Increased polymorphonuclear leucocyte rigidity in HIV infected individuals

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Abstract

Aim—Individuals with human immunodeficiency virus (HIV) infection were evaluated for evidence of abnormal polymorphonuclear leucocyte (PMN) rigidity, which can alter capillary blood flow.

Methods—The transit time of individual PMN through 8 μm pores in a cell transit analyser was used as a measure of cell rigidity. PMN transit time was compared between HIV infected individuals (n=45) with and without CMV retinitis and HIV negative controls (n=17).

Results—Transit times were longer for PMN from HIV infected individuals than for PMN from controls (p<0.001). PMN from HIV infected individuals with CMV retinitis (n=13) had longer transit times than PMN from those without CMV retinitis (n=32, p<0.001). Transit times were longer in HIV infected individuals with lower CD4+ T lymphocyte counts (p<0.001). Regression analysis indicated that the relation between transit times and the presence of CMV retinitis could not be explained solely on the basis of low CD4+ T lymphocytes. In HIV infected individuals, mean transit time was not correlated with age, blood pressure, or serum creatinine, cholesterol, or triglycerides.

Conclusions—HIV infected individuals appear to have increased PMN rigidity, a cellular change that might be involved in the pathogenesis of HIV related retinal microvasculopathy. PMN rigidity appears to be related to severity of immune dysfunction. PMN rigidity may remain high in patients with CMV retinitis after elevations of CD4+ T lymphocyte counts that result from potent antiretroviral therapy.

(*Br J Ophthalmol* 2000;**84**:727–731)

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Retinal microvascular changes and abnormalities in blood flow occur commonly in human immunodeficiency virus (HIV) infected individuals.[1][2] These changes are believed to be among the factors contributing to the development of cotton wool spots and retinal haemorrhages (also known as "HIV retinopathy"), which occur primarily in patients with acquired immunodeficiency syndrome (AIDS), who have the most severe degrees of immunosuppression. HIV related retinal microvascular changes are ultrastructurally similar to those seen in patients with diabetic retinopathy.[2] The cause of these changes is unknown.

Activated polymorphonuclear leucocytes (PMN) are capable of causing direct microvascular damage by the release of proteases and toxic oxygen radicals.[3] In addition, leucocytes have been shown to have a strong influence on microvascular blood flow,[4] in part because they are larger and more rigid than erythrocytes. It is known that PMN in diabetics have increased rigidity,[5] a sign of activation, and a factor that may slow capillary blood flow.

Little is known about the rheological behaviour of leucocytes from HIV infected individuals. We used an in vitro technique to study PMN rigidity in HIV infected individuals, and to determine whether changes in PMN rigidity are related to levels of immune function, as reflected by CD4+ T lymphocyte counts. Understanding haemorheological changes might have clinical implications for understanding retinal and visual problems that occur in HIV infected patients.

Materials and methods

A cell transit analyser was used to measure the time required for individual PMN to pass through 8 μm pores in vitro. Increased pore transit time is attributable to decreased PMN deformability (that is, the ability of the entire cell to adopt a new shape in response to deforming forces), and thus is a measure of PMN rigidity. PMN transit times were studied for 45 HIV infected individuals and 17 HIV negative controls.

Volunteer HIV infected individuals were recruited from the consultation suites of the Jules Stein Eye Institute and from the University of California, Los Angeles (UCLA) Center for Clinical AIDS Research and Education. Individuals with HIV disease were recruited without regard to the presence or absence of HIV related retinal disease. Patients with diabetes mellitus and individuals who had received blood transfusions within the previous month were excluded from the study. The study was approved by the UCLA human subjects protection committee, and all subjects gave informed consent.

The presence or absence of CMV retinitis or other retinal lesions was determined for each HIV infected individual by indirect ophthalmoscopy. The characteristics of CMV retinitis (duration and activity of disease) and the use of systemic medications were not considered in analyses. Blood pressure was measured at the time of venepuncture. The following studies were performed on each blood specimen: PMN deformability, leucocyte count, and serum creatinine, cholesterol, and triglyceride levels. Tests other than PMN deformability were performed using standard methods in the clinical laboratories of the UCLA Medical Center.

Table 1 Comparison of HIV infected individuals and HIV negative controls

	HIV infected individuals (n=45)	HIV negative controls (n=17)	p Value*
Age (years)	39.1 (1.2)†	37.5 (2.2)	0.47
sBP (mm Hg)	130 (2.1)	128 (3.7)	0.67
dBP (mm Hg)	76 (1.6)	74 (3.8)	0.67
Transit time‡ (ms)	5.56 (0.23)	4.02 (0.16)	0.0002

sBP=systolic blood pressure.
dBP=diastoloic blood pressure.
*Two sample *t* tests allowing for unequal variances.
†Data are means (SE).
‡In vitro polymorphonuclear leucocyte transit time in a cell transit analyser as a measure of leucocyte rigidity.

