Comparison of effects of two hemoglobin-based O2 carriers on intestinal integrity and microvascular leakage

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Baldwin, Ann L., Elizabeth B. Wiley, and Abdu I. Alayash. Comparison of effects of two hemoglobin-based O2 carriers on intestinal integrity and microvascular leakage. Am J Physiol Heart Circ Physiol 283: H1292–H1301, 2002.—Two “blood substitutes,” a diaspirin cross-linked human hemoglobin [bis(3,5 dibromosalicyl)formurate, DBBF-Hb] and a bovine polymerized hemoglobin (PolyHbBv), advanced to clinical trials, are used in this study. Previously, we have shown that injection of DBBF-Hb into the rat circulation produces venular leakage and intestinal epithelial disruption. The purpose of this study was to determine whether PolyHbBv, currently approved for veterinary use in the United States, shows similar effects. In anesthetized Sprague-Dawley rats, the mesenteric microvasculature was perfused with DBBF-Hb (n = 6), PolyHbBv (n = 5), cyanomet Hb (CNmet-DBBF-Hb), or HEPES-buffered saline with 0.5% bovine serum albumin (HBS-BSA) (controls, n = 7) for 10 min, followed by FITC-albumin for 3 min, and then fixed for microscopy. For DBBF-Hb, the mean leak number per micrometer venule length [2.41 ± 0.33 (± SE) × 10^{-3}] was significantly greater than for PolyHbBv (0.53 ± 0.14 × 10^{-3}), CNmet-DBBF-Hb (0.36 ± 0.14 × 10^{-3}), and HBS-BSA (0.12 ± 0.08 × 10^{-3}) (P < 0.01). Corresponding quantities for leak area were 0.10 ± 0.03, 0.010 ± 0.003, 0.005 ± 0.003, and 0.02 ± 0.02 μm²/μm. In rats injected with DBBF-Hb (n = 8), intestinal epithelial integrity was significantly compromised compared with those injected with PolyHbBv (n = 5) or saline (n = 6). These results indicate that intravascular PolyHbBv produces significantly less disruption of the intestinal exchange barrier than does DBBF-Hb, probably because the heme is not so easily oxidized.

blood substitutes; fluorescence microscopy; venular leakage; electron microscopy

CELL-FREE HEMOGLOBIN-BASED oxygen carriers, such as diaspirin cross-linked human hemoglobin [bis(3,5 dibromosalicyl)formurate, DBBF-Hb] and bovine polymerized hemoglobin PolyHbBv (Oxyglobin), have been proposed as blood substitutes for transfusions due to their plasma expansion and oxygen transport capabilities. Apart from their use after accidents or major surgery, such substitutes could also be employed to alleviate anemia in patients with hematocrits too high to qualify for blood transfusions. Hemoglobin-based blood substitutes have the added advantages that they can be easily purified, stored for relatively long periods of time, and used in patients of all blood types. However, a number of largely unresolved problems were found during preclinical trials and during the development of some of these hemoglobin-based substitutes. These include cardiovascular/hemodynamic effects, gastrointestinal changes, immune cell activation, coagulation changes, oxidative stress, and decreased host resistance to overwhelming infection (18).

Previously, Baldwin’s laboratory (5) showed that bolus injection of DBBF-Hb increases venular permeability and mast cell degranulation in the rat mesentery. Baldwin and colleagues also demonstrated that polyethylene glycol (PEG)-conjugated hemoglobin (PEG-HB), another hemoglobin-based blood substitute, extravasates from the intestinal mucosal capillaries (4) and that PEG-Hb and DBBF-Hb cause ultrastructural changes in the intestinal epithelium (4, 9). The leakage of these modified hemoglobins through mucosal capillaries into the tissue is associated with the degranulation of mucosal mast cells and the activation of mucin-secreting goblet cells. The responses induced by injection of DBBF-Hb into healthy animals in the mesentery are similar to those responses produced by administration of the mast cell mediator histamine (6). This is not surprising because mast cell degranulation, as observed after injection of DBBF-Hb, causes release of histamine and other inflammatory mediators, which then damage cell membranes. This inflammatory response is also consistent with our finding that injection of DBBF-Hb produces detachment of intestinal epithelial cells, from each other, and from the basement membrane.

Although it is known that DBBF-Hb stimulates the release of mast cell mediators, the mechanism by which this occurs has not been determined. One possibility is that the DBBF-Hb spontaneously autooxidizes to produce the ferric form of Hb (met Hb), together with reactive oxygen species (ROS), such as superoxide (O2⁻) and hydrogen peroxide (H2O2). If this is the case, then

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high concentrations of ROS in the tissue will be associated with the tissue damage produced by DBBF-Hb.

