep serum.

located within these high molecular weight fractions.

The whole antigen also contained lower molecular weight components which reacted in the immunodiffusion test with sera from all three species and to a lesser degree with serum from rabbits and sheep in ELISA.

Gel-filtration only separates components of a mixture according to their molecular size and is not capable of distinguishing different components of a mixture which have similar molecular sizes. Thus these experiments were not able to distinguish different antigens with similar molecular weights.

However, the extent of the overlap in the activity of the various fractions within the high and low molecular weight groups with sera from the different species indicated that there were probably a range of different antigenic components within each of these broad categories.

These observations thus tended to confirm the view that the whole metabolic antigen was composed of a variety of different antigenically active substances. In view of this, it would seem to be unlikely that such an antigen would be suitable for use in the serodiagnosis of field cases of fascioliasis without further purification to try and isolate any species-specific components.

CHAPTER EIGHTEEN

CONCLUSIONS AND DISCUSSION

This series of experiments revealed a number of interesting facts about the metabolic antigens of F. hepatica, including some which were of significance to the application of such antigens to the serodiagnosis of fascioliasis, or to attempts to immunise the host species.

The initial experiment showed that adult flukes could be maintained in large groups for reasonably prolonged periods of time in a recirculating continuous-flow culture apparatus, in which they continued to produce metabolites with antigenic properties during at least a nine day culture period, even when maintained in a protein-free medium. However, it was apparent that the rate of production of these antigenic substances declined during this period. From the evidence obtained in Section 1, it would seem likely that the production of such metabolites would be maintained at a higher level if the medium contained a high proportion of serum. However, as previously noted, the inclusion of large amounts of serum in the medium creates problems, in that it is then impossible to concentrate the medium to the extent necessary to allow further studies on the protein antigens.

Although it was evident that flukes maintained in this way were liberating materials with antigenic properties into their environment, it was of some interest to know whether these substances were similar in nature to metabolic antigens produced by flukes in vivo. It might

be expected that flukes maintained in a protein-free medium would be utilising their endogenous food reserves to maintain essential processes and would thus be in a state of overall catabolism. Under these circumstances, it would seem likely that the products liberated into the environment would differ from those resulting from the normal metabolic activities of the organism *in vivo*. However, comparative immunodiffusion of metabolic antigen obtained from flukes maintained *in vitro* with that present in the bile of a rabbit carrying a patent fluke infection revealed apparent immunological identity between these two entities. Although this simple test does not prove conclusively that the metabolic antigen produced *in vitro* is identical to that produced *in vivo*, it does provide evidence in favour of that hypothesis. Furthermore, if the antigens produced by flukes *in vitro* were the result merely of continuing abnormal catabolic activities, or even autolysis, it might be expected that the quantities produced would increase with time. The studies on the rate of production of metabolic antigen *in vitro* revealed in fact that there was a fairly rapid decline in their output.

This evidence that the metabolic antigen produced *in vitro* had properties in common with that produced *in vivo* was of particular significance to the intended use of the *in vitro* derived product in immunisation studies. Obviously, there would be more likelihood of successful immunisation of the host animal with metabolic antigen produced *in vitro* if this was known to share immunological properties with the metabolic antigens produced by flukes *in vivo*.

The fact that metabolic antigen produced *in vitro* was inevitably

contaminated with materials originating from the host species was also of significance in immunisation studies. As previously noted, the simplest method of avoiding the introduction of such foreign species' materials into the animal to be immunised would be to produce the antigen from flukes taken from the same species of host as that to be immunised. Another possibility would be to attempt to remove the foreign species' materials from the antigen prior to immunisation.

Several of the experiments in this series indicated that the metabolic antigen derived from flukes maintained in vitro was comprised of a number of components which were capable of reacting to different degrees with sera from infected animals. In particular, it was apparent that there were high molecular weight non-protein substances which would react with sheep and rat sera in the immunodiffusion test but not with rabbit serum. However, these substances would react with the sera of all three species in ELISA. The metabolic antigen also contained lower molecular weight heat-labile substances, which showed activity in the immunodiffusion test with serum from all three species and in ELISA with rabbit and sheep sera.

It was shown that metabolic antigen could be used to follow the serological response which developed in animals experimentally infected with F. hepatica, using both the immunodiffusion technique and ELISA, a serodiagnostic method which has not previously been applied to fascioliasis.

On the basis of the present experiments, there was no evidence that metabolic antigen was preferable to somatic antigen in either of the serodiagnostic methods studied. As previously noted, problems associated with the serodiagnosis of helminth infections frequently become more apparent in studies on field cases, rather than on experimentally infected animals maintained under controlled conditions. The apparently complex nature of the metabolic antigen would seem to indicate that it would not be of particular value for the serodiagnosis of field cases of fascioliasis, without first undergoing additional purification, with the aim of identifying antigenic components specific to F. hepatica. However, metabolic antigen is probably less complex than somatic antigen and may therefore be a more suitable preparation on which to undertake further purification procedures. In order to assess the value of such purified antigens for the serodiagnosis of fascioliasis, it would be necessary to undertake field studies on a wide scale and correlate serological findings with alternative evidence of infection such as from coprological or post mortem examinations.

ELISA appeared to be a promising serodiagnostic technique, on which further studies would be justified. The present studies confirmed earlier reports that the technique was sensitive and highly economical in its use of reagents. In view of these properties and the fact that the assay lends itself to the bulk handling of serum samples, it would seem that ELISA could be most useful in extensive field studies.

SECTION THREE

IMMUNISATION OF THE MAMMALIAN HOST WITH METABOLIC ANTIGENS OF FASCIOLA HEPATICA

CHAPTER NINETEEN

REVIEW OF THE LITERATURE

The use of helminth metabolic antigens as immunogens

The development of adequate methods of maintaining helminths *in vitro* has provided a means for the collection of metabolic antigens, which can then be used to immunise the host species. There is now extensive evidence to indicate that such antigens can initiate protective immunity to a greater or lesser degree.

Spindler (1937) appears to have made the first attempt to use metabolic antigens for immunisation. He used clarified digests of rabbit muscle, which had contained *Trichinella spiralis* cysts. Following oral administration to rats, rabbits and guinea pigs, these provoked a degree of resistance to subsequent experimental intestinal trichinosis.

Amongst early reports on successful immunisation with metabolic antigens derived from nematodes maintained *in vitro* were those of Thorsen (1951, 1953) who used *Nippostrongylus muris*, Campbell (1955), Chute (1956), Chipman (1957) and Ewart and Olsen (1961), all using *Trichinella spiralis*, Thorsen (1956a,b) using *Ancylostomum caninum* and Soulsby (1957a,b) who used *Ascaris lumbricoides*. Silverman, Poynter and Podger (1962) claimed that guinea pigs could be successfully immunised against *Dictyocaulus viviparus* and *Trichostrongylus colubriformis* and rabbits against *Strongyloides papillosus*, using antigens derived from *in vitro* cultures. Soulsby (1963), using antigens from cultured third stage larvae of *Ascaris*

suum, was able to demonstrate resistance of immunised guinea pigs to oral challenge. He noted that the antigens were labile and their activity was lost if they were frozen or freeze dried. Denham (1967) also recorded that freeze drying of metabolic antigens of Trichinella spiralis destroyed their ability to induce protective immunity in mice and Robinson (1967) similarly found that fresh metabolic antigens were more effective than lyophilised antigens for protecting calves against infection with Dictyocaulus viviparus. As previously discussed, there is evidence that metabolic antigens have enzymic properties and this may account for their apparent lability.

Crandall and Arean (1965) were able to partially immunise mice using secretions and excretions of second stage larvae of Ascaris suum and likewise Guerrero and Silverman (1969) reported that metabolic antigens harvested from third stage Ascaris suum larvae in vitro were capable of inducing resistance in mice, which was almost comparable to the resistance which resulted from a combined primary and rechallenge infection programme. Guerrero and Silverman (1972) also showed that metabolic antigens from second and third stage larvae would protect mice against a normally lethal challenge of Ascaris eggs.

Ozerol and Silverman (1970) collected metabolites from third and fourth stage Haemonchus contortus larvae maintained in vitro, which they then characterised by biochemical, serological and physical means. Lambs which had been immunised with such antigens showed reduced faecal egg counts after challenge, as compared to control animals. The most active metabolites were proteins. Scott,

Silverman, Mansfield and Levine (1971) made similar findings. However, Neilson (1975) failed to stimulate protective immunity in lambs vaccinated with metabolic antigens of Haemonchus contortus, the activity of which had been identified by immunoelectrophoresis.

Rose (1976) attempted to immunise lambs by the intra-muscular injection of concentrated metabolites of Ostertagia circumcincta and also by the oral administration of lyophilised metabolites. There was evidence for protective immunity in each case.

Rickard and Bell (1971b) demonstrated a high degree of immunity in lambs challenged with Taenia ovis eggs after immunisation with antigens collected during eight days of in vitro cultivation of Taenia ovis and Taenia hydatigena. Herd, Chappel and Biddell (1975) reported that immunisation of dogs with metabolic antigen derived from adult Echinococcus granulosus led to a highly significant reduction in egg production on challenge.

There are very few accounts of attempts to immunise animals with antigens derived from cultured F. hepatica. Healy (1955) injected pooled regurgitated caecal contents into rabbits, but failed to demonstrate any immunity on challenge. Lang (1976) immunised mice with a single intraperitoneal injection of the culture medium (Medium 199) in which 16-day-old flukes had been incubated for four hours. Worm recovery after challenge was not significantly less than from control mice, but host mortality was significantly reduced. However, when mice were immunised with medium in which such flukes had been incubated for a total of 24 hours, worm recovery in the immunised

group was reduced by 83.3%. The mice were immunised by the intraperitoneal route with aliquots of medium taken after 2,7,12 and 24 hours of culture. The mice were challenged 30 days later. This report is of great interest since it implies that the juvenile intrahepatic stage of F. hepatica can initiate protective immunity. Lang also showed that a somatic antigen derived from 16-day-old flukes would not stimulate protective immunity.

In addition to these attempts to induce protective immunity by vaccination with metabolic antigens derived from helminths maintained in vitro, a number of workers have tried to expose the host animal to metabolic antigens produced in vivo, whilst at the same time avoiding exposing the host to other pathological effects associated with the infection. This may be achieved by implanting the parasite at a site removed from its normal location within the host, where it will survive for some time but is unable to significantly damage the host. This technique obviously has technical difficulties and would be of no practical significance as a means of immunising against helminth infections. However it is of great academic interest. Soulsby (1957b) implanted viable Ascaris eggs subcutaneously into guinea pigs. The eggs hatched and underwent a modified life cycle. Significant resistance to a subsequent challenge infection was recorded. Crandall and Arean (1965) implanted live second stage Ascaris suum larvae contained in a diffusion chamber into the peritoneal cavities of mice and produced partial immunity to challenge. Similarly Rickard and Bell (1971a) demonstrated that antigens released by larvae of Taenia ovis contained in diffusion chambers in the peritoneal cavities of lambs would stimulate protective immunity.

Smithers and Terry (1967a,b) induced immunity to Schistosoma mansoni in rhesus monkeys by direct surgical transfer of live worms to the hepatic portal system of previously uninfected animals. Transfer of dead worms did not result in protective immunity.

Gold and Lengy (1972) transferred adult flukes to the peritoneal cavities of mice and challenged the mice with metacercariae 10 days later. No immunity was demonstrated, but it was shown that the flukes would survive for prolonged periods. Ross (1967c) implanted flukes into the intercostal muscles of sheep, but was able to demonstrate only slight evidence of protective immunity on challenge. Hughes and Harness (1972) described a method of transferring F. hepatica from the bile ducts of infected animals to the body cavities of recipient animals. They showed that such flukes would survive for prolonged periods. Eriksen and Flagsted (1974) transferred flukes from cattle, sheep and goats into the subcutis of rats. On challenge four weeks later, there was a 50% reduction in the infection rate as compared to control animals. They suggested that this immunity could only have arisen from stimulation of the host's immune system by antigens liberated from the transplanted flukes.

Hughes, Anderson and Harness (1975) and Anderson, Hughes and Harness (1975) also implanted flukes subcutaneously into rats. They studied the subsequent pathology of the adjacent lymph nodes, noting the characteristic changes associated with a cell-mediated immune response. The rats also developed positive skin hypersensitivity and formed precipitating antibodies. Challenge of these animals resulted in a 34% reduction in the number of adult flukes developing

in the bile ducts, although histopathological changes in the livers of immunised and control animals were equally severe.

Hughes, Anderson and Harness (1976) further showed that if rats were sensitised by subcutaneous implantation of adult flukes and then challenged by intraperitoneal implantation of adult flukes, 23% of the challenge infection were killed. Similarly, if rats were sensitised by a normal oral infection of F. hepatica, 71% of the intraperitoneal challenge flukes were killed. In contrast, neither of these sensitisation routes was effective against a subcutaneous challenge with adult flukes.

Such experiments are of great significance, since they clearly demonstrate that resistance to fascioliasis can occur, even in the absence of pathological changes in the liver resulting from previous infections.

Evidence for the development of acquired resistance to F. hepatica by host species

Although there are now a number of very effective anthelmintic drugs, which will kill F. hepatica, even at a very early stage in its development in the mammalian host, there can be little doubt that an effective vaccine against fascioliasis would be of major importance.

As was pointed out by Stoll (1961), attempts to induce protective immunity in a host species can only succeed in those cases where the host-parasite relationship favours the development of immunity in nature. It is only within relatively recent years that

it has been conclusively demonstrated that certain species of host can develop significant natural immunity to fascioliasis. This has been achieved by administering repeated infections, with or without the elimination of the previous infection by anthelmintics. In those animals in which immunity has developed, the repeat infections give rise to a smaller population of flukes than is seen in previously uninfected control groups. Unfortunately, such experiments do not distinguish between purely immunological resistance and resistance due to other factors such as pathological changes in the liver resulting from the earlier infections. However, the technique of fluke transfer, which was discussed earlier, has somewhat resolved this problem.

It is well established that different species of hosts have differing susceptibilities to natural infection with F. hepatica. Thus Ross (1967a) recorded that cattle and pigs have a higher natural resistance than sheep, possibly to some extent due to the more fibrous type of liver. Boray (1969) classified host species according to their resistance, placing the pig, dog and cat in an early resistance group, bovine, man and horse in a delayed resistance group, and sheep, goat, rabbit, mouse and rat in a low resistance group. Doyle (1971, 1972) showed that flukes were not maintained for prolonged periods in the bovine, with the majority of the fluke burden being shed 16-30 weeks after infection. This phenomenon was apparent prior to the well documented calcification of the bile ducts which occurs in infected cattle and was associated with an increase in immunologically competent cells in the biliary tissues. In contrast, Durbin (1952) demonstrated that flukes can survive for

extremely prolonged periods in sheep, probably equal to the life span of the host.

In addition to the variation in susceptibility to F. hepatica seen in different host species, a number of workers have noted differences relating to age, sex and strain within the same species. Thus Armour and Dargie (1973) and Goose and MacGregor (1974) noted that female rats were more resistant than males and that old rats were more resistant than young rats. Hughes and Harness (1974) again found that male rats were more susceptible and also reported differences between two strains of rats. However Urquhart (1954) found that the sex or breed of rabbit was immaterial. Hayes, Bailer and Mitrovic (1974a) reported differences in the susceptibility to infection of rats of different ages.

There have been a number of reports of successful demonstration of acquired resistance by the repeated infection method. Lang (1967) stimulated mice with two infections of two metacercariae each and then challenged these mice, along with control animals. He recorded that in the previously infected mice the flukes migrated more quickly to the bile ducts and significantly fewer flukes were recovered from the bile ducts 40 days after challenge. Lang suggested that a delayed hypersensitivity reaction was involved. Lang and Dronen (1972) and Lang (1974a) transferred young flukes of different ages into mice, prior to challenge, and concluded that a migration period of at least 10-11 days within the liver was necessary to stimulate subsequent immunity.

Similar results have been obtained in rats by Hayes, Bailer and Mitrovic (1972, 1973), Goose and MacGregor (1973a,b), Armour and Dargie (1974) and Čorba amd Špaldonová (1975). Goose and MacGregor (1973a,b) showed that if adult flukes were transferred to the peritoneal cavities of previously infected rats, they were rapidly killed, whereas the flukes which were already present in the bile ducts remained unharmed. They considered that this indicated that the flukes in the bile ducts were protected by some means, possibly by host mimicry, as is the case with schistosomes. However, the studies of Hughes and Harness (1973a,b) failed to show a 'host-antigen' effect with F. hepatica. Hayes, Bailer and Mitrovic (1975) further showed that resistance could develop in splenectomised rats.

Attempts to demonstrate acquired immunity to F. hepatica in rabbits have generally shown that this species of host is not capable of achieving resistance to any great extent. Although a number of workers (e.g. Ross, 1966b; Kendall 1967a,b; Kendall, Herbert, Parfitt and Peirce, 1967; Fortmeyer, 1973) have been able to demonstrate a reduced number of flukes developing from a challenge infection administered subsequent to a primary infection, the reduction has rarely been of statistical significance. Kendall (1967a,b) and Kendall, Herbert, Parfitt and Peirce (1967) suggested that the differences seen between immunised and control groups of rabbits after challenge were probably the result of liver pathology and the adverse effects of overcrowding of flukes in those animals which had received the primary infection, rather than any purely immunological factor.

Kendall and Sinclair (1971) were unable to demonstrate resistance in rabbits which had received a primary infection, followed by challenge. However, if the primary infection was terminated with hexachlorophene two days prior to the challenge infection, significantly fewer flukes were recovered from the challenge. It was suggested that the destruction of the existing parasites by the drug might have led to an outpouring of antigen which provoked a delayed hypersensitivity response, or that destruction of the flukes led to the release of toxic substances, which had an adverse effect on the challenge infection. Bolbol (1975) was unable to demonstrate significant resistance in rabbits which had received a single preliminary infection prior to challenge, but if two preliminary infections were given, significant resistance to challenge could be demonstrated.

Similarly, attempts to demonstrate acquired immunity in sheep have often yielded poor results. Boray (1967a,b) reported that previous exposure of sheep to high level infections of normal metacercariae did not lead to an appreciable resistance to reinfection. However, repeatedly infected sheep lived longer, developed anaemia later, and showed a less severe tissue reaction on challenge than did the control animals. He concluded that the hepatic fibrosis resulting from the earlier infections was influencing the course of the challenge infection.

Sinclair (1962) was unable to demonstrate significant resistance to reinfection on challenging sheep which had received four primary

infections of 100 metacercariae at weekly intervals. However, there was some evidence for later fluke maturation, and diminished egg production in the immunised animals. Sinclair (1968) demonstrated accelerated development and enhanced pathogenicity of F. hepatica in lambs which had received daily doses of corticosteroids prior to infection. Splenomegaly and hepatic fibrosis were diminished in the treated lambs, as compared to the controls. A further indication of an immunological factor in the pathogenesis of fascioliasis in sheep was the finding of Sinclair (1970b) that splenectomy led to reduced resistance to a primary infection. Splenectomised sheep developed a higher fluke burden, with a greater proportion of large flukes and more severe clinical signs and pathological changes than did normal control animals.

Sinclair (1970a, 1971a, 1973, 1975) in a series of experiments involving repeated administration of metacercariae to sheep, followed by challenge infection, was able to demonstrate evidence for an acquired immunity, in terms of alterations in the pathological response to challenge in the immunised animals, together with retardation of fluke development during the challenge infection. However in most cases the number of flukes which developed in the previously infected animals was not significantly less than that in the control animals. Sinclair (1973, 1975) was able to demonstrate that resistance in sheep was not solely a result of the pathological changes in the liver due to the previous infections. He compared animals which had had a single long-term stimulating infection with animals which had had a series of stimulating infections, each of which had been terminated after one week by anthelmintic treatment,

so as not to cause significant liver damage. Both these groups showed a similar response to challenge, with evidence of immunity as previously noted.

Rushton, Murray, Armour and Dargie (1974) found that fluke migration to the bile ducts of sheep reinfected after a primary infection was delayed in those animals which were treated with anthelmintic prior to challenge, but not in untreated animals. Since the degree of hepatic fibrosis was the same in each case, they suggested that there must have been an immunological element involved in the difference, resulting from the stimulus associated with the destruction of the flukes by the anthelmintic.

There is much stronger evidence for effective acquired immunity in cattle. Kendall (1967a,b) showed that whereas cattle given repeated doses of metacercariae had a diminishing output of fluke eggs, with a very small residual fluke population, sheep similarly treated showed increasing faecal egg counts and a large fluke population. Boray (1967a) showed that if cattle were infected with 1000 metacercariae and the infection was then terminated by anthelmintic treatment 14 to 19 weeks later, they had appreciable resistance to a challenge dose of 5000 metacercariae, as compared to previously uninfected control animals. Ross (1966a, 1967b) reported that if cattle were given a second infective dose (300 metacercariae) 18 weeks after a preliminary dose (200 metacercariae), the existing infection was eliminated from the host animal. Ross described this phenomenon as a 'self-cure' reaction. Ross (1966a) also reported that calves which had been previously infected developed a lighter fluke burden on challenge

than did the control animals. Ross (1967c) further showed strong acquired immunity in calves challenged with 200 metacercariae after a previous infection of 1500 metacercariae given 30 weeks earlier. There was a reduction of more than 60% in the numbers of flukes developing and those which did develop were retarded. The number of animals used was too small to make the results statistically reliable, but Ross claimed that they were strongly suggestive. Doyle (1971, 1972, 1973) was also able to demonstrate the development of acquired resistance to F. hepatica in calves in a series of experiments which involved the administration of challenge infections subsequent to preliminary infections. The development and magnitude of the acquired resistance was found to be related to the degree of development of the initial infection. Thus calves challenged 17 weeks after the first infection showed greater resistance than calves reinfected after 12 weeks, whilst calves challenged only seven weeks after the primary infection did not develop appreciable immunity.

There is thus ample evidence that various species of host animals can develop acquired resistance to infection with F. hepatica and therefore attempts to induce immunity by artificial methods may be undertaken with some hope of success. Amongst those methods which have been used are the administration of attenuated forms of the infective stage, vaccination with fluke antigens, transfer of immune serum and transfer of lymphoid tissue.

One of the major problems facing workers within this field is the well documented variability in the host response which is seen when similar animals are given similar infective doses of F. hepatica.

This variability covers not only such parameters as the haematological and pathological changes in the host, but also the numbers and sizes of flukes which eventually reach maturity. Thus in order to obtain results which are of statistical significance, it is necessary to use adequate numbers of animals. A number of reports have failed in this respect, which may account for the apparently conflicting results obtained by different groups of workers who have undertaken similar experiments.

The use of irradiated metacercariae to stimulate resistance to F. hepatica

The development of the successful vaccine against Dictyocaulus viviparus, which utilises X-ray attenuated larvae of that species (Jarrett, Jennings, Martin, MacIntyre, Mulligan, Sharp and Urquhart, 1958) has stimulated the search for a similar vaccine against F. hepatica. The principle behind such a vaccine is that the infective stage of the parasite which has been exposed to X-irradiation fails to develop to maturity within the host animal, but still stimulates an immune response as a result of exposure of the host to the necessary functional antigens.

Although various workers have shown that metacercariae of F. hepatica may be attenuated by irradiation (Hughes, 1962a,b, 1963; Wikerhauser, 1961b; Dawes, 1963e, 1964; Movsesijan and Čuperlović, 1970), attempts to demonstrate protective immunity at subsequent challenge have provided varied results. Thorpe and Broome (1962) claimed to have demonstrated acquired immunity in rats using this technique, but their results have been questioned on statistical

grounds (Dawes and Hughes, 1964). Dargie, Armour, Rushton and Murray (1974) reported that sheep given repeated doses of irradiated metacercariae showed a marked, but not statistically significant, reduction in the numbers of flukes developing after a challenge infection as compared with control animals. Armour and Dargie (1974) showed that rats exposed to normal or irradiated metacercariae developed resistance of a similar magnitude. Bitakiramire (1970) reported that a high level of protection (98%) was possible in cattle challenged with F. gigantica subsequent to the administration of two doses of irradiated metacercariae of that species. However, Armour and Dargie (1973) cited unpublished work of Armour, Dargie and Doyle, who were only able to obtain a 68% level of protection using a similar immunising schedule, but on a different breed of cattle and using F. hepatica rather than F. gigantica. Nansen (1975) reported immunity of a similar level in calves challenged after administration of irradiated cysts of F. hepatica.

Immunisation with antigens derived from F. hepatica

A number of reports have been made on attempts to immunise animals by injection of somatic antigens derived from F. hepatica and rather fewer on the use of metabolic antigens (discussed earlier).

The majority of experiments which have used somatic fluke antigens have failed to demonstrate significant immunity, and reports which have claimed success have often failed to be repeatable.

Kerr and Petkovitch (1935) used a whole worm extract of adult flukes to immunise rabbits by the intraperitoneal route. They

reported that on challenge five weeks later the immunised animals developed significantly fewer flukes than non-immunised control rabbits. Also some of the flukes in the immunised animals were seen to be calcified. These results have been questioned on statistical grounds and Hughes (1963) repeated this experiment, using the same antigen. The first time that the repeat experiment was undertaken, the immunised animals appeared to develop strong and statistically significant resistance to a challenge infection. However, Hughes was unable to confirm these results in a subsequent experiment and he concluded that his first success, although statistically significant, must have arisen by chance.

