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Major Histocompatibility Complex Control of Immunity Elicited by Genetically Engineered *Eimeria tenella* (Apicomplexa) Antigen in Chickens†

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The immunogenicity of a recombinant *Eimeria tenella* coccidial antigen was studied in $6_1.B$ congenic chickens derived from B^2B^2 and B^5B^5 parents segregating for haplotypes B^2 and B^5. Five-week-old chickens were immunized with 2.4 μg of recombinant protein (designated 5401) in Freund complete adjuvant and challenged with 75,000 oocysts at 28 days postimmunization (DPI) to determine the degree of elicited protective immunity. Serum samples were collected weekly for 5 weeks postimmunization for analysis by enzyme-linked immunosorbent assay, immunofluorescence assay, and Western blotting. Lesion scores following oocyst challenge were significantly reduced in B^5B^5 chickens compared with those in B^2B^2 chickens. Immunization induced a sporozoite-specific immunoglobulin G (IgG) titer in serum detected by the enzyme-linked immunosorbent assay that peaked at 28 DPI, the day of challenge, in B^5B^5 chickens and at 42 DPI in B^2B^2 chickens. After challenge, this titer declined for each genotype. Anti-sporozoite IgG detected by the immunofluorescence assay attained a peak titer at 21 DPI in B^2B^2 chickens and 28 DPI in B^5B^5 chickens. Serum from immunized B^5B^5 chickens reacted strongly in Western blots with several high-molecular-weight (>100,000), soluble proteins prepared from sporozoites. Serum from B^2B^2 chickens reacted with similar proteins as well as with a 51- to 53-kilodalton protein that was not labeled by serum from B^5B^5 chickens. These results demonstrate further the role of host genetics on anticoccidial immunity and suggest that a peak anti-sporozoite IgG titer in B^5B^5 chickens on the day of challenge may signal a state of immunocompetence to that challenge.

Eimeria tenella, an obligate intracellular protozoan parasite, is the etiologic agent of cecal coccidiosis in domestic chickens. Avian coccidia (family Eimeriidae) belong to the phylum Apicomplexa, as do the plasmodia (family Plasmodiidae). Currently, both genera are the subject of intense vaccine research (21; H. D. Danforth and P. C. Augustine, *in* I. G. Wright, ed., *Veterinary Protozoan and Hemoparasite Vaccines*, in press), with considerable emphasis being placed on the ability to clone reactive sporozoite surface antigens (8, 29). The sporozoite is the invasive stage initiating infection of host cells and a probable candidate for the stimulation of the immune response.

Recent studies have been concerned with the host response to malarial antigens at the level of the major histocompatibility complex (MHC) in congenic mice (10, 11). Results of these studies demonstrated that high and low responders to antigen occur in a given inbred mouse population and that these responses are restricted by MHC. Similarly, *H-2* genes as well as non-*H-2* genes have been shown to affect immunity to the murine coccidia *Eimeria falciformis* (18). Avian host immune responses to coccidiosis have also been shown to be mediated by MHC (5, 13, 17). Specifically, the B^5 haplotype (1) of the chicken MHC (25) or *B* locus (2) has been implicated in conferring immunocompetence against *Eimeria tenella* (5). In contrast, chicken hosts possessing the B^2 haplotype were not protected against parasite challenge. The recent development of congenic lines of chickens (highly inbred lines differing only at the chicken *B* complex) permits further clarification of the genetic control of immunity to coccidia.

A recombinant *E. tenella* antigen cloned from the oocyst, the transmission stage of this parasite, has been produced and characterized (8). This 35-kilodalton (kDa) protein is designated 5401 and contains several amino acid repeat sequences, a trait that has also been observed in the circumsporozoite antigen of *Plasmodium falciparum* (7). Immunization of chickens with this antigen induces partial protection against the clinical effects of *E. tenella* infection in a commercial outbred line of chickens (8). Here we report the differential humoral and associated clinical responses elicited by antigen 5401 in congenic chickens segregating for haplotypes B^2 and B^5.

