PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 167, 30–35 (1981)

Effect of Erythrocyte Debris on Reticuloendothelial Function and Susceptibility to Experimental Peritonitis[1] (41120)

GARY J. GROVER[2] AND DANIEL J. LOEGERING

Department of Physiology, Neil Hellman Medical Research Building, Albany Medical College of Union University, Albany, New York 12208

Abstract. Previous work from this laboratory has demonstrated that injection of hemolyzed blood or erythrocyte stroma depressed reticuloendothelial system (RES) phagocytic function and increased susceptibility to hemorrhagic and endotoxin shock. The present study was done to determine if RES depression induced by erythrocyte stroma or foreign particulates will increase susceptibility to experimental peritonitis in rats. RES function, determined from the clearance rate and organ localization of formalinized sheep red blood cells, was depressed following the injection of 0.5 or 0.75 ml/100 g of erythrocyte stroma but was unchanged after 0.3 ml/100 g. Peritonitis was induced by cecal ligation and puncture, and mortality was assessed at 8-hr intervals for 72 hr after cecal ligation. Stroma, at 0.3 ml/100 g, had no effect on time to death, but time to death was decreased with 0.5 ml/100 g ($P < 0.05$) and with 0.75 ml/100 g ($P < 0.001$). Nonhemolyzed, washed erythrocytes and hemoglobin (100 mg/100 g) injected iv had no effect on time to death; however, hemoglobin injected ip decreased time to death. RES depression with gelatinized lipid emulsion or colloidal carbon also decreased time to death following cecal ligation. Thus, RES depression with erythrocyte stroma was associated with increased susceptibility to infection. These data suggest that hemolysis and phagocytosis of erythrocyte debris after thermal injury may contribute to the increased susceptibility to infection which is seen following this form of injury.

Depression of reticuloendothelial system (RES) phagocytic function is well known to be associated with shock (1–3). Several studies have also shown that depression of RES function by injection of foreign particulate material (RES blockade) increases susceptibility to shock and infection (1, 4–7). More recent work has shown that the phagocytosis of a sufficient amount of homologous erythrocyte stroma can depress RES function and increase shock susceptibility (8, 9). Additionally, the degree of hemolysis following thermal injury can be sufficient to generate a phagocytic load of erythrocyte debris which will depress RES function (10).

The present study was carried out to determine if the RES depression induced by the phagocytosis of erythrocyte debris is associated with increased susceptibility to experimental peritonitis. The data show that depression of the RES in this way increases susceptibility to peritonitis suggesting that hemolysis may contribute to the increased susceptibility to infection seen after experimental thermal injury (11, 12).

Methods. *Preparation of washed erythrocytes, erythrocyte stroma, and hemoglobin.* Inbred male Sprague–Dawley rats weighing 250–300 g were used for all experiments. Rats were anesthetized with ether and blood was collected in ACD. The erythrocytes were washed twice in phosphate-buffered saline (PBS: 0.9% NaCl and 5 m*M* phosphate buffer, pH 7.4) and the buffy coat was removed. After the last wash the packed cells were resuspended in an equal volume of PBS and the hemoglobin (Hb) content was measured using the cyanmethemoglobin assay (Hycel Inc., Houston, Tex.). The volume of washed erythrocytes injected was expressed as the milliliters of washed erythrocytes containing 15 g/dl Hb.

Erythrocyte stroma was prepared by lysing washed erythrocytes by freezing at

[1] This work was supported by NIH research Grant GM 26102.

[2] Gary J. Grover is a Predoctoral Trainee supported by HL 07194.

0037-9727/81/050030-06$01.00/0

−20° and thawing at 37°. After centrifuging at 1000g for 15 min the particulate stroma was washed three times in PBS, and the pellet was resuspended in a volume of PBS equal to the volume of the pellet. This preparation contained 9.6 ± 1.0 g/dl Hb and 11.2 ± 1.2 g/dl protein. Protein content was measured using the Lowry method.

A crude preparation of rat hemoglobin was prepared using the method of Drabkin (13). Packed, washed erythrocytes were lysed in 1 vol of water and 0.4 vol of toluene. After refrigeration overnight, the material was centrifuged and the clear Hb solution was dialyzed against 0.9% NaCl at 4°. Any particulate or crystalline material was removed by centrifugation and the resultant solution contained about 7 g/dl Hb.

