INFECTION AND IMMUNITY, May 1991, p. 1703–1708
0019-9567/91/051703-06$02.00/0
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Vol. 59, No. 5

Characterization of Microneme Antigens of *Cryptosporidium parvum* (Protozoa, Apicomplexa)

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Received 22 October 1990/Accepted 1 February 1991

Two monoclonal antibodies (MAbs) raised against purified excysted oocysts and sporozoites of *Cryptosporidium parvum* reacted in an immunofluorescence assay with antigens located at the anterior pole of the zoites. On Western blots of purified oocysts, these MAbs reacted with a series of bands between 210 and 40 kDa; several of these bands were recognized by both MAbs; others were specific. One MAb (TOU) did not react after periodic acid treatment and was therefore considered to recognize a carbohydrate epitope; as determined by immunoelectron microscopy, this MAb reacted on micronemes of sporozoites and merozoites and also with the peripheral cytoplasm and the parasitophorous vacuole of trophozoites and macrogametes. The other MAb (HAD) reacted with an epitope that was insensitive to periodate treatment but did not react in the immunoelectron microscopy assay. However, the similar labeling pattern obtained with the immunofluorescence assay with both MAbs and the fact that the two antibodies share common bands on Western immunoblots suggest that both MAbs react with molecules located in *Cryptosporidium* micronemes, one reacting with a glycannic epitope and the other reacting with a peptidic epitope.

Cryptosporidium sp. (Protozoa, Apicomplexa) is a parasitic protozoan that develops in the brush border of epithelial cells in the digestive and respiratory tract of many animal species, including humans. Host cell invasion by *Cryptosporidium* zoites occurs by progressive evagination of the host cell plasmalemma and enclosure of the parasite in a parasitophorous vacuole (14, 15), a process that is common among other apicomplexa. Among other ultrastructural features specific to the apicomplexa, *Cryptosporidium* zoites possess two sets of apical organelles, the rhoptries and the micronemes (6, 7), which are believed to be secretory and to be involved in host cell invasion (6). The role of micronemes remains unknown, and few data are available on the contents of these organelles and on their fate during or after host cell invasion.

During a study on antigens of *Cryptosporidium parvum* we produced monoclonal antibodies (MAbs) against excysted sporozoites and used them to localize antigens in developing stages of the parasite. In the present paper we report on the characterization of an antigen family located in the micronemes of *C. parvum* zoites. These molecules were also detected in the peripheral cytoplasm and the parasitophorous vacuole of trophozoites and macrogametes, suggesting that the contents of the micronemes might be released in the parasitophorous vacuole by the invading zoites.

MATERIALS AND METHODS

Stool specimens. Fecal samples positive for *C. parvum* were obtained from naturally infected humans, calves, and lambs and stored at 4°C in 2.5% potassium dichromate for up to 12 months before use.

Oocyst purification and excystation procedure. Oocysts were extracted from feces either by centrifugation in a phosphate-buffered saline (PBS)–ether solution followed by separation on a discontinuous Percoll density gradient (23) or by flotation in distilled water saturated with NaCl (22). In both cases the oocysts recovered were excysted in a sodium taurocholate solution as described previously (5).

Production of hybridomas. Mouse immunization and cell fusion were carried out as previously described (4). Briefly, BALB/c mice were immunized by subcutaneous injection with the postexcystation preparation obtained from 10^6 oocysts emulsified in Freund complete adjuvant. Two additional subcutaneous injections with the same preparation emulsified in Freund incomplete adjuvant were given at 3-week intervals. A final boost was given intravenously 3 and 7 days before fusion. At 10 days after the fusion, supernatant fluids were screened for antibody production by an indirect immunofluorescence assay with a 1/400 dilution of fluorescein-conjugated goat anti-mouse immunoglobulin G (IgG), IgA, and IgM antibodies (Sigma) by using air-dried acetone-fixed purified oocysts and sporozoites as antigens. Secreting hybridomas were cloned twice by limiting dilution. The isotypes of immunoglobulins were determined by an immunoenzymatic assay (Behring Diagnostics).

