Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 8734–8738, November 1986
Medical Sciences

In vitro modification of human immunodeficiency virus infection by granulocyte–macrophage colony-stimulating factor and γ interferon

(U-937 cells)

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Communicated by John J. Burns, June 27, 1986

ABSTRACT **The ability of recombinant human granulocyte–macrophage colony-stimulating factor (GM-CSF) and γ interferon (IFN-γ) to modify human immunodeficiency virus (HIV; also called HTLV-III/LAV) infection in the monocytic cell line U-937 was examined. When added to persistently infected cell cultures, GM-CSF at 30–300 units per ml produced maximal reductions in reverse transcriptase activity of 37–55% 10–14 days after its addition, whereas IFN-γ produced reductions of 64–68% 10–17 days after addition. When used prior to acute HIV infection and maintained in the cell culture system, these cytokines reduced reverse transcriptase activity 90–100% and nearly eliminated viral antigen expression but did not prevent return of productive infection after their removal. These results indicate that, in a monocyte model of HIV infection, GM-CSF and IFN-γ substantially restrict HIV expression and that these cytokines deserve further evaluation as therapeutic alternatives in HIV-related disorders.**

The human immunodeficiency virus [HIV; also called T-cell lymphotropic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV)] is now recognized as the etiologic agent of the acquired immunodeficiency syndrome (AIDS) (1–7). Although the T4 lymphocyte is a primary target of infection, there is increasing evidence that other cell types may be involved in the pathophysiology of this disease. Specifically, the neurotropism of the virus and the reports of the isolation of HIV from cells of the monocyte/macrophage lineage support this concept (8–12). Such cells may serve as reservoirs of infection, and their involvement may complicate the potential therapeutic approaches to HIV-related diseases. Investigative efforts directed at the therapy of AIDS have concentrated on specific antiviral agents as well as immune-system modulators (13–21). Although interleukin-2 and γ interferon (IFN-γ) have been disappointing in early trials (20, 21), their full therapeutic potential has not been evaluated. Further, newer cytokines such as granulocyte–macrophage colony stimulating factor (GM-CSF) have only recently been cloned (22, 23) and, therefore, are just arriving on the threshold of clinical use. GM-CSFs of murine and human origin are glycoproteins that may affect hematopoietic precursors of all lineages and have been described to have proliferative, differentiating, and activating effects on monocytes/macrophages (24, 25). Thus, they have potential importance as agents that may restrict HIV infection of these cells. In this study we investigate the effects of GM-CSF and IFN-γ on both chronic and acute HIV infection of the U-937 cell line, a convenient model of monocyte infection.

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METHODS

Virus Strain and Cell Lines. The virus strain HTLV-III$_B$ was obtained from R. C. Gallo (Bethesda, MD) and propagated in H9 cells (2). The latter were maintained in RPMI 1640 medium supplemented with 250 units of penicillin and 250 μg of streptomycin per ml, 2 mM L-glutamine, 10 mM Hepes buffer, and 20% (vol/vol) heat-inactivated fetal calf serum (M.A. Bioproducts, Walkersville, MD). The U-937 cell line was obtained from H. S. Koren (Durham, NC) and maintained in RPMI 1640 medium supplemented with penicillin, streptomycin, L-glutamine, and 10% fetal calf serum. Both cell lines were mycoplasma-free. Cell viability was determined by the trypan blue dye-exclusion technique.

Cytokine Preparations. Recombinant human GM-CSF was obtained from Genetics Institute (Cambridge, MA). It possessed a specific activity of 1.0×10^7 units per mg of protein and was >99% pure. A unit of GM-CSF is defined as the amount that produces a half-maximal response in a colony-forming assay in which an internal laboratory reference standard is included. This activity was measured in the laboratories of Genetics Institute.

The recombinant human IFN-γ was obtained from Biogen (Cambridge, MA), possessed a specific activity of 1.6×10^7 units per mg of protein, and was 97.8% pure. A unit of this material corresponds to a unit of the National Institutes of Health reference standard IFN-γ (Gg 23-901-530) as determined in a cytopathics effect reduction assay utilizing encephalomyocarditis virus and WISH cells as the test virus and target cells, respectively (performed at Biogen).

