Hydroxyurea (HU) significantly increases fetal hemoglobin (Hb F) production significantly in patients with sickle cell anemia. Epidemiologic studies show that high levels of Hb F are associated with reduced disease severity and potentially may decrease the number of vaso-occlusive crises. Administration of HU to patients with severe cases of sickle cell anemia increases Hb F, F reticulocyte count, Hb F red blood cells (RBCs), mean corpuscular volume, RBC survival, oxygen affinity, and total RBC cation content, with additional improvement of RBC deformability and its hydration status. In contrast, mean corpuscular Hb concentration, hemolysis, percent of irreversibly sickled RBCs, and, notably, KCl cotransport are decreased by treatment with the drug.

HU, an antitumor agent, inhibits DNA synthesis by destroying the tyrosyl free radical of the enzyme ribonucleotide reductase. This enzyme catalyzes the formation of deoxyribonucleotides from ribonucleoside diphosphate precursors and is a rate-limiting step in DNA synthesis. In addition, DNA synthesis has been associated with an increase in Na influx, Na/K pump activity, Ca efflux, and intracellular pH (pHi) in cultured fibroblasts. Furthermore, HU (1 mmol/L) has been used to synchronize cultured bovine aortic endothelial cells. However, the effect of HU on DNA synthesis and cell cycle progression depends on its concentration, treatment times, and other unknown factors.

The mechanism of vaso-occlusion involves blood cells, vascular endothelial cells (VECs), and plasma factors. Recent studies have demonstrated that RBCs with Hb S adhere better to endothelial cells than do RBCs with Hb A, and that this effect is increased in the presence of autologous plasma or autologous platelets. Adhesion of erythrocytes and other blood cells appears to induce local hypoxia and stasis, which lead to vaso-occlusion when accompanied by vasoconstriction. In addition, hypoxia induces the production of platelet-derived growth factor (PDGF) and endothelin, two potent vasoconstrictors secreted by VECs.

In light of the above information, the present study was designed to assess the effect of HU on VEC function, which, as in other cells (RBCs and fibroblasts), should be reflected.

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in morphologic and biophysical properties, as well as in RBC adhesion.

MATERIALS AND METHODS

Materials. The endothelial cell line BFAE-39 originated from bovine thoracic aorta of normal 4- to 5-month-old male fetuses (AG 07680, NIA Aging Cell Repository, Coriell Institute for Medical Research, Camden, NJ). Media and chemicals used were as follows: F-12 nutrient mixture (Ham, HF12), cell culture media supplements, trypsin, penicillin, streptomycin, amphotericin B, and EDTA (GIBCO, Grand Island, NY); defined fetal bovine serum (HyClone Laboratories, Logan, UT); bovine serum albumin (catalog no. A7030), tris(hydroxymethyl)aminomethane base (Tris), and HU (Sigma Chemical, St Louis, MO); and Dulbecco’s phosphate-buffered saline and Hanks balanced salt solution without calcium and magnesium (Hanks medium [HM], Whittaker, M.A. Bioproducts, Walkersville, MD).

Cell culture. Bovine aortic endothelial cells were propagated in HF12 (passages 3 through 6, cumulative population doublings [CPD] < 30) supplemented with antibiotics and 10% fetal bovine serum. Cells were subcultured with trypsin-EDTA as described else-

Fig 2. Effect of HU on VEC morphology. (A) Control cells at passage 3 after 10 days in culture and in the absence of HU were approximately 90% confluent, with some cells undergoing division. Aging of the cultures was manifested by the presence of vacuoles. (B) Cells were cultured for 10 days and treated for 7 days with 0.3 mmol/L HU; cultures are at approximately 60% confluence. Note the larger cell size, more binucleated cells, and the larger and higher number of vacuoles than in control cells.
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Fig 2. (Cont’d) (C) Cells were cultured for 10 days and treated for 7 days with 3.0 mmol/L HU; cultures are at approximately 30% to 40% confluence. Note the larger cell size and higher number of binucleated and trinucleated cells as compared with A and B; however, the number of vacuoles was smaller than in B. (Original magnification × 200; Nikon Diaphot microscope; Nikon, Tokyo, Japan.)

where, and passaged at a 1:2 or 1:5 ratio in 81-cm² flasks. For cation transport measurements, cells were cultured in 35-mm diameter dishes, fed every other day, and used after 2 to 11 days in culture. The splitting ratios were one 25-cm² flask into 15 dishes or one 81-cm² flask into 50 dishes; three dishes per point were used. For experiments on RBC-endothelial cell adhesion, cells were seeded in six-well plates of 35-mm diameter (Costar, Cambridge, MA) and used after confluence (between 5 and 12 days after seeding).

