Free Radical Research, Volume 38 Number 5 (May 2004), pp. 431–437

Taylor & Francis
healthsciences

The Neurotoxic Effect of Sickle Cell Hemoglobin

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Accepted by Professor B. Halliwell

(Received 4 September 2003; In revised form 14 October 2003)

A growing body of experimental evidence suggests that the oxidative neurotoxicity of hemoglobin A may contribute to neuronal loss after CNS hemorrhage. Several hemoglobin variants, including hemoglobin S, are more potent oxidants in cell-free systems. However, despite the increased incidence of hemorrhagic stroke associated with sickle cell disease, little is known of the effect of hemoglobin S on cells of neural origin. In the present study, its toxicity was quantified and directly compared with that of hemoglobin A in murine cortical cell cultures. Reactive oxygen species production, as assessed by cellular fluorescence after treatment with dihydrorhodamine 123, was significantly increased by exposure to 10 μM hemoglobin S for 2–4 h. Neuronal death, as measured by propidium iodide staining and lactate dehydrogenase release, commenced at 4 h; for a 20-h exposure, the EC_{50} was approximately 0.71 μm. Glial cells were not injured. Cell death was completely blocked by iron chelation with deferoxamine or phenanthroline. Direct comparison of sister cultures exposed to either hemoglobin A or hemoglobin S revealed a similar amount of cell injury in both groups. A significant difference was consistently observed only after treatment with 1 μM hemoglobin for 20 h, which resulted in death of approximately one third more neurons with hemoglobin S than with hemoglobin A. The results of this study suggest that sickle cell hemoglobin is neurotoxic at physiologically relevant concentrations. This toxicity is iron-dependent, oxidative, and quantitatively similar to that produced by hemoglobin A.

Keywords: Brain injury; Free radical; Iron; Oxidative injury; Sickle cell anemia; Stroke

INTRODUCTION

Sickle cell anemia is an autosomal recessive disease caused by alteration of the β-hemoglobin chain by a single amino acid mutation. This results in the production of hemoglobin S (Hb S), which is less stable than hemoglobin A (Hb A). When deoxygenated, Hb S tends to polymerize, leading to erythrocyte sickling and subsequent vaso-occlusion. An unfortunate consequence of this disease is an increased incidence of stroke,[1] particularly in children and young adults,[2] with a prevalence of 12% by age 21. Although most of these strokes are primarily ischemic, hemorrhagic stroke is surprisingly common in this population. In the Cooperative Study on Sickle Cell Disease, 38% of strokes in Hb S homozygotes were hemorrhagic,[1] compared with approximately 15% in the general population.[3] Hemorrhagic stroke in sickle cell disease has a higher morbidity than ischemic stroke, and a two week mortality of 26%.[1]

A growing body of experimental evidence suggests that blood components may contribute to cell injury following CNS hemorrhage.[4–7] One potential cytotoxin is hemoglobin, which is released from erythrocytes in the hours following hemorrhage via a complement-mediated mechanism.[8] Although reduced hemoglobin effectively sequesters its reactive heme in a hydrophobic pocket, it tends to oxidize in the extracellular space to methemoglobin.[9] This reaction generates superoxide, and also decreases the affinity of globin for its heme group.[10,11] Oxidized heme is then readily transferred to membrane lipids, where it or the iron released from it can effectively catalyze oxidative reactions.[12,13] In cell free systems, this phenomenon occurs more rapidly with Hb S than with normal adult hemoglobin (Hb A),[14–16] leading to an increased rate of lipid peroxidation.

The toxicity of Hb A has been investigated in a variety of *in vitro* and *in vivo* models.[4,17–20]

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2004 Taylor & Francis Ltd
DOI: 10.1080/10715760310001638010

In contrast, relatively little is known of the effect of Hb S on the redox state and viability of cells other than erythrocytes. Nath *et al.* observed increased lipid peroxidation in kidney tissue obtained from sickle cell patients, which was associated with increased heme content and heme oxygenase-1 induction.[21] The authors concluded that heme released from Hb S mediated sickle cell nephritis. However, despite its possible relevance to hemorrhagic stroke in sickle cell patients, the neurotoxicity of Hb S has not been quantified. Given its greater pro-oxidant effect in cell-free systems, such investigation seemed warranted. The primary goal of the present study was therefore to test the hypothesis that Hb S is neurotoxic, and that an oxidative mechanism is responsible for this toxicity. A second goal was to directly compare the toxicity of Hb S and Hb A on cultured cortical neurons.