In the present study, we compared the relative toxicities of DBBF-Hb and PolyHbBv (Oxyglobin), a blood substitute currently approved by the Food and Drug Administration for veterinary use in the United States, and the human counterpart of which has recently been approved for clinical use in humans in South Africa. A dose-response study performed on dogs by Biopure has shown that Oxyglobin increases arterial oxygen content in the face of normovolemic anemia and produces transient clinical signs (skin discoloration, discolored stools, nausea, and vomiting). In addition, histopathology of Oxyglobin administration includes activation of tissue macrophages in multiple organs. DBBF-Hb, produced by the United States Army is similar to diaspirin cross-linked hemoglobin (DCLHb), the commercial analog produced by Baxter Health Care. Baxter has recently terminated its clinical development due to increased fatalities in the test group (24). Our aim was to determine the level of toxicity of PolyHbBv, compared with DBBF-Hb, in the intestine and in the mesenteric microcirculation of healthy rats. For comparison, some experiments were also performed with cyanomet-DBBF-Hb, a hemoglobin in which the CN groups are tightly bound to the heme such that the molecule is unable to participate in redox reactions.

MATERIALS AND METHODS

Hemoglobin Solutions

The human hemoglobin cross-linked by bis(3,5 dibromosalicyl)fumarate (DBBF-Hb) was a kind gift from the Walter Reed Army Institute of Research, Washington, DC. A spectral analysis of hemoglobin oxidation products was performed using a Beckman DU640 spectrophotometer. The ratio of oxy (Fe2+) to met (Fe3+) forms of hemoglobin was calculated as previously described (28) and found to be 0.21. The DBBF-Hb was filtered using a Sephadex G-50 column that was equilibrated with HEPES-buffered saline (HBS), pH 7.4. The molarities of the components of the HBS were as follows (in mM): 10 HEPES sodium salt, 11 HEPES acid, 132 NaCl, 4.7 KCl, 2.0 CaCl2, and 1.2 MgSO4, giving a total molarity of 160.9 mM, and a total osmolarity of 323.8 mosM. To increase the concentration of oxyHb to ferric hemoglobin (metHb), the DBBF-Hb was reduced by adding sodium dithionite (50 mg/100 ml). The sample was then immediately applied to a Sephadex G-50 column, and the eluant, recognized as reduced hemoglobin by its cherry-red color, was collected. Spectral analysis of this solution gave a ratio of 6.8 oxy to met forms of hemoglobin. Glutaraldehyde-polymerized hemoglobin (Poly-HbBv) was purchased from BioPure (Boston, MA). Spectral analysis of the solution just before injection demonstrated the presence of 5–8% of the met form, which is acceptable. PolyHbBv contains a heterogeneous mixture of polymeric (–95%) and nonpolymeric (–5%) species ranging in size from 32 to 500 kDa (23). Both the oxygen-carrying and redox properties of these two hemoglobins have been well documented (2, 13). Cyanomet-DBBF-Hb was synthesized at the Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD, by using the technique described below. Treatment of metHb with potassium cyanide results in the formation of the ferric cyanide derivative (cyanometHb). Cyanide is a strong ligand with respect to metHb, giving highly stable cyanometHb. We used the ferric form of DBBF-Hb to prepare cyanomet-DBBF-Hb (CNmet-DBBF-Hb) as previously described (3). Briefly, 1 vol of 1 mM DBBF-Hb solution was mixed with 2 vol of Drabkin reagent (nominally used to determine of Hb in blood and is prepared by dissolving 1 g of NaHCO3, 50 mg of KCN, and 200 mg of KF/Fe(CN)6 in water bringing the volume to 1 liter). After 5 min of incubation, the mixture was passed through two successive cycles of Sephodes G-25 columns to remove excess ferri cyanide and any bound ferrocyanide. Millimolar extinction at 540 nm of 12.5 mM−1•cm−1 was used to calculate the concentration of CNmet-DBBF-Hb. All hemoglobin solutions were equilibrated with room air during the experiments. However, the concentration of hemoglobin used in these experiments was less than one-tenth of that found in blood, and in addition, oxygen has a low solubility in water. Therefore, the tissues were not exposed to higher concentrations of oxygen than they would experience in vivo.

Preexperimential Treatment of Rats

Male Sprague-Dawley rats, weighing 300–350 g, were obtained from Harlan. Monthly serology, bacteriology, and parasitology evaluations are performed on animals from each virus-free barrier at Harlan. The rats were transported to the animal facility at Tucson Veterans Affairs Medical Center by truck. The animal facility is small with a low personnel activity, and monthly tests are performed on sentinel rats. On arrival the animals were housed two per cage in a room (3 m × 4 m) deliberately chosen so as to be remote from noisy air vents and cage washers, etc. The cages were 45 cm long and 24 cm wide and contained standard Harlan sanichip bedding. Ten to twenty rats were housed in the room at any given time, and no other rats, apart from those participating in this study, were housed with them. A technician entered both rooms once a day to feed and tend to the rats. The temperature ranged between 72 and 74°F, and the humidity was kept between 55 and 60%. The rats were fed Harlan Tech Lab 485 rat chow and placed on a light cycle with lights on between 6:00 AM and 6:00 PM.

Anesthesia

Sprague-Dawley rats were preanesthetized with 1 mg/kg body wt of the following mixture: ketamine hydrochloride (5 ml of 100 mg/ml), acepromazine maleate (2 ml of 10 mg/50 ml), and xylazine (8 ml of 20 mg/ml). This was followed by an intraperitoneal injection of pentobarbital sodium (30 mg/kg). In each rat a tracheostomy was performed for artificial ventilation.

