Inhibition of Penetration of Cultured Cells by *Eimeria bovis* Sporozoites by Monoclonal Immunoglobulin G Antibodies against the Parasite Surface Protein P20†

WILLIAM M. WHITMIRE, JEAN E. KYLE, C. A. SPEER,* AND DONALD E. BURGESS
Veterinary Research Laboratory, Montana State University, Bozeman, Montana 59717

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Five monoclonal antibodies (MAbs) were partially characterized and tested for their ability to inhibit penetration of Madin-Darby bovine kidney (MDBK) cells by sporozoites of *Eimeria bovis*. By indirect fluorescent-antibody assays, all MAbs reacted with acetone-fixed sporozoites, but only two MAbs, EbS9 (immunoglobulin G1) and EbS11 (immunoglobulin G2a), localized specifically on the plasmalemma of live sporozoites. Two of the five MAbs also reacted with acetone-fixed first-generation merozoites of *E. bovis*; however, none of the MAbs reacted with live merozoites. Treatment of live sporozoites with EbS9 or EbS11 resulted in 79 and 73% decreases, respectively, in sporozoite penetration of MDBK cells. No significant differences in cell penetration occurred in MDBK cells inoculated with sporozoites that had been treated with the other three MAbs. Both EbS9 and EbS11 reacted in Western blots (immunoblots) of sporozoites with the same 20,000-relative-molecular-weight protein. The antigens against which these neutralizing MAbs react might be useful in immunizing against bovine coccidiosis.

Inhibition of sporozoite penetration of host cells in vitro by MAbs directed against a 20,000-M⁺ surface antigen.

**MATERIALS AND METHODS**

**Parasite.** Sporulated *E. bovis* oocysts were suspended in calcium- and magnesium-deficient Hanks balanced salt solution (HBSS; pH 7.4) and broken by grinding with a Teflon-coated tissue grinder. Sporozoites were excysted from sporocysts with excysting fluid (0.25% trypsin, 1:250 [GIBCO Laboratories, Grand Island, N.Y.], and 0.75% sodium taurocholate [Difco Laboratories, Detroit, Mich.] in HBSS, pH 7.2). Free sporozoites were washed in HBSS, purified by passage through a nylon wool column (12), and inoculated into cultured bovine cells for the production of first-generation merozoites (14).

**MAbs.** Female BALB/cByJ mice were each immunized by intraperitoneal inoculation of 4 × 10⁵ *E. bovis* sporozoites that had been emulsified (1:1) in 0.5 ml of HBSS containing Freund complete adjuvant (Difco). After 4 weeks, immunized mice were boosted by intraperitoneal inoculation with a similar dose of live sporozoites in 0.5 ml of HBSS. Three days later, the spleens of the immunized mice (usually two per fusion) were removed aseptically and teased apart in sterile HBSS. The splenocytes were fused (6) with P3-X63-Ag8.653 (Ag8) BALB/c plasmacytoma myeloma cells (11; American Type Culture Collection, Rockville, Md.). Hybrids were grown in Dulbecco modified Eagle medium (DMEM; GIBCO) containing 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (Sigma Chemical Co., St. Louis, Mo.) with 15% heat-inactivated horse serum (Hyclone, Logan, Utah). Sporozoite-specific-antibody-secreting hybrids were detected by an indirect fluorescent-antibody (IFA) assay (2) with acetone-fixed sporozoites. Positive hybrids were cloned by limiting dilution and screened by the IFA assay. Hybrid clones which secreted sporozoite-specific MAbs were then expanded in DMEM with 15% heat-inactivated horse serum and subsequently frozen at −195°C in liquid nitrogen. Culture medium (CM) from the cloned hybrids as well as heat-inactivated ascites fluid from pristane

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* Corresponding author.
† Contribution no. J-2174 from the Montana Agricultural Experiment Station.
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BALB/cByJ mice inoculated

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MAbs. MAbs

min

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(pH 7.4). Immunoglobin classes and subclasses of

the parasite-specific MAbs were determined with a

commercial enzyme-linked immunosorbent assay murine-MAB

isotyping kit (Hyclone).

