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Recombinant Proteins of *Cryptosporidium parvum* Induce Proliferation of Mesenteric Lymph Node Cells in Infected Mice

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Recombinant antigens of *Cryptosporidium parvum*, Cp900 and Cp40 but not Cp15, stimulated *C. parvum*-specific proliferative immune responses of mesenteric lymph node cells in C57BL/6J mice infected with different isolates (MD, GCH1, UCP, and IOWA) of *C. parvum*, indicating that both Cp900 and Cp40 are immunodominant targets of cellular immune responses during *C. parvum* infection.

Cryptosporidium parvum is an important enteropathogen that infects the gastrointestinal tracts of humans and animals (14). In immunocompromised hosts, cryptosporidiosis can lead to persistent life-threatening disease (40) against which no therapy is available. This is in part due to the lack of understanding of the precise nature of protective immunity.

Studies on *Cryptosporidium* antigens have focused mainly on the humoral response (17, 19, 21, 22, 24, 29). Antibodies against several surface antigens of sporozoites have been shown to diminish *Cryptosporidium* infection in mice and in other animals (3, 13, 27, 30, 36). While several studies suggest that clearance of the infection requires T-cell response (2, 20, 26, 43, 44), most of the published ex vivo studies have used sporozoite or oocyst extracts (10, 11, 16, 32, 33, 35, 37, 45, 46). Only a few recombinant proteins (a 23-kDa protein and a combined 15/60-kDa protein) have been studied for their abilities to induce cell-mediated immune responses (5, 15, 18).

Three *C. parvum* recombinant antigens, Cp900 (domain 3), Cp40, and Cp15, have been cloned and sequenced, and the antibody responses to them were characterized (4, 7, 8, 28). The cellular immune response to these antigens, however, has not been determined. The surface localization of these proteins and their involvement in the host-parasite interaction suggest that they probably are targets for T-cell response as well. Competitive inhibition of *C. parvum* infection by the binding of purified native Cp900 to intestinal epithelial cells in vitro (4, 34) and the neutralization of *C. parvum* infection in vitro by Cp40-specific antibodies suggest that these proteins are involved in adhesion and invasion of *C. parvum* (4, 8). Cp15, which appears to be associated with Cp40 (47), may serve as a "stalk" to link gp40 to the surface of the parasite.

In this study, the recombinant proteins Cp900, Cp40, and Cp15 were used to induce *C. parvum*-specific proliferative immune responses against infections with each of four well-characterized *C. parvum* isolates.

Forty-eight C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were divided into four groups of five mice each and were challenged with 1×10^6 *C. parvum* MD (23) oocysts, and four groups of seven mice each were used as controls. An additional 15 C57BL/6J mice were divided into three groups of 5 mice each and challenged with 1×10^6 oocysts of GCH1 (41), IOWA (1), and UCP (6) isolates of *C. parvum*, respectively. All mice except the controls were given 1 mg of interferon-γ-neutralizing rat anti-mouse immunoglobulin G1 intraperitoneally, 2 h prior to challenge with *C. parvum* (35). Each mouse in the control group received a single intraperitoneal injection of 1 mg of normal rat immunoglobulin G (Sigma). Oocyst shedding in feces was measured throughout the course of the study (42). Groups of *C. parvum* MD-infected ($n = 5$) and control ($n = 7$) mice were killed on days 7, 14, 21, and 28 of infection. Single-cell suspensions of mesenteric lymph node (MLN) cells isolated from *C. parvum* MD, GCH1, IOWA, and UCP isolate-infected mice along with their respective controls were plated at concentrations of 8×10^5 cells per well in 96-well flat-bottomed microtiter plates (Costar, Cambridge, MA) and restimulated ex vivo with single concentrations (5 μg/ml) of recombinant proteins (Cp900, Cp40, or Cp15) in triplicate in total volumes of 200 μl of RPMI medium/well. The plates were incubated in a humidified 5% CO_2 atmosphere at 37°C for 5 days. The concentrations of recombinant antigens used were standardized by assessing the proliferative responses of MLN cells from *C. parvum* MD-infected mice to different concentrations of recombinant proteins (data not shown). To assess the antigenic specificity of the proliferative response, wells containing 100 μg of ovalbumin per ml and sporozoite antigens were included. Proliferation was determined by using a colorimetric 5-bromo-2′-deoxyuridine cell proliferation enzyme-linked immunosorbent assay (Roche Molecular Biochemicals, Mannheim) as per the manufacturer's instructions. The reaction was read at 450 nm using an enzyme-linked immunosorbent assay reader (Molecular Devices, Sunnyvale, CA). The results are expressed as means ± standard deviations for each recombinant antigen.

