Human CD8⁺ T Cells Clear *Cryptosporidium parvum* from Infected Intestinal Epithelial Cells

Birte Pantenburg, Alejandro Castellanos-Gonzalez, Sara M. Dann, Rhykka L. Connelly,
Dorothy E. Lewis, Honorine D. Ward, and A. Clinton White Jr.*

Division of Infectious Diseases, Department of Internal Medicine, Department of Microbiology, University of Texas Medical Branch, Galveston, Texas; Center for Electromechanics, University of Texas, Austin, Texas; Division of Geographic Medicine and Infectious Diseases, Tufts Medical Center, Boston, Massachusetts

Abstract. Intracellular protozoans of the genus *Cryptosporidium* are a major cause of diarrheal illness worldwide, especially in immunocompromised individuals. CD4⁺ T cells and interferon-gamma are key factors in the control of cryptosporidiosis in human and murine models. Previous studies led us to hypothesize that CD8⁺ T cells contribute to clearance of intestinal epithelial *Cryptosporidium* infection in humans. We report here that antigen expanded sensitized CD8⁺ T cells reduce the parasite load in infected intestinal epithelial cell cultures and lyse infected intestinal epithelial cells. These effects are most likely mediated by the release of cytotoxic granules. Elimination of parasites seems to require antigen presentation through both human leukocyte antigen (HLA)-A and HLA-B. These data suggest that cytotoxic CD8⁺ T cells play a role in clearing *Cryptosporidium* from the intestine, a previously unrecognized feature of the human immune response against this parasite.

INTRODUCTION

Cryptosporidium is an obligatory intracellular parasite that infects epithelial cells of the small intestine causing acute watery diarrhea. The two species responsible for most cases of human cryptosporidiosis worldwide are *Cryptosporidium parvum* and *Cryptosporidium hominis*. The course of the disease depends on the immune status of the host: While immunocompetent individuals experience acute watery diarrhea lasting about a week, immunocompromised persons (e.g., HIV/AIDS patients), may develop chronic or persistent diarrhea that can lead to wasting and even death. Current anti-parasitic drugs have sub-optimal efficacy in immunocompromised individuals who suffer most from the disease. To develop effective treatment alternatives or vaccines, it is indispensable to study the protective immune response against the parasite. The factors contributing to the human immune response against *Cryptosporidium* are not yet completely determined.¹ Among others, CD4⁺ T cells appear to play a pivotal role in both mice and humans, as the risk to experience severe and chronic disease increases with CD4⁺ T cell depletion.^{2–4} Natural killer cells have shown to be able to clear infected human epithelial cells.⁵ Murine models suggest that CD8⁺ T cells may not be important for elimination of the parasite.^{6,7} However, the role of CD8⁺ T cells in the human immune response against *Cryptosporidium* has not yet been studied. Although animal models have been important for dissecting the anti-cryptosporidial immune response, immune responses often differ between mice and men. For example, while interferon-gamma (IFN- γ) seems to be involved in both the innate and adaptive murine immune response,^{8–11} we and others have previously shown that IFN- γ production in human infection is largely restricted to the memory response against the parasite.^{12–14}

A recent study by our group showed that both CD4⁺ and CD8⁺ T cells from previously sensitized donors produce IFN- γ after *in vitro* re-stimulation with *Cryptosporidium* antigen.¹⁵ Another study revealed that cryptosporidiosis was associated with certain human leukocyte antigen (HLA) types, including

class II alleles (which are necessary for antigen presentation to CD4⁺ T cells) but also HLA class I alleles (which are necessary for antigen presentation to CD8⁺ T cells).¹⁶ This led us to the hypothesis that CD4⁺ and CD8⁺ T cells are both important for clearance of *Cryptosporidium* infection in humans. In this study, we provide further evidence that antigen expanded sensitized CD8⁺ T cells are able to significantly reduce the parasite load in infected intestinal epithelial cell cultures.

MATERIALS AND METHODS

Cells and cell culture. Cells of the human colon carcinoma cell line CaCo-2 (American Type Culture Collection [ATCC], Manassas, VA) were cultured (37°C, 5% CO₂) in complete Eagle's Minimum Essential Medium (EMEM, ATCC, Manassas, VA) containing 20% fetal bovine serum (FBS; not heat-inactivated), 100 IU/mL penicillin, and 100 μ g/mL streptomycin in 150 cm² BD Falcon Tissue Culture Treated Flasks (Fisher Scientific, Houston, TX).

