

IMMUNOLOGY

Immunogenic Characterization of a Tissue Culture-Derived Vaccine That Affords Partial Protection Against Avian Coccidiosis

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ABSTRACT The immunogenicity of a tissue culture-derived vaccine generated from an *Eimeria tenella*-infected cell line in a serologically defined bird line, and the ability to confer protection against homologous challenge in young chicks was examined. The cell line, SB-CEV-1/F7, was infected with *E. tenella* sporozoites and the resulting 72-h postinfection cell-free supernatants were adjuvanted and used to immunize Leghorn chicks homozygous for the B¹⁹ haplotype. Peripheral blood and splenic lymphocytes from these immunized birds proliferated *in vitro* in response to both sporozoite and SB-CEV-1/F7 tissue culture-derived parasite antigens. In addition, splenic immune lymphocytes obtained from birds previously exposed to *E. tenella* *in vivo* responded to these tissue culture-derived parasite antigens *in vitro*. To evaluate the efficacy of the vaccine,

B¹⁹B¹⁹ chicks were vaccinated s.c. with adjuvanted 72-h postinfection cell-free supernatants or an ammonium sulfate precipitate derivative thereof, orally boosted, and then subjected to homologous parasite challenge at 10 d of age. The level of protection (body weight gain, cecal lesions) was assessed 6 d after challenge. Performance results from four battery trials demonstrated that vaccinated birds were significantly protected against weight loss compared to unimmunized, challenged controls. In addition, in two of the four trials, vaccinated birds were significantly protected against lesions. These results provide strong evidence that tissue culture-derived parasite antigens obtained from the *E. tenella*-infected SB-CEV-1/F7 cell line are immunogenic in birds and can provide partial protection against *E. tenella* clinical coccidiosis.

(Key words: coccidiosis, tissue culture, vaccine, immunity, *Eimeria*)

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INTRODUCTION

Clinical avian coccidiosis, caused by several species of the parasitic protozoan genus *Eimeria*, is a complex disease that continues to plague the poultry industry worldwide. Replication of the parasite in the epithelial and lamina propria regions lining the intestinal tract leads to severe enteritis, weight loss, growth depression, and in some instances death (Fernando, 1990). Prophylactic chemotherapy traditionally has been, and continues to be, the most widely preferred mode of

treatment. Unfortunately, the rapid emergence of drug-resistant parasite strains and increased development costs associated with new anticoccidial drugs have prompted renewed interest in alternative methods to coccidiosis control. For example, live or attenuated modes of vaccination have proven to be both efficacious and commercially feasible (Shirley and Long, 1990; Williams, 1994). However, several significant safety and practical obstacles remain with this approach, including controlled administration, strain pathogenicity, flock management, and worldwide inventory demand. The cloning and expression of protective *Eimeria* antigens over the past several years has held great promise as a safer and more cost-effective strategy. Nonetheless, the identification of numerous *Eimeria* sporozoite, merozoite, and gametocyte genes (reviewed by Danforth and Augustine, 1990) has yet to successfully result in an efficacious commercial product. Although the utility of molecular biology is potentially enormous, it is readily apparent that the probability of success of developing an anticoccidial vaccine would be significantly increased through a further understanding of the host-parasite relationship. Perhaps recombinant DNA methodologies restricted to *Eimeria* antigens recognized by local

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mucosal immune responses (Lillehoj, 1994) could augment approaches reported previously of identifying parasite antigens using hyperimmune sera (Clarke *et al.*, 1987; Jenkins *et al.*, 1988; Danforth and Auustine, 1989).

At each point during the endogenous eimerian life cycle, different stage-specific antigens become the potential target of host protective immune responses. The host protective immune response appears to be mainly cell-mediated in origin (Rose and Wakelin, 1990; Lillehoj, 1991) and directed at parasite gene products produced during intracellular asexual development (Horton-Smith *et al.*, 1963; Rose, 1967; Rose *et al.*, 1984; McDonald *et al.*, 1986). In order to obtain sufficient quantities of these intracellular parasite products, a suitable cell culture system to propagate *Eimeria* species to high density levels is required. Although *Eimeria* species will invade a variety of avian and nonavian cell types *in vitro* (Doran, 1982; Strout and Schmatz, 1990), high rates of intracellular development have been historically accompanied by increased cell clusters and subsequent host cell monolayer peeling from the matrix (Schmatz, 1987). To this end, an established cloned cell line that supports high levels of *Eimeria tenella* intracellular development and merogony *in vitro* has been recently reported (Danforth *et al.*, 1994). This cell line, designated SB-CEV-1/F7, survives multiple rounds of *E. tenella* sporozoite invasion and development. More importantly, intracellularly produced parasite-specific proteins were readily detected in the SB-CEV-1/F7 tissue culture supernatant by ELISA at various time points post infection (J. Lineberger, unpublished data).

The objective of the present study was to determine the immunogenicity of *E. tenella*-infected SB-CEV-1/F7 tissue culture parasite antigens in a serologically defined (*B19*-locus) bird line (Briles *et al.*, 1950; Clare *et al.*, 1985; Clare and Danforth, 1989). Peripheral blood lymphocytes (PBL) and splenic lymphocytes from both tissue culture-vaccinated as well as naturally infected birds were assayed for their ability to respond to parasite antigens from different sources *in vitro*. In addition, the ability of these *E. tenella* tissue culture-derived parasite antigens to confer protection against homologous parasite challenge in young naive chicks was investigated.

MATERIALS AND METHODS

Birds and Housing

Noninbred, 1-d-old Leghorn chicks⁷ homozygous for the *B19* major histocompatibility haplotype, were vacci-

nated against Marek's disease virus and wing-banded. Chicks were housed in wire-floor battery cages in an AAALAC (American Association for Accreditation of Laboratory Animal Care) facility, and a commercial starter-growing ration⁸ and water were provided for *ad libitum* consumption. The standard ration contained 3,020 kcal of ME/kg, 21.6% CP, 0.92% Ca, and 0.73% available P. Birds were housed 10 per cage and used between 1 and 45 d of age.

Chickens used in all experiments were handled according to the guidelines of the site Institutional Use and Care Committee. Prior to lesion scoring and harvesting of spleens, chickens were euthanatized by cervical dislocation.

