A Glycoprotein Inhibitor of In Vitro Granulopoiesis Associated With AIDS

By Ira Z. Leiderman, Michael L. Greenberg, Bernard R. Adelsberg, and Frederick P. Siegal

Patients infected with the human immunodeficiency virus (HIV) often present with neutropenia. To elucidate the mechanism(s) of this HIV-related neutropenia, we assessed the proliferative capacity of the granulocyte-macrophage progenitor cell (CFU-GM) from the bone marrow (BM) of 78 patients within the AIDS spectrum manifesting symptoms or signs related to HIV infection. Of these, 70 had a significant deficit in the growth of this committed progenitor when compared with normal controls (P < .01). Further analysis revealed that the nucleated bone marrow cells from AIDS and AIDS-related complex (ARC) patients inhibited the growth of CFU-GMs from normal individuals when cocultured in agar (P < .001). Control CFU-GMs were also inhibited when they were cultured over feeder layers containing patients' BM cells (P < .001). Conditioned media obtained from the liquid culture of patients' BM cells did not inhibit normal control CFU-GM growth to a degree different from that of the cells themselves (P > .4). Analysis of these conditioned media by polyacrylamide gel electrophoresis (PAGE) revealed a unique glycoprotein (gp) with a mol wt of 84 kd. Further studies revealed that this gp possessed the inhibitory activity. These data suggest that this gp may be an important factor in HIV-related neutropenia. The presence of gp44 was independent of drugs administered to the patients.

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were allowed to clot at 4°C. The samples were centrifuged at 2,000 g for 10 minutes at 4°C, and the serum was collected and frozen at −70°C until used.

Cell separation. The anticoagulated BM was transferred from the syringe to a sterile polystyrene centrifuge tube (Corning, Corning, NY) and centrifuged at 250 g for 10 minutes at ambient temperature (22 ± 2°C). The buffy coat was aspirated and diluted with 2.5 mL McCoy's 5a medium. A nucleated cell count was performed manually, and the cell suspension was diluted to the appropriate concentration with McCoy's 5a.

Anticoagulated PB was transferred to a sterile disposable centrifuge tube (Corning) and diluted with an equal volume of sterile Hank's balanced salt solution (HBSS, Gibco). Ten milliliters of a Ficoll-Hypaque solution (sp gr = 1.077) (LSM, Litton Bionetics, Kensington, MD) was layered under 40 mL diluted blood. The gradient was centrifuged at 250 g for 20 minutes at 4°C. The mononuclear cells (PB-MNCs) were aspirated from the Ficoll-Hypaque/plasma interface and washed twice with HBSS and were then diluted with 2.5 mL McCoy's 5a to the appropriate concentration.

Granulocyte-macrophage progenitor cell culture. A short-term, similar to the system originally described by Pike and Robinson. Cells in McCoy's 5a were cultured in a final concentration of 10% heat-inactivated fetal bovine serum (FBS, Gibco), 10% giant cell tumor-conditioned medium (GCT-CM; Gibco) as a source of colony-stimulating factor (CSF), and 2 mmol/L l-glutamine and 1% antibiotic solution (100X PSF; Gibco). Warm bacto agar (Difco, Detroit) was added to a final concentration of 0.3%, and the suspension was well mixed. Then, 1.0 mL was dispensed into each of four 35 × 10-mm tissue culture dishes (Corning) and allowed to solidify. Final cell concentrations were 2.0 × 10^8/mL for BM cells and 1.0 × 10^9/mL for PB-MNCs. The cultures were incubated in an atmosphere of 5% CO₂/95% air at 37°C for 10 days. Colonies were scored as ≥40 cells/aggregate. All cultures were scored in triplicate on day 10. These final concentrations of BM nucleated cells and PB-MNCs were chosen because they produced approximately the same CFU-GM growth in healthy controls.

Conditioned medium. Nucleated BM cells from patients and controls were prepared as described above. The nucleated cells were diluted to a concentration of 1.0 × 10^6/mL in McCoy's 5a supplemented with 20% heat-inactivated FBS. The cell suspension was dispensed into 25 cm² or 75 cm² tissue culture flasks (Corning), depending on the final volume. The cells were cultured in an atmosphere of 5% CO₂/95% air at 37°C for 7 days. The CM was harvested by double centrifugation to remove all cells. The cell-free CM was frozen at −20°C until used.