MATERIALS AND METHODS

Chickens. Inbred single-comb White Leghorn chicken line 6_1 (B^2B^2), which was developed at the Regional Poultry Research Laboratory (East Lansing, Mich.), was selected as the background strain for the developing *B* congenic line. This line is histocompatible; resistant to Marek disease; susceptible to subgroup A, B, and C leukosis viruses, and regresses Rous sarcoma virus-induced tumors (27). Females of inbred single-comb White Leghorn line 15_1 (B^5B^5), which were also developed at the Regional Poultry Research Laboratory, were crossed with line 6_1 to produce the F_1 generation. Line 15_1 is susceptible to Marek disease and subgroup A leukosis virus and progresses Rous sarcoma virus-induced tumors (6). For subsequent backcross generations, hetero-

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zygous B^2B^5 females were mated to line 6_1 males. Eighth backcross generation heterozygous males and females were mated to produce B^2B^2 or B^5B^5 homozygous progeny with >99% background gene uniformity (unpublished data).

A total of 55 chickens, which were divided among three trials, were used in this study. All progeny were typed for *B* alloantigens by hemagglutination (1). Chicks were raised to be free of coccidia to 5 weeks of age on wire floor brooding batteries and fed a nonmedicated, all-mash starter diet ad libitum. Because of the highly inbred character of these lines, younger birds are very small and difficult to use in this type of study.

Recombinant antigen and parasite. The recombinant coccidial antigen 5401, which was kindly provided by Genex Corporation (Gaithersburg, Md.), was cloned from nonsporulated and sporulated oocysts of *E. tenella* LS24 (Animal Parasitology Institute, Beltsville, Md.) and was produced as an extract in *Escherichia coli* (8). The gene coding for this antigen was expressed in a λgt11 bacteriophage vector, yielding a fusion protein of approximately 150 kDa corresponding to about 31 kDa of coccidial antigen fused to β-galactosidase. Sequencing indicated that the coccidial antigen contains a primary structure of five repeating segments. The encoded region is also highly hydrophilic and negatively charged (H. D. Danforth, unpublished data).

Immunization of chickens. Five-week-old chickens were immunized individually with 2.4 μg of antigen 5401 from *Escherichia coli* extract emulsified in 50% Freund complete adjuvant (FCA) and injected in a 0.5-ml volume subcutaneously at the base of the neck. Previous titration studies showed that a single dose of 2.4 μg is maximally protective in a commercial line of chickens (Danforth and Augustine, in press). Unimmunized chickens of each genotype were maintained as controls. Chickens were challenged at 28 days postimmunization (DPI) by oral inoculation of 75,000 *E. tenella* LS24 oocysts, to determine the efficacy of antigen 5401 immunization. Preliminary studies have shown that this challenge dose is lethal in 10% of naive birds.

Clinical criteria. Clinical disease was monitored 6 days after oral challenge by cecal lesion scores (scored 0 to 4) (12) and body weight gain.

ELISA. Pooled serum samples within genotypes were taken weekly, beginning on the day of immunization (day 0) through the day of termination of the study (day 42), to assess sporozoite-specific immunoglobulin G (IgG) titers. Control serum was obtained from chickens that were immunized with saline emulsified 1:1 in FCA. Enzyme-linked immunosorbent assay (ELISA) analysis was carried out essentially as described previously (23). Briefly, sporozoites were excysted by standard aseptic procedures, passed over a scrubbed nylon wool column to remove debris, centrifuged, and suspended in phosphate-buffered saline (PBS). Sporozoites were then disrupted with glass beads (diameter, 100 μm) by vortexing them for 2 min. The material was centrifuged at 11,600 × *g* for 2 min, and the supernatant was collected. The protein concentration was determined by the method outlined by Bio-Rad Laboratories (Richmond, Calif.). Soluble sporozoite antigen was then diluted in carbonate-bicarbonate buffer (pH 9.6) and adsorbed to 96-well enzyme immunoassay plates (Costar, Cambridge, Mass.) at a concentration of 1 μg per well overnight at 4°C. All subsequent incubations were carried out at 41°C for 1 h. Plates were blocked with 3% bovine serum albumin in PBS and then rinsed three times with PBS-Tween 20. Serial dilutions of individual serum samples in PBS-Tween 20 were reacted with adsorbed antigen followed by three rinses in PBS-Tween 20. Plates were then exposed to a 1:1,000 dilution of goat anti-chicken IgG horseradish peroxidase conjugate (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) in PBS. Plates were washed three times with PBS-Tween 20 and developed with tetramethyl benzidine reagent (ICN Biomedicals, Costa Mesa, Calif.). Absorbances were read on a micro-ELISA reader (Dynatech Laboratories, Inc., Alexandria, Va.) at 450 nm and corrected for background. Mean absorbances from quadruplicate wells per trial are reported for 1/1,600 serum dilutions.

