THE PRODUCTION AND EVALUATION OF IgA MONOCLONAL ANTIBODIES DIRECTED AGAINST *EIMERIA TENELLA* SPOROZOITES.

Thesis submitted in accordance with the requirements
of the University of Liverpool
for the degree of Doctor in Philosophy by

Jennifer Ann Hoare, October, 1989.

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Jennifer A. Hoare

ABSTRACT

The production of IgA monoclonal antibodies directed against Eimeria tenella sporozoites from fusions involving different immunisation routes, different lymphocyte sources and conventional and germ-free mice was compared. Intra-enteric immunisation of germ-free mice with live sporozoites generated eight specific IgA monoclonal antibodies, seven derived from spleen and one from mesenteric lymph node (MLN), while only one IgA monoclonal antibody was generated from five conventional fusions. MLN offered no advantage over spleen as lymphocyte source in the production of specific IgA secreting hybridomas in this study. In addition to the specific IgA monoclonal antibodies, a further 37 IgG and 17 IgM specific monoclonal antibodies were generated from these fusions.

Specific antibodies were identified utilising a three layer immunofluorescent antibody assay using air-dried, acetone fixed sporozoites as antigen. Agglutination and surface fluorescence tests with live sporozoites in suspension indicated those antibodies directed to surface antigens. Surface active monoclonal antibodies of IgG and IgA isotypes did not cross-react with sporozoites of E.acervulina. Two IgA and one IgG surface active monoclonal antibodies induced direct perturbation of the sporozoite membrane in the absence of complement, revealed by transmission electron microscopy, and one of the IgA monoclonal antibodies tested, consistently and significantly reduced infectivity of sporozoites assessed in vivo. This is the first description of the production of specific anti-eimeria IgA antibodies and the first description of antibody induced, direct neutralisation of sporozoite infectivity in the absence of complement.

Identification of the surface antigens involved, by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Western blotting was unsuccessful, but an internal antigen of 76kD recognised by one of the IgA monoclonal antibodies was identified using these techniques.

Attempts were made to obtain secretion of the mouse IgA monoclonal antibodies into chick bile in order to assess passive protective effects of specific secretory IgA antibodies. Mouse monoclonal antibodies were injected intravenously into recipient chicks; an IgG monoclonal antibody was lost rapidly from the serum, but did not appear in the bile. The IgA monoclonal antibody activities in serum declined more slowly, but an IgA monoclonal antibody, shown to be polymeric in Western blotting and radiolabelling studies, was detected in the chick bile. As this antibody was directed to internal antigens and had no apparent effect on live sporozoites, it was not considered for passive protection studies. A surface active, sporozoite neutralising IgA monoclonal antibody also appeared in bile, but in monomeric form. Failure to achieve the secretory form of the IgA monoclonal antibody in the recipient chick bile and intestinal lumen precluded evaluation of this potentially protective antibody against oral oocyst challenge in chicks.

ACKNOWLEDGEMENTS

I acknowledge with thanks the technical advice of Ms.J.Gale, who introduced me to the field of monoclonal antibody production, and Ms.S.Crozier, Ms.S.McKellar and Ms.B.Johnson who taught me the parasitological techniques. I am grateful to Dr.H.Townson for advice on experimental design and statistical analyses; to the staff of the LSTM animal unit for expert care of experimental animals and assistance with surgical procedures; to Mr.P.Young and the staff of the electron-microscopy unit for their expert assistance in the preparation and staining of samples for E.M. studies, and to Ms.P.Stafford and the photography department for preparing the photographic prints.

I acknowledge the help and advice of my supervisor Dr.A.J.Trees, and I am grateful for his patience and constant encouragement, particularly during times of difficulty.

I am grateful to the Agricultural and Food Research Council for its financial support and the research opportunity afforded me by the award of a veterinary schools training fellowship.

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INTRODUCTION

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The Problem.

Coccidiosis is a disease caused by protozoa of the subclass Coccidia, affecting many mammal and bird species. Most of the coccidia are parasites of the epithelia of the intestines and those of importance in the domesticated species belong to the genus Eimeria. Infection in poultry causes the broiler poultry industry widespread economic losses due to death, decreased weight gains, and subclinical effects reducing feed conversion efficiency. In Great Britain, control of this disease is effected largely by chemo-prophylaxis with a variety of anti-coccidial drugs, costing the British industry an estimated £5m/year (Trees, 1987), and on a world-wide scale, $ U.S. 250 million/year (Long and Jeffers, 1986).

The major species involved in clinical and sub-clinical disease are Eimeria acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox and E. tenella. These are rarely encountered as pure infections, but generally, two or more species are involved. The different species vary in their reproductive capacity, degree of mucosal damage produced, pathogenic effects, sites of infection along the gut and immunogenicity.

Following natural or experimental infection in normal immunocompetent birds, the life cycle of the parasite is completed in 6-9 days following infection with the sporulated oocyst stage, and immunity to reinfection may be stimulated to varying degrees depending on the species of Eimeria involved and the dose of oocysts received both in the primary, immunising dose and secondary

challenge inoculum. For example, E. tenella is only poorly immunogenic while a single oocyst of E. maxima produces marked immunity to subsequent challenge (Tyzzer, 1929).

Immunity to eimerian infections has been studied in many host species and has been demonstrated in bursectomised birds and thymectomised mammals indicating possible roles for both humoral and cell-mediated (CMI) protection mechanisms, and these will be discussed in further detail elsewhere. Of particular relevance to this study has been a proposed role for the mucosal defence system in resistance to reinfection based on studies in vitro (Davis et al, 1978; Davis and Porter, 1979a; 1979b; Davis et al, 1979; Wiesner, 1979a; 1979b; Davis, 1981; Davis and Porter, 1983). From in vivo studies there is further recent evidence of a reduction in the numbers of sporozoites penetrating and/or developing in immune hosts, correlating with the presence of specific secretory antibodies in the intestinal lumen (Augustine and Danforth, 1986a; Rose and Hesketh, 1987), but since a proportion of sporozoites are able to develop normally in the presence of such antibodies, it has been suggested that mucosal immunity is of only minor importance in natural immunity (Rose, 1987; Rose and Hesketh, 1987). However, a mechanism of specific inactivation of sporozoites at the mucosa has been demonstrated in the studies cited, and attempts to increase this phenomenon by artificial immunisation appear valid. Induction of specific antibody in conjunction with non-specific mucosal protection mechanisms, could provide an effective barrier to sporozoite penetration and the initiation of infection, disease and pathogenic effects. Although the chicken shows a poor response to

certain non-living antigens delivered by the oral route (Parry et al, 1977; Davis et al, 1979) some protection was demonstrated using a non-living sporozoite antigen mixture from E.tenella and E.acervulina delivered orally (Murray et al, 1986), although the mucosal response to the vaccine was not investigated. Oral or aerosol vaccination would be of most practical use to the poultry industry and warrants further detailed investigation.

The Approach.

The principle of monoclonal antibody production has now reached many research fields and has proved a valuable tool in the dissection of complex antigenic mixtures (such as parasites) into their constituent molecules from which potentially protective antigens may be identified, isolated and purified. This has been the approach used in studies of several parasitic protozoal diseases (e.g. Boyle et al, 1982; Knopf et al, 1982; Jakobsen et al, 1987; Winger et al, 1987; Dusanic, 1988; Krettli et al, 1988) and in the case of plasmodial infections of man, molecular vaccines have been developed and preliminary immunisation studies initiated (reviewed by Cattani, 1989).

In order to investigate mucosal immunisation of chicks, it was desirable that potentially protective antibodies should be evaluated in the intestines of chicks where they could act in concert with non-specific protection mechanisms (e.g. mucins, lysosyme, proteases). It was for this reason that monoclonal antibodies of the IgA class were sought for passive protection studies, the secretory form of this isotype having an intrinsically high protease

resistance and survival in the gut lumen. Protective antibodies and the antigens recognised by them could then be identified, purified, characterised, and with an appropriate immunisation schedule such antigens could be used as subunit vaccines to stimulate mucosal immunity.

It is noteworthy that monoclonal antibodies have been successfully raised against parasite stages of avian eimeria by groups of workers funded by drug companies, indicating a high level of commercial interest in this approach. The effects of these antibodies on the parasites have been investigated in vitro (agglutination, surface fluorescence, ferritin/gold labelling and electron-microscopy) (Danforth, 1983a; 1983c; Speer et al, 1983a; 1983b; Danforth et al, 1985; Speer et al, 1985a; 1985b; Crane et al, 1986a; 1986c; Danforth, 1986a; 1987; Augustine and Danforth, 1987b; Crane et al, 1988; Speer et al, 1989), in an in vitro infectivity test employing cell monolayers (Danforth, 1982b; 1983a; 1983b; 1986b; Augustine and Danforth, 1987a; 1987b; Danforth and Augustine, 1989), and in vivo, following in vitro incubation of invasive stages in the antibodies of interest (Crane et al, 1986a; 1986b; 1988). There are no reports that any of the antibodies generated are of IgA isotype, and no attempts have been made to passively transfer protective monoclonal antibodies to the chick mucosa and evaluate them there against oocyst challenge. This approach has been successfully applied in identification and assessment of potentially protective IgA monoclonal antibodies at the nasal mucosa in mice challenged with Sendai virus (Mazanec et al, 1987), and at the intestinal mucosa against Trichuris muris infection in mice (Roach, 1988).

Published reports describe several useful monoclonal antibodies directed against Eimeria tenella antigens, some conferring a degree of passive protection against intra-caecal sporozoite challenge, on the basis of lesion scores and oocyst output (Schenkel et al, 1987a; 1987b; Crane et al, 1988). Partially protective antigens have been isolated and used as sub-unit vaccines in preliminary trials, with some success being reported following parenteral immunisation (Danforth, 1986b). A report of successful oral (and parenteral) immunisation of chicks with crude sporozoite antigen has been published (Murray et al, 1986), and this route is apparently receiving considerable attention from commercial organisations.

The aims of the project were to produce IgA monoclonal antibodies directed against E. tenella sporozoites and then to evaluate them for in vitro and in vivo effects on live sporozoites. From here, the work could be developed by using these IgA antibodies to passively protect chicks against oocyst challenge and to identify antigens recognised by protective antibodies. These antigens could be utilised in the active immunisation of the chick mucosa.

Infection is initiated when the mucosa is penetrated by sporozoites which have excysted in the gut lumen. The crucial step in infection is a membrane interaction between epithelial cell and sporozoite, probably involving specific receptor sites on the surface of the parasite and host cells (Augustine and Danforth, 1982; 1984a; Augustine, 1985a; 1985b; 1986; Augustine and Danforth, 1987a). Hence attention was directed towards the surface antigens of live sporozoites where it would be anticipated that these antigens

would retain their native structure and epitopes. By examination of effects of monoclonal antibodies on sporozoites in vitro, attention could be focussed on those potentially useful antibodies directed against surface antigens. In vivo infectivity of sporozoites after treatment with monoclonal antibodies in vitro would verify whether observed in vitro effects equated with alteration in infectivity of sporozoites.

Passive protection studies and evaluation of parasite infectivity in vivo, taking advantage of the non-specific barrier effects of the gut mucosa require the antibodies to be active in the gut milieu. It is the secretory component that confers protease resistance on IgA antibodies and this would be required by potentially protective monoclonal IgA antibodies to allow their survival in the intestinal lumen. Secretory component is attached to polymeric IgA as the antibody is delivered to the intestinal lumen either across hepatocytes or across the mucosal epithelium. Homologous and heterologous binding of secretory component and polymeric IgA has been described for several species (Mach, 1970), and specific secretion of donor polymeric IgA from blood to bile with incorporation of the recipient secretory component in the secreted immunoglobulin has been described (Hall et al, 1980; 1981; Orlans et al, 1978; 1983; Peppard et al, 1983; 1984; 1986; Rose et al, 1981). The possibility of transferring monoclonal mouse IgA to chicks in this way to achieve a hybrid secretory antibody was investigated with a view to carrying out protection studies in vivo. Antigen analysis and immunisation studies would be indicated if monoclonal antibodies protective in the gut milieu could be obtained.

CHAPTER 1

THE IMMUNOLOGY OF AVIAN COCCIDIOSIS - A REVIEW

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Host immune responsiveness and protective immunity to avian coccidiosis have been recognised since the earliest descriptions of the pathogenic species of avian eimeria were published (see Tyzzer, 1929; Tyzzer et al, 1932), but it was also recognised by these workers that not all the responses observed are protective or necessary in the development of protective immunity. Gradually the various processes which may be involved in protective mechanisms have been elucidated and models of protection have been proposed (reviewed by Rose, 1986; 1987). From a sound knowledge of parasite behaviour and susceptibility to immune mechanisms, and from studies on the avian immune system, different immunisation strategies have been considered and developed, aimed at stimulating known protective mechanisms, some to a degree of commercial success.

Avian coccidiosis has been of significant economic importance since intensification of poultry rearing generated conditions conducive to the survival and rapid dissemination of the parasites amongst young, susceptible hosts. The species specificity of immunity was identified in some of the earliest studies (Tyzzer, 1929; Tyzzer et al, 1932), although more recently, some immunological variation within species has been recognised and incomplete cross-immunity between different strains has been described (Joyner, 1969-E. acervulina; Norton and Hein, 1976-E. maxima; Karim, 1988-E.tenella). The different species vary in

immunogenicity but in all cases the degree of immunity induced is dependent on the size of the immunising dose. The most immunogenic species (e.g. E.maxima) induce a more rapid and enduring protective response, but again this can be modified in all species depending on the immunisation schedule (reviewed by Rose, 1987).

Host responses to the parasite have been well documented, initially work concentrating on the circulating humoral response and latterly, the secretory immune system and cell-mediated immunity. It is not wise to extrapolate data derived from different host and parasite species to others, but certain generalisations based on studies of avian and mammalian eimeria are widely accepted. A humoral response to the asexual stages is seen in all species, the magnitude and onset dependent on the infective dose. Response to primary infections in chickens is primarily IgM isotype, with a later IgG (and IgA) response ; secondary responses are not seen in the fully immune bird, but in the partially immune the anamnestic response is seen, with IgG isotypes predominant (Rose and Mockett, 1983; Trees et al, 1985; Mockett and Rose, 1986; Rose, 1987). An increased number of IgA-containing plasma cells in the caecal lamina propria (Davis et al, 1978), increased total IgA in bile (Clare et al, 1987), and specific secretory IgA in bile, intestinal contents and mucosal scrapings from birds infected with Eimeria tenella have been described (Davis et al, 1978; Wiesner, 1979a; 1979b; Mockett and Rose, 1986; Trees et al, 1989), similar to results obtained with mice infected with Eimeria falciformis (Douglass and Speer, 1985; Nash and Speer, 1988). Following pulse infections with Eimeria tenella in chicks, specific bile IgA was detected at five days post-

infection (dpi), peaking at 9 dpi, and falling by 18 dpi (Trees et al, 1989). Similar results were recorded by Mockett and Rose (1986) for both primary and secondary inoculations. A secretory response also occurs in response to trickle infection in caged birds (Davis et al, 1986), and birds kept on litter in simulated field conditions (Mkonyi and Trees, pers. comm.), and is sustained until chicks reach 7-8 weeks of age.

Cellular responses comprise induction of specific T-cell and B-cell development, and circulating and local leukocyte responses associated with generalised inflammation and repair at the gut mucosa (reviewed by Rose, 1978; 1982; 1987). Specific mechanisms inducing hypersensitivity reactions at the mucosa such as described by McKenzie and co-workers (1986), with exudation of macromolecules into the gut lumen and infiltration of inflammatory cells have been ascribed to the action of locally released histamine (Rose, 1978; 1987).

The various host responses decribed have differing effects in modulating parasite behaviour and in contributing to protective immunity. Tyzzer (1929) recognised that serum antibody levels do not equate with levels of protection. More recent studies in surgically or chemically bursectomised birds indicated a role for antibody in limiting primary infections. However, the bursectomised birds displayed a protective memory to subsequent challenge, presumably due to adequate T-cell activity (e.g. Joyner and Norton, 1974), and such specific T lymphocyte clones specific for Eimeria tenella have been characterised (Bhogal et al, 1986). In studies of mammals following thymectomy, T-cell function was shown to be required in

abrogation of primary infection, and in memory to challenge reinfection, substantiating the work on bursectomised birds (e.g. Mesfin and Bellamy, 1979-mice; Rose and Hesketh, 1979-rats; Rose et al, 1988-mice). Although indicating a crucial role for T-cell activity in protective immunity, an important role for protective antibodies cannot be entirely excluded on the basis of these results: bursectomy does lead to the induction of T-cells not normally stimulated during immune responses (Bhogal et al, 1986), and clearly antibody production is adversely affected by thymectomy (Moticka, 1977; Mesfin and Bellamy, 1979; Rose and Hesketh, 1979). Notably, in studies of mucosal immunity to Giardia muris, specific IgA responses were abrogated in mice depleted of T-helper cells (Heyworth, 1989).

It has been demonstrated that immunity is entirely species specific and for this reason Rose (1987) excludes non-specific effectors as mediators of important protective effects. Although this would appear logical, the anti-parasitic effects of interferon (Fayer and Baron, 1971; Long and Milne, 1971; Kogut and Lange, 1989), soluble 'T-cell factors' (Klesius et al, 1978, Klesius and Giambrone, 1984), and histamine-induced local hypersensitivity (Rose, 1978), may be common end-points of specifically stimulated pathways.

As mentioned earlier, the presence of specific serum antibodies is not sufficient to confer immunity; passive transfer of serum antibodies by oral or subcutaneous routes was inconsistent in conferring immunity to oral challenge with Eimeria maxima oocysts (Rose, 1974). Induction of serum antibody responses by immunisation

with non-viable parasite material has been described by several workers since Tyzzer (1929), but such antibodies are not protective unless parasites are introduced intravenously (Long and Rose, 1965; and see Rose and Long, 1980). Also, solidly immune birds may demonstrate no specific circulating antibody, and show no secondary serum response in resisting a further challenge (see Rose, 1987). Unless the serum antibodies encounter the parasite, for example, when sporozoites escape extraintestinally (Kogut and Long, 1984; Fernando et al; 1987), or are inoculated intravenously (Long and Rose, 1965), or the gut mucosa becomes permeable to antibody leakage (McKenzie et al, 1986; Trees et al, 1989), they can exert no anti-sporozoite effect. Local antibodies are more significant since they can encounter the invasive stages prior to establishment of the infection, and a role for the secretory immune response in protective immunity to Eimeria tenella has been proposed (Davis et al, 1978; Davis and Porter, 1979a; 1979b; Davis, 1981), which will be further discussed later.

An examination of the results relating to the modulated activity of parasites in the immune and partially immune bird provides more revealing insights into possible protective mechanisms occurring in natural immunity and indicates the research areas which should be focussed on in immunisation studies.

Partially protective immune responses generated by the sporozoite stage have been demonstrated in some studies (Jeffers and Long, 1985; Bhanushali and Long, 1986), although Rose (1987) summarises such results stating that both invasive and sexual stages do not stimulate full immunity and do not play an important role in normal,

fully protective immune mechanisms. Recent evidence indicates that there is immunological diversity between the different generation merozoites (McDonald et al, 1988) and that they may generate a stage specific immunity. Also, monoclonal antibodies directed against microgametocytes have shown interference with the development of parasites in vitro (Laxer et al, 1987) but these stage specific effects are not characteristic of protective immunity. However, the immunity generated by the asexual second stage schizont is protective, and clearly acts on other (invasive) stages of the life cycle (reviewed by Rose, 1987). Even if sporozoites do not elicit a a fully protective response, this stage is subject to protective mechanisms elicited by the second generation schizont (see below) indicating some shared protective antigens between these stages. Studies with polyclonal and monoclonal antibodies have revealed both specific and common antigens between the different stages (Danforth, 1983a; Danforth and Augustine, 1983a; Speer et al, 1983a; Thammana and Schenkel, 1984; Danforth et al, 1985; Banushali and Long, 1986; McAndrew et al, 1986; Wong and Thammana, 1986; Danforth, 1987; Danforth and McAndrew, 1987; Laxer et al, 1987; Schenkel et al, 1987a; 1987b; Danforth and Augustine, 1989; Speer et al, 1989) and between the different species (Danforth and Augustine, 1981, Danforth, 1982a; Danforth and Augustine, 1982; 1983b; Augustine and Danforth, 1984b; 1984c; Thammana and Schenkel, 1984; Danforth et al, 1985; McAndrew et al, 1986; Danforth, 1987; Augustine et al, 1988; Speer et al, 1989). Clearly, protective antigens must be shared between the second generation schizont and sporozoite for any eimerian species, but since protective immunity is species specific

these antigens are presumably also species specific, or demonstrate differing expression in the different species (see Murray et al, 1986).

In early studies of protective immunity it was stated that activity is directed against the invasive sporozoite stage (Tyzzer et al, 1932). Subsequent work has endorsed this statement, although there are some differences in the mechanisms described, which may be a reflection of parasite species variation per se, differing protective mechanisms occurring in different regions of the chick intestine, or different immunisation procedures and degrees of immunity elicited. A total failure of sporozoites to invade the mucosa was reported for E. nieschulzi and E. tenella (Morehouse, 1938; see Leathem and Burns, 1967); a reduction in numbers invading (Tyzzer et al, 1932- E.necatrix; Augustin and Ridges, 1963- E.meleagrimitis; Leathem and Burns, 1967-E. tenella; Augustine and Danforth, 1986a-E. tenella and E. adenoides); no reduction in sporozoite invasion (Mesfin and Bellamy, 1979-E. falciformis; Augustine and Danforth, 1984c-E. meleagrimitis, E.tenella, E.adenoides, E.acervulina); and increased sporozoite invasion in the immune host (Augustine and Danforth, 1986a-E. acervulina and E. meleagrimitis). There is however, almost uniform acceptance of at least some degree of impediment to sporozoite development in the immune host, not observed in the non-immune host (Tyzzer et al, 1932; Augustin and Ridges, 1963; Horton-Smith et al, 1963-E. tenella; Leathem and Burns, 1967; Mesfin and Bellamy, 1979). Interestingly, this developmental arrest is initially reversible since arrested sporozoites will re-establish infection when

transferred to susceptible hosts (reviewed by Rose, 1978). Failure to develop in the non-specific host is somewhat similar, since arrested sporozoites will re-establish infection upto 48 hours after infection if transferred to a susceptible host of the appropriate species (quoted in Long and Speer, 1977).

Antibody mediated inhibition of parasite development has been demonstrated using a variety of antibody sources. Caecal contents from immune chicks reduced invasion and development of E. tenella sporozoites in cell mono-layers (Davis et al, 1978; 1979; Davis and Porter, 1979a; 1979b), and immune serum similarly blocked cell penetration (Long and Rose, 1972), acting both on the sporozoites and on the cell monolayers, as later described with monoclonal antibodies directed to cell-monolayers (Augustine, 1985b, 1986). Monoclonal antibodies raised to sporozoites have been described both inhibiting cell penetration (sometimes inducing agglutination) (Danforth, 1983a; Augustine and Danforth, 1985; 1986b; Crane et al, 1986a; 1986c; Danforth, 1986a; Augustine and Danforth, 1987a), and also apparently enhancing cell penetration, the agglutinated sporozoites invading en masse (Crane et al, 1986c). Monoclonal antibodies preventing in vitro development were apparently more effective when maintained in the culture system, but not invariably so (Danforth, 1982b; 1982c; 1983a).

Various theories as to the mechanism of arrest have been proposed, and it is likely that more than one may be involved in protective immunity, or that different mechanisms may be involved in differing circumstances. Davis and co-workers (1978) proposed that secretory antibody immobilises and delays penetration of E. tenella

sporozoites, increasing their exposure to non-specific proteases resulting in stripping of surface differentiation antigens. Thus, there is reduced invasion and subsequently reduced development of invading parasites. Although not disregarding this hypothesis, Rose (1987) indicates that such a mechanism of developmental inhibition must be reversible at least initially (as mentioned earlier), and also states that achieving the correct final host cell may be the crucial step which is blocked. This block to migratory behaviour has been suggested by other workers (Long and Speer, 1977), and failure to reach appropriate host cells may be involved in blocking development in the non-specific host. How these models relate to results achieved *in vitro* is unclear.

To summarise: protective effects at the mucosa directed against sporozoites comprise antibody, and non-specific effectors acting in the lumen and at the intestinal mucosa. These antibodies are largely IgA isotype, and are secreted into the gut lumen *via* the bile and across the mucosa, (although exudation of serum antibodies could occur in some circumstances) reducing absolute numbers of sporozoites in the lumen (Rose and Hesketh, 1987) and at least causing a partial reduction in the numbers of sporozoites successfully invading the mucosa, and a further reduction in sporozoites continuing their development. Although the sporozoite arrest is initially reversible, eventually degradation of both parasite and cell occurs, or the parasitised epithelium is sloughed in the normal way, removing the parasite. Failure to develop may reflect damage to differentiation antigens, failure to reach the appropriate final host cell type, or interference by soluble factors

within the parasitised cell, or from the extracellular spaces.

In hosts with incomplete immunity, more sporozoites successfully develop, and the parasite cycle progresses further as immunity wanes and more parasites eventually complete their infective cycle (reviewed by Rose, 1987). Protective immune mechanisms acting at these later stages are most likely to be cell-mediated, since antibody presumably has no access to the parasites once established in intra-cellular sites.

Vaccination procedures are required which will stimulate and even specifically enhance those responses which are protective. For practical reasons, immunisation is required in the first days of a chick's life, and should give lasting immunity for the 6-8 week life span of a broiler bird. The vaccine should generate immunity to all the pathogenic species, but without inducing disease or decreased performance, and should be in a form which is easily and practically delivered and stored (see Rose and Long, 1980).

As described earlier, immunity is more or less easily acquired depending on the species of eimeria involved, following experimental and field infections. The same number of oocysts administered orally as a trickle infection produces a higher degree of immunity than a similar dose given as a single pulse, and with reduced pathological effects (Joyner and Norton, 1973). Trickle immunisation is of practical consideration, the immunising dose administered either in feed or in water. Obviously, all species must be included to cover the species variations observed, and a variety of strains of some species may be necessary. Utilising trickle infection results in contamination of the litter with passaged organisms, and auto-

reinfection which helps to boost immunity. However, accumulation of oocysts in the litter could cause the occasional out-break of clinical disease in susceptible birds, and thorough cleaning of the environment between crops of broilers would be indicated. Although theoretically straightforward, the administration of small numbers of oocysts *via* water or food is not practically simple and birds may fail to acquire the correct dose for a variety of reasons. However, this scheme has been used with some success in the USA. The vaccine Coccivac (Sterwin Laboratories Inc. USA) is administered in the drinking water to chicks from 4 to 14 days old, and a vaccine developed by Unilever, (Bedfordshire, England) relies on the delivery of small numbers of oocysts incorporated into alginate beads and administered to chicks *via* the feed from day old to three weeks (Davis *et al*, 1986). However, the seeding of virulent organisms of all pathogenic species into poultry houses is open to justifiable criticism (Rose, 1987).

Attenuated species have been successful in experimental immunisation studies protecting against disease caused by the parent virulent strain. Most success has come from strains selected for precocious development, completing their life cycle with smaller schizonts, reduced asexual multiplicative stages, and reduced pathogenicity. Attenuated 'precocious' strains for the seven major pathogenic species have been developed, which retain their immunogenicity (Jeffers, 1975; McDonald *et al*, 1982; McDonald and Ballingall, 1983; Shirley and Bellatti, 1984; Shirley *et al*, 1984; Johnson and Long, 1985; reviewed by Jeffers, 1986; and Shirley and Bellatti, 1988). Use of such strains in trickle immunisation, with

auto-reinfection offers considerable advantages over the use of virulent oocysts and experimental trials using the alginate bead delivery have been successful (Johnson et al, 1986). The possibility of reversion to normal pathogenicity has not been observed following multiple passage (unlike reversion noted with attenuated strains developed from embryos), and these lines are apparently stable.

Non-living antigens have been of limited success in immunisation procedures. In most studies utilising crude parasite antigens, a serum response has been generated but with little evidence of protective immunity. The recent exception is of a sporozoite derived 'vaccine' which induced a significant protective immunity to oocyst challenge as assessed by lesion scores and the induced immunity was effective against heterologous challenge with two further species of Eimeria (Murray et al, 1986). The latter finding is most interesting and potentially advantageous in considerations on vaccine production, since it implies the presence of shared protective antigens between species, which might not be expressed by the parasite or recognised by the host in active infections, but are revealed using non-living antigens. This area is clearly of future interest.