Proliferative responses to recombinant antigens Cp900 and Cp40 were observed in MLN cells isolated from *C. parvum* MD-infected mice on days 7, 14, and 21 but not on day 28; no proliferative response to Cp15 was observed at any time point (Fig. 1). The proliferative response to Cp900 was consistently greater than that to Cp40, with no differences in the responses at days 7, 14, and 21 after infection. No further increase in the

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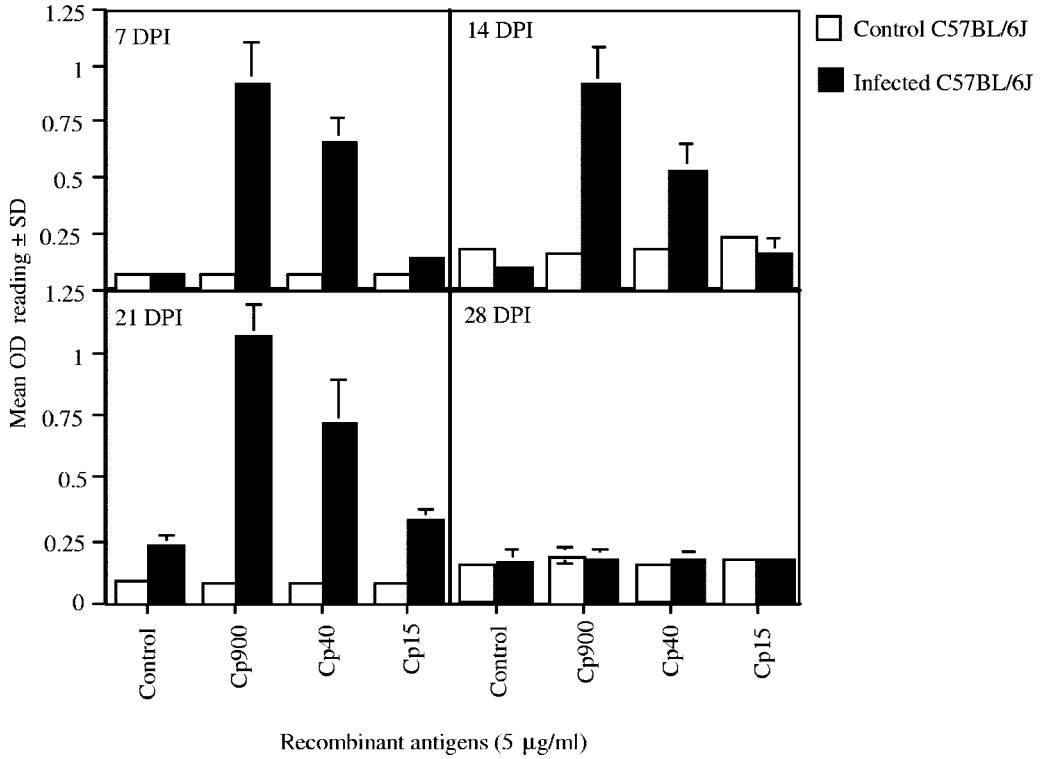

FIG. 1. In vitro proliferation of MLN cells isolated from *Cryptosporidium parvum* MD-infected and control C57BL/6J mice following restimulation with recombinant antigens (Cp900, Cp40, and Cp15). MLN cells were isolated from control (n = 7) and infected (n = 5) C57BL/6J mice at 7, 14, 21, and 28 days postinfection. Results are expressed as mean optical densities (OD) of triplicate wells ± standard deviations (SD).

proliferation of MLN cells was observed with increased concentrations of any of the recombinant proteins (data not shown). Similarly, MLN cells isolated from mice infected with other isolates (GCH1, IOWA, and UCP) of *C. parvum* also proliferated in response to Cp900 and Cp40 but not to Cp15 (Fig. 2). As with the MD isolate, the predominant response was against Cp900, with a lesser response against Cp40. Similar responses to Cp900 and Cp40 among mice infected with MD, IOWA, and UCP isolates were observed and were consistently higher than that for GCH1 (Fig. 2). Comparable responses by MLN cells to sporozoite antigen (freeze-thaw extract of excysted sporozoites from *C. parvum* oocysts) were observed (data not shown). The proliferative response was specific, as no such responses were seen with unrelated ovalbumin (data not shown) or in MLN cells isolated from control mice stimulated with recombinant antigens.

