Histopathologic study following administration of liposome-encapsulated hemoglobin in the normovolemic rat

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Liposome encapsulated hemoglobin is being developed as an artificial resuscitative fluid for *in vivo* oxygen delivery. In the present report, we examine the effect of accumulation of liposome encapsulated hemoglobin on the structure of reticuloendothelial organs following administration of liposome encapsulated bovine hemoglobin in the normovolemic rat. We have also examined the administration of the liposome vehicle, tetrameric bovine hemoglobin, and liposome encapsulated bovine hemoglobin that had been lyophilized with 300 mM trehalose and rehydrated just before injection. Following injection into the tail vein, rats were sacrificed and liver, spleen, kidney, and lung harvested at 2 h, 24 h, 1 week, and 2 weeks for analysis. Gross pathologic findings of animals injected with liposome encapsulated hemoglobin showed statistically significant splenomegaly with a waxy parenchymal pallor at early time points. Microscopic findings indicate that the liver and spleen are principally involved with liposome encapsulated hemoglobin removal over the course of 24 h with transient cytoplasmic vacuolization in tissue resident phagocytes as evidenced by both light and electron microscopic examination. Presence of liposome encapsulated hemoglobin in these vacuoles was confirmed by oil red O and prussian blue stains. Splenic weight was observed to decline after 24 h but still remained significant above sham-treated controls at 2 weeks and could be correlated with increased hematopoietic activity. Other findings only in animals injected with lyophilized liposome encapsulated hemoglobin included transient loss of laminae rara in the basal lamina, podocyte fusion in the kidney, and small pulmonary infarcts in the lung over the course of 24 h. This latter finding may be associated with trapping of large particles or agglutinated liposome encapsulated hemoglobin. These data indicate that the administration of liposome encapsulated hemoglobin causes transient changes in the organs of the reticuloendothelial system. © 1995 John Wiley & Sons, Inc.

INTRODUCTION

The development of an artificial resuscitative oxygen-carrying fluid, or blood substitute, has been driven by the unpredictable supply of blood during times of both civilian and military need, and the risk of transmittable blood-borne pathogens. Blood substitutes have taken different forms with the majority based on the dissolution of oxygen into a fluorocarbon emulsion, or allosteric binding of oxygen to hemoglobin.[1,2] Hemoglobin-based blood substitutes have had a long history of preclinical development and human testing with human testing as recent as the late 1980s.[3,4] Early toxicities identified with tetrameric hemoglobin have led to intramolecular cross linking of hemoglobin dimers (chemically and through genetic modification).[5–7] This was done to prevent dissociation of the tetramer and subsequent filtration by the kidney, which results in nephrotoxicity.[3,8,9] Toxicities associated with modified hemoglobins (crosslinked or polymerized) remain a question largely because of the potential vasoactivity of these preparations.[10] Some hypertensive response indicative of vasoconstriction following administration of cell-free modified hemoglobins persist despite removing possible stromal contaminants.[10,11] The binding of hemoglobin to endothelium-derived relaxation factor (nitric oxide) has been implicated in this response.[12,13] Previous studies of stroma-free and cross-linked hemoglobin have indicated damage to the kidney, liver, and central nervous system.[9,10,14–16]

