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Monocyte Activation by Circulating Fibronectin Fragments in HIV-1-Infected Patients

JoAnn Trial,*† Jose A. Rubio,*† Holly H. Birdsall,*†§ Maria Rodriguez-Barradas,† and Roger D. Rossen²*‡§

To identify signals that can alter leukocyte function in patients receiving highly active antiretroviral therapy (HAART), we analyzed single blood samples from 74 HIV-1-infected patients and additional blood was collected at 90-day intervals from 51 HIV-1-infected patients over a 516 ± 172 (mean ± SD) day interval. Despite the absence of circulating immune complexes and normalization of phagocytic function, compared with controls, the proportion of patients’ monocytes expressing CD49e and CD62L was decreased and expression of CD11b and CD86 increased. Plasma from 63% of patients but none from normal controls contained 110–120 kDa fibronectin fragments (FNf). Presence of FNf did not reflect poor adherence to therapy. Addition of FNf to normal donor blood in vitro replicated changes in monocyte CD49e, CD62L, CD11b, and CD86 seen in vivo. FNf also induced monocytes to release a serine proteinase, nominally identified as proteinase-3, that hydrolyzed cell surface CD49e. α1-Antitrypsin blocked FNf-induced shedding of CD49e in a dose-dependent manner. Plasma with a normal frequency of CD49e+ monocytes contained antiproteases that partially blocked FNf-induced monocyte CD49e shedding, whereas plasma from patients with a low frequency of CD49e+ monocytes did not block this effect of FNf. Electrophoretic analyses of plasma from the latter group of patients suggested that a significant fraction of their α1-antitrypsin was tied up in high molecular mass complexes. These results suggest that monocyte behavior in HIV-1-infected patients may be influenced by FNf and the ratio of protease and antiproteases in the cells’ microenvironment. The Journal of Immunology, 2004, 173: 2190–2198.

Before highly active antiretroviral therapy (HAART),¹ leukocyte functions declined significantly during the progression of HIV-1 infections (1–3). As the disease continues, leukocyte phagocytic activity, which is normal or even enhanced in asymptomatic (stage A) patients (1, 2), becomes defective (3) as do monocyte chemotactic responses, oxidative burst, and transendothelial migration (3–5). By reducing viral burden (6), HAART may remove stimuli that alter leukocyte functions. This hypothesis is supported by observations of the effect of antiretrovirals on infected monocytes (7, 8) and by reports that HAART improves patients’ leukocyte function in vivo (9). Although the agents that cause these leukocyte function defects remain poorly characterized, viral proteins, debris from apoptotic leukocytes, soluble immune complexes, and activated complement fragments have all been implicated (3, 7, 10). In the pre-HAART era, circulating soluble Ag-Aβ complexes, in particular, appeared to be responsible for the decline in monocyte phagocytic function. Immune complexes were not detected in normal donors. Monocytes were placed in decreased monocyte VLA-5 (α5β1), CD29/CD49e and L-selectin (CD62L) expression and increased cell surface display of β2 integrins in stage B and C patients (3). In this study, we report that, with HAART, circulating immune complexes are no longer in evidence. Monocyte phagocytic activity has normalized. But flow cytometric analyses of blood mononuclear leukocytes suggest that patients’ monocytes remain activated. Activation appears to result, in part, from stimulation by circulating cell-binding 110–120 kDa fibronectin fragments (FNf). We postulate that continued viral replication, even at the low levels (11), stimulates release of enzymes that degrade fibronectin (FN) and other proteins (12–14), producing fragments with novel biological properties (15–19), including some that modify monocyte/macrophage behavior (20).

