Protective monoclonal antibody defines a circumsporozoite-like glycoprotein exoantigen of Cryptosporidium parvum sporozoites and merozoites.

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J Immunol 1997; 158:1787-1795;
http://www.jimmunol.org/content/158/4/1787
Circumsporozoite-like Glycoprotein Exoantigen of Cryptosporidium parvum Sporozoites and Merozoites

Michael W. Riggs, Alice L. Stone, Phaedra A. Yount, Rebecca C. Langer, Michael J. Arrowood, and David J. Bentley

The apicomplexan protozoan parasite Cryptosporidium parvum causes a diarrheal disease in humans and other mammals for which specific therapy and immunoprophylaxis are unavailable. Passive immunization with Abs against whole C. parvum organisms has variable efficacy in immunocompromised or neonatal hosts. Because apical and surface-exposed zoite Ags of the Apicomplexa are critical to infectivity and targets of protective immunity, we examined the ability of mAbs generated against such Ags in C. parvum sporozoites to passively protect against infection and identify biologically relevant parasite molecules. A panel of mAbs was produced against affinity-purified native Ags using sporozoite apical- and surface-reactive mAb C4A1 as binding ligand. One resulting mAb, designated 3E2, elicited prominent morphologic changes in sporozoites and merozoites characterized by rapid and progressive formation, posterior movement, and release of membranous Ag-mAb precipitates. These changes had a striking resemblance to the malarial circumsporozoite precipitate (CSP) reaction. Sporozoite infectivity was completely neutralized after in vitro exposure to 3E2 and the CSP-like reaction. Furthermore, orally administered 3E2 completely prevented or markedly reduced infection in neonatal BALB/c mice. 3E2 bound to apical complex and surface molecules of zoites and was demonstrated in membranous precipitates by immunoelectron microscopy. In Western blots, 3E2 recognized multiple 46 to 770 kDa sporozoite Ags and an 1300-kDa Ag designated CSL, also expressed by merozoites. CSL was characterized as a soluble glycoprotein exoantigen released by infectious sporozoites. Further, CSL was determined to be the molecular species mechanistically involved in the CSP-like reaction by its identification in SDS-PAGE gels and Western blots of purified membranous precipitates. These findings indicate that CSL has a functional role in sporozoite infectivity and is a candidate molecular target for passive or active immunization against cryptosporidiosis.

The Journal of Immunology, 1997, 158: 1787-1795.

Cryptosporidium parvum is an apicomplexan parasite that infects intestinal epithelium and causes diarrhea in humans as well as economically important food animals including calves, lambs, and goat kids (1, 2). While cryptosporidiosis is usually self-limiting in immunocompetent hosts, the disease may persist in immunocompromised hosts including those with congenital T cell, B cell, or combined T and B cell deficiencies; chemotherapy-induced immunodeficiency; or AIDS (3-13). The role of C. parvum in diarrhea-related morbidity and mortality in AIDS patients and its economic impact on livestock production worldwide are now well recognized (1, 2, 12). Specific prophylaxis and treatment are currently hampered by the absence of approved vaccines, immunotherapies, or pharmaceuticals, although progress in each approach has been made (13, 14). Because C. parvum infection is prevented or terminated by normal immune responses, immunologic control strategies are under investigation. Abs produced against whole C. parvum organism preparations can neutralize the infective sporozoite and merozoite stages in vitro and diminish infection levels in vivo (15-37). These observations have provided the rationale to investigate passive immunoprophylactic and immunotherapeutic regimens for cryptosporidiosis in neonates and immunodeficient individuals. In these hosts, suboptimal active immune responses increase susceptibility to primary infection and delay or prevent termination of established infection.

We hypothesize that passive immunization against cryptosporidiosis can be optimized by identifying and targeting zoite molecules essential for infectivity by neutralizing mAbs, and defining mechanisms of mAb-mediated neutralization. Apical complex and surface molecules of apicomplexan parasites are involved in attachment, invasion, and intracellular development and may be targeted by protective immune responses (38-40). We previously reported that mAb C4A1, produced against whole C. parvum, reacts with apical and surface Ags of sporozoites and significantly reduces intestinal infection in neonatal mice (19, 41). In the present study, apical and surface Ags were isolated from sporozoites by C4A1-affinity chromatography and used to prepare an extensive panel of mAbs reactive with multiple distinct epitopes. Ability of the mAbs to neutralize C. parvum infectivity and identify parasite molecules relevant to passive immunization was determined. One resulting mAb designated 3E2 elicited pronounced morphologic changes with both sporozoites and merozoites, which
closely resembled the neutralization-associated circumsporozoite precipitate (CSP) reaction of malaria (42–44). We previously reported that an Ab-enriched fraction from hyperimmune bovine colostrum (HBC Ab) prepared against whole C. parvum formed membrane surface precipitates on sporozoites in vitro and significantly reduced persistent infection in adult SCID mice (35). The 3E2-mediated reaction reported here was similar to that described for HBC Ab, but much more prominent and rapid in development. Sporozoite infectivity was neutralized after in vitro incubation with 3E2 and occurrence of the CSP-like reaction. Furthermore, orally administered 3E2 controlled infection in vivo. Lastly, 3E2 was used to identify CSL, a sporozoite glycoprotein exoantigen demonstrated herein to be the Ag species mechanistically involved in the CSP-like reaction. We conclude that CSL is a rational molecular target for immunologic control of cryptosporidiosis.

