Comparison of PEG-modified albumin and hemoglobin in extreme hemodilution in the rat

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OXYGEN-CARRYING RED CELL SUBSTITUTES have been developed in many forms, including human, animal, and recombinant hemoglobins (Hb), which may then be cross-linked, polymerized, or conjugated to various polymers including starches and polyethylene glycol (PEG) (24). The goals of such modifications are indirect and invasive procedures cannot be used in many animal and most human models. A major hurdle remaining in the development of Hb-based products is vasoconstriction, i.e., the tendency of Hb to cause narrowing of vessels in the arterial circulation. Vasoconstriction induced by low molecular weight Hb derivatives can lead to hypertension, reduced cardiac output, increased resistance, reduced tissue perfusion, and, paradoxically, reduced tissue oxygenation with consequent lactic acidosis (26). If used to resuscitate trauma patients with penetrating injuries, rapid restoration of blood pressure could also cause significant rebleeding (2). Clinical trials with an example of this type of product were discontinued because of excessive adverse reactions (14, 16). One unifying hypothesis to explain these clinical failures is that vasoconstriction may be responsible for a wide variety of toxic effects that are mediated by smooth muscle contraction, including hypertension and esophageal spasm (25).

The most popular theory to explain Hb-induced vasoconstriction is scavenging the endothelium-derived relaxing factor nitric oxide (NO) by Hb, which is known to have a very high NO affinity (6). With this working hypothesis, mutant Hbs have been expressed in bacterial and yeast systems that have reduced NO-heme affinity, and these mutants demonstrate a reduced tendency to produce vasoconstriction (5). Our group found, in contrast, that several examples of modified Hb had different effects on blood pressure in rats, even though the measured rates of reaction with NO were identical (13). Among these products was Hb modified with PEG, which had no significant hypertensive effect (26).

With the need for an explanation of Hb-induced vasoconstriction as an alternative to the NO hypothesis, we explored the possibility that this phenomenon could be a normal autoregulatory overreaction to excessive O2 supply to controlling arterioles (27). This theory was based on the ability of cell-free Hb to participate in “facilitated diffusion” (15) and suggested that the way to control vasoconstriction was to control the diffusive properties of Hb, which include molecular size, viscosity, and O2 affinity (19). This concept was tested in a simple artificial capillary system (11) in which PEG-modified Hb with a Po2 necessary to obtain 50% O2 (P50) of ~12 Torr released O2 in a manner very similar to that of native red blood cells, whereas both unmodified Hb and Hb cross-linked between the α chains demonstrated markedly increased release.

Based on these theoretical and experimental findings, we have developed a new PEG-modified Hb that employs novel sulphydryl-maleimide conjugation chemistry (1). The modified Hb (MalPEG-Hb), as formulated at 4.2 g/dl in lactated Ringer...
USP, is designated MP4 (20). MP4 is produced from human Hb obtained from outdated human red blood cells. Its unique properties include high $O_2$ affinity ($P_50$ of ~6 Torr), increased onotic pressure (~50 Torr), and viscosity (~2.5 cPs), all of which are counter to conventional recommendations for the production of Hb-based $O_2$ carriers (23). Because this product has such unusual properties, we wished to determine whether its beneficial effects are a result of $O_2$ delivery by Hb or whether they are merely a reflection of onotic volume expansion or some other property of PEG. In the present study, critical differences were compared in an animal model of extreme hemodilution. MP4 was compared with hydroxyethyl starch [pentastarch (PS), Pentaspan] and PEG-modified human albumin (MPA), oncotically matched to MP4. The primary objective end point was determination of the hematocrit at which lactic acid begins to accumulate (the critical hematocrit).

