INFECTION AND IMMUNITY, July 1989, p. 2014–2020
0019-9567/89/072014-07$02.00/0

Vol. 57, No. 7

Characterization and Vaccine Potential of a Novel Recombinant Coccidial Antigen

GLENN A. MILLER,[1]* BALBIR S. BHOGAL,[1] RUSSELL McCANDLISS,[2] ROBERT L. STRAUSBERG,[2] ELLEN J. JESSEE,[1] ALICE C. ANDERSON,[1] CINDY K. FUCHS,[1] JAMES NAGLE,[2] MELODIE H. LIKEL,[2] JEANNE M. STRASSER,[2] AND SUSAN STRAUSBERG[2]

Molecular Biology Department, A. H. Robins Co., Richmond, Virginia 23220,[1] and Biochemical Genetics Department, Genex Corporation, Gaithersburg, Maryland 29877[2]

Received 21 February 1989/Accepted 15 April 1989

A cDNA clone derived from sporulated oocysts of *Eimeria tenella* and encoding the expression product GX3262 was identified using a monoclonal antibody (12-09) raised against *Eimeria acervulina* sporozoites. The cDNA fragment containing the coccidial antigen gene was cloned in bacteriophage lambda gt11, transferred to a plasmid, and introduced into *Escherichia coli* for analysis of the gene products. The strain carrying the plasmid produced GX3262 as part of a fusion protein consisting of the first 1,006 amino acids of *E. coli* β-galactosidase and 112 amino acids of the *E. tenella* protein of approximately 12 kilodaltons. Partially purified antigen, heat-killed recombinant bacterin, and live *E. coli* containing the recombinant coccidial antigen were used to immunize 1-week-old or newly hatched broiler chicks. Several immunization protocols were utilized, including boosts with partially purified β-galactosidase–GX3262, bacterin, or small numbers of live *E. tenella* oocysts. After challenge with an experimental *E. tenella* infection, the birds were evaluated by scoring cecal lesions to determine the level of protection. The greatest degree of protection was seen after only a single immunization of 2-day-old birds with a live recombinant *E. coli* preparation. The results presented here identify GX3262 as a potential candidate coccidial vaccine antigen and provide evidence for the first time that newly hatched chickens can be successfully vaccinated with a recombinant antigen.

Coccidiosis, an intestinal disease caused by protozoan parasites of the genus *Eimeria*, is characterized by bloody diarrhea, morbidity, and mortality in young chickens. Although chemoprophylaxis is almost the exclusive means of control, over the years a number of methods have been explored, such as the use of genetically resistant birds (13); nutritional techniques including the use of vitamin supplements (19); sanitation and quarantine (19); and live vaccines (21). In spite of the availability of a number of chemotherapeutic agents (14), coccidiosis remains one of the major disease problems confronting the poultry industry. Coccidial chemoprophylaxis is complicated by the frequent and unpredictable development of drug resistance, the cost of synthesis and testing of new anticoccidial agents, the necessity for drug withdrawal periods, and the possibility that restraints will be placed on medication used in feed given animals grown for human consumption. Therefore the development of an alternative control method, such as a vaccine, could clearly be useful and practical. It has long been known that chickens recovered from *Eimeria* infections show a strong immunity to subsequent exposure to the parasite (20, 21). On the basis of such observations, a live oocyst vaccine known as CocciVac (Sterling) has been available for controlling infections in breeder chickens and egg layers. Until recently, attempts to vaccinate chickens with nonviable parasites have been unsuccessful (20), and the prospects for developing a subunit vaccine have appeared dim. Recently, however, Murray et al. (17) have demonstrated protection against virulent experimental coccidial infections of day-old broilers by vaccination with coccidial extracts devoid of living parasites. Similarly, Danforth et al. (5; H. D. Danforth, P. C. Augustine, M. D. Ruff, R. McCandliss, R. L. Strausberg, and M. Likel, submitted for publication) have reported the discovery of a coccidial gene expression product with the ability to impart some protection against *Eimeria tenella* infections when administered as a Freund complete adjuvant emulsion to 4-week-old birds. Using a monoclonal antibody (MAb) raised against *Eimeria acervulina* sporozoites, we have identified and cloned a gene from *E. tenella* coding for an antigen that is capable of imparting significant protection against *E. tenella* after a single immunization of 2-day-old broilers. In the present report, we describe the identification and characterization of this antigen, designated GX3262, and demonstrate its potential as a candidate vaccine for broilers.

MATERIALS AND METHODS

Parasites. Sporulated *E. tenella* oocysts, batch USDA-API no. 24 for isolation of mRNA and batch AHR no. 855B for use in in vivo studies, were prepared from cecal washings (3). In general, oocyst suspensions were filtered through several layers of cheesecloth to remove fecal material and were then collected by centrifugation, suspended in 2.5% potassium dichromate to induce sporulation, and maintained in potassium dichromate until used. Before administration to chickens, the oocysts were washed free of dichromate, treated for 2 min on ice with 5.0% sodium hypochlorite (The Clorox Co., Oakland, Calif.), and centrifuged at 2,600 × *g* to pellet digested material and float clean oocysts. The oocyst-containing supernatant was saved, and the oocysts were washed repeatedly with water and adjusted to appropriate concentration in phosphate-buffered saline (PBS) (pH 7.3). Before use in mRNA isolation, the oocysts were washed, suspended in 5.0% sodium hypochlorite (Clorox), washed, and layered over sucrose. Sporulated oocysts were then collected after centrifugation from the interface and from the layer just above the interface.

