Efficacy of Monoclonal Antibodies against Defined Antigens for Passive Immunotherapy of Chronic Gastrointestinal Cryptosporidiosis

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Received 7 May 2001/Returned for modification 14 July 2001/Accepted 13 October 2001

Cryptosporidium parvum is an important cause of diarrhea in humans and calves and can persistently infect immunocompromised hosts. Presently, there are no consistently effective parasite-specific drugs for cryptosporidiosis. We hypothesized that neutralizing monoclonal antibodies (MAbs) targeting the apical complex and surface antigens CSL, GP25-200, and P23 could passively immunize against cryptosporidiosis. We recently reported that a formulation of MAbs 3E2 (anti-CSL), 3H2 (anti-GP25-200), and 1E10 (anti-P23) provided significant additive prophylactic efficacy over that of the individual MAbs in neonatal ICR mice. In the present study, these MAbs were evaluated for therapeutic efficacy against persistent infection in adult gamma interferon-depleted SCID mice. 3E2 demonstrated the most significant and consistent therapeutic effect, reducing intestinal infection in two experiments. In one experiment, 3E2 plus 3H2 and 3E2 plus 3H2 plus 1E10 also significantly reduced infection; however, no significant increase in efficacy over 3E2 alone was apparent. The results indicate that anti-CSL MAb 3E2 has highly significant efficacy in reducing, but not eliminating, persistent C. parvum infection.

The apicomplexan parasite Cryptosporidium parvum infects the intestinal tract in humans and calves and other agriculturally important animals and is a leading cause of diarrhea throughout the world (30). In neonates, the elderly, and hosts having congenital or acquired immunodeficiencies, the disease may become chronic and life-threatening (30, 59). Dissemination to extraintestinal sites may occur in immunocompromised hosts and contribute to morbidity (30, 79). While knowledge of the biology of C. parvum has advanced in recent years, there are presently no consistently effective parasite-specific drugs, vaccines, or immunotherapies for cryptosporidiosis (3, 39, 52, 64, 67). MAbs determined to have the highest neutralizing activity and to react with distinct epitopes on each antigen were then evaluated, individually and in multiple epitope-specific combinations, for the ability to prevent infection in oocyst-challenged neonatal ICR mice. Anti-CSL MAb 3E2 had the highest protective activity of all MAbs, reducing infection levels by 62 to 92%. 3E2 combined with anti-GP25-200 MAb 3H2 and anti-P23 MAb 1E10 conferred significant additive protection over that provided by the individual MAbs and reduced infection levels by 86 to 93% (67). Complete prevention of infection was observed in up to 40% of mice administered 3E2, alone or in combination with 3H2 and 1E10.

In view of the profound prophylactic efficacy of 3E2 and combinations of MAbs containing 3E2 observed in neonatal ICR mice, the objective of the present study was to determine
the therapeutic efficacy of the MAbs against chronic, fulminating gastrointestinal cryptosporidiosis. Because chronic disseminated *C. parvum* infection does not develop in neonatal ICR or other immunocompetent mice, a fundamentally different adult gamma interferon (IFN-γ)-depleted SCID mouse model was used. 3E2 had the most significant therapeutic effect, consistently reducing intestinal infection in each of two experiments. 3E2 combined with 3H2 and/or 1E10 also significantly reduced intestinal and/or biliary infection and fecal oocyst shedding in one experiment. However, the observed reductions were not significantly greater than those in mice treated with 3E2 alone. While the explanation for the apparent lack of increased therapeutic efficacy of the combined MAbs is not entirely clear, the results provide unequivocal evidence that passive immunotherapy with anti-CL1 MAb 3E2 can significantly reduce intestinal infection in an immunodeficient-adult-rat model of persistent cryptosporidiosis.

**MATERIALS AND METHODS**

**Parasites.** The Iowa *C. parvum* isolate (35) was used in all experiments. Oocysts were obtained from Pleasant Hill Farm (Troy, Idaho) following isolation from experimentally infected newborn Cryptosporidium-free calves as previously described (63). Oocysts were stored in phosphate-buffered saline (PBS) containing 1,000 U of penicillin and 1 mg of streptomycin per ml (4°C) and used within 6 weeks of isolation. Immediately prior to use, oocysts were washed in medium. In vitro excystation (37°C, 0.15% [wt/vol] taurocholate, 2 h) of oocysts used for all mouse inoculations was >90% (53).