Protective monoclonal antibodies have been developed (Crane et al, 1986a; 1986c; Schenkel et al, 1987a; 1987b) and limited protection with a single antigen vaccine (Danforth, 1986b; Danforth and Augustine, 1986), and a mixed vaccine (Danforth et al, 1988) given parenterally has been described. A short report of partial protection following in ovo vaccination with non-living antigens (Ruff et al, 1988) is an entirely new approach and further details

of this study are awaited with interest. The existence of United States patents (see Schenkel et al, 1987a; 1987b) for monoclonal antibodies and the antigens recognised by them is indicative of the considerable commercial benefits that are at stake in the development of a molecular vaccine. Such a vaccine is confidently expected (Rose, 1987) and will establish a major niche in prophylactic control of coccidiosis. It is clear that identifying the appropriate antigens will only be a start: presentation of the antigen, and aspects of mucosal immunisation, particularly of the very young chick require further detailed investigations. The potential to halt sporozoite penetration and development with specific intestinal antibodies has been indicated from several studies, and by appropriate immunisation this could be enhanced to prevent parasite development entirely.

CHAPTER 2

GENERAL MATERIALS AND METHODS

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Experimental birds:

day-old chicks of either sex were reared in a brooder in a room kept specifically coccidia-free. Chicks were caged at 7-10 days old, and from there were moved into a separate room where all procedures with infective parasites were performed. Periodic examination for oocysts in faeces from chicks in the coccidia-free room was performed, and the room was cleaned and gassed with ammonia between crops of birds, as added protection against accidental infections. No contamination of the room was noted throughout the course of the study. Birds were maintained on standard layer mash and water ad libitum. Hisex (layer-type) birds (Midmoor Hatchery, Frodsham) were used for all procedures except for the infectivity assay which was performed with Cobb-1 (broiler-type) birds, kindly donated by Sun Valley Hatchery, Leominster.

Maintenance of parasites:

the Houghton strain of Eimeria tenella, a kind gift from Dr.M.E.Rose, was used throughout. Passage of the parasite in 7-day old chicks was performed in an isolator 3-4 times a year. General production of oocysts was performed under normal housing conditions using birds aged 7-21 days. A dose of 10^3 - 10^4 sporulated oocysts in a volume up to 1ml was given to each bird by crop intubation. Birds were placed in wire cages with plastic trays beneath flooded with 2% w/v potassium dichromate solution.

Faecal material was collected daily from days 6-9 post-infection inclusive, and stored at 4°C until use, the dichromate serving to limit bacterial growth without damaging the oocysts.

Purification of oocysts from faeces:

the pooled faecal slurry was homogenised in a food blender and pelleted in 1 litre polypropylene containers by centrifugation. The supernatant was discarded and the pellet resuspended in saturated salt (NaCl) solution. Centrifugation at low speed gave separation of an oocyst rich scum from the more dense food material. Resuspension of the pellet in saturated salt for a further two extractions was sometimes performed. The oocyst rich supernatant was diluted at least 5-fold with tap water and the oocysts concentrated by centrifugation. The cleaned oocysts were then resuspended in 2% dichromate and prepared for sporulation.

Sporulation:

cleaned oocysts in 2% dichromate were aerated via a diaphragm pump for 2-4 days at room temperature. The oocyst concentration was adjusted to $<2 \times 10^6$. After 2 days , sporulation was assessed by microscopic examination. At least 100 oocysts were examined for the presence of four distinct sporocysts, each sporocyst containing two fully developed sporozoites. When sporulation exceeded 90%, the oocyst suspension was placed in a sealed container and stored at 4°C.

Preparation of sporozoites:

sporulated oocysts were washed free of dichromate solution with tap water, and were then resuspended in 'Miltons', a commercial sterilising solution of sodium hypochlorite (2% w/v) and sodium chloride (16% w/v). The suspension was left at room temperature for 30 minutes, this reducing the bacterial contamination and rendering the oocyst wall more fragile (see Wagenbach et al, 1966). A low speed centrifugation yielded an oocyst rich white scum which was removed and diluted at least ten-fold in tap water. The oocysts were recovered by high speed centrifugation, washed extensively to remove all traces of hypochlorite, and finally resuspended in PBS. Oocysts were either stored overnight at 4°C or the procedure continued in the same day.

Oocysts were suspended in PBS (20 million oocysts in 2-3ml PBS) in a glass 'universal' bottle, and a similar volume of glass beads, 400-500um diameter (Sigma Chemical Co. Ltd., Dorset), was added. Oocysts were disrupted by vortexing the mixture for 45-75 seconds. The degree of disruption was assessed by microscopic examination of small samples taken at 10 second intervals after a minimum 45 seconds. Vortexing was continued until at least 75% disruption was achieved as determined from the formula:

$$\frac{\text{no. sporocysts}}{\text{no. sporocysts} + (\text{no. oocysts} \times 4)} \times 100$$

The beads settled out rapidly and the cloudy supernatant was removed and the sporocysts and parasite debris pelletted by centrifugation. To this pellet was added 5-10 ml excystation fluid, comprising 0.25% trypsin and 2.5% sodium taurocholate in PBS, final pH 7.3-7.5. The

suspension was incubated at 41°C for 90 minutes, and then the reaction stopped by the addition of cold PBS containing the protease inhibitor aprotinin (Sigma Chemical Co. Ltd., Dorset)(100 kallikrein iu/ml). The parasite mixture was pelletted and washed twice with PBS and sporozoites were purified from the mixture by passage down a column of 200um glass beads (Potters Ballotini Ltd., Barnsley), in a 20ml syringe barrel (after Wagenbach, 1969). Sporozoite rich fractions were pooled and counted using a Fuchs-Rosenthal counting chamber. Viability was assessed by trypan blue exclusion. Sporozoites were suspended in PBS and left at room temperature for 1 hour prior to use, and were then used fresh, or frozen at -20°C without cryoprotectant, for subsequent use in antigen analysis by SDS-PAGE.

Preparation of antigen slides-
immunofluorescent antibody tests:

sporozoites of Eimeria tenella were suspended in PBS and a 5ul drop placed in the well of a teflon coated 15- or 30- well antigen slide (C.A.Hendley, Essex). The concentration of sporozoites was adjusted to give adequate numbers of parasites per well, as judged by microscopic examination, and then several hundred slides were loaded with 5ul of the suspension in each well. The slides were allowed to air-dry, were placed in labelled slide boxes and stored at -20°C. Similarly prepared antigen slides for E. acervulina were kindly donated by Dr.M.J.Karim.

On the day of use the appropriate numbers of slides were removed from the freezer, allowed to reach room temperature in a dessicator and then fixed in acetone for 5 minutes.

Assays for specific anti-Eimeria mouse antibodies- immunofluorescent antibody test (IFAT) and three-layer immunofluorescent antibody test (TLIFA):

the procedures for the IFA and TLIFA for specific anti-Eimeria mouse antibodies were based on those described for specific chick antibodies directed against E.tenella sporozoites (Trees et al, 1985).

All antisera were purchased from Nordic Immunochemicals, (Maidenhead, Berkshire) and stored in 5, 10 and 50ul aliquots at -20°C until use. Dilutions of the antisera, made with PBS, were based on those used in the chick system, and the assays detected sporozoite specific IgG and IgM in immunised mouse serum, without false positives in unimmunised controls. Immune mouse serum was used as positive control material in assays used for screening mice after different immunisation routes and in screening supernatants from early fusions. No sporozoite specific mouse IgA was available and the TLIFA for specific mouse IgA was initially uncontrolled, being based empirically on that used for detection of specific IgG and IgM. An IgA secreting hybridoma was identified using the system and the secreted antibody from this and two other hybridoma lines were used as positive controls in subsequent class-specific assays. These were B5G2 (IgG); G12B7 (IgM); and F11B4E12 (IgA). For tests on tissue culture supernatants, neat supernatant was used, with complete medium as negative control. For tests on ascites, control ascites at 1/100 dilution in PBS was used from a pool made from mice bearing one of the control hybridomas or NS-1 cell myeloma.

Test and control solutions (5ul) were applied to the wells of the

antigen slides and incubated for 30 minutes at room temperature in a humid chamber. The slides were washed for 10 minutes in two changes of PBS and allowed to dry. For the IFA, a rabbit anti-mouse fluorescein isothiocyanate (FITC) conjugated antiserum was applied, incubated in the dark for 30 minutes and the slide washed as before. In the TLIFA, the second antibody was a class-specific (7S affinity purified, Fc-specific) antiserum or unconjugated anti-mouse all-isotype antiserum, (all raised in goats), and the fluorescent probe a FITC-conjugated donkey anti-goat IgG antiserum. Incubations and washes were carried out as described above. In the IFA and TLIFA, the slides were shaken dry after the last wash and examined under PBS-glycerol immersion, under incident UV-light illumination (Zeiss Epifluorescent microscope) x 25 objective. Slides not examined immediately were stored in the dark for 1-2 hours without detriment.

Where possible, tests were performed in duplicate on different slides, but this was not practical for the initial screening of several hundred hybridomas, and was only performed when numbers became manageable.

Isotyping of antibodies-

Ouchterlony double diffusion in agar:

isotyping of monoclonal antibodies was performed according to standard techniques, initially using a commercial kit (Serotec, Ltd., Kidlington, Oxon) and later using slides prepared in the laboratory (see appendix 3).

Briefly, wells were cut in agar on a glass slide and test material (neat or concentrated supernatant) was placed in one well and class-

specific antiserum placed in a neighbouring well. A specific precipitin reaction was generally observed after incubation at room temperature, over-night, particularly when the slide was viewed by trans-illumination on a light box. Slides were kept and examined for up to 3 days if lines were not observed sooner. In the commercial test, each supernatant was screened against 5 antisera; in the 'home-made' test, each supernatant was tested against the three class-specific antisera used in the TLIFA, and sub-typing of IgG antibodies was not performed.

CHAPTER 3

PRODUCTION OF MONOCLONAL ANTIBODIES - A COMPARISON OF METHODS TO OPTIMISE NUMBERS OF IgA SECRETING HYBRIDOMAS

CHAPTER 3.

PRODUCTION OF MONOCLONAL ANTIBODIES - a comparison of methods to optimise numbers of IgA secreting hybridomas.

INTRODUCTION

The theory of monoclonal antibody production is founded on the clonal selection theory of antibody production as proposed by Burnet in 1957 and later expanded and verified by other workers. The theory states that antibody production is the result of a specific encounter between antigen and preformed antibody on the B-cell surface resulting in clonal expansion of that cell and differentiation of some of the progeny cells into antibody secreting (plasma) cells. The antibody produced by such a single clone of cells may be defined as 'monoclonal' and the normal 'polyclonal ' humoral response may then be thought of as a composite of very many monoclonal responses. If a single B-lymphocyte or plasma cell could be induced to clonal expansion this might be a source of monoclonal antibody. However, B-lymphocytes have a short life span in culture making them a poor source of in vitro produced antibody. Their malignant counterparts, the myelomas have been adapted to long term culture but the specificity of their monoclonal antibody products is often impossible to establish and they have had limited usefulness as sources of specific monoclonal antibodies.

When a technique for fusing cells of dissimilar origin was developed in which preservation of parental characteristics was

demonstrated (Cotton and Milstein, 1973) this paved the way for the production of monoclonal antibodies of defined specificity (Kohler and Milstein, 1975) and became the basis of monoclonal antibody production as practised so widely today.

Fusion of cells and fusion agents

The first cell fusions were carried out using Sendai virus as fusogen. Now, polyethylene glycol, (PEG), is more widely used. The underlying mechanism of fusion appears to be similar in both cases: an increased fluidity of the plasma membrane is induced and the membranes of adjacent cells 'flow' together to form a single membrane bound unit. Fusion between two cells initially results in the formation of a bi-nucleate cell, but fusion of nuclear membranes occurs later with a resultant doubling in the chromosomal complement. Subsequent rearrangement of the DNA and reduction in chromosome number may result in the loss of desired function(s) as the hybrid cell apparently returns to the more stable state (Goding, 1983).

Selection of hybrids from parent cells

The process of fusing cells is essentially random and the desired fusion product, a hybrid formed between a single B-cell and a single myeloma cell, must be selected from the other randomly produced combinations and the unfused parent cells. The hypoxanthine/ aminopterin/thymidine ('HAT') selection principle of Littlefield (1964) is most commonly used. To summarise the principle of 'HAT' selection: aminopterin-sensitive parent myeloma cells, lacking the

enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT), are killed by HAT medium, while aminopterin resistant hybrid cells, acquiring HGPRT from the lymphocyte source, survive and grow. Unfused lymphocytes have a poor survival time in culture and die naturally in 3-4 days, without selection procedures.

Isotypes of antibodies secreted by hybridomas.

The majority of mouse monoclonal antibodies reported in the literature are of IgM or IgG isotypes while IgA monoclonal antibodies are apparently rare. Small numbers of IgA monoclonal antibodies have been identified following extensive screening of hybridomas generated by conventional fusion procedures (Hurwitz et al, 1980; Claflin et al, 1981; Sharon et al, 1981), and a variety of immunisation/infection regimes with enteric pathogens or parasites were employed in generating IgA secretors specific for bacterial antigens (Colwell et al, 1982; 1983; Komisar et al, 1982; 1983), viral antigens (Mazanec et al, 1987) and helminths (Roach, 1988). The use of gut-associated lymphoid tissue (GALT) as lymphocyte source (Komisar et al, 1982; 1983; Roach, 1988) and in vitro immunisation techniques (Gale, 1987) has also produced some success. However, these murine IgA secreting hybridomas have come from many laboratories using different antigens, immunisation/infection procedures, strains of mice, organ sources of lymphocytes, myeloma fusion partners and fusion protocols, and it is difficult to draw conclusions as to the best protocol for IgA hybridoma production from these results.

In this study the effects of using germ-free and conventional

mice with different immunisation schedules and lymphocyte origin on the generation of anti-Eimeria tenella sporozoites of IgA isotype are described. This being the work of a single operator, utilising a single antigen source, myeloma cell-line and fusion protocol validates a comparison of different procedures in the production of IgA secreting hybridomas, and a rationalised approach to their production is presented and discussed.

MATERIALS AND METHODS

Experimental animals:

conventional Balb-c mice of either sex were used in all immunisation studies, as organ donors in fusion procedures and for the production of ascites. These were obtained from the Liverpool School of Tropical Medicine (LSTM) breeding colony or Harlan Olac, Bicester, England. CD-1 mice from the LSTM breeding colony were the source of peritoneal feeder layer cells. Conventional mice were housed under standard laboratory conditions and were maintained on dry pellet food and water ad libitum. Germ free mice were purchased from Harlan Olac (Bicester, England), and were isolator reared and maintained with sterilised food (Harlan Olac) and water. Germ-free mice were only used for intra-enteric immunisation studies. All mice were aged 4-6 weeks at the commencement of immunisation procedures.

Blood sampling:

blood samples were obtained by 'tail-snip' into plain or heparinised capillary tubes, and plasma or serum samples were obtained by centrifugation and stored at -20°C.

Preparation of antigens:

a) oocysts: sporulated oocysts were obtained as described (see General Materials and Methods), washed free of preservative (2% potassium dichromate solution) and resuspended at the appropriate dilution in tap water.

b) sporozoites: freshly excysted sporozoites, prepared as described

previously (Chapter 2, General Materials and Methods) were suspended at the required concentration in sterile PBS for all parenteral injections.

Inoculation routes:

subcutaneous, intraperitoneal and intravenous injections were delivered by sterile syringe and 25g needle, with manual restraint, with maximum dose volumes of 0.2ml, 0.5ml and 0.2ml respectively. Gastric intubation was performed using a syringe and 2" stainless steel mouse dosing tube, to a maximum dose volume of 0.5ml. Intra-jejunal injections (0.5ml) were carried out using aseptic technique, *via* laparotomy under general anaesthesia.

Chemical restraint:

anaesthesia of conventional mice was carried out with halothane in oxygen *via* a mini-mask and Ayers T-piece. Anaesthesia and surgery of germ-free mice was performed in a sterile hood using 0.6% w/v sodium pentobarbitone in 10% v/v ethanol in water, by intraperitoneal injection (0.135ml/15-20g mouse).

Immunisation studies:

a) parenteral:

three groups of five conventional mice per group were immunised with 250,000 live sporozoites *via* subcutaneous, intraperitoneal or intravenous routes, and booster injections administered *via* the same route six weeks later (d42). Blood samples were obtained and serum samples assayed on days 0, 7, 14, 21, 41, 66,

and 72 days after the primary injection, the latter two samples being 24d and 30d after the second injection. Samples were assayed for antigen-specific activity in the immunofluorescent antibody test (IFAT) as described earlier (Chapter 2, General Materials and Methods), utilising acetone fixed sporozoites as antigen. Five uninjected control mice were bled at the same time points, and serum samples examined similarly.

Parenteral immunisation for fusion experiments comprised two intraperitoneal injections as described above, 6 weeks apart, followed 4-6 weeks later by an intravenous booster injection of 250,000 live sporozoites.

b) oral:

seventeen conventional mice were dosed with 1 million sporulated oocysts by gastric intubation. Nine mice were housed on wire bottomed cages for 72 hours after dosing, their faeces collected and the number of oocysts passing unharmed through the intestine was quantified, using standard salt flotation techniques. Two mice were sacrificed 1 hour after dosing and small intestinal contents and mucosal scrapings were examined for parasitic stages by light microscopy. Antigen specific plasma antibodies were assayed at 7 days (15 mice) and 16 days (9 mice) post-inoculation, using the TLIFA, as described in General Materials and Methods. Selected mice were examined at both time points for gross lesions, or parasitic forms in mucosal scrapings.

c) intra-enteric:

a dose of 2.5 million sporozoites was injected

intra-jejunally into each of three conventional mice and three germ-free mice, in two separate experiments. Specific plasma antibody levels were determined by TLIFA for total antibody response and for specific antibodies of IgG, IgM and IgA isotypes, at 7 and 16 days post-injection (pi). On day 16 pi two mice from each group received an intravenous booster injection of 1 million live sporozoites, and plasma was sampled three days later, when the mice were used as organ donors in fusion experiments.

Hybridoma techniques

Immunisation:

mice received parenteral and intra-enteric priming immunisations as described above. Booster injections of 1 million live sporozoites were administered intravenously 3 days prior to all fusions.

Preparation of lymphocyte suspensions from:-

a) spleen: this was performed according to standard techniques (Goding, 1983) with some modifications outlined in appendix 1. A single cell suspension was generated, yielding 1-3 x 10^8 lymphocytes/ spleen.

b) mesenteric lymph node: the mesenteric lymph node or chain of nodes (MLN) was located in the centre of the fan shaped mesentery of the jejunum. The organ was removed from the adherent mesenteric fat by sharp dissection, taking care to avoid damage to and contamination from the intestines. The MLN was more difficult to disrupt than the spleen, having no well defined capsule, and MLN showed marked

variation in size between individual mice. Cell counts of 3-8 x 10^7 lymphocytes/MLN were obtained.

Cell counts and viability determination:

cell counts were performed utilising the improved Neubauer haemocytometer, and viability estimates were based on the dye exclusion test using 0.3% trypan blue in saline (Sigma Chemical Co. Ltd., Dorset).

Fusogen:

the chemical fusogen polyethylene glycol (PEG), approximate molecular weight 1500, (BDH Ltd.) was used throughout.

Media:

all media were based on Dulbeccos Modified Eagles Medium (Gibco Ltd., Paisley), and full details are given in appendix 1.

Myeloma parent cell line:

the light-chain producing (non-secreting) myeloma cell-line designated P3-NS1-Ag4-1 was used throughout, a kind gift from Ms.J.Gale of the Dept. Tropical Medicine, LSTM.

Fusion technique:

this was performed according to established techniques (Galfre and Milstein, 1981) with minor modifications, and a detailed protocol is given in appendix 1. Briefly, fusion was performed at room temperature, myeloma cells and lymphocytes were

mixed in the ratio 1:10, a 50% solution of PEG 1500 was used as fusogen in serum-free medium, and the final cell suspension was made up in HAT-selective medium, and plated out into 96-well plates. MLN fusions were plated onto 24-hour old feeder layers in 96-well plates.

Hybridoma screening:

at 12 days post-fusion, wells were examined for hybridoma growth using an inverted microscope; supernatants from the wells displaying growth were assayed for specific antibody using the TLIFA. Neat supernatants were appled to the antigen slides and on initial screening, anti-serum directed to all mouse isotypes was used. Subsequently, TLIFA was set up in triplicate for each positive sample and assayed for IgA, IgM and IgG activity. Results are only presented in which spleen fusions generated at least 100 hybrids for assay, and MLN fusions generated at least 25 hybridomas. Only those secretors which were positive in the TLIFA on at least 2 separate occasions have been recorded, although not all of these secreting lines survived to cryopreservation. The initial isotyping result from the class-specific TLIFA was confirmed by Ouchterlony double diffusion in agar, as described earlier (Chapter 2, General Materials and Methods). Positive secretors were checked before and after cloning. The isotypes of antibodies secreted by hybridomas of unknown specificity were also determined by Ouchterlony double diffusion, on 'home-made' slides (see appendix 3). Supernatants were removed from the master colonies, selecting 25 from the centre of each 96-well plate. It is possible that more than one secreting hybrid was present in each well tested, particularly for splenic fusions, giving a

slight over-estimate for infrequent isotypes. However, the low seeding density of MLN fusions rarely resulted in more than one hybridoma visible in each well.

Cloning:

this was performed by the method of 'limiting dilution',(see appendix 1), on one or two occasions for selected hybridoma cell-lines. The designation of the antibody F11B4E12 is derived from the 96-well plate position: F11 - the original plate, B4 - the position on first cloning, and E12 - the position at the second cloning. Where all wells were positive on first cloning, a second cloning was not performed and the designation of such antibodies has two components only, e.g. B5G2.

Ascites production:

this was performed according to established procedures, details outlined in appendix 1. Balb-c mice were pristane primed at least 7 days prior to the injection of hybridoma cells and ascites was collected by aseptic paracentesis. Ascites was centrifuged to remove cells, fibrin clots where present were removed, some aliquots were heat treated (56°C, 30') and the material was then labelled and stored at -20°C.

RESULTS.

Systemic responses to parenteral immunisation:

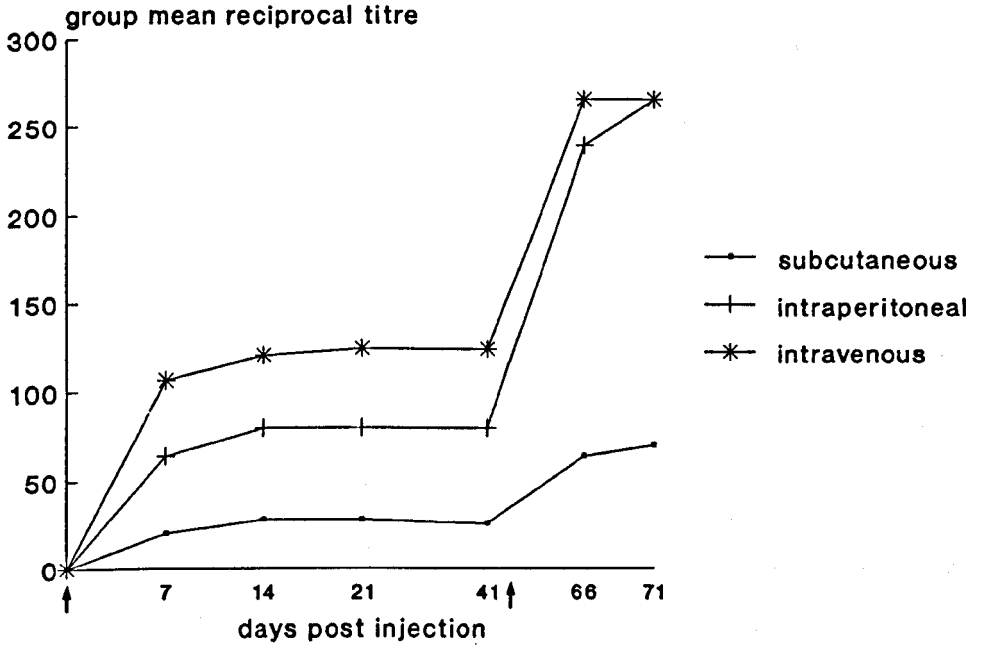
The specific serum antibody responses in mice inoculated with sporozoites by subcutaneous (s/c), intraperitoneal (i/p) or intravenous (i/v) routes are presented in Figure 3.1. Primary responses to the three routes of inoculation were highest in mice receiving an intravenous injection, lowest with subcutaneous injection and intermediate for intraperitoneal. The secondary response to s/c injection was below the primary response levels for i/p and i/v, but titres rose to similar levels in the other two groups. Using the class-specific TLIFA, specific antibodies of IgG and IgM isotypes were detected in all mice, but no specific serum IgA was detected (results not shown).

Systemic responses to gastro-intestinal immunisation

a) Oral oocysts:

A total of approximately 150,000 oocysts per mouse was detected in the faeces in the first 24 hour collection. Any passed subsequently were below the level of detection (<600 oocysts), giving an estimated recovery of 1.5% of the initial dose of 1 million per mouse. Disruption of the oocyst wall was confirmed when sporocysts were found in the small-intestinal contents of mice 1 hour after dosing. Intact oocysts were also observed, but sporozoites were not. There was no evidence of a systemic response to the parasites when plasma samples were examined at 7 days (15 mice) post-inoculation and

Figure 3.1 Serum antibody levels in mice immunised by three parenteral routes



arrows (↑) represent priming injection (d0) and secondary injection (d42).

no gross or microscopic evidence of parasite developmental stages in mucosal scrapings taken at this time. At 16 days post-inoculation 2 of 9 mice showed a trace response in the TLIFA (all isotypes) at a 1/20 screening dilution, but no specific activity was detected when isotype-specific antisera were used.

b) Intra-enteric sporozoites:

Reciprocal plasma antibody titres at 7 and 16 days post-injection and, where appropriate, at 19 days (fusion day) are given for the individual germ-free and conventional mice (Table 3.1). It should be noted that mouse 14a did not show a systemic response to intra-enteric priming, but showed a good response by three days after intravenous booster injection and subsequently yielded 6 specific IgA secreting hybridomas (see Discussion).

Hybridoma production:

The results of fusions from which at least 100 hybridomas were generated from spleen, or at least 25 hybridomas from mesenteric lymph node, are summarised in Tables 3.2 and 3.3. Fusions in which lower numbers of hybrids were achieved, or in which failure could be attributed to technical problems have not been included. Some hybridomas were lost from culture before their isotypes had been determined, and are recorded as ND (not determined).

Table 3.4 summarises the comparative yields of specific IgA anti-Eimeria monoclonal antibodies and IgA antibodies of undetermined specificity from the different groups of fusions.

Table 3.5 summarises all the monoclonal antibodies produced.

TABLE 3.1

SYSTEMIC RESPONSES TO INTRA-ENTERIC PRIMING AND INTRAVENOUS BOOSTER INJECTION - reciprocal serum titres.

