Human immunodeficiency virus causes mononuclear phagocyte dysfunction

(AIDS/3′-azido-3′-deoxythymidine/macrophage/cytotoxicity)

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ABSTRACT There is compelling clinical evidence for dysfunction of the mononuclear phagocyte system in patients with AIDS, which is believed due in part to loss of T-cell cooperativity. The direct consequences of human immunodeficiency virus infection on macrophage function are unknown. To address this question we infected normal human macrophages *in vitro* with a monocytotropic strain of human immunodeficiency virus and performed assays to quantify their extra- and intracellular killing ability. Human immunodeficiency virus-infected macrophages were significantly less effective than control cells in mediating antibody-dependent cell-mediated cytotoxicity against leukemic cell targets and intracellular killing of *Candida pseudotropicalis*. The functional defects were profound, related temporarily to active virus production by the macrophages, and could not be overcome by granulocyte–macrophage colony-stimulating factor. Treatment of macrophages with 3′-azido-3′-deoxythymidine (AZT) 6 days after infection caused a marked decrease in virus production and prevented development of the intracellular killing functional defect. The results suggest that early antiviral therapy may be useful in preventing or mitigating some virus-induced mononuclear phagocyte dysfunction.

The immunopathogenesis of AIDS has been attributed primarily to a selective depletion of T cells with resultant B-lymphocyte and mononuclear phagocyte dysfunction (1–5). Clinically, the pattern of opportunistic infections seen in patients with AIDS relates to impairment of the T-lymphocyte/monocyte axis as well as diminished antibody responses (3–8). Although mononuclear phagocytes may harbor the human immunodeficiency virus (HIV) *in vivo*, the incidence and consequences of infection with monocytotropic HIV strains are uncertain (9, 10). Despite compelling clinical evidence of mononuclear phagocyte dysfunction in AIDS, the direct effects of HIV infection on macrophage function are unknown. To address this question we infected normal human monocytes with HIV *in vitro* and quantified intra- and extracellular killing capacity. Here we show that HIV infection of mononuclear phagocytes causes markedly defective cellular function that may be prevented by treatment with the antiviral agent 3′-azido-3′-deoxythymidine (AZT).

MATERIALS AND METHODS

Cells and Virus. Peripheral blood was obtained from healthy laboratory volunteers and monocytes were isolated by Ficoll/Hypaque separation and adherence to culture dishes in the presence of 5% (vol/vol) human type AB serum. After successive washing procedures cultures contained

greater than 97% pure monocytes. Adhered monocytes were cultured in Iscove's modified Dulbecco's medium (Irvine Scientific) supplemented with 15% (vol/vol) heat-inactivated fetal calf serum, 5% human AB serum, 1% glutamine, and antibiotics at 37°C in a humidified 5% CO_2/95% air incubator.

The monocytotropic HIV-JRFL virus has been described (11). Two million monocytes were infected with 0.5 ml of a filtered cell-free infecting inocula estimated at 50 ng of JRFL viral p24 antigen. Control monocytes were inoculated with either a cell-free supernatant from uninfected CEM cells or a cell-free supernatant isolated from HIV strain HTLV-IIIB-infected CEM cells (estimated at 50 ng of strain HTLV-IIIB viral protein). After a 2-hr virus absorption the cells were washed and cultured. Productive infection of the monocyte-derived macrophages was confirmed by assaying the supernatant of infected monocytes for the release of HIV p24 antigen using an HIV antigen enzyme immunoassay (Abbott).