MATERIALS AND METHODS

Cell Culture Preparation

Cortical cell cultures containing both neurons and astrocytes were prepared from gravid B6/129 mice from our breeding colony. After 16–17 days gestation, mice were administered isoflurane anesthesia, and were then rapidly euthanized. Fetuses were then removed and the cortices of these fetuses were dissected from surrounding tissue. Following dissection, tissue was minced with forceps and placed in medium containing 0.075% acetylated trypsin at 37°C for 1 h. Following low speed centrifugation for 5 min, tissue was dissociated by trituration through a flamed Pasteur pipette in plating medium consisting of Eagle's minimal essential medium (MEM), 5% fetal bovine serum (Hyclone), 5% heat inactivated horse serum (Hyclone), glutamine (2 mM), and glucose (23 mM). Additional plating medium was then added to the cell suspension, which was then seeded on a confluent monolayer of astrocytes at a density of 3 hemispheres per 24 well plate. Astrocyte cultures were prepared in a similar fashion from 1 to 3 day old postnatal mice. All cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere. Two-thirds of the culture medium was replaced with fresh medium lacking fetal bovine serum twice weekly for the first 10 days and daily thereafter.

Cytotoxicity Experiments

Cultures were used for experiments 12–16 days after plating. At this time, neurons are easily differentiated from astrocytes by their phase-bright cell bodies and extensive processes. The accuracy of this method of cell differentiation has been confirmed in a prior study using immunohistochemical-staining techniques with antibodies to neuron-specific enolase and glial fibrillary acidic protein.[22] MEM (iron-free) with 10 mM glucose (MEM10) was added to the cell cultures following aspiration of the feeding media. The cultures were then exposed to 0.3, 1.0, 3.0, and 10 μM concentrations of Hb S or Hb A, and subsequently were returned to the incubator. The concentrations used were based on prior experiments indicating that 3–10 μM Hb A is sufficient to induce widespread neuronal injury.[17] Following a twenty-hour incubation period, the cell cultures were examined for evidence of neuronal injury.

Assessment of Injury

Neuronal death was determined following examination of the cell cultures under phase contrast microscopy. Measurement of lactate dehydrogenase (LDH) activity in the culture medium was used to biochemically quantitate the degree of neuronal injury. Past studies have detailed the use of LDH as an accurate indicator of necrotic and apoptotic cell death in this culture system.[23,24] It is noteworthy that astrocytes are not injured by micromolar concentrations of hemoglobin,[17] and therefore the contribution of astrocytic LDH to the total signal is negligible. The LDH activity in the media that was associated with near-complete neuronal death was approximated by using sister cultures from the same plating continuously exposed to 300 μM NMDA, which kills roughly 92% of neurons[23] but does not injure astrocytes. Stock solutions of sterile 100 mM potassium phosphate buffer and 27.2 mM sodium pyruvate in potassium phosphate buffer (pH 7.4) were prepared in advance and stored at 4°C until used. A solution of NADH (3 mg/10 ml phosphate buffer) was prepared immediately prior to use. Following incubation, 25 μl samples of medium were removed from each culture. Next, 125 μl phosphate buffer and 100 μl NADH solution were added into each well. Then, 25 μl pyruvate was rapidly added using an Eppendorf repeater pipette with 8 well adapter. The absorbance of the reaction mixture at 340 nm was determined at 6-second intervals for 2 min, using a kinetic plate reader (Molecular Devices).

In some experiments, cell death was also quantified by measuring fluorescence intensity of cultures stained with propidium iodide, which stains the nuclei of cells with disrupted membranes but is excluded from viable cells. At defined time points, cultures were incubated with 13 μg/ml of propidium iodide (PI) for 15 min. Extracellular PI was then washed from the cultures and a HEPES-buffered salt solution was added containing (in mM): NaCl, 120; KCl, 5.4; $MgCl_2$, 0.8; $CaCl_2$, 1.8; HEPES, 20; glucose, 5.5, pH 7.4. These cultures were then

visualized using a Nikon Diaphot 300 fluorescent microscope at 100× magnification. Randomly selected fields were viewed through a rhodamine filter. The fluorescence intensity of 6 integrated frames per field was analyzed using Scanalytics IPLab software. Background fluorescence was calculated by analyzing cultures subjected to only a sham wash and dye incubation.