HU treatment. HU was added to the culture medium either during or 2 days after cell seeding, at final concentrations of 0.3 or 3.0 mmol/L to simulate therapeutic and toxic doses, respectively.

Endothelial cell cation and protein determinations. Na and K levels were determined by atomic absorption spectrophotometry (Perkin Elmer 5000; Perkin Elmer-Cetus, Norwalk, CT), and protein content was determined by the method of Lowry et al, as described previously.

RBC adhesion to VECs. The methods of Hebbel et al and Wautier et al were used with some modifications, as described below, to determine RBC adhesion to VECs.

RBC loading with ⁵¹Cr. Blood was obtained by venipuncture into heparinized vacuum tubes from two healthy individuals (homozygous for Hb A) and from two individuals with sickle cell disease (homozygous for Hb S, courtesy of Dr D. Rucknagel, Comprehensive Sickle Cell Center, University of Cincinnati, College of Medicine, Cincinnati, OH) and processed within 1 to 2 hours. After centrifugation for 10 minutes at 3,000g and 4°C, the whole-blood sample was divided into three fractions, plasma,uffy coat resuspended in plasma, and packed RBCs. The first two fractions were maintained on ice while the RBCs were washed three times with an isotonic phosphate-buffered saline solution containing the following (mmol/L): 137 NaCl, 2.7 KCl, 0.9 CaCl₂, 0.5 MgCl₂, 8.1 Na₂HPO₄, and 1.5 KH₂PO₄. After the last wash, packed cells were resuspended at approximately 50% hematocrit in phosphate-buffered saline and labeled for 40 minutes at 37°C with ¹⁵¹Cr (sodium chromate, Amersham, Arlington Heights, IL; 50 µCi/mL packed RBCs). The labeled RBCs were washed an additional three times with HM, divided into three aliquots, washed once more, and resuspended to hematocrit 25% (vol/vol) in either HM containing 0.5% bovine serum albumin, plasma alone, or plasma containing theuffy coat.

RBC adhesion. Because adherence of endothelial cells appears to be independent of the species, we used VECs of bovine origin (cell line BFAE-39). Endothelial cells cultured in six-well plates to confluence and in the presence or absence of HU were washed twice in HM with 0.5% bovine serum albumin. Labeled RBCs (0.7 mL of 25% [vol/vol] suspension) were layered on duplicate wells and incubated for 30 minutes at 37°C. After incubation, nonadhered cells were removed by aspiration, and the cultures were washed five times with 0.7-mL aliquots of HM with albumin and once with distilled water to remove residual radioactivity (from adhered RBCs) in the plates. Radioactivity from each sample was measured in a Packard Auto-Gamma 5650 counter (Packard Instrument, Downers Grove, IL). Results were expressed as the percentage of total radioactivity that remained in the cultures after each wash, as described by Wautier et al.

Statistics. Results are expressed as the mean ± standard deviation (X ± SD). Statistical significance was assessed by two-sample paired or unpaired t test analysis with computer software.

RESULTS

Exposure of endothelial cells to HU before and after cell attachment. Seeding of VECs in the presence of HU decreased the protein content per dish after 3 days of culture in a dose-dependent manner (Fig 1) with no effect on Na and K contents (data not shown). Figure 1 shows three independent experiments, two of them on VECs at passage 6 and one at passage 3. Under these conditions, HU caused, again in a dose-dependent manner, significant changes in cell volume and extensive vacuolization associated with initial cell death (data not shown). To determine whether HU interfered with cell attachment, growth, or both, the effect

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of HU on cell morphology was investigated after cell attachment. Figure 2A through C shows the effect of HU on cell morphology when added 2 days after seeding. Cells at passage 3 or 6 were incubated for 10 days in the absence (control) and presence of 0.3 and 3.0 mmol/L HU, respectively. HU increased cell volume and caused appearance of binucleated and trinucleated cells with extensive vacuolization (both at passages 3 and 6) in a dose-dependent manner (Fig 2B and C).

