Role of Immunoglobulin A Monoclonal Antibodies against P23 in Controlling Murine Cryptosporidium parvum Infection

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Cryptosporidium parvum is an important diarrhea-causing protozoan parasite of immunocompetent and immunocompromised hosts. Immunoglobulin A (IgA) has been implicated in resistance to mucosal infections with bacteria, viruses, and parasites, but little is known about the role of IgA in the control of C. parvum infection. We assessed the role of IgA during C. parvum infection in neonatal mice. IgA-secreting hybridomas were developed by using Peyer’s patch lymphocytes from BALB/c mice which had been orally inoculated with viable C. parvum oocysts. Six monoclonal antibodies (MAbs) were selected for further study based on indirect immunofluorescence assay reactivity with sporozoite and merozoite pellicles and the antigen (Ag) deposited on glass substrate by gliding sporozoites. Each MAb was secreted in dimeric form and recognized a 23-kDa sporozoite Ag in Western immunoblots. The Ag recognized conigrated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis with P23, a previously defined neutralization-sensitive zoite pellicle Ag. MAbs were evaluated for prophylactic or therapeutic efficacy against C. parvum, singly and in combinations, in neonatal BALB/c mice. A combination of two MAbs given prophylactically prior to and 12 h following oocyst challenge reduced the number of intestinal parasites scored histologically by 21.1% compared to the numbers in mice given an isotype-matched control MAb (P < 0.01). Individual MAbs given therapeutically in nine doses over a 96-h period following oocyst challenge increased efficacy against C. parvum infection. Four MAbs given therapeutically each reduced intestinal infection 34.4 to 42.2% compared to isotype-matched control MAb-treated mice (P < 0.05). One MAb reduced infection 63.3 and 72.7% in replicate experiments compared to isotype-matched control MAb-treated mice (P < 0.0001). We conclude that IgA MAbs directed to neutralization-sensitive P23 epitopes may have utility in passive immunization against murine C. parvum infection.

Since the first case of human cryptosporidiosis was described in 1976, the coccidian parasite Cryptosporidium parvum has become recognized as an important diarrhea-causing agent worldwide (13, 41). Immunocompromised individuals such as AIDS patients are particularly susceptible and exhibit an unrelenting infection which may progress to death (13, 41). No commercially available antiparasite chemotherapy is consistently effective in treating such patients (6). Passive immunization with antibodies (Abs) against whole C. parvum organisms has variable efficacy in immunocompromised or neonatal hosts (1, 5, 9, 12, 24, 25, 31, 37, 39, 40, 42). Recovery from and resistance to cryptosporidiosis require principally cellular, but also humoral, immune components in immunocompetent hosts (31, 45). Despite anti-C. parvum Ab responses, AIDS patients with cryptosporidiosis fail to clear the infection (4). However, the relative success of orally administered Abs to immunodeficient hosts suggests that passive humoral immunization can control intestinal C. parvum infection (5, 24, 25, 31, 32, 39, 40, 42, 45).

Although C. parvum is a mucosal pathogen, the role of immunoglobulin A (IgA) during infection has only recently received attention. IgA to 15- to 17-, 23-, 26-, and 33-kDa antigens of C. parvum sporozoites has been detected in intestinal washes and serum from infected humans and mice (4, 30, 36). In addition, the level of parasite-specific IgA in serum, saliva, and feces was higher in AIDS patients with chronic C. parvum infection than in uninfected AIDS patients or normal individuals (4, 10, 14). While some studies concluded that IgA has little or no protective effect against cryptosporidiosis (4, 10), IgA responses to neutralization-sensitive epitopes have not been evaluated in such patients (31). Epitope specificity of Abs is clearly important in neutralization of C. parvum zoites (reviewed in reference 31). The epitope specificity of secretory IgA responses in AIDS patients may be defective; Abs against neutralization-sensitive epitopes either may not be generated or may be insufficient to control C. parvum in the presence of cellular and/or other immune dysfunctions (14).

IgA has been associated with resistance to a number of mucosal pathogens (8, 22, 23, 29, 44). For example, treatment with IgA MAbs specific to rotavirus, Sendai virus, Vibrio cholerae, or Salmonella typhimurium controlled otherwise lethal challenges in mice (8, 19, 22, 44). Because IgA conferred protection against these mucosal pathogens and Ag-specific IgA responses occur in hosts with cryptosporidiosis, we hypothesized that IgA directed to neutralization-sensitive epitopes may be useful in passive immunization against C. parvum. To determine whether IgA can influence the course of C. parvum infection, we produced dimeric IgA MAbs to P23, a previously defined C. parvum Ag containing neutralization-sensitive epitopes (1, 3, 21, 26). Here we report that dimeric anti-P23 IgA MAbs have efficacy against intestinal C. parvum infection in neonatal mice.

