Polymorphonuclear Neutrophils From Human Immunodeficiency Virus-Infected Patients Show Enhanced Activation, Diminished fMLP-Induced L-Selectin Shedding, and an Impaired Oxidative Burst After Cytokine Priming

By C. Elbim, M.H. Prevot, F. Bouscarat, E. Franzini, S. Chollet-Martin, J. Hakim, and M.A. Gougerot-Pocidalo

Impaired polymorphonuclear neutrophil (PMN) function may contribute to the onset of certain life-threatening bacterial and fungal infections in human immunodeficiency virus (HIV)-infected patients. Published data on PMN functional activity in HIV infection are controversial, possibly because most studies have involved PMNs isolated from their blood environment by means of various procedures that may differently affect surface receptor expression and thereby alter cell responses. We therefore used flow cytometry to study the expression of adhesion molecules at the PMN surface, actin polymerization, and the oxidative burst of whole-blood polymorphonuclear neutrophils in 42 HIV-infected patients at different stages of the disease. These PMNs were activated in vivo, as demonstrated by increased expression of the adhesion molecule CD11b/CD18, reduced L-selectin antigen expression, increased actin polymerization, and increased H$_2$O$_2$ production. The alterations were present in asymptomatic patients with CD4* cell counts greater than 500/$\mu$L and did not increase with the progression of the disease. Stimulation by bacterial N-formyl peptides showed dysregulation of L-selectin shedding and decreased H$_2$O$_2$ production after ex vivo priming with tumor necrosis factor alpha or interleukin-8 (IL-8). These latter impairments, which correlated with the decrease in CD4* lymphocyte numbers and with IL-8 and IL-6 plasma levels, could contribute to the increased susceptibility of HIV-infected patients to bacterial infections.

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MATERIALS AND METHODS

Reagents. The reagents and sources were as follows: human recombinant TNFα (rhTNFα; 2 x 10$^6$ U/mL) and human recombinant endothelial cell-derived IL-8 (Genzyme, Cambridge, MA); 2',7'-dichlorofluorescin-diacetate (DCFH-DA; Eastman Kodak, Rochester, NY); N-formyl-methionyl-leucyl-phenylalanine (fMLP), unlabeled phalloidin, fluorescein isothiocyanate (FITC)-phalloidin, and L-α lysophosphatidylcholine (Sigma Chemical Co, St Louis, MO); R-phycocerythrin–conjugated monoclonal mouse antihuman CD11b antibody (PE-anti CD11b; Dakopatts, Glostrup, Denmark); R-fluorescein–conjugated monoclonal mouse antibody to human LAM-1, L-selectin (FITC-anti Leu8), and SimultEST reagents (FITC/PE), ie, FITC-HLE1/PE-LeuM3 (CD45/CD14, leucogate), FITC-y/PE-γ 2 (control), FITC-Leu4/PE-Leu12 (CD3/CD19), FITC-Leu4/PE-Leu11 + Leu19 (CD3/CD16 + CD56), FITC-Leu4/Pe-Leu13 (CD3/CD43), FITC-Leu4/PE-Leu2 (CD3/CD8), FITC-Leu2/PE-HLAd (CD8/NA), FITC-Leu2/PE-Leu17 (CD8/CD38) (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Stock solutions of DCFH-DA (50 mol/L) and fMLP (10$^{-2}$ mol/L) were prepared in dimethylsulfoxide (DMSO) and stored at −20°C. Lyophilized IL-8 was dissolved in phosphate-buffered saline (PBS; Pharmacia Fine Chemicals, Uppsala, Sweden) containing 0.1% human albumin serum and stored at −80°C. The different solutions were diluted in PBS immediately before use.


