Human Immunodeficiency Virus–Induced Cell Death in Cytokine-Treated Macrophages Can Be Prevented by Compounds that Inhibit Late Stages of Viral Replication

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The basis of the cytopathic effect induced by a laboratory strain and several clinical isolates of human immunodeficiency virus (HIV) in human macrophages cultured in the presence of macrophage colony-stimulating factor was studied. Infected macrophages die of necrosis, the consequence of the production of mature virions in infected cells. Cell death can be prevented by antiviral compounds that interfere with the assembly and budding of virions. Programmed cell death (apoptosis), a potential mechanism of HIV-mediated cell death in CD4 T lymphocytes, does not occur in infected macrophages as shown by electron microscopy, cytofluorometric and gel electrophoretic DNA analysis, and nuclear fluorescent staining by Hoechst and terminal dUTP-nick-end-labeling (TUNEL) assay. The data suggest that macrophage killing by HIV may occur in vivo. Thus, combination therapies that include compounds that inhibit the cytopathic effect of HIV in macrophages should be considered for AIDS patients.

Human immunodeficiency virus (HIV), the causative agent of AIDS, infects and replicates in immunocompetent cells bearing the cell surface receptor CD4 (e.g., T lymphocytes and monocytes/macrophages [macrophages]) [1, 2]. Although early studies focused on CD4 T lymphocytes as the principal target cell, there is increasing evidence that infection of macrophages plays a crucial role in the pathogenesis and progression of this disease [3, 4]. Macrophages are a major target for human HIV infection as shown by infection of blood monocytes, macrophages in spleen and lymph nodes, alveolar macrophages of the lung, Langerhans cells of the skin, Kupffer cells of the liver, and macrophage-like cells in the central nervous system [5–12]. More importantly, macrophage infection and neuronal dysfunction in the brain play a crucial role in the pathogenesis of HIV-related dementia complex [13, 14]. In addition, since macrophages may differ from T cells in their susceptibility to the HIV-induced cytopathic effect, it is important to consider this important aspect of macrophage-HIV interaction.

In a preliminary report [15], our group recently showed that human macrophages infected with HIV and cultured in the presence of macrophage colony-stimulating factor (M-CSF; a human cytokine that promotes the growth and differentiation of progenitor cells into macrophages and the survival of macrophages in culture [16, 17]) undergo massive cell death. Because of these observations, we did an in vitro study to analyze the basis of HIV-induced cytopathology.

Materials and Methods

Cells. Peripheral blood from HIV-negative donors was enriched for mononuclear cells by centrifugation over ficoll-hypaque. Macrophages were obtained by adherence of peripheral blood mononuclear cells (PBMC) to plastic as described [18, 19]. Cells obtained by this method are >95% pure as determined by nonspecific esterase activity [18]. Each experiment was done using PBMC from a single donor.

Compounds. M-CSF, purchased from Medical System (Genoa, Italy), contains 2 × 10^6 U/mg of protein. U75875, a synthetic peptidomimetic (Upjohn Laboratories, Kalamazoo, MI), inhibits HIV-1 protease and blocks HIV replication in de novo and chronically infected macrophages [20]. U75875 is for laboratory use only and cannot be administered to humans or food-producing animals or plants. Zidovudine and cystamine were obtained from Sigma (Milan, Italy). OKT4a, a monoclonal antibody directed against the gpl20-linking domain of the CD4 receptor, was purchased from Ortho Pharmaceuticals (Raritan, NJ).

Viruses. A laboratory strain (Ba-I) and 10 clinical HIV isolates were used to infect macrophages. Supernatants of infected macrophages were used as the source of HIV Ba-I and were filtered and stored in liquid nitrogen before use. Five syncytium-inducing (SI) variants (MA107, RA16, PG16, MG8, and R9) and 5 non-SI (NSI) HIV-1 variants (ZB7, CF35, PA42, GA37, and MD31; gift of M. Andreoni, University of Rome “Tor Vergata”) were ob-
tained from HIV-seropositive men. The SI-NSI phenotype was determined by growth in a T cell line (MT-2). The strains were isolated in PBMC cultures, and the culture supernatant was used as the virus source. Titers to determine infectivity of Ba-l and primary isolates were done, respectively, in a primary macrophage system and in PBMC as described [18, 21]. We determined the titers of the virus stocks, expressed as TCID$_{50}$, as described [22].

