Eimeria maxima: ELISA and Western blot analyses of protective sera

MICHAEL WALLACH¹, NICHOLAS C.SMITH², CATHERINE M.D.MILLER¹, JOHANNES ECKERT¹ & M.ELAINE ROSE³

¹Institut für allgemeine Mikrobiologie, Universität Bern, Baltzerstrasse 4, CH-3012, Bern, Switzerland
²Institut für Parasitologie, Universität Zurich, Winterthurerstrasse 266a, CH-8057, Zurich, Switzerland
³AFRC Institute for Animal Health, Compton Laboratory, Compton, Nr Newbury, Berkshire, RG16 0NN, UK

SUMMARY

Infection of chickens with Eimeria maxima induces the production of parasite-specific antisera which can be used passively to protect naïve chickens against infection. Globulin fractions of these antisera can also be used passively to protect chickens. Similarly, intramuscular injection of soybean lectin affinity purified gametocyte antigens of E. maxima in Freund's Complete Adjuvant induces production of antibodies which are maternally transferred and thereby protect hatchlings against E. maxima. ELISA analyses of serum pools having varying protective capacities revealed good correlations between passive protection and levels of anti-unsporulated oocyst, anti-sporulated oocyst, anti-merozoite and anti-gametocyte antibodies. Western blotting demonstrated that the sera mainly recognized a number of high molecular weight antigens in all developmental stages and that the intensity of the reactions reflected the degree of protection induced by the sera. Sera from birds immunized with gametocyte antigens also recognized high molecular weight antigens from all the developmental stages, with banding patterns remarkably similar to those observed for sera from infected birds. Taken together, these results indicate that antibodies can protect against infection with E. maxima and these antibodies may recognize and act against asexual and/or sexual stages of the parasite.

Keywords E. maxima, coccidiosis, stage-specificity, ELISA, Western blotting, antibodies, immunity

INTRODUCTION

The immune mechanisms that govern resistance to infection with species of Eimeria are still not well understood. It is generally believed that the early, asexual stages of development are crucial in the induction of resistance to a variety of Eimeria species and that this resistance is primarily cell-mediated with antibody playing only a minor contributory role (McDougal & Jeffers 1976, Rose 1987). However, several studies indicate that antibodies induced by infection (Rose 1971, 1972, 1974, Rose & Long 1971) or by vaccination with purified gametocyte antigens (Wallach et al. 1992) can transfer significant protection either passively or maternally. Hence, antibodies directed against the sexual as well as the asexual stages of Eimeria may play an important role in the immunological control of these parasites. Therefore, we have taken sera from chickens after infection with Eimeria maxima or after immunization with gametocyte antigens, for analysis of their stage and antigen specificity and attempted to correlate specific antibody levels with the ability of the sera to transfer resistance to infection.

The antisera used in these trials were obtained during passive immunization experiments carried out over several years (Rose 1971, 1974, Rose & Mockett 1983). Pools of sera were made from blood taken 10 to 14 days post-infection (p.i.) with E. maxima oocysts and were stored at −20°C. Sera from birds infected with E. maxima can possess extraordinary protective capabilities. However, sera from different experiments may be distinctly different in their ability to transfer resistance passively (via daily subcutaneous injection) to naïve chickens, depending on the time after infection that the sera are collected (Rose 1971). In general, sera isolated between days 10 and 14 post-primary infection (p.p.i) are the most protective. However, even in this period, the level of passive protection conferred by different pools of
sera is variable. In total, 24 experiments were conducted using sera collected from days 10–14 p.p.i. with *E. maxima* (Rose 1971). Eleven of these resulted in better than 76% protection (as assessed by oocyst excretion), eight scored 51–75% protection, three scored 26–50% protection and two scored 0–25% protection. We have selected five of these serum pools, with a wide variety of protective abilities ranging from essentially zero (11%) to almost total (97%) protection. We have not used serum pools from other time points p.i., believing it is better to test sera from a period where good passive protection is a possibility but not a certainty. Antisera produced by immunization of six-week-old chickens with soybean lectin affinity purified, *E. maxima* gametocyte antigens in Freund’s Complete Adjuvant (FCA), were also used. Maternal transfer of antibodies against gametocyte antigens confers a relatively high level of immunity to hatchlings (70–80% inhibition of oocyst excretion compared to the progeny of sham-immunized controls) (Wallach et al. 1992).

