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KEYWORDS: endotoxemia, ulcerative colitis, radioimmunoassay lysozyme, Crohn's disease

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A STUDY OF ENDOTOXEMIA IN ULCERATIVE COLITIS AND CROHN'S DISEASE. I. CLINICAL STUDY

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Abstract. Endotoxin (lipopolysaccharide, LPS) and LPS antibody in the blood were studied in 61 cases of ulcerative colitis (U. C.) by radioimmunoassay. Lysozyme (LZM) concentration was also studied by the turbidimetric method. As a result, it was found that the blood LPS value as well as serum LZM concentration reflects the clinical observations. The case of endotoxemia in the active phase group showed a positive correlation between the LPS value and LZM concentration. LPS antibody which could not be detected in many cases of the active phase, had a high titer in cases of remission with a long history of the disease. These results would suggest that in U. C. with damaged intestinal mucosal barrier, LPS originating from intestinal flora enters into the blood and aggravates the disease and further that this invading LPS releases LZM into the blood. The same studies were performed on 7 cases of Crohn's disease and the same result was obtained.

Key words: endotoxemia, ulcerative colitis, radioimmunoassay. lysozyme, Crohn's disease.

Although the pathogenesis of ulcerative colitis and Crohn's disease remains mostly unknown, one of the current hypothesis is that, when there is local intestinal inflammation, mucosal ulceration, or granulomatous reaction, bacterial antigens are easily taken up from the intestine and cause local hypersensitivity of the intestine (1). Perlmann, P. *et al.* (2) reported that there could be a cross-reaction of E-coli 014: LPS with colon mucosal antigen and suggested that bacterial penetration through the gut wall predisposes an autoimmune response. On the other hand, C. Tai detected LPS in the blood of patients with U. C. by radioimmunoassay (3, 4) and proposed the possible role of LPS in aggravation of this disease. In the present study, LPS values and LRS antibody titer were followed up over a long period by radioimmunoassay and compared with the clinical course in order to ascertain how the invading LPS aggaravates this disease. Much attention has been paid to the high activity of LZM, one of lysosomal enzymes, in U. C. and Crohn's disease (5). In order to make sure whether the

invaded LPS actually releases LZM into the blood, the possibility of a correlation between LZM concentration and blood LPS value was investigated.

MATERIALS AND METHODS

Subjects. Sixty-one U. C. patients (27 male, 34 females) with a mean age of 39 years (14–72 years) living in Okayama and Hiroshima district which meet the diagnostic criteria of the Ulcerative Colitis Research Unit of the Health and Welfare Ministry Japan (6) were selected as subjects. Activity of the disease process was judged according to the severity index as described by C. Tai *et al.* (7). Both LPS value and LPS antibody titer were studied on 102 samples in which 30 samples were obtained from active phase and 72 samples from remission of 61 non-operative cases. Both LPS value and LZM concentration were studied on 77 samples (23 samples from active phase, 54 samples from remission) from 46 non-operative cases.

All of LPS, LPS antibody and LZM data was obtained from 7 postoperative cases of Crohn's disease (6 males, 1 female) with a mean age of 34 years (29–42 years) whose diagnosis were histlogically confirmed. The control population consisted of 10 normal subjects of the hospital and laboratory staff (7 males, 3 females) with a mean age of 28 years (19–38 years).

Preparation of labeled LPS. E. coli 0111: B_4 (B) LPS (control No. 616326 Difco) and E. coli 0111 B_4 (W) LPS (control No. 1598148, Difco) were used as antigen sources. These LPS were washed by dialysis against 0.5 M phosphate buffer (pH 7.0) and subjected to iodinated as follows. The LPS was labeled with carrier free Na ^{125}I (CEA-IRE-SORIN) by the method of Hantu and Greenwood (8). After iodination, the mixture of the iodinated endotoxin and chemical reagents was applied to a Sephadex G-50 (Coarse) Column (1.5×25cm) and eluted with 0.1 M phosphate buffer (pH 7.4) to separate LPS bound ^{125}I from free ^{125}I. The labeled LPS was stored at −20°C before use.