**Infection.** After purification, macrophages were cultured under different experimental conditions (see below) for 7 days in RPMI 1640 medium supplemented with 1000 U/mL M-CSF, 20% heat-inactivated fetal calf serum, 2 mM L-glutamine, 50 U/mL penicillin, and 50 mg/mL streptomycin (complete medium) at 37°C in a humidified atmosphere (5% CO$_2$ in air) and then exposed to 100 TCID$_{50}$ of the different HIV preparations. Appropriate mock-infected cultures were run as negative controls. Two hours after infection, cells were extensively washed to remove excess virus and grown under the same conditions. Cells were then fed every 4 days.

**Quantification of HIV-induced cytolysis.** Macrophages were seeded in 48-well plates (Costar, Cambridge, MA) at 1.5 × 10$^5$ cells/well in 1 mL of complete medium and infected as described above. At given intervals, macrophages were incubated with 5 μCi of Na$_2^{35}$CrO$_4$/mL for 90 min at 37°C in 5% CO$_2$. The cells were then washed three times in PBS and fed with complete medium. After 4 days, the plates were centrifuged at 200 g for 10 min, and 50 μL of the supernatant was removed and counted in a gamma counter. Cells were washed in PBS and lysed with 100 μL of a solution containing 10 mM TRIS, 1 mM MgCl$_2$, 1 mM CaCl$_2$, and 1% Triton X-100. Cell lysate (50 μL) was removed for radioisotope monitoring. The percentage of cytolysis was determined as

$$\text{HIV production was undetectable in uninfected controls (SI-NSI phenotype was determined in lymphocytic cell lines). Virus production was undetectable in uninfected controls (<15\% syncytia). Data are mean of 3 separate experiments.}$$
pellet was gently resuspended in 1.5 mL of hypotonic fluorochrome solution containing propidium iodide as described [24]. Propidium iodide fluorescence of individual nuclei was measured by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA), as described by Nicoletti et al. [25]. The red fluorescence due to propidium iodide staining of the DNA was registered on a logarithmic scale. The forward and side scatter of particles was simultaneously measured. Cell debris was excluded from analysis by appropriately raising the forward scatter threshold. The residual cell debris had a very low DNA fluorescence emission and a low side scatter signal. We analyzed $\approx 10^4$ cells in each sample.

**Nuclear fluorescent staining.** Cells were fixed in 1% paraformaldehyde, stained with Hoechst 33258 (Sigma), rinsed, and observed under a fluorescent microscope with filter A.

**Terminal dUTP-nick-end-labeling (TUNEL) assay.** The TUNEL assay was done as described [26] with minor modification. In brief, cell cultures were fixed with a freshly prepared buffered formalin (10% in PBS, pH 7.2) for 30 min at room temperature. Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. Endogenous peroxidase was inactivated by covering the sections with 2% hydrogen peroxide for 5 min at room temperature. Cultures were rinsed in tap and distilled water and covered with terminal deoxynucleotidyl transferase buffer containing terminal transferase (Boehringer Mannheim, Milan, Italy) and biotinylated dUTP. Cultures were then rinsed with PBS and covered with extra avidin peroxidase (Ylem, Avezzano, Italy) diluted 1:20 in PBS and stained with diaminobenzidine.

**Results**

**HIV-induced cytopathic effect.** A laboratory strain (Ba-l) and 10 HIV clinical isolates—5 SI variants (MA107, RA12, DG10, MG8, and SM22) and 5 NSI variants (ZB7, CF35, PA42, GA37, and MD31)—were used to infect macrophages. As shown in table 1, productive infection of macrophages (as documented by HIV p24 antigen production) was obtained with all HIV strains. In agreement with previously reported data [15], HIV$_{ba_1}$ replication was toxic for M-CSF–stimulated macrophages. Similarly, 4 of 5 SI variants and 1 of 5 NSI variants caused substantial cell loss in macrophages after 28 days of culture (table 1).

In further experiments at days 12–28 after infection, we monitored increases in percentages of HIV-positive cells in the cultures, formation of syncytia, cytolysis, and cell loss. Macrophages were infected with 2 cytopathic HIV strains (laboratory strain Ba-l and SI clinical isolate MA107). Table 2 illustrates the time-dependent increases in percentages of HIV-positive cells and syncytia. The progressive spread of infection was associated with increased percentages of cells undergoing cytolysis (figure 1A) and decreased numbers of viable cells (figure 1B).