Mesenteric Preparation to Measure Microvascular Leakage and Mast Cell Degranulation

Surgical procedures. The following study was performed to characterize the mesenteric microvascular leakage to albumin caused by intravenous injection of DBBF-Hb or PolyHbBv compared with HBS, pH 7.4, containing 0.5% BSA (HBS-BSA). The animal procedure was similar to that described previously (7, 26) and is summarized here. After anesthesia, the abdomen was opened and several contiguous well-vascularized mesenteric windows were selected and spread out flat over a Plexiglas platform. A mesenteric window is defined as the tissue extending between two adjacent feeding arterioles in the mesentery. The superior mesenteric artery was then cannulated, arterioles and venules surrounding the chosen windows were ligated, and the micro-
vascular network was perfused with HBS-BSA and 1 U/ml heparin at 37°C, followed by 2 mg/ml DBBF-Hb in HBS-BSA. When the microvascular network was filled with DBBF-Hb, the portal vein was clamped to prevent backflow of blood, and the microvascular pressure was adjusted to 40 mmHg. After 10 min, the portal clamp was removed, and the network was perfused with fluorescent tracer, BSA labeled with FITC (Sigma Chemical; St. Louis, MO). As soon as the vasculature of the windows was filled with FITC-albumin, as judged by the color, the pressure was adjusted to 40 mmHg, and the portal vein was clamped. After 1 min, the clamp was removed, and 3 ml of fixative (3% formaldehyde in HBS) were perfused at a pressure of 100 mmHg. This pressure was necessary to ensure that the venules and veins received adequate perfusion. The pressure was then reduced to 40 mmHg, the portal vein was clamped, and fixation was continued at 4°C. After 1 h, the mesenteric tissue was carefully excised, and each window from the preparation was mounted between two thin glass cover-slips by using aqueous mounting medium (Vectashield, Vector Laboratories; Burlingame, CA). Identical microvascular networks were performed on three more groups of rats except that HBS-BSA (5 rats), PolyHbBv (5 rats), or CNet-DDBF-Hb (5 rats) was substituted for DBBF-Hb (6 rats). These numbers of animals were justified to be sufficient by utilizing a sample size nomogram in conjunction with estimates of the difference in means that needed to be detected and the mean standard deviation for each parameter (29). The concentrations of PolyHbBv and CNet-DDBF-Hb were 2 mg/ml.

Assessment of venular leakage. An assessment of overall vascular leakage was made by measuring the number and area of regions with extravascular FITC-albumin. Slides were examined using a Zeiss Axioplan microscope with ×10 objective, numerical aperture 0.6, fitted for epifluorescence. The resolution obtained with this system was such that the smallest leak that could be detected was 2 μm² in area. The light source was a 100-W mercury lamp for epifluorescence and a halogen lamp for transmitted illumination. A video camera (Optronix 750D) was mounted at the camera port of the microscope. Ten images of leaky vessel networks from each mesentery, produced by epifluorescence with the appropriate FITC excitation and emission filters (λ = 488 nm and 515 nm, respectively), were viewed on a black and white monitor and also recorded on a video recorder. Each slide was only exposed to the excitation wavelength for 5 s. Recordings were also made of the networks under transillumination. Videotaped images were later analyzed using an analog-to-digital converter and appropriate software (National Institutes of Health Image) to measure the length and diameter of each venule, the number of leaks per venule, and the area of each leak. If a leak was positioned at a vascular junction, the leak area was divided by the number of venules involved. Data were pooled within each group, and the values calculated were the following: 1) average number of leaks per length of venule, and 2) average leak area per micron of venule.

Assessment of mast cell degranulation. After the fluorescent leaks were videotaped, the tissue was suspended with 1% toluidine blue for 20 s, rinsed with HBS-BSA, and then remounted. Toluidine blue was used to stain the mast cells to determine the numbers that had degranulated. Degranulated mesenteric mast cells, identified by the presence of intracellular granules released into the surrounding tissue, were counted within each circular ×20 microscopic field of view (field area 1.13 mm²). Rows of fields were counted systematically from left to right. Cells located in the periphery of the field were only counted if at least one-half of the cell area was within the field. The error of repeat counting was <2%. About 30 fields were counted for each mesenteric window.

Data analysis. Each parameter was compared between different groups using one-way analysis of variance. If a significant difference was found between groups, pairs of groups were compared by using the Student t-test with a P value <0.05 to determine statistical significance. All values are presented as means ±SE. The n used in the leakage studies was the number of venules examined per group, and the n used for the mast cell degranulation was the number of mesenteric fields of view examined per group.

