

Acta Medica Okayama

Volume 32, Issue 3

1978

Article 3

JULY 1978

A study of endotoxemia in ulcerative colitis and Crohn's disease. II. Experimental study

Kunitake Aoki*

*Okayama University,

Copyright ©1999 OKAYAMA UNIVERSITY MEDICAL SCHOOL. All rights reserved.

A study of endotoxemia in ulcerative colitis and Crohn's disease. II. Experimental study*

Kunitake Aoki

Abstract

The intestinal mucosal barrier of rabbits was damaged by carrageenan-induced ulceration of the colon, superior mesenteric artery occlusion (SMAO) and hemorrhagic shock and the values of Endotoxin (lipopolysaccharide, LPS) were determined by radioimmunoassay and the concentration of lysozyme (LZM) by the turbidimetric method. As a result, endotoxemia was observed in 13 out of 15 carrageenan rabbits, and in all of the SMAO and hemorrhagic shocked rabbits. Serum LZM concentration rose with time in all cases. As to the correlation of LPS and LZM, they changed almost in parallel in carrageenan rabbits, SMAO and hemorrhagic shock. LPS value and LZM concentration in blood were also determined in LPS injected rabbits. It was confirmed that injected LPS increased the LZM concentration of blood. On the basis of these results, it can be concluded that destruction of intestinal mucosal barrier permits an invasion of LPS into blood and then releases LZM into the blood stream..

KEYWORDS: endotoxemia, ulcerative colitis, radioimmunoassay, lysozyme, mucosal barrier

Acta Med. Okayama 32, (3), 207—216 (1978)

A STUDY OF ENDOTOXEMIA IN ULCERATIVE COLITIS AND CROHN'S DISEASE. II. EXPERIMENTAL STUDY

Kunitake AOKI

*Department of Surgery, Okayama University Medical School,
Okayama 700, Japan (Director: Prof. S. Tanaka)*

Received February 14, 1978

Abstract. The intestinal mucosal barrier of rabbits was damaged by carrageenan-induced ulceration of the colon, superior mesenteric artery occlusion (SMAO) and hemorrhagic shock, and the values of Endotoxin (lipopolysaccharide, LPS) were determined by radioimmunoassay and the concentration of lysozyme (LZM) by the turbidimetric method. As a result, endotoxemia was observed in 13 out of 15 carrageenan rabbits, and in all of the SMAO and hemorrhagic shocked rabbits. Serum LZM concentration rose with time in all cases. As to the correlation of LPS and LZM, they changed almost in parallel in carrageenan rabbits, SMAO and hemorrhagic shock. LPS value and LZM concentration in blood were also determined in LPS injected rabbits. It was confirmed that injected LPS increased the LZM concentration of blood. On the basis of these results, it can be concluded that destruction of intestinal mucosal barrier permits an invasion of LPS into blood and then releases LZM into the blood stream.

Key words: endotoxemia, ulcerative colitis, radioimmunoassay, lysozyme, mucosal barrier

The author has carried out a determination of LPS values in human blood by radioimmunoassay (RIA) (1, 2) and previously described the levels of LPS and LZM in the blood of a patient with ulcerative colitis (U.C.) and Crohn's disease (3). It was pointed out that blood LPS values as well as LZM concentrations reflected well the clinical symptoms and, in the active phase of U.C., the values showed a positive correlation. These results support the hypothesis proposed by C. Tai *et al.* (4) that in U.C. with damaged intestinal mucosal barrier, LPS originating from intestinal flora enters into the blood and aggravates the disease. In addition, it can be suggested that this invading LPS would release LZM into the blood.

In order to investigate the hypothesis mentioned above, various experimental systems with damaged intestinal mucosal barrier were examined. The systems used were composed of carrageenan-induced ulceration of the colon (5), superior mesenteric artery occlusion (SMAO) (6, 7) and hemorrhagic shock (8). Another experiment was conducted on the LZM concentration in blood of

rabbits with injection of small amounts of LPS (7). Experimental data was obtained suggesting that entrance of LPS into the blood and the rise in blood LZM occurred coincidentally with damaged intestinal mucosa.

