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Characterization of a high molecular weight antigen of *Cryptosporidium parvum* micronemes possessing epitopes that are cross-reactive with all parasitic life cycle stages

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**Summary** — Crossreacting antigens between life cycle stages of *Cryptosporidium parvum* (Protozoa, Apicomplexa) were detected using monoclonal antibodies (mAbs). Shared epitopes were demonstrated by immunoelectron microscopy, at the level of micronemes of the sporozoite and merozoite stages; some dense granules were also labelled but not so intensively. The parasitophorous vacuole membranes of all intracellular stages, the wall-forming bodies of macrogametes and the outer oocyst walls all shared these epitopes. The antigens that bear these epitopes were characterized using the whole oocyst and sporozoite stages as sources of antigenic material. Complex labelling patterns were observed on Western blots. However, all the mAbs used in this study recognized an antigen of more than 500 kDa. The glycoproteinic nature of this antigen was demonstrated by its sensitivity to pronase and periodate treatments. The expression of this high molecular weight immunoreactive antigen in the intracellular stages of *C. parvum* was not investigated and remains to be found.

*Cryptosporidium parvum* / microneme / parasitophorous vacuole / wall-forming bodies / oocyst wall

**Résumé** — Caractérisation d'un antigène de haut poids moléculaire localisé au niveau des micronèmes des zoites et possédant des épitopes communs avec tous les autres stades de *Cryptosporidium parvum*. Des antigènes communs à tous les stades du cycle parasitaire de *Cryptosporidium parvum* (Protozoa, Apicomplexa) ont été mis en évidence à l'aide d'anticorps monoclonaux (mAbs). Par immunoélectromicroscopie, des épitopes communs ont été démontrés au niveau des micronèmes des stades sporozoïtes et mérozoïtes ; le marquage des granules

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Invasive stages of Apicomplexan parasites possess characteristic organelles which are thought to be associated with the penetration / invasion of the host cell (Scholtyseck and Mehlhorn, 1970). Three types of membrane-bound organelles have been described: (i) rhoptries, which are amphorashaped, secretory granules (Perkins, 1992), connected by ductules to the tip of the apical complex where secreted substances can be discharged (Sinden, 1985); (ii) micronemes, which are smaller, more numerous and may occupy much of the anterior third of the parasite; (iii) dense granules, which are round, densely staining bodies (Bannister et al, 1975). Entzeroth et al (1986) have shown that dense granule content was released through the pellicle into the secondary parasitophorous vacuole of Sarcocystis muris. Dense granules have been described in only few species, such as Plasmodium knowlesi, Sarcocystis muris and Toxoplasma gondii (Torii et al, 1989b).

Rhoptries, numerous micronemes and dense granules have been described in the zoites of the Cryptosporididae family (Vetterling et al, 1971; Current and Reese, 1986; Uni et al, 1987; Ostrovská and Paperna, 1990). Bonnin et al (1991) have characterized an antigen family located in the micronemes. They suggest that the contents of micronemes might be released in the parasitophorous vacuole by invading zoites. Cross-reactive epitopes shared by micronemes and macrogametes were also described by Bonnin et al (1993).

Using monoclonal antibodies, immuno-electron microscopy and Western blotting, we have investigated the antigenic structure of micronemes of zoites, and we have tried to show that epitopes associated with these membrane-bound organelles were also expressed at all other life cycle stages of Cryptosporidium parvum.
Immunization of mice, production of monoclonal antibodies

