The Clinical Sequelae of Intravascular Hemolysis and Extracellular Plasma Hemoglobin
A Novel Mechanism of Human Disease

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Context
The efficient sequestration of hemoglobin by the red blood cell membrane and the presence of multiple hemoglobin clearance mechanisms suggest a critical need to prevent the buildup of this molecule in the plasma. A growing list of clinical manifestations attributed to hemoglobin release in a variety of acquired and iatrogenic hemolytic disorders suggests that hemolysis and hemoglobinemia should be considered as a novel mechanism of human disease.

Evidence Acquisition
Pertinent scientific literature databases and references were searched through October 2004 using terms that encompassed various aspects of hemolysis, hemoglobin preparations, clinical symptoms associated with plasma hemoglobin, nitric oxide in hemolysis, anemia, pulmonary hypertension, paroxysmal nocturnal hemoglobinuria, and sickle-cell disease.

Evidence Synthesis
Hemoglobin is released into the plasma from the erythrocyte during intravascular hemolysis in hereditary, acquired, and iatrogenic hemolytic conditions. When the capacity of protective hemoglobin-scavenging mechanisms has been saturated, levels of cell-free hemoglobin increase in the plasma, resulting in the consumption of nitric oxide and clinical sequelae. Nitric oxide plays a major role in vascular homeostasis and has been shown to be a critical regulator of basal and stress-mediated smooth muscle relaxation and vasomotor tone, endothelial adhesion molecule expression, and platelet activation and aggregation. Thus, clinical consequences of excessive cell-free plasma hemoglobin levels during intravascular hemolysis or the administration of hemoglobin preparations include dystonias involving the gastrointestinal, cardiovascular, pulmonary, and urogenital systems, as well as clotting disorders. Many of the clinical sequelae of intravascular hemolysis in a prototypic hemolytic disease, paroxysmal nocturnal hemoglobinuria, are readily explained by hemoglobin-mediated nitric oxide scavenging.

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A growing body of evidence supports the existence of a novel mechanism of human disease, namely, hemolysis-associated smooth muscle dystonia, vasculopathy, and endothelial dysfunction.

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H EMOGLOBIN IS A HIGHLY CONSERVED MOLECULE FOUND IN SPECIES RANGING FROM SINGLE-CELL ORGANISMS TO MAMMALS, BUT THE ROLE OF HEMOGLOBIN IN DIFFERENT ORGANISMS VARIES. WHILE HEMOGLOBIN IN BACTERIA FUNCTIONS AS A NITRIC OXIDE SUMP BY OXIDATION OF NITRIC OXIDE TO NITRATE, HEMOGLOBIN FUNCTIONS TO REMOVE OXYGEN IN NEMATODES, A CRITICAL TASK FOR ANAEROBES. BY CONTRAST, IN MAMMALS, HEMOGLOBIN PRIMARILY SERVES A RESPIRATORY FUNCTION IN THE DELIVERY OF OXYGEN AND REMOVAL OF CARBON DIOXIDE. BASED ON THE RECENT DISCOVERY THAT NITRIC OXIDE IS A CRITICAL REGULATOR OF VASODILATION AND VASCULAR HOMEOSTASIS, THE INTERACTIONS OF NITRIC OXIDE WITH HEMOGLOBIN IN MAMMALS HAS DRAWN INCREASING INTEREST. BECAUSE THE REACTION OF NITRIC OXIDE WITH THE VAST AMOUNTS OF INTRAVASCULAR OXYHEMOglobin (16 g/dL) IS FAST (107 M−1s−1) AND IRREVERSIBLE, IT WOULD BE EXPECTED THAT NITRIC OXIDE PRODUCED BY ENDOTHELIUM IS LIMITED BY COMPARTMENTALIZATION OF HEMOGLOBIN INSIDE THE ERYTHROCYTE.7,9 THE EVOLUTION OF THE ERYTHROCYTE MAY BE CONSIDERED AS A MECHANISM OF REDUCING TOXICITY WHILE"

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ensuring separation of the critical respiratory transport machinery needed for efficient oxygen delivery from the endothelium. Moreover, multiple systems have evolved to control the level of cell-free hemoglobin in the plasma during normal physiological hemolysis, presumably to curtail the deleterious effects of plasma hemoglobin on nitric oxide bioavailability and endothelial function.

