Macrophenage Colony-Stimulating Factor Enhances the Susceptibility of Macrophages to Infection by Human Immunodeficiency Virus and Reduces the Activity of Compounds That Inhibit Virus Binding

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The effects of macrophenage colony-stimulating factor (M-CSF) on CD4 receptor expression, susceptibility to human immunodeficiency virus type 1 (HIV) infection, and anti-HIV activity of dextran sulfate and soluble CD4 were studied in cultured, human primary macrophages. M-CSF stimulated macrophenage cells to express the CD4 receptor, and this resulted in an increase of both the number of CD4" cells and the density of the receptor on the cell surface. M-CSF also significantly enhanced the susceptibility of macrophenage cells to HIV infection. Interestingly, the anti-HIV activity of dextran sulfate and soluble CD4 (two compounds that interfere with HIV-CD4 binding with different mechanisms) was reduced 100-fold and fivefold, respectively, in M-CSF-treated macrophages. Human blood concentrations of M-CSF are reported to be similar to those used in this work (1,000 U/mL); thus, it is conceivable that also in vivo this cytokine may modify the susceptibility of macrophages to HIV and the ability of dextran sulfate and soluble CD4 to inhibit HIV replication. These results suggest that the in vitro study in M-CSF-treated macrophages of promising drugs inhibitors of HIV-CD4 binding could provide further insights into the potential efficacy of these compounds in patients.

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HUMAN IMMUNODEFICIENCY virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS), infects and replicates efficiently in CD4 lymphocytes and in cells of monocyte/macrophage lineage (macrophages).1-2 Macrophages are widely recognized as a major target for HIV infection in the body. Tissue macrophages are a selective target for HIV, as shown by prominent infection of Langerhans cells of the skin, Kupffer cells of the liver, alveolar macrophages of the lung, and reticuloendothelial cells in lymph nodes.3-7 More importantly, macrophage-like cells account for the large majority of cells infected by HIV in the central nervous system, and macrophage infection and neuronal dysfunction in the brain play a crucial role in the pathogenesis of HIV-related dementia complex.8-10

CD4 lymphocytes require activation and cell replication to produce substantial amounts of the virus.11,12 By contrast, the ability of HIV to replicate in macrophages seems to be mainly dependent on cell differentiation, as in the case of other lentiviral infections.13-15 Indeed, several cytokines that induce changes in macrophage differentiation also enhance HIV production in these cells.16,17 Macrophage colony-stimulating factor (M-CSF) is a cytokine that has a variety of trophic and enhancing effects on macrophages, including the ability to stimulate HIV replication.17-20 The mechanism(s) by which M-CSF induces HIV production in macrophages is not fully understood. One possibility could be an enhancing effect of M-CSF on the expression of the HIV-specific receptor CD4 and, consequently, an increase of the number of potentially infectable cells. CD4 expression at the time of infection is a necessary event for infection of macrophages and correlates strictly to the susceptibility to HIV and to the ability of these cells to produce extracellular virus.21-26

Based on these observations, we undertook an in vitro study to assess the ability of M-CSF to interfere with the expression of the CD4 receptor and to increase the susceptibility to HIV infection of macrophage cells. We found that the treatment with M-CSF stimulates cultured macrophages to increase the expression of the CD4 receptor. M-CSF also enhances the susceptibility of these cells to HIV infection. The effects of M-CSF on CD4 expression and susceptibility to HIV infection prompted us to evaluate the ability of this cytokine to modulate the antiviral activity of compound inhibitors of the binding of HIV with the CD4 receptor, such as dextran sulfate and soluble CD4.27,28 Interestingly, we found that the antiviral activity of both compounds is dramatically reduced in macrophages stimulated with M-CSF, but not in those stimulated with granulocyte-macrophage colony-stimulating factor (GM-CSF) or granulocyte colony-stimulating factor (G-CSF), two other bone marrow stimulatory cytokines.

MATERIALS AND METHODS

Cells. Peripheral blood obtained from HIV-negative donors was enriched for mononuclear cells (PBMCs) by centrifugation over Ficoll Hypaque. Macrophages were obtained by adherence of PBMCs to plastic, as previously described.29-30 Cells obtained by this method are greater than 95% pure, as determined by nonspecific esterase activity.14 Each experiment was performed using PBMCs from a single donor.