The author regards the existence of blood LPS as an important factor in aggravation of U.C. and Crohn's disease.

MATERIALS AND METHODS

Animals. Mature rabbits (1.8kg-2.3kg, average 2.0kg) were maintained on a diet of RC4 chow (Oriental Yeast Industries, Ltd.) for at least one month before the experiment so that they be in a good nutritive condition. Antibiotics and any other drugs were not administered before the experiment.

Assay of endotoxin. Endotoxin was measured by the dextran coated charcoal method using RIA, and immunoreactive LPS ($\mu\text{g/ml}$) was referred as endotoxin (1-3).

Assay of lysozyme. LZM was measured by the turbidimetric method (9) and its activity was expressed as $\mu\text{g/ml}$. M. Lysodeikticus (lot No. ML16C101, Worthington Chemical Co., Freenold, N. J. U.S.A.) was used as the substrate and egg white lysozyme (lot No. 0104, Eisai Co., Tokyo, Japan) as the standard lytic enzyme. The concentration of blood LZM in non-treated rabbits was measured just prior to experiments and their average value was $2.4 \pm 1.5 \mu\text{g/ml}$ ($n=50$).

Experimental Procedures. All experimental procedures were done aseptically under Ketalar anesthesia (40 mg/kg); instruments were sterilized by dry heat at 160°C for 60 min and siliconized before use. The blood pressure was recorded with the femoral artery cannulated down to the aorta and connected with a manometer for measuring the blood pressure. Heparinized blood was collected from the femoral artery; the serum was separated and used for estimation of blood LPS and LZM.

Experimental ulceration. For the purpose of inducing colon ulcer (5), 5% degraded iota carrageenan (Lot 84C-1265-1 Sigma U.S.A) (1.5Kg/kg/day) was administered to 15 mature rabbits in the drinking water continually every day, and measurements were made of the body weight, occult blood, Ht and serum protein. Also, at intervals, heparinized blood was collected from the central artery of the ear. After 6-8 weeks, anemia intensified, and the abdomen was opened to confirm the presence of an ulcerous lesion. At autopsy, the entire gastrointestinal tract was examined and the ulcerated portions were fixed in 10% formalin and sections stained with hematoxylin and eosin.

Hemorrhagic shock. With 10 mature rabbits, femoral arteries on both sides were exposed and cannulated to the aorta; one femoral artery was used for measuring the blood pressure and collecting blood and the other was connected with a reservoir bottle containing 30mg of heparin for regulation of blood pressure. Prior to hemorrhage, 2mg/kg of heparin were given to each rabbit. The shock preparation consisted of rapid bleeding (5min) into the bottle, until a level of 35 to 40 mmHg arterial pressure was reached. This pressure was maintained for 120 min by regulating the level of the bottle. The blood in the reservoir was

then carefully reinfused over 15 min (8).

Bowel ischemia shock (SMAO). Ten mature rabbits were subjected to 60 min of superior mesenteric artery occlusion (10). Heparinized blood was collected every 30 min, blood was collected every 30 min, and blood pressure was recorded continually.

Administration of a non-lethal dose of LPS. One hundred $\mu\text{g}/\text{kg}$ of endotoxin, *E. coli* 0111 B₄ (B) LPS (Control No. 624094 Difco) was injected into nine mature rabbits through the femoral vein. Blood was collected at intervals till the 7th hour. There was no change in the blood pressure nor was any rabbit lost throughout this experiment.

RESULTS

Appearance of endotoxin in the blood stream.