Microscopy of Intestinal Mucosa to Assess Mast Cell Degranulation, Goblet Cell Secretion, and Epithelial Damage

Surgical procedures. The following study was performed to compare the intestinal tissue damage caused by intravenous injection of DBBF-Hb (6 rats), PolyHbBv (5 rats), and HBS-2% BSA (5 rats). After anesthesia, a midline abdominal incision was made to expose the aorta. The aorta was cannulated just downstream from the superior mesenteric artery in a retrograde direction. The free end of the catheter tubing was connected to a reservoir of HBS-2% BSA, pH 7.4, at 37°C. A loop of intestinal ileum, close to the cecum, was pulled outside the body cavity and arranged on a Plexiglas pillar attached to the plastic stage on which the rat was situated. Fifty milligrams of DBBF-Hb or PolyHbBv in a 5-ml bolus of HBS-2% BSA were injected through a 0.2-μm filter via the aortic cannula and allowed to circulate with the blood for 30 min. We assumed that the animal’s blood volume in milliliters is equivalent to 8% of the animal’s weight in grams, and injection of this amount of blood substitute produced a concentration that was similar to that used in the mesenteric experiments (2 mg/ml). Rats in the control group received HBS-2% BSA alone. After 30 min, the aorta was clamped proximal to (upstream of) the superior mesenteric artery, and the intestinal circulation was perfused with Karnovsky’s fixative in phosphate buffer, pH 7.4, at 4°C. When perfusion was complete, the inlet pressure was dropped to 40 mmHg, and the portal vein was clamped. The animal was killed with an intravenous injection of Beuthanasia. Fixation continued for 60 min after which the intestinal loop was excised and cut into several segments, each containing a Peyer’s patch; these segments were washed in buffered saline.

Tissue preparation for histology. Tissue squares were immersed in diaminobenzidine (DAB) overnight in the dark to stain specific granules in immune cells and thus make the cells easier to identify. The DAB was prepared as follows (19): DAB (0.1 g) was added to 50 ml 0.1 M monobasic phosphate buffer, and the pH was adjusted to 7.2 very gradually with concentrated NH₄OH. The solution became a light tannish-pink color. The tissue squares were then rinsed in distilled water. Meanwhile, 25 ml DAB solution were added to 1.66 ml of 3% H₂O₂ to give final concentration of 0.2%. The tissue was placed in this solution for 60 min and then rinsed three times in 0.15 M sodium cacodylate buffer. Finally, the tissue was dehydrated in increasing concentrations of ethanol and embedded in Spurr’s resin. The pieces of tissue were oriented in the resin so that the blocks could be sectioned perpendicular to the villus plane. Thick sections (2 μm) were cut for light microscopy, mounted on slides, and stained with toluidine blue. Ultrathin sections were cut for electron microscopy (Phillips CM12). Before the sections were examined under electron microscopy, the grids were stained with lead citrate and uranyl acetate.
Data analysis. Thick sections were examined under light microscopy to count the numbers of degranulated mast cells and secreting goblet cells, per villus cross section, in 30–60 villi per animal taken from four different regions of the tissue sample. Only villus sections that contained a central lacteal were included because these villi were centrally sectioned. Mast cells were considered degranulated if they exhibited empty vacuoles. For collection of data, the slides were coded and the code was not revealed until the data were analyzed, as previously described, using n as the number of villi in each group. Electron microscopy (×3,000) was used to assess the integrity of the epithelium (EI) in each villus cross section examined. Intact epithelium was assigned an EI of 1; epithelium with some cell-cell separation was assigned 2, and epithelial cells detaching from the basement membrane scored 3.

In Vivo Estimation of ROS in Intestinal Mucosa

Surgical procedures. Experiments were performed on 14 rats (5 DBBF-Hb, 5 PolyHbBv, and 4 control rats). The surgical procedure was similar to that used for electron microscopy except that the segment of intestine was cauterized longitudinally and the mucosal surface was spread over a Plexiglas. The mucosal segment was kept moist with a HBS-0.5% BSA drip heated to 37°C, and autofluorescence from the tissue was recorded from six to eight villi surrounding the Peyer’s patch. The HBS-BSA was then replaced by HBS-BSA containing 0.001 g/dl dihydrorhodamine 123 (DHR). This substance is not fluorescent until oxidized by a high-energy radical. The rate of accumulation of the fluorescent product for a constant suffusate concentration of DHR provides a quantitative measure of oxidant formation in the tissue (17). An image of fluorescence from the same villi was recorded. This preheme-protein image served as a background for later digital subtraction. A 5-ml bolus of 10 mg/ml DBBF-Hb, PolyHbBv in HBS-2%BSA, or of HBS-2% BSA alone was then injected into the aorta through a 0.2-μm filter, and a timer was started on the video monitor. Every 2 min, for the next 30 min, several drops of DHR were applied to the preparation, and an epifluorescent image was recorded. During acquisition of images, the following precautions were taken. First, each fluorescence observation was limited to a few seconds, after which a barrier filter was applied to block the fluorescence and prevent photobleaching. Second, the quantity of DHR that was dripped on the preparation between observations was carefully controlled so that the fluorescence intensity obtained was not affected by variations in DHR sulfusate volume. Third, the offset and gain of the camera was kept constant for all experiments. Finally, the animal was euthanized with an overdose of anesthetic.

Data analysis. The mean intensity of the epithelial fluorescence of the villi contained in each image was measured using NIH Image, and the background value (DHR before injection) was subtracted. Data were pooled among the six animals in each group for each time point, and the means and standard deviations were calculated. Similar measurements were made on the villus lamina propria. The data were then analyzed as described previously.