MATERIALS AND METHODS

Parasite isolation and Ag preparation. The Iowa isolate of C. parvum (originally obtained from H. Moon, Ames, Iowa) was used in the present study and maintained by passage in 2-day-old Holstein bull calves (2, 33). Oocysts were isolated from feces by sequential centrifugation involving discontinuous sucrose and isonicyclic Percoll gradients as previously described (2). Oocysts were stored in 2.5% (wt/vol) KCl2O4 at 4°C for up to 3 months prior to use. Prior to excystation, oocysts were washed with sterile phosphate-buffered
saline (PBS) containing 1.75% (wt/vol) sodium hypochlorite, followed by sterile PBS (4°C). Oocysts were then incubated (45 min, 37°C) in Hanks’ balanced salt solution containing 0.1% (wt/vol) taurocholic acid. Excysted sporozoites were isolated by DEAE-cellulose anion-exchange chromatography as previously described (22). For use in mouse immunization, enzyme-linked immunosorbent assays (ELISAs), and Western immunoblots, sporozoites were disrupted by freeze-thaw cycles and sonication (20 10-s pulses, 1 min intervals) in lysis buffer (50 mM Tris, 5.0 mM EDTA, 0.1% IgG, 5% [vol/vol] glycerol, and 5% [vol/vol] aprotinin). MAb C23 MAb C6B6 (IgG1) (1, 3). Following washing, nitrocellulose lanes were incubated (2 h, 37°C) with affinity-purified, peroxidase-labelled goat anti-mouse IgA Abs (Kirkegaard & Perry) and developed with 4-chloronaphthol.

The monomeric-polymeric state of IgA MAbs was evaluated by Western immunoblotting. Briefly, formalin-fixed, detergent-fractionated hybridoma supernatants were separated by nonreducing SDS-PAGE (15% acrylamide gel electrophoresis and electroblotted to nitrocellulose membranes (22). Membranes were incubated (2 h, 37°C) with peroxidase-labelled rat anti-mouse IgA MAbs (Pharmingen) and developed with 4-chloronaphthol.

Experimental design. (i) Prophylactic effect of IgA MAbs on intestinal infection. Six anti-C. parvum IgA MAbs (G9H4, H8H6, H8H2, F3H6, H8H12, and G9H9) were selected for further study based on their strong reactivity with sporozoites by IFA. MAbs with IgA Ab (Kirkegaard & Perry), and developed with 4-chloronaphthol.

FIG. 1. Sporozoites stained by IFA with IgA MAb G9H4. Note the reactivity of MAb G9H4 with the sporozoite cell body (arrows). The staining patterns of MAbs G9H4, H8H12, G9H9, H8H2, F3H6, and H8H6 were the same. Bar, 5 μm.

RESULTS

Mucosal immunization with C. parvum results in a preferential Peyer’s patch IgA response to P23. From 20 fusions, six anti-C. parvum IgA MAb-producing hybridomas (G9H4, H8H6, H8H2, F3H6, H8H12, and G9H9) were selected for further study based on their strong reactivity with sporozoites by ELISA and IFA. By IFA, each MAb bound diffusely to the cell membrane of C. parvum sporozoites during gliding motility (Fig. 1).

Effect of IgA MAb combination on intestinal infection. To determine the effect of IgA MAbs on intestinal infection, groups of 10 S. mansoni-infected BALB/c mice were inoculated with 10^6 oocysts per mouse intragastrically by using a blunt, curved 30-gauge needle. Four hours prior to challenge and 12 h postchallenge, mice received combinations of ascites containing IgA MAbs (G9H4 and H8H6, H8H2 and F3H6, or H8H12 and G9H9). Control groups received isotype-matched control Abs (MOCP-318 or MAB 92.07t), or anti-C. parvum P23 MAB C6B6 (IgG1) (1, 3). Following washing, nitrocellulose lanes were incubated (2 h, 37°C) with affinity-purified, peroxidase-labelled goat anti-mouse IgA Abs (Kirkegaard & Perry), and developed with 4-chloronaphthol.
Hybridomas maintained in serum-free medium secreted IgA MAbs in dimeric form, as demonstrated by Western immunoblot analysis of supernatants separated under nonreducing conditions (Fig. 2). Each MAb contained monomers and polymers. All six MAbs recognized a 23-kDa *C. parvum* sporozoite molecule in a Western immunoblot (Fig. 3). This molecule comigrated with P23, previously defined by MAb C6B6 (1, 3).