During intravascular hemolysis, cell-free plasma hemoglobin may overwhelm homeostatic systems in place to remove it. Hemolytic conditions with substantial intravascular hemolysis include paroxysmal nocturnal hemoglobinuria (PNH), sickle-cell disease (SCD), thalassemias, hereditary spherocytosis and stomatocytosis, microangiopathic hemolytic anemias, pyruvate kinase deficiency, ABO mismatch transfusion reaction, paroxysmal cold hemoglobinuria, severe idiopathic autoimmune hemolytic anemia, infection-induced anemia, malaria, cardiopulmonary bypass, mechanical heart valve-induced anemia, and chemical-induced anemias. Although the various hemolytic diseases each have unique symptoms, they often share hemoglobinemia-related sequelae. In addition, observations from the clinical administration of artificial, purified, and recombinant hemoglobin solutions have provided further support for the causal relationship between excess cell-free hemoglobin in the bloodstream, symptoms, and cardiovascular events.

Nitric oxide scavenging by excess plasma hemoglobin has been implicated in various clinical manifestations of intravascular hemolysis. Nitric oxide is a regulator of smooth muscle tone and platelet activation, and reductions in nitric oxide plasma levels lead to smooth muscle dystonias, including hypertension, gastrointestinal contractions, and erectile dysfunction, as well as clot formation. Hemoglobin also exerts direct cytotoxic, inflammatory, and pro-oxidant effects that adversely affect endothelial function.

EVIDENCE ACQUISITION
Multiple searches were performed with the New England Research Application Center and PubMed through October 2004, including various combinations of the following key search terms: hemoglobin, recombinant, cell-free, stroma-free, artificial, heme, hemolysis, marker, lactate dehydrogenase, LDH, anemia, pulmonary hypertension, abdominal, abdomen, pain, erectile dysfunction, dysphagia, smooth muscle, nitric oxide, paroxysmal nocturnal hemoglobinuria, and sickle-cell disease. References cited in textbooks or articles were also used in some cases. Plasma hemoglobin concentrations are reported throughout this review in terms of hemoglobin tetramer. Because each tetramer contains 4 heme groups, hemoglobin can react with and inactivate 4 nitric oxide molecules.

EVIDENCE SYNTHESIS
The Removal of Hemoglobin During Intravascular Hemolysis
When red blood cells (RBCs) are destroyed within the vascular compartment, hemoglobin escapes into the plasma, dimerizes, and is rapidly bound by the serum protein haptoglobin. The haptoglobin-hemoglobin complex exposes a neoepitope that is recognized by the hemoglobin scavenger receptor, CD163 on the surface of monocytes/macrophages, which binds the complex with high affinity and mediates haptoglobin-hemoglobin endocytosis and degradation. Since haptoglobin is not recycled, formation of large amounts of haptoglobin-hemoglobin complexes leads to rapid haptoglobin depletion. Thus, in severe hemolytic diseases such as PNH and SCD, serum haptoglobin is typically undetectable.

Ferrous heme (Fe(II)), the oxygen-binding component of hemoglobin, can be oxidized to ferric heme (Fe(III)), which is then released from hemoglobin and binds with high affinity to a plasma glycoprotein, haptoglobin. Heme bound to haptoglobin is degraded in a series of enzymatic steps in the liver. Heme oxygenase 1 (HO-1) subsequently breaks down the pro-oxidant and pro-inflammatory heme into carbon monoxide, biliverdin, and iron. Carbon monoxide has vasodilatory, antiproliferative, anti-inflammatory, and antioxidant properties, while biliverdin is an antioxidant that is converted by biliverdin reductase to bilirubin. Biliverdin reductase itself has catalytic antioxidant properties. The heme-derived oxidant iron is directly sequestered and inactivated by ferritin. Additionally, haptoglobin-hemoglobin binding to CD163 signals anti-inflammatory IL-10 and HO-1 induction in circulating monocytes. Thus, the antioxidant, anticoagulant, antiproliferative, and vasodilating effects of the CD163/HO-1/biliverdin reductase systems likely represent an evolved compensation for the nitric oxide scavenging, vasoconstrictive, proliferative, inflammatory, and pro-oxidant effects of extracellular hemoglobin, heme, and heme-iron.

When the capacity of these scavenging mechanisms has been saturated during acute or chronic hemolysis, levels of hemoglobin and heme increase in the plasma and urine. Plasma hemoglobin has the ability to scavenge nitric oxide while heme possesses multiple proinflammatory and pro-oxidant properties.

