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INTRAVASCULAR CIRCULATION AND DISTRIBUTION OF HUMAN ^{51}CR-DBBF STROMA-FREE HEMOGLOBIN, ^{51}CR-PLASMA, ^{51}CR-SALINE, ^{59}FE-PLASMA, AND ^{125}I-ALBUMIN IN THE MOUSE

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Male B6C3HF1 mice were infused with ^{51}Cr labeled DBBF (bis 3,5-dibromosalicyl fumarate) crosslinked stroma-free hemoglobin (SFH). The intravascular halftime (T50) of SFH, determined from plasma hemoglobin levels, was 0.5 hours in the first 10 minutes and 4.3 hours during the next 50 minutes. Twenty-four hours post-infusion, less than 5% of the SFH remained. Elution of ^{51}Cr was reflected in a lower T50 determined from the radioactivity levels: during the first 10 minutes the T50 was 0.3 hours; in the next 50 minutes it was 1 hour. In the first hour following SFH infusion, 11.2 of the infused

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radioactivity was sequestered in the skin, 11.4% in muscle, 9.1% in the skeleton, and 5% in the liver. Twenty-four hours after infusion, 15.4% of the radioactivity was sequestered in the skin, 10.3% in the muscle, 16.6% in the skeleton and 6.7% in the liver. Within the first 24 hours after infusion, the percentages of infused radioactivity in the gastrointestinal tract and kidney ranged from 3.5 to 5.5%, less than 0.4% was found in the spleen and lung, 25% was found in the urine, and 3% in feces.

The circulation and distribution of ^{51}Cr-labeled DBBF-SFH were compared to the values for ^{51}Cr labeled plasma, ^{51}Cr in saline, ^{59}Fe labeled plasma, and ^{125}I albumin. The radioactivity in the intravascular circulation was similar for ^{51}Cr-DBBF-SFH, ^{51}Cr-plasma, and ^{59}Fe-plasma. Within the 24-hour post-transfusion period, the percentage of ^{125}I-albumin in the circulation was significantly higher than that of ^{51}Cr-DBBF-SFH. Only 10% of the ^{51}Cr radioactivity was present in the plasma ten minutes following the infusion. During the 24-hour post-infusion period, extravascular distribution of the ^{51}Cr-saline, ^{51}Cr-plasma, and ^{125}I-albumin within the organs was similar to that of ^{51}Cr-DBBF-SFH: levels were highest in skin, muscle, skeleton and liver; levels in lung and spleen were not increased. The difference in the distribution of ^{59}Fe compared to that of ^{51}Cr-DBBF, ^{51}Cr-plasma, ^{51}Cr-saline, and ^{125}I-albumin can be explained by the fact that the ^{59}Fe was reutilized.

ABSTRACT

Male B6C3HF$_1$ mice were infused with ^{51}CR labeled DBBF (bis 3,5-dibromosalicyl fumarate) crosslinked stroma-free hemoglobin (SFH). The intravascular halftime (T50) of SFH, determined from plasma hemoglobin levels, was 0.5 hours in the first 10 minutes and 4.3 hours during the next 50 minutes. Twenty-four hours post-infusion, less than 5% of the SFH remained. Elution of ^{51}Cr was reflected in a lower T50 determined from the radioactivity levels: during the first 10 minutes the T50 was 0.3 hours; in the next 50 minutes it was 1 hour. In the first hour following SFH infusion, 11.2% of the infused radioactivity was sequestered in the skin, 11.4% in muscle, 9.1% in the skeleton, and 5% in the liver. Twenty-four hours after infusion, 15.4% of the radioactivity was sequestered in the skin, 10.3% in the muscle, 16.6% in the skeleton and 6.7% in the liver. Within the first 24 hours after infusion, the percentages of infused radioactivity in the gastrointestinal tract and kidney ranged from 3.5 to 5.5%, less than 0.4% was found in the spleen and lung, 25% was found in the urine, and 3% in feces.

The circulation and distribution of ^{51}Cr-labeled DBBF-SFH were compared to the values for ^{51}Cr labeled plasma, ^{51}Cr in saline, ^{59}Fe labeled plasma, and ^{125}I albumin. The radioactivity in the intravascular circulation was similar

for ^{51}Cr-DBBF-SFH, ^{51}Cr-plasma, and ^{59}Fe-plasma. Within the 24-hour post-transfusion period, the percentage of ^{125}I-albumin in the circulation was significantly higher than that of ^{51}Cr-DBBF-SFH. Only 10% of the ^{51}Cr radioactivity was present in the plasma ten minutes following the infusion. During the 24-hour post-infusion period, extravascular distribution of the ^{51}Cr-saline, ^{51}Cr-plasma, and ^{125}I-albumin within the organs was similar to that of ^{51}Cr-DBBF-SFH: levels were highest in skin, muscle, skeleton and liver; levels in lung and spleen were not increased. The difference in the distribution of ^{59}Fe compared to that of ^{51}Cr-DBBF, ^{51}Cr-plasma, ^{51}Cr-saline, and I^{125}-albumin can be explained by the fact that the ^{59}Fe was reutilized.

INTRODUCTION

Homologous blood has several disadvantages, e.g., limited storage periods in the liquid state at +4C, necessity of crossmatching, hemolytic reactions, allergic reactions, and transmission of infectious diseases. Stroma-free hemoglobin (SFH) as a red blood cell substitute has the disadvantages of insufficient intravascular circulation and increased oxygen affinity, as well as the potential toxicity of the contaminants and the vasoconstrictor effect of hemoglobin itself. [1-4] The advantages of SFH are its oncotic activity and ability to transport oxygen.