Immunofluorescence assay. Sporozoites were obtained as described previously and fixed, at a concentration of 10^6/ml, to *Toxoplasma* titer slides (Belco Glass Inc., Vineland, N.J.) by air drying. Sporozoites were reacted with serial dilutions of immune serum and incubated at room temperature in a humid chamber for 1 h. Slides were washed for 10 min in PBS and then reacted with rabbit anti-chicken IgG (Kirkegaard and Perry Laboratories) that was diluted 1:30 in PBS and were incubated for 1 h in a humid atmosphere. Slides were washed again for 10 min in PBS and then reacted with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Kirkegaard and Perry Laboratories) diluted 1:30 for 1 h in a humid atmosphere. Slides were washed a final time in PBS, mounted with glycerol, and observed by microscopy under UV light.

Polyacrylamide gel electrophoresis and Western blot analysis. Approximately 2.0 × 10^7 *E. tenella* sporozoites were disrupted with glass beads (diameter, 100 μm) by vortexing them for 2 min in sample buffer (15). The suspension was centrifuged at 11,600 × *g* for 2 min, and the supernatant was applied to a sodium dodecyl sulfate-10% polyacrylamide gel. The gel was electrophoresed at 35 mA for 6 h. Separated sporozoite proteins were transferred from sodium dodecyl sulfate-10% polyacrylamide gels onto nitrocellulose paper overnight at 30 V (28). The nitrocellulose paper was blocked with 3% gelatin in Tris-buffered saline for 2 h at 37°C, rinsed in water, and exposed for 2 h at room temperature to 1:1,000 dilutions of 5401-immunized chicken serum in antibody buffer. All subsequent incubations were for 2 h at room temperature. The paper was rinsed twice in PBS-Tween 20 and exposed to a 1:1,000 dilution of rabbit anti-chicken IgG in antibody buffer. Following another two rinses in PBS-Tween 20, the nitrocellulose paper was labeled with goat anti-rabbit horseradish peroxidase conjugate diluted 1:1,000 in antibody buffer and was then washed twice in PBS-Tween 20 and developed with 4-chloronaphthol reagent.

Statistics. All data were analyzed by analysis of variance, and means were tested by the Student-Newman-Keul test at a 0.05 level of probability.

RESULTS

Clinical response to parasite challenge. Oral challenge at 28 DPI induced moderately severe cecal lesions 6 days later (34 DPI) in 5401-immunized B^2B^2 chickens, but there was essentially no infection in B^5B^5 chickens (Table 1). All unimmunized controls had lesion scores of +4. Although immunized birds of each genotype gained more weight than unimmunized controls, this difference was not significant ($P < 0.05$) (Table 1).

ELISA for antibody production in serum. A sporozoite-specific IgG titer was first detected by ELISA in B^5B^5 chickens at 14 DPI and continued to increase through 21 DPI, with a peak at 28 DPI, the day of oral challenge (Fig. 1). In unchallenged B^5B^5 chickens, the IgG titer fell sharply by 35 DPI and continued its decline to 42 DPI. The IgG titer of

TABLE 1. Mean cecal lesion scores as a distribution of individual scores and body weight gain[a]

Cecal lesion score	No. of chickens		
	B^2B^2	B^5B^5	Control
0	0	6	0
1	0	3	0
2	2	0	0
3	4	0	0
4	4	0	10

[a] Scores were determined 6 days following oral challenge with *E. tenella* oocysts (28 DPI) of $6_1.B$ congenic chickens that were previously immunized with recombinant *E. tenella* antigen 5401. The mean weight gains ± standard errors of the mean for B^2B^2, B^5B^5, and control chickens were 99.5 ± 17.3, 86.7 ± 14.6, and 66.1 ± 3.7 g, respectively.