Urquhart, Mulligan and Jennings (1954) immunised rabbits by the intra muscular route with an alum-precipitated protein antigen derived from adult flukes. These workers noted that the flukes recovered from the immunised animals 63 days after challenge contained less tissue nitrogen and were smaller than those recovered from the control animals. However, the same number of flukes were recovered from each group and the liver pathology was similar, although the livers containing the most retarded flukes showed the most marked inflammatory response. It was suggested that this was the result of a delayed hypersensitivity response resulting from the immunisation. Healy (1955) attempted to immunise rabbits with an extract of adult flukes, but was unable to demonstrate acquired immunity. There was some evidence of retardation of flukes recovered from immunised animals 35 days after challenge and it was also noted that some flukes from the immunised animals were producing abnormal eggs.

Shibanai, Tozawa, Takahashi and Isoda (1956) also immunised rabbits with antigen derived from adult flukes, which was administered by a variety of routes. The route of administration of the antigen was claimed to affect the severity of the lesions resulting from a subsequent challenge infection.

Babadzhanov and Tukhmanynts (1958) cited by Geyer (1967) claimed to have produced complete resistance in rabbits immunised by intra-venous administration of a polysaccharide antigen derived from adult flukes. Full details were not given, and the work does not appear to have been repeated.

Ershov (1959) prepared a polysaccharide (60%) and albumin (30-40%) antigen from adult flukes, which gave rise to short term immunity in lambs. 10-25% of lambs were immune to infection, whilst others showed lower susceptibility. However on challenge 45 days after immunisation none of the animals were immune.

Hughes (1963) immunised sheep and rabbits with a variety of antigens including whole powdered flukes, a metacercarial antigen, Kerr and Petkovitchs' antigen (q.v.), Ershov's antigen (q.v.) and a metabolic antigen. All proved negative in inducing protective immunity.

Geyer (1967) failed to produce resistance in rabbits immunised by various routes with soluble saline material containing protein, carbohydrate and lipid. A light inhibition of the flukes recovered from immunised animals was recorded. Similarly Ross (1967c) reported retarded growth of flukes recovered from rabbits immunised with

extracts of six week old immature flukes. Ross (1967c) also combined subcutaneous injection of fluke homogenate with intramuscular implantation of live six week old flukes in rabbits, lambs and calves, but again was unable to demonstrate protective immunity on challenge.

Transfer of immune serum

A number of reports have indicated that serum from animals infected with F. hepatica, if transferred to recipient animals, may provide a degree of protection against a subsequent challenge infection.

Thus Hayes, Bailer and Mitrovic (1974b,c) demonstrated significant immunity in rats given intraperitoneal serum from a subacute seven to eight week old infection. Serum from a chronic infection (25 weeks) failed to provide protection. Significant immunity was only seen if the serum was given close to the time of challenge, being of no value if given six to eight days post-challenge. The protective effect of the serum was found to be diminished by heating or adsorption with fluke antigens.

Armour and Dargie (1974) demonstrated that the degree of protection obtainable by transfer of immune serum to rats was dependent on the volume given, with the greatest effect being seen in those individuals receiving the largest volume. Homologous or heterologous sera gave comparable effects. These workers noted that when there was a high degree of immunity, there was no evidence of liver damage, indicating that the young flukes had been killed before penetrating the liver.

Dargie, Armour and Urquhart (1973) and Armour and Dargie (1973) have reported the successful demonstration of passive immunity to F. hepatica by transfer of immune serum in rats, cattle and sheep. In the case of sheep and cattle, it was necessary to administer very large volumes of immune serum. It was shown that IgG was necessary for protection, no effect being seen with IgM.

Transfer of lymphoid tissue

A few attempts have been made to demonstrate protective immunity to F. hepatica by administering lymphoid tissue taken from a fluke-infected animal to a previously uninfected recipient animal, which is then challenged. In order to avoid the problems of tissue rejection, it is necessary to use either highly inbred strains or monozygotic twins in such experiments.

Lang, Larsh, Weatherly and Goulson (1967) first demonstrated the transfer of immunity by this technique, using mice. Corba, Armour, Roberts and Urquhart (1971) successfully used rats and a pair of monozygous twin calves. In the experiments using rats, these workers utilised donor cells from three types of infection, namely pre-bile duct, bile duct and from rats immunised with three weekly doses of irradiated metacercariae. It was found that lymphoid cells failed to confer immunity if taken four weeks after infection, but they did confer immunity if transferred after eight to ten weeks. Lymphocytes from the animals infected with irradiated cysts also provided immunity if transferred 11 weeks after infection. Armour and Dargie (1974) further showed that the extent of the protection extended to

rats by cell transfer was related to the magnitude and persistance of the antigenic stimulus given to the donors.

Sinclair (1971b) was unable to demonstrate appreciable resistance to challenge in sheep which were given homogenates of lymph nodes and spleen obtained from sheep infected eight weeks previously with 500 metacercariae. However, Armour and Dargie (1973) reported success in an experiment involving the transfer of hepatic lymph node tissue from infected sheep to highly inbred recipients, which developed a high degree of resistance to subsequent challenge.

CHAPTER TWENTY

MATERIALS AND METHODS

Removal of adult flukes from rats

In certain experiments adult flukes were removed from rats and maintained *in vitro*. The method used to obtain the flukes in a sterile condition, suitable for subsequent axenic culture, was as described in Section 1 for the removal of adult flukes from rabbits.

The recovery of intrahepatic stages of F. hepatica

In certain experiments metabolic antigens derived from the *in vitro* culture of 16-day-old flukes were used to immunise laboratory animals.

These flukes were obtained from the livers of rats (which had been infected with 100 metacercariae) or mice (which had been infected with 20 metacercariae). Special precautions were taken in order to extract the flukes in a sterile manner, so as to allow for the setting up of axenic cultures.

The animals were killed by cervical fracture and the ventral thorax and abdomen shaved and prepared for surgery. The abdominal cavity was incised to reveal the liver and, after clamping the common bile duct with fine artery forceps, the whole organ was carefully removed from the body by gently freeing it from its various attachments. The liver was placed in a sterile 60 x 15 mm plastic petri dish (Becton Dickinson U.K. Ltd., Wembley, Middlesex) containing approximately 10 ml of Medium 199 at 37°C. The liver was

gently teased apart with forceps and incubated at 37°C for some ten minutes. On examination under a low power stereoscopic microscope, the young flukes could be seen emerging from the macerated liver. Vigorous and intact specimens were taken up with a pasteur pipette and transferred to a beaker containing a small volume of Medium 199 at 37°C. The young flukes were washed three times by decanting the Medium 199 in the beaker and replacing a similar volume of fresh medium. They were then transferred to the culture in a pasteur pipette.

This process was found to be very much easier in mice than in rats, where the relatively large liver made it difficult to find the young flukes. However, since the flukes recovered in this way were inevitably contaminated with host liver tissue, it was necessary to immunise animals with antigens obtained from flukes taken from the same species of host, so as to avoid the introduction of foreign species' material into the system.

Immunisation procedures

The metabolic antigens were administered either alone, or in Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan). All antigen solutions were passed through a 0.22 µm membrane filter (Millipore U.K.Ltd., London) in order to remove particulate material prior to injection or incorporation with adjuvant. When adjuvant was used, equal volumes of the antigen solution and the well shaken adjuvant were vigorously mixed by being repeatedly drawn into a syringe and then expelled through a wide bore needle, until the

required water-in-oil emulsion had been established. The emulsion was then administered by deep intra-muscular injection through a 20 gauge needle.

Administration of challenge infections

When immunised and control animals were to be challenged, metacercariae originating from the same snail-shedding were used for all the animals, so as to avoid possible differences in the infectivity of different batches of metacercariae. Metacercariae were scraped off the polythene on which they had encysted as previously described. The required number were placed in a watch glass, rejecting any which did not show the characteristic granular appearance of viability.

Rabbits were infected using the gelatine capsule technique, whilst rats and mice were infected by means of a stomach tube, as previously described.

Immunoadsorption of sheep serum proteins from metabolic antigen

As previously noted, metabolic antigen was invariably contaminated with materials originating from the host animal from which the flukes had been taken. In order to diminish interference by such foreign materials, prior to the immunisation of rats with metabolic antigen derived from flukes which had been obtained from sheep, an attempt was made to remove sheep serum proteins from the crude antigen by immunoadsorption.

Rabbit-anti-sheep whole serum was prepared by immunising rabbits

with sheep serum, using the same regime as that described for the immunisation of rabbits with sheep IgG in the preparation of peroxidase-labelled antiglobulin. Each injection consisted of 0.5 ml of sheep serum (8.0 mg/ml) emulsified in an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan). The rabbit-anti-sheep serum so obtained was insolubilised with glutaraldehyde according to the method of Avrameas and Ternynck (1969). Ten millilitres of the serum were dialysed overnight at 4°C against normal saline and 1.0 ml of 1M acetate buffer (pH 5.0) was then added. Three millilitres of a 2.5% aqueous solution of glutaraldehyde were then added dropwise, with stirring and the resulting gel allowed to stand for three hours at room temperature. The insolubilised protein was homogenised and prepared for immunoadsorption as previously described.

Sheep serum proteins were adsorbed from the metabolic antigen by mixing an appropriate volume of the whole antigen with the insolubilised rabbit-anti-sheep serum. After stirring at room temperature for 30 minutes, the mixture was centrifuged at 2,500 g for 15 minutes. The supernatant was tested by immunodiffusion against rabbit-anti-sheep serum to indicate the effectiveness of the removal of sheep serum proteins from the metabolic antigen.

Recovery of flukes from immunised and control animals

The animals were killed seven or eight weeks after challenge with metacercariae. The abdominal cavity was opened to expose the liver and the common bile duct clamped with a fine pair of artery forceps at its point of attachment to the duodenum. The whole liver

was then carefully freed from its attachments, and removed from the body. The bile ducts were opened and flukes removed. The liver was then cut into pieces approximately 1 cm square, which were incubated at 37°C for two hours in a small volume of normal saline. At the end of this period, the saline was carefully examined and any flukes which had emerged from the hepatic tissue were recovered. By gently squeezing the pieces of liver, which had been softened by the incubation process, it was possible to liberate any remaining flukes.

The flukes thus recovered were counted and measured. The flukes were laid on a piece of paper, taking care not to distort their natural shape. Lengths and breadths were measured using a pair of geometrical dividers.

In the experiment involving the immunisation and challenge of mice, it was necessary to recover flukes from animals which died during the course of the challenge infection, at a time when the flukes were still within the hepatic parenchyma. The abdominal cavity, having been opened as described above, was washed out several times with saline in order to recover any flukes which had migrated out of the liver on the death of the host. Those flukes which had remained within the liver were then recovered by a process similar to that described above.

Haematological techniques

Total leucocyte counts were performed on well mixed blood samples, using an electronic cell counter (Model FN, Coulter Electronics, South Dunstable, Bedfordshire) in accordance with the

manufacturer's instructions.

Blood films were prepared and stained by the Undritz modification of the peroxidase reaction as described by Archer (1965), in order to distinguish the eosinophils, which stain greenish yellow. The percentage of eosinophils was then assessed by performing a differential count and the total eosinophil count derived from the previously assessed total leucocyte count.

Glutamate dehydrogenase assay

Estimation of serum glutamate dehydrogenase was shown by Sewell (1967c) to be a useful indicator of the severity of the liver damage in fascioliasis.

The assay was carried out using the technique described by Ford and Boyd (1962). The reduction in the optical density of the enzyme/substrate mixture was measured at 340 nm on an 'SP 1800' spectrophotometer (Pye Unicam, Cambridge) and recorded graphically on an 'AR 2' Linear Recorder (Pye Unicam, Cambridge).

A reduction in the optical density of 0.001 is equivalent to the oxidation of 4.83×10^{-4} moles of the diphosphopyridine nucleotide (DPNH) and the enzyme activity of the serum was expressed in micromoles of product (i.e. DPN) produced per litre of serum per minute (i.u.s).

CHAPTER TWENTY-ONE

IMMUNISATION OF RABBITS WITH METABOLIC ANTIGEN DERIVED FROM ADULT F. HEPATICA

Introduction

An experiment was undertaken to determine whether rabbits immunised with metabolic antigens obtained from adult flukes maintained *in vitro* would respond with the production of antibodies and if so, whether such antibodies provided any protection against a challenge infection with *F. hepatica*.

In order to obtain adequate quantities of antigen, it was anticipated that a relatively large number of flukes would be required for the culture. These would have been most conveniently obtained from a heavily infected sheep. However, as was noted in Section 2, metabolic antigen was invariably contaminated with materials originating from the host animal from which the flukes were taken. Thus in order to avoid the introduction of such foreign species' material into the immunised rabbits, it was considered preferable to make use of flukes taken from infected rabbits as the source of the antigen, even though a number of animals would have to be used in order to obtain sufficient flukes.

In addition, in order to ensure that only the response to antigens specifically of fluke origin was being measured, the antigen used in the subsequent serological studies on the immunised rabbits was a metabolic antigen derived from adult flukes which had been taken from a sheep.

Experimental design

Approximately 90 adult flukes recovered from rabbits were maintained for seven days in a continuous-flow recirculating culture system containing 2 l of Medium 199. At the end of this period the medium was concentrated to a volume of 3 ml, the protein concentration of which was 2.0 mg per ml.

Eleven female New Zealand White rabbits (mean weight 2.5 kg) were randomly divided into two groups of six and five animals respectively. The first group (six animals) were each injected with 0.5 ml of the concentrated medium emulsified in an equal volume of Freund's complete adjuvant. The control group (five animals) were injected with 0.5 ml of Medium 199 emulsified in an equal volume of Freund's complete adjuvant. All injections were made into the muscles of the hind limb.

Four weeks later this immunising schedule was repeated exactly, using antigen of the same protein concentration, prepared as above. After a further four weeks the immunised animals were each given a subcutaneous injection of 0.5 ml of the same antigen without adjuvant, the control animals receiving 0.5 ml of Medium 199. A similar subcutaneous injection was administered after a further two weeks and the animals were challenged with 100 metacercariae each two weeks later.

Eight weeks after challenge the rabbits were killed and the flukes recovered and measured.

The rabbits were bled at fortnightly intervals during the experiment and the serological response to immunisation and challenge measured by immunodiffusion and ELISA. Antigen was used in immunodiffusion at a concentration of 600 µg of protein per ml and in ELISA at a concentration of 6.0 µg of protein per ml, with serum diluted 1/50 and the commercial peroxidase-conjugated goat-anti-rabbit IgG diluted 1/700.

The host response to the challenge infection and the severity of the liver damage were monitored by means of the eosinophilia and plasma glutamate dehydrogenase assay respectively.

A number of studies were made on pooled serum taken from immunised animals prior to challenge (termed immune serum), so as to compare the antibodies present with those in serum of rabbits carrying a patent fluke infection (termed infected serum). Thus, the immune serum was compared with the infected serum by immunodiffusion against metabolic antigen in order to see whether there was any serological identity. The activities of the two types of serum were also studied by immunoadsorption, each serum sample being completely adsorbed with metabolic antigen and then tested by immunodiffusion against the other serum.

The immune serum was tested by immunodiffusion against the fractions of metabolic antigen which were obtained by gel filtration on Sephadex G-200 (Section 2), in order to see which fractions had stimulated an immune response. The immune serum was also tested against the non-protein component of the metabolic antigen in order

to see whether the rabbits had responded to this part of the immunising antigen.

Newly excysted flukes were incubated in normal, infected and immune sera and examined for the development of immune precipitates.

Results

Serology

The immunised animals all produced antibodies to the metabolic antigen, which were detectable by immunodiffusion and ELISA. A serological response was not evident in all animals until six weeks after the initial immunisation. Titres continued to rise thereafter, although there was no evidence of an anamnestic response to the subcutaneous booster injections. After challenge, the titres of the immunised rabbits contined to rise, whilst the titres of the control animals showed the normal expected response, similar to that noted in Section 2.

Individual titres of immunised and control rabbits are recorded in Appendix Tables 21.1 and 21.2, whilst group mean titres are shown in Figs. 21.1 and 21.2. Wide individual variations in titres were noted for animals within the same group at any particular time during the experiment.

Fluke recovery

The numbers of flukes recovered from individual animals are recorded in Appendix Table 21.3 and group means shown in Table 21.1. Again there were wide variations in the numbers of flukes recovered

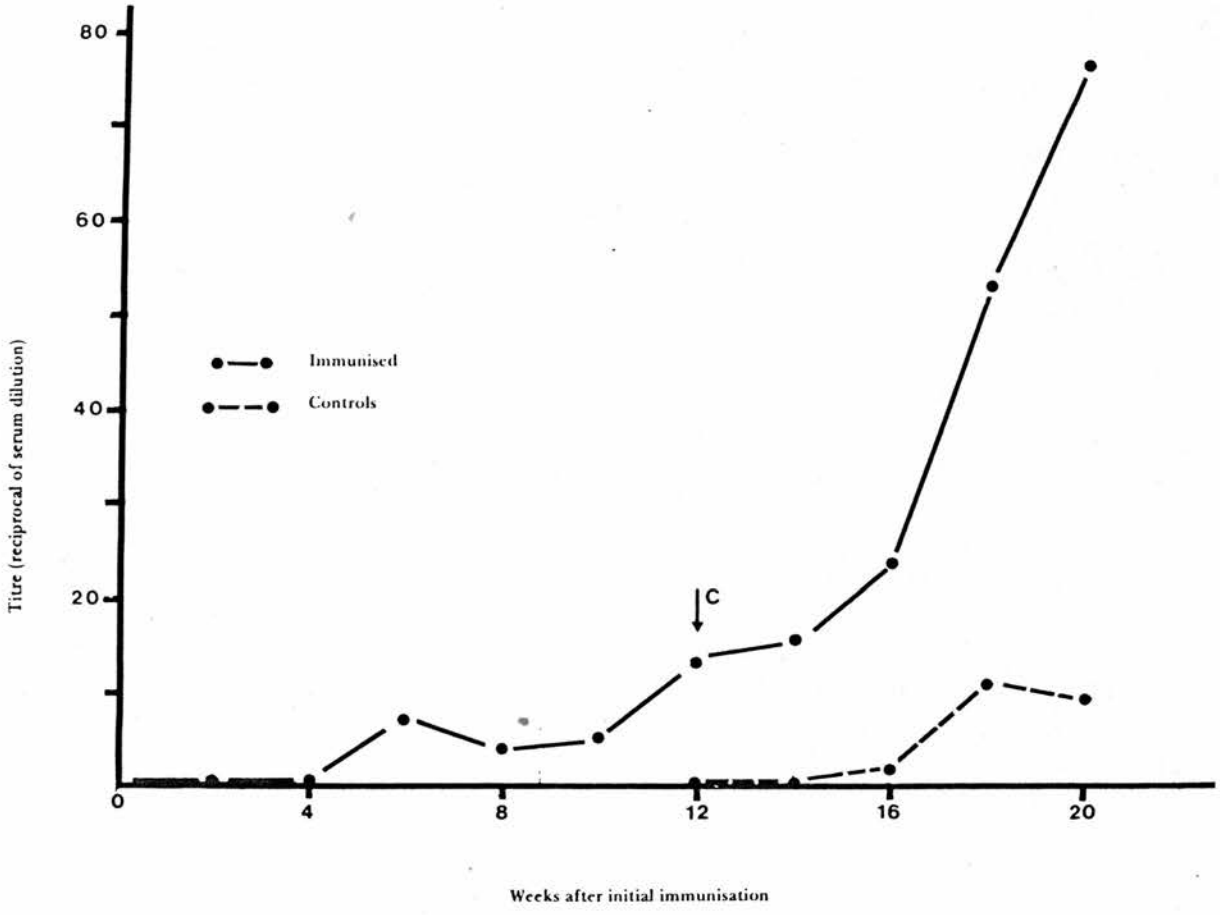

Fig. 21.1. Group mean immunodiffusion titres of rabbits immunised with metabolic antigen derived from adult flukes and of control rabbits.

C = administration of challenge infection.

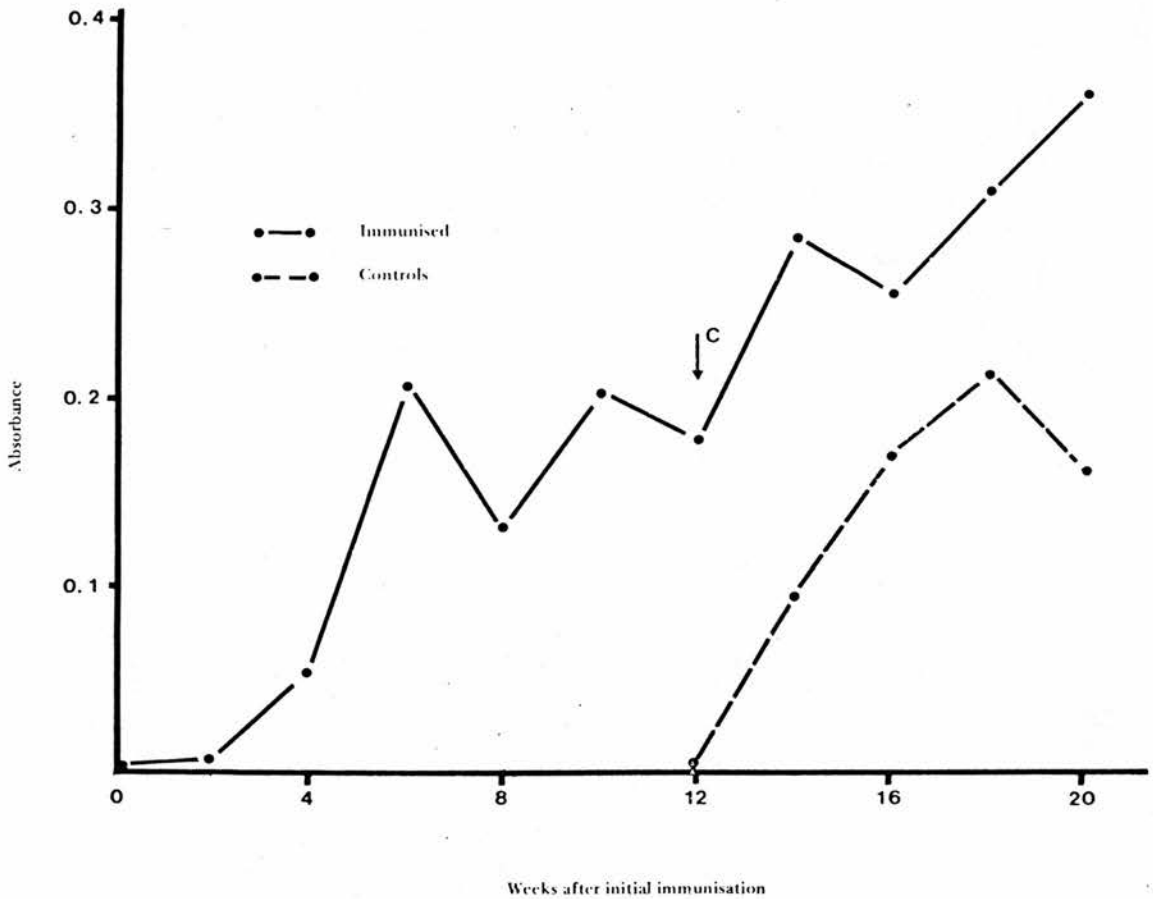

Fig. 21.2. Group mean ELISA values of rabbits immunised with metabolic antigen derived from adult flukes and of control rabbits.

C = administration of challenge infection.

from animals within the same group, but the difference between the two groups was of no statistical significance.

The sizes of the individual flukes recovered are recorded in Appendix Table 21.3 and group means shown in Table 21.1. The differences in sizes of individual flukes recovered from the same rabbit were extremely marked and analysis of variance showed that there were significant differences in the mean sizes of flukes recovered from rabbits in both the immunised and control groups ($F = 3.89$; degrees of freedom = 5, 171; $p < 0.005$ for the immunised group; $F = 12.45$; degrees of freedom = 4, 149; $p < 0.001$ for the control group). However, it was also apparent that the mean size of flukes recovered from the immunised group was significantly smaller than that of the flukes recovered from the control animals ($F = 5.47$; degrees of freedom = 1, 8; $p < 0.05$).

Host response to challenge

Individual haematological results of immunised and control rabbits are recorded in Appendix Table 21.4 and group mean eosinophil counts shown in Fig.21.3. In each case the eosinophilia showed an increase from the second week after challenge. The immunised animals showed lower mean eosinophil counts than did the control animals at four, six and eight weeks after challenge, but the differences were not significant.

Individual plasma glutamate dehydrogenase assays are recorded in Appendix Table 21.5 and group mean values shown in Fig.21.4. In each case the enzyme levels increased from the second week after

	Mean no. flukes recovered per rabbit ± S.D.	Mean size (length x breadth) of flukes recovered (mm^2) ± S.D.
Immunised group	29.5 ± 7.5	30.7 ± 12.2
Control group	30.8 ± 9.7	39.9 ± 14.4
	t = 0.25	F = 55.26 (degrees of freedom = 1, 319)
Significance	$p > 0.3$	$p < 0.001$

Table 21.1. The number and sizes of flukes recovered from rabbits immunised with metabolic antigen derived from adult flukes and from control rabbits eight weeks after challenge.

(See Statistical Corrigenda).

challenge. The immunised animals again showed lower mean glutamate dehydrogenase levels at four and six weeks after challenge, but the differences were not statistically significant.

