Mechanisms of Impaired Anticryptococcal Activity of Monocytes from Donors Infected with Human Immunodeficiency Virus

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The mechanisms by which monocytes from patients infected with human immunodeficiency virus (HIV) have reduced growth inhibitory activity against Cryptococcus neoformans was examined. Monocyte-enriched peripheral blood mononuclear cells from 12 HIV-seropositive donors with CD4 cell counts of 10–210 cells/mm³ (median, 85) and HIV-seronegative donors were compared in assays to determine the binding and phagocytosis of C. neoformans and the respiratory burst and degranulation in response to C. neoformans and zymosan. Monocytes from HIV-infected and uninfected persons bound and ingested C. neoformans equally well; however, generation of hydrogen peroxide and specific release of β-glucuronidase in response to C. neoformans was significantly reduced in monocyte-enriched cells from the HIV-infected donors. The impaired anticryptococcal activity of monocytes from persons with HIV may be related to defects in both oxidative and nonoxidative effector pathways that occur after the binding and internalization of the organism.

Cryptococcosis remains a frequent and life-threatening infection in patients infected with human immunodeficiency virus (HIV), and, despite advances in therapy, treatment of some patients remains unsatisfactory [1]. It is clear that cell-mediated immunity is critical in host defense against Cryptococcus neoformans, but the specific mechanisms involved and how these are affected by HIV are still incompletely understood.

The anticryptococcal activity of monocytes from healthy donors has been recognized for some time [2]. Both oxidative (in particular the myeloperoxidase–hydrogen peroxide–halide system) and nonoxidative mechanisms may be involved. The activity of monocytes declines with time in culture coincident with the known loss of myeloperoxidase activity. In addition, we have found that sodium azide, an inhibitor of myeloperoxidase, partially inhibits the anticryptococcal activity of freshly isolated monocytes (Tabuni A, Harrison TS, Levitz SM, unpublished observations). More recently, we have demonstrated that in an 18-h assay, monocytes from HIV-infected donors had reduced anticryptococcal activity compared with that for control monocytes [3]. Herein we examine the mechanisms of this deficit, comparing the binding and phagocytosis of C. neoformans by monocytes from HIV-seropositive donors with those of monocytes from HIV-seronegative donors. We determined hydrogen peroxide generation and the release of the lysosomal enzyme β-glucuronidase, as measures of oxidative and nonoxidative effector mechanisms, in response to C. neoformans and to zymosan by monocyte-enriched cells from HIV-seropositive and -seronegative donors.

Materials and Methods

Materials. All reagents were obtained from Sigma Chemical (St. Louis), except as otherwise noted. PBS was supplemented with 1 g/L glucose, 100 mg/L CaCl₂, and 100 mg/L MgCl₂. Pooled human serum was obtained by combining serum from ≥10 healthy volunteers under ice-cold conditions and stored at −70°C to preserve complement activity [3].

Blood donors. Peripheral blood was obtained by venipuncture from HIV-seropositive donors and -seronegative healthy volunteers. HIV-seropositive donors were recruited from the immunodeficiency clinics at Boston Medical Center. HIV-seropositive donors with relatively low CD4 cell counts were studied because it is these patients who are susceptible to cryptococcosis and other opportunistic infections. The 12 HIV-infected donors had CD4 cell counts ranging from 10 to 210 cells/mm³ (median, 85), and none had an acute intercurrent illness or a history of cryptococcosis. At the time of the study, 10 were taking reverse transcriptase inhibitors, but none had begun taking protease inhibitors. Healthy volunteers were staff and students at Boston University Medical Center.

Isolation of monocyte-enriched cells. All experiments comparing monocytes from HIV-seropositive and -seronegative donors were conducted side by side. Thus, blood was obtained from HIV-seropositive and -seronegative donors at approximately the same time, and all subsequent steps were performed in parallel. Blood was anticoagulated with heparin and centrifuged at 500 g for 15 min. Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation of the leukocyte-rich buffy coat cells on a ficoll-hypaque density gradient. PBMC were enriched for monocytes by depletion of T cells by rosetting with neuraminidase-treated sheep red blood cells [3]. This step also approximately equalized the proportion of monocytes in cells from HIV-seropositive and -sero-
negative donors. In previous studies using these conditions, monocyte-enriched cells from HIV-seropositive and -seronegative donors contained 51% ± 4% and 47% ± 3% cells, respectively, that stained for the monocyte marker CD14 or CD13 (or both) [3]. The remainder of the cells were B and NK lymphocytes with <3% T cells.