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0006-4971/94/4808-0034$3.00/0
Subjects. Forty-two HIV-infected adults (18 females and 34 males; mean age, 38 ± 10 years) were studied. HIV seropositivity was determined by enzyme-linked immunosorbent assay (ELISA) and confirmed by Western blot analysis. Patients with ongoing infections, in particular opportunistic infections, which could affect neutrophil functions, were excluded. HIV infection was classified according to the Centers for Disease Control criteria. Four groups of patients were studied: CDC class A1 (n = 10; asymptomatic), CDC class A2 (n = 10; asymptomatic or generalized lymphadenopathy); CDC class C4 (cell count <200/μL; none of these patients received any significant medication); CDC class C5 (n = 10; asymptomatic or documented opportunistic infection); 7 patients were on antiretroviral therapy: CDC class C2 (n = 5) or CDC class C3 (n = 2). Twenty patients (10 with an AIDS-related complex and 10 with AIDS) were receiving prophylaxis of opportunistic infections: aerosolized Pentamidine with or without either stavudine [ddC] or didanosine [ddI]; CDC class B2 and B3 (n = 12; acquired immunodeficiency syndrome [AIDS]-related complex; 11 patients were on antiretroviral therapy: AZT [n = 7], ddI [n = 3], or AZT + ddI [n = 1]); CDC class C5 (n = 10; asymptomatic or documented opportunistic infection; 7 patients were on antiretroviral therapy: AZT [n = 5] or ddI [n = 2]). Twenty patients (10 with an AIDS-related complex and 10 with AIDS) were receiving prophylaxis of opportunistic infections: aerosolized Pentamidine with or without pyrimethamine in 15 cases, Cotrimoxazole in 1, and Dapsone with or without Pyrimethamine in 4. Three AIDS patients were receiving treatment for tuberculosis and 2 AIDS patients were on antiangioprophylaxis. Blood samples were obtained during a regular clinical consultation. Ten HIV-negative members of the laboratory staff served as controls. Whole-blood samples were kept on ice and transported immediately to the laboratory.

Assay of lymphocyte subsets. Samples of 100 μL of fresh blood collected in EDTA tubes were mixed with 20 μL of the monoclonal antibodies, incubated for 15 minutes at room temperature. Red blood cells were lysed with fluorescence-activated cell sorting (FACS) lysing solution (Becton Dickinson). After one wash in FACSflow buffer (400g for 5 minutes), white blood cells were resuspended in 1% paraformaldehyde-PBS. The samples were stored at 4°C and analyzed by means of flow cytometry within 24 hours of fixation.

H2O2 production. H2O2 production was measured by using a flow cytometric assay derived from the technique described by Bass et al.29 One milliliter of fresh blood collected onto preservative-free lithium heparinate (10 U/mL) was preincubated for 15 minutes with 2',7'-DCFH-DA (100 μM/μL in a water bath with gentle horizontal agitation at 37°C. DCFH-DA diffuses into the cells and is hydrolysed into 2',7'-dichlorofluorescin (DCFH), during the PMN oxidative burst, nonfluorescent intracellular DCFH is oxidized to highly fluorescent dichlorofluorescein (DCF) by H2O2 in the presence of peroxidase.29 The samples were then incubated with either TNF (100 U/mL) or IL-8 (50 ng/mL) and diluted in PBS, or with PBS alone, at 37°C for 30 minutes. FMLP diluted in PBS (10−6 mol/L final concentration) or a similar dilution of DMSO was added for 5 minutes at 37°C. The reaction was stopped and red blood cells were lysed with FACS lysing solution. After one wash (400 g for 5 minutes), white blood cells were suspended in 1% paraformaldehyde-PBS. The fixed samples were kept on ice until cytofluorometric analysis on the same day.

F-actin content of PMNs. Whole-blood samples were either kept on ice or incubated with FMLP (10−6 mol/L) or PBS for 1 minute at 37°C. Whole blood cells obtained after red blood cell lysis were fixed with 1% paraformaldehyde-PBS, and F-actin content was measured using a flow cytometric assay.30 One hundred microliters of the cell suspension was incubated for 30 minutes at 0°C in 100 μL of 8% paraformaldehyde and 200 μL of L-α lysophosphatidylcholine in PBS alone or supplemented with 1 mmol/L unlabeled phalloidin to measure nonspecific binding of FITC-phalloidin. FITC-phalloidin (20 μmol/L) was then added to the suspension and incubation was continued for 30 minutes at 0°C. After one wash in PBS, the cells were resuspended in 1% paraformaldehyde-PBS.

Determination of CD11b and L-selectin expression. Whole-blood samples were either kept on ice or incubated with FMLP (10−6 mol/L) or PBS at 37°C for 5 minutes. One hundred microliters of sample was then incubated with a combination of PE-anti-CD11b and FITC-anti-Leu7 antibodies for 30 minutes at 4°C; red blood cells were lysed with FACS lysing solution. After one wash with ice-cold PBS, the cells were resuspended in 1% paraformaldehyde-PBS and kept on ice until flow cytometric analysis. Nonspecific binding of antibody was determined on cells incubated with the same concentration of an isotypic control.

