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Effect of Hemolyzed Blood on Reticuloendothelial Function and Susceptibility to Hemorrhagic Shock[1] (40361)

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Severe depression of reticuloendotehlial system (RES) phagocytic function is considered to contribute to the deterioration of an organism during circulatory shock (1–4). One aspect of the data supporting this concept is the finding that the injection of various foreign colloids including colloidal carbon, thorotrast, saccharated iron oxide and gelatinized lipid emulsion will induce a period of RES depression or blockade which is associated with increased susceptibility to various forms of shock (5–9). Additionally, RES blockade has been shown to be associated with the depletion of a plasma opsonic α-2-glycoprotein and the circulating levels of this opsonic protein have been implicated in the control of RES phagocytic function (3, 8, 10–12).

Few studies have been carried out using altered homologous material as a potentially blockading substance even though the RES avidly clears such material from the circulation (13). RES blockade induced with altered homologous material would represent a much less artificial condition than the use of foreign or inert colloidal material. The present study was carried out to determine if a blockade-like depression of RES phagocytic function and increased susceptibility to shock is induced following the RES clearance of homologous erythrocyte cellular debris. Additionally, α-2-glycoprotein opsonic activity was measured to determine its potential role in this form of RES depression.

Methods. Male Sprague-Dawley rats weighing 250–300 g were used for all experiments. Blood to be hemolyzed was collected in a plastic heparinized syringe from animals under ether anesthesia. The blood was hemolyzed by freezing at −20° for 30 min and rapid thawing and warming to 37°. Hemolyzed blood was injected over 1–2 min at a dose of 0.3 ml/100 g and control animals received an equal volume of heparinized non-hemolyzed blood.

Animals receiving injections of hemolyzed or non-hemolyzed blood were anesthetized with sodium pentobarbital (30 mg/kg, iv) and a femoral artery was cannulated. The animals were heparinized (100 USP units/100 g) and colonic temperature was monitored and maintained at 36–37°. Arterial blood pressure was monitored throughout the experiments. Thirty minutes after the injection of hemolyzed or nonhemolyzed blood, phagocytic index was determined, or a blood sample was taken for the determination of plasma opsonic activity, or hemorrhagic shock was induced for the evaluation of shock susceptibility.

Hemolyzed blood was separated into a particulate stroma and soluble supernatant fraction by centrifugation at 2000*g* for 15 min. The stroma fraction was washed three times in isotonic saline and resuspended in sufficient saline to bring the volume to the original blood volume. This stroma preparation contained approximately 13.5 mg of stroma protein/ml as determined with the Lowry assay. Similarly, the supernatant fraction was diluted with sufficient saline to bring the volume to the original blood volume. The fractions were injected iv at a dose of 0.5 ml/100 g, into animals prepared as described above, and phagocytic index was determined 30 min after injection.

Erythrocytes and erythrocyte stroma were labelled with ^{125}I using a slight modification of the method of Hynes (14). Washed erythrocytes were suspended in phosphate buffered saline (PBS) (pH 7.2) plus 5 m*M* glucose to a hematocrit of approximately 50%. Carrier free $Na^{125}I$ was added to a final concentration of 400 μCi/ml and the reaction was started by the addition of 3.2 units/ml of lactoperoxidase (Boehringer Mannhein, E.C. 1.11.1.7) and 0.1 units/ml of glucose oxidase (Boehringer Mannhein, grade I, E.C. 1.1.3.4). The mixture was incubated for 30 min at 37°.

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The reaction was stopped by the addition of 6 vol of 0.9% NaI, and the cells were washed six times and resuspended in an equal volume of PBS. Erythrocyte stroma was prepared as described above. The clearance rate of erythrocytes and erythrocyte stroma was determined following the iv injection into anesthetized and heparinized rats by taking blood samples (0.1 ml) at 5 min intervals for 30 min. At 30 min, the distribution of the ^{125}I was determined in liver, spleen, lungs and kidneys. Half-time was determined from semilogarithmic plots of blood radioactivity against time.

Phagocytic index for the hemolyzed blood and the unlabelled stroma was determined from the clearance rate of gelatinized lipid emulsion labelled with ^{131}I triolein as previously described (11, 12). The gelatinized lipid emulsion was injected iv at a dose of 50 mg/100 g. Sequential blood samples were taken over 5 min and the half-time determined from semilogarithmic plots of blood radioactivity against time. Phagocytic index was calculated from the formula: phagocytic index = .301/half-time, where .301 is the $\log_{10}$ of 2, and half-time is expressed in min. Five min after the colloid injection, the distribution of the colloid in the liver, lungs and spleen was determined.