**Materials and Methods**

**Oocyst, sporozoite, and merozoite isolation**

The Iowa C. parvum isolate (H. W. Moon, National Animal Disease Center, Ames, IA) previously shown to be pathogenic for calves and humans, was passaged in newborn Cryptosporidium-free Holstein calves every 2 mo to obtain parasite material for study (17).

Oocysts were isolated from calf feces by sucrose density gradient centrifugation, stored in 2.5% KClO3 (4°C) for up to 2 mo and hypochlorite-treated immediately prior to excystation (17, 45). Sporozoites were isolated from excysted oocysts by anion exchange chromatography (17). Merozoites were isolated from intestinal contents of neonatal BALB/c mice at 65 h after oocyst inoculation by Percoll density gradient centrifugation (35). Identity of isolated merozoites was confirmed as previously described using mAbs that bind to merozoites but not sporozoites (35). Sporozoite and merozoite viability was determined by fluorescein diacetate assay (17).

**Ab production**

Production and characterization of mAb CA1 have been previously described (19, 41). This murine IgM mAb prepared against excysted C. parvum oocysts binds to the surface and apical region of sporozoites and merozoites in immunofluorescence assays and recognizes an epitope expressed on multiple ~25 to 230 kDa sporozoite Ags in Western blots. These Ags were purified from sporozoites by CA1-affinity chromatography and used to prepare a panel of mAbs against multiple epitopes as follows: CA1 was purified from ascites fluid by mixed mode resin cation exchange, affinity chromatography, and HPLC to 96% purity then coupled to cyanogen bromide-activated Sepharose 4B according to the manufacturer’s protocol (Pharmacia, Piscataway, NJ). After determination of optimal conditions for Ag binding and elution, preparative purification of sporozoite Ags was performed (4°C, all steps). Sporozoites were solubilized by boiling buffer (100 mM Tris, pH 7.5, 1 mM MLCK, 1 mM PMSF, 1% (v/v) octyl glucoside, centrifuged (50,000 × g, 30 min) to remove the insoluble fraction, then bound to CA1-coupled Sepharose. After extensive washing (40 column volumes binding buffer), specifically bound Ags were eluted (0.1 M glycine-HCl, pH 3.3), dialyzed (3500 m.w. exclusion limit) against PBS and stored at −80°C prior to use. Partly and immunoactivity of eluted Ags were determined by SDS-PAGE silver stain and Western blot as described below. The concentration of eluted Ags was determined by bichinchonic acid protein assay (Pierce, Rockford, IL).

Eight-week-old female BALB/c mice (Harlan Sprague Dawley, Indianapolis, IN) were immunized i.p. and s.c. with affinity-purified Ags (35 μg/mouse) emulsified in monophosphoryl lipid A-trehalose dimycolate adjuvant (Ribi, Hamilton, MT) and boosted (2 to 5 μg Ags in adenovirus per mouse) by the same routes 3, 6, 10, and 14 wk later. From 4 to 6 wk following the last boost, a single i.v. injection (5 μg Ags in PBS/mouse) was administered. Spleen cells were fused 3 days later with SP2/0 myeloma cells (18). Hybridomas were screened by indirect immunofluorescence using viable (4°C) or air-dried sporozoites and merozoites to determine if Abs reacted with surface-exposed or internal epitopes, respectively (17, 19). Positive hybridomas were cloned and isoy ted as previously described (18). mAb concentrations in culture supernatants or ascites was determined by radial immunodiffusion (Binding Site, San Diego, CA). mAb 3E2 (IgM, κ) was selected for the present study. Polyclonal HBC Ab and control Ab-enriched fraction from normal bovine colostrum (NBC Ab) used in the present study were from the same lots previously reported (35).

**C. parvum neutralization assays**

Assays to determine in vitro sporozoite-neutralizing activity of 3E2 were performed as previously described, using hybridoma culture supernatant to simplify interpretation of the role of mAb (17, 18). Purified sporozoites were incubated (30 min, 37°C, 5% CO2) with 3E2 (25 μg/ml) or isotype- and concentration-matched control mAb of irrelevant specificity prior to intraintestinal inoculation of 6-day-old BALB/c mice with 2 × 103 sporozoites (25 X mouse ID50) (17). In parallel, sporozoite morphology during incubation with mAb (0.5, 1, 5, 10, 15, and 30 min) was examined by phase-contrast microscopy. The jejunum, ileum, cecum, and colon were collected 92 to 94 h after inoculation and examined histologically without knowledge of treatment group for the presence and number of C. parvum stages in mucosal epithelium. Scores of 0, 1, 2, or 3 (0, no infection; 1, <33% of mucosa infected; 2, 33% to 66% of mucosa infected; and 3, >66% of mucosa infected) were assigned to longitudinal sections representing the entire length of 1) terminal jejunum and ileum, 2) cecum, and 3) colon, then summed to obtain an infection score for each group. Individual scores were analyzed by Student’s one-tailed t test for significant differences.