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An alternative strategy toward preventing the exposure of free hemoglobin to the vascular compartment, preventing its rapid dissociation and accumulation in the kidney, and extending the circulation persistence is the encapsulation of hemoglobin within a biodegradable carrier.[17,18] Liposome encapsulated hemoglobin is one form of encapsulated hemoglobin that has been investigated *in vitro* and in small animal models.[19–22] The efficacy of liposome encapsulated hemoglobin preparations has been demonstrated in total and partial exchange transfusions in small animals.[23–27] Recent efficacy studies have also included demonstration of oxygen delivery in peripheral tissues and increased survival in a 70% hypovolemic hemorrhagic shock model in the rat.[27] Previous hemodynamic studies in normo-volemic conscious rats following administration of moderate doses of liposome encapsulated hemoglobin showed mild transient effects upon administration (thrombocytopenia, leukocytosis, hemoconcentration, and tachycardia), which returned to normal after 30 min.[28] Many of these effects were alleviated by the substitution of more pure phospholipids and by coadministration of a platelet-activating factor antagonist.[29,30] Recent studies with Tc^{99m}liposome-encapsulated hemoglobin have demonstrated that the transient thrombocytopenic event is associated with sequestration of the liposomes in the lung with subsequent release (manuscript in preparation). We are currently examining the role of complement in this effect, which has been demonstrated with other liposome preparations.[25,26] The organ biodistribution of Tc^{99m}liposome-encapsulated hemoglobin in the rabbit shows localization of liposome encapsulated hemoglobin in reticuloendothelial organs over a 20-h period after injection with principal accumulation in the liver and spleen and no significant distribution to the kidney.[33] Thus, the encapsulation of hemoglobin in liposomes clearly shunts the distribution of a hemoglobin-based blood substitute away from the kidney. In the present study, we have extended our investigation of the interaction of this blood substitute with the reticuloendothelial system and present the first pathologic examination of the consequences of administration of this blood substitute. This study examines the effect of liposome encapsulated hemoglobin administration on the liver, spleen, kidney, and lungs in the normovolemic rat.

MATERIALS AND METHODS

Liposome-encapsulated hemoglobin fabrication

Fabrication of liposome-encapsulated hemoglobin has been described elsewhere.[21] All of the solutions injected were characterized before injection. Liposome-encapsulated hemoglobin and lyophilized liposome-encapsulated hemoglobin were characterized for vesicle size, concentration of hemoglobin and methemoglobin, lipid concentration, endotoxin level, and sterility by inoculation in thioglycollate broth followed by plating on agar and blood agar. All of the samples were sterile and had <60 EU/ml. Other characteristics of the injected samples are presented in Table I.

TABLE I
***In Vitro* Characteristics of Liposome-Encapsulated Hemoglobin, Lyophilized Liposome-Encapsulated Hemoglobin, Liposome Vehicle (LV), and Hemoglobin and Hb Administered Experimental Groups**

Sample	Diameter (nm)	Hemoglobin (g/dl)	MetHb (%)	Lipid conc. (mM)
LEH	400 ± 100	3.0 ± 0.6	16.0 ± 3.0	77.5 ± 7.5
LLEH	280 ± 70	2.6 ± 0.3	11.5 ± 0.7	95.0 ± 5.0
LV	220 ± 30			82.5 ± 2.5
Hb		5.5 ± 0.5	8.0 ± 2.0	

Animal protocol

National Institutes of Health guidelines for the care and use of laboratory animals (NIH Publication no.

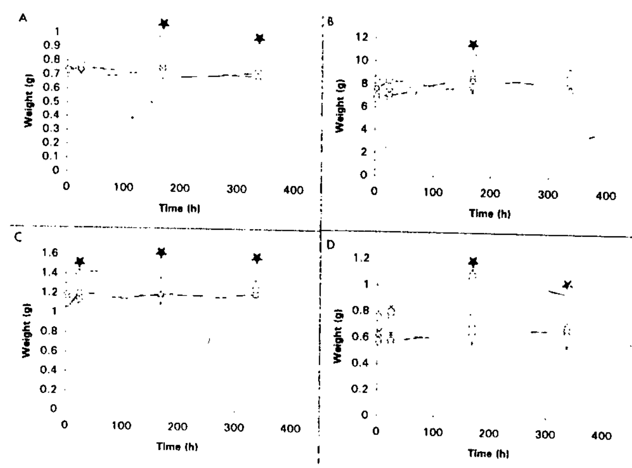

Figure 1. Organ weights over a 2-week period following the injection of test groups. The lyophilized and nonlyophilized liposome-encapsulated hemoglobin-treated animals showed statistically significant increased weights in the spleen over the 2-week period, with increased hematopoietic activity after 1 week. The lyophilized liposome-encapsulated hemoglobin was the only group that showed statistically increase weights in the kidney, liver, and lung. ✱ = sham, ○ = saline, △ = liposome, x = liposome encapsulated hemoglobin, □ = lyophilized liposome encapsulated hemoglobin, ◇ = hemoglobin. *Statistical significance ($P < .05$).