RES blockade. Blockade was induced by injection of colloidal carbon or gelatinized lipid emulsion. Colloidal carbon (C11/143a, Pelikan, Accurate Chemical Corp., Hicksville, N.Y.) was injected iv at a dose of 32 mg/100 g. Gelatinized lipid emulsion was prepared as previously described (14). The anhydrous lipid base containing lecithin, glycerol, and triolein (1:10:10 by weight) was suspended in a 5% dextrose, 0.3% gelatin solution (pH 7.4) and was injected iv at a dose of 50 mg/100 g.

Determination of RES Function. Animals were anesthetized with sodium pentobarbital (30 mg/kg) and a carotid artery was cannulated for taking blood samples. Erythrocyte stroma, colloidal carbon, gelatinized lipid emulsion, or an equal volume of saline was injected iv via the dorsal penis vein. Thirty minutes after injection, RES phagocytic function was assessed from the determination of the phagocytic index and test particle organ localization.

Phagocytic index was determined from the clearance of formalin-fixed sheep red blood cells (SRBC; MA Bioproducts, Walkersville, MD.). SRBC were washed twice in saline and labeled with ^{51}Cr. Packed SRBC were suspended in 10 vol of PBS, and a dialysis bag containing 2 vol of 47% formalin was placed in the cell suspension. After 3 hr of gentle oscillation at room temperature, the dialysis bag was opened and incubation was continued overnight (15). The fixed cells were washed, resuspended in PBS, and injected at a dose of 3×10^9 cells/100 g. Arterial blood samples (0.1 ml) were taken each minute for 5 min after SRBC injection and blood radioactivity was determined. Clearance half-time was determined and phagocytic index (K) was calculated from: $K = 0.301/\text{half-time}$. SRBC showed linear clearance kinetics on semilogarithmic plots during the observation period. At 5 min after SRBC injection, the rats were killed and the liver, spleen, and lungs were removed and weighed. Radioactivity present in these organs was determined and expressed as percentage of the injected dose per total organ (%ID/TO).

Production of peritonitis. Rats were anesthetized with ether and through a midline incision, the cecum was exposed and ligated just below the ileocecal valve. The cecum was punctured twice with an 18-gauge needle and a small amount of cecal contents (0.05 ml) was squeezed out of each puncture. This procedure is identical to that described by Chaudry and co-workers except our animals were not fasted before the experiment (16, 17).

At 1 hr after cecal ligation, rats were injected with RBC stroma, colloidal carbon or gelatinized lipid emulsion, an equal volume of vehicle, washed erythrocytes, or Hb. Sham-operated controls (abdominal incision and cecal manipulation without ligation and puncture) received injections of equal volumes of stroma, colloidal carbon, or gelatinized lipid emulsion. As a control for the Hb content of the stroma, Hb was injected iv or ip at a dose of 100 mg/100 g 1 hr after cecal ligation. Mortality was assessed at 8-hr intervals after surgery for 72 hr. The time to death was ranked according to these 8-hr intervals.

Statistical analysis. Data for phagocytic index and organ test particle localization were expressed as the mean ± SE and statistically analyzed using Student's t test with the confidence level placed at 99%. Differences in death rates were statistically analyzed using the Mann−Whitney U test for tied, ranked data with the confidence level placed at 95%.

Results. Injection of colloidal carbon (32 mg/100 g) or gelatinized lipid emulsion (50 mg/100 g) resulted in a substantial reduction

TABLE I. EFFECT OF COLLOIDAL CARBON AND GELATINIZED LIPID EMULSION ON PHAGOCYTIC INDEX AND TEST PARTICLE ORGAN LOCALIZATION[a]

	Phagocytic index	%ID/TO		
		Liver	Spleen	Lungs
Control	0.0968 ±0.0109	52.9[b] ±1.1	2.6 ±0.4	7.0 ±0.7
Colloidal carbon (32 mg/100 g)	0.0561 ±0.0038	22.1[c] ±2.5	1.8[c] ±0.2	13.6[c] ±0.7
Gelatinized lipid emulsion (50 mg/100 g)	0.0423 ±0.0086	23.9[c] ±2.2	3.3 ±0.3	12.2[c] ±0.9

[a] Formalinized SRBC were used as the test particle and were injected at the dose of 3×10^9/100, 30 min after the injection of colloidal carbon or gelatinized lipid emulsion. Organ localization was determined 5 min after SRBC injection and expressed as the percentage of the injected dose per total organ (%ID/TO).
[b] Values are expressed as the mean ± SE with six animals per group.
[c] $P < 0.01$ compared with the control group.

in phagocytic index (Table I). The reduction in phagocytic index was associated with a decrease in hepatic test particle localization and an increase in lung particle localization (Table I). There was no change in splenic SRBC localization with gelatinized lipid emulsion; however, colloidal carbon decreased splenic uptake of SRBC.

