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Maternal Immunization with Gametocyte Antigens as a Means of Providing Protective Immunity against *Eimeria maxima* in Chickens

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In the present study, we wished to demonstrate the ability of surface gametocyte antigens to induce protective immunity against *Eimeria maxima* infections in chickens. In order to accomplish this goal, we employed maternal immunization as a means of providing large amounts of specific antibodies to offspring chicks. Upon challenge with sporulated *E. maxima* oocysts, chicks from hens immunized with affinity-purified gametocyte antigens showed greatly reduced oocyst production compared with chicks from sham-immunized hens. These results suggest that maternal immunization with gametocyte antigens can be used as a means to provide transmission-blocking immunity against *E. maxima* infections.

Parasites of the class *Sporozoa* cause a variety of diseases of great veterinary and medical importance, such as malaria, toxoplasmosis, and babesiosis. Coccidiosis, caused by a variety of species of the genus *Eimeria*, is an important disease of this class of parasites. The *Eimeria* parasite undergoes a complex life cycle in the intestinal mucosa of the infected host. Transmission of the disease occurs through the production of oocysts which develop as a result of fertilization of macrogametes by the microgametes. We hypothesized that, as shown for *Plasmodium gallinaceum* and *P. falciparum* (1–3), antibodies raised against surface gametocyte antigens of *Eimeria* spp. could inhibit the growth, development, and/or fertilization of gametes and thereby block transmission of the disease.

We have recently shown that a monoclonal antibody raised against a 56-kDa gametocyte surface glycoprotein of the avian parasite *Eimeria maxima* can significantly reduce oocyst production in passively immunized chickens (10). In addition, sera from convalescent chickens that also provided protective immunity against infections with *E. maxima* (5, 10) recognized three major gametocyte surface glycoproteins with molecular masses of 56, 82, and 250 kDa. Thus, we wished to test the effect of these three gametocyte antigens on the induction of a protective immune response in immunized chickens.

In the present study, we employed maternal immunization as a means of providing protection against *E. maxima* infections in offspring chicks. Affinity-purified 56-, 82-, and 250-kDa *E. maxima* gametocyte antigens were used to immunize laying hens which, via the yolk, provided very large amounts of maternal anti-gametocyte antibodies to their offspring. Chicks that were challenged per os with sporulated *E. maxima* oocysts showed greatly reduced oocyst production compared with chicks from sham-immunized hens, suggesting that the anti-gametocyte maternal antibodies provided transmission-blocking immunity against *E. maxima*. These results also indicate the potential use of transmission-blocking immunity in the control of other sporozoan parasites of veterinary and/or medical importance (8).

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MATERIALS AND METHODS

Parasites. The Houghton strain of *E. maxima* was used throughout these experiments. The oocysts were passaged in Anak 180 Israeli commercial broiler-breed chickens, and clean sporulated oocysts were prepared as described previously (7).

Preparation of gametocyte affinity-purified antigens. *E. maxima* gametocytes were purified from infected chicken intestines as described previously (9). Detergent extracts were prepared, and the 56-, 82-, and 250-kDa antigens were isolated by using columns containing monoclonal antibody 1E11-11 bound to Sepharose 4B or soybean lectin bound to agarose as described previously (10). The antigens were lyophilized and stored dessicated at −20°C.

Western blotting. Western blotting (immunoblotting) of gametocyte extracts was carried out as described previously (9). Chicken sera used in the immune detection were diluted in phosphate-buffered saline (PBS) containing 2.5% fetal calf serum and 2.5% powdered milk (blocking buffer) and incubated at 37°C for 2 h. The blots were washed two times in blocking buffer diluted 1:1 with PBS and two times in blocking buffer diluted 1:4 with PBS. Peroxidase-linked rabbit anti-chicken immunoglobulin G (Biomakor, Rehovot, Israel) was used as the second antibody reagent, and the color reaction was performed as described previously (9).