Detection of Reactive Oxygen Species (ROS)

Cellular ROS generation was quantified with dihydrorhodamine 123, which is a cell-permeable, nonfluorescent compound that fluoresces only after being oxidized by ROS. Therefore, fluorescence intensity is directly proportional to cellular oxidative stress. In order to prevent oxidation of dihydrorhodamine in the culture medium, that might then stain cells, hemoglobin was washed thoroughly from cultures prior to adding the reduced probe. Images of dihydrorhodamine stained cultures were captured immediately after illumination; six frames were integrated per field. Photomicrographs of random fields were taken and analyzed as described above for propidium iodide staining.

Materials

MEM and glutamine were purchased from Gibco (Invitrogen Corp., Carlsbad, CA), and serum was purchased from Hyclone Inc., Logan, Utah. Human Hb S was purchased from Sigma (Sigma–Aldrich Co., St. Louis, Mo), and human Hb A was kindly provided as a gift by Hemosol (Hemosol Inc., Mississauga, Ontario). All other reagents were purchased from Sigma.

RESULTS

Hb S Rapidly Generates Neuronal ROS

Dihydrorhodamine 123 (DHR) was chosen as a marker of oxidative stress because of its ability to rapidly enter and be retained by neural cells. Its oxidation product, cationic rhodamine 123, is likewise trapped within cells. Since hemoglobin solutions generate ROS due to autoxidation at 37°C and neutral pH,[10,25] the culture medium was replaced with hemoglobin-free medium at defined intervals prior to adding DHR. It is therefore unlikely that direct oxidation of DHR by hemoglobin in the medium contributed significantly to the cellular fluorescent signal. Consistent with our recent observations using hemoglobin A,[26,27] Hb S induced cellular fluorescence in DHR-treated cultures (Fig. 1). A significant increase compared with sham-washed sister cultures was noted by 2 h and peaked at 4 h. Fluorescence was

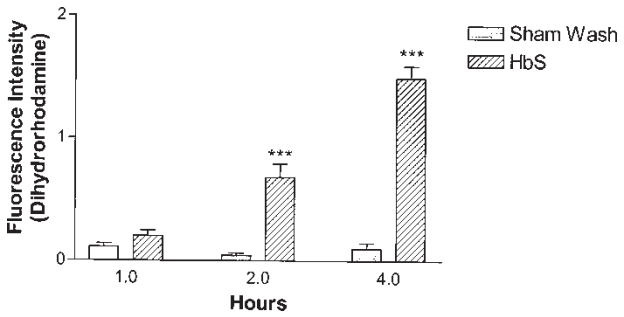

FIGURE 1 Time course of reactive oxygen species formation after exposure to sickle cell hemoglobin. Cultures (9–16/condition) were exposed to either a 10 μM concentration of Hb S or to MEM with 10 mM glucose alone (sham wash) for indicated intervals. The cultures were then washed and incubated with a 20 μM concentration of dihydrorhodamine 123 for 15 min. The dihydrorhodamine was then washed from the cultures, and fluorescent images of the cultures were taken using a Nikon Diaphot 300 fluorescent microscope at 100× magnification. Finally, IPLab software was used to directly quantify the fluorescent intensity of these images. $^{***}P < 0.001$ versus mean (±S.E.M.) in cultures subjected to sham wash alone, Bonferroni multiple comparisons test.

concentrated in neuronal cell bodies, with little specific signal observed in the background glial monolayer. At later time points, cell injury and membrane disruption reduced dye retention, producing a weak, diffuse signal with a lower overall intensity. Detailed investigation at these time points was not undertaken.

Time Course and Concentration-Dependence of Neuronal Injury

The time course of neuronal injury was assessed after 2–6 h exposure to 10 μM Hb S. The membrane-impermeant fluorescent dye propidium iodide (PI), which stains the nuclei of cells with disrupted membranes, was used for these studies. No significant fluorescence was detected at 2 h (Fig. 2).