It was very noticeable that different individuals within the same group showed marked variations in both eosinophil counts and glutamate dehydrogenase levels at any particular time during the course of the challenge infection.

Comparison of immune serum with infected serum.

The two types of serum showed apparent immunological identity on immunodiffusion against metabolic antigen (Plate 21.1). The immunodiffusion reactions of the fractions of metabolic antigen with infected and immune sera indicated that each type of serum contained antibodies against the same range of antigens, activity being noted with Fractions 10-14 in each case. Immunoadsorption studies provided additional evidence for similarity between the two types of sera, as metabolic antigen completely adsorbed with infected serum gave no reaction with immune serum and similarly, after being adsorbed with immune serum, the antigen gave no reaction with infected serum.

There was no reaction between immune serum and the non-protein component of the metabolic antigen.

Newly excysted flukes incubated in infected serum developed very marked precipitates, which virtually covered the whole body within 24 hours. Flukes incubated in immune serum showed only slight evidence of precipitate formation about the oral cavity, whilst flukes

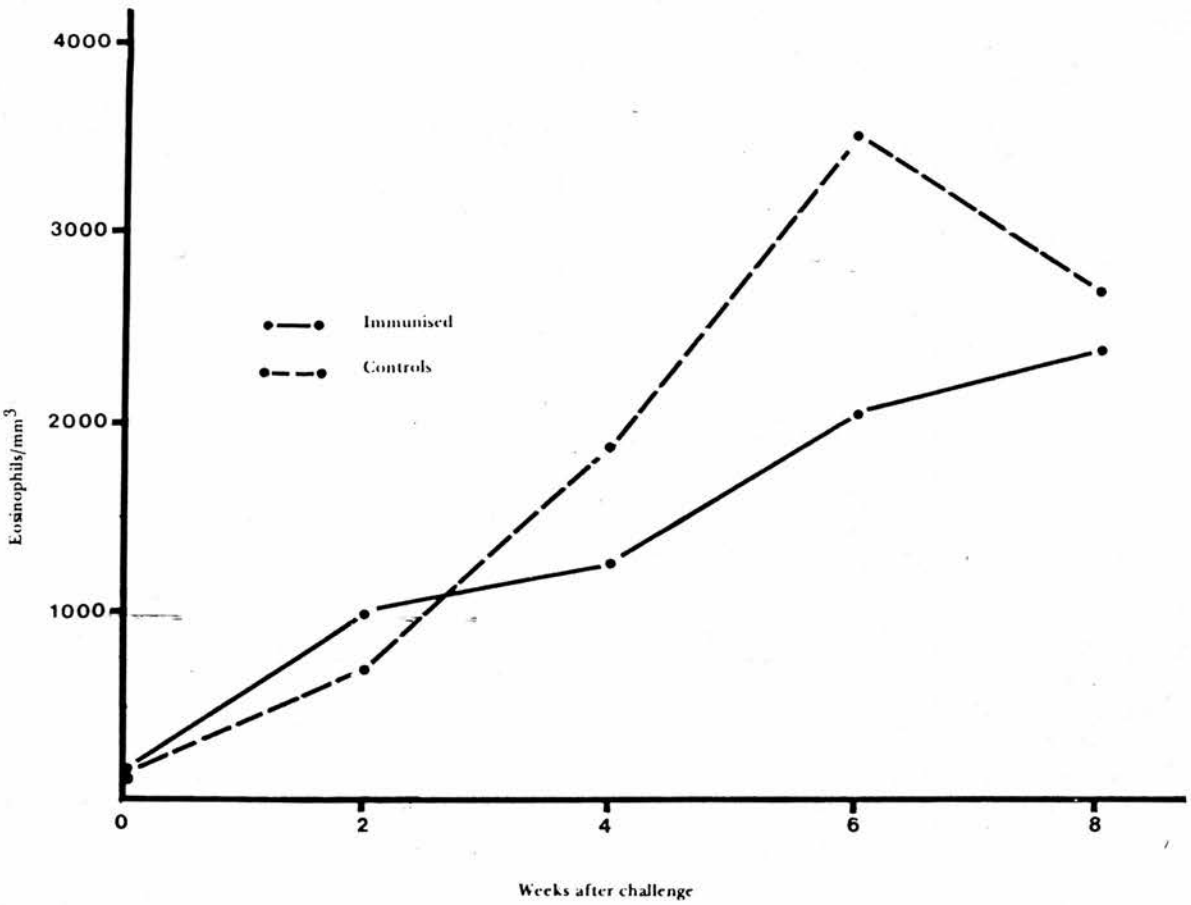

Fig. 21.3. Group mean eosinophil counts of rabbits immunised with metabolic antigen derived from adult flukes and of control rabbits.

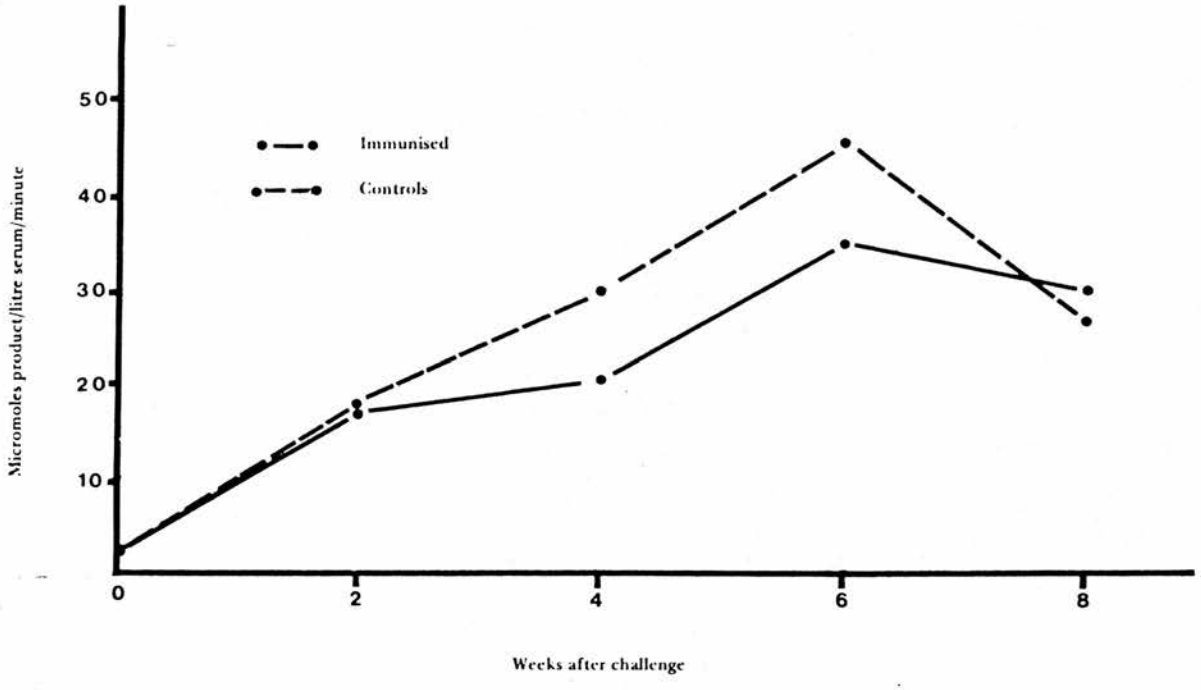

Fig. 21.4. Group mean plasma glutamate dehydrogenase levels of rabbits immunised with metabolic antigen derived from adult flukes and of control rabbits.

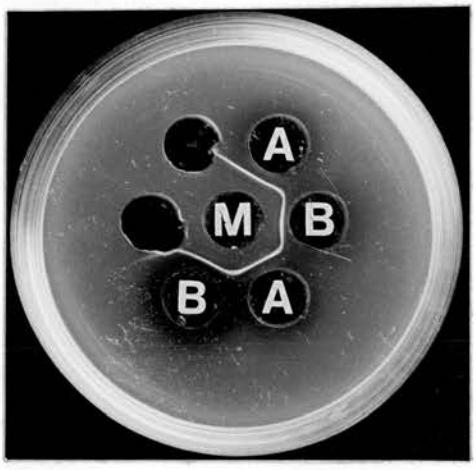

Plate 21.1. Immunodiffusion reactions between metabolic antigen (M) and infected rabbit serum (A) and immune rabbit serum (B).

incubated in normal rabbit serum did not develop precipitates.

Discussion

It was apparent that immunisation of rabbits with metabolic antigen derived from adult F. hepatica stimulated a serological response. However, the antibodies associated with this response provided no protection against a challenge of 100 metacercariae, in terms of the numbers of flukes recovered eight weeks after challenge. There was evidence that the flukes which developed in the immunised rabbits were inhibited to some extent, since the mean size of flukes recovered was significantly smaller than that of the flukes recovered from the control group. There was also evidence that the response to challenge of the immunised rabbits, as indicated by the eosinophilia and plasma glutamate dehydrogenase assays, was somewhat less marked than that of the control rabbits.

These results are similar to those of other workers who have immunised rabbits with somatic fluke antigens. Thus, Urquhart, Mulligan and Jennings (1954), Healy (1955), Geyer (1967) and Ross (1967c) all recorded a degree of inhibition of flukes recovered from immunised rabbits, but were unable to show any differences in the numbers of flukes recovered from immunised or control animals.

The comparative studies on the immune and infected sera gave results which were of some interest, in that they revealed a number of similarities between these two entities. However, the fact that

the precipitation reaction about newly excysted flukes was much weaker in the case of the immune serum indicated that there were also important differences between the two types of sera. As previously noted, it has been shown with certain host species that transfer of serum from infected animals to recipients prior to challenge can provide significant immunity. Thus it is possible that if the serum resulting from immunisation with metabolic antigen had sufficient features in common with the serum resulting from a normal infection, the immune serum might be able to provide immunity, either if transferred to another animal, or more importantly, to the immunised animal itself.

Since the evidence for natural immunity to F. hepatica in rabbits is only slight, it would perhaps have been surprising if immunisation with metabolic antigen had resulted in protection against the challenge infection. However, this experiment had shown that the immunised animals would produce antibodies, which had certain features in common with those present in infected serum and which apparently caused inhibition of the flukes of the challenge infection. It therefore seemed sufficiently encouraging to warrant further experiments on rats and mice, which have both been shown to be more capable of developing resistance to F. hepatica. It was very evident that there was marked individual variation between animals within the same group in the various parameters measured. This has been a common finding of other workers who have studied fascioliasis in the rabbit and other host species and underlines the importance of using adequate numbers of experimental animals if results are to be of statistical value. It is probable that the numbers of rabbits used in this experiment represented the lowest acceptable number and in future experiments larger numbers of animals were used.

CHAPTER TWENTY-TWO

IMMUNISATION OF RATS WITH METABOLIC ANTIGEN DERIVED FROM ADULT F. HEPATICA

Introduction

This experiment was in large measure a repeat of the previous one, but involved the use of rats rather than rabbits.

The rats used in this and subsequent experiments were of a strain which had been shown by the administration of repeated infections, the transfer of infected serum and the subcutaneous transplantation of adult flukes to be capable of acquiring immunity to F. hepatica (Haroun, E.M., personal communication).

As previously noted, in order to avoid the introduction of foreign species' materials into the immunised animals, it would be preferable to make use of metabolic antigen derived from flukes taken from the same species of host as that to be immunised. However, when using rats, it was anticipated that there might be problems in obtaining the large numbers of flukes required to produce the quantities of antigen needed to immunise the necessary number of experimental animals. In this experiment it was therefore decided to immunise the rats with antigens derived from flukes taken from a sheep and to try to minimise the effects of the foreign species' antigens by first adsorbing out the sheep serum proteins, which would be likely to constitute one of the major contaminants. As in the previous experiment, precautions were taken to ensure that only the serological response to antigens specifically of fluke origin was

being measured, the antigen used in the serological studies being a metabolic antigen derived from adult flukes obtained from rabbits. Thus, even if the immunised rats did respond to residual sheep contaminants in the immunising antigen, this response would not be measured.

Experimental design

Approximately 120 adult flukes taken from a sheep were maintained for seven days in a recirculating continuous-flow culture system containing 2 l of Medium 199. At the end of the culture period, the medium was concentrated to a volume of 2.5 ml. Sheep serum proteins were removed from the concentrate by immunoadsorption with insolubilised rabbit-anti-sheep serum, the process being repeated until the concentrate gave no detectable reaction on immunodiffusion against rabbit-anti-sheep serum. The resulting solution had a protein concentration of 6.0 mg per ml. Twenty male Porton Wistar rats (mean weight 270 g) were randomly divided into two groups of ten. Each member of the first group was injected with 0.25 ml of the concentrated medium emulsified in an equal volume of Freund's complete adjuvant. The control group were each injected with 0.25 ml of Medium 199 emulsified in Freund's complete adjuvant. All injections were made into the muscles of the hind limb.

Four weeks later this immunising schedule was repeated. After a further four weeks the immunised animals were each given a subcutaneous injection of 0.25 ml of similar antigen without adjuvant, the control animals each receiving 0.25 ml of Medium 199 subcutaneously. Two weeks after this injection the rats were each

challenged with 20 metacercariae. One of the rats in the control group died at the time of challenge due to inhalation of the infective dose.

The animals were bled fortnightly throughout the experiment. The serological response to immunisation and challenge was measured by immunodiffusion, using antigen at a concentration of 600 µg of protein per ml. The response to the challenge infection was monitored by means of the eosinophilia and plasma glutamate dehydrogenase assay.

The rats were killed seven weeks after challenge and flukes recovered, counted and measured.

Pooled serum from immunised animals prior to challenge was compared with serum from rats carrying a patent fluke infection, using immunodiffusion, immunoadsorption and the juvenile fluke precipitin test.

Results

Serology

The immunised rats developed antibodies to the metabolic antigen, which could be detected in all animals by six weeks after the initial immunisation. Again there was no anamnestic response to the subcutaneous booster injection. After challenge, the titres of the immunised animals continued to rise, whilst those of the control animals showed the normal expected response. It was noticeable that the titres of the immunised group did not rise in proportion to those

of the control group, as was seen in the previous experiment.

The individual titres of immunised and control animals are recorded in Appendix Table 22.1 and group mean titres shown in Fig.22.1. Once again, there were marked individual variations between animals in the same group at any time.

Sera from immunised animals gave no immunodiffusion reaction with sheep serum, which indicated that the immunoadsorption of sheep serum contaminants from the metabolic antigen had been effective.

Fluke recovery

The numbers of flukes recovered from individual animals is recorded in Appendix Table 22.2 and group means shown in Table 22.1. There was clearly no significant difference between the two groups.

The sizes of individual flukes recovered are recorded in Appendix Table 22.2 and group means shown in Table 22.1. In this case there was no evidence for inhibition of the flukes recovered from the immunised animals, as their mean size was slightly larger than that of the flukes recovered from the control animals, although the difference was of no statistical significance.

As in the previous experiment, there were very marked individual variations in the numbers and sizes of flukes recovered from animals within the same group.

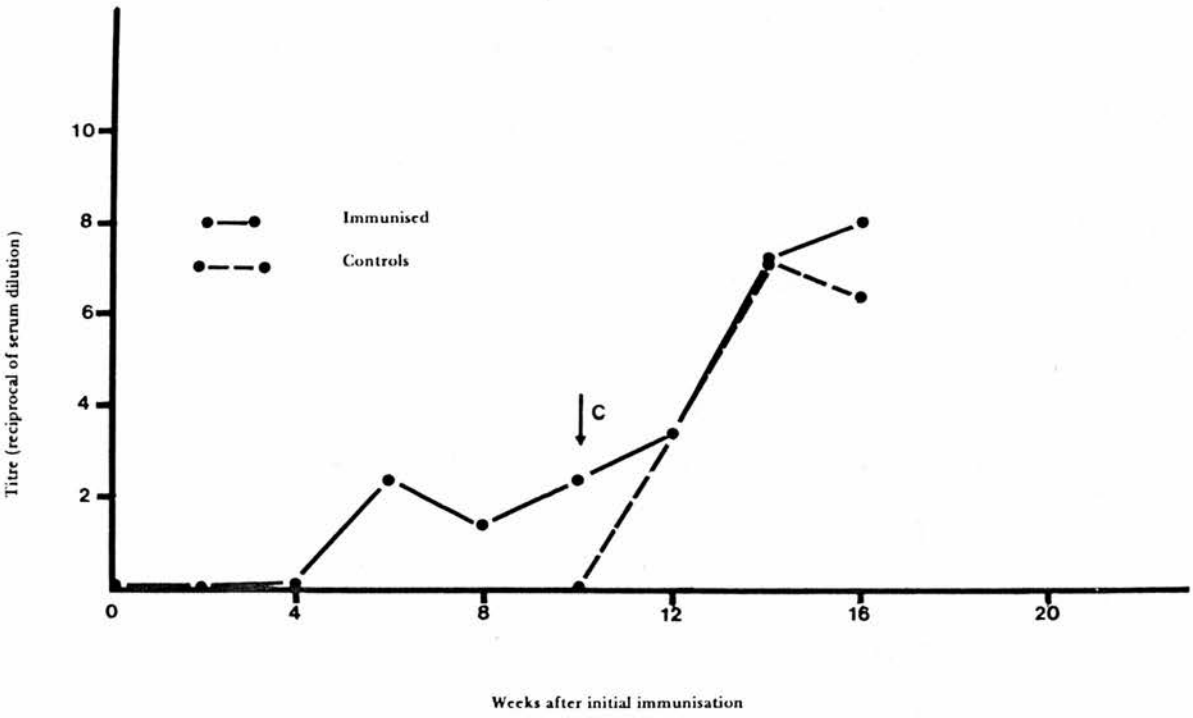

Fig. 22.1. Group mean immunodiffusion titres of rats immunised with metabolic antigen derived from adult flukes and of control rats.

C = administration of challenge infection.

	Mean no. flukes recovered per rat ± S.D.	Mean size (length x breadth) of flukes recovered (mm^2) ± S.D.
Immunised group	1.9 ± 1.3	32.2 ± 25.0
Control group	1.9 ± 1.1	28.4 ± 17.0
	t = 0.0	F = 0.52 (degrees of freedom = 1, 16)
Significance	$p > 0.3$	$p > 0.1$

Table 22.1. The numbers and sizes of flukes recovered from rats immunised with metabolic antigen derived from adult flukes and from control rats seven weeks after challenge.

Host response to challenge

Individual haematological results from immunised and control animals are recorded in Appendix Table 22.3 and group mean eosinophil counts shown in Fig.22.2. In each case the rats showed the normal expected increase in eosinophil counts from the second week after challenge. There were no significant differences between the two groups at any time and in this case there was no evidence to suggest a lesser response in the immunised animals.

Individual plasma glutamate dehydrogenase assays are recorded in Appendix Table 22.4 and group mean values shown in Fig.22.3. Both immunised and control groups showed the normal expected increases in enzyme activity during the challenge infection. Although the immunised rats showed lower glutamate dehydrogenase levels at four and six weeks after challenge, the individual within-group variation was so high that the differences between the two groups were not statistically significant.

Comparison of immune with infected serum

The two types of serum showed only partial immunological identity on immunodiffusion against metabolic antigen (Plate 22.1), the immune serum showing only a single precipitin line, as compared to the two lines seen with the infected serum. It was evident that the immune serum was showing no activity with the non-protein component of the metabolic antigen, in marked contrast to the infected serum, which showed strong activity with this part of the antigen.

Immunoadsorption studies confirmed the differences between the

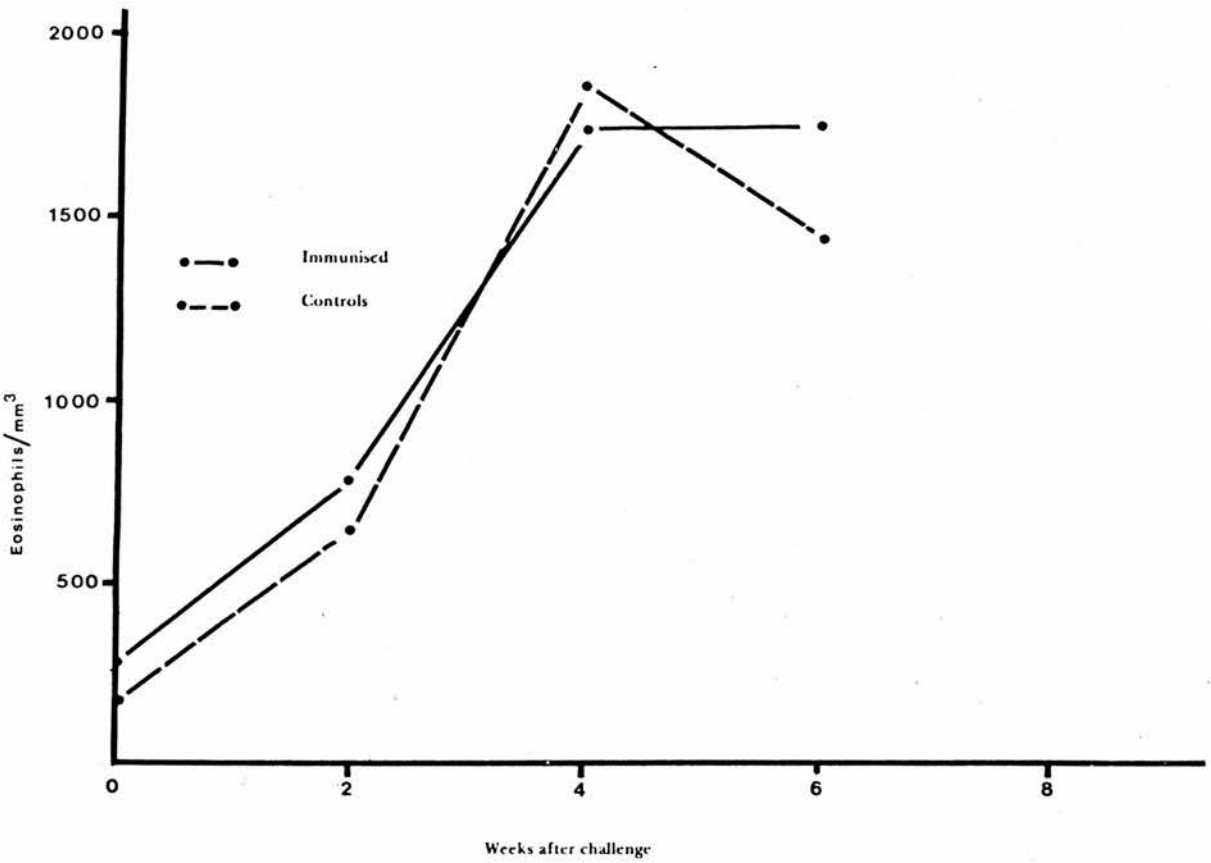

Fig. 22.2. Group mean eosinophil counts of rats immunised with metabolic antigen derived from adult flukes and of control rats.

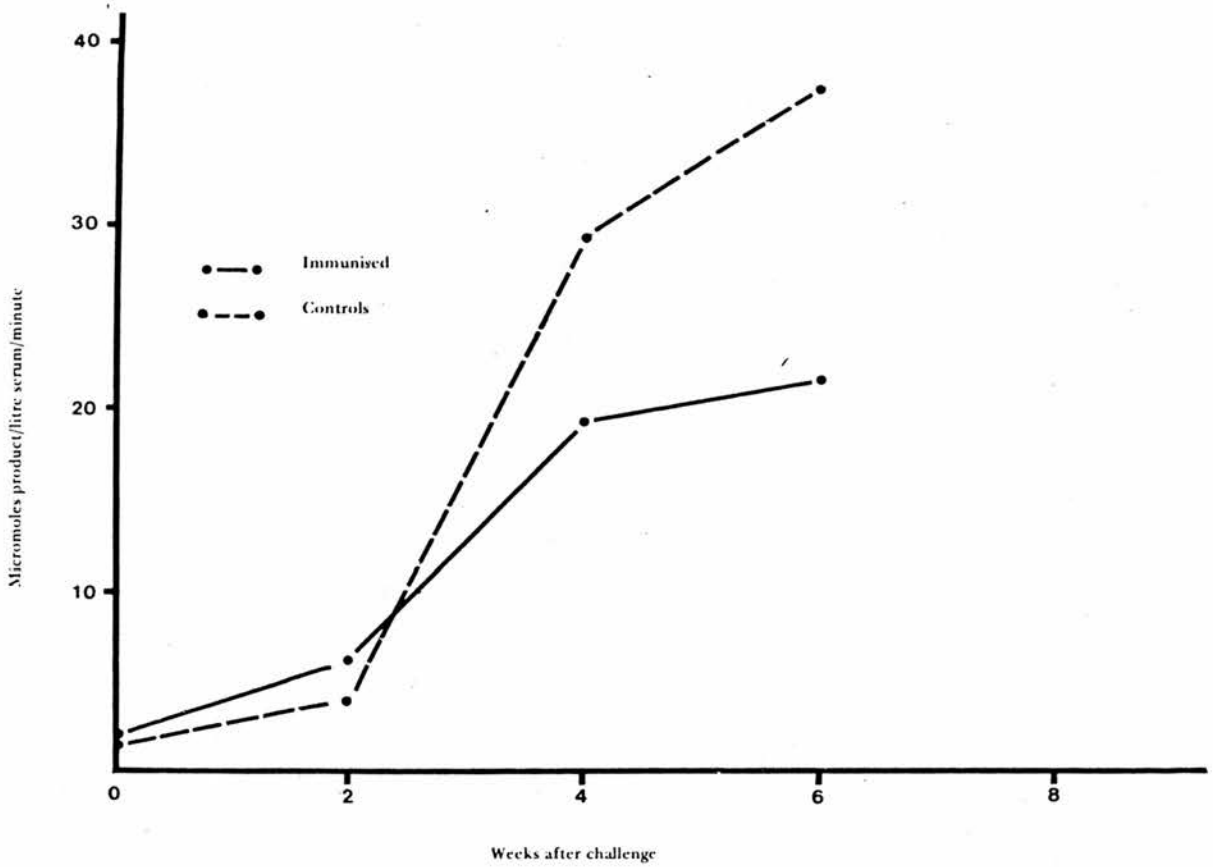

Fig. 22.3. Group mean plasma glutamate dehydrogenase values of rats immunised with metabolic antigen derived from adult flukes and of control rats.