IFA assays. The IFA assay with acetone-fixed sporozoites

was used to detect parasite-specific MAbs and to
determine their titers in ascites fluid. Titters are reported as the

reciprocal of the highest dilution in which a positive IFA assay

result was obtained.

The ability of the MAbs to react with parasite surface

antigens was determined by IFA assay on live sporozoites

called live IFA; 6). Approximately 3 × 10⁶ live sporozoites

or merozoites were reacted with 0.5 ml of diluted ascites

fluid in Microfuge tubes (Sarstedt, Inc., St. Louis, Mo.) for

45 min at room temperature (RT), washed in HBSS, fixed

with 0.2% (vol/vol) glutaraldehyde in Millonig phosphate

buffer for 30 min, washed in HBSS, incubated with fluores-

ccein-conjugated goat antimouse immunoglobulin G (IgG)

(heavy and light chain specific; United States Biochemical

Corp., Cleveland, Ohio) for 30 min at RT, washed twice in

HBSS, applied to microscope slides, covered by glass cover

slips, and examined by fluorescence microscopy. Ascites

fluid containing individual MAbs was also reacted against

acetone-fixed merozoites in the standard IFA assay.

Cell line. Madin-Darby bovine kidney (MDBK) cells

(American Type Culture Collection), used as host cells for

the parasite, were maintained in CM that consisted of

DMEM plus 15% heat-inactivated horse serum-2 mM t-

glutamine-50 U of penicillin G per ml-50 µg of dihydrostrept-

omycin (GIBCO) per ml and incubated at 38°C in 5% CO₂-

95% air.

Sporozoite penetration inhibition assay. DMEM (0.3 ml;

15% heat-inactivated horse serum) containing 3 × 10⁴

MDBK cells was inoculated into each chamber of eight-

chamber tissue culture microscope slides (Miles Scientific,

Div. Miles Laboratories, Inc., Naperville, Ill.) and incubated

as described above for 24 h. Freshly excysted sporozoites

were exposed for 30 min at RT to CM containing individual

MAbs or to CM from Ag8 myeloma cells, washed in HBSS,

and suspended in DMEM (2% heat-inactivated horse

serum). DMEM (0.3 ml) containing 1.5 × 10⁵ MAb- or

Ag8-treated sporozoites was inoculated into each chamber

of eight-chamber slides and incubated at 38°C in 5% CO₂-

95% air. Experiments 1 and 2 were conducted with different

batches of freshly excysted sporozoites. These experiments

were repeated several times with MDBK cells as well as with

an additional bovine cell line, M617 (17).

To determine if pretreatment of MDBK cells with CM

containing EbS9 or EbS11 would have an effect on the

numbers of intracellular sporozoites, monolayers of MDBK

cells in four-chamber slides were exposed to CM containing

EbS9, EbS11, or HBSS for 30 min at RT and rinsed in

HBSS. Each chamber was then inoculated with 1.5 × 10⁴

freshly excysted sporozoites in DMEM (2% heat-inactivated

horse serum) and incubated at 38°C in 5% CO₂-95% air. At

24 h after sporozoite inoculation, all of the cultures de-
scribed above were rinsed in HBSS, fixed in Bouin fluid,

Giemsa stained, and examined by bright-field microscopy for

intracellular sporozoites. The number of intracellular spor-

zoites in each of 5 or 10 microscopic fields per chamber at a

magnification of ×400 was recorded as one count, and the

means for four separate counts from four separate chambers

were then recorded for each experimental group. In live or

fixed-and-stained specimens, intracellular sporozoites could

be distinguished from extracellular sporozoites because the

former are less refractile in phase-contrast as well as in

bright-field microscopy. Also, in fixed-and-stained speci-

mens, a small parasitophorous vacuole can be seen sur-

rounding intracellular sporozoites. The data were statisti-

cally analyzed by the Student t test and the Tukey

(Studentized range) single-factor analysis of variance (13).

