Filterability of Subpopulations of Leukocytes: Effect of Pentoxifylline

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Human leukocytes were separated by density into two fractions, one containing predominantly granulocytes (FI) and the other, lymphocytes and monocytes (FII). The filterability of these fractions and their mixture was determined from the pressure measured during constant flow through 5-μm Nuclepore filters. The pressure-time curve of FI indicates the behavior of a relatively homogeneous cell population. The FI pressure-time curve can be analyzed to distinguish the effect of the more numerous and more filterable lymphocytes from that of the sparser but less filterable monocytes. Pressure generated by mixed leukocytes, which had been treated with 1 and 10 mmol/L of pentoxifylline (PTX) or its metabolite I, was substantially less than untreated control; at 10 mmol/L, the pressure was reduced to about 50% of control. PTX appears to affect the filterability of monocytes and polymorphonuclear leukocytes but not lymphocytes. Scanning electron microscopy showed an inhibition of protopod formation in the treated granulocytes. The degree of cell adhesion to the filter, as measured by the number of cells remaining on the outflow side of the filter, was similar in all groups (FI and FII, treated and untreated). The results indicate that the variations in filterability between the leukocyte subpopulations and the improvement by PTX treatment reflect differences in the cells’ ability to deform under the test conditions.

Materials and Methods

Normal human blood, taken from healthy volunteers in accordance with the Helsinki Declaration and with the approval of the local Human Investigations Committee, was placed on a Ficoll-Hypaque gradient (Mono-Poly Resolving Medium, Flow Laboratories, McLean, Va). After 30 minutes of centrifugation at 300 g, two layers of leukocytes appeared above the erythrocytes and were separately removed by pipetting. The lower layer was designated fraction I (FI) and the upper layer, fraction II (FII). As assessed by light microscopy of Wright-stained slides, 95.5% ± 1.3% (SD) of FI cells were polymorphonuclear neutrophils (PMNs), with 3.0% ± 2.2% lymphocytes and 1.7% ± 1.5% eosinophils. The leukocytes in FII were composed of 82.3% ± 5.7% lymphocytes, 12.2% ± 3.5% monocytes and 5.5% ± 2.9% granulocytes; platelets were also seen in FII. For experiments on mixed leukocytes, cells were either obtained from the plasma layer after gravity sedimentation for one hour or were pooled from FI and FII after Ficoll-Hypaque sedimentation. The mixed populations of cells obtained by either method were found to contain about 60% PMNs, 30% lymphocytes and monocytes, and 5% eosinophils.

All leukocyte suspensions were washed three times in a Tris-buffered (pH 7.4) Ringer-albumin solution consisting of 0.9 g/dL NaCl, 0.03 g/dL KCl, 0.1 g/dL glucose, 27.5 mg/dL CaCl₂ (2.2 mmol/L), 0.5 g/dL albumin, and 7.5 mmol/L Tris. The cells were resuspended at a concentration of 3,000 to 4,500/μL.

PTX (supplied by Hoechst-Roussel Pharmaceuticals, Inc, Somerville, NJ) was dissolved in saline at concentrations from 0.1 to 100 mmol/L and added to the cell suspension at a volume ratio of 1:9, thus diluting the drug to one tenth of its initial concentration. Metabolite I, which is not soluble at 100 mmol/L, was dissolved in buffer at 10 mmol/L. The washed and resuspended cells were centrifuged, the supernatant removed as completely as possible, and the suspension reconstituted by adding the 10 mmol/L metabolite I solution or the buffer control.
FILTERABILITY OF WBC SUBPOPULATIONS

The cell count for each prepared suspension was determined on a Coulter Model ZB Counter (Coulter Electronics, Hialeah, Fla.). Final white cell concentrations for all studies ranged from 2,500 to 4,500 cells/μL, but for any given experiment, the variation among different samples was within ± 200/μL. All samples (including the controls) were incubated at 37 °C for one hour and then allowed to return to room temperature for 30 minutes before the beginning of the filtration test.

The filtration device has been described previously.10 With the use of a Harvard pump (Harvard Apparatus, Millis, Mass.), the cell suspension was pumped at a steady flow rate (1.6 mL/min) through a polycarbonate filter (Nuclepore Corp, Pleasanton, Calif) housed in a lucite block. Pressure measurements were made from a 0.5-cc chamber located just before the filter with a Statham transducer and a lucite block. Pressure measurements were performed on the saline pressure (P0) to attain a polycgraph recorder (Grass Instrument Co., Quincy, Mass). The filters used had 4.8 × 10³ pores/cm²; the pore diameter averaged 4.5 ± 0.5 (SD) μm.