**Production of MAbs.** Immunofluorescence and neutralizing antibody titers of MAbs were assessed by indirect immunofluorescence (IIF) assay and neutralization assays, respectively. MAbs were produced in bioreactors to obtain the quantities required for therapeutic studies in pure form. MAbs were produced in bioreactors to obtain the quantities required for therapeutic studies in pure form.

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says, in vitro sporozoite-neutralizing activity, or in vivo neutralizing activity in oocyst-challenged neonatal ICR mice. Western blot reactivities of the MAbs were also unaffected, being indistinguishable from those reported previously (67).

Passive immunotherapy with anti-CSL MAb 3E2 alone has highly significant efficacy, with consistent therapeutic predictability, against persistent intestinal infection with *C. parvum*. (i) Reduction of tissue infection levels. In experiment 1, treatment with 3E2 resulted in a highly significant ~39% reduction of intestinal infection levels compared to isotype control treatment (Fig. 1A). Pyloric and biliary infection levels were also lower in 3E2-treated mice, but the reductions were not statistically significant. In experiment 2, 3E2 efficacy against intestinal infection was confirmed: 3E2 reduced infection by ~38% compared to the isotype control (Fig. 1B). In addition, biliary infection levels were ~46% lower in 3E2-treated mice (Fig. 1B). Unlike treatment with 3E2, treatment with either 3H2 or 1E10 had no significant effect on infection in any anatomic site examined (Fig. 1A).

(ii) Reduction of fecal oocyst production. To provide a quantitative index of parasite load, experiment 1 was designed to determine the total number of oocysts excreted by mice in each group. However, during the experiment it was observed that mice housed on raised wire mesh flooring without bedding frequently fed, defecated, and huddled in one area of the cage. This behavior resulted in the presence of variable amounts of food and debris in fecal trays and desiccation of fecal pellets, influencing oocyst recovery and precluding accurate quantitation. Statistical comparisons of the mean total number of oocysts recovered and detected in 24-h collections prior to and after all MAb treatments were nevertheless performed and revealed (i) significantly higher oocyst numbers in the isotype control group on days 5, 17, 19, and 21 than on day 3 (*P* < 0.05), (ii) a significantly higher number of oocysts in the 3H2 group on day 21 than on any preceding time point (*P* < 0.05), (iii) a significantly lower number of oocysts in the 3E2-plus-3H2-plus-1E10 group on day 5 than on day 14 (*P* < 0.05), and (iv) no significant differences in the 3E2, 1E10, 3E2-plus-3H2, and 3E2-plus-1E10 groups at any time point. In experiment 2, mice were housed in standard cages and bedding and fecal pellets were collected directly from individual mice to facilitate accurate oocyst quantitation. Statistical comparison of the mean number of oocysts per milligram of feces prior to and after treatment with 3E2 indicated a significant ~55% reduction in oocyst production within the group by day 18 of treatment (Fig. 2). Comparison of fecal oocyst production by mice prior to and after treatment with isotype control MAbs re-

![FIG. 1. Therapeutic efficacy of MAbs 3E2, 3H2, and 1E10, individually and in combinations, against persistent *C. parvum* infection in the intestinal tract (solid bars), pylorus (open bars), and gall bladder-common bile duct (hatched bars) in experiments 1 (A) and 2 (B). *, **, ***, and ****, *P* < 0.05, 0.01, 0.0025, and 0.0001, respectively, compared to results for the isotype control MAb-treated group. Error bars, standard deviations (SD).](http://aac.asm.org/)

![FIG. 2. Oocyst-shedding patterns in persistently infected SCID mice treated with anti-*C. parvum* or isotype control MAbs. The mean number of fecal oocysts was significantly (*) lower in mice treated with 3E2 (day 18, *P* < 0.05), 3E2 plus 3H2 (day 18, *P* < 0.025), 3E2 plus 1E10 (day 4, *P* < 0.01, and day 11, *P* < 0.005), or 3E2 plus 3H2 plus 1E10 (days 11 and 18, *P* < 0.005) than the pretreatment (day −1) values for each group. Error bars, standard deviations.](http://aac.asm.org/)
revealed no significant differences within the group at any time point examined (Fig. 2).