Erythrocyte stroma, when injected at a dose of 0.3 ml/100 g, had no effect on phagocytic index or test particle organ localization (Table II). The 0.5 m /100 g dose decreased phagocytic index and hepatic SRBC localization. The 0.75 ml/100 g dose of erythrocyte stroma caused a depression of phagocytic index, hepatic SRBC localization, and splenic SRBC localization and an increase in lung SRBC localization. Since the liver accounted for most of the SRBC clearance, it is apparent that the depression of phagocytic index was due primarily to a depression of hepatic phagocytosis.

The mortality rate following cecal ligation and puncture was increased compared to vehicle-injected controls when colloidal carbon ($P < 0.01$) or gelatinized lipid emulsion ($P < 0.05$) was injected 1 hr after cecal ligation (Figs. 1 and 2). Sham-operated controls which received injections of col-

TABLE II. PHAGOCYTIC INDEX AND TEST PARTICLE ORGAN LOCALIZATION FOLLOWING ERYTHROCYTE STROMA INJECTION[a]

	Phagocytic index	%ID/TO		
		Liver	Spleen	Lungs
Control	0.0948[b] ±0.0078	51.3 ±2.2	6.4 ±0.6	6.5 ±0.4
Stroma				
0.3 ml/100 g	0.1132 ±0.0111	51.5 ±2.2	4.8 ±0.5	5.7 ±0.5
0.5 ml/100 g	0.0605[c] ±0.0058	34.3[c] ±2.9	6.0 ±0.6	7.9 ±0.8
0.75 ml/100 g	0.0641[c] ±0.0084	24.2[c] ±2.5	2.6[c] ±0.3	12.6[c] ±0.5

[a] Phagocytic index was determined 30 min after stroma injection. Formalinized SRBC were used as the test particle at a dose of 3×10^9/100 g. Organ localization was determined at 5 min after SRBC injection, and is expressed as percentage injected dose per total organ (%ID/TO).
[b] Values are expressed as the mean ± SE, with 6–10 animals per group.
[c] $P < 0.01$ compared with the control group.

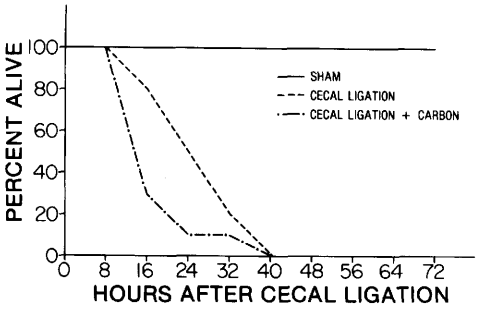

FIG. 1. Effect of colloidal carbon on time to death following cecal ligation and puncture. Colloidal carbon (32 mg/100 g) was injected 1 hr after cecal ligation or sham surgery. Cecal ligation control animals received an equal volume of colloidal carbon vehicle (saline). Colloidal carbon decreased the time to death following cecal ligation ($P < 0.01$). No sham-operated animals died (10 animals per group).

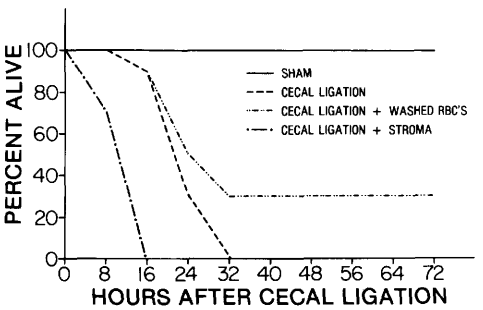

FIG. 3. Effect of erythrocyte stroma on time to death following cecal ligation and puncture. Erythrocyte stroma (0.75 ml/100 g) or washed RBC (0.75 ml/100 g) were injected 1 hr after cecal ligation. Cecal ligation control animals received an equal volume of saline. Sham-operated animals received 0.75 ml/100 g stroma 1 hr after surgery. Stroma decreased time to death after cecal ligation ($P < 0.001$). No sham-operated animals died (10 animals per group).

loidal carbon or gelatinized lipid emulsion showed no mortality.

