CONCISE COMMUNICATIONS

Effect of Immune Activation Induced by Cryptosporidium parvum Whole Antigen on In Vitro Human Immunodeficiency Virus Type 1 Infection

Lihua Xiao,1 Renu B. Lal,2 and Altaf A. Lal1

1Immunology Branch, Division of Parasitic Diseases, and 2Retrovirus Diseases Branch, Division of AIDS, Sexually Transmitted Diseases, and Tuberculosis Laboratory Research, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia

Previous epidemiologic investigations have suggested that persons with AIDS who are infected with Cryptosporidium parvum have a shorter survival time than those with other opportunistic infections. In this study, the effect of immune activation by a crude Cryptosporidium whole antigen on human immunodeficiency virus type 1 (HIV-1) infection was evaluated. Peripheral blood mononuclear cells from healthy persons without HIV-1 infection had increased proliferative and cytokine (interleukin-4, interferon-γ, and tumor necrosis factor [TNF]-α) responses to stimulation with the crude Cryptosporidium whole antigen. This stimulation increased HIV-1 p24 antigen production in vitro infection by >30-fold. A similar increase in p24 production was also seen when stimulation was done after cells were infected with HIV-1. Neutralization of TNF-α reduced Cryptosporidium antigen–induced p24 production by >50%. Results of this study suggest that Cryptosporidium-induced immune activation may be a cofactor in regulating HIV-1 production.

Opportunistic infections are a major threat to persons infected with human immunodeficiency virus (HIV) and are responsible for most of the morbidity and mortality associated with HIV type 1 (HIV-1) diseases. Results of recent studies indicate that opportunistic infections may promote HIV-1 replication, therefore, accelerating the progression from HIV infection to the development of AIDS [1, 2]. It is suggested that the increased viral replication after infection is the result of immune activation by these agents, because replication of HIV occurs primarily in the activated cells. This opportunistic infection–induced viral replication causes rapid CD4 depletion and accelerated death [3].

Cryptosporidiosis, an opportunistic infection in HIV-infected persons, causes chronic and sometimes life-threatening diarrhea in AIDS patients [4]. Circumstantial evidence suggests that Cryptosporidium infection may also promote AIDS progression. Cryptosporidium-infected ileum has higher tumor necrosis factor (TNF)-α expression and macrophage infiltration [5, 6]. Also, Cryptosporidium oocyst antigen induces increased cellular proliferation and cytokine production in peripheral blood mononuclear cells (PBMC) and splenocytes from both exposed and nonexposed humans and mice [7–9]. In addition, AIDS patients with cryptosporidiosis have a much shorter survival time than those with other opportunistic infections, after adjustment for age and CD4 cell counts [10, 11].

Because the gastrointestinal tract is the major site of viral replication [12], it is possible that the immune activation induced by cryptosporidiosis can promote HIV-1 replication. As a first step in the investigation of the interaction between Cryptosporidium infection and HIV-1, we evaluated the effect of a Cryptosporidium oocyst whole antigen stimulation on HIV-1 infection of PBMC of healthy donors and investigated the mechanism involved in Cryptosporidium antigen up-regulated HIV-1 production.

Materials and Methods

Cryptosporidium whole antigen preparation. The crude Cryptosporidium whole antigen used in this study was prepared from Cryptosporidium parvum oocysts purified by the freezing-thaw technique from naturally infected calves. In brief, oocysts were purified from fecal samples by sucrose and Percoll centrifugation, treated in 0.5% sodium hypochloride solution (undiluted commercial bleach) at 4°C for 10 min, washed 6× in sterile water, and resuspended in PBS at 2 × 10⁸ oocysts/mL. Purified oocysts were then subjected to 10 freezing-thaw cycles and stored at –80°C before use within 6 months. The presence of endotoxin in the whole oocyst
antigen preparation was determined to be <0.05 U/mL by E-TOXATE kit (Sigma, St. Louis).