PREPARATION OF POLYMORPHONUCLEAR LEUCOCYTES

Whole blood was gently drawn into a plastic syringe containing sodium heparin (10 U/ml) using a 21 gauge needle, with the tourniquet having been removed immediately after insertion of the needle. Pure PMN suspensions were prepared using a density based centrifugal technique; endotoxin free sterile media and plasticware were used to avoid in vitro PMN activation. Heparinised whole blood (7 ml) was mixed with 3 ml of phosphate buffered saline (PBS, pH = 7.4, 285 mOsm/kg), gently layered onto 5 ml of 54% Percoll-PBS (final density = 1.076 g/cm^3, Sigma Chemical Co, St Louis, MO, USA), and then centrifuged at 400 *g* for 25 minutes at 22°C. The top 3 ml of the resulting PMN and red blood cell interface and pellet were transferred into distilled water for 30 seconds, to remove residual erythrocytes by hypotonic lysis. Tonicity was then restored by the addition of 10× PBS, and the suspension spun at 220 *g* for 6 minutes at 22°C. The PMN were washed and maintained at room temperature in PBS. Purity was assessed by optical microscopy (>96% PMN), and leucocyte count was determined using an automated device.

RIGIDITY MEASUREMENTS

PMN rigidity was assessed using the cell transit analyser (CTA; ABX Hematologie, Montpellier, France).[6] The central feature of the CTA is a special filter that separates two fluid filled reservoirs. The filter is a specially fabricated thin sheet of polycarbonate plastic containing 30 identical 8 μm diameter by 21 μm length pores, and thus represents a simple, yet geometrically stable, in vitro model of a capillary bed.[6] Hydrostatic pressure generated by the 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. 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).[6]

The following experimental variables were employed: (1) pressure gradient of 8 cm water; (2) measurements at 25°C; (3) a final PMN concentration in sterile PBS of 10^5 cells/ml; (4) at least 1000 cells per run; and (5) 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 summarise 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 measurements used the same filter and were completed within 4 hours of venepuncture. To check for possible temporal changes of the CTA filter characteristics with repeated use, control samples with a known range of PMN transit times were processed with each batch of samples.

STATISTICAL ANALYSIS

Comparisons between groups were performed by using two sample *t* tests allowing for unequal variances. Relations between variables were analysed by product moment Pearson correlation. Multiple linear regression was used to study in more depth relations between CD4+ T lymphocyte counts, CMV retinitis, and mean transit times.

Results

All patients with CMV retinitis were receiving systemic anti-CMV drugs at the time of examination. No patients had cotton wool spots or retinal haemorrhages.

PMN from HIV infected individuals had longer mean transit times (5.56 (SE 0.23) ms) than HIV negative controls (4.02 (0.16) ms, p<0.001) (Table 1). There were no significant differences between these groups in terms of age or blood pressure (all p values ≥0.47). PMN from patients with CMV retinitis had longer mean transit times (6.91 (0.33) ms) than HIV infected patients without CMV retinitis (5.01 (0.24) ms, p<0.001) (Table 2). There were no significant differences between these subgroups in terms of age, systolic blood pressure, or serum creatinine, cholesterol, or triglycerides (all p values ≥0.20). Individuals with CMV retinitis had higher diastolic blood pressure (p=0.02) and lower CD4+ T lymphocyte counts (p=0.04) than those without CMV retinitis. Mean HIV blood levels were higher in patients with CMV retinitis, although the difference was not significant (p=0.26).

The mean transit time of PMN from HIV infected patients with CD4+ T lymphocyte

Table 2 Comparison of HIV infected individuals with and without CMV retinitis

	Individuals with CMV retinitis (n=13)	Individuals without CMV retinitis (n=32)	p Value*
Age (years)	41.1 (2.3)	38.2 (1.4)†	0.26
sBP (mm Hg)	131 (3.3)	129 (2.7)	0.75
dBP (mm Hg)	81.7 (1.8)	73.5 (2.0)	0.02
Serum creatinine (mg/dl)	1.27 (0.22)	0.93 (0.05)	0.20
Serum cholesterol (mg/dl)	189 (24.0)	191 (13.5)	0.96
Serum triglycerides (mg/dl)	392 (226)	468 (68.5)	0.69
CD4+ T lymphocyte count (cells ×10^6/l)	68.4 (26.5)	175 (27.3)	0.04
HIV blood level (genomes per ml)	217 000 (126 000)	50 400 (25 600)	0.26
Transit time‡ (ms)	6.91 (0.33)	5.01 (0.24)	0.0001