Experimental ulcer of colon. In rabbits administered 5% degraded iota carrageenan in drinking water, stools became positive for occult blood from the third week on average and marked anemia and hypoproteinemia were observed from the 4th week onward. Twelve out of 15 rabbits showed ulceration mainly of the caecum. Microscopically, the ulceration occasionally extended deeply into the submucosa. The lamina propria was heavily infiltrated by inflammatory cells and the mucosal glands showed varying degrees of hyperplasia. Table 1 shows the relationship between the LPS value and ulceration. Thirteen out of

TABLE 1. CHANGES IN BLOOD LPS VALUES OF RABBITS WITH ULCER OF THE COLON FOLLOWING ADMINISTRATION OF CARRAGEENAN

Rabbit no.	Serum LPS ($\mu\text{g/ml}$)							Ulceration
	Days after administration							
	0	10	20	30	40	50	60	
C-1	0	0	6.0					+++
2	0	0.16	0	0.36	0	0.34		+++
3	0	3.0	0.55	1.8	2.1	0	1.5	+++
4	0	0	1.05	2.7	1.62	1.87	3.2	+++
5	0	5.0	3.1					++
6	0	0	1.1	0.5	1.0	0.64		++
7	0	0	1.08	1.0	0.36	0.34	0.5	++
8	0	0	0	2.64	3.01	1.76	2.86	++
9	0	0.36	0.07	0.05	0	0.1		+
10	0	0	1.02	0	1.6	0	2.7	+
11	0	2.4	1.6	4.0	2.8	0	0	+
12	0	0.32	0.38	0.26	2.68	1.6	2.88	+
13	0	3.0	0.13	1.25	0.24	0.25		—
14	0	0	0	0	0	0		—
15	0	0	0	0	0			—

The degree of ulceration was judged macroscopically. (+++), Myriads of ulcers; (++) , 20 or more of ulcers; (+), Not more than 20 ulcers; (-), Neither ulceration nor erosion observed.

15 rabbits showed endotoxemia; and of these 13 rabbits, 12 were rabbits with an ulcer. Two rabbits showing no endotoxemia were free of ulceration. Therefore, it can be said that there is a significant correlation between appearance of endotoxemia and presence of ulcer. Further, there also is correlation between LPS values and the degree of ulceration. However, this correlation cannot be established in all cases, *e.g.* C-2 and C-13 are the cases without this correlation.

Bowel ischemia shock (SMAO). There were almost no changes in aortic blood pressure during SMAO. Immediately after removal of SMAO, however, the blood pressure fell off sharply and thereafter recovers temporarily, but hypotension prevailed, and the animals died at 120–240 min after initiation of SMAO. Blood LPS detected was in the range of 0.04 to 2.8 $\mu\text{g}/\text{ml}$ in all of the 10 rabbits. In seven out of the 10 rabbits, LPS was detected during SMAO. There was no correlation between the blood LPS levels and the survival time. Fig. 1)

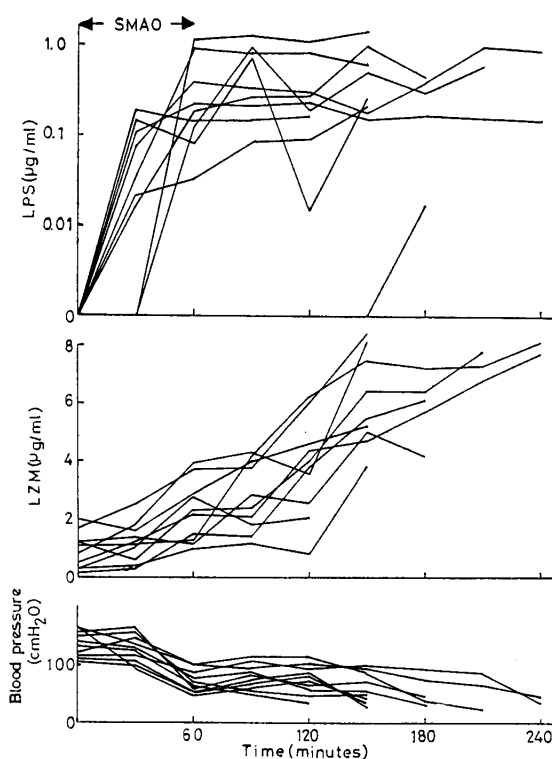


Fig. 1. Changes in blood LPS values, LZM concentrations and blood pressure of bowel ischemia shocked rabbits. Superior mesenteric artery occlusion (SMAO) was maintained for 60 minutes in each case.