Materials and Methods

Human subjects

Seventy-four antiretroviral drug-experienced HIV-1-infected men were recruited at the Michael E. DeBakey Veterans Affairs Medical Center under protocols approved by the Institutional Review Boards of Baylor College of Medicine. CD4 T cell counts and viral RNA load measurements in these patients were performed by the hospital clinical laboratory. Centers for Disease Control and Prevention (CDC) disease stages for these patients were A1 = 10, A2 = 9, B1 = 9, B2 = 10, C1 = 2, C2 = 13, and C3 = 21. Seventeen healthy sex- and age-matched individuals served as controls. Venous blood was collected in polypropylene syringes using preservative-free heparin (Squibb-Marsam, Cherry Hill, NJ) that was free of endotoxin to the detection limit (10 pg/ml) of the Limulus amebocyte assay (Associates of Cape Cod, Woods Hole, MA). Aliquots of heparinized plasma were stored at −120°C in the vapor phase of liquid nitrogen. Some were stabilized with 1 mM EDTA and 0.2 mM PMSF to retard degradation of plasma proteins. Other aliquots, destined for use in cell stimulation assays, were stored at −120°C without inhibitors to avoid potentially confounding toxicity. Adherence to antiretroviral therapy was evaluated in each case by reviewing the patients’ timeliness in collecting monthly refills of their medications during the 90-day interval before blood collection. Patients were judged adherent if they collected refills of their prescriptions within a ±14-day interval before or after the expiration date of the prescription. Patients who failed to collect their monthly refills within that interval were considered poorly compliant.
Flow cytometry

Flow cytometric analyses were performed as previously described (3) on a Beckman Coulter FACSCaliber XL cytometer, which was calibrated daily with Flow-Check fluorospheres (Beckman Coulter, Miami, FL). Photomultiplier tube voltages were adjusted each day to a target range of fluorescent intensities by means of Flow-Set fluorospheres (Beckman Coulter) to remove variability attributable to day to day fluctuations in instrument performance. Normal donor samples were obtained among patient samples to monitor and forestall drift in assay results attributable to inadvertent changes in sample handling or reagents. We used two-color fluorescence to measure surface markers on CD14-positive monocytes. mAbs from Beckman Coulter included anti-CD14 (clone M5E2), anti-CD11b (CR3, clone Bear1), anti-CDF16 (clone 3G8), anti-CDF64 (clone 22), anti-CDF62L (DERG56), and anti-CDF49c (clone SAM1). Anti-CD32 (clone 7.3) was obtained from Ancell (Bayport, MN) and anti-CD49e (clone IT2.2) from Bio-Rad Laboratories (Hercules, CA). FITC-labeled anti-CD14 (clone M5E2) from BD Pharmingen (Los Angeles, CA). Isotype control Abs were obtained from BD Biosciences (San Jose, CA) and anti-human proteinase-3 mAbs from Weislab (Lund, Sweden) and from Research Diagnostics (clone IB10, Research Diagnostics, Flanders, NJ). Goat F(ab)_2 anti-rabbit Ig, human Ig absorbed and conjugated to PE, was obtained from BioSource International (Camarillo, CA).

Phagocytosis and immune complexes

Staphylococcus aureus, ATCC strain 25923, a gift from Dr. J. Cillardre (Veterans Affairs Medical Center, Houston, TX), was stained with Texas Red (Molecular Probes, Eugene, OR). Uptake and phagocytosis of the opsonized bacteria by CD14+ monocytes in whole blood was measured as previously described (1). Soluble immune complexes were measured by the CIE-C1q ELISA; results are presented in micrograms per milliliter equivalents of heat-aggregated IgG (Quidel, San Diego, CA).

FN assays

Total plasma FN was measured by rocket electrophoresis in 1% Seakem ME agarose at pH 8.6 in Tris-barbital buffer containing rabbit anti-FN Abs, a gift from Dr. R. Baugh (Baylor College of Medicine). Serial dilutions of purified full-length human FN (Chemicon, Temecula, CA) were used to generate a standard curve that related FN concentration to the length of the rocket formed in anti-FN Ab-impregnated gels. Rockets were identified in images of washed gels that had been stained with Coomassie blue, destained, and dried.

To identify and measure FN fragments, plasma samples were fractioned by electrophoresis under reducing conditions in 7.5% precast polyacrylamide gels purchased from Bio-Rad (Hercules, CA). Having determined the quantity of FN in the transblots a single gel, the bands resulting from the transfer of reduced FN were destained, and dried. Images of washed gels that had been stained with Coomassie blue, destained, and dried. To identify and measure FN fragments, plasma samples were fractionated by electrophoresis under reducing conditions in 7.5% precast polyacrylamide gels purchased from Bio-Rad (Hercules, CA). Having determined the quantity of FN in the transblots no longer increased in proportion to the increase in quantity of FN. Thereafter, to increase the precision of measurement of FN110 and to estimate the uniformity or lack of uniformity in the loading of these gels, we included three controls in each gel (Fig. 1). Controls contained 0.6 μg of native FN and either 0.06, 0.12, or 0.18 μg of FN110; these concentrations of FN110 corresponded to the load of protein that would be delivered by adding 30 μl of plasma diluted 1/15 that contained, respectively, 30, 60, and 90 ng/mL FN.