**METHODS**

**Solutions.** Hemoglobin solutions were prepared and characterized as described previously (20). Briefly, outdated human packed red blood cells, obtained from the San Diego Blood Center, were washed with saline, lysed with distilled water, and diafiltered to achieve a Hb concentration of 4.2 g/dl in lactated Ringer solution (stroma-free hemoglobin). MalPEG-Hb was produced by the reaction of human Hb with 2-iminothiolane to introduce sulfhydryl groups at surface $\epsilon$-amino groups (lysine), which were then conjugated to six strands with 2-iminothiolane to introduce sulfhydryl groups at surface $\epsilon$-amino groups (lysine), which were then conjugated to six strands

<table>
<thead>
<tr>
<th>Solution</th>
<th>PS</th>
<th>MP4</th>
<th>MPA</th>
<th>SFH</th>
<th>Albumin</th>
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</thead>
<tbody>
<tr>
<td>Hemoglobin, g/dl</td>
<td>4.2</td>
<td>4.2</td>
<td>4.2</td>
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<tr>
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<td>Albumin, g/dl</td>
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<tr>
<td>Viscosity, cPs</td>
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<td>2.2</td>
<td>2.2</td>
<td>2.2</td>
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<tr>
<td>COP, mmHg</td>
<td>50</td>
<td>49</td>
<td>49</td>
<td>49</td>
<td>49</td>
</tr>
<tr>
<td>$P_{50}$, Torr</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>*Peak retention, min</td>
<td>43.7</td>
<td>43.0</td>
<td>55.7</td>
<td>50.2</td>
<td>50.2</td>
</tr>
</tbody>
</table>

**RESULTS**

**Table 1. Characteristics of the solutions studied**

- PS, pentastarch; MP4 and MPA, polyethylene glycol-modified hemoglobin and albumin, respectively; SFH, stroma-free hemoglobin; COP, colloid osmotic pressure; $P_{50}$, $P_{O_2}$ necessary to obtain 50% $O_2$. *Size exclusion chromatography. See METHODS for details.
tored for an additional 70 min before euthanasia. Blood samples (0.3 ml) were taken every 10 min for hematologic and blood-gas analysis.

Statistical and survival analysis. Statistical analyses were done using Sigmaplot (SPSS). Unless otherwise noted, values are expressed as means ± SE. Differences between means were considered significant at \( P < 0.05 \). The time of death was determined to be the point at which pulse pressure (systolic – diastolic) was <5 mmHg.

**RESULTS**

**Solutions.** The properties of the solutions used in the experiments are summarized in Table 1. MP4 and MPA were closely matched in oncotic pressure and viscosity. The concentrations given (4.2 and 5.0 g/dl) are the concentrations of protein in both cases. Because of the conjugation to PEG, the molecular mass of each is ~95 kDa. We have expressed the concentration on protein basis so that \( O_2 \) transport capacity can be compared directly with native Hb. The \( P_{50} \) of MP4 is ~6 Torr.

The PEG modification reactions had similar effects on the properties of Hb and albumin (Table 1). In both cases, the viscosity increased from 1.0 to 2.2 cPs and the oncotic pressure from 15 to 49 Torr for Hb and from 20 to 50 Torr for albumin. Although the number of PEG strands per molecule was not measured directly for MPA, the reduction in peak retention time in size-exclusion chromatography from 50.2 to 43.0 min is similar to the change found for PEG modification of Hb (55.7–43.7 min). We thus conclude that, like MP4, MPA contains approximately six strands of 5,000-kDa PEG (20).

**Survival.** The end of the survival period was defined as a pulse pressure (systolic – diastolic) of <5 mmHg (Fig. 1, top). This allowed an unambiguous time of death for the Kaplan-Meier analysis of survival. (Fig. 1, bottom). All of the animals that received MP4 survived for the entire 130-min period of exchange transfusion, whereas none of the PS or MPA animals did. There was no difference in survival for the latter two groups, suggesting that the presence of PEG did not confer any protection. Because the oncotic pressures of all three solutions were similar (Table 1), we conclude that prolonged survival for the MP4 animals is not a result of greater volume expansion.
Hematology. The hematocrit fell exponentially (Fig. 2, top), reaching virtually undetectable levels at 100 min after the exchange process was started in the MP4 animals. The hematocrit level in the PS and MPA animals did not fall as low as it did in the MP4 animals because animals in these groups did not survive as long. The hematocrit fall was slightly greater for the MP4 compared with MPA or PS animals, suggesting greater volume expansion in those animals. In the animals that received either PS or MPA, total Hb declined to ~2 g/dl at the end of the exchange procedure, whereas in the animals that received either MP4 total Hb was significantly higher (Fig. 2, bottom). The total Hb was a linear function of hematocrit in all animals (Fig. 3, top), as was plasma Hb in the MP4 animals (Fig. 3, bottom).