Macrophage Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) Assays. Uninfected monocytes were cultured *in vitro* in u-bottom microtissue culture plates (no. 3072, Falcon Plastics) with either recombinant granulocyte–macrophage colony-stimulating factor (GM-CSF) or protein diluent (endotoxin-free isotonic phosphate-buffered saline containing 0.01% bovine serum albumin) for periods up to 54 hr. At 2, 6, 24, or 54 hr of culture, monocytes were stimulated with endotoxin (100 ng/ml, final concentration) for 6 hr at 37°C. HL-60 target cells were radioactively labeled by incubating 1.8×10^7 cells in 1.5 ml of assay medium (12) containing 300 μCi of $Na^{51}CrO_4$ (NEN; 1 Ci = 37 GBq) for 1.5 hr at 37°C. Labeled HL-60 target cells were washed once, incubated with fresh assay medium for 30 min at 37°C, washed twice, and incubated with a 1:50 dilution of rabbit anti-HL-60 antisera (a gift from H. P. Koeffler, UCLA School of Medicine) for 1 hr at 37°C. After incubation with antibody, target cells were washed twice and added (in 0.2 ml) to monocytes (0.5–1.0×10^6 cells per well) at an effector to target ratio of 25:1.

Cytotoxicity was determined after a 6-hr incubation (37°C) by measuring the amount of released chromium in 125 μl of supernatant and expressing this value as a percent. Percent lysis = $[(A - B)/C] \times 100\%$, where A is the mean cpm in the supernatant from wells containing target and macrophage effector cells, B is the mean cpm from wells containing targets alone, and C is the total cpm added to each well. Values are the mean and SEM of eight experiments done in triplicate.

Abbreviations: HIV, human immunodeficiency virus; ADCC, antibody-dependent cell-mediated cytotoxicity; AZT, 3′-azido-3′-deoxythymidine; GM-CSF, granulocyte–macrophage colony-stimulating factor.
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For infected macrophage ADCC assays, targets were human T-cell leukemia virus type II-infected B-lymphoblastoid cells (JWIL2D), which were labeled, washed, and preincubated (1 hr, 37°C) with the monoclonal antibody W632B (Pel-Freez Biologicals). W632B is specific for HLA monomorphic determinants and is highly effective in mediating neutrophil ADCC at a dilution of 1:500 (12). Effectors were either JRFL-, HTLV-IIIB-, or mock-infected macrophages, prepared as described above. Viability was confirmed by trypan blue dye exclusion, and experiments were performed with equal numbers of viable uninfected and JRFL-infected macrophages. JRFL-infected macrophages were larger and often multinucleated. Syncytia formation was noted in some of the infected cultures, but viability remained >80%. In the infected macrophage ADCC assays, the effector to target ratio was 25:1 and cytotoxicity was determined after a 12-hr incubation period (37°C) by measuring the amount of released chromium in the supernatant. At the time of each ADCC assay, cell cultures contained >98% monocytes/macrophages and no detectable contaminating lymphocytes. Spontaneous release of ^{51}Cr was 10.4 ± 5.63% of total chromium. Background chromium release was subtracted in the final calculation of percent cytotoxicity.

Intracellular Killing Assays. Peripheral blood monocytes were purified and infected as described. At day 14 after infection, control and JRFL-infected macrophages were removed from tissue culture dishes with EDTA, washed, and resuspended in Hanks' balanced salt solution (HBSS) at 3–5 × 10^6 cells per ml. Viability was determined by trypan blue dye exclusion and equal cell numbers were adjusted accordingly. *Candida pseudotropicalis* (3–5 × 10^5 cells per ml) and macrophages were rocked gently at 37°C. At 0, 30, 90, and 180 min, a 0.075-ml sample was removed from the mixture, sonicated, diluted serially, and plated on Sabouraud agar (Difco). Plates were incubated overnight in a dry incubator (37°C) and colonies were counted the next day.

To assay the effect of AZT on the ability of infected macrophages to kill *C. pseudotropicalis*, monocytes were purified and infected as described. At day 6 after infection when viral production was 620 pg/ml as determined by p24 antigen release, one-half of duplicate cultures of infected macrophages were treated with 10 μM AZT (Sigma). In the AZT-treated macrophages viral production peaked at day 8 and thereafter decreased until day 14 after infection (<100 pg/ml) when AZT-treated cells were harvested and assayed for intracellular killing activity as described. Untreated infected macrophages continued to produce high levels of virus (as quantitated by p24 antigen release, >1 ng of viral protein per ml). Viability of infected macrophages (with or without AZT) was greater than 80%.