Free hemoglobin binds to plasma haptoglobin, and the hemoglobin-haptoglobin complex is removed from the circulation primarily by the parenchymal cells of the liver[5] or macrophages of the reticuloendothelial system.[6] Free tetrameric hemoglobin molecules dissociate into dimers and monomers and these are excreted through renal glomerular filtration into the urine.[6-7] Chemical modification of the hemoglobin molecule by intramolecular or intermolecular crosslinking has been studied as a means of reducing the rate of tetramer dissociation and renal excretion.[1,8] Although the intravascular circulation of SFH is improved by chemical modification of the hemoglobin molecule, the ultimate fate of SFH following transfusion is not well understood. We have conducted studies in mice to evaluate the intravascular circulation and distribution within the organs of radiolabeled intramolecularly crosslinked human DBBF-SFH, ^{51}Cr-plasma, ^{51}Cr-saline, ^{59}Fe-plasma, and ^{125}I-albumin.

METHODS

Animals

Male B6C3HF$_1$ (C57BL/6) and female (X C3H/He male) mice up to 6 months of age were used in the experiments (Jackson Laboratory, Bar Harbor, ME).

^{51}Cr Radiolabeling of DBBF Crosslinked Human SFH

Human SFH crosslinked by bis 3,5-dibromosalicyl fumarate, (DBBF-SFH) was supplied by Baxter Healthcare Corporation (Deerfield, IL). Fifteen ml of DBBF-SFH, with a mean hemoglobin concentration of 12.6 g/dl, was incubated at 37C for 30 minutes with 30 uCi of disodium chromate (ER Squibb & Son, Princeton, NJ). To remove any excess non-bound ^{51}Cr, the DBBF-SFH sample was transferred to a Centriprep^{tm} concentrator (Centricon, Amicon Division, WR Grace Co, Danvers, MA) with a filtration membrane cutoff of 10,000 MW and centrifuged for 30 minutes at 3000 x g. Approximately 5 ml of the sample passed through the Centriprep membrane; the remainder was diluted to a 15 ml volume with 0.9% sodium chloride and an aliquot of this was counted for gamma radioactivity and the remainder infused into the mice.

The *in vitro* elution of ^{51}Cr from the ^{51}Cr-DBBF-SFH sample was measured throughout the 8 to 24 hours of room temperature storage. A 5 ml aliquot of the sample diluted with saline to a volume of 15 ml was centrifuged in Centriprep concentrators to separate the ^{51}Cr bound to protein from the free ^{51}Cr. The ^{51}Cr radioactivity in the filtrate and retentate portions was measured, and the percentages of DBBF-SFH-bound ^{51}Cr and free ^{51}Cr were calculated.

Radiolabeling of Plasma, Albumin, and Saline Infusates

A 5 ml volume of heparinized mouse plasma was incubated at 37C for 30 minutes with 20 uCi of ^{51}Cr-disodium chromate (^{51}Cr-Plasma) or with 5 uCi of ^{59}Fe citrate (New England Nuclear, Boston, MA) (^{59}Fe-Plasma); ^{51}Cr was incubated with saline alone to achieve 2 uCi/ml ^{51}Cr dissodium chromate (^{51}Cr-Saline); and an ^{125}I human albumin solution (Mallinkrodt) was diluted to 0.02 uCi/ml with normal saline (^{125}I-Albumin).

To determine the percentages of free and protein-bound radioactivity in the infusates, approximately 1 ml of the radiolabeled albumin or radiolabeled plasma was diluted with normal saline to a final volume of 15 ml, and centrifuged in a Centriprep microconcentrator with a filtration membrane cutoff of 10,000 MW.

51Cr-Labeling of Fresh and Stored Mouse Red Blood Cells

Blood was collected from 20 mice into heparinized syringes by aortic puncture through a laparotomy-incision and pooled to a volume of approximately 20 ml. The blood was centrifuged in a Sorvall GLC-1 at 2400 RPM (590 X g) for 6-8 minutes, and the plasma was removed. The red blood cells were washed twice as follows: the red cell concentrate was diluted to approximately 3 times its volume with 0.9% sodium chloride then centrifuged at 2400 RPM (590 X g) for 6-8 minutes and the supernatant removed. The diluted red blood cells were resuspended with 0.9% sodium chloride to a hematocrit of 80% and incubated for 30 minutes at 37C with 1 to 2 uCi ^{51}Cr disodium chromate per ml. The ^{51}Cr-labeled red blood cell suspension was centrifuged at 2400 RPM (590 X g) for 6-8 minutes and the supernatant removed. The red cells were washed twice with 0.9% sodium chloride and resuspended with 0.9% sodium chloride to a hematocrit of 40%. Heparinized red

blood cell samples that had been stored at +4C for 14 days were washed up to 4 times before ^{51}Cr-labeling and 3 times after labeling to remove the supernatant hemoglobin. Four ml of the ^{51}Cr-labeled red cell suspension were used for gamma counting, and the remainder for infusion.

Experimental Protocol

Each of 20 mice was weighed, anesthetized by intraperitoneal injection of chloral hydrate (0.5 mg/g body weight), and infused retro-orbitally with 0.25 ml of ^{51}Cr-DBBF-SFH, ^{51}Cr-plasma, ^{59}Fe-plasma, ^{125}I-albumin, or ^{51}Cr-saline, after which blood and organ samples were obtained for radioactivity measurements. Ten minutes and 1 hour and 24 hours following infusion, blood samples were collected into heparinized syringes by aortic puncture through a laparotomy incision under anaesthesia. The blood samples from the 20 mice were pooled to produce a sample of approximately 4 ml for each collection time. One hour and 24 hours, and sometimes 5 hours, after infusion, the mice were sacrificed by cervial dislocation and the liver, spleen, kidneys, lungs, gastrointestinal tract, and skin were removed as quickly as possible. Samples of skeleton and muscle were also obtained by cleaning off as much tissue from bone as possible by scissors and scalpel. Weights on the organ samples were recorded. Urine and feces samples were collected for 24 hours following infusion from 10 mice that were kept in metabolic cages. Hematocrits were measured in post infusion blood samples. Aliquots of the post-infusion blood samples were centrifuged at 2400 RPM (590 x g) for 6-8 minutes, the plasma was isolated, and protein-bound radiolabel in the plasma was measured using the Centriprep microconcentrators with a

10,000 MW cutoff. The Centricon^{tm} microconcentrator (Amicon Division, WR Grace Co, Danvers, MA) was used when the volume of the sample was 2 ml or less. Each of the organs was weighed. The infusate, the postinfusion blood and plasma samples, 24-hour urine and feces samples, and the filtrate and retentate obtained following microconcentration, were counted in a well-type gamma counter (Model 1185, TM Analytic, Elk Grove Village , IL).