Parasite Excystation and Antigen Production

Eimeria tenella (L.S. 65; a gift from R. G. Strout, University of New Hampshire, Durham, NH 03824) was used throughout the entire study. Oocysts were produced and maintained by routine passage in Petersen × Arbor Acres broilers, and inoculum was prepared as described by Dulski and Turner (1988) with the following exceptions. Oocysts were broken on ice for 3 min using a Biospec bead beater,⁹ and sporocysts were purified using 0.75 M sucrose instead of 1 M sucrose.

Sporozoites or merozoites produced *in vitro* in SB-CEV-1/F7 were resuspended in Dulbecco's PBS containing 0.05 M phenylmethylsulfonyl fluoride,¹⁰ freeze-thawed three times on dry ice, and sonicated¹¹ on ice using a 1 s pulse, 80% duty cycle. After five cycles, each 1 min long, samples were transferred to microcentrifuge tubes and centrifuged at 10,000 × *g* for 10 min at 4 C. Soluble material above the pellet was collected and total protein concentration was determined¹² (Bradford, 1976). Sonicated parasite preparations were adjusted to 1 mg/mL in serum-free Medium 199,¹³ aliquoted, and stored at -20 C until use.

Tissue culture vessels (150 cm²) were seeded with cells of the SB-CEV-1/F7 cell line, an established fibroblast cell line (ATCC No. CRL10495) described previously (Danforth *et al.*, 1994), at a density of 1.0 × 10⁵ per milliliter suspended in 30 mL Medium 199 with 5% fetal bovine serum, and incubated at 40.5 C in 5% CO₂. After an overnight incubation, medium was aspirated and sporozoites were added at a density of 1.0 × 10⁶ sporozoites per milliliter in 30 mL of Medium 199 with 1% fetal bovine serum. Culture medium was collected at 24, 48, and 72 h postinfection, and centrifuged at 800 × *g* for 30 min to remove cell debris and any parasite forms. Supernatants were then designated as 24-, 48-, and 72-h antigens (AG). Samples from each time point were quantified for parasite-specific protein (PSP) using a direct sporozoite ELISA described below. Cell-free supernatants were aliquoted and frozen at -20 C until use. Samples were thawed immediately prior to use for *in vitro* assays, ammonium sulfate precipitation, or vaccine formulation.

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Characterization of Tissue Culture-Derived Parasite AG

The tissue culture-derived parasite AG were quantified for total protein (Bradford, 1976). In addition, parasite-specific protein was determined by a sporozoite-specific ELISA. To produce the sporozoite-specific antibody, purified sporozoites were injected at two weekly intervals into rabbits using Freund's complete adjuvant. Vaccine antigens (100 ng per well or 1,000 ng per well if fetal bovine serum was present) or purified sporozoites (10 ng per well) were adsorbed to Nunc¹⁴ plates overnight at 4 C. After washing with PBS, the rabbit anti-sporozoite antibody (diluted 1:20,000) was added and incubated at 37 C, then washed with PBS. Biotin-labeled goat anti-rabbit¹⁵ was added and incubated 1 h at 37 C, then washed with PBS. Peroxidase-labeled streptavidin¹⁵ in 2% skim milk was added, and incubated for 1 h at 37 C, washed, then 3,3',5,5'-tetramethyl benzidine-peroxidase¹⁵ in hydrogen peroxide was added. The reaction was stopped with 1 M hydrochloric acid, and plates were read at 450 nm.

Cell Isolations

Wing-web bleeds from all birds in each group were collected and pooled into heparinized Vacutainer¹⁶ tubes. Blood samples were diluted 1:3 in Ca²⁺ and Mg²⁺-free Hank's balanced salt solution containing 25 mM HEPES,¹³ pH 7.5 (CHBSS), and washed two times at 150 × g for 10 min at room temperature. Samples were diluted to twice the original volume in CHBSS and 3-mL suspensions layered over an equal volume of Histopaque 1077.¹⁰ Samples were centrifuged at 400 × g, for 15 min at room temperature, and cells from the interface were collected.

Spleens were removed and placed in ice-cold sterile CHBSS. Spleen tissue was disrupted in CHBSS using a sterile scalpel, followed by syringe 14-gauge cannulation. Cell suspensions were then centrifuged at 50 × g for 10 min at room temperature. Supernatants were removed and centrifuged at 150 × g for 10 min at room temperature. Cell pellets were resuspended in CHBSS and 3-mL suspensions were layered over Histopaque and centrifuged as described above. Cell interfaces from blood and spleen suspensions were collected, diluted in CHBSS, and washed three times at 150 × g for 10 min at room temperature. Viable cells were enumerated using trypan blue and a hemacytometer and kept on ice until ready for plating. Cell viability was typically greater than 95%.

In Vitro Proliferation Assays

Harvested PBL and spleen cells were added to complete serum-free Leibovitz's Modified Hahn's media (equal parts McCoy's 5A and Leibovitz's media, 5 × 10⁻⁵ M 2-mercaptoethanol, 5 μg/mL insulin, 2 mM L-glutamine, 100 U/mL penicillin and streptomycin, 0.25 μg/mL amphotericin B, 2% tryptose phosphate, 1 mM sodium pyruvate) (CLMH)¹⁷ containing 2% heat-inactivated autologous chicken serum and 0.1 mL was added to 96-well round-bottomed microtiter plates in quadruplicate. Preliminary studies (not shown), determined that the optimal cell concentrations from 72-h AG immunized and trickle immunized birds were 5 × 10⁵ and 2 × 10⁵ cells per well, respectively (see below). *Eimeria* AG were adjusted to the specified protein concentrations in CLMH, and 0.1 mL was added to wells. In pilot experiments, no significant differences in mean cpm between fresh medium and SB-CEV-1/F7 conditioned medium were detected; thus, control wells received 0.1 mL fresh CLMH/2% heat-inactivated autologous chicken serum. Cultures were incubated at 40.5 C, 5% CO₂ for 96 h and pulsed with 1 μCi per well ³[H]-thymidine¹⁸ during the final 18 h of culture. Cells were harvested onto glass fiber mats using an automated cell harvester¹⁹ and radioactivity determined in a direct beta counter.²⁰