Hemorrhagic shock. After reinfusion, the blood pressure was restored temporarily but fell gradually and six out of 10 rabbits died on the 8th hour after hemorrhage, three rabbits on the 20th hour and one rabbit on the 29th hour. Blood LPS was detected in the range of 0.01 to 0.78 $\mu\text{g/ml}$ in all rabbits, and it appeared as early as one hour after hemorrhage in four rabbits. No correlation was observed between blood LPS levels and the survival time (Table 2).

TABLE 2. CHANGES IN SERUM LPS VALUE OF RABBITS DURING HEMORRHAGIC SHOCK

Rabbit no.	Serum LPS ($\mu\text{g/ml}$)						Survival time (h)
	Hours after bleeding						
	bleeding ↓ 0	1	reinfusion ↓ 2	3	6	10	
H-1	0	0		0	0.26	0	20
2	0	0.78		0	0.70	0.54	29
3	0	0		0.08	0.10	0	20
4	0	0		0.19	0.40		7
5	0	0.09		0	0.31		8
6	0	0.01		0.07	0.01		7
7	0	0.02		0.06	0.18		8
8	0	0		0.12	0.22		8
9	0	0		0	0.14	0.21	13
10	0	0		0.10	0.12		8

Hemorrhage (45-65 ml, average 55 ml) was done and the blood pressure was maintained at 40 mmHg for 120 min, thereafter blood was reinfused.

Appearance of lysozyme in the blood

Experimental ulcer of colon. Ulceration was observed in 12 out of 15 rabbits and their blood LZM concentrations rose to more than twice that of the control. The remaining three rabbits had no ulceration and their blood LZM concentrations were similar to that of the control. There was very close correlation between the presence of ulceration and blood LZM levels. But no difference in the LZM concentration was observed between the group of rabbits with marked ulceration (+++) and those with erosion alone (+) (Table 3).

Bowel ischemia shock (SMAO). Blood LZM level rose with time, and immediately before death it reached a level more than 10 times that before SMAO. No significant correlation was noted between the survival time and blood LZM levels (Fig. 1).

Hemorrhagic shock. Blood LZM increased gradually from 30 min after hemorrhage. LZM concentrations at the third hour was 3-8 times higher than that in the control and these levels were maintained till death. In this experi-

ment too, no correlation was observed between blood LZM concentrations and the survival time (Table 4).

TABLE 3. CHANGES IN SERUM LZM CONCENTRATIONS OF RABBITS WITH
ULCER OF THE COLON FOLLOWING ADMINISTRATION OF CARRAGEENAN

Rabbit no.	Serum LZM ($\mu\text{g/ml}$)							Ulceration
	Days after administration							
	0	10	20	30	40	50	60	
c-1	4.8		11.6					+++
2	3.2	3.2	7.8	9.0	9.1			+++
3	7.4		7.2	8.6	10.3	11.9	15.2	+++
4	2.6	2.7	7.1	8.0	6.3	5.9	8.8	+++
5	2.7	7.0	4.6					++
6	1.2		1.0	6.3	3.2	5.3		++
7	1.7	1.7		3.7	5.1	6.7	4.7	++
8	2.8	4.1	4.2	4.8	9.6	9.2	9.0	++
9	2.4	1.0	5.3	1.0	3.2	6.3		+
10	3.9	2.8	3.0	8.4	7.7	3.2	6.6	+
11	5.0	5.5		9.4	8.6	10.1	8.9	+
12	5.0	4.6	5.2	3.2	6.0	7.5	7.1	+
13	1.7	1.0	1.7	3.2	1.7			—
14	3.2	3.8	1.0					—
15	1.7	7.0	1.0	1.0	9.9			—

