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STUDIES ON THE EFFECTS OF LIPOPOLYSACCHARIDE ON LIPID PEROXIDATION OF ERYTHROCYTE AND ITS REVERSAL BY MANNITOL AND GLYCEROL

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Gram-negative sepsis often produces endotoxin (LPS) which causes infection. Reduction in tissue perfusion due to microcirculatory failure may lead to septic shock. We studied the effect of LPS on lipid peroxidation of erythrocyte. *In vitro* studies using 50 μg to 250 μg LPS/ml blood showed increased lipid peroxidation of erythrocyte in a dose-dependent manner. The increased effect of lipid peroxidation does not occur with LPS when erythrocytes were washed to remove plasma and leukocytes. Mannitol and glycerol, known scavengers of hydroxyl radical, arrest the elevation in lipid peroxidation of erythrocytes after LPS treatment. Hemolysis of erythrocytes was reduced with low doses of LPS. Plasma lipid peroxidation was elevated after treatment of blood with LPS. From the results we suggest that the peroxidation of erythrocyte lipid caused by LPS may probably play a role in the production of septic shock.

Key words: *sepsis, erythrocyte, lipid peroxidation, lipopolysaccharide.*

INTRODUCTION

Sepsis has often been associated with bacterial infection caused by the liberation of endotoxin (lipopolysaccharide, LPS) from the outer membrane of bacteria which produces lethal complication, after being produced from the organism. This lipopolysaccharide often causes biochemical, hemodynamic and physiological alteration in a system. For effective microcirculatory function, erythrocyte deformability is very critical. Impairment of erythrocyte deformability is believed to alter the blood circulation. This defective hemodynamic pattern is often observed in septic shock patients. Thus alteration in red cell mechanics may be partially responsible for inadequate oxygen delivery system often observed during septic shock (1, 2). Evidences

have also shown by some investigators (3, 4) that erythrocytes become less deformable during infection. Microcirculatory blood flow is dependent upon many factors among which rheological properties of blood elements play an important role in effective tissue circulation. Both erythrocytes and leukocytes have to deform themselves in order to pass through the capillary network which are often smaller in diameter than these cells. In sickle cell crisis erythrocytes were found to have decreased deformability which can impair microcirculation (5). Endotoxin and tumor necrosis factor have been shown to have effects on erythrocyte and leukocytes deformability *in vitro* (6). Later, Butterfield *et al.* (7) and Bellary *et al.* (8) have studied the effect of endotoxin on lipid order, motion and physical conformation of the erythrocyte cytoskeletal proteins and showed decreased values of these parameters, indicating that LPS influences those parameters in erythrocytes.

Recently, we have studied the effect of LPS on protein degradation and lipid peroxidation in erythrocytes (9) and found degradative changes by LPS. Lipid peroxidation has been observed in pathological states *in vivo* by Plaa and Witschi (10) and once started, it is a physiologically ongoing process. Hence in this communication, *in vitro* effect of LPS on lipid peroxidation in erythrocytes and its protection by some oxygen-radical scavengers have been studied.

MATERIALS AND METHODS

LPS (*Escherichia coli* 026:B6) was purchased from Sigma Chemicals Co. Thiobarbituric acid was obtained from E. Merck. All other reagents used were of reagent grade.

Cell preparations and incubation

Rat blood was collected by cardiac puncture in Acid-Citrate-Dextrose solution as anticoagulant. For *in vitro* experiments, blood containing same amount of protein from control and experimental group was taken and incubated with LPS (50 μg to 250 μg/ml blood) at 37°C for 90 min with shaking. After incubation, packed erythrocytes were isolated by centrifugation at 1000 × g for 10 min at 4°C. The plasma and buffy coat were removed by aspiration and the erythrocytes obtained were washed three times with suspension medium containing 15 mM Tris — 0.9 NaCl solution, pH 7.0 and 20% (w/v) suspension was made with the same suspension medium.

