Impaired Interleukin 12 Production in Human Immunodeficiency Virus–infected Patients

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Summary

Peripheral blood mononuclear cells (PBMC) from human immunodeficiency virus (HIV)-infected patients, asymptomatic or with acquired immunodeficiency virus, produced 10-fold less interleukin 12 (IL-12) free heavy chain and fivefold less biologically active IL-12 heterodimer than PBMC from uninfected healthy donors when challenged in vitro with the common human pathogen Staphylococcus aureus. In contrast, PBMC from HIV-infected individuals and uninfected control donors produced similar levels of tumor necrosis factor α, IL-1β, and IL-10, and PBMC from HIV-infected individuals produced three- to fourfold more IL-6 compared with PBMC from uninfected control donors. The defect in IL-12 production is not due to hyperproduction of IL-10, a cytokine exerting an autocrine-negative feedback on IL-12 production, but was directly related to HIV infection, as suggested by the reduced ability of monocytes infected in vitro with HIV to produce IL-12. IL-12 deficiency may be an important component of the immunodeficiency associated with HIV infection.

Patients with HIV infection at both early or late stages have immunoregulatory defects at several levels of the immune system, and the in vivo progression of the infection is closely related to abnormal functioning of the immune system (1, 2). The nature and role of the humoral and cell-mediated immune responses against HIV have not been fully characterized, and it remains to be established whether immune responses against HIV prevent primary infection and/or protect against disease progression in infected individuals. Defining immune responses against HIV that are protective is critical for the rational design of effective vaccines and other forms of immunotherapy.

A great deal of information on the role of several cytokines during HIV infection has accumulated, although the relative extent of their expression remains unclear (1). Natural killer cell stimulatory factor (NKSF) or IL-12 is a heterodimeric cytokine of 70 kD formed by a H chain (p40) and a L chain (p35) encoded by two separate genes (3). IL-12 is produced in response to bacteria, bacterial products, and intracellular parasites by cells with accessory or antigen-presenting ability (4). When human PBMC are stimulated in vitro, IL-12 is mostly produced by monocytes, but, in part, also by B cells, and probably by other non-B, nonmonocytic HLA-DR+ cells (4). Accumulation of p40 mRNA is highly regulated and rapidly induced in vitro and in vivo after stimulation of the producer cells with bacteria or bacterial products (4). Although only the p70 heterodimer has been shown to have biologic activity, the p40 H chain is always produced in large excess over the p70 heterodimer, both in vitro (4) and in vivo (Wysocka, M. and G. Trinchieri, unpublished results).

IL-12 has potent and pleomorphic activities on NK and T cells, inducing production of cytokines, particularly IFN-γ, enhancement of cytotoxic activity of NK cells (3–5), and generation of cytotoxic lymphocytes (6). IL-12 has a proliferative effect on T and NK cells, acting synergistically with various stimuli, including specific antigen stimulation (7). Both in vivo and in vitro, IL-12 is a powerful inducer of T helper cells type I (Th1) responses, whereas it inhibits Th2-type responses (8). Emerging evidence indicates a central role for IL-12 in resistance to bacterial and parasitic infections (9, 10). In light of its production by phagocytic cells shortly after exposure to infectious agents and its powerful effect on the regulation of T and NK cell functions, IL-12 represents an important link between natural resistance and adaptive immunity (8).

Because emerging data suggests a relationship between disease progression and the nature of the cellular immune response in HIV-infected patients (1, 2, 11), we evaluated the ability of HIV-infected patients to produce IL-12 in response to bacterial stimulation.
Materials and Methods

Study Population. 73 consenting adult patients (22-45-yr-old, 17 females and 56 males), HIV seropositive and at various stages of the disease, were enrolled in this study. HIV serology was determined using commercially available assays. All patients were repeatedly positive for HIV antibodies by HIV ELISA, and confirmed by Western blot analysis. Patients were classified according to their absolute CD4+ cell counts (<200/mm3, between 200 and 500/mm3 higher than 500/mm3). Healthy HIV-seronegative donors, matched for age and sex, were included as controls. At the time of the study, none of the patients was receiving cytokine therapy.