Injection of erythrocyte stroma at a dose of 0.75 ml/100 g 1 hr after cecal ligation and puncture resulted in an increase in mortality rate compared with groups of animals subjected to cecal ligation plus saline injection ($P < 0.01$) and cecal ligation plus injection of washed erythrocytes (0.75 ml/100 g) ($P < 0.01$) (Fig. 3). The washed erythrocytes were prepared simultaneously with the stroma except for the cell lysis, and served as controls for bacterial or pyrogen contamination of the preparation. Sham-operated animals injected with stroma showed no mortality.

The effect of various doses of stroma on mortality following cecal ligation and puncture was evaluated in a separate experiment (Fig. 4). The injection of 0.3 ml/100 g of erythrocyte stroma after cecal ligation had no effect on mortality rate; however, mortality rate increased with 0.5 ml/100 g ($P < 0.05$) and 0.75 ml/100 g ($P < 0.001$). These data indicate that the doses of

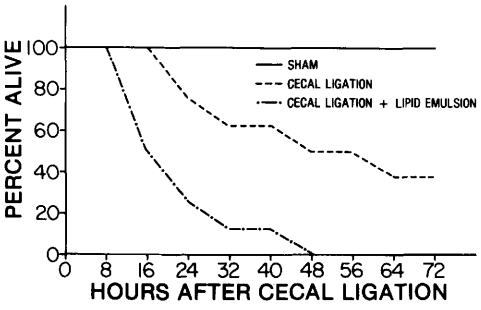

FIG. 2. Effect of gelatinized lipid emulsion on time to death following cecal ligation and puncture. Gelatinized lipid emulsion (50 mg/100 g) was injected 1 hr after cecal ligation or sham surgery. Cecal ligation control animals received an equal volume of vehicle (5% dextrose). Gelatinized lipid emulsion decreased time to death following cecal ligation ($P < 0.05$). No sham-operated animals died (10 animals per group).

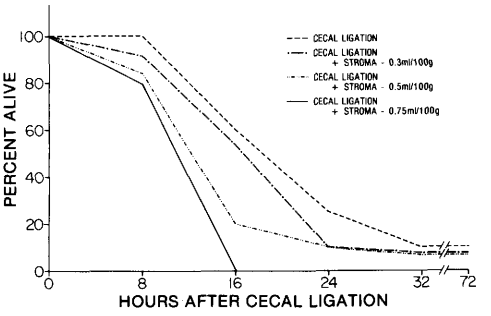

FIG. 4. Effect of various doses of erythrocyte stroma on time to death after cecal ligation. Stroma was injected at doses of 0.3, 0.5, or 0.75 ml/100 g at 1 hr after cecal ligation. Cecal ligation control animals received 0.75 ml/100 g saline 1 hr after cecal ligation. Time to death was decreased after 0.5 ml/100 g ($P < 0.5$) and after 0.75 ml/100 g ($P < 0.01$) of erythrocyte stroma (15 animals per group).

stroma that depressed phagocytic index increased the mortality rate following cecal ligation.

Since the erythrocyte stroma preparation contained Hb, the effect of soluble Hb on mortality following cecal ligation was evaluated. The iv injection of 100 mg/100 g Hb after cecal ligation had no effect on mortality rate while the ip injection of the same dose of Hb increased the mortality rate ($P < 0.05$).

Discussion. The present study is consistent with previous work on the effect of phagocytosis of homologous erythrocyte debris on RES phagocytic function (8, 9). Our previous work indicated, however, that 0.3 ml/100 g stroma caused a depression of the RES while the present study failed to show an effect with this dose. Since earlier studies used different test particles (gelatinized lipid emulsion and nonfixed SRBC) this may be due to slight differences in the sensitivity of the test particles. Higher doses of stroma consistently resulted in substantial RES depression. The injection of colloidal carbon or gelatinized lipid emulsion, which are well-known inducers of RES depression or blockade (14), did so with the methods employed here.

A consistent relationship was observed between the ability of a preparation to depress the RES and to increase the mortality rate with experimental peritonitis. While previous studies have shown that RES depression with foreign particulates will depress the RES and increase susceptibility to infection (6, 7), the present study extends these observations by demonstrating that phagocytosis of homologous erythrocyte debris can also exert such an effect.

The injection of washed intact erythrocytes served as a control for the potential contamination of the preparations of erythrocyte stroma. The erythrocytes were prepared in parallel with the stroma, except for lysis, and had no effect on the susceptibility to peritonitis. This indicates that if the preparation was contaminated, this was not responsible for the difference in mortality observed with the stroma. Also, the iv injection of hemoglobin, simulating the amount of hemoglobin bound to the stroma, had no effect on mortality rate as previously reported (18). However, the ip injection of hemoglobin increased the mortality rate. This effect of ip hemoglobin was probably due to impaired local defense mechanisms in the form of depressed neutrophil chemotactic, phagocytic, and bactericidal functions (18–21).