	Days post primary immunisation											
	7				16				19			
Isotype	G	A	M	Ig	G	A	M	Ig	G	A	M	Ig
Mouse												
CONV.13a	-	T	40	40	20	-	20	40	160	-	40	160
13b	-	-	20	20	40	-	20	40	320	-	40	320
13c	-	-	T	-	-	-	-	20		NA		
G.F. 14a	-	-	-	-	-	-	-	-	320	-	160	80
14b	-	-	40	40	20	-	20	-	160	-	320	320
14c	20	-	20	20	40	-	20	20		NA		

NA - not applicable
T - trace at 1/20 dilution

CONV.- conventional mice
G.F.- germ-free mice

TABLE 3.2

PRODUCTION OF ANTI-EIMERIA HYBRIDOMAS-
summary of splenic fusions.

fusion no.	mouse type	lymphocytes fused, x 10^7	immunisation route	hybrid ratio	isotypes A	G	M	ND
1	conv	30	parenteral	0.13	0	3	0	1
6	"	13	"	0.54	1	4	1	1
8	"	14	"	0.29	0	3	1	
9	"	10	"	0.20				2
10	"	18	"	0.28	0	4	1	
13a	"	24	intra-enteric	0.08	0	1	1	
13b	"	20	"	0.20	0	3	1	
14a	germ-free	22	"	0.36	5	3	0	
14b	"	16	"	1.19	2	9	8	

ND - not determined conv - conventional mice

Hybrid ratio -
number of specific hybridomas produced per 10^7 lymphocytes fused

TABLE 3.3

PRODUCTION OF ANTI-EIMERIA HYBRIDOMAS-
summary of mesenteric lymph node fusions

fusion no.	mouse type	lymphocytes fused, x 10^7	immunisation route	hybrid ratio	isotypes A	G	M	ND
8	conv	4.0	parenteral	0				
9	"	2.0	"	0.50				1
10	"	1.8	"	0				
13a	"	3.6	intra-enteric	0.28			1	
13b	"	7.5	"	0.27		1	1	
14a	germ-free	2.7	"	2.59	1	5	1	
14b	"	1.8	"	1.11	0	1	1	

ND - not determined conv - conventional mice

Hybrid ratio -
number of specific hybridomas produced per 10^7 lymphocytes fused

TABLE 3.4

COMPARATIVE YIELDS OF SPECIFIC AND NON-SPECIFIC IgA SECRETING HYBRIDOMAS FROM DIFFERENT FUSION PROTOCOLS

mouse type	immunisation route	lymphocyte source	non-specific secretors IgA/total	specific secretors IgA/total
conv	parenteral	spleen	ND	1/22
"	"	MLN	4/22	1*
"	intra-enteric	spleen	0/24	0/6
"	"	MLN	2/28	0/3
germ-free	"	spleen	0/76	7/27
"	"	MLN	2/17	1/9

MLN - mesenteric lymph node
* - isotype not determined
conv - conventional mice

TABLE 3.5

SUMMARY OF ALL POSITIVE HYBRIDOMAS IDENTIFIED

code no. JH/Et	fusion no.	mice/ immunisation	organ source	name	clone name	isotype	frozen stock*
1	1	conv/par	spleen			G	
2	"	"	"	F1		G	
3	"	"	"	G2	G2C5	G	
4	6	"	"	B5	B5G2	G	+
5	"	"	"	B6	B6E3	G	
6	"	"	"	D9		G	
7	"	"	"	F11	F11B4E12	A	+
8	"	"	"	G12	G12B7	M	+
9	"	"	"		H4H6	G	+
10	8	"	"	E6		M	
11	"	"	"	E9		G	+
12	"	"	"	F11		G	+
13	"	"	"	G12		G	+
14	10	"	"	E7		G	
15	"	"	"	E8		M	
16	"	"	"	E12		G	
17	"	"	"	F4		G	
18	"	"	"	F10		G	
19	13a	conv/ie	"	B4		G	
20	"	"	"	D1		M	
21	"	"	MLN	A4		M	
22	13b	"	spleen	A2		G	
23	"	"	"	A9		G	
24	"	"	"	B11		M	
25	"	"	"	F11		G	
26	"	"	MLN	B11		M	
27	"	"	"	E9		G	
28	14a	gf/ie	spleen	B4	B4A6	A	+
29	"	"	"	B7	B7C10	A	+
30	"	"	"	B10		G	+
31	"	"	"	D4i	D4E8	A	+
32	"	"	"	D4iii		G	+
33	"	"	"	D7	D7C4	A	+
34	"	"	"	D8		G	+

conv - conventional mice
gf - germ free mice
MLN - mesenteric lymph node

par - parenteral priming
ie - intraenteric priming

* cryopreserved cells from master colonies/clones in liquid nitrogen
JH/Et code no. - external laboratory reference (not used in text)

continued...

TABLE 3.5 (cont)

SUMMARY OF ALL POSITIVE HYBRIDOMAS IDENTIFIED

code no. JH/Et	fusion no.	mice/ immunisation	organ source	name	clone name	isotype	frozen stock*
35	14a	gf/ie	spleen	E4	E4E3	A	+
36	"	"	MLN	B11		G	
37	"	"	"	D8		M	
38	"	"	"	D9		G	
39	"	"	"	D10	D10B4	A	+
40	"	"	"	E8		G	
41	"	"	"	F2		G	
42	"	"	"	G6		G	
43	14b	"	spleen	A5		M	+
44	"	"	"	B8		G	+
45	"	"	"	B9		G	
46	"	"	"	C6		G	+
47	"	"	"	C10		G	
48	"	"	"	C11		M	
49	"	"	"	D5iii		M	+
50	"	"	"	D5iv		M	
51	"	"	"	D6		G	
52	"	"	"	D7		G	+
53	"	"	"	D9		G	
54	"	"	"	D12		M	
55	"	"	"	E2		M	+
56	"	"	"	E4		G	
57	"	"	"	E6		M	+
58	"	"	"	F6	F6D4	A	+
59	"	"	"	F8		M	
60	"	"	"	G6		G	
61	"	"	"	H3		A	
62	"	"	MLN	D4		G	+
63	"	"	"	D9		M	

conv - conventional mice
gf - germ free mice
MLN - mesenteric lymph node

par - parenteral priming
ie - intraenteric priming

* cryopreserved cells from master colonies/clones in liquid nitrogen
JH/Et code no. - external laboratory reference (not used in text)

DISCUSSION

Monoclonal antibodies of IgA isotype directed against Eimeria tenella were sought for investigations of mucosal immunity to E.tenella by passive transfer of such antibodies to recipient chicks. Monoclonal antibodies have been produced against avian Eimeria species by several groups of workers (Danforth, 1982a - E.tenella and E.mitis; Danforth and Augustine, 1983a; 1983b - E.mivati, E.adenoides and E.meleagrimitis; Speer et al, 1983a; 1983b - E.tenella; Thammana and Schenkel, 1984 - E.tenella; Crane et al, 1986a; 1986c; 1988 - E.tenella; Schenkel et al, 1987a; 1987b - E.tenella). However, none have been reported of IgA isotype and none have been investigated for protective effects at the chick intestinal mucosa. Those IgA monoclonal antibodies that have been reported in the literature have come from a wide variety of sources and comparative aspects of IgA production have not been described. The low representation of IgA secretors amongst specific secreting hybridomas may be due to a failure to seek or detect specific IgA antibodies, compounded by the use of inappropriate immunisation procedures in the stimulation of IgA bearing B-cells to blastogenesis, and the use of inappropriate tissues in the harvesting of such cells for fusions.

Monoclonal antibodies directed to surface antigens were required to investigate effects on penetration of epithelial cells and initiation of infection in the chick intestine, by blocking or altering specific recognition sites between sporozoites and cells as described in studies in vitro (e.g.Augustine and Danforth, 1982; 1984a; Augustine, 1985a; 1986). Consequently, live sporozoites were used as immunising

antigen, since in this form antigens would be presented in native form, on or in the parasite surface membrane. Ideally, an assay system for specific antibodies generated should have employed antigen in similar form (e.g. live parasites in agglutination assay or surface fluorescence test), but as a compromise between this ideal and the practicalities of screening hundreds of culture supernatants, air-dried, acetone fixed sporozoites were used. Many of the monoclonal antibodies detected did demonstrate surface activity against live sporozoites (see Chapter 4), demonstrating at least partial adequacy of the screening system in identifying antibodies directed to surface antigens. Fixation procedures have been shown to alter eimerian antigen recognition by monoclonal antibodies (Augustine et al, 1988) and antigen distribution in sporozoites (Augustine and Danforth, 1987b) which could have precluded identification of some monoclonal antibodies. The TLIFA was used in preference to the (two-step) IFA, for its increased sensitivity.

Clearly, IgA antibodies are not detected in some studies since Protein-A or anti-IgG antisera are used in screening assays (e.g. Danforth, 1982a), as advocated by some workers to avoid detection of low-specificity IgM antibodies and ensure a means of purifying useful antibodies (Pearson and Clarke, 1983). In order to evaluate the problem of lack of detection, a conventional fusion protocol was selected. In preliminary immunisation studies, a systemic response to parenteral immunisation was detected in all mice for sub-cutaneous, intraperitoneal and intravenous priming doses and following a secondary injection, titres rose in all groups with similar titres reached 30 days after two intraperitoneal or intravenous injections.

Intravenous injections were prone to technical failure, and local tissue damage could preclude second and third injections by the same route while intraperitoneal injections were consistently simple. Since administration of two intraperitoneal injections produced good serum responses, this was used to immunise mice for conventional fusions, with an intravenous booster 3 days prior to fusions. None of the parenteral priming routes produced a detectable serum IgA response although it was conceivable that low levels of serum IgA would not be detected in the face of high levels of IgG due to swamping of antigenic sites at low dilutions.

The conventional fusion protocol generated 22 specific hybridomas from 5 fusions. A single IgA secretor was identified by the class-specific TLIFA, (the isotype confirmed by Ouchterlony double diffusion) indicating adequacy of the screening assay and suggesting that a failure to detect IgA secretors was not responsible for their low representation in fusions in general. The IgA monoclonal antibody obtained (F11B4E12) was used thereafter as positive control in the IgA specific TLIFA.

Clearly, generation of useful numbers of IgA monoclonal antibodies would necessitate screening considerable numbers of hybridomas (as performed by Hurwitz et al, 1980), or employing techniques to increase the proportion of IgA secretors generated.

It is generally accepted that the active lymphocyte partners giving rise to secreting hybridomas are rapidly dividing immature B-cells undergoing antigen driven blastogenesis (e.g. Zola and Brooks, 1985). The isotype and specificity of the resultant hybridoma is that borne by the lymphocyte at the time of fusion. The evidence in support of

the use of gut-associated lymphoid tissue (GALT) as lymphocyte source in IgA hybridoma production is conflicting. On the basis that GALT is an enriched source of IgA B-cell precursors (Craig and Cebra, 1971), Komisar and co-workers (1982) used pooled mesenteric lymph node (MLN) and Peyer's patch (PP) as lymphocyte sources, and produced 8 IgA monoclonal antibodies. However, the number of IgA bearing B-cells from PP and MLN is only around 5% of B-cells, a little higher than for spleen (McWilliams et al, 1974) which suggests the B-cell array is not fundamentally different from that of spleen. Recent evidence suggests that pre-committment to IgA production occurs in PP due to the presence of 'switch T-cells' (Kiyono et al, 1982; Cebra et al, 1983; Kawanishi et al, 1983; Michalek et al, 1983; Campbell and Vose, 1985) possibly via soluble factors (Mayer et al, 1983), and these switch cells may also induce spleen cells to IgA production (Kawanishi et al, 1983). The important factor would appear to be the stimulation of such T-cells by mucosal immunisation, and for IgA hybridoma production, the harvesting of B-cells which have undergone such a class switch. Intra-enteric priming has been demonstrated to induce specific IgA memory cells in the spleen as well as MLN (Jeurissen et al, 1985) and a model of IgA induction is proposed in which mucosal immunisation activates local IgA precursor cells (via class switch T-cell activity) which leave PP and migrate via MLN, lymph and blood to distant sites, including mucosae and spleen. These cells are then re-activated by subsequent specific antigenic stimulation to differentiation and IgA production. This evidence indicated that intra-enteric priming might be most efficient in promoting IgA memory cells in the spleen and MLN, which could be

reactivated by parenteral immunisation and used as fusion partners. It was for these reasons that fusions were carried out using MLN and spleen cells with intra-enteric immunisations and comparisons made between the different fusion protocols.

The first aspect of the comparative study using conventionally reared mice was an investigation of GALT as lymphocyte source. Collection of PP cells was not possible without contamination of cultures with bacteria and yeasts, and there was initial failure to produce hybridomas from MLN. The latter problem was overcome by plating the newly fused cells on to 24-hour old feeder layers. Only one specific secreting hybridoma was obtained from MLN (of 230 hybrids screened), and this died before isotyping was carried out. The low numbers of lymphocytes generated from MLN compared to spleen, and the resultant low numbers of hybridomas obtained was not encouraging. The hybrid ratio (number of hybridomas secreting antigen specific antibody as a proportion of the number of cells fused) was also low, suggesting that antigen was not being encountered by MLN following intraperitoneal and intravenous injections.

The second aspect of the study was to increase specific mucosal stimulation and assess the effects on spleen and MLN derived hybridoma production.

Oral administration of sporulated oocysts of Eimeria tenella to mice has been shown to result in excystation and partial, although aberrant development in the non-specific host (Naciri and Yvore, 1982; Naciri, 1985; Naciri and Yvore, 1986). In this present study, a similar level of oocyst disruption and excystation is inferred with passage of only 1.5% of the original dose through the mouse gut.

Immune responses to E.tenella infection in mice have not been described, and in the present study, specific serum antibodies were not detected and these mice were not used for fusions. However, the absence of a primary serum antibody response was shown subsequently to be a poor indicator of suitability for hybridoma production of IgA isotypes and results of fusions following oral oocyst immunisation could have been most interesting.

It became clear that the conventional mice used were receiving a heavy enteric parasite challenge with the identification of eggs of Hymenolepis spp., Aspiculuris spp. and Trichuris spp. in the mice faeces. Germ-free mice were used to eliminate this unwanted mucosal challenge with 'competitor' antigens, and fusion results were compared with conventional mice following intra-enteric priming, and intra-enteric priming compared with conventional immunisation. Intra-enteric immunisation with sporozoites was performed to ensure large amounts of the appropriate antigens were encountered at the mucosa, as described by Jeurissen and co-workers (1985). Mucosal stimulation by a single priming dose induced a systemic response in all three conventional mice, and two of the three germ-free mice; this suggested that the sporozoites had invaded the host tissues via the gut wall and thereby presented a mucosal antigenic challenge, although specific serum IgA was rarely detected. The reason for the apparently improved response using the equivalent of a third of the sporozoite dose (2.5 million) compared to the oocyst dose used (1 million oocysts ≡ 8 million sporozoites) is not clear, although it is suggestive of poor excystation in the foreign host following adequate oocyst wall disruption.

Results of fusions with intra-enteric priming of conventional mice were similar to results for the conventional fusions. MLN gave slightly improved hybrid ratios compared to spleen from the same mice, but amongst the small number of positive hybridomas, none of IgA isotype.

The most remarkable success came from the intra-enteric immunisation of germ-free mice. Hybrid ratios were improved for spleen and MLN. There was an absolute increase in the number of IgA secretors generated, and for spleen fusions, an increased relative proportion of IgA secretors amongst the specific secretors of other isotypes. Although increased hybridomas were generated from MLN, the proportion of IgA secretors was low.

The four mice used for intra-enteric priming and fusions were selected prior to the determination of their systemic response to antigen priming. It is interesting to note that the mouse which gave 5 IgA secretors from spleen and 1 from MLN did not show a primary serum response. The serum response 3 days after the i.v. booster injection comprised both IgG and IgM, indicative of a secondary response, but no IgA was detected. Clearly, serum antibody levels and isotypes are not relevant in the production of specific IgA monoclonal antibodies. It was unfortunate that orally infected/immunised mice were not studied further for reasons based on false assumptions, as oocyst administration by gastric intubation was the most simple of the immunising procedures. Further comparisons using this infection route in germ-free mice could have proved rewarding.

Taken together, these results demonstrate no advantage in the use

of MLN as lymphocyte source in the generation of IgA monoclonal antibodies for this antigen system, contrary to the results of Komisar and co-workers (1982). For general monoclonal antibody production, MLN was a poorer lymphocyte source, disrupting to give fewer lymphocytes and fewer hybridomas, (even when the hybrid ratio was elevated using germ-free mice), although this disadvantage could be offset by pooling MLN from 5-10 mice. The theoretical possibility of obtaining large numbers of specific IgA secretors from MLN was not borne out in this study, but isotyping of hybridomas of undetermined specificity did show a tendency for this tissue to generate IgA hybridomas in all the mice. These IgA secreting hybridomas were presumably derived from B-cells precommitted to antigens derived from other enteric pathogens, ingested material etc. Spleen fusions were most successful when intra-enteric priming of germ-free mice was carried out. Whether the germ-free status exerted its effect by promoting increased specific antigen presentation at the mucosa, or in a generalised increased responsiveness can not be stated, although use of 'healthy' animals is advised in immunisation studies (Dresser, 1983). From the isotype analysis of non-specific hybridomas from these mice, it would appear that the induction of IgA bearing spleen cells has been antigen specific, as predicted by Jeurissen and co-workers (1985) since no other IgA secretors were identified from 100 tested.

To summarise, for this antigen and fusion procedure, there is evidence to indicate the value of germ-free mice in the generation of large numbers of specific hybridomas. Although the use of germ-free

mice is sometimes specified (e.g. Colwell et al, 1982), conventional mice are usually used since handling and housing procedures are more simple. However, for some fusion procedures the use of germ-free mice may reduce the number of fusions required, reduce costs and prevent unnecessary waste of mice, labour and time.

Intra-enteric priming was effective in stimulating IgA memory cells in the spleens of germ-free mice, but not conventional mice. MLN did not prove advantageous in the production of specific IgA secreting hybridomas.

A rationalised approach to the production of specific IgA secreting hybridomas would employ intra-enteric priming and intravenous boosting of young, germ-free mice, and would utilise spleen alone, in an established fusion protocol.

CHAPTER 4

CHARACTERISATION OF MONOCLONAL ANTIBODIES

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CHARACTERISATION OF MONOCLONAL ANTIBODIES

INTRODUCTION

Hybridoma cell lines secreting anti- Eimeria tenella sporozoite antibodies were identified by screening of culture supernatants as described earlier. Using IFA and TLIFA, different binding patterns to the fixed sporozoites were noted and the different isotypes were determined using the TLIFA, and confirmed by Ouchterlony double diffusion. Initially, all the cell lines identified were cryopreserved and supernatants and ascites derived from them examined in tests in vitro. When the desired IgA secretors were obtained attention was focussed on these.

The aim of the characterisation procedures was to identify monoclonal antibodies which might have applicability in passive protection studies and involved an examination of effector properties, reflecting both antigen specificity and isotype; analysis of the antigen distribution on/in the sporozoite, species specificity and some attempts to characterise the antigens themselves; and for the IgA monoclonal antibodies, analysis of their degree of polymerisation (of importance in consideration of passive transfer techniques).

MATERIALS AND METHODS

Monoclonal antibody screening assays.

i.Immunofluorescent antibody tests (IFAT and TLIFA).

Culture supernatants or ascites were tested by IFAT and TLIFA, with air- dried acetone fixed sporozoites of E.tenella or E.acervulina as antigen, as previously described (Chapter 2, General Materials and Methods).

ii.Surface fluorescence test (IFA using live sporozoites).

Examination for antibody binding to surface membrane antigens of live sporozoites of E. tenella was performed as since described(Trees et al, 1989). Freshly excysted live sporozoites were resuspended in PBS + 2% bovine serum albumin (BSA) + 0.2% sodium azide, at 10 million cells /ml. Equal volumes of test medium (50-100ul) and sporozoite suspension were mixed in a 1.5ml Eppendorf tube and incubated at 4°C for 30 minutes (final azide concentration 0.1%). To this was added 1ml cold washing buffer (PBS + 1% BSA + 0.1% azide), and the cells pelleted (2', 13,000g) and the supernatant discarded. The cells were washed again by resuspension in washing buffer and repelletted by centrifugation. The fluorescent probe, rabbit anti-mouse Ig, (Nordic Immunochemicals) was applied to the cell pellet (diluted 1/50-1/100 in washing buffer) and the sporozoites gently resuspended and incubated at 4°C for 30 mins. Two washes were performed as before and the cells finally resuspended in 1/2 the original total volume (50-100ul) and placed on ice in a light proof container until examined.

A 5ul drop of cell suspension was placed on a clean glass slide, a cover slip applied and the cells examined by ultra-violet incident illumination ('Standard' microscope, equipped with an epifluorescence condenser, Zeiss, Oberkochen, West Germany), x 25 objective. Photographs were taken at x 60 objective.

iii.Agglutination of sporozoites

Agglutination of sporozoites was examined as described recently by Trees and co-workers (1989) in tests with chick antibody sources. Freshly excysted live sporozoites were suspended at 100 million /ml in PBS. A 5ul drop of cell suspension was placed on a clean glass slide. To this, 5ul of test material was added, gently mixed with the pipette tip, and covered with a glass cover slip (16mm diameter). The slide, appropriately labelled, was placed in a humid chamber at 37°C and the cells examined following different incubation periods, and the degree of agglutination and /or alteration in shape noted. A PBS control was performed simultaneously with any test samples. Initially, mouse hyperimmune serum was used as positive control, but following the identification of the IgG monoclonal antibody designated B5G2, a powerful agglutinator, aliquots of a batch of heat-treated supernatant were prepared and stored at -20°C and this antibody was subsequently included as a positive control.

Viability was assessed by trypan blue exclusion as described previously (Wagenbach, 1969), viable cells being those able to exclude the dye.

iv.Transmission Electron Microscopy

This was performed in a manner similar to other studies with sporozoites of E.tenella (Speer et al, 1983a). Three to 5 million sporozoites were suspended in 100ul PBS in 1.5ml Eppendorf tubes to which an equal volume of test supernatant or control material was applied. Incubation, washing and centrifugation steps were performed as described for surface fluorescence test. A solution of 2% gluteraldehyde in 0.1M cacodylate buffer, pH 7.4, was slowly trickled on to the pellet, taking care not to disturb the cells, and fixation carried out for 1 hour at room temperature. The fixative was aspirated and replaced with 0.1M cacodylate buffer, stored overnight at 4°C and then post-fixed in 1% osmium tetroxide (1 hour, room temperature). Following a wash in cacodylate buffer, en bloc staining with 2% uranyl acetate for 20 minutes was carried out. The pellet was washed rapidly with distilled water, progressively dehydrated, treated with propylene oxide, propylene oxide/resin mixture and finally resin alone. Spurr's resin-medium mix was used and polymerisation carried out at 70°C for 9 hours. Sections were cut with glass knives on an LKB - Ultratome III, collected on copper grids and stained in situ with 2% uranyl acetate for 20 minutes and Reynold's lead citrate for 3 minutes.

Sections were examined in a Philip's CM-10 electron microscope.

Isotype determination of monoclonal antibodies.

a) Three layer immunofluorescent antibody test (TLIFA)

The antibody screening IFAT was modified to include class-specific second antibodies (mouse Fc-specific, 7S affinity purified antisera

raised in goats, purchased from Nordic Immunochemicals) and an anti-goat Ig fluorescent probe (Nordic Immunochemicals) giving the three layers: mouse monoclonal, goat anti-mouse class specific antiserum and donkey anti-goat-FITC applied to the acetone fixed sporozoite antigen. The test was performed as described in Chapter 2,General Materials and Methods.

b) Ouchterlony double diffusion.

This was performed as previously described (Chapter 2, General Materials and Methods), initially utilising a commercial kit (Serotec Ltd.) and later a simple laboratory version (see appendix 3) utilising the Fc-specific antisera (Nordic Immunochemicals).

Sporozoite antigen analysis

The component proteins of E.tenella sporozoites were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with or without reducing agents, blotted on to nitrocellulose paper and the antigens recognised by monoclonal antibodies identified by immunoperoxidase staining. Techniques employed were based on published techniques (Towbin et al, 1979; Tsang et al, 1983; Wisher, 1986) with minor modifications as detailed in appendix 2.

Protein preparation:-

sporozoites of E.tenella were pelleted and stored in small Eppendorf tubes in PBS at -20°C until use. The thawed pellet was resuspended by the addition of an equal volume of sample buffer with or without reducing agent, to give a final volume of 100ul per 5-10 million sporozoites, the tube then placed in a water bath at 100°C for 3 minutes or left at room temperature, before

centrifugation (13,000g for 2') to remove insoluble material. Sample volumes up to 40ul could be applied to each well.

Markers:-

heavy and light molecular weight marker proteins (BioRad, Hemel Hempstead, Herts.) were included in at least one track of all gels. The markers (MW) comprised: myosin (200kD), beta-galactosidase (116.25kD), phosphorylase B (92.5kD), bovine serum albumin (66kD), ovalbumin (45kD), carbonic anhydrase (31kD) and soybean trypsin inhibitor (14.4kD) and were prepared and used according to the manufacturer's instructions.

Gels:-

slab gels comprising a 3% stacking gel and 5-15% gradient separating gel were poured and run using a commercial gradient mixer,gel former and electrophoresis cell (BioRad). Electrophoresis was initiated at 25mA per gel until the bromophenol blue formed a thin blue band and was continued overnight at 5mA/gel, at room temperature, until the dye front could be seen to be approaching the bottom of the gel.

Gels or parts of gels were stained for protein with Coomassie blue stain, destained and photographed and/or dried on Whatman No.3 paper to give a permanent record of the protein bands. Alternatively, gels were prepared for Western blotting.

Western blotting:

this was performed according to the trans-blot cell operating instructions (Bio-Rad, Richmond, California, USA) with minor modifications. Polyacrylamide gels were soaked in two changes

of transfer buffer for 1 hour prior to electrophoretic transfer, during which time the gels would swell considerably. The nitrocellulose paper (NCP) was of pore size 0.45um (Schleicher and Schuell) supplied through Anderman (Kingston, Surrey). The NCP and the Whatman No.3 paper were pre-soaked in transfer buffer for 5 minutes. The 'sandwich' of gel and paper was constructed in the gel holders ensuring that no air bubbles were trapped between the different layers and that the gel was placed cathodic to the NCP in the trans-blot cell (BioRad). Transfer was performed either overnight (15-18hrs) at 90mA, 30V; or in 3 hours (220mA, 60V) in both cases, at 4°C.

Following transfer the NCP was temporarily stained with the water soluble red dye Ponceau-S (to identify and allow marking of antigen tracks and positions of protein markers) and then the remaining protein binding sites were blocked using 10% horse serum in PBS. Blocking and antibody incubations for 1 hour and washing steps, four times each of 10 minutes were each performed at room temperature and with gentle agitation on a rocker.

The NCP was sometimes retained as a single sheet (e.g different antigen tracks probed with single antibody) or divided into strips with a sharp blade (same antigen mixture probed with different antibodies). Volumes of antibody applied varied according to the size of the NCP, but were always enough to cover the paper.

Tissue culture supernatants were applied neat, while ascites and serum samples were diluted in PBS before addition. Monoclonal antibodies were probed with the appropriate class-specific antibody followed by rabbit anti-goat peroxidase (Nordic Immunochemicals)

(three step blot) or probed directly with a rabbit anti-mouse Ig peroxidase (Miles Immunochemicals) (two step blot). Following the addition of the final antiserum and four washes, two washes in PBS alone (to remove extraneous protein) and then Tris (to change the buffer conditions) were carried out. The diamino-benzidine substrate (DAB) solution was freshly prepared during the final wash, observing strict safety precautions in the handling of DAB. The incubation in substrate was normally carried out for 1-2 minutes in subdued lighting by which times bands were visible, and undue background colouration was acceptable. The NCP was then washed extensively in large volumes of tap water and the specific red-brown colour reaction recorded and/or photographed as soon as possible. Where specific bands were not detected in 1-2 minutes, the paper was incubated longer, but this generally resulted in an overall brown colouration of the paper, and did not aid resolution of specific bands.

Control solutions included as appropriate comprised: NS-1 ascites, NS-1 culture supernatant, OPI-HT, washing buffer, and an IgA monoclonal antibody raised to beta-spectrin in culture supernatant (a kind gift from J.Gale, N.McCallum-Deighton and M.Hommel, Dept. Tropical Medicine, LSTM).

IgA monoclonal antibodies - polymerisation analysis:

the approximate molecular weights of the IgA monoclonal antibodies were estimated from SDS-PAGE of culture supernatants. The specific antibody was either identified by autoradiography of the dried gel following biosynthetic radiolabelling or by immunoblotting of

unlabelled tissue culture supernatant using the sensitive three stage blot.

Biosynthetic radiolabelling:

10^7 viable hybridoma cells were incubated in 1 ml methionine- deficient RPMI tissue culture medium (Selectamine Kit, Gibco) supplemented with 10% FCS and 75uCi ^{35}S-methionine (Amersham Labs., Amersham, Bucks.) for 2-4 hours at 37°C, 8% CO_2 in a humidified incubator. The cells were then removed by centrifugation and the labelled supernatant either used directly or dialysed extensively against PBS to remove the excess labelled methionine.

SDS-PAGE:

supernatants from over-grown cultures and biosynthetically radiolabelled supernatants were subjected to SDS-PAGE under reducing and non-reducing conditions. Equal volumes of supernatant and sample buffer were mixed, heated to 100°C in a water bath for 3 minutes, and electrophoresed as described above.

Radiolabelled gels were stained with Coomassie blue, soaked for 30 minutes in a liquid fluor, 'Amplify' (Amersham Labs.), dried, and the test tracks and MW marker positions indicated with radioactive or fluorescent ink on the dried gel. Blue light-sensitive radiographic film (Ilford) was preflashed, placed in a cassette (without screens) apposed to the dried gel and then placed at -70°C for 2-4 weeks after which the film was developed.

