Acta Medica Okayama

Volume 32, *Issue* 2 1978 *Article* 7

JUNE 1978

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Setsuo Washida*

*Okayama University,

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KEYWORDS: endotoxin, platelet, receptor, binding, diffusion dialysis

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*PMID: 150201 [PubMed - indexed for MEDLINE]

Acta Med. Okayama 32, (2), 159—167 (1978)

ENDOTOXIN RECEPTOR SITE. I. BINDING OF ENDOTOXIN TO PLATELETS

Setsuo WASHIDA

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

Received February 17, 1978

Abstract. Binding of bacterial endotoxin to platelets, erythrocytes, lymphocytes and granulocytes was examined by using diffusion dialysis. Platelets, erythrocytes, lymphocytes and granulocytes were fractionated from normal human blood and the binding of endotoxin (LPS: Lipopolysaccharide of E. coli) to each cell fraction wss measured at 4°C and the binding efficiency was expressed as a binding index (%d4°C ± SD). The binding index for each cell fraction was as follows; 10.2 ± 1.6 for platelets, 1.0 ± 0.9 for erythrocytes, 4.3 ± 1.6 for lymphocytes and 10.0 ± 1.5 for granulocytes (n = 11) respectively. Since a platelet possesses a small cell surface area compared with other cells, it was clear that the endotoxin bound preferentially to platelets *in vitro*. The binding mechanism to the platelet cell surface was suggested to be direct binding of endotoxin to the receptor on platelet cell membrane rather than through an immunologically activated mechanism.

Key words: endotoxin, platelet, receptor, binding, diffusion dialysis

Platelets, one of the blood components, play an important role in hemostatic and defense mechanisms by the fixation of various pharmacological substances in circulating blood (1-4).

The endotoxin of gram negative bacilli has been frequently detected in the blood of ileus or peritonitis patients (5). The relationship between platelets and the bacterial endotoxin is suspected to be an important factor in the course of endotoxemia (6).

The binding of the endotoxin to platelets was first shown by administration of chromium^{51} labelled endotoxin (7, 8), suggesting that there was specific affinity between platelets and endotoxin. Two different mechanisms of binding have been proposed; one is by activation of the complement system (9-11) and the other by direct binding of endotoxin to membrane receptor. The direct binding was assumed to be by a lipid-lipid interaction between endotoxin and platelet cell membrane whose surface seemed to have an endotoxin sensitive component (endotoxin receptor) (12, 13).

In this study, the author attempted to examine the binding efficiency of endotoxin (Lipopolysaccharide of E. coli) to blood cell components by binding

index (%d) (14) using plexiglas equilibrium dialysis units and confirmed the preferential binding of endotoxin to the platelet cell surface.

MATERIALS AND METHODS

Bacterial endotoxin. The following commercially available endotoxins were used: E. coli 0111: B4(B) lipopolysaccharide (control No. 616326. Difco Laboratories, Detroit, Michigan) and E. coli 0111: B4(W) lipopolysaccharide (No. 1598148, Difco).

Diffusion dialysis system. A pair of plexiglas dialysis chambers (Chemical Rubber Co. Cleveland, Ohio) of 1.1ml capacity separated by a cellulose polyacetate membrane (Sephraphore III membrane, Gelman, Ann Arbor, Mich.) were used. Exactly 1 ml of a cell suspension in physiological saline containing 15mM sodium azide (abbreriated NaN_3 saline) was injected into one chamber and 1 ml of NaN_3 saline into the other. For one experiment, two chamber sets were used as shown in Fig. 1. Set 1: chamber A contained endotoxin (^{125}I-LPS) and cells, whereas chamber B contained only the buffer. Set 2: chamber A contained the buffer and cells, whereas chamber B contained the endotoxin (^{125}I-LPS). Dialysis

