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Characterization of a monoclonal antibody reacting with antigen-4 domain of gp900 in *Cryptosporidium parvum* invasive stages

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Abstract *Cryptosporidium parvum* (Protozoa, Apicomplexa) infects the apical surface of intestinal epithelial cells, where it grows and divides within a membrane-bound parasitophorous vacuole. gp900, an abundant glycoprotein of *C. parvum* merozoites and sporozoites, is localized in micronemes and at the surface of invasive stages and participates in the invasion process. Here, we describe a new monoclonal antibody (mAb) against gp900. As shown by immunofluorescence of excysted parasites and immunoelectron microscopy of infected tissues, the mAb reacted with micronemes present in the apical pole of invasive stages. In immunoprecipitation experiments, the mAb was shown to react with a high molecular weight antigen co-migrating with gp900. Finally, three reactive clones were selected upon screening of a *C. parvum* genomic expression library with the mAb; and sequencing of the insert from one of them showed a 596 bp sequence identical to the DNA region encoding a domain of gp900 identified as antigen 4.

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Introduction

Cryptosporidium parvum is an Apicomplexan protozoan that develops in the brush border of epithelial cells in the digestive tract of mammals and causes chronic diarrhea in immunocompromized humans, especially in HIV-infected individuals or in malnourished children. The parasite may also cause acute diarrhea in normal adults and children (Current and Garcia 1991; Guerrant 1997; Agnew et al. 1998). Moreover, *C. parvum* is recognized as one of the most ubiquitous and difficult to control agents of water-borne diarrhea (Smith and Rose 1998).

Invasion of intestinal epithelial cells by *C. parvum* sporozoites and merozoites is a critical step for initiation of the infectious cycle. Like other members of the phylum Apicomplexa, such as *Plasmodium* spp. and *Toxoplasma gondii*, invasive *C. parvum* zoite stages possess a highly differentiated complex of secretory apical organelles, the micronemes, rhoptries, and dense granules, whose contents are sequentially released during the events of attachment, invasion, and establishment of the parasitophorous vacuole (PV; Joiner and Dubremetz 1993; Dubremetz et al. 1998). Invasion occurs by zoite attachment to the enterocyte, followed by discharge of the microneme and rhoptry contents, which induce fusion of the enterocyte microvillar membranes over the parasite pellicle. This encloses the parasite in an intracellular, but extracytoplasmic PV that remains in the brush border, at the surface of the enterocyte (Current and Reese 1986; Marcial and Madara 1986).

To examine the molecular basis of these *C. parvum*-enterocyte interactions, we produced monoclonal antibodies (mAbs) that recognized antigens within the zoite apical organelles; and we used them to characterize the identity and fate of cognate antigens during epithelial cell invasion and intracellular development (Bonnin et al. 1991, 1993, 1995). In this paper, we describe mAb IRM that recognizes a *C. parvum* microneme antigen and demonstrate that this mAb reacts with gp900, an

abundant mucin-like glycoprotein previously shown to be involved in host-cell invasion (Barnes et al. 1998).

Materials and methods

Hybridomas secreting mAb IRM were produced and selected in a series of experiments that were described previously (Bonnin et al. 1993, 1995). The IgG isotype of mAb IRM was determined by an immunoenzymatic assay (Behring Diagnostics). For immunoelectron microscopy, small pieces of terminal ileum were obtained from an immunosuppressed rat experimentally infected with a lamb isolate of the parasite. Processing of tissue samples and immunolocalization were performed as described previously (Bonnin et al. 1993, 1995). For immunoprecipitation, proteins from 1×10^7 oocysts/100 µl were diluted with nine volumes of hybridoma culture supernatants from either mAb IRM or mAb 10C6, a previously described anti-gp900 mAb (Petersen et al. 1992; Barnes et al. 1998), protease inhibitors (Doyle et al. 1993) and 1% Triton X-100. After 1 h incubation at room temperature, protein A Sepharose was added to the samples and incubated for 2 h at 37 °C to ensure