Hemolysis Causes Local and Systemic Nitric Oxide Deficiency Through the Release of Hemoglobin in Plasma
Nitric oxide reacts with hemoglobin in an extremely fast and irreversible reaction (10^7 M^-1 s^-1) that produces an inactivating product nitrate (NO_3^-) and methemoglobin. The speed and irreversibility of this reaction is such that very little hemoglobin can completely inhibit endothelial nitric oxide and produce endothelial dysfunction. For example, 0.01 g/dL of hemoglobin is sufficient to completely inhibit aortic ring dilation on exposure to acetylcholine. Under normal physiological conditions, the reaction rate of nitric oxide and hemoglobin is severely limited by approximately 600-fold due to multiple diffusional barriers to nitric oxide around the RBC membrane and along
the endothelium in laminar flowing blood.3,5 A tting to this model, vascular homeostasis is dependent on the compartmentalization or physical separation of hemoglobin from endothelium.5 During intravascular hemolysis, this separation and the diffusional barriers are disrupted, resulting in efficient nitric oxide scavenging and endothelial dysfunction.39,40

Haptoglobin can bind approximately 0.07 to 0.15 g/dL of hemoglobin depending on the haptoglobin al lotype.41 Once the capacity of this hemoglobin-scavenging protein is exceeded, consumption of endogenous nitric oxide intensifies. Plasma hemoglobin levels in patients with PNH are commonly in the range of 0.05 to 0.2 g/dL and can exceed 1.0 g/dL during severe hemolytic episodes.42 Similarly, plasma hemoglobin levels range from 0.001 to 0.033 g/dL in SCD and can exceed 1.0 g/dL during severe episodes.43,44 Because the haptoglobin/hemoglobin complex is degraded in the liver, the occurrence of steady state intravascular hemolysis in diseases such as PNH and SCD typically generates sufficient plasma hemoglobin to completely deplete haptoglobin. It has been shown that quantities of plasma hemoglobin greater than 0.01 g/dL can potently inhibit nitric oxide–dependent vasodilation in vivo.39,44

In vitro consumption of nitric oxide in patients with SCD is highly correlated with measured plasma hemoglobin levels (R = 0.92), and immunodepletion of hemoglobin from plasma eliminates the ability of the plasma to consume nitric oxide.39 Consistent with an in vivo effect of elevated circulating plasma hemoglobin on endothelial function, patients with SCD exhibit blunted vasodilatory responses to infusions of the direct-acting nitric oxide donor sodium nitroprusside.39 In fact, patients with plasma hemoglobin levels higher than 0.01 g/dL have an 80% reduction in nitric oxide–dependent blood flow responses. Moreover, nitroglycerin-induced vasodilation is impaired in SCD patients,45 and diminished vasomotor response to nitric oxide donors is observed in transgenic sickle-cell mice.46,47 These mice also exhibit systemic resistance to nitric oxide donors, which correlates with hemolytic rate and plasma hemoglobin levels.48

While there is clearly a state of nitric oxide–mediated resistance in SCD patients and transgenic sickle-cell mouse models, overall blood flow and cardiac output are high in the face of the associated anemia. This increase in cardiac output in response to anemia appears to be mediated by up-regulation of non–nitric oxide vasodilators such as prostacyclin and endothelium-derived hyperpolarizing factor. This is illustrated in SCD patients by normal or high basal blood flow responses to acetylcholine, an endothelium-dependent vasodilator, which is not affected by simultaneous nitric oxide synthase inhibition.39,48 The hypothesis of a compensatory increase in non–nitric oxide vasodilators was recently confirmed by Kaul and colleagues who demonstrated that transgenic mice expressing exclusively human hemoglobin S exhibit complete resistance to nitric oxide–mediated vasodilation accompanied by an increase in cyclooxygenase 2 (COX-2) levels and non–nitric oxide–dependent blood flow.48

In addition to hemoglobin decompartmentalization and nitric oxide scavenging, hemolysis also releases erythrocyte arginase, an enzyme that converts L-arginine, the substrate for nitric oxide synthesis, to ornithine, thereby further reducing the systemic availability of nitric oxide.50-52 Consistent with this observation, the arginine-to-ornithine ratio decreases significantly as pulmonary pressures increase in SCD patients.22

The biological effects of nitric oxide and its removal during intravascular hemolysis are depicted in the Figure. We propose that the release of hemoglobin during intravascular hemolysis results in excessive consumption of nitric oxide, subsequent reduction in guanylate cyclase activity, smooth muscle contraction, vasoconstriction, and platelet activation/aggregation. The systemic removal of nitric oxide has been shown to contribute to clinical morbidities, including severe esophageal spasm and dysphagia, abdominal pain, erectile dysfunction, and thrombosis.16,17,23-26 In addition, systemic release of hemoglobin is associated with pulmonary and systemic hypertension.17,20,53-57 Decreased organ perfusion, and increased mortality.53-58 Plasma hemoglobin and its breakdown product heme can also directly activate endothelial cells and further promote inflammation and coagulation.27