To assess anticyryptosporidial activity in vivo, 3E2 ascites fluid (75 μl, 1.7 mg IgM/ml) was administered concurrently with 107 peracetic acid-disinfected oocysts (10 X mouse ID50) (17) to 6-day-old BALB/c mice by gastric intubation. At 2 h and every 12 h thereafter, mice received an additional 75 μl ascites fluid by gastric intubation. Six-day-old control mice were treated identically with ascites containing isotype- and concentration-matched mAb of irrelevant specificity. Intestinal tracts were examined histologically at 92 to 94 h after inoculation and scored as described above.

**Electron microscopy**

Viable sporozoites were incubated (2 min, 37°C, 5% CO2) with hybridoma culture supernatant containing either 3E2 or isotype control mAb (25 μg/ml). The preparations were then cooled (4°C), washed four times with HBBS, fixed, and processed for either transmission (TEM) or immunoelectron (IEM) microscopy as previously described (35). TEM samples were blocked (0.1% (w/v) BSA, 0.1% (v/v) Tween-20), incubated with affinity-purified rabbit anti-mouse IgM (Zymed, San Francisco, CA), washed, incubated with affinity-purified goat anti-rabbit IgG (Zymed, 20 nm, EM grade), washed and post-fixed (4% (w/v) formaldehyde, 1% (v/v) glutaraldehyde, 3 min). TEM and IEM samples were examined and photographed with a JEOL 100 CX transmission electron microscope at 80 kV. For scanning electron microscopy, viable sporozoites were incubated (2 min, 37°C, 5% CO2) with 3E2 or isotype control mAb (100 μg/ml), collected on a poly-L-lysine-coated nucleopore filter, washed with HBBS, and fixed (4% (w/v) formaldehyde, 1% (v/v) glutaraldehyde, 12 h). Samples were then ethanol-dehydrated, critical point dried with CO2, sputter coated with 30 nm gold, and examined with an ISI DS-130 scanning electron microscope at 10 kV.

**Ag preparation and characterization**

Purified sporozoites, merozoites, or excysted oocysts were solubilized (30 min, 4°C) in lysis buffer (50 mM Tris, 5 mM EDTA, 5 mM dithiothreitil, 0.1 mM TLCK, 1 mM PMSF, 1% (v/v) octyl glucoside) with or without the neutral protease inhibitor disopropylfluorophosphat (0.5 mM) (Sigma, St. Louis, MO) and stored at −80°C prior to use in SDS-PAGE and Western blots.

To determine if exoantigens are released by C. parvum, incubation medium of viable sporozoites was analyzed by SDS-PAGE and silver stain, radioimmunoprecipitation, and Western blot as follows. Purified sporozoites were incubated (1.5 h, 37°C) in HBBS, after which viability was 95%, then cooled (4°C, 10 min) and removed by centrifugation (5000 × g, 10 min, 4°C). After addition of protease inhibitors (1 mM PMSF, 0.1 mM TLCK, and 5 mM dithiothreitol) the supernatant was ultracentrifuged (100,000 × g, 30 min, 4°C) and filtered (0.2 μm pore size). The soluble phase was then dialyzed (3500 m.w. exclusion limit, 4°C) against lysis buffer for SDS-PAGE and Western blots (described below), or PBS for radioimmunodistion, and stored at −80°C prior to use. For radioimmunodistion, the solubie phase of incubation medium from 3 × 105 sporozoites was added

1 Abbreviations used in this paper: CSP, circumsporozoite precipitate; HBC Ab, hyperimmune bovine colostral antibody; NBC Ab, normal bovine colostral antibody; TLCK, N alpha-p-tosyl-L-lysyl chloromethyl ketone; TEM, transmission electron microscopy; IEM, immunoelectron microscopy; TEN buffer, 20 mM Tris-HCl, 5 mM EDTA, 100 mM NaCl, 15 mM NaN3 and 1% (v/v) octyl glucoside.
to a mixture containing four iodobeads (Pierce), 300 μl PBS, and 250 μCi ([125I]iodo-L-tyrosine; Du Pont-NEN, Wilmington, DE) then incubated (30 min, 4°C) with rocking. After iodobead removal, protease inhibitors (5 mM iodoacetamide, 0.1 mM TLCK, and 1 mM PMSF) were added and the solution was dialyzed (12,000 to 14,000 m.w. exclusion limit, 4°C) against lysis buffer, centrifuged (20,000 × g, 30 min, 4°C) and stored at −80°C. For immunoprecipitation, Sepharose 4B was coupled to individual purified mAbs according to the manufacturer’s protocol, washed in TEB buffer (20 mM Tris-HCl, 5 mM EDTA, 100 mM NaCl, 15 mM NaN3, and 1% (w/v) octyl glucoside, pH 7.6) and blocked (1 h, 21°C) with TEB-BSA (0.1% (w/v)). Radiolabeled samples (2 × 10^6 TCA-precipitable cpm) were incubated (1 h, 21°C) with Sepharose-coupled-3E2, C4A1, or isotype control mAb in TEB buffer, washed seven times in TEB buffer, and processed for SDS-PAGE/autoradiography as described below.