challenged B^5B^5 chickens at 34 DPI, the day that these chickens were sacrificed for cecal lesion scoring, declined 50% (0.141) following challenge, but at a slower rate than that in unchallenged B^5B^5 chickens. A sporozoite-specific IgG titer was barely detectable in B^2B^2 chickens at 14 DPI but continued to rise slowly to a peak concentration at 42 DPI in unchallenged birds. Following oral challenge on 28 DPI, the IgG titer of B^2B^2 chickens fell sharply at 34 DPI (the day of lesion scoring), to a level (0.082) that was approximately one-half of that measured in B^5B^5 chickens. No anti-sporozoite titers were measured in chickens of either genotype following immunization with saline and FCA (data not shown).

Immunofluorescence assay for antibody production in serum. Immune sera from chickens of both genotypes produced a surface-internal immunofluorescent antibody pattern on air-dried sporozoites. No immunofluorescence assay (IFA) titers were detected at 7 DPI from chickens of either genotype, while equivalent IFA titers were measured for chickens of each genotype at 14 DPI (Table 2). In the absence of challenge, the anti-sporozoite titer in serum from B^2B^2 chickens peaked at 21 DPI and then declined from 28 to 35 DPI. In contrast, the response in B^5B^5 chickens, which was lower than that of B^2B^2 chickens peaked at 28 DPI and then declined. Following challenge at 28 DPI, however, titers in B^2B^2 chickens were elevated to their highest levels on 35 DPI, while titers in B^5B^5 chickens remained at their prechallenge level.

Profiles of sporozoite antigen detected by serum. Western blot analysis was used to characterize differential serum antibody reactivities following immunization with 5401. Soluble sporozoite antigen was resolved on a 10% polyacrylamide slab gel into several protein bands ranging in molecular weight between 15,000 and >200,000. On transfer to Western blots, serum from 5401-immunized B^5B^5 chickens consistently labeled several high-molecular-weight bands (>100,000) beginning at 14 DPI, with the strongest labeling or signal seen at 28 DPI (Fig. 2). A weaker signal was observed with serum from unchallenged B^5B^5 chickens at 35 and 42 DPI. Serum from 5401-immunized B^2B^2 chickens labeled similar high-molecular-weight proteins, although labeling was not detected until 21 DPI. Serum from unchallenged B^2B^2 chickens gave the strongest signal at 35 and 42 DPI. Serum from B^2B^2 chickens also reacted with a lower-molecular-weight band (approximately 51,000 to 53,000) that was not labeled with serum from B^5B^5 chickens. Serum from chickens of both genotypes immunized with 5401 reacted with Western blotted antigen 5401, indicating a band of approximately 150 kDa; however, serum raised against saline and FCA did not react in Western blots (data not shown).

TABLE 2. Anti-sporozoite IgG titers in serum detected by IFA and reported as reciprocal dilutions[a]

B genotype	Titer on the following days:						
	0	7	14	21	28	35	34C[b]
B^2B^2	0	0	80	320	160	40	500
B^5B^5	0	0	80	160	160	80	160

[a] Titers were of serum from $6_1.B$ congenic chickens immunized with recombinant *E. tenella* antigen 5401 on day 0 and challenged with 75,000 *E. tenella* oocysts at 28 DPI.

[b] IgG titers in serum of chickens challenged at 28 DPI and scored for cecal lesions at 34 DPI.

DISCUSSION

Results of the present study confirm the results of earlier clinical studies implicating the role of the chicken *B* (MHC)

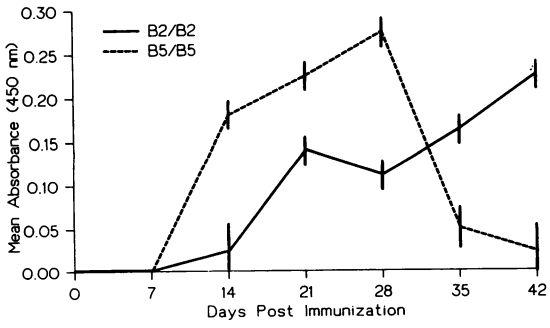

FIG. 1. Anti-sporozoite IgG titers in serum detected by ELISA and reported as the A_{450} of $6_1.B$ congenic chickens immunized with recombinant *E. tenella* antigen 5401.