RESULTS

GM-CSF Enhancement of Macrophage ADCC. We developed an *in vitro* mononuclear phagocyte ADCC assay system permitting quantitation of effector cell function in mononuclear phagocytes. Peripheral blood monocytes were cultured *in vitro* with and without GM-CSF and exposed to endotoxin for 6 hr before assaying their ability to cause radioactive chromium release from antibody-coated target cells. Exposure to GM-CSF induced a 2- to 3-fold increase in macrophage cytotoxicity above control levels (Fig. 1). Maximal enhancement was seen at concentrations of 150–250 pM GM-CSF, a range comparable to concentrations of GM-CSF that augment neutrophil functions *in vitro* (12). In this system, GM-CSF had no effect on monocyte cytotoxicity by itself but significantly increased endotoxin-induced cytotoxicity. The enhancement in cytotoxicity was dependent upon culturing monocytes in the presence of GM-CSF for at least 24 hr. The supernatants from the stimulated macrophages did not appear to have cytotoxic activity against the target cells.

HIV Infection Compromises Macrophage ADCC Function. The effect of *in vitro* HIV infection on the ability of mononuclear phagocytes to mediate cytotoxicity was examined using as targets JWIL2D, a human T-leukemia virus type II-infected B-lymphoblastoid cell line (13). Peripheral blood monocytes were cultured for 24 hr and then infected with the HIV strain JRFL, a monocytotropic HIV strain, as described in detail (11). Control monocytes were either mock-infected or infected with HTLV-IIIB, which does not productively infect freshly isolated monocytes *in vitro* (14). JRFL-infected macrophages showed that GM-CSF-enhanced cytotoxicity
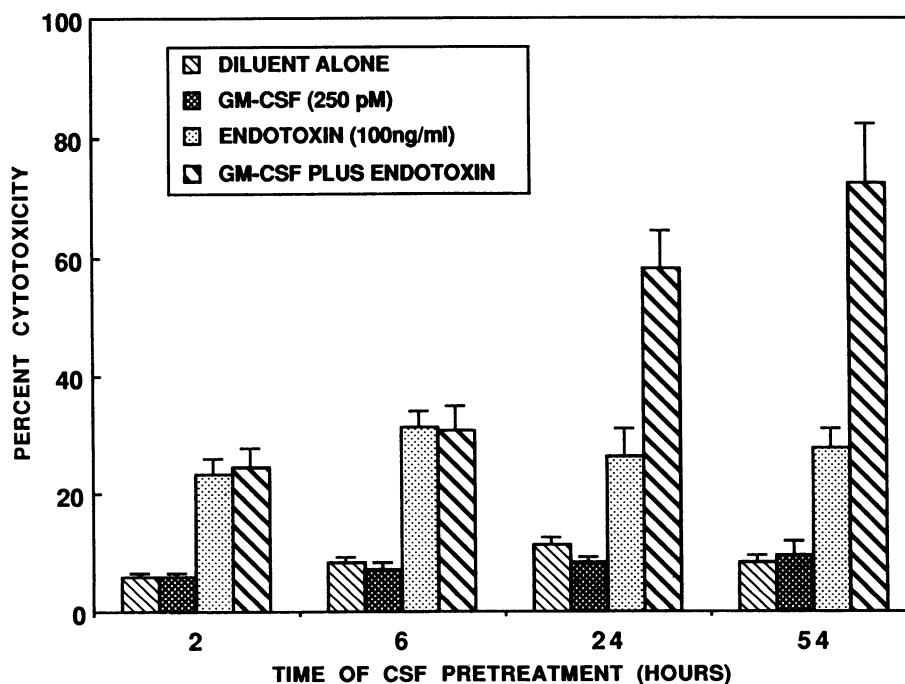

FIG. 1.   GM-CSF enhances endotoxin-stimulated mononuclear phagocyte ADCC.

comparable to control macrophages and did not exhibit functional compromise in ADCC toward JWIL2D target cells when tested 3 days after infection (Fig. 2A). Virus production was undetectable by p24 release at day 3; however, 100,000 mock- and JRFL-infected cells were maintained in culture, and the JRFL-infected macrophages were found to produce virus (400 pg of viral p24 antigen per ml) at 14 days, demonstrating that productive infection had occurred.

