Am. J. Trop. Med. Hyg., 77(3), 2007, pp. 583–585

Seropositive Human Subjects Produce Interferon Gamma after Stimulation with Recombinant *Cryptosporidium hominis* gp15

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Abstract. Cryptosporidiosis is an important cause of diarrhea worldwide. In normal hosts, infection is self-limited and associated with seroconversion and partial immunity to reinfection. Immunity is associated with interferon gamma (IFNγ) production. *Cryptosporidium* surface proteins gp15 and gp40 are among the immunodominant proteins in terms of antibody responses. We asked the question of whether these antigens also stimulate production of IFNγ in patients who have serologic evidence of prior infection. Whole blood from seropositive donors was stimulated with recombinant gp15 and gp 40 from *Cryptosporidium hominis* and *Cryptosporidium parvum* or His-tag controls. *C. hominis* gp15 stimulated increased production of IFNγ. By contrast, there was no significant increase after stimulation with *C. parvum* gp15 or either gp40 preparation. IFNγ production in response to *C. hominis* gp15 was noted in both $CD4^+$ and $CD8^+$ cells. This highlights the potential for *C. hominis* gp15 as a vaccine candidate for human cryptosporidiosis.

Cryptosporidiosis is an important cause of diarrhea worldwide.[1] The host immune response controls the disease in immunocompetent patients, and recovery is associated with resistance to reinfection. By contrast, cryptosporidiosis can be chronic and fatal in AIDS patients and malnourished children. Cytokines, particularly interferon gamma (IFNγ), are critical in the immune response controlling cryptosporidiosis. IFNγ-knockout mice develop chronic, severe infections.[2] Lymphocytes from people who have recovered from cryptosporidiosis produce IFNγ after antigen stimulation in vitro.[3,4] In volunteer studies, expression of IFNγ was associated with resistance to infection and prior sensitization.[5] Thus, expression of IFNγ is an important marker of immunity to infection.

Among the immunodominant antigens of *Cryptosporidium* species, several investigators identified a family of glycoproteins coded by a single-copy gene termed Cpgp15/60, gp60/45/15, S60, or Cp17.[6–9] The gene product is proteolytically cleaved into two surface proteins. The larger protein (gp40) is highly polymorphic with separate sequences for each of several *C. parvum* and *C. hominis* subtypes. The smaller protein (gp15), although variable, is more conserved.[10]

Cryptosporidium gp15 and gp40 sequences from *C. parvum* and *C. hominis* (subtype 1a) genomic DNA were cloned into the pET-32 Xa/LIC vector (Novagen, Madison, WI), which contains an S-tag, two His tags, and a thioredoxin tag.[7] The recombinant proteins were overexpressed in *Escherichia coli* and purified by metal affinity chromatography (Clontech, Palo Alto, CA). Blood samples were collected in heparinized tubes from healthy volunteers in Houston, TX, who had provided informed consent. Either 1 or 5 μg/mL of recombinant protein was added to 1-mL blood samples from 10 seropositive and 10 seronegative volunteers, as determined by the presence of serum antibodies to crude antigen from *C. parvum* oocysts detected by ELISA. Serologic responses rather than documented infections were used to identify presumed immune and naïve subjects. Controls included no antigen, recombinant protein containing only the fusion tags (Novagen), and mitogen (positive control) from the QuantiFERON-CMI kit (Cellestis, Valencia, CA). After overnight incubation (37°C, 5% CO_2), the plasma was removed (300 μL per tube), and the quantity of IFNγ was measured by ELISA using the QuantiFERON-CMI kit (Cellestis). We noted some nonspecific stimulation with the fusion-tag control, especially at 5 μg/mL. After subtracting nonspecific reactions stimulated with the 1 μg/mL fusion-tag control protein from samples stimulated with 1 μg/mL of recombinant protein, we observed an increased production of IFNγ by seropositive and seronegative donors (Figure 1). In response to *C. hominis* r-gp15, seropositive volunteers produced more IFNγ than seronegative volunteers ($P = 0.05$, one-tailed Mann–Whitney test; Figure 1). A nonsignificant increase in production was observed with *C. parvum* r-gp40. Only two individuals produced IFNγ in response to *C. hominis* r-gp40, and very little IFNγ was elicited with *C. parvum* r-gp15.