Preparation of LPS antibody. Antiserum was prepared from rabbit immunized with LPS mixed with complete Freund's adjuvant (control No. 61152 Difco). Rabbits were injected in the gluteal muscle with 4 mg of LPS (2 mg of E. coli 0111; B_4 (B) LPS + 2 mg of E. coli 0111; B_4 (W) LPS) once a week for 3 months. Plasma which was decomplemented at 56°C and centrifuged for 30 min was used as standard antiserum.

Radioimmunoassay of LPS. Assay was performed according to the method of Kimura (4) using dextran-coated charcoal. The set-up was routinely duplicated each time for the specimen, and triplicated for the calibration curve. Radioactivity were determined by autogammacounter (RDI-212-A, Toshiba). The units used were μg/ml.

Radioimmunoassay of LPS antibody. LPS antibody was measured by determining the amount of ^{125}I-LPS in the LPS-LPS antibody complex. For convenience, a titer of a 20-fold dilution of a standard antiserum was taken to be 10 units, a titer over 10 units was expressed as +++, over 5 units as ++ and under 5 units as +.

Measurement of lysozyme. Serum LZM concentration was measured by the turbidimetric method (9) and M. Lysodeikticus (lot No. ML 15C 101, Worthington Chemical Co. Freenold, N. J. U. S. A.) was used as a substrate and egg white lysozyme (lot No. 0104, Eisai Co., Tokyo, Japan) as the standard lytic enzyme. The concentration were expressed in μg/ml.

RESULTS

Blood LPS value related to the activity of the disease. LPS was positive in 38 (62%) out of 61 cases of U. C. and three (43%) out of seven cases of Crohn's disease. To analyse this data in further detail, patients with U. C. (61 cases, 102 samples) were classified in subgroups on the basis of two different parameters—this is, activity of disease and extent of lesion. Patients at active phase (n=30) had a blood LPS value (means $\pm$ sd) of 0.68 ± 0.42 μg/ml and positive LPS in 87 per cent of the cases; those in remission (n=72) had a value of 0.17 ± 0.28 μg/ml and positive LPS in 35 per cent of the cases (Fig. 1).

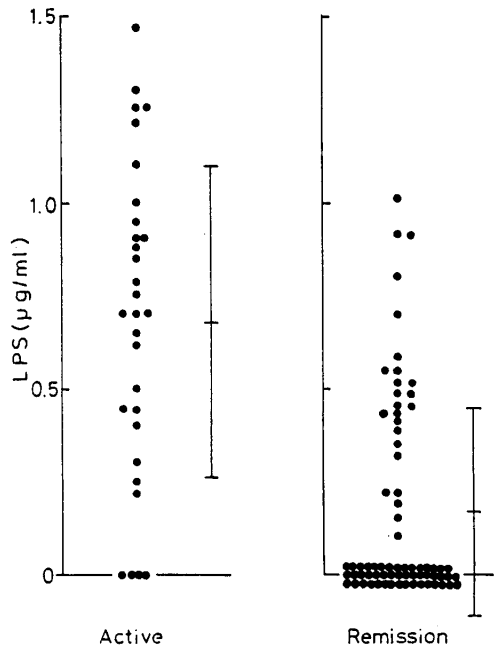

Fig. 1. Comparison of LPS values of the active phase and the remission of U. C.. LPS values of active phase were determined from 30 samples and their mean value was 0.68 ± 0.42 μg/ml. Those of remission were from 72 samples and the mean value was 0.17 ± 0.28 μg/ml.

Classification of the patients with U. C. on the basis of extent of the lesion showed that patients with total colitis (n=45) had a blood LPS value of $0.28 \pm$

0.39 μg/ml and positive LPS in 44 percent; those with left-sided colitis (n=38) a value of 0.33±0.36 μg/ml and positive in 55 percent; those with proctitis (n=19) a value of 0.40±0.46 μg/ml and positive in 53 percent. In relation to the activity of the disease, both the detectability of LPS and LPS value were statistically significant ($p<0.001$), on the other hand with the extent of the lesion, neither of them were statistically significant.

