

Human Immunodeficiency Virus Type 1 Infection of Alveolar Macrophages Impairs Their Innate Fungicidal Activity

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Impaired adaptive immunity is the hallmark of AIDS, but the effects of human immunodeficiency virus type 1 (HIV-1) infection on innate immunity are less clear. *Cryptococcus neoformans* (CN) is a common AIDS-related fungal pathogen acquired by inhalation. Alveolar macrophages (AM ϕ) comprise the initial host defense in cryptococcosis and they may arrest infection before dissemination occurs. We hypothesized that HIV-1 infection of AM ϕ impairs their anti-cryptococcal activity. This was tested by infection of normal AM ϕ with the M-tropic strain HIV-1_{Bal}. Two weeks postinfection we measured fungistatic activity against CN by colony counting, binding, and internalization of CN by confocal microscopy and AM ϕ cell viability by Alamar Blue assay. Uninfected AM ϕ from most donors demonstrated innate fungicidal activity against CN. In HIV-1-infected AM ϕ , there was a significant reduction, and in most cases loss, of fungicidal activity compared with the uninfected AM ϕ . The reduced antifungal activity was not due to any cytotoxic effect of HIV-1, and HIV-1 infection did not impair binding or internalization of yeast by AM ϕ . Thus, the innate fungicidal activity of primary human AM ϕ is impaired after HIV-1 infection *in vitro* by a mechanism involving a defect of intracellular antimicrobial processing.

In the lung, the alveolar macrophage (AM ϕ) is a key mediator of innate immunity and plays a central role in cell-mediated adaptive immunity. In contrast to the well studied deleterious effects on T cell numbers and function, the impact of acquired immunodeficiency syndrome (AIDS) on innate immunity mediated by monocytes/macrophages is unclear. A variety of monocyte/macrophage functional perturbations in human immunodeficiency virus type 1 (HIV-1)-infected persons have been reported, including augmented cytokine release (e.g., interleukin 1 [IL-1], IL-6, and tumor necrosis factor [TNF]) (1, 2) and impaired phagocytosis of certain AIDS copathogens including *Pneumocystis carinii* (3). Analysis of AM ϕ function in the setting of AIDS is complicated by a plethora of uncontrolled clinical variables in addition to the well-recognized donor variability of many AM ϕ responses measured *in vitro* or *in vivo*. For that reason, *in vitro* HIV-1 infection of AM ϕ from healthy donors has been employed to investigate mechanisms of viral pathogenesis. Given the relatively low level of HIV-1 infection and expression in AM ϕ from HIV-1-infected persons without clinical lung disease (4), the physiological rele-

vance of *in vitro* studies has been questioned. However, studies by Koziel and coworkers (5), and others (6–8), suggest that pulmonary viral load and the proportion of AM ϕ infected with HIV-1 may be greatly increased during coinfection with a variety of bacterial and fungal pathogens.

Cryptococcus neoformans (CN) is one of the most common causes of fatal fungal infection in AIDS (9). Cryptococcosis is acquired by inhalation, and AM ϕ are the initial effector cells of host defense. The yeast bind to several different receptor types on AM ϕ , and phagocytosis may be followed by growth suppression and killing of internalized yeast even in the absence of T lymphocytes (10, 11). Cryptococcosis is therefore a relevant infection for the investigation of HIV-1-mediated macrophage dysfunction, and one that may reflect AM ϕ mechanisms directed at a variety of other pathogens. We investigated the antifungal performance of AM ϕ infected with HIV-1_{Bal} challenged with CN, and found evidence of a virus-mediated impairment of antimicrobial activity.

METHODS

Reagents

Culture medium consisted of RPMI 1640 with penicillin (50 U/ml), streptomycin (50 μ g/ml) (BioWhittaker, Walkersville, MD), and 10% fetal bovine serum (FBS; GIBCO, Grand Island, NY). Pooled human serum (PHS) was obtained by combining under ice-cold conditions serum from 10 healthy donors, and storing it at -70° C to preserve complement activity.