Lipid Peroxidation

To a suitable aliquot of erythrocyte cell suspension (20%), thiobarbituric acid — trichloroacetic acid — HCl reagent was added and lipid peroxidation of erythrocytes was measured according to Chattopadhyay *et al.* (11) following the principle of Buege and Aust (12). Malondialdehyde (MDA) formed from the breakdown of polyunsaturated fatty acids, serves as a convenient index for determining the extent of peroxidation reaction. The MDA produced from

the reaction with LPS was measured at 535 nm in spectrophotometer (Beckman DU6). The concentration of the MDA in the sample was calculated using an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ and expressed as nmoles of MDA per μg protein. Plasma MDA was also estimated by using trichloroacetic acid-soluble supernatant of respective plasma samples.

Hemolysis Studies

Lipid peroxidation involves peroxidative deterioration of cell membrane, hence we were interested to study any damaging effect of LPS on hemolysis of erythrocytes. Packed erythrocytes free from plasma and leukocytes were suspended in Tris-NaCl buffer to a final 6% hematocrit. We tested hemolysis at 37°C, instead of using a variation of temperatures. To a suitable amount of erythrocyte suspension, LPS was added and the reaction mixture was incubated at 37°C for 15 min. Later, a hypotonic shock was induced by addition of 15 mM Tris, pH 7.0 to the reaction mixture. The mixture was then incubated for another 10 min and centrifuged at $12.000 \times g$ for 10 min. The supernatant was taken for absorbance at 540 nm. Results were expressed as a percentage of control hemolysis, determined in the absence of endotoxin as followed by Godin *et al.* (13). Protein of the samples was estimated according to Lowry *et al.* (14).

Statistics

All values were expressed as mean ± SD, and Student's t-test was used for statistical significance.

RESULTS

To examine *in vitro* effect of LPS on lipid peroxidation, rat's blood was incubated with LPS at 37°C for 90 min. Various workers have used different types of *Escherichia coli* strain for experiments. Among them Godin *et al.* (13) studied the interaction of *E. coli* endotoxin (serotype 026:B6) with erythrocyte membrane. We have used the same strain of *E. coli* endotoxin in our previous experiments (9). In order to maintain continuity this particular strain of LPS has been used in the present study. The LPS effect was studied ranging from 50 μg to 250 μg/ml blood and a dose dependent increase (*Fig. 1*) in MDA production was observed. We found little change in lipid peroxidation with lower doses of endotoxin and the difference from the control value is not significant. However at higher concentration the changes are quite high and the results are statistically significant. In this experiment MDA production was measured upto 90 min, beyond which experiments were not performed. The interaction between LPS and erythrocytes is a slow process and we have observed very little effect of LPS in the early stages of incubation (unpublished observation). The effect is not linear and significant change in MDA production could only be observed after 90 minutes of incubation. Other workers (7, 8) have reported similar effects of LPS at 90 min. When washed

erythrocytes free from plasma and leukocytes were treated with 250 μg LPS, the increased effect of LPS was attenuated and the value obtained was more or less comparable to the control level without LPS (*Fig. 1*). Results show that *in vitro* MDA production, as an index of lipid peroxidation, may be dependent on the factors present in plasma and/or leukocytes.

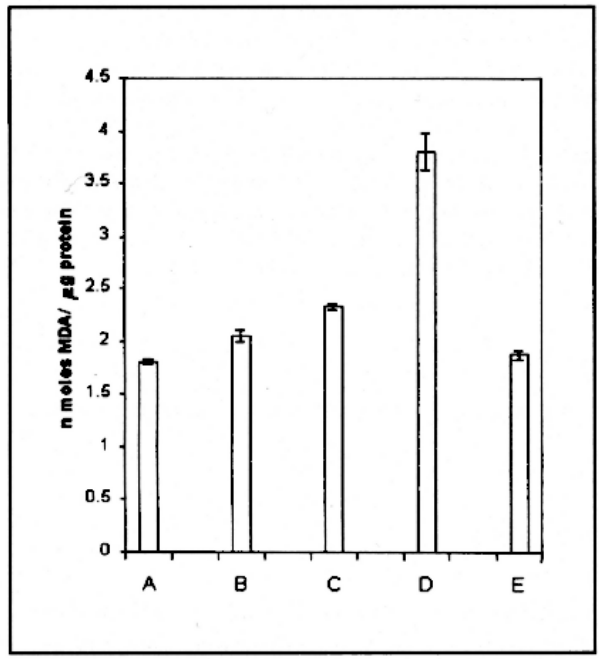

Fig. 1. Increasing concentrations of LPS on lipid peroxidation of erythrocytes. A: Control erythrocyte, B: 50 μg LPS/ml blood, C: 150 μg LPS/ml blood, D: 250 μg LPS/ml blood, E: Washed erythrocytes + 250 μg LPS/ml suspension.