Western blotting and immunodetection of sporozoite anti-
gens. Sporozoites were solubilized in sodium dodecyl sulfate

(SDS) solubilizing solution (2% SDS, 10% glycerol, and 6.25

× 10⁻² M Tris, with or without 4% 2-mercaptoethanol) in

a boiling water bath for 10 min at 6 × 10⁵ parasites per µl of

solubilizing solution. Reduced and nonreduced sporozoite

proteins were electrophoretically transferred from an SDS-

polyacrylamide slab gel containing 12.5% acrylamide to

nitrocellulose paper in a Trans-Blot Cell (Bio-Rad Labora-

tories, Richmond, Calif.) (19). Following transfer, the nitro-

cellulose sheet was fixed (20:10:70:1, ethanol-acetic acid-

distilled H₂O) for 15 min (9), washed twice in distilled H₂O,

and incubated in bovine lacto-transfer technique optimizer

for 1 h at RT to block nonspecific binding sites (10). The

nitrocellulose sheet was then probed with concentrated

EbS9, EbS11, or Ag8 (diluted 1:20 in bovine lacto-transfer

technique optimizer) in a moist chamber at 4°C overnight

followed by a 1:200 dilution of horseradish peroxidase-

conjugated goat antimouse IgG (United States Biochemical

Corp.) in bovine lacto-transfer technique optimizer. Bound

peroxidase activity was developed with peroxidase substrate

solution (7). The Mr of the sporozoite antigens were esti-

mated by comparing their R₀ to the Rₚ of prestained

molecular weight standards (Bethesda Research Laborato-
ies, Inc., Gaithersburg, Md.) which had been transferred to

the same nitrocellulose sheet from the 12.5% SDS-polyacryl-

amide gel.

RESULTS

MAbs and IFA assays. After completion of cell fusion,

screening, and cloning, five E. bovis-specific, MAb-secreting

hybridomas were obtained (Table 1). All of these MAbs were

TABLE 1. Immunoglobulin subclass and IFA assays of MAbs

with sporozoites and merozoites of E. bovis

<table>
<thead>
<tr>
<th>Antisporozoite MAb</th>
<th>IgG subclass</th>
<th>Sporozoite*</th>
<th>Merozoiteb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fixed Live</td>
<td>Fixed Live</td>
<td></td>
</tr>
<tr>
<td>EbS7</td>
<td>1</td>
<td>Whole</td>
<td>+</td>
</tr>
<tr>
<td>EbS9</td>
<td>1</td>
<td>Apical</td>
<td>Whole</td>
</tr>
<tr>
<td>EbS11</td>
<td>2a</td>
<td>Apical</td>
<td>Whole</td>
</tr>
<tr>
<td>EbS14</td>
<td>2a</td>
<td>Whole</td>
<td></td>
</tr>
<tr>
<td>EbS15</td>
<td>2a</td>
<td>Whole</td>
<td>-</td>
</tr>
</tbody>
</table>

* IFA pattern exhibited by sporozoites. Whole, Immunofluorescence of entire sporozoite; apical, regional immunofluorescence of sporozoites.

b Cross-reactivity of antisporozoite MAbs with in vitro-produced first-generation merozoites.

c Sporozoites and merozoites were fixed in acetone before exposure to MAb.

d Sporozoites and merozoites were fixed in 0.2% glutaraldehyde after exposure to MAb.
found to be subclasses of murine IgG by enzyme-linked immunosorbsorbent assay and to demonstrate fluorescence on acetone-prefixed parasites by the IFA assay (Table 1; Figs. 1–3 and 5). Both EbS7 and EbS15 reacted with acetone-fixed sporozoites and merozoites (Fig. 1 and 2) but not with these parasite stages by live IFA, indicating that both stages contain common internal antigens. None of the other MAbs cross-reacted with acetone-fixed or live merozoites (Table 1). None of the MAbs appeared to cross-react with other bovine eimerian sporozoites which were sometimes present in sporozoite preparations of E. bovis (Fig. 4 and 5).

