Comparative Evaluation of Macrophage Inactivation of Endotoxin[1] (37707)

RAFAEL A. TREJO[2] AND N. R. DI LUZIO

Department of Physiology, Tulane University School of Medicine, New Orleans, Louisiana 70112

The concept of reticuloendothelial system (RES) involvement during endotoxemia developed when Beeson (1, 2) showed that blockade of the RES with colloidal materials abolished the acquired resistance of rabbits to the pyrogenic and Shwartzman effect of endotoxin. Additional studies indicated that colloidal RES blockade enhanced the susceptibility of rats to endotoxin shock (3). The observations that intravenously administered endotoxins are rapidly removed from the circulation and localized almost exclusively in the reticuloendothelial (RE) cells of the liver and spleen further emphasized the importance of the RES in endotoxemia (4, 5). Moreover, liver and spleen, organs which possess extensive RE cell components, have been shown to possess a potent endotoxin-detoxifying activity (6–10). In contrast, tissues such as brain, kidney, and heart did not detoxify endotoxins (9). These findings suggest that the RES, by virtue of both its phagocytic and endotoxin-detoxifying properties, comprises a fundamental host defense mechanism against endotoxins.

The precise nature of RES involvement in host defense against endotoxin has not as yet been precisely defined. Experiments in this laboratory employing both RES stimulating and depressing agents indicated that altered phagocytic states do not correlate with altered host susceptibility to endotoxin (11, 12). The studies of Stuart and Cooper (13) and Crafton and Di Luzio (11) in conjunction with the recent observations of Palmerio and Fine (14), Filkins (15), and Trejo and Di Luzio (10) have accented the detoxification activity of macrophages as a factor in endotoxin shock.

Previous attempts to ascertain the endotoxin-detoxifying capacity of isolated Kupffer cells and splenic macrophages have been hampered by the lack of techniques for isolating functional macrophages devoid of other cell populations. In addition, methods were not available to detect minute amounts of endotoxins.

Recently, Filkins (15, 16) reported endotoxin detoxification by rat Kupffer cells isolated by magnetic means 1 hr following the administration of particulate iron. However, since in a preliminary study we found that the macrophages isolated by this technique did not manifest phagocytic activity *in vitro* and that the iv administration of 3-μm iron particles enhanced the susceptibility of rats to endotoxins, the appropriateness of employing the iron technique for the evaluation of endotoxin-detoxifying capacity of Kupffer cells is a matter of significant concern.

The development of procedures for the enzymatic isolation of hepatic macrophages which do not employ the incorporation of particulate iron within the macrophages obviously permits a more appropriate evaluation of Kupffer cell endotoxin-detoxifying ability. Similarly, the refinement of techniques to isolate other macrophage populations coupled with the recent development of a bioassay procedure capable of detecting submicrogram levels of endotoxin (17) permits the evaluation of the functional capacity of isolated macrophages and hepatic parenchymal cells to detoxify endotoxins.

The present study was, therefore, under-

[1] This study was supported in part by the Louisiana Heart Association and American Heart Association.

[2] On leave of absence from the Department of Physiology, University of Zulia, School of Medicine, Maracaibo, Venezuela.

taken to define the hepatic cellular site of endotoxin inactivation as well as to comparatively evaluate the endotoxin-detoxifying ability of macrophages derived from liver, lung, spleen, and the peritoneal cavity.

The further delineation of endotoxin inactivation is of obvious concern in view of the recent observations that more than 100,000 fatalities from gram-negative bacteremia may occur each year in the United States (18).

Materials and Methods. In an evaluation of the influence of particulate iron on the susceptibility of rats to endotoxin, carbonyl iron (3 μm) was prepared in 0.9% saline solution and administered intravenously at a dose of 100 mg/hg. Control animals received 0.9% saline solution, isovolumetrically. One hour following the administration of iron, the susceptibility of animals to intravenously administered *S. enteritidis* endotoxin (lipopolysaccharide B. Difco Laboratories) was ascertained. Mortality was recorded at 24 hr.