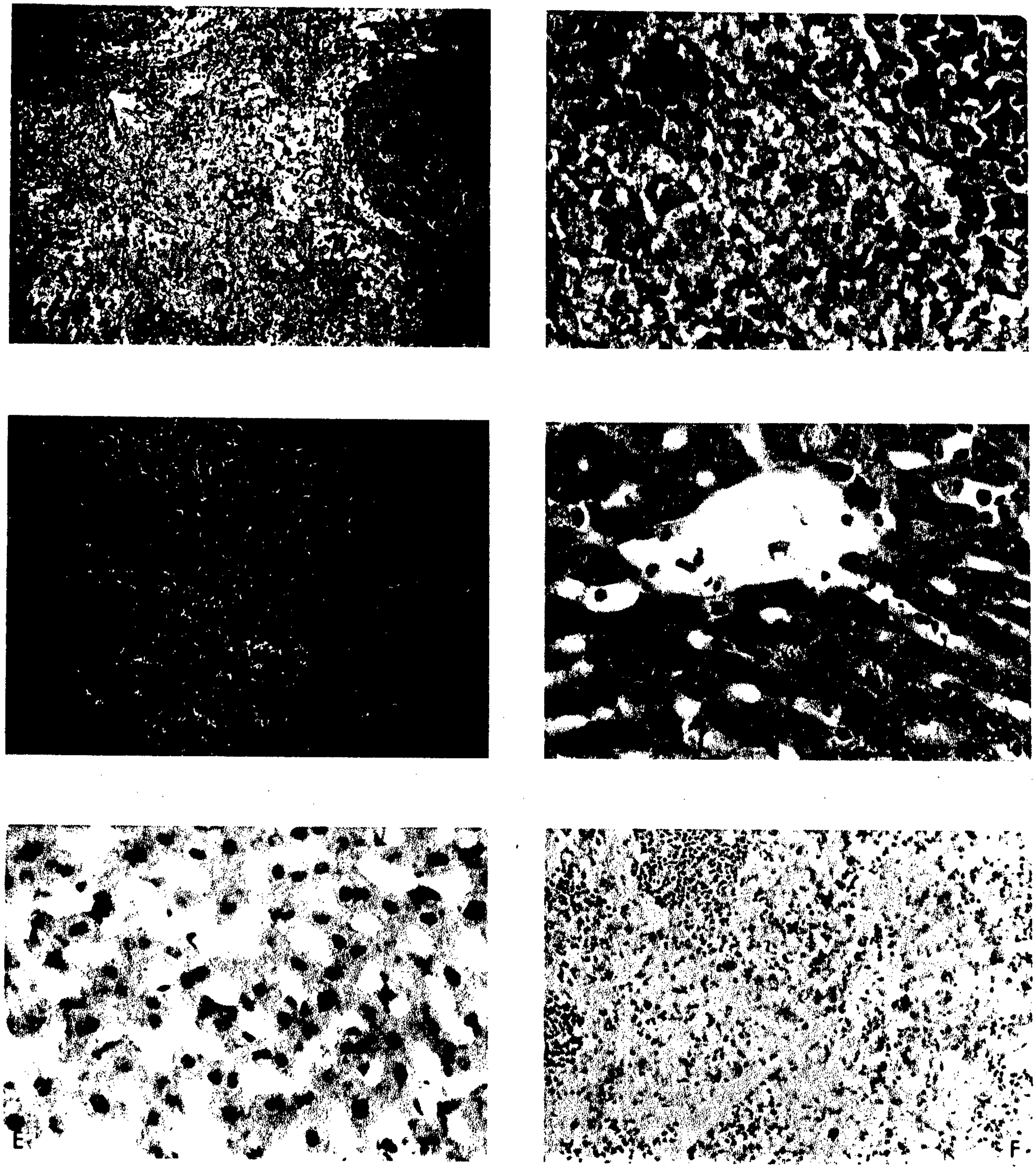

Figure 2. Lipid trapping in the reticuloendothelial system was a prominent but transient finding. (A) early lipid trapping in macrophages at 24 h results in loss of red pulp appearance (hematoxylin/eosin, original magnification ×60). (B) Lipid trapping at 24 h is evident by granular background of splenic tissue (hematoxylin/eosin, original magnification ×125). (C) After 1 week, significant hematopoietic activity is observed in the spleen, which may account for persistent increased splenic weight at 1 week that persists at 2 weeks (hematoxylin/eosin, original magnification ×60). (D) Kupffer cells in the liver show cytoplasmic vacuolization at early time points (24 h) (hematoxylin/eosin, original magnification ×125). (E) Oil Red O stain for neutral lipid confirms the presence of lipid material accumulated in phagocytic cells of the liver at 24 h (original magnification ×125). (E) Prussian blue stain in the spleen 24 h after the injection of liposome-encapsulated hemoglobin shows prominent iron deposition (original magnification ×60).

85-23 Rev. 1985) were observed. Female Sprague–Dawley rats (approximately 200 g) were divided into six experimental groups (n = 6 for each group): untreated sham controls, phosphate-buffered saline, liposome-encapsulated hemoglobin, lyophilized liposome-encapsulated hemoglobin, liposome vehicles, and unencapsulated hemoglobin. The proximal end of the tail vein was prepared under sterile conditions, and a 27-gauge lymphograph catheter inserted (Bekton and Dickinson, Rotherfort, NJ). The approximate volume injected was calculated based on the weight of the animals and represented approximately 30% of the blood volume (5.2 ml). Each animal was injected into the tail vein at 1 cc/min. This volume of liposome encapsulated hemoglobin represents a dose of 2.5 g phospholipid and 1.25 g Hb/kg rat.

Animals were sacrificed at 2 h, 24 h, 1 week, or 2 weeks after injection. Organs were harvested, weighed, and fixed in Hank's fixative, and paraffin sections were prepared for histologic analysis by routine light microscopy. Special stains (immunoDAB for hemoglobin, Oil red O, prussian blue) were used to visualize the protein and neutral lipid components of liposome encapsulated hemoglobin