A dextran-based warming method for preparing leukocyte-rich plasma and its clinical application for endotoxin assay

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ABSTRACT

We devised a method using dextran for obtaining leukocyte-rich plasma (LRP) to measure endotoxin in blood. In order to find the optimal temperature for obtaining LRP, the measurement results were examined using samples prepared at 37 and 0°C. Sample separation time of LRP was significantly shorter at 37°C than at 0°C. Endotoxin measurement values showed a strong correlation between the two groups but many of the LRPs made at 37°C had measurements above those of the LRPs prepared at 0°C. The diagnostic accuracy for gram-negative bacterial infection was superior for LRP prepared at 37°C, with sensitivity and specificity of 96.8 and 100%, respectively.

METHOD SUMMARY

Plasma is usually used for measurement of blood endotoxin. Here, leukocyte-rich plasma (LRP) was obtained using an erythrocyte aggregation agent, dextran, and used for the assay. It was found that when LRP was separated at 37°C (referred to here as the LRP37 method), endotoxin values were higher than those obtained at 0°C (LRP0 method). The sensitivity and specificity of the LRP37 method were superior to those achieved using the LRP0 method.

KEYWORDS

dextran • endotoxin • endotoxin-specific limulus amoebocyte lysate assay • leukocyte-rich plasma • platelet-rich plasma • polymyxin B • sepsis • turbidimetric kinetic assay

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Endotoxins are cell wall components of gram-negative bacteria with various biological activities. In patients with bacterial infections, sepsis is typically diagnosed by measuring blood endotoxin concentrations after exposing samples to blood cell extracts from the horseshoe crab. In Japan, the standard practice for measuring blood endotoxin concentrations is an endotoxin-specific limulus amoebocyte lysate (LAL) assay using platelet-rich plasma (PRP). However, the sensitivity of this assay is too low to be considered diagnostically efficient [1].

Endotoxins bind to leukocytes through TLR4 [2] or CD18/CD11b [3], after which the endotoxins are engulfed into the phagocytic cells [4]. It follows that endotoxin levels should be measured on the surface as well as inside the cells during LAL assays. We first attempted to use the leukocyte fraction (buffy coat) from centrifuged patient blood for the assay [5] and found that the leukocyte fractions had higher endotoxin levels than the plasma fractions in some patients. However, this method is too complicated and impractical for routine use. Therefore, we attempted to use leukocyte-rich plasma (LRP) for the endotoxin assay [1,6] and found once again that the measurement of leukocyte-bound endotoxins was necessary. Separation of LRP was previously performed by adding the hemagglutinating agent hydroxyethyl starch (HES) to the blood [7,8]. However, it has become difficult to obtain HES in recent years; thus, we attempted to determine whether separation with dextran is possible. It has been reported that ice-cold conditions are important for restoring sufficient endotoxin activity (compared with incubation at 37°C and based on lipopolysaccharide (LPS) spike experiments on plasma from sepsis patients) [9]. Therefore, in this study, we also investigated whether the measurement accuracy of endotoxins is sufficiently high under ice-cold conditions.

MATERIALS & METHODS

Reagents

- Dextran T-500 (Pharmacosmo, Holbaek, Denmark), with an average molecular weight of 500,000 Da, was dissolved in physiological saline (6% w/v) and autoclaved at 121°C for 90 min to ensure the endotoxin level was below the detection limit (0.04 pg/ml) of the limulus test (endotoxin-free);
- Phosphate-buffered saline (-): The compound powder (Nissui Pharmaceutical, Tokyo) was dissolved in water to a concentration of 0.01 M and autoclaved at 121°C for 90 min to ensure it was endotoxin-free. The solution does not contain calcium or magnesium;
- LPS: LPS from *Escherichia coli* O111:B4 (Sigma-Aldrich, MO, USA) was suspended in phosphate-buffered saline (-) at a concentration of 1 mg/ml. The LPS suspension was dispersed using an ultrasonic generator (UR 21-P, Tommy Seiko, Tokyo), dispensed in small volumes, and stored at -80°C until use;
- Plasma pretreatment solution and LAL reagent: a plasma pretreatment solution (0.02% Triton X-100 Solution, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and Endotoxin Single Test Wako® (Lyophilized product, FUJIFILM Wako Pure Chemical Corporation) were used. Endotoxin Single Test Wako is a reagent specific for endotoxins [10,11];
- Polymyxin B was purchased from Sigma-Aldrich.

