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# A mechanism for secretory IgA-mediated inhibition of the cell penetration and intracellular development of *Eimeria tenella*

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**Summary.** The ability of *Eimeria tenella* sporozoites to develop normally in cultured chick kidney cells was used as an indicator of the anticoccidial effects of sera and extracts of caecal contents or tissue. Pre-treatment of sporozoites with normal serum globulin enhanced the frequency of intracellular development but pre-treatment in balanced salt solution, without protein, damaged sporozoites so that most had lost the ability to differentiate, even when they were able to invade host cells. The same inhibitory effect was seen when sporozoites were incubated in extracts of caecal contents from non-immunized chickens, although parasitic development was unaffected when sporozoites were pre-treated in similar extracts of mucosae. Extracts of immune caecal contents impaired both cell penetration and subsequent development. These results show that sporozoites can lose the ability to differentiate before the ability to penetrate cells and provide evidence of a possible synergism between non-specific factors and secretory antibodies in anticoccidial immunity.

## INTRODUCTION

Current views on the role of the secretory immune system centre on the importance of the phenomenon of antigen exclusion, and a primary function of secretory antibodies is seen as the immobilization of antigen on the mucous surface (Buckley & Dees, 1969; Walker, Isselbacher & Bloch, 1972; Heremans, 1974; Andre, Lambert, Bazin & Heremans, 1974; Bazin, 1976). An important aspect of local defence is that this mechanism provides an opportunity for further enzymic degradation to take place, before antigen can be absorbed (Walker, Wu, Isselbacher & Bloch, 1975). Furthermore, with pathogenic bacteria, agglutination by mucous antibody facilitates dislodgement and clearance by peristalis (Porter, Parry & Allen, 1977).

Although such mechanisms must be of great significance in intestinal parasitic infections, their role in anti-parasitic immunity has not been fully examined. In particular, coccidial parasites provide an interesting challenge to the concept of immune exclusion since their life cycle involves penetration of the mucosa not by macromolecules but by intact sporozoites and merozoites, from the lumen. Thus, the invasive forms of this parasite imitate at the cellular level, the events apparently taking place at the molecular level in antigen uptake. It is plausible that the mechanisms shown to be involved in antigen exclusion are also effective against the sporozoites and merozoites of the intestinal coccidia.

The known behaviour of *Eimeria tenella* (a typical coccidial parasite which infects the caeca of chickens)

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poses an enigma, for it has long been known that upon challenge of a solidly immune host with infective oocysts, large numbers of sporozoites manage to invade mucosal cells but do not develop further (Tyzzer, Theiler & Jones, 1932; Horton-Smith, Long & Pierce, 1963; Leathem & Burns, 1967). Recently the sporozoites of *E. maxima* have been shown to behave in an identical manner (Rose & Hesketh, 1976). Yet this happens in the presence of a well characterized secretory immune system (Lebacq-Verheyden, Vaerman & Heremans, 1972; Orlans & Rose, 1972; Porter & Parry, 1976; Parry & Porter, 1978) which has been shown to respond to coccidial infection (Orlans & Rose, 1972) with the production of antibodies having the capacity to prevent the invasion of host cells by the parasite (Davis, Parry & Porter, 1978).

This paper describes an evaluation of whether or not the mechanism implicated in antigen exclusion is also effective against whole organisms, as exemplified by *E. tenella*. An *in-vitro* system, based upon the cultivation of *E. tenella* in cell culture, made it possible to study the behaviour and development of the parasite in an environment free of the host's own immune response, thus allowing the anti-coccidial effects of serum and secretions to be observed and measured precisely.

## MATERIALS AND METHODS

The main details of the techniques used in this study have been given previously (Davis *et al.*, 1978) and are, therefore, only briefly described here.

### *General procedure*

Three-week-old Cobb broiler chicks were immunized by means of an initial light, oral dose of oocysts followed by a second, larger dose 2 or 3 weeks later. Sera and caecal samples were taken at suitable times and their effects on sporozoite behaviour in primary cultures of chick kidney cells were examined.