The Houghton strain of *E. maxima* was used to induce infections for the preparation of the various stages of development. For the preparation of oocysts, chickens three to four weeks of age were infected with 2000 sporulated *E. maxima* oocysts, faeces were collected six to eight days p.i., and unsporulated and sporulated oocysts were purified by centrifugation, salt floatation and treatment with sodium hypochloride as previously described (Wagenbach, Challey & Burns 1966). For antigen preparation the oocysts were suspended in an equal volume of phosphate-buffered saline (PBS) and an almost saturating quantity of 1 mm glass beads was added. The oocysts were then vortexed with the glass beads for five mm. Rupture of the oocysts was confirmed microscopically. Residual, unruptured sporocysts and sporozoites were lysed by three cycles of freeze/thawing (liquid nitrogen/4°C) followed by sonication in a Branson sonifier at 20 watts for 20 s at 4°C. The homogenate was then centrifuged at 12,000g for 5 min and the supernatant retained. The protein concentration was determined using a Biorad protein determination kit.

For the preparation of merozoites, chickens were infected at three to four weeks of age with 200,000 *E. maxima* sporulated oocysts and at 96 h post infection the chickens were killed in accordance with Swiss animal protection regulations, their intestines removed, flushed with cold PBS, and slit open. They were then cut into 1 cm² pieces which were incubated for a maximum of 30 min at 40°C in Hanks’ medium (pH 7-6) containing 0.025% trypsin (Difco), 1% taurocholic acid (Fluka) and 10 mM MgCl₂. For every 20 intestines, 400 ml of media were used. The number of merozoites liberated from the enterocytes was checked, microscopically, every five min until the total number had plateaued. The large intestinal debris were then separated by filtering through gauze and smaller debris were removed by filtration through a 17 μM polycrylon mesh (Swiss Silk Boling Cloth Mfg. Co. Ltd., Zurich, Switzerland). The merozoites were then centrifuged at 1000g for 10 min, the supernatant discarded and the merozoites resuspended in PBS. The merozoites were then filtered through a 10 μM mesh and the centrifugation and washing steps repeated three times. This procedure produced approximately 10⁸ *E. maxima* merozoites per bird. Merozoite extracts were prepared by suspending merozoites in PBS at 1–2 x 10⁸/ml and freeze-thawing and sonication was done as described for the oocysts.

For the preparation of gametocytes, chickens were infected with 10,000 oocysts at four weeks of age. At 136–138 h p.i., the chickens were killed, the intestines removed and washed with cold SAC (170 mM NaCl, 10 mM Tris-HCl pH 7, 10 mM glucose, 5 mM CaCl₂, 1 mM phenylmethanesulphonyl fluoride, 1 mg/ml bovine serum albumin). One end of the intestine was tied with string and the gut filled with 0.5 mg/ml hyaluronidase in SAC. The other end of the intestine was then tied and the filled gut was placed in 37°C PBS in a shaking water bath for 20 min. At the end of this incubation the intestines were slit open and the contents discarded. The intestines were placed on top of a 17 μm mesh and the mucosa washed through with SAC (room temperature). The material left on the mesh was discarded and the flow through filtered through a 10 μm mesh. The gametocytes accumulated on the filter and were washed off with SAC and centrifuged at 800g for 5 min. The gametocyte extract was made by incubating the gametocytes at room temperature for 30 min in 0.5% deoxycholate in DEB (10 mM Tris-HCl pH 7.4, 50 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 1 mM phenylmethanesulphonyl fluoride)–2 million gametocytes per ml. The ruptured parasites were then centrifuged at 1500g for 20 min and the supernatant collected and dialysed against 10 mM phosphate buffer (pH 8) plus 1 mM phenylmethanesulphonyl fluoride at 4°C overnight. The extract was then lyophilized and stored desiccated at -20°C.