Parasite preparation and labeling with carboxyfluorescein diacetate succinimidyl ester. Purified *C. parvum* oocysts (Iowa isolate) were purchased from the University of Arizona (Tucson, AZ) or Bunch Grass Farm (Deary, ID) and stored in 2.5% potassium dichromate at 4°C until use. For infection of CaCo-2 cells, oocysts were washed three times with phosphate buffered saline (PBS) (pH 7.2), centrifuged (room temperature, 16,000 $\times$ g, 2 min), and treated with acidic H₂O (pH 2.5; 4°C, 10 min). Oocysts were centrifuged again, resuspended in 1 mL PBS/0.8% Na-taurocholate (Sigma, St. Louis, MO) and incubated (37°C, 5% CO₂). Parasites were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) according to a previously published protocol with modifications.¹⁷ After 15 min of incubation in PBS/0.8% Na-taurocholate, 6 μ L of CFSE solution (Invitrogen, Carlsbad, CA) were added, and parasites were incubated for another 30 min. Parasites were washed and added to 0.4 $\times$ 10⁶ CaCo-2 cells (sporozoite:cell ratio at least 15:1) that had been cultured in 25 cm² flasks in serum- and antibiotic-free EMEM media overnight. Cells and parasites were incubated for approximately 18 hrs (37°C, 5% CO₂) before cells were harvested for experiments. Infection rates were usually between 25% and 50% as analyzed by flow cytometry after infection of target cells with CFSE stained parasites.

*Address correspondence to A. Clinton White Jr., Division of Infectious Diseases, Department of Internal Medicine, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555. E-mail: acwhite@utmb.edu

Isolation and culture of peripheral blood mononuclear cells. Blood samples were collected from consenting healthy volunteers, as approved by the Institutional Review Board of the University of Texas Medical Branch (UTMB) in Galveston. The HLA typing was performed in the tissue typing laboratory at UTMB. Donors were screened for prior exposure by testing serum for anti-*Cryptosporidium*-antibodies by enzyme-linked immunosorbent assay (ELISA) with slight modifications as described elsewhere.¹⁸ Peripheral blood mononuclear cells (PBMCs) from two seronegative and three seropositive donors were separated from heparinized blood by density-gradient centrifugation using CPT tubes (Fisher, Houston, TX) or Histopaque 1077 (Sigma). The PBMCs were resuspended at 1×10^6 cells/mL of complete RPMI 1640 (Invitrogen), containing 10% heat-inactivated fetal bovine serum (FBS), 25 mM HEPES buffer, 100 IU/mL penicillin, 100 μ g/mL streptomycin, and 250 ng/mL amphotericin B.

The 1.5×10^6 PBMCs/well were cultured in 24-well plates with human recombinant IL-15 (rIL-15; 10 ng/mL; Millipore, Billerica, MA), and IL-2 (rIL-2; 20 IU/mL; Millipore) with or without recombinant glycoprotein 15 antigen (gp15; 1 μ g/mL). The gp15 was produced at Tufts University as previously described.^{15,19} We used *C. hominis* gp15, which is highly homologous to *C. parvum* gp15,^{20,21} and has been shown to elicit stronger IFN- γ responses in sensitized persons.¹⁵ Cells were incubated (37°C, 5% CO₂) for 6 days.

Isolation of CD8⁺ T cells. After 6 days of culture, PBMCs were harvested and CD8⁺ T cells isolated by negative selection using magnetic beads (Dynabeads Untouched Human CD8 T Cells kit, Invitrogen). Briefly, PBMCs were washed with and resuspended in cold isolation buffer (PBS without Ca²⁺ and Mg²⁺, supplemented with 2% FBS and 2 mM EDTA) and incubated with an antibody-mix (4°C, 20 min) labeling the non-CD8⁺ T cells. Cells were washed again and pre-washed magnetic Dynabeads were added. Cells and beads were incubated (room temperature, 15 min) in 15 mL tubes with gentle tilting and rotation allowing the beads to bind to antibody-labeled cells. The tubes were then put into a DynaMag-15 magnet (Invitrogen), allowing collection of non-bead bound CD8⁺ T cells with the supernatant while capturing all other cells at the tube walls. The CD8⁺ T cells were resuspended in complete RPMI 1640 and used for subsequent assays. Isolated CD8⁺ T cells were > 93% pure as analyzed by flow cytometry after staining with anti-CD8 antibody (Biolegend, San Diego, CA).