Flow cytometry analysis. We used a Becton Dickinson FACScan with a 15-mW, 488-nm argon laser. PMN functions were analyzed using LysisII software. Forward and side scatter were used to identify the granulocyte population and to gate out other cells and debris. The purity of the gated cells was assessed using FITC- or PE-conjugated CD3, CD45, CD14, and CD15 antibodies (Becton Dickinson). Ten thousand events were counted per sample and the fluorescence pulses were amplified by 4-decade logarithmic amplifiers. The green fluorescence of DCF, FITC-phalloidin, and FITC-anti-Leu7 was recorded from 515 nm to 545 nm; the red fluorescence of PE-anti-CD11b was recorded from 563 to 607 nm. In all cases, unstained cells were used and the photomultiplier settings were adjusted so that the unstained cell population appeared in the lower left-hand corner of the fluorescence display. In the dual-color analysis, single-cell controls were used to optimize signal compensation. All the results were obtained with a constant photomultiplier gain. The median fluorescence intensity was used to quantify the responses. The effect of agonists on H2O2 production was calculated using a stimulation index (SI = ratio of median fluorescence intensity of stimulated cells to that of unstimulated cells). Dual-color identification of lymphocyte subsets was performed using SimulSET software. Before acquisition, Autocomp was run to adjust gain settings and fluorescence compensation with caliBRITE beads (Becton Dickinson). Leucogate lets SimulSET automatically "gate" on lymphocytes in samples. The simulTEST control reagent was used to establish the marker settings for negative immunofluorescence.

Cytokine assays. Blood was collected into sterile EDTA-treated vacuum tubes, transported on ice to the laboratory, and immediately centrifuged at 1,500g for 15 minutes at 4°C to avoid cytokine synthesis in vitro.31 Plasma samples were stored at −70°C for no longer than 15 days before assay. Cytokines in plasma were assayed in duplicate using an immunoradiometric assay for TNFa with a detection limit of 15 pg/mL (Medgenix, Brussels, Belgium) and immunoenzymatic assays for IL-6, IL-10, and IL-8 with detection limits of 3 pg/mL (Medgenix for IL-6 and IL-10; British Biotechnology [Abingdon, UK] for IL-8). The assays were standardized by comparing the results to those for standards from the National Institute for Biological Standards and Control (UK). Cytokine recovery was 102% ± 4% from normal plasma spiked with recombinant human cytokines.

Statistical analysis. All results are expressed as the mean ± standard error of the mean (SEM). The group means were compared by analysis of variance followed by a multiple comparison of means by Fisher’s least significant difference procedure. P values of .05 or less were considered significant. Correlations were identified by the Spearman rank correlation coefficient ρ.

RESULTS
PMNs and lymphocyte counts in HIV-infected patients. As shown in Table 1, although PMN counts were significantly decreased in groups 2 and 4 relative to healthy controls, the mean PMN count for the four groups was within...
Table 1. Characteristics of the Study Population

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 10)</th>
<th>Group 1 (n = 10)</th>
<th>Group 2 (n = 10)</th>
<th>Group 3 (n = 10)</th>
<th>Group 4 (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PMN count</strong></td>
<td>3,657 ± 265</td>
<td>3,471 ± 395</td>
<td>2,047 ± 212†</td>
<td>2,889 ± 604</td>
<td>2,184 ± 517†</td>
</tr>
<tr>
<td><strong>Lymphocyte count</strong></td>
<td>2,104 ± 178</td>
<td>2,206 ± 160†</td>
<td>2,024 ± 245</td>
<td>1,319 ± 197†</td>
<td>867 ± 166†</td>
</tr>
<tr>
<td><strong>CD4</strong> count</td>
<td>991 ± 92</td>
<td>798 ± 93</td>
<td>380 ± 33†</td>
<td>150 ± 37†</td>
<td>63 ± 19†</td>
</tr>
<tr>
<td><strong>CD8</strong> count</td>
<td>670 ± 89</td>
<td>1,436 ± 160†</td>
<td>1,131 ± 214</td>
<td>818 ± 146</td>
<td>580 ± 143</td>
</tr>
<tr>
<td><strong>CD8/CD38</strong></td>
<td>17 ± 1</td>
<td>38 ± 61†</td>
<td>42 ± 41</td>
<td>57 ± 41</td>
<td>60 ± 41†</td>
</tr>
<tr>
<td><strong>CD8/HLA-DR</strong></td>
<td>5 ± 1</td>
<td>32 ± 51†</td>
<td>31 ± 41</td>
<td>42 ± 51</td>
<td>43 ± 41†</td>
</tr>
</tbody>
</table>

Values are means ± SEM.