Patients

The total number of patients was 67; the patient backgrounds are presented in Table 1. Of these, 47 patients were suspected of sepsis or infection, and no infection was found in the other 20 patients. Among those suspected of sepsis or infection, bacteria were detected in cultures of blood or urine, sputum, ascitic fluid, spinal fluid or wound

for 45 patients. Gram-negative bacteria were detected in 33 cases, and gram-positive bacteria were detected in 12 cases. Informed consent was obtained from all participants.

Preparation of PRP

As soon as the blood sample was drawn with heparin, the tube was placed in crushed ice to equilibrate it at 0°C (within 5–10 min). To obtain PRP, the blood samples were centrifuged at 1200 *g* (3000 r.p.m.) for 40 s [12]. The endotoxin assay using PRP is herein after referred to as the plasma method.

Preparation of LRP

LRP was prepared under two conditions: at 37°C and on ice. A dextran solution (6%) was equilibrated to the experimental temperature in advance. Immediately after blood collection, the heparinized sample was transported under ambient temperature to our endotoxin research laboratory and equilibrated for 5–10 min at the experimental temperature. Thereafter, the LRP was collected according to the following protocol. Briefly, 800 μ l of the equilibrated blood was added to 400 μ l of 6% dextran T-500, which was also equilibrated to the same temperature, and the mixture was incubated. The supernatant layer separated from the sedimented erythrocytes after 30 min was designated as the LRP. The methods used to prepare the LRP under ice-cooling and at 37°C are referred to as the LRP0 and LRP37 methods, respectively.

Endotoxin measurement by turbidimetric kinetic assay

PRP or LRP (100 μ l) obtained using the LRP37 or LRP0 method was added to 900 μ l of plasma pretreatment solution (i.e., a 10 $\times$ dilution) and mixed in a vortex mixer (MS-3, IKA Japan, Osaka, Japan). The diluted LRP was mixed five-times in a vortex mixer at 3000 r.p.m. for 10 s to ensure the destruction of white blood cells. The mixture was heated at 70°C for 10 min and then cooled in iced water for 5 min. The Endotoxin Single Test Wako reagent was dissolved with 200 μ l of the pretreated specimen. The Toxinometer MT-5500® (Wako Pure Chemical Corporation) was used to obtain measurements. The time taken for the amount of transmitted light to be reduced by 8% was defined as the gelation time. The endotoxin concentration was calculated using a calibration curve according to the gelation time for standard LPS (*E. coli* 0111: B4, Wako Pure Chemical Corporation) [10,11]. The detection limit for this assay was approximately 0.05 pg/ml; this value was calculated at a maximum set time of 200 min (this value varied depending on the lot number for the LAL). Since the plasma was diluted to tenfold, the detection limit for endotoxins was approximately 0.5 pg/ml. Average values were obtained by duplicate measurements of each specimen.

Correction of endotoxin content of LRP considering hematocrit value

Since half the volume of dextran was added to one volume of blood to obtain the LRP, the LRP endotoxin content was corrected using the following formula. The LRP endotoxin value (pg/ml) was obtained by multiplying the actual value obtained with the LRP method by the coefficient $([A + \text{dextran amount}]/A)$, wherein $A = \text{blood volume} - \text{blood volume} \times \text{hematocrit value}/100$. For example, if the blood volume and hematocrit value are 800 ml and 30, respectively, A is calculated as 560 $(800 - 800 \times 0.3)$. If the dextran volume is 400 ml, a multiplying factor of 1.71 is obtained by $(560 + 400)/560$. Since the endotoxin levels of LRP and PRP were diluted tenfold in the treatment stage, they were finally multiplied by 10.

Statistical analysis

Student's *t*-test, Mann–Whitney *U* test, Wilcoxon test, creation of receiver operating characteristic (ROC) curves and calculation of specificity and sensitivity were performed using SPSS software. Significant differences were regarded as those with *p*-values of less than 0.05.

RESULTS & DISCUSSION

Incubation conditions for LRP preparation

First, we performed an experiment to obtain LRP under ice-cooled conditions. For this experiment, room temperature was set as the control condition because a previous study reported that endotoxin inactivation in patient plasma occurred at 37°C [9]. However, endotoxin levels in the LRP prepared at room temperature tended to be higher than those in the LRP prepared under ice-cooled conditions, contrary to our expectations (data not presented). Therefore, we set the preparation temperature for LRP as 37°C (LRP37 method) and compared the endotoxin levels with the values obtained under ice-cooled conditions (LRP0 method). The times to obtain the LRP were 9 min and 12 s with the LRP37 method and 12 min and 34 s with the LRP0 method (no infection group, $n = 20$, $p < 0.001$, *t*-test). The results for the 67 samples measured indicated that both values were significantly correlated ($R^2 = 0.97$). As shown in Figure 1, the values obtained using the LRP37 method were higher than those obtained using the LRP0 ►

Table 1. Patient characteristics.