Monoclonal antibodies (mAbs) were obtained by fusion of Sp2-0-Ag14 myeloma cells with splenocytes of Balb/c mice immunized with C. parvum. We performed 3 different cellular fusions (Kohler and Milstein, 1975). The first immunization was realized by intraperitoneal injections of at least 10^7 sonicated oocysts (Ungar et al., 1986). Immunizations were performed on day 0 and on days 14, 31, 149, and 156. The first was given with complete Freund adjuvant (CFA), the others with incomplete Freund adjuvant (IFA). The fusion was carried out 4 d after the last booster, and mAb S1G12 was obtained. For the second fusion, called Y (production of Y3A5 and Y14F3 mAbs), the mouse received 5 intraperitoneal immunizations. Intact oocysts (20 x 10^6) were infected, in CFA, 230 d before the fusion; 2 booster doses, in IFA, were given 13 and 29 d later. The final boosters consisted of viable sporozoites (> 4 x 10^5) and oocysts (12 x 10^6) injected intraperitoneally, in PBS, 6 and 2 d before the fusion. For the third fusion, called AT (production of AT7G10, AT7F7 and AT9H6 mAbs), 8 x 10^6 intact oocysts were injected intraperitoneally in CFA 105 d prior to the fusion; followed 10 d later by an intraperitoneal injection of 15 x 10^6 viable purified sporozoites in PBS. An intrasplenic immunization (Spitz et al., 1984) was performed, 4 d before the fusion, using 11 x 10^6 sporozoites and oocysts fixed in 4% paraformaldehyde at 4°C for 1 h and washed twice with PBS. Screening of hybridoma supernatant was performed by an indirect immunofluorescent assay on air-dried or acetone-fixed sporozoites and oocysts. Hybridoma specific to C. parvum were cloned by limiting dilution. The isotype of secreted immunoglobulines was determined by an immunoenzymatic assay (Amer-sham Life Science, UK).

Immunoelectron microscopy

Labelling after embedding

Ileum segments of OF1 mice experimentally infected in their first week of age, were taken 7 d after oral inoculation of 10,000 oocysts. In the first experiment, small ileum segments were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 4 h at 4°C, and then embedded. Osmicated tissues were prepared for a second labelling experiment: ileum pieces were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h, 30 min at 4°C, then washed 3 times with phosphate buffer and post-fixed with phosphate-buffered 1% osmium tetroxide for 1 h at 4°C. After several rinses in buffer, the tissues were dehydrated through a grade series of ethanol to 100% and then embedded in LR-White resin (London Resin Co) (Bendayan et al., 1987). Thin sections were cut with a diamond knife and collected on nickel grids (coated, in some cases, with form-var) and floated for 10 min in PBS/2% BSA, then transferred onto hybridomas culture medium, diluted (v/v) with PBS/BSA for 1 h. After washing in PBS 0.01% Tween 20, the grids were successively floated on rabbit anti-mouse immunoglobulin diluted to 10 μg/ml in PBS/BSA and 15 nm gold-protein A conjugate (Amer-sham) for 30 min and 1 h, respectively. The gold conjugate was diluted 1/50 in PBS/BSA with 1/20 gelatin. After 2 washes in PBS-0.01% Tween 20, grids were rinsed with water and stained for 20 min in 4% uranyl acetate in water. Control sections were processed in the same manner except that mAbs were omitted. Sections were examined with a Philips electron microscope.

SDS-polyacrylamide gel electrophoresis

Antigens were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Three different antigen preparations were used for these analyses: boiled oocysts (BO Ag), sporozoites (SP Ag), and a parasite extract (PE Ag) obtained from ultrasonicated oocysts. BO Ag was obtained from oocysts (16 x 10^4 oocysts/μl) diluted (v/v) in sample buffer (20 mM Tris, pH 7.4, 2 mM EDTA, 5% SDS, and either 5% mercapto-2-ethanol or no mercaptoethanol), boiled for 15 min, centrifuged at 8 000 g for 5 min to eliminate insoluble material. It was analyzed on Phastgel homogeneous 7.5% or 4–15% acrylamide gradient with a Phastsys-
tem aparatus (Pharmacia Fine Chemicals, Uppsala, Sweden). The running conditions were 250 V; 10 mA; 3 W; 63, 90, or 200 Vh. SP Ag was obtained by excystation. After 2 washes with PBS, excysted sporozoites were lysed by NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.02% sodium azide, 0.5% Nonidet P-40, 1 mM PMSF, 5 mM iodoacetamide, pH 7.4) for 30 min at room temperature. Intact oocysts and oocyst walls were removed by centrifugation at 1500 g for 15 min. The supernatant, which contained sporozoite antigen, was analyzed by electrophoresis. PE Ag was obtained according to Achbarou et al. (1991) with some modifications. Briefly, 2 x 10⁸ purified oocysts were sonicated in 800 μl of NET buffer without NP-40. Sonicated parasites were centrifuged at 1500 g for 10 min; the supernatant was mixed with 200 μl of 5% SDS in NET buffer and left 30 min under agitation at room temperature; after ultrasonication for 1 min, 1 ml of NET containing 2% Triton X-100 and 2% sodium deoxycholate was added. After agitation for 30 min at room temperature, the preparation was ultrasonicated for 1 min and centrifuged at 10 000 g for 15 min at 4°C. The supernatant was collected and analyzed by SDS-PAGE or used for purification by chromatography.