Plasma Hemoglobin Causes Dose-Dependent Increases in Adverse Clinical Signs and Symptoms
Stroma-free hemoglobin, cross-linked human or bovine hemoglobin, purified human hemoglobin, or recombinant human hemoglobin preparations have been administered to patients, human volunteers, and animals. As summarized in the Table, intravascular plasma hemoglobin is associated with a dose-dependent increase in adverse clinical signs and symptoms, including hemoglobinuria, abdominal pain, splanchnic pain, esophageal spasm, and dysphagia, as well as increases in blood pressure, platelet activation, creatine kinase level, and mortality.

Hemoglobinuria and Renal Dysfunction. Hemoglobinuria is one of the most prominent clinical signs of excessive intravascular hemolysis and is commonly associated with the administration of hemoglobin solutions.11,12 Plasma hemoglobin is normally filtered through the glomerulus and actively reabsorbed in proximal tubule cells where it is catabolized with release of iron in the form of hemosiderin. When the kidney’s reabsorption capacity is exceeded, clinically significant hemoglobinuria occurs. Acute renal failure may occur during severe episodes of hemoglobinuria.29 Persistent severe hemoglobinuria is also associated with substantial proximal tubule hemosiderin deposition, Fanconi syndrome (defec tive renal reabsorption of small molecules leading to hyperaminoaciduria, glycosuria, hyperphosphaturia, and...
During intravascular hemolysis, hemoglobin is released into the plasma where it is normally cleared by the hemoglobin scavengers haptoglobin, CD163, and hemopexin. Haptoglobin-hemoglobin complexes bind to CD163 on the surface of macrophages/monocytes initiating endocytosis and degradation of the complex. Hemoglobin also releases ferric heme on oxidation, which is bound by hemopexin and degraded by hepatocytes in the liver. Excessive hemolysis saturates and depletes these hemoglobin removal systems and leads to a buildup of hemoglobin and heme in the plasma. Plasma hemoglobin and heme mediate direct proinflammatory, proliferative, and pro-oxidant effects on vessel endothelial cells. NO is normally generated from L-arginine in vessel endothelial cells by the enzyme nitric oxide synthase (NOS). NO maintains smooth muscle relaxation and inhibits platelet activation and aggregation, thereby regulating vessel tone and promoting organsystem homeostasis. During intravascular hemolysis, NO availability can be severely limited by its reaction with oxyhemoglobin (NO scavenging) and by the breakdown of the substrate for NO synthesis, L-arginine, by the red cell enzyme arginase, despite elevated levels of NOS (decreased NO synthesis). NO depletion results in decreased activation of guanylate cyclase, an enzyme required for the generation of cyclic guanine monophosphate (cGMP). Decreased cGMP levels disrupt regulation of smooth muscle tone resulting in dystonias, including systemic and pulmonary hypertension, erectile dysfunction, dysphagia, and abdominal pain. Decreased cGMP levels through the depletion of NO can also lead to platelet activation and aggregation, promoting clot formation. GTP indicates guanosine 5’-triphosphate.
bicarbonate and water loss), and chronic renal failure.\textsuperscript{79,80}

**Gastrointestinal Dystonias and Pain.** Administration of hemoglobin preparations to healthy human volunteers is associated with dose-dependent gastrointestinal symptoms, including abdominal pain, esophageal spasms, and dysphagia.\textsuperscript{11,12,14,16,17} Increasing doses of recombinant hemoglobin result in increases in the duration of esophageal contractions.\textsuperscript{16} Further, acute episodes of intravascular hemolysis in patients undergoing long-term dialysis with plasma hemoglobin levels ranging from approximately 0.3 to 2.1 g/dL have also been associated with abdominal pain.\textsuperscript{61}

Hemoglobin-induced esophageal spasms are most likely attributable to nitric oxide consumption, as inhibition of this molecule in healthy human volunteers results in an increase in esophageal peristaltic amplitude and velocity (spasms) and a decrease in gastric distention-triggered transient lower esophageal sphincter relaxation.\textsuperscript{62} Consistent with this hypothesis, augmentation of the downstream effect of nitric oxide via inhibition of phosphodiesterase type 5 (PDE5) with sildenafil relieves spasms in patients with esophageal motor disorders.\textsuperscript{63,64}