Immunogenicity and In Vitro Proliferation Experimental Design

Immunization with 72-h AG. Control (n = 3) and experimental (n = 3) B¹⁹B¹⁹ birds were immunized at 24 d of age. Control groups received 1.0 mL uninfected SB-CEV-1/F7 tissue culture Medium 199 adjuvanted by vortexing, to 5% with 30% aqueous Amphigen.²¹ Individual experimental birds received 4.7 μg PSP in 1 mL of 72-h AG with Amphigen. Control and experimental groups were immunized either orally by intubation or s.c. in the back of the neck using a 21-gauge needle. In all experiments, a sufficient amount of adjuvant control and 72-h AG vaccine was prepared and stored at 4 C for boost immunizations. Groups were boosted by the same route and volume 11 d after the primary immunization. Four days postboost, pooled blood and individual spleen samples were collected and lymphocytes isolated. For the *in vitro* proliferation assay, PBL and spleen cells were adjusted to 5 × 10⁶ cells per milliliter in CLMH containing 2% heat-inactivated autologous chicken serum. The SB-CEV-1/F7 24, 48, and 72 h infected tissue culture supernatants were adjusted to 20 μg/mL total protein in CLMH and sonicated sporozoite antigen was adjusted to 5 μg/mL total protein in CLMH. Assays were performed as described above. Results are expressed as mean cpm ± SD where mean cpm equals [(mean cpm "experimental" + antigen) - (mean cpm "experimental" + media)] - [(mean cpm "control" + antigen) - (mean cpm "control" + media)].

Trickle Immunization and Challenge at 10 d. One-day-old experimental birds (n = 4) were trickle-immunized daily for 5 consecutive d with 500 *E. tenella*

¹⁴Nunc Inc., Naperville, IL 60566.

¹⁵Kirkegaard and Perry Labs, Inc., Gaithersburg, MD 20879.

¹⁶Becton-Dickinson and Co., Franklin Lakes, NJ 07417-1885.

¹⁷ICN Biomedicals, Inc., Costa Mesa, CA 92626.

¹⁸Amersham, Arlington Heights, IL 60005.

¹⁹TomTech, Orange, CT 06477.

²⁰Packard Instrument Co., Meriden, CT 06450.

²¹Hydronics, Lincoln, NE 68510.

oocysts. Control birds ($n = 4$) were mock immunized in a similar fashion using distilled water. At 10 d of age, experimental and control groups were challenged with 35,000 homologous oocysts or distilled water, respectively. Twenty days later, spleens were collected and lymphocytes were isolated. For the *in vitro* proliferation assay, spleen cells were adjusted to 2×10^6 cells per milliliter in CLMH containing 2% heat-inactivated autologous chicken serum. Serial dilutions in CLMH of the 72-h AG, 72-h AG-ASP (ASP = 30% $(\text{NH}_4)_2\text{SO}_4$ precipitation) and sonicated sporozoite or merozoite AG were prepared. Assays were performed as described above. Results are expressed as mean cpm \pm SD where mean cpm equals [(mean cpm + antigen) - (mean cpm + media)].

Trickle Immunization and Challenge at 16 or 43 d.

One-day-old experimental birds ($n = 5$) were trickle-immunized daily for 5 consecutive d with 500 *E. tenella* oocysts. At 16 and 43 d of age, experimental birds were challenged with 35,000 homologous *E. tenella* oocysts. Control birds ($n = 5$) were mock-immunized and boosted with distilled water at the same ages as the experimental group. At 45 d of age, spleens were collected and lymphocytes isolated. For the *in vitro* proliferation assay, spleen cells were adjusted to 2×10^6 cells per milliliter in CLMH containing 2% heat-inactivated autologous chicken serum. Twofold dilutions in CLMH of 72 h AG-ASP [30% $(\text{NH}_4)_2\text{SO}_4$ precipitation] and sonicated sporozoite or merozoite antigen were prepared. Assays were performed as described above. The results are expressed as mean cpm \pm SD where mean cpm equals [(mean cpm + antigen) - (mean cpm + media)].

Protection Studies

Immunization with 72-h AG. The 72-h AG mixed in Amphigen was used to immunize two different groups of 4-d-old birds. One group ($n = 8$) was vaccinated s.c. with 1 mL of 2 μg PSP/mL vaccine and the other group ($n = 8$) vaccinated s.c. with 1 mL of 19 μg PSP/mL vaccine. Both groups were boosted orally 3 d later using the same dose and volume as the primary immunization. Another group ($n = 8$) of 2-d-old birds were trickle-immunized with 500 *E. tenella* oocysts in 1 mL daily for 5 consecutive d. At 10 d of age, vaccinated, trickle-immunized, and unimmunized, naive ($n = 8$) birds were weighed and orally challenged with 35,000 *E. tenella* sporulated oocysts. A 10-d-old unimmunized group ($n = 7$) was also weighed and mock-challenged with distilled water. At 6 d postchallenge, weight gain and lesions scores were determined (Johnson and Reid, 1970).

Immunization with Ammonium Sulfate-Fractionated 72-h AG. The 72 h AG-ASP mixed in Amphigen was used to immunize 4-d-old *B*¹⁹*B*¹⁹ chicks. One group ($n = 8$) was vaccinated s.c. with 1 mL of 9 μg PSP/mL vaccine and a control group ($n = 8$) was immunized with Amphigen mixed with uninfected 72 h SB-CEV-1/F7 tissue culture media. Both groups were boosted orally 3 d later using the same dose and volume as the primary immunization. Another group ($n = 8$) of 1-d-old birds were trickle-immunized with 500 *E. tenella* oocysts in 1 mL daily for 5

consecutive d. At 10 d of age, vaccinated, trickle-immunized, and control groups were weighed and orally challenged with 35,000 *E. tenella* sporulated oocysts. A 10-d-old unimmunized group ($n = 4$) was also weighed and mock-challenged with distilled water. At 6 d postchallenge, weight gain and lesions scores were determined.

