Distribution of Spermidine and Spermine in Blood From Cystic Fibrosis Patients and Control Subjects

By Lawrence F. Cohen, David W. Lundgren, and Philip M. Farrell

Previous studies have shown an abnormality of the spermidine-to-spermine (Spd/Spm) ratio in whole blood of cystic fibrosis homo- and heterozygotes. To investigate Spd and Spm distribution among blood components as a possible cause of the abnormality, blood was fractionated using Rabinowitz's glass bead technique and Boyum's Ficoll-Hypaque method. Free (unconjugated) polyamines were extracted with perchloric acid and quantitated on an amino acid analyzer. In controls, mean ± SEM concentrations in nmoles/10^9 cells of Spd and Spm, respectively, were 1.02 ± 0.08 and 0.89 ± 0.28 for erythrocytes; 126 ± 31 and 357 ± 105 for lymphocytes; 36 ± 16 and 240 ± 33 for granulocytes; and <0.5 and <0.5 nmoles/ml for plasma. When converted to the concentration in whole blood, it was found that greater than 90% of Spd and over 70% of Spm was associated with erythrocytes. While the higher cellular concentration in leukocytes was not unexpected, the fact that Spd and Spm in whole blood were primarily associated with erythrocytes was a new finding. Comparison with controls revealed that the Spd/Spm ratio in both whole blood and erythrocytes was significantly higher in the group of cystic fibrosis patients.

Polyamines have received increasing attention in mammalian systems over the past decade, due in part to the work of Tabor and Tabor leading to elucidation of biosynthetic pathways, as well as the findings of several groups suggesting that polyamines may have a significant role in growth and differentiation. These compounds include spermidine (Spd) and spermine (Spm), both of which are formed from the diamine, putrescine. The latter is derived from the urea cycle, via ornithine, and its synthesis is catalyzed by the important regulatory enzyme, ornithine decarboxylase. In addition to numerous observations on tissues in lower animals, measurements in human fluids have revealed that these compounds are found in whole blood and urine. Recently, it has become apparent that polyamines may have useful medical applications. Clinical studies in patients with neoplastic states (e.g., leukemia and lymphoma) indicate that serum and urinary polyamine levels are increased in such a way that their analysis may be employed for both diagnosis and monitoring of therapy.

More recently, polyamine abnormalities have been detected in whole blood samples from patients with cystic fibrosis (CF). These alterations, as reported by Lundgren et al., consist of an elevated Spd/Spm ratio, a change especially consistent in male CF homo- and heterozygotes. No previous reports of circulating polyamines in human disease have determined the distribution among the various blood components. The present study was conducted to establish...
the cellular distribution of these substances in fractionated blood samples, and
to identify which hematologic component was responsible for the abnormality
in CF patients.

MATERIALS AND METHODS

Subjects

Thirteen male CF subjects were selected for evaluation. All had been diagnosed on the basis of
a positive sweat test (sweat chloride range 70-130 mEq/liter) and compatible clinical manifesta-
tions. Progression of the disease, judged by the NIH Clinical Score,\(^*\) was mild in 9 (score >70) and severe in 4 (score <70). All results for CF subjects refer to the mildly involved
group, unless otherwise stated. The control group was composed of 14 healthy males. Mean ± SEM
leukocyte counts in whole blood were 7015 ± 608 and 6171 ± 331, and ages were 20.3 ± 1.12
and 32.2 ± 2.62 yr for CF and control groups, respectively.

