

# Hemoglobin stimulates the release of proinflammatory cytokines from leukocytes in whole blood

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Isolated mononuclear leukocytes, when incubated with purified hemoglobin Ao (HbAo), release the proinflammatory cytokines interleukin-8 (IL-8) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). In this study we examined whether leukocytes in whole blood, when incubated with HbAo, release IL-8, TNF- $\alpha$ , and IL-6. Leukocytes in whole blood incubated with HbAo for 4 hours at 37°C, 5% CO<sub>2</sub>, and 95% humidity released 187, 1313, and 50 pg/mL of IL-6, IL-8, and TNF- $\alpha$ , respectively, as compared with 6, 192, and 2 pg/mL released by leukocytes in blood incubated with human serum albumin (HSA). Furthermore, plasma from blood incubated with HbAo exhibited chemotactic activity and stimulated human umbilical vein endothelial cells to become adherent to neutrophils. These activities were 3.3 and 2.6 times those measured in plasma from blood incubated with HSA. Hydrocortisone (0.05  $\mu$ mol/L to 50  $\mu$ mol/L) inhibited cytokine release in a dose-dependent manner with ED<sub>50</sub> values of 0.23  $\mu$ mol/L, 0.19  $\mu$ mol/L, and 0.10  $\mu$ mol/L for IL-6, IL-8, and TNF- $\alpha$ , respectively. The release of proinflammatory cytokines in whole blood after exposure to hemoglobin solutions is consistent with the possibility that an inflammatory reaction could develop on infusion of hemoglobin, whereas inhibition of cytokine release by hydrocortisone suggests that the inclusion of anti-inflammatory compounds in hemoglobin solutions may prevent undesirable effects caused by inflammation after infusion. (J Lab Clin Med 2000;135:263-9)

**Abbreviations:** ED<sub>50</sub> = effective dose necessary to produce 50% inhibition; ELISA = enzyme-linked immunosorbent assay; HbAo = hemoglobin Ao; HEPES = N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; HSA = human serum albumin; HUVEC = human umbilical vein endothelial cell; LPS = lipopolysaccharide; MNL = mononuclear leukocyte; MPRM = mono-poly resolving medium; PMN = neutrophil; RANTES = regulated on activation with normal T-cell expressed and secreted; RBC = red blood cell

**F**or over 60 years, cell-free hemoglobin has been under development for use as a large volume resuscitation fluid.<sup>1-4</sup> Unlike saline or colloid-

based solutions, hemoglobin solutions carry and deliver oxygen to tissues in addition to restoring intravascular volume and pressure,<sup>5</sup> and unlike RBCs, hemoglobin solutions do not need cross matching and can be prepared virus free. Furthermore, because of the oxygen-carrying capacity and low viscosity of cell-free hemoglobin, solutions of cell-free hemoglobin potentially should preserve tissues better than conventional solutions.<sup>6</sup>

The development of cell-free hemoglobin as a clinically relevant resuscitation fluid has been hindered, however, by observations that hemoglobin has an increased affinity for oxygen outside of the erythrocyte, is excreted quickly with a half-life of 2 to 4 hours,<sup>5-7</sup> and exhibits several kinds of toxicity including kidney tubular epithelial cell injury<sup>8</sup>; hepatic necrosis<sup>9</sup>; neuronal cytotoxicity<sup>10</sup>; activation of mononuclear leuko-