Chromium release assay to measure cytolytic activity. *Cryptosporidium parvum*-infected (as described previously) and non-infected Caco-2 target cells (0.4×10^6 cells/25 cm² flask) were rinsed twice without disrupting the monolayer to remove any unattached sporozoites and oocysts. Cells were harvested by using a nonenzymatic cell-dissociation solution (Sigma) or sometimes a cell scraper. After washing once, the target cells (5×10^5 cells/mL) were labeled with 100 mCi of Na₂CrO₄ (⁵¹Cr; MP Biomedicals, Solon, OH) in 1 mL of complete RPMI 1640 medium (37°C, 5% CO₂, 3 hrs). The cells were washed 3 times (22°C, 400 $\times$ g, 10 min), resuspended in complete RPMI 1640 at 75,000 cells/mL, and 100 μ L of the cell suspension were added to each 0.5 mL eppendorf tube. Effector CD8⁺ T cells were resuspended in complete RPMI 1640 and used at different effector:target ratios (50:1; 25:1; 12.5:1; 6.25:1; 1:1). Tubes were briefly centrifuged and incubated (37°C, 5% CO₂) for 4 hrs. After incubation, 180 μ L of supernatants were carefully harvested and released ⁵¹Cr was measured with a

gamma counter (Cobra Auto-Gamma, Packard Instrument Company, Meriden, CT). Spontaneous lysis was determined from the supernatants of tubes containing target cells in media only; the total amount of ⁵¹Cr was determined after target cell lysis with 1% sodium dodecyl sulfate (SDS) solution. Results from duplicate wells were averaged, and percentage of specific cytotoxicity was calculated using the following formula:

$$[(\text{CPM}_{\text{experimental}} - \text{CPM}_{\text{spontaneous}}) / (\text{CPM}_{\text{total}} - \text{CPM}_{\text{spontaneous}})] \times 100.$$

RNA isolation and real-time polymerase chain reaction to measure elimination of parasites from cell cultures. The CaCo-2 cells were infected and harvested as described previously and resuspended at 250,000 cells/mL in complete RPMI 1640 supplemented with recombinant human IL-7 (rIL-7; 10 ng/mL; R&D Systems, Minneapolis, MN). The CD8⁺ T cells were isolated as described previously, resuspended at 6.25×10^6 cells/mL in complete RPMI 1640 supplemented with recombinant human IL-7 and added to CaCo-2 cells in 0.5 mL eppendorf tubes (effector:target ratio 25:1). Tubes were briefly centrifuged and incubated for 24 hrs (37°C, 5% CO₂). After incubation, cells were centrifuged again, 50 μ L of the supernatant carefully harvested and pellet and supernatant stored at -80°C until analysis. The RNA was extracted from the pellets using a commercial kit (RNeasy Plus Mini Kit, Qiagen, Valencia, CA). Briefly, cell pellets were homogenized using the QIAshredder (Qiagen), genomic DNA was removed with a gDNA eliminator column and after several washing steps the RNA eluted. Quantity and quality of the RNA was assessed with a NanoDrop 1000 (Thermo Scientific, Asheville, NC). Reverse transcription and amplification of the target gene *C. parvum* 18 ribosomal sub-unit (Cp18s, access no. AB089290) RNA was performed with the SYBR Green SuperScript One-Step Kit (Invitrogen). To quantify *C. parvum* RNA, we compared the same volume (in μ L) from each experimental sample (infected target cells plus effector cells) to positive controls (infected target cells only). The CD8⁺ T cells alone were used as negative control (no *C. parvum* RNA). Specific primers (Integrated DNA Technologies, Coralville, IA) for the Cp18s were used as previously published²²: AWA995F 5'-TAG AGA TTG GAG GTT GTT CCT-3' (forward) and AWA1206R 5'-CTC CAC CAA CTA AGA ACG GCC-3' (reverse).

Reactions were performed with the real-time polymerase chain reaction (rtPCR) ABI PRISM 7300 fast-system under the following conditions: complementary DNA (cDNA) synthesis: 50°C for 30 min and 94°C for 5 min; PCR amplification: 94°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec, for 40 cycles; dissociation step: 95°C for 15 sec and 60°C for 1 min. The cycle threshold (Ct) values were determined using the 7500 system SDS software. Reduction of parasite presence was calculated based on comparing Ct values for Cp18s RNA of experimental samples with Ct values for Cp18s RNA of positive and negative control samples. In experimental samples, increasing Ct values reflect decreasing amounts of Cp18s RNA amplification and correlate with fewer numbers of *C. parvum* organisms.

Inhibition studies. For inhibition studies CD8⁺ T cells were pre-incubated with anti-CD8-antibody (LT8, Abcam, Cambridge, MA) at 1:2500. For blocking the release of cytotoxic granules, CD8⁺ T cells were incubated with Concanamycin A (100 nM; Sigma)²³ and for blocking of Fas ligand, CD8⁺ T cells were incubated with anti-Fas ligand-antibody (5 μ g/mL; R&D

Systems).²³ For blocking of HLA-A or HLA-B on the target cells, CaCo-2 cells were incubated with an anti-HLA-A2-antibody (BB7.2; 10 µg/mL; AbD Serotec, San Diego, CA)^{24,25} or anti-HLA-B/C-antibody (B1.23.2; very generous gift from Prof. Le Bouteiller from INSERM, Toulouse, France),^{24,26} respectively. Cells and blocking antibodies or Concanamycin A were incubated at room temperature for 1 hr and washed once before being used in the assays.