* Values per microliter.
† Significantly different from control values (P < .05).
‡ CD8+ cells that were CD38- or HLA-DR-positive as expressed in percentage of the total lymphoid cells.

Table 2. L-Selectin Expression at the Surface of PMNs From HIV-Infected Patients and Healthy Controls

<table>
<thead>
<tr>
<th></th>
<th>PBS 4°C</th>
<th>PBS 37°C</th>
<th>fMLP 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Controls</strong></td>
<td>15 ± 1.65</td>
<td>14.7 ± 1.63</td>
<td>0</td>
</tr>
<tr>
<td>Group 1</td>
<td>9.5 ± 1.75†</td>
<td>9.5 ± 1.80†</td>
<td>0.2 ± 0.10†</td>
</tr>
<tr>
<td>Group 2</td>
<td>13.3 ± 2.50</td>
<td>17.5 ± 2.80†</td>
<td>1.76 ± 0.70†</td>
</tr>
<tr>
<td>Group 3</td>
<td>17.5 ± 3.70†</td>
<td>16.8 ± 2.60†</td>
<td>3.1 ± 1.10†‡</td>
</tr>
<tr>
<td>Group 4</td>
<td>19.2 ± 2.90†</td>
<td>13.1 ± 1.86</td>
<td>3.4 ± 0.70†‡</td>
</tr>
</tbody>
</table>

* Whole blood was incubated with FITC-Leu8 antibody at 4°C for 30 minutes either after maintaining the samples at 4°C after incubation with PBS or fMLP (10−6 mol/L) at 37°C for 5 minutes. Median fluorescence intensity was recorded as described in Materials and Methods. Values obtained with an irrelevant antibody of the same isotype were subtracted. Values are means ± SEM.
† Significantly different from control values (P < .05).
‡ Significantly different from group 1 (P < .05).

Table 3. CD11b Expression at the Surface of PMNs From HIV-Infected Patients and Healthy Controls

<table>
<thead>
<tr>
<th></th>
<th>PBS 4°C</th>
<th>PBS 37°C</th>
<th>fMLP 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Controls</strong></td>
<td>145 ± 2</td>
<td>183 ± 12</td>
<td>2,237 ± 198</td>
</tr>
<tr>
<td>Group 1</td>
<td>355 ± 50†</td>
<td>925 ± 102†</td>
<td>2,072 ± 98</td>
</tr>
<tr>
<td>Group 2</td>
<td>387 ± 130†</td>
<td>727 ± 208†</td>
<td>2,322 ± 270</td>
</tr>
<tr>
<td>Group 3</td>
<td>355 ± 74†</td>
<td>663 ± 153†</td>
<td>2,428 ± 207</td>
</tr>
<tr>
<td>Group 4</td>
<td>352 ± 21†</td>
<td>512 ± 32†‡</td>
<td>2,076 ± 203</td>
</tr>
</tbody>
</table>

* Whole blood was incubated with phycoerythrin-CD11b antibody as described in the legend of Table 2. Values are means ± SEM.
† Significantly different from control values (P < .05).
‡ Significantly different from group 1 (P < .05).
cence. As shown in Fig 1, DCF fluorescence intensity was significantly higher with unstimulated PMN from HIV-infected patients.