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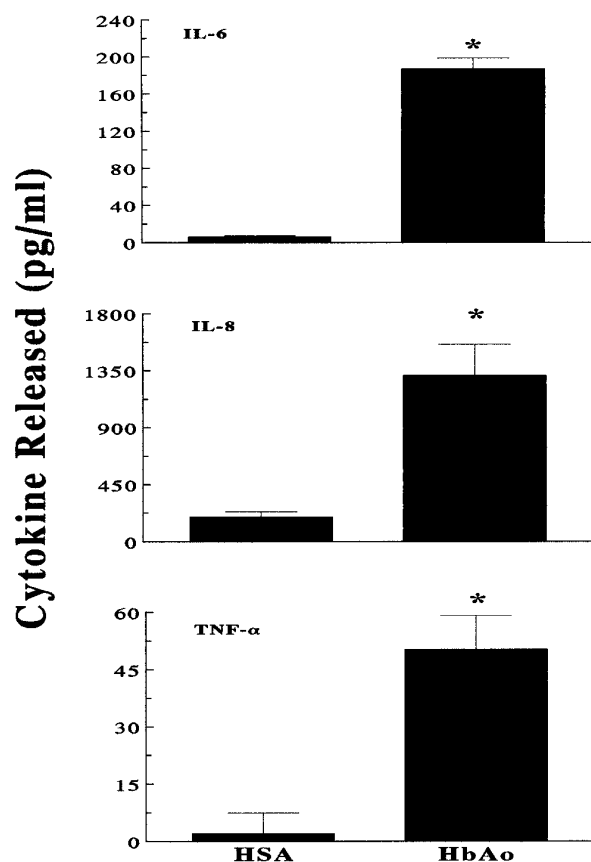
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**Fig 1.** Cytokine release from leukocytes in blood after exposure to hemoglobin. Heparinized blood was incubated with 1 g/dL of either HSA or HbAo for 4 hours as described in Methods. Plasma supernatant fluids were then analyzed for IL-6, IL-8, and TNF- $\alpha$  by ELISA. Blood was also incubated with LPS from *E. coli* as a positive control and released  $4899 \pm 59$ ,  $19,075 \pm 1505$ , and  $24,944 \pm 2917$  pg/mL of IL-6, IL-8, and TNF- $\alpha$ , respectively. Values are expressed as mean  $\pm$  SEM for four, seven, and six experiments for IL-6, IL-8, and TNF- $\alpha$ , respectively. \* $P < .05$ , significance level for differences from HSA data.

cytes to cause disseminated intravascular coagulation through the expression of procoagulant factors<sup>11,12</sup> and to release proinflammatory cytokines<sup>13</sup>; coronary and pulmonary vasoconstriction<sup>14-16</sup>; and increased mortality in conjunction with endotoxemia.<sup>17, 18</sup> Through chemical modification,<sup>19</sup> cross-linking,<sup>20-22</sup> genetic engineering,<sup>23</sup> and polymerization,<sup>24</sup> the oxygen affinity has been reduced to that comparable to intra-erythrocytic hemoglobin, and circulation half-life has been extended.<sup>25-26</sup>

Toxicity, however, still remains an unresolved issue. Of significant concern is the hypertensive effect hemoglobin solutions exhibit in animals, which is thought to be due in part to its affinity for nitric oxide.<sup>14,27,28</sup> Other mechanisms for hemoglobin-mediated vasoconstriction have also been proposed, including contraction medi-

ated by eicosanoids and by the sustained accumulation of intracellular calcium through the activation of phospholipase C and the release of inositol 1,4,5-triphosphate.<sup>16,29-32</sup> Another significant concern is that hemoglobin solutions potentially could invoke inflammatory reactions in recipients. In our earlier work we demonstrated that hemoglobin solutions stimulated isolated human peripheral blood MNLs to release the proinflammatory cytokines IL-8 and TNF- $\alpha$  and that quantities of these cytokines released during the 16-hour incubation were sufficient to induce significant PMN chemotaxis and PMN adherence to HUVECs.<sup>13</sup> Previous studies were carried out in RPMI media in the absence of erythrocytes, PMNs, platelets, and plasma, however, so it is unclear what effects these blood components would have on the release or activity of various cytokines. Erythrocytes have been shown to bind IL-8,<sup>33</sup> and plasma antioxidants such as ascorbic acid may inhibit hemoglobin-mediated stimulation of MNLs by quenching oxidative reactions.<sup>34,35</sup> In the current study we examine the effects of hemoglobin on leukocytes in whole blood and demonstrate that leukocytes incubated with HbAo in the presence of other blood components release IL-6 and biologically active concentrations of IL-8 and TNF- $\alpha$  and that hydrocortisone blocks the release of these cytokines.