RESULTS

Mesenteric Preparation to Measure Microvascular Leakage and Mast Cell Degranulation

Distribution of leaks. Microscopic examination of control mesenteric preparations (that were perfused with HBS-BSA) by epifluorescence revealed very few leaky sites, but preparations treated with DBBF-Hb showed many leaks. The leakage occurred in venules but not in arterioles or capillaries. The total number of venules examined for HBS-BSA (control) and DBBF-Hb was 374 and 299, respectively, and the percentage of venules demonstrating leaks for these two categories was 2.6% and 38.4%, respectively. Most of the leaks were <100 μm² in area; 17/20 (85%) in the case of HBS-BSA and 290/309 (93.8%) in the case of DBBF-Hb. This compares with a luminal surface area of individual endothelial cells that ranges from 400 to 900 μm² (6). Preparations treated with PolyHbBv or with CNmet-DBBF-Hb resembled controls with respect to venular leaks. For PolyHbBv, out of 298 venules examined, 23 venules contained leaks (7.7%). Once again, most of the leaks (62/67; 92.6%) were <100 μm² in area. For CNmet-DBBF-Hb, out of 303 venules examined, 10 venules contained leaks and most of the leaks (44/45, 97.8%) were <100 μm² in area. Typical networks from rats after perfusion with PolyHbBv or DBBF-Hb, followed by FITC-BSA, can be seen in Fig. 1, A and B, respectively.

Quantification of leaks. The mean numbers and areas, respectively, of leaks per venule length for networks from rats perfused with DBBF-Hb, PolyHbBv, CNmet-DBBF-Hb, or with HBS-BSA are shown in Fig. 2, A and B. In rats given DBBF-Hb, the mean microvascular leak number [2.41 ± 0.33 (SE) × 10⁻³ leaks/μm venule length] and area (0.10 ± 0.03 μm²/μm) were both significantly greater than corresponding values in controls (0.12 ± 0.08 × 10⁻³ leaks/μm and 0.02 ± 0.02 μm²/μm, respectively). In rats perfused with PolyHbBv, the mean leak number per micron length of venule (0.53 ± 0.14 × 10⁻³) was significantly lower than that for DBBF-Hb treatment and was slightly, but significantly, higher than that for controls. The mean leak area (0.010 ± 0.003 μm²/μm) was significantly lower than that for DBBF-Hb treatment and was not significantly different from controls. In rats perfused with CNmet-DBBF-Hb, the mean leak number per micron length of venule (0.36 ± 0.14 × 10⁻³) and the mean leak area (0.005 ± 0.003 μm²/μm) were significantly lower than those for DBBF-Hb treatment but not significantly different from controls.

Mesenteric mast cell degranulation. Degranulated mast cells were easy to identify in mesenteric tissue stained with toluidine blue, because the granule contents were expelled into the surrounding tissue and were stained a dark red against a blue background. Intact mast cells formed compact, oval-shaped disks that also stained dark red. The numbers of microscopic fields of view observed were 74, 107, 105, and 83 for DBBF-Hb, PolyHbBv, CNmet-DBBF-Hb, and HBS-BSA preparations, respectively. In the mesenteries of animals perfused with DBBF-Hb, the number of degranulated mast cells per field of view [12.88 ± 1.61 (±SE)] was significantly greater than that for control animals (8.24 ± 1.09) (Fig. 3). Mesenteric mast cell degranulation in mesenteries from animals treated with PolyHbBv (3.21 ± 0.52) was significantly lower.
than that for DBBF-Hb and for control groups. Although mesenteric mast cell degranulation in animals treated with CNmet-DBBF-Hb (6.85 ± 0.70) was significantly lower than that for DBBF-Hb and control groups, it was significantly greater than that for animals treated with PolyHbBv.

Fig. 2. Mesenteric preparation to measure microvascular leakage. All hemoglobins used at a concentration of 2 mg/ml. A: average number of leaks per venule length (µm⁻¹) for different treatments. Mean numbers of leaks per micron vessel length for the HBS-BSA, DBBF-Hb, and PolyHbBv groups are all significantly different from each other (P < 0.05). Cyanomet-DBBF-Hb group has significantly fewer leaks than the DBBF-Hb group but is not significantly different from PolyHbBv or HEPES-buffered saline (HBS)-BSA. B: average total leak area per venule length (µm²/µm) for different treatments. DBBF-Hb is significantly greater than the other three groups, which are not statistically different from each other. Error bars indicate means ± SE.

Fig. 1. Mesenteric preparation to measure microvascular leakage. A: light microscopic image of rat mesenteric microvasculature after perfusion with bovine polymerized hemoglobin (PolyHbBv, 2 mg/ml) followed by FITC-albumin. A few leakage sites of FITC-albumin from the microvasculature are visible. Scale bar = 25 µm. B: mesenteric preparation after perfusion with diaspirin cross-linked human hemoglobin (DBBF-Hb, 2 mg/ml) followed by FITC-albumin. A greater number of leakage sites are visible compared with PolyHbBv. Scale bar = 25 µm.
Microscopy of Intestinal Mucosa to Assess Mast Cell Degranulation, Goblet Cell Secretion, and Epithelial Damage

Degranulated mast cells. Mucosal degranulated mast cells were easy to identify by light microscopy because they stained intensely with toluidine blue and demonstrated empty vacuoles. A comparison of the degree of mast cell degranulation seen in the three groups is shown in Fig. 4A. The numbers of villi from which degranulated mast cell counts were obtained were 154, 187 and 144 for control, DBBF-Hb, and PolyHbBv preparations, respectively. The mean number of degranulated mucosal mast cells observed per villus was significantly higher for DBBF-Hb preparations than for controls (0.64 ± 0.09 vs. 0.36 ± 0.06). Perfusion with PolyHbBv produced significantly more mucosal mast cell degranulation than DBBF-Hb (1.41 ± 0.12). This result was obtained from counts for 144 villi.