As we have previously reported,15 FMLP (10^{-6} mol/L for 5 minutes), TNF (100 U/mL for 30 minutes), and IL-8 (50 ng/mL for 30 minutes) added separately to whole blood induced barely detectable H_{2}O_{2} production by PMNs from the control subjects. No significantly higher H_{2}O_{2} production by PMNs from HIV-infected patients were observed. The SI did not differ significantly from 1 in all the groups of patients (data not shown). Preincubation of whole blood from the control subjects with 100 U/mL TNF for 30 minutes, followed by treatment with 10^{-6} mol/L FMLP for 5 minutes, induced a bimodal response in terms of DCF fluorescence intensity that has been shown by us earlier15 (Fig 2). Indeed, 51% ± 11% of PMNs had an SI of 7.50 ± 1.38, whereas the remainder had an SI of 1.47 ± 0.26. Such a bimodal priming effect was also observed after preincubation of whole blood with 50 ng/mL IL-8 for 30 minutes, followed by FMLP. In these priming conditions, the hyperresponsive subpopulation was detectable with PMNs from 8 of the 10 patients in group 1 and 2 of the 10 patients in group 2. A unimodal pattern was observed in the other patient groups (Fig 2). In addition, after TNF or IL-8 priming, the SI of the total PMN population gradually decreased with the stage of HIV infection, with a decrease of about 50% in the AIDS patients (Fig 3). The gradual decrease in SI values with the stage of HIV infection resulted primarily from the decrease in the median fluorescence intensity of stimulated cells. In fact, in the case of TNF priming, the median fluorescence intensity was 93 ± 7.1, 55 ± 8.6, 48 ± 6.3, 37 ± 5.2, and 44 ± 6.6, respectively, for the controls and groups 1 through 4. With IL-8 priming, the median fluorescence intensity was 73 ± 4.7, 69 ± 11, 32 ± 6.2, 26 ± 4.4, and 29 ± 2.9, respectively. In addition, slight (not significant) differences between the baseline levels of H_{2}O_{2} production were observed in the different groups of patients (22 ± 3.4, 16 ± 2.1, 17 ± 1.6, and 23 ± 7.3, respectively, for groups 1 through 4). However, the higher SI value of the control subjects also results, in part, from a lower baseline value (13 ± 0.5). The decline in SI values correlated with the decrease in CD4+ cell numbers and with IL-8 and IL-6 plasma levels (Table 4).

**Actin polymerization in whole-blood PMNs.** Because actin polymerization has been involved in the upregulation of CD11b/CD18 molecules16 and in the oxidative burst15,16 after neutrophil activation, we studied PMN F-actin content. As shown in Table 5, median fluorescence intensity of FITC-phalloidin binding was significantly higher in patients from groups 1, 2, and 4 and close to the threshold of significance in group 3 relative to the control subjects. There was no significant increase in fluorescence intensity with the stage of HIV disease. However, after 1 minute of incubation with PBS at 37°C, the intensity of actin polymerization increased in PMNs from patients in groups 1 and 2 but not in PMNs from patients in groups 3 and 4. After stimulation with 10^{-6} mol/L FMLP for 1 minute, FITC-phalloidin binding was similar in the patients and controls, showing that maximal F-actin polymerization was unaffected by HIV infection.
PMN FUNCTIONS IN HIV-INFECTED PATIENTS

Table 4. Correlation Between fMLP-Induced H2O2 Production After TNF or IL-8 Priming, With CD4+ Cell Counts and IL-8, IL-6, and TNF Plasma Levels in HIV-Infected Patients

<table>
<thead>
<tr>
<th>CD4</th>
<th>IL-8</th>
<th>IL-6</th>
<th>TNF</th>
</tr>
</thead>
<tbody>
<tr>
<td>s*</td>
<td>P</td>
<td>s*</td>
<td>P</td>
</tr>
<tr>
<td>TNF priming</td>
<td>.37</td>
<td>.01</td>
<td>.62</td>
</tr>
<tr>
<td>IL-8 priming</td>
<td>.45</td>
<td>.03</td>
<td>.42</td>
</tr>
</tbody>
</table>

Abbreviation: NS, not significant.
* Spearman's rank-correlation coefficient.

Cytokine plasma levels. As shown in Table 5, TNF, IL-8, and IL-6 plasma levels increased with the stage of HIV disease. In particular, IL-8 plasma levels were significantly elevated in group 1 patients and correlated with the HIV disease stage. In particular, IL-8 plasma levels were significantly higher in the AIDS patients than in the control subjects. The largest increase was in IL-8, followed by IL-6 and TNF (5.7-, 3.5-, and 2.9-fold increases, respectively). IL-1 was not significantly detectable in the controls or HIV-infected patients.

DISCUSSION

Our results show that circulating PMNs from HIV-infected patients are activated, even in the early, asymptomatic stage of the disease, when the CD4+ cell count is still greater than 500/µL. In fact, unstimulated PMNs from these patients showed increased CD11b/CD18 adhesion molecule expression, actin polymerization, and H2O2 production, and decreased L-selectin expression. These alterations did not increase with HIV disease severity; on the contrary, decreased L-selectin expression was only found at the earliest stage of HIV infection. In contrast, after stimulation with bacterial N-formyl peptides, PMNs from HIV-infected patients showed normal maximal CD11b expression and actin polymerization, but reduced L-selectin antigen shedding. Furthermore, after TNF and IL-8 priming, H2O2 production was impaired. These impairments correlated with the clinical stage of the disease, reflected by the CD4+ cell counts, and also with IL-8 and IL-6 plasma levels.