In vitro treatment of cells with FNf

To evaluate the stimulatory influences of FNf, 100-μl aliquots of heparin-anticoagulated whole blood were treated with the indicated quantities of purified FNf, in polypropylene tubes, at 37°C for 90 min, unless otherwise noted. To investigate the effect of serine proteinase inhibition on the impact of FNf on expression of CD49e, CD62L, CD11b, and CD86 on monocytes, we added 10 μg/mL FNf for 1 h with or without 0.2 mM PMSF or increasing concentrations of Prostain (purified human α1-antitrypsin (α1-proteinase inhibitor, AIAT; Bayer Research, Triangle Park, NC). U937 cells (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 plus 10% FBS (HyClone, Logan, UT) without other stimuli.

AIAT and proteinase-3 assays

AIAT was identified by probing immunoblots of patient plasma, fractionated on Superase 12 anion exchange column, with rabbit anti-human AIAT (catalogue no. A-0409; Sigma-Aldrich); serial dilutions of highly purified human AIAT (catalogue no. A-6150; Sigma-Aldrich) provided standards for these experiments. The adequacy and reproducibility of sample loading of these gels was verified by densitometric analysis of the 45-kDa AIAT in the developed immunoblots. To identify some of the enzymes that may be associated with AIAT in plasma, A1AT was immunoprecipitated from plasma with rabbit anti-human AIAT (catalogue no. A-0409; Sigma-Aldrich) adsorbed to staphylococcal protein A-coated polystyrene beads (Roche, Mannheim, Germany). The plasma samples (samples 1, 3, 4, and 5, see Fig. 4) used in these experiments were preclared with Pansorbin (Calbiochem, San Diego, CA) (killed S. aureus rich in protein A) coated with mouse anti-rabbit IgG (20). Protein A-coated beads used to collect the AIAT from the patient plasma samples were washed repeatedly with 0.1% Tween 20 in PBS to remove unbound plasma proteins and then stripped with reducing buffer. Eluted proteins were fractionated by electrophoresis in 5–20% gradient polyacrylamide gels and blotted to nitrocellulose. Transblots were developed with rabbit antiserum against human proteinase-3 (Elastin Products, Owensville, MO), sheep anti-cathepsin G (Biogenesis, U.K.), or rabbit anti-leukocyte elastase (Research Diagnostics). The Abs to cathepsin G, elastase, and proteinase-3 did not react with purified AIAT in a dot blot assay or after fractionation by PAGE and transfer to nitrocellulose paper.

Statistical analysis and graphic representations of data were performed using STATISTICA 6 (StatSoft, Tulsa, OK).

Results

Monocyte cell surface markers, phagocyte function, and potential activating stimuli

Circulating monocytes were activated in CDC stage A patients, even in those who had undetectable viral loads (viral load, <400 copies/ml; Table I). Expression of CD49e and CD62L was significantly decreased, and cell surface display of CD11b, CD40, CD86, and CD64 (FcyRII) was increased. Expression of CD32 (FcyRII) and CD16 (FcyRIII) remained within normal limits. Monocytes of patients that had progressed to CDC stage C displayed even further abnormalities of CD11b and CD64. However, monocyte phagocytosis remained within normal limits and C1q binding immune complex levels were not different in normal control plasmas and samples from patients at any stage of the disease (Table I).

Initial plasma samples from 47 (63%) of the 74 patients contained 110–120 kDa FNf. Representative immunoblots of patients’ plasma samples (Fig. 1, C and D) illustrate the wide range of FNf found in these plasma samples. Sample 4 (Fig. 1C) is indicative of what we saw in transblots of normal donor plasma. These had no detectable FNf. Some plasmas like samples 3, 5, and 9 lacked 110-kDa FNf but they did contain lower molecular mass fragments. Fragments <90–100 kDa were also found in samples like no. 1 that we estimated contained 13 μg/ml and in samples like no. 6 that contained 38.1 μg/ml of the 110–120-kDa fragment. The
estimated quantities of 110–120 kDa FN in samples 2, 7, 8, and 10 were <10 μg/ml. Some plasma samples contained a discreet 40-kDa fragment as seen in samples 6 through 10. Although we could not assign a protein weight to these low molecular mass fragments for lack of defined purified standards, we measured and recorded their density. The cumulative density of the <90- to 100-kDa fragments correlated significantly with the quantity of 110–120 kDa FNf in these plasma samples (r = 0.51, p < 0.01), consistent with the notion that the processes that caused plasma to contain relatively high molecular mass fragments of FN are also likely to create smaller degradation products as well. However, in contrast to the 110- to 120-kDa FNf, we were unable to show any relationship between the independent density measurements for these low molecular mass fragments and monocyte expression of CD49e and other cell surface molecules discussed below.

