An Assessment of Polymorphonuclear Leukocyte Rigidity in HIV-Infected Individuals after Immune Recovery

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PURPOSE. To determine whether polymorphonuclear leukocytes (PMNs) remain rigid after immune reconstitution in human immunodeficiency virus (HIV)-infected individuals with a history of severe immunosuppression.

METHODS. PMN rigidity was measured in vitro in three groups: (1) HIV-infected individuals with a history of CD4+ T-lymphocyte counts of less than 50/μL, but with current counts of more than 200/μL attributable to potent antiretroviral therapy (group 1); (2) HIV-infected individuals whose CD4+ T-lymphocyte counts had always been more than 200/μL (group 2); and (3) HIV-negative control subjects. Rigidity was determined with a cell transit analyzer (containing a micropore filter with 30 identical, 8-μm diameter pores), representing a simple in vitro model of a capillary bed. A longer PMN pore transit time reflects increased PMN rigidity.

RESULTS. PMN transit time (median) in group 1 (n = 11) was 3.54 ms, in group 2 (n = 9) was 3.19 ms, and in control subjects (n = 15) was 2.66 ms. PMN rigidity was significantly greater in groups 1 (P = 0.014) and 2 (P = 0.046) than in control subjects (Wilcoxon rank-sum test). A significant difference was not identified between groups 1 and 2 (P = 0.518).

CONCLUSIONS. The increased PMN rigidity known to occur in severely immunosuppressed HIV-infected individuals persists after immune reconstitution. Furthermore, PMN rigidity is increased in those HIV-infected individuals who do not have a history of severe immunosuppression. Because PMN rigidity can alter microvascular blood flow, HIV-infected individuals may remain at risk for retinal vascular damage in the era of potent antiretroviral therapy. (Invest Ophthalmol Vis Sci. 2002;43:1857–1861)

Changes in retinal blood flow have been well documented in human immunodeficiency virus (HIV)-infected individuals.1–4 It has been hypothesized that leukocytes, which are known to have a strong influence on microvascular blood flow,5 play an important role in these changes.6 Polymorphonuclear leukocyte (PMN) rigidity has been found to correlate inversely with CD4+ T-lymphocyte counts in severely immunosuppressed HIV-infected individuals.6 These rigid cells may be less able to pass through the retinal microvasculature.

The flow of leukocytes in macular capillaries appears to remain impaired in HIV-infected individuals after immune reconstitution attributable to potent antiretroviral therapy.2 Tufail et al.6 have speculated that PMNs may remain rigid in HIV-infected individuals, even after immune reconstitution. Such PMN rigidity may be a factor that contributes to the persistent hemorheologic abnormalities.

We determined PMN rigidity in immune-reconstituted individuals, using an in vitro technique. Data were compared with those from HIV-negative control subjects and from HIV-infected individuals who had never been severely immunosuppressed.

MATERIALS AND METHODS

Subjects

Volunteer HIV-infected individuals were recruited from the consultation suites of the Doheny Eye Institute and the Maternal-Child HIV Management and Research Center, University of Southern California (USC), Los Angeles, California, without regard to age, gender, or ethnicity. Control subjects were recruited by means of flyers at the USC Health Sciences Campus. Individuals with diabetes mellitus and those who had received blood transfusions within the prior month were excluded from the study. The study was approved by the USC Institutional Review Board, and all subjects gave informed consent. The study also conformed to requirements of the Human Subjects Protection Committee at UCLA, where data were analyzed. The study’s protocol adhered to the provisions of the Declaration of Helsinki for research involving human subjects.

Studies were performed in 20 HIV-infected individuals and 15 HIV-negative control subjects. Among the HIV-infected individuals, 11 had had a previous CD4+ T-lymphocyte count less than 50/μL and a current count more than 200/μL (group 1), and nine had always had CD4+ T-lymphocyte counts more than 200/μL (group 2).