present in tissues. The slides were examined by conventional light microscopy using a Nikon Optiphot equipped with a 25-W halogen light source. Each tissue prepared for electron microscopy was postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer, water rinsed, and dehydated in graduated steps with ethyl alcohol, exchanged in acetone, and embedded in Spur's resin. Thick sections (0.5 μm) were cut and stained with toluidine blue for evaluation by light microscopy. Thin sections stained with methanolic uranyl acetate and Reynold's lead citrate for evaluation by transmission electron microscopy (Zeiss 10C).

Data analysis

The prepared slides were read in a blinded manner, and the changes were noted by entering into a labcat pathology data system. Organ weight data was analyzed for mean ± SEM. One-way analysis of variance (ANOVA) followed by a Student-Newman-Kuels test was used for statistical analysis. $P < .05$ was considered significant.

RESULTS

Morphologic changes were seen in all four organ systems. Statistically significant ($P < .05$) increases in organ mass were most apparent in the spleen at 1 week in the liposome-encapsulated hemoglobin groups. Gross visible changes in the splenic parenchyma consisting of pallor and a waxy texture were also noted. The light microscopic morphologic changes observed in the filter organs were limited principally to the reticuloendothelial components, were transient with resolution after 1 week, and were most closely associated with the lipid component of the test materials. Ultrastructural changes, aside from lipids in the reticuloendothelial system, were noted focally and transiently in the glomerular basement membrane and epithelial cell foot processes of the lyophilized liposome-encapsulated hemoglobin and liposome-encapsulated hemoglobin exposed groups.

Gross pathology

Among the six groups of rats, only those injected with lipids (liposome-encapsulated hemoglobin, lyophilized liposome-encapsulated hemoglobin, and liposome vehicle) showed gross organ changes. In

Figure 3. Transient lipid accumulation within phagocytic cells of the renal glomerulus were noted most prominently in the lyophilized liposome encapsulated hemoglobin group at 24 h (hematoxylin/eosin, original magnification ×125).