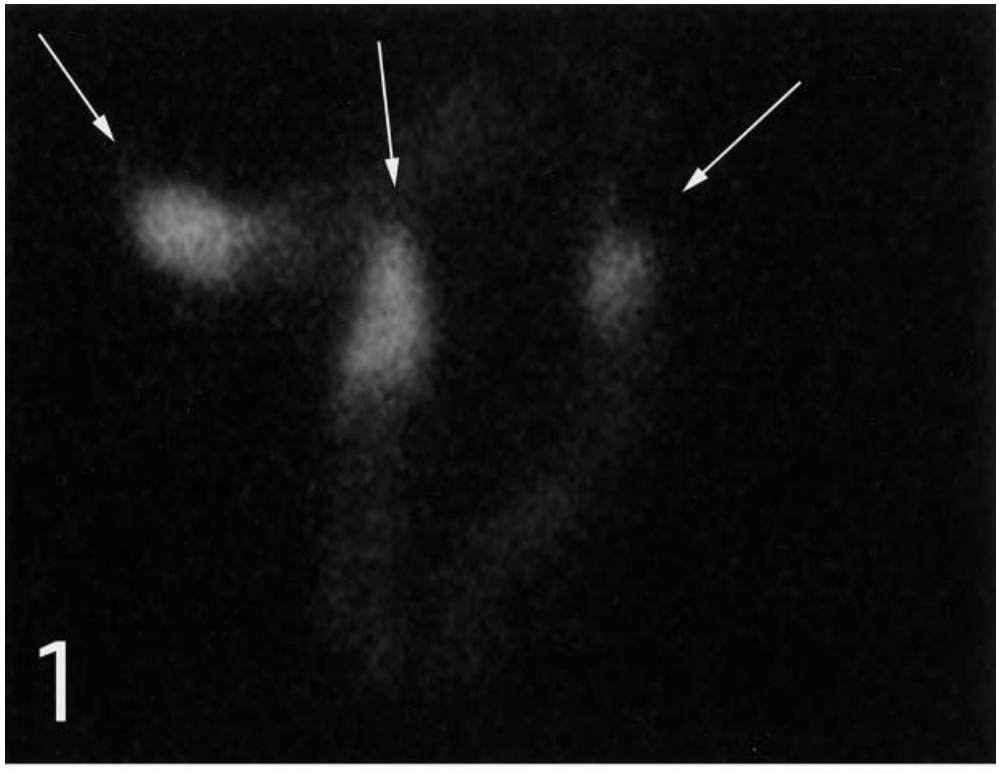

Fig. 1 Immunofluorescence of air-dried, acetone-fixed, excysted sporozoites of *Cryptosporidium parvum* labelled with mAb IRM, followed by a fluorescein-isothiocyanate-conjugated secondary antibody. Evans blue counterstaining. *White arrows* point to the IRM antigen at the apical pole of the parasite