## METHODS

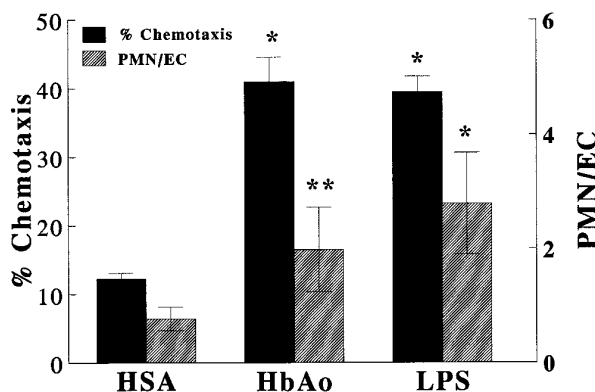
**Reagents.** HbAo was prepared from outdated packed RBCs as described previously.<sup>36</sup> Hemoglobin preparations contained less than 0.06 endotoxin units/mL of endotoxin, as determined by the limulus amoebocyte lysate gel clot test, and no detectable level of organic phosphorous ( $<0.2$   $\mu\text{g/mL}$ ). Furthermore, membrane contamination could not be detected with sodium dodecyl sulfate-polyacrylamide gel electrophoresis. RPMI medium without phenol red, glutamine, antibiotic-antimycotic mixture ( $\times 100$ ), and 1 mmol/L HEPES buffer were purchased from Gibco BRL, Grand Island, NY. Clinical grade HSA (25%) was purchased from Miles Inc, Elkhart, IN. Quantikine ELISA kits for IL-6, IL-8, and TNF- $\alpha$  were purchased from R&D Systems, Minneapolis, MN. Hydrocortisone and LPS from *E. coli* 0111:B4 were purchased from Sigma Chemical Co, St Louis, MO.

**Blood incubation.** Blood was drawn from healthy volunteers into heparin-coated vacuum tubes and maintained on a rocker until used (not longer than 30 minutes). Either 1.6 mL or 0.4 mL of blood was mixed in 12-well plates (Costar) with RPMI 1640 medium containing 25 mmol/L HEPES buffer, 100 U/mL penicillin G, 100  $\mu\text{g/mL}$  streptomycin sulfate, 2.5  $\mu\text{g/mL}$  amphotericin B (incubation medium), and different treatments as indicated in the figure legends to a total volume of 2.0 mL. The final suspensions contained 80% blood by volume for experiments where cytokines were measured and 20% blood for experiments where plasma was used in chemotaxis and PMN/EC adherence measurements. The reason for this difference is that chemotaxis assays could not be per-

formed reliably in supernatant fluids that contained greater than 20% plasma. Larger percentages of plasma caused anomalies with the particle counter used to quantify chemotaxis results (see the section titled "Chemotaxis assays"). Blood mixtures were incubated on a platform shaker (IKA-Schuttler, 300 rpm) for 4 hours at 37°C, 5% CO<sub>2</sub>, and 95% humidity. The plates were then centrifuged at 2000g for 10 minutes, and the supernatant fluids were transferred to a clean multi-well plate and kept frozen at -20°C until they were analyzed for cytokines by ELISA. Supernatant fluids used for chemotaxis and PMN/EC adherence assays were analyzed immediately.

**Chemotaxis assays.** Chemotaxis was assayed as described previously.<sup>13</sup> In brief, the number of PMNs that migrated through 3.0 μm pores in polycarbonate membranes (Transwell cell culture inserts; Costar Inc, Cambridge, MA) was measured. PMNs were isolated from 30 mL of human blood collected into EDTA by layering over 15 mL of Mono-Poly Resolving medium (ICN Biochemicals) and centrifuging at 750g for 50 minutes. Contaminating erythrocytes were removed from the PMN suspension by underlayering with 5 mL of MPRM and centrifuging at 750g for 15 minutes. Supernatant fluids from the blood incubation (300 μL) were mixed with 300 μL of incubation medium that contained 0.25% HSA in 24-well plates. Then 1 × 10<sup>5</sup> PMNs in 100 μL of incubation medium was added to each Transwell, and the Transwells were placed into wells that contained diluted supernatant fluid. The plates were incubated for 40 minutes at 37°C, 5% CO<sub>2</sub>, and 95% humidity. Then the bottom of each Transwell was rinsed with 2 mL of cold Hanks' balanced salt solution without calcium and magnesium that contained 0.25% EDTA into each respective well, and the plates sat at room temperature for 30 minutes to dislodge any adherent PMNs. The number of PMNs that migrated through the Transwell membranes into each well was counted with an Elzone 282 PC particle counter (Particle Data Inc, Elmhurst, IL). Chemotaxis activity is expressed as the percent of the 1 × 10<sup>5</sup> PMNs added initially that migrated through the membrane into the well.