Construction of the *E. tenella* cDNA library. Total RNA

* Corresponding author.

was prepared from oocysts by the method of Pasternak et al. (18). To approximately 0.75 g of *E. tenella* oocysts (USDA-API no. 24) in 1.5 ml of lysis buffer (10 mM Tris acetate, 75 mM sodium acetate, 2 mM EDTA, 1% sodium dodecyl sulfate [SDS], 200 μg of proteinase K per ml, pH 7.5), an equal volume of sterile glass beads (0.45 to 0.50 mm in diameter) was added, and the oocysts were broken by vortexing for approximately 2 min. The preparation was centrifuged at 4,302 × *g* for 10 min, and the supernatant was recovered and incubated at 37°C for 20 min. The solution was extracted with a saturated phenol solution containing 10 mM Tris acetate, 75 mM sodium acetate, and 2 mM EDTA (pH 7.5) and then extracted with chloroform, and the total RNA was precipitated by addition of 0.1 volume of 2.4 M sodium acetate (pH 5.5) and 2.5 volumes of 95% ethanol at −20°C overnight. The RNA was then pelleted at 11,950 × *g* for 15 min in an SS-34 rotor, desiccated, suspended in sterile distilled water, and stored at −80°C. Poly(A)$^+$ RNA was separated from total RNA by oligo(dT)-cellulose chromatography (1). Bound RNA was eluted with 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA. Fractions containing poly(A)$^+$ RNA were combined, and the RNA was ethanol precipitated as described above. Methods for double-stranded cDNA preparation were adapted from those of McCandliss et al. (16).

For cloning of the cDNA into the *Eco*RI site of bacteriophage lambda gt11, *Eco*RI linkers were added to the ends of the cDNA molecules. After ligation of the cDNA with lambda gt11, the DNA was packaged into phage heads (8). *Escherichia coli* Y1088 (ATCC 37195) was used as a host for titration and propagation of the phage. Bacteriophage lambda gt11 and all bacterial strains used with it were obtained from Ron Davis (Stanford University, Stanford, Calif.) and are described by Hunyh et al. (10).

Plaque screening using antibodies. Phage from an amplified library of *E. tenella* cDNA in lambda gt11 were used to transfect host *E. coli* Y1090 (ATCC 37197) for plaque formation. The plaques were then transferred to nitrocellulose filters saturated with 0.01 M isopropyl-β-D-thiogalactopyranoside (IPTG) and screened for their ability to bind MAb 12-09 by a modification of the method used by Danforth et al. (submitted). A bacteriophage giving a positive response was transfected into *E. coli* Y1089 to produce a lysogen. The antigen encoded by the phage was designated GX3262.

Analysis of GX3262 cDNA. Phage DNA was prepared using methods described by Maniatis et al. (15). The purified phage DNA was digested with restriction endonuclease *Eco*RI. The fragment encoding the GX3262 antigen was subcloned into bacteriophage M13 mp8 for DNA sequence analysis by the dideoxy method (2, 24).

Transfer of GX3262 cDNA coding sequence to an *E. coli* expression plasmid. The *lac* promoter, β-galactosidase gene, and GX3262 coding sequence cloned in lambda gt11 were excised with restriction endonucleases *Kpn*I and *Bam*HI and inserted between the unique *Kpn*I and *Bam*HI sites in plasmid pGX3213. Plasmid pGX3213 is a modified form of pGX1066 (23) carrying the set of cloning sites from pUC18. The newly assembled plasmid was designated pGX3262.

Production and purification of the β-gal–GX3262 antigen. To produce the β-galactosidase–GX3262 fusion protein (β-gal–GX3262), recombinant *E. coli* cells, grown overnight in Luria-Bertani broth with ampicillin and induced with IPTG, were lysed by sonication and centrifuged, and the insoluble cell pellet was washed extensively with PBS. It was found that the fusion antigen produced from pGX3262 was primarily insoluble in the cells. The insoluble cell material was dissolved in 6 M guanidine hydrochloride, and after centrifugation, the soluble fraction was dialyzed against PBS to remove guanidine hydrochloride, at which point the antigen precipitated. The purity of these preparations, as indicated by SDS-polyacrylamide gel electrophoresis and laser scanning, ranged from 40 to 60%. For use in certain studies a control antigen preparation, GX3217, was also prepared. Plasmid pGX3217 carries all of the genetic material of pGX3262 except that it has no cloned cDNA. The GX3217 antigen preparation was made in a manner identical to β-gal–GX3262 but from *E. coli* carrying pGX3217.