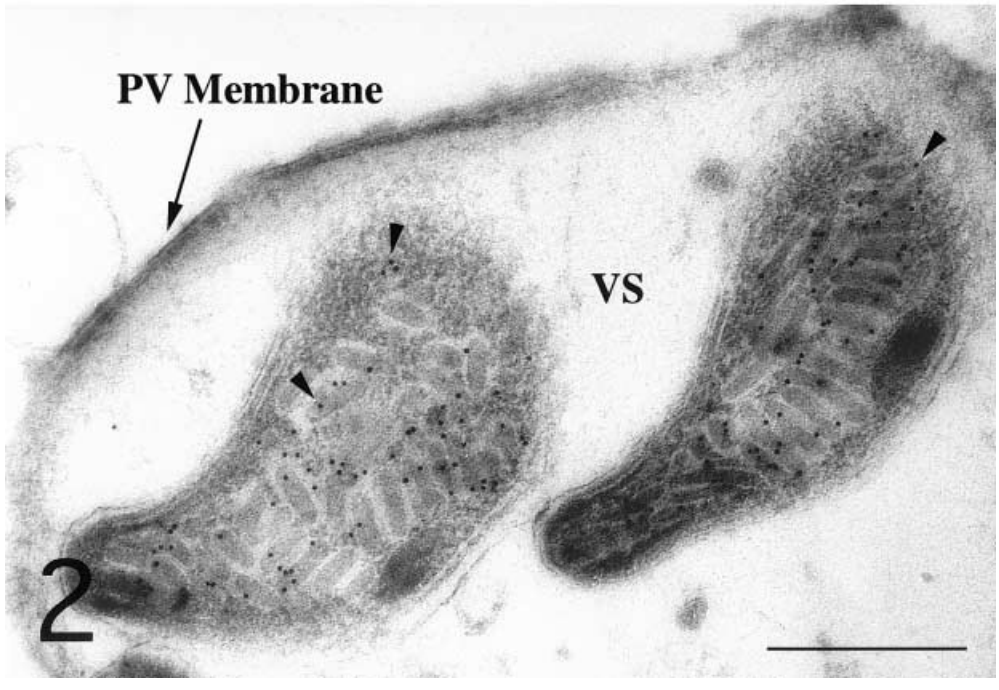

Fig. 2 Immunogold electron microscopy showing reactivity of mAb IRM with a developing meront of *C. parvum* in infected intestinal tissue. Infected tissues were embedded in LR White (London Resin Co.) and thin sections were labelled with mAb IRM, followed by rabbit anti-mouse immunoglobulin serum (Tago) and 8 nm protein A-coated gold beads. The gold particles (*arrowheads*) are clustered in the microneme region of the two merozoites shown in this field. *PV* Parasitophorous vacuole, *VS* vacuolar space. *Scale bar* 0.5 µm

immobilization of the immune complexes. Immunoprecipitated proteins were solubilized by boiling for 5 min in sample buffer; and proteins from approximately 10^7 oocysts/slot were separated by a 5–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gradient and transferred to nitrocellulose. The membrane was cut into two pieces that were incubated with either mAb IRM or polyclonal serum against gp900 and were revealed using anti-mouse IgG conjugated with alkaline phosphatase and colorimetric development with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. In order to clone the genomic DNA region encoding the IRM antigen, we screened a *Cryptosporidium parvum* λgt11 genomic DNA expression library (Kim et al. 1992) with hybridoma culture supernatant containing mAb IRM. Three clones were identified in a screen of 10^6 plaque-forming units. They were purified and the clone IRM 15 was sequenced by methods employed previously (Barnes et al. 1998).

Results and Discussion

In Apicomplexan protozoans such as *Plasmodium* spp., *Toxoplasma gondii*, and *Eimeria tenella*, micronemes have been shown to contain molecules used for recognition of, adhesion to, and invasion of host-cells, and for zoite motility (Dubremetz et al. 1998). In *Cryptosporidium parvum*, two microneme proteins have been identified to date, the thrombospondin-related adhesive protein of *Cryptosporidium* 1 (TRAP-C1; Spano et al. 1998), which shares structural features with other microneme molecules such as the TRAP molecules of *Plasmodium* spp. (Robson et al. 1988), the MIC 2 protein of *T. gondii* (Wan et al. 1997), the Etp 100 protein of *E. tenella* (Tomley et al. 1991), and gp900, a highly glycosylated mucin-like protein, which is the target of antibodies that inhibit the invasion and intracellular development of *C. parvum* in vitro (Barnes et al. 1998).

Here, we characterize a mouse IgG mAb that reacts with the micronemes of *C. parvum* invasive stages. The apical immunofluorescence observed with mAb IRM (Fig. 1) was identical to that previously observed using other mAbs that recognize *C. parvum* micronemes (Bonnin et al. 1991, 1993). When mAb IRM was assayed by immunoelectron microscopy on *C. parvum*-infected tissues, strong and specific labelling of micronemes was observed (Fig. 2). No labeling of rhoptries or dense granules was observed, nor was any surface-labelling of sporozoites or merozoites detected. Moreover, no gold particles were observed in the PV or over the vacuolar wall, and no IRM antigen was detected in the host-cell cytoplasm.