sBP=systolic blood pressure.
dBP=diastoloic blood pressure.
*Two sample *t* tests allowing for unequal variances.
†Data are means (SE).
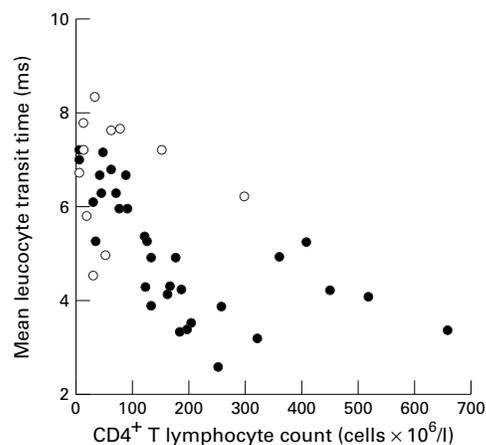‡In vitro polymorphonuclear leucocyte transit time in a cell transit analyser as a measure of leucocyte rigidity.

Figure 1 Polymorphonuclear leucocyte rigidity (expressed as in vitro transit times in a cell transit analyser) versus CD4+ T lymphocyte count in HIV infected individuals. There is a statistically significant correlation (r=−0.61, p<0.0001). Individuals without CMV retinitis are indicated with solid symbols; individuals with CMV retinitis are indicated with open symbols.

counts >100 ×10⁶/l (4.36 (0.23) ms) was not significantly different from that of controls (4.02 (0.16) ms, p=0.25). There were no significant differences between these two groups in terms of age or blood pressure (all p values ≥0.36).

Among all HIV infected individuals, transit times were inversely correlated with CD4+ T lymphocyte counts (r =−0.61, p<0.001) (Fig 1) Transit times were not correlated with age, blood pressure, HIV blood level, or serum creatinine, cholesterol, or triglycerides (all p values ≥0.09). The relations of CMV retinitis and CD4+ T lymphocyte count to pore transit times were considered in a regression analysis; both CD4+ T lymphocyte count and CMV retinitis were significant predictors of increased transit time when all patients were considered. Thus, the relation between CMV retinitis and transit time could not be explained solely on the basis of low CD4+ T lymphocyte counts.

Although sample sizes are small, it was informative to look at subgroups based on CD4+ T lymphocyte counts. In five of the 11 patients with CMV retinitis for whom CD4+ T lymphocyte counts were available, counts were >50 ×10⁶/l, the threshold above which CMV retinitis usually does not occur. Values for these five patients were 52, 61, 78, 154, and 300 ×10⁶/l. It is therefore likely that these patients were receiving combination antiretroviral therapy and had experienced elevations in CD4+ T lymphocyte counts after CMV retinitis developed. PMN from these five individuals had a mean transit time (6.80 (0.48) ms) that was nearly identical to the value for patients with CMV retinitis whose CD4+ T lymphocyte counts were <50 ×10⁶/l (n=6, 6.76 (0.57) ms, p=0.96). Among HIV infected individuals whose CD4+ T lymphocyte counts were <50 ×10⁶/l , the mean transit time for PMN from those without CMV retinitis (n=7, 6.56 (0.27) ms) was not significantly different from mean transit time for patients with CMV retinitis (n=6, 6.76 (0.57) ms, p=0.76). The relation between CD4+ T lymphocyte count

and pore transit time remained significant when only HIV infected individuals without CMV retinitis were considered (r = −0.63; p=0.0001).

Discussion

Studies of the rheological behaviour of blood and its formed elements may provide insight into the pathogenesis of HIV related diseases of the retina. When PMN are activated, f-actin content increases, the cells become more rigid, and they can obstruct flow in capillaries.[4] Activated PMN also tend to adhere to endothelial cells and may degranulate, leading to local vascular endothelial damage mediated through the release of potent proteases and oxygen radicals. Thus, rigid PMN might damage microvasculature with permanent alterations of blood flow.

Abnormal PMN rigidity has been found to occur in patients with diabetes mellitus,[5] who have microvascular changes similar to those seen in HIV infected individuals. Similar PMN abnormalities occur in a feline model of diabetes mellitus.[7] The increased PMN transit times found in our study indicate that PMN are also more rigid in HIV infected individuals. A relation between abnormal PMN rigidity and microvasculopathy associated with HIV disease remains speculative. The microvasculopathy of HIV disease cannot be seen clinically; thus, although we have identified a factor that could contribute to vascular damage, we cannot confirm the relation in this study.