Cell Separation. All reagents used for this study were selected for low levels of endotoxin contamination by the Limulus amebocyte assay. PBMC were separated by Ficoll-Hypaque density gradient centrifugation (Lymphoprep, Nyaagard and Co., Oslo, Norway), and resuspended in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (complete medium). Fresh monocytes were isolated by incubating PBMC in plastic flasks at 37°C for 2 h in RPMI-1640 supplemented with 20% FCS. Nonadherent cells were removed by several washes with PBS, and adherent cells were incubated for 20 min in ice-cold PBS and resuspended at 0.5 x 10° cells/ml. Adherent cells were then incubated for 2-3 d before viral infection. These monocyte preparations contained >90% CD14+ cells, and >90% nonspecific esterase-positive cells.

Virus Infection. HUT-78 and CEM-X174 cell lines were maintained in RPMI-1640 supplemented with 10% FCS (12). Culture supernatants of the following HIV-1 strains, displaying a different pattern of cellular tropism, were used: lymphocyte-tropic strains IIIB and MN, grown in HUT78 cells (provided through the AIDS Research and Reference Reagent Program, Rockville, MD), and monocytotropic strain 89.6 (kindly provided by R. Collman and H. Friedman, University of Pennsylvania) grown in CEM-X174 cells (12). Virus stocks were titered for p24 core Ag production using an Ag capture assay (Coulter Immunology, Hialeah, FL) and for tissue culture infective dose. Monocytes (2 x 10° cells/well) were mock infected (with media from the cell lines used to grow HIV), or infected with cell-free HIV isolates containing 20-30 ng/ml of p24 Ag or equivalent amounts of heat-inactivated viruses (1 h at 56°C). After 3-4 h of incubation at 37°C, cells were gently washed, resuspended with complete medium, and maintained for 5-7 d. At the end of the incubation period, culture supernatants and cells were harvested for p24 Ag determinations.

Induction of Cytokine Production. To induce cytokine production, PBMC (2.5 x 10° cells/ml) from seronegative controls and HIV-infected individuals were cultured for 24 h in complete medium alone or in the presence of heat-inactivated Staphylococcus aureus Cowan strain 1 (Pansorbin; Calbiochem-Behring Corp., La Jolla, CA, diluted 10^- vol/vol). Cell-free supernatants were then harvested, filtered, and stored at -80°C until assayed for cytokines. Mock- and HIV-infected monocytes were washed and stimulated with S. aureus for 24 h. Cell-free supernatants were harvested, filtered, and stored at -80°C until assayed.

Cytokine Assays. The production of IL-12 p40 (either alone or associated with p35) was measured by specific RIA using the mAb pair C11.79/C8.6 (sensitivity, 10 pg/ml) as described (4). Biologically active IL-12 p70 was quantitated using a newly developed Ab capture bioassay (sensitivity, 10 pg/ml) as described by D'Andrea et al. (13). TNF-α and IL-1β were determined by RIA using mAbs B154.7/B154.9 and P16.609/P16.206 (kindly provided by M.A. Cousin, Roussel Uclaf, Rompsoreilles, France), respectively. All determinations were done in triplicate. IL-4, IL-6, and IL-10 were quantitated using commercially available specific ELISA assays (Quantikine, R & D Systems, Inc., Minneapolis, MN for IL-4; Biosource International, Camarillo, CA for IL-6 and IL-10). In indicated experiments, PBMC were stimulated with S. aureus in the presence of optimal concentrations of neutralizing anti-IL-10 mAb 19F1 (10 µg/ml, kindly provided by K. Moore, DNAX, Palo Alto, CA).

Results and Discussion

We have previously reported that short-term incubation of lymphocytes from HIV-infected patients with IL-12 restored the NK cell cytotoxic activity to levels mediated by unstimulated PBMC of healthy donors (5). IL-12 also stimulated IFN-γ production by lymphocytes of HIV-infected patients, although their ability to produce IFN-γ in response to both IL-12 and other inducers was reduced compared with lymphocytes from healthy seronegative controls (5). Furthermore, we have reported enhancement of NK cell-mediated cytolysis against herpesvirus (CMV, varicella Zoster virus, HSV) and HIV-infected target cells with concentrations of IL-12 ~100-1,000-fold lower than concentrations of IFN-α, IFN-β, and IL-2 required for a similar effect (14). It is interesting to note that Clerici et al. (15) recently demonstrated that in vitro addition of IL-12 to HIV-infected individuals can restore HIV-specific IL-2 and IFN-γ production as well as antigen-specific lymphocyte proliferation.