*C. neoformans*. Serotype A strain 145 was grown in RPMI 1640 without bicarbonate (pH 6.0) at 37°C in air not supplemented with CO₂. Under such conditions, capsule thickness averages 1.2 μm. Fungi were harvested after 4 days growth, heat-killed at 60°C for 30 min, washed 6 times in PBS, and stored at 4°C until use. For assays of hydrogen peroxide generation and lysosomal enzyme release, *C. neoformans* and zymosan particles were pre-opsonized by being incubated in pooled human serum for 30 min at 37°C and then washed in supplemented PBS prior to use. In preliminary experiments, we found that hydrogen peroxide generation was not significantly different when live versus heat-killed organisms were used.

**Binding and phagocytosis.** Binding and phagocytosis assays were done as previously described [4]. Monocyte-enriched cells (5 × 10⁷) in 100 μL of supplemented PBS containing 10% pooled human serum were incubated for 1 h at 37°C with 2.5 × 10⁵ fluorescent isothiocyanate–labeled *C. neoformans*. Wells were washed with PBS to remove nonadherent cells and unbound fungi, and the cells were fixed with 1% formaldehyde. By use of an inverted microscope, 100–200 monocytes were randomly selected using a bright field and then examined using epifuorescence to identify yeast cells. Results are expressed both as the percent of monocytes with ≥1 associated yeast and as the binding index, which is defined as the number of cell-associated yeasts per 100 monocytes. To distinguish attached from internalized organisms, the Uvitex assay was performed as described [4]. In brief, monocytes were incubated with rhodamine isothiocyanate–labeled *C. neoformans*. After fixation, cells were incubated with 0.1% Uvitex (Fungiaqual A; Specialty Chemicals for Medical Diagnostics, Kandern, Germany) for 1 min and then washed with PBS. At least 100 cell-associated yeasts were identified and then examined with excitation to determine which yeasts stained. Organisms that stain with Uvitex under these conditions are bound but not internalized, whereas those that do not stain are fully internalized. Results are expressed as the percent of cell-associated yeasts internalized. In all binding and phagocytosis experiments, researchers who read the wells were blind as to the HIV status of the donor.

**Hydrogen peroxide generation.** Hydrogen peroxide generation was measured by the hydrogen peroxide–dependent, horseradish peroxidase–mediated oxidation of homovanillic acid to the fluorescent dimer 2,2′-dihydroxy-3,3′-dimethoxydiphenyl-5,5′-diametic acid [5]. Monocyte-enriched cells (10⁶) in 1 mL of supplemented PBS were left unstimulated or stimulated for 2 h at 37°C in suspension with 10 × 10⁵ pre-opsonized *C. neoformans* or zymosan particles in the presence of horseradish peroxidase and homovanillic acid. The fluorescence of the supernatants was compared with a series of standard dilutions and results were expressed as nanomoles of hydrogen peroxide generated per 10⁶ cells.

**Lysosomal enzyme release.** β-glucuronidase activity was assayed by use of the β-glucuronidase–dependent cleavage of 4-methylumbelliferyl-β-D-glucuronic acid to form fluorescent 4-methylumbelliferyl [6]. In 96-well plates, 2 × 10⁵ monocyte-enriched cells in 100 μL of supplemented PBS were left unstimulated or stimulated for 2 h at 37°C with 2 × 10⁶ pre-opsonized *C. neo-

<table>
<thead>
<tr>
<th>Donor</th>
<th>% monocytes binding ≥1 yeast</th>
<th>Binding index (bound yeasts/100 monocytes)</th>
<th>% bound yeasts internalized</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-seronegative</td>
<td>78 ± 2</td>
<td>140 ± 10</td>
<td>43 ± 3</td>
</tr>
<tr>
<td>HIV-seropositive</td>
<td>80 ± 2</td>
<td>152 ± 12</td>
<td>41 ± 3</td>
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**Table 1. Binding and phagocytosis of Cryptococcus neoformans by monocytes from HIV-seropositive and HIV-seronegative donors.**

**Results**

**Binding and phagocytosis of *C. neoformans* by monocytes from HIV-seropositive and -seronegative donors.** In initial experiments, we examined whether monocytes from HIV-seropositive donors had any defect in the binding and internalization of *C. neoformans*. Binding, whether measured by the percent of cells with associated yeasts or the binding index, was similar with cells from HIV-seropositive and -seronegative donors (table 1). Similar results were obtained when the assays were done in suspension (data not shown). Furthermore, using the Uvitex assay to distinguish between internalized organisms and those that are attached to the surface of the cell, we also found no difference in the percent of bound organisms internalized by cells from HIV-seropositive and -seronegative donors (table 1).