Immunofluorescent staining of parasites grown in cell culture. Development of asexual stages of *C. parvum* was achieved in LGA cells (a cell line derived from a carcinoma of the small intestine in Lewis rats) as previously described (5). Briefly, excysted sporozoites from a single bovine isolate were washed in RPMI 1640 medium and suspended in the medium supplemented with 10% fetal calf serum. Approximately 200,000 sporozoites were added to each confluent cell monolayer growing on a 2-cm^2 plastic coverslip. After incubation for 4 h at 37°C in a 5% CO_2 atmosphere, cell monolayers were rinsed with RPMI 1640 and the medium was replaced. Infected cell cultures were then kept at 37°C in a 5% CO_2 atmosphere. At 14 h after inoculation, cell monolayers infected with *C. parvum* were fixed in 2% formaldehyde in PBS for 30 min at room temperature, rinsed in PBS, and treated with 50% acetone in PBS (2 min, 4°C), pure acetone (2 min, −20°C), and 50% acetone in PBS (2 min, 4°C). After fixation the coverslips were exposed to

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undiluted hybridoma culture supernatant for 30 min at 37°C, washed in PBS, and incubated with a 1/50 dilution of fluorescein-conjugated sheep anti-mouse IgG (Diagnostic Pasteur) for 30 min at 37°C. The coverslips were then washed in PBS, mounted onto a microscope slide, and viewed under a Zeiss epifluorescence microscope. Controls were made with unrelated hybridoma culture supernatants.

Immunoelectron microscopy on parasites grown in rat intestine. Small pieces of terminal ileum were obtained from an immunosuppressed rat experimentally infected with a lamb isolate of the parasite (18). Tissue samples were fixed with 2% formaldehyde–0.1% glutaraldehyde in PBS for 2 h at room temperature. They were washed in PBS, dehydrated in ethanol at −20°C, and embedded in LR White (London Resin Co.). After polymerization at 37°C for 5 days, thin sections were cut with a diamond knife and collected on carbon-coated nickel grids. They were floated for 30 min on 1% ovalbumin in PBS (PBSO) and then transferred on MAbs (undiluted culture medium) for 1 h. After the grids were washed with PBS, they were floated on rabbit anti-mouse-immunoglobulin serum (Tago) diluted 1/200 in PBSO for 1 h, washed, and then transferred for 1 h on 8- or 5-nm protein A-coated gold beads diluted 1:50 in PBSO (optical density at 525 nm, 0.05). Thin sections were stained with 3% uranyl acetate in water and observed with a Hitachi H600 electron microscope. Control sections were incubated with unrelated MAbs.

In another experiment 25×10^6 purified oocysts were excysted as described previously (5). Intact oocysts and free sporozoites were washed twice in PBS, fixed in 4% paraformaldehyde–0.05% glutaraldehyde in PBS for 30 min at room temperature, and washed twice in PBS before being suspended in 2 ml of PBS. Vero cells trypsinized from a 25-cm^2 confluent monolayer were processed similarly, and the resulting suspension was added to that containing the parasites. The mixed suspension was pelleted by centrifugation and washed twice in PBS. The final pellet was dehydrated in ethanol at −20°C, embedded in LR White resin, and processed for ultrastructural immunolocalization as described above.

SDS-PAGE. Intact oocysts purified from feces were suspended in sample buffer containing 65 mM Tris (pH 6.8), 1% sodium dodecyl sulfate (SDS), 5% sucrose, and either 0.1 M dithiothreitol or no dithiothreitol to produce a parasite concentration of approximately 5×10^8 oocysts per ml; the oocysts were immediately frozen at −80°C. After three freeze-thaw cycles, samples were heated for 5 min at 100°C and centrifuged for 20 min at 10,000 rpm (Jouan MR 14-11 centrifuge) to remove particulate matter. SDS-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli (9) with 8% acrylamide separating gels. Molecular weight markers (Pharmacia LMW Calibration kit) were used for calibration.