None of the patients in this study had cotton wool spots or retinal haemorrhages ("HIV retinopathy"). A distinction should be made between these lesions, which are seen in patients with the most severe degrees of immunosuppression, and the microvasculopathy associated with HIV disease. Ultrastructural abnormalities of retinal capillaries can be found diffusely in most HIV infected individuals at necropsy, even in the absence of clinically apparent retinal lesions. Cotton wool spots are believed to be caused by focal ischaemia. Although microvascular disease may be necessary for development of cotton wool spots, other factors probably contribute to the development of focal ischaemia; for example, a relation has been found between cotton wool spots and elevated fibrinogen levels.[1] The absence of cotton wool spots or retinal haemorrhages does not rule out the presence of microvascular disease in our patients. By analogy, microvascular abnormalities can occur in diabetics without clinical signs of diabetic retinopathy.[8] Because none of the patients in this study had clinical signs of HIV retinopathy, we cannot evaluate whether or not PMN rigidity plays a part in the pathogenesis of focal lesions associated with ischaemia. The absence of clinically apparent HIV retinopathy does not, however, rule out a role for PMN rigidity in the pathogeneses of HIV related microvasculopathy.

The mechanism of decreased PMN deformability in HIV infected individuals is unknown. It might be due to changes in membrane lipid composition, as seen in diabetic rats.[9] Another

possibility is a state of chronic PMN activation, caused by HIV infection or other stimuli. PMN from HIV infected individuals have been shown to express increased levels of CDI Ib/CD18 adhesion molecule, decreased levels of L-selectin antigen expression, and increased actin polymerisation, all of which suggest chronic PMN activation.[10] Although factors such as age, blood pressure, and serum creatinine, cholesterol, and triglyceride might affect PMN rigidity, the relation identified in this study could not be explained on the basis of those factors.

Use of filgrastim (granulocyte colony stimulating factor) to elevate PMN counts is common among HIV infected patients receiving ganciclovir or other drugs that cause bone marrow suppression. Subjects were not questioned about the use of filgrastim or other leucocyte growth factors, and therefore any effect of such agents on PMN rigidity is unknown.

PMN rigidity was related to CD4+ T lymphocyte count, but no association was found with HIV blood levels, another indicator of the severity of HIV related immunosuppression. The relation between CMV retinitis and PMN rigidity was not solely a reflection of the fact that CMV retinitis occurs at low CD4+ T lymphocyte counts. This observation should be interpreted with caution, however, because CD4+ T lymphocyte counts could have changed during the course of the retinal infection, attributable to initiation of potent antiretroviral therapy. This study was performed in 1996 before many patients were receiving protease inhibitors, and we did not control for use of antiretroviral therapy, which increases CD4+ T lymphocyte counts. We did observe, however, that counts were above 50×10^6/l (the threshold above which CMV retinitis usually does not develop) in five of 11 patients with CMV retinitis. Information was not available about whether or not patients were receiving potent antiretroviral therapy at the time of the study; from these data, however, we can infer that their counts had risen after development of CMV retinitis, in response to antiretroviral therapy. Before the era of potent antiretroviral therapy, it would have been highly unusual for five of 13 patients to have CMV retinitis with CD4+ T lymphocyte counts above 50×10^6/l. Transit times remained high in the subgroup of patients with CMV retinitis and high CD4+ T lymphocyte counts. If one hypothesised that CD4+ T lymphocyte counts were the sole predictor of PMN rigidity, then elevation of counts should return transit times to lower values, and one would expect to see transit times that are lower in patients with CMV retinitis and counts >50×10^6/l than in those patients with CMV retinitis whose counts remain <50×10^6/l. We did not see evidence of this pattern, however. The fact that transit times were nearly identical for these two subgroups of patients suggests that once PMN rigidity is increased, it may not return to normal, even with improved immune function, placing HIV infected patients at continued risk for retinal vascular disease. The half life of a PMN in the circulation is measured in hours,

indicating that there must be a state of chronic PMN activation in the subpopulation of patients with CMV retinitis. The cause and duration of this phenomenon is unknown. Additional studies should be undertaken to determine whether PMN rigidity will eventually return to normal after longer periods of immune reconstitution than were present in our patients.