C. parvum-specific proliferative immune responses to recombinant antigens Cp900 and Cp40 were detected in MLN cells in infected mice during the active *C. parvum* infection. The absence of cellular immune response at 4 weeks of infection correlated with the elimination of the parasite from the gut, as no oocyst shedding was observed after 20 to 21 days of infection (data not shown). As observed previously, the level of parasite-specific immune response achieved was sufficient to clear the infection in 3 weeks in these mice (2, 35). The major proliferative response to recombinant proteins was against Cp900, followed by that against Cp40, indicating that Cp900 and Cp40 are more-immunogenic proteins and may contain greater numbers of antigenic determinants which induced the

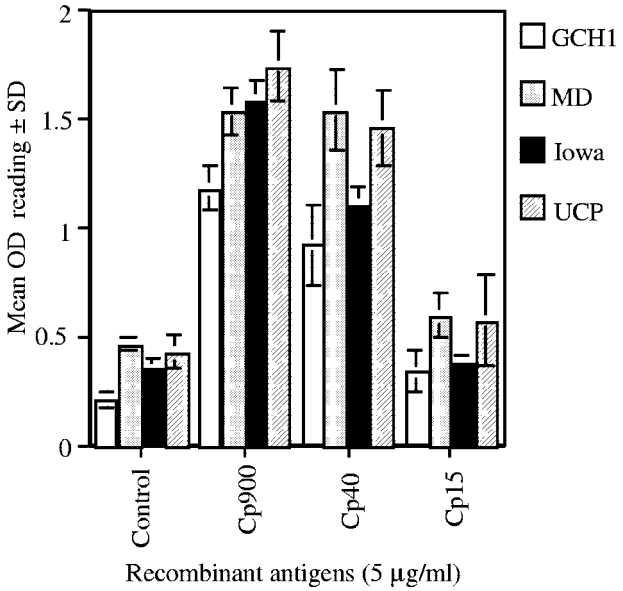

FIG. 2. In vitro proliferation of MLN cells isolated from C57BL/6J mice infected with GCH1, MD, IOWA, and UCP isolates of *Cryptosporidium parvum* (n = 5) and control C57BL/6J mice (n = 7) following restimulation with recombinant antigens (Cp900, Cp40, and Cp15). MLN cells were isolated from infected and control mice at day 14 of infection. Results are expressed as mean optical densities (OD) of triplicate wells ± standard deviations (SD).

observed T-cell responses in vitro. The absence of T-cell response to Cp15 may be due either to the presence of a suppressor epitope within the Cp15 protein or to defective presentation of antigen to induce T-cell response. Townsend et al. (39) demonstrated that some peptides can associate with class I antigen as targets for cytotoxic T lymphocytes but were not able to induce cytotoxic T lymphocytosis. Similar observations pertaining to T-cell responses to recombinant peptides have been made elsewhere (38). Bonafonte et al. (5) showed similar specific proliferative responses in splenocytes and MLN from infected BALB/c mice to a 23-kDa recombinant protein of *C. parvum*. Similarly, Gomez Morales et al. (15) described proliferation of human peripheral blood mononuclear cells with a 190-kDa recombinant antigen of *C. parvum*. Although most of the recombinant antigens were efficient in generating T-cell responses, it is not clear which antigen(s) is important in generating the protective response. Some studies used recombinant proteins (23 kDa and 15 kDa) of *C. parvum* for the immunization of mice (31) and calves (25). While the precise immune mechanism of protection remains undetermined, the immune response to Cp900 and Cp40 appears to correlate with the clearance of infection in mammals.

The similar proliferative responses to recombinant antigens (Cp900, Cp40, and Cp15) of mice infected with different isolates of *C. parvum* demonstrated that the epitopes present in Cp900 and Cp40 are highly conserved among *C. parvum* isolates. The level of proliferative response of GCH1 was lower than those of the other three isolates and may have been due to the milder infection and its impact on the time point analyzed during the process of infection. Although intraspecies antigenic variation has been reported for several other parasites (9, 12), no antigenic variation was observed for the recombinant proteins studied here among *C. parvum* isolates. However, it is not clear that this has been studied for any isolate over a period of time. Antigenic variation in proteins that nonetheless keep conserved domains may be possible, with the conserved domain inducing the observed cell-mediated immune responses. Similar observations were reported by others using specific *Cryptosporidium* antibodies against the same and other epitopes, in which no differences were detected among isolates from different mammalian species (20, 21, 25). The lack of apparent antigenic variation in *C. parvum* proteins simplifies the prospect of developing immunization strategies against cryptosporidiosis. Immunization with recombinant proteins may indicate whether this antigen(s) is an immunogen(s) suitable for the induction of protective immune responses against cryptosporidiosis in mammals.

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