To identify the Ag species bound by 3E2 during the CSP-like reaction, membranous precipitates shed from sporozoites were isolated by density gradient centrifugation as follows. Excysted oocysts containing 10^6 free sporozoites were incubated (1 h, 37°C) with bioreactor-derived 3E2 (300 μg) in 1 ml serum-free medium (WEC 935; Amicon, Beverly, MA), gently vortexed (1 min), then layered onto 9 ml Percoll (Pharmacia) solution (Percoll 9 parts, 10% Alsever’s solution (1 part, 1% Alsever’s solution (9 parts)) and centrifuged (40,000 × g, 30 min, 4°C). Gradient fractions (1 ml) were examined by phase-contrast microscopy and zones containing predominantly oocyst shells, shed precipitates, precipitates attached to sporozoites, sporozoites, or intact oocysts, in order of increasing density were identified. Fractions containing shed precipitates were pooled, diluted in protease inhibitor buffer (PBS with 5 mM EDTA, 5 mg/ml bovine serum albumin, 0.1 mM TLCK, and 1 mM PMSF) and centrifuged (16,000 × g, 10 min, 4°C). Pelleted precipitates were washed four times in protease inhibitor buffer (4°C), further fractionated through successive (6 to 7) Percoll gradients until relatively free of oocyst shells, sporozoites, and intact oocysts, then washed as above, solubilized (30 min, 4°C) in lysis buffer and stored at −80°C prior to analysis.

**SDS-PAGE and Western blotting**

Preparations for SDS-PAGE were boiled (4 min) in sample buffer (25 mM Tris, pH 6.8, 2.2% (w/v) SDS, 15% (v/v) glycerol, 2.5% (v/v) 2-ME, and 0.001% (w/v) bromophenol blue), centrifuged (20,000 × g, 15 min) to remove insoluble material and resolved in 10 to 20% gradient gels with 4% stacking gels (35), or in 2 to 12% gradient gels. Sample type and quantity per lane were as follows: sporozoites (3 × 10^7); excysted oocysts (5 × 10^6); C4A1-affinity-purified sporozoite Ags (2 μg); soluble phase of sporozoite incubation medium (from 6 × 10^7 sporozoites); 125I-labeled Ags immunoprecipitated from the soluble phase of sporozoite incubation medium; and purified membranous precipitates (shed from 10^7 sporozoites).

Molecular weight standards consisted of titron (2450 Da) and nebulin (770 kDa) (a gift from Kuan Wang, University of Texas, Austin, TX), and prenatized or 14C-labeled myosin (200 kDa), phosphorylase b (97.4 kDa), BSA (69 kDa), OVA (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.3 kDa) (Amersham, Arlington Heights, IL). Gels were silver stained according to the manufacturer’s protocol (Bio-Rad, Hercules, CA) or processed for autoradiography as described previously (18).

Western blotting (reducing, denaturing) was performed as previously described for mAbs (37) or HBC Ab (35), except that cellulose acetate (1.2 μm pore size; Schleicher & Schuell, Keene, NH) was placed between 2 to 12% gels and nitrocellulose to reduce gel adherence. Sample type and proteinase K treatments of Ag in lysis buffer were determined by dot-immunoblot assay. Ag in lysis buffer was loaded with sample buffer to identify silver stain artifacts.

**Results**

**C4A1 affinity-purified sporozoite Ags express multiple highly immunogenic epitopes**

A subset of sporozoite Ags was isolated by C4A1 affinity chromatography and co-migrated with Ags in whole sporozoite preparations as demonstrated by SDS-PAGE and silver stain (Fig. 1). Comparison of affinity-purified Ags identified in silver-stained gels with those recognized by C4A1 in Western blots indicated relative freedom from contamination with nonimmunoreactive proteins.

Three fusions produced 112 stable hybridomas secreting mAbs that bound in heterogeneous immunofluorescence patterns to the sporozoite pellicle, internal apical, and mid regions of sporozoites, oocyst shells, and residual bodies. mAbs also reacted in heterogeneous binding patterns with the sporozoite Ags defined by C4A1 in Western blots (data not shown). One mAb group reacted identically with C4A1-affinity-purified sporozoite Ags, isotype control mAbs, C. parvum peptidase-reactive control mAb 1G12, or carbohydrate-reactive control mAb 4H5. Blots were then washed and incubated with affinity-purified alkaline phosphatase-conjugated rabbit anti-mouse IgM or IgG (Zymed) followed by substrate as previously described (31).