Secreting goblet cells. The extent of goblet cell secretion following injection of HBS-BSA, DBBF-Hb, or PolyHbBv is shown in Fig. 4B. Injection of HBS-BSA caused negligible goblet cell secretion (0.04 ± 0.02), whereas injection of DBBF-Hb or PolyHbBv produced significantly greater secretion: 4.80 ± 0.37 and 2.27 ± 0.24, respectively. Goblet cell secretion in rats perfused with DBBF-Hb was significantly greater than that produced by PolyHbBv.

Epithelial damage. Electron micrographs of transverse sections through the intestinal mucosa from rats perfused with DBBF-Hb for 30 min, showed some disrupted epithelium and edematous subepithelial interstitium, particularly in tissue remote from Peyer’s patches. The epithelial cells were sometimes separated from each other at the basal aspects of their intercellular junctions and were linked only by long, cytoplasmic protuberances. The cells were also occasionally partially separated from the basement membrane. In control preparations, the epithelium was usually intact and the subepithelial interstitium was compact. Electron micrographs of interstitial mucosa from rats perfused with PolyHbBv looked very similar to those from controls. An example is shown in Fig. 5. The epithelial intercellular junctions (arrows) are tight, and the cells are closely adhering to the basement membrane. No edematous areas (extensive electron lucent regions) are evident in the interstitial tissue. This electron micrograph shows a nondegranulated mast cell and an eosinophil. The average EI indexes for each of the three groups are depicted in Fig. 6. In Fig. 6, the results from tissue away from Peyer’s patches, as well as adjacent to Peyer’s patches, are also included because EI differed between the two areas in some cases. Away from Peyer’s patches the EI of DBBF-Hb preparations was sig-

Fig. 3. Mesenteric preparation to measure mast cell degranulation. Average number of mesenteric degranulated mast cells per microscopic field of view for different treatments. All hemoglobins were used at a concentration of 2 mg/ml. All groups are significantly different from each other (P < 0.01).

Fig. 4. Mesenteric preparation to measure mast cell degranulation and goblet cell secretion. A: average number of degranulated mucosal mast cells (DMC) per villus cross section for different treatments. DBBF-Hb and PolyHbBv were each injected as a 5-ml bolus at a concentration of 10 mg/ml. All groups are significantly different from each other (P < 0.01). B: average number of secreting goblet cells per villus cross section for different treatments. All groups are significantly different from each other (P < 0.01).
nificantly worse than that of controls (1.37 ± 0.04 vs. 1.18 ± 0.03). The EI of preparations perfused with PolyHbBv (1.07 ± 0.02) was significantly better than that of preparations perfused with DBBF-Hb or with HBS-BSA (controls). Near Peyer's patches the EI of DBBF-Hb preparations was significantly worse than that of controls (1.20 ± 0.03 vs. 1.11 ± 0.02). The EI of preparations perfused with PolyHbBv (1.08 ± 0.03) was significantly better than that of preparations perfused with DBBF-Hb but not better than that of controls.

**DISCUSSION**

In the present study, we show that mesenteric microvascular leakage is significantly lower in animals treated with PolyHbBv compared with DBBF-Hb. Strengthening this finding, we also show a lower number of degranulated mast cells present in the mesentery following perfusion with PolyHbBv compared with controls.

**In Vivo Estimation of ROS in Intestinal Mucosa**

In preliminary experiments, in which no systemic injection was given to the animal and the mucosal epithelium was exposed and suffused with DHR 123, the background fluorescence was low and did not increase over a time period of 30 min. However, in experiments in which the rats were injected with HBS-2% BSA, the fluorescence in the epithelium (Fig. 7) and in the lamina propria (Fig. 8) gradually increased and peaked between 16 and 18 min after injection. When rats were injected with DBBF-Hb, the fluorescence in the epithelium and in the lamina propria rapidly increased from 2 to 10 min after injection and remained intense for the whole observation period. When DBBF-Hb and control preparations were compared, a significantly greater fluorescence was seen in the epithelium of DBBF-Hb preparations between 6 and 12 min after injection and in the lamina propria between 6 and 14 min after injection. Animals injected with PolyHbBv showed epithelial fluorescence that was not significantly different from controls at any time point after injection. However, fluorescence generated in the lamina propria was significantly less than that for controls between 8 and 18 min after injection. In addition, the PolyHbBv fluorescence in the lamina propria was significantly less intense than that produced by DBBF-Hb from 4 min after injection until the end of the observation period (22 min).

**Fig. 5.** Electron microscopy of intestinal mucosa to assess epithelial damage. Typical electron micrograph of transverse section through intestinal villus from rat perfused with a bolus of 10 mg/ml PolyHbBv. Note the intact epithelium (E) in which adjacent cells adhere closely to each other and to the basement membrane. Subepithelial interstitium is compact and contains an intact mast cell (mc) and an eosinophil (eo). Scale bar = 2 μm.