Western immunoblotting. Oocysts obtained from a single bovine isolate of *C. parvum* were analyzed by SDS-PAGE (approximately 10^7 oocysts per 1-cm-wide slot) and electrophoretically transferred to nitrocellulose (1 h, 0.8 mA/cm^2). The nitrocellulose sheet was then saturated for 30 min in 5% nonfat dry milk in TNT buffer (140 mM NaCl and 0.05% Tween 20 in 15 mM Tris-HCl [pH 8]). It was then incubated in MAbs (hybridoma culture supernatant diluted 1/3 in TNT) for 1 h at 37°C. After the sheet was washed, it was incubated in alkaline phosphatase-conjugated anti-mouse IgG antibodies diluted 1/6,000 in TNT and then revealed with Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Determination of carbohydrate epitopes. We used the procedure developed by Woodward et al. for antigens on Western blots (24). Briefly, after saturation nitrocellulose strips were incubated with 10 mM sodium orthoperiodate in 50 mM sodium acetate buffer (pH 4.5) in the dark at room temperature and then exposed to 50 mM sodium borohydride in PBS for 30 min. Controls were performed by incubating strips in the same buffer without periodate treatment. After three washes with TNT, the nitrocellulose strips were probed with MAbs as described above.

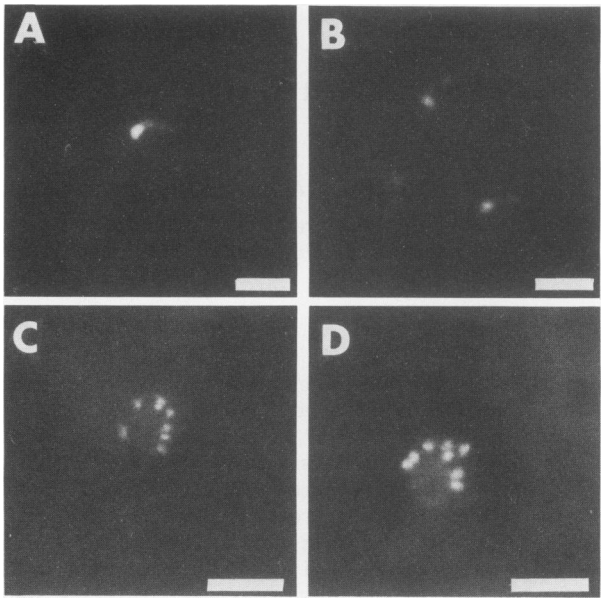

FIG. 1. Immunofluorescent staining patterns of MAbs HAD (A, C) and TOU (B, D) with isolated sporozoites (A, B) and schizonts grown in cell culture (C, D). Bars, 5 μm.

RESULTS

Two IgG antibodies (MAbs TOU and HAD) raised from fusions described in Materials and Methods reacted with the anterior pole of the zoite in an immunofluorescence assay performed on air-dried, acetone-fixed excysted sporozoites (Fig. 1A and B). Both antibodies recognized sporozoites from the six different mammalian strains of the parasite evaluated so far (one human isolate, one lamb isolate, and four calf isolates), including the isolates used for cell culture, immunoelectron microscopy, and Western blotting experiments. When the immunofluorescence procedure was performed on *C. parvum*-infected cell monolayers, the two MAbs clearly labeled the apical region of merozoites in mature first-generation schizonts; the labeling pattern obtained with both antibodies was essentially the same (Fig. 1C and D).

To localize precisely the subcellular structures recognized, culture supernatants corresponding to these hybridomas were assayed on LR White sections of ileum from experimentally infected rats that showed all developmental stages of the parasite. MAb TOU was shown to react with micronemes in merozoites (within developing schizonts; Fig. 2A and B) and sporozoites (inside oocysts or released by excystation; Fig. 2C). Rhoptries and other zoite organelles were not labeled. No microneme antigen could be detected in the host cell cytoplasm. Some gold particles were also found over the peripheral cytoplasm of trophozoites and