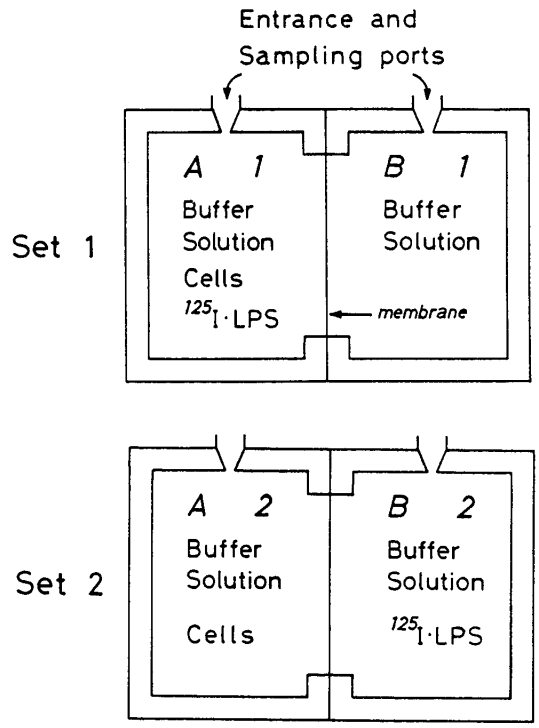

Fig. 1. Schematic illustration of dialysis chambers used for the measurement of binding index of ^{125}I-LPS to blood cells.

chambers were set on a rotating disk and gently rotated (12 rpm) at 4°C. After an adequate reaction time, a 100 μl sample was taken using with a micropipette and the radioactivity was measured in counts/minute using a spectro scaler (Toshiba). The binding index was calculated by the following equation;

$$\left(\frac{A_1-B_1}{A_1+B_1}+\frac{A_2-B_2}{A_2+B_2}\right)\times 100=\%d \quad (14)$$

where, A_1, A_2 were LPS concentrations of the cell-containing chamber of set 1 and 2 respectively and B_1, B_2 were the LPS concentrations of the cell free chamber of set 1 and 2 respectively. The binding index was calculated as the mean value of duplicate experiments.

^{125}I-LPS preparation method. ^{125}I-LPS was prepared using the method of Hunter and Greenwood (15) as described by Kimura (16). The specific activity of the ^{125}I-LPS preparation was 2×10^4 cpm/ng/0.1 ml.

Preparation of whole blood cells. Two ml of fleshly drawn citrated blood from a healthy human donor was mixed with 8 ml of physiological saline solution containing 15 mM sodium azide and 0.1 mM EDTA following by centrifugation at 3000 rpm (1600 g) for 10 min. The precipitate was suspended in physiological saline containing 15 mM sodium azide (abbreriated NaN_3 saline) and centrifuged. The procedure was repeated three more times in order to eliminate serum contamination of the cell fraction. The cells were suspended in NaN_3 saline at the cell numbers of 10^6, 10^7, 10^8 cells/ml on the basis of the number of erythrocytes.

Fraction of blood cell components. Two ml of fleshly drawn citrated blood from a healthy human donor was mixed with 6 ml of NaN_3 saline and layered on top of 3 ml of Lymphoprep (Nyegard & As Oslo) followed by centrifugation at 1550 rpm (400g) for 30 min. Platelets, lymphocytes and erythrocytes were seperated as layers. Each layer was collected and then washed three times. The cell number was adjusted by microscopic observation using a cell counting chamber, and no aggregation of platelets was observed. Granulocytes were isolated from citrated blood containing 6% dextran by using the Lymphoprep density gradient method. The remaining erythrocytes in the granulocyte layer were removed by hemolysis. All the procedures described above were performed aseptically at 4°C. All reagents used for the experiments were superfine grade.

RESULTS

Diffusion dialysis in the absence of cells. The cell free diffusion rate of ^{125}I-LPS was measured in the case of the standard as shown in Fig. 2. The diffusion rate reached equilibrium after 6 h incubation at 4°C. The binding index of cells was, therefore, measured after 6 h incubation hence forth.