Virus. A monocytotropic strain of HIV-1, human T-lymphotrophic virus type III (HTLV-III_LAV; gift of Drs S. Garmer, R.C. Gallo, and M. Popovic, National Cancer Institute, Bethesda, MD) was used. This will be referred to as HIV-1LAV. Supernatants from infected cultures of fresh macrophages were used as the source of HIV-1LAV; these were filtered and stored in liquid nitrogen before use. Titration to determine infectivity was performed in a primary macrophage system as previously described.31 The titer of virus stock, expressed as 50% tissue culture infectious dose (TCID50), was determined as previously described.32

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Compounds. Soluble CD4 was obtained from Genentech Inc (San Francisco, CA). Dextran sulfate (molecular weight [MW] 8,000) and zidovudine (AZT) were purchased from Sigma (St Louis, MO). OKT4a, an antibody that reacts with the HIV-specific domain of CD4, was from Ortho Pharmaceuticals (Raritan, NJ). Recombinant M-CSF was kindly provided by Genetics Institute (Cambridge, MA) and contains 2 × 10^6 U/mg of protein; recombinant GM-CSF was obtained from Sandoz Research Institute (East Hanover, NJ) and contains 5.4 × 10^6 chronic myelogenous leukemia (CML) units per milligram of glycoprotein; recombinant G-CSF (Angen Biologics, Thousand Oaks, CA) had a specific activity of 1 × 10^6 U/mg protein.

Cytotoxicity analysis. Just after purification, 1 × 10^6 macrophages were seeded in Petri dishes in 3 mL of complete medium with or without 1,000 U/mL M-CSF or 100 U/mL GM-CSF. On day 7, adherent cells were detached by 20 minutes of incubation with 0.2% EDTA, followed by vigorous pipetting. The expression of CD4 protein was assessed by flow cytometry using a fluoresceinated Leu3a + b monoclonal antibody (Becton Dickinson, Mountain View, CA). Pairwise isotype-specific control antibodies (Becton Dickinson) were run with each sample. Macrophages were differentiated from lymphocytes and dead cells on the basis of forward angle and 90° scatter, as previously described.

Assay of antiviral activity. For the evaluation of the antiviral activity of dextran sulfate and soluble CD4, macrophage cells were plated at 1.5 × 10^5/mL in 48-well plates (Costar, Cambridge, MA) with or without 1,000 U/mL M-CSF or 100 U/mL GM-CSF. On day 7, cells were exposed to various concentrations of the compounds and 30 minutes later were exposed to 100 TCID_50 HIV-1_xla.. Appropriate mock-infected cultures were run as negative controls. Two hours after infection, macrophages were extensively washed to remove excess virus and cultivated in 1 mL of RPMI-1640 medium supplemented with 20% heat-inactivated fetal calf serum, 2 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin (complete medium) at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were washed and fed and the same concentrations of M-CSF, GM-CSF, and drugs as before were added every 7 days.

Viral detection. HIV-p24 antigen production in supernatants was assessed by a sandwich enzyme-linked immunosorbent assay (ELISA; Abbott, Pomezia, Italy). The number of macrophages expressing the viral antigen p24 was evaluated by indirect immunofluorescence (IF). For this purpose, an anti-p24 monoclonal antibody (Becton Dickinson, Mountain View, CA) was diluted to a concentration of 1 μg/mL and stored at −20°C until used. The neutralization assay was performed by preincubating for 2 hours at 37°C 1,000 U of M-CSF with 50 μg of anti-M-CSF antibody.

Enzymatic amplification. Macrophages were infected as described above and detached from the wells 24 hours after viral challenge. DNA was extracted as previously described. After ethidium bromide fluorescent quantitation of the amount of DNA, equivalent amounts of each sample were subjected to 30 cycles of polymerase chain reaction (PCR) amplification using either the HIV gag primer pair SK 38/39 or the HLA-DQA primer pair GH26/27. Amplified products, a 115-bp sequence in the gag region of the HIV genome, and a 242-bp fragment (or 239-bp fragments from some alleles) from the second exon of the HLA-DQA locus were specifically detected by the oligomer-hybridization procedure. The SK 19 and RH 54 oligonucleotide probes were end-labeled with 32P adenosine triphosphate as previously described. X-ray films of polyacrylamide gels were quantified by analysis with an LKB Ultrascan XL laser densitometer (LKB-PharMacia, Rome, Italy).

Toxicity. Toxicity of dextran sulfate and soluble CD4 in macrophages was evaluated by trypan blue dye exclusion.

Protein determination. Proteins were measured by the method of Lowry et al using bovine serum albumin as standard.