**Generation of hydrogen peroxide in response to *C. neoformans* and zymosan by monocyte-enriched cells from HIV-seropositive and -seronegative donors.** Oxidative mechanisms have been implicated in the killing of *C. neoformans* by human neutrophils, and *C. neoformans* has been shown to be killed in vitro by concentrations of hydrogen peroxide thought to be attainable within phagocyte phagolysosomes [2]. Therefore, we next examined whether monocytes from HIV-seropositive patients were defective in their ability to generate a respiratory burst, as measured by hydrogen peroxide release, in response to *C. neoformans*. Monocyte-enriched cells from HIV-seropositive donors generated 10-fold less hydrogen peroxide in response to *C. neoformans* than did cells from HIV-
HIV-seropositive and -seronegative donors. Degranulation of low CD4 cell counts because it is these individuals who are additive (data not shown). Intercurrent illnesses and use of medications. Despite this heterogeneity, the results obtained were consistent and could not be linked to any one of these factors. Some previous studies have found an impairment of the respiratory burst in monocytes from HIV-infected patients [7] and by monocytes from normal donors infected in vitro with HIV [8]. The defects in respiratory burst and degranulation in response to C. neoformans seen in monocytes from HIV-infected donors cannot be explained by defects in recognition and internalization of the organism.

We cannot completely exclude the possibility that the impairment of the respiratory burst was related to some factor (other than HIV) associated with the HIV-infected subjects. However, the subjects studied were diverse in terms of risk factors for the acquisition of HIV, previous substance abuse, and use of medications. Despite this heterogeneity, the results obtained were consistent and could not be linked to any one of these factors. Some previous studies have found an impairment of the respiratory burst in monocytes from HIV-infected donors, while others have not (reviewed in [9, 10]). Differences in assay methodologies may explain some of these discrepancies.

In addition, impairment of the respiratory burst may be specific to particular stimuli or patient populations. Prior studies have used a variety of stimuli, and some earlier reports appear to have included HIV-infected inpatients during acute opportunistic infections, which may have activated monocyte functions and thereby obscured a deficit in baseline function in cells from HIV-infected donors. We examined patients without any intercurrent illnesses and used C. neoformans, a stimulus of particular relevance to HIV disease. We studied patients with low CD4 cell counts because it is these individuals who are susceptible to cryptococcosis, and most prior studies have

**Discussion**

The results demonstrate that monocytes from HIV-infected patients with low CD4 cell counts bind and internalize C. neoformans as well as cells from uninfected healthy donors. However, in response to C. neoformans, monocyte-enriched cells from HIV-infected donors generate significantly less hydrogen peroxide than do cells from uninfected donors, and their release of the lysosomal enzyme β-glucuronidase is impaired compared with that by cells from donors without HIV.

Our results demonstrating intact binding and phagocytosis of C. neoformans by monocytes from HIV-infected patients are in agreement with previous studies of the phagocytosis of C. neoformans by monocytes from AIDS patients [7] and by monocytes from normal donors infected in vitro with HIV [8]. The defects in respiratory burst and degranulation in response to C. neoformans seen in monocytes from HIV-infected donors cannot be explained by defects in recognition and internalization of the organism.

Lysosomal enzyme release by monocyte-enriched cells from HIV-seropositive and -seronegative donors. Degranulation of lysosomal enzymes is critical for both oxidative and nonoxida-
found more marked deficits in monocyte function in late- than in early-stage HIV disease [9, 10].

In future studies, we hope to examine monocyte function before and after the initiation of protease inhibitor therapy in order to examine the relationship of the observed defects to HIV virus load. To our knowledge, no prior studies have examined lysosomal enzyme release in monocytes from HIV-infected donors. However, phagolysosomal fusion has been reported to be impaired in monocytes from HIV-infected donors, especially those with low CD4 cell counts [11].

The impaired responses of monocytes from HIV-infected persons to *C. neoformans* could be due to direct effects of the virus or viral products on monocyte function. In vitro infection with HIV of monocytes from normal donors caused an impairment of anticryptococcal activity [8]. Some evidence also suggests that the HIV envelope glycoprotein gp120, which is found in the serum of HIV-infected patients, may impair the respiratory burst [12] and phagolysosomal fusion [11]. Alternatively, monocyte dysfunction in HIV infection may result from dysregulation of macrophage-activating and -deactivating cytokines. Alterations of the cytokine milieu have been associated with changes in the surface phenotype of monocytes from AIDS patients [13]. Production of interferon-γ (IFN-γ) in response to specific stimuli, including *C. neoformans* [14], is reduced in PBMC from HIV-infected donors. IFN-γ has been shown to restore the deficient antimicrobial activity of monocytes from HIV-infected patients against *Toxoplasma gondii* [15].

Thus, our data demonstrate that the impaired anticryptococcal activity of monocytes from HIV-infected patients may be related to defects in both oxidative and nonoxidative effector pathways that occur after the binding and internalization of the organism. This impaired activity could contribute to the extraordinarily high prevalence of cryptococcosis in AIDS.

Acknowledgments

We thank the patients of Boston Medical Center for their cooperation, Colleen Labelle for helping to identify potential donors, and Abdul Tabuni for helping with the binding and phagocytosis assays.

References