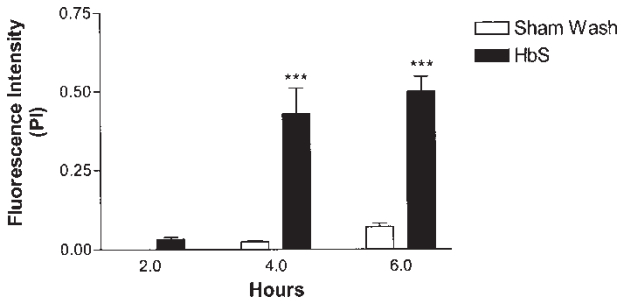

FIGURE 2 Time course of cell death after exposure to sickle cell hemoglobin. Cultures (6–11/condition) were exposed to either a 10 μM concentration of Hb S or to MEM with 10 mM glucose alone (sham wash) for indicated intervals. The cultures were then stained with 13 μg/ml propidium iodide for 15 min. After dye washout, fluorescent images of the cultures were captured and fluorescence intensity was calculated. PI values are scaled to that in sister cultures exposed to Hb for 24 h (= 1.0), which results in death of all neurons without injuring glia. $^{***}P < 0.001$ versus mean in cultures subjected to sham wash alone, Bonferroni multiple comparisons test.

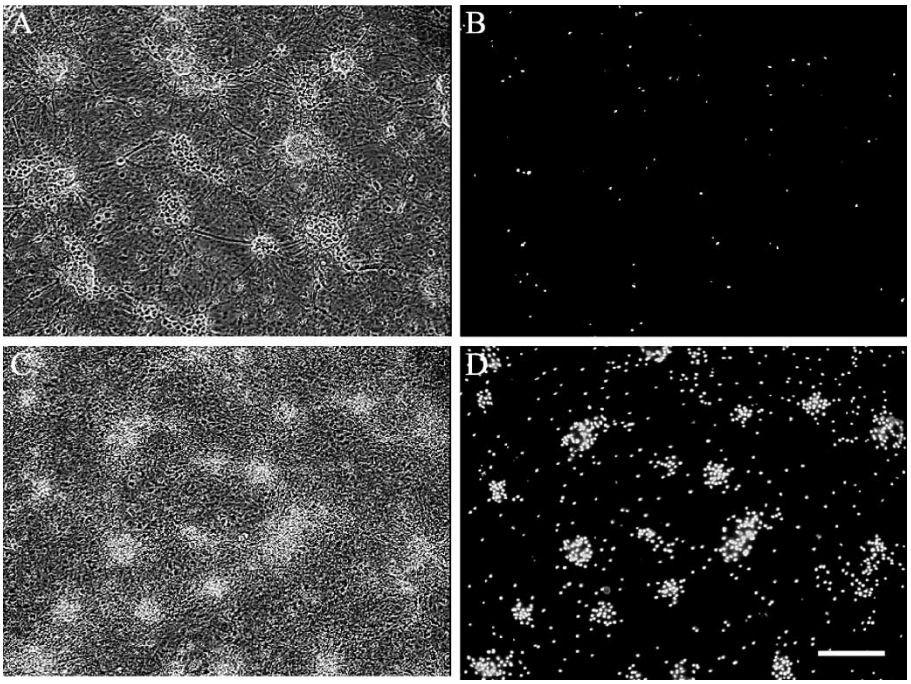

FIGURE 3 Morphologic appearance of control and Hb S exposed cultures. Phase contrast (A,C) and fluorescent (B,D) photomicrographs of sister cultures incubated for 20 h with: A, B: MEM with 10 mM glucose only; most cells appear healthy and exclude propidium; C, D: 3 μM HbS; most neurons have degenerated and stain with propidium. Scale bar = 200 μm.

However, a marked increase was observed by 4–6 h, which was about half of that observed in sister cultures from the same plating that had sustained death of all neurons. Staining was localized to neuronal cell bodies, with minimal specific fluorescence observed in the background glial monolayer (Fig. 3).