Figure 3 shows a representative experiment demonstrating the effect of HU, added 2 days after seeding, on the protein content per dish at various days in culture and in the presence or absence of HU. The drug decreased the protein content per dish after 24 hours of treatment (day 3 in culture), an effect that became dose-dependent after 48 hours (day 4 in culture), reaching a maximum after 9 days of treatment. Thus, the effect of HU on cell growth was not mediated by inhibition of cell attachment.

Under experimental conditions identical to those of Fig 3, HU increased the Na content of VECs in a dose-dependent manner between 4 and 8 days in culture and partially decreased the peak of Na content characteristic for VECs at about 10 to 11 days of culture.21 Figure 4A shows the typical decrease of cell K content after seeding, 19 which was prevented by HU in a dose-dependent manner after day 4 in culture, ie, 2 days after HU treatment was started. The effect of HU on total cation content (a measure of VEC volume) was similar to that on K, the major osmolyte in these cells (Fig 4C).

A clear insight into the dynamics of ionic shifts can be obtained by plotting the HU-related changes as a function of time. Figure 5A through C shows the mean values of HU-dependent Na, K, and total cation changes, respectively, versus days in culture from three independent experiments. The effect of HU on Na content was transient, with a maximum increase of about 70% at 0.3 mmol/L and 150% at 3.0 mmol/L HU between 4 and 7 days in culture (Fig 5A). The HU-induced increase of K (Fig 5B) and total cation contents (Fig 5C) occurred after day 4, with a maximum (up to 40% to 50% at 3.0 mmol/L HU) after day 8. Hence, HU caused statistically significant and sequential changes in Na and K contents of cultured VECs.

**RBC adhesion to HU-treated VECs.** Figure 6 plots the percentage of $^{51}$Cr radioactivity associated with VECs in a wash-out experiment to determine Hb A RBC adhesion to untreated and HU-treated VECs (0.3 mmol/L HU for 7 days) for three conditions in which cells were incubated in either HM, autologous plasma, or autologous plasma containing the buffy coat (for further details, see legend to Fig 6). The rationale for using autologous plasma containing white blood cells (in the buffy coat) was to test for competition between these and VECs for RBC adherence. No difference was observed in RBC adhesion to either treated or untreated VECs in buffered saline solution, whereas a slight and probably nonsignificant decrease was observed in the adhesion of RBCs to HU-treated VECs in autologous plasma and plasma containing white blood cells. However, in comparison to plasma alone, the presence of white blood cells in autologous plasma appeared to produce a small but significant difference ($P < .05, n = 3$ independent experiments) in RBC adhesion, which disappeared as the washing procedure continued.

Table 1 summarizes the fractional $^{51}$Cr radioactivity remaining in VECs during wash-out experiments to determine the effect of HU on Hb A and S RBC adhesion. Mean values per wash were calculated from three experiments with Hb A and two experiments with Hb S RBCs. On average, incubation in plasma left higher counts along the wash-out curve, although statistical significance was reached only for the first two washes both in control and HU-treated VECs.

**Determination of the radioactive hemolysates (obtained by addition of distilled water to the dishes after the five washes were completed) is another means to estimate VEC-bound RBC $^{51}$Cr. Figure 7 summarizes the hemolysate counts for the first four conditions described in Fig 6 for two subjects with Hb A and two subjects with Hb S. For the first subject with Hb A (A1), HU treatment of VECs in culture lasted 3 days [A1(3d)]. For the second Hb A subject (A2), the experiments were performed after 4 [A2(4d)] and 7 [A2(7d)] days of treatment with HU. Note that at day 4 [A2(4d)] no HU-induced decrease was observed for cells incubated in HM and only a small decrease (18%) was shown by those incubated in autologous plasma, whereas at day 7 [A2(7d)], HU decreased by 30% and 50% the hemolysate counts in cells incubated in HM and plasma, respectively.

In light of the above findings, VECs were treated for 7 days with 0.3 mmol/L HU to study Hb S RBC adhesion. For the first Hb S subject (S1), HU decreased the counts by 0% and 20% for cells incubated in HM and plasma, respectively, and for the second Hb S subject (S2), by 29% and 58%. Despite the small number of samples, the difference in hemolysate radioactivity between control and HU-treated endothelial cells incubated in plasma was significant ($P < .05, n = 5$).