In order to ascertain the endotoxin-detoxifying capacity of hepatic macrophages, Kupffer cells were isolated from the liver of normal male rats weighing approximately 150–200 g. The method selected for Kupffer cell isolation was that of Pisano *et al.* (19) as modified by Lentz and Di Luzio (20). This method is based on the enzymatic digestion of liver tissue by 0.01% (w/v) collagenase (Type I, Sigma) and 0.005% (w/v) trypsin (Type I, Sigma).

The method of Berry and Friend (21), as modified by Lentz and Di Luzio (20), was employed to isolated rat hepatic parenchymal cells. This method is based on the enzymatic digestion of liver tissue by 0.05% (w/v) collagenase (Sigma, Type I) and 0.1% (w/v) hyaluronidase (Sigma, Type I).

In agreement with previous observations (19, 20), the purity of the Kupffer cell population was reflected by less than 1% contamination with parenchymal cells. Some small "lymphocyte-like" cells were, however, present. The isolated Kupffer cells were phagocytically active as reflected by the uptake of particulate lipid emulsions (19).

The parenchymal cells preparation contained less than 1% contaminating cells with a small population of monocytes (20). The isolated parenchymal cells exhibited no phagocytosis of the reticuloendothelial test lipid emulsion but did manifest uptake of bromsulphalein, an event which did not occur with the isolated Kupffer cells. Fibroblasts and bile ductule cells were not observed to be present in either isolated cell preparation.

Rat spleen macrophages were isolated by the technique of Mosier (22). Rat alveolar macrophages were isolated by a modification of the technique of Myrvik *et al.* (23). Isolation of peritoneal macrophages was performed as previously described (24).

All isolated macrophages were suspended in buffered saline solution (pH 7.4), counted in a hemocytometer, and cell viability determined with 0.5% trypan blue. The cells were sonified and the sonicates centrifuged to remove cell debris. Protein concentration of the supernatant was estimated by ultraviolet light absorption at 280 and 260 nm as described by Layne (25). The final protein concentration was adjusted with phosphate-buffered saline to 2.0 mg of protein/ml.

Endotoxin inactivation by macrophage sonicates was determined by employing endotoxin-sensitive actinomycin D-treated mice (17) as previously described (9, 10). The designated mortality was that occurring within 48 hr after the ip injection of the various preparations. Mortality data were analyzed using the Chi-square test corrected by Yates' factor with a 95% confidence interval to designate differences between the groups.

Results. The intravenous administration of carbonyl iron markedly enhanced the susceptibility of rats to *S. enteritidis* endotoxin (Table I). The administration of 0.1 mg/hg of endotoxin, which produced no mortality in saline-injected (control) animals, induced in iron-treated animals a 67% mortality at 24 hr. Enhanced sensitivity to endotoxin of iron-injected rats was also indicated by the finding that 0.0001 mg/hg of endotoxin induced in this group a 20% mortality, while a 1000-fold increase of this dose in normal saline-injected animals induced no mortality. The LD_{50} of normal animals was 0.24 mg/

TABLE I. Influence of Carbonyl Iron on the Susceptibility of Rats to *S. enteritidis* Endotoxin.[a]

Pretreatment	Endotoxin (mg/100 hg)	Deaths / Total	Percent mortality
Saline	1.25	8/8	100
	1.0	9/10	90
	0.5	8/10	80
	0.125	3/10	30
	0.1	0/10	0
Carbonyl iron	—	0/13	0
	0.5	12/12	100
	0.125	11/12	92
	0.1	8/12	67
	0.01	6/14	43
	0.001	4/12	33
	0.0001	2/10	20
	0.00001	0/8	0

[a] *S. enteritidis* endotoxin was administered intravenously 1 hr after the intravenous administration of either saline solution or 100 mg of carbonyl iron/hg. Mortality was recorded 24 hr after the administration of the endotoxin preparations.

hg, as compared to 0.008 mg/hg in iron-injected rats, indicating a 30-fold increase in endotoxin susceptibility.

The study of the contribution of specific liver cells to endotoxin inactivation indicated that liver parenchymal cell sonicates did not detoxify *S. enteritidis* endotoxin, as denoted by an inability of parenchymal cell sonicates to modify the lethality induced by the endotoxin preparation (Table II). Indeed, the 82% mortality in the parenchymal cell endotoxin group was identical to that observed in the buffer endotoxin group. In marked contrast, when endotoxin was incubated in Kupffer cell sonicates, there was a 76% reduction of the mortality compared to the buffer group, indicating a significant ($p < 0.001$) endotoxin-detoxifying activity by Kupffer cells. These results indicate that the endotoxin-detoxifying capacity of the liver is a specific property of its macrophage component.