In the present study, PBMC from 73 adult HIV-seropositive individuals at different stages of disease, stimulated in vitro with the common human pathogen S. aureus (heat fixed) for 24 h, produced 10-fold less IL-12 p40 chain on average than cells from healthy control donors (Fig. 1 A). Statistical analysis of IL-12 p40 production in the 73 patients grouped according to absolute CD4+ cell count revealed significantly less IL-12 p40 production for each group as compared with controls (Student's t test, p <0.001). p40 production by PBMC of asymptomatic individuals with >500 CD4+ cells/mm3 was not significantly different from that of AIDS patients with <200 CD4+ cells/mm3. Unlike S. aureus–induced p40 production, low constitutive production of p40 detected in unstimulated cultures was not statistically different for controls (58 ± 10 pg/ml, n = 61) and patients (54 ± 9 pg/ml, n = 73). The ability of normal donors and patients to produce IL-12 p70 in response to S. aureus was determined in 32 patients. Control donors produced fivefold more IL-12 p70, on average, in response to S. aureus than did the HIV-infected patients tested (Fig. 1 B, p <0.001). For the same group of HIV-infected and control donors, the difference in IL-12 p40 production in response to S. aureus was ~20-fold. Therefore, although the PBMC from the patients were significantly and severely depressed in their ability to produce biologically active IL-12, this defect was not as severe as that involving the production of p40. This partial dissociation in the ability to produce p40 and p70 might depend on the state of activation of monocyte/macrophages in HIV-infected individuals. In contrast, production of TNF-α and IL-1β (under the same culture conditions) was equivalent for PBMC from patients and control donors (Fig. 1, C and D). In addition, PBMC from HIV-infected patients produced three- to fourfold more IL-6 both constitutively (data not
shown, $p < 0.001$) and after *S. aureus* stimulation than control donors (Fig. 1 E, $p < 0.001$). These data suggest that the defect in IL-12 production by PBMC of HIV-infected patients is relatively specific and not secondary to a generalized inability of their monocytes to produce cytokines.

IL-10, a cytokine produced by various cell types including monocytes, B and T cells, acts at both the accessory and T cell levels to inhibit Th1-like functions such as cytokine production and proliferation. IL-10 also efficiently inhibits IL-12 production (13). Because IL-10 has been suggested to be produced at elevated levels in HIV-infected patients (16), we investigated whether the decreased ability of PBMC from HIV-infected patients to produce IL-12 might be secondary to enhanced production of IL-10. Analysis of IL-10 production by PBMC in response to *S. aureus* stimulation revealed no significant difference between HIV-infected patients and control donors (Fig. 2 A), in fact controls produced more IL-10 than patients (5,515 pg/ml vs. 3,267 pg/ml), although this difference was not statistically different. In the absence of stimulation, a low spontaneous production of IL-10 was detected by cells from controls and patients (NS, data not shown). Addition of neutralizing anti-IL-10 mAb to the cultures at concentrations sufficient to inhibit the IL-10 produced in response to *S. aureus*, enhanced IL-12 production in PBMC cultures of HIV-infected patients (636 pg/ml vs. 267 pg/ml, $t = 1.898$, NS) and control donors (3,258 pg/ml vs. 1,986 pg/ml, $t = 3.408$, $p = 0.011$, Fig. 2 B). Addition of exogenous IL-10 to patients’ PBMC cultures induced a dose-dependent decrease in IL-12 production to undetectable levels (data not shown), similar to results obtained with normal donors (13 and data not shown). Thus IL-10 induced by *S. aureus* stimulation has an autocrine-negative feedback on IL-12 production, and neutralization of IL-10 enhances IL-12 production. However, this relative enhancement was not significantly different for HIV-infected patients and control donors ($1.8 \pm 0.36$-fold vs. $1.93 \pm 0.23$-fold, $t = 1.291$, NS), indicating that production of IL-10 in vitro during *S. aureus* stimulation is not responsible for the fact that PBMC from HIV-infected patients produce less IL-12 than those from uninfected donors. Although continuous exposure of monocytes to elevated levels of IL-10 in vivo, proposed to be pro-inflammatory at detectable levels (<30 pg/ml; 24 patients and 36 controls) in the presence of heat-inactivated *S. aureus*, PBMC from healthy donors (Fig. 1 A) in the presence of neutralizing anti-IL-10 mAb 19F1, and IL-12 p40 was measured in cell-free supernatants by RIA.

