Characterization of an Immunogenic Glycocalyx on the Surfaces of Cryptosporidium parvum Oocysts and Sporozoites

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Cryptosporidium parvum is a coccidian protozoa which causes severe diarrhea in patients with AIDS (3, 14). In parasitic infections, the surface coat of the parasite, which forms the interface between the parasite and its environment, must facilitate parasite survival in both the extracellular and intracellular stages of the life cycle. The principal components of this surface coat, for example, the glycoproteins in Trypanosoma brucei (9), the lipophosphoglycans (13), and the glycosinolophospholipids in Leishmania spp. (8), form a dense glycocalyx (GX) which effectively covers the entire surface of the parasite. The GX plays an important role in several organisms by modulating resistance to proteolysis (13), antibody binding (12), and adhesion (4). The present investigation is centered on the morphological, biochemical, and immunological characterization of the surface of the C. parvum oocyst.

Oocysts collected from stools from AIDS patients diagnosed with active cryptosporidiosis were purified (2), fixed, and stained with ruthenium red to characterize the carbohydrate-rich GX (6, 7). Transmission electron micrographs of an osmium-fixed oocyst show three visible sporozoites parallel to one another with their anterior ends all pointing in the same direction (Fig. 1A). Higher magnification shows that the oocyst is composed of two electron-dense layers (50 nm thick) (Fig. 1C) separated by a thin electron-lucent space. Ruthenium red staining of the oocyst shows a regularly spaced array of dense aggregates (20 to 30 nm thick) (Fig. 1B and D). In addition, some electron-dense stained material was seen inside the oocyst on the surfaces of sporozoites, suggesting that the GX may be present throughout sporozoite development. To confirm this, sporozoites were isolated, fixed, and stained with ruthenium red. Transmission electron micrographs (Fig. 2A) show crescent-shaped sporozoites averaging 4.8 by 1.2 μm in size with prominent nuclei and dense granules. Higher magnification shows that each surface is comprised of a trilaminar membrane (Fig. 2a, inset). The ruthenium red staining pattern was restricted to irregularly spaced 15- to 20-nm electron-dense bodies (Fig. 2b).

To characterize the ruthenium red-stained material on the surfaces of oocysts, we used a reductive procedure employing NaBH₄ and periodate oxidation, which is known to label only the surface of an organism (11). Labeled oocysts were subject to 85% phenol to disassociate the GX into its aqueous phase, dialyzed, and chromatographed on Sepharose Cl-6B in the presence of 0.1% sodium dodecyl sulfate (SDS) (1, 10). About 90% of the dialyzed labeled material eluted in the void volume, indicating that it had a molecular mass of >10⁶ Da (Fig. 3). The yields of protein and carbohydrate from 2 × 10⁷ oocysts averaged 8 and 40 μg, respectively, after SDS chromatography. The high-molecular-weight material was highly resistant to proteases (trypsin, Proteinase K, pronase, and thermolysin) and remained totally excluded from the running gel in SDS-polyacrylamide gel electrophoresis with or without proteolytic treatments (data not shown). Carbohydrate composition analysis indicated that glucose was the predominant sugar (65%), followed by galactose (12%). Mannose, xylose, and ribose were present in small amounts (4 to 8%). Both an alditol acetate derivative and a trimethylsilyl method showed that GalNAc was the only amino sugar present. In addition, trace amounts of a C₄₉ fatty acid was identified in the preparations by its characteristic fragmentation pattern. Studies of the antigenic composition of the oocyst wall have shown that carbohydrate moieties comprise a significant proportion of the epitopes that bind to antibodies in the immune response to C. parvum (15). The GX reacted positively on immunoblots with cryptosporidium-infected human sera (1/25 dilution) (Fig. 4B), indicating that it is antigenic. No such reactivity was observed with normal human sera (Fig. 4A). In addition, the purified GX also recognized two monoclonal antibodies (3D8 2B11 and 3F101G3) raised in BALB/c mice by repeated infections of C. parvum oocyst extracts (5) in a standard enzyme-linked immunosorbent assay and this reactivity

<p>| Mean level of reactivity (OD₄₀₅) ± SD to: |</p>
<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>IgM</th>
<th>IgG</th>
<th>IgA</th>
</tr>
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<tbody>
<tr>
<td>3D8 2B11</td>
<td>0.20 ± 0.08</td>
<td>0.19 ± 0.05</td>
<td>1.22 ± 0.175</td>
</tr>
<tr>
<td>3F101G3</td>
<td>0.13 ± 0.042</td>
<td>0.14 ± 0.038</td>
<td>1.13 ± 0.148</td>
</tr>
<tr>
<td>2F4 2H8</td>
<td>0.31 ± 0.02</td>
<td>0.31 ± 0.04</td>
<td>0.04 ± 0.05</td>
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TABLE 1. Monoclonal antibody reactivity with high-molecular-weight material

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was found to be specifically of the immunoglobulin A (IgA) type (Table 1). Treatment of GX with periodate at a concentration (10 mM) known to cleave specifically carbohydrate vicinal hydroxyl groups (16) abolished the reactivities of both the antibodies by about 50% (data not shown), suggesting that the epitope is glycosylated. Higher concentrations of periodate did not show any further decrease in the inhibition of binding. Partial inhibition of binding may have been due to incomplete removal of hydroxyl groups due to stearic hindrance. The high molecular mass of the GX distinguishes it from previously reported oocyst surface antigens ranging in molecular mass from 40 to 250 kDa (15).

In this study, we identified a polysaccharide matrix on the surface of a *C. parvum* oocyst that meets all the characteristics of a GX and is antigenic. First, ruthenium red-stained preparations of oocysts and sporozoites viewed by electron microscopy revealed uniformly distributed aggregates on the surfaces of *C. parvum* oocysts and randomly distributed vesicles on the surfaces of *C. parvum* sporozoites. Second, the GX was labeled by a periodate-NaB₃H₄ procedure which labels only the surface of a parasite. Third, 90% of the labeled material had an apparent molecular mass of >10⁶ Da in the presence of SDS, indicating that the material is not likely due to aggregation. Fourth, compositional analyses showed that 82% of the total mass was carbohydrate, with glucose being the abundant sugar. The resistance of the GX to proteases may be due to a putative peptide backbone being concealed by the abundance of carbohydrate. Resistance may be of biological importance, as it may impart structural and functional stability under gastrointestinal conditions. However, apart from protein estimation, there is no evidence that the GX includes a peptide backbone. Indeed it is possible that a glycolipid moiety is responsible for anchoring the GX of an oocyst.

Of particular interest, the high-molecular-weight carbohydrate material from *C. parvum* oocysts reacted positively with sera from cryptosporidium-infected patients and with IgA...
iodate-NaB₃H₄. Dialyzed material labeled with iodate-NaB₃H₄ was fractionated bodies, which are 15 to 20 nm in size.

monoclonal antibodies raised against C. parvum oocyst extracts, demonstrating that it is antigenic. Additionally, partial loss of the antigenicity of the GX after mild periodate oxidation treatment indicated that carbohydrate is the major antigenic determinant. Since GX is the first protein with which the host comes into contact and because of its carbohydrate antigenicity, it may be an important immunological target.

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REFERENCES