(iii) Effect on body weight. In experiment 1, comparison of the mean body weights of mice prior to and after treatment with individual MAbs 3E2, 3H2, and 1E10 or isotype control MAbs revealed no significant changes within each group during the treatment period. However, in experiment 2, comparison of the mean body weights of isotype control mice prior to and after treatment revealed significant weight loss beginning at treatment day 11 ($P < 0.0005$) and continuing until day 17 ($P < 0.025$). In contrast, no significant changes in the mean body weight of 3E2-treated mice were observed during the treatment period. From day 14 to the end of the experiment, the mean body weight of 3E2-treated mice was greater than that of the isotype control mice, and these differences approached significance.

Passive immunotherapeutic efficacy of MAb 3E2, 3H2, and 1E10 combinations against persistent *C. parvum* infection. The rationale for selection of the MAb combinations evaluated was twofold. First, 3E2 was considered an essential component based on results of previous studies demonstrating its superior activity against *C. parvum* (64, 67). Second, of all possible combinations, those selected had consistently demonstrated the greatest prophylactic efficacy against oocyst challenge (67).

(i) Effect on tissue infection levels. In experiment 1, each of the three combinations evaluated had no significant effect on infection levels, although intestinal and biliary scores in mice treated with 3E2 plus 3H2 were lower than those in the isotype control group mice (Fig. 1A). Therefore, the second experiment was performed to determine if the apparent reduction of infection by 3E2 plus 3H2 was significant and to further investigate the observation that no increase in efficacy over 3E2 alone was conferred by any of the combinations. Larger treatment groups were used to increase the power of statistical analyses. Mice were also housed conventionally on standard bedding for the duration of experiment 2. In this experiment, treatment with 3E2 plus 3H2 plus 1E10 or 3E2 plus 3H2 significantly reduced intestinal infection levels by $\sim 31$ and $\sim 48\%$, respectively, compared to the isotype control group treatment (Fig. 1B). Mice treated with 3E2 plus 3H2 or 3E2 plus 1E10 also had significantly lower biliary infection levels, representing $\sim 53$ to $\sim 56\%$ reductions (Fig. 1B). Mice treated with 3E2 plus 3H2 had lower intestinal and biliary infection scores than mice treated with 3E2 alone; however, the reductions observed with this and the other combinations evaluated were not statistically different from those observed with 3E2 alone (Fig. 1B).

(ii) Effect on fecal oocyst production. Based on comparison of the mean numbers of oocysts per milligram of feces prior to and after treatment in experiment 2, all MAb combinations significantly reduced oocyst production within each group over time (Fig. 2). Significant reductions were observed as early as day 4 ($\sim 46\%$) and continued to day 11 ($\sim 57\%$) in mice treated with 3E2 plus 1E10 but were no longer evident at day 18 (Fig. 2). In mice treated with 3E2 plus 3H2, a significant $\sim 59\%$ reduction was evident by day 18 (Fig. 2). Oocyst production in mice treated with 3E2 plus 3H2 plus 1E10 was significantly reduced by day 11 ($\sim 69\%$), and this reduction continued until day 18 ($\sim 59\%$) (Fig. 2).

(iii) Effect on body weight. In experiment 1, comparison of the mean body weights of mice prior to and after treatment with each MAb combination revealed no significant changes within each group during the treatment period. On day 14 of experiment 1, mice treated with 3E2 plus 1E10 or 3E2 plus 3H2 were observed to have significant weight reductions compared to the isotype control group ($P < 0.005$). In experiment 2, the mean body weights of mice treated with each MAb combination did not change significantly during the treatment period, unlike those of the isotype control group, which lost weight. Beginning on day 11 and continuing to the end of the experiment, the mean body weights of mice treated with 3E2 plus 3H2 or 3E2 plus 3H2 plus 1E10 were significantly greater than those of the isotype control mice ($P < 0.05$). On days 11 and 14, the mean body weights of mice treated with 3E2 plus 1E10 were also significantly greater than those of the isotype control mice ($P < 0.025$).

**DISCUSSION**

Passive immunization by locally delivered antibody is a valid means for preventing or treating infection by a variety of enteric and other pathogens (6, 16, 17, 31, 38, 70, 71, 82, 83). Its feasibility has been extended for use in cryptosporidiosis by results of initial studies evaluating antibodies generated against uncharacterized whole *C. parvum* preparations (9, 54, 60). In the present study, we hypothesized that passive immunotherapy against persistent *C. parvum* infection could be enhanced by targeting antigens known to function in the infection process. Because surface-exposed and apical complex antigens are critical to infection, they are opportune targets for immunization against *C. parvum* (7, 10, 18, 34, 36, 37, 39, 42, 46, 47, 53, 57, 61, 62, 64, 66, 67, 74, 80) as well as other apicomplexan parasites (5, 14, 15, 23, 25, 46, 68). Therefore, neutralizing MAbs against the surface pellicle antigen P23, the apical glycoprotein antigen complex GP25-200, and the apical complex glycoprotein CSL were prepared for therapeutic evaluation.