Plasma opsonic activity (α-2-glycoprotein activity) was determined using the rat liver slice bioassay as previously described (11, 12). This assay evaluates the plasma opsonic stimulation of phagocytosis of gelatinized lipid emulsion by rat liver slices *in vitro*. The liver slices were incubated for 30 min in the presence of heparin, 1 ml of plasma, 2 ml of Krebs-Ringer phosphate buffer (pH 7.4) and 2 mg of ^{131}I labelled gelatinized lipid emulsion. At the end of the incubation, the liver slices were evaluated for the presence of ^{131}I, and opsonic activity was expressed as μg of lipid emulsion phagocytized per 100 mg hepatic tissue (μg/100 mg). Each plasma sample was assayed in triplicate.

Hemorrhagic shock was induced as previously described (15) by withdrawing sufficient blood via a cannulated femoral artery to decrease the mean arterial blood pressure to 40 mm Hg within 10 min. The arterial blood pressure was then maintained at 40–45 mm Hg by withdrawing small volumes of blood until the point of initial decompensation, that is, when it was first necessary to return some of the withdrawn blood to maintain the blood pressure. Shock susceptibility was evaluated on the basis of the duration of hypotension required to reach the point of initial decompensation and the maximum shed volume.

Data were statistically analyzed using the unpaired Student's t test, placing the confidence level at 95%. All data are expressed as the mean and standard error of the mean.

Results. Phagocytic index, determined 30 min following the injection of hemolyzed whole blood, was decreased 44.7% ($P < 0.01$) compared with control animals injected with an equal volume of nonhemolyzed blood (Fig. 1). Evaluation of the distribution of the test colloid 5 min after colloid injection revealed a 30.7% decrease ($P < .01$) in liver phagocytosis and no change in the colloid localization in the spleen and lungs.

Following the injection of the particulate stroma fraction of hemolyzed blood phagocytic index was decreased 41.4% when compared to the saline controls (Table I). The injection of the soluble supernatant fraction of hemolyzed blood had no effect on phagocytic index. Tissue distribution of the test colloid showed that hepatic phagocytosis was depressed 37.7% following stroma injection and was unchanged after injection of the supernatant fraction. Localization of the colloid in the spleen was not changed. The stroma injection was associated with an increase in lung colloid localization, however,

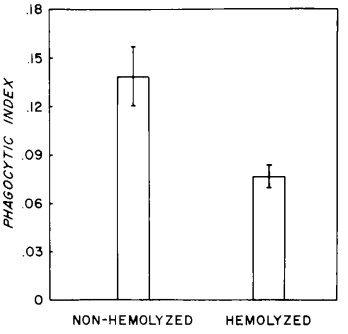

FIG. 1. Phagocytic index determined 30 min after the injection of hemolyzed or nonhemolyzed blood at a dosage of 0.3 ml/100 g. The values are different at $P < .01$. The values are expressed as mean ± the SEM of eight animals per group.

the lungs contained only a small proportion of the injected colloid.

The intravenous injection of hemolyzed whole blood resulted in a large but transient decrease in arterial blood pressure. The rate of injection was adjusted so that the blood pressure was not reduced below 50 mm Hg which required that the blood be injected over 1–2 min. The pressure recovered to the preinjection level within 2.3 ± 0.3 min after the start of injection. The pressure then increased to and remained at or above control levels for the remainder of the 30 min observation period. The injection of the supernatant fraction of hemolyzed blood resulted in a blood pressure response that was identical to that seen following the injection of whole blood. The injection of non-hemolyzed blood or the stroma fraction did not change arterial blood pressure.

Intact erythrocytes labelled with ^{125}I were not cleared from the circulation at a sufficient rate to allow the determination of half-time over the 30 min observation period (Table II). The organ distribution of the erythrocytes is consistent with a very slow clearance rate. On the other hand, the erythrocyte stroma was rapidly removed from the circulation. A very substantial amount of the stroma was cleared by the liver with lesser amounts present in the spleen and lungs. This pattern of particulate clearance is very similar to that observed with the test colloid clearance (Table I). The minimal amount of labelled erythrocyte stroma present in the kidney indicates little non-specific trapping in vascular beds.

Plasma opsonic activity determined 30 min after the injection of hemolyzed blood is presented in Table III. No differences were observed in plasma opsonic activity in animals injected with hemolyzed whole blood when compared with animals injected with non-hemolyzed blood.

Evaluation of the response to hemorrhagic shock revealed that the time to initial decompensation during hypotention was decreased 50.1% ($P < .01$) in the animals injected with hemolyzed blood 30 min before initiation of hemorrhage (Fig. 2). There was no difference in maximum shed volume in animals injected with hemolyzed or nonhemolyzed blood. The large decrease in time to initial decompensation is interpreted as indicating an increased susceptibility to hemorrhagic shock in animals injected with hemolyzed blood.