EbS9 and EbS11 reacted with the apical region of acetone-fixed sporozoites and caused whole-cell fluorescence of sporozoites in the live IFA assay (Table 1; compare Fig. 3 and 5 with Fig. 6 and 7) but did not react with acetone-fixed or live merozoites. In the live IFA, EbS9 and EbS11 caused a low degree of sporozoite agglutination but only in the presence of the secondary antibody (i.e., fluorescein-conjugated goat antimouse IgG) (Fig. 6). In the live IFA, EbS9 and EbS11 also reacted with the sporocyst wall, especially at one pole of the sporocyst near the gap created by the dissolution of the Stieda body (Fig. 7). These MAbs reacted with sporozoites within sporocysts with no Stieda body (Fig. 7) but did not react with the sporocyst wall or with sporozoites in intact sporocysts.

**Sporozoite penetration inhibition assay.** At 24 h after sporozoite inoculation, cultures of MDBK cells inoculated with sporozoites pretreated with EbS9 or EbS11 contained significantly fewer (P < 0.05) intracellular sporozoites (75 or 73% decrease, respectively) than did cultures that were inoculated with Ag8-pretreated sporozoites (Fig. 8 and 9; Table 2, experiment 1). There were no significant differences in mean numbers of intracellular sporozoites in MDBK cell cultures inoculated with sporozoites pretreated with EbS7, EbS14, EbS15, or Ag8 (Table 2, experiment 2). Additional experiments with these MAbs and MDBK and M617 cells produced similar results (data not shown); i.e., EbS9 and EbS11 caused inhibition of sporozoite penetration, but Ag8, EbS7, EbS14, and EbS15 had no inhibitory effect. Differences in the numbers of intracellular sporozoites treated with Ag8 in experiments 1 and 2 were probably due to variations in viability between the two batches of excysted sporozoites. No significant differences in mean numbers of intracellular sporozoites were detected in cultures in which the MDBK cells (and not the sporozoites) had been pretreated with EbS9, EbS11, or DMEM (Table 3).

**Immunodetection of sporozoite antigens.** SDS-polyacrylamide gel electrophoresis of E. bovis sporozoite proteins revealed a profile of proteins ranging in Mr from approximately 15,000 to more than 200,000 (Fig. 10). Western blot analysis showed that both EbS9 and EbS11 reacted with an antigen(s) of approximately 20,000 Mr (P20; Fig. 10). Neither EbS9 nor EbS11 reacted with Western blots of sporozoites that had been solubilized under reducing conditions in the presence of 2-mercaptoethanol (data not shown).

**DISCUSSION**

Five MAbs (EbS7, -9, -11, -14, and -15) which were elicited against sporozoites of E. bovis reacted either with internal sporozoite antigens only or with surface sporozoite antigens that were also expressed internally. EbS7 and EbS15 reacted against acetone-fixed first-generation merozoites of E. bovis, indicating that both stages share some antigenic determinants. MAbs EbS7 and EbS15 did not react with live IFA preparations of E. bovis sporozoites or merozoites, indicating that these shared antigenic determinants are located internally but not externally. The other MAbs proved to be sporozoite specific, and only two of these, EbS9 and EbS11, reacted against the sporozoite surface antigen, P20, by live IFA assay. These MAbs also reacted with the inner surface of the sporocyst wall, especially that portion near the Stieda body, demonstrating that the sporocyst wall and sporozoite share the P20 antigen.