**Vasoconstriction and Systemic and Pulmonary Hypertension.** The administration of cell-free hemoglobin solutions to healthy volunteers and patients is commonly associated with a dose-dependent increase in systolic and diastolic blood pressure.\textsuperscript{11,15,17,18} which is reversed by the administration of the nitric oxide donor, sodium nitroprusside, confirming the importance of nitric oxide scavenging in vasoregulation.\textsuperscript{19} Plasma hemoglobin and erythrocytes also augment hypoxic pulmonary vasoconstriction by scavenging nitric oxide, with plasma hemoglobin demonstrating an approximate 1000-fold greater nitric oxide scavenging potency in these models.\textsuperscript{20}

Pulmonary arterial hypertension is an increasingly recognized complication of chronic hereditary and acquired hemolytic anemias, including SCD,\textsuperscript{22,31,63-69} thalassemia intermedia and major,\textsuperscript{70-76} PNH,\textsuperscript{77,78} hereditary spherocytosis and stomatocytosis,\textsuperscript{79-84} microangiopathic hemolytic anemias,\textsuperscript{85-91} and pyruvate ki-

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**Table. Clinical Signs and Symptoms Associated With the Administration of Hemoglobin Solutions**

<table>
<thead>
<tr>
<th>Source</th>
<th>Model</th>
<th>Hemoglobin Level</th>
<th>Estimated Blood Hemoglobin Level*</th>
<th>Clinical Signs and Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Przybelski et al, 1996</td>
<td>24 Healthy adult volunteers</td>
<td>0.05</td>
<td>7.8</td>
<td>Abdominal pain and sternal pain; increased CK level; increased BP with high dose</td>
</tr>
<tr>
<td>Savitsky et al, 1978</td>
<td>10 Healthy adult men</td>
<td>0.23</td>
<td>35.9</td>
<td>Hemoglobinuris; abdominal pain; increased BP; creatinine clearance reduction</td>
</tr>
<tr>
<td>Sloan et al, 1999</td>
<td>112 Patients with traumatic hemorrhagic shock</td>
<td>0.71-1.42</td>
<td>110.9-221.8</td>
<td>Increased mortality</td>
</tr>
<tr>
<td>Carmichael et al, 2000</td>
<td>42 Healthy adult male volunteers</td>
<td>0.02-0.60</td>
<td>3.7-93.7</td>
<td>Hemoglobinuris moderate &gt;0.10 g/dL; increased BP over 0.05 g/dL with increased duration over 0.10 g/dL; abdominal pain and dysphagia: &lt;0.40 g/dL (12/32 mild); &gt;0.40 g/dL (10/10 moderate-severe); dysphagia relieved by smooth muscle relaxants</td>
</tr>
<tr>
<td>Murray et al, 1995</td>
<td>9 Volunteers</td>
<td>0.11-0.15</td>
<td>17.1-23.4</td>
<td>Dose-dependent increase in duration of esophageal contractions; dysphagia and CP: 0.15 g/dL (4/6 participants)</td>
</tr>
<tr>
<td>Larney et al, 2000</td>
<td>209 Postcardiac bypass patients</td>
<td>0.36</td>
<td>56.2</td>
<td>Hemoglobinuris; significant increase in BP; SVR, PVR after 0.36 g/dL; increased pancreatic enzyme levels; jaundice</td>
</tr>
<tr>
<td>Viele et al, 1997</td>
<td>48 Healthy adult male volunteers</td>
<td>0.02-0.32</td>
<td>2.3-50.0</td>
<td>Hemoglobinuris; dose-dependent gastrointestinal symptoms including abdominal pain and dysphagia: 0.02-0.11 g/dL (6/16 participants), 0.15-0.22 g/dL (11/12 participants), 0.25-0.32 g/dL (6/6 participants); prophylactic smooth muscle relaxants required in 2 highest dose groups; increased amylase/lipase levels; transient modest increase in BP and heart rate</td>
</tr>
<tr>
<td>Lamuraglia et al, 2000</td>
<td>72 Vascular surgery patients</td>
<td>0.86</td>
<td>134.3</td>
<td>Significant increases in BP and BUN; trends for increases in creatinine and amylase levels</td>
</tr>
<tr>
<td>Saxena et al, 1999</td>
<td>85 Stroke patients</td>
<td>0.03</td>
<td>3.9</td>
<td>Hemoglobinuris; jaundice at 0.10 g/dL; dose-dependent increase in BP duration</td>
</tr>
<tr>
<td>Olsen et al, 1996</td>
<td>66 Rats CEA model</td>
<td>0.02</td>
<td>96.8</td>
<td>71% Increased platelet activation; 26% increase in BP</td>
</tr>
</tbody>
</table>

Abbreviations: BP, blood pressure; BUN, blood urea nitrogen; CEA, carotid endarterectomy; CK, creatine kinase; CP, chest pain; PVR, pulmonary vascular resistance; SVR, systemic vascular resistance.