Blood Fractionation

Five hundred milliliters of whole blood were aseptically collected in Fenwal bags containing
2200 units sodium heparin and sedimented with 1 ml dextran (5 g/dl, MW 170,000, Sigma Chemi-
cal Co., St. Louis, Mo.) per 5 ml whole blood for 35 min. The leukocyte-rich supernatant fluid was
withdrawn, concentrated by centrifugation for 10 min at 150 g and 17°C and added to a column of
glass beads (0.2 mm, Pyrex, from Arthur H. Thomas Co., Philadelphia, Pa.) for separation ac-
cording to the method of Rabinowitz.\(^8\) After 30 min incubation at 37°C, blood cell fractions
were eluted by successive washings with (1) autologous undiluted plasma, (2) 20\% (v/v) plasma in
Hanks’ balanced salt solution, and (3) calcium magnesium-free EDTA solution.\(^8\) Respective eluates
contained (1) lymphocytes plus erythrocytes, (2) residual erythrocytes, and (3) granulocytes.
Granulocytes were concentrated by centrifugation, while lymphocytes were freed of most of the
contaminating red cells by the Ficoll-Hypaque technique of Boyum.\(^9\) To obtain the red cell fraction,
whole blood anticoagulated with sodium citrate was centrifuged (150 g) and the plasma and
buffy coat removed.

In addition to blood fractions, polyamines were extracted from samples of whole blood. For
this purpose, 10 ml were collected with 0.1 ml sodium citrate (23 mg/dl).

Erythrocyte, total leukocyte and differential leukocyte counts were done with a hemocytometer
on whole blood and blood fractions.

Polyamine Extraction and Quantitation

Free polyamines were extracted with perchloric acid and were quantitated on an amino acid
analyzer, as previously described in detail.\(^6\) Results represent levels of free (or nonconjugated)
polyamines, since hydrolysis with concentrated hydrochloric acid is required to release covalently
bound polyamines.

RESULTS

Anticoagulation

Anticoagulation of whole blood for polyamine determination was done with
sodium citrate,\(^6\) while heparin was used for blood cell fractionation.\(^8\) As shown
in Fig. 1, parallel samples drawn from the same subject at the same time, anticoagulated with either citrate or heparin, and then extracted for Spd and Spm, produced almost identical concentrations. Similar results were obtained when
erthrocytes were extracted in a parallel fashion. When erythrocytes from

\(^*\)With this scoring system, a clinical score of less than 50 was associated with 100\% 3-yr mort-
tality in 11 cases.
citrate-anticoagulated blood were separated by dextran sedimentation, Spd extraction was lower than that obtained without this procedure, while Spm concentration was unaffected.

\(^{14}\text{C}\)-spermidine and \(^{14}\text{C}\)-spermine were found to elute separately from dextran on a glass bead column. Thus, the dextran effect was not due to direct binding, but may have been related to rouleaux formation. Therefore, dextran was not used for routine separation of erythrocytes.

**Cell Separation**

Erythrocyte purification resulted in cell preparations with mean ± SEM hematocrit of 91.3 ± 0.66 and an average of 11 ± 3.0 leukocytes/10^5 RBC, compared to 132 ± 7.1 leukocytes/10^5 RBC in whole blood; thus, elimination of >90% of leukocytes was achieved. Mean contamination of the isolated lymphocyte fraction was 1 ± 0.28 nonlymphocyte/100 leukocytes and 39 ± 11 red cells/100 lymphocytes. Mean contamination of isolated granulocytes was 3 ± 0.9 mononuclear cells/100 leukocytes and 18 ± 12 red cells/100 granulocytes. Representative photomicrographs of lymphocyte and granulocyte preparations are shown in Fig. 2.
Fig. 2. Photomicrographs of blood fractions. (A) Lymphocytes. x 62.5. (B) Granulocytes. x 25.

Polyamine Concentrations in Blood Fractions

Table 1 shows the Spd and Spm concentration for specific cell types. Putrescine was not detected in whole blood, nor in blood fractions. Erythrocytes were found to contain more Spd than Spm. Lymphocytes and granulocytes, on the other hand, contained more Spm, and concentrations of Spd and Spm per 10⁹ cells were one to two orders of magnitude greater than levels present in erythrocytes. Small peaks were obtained for granulocytes because of the low yield and the sensitivity of our assay. Therefore, the results given for this fraction are approximations. With our techniques, neither polyamine was detected in plasma.