Diagnosis	Noninfection (n)	Infection or sepsis (n)
Respiratory disease	0	16
Peritonitis	0	12
Streptococcal toxic shock syndrome	0	6
Bacterial meningitis	0	4
Burns	3	7
Liver abscess	0	1
Tetanus	0	1
Trauma	9	1
Acute pancreatitis	3	0
Malignant hyperthermia	2	0
Healthy	3	0
Total (n = 67)	20	47

method in more than 80% of the cases and especially in cases where gram-negative bacteria were detected, and the average value also tended to be higher (LRP0 method: 31.42 ± 14.12 pg/ml, LRP37 method: 41.46 ± 14.12 pg/ml).

Effect of dextran

The limulus activity of endotoxin in LPS-spiked physiological saline and in endotoxemic patient plasma was observed after incubation in the presence of dextran for 15 min at 0°C (ice-cooled) or 37°C (Figure 2). The limulus activity of LPS appeared to be enhanced depending on the concentration of dextran, especially at 37°C, in both LPS-spiked physiological saline and endotoxemic patient plasma. This enhancement was completely inhibited in LPS-spiked saline by polymyxin B, confirming that the reactions were not false positives. In addition, when the dextran concentration was set to 6% or higher, the viscosity of the specimen increased, and accurate measurement could not be performed. Therefore, in this study, the dextran concentration was set to 6%.

Examination using patient samples

Three methods were compared: the LRP0, LRP37 and plasma methods. The measured values were significantly higher for gram-negative bacteria with all methods. The measured values tended to be slightly higher with the LRP method than with the plasma method. In addition, for cases where only gram-positive bacteria were detected with the LRP method, the values were slightly higher than those obtained using the other two methods (Figure 3). ROC curves were created for the groups infected by gram-negative bacteria and other diseases (including gram-positive bacterial infection), and the sensitivity and specificity were calculated (Table 2). Groups in which gram-negative bacteria were detected in the blood culture were defined as true positive groups, whereas groups in which gram-positive bacteria or no infection were detected in the blood culture were identified as true negative groups. ROC curves were subsequently created between the two groups to calculate the sensitivity and specificity (Table 3). The LRP37 method showed the best diagnostic accuracy of the three measurement methods.

The sensitivity and specificity of techniques for diagnosing sepsis using conventional plasma endotoxin assays are too low, and plasma endotoxins are not detectable in many cases of gram-negative bacterial infection [1]. Kan *et al.* demonstrated that LRP is useful for the diagnosis of endotoxemia and found a clear improvement in the sensitivity and specificity of this assay compared with the plasma method [1,6]. Their experiments were performed with a commercially available HES solution, which is used as a cryopreservation agent for cultured cells. They incubated a mixture of the HES solution and blood at room temperature to obtain the supernatant (LRP). However, properties such as the molecular weight of the HES solution are not disclosed by the manufacturer. The production of HES was also discontinued recently. In addition, HES is less soluble in water, and preparing a saline solution can be difficult. Therefore, we used dextran instead of HES in this study.

The results indicated that dextran enhanced endotoxin activity, and that the enhancement was greater at 37°C. Based on the study results, the most important question is how dextran increases the endotoxin activity. Endotoxins have a micellar structure and constitute gigantic associates of 100,000–1,000,000 Da [13]. We think that these gigantic associates moderately decrease in size in the presence of dextran. Thus, dextran may enter the micellar structure and break the endotoxin into a large number of smaller micellar struc-

Figure 1. Measurement values of both methods in gram-negative bacteria detection examples. The measurement value of the LRP37 method was high in 27 of 33 cases. LRP: Leukocyte-rich plasma.

tures. When the micelles increase in number, and convection occurs in an environment of 37°C, it becomes easier for them to bind to the target molecule, that is, factor C in the LAL reagent. Furthermore, it is possible that the causative bacteria proliferated in the sample during culture at 37°C and the endotoxin levels may have increased. However, as illustrated in Figure 1, since the increase in the measured value was apparent even in the experiment using LPS, the reason for the increase in the measured value is unclear.