### *Sporozoites*

*E. tenella* sporozoites were excysted under aseptic conditions and were then washed free of excystation fluid and debris by differential centrifugation. A sample of each batch was immediately inoculated into cell cultures to establish the frequency of cell penetration and subsequent development in the absence of any pre-treatment. The remainder of the batch were incubated in various test and control samples for 30 min before inoculation, to determine their effects on the sporozoites in terms both of cell penetration and their subsequent development into trophozoites and schizonts.

### *Cell culture*

Primary cell cultures from kidneys of three-week-old chicks were established in Leighton tubes, by means of the procedures described by Doran (1971), 2 or 3 days before the sporozoites were to be inoculated. The culture medium consisted of: Hanks's BSS 70% (v/v); lactalalbumin hydrolysate (0·5% in Hanks's BSS) 25%; foetal calf serum 5% (v/v); penicillin/streptomycin, 10,000 units/ml 1% (v/v).

### *Sporozoite treatment and infection of the cultures*

Prior to inoculation into the cell cultures, aliquots of the sporozoite preparation were incubated at 37° for 30 min in immune or control samples. The samples from immunized birds were as set out in Table 1 and the control samples were from uninfected control birds of similar age. An additional control was provided by incubation of sporozoites in a protein-free balanced salt solution.

After 30 min incubation in these samples, the sporozoites were washed and resuspended in complete cell culture medium in readiness for inoculation into the cell culture tubes. They were left in contact with the cell monolayer for 4 h and then either the cover-slip was removed for histological examination, or the medium was changed and the cultures left undisturbed for a further 4 days, to allow development of the parasite.

### *Enumeration of parasites*

The ability of sporozoites to penetrate host cells was determined by sampling the cultures 4 h after inoculation and then counting the intracellular parasites. The frequency of normal development was determined as the mean number of trophozoites and schizonts per microscope field (magnification × 500) 4 days after the inoculation of sporozoites. These mean values were based on parasite counts in twenty-four such fields from each of four separate cultures per treatment (i.e. a total of 96 counts per sample). The results were subjected to statistical analysis by means of Duncan's multiple range test.

**Table 1.** Immunization procedures used to produce immune sera and secretions

| Sample | 1° Oocyst dose[a] | 2° Oocyst dose[a] | Interval (days) | Adjuvant[b] | Time of sampling |
|---|---|---|---|---|---|
| 2 Serum | 5000 | — | — | — | 14 days post infection |
| 3 Serum | 5000 | 50,000 | 14 | — | 7 days post challenge |
| 9 C.M.EX.* | 10,000 | 100,000 | 21 | — | 7 days post challenge |
| 11 C.C.EX.** | 10,000 | 100,000 | 21 | — | 7 days post challenge |
| 12 C.C.EX. | 10,000 | 100,000 | 21 | FCA | 7 days post challenge |

[a] Oral dose.
[b] Given on day 0.
* C.M.EX, caecal mucosae extract.
** C.C.EX, caecal contents extract.

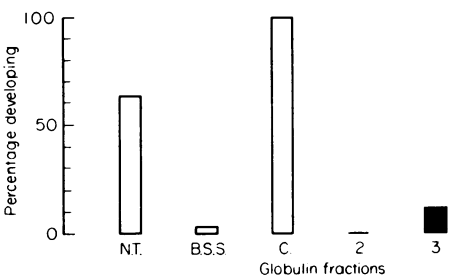
## RESULTS

This experimental design allowed the detection of two distinct types of anticoccidial activity. The first was a typical parasite-neutralizing activity, mediated by antibodies on the surface membranes of the parasite, and was detected as an inhibition of sporozoite invasion into host cells. The second type of activity, which is more difficult to define exactly, is revealed as an impairment in the normal development of sporozoites into schizonts. The sporozoite-neutralizing activities of the samples derived by the immunization procedures shown in Table 1 have been described previously (Davis *et al.*, 1978). The results presented in this text relate to the effect of these samples on the subsequent intracellular, parasitic development.

The results from the cell culture studies on the effects of various pre-incubation treatments on the intracellular development of *E. tenella* are shown in Figs 1 and 2. The ordinate gives the number of developing parasites after 4 days, relative to the maximum control sample. This maximum value is designated as 100% and represents the maximum frequency of parasite development which could be obtained under any of the conditions used.