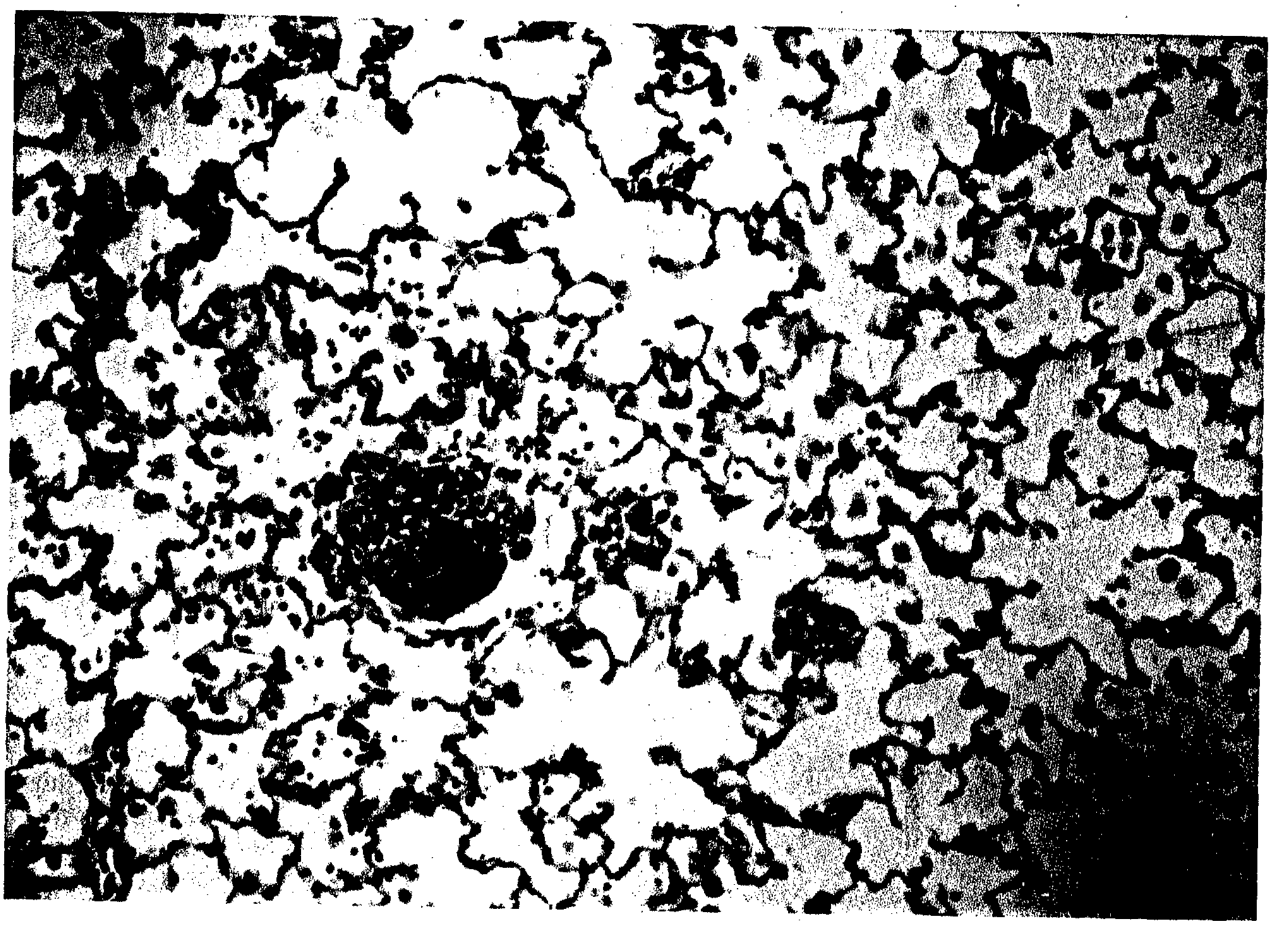

Figure 4. Rare organizing pulmonary infarcts were seen in the lyophilized liposome-encapsulated hemoglobin group at 2 weeks (hematoxylin/eosin, original magnification ×12).

these three groups, splenomegaly and parenchymal pallor on section were noted within 2 h following injection, with splenomegaly persisting at 2 weeks (Fig. 1). Statistically significant increases over the full 2-week study period were noted in liver weights for the liposome-encapsulated hemoglobin groups, and significant increases in kidney and lung weights were noted for the lyophilized liposome-encapsulated hemoglobin group primarily. Organ weights were observed to decrease after 24 h but remained increased over baseline at 2 weeks.