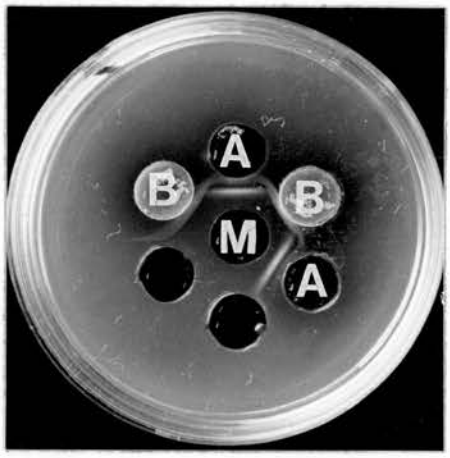

Plate 22.1. Immunodiffusion reactions between metabolic antigen (M) and infected rat serum (A) and immune rat serum (B).

two types of sera. Thus, metabolic antigen, having been completely adsorbed with the infected serum, gave no reaction with the immune serum, whereas having been completely adsorbed with the immune serum, a reaction was still obtained on immunodiffusion against the infected serum. This indicated that the infected serum contained additional antibody components, which were lacking from the immune serum.

Newly excysted flukes incubated in infected rat serum developed marked precipitates, whereas flukes incubated in the immune serum showed only very slight evidence of precipitates and flukes incubated in normal rat serum showed no precipitates.

Discussion

The results of this experiment were rather disappointing, in that although it was shown that rats would respond to immunisation with metabolic antigen derived from adult F. hepatica, there was no evidence that the antibodies associated with this response had any influence on a subsequent challenge infection.

In contrast to the findings in the previous experiment, in the present case there was no evidence of inhibition of flukes in the immunised animals, nor any evidence that the host response to challenge, as indicated by the eosinophilia and plasma glutamate dehydrogenase assays, was reduced in the immunised rats.

The comparative studies on the immune and infected sera revealed that although there were some common features between the two types of sera, there were also marked differences, which were

apparently sufficient to render the immune serum incapable of protecting the immunised animals against the challenge infection. The major difference appeared to be the failure of the immunised animals to respond to the non-protein component of the metabolic antigen.

These results were particularly disappointing since it was known that the strain of rats used was capable of demonstrating acquired resistance to F. hepatica, either by administration of repeated infections, the transfer of immune serum, or by subcutaneous transplantation of adult flukes prior to challenge.

It might therefore be concluded that administration of metabolic antigen alone is insufficient to induce protective immunity and that there is a need for some additional or alternative stimulus. However, it is possible to offer a number of alternative suggestions as to why immunisation of rats in this experiment failed to induce protective immunity. A number of workers (Soulsby, 1963; Denham, 1967; Robinson, 1967) have noted that fresh helminth metabolic antigens were more effective in inducing protective immunity than were antigens which had been subjected to excessive handling or adverse physical conditions. It is possible that the method of preparing metabolic antigen in the present experiment, where flukes were maintained in a recirculating culture system for seven days before the medium was concentrated, may not be very suitable if certain of the antigen components are labile. Furthermore, the antigen used to immunise the rats underwent further extensive handling in order to adsorb the sheep serum contaminants. In subsequent experiments, precautions were

therefore taken to ensure that the antigen was used as soon as possible after it had been produced and that it was protected from adverse physical conditions, in particular by collecting the medium as it passed over the flukes and rapidly cooling it to 4°C.

It is possible that metabolic antigens derived from adult flukes are not effective in stimulating protective immunity to challenge. Further experiments were therefore undertaken in which antigens were obtained from immature flukes which were still migrating through the hepatic parenchyma.

CHAPTER TWENTY-THREE

IMMUNISATION OF RATS WITH FRESHLY COLLECTED METABOLIC ANTIGEN DERIVED FROM ADULT F. HEPATICA

Introduction

The purpose of this experiment was to immunise rats with metabolic antigens derived from adult flukes, with special precautions being taken to protect the antigens against adverse physical conditions and excessive handling.

The flukes used to produce the antigens in this experiment were taken from experimentally infected rats. As noted previously, the use of flukes taken from the same host species as that to be immunised prevents the introduction of foreign species' material into the system. In the case of experiments involving the use of rats, there was the problem of obtaining sufficient flukes to produce the required quantities of antigen for immunisation. In the previous experiment, flukes were taken from sheep and sheep serum proteins removed from the antigen by immunoadsorption. Although this technique was apparently effective, it entailed subjecting the antigen to extensive handling. In the present experiment it was therefore decided to make use of flukes obtained from rats for the production of the vaccine and to accept the problems of acquiring sufficient numbers. In order to ensure that only the response to specifically fluke antigens was being measured, the antigen used in the serological studies in this experiment was a metabolic antigen derived from adult flukes taken from rabbits.

Experimental design

Approximately 30 adult flukes were collected from experimentally infected rats and placed in a continuous-flow culture apparatus containing 2 l of Medium 199. The apparatus was of the type used in Section 1 for the studies on the *in vitro* culture of adult flukes (see Plate 2.1). The culture was so arranged that the collection flask was placed in a refrigerator, the silicone tubing leading to the flask passing through a small hole in the door-seal. Thus, culture medium, having passed over the flukes was quickly cooled to 4°C. Eight hours after setting up the culture, the medium which had been collected in the flask was removed and concentrated to a volume of 2 ml by dialysis against polyethylene glycol 6000, the whole procedure being undertaken at 4°C. The concentrated medium was emulsified in an equal volume of Freund's complete adjuvant. Twenty male Porton Wistar rats (mean weight 265 g) were randomly assigned to two groups of ten animals each. Each animal in the first group was injected intramuscularly with 0.4 ml of the emulsified antigen. Each animal in the control group was injected intramuscularly with 0.4 ml of Medium 199 emulsified in Freund's complete adjuvant.

After a further 16 hours of culture (i.e. after a total of 24 hours from the start), the medium collected was removed and concentrated to a volume of 2 ml as before. Each rat in the immunised group was injected intramuscularly with 0.2 ml of this concentrate without adjuvant, whilst each of the control animals received 0.2 ml of Medium 199. This process was then repeated after 48, 72, 96, 120 and 144 hours from the start of the culture. Four weeks after the first injection, this immunising schedule was repeated, using antigen

prepared in a similar manner from another 30 adult flukes recovered from rats and maintained as above.

In view of the apparent failure of subcutaneous booster injections to induce an anamnestic response in the two previous experiments, such injections were not given in the present experiment. Four weeks after the second series of injections the rats were each challenged with 20 metacercariae. One of the rats in the control group died prior to challenge.

The rats were bled fortnightly throughout the experiment. The serological response to immunisation and challenge was followed by means of immunodiffusion, using antigen at a concentration of 600 µg of protein per ml. The host response to challenge was monitored by means of the eosinophilia and plasma glutamate dehydrogenase assays.

The rats were killed eight weeks after challenge and flukes recovered and measured.

Pooled serum from immunised animals prior to challenge was compared with serum from rats harbouring a patent fluke infection by immunodiffusion, immunoadsorption and the juvenile fluke precipitin technique.

Results

Serology

All the immunised animals developed a serological response, which was detectable in all cases by six weeks after the initial

immunisation. It was again noticeable that although the titres of the immunised animals continued to rise after challenge, the rise was proportionally less than that of the control animals.

Individual titres of immunised and control rats are recorded in Appendix Table 23.1 and group mean titres are shown in Fig.23.1.

Fluke recovery

The numbers of flukes recovered from individual rats are recorded in Appendix Table 23.2 and group means summarised in Table 23.1. Again there was marked individual variation in the numbers of flukes recovered from rats within the same group, but there was no significant overall difference between the two groups.

The sizes of individual flukes recovered are shown in Appendix Table 23.2 and group means in Table 23.1. There was no significant difference between the two groups.

Host response to challenge

Individual haematological results are recorded in Appendix Table 23.3 and group mean eosinophil counts shown in Fig.23.2. There were no significant differences between the two groups. Individual glutamate dehydrogenase assays are recorded in Appendix Table 23.4 and group mean values in Fig.23.3. In this case, the glutamate dehydrogenase values were very similar for each group, with no significant differences occurring at any time.

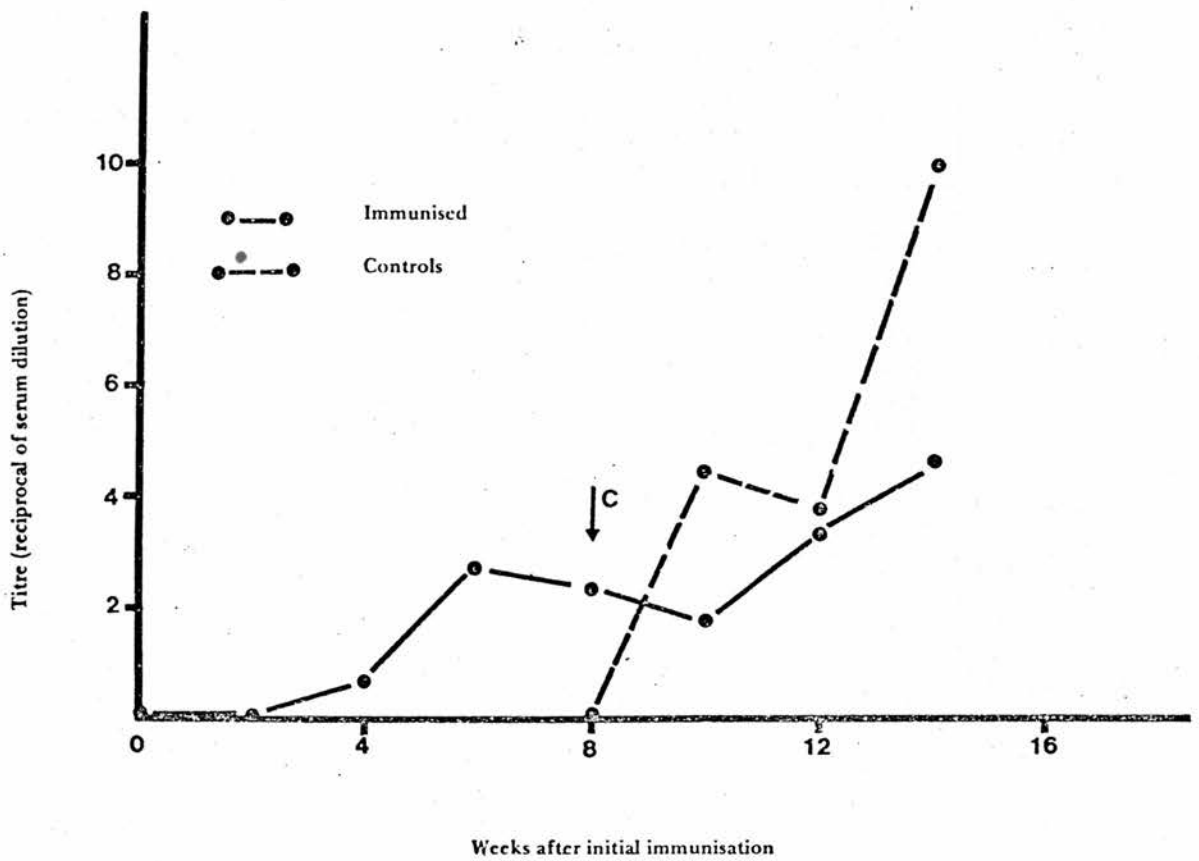

Fig. 23.1. Group mean immunodiffusion titres of rats immunised with freshly collected metabolic antigen derived from adult flukes and of control rats.

C = administration of challenge infection.

	Mean no. flukes recovered per rat ± S.D.	Mean size (length x breadth) of flukes recovered (mm^2) ± S.D.
Immunised group	2.7 ± 1.6	46.5 ± 30.8
Control group	3.1 ± 1.5	41.7 ± 25.3
	t = 0.52	F = 0.53 (degrees of freedom = 1, 35).
Significance	$p > 0.3$	$p > 0.1$

Table 23.1. The number and sizes of flukes recovered from rats immunised with freshly collected metabolic antigen derived from adult flukes and from control rats, eight weeks after challenge.

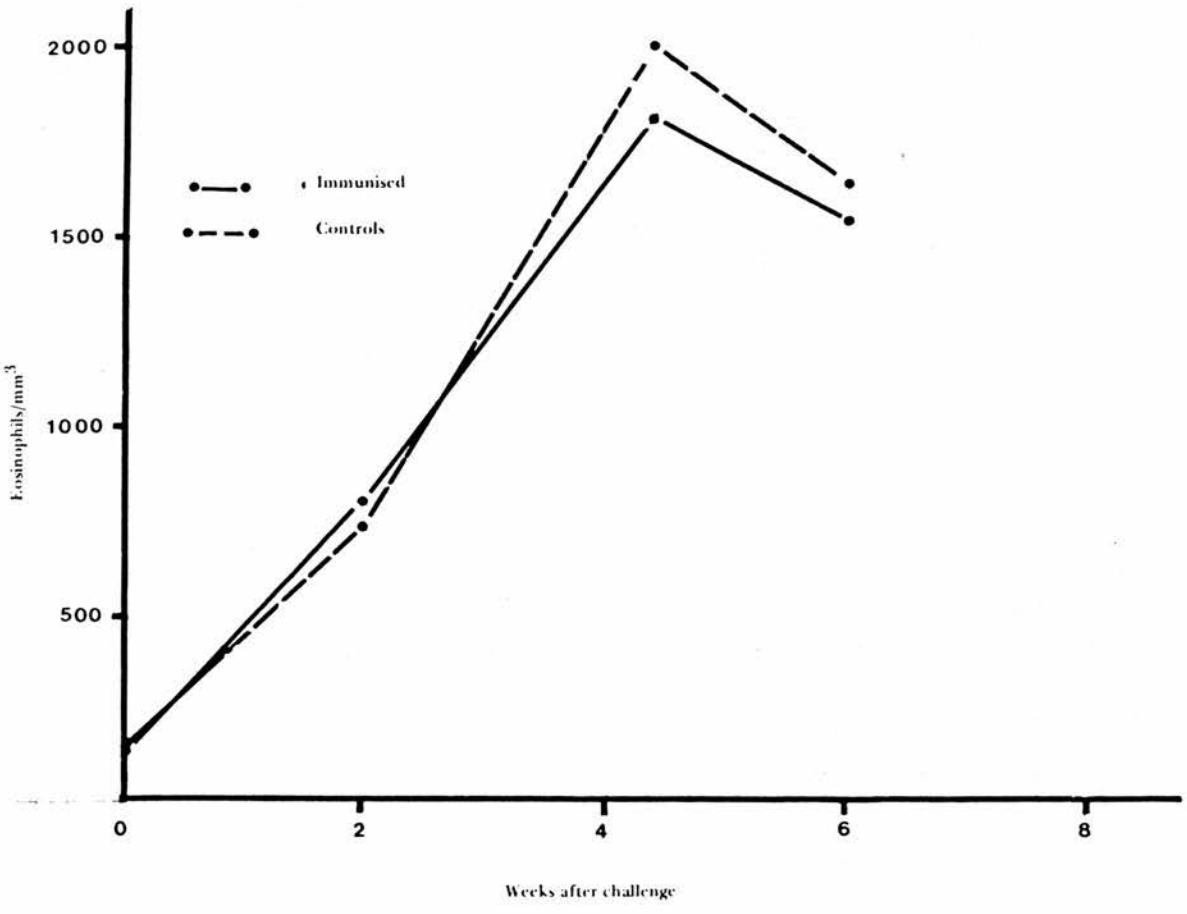

Fig. 23.2. Group mean eosinophil counts of rats immunised with freshly collected metabolic antigen derived from adult flukes and of control rats.

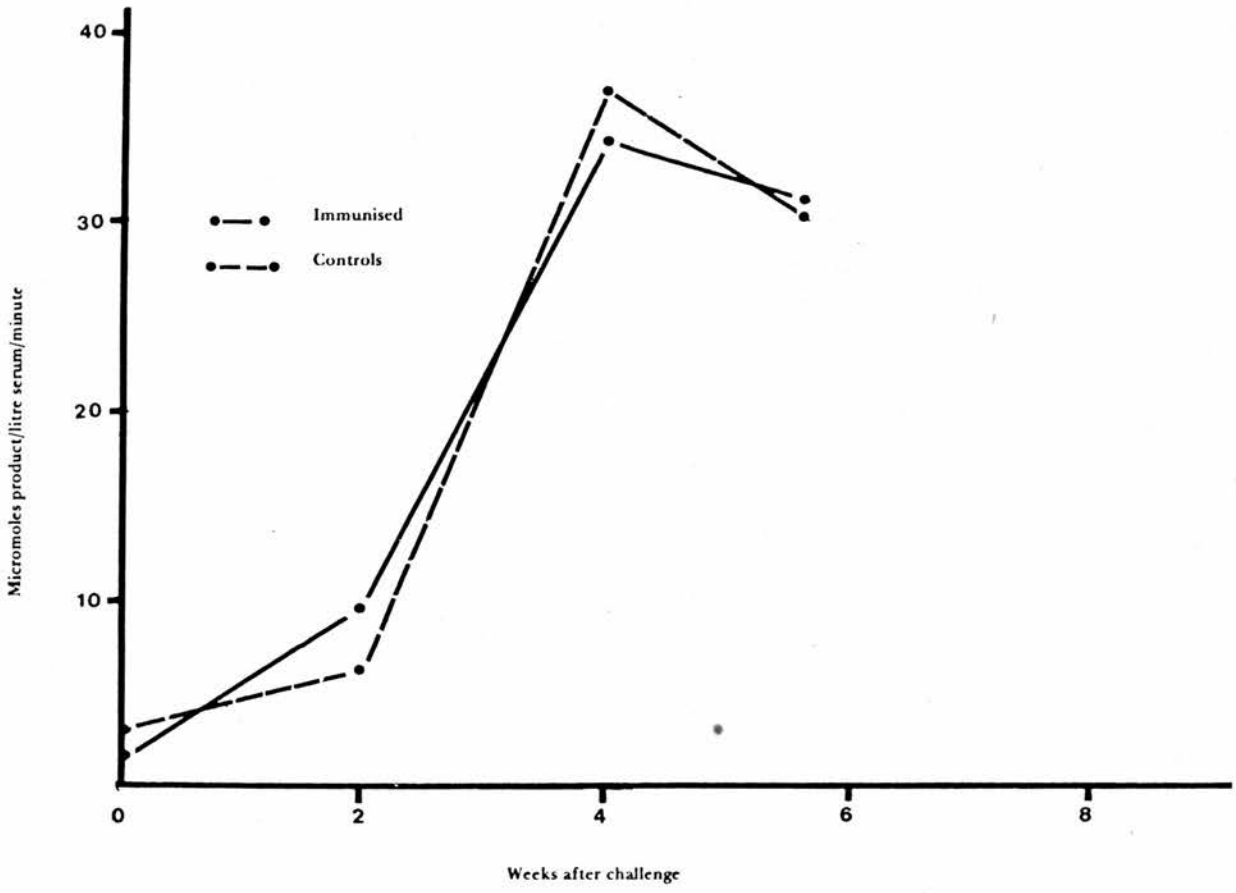

Fig. 23.3. Group mean plasma glutamate dehydrogenase values of rats immunised with freshly collected metabolic antigen derived from adult flukes and of control rats.

Comparison of immune and infected sera

Immunodiffusion of the two types of sera against metabolic antigen showed that in this case the immune serum gave a very weak precipitin reaction with the non-protein component of the antigen, as compared to the strong reaction seen between the infected serum and the non-protein antigen. Immunoadsorption studies again indicated that the immune serum lacked certain antibody components which were present in the infected serum. Thus, metabolic antigen, having been completely adsorbed with the infected serum, gave no reaction with the immune serum, whereas when the antigen had been adsorbed with the immune serum, a reaction still occurred with the infected serum.

Incubation of newly excysted flukes in immune serum again resulted in the formation of only very weak precipitate, as compared to the strong precipitates seen on incubation in infected serum.

Discussion

The results of this experiment were very similar to those of the previous experiment and it was apparent that the measures taken to use freshly prepared metabolic antigen, which had been protected from adverse conditions and excessive handling, had no influence on the previously noted failure of the antigen to induce protective immunity in rats.

It must therefore be concluded that metabolic antigen derived from adult flukes in the manner described is not capable of stimulating protective immunity, even in a strain of animals which was known to be capable of developing acquired immunity to

F. hepatica. However, it was thought possible that metabolic antigens produced by flukes during relatively long-term cultures, as used in the previous three experiments, undergo changes in composition, such that they are no longer effective in stimulating protective immunity. In subsequent experiments, animals were therefore immunised with metabolic antigens collected during shorter periods of culture, in the hope that these might be more effective.

It was also thought possible that metabolic antigen derived from adult flukes is not capable of inducing protective immunity and in subsequent experiments use was made of antigen derived from immature rather than adult flukes.

CHAPTER TWENTY-FOUR

IMMUNISATION OF MICE WITH METABOLIC ANTIGEN DERIVED FROM IMMATURE F. HEPATICA

Introduction

The purpose of this experiment was to try and verify the claim of Lang (1976) that mice could be protected against F. hepatica by immunisation with culture medium in which 16-day-old flukes had been maintained for relatively short periods.

Lang reported that intraperitoneal injection of medium in which 16-day-old flukes had been incubated for four hours induced no protective immunity, in terms of the number of flukes recovered from a two metacercariae challenge administered 30 days later, but the host mortality was significantly lowered as compared to control animals. However, injection of the medium in which 16-day-old flukes had been incubated for 24 hours did stimulate protective immunity. In Lang's experiment, 80 16-day-old flukes were incubated in 40 ml of Medium 199. Each of 20 mice were injected intraperitoneally with a total of 1.0 ml of the medium after 2 (0.2 ml), 7 (0.2 ml), 12 (0.2 ml) and 24 (0.4 ml) hours of incubation. Thirty days after the last injection the mice were each challenged with two metacercariae and killed after a further 34 days. Only 25% of the immunised mice were found to be infected, as compared to 100% of the control animals, the worm recovery was reduced by 83.3% in the immunised group and host mortality reduced from 33.3% to 10%.

Lang also noted that administration of a single injection of the

medium in which 16-day-old flukes had been incubated for 24 hours was of no value in inducing protective immunity and therefore concluded that the sequential injection of the continuously elaborated antigens was of primary importance. It was suggested that different antigens were being produced during the 24 hour culture period and that those produced in the initial four hour period were not sufficient to stimulate protective immunity.

It is inevitable that immature flukes recovered from the hepatic parenchyma will be contaminated with host tissues. Thus in order to avoid the introduction of foreign species' material into the immunised mice, the flukes used to produce the antigen for this attempt to repeat Lang's experiment were obtained from mice.

Experimental design

Forty 16-day-old flukes were recovered from mice and incubated at 37°C in a 35 x 10 mm sterile plastic petri dish (Becton, Dickinson U.K. Ltd., Wembley, Middlesex) containing 15 ml of Medium 199 and antibiotics as previously described. Forty male M.R.C. strain white mice (mean weight 28 g) were randomly divided into two groups of 20. Each member of the first group was injected sequentially with a total of 0.75 ml of the medium in which the flukes had been incubated for 2 (0.15 ml), 7 (0.2 ml), 11 (0.2 ml) and 24 (0.2 ml) hours. The control group of mice were given equivalent injections of Medium 199 alone. All injections were made intraperitoneally.

Four weeks later this immunising schedule was repeated exactly and after a further three weeks the mice were each challenged with

two metacercariae. One member of the immunised group and two members of the control group died prior to challenge.

Mice which died during the course of the challenge infection were autopsied as soon as possible after death, an attempt being made to recover any flukes present. The remaining mice were killed 30 days after challenge and flukes recovered from the bile ducts.

Results

The survival times of the mice and the number of flukes recovered at post mortem are recorded in Appendix Table 24.1. The results of the experiment are summarised in Table 24.1. It was apparent that there was no significant differences between the immunised and control groups in terms of the mortality rate, infection rate, survival time or number of flukes recovered. Although flukes were not recovered from all the animals, this was almost certainly due to failure of the recovery technique rather than actual absence of flukes. Only one animal (in the immunised group) showed no pathological evidence of having been infected. The problem of recovering such small numbers of flukes from the mice was especially marked in the case of those animals which died during the course of the infection and were not examined until some hours after the time of death. There was a tendency for flukes to migrate out of the liver after the death of the host and although the abdominal cavity was carefully washed out with saline, flukes were often not found, although the liver pathology indicated quite clearly that infection had occurred.