Thus, the HU-induced decrease in hemolysate counts when cells were incubated in either HM or autologous plasma showed time and interindividual dependence. The time dependence is clearly shown for subject A2 when com-
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Fig 4. Effect of HU on cation content in bovine aortic endothelial cells after several days in culture. (A) Na; (B) K; (C) total cations. Cell passage number, symbols, and expression of results are as in Fig 3.

Fig 5. HU-dependent cation changes in bovine aortic endothelial cells as a function of days in culture. (A) Na; (B) K; (C) total cations. Cells were at passages 3 through 6. (A) 0.3 mmol/L HU; (C) 3.0 mmol/L HU. Results are expressed as X ± SD; means represent the percent change in three independent experiments with triplicate samples each.

DISCUSSION

This study reports for the first time prominent effects of therapeutic and toxic doses of HU on the morphology and steady-state ionic composition of cultured VECs. The HU-induced formation of giant, multinuclear VECs with reduced confluency (Fig 2) and hence decreased protein content per dish (Fig 3) is consistent with the fact that HU inhibits DNA synthesis and that large RBCs appear in the circulation after HU treatment of Hb S patients. Our data rule out interference by HU solely on cell attachment (Fig
The substantial changes in cellular morphology may reflect endothelial transformation, failure of cell division, or a combination of these effects, and may be at the root of or causal to activation/inhibition of ion transport pathways as evident from the cellular cation changes specifically due to HU treatment (Fig 5).

HU altered the Na and K composition of cultured VECs when added after cell attachment (Figs 4A through C and 5A through C), but not when added during cell seeding (data not shown). At first glance, these results appear to indicate that HU affects cation content differently depending on the time of exposure to the drug, i.e., before or after cell attachment occurred. However, when cells were exposed to HU during seeding, the changes in both protein and cation content were studied only after 3 days in culture (Fig 1; other results not shown). At this time point, Fig 4A through C also shows no statistical difference in cation content between HU-treated and untreated cells, while the effect appears beyond 4 days in culture. Thus, the changes in cation content were not a consequence of HU effect(s) on cell attachment, but rather of time- and concentration-dependent effect(s) on cellular ionic transport.

The increase in Na content was transient and lasted less than 72 hours (Figs 4A and 5A). This phenomenon may underlie an increase in the passive permeability of VECs to Na. Since this effect preceded the changes in K content, it is possible that the increased cellular Na content resulted in (1) activation of already-existing Na/K pumps, (2) synthesis of new pump molecules, (3) a sum of these two effects, or (4) a mediated passive Na entry (through Na-K-Cl cotransport or Na/H exchange?). Such mechanisms should increase cellular K levels. However, if this was the only mechanism of increase in K content, the total cation content or cell volume would be expected to remain the same or decrease since the pump operates at a 3 Na:2 K stoichiometry. Thus, these results suggest that in addition to direct or indirect effects on the Na/K pump, HU affects K content either by inhibiting other pathway(s) for K exit or by stimulating alternative routes for K influx. The first possibility seems more likely in light of the effects of HU on K-Cl cotransport and cell volume in RBCs. Although at this point K-Cl cotransport has not been shown in VECs, Fig 4B and unpublished evidence from this laboratory points in this direction, since HU inhibited in a dose-dependent manner and as a function of time in culture the normal decrease in K content reported earlier. This effect of HU on K content was accompanied by an increase of cation content and thus of cell volume (Fig 4C and 5C), which resembles the effects of the drug in RBCs of patients under treatment.

HU significantly decreased RBC adherence to VECs during analysis of the radioactivity remaining in the hemolysates after the fifth wash (Fig 7). Furthermore, in 51Cr wash-out experiments (Fig 6 and Table 1), adhesion of RBCs to VECs was higher in the presence of plasma than in HM both with or without HU treatment. However, statistical significance was obtained only for the two initial washes, whereas the HU effect was statistically nonsignificant. This result may be accounted for by several factors. First, the high number of counts in the wash-out experiments produced a larger error than the one inherent to the small number of counts extracted in the hemolysate, with the consequent effect on statistical significance. Second, the effect of HU was time-dependent; however, selection for computation of the experiments performed after 7 days of treatment with HU did not improve the statistical significance in the wash-out curves. Third, the effect of HU on RBC adherence showed interindividual variability, suggesting that RBC-VEC adhesion is a specific process.