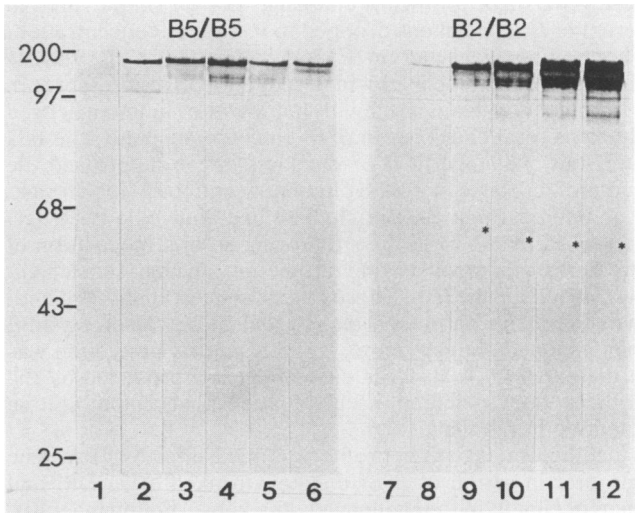

FIG. 2. Western blot analysis of serum from B^5B^5 (B5/B5) or B^2B^2 (B2/B2) chickens immunized with antigen 5401 on an *E. tenella* sporozoite preparation. Lanes 1 and 7, 7 DPI; lanes 2 and 8, 14 DPI; lanes 3 and 9, 21 DPI; lanes 4 and 10, 28 DPI; lanes 5 and 11, 35 DPI; lanes 6 and 12, 42 DPI. Asterisks in lanes 9 to 12 are placed beside the 51- to 53-kDa antigen that reacted with serum from immunized B^2B^2 chickens. Positions of the molecular weight markers are identified on the left and correspond to the following from top to bottom: myosin, 200,000; phosphorylase *b*, 97,000; serum albumin, 68,000 ovalbumin, 43,000; and α-chymotrypsinogen, 25,000.

complex on immunity to coccidiosis (5, 24). Chickens congenic for the *B* complex exhibited a differential response to a cloned coccidial protein administered in FCA following both immunization and parasite challenge. Immunization with recombinant antigen 5401 reduced cecal pathology following challenge in B^5B^5 chickens, while it did not elicit the same level of protection in B^2B^2 hosts. Body weights were not significantly influenced by either parasite challenge or genotype, although immunized birds gained more weight than unimmunized birds. This apparent uniformity in weight gain may reflect the highly inbred character of these lines.

Previous immunity studies with antigen 5401 in a commercial outbred line demonstrated its antigenicity by measuring high IgG titers following immunization (Danforth and Augustine, in press). In the present study, the anti-sporozoite IgG titer elicited by 5401, which was measured weekly by both ELISA and IFA, differed between both genotypes and assays. Since ELISA and IFA exhibit inherently different antibody concentration sensitivities and specificities, these patterns may explain the interassay differences in the observed titer profiles. The ELISA technique used in this study would detect antibody specific for soluble sporozoite antigen only, while the IFA technique with air-dried sporozoites would measure the antibody directed against antigens present in or on the intact parasites. There may be several antigens in common for each assay, and the different antigen preparations may select for or eliminate certain antigenic moieties. ELISA and IFA may measure an IgG titer to different antigens. In addition, the more sensitive ELISA may detect small quantities of antigens that are not detected by IFA. Both assays detected genotype differences in the magnitude and kinetics of the response. B^5B^5 chickens exhibited a peak IgG titer by ELISA at 28 DPI, the day of challenge with 75,000 oocysts, and showed protection against that challenge 6 days later (34 DPI). On the other hand, B^2B^2 chickens produced a significantly lower IgG titer at 28 DPI and were not protected against this same challenge dose. Six days following challenge (34 DPI), the titer in serum of B^2B^2 chickens dropped to its lowest concentration. Therefore, a peak serum ELISA IgG titer at the day of challenge was associated with a reduction in lesion scores in B^5B^5 chickens. Compared with the IgG titer in unchallenged chickens, oral challenge in B^5B^5 chickens sustained the IgG titer, but challenge in B^2B^2 chickens either suppressed the humoral response or bound circulating antibody. In contrast, a noninbred Light Sussex chicken line showed a peak IgG titer in serum 11 to 15 days following an oral inoculation of 10^4 *E. tenella* oocysts and protection against subsequent parasite challenge (20). These results suggest that the natural parasite elicits both a protective and more rapid response than a cloned antigen. However, this natural protection was at the expense of a severe clinical disease produced by the primary oocyst infection, a situation that could not be tolerated in the field.