From the data presented in Fig. 1 it is obvious that the cell penetration and the normal development of sporozoites pre-incubated in normal serum globulins were unimpaired. In fact, this treatment gave rise to a population of sporozoites which exhibited a frequency of successful infection higher, even, than that of the sporozoites inoculated directly into the cultures (N.T.).

The sporozoites maintained at 37° in a protein-free balanced salt solution (BSS) were rather badly damaged; despite a relative frequency of sporozoite penetration of nearly 70%, the numbers of developing parasites 4 days later amounted to only 3% of that found in the normal serum control. The rate of this sporozoite degeneration is clearly influenced by temperature, for Millard & Long (1974) have shown that sporozoites can remain fully infective for more than 5 days at +4° in buffered salt solution. These workers also reported that, ultimately, under these conditions sporozoites could become damaged in such a way that they might readily invade cells but could not develop.




| | N.T. | B.S.S. | C. | 2 | 3 |
|---|---|---|---|---|---|
| Relative frequency of sporozoite penetration | 100 | 69 | 100 | 1·4 | 0·9 |
| Predominant immunoglobulin | none | none | IgG | IgG | IgG |

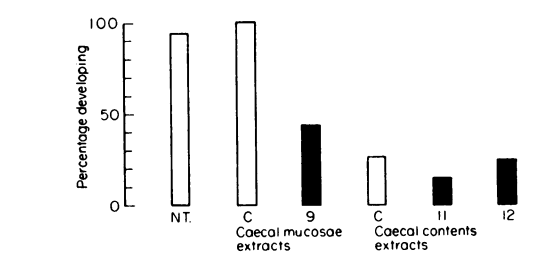
**Figure 1.** The effect of pre-incubation on the intracellular development of *E. tenella* sporozoites *in vitro*; serum globulins and protein-free salt solution. (Open bars indicate non-immune samples, solid bars indicate immune samples). N.T., no treatment. Sporozoites were excysted and immediately inoculated into the cell culture. B.S.S.: sporozoites were incubated in a protein-free, balanced salt solution before inoculation into the cell culture. Globulin fractions: Sporozoites were incubated in globulin fractions of control (C) or immune sera (2 and 3, see Table 1).

Pre-incubation of sporozoites in immune globulin brought about a very effective inhibition of sporozoite penetration and consequently profoundly affected the numbers of developing parasites.

Figure 2 displays the results from sporozoites pre-incubated in extracts of caecal mucosae and caecal contents. The highest frequency of developing parasites was found in cultures infected with sporozoites treated with the extract of caecal mucosae from coccidia-free birds. This correlated with the high frequency of sporozoite penetration. By both of these criteria, the performance of sporozoites pre-incubated in this sample was very similar to the performance of sporozoites inoculated directly into the cultures (N.T.).

The results obtained with the extract of immune caecal mucosae suggest that, in this case, sporozoite neutralizing activity was the only influence on the subsequent development of parasite infection, since the value obtained with sporozoite penetration was close to the value obtained for parasites surviving at 4 days post-infection. Hence, antibody could not be expected to impair parasite development subsequent to penetration of the host cell.

On the other hand, surprising results were obtained with the caecal contents extracts, all of which, regardless of immunoglobulin content and sporozoite neutralizing activity, seriously impaired parasite development. The sporozoites pre-treated in the extract of normal caecal contents exhibited maximal (100%) penetration but, despite this, the relative frequency of subsequent development was only 27%, a value which differed significantly ($P = <0{\cdot}05$, Duncan's multiple range test) from control sample values and was very close to that of the immune caecal contents. The extracts of immune caecal contents had the capacity both to inhibit the invasive ability of sporozoites and to impair their subsequent development.




| | | | | | | |
|---|---|---|---|---|---|---|
| Relative frequency of sporozoite penetration | 94 | 91 | 53 | 100 | 62 | 45 |
| Predominant immunoglobulin | none | IgG | IgG | IgA | IgA | IgA |

**Figure 2.** The effect of pre-incubation on the intracellular development of *E. tenella* sporozoites *in vitro*; extracts of caecal tissue and caecal contents. (Open bars indicate non-immune samples, solid bars indicate immune samples.) N.T., no treatment. Sporozoites were excysted and immediately inoculated into the cell culture. Caecal mucosae and contents extracts: sporozoites were incubated in control (C) or immune extracts (9, 11 and 12, see Table 1) before inoculation into the cell culture. The effect of each caecal contents extract differed significantly ($P = <0{\cdot}05$) from both N.T. and control caecal mucosae extract.