**Epitope characterization**

Ability of mAbs to bind sporozoite Ag in the soluble fraction of excysted oocysts after treatment with sodium periodate, N-glycosidase F, or proteinase K was determined by dot-immunoblot assay. Ag in lysis buffer was dotted onto nitrocellulose and treated with sodium periodate (2 mM, 5 mM) or control buffer (46). For glycosidase treatment, Ag in treatment buffer (100 mM NaF, 7 mM EDTA, 1% (w/v) octyl glucoside, and 1% (v/v) 2-ME, pH 7.0) was incubated (12 h, 37°C) with or without N-glycosidase F (10 U/10^6 excysted oocysts) (Boehringer Mannheim, Indianapolis, IN) then dotted onto nitrocellulose after addition of SDS (0.3% (w/v)). For proteinase K treatment, Ag in TBE buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA, 0.5% SDS) was incubated (1 h, 37°C) with or without proteinase K (5 × 10^-3 U/10^6 excysted oocysts) (Sigma) then dotted onto nitrocellulose after addition of protease inhibitors (1 mM PMSF and 0.1 mM TLCK). Replicate dot-blotts from each treatment group were incubated with 3E2 or other mAbs prepared against C4A1-affinity-purified sporozoite Ags, isotype control mAbs, C. parvum peptidase-reactive control mAb 1G12, or carbohydrate-reactive control mAb 4H5. Blots were then washed and incubated with affinity-purified alkaline phosphatase-conjugated rabbit anti-mouse IgM or IgG (Zymed) followed by substrate as previously described (31).

**FIGURE 1.** Silver-stained SDS-PAGE gel of solubilized sporozoites before (lane 1 and lane 2) and after (lane 3 and lane 4) C4A1-affinity chromatography purification. Lane 2 contains solubilized oocysts (2.5 × 10^6) for comparison. Lane 4 was loaded with sample buffer to identify silver stain artifacts.
pooled around individual sporozoites, and the apical complex region and pellicle of sporozoites. Reactivity with merozoites and surrounding amorphous Ag deposits was indistinguishable from that observed with sporozoites. In immunofluorescence assays using viable sporozoites, 3E2 reacted with surface epitopes in a posteriorly aggregated pattern, membranous material extending from the posterior sporozoite and detached aggregates of membranous material.

When viable sporozoites or merozoites were incubated with 3E2, distinct morphologic changes resembling the malarial CSP reaction (42, 43) were observed. These changes consisted of progressive formation, posterior movement, and eventual release of elongated membranous surface precipitates (Figs. 2, 3). Precipitate formation was observed within 30 s of sporozoite or merozoite exposure to 3E2, and was morphologically indistinguishable between the two stages. 3E2 specifically bound in precipitates was demonstrated by IEM (Fig. 4). No CSP-like reaction was observed when viable zoites were incubated with 1) isotype control mAb (Figs. 2 to 4), 2) C4A1, 3) all other mAbs prepared against C4A1 affinity-purified Ags which recognized different CSL epitopes than 3E2, or 4) mAbs recognizing a 23-kDa C. parvum sporozoite surface pellicle Ag (P23) (37).

Sporozoite infectivity for mice was completely neutralized after in vitro incubation with 3E2 and occurrence of the CSP-like reaction, whereas sporozoites incubated with isotype control mAb infected ileum, cecum, and colon of all inoculated mice (Table I). In a more stringent efficacy assay, orally administered 3E2 completely protected 6 of 15 oocyst-challenged mice against infection in two experiments. In 9 of 15 3E2-treated mice, only scattered C. parvum stages limited to the ileum were observed histologically (Table II). In contrast, all mice treated with isotype control mAb had well-established infections in terminal jejunum and ileum, cecum, and colon. Anterior jejunal infection was not observed in any mice. The mean infection score reduction in 3E2-treated mice was highly significant (p < 0.00001) in each experiment.

A 1200 to 1400-kDa sporozoite glycoprotein exoantigen is mechanistically targeted by Ab in the CSP-like reaction

In Western blots, 3E2 recognized multiple sporozoite Ags ranging from 46 to ~230 kDa (Fig. 5, lane 4), ~230 to ~770 kDa, and a prominent ~1300 kDa Ag designated CSL (Fig. 6, lane 4). 3E2, C4A1, and HBC Ab recognized multiple comigrating sporozoite Ags, including CSL, as well as Ags specific for each Ab (Figs. 5, 6). In Western blots of sporozoites solubilized in the presence or absence of diisopropylfluorophosphate, banding patterns of the mAbs were indistinguishable (data not shown).

In silver-stained gels of sporozoite incubation medium, an ~1300-kDa band was identified as the predominant protein species (Fig. 7, lane 4). When this preparation was radioiodinated and incubated with 3E2- or C4A1-Sepharose, an ~1200-kDa Ag was specifically precipitated (Fig. 8). In Western blots, 3E2, C4A1, and HBC Ab recognized an ~1300-kDa Ag in sporozoite incubation medium, which comigrated with an Ag recognized in whole sporozoites (Fig. 6). The ~1300-kDa Ag was the only Ag recognized by 3E2 in Western blots of sporozoite incubation medium resolved in either 2 to 12% gels (Fig. 6, lane 9) or to 20% gels (data not shown). These observations indicated that CSL is a soluble, iodo-natal sporozoite exoantigen bearing epitopes recognized by 3E2, C4A1, and HBC Ab.

