Effect of Cyanate on Erythrocyte Deformability

By John R. Durocher, Bertil E. Glader, Lelia T. Gaines, and Marcel E. Conrad

Cyanate is undergoing study as a drug to prevent occlusive sickle cell crises. Carbamylation of hemoglobin S increases its oxygen affinity, thereby decreasing its tendency to aggregate at low oxygen tensions. The cell membrane has also been shown to be carbamylated. We studied the effect of carbamylation on deformability of sickle, normal, and stored normal erythrocytes. Deformability was measured as filtration time of erythrocyte suspensions through 3-μm polycarbonate filters. Oxygenated carbamylated sickle erythrocytes had a marked shortening of filtration time that could not be explained by changes in morphology. In addition, normal erythrocytes became more filterable with carbamylation. Decreased filterability after storage at 4°C for 48 hr was returned to normal following carbamylation and incubation with glucose. No correlation was made with changes in either morphology, cell size, or ATP levels. Therefore, it was postulated that carbamylation affected filterability by binding either the cell membrane or cell contents or both.

Cyanate is currently an investigational drug purported to prevent occlusive sickle cell crises. This agent irreversibly carbamylates the N-terminal amino group of proteins and the free amino group of lysine. The salutory effect of cyanate in sickle cell disease presumably is related to the carbamylation of the terminal valine residue of hemoglobin S. Carbamylation at this locus increases hemoglobin's affinity for oxygen. It is this increased oxygen affinity that is believed to reduce the tendency of hemoglobin S to aggregate at low oxygen tensions.

Reaction of red blood cells with cyanate is not limited to hemoglobin, since in vitro carbamylation of erythrocytes also reduces G6PD activity and significantly labels the erythrocyte membrane. In this study, we investigated the possible functional significance of membrane carbamylation. Specifically, we studied the effect of cyanate on the deformability of fresh erythrocytes from patients with sickle cell disease and fresh and stored red blood cells from normal individuals.

MATERIALS AND METHODS

Blood was collected in EDTA from healthy laboratory personnel and patients with sickle cell disease who had not been transfused within 4 mo. Samples either were used immediately or were stored at 4°C for 48 hr. Erythrocytes were separated from plasma by centrifugation and then washed three times in a pH 7.4 phosphate-buffered salt solution (275 milliosmols) containing 110 mM NaCl, 20 mM NaHPO₄, and 4 mM KH₂PO₄. After the final wash, a 20% red blood cell suspension was made in the phosphate-buffered salt solution. Neutralized NaCNO (recrystallized from ethanol) was added such that the final concentration was 50mM. An equimolar amount of NaCl was added to control samples so that the final osmolality of both cyanate and control solutions was 314 milliosmols. Glucose when present was added to a final concentration of 10 mM. The erythrocyte suspensions were incubated at 37°C in an Eberbach
waterbath shaker at 100 oscillations/min. After 2 hr incubation, an aliquot of the red blood cell suspension was removed for determination of ATP concentration to ascertain if deformability was related to the ATP in erythrocytes. The remaining cells were washed three times in Ringer's solution containing 0.25% albumin and 12 mM tris (297 milliosmols), adjusted to pH 7.4, and then diluted to a 2% suspension for the determination of erythrocyte deformability. In these studies, deformability was measured by the filtration properties of the red blood cell suspension as determined by the time (sec) required for 2 ml of the 2% erythrocyte suspension to filter through a 3-μ polycarbonate (Nuclepore) filter under 0 cm water negative pressure at room temperature. Immediately prior to the filtration of sickle erythrocytes, a mixture of 95% oxygen and 5% carbon dioxide was gently bubbled through the sample for 5 min (O2 tension - 450 mm Hg). All samples were coded and filtered in triplicate. Mean corpuscular volume was determined from the microhematocrit and red blood cell count (Coulter Model B). Morphology was assessed in wet preparations with a phase microscope. Osmolality was measured with an Osmette osmometer.

RESULTS

Freshly collected normal erythrocytes had a filtration time of 10.7 ± 0.7 sec in our experimental system. Fresh erythrocytes from patients with sickle cell disease, incubated with or without glucose for 2 hr, had a markedly prolonged filtration time (Table I). This decreased deformability was partially corrected by 50 mM cyanate and further improved when both glucose and cyanate were present in the incubation medium. At the time of filtration, there were less than 1% sickle cells observed under phase microscopy; almost all sickled cells were irreversibly sickled because they were unaffected by treatment with oxygen.

Also seen in Table I, freshly obtained normal erythrocytes processed in the same manner as sickle red blood cells had a similar response to cyanate and glucose, although the differences were small. The addition of glucose produced no significant change in filterability, but cyanate produced significant changes with or without added glucose (p < 0.02). When normal erythrocytes were stored at 4°C for 48 hr prior to study, decreased deformability was observed which was partially

Table 1. Effect of Cyanate on Filtration of Sickle, Normal, and Stored Normal Erythrocytes