Gels containing the unlabelled supernatants were blotted as

described above and the specific antibody was detected by using the IgA specific antiserum in the three layer blot.

RESULTS

Homologous TLIFA

All the monoclonal antibodies examined were positive on TLIFA employing E.tenella sporozoites as antigen, this being the antigen system against which the antibodies were raised and which initially identified them. However, the titres of over-grown supernatant and ascites varied between different cell-lines and also between different cultures of the same cell-line. Antibody levels rose in ascites from the same mouse with successive taps and showed variation between mice for the same cell-line. Supernatant titres were in the range neat-1/64; ascites titres ranged from $1/10^3$-$1/10^4$.

The patterns of fluorescence included: 'all over' (even distribution outlining the whole sporozoite; 'internal' (uneven distribution delineating internal organelles such as refractile bodies); 'polar' (bright fluorescence of one pole only) and 'semi-polar' (brighter fluorescence towards one half of the sporozoite) and combinations of the above, and are illustrated in Figure 4.1. Omission of the acetone fixation did not alter these binding patterns.

Heterologous TLIFA

A pool of serum from mice parenterally immunised with E.tenella failed to show cross-reactivity to sporozoites of E.acervulina in the TLIFA. This serum showed an IgG titre of 1/800 in the homologous assay but only a trace of specific IgM. Monoclonal antibodies of all isotypes were tested for cross-reactivity to E.acervulina sporozoites and results are presented in Table 4.1. None of the 13 IgG McAbs

FIGURE 4.1

Patterns of fluorescence observed with different monoclonal antibodies, on air-dried acetone-fixed sporozoites of *Eimeria tenella*.

a) polar
b) all over
c) semi-polar
d) semi-polar
e) internal
f) internal
(delineating refractile bodies)

FIGURE 4.1

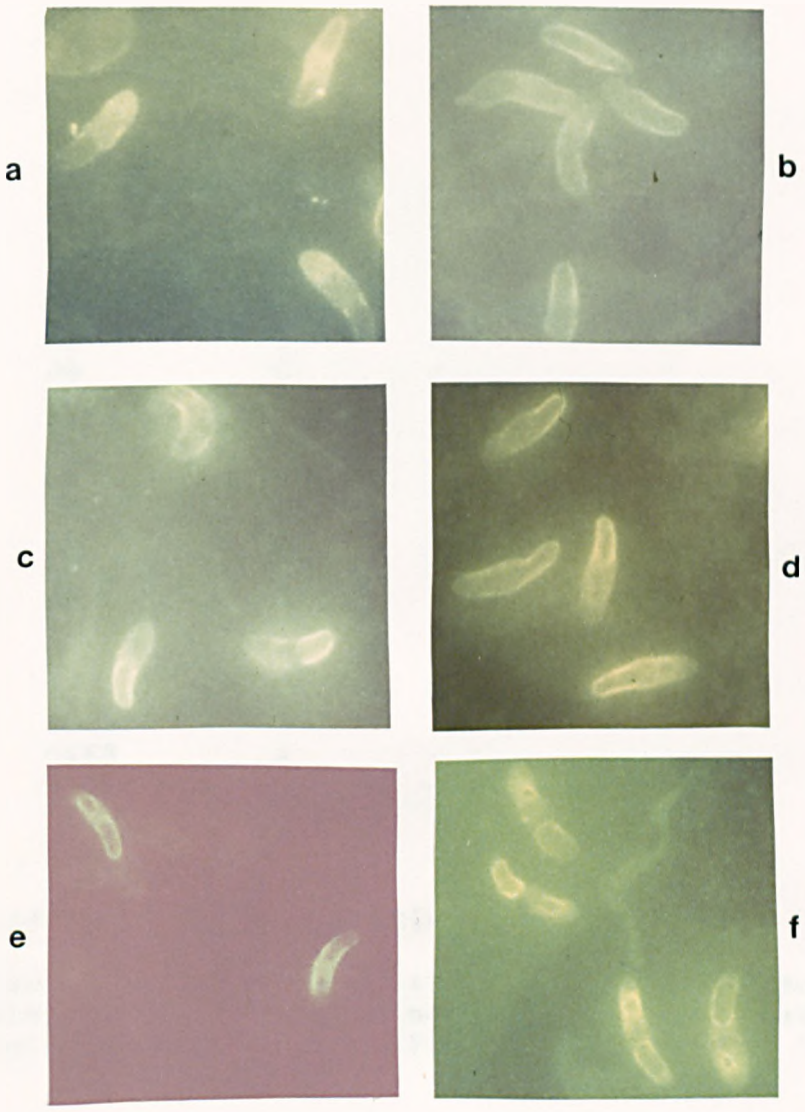


TABLE 4.1

CROSS-REACTIVITY OF MONOCLONAL ANTIBODIES RAISED TO EIMERIA TENELLA WITH EIMERIA ACERVULINA SPOROZOITES.

Fusion No.	Monoclonal antibody	Isotype	E.tenella	E.acervulina
F6	B5G2	G	+	-
"	H4H6	G	+	-
F8	E9	G	+	-
"	F11	G	+	-
"	G12	G	+	-
F9	E7	G	+	-
"	F10	G	+	-
"	F4	G	+	-
F14a	B10	G	+	-
"	D4	G	+	-
"	D8	G	+	-
F14b	D4	G	+	-
"	D9	G	+	-
F6	G12B7	M	+	+
F8	E6	M	+	+
F9	E8	M	+	+
F14b	E6	M	+	-
"	E2	M	+	-
F6	F11B4E12	A	+	+
F14a	B4A6	A	+	-
"	B7C10	A	+	-
"	D4E8	A	+	-
"	D10B4	A	+	-
"	E4E3	A	+	-
F14b	F6D4	A	+	-

No cross-reactive IgG monoclonal antibodies.

3 IgM and 1 IgA cross-reactive monoclonal antibodies gave similar patterns of fluorescence and titres with both antigen sources in TLIFA.

tested showed cross-reactivity, while 3 of 5 IgM monoclonal antibodies and 1 of 7 IgA monoclonal antibodies showed the same pattern of fluorescence and similar titres in the heterologous antigen system.

Surface activity (agglutination and surface fluorescence)

The appearance of a positive surface fluorescence test and agglutination of fluorescing sporozoites by IgG monoclonal antibody B5G2 is illustrated in Figure 4.2. Tests for antibody activity against sporozoite surface antigens were applied to 30 culture supernatants and in addition, 14 were tested for agglutination alone. Of the 30 supernatants, 19 were positive by surface fluorescence, but only 11 of these, and 6 out of a further 14 caused direct sporozoite agglutination. Although not all monoclonal antibodies showing surface binding by the fluorescence assay agglutinated sporozoites, all but one of the 11 agglutinating antibodies gave positive surface fluorescence, as shown in Table 4.2a, and summarised in Table 4.2b. As judged by results of both tests, two-thirds of the monoclonal antibodies tested showed activity against surface antigens.

In Table 4.3, the cross-reactivity of some of the monoclonal antibodies in relation to their isotypes and surface activity is presented. Surface activity (as assessed by surface fluorescence) and cross-reactivity to E. acervulina sporozoites appear to be mutually exclusive since 8/9 IgGs are surface active and none are cross-reactive; 2 surface active IgMs are species specific while the 2 cross-rective IgMs do not show surface activity; 7 of 8 IgAs are surface active and do not cross-react and 1 IgA without surface activity does cross-react.

FIGURE 4.2

Positive surface fluorescence with agglutination of live sporozoites induced by IgG monoclonal antibody B5G2.

TABLE 4.2a

SURFACE ACTIVITY OF MONOCLONAL ANTIBODIES

a) tested by surface fluorescence and agglutination tests

SURFACE ACTIVE (S+ve/A+ve) JH/Et code no.	SURFACE INACTIVE (S-ve/A-ve) JH/Et code no.
3	5
4	6
11	7
30	8
31	9
32	43
34	44
35	60
39	62
53	

SURFACE ACTIVE (S+ve/A-ve) JH/Et code no.	SURFACE ACTIVE (S-ve/A+ve) JH/Et code no.
12	10
13	
28	
29	
33	
46	
52	
55	
57	

b) tested by agglutination alone

AGGLUTINATION +ve JH/Et code no.	AGGLUTINATION -ve JH/Et code no.
16	14
17	15
20	18
22	19
25	21
26	23
	24
	27

S surface fluorescence
A agglutination

JH/Et No. - external laboratory code (not used in text) given in Table 3.5

TABLE 4.2b

SUMMARY OF SURFACE ACTIVITY OF 30 MONOCLONAL ANTIBODIES AGAINST LIVE SPOROZOITES

Surface active		No surface activity	
S+ve A+ve	10/30	S-ve A-ve	10/30
S+ve A-ve	9/30		
S-ve A+ve	1/30		
Total	20/30	Total	10/30

S: surface fluorescence
A: agglutination

TABLE 4.3

SURFACE FLUORESCENCE AND SPECIES CROSS-REACTIVITY
of anti-*E.tenella* sporozoite monoclonal antibodies

monoclonal antibody	cross-reactive to *E.acervulina*	surface fluorescence	isotype
B5G2	-	+	G
(F8)G12	-	+	G
(F8)E9	-	+	G
(F8)F11	-	+	G
E4E3	-	+	A
B7C10	-	+	A
D4E8	-	+	A
D7C4	-	+/-	A
B4A6	-	+	A
F6D4	-	+	A
D10B4	-	+	A
(F14a)D4	-	+	G
(F14a)D8	-	+	G
(F14a)B10	-	+	G
(F14b)D9	-	+	G
(F14b)E6	-	+	M
(F14b)E2	-	+	M
(F14b)D4	-	-	G*
G12B7	+	-	M
F11B4E12	+	-	A
(F8)E6	+	-	M

* - surface fluorescence and cross-reactivity to *E.acervulina* are mutually exclusive except for this monoclonal antibody, negative in both tests.

Antigen analysis by SDS-PAGE

The sporozoite polypeptides separated by SDS-PAGE and stained with Coomassie blue are shown under reducing and non-reducing conditions, the latter, with and without boiling (Figure 4.3). The different preparations did produce differing patterns and boiling in the presence of the reducing agent dithiothreitol apparently increased the solubilisation of the sporozoites. However, in all cases many discrete polypeptide bands were separated with molecular weights ranging from <14kD - >200kD.

Of the eight IgA monoclonal antibodies tested by Western blotting, only one (F11B4E12) showed binding to electroblotted antigens, the proteins recognised having approximate molecular weights of 76kD, 61kD and 47kD (Figure 4.4). A poorly stained band at 49kD and two stronger bands at the top of the gel were also present, to a lesser extent, in the control track in which an IgA monoclonal antibody raised to beta-spectrin was applied.

The remaining seven surface reactive IgA monoclonal antibodies were tested against sporozoite antigens prepared with and without reducing agent, and in the latter case, also without boiling. On no occasion could specific binding be detected. Similarly, the surface active IgG monoclonal antibody B5G2 did not show a consistent specificity, and no surface antigens were detected by SDS-PAGE and Western blotting.

FIGURE 4.3

SDS-PAGE of *Eimeria tenella* sporozoites prepared under differing conditions

Track a) cold solubilisation; non-reduced
b) boiled without reducing agent
c) boiled with reducing agent

Relative positions of molecular weight markers indicated:(kD).

FIGURE 4.3

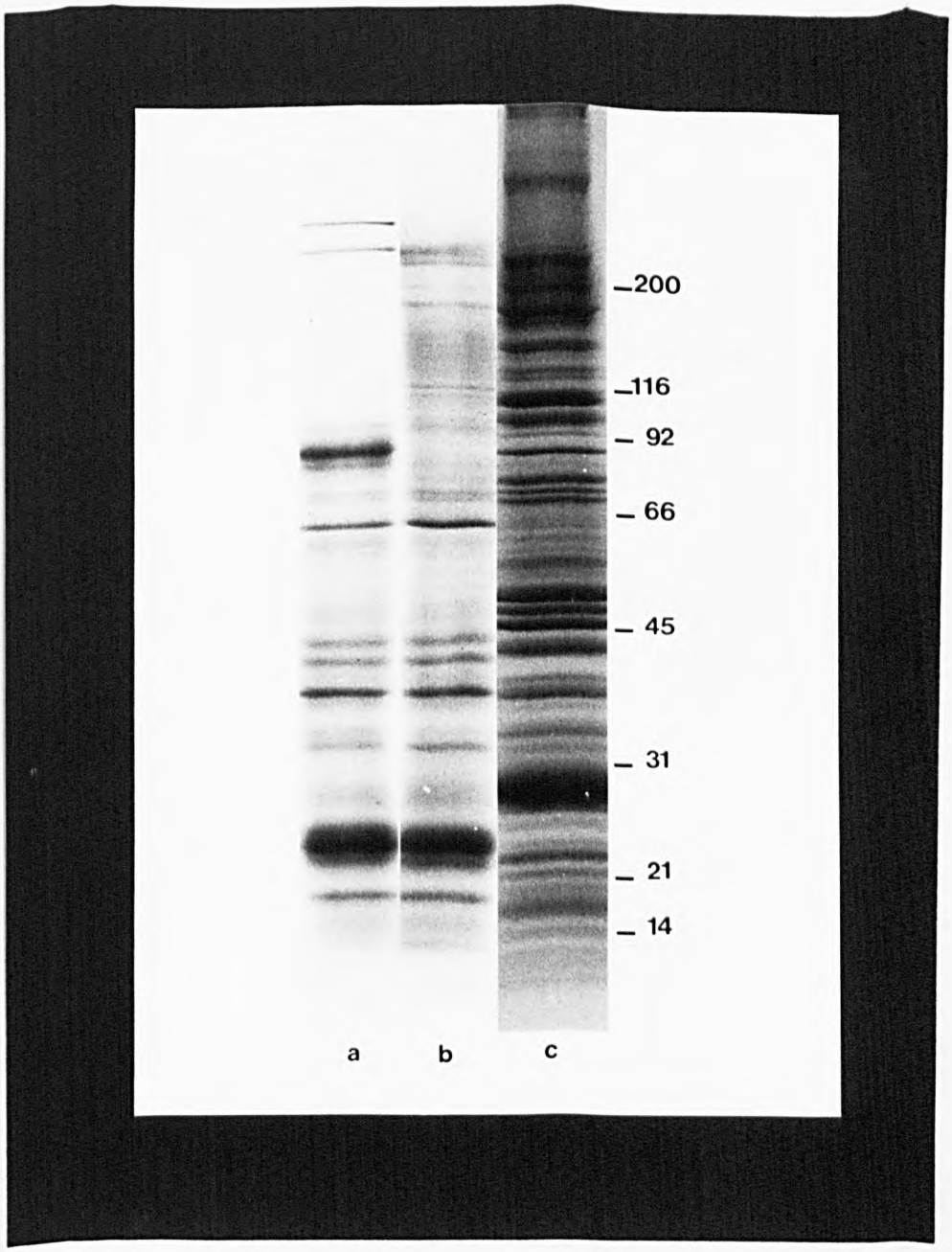


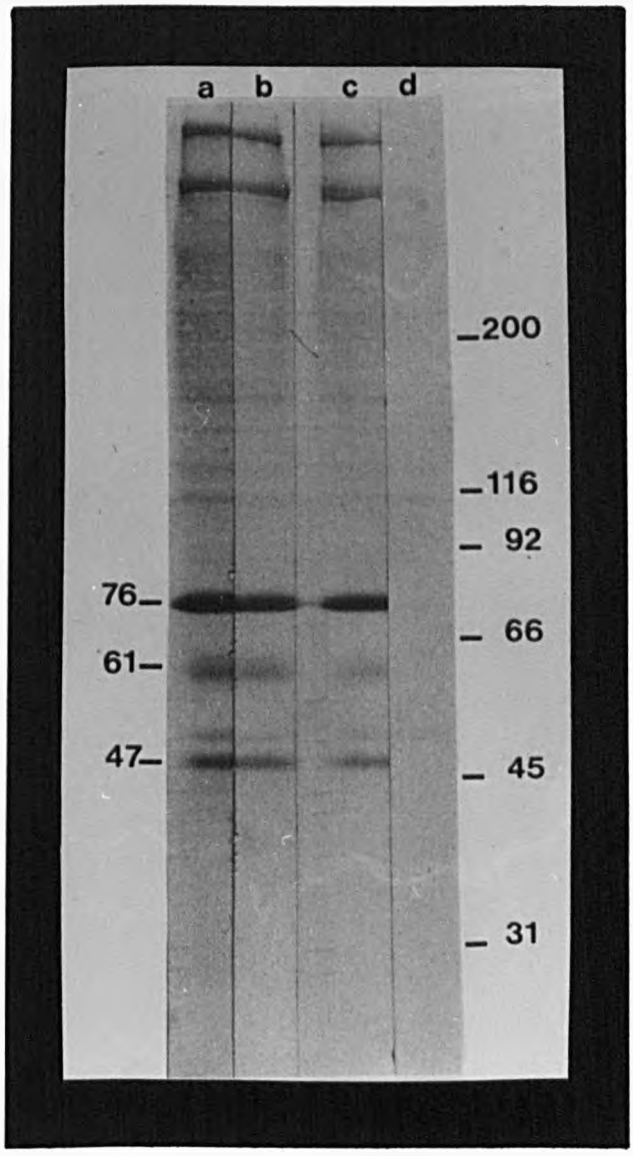
FIGURE 4.4

Specificity of IgA monoclonal antibody F11B4E12 on Western blotting of sporozoite antigens of *Eimeria tenella* separated by SDS-PAGE, under reducing conditions.

Track
a) F11B4E12 ascites (1/10); 2nd antibody (1/200)
b) F11B4E12 ascites (1/50); 2nd antibody (1/400)
c) F11B4E12 ascites (1/50); 2nd antibody (1/400)
d) Control IgA ascites (1/50); 2nd antibody (1/400)

Relative positions of molecular weight marker proteins indicated (kD).

FIGURE 4.4



Surface Activity - Transmission Electron Microscopy (TEM).

Three surface active monoclonal antibodies were examined by TEM with a non-surface active IgA control and also PBS and OPI-HT controls.

B5G2 IgG: at low power the sporozoites showed a greater packing density when compared with controls. At higher magnifications the following features specific to this antibody treatment, not observed in controls, were observed and are indicated in Figures 4.5a-4.5c:

extensive areas of membrane apposition between adjacent sporozoites, with the double membranes of each sporozoite intact and forming four parallel 'tram-lines' with electron dense material lying between the sporozoites, and also electron-dense material covering large areas of the parasite outer membrane giving a fuzzy appearance (Figure 4.5a);

sheets of sloughed membrane showing degrees of detachment but without apparent loss of parasite integrity (Figure 4.5a);

single membrane bound vesicles shedding from both anterior and posterior poles, with electron-dense material both coating and within them (Figures 4.5a and 4.5b);

occasional loss of refractile body, the electron-dense organelle replaced by a lucent misshapen structure (Figure 4.5c).

F6D4 IgA: when examined at lower magnification there was extensive apposition of sporozoites but less overall packing than for B5G2. Specific details noted at higher magnification comprised:

an electron dense layer coating the sporozoites and lying between apposed membranes as described for B5G2 (Figure 4.6a);

in some regions,an apparent loss of the inner double membrane of two adjacent apposed sporozoites (Figure 4.6a);

the membranes were frequently undulating, more similar to controls than B5G2 (Figure 4.6b);

vesicles were rarely seen at sporozoite poles, and were not numerous at sites seen (Figure 4.6b).

E4E3 IgA:there was again multiple and extensive apposition of sporozoites along their membranes. A fuzzy coat and slightly undulating membrane was observed and at areas of apposition the double membranes of participating sporozoites appeared intact. Some discontinuity of the inner membrane was noted occasionally (Figure 4.7a). Vesicle formation was again rare, and those observed seemed more variable in size and shape than for B5G2 (Figure 4.7b).

Controls: the protein rich media containing F11B4E12 or OPI-HT did not produce tight packing of parasites and although some 'welding' of the adjacent membranes was observed this occurred over small areas, did not produce a 'tram-line' effect and the parasite membranes appeared to project towards each other (Figure 4.8a). The external surfaces were free of electron-dense material but the membranes were undulating and difficult to observe in true cross-section (Figure 4.8b). The refractile bodies were uniformly stained and showed no degenerative signs.

Figure 4.5a
TEM of sporozoites treated with monoclonal antibody B5G2 IgG. mag. x 40,000.
Tram line appearence to apposing membranes, a few membranous vesicles, a fuzzy surface coat to sporozoites, and sloughed sheets of membrane (▼).

Figure 4.5b.
TEM of sporozoites treated with monoclonal antibody B5G2 (IgG). mag. x 58,000
Vesicles (v) shed from sporozoite pole.

FIGURE 4.5a

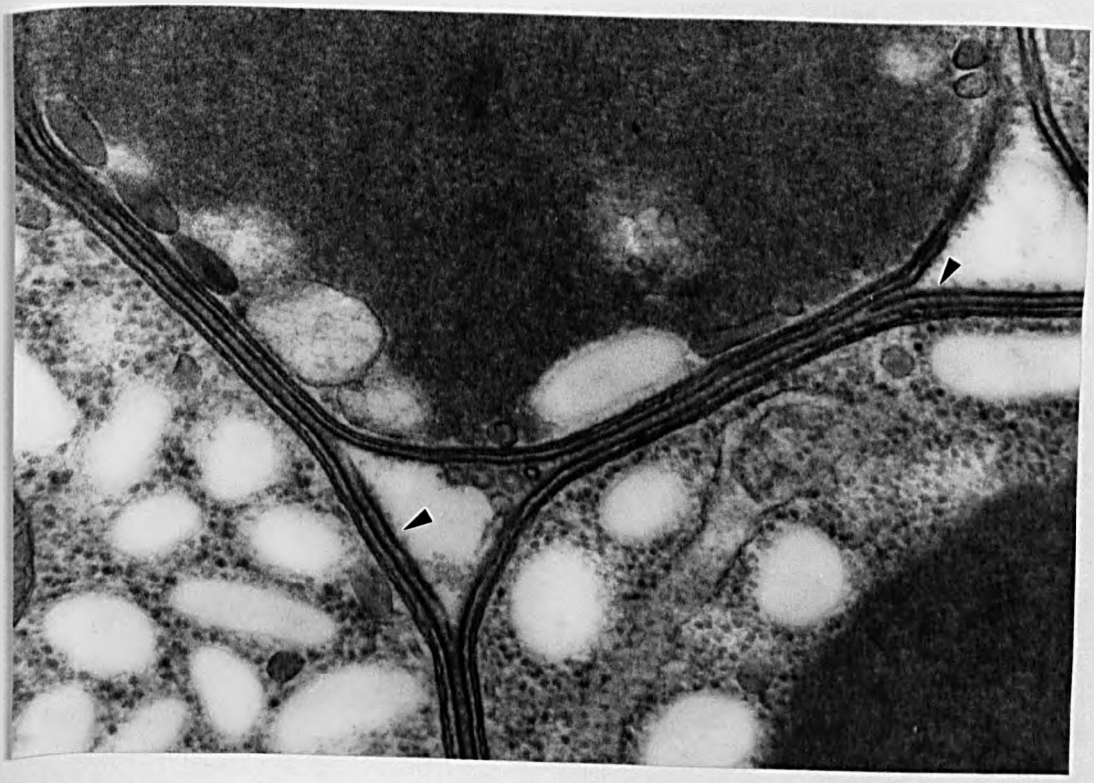


FIGURE 4.5b

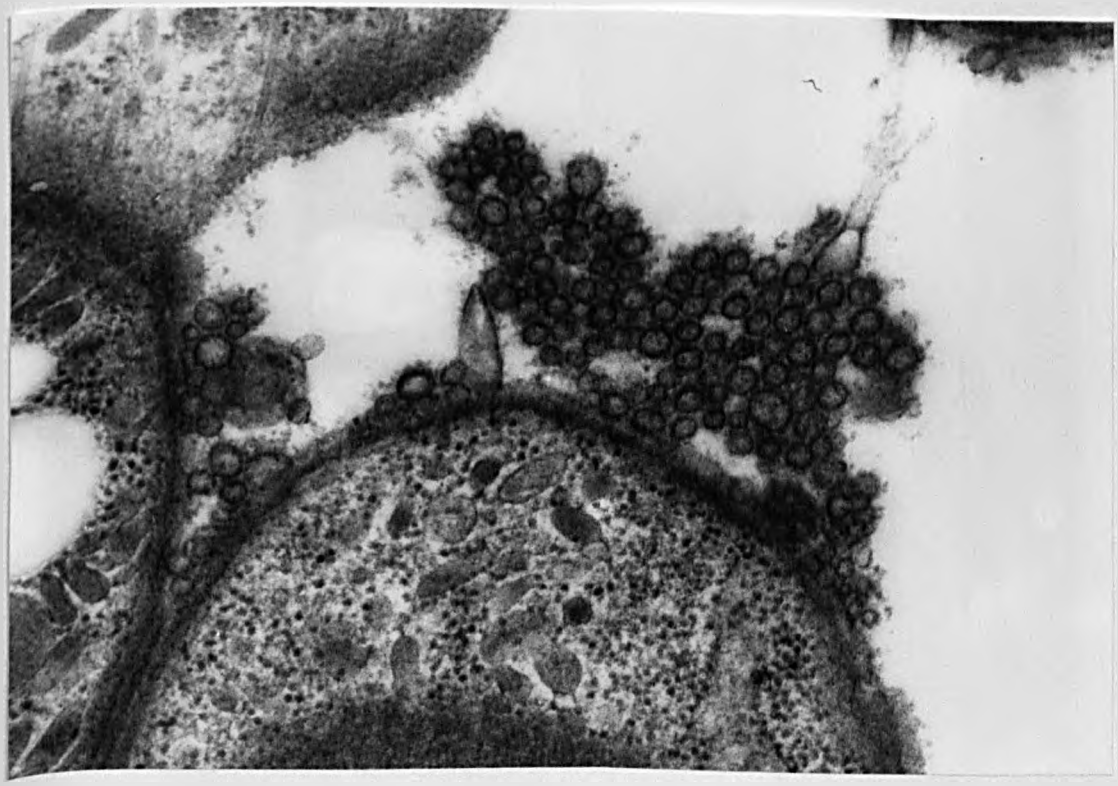


Figure 4.5c
TEM of sporozoites treated with monoclonal antibody B5G2 (IgG). mag. x 14,000
Sporozoite with normal electron dense refractile body (rb), and degenerated, lucent refractile body (drb).

FIGURE 4.5c

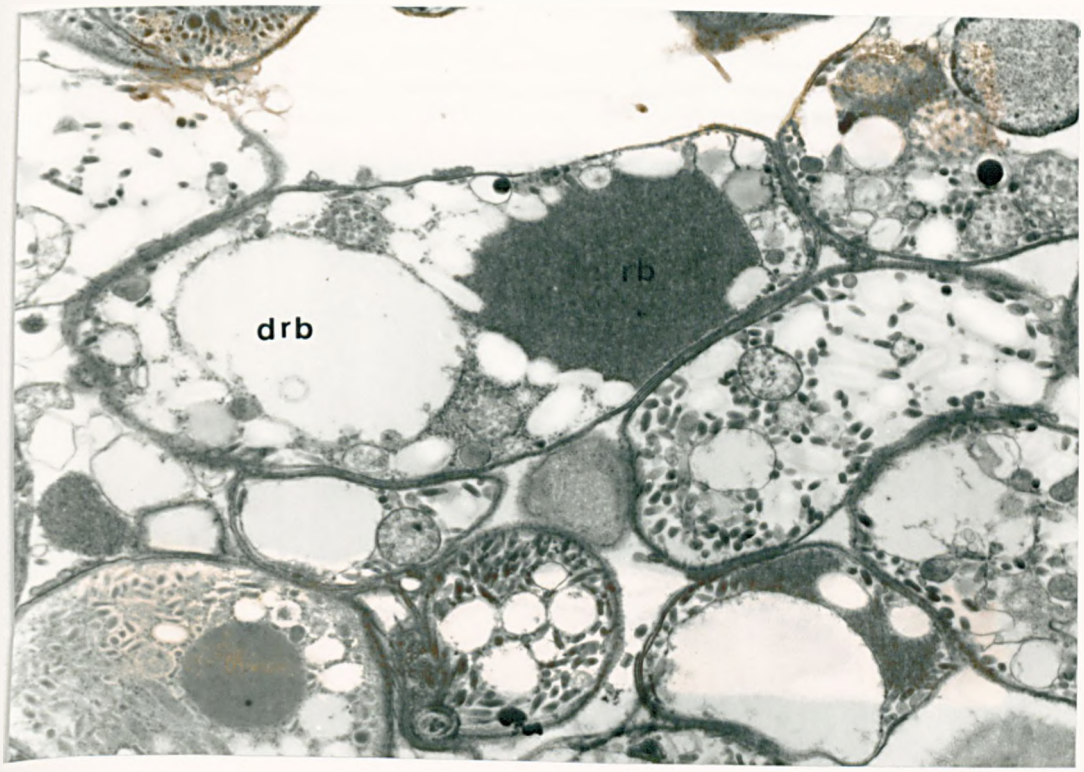


Figure 4.6a
TEM of sporozoites treated with monoclonal antibody F6D4 (IgA). mag. x 135,000
Loss of inner membrane staining in two apposed sporozoites.(▼)

Figure 4.6b
TEM of sporozoites treated with monoclonal antibody F6D4 (IgA). mag. x 135,000
Presence of some vesicles with fuzzy coating, and slightly undulating plasma membranes at apposition site.