**Western blotting**

Antigens were analyzed by SDS-PAGE and electrophoretically transferred to nitrocellulose (Towbin et al., 1979), on a PhastTransfert apparatus (Pharmacia, Uppsala, Sweden). Low and high molecular weight markers (Pharmacia), and phosphorylase b (Sigma Chemical Co, St Louis, MO) were used. The nitrocellulose sheet was saturated with a casein solution (Pearce-Pratt and Roser, 1989) for 30 min at room temperature, then incubated in hybridoma culture medium diluted (v/v) in casein for 40 min at room temperature. After washing in PBS-1% Tween 20, the sheet was incubated in biotinylated antimouse-immunoglobulins (Amersham), diluted 1/500 in casein for 40 min, washed again and finally incubated in avidin-biotinlated peroxidase complex (Amersham) diluted 1/500 in casein. The blot was revealed by chemiluminescence using ECL (Amersham), and exposed to Hyperfilm MP (Amersham). The photographic film was developed after 30 s to 5 min exposure time.

**Determination of proteinic and carbohydrate epitopes**

PE Ag or affinity-purified antigens were exposed to periodate or to pronase (Beeley, 1985) and then analyzed by SDS-PAGE and Western blotting. For periodate oxidation, the preparation was incubated with 33 mM NaIO₄ in 20 mM sodium acetate buffer (pH 4.5) for 24 h in the dark. Addition of ethylene glycol was followed by treatment with 60 mM sodium borohydride in 60 mM sodium borate buffer, pH 8.0, in the dark for 4 h. Controls were incubated in the same way except that NaIO₄ was omitted from the buffer. For pronase treatment, predigested pronase (60°C, 30 min) was used at the working concentration of 1 mg/ml in 100 mM Tris-HCl, 1 mM CaCl₂, pH 8.0. Digestion was performed over 30 h at 37°C; the same incubation procedure, but without pronase, was applied to the control antigen. A third type of control was the same antigen preparation at the corresponding dilution stored at -20°C without any incubation. All samples were analyzed by SDS-PAGE and Western blotting.

**Purification of mAbs from ascitic fluids**

A large amount of mAbs of the Ig G isotypes were purified from ascitic fluids on a protein G-Sepharose 4B column (Pharmacia, Uppsala, Sweden). In the case of Ig M isotype, the ascitic fluid was applied on a protein G column, and the flow-through, which contained Ig M and Ig A isotypes, was applied on a Sepharose 4B column (Pharmacia) where rat mAbs (Ref ATCC HB58) that were specific to mouse kappa light chain were covalently linked. For all these chromatographic steps, equilibration buffer was 20 mM NaH₂PO₄/Na₂HPO₄, pH 7.2; and elution buffer was 100 mM glycine hydrochloride, pH 2.7.

**Isolation of antigens by affinity chromatography**

Purified mAbs Y3A5 were covalently linked to CNBr-activated Sepharose 4B (Pharmacia), according to the manufacturer's recommendation. Parasite extracts (oocysts and sporozoites) in NET buffer and detergents, prepared as described above, were mixed with the mAbs-
Identification of antigens by ELISA

The presence of specific antigen in solution or in fractions obtained by chromatography was determined by ELISA; immunoplates (maxisorb-Nunc, Rockilde, Denmark) were coated overnight at 4°C with purified mAbs (mAbs Y3A5, S1G12, S7H6, AT9H6) diluted in PBS-0.1% (w/v) thimerosal (Sigma) at 1 μg/well. Saturation was performed with casein at room temperature for 30 min. After washing in PBS-0.1% Tween 20, 100 μl of each fraction was applied per well for 1 h at room temperature. The wells were washed and the test was revealed using tetramethylbenzidine (TMB Sigma) as a chromogen. The colour reaction was stopped with 100 μl 1 M H₂SO₄ per well and optical densities (OD) were recorded at 450 nm. Positive fractions (OD > 0.3) were pooled, concentrated by ultrafiltration using immersible cX10 and ultrafree-Mc (Millipore Corp, USA), and dialysed overnight against PBS. This purified antigen was analyzed by Western blotting as done for purified oocyst preparation.

