Effect of Cyanate on Erythrocyte Membrane Surface Charge (37566)

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Cyanate has been proposed as a therapeutic agent for the prevention of occlusive sickle cell crises (1). This agent irreversibly carbamylates the N-terminal amino groups of proteins and the free epsilon amino group of lysine (2). The beneficial effect of cyanate (CNO) in sickle cell disease presumably is related to carbamylation of the terminal amino-valine residue of hemoglobin (1). Carbamylation at this locus interferes with the binding of 2,3-DPG to deoxyhemoglobin, thereby increasing oxygen affinity and decreasing the tendency of sickle hemoglobin to aggregate at low oxygen tensions (3). Reaction of the erythrocyte with CNO is not limited to hemoglobin, since intracellular enzymes (4) and the cell membrane (5) are also carbamylated. In this report we measured the *in vitro* effect of CNO on the membrane surface charge of red blood cells from humans, rats, and rabbits.

Materials and Methods. Blood was collected in EDTA from healthy human adults and two patients with sickle cell disease. Red blood cells were separated from plasma by centrifugation and then washed three times in a phosphate-buffered salt solution (pH 7.4) containing 110 m*M* NaCl, 20 m*M* Na_2HPO_4, and 4 m*M* KH_2PO_4. After the final wash, a 20% erythrocyte suspension was made and glucose was added to a final concentration of 10 m*M*. Neutralized NaCNO (K and K Laboratories) recrystallized from ethanol was added to a final concentration of 50 m*M*. When cyanate was added, an equivalent amount of chloride was withheld from the incubation medium to maintain a constant ionic strength. Blood was collected in heparin (14 USP units/ml of blood) from rats (Walter Reed Carsworth Farm Strain) and rabbits (New Zealand white) and then processed in the same manner as the human cells. The reconstituted human, rat, or rabbit red blood cell suspensions were covered after exposure to room air and incubated at 37° in an Eberbach metabolic shaker at 100 oscillations/min. Aliquots of the erythrocyte suspensions were removed for measurement of membrane surface charge at the beginning of the experiment and again after 30 and 120 min incubation. These aliquots were washed three times with 150 m*M* NaCl and then diluted to a 0.25% suspension in a solution containing 4 vol of 5% sorbitol and 1 vol of 67 m*M* Sorensen's buffer, pH 7.2 (6). The erythrocyte electrophoretic mobility was determined at 23° in the rectangular cuvette of a Zeiss cytopherometer (7) fitted with platinum electrodes. A current of 2 mA with 17.5 V/cm was used. Samples were coded and the mobility of at least 10 cells in each sample were measured. The movement of each erythrocyte was measured twice, with the second measurement after the polarity of the field was reversed. All determinations were made in the frontal plane. To determine the extent of membrane carbamylation, $K^{14}CNO$ (Amersham/Searle Corp.) was used in separate incubations with human, rat, and rabbit erythrocytes. Hemoglobin-free ghosts were prepared (8) and protein was estimated (9). Scintillation counting of ghosts was done in PCS solubilizer (Amersham/Searle Corp.) using a Beckman LS-345 liquid scintillation system. Quenching was corrected with a standard quench curve using an internal standard.

Results and Discussion. The erythrocyte has a negative surface charge as determined

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by migration in an electrical field (10). The bulk of the charge is due to the exposed carboxyl groups of sialic acid (11). Presumably, the net surface charge is also influenced by other exposed carboxyl (COOH) and amino (NH_2) groups. Since the pK of amino groups is greater than pH 7.4, many of the NH_2 groups would be positively charged at physiologic pH. Blockade of exposed membrane amino groups, therefore, could increase the net negative charge of the membrane. CNO has previously been demonstrated to increase the electronegativity and hence electrophoretic mobility of hemoglobin peptide chains (12) and other proteins (13, 14).

As shown in Table I, cyanate-treated human erythrocytes had a small but significant increase in electrophoretic mobility as compared to controls. This effect was observed after 30 min incubation in cyanate and did not significantly change during an additional 90 min incubation. The mobility of untreated erythrocytes from patients with sickle cell disease was the same as controls, and there was a similar increase in their charge when these cells were carbamylated.

Confirming previous observations, rabbit red blood cells have a much slower (15) and rat erythrocytes a faster (16) electrophoretic mobility than human cells (Table II). While there are many differences between the red blood cells of these species, it is only the membrane surface charge density that influences the rate of cellular migration in an electric field (16). The differences in the chemical composition of these membranes is further manifested by the CNO-induced increase in human and rabbit red blood cell mobility, and the lack of any significant effect on the surface charge of rat erythrocytes. Experiments with ^{14}CNO showed incorporation into human, rat, and rabbit ghosts of 85.0 ± 6.2, 77.4 ± 7.6, and 90.1 ± 1.8 μmoles cyanate/mg membrane protein ± 1 SE, respectively. Therefore, absence of carbamylation of rat erythrocyte membranes could not explain the lack of change in surface charge of carbamylated rat erythrocytes.

TABLE I. Effect of CNO on the Electrophoretic Mobility[a] of Human Erythrocytes.

	Control	50 m*M* CNO
Normal RBC	2.00 ± 0.01	2.10 ± 0.01 ($n = 19$)
Sickle RBC	2.01	2.12 ($n = 2$)

[a] μm/sec/V/cm ± 1 SE.

TABLE II. Effect of CNO on the Electrophoretic Mobility[a] of Rat and Rabbit RBC.

	Control	50 m*M* CNO
Rats	2.26 ± 0.02	2.27 ± 0.02 ($n = 9$)
Rabbits	0.84	0.93 ($n = 3$)

[a] μm/sec/V/cm ± 1 SE.

These data indicate that CNO may be a useful agent in understanding the nature and functional significance of the membrane surface charge. Previous attempts to increase membrane electronegativity either by blockade of cationic groups or the introduction of anionic ligands have been largely inconclusive (17, 18). The one exception is glutaraldehyde, a fixative, which increases the rate of human red blood cell electrophoretic mobility by 14% (19). This agent, however, seriously disrupts membrane architecture (20) and subsequently makes it difficult to evaluate the functional role of the membrane surface charge.

We have previously demonstrated that reducing the membrane surface charge by removing sialic acid with neuraminidase is associated with a shortened RBC survival of these cells *in vivo* (21). Whether the RBC surface charge is one of the determinants of normal RBC survival is an interesting possibility, since the surface charge is known to decrease as the cell ages (22). It is reasonable to assume that the destruction of senescent erythrocytes by cells of the reticuloendothelial system may be influenced by the electrical interaction of the juxtaposed membranes.

It remains to be determined whether CNO-induced changes in membrane surface charge have any relationship to the reported increased erythrocyte survival of carbamylated cells from patients with sickle cell disease. The observed changes in surface charge may merely represent a more generalized alteration in the membrane structure and function.

Summary. Erythrocytes carbamylated *in*

vitro with sodium cyanate have significantly increased electrophoretic mobility. Species differences were noted between human, rabbit, and rat erythrocytes. In contrast to other agents' increasing net negative surface charge, cyanate does not destroy erythrocyte viability. It is postulated that carbamylation of surface amino groups results in an increased net negative charge.

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