To characterize which cells produced IFNγ, peripheral blood mononuclear cells (PBMCs) from three seropositive donors were incubated with *C. hominis* r-gp15 (1 μg/mL) or fusion-tag control (2×10^6 cells/mL, overnight). GolgiStop (1.3 μL/mL, BD Biosciences Pharmingen, San Diego, CA) was added (4–6 h). Next, the cells were stained with PE anti-CD3 and Cy5 anti-CD8 (BD Biosciences Pharmingen), washed, fixed with Cytofix/Cytoperm (20 min, 25°C, BD Biosciences Pharmingen), stained with FITC anti-IFNγ or isotype control (20 min, 4°C, BD Biosciences Pharmingen), and analyzed by flow cytometry (Coulter EPICS XL-MCL, Beckman Coulter, Fullerton, CA). Stimulation with *C. hominis* r-gp15 led to expression of IFNγ by 0.09–0.14% of $CD8^-$ cells (primarily CD4 cells) and by 0.07–0.11% of $CD8^+$ cells (about three times the fusion-tag controls, $P < 0.05$, paired *t*-test; Figure 2a). Minimal staining was noted with the fusion-tag control (Figure 2b).

We demonstrated that recombinant *C. hominis* gp15 stimulates production of IFNγ by lymphocytes from sensitized volunteers. Interestingly, both CD4 and CD8 cells responded to

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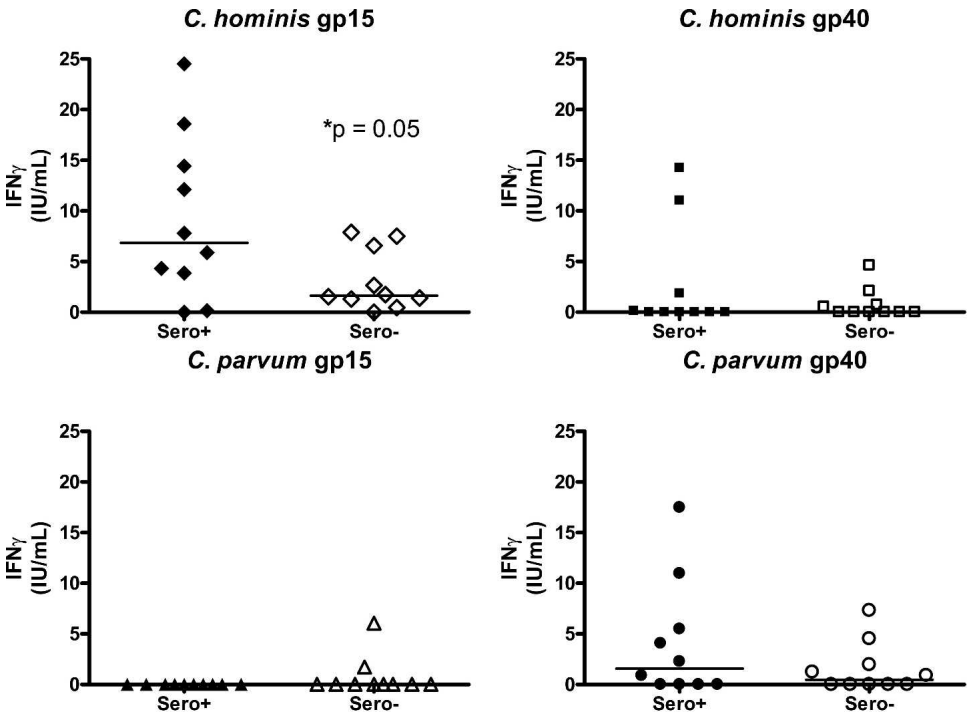

FIGURE 1. Interferon gamma (IFNγ) levels were measured in plasma after incubation of blood from 10 anti-*Cryptosporidium* seropositive (solid shapes) and 10 seronegative (open shapes) donors with antigens. Each line shows the median results of either seropositive or seronegative donors incubated with 1 μg/mL of recombinant *C. hominis* r-gp15 (diamonds), *C. hominis* r-gp40 (squares), *C. parvum* r-gp15 (triangles), or *C. parvum* r-gp40 (circles). Note the significant response to *C. hominis* r-gp15 compared with the other antigens.

the recombinant gp15. The responses to recombinant gp40 preparations were less robust than those to r-gp15. Because natural exposures likely reflect several parasite gp40 variants, the volunteers may not be sensitized to all gp40 variants.

Antibody to gp15 can be detected in nearly all people after *Cryptosporidium* infection.[11] Pre-existing antibody levels correlate with decreased rates of diarrhea after waterborne exposure to *Cryptosporidium*.[12] Similarly, development of anti-