Our previous studies11 have shown that a mixture of two populations of cells, one with a higher concentration and a greater deformability than the other (eg, red cells at 5% hematocrit and leukocytes at 3,000/μL), would generate a pressure-time curve with the following pattern. An initial pressure rise is seen within the first second to attain a transient plateau (Pt), and this is followed by a secondary pressure rise. The ratio of Pt to the saline pressure (P0) reflects primarily the resistance of the cells present in high concentration. The secondary rise in pressure reflects mainly the resistance of the less deformable cells existing in a lower concentration. This pressure rise can be explained on the basis of pore plugging,15 when the pressure reaches a plateau, the shear stress is sufficient to cause the cells to pass through the filter, and there is no further increase in plugging. The ratio of pressure readings taken at the later phase of filtration to the saline pressure (P0) can be used as a measure of the rigidity of the cells existing in the lower concentration.

After one hour of incubation in either the control or experimental medium, an aliquot of the leukocyte suspension was prepared for scanning electron microscopy by overnight fixation in 20 vol of 1% glutaraldehyde in cacodylate buffer at pH 7.4. The samples were then washed in cacodylate buffer, postfixed in 1% OsO₄ for one hour, and dehydrated in ascending ethanol series. The cells were spread on 0.2-μM Millipore filters and critical-point dried. The Millipore filters used in the filtration test were removed after 30 seconds of pumping, placed in the glutaraldehyde-cacodylate buffer, and processed in the same way as the suspensions. The filters were coated with gold-palladium and viewed in a Jeolco model JSM-25 scanning electron microscope at 15 kV. Transmission electron microscopy was also performed on the dehydrated specimens after embedding in Spurr's low viscosity embedding medium. The blocks were stained with uranyl acetate and lead citrate, and thin sections were observed in a Zeiss JSM transmission electron microscope.

Leukocyte adhesion was quantified by counting the cells remaining on the outflow side of the filter. Adherent cells move out of the pore with the flow under the shear stresses (400 to 700 dyne/cm²) used in the filtration test, but remain attached to the downstream side of the filter by a "tether" of membrane unless the force becomes great enough to break the tether.16 Adhesion is therefore represented by teardrop-shaped cells on the downstream side of the filter with an attachment to the pore. Generally, several cells are tethered to the same pore, and these appear as grape-like clusters hanging below the filter, similar to those described by Lessin et al.17 for the filtration of sickle erythrocytes. Some apparently nonadherent cells were also found to remain on the filter. For this analysis, the filters were removed after precisely 30 seconds of filtration of the suspension and placed immediately in glutaraldehyde. Ten adjacent fields (approximately 2,600 pores) from the downstream side of the filter were photographed under the scanning electron microscope at a magnification of 260x. The number of cell clusters per pore, cells per cluster, and total number of cells (tethered and untethered) per pore were ascertained.

RESULTS

The shapes of the pressure–time curves for the filtration of Fl and F II are distinctly different (Fig 1). For Fl (primarily PMNs), the pressure rose abruptly to become almost constant at ten seconds. For F II (82% lymphocytes and 12% monocytes), the pressure was lower than that for Fl during the first ten seconds, but it rose progressively to become higher than that for Fl at 30 seconds.

The filtration pressure of both fractions was reduced by 10 mmol/L PTX (Fig 2). The effect of PTX on Fl was evident as early as five seconds after the onset of
filtration, whereas with FII, a significant effect did not appear until 20 seconds had elapsed.

PTX at 10 mmol/L concentration lowered the filtration pressure of mixed leukocytes to about 50% of control; the effect was not significant at lower doses. Metabolite I of PTX had an effect similar in magnitude to the parent drug (Fig 3).

After one hour incubation without PTX, the control FI cells underwent spontaneous deformation with the formation of protopodia (Fig 4A). FI cells concurrently incubated with 10 mmol/L PTX did not form protopodia (Fig 4B). The treated and untreated FII cells did not show a visible difference in morphology. Cell size distribution in both FI and FII fractions, as measured by the Coulter Channelizer (Coulter Electronics) showed no alteration with PTX treatment. The amounts of cells retained on the filters are similar between control and PTX-treated cells, as examined by scanning electron microscopy (Table 1). Transmission electron microscopy of both PTX-treated and control FI cells showed normal intracellular organelles (Fig 5).

**DISCUSSION**

Analysis of the pressure–time curves obtained with the two fractions of leukocytes can help to determine the relative filterability of different types of cells. The height of the pressure rise at any given time after onset of filtration is determined by the characteristics of the individual cells and their concentrations. The cells existing in highest concentrations were granulocytes in FI and lymphocytes in FII; these cells are probably responsible for the pressure rise in the early phase when filtering these respective fractions. When the FI pressure rise has peaked, the pressure for FII is only about one half as high. These results suggest that lymphocytes pass more easily through 5-μm pores than granulocytes. After 20 to 30 seconds of filtration, however, the pressure becomes higher for FII than FI cells. The monocytes, which exist in FII at a relatively low concentration, are probably responsible for this late pressure rise. The monocytes can be expected to have a low filterability because of their large size.