Several adult rodent models have been developed for characterization of immune responses to *C. parvum* and evaluation of potential therapeutic agents (1, 2, 20, 33, 72, 73; earlier studies reviewed in references 41 and 59). C57BL/6 mice having functional B and T lymphocytes but disruption of the IFN-$\gamma$ gene develop acute overwhelming *C. parvum* infection (73). Severe small-intestine infection and extensive mucosal destruction result in rapidly progressive wasting and death within 2 to 3 weeks; therefore, dissemination to the biliary tract does not occur and only mild pyloric infection is observed (73). IFN-$\gamma$ gene knockout mice are being used to further define *C. parvum*-specific T- and B-lymphocyte responses and to distinguish protective from nonprotective active immune responses (73). These mice also provide a sensitive model to test potential therapeutic agents for acute life-threatening *C. parvum* infection (33, 73). Athymic nude mice used in early studies demonstrated the central role of T lymphocytes and IFN-$\gamma$ in controlling *C. parvum* infection (41, 59). More recently, athymic mouse strains have been used to phenotype endogenous intestinal intraepithelial lymphocyte populations involved in resistance to *C. parvum* (1). SCID mice lack functional T and B lymphocytes and have been useful for defining the role of specific cytokines and adoptively transferred peripheral and
intestinal intraepithelial lymphocyte populations in controlling C. parvum infection (2, 41, 59). SCID mice have also been used for evaluation of passive immunization strategies (24, 41, 59). Of these models, adult SCID mice, predisposed to disseminated C. parvum infection by anti-IFN-γ MAb treatment (20, 72), were selected for therapeutic evaluation of MAbs recently shown to protect against primary infection (67). Chronic C. parvum infection can be consistently established in such mice, providing a stringent model that is especially suitable for evaluation of passive immunotherapy because of its independence from the influence of specific cellular or humoral host immune responses (20, 54, 60, 72, 76). Unlike the neonatal ICR mouse model widely used for initial studies to evaluate potential anticytost ospidal agents, adult SCID mice have a physiologically and anatomically mature gastrointestinal system through which MAbs must survive transit. In addition, neutrophilic enterocolitis with crypt microabscessation and hyperplasia occur in chronically infected SCID mice but not in neonatal ICR mice (60, 76). The physical presence of exudate, as well as inflammatory proteases, may impede delivery of MAbs to mucosal surfaces where C. parvum propagates. Finally, in SCID mice C. parvum may disseminate to the hepatobiliary tract and pylorus after the infection becomes chronic. Such extraintestinal sites may then provide reservoirs of parasites that can reinfect intestinal mucosa (54, 60, 76).

Treatment with anti-CSL MAb 3E2 alone resulted in the most significant and consistent therapeutic effect, reducing both intestinal infection and fecal oocyst production. These observations parallel its previously reported sporozoite-neutralizing activity in vitro and ability to passively protect neonatal mice against oocyst challenge (39, 67), which are greater than those of all other MAbs against C. parvum that we have produced (27, 52, 55, 61, 62, 64, 67). In one experiment, 3E2 in combination with either anti-GP25-200 MAb 3H2 or 3H2 and anti-P23 MAb 1E10 also significantly reduced intestinal infection and oocyst production, while 3E2 in combination with 1E10 was only effective in reducing oocyst production. The lower intestinal infection score for mice treated with the combination of 3E2 plus 3H2 versus that for mice treated with 3E2 alone was similar to previous results for neonatal ICR mice administered this combination prophylactically (67). However, unlike the results for neonatal mice, the difference was not statistically significant. We also observed in this experiment that biliary infection in mice treated with 3E2, 3E2 plus 3H2, or 3E2 plus 1E10 was significantly reduced. However, because the biliary tract is anatomically sheltered from orally delivered MAbs and because ascending infection to this site is a sequela to chronic gastrointestinal infection (30, 41, 79), the observed reductions are considered secondary treatment effects associated with lower gastrointestinal infection levels.