A different IgG titer profile was measured by IFA. The IgG titer in serum of B^2B^2 chickens peaked at 21 DPI and then declined in unchallenged chickens. Following challenge, the titer in serum of B^2B^2 chickens increased to its highest level while the IgG titer in serum of B^5B^5 chickens remained unchanged. An elevated IgG titer measured by IFA was not associated with a reduction in lesion scores in B^2B^2 chickens. These results may indicate that the B^2 haplotype mediates a strong response to a nonprotective antigen. A similar decoy scenario has been suggested in response to the circumsporozoite antigen of malaria (19). Moreover, B^2B^2 chickens responded most strongly to sporozoite antigen after challenge, a phenomenon which appears to abrogate a protective response.

Western blot analysis indicated a response similar to that measured by ELISA, because the magnitude of antibody labeling or signal corresponded directly with measured titers; in both assays similar solid support antigens were used. Immune serum from both genotypes labeled similar high-molecular-weight protein bands. Serum from B^2B^2 chickens that was collected at 21, 28, 35, and 42 DPI also labeled a 51- to 53-kDa band that was not labeled by serum from B^5B^5 chickens. Again, the B^2 haplotype may mediate a response against an antigen which either misdirects or inhibits protection.

Immunity to the avian *E. tenella* has been shown to be a T-cell-mediated response (14). Previously, there were few data to support a direct humoral role in the protective response to coccidiosis, although immunity to *E. tenella* may be augmented by secretory IgA (4, 9). The present data demonstrate the association between a peak anti-sporozoite IgG ELISA titer in serum on the day of parasite challenge and reduced cecal pathology following immunization with a recombinant antigen in B^5B^5 chickens.

In addition, the B^5 haplotype appears to impart a greater efficiency in mounting the IgG response than does the B^2 haplotype. Although parasite neutralization may ultimately involve T cells, the efficiency of the response may be inherent in antigen presentation as a result of the interaction between parasite antigen and MHC gene products on antigen-presenting cells (26). In this system, antigen 5401 may have a stronger affinity for the class II molecule encoded by the B^5 haplotype. Thus, B^5B^5 chickens can be considered high responders to antigen 5401 and B^2B^2 chickens can be considered low responders. Similar genetic responsiveness has been described in mice immunized with a *P. falciparum* sporozoite vaccine and has been shown to map to the *I-A* region of the murine MHC (11).

The initial response to antigen 5401, a soluble antigen, may be B-cell mediated, as the B cell may serve as the antigen-presenting cell along with an obligatory production of parasite-specific IgG and subsequent secretory IgA (3). It is not known what direct role these antibodies may have, if any, in arresting parasite development. Antigen presentation during a natural infection may involve macrophages (22) or intraepithelial lymphocytes (16). Therefore, the use of a recombinant antigen such as 5401 may invoke an additional cellular component, B cells, which may serve in some capacity to neutralize parasitism.

These results demonstrate the role of host genetics in ascertaining the efficacy of a recombinant protein as a vaccine. The use of these congenic lines of chickens suggests that a high anti-sporozoite IgG ELISA titer at the time of challenge may signal a level of immunocompetence that is necessary for protection. The ability of a recombinant coccidial protein to stimulate this IgG response in chickens may indicate an effective vaccine component. Further studies must be carried out to determine whether this antibody signal is specific only for B^5B^5 chickens or whether B^2B^2 chickens would show similar responses when challenged at 42 DPI, the time of their peak ELISA titer.

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