FIGURE 4.6a

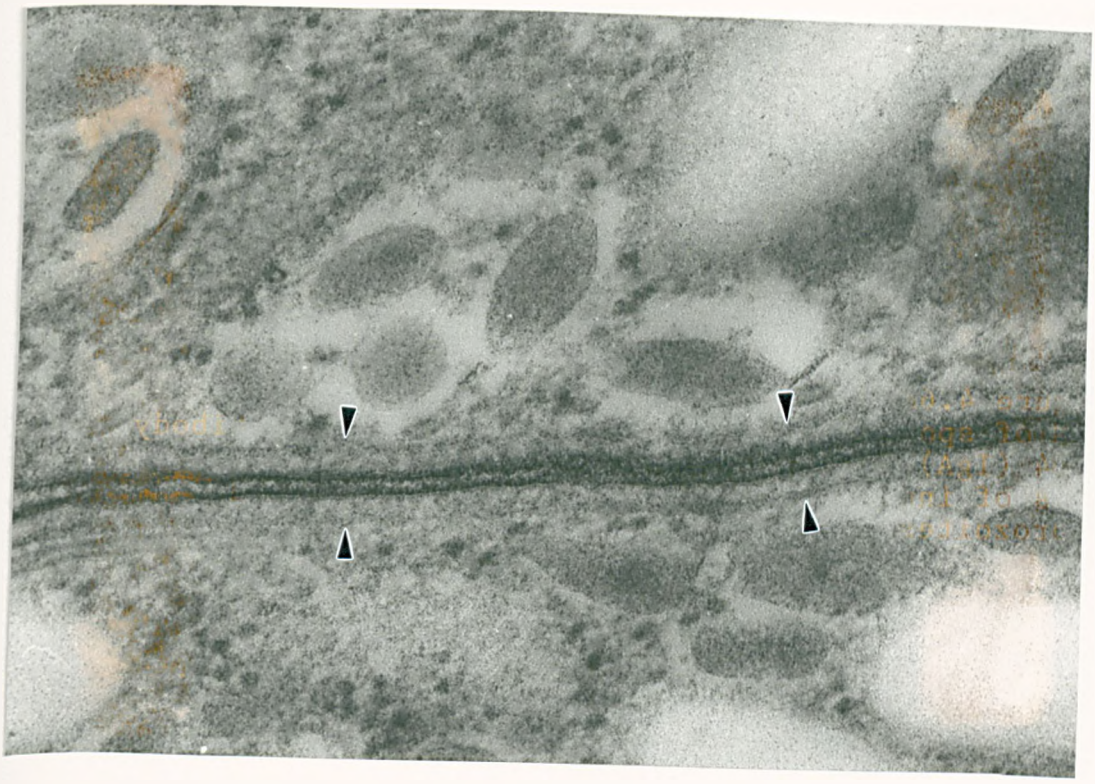


FIGURE 4.6b

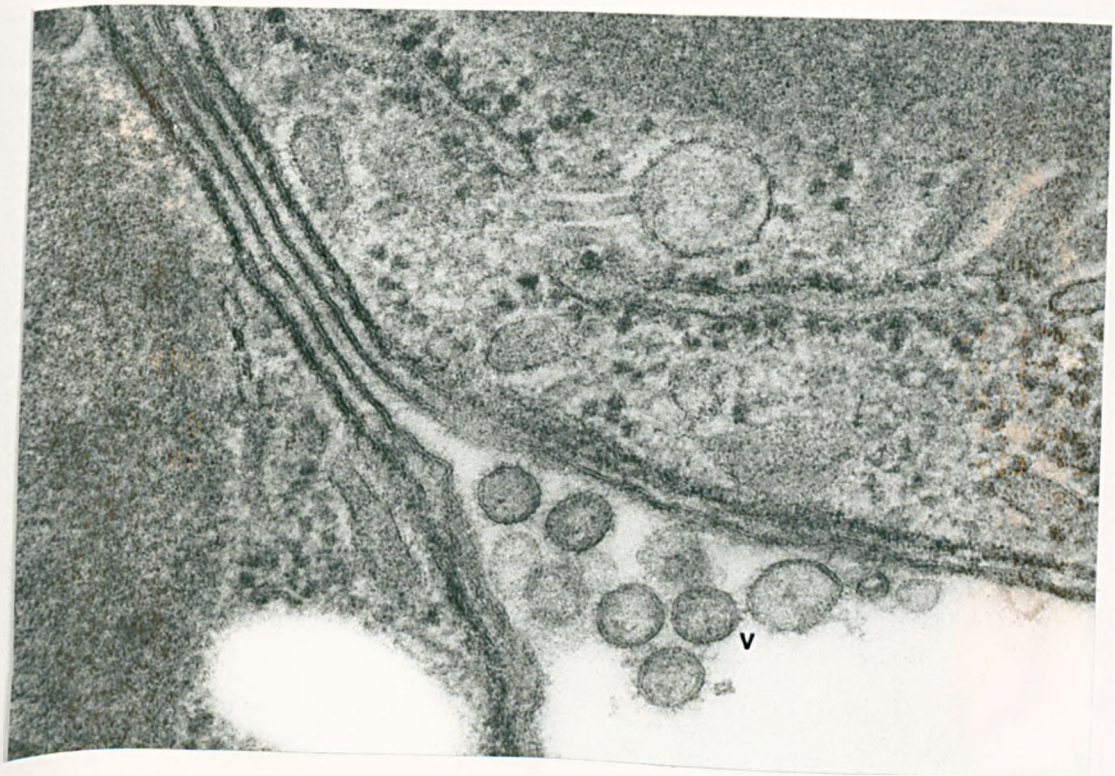


Figure 4.7a
TEM of sporozoites treated with monoclonal antibody E4E3 (IgA). mag. x 195,000
Presence of occasional areas of inner membrane discontinuity (▼).

Figure 4.7b
TEM of sporozoites treated with monoclonal antibody E4E3 (IgA). mag. x 58,000
Vesicles (v) at conoid region (c) of sporozoite.

FIGURE 4.7a

FIGURE 4.7b

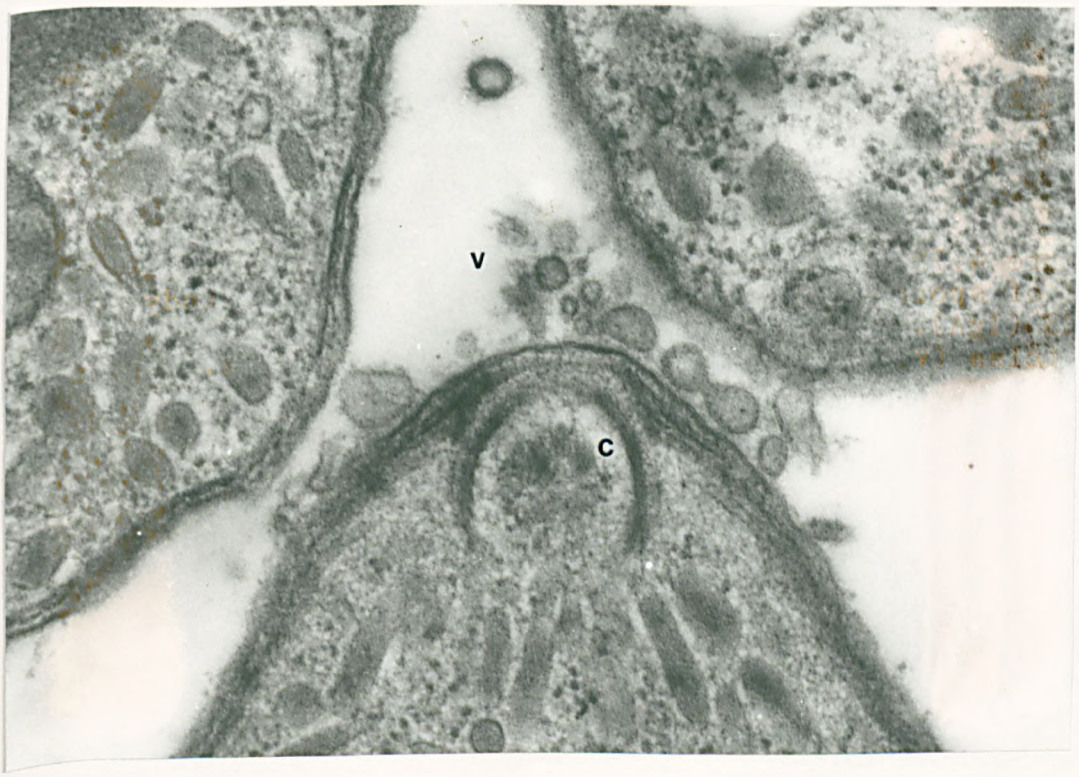


Figure 4.8a
TEM of sporozoites treated with tissue culture medium OPI-HT. mag. x 13,000
Low packing density and membrane welding at apposition sites (▼).

Figure 4.8b
TEM of sporozoites treated with tissue culture medium OPI-HT. mag. x 130,000
Undulating plasma membrane (pm) at apposition of sporozoites, and membrane 'welding'.

FIGURE 4.8a

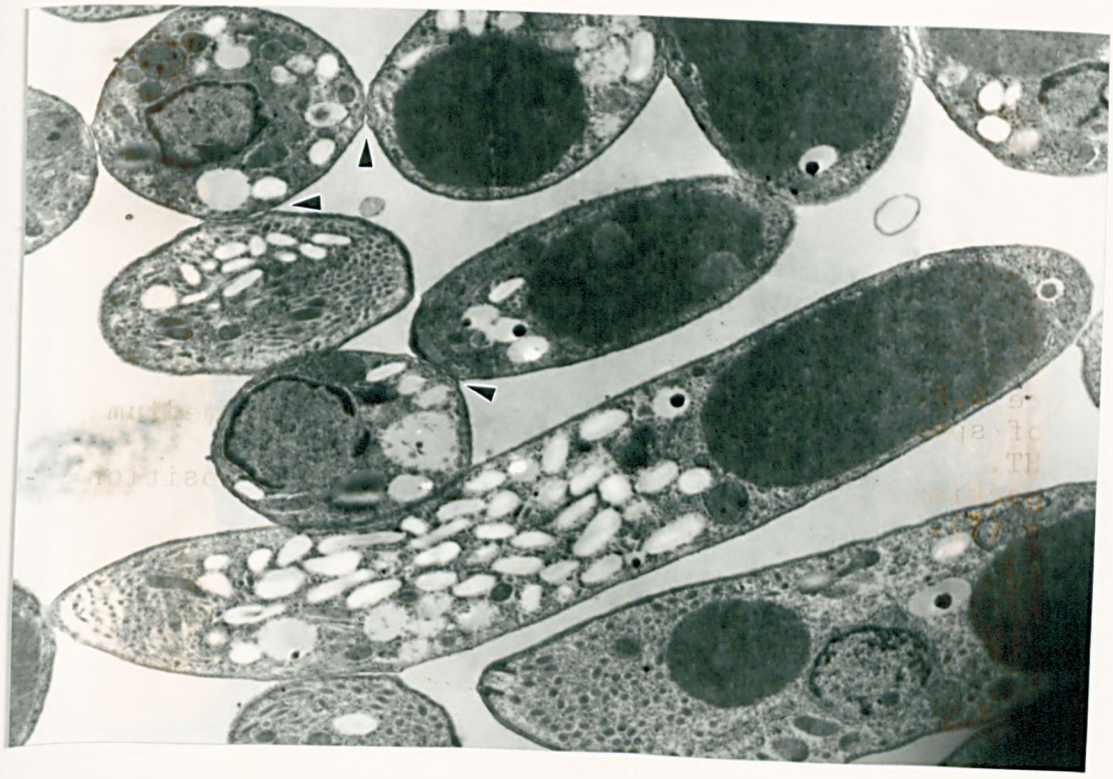
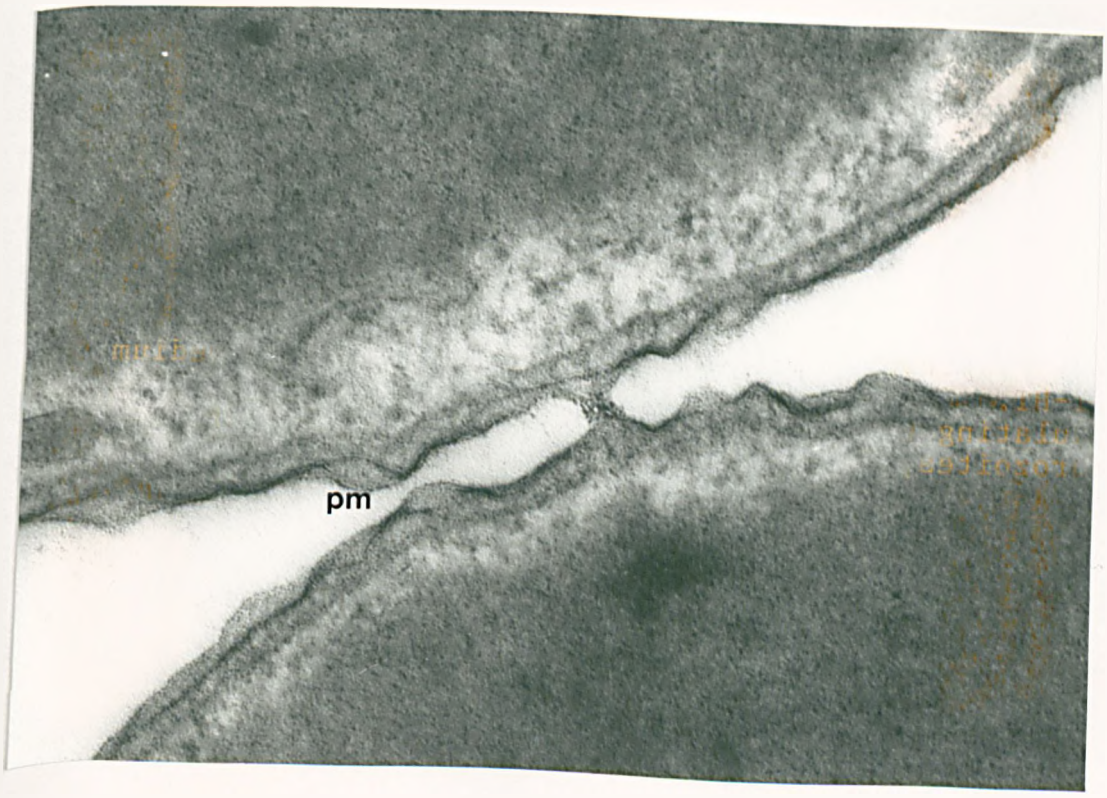


FIGURE 4.8b



Polymerisation of IgA antibodies

F11B4E12 IgA: results of blotting and radiolabelling (Figures 4.9a and 4.9b) demonstrate the presence of three heavy polypeptides, with relative molecular mass greater than the heaviest marker protein (200kD). By extrapolation from a standard curve, the MW of the lightest of the three may be estimated at approximately 230kD. This would be the approximate size of dimeric mouse IgA in which the light chains had detached. The heavier bands are presumably higher polymers of IgA.

E4E3 IgA: although there was some evidence of heavy bands in the overgrown supernatant used for blotting studies the radiolabelling demonstrates the presence of a prominent band of 170kD, which on reduction resolves to give bands of 55kD and (not shown) 25kD, (Figure 4.10). These bands represent intact monomeric IgA and its constituent heavy and light chains.

B5G2 IgG: the monomeric IgG is shown non-reduced, and reduced in Figure 4.11.

FIGURE 4.9a

IgA monoclonal antibody F11B4E12 examined by SDS-PAGE/Western blotting, under non-reducing conditions
Track a) F11B4E12 supernatant
b) OPI-HT tissue culture medium

FIGURE 4.9b

Biosynthetically radiolabelled IgA monoclonal antibody F11B4E12 examined by SDS-PAGE/autoradiography.

Labelling performed for 1, 2 and 4 hours.
Supernatants examined under reducing (A) and non-reducing (B) conditions.

Relative positions of molecular weight markers indicated: (kD).

FIGURE 4.9a

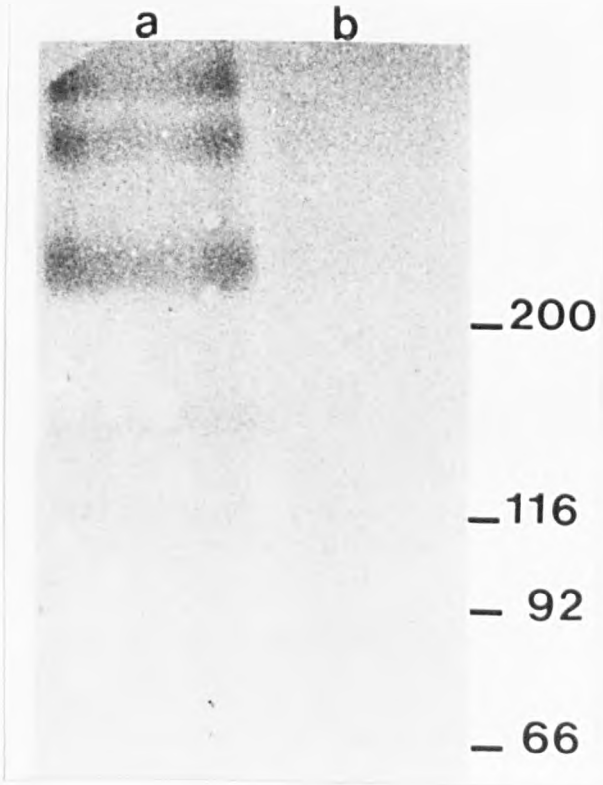


FIGURE 4.9b

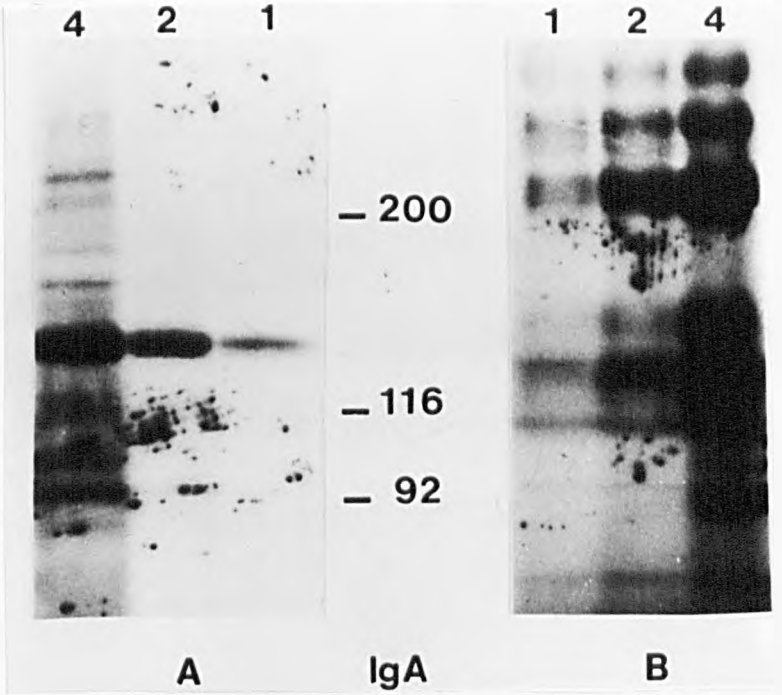


FIGURE 4.10

Biosynthetically radiolabelled IgA monoclonal antibody E4E3 examined by SDS-PAGE/autoradiography.

i) non-reduced
ii) reduced

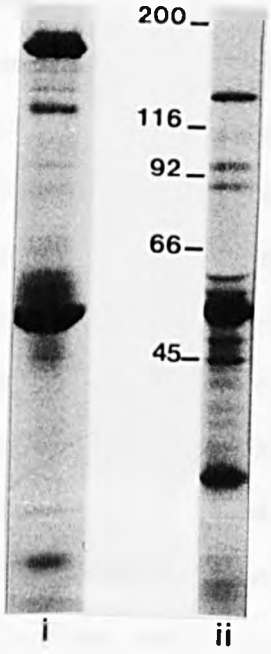
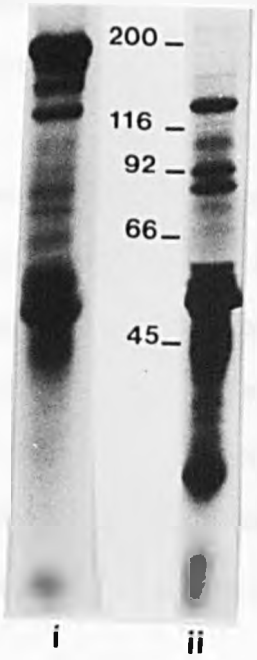


FIGURE 4.11

Biosynthetically radiolabelled IgG monoclonal antibody B5G2 examined by SDS-PAGE/autoradiography.

i) non-reduced
ii) reduced



DISCUSSION

The immunofluorescent antibody test proved a useful and relatively rapid screening test for specific anti-sporozoite monoclonal antibodies. It was the method of choice in most other similar studies although Crane and co-workers (1988) employed a solid phase radio-immuno-assay, but to no immediate advantage. The TLIFA does have the necessary rapidity and convenience for screening large numbers of culture supernatants, but it has the possible disadvantage that some potentially useful antibodies may not be detected; there may be some alteration in the antigen structures from the immunising form (live sporozoites) to the fixed antigen (air-dried, acetone treated sporozoites) used in the detection system and failure to detect antibodies raised to native antigens. Chemical treatments have been shown by other workers to alter and in some cases abolish antibody binding to sporozoites e.g. gluteraldehyde fixation (Augustine and Danforth, 1987b) and ethanol or methanol fixation (Augustine et al, 1988). However, there was no alteration in TLIFA results with or without acetone treatment in the present study, and two-thirds of the monoclonals identified by TLIFA also recognised antigens on live sporozoites.

Although several patterns of fluorescence were observed similar to those previously described (Danforth, 1982a), only 'tip' and an 'all-over' fluorescence (denoted as 'surface' by Danforth) were seen uniformly in samples. The 'internal' and 'semi-polar' (denoted as 'anterior 1/2' or '1/3' by Danforth) patterns seemed to be variable in appearance, according to the orientation of the fixed sporozoite on the slide. That it may be un-wise to over-interpret the different

fluorescence patterns produced on fixed antigen slides was shown by Danforth's own work, in which an antibody displaying an 'internal anterior 1/2' binding pattern, (Danforth, 1982a), was later shown to give uniform surface binding on TEM, (Danforth, 1983c; 1986a) and to agglutinate sporozoites and inhibit cell penetration in vitro (Augustine and Danforth, 1985; 1987a). Consequently, in this study IFA patterns were described as 'polar', 'all-over' and 'internal', but these patterns were not used to make predictions as to which monoclonal antibodies might be protective. Clearly, from this and previous studies, an apparent 'internal' TLIFA binding pattern does not exclude surface activity as shared antigenic sites may occur between internal and membrane antigens as demonstrated by Speer and co-workers (1983a). Also, it is possible that membrane fluorescence may not be discerned in the presence of bright cytoplasmic fluorescence. In order to firmly identify surface active monoclonal antibodies with potentially protective effects, further in vitro tests were necessary.

Agglutination and surface fluorescence together identified 20 of 30 antibodies tested as surface active. Surface fluorescence identified all but one, while agglutination tests failed to identify nine. Surface fluorescence was the more satisfactory test of surface activity and by conducting the test at 4°C, in the presence of azide, capping and shedding of surface antigens was limited. Despite this precaution, during observation of the parasites the cell suspension inevitably warmed and capping/patching of fluorescent material was frequently observed and in some cases fluorescent debris was shed from the sporozoites as has been described for other eimerian

sporozoites (Chbouki and Dubremetz, 1981). This could account for a single negative surface fluorescence result for an agglutinating antibody. However,it has been noted previously that monoclonal antibodies directed to surface antigens may not produce agglutination, with only one of five tested producing agglutination in the study of Augustine and Danforth (1987a). For successful agglutination of sporozoites by a surface active monoclonal antibody, the relative amounts of sporozoite, sporozoite antigen and antibody must be appropriate, since the interaction is initially dependent on random encounter of antigen and antibody. An excess antibody level could theoretically block cross-linking, in a prozone-type reaction. Dilution of antibody in this study did not promote agglutination so this possibility is not supported by the results. Since the sporozoite cell concentration was standardised and a positive control antibody included in each set of tests, a failure to cross-link the sporozoites more likely represents the distribution and relative density of the surface antigens/epitopes involved. If the antigen(s) is relatively fixed in the membrane and the antigen or epitope infrequently represented, effective cross-linking would not occur. In the surface fluorescence test, agglutinated clumps of sporozoites were seen in most positive samples, including those with non-agglutinating antibodies. The second antibody (fluorescent probe) effectively increased the cross-linkages between sporozoites and promoted agglutination. However, a positive surface fluorescence test was not dependent on the cross-linkaging and it was not surprising that more surface active monoclonal antibodies should be detected in this way.

The isotype of the monoclonal antibodies had no apparent bearing on their ability to agglutinate sporozoites; (monomeric) IgG antibodies performed as well as IgA and IgM antibodies. The one agglutinating, non-surface fluorescent antibody did not give consistent results and was not given detailed attention. Possible explanations for the result might be that the antibody did not bind at the low temperature at which the surface fluorescence test was carried out, or as mentioned earlier, antibody may have been 'capped off' during the test.

In the present study, antibodies positive in the surface fluorescence test did not cross-react with E.acervulina sporozoites in the TLIFA test. Since natural immunity and probably the protective antigens involved are species specific, such surface reactive monoclonal antibodies are of great interest in the study of potentially protective effects and evaluation of the specific antigens recognised. Danforth (1986b) reported 'surface' IFAT cross-reactivity between species, but since inhibition of cell penetration by monoclonal antibodies was species specific (Augustine and Danforth, 1987a) this would apparently endorse the view that surface fluorescence and inhibition of invasion provide better criteria for determining surface activity than apparent surface fluorescence on IFAT/TLIFA, and therefore in identifying potentially protective surface antigens.