Hemoglobin and Methemoglobin Measurements

The hemoglobin levels in the DBBF-SFH infusate and the post-infusion plasma samples were measured by the cyanmethemoglobin method. When plasma hemoglobin concentrations were below 500 mg/dl, the measurements were repeated using a dual beam spectrophotometric method[9]. Methemoglobin measurements were made using the Co-oximeter (Instrumentation Laboratories, Lexington, MA, Model 282).

Intravascular Circulation Of Infused Radioactivity

In all calculations, radioactivity is expressed as counts per minute (CPM), and hematocrit (Hct) as a decimal.

The intravascular circulation of the infused radioactivity was calculated from the radioactivity in the injectate (INJ) and the blood samples at each of the sampling times. Post-infusion red cell volume (RCV) and total blood volume (TBV) were estimated from the body weight (BW) and hematocrit (Hct) as follows:

$$RCV\ (ml) = 0.017\ BW + 0.29$$

$$TBV\ (ml) = RCV\ (ml)\ /Hct$$

$$PV\ (ml) = TBV\ (ml) - RCV\ (ml)$$

The percent recovery of radioactivity in the whole blood (WB), plasma, and red blood cells (RBC) was calculated as follows:

Whole blood (%) = WB (cpm/ml) X TBV (ml) X 100/ INJ (total cpm)

Plasma (%) = Plasma (cpm/ml) X PV (ml) X 100/ INJ (total cpm)

Red Blood Cells (%) = Whole Blood (%) - Plasma (%)

The percent circulation of the DBBF-SFH was also determined using the hemoglobin concentrations of the injectate and post-infusion plasma samples as follows:

Plasma (%) = Plasma (mg/dl) X PV (ml) /INJ (total mg)

Distribution of Infused Radioactivity

The radioactivity sequestered in each organ was determined by subtracting the estimated radioactivity due to the blood content of the organ from the measured total organ radioactivity. The organ blood volume (BV) was estimated from the organ weight as previously described.

The organ intravascular blood volume radioactivity was estimated as follows:

Organ intravascular radioactivity (CPM) =

Blood radioactivity (cpm/ml) X Organ Blood Volume (ml)

The extravascular radioactivity sequestered in each organ as a percentage of infused radioactivity was calculated as follows:

Sequestered (%)=

[100 X (total organ (cpm)-organ intravascular (cpm))] / INJ (cpm)

The % sequestered radioactivity was expressed per gram of organ weight and per gram of femur marrow. Seventeen percent of the femur weight was estimated to be attributable to marrow.

Distribution of Radioactivity Within the Femur

The distribution of radioactivity within the femur was determined in mice sacrificed 24 hours after infusion. The femur was

cleaned of muscle tissue as described above. The remaining muscle and connective tissues were removed by incubating the femur for 30 minutes at 37C in a 2% collagenase solution (Millipore Corporation, Freehold, NJ, 126 u/mg), washing it with saline, and then incubating it in a 0.3% trypsin solution (Flow Laboratories, McLean, VA) at 37C for 30 minutes. After washing with saline, both ends of the femur were cut and the bone marrow was flushed four times with saline. The bone was incubated in collagenase solution and then in trypsin solution as described above, flushed out with saline, and counted for radioactivity. Radioactivity was also counted in all the solutions used. The radioactivity in the bone, bone marrow, extra-osseus muscle and connective tissue was reported as a percent of total femur. For comparison, the distribution of comparison, the distribution of radioact measured in the mice transfused with ^{51}Cr-labeled fresh red blood cells and heparinized red blood cells stored at +4 C for 15 days.

Statistical Analysis

The differences in the circulation of radioactivity associated with ^{51}Cr-DBBF-SFH, ^{51}Cr-plasma, ^{51}Cr-saline, ^{125}I-albumin, and ^{59}Fe-plasma were determined by analysis of variance, and the least-squares means method was used for multiple comparisons using SAS (Statistical Analysis Systems, Cary, NC) licensed to Boston University.

RESULTS

In Vitro Elution of 51Cr-DBBF-SFH

The elution of ^{51}Cr from ^{51}Cr-DBBF-SFH was 6% during the first 8 hours of storage at room temperature and 7% 24 hours after infusion.

Plasma Hemoglobin and Methemoglobin Levels Following DBBF-SFH Infusion

The half-time of the DBBF-SFH 10 minutes after infusion was 0.5 hours (Figure 1, Tables 1, 2, 3); the half-time was 4.3 hours 1 hour post-infusion, and 4.0 hours during the 1 to 5 hour post-infusion period. The methemoglobin level in the infused DBBF-SFH was 15.4%: 10 minutes after infusion the value was 16%; 1 hour after infusion it was 11.9%, and 5 hours after infusion it was 8.7% (Table 1).

Intravascular Circulation of Infused Radioactivity

The disappearance of DBBF-SFH was faster when it was determined from the circulation of infused radioactivity than when it was determined from the hemoglobin level (Figures 1 and 2, Tables 2 & 3). Fifty-nine percent of the radioactivity associated with ^{51}Cr-DBBF-SFH disappeared from the circulation within 1 hour of infusion and only 4% remained in the blood 24 hours post-infusion. The half-time was 0.3 hours during the first 10 minutes post-infusion, the half-time was 1.0 hour during the 10 minute to one-hour post-infusion period and 12.6 hours during the 1 to 24 hours post-infusion (Table 3).

Approximately 50% of the ^{51}Cr-plasma was removed from the bloodstream 10 minutes after infusion; and only 6% remained in the circulation 24 hours after infusion (Figure 2, Table 2). The half-time of the ^{51}Cr-plasma was 0.2 hours for the first 10 minutes, and 13.9 hours during the 24 hour post-infusion period.