**PMN adherence to ECs.** PMN adherence to HUVECs was assayed as described previously.<sup>37</sup> Second passage HUVECs (Clonetics, San Diego, CA) were grown to confluence in 24-well plates with endothelial growth medium supplied with the cells. The medium was replaced with 250 μL of RPMI 1640 medium containing 25 mmol/L HEPES plus 250 μL of supernatant fluid from the blood incubation, and the plates were incubated for 4 hours at 37°C, 5% CO<sub>2</sub>, and 95% humidity. Supernatant fluids were then removed from each well, and the HUVECs were washed 3 times with HEPES-buffered RPMI 1640 medium. Then 250 μL of RPMI medium containing 1 × 10<sup>6</sup> PMNs was placed into each well and incubated for 20 minutes at 37°C, 5% CO<sub>2</sub>, and 95% humidity. Nonadherent PMNs were removed by washing HUVECs two times with HBSS. Adherent PMNs and HUVECs were dislodged by treatment with 0.25% trypsin/0.01% EDTA (about 5 minutes) and were dispersed into single cells by pipetting up and down several times. The single cells were fixed by the addition of an equal volume of 1% glutaraldehyde, and PMNs and HUVECs were counted simultaneously with the Elzone 282 particle counter



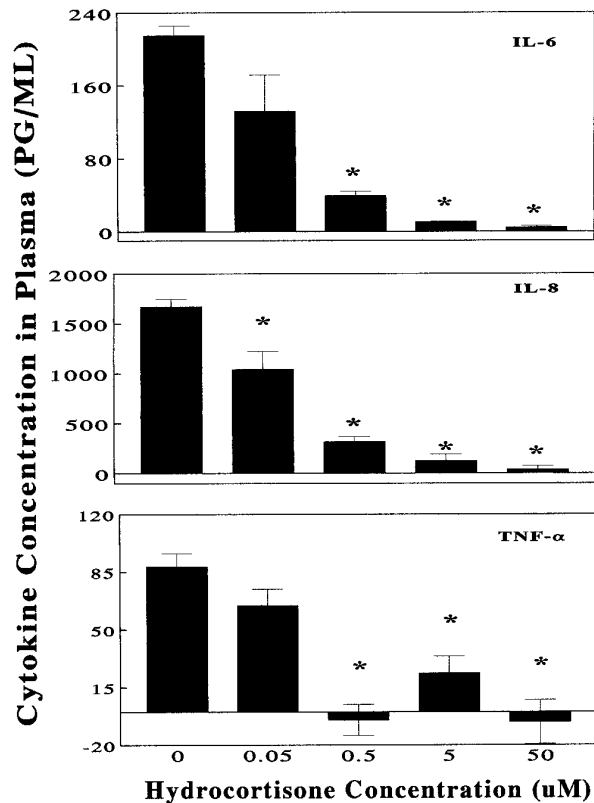
**Fig 2.** Inflammatory activities of blood plasma after exposure to hemoglobin. Heparinized blood was incubated with 1 g/dL of either HSA, HbAo, or LPS (*E. coli*) for 4 hours, then plasma supernatant fluids were assayed for chemotactic and PMN adherence activities as described in Methods. Chemotactic activity is expressed as the percent of PMNs that migrated thru 3.0-μm pores of a cellulose acetate membrane, and PMN adherence activity is expressed as the average number of PMNs attached per endothelial cell (PMN/EC). Values are expressed as mean ± SEM for four and five experiments for chemotaxis and PMN adherence activities, respectively. \**P* < .05, \*\**P* < .13, significance levels for differences from HSA data.

used in the chemotaxis assays. PMNs and HUVECs were delineated by size, where PMNs ranged from 7.02 μm to 11.63 μm and HUVECs ranged from 11.63 μm to 25.66 μm. PMN adherence is expressed as the PMN/EC ratio.