Bronchoalveolar Lavage Cells

Bronchoalveolar lavage (BAL) of nonsmoking healthy volunteers was performed by a standard protocol (12) approved by Institutional Review Board of Boston Medical Center. After informed written consent was obtained, 240 ml of sterile saline was instilled in 60-ml aliquots through a bronchoscope and recovered by gentle aspiration. BAL fluid was strained through a single layer of gauze, then centrifuged for 12 min at $300 \times g$. BAL cells (BALCs) were washed once in RPMI 1640. Differential counts were made on Diff-Quik- and non-specific esterase (Sigma, St. Louis, MO)-stained cell preparations. AM ϕ were isolated by adherence. BALCs (5×10^5) were plated in 24-well cell culture plates (Corning, Corning, NY) in a final volume of 2 ml and incubated for 24 h at 37° C in humidified air supplemented with 5% CO₂. Nonadherent cells were then removed by vigorous washing and the remaining adherent cells were, on average, > 98% viable (trypan blue dye exclusion), > 95% esterase positive, and > 95% phagocytic (latex beads).

HIV-1 Infections

M-tropic HIV-1_{Bal} was obtained from G. Viglianti (Boston University School of Medicine, Boston, MA) as cell-free supernatant of infected human peripheral blood mononuclear cell cultures. AM ϕ were inoculated with 3 ng of p24 antigen/ 10^5 cells. Control AM ϕ received only medium. Cells were placed on a rocker and incubated for 4 h at 37° C in humidified air supplemented with 5% CO₂. The cells were then washed and cultured in fresh medium. Unless otherwise specified, all *in vitro* infections were conducted for 2 wk. Every 3 d, media were aspirated, filtered, and stored at -70° C for later analysis.

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HIV-1 p24 Antigen Detection

Two techniques were employed to measure p24 antigen. To confirm HIV-1 infection of AM ϕ , measurement of p24 antigen in culture supernatant was performed by enzyme-linked immunosorbent assay (ELISA) (Beckman-Coulter, Brea, CA) according to the manufacturer instructions. Results are expressed as picograms of antigen per milliliter of supernatant. To determine the fraction of HIV-1-infected AM ϕ in culture, intracellular p24 was measured by flow cytometry. After aspirating the supernatant, HIV-1-infected AM ϕ and uninfected controls were detached from culture wells by vigorous pipetting with 1 ml of phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA) and scraping with the pipette tip. The cells were pelleted, resuspended in 100 μ l of Fixation Medium A (Caltag, Burlingame, CA), incubated at room temperature for 15 min, and washed. Cells were permeabilized in 100 μ l of Permeabilization Medium B (Caltag), blocked with murine IgG₁ (Beckman-Coulter) for 15 min, and stained with KC57-phycoerythrin (PE) antibody (20 μ g/ml, Beckman-Coulter), which recognizes HIV-1 core antigens, or with PE-conjugated isotype control antibody (20 μ g/ml; Beckman-Coulter) for 15 min (13). The monoclonal KC57-PE antibody is specific for the intracytoplasmic staining of HIV-infected cells as confirmed by correlate reverse transcriptase activity (14). Cells were washed, resuspended, and fixed in 1% formalin (Fisher Scientific, Fair Lawn, NJ). Stained AM ϕ were analyzed on a Becton Dickinson (Mountain View, CA) FACScan. Live gating on AM ϕ by forward-versus-side scatter characteristics was used to collect 10,000 events. Collected data were analyzed by CellQuest software (Becton Dickinson), generating histograms for the FL-2 channel. Isotype controls for each specimen were used to determine the 95% confidence interval of nonspecific fluorescence.

Cryptococcus neoformans

Serotype A strain 145 was maintained by serial passages on Sabouraud dextrose plates (Remel, Lenexa, KS) at 30°C. For the antifungal assay, a single colony was harvested from Day 5 cultures and resuspended in 1.0 ml of RPMI 1640. A yeast suspension was obtained by vortexing for 30 s and repeated aspirations through a 25-gauge needle. The fungi were then counted on a hemacytometer and diluted to a final concentration of 1.6×10^4 fungal cells per milliliter of RPMI 1640 with 10% PHS.