Approximately 56% of the radioactivity associated with ^{125}I-albumin remained in the circulation 1 hour after infusion and 16% remained 24-hours after infusion: the half-time was 0.3 hours 10 minutes post-infusion and 16.0 hours 24 hours post-infusion.

About 90% of the radioactivity associated with the ^{51}Cr-saline disappeared from the bloodstream within 10 minutes after infusion (Figure 2, Table 2). Approximately 56% of the radioactivity associated with ^{59}Fe was removed during the first hour post-infusion: the half-time time was 0.3 hours during the first 10 minutes (Figure 2, Table 2).

The distribution of free (<10,000 MW) and bound radiolabel (>10,000 MW) in the infusate and the post-infusion samples is reported in Tables 4, 5, and 6. During the 24-hour post-infusion period, a mean of 8% of the ^{51}Cr in the DBBF-SFH in the injectate was free (<10,000 MW), and between 5% and 9% in the circulation was free (<10,000 MW) (Table 4). Thirty percent of the ^{51}Cr in the ^{51}Cr-plasma infusate was free, 14% was free 10 minutes after infusion, 11% was free 1 hour after infusion, and 3% was free 24 hours after infusion. Less than 5% of the radioactivity in the ^{59}Fe plasma and the ^{125}I albumin was free. Twenty-nine percent of the radioactivity in the saline was free 10 minutes after infusion, 14% was free 1 hour after infusion, and 2% was free 24 hours after infusion.

Twenty-four hours after infusion, 1% or less of ^{51}Cr-DBBF-SFH or ^{51}Cr-plasma, 5% of ^{51}Cr-saline, and 46% of ^{59}Fe-plasma was associated with the red cells (Table 7).

Distribution of Infused Radioactivity in Organs

Large proportions of ^{51}Cr radioactivity were found in the skin, muscle, and skeleton following the infusion of ^{51}Cr-DBBF-SFH, (Figure 3, Table 8). One hour after infusion, 11% of the radioactivity was in the skin and 9% in the skeleton. Twenty-four hours after infusion, 15% of the radioactivity was in the skin, 17% in the skeleton, and 11% was in muscle. The uptake in the liver was 5% one hour after infusion and 7% 24 hours after infusion. One hour, 5 hours, and 24 hours after infusion, less than 1% of the infused ^{51}Cr-DBBF-SFH radioactivity was observed in the spleen and lung. Three to 6% of the infused ^{51}Cr-DBBF-SFH radioactivity was in the gastrointestinal tract or kidneys 1 hour, 5 hours, and 24 hours after infusion (Table 8).

One hour after the infusion of ^{125}I albumin, 12% of the radioactivity was found in the muscle, 9% in the skin, and 5% was in the skeleton (Table 8). Twenty-four hours after infusion, 15% of the radioactivity was in the muscle, 11% was in the skin, and 4% in the skeleton. Uptake of ^{125}I-albumin in the liver was 6% one-hour after infusion and 2% twenty-four hours after infusion. Between one and 24 hours after infusion, less than 1% uptake was in the spleen and lung, and less than 6% in the gastrointestinal tract or kidneys.

Between one and 24 hours after the infusion of ^{59}Fe-plasma, 23% of the radioactivity was in the skeleton and 13% was in the muscle (Table 8). The uptake in the liver one hour after infusion was 7%, and 24 hours after infusion was 13%. The uptake in the spleen and lungs was 2% twenty-four hours after infusion. From 1 to 24 hours after infusion, the uptake in the gastrointestinal tract was approximately 10%, and in the kidneys uptake was 2%.

One hour after the infusion of ^{51}Cr-saline, 19% of the radioactivity was in the muscle, 7% in the skin, and 9% in the skeleton. Twenty-four hours after infusion, the uptake was 5% in the muscle, 5% in the skin, and 11% in the skeleton. From one to 24 hours after infusion, less than 1% of the ^{51}Cr radioactivity was in the lungs and spleen, 5 to 9% was in the gastrointestinal tract, and 4 to 11% was in the kidneys.

Tables 9 and 10 report the uptake of radioactivity per gram of tissue following the infusion of ^{51}Cr-DBBF-SFH. One hour after the infusion of the ^{51}Cr-DBBF-SFH, the uptake of radioactivity was 36% in the bone marrow, 9% in the kidneys, 3% in the liver, 3% in the lung, and less than 1% in the spleen. Twenty-four hours after infusion the uptake was 67% in the marrow; less than 5% in the liver, spleen, and lung; and 13% in the kidneys reflecting the free ^{51}Cr excreted in the urine.

Twenty-four hours after infusion of ^{51}Cr-DBBF-SFH 25% of the radioactivity was in the urine and 3% in feces; total recovery of the ^{51}Cr-DBBF radioactivity infused was 88% (Table 11). Twenty-four hours after infusion of ^{51}Cr-plasma and ^{51}Cr-saline, 30% of the radioactivity was in the urine and 8% was in the feces; whereas 24 hours after infusion of ^{125}I-albumin, 19% of the radioactivity was in the urine and 7% in the feces. The total recovery of infused radioactivity associated with ^{51}Cr-plasma, ^{51}Cr-saline, and ^{125}I-albumin was approximately 80%. Twenty-four hours after infusion of ^{59}Fe-plasma, less than 2% of the radioactivity was in the urine and feces, and total recovery of the infused radioactivity was 106% (Table 11).

Twenty-four hours following infusion of the ^{51}Cr-DBBF-SFH, 66% of the radioactivity in the femur was associated with bone, 17% with bone marrow, and 20% with the muscle and connective tissue (Table 12). Twenty-four hours following the infusion of ^{51}Cr-saline, 85% of the radioactivity in the femur was associated with bone. Twenty-four hours following ^{125}I-albumin infusion, 75% of the radioactivity in the femur was associated with muscle and connective tissue. Twenty-four hours following the infusion of ^{59}Fe-plasma, 64% of the radioactivity in the femur was in the bone marrow and 24% in the bone. Twenty-four hours following the infusion of 14-day-old ^{51}Cr-labeled red blood cells, 50% of the radioactivity in the femur was in the bone marrow and 38% in the bone (Table 12).