Persistent Infection. HIV infection of U-937 cells was initiated by incubating 5×10^6 cells in 1 ml of a filtered, cell-free supernatant harvested from the strain HTLV-III$_B$-infected H9 cell culture for 2 hr at 37°C. The reverse transcriptase activity of the inoculum was 1.0×10^6 cpm/ml. Control cells were mock-infected with a filtered, cell-free supernatant from an uninfected H9 cell culture. After virus adsorption, the cells were pelleted and resuspended in fresh medium to a concentration of 2×10^5 cells per ml. A persistent infection was readily established (see below), and after 40 days of stable infection, the cell cultures were divided and either left untreated or exposed to one of the following: recombinant human GM-CSF at 30–300 units per ml or recombinant human IFN-γ at 100–1000 units per ml. Every 3–4 days for 21 days, the cells were counted and passed to a concentration of 2×10^5 cells per ml with replenishment of the appropriate cytokine in the treated cultures.

Abbreviations: HIV, human immunodeficiency virus; IFN-γ, γ interferon; GM-CSF, granulocyte–macrophage colony-stimulating factor; AIDS, acquired immunodeficiency syndrome.

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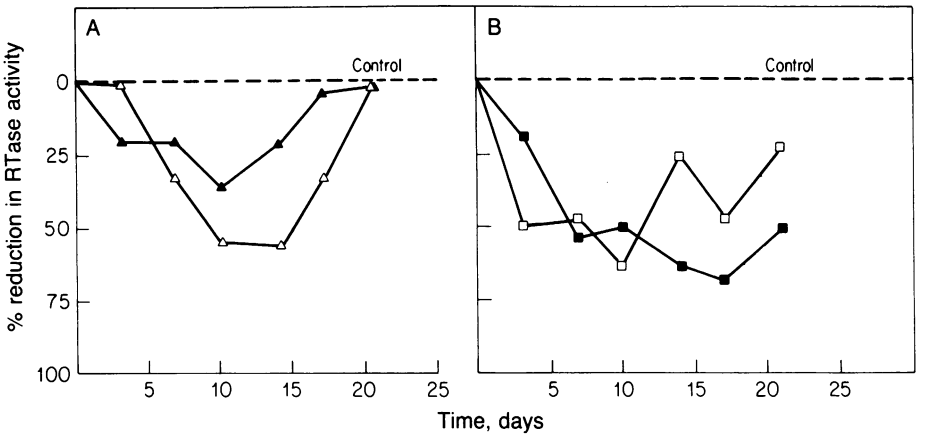

FIG. 1. Effect of GM-CSF at 30 units (▲) and 300 units (△) per ml (*A*) or IFN-γ at 100 units (□) or 1000 units (■) per ml (*B*) on reverse transcriptase activity in persistently HIV-infected U-937 cells.

Acute Infection. For evaluation of the possible prophylactic effect of these cytokines, uninfected U-937 cells were left untreated or were exposed to GM-CSF or IFN-γ at the above concentrations for 4–7 days. The cells were pelleted and infected as detailed above using differential inocula (reverse transcriptase activities of 1.2×10^6 cpm/ml or 7.6×10^5 cpm/ml). After the period of virus adsorption or mock infection, the cells were pelleted and resuspended to a concentration of 2×10^5 cells per ml. Cultures previously exposed to GM-CSF or IFN-γ were either left untreated after infection or were maintained in the presence of the cytokines at the same concentrations. Cultures were split every 3–4 days with replenishment of the cytokines in the treated cultures.

The studies described were replicated two to four times with reproducible results. The figures shown illustrate representative experiments.

Reverse Transcriptase Activity. Reverse transcriptase activity in the supernatant was determined by standard techniques (26) at every cell passage.

Immunofluorescence. The expression of HIV antigens was determined by indirect immunofluorescence. At the time of each culture passage, infected and control cells were washed in phosphate-buffered saline, air-dried on glass slides, and fixed in 50% methanol/50% acetone (vol/vol). Slides were incubated with a known positive anti-HIV human serum for 30 min at 37°C in a humidified atmosphere and were stained with a fluorescein isothiocyanate-conjugated $F(ab')_2$ goat anti-human immunoglobulin (Cooper Biomedical, Malvern, PA). After an Evans blue counterstain, the slides were examined under an Olympus BH-2 fluorescence microscope, and the percentage of immunofluorescent positive cells was quantitated.

RESULTS

Cytokine Treatment of Persistently HIV-Infected U-937 Cells. A persistent infection was readily established in U-937 cells and has been maintained for over 65 days. The level of virus expression has been high, with a mean supernatant reverse transcriptase activity of 3.0×10^6 cpm/ml and an average of 89% of the cells positive for HIV antigens by indirect immunofluorescence. Cell growth, as measured by viable-cell count, and percent cell viability were equivalent between infected and control cultures: HIV-infected, mean of $10^{6.1}$ cells per ml with 90% viability; uninfected control, mean of $10^{6.2}$ cells per ml with 96% viability.