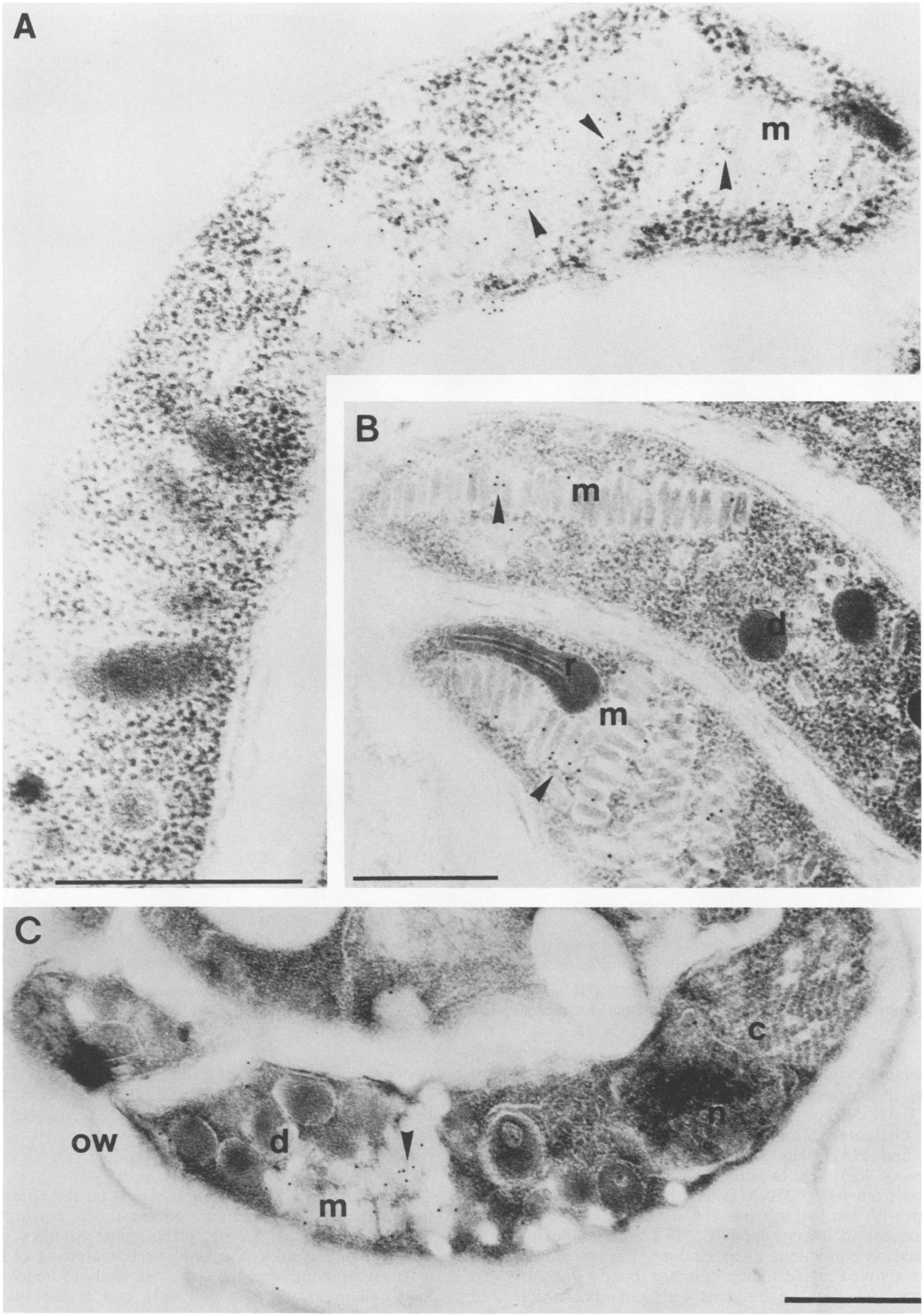

FIG. 2. Immunoelectron micrograph of monoclonal antibody TOU with merozoites (A, B) and sporozoites (C). Gold particles are clustered over micronemes (m) (arrowheads); c, crystalloid body; d, dense granules; n, nucleus; ow, oocyst wall, r, rhoptries. Bars, 0.5 μm.