Feeder layers. Feeder layers (FLs) were prepared from BM cells or CM. The cells or CM was diluted in McCoy's 5a and supplemented with a final concentration of 20% heat-inactivated FBS, 2 mmol/L l-glutamine, and 1.0% antibiotic solution (100X PSF). Warm bacto agar was added to a final concentration of 0.5%. After thorough mixing, 1.0 mL of suspension was dispensed into 35 × 10-mm tissue culture dishes and allowed to solidify. FLs were overlaid with normal target cells in 0.3% agar as described above. The amount of CM, or CM precipitate or PAGE eluate (below), used in these cultures was the amount calculated to be produced by 0.2 × 10^8 BM cells.

Ammonium sulfate precipitation. CM was precipitated with saturated ammonium sulfate, which was added dropwise to an equal volume of CM with continuous stirring at 4°C. The stirring was continued for 2 hours. The CM/salt solution was then allowed to sit undisturbed overnight at 4°C. The precipitate was then pelleted by centrifugation at 15,000 g for 20 minutes at 4°C. The supernatant was saved, and the pelleted precipitate was resuspended in McCoy's 5a to a volume equal to the starting volume of the CM. Both the resuspended precipitate and the supernatant were dialyzed against 0.015 mol/L phosphate-buffered saline (PBS) at 4°C. Three buffer changes were made over 48 hours of dialysis. The dialyzed material was then removed and stored at −20°C until used.

PAGE. Ammonium sulfate precipitates of the CM were diluted 1:5 with sample buffer pH 6.8 (0.5 mol/L Tris-Cl, 1.25% glycerol, 0.05% bromophenol blue) and then run on 7.5% native polyacrylamide gels (PAGE); mol wt was determined in 7.5% gels containing 1% sodium dodecyl sulfate (SDS). All gels were run at a constant current as a discontinuous system with the 7.5% running gel at pH 8.8, the 3% stacking gel at pH 6.8, and the Tris-glycine running buffer at pH 8.8. Proteins running into the gel were visualized by silver staining. Glycoprotein (gp) bands were visualized by periodic acid-Schiff (PAS) staining.

Elution of proteins from PAGE. Proteins were eluted from the gel slices in an ISCO sample concentrator (Model 1750, ISCO, Lincoln, NB). In brief, lanes containing CM from both patients and controls were cut from the gels, and the individual lanes were sectioned. The gel sections were placed in individual sample cups to which 10 mL Tris-glycine buffer (pH 8.8) was added. Elution of the proteins was achieved at 3 W constant power for 2 hours. At that time, the protein/buffer solution was removed and dialyzed as described above. After dialysis, the protein concentration was determined by the method of Bradford. This dilute protein solution was concentrated by centrifugation through a Centricon-10 microcentrifuge (Amicon, Danvers, MA), and the final protein concentration was redetermined. Inhibitory activity was assayed by adding the concentrated eluate to the FL in place of the CM.

Western blot. The Western blot technique was adapted from the EIA described by Tsang and colleagues. Antigen is run into a native or SDS-PAGE. A nitrocellulose membrane (BA-85; Schleicher and Schuell, Keene, NH) is placed over the gel, and the gel/nitrocellulose is sandwiched between two plastic pads. The sandwich is then immersed into cold Tris-glycine/methanol buffer and electrophoresed with constant voltage for 12 to 14 hours at 4°C. The nitrocellulose membrane is then incubated with the first antibody, rewarshed, and then reincubated with a horseradish peroxidase-conjugated second antibody. Diaminobenzidine is then added, and any positive bands are visualized.

Statistics. Data are presented as the mean ± 1 SD. Statistical significance of the differences between groups was determined by Student's t test.

RESULTS

Leukocyte counts. WBC and neutrophil counts for all patients studied are summarized in Table 1. The normal WBC count for normal adults in this laboratory is 4.8 to 10.8 × 10^9/mL, and the normal neutrophil count is 1.9 to 8.0 × 10^9/mL. The mean ± SD WBC count of all AIDS patients studied was 3.3 ± 1.8 × 10^9/mL, for ARC patients 4.5 ± 1.7 × 10^9/mL, and for PGL patients 5.0 ± 0.6 × 10^9/mL. The mean ± SD absolute neutrophil counts were 2.1 ± 0.9 × 10^9/mL, 2.5 ± 1.2 × 10^9/mL, and 3.0 ± 0.6 × 10^9/mL for the patient groups, respectively. The WBC and neutrophil counts were not significantly different between the three HIV-infected groups. There was no significant difference between the WBC or neutrophil counts of the homosexual and drug abuser groups studied or between homosexual patients with Pneumocystis carinii pneumonia (PCP), Kaposi's sarcoma (KS), or other opportunistic infections (OIs). The WBC and neutrophil counts of AIDS and
ARC groups were significantly reduced when compared with those of the normal population \((P < .01)\).