Cell count. Nuclei were extracted from macrophages by lysing buffer and counted in a cell counting chamber under a phase contrast microscope.

RESULTS

Enhancement of virus replication by cytokines. Macrophages were exposed to 1,000 U/mL M-CSF, 100 U/mL GM-CSF, or 500 U/mL G-CSF and then challenged with HIV-1. Viral production was assessed as p24 gag protein release in the supernatants at various time points. In agreement with previously reported data, both M-CSF and GM-CSF potently enhanced the replication of HIV in macrophages as compared with control cells (Fig 1). By contrast, G-CSF had no effect on HIV replication in macrophages, with the overall curve of virus production in supernatants being superimposable with that obtained in control macrophages.

CD4 expression in normal and M-CSF-treated macrophages. Macrophage cells cultured in the absence or in presence of M-CSF or GM-CSF were analyzed for CD4 expression at day 7 of culture. Figure 2A shows that, of the unstimulated LeuM3-positive monocytes, 56 ± 13% expressed CD4 (mean channel difference +23). In contrast, 89% ± 8% of M-CSF-treated macrophages showed CD4 expression (mean channel difference +52; Fig 2B). To confirm the role of M-CSF in the induction of CD4 expression in macrophages, we performed a neutralization assay by using a specific antihuman M-CSF antibody. Figure 2C shows that the increased expression of CD4 induced by M-CSF on macrophages was abolished when M-CSF was preincubated with the anti-M-CSF antibody (63% ± 18% of positive cells, +30 mean channel difference). In contrast with M-CSF, GM-CSF treatment did not modify significantly the expression of CD4 on macrophages; 49 ± 10 GM-CSF–treated macrophages expressed CD4, with a mean channel difference of +19. These data indicate that M-CSF increases the number of macrophage cells expressing CD4 as well as the overall expression of this receptor.

Effect of M-CSF on HIV DNA formation. DNA was ex-
M-CSF INCREASES HIV INFECTION ON MACROPHAGES

Fig 1. Enhancement of virus replication by cytokines. (■) Control M/M; (▲) M-CSF M/M; (●) GM-CSF M/M; (●) G-CSF M/M. The data represent the average of three experiments, each performed in triplicate.

Fig 2. CD4 expression in normal and M-CSF-treated macrophages. More than 90% of the cells analyzed were LeuM3-positive. The percentage of CD4+ cells was calculated by straight channel integration, with the integration channel set so that less than 1% of the isotype controls cells appeared positive. The density of CD4 on the surface of the cells was calculated comparing the Leu 3a + b fluorescence with appropriate fluorescein-labeled isotype controls. The histograms showed in this figure represent data obtained from a representative experiment performed. (—) Control; (—) Leu3a + b-stained macrophages. (A) Normal macrophages; (B) M-CSF–treated macrophages; (C) macrophages treated with M-CSF plus anti–M-CSF antibody.

Fig 3. Detection of HIV DNA in infected macrophages. Oligomer-hybridization analysis of HIV gag-amplified (115-bp) products. The HIV control was plasmid DNA (Perkin-Elmer Cetus, Norwalk, CT) containing the entire rearranged genome of the HIV-Z6 isolate. Lane 1, mock-infected macrophages; lanes 2 through 5, amplification of 104, 105, 106, and 107 copies, respectively, of the HIV control plasmid in 900 ng of human placental DNA; lanes 6 through 10, amplification of 900 ng of the following DNA samples: HIV-infected, unstimulated macrophages (lane 6); HIV-infected, M-CSF–treated macrophages exposed to 5 μg/mL OKT4a (lane 8); HIV-infected, normal macrophages exposed to 5 μg/mL OKT4a (lane 9); and HIV-infected, M-CSF–treated macrophages exposed to 150 μg/mL OKT4a (lane 10). Densitometric analysis of this representative x-ray film gave the following results (measured as absorbance values × mm2): lane 1, 0.028; lane 2, 108.9; lane 3, 31; lane 4, 11; lane 5, 3.5; lane 6, 10.2; lane 7, 42.4; lane 8, 15.35; lane 9, 1.15; lane 10, 2.15. Consistent results in term of DNA amplification were achieved in all experiments.

150 μg/mL of the antibody had to be used to obtain a comparable inhibition in M-CSF–treated macrophages. These data suggest that there is a direct correlation between the M-CSF–induced increased expression of CD4 and the ability of HIV to enter within macrophage cells and to synthesize its DNA.