Electrophoresis and Western blotting (immunoblotting). Prior to animal studies, all antigens were analyzed by SDS-polyacrylamide gel electrophoresis, laser densitometry, and Western blot, and for total protein concentration. The antigens were electrophoresed under reducing conditions on 6% stacking and 10 or 12.5% resolving SDS-polyacrylamide gels by the method of Laemmli (12). Pairs of identical gels were run; one was stained with Coomassie blue R-250 for scanning with an Ultroscan XL Enhanced Laser Densitometer (LKB Instruments), and the other was transferred to nitrocellulose membrane for immunoassay. The protein transfer was performed according to the method of Towbin et al. (24), using Blotto instead of bovine serum albumin (10). The blot was probed with MAb 12-09 and horseradish peroxidase-conjugated secondary antibody (Kirkegaard and Perry Laboratories, Gaithersburg, Md.). The transferred molecular weight standards were stained with amido black 10B. Protein concentrations were determined with the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, Ill.).

Preparation of heat-killed bacterin carrying the GX3262 antigen. Recombinant bacteria grown for approximately 5 h in Luria-Bertani broth were collected by centrifugation, suspended in PBS, and subjected to heat treatment at 65°C for 1 h. After inactivation, the cells were tested for viability and evaluated for total protein and antigen content. These preparations contained from 5 to 10% of total protein as the β-gal–GX3262 fusion protein.

In vivo evaluation of GX3262. Male Hubbard × Hubbard broilers (Hubbard Farms, Statesville, N.C.) at either 2 days or 1 week of age were used in these studies. Chickens with body weights within 10% of the mean were randomized into groups of 10 to 15 before use and housed in a brooder (Petersime Incubator Co., Gettsyburg, Ohio). At 14 days post-hatch, chicks were moved to large wire-floored cages for the duration of the study. The basic experimental design involved immunizing birds subcutaneously or orally with partially purified recombinant antigen preparations, heat-killed bacterins, live *E. coli* cells, or control materials; challenging them with *E. tenella* oocysts; and then evaluating them for cecal lesions as described by Johnson and Reid (11). This involves evaluating the level of infection in both ceca according to a score range of 0 (no pathology) to 4 (maximal pathology). For all immunizations, antigen samples were diluted in 30% Alhydrogel (Superfoss; Sergeant Chemical Co., Clifton, N.J.) based on percent fusion protein as determined by densitometric scanning of SDS gels. Statistical evaluation of data was accomplished by using the Dunnett one-tailed test (7).

RESULTS

Identification and characterization of GX3262. The apparent molecular mass of the β-galactosidase fusion protein as

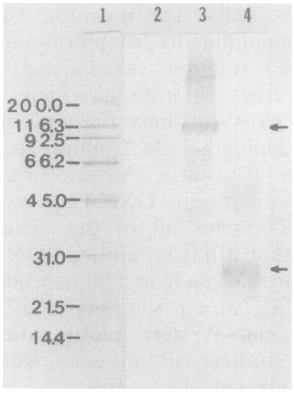

FIG. 1. Western blot analysis of coccidial proteins. Lane 1, Molecular size markers; lane 2, GX3217 antigen; lane 3, GX3262 antigen; lane 4, *E. tenella* sporulated oocyst antigen. The blot was probed with MAb 12-09. Molecular size markers, indicated in kilodaltons at the left, were used to estimate the apparent molecular masses of coccidial proteins. Upper arrow indicates the 115-kDa β-gal–GX3262 recombinant fusion protein in lane 3, and lower arrow indicates the 28-kDa coccidial protein identified by MAb 12-09 in sporulated oocysts (lane 4).

determined by SDS-polyacrylamide gel electrophoresis and Western blotting (Fig. 1) was about 115 kilodaltons (kDa). The DNA sequence and predicted translation product for the GX3262 antigen are presented in Fig. 2. The sequence encoding the GX3262 antigen was determined by the method of Sanger et al. (22) and revealed that the antigen without β-galactosidase is composed of 112 amino acids with a predicted molecular mass of 12 kDa. It is therefore considerably smaller than the 28-kDa sporozoite refractile body protein shown by Western blot analysis to be detected by MAb 12-09 (Fig. 1). The DNA sequence also established that the cloned segment encodes the carboxy terminus of the *E. tenella* protein. The repeated regions observed in *Plasmodium* parasite-derived proteins (4, 25) and also in the 5401 antigen described by Danforth et al. (submitted) were not observed in GX3262. The segment directly adjacent to the β-galactosidase coding region is unusual in that it begins with 10 leucine residues. Amino acid composition analysis revealed that five amino acids, alanine, leucine, serine, glycine, and glutamine, are prominent in this antigen.

Protective immunity by partially purified β-gal–GX3262. Initial studies with GX3262 involved determining whether partially purified antigen could induce a protective response against *E. tenella* infection. One-week-old birds were selected for these studies, and multiple immunization protocols were utilized in an effort to maximize the ability to detect a protective response. On day 7 post-hatch, various groups of birds were immunized subcutaneously with 10, 50, or 100 μg of partially purified β-gal–GX3262 fusion protein in 30% Alhydrogel. Control birds were given Alhydrogel alone or PBS. Two weeks later, 100 μg of the fusion protein GX3262 was administered to three of the groups, and the birds were challenged orally with sporulated *E. tenella* oocysts 1 week later. Cecal lesions were evaluated 7 days after challenge (Table 1, study A). Immunization with 100 μg of β-gal–GX3262 resulted in significant protection (mean lesion score of 1.41 ± 0.01, which is a reduction of 37% compared with the control).