In silver-stained gels of purified membranous precipitates, an ~1400 kDa Ag was identified as the predominant sporozoite-derived species (Fig. 9) and comigrated with an Ag in whole sporozoites, C4A1-affinity chromatography-purified sporozoite preparations, and sporozoite incubation medium (Fig. 7). Several faint lower molecular mass bands (~<380 kDa) in shed precipitates (Fig. 7, lane 5; Fig. 9, lane 3) were possibly from trace contamination with sporozoites or proteins in 3E2 bioreactor medium. Prominent lower molecular mass bands in shed precipitates comigrated with bands in bioreactor-derived 3E2, indicating the presence of medium proteins (164 kDa, 158 kDa) and IgM heavy chains (82 kDa) (Fig. 9, compare lanes 2 and 3). In Western blots, 3E2, C4A1 and HBC Ab recognized a comigrating ~1400-kDa Ag in shed precipitates and whole sporozoites (Fig. 10). Bands at ~82 kDa in precipitates (Fig. 10, lanes 9 and 10) comigrated with bioreactor-derived 3E2 bands recognized by anti-mouse IgM secondary Ab (Fig. 10, lanes 6 and 11), confirming IgM heavy chain origin. The faint <~380 kDa silver-stained bands in precipitates described above (Fig. 7, lane 5; Fig. 9, lane 3) were not recognized by 3E2 in Western blots of precipitates (Fig. 10, lane 9). Finally, in Western blots of merozoites, 3E2, C4A1, and HBC Ab recognized an ~1200-kDa Ag that comigrated with an Ag recognized in sporozoites (Fig. 11). These observations indicated that CSL is the Ag species bound by 3E2 to elicit the CSP-like reaction, and is conserved in both infective stages of C. parvum.

3E2 did not bind Ag treated with 5 mM periodate or N-glycosidase F whereas binding to proteinase K-treated Ag was reduced but not eliminated (Fig. 12). Reactivity of carbohydrate- or peptide-specific control mAbs with treated Ags validated the specific activities of periodate and N-glycosidase F for carbohydrate, and proteinase K for polypeptides (data not shown).
FIGURE 3. TEM of sporozoites after incubation with 3E2 (A) or isotype control mAb (B). Note membranous precipitate (arrows) extending from a sporozoite undergoing the CSP-like reaction (A), and absence of precipitate in the control preparation (B). Bar, 1 μm.

FIGURE 4. IEM of sporozoites after incubation with 3E2 (A and B) or isotype control mAb (C). Note dense immunogold labeling of 3E2 in precipitate (arrows) extending from the sporozoite posterior (A) or shed from sporozoites (B). Bar, 1 μm.

Table I. Neutralization of C. parvum sporozoite infectivity by mAb 3E2

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>No. Infected/ No. Inoculated</th>
<th>Mean Infection Score ± SD</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sporozoites + 3E2</td>
<td>0/10</td>
<td>0</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>Sporozoites + control IgM</td>
<td>10/10</td>
<td>4.5 ± 1.2</td>
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</table>

Discussion

In the present study, a mAb panel was prepared to identify C. parvum molecules essential for infectivity as targets for passive Ab-based immunization against cryptosporidiosis. Native apical and surface Ags were purified from whole sporozoites by immunoaffinity chromatography for mouse immunization. The strategy was to focus the Ab response on immunologically relevant Ags and optimize the probability of identifying potentially functional epitopes. One of the resulting mAbs, designated 3E2, elicited a CSP-like reaction, neutralized sporozoite infectivity in vitro, and controlled infection in vivo. 3E2 allowed the identification of CSL, a biologically relevant zoite molecule involved in the pathogenesis of infection and mechanistically targeted by Ab in the CSP-like reaction.

Progressive formation, posterior movement and release of membranous surface precipitates observed in 3E2-treated C. parvum sporozoites closely resembles the malarial CSP reaction. In this reaction the circumsporozoite protein of Plasmodium spp., an apical complex-derived sporozoite exoantigen, forms a progressively elongated posterior precipitate when cross-linked by neutralizing
mAb VS CIRCUMSPOROZOITE-LIKE EXOANTIGEN PROTECTS AGAINST *C. parvum*

Table II.  **Passive protection against C. parvum oocyst challenge by mAb 3E2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. Infected/No. Inoculated</th>
<th>Mean Infection Score ± SD</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3E2</td>
<td>5/8</td>
<td>0.6 ± 0.5</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>Control IgM</td>
<td>8/8</td>
<td>4.5 ± 1.5</td>
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</tr>
<tr>
<td>Experiment 2</td>
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<td></td>
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<tr>
<td>3E2</td>
<td>4/7</td>
<td>0.9 ± 0.9</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>Control IgM</td>
<td>8/8</td>
<td>5.1 ± 1.5</td>
<td></td>
</tr>
</tbody>
</table>