Hemodynamics. Systolic and diastolic blood pressures are plotted on a minute-by-minute basis in Fig. 4. In the figure, the start of the exchange procedure is indicated by an arrow, showing a slight rise in both diastolic and systolic pressures when the procedure begins. As reflected in Fig. 1, the pulse pressure diminishes in both the PS and MPA animals but is maintained in the MP4 animals. It appears that, in both the PS and MPA animals, there is the beginning of hemodynamic recovery at ~90 min, but it is short lived and ineffective.

After initiation of the exchange procedure, the MAP of the animals that received MP4 increased slightly, but there were no significant between-group differences until ~40 min of exchange (Fig. 5, top). At that point, MAP in both the PS and MPA groups began to fall. Although the MAP tended to drift downward for the duration of the experiment in the MP4 animals, it remained >75 mmHg at the time of euthanasia.

The heart rate rose almost immediately in the animals that received the non-O2 carriers (MPA, PS) and only somewhat later in the animals that received MP4 (Fig. 5, bottom). The heart rate in the PS and MPA animals fell sharply at ~40 min, even though the first death in these two groups did not occur until 60 min. Maintenance of heart rate was best in the animals that received MP4. Shock index (heart rate/systolic blood
pressure, not shown) is a general index of shock stress. Significant between-group differences are seen as early as 20 min after initiation of the exchange transfusion, where the values for animals that received MP4 are lower than those in the animals that received either PS or MPA.

Acid-base and blood gas regulation. As the exchange transfusion progressed, animals hyperventilated, as reflected in rising arterial \( P_{O_2} \) (Fig. 6, top) and falling arterial \( P_{CO_2} \) (Fig. 6, bottom). This effect appears to be least pronounced for the animals that received MP4. At the last time point at which all animals are alive (50 min), \( P_{O_2} \) is significantly lower and \( P_{CO_2} \) significantly higher in the MP4 animals compared with all other groups, and there are no significant differences among the PS and MPA groups. At that point, total Hb (Fig. 2, bottom) is highest in the MP4 animals. These findings suggest the stimulus to hyperventilate was less in the MP4 animals.

Critical hematocrit. The “critical” hematocrit is indicated by many of the variables measured in these experiments, all of which demonstrate a change at \( \sim 40 \) min after start of the exchange transfusion. This is true of MAP (Fig. 5, top), heart rate, \( P_{ACO_2} \) (Fig. 6, bottom), pH (Fig. 7, top), and BE (Fig. 7, bottom). With the use of lactic acid as an indicator of global \( O_2 \) sufficiency, the critical hematocrit in animals hemodiluted with either PS or MPA is \( \sim 15\% \) (Fig. 8, top). In contrast, the animals that received the \( O_2 \) carrier demonstrated a lowered critical hematocrit, by \( \sim 10 \) percentage points, to \( \sim 5\% \). Viewed as a function of total Hb concentration (Fig. 8, bottom), the critical point in \( O_2 \) delivery is reached at \( \sim 5 \) g/dl, regardless whether the Hb is located in red blood cells or plasma. This equivalence is evidence that the Hb component of MP4 (MalPEG-Hb) is as effective at \( O_2 \) delivery as red cell Hb.

Submaximal hemodilution. At 40 min of exchange transfusion, all animals in all groups are still alive, but some differences are clearly apparent (Table 2). Among these, the hematocrit and total Hb are significantly lower in the MP4 animals compared with PS and MPA, whereas plasma Hb is 2.38 g/dl.

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Fig. 5. Hemodynamic response to continuous exchange transfusion. Top: mean arterial pressure (MAP) increases slightly after initiation of the exchange but is better maintained for the duration of the experiment in the animals that received MP4 compared with those that received non-\( O_2 \) carriers (MPA, PS). Bottom: heart rate response is more immediate in the PS and MPA animals. Values are means \( \pm \) SE.