TABLE 4. CHANGES IN SERUM LZM CONCENTRATION OF RABBITS
DURING HEMORRHAGIC SHOCK

Rabbit no.	Serum LZM ($\mu\text{g/ml}$)						Survival time (h)
	Hours after bleeding						
	Bleeding	Reinfusion					
	↓ 0	1	↓ 2	3	6	10	
H-1	5.5	7.6		25.8	18.9	20.6	20
2	2.9	5.5		5.5	8.9	15.5	29
3	2.1	2.9		7.5	12.3	12.9	20
4	1.4	2.3		8.3	5.3		7
5	2.9	5.5		20.0	23.5		8
6	3.8	16.2		25.5	25.2		7
7	2.6	4.5		8.8	9.6		8
8	3.6	7.2		8.4	9.2		8
9	2.8	2.2		21.6	20.0	17.6	13
10	3.2	6.9		18.0	15.8		8

Administration of a non-lethal dose of LPS. The effect of a non-lethal dose of LPS injected intravenously on changes in serum LZM concentration was monitored for seven hours. Serum LZM started rising from the first hour after injection: two and three hours later, LZM activity was 3-5 times higher than the control. Thereafter, serum LZM concentration decreased and at the fifth hour returned to the level of the control (Table 5).

TABLE 5. CHANGES IN BLOOD LZM CONCENTRATION OF RABBITS AFTER INJECTION OF VERY SMALL AMOUNTS OF LPS

Rabbit no.	Serum LZM ($\mu\text{g/ml}$)				
	Hours after injection				
	0	1	3	5	7
L-1	1.9	1.9	9.5	3.5	1.5
2	2.1	3.8	11.3	3.8	3.2
3	1.5	2.5	4.8	3.0	1.9
4	2.9	5.0	3.8	2.9	3.1
5	1.9	2.2	5.0	3.8	3.4
6	1.1	3.4	6.0	2.2	2.8
7	1.2	3.1	5.3	2.8	1.2
8	2.0	4.6	6.0	1.7	2.3
9	2.4	3.4	5.4	2.0	2.1

100 $\mu\text{g/kg}$ of LPS was intravenously injected.

Comparison of blood endotoxin values with lysozyme concentrations

In the case of experimental ulcer of the colon, blood LPS values and serum LZM concentrations in the carrageenan administered group were semilogarithmically plotted and the correlation between the two was evaluated. Marked endotoxemia tended to be accompanied by an increased LZM concentration as shown in Fig. 2. However, there were eight samples in which serum with no LPS detected shows high LZM concentration. It could not, therefore, be concluded that there was significant correlation between the two. In case of SMAO, changes in LPS and LZM showed almost the same tendency (Figs. 1, 2). In individual samples, however, ones showing high LPS levels do not always show high LZM levels and *visa versa*. Thus it is not likely that there is a significant correlation between LPS and LZM. As to hemorrhagic shock, a similar result as in SMAO was obtained.

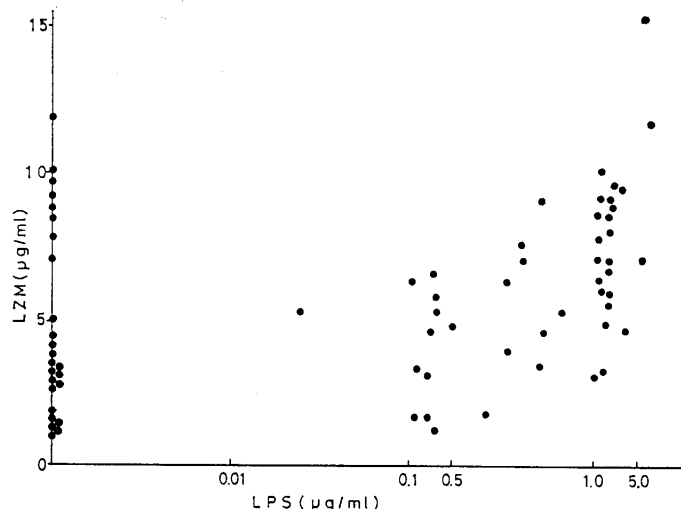


Fig. 2. Correlation between blood LPS values and LEM concentrations in rabbits with ulcer of the colon following administration of carrageenan. Blood LZM was plotted along the ordinate and LPS on logarithmic paper along the abscissa.