The effect of adding GM-CSF to this stable, persistently infected culture for 21 days is illustrated in Fig. 1*A*. GM-CSF at 30 units per ml resulted in a 37% reduction in supernatant reverse transcriptase activity on day 10 after addition, whereas 300 units per ml resulted in a 55% reduction on day 14. Supernatant reverse transcriptase activity returned to control levels by day 21, however, despite replenishment of the cytokine at the time of each cell passage. Viable cell counts and percent cell viability were equivalent in infected and uninfected cultures exposed or unexposed to GM-CSF. In parallel studies a mock preparation of GM-CSF (provided by Genetics Institute) was shown to have no effect on HIV replication or U-937 cell growth (data not shown).

The effect of adding IFN-γ is illustrated in Fig. 1*B*. A concentration of 100 units per ml resulted in a maximal 64% reduction in reverse transcriptase activity on day 10 after its addition; 1000 units per ml produced a 68% reduction on day 17. On day 21 a trend toward a return to control levels was noted, but reductions in reverse transcriptase activity of 23% and 52% were still seen with concentrations of 100 and 1000 units per ml, respectively. With a concentration of 100 units per ml, no differences in viable cell counts or percent cell viabilities were seen between treated or untreated infected and uninfected cultures. At 1000 units per ml, there were decreases of 0.22 and 0.54 $\log_{10}$ in total viable cell count noted on days 17 and 21 in HIV-infected cultures compared to untreated infected cultures. Therefore, the reverse transcriptase activity is represented as cpm per 10^5 viable cells to adjust for any variations due to alterations in cell count (see Figs. 1, 2, and 3).

The percentage of cells expressing HIV antigens was not altered by exposure to GM-CSF or IFN-γ (Table 1).

Cytokine Treatment of Acutely HIV-Infected U-937 Cells. To determine if these cytokines could protect U-937 cells from acute infection, uninfected cells were exposed to GM-CSF or IFN-γ for 4–7 days prior to infection. After infection the cultures were divided and placed in culture with

Table 1. Effect of cytokine addition on the percentage of cells expressing HIV antigens in persistently infected U-937 cell cultures

Time after addition, days	HIV antigen-positive cells in persistently infected cultures, %				
	No cytokine	GM-CSF		IFN-γ	
		30*	300*	100*	1000*
3	96	93	95	95	95
10	92	90	84	84	72
14	94	95	87	86	88
17	95	95	94	90	88
21	71	76	83	71	64

*Units added per ml.