Diffusion dialysis of whole blood components. The binding indices of whole blood cells whose number was adjusted on the basis of erythrocytes were 1.3 ± 1.0 for 10^6 cells/ml, 3.6 ± 1.7 for 10^7 cells/ml, and 9.4 ± 1.9 for 10^8 cells/ml ($n=7$, n represents experimental numbers)as shown in Fig. 3. The blood cell number and the binding indices showed an apparent linear relationship, suggesting that

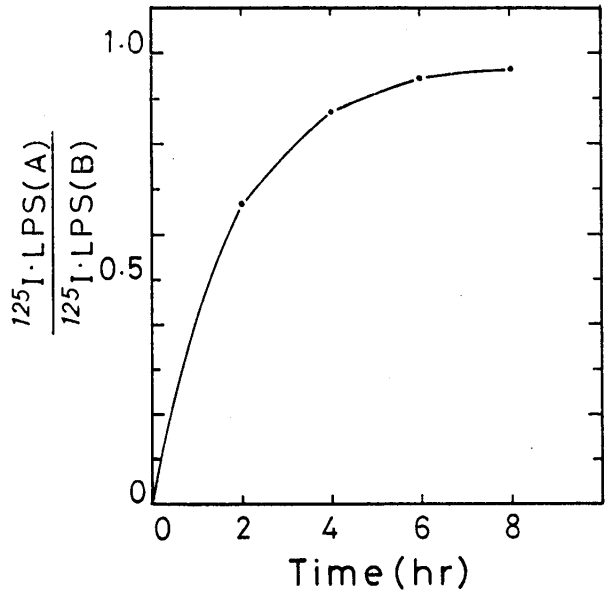

Fig 2. Standard curve of the diffusion dialysis in the cell free system. Ratio of ^{125}I-LPS in chamber B to chamber A. Diffusion dialysis equilibrium was attained about 6 h (0.95±0.055, n=11).

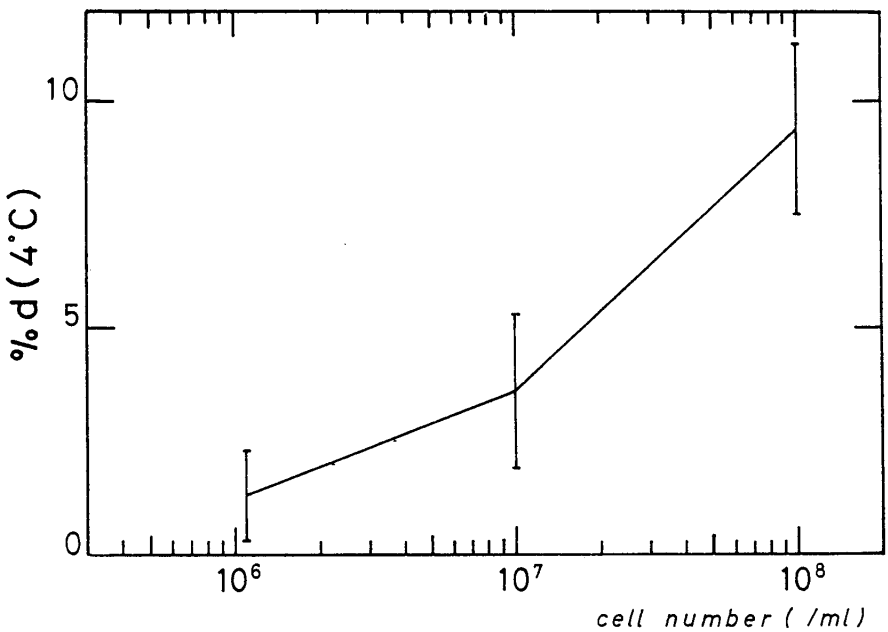

Fig. 3. Binding indices (%d 4°C) of ^{125}I-LPS to whole blood cell components. The number of cells was adjusted on the basis of erythrocytes. The binding indices were 1.3±1.0 for 10^6 cells/ml, 3.6±1.7 for 10^7 cells/ml, and 9.4±1.9 for 10^8 cells/ml (n=7).

either whole blood cells or some components of cells are able to bind endotoxin. Further studies were carried out to ascertain which blood cells had specific binding site to endotoxin.