Antifungal Assay

Antifungal activities were measured on selected days in cultures of uninfected and HIV-1-infected AM ϕ as previously described (15, 16). Briefly, culture medium was replaced with RPMI 1640 plus 10% PHS. AM ϕ were infected with CN 145 (1:50 yeast:macrophage) and incubated at 37°C in humidified air supplemented with 5% CO₂. After 18 h cell cultures were lysed with 0.1% Triton X-100 (Fisher Scientific), diluted 1:20 in sterile water with chloramphenicol (50 mg/ml; Sigma), vortexed, and spread on Sabouraud dextrose agar plates. Plates were incubated for 3 d at room temperature and colonies were counted. Each condition was incubated in duplicate and subsequently plated in duplicate. For each experiment, two sets of cell wells containing *C. neoformans*, medium, and PHS, but no effector cells, were included. One set was incubated at 4°C, thereby inhibiting CN growth and representing the inoculum size; the second set was incubated at 37°C to determine unrestricted fungal growth. Both were processed and plated, and colony-forming units determined, as described for the cell cultures. The results of each experiment are expressed as percent growth according to the following formula: $[(CFU_{\text{experimental group}} / CFU_{4^\circ C}) - 1] \times 100$. Thus, a value of 0 indicates that the number of colony-forming units at the conclusion of incubation was the same as that at the start and that no net fungal growth occurred. Positive values denote fungal growth, with values of 100 and 300% indicating averages of one and two replications per fungal cell, respectively. Negative values mean a decrement in colony-forming units occurred during the course of incubation; therefore, fungal killing took place. It must be recognized, however, that during the incubation of fungi with effector cells, some fungi may be killed while others replicate. Therefore, fungal killing could still take place even if a positive value for percent growth is obtained.

Binding and Internalization Assays

BALCs (5×10^5) were placed in each well of a two-well Lab-Tek chamber slide (Nunc, Naperville, IL) in serum-free culture medium for 24 h and

then infected *in vitro* with HIV-1 or left uninfected as described earlier. Chamber slides were then incubated for 14 d (37°C, 5% CO₂) with periodic aspiration of supernatants as described. Rhodamine B isothiocyanate (RITC; Sigma)-labeled heat-inactivated CN prepared as described (15) was then added to each well at a macrophage:yeast ratio of 1:10 in 1.0 ml of medium with PHS. Cultures were incubated for 30 min for the binding assay or for 1 h for the internalization assay. After incubation, AM ϕ were washed three times in PBS to remove unbound fungi. The AM ϕ were then fixed, permeabilized, and stained with fluorescent phalloidin (Molecular Probes, Eugene, OR) for 30 min. Confocal laser scanning microscopy (Leica, Deerfield, IL) was utilized to count bound and internalized yeast. Binding was measured by counting the number of yeast adhering to 200 macrophages, excluding internalized yeast. Adherent yeast are discriminated from internalized yeast by examining incremental 1- μ m optical sections through each macrophage. The binding index was calculated as the number of bound yeast divided by the total number of macrophages counted. To measure internalization, 100 macrophages and any associated intracellular yeast were counted by examining incremental 1- μ m optical sections through each macrophage by confocal microscopy. The internalization index was calculated as the number of internalized yeast divided by the total number of macrophages counted.

Macrophage Viability Assay

The viability of HIV-1-infected and uninfected AM ϕ was measured on Day 14 by colorimetric Alamar Blue assay. Alamar Blue dye undergoes a colorimetric change from blue to red when exposed to viable cells (17). Uninfected and HIV-infected cell cultures were exposed for 4 h to Alamar Blue dye. Absorbance at wavelength 570 and 600 nm was determined with an optical density colorimeter plate reader (Molecular Devices, Menlo Park, CA). Specific absorbance was determined by subtracting the latter value from the former.

Statistical Analysis

Results of experiments comparing uninfected and HIV-1-infected AM ϕ were analyzed by paired, Wilcoxon signed rank test, using Graphpad (San Diego, CA) InStat statistical software.