## DISCUSSION

The way in which secretory antibodies exert their protective effect against coccidial parasites is still not completely defined. One can appreciate the simple intervention of antibody on the host's mucous membrane, inhibiting penetration of epithelial cells by the parasite. This particular feature of host defence has been demonstrated in our earlier studies (Davis *et al.*, 1978) but the point of further interest is the fact that parasitic organisms breaching this first line of defence do not subsequently develop and proliferate. In short, the first line of defence is not limited to function outside the host cell.

Under *in vivo* conditions it is certain that the effective antibody is also inside the epithelial cell, because that is its normal route of transport. In the present *in vitro* system, however, antibody in the host cell must be discounted and the effects on parasitic development are attributable entirely to the events prior to penetration.

In recent studies, Walker *et al.* (1975) have demonstrated that secretory antibodies act in concert with proteolytic enzymes from the pancreas to bring about the destruction of protein antigens on the mucosal surface. A similar mechanism may be responsible for the protection afforded to the mucosa of chickens immunized against *E. tenella*. We suggest, by analogy and on the basis of the data presented in this paper, that for *E. tenella* sporozoites to initiate a successful infection they must rapidly leave the lumen and enter host cells (because the normal caecal contents provide a potentially damaging environment). In the immune chicken, the presence of secretory antibodies in the apical mucin impedes their entry into cells and during this delay they are damaged by the lytic components of the caecal fluid. Many subsequently break free from the

antibodies, perhaps by proteolytic cleavage of surface antigens, to enter cells but most are no longer able to develop.

Essential to this argument is the assumption that the removal of surface membrane 'differentiation antigens' would result in an inhibition of differentiation. Such a denuded parasite would no longer be able to receive stimuli from its new micro-environment and would remain static until the eventual shedding of the host enterocyte into the gut lumen. Probably, this inhibition would hold so long as the relevant surface proteins were not replaced, and it is likely that this could not happen unless the sporozoite was subsequently released from that particular cell.

It is already established that, in mammals at least, cell–cell recognition is mediated by cell surface antigens, and that such antigen-mediated recognition is crucial in differentiation and morphogenesis (Boyse & Old, 1969; Bodmer, 1972). These antigens, named 'differentiation antigens', must be recognized by complementary surface protein molecules on the other interacting cell; these have been styled 'recognizers'. Striking similarities exist between this type of system and the system composed of lymphocyte surface membrane antigens and their recognizers, as represented by surface membrane immunoglobulin and the putative T-lymphocyte receptor (Bodmer, 1972; Burnet, 1973; Artze & Bennett, 1975).

Observations in this field provide several interesting analogies to the mechanism which we postulate for sporozoite inhibition in immune hosts. For example, Weksler & Birnbaum (1972) found that after trypsin treatment, cultured lymphoblasts had lost the ability to stimulate autologous lymphocytes in the mixed leucocyte culture, but this ability was restored after a further 48 h in culture. Similarly, histocompatibility antigens can be stripped from the surface membranes of lymphocytes by papain treatment, but are actively replaced within about 6 h (Schwartz & Nathensen, 1971). Again, B-lymphocytes lose their surface immunoglobulin receptors upon trypsinization, but these are then actively replaced over a period of about 6–8 h (Greaves, 1970).

Our basic assumption seems, therefore, to be well founded on principles which, although established in species phylogenetically very remote from *E. tenella*, may well apply generally.