We did not confirm previous findings of increased Hb S RBC adherence to VECs, which may be due to the small sample size and interindividual variability. The use of bovine instead of human serum albumin during the incubations with HM could explain the differences observed between this medium and the conditions using human

<table>
<thead>
<tr>
<th>Medium</th>
<th>Wash No.</th>
<th>Control (%)</th>
<th>HU (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HM</td>
<td>1</td>
<td>8.5 ± 2.5</td>
<td>9.1 ± 2.6</td>
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<tr>
<td></td>
<td>2</td>
<td>4.9 ± 1.8</td>
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<tr>
<td></td>
<td>3</td>
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<td>3.3 ± 1.9</td>
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<tr>
<td></td>
<td>4</td>
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<td>2.7 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2.2 ± 1.8</td>
<td>2.3 ± 1.9</td>
</tr>
<tr>
<td>Plasma</td>
<td>1</td>
<td>12.8 ± 3.3*</td>
<td>14.6 ± 4.21</td>
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<tr>
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<td>2</td>
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<td>9.5 ± 2.41</td>
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<tr>
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<td>3</td>
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<tr>
<td></td>
<td>5</td>
<td>4.5 ± 2.2</td>
<td>5.8 ± 3.2</td>
</tr>
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</table>

Results are presented as fractional 51Cr radioactivity remaining in VECs after each wash (n = 5, X ± SD).

* P < .01 with respect to HM.

† P < .05 with respect to HM.
plasma. However, based on the finding that bovine and human VECs behave similarly for RBC adhesion, it is to be expected that the differences observed between HM and plasma are due to the presence of other plasma components, ie, Ca, growth factors, vasoactive agents, and hormones. Our results instead confirm the suggestion by Narla and Evans of a lack of an effect of plasma-free suspending medium on RBC-VEC adhesion, which was shown by Hebbel et al and Antonucci et al to be due to a calcium effect. Furthermore, our results on RBC adherence to HU-treated VECs are commensurate with in vivo studies indicating a beneficial effect of HU on the mechanism of vaso-occlusion.

Our results on cell morphology thus appear to indicate that by inhibiting DNA synthesis, HU provoked initial cell death. However, by an as-yet-unknown mechanism (transformation? adaptation?), the surviving cells were able to multiply and grow, although a higher percentage of binucleated and trinucleated cells was observed. If massive VEC death or cell injury also occurred in vivo, this would be anticipated to initiate clotting, which is especially disadvantageous for sickle cell anemia. However, the fact that the increase in Na content was transient and that the cells, rather than losing K, increased their cation content after 4 days of treatment (Figs 4 and 5) is an indicator of good cell health, since dying cells cannot maintain their ionic gradients. Furthermore, HU has been shown to heal chronic leg ulcers in vivo, which suggests that the potentially negative effects of the drug are a function of dose, length of treatment, and interindividual responses, and that by controlling these factors its positive effects can be maximized.

Finally, the HU-induced effects on the morphologic and functional properties of VECs, as reported in this study, may be caused or accompanied by alterations in cell membrane permeability, transformation of endothelial cells, or decreased number/density of VEC adhesion molecules. Elucidation of such mechanisms may have significant importance not only for sickle cell anemia, but also for chemotherapy, since cell enlargement seems to be a generalized phenomenon induced by HU and also by other drugs. HU is not only a cytostatic drug, but also a carcinogen. Knowing the mechanism of toxicity of the drug at both cellular and molecular levels could provide relevant information for the treatment of sickle cell anemia and of cancer as well.

The decrease in RBC adhesion could result either from cell enlargement by decreasing the relative density of adhesion molecules anchoring the RBC, from a drug-induced concomitant decrease in the absolute number of adhesion molecules, or from HU effects on their normal function. A combination of all these factors is also possible. These factors may appear as a consequence of (1) changes in membrane potential affecting the assembly of the adhesion complexes that insert in the membrane, (2) altered surface potential, local pH, ionic microenvironment, or cell swelling, or (3) any other change affecting production of second messengers. All of these phenomena can be reflected in an altered ion transport activity of the cell. Understanding the mechanism of decreased adhesion can provide significant information on the process of RBC-endothelial cell adhesion that is at the root of the vaso-occlusive process, which is of high significance in sickle cell anemia.

In conclusion, our results show that HU induced significant morphologic and membrane transport changes in cultured VECs. In addition, this study provokes interesting questions that could stimulate further studies to determine the effect of HU on VEC function in vivo.

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Hydroxyurea affects cell morphology, cation transport, and red blood cell adhesion in cultured vascular endothelial cells

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