**PBMC isolation and depletion of CD8 cells.** PBMC were isolated by the standard Ficoll-Paque (Pharmacia Biotech, Piscataway, NJ) from healthy donors who had never been exposed to HIV density-gradient centrifugation. The samples were depleted of CD8 T cells by incubation with magnetic beads coated with anti-CD8 antibody (Dynabeads; Dynal, Lake Success, NY), according to the manufacturer’s instructions. Residual CD8 cells were checked by flow cytometry before the experiment was started and usually had <1% contamination. Depletion of CD8 cells in the culture system allows for more-consistent and stable infection of CD4 lymphocytes.

**Stimulation and acute HIV-1 infection of PBMC.** CD8-depleted PBMC were placed in 24-well plates (Costar, Cambridge, MA) at 2 × 10⁶ cells/well in a total volume of 2 mL. They were stimulated with the *Cryptosporidium* whole antigen at the equivalent of 0.02 oocyst/cell for 12 h and simultaneously inoculated with HIV-1 Lai strain (HIV-1Lai) (MOI = 0.01), as described elsewhere [13]. Unstimulated cells (medium only) were used as the treatment control. Cultures were maintained in RPMI 1640, supplemented with 10% fetal bovine serum in the absence of exogenous mitogens or cytokines. Cells were washed 3 × after stimulation and HIV inoculation and replaced in 24-well plates. *Cryptosporidium* antigen was not replenished after cell washing. Half of the culture supernatant was collected at days 2, 4, 6, and 8 and replaced with fresh medium. Culture supernatants were stored at −70°C before cytokine and p24 analyses. To determine whether modulation in p24 production by *Cryptosporidium* antigen stimulation can occur in PBMC preinfected with HIV-1, cells from healthy donors were infected with HIV-1Lai for 12 h and washed 3 × . One day after the infection, these cells were stimulated with the *Cryptosporidium* whole antigen at 0.02 oocyst/cell. Culture supernatant was collected at days 2, 4, 6, and 8 for p24 determination. Levels of p24 antigen in the culture supernatant were determined by commercial EIA kits (Coulter Immunology, Hialeah, FL). To evaluate the role of cytokines in *Cryptosporidium* antigen up-regulated HIV-1 infection, neutralizing monoclonal antibodies (MAbs) against TNF-α (0.25 μg/mL) and interleukin (IL)-10 (1 μg/mL) (R&D Systems, Minneapolis) were added to the culture system at the time of stimulation and HIV-1 inoculation. After being washed, MAbs were added back to PBMC cultures but were not supplemented at the time of supernatant collection and medium replenishment.

**Cellular proliferation and cytokine induction.** Proliferative responses of PBMC to the *Cryptosporidium* whole antigen were assessed by a conventional [HI]thyminde incorporation 6-day assay, using the medium as control. The effect of stimulations on the production of cytokines was evaluated by stimulation of PBMC from 6 healthy donors with the *Cryptosporidium* whole antigen (equivalent of 0.02 oocyst/cell) in 24-well plates for 2, 4, 6, and 8 days in the absence of HIV-1 infection. Levels of TNF-α, interferon (IFN)γ, RANTES, and IL-4, -6, and -10 in the culture supernatants were determined by EIA kits (BioSources, Camarillo, CA).

**Statistical analysis.** Data were presented as mean ± SEM of 4 subjects for infection studies and 6 persons for proliferative and cytokine responses. Differences between groups were compared by Student’s *t* test and were considered significant at *P* ≤ .05.

**Results**

We first examined the effect of *Cryptosporidium* whole antigen stimulation on the infection of PBMC with HIV-1. As expected, PBMC had low infection intensities when they were inoculated with a T cell–tropic HIV-1 strain (HIV-1Lai) in the absence of stimulation with exogenous mitogens or cytokines, as judged by production of p24 HIV-1 core antigen (figure 1A). Stimulation with an equivalent of 0.02 oocyst/cell of the *Cryptosporidium* whole antigen increased p24 production after the cells were simultaneously inoculated with HIV-1Lai. This increase in p24 production was first seen at day 4, when an average of 4.9 ± 2.3-fold increase was seen. Increases in p24 production after stimulation were more obvious at days 6 and 8, when p24 production in the *Cryptosporidium* antigen–stimulated group was 31.7 ± 47.5 and 36.6 ± 26.9 times higher. The differences between the unstimulated cells and those stimulated with the *Cryptosporidium* whole antigen was significant at days 4, 6, and 8 (*P* < .05).