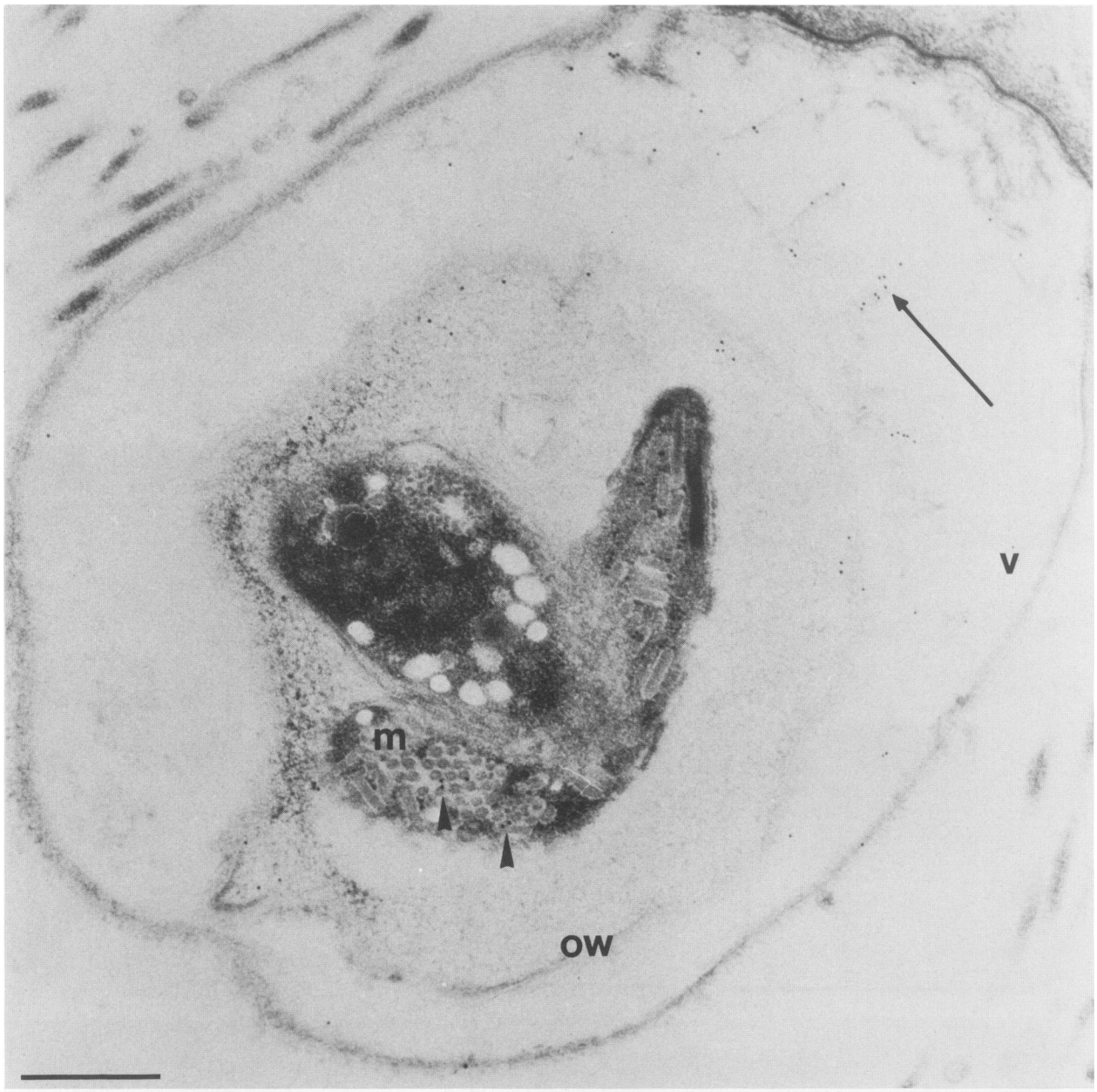

FIG. 3. Immunoelectron micrograph of MAb TOU with a sporulated oocyst in the parasitophorous vacuole. Gold particles appear over the micronemes (m) (arrowheads) and in the parasitophorous vacuole (v) (arrow). ow, Oocyst wall. Bar, 0.5 μm.

macrogametes and in the parasitophorous vacuole of trophozoites, macrogametes, and sporulating oocysts (Fig. 3). MAb HAD did not react in electron microscopy.

The characterization of the antigens recognized by MAbs TOU and HAD was achieved by immunoblotting. The pattern of MAb HAD showed a series of distinct bands ranging from 63 to 210 kDa. MAb TOU gave a complex pattern with two major bands at 210 and 130 kDa and several components of lower reactivity. The immunoblot patterns obtained on contiguous nitrocellulose strips with both antibodies showed at least nine common bands (Fig. 4A). To determine whether the antigenic determinants characterized were located on carbohydrate or amino acid chains, the reactivity of the MAbs to nitrocellulose strips treated with periodic acid was studied. The binding of MAb TOU was completely abolished by mild periodate oxidation, whereas the binding of MAb HAD was unaffected (Fig. 4B).