The two groups of HIV-infected individuals were chosen to represent the ends of a spectrum of HIV-related immunosuppression. A CD4+ T-lymphocyte count of 200 cells/μL is the level at which patients are defined as having AIDS, and a level above which patients rarely...
manifest ocular disorders, including HIV retinopathy. A CD^4^+ T-lymphocyte count of 50 cells/μL is the level of severe immunosuppression below which there is a high rate of HIV retinopathy, cytomegalovirus (CMV) retinitis, and other ocular complications of HIV. These groups are consistent with those studied in previous studies of hemorheologic abnormalities in HIV-infected individuals.

The following clinical and laboratory data were collected for each patient: presence or absence of CMV retinitis, use and duration of potent antiretroviral therapy, current HIV blood levels, current CD^4^+ T-lymphocyte count, nadir CD^4^+ T-lymphocyte count (defined as the lowest previously identified CD^4^+ T-lymphocyte count at any point in the course of a subject’s HIV infection). In addition, in group 1, time intervals since CD^4^+ T-lymphocyte counts had risen above threshold levels of 100/μL and 200/μL were determined. The following studies were performed on a venous blood specimen from each subject: PMN deformability, complete blood count, and differential leukocyte analysis.

Potent antiretroviral therapy was defined as use of any of the following drug combinations: two nucleoside reverse transcriptase inhibitors and one protease inhibitor, one nucleoside reverse transcriptase inhibitor and two protease inhibitors, or one nucleoside and one protease inhibitor, one nucleoside and one nonnucleoside reverse transcriptase inhibitor and one or more protease inhibitors.

**Isolation and Preparation of PMNs**

After venipuncture was performed with a 19-gauge needle, 20 mL of whole blood was gently drawn into a plastic syringe containing sodium heparin (10 U/mL), with the tourniquet removed immediately after insertion of the needle. Pure PMNs suspensions were prepared by a density-based centrifugation technique, as described previously. Endotoxin-free sterile media and plastic ware were used to avoid in vitro PMN activation. Heparinized whole blood (7 mL) was mixed with 3 mL of phosphate-buffered saline (PBS, pH 7.4, 285 mOsm/kg), gently layered onto 3 mL of 54% Percoll-PBS (final density, 1.076 g/cm^3; Sigma Chemical Co., St. Louis, MO), and then centrifuged at 400g for 25 minutes at 22°C. The top 3 mL of the resulting PMN-red blood cell interface were transferred into distilled water for 40 seconds, to remove residual erythrocytes by hypotonic lysis. Toxicity was then restored by the addition of 10X PBS and the suspension spun at 220g for 6 minutes at 22°C. The PMNs were washed and maintained at room temperature in PBS. Purity was assessed by optical microscopy (>96% PMNs), and leukocyte count was determined using a hematology analyzer (Micros; ABX Hematology, Montpellier, France).

**PMN Rigidity Measurements**

PMN rigidity was assessed with a cell-transit analyzer (CTA; ABX Hematology). The central feature of the CTA is a special micropore filter that separates two fluid-filled reservoirs. The filter is a specially fabricated thin sheet of polycarbonate plastic containing 50 identical 8-μm diameter by 21-μm-length pores, thus representing a simple, yet geometrically stable, in vitro model of a capillary bed. Hydrostatic pressure generated by the pressure difference in height between the two fluids forces the cell suspension through the filter pores, and the transit time for each complete cell passage is obtained by monitoring the change in electrical resistance of the filter as a cell traverses a pore. Accompanying software for the computer system corrects for possible simultaneous pore occupancy and provides mean and median transit times for the cell population being tested. A longer PMN pore transit time reflects a decrease of PMN deformability (increased PMN rigidity).