LPS value was followed over time on 11 cases. LPS was positive in all samples from the active phase, but negative or of lower value in the samples from remission (Table 1).

Among 7 cases of Crohn's disease, 0.15, 0.01 and 0.2 μg/ml of LPS were detected in the first case, second case and third case respectively. In the first, postoperative recurrence developed twice. The second was complicated with rupture of gut anastomosis. The third had no complication (Fig. 2).

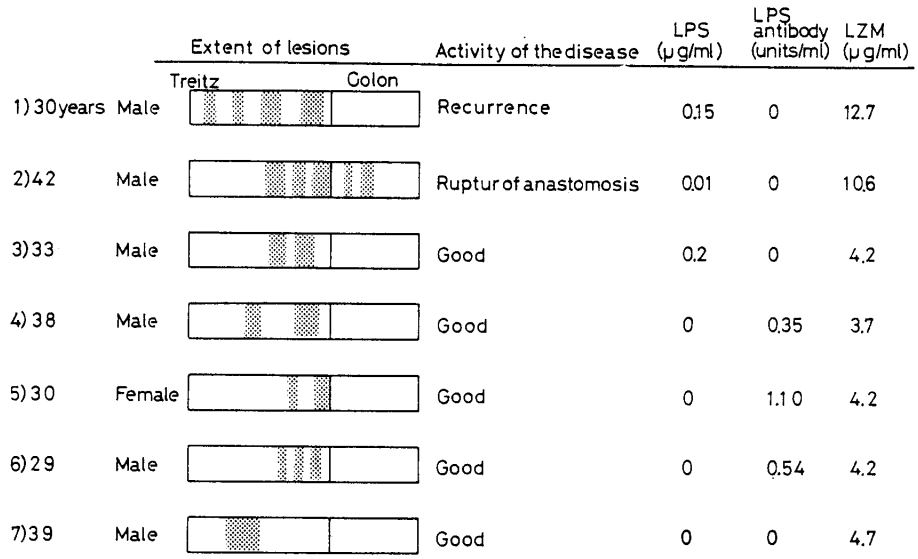

Fig. 2. Dotted areas represent the site of lesions. Determination of LPS values, LPS antibody titers and LZM concentrations was carried out at the time indicated by the activity of the disease.

Blood LZM concentration related to the activity of the disease. The mean LZM concentration (means±sd) was 6.4±4.5 μg/ml (n=77) in the patients with U. C.; 6.3±3.4 μg/ml (n=7) in the patients with Crohn's disease and 2.1±1.5 μg/ml (n=10) in the normal subjects. Further detail analysis of LZM concentration was carried out in U. C. The mean LZM concentration was 10.9±4.6 μg/ml (n=23) in the active phase, and 4.5±3.2 μg/ml (n=54) in the remission.

The differences are statistically significant. ($p<0.001$) (Fig. 3). With the extent of the lesion of U. C., the serum LZM concentration was 6.2 ± 3.9 μg/ml in total colitis ($n=34$), 6.8 ± 5.7 μg/ml in left-sided colitis ($n=29$), and 6.0 ± 3.6 μg/ml in proctitis ($n=14$). These differences were statistically insignificant.