To assess whether *Cryptosporidium* antigen–induced immune activation could increase HIV-1 production after cells were already infected with HIV-1, we examined the effect of *Cryptosporidium* antigen stimulation on p24 production by HIV-1–infected PBMC. CD8-depleted PBMC were first infected with HIV-1Lai. They were then stimulated with the *Cryptosporidium* whole antigen at the equivalent of 0.02 oocyst/cell. Stimulations with the *Cryptosporidium* whole antigen increased the production of p24 by infected cells (figure 1B). The stimulatory effect was first seen at day 4 with an average 2-fold increase. This increase reached 75.8 ± 129.7-fold and 39.2 ± 28.2-fold at days 6 and 8, respectively, suggesting that the *Cryptosporidium* whole antigen probably can also affect postintegration events in HIV-1 infection. The differences between the unstimulated cells and those stimulated with the *Cryptosporidium* whole antigen was significant at days 6 and 8 (*P* < .05).

To evaluate the role of cytokines in *Cryptosporidium* antigen–induced HIV-1 p24 antigen production, we examined changes in cytokine production by PBMC after antigen stimulation. Antigen stimulation increased the production of IFN-γ by 10.2-, 11.5-, 9.7-, and 8.2-fold at days 2, 4, 6, and 8, respectively (figure 2A; *P* < .05). *Cryptosporidium* whole antigen stimulation also increased the production of IL-4, albeit to a lesser degree (figure 2B). Likewise, stimulations with the *Cryptosporidium* whole antigen increased the production of TNF-α (figure 2C). The level of TNF-α production by *Cryptosporidium* antigen–stimulated cells at each time point was higher than by unstimulated cells (84.0 vs. 0.3, 553.7 vs. 113.0, 674.3 vs. 330.0, and 375.5 vs. 207.5 pg/mL at days 2, 4, 6, and 8, respectively; *P* < .05 at days 2 and 4), although the unstimulated control cells also had increases in TNF-α production. The latter probably resulted from nonspecific activation due to monocyte adherence to the plate. Small increases in IL-6, IL-10, and RANTES were also seen after stimulation (data not shown). Stimulations of PBMC with the *Cryptosporidium* whole antigen also in-
Figure 1. Cryptosporidium whole antigen (Crypto) up-regulated human immunodeficiency virus type 1 (HIV-1) production. Peripheral blood mononuclear cells isolated from 4 healthy donors were inoculated with HIV-1 Lai strain (HIV-1Lai) in presence of Crypto (A) or infected first and stimulated with Crypto 1 day after viral infection (B). Culture supernatant was collected at days 2, 4, 6, and 8 and analyzed for p24 antigen. Data are expressed as mean ± SEM pg/mL of p24 antigen in control (HIV only) and Cryptosporidium antigen–stimulated (Crypto) groups (P < .05 at days 4, 6, and 8 [A]; and at days 6 and 8 [B]).

duced proliferative responses (counts/min ± SD of means = 24,601 ± 6524 in the stimulated group vs. 1728 ± 316 in the control group; P < .05).

Because TNF-α plays an important role in controlling HIV-1 replication and stimulation with the Cryptosporidium whole antigen increased the production of TNF-α, we next examined whether Cryptosporidium antigen–promoted virus production was mediated through the induction of TNF-α. The addition of TNF-α neutralizing MAb reduced the Cryptosporidium antigen–induced HIV-1 p24 antigen production in CD8-depleted PBMC by averages of 58.3%, 54.0%, and 36.5% at days 4, 6, and 8, respectively (figure 2D; P < .05 for days 4 and 6). Neutralization of TNF-α also reduced Cryptosporidium antigen–promoted p24 antigen production in cells preinfected with HIV-1 by 43.7% on day 6 (P < .05; data not shown). Neutralization of IL-10 had no significant effect on Cryptosporidium antigen–induced p24 production (data not shown).