Pulmonary alveolar, peritoneal, and splenic macrophages manifested a significant endotoxin-detoxifying activity (Table II). Alveolar, splenic, and peritoneal macrophage preparations possessed comparable endotoxin-detoxifying activity. In this study, Kupffer cells possessed the highest endotoxin-detoxifying capacity of all macrophage populations, as determined by the observed reduction in mortality.

Discussion. The present observation of enhanced susceptibility of iron-injected animals to endotoxin, while apparently possessing unimpaired endotoxin-detoxifying activity (15), stresses the involvement of factors other than endotoxin inactivation in host susceptibility to endotoxin. Recently, Shands and Senterfitt (26) found that BCG infection, which produces marked hypersensitivity to endotoxin, induced hepatic parenchymal cell damage. Thus, the enhanced susceptibility of iron-injected rats to endotoxin may be associated with a metabolic derangement due to the acute hepatic injury which does not involve macrophages.

The present observations clearly demonstrate the endotoxin-detoxifying capacity of diverse populations of RE cells. These findings are in basic accordance with a recent observation of Filkins (15) who demonstrated endotoxin detoxification by macrophage sonicates using the lead-sensitized rat for the bioassay of endotoxin. The inability of endotoxin detoxification by rat hepatic parenchymal cell sonicates found in the present study also support Filkins' observation (16). The lack of endotoxin detoxification by hepatic parenchymal cells, as opposed to the potent endotoxin detoxification present in Kupffer cells, demonstrate that the endotoxin-detoxifying capacity of liver homogenates (7, 9, 10, 27) is a function of the RE component. Similarly, the high-endotoxin-detoxifying ability of spleen homogenates (7, 9, 10) appears to be dependent on its macrophage component, since splenic macrophage sonicates were indeed effective in modifying the endotoxin-induced lethality. The inability of leukocytes to detoxify endotoxin (15) further supports this concept of endotoxin inactivation to be the property of the fixed macrophage population.

During the present study, attempts were not made to localize the subcellular site, nor to characterize the agent(s) present in macrophages which are involved in endotoxin de-

TABLE II. Comparison of Detoxification of *S. enteritidis* Endotoxin by Diverse Macrophage and Hepatic Parenchymal Cell Sonicates.

Group	Sonicate[a]	Endotoxin (0.1 μg)	Deaths / Total	Percent mortality		p^b
1	Buffer	+	54/65	83.1		
2	Hepatic Kupffer cells	+	9/47	19.1	2 vs 1	<0.001
		--	4/44	9.1		
3	Hepatic parenchymal cells	+	19/23	82.6	2 vs 3	<0.001
		—	'23	17.4	3 vs 1	NS
4	Alveolar macrophages	+	12/37	32.4	4 vs 1	<0.001
		—	1/37	2.7		
5	Splenic macrophages	+	8/21	38.1	5 vs 1	<0.02
		—	2/21	9.1		
6	Peritoneal macrophages	+	9/27	33.3	6 vs 1	<0.01
		—	0/27	0		

[a] Four milliliters of macrophage sonicates with a protein concentration of 2 mg/ml were incubated with 1.0 μg (0.5 ml) of endotoxin at 37° for 3 hr. Following incubation, 200 μg (0.5 ml) of actinomycin D were added. Assay mice received 0.5 ml of these preparations intraperitoneally. Mortality was recorded 48 hr after the administration of the preparation.

[b] As determined by Chi-square test. NS = not significant.

toxification. Previous investigators (6, 27), employing liver homogenates, have reported endotoxin detoxification by mitochondrial, microsomal, or supernatant fractions. Recent studies by Filkins (16) employing subcellular fractions isolated from whole liver indicated that the lysosomes are the site of endotoxin detoxification. Since our studies indicate that Kupffer cells are the site of endotoxin inactivation, it is obvious the lysosomal profile on the Kupffer and parenchymal cell must differ enzymatically.