DISCUSSION

The circulation of stroma-free hemoglobin following infusion is influenced by the method of preparation and the concentration of the preparation; the species of animal studied also influence the results.[4,6,7] Bunn et al[10] studied rats and found that the intravascular half-time of unmodified SFH was less than 30 minutes, whereas DeVenuto et al[11] reported a half-time of 3.5 hours. The half-time of pyridoxalated and polymerized SFH in rats has been reported to be 25 to 30 hours in rats,[12,13] whereas the half-time of DBBF crosslinked SFH was found to range from 4 to 24 hours depending on the dose of DBBF that was infused.[14]

In our study, the circulation of ^{51}Cr-DBBF and ^{59}Fe-plasma were similar during the first 10 minutes after infusion; infused ^{51}Cr-plasma was cleared at a faster rate, probably because it contained 30% free ^{51}Cr. During the twenty-four hour post-infusion period, half-times were similar for ^{51}Cr-DBBF-SFH, ^{125}I-albumin, and ^{51}Cr-plasma.

Twenty-four hours following ^{51}Cr-DBBF-SFH infusion, 25 percent of the ^{51}Cr radioactivity was excreted in the urine. However, this value could not be used as an indication of the urinary excretion of DBBF-SFH both because of the elution of ^{51}Cr from DBBF and because most of the ^{51}Cr excreted in the urine was free and not associated with hemoglobin molecule. Velkey and associates[15] reported that 8% of ATP modified SFH was excreted in the urine, and Hess and associates[14] reported that less than 1% of DBBF-SFH infused in pigs was excreted in the urine.

During the first 24 hours following the infusion of human DBBF-SFH, almost 50% of the ^{51}Cr radioactivity was retained in the organs,

the main sites of sequestration being the skin, muscle, and skeleton. Of the ^{51}Cr-DBBF-SFH radioactivity seen in the femur, the bone contained four times more than the bone marrow. During this time, the uptake of the ^{51}Cr-DBBF-SFH in the liver was only 5 to 7%, and little uptake was observed in the spleen. Bleeker and associates[16] have reported intramolecularly crosslinked hemoglobin distributed into the extravascular space shortly after exchange transfusion. We found the distribution of the infused ^{51}Cr-labeled DBBF crosslinked SFH with a molecular weight of 64,000 was similar to that of ^{125}I-albumin with a molecular weight of 69,000.[20] Rothschild and associates[17] reported that 18% of the ^{131}I-human albumin accumulated in the skin and 15% of the ^{131}I-human albumin accumulated in muscle following infusion into humans. Feola and associates[18] observed hemoglobin distribution into the pulmonary interstitial space following infusion of unmodified bovine SFH into rabbits. Marks and Brown[19] found that pyridoxalated polymerized human SFH had distributed into the extravascular space in dogs. Velky and associates[15] reported that 2 to 3% of unmodified or ATP-modified SFH distributed into the peritoneal cavity 4 hours after infusion in rats.

Twenty-four hours following the infusion of ^{125}I-albumin, the uptake in the skeleton, liver, and spleen was significantly less than that seen with the ^{51}Cr-labeled DBBF-SFH, ^{51}Cr-plasma, and ^{51}Cr-saline. One-hour following ^{59}Fe-plasma infusion, 24% of radioactivity was in the skeleton, and 24-hours after transfusion, 46% of the radioactivity was in the red blood cells. Iron binds to plasma transferrin, which has the molecular weight of 76,000[20]. One hour and

24-hours after infusion of ^{59}Fe-plasma, 13% of the Fe was found in the muscle which contains myoglobin-an iron-porphyrin complex.

The rapid removal of ^{51}Cr-DBBF-SFH from the circulation was by distribution into the extravascular volume and not through urinary excretion. Since the DBBF crosslined human SFH infused intravenously into mice was distributed mainly in the extravascular volume of the skin, muscle, and skeleton, the potential toxicity of DBBF-SFH in the extravascular volume should be investigated.

FIGURE 1

Plasma hemoglobin and ^{51}Cr radioactivity following infusion of ^{51}Cr labeled DBBF-SFH.

FIGURE 2

Intravascular circulation of radioactivity following infusion of ^{51}Cr-DBBF- SFH, ^{51}Cr-plasma, ^{59}Fe-plasma, ^{125}I-albumin, and ^{51}Cr-saline

FIGURE 3

Recovery of radioactivity in organs and plasma at 1-hour and 24-hours following infusion of ^{51}Cr-labeled DBBF-SFH.

TABLE 1

PLASMA HEMOGLOBIN AND METHEMOGLOBIN LEVELS FOLLOWING INFUSION OF ^{51}CR-DBBF STROMA-FREE HEMOGLOBIN SOLUTION

Time following Infusion:		10 Min	1 Hour	5 Hour	24 Hour
Hemoglobin Concentration (mg/dl):	Mean:	2393	2148	1069	230
	SD:	340	252	98	171
	n:	8	8	2	6
Intravascular Circulation (% of infused):	Mean:	82.5	74.1	36.9	<5
	SD:	11.7	8.7	2.1	-
	n:	8	8	2	6
Methemoglobin* Circulation (%):	Mean:	16.0	11.9	8.7	-
	SD:	3.0	2.1	2.3	
	n:	7	6	2	

*Infused DBBF: 15.4 $\pm$ 4% methemoglobin

TABLE 2

INTRAVASCULAR CIRCULATION OF ^{51}CR RADIOLABELED DBBF STROMA FREE HEMOGLOBIN, 51CR PLASMA, 59FE PLASMA, 125I ALBUMIN, AND 51CR SALINE