Discussion. The present study has demonstrated that the injection of hemolyzed whole blood results in a large decrease in phagocytic index. This depression of RES phagocytic function was associated with a large reduction in the test colloid localization in the liver, with no change in the spleen and lung local-

TABLE I. PHAGOCYTIC INDEX AND ORGAN LOCALIZATION OF TEST COLLOID 30 MIN FOLLOWING INJECTION OF HEMOLYZED BLOOD STROMA OR SUPERNATANT FRACTIONS.[a,b]

	Phagocytic index (K)	Liver (%ID/TO)	Spleen (%ID/TO)	Lungs (%ID/TO)
Sham (saline)	.0947 ± .0070[c]	46.10 ± 2.26	2.88 ± 0.31	0.75 ± 0.08
Stroma	.0555 ± .0086[d]	28.70 ± 3.80[d]	3.09 ± 0.29	1.28 ± 0.16[d]
Supernatant	.0942 ± .0167	43.96 ± 4.03	2.39 ± 0.22	0.73 ± 0.06

[a] Stroma and supernatant fraction injected volume was 0.5 ml/100 g.

[b] Colloid localization was determined 5 min after injection of 50 mg/100 g ^{131}I labelled gelatinized lipid emulsion and is expressed as the percent of the injected dose per total organ (%ID/TO).

[c] Values expressed as mean ± SE; $n = 7–10$ for all groups.

[d] $P < .01$ compared with the sham group.

TABLE II. CLEARANCE RATE AND ORGAN LOCALIZATION OF LABELLED ERYTHROCYTES AND ERYTHROCYTE STROMA.[a]

	Half-time (min)	Liver %ID/TO	Spleen %ID/TO	Lungs %ID/TO	Kidneys %ID/TO
Erythrocytes	—[b]	6.0 ± 0.4	1.9 ± 0.2	3.1 ± 0.2	0.34 ± 0.01
Erythrocyte stroma	1.86 ± 0.16	71.5 ± 2.2	5.6 ± 0.8	8.1 ± 0.9	0.42 ± 0.03

[a] Organ distribution was determined 30 min after iv injection of 0.5 ml/100 g and expressed as the percentage of the injected dose per total organ.

[b] Erythrocyte clearance was too slow to estimate the half-time over the 30 min observation period.

TABLE III. PLASMA OPSONIC ACTIVITY 30 MIN FOLLOWING THE INJECTION OF HEMOLYZED BLOOD[a]

	n	Plasma Opsonic Activity (μg/100 mg[b])
Nonhemolyzed blood	6	268 ± 17[c]
Hemolyzed blood	6	291 ± 19

[a] Volume of hemolyzed and nonhemolyzed blood injected was 0.3 ml/100 g.

[b] Opsonic activity is expressed as μg of gelatinized lipid emulsion phagocytized per 100 mg hepatic tissue.

[c] Values are expressed as the mean ± SE of the mean with six animals per group.

ization. Since the bulk of the colloid cleared from the circulation was removed by the liver, the depression of RES clearance was due primarily to a reduction in hepatic phagocytosis.

The fraction of whole hemolyzed blood which is responsible for the depression of RES phagocytic function appears to be the particulate stroma fraction. The depression of phagocytic index due to stroma injection is associated with a pattern of tissue colloid localization which is similar to that observed following whole hemolyzed blood injection. Additionally, the pattern of colloid distribution in the animals injected with hemolyzed blood or erythrocyte stroma was similar to that previously seen during RES depression due to RES colloidal blockage (11). The soluble fraction of hemolyzed blood had no effect on RES function which indicates that while hemoglobin may or may not be removed from the circulation by the hepatic Kupffer cells (16, 17), the presence of free hemoglobin in the circulation does not depress RES function.

The response to the fractions of hemolyzed blood, in addition to demonstrating that the RES depressing substance is present in the particulate stroma fraction, also showed that the RES depression was independent of the vasoactive components of whole hemolyzed blood. This indicates that the RES depression was not due to the vasoactive material decreasing the hepatic blood flow sufficiently to limit colloid delivery to the hepatic Kupffer cells.

Data obtained from the clearance of labelled erythrocyte stroma suggests that the preparation of stroma employed in this study is cleared by the RES. This is based on (a) the rapid rate of clearance from the circulation; (b) organ localization pattern which is very similar to that of the test colloid; and (c) minimal localization in the kidneys. The dose of erythrocyte stroma which was used to evaluate stroma clearance characteristics was identical to the dose which depressed RES phagocytic function. The persistence of the labelled intact erythrocytes in the circulation indicates that the rapid clearance of the stroma was not due to an alteration of the membrane during the labelling process. Thus, the particulate erythrocyte stroma fraction of hemolyzed blood is rapidly cleared from the circulation by the RES, and appears to be responsible for a blockade-like depression of RES phagocytic function.