DISCUSSION

Tai and Tanaka detected endotoxin in blood of u.c. patients by RIA method (4). In the previous paper (3), the author reported that blood LPS of u.c. patients estimated by RIA reflected well the clinical symptoms. J. Fine *et al.* detected blood endotoxin in SMAO shocked rabbits by the limulus gelatin test (6, 11, 12). However, this limulus test is complicated by inhibitors present in blood and the difficulty in judging gelatination. More specifically, the problem of inhibitors in blood has not been solved yet, and thus the limulus test cannot be regarded as a method suitable for determining endotoxin in blood. As a matter of fact, it was pointed out that results of limulus test are not always in agreement with clinical symptoms and prognosis (13). The biological activities of LPS are multifarious and complex. Thus, no better method than RIA is available for measuring blood endotoxin. LPS used has a bacterial component which crossreacts some intestinal mucosal antigen (14). The RIA method also has an unsolved problem involving the diversity of antibody (15).

In the present study, the author introduced the RIA method to experimental systems such as carrageenan-induced ulceration, SMAO, and hemorrhagic shock. These systems are induced by different mechanisms but eventually bring about the destruction of the intestinal mucosal barrier. As a result, the existence of blood LPS was confirmed in all of the animals and it was demonstrated that LPS in the intestinal tract can enter into the blood stream when the intestinal

mucosal barrier is damaged.

With regard to cytotoxicity of LPS, A Jnoff *et al.* (7, 16) found experimentally that LPS works directly on the lysosome membrane as a labiliser and induces the release of hydrolytic enzymes. In the previous paper, the author reported that LPS values are proportional to LZM concentrations in blood of u.c. and surmised that LZM, a lysosomal enzyme could be released into the blood stream by invading LPS. Experiments were performed for the purpose of corroborating this hypothesis. The changes in serum LZM concentrations were shown to be in parallel with the change in blood LPS values in experiments of SMAO, carrageenan-induced ulcer of the colon and hemorrhagic shock. However, this correlation was not found in a few samples. In animals subjected to SMAO and hemorrhagic shock, there may be some intracellular anoxia which may make the lysosomal membrane labile, causing lysosomal enzymes to be released (8). In animals with carrageenan-induced ulcer too, carrageenan itself works as a labiliser on the lysosome membrane (17, 18). Such factors seem to give some cases in which LPS values are not correlated with LZM concentrations. This is also applicable to clinical cases which showed no correlation between blood LPS level and LZM concentration (3). When the intestinal mucosal barrier is destroyed, LPS enters into the blood, and at the same time serum LZM concentration rises. When LPS was injected intravenously, serum LZM concentration was also high. With these findings and aforementioned studies of A. Janoff *et al.* put together, it is clear that the LPS which entered from the intestinal tract is responsible for the release of LZM into the blood stream. It is not known what significance the rise in the blood lysosomal enzyme activity has in relation to aggravation of u.c. and Crohn's disease, although a detoxifying function and harmful actions of LZM has been reported (19, 20). These points require further detail study. It remains to be ascertained whether the appearance of blood LPS is responsible for aggravation of u.c. or if it is a secondary effect due to destruction of the intestinal mucosal barrier.

Acknowledgment. The author wishes to thank Prof. Sanae Tanaka for reviewing this manuscript and Dr. Chiaki Tai for helpful advice throughout this study.