*All blood hemoglobin values are estimates except for those of Przybelski et al,\textsuperscript{17} which reflect actual plasma values; calculations assume a weight of 70 kg and a blood volume of 7 L.

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SEQUELAE OF INTRAVASCULAR HEMOLYSIS AND EXTRACELLULAR PLASMA HEMOGLOBIN

nase deficiency. There are a number of pathophysiological features shared by these disparate disorders, including intravascular hemolysis, iron overload, a propensity toward thrombosis, and surgical or autosplenectomy.

In a recent study of 195 adult patients with SCD, 32% had mild to severe pulmonary hypertension. Markers of hemolysis, including anemia, bilirubin, lactate dehydrogenase (LDH), and aspartate aminotransferase, were associated with pulmonary hypertension. In addition to limiting nitric oxide bioavailability via hemoglobin-based nitric oxide scavenging and dysregulated arginine metabolism, hemolysis is associated with activation of downstream adhesion, prothrombotic, and pro-oxidant pathways that may further contribute to endothelial dysfunction and vasculopathy. Other mechanisms may also contribute to the development of pulmonary hypertension, including chronic thromboembolism and in situ thrombosis, asplenia, pulmonary fibrosis, liver cirrhosis secondary to iron overload and hepatitis C, and induction of hypoxia-inducible factor 1α–dependent factors such as vascular endothelial growth factor, endothelin 1, and erythropoietin.

Thrombosis and Platelet Activation. Excessive plasma hemoglobin may contribute to platelet activation and thrombosis. The infusion of cross-linked hemoglobin increases platelet aggregation and adhesion in vivo on prothrombotic surfaces such as an injured vessel wall. Additionally, administration of heme in healthy volunteers is associated with thrombopilebitis, demonstrating that heme can cause vascular inflammation followed by vascular obstruction in vivo. Interestingly, the addition of cell-free hemoglobin to human serum at concentrations of 0.2 to 2.0 g/dL causes a dose-dependent inhibition of the metalloprotease ADAMTS13, an enzyme critical in limiting platelet thrombus formation. The major untoward effects of plasma hemoglobin on platelet function are most likely mediated by the scavenging of nitric oxide. Nitric oxide has been shown to inhibit platelet aggregation, induce disaggregation of aggregated platelets, and inhibit platelet adhesion through increasing cyclic guanine monophosphate (cGMP) levels. In fact, nitric oxide donor drugs (S-nitrosothiols) that increase systemic levels of nitric oxide have been shown to inhibit platelet aggregation. Conversely, nitric oxide scavenging by hemoglobin or the reduction of nitric oxide generation by the inhibition of arginine metabolism results in an increase in platelet aggregation.

Nitric oxide interacts with components of the coagulation cascade to down-regulate clot formation. For example, nitric oxide has been shown to chemically modify and inhibit factor XIII, which suggests that nitric oxide deficiency would enhance clot stability and reduce clot dissolution. In animal models, reduction of nitric oxide causes increases in fibrin split products and thrombin-antithrombin complexes leading to significant fibrin deposition and thrombus formation. Moreover, in a patient with l-arginine deficiency, reduced nitric oxide production is associated with increased thrombin-antithrombin complexes and fibrin split products, while reversal of nitric oxide deficiency with l-arginine causes a reduction in intravascular coagulopathy.

Erectile Dysfunction. Hemoglobin release during intravascular hemolysis has been implicated in the pathogenesis of erectile dysfunction in patients with PNH, presumably through the scavenging of nitric oxide. It has been well established that local nitric oxide deficiency due to decreased synthesis, impaired release, or premature destruction is one of the factors responsible for erectile dysfunction. The capacity of PDE5 inhibitors such as sildenafil to improve erectile dysfunction via the accumulation of cGMP is dependent on the availability of nitric oxide.

Inflammation and Oxidation. Plasma hemoglobin and heme may possess inflammatory properties. The presence of large amounts of vascular heme results in inflammatory infiltrates in various organs in mice and induction of neutrophil activation and migration in vitro. Heme stimulates the expression of the adhesion molecules ICAM-1 (intracellular adhesion molecule 1), VCAM-1 (vascular cell adhesion molecule 1), and E-selectin on endothelial cells in vitro. Plasma hemoglobin promotes formation of the biologically hazardous hydroxyl-radical, a process that may be regulated by the hemoglobin scavenger haptoglobin. Many of the proinflammatory effects of plasma hemoglobin and heme may involve consumption of nitric oxide. Studies have shown that nitric oxide inhibits cytokine-induced induction of VCAM-1, ELAM-1 (endothelial leukocyte adhesion molecule 1), and ICAM-1 resulting in an anti-inflammatory effect. The consumption of nitric oxide by hemoglobin circumvents the anti-inflammatory properties of nitric oxide.