Ultra-structural examination of the the interaction of monoclonal antibodies and the membranes of sporozoites was performed by transmission electron-microscopy. It has been stated that surface directed monoclonal antibody can not be visualised by TEM (Speer et

al, 1983a) despite reports of detection in other protozoal species (Cochrane et al, 1976). However, Speer and co-workers (1985b) have since reported the successful visualisation of the same antibody, attributing the previous failure to a low titre antibody source and equipment of poorer resolving power. Danforth (1983c) also demonstrated a 'fuzzy coat', attributing it to the presence of antibody binding alone. Each of the three antibodies tested in this study gave a surface 'fuzzy coat' not seen in the controls, but further apparent effects on the sporozoites varied. The titres of the test supernatants were similar by IFA (1/16-1/64) and the different effects can be attributed to the specificty and/or isotypes of the antibodies involved. Again, although B5G2 and E4E3 produced similar IFA patterns, SF and agglutination (the last test weaker for E4E3) the TEM results were different. Capping and shedding of surface membrane and the bound antibody was a startling feature of the B5G2, with vesicles visible at anterior and posterior poles of sporozoites. In other studies capping and shedding of vesicles was only observed when second antibodies were applied and always to the posterior pole (Speer et al, 1985b), but in this study vesicles seen at the anterior poles (adjacent to the conoid) may have come from the posterior poles of adjacent sporozoites. Capping phenomena have been described in several studies of parasitic protozoa: e.g. Doyle et al, 1974, [Leishmania enriettii]; Kloetzel and Deane, 1977, [Trypanosoma cruzi]; Ferranti and Thong, 1979, [Naegleria fowleri]; Dubremetz et al, 1985, [Toxoplasma gondii]. The following features promoting capping have been noted (King, 1988): a) the presence of repeating epitopes on a single surface antigen to facilitate cross-linkage by

monoclonal antibodies; b) the use of polyclonal antisera to again promote cross-linkage formation; c) the presence of a second cross-linking antibody (as in indirect immunofluorescent/ferritin labelling studies. The circumsporozoite precipitin reaction observed with malaria sporozoites seems to be related, but may occur independently of specific ligand binding (Stewart and Vanderberg, 1988). Capping is energy dependent and inhibited by metabolic inhibitors, and although manifest at the parasite surface is probably directed by intracellular microtubules (Russell and Dubremetz, 1986) or intramembranous/intracellular actin/myosin filaments (King, 1988). The significance of capping and resultant loss of surface antigen/antibody complexes (by exo/endocytosis) on the subsequent infectivity of the zoites apparently varies between different studies, this presumably reflecting the relative importance of the different surface antigens capped to recognition, invasion and subsequent development of the invasive parasitic stage. The B5G2 IgG antibody treatment resulted in the formation of capped vesicles similar to those described by Russell (1983) shed in the wake of sporozoites invading cells. This was apparently due to a single membrane (antigen)-membrane interaction similar to an antigen-antibody reaction. It is therefore conceivable that antibody induced capping and shedding of such a recognition surface antigen could interfere with subsequent invasion. Equally, capping might result in an evasion of the sporozoite from constraining antibody, and the observation that capping occurs with this antibody does not indicate whether it would affect sporozoite infectivity. The B5G2 treatment resulted in both vesicle formation and agglutination in the same

sporozoite, but Speer and co-workers (1985b) stated that agglutination prevented capping and shedding. It is probable that capping and agglutination are related phenomena and either may occur depending on whether cross-linkages are established between different sporozoites or between adjacent binding sites on a single sporozoite. The B5G2 treatment also produced a unique degeneration of one or both refractile bodies in some sporozoites, although such parasites showed no pellicular lesions in the same cross-section. Although this study does not rule out at least functional damage to the membrane, B5G2 has not been shown to promote uptake and staining by trypan blue in the absence of complement.

The two surface active, IgA antibodies E4E3 and F6D4 produced similar TEM results although the different TLIFA patterns suggested that the two antibodies are directed towards different antigenic sites, probably shared between surface and internal sites, with only surface sites accessible for the TEM studies. Ultrastructural examination revealed pellicular lesions similar to those described previously (Speer et al, 1983b), although the complete loss of inner membranes was a unique feature of F6D4 treatment, and in both cases vesicle formation was rare.

The presence of the 'fuzzy coats' confirmed the surface binding of the three antibodies by TEM. The differing results are suggestive that different antigens may be involved in each case, the difference in capping probably reflecting the distribution of the antigenic determinants involved. Different preparative techniques as well as differences in antigen specificity and/or effector mechanisms make comparisons between these results and other published work difficult.

The *in vitro* effects of these antibodies were attributed to direct activity of the antibodies in the absence of complement mediated effects and the potentially protective effects of these antibodies was studied further *in vivo* (see Chapter 5).

SDS-PAGE and Western blotting have been used successfully in the preliminary identification of antigens recognised by monoclonal antibodies in many published reports. Since techniques have been well standardised it is possible to compare results between different groups of workers and identify similar specificities between different monoclonal antibodies from a variety of sources.

The IgA monoclonal antibody F11B4E12 gave a polar tip fluorescence on IFA, was not surface active and showed binding to four major antigens: MW 76kD, 66kD, 47kD and 45kD. A monoclonal antibody designated 'C8' produced by Danforth and McAndrew (1987) appeared to show very similar antigen binding characteristics both on IFA and Western blotting and in species cross-reactivity.

The IgM antibodies showed a broad specificity, binding to multiple bands over the full range of MW. This is a common feature of IgM monoclonal antibodies (Pearson and Clarke, 1983) and the reason for the use of IgG specific screening assays to avoid their detection (as discussed in Chapter 3).

The surface antigens recognised by the seven surface active IgA monoclonal antibodies could not be identified by SDS-PAGE/Western blotting, although the electrophoresis and detection system had been shown to work effectively for internal antigens and the F11B4E12 IgA antibody. Samples prepared under reducing and non-reducing conditions

with and without boiling did show changes in the MW of the polypeptides and bands seen on Coomassie stained gels, but the surface antigens were not identified under any of these conditions. The surface antigen(s) recognised by the IgG monoclonal antibody B5G2 was also not identified. Surface antigens have been recognised by SDS-PAGE/Western blotting by other workers, having relatively low molecular weights of 5-10kD and in the region 17-25kD (e.g. Danforth et al, 1985; Danforth, 1986b; Files et al, 1987; Jenkins and Dame, 1987; Brothers et al, 1988; Jenkins et al, 1989). However, failure to detect antigens with monoclonal antibodies is a common problem (e.g. Augustine, 1985b; Burgess et al, 1988; East et al, 1988), and may be due to destruction of antigenic sites during the preparation of the antigen mixture, the SDS incorporation destroying secondary and tertiary structure of proteins. Although noting such a possible loss of epitope/antigenic site, Tsang and co-workers (1983) state that proteins lose SDS and regain their native configuration on bonding to nitrocellulose paper. However, in a study of monoclonal antibodies raised to several species of Eimeria, one raised to E.tenella and cross-reactive on IFA, failed to detect antigen from any of the cross-reacting species, including E.tenella on Western blotting (Augustine et al, 1988). In studies of nine monoclonal antibodies, there was failure to detect antigens by Western blotting of adult or third stage larval extracts of Nematospiroides dubius (East et al, 1988), and Burgess and co-workers (1988) reported a failure of a surface active monoclonal antibody raised to Sarcocystis cruzi merozoite to detect antigen(s) on blotting, and suggested not only SDS denaturation of epitopes, but also possible loss of antigen due

to heat lability or proteolysis by endogenous enzymes.

In this present study, it is unlikely that all eight surface active monoclonal antibodies investigated are directed against the same epitope or antigen on the basis of their differing IFA patterns, agglutination results, and for three antibodies, their TEM results. Therefore, this apparently represents a failure to detect several antigens/antigenic sites. Since these antigens are presumably situated on or in the membrane, it is most likely that the antigenic sites are highly organised and dependent on tertiary structure which is unlikely to be attained in refolding on nitrocellulose paper rather than within a fluid bilayer. However, identification of potentially protective antigens is of central importance to studies such as these, and alternative approaches to identification of the antigens might include separation of proteins under non-denaturing conditions, the inclusion of enzyme inhibitors in the preparation of the antigen mixture and immunoprecipitation of surface or biosynthetically radiolabelled parasite proteins.

In the study of the IgA monoclonal antibody F11B4E12, biosynthetic radiolabelling/SDS-PAGE and also SDS-PAGE/Western blotting gave similar results, demonstrating polymerisation of the IgA in culture supernatant. Radiolabelling produced many more bands than those confirmed as IgA on Western blotting, presumably as a result of some cell death and liberation of labelled cytoplasmic proteins, particularly as the number and relative density of the bands increased with time. It is has been reported that in Balb-c mouse myeloma IgA, the light (L) chains are non-covalently linked to the

heavy (H) chains (Abel and Grey, 1968; Goding, 1983). Therefore, dimeric IgA (comprising H4L4) would be expected to lose light chains on SDS-PAGE and show a MW of around 230kD (4xH,@55kD). However, the determination of MW of IgA by SDS-PAGE is complicated further: firstly, 10-15% of the mass of IgA may be accounted for by carbohydrate moieties and this effectively reduces the binding of the SDS and reduces the charge/mass ratio normally achieved; secondly, in the absence of reducing agents, protein un-folding is incomplete and SDS binding is diminished. These effects would tend to cause retardation of migration of the protein bands. Determination of MW is not accurate at either end of a standard curve and extensive extrapolation is unacceptable. All of the three heavy bands seen in F11B4E12 fall outside the range of the heavy MW markers and probably represent dimer and higher polymers. With the radiolabelled material, reduction to the heavy and light chain components was demonstrated.

E4E3 and F6D4 showed evidence of heavy bands on SDS-PAGE and Western blotting, but radiolabelling showed secretion of a single band by E4E3 while F6D4 failed to show any specific labelling. The E4E3 label appeared at around 170kD. Clearly, this is less than intact dimeric IgA (280kD) or light chain depleted dimeric IgA (230kD). Intact monomeric IgA would have an approximate MW of 160kD and the apparent MW of 170kD could reflect the retardation in the gel because of decreased SDS incorporation as discussed earlier. The 170kD band was reduced to constituent heavy (55kD) and light (25kD) bands.

The IgG B5G2 was successfully labelled and showed a similar 'monomeric' form comprising heavy and light chains as demonstrated in reducing gels. The relevance of these various structural forms is

discussed further in relation to their potential use in passive transfer studies in chicks, in Chapter 6.

This is the first description of IgA monoclonal antibodies directed against Eimeria tenella sporozoites. These in vitro tests have indicated that one is directed to internal antigens, and seven are directed against surface membrane antigen(s). The surface active IgA monoclonal antibodies were species specific indicating that they may be directed against 'protective' antigens and that they warrant further investigation of potentially protective effects in vivo.

CHAPTER 5

IgA MONOCLONAL ANTIBODIES NEUTRALISE SPOROZOITES IN INFECTIVITY ASSAY *IN VIVO*

CHAPTER 5

IgA MONOCLONAL ANTIBODIES NEUTRALISE SPOROZOITES IN INFECTIVITY ASSAY IN VIVO.

INTRODUCTION

The potentially protective effects of selected monoclonal antibodies were assessed by an in vivo infectivity assay. Three anti-E.tenella sporozoite specific monoclonal antibodies, generated and assessed in vitro as described earlier (Chapters 3 and 4) were selected on the basis of their surface reactivity and isotypes. It has been shown that, in immune birds, sporozoites may succeed in invading the mucosa but their subsequent development may be arrested (reviewed in Chapter 1), and a similar phenomenon has been demonstrated on cell monolayers treated with different monoclonal antibodies in vitro (Danforth, 1982b; 1982c). Clearly, the effects on parasite development must be considered in the evaluation of potentially protective antibodies, and assessment of penetration of monolayers alone, as described in several studies (Danforth, 1982b; 1983b; Augustine and Danforth, 1984a; 1985; Crane et al, 1986a) may not be a sufficient indicator of protective effects. Normal caecal contents can reduce sporozoite development in cell monolayers (Davis and Porter, 1979a; 1979b), and to take advantage of the non-specific effectors which undoubtedly potentiate the effects of local antibodies, assessment of sporozoite infectivity in vivo seemed most appropriate. The in vivo assay used in this study was based on that used previously in this laboratory (Trees, 1987, AFRC Project report), and by others (Crane et al, 1986c; Schenkel et al, 1987a;

1987b), with some modifications. The infectivity of the sporozoites was assessed on the basis of oocyst output by birds infected intra-enterically with sporozoites previously exposed to various treatments *in vitro*. In taking account of possible specific effects on penetration and development and participation of non-specific effector mechanisms at the mucosa, the assay provided a useful and meaningful assessment of potentially protective effects of monoclonal antibodies.

MATERIALS AND METHODS

Chicks: day-old Cobb-1 broiler chicks (Sun Valley Hatchery, Leominster) were reared and maintained coccidia-free until used at 14 days old.

Test materials: aliquots of culture supernatants were stored sterile at -20°C until day of use. TLIFA titres determined on such aliquots were: B5G2 IgG 1/64

F6D4 IgA 1/16

E4E3 IgA 1/16

Supernatants were heat treated (56°C, 30')and their pH adjusted to 7.5 prior to incubations. Similar aliquots were used in TLIFA to determine antibody titre. Similarly treated culture medium (OPI-HT) was used as control, and additionally, an IgG monoclonal antibody raised to Taenia pisiformis (PC-1) was used as control in Expt.1.

Infective dose: excystation and preparation of sporozoites was performed as previously described (Chapter 2, General Materials and Methods). Sporozoites were stored at room temperature for 1hr, then suspended in Eagles Minimum Essential Medium (EMEM), pH7.5, at a concentration of 15 x 10^3/ml. Viability was assessed at the beginning and end of the inoculation period, by trypan blue exclusion. Agglutination tests were performed after the inoculation period, as previously described.

200ul of test medium and 200ul of well mixed sporozoite suspension containing 3 x 10^3 sporozoites were mixed in a small Eppendorf tube and incubated at 41°C for 30'. The dose was then gently mixed and

drawn into a sterile 1ml syringe with a 25g needle. Individual doses were prepared at 2-3 minute intervals, taking one dose of each test supernatant in turn.

Inoculation procedure:

anaesthesia was induced and maintained by halothane in oxygen, delivered by mask. The surgical approach used was as previously described (Burns and Challey, 1959). The bird was positioned in dorsal recumbency, a longitudinal incision made cranial to the cloaca and the distal ileum exteriorised. The infective dose was injected into the distal ileum at the level of the blind extremity of the caeca, the intestines were returned to the abdominal cavity and the wound closed with 1 or 2 surgical clips. The bird was allowed to recover under a heat lamp and then individually housed in a clean room. One bird from each treatment group (omitting sentinels) was inoculated in turn to eliminate possible effects of time on infectivity.

Experimental design. Expt.1. Five test groups of 7 birds per group received sporozoites treated with one of the following: B5G2 (IgG); E4E3 (IgA); F6D4 (IgA); OPI-HT; PC-1 (IgG) and five untreated birds acted as sentinels.

Expt.2. Four test groups of 9 birds per group received sporozoites treated with the same test media, omitting PC-1. Four untreated birds acted as sentinels

The 40 chicks used in each experiment were individually housed in wire mesh cages with individual collection trays beneath each cage. The cages were arranged in four rows of ten with the birds housed in

the same sequence they were operated on, the sentinel birds scattered amongst them.

Faecal material was collected daily from individual birds on days 6-10 inclusive,post inoculation. Faecal material was made up to a standard volume, homogenised in a food processor for 20 seconds and aliquots of the homogenate removed, diluted in saturated salt solution and oocysts counted by flotation in a McMaster counting chamber. In Expt.1, 2 x 1ml aliquots were separately suspended in saturated salt solution and 2 counts per sample made (4 counts per bird per day). In Expt.2, 5 x 1ml aliquots were pooled from each sample and diluted in saturated salt solution and counted as before (2 counts per bird per day).

Statistical analysis: the Mann-Whitney U-test was performed on total oocyst output per bird for each treatment group.

RESULTS

The total oocyst output and the mean oocyst output per bird for the five days (6-10 days post infection) are presented for each treatment group in Table 5.1 (Expt.1) and Table 5.2 (Expt. 2).

The sentinel birds provided no evidence of extraneous infection occurring from the environment.

Untreated sporozoites examined before and after the inoculation procedure were 100% viable by trypan blue exclusion.

The same batches of sporozoites and test media used in the inoculations, tested immediately following the surgical procedures, gave agglutination results consistent with results previously described: B5G2 (IgG) produced marked and rapid agglutination, with no evidence of stumping or lysis of sporozoites; F6D4 (IgA) produced moderate agglutination and E4E3 (IgA) weak agglutination, but again without stumping or lysis.

Statistical analysis of the results of Expt.1.

One bird from each of two groups was destroyed during the experiment (due to respiratory illness and surgical hernia) hence two groups were reduced to 6 birds only.

There was a great variability in results both within groups and between groups. However, the IgA antibody F6D4 gave significant neutralisation as assessed by oocyst output compared with control medium (OPI-HT) ($p < 0.05$), and a just significant result ($p = 0.05$) compared with control antibody PC-1. Differences between the remaining results were not statistically significant.

TABLE 5.1

SPOROZOITE NEUTRALISING EFFECTS OF MONOCLONAL ANTIBODIES ASSESSED BY OOCYST OUTPUT IN VIVO.

Experiment 1.

Treatment / Isotype	No.birds/ group	Total oocyst output/group (millions)	Mean oocyst output/bird (millions)	Standard error +/-	Significance *
B5G2/IgG	6	23.2	3.9	2.4	c
E4E3/IgA	7	5.6	0.8	0.5	bc
F6D4/IgA	7	1.2	0.2	0.1	ab
Control IgG	6	14.8	2.5	1.0	c
OPI-HT	7	18.2	2.6	1.1	c

* - results marked with different letters are significantly different (for levels of significance, see text).

Oocyst counts given rounded up to one decimal place.

TABLE 5.2

SPOROZOITE NEUTRALISING EFFECTS OF MONOCLONAL ANTIBODIES ASSESSED BY OOCYST OUTPUT IN VIVO.

Experiment 2.

Treatment / Isotype	No.birds/ group	Total oocyst output/group (millions)	Mean oocyst output/bird (millions)	Standard error +/-	Significance *
B5G2/IgG	9	96.6	10.7	3.7	a
F6D4/IgA	9	52.5	5.8	1.8	b
E4E3/IgA	9	58.9	6.5	4.2	ab
OPI-HT	9	224.2	24.9	9.4	c

* -results marked with different letters are significantly different (for levels of significance, see text).

Oocyst counts given rounded up to one decimal place.

Statistical analysis of the results from Expt.2 demonstrated a highly significant reduction in oocyst output following treatment of sporozoites with both IgA antibodies compared with controls ($p < 0.001$) and a significant reduction following B5G2 (IgG) treatment ($p < 0.05$) compared to controls. F6D4 (IgA) produced a significant reduction in infectivity compared with B5G2 (IgG) ($p < 0.05$), whereas no significance could be attributed to the difference between E4E3 (IgA) and B5G2 (IgG). Analysis of log transformed data by a 't'-test gave similar levels of significance. The pattern of oocyst production was similar in all birds with patent infection and there was no relationship between infectivity and order of inoculation.

DISCUSSION

Investigation of potentially protective antigens of avian eimerian parasites is being carried out by several laboratories using different experimental approaches. Firstly, by identification and *in vitro* production of purified parasite proteins for use in vaccination trials (e.g. Clarke *et al*, 1987a; 1987b; Brothers *et al*, 1988; Jenkins and Dame, 1987; Jenkins *et al*, 1989), and secondly, by assessment of potentially protective antibodies in infectivity assays and subsequently identifying the antigens recognised and embarking on immunisation trials (Danforth and Augustine, 1986; Crane *et al*, 1988). Evaluation by passive protection studies in living hosts has been used successfully in studies of a variety of other infections (e.g. Boyle *et al*, 1982 [*Plasmodium chabaudi*]; Harrison and Parkhouse, 1986 [*Taenia saginata*]; Mazanec *et al*, 1987 [Sendai virus]; Winger *et al*, 1987 [*Babesia divergens*]; Roach, 1988 [*Trichuris muris*]), and was the approach used in this study.

As mentioned earlier, an *in vivo* infectivity assay was selected to assess the monoclonal antibodies, to take account of possible effects on penetration and subsequent development up to oocyst production in the naturally hostile environment of the chick intestine.

Infectivity of avian *Eimeria* in the natural host may be assessed by measurement of a variety of parameters: weight gain, packed cell volume (PCV), total oocyst output, lesion scores and some combinations of these. Clearly the choice of assay is crucial to such a study, and ideally, antibodies should be assessed against different infective doses, and the appropriate parameters measured in each

case.

Weight gain, PCV and lesion scores may only be used to assess protection against high infective doses of E.tenella causing severe mucosal damage. Lesion scoring has the added disadvantage that it can only be performed on any chick at a fixed time-point post infection and requires some subjectivity. However, it has been used to demonstrate partial protection by monoclonal antibodies (Crane et al, 1986b; 1988; Schenkel et al, 1987a; 1987b).

Oocyst output is not a reliable measure of infectivity at high infective doses, since pathological effects result in mucosal damage, and epithelial sloughing and a failure of some parasites to complete their developmental cycle. The 'crowding' effect first suggested by Tyzzer and co-workers (1932) reflects the reduced availability of cells for parasitism in the face of heavy challenge, and the formation of caecal cores in E.tenella infections may prevent oocyst discharge and reduce apparent oocyst output. Consequently, reduced infectivity may result in elevated oocyst output (Hein, 1968; Joyner, 1969; Norton and Hein, 1976; Norton and Joyner, 1980), or at least an increased reproductive index (Krassner, 1963). However, at low infective doses, oocyst output is considered a valid means of assessing infectivity (Reid, 1975; Crane et al, 1986a; Rose, 1987). From the linear relationship between oocyst output and low sporozoite doses demonstrated by Crane and co-workers (1986a) for E.tenella, a dose of 3 thousand sporozoites was selected for this present study, a level that would have minor effects on weight gain, PCV or lesion scores.

Other factors shown to influence infectivity (age and strain of

chicken, gut fill, nutritional status and diet) (Williams, 1973) could be controlled.

Auto-reinfection could occur in chicks as soon as infections became patent (days 5-6 PI) and therefore oocyst counts were discontinued after day 10 to avoid the effect of a secondary wave of infection.

The inoculation time took 2-3hr and over this period there was no loss of viability of sporozoites stored in EMEM. The three test monoclonal antibodies produced various degrees of agglutination as described previously, but no lysis or stumping was observed, while control media and PBS had no observable effect. Deleterious effects of the supernatants could be attributed to the antibody binding alone and were not due to lysis of sporozoites by antibody and complement prior to inoculation.

The IgA monoclonal antibody F6D4, reduced the infectivity of the sporozoite inoculum to a significant degree in both experiments. The B5G2 (IgG) produced inconsistent results. Since the antigen(s) recognised by the three antibodies have not been identified, it is difficult to ascribe the different effects on infectivity to antigen specificity, isotype or both. There is evidence to suggest the three antigens are different on the basis of differing IFAT results, agglutination and capping, and ultrastructural changes on sporozoites, but definitive proof is lacking. This also makes comparisons with other published results more difficult.

The common features of protective antibodies described by other workers in their *in vitro* assays has been their surface reactivity to sporozoites (Danforth, 1982a;1982b; Crane *et al*, 1986a; 1986c), but not all surface reactive antibodies prevent penetration of cell

monolayers, and further, sporozoite stage-specific antibodies were protective while surface reactive antibodies cross-reactive to merozoites failed to prevent cell penetration (Augustine and Danforth, 1985).

Danforth (1982a; 1982b) demonstrated protective effects of agglutinating and non-agglutinating monoclonal antibodies, but agglutination itself appears to have variable effects. No protective effect of polyclonal IgA agglutinating antibodies in caecal contents was observed on weight gains, (Trees, AFRC 1987 project report). Similarly, a specific mouse antiserum showed no protection at dilutions still capable of inducing agglutination (Crane et al, 1986c), non-specific agglutination by cationised ferritin did not impede penetration (Augustine and Danforth, 1984a), and even enhanced infection rates have been observed in vitro with agglutinated sporozoites invading cultured cells en masse (Crane et al, 1986c).

Monoclonal antibody induced, complement mediated lysis of sporozoites was responsible for the protective effects of an IgG antibody described by Crane and co-workers (1986a; 1986c) but in the absence of complement, no protective effect was obtained. These workers reported a failure to produce any monoclonal antibodies capable of passive protection by any direct effect, although they stated that complement-independent protective antibodies were present in a protective mouse antiserum. Monoclonal antibodies protected by U.S. patents were apparently protective, but experimental details are scant, and it is unclear whether complement mediated effects are involved (Schenkel et al, 1987a; 1987b).

The two IgA monoclonal antibodies described in this study are

protective in the in vivo infectivity assay. They did not induce lysis of the sporozoites prior to inoculation and their protective effects may be ascribed to their direct binding with or without non-specific helper activity at the mucosa. EM results (Chapter 4) demonstrate direct alteration in the sporozoite membrane ultrastructure and in both cases, little evidence of capping and shedding of surface membrane. The isotype of the antibodies may not have had any bearing since the IgA was not in a secretory form, and hence did not have inherent protease resistance which could have enhanced protective effects within the gut milieu. However, this is the first description of anti-Eimeria IgA monoclonal antibodies which neutralise sporozoite infectivity in the absence of complement. It provides some indirect support for the suggestion that non-complement dependent IgA neutralisation of sporozoites can occur in the gut lumen.

The mechanism(s) by which the IgA monoclonal antibodies exert their protective effects is unclear; examination of the mucosa of serially killed birds similarly inoculated with treated sporozoites might reveal if infectivity has been affected at the penetration stage, or whether one or more developmental stage has been affected, or if a combination of effect has taken place.

Monoclonal antibodies are renowned for their 'all or nothing' effects, but in this study only partial protection occurred; apparently sporozoites were not equally affected during the incubation period, or were subsequently released from constraints on their infectivity. This may reflect breakdown of antibody in the gut lumen, antibody capping (as observed, in particular with the IgG

monoclonal antibody, B5G2), or possibly innate variation in sporozoite infectivity, as described by Augustine and Danforth, (1984a).

It is not known whether the antigens recognised are naturally expressed in sporozoites released at the chick intestinal mucosa during natural challenge. This is an important consideration, since in vitro excysted sporozoites have been shown to display a modified array of antigenic determinants compared with sporozoites harvested from the chick intestinal lumen after in vivo excystation (Wisher and Rose, 1987). It is possible that the protective antibodies described would be ineffective against natural challenge, and this would require further investigation; the use of in vivo excysted sporozoites in a similar infectivity assay would clarify this.

CHAPTER 6

PASSIVE TRANSFER OF MOUSE MONOCLONAL ANTIBODIES TO RECIPIENT CHICKS

CHAPTER 6

PASSIVE TRANSFER OF MOUSE IgA MONOCLONAL ANTIBODIES TO RECIPIENT CHICKS

INTRODUCTION

Potentially protective properties of monoclonal antibodies can be demonstrated by infectivity assays both *in vitro* and *in vivo*, as outlined in Chapter 5. However, definitive proof of protective activity against *E.tenella* infection requires that passively administered antibody confers protection on chicks against natural oocyst challenge.

In 1978, Davis and co-workers first indicated a role for secretory antibodies in immunity to *E.tenella* infection, and in 1979, Davis and Porter proposed that the specific secretory IgA may act within the intestinal lumen by preventing or delaying sporozoite invasion of the mucosa and allowing non-specific protease activity to strip surface antigens. Even if sporozoites subsequently penetrate the mucosa they suggested that further development is halted owing to loss of surface (differentiation) antigens as has been described for the neutralisation of sporozoites of other protozoal parasites (Fine *et al*, 1980; Aikawa *et al*, 1981). A similar complement independent blocking or stripping of surface antigens could be envisaged for the IgA monoclonal antibodies raised to *E. tenella* sporozoites which showed anti-parasitic activity in the *in vivo* infectivity assay (Chapter 5). However, to evaluate their possible protective effects against natural challenge *in vivo* it would require the antibodies to bathe the gut mucosa during excystation and mucosal attack by

sporozoites. Also, in order to take full advantage of the isotype, the IgA monoclonal antibodies should be secreted across the epithelial cell layer, acquire secretory component and enter the gut lumen in secretory form, the antibodies thereby acquiring protease resistance and a prolonged effective life in the hostile gut milieu.

Passive transfer of antibodies between animals of the same species (homologous transfer) and between animals of different species (heterologous transfer) has been carried out in a number of different systems.

In passive transfer studies with homologous antibodies in chicks, passive protection was achieved using immune serum (IgG) given subcutaneously and sometimes orally against oocyst challenge with E. maxima (Rose, 1974). Similarly caecal extracts from immune birds given 'parenterally' produced marked depression in oocyst output in challenged chicks when compared with controls (Orlans and Rose, 1972). These authors demonstrated the presence of chick IgA in the donor material but not its specificity, and did not expand on these interesting findings. Conversely, immune chick serum IgA did not confer protection despite reaching detectable levels in recipient chick caecal contents (Trees, AFRC project report), although weight gain determination (a relatively insensitive parameter) was not ideal in assessing passive protection (see Chapter 5). Homologous transfer and protection by IgA antibodies has been performed occasionally with other pathogens: e.g. orally administered colostral IgA produced passive protection of calves against Taenia saginata infection (Lloyd and Soulsby, 1976); intraduodenal injection of intestinal IgA protected mice against Taenia taeniaeformis infection (Lloyd and

Soulsby, 1978); and recently, intra-nasal administration of monoclonal IgA antibody protected mice against respiratory infection with Sendai virus (Mazanec et al, 1987).