The following experimental parameters were used for the CTA: pressure gradient of 8 cm water, measurements at 25°C, a final PMN concentration in sterile PBS of 10^5 cells/mL, at least 500 cells per run, and triplicate runs for each sample. The average of the median values for the three runs was used for subsequent data analyses. Median, rather than mean, values were used to summarize each run, because occasional outliers are seen, presumably because of PMN activation during handling of specimens. The median is not unduly influenced by these outliers. All studies were performed in duplicate using two micropore filters (A and B) and were completed within 4 hours of venipuncture. To avoid possible bias associated with temporal changes of the CTA filter with repeated use, which might alter transit time results, samples from HIV-infected individuals and control subjects were included with each batch of samples tested, rather than testing groups sequentially. Investigators performed PMN rigidity measurements without knowledge of the patient’s identity or group assignment for individual specimens.

**Statistical Analyses**

Pair-wise comparisons between groups were performed with the Wilcoxon rank-sum test. Three group comparisons were performed with the Kruskal-Wallis ANOVA. Linear regression and correlation analyses were used to evaluate relationships between transit times for both HIV-infected groups and the following factors: current and nadir CD^4^+ T-lymphocyte counts, duration of potent antiretroviral therapy, intervals since CD^4^+ T-lymphocyte counts had risen above 100/μL and 200/μL, the presence or absence of CMV retinitis, and current HIV blood level. Each analysis was performed on data from filter A and then repeated on data from filter B.

**Results**

Characteristics of the HIV-infected subjects are shown in Table 1. All subjects were adults. Mean ages for the two groups of HIV-infected individuals were not statistically different. Mean age for the control group was significantly less than the means for groups 1 (P = 0.002) and 2 (P = 0.017), but in our
TABLE 2. Leukocyte Transit Times

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Filter A (ms)</th>
<th>Filter B (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1†</td>
<td>11</td>
<td>3.40 ± 0.523</td>
<td>2.96 ± 0.740</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>3.54 (3.08, 3.75)</td>
<td>2.82 (2.38, 3.67)</td>
</tr>
<tr>
<td>Median</td>
<td></td>
<td>3.24 ± 0.409</td>
<td>2.86 ± 0.558</td>
</tr>
<tr>
<td>Group 2‡</td>
<td>9</td>
<td>3.19 (2.99, 3.59)</td>
<td>2.81 (2.52, 3.16)</td>
</tr>
<tr>
<td>Control subjects</td>
<td>15</td>
<td>2.80 ± 0.713</td>
<td>2.26 ± 0.505</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>2.66 (2.13, 2.20)</td>
<td>2.16 (1.99, 2.46)</td>
</tr>
<tr>
<td>Median</td>
<td></td>
<td>2.04</td>
<td>0.16</td>
</tr>
<tr>
<td>Three-way comparison (P)‡</td>
<td></td>
<td>0.025</td>
<td>0.011</td>
</tr>
<tr>
<td>Pair-wise comparisons (P)§</td>
<td></td>
<td>0.014</td>
<td>0.016</td>
</tr>
<tr>
<td>Group 1 vs. control subject</td>
<td></td>
<td>0.046</td>
<td>0.010</td>
</tr>
<tr>
<td>Group 2 vs. control subject</td>
<td></td>
<td>0.518</td>
<td>0.676</td>
</tr>
<tr>
<td>Group 1 vs. group 2</td>
<td></td>
<td>0.89</td>
<td>0.051</td>
</tr>
</tbody>
</table>

*Leukocyte transit time is a measure of cellular rigidity. For each determination of leukocyte transit time on a single specimen, the CTA provided a median value for multiple measurements. Each specimen was tested three times in the CTA, with each filter. For each specimen, the three medians produced by the CTA were averaged and used for group comparisons. Summary data for each group and filter are presented as mean ± SD and median (25th quartile, 75th quartile).