<table>
<thead>
<tr>
<th></th>
<th>Filtration Time (sec)</th>
<th>ATP (μ mole/ml Erythrocytes)</th>
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<tbody>
<tr>
<td>Fresh normal erythrocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.7 ± 0.7 (8)</td>
<td>1.41 ± 0.37 (8)</td>
</tr>
<tr>
<td>Incubated sickle erythrocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>183.1 ± 35.7 (3)</td>
<td>1.54 ± 0.33 (3)</td>
</tr>
<tr>
<td>50 mM CNO</td>
<td>89.1 ± 36.8 (3)</td>
<td>1.41 ± 0.14 (3)</td>
</tr>
<tr>
<td>10 mM glucose</td>
<td>155.8 ± 33.6 (3)</td>
<td>1.72 ± 0.24 (3)</td>
</tr>
<tr>
<td>10 mM CNO + 50 mM glucose</td>
<td>39.2 ± 10.4 (3)</td>
<td>1.54 ± 0.10 (3)</td>
</tr>
<tr>
<td>Incubated normal erythrocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>13.9 ± 1.8 (9)</td>
<td>1.44 ± 0.32 (22)</td>
</tr>
<tr>
<td>50 mM CNO</td>
<td>10.4 ± 0.7 (9)</td>
<td>1.40 ± 0.24 (18)</td>
</tr>
<tr>
<td>10 mM glucose</td>
<td>11.0 ± 0.5 (11)</td>
<td>1.38 ± 0.33 (10)</td>
</tr>
<tr>
<td>10 mM glucose + 50 mM CNO</td>
<td>9.4 ± 0.2 (11)</td>
<td>1.34 ± 0.32 (11)</td>
</tr>
<tr>
<td>Incubated normal stored erythrocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>28.5 ± 6.3 (6)</td>
<td>1.05 ± 0.40 (9)</td>
</tr>
<tr>
<td>50 mM CNO</td>
<td>17.1 ± 2.0 (6)</td>
<td>1.10 ± 0.37 (9)</td>
</tr>
<tr>
<td>10 mM glucose</td>
<td>17.5 ± 1.8 (8)</td>
<td>1.21 ± 0.37 (9)</td>
</tr>
<tr>
<td>10 mM glucose + 50 mM CNO</td>
<td>11.5 ± 0.6 (8)</td>
<td>1.27 ± 0.62 (9)</td>
</tr>
</tbody>
</table>

* Mean ± 1 SE
† Mean ± 1 SD
‡ Numbers in parentheses are number of individual tests performed.
reversed with the addition of either glucose or cyanate to the suspending media. Normal deformability was observed when the cells were incubated with both glucose and cyanate. No significant difference in the mean corpuscular volume of sickle, normal, or stored erythrocytes was observed when cells were incubated with or without the addition of sodium cyanate.

The concentration of ATP in these variously treated erythrocyte preparations is recorded in Table I. Stored red blood cells had slightly less ATP, but the changes in filterability related to glucose or cyanate occurred without any significant difference in cellular ATP concentration.

DISCUSSION

The pathophysiologic events in sickle cell disease have been related to the propensity of sickle hemoglobin to aggregate at low oxygen tensions and distort the cell membrane, thus forming a rigid cell. If the cell is not trapped in the microvasculature, the continuous sickling–desickling process reaches a state where it is no longer reversible, and the cell is irreversibly sickled. Jensen has suggested that the property of reversibility is intrinsic to the membrane rather than the hemoglobin molecule. More recently it has been found that the formation of irreversibly sickled cells can be prevented by avoiding the accumulation of calcium in the membrane.

The demonstration that 14C-labeled cyanate labels the erythrocyte membrane as well as hemoglobin suggests that cyanate might alter membrane function, since carbamylation of other proteins alters protein function. Membrane deformability is a property alleged to be important in cellular integrity and lifespan. A technique for measuring deformability is the time required for a specified volume of cells to traverse a filter of known pore size. As previously reported by others, sickle erythrocytes have decreased deformability when tested in a microfiltration system.

In our studies, glucose added to sickle cells did not significantly alter deformability. Cyanate, however, was capable of improving filtration. The sickle cells used in our experiments were well oxygenated prior to filtration, and we observed less than 1% irreversibly sickled forms in each study. Nevertheless, we observed marked changes in the deformability of these cells which were improved by the addition of cyanate to the incubation mixture. Since these changes could not be attributed to visible changes in cell shape, we studied the effect of cyanate on the filterability of normal erythrocytes and found altered filterability. This effect was small but significant with fresh erythrocytes, and was increased in cells stored at 4°C for 48 hr before study. This decreased filterability was partially reversed by incubation with either cyanate or glucose, but when these agents were added together, the deformability of stored cells returned to normal. In stored red blood cells, the ATP levels were lower than in fresh erythrocytes, but there were no significant differences between the experimental groups of the same age when incubated with 10 mM glucose for 2 hr. Prior to this study, the deformability of normal erythrocytes has been correlated with the maintenance of intracellular ATP concentrations. One of the functions of intracellular ATP presumably is to chelate calcium and prevent the formation of calcium complexes with membrane proteins or lipids. These results with cyanate, however, indicated that deformability of the erythrocyte can be improved without significant changes in cellular ATP. Enzymes responsible for ATP production are partially located in close association with the
An increase in local ATP may explain changes in filtration time following incubation in glucose.

The influence of cyanate on the deformability of normal erythrocytes suggests that part of the reported increase in survival of carbamylated erythrocytes in patients with sickle cell disease may be related to this rheologic change, in addition to the known effect on the hemoglobin molecule.

The mechanism by which cyanate alters cellular deformability remains to be determined. Since this compound also binds to hemoglobin A, it is possible that carbamylation of hemoglobin could alter the normal internal viscosity of the erythrocyte due to hemoglobin. Carbamylation of hemoglobin may affect the binding of ATP to hemoglobin, thereby increasing the amount of free ATP. On the other hand, cyanate may directly alter the cell membrane by interfering with calcium accumulation in the membrane.

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REFERENCES

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