The demonstrated ability of specific cell populations to inactivate endotoxin should contribute to defining cellular mechanisms of host defense against endotoxins.

Summary. The comparative evaluation of the endotoxin-detoxifying capacity of diverse populations of rat macrophages was undertaken in order to define the relative role of macrophages in endotoxemia. Actinomycin D-treated mice were employed in the bioassay of submicrogram amounts of endotoxin. Sonicate preparations of rat pulmonary alveolar, peritoneal, hepatic, and splenic macrophages were found to possess significant endotoxin-detoxifying activity. Kupffer cells manifested the greatest activity. In contrast, liver parenchymal cell sonicates did not detoxify endotoxin. The demonstrated endotoxin-detoxifying capacity of macrophages further accept the protective role of macrophages in host defense against endotoxins.

1. Beeson, P. B., J. Exp. Med. **86**, 39 (1947).
2. Beeson, P. B., Proc. Soc. Exp. Biol. Med. **64**, 146 (1947).
3. Zweifach, B. W., Benacerraf, B., and Thomas, L., J. Exp. Med. **106**, 403 (1957).
4. Braude, A. E., Carey, F. J., and Zalesky, M., J. Clin. Invest. **34**, 858 (1955).
5. Di Luzio, N. R., and Crafton, C. G., Proc. Soc. Exp. Biol. Med. **132**, 686 (1969).
6. Farrar, W. E., Proc. Soc. Exp. Biol. Med. **118**, 218 (1965).
7. Filkins, J. P., Proc. Soc. Exp. Biol. Med. **134**, 610 (1970).
8. Rutenburg, S., Skarnes, R., Palmerio, C., and Fine, J., Proc. Soc. Exp. Biol. Med. **125**, 455 (1967).
9. Trejo, R. A., and Di Luzio, N. R., Proc. Soc. Exp. Biol. Med. **136**, 889 (1971).
10. Trejo, R. A., and Di Luzio, N. R., J. Reticuloendothel. Soc. **10**, 5 (1971).
11. Crafton, C. G., and Di Luzio, N. R., Amer. J. Physiol. **217**, 736 (1969).
12. Di Luzio, N. R., and Crafton, C. G., *in* "Shock: Biochemical Pharmacological and Clinical Aspects" (A. Bertelli and N. Back, eds.), p. 27. Plenum Press, New York (1970).

13. Stuart, A. E., and Cooper, G. N., J. Pathol. Bacteriol. **83**, 245 (1962).

14. Palmerio, C., and Fine, J., Arch. Surg. **98**, 679 (1965).

15. Filkins, J. P., Proc. Soc. Exp. Biol. Med. **137**, 1396 (1971).

16. Filkins, J. P., J. Reticuloendothel. Soc. **9**, 480 (1971).

17. Pieroni, R. E., Broderik, E. J., Bundeally, A., and Levine, L., Proc. Soc. Exp. Biol. Med. **133**, 790 (1970).

18. McCabe, W. R., Kreger, B. E., and John, M., New Eng. J. Med. **287**, 261 (1972).

19. Pisano, J. C., Filkins, J. P., and Di Luzio, N. R., Proc. Soc. Exp. Biol. Med. **128**, 917 (1968).

20. Lentz, P. E., and Di Luzio, N. R., Exp. Cell Res. **67**, 17 (1971).

21. Berry, M. N., and Friend, D. S., J. Cell Biol. **43**, 506 (1969).

22. Mosier, D. E., Science **158**, 1573 (1967).

23. Myrvik, Q. N., Leake, E. S., and Fariss, B., J. Immunol. **86**, 128 (1961).

24. Reed, P. W., and Tepperman, J., Amer. J. Physiol. **216**, 223 (1969).

25. Layne, E., *in* "Methods in Enzymology" (S. Colowick and N. Kaplan, eds.), Vol. III, p. 447. Academic Press, New York (1957).

26. Shands, J. W., and Senterfitt, V. C., Amer. J. Pathol. **67**, 23 (1972).

27. Trapani, R. J., Waravdekar, V. S., Landy, M., and Shear, M. J., J. Infec. Dis. **110**, 135 (1962).

Received July 9, 1973. P.S.E.B.M., 1973, Vol. 144.