† Group 1, immune reconstituted, is defined as CD4+ T-lymphocyte counts currently ≥200/μL with lowest previous value <50/μL.
‡ Group 2, never severely immunosuppressed, is defined as CD4+ T-lymphocyte counts always >200/μL.
§ Comparison of all groups, using Kruskal-Wallis test.
|| Wilcoxon rank-sum test.

experience, the magnitude of the differences did not have a substantial effect on PMN rigidity. Neither current mean CD4+ T-lymphocyte counts nor the duration of potent antiretroviral therapy differed between the two HIV groups. In accordance with the experimental design, the mean nadir CD4+ T-lymphocyte count was lower in group 1. A comparison of PMN transit times measured by two filters is shown in Table 2. All probabilities shown in the following text are for filter A. Identical analyses of data generated by filter B yielded results similar to those for filter A, with identical conclusions. PMN transit times in group 1 (immune-reconstituted individuals) were significantly longer than in control subjects (P = 0.014). PMN transit times in group 2 (individuals who were never severely immunosuppressed) were also significantly longer than in control subjects (P = 0.046). A stronger difference between group 2 and control subjects was identified using filter B (P = 0.010). PMN transit times were not statistically different between the two HIV-infected groups (P = 0.518).

CMV end organ disease was present in four individuals (all in group 1, with inactive CMV retinitis). HIV was detected in the blood of seven individuals (four individuals in group 1 and three individuals in group 2). By comparing subgroups based on these factors, we investigated whether they influenced PMN rigidity. PMN transit time was not significantly different between individuals with CMV retinitis and all other HIV-infected individuals without CMV retinitis (P = 0.657). PMN transit time was not significantly different between individuals with detectable HIV in the blood and those without (P = 0.905).

Among the HIV-infected individuals, current CD4+ T-lymphocyte counts did not correlate with PMN transit times (P = 0.649). In addition, nadir CD4+ T-lymphocyte counts did not correlate with PMN transit times, when all HIV-infected individuals were considered together (P = 0.557) or in group 1 alone (P = 0.341). Given the limited data, we found no evidence that PMN transit times were greater in the three HIV-infected individuals who were not receiving potent antiretroviral therapy (2.61, 2.90, and 3.75 ms) than the PMN transit times in the 17 individuals who were receiving potent antiretroviral therapy (mean, 3.37 ± 0.452 ms; median, 3.34). PMN transit time was not associated with the duration of therapy when all HIV-infected individuals were considered together (P = 0.199) or when group 1 was considered alone (P = 0.264). PMN transit time was not related to the intervals, in that CD4+ T-lymphocyte counts rose above thresholds of 100/μL (P = 0.493) or 200/μL (P = 0.394) in group 1.

DISCUSSION

It has been hypothesized that changes in retinal blood flow, in the setting of the microvascular abnormalities associated with HIV, may contribute to HIV retinopathy (cotton-wool spots, retinal hemorrhages), to subtle vision changes in HIV-infected individuals (color vision abnormalities, altered contrast sensitivity), and to an increased risk of retinal infections in patients with AIDS.2-6 A distinction should be made between the development of cotton-wool spots and retinal hemorrhages (which occurs in patients with the most severe degrees of immunosuppression) and the retinal microvasculopathy associated with HIV disease. The latter is characterized by ultrastructural abnormalities of retinal capillaries (loss of pericytes, thickening of basal laminae, narrowing of capillary lumina) that can be found diffusely in most HIV-infected individuals at autopsy,7 even in the absence of the clinically apparent retinal lesions of HIV retinopathy. HIV retinopathy is uncommon in individuals with CD4+ T-lymphocyte counts above 200/μL,7 but the ultrastructural status of the microvasculature has not been studied at autopsy in this group of patients, in whom HIV-related mortality is low. Cotton-wool spots are believed to be caused by focal ischemia. Although microvasculopathy is probably necessary for development of cotton-wool spots in HIV-infected individuals, other dynamic factors must be involved in development of transient, focal ischemia, in that cotton-wool spots develop episodically, even in profoundly immunosuppressed individuals. There are probably multiple factors that contribute to such ischemic events. One such factor is erythrocyte aggregation in small vessels. A relationship has been found between cotton-wool spots and fibrinogen levels, the major determinant of erythrocyte aggregation.5 Thus, the absence of cotton-wool spots or retinal hemorrhages does not rule out the presence of microvascular disease, nor does it imply hemorheologic function in immune-reconstituted individuals.