Maternal immunization studies. In order to perform maternal immunization experiments, the first step was to produce a flock of hens which contained low levels of naturally occurring anti-gametocyte antibodies. Therefore, Anak 180 layers were selected from Israeli breeding flocks on the basis of the reactivity of their sera with gametocyte antigens on Western blots. Hens with low titers were then maintained free of coccidia for 2 months prior to the start of the experiment. At 5 to 6 months of age, the hens were either immunized with 3 weekly injections of affinity-purified ga-

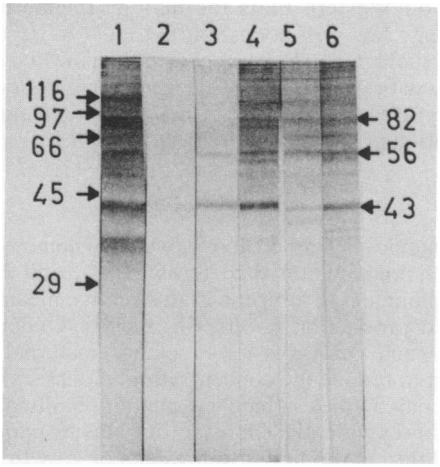

FIG. 1. Western blot analysis of chicken anti-gametocyte antibodies in sera from hens reared under field conditions. Sera were taken from 150 Anak 180 hens selected at random from Israeli breeding flocks. They were used at a dilution of 1:50 to detect *E. maxima* gametocyte antigens by Western blotting as described previously (9). The results of six representative serum samples are shown (lanes 1 through 6). Numbers at left denote the following molecular mass marker proteins: carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine serum albumin (66 kDa), phosphorylase *b* (97 kDa), and β-galactosidase from *Escherichia coli* (116 kDa). Numbers at right indicate the molecular masses in kilodaltons of the major gametocyte antigens.

metocyte antigen (approximately 50 to 100 ng per booster injection as determined by optical density and Coomassie blue staining on polyacrylamide gels), sham immunized with PBS in Freund's adjuvant, or given a live immunization with 50,000 sporulated *E. maxima* oocysts. The live-immunized group was housed separately from the hens immunized with PBS or gametocyte antigen. During the immunization period, hens were artificially inseminated every 3 to 4 days. In addition, their anti-gametocyte antibody titers were monitored both in the sera and egg yolks prior to and after immunization. Two weeks after the final booster injection with antigen (see Fig. 2A) or 3 weeks postinfection, the fertilized eggs were collected, incubated, and hatched. The offspring chicks were tagged and housed in the same cage for a period of 3 to 4 days. They were then challenged with 100 sporulated *E. maxima* oocysts and 5 days later were moved into individual cages for collection of feces into trays containing 2% potassium dichromate. On day 6 or 9 postinfection (i.e., prior to the time when the chicks may themselves respond to the gametocyte antigens in the live infection), serum samples were taken and analyzed by Western blotting. From days 6 to 8 postinfection, chicks were put on limited feeding (30 min/day) while the feces were being collected. On day 8 postinfection, total 3-day oocyst counts were performed as described previously (10).

RESULTS

Immunization of laying hens with *E. maxima* affinity-purified gametocyte antigens. Sera from laying hens taken at random from field flocks were analyzed by Western blotting. Figure 1 shows the results with some representative sera; it can be seen that under field conditions, several hens produced high titers of anti-gametocyte antibodies (lanes 1 and 4 to 6), while others produced intermediate or low amounts of antibody to gametocyte antigens (lanes 2 and 3). Laying hens with low anti-gametocyte titers were reared free of coccidia for 2 months in order to further reduce the background maternal antibody levels. Their sera were retested by Western blotting, and those hens showing virtually no anti-gametocyte titers were immunized intramuscularly with affinity-purified 56-, 82-, and 250-kDa *E. maxima* gametocyte antigens in Freund's adjuvant. As negative controls, a group of layers were sham immunized with PBS in Freund's adjuvant. A third group of layers, which received a live infection with 50,000 sporulated *E. maxima* oocysts, were used as positive controls.

As was shown previously (9, 10), the sera from most hens immunized with the affinity-purified antigens or with live infection reacted very strongly with the 56- and 82-kDa antigens. Figure 2A shows a Western blot analysis of yolk antibody titers from three representative samples; it can be seen that very high titers were present in the yolks of eggs from most of the immunized layers, while yolks from the sham-immunized control group contained little or no anti-gametocyte antibody. Thus, we concluded that these groups of layers could now be employed to carry out the maternal immunization experiments described below.