**Thin-section EM of HIV-infected cultures.** At day 28 after infection, >80% of the virus-producing cells were large multinucleated cells showing massive release of mature virus particles in intracellular vacuoles (figure 2A) and evidence of viral output in the extracellular compartment (figure 2B). Infected multinucleated cells go through a morphologic evolution in which pedunculated protuberances of varying size, which do not contain cytoplasmic organelles, develop on the cell surface (figure 2C, D). Most of the infected syncytia also showed typical cellular changes associated with necrosis (disorganization of cytoplasm and loss of integrity of the cellular membrane; figure 2E–G). Scarce virus production and no sign of cytotoxicity were observed in infected mononuclear cells (figure 2H, I).

![Figure 1](https://academic.oup.com/jid/article-abstract/173/6/1367/1010051)
These data indicate that the HIV-induced cytopathic effect is associated with multinucleated giant cell formation and massive intracellular virus accumulation.

**DNA laddering.** Cells undergoing apoptosis massively digest their DNA in the internucleosomal region, displaying the characteristic nucleosomal ladder on agarose gels [27]. To ascertain whether apoptosis plays a role in HIV-induced cytopathology in macrophages, we determined whether DNA laddering occurred in infected cells. As shown in figure 3, a typical ladder was obtained from control macrophages treated with the apoptosis-inducing factor SKT [28]. In contrast, at day 28 after viral challenge, neither uninfected nor HIV-infected macrophage cultures showed any DNA laddering. Similar results were obtained at days 20 and 24 (not shown).

**Flow cytometry.** We further analyzed HIV-infected macrophages for the presence of apoptosis by flow cytometry of cells after propidium iodide staining. With this method, apoptotic cell nuclei result in an unequivocal hypodiploid DNA peak in the red fluorescence channels (figure 4A) and are easily distinguishable from debris by the high side scatter value (figure 4B) [25]. A typical diploid DNA peak and a minor one in the polyploid range (which could be explained either by very sticky nuclei that have not been separated by the extraction procedure or by the presence of dividing cells in the G2 phase
Figure 2. Continued.
of the cell cycle) were observed in both uninfected and infected samples at day 28 after infection (figure 4C, D). A subdiploid DNA peak due to fragments of dead cells, with a very low red fluorescence emission (figure 4D), and a low side scatter signal (figure 4E) also appeared in HIV-infected cultures. However, no DNA peaks were observed in the hypodiploid range due to apoptotic nuclei. Similar results were obtained at days 20 and 24 (not shown).

Nuclear fluorescent staining and TUNEL assay. Macrophages clear apoptotic cells and nuclei very rapidly (in 4 h), so cells undergoing apoptosis may be too rare to detect by DNA laddering or propidium iodide staining. To overcome these problems, we analyzed infected cultures with two sensitive assays that can detect even a small number of cells undergoing apoptosis — nuclear morphologic analysis after staining with the DNA-specific fluorochrome Hoechst 33258 and the TUNEL assay. The latter labels DNA strand-breaks that are generated during apoptosis [26]. Upon fluorescent nuclear staining (figure 5A–C), no apoptosis was detected in infected or uninfected cultures. The TUNEL assay produced a negative reaction in both uninfected and infected cultures (figure 5D, E), unlike the positive reaction obtained in human breast cancer cells undergoing apoptosis (figure 5F). Finally, EM confirmed that apoptosis did not take place in infected macrophages. We did not observe chromatin condensation, nuclear convolution, or budding or cytoplasm condensation (figure 2).

Effect of antiviral compounds that inhibit HIV assembly and budding on the HIV-induced cytopathic effect. We assessed the ability of some antiviral compounds acting at different stages of the HIV replication cycle to interfere with virus-induced cytotoxicity. Infected macrophages were exposed to substances that interfere with viral assembly and budding, the thiolic compound cystamine (500 µM) and the inhibitor of the viral protease U75875 (10 µM) [20, 29]; to OKT4a (5.6 µg/mL), a monoclonal antibody directed against the gp120-linking domain of CD4 that prevents gp120-CD4 binding and virus entry [30]; or to zidovudine (10 µM), an inhibitor of HIV reverse transcriptase that blocks proviral DNA formation but does not interfere with virus entry or assembly and budding [31]. To avoid possible interference by the antivirals with spread of infection in cultures, the compounds were added to infected cells at day 24, when HIV infection was well established (75%–69% of HIV-positive cells and 77%–72% of syncytia, respectively, in Ba-I and MA107-infected cells; see also table 2). The virus-induced cytopathic effect was evaluated at day 28.