Ab (42–44). The CSP reaction may mimic a process triggered by sporozoite attachment to host cell receptors during infection and result in premature shedding of attachment and invasion molecules on the sporozoite surface (44). The CSP-like reaction elicited by 3E2 was more rapid and morphologically prominent than a similar reaction observed when *C. parvum* sporozoites were incubated with HBC Ab (35). Elongated membranous precipitates extended from the posterior sporozoite within 30 s of exposure to 3E2. In contrast, sporozoite precipitate formation did not begin until 8 min of exposure to HBC Ab (35). Demonstration of 3E2 specifically bound in precipitates by IEM, SDS-PAGE/silver stain, and Western blot indicates mechanistic involvement of mAb in the CSP-like reaction, as previously demonstrated for *Plasmodium* spp. Because *Plasmodium* spp. sporozoites are neutralized after the CSP-like reaction (44) and region II-plus of the *P. falciparum* circumsporozoite protein contains a sporozoite ligand for hepatocyte receptors (47–49), we investigated the CSP-like reaction of *C. parvum* further.

When sporozoites incubated with 3E2 in vitro underwent the CSP-like reaction, approximately 25 times the mouse ID₅₀ was completely neutralized. Neutralizing Abs against the circumsporozoite protein of *Plasmodium* spp were previously reported to block sporozoite attachment and invasion in Caco-2 cell cultures were inhibited after the CSP-like reaction (52). Furthermore, orally administered 3E2 significantly protected BALB/c neonates against challenge with approximately 10 times the mouse ID₅₀ of oocysts, indicating that specific activity was retained and neutralization could be mediated in the gastrointestinal milieu during the brief period (38) that zoites are extracellular. Studies are in progress to determine if complete protection against primary infection or termination of established infection can be achieved by treatment with formulations comprised of 3E2 and additional neutralizing mAbs reactive with different epitopes. Ab-mediated neutralization of *C. parvum* is dependent on epitope specificity, Ab concentration, and duration of exposure to zoites (13, 23, 33, 36). Therefore, neutralizing mAb formulations targeting multiple...
FIGURE 8. SDS-PAGE gel autoradiograph demonstrating an ~1200-kDa \(^{125}\)I-labeled soluble Ag (arrow) immunoprecipitated from sporozoite incubation medium by 3E2 (lane 1) and C4A1 (lane 4). Lane 2 was loaded with sample buffer and lane 3 with isotype control mAb-precipitate.

FIGURE 9. Silver-stained SDS-PAGE gel demonstrating an ~1400-kDa Ag (arrow) in precipitates shed during the CSP-like reaction (lane 3) and whole sporozoites (lane 4, 3 x 10^7; lane 5, 7 x 10^7). Lane 1 was loaded with sample buffer to identify silver stain artifacts, and lane 2 with bioreactor-derived 3E2 (15 μg).

FIGURE 10. Western blot recognition of an ~1400-kDa Ag (arrow) in precipitates shed during the CSP-like reaction (lanes 6 to 10) and a comigrating Ag in whole sporozoites (lanes 1 to 5) by HBC Ab (lanes 3 and 8), 3E2 (lanes 4 and 9), and C4A1 (lanes 5 and 10). Lanes 1 and 6 were probed with isotype control mAb and lanes 2 and 7 with NBC Ab. Lane 11 was loaded with bioreactor-derived 3E2 (25 μg).

FIGURE 11. Western blot recognition of an ~1200-kDa merozoite Ag (lanes 1 to 5) and a comigrating sporozoite Ag (lanes 6 to 10) by HBC Ab (lanes 3 and 8), 3E2 (lanes 4 and 9), and C4A1 (lanes 5 and 10). Lanes 1 and 6 were probed with isotype control mAb and lanes 2 and 7 with NBC Ab.

sporozoite and merozoite epitopes will likely have an additive effect and be required to optimally control infection, particularly in diarrheic hosts having accelerated gastrointestinal transit. In addition, the recent observation that IL-12 administered prophylactically can prevent or reduce C. parvum infection in neonatal SCID and BALB/c mice suggests that immunization against cryptosporidiosis may be enhanced by regimens which include this cytokine (53).

Accurate comparisons between the present study and others reporting efficacy of mAbs (19, 23, 28, 37) or polyclonal Abs (20, 22, 23, 30) against infection in oocyst-challenged BALB/c neonates are difficult to make due to variations in experimental design. The marked efficacy of 3E2 against infection is attributed to its ability to elicit the CSP-like reaction and to the rapidity and magnitude of this reaction with the life cycle-initiating sporozoite stage. Occurrence of the CSP-like reaction with merozoites may also have contributed to 3E2 efficacy although merozoite neutralization was not examined directly. Evidence that the CSP-like reaction has biologic significance and relevance to Ab-mediated control provided the rationale to determine which of the multiple sporozoite Ags recognized by 3E2 is mechanistically involved.

