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 coccidial 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 unremitting 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 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 isopicryl Percoll gradients as previously described (2). Oocysts were stored in 2.5% (wt/vol) K2Cr2O7 at 4°C for up to 3 months prior to use.

Prior to exocystation, oocysts were washed with sterile phosphate-buffered...
saline (PBS) containing 1.75% (wt/vol) sodium hypochlorite, followed by sterile PBS (4°C). Oocytes 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 (12). For use in mouse immunization, enzyme-linked immunosorbent assays (ELISAs), and Western immunoblotting, sporozoites were disrupted by freeze-thaw cycles and sonication (20-10 pulses, 1-min intervals) in lysis buffer (50 mM Tris, 5.0 mM EDTA, 0.1 mM Na-p-tosyl-l-lysine chloride, 5.0 mM phenylmethylsulfonfyl fluoride, 0.01 mM leupeptin, 0.01 mM pepstatin, 1.0% (wt/vol) oxytetracycline) and then centrifuged (10,000 × g, 30 min) to remove the insoluble fraction. For mouse immunizations and ELISA, the supernatant was dialyzed against PBS (12,000 to 14,000 molecular weight exclusion limit, 4°C) and the protein concentration was determined by the bicinchoninic acid assay (Pierce, Rockford, Ill.). Sporozoite Ag was stored at −80°C prior to use.

Merozoites were isolated by Percoll density gradient centrifugation of intestinal contents from neonatal BALB/c mice at 65 h postinoculation with oocysts (32).

IgA MAb production and characterization. Six-week-old BALB/c mice (Jackson Laboratory, Bar Harbor, Maine) were housed in microisolation cages and maintained with 12-h photoperiod cycles. Mice were inoculated once or twice at 3-week intervals with 5 × 10³ viable C. parvum oocytes. Three weeks following the second oocyst inoculation, mice were injected intraperitoneally with 5 µg of sporozoite Ag in PBS. Sera were screened by ELISA and indirect immunofluorescence assay (IFA) for antibody responses to anti-C. parvum IgA antibody bodies. Positive sera were stored at −80°C until used as controls in immunoadsays.

Four days after the final immunization, mice were euthanized by CO₂ inhalation. Their spleens were then dissected and removed aseptically, pooled, and incubated (1 h, 37°C) in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gemini Bioproducts), 0.1% (wt/vol) collagenase type IV (Sigma), and 100 U of penicillin, 100 µg streptomycin, and 0.25 µg of amphotericin B (Sigma) per ml. Peyer’s patches were then transferred into a glass homogenizer and gently disrupted. Peyer’s patch cells were fused with SP2/O myeloma cells and resuspended in monocyte-macrophage-thymocyte-conditioned medium as previously described (11). Hybridomas producing IgA antibodies were identified by ELISA. Briefly, plates were coated (12 h, 4°C) with an affinity-purified anti-mouse IgA Ab (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) and blocked with 1% (wt/vol) nonfat dry milk in PBS (30 min, 21°C). Hybridoma supernatants were added to plates, which were then incubated (2 h, 37°C), washed, and incubated (2 h, 37°C) with a peroxidase-labelled goat anti-mouse IgA Ab (Kirkegaard & Perry). Following washing, the plates were developed with 2,2'-azino-di(3-ethylbenzthiazoline sulfate) (ABTS; Kirkegaard & Perry), and developed with ABTS. Hybridomas secreting IgA were then assessed for sporozoite specificity by ELISA and IFA as follows.

ELISA plates were coated with sporozoite Ag (5 µg/ml of carbonate buffer), blocked, and incubated with IgA-positive hybridoma supernatants. Immune serum and IgA Ab MOPC-318 were included as controls. Plates were then washed, incubated, washed, incubated with peroxidase-labelled goat anti-mouse IgA Ab (Kirkegaard & Perry), and developed with ABTS. Hybridomas secreting C. parvum-specific IgA were assayed further by IFA to identify patterns of binding to sporozoites and merozoites. For IFA, purified sporozoites or merozoites were gently heat fixed in multistrips of previously air-dried NR8383 cells previously treated with boiling physiological saline (3.8% NaCl).