Lipid peroxidation is thought to occur due to deterioration of cell membrane and mostly mediated by the production of free-radicals. Hence we were interested to examine the effect of mannitol (2 mM, 5 mM) and glycerol (10%) which are known oxyradical scavengers in lipid peroxidation. Results show (*Fig. 2*) that 2 mM mannitol with 250 μg LPS, attenuates the increased effect observed with 250 μg LPS alone. With 5 mM mannitol plus 250 μg LPS, the values obtained were comparable to the control values without LPS (*Fig. 2*). From these studies it is evident that mannitol with the above mentioned doses, may scavenge the oxygen-radical production, responsible for peroxidation of lipids. When we used 10% glycerol, we found effect like 5 mM mannitol (*Fig. 2*). Thus in our system both mannitol and glycerol were found to act as oxygen-radical scavengers of lipid peroxidation of erythrocytes.

As lipid peroxidation is a membrane related phenomenon, we studied the effect of LPS on hemolysis of erythrocytes. As shown in *Table 1*, with the two

doses of LPS concentration (100 μg, 200 μg) the hemolysis effect was less as compared to the control sample without LPS.

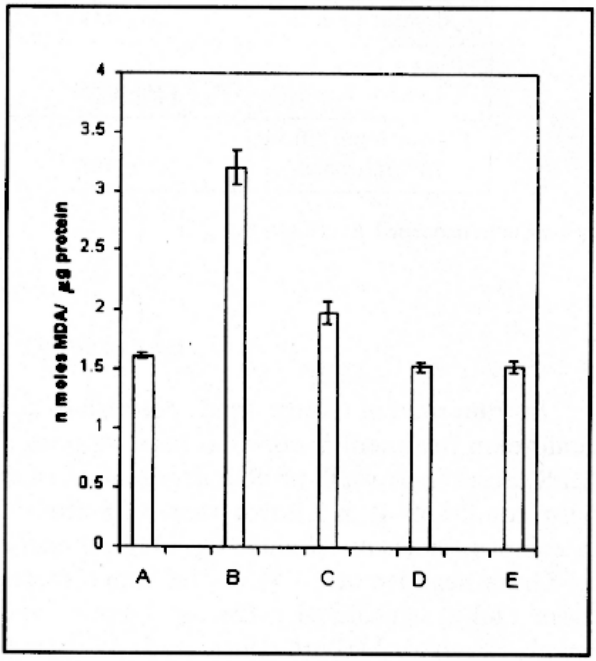

Fig. 2. Effect of LPS on lipid peroxidation of erythrocytes and its protection by mannitol and glycerol. A: Control erythrocyte, B: 250 μg LPS/ml blood, C: 250 μg LPS ml blood + 2 mM mannitol, D: 250 μg LPS/ml blood + 5 mM mannitol, E: 250 μg LPS + 10% glycerol.

We also studied the effect of increasing doses of LPS on blood plasma. Results (*Table 2*) show that plasma from LPS (50 μg/ml blood) treated blood shows stimulation of plasma lipid peroxidation by 13.71% whereas plasma lipid peroxidation is increased 60% over control treated with 250 μg LPS/ml blood. The significance of this increased effect of LPS on plasma is not fully known.

Table 1. Effect of *in vitro* LPS on hemolysis of rat blood.

System	Absorbance at 540 nm	Reduction in (%) hemolysis	p
Control	0.50 ± 0.028		
100 μg LPS	0.47 ± 0.011	6.0	< 0.1
200 μg LPS	0.43 ± 0.014	14.2	< 0.01

Results are mean ± SD.