The characterization of the antigenic molecules recognized by mAb IRM was achieved by immunoprecipitation of *Cryptosporidium* antigens with IRM or with anti-gp900 mAb 10C6, followed by immunoblotting with IRM or polyclonal antibodies against gp900 (Fig. 3). These experiments showed that mAb IRM precipitated and reacted with several antigenic species, including a high molecular weight antigen that co-migrated with gp900. IRM also reacted with the same molecule in immunoprecipitates obtained using the anti-gp900 mAb 10C6. Taken together, these data suggest that IRM reacts with an epitope of gp900.

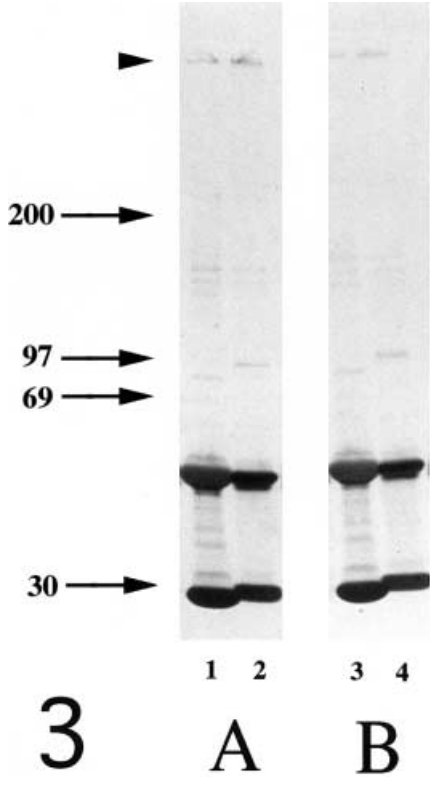

Fig. 3 Immunoprecipitation of *C. parvum* antigens showing the reactivity of mAb IRM with gp900. The immunoprecipitation step was done with mAb IRM (*lanes 1, 3*) or mAb 10C6 (*lanes 2 4*). Western blots were probed with a polyclonal antibody against gp900 (*panel A*) or mAb IRM (*panel B*) and developed with anti-mouse IgG conjugated with alkaline phosphatase, with colorimetric development. *Arrows* refer to molecular weight markers in kilodaltons, while the *arrowhead* corresponds to the expected molecular weight for the gp900 antigen. Reactive bands at about 30 kDa and 50 kDa correspond to the light and heavy chains of the antibodies used for immunoprecipitation, which are recognized by the secondary anti-IgG antibody conjugate used to develop the Western blot

When immunoblotted *C. parvum* antigen was oxidized with periodate under conditions that ablate the antigenicity of carbohydrate epitopes (Bonnin et al. 1991, 1993, 1995), the profile of mAb IRM reactivity was not altered (data not shown), suggesting that IRM binds a protein epitope. The mAb IRM was thus used to screen a genomic DNA *C. parvum* expression library; and three reactive clones were selected, each containing a 600-bp insert. Sequencing of the insert of IRM clone 15 produced a 596 nucleotide sequence identical to the DNA sequence encoding "antigen 4" , one of the previously sequenced regions of gp900 (Barnes et al. 1998). Clone IRM 15 contained a single open reading frame whose predicted sequence was identical to the sequence of amino acids 1,050–1,246 of gp900 from the NINC isolate of *C. parvum* (Petersen et al. 1997).

Although previous studies showed that gp900 surrounds the developing merozoite and is ^{125}I surface-labelled in sporozoites (Petersen et al. 1992; Barnes et al. 1998), no surface-labeling of sporozoites or merozoites

occurred with IRM in immunoelectron microscopy experiments performed in the current study. Similarly, no surface labeling of invasive stages was detected in previous immunoelectron microscopy studies using polyclonal anti-gp900 antibodies (Barnes et al. 1998). This discrepancy could be due to the fixation technique used for immunoelectron microscopy; and ultrastructural immunodetection of gp900 in cryosections of frozen samples or tissues processed with different fixation methods will be required to clarify this matter.

In this study, we describe the characterization of IRM, a mAb that recognizes gp900 in the micronemes of *C. parvum*, and show that the epitope recognized by this mAb resides in the "antigen 4" region of the polypeptide backbone of gp900. This new reagent will provide a useful tool for investigating the biological properties of distinct domains of gp900 and the fate of this molecule during the *C. parvum* enterocyte interaction.

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