**CFU-GM proliferation.** As shown in Fig 1, BM cells from 53 AIDS patients, 20 ARC patients, and 5 PGL patients each generated significantly fewer CFU-GM colonies than did controls \((P < .01)\). No significant differences were noted among AIDS, ARC, and PGL patients, among the various risk groups with AIDS described above, or when the patients with AIDS who had PCP, other OIs, or KS without OI were compared \((P > .5\) for each comparison), although each group was significantly reduced when compared with normal subjects \((P < .01)\).

**Coculture and FLs.** Control PB-MNCs were cultured for CFU-GM in the presence of 10% serum from AIDS

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**Table 1. Leukocyte and Neutrophil Counts of Patients**

<table>
<thead>
<tr>
<th>Patients* (n)</th>
<th>Leukocyte Count (Range)</th>
<th>Neutrophils (Range)</th>
</tr>
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<tbody>
<tr>
<td>AIDS (53)</td>
<td>3.3 ± 1.8 (1.0-8.6)</td>
<td>2.1 ± 0.9 (0.1-7.5)</td>
</tr>
<tr>
<td>ARC (20)</td>
<td>4.5 ± 1.7 (2.8-7.0)</td>
<td>2.5 ± 1.2 (1.2-4.8)</td>
</tr>
<tr>
<td>PGL (5)</td>
<td>5.0 ± 0.6 (4.2-5.9)</td>
<td>3.0 ± 0.6 (2.4-3.8)</td>
</tr>
<tr>
<td>AIDS, by risk group†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homosexual (38)</td>
<td>3.3 ± 1.6 (1.0-7.7)</td>
<td>2.1 ± 0.98 (0.13-4.2)</td>
</tr>
<tr>
<td>IVDA (13)</td>
<td>3.6 ± 3.4 (1.6-8.6)</td>
<td>3.4 ± 3.1 (1.2-7.5)</td>
</tr>
<tr>
<td>Hemophiliac (1)</td>
<td>1.5</td>
<td>0.7</td>
</tr>
<tr>
<td>Wife of IVDA (1)</td>
<td>5.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Homosexuals with AIDS, by infection/neoplasm‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCP (16)</td>
<td>2.9 ± 1.4 (1.1-5.2)</td>
<td>2.1 ± 1.3 (0.13-4.2)</td>
</tr>
<tr>
<td>Other OI (14)</td>
<td>3.5 ± 2.2 (1.0-7.7)</td>
<td>1.9 ± 0.8 (0.67-3.3)</td>
</tr>
<tr>
<td>KS (8)</td>
<td>3.9 ± 0.6 (1.1-5.2)</td>
<td>2.3 ± 0.4 (1.8-2.6)</td>
</tr>
</tbody>
</table>

Neutrophil counts are mean count \(\times 10^6\) ± 1 SD/mL. Normal leukocyte count = 7.8 (4.8-10.8) \(\times 10^6\)/mL; normal neutrophil count = 4.0 (1.9-8.0) \(\times 10^6\)/mL.

* Differences between AIDS and ARC counts were not significant.
† Differences between leukocyte and neutrophil counts of homosexuals and IVDA were not significant.
‡ Differences between leukocyte and neutrophil counts of homosexuals with PCP, and other OIs, and KS were not significant.

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**Fig 1.** Comparison of growth of the granulocyte-macrophage progenitor cell colonies (CFU-GMs, mean ± SEM) from 9 healthy controls (35.7 ± 1.8), 5 patients with PGL (8.8 ± 1.1), 20 ARC (16.4 ± 3.2), and 53 AIDS (11.32 ± 1.7). There was a statistically significant difference \((P < .001)\) in the number of CFU-GM colonies between the control group and each of the three groups of patients, but not between the patient groups. There was no significant difference between the patient groups. Within the AIDS group, there was no significant difference between the patients with an opportunistic infection (treated or untreated) and those with a neoplasm characteristic of AIDS (not shown). No patients had received antineoplastic chemotherapy prior to being studied.