The binding indices of endotoxin to platelets, erythrocytes, lymphocytes and granulocytes. The binding indices (%d 4°C) of each cell fraction from whole blood were examined. They were 10.2 ± 1.6 for platelets, 1.0 ± 0.9 for erythrocytes, 4.3 ± 1.4 for lymphocytes and 10.0 ± 1.5 for granulocytes ($n = 11$) as shown in Fig. 4. ^{125}I-LPS had high binding affinity for platelets and granulocytes. If the cell membrane surface area is taken into account, platelets seemed to have more affinity for endotoxin than any of the other blood cell components.

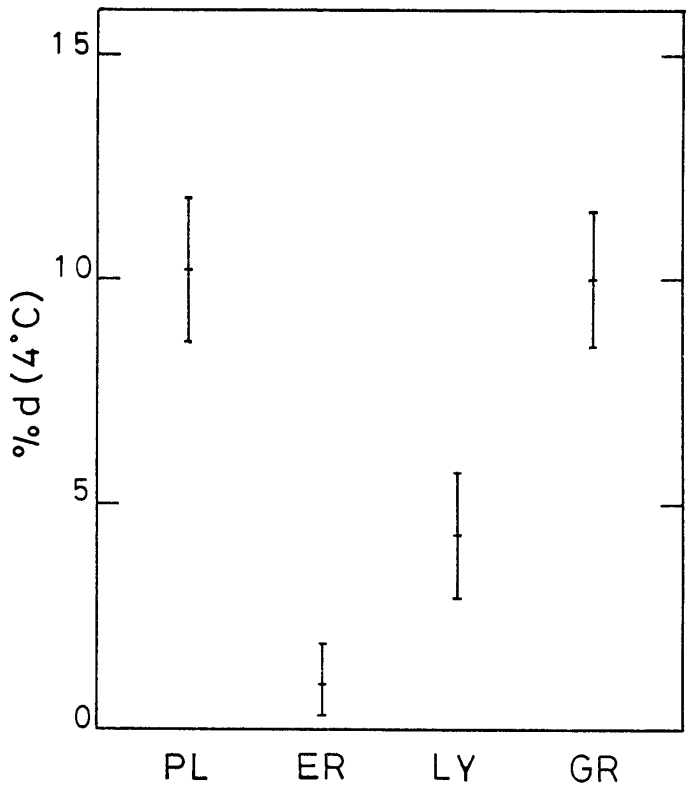

Fig. 4. Binding indices (%d4°C) of ^{125}I-LPS to platelets, erythrocytes, lymphocytes and granulocytes. PL, platelets; ER, erythrocytes; LY, lymphocytes; GR, granulocytes. The binding indices of ^{125}I-LPS to 10^6 cells/ml suspensions were 10.2 ± 1.6 for platelets, 1.0 ± 0.9 for erythrocytes, 4.3 ± 1.4 for lymphocytes and 10.0 ± 1.5 for granulocytes. Platelets and granulocytes showed high affinity for endotoxin.

The binding indices depend upon the amount of platelets, erythrocytes, lymphocytes or granulocytes present. Fig. 5 shows the changes of the binding indices of each of the components. The binding indices of platelets varied from 3.5 for 10^5 cells/ml, and 10.9 for 10^6 cells/ml, to 16.5 for 10^7 cells/ml. Those of erythrocytes, however, were 1.0 for 10^5 cells/ml, 1.6 for 10^6 cells/ml and 1.4 for 10^7 cells/ml, lymphocytes binding indices were 1.7 for 10^5 cells/ml, 4.5 for 10^6 cells/ml and 5.6 for 5×10^6 cells/ml. Those of granulocytes were 4.6 for 10^5 cells/ml and 9.8 for 10^6 cells/ml (n=7). Clearly, as the cell number increased, the binding indices rose in platelets and granulocytes but not in erythrocytes and lymphocytes. Evidently, platelets and granulocytes have specific binding affinity for endotoxin.