	% of mice which died during challenge period	Mean survival time (days) ± S.D. of mice dying during challenge period	Mean no. of flukes recovered from all mice ± S.D.
Immunised group	68.0	24.8 ± 2.4	1.0 ± 0.8
Control group	67.0	25.3 ± 1.2	1.1 ± 0.7
t		0.81	0.4
Significance		$p > 0,3$	$p > 0.3$

Table 24.1. Mortality rate, survival time and fluke recoveries for immunised and control mice.

Discussion

The results of this experiment were in marked contrast to the findings of Lang (1976). Although the experiment was modelled on that of Lang, it was not identical. Lang administered only a single series of immunisations of antigen collected over a 24 hour period, whereas in the present experiment two series of immunisations were given. This immunising regime was used in view of the findings in the earlier experiments in this series that the host serological response to metabolic antigen was maximal a few weeks after the administration of a second antigen injection given four weeks after the initial injection.

It is possible that the administration of the second series of antigen injections to the mice caused an immunological paralysis due to over exposure to the antigen. However, in view of the extremely small quantities of antigen administered, this is considered to be rather unlikely. Unfortunately serological studies were not undertaken in this experiment and therefore the extent to which the mice responded to the immunisations was not known.

It is known that different strains of the same species may show marked differences in their susceptibilities to infection with F. hepatica and it is possible that the strain of mice used by Lang was capable of acquiring protective immunity following the administration of metabolic antigens, whereas the strain used in the present experiment were not. In view of this, similar experiments were then carried out using a strain of rats which was known to be capable of acquiring immunological resistance to F. hepatica.

CHAPTER TWENTY-FIVE

IMMUNISATION OF RATS WITH METABOLIC ANTIGEN DERIVED FROM IMMATURE F. HEPATICA

Introduction

Despite the failure of the previous experiment in which mice were immunised with metabolic antigens obtained from 16-day-old flukes, it was considered worthwhile to undertake similar experiments in rats, using a strain of animals which were known to be capable of acquiring immunity to F. hepatica.

Again, in order to prevent the introduction of foreign species' materials into the immunised animals, the immature flukes used to produce the antigen in this experiment were taken from experimentally infected rats. The antigen used to follow the serological response was a metabolic antigen derived from adult flukes taken from rabbits.

Experimental design

Sixty-five flukes were recovered from the livers of rats which had been infected with 100 metacercariae 16 days previously. The flukes were placed in a continuous-flow culture apparatus containing 2 l of Medium 199. Medium which had passed over the flukes was collected at 4°C as described in Chapter 23. Ten hours after setting up the culture, the medium which had been collected was removed and concentrated to a volume of 2.0 ml by dialysis against polyethylene glycol at 4°C. The concentrate was then emulsified in Freund's complete adjuvant.

Twenty male Porton Wistar rats (mean weight 220 g) were randomly divided into two groups of ten. Each animal in the first group was injected intramuscularly with 0.4 ml of the emulsified antigen. Each animal in the control group was injected similarly with Medium 199. After a further 14 hours of culture (i.e. after a total of 24 hours from the start) the medium which had been collected was removed and concentrated to 2.0 ml as before. Each member of the immunised group was injected intramuscularly with 0.2 ml of the concentrate, whilst the control animals received 0.2 ml of Medium 199. This process was repeated after 48,72,96 and 120 hours of culture.

Four weeks after the last injection this immunising regime was repeated exactly, using antigen prepared in a similar manner from 50 16-day-old flukes recovered from rats and maintained as above.

After a further four weeks the rats were each challenged with 20 metacercariae. The rats were bled fortnightly throughout the experiment, the serological responses being measured by immunodiffusion, using antigen at a dilution of 600 µg of protein per ml. The response to the challenge infection was again monitored by means of the eosinophilia and plasma glutamate dehydrogenase assay.

The rats were killed eight weeks after challenge and flukes recovered and measured. One rat in the control group died six weeks after challenge.

Pooled serum from immunised animals prior to challenge was compared with serum from rats carrying a patent fluke infection by

immunodiffusion, immunoadsorption and the juvenile fluke precipitin technique.

Results

Serology

The serological response of the immunised rats was rather weaker than that seen in the earlier experiments. This was almost certainly due to the fact that the amount of antigen administered in the present experiment was considerably less than that derived from adult flukes in previous experiments.

Overall, nine of the ten immunised animals showed precipitating antibodies at some stage of the pre-challenge period, but in several cases the response was apparently only transient.

Both immunised and control groups showed a similar serological response to challenge.

Individual titres of immunised and control rats are shown in Appendix Table 25.1 and mean titres in Fig.25.1

Fluke recovery

The numbers and sizes of flukes recovered eight weeks after challenge are recorded in Appendix Table 25.2 and group means in Table 25.1. It was evident that there were no significant differences in the number or sizes of flukes recovered from the immunised or control animals.

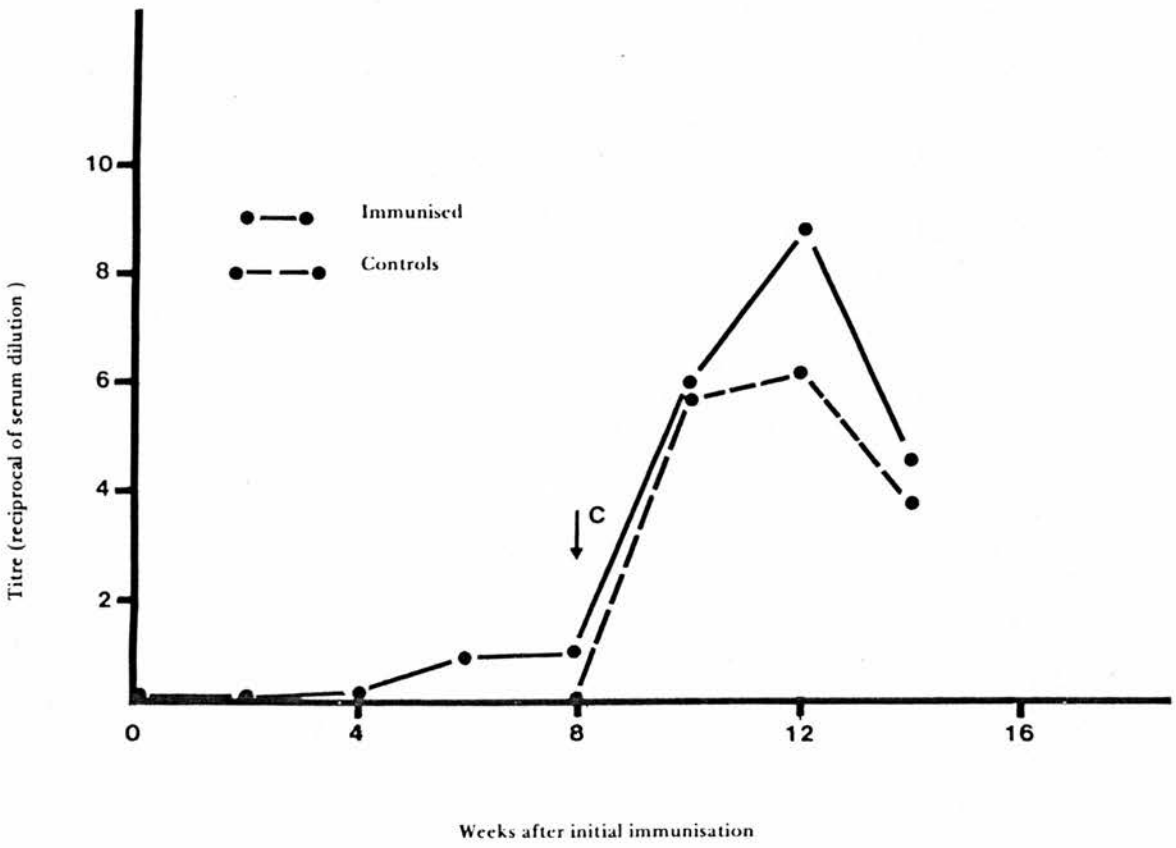

Fig. 25.1. Group mean immunodiffusion titres of rats immunised with metabolic antigen derived from immature flukes and of control rats.

C = administration of challenge infection.

	Mean no. flukes recovered per rat ± S.D.	Mean size (length x breadth) of flukes recovered (mm^2) ± S.D.
Immunised group	3.6 ± 1.3	32.9 ± 17.8
Control group	3.8 ± 1.7	34.1 ± 15.3
	t = 0.25	F = 0.1 (degrees of freedom = 1,50).
Significance	$p > 0.3$	$p > 0.1$

Table 25.1. The number and sizes of flukes recovered from rats immunised with metabolic antigen derived from 16-day-old flukes and from control rats eight weeks after challenge.

Host response to challenge

Individual haematological results are shown in Appendix Table 25.3 and group mean eosinophil counts in Fig.25.2. There were no significant differences between the immunised and control groups at any time. The individual glutamate dehydrogenase assays are recorded in Appendix Table 25.4 and group mean values in Fig.25.3. In this case, the immunised animals showed higher glutamate dehydrogenase levels during the challenge infection, but due to the very wide within-group variation, the differences were at no time of statistical significance.

Comparison of immune and infected sera

Immunodiffusion of the two types of serum again revealed partial identity as in the previous experiments. There was no evidence of a response to the non-protein component of the metabolic antigen. Once again, immunoadsorption studies revealed a difference in the antibody constituents of the two types of sera, with metabolic antigen which had been adsorbed with the immune serum still showing activity against the infected serum.

Incubation of newly excysted flukes in the immune serum resulted in the formation of weak precipitates, as compared to the strong precipitates seen on incubation in infected serum.

Discussion

The results of this experiment were virtually identical to those of the earlier experiments, in which rats had been immunised with metabolic antigen derived from adult flukes. There was thus no

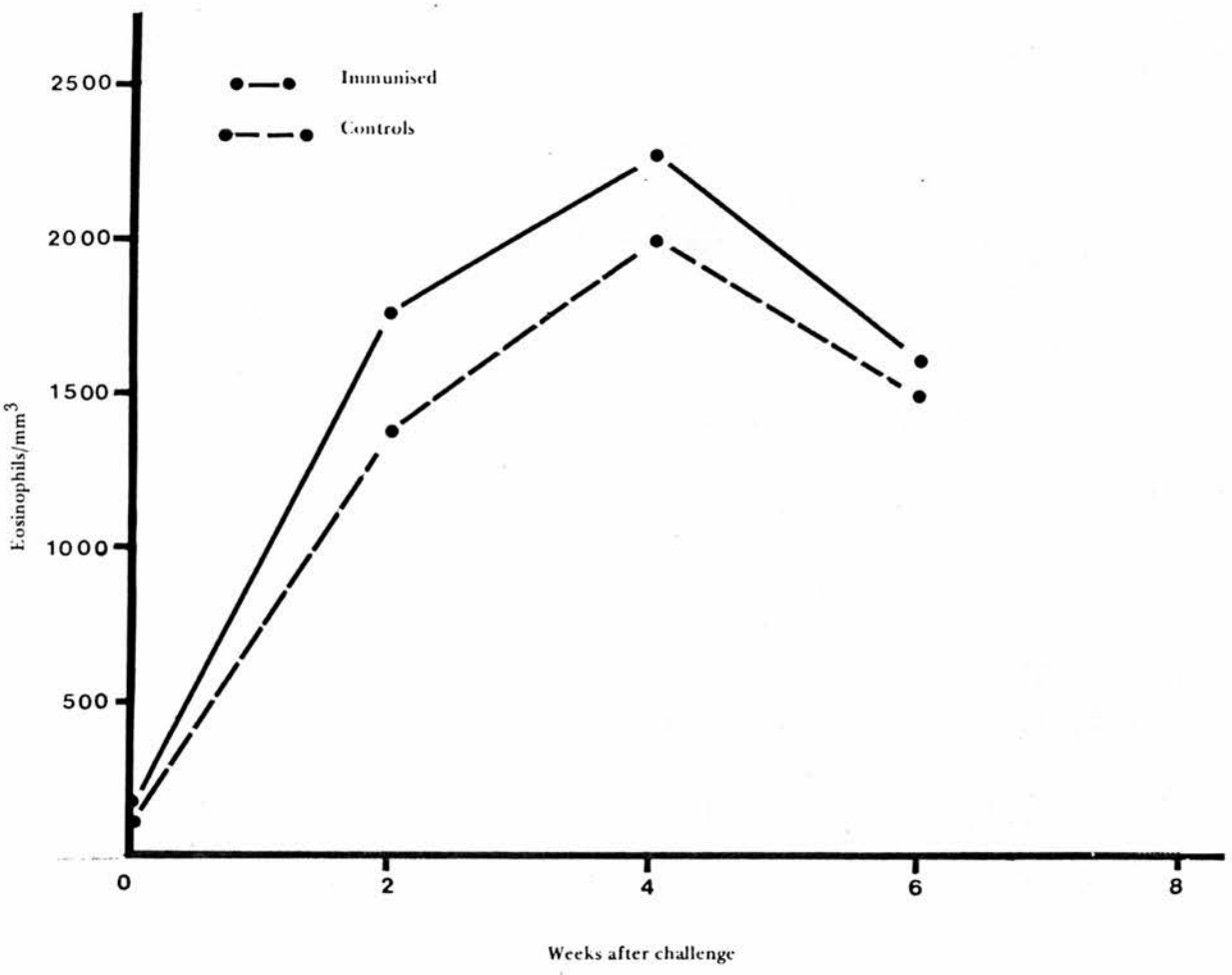

Fig. 25.2. Group mean eosinophil counts of rats immunised with metabolic antigen derived from immature flukes and of control rats.

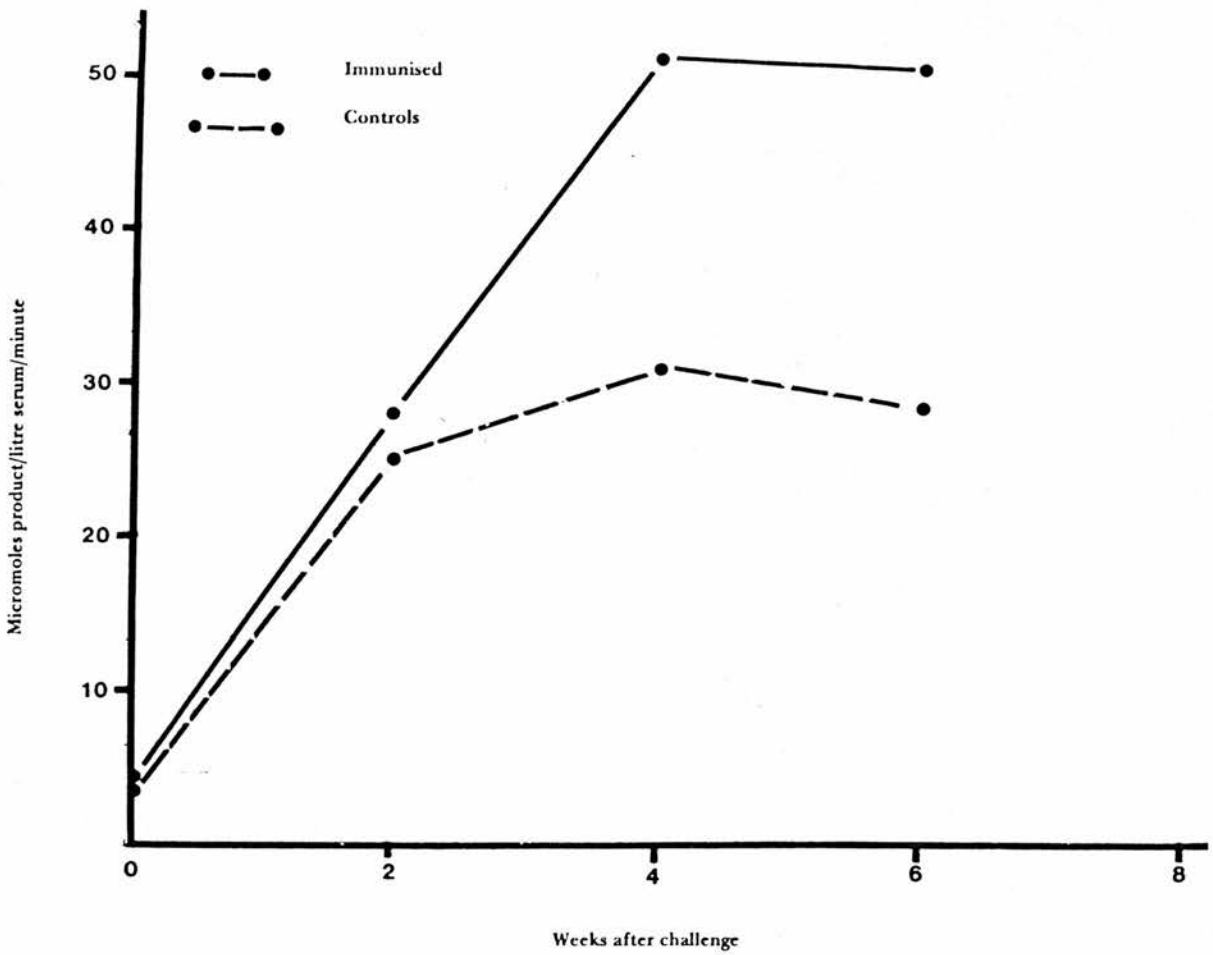

Fig. 25.3. Group mean plasma glutamate dehydrogenase values of rats immunised with metabolic antigen derived from immature flukes and of control rats.

evidence to suggest that metabolic antigen derived from immature flukes taken from the hepatic parenchyma was of any value in stimulating protective immunity.

It was still thought possible that the composition of the metabolic antigen produced during the relatively long-term culture used in this experiment had undergone changes, which made it ineffective in inducing protective immunity. A further experiment was therefore undertaken, in which rats were immunised with metabolic antigen derived from 16-day-old flukes maintained in short-term culture, as was successfully used by Lang (1976).

CHAPTER TWENTY-SIX

IMMUNISATION OF RATS WITH METABOLIC ANTIGEN DERIVED FROM IMMATURE F. HEPATICA MAINTAINED IN SHORT-TERM CULTURE

Introduction

In view of the failure of the previous experiment, in which rats were immunised with metabolic antigen derived from 16-day-old flukes maintained for 120 hours in a flow-culture, an attempt was made to immunise rats with antigen derived from similar flukes, maintained for a shorter period, in the hope that the antigens liberated during the early period of the culture might be more effective in stimulating acquired immunity, as was suggested by Lang (1976).

The flukes used to produce the antigen were taken from experimentally infected rats, whilst the antigen used to follow the serological responses was a metabolic antigen obtained from adult flukes taken from rabbits.

Experimental design

The design of this experiment was essentially the same as that in Chapter 24, where mice were immunised with antigen obtained from immature flukes. Sixty 16-day-old flukes were obtained from rats and incubated at 37°C in a plastic petri dish containing 20 ml of Medium 199. Twenty male Porton Wistar rats (mean weight 235 g) were randomly divided into two groups of ten. Each rat in the first group was injected sequentially with a total of 2.0 ml of the medium in which the flukes had been cultured for 2 (0.2 ml), 7 (0.3 ml), 11 (0.3 ml), 24 (0.3 ml), 32 (0.4 ml) and 48 (0.5 ml) hours of culture.

The initial 0.2 ml of medium was emulsified in an equal volume of Freund's complete adjuvant prior to administration. All injections were made intramuscularly. The control group were given equivalent injections of Medium 199.

After four weeks this immunising schedule was repeated exactly, using antigen produced by 45 16-day-old flukes collected and maintained as above. Four weeks later the rats were each challenged with 20 metacercariae.

The rats were bled fortnightly, the serological responses being measured by immunodiffusion, using antigen at a concentration of 600 µg of protein per ml. The response to challenge was measured by means of the eosinophilia and plasma glutamate dehydrogenase assays.

The rats were killed eight weeks after challenge and flukes recovered and measured. Pooled serum from immunised animals prior to challenge was compared with serum from rats carrying a patent fluke infection by means of immunodiffusion, immunoadsorption and the juvenile fluke precipitin technique.

Results

Serology

The immunised animals again showed rather weak serological responses to the immunising antigen, almost certainly due to the fact that only small amounts of antigen were being obtained from the relatively small number of immature flukes used. Overall, nine of the ten immunised rats showed precipitins to the antigen prior to challenge.

Both immunised and control groups showed a similar serological response to challenge.

Individual titres of immunised and control rats are recorded in Appendix Table 26.1 and group mean titres are shown in Fig.26.1.

Fluke recovery

The numbers and sizes of flukes recovered from individual rats are recorded in Appendix Table 26.2 and group means shown in Table 26.1. It was clear that there were no significant differences in the numbers or sizes of flukes recovered from the two groups.

Host response to challenge

Individual haematological results are recorded in Appendix Table 26.3 and group mean eosinophil counts shown in Fig.26.2. There were no significant differences between the two groups at any time.

The individual glutamate dehydrogenase assays are recorded in Appendix Table 26.4 and group mean values shown in Fig.26.3. There were no significant differences between the two groups at any time.

Comparison of immune and infected sera

Immunodiffusion of immune serum against metabolic antigen revealed a weak response to the non-protein component of the antigen, as compared to the strong reaction occurring between the infected serum and this component of the antigen. Immunoadsorption studies once again confirmed that certain antibody components present in the infected serum were lacking from the immune serum, metabolic antigen

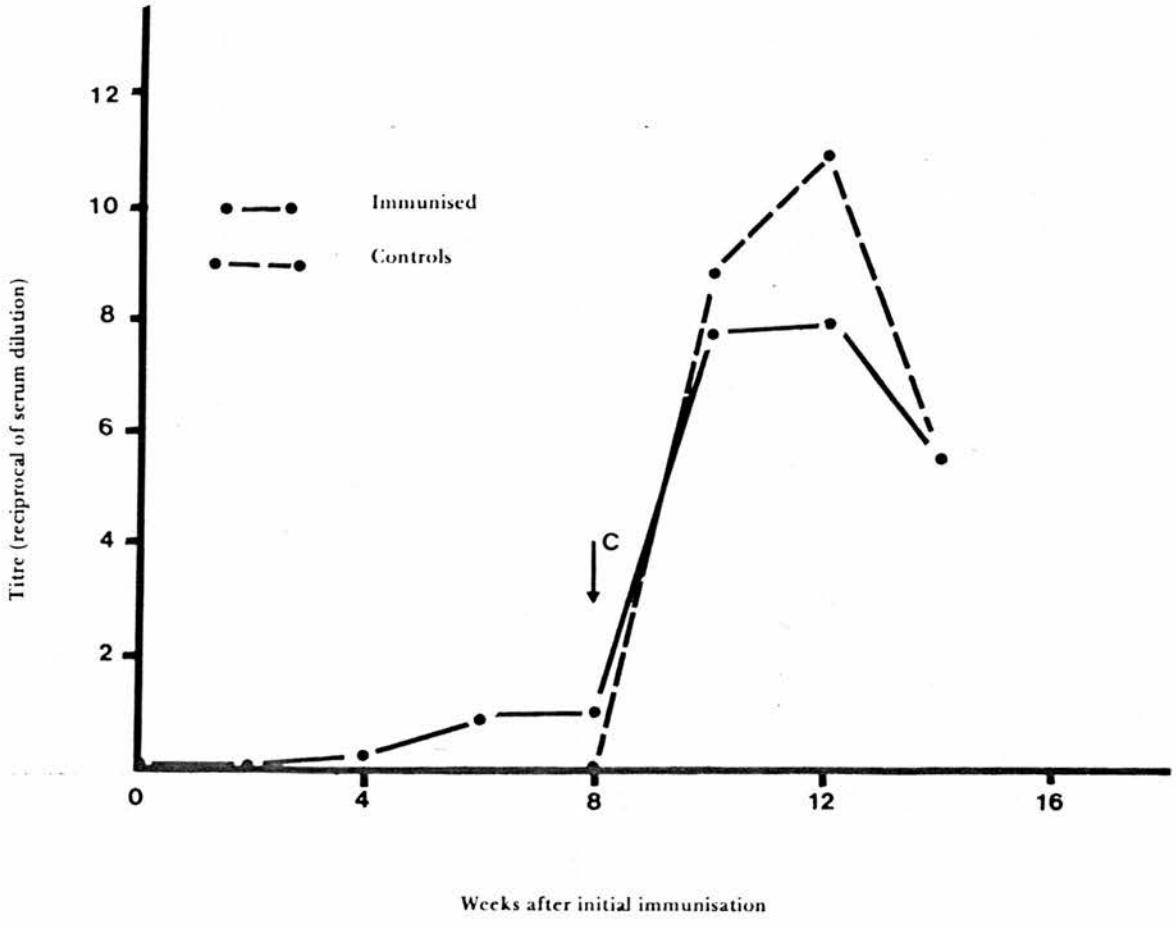

Fig. 26.1. Group mean immunodiffusion titres of rats immunised with metabolic antigen derived from immature flukes maintained in short-term culture and of control rats.

C = administration of challenge infection.

	Mean no. flukes recovered per rat ± S.D.	Mean size (length x breadth) of flukes recovered ± S.D.
Immunised group	3.5 ± 2.1	34.8 ± 19.1
Control group	3.4 ± 2.2	31.3 ± 17.2
	t = 0.11	F = 0.68 (degrees of freedom = 1, 48)
Significance	$p > 0.3$	$p > 0.1$

Table 26.1. The number and sizes of flukes recovered from rats immunised with metabolic antigen derived from short-term culture of 16-day-old flukes and from control rats eight weeks after challenge.