In plasma collected at 90- to 100-day intervals over a 516 day period (mean ± SD) from 51 patients and 17 controls, we found 110–120 kDa FNf in all serial samples from 17 patients and in 26 (46%) of 57 serial samples from another 16 patients. FNf were never detected in plasma from 18 patients nor in any control donor plasmas. FNf were found in 47 (49%) of 95 plasma samples from stage B patients (Table I) and another (Fig. 2A) in which plasma FNf correlated with the percentage of CD49e monocytes. This replicates what we have demonstrated previously (20), that in vitro FNf causes a substantial dose-dependent decrease in the fraction of monocytes expressing CD49e. Fig. 1E also shows, in comparison, the percentage of CD49e+ monocytes in the blood of patients whose plasma contained FNf. With two exceptions, the percentage of patients’ monocytes expressing CD49e was much higher than one would see if the same concentrations of FNf were introduced into normal donor blood in vitro. Indeed, in one-half of the samples shown in Fig. 1E, the fraction of monocytes displaying CD49e− was within normal limits. Consequently, there was no correlation between plasma FNf levels and the percentage of CD49e− monocytes in patient blood. The summary data in Table II, however, suggests that the presence of FNf in the plasma was associated with a significant decrease in the percentage of CD49e+ monocytes. To understand this apparent discrepancy, we plotted the relationship between FNf and the percentage of monocytes that expressed CD49e in serial samples from single patients. Fig. 2 shows representative data from this analysis and reveals two categories of patients: Those in whom plasma FNf levels bore no relationship to the percentage of monocytes that express CD49e (Fig. 2A) and another (Fig. 2B) in which plasma FNf correlated reciprocally with the percentage of CD49e monocytes.

Table II also suggests that the frequency of monocytes expressing CD62L was significantly decreased and expression of both CD11b and CD86 was significantly increased, whereas monocyte expression of CD40 and CD64 was unaffected by circulating FNf. These abnormalities in monocyte cell surface phenotype could also be induced, in vitro, by FNf stimulation. Addition of as little as 10 μg/ml FNf to blood from three normal donors significantly reduced the fraction of monocytes expressing CD62L and increased CD11b fluorescence intensity and the fraction of monocytes expressing CD86 (data not shown). Since FNf reduces CD49e expression by inducing monocytes to secrete a serine proteinase that cleaves some of the cell surface CD49e (20), we evaluated the possibility that changes, particularly in CD62L, that follow FNf stimulation might have a similar mechanism. Although treatment with the serine proteinase inhibitor PMSF prevented the loss of CD49e as we had seen before (20), this treatment did not affect the FNf-induced loss of CD62L nor the increased expression of CD86 and CD11b (data not shown).

**Investigations of mechanisms that regulate monocyte CD49e expression in the blood of HIV-infected patients that contain FNf**

Since reduction in the number of monocytes expressing CD49e after FNf treatment depends on the induction of a proteolytic...
enzyme that removes α4 molecules from these cells, we postulated that blood of patients who have a normal frequency of CD49e+ monocytes may contain inhibitors that interfere with the activity of these proteases. To test this hypothesis, we analyzed plasma from 16 patients. These samples contained between 17 and 98 μg/ml FNf. Eight were selected from blood that contained 94–99%
CD49e⁺ monocytes; the other eight were from blood that contained 68–86% CD49e⁺ monocytes. The last set of values is well outside the 95% confidence limits of the mean for normal controls. We tested the ability of these plasmas to induce U937 cell shedding of CD49e. U937 cells were chosen for these experiments for two reasons: Cells of this monocytoid line provide a uniform test platform that displays a far greater density of CD49e than normal monocytes and, because they lack CD14, they are insensitive to bacterial LPS, which can also cause human monocytes to shed CD49e (20). FNf-rich plasma from only 3 of the 16 patients induced a dose-dependent decrease in CD49e expression reminiscent of what we saw when increasing concentrations of FNf are added to normal donor blood samples (Fig. 1E). Results with one such plasma sample are shown in Fig. 3A. Notably, however, 13 of the 16 patient samples, regardless of FN content, had no effect on U937 cell expression of CD49e.