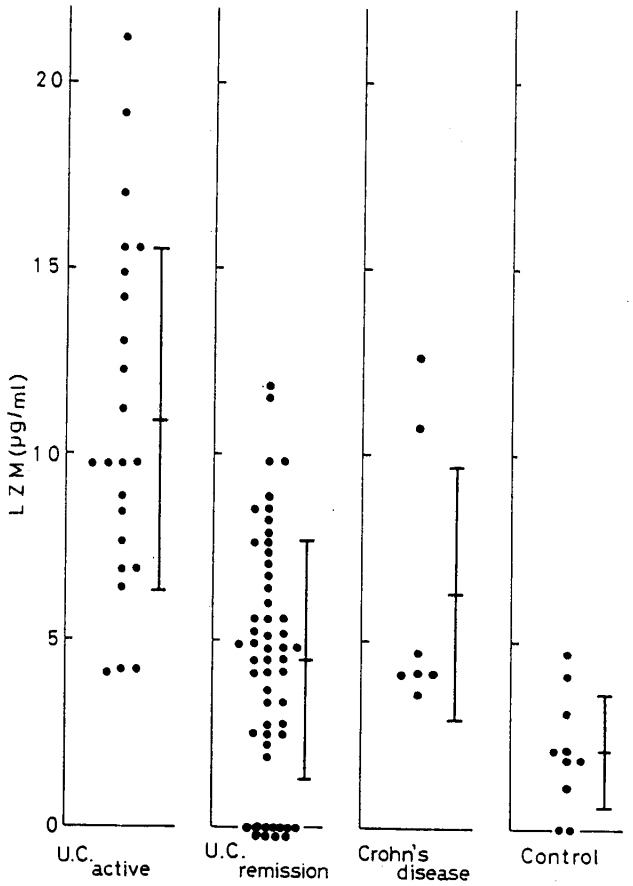

Fig. 3. Distribution of LZM concentrations of U. C., Crohn's disease and control. In U. C. 23 samples from active phase and 54 samples from remission were analyzed and their LZM concentrations were 10.9 ± 4.6 and 4.5 ± 3.2 μg/ml, respectively. In Crohn's disease and the control, they were 6.3 ± 3.4 and 2.1 ± 1.5 μg/ml, respectively.

The changes of LZM concentration with the lapse of time reflected well the activity of the disease process, as the LPS value did (Table 1).

In Crohn's disease, the first case in active phase and the second complicated with rapture of gut anastomosis had high concentrations, of LZM 12.7 μg/ml and 10.6 μg/ml respectively (Fig. 2).

TABLE 1. CHANGES IN BLOOD LPS VALUE, LPS ANTIBODY TITER AND SERUM LZM CONCENTRATION DURING THE CLINICAL COURSES OF U. C.

Patient no.	Years	Sex	Extent of lesion		Active→Remission→Active→Remission			
1	16	Female	Total colitis	LPS	0.9	0	1.6	
				LPS antibody		++	−	
				LZM	6.5	5.0		
2	42	Male	Proctitis	LPS	1.3		0.69	0.22
				LPS antibody	−		−	+
				LZM	15.5		11.1	4.8
3	65	Female	Left-sided colitis	LPS		0	1.21	
				LPS antibody		++	−	
				LZM		2.2	14.3	
4	50	Male	Left-sided colitis	LPS	1.0	0	1.47	
				LPS antibody	−	++	−	
				LZM	9.6	2.7	21.2	
5	34	Female	Left-sided colitis	LPS		0	0.69	0.33
				LPS antibody		+	−	
				LZM			14.7	5.4
6	17	Male	Total colitis	LPS	0.5	0		
				LPS antibody	−	+		
				LZM	9.6	5.5		
7	21	Female	Total colitis	LPS		0	0.45	
				LPS antibody		+	−	
				LZM		2.9	8.1	
8	42	Male	Left-sided colitis	LPS		0	0.24	
				LPS antibody		++	−	
				LZM		4.4	4.2	
9	48	Male	Total colitis	LPS	0.21	0		
				LPS antibody	−	+		
				LZM	7.7	2.9		
10	38	Female	Proctitis	LPS		0.55	1.27	
				LPS antibody		−	−	
				LZM		1.9	12.2	
11	64	Female	Left-sided colitis	LPS	1.26	0.06 ↓ ope.	1.5	
				LPS antibody	+	+	−	
				LZM	19.1	4.83	3.7	

Correlation between LPS value and LZM concentration in U. C.. The correlation between LPS value and LZM concentration was compared in 77 samples from 46 non-operative cases of U. C..

First, 23 samples from the active phase were studied. The samples from the cases with LPS value more than 1.0 μg/ml had LZM concentrations of more than

10 μg/ml. The samples from the cases with LPS value less than 0.3μg/ml had normal LZM concentrations as shown in Fig. 4. There was a positive correlation between LPS value and LZM concentration (correlation coefficient $r = +0.72$, $p < 0.01$).