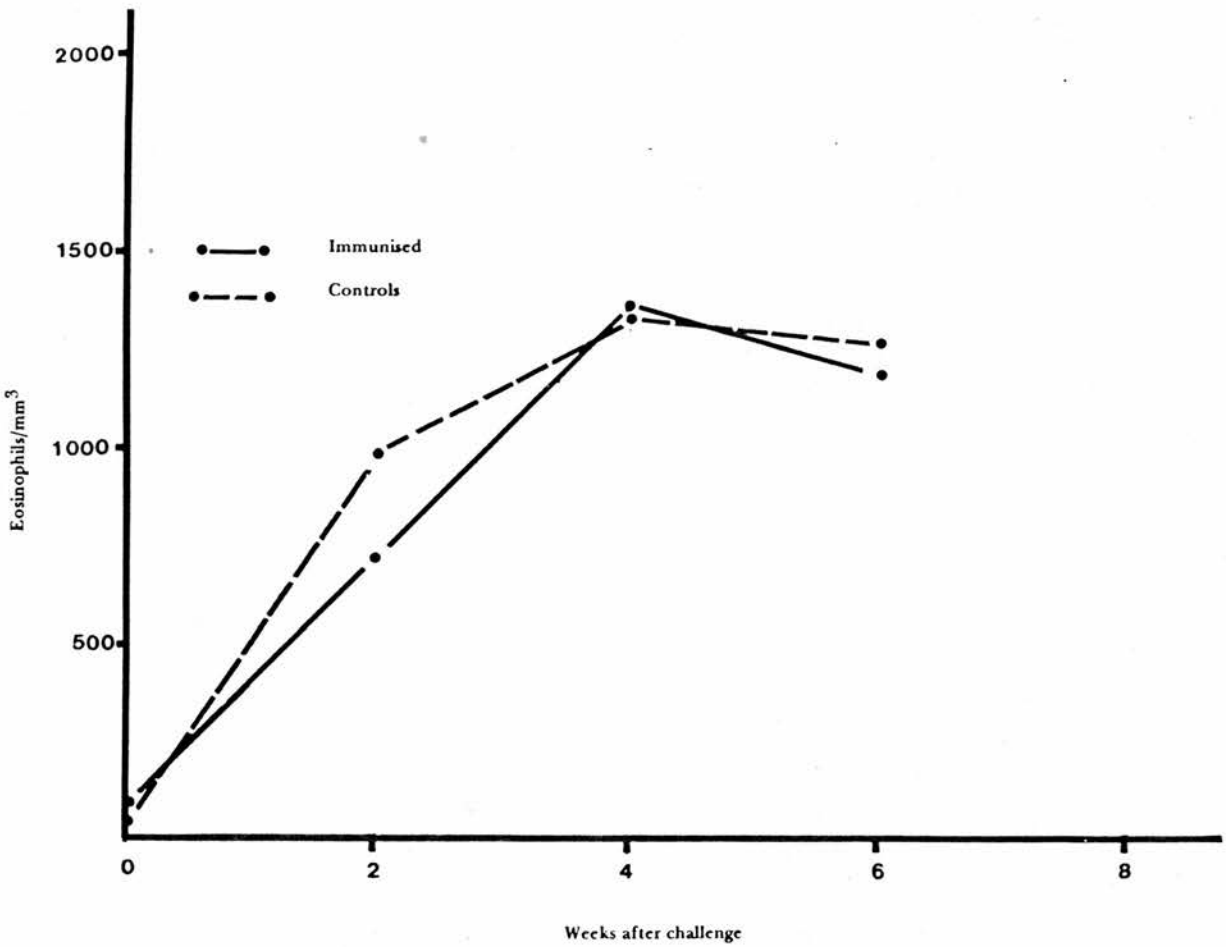

Fig. 26.2. Group mean eosinophil counts of rats immunised with metabolic antigen derived from immature flukes maintained in short-term culture and of control rats.

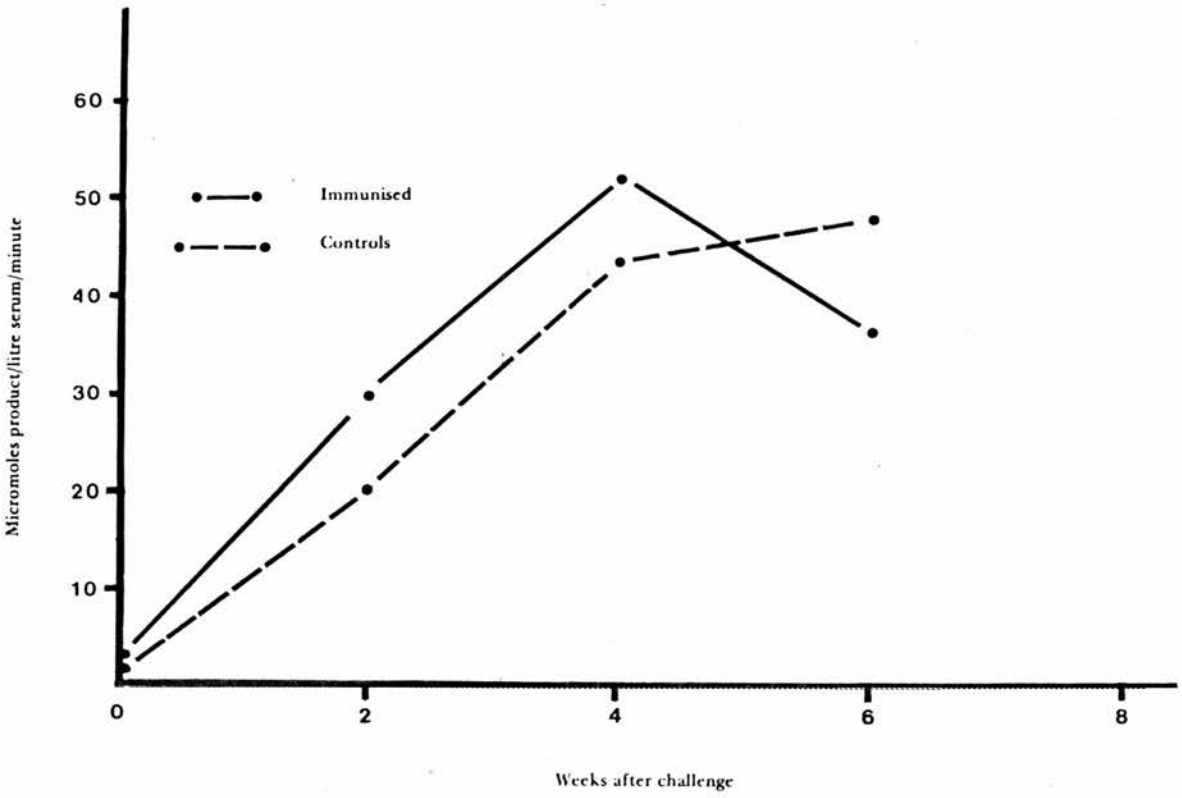

Fig. 26.3. Group mean plasma glutamate dehydrogenase values for rats immunised with metabolic antigen derived from immature flukes maintained in short-term culture.

which had been completely adsorbed with the immune serum still showing activity with the infected serum.

In this case incubation of newly excysted flukes in the immune serum did not result in the formation of any precipitates, whereas incubation in infected serum resulted in the formation of strong precipitates.

Discussion

The results of this experiment were similar to those of the previous ones in which rats were immunised with metabolic antigen derived either from adult or immature flukes.

The immunised rats again showed a serological response to the immunising antigen, but there was no evidence to suggest that the antibodies associated with this response had any influence on the course of a subsequent challenge infection.

It was again apparent that there were major differences between serum from the immunised rats and serum from infected rats.

It must therefore be concluded that immunisation of rats with metabolic antigen derived from the short-term culture of 16-day-old F. hepatica as described did not stimulate protective immunity, in contrast to the findings of Lang (1976), who was able to successfully immunise mice using antigen prepared in a similar manner.

CHAPTER TWENTY-SEVEN

CONCLUSIONS AND DISCUSSION

The series of experiments involving the immunisation of rabbits and rats with metabolic antigen derived from F. hepatica maintained in vitro showed consistent results, in that on each occasion the immunised animals were shown to have produced antibodies to the immunising antigen, but these antibodies had no influence on the numbers of flukes developing from a subsequent challenge infection. Only in the case of the immunised rabbits was there any evidence that the flukes of the challenge infection had been inhibited and that the response of the immunised animals was less severe than that of the controls. It is rather surprising that it should be the rabbits which provided the only evidence for any form of immunity resulting from the administration of metabolic antigen, since in general, the evidence for immunity to F. hepatica in rabbits is slight, as compared to the strong evidence that rats are able to become immune to this parasite. Indeed, the particular strain of rats used in this series of experiments was known to be capable of demonstrating acquired resistance to F. hepatica.

The experiments involving the adminstration of freshly prepared metabolic antigen, or metabolic antigen derived from immature flukes provided little indication that the response to such antigen preparations differed from that to the original antigen, which was derived from adult flukes maintained for relatively prolonged periods in a continuous-flow recirculating system. However, there was some evidence that the non-protein component of the antigen was more immunogenic to rats in the case of the freshly prepared antigen.

The results of the experiment in which mice were immunised with metabolic antigens resulting from the short-term culture of 16-day-old flukes were in marked conflict to those of Lang (1976), who undertook a similar, but not identical experiment. Whereas Lang was able to demonstrate substantial differences between immunised and control mice, in terms of mortality rates and fluke recoveries, in the present experiment there was no evidence that immunisation had any influence on the subsequent challenge infection.

It might be suggested that the differences between Lang's experiment and the present experiment were the result of the use of different strains of mice, which differed in their ability to develop an acquired resistance to F. hepatica. However, in view of the consistently negative results obtained in the present series of experiments, even when Lang's technique was applied to rats of a strain known to be capable of developing acquired resistance to F. hepatica, the validity of Lang's results must be questioned. Lang concluded his report by stating that further similar experiments were in progress and the results of these will be awaited with interest.

Throughout the present series of experiments there were certain similarities between the serum obtained from the immunised animals prior to challenge and serum obtained from animals carrying a patent fluke infection. However, it was apparent that there were also significant differences between the two types of sera. In particular, the immunised rats did not respond well to the non-protein component of the metabolic antigen, although the response appeared to be better when freshly prepared antigen was used. In addition, the antibodies

which were associated with the development of immune precipitates about newly excysted flukes were absent or present in only small amounts in the immune sera. If it had been possible to produce an immune serum which more closely resembled the infected serum, the likelihood of being able to demonstrate subsequent immunity to challenge would have been increased, since the passive transfer of such resistance has been demonstrated in the strain of rats used (Haroun, E.M., personal communication).

The experiments previously discussed, in which flukes were subcutaneously transplanted into recipient rats, which then became resistant to a subsequent challenge infection (Eriksen and Flagsted, 1974; Hughes, Anderson and Harness, 1975; Anderson, Hughes and Harness, 1975) provide evidence that antigens produced by the living parasite are of major importance in the stimulation of the protective immune response. In view of the failure of metabolic antigens derived from flukes maintained *in vitro* to stimulate protective immunity it would seem that either there is a need for some alternative or additional stimulus to the host, or that the metabolic antigens obtained *in vitro* were significantly different from those produced *in vivo*, to the extent that they no longer retained their ability to stimulate protective immunity.

It should be possible to show whether metabolic antigens produced by transplanted flukes were the sole necessary stimulus for protective immunity by enclosing the flukes in diffusion chambers, as has been described for other helminth species by Crandall and Arean (1965) and Rickard and Bell (1971a). If the transplanted flukes

failed to stimulate protective immunity when enclosed in such chambers, it would indicate that further studies on the use of metabolic antigens derived from *in vitro* cultures as immunogens would be unlikely to be rewarding. On the other hand, if protective immunity still arose under such circumstances, it would indicate that the metabolic antigens obtained *in vitro* were significantly different from those produced *in vivo*.

It is only to be expected that flukes maintained *in vitro* will, to a greater or lesser degree, be undergoing metabolic processes which differ from those normally occurring *in vivo* and as a result, the composition and rate of production of those excretory and secretory products having antigenic properties may also be changed. It would seem likely that the extent of such changes would be least under those *in vitro* conditions which were most favourable for the maintenance of the parasite. It was shown in Section 1 that flukes are able to survive and continue their metabolic processes for reasonably prolonged periods of time in Medium 199. However, it was also apparent that protein metabolism was maintained at a more constant rate if the medium was supplemented with increasing amounts of serum. It is therefore possible that the maintenance of flukes in Medium 199 alone did not provide a sufficiently favourable environment for the prolonged production of metabolic antigens sufficiently similar to those normally produced *in vivo*, such that they were capable of stimulating protective immunity in the immunised animals. There can be little doubt that it would have been preferable to have maintained the flukes in a medium supplemented with serum, but as previously noted, this would introduce major problems, in that it is

not possible to greatly concentrate media containing large amounts of serum. In addition, the serum in the medium would tend to stimulate the immune system of the immunised animal and this might lead to a diminished response to the antigens of purely fluke origin.

In the present series of experiments, efforts were made in some cases to overcome the potential problems associated with alterations in metabolic processes during the course of the culture, by concentrating attention on the metabolic antigens produced during the initial period *in vitro*, in the hope that these antigens might more closely resemble the antigens produced *in vivo*. However, the results of such experiments did not indicate that the antigens produced during the early stages *in vitro* were any more favourable than antigens derived from long-term cultures.

It was noticeable that there were always very marked individual variations between animals within the same treatment group with regard to the numbers and sizes of flukes recovered after challenge, serological titres, eosinophilia and plasma glutamate dehydrogenase assays. These differences emphasise the importance of using adequate numbers of experimental animals if results are to be of statistical value. However, the numbers of animals which could be used in these experiments were limited by the difficulties in producing sufficient metabolic antigen, particularly in those experiments which involved the use of immature flukes. It was apparent throughout this series of experiments that the serological responses to the metabolic antigen were usually relatively low, as compared to those associated with infections. It is possible that higher titres might

have been produced if greater quantities of antigen had been administered and it is also possible that such higher titres might have been associated with a greater degree of protective immunity.

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APPENDIX

APPENDIX
TABLE 3.1

The production of ammonia by adult F. hepatica maintained in basal saline.

Culture 1 - Modified Earle's salts solution + live flukes

Culture 2 - Modified Earle's salts solution + dead flukes

Time (hours)	Culture	Volume of medium collected (mls)	Ammonia conc. (mg/l)		Rate of ammonia production (μg NH_3/fluke/hr)
			Pre flukes	Post flukes	
24	1	123	0.0	8.0	8.2
	2	125	0.0	0.0	0.0
48	1	130	0.0	4.0	4.3
	2	134	0.0	0.0	0.0
69	1	110	0.0	2.3	2.4
	2	116	0.0	0.0	0.0
93	1	130	0.0	1.8	1.9
	2	131	0.0	0.0	0.0
118	1	116	0.0	1.0	0.9
	2	121	0.0	0.0	0.0

APPENDIX
TABLE 4.1

The influence of increasing the serum content of the medium on the protein metabolism of adult F. hepatica maintained in vitro.
Culture 1 - 90% serum/10% modified Earle's salts solution
Culture 2 - 60% serum/40% modified Earle's salts solution
Culture 3 - 30% serum/70% modified Earle's salts solution
Culture 4 - 10% serum/90% modified Earle's salts solution

Time (hours)	Culture	Volume of medium collected (mls)	Ammonia conc. (mg/l)		Rate of ammonia production (μg NH_3/fluke/hr)
			Pre flukes	Post flukes	
21	1	91	19.0	29.0	8.7
	2	89	14.5	24.0	8.1
	3	107	6.1	14.5	8.6
	4	95	2.0	14.0	10.9
45	1	93	23.0	35.0	9.3
	2	89	17.0	31.0	10.4
	3	119	6.2	13.5	7.2
	4	113	2.0	8.8	6.4
69	1	86	30.0	41.2	8.0
	2	83	21.0	28.0	4.8
	3	123	8.4	13.0	4.7
	4	115	3.4	7.4	3.8
93	1	83	35.0	45.0	6.9
	2	80	24.0	32.0	5.3
	3	115	9.0	12.0	2.9
	4	111	3.9	6.8	2.7
116	1	76	39.0	50.0	7.3
	2	74	28.0	34.0	3.9
	3	108	11.0	14.5	3.3
	4	105	5.2	6.2	1.0

APPENDIX
TABLE 5.1

Comparison of modified Earle's salts solution with Medium 199 for the maintenance of adult F. hepatica in vitro.

Culture 1 - Modified Earle's salts solution

Culture 2 - Medium 199

Time (hours)	Culture	Volume of medium collected (mls)	Ammonia conc. (mg/l)		Rate of ammonia production (μg NH_3/fluke/hr)
			Pre flukes	Post flukes	
24	1	180	0.0	7.6	11.4
	2	185	3.0	11.0	12.3
48	1	184	0.0	3.2	4.9
	2	187	3.2	7.5	6.7
70	1	173	0.0	2.4	3.7
	2	178	3.3	6.3	4.8
93	1	186	0.0	1.0	1.6
	2	190	3.3	4.8	2.5
119	1	194	0.0	0.9	1.3
	2	196	3.8	5.4	2.4

APPENDIX
TABLE 6.1

The influence of including whole blood in the medium on the protein metabolism of adult F. hepatica maintained in vitro.

Culture 1 - 80% serum/20% modified Earle's salts solution

Culture 2 - 80% serum/20% modified Earle's salts solution + blood

Culture 3 - 80% serum/20% modified Earle's salts solution + blood (without flukes).

Time (hours)	Culture	Volume of medium collected (mls)	Ammonia conc. (mg/l)		Rate of ammonia production (μg NH_3/fluke/hr)
			Pre flukes	Post flukes	
20	1	150	19.5	32.0	10.4
	2	159	18.0	32.5	11.5*
	3	152	19.5	21.0	
44	1	163	18.5	27.5	6.8
	2	175	19.5	29.5	6.5*
	3	171	19.5	21.5	
66	1	150	25.0	35.0	7.6
	2	155	25.0	38.0	8.6*
	3	153	23.5	25.5	
90	1	155	26.5	32.5	4.3
	2	169	26.5	35.5	4.7*
	3	169	27.5	30.5	

* Corrected for NH_3 produced by blood.

APPENDIX
TABLE 7.1

The influence of medium flow-rate on the protein metabolism of adult *F. hepatica* maintained *in vitro*

Culture 1 - 80% serum/20% modified Earle's salts solution
Slow flow-rate

Culture 2 - 80% serum/20% modified Earle's salts solution
Fast flow-rate

Time (hours)	Culture	Volume of medium collected (mls)	Ammonia conc. (mg/l)		Rate of ammonia production (μg NH_3/fluke/hr)
			Pre flukes	Post flukes	
24	1	127	22.0	33.0	9.7
	2	275	21.0	31.5	20.1
47	1	133	22.0	32.5	10.1
	2	275	23.0	33.5	20.9
71	1	127	24.5	32.5	7.1
	2	280	26.5	33.5	13.6
95	1	133	26.0	31.0	4.6
	2	275	27.0	32.0	9.6

APPENDIX
TABLE 8.1

The influence of crowding on the protein metabolism of adult *F. hepatica* maintained *in vitro*.

Culture 1 - 80% serum/20% modified Earle's salts solution, 5 flukes

Culture 2 - 80% serum/20% modified Earle's salts solution, 23 flukes

Time (hours)	Culture	Volume of medium collected (mls)	Ammonia conc. (mg/l)		Rate of ammonia production (μg NH_3/fluke/hr)
			Pre flukes	Post flukes	
23.5	1	185	24.0	31.0	11.0
	2	177	25.5	63.5	12.4
50.0	1	219	26.5	33.5	11.6
	2	211	27.0	52.0	8.6
70.0	1	183	30.0	34.0	7.3
	2	175	30.5	47.5	6.8
93.5	1	200	31.5	34.5	5.1
	2	195	32.0	43.0	4.0

APPENDIX
TABLE 9.1

Fluke sizes ($\mu m^2 \times 10^3$) during *in vitro* maintenance of juvenile flukes.

Experiment 1 - Earle's salts solution; Experiment 2 - Whole sheep blood

Experiment	Days			Experiment	Days		
	0	1	2		0	3	7
1	27.0	19.2	18.0	2	21.6	22.4	28.0
	22.4	18.7	20.8		18.0	33.6	16.2
	28.5	18.0	16.9		26.6	28.0	28.5
	33.0	22.5	25.2		32.0	23.4	27.2
	22.4	16.8	20.4		25.2	25.2	25.5
	28.8	29.4	24.0		25.2	22.4	24.0
	23.8	24.0	25.5		28.0	22.8	22.0
	18.0	32.4	20.8		26.0	22.4	14.4
	25.5	25.3	30.0		14.4	22.1	25.2
	25.2	20.8	24.0		17.0	22.4	20.4
	24.0	25.2	25.5		26.4	28.6	27.0
	21.0	30.4	19.5		19.8	25.5	25.2
	21.0	20.0	22.5		21.6	18.0	26.6
	16.8	21.0	24.0		17.1	19.6	22.4
	23.8	23.8	22.1		18.0	21.6	22.0
	22.5	24.0	30.8		25.5	26.6	18.0
	28.5	37.5	25.2		21.6	31.5	21.0
	21.0	33.0	21.6		21.0	24.0	19.5
	33.0	49.0	15.0		19.8	26.0	20.0
	21.0	28.8	16.8		17.0	28.0	21.6
Mean size	24.36	25.99	22.43		22.09	24.71	22.74
$\pm$ S.D.	$\pm$4.39	$\pm$7.78	$\pm$4.13		$\pm$4.59	$\pm$3.86	$\pm$3.97

APPENDIX
TABLE 9.2

Fluke sizes during *in vitro* maintenance of juvenile flukes ($\mu m^2 \times 10^3$).

Experiment 3 - Calf serum.

Experiment	Days				
	0	3	6	8	10
3	18.0	28.5	22.4	28.0	28.5
	22.0	16.8	30.0	24.0	23.8
	30.0	19.5	25.5	27.0	29.9
	24.2	33.0	22.4	33.0	30.0
	20.4	22.0	23.8	23.8	33.6
	21.6	24.0	24.7	37.5	31.5
	19.2	28.0	20.9	30.8	16.8
	24.2	25.5	29.9	28.5	27.0
	20.4	23.8	28.6	30.0	23.1
	26.4	22.0	23.8	28.0	21.6
	18.7	28.5	24.0	30.0	27.0
	20.4	24.0	23.0	24.0	27.3
	28.8	25.5	23.8	23.4	27.0
	18.0	16.0	24.2	29.4	26.0
	19.2	25.0	28.0	26.6	25.5
	29.4	22.1	23.8	26.0	28.5
	17.0	22.5	30.8	30.4	27.0
	18.0	24.0	28.8	27.3	25.2
	18.0	27.0	25.5	31.5	31.5
	28.8	15.6	25.5	28.5	25.3
Mean size	22.13	23.66	25.47	28.38	26.80
± S.D.	± 4.37	±4.41	±2.88	±3.45	±3.79

APPENDIX
TABLE 9.3

Fluke sizes ($\mu m^2 \times 10^3$) during <u>in vitro</u> maintenance of juvenile flukes.

<u>Experiment 4</u> - MEM + 10% calf serum.

Experiment	Days				
	0	3	6	8	11
4	19.5	28.6	29.9	26.6	24.0
	18.2	31.5	34.5	28.0	30.0
	18.0	25.5	30.0	34.2	23.8
	16.8	30.8	30.0	26.6	37.8
	18.0	22.5	27.5	28.8	22.4
	19.2	24.0	25.2	21.6	28.6
	23.4	22.1	31.2	35.0	32.5
	13.0	28.0	33.0	36.4	22.4
	18.2	25.2	21.6	26.6	32.5
	26.6	26.0	26.4	37.5	33.6
	26.0	30.8	26.4	28.8	24.2
	18.0	28.0	43.2	30.0	23.4
	19.5	23.8	21.0	28.6	25.0
	12.0	25.0	30.0	28.0	25.2
	18.0	26.4	20.4	30.0	21.6
	20.4	30.0	18.0	30.0	26.4
	20.4	24.0	18.0	25.5	25.2
	22.4	28.8	31.5	27.0	26.0
	25.5	27.0	20.4	35.0	39.0
	17.0	23.1	24.0	30.0	33.6
Mean size	19.50	26.55	27.11	29.71	27.86
± S.D.	±3.83	±2.91	±6.30	±4.04	±5.27

APPENDIX
TABLE 9.4

Fluke sizes ($\mu m^2 \times 10^3$) during *in vitro* maintenance of juvenile flukes.
Experiment 5 - MEM + 50% liver extract + 10% calf serum.

Experiment	Days			
	0	3	5	8
5	16.0	28.0	25.2	28.5
	19.5	26.0	27.0	30.4
	16.8	23.4	26.0	26.6
	18.0	22.1	23.1	23.0
	18.7	18.0	27.3	28.0
	28.5	19.2	24.0	27.3
	21.0	21.6	26.6	30.0
	21.0	14.4	30.4	31.5
	17.0	25.2	23.8	27.3
	19.2	22.4	20.9	26.0
	28.5	17.0	22.4	25.2
	16.8	26.4	22.0	31.5
	15.0	26.4	25.2	25.3
	25.2	19.8	31.5	28.5
	21.6	26.6	25.3	28.8
	22.5	28.0	25.5	24.2
	22.4	17.1	28.8	27.3
	28.5	21.6	23.0	26.0
	33.0	14.4	23.8	25.5
	24.0	26.0	20.9	28.5
Mean size ± S.D.	21.66 ±4.96	22.18 ±4.41	25.14 ±2.90	27.47 ±2.32

APPENDIX
TABLE 9.5

Fluke sizes ($\mu m^2 \times 10^3$) during *in vitro* maintenance of juvenile flukes.
Experiment 6 - MEM + 10% calf serum + Chang liver cell monolayer.