To investigate whether these plasma samples contained substances that opposed the effects of FNf, we evaluated their ability to block the ability of exogenous FNf to induce shedding of CD49e from U937 cells. We first identified a concentration (160 µg/ml) of FNf that, when added to U937 cells in serum-free medium, reproducibly caused a 50% decrease in CD49e fluorescence intensity under the standardized conditions of the assay. We then measured the ability of equal quantities of individual patients’ plasma to modulate the effect of the FNf. Fig. 3B shows that adding plasma from patients with a low frequency of CD49e⁺ monocytes had little influence, whereas plasma from patients with a high frequency of CD49e⁺ monocytes significantly inhibited the loss of CD49e induced by the added FNf, consistent with the notion that the latter samples contained an agent that partially blocked the activity of the protease induced by FNf.

**Proteolytic enzymes induced by FNf stimulation**

Although we knew that treatment with 110–120 kDa FNf or with GRGDSP, a peptide that contains the central RGD sequence in FNf, induces monocytes to produce a serine protease that cleaves cell surface-associated α5 molecules (20), the specific identity and localization of this enzyme and the kinetics of its appearance remained to be defined. Considering that stimulation with as little as 2 ng/ml TNF-α significantly increases monocytes’ cell surface display of proteinase-3 (21) and since FNf induces monocytes to release TNF-α (22), we postulated that the proteolytic enzyme induced by FNf may be proteinase-3 or another of the granular proteases that are up-regulated and brought to the cell surface by TNF-α stimulation (23). To evaluate this hypothesis, we added

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**Table II. Phenotypic differences among healthy controls and HIV⁺ patients with and without FNf**

<table>
<thead>
<tr>
<th>Monocyte markers</th>
<th>Healthy Controls</th>
<th>p (t test) ↔</th>
<th>FNf⁻ Samples</th>
<th>p (t test) ↔</th>
<th>FNf⁺ Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>% CD49e⁺</td>
<td>95.3 ± 4.3</td>
<td>&lt;0.01</td>
<td>91.7 ± 7.1</td>
<td>&lt;0.01</td>
<td>85.5 ± 13.9</td>
</tr>
<tr>
<td>% CD62L⁺</td>
<td>86.8 ± 7.9</td>
<td>&lt;0.01</td>
<td>80.2 ± 10.3</td>
<td>0.02</td>
<td>76.0 ± 14.1</td>
</tr>
<tr>
<td>% CD86⁺</td>
<td>9.0 ± 5.1</td>
<td>NS</td>
<td>10.8 ± 9.0</td>
<td>0.02</td>
<td>16.5 ± 14.2</td>
</tr>
<tr>
<td>CD11b mcf</td>
<td>2.3 ± 0.9</td>
<td>&lt;0.01</td>
<td>3.4 ± 1.3</td>
<td>0.05</td>
<td>4.0 ± 2.1</td>
</tr>
<tr>
<td>CD40 mcf</td>
<td>0.50 ± 0.1</td>
<td>&lt;0.01</td>
<td>0.7 ± 0.1</td>
<td>NS</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>% CD40⁺</td>
<td>29.3 ± 18.6</td>
<td>0.02</td>
<td>45.2 ± 22.0</td>
<td>NS</td>
<td>44.7 ± 23.5</td>
</tr>
<tr>
<td>CD64 mcf</td>
<td>2.9 ± 0.8</td>
<td>&lt;0.01</td>
<td>3.9 ± 0.9</td>
<td>NS</td>
<td>4.0 ± 1.3</td>
</tr>
<tr>
<td>Other parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>450 kDa FN (µg/ml)</td>
<td>336 ± 76</td>
<td>&lt;0.01</td>
<td>388 ± 113</td>
<td>&lt;0.01</td>
<td>344 ± 76</td>
</tr>
<tr>
<td>Viral load (HIV RNA cps/ml)</td>
<td>35,800 ± 129,400</td>
<td>NS</td>
<td>31,000 ± 94,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of CD4 T cells/µl</td>
<td>374 ± 267</td>
<td>NS</td>
<td>411 ± 273</td>
<td>NS</td>
<td>1.1 ± 0.8</td>
</tr>
<tr>
<td>No. of reverse transcriptase inhibitors</td>
<td>2.1 ± 0.8</td>
<td>NS</td>
<td>1.9 ± 0.9</td>
<td>NS</td>
<td>1.1 ± 0.8</td>
</tr>
<tr>
<td>No. of protease inhibitors</td>
<td>1.1 ± 0.8</td>
<td>NS</td>
<td>1.9 ± 0.9</td>
<td>NS</td>
<td>1.1 ± 0.8</td>
</tr>
</tbody>
</table>