TABLE 1. Protection induced by partially purified β-gal–GX3262 fusion protein[a]

Study	Treatment		Lesion score ($\bar{X}$ ± SD)
	Day 7	Day 21	
A	GX3262 (10 μg)	GX3262 (100 μg)	2.30 ± 1.03
	GX3262 (50 μg)	GX3262 (100 μg)	2.21 ± 0.52
	GX3262 (100 μg)	GX3262 (100 μg)	1.41 ± 1.01
	Alhydrogel	Alhydrogel	2.24 ± 0.85
	PBS	PBS	2.11 ± 1.07
B	GX3262 (100 μg)	GX3262 (100 μg)	1.96 ± 1.05
	Alhydrogel	Alhydrogel	3.11 ± 0.35
	PBS	PBS	3.04 ± 0.50

[a] One-week-old birds were treated subcutaneously with partially purified β-gal–GX3262 fusion protein (GX3262) as indicated and then challenged on day 28 with *E. tenella* oocysts. Lesions were scored on day 35. Values underlined indicate significant reduction compared with Alhydrogel-treated controls at $P \leq 0.05$.

In a follow-up study (Table 1, study B), in which birds were likewise given two 100-μg doses of β-gal–GX3262, very similar results were observed. However, in this case the birds were challenged with enough oocysts to achieve a control lesion score of 3.11 ± 0.35, and immunized birds showed a reduction in their lesion scores to 1.96 ± 1.05, again a 37% reduction in lesion score even though the infection level was high. To ensure that the recombinant antigen alone was responsible for the protection, a group of birds were immunized with the control antigen preparation, GX3217. With such a preparation no protection was observed (data not shown).

In subsequent studies we evaluated dosage requirements of the β-gal–GX3262 fusion antigen (Table 2). Two immuni-

```
leu leu leu leu leu leu leu leu leu gln gly ala glu cys leu leu arg ser ser lys  20
CTG CTG CTG CTG CTG CTG CTG CTG CTG CAG GGC GCG GAG TGC CTC TTG AGG AGC AGC AAA  60

leu ala leu glu ala leu leu glu gly ala arg val ala ala thr arg gly leu leu leu  40
CTG GCC CTC GAG GCC CTC CTC GAG GGG GCC CGC GTT GCA GCA ACG CGG GGT TTG CTG CTG 120

val glu ser ser lys asp thr val leu arg ser ile pro his thr gln glu lys leu ala  60
GTC GAG AGC AGC AAA GAC ACG GTG CTG CGC AGC ATT CCC CAC ACC CAG GAG AAG CTG GCT 180

gln ala tyr ser ser phe leu arg gly tyr gln gly ala ala ala gly arg ser leu gly  80
CAG GCC TAC AGT TCT TTC CTG CGG GGC TAC CAG GGG GCA GCA GCG GGG AGG TCT CTG GGC 240

tyr gly ala pro ala ala ala tyr gly gln gln gln gln pro ser ser tyr gly ala pro 100
TAC GGG GCC CCT GCT GCT GCT TAC GGC CAG CAG CAG CAG CCC AGC AGC TAC GGG GCG CCC 300

pro ala ser ser gln gln pro ser gly phe phe trp ***                          112
CCC GCC TCC AGC CAG CAG CCC TCC GGC TTC TTC TGG TAG                          336
```

FIG. 2. Nucleotide sequence encoding the GX3262 antigen. The GX3262 DNA sequence is shown in capital letters, and the antigen amino acid sequence is shown in lowercase letters.

TABLE 2. Protection induced by single or multiple immunizations with partially purified β-gal–GX3262 fusion protein[a]

Treatment		Lesion score ($\bar{X}$ ± SD)
Day 7	Day 21	
GX3262 (100 μg)		3.58 ± 0.44
GX3262 (100 μg)	GX3262 (100 μg)	2.21 ± 0.69
GX3262 (200 μg)		2.87 ± 0.69
GX3262 (200 μg)	GX3262 (200 μg)	2.88 ± 0.80
PBS	PBS	3.71 ± 0.22

[a] One-week-old birds were treated subcutaneously with partially purified β-gal–GX3262 fusion protein (GX3262) as indicated and then challenged on day 28 with *E. tenella* oocysts. Lesions were scored on day 35. Values underlined indicate significant reduction compared with PBS-treated controls at $P \leq 0.05$.

zations with 100 μg of partially purified β-gal–GX3262 resulted in significant protection, whereas a single immunization of 100 μg failed to provide such protection and the administration of 200 μg of antigen failed to increase the level of protection above that observed with two independent 100-μg doses.

In another series of studies, the partially purified β-gal–GX3262 fusion antigen was evaluated in an effort to determine its ability to induce protective immunity, beginning with the immunization of 1-day-old broilers. These birds were given three separate immunizations of 100 μg of β-gal–GX3262 on days 1, 7, and 21 post-hatch. After the standard challenge with *E. tenella* oocysts and subsequent lesion evaluation, it was observed that such treatment resulted in a substantial reduction in the severity of cecal lesions as compared with lesion scores observed in the nonimmunized controls. Chickens vaccinated three times with 100 μg of β-gal–GX3262 had lesion scores reduced significantly (mean lesion score of 1.56 ± 0.53) as compared with nonvaccinated counterparts (mean lesion score of 3.43 ± 0.54). In addition, when distribution patterns of lesion scores in vaccinated and control chickens were observed (Fig. 3), it was clear that vaccination with the recombinant protein induced a significant shift to the left, resulting in about 70% of chickens being scored in the 0 to 1.9 range of cecal lesions, in contrast to the control birds, where over 80% of chickens scored in the 3 to 4 range.