Discussion

In our in vitro model of HIV-1 infection, the immune activation due to exposure to the crude Cryptosporidium whole antigen promoted HIV-1 production. Stimulation of PBMC from Cryptosporidium-naive donors with crude Cryptosporidium antigen increased the production of p24 antigen, after these cells were infected with HIV-1. Increases in p24 production were seen regardless of whether the cells were stimulated at the time of in vitro HIV-1 infection or after infection. The role of cellular activation in the initiation and propagation of HIV-1 infection of CD4 T cells in vitro has been well defined. Thus, T cell activation resulting from exposure to a common pathogenic stimulus, such as Cryptosporidium infection, could prove to be an important determinant of HIV-1 replication. The increased HIV-1 production probably resulted from immune stimulation induced by the Cryptosporidium whole antigen. We found that stimulation of PBMC from healthy donors with the Cryptosporidium whole antigen resulted in the immune activation of cells, as judged by proliferative responses and cytokine production. In previous studies in mice and humans, PBMC from Cryptosporidium-naive persons proliferated in response to stimulation with crude Cryptosporidium antigen [7–9]. These subjects also had increased production of IFN-γ [9]. Results from our study confirm that the Cryptosporidium antigen can activate PBMC from persons with no recent exposure. In addition to the increased proliferative responses and IFN-γ production, we found that the production of TNF-α and IL-4 was also increased. Thus, the production of Th1, Th2, and proinflammatory cytokines can all be activated in cells after exposure to the crude Cryptosporidium antigen. Previous studies in humans infected with C. parvum showed that both Th1 and Th2 cytokine responses were induced after lymphocytes were exposed to a crude Cryptosporidium antigen [7]. We found that TNF-α appears to play a role in Cryptosporidium antigen–promoted virus production, since neutralizing antibodies to TNF-α reduced Cryptosporidium–induced HIV-1 p24 antigen production. However, since only a partial inhibitory effect on Cryptosporidium antigen–stimulated p24 antigen production was achieved by neutralization of TNF-α, the role of other
Cryptosporidium antigen–induced immune activation and its role in human immunodeficiency virus type 1 (HIV-1) production. Peripheral blood mononuclear cells (PBMC) from 6 healthy donors were stimulated with Cryptosporidium whole antigen (Crypto), with medium only as control (Ctr). Levels (pg/mL) of interferon (IFN)-γ (A), interleukin (IL)-4 (B), and tumor necrosis factor (TNF)-α (C) in culture supernatants were determined at days 2, 4, 6, and 8. To evaluate role of TNF-α in Cryptosporidium antigen–induced HIV-1 production (D), neutralizing monoclonal antibodies (MAbs) against TNF-α were added to infection experiments at time of antigen stimulation. PBMC were then stimulated and infected as in figure 1A. Addition of TNF-α neutralizing MAb (Crypto + TNF MAb) reduced Cryptosporidium-stimulated HIV-1 infection (Crypto) by averages of 58.3%, 54.0%, and 36.5% at days 4, 6, and 8, respectively (P < .05 at days 4 and 6).

Factors, such as IL-6 and IFN-γ, remains to be determined. Some of these cytokines (e.g., IL-6) up-regulate HIV-1 replication, although conflicting results have been reported regarding IFN-γ [14, 15].

In conclusion, the results of this in vitro study suggest that Cryptosporidium-induced immune activation can potentially modulate HIV-1 infection. These in vitro observations, together with the results of prior epidemiologic studies, suggest that dual infection of Cryptosporidium and HIV-1 may potentially trigger immunologic events that modulate the infection of both the virus and the parasite. Although the effect of HIV-1 on cryptosporidiosis has been investigated in many epidemiologic studies, further in vitro studies with intraepithelial lymphocytes and in vivo studies are required, to explore the impact of cryptosporidiosis on HIV/AIDS.

References
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