Table 1. Concentration of Spermidine and Spermine in Blood Components From Control and Cystic Fibrosis Subjects

<table>
<thead>
<tr>
<th></th>
<th>Spermidine</th>
<th>Spermine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>CF</td>
</tr>
<tr>
<td>Erythrocyte</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Granulocyte†</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Plasma</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

* p < 0.5 vs. control.
†Granulocyte results approximated from small peaks (see text).

Cellular concentrations are expressed as nmoles/10⁹ cells and plasma concentrations as nmoles/ml. Mean ± SEM.
Table 2. Whole Blood Concentrations of Spermidine and Spermine by Blood Components in Control and Cystic Fibrosis Subjects

<table>
<thead>
<tr>
<th></th>
<th>Spermidine</th>
<th>Spermine</th>
<th>Spd/Spm Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Erythrocyte</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.26 ± 0.43</td>
<td>4.68 ± 1.50</td>
<td>1.52 ± 0.28</td>
</tr>
<tr>
<td>CF</td>
<td>7.07 ± 0.83*</td>
<td>3.18 ± 0.33</td>
<td>2.30 ± 0.26*</td>
</tr>
<tr>
<td><strong>Lymphocyte</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.231 ± 0.067</td>
<td>0.752 ± 0.021</td>
<td>0.370 ± 0.042</td>
</tr>
<tr>
<td>CF</td>
<td>0.224 ± 0.054</td>
<td>0.480 ± 0.242</td>
<td>1.25 ± 0.97</td>
</tr>
<tr>
<td><strong>Granulocyte†</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.136 ± 0.058</td>
<td>1.02 ± 0.251</td>
<td></td>
</tr>
<tr>
<td>CF</td>
<td>0.110</td>
<td>0.255</td>
<td></td>
</tr>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>&lt;0.3</td>
<td>&lt;0.3</td>
<td></td>
</tr>
<tr>
<td>CF</td>
<td>&lt;0.3</td>
<td>&lt;0.3</td>
<td></td>
</tr>
<tr>
<td><strong>Whole Blood</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6.12 ± 0.39</td>
<td>5.57 ± 2.85</td>
<td>1.27 ± 0.13</td>
</tr>
<tr>
<td>CF</td>
<td>8.04 ± 1.05*</td>
<td>4.72 ± 0.85</td>
<td>1.89 ± 0.23*</td>
</tr>
</tbody>
</table>

* p < 0.05 vs. control, for respective fraction.
† See note on granulocytes in Table 1.
Concentrations in nmoles/ml whole blood. Mean ± SEM of cell count × cellular concentration for each fraction.

Distribution in Whole Blood

With the cell count per milliliter whole blood and cellular concentration of Spd and Spm known, the polyamine contribution of each cell type in 1 ml whole blood was calculated (Table 2). While erythrocytes contained far less of each compound on a cell-for-cell basis, this fraction accounted for >90% of Spd and >70% of Spm in the circulation (Table 3). Leukocytes contributed proportionately less of each polyamine.

Since there were only 10–12 leukocytes/10⁵ red cells in the erythrocyte fraction, the contribution of leukocyte spermidine and spermine to this fraction amounted to <5%. By similar calculations, erythrocyte spermidine and spermine were found to contribute less than 0.8% to lymphocyte and granulocyte results.

Table 3. Distribution of Spermidine and Spermine in Whole Blood Components in Control and Cystic Fibrosis Subjects

<table>
<thead>
<tr>
<th></th>
<th>Spermidine (%)</th>
<th>Spermine (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Erythrocyte</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>91.4</td>
<td>77.4</td>
</tr>
<tr>
<td>CF</td>
<td>97.9</td>
<td></td>
</tr>
<tr>
<td><strong>Lymphocyte</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.0</td>
<td>11.5</td>
</tr>
<tr>
<td>CF</td>
<td>(&lt;1.6)</td>
<td>(22.0)</td>
</tr>
<tr>
<td><strong>Granulocyte†</strong></td>
<td>(2.0)</td>
<td></td>
</tr>
<tr>
<td><strong>Plasma</strong></td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Spermidine (%)</th>
<th>Spermine (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total</strong></td>
<td>95.4</td>
<td>110.9</td>
</tr>
</tbody>
</table>

* See note on granulocytes in Table 1.