The concentration-dependence of neuronal injury was further defined by assaying LDH activity in the culture medium after exposing cultures to 0.3–10 μM Hb S for 20 h (Fig. 4). Release of all neuronal LDH was observed at both 3 and 10 μM, consistent with death of all neurons. The calculated EC_{50} for a 20-h exposure was 0.71 μM.

Iron-dependence of Hemoglobin S Neurotoxicity

In order to determine if hemoglobin S neurotoxicity is mediated by iron release, the effect of iron chelators on cell injury was determined. Deferoxamine is a water-soluble chelator; phenanthroline is lipid soluble. Both sequester iron in a form that makes it less available for participation in cellular

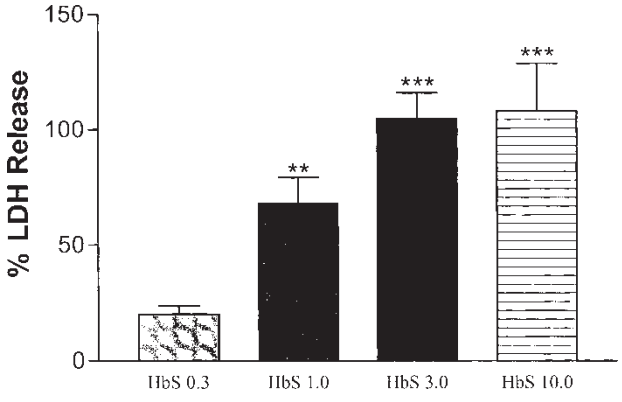

FIGURE 4 Concentration-toxicity relationship for Hb S. Cell cultures were exposed to specified concentrations of Hb S and incubated for 20 h. Medium was then sampled for LDH assay. LDH values (mean ± S.E.M, $n = 7–8$/condition) are scaled to the mean value in sister cultures exposed to 300 μM NMDA (= 100% LDH release), which results in release of almost 100% of neuronal LDH without injuring glia. $^{***}P < 0.001$, $^{**}P < 0.01$ versus sister cultures exposed only to culture medium.

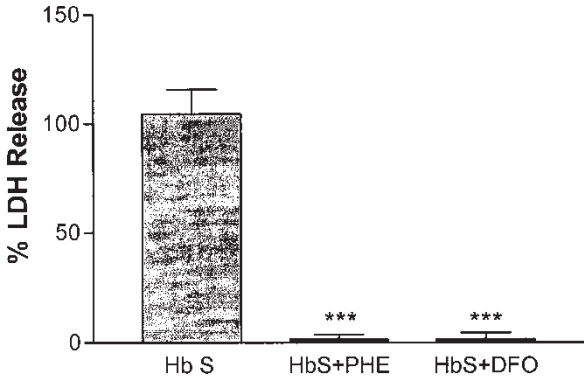

FIGURE 5 Efficacy of treatment with iron chelators. Cultures were treated for 20 h with either 3 μM Hb S alone, or with 30 μM of either deferoxamine (DFO) or 1,10 phenanthroline (PHE). LDH values (mean ± S.E.M, $n = 8$/condition) are scaled to that in sister cultures exposed to 300 μM concentration of NMDA (= 100), which results in release of almost 100% of neuronal LDH without injuring glia. $^{***}P < 0.001$ v. cultures treated with Hb S alone, Bonferroni multiple comparisons test.

free radical reactions.[28,29] The cell death produced by Hb S was completely prevented by concomitant incubation with either chelator (Fig. 5).

Direct Comparison of the Neurotoxicity of Hemoglobin A and Hemoglobin S

The EC_{50} of 0.71 μM for Hb S was lower than our previous observation of 2.1 μM for Hb A.[17] However, in the latter study, cultures were prepared from Swiss-Webster mice; in the present study, B6/129 mice were used. Since mouse strains vary considerably in their vulnerability to oxidative injury, cell injury induced by Hb A and Hb S was directly compared in sister cultures prepared from B6/129 mice from the same plating. Increased cell death, as assessed by LDH release, was observed in cultures treated with Hb S (Fig. 6). However, the difference was modest, and achieved statistical significance only in cultures treated with 1 μM Hb for 20 h. A trend toward increased injury with Hb S was observed in other conditions. Similar results were observed when cell death was assessed with fluorescence intensity after propidium iodide staining (Fig. 7). In these experiments, significantly more cell death was also observed in cultures treated with 10 μM Hb S for 4 h. However, this difference was no longer observed by 8 h.