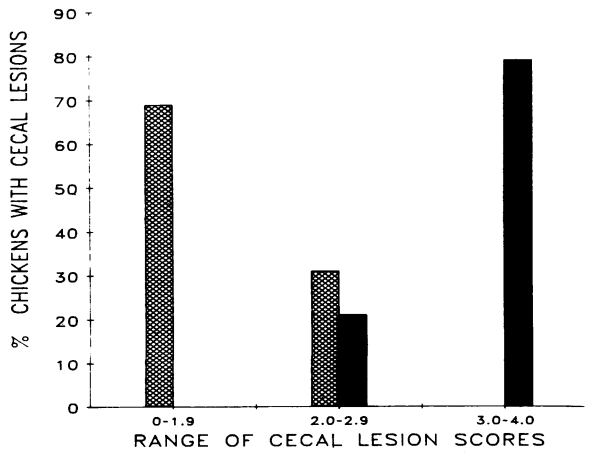

FIG. 3. Distribution patterns of lesion scores. Doses (100 μg) of partially purified recombinant β-gal–GX3262 antigen were administered on days 1, 7, and 21 post-hatch. An *E. tenella* challenge was administered on day 28, and cecal lesions were scored on day 35. Hatched bars, β-Gal–GX3262 antigen; solid bars, PBS.

TABLE 3. Lack of protection with partially purified β-gal–GX3262 fusion protein when administered on day 2 post-hatch[a]

Treatment			Lesion score ($\bar{X}$ ± SD)
Day 2	Day 7	Day 21	
GX3262 (100 μg)			3.16 ± 0.87
GX3262 (100 μg)		GX3262 (100 μg)	3.08 ± 0.87
	GX3262 (100 μg)		2.04 ± 1.29
	GX3262 (100 μg)	GX3262 (100 μg)	2.79 ± 0.94
PBS		PBS	3.57 ± 0.50

[a] Two-day- or 7-day-old birds were treated subcutaneously with partially purified β-gal–GX3262 fusion protein (GX3262) as indicated and then challenged on day 28 with *E. tenella* oocysts. Lesions were scored on day 35. Values underlined indicated significant reduction compared with PBS-treated controls at $P \leq 0.05$.

Although the GX3262 antigen is active, results thus far obtained failed to show whether the protective immunity was induced by the immunization administered on day 1, or day 7, or day 21, or a combination thereof. Therefore a study was designed to demonstrate the feasibility of immunization of 1-day-old broilers (Table 3). A single immunization with 100 μg of β-gal–GX3262 on day 7 post-hatch induced partial protective immunity, as evidenced by a significant reduction in severity of cecal lesions to 2.04 ± 1.29, compared to the mean cecal lesion scores of 3.6 ± 0.50 in the control group. An additional injection of 100 μg of antigen on day 21 failed to increase this level of protection, and no protection was induced by a single antigen immunization of 1- or 2-day-old chickens.

Protective immunity with heat-killed bacterins. Although partially purified preparations of β-gal–GX3262 proved to be active against *E. tenella* challenges, protection was not optimum. Therefore we immunized groups of 1-week-old birds with heat-killed recombinant *E. coli* cells (bacterin) containing 100, 200, or 500 μg of β-gal–GX3262 and followed this 2 weeks later with an identical immunization. Immunization of broilers with bacterin carrying 100 μg of β-gal–GX3262 induced a partial immunity against challenge infection, manifest as a reduction of nearly a full lesion score as compared with nonimmunized controls when scored 6 days after challenge. The distribution pattern of cecal lesion scores for the various treatment groups on the basis of severity was skewed to the left (Fig. 4) in comparison with the control birds, which were skewed to the right. Increasing the dose to 200 μg appeared to be slightly more effective, as suggested by an average cecal lesion score reduction of 1.2; however, the distribution pattern was comparable to that observed in the group given 100 μg. A 500-μg dose of antigen, in the form of *E. coli* bacterin, also showed some effect in reducing cecal lesions as compared with the lesions of the control chickens, but protection was less marked in comparison with that seen with the 100- or 200-μg doses.

Subclinical *E. tenella* exposure. It was speculated that it might be possible to prime 1- or 2-day-old chickens and then expect a booster effect (anamnestic response) from any subclinical exposure of the chickens to the parasite in the field. Owing to the considerable degree in variability in the parasite load in the field a consistent low-level natural exposure to the parasite may not be achieved. Therefore, to simulate natural parasite exposure in a controlled manner, a titration was performed to determine the minimum number of oocysts that should be used during the boost so that no protection was afforded without an initial priming with the recombinant β-gal–GX3262 bacterin. The titration suggested