Paroxysmal Nocturnal Hemoglobinuria: a Prototypic Disease of Intravascular Hemolysis

Paroxysmal nocturnal hemoglobinuria is an acquired clonal disorder of the hematopoietic stem cell that is characterized by chronic intravascular hemolysis, as indicated by the grossly elevated levels of LDH in almost all patients. Episodes of severe intravascular hemolysis are manifest clinically by hemoglobinuria (the hallmark of PNH) and are also frequently associated with dysphagia, abdominal pain, erectile dysfunction, thrombosis, and disabling fatigue.

The biochemical defect underlying PNH occurs in the synthesis of the glycosyl-phosphatidylinositol (GPI) anchor. This glycolipid structure is the means by which many proteins are attached to the plasma membrane. Two GPI-linked proteins missing from PNH cells are the complement regulatory proteins CD55 (also called “decay-accelerating factor”) and CD59 (also...
called “membrane inhibitor of reactive lysis”). The lack of complement regulation on the PNH RBC surface renders these cells extremely sensitive to complement-mediated lysis resulting in systemic hemoglobin release.\(^{115}\)

Lactate dehydrogenase catalyzes the reversible reduction of pyruvate to lactate by nicotinamide adenine dinucleotide. Red blood cells contain large amounts of LDH, and quantitation of total LDH and hemoglobin in osmotically lysed RBCs shows a near uniform correlation between these parameters in vitro.\(^{116}\) Lactate dehydrogenase is released into the plasma during hemolysis, and levels of this enzyme are generally elevated in patients with intravascular hemolysis.\(^{10,117}\)

Elevated levels of LDH are common among PNH patients due to ongoing chronic intravascular hemolysis.\(^{10,118,119}\) Lactate dehydrogenase is accepted as an accurate measure of intravascular hemolytic rate in PNH patients, and resolution of hemolysis in these patients results in an immediate reduction in LDH levels to near normal values.\(^{118}\)

A paroxysm, from which PNH derives its name, occurs when there is a sudden marked increase in the rate of intravascular hemolysis. During such paroxysms, LDH levels can reach more than 25 times that of normal.\(^{118}\) Paroxysms can be precipitated by events such as infection, drugs, and trauma, or they can occur spontaneously.

Paroxysms are characterized by severe bouts of hemoglobinuria, and more than 90% of PNH patients exhibit hemoglobinuria at some point in the disease, with approximately 50% of patients presenting with this clinical sign.\(^{120}\) During a severe paroxysm, hemoglobin filtered through the kidney can reach sufficient levels to turn the urine black. Severe hemoglobinuria typically lasts 3 to 7 days, but extended episodes can occur. The incidence of hemoglobinuria is increased in patients with a large proportion of PNH blood cells (large PNH clone), as clone size often correlates with the degree of hemolysis.\(^{23}\) In addition, it is well documented that intense hemoglobinuria during a paroxysm can be associated with acute renal failure.\(^{39,121}\)

As described above, many organ systems that are innervated by smooth muscle are adversely affected by administration of exogenous hemoglobin preparations, most likely due to the systemic removal of nitric oxide. Similarly, during paroxysms, PNH patients exhibit symptoms that are consistent with smooth muscle perturbation through the release of hemoglobin and nitric oxide scavenging, including abdominal pain, esophageal spasms, and erectile dysfunction.

Abdominal pain is experienced by approximately 35% of PNH patients during a paroxysm, and episodes are more common in patients with a large PNH clone.\(^{23}\) Although thrombosis of the mesenteric venous tree has been implicated in recurrent episodes of abdominal pain,\(^{122}\) many such cases do not show evidence of thrombosis. Further, abdominal pain usually rapidly resolves when the paroxysm abates, supporting the hypothesis that nitric oxide scavenging causes intestinal dystonia and spasm.