At 14 days after infection, normal macrophages productively infected with the HIV strain JRFL were not capable of mediating significant cytotoxicity ($P < 0.001$, Fig. 2B). Both JRFL-infected and control macrophages were >80% viable, as determined by trypan blue dye exclusion. Thus, defective mononuclear phagocyte ADCC was related temporally to active virus production. Although GM-CSF enhanced uninfected macrophage cytotoxicity toward selected targets 2- to 3-fold, addition of GM-CSF to productively infected effector cells did not overcome the defect in cytotoxic function. We

considered the effect of GM-CSF in stimulating viral replication in HIV-infected macrophages (15); however, when assays were performed in the absence of GM-CSF a similar reduction in cytotoxicity was seen. Maximal levels of endotoxin-mediated cytotoxicity for JRFL-infected macrophages were $8.3 \pm 0.72\%$, which is significantly less ($P < 0.001$) than values for control macrophages ($24.67 \pm 2.85\%$). The HIV-induced macrophage dysfunction was not target cell or antibody specific, as similar results were obtained when experiments were performed using B-lymphoblastoid cell targets, JWIL2D, or myeloid leukemic cell targets, HL-60 and KG-1 cells (Table 1). By day 14, levels of cytotoxicity for productively infected JRFL macrophages were 7–10 times lower than the values obtained with control macrophages.

Intracellular Killing Capability of HIV-Infected Macrophages. The defect in macrophage function occasioned by HIV infection was not restricted to extracellular cytotoxicity since JRFL-infected mononuclear phagocytes had diminished capacity for intracellular killing of the fungus *C. pseudotropicalis* (Fig. 3A). At 14 days after infection when HIV p24 antigen release was high (≥ 600 pg/ml), macrophages were harvested and incubated with opsonized *C. pseudotropicalis*. Uninfected cultured macrophages killed *C. pseudotropicalis* actively with an average percent reduction in colony number of 80.01 ± 1.50 over 3 hr. JRFL-infected macrophages were significantly impaired ($P < 0.001$) showing between 50 and 60% less candidacidal activity than uninfected control cells ($47.20 \pm 1.8\%$ reduction in colony number). Macrophages assayed prior to significant viral antigen release (5 days) were as effective in killing *Candida* as uninfected control macrophages (85% reduction in *Candida* colony number at 180 min). Moreover, treatment of JRFL-infected macrophages with AZT inhibited viral production and restored the intracellular killing capability of HIV-infected macrophages to control levels (Fig. 3B).

The defect in macrophage killing was not due to a defect in phagocytosis as both mock-infected and JRFL-infected cultured macrophages (14 days after infection, viral p24 ≥ 600 pg/ml) efficiently phagocytosed *Candida* organisms. In phagocytosis assays done simultaneously, individual macrophages were evaluated as having 0, 1–10, 11–20, 21–30, or >30 organisms associated with them. A weighted phagocytic index (WPI) was calculated by multiplying the number of macrophages in each category by 0, 1, 2, 3, or 4, respectively, and dividing the total score by the number of macrophages examined (usually 100). For uninfected macrophages, the WPI was 0.84 at 30 min; and, for JRFL-infected macrophages, the WPI was 0.81 at 30 min.