The exact mechanism by which erythrocyte stroma increases the susceptibility to peritonitis is unknown, but we suggest that this is due, in part, to the phagocytosis of erythrocyte stroma and subsequent depression of RES function. Another contributing factor to this effect may be the procoagulant properties of erythrocyte stroma (22, 23). Since induction of intravascular coagulation alone can depress the RES (24) any procoagulant effect of the stroma may magnify the RES depression, especially in the face of a developing infection (25).

Following thermal injury in rats, depression of RES function is associated with an increase in susceptibility to infection (12, 26). The present study indicates that the phagocytosis of sufficient erythrocyte debris to depress RES phagocytic function increases the susceptibility of rats to infection. Such a phagocytic load of erythrocyte debris may be generated immediately following experimental thermal injury (11). The role of hemolysis in burn patients that develop sepsis several days after injury is unknown; however, many burn patients demonstrate progressive anemia with increased erythrocyte phagocytosis by the spleen and liver (27). Further studies are required to determine the amount of hemolysis and/or erythrocyte phagocytosis required to impair bacterial defense in order to clarify the role of hemolysis in the susceptibility of burn patients to infection.

The authors acknowledge the technical assistance of Linda Garrett and Lisa Gary.

1. Altura, B. M., Advan. Microcirc. 9, 252 (1980).
2. Schildt, B. E., Advan. Exp. Med. Biol. 73A, 375 (1976).
3. Saba, T. M., Circ. Shock 2, 91 (1975).
4. Altura, B. M., and Hershey, S. G., J. Reticuloendothel. Soc. 10, 361 (1971).
5. Fine, J., Rutenburg, S., and Schweinburg, F. R., J. Exp. Med. 110, 547 (1959).

6. Murray, E. G. D., J. Hyg. **22**, 175 (1923).
7. Howard, J. G., Scot. Med. J. **6**, 60 (1961).
8. Schneidkraut, M. J., and Loegering, D. J., Proc. Soc. Expt. Biol. Med. **159**, 418 (1978).
9. Schneidkraut, M. J., and Loegering, D. J., Advan. Shock Res. **3**, 273 (1980).
10. Loegering, D. J., J. Trauma, in press (1981).
11. Alexander, J. W., J. Surg. Res. **8**, 128 (1968).
12. McRipley, R. J., and Garrison, D. W., J. Infect. Dis. **115**, 159 (1965).
13. Drabkin, D. L., J. Biol. Chem. **164**, 703 (1946).
14. Saba, T. M., and Di Luzio, N. R., Amer. J. Physiol. **216**, 197 (1969).
15. Csizmas, L., Proc. Soc. Exp. Biol. Med. **103**, 157 (1960).
16. Chaudry, I. H., Wichterman, K. A., and Baue, A. E., Surgery **85**, 205 (1979).
17. Wichterman, K., A., Baue, A. E., and Chaudry, I. H., J. Surg. Res. **29**, 189 (1980).
18. Lee, Jr., J. T., Ahrenholz, D. H., Nelson, R. D., and Simmons, R. L., Surgery **86**, 41 (1979).
19. Hau, T., and Simmons, R. L., Surgery **87**, 588 (1980).
20. Hau, T., Nelson, R. D., Fiegel, V. D., Levenson, R., and Simmons, R. L., J. Surg. Res. **22**, 174 (1977).
21. Hau, T., Hoffman, R., and Simmons, R. L., Surgery **83**, 223 (1978).
22. Rabiner, S. F., and Friedman, L. H., Brit. J. Haematol. **14**, 105 (1968).
23. Birndorf, N. I., Lopas, H., and Robboy, S. J., Lab. Invest. **25**, 314 (1971).
24. Kaplan, J. E., Saba, T. M., Amer. J. Physiol. **234**, H323 (1978).
25. Effeney, D. J., Blaisdell, W., McIntyre, K. E., and Graziano, C. J., J. Trauma **18**, 689 (1978).
26. Di Maio, A., Di Maio, D., and Jacques, T., J. Surg. Res. **21**, 437 (1976).
27. Loebl, E. C., Baxter, C. R., and Curreri, P. W., Ann. Surg. **178**, 681 (1973).

Received September 15, 1980. P.S.E.B.M. 1981, Vol. 167.