Table 2. Effect of *in vitro* LPS on plasma lipid peroxidation of rat blood.

System	n moles MDA/μg protein	% change	p
Control plasma	1.75±0.11		
Plasma from 50 μg LPS/ml blood	1.99±0.05	13.71	< 0.01
Plasma from 250 μg LPS/ml blood	2.80±0.08	60.00	< 0.001

Results expressed as mean±SD.

DISCUSSION

In the present study lipid peroxidative changes in erythrocytes after endotoxin treatment *in vitro* has been reported. We have shown earlier (9) that LPS causes increase in protein degradation of erythrocytes both *in vitro* and *in vivo* condition. It is known that LPS from Gram-negative bacteria elicits a complex biochemical and physiological alterations which are characteristics of Gram-negative sepsis (15). The actual mechanism of septic shock has not been fully elucidated. During septic shock there has been often micro-circulatory failure which leads to reduction in tissue perfusion causing less oxygen supply to tissues and produces multiple organ failure or shock. Thus the state of septic shock may be dependent on the mechanistic structure of erythrocyte. Reports from earlier workers (16) indicated that malondialdehyde, byproduct of lipid peroxidation, has effect on the deformability of erythrocyte.

Peroxidative damage of erythrocyte as we observed after LPS treatment may change rheological properties of erythrocyte, which are essential to maintain tissue circulation properly. Presently, there are few reports concerning peroxidative damage of erythrocyte *in vitro* during sepsis or endotoxemia, are available.

In our previous communication (9) we have reported that washed erythrocytes free from plasma and leukocytes did not show increase in protein degradation of the erythrocytes after incubation with LPS. Similarly, in our present report we could not find any elevation in lipid peroxidation of washed erythrocytes after LPS treatment. It is expected that there will be no change in lipid peroxidation in washed erythrocytes, as the preparation was free from leukocytes and other cells. In our present preparation leukocytes are present, as we have used whole blood. Microscopic examination also reveals the same. Our observation supports the concept obtained by Butterfield *et al.* (7) where

they showed that if endotoxin was added to washed plasma and leukocyte free intact erythrocytes, no change in the motion of the spin level was found. Data from their paper together with our observation suggest that plasma soluble substances and/or leukocytes are required to produce the change in lipid peroxidation of erythrocyte. Our result also supports the finding of other groups (6) who showed that incubation with endotoxin and tumor necrosis factor in physiological concentrations increased the rigidity of mixed blood cells and of isolated polymorphonuclear leukocytes in a dose dependent manner. The exact mechanism of interaction of LPS with erythrocyte membrane may be specific, direct or may be non-specific (17,18). Our results obtained in lipid peroxidation may be non-specific mediated via cytokines, reactive oxygen species etc.

Mannitol is widely used as scavenger of hydroxyl radicals (19). Tien *et al.* (20) reported that mannitol (10 mM) produced more than 95% inhibition of lipid peroxidation initiated by Fenton reaction generated · OH radicals. Studies with free radical scavengers (Mannitol, Glycerol) in our system show 56% inhibition with 5 mM mannitol and glycerol, suggesting that may be hydroxyl radical is the mediator of peroxidation induced with LPS. Others have also showed the effect of glycerol as oxygen-radical scavenger (21) in erythrocyte lipid peroxidation. The influence of *E. coli* LPS on the stability of rat erythrocytes to hypotonic challenge has also been studied in our recent report. From our studies stabilization was observed with two concentrations (100 μg and 200 μg) of LPS used at a fixed incubation temperature of 37°C. Our results using erythrocyte as model system indicate that interaction of *E. coli* LPS with membrane alters osmotic fragility, the effect being influenced by the LPS concentrations. Though Godin (13) established that with higher concentration of LPS hemolysis increased, we did not repeat this as higher doses (above 200 μg) will be unphysiological for the system. The cause of increased plasma lipid peroxidation in our study is also not clear. Further studies are required to elucidate the role of both plasma and leukocyte factors on LPS-mediated peroxidative damage of erythrocytes.