DISCUSSION

The present paper is the first report on the characterization of subcellular organelle contents of *Cryptosporidium* zoites. Micronemes belong to the apical complex, a highly differentiated system of organelles characteristic of invasive stages of apicomplexa and therefore likely to be involved in host cell invasion. Although only one of the two MAbs described here reacted by immunoelectron microscopy, identical IFA patterns and partial identity on Western blots

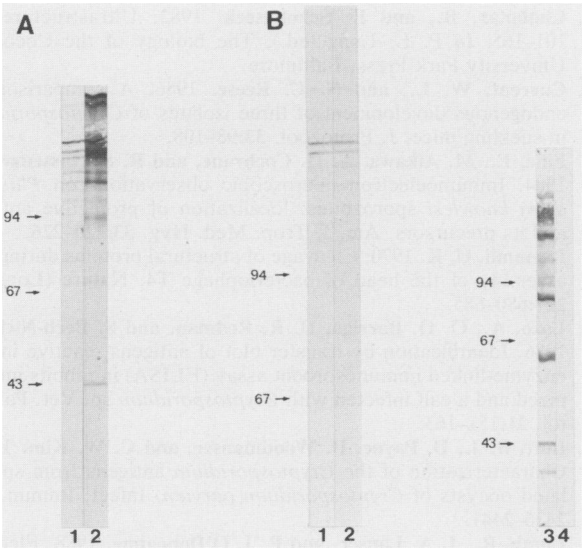

FIG. 4. Immunoblotting of *C. parvum* oocysts probed with MAbs TOU and HAD. (A) Samples incubated in MAb HAD (lane 1) and MAb TOU (lane 2) and standards were not reduced before SDS-PAGE. (B) Effect of periodate oxidation on the binding of MAbs TOU and HAD. The strips in 1 and 2 were incubated with MAb HAD (SDS-PAGE under nonreducing conditions). The strips in lanes 3 and 4 were incubated with MAb TOU (SDS-PAGE under reduced conditions). Lanes 2 and 4 were treated with 10 mM periodic acid in acetate buffer (pH 4.5), whereas lanes 1 and 3 were incubated in buffer only. All strips were then treated with sodium borohydride and washed before immunostaining. The apparent molecular weights ($\times 10^3$) of standard proteins are given.

led us to think that both MAbs are likely to react with different epitopes of the same molecules located in the same organelles. We therefore discuss them together according to this working hypothesis, which remains to be investigated further.

Both MAbs characterized reacted identically in the immunofluorescence assay with the apical region of sporozoites from the six different strains of the parasite evaluated, suggesting that the antigenic determinants recognized are probably preserved among different isolates of *C. parvum*. Immunoelectron microscopy demonstrated that the antigen recognized by MAb TOU was present in micronemes of both invasive stages of *C. parvum*, indicating at least a partial biochemical homology of these organelles, which were previously considered identical based on morphologic features only. Thus, being conserved in all invasive stages, this antigen might be implicated in a basic microneme function. Some micronemes were not labeled; Pohl et al. reported a similar finding in *Sarcocystis muris* (17) and suggested that such nonhomogenous labeling was due to the small size of the micronemes relative to the thickness of the electron microscope sections.

Some reactivity of MAb TOU was observed with the vacuolar space and peripheral cytoplasm in trophozoites, macrogametes, and developing oocysts. Vacuolar reactivity could support the hypothesis of microneme contents being exocytosed into the vacuolar space after invasion. Indeed, detection of a microneme antigen at the periphery of the gamont after host cell invasion has been reported in *S. muris* (17). Furthermore, *Plasmodium brasilianum* microneme antigens were found in membranous structures and in the cytoplasm of infected erythrocytes (20), and *Plasmodium knowlesi* micronemes have been shown to contain precursors of a protective surface antigen of the sporozoite (8) and the Duffy receptor family (1), suggesting that micronemes might be exocytosed. Peripheral cytoplasm labeling is not easy to interpret, and we cannot decide whether it corresponds to diffusion of vacuole labeling, to parasite surface labeling, to the actual presence of the antigen in the cytoplasm, or to the detection of a cross-reacting epitope undetected in the immunofluorescence assay because the sexual stages were not found in vitro. We must emphasize, however, that this labeling was faint and that additional studies and probably other probes are needed to evaluate these hypotheses further.