Experiment	Days						
	0	3	5	7	10	13	21
6	23.4	30.6	39.0	56.7	63.0	66.0	80.0
	22.1	35.7	28.8	39.9	65.1	56.7	50.0
	20.7	29.4	67.6	42.5	60.0	54.0	52.0
	22.4	33.6	44.0	58.9	63.0	56.0	92.4
	22.4	32.2	50.0	54.0	61.2	42.0	78.2
	22.1	39.0	60.0	46.0	60.9	67.2	73.5
	17.0	32.2	40.5	51.0	60.0	64.0	57.2
	28.0	31.2	50.0	40.8	80.0	58.8	65.1
	20.4	35.0	64.0	38.4	57.0	78.3	75.9
	26.6	34.5	33.6	60.0	46.0	58.0	48.6
	18.7	32.0	38.4	46.8	50.4	80.0	86.0
	24.0	31.5	32.0	33.6	54.6	66.0	96.6
	22.4	33.6	36.0	45.0	52.0	109.2	51.0
	21.0	27.3	49.4	37.4	48.0	54.0	68.0
	18.7	33.6	46.0	56.7	40.8	74.8	56.0
	26.4	37.8	47.5	54.0	61.2	82.8	58.0
	27.6	31.5	36.0	72.0	52.0	71.4	52.5
	29.7	37.4	45.0	51.0	50.0	68.4	79.2
	27.6	34.5	46.8	43.2	57.0	54.0	70.4
	26.0	39.1	30.0	50.0	46.8	61.6	50.0
Mean size ± S.D.	23.36 ±3.52	33.58 ±3.14	44.23 ±10.79	48.87 ±9.34	56.45 ±14.49	66.16 ±14.41	67.03 ±15.16

APPENDIX
TABLE 9.6

Fluke sizes ($\mu m^2 \times 10^3$) during *in vitro* maintenance of juvenile flukes.

Experiment 7 - MEM alone + Chang liver cell monolayer.

Experiment	Days		
	0	3	5
7	20.4	22.1	24.4
	20.8	23.8	16.0
	19.6	20.8	18.0
	23.8	20.4	16.8
	20.4	30.4	23.8
	22.1	20.4	16.8
	20.8	22.0	23.8
	18.0	26.6	16.8
	19.2	22.4	15.6
	23.4	18.0	18.0
	14.4	22.5	25.2
	22.0	21.6	18.0
	24.0	18.0	21.0
	24.2	39.1	19.5
	28.0	21.0	20.8
	22.0	23.8	25.2
	25.2	22.5	21.6
	21.6	21.6	24.0
	24.0	18.0	25.2
	20.4	23.8	16.8
Mean size	21.71	22.94	20.36
± S.D.	±2.91	±4.77	±3.52

APPENDIX
TABLE 9.7

Fluke sizes ($\mu m^2 \times 10^3$) during *in vitro* maintenance of juvenile flukes.

Experiment 8 - MEM + 10% calf serum + Chang liver cell monolayer (under nitrogen).

Experiment	Days					
	0	2	5	7	10	14
8	22.5	22.4	21.6	29.4	33.6	25.2
	21.6	16.8	28.8	30.0	31.5	35.0
	25.5	16.8	27.6	29.9	26.6	28.8
	20.8	22.8	24.0	25.5	21.0	20.4
	27.3	25.2	25.5	31.5	33.0	37.5
	24.0	19.5	22.1	30.0	21.0	26.4
	21.0	22.4	30.0	35.7	19.5	24.0
	30.0	19.5	24.0	23.8	27.2	22.5
	20.8	18.2	20.9	37.4	26.4	29.4
	23.8	22.4	19.2	33.6	30.0	23.1
	20.4	19.6	32.2	30.0	36.0	25.2
	19.2	20.4	25.6	37.5	32.6	42.0
	22.4	19.2	29.4	37.4	23.8	20.4
	18.0	21.0	25.5	36.0	37.4	30.8
	18.2	22.1	22.5	33.0	26.4	23.8
	21.0	24.0	21.0	28.0	23.8	24.0
	19.2	19.5	25.5	36.4	36.0	28.6
	27.0	25.2	24.0	40.5	28.0	22.8
	19.5	20.4	24.3	27.3	30.8	33.0
	24.7	21.0	25.6	26.4	45.9	34.5
Mean size	22.34	20.92	24.96	31.96	29.52	27.87
± S.D.	±3.26	±2.41	±3.36	±4.68	±6.51	±5.97

APPENDIX
TABLE 9.8

Fluke sizes ($\mu m^2 \times 10^3$) during *in vitro* maintenance of juvenile flukes

Experiment 9 - MEM + 10% calf serum + Chang liver cell monolayer (under 97.6% nitrogen/1.9% oxygen/0.5% carbon dioxide).

Experiment	Days							
	0	2	5	7	9	12	15	20
9	20.4	24.0	23.8	26.4	37.4	40.0	100.0	51.0
	20.4	22.5	26.4	34.0	42.5	39.6	74.8	101.5
	19.2	30.8	28.5	38.4	39.6	28.6	67.6	44.2
	22.0	22.5	22.5	34.0	34.0	38.0	67.2	76.8
	21.0	28.0	28.0	30.8	33.6	40.0	67.2	63.0
	21.0	25.5	28.8	36.4	41.4	52.5	42.0	63.0
	14.4	15.0	20.8	34.0	24.0	39.9	52.5	67.2
	16.5	30.8	32.3	27.4	41.8	50.6	69.0	69.0
	18.7	21.0	30.8	40.0	45.0	48.0	48.3	57.2
	18.7	30.4	37.4	36.4	60.9	40.8	37.5	98.6
	28.5	20.6	27.3	28.9	42.5	28.9	63.0	108.0
	12.8	27.0	28.0	40.5	45.0	52.8	50.4	67.2
	15.6	23.8	29.4	26.4	60.0	42.0	50.6	101.5
	15.0	24.0	36.0	29.4	30.0	39.6	40.0	59.5
	18.0	26.6	28.8	40.0	36.4	43.2	59.8	87.5
	16.8	22.4	32.0	30.0	31.5	36.0	64.4	72.9
	16.0	30.8	33.0	39.6	52.5	52.5	66.0	55.2
	19.5	28.8	32.0	34.0	30.4	30.0	55.0	49.4
	20.6	18.0	37.5	24.0	50.0	27.3	110.0	72.8
	18.2	27.0	28.0	34.0	41.6	40.8	71.3	59.5
Mean size ± S.D.	18.66 ±3.40	24.97 ±4.42	29.56 ±4.49	33.23 ±5.11	41.00 ±9.62	40.56 ±7.94	62.83 ±18.09	71.25 ±18.86

APPENDIX
TABLE 16.1

The immune response of rabbits experimentally infected with F. hepatica. Immunodiffusion titres (reciprocals of serum dilutions), using whole metabolic antigen in the test.

Rabbit no.	Weeks post infection					
	-1	0	2	4	6	8
1	0	0	0	4	8	16
2	0	0	2	4	16	16
3	0	0	0	4	8	16
4	0	0	2	4	8	8
Mean	0	0	1.0	4.0	10.0	14.0
± S.D.			±1.2	±0.0	±4.0	±4.0

APPENDIX
TABLE 16.2

The immune response of rabbits experimentally infected with F. hepatica. ELISA values (units of absorbance), using whole metabolic antigen in the test.

Rabbit no.	Weeks post infection					
	-1	0	2	4	6	8
1	0.00	0.00	0.30	0.49	0.43	0.45
2	0.00	0.00	0.34	0.41	0.43	0.45
3	0.00	0.00	0.17	0.46	0.54	0.36
4	0.00	0.00	0.21	0.36	0.45	0.41
Mean	0.00	0.00	0.255	0.430	0.462	0.417
± S.D.			±0.078	±0.057	±0.052	±0.043

APPENDIX
TABLE 16.3

Immune response of rabbits experimentally infected with F. hepatica. ELISA values (units of absorbance), using non-protein component of the metabolic antigen in the test

	Weeks post infection					
Rabbit no.	-1	0	2	4	6	8
1	0.00	0.00	0.27	0.45	0.40	0.43
2	0.00	0.00	0.28	0.40	0.41	0.42
3	0.00	0.00	0.19	0.43	0.46	0.35
4	0.00	0.00	0.20	0.39	0.42	0.39
Mean	0.00	0.00	0.235	0.418	0.423	0.398
± S.D.			±0.046	±0.027	±0.026	±0.036

APPENDIX
TABLE 16.4

The immune response of rabbits experimentally infected with F. hepatica. Immunodiffusion titres (reciprocals of serum dilutions), using somatic fluke antigen in the test.

Rabbit no.	Weeks post infection					
	-1	0	2	4	6	8
1	0	0	0	4	8	16
2	0	0	2	4	16	16
3	0	0	2	8	8	16
4	0	0	2	4	16	8
Mean	0	0	1.5	5.0	12.0	14.0
± S.D.			±1.0	±2.0	±4.6	±4.0

APPENDIX
TABLE 16.5

The immune response of rabbits experimentally infected with F. hepatica. ELISA values (units of absorbance) using somatic fluke antigen in the test.

Rabbit no.	Weeks post infection					
	-1	0	2	4	6	8
1	0.00	0.00	0.26	0.39	0.40	0.42
2	0.00	0.00	0.29	0.41	0.38	0.39
3	0.00	0.00	0.15	0.41	0.44	0.50
4	0.00	0.00	0.24	0.43	0.35	0.45
Mean	0.00	0.00	0.235	0.410	0.393	0.440
± S.D.			±0.060	±0.160	±0.038	±0.047

APPENDIX
TABLE 16.6

Immune response of sheep experimentally infected with F. hepatica. Immunodiffusion titres (reciprocals of serum dilutions), using whole metabolic antigen in the test.

Sheep no.	Weeks post infection					
	-1	0	2	4	6	8
1	0	0	0	8	16	16
2	0	0	2	16	16	8
3	0	0	2	16	16	16
4	0	0	0	2	16	8
Mean	0	0	1.0	10.5	16.0	12.0
± S.D.			±1.2	±6.8	±0.0	±4.6

APPENDIX
TABLE 16.7

Immune response of sheep experimentally infected with F. hepatica. ELISA values (units of absorbance), using whole metabolic antigen in the test.

Sheep no.	Weeks post infection					
	-1	0	2	4	6	8
1	0.08	0.09	0.31	0.30	0.17	0.27
2	0.09	0.08	0.27	0.35	0.30	0.30
3	0.08	0.08	0.24	0.22	0.28	0.30
4	0.07	0.08	0.30	0.33	0.17	0.22
Mean	0.080	0.082	0.280	0.300	0.230	0.273
± S.D.	±0.008	±0.005	±0.031	±0.057	±0.070	±0.038

APPENDIX
TABLE 16.8

Immune response of sheep experimentally infected with F. hepatica. Immunodiffusion titres (reciprocals of serum dilutions), using non-protein component of the metabolic antigen in the test.

	Weeks post infection					
Sheep no.	-1	0	2	4	6	8
1	0	0	0	8	16	16
2	0	0	2	16	16	8
3	0	0	2	16	16	16
4	0	0	0	4	16	8
Mean	0	0	1.0	11.0	16.0	12.0
± S.D.			±1.2	±6.0	±0.0	±4.6

APPENDIX
TABLE 16.9

Immune response of sheep experimentally infected with F. hepatica. ELISA values (units of absorbance), using non-protein component of the metabolic antigen in the test.

	Weeks post infection					
Sheep no.	-1	0	2	4	6	8
1	0.08	0.09	0.30	0.31	0.19	0.28
2	0.08	0.08	0.28	0.29	0.30	0.30
3	0.08	0.07	0.26	0.25	0.28	0.33
4	0.07	0.09	0.31	0.31	0.16	0.26
Mean	0.077	0.082	0.288	0.290	0.233	0.293
± S.D.	±0.005	±0.010	±0.022	±0.028	±0.068	±0.030

APPENDIX
TABLE 16.10

Immune response of sheep experimentally infected with F. hepatica.

Immunodiffusion titres (reciprocals of serum dilutions), using somatic fluke antigen in the test.

	Weeks post infection					
Sheep no.	-1	0	2	4	6	8
1	0	0	1	8	16	16
2	0	0	2	16	16	16
3	0	0	2	16	16	16
4	0	0	0	16	16	8
Mean	0	0	1.3	14.0	16.0	14.0
± S.D.			±1.0	±4.0	±0.0	±4.0

APPENDIX
TABLE 16.11

Immune response of sheep experimentally infected with F. hepatica.

ELISA values (units of absorbance), using somatic fluke antigen in the test.

	Weeks post infection					
Sheep no.	-1	0	2	4	6	8
1	0.09	0.08	0.33	0.31	0.30	0.26
2	0.09	0.09	0.29	0.35	0.33	0.29
3	0.08	0.08	0.26	0.30	0.33	0.32
4	0.08	0.08	0.28	0.33	0.31	0.30
Mean	0.085	0.083	0.290	0.322	0.318	0.293
± S.D.	±0.006	±0.005	±0.029	±0.022	±0.015	±0.025

APPENDIX
TABLE 16.12

Immune response of rats experimentally infected with F. hepatica.

Immunodiffusion titres (reciprocals of serum dilutions), using whole metabolic antigen in the test.

Rat no.	Weeks post infection					
	-1	0	2	4	6	8
1	0	0	4	16	8	8
2	0	0	4	16	4	4
3	0	0	4	8	8	8
4	0	0	8	8	8	8
Mean	0	0	5.0	12.0	7.0	7.0
± S.D.			±2.0	±4.6	±2.0	±2.0

APPENDIX
TABLE 16.13

Immune response of rats experimentally infected with F. hepatica.

ELISA values (units of absorbance), using whole metabolic antigen in the test.

Rat no.	Weeks post infection					
	-1	0	2	4	6	8
1	0.00	0.00	0.12	0.22	0.17	0.19
2	0.00	0.00	0.16	0.19	0.22	0.23
3	0.00	0.00	0.14	0.21	0.21	0.16
4	0.00	0.00	0.14	0.17	0.19	0.20
Mean	0.00	0.00	0.140	0.198	0.198	0.195
± S.D.			±0.016	±0.022	±0.022	±0.028

APPENDIX
TABLE 16.14

Immune response of calves experimentally infected with F. hepatica. Immunodiffusion titres (reciprocals of serum dilutions), using whole metabolic antigen in the test.

	Weeks post infection					
Calf no.	-1	0	2	4	6	8
1	0	0	0	1	1	1
2	0	0	0	1	2	2
Mean	0	0	0	1.0	1.5	1.5
± S.D.				±0.0	±0.7	±0.7

APPENDIX
TABLE 16.15

Immune response of calves experimentally infected with F. hepatica. ELISA values (units of absorbance), using whole metabolic antigen in the test.

	Weeks post infection					
Calf no.	-1	0	2	4	6	8
1	0.03	0.05	0.08	0.09	0.06	0.04
2	0.04	0.04	0.12	0.12	0.08	0.05
Mean	0.035	0.045	0.100	0.105	0.070	0.045
± S.D.	±0.007	±0.007	±0.028	±0.021	±0.014	±0.007

APPENDIX
TABLE 21.1

Serology of immunised and control rabbits. Immunodiffusion titres (reciprocal of serum dilution). Rabbits numbers 1-6, immunised; numbers 7-11, controls. Pre-challenge.

Rabbit number.	Weeks after initial immunisation					
	0	2	4	6	8	10
1	0	0	1	16	8	8
2	0	0	0	8	4	4
3	0	0	0	8	4	4
4	0	0	0	4	4	4
5	0	0	0	4	4	4
6	0	0	0	4	4	8
Group mean ± S.D.	0	0	0.2 ±0.4	7.3 ±4.6	4.6 ±0.2	5.3 ±2.1
7	0	0	0	0	0	0
8	0	0	0	0	0	0
9	0	0	0	0	0	0
10	0	0	0	0	0	0
11	0	0	0	0	0	0
Group mean ± S.D.	0	0	0	0	0	0

APPENDIX
TABLE 21.1 (contd.)

Serology of immunised and control rabbits. Immunodiffusion titres (reciprocal of serum dilution). Rabbits numbers 1-6, immunised; numbers 7-11, controls. Post-challenge.

Rabbit number.	Weeks after initial immunisation				
	12*	14	16	18	20
1	16	16	16	32	64
2	8	8	16	32	64
3	8	8	16	64	64
4	8	16	32	64	64
5	32	32	32	64	128
6	-	16	32	64	-
Group mean	13.3	16.0	24.0	53.3	76.8
± S.D.	±9.7	±8.8	±8.8	±16.5	±28.6
7	0	0	8	16	16
8	0	0	2	8	8
9	0	0	2	8	8
10	0	0	0	8	8
11	0	0	0	16	8
Group mean	0	0	2.4	11.2	9.6
± S.D.			±3.3	±4.4	±3.6

* Administration of challenge infection.

APPENDIX
TABLE 21.2

Serology of immunised and control rabbits. ELISA values (units of Absorbance). Rabbits numbers 1-6 immunised; numbers 7-11 controls - Pre-challenge .

Rabbit no.	Weeks after infection					
	0	2	4	6	8	10
1	0.0	0.0	0.03	0.16	0.08	0.27
2	0.0	0.0	0.13	0.20	0.30	0.16
3	0.0	0.0	0.04	0.21	0.07	0.14
4	0.0	0.05	0.06	0.29	0.14	0.11
5	0.0	0.0	0.02	0.26	0.09	0.34
6	0.0	0.0	0.05	0.12	0.11	0.18
Group mean ± S.D.	0.0	0.008 ±0.020	0.055 ±0.039	0.207 ±0.062	0.132 ±0.086	0.203 ±0.086
7	0.0	0.0	0.0	0.0	0.0	0.0
8	0.0	0.0	0.0	0.0	0.0	0.0
9	0.0	0.0	0.0	0.0	0.0	0.0
10	0.0	0.0	0.0	0.0	0.0	0.0
11	0.0	0.0	0.0	0.0	0.0	0.0
Group mean ± S.D.	0.0	0.0	0.0	0.0	0.0	0.0

APPENDIX
TABLE 21.2 (contd.)

Serology of immunised and control rabbits. ELISA values (units of Absorbance). Rabbits 1-6 immunised; numbers 7-11 controls - Post challenge.

Rabbit no.	Weeks after infection				
	12*	14	16	18	20
1	0.11	0.08	0.23	0.24	0.36
2	0.17	0.36	0.41	0.37	0.49
3	0.17	0.26	0.23	0.26	0.34
4	0.17	0.37	0.24	0.31	0.32
5	0.27	0.39	0.30	0.34	0.30
6	-	0.27	0.16	0.34	-
Group mean ± S.D.	0.178 ±0.057	0.285 ±0.112	0.261 ±0.085	0.310 ±0.051	0.362 ±0.074
7	0.0	0.16	0.19	0.19	0.16
8	0.0	0.18	0.25	0.19	0.17
9	0.0	0.05	0.11	0.24	0.15
10	0.0	0.06	0.14	0.23	0.20
11	0.0	0.02	0.15	0.22	0.13
Group mean ± S.D.	0.0	0.094 ±0.071	0.168 ±0.054	0.214 ±0.023	0.162 ±0.025

* Administration of challenge infection

APPENDIX
TABLE 21.3

Numbers and sizes (mm^2) of flukes recovered from immunised and control rabbits. Rabbits numbers 1-6, immunised group.

Rabbit no.	Fluke sizes	Mean size ± S.D.
1	55.0, 21.0, 36.0, 30.0, 20.0, 24.0, 18.0, 40.0, 28.0, 32.0, 18.0, 21.0, 40.0, 15.0, 24.0, 24.0, 18.0, 40.0, 36.0, 24.0, 39.0, 24.0.	28.5 ±10.1
2	36.0, 36.0, 36.0, 40.0, 45.0, 20.0, 20.0, 27.0, 21.0, 40.0, 40.0, 45.0, 21.0, 27.0, 21.0, 24.0, 45.0, 28.0, 28.0, 15.0, 15.0, 18.0, 18.0.	29.0 ±10.2
3	32.0, 18.0, 18.0, 28.0, 9.0, 18.0, 21.0, 27.0, 32.0, 24.0, 36.0, 21.0, 32.0, 18.0, 35.0, 21.0, 28.0, 21.0, 18.0, 28.0, 28.0, 24.0, 18.0, 18.0, 18.0, 27.0, 3.0.	23.0 ±7.6
4	20.0, 18.0, 27.0, 18.0, 32.0, 30.0, 24.0, 21.0, 50.0, 75.0, 66.0, 45.0, 44.0, 40.0, 40.0, 32.0, 21.0, 21.0, 24.0, 25.0, 24.0, 54.0, 55.0, 40.0, 21.0, 30.0, 28.0, 36.0, 24.0, 32.0, 14.0, 14.0, 24.0, 14.0, 55.0, 40.0.	32.7 ±15.0
5	40.0, 44.0, 44.0, 40.0, 40.0, 30.0, 32.0, 45.0, 44.0, 27.0, 55.0, 27.0, 36.0, 32.0, 40.0, 24.0, 35.0, 24.0, 32.0, 36.0, 18.0, 18.0, 32.0, 18.0, 24.0, 48.0, 27.0, 36.0.	33.8 ±9.5
6	52.0, 30.0, 42.0, 55.0, 52.0, 40.0, 36.0, 40.0, 68.0, 52.0, 48.0, 27.0, 32.0, 52.0, 44.0, 56.0, 40.0, 16.0, 18.0, 28.0, 36.0, 28.0, 40.0, 40.0, 24.0, 40.0, 32.0, 32.0, 36.0, 21.0, 36.0, 28.0, 16.0, 35.0, 18.0, 12.0, 21.0, 32.0, 12.0, 24.0, 12.0.	34.2 ±13.5

APPENDIX
TABLE 21.3 (contd.)

Numbers and sizes (mm^2) of flukes recovered from immunised and control rabbits. Rabbits numbers 7-11, control group.

Rabbit no.	Fluke sizes	Mean size ± S.D.
7	24.0, 60.0, 75.0, 40.0, 50.0, 66.0, 50.0, 44.0, 52.0, 40.0, 60.0, 50.0, 50.0,75.0, 50.0, 60.0, 32.0, 54.0, 40.0, 56.0, 40.0, 54.0, 60.0, 60.0, 65.0, 36.0, 55.0, 28.0, 55.0, 50.0, 28.0, 35.0, 72.0, 48.0, 32.0, 55.0, 72.0, 44.0, 40.0, 36.0, 42.0, 55.0, 52.0, 56.0, 66.0, 40.0, 50.0, 32.0.	49.7 ±12.7
8	40.0, 32.0, 30.0, 40.0, 32.0, 50.0, 40.0, 16.0, 50.0, 40.0, 44.0, 36.0, 36.0, 24.0, 40.0, 21.0, 55.0, 40.0, 44.0, 50.0, 40.0, 55.0, 44.0, 33.0, 24.0, 40.0, 40.0, 36.0.	38.3 ±9.6
9	65.0, 50.0, 50.0, 45.0, 45.0, 60.0, 15.0, 30.0, 21.0, 18.0, 32.0, 40.0, 24.0, 18.0, 60.0, 45.0, 40.0, 70.0, 40.0, 21.0, 21.0, 28.0, 32.0, 21.0, 24.0, 24.0, 24.0, 24.0.	35.2 ±15.7
10	32.0, 36.0, 18.0, 32.0, 40.0, 45.0, 24.0, 45.0, 36.0, 24.0, 32.0, 15.0, 15.0, 45.0, 28.0, 36.0, 32.0, 28.0, 30.0, 36.0, 32.0, 8.0, 24.0, 32.0, 18.0.	29.7 ±9.7
11	40.0, 50.0, 50.0, 44.0, 45.0, 75.0, 60.0, 18.0, 40.0, 24.0, 28.0, 35.0, 32.0, 40.0, 60.0, 50.0, 44.0, 28.0, 45.0, 36.0, 24.0, 28.0, 20.0, 28.0, 20.0.	38.6 ±14.2

APPENDIX
TABLE 21.4

Eosinophil counts of immunised and control rabbits (cells per mm^3).

Rabbits numbers 1-6 immunised; numbers 7-11 controls.

Rabbit no.	Weeks post infection				
	0	2	4	6	8
1	85	1524	2054	3616	3156
2	106	1136	798	1890	1242
3	272	393	1656	815	2820
4	348	1599	1290	2839	1980
5	230	760	765	1162	2745
6	90	540	984	2013	-
Group mean	188.5	992.0	1257.8	2055.8	2388.1
± S.D.	±110.7	±507.8	±514.3	±1040.7	±771.9
7	96	1232	1470	4338	906
8	144	837	1876	2016	975
9	93	520	2688	5852	3255
10	222	624	1239	2520	5025
11	257	321	2080	2800	3354
Group mean	162.4	706.8	1870.6	3505	2703.0
± S.D.	±74.3	±347.6	±563.8	±1572.2	±1756.1
t	0.45	1.06	1.88	1.81	0.39
Significance	n.s.	n.s.	n.s.	n.s.	n.s.

APPENDIX
TABLE 21.5

Glutamate dehydrogenase assays of immunised and control rabbits (micro moles product per litre of serum per minute).