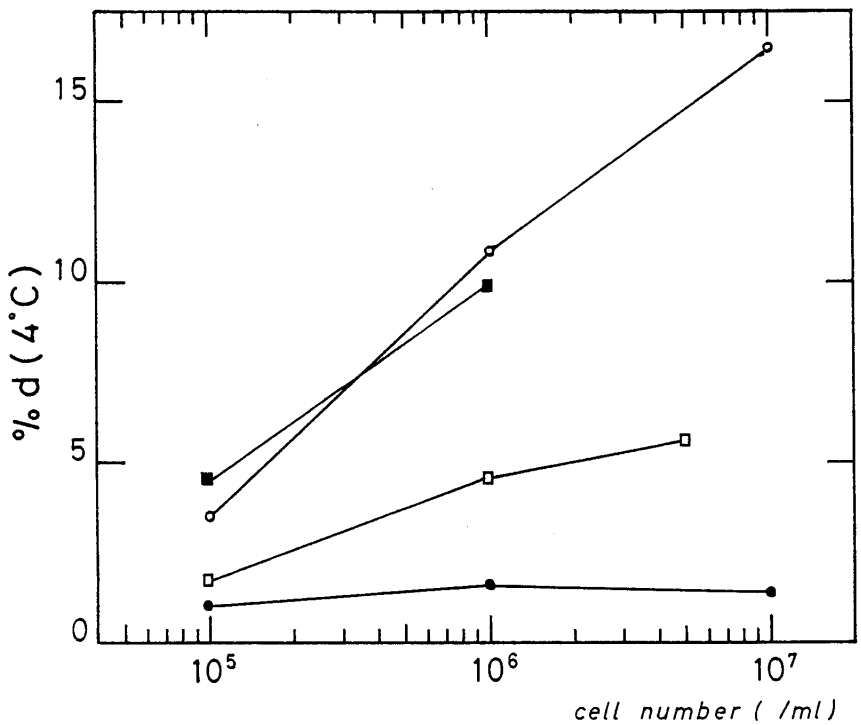

Fig. 5. Binding indices (%d 4°C) versus cell number. The binding indices were measured at 4°C. Platelets; 3.5 for 10^5 cells/ml, 10.9 for 10^6 cells/ml and 16.5 for 10^7 cells/ml. Erythrocytes; 1.0 for 10^5 cells/ml, 1.6 for 10^6 cells/ml and 1.4 for 10^7 cells/ml. Lymphocytes; 1.7 for 10^5 cells/ml, 4.5 for 10^6 cells/ml and 5.6 for 5×10^6 cells/ml. Granulocytes; 4.6 for 10^5 cells/ml and 9.8 for 10^6 cells/ml (n=7). It is clear that the binding indices depended on the number of platelets and granulocytes but not on the number of erythrocytes and lymphocytes. ○——○ Platelets; ●——● Erythrocytes; □——□ Lymphocytes; ■——■ Granulocytes.

Effect of temperature on binding indices. The effect of temperature on the binding of endotoxin to cells was studied. The binding indices were measured at reaction temperatures of 4°C, 17°C and 27°C. The platelets binding indices (%d) were 10.2 at 4°C. 13.8 at 17°C and 8.8 at 27°C. Those of erythrocytes were 1.0 at 4°C, 2.0 at 17°C and 1.8 at 27°C: while those of lymphocytes were 4.3 at 4°C, 4.8 at 17°C, 2.5 at 27°C: that of granulocytes was 10.0 at 4°C as shown in Fig. 6. In the case of granulocytes, it was impossible to measure the binding indices at 17°C and 27°C because of chemotaxis of granulocytes. The binding indices were higher at 17°C than at 4°C, and then slightly lower at 27°C. However, these results are not conclusive evidence of a dependency of binding efficiency of endotoxin to cells on temperature.