Esophageal spasm and dysphagia due to strong peristaltic waves are a common occurrence in PNH patients, with a reported incidence of approximately 23%.\(^{23}\) Dysphagia in these patients has also been shown to be closely linked to a large PNH clone and hemolysis.\(^{23}\) Similarly, episodes of dysphagia are most commonly associated with paroxysms and tend to resolve as the hemolysis subsides.\(^{30}\)

Erectile dysfunction occurs in 35% of male patients with PNH and is associated with paroxysms and PNH clone size, although erectile dysfunction can persist beyond hemolytic episodes and in many cases become permanent.\(^{23,60}\) Erectile dysfunction in these patients appears to improve with PDE5 inhibitor therapy, as long as macroscopic hemoglobinuria is absent, suggesting a role for nitric oxide scavenging in the pathogenesis (P.H., unpublished data, 2005).

Thrombosis is the most common cause of death in PNH patients. In a series of 80 PNH patients, 50% of PNH-related deaths were attributed to venous thrombosis, and approximately 40% of patients experienced thrombosis at some point in the disease.\(^{122}\) The most frequent types of thrombosis in this study included hepatic, pulmonary, deep, cerebral, and superficial veins, and inferior vena cava. Interestingly, there is a tight correlation between thrombosis and a large PNH clone, and clone size correlates with hemolytic rates.\(^{23,124}\)

As described above, nitric oxide plays an important role in the maintenance of normal platelet functions through the down-regulation of platelet aggregation and adhesion and the regulation of molecules in the coagulation cascade. Accordingly, the chronic consumption of nitric oxide by plasma hemoglobin has been implicated in the formation of clots in PNH patients. Indeed, thrombotic events such as Budd-Chiari syndrome increase in PNH patients during severe bouts of hemolysis (Wendell F. Rosse, MD, oral communication, August 6, 2003). The lack of the complement regulatory proteins CD55 and CD59 on the surface of PNH platelets, which renders these cells more sensitive to complement-mediated activation,\(^{125,126}\) may also contribute to thrombotic tendency in these patients.

The relationship between intravascular hemolysis and plasma hemoglobin/nitric oxide–dependent symptoms was recently evaluated with a specific drug intervention in PNH patients. A monoclonal antibody that blocks cleavage of the complement component C5, thereby preventing complement-mediated red cell lysis, was administered to hemolytic PNH patients over a 3-month period.\(^{118}\) This study demonstrated a dramatic reduction in intravascular hemolysis as LDH levels dropped from 3111 to 594 U/L (normal range, 150-480 U/L; P = .002). Similarly, the rate of hemoglobinuria (paroxysms) was reduced from 2.9 days per patient per month before treatment to 0.12 day per patient per month during treatment (P < .001). Therapeutic resolution of intravascular hemoly-
sis in these patients also resulted in a significant improvement in objectively studied quality of life measurements. Furthermore, clinical assessment of symptoms in these patients prior to drug therapy and after the resolution of hemolysis during treatment showed that symptoms attributed to smooth muscle dystonias, including abdominal pain, dysphagia, and erectile failure, were either completely resolved, or at least dramatically reduced, in most patients. These improvements in quality of life and clinical symptoms occurred despite the observation that total hemoglobin levels were unchanged during treatment, owing to a reduction in hemolytic rate and transfusion requirements. These data suggest that intravascular hemolysis and hemoglobin release mediate much of the symptoms of PNH patients, and further imply that smooth muscle constriction and prothrombotic effects of nitric oxide scavenging by plasma hemoglobin may be involved.

CONCLUSIONS

Intravascular hemolysis represents a severe pathological condition in a number of vital organ systems. Importantly, in a variety of human volunteer, patient, and animal studies, the presence of cell-free plasma hemoglobin is reproducibly associated with adverse clinical signs and symptoms, including gastrointestinal, cardiovascular, pulmonary, urogenital, hematologic, and renal abnormalities. Further, use of drugs that affect systemic levels of nitric oxide has revealed a strong relationship between homeostasis of these organ systems and the availability of nitric oxide. The demonstration that plasma hemoglobin is an efficient scavenger of nitric oxide, and that hemoglobin levels characteristic of intravascular hemolytic diseases are sufficient to effectively deplete nitric oxide, strongly support a critical role of nitric oxide scavenging in many of the clinical manifestations of hemoglobin release. Indeed, inhibition of complement-mediated hemolysis in PNH results in a reduction in plasma hemoglobin level and resolution of various clinical symptoms, thus demonstrating the close association between hemoglobinemia and these symptoms.

These data support the existence of a novel mechanism of human disease, hemolysis-associated smooth muscle dystonia, vasculopathy, and endothelial dysfunction. The definitive link between cell-free plasma hemoglobin, nitric oxide consumption, and symptoms characteristic of intravascular hemolytic diseases is now a topic of intense investigation.

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