Table 5. Actin Polymerization in PMNs From HIV-Infected Patients and Control Subjects

<table>
<thead>
<tr>
<th></th>
<th>PBS 4°C</th>
<th>PBS 37°C</th>
<th>fMLP 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>52 ± 7</td>
<td>54 ± 8</td>
<td>248 ± 32</td>
</tr>
<tr>
<td>Group 1</td>
<td>45 ± 8t</td>
<td>127 ± 13t</td>
<td>207 ± 17</td>
</tr>
<tr>
<td>Group 2</td>
<td>96 ± 9t</td>
<td>97 ± 121t</td>
<td>262 ± 25</td>
</tr>
<tr>
<td>Group 3</td>
<td>70 ± 7t</td>
<td>61 ± 74</td>
<td>185 ± 24</td>
</tr>
<tr>
<td>Group 4</td>
<td>78 ± 5t</td>
<td>80 ± 74</td>
<td>222 ± 23</td>
</tr>
</tbody>
</table>

* Whole blood was incubated with FITC-phalloidin at 4°C for 30 minutes either after maintaining the samples at 4°C or after incubation with PBS or fMLP (10−8 mol/L) at 37°C for 1 minute. Values for nonspecific binding of FITC-phalloidin (samples preincubated with unlabeled phalloidin) were subtracted. Values are means ± SEM.
† Significantly different from control values (P < .05).
‡ Significantly different from group 1.

There have been conflicting reports on the activation status of circulating PMNs from HIV-infected patients. PMN activation is reflected by L-selectin shedding and an increase in CD11b/CD18 expression. L-selectin expression at the PMN surface has not, to our knowledge, previously been studied in HIV-infected patients. We found decreased L-selectin expression by unstimulated PMNs in the asymptomatic stage of HIV infection. An increase in CD11b/CD18 expression has been reported with whole-blood PMNs from CDC class IV HIV-infected patients.3,7 However, we extended this observation to all the patient groups including asymptomatic patients with CD4+ greater than 500/µL and no significant differences were found between the different groups of patients. In addition, we found an increase in spontaneous H2O2 production by whole-blood PMNs from HIV-infected patients relative to healthy control subjects. This increase did not differ according to the stage of HIV infection. A similar pattern of increase in F-actin content was also observed in the HIV-infected patients. These latter results contrast with another report of similar oxidative burst level and F-actin content in isolated unstimulated PMNs from AIDS patients and healthy control subjects.3,5 The apparent discrepancy could at least in part be due to methodologic differences. In particular, PMN isolation procedures have been shown to modify the surface expression of molecules that are not detectable in whole blood and that may be markers of PMN activation.9 Activation due to the isolation procedures might thus mask differences between patients and healthy controls. Taken together, our findings obtained with whole-blood PMNs clearly demonstrate that these cells are activated in HIV-infected patients.

To assess PMN functions after stimulation, whole-blood PMNs were treated ex vivo with fMLP, a structural analog of bacterial metabolic products. In keeping with data reported by Capsoni et al10 for TNF- and granulocyte-macrophage colony-stimulating factor (GM-CSF)-treated PMNs, there was a

Table 6. Cytokine Plasma Levels

<table>
<thead>
<tr>
<th></th>
<th>TNFα</th>
<th>IL-8</th>
<th>IL-6</th>
<th>IL-10</th>
</tr>
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<tbody>
<tr>
<td>Controls</td>
<td>&lt;15</td>
<td>&lt;15</td>
<td>&lt;15</td>
<td>&lt;15</td>
</tr>
<tr>
<td>Group 1</td>
<td>25 ± 4</td>
<td>38 ± 1*</td>
<td>&lt;15</td>
<td>&lt;15</td>
</tr>
<tr>
<td>Group 2</td>
<td>36 ± 7</td>
<td>46 ± 3*</td>
<td>35 ± 9</td>
<td>15.4 ± 0.4</td>
</tr>
<tr>
<td>Group 3</td>
<td>44 ± 10*</td>
<td>47 ± 4*</td>
<td>32 ± 41</td>
<td>16.2 ± 1.05</td>
</tr>
<tr>
<td>Group 4</td>
<td>44 ± 17*</td>
<td>86 ± 10*</td>
<td>52 ± 14*</td>
<td>&lt;15</td>
</tr>
</tbody>
</table>