For ELISA, the various stage-specific antigen extracts were diluted in 0.1 M carbonate/bicarbonate buffer (pH 9.6) plus 0.02% sodium azide. 100 µl aliquots of diluted antigens were allowed to absorb onto NUNC 'maxisorp' 96 well plates overnight at 4°C. (Unsporulated oocyst, sporulated oocyst and merozoite antigens were applied at 0.5 µg/well whereas gametocyte antigen was applied at 1 µg/well.) The plates were then washed
three times with 0.9% saline containing 0.3% Tween 20, prior to addition of 100 μl aliquots of samples diluted in PBS plus 0.3% Tween 20, 0.02% azide, and 0.05% bovine haemoglobin. After 120 min of incubation at 41°C, plates were again washed three times and 100 μl of diluted (1:2000) alkaline phosphatase-conjugated antibody to chicken IgG (Fc fragment specific, affinity purified, Bethyl Laboratories, Montgomery, TX, USA) was added to each well. After incubation at 41°C for 120 min the plates were again washed three times and 100 μl of a 1 mg/ml solution of p-nitrophenylphosphate (Sigma Chemical Ltd., St. Louis, MO, USA) in 0.05 M carbonate/bicarbonate (pH 9.8) was added to each well. After incubation at 37°C for 30 min the reaction was stopped by addition of 50 μl of 3 M NaOH to each well. Absorbance values were read at 405 nm in an automatic microelisa reader (MR610 autoreader, Dynatech Laboratories, Denkendorf, Germany).

For Western blot analyses, antigens extracted from the various stages of development were separated on SDS-PAGE and Western transfer was performed as previously described (Towbin & Gordon 1984). Approximately 5–10 μg of protein from unsporulated oocysts, sporulated oocysts, or merozoites, or 50 μg gametocyte protein, was electrophoresed in each lane (the use of a larger amount of gametocyte proteins was due to host contamination of this intracellular stage). These amounts were chosen according to the results of a titration experiment where the optimal amounts of the stage-specific proteins per lane were determined. Immune or normal chicken sera were used to detect antigens on the Western blot as described previously (Wallach et al. 1989). The immune sera were used at various dilutions and incubated for two h at room temperature.

Figure 1 shows the ELISA results obtained using samples from five pools of sera which were previously shown to provide varying levels of passive protection against *E. maxima* infections (Rose & Mockett 1983). There was a good correlation between reactivity with all of the stages of development and protection, whether tested at a dilution of 1:100 or 1:500. However, the 1:100 dilution allowed excellent differentiation between the sera which protected by 77, 90 and 97% and the sera which conferred only 11 or 56% protection whereas the dilution of 1:500 allowed differentiation amongst the more protective sera. Furthermore, at the 1:100 dilution, the reactivity with the different developmental stages of the parasite appeared more-or-less equal but analyses at dilutions of 1:500 revealed that the strongest reactivity of the sera was with the unsporulated oocyst and merozoite stages, with lower reactivity with sporulated oocysts and gametocytes. In contrast, antiserum raised against the affinity purified gametocyte antigens, tested at a dilution of 1:500, reacted most strongly with the gametocyte (absorbance=1.388) and unsporulated oocyst (absorbance=1.527) extracts and reactivity with the sporulated oocyst (absorbance=0.619) and merozoite (absorbance=0.562) extracts was somewhat lower, though still quite high (compare with Figure 1). At a dilution of 1:500, sera from sham-immunized birds (that is, PBS in FCA) was virtually non-reactive, recording absorbance values of only 0.018–0.031 for the extracts of the different parasite stages.

The antigen specificity of protective sera was analysed by Western blotting. Antibodies from all of the immune sera reacted with a large number of protein bands in all four stages of development (Figure 2), while sera from untreated control chicks showed little or no reactivity (data not shown). Within each stage of development tested, similar banding patterns were observed for the high and low protective sera (whereas between stages the banding patterns were very different), with the majority of the proteins recognized being in the high molecular weight range. Indeed, in many cases there were very few proteins recognized that had molecular weights lower than 66 kDa. However, the sera that provided the highest levels of passive protection reacted with much greater intensity than sera which conferred only partial protection.

Within each stage of development analysed, the banding patterns obtained using antisera induced by immunization against affinity purified gametocyte antigens were similar to those induced by infection with *E. maxima* (schematically summarized in Figure 3). This is particularly true for the merozoite stage where the banding patterns were nearly identical to that obtained with one of the convalescent sera. There were, however, some important differences between the antigenic profile induced by immunization with gametocyte proteins and infection with *E. maxima*. Thus, in the gametocyte and unsporulated oocysts stages there were two major protein bands recognized by the anti-gametocyte sera which were not recognized by sera from infected birds. In the gametocyte stage these proteins had molecular weights of 56 kDa and 82 kDa, and in the unsporulated oocyst stage they had molecular weights of about 30–35 kDa.