**Fig. 6.** Electron microscopy of intestinal mucosa to assess epithelial damage. Typical electron micrograph of transverse section through intestinal villus from rat perfused with a bolus of 10 mg/ml PolyHbBv. Note the intact epithelium (E) in which adjacent cells adhere closely to each other and to the basement membrane. Subepithelial interstitium is compact and contains an intact mast cell (mc) and an eosinophil (eo). Scale bar = 2 μm.

**Fig. 7.** Electron microscopy of intestinal mucosa to assess epithelial damage. Histogram to show epithelial integrity index of villi from tissue within (A) and remote from (B) Peyer's patch after injection of HBS-2% BSA (control), DBBF-Hb (5 ml bolus at 10 mg/ml), or Oxyglobin (PolyHbBv) (5 ml bolus at 10 mg/ml). Scale ranges from 1 to 3; a score of 1 means the cells are intact, 2 means the cells show some separation from each other, and 3 means there is some separation of cells from the basement membrane. *Value is significantly lower than DBBF-Hb (P < 0.05). Saline and PolyHbBv values are not significantly different from each other. In B, all values are significantly different from each other.
DBBF-Hb, BSA and DBBF-Hb have Stokes-Einstein diameters of 7.4 and 5.6 nm, respectively, and the Stokes-Einstein diameter of PolyHbBv is expected to be much greater than those values, because, in this case, the Hb is polymerized. Because the PolyHbBv caused very few microvascular leaks wide enough for the subsequent escape of albumin, it is very unlikely that the PolyHbBv could, itself, extravasate from the mesenteric microcirculation during the 10-min perfusion period. Thus the PolyHbBv probably did not contact the mast cells in the mesenteric interstitium. On the other hand, the DBBF-Hb caused extensive microvascular leakage to albumin, and so it is very likely that the leakage it produced were large enough for the DBBF-Hb itself to extravasate and then stimulate the mesenteric mast cells to degranulate.

In the intestinal mucosa, treatment with PolyHbBv resulted in more mast cell degranulation and less goblet cell activation than produced by DBBF-Hb. The mucosal microvessels, however, are known to be somewhat permeable to albumin (10) and to PEG-Hb, which has a Stokes-Einstein diameter of 25 nm (4). Thus it is very likely that both DBBF-Hb and PolyHbBv escaped into the mucosal interstitium during the perfusion period and contacted the mast cells. The PolyHbBv produced more mast cell degranulation than the DBBF-Hb, indicating that the mucosal mast cells were more sensitive to this product. Although there was greater release of inflammatory mediators from mast cells, and less protective secretion of mucin by goblet cells in animals treated with PolyHbBv, compared with DBBF-Hb, the epithelium of these animals was apparently protected from ultrastructural damage. We also observed that intestinal villi of animals injected with PolyHbBv demonstrated significantly lower concentrations of ROS in the epithelium and in the lamina propria than those from animals injected with DBBF-Hb.

Differences between the structures of DBBF-Hb and PolyHbBv may account for the differences in the intestinal toxicities observed in the treatment of healthy experimental animals with these products. DBBF-Hb, an intramolecularly cross-linked tetramer, has been extensively studied in vitro and in animal models. In vitro studies on DBBF-Hb revealed that besides modifying oxygen-carrying properties, subunit cross-linking with DBBF can also affect the tendency of this hemoglobin to undergo autooxidation and subsequent oxidative side reactions. Polymerization of DBBF-Hb into larger molecular sizes has not overcome the tendency of this hemoglobin to undergo autooxidation and damaging oxidative modiﬁcations (24). Polymerization with glutaraldehyde is known to alter the oxygen afﬁnity, redox potential, and autooxidation kinetics of bovine hemoglobin (1). However, recently published work (2) has shown that PolyHbBv is indeed more resistant than DBBF-Hb to autooxidation and oxidative side reactions than DBBF-Hb in vitro. The autooxidation process is known to be a source for metHb as well as ROS. Thus this difference between the chemistries of the two hemoglobins may account for our observations regarding ROS production and tissue damage. Because perfusion with cyanometHb, which is resistant to oxidation, did not produce mesenteric microvascular leakage signiﬁcantly different from controls, this lends further support to our hypothesis: DBBF-Hb causes more damage to tissue than

![Fig. 7.](http://ajpheart.physiology.org/) In vivo estimation of reactive oxygen species in intestinal mucosa. Histogram to show ﬂuorescence intensity of dihydrorhodamine 123 in the epithelium of intestinal villi after injection of HBS-2% BSA, DBBF-Hb (5 ml bolus at 10 mg/ml), or Oxyglobin (PolyHbBv) (5 ml bolus at 10 mg/ml). On the abscissa is plotted the time after injection in minutes. *Value is signiﬁcantly greater than for PolyHbBv and saline (P < 0.01). Values for PolyHbBv and saline are not signiﬁcantly different from each other.