Changes in leukocyte rigidity may affect retinal blood flow. Because of their large size and high cytoplasmic viscosity,11,12 leukocytes are believed to have an influence on hemodynamics that is disproportionate to their number in the circulation.5 The diameter of a leukocyte exceeds that of an average capillary,5,8 which can lead to capillary obstruction.5,8

Tufail et al.6 have shown that PMNs of HIV-infected individuals are more rigid than PMNs of HIV-negative control subjects. We reanalyzed data presented graphically in Figure 1 of Tufail et al.,6 which show that PMNs from the subgroup of patients with CD4+ T-lymphocyte counts less than 50/μL are significantly more rigid than PMNs from HIV-negative control subjects (P < 0.0001, Wilcoxon rank-sum test). Based on indirect evidence, Tufail et al.6 also speculated that these rigid PMNs may not recover normal deformability when CD4+ T-lymphocyte counts increase in response to potent antiretroviral therapy, suggesting a state of chronic PMN activation, despite improved immune function. Our study was undertaken to

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investigate this question. Our results indicate that HIV-infected individuals whose CD4 T-lymphocyte counts had increased as a result of potent antiretroviral therapy had PMNs that were significantly more rigid than PMNs of HIV-negative control subjects. Furthermore, PMN rigidity did not appear to be less in individuals who had been receiving potent antiretroviral therapy for longer periods or who had elevated CD4 T-lymphocyte counts for longer periods. Our findings are thus consistent with the hypothesis that PMN rigidity does not improve with immune reconstitution.

Lim et al. found reduced leukocyte velocity in macular capillaries in HIV-infected, severely immunosuppressed individuals and in those who had experienced immune reconstitution. The duration of potent antiretroviral therapy and the duration of elevated CD4 T-lymphocyte counts did not affect velocity measurements. There are probably many factors that affect macular leukocyte flow, including permanent damage to the microvasculature, but leukocyte rigidity may be one factor contributing to the persistence of reduced blood flow in immune-reconstituted individuals. Although an effect of rigid PMNs on macular blood flow is consistent with our findings, a direct causal relation in HIV-infected individuals has yet to be demonstrated.

PMN from HIV-infected subjects exhibit enhanced activation, yet the specific cause of this activated state is not known. In the present study, an unexpected finding was increased PMN rigidity in those individuals whose CD4 T-lymphocyte count had never been below 200/μL. This finding suggests that factors other than the level of immune function (as reflected by CD4 T-lymphocyte count) may influence PMN activation.

The cause of PMN activation is probably multifactorial, with more than one agent causing the observed increase of PMN rigidity. For example, PMNs can be activated by a variety of cytokines. Interleukin (IL)-15 has been described as a potent PMN activator in HIV-negative individuals, by causing direct morphologic cell changes and by stimulating PMNs to produce IL-8, a cytokine that induces both recruitment and activation of PMNs. Mastroiani et al. have suggested that PMNs from the following HIV-infected groups are activated in vitro by IL-15: those that are antiretroviral drug naïve, those successfully treated with potent antiretroviral therapy (with sustained increases in CD4 T-lymphocyte counts), and those in whom treatment with potent antiretroviral therapy has failed. PMN activation by IL-15 was dose dependent. Kacani et al. observed a significant increase in serum levels of IL-15 in HIV-infected individuals compared with healthy control subjects. Future studies should investigate whether IL-15 remains elevated in HIV-infected individuals after immune reconstitution.