Haematogenous spread of CMV to retinal tissue is supported by the findings that CMV retinitis frequently develops adjacent to retinal blood vessels, and the fact that development of CMV retinitis in both patients with AIDS and other immunosuppressed patients is related to CMV viraemia. It is interesting to speculate that haemorheological abnormalities might facilitate the development of CMV retinitis, by slowing the transit of infected leucocytes through the retinal vasculature or by increasing the interaction between CMV infected leucocytes and vascular endothelium. Although a relation between CMV retinitis and transit time that is separate from the effect of low CD4+ T lymphocyte counts was not found when evaluating only patients with counts <50×10^6/l, sample sizes were small, and our study was not designed to investigate a causal relation between PMN rigidity and CMV retinitis. Our population was not necessarily representative of patients at the onset of CMV retinitis; CD4+ T lymphocyte counts and transit times may have changed subsequent to disease onset. Conversely, CMV disease might be a cause, rather than the effect, of PMN activation and increased rigidity. The activation state of PMN in patients with AIDS and CMV retinitis is currently unknown, but PMN have been identified as the site of early replication of CMV during reactivation from latency.[11]

Even in the absence of intraocular opportunistic infections or other clinically apparent retinal lesions, HIV infected patients can have visual abnormalities, including visual field defects, changes in colour vision, and altered contrast sensitivity. It is possible that these subtle changes in vision might be attributable to haemorheological abnormalities and the retinal microvasculopathy of HIV infection. In diabetics, loss of contrast sensitivity has been related to retinal capillary dropout.[12] With the increased survival of HIV infected individuals attributed to potent antiretroviral therapies, it is increasingly important to understand the pathogenesis of HIV related retinal microvasculopathy and its associated clinical abnormalities.

In summary, we have identified abnormal rigidity of PMN in HIV infected individuals, a factor that could alter blood flow through retinal capillaries and could lead to retinal microvascular damage. Abnormalities in leucocyte rheology may be only one of many factors contributing to HIV related retinal diseases, but it potentially offers a novel approach to the study of retinal microvasculopathy and its associated clinical disorders in HIV infected individuals. Our data have also identified an association between the mechanical behaviour of PMN and chronic CMV retinitis that should be

clarified in future studies. Furthermore, leucocyte rheology may provide a link to understanding the similarity between the retinal microvasculopathy of AIDS and diabetic retinopathy.

1 Engstrom RE, Holland GN, Hardy WD, *et al*. Hemorheologic abnormalities in patients with human immunodeficiency virus infection and ophthalmic microvasculopathy. *Am J Ophthalmol* 1990;**109**:153–61.
2 Pepose JS, Holland GN, Nestor MS, *et al*. Acquired immune deficiency syndrome. Pathogenic mechanisms of ocular disease. *Ophthalmology* 1985;**92**:472–84.
3 Weiss SJ. Tissue destruction by neutrophils. *N Engl J Med* 1989;**320**:365–76.
4 Sutton DW, Schmid-Schonbein GW. Elevation of organ resistance due to leukocyte perfusion. *Am J Physiol* 1992;**262**:H1646–50.
5 Pecsvarady Z, Fisher TC, Darwin CH, *et al*. Decreased polymorphonuclear leukocyte deformability in NIDDM. *Diabetes Care* 1994;**17**:57–63.
6 Pecsvarady Z, Fisher TC, Fabok A, *et al*. Kinetics of granulocyte deformability following exposure to chemotactic stimuli. *Blood Cells* 1992:333–52.
7 Braun RD, Fisher TC, Meiselman HJ, *et al*. Decreased deformability of polymorophnuclear leukocytes in diabetic cats. *Microcirculation* 1996;**3**:271–8.
8 Arend O, Wolf S, Jung F, *et al*. Retinal microcirculation in patients with diabetes mellitus: dynamic and morphological analysis of perifoveal capillary network. *Br J Ophthalmol* 1991;**75**:514–18.
9 Masuda M, Murakami T, Egawa H, *et al*. Decreased fluidity of polmorphonuclear leukocyte membrane in stroptozocin-induced diabetic rats. *Diabetes* 1990;**39**:466–70.
10 Elbim C, Prevot MH, Bouscarat F, *et al*. 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. *Blood* 1994;**84**: 2759–66.
11 Gerna G, Zipeto D, Percivalle E, *et al*. Human cytomegalovirus infection of the major leukocyte subpopulations and evidence for initial viral replication in polymorphonuclear leukocytes from viremic patients. *J Infect Dis* 1992;**166**: 1236–44.
12 Remky A, Arend AR, Evans D, *et al*. Contrast sensitivity loss is coupled with capillary dropout in patients with diabetes. *Invest Ophthalmol Vis Sci* 1997;**38**:1819–24.