Several lines of evidence indicate that CSL is the molecular species mechanistically bound by Ab in the CSP-like reaction. An ~1400-kDa species compatible with CSL was the principle sporozoite Ag identified in silver-stained gels of purified precipitates shed during the reaction, and the only sporozoite Ag recognized by 3E2 in Western blots of the precipitates. This Ag was also recognized by 3E2 in Western blots of sporozoites that had not undergone the reaction. Finally, HBC Ab previously shown to elicit a morphologically similar reaction recognized this Ag in shed precipitates and in sporozoites.

CSL was further characterized as a soluble exoantigen released by infectious sporozoites in the absence of Ab. 3E2 specifically
recognized amorphous pools of Ag immediately surrounding air-dried sporozoites in immunofluorescence assays. In sporozoite incubation experiments, high viability indicated that exoantigen was from nonviable sporozoites. An amorphous pool of Ag immediately surrounding air-dried Ag, and comigrated with CSL identified in silver-stained gels released by an active metabolic process and not by passive leakage. This exoantigen was the only species 3E2 specifically recognized by radiographic immunoprecipitation or Western blot of sporozoite incubation medium. Furthermore, this exoantigen was not previously recognized (19) because this Ag was not recognized by HBC Ab in Western blots of sporozoite incubation medium.

At least five C. parvum Ags bearing neutralization-sensitive epitopes have been identified with mAbs possessing significant anticyptosomal activity, either alone or in combinations (18, 19, 24, 28, 37, 41, 54). An additional neutralization-sensitive epitope and the biologically relevant Ag on which it is expressed have now been identified by 3E2. Reactivity of C4Al with CSL was not previously recognized (19) because this Ag was not resolved in 10 to 20% SDS-PAGE. However, C4Al and 3E2 define distinct CSL epitopes based on differences in Western blot and immunofluorescence reactivities and the ability of 3E2 but not C4Al to elicit the CSP-like reaction. The epitope recognized by 3E2 was destroyed by periodate or N-glycosidase F, but not protease K, indicating a carbohydrate or carbohydrate-dependent structure. Protease resistance of the CSL epitope defined by 3E2 may impart structural and functional stability under gastrointestinal conditions. Indeed, three of six neutralization-sensitive epitopes previously reported are carbohydrate or carbohydrate-dependent moieties (18, 19, 24) and other studies suggest that glycoconjugate Ags are important targets of the immune response to C. parvum (28, 32, 55, 56).

CSL is a glycoprotein based on the presence of carbohydrate and iodinatable tyrosine residues, detection by protein-staining reagents, and slow migration in SDS-PAGE resulting in variable molecular mass estimation (1200 to 1400 kDa). The epitope defined by 3E2 is expressed on multiple sporozoite glycoproteins based on Western blot reactivity. It is unlikely that the lower $M_r$ species are proteolytic fragments of higher $M_r$ species because Ags were processed at 4°C with protease inhibitors, including disopropylfluorophosphate, and stored at ~80°C prior to use. However, it is possible that Western blot patterns reflect in part deglycosylation artifacts because glycosidase inhibitors are not routinely used. Some species are likely disulfide-linked subunits of higher $M_r$ multimers based on recognition of fewer bands by 3E2 and C4Al in Western blots performed under nonreducing conditions (M. W. Riggs, unpublished observations). The molecular relationship between Ags recognized by 3E2 requires additional study. Relationships, if any, between CSL and other high molecular mass C. parvum Ags, including >500-kDa and >900-kDa glycoproteins localized to microneme organelles by IEM, have not been determined (32, 56, 57–59). However, in IEM of fixed sporozoites, dense granule organelles of the apical complex are predominantly labeled by 3E2 in addition to a subpopulation of micronemes (M. W. Riggs, manuscript in preparation).

Preliminary studies suggest functional similarities between CSL and the circumsporozoite protein exoantigen of P. falciparum (M. W. Riggs, unpublished observations). CSL, like the malarial circumsporozoite protein (60), is initially released from the apical sporozoite then translocated posteriorly on the surface pellicle. Cytchalasin D inactivation of sporozoite microfilaments does not inhibit apical release of CSL but does inhibit its posterior translocation and the CSP-like reaction. Furthermore, preliminary findings indicate that native CSL purified by isoelectric focusing binds specifically to viable Caco-2 cells and significantly reduces their permissiveness to infection by C. parvum sporozoites in vitro, suggesting that CSL may be a sporozoite ligand (52). A functional role for CSL is further supported by its conservation in both infective stages of C. parvum.

Results reported herein indicate that CSL is a rational target for passive immunization against cryptosporidiosis. In addition, the results suggest that CSL is a candidate Ag for recombinant eukaryotic expression and active immunization of immunocompetent hosts. Finally, the results validate the strategy of preparing mAbs against defined native apical and surface Ags to identify and characterize functional parasite epitopes amenable to immunologic intervention.

Acknowledgments

We thank Lisa Krug-Hepler, Diane Duke, Kathryn E. Huey, Janine M. Bahle, and Nanette C. Westhof for excellent technical assistance.

References