Values are in picograms per milliliter. Values are means ± SEM.
* Significantly higher than control values.
† Significantly different from group 4.
in whole blood, fMLP-stimulated PMNs from HIV-infected patients showed normal maximal CD11b surface expression. In contrast, increased CD11b/CD18 expression in response to zymosan stimulation has been reported by Ellis et al. with isolated PMNs from CDC class IV HIV-infected patients. We also found normal maximal actin polymerization in PMNs from HIV-infected patients after stimulation with \(10^{-4}\) mol/L fMLP for 1 minute, whereas Ryder et al. reported an increased response of isolated PMNs to fMLP. As discussed above, isolation procedures could explain the differences observed between isolated and whole-blood PMNs. Changes in L-selectin expression after stimulation with fMLP differed from those in CD11b/CD18 expression and actin polymerization. Whereas fMLP-stimulated PMNs from HIV-infected patients expressed normal amounts of CD11b at their surface and normal actin polymerization, L-selectin expression was dysregulated. In contrast to PMNs from healthy control subjects, L-selectin expression by PMNs from HIV-infected patients failed to decrease after fMLP stimulation. The extent of this abnormality increased with the stage of HIV infection, as shown by the correlation between median fluorescence intensity, the decrease in the CD4+ cell count, and the increase in T-cell activation markers. There have been conflicting reports on the oxidative burst of PMNs isolated from HIV-infected patients in response to various stimuli. Some investigators have reported that in vitro \(O_2^\bullet\) production is significantly depressed after stimulation with fMLP. In contrast, others have reported normal or even enhanced oxidative metabolism. \(^{1,3,6,10}\) We \(^{33}\) and others \(^{32}\) have previously reported that fMLP, TNF, and IL-8 alone induce barely detectable \(H_2O_2\) production by whole-blood PMNs from healthy control subjects, whereas pretreatment of blood with TNF or IL-8 strongly primes PMNs to produce \(H_2O_2\) in response to fMLP. \(^{30}\) Here, fMLP, TNF, or IL-8 alone barely increased \(H_2O_2\) production by PMNs from HIV-infected patients over baseline. These results point to an absence of in vivo PMN priming by cytokines and other inflammatory mediators, as described in other clinical settings such as bacterial infections. \(^{36}\) Incidentally, these data also confirm the absence of ongoing bacterial infections in our patients. No data on the oxidative burst of PMNs from HIV-infected patients in response to stimulation after cytokine priming have previously been reported. In this study, a TNF- or IL-8–primed hyperresponsive PMN subpopulation was found after fMLP stimulation in control subjects in accordance with our previous data, \(^{33}\) but not in HIV-infected patients. Furthermore, the stimulation index of the overall PMN population was strongly decreased in AIDS patients relative to the control subjects. This impairment increased as HIV disease progressed, as shown by the strong correlation between the SI and the absolute CD4+ lymphocyte count (Table 4).