**IgA MAbs significantly reduce *C. parvum* infection in mice.** A combination of MAbs G9H4 and H8H6 administered prophylactically to mice significantly reduced the mean infection score compared to that of mice receiving an isotype-matched control MAb (P = 0.008; 21.1% reduction) (Table 1). A combination of MAbs H8H2 and F3H6 or H8H12 and G9H9 did not significantly reduce infection (Table 1). Because neutralization of *C. parvum* by MAbs is dependent on time and MAb concentration (27, 31), a second experiment was performed in which the number and duration of treatments with individual MAbs were increased. In the second experiment, all MAb treatments were given per os.

Individual anti-P23 IgA MAbs H8H2, F3H6, G9H9, and H8H6 significantly reduced mean intestinal infection scores (range, 34.4 to 42.2%) compared to those of isotype-matched control MAb-treated mice (Table 2). MAb G9H4 reduced infection 63.3 and 72.7% in replicate experiments compared to those of isotype-matched control MAb-treated mice (Table 2). MAb H8H12 did not reduce the mean infection score (Table 2).

**DISCUSSION**

The anti-*C. parvum* P23 IgA MAbs presented herein were produced following a combination of oral and intraperitoneal immunizations. One or both of these routes of immunization preferentially induced an anti-P23 IgA response in Peyer’s patches. Other studies have suggested that P23 is immunodominant based on serum IgG responses following infection (21, 31). The six IgA MAbs selected recognized surface P23 on sporozoites and merozoites, as well as in sporozoite Ag deposits.

**TABLE 1. Prophylactic effect of oral and intraperitoneal administration of anti-P23 IgA MAb combinations against *C. parvum* challenge in neonatal mice**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean infection score ± SD&lt;sup&gt;a&lt;/sup&gt;</th>
<th>P value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5.9 ± 1.2</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>92.07t</td>
<td>5.2 ± 0.8</td>
<td>0.0001</td>
</tr>
<tr>
<td>G9H4 + H8H6</td>
<td>4.1 ± 0.9</td>
<td>0.008</td>
</tr>
<tr>
<td>H8H12 + G9H9</td>
<td>5.0 ± 0.7</td>
<td>&gt;0.25</td>
</tr>
<tr>
<td>H8H2 + F3H6</td>
<td>4.8 ± 1.6</td>
<td>&gt;0.25</td>
</tr>
</tbody>
</table>

<sup>a</sup> Scores are based on ileum and cecum infection levels (0, absence of infection; 1, to 33% of mucosa parasitized; 2, 34 to 66% of mucosa parasitized; 3, greater than 66% of mucosa parasitized). Cumulative scores that included both intestinal regions were calculated for each mouse. The maximum cumulative score per mouse was 6.

<sup>b</sup> Compared to *C. parvum*-challenged mice given isotype-matched control IgA MAb 92.07t.

**TABLE 2. Treatment effect of multiple oral doses of individual anti-P23 IgA MAbs on *C. parvum* infection in neonatal mice**

<table>
<thead>
<tr>
<th>Treatment per os</th>
<th>Mean infection score ± SD&lt;sup&gt;a&lt;/sup&gt; (P value)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt 1</td>
</tr>
<tr>
<td>92.07t</td>
<td>9.0 ± 1.0</td>
</tr>
<tr>
<td>H8H2</td>
<td>5.2 ± 1.1 (0.0001)</td>
</tr>
<tr>
<td>H8H12</td>
<td>9.6 ± 2.0 (&gt;0.5)</td>
</tr>
<tr>
<td>F3H6</td>
<td>5.8 ± 2.0 (0.025)</td>
</tr>
<tr>
<td>G9H9</td>
<td>5.2 ± 1.8 (0.0005)</td>
</tr>
<tr>
<td>G9H4</td>
<td>3.3 ± 1.1 (0.0001)</td>
</tr>
<tr>
<td>H8H6</td>
<td>5.9 ± 1.8 (&lt;0.0005)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Scores are based on infection levels in the terminal jejunum, ileum, cecum, and proximal colon (0, absence of infection; 1, to 33% of mucosa parasitized; 2, 34 to 66% of mucosa parasitized; 3, greater than 66% of mucosa parasitized). Cumulative scores that included each intestinal region were calculated for each mouse. The maximum cumulative score per mouse was 12.