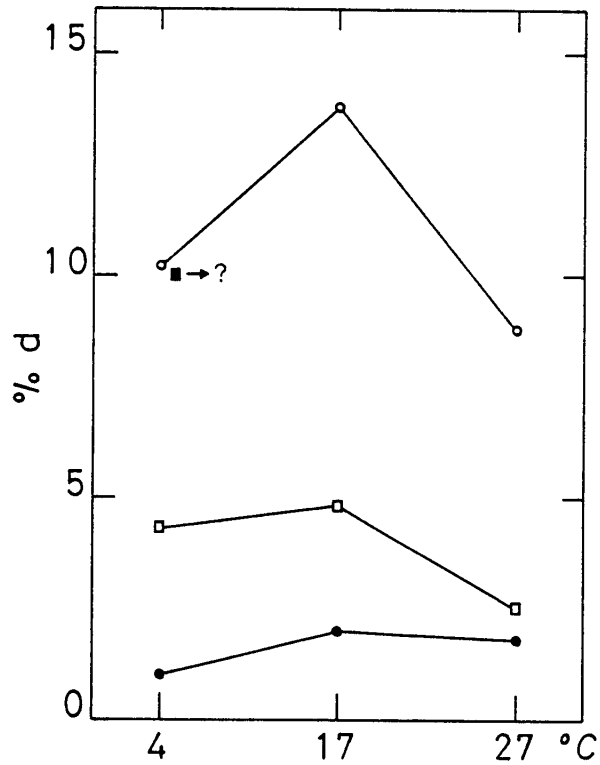

Fig. 6. Effect of temperature on the binding indices (%d). The binding indices were measured at 4°C, 17°C and 27°C with a cell number of 10^6/ml. Platelets; 10.2 at 4°C, 13.8 at 17°C and 8.8 at 27°C. Erythrocytes; 1.0, 2.0 and 1.8, lymphocytes; 4.3, 4.8 and 2.5 respectively. Granulocytes gave a figure of 10.0 at 4°C however at 17°C and 27°C, chemotactic activity of leukocytes caused difficulty in measuring accurately the binding index.
○—○ Platelets; ●—● Erythrocytes; □—□ Lymphocytes;
■—■ Granulocytes.

DISCUSSION

In examining the binding affinity of endotoxin to blood components, the binding indices (%d) of radioactive lipopolysaccharide (^{125}I-LPS) to each cell component were measured by the diffusion dialysis method (14). The binding indices (%4°C) (14) of LPS to platelets, erythrocytes, lymphocytes and granulocytes were 10.2, 1.0, 4.3 and 10.0 respectively. It is clear that lymphocytes and erythrocytes had a little or no binding affinity when compared with platelets or granulocytes. Since, lymphocytes have been reported to have no binding affinity for endotoxin (8), the author's results which showed a low affinity of lymphocytes to endotoxin could be attributed to the presence of platelets in the lymphocyte fraction.

Platelets and granulocytes had high binding affinities for endotoxin. In this experiment, however, this was caused by neither pinocytosis nor phagocytosis (17) because the reaction mixure contained 15 mM sodium azide which was enough to inhibit cell functions. Therefore, the high binding index indicated direct binding of endotoxin to the cell. The involvement of the complement system in binding was also negligible. When the binding index was expressed as a fraction of cell number in the assay system both platelets and granulocytes gave about the same binding indices. In other words, since the binding takes place on the cell membrane, platelets have a higher binding affinity per unit cell membrane area. For instance, if both cells are assumed to be a sphere of $3.0 \pm 2.1\,\mu$ for platelets and $14.0 \pm 2.5\,\mu$ for granulocytes in diameter (18), the ratio of their surface areas would be about 1/22. The binding of platelets per unit surface area is about 22 times higher than that of granulocytes. It is clear that endotoxin has a very high binding affinity for platelet membrane. The effect of temperature on the binding indices was not so remarkable. The low value of the binding index at 27°C may be due to the degeneration of platelets during the 6 h incubation.

The existence of endotoxin receptor on platelets was suggested by Hawiger *et al.* (13) and this report confirmed it. The high binding affinity of platelets for endotoxin may be an important first defense mechanism in endotoxemia. The significance of the platelets binding affinity to endotoxin *in vivo* will be studied in the following paper (4).

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

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