* There were 38 samples from 17 healthy controls. The 89 FNf⁻ samples included all samples from the 18 patients who were consistently negative and negative samples from the 16 patients with fluctuating levels. Likewise, the 88 FNf⁺ samples included all samples from the 17 patients who were consistently positive plus positive samples from the 16 patients with fluctuating levels. All of the values that differed significantly between controls and FNf⁻ samples were also significantly different when controls were compared solely to samples taken from donors that were consistently negative. In 13 patients with fluctuating levels, the percentage of CD49 was significantly lower on days that FNf were present, based on paired t test analysis of 15 paired samples, taken sequentially from times when the FNf status changed (ρ = 0.047). Expression of CD62L, CD64, CD11b, CD64, and CD40 and total FN were significantly different only when all samples were considered. Data are presented as mean ± SD.

* mcf, Mean channel fluorescence.

* Average number of nucleoside or non-nucleoside reverse transcriptase inhibitors prescribed per patient during course of study.

* Average number of protease inhibitor per patient during the course of the study.
human A1AT immediately before adding FNf. D, in a dose-dependent manner, by pretreating the cells with 25 \mu l/mL normal plasma. E, pretreating patients caused a dose-dependent decrease in CD49e expression when added to U937 monocytoid cells. Normal donor plasma had no effect on CD49e expression. B, To evaluate whether patients’ plasma contained substances that inhibited the effects of FNf, we measured their ability to interfere with the shedding of CD49e induced by FNf. U937 cells, incubated in the presence of normal donor plasma and 160 \mu g/mL purified 110–120 kDa of FNf for 1 h, reliably shed 50% of cell surface CD49e. This loss of CD49e was unaffected by adding FNf-rich plasma from patients who had reduced numbers of CD49e+ monocytes (FNf pos; low CD49e). However, FNf-containing plasma from patients who had a normal fraction (95 ± 4%) of CD49e+ monocytes (FNf pos; high CD49e) suppressed the effects of added FNf. C, FNf-induced loss of CD49e by U937 cells could be reduced, in a dose-dependent manner, by pretreating the cells with 25–200 \mu g/mL human A1AT immediately before adding FNf. D, Treatment with a mAb to proteinase-3 suppressed the FNf-induced loss of CD49e, whereas secretory leukoprotease inhibitor (SLPI) to U937 cells before stimulation with FNf. SLPI inhibits leukocyte elastase and cathepsin G activity but does not inhibit proteinase-3 (24). We found that SLPI did not affect the loss of CD49e induced by FNf. However, A1AT, the physiological inhibitor of proteinase-3 (25), effectively suppressed FNf-induced loss of CD49e in a dose-dependent manner (Fig. 3C). Addition of Abs to proteinase-3 also blocked the loss of CD49e caused by FNf (Fig. 3D). Flow cytometric analyses demonstrated that within 5 min after addition of FNf, monocyte cell surface expression of proteinase-3 increased 2.5-fold (Fig. 3E). From its apogee, cell surface expression of this enzyme subsided somewhat after 20 min but remained above baseline. Over the same time interval, cell surface expression of proteinase-3 slowly rose in the unstimulated controls as well (Fig. 3E).

Evidence that A1AT could suppress the FNf-induced loss of monocyte CD49e suggested that, in plasma samples that fail to block the effect of added FNf, relatively more of the A1AT might be tied up irrevocably in complexes with proteolytic enzymes and therefore are unavailable to interact with newly added proteases (26). As a result, the quantity of uncomplexed A1AT might be insufficient to block proteases induced by FNf treatment. To evaluate this hypothesis, we analyzed the 8 normal donor and 16 patient samples used in the experiments illustrated in Fig. 3B for monomeric 45-kDa A1AT and complexed A1AT. Representative data from these experiments are shown in Fig. 4A and the results of densitometric analyses of all samples are summarized in Fig. 4, B–D.