**Fig 2.** Dose-related inhibition of control CFU-GMs by BM-nucleated cells from AIDS patients. BM-nucleated cells from patients \(n = 10\) with AIDS were isolated in FLs at concentrations of 0.05, 0.1, 0.2, and 0.4 \(\times 10^6\) cells/mL and overlaid with 0.2 \(\times 10^6\) control BM-nucleated cells (○) or PB-MNCs (○). These control cells had progressive decreases in colony growth of 20.2%, 44.2%, 68.1%, and 80.1%, respectively, when compared with control cells grown over FLs devoid of cells (heavy lines are the mean number of colonies; broken lines are the SEM). Mean number of colonies grown over FLs containing normal control BM nucleated cells (○) (shaded area denotes SEM). The decrement seen for each group was significantly different \((P < .001)\) from values for control cells grown over cell-free FLs or FLs containing 0.2 \(\times 10^6\) control BM cells.
patients. No difference was seen between these cultures and those cultured with or without heterologous control serum (data not shown). In contrast, coculture of AIDS BM-nucleated cells with control PB-MNCs in 0.3% agar revealed significant, sometimes total, colony growth inhibition \((P < .001)\).

When FLs containing varying numbers of AIDS-BM nucleated cells were overlaid with \(1 \times 10^6\) control PB-MNCs, CFU-GM growth at maximal inhibition was similar to that from the BM cells from the AIDS patients. Decreased colony formation by the control cells was seen in a dose-response fashion with increasing concentrations of patients’ cells (Fig 2).

**Conditioned media.** Cell-free CM prepared from the BM-nucleated cells from eight patients with AIDS and two controls were added to the FLs at a final concentration of 10% in place of BM cells. The CM from all the AIDS patients significantly \((P < .001)\) inhibited the CFU-GM growth of normal PB-MNCs in the same fashion as did AIDS BM-nucleated cells. Control CM had no effect on the cultures. After ammonium sulfate precipitation of the AIDS CM, the inhibitory activity was recovered from the precipitates (Fig 3) but not from the supernatants (not shown). Again, control CM showed no inhibitory activity in either fraction.

**PAGE.** The ammonium sulfate precipitates of the patients’ and controls’ CM were run in 7.5% polyacrylamide gels. A unique band was observed in the lanes containing the patients’ CM but was absent in the control lanes (Fig 4). This protein, absent in the control lanes, had a relative molecular mass of 84 kd as determined by SDS-PAGE (Fig 4). PAS staining of these gels revealed a gp band in the same region as the unique band, but the 84-kd region was PAS negative in the control lanes.

The proteins were electrophoretically eluted from serial slices of 7.5% preparative PAGE. The proteins isolated in this manner had inhibitory activity in the CFU-GM assay in the same fashion as described for the CM. The inhibitory activity was recovered from proteins eluted from the region of the gel that contained the unique band (Fig 3). No other proteins with inhibitory activity were recovered from any other region of these lanes or from control lanes.

**Western blotting.** Resolubilized ammonium sulfate precipitates of CM were electrophoresed into 7.5% SDS-PAGE and were then blotted as described. The blotted proteins were then probed with polyclonal and monoclonal antibodies raised against HIV antigens (generously provided by Drs Robert Gallo and S. Zaki Salahuddin of the National Cancer Institute). The 84-kd band was not recognized by any of these antibodies.

**DISCUSSION**

Leukopenia and neutropenia are frequent findings in patients within the AIDS spectrum. To elucidate the mechanism of the neutropenia, we assessed the proliferation of CFU-GMs from the BM cells of 78 patients within the AIDS spectrum (AIDS, ARC, and PGL). BM cells from 70 of 78 patients made significantly fewer CFU-GM colonies than did controls \((P < .01)\).

The number of CFU-GM colonies from groups of patients with AIDS, ARC, or PGL were not statistically significantly different from each other, indicating that this decrease was not related to the severity of clinical disease. Statistical comparison of the number of CFU-GMs among the various risk groups also showed no significant differences. Homosexual men, the largest group of AIDS patients we studied, were further divided into three subgroups: those with PCP, other OIs, or with KS with no OI. No significant differences were seen between these subgroups in the number of CFU-GM.
colonies, indicating that there was no relationship to any type of secondary infection or malignancy. Of particular interest, 60% of the patients with PCP but none of the others had been treated with trimethoprim-sulfamethoxazole. Although this drug combination has been well documented to cause neutropenia and AIDS patients have been reported to be especially sensitive, these results from the various subgroups indicate that the neutropenia and reduced CFU-GMs in our patients were not consequent to such drug therapy. The apparent exquisite sensitivity to the drug may be the result of a background of preexisting defective granulopoiesis with little or no reserve capacity when even minor additional suppression is superimposed.