Both cystamine and U75875, but not OKT4a or zidovudine, significantly reduced cytolysis and cell loss (figure 6). By EM, macrophage multinucleated cells in cystamine- and in U75875-treated cultures, but not in OKT4a- or zidovudine-treated or in untreated cells, did not display necrotic features or cellular changes associated with productive infection (e.g., pedunculate protuberances on the cellular membrane; figure 7). Also, in cystamine- and in U75875-treated cultures, syncytia did not contain mature virus (figure 7).

These results indicate that the production (and possibly the consequent intracellular accumulation) of mature virions in infected syncytia is a toxic event. In contrast, binding of gp120 to the CD4 receptor, entry of virus, and formation of proviral DNA do not seem to mediate the HIV-induced cytopathogenicity.

Discussion

This study showed that human macrophages infected with a laboratory strain or with primary clinical HIV isolates and cultured in the presence of M-CSF undergo cell death by necrosis as a direct consequence of viral replication. Apoptosis does not take place in infected macrophages. The HIV-induced cytopathic effect occurred as a result of massive production of intact virions and could be prevented by antiviral compounds that inhibit viral assembly and budding.

There is considerable controversy about whether all or only certain HIV isolates have the ability to replicate in macrophages. Schuitemaker et al. [32] reported that most SI HIV isolates do not replicate in macrophages. In contrast, others have found that dual tropism for macrophages and lymphocytes is a common feature of HIV-1 and -2 isolates [33–36]. We found that isolates of both phenotypes (NSI and SI) can infect and replicate in macrophages. In support of our findings, Connor et al. [37] reported that all 28 HIV-1 isolates sequentially obtained from 4 persons replicated in macrophages, despite differences in their ability to replicate in tumor cell lines. The seemingly conflicting data on the ability of HIV-1 isolates to replicate in macrophages are probably due to technical differences in the methods used to isolate and cultivate these cells. Schuitemaker et al. [32] isolated blood monocytes by counter-current centrifugal elutriation and cultured cells in the presence of human serum. In contrast, we isolated cells by their adher-
Figure 4. DNA fluorescence flow cytometry profiles (logarithmic mode) of propidium iodide (PI)–stained HIVΔM–infected or uninfected macrophages. A, DNA fluorescence histograms of PI-stained thymocytes after incubation 24 h with apoptosis-inducing agent dexamethasone (10⁻⁷ M). Note hypodiploid DNA peak typical of apoptosis. B, Forward scatter (FSC) and side scatter (SSC) measurements of same thymocytes as in A. Particles that gave rise to hypodiploid peak presented relatively high SSC signal (arrow). C, Uninfected macrophages. D and E, Infected macrophages. Hypodiploid peak shows very low DNA red fluorescence (too low to be due to apoptotic cells) caused by necrotic cell debris. E, FSC and SSC measurements. Particles that gave rise to hypodiploid peak had low SSC signal (arrow) typical of necrotic cell debris. Similar results were obtained with HIVΔMA107–infected cells.

ence to plastic, and the differentiation process of monocytes took place in the presence of M-CSF, a cytokine that induces maturation of monocytes into macrophages.

Several investigators have shown that the susceptibility of macrophages to HIV infection is greatly influenced by their stage of maturation. Our group recently showed that M-CSF increases the susceptibility of macrophages to HIV-1 infection by increasing the expression of the CD4 receptor on these cells [38]. Thus, in view of the importance of the stage of maturation of macrophages, it is not surprising that different isolation and culture procedure may give seemingly contradictory results on the susceptibility of macrophages to HIV infection. Nevertheless, further experiments are needed to determine which culture procedure is most relevant in vivo.