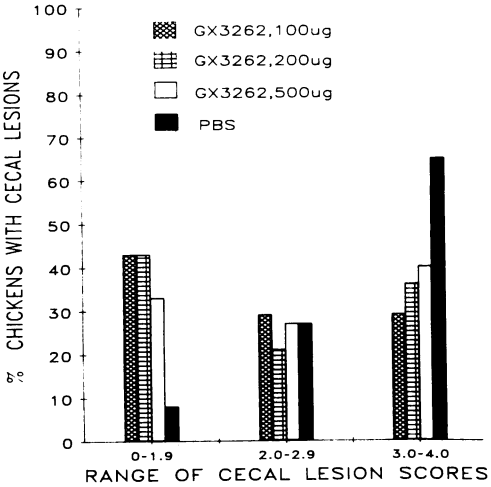

FIG. 4. Distribution patterns of lesion scores. Heat-killed recombinant *E. coli* cells carrying the β-gal–GX3262 fusion protein were administered in sufficient quantities to deliver the β-gal–GX3262 antigen as indicated in the figure. Immunizations were given on days 7 and 21 post-hatch. An *E. tenella* challenge was administered on day 28, and cecal lesions were scored on day 34.

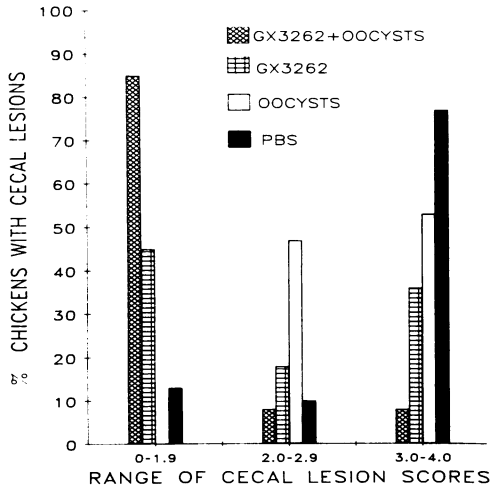

FIG. 5. Distribution patterns of lesion scores. Heat-killed recombinant *E. coli* cells carrying the β-gal–GX3262 fusion protein were administered on day 2 post-hatch to groups indicated and in sufficient quantities to deliver 100 μg of the β-gal–GX3262 antigen. Twenty-five live sporulated oocysts were administered on day 10 post-hatch. An *E. tenella* challenge was administered on day 24, and cecal lesions were scored on day 30.

the minimum number of oocysts to be less than 50 per bird. A study was then initiated in which 2-day-old birds were immunized with heat-killed bacterin carrying the β-gal–GX3262 fusion protein and then some groups were boosted orally with 25 live *E. tenella* oocysts (Table 4). Immunization of birds on day 2 of life with 100 μg significantly reduced the lesion score level (mean lesion score of 2.16). A subclinical infection of 25 *E. tenella* oocysts administered to birds so primed reduced the level of the mean lesion score substantially further (mean lesion score of 1.02 ± 0.91). Administration of 25 oocysts in the absence of a priming immunization had no significant influence on lesion scores. An evaluation of the percentage of chickens in each treatment group showing lesions in various ranges (Fig. 5) supports the contention that an initial immunization with bacterin, followed by a live boost with oocysts, results in the greatest level of protection.

Protective immunity with live recombinant *E. coli*. Since the possibility exists that the appropriate number or strains of coccidia may not be available naturally at the time they are required, a vaccine that would induce maximum protection with only a single immunization given on day 1 post-hatch, without the requirement for a boost, would be preferable. To explore the feasibility of such a system, we delivered the β-gal–GX3262 by subcutaneous inoculation of live recombinant *E. coli* cells and examined the effects on subsequent infection. In this study, IPTG-induced recombinant *E. coli* were harvested; one half were heat killed, and the remainder were stored at 4°C. Both preparations were then used for immunization. A single immunization with heat-killed bacterin at either 25 or 100 μg reduced cecal lesions to 2.68 ± 0.81 and 2.52 ± 1.07, respectively, compared with a score of 3.67 ± 0.38 in the nonimmunized group. However, a single vaccination with live *E. coli* designed to deliver antigen at comparable doses reduced the lesion scores to 1.86 ± 1.01 and 1.42 ± 1.06, respectively. The level of protection obtained using the live *E. coli* was particularly striking on examination of the distribution pattern of the percentage of chickens in each group showing lesions in various ranges (Fig. 6). With the live *E. coli*, about 70% of the birds given 100 μg of fusion antigen had lesion scores in the 0 to 1.9 range; 97% of nonimmunized birds showed lesions in the 3 to 4 range. In a subsequent study, live *E. coli* producing GX3217 was used to immunize birds and no effect on lesion scores was observed. For example, birds immunized with live *E. coli* carrying 100 μg of GX3217 showed average cecal lesions of 4.00 ± 0 6 days after challenge, as compared to 3.98 ± 0.10 for the nonimmunized controls. In this same study, 100 μg of GX3262, also administered in live *E. coli*, reduced the average lesion score to 2.78 ± 1.20.