Effect of M-CSF on the susceptibility of macrophages to HIV infection. The effect of M-CSF on the susceptibility of macrophages to HIV infection is depicted in Fig 1. The M-CSF dose–response relationship was determined using concentrations of M-CSF ranging from 5 to 1500 ng/mL. The data indicate that M-CSF enhances HIV replication in a dose-dependent manner.

Relative cell number

Days of culture

HIV-p24 gag (ng/ml)

0 20 40 60 80 100 120 140

7 14 21

Densitometric analysis of this representative x-ray film gave the following results (measured as absorbance values × mm2): lane 1, 0.028; lane 2, 108.9; lane 3, 31; lane 4, 11; lane 5, 3.5; lane 6, 10.2; lane 7, 42.4; lane 8, 15.35; lane 9, 1.15; lane 10, 2.15. Consistent results in term of DNA amplification were achieved in all experiments.
ties of infection, M-CSF-treated macrophages produced about 10-fold more extracellular virus than normal macrophages (Fig 4).

Thus, overall data suggest that the increased replication of HIV in M-CSF-treated macrophages is not caused by the increased number of target cells.

Anti-HIV activity of dextran sulfate and soluble CD4 in normal and M-CSF-treated macrophages. The anti-HIV activity of dextran sulfate and soluble CD4, two compounds that interfere with the binding of HIV to the CD4 receptor, to HIV infection was further investigated by infecting normal or M-CSF-treated macrophages with HIV at different multiplicities of infection. As shown in Fig 4, infection of normal macrophages (as assessed by p24 production) was obtained with 100, 10, and 1 TCID<sub>50</sub> of the HIV-1<sub>la</sub> stock. In contrast, M-CSF-treated macrophages could be infected with TCID<sub>50</sub> as low as 0.01. When infected at the same multiplicities of infection, M-CSF-treated macrophages produced about 10-fold more extracellular virus than normal macrophages (Fig 4).

It has been reported that M-CSF may induce some replication of cultured macrophages. Thus, to further exclude that the enhanced susceptibility of M-CSF-treated macrophages to HIV infection (assessed as the production of extracellular virus) was the simple consequence of an increase in the number of target cells, we assessed the total number of cells (by counting the nuclei after cell lysis and by determining the amount of total cellular proteins) and the relative number of virus-expressing cells by EM and IF analysis. As shown in Table 1, at day 21 of culture, M-CSF treatment was associated with an increase in the production of p24-antigen in the supernatants and in the number of virus-expressing cells with respect to untreated cells. However, no substantial differences were found between treated and untreated cells regarding the total number of cells (Table 1). EM analysis also shows that, at a single-cell level, M-CSF treatment of macrophages was associated with an increased release of mature virus particles both in the extracellular compartment and in intracellular vacuoles with respect to untreated cells (Fig 5).

Thus, overall data suggest that the increased replication of HIV in M-CSF-treated macrophages is not caused by the increased number of target cells.

**DISCUSSION**

In this report, we show that M-CSF treatment stimulates cultured macrophages to increase the expression of the CD4 receptor. Also, the susceptibility of these cells to HIV infection is significantly enhanced by the treatment with M-CSF, whereas the antiviral activity of compounds that inhibit

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**Table 1. Effect of M-CSF on Culture Viability and Susceptibility to HIV Infection in Macrophages**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>p24</th>
<th>No. of Nuclei</th>
<th>Proteins</th>
<th>% of HIV' Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>7.5</td>
<td>37,000</td>
<td>315</td>
<td>EM 19, IF 15</td>
</tr>
<tr>
<td>M-CSF</td>
<td>35</td>
<td>43,000</td>
<td>330</td>
<td>83, 88</td>
</tr>
</tbody>
</table>

The number of nuclei (no. of cells/well), p24 (ng/mL), and total cellular proteins (micrograms of proteins/well) as well as EM and IF analyses were performed at day 21 of culture. Less than 5% macrophages stained positive by IF in uninfected cultures. The data represent the average of three experiments, each performed in triplicate. The differences among the experiments were less than 15% for all the parameters analyzed.
M-CSF INCREASES HIV INFECTION ON MACROPHAGES

Fig 5. Electron microscopy analysis of HIV production in unstimulated and M-CSF–treated macrophages. (A and B) Electron micrographs of an unstimulated macrophage showing no extracellular virions (A) and scarce intracytoplasmatic HIV accumulation (B). (Original magnifications: [A] × 7,000; [B] × 40,000.) (C, D, and E) Electron micrographs of an M-CSF–stimulated macrophage showing substantial extracellular HIV release (C and D) and massive intracytoplasmatic virus accumulation (E). (Original magnifications: [C] × 5,500; [D] × 18,000; [E] × 31,000.)

viral binding, such as dextran sulfate and soluble CD4, is dramatically reduced.