Fluorescent microscopy. The CaCo-2 cells (20,000 cells/well) in an 8-well LabTek chamber slide (Labtek Inc., Grand Rapids, MI) were infected with CFSE labeled *C. parvum* as described previously. After 24 hrs, CaCo-2 cells were carefully washed three times without disrupting the monolayers to remove any unattached sporozoites and oocysts. To label the nuclei of the cells, 250 µL of Hoechst 33342 (1 µM in complete EMEM, Invitrogen) was added to the well and incubated for 20 min (37°C, 5% CO₂). PKH-26 (Sigma) was then used to stain the cell membrane: After washing, cell monolayers were briefly incubated with Diluent C (provided with the kit), which was replaced by 250 µL of dye solution (20 µL of PKH-26 plus 2 mL of Diluent C) and cells were incubated (room temperature, 5 min). Then 250 µL of 100% FBS was added and cells were incubated for 1 min at room temperature. Cells were washed three times with complete EMEM.

To stain the effector cells, 1×10^6 CD8⁺ T cells were resuspended in 1 mL complete RPMI 1640 and red LysoTracker (Invitrogen) was added at 50 nM to stain the acidic compartments of the cells. Cells were incubated at 37°C for 1 hr. For the last 20 min, Hoechst 33342 was added (1 µM) to the medium. After incubation, cells were washed and added to the target cells. Cells were analyzed with a Zeiss Axiovert 200M inverted microscope (Thornwood, NY) using a stage incubator over a time-course of up to 4 hrs. The Zeiss LSM 510 w.s. software was used to render the 3D images.

Statistical analysis. Statistical analysis was performed with GraphPad Prism (GraphPad Software, Inc., San Diego, CA). The results are presented as means $\pm$ SEMs. Statistical significance was determined using Student's *t* test. Differences were considered significant at *P* < 0.05.

RESULTS

Donor HLA types. Three anti-*Cryptosporidium*-antibody positive (sensitized) and two anti-*Cryptosporidium*-antibody negative (non-sensitized) healthy donors (Table 1) were identified who matched the CaCo-2 cell line in HLA-A (HLA-A2). Because only CD8⁺ T cells from Donor 1 consistently lysed infected CaCo-2 cells (see below) and a recent study

suggested that certain HLA-B alleles might be associated with *C. parvum* infection,¹⁶ additional HLA typing of all donors was performed. We found that Donor 1 was the only sensitized donor who matched the cell line in both HLA-A and HLA-B (HLA-B62). This finding suggests that antigen recognition through both HLA-A and HLA-B might be important for CD8⁺ T cells to clear *Cryptosporidium* from infected intestinal epithelial cells. Most of the experiments were therefore performed exclusively with sensitized Donor 1 and non-sensitized Donor 4, who both matched the CaCo-2 cells in HLA-A and HLA-B.

Antigen expanded sensitized CD8⁺ T cells reduce the *C. parvum* load in infected cell cultures. To determine whether CD8⁺ T cells are able to eliminate *C. parvum* from infected epithelial cell cultures, CD8⁺ T cells were isolated by negative selection from gp15 expanded PBMCs of Donor 1 and 4 and incubated with *C. parvum*-infected CaCo-2 cells for 24 hrs in complete medium containing IL-7, which has recently been shown to increase antigen-specific immune responses.²⁷

Although non-expanded (no gp15) CD8⁺ T cells from sensitized Donor 1 eliminated > 20% of *C. parvum* organisms from the infected cell cultures, the anti-parasitic effect increased to > 60% after gp15 antigen expansion (Figure 1). Pre-treatment of the same CD8⁺ T cells with anti-CD8-antibody completely inhibited the effect. The CD8⁺ T cells from non-sensitized Donor 4 had no significant effect on parasite presence independent of whether PBMCs were antigen expanded or not. These results indicate that gp15 expanded sensitized CD8⁺ T cells have the potential to reduce the *C. parvum* load in infected intestinal epithelial cell cultures.

Lysis of *C. parvum*-infected cells by gp15 expanded CD8⁺ T cells from a sensitized donor. Antigen expanded CD8⁺ T

FIGURE 1. CD8⁺ cells from a sensitized donor clear *Cryptosporidium parvum* messenger RNA (mRNA) from infected cells. Antigen expanded sensitized CD8⁺ T cells reduced the amount of *C. parvum* mRNA in infected intestinal epithelial cell cultures. CD8⁺ T cells were isolated from a sensitized and a non-sensitized donor (HLA-A and HLA-B matched with the CaCo-2 cells) and used as effector cells in co-cultures with infected CaCo-2 target cells (effector:target ratio of 25:1) in complete RPMI supplemented with IL-7. After 24 hrs, cell pellets were harvested, and *C. parvum* RNA was quantified by real-time polymerase chain reaction (rtPCR). The experiment was repeated two times. The results of one representative experiment are shown. * *P* < 0.05