![Fig. 8.](http://ajpheart.physiology.org/) In vivo estimation of reactive oxygen species in intestinal mucosa. Histogram to show ﬂuorescence intensity of dihydrorhodamine 123 in the lamina propria of intestinal villi after injection of HBS-2% BSA, DBBF-Hb (5 ml bolus at 10 mg/ml), or Oxyglobin (PolyHbBv) (5 ml bolus at 10 mg/ml). On the abscissa is plotted the time after injection in minutes. *Value for DBBF-Hb is signiﬁcantly greater than for HBS-BSA (P < 0.01). #Values for PolyHbBv are signiﬁcantly smaller than for DBBF-Hb (P < 0.01). †Values for PolyHbBv are signiﬁcantly smaller than for HBS-BSA (P < 0.01).
PolyHbBv because DBBF-Hb is more easily oxidized and stimulates a higher rate of production of ROS in the tissue. In support of this hypothesis, our previous studies have shown that administration of scavengers of H$_2$O$_2$ (catalase) and O$_2$ (superoxide dismutase) temporarily reduce the tissue damage caused by subsequent injection of DBBF-Hb (8). It is known that ROS are responsible for lipid peroxidation and for activating eosinphils, neutrophils, and mast cells to release more inflammatory mediators.

Surprisingly, it was not necessary to inject rats with a modified hemoglobin to increase the presence of excess ROS in the mucosa. Even when control animals were perfused with HBS-2% BSA, DHR fluorescence gradually increased in the epithelium and lamina propria. It is possible that this increased fluorescence and associated ROS production occurred as follows. Epithelial cells, due to their high density of mitochondria, produce high quantities of ROS (O$_2$ and H$_2$O$_2$) as products of respiration. Normally, the excess ROS are scavenged by endogenous antioxidants such as catalase, superoxide dismutase, and glutathione peroxidase. However, in the experiments described above, injection of either HBS-2% BSA or modified Hb diluted the concentration of these scavengers in the blood and thus resulted in excess ROS in the circulation. This phenomenon has been noted previously in another study in which it was shown that when resuscitation fluids lacking ROS scavengers, such as pasteurized plasma protein solution or 0.9% saline, are injected into the circulation, peroxyl radical trapping capacity is decreased (21). This does not occur when fresh plasma or donor red blood cells are used. It was suggested that the decreased antioxidant capacity was a result of dilution of the blood by the resuscitation fluids. This hypothesis is consistent with the fact that no significant increase in fluorescence was observed in the intestinal mucosa when DHR was suffused in the absence of any systemic injections.

Injection of DBBF-Hb into the systemic circulation produced a more rapid and significantly greater increase in DHR fluorescence in the epithelium and lamina propria than seen after injection of HBS-BSA, indicating a reaction of the ROS with DBBF-Hb to produce more ROS. Oxidation of hemoglobin results in the production of the ferric form of hemoglobin (20) methemoglobin, which destabilizes the protein and allows the heme group to become detached (11). The heme then releases free iron that may catalyze production of the hydroxyl radical by the Fenton reaction, thus increasing the ROS concentration. Alternatively, hemoglobin may react with H$_2$O$_2$ to produce the ferryl hemoglobin radical and a ferryl radical (13, 16). This higher oxidation state of hemoglobin (Fe$^{4+}$) is a potent oxidant capable of promoting oxidative damage to most classes of biological molecules (15). Oxidative reactions between hemoglobin and oxidants such as H$_2$O$_2$ (generated by Hb autooxidation or from other intracellular sources) may potentially cause a damaging vascular inflammatory cascade of reactions. The formation of DBBF-HbFe$^{4+}$ was correlated to cytotoxicity in an endothelial cell culture model of ischemia-reperfusion and in cells that lack their antioxidative mechanisms such as glutathione (13). In addition, we have recently demonstrated that under mild oxidative stress, DBBF-Hb can induce growth arrest in cultured endothelial cells, ultimately leading to a apoptotic and necrotic cell death (14). Interestingly, recently published preclinical studies reported the detection of myocardial lesions in a number of animal models infused with DCLHb. These lesions are characterized by a mild to moderate focal-to-multifocal myocardial degeneration and/or necrosis in a highly vascularized portion of the myocardium (12).

The hemoglobin cross-linking and polymerization involved in producing hemoglobin-based blood substitutes can affect these oxidative reactions. Indeed, we have compared the oxygen-carrying and redox reactions of DBBF-Hb and PolyHbBv and found that generally DBBF-Hb exhibits superior oxygenation characteristics, but PolyHbBv was found to be more resistant to irreversible oxidative processes, which include the formation of long-lived ferryl species and subsequent heme degradation and iron loss (22).

In summary, the studies reported here indicate that in terms of redox-mediated cytotoxicity, PolyHbBv is superior to DBBF-Hb in that it produces significantly less damage to microvascular networks and to the intestinal epithelium. However, there are other factors that must also be considered in the development of successful blood substitutes. For example, oxygen affinity and rate constants for oxygen uptake and release will affect rates of oxygen delivery to tissues. Recent studies on the influence of PolyHbBv on local tissue perfusion and oxygenation in acute anemia after isovolemic hemodilution caused abnormally low tissue oxygenation and functional capillary density, whereas the same level of hemodilution with dextran maintained normal microvascular conditions (27). Partial oxygenation (~80%) at normal oxygen pressure and the insensitivity of PolyHbBv to normal allosteric modifiers of bovine hemoglobin may have contributed to the observed poor tissue oxygenation under these experimental conditions (2).

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REFERENCES