Rabbits numbers 1-6 immunised; numbers 7-11 controls.

Rabbit no.	Weeks post infection				
	0	2	4	6	8
1	2.4	16.9	31.4	38.6	31.4
2	1.2	31.4	21.7	53.1	33.8
3	2.4	7.2	14.5	29.0	9.7
4	2.4	9.7	19.3	38.6	55.5
5	3.6	12.1	7.2	16.9	21.7
6	2.4	24.2	31.4	33.8	-
Group mean ± S.D.	2.40±0.76	16.92±9.30	20.92±9.51	35.00±12.00	30.42±16.93
7	2.4	14.5	29.0	62.8	7.2
8	2.4	12.1	24.2	36.2	16.9
9	3.6	16.9	38.6	26.6	14.5
10	1.2	29.0	31.4	36.2	62.8
11	2.4	14.5	26.6	67.6	33.8
Group mean ± S.D.	2.40±0.85	17.40±6.70	29.96±5.52	45.88±18.15	27.04±22.24
t	0.0	0.10	1.88	1.20	0.27
Significance	n.s.	n.s.	n.s.	n.s.	n.s.

APPENDIX
TABLE 22.1

Serology of rats immunised with metabolic antigen derived from adult flukes and of control rats. Immunodiffusion titres (reciprocal of serum dilution). Rats numbers 1-10, immunised; numbers 11-19 controls

Rat no.	Weeks after initial immunisation								
	0	2	4	6	8	10*	12	14	16
1	0	0	0	1	2	0	1	8	8
2	0	0	0	2	1	1	2	8	8
3	0	0	1	4	2	4	4	8	8
4	0	0	0	1	1	1	2	4	8
5	0	0	0	2	2	2	2	4	16
6	0	0	0	4	2	2	4	4	8
7	0	0	0	4	1	4	8	8	8
8	0	0	0	2	1	4	4	8	8
9	0	0	0	2	1	4	4	4	4
10	0	0	0	2	1	2	2	16	4
Group mean ± S.D.	0	0	0.01 ±0.3	2.4 ±1.2	1.4 ±0.5	2.4 ±1.5	3.3 ±2.0	7.2 ±3.7	8.0 ±3.3
11	0	0	0	0	0	0	8	8	4
12	0	0	0	0	0	0	0	16	8
13	0	0	0	0	0	0	0	8	8
14	0	0	0	0	0	0	4	8	4
15	0	0	0	0	0	0	4	8	4
16	0	0	0	0	0	0	4	2	8
17	0	0	0	0	0	0	4	4	4
18	0	0	0	0	0	0	4	2	2
19	0	0	0	0	0	0	2	8	16
Group mean ± S.D.	0	0	0	0	0	0	3.3 ±2.4	7.1 ±4.2	6.4 ±4.2

* Administration of challenge infection

APPENDIX
TABLE 22.2

Numbers and sizes of flukes recovered from rats immunised with metabolic antigen derived from adult flukes and from control rats.

Rats numbers 1-10, immunised; numbers 11-19, controls.

Rat no.	No. flukes recovered.	Fluke sizes (mm^2).	Rat no.	No. flukes recovered.	Fluke sizes (mm^2).
1	1	85.0.	11	1	24.0.
2	3	28.0, 8.0, 4.0.	12	1	24.0.
3	4	40.0, 21.0, 8.0, 8.0.	13	4	21.0, 15.0, 12.0, 9.0.
4	1	30.0.	14	1	40.0.
5	1	24.0.	15	3	50.0, 40.0, 24.0.
6	4	65.0, 18.0, 15.0 9.0.	16	2	52.0, 15.0.
7	2	48.0, 50.0.	17	2	32.0, 27.0.
8	1	65.0.	18	1	66.0.
9	1	14.0.	19	2	20.0, 6.0.
10	1	72.0.			

TABLE 22.3

Eosinophil counts of rats immunised with metabolic antigen derived from adult flukes and of control rats (cells per mm^3). Rats numbers 1-10, immunised; numbers 11-19, controls.

Rat no.	Weeks post infection			
	0	2	4	6
1	0	756	2414	640
2	100	1143	2167	1776
3	242	1898	2640	3795
4	260	444	880	630
5	490	1008	2299	2975
6	318	276	-	740
7	264	625	2775	2277
8	258	515	1188	2329
9	396	246	92	0
10	504	972	1240	2375
Group mean ± S.D.	283.2 ±157.2	788.3 ±496.3	1743.8 ±924.7	1753.7 ±1213.6
11	101	344	1800	744
12	184	979	2223	1540
13	110	615	1776	1376
14	86	552	990	1551
15	308	581	1300	1197
16	336	535	1727	1419
17	188	369	3636	2376
18	224	273	1652	704
19	81	1599	1694	2100
Group mean ± S.D.	179.8 ±95.2	649.6 ±411.1	1866.4 ±746.3	1445.2 ±550.3
t	1.71	0.66	0.31	0.75
Significance	n.s.	n.s.	n.s.	n.s.

Plasma glutamate dehydrogenase assays of rats immunised with metabolic antigen derived from adult flukes and of control rats (micromoles of product per litre of serum per minute. Rats numbers 1-10, immunised; numbers 11-19, controls.

Rat no.	Weeks post infection			
	0	2	4	6
1	7.2	14.5	26.6	16.9
2	1.2	14.5	7.2	12.1
3	2.4	14.5	12.1	19.3
4	2.4	4.8	24.1	74.9
5	1.2	2.4	43.5	36.2
6	1.2	0.0	-	4.8
7	2.4	4.8	4.8	4.8
8	1.2	0.0	12.1	33.8
9	2.4	0.0	14.5	7.2
10	0.0	7.2	29.0	7.2
Group mean ± S.D.	2.16±1.94	6.26±6.15	19.32±12.44	21.72±21.85
11	2.4	2.4	25.7	7.2
12	0.0	2.4	12.1	86.9
13	0.0	7.2	24.1	77.3
14	0.0	9.7	53.1	7.2
15	9.7	1.2	19.3	4.8
16	0.0	4.8	24.1	72.4
17	0.0	4.8	50.7	62.8
18	1.2	2.4	38.6	7.2
19	0.0	2.4	21.7	12.1
Group mean ± S.D.	1.48±3.19	4.14±2.78	29.93±14.27	37.54±35.97
t	0.58	0.95	1.68	1.17
Significance	n.s.	n.s.	n.s.	n.s.

APPENDIX
TABLE 23.1

Serology of rats immunised with freshly collected metabolic antigen derived from adult flukes and of control rats. Immunodiffusion titres (reciprocal of serum dilution). Rats numbers 1-10, immunised; numbers 11-19, controls.

Rat no.	Weeks after initial immunisation							
	0	2	4	6	8*	10	12	14
1	0	0	1	4	4	1	8	8
2	0	0	2	2	4	4	2	8
3	0	0	2	1	2	2	1	2
4	0	0	0	1	2	1	2	2
5	0	0	1	4	1	1	4	4
6	0	0	0	4	2	1	4	4
7	0	0	0	1	4	2	2	8
8	0	0	0	4	1	2	4	4
9	0	0	0	4	1	2	2	2
10	0	0	1	2	2	2	4	4
Group mean ± S.D.	0	0	0.7 ±0.8	2.7 ±1.4	2.3 ±1.2	1.8 ±0.9	3.3 ±2.0	4.6 ±2.5
11	0	0	0	0	0	4	2	4
12	0	0	0	0	0	1	4	2
13	0	0	0	0	0	4	4	32
14	0	0	0	0	0	4	4	32
15	0	0	0	0	0	4	8	4
16	0	0	0	0	0	8	2	2
17	0	0	0	0	0	8	4	8
18	0	0	0	0	0	4	2	2
19	0	0	0	0	0	4	4	4
Group Mean ± S.D.	0	0	0	0	0	4.5 ±2.2	3.8 ±1.8	10.0 ±12.6

* Administration of challenge infection.

APPENDIX
TABLE 23.2

Numbers and sizes of flukes recovered from rats immunised with freshly collected metabolic antigen derived from adult flukes and from control rats. Rats numbers 1-10, immunised; numbers 11-19, controls.

Rat no.	No.flukes recovered	Fluke sizes (mm^2)	Rat no.	No.flukes recovered	Fluke sizes (mm^2)
1	2	60.0, 21.0	11	4	30.0, 70.0, 78.0,
2	2	36.0, 32.0			60.0
3	1	98.0	12	4	14.0, 12.0, 28.0, 45.0
4	6	54.0, 77.0, 60.0, 21.0, 24.0, 8.0	13	3	28.0, 45.0, 24.0
5	1	128.0	14	3	36.0, 24.0, 40.0
6	3	60.0, 12.0, 8.0	15	2	50.0, 36.0
7	4	84.0, 21.0, 12.0,	16	6	84.0, 72.0, 78.0, 24.0,
		4.0			6.0, 8.0
8	3	78.0, 84.0, 40.0	17	2	24.0, 66.0
9	4	40.0, 40.0, 44.0,	18	3	28.0, 45.0, 14.0
		50.0	19	1	98.0
10	1	60.0			
Group means ± S.D.	2.7 ±1.6	46.5±30.8		3.1 ±1.5	41.7±25.3

APPENDIX
TABLE 23.3

Eosinophil counts of rats immunised with freshly collected metabolic antigen derived from adult flukes and of control rats. Cells per mm^3. Rats numbers 1-10, immunised; numbers 11-19 controls.

	Weeks post infection			
Rat no.	0	2	4	6
1	230	320	1580	1030
2	117	890	2540	1070
3	0	620	1490	1450
4	81	1370	3420	1976
5	120	250	1450	1260
6	180	1250	1660	1150
7	88	1570	2230	3250
8	270	500	1270	880
9	224	620	760	1850
10	0	630	123	1510
Group mean ± S.D.	131.0 ±93.8	802.0 ±452.8	1652.3 ±918.9	1542.6 ± 696.5
11	102	740	1600	1220
12	0	700	2810	2448
13	180	230	2860	1520
14	350	750	2626	2460
15	310	1020	2641	1040
16	103	1310	1460	1650
17	330	830	2880	2150
18	0	550	1020	970
19	0	570	180	1300
Group mean ± S.D.	152.0 ±146.1	744.4 ±303.7	2008.5 ±980.8	1639.7 ± 581.0
t	0.38	0.32	0.77	0.32
Significance	n.s.	n.s.	n.s.	n.s.

APPENDIX
TABLE 23.4

Plasma glutamate dehydrogenase assays of rats immunised with freshly collected metabolic antigen derived from adult flukes and of control rats. (micromoles of product per litre of serum per minute). Rat numbers 1-10, immunised; numbers 11-19, controls.

Rat no.	Weeks post infection			
	0	2	4	6
1	0.0	2.4	55.5	24.0
2	0.0	12.1	21.7	33.8
3	7.2	2.4	19.3	0.0
4	2.4	19.3	24.0	19.3
5	0.0	12.1	48.0	29.0
6	0.0	2.4	2.4	2.4
7	2.4	12.1	33.8	4.8
8	4.8	12.1	87.0	62.8
9	0.0	12.1	45.9	130.4
10	2.4	-	4.8	4.8
Group mean ± S.D.	1.92 ±2.48	9.66 ±5.92	34.24 ±25.66	31.13 ±39.78
11	2.4	2.4	19.3	4.8
12	7.2	4.8	45.9	4.8
13	0.0	12.1	70.0	89.3
14	2.4	9.7	-	12.1
15	0.0	2.4	2.4	14.5
16	7.2	12.1	16.9	58.0
17	7.2	9.7	67.6	67.6
18	2.4	2.4	4.8	9.7
19	0.0	2.4	67.6	9.7
Group mean ± S.D.	3.20 ±3.17	6.44 ±4.37	36.81 ±29.26	30.05 ±32.34
t	0.98	1.31	0.20	0.06
significance	n.s	n.s	n.s	n.s

APPENDIX
TABLE 24.1

Survival times and flukes recovered from immunised and control mice.

Days after challenge	No. of mice dying		No. of flukes recovered/mouse	
	Immunised group	Control group	Immunised group	Control group
19	1		2	
24	5	3	2,2,0,0,0	2,2,0.
25	4	5	1,2,1,0.	1,2,1,1,1.
26	1	1	1	1
27		3		2,1,1.
28	1		1	
29	1		1	
30*	6	6	0,2,2,1,1,0.	1,1,1,0,0,2

* All remaining mice were killed at 30 days after challenge.

APPENDIX
TABLE 25.1

Serology of rats immunised with metabolic antigen derived from 16-day-old flukes and of control rats. Immunodiffusion titres (reciprocal of serum dilution). Rats numbers 1-10, immunised; numbers 11-20, controls.

	Weeks after initial immunisation							
Rat no.	0	2	4	6	8*	10	12	14
1	0	0	0	0	1	8	16	4
2	0	0	0	2	1	4	8	2
3	0	0	1	1	4	4	4	4
4	0	0	0	2	1	4	8	8
5	0	0	0	1	0	8	8	2
6	0	0	0	0	1	8	16	4
7	0	0	0	1	0	8	8	4
8	0	0	0	0	1	8	4	8
9	0	0	0	0	0	4	8	8
10	0	0	0	2	1	4	8	2
Group mean ± S.D.	0	0	0.1 ±0.3	0.9 ±0.9	1.0 ±1.1	6.0 ±2.1	8.8 ±4.1	4.6 ±2.5
11	0	0	0	0	0	2	4	4
12	0	0	0	0	0	8	8	4
13	0	0	0	0	0	16	4	4
14	0	0	0	0	0	8	8	2
15	0	0	0	0	0	4	8	2
16	0	0	0	0	0	2	2	2
17	0	0	0	0	0	4	8	8
18	0	0	0	0	0	8	8	8
19	0	0	0	0	0	4	4	2
20	0	0	0	0	0	2	8	2
Group mean ± S.D.	0	0	0	0	0	5.8 ±4.4	6.2 ±2.4	3.8 ±2.4

* Administration of challenge infection

APPENDIX
TABLE 25.2

Numbers and sizes of flukes recovered from rats immunised with metabolic antigen derived from 16-day-old flukes and from control rats. Rats numbers 1-10, immunised; numbers 11-20, controls.

Rat no.	No. flukes recovered	Fluke sizes (mm^2)	Rat no.	No. flukes recovered	Fluke sizes (mm^2)
1	5	40.0, 55.0, 36.0, 5.0, 21.0.	11	1	15.0.
			12	4	55.0, 20.0, 21.0, 12.0.
2	3	12.0, 24.0, 10.0.			
3	3	66.0, 24.0, 21.0.	13	3	32.0, 10.0, 12.0.
4	5	50.0, 45.0, 16.0, 32.0, 14.0.	14	5	55.0, 40.0, 16.0, 10.0,
5	4	60.0, 36.0, 40.0, 32.0.			30.0.
			15	6	66.0, 60.0,
6	4	45.0, 50.0, 32.0, 28.0.			45.0, 50.0, 32.0, 40.0.
7	5	4.0, 8.0, 18.0, 45.0, 40.0.	16	2	10.0, 28.0.
			17	4	45.0, 32.0,
8	4	48.0, 72.0, 32.0, 6.0.			32.0, 40.0.
			18	6	40.0, 40.0,
9	1	45.0.			32.0, 28.0, 40.0, 32.0.
10	2	50.0, 21.0.			
			19	3	50.0, 50.0, 40.0.
			20	-	
Group means	3.6	32.9		3.8	34.1
± S.D.	±1.3	±17.8		±1.7	±15.3

APPENDIX
TABLE 25.3

Eosinophil counts of rats immunised with metabolic antigen derived from 16-day-old flukes and of control rats. Cells per mm^3. Rats numbers 1-10, immunised; numbers 11-20, controls.

Rat no.	Weeks post infection 0	2	4	6
1	160	1991	2720	1010
2	0	1380	2500	2150
3	118	2610	2330	1460
4	248	1640	1380	420
5	135	960	1930	2720
6	75	2480	3370	1270
7	440	1700	3130	2170
8	152	1300	2590	2490
9	0	1660	1790	960
10	312	1800	870	1410
Group mean ± S.D.	164.0 ±137.4	1752.1 ±507.6	2261.0 ±775.2	1606.0 ± 744.4
11	0	950	2400	1800
12	140	1990	2480	1490
13	106	1300	2180	980
14	108	1920	2110	1310
15	270	2460	1490	1710
16	0	1010	1730	860
17	240	1177	3640	2090
18	0	1430	2170	900
19	0	570	680	2660
20	131	1230	1070	1140
Group mean ± S.D.	99.5 ±100.6	1403.8 ±556.2	1995.0 ± 822.8	1494.0 ± 580.5
t	1.20	1.45	0.74	0.36
Significance	n.s.	n.s.	n.s.	n.s.

APPENDIX
TABLE 25.4

Plasma glutamate dehydrogenase assays of rats immunised with metabolic antigen derived from 16-day-old flukes and of control rats. (micromoles of product per litre of serum per minute). Rats numbers 1-10, immunised; numbers 11-20, controls.

	Weeks post infection			
Rat no.	0	2	4	6
1	9.6	58.0	86.9	67.6
2	0.0	14.5	9.7	33.8
3	0.0	0.0	9.7	14.5
4	4.8	38.6	72.4	55.5
5	9.6	50.7	101.4	28.9
6	0.0	0.0	33.8	7.2
7	4.8	15.0	41.0	74.9
8	12.0	48.3	50.7	101.4
9	0.0	45.9	91.8	101.4
10	7.2	12.1	16.9	21.7
Group mean ± S.D.	4.80 ±4.66	28.31 ±22.19	51.43 ±34.83	50.69 ±34.70
11	0.0	19.3	16.9	2.4
12	9.6	48.3	41.0	77.3
13	0.0	33.8	38.6	33.8
14	0.0	24.1	9.6	14.5
15	7.2	0.0	9.6	4.8
16	2.4	48.3	55.5	70.0
17	7.2	24.1	53.1	26.5
18	0.0	12.1	4.8	28.9
19	4.8	0.0	33.8	24.1
20	4.8	41.0	48.3	4.8
Group mean ± S.D.	3.60 ±3.62	25.10 ±17.86	31.12 ±19.31	28.71 ±26.15
t	0.64	0.36	1.61	1.60
Significance	n.s.	n.s.	n.s.	n.s.

APPENDIX
TABLE 26.1

Serology of rats immunised with metabolic antigen derived from short-term culture of 16-day-old flukes and of control rats. Immunodiffusion titres (reciprocals of serum dilution). Rats numbers 1-10, immunised; numbers 11-20, controls.

	Weeks after initial immunisation							
Rat no.	0	2	4	6	8*	10	12	14
1	0	0	0	1	2	16	16	8
2	0	0	0	1	2	8	8	16
3	0	0	2	0	2	2	8	4
4	0	0	0	2	0	2	8	2
5	0	0	0	1	0	4	4	-
6	0	0	0	1	1	4	4	2
7	0	0	0	1	0	2	8	4
8	0	0	0	1	1	16	8	8
9	0	0	0	0	0	16	8	4
10	0	0	0	1	2	8	8	2
Group mean ± S.D.	0	0	0.2 ±0.6	0.9 ±0.6	1.0 ±0.9	7.8 ±6.1	8.0 ±3.3	5.6 ±4.6
11	0	0	0	0	0	16	8	4
12	0	0	0	0	0	8	16	8
13	0	0	0	0	0	8	8	8
14	0	0	0	0	0	8	16	8
15	0	0	0	0	0	4	2	2
16	0	0	0	0	0	4	4	2
17	0	0	0	0	0	8	16	8
18	0	0	0	0	0	16	8	4
19	0	0	0	0	0	8	16	4
20	0	0	0	0	0	8	16	8
Group mean ± S.D.	0	0	0	0	0	8.8 ±4.1	11.0 ±5.6	5.6 ±2.6

* Adminstration of challenge infection

APPENDIX
TABLE 26.2

Numbers and sizes of flukes recovered from rats immunised with metabolic antigen derived from short-term culture of 16-day-old flukes and from control rats. Rats numbers 1-10, immunised; numbers 11-20, controls.

Rat no.	No. flukes recovered	Fluke sizes (mm^2)	Rat no.	No. flukes recovered	Fluke sizes (mm^2)
1	8	72.0, 32.0, 40.0 32.0, 3.0, 48.0, 50.0, 15.0.	11	2	36.0, 4.0.
			12	6	4.0, 50.0,45.0, 24.0, 55.0,
2	2	32.0, 35.0.			12.0.
3	2	65.0, 54.0.	13	2	32.0, 24.0.
4	4	40.0, 40.0,40.0, 35.0.	14	2	50.0, 40.0.
			15	1	24.0.
5	2	65.0, 45.0.	16	5	45.0, 54.0,
6	3	45.0, 50.0,6.0.			40.0, 28.0, 18.0.
7	2	72.0, 40.0.	17	3	30.0, 6.0,
8	2	6.0, 8.0.			45.0.
9	4	48.0, 12.0,20.0, 16.0.	18	7	24.0, 48.0, 15.0, 24.0, 18.0, 18.0,
10	6	45.0, 32.0,28.0, 21.0, 8.0, 18.0.			40.0.
			19	5	77.0, 50.0, 40.0, 12.0, 15.0.
			20	1	18.0.
Group mean	3.5	34.8		3.4	31.3
± S.D.	±2.1	±19.1		±2.2	±17.2

TABLE 26.3

Eosinophil counts of rats immunised with metabolic antigen derived from short-term culture of 16-day-old flukes and of control rats. Cells per mm^3. Rats numbers 1-10, immunised; numbers 11-20, controls.

	Weeks post infection			
Rat no.	0	2	4	6
1	288	260	1312	1204
2	110	570	1340	748
3	0	411	1050	1440
4	0	1188	931	2167
5	150	282	1206	-
6	135	156	1860	1320
7	108	1005	1890	543
8	0	630	1690	918
9	90	1788	1280	968
10	118	1050	1120	1344
Group mean ±S.D.	99.9 ±88.0	734.0 ±515.9	1367.9 ±334.9	1183.5 ±473.6
11	0	2015	1870	1737
12	0	1216	1022	1416
13	96	846	1265	1134
14	226	1070	414	684
15	0	798	1474	1200
16	78	870	828	1080
17	0	1071	1932	1998
18	108	615	1584	1568
19	0	1040	2100	840
20	121	332	912	1074
Group mean ± S.D.	62.9 ±76.8	987.3 ±442.5	1340.1 ±547.3	1273.1 ± 406.1
t	1.00	1.18	0.13	0.43
Significance	n.s.	n.s.	n.s.	n.s.

APPENDIX
TABLE 26.4

Plasma glutamate dehydrogenase assays of rats immunised with metabolic antigen derived from short-term culture of 16-day-old flukes and of control rats (micromoles of product per litre of serum per minute). Rats numbers 1-10, immunised; numbers 11-20, controls.

Rat no.	Weeks post infection			
	0	2	4	6
1	0.0	38.6	77.3	-
2	2.4	0.0	9.6	29.0
3	2.4	9.6	96.6	38.6
4	9.6	38.6	86.9	9.6
5	4.8	19.3	43.5	58.0
6	0.0	4.8	48.3	67.6
7	4.8	4.8	4.8	4.8
8	4.8	29.0	53.1	9.6
9	2.4	135.2	67.6	19.3
10	2.4	14.5	33.8	91.8
Group mean ± S.D.	3.36 ±2.82	29.44 ±39.65	52.15 ±30.79	36.47 ±30.20
11	9.6	4.8	33.8	53.1
12	2.4	33.8	48.3	14.5
13	2.4	33.8	58.0	43.5
14	0.0	14.5	19.3	96.6
15	0.0	9.6	14.5	24.1
16	0.0	24.1	115.9	19.3
17	0.0	24.1	62.8	91.8
18	0.0	19.3	19.3	62.8
19	0.0	4.8	4.8	72.5
20	2.4	29.0	58.0	4.8
Group mean ± S.D.	1.68 ±3.00	19.78 ±11.03	43.47 ±32.76	48.3 ±32.54
t	1.29	0.74	0.61	0.84
Significance	n.s.	n.s.	n.s.	n.s.

STATISTICAL CORRIGENDA

1. The data on pages 61, 62, 66, 70, 74 and 79 should not have been subjected to correlation analysis.

2. The data on page 83 should have been analysed using non-parametric statistics, as it is clear that the variance is not independent of the mean.

3. The data on pages 227, 240, 248, 254 and 263 should have been analysed using non-parametric methods, as the distribution cannot be normal.

4. Geometric means should have been used for the titres shown in the graphs on pages 159, 162, 163, 164, 166, 212, 226, 239, 253 and 262 and Appendix Tables on pages 308, 310, 311, 312, 313, 314, 315, 316, 317, 324, 328, 333 and 337.

5. The data on pages 215, 227, 240, 254 and 263 should have been subjected to a nested analysis of variance and incorrect variances have been used for the F tests. These changes do not alter the conclusions, although the significance of the relationship between fluke sizes and immunisation shown in Table 21.1 (page 215) is greatly reduced:-

Preliminary Anova

	d.f.	s.s.	M.S.	F
Between groups	1	6955.052	6965.05	5.98*
Between rabbits within groups	9	10642.433	1182.492	7.97
Error	320	47448.213	148.276	
Total	330	65055.698		

*Group sizes unequal - corrected F = 5.47

corrected d.f. = 8.8 $\approx$ 8

$F_{.05(1,8)} = 5.32$ $F_{.001(9,120)} = 3.38$