PTX affected both subpopulations of leukocytes separated by the Ficoll-Hypaque gradient. For FI, the entire pressure curve was lowered by PTX treatment, indicating that PTX improved the filterability of granulocytes. For FII, the pressure at 20 and 30 seconds became lower for the PTX-treated cells than their untreated control, suggesting that the filterability of

**Table 1. Adherence of Leukocytes to Nuclepore Filters**

<table>
<thead>
<tr>
<th>Percent Pores With Clusters</th>
<th>Cells/Cluster</th>
<th>Cells/100 Pores*</th>
</tr>
</thead>
<tbody>
<tr>
<td>FI Control</td>
<td>6.3 ± 3.3</td>
<td>3.17 ± 0.42</td>
</tr>
<tr>
<td>FI PTX 10⁻³ mmol/L</td>
<td>6.2 ± 1.7</td>
<td>3.24 ± 0.40</td>
</tr>
<tr>
<td>FII Control</td>
<td>7.0 ± 1.6</td>
<td>2.88 ± 0.70</td>
</tr>
<tr>
<td>FII PTX 10⁻³ μmol/L</td>
<td>5.7 ± 1.6</td>
<td>3.18 ± 0.50</td>
</tr>
</tbody>
</table>

ANOVA showed no significant difference among groups in any column.

*Including individual cells as well as cells in clusters.
monocytes is improved by PTX. The close agreement between the curves for control and PTX-treated FiI cells in the early phase, when the contribution of lymphocytes is predominant, indicates that lymphocyte filterability is not affected by the drug. Earlier reports\(^4\)\(^5\) on PTX-induced improvement in the filter-ability of red blood cells did not take into account the effect on the WBC.

The filterability of a cell may be affected by its adheriveness to the filter. When cell adhesion is marked, eg, during the filtration of sickle erythrocytes,\(^6\) clusters of cells develop around the outflow of the pore, with each cell linked by a filament to the inside of the pore. Cell adhesion to the pore can influence filtration pressures in several ways. First, the force required to cause plastic deformation and/or breakage of the tether is in addition to that needed merely to induce shear deformation of the cell. Second, the presence of traumatized cell membrane in the pore probably facilitates the adhesion of subsequent cells. Finally, the tethers themselves slightly diminish the lumen of the pore, thus impeding cell passage. Scanning electron microscopy of the filter at 30 seconds, when plateau pressures had been reached, showed a moderate degree of cluster formation, which was comparable between the PTX-treated and control cells (Table 1). These results suggest that cell adhesion to the filter or other cells is not the primary cause of the difference in filtration behavior between these groups. Hence, it appears that the size and deformability of the cells are the main determinants of their filterability in these tests.

Cell deformability is influenced by the cell geometry (the ratio of surface area to volume and the overall shape), the viscoelastic properties of the membrane, and the viscoelastic properties of the cell interior.\(^7\)\(^8\) We have not performed morphometric measurements to quantitate the geometry of the cells studied. However, the presence of a large amount of excess membrane area (membrane folding)\(^9\) in both PTX-treated and control samples, as seen under electron microscopy (Figs 4 and 5), suggests that cell geometry is probably not a limiting factor.

The rheologic behavior of leukocytes is dominated by the viscoelastic properties of the cell interior rather than those of the membrane.\(^8\) The most probable cause of the effect of PTX on leukocyte filterability is a change in the cytoplasm. Protopods have been found to be more rigid than the remainder of the cell,\(^10\) probably reflecting the rigidity of the meshwork of microfilaments in the substructure. Protopods are present in the control but not the PTX-treated cells. PTX has been shown to change the adenosine triphosphate (ATP) content\(^1\) and \(\text{Ca}^{++}\) levels\(^3\) in the cell, constituents that are instrumental in the actin gelation that forms the protopod.\(^1\)\(^9\) These biochemical findings, together with our biophysical data and electron microscopy, suggest that PTX improves leukocyte filterability by reducing protopod formation.

The increase in leukocyte filterability by PTX occurs only at doses higher than that expected in vivo following PTX administration. It is possible that PTX can exert beneficial effects on leukocytes in vivo at lower circulating concentrations. In the present study, metabolite I is found to have effects similar to those of PTX on leukocytes. Other metabolites may also act in a similar manner. Furthermore, there is evidence that the release of prostacyclin by the vascular endothelium may be increased during vessel perfusion with PTX;\(^2\) exposure to this and other endogenous substances may
also affect the deformability of leukocytes. The in vivo action of PTX may thus be amplified at lower doses.

A number of clinical situations are associated with vascular stasis because of increased leukocyte concentration. Most prominent are the leukemic disorders.23

The more moderate leukocytosis of sickle cell crisis or other acute infarctions may also contribute to rheologic difficulties in the microvasculature. Improving the deformability of the leukocytes could facilitate microcirculatory perfusion in such conditions.

REFERENCES

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