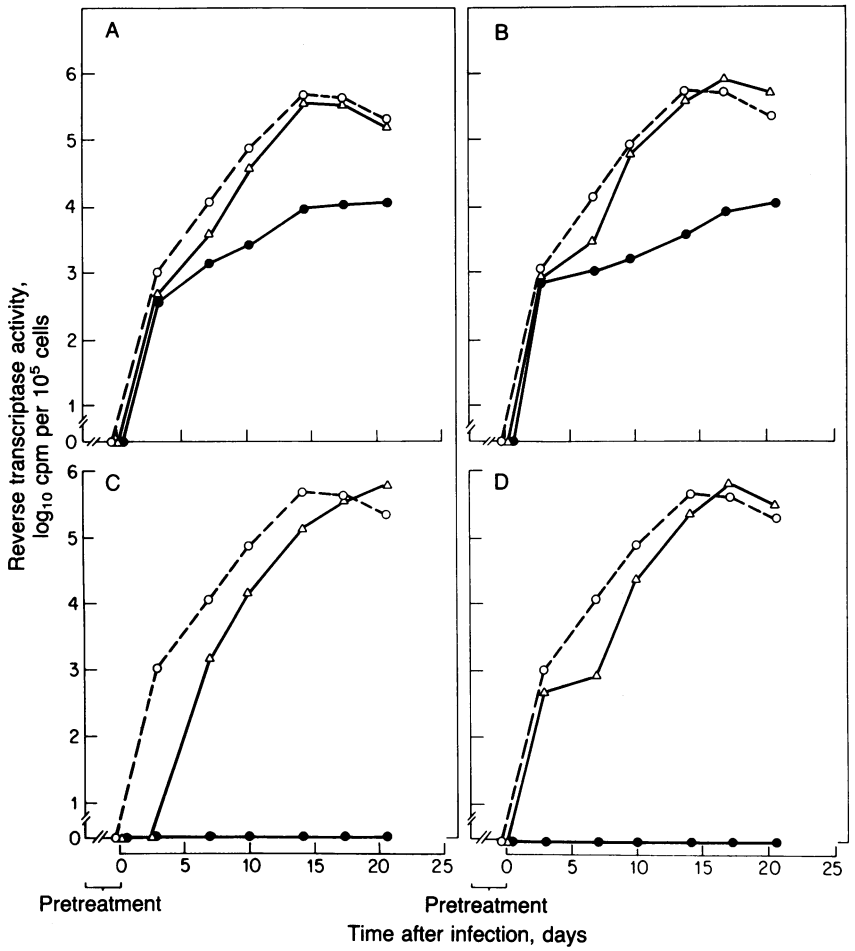

FIG. 2. Effect of cytokine treatment on acute infection of U-937 cells with HIV. (*A*) GM-CSF at 30 units per ml. (*B*) GM-CSF at 300 units per ml. (*C*) IFN-γ at 100 units per ml. (*D*) IFN-γ at 1000 units per ml. Cultures were untreated (○) or were exposed to the respective cytokine for (*i*) 4–7 days prior to infection only (△) or (*ii*) 4–7 days prior to infection and throughout the culture period (●).

or without replenishment of the respective cytokine. Untreated acutely infected cells were included as controls. The results of GM-CSF treatment are illustrated in Fig. 2 *A* and *B*. Pretreatment alone with either 30 or 300 units per ml did not protect U-937 cells from infection, with supernatant reverse transcriptase activity nearly equivalent to untreated infected cultures. As measured by the percentage of immunofluorescent positive cells, however, some protection was evident through day 10 after infection (Table 2). In contrast, cultures exposed to GM-CSF before and after infection demonstrated substantial protection from HIV infection. In cultures exposed to 30 or 300 units of GM-CSF per ml, supernatant reverse transcriptase activity was lower on each assay day in continuously exposed cultures, with average reductions of 1.8 and 1.5 $\log_{10}$ cpm per 10^5 cells after day 7, respectively. The reduction in antigen-positive cells with continuous GM-CSF exposure was dramatic, with an average of <1% immunofluorescent positive cells throughout the culture period (Table 2).

Attempts to modify acute HIV infection with IFN-γ produced qualitatively similar results to GM-CSF treatments. Cytokine pretreatment alone did not prevent infection and resulted in modest reductions in reverse transcriptase activity and reductions in the percentage of antigen-positive cells early after infection (Figs. 2 *C* and *D*; Table 2). By day 17 after infection, the levels of infectivity were equivalent between pretreated and untreated cultures. Cultures continuously exposed to IFN-γ at either 100 or 1000 units per ml showed no supernatant reverse transcriptase activity and 1.2% antigen-positive cells or fewer throughout the culture

Table 2. Effect of cytokine addition on the percentage of cells expressing HIV antigens in acutely infected U-937 cell cultures

Time after infection, days	HIV antigen-positive cells in acutely infected cultures, %								
	No cytokine	GM-CSF				IFN-γ			
		30*		300*		100*		1000*	
		Pre	Pre/post	Pre	Pre/post	Pre	Pre/post	Pre	Pre/post
3	1.5	1.6	<1	1.6	<1	<1	<1	<1	1.1
10	31.7	17.8	<1	18.8	<1	11.6	<1	12.4	<1
17	68.3	50.6	1.7	56.5	<1	38.7	<1	56.9	1.2

Pre, cytokine treatment of cell cultures before infection with HIV; Pre/post, cytokine treatment of cell cultures before and after infection with HIV.
*Units added per ml.

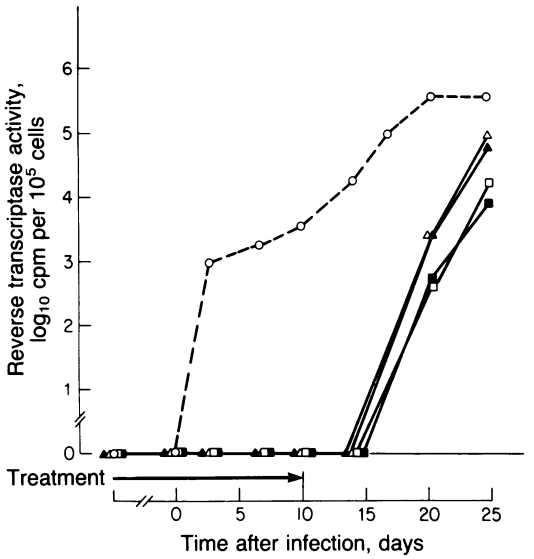

FIG. 3. Effect of cytokine treatment on acute infection of U-937 cells with HIV. Cultures were untreated (○) or pretreated and maintained in the respective cytokine for 10 days after infection: △, GM-CSF at 300 units per ml; ▲, GM-CSF at 30 units per ml; □, IFN-γ at 100 units per ml; ■, IFN-γ at 1000 units per ml.

period. The reduction in reverse transcriptase activity averaged 5.4 $\log_{10}$ cpm/10^5 cells after day 7 postinfection.