The results presented here, when considered in combination with previously published data, provide compelling evidence that antibodies can mediate resistance to *Elmeria*. Thus, it was demonstrated some time ago that globulin fractions of sera (Rose 1974) or gamma-livetin fractions of egg yolks (Rose 1972) from chickens infected with *E. maxima* were able to transfer protection against coccidiosis passively to naïve chickens. These results indicated that the protective effect of passively trans-
ferred sera or maternally transferred yolk was almost certainly antibody-mediated, although the protective effects had not been definitively correlated with parasite-specific antibody levels. The present study provides these necessary correlations. ELISA analyses of sera which protect against infection with *E. maxima*, have revealed good correlations between their ability to reduce total oocyst output, and levels of antibodies to unsporulated oocysts, sporulated oocysts, merozoites and gametocytes. However, the relationship is not linear. Rather, there is a threshold effect whereby there are relatively low levels of antibodies to all developmental stages in low–moderately protective sera (that is, 11% or 56% protective capability), higher levels in the pool which is 77% protective and very high levels in sera which confer better than 90% passive resistance to infection. These findings confirm and extend previous studies which indicated that there may exist a correlation between reactivity with asexual (Rose & Mockett 1983, Gilbert, Bhanushali & McDougald 1988) or sexual (Wallach et al. 1992) stage antigens and protection in vivo.

Within a given stage of development analysed, most of the sera appear to recognize very similar high molecular weight antigens (Figure 2). Therefore, it does not appear

---

**Figure 1** ELISA absorbance (405 nm) and passive protection by sera from chickens after infection with *E. maxima*. ●—Sera tested at a dilution of 1:500. ○—Sera tested at a dilution of 1:100.
that protective capacity can be correlated with the recognition of particular stage-specific antigens. Rather, the absolute concentration of antibodies present appears to govern the degree of protection since highly protective sera reacted with much greater intensity than did less protective sera.

These results indicate that both asexual and sexual stages are immunogenic and may contain protective antigens. The results of analyses of sera from birds immunized with *E. maxima* gametocyte proteins also suggest this conclusion. Surprisingly, antisera raised against soybean lectin affinity purified gametocyte antigens (and which react almost exclusively with the 56 and 82 kDa gametocyte proteins) also reacted with the asexual stages of the parasite, although previous results had indicated that these antigens are expressed in a stage specific manner which is controlled at the level of mRNA transcription (Mencher, Pugatsch & Wallach 1989, Fried et al. 1992). There are a few possible explanations for this phenomenon. First, natural antibodies to these antigens may be present in the sera of uninfected chickens as suggested by Mencher et al. (1989), and increased production of these antibodies may be induced by very strong antigenic stimulation. Second, there may exist...
cross-reactive epitopes within proteins from different stages (or even different species) as suggested by Rose & Mockett (1993). Third, some gametocyte antigens may be expressed throughout parasite development. Work is currently in progress to try and elucidate the relationship of the various protein bands identified with the anti-gametocyte sera.

It was surprising to find that, in contrast to previous observations (Wallach et al. 1989), the sera from chickens immunized by infection with *E. maxima* did not react strongly with the 56 kDa and 82 kDa gametocyte antigens. This finding may be due to the use of a different breed of chicken in the preparation of the immune sera. Thus, there may be an influence of genetic factors on the specificity of the immune response which occurs during an infection with *Eimeria*.

In conclusion, these results indicate that antibodies can protect against infection with *E. maxima* and these antibodies recognize and may act against both asexual and sexual stages of the parasite. However, it seems unlikely that this is the sole mechanism by which immunity to *Eimeria* can be achieved since not all pools of sera are protective. The results also give some indications that ELISA and Western blotting techniques may be useful in the prediction of resistance against infection with *E. maxima*, at least in some circumstances, including monitoring of maternally-derived immunity. The key question which remains is to determine which of the many antigens recognized by these protective sera are crucial for providing immunity against infection.

ACKNOWLEDGEMENTS

This work was supported by the Bundesamt für Bildung und Wissenschaft, Bern (Project: Coccdiosis, COST 89), the Bundesamt für Veterinärwesen, Liebefeld-Bern (Project No. 012.92.8), the Swiss National Science Foundation (Project No. 31-35656.92) and by the Roche Foundation.

REFERENCES


correlation of antibody levels with prior exposure to coccidia in the laboratory and in the field. *Avian Diseases* 32, 688–694