REFERENCES

1. Baker CH, Wilmoth FR, Shutton ET. Reduced RBC versus plasma microvascular flow due to endotoxin. *Circulatory Shock* 1986; 20: 127—139.
2. Rogers F, Dunn R, Berrett J, Meriotti G, Sheaff C, Nolan P. Alterations of capillary flow during sepsis. *Circulatory Shock*, 1985; 15: 105—110.
3. Langenfeld JE, Livingston DH, Machiedo GW. Red cell deformability is an early indicator of infection. *Surgery* 1991; 110: 398—404.
4. Parrillo JE, Parker MM, Natanson C, Suffredini AF, Danner RL, Cunnion RE, Ognibere FP. Septic shock in humans. Advances in the understanding of pathogenesis, cardiovascular dysfunction and therapy. *Ann Int Med* 1990; 113: 227—238.

5. Stuart J, Johnson CS. Rheology of the sickle cell disorders. *Baill Clin Haematol* 1987; 1: 747—776.
6. Betticher CD, Keller H, Maly FE, Reinhart WH. The effect of endotoxin and tumor necrosis factor on erythrocyte and leukocyte deformability *in vitro*. *Brit J Haematol* 1993; 83: 130—137.
7. Butterfield DA, Sun B, Bellary S, Arden WA, Anderson KW. Effect of endotoxin on lipid order and motion in erythrocyte membranes. *Biochim Biophys Acta* 1994; 1225: 231—234.
8. Bellary SS, Anderson KW, Arden WA, Butterfield DA. Effect of lipopolysaccharide on the physical conformation of the erythrocyte cytoskeletal proteins. *Life Sciences* 1995, 56: 91—98.
9. Bhattacharyya J, Das Chowdhury T, Datta AG. Effect of endotoxin on protein degradation and lipid peroxidation of erythrocytes. *J Physiol Pharmacol* 1999; 50: 321—326.
10. Plaa GL, Witschi H. Chemicals, drugs and lipid peroxidation. *Ann Rev Pharmacol Toxicol* 1976; 16: 125—141.
11. Chattopadhyay A, Das Chowdhury T, Basu MK, Datta AG. Effect of Cu^{2+}-ascorbic acid on lipid peroxidation, Mg^{2+}-ATPase activity and spectrin of RBC membrane and reversal by erythropoietin. *Mol Cell Biochem* 1992; 118: 23—30.
12. Buege JA, Aust SD. Microsomal lipid peroxidation. *Methods Enzymol* 1978; 52: 302—310.
13. Godin DV, Tuchek JM, Garnett ME. Studies on the interaction of *Escherichia coli* endotoxin with erythrocyte membrane. *Can J Physiol Pharmacol* 1982; 60: 977—985.
14. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with Folin phenol reagent. *J Biol Chem* 1951; 193: 266—275.
15. Young LS, Martin WJ, Meyer RD, Weinstein RJ, Anderson ET. Gram-negative rod bacterimia: microbiologic, immunologic and therapeutic consideration. *Ann Intern Med* 1977; 86: 456—471.
16. Pfafferott C, Meiselman HJ, Hochstein P. The effect of malondialdehyde on erythrocyte deformability. *Blood* 1982; 59: 12—15.
17. Cavaillon HN, Cavaillon JM, Sjabo L. Cellular receptors for endotoxin. In Handbook of endotoxin: Cellular biology of endotoxin. LJ Berry (ed.) Elsevier 1985, pp. 1—24.
18. Morrison DC. Nonspecific interaction of bacterial lipopolysaccharides with membrane and membrane component. In Handbook of endotoxin Cellular Biology of endotoxin LJ Berry (ed.), Elsevier 1985, pp. 25—53.
19. Chan PC, Peller OG, Kesner L: Copper (II) catalyzed lipid peroxidation in liposomes and erythrocyte membranes. *Lipids* 1982; 17: 331—337.
20. Tien M, Svengen BA, Aust SD: Superoxide dependent lipid peroxidation. *Fed Proc* 1981; 40: 179—182.
21. Davis KJA, Goldberg AL. Oxygen radicals stimulate intracellular proteolysis and lipid peroxidation by independent mechanisms in erythrocytes. *J Biol Chem* 1987; 262: 8220—8226.

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