Immunization of Birds of Different Ages with Ammonium Sulfate-Fractionated 72-h AG. The 72-h AG-ASP mixed with Amphigen was used to immunize either 1-d-old ($n = 5$) or 4-d-old ($n = 7$) *B*¹⁹*B*¹⁹ chicks. Groups were vaccinated s.c. at the base of the neck with 1 mL of 15 μg PSP/mL vaccine and boosted orally 3 d later using the same dose and volume used for the primary immunization. Two control groups ($n = 8$) were immunized with Amphigen adjuvanted with uninfected 72 h SB-CEV-1/F7 tissue culture media at either 1 or 4 d of age, and birds were orally boosted 3 d later. Another group ($n = 8$) of 1-d-old birds were trickle-immunized with 500 *E. tenella* oocysts in 1 mL daily for 5 consecutive d. At 10 d of age, the two vaccinated, one trickle-immunized, and two control groups were weighed and challenged orally with 35,000 *E. tenella* sporulated oocysts. At 6 d postchallenge, weight gain and lesions scores were determined.

Immunization with Ammonium Sulfate-Fractionated 72-h AG Following Prolonged Storage. The 72-h AG-ASP was stored at 4 C for 24 d then mixed with Amphigen to immunize 4-d-old *B*¹⁹*B*¹⁹ chicks. One group ($n = 5$) was vaccinated s.c. with 1 mL of 15 μg PSP/mL vaccine and a control group ($n = 6$) was immunized with Amphigen containing uninfected 72-h SB-CEV-1/F7 tissue culture media. Both groups were boosted orally 3 d later using the same dose and volume as the primary immunization. Another group ($n = 5$) of 1-d-old birds were trickle-immunized with 500 *E. tenella* oocysts in 1 mL daily for 5 consecutive d. At 10 d of age, vaccinated, trickle-immunized, and control birds were weighed and challenged orally with 35,000 *E. tenella* sporulated oocysts. A 10-d-old unimmunized group ($n = 5$) was weighed and mock-challenged with distilled water. At 6 d postchallenge, weight gain and lesions scores were determined.

Statistical Analysis. Bird performance data, specifically weight gain (Days 0 to 6 postchallenge) and lesion scores (Day 6 postchallenge), were analyzed using the General Linear Models (Version 5.1) procedure of the SAS Institute (1988). Least squares statistical comparisons for weight gain and lesion score were made and shown only between the control (unimmunized/challenged or sham-immunized/challenged) and vaccinated (immunized/challenged) groups.

RESULTS

Immunogenicity Studies

Immunization with 72-h AG. The immunogenicity of the 72-h AG was initially examined in *B*¹⁹*B*¹⁹ birds using two different immunization routes. Results indicate that

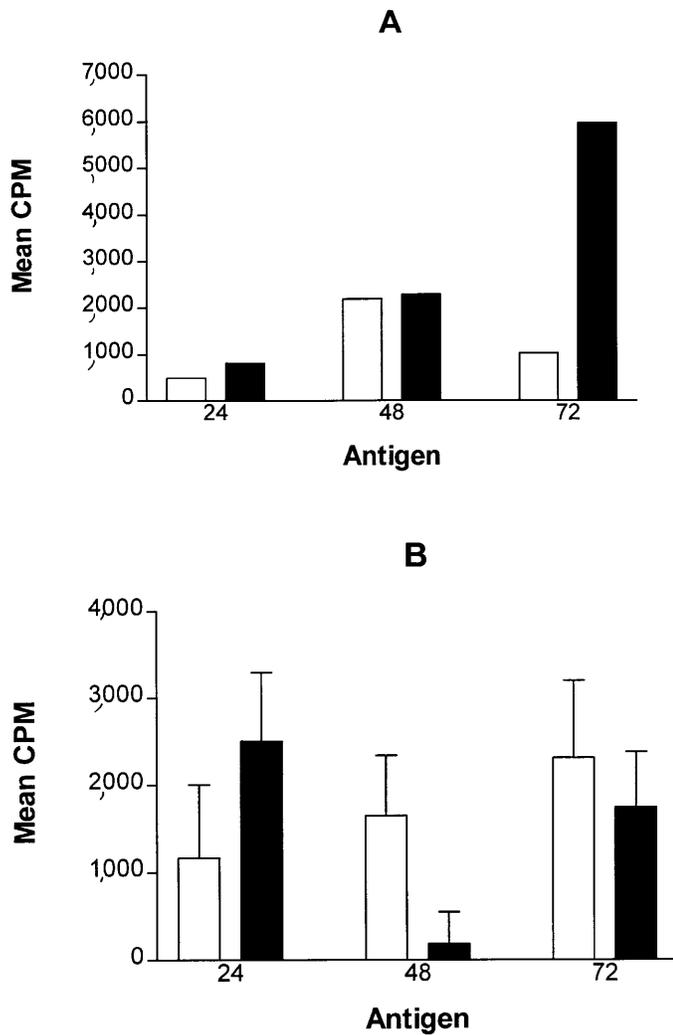


FIGURE 1. A) Pooled peripheral blood lymphocyte ($n = 1$) responses to *Eimeria tenella*-infected SB-CEV-1/F7 tissue culture antigens harvested 24, 48, or 72 h after infection from either orally (■) or s.c. (□) vaccinated birds. B) Splenic lymphocyte ($n = 3$) responses ($\bar{x} \pm SD$) to *E. tenella*-infected SB-CEV-1/F7 tissue culture antigens harvested 24, 48, or 72 h after infection from either orally (■) or s.c. (□) vaccinated birds.

PBL (Figure 1A) and splenic lymphocytes (Figure 1B) from either orally or s.c. vaccinated birds responded to 24-, 48-, and 72-h AG following *in vitro* stimulation. The highest proliferative response was measured in the orally vaccinated group following PBL stimulation with 72-h AG. These results indicate that the 72-h AG is immunogenic in $B^{19}B^{19}$ birds. In addition, the results demonstrate that lymphocytes primed with 72-h AG *in vivo* can proliferate to 24- and 48-h AG *in vitro*.