Plasma samples like nos. 4, 5, and 6 in Fig. 4A that were among those that failed to block FNf-induced loss of CD49e were replete in <45-kDa fragments of A1AT. They also contained abundant high molecular mass complexes that incorporated A1AT. Complexes and fragments of A1AT were less evident in samples like those shown in lanes 1–3 of Fig. 4A that were obtained from the group of plasmas that blocked FNf-induced shedding of CD49e.

Densitometric analyses confirmed that A1AT-containing complexes were abundant in plasma from patients with <87% CD49e+ monocytes. This is the group whose plasma, when tested in the experiment shown in Fig. 3B, did not suppress the loss of CD49e induced by exogenous FNf. The relative quantity of A1AT in complexes in samples from normal controls and patients with >94% CD49e+ monocytes was similar and less than that found in plasma of patients with <87% CD49e+ monocytes. The average quantity and variance of monomeric 45-kDa A1AT (Fig. 4D) in each group overlapped completely, showing that the differences in high molecular mass A1AT complexes among these three groups of samples could not be explained by differences in sample loading.

To further compare the relative quantities of high molecular mass A1AT complexes in these samples, serial dilutions of each plasma shown in Fig. 4A were fractionated, transferred to nitrocellulose, and probed with anti-A1AT. Representative data shown in Fig. 4, E and F, suggest that high molecular mass complexes were more abundant in transblots of plasma from donors 4 and 5 than in samples from donors 1 and 2. This provides additional evidence that more of the A1AT in plasma from patients with a medium alone (none) and nonspecific mouse IgG (control) had no effect. E, Within 5 min after adding FNf (solid line), the fraction of monocytes expressing proteinase-3 at the cell surface transiently increased 2.5-fold. Proteinase-3 expression on cells treated with medium alone rose slowly from baseline over the same interval. In all panels, asterisks indicate values significantly different from those of controls (p < 0.05, paired t test). The y-axis in A–D measures CD49e fluorescence intensity on U937 cells; E shows the percentage of monocytes that express proteinase-3.
low (<87%) frequency of CD49e⁺ monocytes is incorporated in complexes with other proteins.

We know that much of the A1AT in human plasma, regardless of source, is bound up in complexes with leukocyte granular proteases such as cathepsin G, elastase, and proteinase-3 (27). The A1AT-containing complexes identified in this study were not unique in this regard. Immunoactive proteinase-3, elastase, and cathepsin G were each found in immunoprecipitates prepared with anti-A1AT from plasma samples 1, 3, 4, and 5 (Fig. 4A). In each sample, the dominant protein identified in these immunoprecipitates had the relative mobility of a 45-kDa protein, suggesting that in many cases immunoreactive fragments of these enzymes that bind to A1AT are small enough that they do not substantially affect the mobility of the A1AT in this fractionation medium. Less prominent but much in evidence were bands with both higher and lower relative mobilities, consistent with the presence of both breakdown products and complexes incorporating both A1AT and these enzymes (data not shown).

Discussion
Advancing HIV-1 infections degrade innate leukocyte functions, increasing the risk of secondary infections (1–3, 28). Neutrophil and monocyte phagocytic activity, which is normal or even enhanced in asymptomatic (stage A) patients (1, 2), can become defective as the disease progresses (3). Leukocyte responses to chemotactic stimuli decrease and the fraction of phagocytes that can produce reactive oxygen intermediates and carry out other immunosurveillance activities decline (3–5). Some of the agents that
impair leukocyte function have been identified; these include HIV-1 viral proteins, debris from apoptotic leukocytes, soluble immune complexes, and activated complement fragments (3, 7, 10). Circulating immune complexes, in particular, induce changes in expression of monocyte surface molecules that diminish phagocytic function and impair migration in tissue matrices (3). HAART decreases the risk of opportunistic infections (29), presumably by reducing viral load (6) and the prevalence of stimuli that alter leukocyte function (7–9). However, normalization of function is incomplete (9) and defects in monocyte intracellular signal transduction persist (30). At the cellular and molecular level, it has been unclear what changes in the leukocyte microenvironment must occur in order for functions to normalize (30). It has also been unclear what stimuli remain after optimum treatment that may continue to subvert normal leukocyte functioning.