TABLE 1
HLA typing and sensitization of cells*

Name	Sensitized	HLA-A	HLA-B	HLA-Cw	HLA-DR	HLA-DQ
CaCo-2	Not applicable	02	62	04,12	04	08
Donor 1	Yes	02	60,62	03	04,15	06,08
Donor 2	Yes	02,29	07,44	07,16	07,15	02,06
Donor 3	Yes	02,03	07,44	07,16	07,15	02,06
Donor 4	No	02,24	39,62	03,07	01,07	02,05
Donor 5	No	02,24	35,61	03,12	04,11	07,08

*All donors matched the CaCo-2 cells at HLA-A, but only sensitized Donor 1 and non-sensitized Donor 4 matched the cell line in both HLA-A and HLA-B. The human leukocyte antigen (HLA) typing was performed in the tissue typing laboratory at the University of Texas Medical Branch (UTMB). Donors were screened for sensitization to *Cryptosporidium* by testing the serum for anti-*Cryptosporidium*-antibodies by enzyme-linked immunosorbent assay (ELISA) (sensitized – seropositive; non-sensitized – seronegative).

FIGURE 2. Antigen expanded sensitized CD8⁺ T cells lyse intestinal epithelial cells from a culture infected with *Cryptosporidium parvum*. CD8⁺ T cells were isolated from sensitized Donor 1 and non-sensitized Donor 4 (HLA-A and HLA-B matched with CaCo-2 cells) and used as effector cells in co-cultures with ⁵¹Cr-labeled CaCo-2 target cells at various ratios of effector CD8⁺ T cells to CaCo-2 target cells (E:T ratio). Four hours after incubation, lysis of target cells was determined by ⁵¹Cr release. The experiment was repeated at least three times. The results of one representative experiment are shown. ▲ = expanded sensitized; ● = non-expanded sensitized; △ = expanded non-sensitized; ○ = non-expanded non-sensitized.

cells of sensitized Donor 1 lysed-infected target cells in an effector:target (E:T) cell ratio-dependent manner (Figure 2). In this experiment, gp15 expanded CD8⁺ T cells lysed 3.5% of CaCo-2 cells from cultures infected with *C. parvum* at an E:T ratio of 25:1. Expansion with gp15 elicited some non-specific cytotoxic response with uninfected CaCo-2 cells, but only at the E:T ratio of 50:1 (data not shown). In contrast, expanded CD8⁺ T cells from a non-sensitized donor did not exhibit a cytotoxic effect on CaCo-2 cells from cultures infected with *C. parvum* at the E:T ratio of 1:25. Thus, the effect was specific to sensitized donors and not an effect of non-specific stimulants (e.g., LPS). In contrast to Donor 1 who matched the cell line in both HLA-A and HLA-B, gp15 antigen expanded CD8⁺ T cells from sensitized Donor 2 and Donor 3 did not consistently lyse infected target cells (data not shown).

Antiparasitic effect depends on antigen presentation through HLA-A and HLA-B. The finding that gp15 expanded CD8⁺ T cells from sensitized Donor 1 who matched the CaCo-2 cell line in HLA-A and HLA-B consistently lysed infected target cells in contrast to CD8⁺ T cells derived from sensitized Donors 2 and 3 who matched CaCo-2 cells only in HLA-A, suggests that both HLA-A and HLA-B might be necessary for antigen presentation in our model. To confirm this hypothesis, inhibition studies were performed by pre-treating target cells with blocking antibody against either HLA-A2 or HLA-B/C. The CD8⁺ T cells from sensitized Donor 1 were isolated after antigen expansion with gp15 and incubated with pre-treated infected target cells. By rtPCR, we found complete inhibition of clearance of *C. parvum* organisms from infected cell cultures after blocking HLA-A or HLA-B (Figure 3). Similarly, in ⁵¹Cr release assays pre-treatment with either antibody inhibited lysis of target cells (data not shown). Taken together, these data suggest that antigen presentation through HLA-A and HLA-B is equally necessary in order for CD8⁺ T cells to be

FIGURE 3. Parasite clearance is blocked by antibody to HLA-A or HLA-B/C. CD8⁺ T cells were isolated from gp15 antigen expanded peripheral blood mononuclear cells (PBMCs) from sensitized Donor 1 (HLA-A and HLA-B matched with the CaCo-2 cells) and used as effector cells in co-cultures with *C. parvum*-infected CaCo-2 target cells at an effector:target ratio of 25:1 in complete RPMI supplemented with IL-7. Before co-culture, CaCo-2 cells were incubated with either anti-HLA-A or anti-HLA-B/C-antibody. After 24 hrs, cell pellets were harvested and *C. parvum* RNA was quantified by real-time polymerase chain reaction (rtPCR). * $P < 0.05$

able to clear parasites from infected epithelial cell cultures and to lyse *C. parvum*-infected CaCo-2 cells.