RESULTS

Infection of Human Alveolar Macrophages with HIV-1_{Bal} *In Vitro*

To investigate the effect of HIV-1 on the fungistatic capacity of human macrophages, AM ϕ were infected with HIV-1_{Bal} as described in METHODS. The extent of virus replication in these cultures was determined by measurement of p24 antigen in culture supernatant on Day 14. The level of p24 antigen in supernatant of Day 14-infected AM ϕ ranged from 1.0×10^4 to 9.4×10^4 pg/ml (mean \pm SD, $4.4 \times 10^4 \pm 1.6 \times 10^4$ pg/ml). No

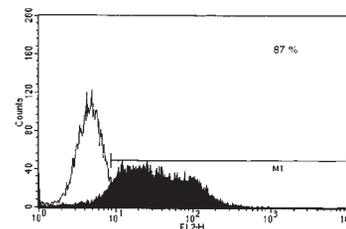


Figure 1. Detection of HIV-1-infected AM ϕ by flow cytometry. Alveolar macrophages of healthy donors were infected with HIV-1_{Bal} *in vitro* and cultured for 14 d. Cells were then detached, permeabilized, and stained with PE-conjugated KC57 antibody, which recognizes HIV-1 core antigens, or with PE-conjugated isotype control. Stained cells were analyzed by flow cytometry. Live gating on AM ϕ by forward-versus-side scatter characteristics was used to collect 10,000 events. A representative histogram of PE, or FL2 fluorescence, is presented here, where 87% of the AM ϕ were found to be p24 positive. M1 defines that which is beyond the 95% confidence interval for nonspecific fluorescence. Six donors were included in our analysis, with $79 \pm 15\%$ p24-positive cells (mean \pm SD) observed.

p24 antigen was detected in any of the uninfected AM ϕ cultures. The proportion of HIV-1-infected AM ϕ from six donors was determined by flow cytometry after permeabilization and staining for intracellular p24 (Figure 1). At 14 d postinfection, the majority of AM ϕ were p24 antigen positive (mean percent p24-positive cells \pm SD, $79 \pm 15\%$).

Effect of HIV-1 Infection on Alveolar Macrophage Fungistatic Activity

To measure fungistatic activity, control AM ϕ cultures and HIV-1-infected AM ϕ cultures were challenged with CN on Day 14 postinfection. Each experiment used AM ϕ from a single donor. The AM ϕ from 7 of 10 donors exhibited fungicidal activity against CN as demonstrated by a reduction in colony-forming units below the infecting dose (negative percent CN growth; Figure 2). Infection with HIV-1 reduced fungicidal or fungistatic activity mediated by AM ϕ from all 10 donors, causing a switch from negative percent CN growth to positive percent CN growth in six cases. By combining all experiments, there was a significant decrease of fungistatic activity in the HIV-1-infected AM ϕ when compared with the uninfected AM ϕ (mean percent fungistatic growth \pm SD, $-9 \pm 70\%$ for uninfected cells versus $79 \pm 75\%$ for HIV-1 infected cells; $p = 0.0020$). There was no evident correlation between the peak p24 antigen level and the measured impairment in fungistatic activity (data not shown).

A time-course experiment was conducted to determine the kinetics of impaired fungistatic activity in HIV-1-infected AM ϕ cultures. Antifungal assays were performed on HIV-1-infected and uninfected AM ϕ from a single donor on Days 4, 7, 11, and 13 after HIV-1 infection (Figure 3). On Day 4 there was no difference in percent CN growth in comparing control and HIV-1-infected AM ϕ , with both cultures demonstrating fungicidal activity. Although maximal impairment of antifungal activity was not observed in the HIV-1-infected AM ϕ until Day 13, there was a degree of impairment noted by Day 7, at which time 34% of the AM ϕ exhibited intracytoplasmic p24 by flow cytometry. The development of reduced fungistatic activity correlated with the spread of virus through the culture, but it cannot be determined from these data whether the effect of HIV-1 on AM ϕ function is a direct consequence of infection or if it is caused by a soluble factor released from infected cells that also acts on the uninfected AM ϕ population. While the HIV-1-infected AM ϕ had reduced anticryptococcal