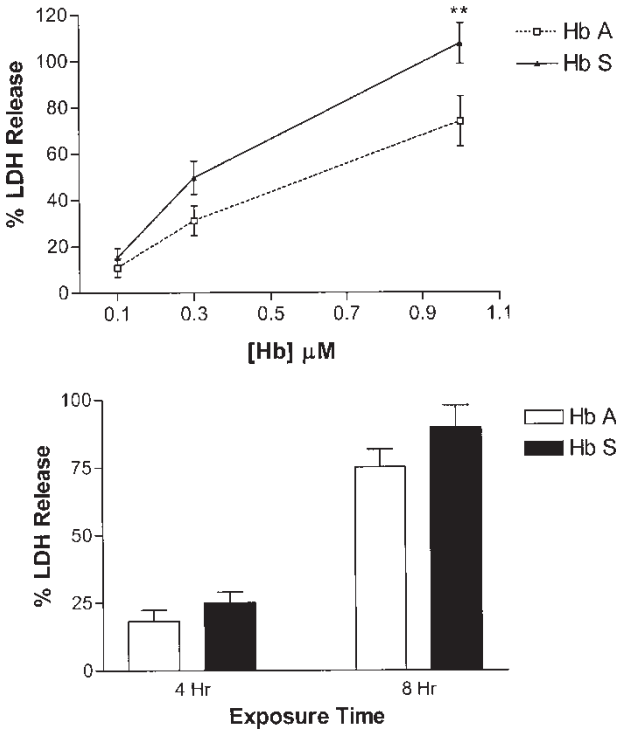

FIGURE 6 Comparison of the neurotoxicity of hemoglobin A and hemoglobin S, assessed by LDH release. *Top*: Sister cultures ($n = 10$/condition) were exposed to indicated concentrations of Hb A or Hb S for 20 h. *Bottom*: Sister cultures ($n = 11–12$/condition) were exposed to 10 μM Hb A or Hb S for indicated intervals. $^{**}P < 0.01$ v. mean value in cultures exposed to same concentration of Hb A, Bonferroni multiple comparisons test.

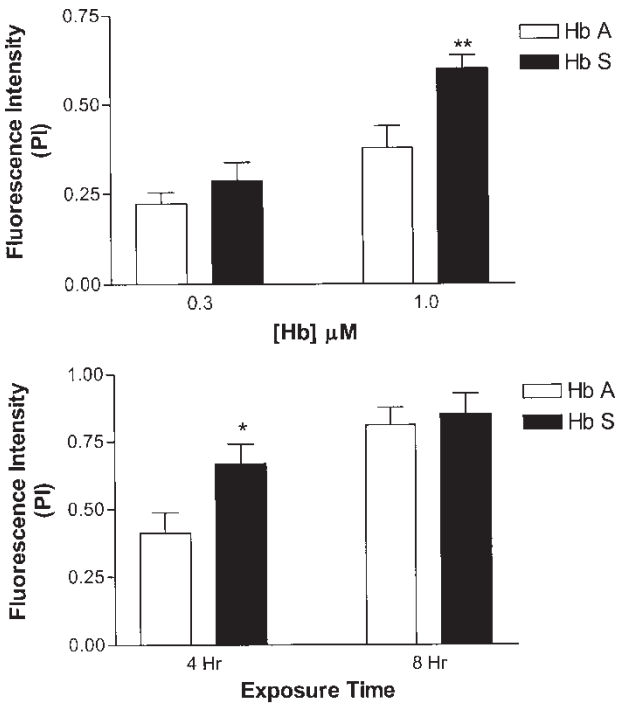

FIGURE 7 Comparison of the neurotoxicity of hemoglobins A and S, assessed by fluorescence intensity after propidium iodide staining. *Top*: Sister cultures ($n = 8–10$/condition) were exposed to indicated concentrations of hemoglobins for 20 h. *Bottom*: Sister cultures (13/condition) were exposed to 10 μM Hb A or Hb S for indicated intervals. Fluorescence intensity values are scaled to that in sister cultures exposed to Hb A 10 μM for 24 h (= 1.0), which results in death of all neurons without injuring glia. $^{*}P < 0.05$, $^{**}P < 0.01$ v. mean value in cultures exposed to corresponding Hb A condition, Bonferroni multiple comparisons test.