Maternal immunization experiments using offspring chicks from hens immunized with gametocyte antigens. The results of the first maternal immunization experiment are summarized in Table 1 and Fig. 2B; it can be seen that all of the 12-day-old immune chick sera contained a strong titer of anti-gametocyte maternal antibodies reacting predominantly with the 56- and 82-kDa antigens, as well as with the 250-kDa antigen (Fig. 2B, lanes 1 through 7). None of the chick sera from negative controls reacted with the gametocyte antigens (Fig. 2B, lanes 8 to 10). By oocyst counts (Table 1, experiment 1), it was found that chicks from hens immunized with the affinity-purified gametocyte antigens showed an 83% reduction in oocyst output compared with that of the unimmunized controls. Furthermore, 2 of 11 of the chicks from hens immunized with the affinity-purified antigens had oocyst outputs below our limit of detection. This level of inhibition was equal to or even greater than that obtained by using a live oocyst infection to immunize the hens (76.5%).

The second maternal immunization experiment was designed to determine the duration of the effect of the immune response to the gametocyte antigens in these hens (Table 1, experiment 2). The same immunized layers used in the first maternal immunization experiment were kept free of coccidia in cages for an additional 4 months prior to artificial insemination and collection and hatching of the eggs. As can be seen in Table 1, even after 4 months with no exposure to the parasite, chicks from hens immunized with the affinity-purified antigens still showed a statistically significant level of inhibition in oocyst output of about 25%.

The third maternal immunization experiment was performed by using the same layers; however, 2 weeks after the end of experiment 2, each bird was given one additional boost of 50 to 100 ng of affinity-purified gametocyte antigen prior to collection of the fertilized eggs. As can be seen in Table 1, the results of experiment 3 showed that a more significant level of inhibition of oocyst output was achieved (compare with the results of experiment 2).

Serum samples were taken at random from nine of the chicks from gametocyte antigen-immunized hens and five of the chicks from the sham-immunized hens of experiment 3 and were analyzed by Western blotting. It was found that seven of the nine chicks from immunized hens had high titers of antibody to the 56- and 82-kDa gametocyte antigens and