RESULTS

Monoclonal antibodies

Molecular weights of the antigens recognized by mAbs

The Western blot reactivities of S1G12 (IgG3), Y14F3 (IgG3), AT7G10 (IgM), AT7F7 (IgG1), AT9H6 (IgG2a), Y3A5 (IgG3) and unrelated (negative control) mAbs to solubilized C. parvum sporozoite antigens (SP Ag) are presented in figure 1. All these anti-Cryptosporidium mAbs recognized a high molecular weight antigen but the bands were weak in the cases of mAbs Y14F3 and AT7F7. Moreover, mAbs AT7G10 and AT9H6 recognized several additional antigens. Different immunoblot reactivities were observed for the 3 types of antigen preparation.

MAb AT7G10 recognized 8 antigenic bands in BO Ag (fig 2a, lane 1; fig 2b, lane 1; fig 2c, lane 1). Due to the large number of immunoreactive bands, different preparations of BO Ag were analyzed to verify that reproducible results could be obtained. Using chemiluminescence, even a faint immunoreactive band could be seen, but in this case longer exposure times were needed and the strong immunoreactive antigen appeared as overexposed. The approximate molecular weight of these bands ranged from 50 to more than 500 kDa (fig 2b, lane 1; fig 2c, lane 1). Unreduced preparations (data not shown), or PE Ag (fig 2c, lane 2) gave the same pattern. With mAb AT7G10, only 3 antigenic bands were clearly observed in the case of SP Ag (fig 1, lane 3; fig 2a, lane 2). Analysis of sporo-
zoites isolated by DEAE-cellulose anion-exchange chromatography (data not shown), according to Riggs and Perryman (1987), gave the same results as those obtained with Sp Ag.

MAb Y3A5 reacted with 2 antigens of different molecular weight (> 500 and 340 kDa) when BO Ag was analyzed (fig 2a, lane 3). The antigenic reactivity was lost when PE Ag was tested (fig 2c, lane 3) and the > 500
kDa antigen was the only one detected by mAb Y3A5 with SP Ag (fig 1, lane 6). When negative results were obtained, the immunoreactivity of mAb Y3A5 was checked by an indirect immunofluorescent assay. In spite of the absence of reactivity of mAb Y3A5 in the Western blot of PE Ag, this type of preparation still contained antigenic structures recognized by an Y3A5-HRP conjugate in ELISA.

The antigen of > 500 kDa was associated with a soluble ultrasonicated oocyst extract, because it was still present in the supernatant after a centrifugation at 135 000 g for 30 min at 4°C. This high molecular weight size was corroborated by separation of PE Ag on a Superdex 200 column (exclusion limit of 600 kDa). The last fraction of the void volume and the early post-void volume fractions gave the maximum OD in an ELISA performed with mAb Y3A5 conjugate.

**Analysis of the antigens purified by affinity chromatography**

Figure 3b shows the immunoblot reactivity of the molecules purified by affinity chromatography on a sepharose 4B column linked to mAb Y3A5. To make the comparison easier, a preparation of BO Ag (fig 3a, lane 1) was also run on the same blot. A more concentrated preparation of purified antigen was separated by electrophoresis using a shorter run time (fig 3c). In this case, all bands were clearly seen and less diffusion occurred. The reactivity against affinity-purified antigens was as follows: mAb Y3A5 did not recognize any band (fig 3b, lane 2; fig 3c, lane 2); while a complex labelling pattern was obtained with mAb AT7G10 (fig 3b, lane 4; fig 3c, lane 1). The antigen of > 500 kDa was heavily marked and several bands of the same molecular weight range as those recognized by mAb AT7G10 in BO Ag (fig 3a, lane 1) were observed. Two other