Hybridoma supernatants, along with immune and preimmune control sera (1:10 dilution), were added to individual wells and incubated (40 min, 37°C) at 1:10 dilution. Following washing, each well was incubated sequentially (40 min, 37°C) with a biotinylated rat anti-mouse IgA MAb (Pharmingen, San Diego, Calif.) and streptavidin-fluorescein (Pharmingen) and then examined with an Olympus epifluorescence microscope. Following IFA evaluation, antisporeozoite and antimerozoite IgA-secreting hybridomas were cloned three times by limiting dilution. A single IgA-secreting hybridoma (92.07t) not recognizing C. parvum was selected for use as an isotype control and cloned. Cloned hybridomas were adapted to serum-free medium (Ultradoma; Biowhittaker) and then cryopreserved. Ascites were produced for all IgA MAbs as previously described (11). The IgA MAb ascites titers against C. parvum sporo- zotes were determined by IFA. The IgA MAb concentration in ascites was determined by IFA with IgA MAb G9H4. Note the reactivity of G9H4 with the sporozoite pellicle and Ag trails (arrows). The staining patterns of MAbs G9H4, H8H12, F3H6, and H8H6 were the same. Bar, 5 µm.

FIG. 1. Sporozoites stained by IFA with IgA MAb G9H4. From the reactive of G9H4 with the sporozoite pellicle and Ag trails (arrows). The staining patterns of MAbs G9H4, H8H12, 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 pellicle of both sporozoites (Fig. 1) and merozoites (data not shown). In addition, each MAb also bound to antigen trails deposited on a glass substrate over the course traveled by sporozoites during gliding motility (Fig. 1).
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.

**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</th>
<th>P value</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></td>
</tr>
<tr>
<td>G9H4 + HSH6</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>

<table>
<thead>
<tr>
<th>Expt 1</th>
<th>Expt 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>92.07t</td>
<td>9.0 ± 1.0</td>
</tr>
<tr>
<td>H8H2</td>
<td>5.2 ± 1.1 (&lt;0.001)</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 (&lt;0.025)</td>
</tr>
<tr>
<td>G9H9</td>
<td>5.2 ± 1.8 (&lt;0.005)</td>
</tr>
<tr>
<td>G9H4</td>
<td>3.3 ± 1.1 (&lt;0.0001)</td>
</tr>
<tr>
<td>H8H6</td>
<td>5.9 ± 1.8 (&lt;0.005)</td>
</tr>
</tbody>
</table>

* a Scores are based on ileum and cecum infection levels (0, absence of infection; 1, 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 12.

* b 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</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt 1</td>
<td>Expt 2</td>
<td></td>
</tr>
<tr>
<td>92.07t</td>
<td>9.0 ± 1.0</td>
<td>8.8 ± 0.9</td>
</tr>
<tr>
<td>H8H2</td>
<td>5.2 ± 1.1 (&lt;0.001)</td>
<td>ND</td>
</tr>
<tr>
<td>H8H12</td>
<td>9.6 ± 2.0 (&gt;0.5)</td>
<td>ND</td>
</tr>
<tr>
<td>F3H6</td>
<td>5.8 ± 2.0 (&lt;0.025)</td>
<td>5.4 ± 1.1 (&lt;0.05)</td>
</tr>
<tr>
<td>G9H9</td>
<td>5.2 ± 1.8 (&lt;0.005)</td>
<td>ND</td>
</tr>
<tr>
<td>G9H4</td>
<td>3.3 ± 1.1 (&lt;0.0001)</td>
<td>2.4 ± 1.8 (&lt;0.0001)</td>
</tr>
<tr>
<td>H8H6</td>
<td>5.9 ± 1.8 (&lt;0.005)</td>
<td>ND</td>
</tr>
</tbody>
</table>

* a Scores are based on ileum levels in the terminal jejunum, ileum, cecum, and proximal colon (0, absence of infection; 1, 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.

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

* c ND, not done.
ited in trails. Sporozoite Ag trails have been identified with MAbs against GP15 (16, 38) and P23 (3, 38) by IFA. Ags deposited in trails 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 zoite stages, deposited during zoite 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 human C. parvum 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

20. McGowan, I. C., reviewed in reference 31). IgA directed to a 15- to 17-kDa hum...