Protection studies using heterologous transfer of monoclonal antibodies were successful in cattle given mouse monoclonal IgM intravenously and challenged orally with T.saginata onchospheres (Harrison and Parkhouse, 1986), and most recently, a mouse IgG monoclonal antibody given intraperitoneally protected chicks against E.tenella oral oocyst challenge (Crane et al, 1988). However in both these studies, the antibodies were believed to effect protection via complement mediated lysis of infective forms within the mucosa and did not gain access to the intestinal lumen. Heterologous transfer of mouse monoclonal IgA to the chick intestinal lumen has not been described in the literature but was clearly an important prerequisite in the approach of this study.

Mouse myeloma IgA and other mammalian serum IgA antibodies will bind free secretory component (FSC) from human and bovine sources in vitro, to give a stable complex apparently identical to secretory IgA (Mach, 1970). Mach demonstrated the importance of polymerisation of the IgA, although it was also shown that chemical dimerisation was insufficient, the presence of J-chain and intracellular assembly being critical (Halpern and Koshland, 1970). Further in vitro studies have demonstrated combination of human secretory component and chick serum IgA (Bienenstock et al, 1973), and rat secretory component on cultured hepatocytes and human IgA (Limet et al, 1981). Subsequently, heterologous binding of polymeric IgA to secretory component has been demonstrated in vivo: intravenous administration of polymeric IgA

resulted in the production of a hybrid secretory IgA within the bile, comprising the heterologous (donor) IgA and the recipient secretory component. Thus, rat IgA was secreted by rabbits (Hall et al, 1980; 1981; Orlans et al, 1983); human IgA was secreted by rats and rabbits (Hall et al, 1980; 1981; Peppard et al, 1981; Orlans et al, 1983); and most significantly, human IgA was secreted into the bile of chicks (Rose et al, 1981; Peppard et al, 1983). Successful transfer was again dependent on the IgA being in polymeric form. Some species did not secrete heterologous antibodies (guinea pig and sheep) suggesting that transfer is dependent on the recipient (Orlans et al, 1983), since sheep IgA apparently bound bovine FSC (Mach, 1970).

On the evidence that chick IgA can be transferred from the peritoneal cavity to the gut lumen (Trees, 1987, AFRC project report), this approach was attempted both to verify previous findings using homologous IgA, and to investigate the behaviour of mouse IgA in the same procedure.

Since mouse myeloma IgA will bind FSC (Mach, 1970), and chicks will secrete heterologous IgA into their bile (Rose et al, 1981), attempts to transfer mouse IgA from the serum to the bile of chicks appeared to be well founded and were carried out with five IgA monoclonal antibodies (including potentially protective E4E3 and F6D4) and an IgG monoclonal antibody (B5G2) for comparison.

MATERIALS AND METHODS

Chicks: Hisex chicks were used throughout. Rearing and maintenance in a coccidia free environment was carried out as described in General Materials and Methods.

Antibodies:

Immune chick serum: convalescent serum from chicks recovering from experimental E.tenella infection, stored at -20°C.

Mouse monoclonal antibodies: these were generated and ascites produced as described previously (Chapter 3). The antibodies used were: F11B4E12 (IgA), F6D4 (IgA), E4E3 (IgA), B4A6 (IgA), D10B4 (IgA), and B5G2 (IgG). A pool of ascites for each antibody was filtered (1um, then 0.3um) and frozen in 5ml aliquots for intravenous administration in passive transfer experiments. Radiolabelled supernatants were prepared as described previously (Chapter 4), but in these experiments the free labelled methionine was removed from the donor material by dialysis against PBS overnight.

Injections and samples:

All injections were made with sterile 1ml syringe and 25g needle. Intravenous injections were made via the medial wing vein, injecting towards the heart. Blood samples were collected in heparinised capillary tubes from the contralateral wing vein.

Gut contents were harvested post-mortem, as follows: the intestines were sealed with artery forceps at the anterior rectum and anterior

duodenum, removed from the carcase and placed on a cold glass plate. The duodenum, caeca and remaining intestine were isolated from each other with additional artery forceps and 1ml cold PBS + protease inhibitor (aprotinin) (100KIU/ml) was injected into each compartment. The intestines were massaged gently and the contents expelled into appropriately labelled cold glass universal bottles and kept at 4°C until used. Remaining steps were performed at 4°C, and with minimum delay. Samples were homogenised by vortex mixing, then transferred to polypropylene centrifuge tubes and centrifuged (18,000 rpm, 30',4°C). The supernatant was removed carefully and stored at -20°C until use. Bile was aspirated from the gall bladder immediately post mortem using a 21g needle and 1ml syringe. In collection of all samples, great care was exercised to avoid contamination with blood. If blood contamination was suspected, samples were discarded.

Bile duct cannulation:

this was performed according to the method of Rose and co-workers (1981), on the day prior to attempted transfer. Briefly, the two bile ducts were identified through an incision behind the last rib on the right side. A cannula was inserted towards the liver into each bile duct, through a nick in the duct wall, and tied securely. The free end of each cannula was sutured to the skin and checked for bile flow, which was allowed to drip freely. Large chicks (34d old) were used in these experiments to facilitate cannulation. On recovery from anaesthesia, chicks were individually housed, and the following day 1½ml Eppendorf tubes were attached to

the free end of each cannula and fixed under the wing using a body-sock of tubi-gauze or elastic bandage. Tubes were removed at collection times and replaced with a fresh supply.

TLIFA and SDS-PAGE/autoradiography:

techniques were carried out as previously described for mouse antibodies(Chapters 2 and 4); TLIFA for specific chick antibodies was performed as described by Trees and co-workers (1985).

Single radial immunodiffusion:

this was performed according to standard techniques, (details given in appendix 3) to detect the presence of chick IgG in bile, and mouse IgA in bile and intestinal contents.

Experimental design:

1. Passive transfer of donor antibody by intraperitoneal injection into recipient chicks.

The procedure was carried out according to the successful technique of Trees (1987, AFRC Project Report). In the present study the donor material comprised: a) mouse ascites F11B4E12 (IgA), TLIFA reciprocal titre 10^3.

b) polyclonal immune chick serum (ICS), TLIFA reciprocal titre for IgA:80 (Expt.i); 320 (Expt.ii).

In Expt.i., two groups of six, 19-day old chicks received 1ml donor material by intraperitoneal injection. The birds were killed 6 hours post-injection (p.i.).

In Expt.ii., two groups of six 14-day old chicks received 1½ml donor material by intraperitoneal injection. Birds were killed at 6 and 10 hours p.i. The appropriate TLIFA was used to test for specific antibody in test samples prepared from serum, bile and caecal contents (Expt.i), and additionally, duodenal and intestinal contents (Expt.ii). Samples prepared from uninjected controls were examined similarly.

2. Passive transfer of donor antibody by intravenous injection into recipient chicks.

(i) mouse IgA survival in chick serum.

A dose of 1ml mouse ascites/bird was administered intravenously to 19-day old chicks. Five different antibodies were tested in birds in replicates of four (total, 20 birds). Chick serum titres of donor antibody were determined by TLIFA on blood samples withdrawn within 5'of the injection, and at ½, 1,2,4,7 and 24 hours post-injection. Bile was withdrawn post-mortem and examined neat and diluted 1 in 4 by TLIFA. Samples were examined fresh or after storage at -20°C.

(ii) assessment of heterologous IgA secretion into bile, by bile duct cannulation.

The bile ducts of four 34-day old birds were cannulated and on the following day at least one cannula was patent in each bird.

Intravenous injection of ascites from the standard pool of F11B4E12 was made as described above (2.i). Serum samples were collected immediately after the injection and 1, 2, 4, 7 and 24 hours thereafter, and bile was collected for 1 minute at 15 minutes, and 1, 1½, 2, 4, 7 and 24 hours after the injection. Bile and serum were examined by TLIFA, and bile alone by SRID for chick (serum) IgG and donor mouse IgA content.

(iii) assessment of secretion into bile by serial killing.
Twelve uncannulated 34-day old chicks received 1ml of the F11B4E12 antibody pool by intravenous injection as in Expts.(2.i) and (2.ii). Four birds were killed at ½, 1, and 2 hours post-injection, and serum, bile and duodenal contents were obtained from each bird and examined by TLIFA and SRID. The effect of bile on the apparent sensitivity of the TLIFA was investigated by incubating the donor material in bile or PBS and determining the TLIFA titre thereafter.

(iv) passive transfer of mouse monoclonal IgA and IgG: comparative serum survival profiles.
Three uncannulated 34-day old chicks were injected with F11B4E12, as in Expt.(2.i). A further twelve chicks received 1ml of ascites from a pool of mouse IgG (B5G2), and four birds were killed at ½, 1 and 2 hours post injection, and bile and duodenal contents examined by TLIFA. Serum samples were removed from all chicks remaining alive at the same time points and assayed for the appropriate specific mouse antibody.

(v) assessment of secretion of mouse IgA and IgG from serum to bile by radioactive tracing.

Three IgA monoclonal antibodies (F11B4E12, F6D4, E4E3) and one IgG (B5G2) were radiolabelled as previously described (Chapter 4). Four groups of four 19-day old chicks were intravenously injected with one of the radiolabelled supernatants (1ml/bird), and a further group of four birds received the labelling medium alone, in which no cells had been grown. Bile was collected post-mortem, at 2 hours post injection.

Bile and donor material was examined by SDS-PAGE under reducing and non-reducing conditions, and autoradiographs prepared as described previously (Chapter 4).

RESULTS

1.Passive transfer of donor antibody by intraperitoneal injection into recipient chicks.

There was no detectable transfer of chick polyclonal serum IgA or mouse monoclonal IgA from the the peritoneal cavity to the different gut compartments or to the bile or serum of recipient chicks in either experiment i., or ii. Increasing the actual dose (and relative dose ,since chicks were smaller), and antibody titre of chick IgA in Expt.ii., did not result in detectable transfer of specific IgA of homologous or heterologous origin. No false positive reactions were observed in samples from control birds.

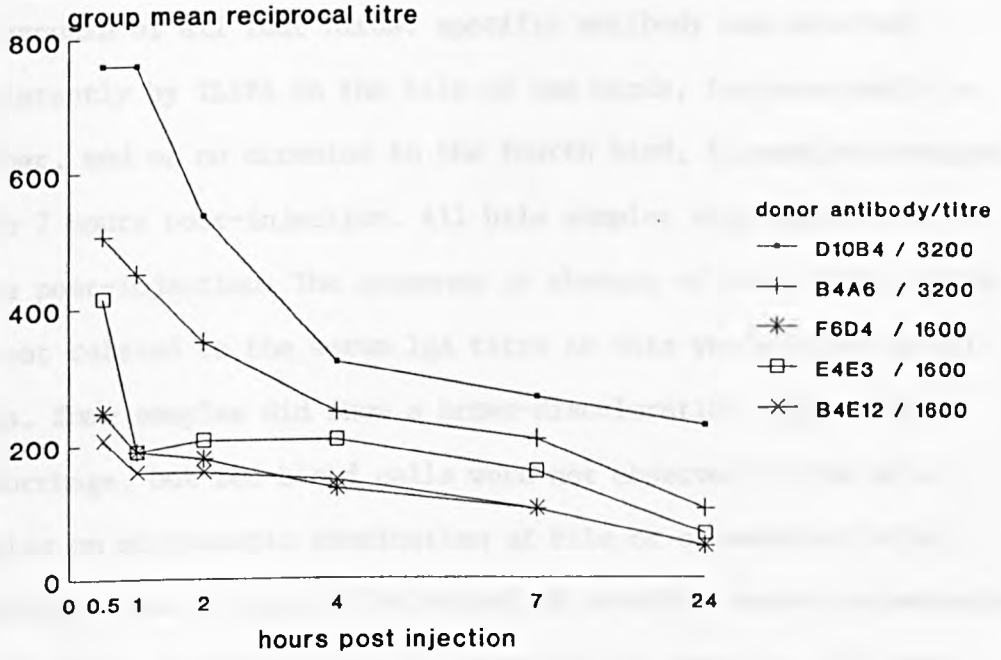
2.Passive transfer of donor antibody by intravenous injection into recipient chicks.

(i) mouse IgA survival in chick serum.

The geometric mean reciprocal titre for specific mouse monoclonal IgA in chick serum up to 24 hours post-injection, is shown in Fig. 6.1. The reciprocal titre of donor material is also indicated. After an initial decline, specific antibody titres remained high and were still readily detected for all samples 24 hours post-injection. Similar patterns of serum decline were observed for all five antibodies. Cystic bile samples of recipient chicks were all negative by TLIFA for specific mouse IgA at 24 hours post-injection.

Figure 6.1

Survival of mouse IgA in chick serum



2.(ii) assessment of heterologous IgA secretion into bile, by bile duct cannulation.

The serum decline profile was similar to that obtained for F11B4E12 in Expt.2(i): an initial decline in titre in three of the four chicks, which continued slowly, specific activity remaining detectable at 24 hours. The cannulation apparently made no difference to the fate of the antibody (Fig.6.2). Bile ran freely from at least one cannula of all four birds: specific antibody was detected consistently by TLIFA in the bile of two birds, inconsistently in another, and on no occasion in the fourth bird, in samples obtained up to 7 hours post-injection. All bile samples were negative at 24 hours post-injection. The presence or absence of mouse IgA in bile was not related to the serum IgA titre as this was similar in all birds. Some samples did show a brown-discoloration suggestive of haemorrhage, but red blood cells were not observed in the bile samples on microscopic examination of bile or of material after centrifugation. To assess the extent of possible serum contamination of the bile, presumably due to trauma by the cannulae, SRID was performed to estimate chick IgG levels in the bile, and to correlate possible leakage of serum IgA into the bile. Normal chick bile was negative for chick IgG or cross-reactivity to mouse IgA in the SRID. Bile collected by cannulation was positive for chick IgG in some samples from all four birds including samples in which mouse IgA was not detected by TLIFA. However, all samples positive by TLIFA and SRID for mouse IgA also contained chick IgG. These results are summarised in Table 6.1.

Figure 6.2

Mouse IgA survival in normal and bile duct cannulated chicks.

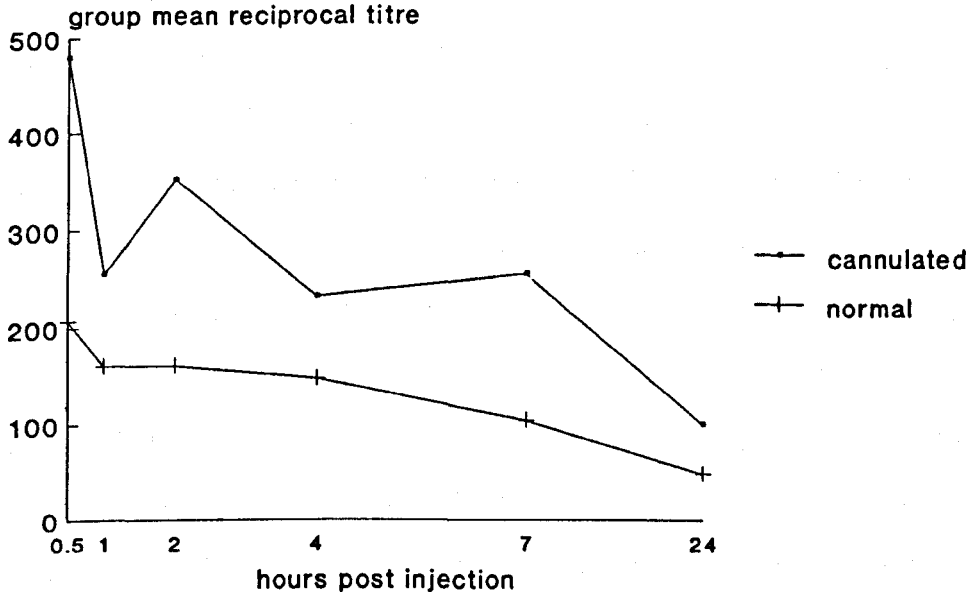


TABLE 6.1

CHICK SERUM IgG AND MOUSE (iv administered) IgA IN BILE COLLECTED BY BILE DUCT CANNULATION

Sample No.	Mouse IgA TLIFA	Mouse IgA SRID	Chick IgG SRID
1.	+	++	++
2.	+	+	+
3.	++	+	+
4.	+	+	++
5.	-	-	+
6.	-	-	-
7.	-	-	+
8.	-	-	+
9.	-	-	+

2.(iii) assessment of secretion into bile by serial killing.

Bile and duodenal contents were collected post-mortem in serially killed groups of birds, precluding leakage of serum immunoglobulins through a traumatised bile duct wall. Specific mouse IgA was not detected by TLIFA in any sample of duodenal contents; in SRID, the control and test samples caused shrinkage and distortion of the agar wells and no precipitin rings were observed. Bile samples tested neat on TLIFA apparently removed large numbers of the sporozoites from the antigen slide, but all samples were negative. At dilutions of 1/4, the antigen appeared normal, and again no specific reactions were observed. SRID results for samples at 1 and 2 hours p.i. are shown in Figure 6.3. Mouse IgA was present in bile from all four birds killed at 2 hours post-injection, 2/4 at 1 hour and 1/4 at ½ hour, but was absent from uninjected control birds.

Donor material was incubated in PBS or bile for 30 minutes before two-fold dilutions in PBS were made and the end-point titre determined in the TLIFA. The donor material incubated in PBS had an apparent titre of 1/1600 (and a weak+/- result at 1/3200). Material preincubated in normal chick bile was positive upto 1/800, and was negative at 1/1600, an apparent 2- to 4-fold loss of specific activity compared to the original sample.

2(iv) passive transfer of mouse monoclonal IgA and IgG: comparative serum survival profiles.

Serum profiles (geometric mean reciprocal titres against time) for the two antibodies are presented in Fig.6.4. The titre of the donor

FIGURE 6.3

Secretion of mouse IgA monoclonal antibody F11B4E12 from serum of recipient chicks, detected in the bile by SRID.

1hr - cystic bile samples from four chicks collected 1hr post iv injection with mouse antibody
2/4 positive

2hr - cystic bile samples from four chicks collected 2hr post iv injection with mouse antibody
4/4 positive

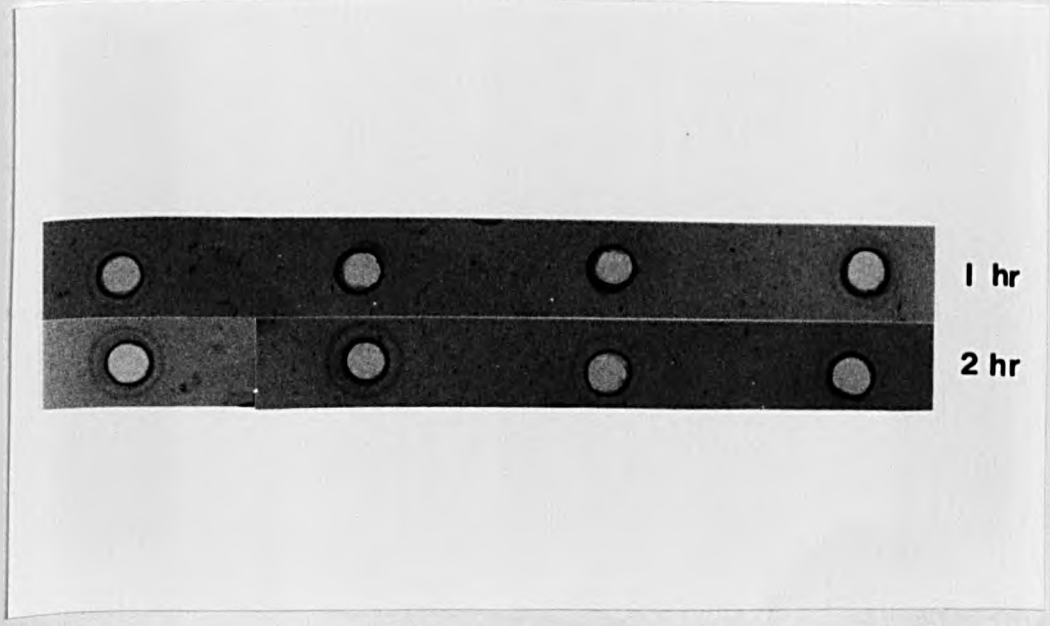
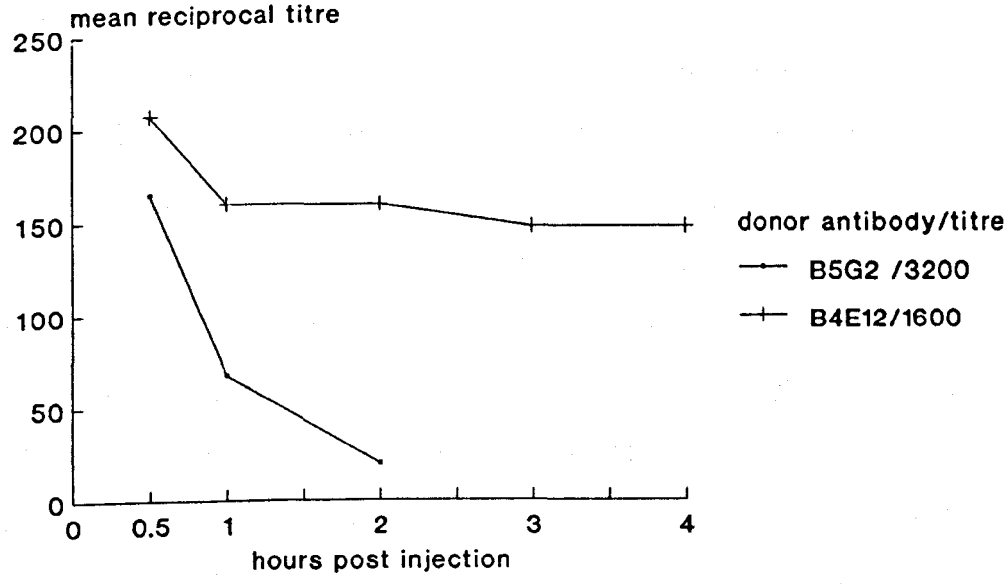


Figure 6.4

Survival of mouse IgG and IgA in chick serum



IgG antibody was twice that of the IgA, but serum specific IgG levels fell rapidly below those for the mouse IgA and were undetectable or base-line at 2 hours post-injection. The typical serum profile for F11B4E12 was obtained, IgA levels remaining high over the test period. Specific IgG was not detected in bile or duodenal contents by TLIFA.

2(v) assessment of secretion of mouse IgA and IgG from serum to bile by radioactive tracing.

Donor material E4E3 and B5G2 were radiolabelled satisfactorily. Poor labelling of F11B4E12 and F6D4 precluded attempts to trace these antibodies. No bile was obtained from one of the chicks receiving E4E3 supernatant. The autoradiographs produced from SDS-PA gels of donor materials and the recipient chicks' bile are reproduced in Figures 6.5 and 6.6. There was no transfer of the IgG B5G2 into the bile. Radiolabelled material was present in all three bile samples of chicks receiving the IgA monoclonal antibody, E4E3. The molecular weight of the major labelled band in donor material and bile was identical, at about 170kD. On reduction this was apparently resolved into two components of 55kD and 25kD in the donor supernatant; only the heavier band was clearly detected in the reduced bile samples. The dialysed labelling medium showed no labelled protein bands, and bile samples from recipient chicks were unlabelled (autoradiographs not shown).

FIGURE 6.5

Passive transfer studies of mouse IgA to chick bile. SDS-PAGE/autoradiography of biosynthetically radiolabelled mouse IgA monoclonal antibody, E4E3.

Tracks a-c: cystic bile from three chicks receiving E4E3 by iv injection. Non-reduced.

Track d: donor supernatant containing radiolabelled E4E3 IgA. Non-reduced.

Track e: donor supernatant containing radiolabelled E4E3 IgA.Reduced.

Track f-h: cystic bile from three chicks receiving E4E3 by iv injection. Reduced.

FIGURE 6.6

Passive transfer studies of mouse IgG to chick bile. SDS-PAGE/autoradiography of biosynthetically radiolabelled mouse IgG monoclonal antibody B5G2.

Tracks a-d: cystic bile from four chicks receiving B5G2 by iv injection. Non-reduced.

Track e: donor supernatant containing radiolabelled B5G2 IgG. Non-reduced.

Track f: donor supernatant containing radiolabelled B5G2. Reduced.

TRacks g-j: cystic bile from four chicks receiving B5G2 by iv injection. Reduced.

FIGURE 6.5
E4E3 IgA

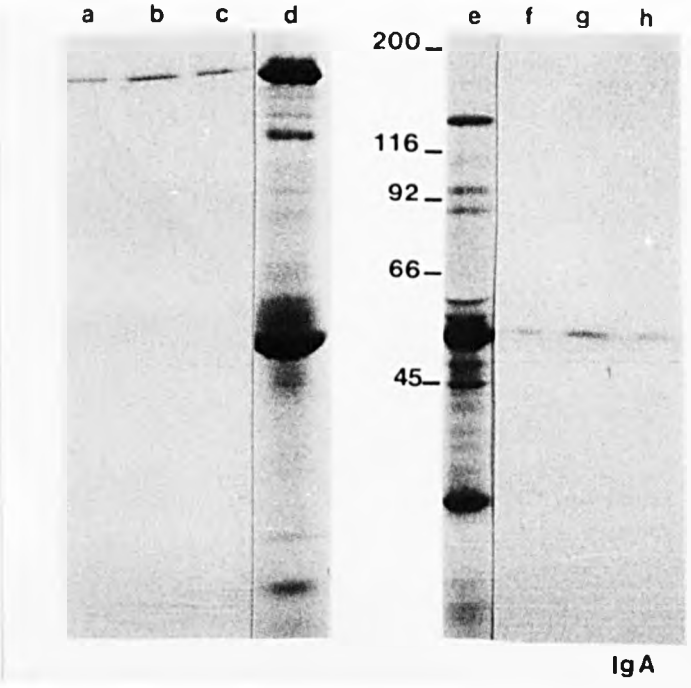
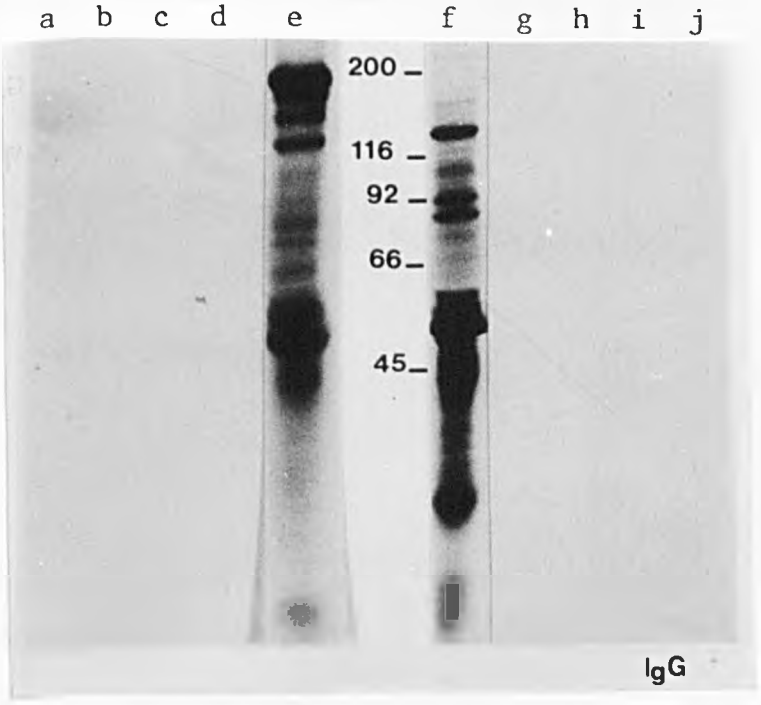


FIGURE 6.6
B5G2 IgG



DISCUSSION

Passive transfer of mouse monoclonal antibodies was investigated to determine whether heterologous transfer of protective immunity might be feasible in this host/parasite system. In order that mouse IgA antibodies could be assessed at the gut mucosa it was necessary that they should retain idiotypic function at that site. Oral administration was not considered, since it was supposed that the antibodies would be destroyed by the detergent activity of bile salts and by proteases of the small intestine. Consequently, passsive transfer of antibodies across the intestinal mucosa was attempted. Passively administered chick serum IgA had been detected in caecal contents of recipient chicks following intraperitoneal injection (Trees, 1987, AFRC project report). However, neither homologous transfer nor heterologous transfer of mouse monoclonal IgA from the peritoneal cavity to the intestinal lumen was achieved in this study. Repetition of the experiment using the same age and strain of chicks as in the previous study, and collaboration with the previously successful operator, even with increased doses of donor antibody, proved equally unsuccessful. The disparity between these passive transfer results is not readily explained. A mechanism for the apparent transfer of homologous chick IgA was not elucidated in the previous studies, but since some IgG was also transferred, a passive 'permeation' of antibody had been assumed (Trees, pers.comm.). However, secretion of polymeric IgA across the mucosa from the extravascular spaces may have occurred (see Orlans et al, 1978), but as the nature of the donor and intra-caecal antibody was not

investigated this is uncertain. Variable results might be obtained if in fact the intra-caecal antibody detected was not in secretory form and was subject to some protease attack. False positive results may have been obtained if the birds were not coccidia-free, if intercurrent infection led to caecal damage and transudation of antibody or if the injections were made intra-enterically. Equally, the negative results obtained in the present study may reflect loss of antigen specificity, either *in vivo* or during sample preparations. The results of homologous transfer remain equivocal, but clearly, high titre polymeric mouse IgA F11B4E12 is not transferred in active form to bile or gut compartments of chicks following intraperitoneal injection.