		Percent of Infused Radioactivity in Plasma		
Time Following Infusion:		10 Min	1 Hour	24 Hour
INFUSATE				
51Cr DBBF-SFH	Mean:	71.0	41.3	3.6
(n=12)	SD:	8.1	6.1	1.2
51Cr Plasma	Mean:	53.7*	35.4	6.2
(n=6)	SD:	9.1	5.3	1.6
59Fe Plasma	Mean:	75.1	44.4	1.1
(n=6)	SD:	10.0	7.8	1.9
125I Albumin	Mean:	64.0	56.2*	15.6*
(n=7)	SD:	13.0	12.4	5.9
51Cr Saline	Mean:	9.7*	6.3*	2.4
(n=9)	SD:	1.8	0.9	0.7

* $p < 0.01$ Significant difference compared to 51Cr-DBBF-SFH by Least Squares Method

TABLE 3

INTRAVASCULAR CIRCULATION OF 51CR-DBBF-SFH AND DBBF-SFH

Time After Infusion:	0-10 Min	10 Min-1 Hr	1-5 Hr	1-24 Hr
Loss of 51CR-DBBF: from the circulation (%)	29%	30%	-	38%
51Cr Halftime: Time for 50% to be removed from the circulation	0.3 Hr	1 Hr	-	12.6 Hr
Loss of Hemoglobin: from the circulation (%)	18%	8%	37%	-
Hemoglobin Halftime: Time for 50% to be removed from the circulation	0.5 Hr	4.3 Hr	4.0 Hr	-

TABLE 4

PERCENTAGE OF FREE RADIOLABEL (<10,000 MW) TO THE FREE AND BOUND RADIOACTIVITY MEASURED IN THE INJECTATE, PLASMA SAMPLES 10-MINUTES, 1-HOUR, AND 24-HOURS FOLLOWING INFUSION, AND IN THE 24 HOUR URINE SAMPLE

Infusate		Radiolabeled Injectate	Plasma samples following infusion 10 Min	1 Hr	24 Hr	24 Hr Urine
51Cr-SFH-DBBF						
	1.	8	-	-	-	-
	2.	6	-	-	5	100%
	3.	10	3	11	3	100%
	4.	12	-	-	-	96%
	5.	10	8	9	7	-
	6.	2	-	-	-	-
	7.	10	12	8	3	100%
	8.	7	5	9	6	100%
	9.	7	6	8	4	100%
Mean:		8	7	9	5	100%

TABLE 5

PERCENTAGE OF FREE RADIOLABEL (<10,000 MW) TO THE FREE AND BOUND RADIOACTIVITY MEASURED IN THE INJECTATE, PLASMA SAMPLES 10-MINTUES, 1-HOUR, AND 24-HOURS FOLLOWING INFUSION, AND IN THE 24 HOUR URINE SAMPLE

Infusate	Radiolabeled Injectate	Plasma samples following infusion 10 Min	1 Hr	24 Hr	24 Hr Urine
^{51}Cr-Plasma					
1.	31	14	10	1	100%
2.	29	13	11	1	-
3.	-	-	-	5	100%
4.	-	-	-	4	100%
Mean:	30	14	11	3	100%
^{59}Fe-Plasma					
1.	<1	-	-	-	*
2.	<1	-	-	-	*
3.	<1	0	0	5	*
4.	0	-	-	0	*
Mean:	0	0	0	3	
^{125} Albumin					
1.	<1	0	0	0	100%
2.	2	0	0	5	100%
3.	2	0	0	3	99%
Mean:	2	0	0	3	100%
^{51}Cr-Saline					
1.	100	30	-	2	100%
2.	93	29	14	2	100%
3.	-	28	13	2	100%
Mean:	96	29	14	2	100%

* Radioactivity too low to measure

TABLE 6

MEAN PERCENT FREE RADIOLABEL (<10,000 MW) TO THE FREE AND BOUND RADIOACTIVITY

		Plasma samples following infusion			24 Hr
	Injectate	10 Min	1 Hr	24 Hr	Urine
51Cr SFH-DBBF (n=9)	8	7	9	5	100
51Cr Plasma (n=2)	30	14	11	3	100
59Fe Plasma (n=2)	0	0	0	3	-
125I Albumin (n=3)	2	0	0	3	100
51Cr Saline (n=3)	96	29	14	2	100

TABLE 7

***IN VIVO* UPTAKE OF RADIOLABEL BY RED BLOOD CELLS AFTER INFUSION OF 51CR-DBBF STROMA FREE HEMOGLOBIN, 51CR PLASMA, 59FE PLASMA AND 51CR SALINE**

		Percent of Infused radioactivity in RBC		
Time following infusion:		10 Min	1 Hr	24 Hr
INFUSATE				
51Cr SFH-DBBF	Mean:	1.0	<1	<1
(n=12)	SD:	1.3		
51Cr Plasma	Mean:	<1	<1	<1
(n=6)	SD:			
59Fe Plasma	Mean:	2.2	7.4	45.5
(n=6)	SD:	2.7	3.1	11.7
51Cr Saline	Mean:	4.8	4.5	4.7
(n=9)	SD:	1.6	1.4	1.1

TABLE 8
DISTRIBUTION OF INFUSED RADIOACTIVITY
Percent of Total Infused Radioactivity