Because individual and combined MAb concentrations tested in the present study were similar to those evaluated in prophylaxis studies (67), the results suggest no additive therapeutic effect of the MAb combinations over 3E2 alone. These observations likely reflect the fundamental model differences in evaluating treatment efficacy of MAbs administered after infection becomes chronic and disseminated in immunodeficient adult mice versus the prophylactic efficacy of MAbs administered concurrently with oocyst challenge in normal neonatal mice. The enhanced efficacy of MAb combinations may be an advantageous property which is more applicable for prevention of infection than treatment of chronic infection. Another possible explanation is that CSL may have a pivotal role in both initiation of primary infection and perpetuation of chronic infection while GP25-200 and/or P23 may function principally in initiating infection. If so, differential expression of these antigens by sexual and asexual parasite stages, including autoinfective sporozoites and merozoites, would be expected during chronic infection and could explain differences in treatment versus prophylactic effects.

The apparent lack of efficacy of MAb combinations observed in experiment 1 could be accounted for by the following variables. In this experiment, treatment began on day 39 postinfection, whereas in experiment 2 treatment was initiated on day 35 postinfection; therefore, infection in experiment 1 was likely more advanced. Higher infection scores in both the control and treatment groups observed in experiment 1 confirmed that infection had indeed progressed beyond that present in experiment 2, a finding consistent with the asynchronous replication of C. parvum and completion of each cycle in 48 to 72 h (30, 75). Higher intestinal infection levels in experiment 1 may also account for the observation that secondary biliary infection was not reduced in 3E2-treated mice, unlike what was found in experiment 2. It is also possible that infection levels in experiment 1 exceeded the threshold for efficacy detection of MAb combinations in the treatment and evaluation regimens used. Related to this, it is highly probable that the smaller group sizes in experiment 1 (13 to 14 mice/group) yielded mean infection scores that were not representative of a larger treatment population such as that used in experiment 2 (20 mice/group). Despite the higher starting dose in experiment 1, pharmacologically active levels of the combined MAbs reaching the intestinal mucosa in some mice may have been lower than in experiment 2 due to increased proteolytic degradation. This possibility is suggested by lesion progression and the likely increase in activity of inflammatory cell and plasma-derived proteases in mucosal exudates due to the longer duration of infection prior to treatment in experiment 1. A final possible explanation, that there was antagonism between the combined MAbs at the higher concentrations used in experiment 1, is inconsistent with the results of previous studies (67). However, antagonism cannot be excluded without additional pharmacokinetics studies (26). Despite the preceding variables, 3E2 significantly reduced intestinal infection in both experiments, further substantiating its activity against C. parvum. Importantly, none of the MAbs evaluated had any significant effect on gastric infection. This is most likely due to the acidic gastric environment, effectively reducing the affinity of, or eliminating, antibody-antigen binding. Nevertheless, reduction of intestinal infection confirmed that sufficient quantities of MAbs can survive transit through the adult gastrointestinal environment to mediate neutralization.

Based on studies of related apicomplexan parasites, therapeutic antibodies for cryptosporidiosis would be expected to bind extracellular stages and prevent their attachment and invasion (43, 49, 59) or disrupt intracellular development of bound stages which retain the ability to invade (43, 45, 49, 59). However, knowledge of specific mechanisms by which antibodies mediate neutralization of C. parvum is currently limited and is needed to further develop immunization strategies (24, 59,
tients or by interruption of treatment in chemically immunosuppressed patients, MAβ therapy would have a higher probability of success. Finally, results of the present and previous studies (67), taken together, suggest that MAβ therapy could have immediate application in certain cases of cryptosporidiosis in immunocompetent, elderly, and neonatal patients in which parasite-specific treatment may be medically indicated.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI 30223 from the National Institutes of Health, Bethesda, Md., and U.S. Department of Agriculture NRCCGP grants 94-37204-0496, 99-35204-8533, and 2000-35204-09960. Costs for bioreactor production of MAbs 3E2, 3H2, and 1E10 by the National Cell Culture Center were subsidized through a Cooperative Agreement Award from the National Center for Research Resources, National Institutes of Health. Deborah A. Dalton was supported in part by funds from the Microbiology and Immunology Graduate Program, University of Arizona.

We thank Deborah Dalton, Beth Auerbach-Dixon, Kathryn Huey Tubman, and Jessica Chodos for excellent technical assistance and E. Havell for providing R46A2 hybridoma cells as a source of anti-mouse IFN-γ antibody.

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