TABLE 4. Enhancement of protection induced by heat-killed recombinant *E. coli* carrying the β-gal–GX3262 fusion protein following a live boost with *E. tenella* oocysts[a]

Treatment		Lesion score ($\bar{X}$ ± SD)
Day 2	Day 10	
GX3262 (100 μg)	25 oocysts	1.02 ± 0.91
GX3262 (100 μg)		2.16 ± 0.83
PBS	25 oocysts	2.88 ± 0.53
GX3262 (25 μg)	25 oocysts	2.18 ± 0.95
PBS		3.27 ± 0.82

[a] Two-day-old birds were immunized subcutaneously with enough heat-killed recombinant *E. coli* to yield β-gal–GX3262 fusion protein (GX3262) as indicated, and various groups were given a subclinical oral dose of *E. tenella* oocysts. All birds were challenged with an infectious dose of oocysts on day 24, and lesions were scored on day 30. Values underlined indicate significant reduction compared with PBS-treated controls at $P \leq 0.05$.

DISCUSSION

The objective of this study was to develop an inexpensive, competitive, safe, and efficacious vaccine to protect poultry from coccidiosis. Using the MAb 12-09, a cloned cDNA sequence has been identified which encodes an antigen (designated GX3262) that may meet these objectives.

Several recent reports (5, 17; Danforth et al., submitted) have indicated the feasibility of using a nonliving subunit

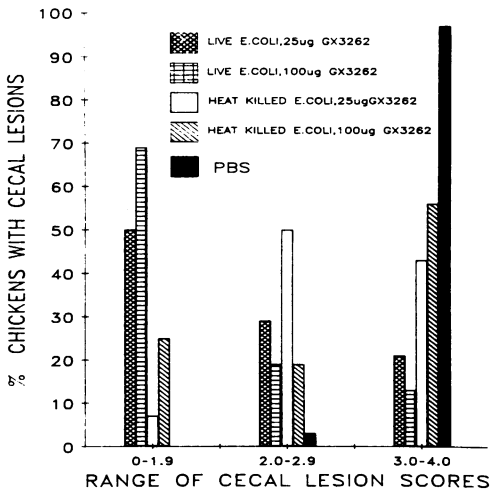

FIG. 6. Distribution patterns of lesion scores. Two-day-old chicks were given either live or heat-killed recombinant *E. coli* cells carrying the β-gal–GX3262 fusion protein (GX3262 in figure) at levels indicated in the figure. An *E. tenella* challenge was given on day 24, and cecal lesions were evaluated on day 30.

vaccine for controlling coccidiosis. The major problem with the use of such preparations obtained from native oocysts is the cost of production. The identification, cloning, and expression of an *E. tenella* cDNA sequence coding an antigen designated 5401 by Danforth et al. (submitted) demonstrated that by using modern genetic engineering techniques it was possible to clone and express an antigen that could provide partial protection against coccidiosis. However, to achieve partial protection with the 5401 antigen, the birds were immunized beginning at 4 weeks of age by antigen emulsified in Freund complete antigen. To comply with current husbandry practices of the poultry industry, due mainly to economic constraints, it would be necessary to immunize chicks at the time of hatching or shortly thereafter and without Freund complete antigen.

The GX3262 antigen provides some major advantages over antigens previously identified. The MAb 12-09 was originally prepared against *E. acervulina*, but it cross-reacts with sporozoites of all the commercially important species of *Eimeria* (6). Therefore, the GX3262 antigen cloned from *E. tenella* is probably found as part of a highly conserved refractile body protein present in most *Eimeria* species and has the potential to provide cross-protection against a number of *Eimeria* species.

We have demonstrated that although partially purified preparations of GX3262 are active, they do not provide optimal protection. In addition, of nine such preparations tested, only three gave consistent positive results. The inconsistency in activity with various preparations may be the result of improper folding of the molecule during the purification procedure or may indicate that the molecule is fairly labile. Utilization of a heat-killed bacterin avoids problems associated with purification and in addition is a more economical means of antigen production. Our studies have shown that GX3262 bacterins have the ability to consistently induce partial protection in newly hatched chicks after only a single immunization. The level of the protection observed with this type of immunization was still only partial, but can be significantly increased by the use of a booster challenge with small numbers of live *E. tenella* oocysts. This booster mimics the low level of infection that would be expected to occur under field conditions. However, because the required level and type of coccidia may not be available in the field at the appropriate time, and because with the use of such a vaccine it would be anticipated that the environmental level of coccidia would decrease with time, a vaccine that would induce maximum protection without the need for an oocyst boost would be preferable. In this regard, consistently better protection was seen with the live vaccine as compared with the heat-killed vaccine. It is possible that the live bacterin was more effective because the GX3262 antigen had not been compromised by heat treatment. It is also possible, however, that the lack of heat treatment permitted the *E. coli* cells to function as a more effective adjuvant. These questions, as well as the development of various other procedures for inactivating the live *E. coli*, are currently being explored.

The structure of GX3262 is different from those of the 5401 antigen and other parasite antigens (4, 25). GX3262 is not particularly hydrophobic or hydrophilic, and it has no repeating sequences. This antigen is less than half the size of the 28-kDa *E. tenella* protein which cross-reacts with MAb 12-09. However, we have recently identified additional cDNA clones that encode longer segments of the coccidial protein from which GX3262 is derived. It is possible that these larger fragments of the complete *E. tenella* protein would constitute an even more active antigen. It will also be interesting to compare the protective capabilities of *E. tenella* and *E. acervulina* variants of the GX3262 antigen, particularly since it is well known that *E. acervulina* is highly immunogenic (20, 21).