In this study, we report that immune complexes have virtually disappeared from the circulation, even of CDC stage C patients, maintained for over 1 year on HAART. Concurrently, monocyte phagocytosis of opsonized bacteria is within normal limits. Yet patients’ circulating mononuclear monocytes remain activated, as shown by flow cytometric analyses of their cell surface molecules. Continued activation may be stimulated, in part, by a heretofore unrecognized agent: circulating cell-binding 110–120 kDa of FNf.

Although in this and previous studies, measurement of 450-kDa plasma FN did not identify subsets of patients with distinctive prognoses (31–33), recent reports suggest that certain fragments, like the III-C FN fragment, and FN polymers, as may be found in tissue matrices, can enhance infectivity of HIV-1 for CD4 T cells (34, 35). FN fragments and native FN have different biological properties (15–20). Some FN fragments that are chemotactic for monocytes (17, 36) can stimulate phagocytosis (37) and production of proinflammatory cytokines (15, 16, 38). Motifs associated with other FN subunits protect leukocytes from proapoptotic stimuli and enable adhesive interactions that facilitate migration in tissue matrices (39, 40). Attachment of FNf to α5β1 integrin (CD29/CD49e, VLA-5) and α6β1 integrin (CD29/CD49e, VLA-5) transduces signals that mediate these and many other biological effects (39, 41–43).

The FNf identified in this study are similar in size to those released in the course of diverse conditions that result in inflammation and tissue necrosis (20, 44–46). A common feature of these conditions is an inflammatory response that produces proteolytic enzymes that break down plasma and tissue FN (47–49). In HIV-1 infections, both host (12, 14) and viral (13, 50) proteases are maintained at 4°C or are pretreated at 37°C with 2-deoxyglucose and sodium azide, treatment with FNf has no effect. Since the effect of FNf could be blocked by adding PMSF but not CDCl2, bestatin, 1,10-phenanthroline, or EGTA, agents which, respectively, block leucine aminopeptidases, arylamidases, and metalloproteases and since monocytes maintained in serum-free medium will shed CD49e in response to FNf, we concluded that the inducible product is a serine protease and that it is produced by the monocytes themselves. Evidence presented here suggests that the enzyme induced by FNf treatment is not elastase or cathepsin G. The fact that its activity is blocked by A1AT and with mAbs to proteinase-3 suggest that it may be proteinase-3, a 29-kDa granule-associated serine proteinase distinct from elastase and cathepsin G. Low levels of this enzyme are found on the membranes of resting monocytes and brief stimulation with a number of agonists, including IL-8, TNF-α, and opsonized particles that cross-link FcγR, dramatically increase its cell surface expression (21, 25). Considering that brief exposure to FNf induces monocytes to release TNF-α (22), this cytokine may be an intermediary in the signaling used by FNf to mobilize cell surface display of this enzyme. Within 2 min following stimulation with as little as 10 μg/ml FNf protein kinase C translocates from the cytosol to the monocyte cell membrane (16). Compared with the effect of phorbols, the activation of protein kinase C induced by FNf is short-lived, but it appears to be responsible for the subsequent release of TNF-α since production of this cytokine following FNf stimulation can be blocked or suppressed by the kinase inhibitors H-7 and spinogosine and by Ca2+ channel blocking agents (16).

In addition to its effect on CD49e, FNf also up-regulates expression of CD11b and CD86 and reduces the fraction of monocytes that express CD62L. These effects are not blocked by serine protease inhibitors and are not, therefore, likely to be influenced by proteinase-3 or other enzymes induced by FNf stimulation. However, the changes in monocyte CD11b, CD62L, and CD86 expression induced by FNf stimulation are nevertheless potentially important. The level of expression of CD86 is likely to affect the ability of CD14+ blood monocytes to provide costimulatory signals to T cells. Similarly, changes in expression of CD62L and CD18/CD11b are likely to influence monocyte migration across vascular barriers, a behavior of HIV-1-infected blood leukocytes that has already been linked to progression of disease, susceptibility to secondary infection, and death (51). Recent data from our laboratory suggest that FNf may be among the stimuli that cause HIV-1-infected leukocytes to migrate across endothelial barriers and distribute virus among infectable leukocytes that accumulate outside the vascular compartment (52).

References


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