Coculture of control PB-MNCs with AIDS BM cells revealed a profound decrease in the number of colonies formed. To determine whether this decrease in CFU-GM colony growth was due to a cell–cell interaction or to a factor released by the cells into the culture milieu, patients' BM-nucleated cells were immobilized in a FL without CSF, over which the control cells were cultured with added CSF. Because the normal CFU-GMs can be progressively inhibited with increasing concentrations of physically separated patients' cells in the FL, we established the humoral nature of the suppression.

To identify further the factor(s) responsible, CM were obtained from liquid cultures of AIDS BM-nucleated cells. CM added to cell-free FLs inhibited growth to a degree comparable to coculture with BM cells (Fig 3). The inhibitory factor was precipitated by 50% ammonium sulfate. PAGE analysis of the CM precipitate and PAS staining revealed that the inhibitory factor is a gp with a relative mol wt of 84 kd. The 84-kd bands electrophoretically eluted from the gels had inhibitory activity identical to that of the CM.

We previously reported that excessive margination of neutrophils was not found in AIDS patients and that five of six AIDS patients had a decreased mobilizable BM neutrophil storage pool in vivo. Morphological observations of BM biopsies confirmed a decrease in the mature neutrophil pool. Because the fraction of BM myeloid precursors in DNA synthesis was within the normal range, BM granulopoiesis may not respond appropriately to the neutropenia. Although our data from in vitro cultures cannot be directly compared with the above observations in vivo, the decrease in the BM storage pool of mature neutrophils could be explained at least in part by inhibition of stem cell activity. The inhibitory activity on BM neutrophil progenitors demonstrated in vitro might also affect more mature precursors. The decrease in the storage pool, in turn, would explain a profound decrease in CFU-GMs associated with a less profound decrease in blood neutrophils since one can maintain blood neutrophils at the expense of the storage pool.

Donahue et al have reported that sera and immunoglobulins from HIV-infected individuals suppressed CFU-GMs and BFU-Es from AIDS and ARC patients but not from healthy controls. They observed that antibodies against the HIV envelope gp130, inhibit CFU-GM, thus suggesting that this antibody reacts with HIV infected progenitors and suppresses them. Similarly, we found that sera from patients with AIDS had no effect on CFU-GMs from healthy controls, but the novel gp that we report here differs from the findings of Donahue et al since it is not an immunoglobulin and is not cross-reactive with antibodies against HIV. In other unpublished studies, we found no evidence of inhibition of BM cells or PB BFU-Es or CFU-Es by control or patient CM. In addition, CM added to control PB-MNCs stimulated with varying concentrations of phytohemagglutinin, pokeweed mitogen, and concanavalin-A did not decrease the blastogenic response to these mitogens. Thus, the specificity of gp84 is different from that reported by Donahue et al and from an inhibitor of lymphocyte proliferation in AIDS patients described by Laurence and Mayer. Our Western blot data do not preclude the possibility that gp84 is an HIV product, although it is more likely a secondary product of HIV infection.

Inhibitors of CFU-GMs have been well described in numerous acute diseases and syndromes. Many of these inhibitors are T lymphocyte-mediated, whereas others are factors released by other specific cell populations. The gp that we demonstrated has different physiochemical characteristics from these previously described factors.

FeLV, a retrovirus with similarities to HIV, induces an immune-deficient state in some infected cats, many of whom are neutropenic as well. These infected, neutropenic animals have a decrease in the number of BM CFU-GMs, although no explanatory mechanism has been proposed. Conceivably, the neutropenia secondary to FeLV infection is analogous to the neutropenia associated with AIDS.

Our demonstration of a gp that inhibits granulopoiesis in vitro possibly provides an explanatory mechanism for previous observations of neutropenia in patients within the AIDS spectrum.

Preliminary experiments suggesting the specificity of the inhibitor raise the possibility that a series of specific viral products or virally induced products may explain some of the many cellular defects observed in these patients. The lack of reactivity with known antibodies to HIV suggests that gp84 is not a viral constituent, although gp84 may be produced by a population of BM cells in response to HIV infection.

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