Heterologous transfer of donor IgA from the serum to bile of recipients has been recorded for a variety of species as mentioned earlier (see Chapter 6, Introduction). However, intravenous administration of specific mouse monoclonal antibodies to chicks and tracing of the donor material has not been described previously.

The decline in mouse IgA levels in recipient chicks was similar for all five IgA monoclonal antibodies, and although there was an initial fall in activity, all the antibodies were still detectable 24 hours after administration. Active secretion of polymeric IgA antibodies has been quantified in some studies as follows: rat IgA in rats:90% in 3 hours (Orlans *et al*, 1978) and 50% in 5 hours (Hall *et al*, 1981); rat IgA in chicks, 50% in 7 hours; rat IgA in rabbits, and human IgA in rats and rabbits, 50% in 5 hours (Hall *et al*, 1981), and human dimeric IgA in chicks, 27% at 2 hours, 43% at 7 hours, compared with 1% monomer at 7 hours (Rose *et al*, 1981). In all cases an

exponential decline in polymeric IgA from the serum was described. However, Balb-c mouse myeloma IgA injected intravenously in the same strain of mice showed a bi-phasic decay curve (Jackson et al, 1977) with an initial decline similar to that described for active secretion, with a secondary prolonged decline with an estimated half life of 2.2 days. This bi-phasic pattern was ascribed to the presence of a mixture of polymers which were actively secreted from serum into the bile and monomeric IgA which was cleared more slowly by an unknown mechanism.

Clearly, the degree of polymerisation of the IgA is crucial to effective binding to secretory component both in homologous and heterologous systems. In studies of naturally occurring IgA myeloma proteins, all of 18 were polymeric (Fahey, 1961), while 4 of 5 IgA monoclonal antibodies were dimeric/polymeric (Komisar et al, 1983), and Colwell and co-workers (1983) described 30 IgA monoclonal antibodies as 'primarily polymeric'. Consequently, heterologous transfer of specific mouse IgA monoclonal antibodies in the chick seemed a feasible proposition. The antibody F11B4E12 was shown to be polymeric by blotting and radiolabelling experiments using spent culture supernatants (Chapter 4). For passive transfer studies, high titre ascites raised from the same cell line was used. Since production of heavy and light chains and assembly and secretion of polymers in conjunction with J-chain is all performed within the same cell, it was assumed that the same degree of polymerisation would be found in the ascites as in the supernatant. Following intravenous administration of the polymeric F11B4E12, the serum decline profile (Figure 6.1) was similar to the bi-phasic curve described above, with

an early initial decline, but with some activity retained to at least 24 hours after injection. The initial decline could have been due to active secretion in to bile and consequently mouse IgA activity in the recipient chick bile was examined by a variety of means.

Cannulation of bile ducts and collection of secreted antibody has been described in rats (Peppard et al, 1981), and chicks (Rose et al, 1981). These workers made no mention of possible contamination of samples with serum, but since specific activity was in fact higher in the bile than in the serum, low level passive exudation would have been masked in the presence of high level transfer. In the present study (Expt. 2.ii), the secretion of polymeric monoclonal IgA antibody, F11B4E12 was examined. The mouse IgA was detected in the bile in 3 of 4 chicks, but at considerably lower activity than in serum samples. However, the presence of chick IgG in the bile of cannulated birds but not unoperated controls is evidence of serum contamination of the bile making interpretation of the results difficult, particularly since all the samples positive for specific mouse IgA were positive for chick IgG. The use of serial killing and collection of cystic bile post mortem precluded serum exudation effects, and allowed assessment of active secretion of mouse IgA F11B4E12. On SRID, mouse IgA was detected in cystic bile from all four birds killed 2 hours post-injection. Surprisingly, none of these samples was positive when the antigen specific assay (TLIFA) was used. It is unlikely that a non-specific IgA is present in the ascites, particularly after two cloning procedures, and endogenous IgA in the ascites would be at trace levels. Therefore the disparity in SRID and TLIFA results suggests that the antigen binding site had

been altered. Incubation of the ascites in bile did reduce specific activity 2-4 fold as determined by TLIFA, but specific IgA had been detected by TLIFA in bile from cannulated birds in the earlier experiment. However, this may represent the higher levels of IgA which may have occurred with accompanying exudation. The apparent loss of specific activity indicates that the transferred antibody is still vulnerable to the effects of bile, or has been damaged by the transfer. The serum decline profile indicated that some antibody was not secreted presumably due to failure to bind to secretory component. This could be due to a J-chain deficiency, as has been described in some naturally occurring polymers (Tomasi et al, 1965), or the presence of incomplete polymers and monomeric IgA ,which would preclude binding to secretory component (Halpern and Koshland, 1970). The serum decline profiles of the four other IgA monoclonal antibodies were similar to that for F11B4E12, while in sharp contrast, the specific activity of the IgG monoclonal antibody, B5G2 was lost rapidly from the circulation. IgG survival in serum is normally prolonged (Orlans et al, 1978; Hall et al, 1981; Harrison and Parkhouse, 1986) and there is no evidence in the literature to suggest that active transport mechanisms occur for this isotype and, in fact, no specific activity was detected in bile or duodenal contents in this study. In order to follow the fate of the antibodies and the nature of any secreted antibodies in bile, radiolabelling of selected antibodies was performed. The two IgA monoclonal antibodies which had shown protective activity in the in vivo infectivity assay F6D4 and E4E3 were chosen; F11B4E12 was selected in order that the apparent secretion of mouse IgA could be studied further, and the IgG

a control. It was unfortunate that only the IgG B5G2 and the IgA E4E3 were successfully radiolabelled for the passive transfer study. The E4E3 IgA was apparently monomeric but also appeared in the bile of recipient chicks in a similar form. The IgG B5G2 did not appear in the bile, confirming the previous result with this isotype. There is evidence in rats that monomeric IgA (unlike IgG) will cross epithelia, although more slowly than polymeric forms (Orlans et al, 1978) which may explain the observed result. Clearly, passive transfer of protective secretory IgA is dependent on the IgA being polymeric and binding to secretory component; this approach would apparently have no application to the monomeric IgA, E4E3. However, further radiolabelling studies are warranted in this area. In view of the specificity of the polymeric IgA F11B4E12 (see Chapter 4)it is not of major importance in protection studies but further studies on its secretion are indicated since demonstration of the binding of polymeric mouse IgA and chick secretory component would validate this approach to passive protection studies. The second potentially protective IgA, F6D4, may be polymeric and secreted similarly, allowing an assessment of passive protection against oral oocyst challenge.

CONCLUDING DISCUSSION

CONCLUDING DISCUSSION

The aims of the project were fulfilled, firstly in the production of specific anti-Eimeria monoclonal antibodies of IgA isotype, and secondly, in the demonstration of sporozoite neutralising activity of two IgA monoclonal antibodies directed against sporozoite surface antigen(s).

An alternative approach to the production of IgA monoclonal antibodies might be to select a hybridoma cell-line secreting antibody of desired specificity (i.e. surface active) and assay extensively for class-switch variants within the cell line, either arising spontaneously or induced by mutagens, as described by Coffino and co-workers (1971), Yelton and Scharff (1982), and Radbruch (1983). A major advantage of generating specific IgA monoclonal antibodies in this way would be that the original parent hybridoma would secrete antibody (e.g. IgG) of identical specificity; variation in anti-parasitic activity between the isotypes could then be attributed to the particular isotype. In the present study, failure to identify the antigens, or define the antigenic determinants recognised by B5G2 (IgG), E4E3 (IgA) and F6D4 (IgA) monoclonal antibodies meant that the different levels of protection afforded could not be attributed to the different isotypes. Even if the same antigen was bound by the different monoclonal antibodies, the precise epitope recognised could also vary, and affect the antibody activity. The main disadvantage in the isolation of class-switch variants is that the reported frequency of IgG switched cells from IgM secreting

cultures is very low, ranging from about 1 in 10^4 (Yelton and Scharff, 1982) to 1 in 10^6 - 1 in 10^7 (Radbruch, 1983). Consequently, extensive screening would be required. Also, none of these reports have described switching as far as IgA production, and such an attempt to produce IgA monoclonal antibodies in this way would be speculative.

The enteric immunisation of germ-free mice in this study gave interesting and rewarding results, although the use of only two mice is of course inadequate to make a full assessment of this approach. Further comparative studies would be required to evaluate this promising technique adequately. The use of oral oocyst immunisation of mice was not pursued on the false assumption that a lack of serum response indicated failure to prime the immune system. Being a relatively simple procedure it might have proved a better, and certainly more convenient route of enteric immunisation than intraenteric immunisation under general anaesthesia. Studies using *in vitro* immunisation techniques and purified antigen would be valuable in assessing the isotype potential of different lymphocyte sources from the same mouse (e.g. spleen, mesenteric lymph node and Peyer's patches) without the complication of individual variation between mice, and different efficiencies of antigen presentation to the cells.

Direct neutralisation of *Eimeria tenella* sporozoites by complement independent mechanisms has not been described previously and the mode of action and the antigen specificities of the antibodies E4E3 and F6D4 are clearly of continuing interest. Identification of the surface antigens was not revealed by standard SDS-PAGE/Western

blotting techniques. Surface radiolabelling and immunoprecipitation is an alternative approach, but might prove equally unsuccessful if the appropriate antigens are not solubilised, or if the specific determinants are readily denatured. Periodate treatment might reveal whether carbohydrate moieties are involved in the antigen binding site.

The mode of action of protective antibodies in neutralisation of sporozoite infectivity could be studied in serially killed birds, with histological examination of the caecal mucosa for developmental stages. An immunofluorescent technique using monoclonal antibody probes as described by Augustine and Danforth (1984b) would provide a means of quantifying the numbers of sporozoites successfully invading, or remaining in arrested form at different time points. Similar techniques could be applied to studies on sporozoite penetration and development using cultured cell monolayers, but extrapolation of such results to the situation in vivo should be made with caution.

In order to assess the neutralising activity of the IgA monoclonal antibodies against natural challenge, it was necessary to transfer the antibodies in secretory form to the chick intestinal lumen, where they could bathe the mucosa during excystation and attack free sporozoites at the earliest opportunity. The antibody E4E3 was monomeric, and although it entered the bile from the blood circulation of recipient chicks, it was not transferred in secretory form. Conversely, the IgA monoclonal antibody F11B4E12 (directed to internal antigens) was polymeric and was secreted from serum to bile of recipient chicks. Further radiolabelling studies are required to

confirm binding of mouse polymeric IgA to chick secretory component and to study the form and fate of the secreted antibody. If successfully secreted in secretory form, it might be possible to fuse F11B4E12 and E4E3 cell-lines to generate polymeric IgA bearing at least some functional (E4E3) antigen binding sites. In this way specific secretory mouse IgA with the specificity of E4E3 could be evaluated. Detailed study of the F6D4 protective IgA monoclonal antibody again requires radiolabelling and tracing studies in recipient chicks. Should this antibody be secreted into the gut lumen of chicks in a secretory form, passive transfer studies against oral oocyst challenge would be indicated. The passive transfer of heterologous IgA antibodies is well documented, but mouse IgA secretion in chicks had not been described previously, and was crucial to an evaluation of potentially protective antibodies in vivo. If chick IgA monoclonal antibodies could be produced, such an evaluation would be more simple. Since no chick myeloma exists to act as a parent cell-line, heterohybridoma production could be carried out as described for the production of rabbit (Raybould and Takahashi, 1988) and human monoclonal antibodies (e.g. Teng et al, 1983) utilising mouse myeloma cell-lines as fusion partners. In a preliminary study (Hoare, unpublished observations) fusing immune chick bursal cells with mouse NS-1 cells, three aminopterin resistant clones were produced, but no chick immunoglobulin secretion was detected. The chromosomal analysis of these cells was indistinguishable from parent myeloma cells (62-64 chromosomes/cell) and the cell-lines may have been aminopterin resistant mutants of the myeloma cells. A further approach in the production of active

antibodies of predominantly chick characteristics is by the production of chaemeric antibodies, where specific variable regions are transferred to the appropriate effector antibody, or just the appropriate antibody binding site is transferred (Riechmann et al, 1988). These would be interesting lines of research in the production and evaluation of potentially protective chick secretory antibodies.

Immunisation of the chick intestinal mucosa is a related area of interest in the development of anti-coccidial vaccines. Potentially protective antigens of Eimeria spp. have been identified by many workers using different experimental approaches, but mucosal immunisation studies are lacking. Delivery of antigen to the mucosae, a study of the relationship between respiratory mucosal immunisation and enteric immunity, and the use of adjuvants which specifically enhance mucosal immunity are clearly of future importance in this field. The commercial incentives associated with the development of a vaccine against the pathogenic Eimeria of poultry have enabled rapid advances towards, firstly, attenuated live vaccines, and recently, towards sub-unit/synthetic peptide vaccines. However, the necessity for experimental secrecy has resulted in a dearth of publications relating to preliminary trials of these vaccines and no doubt is leading to unnecessary repetition of work in different laboratories. It is to be hoped that gaps in our understanding of these parasites and their hosts which require continuing fundamental research will not be overlooked, and that research in these areas will not be under-funded, simply because it offers no immediate financial reward in the form of a commercially viable vaccine.

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APPENDICES

APPENDIX 1

MONOCLONAL ANTIBODY PRODUCTION

MEDIA:

STOCK DMEM (Gibco, Paisley, U.K.)-
prepared according to manufacturers instructions, pH adjusted to 7.3, and stored upto 6 weeks, 4°C.

S.F.M. (serum-free medium):
100ml stock DMEM +
2ml 200mM L-glutamine (Flow Labs.) +
0.2ml gentamicin sulphate, 50mg/ml (Sigma Chemical Co., Poole, Dorset.)

DMEM + FCS:
80ml S.F.M. +
20ml foetal calf serum (FCS) (Gibco, Paisley, U.K.)

OPI-HT:
80ml SFM + 20ml FCS, supplemented with (final concentrations)

oxaloacetic acid	(O)	150mg/l
sodium pyruvate	(P)	50mg/l
insulin	(I)	200U/l
hypoxanthine	(H)	13.6mg/l and
thymidine	(T)	3.8mg/l (all from Sigma).

MEDIA continued:

OPI-HAT (selective medium):

100ml OPI-HT supplemented with

aminopterin (A) 0.18mg/l final conc. (Sigma).

'AZA' (myeloma culture medium):

DMEM + FCS + 8-azaguanine 15mg/l final conc. (Sigma).

REAGENTS:

Red cell lytic solution:

0.85% w/v ammonium chloride solution in distilled water, 0.22um filter sterilised.

Fusogen:'50% PEG':

10ml SFM + 10g autoclaved polyethylene glycol, MW:1500 (BDH, Ltd.)

trypan blue: 0.3% trypan blue in saline (Sigma)

PREPARATION OF CELLS

Myeloma cells:

in the week prior to fusions P3-NS1-Ag4-1 (NS-1) cells were maintained at low cell density in stationary flasks ($<10^6$/ml) and 24 hours before use seeded at 2-3 x 10^5/ml to ensure continued rapid growth upto the time of fusion. Cells were cultured continuously in 'AZA' to ensure aminopterin sensitivity.

Lymphocytes:

mice were killed 3 days after booster immunisations by CO_2 asphyxiation. Spleen and MLN were removed aseptically and washed through three changes of SFM, then disrupted into a single cell suspension in DMEM + FCS. This was performed using two sterile hypodermic needles bent to 90° angles, one to fix the tissue and the other to gently tease out the cells. Spleen cells were pelletted (1000g, 5') and red cells lysed by treating the pellet with ammonium chloride solution for 1-2 minutes and then 10ml DMEM + FCS was added. Treatment of MLN cell suspensions was unnecessary. Viable cell counts were performed using trypan blue and counting the unstained cells in an improved Neubauer haemocytometer.

FUSION (after Galfre and Milstein, 1981)

1.Mix lymphocytes and myeloma cells in a 50ml conical centrifuge tube and pellet the cells (1000g, 5'), pouring off the supernatant.

2.Resuspend the cells in SFM to remove traces of FCS, pellet again and pour off supernatant to give a dry pellet.

3.Loosen the cells by gently tapping the base of the centrifuge tube.

4.Add the pre-warmed 50% PEG over 1 minute (0.8ml for splenic fusions, 0.2ml for MLN), stirring continuously with the pipette tip.

5.Continue stirring for 2 minutes.

6.Add 1ml pre-warmed SFM over 1 minute and continue stirring one minute further, and then repeat.

7.Add 7ml pre-warmed SFM over 2 minutes.

8.Make up volume to 25 ml to dilute out the PEG and harvest the cells by centrifugation.

9.Resuspend cells in 30ml OPI-HAT and plate out into 96 well plates, 0.1ml per well. For MLN, aspirate medium from feeder layers and plate out as for spleen on to the feeder layers in 96 well plates.

10. Place cultures in humid incubator at 37°C, 8% CO_2 in air.

11.Feed with one drop of OPI-HAT 24 hours later and leave undisturbed for 6 days.

12.On day 6, pull and feed all cultures with OPI-HAT

13.On day 12, examine wells for hybridoma growth, and commence screening assay, as appropriate. Continue feeding with OPI-HT.

PRODUCTION OF FEEDER LAYERS.

1.Kill the mouse, pin it out on a dissecting board and swab the abdomen with 70% alcohol.

2.Carefully dissect away the skin to expose the body wall, and swab again.

3.Inject 5ml cold DMEM + FCS into the peritoneal cavity and massage the abdomen.

4.Swab the body wall again and then remove the cell-rich medium, either using a sterile syringe and needle, or sterile glass Pasteur pipette introduced via a small stab incision in to the body cavity.

5.Count the cells obtained, and plate out in DMEM+FCS, at 0.5×10^6 viable cells /ml and place at 37°C, 8%CO_2 in a humidified incubator.

6.Examine 24 hours later for the characteristic flattened appearance of healthy feeder layers.

CLONING, by limiting dilution.

1.Prepare feeder layers 24 hours in advance.

2.Harvest hybridoma cells and determine viable cell concentration.

3.Dilute cells by three ten fold dilutions and express new concentration as n x 100/ml. A volume of 2400/n ul of the suspension contains 240 cells.

4.Mix the 240 cells thoroughly in 4.6ml medium containing 20% FCS, and place 0.1ml on to the feeder layers in the first three rows of the 96 well plate (average about 5 cells per well).

5.To the remaining 1ml add 4ml medium, mix gently but thoroughly and place 0.1ml in each well of the next three rows (average about 1 cell per well).

6.To the remaining 1.4ml add 1.ml medium and plate out in to the remaining wells (around 0.5 cells per well).

7.Examine from 6 days later for hybridoma growth and note those with single colonies before pulling and feeding cultures.

8.Assay 'single' colonies when medium shows signs of yellowing.

9.Expand positive culture(s).

CRYOPRESERVATION OF CELL LINES

Media: SFM + 20%FCS (Medium 1)

SFM + 20% FCS + 10% DMSO (dimethyl sulphoxide) (Medium 2)

1.Harvest hybridoma or myeloma cells and determine viable cell concentration.

2.Pellet cells by centrifugation (1000g, 5') and resuspend in Medium 1, at 5-10 x 10^6/ml.

3.Add drop-wise an equal volume of Medium 2, stirring gently but continuously with the pipette tip.

4.Transfer cells to appropriately labelled cryopreservation tubes (NUNC cryotubes) and place in insulated box at 4°C for 30 minutes.

5.Transfer tubes in the insulated box to -70°C freezer for at least 12 hours.

6.Transfer vials to liquid nitrogen storage vessel and record positions for future recovery.

RECOVERY OF CELLS from liquid nitrogen

1.Locate appropriate vial and thaw rapidly in 37°C water bath.

2.Carefully transfer the cell suspension to a centrifuge tube and dilute out the cryoprotectant ten-fold by the slow addition of warm DMEM + 20% FCS.

3.Determine the viable cell concentration and plate out at several concentrations from 5 x 10^4/ml - 5 x 10^5/ml, or at lower concentrations on to 24 hour old feeder layers (see above). Additionally or alternatively, cells may be introduced in to mice for ascites production (see below), or diluted for cloning (see above).

PRODUCTION OF ASCITES. Balb-c mice were given an intraperitoneal injection of 0.5ml pristane (2,6,10,14-tetramethyl-pentadecane) (Sigma Chemical Co., Poole, Dorset) at least one week prior to injection of cells. Protective clothing and safety spectacles were used when handling pristane, and polypropylene syringes with non-rubber seals to plungers were used for injections, as the mineral oil adversely affected rubber, allowing leakage from some syringes.

Hybridoma cells were harvested, viability assessed, and 5-10 million cells were suspended in a maximum volume of 0.5ml and then administered to pristane primed mice by intraperitoneal injection.

Ascites was collected from mice when abdominal distension was noted, usually from 10 days after cells were injected. Ascites was collected by paracentesis with a sterile 21g needle, inserted either at the umbilicus or in the inguinal region.

APPENDIX 2

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

BUFFERS:

Separating gel 1.5M Tris, pH 8.8

18.15g Tris, dissolved in minimal double distilled water (ddH_2O), pH adjusted to 8.8 with 5M HCl, then made up to 100ml and final pH readjusted as necessary

Stacking gel/Sample cocktail 0.5M Tris pH 6.8

3.0g Tris, dissolved in minimal ddH_2O, pH adjusted with 5M HCl and made up to 50ml.

Running buffer pH 8.3

12.12g Tris

57.68g glycine

4g lauryl sulphate (SDS), made up to 4 litres with distilled water.

STOCK ACRYLAMIDE (30%):

29.2g acrylamide (electrophoresis grade)

0.8g N'N'-bis acrylamide "

made upto 100ml with ddH_2O, filtered and stored at 4°C in the dark, for up to 1 month.

Sample cocktail

1.88ml Tris pH 6.8

6ml 10% SDS w/v in ddH_2O

3ml glycerol

2.12ml ddH_2O

Reducing mixture

1.39g dithiotreitol

6 ml 0.1% bromophenol blue in ddH_2O

filtered and stored at -20°C, in small aliquots.

Non-reducing mixture

0.1% bromophenol blue in ddH_2O, stored room temperature.

SAMPLE BUFFER

i.reducing: 0.65ml sample cocktail + 0.1ml reducing mixture (final conc. 4% SDS, 0.2M DTT)

ii.non-reducing: 0.65ml sample cocktail + 0.1ml non-reducing mixture.

MARKERS: marker proteins were stored and prepared according to the manufacturers instructions (Biorad). They comprised: myosin (200kD), beta-galactosidase (116.25kD), phosphorylase B (92.5kD), bovine serum albumin (66kD), ovalbumin (45kD), carbonic anhydrase (31kD) and soybean trypsin inhibitor (14.4kD).

GEL RECIPES

	Separating gel		Stacking gel
	5%	15%	3%
Acrylamide stock	2.5ml	7.5ml	1ml
Stacking buffer			1.25ml
Separating buffer	5.6ml	5.6ml	
ddH_2O	6.8ml	0.35ml	7.7ml
glycerol		1.5ml	

Following degassing the following were added, the ammonium persulphate (APS) being prepared freshly on the day of use, mixed thoroughly and gels poured immediately:

10%APS in ddH_2O	50ul	50ul	50ul
10%SDS "	0.15ml	0.15ml	0.1ml
TEMED	8ul	8ul	8ul

COOMASSIE BLUE STAIN: 0.1% w/v coomassie brilliant blue R-250 in methanol/glacial acetic acid/water 45:10:45 by volume

DESTAIN: methanol/glacial acetic acid/water 20:73:7 by volume

WESTERN BLOTTING

SOLUTIONS:

Phosphate buffered saline, pH 7.5-8.0

(PBS 7.5)

8.0g NaCl

0.2g KCl

0.2g KH_2PO_4

1.15g Na_2HPO_4

q.s 1 litre distilled water.

Transfer buffer: 20mM Tris, 100mM glycine in dH_2O

Blocking buffer: PBS 7.5 + 10% horse serum

Washing buffer: PBS 7.5 + 2% horse serum + 0.2% Triton X-100

Tris buffer : 100mM Tris + HCl, pH 7.3

Substrate: 5mg diaminobenzidine (DAB), 20ml Tris buffer pH 7.3,

+ 5ul 30% H_2O_2, added immediately before use

APPENDIX 3

STOCK PBS 7.2-7.3

36.0g NaCl

7.4g Na_2HPO_4

2.15g KH_2PO_4

q.s 1 litre with distilled water

This is diluted 1 in 5 for general use, including suspension of sporozoites, dilution of antisera and agar gels.

AGAR GELS (i)

Ouchterlony double diffusion for antibody isotyping:

1.Precoat glass slide with 0.5% agar dissolved in water and dry.

2.Dissolve agar in boiling water bath to 2% in PBS+0.1% azide.

3.Pour the molten agar on to the precoated glass slide and allow to set, and then cut wells in a six well rosette pattern, with a single larger central well using a gel-cutter attached to a vacuum pump.

4.Place neat class-specific antiserum in centre well (8-10ul) and supernatant in outer wells (5-7ul). Supernatants may be concentrated by dessication or freeze-drying to assist detection.

5.Store in a humid chamber at room temperature up to 3 days, and view slides by trans-illumination.

6.Permanent records can be made by photography or

7.Staining: soak slides in thymol-saline and then water to remove soluble protein. Air dry and stain with Coomassie blue for 15 minutes, and destain until precipitin bands are visible.

AGAR GELS (ii)

Single radial immunodiffusion (SRID):

1.Precoat slides as above

2.Dissolve 2% agar in PBS+0.1% azide, pH 7.3 and maintain at 56°C.

3.Add an equal volume of antiserum (goat anti-chick IgG or goat anti-mouse IgA, Nordic Immunochemicals, diluted 1/50 in PBS + azide) and 2% agar. Mix well, and pour the molten mixture immediately.

4.When set, cut wells and fill with test materials.

5.Continue as '5' above.

APPENDIX 4.

PRODUCT SPECIFICATIONS

ANTISERA:

	Product code	Manufacturer
Goat anti-mouse IgA	GAM/IgA (Fc)/7S	Nordic Immunochemicals,
Goat anti-mouse IgG	GAM/IgG (Fc)/7S	Maidenhead, Berkshire.
Goat anti-mouse IgM	GAM/IgM (Fc)/7S	
Goat anti-mouse Ig (all isotypes)	GAM/Ig	
Rabbit anti-mouse Ig (fluorescent conjugate)	RAM/FITC	
Donkey anti-goat Ig (fluorescent conjugate)	DoAG/FITC	
Rabbit anti-goat Ig (peroxidase linked)	RAG/PER	

MEDIA COMPONENTS:

DMEM	074-2100	Gibco, Paisley, UK.
Foetal calf serum	013-06290	"
Aminopterin	A-1784	Sigma, Poole, Dorset.
8-azaguanine	A-8526	"
Gentamicin sulphate	G-3632	"
Hypoxanthine	H-9373	"
Insulin	I-5500	"
Oxaloacetic acid	O-4126	"
Sodium pyruvate	P-5280	"

PRODUCT SPECIFICATIONS (cont.)

	Product code	Manufacturer
Thymidine	T-1895	Sigma, Poole, Dorset.
L-glutamine	16-801-49	Flow Labs., Glasgow.
MISCELLANEOUS:		
Polyethylene glycol	29575 4U	BDH, Speke, Liverpool.
Trypan blue (sterile)	T-9520	Sigma, Poole, Dorset.