A complex pattern of reactivity in Western blots was a striking feature of MAbs HAD and TOU. The reactivity of both MAbs was not altered after a second cloning of the secreting hybridoma cell line, which ruled out the possibility that we were dealing with more than one secreting clone. Similarly, the sample preparation procedure makes proteolysis unlikely, since samples were rapidly frozen, thawed, boiled in sample buffer, and kept at −80°C until electrophoresis. Therefore, the complex pattern could be due to the presence of common carbohydrate epitopes located on oligosaccharide side chains of different glycoproteins. Nitrocellulose strips were then treated with periodate at concentrations known to cleave specifically carbohydrate vicinal hydroxyl groups (24). This procedure completely abolished the reactivity of MAb TOU and therefore supports the hypothesis that this antibody binds to a carbohydrate epitope. However, our data do not exclude the possibility that alteration of the carbohydrate moieties by periodate may have changed the conformation of the proteins and abolished the affinity of MAb TOU for a discontinuous protein antigenic site. The reactivity of MAb HAD was unaffected by periodate treatment. This antibody reacted with several bands comigrating with components identified by MAb TOU. This finding and the similar labeling pattern obtained in the immunofluorescence with both antibodies suggest that MAb HAD recognizes a proteinic epitope on the glycoprotein family identified by MAb TOU. A possible interpretation of our findings is that we are dealing with a family of glycoproteins sharing a common polypeptide backbone but different discrete degrees of glycosylation; however, this hypothesis remains unproven and has to be studied further. We cannot rule out the possibility that MAb HAD reacts with a carbohydrate epitope that is insensitive to periodate oxidation on Western blots. The fact that both MAbs do not share all bands could be due to masking of their respective epitopes on some of the molecules of the family. Another possibility would be that the two antibodies recognize two different epitopes shared by the commonly reacting molecules and that the epitopes are present independently on the bands reacting with only one of them. Few data are available on the biochemical structure of micronemes. The present work thus represents the first characterization of a carbohydrate determinant in the micronemes of a sporozoa.

Several antigens have been recently identified on Western blots of *C. parvum* antigen extract reacted with immune sera from animals and humans (10, 12, 16, 21). The present work confirms the finding of Luft et al. (11), who suggested that carbohydrates form a significant proportion of the epitopes that bind to antibodies in the immune response to *C. parvum*. However, only a few *Cryptosporidium* antigens have been characterized and immunolocalized with MAbs. A 20- to 23-kDa molecule (16, 21) sharing common epitopes with high-molecular-weight proteins (13) was localized in the

pellicle of sporozoites and merozoites by immunoelectron microscopy (13), but no labeling of the micronemes occurred, suggesting that this molecule is distinct from the glycoproteins identified in the present work. Riggs et al. (19) and Bjorneby et al. (3) characterized two neutralization-sensitive epitopes at the surface of sporozoites and merozoites. Thus, unless the molecules identified in the present work were secretory products produced internally and later presented at the surface, a relation with these antigens is unlikely. Indeed, the patterns obtained with these antibodies with Western blotting or after [^{35}S]methionine labeling of sporozoite surface antigens differed from those obtained with MAbs HAD and TOU. Arrowood et al. (2) described an antigen located at the anterior pole of the sporozoites that was also identified on merozoites and showed a complex Western blot pattern with several bands ranging from 25 to 200 kDa. These findings suggested that this antigen could be related to the glycoproteins characterized by MAbs HAD and TOU, but examination of the immunoblot patterns seems to indicate that the reacting molecules have different molecular weights. However, differences in electrophoresis conditions or in the procedure used to obtain soluble antigen may explain this descrepancy, and therefore we may have been dealing with the same antigen. If such is the case, our contribution is to have localized it in micronemes and to have obtained data strongly suggesting a glycoproteinic nature. Further studies are needed to find out whether the presence of glycoproteins is a characteristic of *Cryptosporidium* micronemes and to investigate the biological properties of these molecules.

ACKNOWLEDGMENTS

We thank F. Martin (Institut National de la Santé et de la Recherche Médicale, U252 Dijon, France) for supplying the LGA cell line and G. Harly, Bruno Cornot, M. Naciri, and P. Chavanet for providing infected samples. The constant interest and support of P. Pothier are gratefully acknowledged.

This work was supported in part by a grant from the Institut National de la Santé et de la Recherche Médicale (PNRS 1987–1988).

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