In the IFA assay, EbS9 and EbS11 produced a strong apical fluorescence with acetone-fixed sporozoites. A diffuse whole-cell fluorescence was observed with live sporozoites, indicating that EbS9 and EbS11 reacted against P20 in the plasmalemma of living sporozoites. Sporozoites that were fixed in acetone before exposure to MAbs would have lost the integrity of their plasmalemmae, allowing access by the MAbs to the sporozoite interior, where they evidently reacted with P20 surface antigen precursors, resulting in the apical fluorescence. Precursors of protective surface antigens have been found in association with micronemes and rhoptries in the apical regions of sporozoites of Plasmodium knowlesi (5). A penetration enhancement factor that facilitates the entry of tachyzoites of Toxoplasma gondii into mammalian cells has recently been shown to be associated with its rhoptries (16). Thus, it is possible that the micronemes and rhoptries of E. bovis sporozoites serve to store and transport P20 to the anterior tip of the sporozoite, where it is secreted or inserted into the plasmalemma. Although we did not determine whether P20 was secreted, the live IFA indicated that it was inserted into the sporozoite plasmalemma.

Even though EbS9 and EbS11 belong to different IgG subclasses (IgG1 and IgG2a, respectively), they exhibited similar fluorescence patterns on live and acetone-fixed sporozoites, had similar inhibitory effects on the ability of sporozoites to penetrate cultured cells, and reacted with the same protein band (P20) in Western blots. Neither EbS9 nor EbS11, however, reacted in Western blots with sporozoites that had been solubilized under reducing conditions in the presence of 2-mercaptoethanol. This indicates that the antigenic determinants against which these MAbs react are dependent on the tertiary structure of P20. Because EbS9 and EbS11 reacted with P20 only in Western blots, other sporozoite proteins evidently lack the antigenic determinants recognized by these MAbs. Whether EbS9 and EbS11

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Treatment*</th>
<th>IFA titer</th>
<th>Mean no. ± SD of intracellular sporozoite</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Medium + EbS9</td>
<td>20</td>
<td>39 ± 5f</td>
</tr>
<tr>
<td></td>
<td>Medium + EbS11</td>
<td>20</td>
<td>50 ± 5f</td>
</tr>
<tr>
<td></td>
<td>Ag8f</td>
<td></td>
<td>185 ± 12</td>
</tr>
<tr>
<td>2</td>
<td>Medium + EbS7</td>
<td>20</td>
<td>46 ± 9f</td>
</tr>
<tr>
<td></td>
<td>Medium + EbS14</td>
<td>10</td>
<td>54 ± 9f</td>
</tr>
<tr>
<td></td>
<td>Medium + EbS15</td>
<td>10</td>
<td>44 ± 11f</td>
</tr>
<tr>
<td></td>
<td>Ag8f</td>
<td></td>
<td>46 ± 10f</td>
</tr>
</tbody>
</table>

* Results of two experiments. Data were obtained at 24 h after sporozoite inoculation of MDBK cell cultures.

* Before inoculation to cell cultures, sporozoites were treated for 30 min at RT with CM with or without MAbs.

* Sample size, four counts.

* Significantly different (P < 0.05) from results with Ag8 control.

* CM from unfused Ag8 myeloma cell culture.