When we analyzed macrophage cultures for CD4 expression, we found that the percentage of cells expressing CD4 increased upon M-CSF stimulation. These data suggest that the expression of CD4 on macrophages is under the control of exogenous stimuli that modify cell maturation and functions. M-CSF stimulates macrophages to undergo several cycles of replication and to differentiate in vitro. The effect of M-CSF on CD4 could be the consequence of macro-
Membrane insertment of CD4 in macrophages. Thus, it is consistent with several reports showing that infection was associated to an effect of this cytokine on some late steps of the HIV replication cycle. GM-CSF, a cytokine that, similar to M-CSF, supports the long-term growth and differentiation of macrophages in vitro, has no effect on CD4 expression. It has been reported that posttranslational changes affecting the conformation of the CD4 molecule cause internalization and reduced CD4 expression on macrophages, our results support the hypothesis that it enhances HIV replication in macrophages by upmodulating steps of virus replication later than transcription. In this regard, it has been postulated that cytokines may upregulate the expression of HIV in macrophage cells by the induction of a cellular gene that, in turn, induces the promoter of HIV either through a cis mechanism that depends on the integration site of the provirus or by transacting via a DNA-binding protein. A DNA-binding protein (NF-κB) has been described that binds to the HIV long terminal repeats in infected T lymphocytes and upregulates virus expression.59

Regarding G-CSF, our data show that this cytokine has no effects on HIV replication in macrophages. These data are similar to those reported in the literature by a number of groups and are consistent to the fact that G-CSF affects mainly the granulocyte lineage, whereas its specific receptor is poorly (if at all) expressed in mature macrophages.17,44

The effects of M-CSF on CD4 expression and macrophage susceptibility to HIV could be responsible for the reduced antiviral activity of dextran sulfate and soluble CD4 observed in cytokine-treated macrophages. This hypothesis is also supported by the evidence we obtained that GM-CSF did not show any effect on both the expression of CD4 and the antiviral activity of dextran sulfate and soluble CD4. The ability of M-CSF to increase the overall expression of the CD4 receptor may directly reduce the activity of dextran sulfate, a compound that inhibits HIV replication by blocking the gp120 binding site on cellular CD4.28,45 Also, the enhanced susceptibility to HIV infection induced by M-CSF may reduce the antiviral activity of concentrations of soluble CD4 that sufficiently neutralize HIV infectivity in normal macrophages.

In contrast to tissue macrophages in lung, brain, and lymph nodes, blood monocytes are only rarely infected in AIDS patients.3,8,46,47 Moreover, it has been recently reported that in the early stage of the HIV infection there is a dichotomy between the number of infected monocytes in peripheral blood versus lymphoid tissue.48,49 This enhanced susceptibility to HIV infection of tissue macrophages with respect to blood monocytes could be due, at least in part, to the effect of endogenous M-CSF. In this regard, it should be noted that bioassays performed on human blood have shown that endogenous M-CSF levels are similar to those used in this work (700 to 1,000 U/mL).50,51 Moreover, it has been reported that, during experimentally induced bacterial infection in mice, the blood levels of M-CSF may increase from 700 to about 1,500 U/mL.52

The role of macrophages in the pathogenesis of HIV infection has been overstressed over several years.1,11 Thus, therapeutic strategies used in the therapy of AIDS and related disorders should directly address the virus infection in this special target population. Dextran sulfate and soluble CD4 have been shown to be potent inhibitors of HIV replication in vitro.27,28,53,54 However, in vivo, both compounds have been consistently shown to be ineffective in AIDS patients.55,56 The lack of clinical activity has been attributed to the existence of primary HIV isolates resistant to soluble CD4 neutralization58,59 as well as to the inefficient absorption from the gastrointestinal tract for orally administered dextran sulfate.60 New therapeutic strategies based on the use of sulfated polysaccharides, other than dextran sulfate, with improved absorption and less potent anticoagulant activity are currently under study.61-63 Because it is possible that endogenous M-CSF may be able to decrease the antiviral activity of most...
compounds acting at the level of virus binding, to better predict the in vivo efficacy of these agents on the basis of their in vitro activity, it could be useful to perform selected antiviral assays also in M-CSF-stimulated macrophages systems before reaching clinical trials.

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