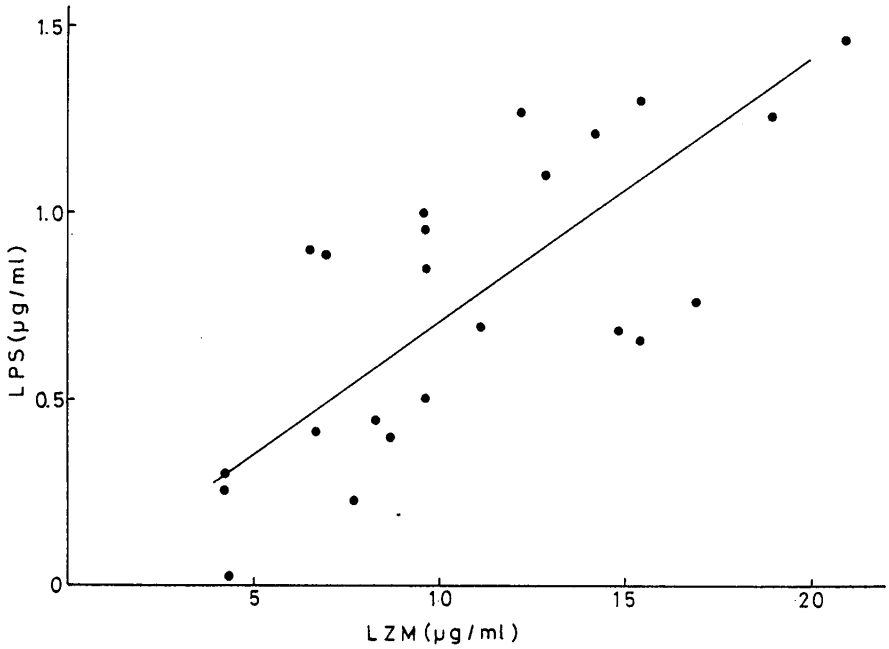

Fig. 4. Correlation between blood LPS values and serum LZM concentrations in the active phase of U. C.. Correlation coefficient $r = +0.72$, $p < 0.01$.

Fifty-four samples from the cases in remission showed no correlation between LPS value and LZM activity (Fig. 5).

LPS antibody titer related to the activity of the disease. LPS antibody titer was studied on 102 samples from 61 cases of U. C. in which no colectomy was done. It was positive in only 7 cases (23%) out of 30 samples from the active phase and in 39 cases (58%) out of 72 samples from the remission. As shown in Table 1, in the remission LPS antibody titer was high, but in active phase it could not be detected.

In Table 2 details of seven patients with very high LPS antibody titer, *i.e.* 10 units or more; all of them were remission cases, their histories were relatively long, averaging 7.1 years. Less than 10 units of LPS antibody titer was observed in 24 remission cases of relative short history, averaging 3.5 years.

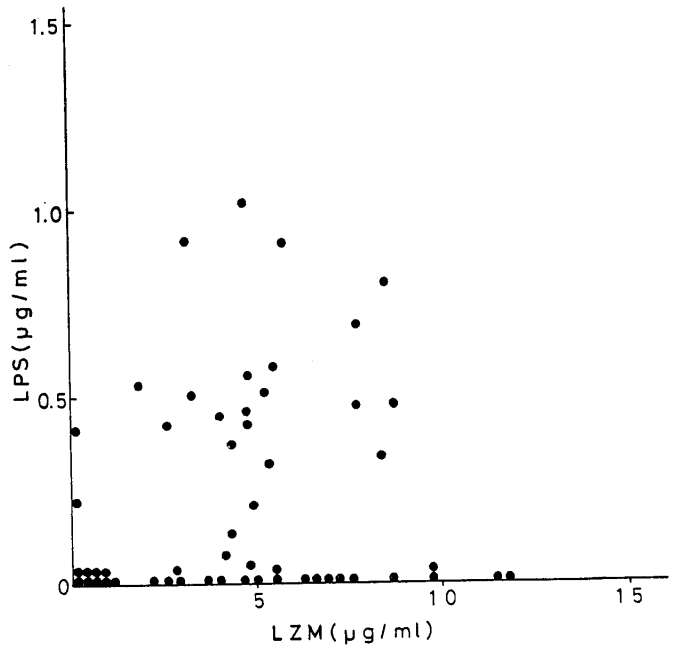

Fig. 5. Correlation between blood LRS values and serum LZM concentrations in the remission of U. C. .