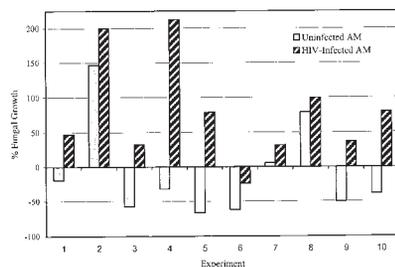


Figure 2. Antifungal activity of HIV-1-infected AM ϕ . Cells were infected with HIV-1_{Bal} and maintained in culture for 14 d. Wells containing HIV-1-infected (hatched bars) or uninfected (solid bars) AM ϕ were then challenged with CN. After 18 h, colony counts in experimental wells were compared with the input number of yeast (4° C). Percent growth was calculated as $[(CFU_{\text{experimental group}}/CFU_{4^\circ C}) - 1] \times 100$. A negative percent growth value represents fungicidal activity. Infection with HIV-1 induced a significant ($p = 0.0020$) reduction in antifungal activity mediated by AM ϕ from all 10 donors, causing a switch from negative percent CN growth to positive percent CN growth in six cases.

activity compared with control AM ϕ , CN growth was still restricted by the HIV-1-infected cells as compared with CN growth in medium alone (data not shown).

The antifungal assay measures the performance of the entire cell culture on the basis of the number of macrophages initially plated. By light microscopy there were no gross cytopathic effects or cell dropout in the HIV-1-infected AM ϕ cultures. Nonetheless, the observed loss of macrophage fungicidal activity could have resulted from cytotoxicity rather than a specific functional derangement induced by virus infection. We therefore examined the viability of uninfected AM ϕ and HIV-1-infected AM ϕ by means of the Alamar Blue assay. No significant difference in AM ϕ viability was observed on comparing uninfected and HIV-1-infected cells (mean optical density [OD] \pm SD, 0.23 ± 0.13 for uninfected cells versus 0.24 ± 0.01 for HIV-1-infected cells; $p = 0.63$). Qualitatively similar results were obtained by trypan blue dye staining (data not shown). Therefore, the effect of HIV-1 to inhibit AM ϕ fungicidal activity is not a consequence of reduced macrophage viability.

Effect of HIV-1 Infection on Alveolar Macrophage Binding and Internalization of *Cryptococcus neoformans*

Our experiments demonstrate that HIV-1 impairs fungicidal activity without reducing AM ϕ viability. This functional derangement could be caused by reduced binding or internalization of yeast. Alternatively, reduced fungicidal activity with intact phagocytosis would suggest a defect of intracellular antimicrobial processing. To examine the first possibility, we challenged control AM ϕ and HIV-1-infected AM ϕ with CN that was labeled with rhodamine B isothiocyanate. Macrophages were incubated with labeled CN for 30 min to measure binding, and for 60 min to measure internalization. After counterstaining with phalloidin, slides were analyzed by confocal microscopy. By examining optical sections through cells, this method permits clear discrimination between extracellular cell-associated CN versus intracellular CN. No significant difference in either binding or internalization was found between HIV-1-infected AM ϕ and uninfected AM ϕ (Table 1).

DISCUSSION

We investigated whether *in vitro* HIV-1 infection could influence the capacity of human AM ϕ to bind, internalize, and kill CN yeast. Our data demonstrate that HIV-1 infection of AM ϕ impairs the normal ability of these cells to kill CN. This effect

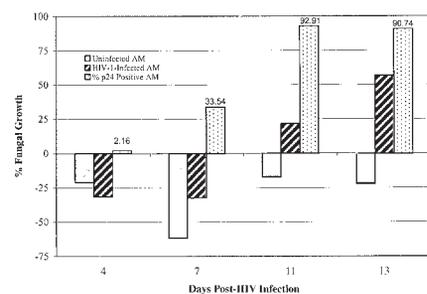


Figure 3. Kinetic analysis of antifungal activity in HIV-1-infected AM ϕ . Antifungal assays were performed on infected (hatched bars) and uninfected (solid bars) AM ϕ from a single donor on Days 4, 7, 11, and 13 after HIV-1 infection. The fraction of p24 antigen-positive cells of parallel cell cultures was determined by flow cytometry after staining with PE-conjugated KC57 antibody, which recognizes HIV-1 core antigens. As the fraction of infected AM ϕ increases with time, a correlate impairment in antifungal activity manifests in the HIV-1-infected AM ϕ as compared with the uninfected population.