Light microscopic findings

Histopathologic analysis of the spleens from rats injected with liposomes, liposome-encapsulated hemoglobin, or lyophilized liposome-encapsulated hemoglobin revealed initial cytoplasmic vacuolization of splenic macrophages 2 h after administration and peak vacuolization at 24 h (Figs. 2a and 2b). Neutral lipids, as demonstrated by Oil-Red O, were major contributors to the vacuolization in all liposome test groups (Fig. 2e) and heme, as demonstrated by the prussian blue reaction, was also present in slightly greater quantities over controls in liposome-encapsulated hemoglobin and lyophilized liposome-encapsulated hemoglobin groups (Fig. 2f). There was an artifactual loss of red pulp prominence associated with the accumulation of lipid at 2 and 24 h. Increased hematopoietic activity was observed in the spleen at 1 and 2 weeks, and seemed to correlate to the persistent increased splenic weight (Fig. 2c). Lipid trapping in Kupffer cells in the liver was noted in a similar chronologic pattern (Fig. 2d). There was no evidence of parenchymal cell changes, focal necrosis, lobular hepatitis, or triaditis at any time period for any of the test groups. In animals injected with free hemoglobin, iron levels in both the spleen and liver were not greater than in the sham and saline controls. Animals injected with free hemoglobin also were observed to excrete hemoglobin in the urine within 15 min following injection.

The lungs and the kidneys by light microscopic examination showed features suggestive of focal glomerular and pulmonary capillary lipid trapping in the three lipid groups, which was confirmed by Oil-red O staining only in the lungs. These were most prominent in the lyophilized liposome-encapsulated hemo-

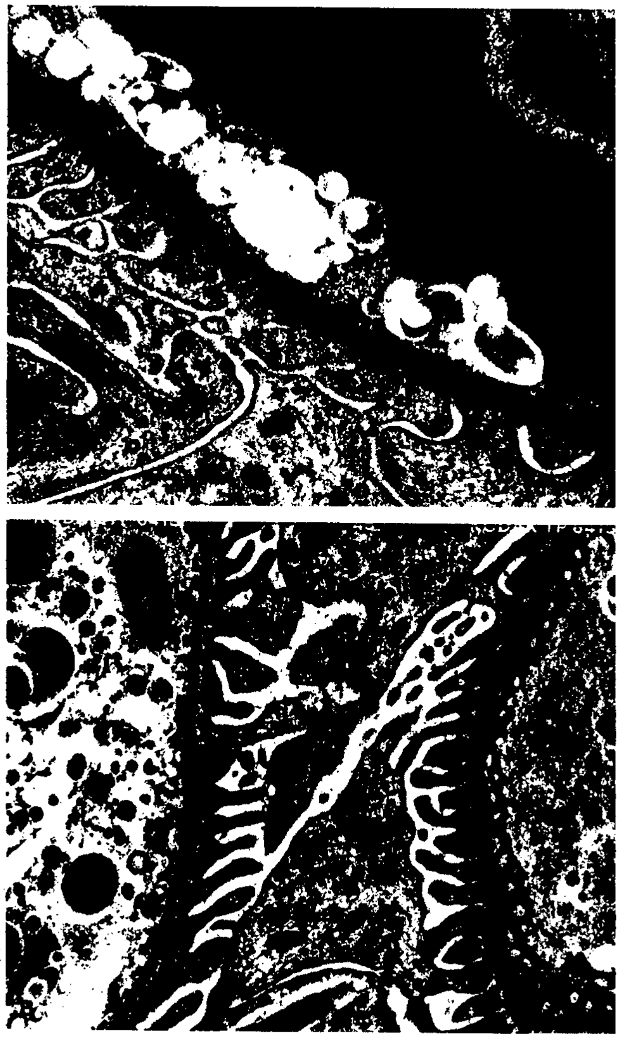

Figure 5. (A) Ultrastructural changes in the lipid groups consisted of transient and reversible loss of laminae rara (interna and externa) with an associated focal fusion of epithelial foot processes (original magnification ×22,000). (B) At 1 week, ultrastructural features were normal (original magnification ×12,000).