TABLE 2. CLINICAL COURSE OF U. C. PATIENTS WITH LPS ANTIBODY (+++)

Patient no.	Years	Clinical history (years)	Activity of the disease
1	51	9	Remission
2	45	9	Remission
3	39	5	Remission
4	65	11	Remission
5	44	7	Remission
6	45	5	Remission
7	39	4	Remission

DISCUSSION

Blood LPS and LPS antibody in U. C. and Crohn's disease were studied by the method of radioimmunoassay and at the same time, serum LZM concentration was measured and these values were compared with the actual clinical observations. Results of this clinical study made it clear that not only the detectability of LPS but also its value was higher in the active phase than in remission

of U. C., reflecting the clinical severity of the disease. Blood LZM concentration was also high in the U. C. patients, especially in the active phase. Blood LPS value and LZM concentration showed a positive correlation. Similar results were obtained in cases of Crohn's disease.

It is very interesting to note that LPS could be detected in these diseases. The reason for this are not clear but they may be as follows. Since many bacteria always exist in the intestinal tract, it may be that blood LPS comes from these bacteria. Although there have been several reports which suggest that macromolecules such as horseradish peroxidase (10), insulin (11), or milk protein (12), can be absorbed through the intestinal mucosal barrier, it is unlikely that a larger molecule such as LPS can do likewise. However, entrance of LPS into blood can occur and may result from local intestinal and systemic disorders, when this barrier is destroyed by factors such as inflamation or ulceration etc; as suggested by W. A. Walker *et al.* (13). The mucous membrane in the active phase of U. C. is characterized by easy bleeding, erosion and ulcer. The fact that both the LPS value and its detectability were high in the active phase of U. C. is consistent with the hypothesis of LPS invasion as deseribed above. Fever, tachycardia, and diarrhea observed in the active phase are probably clinical evidence of systemic disorders caused by the invading LPS.

In the present clinical study, serum LZM concentration was higher than control. There are several reports (14) that serum LZM concentration is correlated white blood cell counts in other diseases. But the author found that, in the patients with U. C., there was no correlation between serum LZM concentration and the white blood cell counts. Since there was a positive correlation between serum LZM concentration and the LPS value, it is more likely that the raised serum LZM activity was due to the labileizing action of LPS on the lysosome membrane (15, 16) of palatelet, macrophage, granulocyte and local mucous cell. Such an explanation can be made in case of active phase of U. C.. On the other hand, high concentrations of LZM were observed in remission of U. C., despite the decrease in LPS. This discrepancy was probably due to the influence of other factors, such as local anoxia, metabolic disorder and administration of steroid (15).

As to LPS antibody, it is considered to be responsible for the aggravation of U. C., since antibodies to colon mucosal antigen (17) and to E-coli 014: LPS (2) have been detected in the blood of patients with U. C.. So, the author determined the LPS antibody titer in U. C. patients. The detectability of LPS antibody was low in the active phase and these results suggest that LPS antibody does not aggravate the disease. This supports the current proposal (18) that they are only a secondary product due to destruction of the mucous membrane and modi-

fication of its antigenicity.

Similar examinations were carried out in 12 patients to evaluate postoperative cases of U. C.. Blood LPS was detected immediately before operation, but it disappeared along with improvement of the disease, supporting the author's hypothesis as described above (data not shown).

There have been no studies so far to clarify the mechanism of aggravation of U. C.. In the present clinical study, the author showed that LPS originated from the intestinal flora entering into the blood of U. C. and Crohn's disease and that this aggravated the disease.

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