TABLE 1

BINDING AND INTERNALIZATION OF *Cryptococcus neoformans* BY HIV-1-INFECTED ALVEOLAR MACROPHAGES*

Condition	Binding Index*†	Internalization Index‡
Uninfected AM ϕ	2.27 \pm 1.14	2.20 \pm 1.16
HIV-1-infected AM ϕ	1.83 \pm 0.64	2.70 \pm 1.29

Definition of abbreviation: AM ϕ = alveolar macrophage.

* Results represent means \pm the standard deviation of six experiments.

† $p = 0.22$ uninfected versus infected AM ϕ .

‡ $p = 0.16$ uninfected versus infected AM ϕ .

does not result from virus-mediated cytotoxicity, or from altered ability of AM ϕ to bind or internalize yeast.

Cameron and coworkers (18) reported that *in vitro* HIV-1 infection inhibits the antifungal activity of human monocyte-derived macrophages and peritoneal macrophages, but not that of AM ϕ . Preservation of AM ϕ function in that study may have been due to lower rates of viral replication in AM ϕ than in other macrophage types. The antifungal assay measures the combined performance of all AM ϕ in culture. To observe a change in this parameter, a large proportion of the AM ϕ must be affected. Both studies employed the same M-tropic HIV-1 isolate, but the spread of infection and the level of viral replication may have differed significantly. A high proportion of AM ϕ were infected in the current study, whereas Cameron and coworkers did not directly measure the proportion of infected cells.

Another variable that may have compromised detection of significant differences in the HIV-1 infected AM ϕ in the study by Cameron and coworkers concerns the level of complement in human serum; complement is an important CN opsonin necessary for the fungicidal activity of AM ϕ (10). The level of complement activity in the serum used in our study, compared with that used by Cameron and coworkers, may have contributed to the differing results. Finally, we used a virulent cryptococcal strain, whereas Cameron and coworkers used a hypovirulent strain that was deficient in its ability to make capsule under physiologic conditions.

The reduction in fungistatic activity that we observed does not manifest until at least 1 wk after HIV-1 infection. The influence of HIV-1 infection on AM ϕ function correlates with the fraction of infected cells as measured by intracellular p24 staining but is not the result of a cytolytic effect of the virus. While the impairment of antifungal activity was maximal 14 d after virus infection, there was evidence of HIV-1 infection as early as 4 d postinoculation. The delayed kinetics of this pathological effect of HIV-1 may be due to the time required for a critical proportion of AM ϕ to become infected. However, our data do not exclude a mechanism involving some cumulative effect of virus infection on intracellular processes.

Binding and phagocytosis of CN is a prerequisite step in the fungicidal activity of AM ϕ . Reduced phagocytic activity of macrophages for *Pneumocystis carinii* and *Staphylococcus aureus* after *in vitro* HIV-1 infection, or of macrophages of HIV-1-infected donors with and without pneumonia, has been reported by others (19, 20). Denis and Ghadirian reported that phagocytic activity for *Mycobacterium avium* of AM ϕ from HIV-1-seropositive donors was no different from that of control subjects (21). We previously reported that treatment of AM ϕ with HIV-1 envelope protein gp120 inhibited antifungal activity and reduced phagocytosis, but not surface binding of CN (11). To evaluate this function we used confocal microscopy to examine optical sections through HIV-1-infected AM ϕ and uninfected control AM ϕ incubated with RITC-conjugated CN. We found no difference between these conditions for either binding or internalization of yeast, indicating

that productive HIV-1 infection affects macrophage antifungal activity at a postphagocytic step.

The effects of HIV-1 infection on CD4⁺ T cell survival and adaptive immunity are well recognized. Our data presented here suggest that a parallel mechanism to incapacitate innate immune function may also be operating in the lung. Our data also support an intracellular viral effect, although what the mechanism of impairment is remains unclear. Important AM ϕ responses involved in the handling of CN include phagosome-lysosome fusion, generation of reactive oxygen and nitrogen intermediates, and production of cytokines (22–25). Investigation of these functions in AM ϕ infected with HIV-1 *in vitro* may reveal the mechanism(s) of reduced antifungal activity and identify potential sites for therapeutic interventions in AIDS-related lung disease.

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