* Not significantly different (P > 0.05) from results with Ag8 control.
FIG. 1-9. Fig. 1-3. Photomicrographs showing immunofluorescence patterns of MAb on sporozoites and merozoites of *E. bovis*. Fig. 1. Whole-cell fluorescence of sporozoites exposed to EbS15. Bar, 20 μm; magnification, ×900. Fig. 2. EbS15 causes a speckled fluorescent pattern on merozoites. Bar, 20 μm; magnification, ×900. Fig. 3. Sporozoites exhibiting apical fluorescence (arrows) after exposure to EbS11. Bar, 20 μm; magnification, ×900. Cells were acetone fixed and treated with MAb and fluorescein-conjugated goat antimouse IgG. Fig. 4 and 5. Phase-contrast (Fig. 4) and fluorescence (Fig. 5) photomicrographs of acetone-fixed sporozoites. Fig. 4. Several sporozoites of *E. bovis* and one sporozoite of *E. ellipsoidalis* or *E. zuernii* (arrow). Bar, 20 μm; magnification, ×900. Fig. 5. Same specimens as in Fig. 4, showing fluorescence with EbS9 on sporozoites of *E. bovis* but not on those of *E. ellipsoidalis* or *E. zuernii*. Note the apical fluorescence of *E. bovis* sporozoites (arrows). Bar, 20 μm; magnification, ×900. Cells were acetone fixed and treated with EbS9 and fluorescein-conjugated goat antimouse IgG. Fig. 6 and 7. Photomicrographs showing immunofluorescence by live IFA with EbS9 on a sporocyst and sporozoites of *E. bovis*. Fig. 6. Whole-cell fluorescence of agglutinated sporozoites. Bar, 20 μm; magnification, ×1,100. Fig. 7. Fluorescence of sporocyst and sporozoite within sporocyst; note that sporocyst wall (Sw) fluoresces intensely at one pole (arrow) near the gap created by dissolution of the Stieda body. Bar, 20 μm; magnification, ×1,100. Cells were glutaraldehyde fixed and treated with EbS9 and fluorescein-conjugated goat antimouse IgG. Fig. 8 and 9. Phase-contrast photomicrographs of live *E. bovis* sporozoites (Sz) in MDBK cells 24 h after sporozoite inoculation. Fig. 8. Several intracellular sporozoites that had been pretreated with Ag8. Bar, 50 μm; magnification, ×400. Fig. 9. MDBK cells with relatively few intracellular sporozoites that had been pretreated with EbS9. Bar, 50 μm; magnification, ×400.
react with the same or different epitopes in P20 remains to be
determined. It is interesting to note that the circumsporo-
zoite antigens of sporozoites of *Plasmodium* spp. also con-
tain a single immunodominant region with two or more
identical epitopes (21).

Both P20-specific MAbS caused a significant decrease in
sporozoite penetration of cultured cells, whereas other
MAbS that reacted against internal antigens only had no
adverse effect on penetration. Similarly, Augustine and
Danforth (1) found that treating sporozoites of *E. tenella* and
*E. adenoideS* with surface-reacting MAbS decreased pen-
etration of cultured cells by 37 to 67%. Because pretreatment
of cultured cells with EbS9 or EbS11 did not prevent sporozoite
penetration, it appears that the surface-reacting
MAbS had a direct inhibitory effect on the ability of *E. bovis*
sporozoites to undergo penetration. Whether EbS9 and
EbS11 exerted their inhibitory effects on cell penetration
by interfering with sporozoite motility or with some other
biological activity of P20 remains to be determined. Eventu-
ally, the P20 antigen as well as other parasite antigens,
especially surface antigens, will be tested for their ability to
protect cattle against coccidiosis induced by *E. bovis*.

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**TABLE 3. Effects of MAb treatment of MDBK cells on
penetration by sporozoites of *E. bovis***

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>IFA titer</th>
<th>Mean no. ± SD of intracellular sporozoites†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium + EbS9</td>
<td>20</td>
<td>286 ± 27</td>
</tr>
<tr>
<td>Medium + EbS11</td>
<td>20</td>
<td>278 ± 17</td>
</tr>
<tr>
<td>DMEM</td>
<td>20</td>
<td>280 ± 30</td>
</tr>
</tbody>
</table>

* MDBK cells were treated for 30 min at RT before inoculation of untreated sporozoites.
† Sample size, four counts. Data were obtained at 24 h after sporozoite
inoculation of MDBK cell cultures. No values are significantly different (*P* >
0.05).

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**FIG. 10. Nonreduced protein profile of *E. bovis* sporozoites from*
a 12.5% SDS-polyacrylamide gel stained with Coomassie brilliant
blue (lane B) and Western blots of sporozoite proteins probed with
EbS9 (lane C), EbS11 (lane D), or A8g (lane E). The arrow indicates
the 20,000-Mr (P20) sporozoite protein band against which EbS9 and
EbS11 react. The M₁ protein bands between 40,000 and 100,000 M₁,
in lanes C through E are due to nonspecific cross-reactivity of serum
proteins in the concentrated CM. Lane A consists of prestained
molecular weight standards (in thousands; Bethesda Research Labor-
atories).**

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