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**Fig 3.** a. Blot of BO Ag probed with mAb AT7G10. Electrophoresis conditions: gradient 4–15%, 200 Vh, high molecular weight markers from Sigma. b. Immunoblots of affinity-purified antigens caught by mAb Y3A5, and probed with the following mAbs: lane 1: S1G12; lane 2: Y3A5; lane 3: Y14F3; lane 4: AT7G10; lane 5: AT9H6. Blots were separated under the same electrophoretic procedure as in a; c. Analysis of a more concentrated preparation of purified antigens (gradient 4–15%, 63 Vh) probed with mAbs AT7G10 (lane 1) and Y3A5 (lane 2).
mAbs always recognized some bands when tested against purified antigen: mAb S1G12 (fig 3b, lane 1) recognized the high molecular weight antigen; and mAb Y14F3 (fig 3b, lane 3) gave approximately the same pattern as the one observed with mAb AT7G10. Mab AT9H6 recognized some bands of lower molecular weight, while the reactivity against the > 500 kDa antigen (as seen in fig 1, lane 5) was absent.

The effect of pronase digestion and periodate oxidation

The reactivity of mAb AT7G10 to antigenic preparations treated in solution with sodium periodate or pronase is shown in figure 4. Two types of preparation were used: PE Ag (fig 4, lanes 1, 2 and 3) and the antigen purified by affinity chromatography on sepharose-Y3A5 mAb (fig 4, lanes 4, 5 and 6). Pronase deeply altered the antigenic structure of these preparations since no labelling could be observed after pronase treatments (fig 4, lanes 1 and 4), except a faint band of lower molecular weight which still appeared in figure 4, lane 4. The binding of mAb AT7G10 to purified antigen was completely abolished by periodate oxidation (fig 4, lane 6), whereas only one antigenic band, corresponding to the 575 kDa antigen, was still recognized by this mAb after treatment of the PE Ag extract (fig 4, lane 3). The 3 control preparations gave the same electrophoretic pattern, and only the results obtained from the control samples incubated during 30 h at 37°C without pronase are shown in figure 4, lane 2 and 5.

Immunolocalization

In immunofluorescent assay mAb Y3A5 recognized the oocyst wall and heavily labelled the sporozoite apex (fig 5). On LR-White sections, mAb Y3A5 recognized the limiting membrane of parasitophorous vacuoles of all stages of *C parvum*; the mero-

Fig 4. Effects of pronase digestion and periodate oxidation. After separation (gradient 4–15%, 63 Vh), the blots were incubated in mAb AT7G10. PE Ag and affinity-purified antigen were analyzed in lanes 1 and 4 (pronase digestion), lane 2 and 5 (untreated samples) and lane 3 and 6 (periodate oxidation).
Fig 5. Immunofluorescent staining pattern of mAb Y3A5 with acetone-fixed sporozoites and oocysts (bar, 5 μm).

Fig 6. Labelling with mAb Y3A5 and Prot A-15 nm gold particles, fixation with paraformaldehyde, some gold particles are associated with structures which seem to be dense granules (arrows). MZ, merozoite; PVM, parasitophorous vacuole membrane; SZ, schizont (bar, 0.5 μm).
**Fig 7.** Labelling with mAb AT7G10 and Prot A-15 nm gold particles, fixation with glutaraldehyde and osmium tetroxide, formvar-coated grid. D, dense granule; FO, feeder organelle; M, microneme; MZ, merozoite; PE, pellicle; PVM, parasitophorous vacuole membrane; RH, rhoptry; SZ, schizont; T, trophozoite (bar, 0.5 μm).

**Fig 8.** Labelling of a macrogamete with mAb AT7G10 and Prot A-15 nm gold particles, fixation with glutaraldehyde and osmium tetroxide, formvar-coated grid. AM, amilopectin-like granule; FO, feeder organelle; MA, macrogamete; PVM, parasitophorous vacuole membrane; WF, wall-forming body (bar, 0.5 μm).