Trickle Immunization and Challenge at 10 Days. In order to determine whether *in vitro* produced *E. tenella* parasite antigens were capable of being recognized by lymphocytes obtained from *E. tenella* immune birds, $B^{19}B^{19}$ splenic lymphocyte proliferative responses were tested in cultures containing either extracellular (sporozoite, merozoite) antigens, the 72-h AG, or the 72-h AG-ASP. Immune splenic lymphocytes co-cultured with either sporozoite (Figure 2A) or merozoite (Figure

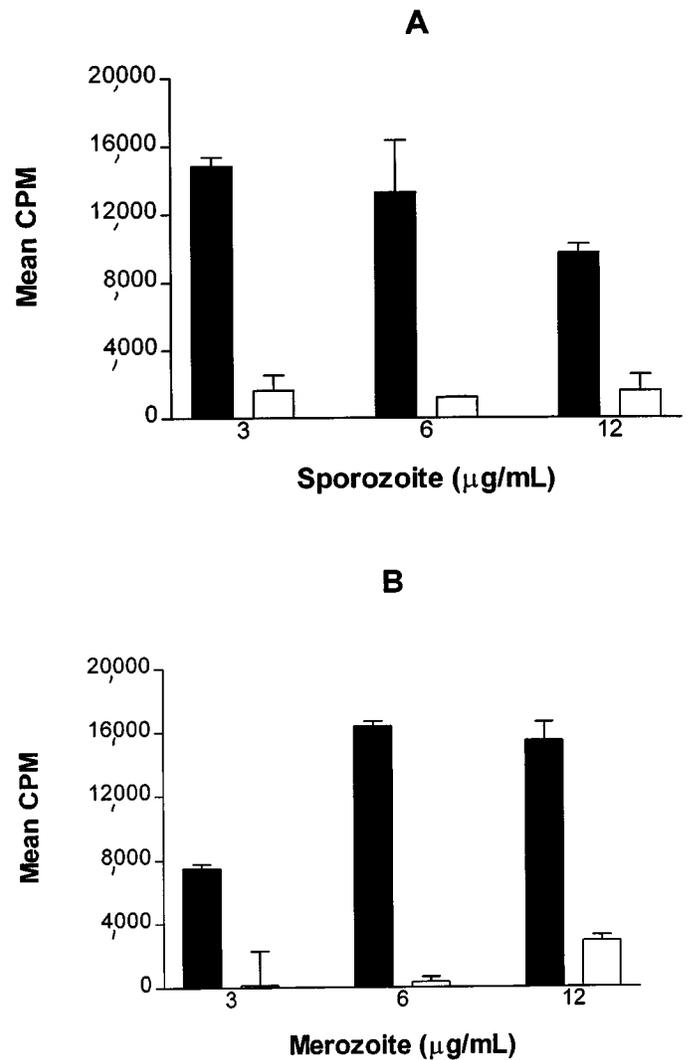


FIGURE 2. Dose-dependent response to *Eimeria tenella* sporozoites (A) and merozoites (B) using splenic lymphocytes. Values ($n = 4$) represent $\bar{x} \pm SD$ from either naturally exposed and challenged (■) or naive (□) birds.

2B) AG showed higher proliferative responses than splenic lymphocytes obtained from naive birds. More importantly, splenic lymphocytes isolated from trickle-immunized, challenged birds showed higher proliferative responses than age-matched naive birds against both 72-h AG (Figure 3A) and 72-h AG-ASP (Figure 3B). However, part of the response to the 72-h AG-ASP appeared to be nonspecific, based on the dose-dependent responses observed using naive splenocytes. These data show that immune lymphocytes obtained from *E. tenella* immune birds proliferated *in vitro* when stimulated with parasite AG contained in the tissue culture supernatant of an *E. tenella*-infected cell line.

Trickle Immunization and Challenge at 16 or 43 d. Based on the previous results, the relative PSP stimulatory activity of the 72-h AG-ASP was compared against both sporozoite and merozoite antigens. Dose response results (Figure 4) indicate that the 72-h AG-ASP added to a final concentration of 8 µg PSP/mL gave the highest prolifera-

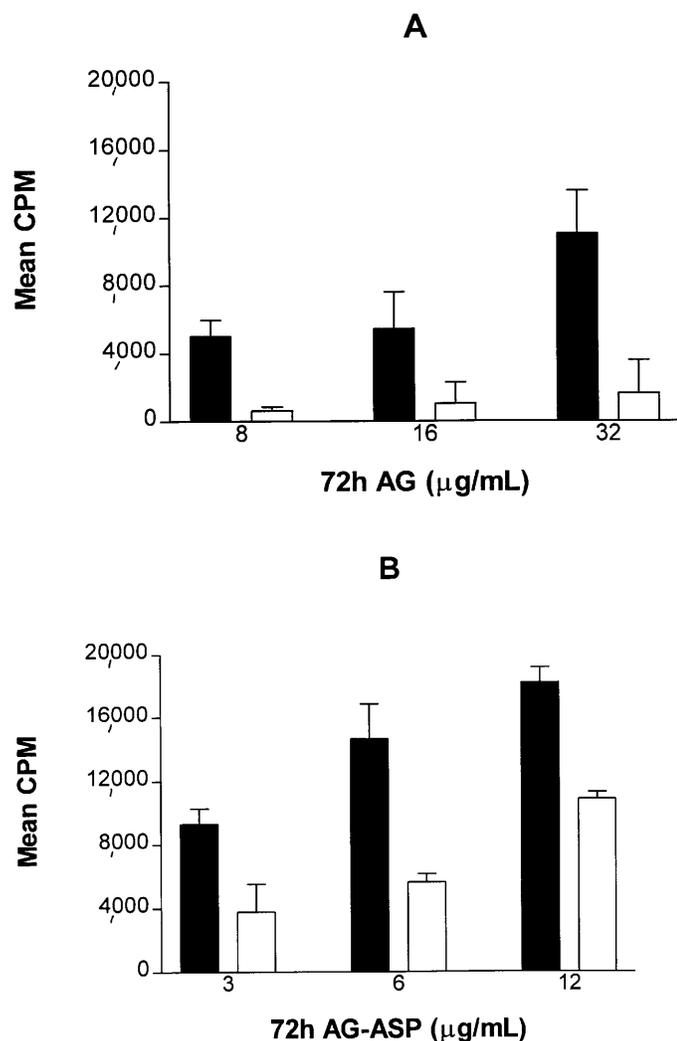


FIGURE 3. Dose-dependent response to *Eimeria tenella*-infected SB-CEV-1/F7 tissue culture supernatant harvested 72 h after infection (72 h AG) (A), and then fractionated with 30% ammonium sulfate (72 h AG-ASP) (B) using splenic lymphocytes. Values ($n = 4$) represent $\bar{x} \pm \text{SD}$ from either naturally exposed and challenged (■) or naive (□) birds.

tive response. In contrast, the highest proliferative concentration for both sporozoite or merozoite antigen was 16 μg PSP/mL.