In separate experiments, using a lower initial inoculum (reverse transcriptase = 7.6×10^5 cpm/ml), both GM-CSF and IFN-γ were shown to completely inhibit expression of reverse transcriptase activity for 10 days after infection. Following removal of the cytokines, this inhibition persisted for 10 additional days before gradual increases in reverse transcriptase activity (Fig. 3) and the percentage of antigen-positive cells (data not shown) were seen.

DISCUSSION

The monocyte/macrophage system is receiving increasing attention as a potentially important target of HIV infection. Although the virus is clearly T4 lymphotropic with the T4a epitope a likely component of the viral receptor (27–29), there is increasing evidence that leukocytes other than lymphoid cells may be infected *in vivo*. Early suggestive data included the ultrastructural evidence of retroviral particles in the macrophages of lymph nodes of individuals with AIDS-related complex (30) and the knowledge of the importance of the monocyte/macrophage lineage as a natural target of infection for visna virus and caprine arthritis–encephalitis virus (31–33), two other members of the lentivirus group. Recent reports have provided more direct evidence with descriptions of the *in vitro* infectibility of normal monocytes/macrophages as well as the isolation of virus from cells of this lineage derived from infected individuals (11, 12).

The importance of the monocyte/macrophage system in AIDS may be its function as a reservoir of ongoing infection of other target cells. If so, therapeutic strategies will need to take this into account. In addition to the potential value of specific antiviral agents, these cells are theoretically ideal targets for activation by cytokines. We have chosen to examine this question by utilizing the U-937 cell line. These cells are monocytic in origin (34), can be functionally activated to restrict *Toxoplasma gondii* and herpes simplex virus infection (35, 36), and have been shown to be susceptible to both acute and persistent HIV infection, perhaps because they express the T4 antigen (12, 27).

In the treatment of persistently infected U-937 cells, both GM-CSF and IFN-γ demonstrated a moderate and transient ability to reduce the level of infectivity. The lack of more pronounced results may be due to the highly productive nature of infection of this cell line, a functional abnormality induced by the viral infection, or the development of resistance to the effects of the cytokines (37). Much more dramatic effects were seen when these agents were used prophylactically and maintained in the cell culture system. Substantial reductions in reverse transcriptase activity and near elimination of all antigen-positive cells were seen. Complete protection was not provided, however, as productive infection returned to cultures after removal of the cytokines.

IFN-α has been shown previously to restrict HIV infection *in vitro* (18). IFN-γ is a potent macrophage-activating factor (38), and, although its antiviral activity is sometimes less than that of IFN-α or -β (39), its effectiveness in this system may relate to its specific activity on macrophages. In addition, the importance of IFNs in lentivirus infections is illustrated by the recent description of a unique IFN induced by visna virus, which is involved in both the restriction of viral infection and possibly in immunopathologic injury (40, 41).

GM-CSF is a member of a group of compounds that can stimulate functional activities in macrophages (24), but its antiviral potential has not been fully evaluated. Our data suggest that GM-CSF may well have a role in restricting HIV infection under certain conditions. The mechanism of GM-CSF's antiviral activity is not clear, although it is likely not mediated by oxidative metabolism (42). The restriction of HIV expression described may relate to the level of monocyte–macrophage differentiation, a factor of known importance in other lentivirus systems. For example, as monocytes mature into macrophages, their permissiveness for caprine arthritis–encephalitis virus increases (31). It is interesting to speculate that the opposite may be true for HIV in that GM-CSF restricts virus replication, while it may simultaneously be leading to increasing differentiation of U-937 cells. Further, GM-CSF has attraction for clinical applications in AIDS patients because of the cytopenias that are common accompaniments of that disease.

These *in vitro* studies suggest that biologic-response modifiers should receive further evaluation in the treatment of AIDS and related conditions despite disappointing results reported in early trials (20, 21). GM-CSF and IFN-γ may prove to be especially useful as the role of the monocyte/macrophage system in the pathophysiology of this disease is further elucidated. Their ultimate utility may be in combination with specific antiviral agents as they may prove useful in maintaining the virus in a nonproductive state.

We thank Ms. Ruth Colman for her invaluable assistance in the preparation of this manuscript. This work was supported in part by Grant HL33774-01 from the National Heart, Lung, and Blood Institute (to J.E.G.).

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