The formation of nonviable syncytia has been proposed as a mechanism of HIV-induced cytopathology in T lymphocytes in vitro and in vivo [39–41]. HIV replication in macrophages was associated with the formation of multinucleated giant cells; however, the lysis of infected syncytia occurs as a result of viral replication rather than as a consequence of syncytia forma-
Figure 5. A–E, Apoptosis analysis by nuclear fluorescent staining with Hoechst 33258 and terminal dUTP-nick-end-labeling (TUNEL) assay in macrophages infected with HIV_p24 or uninfected. A and B, Infected and uninfected cultures, respectively, stained with Hoechst 33258. C, Vascular smooth muscle cells with fragmented chromatin typical of apoptosis (arrow). D and E, TUNEL reaction in infected and uninfected cells, respectively. F, TUNEL reaction in human breast cancer cells showing many apoptotic cells with black stain of fragmented DNA (arrowheads).
with different tropism and toxicity for macrophages may exist in vivo and contribute to a more rapid progression of HIV infection towards AIDS.

Programmed cell death (apoptosis) is a normal mechanism of cell death that was originally described in the context of the response of immature thymocytes to cellular activation [46]. It has been suggested that both qualitative and quantitative defects in T lymphocytes in HIV-infected patients may result from apoptosis [47-49]. We conducted a series of experiments to determine whether programmed cell death may play a role in the cytopathogenicity of HIV in macrophages. None of the characteristic morphologic or biochemical features of apoptosis were found by DNA gel electrophoresis, flow cytometry, Hoechst staining, TUNEL assay, or EM.

Although we previously reported [15] that HIV-infected macrophages express transglutaminase, an enzyme often induced during apoptosis [50], the present data indicate that macrophages die by a nonapoptotic mechanism. Thus, transglutaminase expression can be associated with cell activation, fusion, or both as previously reported [51, 52].

The process of apoptosis in HIV-infected lymphocytes has been related to abortive cell activation: Lethal endonuclease activation may be the consequence of imbalances in deoxynucleotide pools necessary for DNA repair, a sudden cessation of DNA replication, and negative signals elicited by the binding of HIV gp120 to CD4 [47-49]. Unlike T lymphocytes, macrophages are differentiated cells with a limited DNA mechanism. This may account for the absence of an activation-induced death program in this cell population.

Infection of cells of macrophage lineage is now recognized as an important event in the pathogenesis of AIDS. Thus, factors that modify the ability of HIV to induce cell toxicity in these cells may influence the course of HIV infection in patients. While some HIV strains have been reported to replicate in macrophages without inducing substantial cytotoxicity [44, 45], we show here that stimulation of these cells by M-CSF markedly enhances their susceptibility to the cytopathic effect induced by a laboratory strain and several primary clinical isolates of HIV. M-CSF is physiologically present in blood at concentrations similar to those used in this work (700-1000 U/mL) [53, 54] and is considered the key physiologic regulator of blood monocyte levels [55]. These findings suggest the possibility that macrophage killing by HIV might also occur in vivo. It is worth noting that accumulation of HIV within cytoplasmic vacuoles of macrophage-derived, multinucleated giant cells in the brain has been described [12, 13]. These observations made in brain tissue from AIDS patients closely parallel the ultrastructural findings in HIV-infected macrophages reported here. Of interest, monocytes have been infected in vivo with HIV SI variants, and a possible relationship between the development of neurologic disorders and the emergence of SIMonocytotropic variants has been suggested [56].

EM documented large numbers of mature virions in cytoplasmic vacuoles of infected macrophages. In vivo, macrophage killing by HIV could lead to massive release of infectious
Figure 7. Thin-section electron microscopy of effects of cystamine and U75875 on HIV\textsubscript{Ba\textsubscript{a10}}-induced cytopathic changes in macrophages. Similar results were obtained with HIV\textsubscript{MA107}-infected cells. A, Infected multinucleated cell treated with cystamine. Note absence of cytopathic changes associated with virus production (pedunculate protuberances and necrosis) and intravacuolar intact virions. B, C, Magnifications of A show normal cytoplasmatic structure and abnormal virions (arrows). D, Infected multinucleated cell treated with proteinase inhibitor U75875. Note absence of cytopathic changes associated with virus production (pedunculate protuberances and necrosis) and intravacuolar mature virions. E, F, Magnifications of D show intracellular immature virions (arrowheads).
HIV Induces Cell Death in Macrophages

References