Fig. 6. Ventilatory response to hemodilution. Arterial \( P_{O_2} \) (\( P_{A_{O_2}} \); top) and arterial \( P_{CO_2} \) (\( P_{A_{CO_2}} \); bottom) changes are mirror images. Values are means \( \pm \) SE.
in these animals. Although not significant, the lactic acid has begun to rise in both the MPA and PS animals, whereas it remains at baseline in the MP4 animals. PaCO₂ is significantly lower and PaO₂ is higher in the MPA compared with MP4 animals, indicating a greater degree of hyperventilation in the MPA animals. BE is significantly higher in the MP4 compared with PS animals, and MAP in the MP4 animals is significantly higher than that in either of the control groups. Finally, the shock index (heart rate/MAP) is significantly lower in the MP4 compared with the PS or MPA animals. Taken together, these data clearly show that, even at submaximal hemodilution, delivery of O₂ to tissues is more adequate in the MP4 compared with the PS or MPA animals.

DISCUSSION

The purpose of the present experiments was to determine whether a new formulation of PEG-modified human Hb (MalPEG-Hb) could serve as a surrogate for O₂ transport by red blood cells. MPA was prepared using the same reaction chemistry as is used to prepare MalPEG-Hb, and it was formulated so that the solution properties (oncotic pressure and viscosity) would be identical (50 Torr and 2.2 cPs, respectively). Thus study of these two modified proteins allows conclusions to be drawn about one property: the capacity to transport O₂. The main conclusion of the study is that the beneficial effect of MP4 in supporting life at extremely low hematocrit is a result of its ability to deliver O₂ to tissues, not its oncotic pressure or viscosity.

MalPEG-Hb is currently formulated for human clinical development as 4.2 g/dl in lactated Ringer USP (MP4) (20). Two

Table 2. Parameters at 40 min of exchange transfusion

<table>
<thead>
<tr>
<th></th>
<th>PS</th>
<th>MPA</th>
<th>MP4</th>
</tr>
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<tbody>
<tr>
<td>Hematocrit, %</td>
<td>12.6±0.7</td>
<td>10.7±0.7</td>
<td>8.9±0.6*</td>
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<tr>
<td>Total hemoglobin, g/dl</td>
<td>4.18±0.34</td>
<td>3.68±0.29</td>
<td>5.42±0.20†</td>
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<tr>
<td>Plasma hemoglobin, g/dl</td>
<td>0.00</td>
<td>0.00</td>
<td>2.38±0.06†</td>
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<tr>
<td>Lactate, mM</td>
<td>3.09±1.44</td>
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<td>pH</td>
<td>7.43±0.02</td>
<td>7.48±0.01</td>
<td>7.44±0.01</td>
</tr>
<tr>
<td>PaCO₂, Torr</td>
<td>42.5±2.6</td>
<td>35.5±3.8</td>
<td>45.4±1.0†</td>
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<tr>
<td>PaO₂, Torr</td>
<td>94.4±7.0</td>
<td>107.0±5.4</td>
<td>94.4±2.2</td>
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<tr>
<td>Base excess, mEq/l</td>
<td>2.5±0.8</td>
<td>1.7±2.3</td>
<td>5.8±0.6*</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>91.7±4.8</td>
<td>82.6±7.3</td>
<td>105.8±2.6†</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>483.5±13.5</td>
<td>486.7±15.4</td>
<td>455.3±13.1</td>
</tr>
<tr>
<td>HR/MAP, beats</td>
<td>5.25±0.31</td>
<td>6.02±0.74</td>
<td>4.30±0.30*†</td>
</tr>
</tbody>
</table>

Values are means ± SE. All animals (n = 5 for each group) are alive at this point. PaCO₂, arterial PCO₂; PaO₂, arterial PO₂; MAP, mean arterial pressure; HR, heart rate. *MP4 vs. PS, P < 0.05. †MP4 vs. MPA, P < 0.05.
Concerns are specifically addressed in this study: 1) that the $P_{50}$ of MP4 is so low (~6 Torr) as to prevent release of $O_2$ to tissues, and 2) that the oncotic pressure of MP4 (~50 Torr) is such that its efficacy is due solely to its ability to rapidly and effectively expand the blood volume. To satisfy both of these concerns, we compared the effects of MP4 with MPA prepared by using the identical chemistry so that the molecules were comparable in every regard except that MPA does not bind $O_2$. The protocol involved a continuous exchange transfusion, carried out until the hematocrit was nearly unmeasurable. In the case of MP4 animals, essentially all $O_2$ was supplied by the plasma Hb. The study was not designed for long-term effects or survival.