Protection Studies

Immunization with 72-h AG. The ability of 72-h AG to confer protection in naive birds was initially examined using two different vaccine doses. Results (Table 1) show that both high and low doses of 72-h AG-vaccinated groups immunized at 4 d of age and boosted 3 d later were significantly protected against weight loss compared to unimmunized, challenged controls, following challenge at 10 d of age. The mean weight gain in vaccinated birds was identical to the mean value obtained in both challenged trickle-immunized and unimmunized, unchallenged controls. Moreover, the 72-h AG-vaccinated group that received the higher PSP vaccine dose also demonstrated a significant reduction in

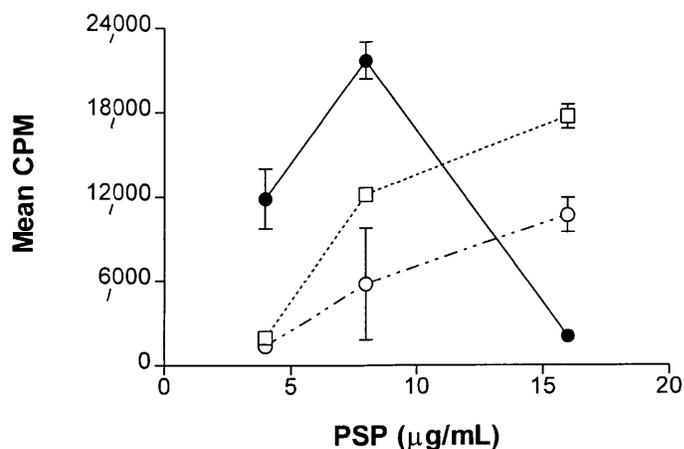


FIGURE 4. Comparison of the dose-dependent response among SB-CEV-1/F7 supernatants harvested 72 h after *Eimeria tenella* infection, then fractionated with 30% ammonium sulfate (■); sporozoite (□); and merozoite (○) antigens using splenic lymphocytes from naturally exposed, challenged birds. Values ($n = 5$) represent $\bar{x} \pm \text{SD}$.

lesions. In contrast, the group immunized and boosted with the lower vaccine dose was not protected against cecal lesions.

Immunization with Ammonium Sulfate-Fractionated 72-h AG. Because the 72-h AG-ASP contained a higher PSP concentration than the 72-h AG, and because the 72-h AG-ASP stimulated lymphocytes from *E. tenella* immune birds (Figure 4), the ability of 72-h AG-ASP to confer protection against parasite challenge was tested. Six days postchallenge performance results (Table 2) indicate that the 72-h AG-ASP vaccinated birds were protected significantly ($P \leq 0.001$) against weight depres-

TABLE 1. Mean weight gain and lesion scores in $B^{19}B^{19}$ birds vaccinated with tissue culture supernatant of SB-CEV-1/F7 cells harvested 72 h after infection with *Eimeria tenella*

Treatment group	Challenge ¹	n	Weight gain ² (g)	Lesion score ²
Not immunized	no	7	43 \pm 2.1	0.0 \pm 0.0
Not immunized	yes	8	31 \pm 1.8	3.0 \pm 0.0
Trickle immunization ³	yes	8	43 \pm 1.9	0.3 \pm 0.2
Vaccine (low PSP) ⁴	yes	8	43 \pm 0.8**	3.3 \pm 0.2
Vaccine (high PSP) ⁵	yes	8	43 \pm 1.9*	1.8 \pm 0.4**

¹Ten-day-old chicks were orally challenged with 35,000 *E. tenella* oocysts.

²Mean weight gain and lesion scores ($\bar{x} \pm \text{SEM}$) were determined 6 d after challenge. Significance values are shown only for vaccinates.

³*Eimeria tenella* oocysts (500 per day) were administered orally for 5 consecutive d to 2-d-old chicks.

⁴Parasite-specific protein (PSP) was administered s.c. at 2 μg PSP per dose to 4-d-old chicks, and orally boosted 3 d later with the same vaccine.

⁵Parasite-specific protein (PSP) was administered s.c. at 19 μg PSP per dose to 4-d-old chicks, and orally boosted 3 d later with the same vaccine.

* $P \leq 0.05$ when compared to the nonimmunized challenge group.

** $P \leq 0.01$ when compared to the nonimmunized challenge group.

TABLE 2. Mean weight gain and lesion scores in *B¹⁹B¹⁹* birds vaccinated with an ammonium sulfate-fractionated supernatant of SB-CEV-1/F7 cells harvested 72 h after infection with *Eimeria tenella*

Treatment group	Challenge ¹	n	Weight gain ²	Lesion score ²
			(g)	
Not immunized	no	4	47 ± 3.7	0.1 ± 0.1
Not immunized	yes	8	42 ± 2.6	2.6 ± 0.8
Trickle immunization ³	yes	8	44 ± 2.7	0.8 ± 0.2
Tissue culture vaccine ⁴	yes	8	51 ± 1.8***	2.4 ± 0.3

¹Ten-day-old chicks were orally challenged with 35,000 *E. tenella* oocysts.

²Mean weight gain and lesion scores ($\bar{x} \pm \text{SEM}$) were determined 6 d after challenge. Significance values are shown only for vaccinates.

³*Eimeria tenella* oocysts (500 per day) were administered orally for 5 consecutive d to 1-d-old chicks.

⁴Parasite-specific protein was administered s.c. at 9 µg per dose to 4-d-old chicks, and orally boosted 3 d later with the same vaccine.

*** $P \leq 0.001$ when compared to the nonimmunized challenge group.

sion when challenged at 10 d of age. In contrast to the previous protection experiment, the vaccinated group failed to demonstrate a significant reduction in lesions.

Immunization of Birds of Different Ages with Ammonium Sulfate-Fractionated 72-h AG. In order to evaluate the influence of bird age with respect to immunization and protection, chicks received a primary immunization of 72 h AG-ASP vaccine at either 1 or 4 d of age, and were boosted 3 d later. Protection results (Table 3) show that only the 4-d-old birds immunized and boosted with 72-h AG-ASP were protected significantly against weight loss compared to 4-d-old immunized controls. As observed in the previous experiment, no protection against lesions was afforded by the 72-h AG-ASP vaccine.