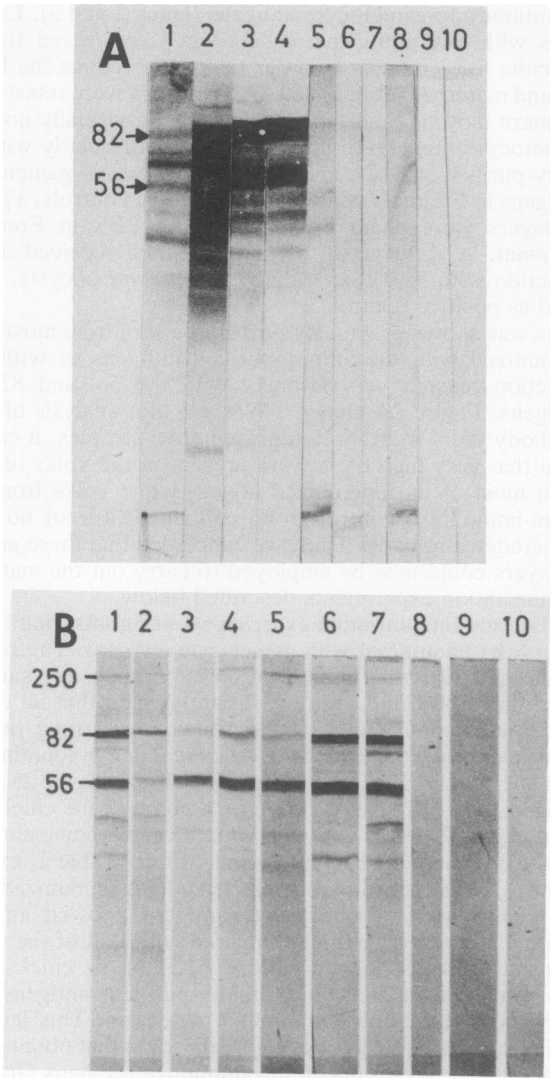

FIG. 2. Western blot analysis of yolk and serum anti-gametocyte antibodies from eggs and offspring chicks of immunized Anak 180 hens. (A) Anak 180 hens were immunized with *E. maxima* gametocyte affinity-purified 56-, 82-, and 250-kDa antigens prepared as described previously (10). Yolk from an egg representative of the results of the immunized hens or from two eggs of sham-immunized hens were used to detect *E. maxima* gametocyte antigens by Western blotting as described previously (6). Serum from a convalescent chicken was used as a positive control. Lanes: 1, serum from a convalescent chicken, 1:50 dilution; 2 to 4, yolk from an egg of a hen immunized with the affinity-purified gametocyte antigens at dilutions of 1:50, 1:500, and 1:1,600, respectively; 5 to 7 and 8 to 10, yolks from eggs of two sham-immunized hens at dilutions of 1:50, 1:500, and 1:1,600, respectively. Numbers at left indicate the molecular masses (in kilodaltons) of the major gametocyte antigens. (B) Sera from 12-day-old offspring chicks from hens immunized with affinity-purified gametocyte antigens (lanes 1 to 7) or unimmunized hens (lanes 8 to 10) were analyzed by Western blotting as described above. Sera were used to detect gametocyte antigens at a dilution of 1:100. Numbers at left indicate the major gametocyte antigen bands of 56-, 82-, and 250 kDa.

had an average oocyst output of 1.87×10^6, 70% below that of the average sham-immunized control and similar to the results obtained in experiment 1. Two of the chicks from immunized hens showed low reactivity with gametocyte antigens on Western blots (as in Fig. 1, lane 3) and had intermediate levels of oocyst output (3.79×10^6 and 4.92×10^6), and the five chicks from control hens which showed no reactivity with gametocyte antigens had high oocyst outputs (average, 7.25×10^6). Thus, there was a relationship between anti-gametocyte titer and protection.

DISCUSSION

In the studies described above, we have demonstrated that maternal immunization with the 56-, 82-, and 250-kDa *E. maxima* gametocyte antigens is an effective means of reducing oocyst production in offspring chicks. Under field conditions, such transmission-blocking maternal antibody would act to reduce the concentration of oocysts in the litter from the day a chick is hatched, thereby limiting the potential for the exponential increase in parasite numbers often observed during the first 3 to 5 weeks of growth (6).

The mechanism by which the maternal antibodies to the 56-, 82-, and 250-kDa gametocyte antigens inhibit oocyst output is not presently understood. In studies carried out by using the immunofluorescence assay on both isolated gametocytes and gametocytes in situ, mouse antibodies raised against these three affinity-purified antigens bound predominantly to the surface of the macrogametocytes and macrogametes, with little or no reactivity with any other stages of development (9). Thus, it would appear that the maternal antibodies may be either blocking an important receptor present on the surface of the macrogametocytes or macrogametes or may be directly preventing the growth of this stage of development. Further work is required to elucidate this issue.

In a previous study (10), we noted that the monoclonal antibody 1E11-11, which on Western blots gave a single 56-kDa band, bound the 56- and 82-kDa proteins by affinity chromatography. Furthermore, monoclonal antibodies raised against the 82-kDa protein switched to recognition of the 56-kDa antigen. In the present work, we have found that maternal antibodies raised against the 1E11-11 monoclonal antibody affinity-purified gametocyte antigens recognized, in addition to the 56- and 82-kDa proteins, a 250-kDa antigen. From these results, we concluded that either these three proteins contain some homologous epitopes or that they exist as a protein complex, as was noted previously (10). Alternatively, the 56- and 82-kDa glycoproteins may be processed from the 250-kDa protein. Work aimed at distinguishing between these possibilities is in progress.

The results presented in this study are similar to those obtained in work on transmission-blocking immunity against malaria (2, 3, 4, 8). In those studies, it was found that antibodies against three or four *P. falciparum* or *P. gallinaceum* gametocyte surface antigens (230 or 250 kDa, 45 or 56 kDa, and 25 kDa) could block transmission of malaria to mosquitos. Furthermore, these malarial transmission-blocking antigens appear to be well conserved between *Plasmodium* strains, as was demonstrated by Kaslow et al. (1). Our previous results have also indicated that gametocyte antigens of *E. maxima* are well conserved between *Eimeria* strains and perhaps even species (9). Therefore, cloning and expressing the genes encoding these gametocyte antigens may lead to the production of a transmission-blocking vaccine for coccidiosis in chickens.