GM-CSF generally increased the killing capability of cultured uninfected macrophages, but the degree of enhancement was not statistically significant ($P > 0.1$). GM-CSF also enhanced the killing activity of infected macrophages, and the levels of enhancement approached significance ($P = 0.05$) in some experiments.
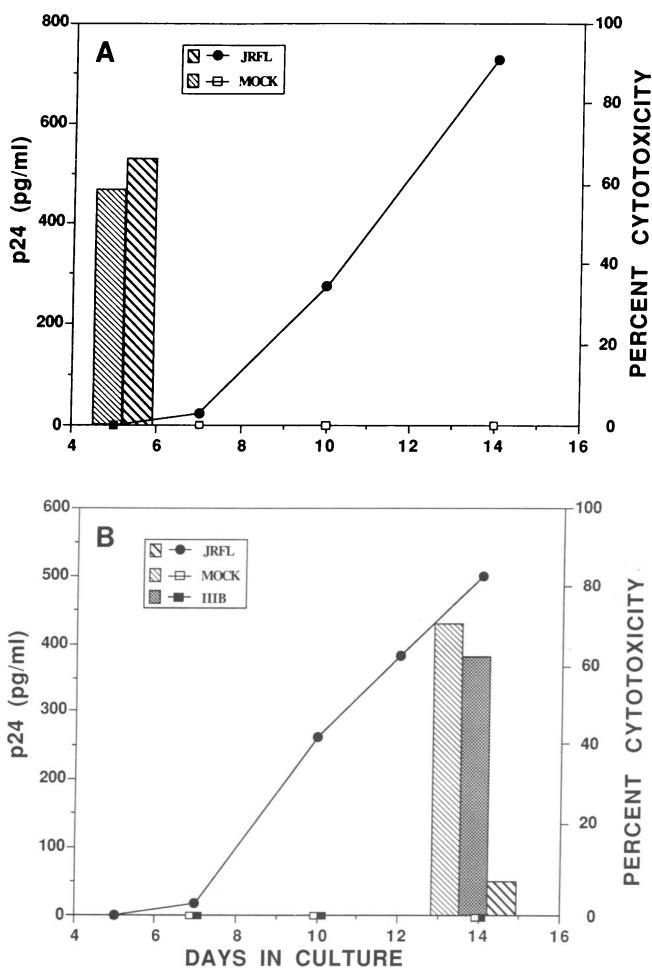
FIG. 2. Effect of productive *in vitro* HIV infection on macrophage ADCC function. (A) Immediately after the 2-hr virus adsorption, equal numbers of viable infected and control monocytes were washed and incubated for 48 hr in the presence of either 250 pM GM-CSF or protein diluent. Basal levels of cytotoxicity in the absence of GM-CSF stimulation were $8.5 \pm 2.7\%$ for JRFL-infected macrophages and $22.4 \pm 5.9\%$ for mock-infected macrophages. (B) At day 12 after exposure to virus, control and JRFL-infected macrophages were stimulated with GM-CSF for 48 hr, and duplicate plates were stimulated with diluent. On day 14 virus production was high (500 pg/ml, as quantitated by p24 antigen release) and 65–80% of the JRFL-infected macrophages expressed viral proteins as detected by immunofluorescence. Viral production was undetectable in controls. Levels of cytotoxicity without GM-CSF stimulation were similar to those described for A.

Table 1. Control and JRFL-infected macrophage ADCC

Effector cells	Target cells	% cytotoxicity
Control macrophages	HL-60	46.50 ± 6.09
	KG-1	58.75 ± 3.77
JRFL-infected macrophages	HL-60	5.52 ± 1.11
	KG-1	7.58 ± 1.49