REFERENCES

1. Tai, C.: Radioimmunoassay of endotoxin. *Igaku no Ayumi* **84**, 78-79, 1973 (in Japanese).
2. Kimura, H.: Measurement of endotoxin. 1. Fundamental studies on radioimmunoassay of endotoxin. *Acta Med. Okayama* **30**, 245-255, 1976.
3. Aoki, K.: The study on endotoxemia in ulcerative colitis and Crohn's disease. I. Clinical study. *Acta Med. Okayama* (in press).
4. Tai, C. and Tanaka, S.: Endotoxemia and clinical severity in ulcerative colitis. *Geka* **36**, 665-670, 1974 (in Japanese).
5. Watt, J. and Marcus, R.: Experimental ulcerative disease of the colon in animals. *Gut* **14**, 506-510, 1973.

6. Tamakura, S., Rojas-Corona, Cuevas, P. and Fine, J.: Demonstration of a lethal endotoxemia of intestinal origin in refractory non-septic shock. *Ann. Surg.* **173**, 219-224, 1971.
7. Janoff, A., Weissmann, G., Zweifach, B.W. and Thomas, L.: Pathogenesis of experimental shock. IV. Studies on lysosomes in normal and tolerant animals subjected to lethal trauma and endotoxemia. *J. Exp. Med.* **116**, 451-466, 1962.
8. N. Gregor Sutherland, N.G., Bounous, G. and Fraser N. Gurd, F.: Role of intestinal mucosal lysosomal enzymes in the pathogenesis of shock. *J. Trauma* **8**, 350-380, 1968.
9. Inai, S., Hirao, F., Kishimoto, S. and Takahashi, H.: Studies on serum lysozyme. II. Lysozyme activity in normal human serum and in serum of patients with various diseases. *Med. J. Osaka Univ.* **9**, 33-43, 1958.
10. Hershey, S.G., Baez, S. and Rovenstine, E. A.: Intestinal ischemia shock in normal and dibenzylamine-protected dogs'. *Am. J. Physiol.* **200**, 1239-1244, 1961.
11. Fine, J., Frank, E. D., Ravin, H. A., Rutenberg, S. H. and Schweinburg, F. B.: The bacterial factor in traumatic shock. *N. Engl. J. Med.* **260**, 214-220, 1959.
12. Cuevas, P. and Fine, J.: Demonstration of a lethal endotoxemia in experimental occlusion of the superior mesenteric artery. *Surg. Gynecol. Obst.* **133**, 81-83, 1971.
13. Stumacher, R. J., Kovnat, M. J. and McCabe, W. R.: Limitations of the usefulness of the limulus assay for endotoxin. *N. Engl. J. Med.* **288**, 1261-1264, 1974.
14. Perlmann, P. and Hammarstrom, S.: Antigen from colon of germfree rats and antibodies in human ulcerative colitis. *Ann. N. U. Acad. Sci.* **124**, 377-394, 1965.
15. Leonard, S. E. and Padians, E.: The synthesis and secretion of immunoglobulins by lymphoid cells in the sheep. The primary response to salmonella LPS. *J. Exp. Med.* **144**, 586-603, 1976.
16. Weissmann, G. and Thomas, L.: Studies on lysosomes. I. The effects of endotoxin, endotoxin tolerance, and cortisone on the release of acid hydrolases from a granular fraction of rabbit liver. *J. Exp. Med.* **116**, 433-450, 1962.
17. Mankes, R. and Abraham, R.: Lysosomal dysfunction in colonic submucosal macrophages of rhesus monkeys caused degraded late carrageenin. *Proc. Soc. Exp. Biol. Med.* **150**, 166-170, 1975.
18. Catanzaro, P. J.: Spectrum and possible mechanism of carrageenin cytotoxicity. *Am. J. Pathol.* **64**, 387-404, 1971.
19. Hill, I. R. and Porter, P.: Studies of bactericidal activity to escherichia coli of porcine serum and colostral immunoglobulins and the role of lysozyme with secretory IgA. *Immunology* **26**, 1239-1250, 1974.
20. Golub, E. S. and Spitznagel, J. K.: The role of lysosomes in hypersensitivity reactions: Tissue damage by polymorphonuclear neutrophil lysosomes. *J. Immunol.* **95**, 1060-1066, 1966.