	Time Following Infusion		Skeleton	Skin	Muscle	Liver	Lung	Spleen	GI	Kidney
^{51}Cr SFH (DBBF) (n=7)	1 Hour	Mean:	9.1	11.2	11.4	5.0	0.4	0.0	5.5	3.5
		SD:	1.0	2.8	3.1	1.6	0.4	0.0	0.6	0.7
	5 Hours	Mean:	14.9	12.8	10.9	4.1	0.2	0.1	4.5	3.6
		SD:	0.1	0.4	0.7	0.6	0.1	0.0	1.0	0.2
	24 Hours	Mean:	16.6	15.4	10.3	6.7	0.4	0.3	4.4	5.2
		SD:	1.2	2.6	1.6	2.0	0.1	0.1	1.5	1.4
^{51}Cr Plasma (n=3)	1 Hour	Mean:	5.9	8.8	11.9	4.9	0.3	0.0	4.4	5.2
		SD:	0.5	0.3	4.5	0.9	0.1	0.0	1.3	0.6
	24 Hours	Mean:	9.8	9.2	6.8	4.0	0.3	0.1	2.9	2.7
		SD:	1.3	1.1	0.7	0.4	0.1	0.0	0.1	0.2
^{59}Fe Plasma (n=4)	1 Hour	Mean:	23.5	3.7	13.7	6.6	0.7	3.0	7.7	2.4
		SD:	3.5	1.3	5.1	0.9	0.9	1.2	1.6	0.7
	24 Hours	Mean:	21.7	2.6	13.3	13.1	1.9	2.3	10.2	1.9
		SD:	2.1	1.5	6.0	1.6	1.0	0.2	4.1	0.6
^{125}I Albumin (n=6)	1 Hour	Mean:	5.2	8.5	11.8	6.0	0.8	0.0	5.9	2.1
		SD:	1.2	2.9	5.8	1.4	0.8	0.0	0.9	0.9
	24 Hours	Mean:	4.3	11.2	14.8	1.6	0.4	0.0	3.8	0.8
		SD:	1.2	3.1	1.2	0.5	0.3	0.0	1.2	0.3
^{51}Cr Saline (n=4)	1 Hour	Mean:	9.1	6.7	19.2	10.9	0.6	0.02	8.9	11.1
		SD:	1.0	0.2	6.1	2.8	0.2	0.0	1.1	0.7
	24 Hours	Mean:	10.9	4.6	5.1	6.3	0.3	0.1	4.8	4.4
		SD:	3.2	0.6	0.6	1.9	0.0	0.0	0.7	2.1

TABLE 9

DISTRIBUTION OF 51CR-DBBF-SFH 1 HOUR AFTER INFUSION

PERCENT OF INFUSED RADIOACTIVITY PER GRAM OF TISSUE

Number	Liver	Spleen	Kidneys	Lungs	Femurs	Marrow in the Femurs
1	2.95	0	6.92	0.62	8.89	44.50
2	2.33	0	6.04	0.07	6.98	34.90
3	2.65	0	5.70	0	6.57	32.85
4	2.59	0	7.82	0	7.40	37.00
5	3.60	0	11.70	6.92	9.89	49.45
6	4.02	0	14.81	8.04	12.80	64.00
7	3.32	0	12.11	5.73	8.98	44.90
8	4.93	4.81	10.79	10.32	10.00	50.00
9	3.47	0	7.84	0	8.78	43.90
10	2.26	0	7.73	4.23	10.00	50.00
11	2.49	0	7.86	0	5.71	28.55
12	3.59	0	8.31	1.42	9.11	45.55
13	2.59	0	6.99	3.98	4.96	24.80
14	2.81	0	9.43	2.82	7.50	37.50
15	2.65	0	8.16	0.76	4.86	24.30
16	2.84	0	7.42	2.17	4.61	20.80
17	2.46	0	6.54	0	9.55	47.75
18	1.96	0	6.97	0.57	7.10	35.50
19	2.30	0	6.75	0	2.05	10.25
20	2.28	0	6.73	0	7.53	37.65
21	2.40	0	7.01	0	4.13	21.50
22	3.69	0	11.25	0	3.50	17.50
23	3.71	0	9.46	0	6.46	32.30
24	5.06	0	9.69	0.50	3.77	18.85
25	5.79	6.55	17.79	11.46	8.91	44.55
26	5.70	0	15.75	6.07	7.66	37.75
27	3.41	0	10.42	0.70	5.80	29.0
28	4.95	0	10.83	0	4.72	23.6
29	4.96	0	15.80	6.96	11.61	58.05
Mean:	3.37	0.39	9.47	2.53	7.24	36.2
SD:	1.12	1.51	3.20	3.41	2.50	12.5

TABLE 10

DISTRIBUTION OF 51CR-DBBF-SFH 24 HOURS AFTER INFUSION

Percent of infused radioactivity per gram of tissue

Number	Liver	Spleen	Kidneys	Lungs	Femurs	Marrow in the Femurs
1	3.13	2.92	10.00	1.62	13.24	66.20
2	3.88	3.65	12.54	2.25	15.85	79.25
3	3.68	3.16	11.47	2.15	13.90	69.50
4	0.20	3.81	12.03	2.64	11.90	59.50
5	3.12	4.52	12.81	2.52	23.65	118.25
6	3.05	3.21	10.32	0.82	19.38	96.90
7	3.05	3.39	8.81	1.56	15.51	77.55
8	3.29	0	10.89	3.08	13.74	68.70
9	3.87	5.12	15.44	2.67	18.38	91.90
10	3.34	0	9.55	1.41	14.88	74.40
11	2.80	2.36	10.09	1.97	12.98	64.90
12	2.78	2.35	7.96	1.68	11.11	55.55
13	3.63	2.86	8.58	1.80	10.69	53.45
14	3.55	2.86	8.68	2.56	10.14	50.70
15	3.78	3.73	7.30	2.46	9.00	45.00
16	4.24	5.76	14.02	2.69	17.95	89.75
17	3.75	5.88	10.16	2.96	11.62	58.10
18	4.28	6.00	12.26	2.84	8.83	44.15
19	3.99	5.45	10.82	2.82	13.48	67.40
20	3.06	4.22	7.88	2.26	7.02	35.10
21	3.37	7.67	9.53	2.50	9.75	48.75
22	3.50	4.13	10.48	2.52	10.00	50.00
23	3.60	5.68	11.37	2.54	11.19	55.95
24	6.44	7.86	23.1	4.50	14.80	74.00
25	7.92	6.15	24.84	4.83	17.66	88.30
26	9.12	6.60	26.54	4.32	17.10	85.50
27	8.31	8.93	26.49	4.40	12.22	61.10
28	8.41	6.03	16.77	3.06	10.68	53.40
Mean:	4.14	4.36	12.79	2.61	13.69	67.26
SD:	2.91	2.02	5.40	0.90	3.76	18.79