Actinomycin-D-treated target cells were preincubated with heterologous antisera prior to incubating with control and JRFL-infected cultured macrophages. JRFL-infected macrophages were used 16–18 days after infection. Percent cytotoxicity reflects GM-CSF-enhanced macrophage ADCC. Data are the mean $\pm$ SEM of two experiments done in triplicate.
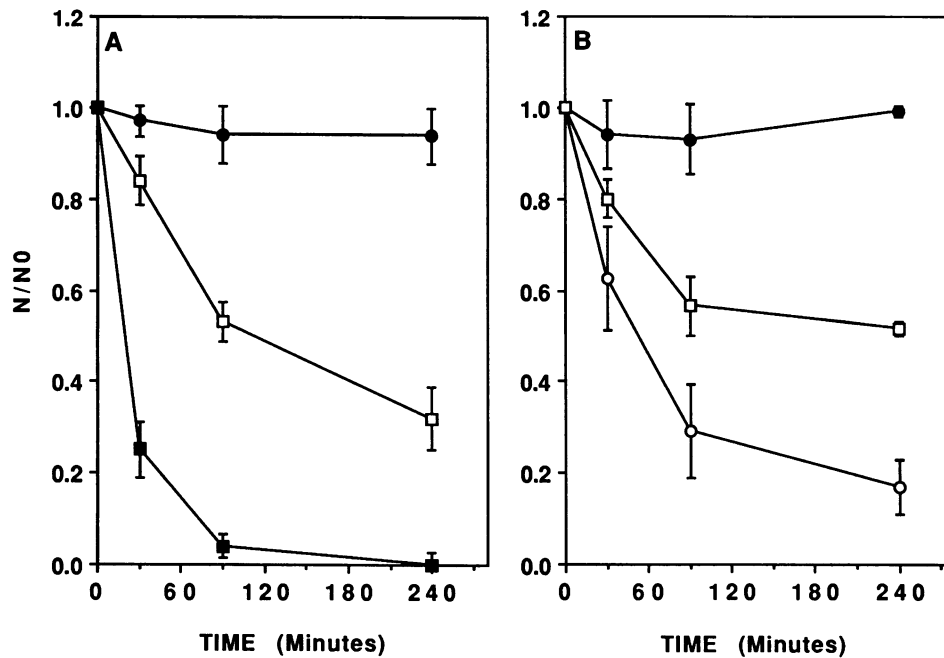
FIG. 3. (A) Intracellular killing of C. pseudotropicalis by HIV-infected macrophages. ●, C. pseudotropicalis alone; □, cultured JRFL-infected cells plus C. pseudotropicalis; ■, cultured, mock-infected cells plus C. pseudotropicalis. (B) Effect of AZT on the intracellular killing of C. pseudotropicalis by HIV-infected macrophages. ●, C. pseudotropicalis alone; □, cultured, JRFL-infected cells plus C. pseudotropicalis; ○, cultured, JRFL-infected and AZT-treated cells plus C. pseudotropicalis. N, number of colonies at each time interval; N_0, number of colonies at time of initial combination of cells and yeast.

DISCUSSION

Despite the clinical evidence for macrophage dysfunction in AIDS patients, in vitro testing of AIDS patients' mononuclear phagocyte function has yielded contradictory results. For example, normal (16) and defective (17, 18) bactericidal capacity has been reported as well as normal (16) and defective (19, 20) chemotactic responses. Also mononuclear phagocytes derived from HIV-infected individuals were reported to have diminished ability to remove ^{51}Cr-labeled opsonized erythrocytes (21), implying a defect in Fc-receptor-mediated clearance. When tested in vitro these cells evidence a normal ability to phagocytose (16–19) and to produce superoxide anion (16, 17, 19). Only a small percentage of mononuclear phagocytes from HIV-infected individuals may be infected, and many, if not most, patients may have no infection of monocytes (10, 22, 23). The variability of monocyte infection in HIV-positive individuals may partially explain the contradictory functional data.

Although GM-CSF enhances the ability of uninfected macrophages to function in ADCC assays, in our studies it did not overcome the defect in cytotoxic activity in infected cells. Likewise, in macrophage intracellular killing assays, cultured HIV-infected macrophages were less capable of killing C. pseudotropicalis than uninfected macrophages, with or without GM-CSF. Both of these functional defects were correlated with a productive HIV infection, as macrophages assayed prior to viral production were not functionally compromised. These results are consistent with a report by Petit et al. (24) indicating HIV infection of U937 cells results in decreased accessory cell functions required for T-cell proliferation. Additionally, we found that administration of AZT to infected macrophages prevented viral replication and p24 release, thereby maintaining functional capability in infected cells.

Our results indicate that productive mononuclear phagocyte infection by monocytotropic HIV leads to both extracellular and intracellular killing defects. Neither are meaningfully overcome by GM-CSF but intracellular killing de-

fects can be prevented by administration of AZT. These findings suggest that HIV infection of mononuclear phagocytes in vivo can cause defective cellular function independent of impairment attributable to a selective depletion of T cells. The data provide a rationale for the early use of antiviral agents to prevent or mitigate mononuclear phagocyte viral dysfunction.

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