PMNs are also activated by other cytokines. Skoutelis et al. have described decreased PMN deformability with the in vitro addition of tumor necrosis factor (TNF)-α, IL-1, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), and, to a lesser degree, IL-6. Before widespread use of potent antiretroviral therapy, it was shown that monocytes from HIV-infected individuals produce spontaneous and significantly increased quantities of IL-1 and TNF-α. Aukrust et al. found that TNF-α levels decrease with effective potent antiretroviral therapy, but levels do not normalize and remain significantly elevated compared with levels in HIV-negative control subjects. In addition, TNF-α levels increase with treatment failure. Using a MedLine search, we were unable to find current literature describing the quantities of IL-1 or -6 in HIV-infected individuals during the era of potent antiretroviral therapy (Medline is provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD, and is available at http://www.ncbi.nlm.nih.gov/PubMed/).

In many HIV-infected individuals, antiretroviral therapy is supplemented with hematopoietic growth factors. Both G-CSF and GM-CSF have been shown to increase PMN rigidity and both are currently being examined as possible supplements to antiretroviral therapy. The use of such agents among our subjects was not determined.

Although infections of PMN by HIV or CMV have been hypothesized as possible causes of cell activation, we found no relationship between PMN rigidity and the presence of detectable HIV in the blood or a history of CMV retinitis. We were unable to draw conclusions regarding a role for CMV infection in PMN activation on the basis of this observation. In all cases, CMV retinitis was clinically inactive, and the persistence of lesions would therefore reflect past, rather than current, viremia. It is unlikely, however, that patients receiving potent antiretroviral therapy with CD4 T-lymphocyte counts more than 200/μL would have circulating CMV. Thus, CMV would be unlikely to explain the common occurrence of persistent PMN activation demonstrated in this study. Previous studies of leukocyte rigidity in HIV-infected individuals have not shown an effect of age, systolic blood pressure, serum creatinine, cholesterol, or triglycerides on PMN rigidity in HIV-infected individuals, and thus these factors were not examined in the present study.

Persistent PMN activation may also have adverse effects on the retina by mechanisms other than its potential direct role in altering blood flow. Activated PMNs are capable of inducing direct microvascular damage by adhering to endothelial cells followed by degranulation. Vascular damage is mediated through the release of proteases and toxic oxygen radicals. Thus, HIV-infected individuals may be at risk of progression of retinal microvascular damage, despite immune reconstitution.

In HIV-infected individuals, alterations in blood flow and microvascular abnormalities may contribute to the subtle forms of visual dysfunction that occur in HIV-infected individuals. Investigation of contrast sensitivity in HIV-infected individuals grouped by the same criteria used in this study found no evidence that contrast sensitivity improves with immune reconstitution. Furthermore, contrast sensitivity is abnormal, even in HIV-infected individuals without a history of severe immunosuppression.

PMNs are also more rigid in individuals with diabetes, which may contribute to the development of diabetic retinopathy. The ultrastructural changes associated with diabetic retinopathy are similar to those of HIV-related retinal microvasculopathy. It has been hypothesized that microvascular abnormalities in diabetic patients lead to changes in contrast sensitivity, as described earlier in HIV-infected individuals. If PMNs remain rigid in HIV-infected individuals, despite improved general health associated with potent antiretroviral therapy, long-term survivors may be at risk for progressive retinal damage, as occurs in diabetes.

Although we could not confirm that PMN rigidity was the same in those who had never been severely immunosuppressed and those who had experienced immune reconstitution, the values and distributions were similar between these groups, and it is unlikely that a true difference exists. Thus, even HIV-infected individuals whose CD4 T-lymphocyte counts have never fallen below 200/μL may be at risk for ongoing damage to the retina. The magnitude of the risk may be different between groups, however, depending on differences in the severity of microvascular damage, which probably would be greater in the group with a history of severe immunosuppression.

In summary, our results suggest that PMNs remain activated and rigid after immune reconstitution in HIV-infected individuals with a history of AIDS. Furthermore, PMN rigidity is increased in HIV-infected individuals, even without a history of severe immunosuppression. Thus, HIV-infected individuals...
may remain at risk for retinal vascular damage in the era of potent antiretroviral therapy. Further investigation of this possibility would be best undertaken in a longitudinal study.

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References