TABLE 11

MEAN TOTAL PERCENT RECOVERY OF RADIOACTIVITY 24 HOURS AFTER INFUSION

	^{51}Cr DBBF-SFH	^{51}Cr PLASMA	^{59}Fe PLASMA	^{125}I ALBUMIN	^{51}Cr SALINE
Number of Experiments	3	2	2	3	2
Blood	2.7	7.4	37.7	15.0	8.4
Organs*	57.2	36.5	66.4	36.9	35.5
Excretion					
Urine	25.1	31.9	0.1	18.6	28.9
Feces	3.1	7.5	1.5	7.3	8.6
TOTAL:	88.1	83.3	105.7	77.8	81.4

*Skeleton, skin, muscle, liver, lungs, spleen, GI, kidneys

TABLE 12

DISTRIBUTION OF INFUSED RADIOACTIVITY IN THE FEMUR AT 24 HOURS

(Percent, Mean ± SD)

	BONE WITHOUT MARROW	MARROW	MUSCLE AND CONNECTIVE TISSUE	MARROW/BONE+MARROW (PERCENT)
^{51}Cr-DBBF-SFH (n=3)	66.0±7.6	17.4±2.6	19.7±10.6	20.9%
^{125}I Albumin (n=3)	8.0±1.7	17.1±3.7	75.0±3.1	68.1%
^{51}Cr Plasma (n=3)	52.6±7.6	23.9±2.6	23.5±5.0	31.2%
^{51}Cr Saline (n=3)	85.0±0.8	5.4±2.2	9.9±1.4	6.0%
^{59}Fe Plasma (n=3)	23.7±7.9	63.5±3.1	12.8±0.1	72.8%
^{51}Cr Fresh RBC (n=3)	38.7±2.2	25.1±10.5	36.5±11.4	39.3%
^{51}Cr 14-day Stored RBC (n=3)	38.1±0.5	50.4±2.5	11.4±2.3	56.9%

REFERENCES

1. Feola M, Simoni J, Canizaro PC, Tran R, Raschbaum G, Behal FJ: Toxicity of polymerized hemoglobin solutions. Surg Gynec Obstet 166:211-222, 1988.

2. White CT, Murray AJ, Greene JR, Smith DJ, Medina F, Makovec GT, Martin EJ, Bolin RB: Toxicity of human hemoglobin solution infused into rabbits. J Lab Clin Med 108:121-131, 1986.

3. Vogel WM, Lieberthal W, Apstein CS, Levinsky N, Valeri CR: Effects of stroma-free hemoglobin solutions on isolated perfused rabbit hearts and isolated perfused rat kidneys. In Blood Substitutes, Eds. TMS Chang and RP Geyer, Marcel Dekker, Inc, New York, NY, 1988, pps 227-236.

4. DeVenuto F: Hemoglobin solutions as oxygen-delivering resuscitation fluids. Crit Care Med 10:238-245, 1982.

5. Weinstein MB, Segal HL: Uptake of free hemoglobin by rat liver parenchymal cells. Biochem Biophys Res Comm 123:489-496, 1984.

6. DeVenuto F, Friedman HI, Neville JR, Peck CC: Appraisal of hemoglobin solution as a blood substitute. Surg Gynec Obstet 149:417-436, 1979.

7. Greenburg AG, Peskin GW, Hoyt DB, Moores WY: Is it necessary to improve the intravascular retention of hemoglobin solutions? Crit Care Med 10:266-269, 1982.

8. Kahn RA, Allen RW, Baldassare J: Alternate sources and substitutes for therapeutic blood components. Blood 66:1-12, 1985.

9. Blakney GB, Dinwoodie AJ: A spectrophotometric scanning technique for the rapid determination of plasma hemoglobin. Clin Biochem 8:96-102, 1975.

10. Bunn HF, Esham WT, Bull RW: The renal handling of hemoglobin. I. Glomerular filtration. J Exp Med 129:909-924, 1969.

11. DeVenuto F, Moores WY, Zegna AI, Zuck TF: Total and partial blood exchange in the rat with hemoglobin prepared by crystallization. Transfusion 17:555-562, 1977.

12. DeVenuto F, Zegna A: Blood exchange with pryidoxalated and polymerized hemoglobin solution. Surg Gynec Obstet 155:342-346, 1982.

13. Keipert PE, Chang TMS: Effects of partial and total isovolemic exchange transfusion in fully conscious rats using pyridoxylated polyhemoglobin solution as a colloidal oxygen-delivering blood replacement fluid. Vox Sang 53:7-14, 1987.

14. Hess J, Fadare S, Tolentino L, Bengal N: The intravascular persistence of DBBF-hemoglobin. Seventh International Conference on Red Cells. Metabolism and Function, Ann Arbor MI (Abst 19), 1988.

15. Velky TS Jr, Lee ES, Maffuid PW, Robinson GT, Yang JC, Greenburg AG: Peritoneal accumulation of infused stroma-free hemoglobin. Arch Surg 122:355-357, 1987.

16. Bleeker WK, van der Plas J, Feitsma RIJ, Agterberg J, Rigter G, de Vries-van Rossen A, Pauwels EKJ, Bakker JC: In vivo distribution and elimination of hemoglobin modified by intramolecular cross-linking with 2-nor-2-formylpyridoxal 5′-phosphate. J Lab Clin Med 113:151-161, 1989.

17. Rothschild MA, Bauman A, Yalow RS, Berson SA: Tissue distribution of I^{131} labeled human serum albumin following intravenous administration. J Clin Invest 34:1354-1358, 1955.

18. Feola M, Gonzalez H, Canizaro PC, Bingham D, Periman P: Development of a bovine stroma-free hemoglobin solution as a blood substitute. Surg Gynec Obstet 157:399-408, 1983.

19. Marks DH, Brown DR: Antibody response to transfusion with pryidoxalated polymerized hemoglobin solution. Milit Med 152:473-477, 1987.

20. Harper HA, Rodwell VW, Mayer PA: Review of physiological chemistry, 17th Ed., Los Altos: Lange Medical Publication, 1979.