The animals used in the present experiments were heparinized in order to eliminate the procoagulant effects of hemolyzed blood (18). This was done because it has been shown that intravascular coagulation induced by the injection of thrombin is associated with a depression of RES phagocytic function (19). Additionally, the high clearance rate of the control animals can be attributed to the heparin because in our hands heparin increases the rate of gelatinized lipid emulsion clearance (20). Other investigators have found that heparin increases (21, 22) or decreases the rate of colloid clearance (23). However, heparin does not reverse RES blockade following the injection of gelatinized lipid emulsion (21).

While the depression of RES phagocytic

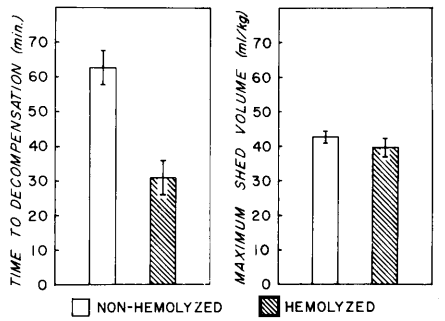

FIG. 2. Time to initial decompensation during hypotension and maximum shed volume in animals injected with hemolyzed and nonhemolyzed blood at a dosage of 0.3 ml/100 g. Hemorrhage was initiated 30 min after injection. Significant difference in time to decompensation ($P < .01$). Values are expressed as the mean ± SEM of 10 animals per group.

function following hemolyzed blood injection is similar to RES colloid blockade in terms of colloid clearance depression and the pattern of tissue colloid distribution, the lack of a depression of plasma opsonic activity is not consistent with the humoral opsonic factor theory of RES blockade. The depletion of plasma opsonic α-2-glycoprotein activity from the circulation is a consistent finding with RES depression due to colloidal blockade (8, 10, 11) and various types of shock and injury (3, 4, 12, 15, 26). Since the RES depression associated with the injection of erythrocyte stroma is not associated with a depression of the circulating activity of this opsonic factor some other mechanism must mediate this RES depression. Such possible mechanisms may include the depletion of some other opsonic factor(s), saturation of phagocytic cell function or a decrease in liver blood flow of sufficient magnitude to limit delivery of the test colloid to the phagocytic cells. The data presented here suggested that a decrease in liver blood flow is not a likely mechanism.

The RES depression induced by the injection of hemolyzed blood was associated with an increased susceptibility to hemorrhagic shock. Since the whole hemolyzed blood contained a vasoactive component, and soluble proteins as well as stroma it is possible that the observed increase in shock susceptibility was not entirely due to the stroma-induced RES depression. Previous work by Hardaway *et al.* has shown that the injection of a small volume of hemolyzed blood into heparinized dogs resulted in an increased mortality with hemorrhagic shock (27). The present study suggests that this increase in mortality was due, in part, to a depression of RES phagocytic function. This notion is consistent with previous studies that have demonstrated that RES blockade with foreign material increased susceptibility to shock induced by hemorrhage, trauma, intestinal ischemia and endotoxin (5–9). Other studies by Subramanian *et al.* showed that intravascular hemolysis associated with experimental cardio-pulmonary bypass was associated with a depression of RES phagocytic function in terms of the clearance of colloidal gold and bacteria (28, 29). Thus, it is possible that hemolysis associated with severe burn or traumatic injury (30, 31) may contribute to RES depression and thereby increase the rate of deterioration of the organism during shock.

Summary. RES phagocytic function and susceptibility to hemorrhagic shock were determined following the injection of hemolyzed blood into heparinized rats. Phagocytic index was severely depressed 30 min following the iv injection of whole hemolyzed blood (0.3 ml/100 g) and was due primarily to an impairment of hepatic phagocytosis of the test colloid. The erythrocyte stroma fraction of hemolyzed blood depressed phagocytic index while the soluble protein fraction had no effect on phagocytic index. Labelled erythrocyte stroma was rapidly cleared from the circulation and localized primarily in the liver with lesser amounts in the spleen and lungs indicating RES clearance of this particulate material. This depression of phagocytic index was associated with normal circulating levels of α-2-glycoprotein opsonic activity. Animals injected with hemolyzed blood showed a 50% decrease in the duration of hypotension required to cause initial decompensation indicating an increased susceptibility to hemorrhagic shock. It is concluded that the hemolysis which accompanied severe injury such as burn or trauma may contribute to RES depression and increased susceptibility to shock states.

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