Further rtPCR experiments revealed that HLA-A or HLA-B RNA expression did not significantly change upon infection of CaCo-2 cells with *C. parvum* (data not shown).

Antiparasitic effect involves release of cytolytic granules from CD8⁺ T cells. To visualize the interaction of antigen expanded sensitized CD8⁺ T cells and infected CaCo-2 cells, we labeled both effector and infected target cells. The CD8⁺ T cells isolated from PBMCs after antigen expansion with gp15 were incubated with *C. parvum*-infected CaCo-2 cells. Cells were followed over a time-course of up to 4 hrs. At 1.5 hrs, we observed effector cells attached to the infected target cell (Figure 4A). Effector cells and infected CaCo-2 cell were in close contact, near the sites where the parasites were located in the target cell membrane. One effector cell was noted releasing acidic granules into the target cell (Figure 4B). These observations suggest that cytotoxic granules may be one of the pathways by which CD8⁺ T cells lyse infected epithelial cells.

Antiparasitic effect depends on cytotoxic granules. To better define the pathways by which the effector functions of antigen expanded sensitized CD8⁺ T cells are being mediated, further inhibition studies were performed. Before functional assays, gp15 expanded sensitized CD8⁺ T cells were treated with neutralizing antibody to Fas ligand or with Concanamycin A, which inhibits the release of cytolytic granules.²⁸ Consistent with the observation during fluorescent microscopy, we found that Concanamycin A completely inhibited clearance of parasite RNA from cell cultures infected with *C. parvum* (Figure 5). Similarly, in ⁵¹Cr release assays Concanamycin A completely inhibited lysis of cell cultures that were infected with the parasite (data not shown). This clearly suggests a major role of cytotoxic granules mediating the cytolytic and antiparasitic effect.

FIGURE 4. CD8⁺ lymphocytes bind infected epithelial cells. Isolated, gp15 expanded CD8⁺ T cells from sensitized donor 1 were co-cultured with *Cryptosporidium parvum*-infected CaCo-2 cells. Cells were analyzed with a Zeiss Axiovert 200M inverted microscope using a stage incubator. *Cryptosporidium parvum* sporozoites were stained yellow (CFSE), CaCo-2 cell membrane stained red (PKH-26), CD8⁺ T cell nuclei stained blue (Hoechst 33342), and CD8⁺ T cell cytolytic granules stained red (LysoTracker). **Panel A:** CD8⁺ T cells associated with infected target cells (1.5 hrs of co-culture). **Panel B:** three-dimensional reconstruction of effector cell releasing granules into an infected target cell (1.5 hrs of co-culture). This figure appears in color at www.ajtmh.org.

Pre-incubation of CD8⁺ T cells with anti-Fas ligand-antibody cut lysis of infected cells to half in ⁵¹Cr release assays (data not shown) and completely inhibited clearance of *C. parvum* parasites from infected cultures (Figure 5). These data indicate that Fas/Fas ligand interaction may be partially involved in the effects antigen expanded sensitized CD8⁺ T cells exert on infected target cells.

DISCUSSION

In this study, we investigated the ability of human CD8⁺ T cells to eliminate parasites from infected cell cultures. Our

FIGURE 5. Parasite clearance is blocked by Concanamycin A or antibody to Fas ligand. CD8⁺ T cells were isolated from gp15 antigen expanded peripheral blood mononuclear cells (PBMCs) from sensitized Donor 1 and used as effector cells in co-cultures with *Cryptosporidium parvum*-infected CaCo-2 target cells at an effector:target ratio of 25:1. Before co-culture CD8⁺ T cells were incubated with either Concanamycin A or antibody to Fas ligand. After 24 hrs of co-culture in complete RPMI supplemented with IL-7 cell pellets were harvested and *C. parvum* RNA was quantified by real-time polymerase chain reaction (rtPCR). * $P < 0.05$

data suggest that gp15 expanded human CD8⁺ T cells are able to significantly reduce the parasite load in infected intestinal epithelial cell cultures, that they lyse *C. parvum*-infected intestinal epithelial cells, that these effects are mediated by cytotoxic granules and may require antigen presentation through both HLA-A and HLA-B.