Immunization with Ammonium Sulfate-Fractionated 72-h AG Following Prolonged Storage. To begin to examine the effect of prolonged storage on vaccine efficacy, the 72-h AG-ASP used in the previous experi-

ment was stored at 4 C for 24 d, mixed with Amphigen, and used to immunize birds. The 72-h AG-ASP vaccine retained weight protective activity (Table 4). In addition, although the mean lesion score in vaccinated birds was greater than 2+, significant protection ($P \leq 0.05$) was observed.

DISCUSSION

Contemporary research toward the development of an effective vaccine against avian coccidiosis has focused on the cloning and expression of *Eimeria* gene products identified with either monoclonal antibodies or sera obtained from hyperimmunized birds. Although the database for *Eimeria* gene sequences continues to increase, many of the parasite proteins encoded by these genes are expressed on the surface of extracellular sporozoite and merozoite forms, as well as gametocytes (Danforth and Augustine, 1990). Perhaps these surface proteins function as decoys to the host's immune system. In contrast, there is a paucity of sequence and biochemical information available on genes and parasite proteins expressed differentially during the intracellular stages of the parasite life cycle. This observation is intriguing because acquired immunity to *Eimeria* appears to be directed toward the metabolizing intracellular parasite. Sporozoites invade target intestinal cells but are prevented from continued development in an immune host (Leatham and Burns, 1967). In addition, challenged birds previously vaccinated with x-irradiated oocysts are protected against weight loss, despite histological evidence that sporozoites from x-irradiated oocysts maintain their capacity to invade epithelial cells but fail to exhibit merogonic development (Jenkins *et al.*, 1991a,b). Therefore, mature first-generation merogonic development is not an essential step for the induction of the immune mechanisms responsible for weight performance protection. Characterization of these intracellular metabolic products has been hampered by the inability

TABLE 3. Mean weight gain and lesion scores in *B¹⁹B¹⁹* birds vaccinated at different ages with an ammonium sulfate-fractionated supernatant of SB-CEV-1/F7 cells infected with *Eimeria tenella*

Treatment group ¹	Age at injection		n	Weight gain ²	Lesion score ²
	Primary	Booster			
(d)			(g)		
Not immunized	1	4	8	21 ± 2.4	3.0 ± 0.0
Not immunized	4	7	8	23 ± 6.0	3.0 ± 0.0
Trickle immunization ³	8	55 ± 2.4	0.0 ± 0.0
Tissue culture vaccine ⁴	1	4	5	31 ± 6.2	3.0 ± 0.0
Tissue culture vaccine ⁴	4	7	7	35 ± 4.7**	2.7 ± 0.2

¹All treatment groups were orally challenged with 35,000 *E. tenella* oocysts at 10 d of age.

²Mean weight gain and lesion scores ($\bar{x} \pm \text{SEM}$) were determined 6 d after challenge. Significance values are shown only for vaccinates.

³*Eimeria tenella* oocysts (500 per day) were administered orally for 5 consecutive d to 1-d-old chicks.

⁴Parasite-specific protein was administered s.c. at 15 µg per dose to 1 or 4-d-old chicks, and orally boosted 3 d later with the same vaccine.

** $P \leq 0.01$ when compared to the respective nonimmunized challenge groups.

TABLE 4. Mean weight gain and lesion scores in *B¹⁹B¹⁹* birds vaccinated with an ammonium sulfate-fractionated vaccine following prolonged storage

Treatment group	Challenge ¹	n	Weight gain ² (g)	Lesion score ²
Not immunized	no	5	35 ± 2.8	0.0 ± 0.0
Not immunized	yes	6	19 ± 3.0	3.3 ± 0.2
Trickle immunization ³	yes	5	34 ± 2.5	0.6 ± 0.4
Tissue culture vaccine ⁴	yes	5	30 ± 1.9**	2.5 ± 0.3*

¹Ten-day-old chicks were orally challenged with 35,000 *E. tenella* oocysts.

²Mean weight gain and lesion scores ($\bar{x} \pm \text{SEM}$) were determined 6 d after challenge. Significance values are shown only for vaccinates.

³*Eimeria tenella* oocysts (500 per day) were administered orally for five consecutive days to 1-d-old chicks.

⁴Parasite-specific protein was administered s.c. at 15 μg per dose to 4-d-old chicks, and orally boosted 3 d later with the same vaccine.

* $P \leq 0.05$.

** $P \leq 0.01$ when compared to the nonimmunized challenge group.

to reproducibly grow *Eimeria* to high densities *in vitro*. This problem has been partially overcome by this laboratory through the recent isolation and characterization of a continuous cell line, designated SB-CEV-1/F7, which supports vigorous *E. tenella* sporozoite invasion and merogonic development (Danforth *et al.*, 1994). The present study was conducted to determine the immunogenic and protective properties of *E. tenella*-infected SB-CEV-1/F7 tissue culture supernatants in a bird line defined serologically at the *B*-locus.

Infected SB-CEV-1/F7 tissue culture supernatant harvested between 48 and 72 h postinfection was selected for immunogenicity studies because this preparation routinely contained the highest amounts of PSP (J. Lineberger, unpublished data). The adjuvant Amphigen was chosen because it has been approved for use in animal biological vaccines and can be administered both parenterally or orally. The PBL and splenic lymphocytes from *B¹⁹B¹⁹* birds vaccinated with 72-h AG proliferated *in vitro* in response to this immunogen. Moreover, these same cell populations were also stimulated in the presence of the 24- and 48-h infected tissue culture supernatants as well as sporozoite AG (data not shown), suggesting the presence of cross-reactive epitopes on these parasite proteins. From the present study, it is difficult to determine which time point possessed the best *in vitro* stimulatory activity, as the supernatants were adjusted to the same total protein, not PSP concentration. In general, more uniform and higher lymphocyte proliferative responses were detected in s.c. vaccinated birds than in orally vaccinated birds.