**Statistics.** The data in this study were tested for significance by either the Student *t* test (Figs 1 and 2) or by the Bonferroni test for multiple comparisons against a single control group (Fig 3). The levels of significance are indicated in the figure legends.

## RESULTS

Leukocytes from whole blood released significantly higher amounts of IL-6, IL-8, and TNF-α in the presence of HbAo than in the presence of HSA (Fig 1). After 4 hours of incubation with HbAo, leukocytes in blood released 187, 1313, and 50 pg/mL of IL-6, IL-8, and TNF-α, respectively, as compared with 6, 192, and 2 pg/mL released by leukocytes in blood incubated with HSA. Furthermore, these cytokine concentrations in plasma were high enough to elicit PMN inflammatory responses in vitro (Fig 2). Supernatant fluid from blood incubated with HbAo elicited a chemotactic response in PMNs through 3-μm pores of polycarbonate membranes that was three times greater than the chemotactic response elicited by supernatant fluid from blood incubated with HSA. Also, supernatant fluid from blood incubated with HbAo stimulated HUVECs to adhere two-and-a-half times more PMNs than did supernatant fluid from blood treated with HSA. Both activities were comparable to that of supernatant fluid from LPS-treated blood.



**Fig 3.** Hydrocortisone inhibition of cytokine release from leukocytes in blood exposed to hemoglobin. Heparinized blood was incubated with 1 g/dL of HbAo for 4 hours in the presence of varying concentrations of hydrocortisone as described in Methods. Cytokine levels in plasma supernatant fluids were quantified by ELISA. Values are expressed as mean  $\pm$  SEM for three experiments for inhibition of IL-8 and two experiments for inhibition of IL-6 and TNF- $\alpha$ . \* $P < .05$ , significance level for differences in cytokine release in the presence of hydrocortisone from cytokine release in the absence of hydrocortisone.

Fig 3 shows that hydrocortisone effectively inhibited the release of IL-6, IL-8, and TNF- $\alpha$  from leukocytes in blood treated with HbAo. Inhibition was dose dependent, and as little as 0.05  $\mu\text{mol/L}$  inhibited IL-8 release, while 0.5  $\mu\text{mol/L}$  was required to inhibit the release of IL-6 and TNF- $\alpha$ . Complete inhibition of the release of IL-6 and IL-8 required 50  $\mu\text{mol/L}$  hydrocortisone, but 5  $\mu\text{mol/L}$  hydrocortisone inhibited the release of these cytokines by 96% and 97%, respectively. The hydrocortisone  $\text{ED}_{50}$  of the release of each cytokine was estimated by fitting the data to an exponential equation of the type  $y = a_0 e^{a_1 x}$ . From this relationship,  $\text{ED}_{50}$  values were calculated to be 0.23  $\mu\text{mol/L}$ , 0.19  $\mu\text{mol/L}$ , and 0.10  $\mu\text{mol/L}$  for inhibition of the release of IL-6, IL-8, and TNF- $\alpha$ , respectively.