**Figure 1.** IL-12, TNF-α, IL-1β, and IL-6 production in response to *S. aureus*. PBMC from 73 HIV-seropositive patients divided into three groups according to their CD4 cell counts, and from healthy HIV-seronegative donors were cultured for 24 h in complete medium in the presence of heat-inactivated *S. aureus*. Cytokine production was measured in the cell-free supernatants. (A) IL-12 p40 was measured by RIA using the mAb pair C11.79/C8.6. (B) Biologically active IL-12 p70 was quantitated using an Ab capture bioassay. (C) TNF-α (42 patients, 28 controls) and (D) IL-1β (28 patients, 26 controls) were determined by RIA. (E) IL-6 (20 patients, 15 controls) was quantitated by ELISA. Control healthy donors (○) and HIV-seropositive donors (●) represent results for individual donors. Horizontal bars indicate mean ± SE.

**Figure 2.** IL-10 production and effect of endogenous IL-10 on IL-12 p40 production. (A) An ELISA assay was used to measure IL-10 in supernatants of 24-h cultures of PBMC from healthy controls (○) or HIV-seropositive patients (●) in the presence of *S. aureus*. (B) PBMC from healthy controls and patients were stimulated for 24 h with *S. aureus* in the presence of neutralizing anti-IL-10 mAb 19F1, and IL-12 p40 was measured in cell-free supernatants by RIA.
and anti-CD3 Abs induced slightly higher production of IL-4 by PBMC from control donors, than from a small number of HIV-infected individuals tested (data not shown). Furthermore, IL-4-mediated inhibition of cytokine production is not selective for IL-12, and pretreatment of PBMC and monocytes from control donors with IL-4 stimulates rather than inhibits IL-12 production (D'Andrea, A. and G. Trinchieri, manuscript in preparation). Thus IL-4 is an unlikely candidate for causing the selective defect in IL-12 production observed in most HIV-infected individuals at all stages of disease.

To determine whether the defect in IL-12 production in HIV-infected patients was dependent on HIV infection, we tested the effect of in vitro infection of monocytes with HIV on their ability to produce IL-12 in response to S. aureus. Monocytes freshly isolated from six different healthy seronegative donors were infected with the duotropic strain 89.6, as well as with the lymphotropic strains IIIB and MN for 5–7 d. 89.6-infection of monocytes was confirmed by p24 Ag determinations (2–5 ng/ml, Fig. 3, right). Monocytes inoculated with IIIB, MN, or with heat-inactivated 89.6 produced negligible amounts of p24 Ag (data not shown). After stimulation with S. aureus for 24 h, 89.6-infected monocytes produced significantly less IL-12 p40 than mock-infected monocytes (Fig. 3, center, p < 0.001). In contrast, monocytes inoculated with lymphocyte-tropic strains IIIB and MN, as well as monocytes inoculated with heat-inactivated 89.6, produced levels of IL-12 similar to those produced by mock-infected monocytes (data not shown). Similarly, in vitro infection of fresh monocytes had no significant effect on their ability to produce IL-10 in response to S. aureus (Fig. 3, left, 3,004 pg/ml by uninfected monocytes vs. 2,112 pg/ml in infected monocytes; NS), although IL-12 p40 production was significantly reduced (Fig. 3, center). These data suggest that in vitro infection of the monocytes and not simple exposure to the virus is required for IL-12 inhibition.