**Fig 9.** Labelling of a microgamont with mAb AT7G10 and Prot A-15 nm gold particles, fixation with paraformaldehyde. MI, micro-gamete; MO, microgamont; PVM, parasito-phorous vacuole membrane (bar, 0.5 μm).
Western blot patterns observed with these mAbs suggest that different epitopes located on a common antigen are concerned. In immunoelectron microscopy stronger labellings were observed with mAbs AT7G10 and Y3A5, so we have emphasized the study of the antigens recognized by these mAbs.

Each stage of *C. parvum* carried the epitopes recognized by mAbs AT7G10 and Y3A5. These epitopes were clearly associated with: (i) the micronemes of sporozoites and merozoites; (ii) the parasitophorous vacuole membrane of trophozoites, schizonts, microgamonts and macrogametes; (iii) the outer oocyst wall; and (iv) the wall-forming bodies in maturating macrogametes.

In Western blots probed with mAb AT7G10, the same patterns were observed with PE Ag and BO Ag: 8 antigenic bands were observed. Two of these bands (> 500 kDa and 340 kDa) were recognized by mAb Y3A5 in BO Ag. Results obtained with mAb Y3A5 suggest that some modifications had occurred after treatment with detergents and sonication because mAb Y3A5 gave no signal on Western blot of PE Ag (antigen purified by affinity chromatography using Y3A5 as capture mAb). This antigenic material still contained antigens recognized by a Y3A5-HRP conjugate in ELISA but this was tested before dilution and boiling in electrophoresis buffer. These last steps must have modified the epitopes recognized by mAb Y3A5. Otherwise, signals could be observed when blots of PE Ag were incubated with mAbs AT7G10, Y14F3 or S1G12. In this case the antigen of > 500 kDa was labelled. These data would suggest that these mAbs and mAb Y3A5 recognize the same antigen and that the treatment with detergents or sonication could modify the antigenic structure but not the molecular weight of the molecule. Surprisingly, the antigen purified using mAb Y3A5 and revealed by mAb AT7G10 on Western blots,
showed a labelling pattern close to that obtained with mAb AT7G10 against BO Ag or PE Ag. This complex pattern could be explained by 3 hypotheses. (i) The epitope recognized by mAb Y3A5 was not only present on 2 antigens of > 500 and 340 kDa, but also on other antigens and these epitopes were altered during preparation for electrophoresis. (ii) Different molecular weight antigens were physically linked to the antigens of > 500 kDa and/or 340 kDa so they would be copurified by affinity chromatography. (iii) Some degradations occurred during the various purification steps.

The comparison of Western blot patterns obtained with SP Ag or BO Ag and mAb Y3A5 showed that the high molecular weight antigen (> 500 kDa) was certainly located at the sporozoite level whereas the 340 kDa antigen was only observed when oocyst walls were present in the preparation. Of the 8 antigens that bear the epitopes recognized by mAb AT7G10, only 3 of them were clearly detected with lysed sporozoites (> 500, 255 and 215 kDa). We were not able to determine whether these 3 antigens were only specific to the sporozoite stage or were also associated with the oocyst wall. Five antigens of 340, 130, 100, 80 and 50 kDa could only be demonstrated when the oocysts walls were present in the sample.

It is difficult to know whether these cross-reacting antigens were linked together in the same metabolic process. In the case of *Plasmodium falciparum*, 4 levels of cross-reactivities were described (Moelans and Schoenmakers, 1992). According to this classification, the antigens of *C parvum* recognized by mAbs AT7G10 and Y3A5 could be cross-reacting epitopes of type III (epitopes of different proteins that are individually expressed at different life cycle stages) or type IV (expression of the same antigen in different life cycle stages). Further studies are needed to find out which molecules bear the epitopes recognized by mAbs AT7G10 and Y3A5 in other stages of *C parvum*.