The whole blood spermidine and spermine concentrations of each fraction for a given subject were compared to that subjects’ whole blood concentration, and individual percentage distributions were obtained. Values indicate the mean of the percentages for each fraction. There were no significant differences between control and CF populations.
Comparison of Cystic Fibrosis Patients and Controls

The Spd/Spm ratio was found to be significantly higher for both CF whole blood ($p < 0.01$) and erythrocytes ($p < 0.05$), as shown in Table 2. Similarly, mean Spd concentration was significantly greater in whole blood and erythrocytes. When four additional subjects with moderate to severe progression of CF were tested, the mean Spd/Spm ratio was significantly higher ($p < 0.05$) for whole blood ($3.04 \pm 0.50$) and erythrocytes ($5.69 \pm 0.76$) than in the mildly involved group. However, for CF subjects with clinical scores $>70$, there was a poor correlation between the exact score and the Spd/Spm ratio ($r = 0.243$ for whole blood and $r = 0.633$ for erythrocytes).

DISCUSSION

Rosenthal and Tabor\textsuperscript{10} noted in 1956 that “spermine . . . [in mouse blood] . . . was in the formed elements, principally the leukocytes, and none could be detected in the plasma.” With these compounds implicated in certain clinical disorders, it became important to clarify their distribution in blood. Accordingly, this investigation was designed to determine polyamine concentrations in plasma and blood cells. Four enriched blood fractions were obtained using Rabinowitz’s glass bead column\textsuperscript{8} and the Ficoll-Hypaque gradient of Boyum.\textsuperscript{9}

Our results demonstrated relatively higher free Spd and Spm levels in leukocytes, lower concentrations in erythrocytes, and undetectable levels in plasma. Since polyamines may be involved in regulation of intracellular metabolism,\textsuperscript{2} one might expect to find higher concentrations in leukocytes. The low level in plasma was not surprising in view of recent studies of Russell and others,\textsuperscript{3} in which total (free plus covalently bound) spermidine and spermine were detected in plasma or serum from cancer patients, but not normal subjects.

While leukocytes contain more Spd and Spm on a cell-for-cell basis, the fact that there are about 600 erythrocytes for every leukocyte in whole blood accounts for the predominant association with erythrocytes. That spermidine and spermine in whole blood are largely associated with the erythrocytes is a new observation, which raises questions about the possible role of these cells in relationship to polyamines. It may be speculated that erythrocytes can either detoxify the highly toxic aldehydes produced when polyamines are oxidized,\textsuperscript{11} or act as carriers in the circulation.

Two recent studies are relevant to this discussion of polyamine concentrations in blood cells. Graziano\textsuperscript{12} measured free polyamine levels in lymphocytes in a culture system before and after phytohemagglutinin stimulation. In 1-day cultures, he found 180 and 244 nmoles/10\textsuperscript{6} cells for spermidine and spermine, respectively, prior to stimulation, and 365 and 253 after. These values approximated our results for lymphocytes. In a study of patients with polycythemia vera,\textsuperscript{13} three control subjects were shown to have mean values of 30 and 129 nmoles/10\textsuperscript{9} cells of free Spd and Spm, respectively, in mixed leukocytes from peripheral blood. Since peripheral blood leukocytes represented 60\textsuperscript{o}, 75\textsuperscript{o} granulocytes, it was interesting that these results were similar to our approximations for granulocytes. No results were given for erythrocytes in either study.

The previous observations of an elevated Spd/Spm ratio in whole blood from cystic fibrosis patients\textsuperscript{6} has been confirmed in a new population of subjects.
Although the overall distribution of polyamines among the components of whole blood is similar for CF and control subjects, the abnormal Spd/Spm ratio in CF whole blood is paralleled by an elevation in erythrocytes. From this and the predominant contribution of red cells to blood polyamine concentrations (Table 3), we conclude that abnormalities in erythrocyte polyamines are responsible for the previously reported increased Spd/Spm ratio in cystic fibrosis.

ACKNOWLEDGMENT

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