ACKNOWLEDGMENTS

We gratefully acknowledge Harry Danforth for providing *Eimeria* oocysts and MAb 12-09. We also thank Harry T. Brenner for excellent technical assistance and Catherine Burton for expert editorial and secretarial assistance.

LITERATURE CITED

1. **Aviv, H., and P. Leder.** 1972. Purification of biologically active globin messenger RNA by chromatography on oligo-thymidylic acid-cellulose. Proc. Natl. Acad. Sci. USA **69**:1408–1412.
2. **Biggin, M. D., T. J. Gibson, and G. F. Hong.** 1983. Buffer gradient gels and ^{35}S label as an aid to rapid DNA sequence determination. Proc. Natl. Acad. Sci. USA **80**:3963–3965.
3. **Bontemps, M., and P. Yvore.** 1974. Technique de purification de suspensions de sporozoites d'*Eimeria* sur colonée de fibres synthetiques. Ann. Rech. Vet. **5**:109–113.
4. **Dame, J. B., J. L. Williams, T. F. McCutchan, J. L. Weber, R. A. Wirtz, W. T. Hockmeyer, W. L. Maloy, J. D. Haynes, I. Schneider, D. Roberts, G. S. Sanders, E. P. Reddy, C. L. Diggs, and L. H. Miller.** 1984. Structure of the gene encoding the immunodominant surface antigen on the sporozoite of the human malaria parasite *Plasmodium falciparum*. Science **225**: 593–595.
5. **Danforth, H. D.** 1986. Use of hybridoma antibodies combined with genetic engineering in the study of protozoan parasites: a review, p. 574–590. *In* L. R. McDougald, L. P. Joyner, and P. L. Long (ed.). Research in avian coccidiosis. University of Georgia Press, Athens.
6. **Danforth, H. D., and P. C. Augustine.** 1983. Specificity and crossreactivity of immune serum and hybridoma antibodies to various species of avian coccidia. Poultry Sci. **62**:2145–2151.
7. **Dunnett, C. W.** 1985. A multiple comparison procedure for comparing several treatments with a control. J. Am. Statist. Assoc. **50**:1096–1121.
8. **Enquist, L., and N. Sternberg.** 1979. *In vitro* packaging of lambda Dam vectors and their use in cloning DNA fragments. Methods Enzymol. **68**:281–298.
9. **Huynh, T. V., R. A. Young, and R. W. Davis.** 1985. Constructing

and screening cDNA libraries in λgt10 and λgt11, p. 49–78. *In* D. Glover (ed.), DNA cloning, a practical approach, vol. 1. IRL Press, Oxford.

10. **Johnson, D. A., J. W. Gautsch, J. R. Sportsman, and J. H. Elder.** 1984. Improved techniques utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Anal. Techn. **1**:3–8.
11. **Johnson, J., and W. M. Reid.** 1970. Anticoccidial drugs: lesion scoring techniques in battery and floor-pen experiments with chickens. Exp. Parasitol. **28**:30–36.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
13. **Long, P. L.** 1968. The effect of breed of chickens on resistance to *Eimeria* infections. Br. Poult. Sci. **9**:71–78.
14. **Long, P. L.** 1984. Modulation of coccidial infections by drugs. Prog. Clin. Biol. Res. **161**:213–219.
15. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 247–268. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **McCandliss, R., A. Sloma, and S. Pestka.** 1981. Isolation and cell-free translation of human interferon mRNA from fibroblasts and leukocytes. Methods Enzymol. **68**:281–298.
17. **Murray, P. K., B. S. Bhogal, M. S. J. Crane, and T. T. MacDonald.** 1986. *Eimeria tenella—in vivo* immunization studies with sporozoite antigen, p. 564–573. *In* L. R. McDougald, L. P. Joyner, and P. L. Long (ed.), Research in avian coccidiosis. University of Georgia Press, Athens.
18. **Pasternak, J., R. J. Winkfein, and M. A. Fernando.** 1981. Ribosomal and translatable messenger RNA of *Eimeria tenella*. Mol. Biochem. Parasitol. **3**:133–142.
19. **Reid, W. M., P. L. Long, and L. R. McDougald.** 1984. Coccidiosis, p. 703–704. *In* M. S. Hofstad (ed.), Diseases of poultry. Iowa State University Press, Ames.
20. **Rose, M. E.** 1982. Host immune response, p. 330–371. *In* P. L. Long (ed.), The biology of the coccidia. University Park Press, Baltimore.
21. **Rose, M. E., and P. L. Long.** 1980. Vaccination against coccidiosis in chickens. Symp. Br. Soc. Parasitol. **18**:57–74.
22. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
23. **Scandella, D., P. Arthur, M. Mattingly, and L. Neubold.** 1985. A tightly regulated modular cloning and expression system. J. Cell. Biochem. **29**(Suppl. 9B):203.
24. **Towbin, H. T., T. Staehelin, and J. Gorden.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets—procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
25. **Zavala, F., J. P. Tam, A.H. Cochrane, I. Quakyi, R. S. Nussenzweig, and V. Nussenzweig.** 1985. Rationale for development of a synthetic vaccine against *Plasmodium falciparum* malaria. Science **228**:1436–1440.