The presence of inhibitory cytokines in the virus preparations used was a possible concern in the interpretation of results with HIV exposed monocytes. IL-4 could not be detected in any of the virus preparations and culture supernatants used for mock infection, whereas IL-10 concentrations were 434 pg/ml in supernatants of CEM-X174 cells, and 90 pg/ml in supernatants of 89.6-infected CEM-X174 cells. Although these concentrations of IL-10 could be responsible for a partial inhibition of IL-12 production if present at all times during the culture, IL-12 production was not inhibited under the experimental condition used, since mock-infected monocytes produced higher levels of IL-12 than 89.6-infected monocytes.

The in vitro results are consistent with the hypothesis that impaired IL-12 production is dependent on HIV infection and not secondary to altered immunoregulation or other infection-associated factors. However, only a minor proportion of the circulating monocytes of HIV-infected individuals harbors HIV (1, 2), and in the cultured monocytes in vitro infected with HIV, only a proportion of cells (~10% as detected by immunofluorescence using serum from HIV-infected patients) reacted positively at the time of stimulation with S. aureus. Thus, it is possible that HIV infection may not directly downregulate IL-12 production, but acts indirectly through viral or cellular products, possibly including immunoregulatory cytokines, thus the exact molecular mechanism involved remains unclear at the present time.

Our data indicate that PBMC from HIV-infected individuals, even at early stages of the disease when no symptoms are present and the number of CD4+ cells is still close to normal levels, are severely impaired in their ability to produce IL-12 in response to S. aureus. Because we analyzed only cells from peripheral blood, and relatively little is known about the major IL-12 producer cells in vivo, it is possible that the defect in IL-12 production is not generalized and that IL-12, at least at early stages of the disease, is still produced at sufficient levels from other tissues. Alternatively, a very early infection-associated deficiency in IL-12 production may not result in an observable pathologic deficiency in the immune response until the decrease in the number of CD4+ cells and of other immune parameters in the symptomatic stages of the disease makes optimal levels of this cytokine necessary for efficient resistance to infection or neoplastic growth. Several previous findings in all or most HIV-infected patients are compatible with a defect in IL-12 production, in particular, early deficiencies in T cell proliferative responses to antigens (18) and in production of IFN-γ (19, 20), defective NK cell-mediated cytotoxic activity (21), and B cell activation with hyper-IgE production (1, 2).

It has been proposed that HIV-infected patients have an in vitro imbalance of the Th1/Th2 responses, with severely depressed Th1 response and enhanced Th2 response (16, 17, 22, 23). Whereas these latter studies described an in vitro...
Th1/Th2-like dysregulation using PBMC or T cell clones from HIV-infected patients, this phenomenon does not appear to occur in lymphoid tissue from patients (24). Several factors, from in vivo anatomic locations to in vitro culture conditions, can influence the pattern of cytokine expression, and might explain these disparate results.

Our recent findings indicate that the major effect of IL-12 on Th1 cell generation is exerted through the induction of a stable priming of IFN-γ for efficient production from Th1 or Th0 cells (producing both IFN-γ and IL-4) (25). In the absence of IL-12, low production of IFN-γ might mask the detection of an efficient Th1 response, whereas a Th0 response would be characterized by low IFN-γ and high IL-4 production, mimicking the phenotype of a Th2-type response (17, 19).

In experimental animals, IL-12 has been shown to have a protective effect in vivo against infection with Toxoplasma gondii, an opportunistic parasite in some AIDS patients (9). A curative effect of IL-12 in vivo has also been observed in susceptible BALB/c mice infected with Leishmania major (10). Because the susceptibility of BALB/c mice to this parasite is due to a predominant Th2 response rather than the Th1 response observed in resistant strains, Leishmania infection has been proposed as a possible model for immune responses in AIDS patients, in which a predominant Th2 response has been postulated (22). It is also of interest that IL-12 acts as a potent adjuvant in vaccination with L. major soluble Ags in BALB/c mice, inducing a Th1 memory immune response and persistent resistance to infection (26). Thus, IL-12 is a potential adjuvant in vaccination for those infectious organisms, including possibly HIV, against which a potent cellular immunity in parallel with a humoral response may provide complete protection. Even though IL-12 has no direct antiviral effect, this cytokine might be considered a potential therapeutic agent in HIV-infected patients, to correct immune defects (5, 15), and to augment resistance mechanisms against opportunistic infections and tumor growth.

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