The puzzling behaviour of *E. maxima* and *E. tenella* sporozoites, transferred from immune to non-immune hosts, may well have its explanation in this theory. Predictably, Leathem & Burns (1967) and Rose & Hesketh (1976) found that sporozoites which had invaded epithelial cells in an immune host were unable to differentiate, but they also observed that upon subsequent release from these host cells and transfer to a non-immune recipient, these same sporozoites could initiate a successful infection. It now seems reasonable to suggest that this is because the missing or damaged surface membrane antigens could be renewed during the time between invasion of the original host cell and the invasion of a fresh cell in the non-immune host.

Ironically, it seems that although the loss of essential membrane proteins might allow the sporozoite to evade the immune defences, this same loss might also inflict serious damage from which the intracellular parasite cannot recover. This mechanism, shown diagrammatically in Figure 3, may be equally effective against merozoites.

The results described here give strong support to this theory and conform exactly to its predictions, in that incubation of sporozoites in any sample of caecal contents for 30 min before exposure to host cells caused a highly significant decrease in the frequency of normal, intracellular development. The behaviour of sporozoites pre-incubated in the absence of protein confirms that these parasites are very sensitive to their environment when they are maintained at 37° and are prevented from entering host cells. It also shows that the ability to differentiate into a trophozoite is lost before the ability to penetrate cell membranes.

Thus a non-specific anti-sporozoite effect has been detected which could be an important factor in the mechanism of the secretory IgA-mediated immune response to coccidia. In view of the well established behaviour of sporozoites in an immune host and the demonstrable local immune response, it is likely that anticoccidial immunity in the chicken is another example of synergism between non-specific lytic (or other) agents normally present in the caecal lumen (Lepkovsky, Wagner, Furuta, Ozone & Koike, 1964) and specific secretory antibodies which are locally produced, only in response to infection.

Finally, this mode of action of secretory IgA suggests that this class of antibody has evolved to operate effectively at the mucous surface of the gut by a special development of the ability to survive in a strongly proteolytic environment, whilst the attached

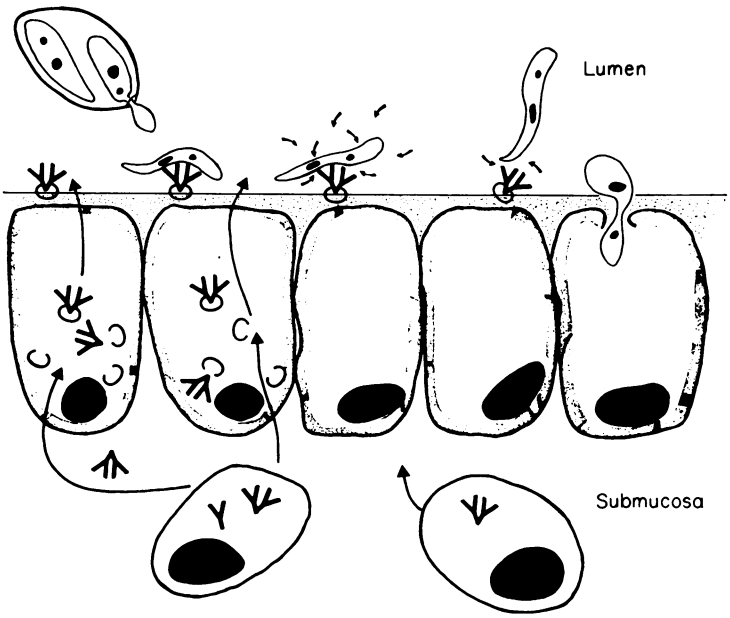



**Figure 3.** Diagrammatic representation of the proposed mechanism of secretory IgA-mediated anticoccidial immunity in the chicken. Subepithelial plasma cells secrete IgA, anti-sporozoite antibodies, and sporozoites become attached to them on the mucosal surface. During this time, the parasite is subject to proteolytic (or other) damage. Eventually many sporozoites break free, perhaps because their membrane antigens have been cleaved by proteolytic enzymes, and are able to penetrate host cells. As with sporozoites pretreated in the absence of soluble protein or in extracts of caecal contents, however, most have lost the ability to differentiate into a trophozoite.

target antigens are destroyed. When judging the overall significance of the secretory immune system, this positive attribute of secretory IgA is, therefore, far more relevant than its apparent lack of involvement with phagocytes or complement etc.

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