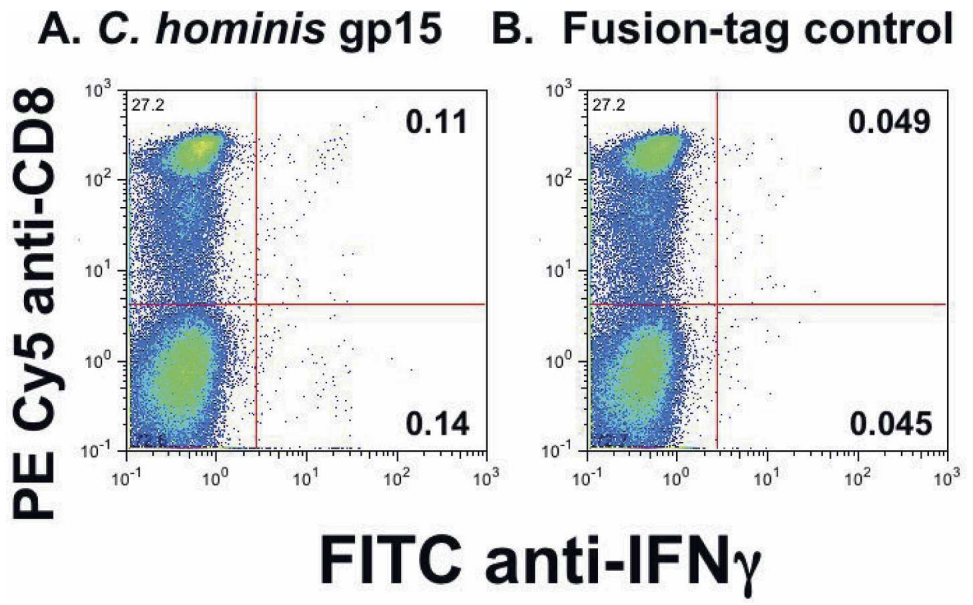

FIGURE 2. Flow-cytometric analysis with intracellular cytokine staining for IFNγ production in response to stimulation with recombinant gp15 antigen in a donor seropositive for anti-*Cryptosporidium* antibody. PBMCs from three anti-*Cryptosporidium* seropositive subjects were stimulated with *C. hominis* r-gp15 (1 μg/mL) or fusion-tag control. After intracellular cytokines were trapped with GolgiStop, cells were stained with PE anti-CD3, Cy5 anti-CD8, and FITC anti-IFNγ and analyzed by flow cytometry. Panel A demonstrates increased expression of intracellular IFNγ by both $CD8^+$ and $CD8^-$ cells. Similar results were noted for all three donors. This figure appears in color at www.ajtmh.org.

body to gp15 was associated with prevention of symptoms after experimental infection.[13] We now demonstrate IFNγ production by sensitized humans in response to recombinant gp15. The fact that this antigen is immunodominant in sensitized individuals further strengthens the rationale for developing gp15 vaccines to prevent human cryptosporidiosis.

Interestingly, we noted that the IFNγ response to r-gp15 of *C. hominis* was greater than that noted for *C. parvum*. Molecular epidemiologic studies in most areas have noted that human infections are more frequently caused by *C. hominis* than by *C. parvum.*[1] It has previously been shown that priming for IFNγ production requires repeated exposures,[5] so the increased response to *C. hominis* r-gp15 may reflect more frequent exposures to or infections with *C. hominis*. Another possibility is that our recombinant *C. parvum* peptides were derived from a strain dissimilar to those previously encountered by our volunteers. Alternatively, the recombinant gp15 peptide from *C. hominis* may be more immunogenic.

Although previous studies of immunity to cryptosporidiosis have focused on CD4 cells, our studies demonstrated that both $CD4^+$ and $CD8^+$ cells produce IFNγ. $CD4^+$ T cells play an established role in the control of cryptosporidiosis. Both susceptibility and severity of cryptosporidiosis in AIDS patients vary with CD4 cell counts.[1] By contrast, the role of CD8 cells is less clear. Our data suggest that CD8 cells as well as CD4 cells may be an important source of IFNγ in human cryptosporidiosis.

In summary, we propose that immunity to cryptosporidiosis may be associated with sensitization to *C. hominis* gp15. Stimulation with recombinant antigen leads to production of IFNγ, which is closely linked to the adaptive immune response. Interestingly, *C. hominis* r-gp15 appears to be more immunogenic than r-gp40 or either *C. parvum* antigen. Overall, these data support further investigation of recombinant *C. hominis* gp15 as a vaccine candidate for human cryptosporidiosis.

Received March 1, 2007. Accepted for publication May 18, 2007.

Acknowledgments: The authors thank Anne Kane of the GRASP Digestive Diseases Center and Geneve Allison of the Tufts–New England Medical Center for preparation of the recombinant antigen. The protocol and consent forms were approved by the Institutional Review Board of Baylor College of Medicine.

Financial support: This work was supported by the National Institutes of Health (grants R01 AI041735 to A.C.W. and R01 AI07389 to H.D.W.), the Baylor UT Houston Center for AIDS Research (P30 AI36211), the Texas Gulf Coast Digestive Diseases Center (P30 DK056338), the GRASP Digestive Diseases Center (P30 DK34928) at Tufts–New England Medical Center, and a training grant (T32 AI07389 to K.R.).

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