globin group (Fig. 3). Last, both the liposome-encapsulated hemoglobin and lyophilized liposome-encapsulated hemoglobin groups were associated with small pulmonary infarcts, with the bulk of the lesions associated with the lyophilized liposome-encapsulated hemoglobin injections (Fig. 4).

Ultrastructural features

Examination of renal parenchyma disclosed glomerular changes in the liposome-encapsulated hemoglobin, lyophilized liposome-encapsulated hemoglobin, and liposome vehicle groups. The glomerular changes consisted of a loss of the laminae rara with a compensatory expansion of the lamina densa, focal electron dense intramembranous deposits, and focal loss of epithelial cell foot processes. These changes were present only at the 2 and 24-h specimens and were not observed at 1 or 2 weeks (Fig. 5). There were no tubular epithelial changes noted in any of the liposome groups at any time points.

DISCUSSION

An optimal blood substitute is expected to exhibit physiologically relevant oxygen binding affinity, reasonable longevity in circulation, and not damage or impair organ function. Previous studies with liposome-encapsulated hemoglobin have demonstrated physiologic oxygen binding affinity and a circulatory half-life of 15–20 h in small animals.[22,33] The present study demonstrated that liposome-encapsulated hemoglobin induces transient alterations in organ morphology in rats over the course of the 2-week observation period.

The trapping of liposome-encapsulated hemoglobin and lyophilized liposome encapsulated hemoglobin is largely manifest in the spleen over the course of 24 h. Rapid accumulation of material was observed in the spleen and liver for all liposome groups. Degradation of the material was observed over the course of a week, as evidenced by the return of normal histopathologic appearance. Splenic involvement included increased hematopoietic activity following the clearance of the material, which accounted for significantly increased splenic weight over the course of the study. Further metabolic studies are needed to define the kinetics of liposome-encapsulated hemoglobin handling in the reticuloendothelial system. The accumulation of liposome-encapsulated hemoglobin in the liver and spleen, however, is consistent with the intravenous administration of other liposome preparations.[35]

It is interesting to note the observations in the kidney of animals injected with lyophilized liposome-encapsulated hemoglobin, which showed an accumulation of electron-dense material within the basal laminae; this was not observed in the unlyophilized liposome-encapsulated hemoglobin group. The negative charge of the laminae rara, which is associated with proteoglycans, appears to be focally compromised by components or metabolic products of lyophilized liposome-encapsulated hemoglobin at 24 h. That this observation is usually associated with podocyte fusion suggests that the mechanism may be attributed to transient filtration of membrane protein complexes from intravascularly damaged or partially phagocytosed lyophilized liposome-encapsulated hemoglobin. Alternatively, the presence of the protective disaccharide trehalose in the lyophilized liposome-encapsulated hemoglobin samples may play an undetermined role in the observed changes.

Pulmonary infarcts observed in the liposome groups represent classic secondary embolic injury associated with trapping of large particles. Light scattering profiles of liposome-encapsulated hemoglobin solutions indicate a bimodal distribution of liposome-encapsulated hemoglobin particle size, with 10–20% of the liposome-encapsulated hemoglobin particles agglutinated at 700–900 nm.[22,34,35] These particles may be trapped and initiate the observed embolic injury. This points out the need for the filtration or other strategies in liposome-encapsulated hemoglobin processing to remove large particles.

These morphologic findings demonstrate that there are only transient changes in the reticuloendothelial organs and nephron. This study must now be supplemented by studies that focus on organ function. Toward this goal, we have begun to evaluate the cytokine response elicited by interactions between liposome-encapsulated hemoglobin and cells of the reticuloendothelial system.

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