DISCUSSION

The present study was designed to accomplish two aims. First, the neurotoxicity of hemoglobin S was quantified in an established cell culture model. At low micromolar concentrations, Hb S was found to produce oxidative stress, detected by cellular oxidation of dihydrorhodamine 123 to its fluorescent product, within a few hours. This was soon followed by neurodegeneration, leading to loss of membrane integrity and increased lactate dehydrogenase activity in the culture medium. As with Hb A, cell injury was completely prevented by concomitant treatment with either a lipid-soluble or a water-soluble iron chelator with demonstrated antioxidant activity.[28]

The lethal injury produced by Hb S to primary cortical neurons likely reflects the vulnerability of these cells to iron. In a recent study,[27] we observed that incubation with 10–30 μM ferrous sulfate, equivalent to the iron content of 2.5–7.5 μM Hb, was sufficient to kill most neurons in this culture

system within 24 h. As previously observed with Hb A, the vulnerability of neurons to Hb S was not shared by glial cells present in the same cultures. These cells showed no morphologic evidence of injury whatsoever, and continued to exclude propidium iodide after the death of all neurons. Compared with glial cells, neurons have limited antioxidant defenses.[30,31] They also are more dependent on the activity of membrane cation pumps to recover from depolarization; these proteins are highly vulnerable to iron-dependent oxidative injury.[32,33] Nevertheless, the vulnerability of neurons to Hb is surprising given their constitutive expression of ferritin,[34] which sequesters iron in a mineral core which usually makes it unavailable for free radical reactions. Further investigation is necessary to explain this paradox. It is noteworthy that neuronal ferritin has a high proportion of H subunits.[34] Compared with L-rich ferritin, H-rich ferritin has a lower iron binding capacity, and it tends to become unstable with iron loading.[35]

Second, the vulnerability of cortical neurons to Hb S and Hb A was directly compared in sister cultures prepared at the same time by the same technician. Consistent with observations made in cell-free systems, Hb S was somewhat more toxic than Hb A. However, in this model, the magnitude of this effect was rather small, resulting in a maximal absolute increase in LDH release of 34%. Although a trend toward increased injury was identified in other conditions, the differences were transient or did not reach statistical significance.

The difference in cytotoxicity produced by Hb S and Hb A was less than expected, given the 72% faster oxidation rate for Hb S observed by Hebbel *et al.*[14] In a subsequent study from the same group, the adventitious association of molecular iron with Hb S, but not with Hb A, accounted for part of this effect.[36] In the present study, neurons were exposed to Hb in a buffer containing 26 mM sodium bicarbonate, which may limit the solubility of adventitious iron and therefore its ability to participate in redox reactions. The present results are consistent with those of Chiu *et al.*[16] These investigators observed a 28% increase in the rate of lipid peroxidation in white erythrocyte ghosts with Hb S compared with Hb A. Similarly, Belcher *et al.* reported a 22% increase in the oxidation rate of low-density lipoprotein from sickle cell patients compared with Hb A controls.[37] Taken together, these and the present studies support the hypothesis that Hb S is somewhat more neurotoxic than Hb A. However, the physiologic relevance of this modest difference is uncertain.

The results of this study nevertheless suggest that Hb S, like Hb A, may be quite deleterious when released after CNS hemorrhage. Its EC_{50} in this simplified cell culture model is very low when compared with the hemoglobin concentration of blood, which is 2–2.5 mM. However, the antioxidant effect of serum and CSF proteins will likely mitigate its toxicity in the intact CNS.[38,39] This protective effect may be countered by potentiation of other injury mechanisms that may be synergistic with hemoglobin toxicity, such as excitotoxicity.[40] The vulnerability of cortical neurons to Hb S supports the potential use of iron-chelating antioxidants in the treatment of hemorrhagic stroke in sickle cell patients. Further study in relevant *in vivo* models seems warranted.

Acknowledgement

This study was supported in part by a grant from the National Institutes of Health (1RO1NS042273).

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