The PMN functional abnormalities observed here may be due to several factors. The enhanced activation of circulating PMNs from HIV-infected patients could be a direct consequence of HIV infection; indeed, HIV DNA has been detected in PMN of HIV-infected patients by Gabrilovich et al. and Spear et al., and mononuclear phagocytes infected in vitro with HIV express increased surface levels of CD18 adhesion molecule. Internalization of HIV by PMNs may occur, at least in part, by phagocytosis after opsonization with specific anti-HIV antibodies present after the seroconversion phase. \(^{30,41}\) This could explain the basal activation of unstimulated PMNs from all the groups of HIV-infected patients tested, even those in the asymptomatic phase with CD4+ cell counts greater than 500/\(\mu\)L, and activation state that did not increase with disease progression. Systemic release by activated monocytes or lymphocytes of cytokines reported to induce functional activation of circulating neutrophils in HIV-infected patients does not seem to be directly related to this basal activation. Indeed, we confirmed the increased levels of TNF, IL-6, and IL-8 in the plasma of HIV-infected patients, but, in contrast to CD11b expression, actin polymerization, and \(H_2O_2\) production, plasma levels of these cytokines increased with the progression of HIV disease. In addition, we have previously found that TNF, IL-6, and IL-8 alone do not enhance \(H_2O_2\) production by PMNs (personal data), suggesting that other products play a role in both the increased oxidative metabolism of unstimulated PMNs and the existence of a hyperresponsive subpopulation in some patients. It has recently been reported that monocytes infected with HIV produce factors other than IL-1, IL-8, and TNF, which augment superoxide anion production by PMNs, particularly in the early stages of HIV infection. \(^{42}\) The decrease in L-selectin expression and the enhanced expression of CD11b and actin polymerization after incubation at 37°C, phenomena observed in the early stages of HIV infection, could be related to such factors. In contrast, after in vitro priming with TNF and IL-8 and stimulation by bacterial peptides, \(H_2O_2\) production by PMNs from HIV-infected patients showed strong negative correlations with IL-8 plasma level and the stage of the disease (Table 4). This decreased \(H_2O_2\) production by IL-8–primed PMNs could be related to a “desensitization” phenomenon. \(^{33}\) The large individual differences between TNF plasma level from HIV-infected patients could explain the lack of correlation between TNF plasma levels and \(H_2O_2\) production by TNF-primed PMNs. However, synergy between the proinflammatory cytokines TNF, IL-6, and IL-8 could contribute to the desensitization phenomenon. The decrease in the shedding of L-selectin antigen after fMLP stimulation, which correlated strongly with HIV disease progression, the decrease in the CD4+ cell count, and IL-8 and IL-6 plasma levels remain to be explained. Impaired CD4+ lymphocyte functions in HIV infection might contribute to PMN defects. \(^{46}\)

In physiologic conditions, circulating neutrophils are in a resting state. Migration of neutrophils to inflammatory sites involves a series of precisely regulated events. The selectin family of adhesion molecules on both neutrophils and endothelial cells are involved in the initial interaction of circulating neutrophils with the endothelium. In particular, L-selectin present on the neutrophil surface has been shown to participate in neutrophil rolling. \(^{22}\) In response to stimuli such as endothelium-derived cytokines, \(^{44}\) L-selectin is rapidly shed from the neutrophil surface and a \(\beta_2\) integrin-dependent phase of adhesion then occurs. In particular, upregulation of CD11b/CD18 molecules at the cell surface is involved in the adhesion of neutrophils to the endothelium and in trans-
endothelial migration. Recent data show that neutrophil activation induces association of the actin-binding protein actinin with the cytoplasmic domain of the β2-subunit of the integrin. This may link the actin cytoskeleton to the neutrophil membrane, a step necessary for enhanced adhesion and transendothelial migration. Actin polymerization has also been implicated in the cycling of receptors regulating the oxidative burst. All these phenomena are regulated in a precise manner, and excessive or inappropriate stimulation of neutrophils could lead to tissue injury, eg, vascular damage. Our results showing that unstimulated PMNs from HIV-infected patients express increased levels of CD11b adhesion molecule, decreased levels of L-selectin, increased actin polymerization, and enhanced H2O2 production strongly suggest that circulating PMNs are activated in HIV-infected patients. Such a hyperactivation state may participate in the pathogenesis of certain leukocytoclastic vasculitis reported in HIV-infected patients. In addition, after bacterial peptide stimulation, L-selectin expression at the PMN surface was dysregulated, a phenomenon that could immobilize PMNs on the endothelium and hinder migration to inflammatory sites, thereby increasing susceptibility to bacterial infections. It has also been suggested that cytokine priming of neutrophils is critical for triggering of the oxidative burst, and our results suggest that susceptibility to infections in HIV-infected patients may be related in part to a failure of PMN oxidative metabolism to increase in response to inflammatory cytokines. In conclusion, we found that whole-blood PMNs from HIV-infected patients are activated, even in the asymptomatic stage of infection when CD4+ cell counts are greater than 500/μL. The degree of activation did not increase with HIV disease progression; on the contrary, some phenomena were more marked in the early asymptomatic phase. This could be related to the early immunologic responses to HIV and could contribute to vascular damage reported in HIV infection. We also found that L-selectin expression was dysregulated and that H2O2 production in response to bacterial peptides after TNF or IL-8 priming ex vivo was diminished. These functional abnormalities, which increased with HIV disease progression, could contribute to the increased susceptibility to bacterial infections in this setting.

ACKNOWLEDGMENT

We are indebted to the clinical services of infectious diseases of Prof F. Vachon and Prof J.P. Coulaud (CHU Bichat-Claude Bernard, Paris, France) and Prof W. Rozenbaum (Hopital Rotschild, Paris, France) for their help with this study.

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