Results from the subsequent immunogenicity study showed a dose-dependent *in vitro* response to the 72-h AG. These data indicate that the SB-CEV-1/F7 cell line supports the *in vitro* expression and production of parasite gene products, which can be recognized by the immune system of birds previously exposed to *E. tenella* infection. Furthermore, the fact that lymphocytes from

immune birds also responded *in vitro* to the 72 h AG-ASP supports the conclusion that ammonium sulfate precipitation does not severely alter or destroy putative T cell epitopes on parasite proteins present in this preparation. However, ammonium sulfate precipitation did appear to alter the immune recognition pattern by naive lymphocytes in some undefined manner, as higher nonspecific stimulation was observed using the ammonium sulfate preparation than that observed with the 72-h AG. Moreover, the fact that splenic lymphocytes harvested 20 d postchallenge were still capable of *in vitro* proliferative responses to the 72-h AG-ASP indicates that the memory response to the infected SB-CEV-1/F7 tissue culture supernatants in naturally exposed, challenged *B¹⁹B¹⁹* birds is at least 3 wk. Furthermore, immune responses to these tissue culture-derived parasite antigens can be detected as early as 4 d postchallenge in immune birds (D. Brake *et al.*, unpublished data).

In order to further study the *in vitro* immune response to the 72-h AG-ASP, splenic lymphocytes were removed from *E. tenella* immune birds 48 h following a challenge infection. Unlike the previous *in vitro* assays, comparison of the stimulatory activities of sporozoite, merozoite, and 72 h AG-ASP was based on PSP rather than total protein. Optimal stimulation with either the sporozoite or merozoite AG required an approximately twofold higher PSP concentration than stimulation with the 72-h AG-ASP. One possible explanation for this result is that the parasite AG present in the 72-h AG-ASP preparation are more efficiently processed and recognized by immune lymphocytes *in vitro*. An alternative interpretation is that the rabbit polyclonal anti-sporozoite sera used in the ELISA to determine PSP concentrations is biased toward sporozoite-specific epitopes and does not efficiently recognize epitopes present on infected SB-CEV-1/F7 tissue culture-derived parasite AG. Western blot results using the rabbit polyclonal antisera support this argument in that different immunoreactivity profiles were observed between sporozoite and tissue culture-derived parasite antigens (D. Brake *et al.*, unpublished data).

Based on the *in vitro* assays, the 72-h AG was tested for the ability to confer protection against disease in naive *B¹⁹B¹⁹* birds. An immunization strategy based on a primary s.c. vaccination followed by an oral boost 3 d later was selected for the protection studies. This strategy was based on results from a series of immunogenicity studies in young chicks using various tissue culture-derived supernatant preparations (D. Brake *et al.*, unpublished data). In the protection experiment using both low and high doses of PSP, vaccinated birds were protected against weight loss following challenge. Although both vaccinated groups had identical mean weight gains, protection against lesions was observed only in the high dose group, suggesting that a higher amount of PSP is required to prevent disease pathology.

In order to rule out the possibility that the Amphigen adjuvant or uninfected SB-CEV-1/F7 derived cell proteins were responsible for the protection observed, a second protection study was performed in which control birds were immunized and boosted with Amphigen containing uninfected SB-CEV-1/F7 tissue culture media. Results clearly showed that only birds immunized with 72-h AG-ASP were protected against weight loss. However, the vaccine failed to induce protection against cecal lesions. One plausible explanation for this result is that the parasite antigens contained in the ammonium sulfate vaccine are processed and presented *in vivo* through a different pathway than untreated parasite AG released directly into the tissue culture media during SB-CEV-1/F7 infection. Alternatively, 30% $(\text{NH}_4)_2\text{SO}_4$ may differentially precipitate only those parasite AG that induce host immune mechanisms responsible for protection against weight loss. Precipitation of additional parasite proteins recognized by immune pathways operative to reduce cecal lesions in *B19B19* birds may require higher salt concentrations. This concept is supported by data that shows that sequential ammonium sulfate precipitation of 72-h AG results in distinct silver staining and Western blot reactivity profiles using *E. tenella* immune sera (J. Lineberger, unpublished data).

Because the target age for immunization against coccidiosis is newly hatched or 1-d-old chicks, the efficacy of the 72-h AG-ASP vaccine was compared in 1-d-old vs 4-d-old chicks. Surprisingly, only 4-d-old vaccinated chicks were protected against weight loss following challenge. In addition, consistent with previous results, no protection against lesions was detected. The failure of 1-d-old birds to be effectively immunized with the tissue culture-derived vaccine is not clear but may be related to neonatal tolerance (Morgan and Tempelis, 1983). Neonatal tolerance mechanisms may interfere with immunization efficacy when killed, non-replicating, or subunit vaccines are utilized, but perhaps are bypassed or blocked when attenuated or live vector vaccine delivery systems are employed.

To begin to address the stability of these tissue culture-derived parasite AG as it relates to protective activity, the 72-h AG was stored at 4 C for 24 d, then formulated and used to immunize 4-d-old birds. Subsequent protection against weight loss suggests that these tissue culture-derived parasite AG are fairly stable with respect to *in vivo* activity. This result is consistent with ELISA data, in which no appreciable loss of PSP activity occurs following a single freeze-thaw cycle (T. Banas, unpublished data).

In summary, the present study provides evidence that *E. tenella*-infected SB-CEV-1/F7 tissue culture supernatants are immunogenic in birds. More importantly, results suggest that the SB-CEV-1/F7 tissue culture-derived vaccine provides partial protection against homologous parasite challenge. Significant protection against weight loss was shown in four independent studies conducted in the serologically defined *B19B19*

bird line. The failure of the vaccine to consistently protect against lesions is presently unclear, but several possibilities are worthy of consideration. First, the lack of correlation between weight loss and moderate lesion scores may be a phenomenon restricted to *E. tenella* based on preferential replication in the cecum, as opposed to other intestinal regions associated with nutrient uptake. Secondly, parasite-induced alterations in the host cytokine network has been postulated to play a role in disease pathogenesis (Cox and Liew, 1992). Overproduction of pro-inflammatory cytokines such as tumor necrosis factor and interleukin-1 directly elicit changes in a variety of target cells and indirectly amplify the magnitude of the inflammatory response (Dinarello, 1989). Production of these immune mediators in response to parasite invasion may contribute to the gross pathology observed during clinical coccidiosis. Lastly, the lack of consistent lesion protection could be attributed to the bird line used in the present study. Additional studies in the laboratory are focused on the characterization of the local mucosal immune responses to these tissue culture-derived parasite AG following challenge in naive and trickle-immunized birds. These experiments should allow for the identification and eventual cloning of SB-CEV-1/F7 intracellular *E. tenella* and other *Eimeria* species genes involved in the induction of host protection.

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