## DISCUSSION

We showed that blood incubated with HbAo for 4

hours released IL-6, IL-8, and TNF- $\alpha$ . Enough IL-8 and TNF- $\alpha$  were released to mediate PMN chemotaxis and PMN adherence to HUVECs in vitro. Earlier work in our laboratory demonstrated the release of IL-8 and TNF- $\alpha$  from isolated MNLs incubated with hemoglobin solutions. The time of earliest detection was also within 4 hours for the release of IL-8 but was not until 10 hours for TNF- $\alpha$ .<sup>13</sup> Furthermore, chemotactic and PMN adherence activities of supernatant fluids from MNLs incubated with HbAo were not detectable until 8 and 10 hours, respectively. The kinetic differences in the release of TNF- $\alpha$  and in the appearance of chemotactic and PMN adherence activities between isolated MNLs and blood may be ascribed to the presence in diluted blood suspensions of either plasma constituents that support leukocyte function or to the presence of PMNs and platelets in addition to monocytes and lymphocytes. The presence of PMNs and platelets may facilitate the activation of monocytes by hemoglobin through intercellular communication pathways or may themselves be activated by HbAo either directly or through some metabolite of activated MNLs to release additional levels of these cytokines. One form of intercellular communication recognized recently is through transcellular metabolism of eicosinoids, where one cell type releases an eicosinoid precursor and another cell type uses it to synthesize a new eicosinoid metabolite. Transcellular metabolism has been observed between neutrophils and platelets<sup>38</sup> and between ECs and platelets.<sup>39</sup> Moreover, eicosinoids released from activated platelets enhance monocyte procoagulant activity.<sup>40</sup> It is conceivable that hemoglobin causes platelets to release factors that enhance the release of cytokines by monocytes.

In addition to transcellular metabolism of eicosinoids, physical contact between cells also regulates activities of inflammatory cells. Contact between monocytes and ECs increases the production of IL-8 and monocyte chemoattractant peptide-1 by ECs,<sup>41</sup> while contact between monocytes and fibroblasts increases production of macrophage inflammatory protein-1 $\alpha$  from monocytes.<sup>42</sup> In contrast, the presence of RBCs inhibits mobilization of the complement receptor for C3b (CR1) by granulocytes that occurs spontaneously at 37°C.<sup>43</sup> Thus, interactions among the different cell types in blood may moderate their individual responses to hemoglobin.

RBCs have been shown to bind IL-8 and thereby decrease the level of circulating IL-8.<sup>33</sup> For that reason, the concentrations of IL-8 in the plasma from diluted blood that we report are probably less than the actual amount of IL-8 secreted by the leukocytes in blood. We measured  $1313 \pm 246$  pg IL-8 (mean  $\pm$  SEM,  $n = 12$ ) per milliliter of supernatant fluid after incubation of

diluted blood with HbAo for 4 hours. The average number of RBCs in human blood is  $5 \times 10^9/\text{mL}$ .<sup>44</sup> Blood used in this study was diluted to 80% by volume and therefore contained about  $4 \times 10^9$  RBC/mL. When using the constants reported by Darbonne et al<sup>33</sup> for IL-8 binding to receptors on RBCs ( $K_d=5\text{nmol/L}$  and 2000 binding sites/RBC), RBCs in our samples were capable of binding an additional 3389 pg of IL-8 per milliliter. Thus the total amount of IL-8 secreted by leukocytes in our blood suspensions (that bound to RBCs plus that free in plasma) could have been as high as 4702 pg/mL.

Our results demonstrate clearly that leukocytes suspended in blood respond similarly to incubation with HbAo, as do isolated MNLs suspended in RPMI, with respect to the release of proinflammatory cytokines. In fact, TNF- $\alpha$  release was detected in the blood/hemoglobin supernatant fluid 6 hours earlier than in RPMI suspensions of MNLs.<sup>13</sup> Furthermore, IL-6 was released within 4 hours. Thus the presence of additional cell types and plasma components does not impede the mechanism by which HbAo stimulates monocytes to release cytokines.

These results are consistent with the possibility that an inflammatory reaction could develop on infusion of hemoglobin solution because of the release of these and possibly other proinflammatory cytokines. Also consistent with this possibility is the fact that such a small amount of HbAo evoked a measurable inflammatory response in blood that contained antioxidants and a number of other constituents that potentially could prevent such a response. Autoxidation of hemoglobin, which occurs during incubation at 37°C, produces the reactive oxygen species superoxide and hydrogen peroxide, and reactive oxygen species have been implicated in the release of IL-8 from blood treated with LPS.<sup>34,35</sup> Furthermore, the work of White et al<sup>11</sup> and Smith and Winslow<sup>12</sup> demonstrated that the infusion of hemoglobin into rabbits caused disseminated intravascular coagulation that resulted from stimulation of monocyte procoagulant activity. Their work demonstrates that hemoglobin solutions can invoke inflammatory reactions in vivo.