The human immune response leading to clearance of *Cryptosporidium* infection is only partially understood. While CD4⁺ T cells clearly play a pivotal role²⁻⁴ and natural killer cells seem to be involved,⁵ the importance of other components of the immune system, e.g., antibodies, remains controversial.¹ The role of human CD8⁺ T cells has never been studied. One of the most important cytokines involved in the immune response against the parasite is IFN- γ . Patients with IFN- γ deficiency may experience severe and fatal *Cryptosporidium* infection.²⁹ Our previous studies of volunteers with experimental cryptosporidiosis have shown that IFN- γ is associated with the human adaptive immune response against the parasite.¹⁴ Similarly, lymphocytes from persons who had recovered from *Cryptosporidium* infection produced IFN- γ after *in vitro* re-stimulation, whereas lymphocytes from human immunodeficiency virus (HIV)-infected individuals with active cryptosporidiosis did not.^{12,13} How IFN- γ exerts its protective effect has not yet been clearly determined. Pretreatment of human intestinal cell lines with IFN- γ led to impaired invasion and intracellular development of the parasite while not having a direct effect on *Cryptosporidium* itself.³⁰ However, inhibition was only observed in human cancer cell lines when high levels of IFN- γ were used, and its efficacy was largely dependent on the type of the cell line.³⁰

We recently found that *Cryptosporidium* antigen re-stimulation of PBMCs from sensitized donors led to IFN- γ production by both CD4⁺ and CD8⁺ T cells.¹⁵ A recent study noted a possible association between *Cryptosporidium* infection and

HLA class I (necessary for antigen presentation to CD8⁺ T cells) and class II alleles (necessary for antigen presentation to CD4⁺ T cells).¹⁶ Together with the fact that cytotoxic CD8⁺ T cells are important for clearing a number of intracellular pathogens in humans,^{31–33} we hypothesized that CD8⁺ T cells may play an important role in the human immune response against *Cryptosporidium*. Furthermore, the parasite primarily infects the small intestine, a site where the intraepithelial T cell pool consists predominantly of CD8⁺ T cells.³⁴

For our experiments, we chose to use the intestinal epithelial cell line CaCo-2 as target cells. The CaCo-2 cells can be infected with *Cryptosporidium* and the cells express major histocompatibility complex (MHC) class I on their surface.³⁵ Ideally, effector cells would have been intestinal epithelial lymphocytes. It is possible that the majority of *Cryptosporidium*-specific T cells may be found in the intestines and only in small numbers in the periphery. However, that was not feasible, so effector cells were isolated from PBMCs after 6-day culture with IL-2 and IL-15 to maintain their viability and expand their numbers. Specific cell populations were expanded by stimulation with the immunodominant gp15 antigen.

To investigate whether cytotoxic CD8⁺ T cells specifically eliminate *C. parvum* organisms from infected intestinal epithelial cell cultures, we performed rtPCR experiments. We used the conditions of a previously published protocol showing increased antigen-specific IFN- γ responses if the culture medium was supplemented with IL-7 and the final volume reduced.²⁷ We showed that antigen expanded sensitized CD8⁺ T cells reduced the quantity of *C. parvum* in intestinal cell cultures by over 60%, whereas CD8⁺ T cells from a non-sensitized donor did not significantly clear the organisms. Pre-treatment of gp15 expanded sensitized effector cells with anti-CD8-antibody completely inhibited reduction of parasite load, confirming that the observed anti-parasitic effect is indeed mediated by CD8⁺ T cells. In ⁵¹Cr release assays we showed that antigen expanded sensitized CD8⁺ T cells lysed infected intestinal epithelial cells in an effector:target ratio-dependant way, although the lytic effect we observed was very modest. Taken together, these observations strongly support our hypothesis that human CD8⁺ T cells contribute to clearance of intestinal *Cryptosporidium* infection.

Although expanded sensitized CD8⁺ T eliminated most of the parasite RNA from infected intestinal epithelial cell cultures, the cytolytic effect on target cells using the ⁵¹Cr release assays was much less pronounced. The incubation time for the chromium release assays was less than that used for the RNA assays and IL-7 was not included in the incubation medium. We speculate that the cytolytic effect could have been increased by a longer incubation time or by the addition of IL-7. Furthermore, we may be underestimating the cytolytic effect of the CD8⁺ T cells and their ability to eliminate parasites as only a subset of the target cells got infected. In addition, we lack information on the time-course of peptide presentation on the surface of the target cells and might have missed the time-point of most efficient antigen presentation. Another explanation may be that the primary effect of anti-cryptosporidial CD8⁺ T cells may not be lysis of infected cells. Instead, CD8⁺ T cells may possibly lead to release of the parasite from the target cells, while the target cell may either continue to live or may undergo non-lytic death.

All donors and the cell line were matched in HLA-A (HLA-A2). Surprisingly, only one seropositive donor consistently

lysed infected CaCo-2 cells. This was the only seropositive donor who matched the CaCo-2 cell line in both HLA-A and HLA-B (HLA-B62). Blocking of either HLA-A or HLA-B on the target cells inhibited lysis of infected cells and reduction of parasite load. Intriguingly, HLA-B62 belongs to one of the HLA class I alleles (B*15) recently found to be associated with *Cryptosporidium*-infected Bangladeshi children,¹⁶ which is in favor of our hypothesis that CD8⁺ T cells are involved in the immune response against the parasite. Importantly, our data suggest that antigen presentation through HLA-A and HLA-B may be necessary for clearance of *Cryptosporidium* by CD8⁺ T cells, which is important for the successful search for vaccine candidates.