We found that animals exchange transfused with MP4, but not MPA or PS, survived the entire exchange period with no deaths. All animals in the MPA and PS groups died within the period of the experiments. The study thus confirms that MP4 is able to oxygenate tissues and that the effect is not due to any alternate property of PEG, because MPA was not effective. Furthermore, the study shows that, on a gram-for-gram basis, MalPEG-Hb is as effective in transporting $O_2$ as red cell Hb, despite its low $P_{50}$. This latter conclusion is in agreement with our laboratory’s previous findings (11) that PEG-Hb and human red blood cells release $O_2$ in approximately the same manner in an artificial capillary exposed to pure $N_2$.

It appears that, given enough time, even the animals exchange transfused with MP4 might not have survived; lactic acid was increasing and BE was decreasing at the end of the observation period. Thus a plasma Hb level of 2–2.5 g/dl may not be sufficient to support life indefinitely in rats. However, due to the elevated oncotic pressure of MP4, it may not be possible to achieve a much higher plasma Hb concentration than this, and it might not be necessary in the majority of clinical cases, whereas hematocrit rarely reaches the low levels of these experiments.

Similar concerns were raised in regard to a different product, PEG-modified bovine Hb developed as a tumor radiation enhancer by Enzon. In response, Enzon conducted a comparison of PEG-bovine Hb, PEGylated human serum albumin, oxidized PEGbVHb, and PEGbVHb liganded to CO (3). These authors performed an 85% hematocrit reduction by hemodilution with test material and showed clearly that PEG-Hb led to superior survival, even 2 wk postdosing. In this case, sufficient red blood cells remained after exchange transfusion to ensure adequate tissue supply of $O_2$.

An additional value of the present experiments is that they show a distinct lowering of the critical hematocrit compared to either PS or MPA. At a plasma Hb of ~2 g/dl, MP4 is able to reduce the critical hematocrit from ~15 to ~7% in this model.

In a theoretical model, Hoeft et al. (8) found that, to maintain normal $O_2$ consumption in the heart, the critical hematocrit in the coronary circulation is 14% and the Hb concentration at this hematocrit is 4.7 g/dl, which are values very close to our findings for PS and MPA. Hyperoxia, raising the $P_{O_2}$ to 400 Torr, was found to lower the hematocrit to 12%. However, increasing the $O_2$ consumption by a factor of 3, as might be seen in stress, raised the critical hematocrit to 21%. In the intestinal microcirculation, Van Bommel et al. (17) found that, at a hematocrit of 16%, tissue oxygenation became supply dependent, suggesting this level for the critical hematocrit of the gastrointestinal tract. Thus it would be expected that the actual value of the critical hematocrit might vary by organ, by patient, and in different age groups (4).

Messmer (12) has recommended that perioperative hemodilution should be performed to 20–25%. The present results suggest that MP4 could be effective for moderate hemodilution. However, its relatively low $O_2$ capacity, compared with blood, suggests that its maximal potential would be realized when some red cells are present. Additional experiments need to be performed to model this clinical scenario.

Our experiments leave open the question of the optimal balance between $O_2$-carrying capacity (Hb concentration) and plasma expansion capability (oncotic pressure). These parameters may be of critical importance when a solution is designed for use in patients with cardiovascular disease, for example, who might have a poor cardiac output response to volume load as opposed to patients with a healthier response. Clinical development of such products should be thus careful and prudent, keeping in mind the needs and limitations of individual patient groups.

ACKNOWLEDGMENTS

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REFERENCES


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