Because cytokines function as signals among immune cells, stimulation of monocytes by hemoglobin may establish an inflammatory cycle of cell activation, cytokine release, and further cell activation. Hemoglobin stimulates monocytes to release IL-6 and IL-8, which in turn activate platelets to express P-selectin,<sup>45</sup> and through engagement of P-selectin with monocytes, monocytes are activated to release more IL-8, and additionally, MCP-1.<sup>46</sup> Furthermore, activated platelets release a host of additional chemokines including platelet factor 4,  $\beta$ -thromboglobulin, neutrophil-

activating peptide-2 variants, and RANTES.<sup>47,48</sup> Such an inflammatory cycle could produce systemic margination, activation, and transendothelial migration of neutrophils, which can lead to systemic inflammatory pathologies such as adult respiratory distress syndrome and multiple organ dysfunction.<sup>49</sup>

Inflammatory reactions such as these have been observed in patients in a recent European trauma trial of diaspirin- $\alpha\alpha$ -cross-linked hemoglobin manufactured by Baxter Healthcare.<sup>50</sup> The trial was terminated prematurely concurrent with the premature termination of their phase III trial in the United States. The phase III trial was terminated because there was increased mortality in the treatment arm: 24 out of 52 treated patients compared with 8 out of 46 control subjects (no hemoglobin). The results of the European trial showed that after treatment of more than 400 patients with hemoglobin and an equal number of control subjects, the treated patients exhibited higher frequencies of respiratory distress syndrome, systemic inflammatory response syndrome, multiple organ dysfunction, and pancreatitis than did control patients. In aggregate the incidence of these disorders was 8% in the treated patients and 3% in the control subjects. Each of these dysfunctions is related to inflammatory reactions and is consistent with the release of IL-6, IL-8, and TNF- $\alpha$ .

Numerous studies have documented the increased risk of postoperative infections and earlier recurrences of solid tumors associated with transfusions of allogeneic blood related to immunosuppression and depression of cytokine production.<sup>51-55</sup> Although it is tempting to speculate that the immunostimulatory effects of hemoglobin that we report could benefit patients over transfusion with allogeneic red cells, the toxicity observed in the phase III clinical trials of the Baxter Healthcare product argues strongly against that. Regulation of immune cells depends on a finely tuned balance of proinflammatory and anti-inflammatory cytokines that work in concert to confer immunity. Under healthy conditions the release of these mediators is under tight regulation. Transfusion of hemoglobin likely causes the uncontrolled release of at least three proinflammatory cytokines systemically that will upset that balance. Results from recent clinical trials of diaspirin- $\alpha\alpha$ -cross-linked hemoglobin support this conclusion and demonstrate that toxicities associated with this hemoglobin formulation outweigh the proposed benefits.

In view of the recent demonstration of inflammation-related dysfunctions in patients receiving hemoglobin, our observation that hydrocortisone blocks the release of IL-6, IL-8, and TNF- $\alpha$  from leukocytes in blood incubated with HbAo is significant. Hydrocortisone, a glucocorticoid with anti-inflammatory properties that

is used therapeutically for a number of allergic conditions,<sup>56</sup> and dexamethasone have been reported to inhibit transcription of mRNA for the cytokines IL-1, IL-4, IL-5, IL-6, IL-8, TNF- $\alpha$ , monocyte-neutrophil chemotactic factor, and granulocyte-macrophage colony-stimulating factor from monocytes, macrophages, eosinophils, ECs, pancreatic beta cell lines, and keratinocytes.<sup>56-65</sup> Effective concentrations ranged from  $10^{-8}$  mol/L to  $10^{-2}$  mol/L, which is consistent with the concentrations used in our study. Previous studies have demonstrated the inhibitory effects of these glucocorticoids on the release of cytokines by cells stimulated with either LPS or a cytokine, usually IL-1, but our results constitute the first report demonstrating that hydrocortisone inhibits the release of cytokines from leukocytes stimulated by hemoglobin. The results suggest that the addition of anti-inflammatory compounds to hemoglobin solutions might prevent any undesirable inflammatory effects after infusion.

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