We also studied mechanisms of effector functions. Cytotoxic CD8⁺ T cells may lyse their targets through various pathways, which among others include release of cytolytic granules, e.g., perforin/granzyme B, Fas/Fas ligand interaction, and signaling through TNF-related apoptosis-inducing ligand (TRAIL).³⁶ Previous work by our group suggested that TRAIL may be involved in clearance of infected epithelial cells.³⁷ In this study, we observed the release of acidic granules from an effector cell into an infected target cell, suggesting that cytolytic granules are one of the mechanisms by which infected intestinal epithelial cells are being lysed. Consistently, lysis of infected cells and elimination of parasites from cell cultures could be completely blocked by inhibiting the release of lytic granules from CD8⁺ T cells with Concanamycin A, which induces accelerated degradation of granule content by increasing the pH.²⁸ Apart from cytotoxic granules forming pores in the target cell membrane leading to destruction of the physiologic membrane potential and allowing other lytic granules to enter and induce the death of the target cell,³⁶ there is also evidence that they may have a direct microbicidal effect on infectious pathogens.^{38,39} The role of cytotoxic granules during cryptosporidiosis may thus be lysis of infected epithelial cells leading to release of live but immature *Cryptosporidium* organisms that are not able to re-infect the host and die because of interruption of their life cycle. Alternatively, the released cytotoxic granules may also have a parasitocidal effect on the organisms themselves, thus directly contributing to their elimination. Our studies indicate that Fas/Fas ligand interaction may also be involved in clearing the parasite as blocking of Fas ligand at least partially inhibited lysis of infected target cells and completely prevented reduction of parasite load in cell cultures. We therefore speculate that CD8⁺ T cells contribute to clearance of the parasite through a variety of mechanisms.

Studies in murine and other animal models have led to important advances in understanding the immune response against *Cryptosporidium*. However, whereas CD4⁺ T cells are clearly important for clearing the parasite in both mice and men,^{2–4,7} the role of other immunologic factors, e.g., IFN- γ , seems to differ between murine and human cryptosporidiosis.¹ Although CD8⁺ T cells may not play an important role in the protective immune response toward *C. parvum* infection in mice,^{6,7} their role in the human immune response against this parasite has never been studied. Recent findings of our group and other groups led us to hypothesize that CD8⁺ T cells may play a previously unrecognized role in clearing the parasite in humans.

Taken together, our data are in favor of this hypothesis and suggest that the human immune response against *C. parvum* involves CD8⁺ T cells in sensitized individuals. We propose the following model: CD8⁺ T cells are involved in the memory

human immune response against the parasite and are attracted to the site of infection. Antigen-specific CD8⁺ T cells recognize *Cryptosporidium*-derived peptides on infected epithelial cells in the context of HLA-A and HLA-B. Besides activating other cytotoxic and/or anti-parasitic mechanisms, CD8⁺ T cells release cytotoxic granules, which may lead to lysis of infected epithelial cells and release of immature parasites. The *Cryptosporidium* organisms die because of interruption of their life cycle and/or because of a direct parasitocidal effect of the cytotoxic granules. Future studies are needed to better define the characteristics and effector mechanisms of the CD8⁺ T cell population involved in the human immune response toward *Cryptosporidium*.

Received September 30, 2009. Accepted for publication January 9, 2010.

Acknowledgments: Our deepest thanks to C. Logsdon at MD Anderson Cancer Center in Houston, Texas and all the members of his laboratory who very generously helped us out after the University of Texas Medical Branch and the island of Galveston were devastated by Hurricane Ike.

Authors' addresses: Birte Pantenburg, Alejandro Castellanos-Gonzalez, and A. Clinton White Jr., Division of Infectious Diseases, Department of Internal Medicine, University of Texas Medical Branch, Galveston, TX, E-mails: birte.pantenburg@upch.pe, alcastel@utmb.edu, and acwhite@utmb.edu. Sara M. Dann and Dorothy E. Lewis, Division of Infectious Diseases, Department of Internal Medicine, and Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX, E-mails: smdann@utmb.edu and dolewis@utmb.edu. Rhykka L. Connelly, Center for Electromechanics, University of Texas, Austin, TX, E-mail: r.connelly@cem.utexas.edu. Honorine D. Ward, Division of Geographic Medicine and Infectious Diseases, Tufts Medical Center, Boston, MA, E-mail: hward@tuftsmedicalcenter.org.

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