Finally, the application of transmission-blocking immunity to the control of chicken coccidiosis may be an excellent model system for the control of diseases caused by other sporozoan parasites. Once the parameters affecting parasite

TABLE 1. Summary of results of the maternal immunization experiments

Expt. no.	Results with chicks:										
	PBS sham immunized[a]			Gametocyte antigen immunized				Live immunized			
	n[b]	$\bar{x}$ (10^6)[c]	SD (10^6)[d]	n	$\bar{x}$ (10^6)	SD (10^6)	% Reduced[e]	n	$\bar{x}$ (10^6)	SD (10^6)	% Reduced
1 (2 wk)	29	1.5	0.9	11	0.2	0.2	83.2	11	0.35	0.35	76.5
2 (4 mo)	27	9.3	4.4	29	7.0	3.4	24.7	ND[f]	ND	ND	ND
3 (booster injection)	17	5.9	3.0	37	2.9	1.2	50.8	ND	ND	ND	ND

[a] In the first maternal immunization experiment, Anak 180 chicks from a commercial hatchery were used.
[b] n, number of chicks per group.
[c] $\bar{x}$, total number of oocysts per chick (average).
[d] SD, standard deviation of average of total oocyst counts.
[e] By Student's t test, the percent reduced results were significant at the $P < 0.001$ level for all of the experiments except experiment 2, which was significant at the $P < 0.05$ level.
[f] ND, not done.

transmission in chickens are better understood, then the application of this concept to the control of diseases caused by other sporozoan parasites may be more easily accomplished.

REFERENCES

1. **Kaslow, D. C., I. A. Quakyi, and D. B. Keister.** 1989. Minimal variation in a vaccine candidate from the sexual stage of *Plasmodium falciparum*. Mol. Biochem. Parasitol. **32**:101–104.
2. **Kaslow, D. C., I. A. Quakyi, C. Syin, M. G. Raum, D. B. Keister, J. E. Coligan, T. F. McCutchan, and L. H. Miller.** 1988. A vaccine candidate from the sexual stage of human malaria that contains EGF-like domains. Nature (London) **333**:74–76.
3. **Kaushal, D. C., R. Carter, J. Rener, C. A. Groten-Dorst, L. H. Miller, and R. J. Howard.** 1983. Monoclonal antibodies against surface determinants on gametes of *Plasmodium gallinaceum* block transmission of malaria parasites to mosquitoes. J. Immunol. **131**:2557–2562.
4. **Rener, J., R. Carter, Y. Rosenberg, and L. H. Miller.** 1989. Anti-gamete monoclonal antibodies synergistically block transmission of malaria by preventing fertilization in the mosquito. Proc. Natl. Acad. Sci. USA **77**:6797–6799.
5. **Rose, M. E.** 1971. Immunity to coccidiosis: protective effect of transferred serum in *Eimeria maxima* infections. Parasitology **62**:11–25.
6. **Voeten, V. A. C., and W. W. Braunius.** 1981. Subclinical coccidiosis in broilers: a comparative investigation of detection methods. Arch. Gefluegelkd. **45**:189–193.
7. **Wagenbach, G. E., J. R. Challcy, and W. C. Burns.** 1966. A method for purifying coccidian oocysts employing Chlorox and sulfuric acid-dichromate solution. J. Parasitol. **52**:1222.
8. **Wallach, M., M. Fried, and D. Mencher.** The importance of transmission-blocking immunity in the control of sporozoan parasites. Immunol. Infect. Dis., in press.
9. **Wallach, M., D. Mencher, S. Yarus, A. Halabi, and T. Pugatsch.** 1989. *Eimeria maxima*: identification of gametocyte protein antigens and their possible role in protective immunity. Exp. Parasitol. **68**:49–56.
10. **Wallach, M., G. Pillemer, S. Yarus, A. Halabi, T. Pugatsch, and D. Mencher.** 1990. Passive immunization of chickens against *Eimeria maxima* infection with a monoclonal antibody developed against a gametocyte antigen. Infect. Immun. **58**:557–562.