The pronase treatment abolished the reactivity of mAb AT7G10. This suggests that the molecule identified was a protein. Results of periodate oxidation demonstrate that the epitopes recognized were localized on a glycoprotein. Periodate treatment of PE Ag suppressed the binding of mAb AT7G10 with all molecules except one. Indeed, the high molecular weight antigen was still labelled but it seemed to migrate further into the gel, which suggests an alteration of this molecule. It is possible that carbohydrate moieties were cleaved by periodate treatment and that a lighter molecule with unchanged epitopes was generated. If this hypothesis is proved, the epitopes recognized are not localized on carbohydrate moieties. As far as the antigens of lower molecular weight are concerned, we hypothesize that periodate oxidation caused a big conformational change. Purified antigens were more susceptible to periodate oxidation since binding to all bands was abolished. During purification, antigens were exposed to acidic pH (glycine buffer pH 2.7), and some molecular changes could have occurred during this step.

The description of antigen of such a high molecular weight is uncommon; there was only one report of a > 900 kDa Cryptosporidium glycoprotein (Petersen *et al.*, 1992). Thus, in order to be sure that molecules were well solubilized, different procedures were used to dissociate the parasite. Even after drastic treatments with detergents, the high molecular weight antigen was still recognized in Western blot by some mAbs (AT7G10, S1G12, Y14F3). The reproduciveness of these results and the solubility of this antigen led us to believe that the epitopes were not carried by an insoluble membrane-associated molecule. The high molecular weight of this antigen was confirmed by its higher concentration
in the last fraction of the void volume and in the first post-void volume fractions of a Superdex 200 column (exclusion limit of 600 kDa). Of course, more accurate determination with molecular weight markers for gel filtration chromatography should be necessary.

Bonnin et al (1991) described mAbs HAD and TOU, which reacted with micronemes of zoites and also with the parasitophorous vacuole of trophozoites and macrogametes. On the other hand, the same authors (Bonnin et al, 1993) described mAbs ABD, BAX and SPO, which reacted with micronemes and macrogametes granules. MAbs AT7G10 and Y3A5, which we described in this paper, exhibited the 2 immunolabelling patterns described by Bonnin et al (1991, 1993) with their 2 sets of mAbs. Moreover, our mAbs recognized the parasitophorous vacuole membrane of microgametes and the outer oocyst wall. The antigens identified by our mAbs could have some homologies with those described by Bonnin et al (1991, 1993) (similarities in the immunolabelling patterns and in the sensitivity to periodate oxidation). However there was a discrepancy between the molecular weight of the antigens recognized. MAb TOU, produced by Bonnin et al (1991), recognized 2 major bands at 210 and 130 kDa.

Bjorneby et al (1990) described epitopes shared by sporozoites and merozoites of C parvum. Riggs et al (1989) have reported the production of mAbs that can bind to sporozoites and the external wall of intact oocysts. Bonnin et al (1991) have suggested that the content of microneme could be released in the parasitophorous vacuole by invading zoites. By electron microscopy, Current and Reese (1986) obtained evidence to suggest the participation of wall-forming bodies in the building of the oocyst wall. Bonnin et al (1993) described cross-reactive epitopes shared by micronemes and macrogamete granules. In the present work, our data suggest that these events are closely linked. By immunolocalization, we observed that the same epitopes are involved in separate development process, which would imply membrane synthesis. These epitopes are expressed at different levels by all parasitic stages of C parvum and therefore could not only be confined to the invasive process. In sporozoites these epitopes are always carried by a greater than 500 kDa antigen which is localized in the micronemes. Dense granules could also contain this antigen because labelling was also observed on some of them, but not so intensively. This antigen could play a role in the colonization of the host cell since the parasitophorous vacuole membrane is a sack of host cell origin (Marcial and Madara, 1986). The role assigned to microneme in the building of parasitophorous vacuole membrane of Cryptosporidium parvum is similar to the function attributed to micronemes of Plasmodium brasilianum merozoites (Torii et al, 1989a), to dense granules and micronemes of Sarcocystis muris (Entzeroth et al, 1986, 1991) and to dense granules of Toxoplasma gondii (Linder et al, 1992). The antigens described in this study appear to be shared by micronemes and dense granules but were not successfully demonstrated at the level of rhoptries of zoites. The antigen of > 500 kDa is very immunogenic since it was recognized by several of our mAbs raised according to different immunization schedules and by bovine immune sera (data not shown). Further studies are needed to know if crucial epitopes, which are the targets for a protective immune response, are carried by this antigen.

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