<sup>b</sup> Compared to *C. parvum*-infected mice treated with isotype-matched control IgA MAb 92.07t per os.

<sup>c</sup> ND, not done.
ited in trials. Sporozoite Ag trails have been identified with MAbs against GP15 (16, 38) and P23 (3, 38) by IFA. Ags deposited in trials may be important functional target Ags because they are deposited during locomotion and are shed during invasion of host cells (3, 16, 38). Spleen-derived MAbs against GP15 (monomeric IgA) and P23 (IgG1) have been shown to decrease infection levels in mouse models, indicating that GP15 and P23 contain neutralization-sensitive epitopes (1, 26, 31, 37). Because P23 is conserved among geographically diverse bovine and human C. parvum isolates (26), present in both infectious zoeite stages, deposited during zoeite motility, and known to contain neutralization-sensitive epitopes, it may be a biologically relevant Ag which can be targeted for immunological intervention. Results of the present study support the relevance of P23 and suggest that dimeric IgA targeted to this Ag may be a functional mucosal immune response to infection.

While Ab responses to C. parvum have been described in humans, mice, sheep, cattle, and other mammals, relatively few studies have examined mucosal IgA responses (4, 10, 14, 30, 36; reviewed in reference 31). IgA directed to a 15- to 17-kDa antigen has been observed in the intestines and sera of infected mice (30). Anti-C. parvum IgA has been demonstrated in the serum and feces of AIDS patients with cryptosporidiosis (4, 10). The saliva of human immunodeficiency virus-positive pre-AIDS patients who cleared a C. parvum infection had higher titers of specific IgA than did that of AIDS patients with persistent cryptosporidiosis, suggesting that C. parvum-specific secretory IgA may contribute to recovery from cryptosporidiosis (14). Results presented here support this hypothesis.

The utility of Abs in the control of intestinal cryptosporidiosis is exemplified by passive immunotherapy studies with polyclonal Abs and MAbs (reviewed in reference 31). Anti-C. parvum MAbs, and polyclonal Abs in hyperimmune hen egg yolk and secretory IgG1-rich hyperimmune bovine colostrum, have been efficacious in the control of intestinal cryptosporidiosis in immunologically immature or immunocompromised rodent models (1, 5, 9, 12, 25, 26, 32, 34, 37). Hyperimmune polyclonal Ab preparations have had variable efficacy against intestinal C. parvum infection in immunocompromised humans (24, 39, 40, 42).

Hepatobiliary cryptosporidiosis is a common complication in AIDS patients having persistent intestinal C. parvum infections (6, 7, 15, 20, 28, 41, 43). While oral Ab-based immunotherapy has shown efficacy against intestinal infection, this approach is unlikely to be effective against hepatobiliary cryptosporidiosis (5, 25). Further, hepatobiliary cryptosporidiosis may provide a reservoir of infection which can contribute to relapse of intestinal infection following clearance with Ab-based immunotherapy (39, 40). Parenterally administered dimeric IgA migrates to hepatobiliary and other extraintestinal mucosal sites which are inaccessible to orally administered Abs. Additionally, IgA acquires a secretory component during migration, thereby enhancing its resistance to the harsh mucosal environment (17, 23). These properties may confer a therapeutic advantage on IgA-based passive immunization against C. parvum infection. Studies assessing the delivery and efficacy of anti-C. parvum dimeric IgA MAbs in hepatobiliary cryptosporidiosis are in progress in our laboratories. In preliminary studies, significant anti-C. parvum IgA MAb concentrations and reduction of infection have been observed in the intestinal tract following parenteral anti-C. parvum IgA MAb administration in mice with cryptosporidiosis (11a).

IGA prevents access of pathogens to mucosal surfaces, precipitates pathogen agglutination and clearance, and effectively protects animals against otherwise lethal infections (8, 17, 19, 22, 29, 44). Results presented here demonstrate that dimeric IgA MAbs can reduce infection by an opportunistic intestinal protozoan parasite. While cellular immunity is required to overcome C. parvum infection in immunocompetent patients (31, 45), IgA directed to neutralization-sensitive zoite epitopes may have utility in passive immunization against cryptosporidiosis in immunocompromised hosts.

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REFERENCES