Yaegashi *et al.* [5] measured endotoxin levels in the white blood cell fraction (buffy coat) and reported the detection of endotoxins in this fraction, but not in the plasma, for some specimens. Kan *et al.* [1] reported that even if the endotoxin levels were below the detection limit for the plasma method, LRP endotoxin levels could be measured ►

Figure 2. Effect of dextran concentration on the limulus activity of lipopolysaccharide in physiological saline and endotoxemic patient plasma. LPS in physiological saline or endotoxemic patient plasma was incubated at 0 or 37°C in the presence of graded doses of dextran for 15 min, followed by endotoxin measurement. PXB (100 µg/ml) was added to the LPS solution and the inhibitory effect was observed.

* $p < 0.05$ (Student's *t*-test against 0 control).

$p < 0.05$ (Student's *t*-test against the same dextran concentration).

LPS: Lipopolysaccharide; PXB: Polymyxin B.

Figure 3. Patient endotoxin values with leukocyte-rich plasma and plasma methods. Box plots show the data for specimens obtained from patients with sepsis due to G(-) and G(+) bacteria, and for noninfectious patients. The figures for the G(-) and G(+) groups represent the median values. G(-): Gram negative; G(+): Gram positive; LRP: Leukocyte-rich plasma.

in some specimens from patients with sepsis. Similar unexplainable results were also obtained in this study in approximately 8% of cases. Endotoxins positively bind to leukocytes through the endotoxin receptor to activate them, but not all endotoxins that enter into the circulation will immediately bind to leukocytes without first floating freely in the circulation. It may be that leukocytes capture endotoxins at the site of infection and subsequently enter into the circulation. Recently, a study demonstrated that although neutrophils are short-lived effector cells that undergo apoptosis in damaged tissues, in some settings they may leave the site of tissue injury and migrate back into the circulation [14]. Thus, the site at which endotoxins interact with leukocytes may also be the site

of inflammation rather than in the circulation. Neutrophils are thought to play a major role in this context, but lymphocytes or platelets, in addition to monocytes, may also be carriers of endotoxins.

Even in cases of gram-positive bacterial infection, we could detect small concentrations of endotoxins around the cut-off value. In such cases, it is possible that gram-negative bacterial infections were hidden. However, if the patient has a serious condition, the strong bonds between the cells of intestinal villi may collapse, or villus cell destruction may occur, resulting in intestinal bacteria invading the site and leukocyte accumulation from blood vessels at the same site. Thereafter, leukocyte binding of

endotoxins and reverse migration into the circulation may occur. As the presence of cell-surface markers for re-entered leukocytes has been reported [14], leukocyte-associated endotoxins may also become a valuable marker. A previous study demonstrated the existence of endotoxin-bound blood leukocytes in patients with Kawasaki disease [15]. There is a need to investigate methods for measuring leukocyte-bound endotoxins in the blood for various diseases with increased intestinal permeability, and the possibility that leukocyte-bound endotoxins are involved in their pathophysiology. Measuring leukocyte-bound endotoxins will not only improve diagnostic efficiency for sepsis, but it may also be a means of clarifying the role of endotoxins in diseases other than sepsis, which have only been estimated so far.

In conclusion, the important findings in this study are as follows:

- The LRP method using dextran T-500 does not require centrifugation, and samples can be produced at any facility;
- The LRP method using dextran T-500 has better measurement accuracy than the plasma method;
- The LRP37 method was superior to the LRP0 method in terms of isolation time and diagnostic accuracy.

The measurement sensitivity of the LRP37 method is expected to improve in the

Table 2. Measurement accuracy by receiver operating characteristic analysis.			
	LRP37 method	LRP0 method	plasma0 method
Area under the curve	0.92	0.88	0.85
Cut-off value	2.69	1.71	1.1
Sensitivity	96.8	89.6	96.8
Specificity	82.1	76.0	67.7
LRP: Leukocyte-rich plasma.			

Table 3. Measurement accuracy by receiver operating characteristic analysis.			
	LRP37 method	LRP0 method	plasma0 method
Area under the curve	0.99	0.97	0.76
Cut-off value	0.97	1.07	0.66
Sensitivity	96.8	90.9	100
Specificity	100	95.0	53.4
LRP: Leukocyte-rich plasma.			

future, and a simple method for endotoxin measurement, such as a point-of-care test, may be developed.

FUTURE PERSPECTIVE

At present, endotoxin has not been measured clinically because of the low accuracy of the assay. However, the accuracy was dramatically improved by the LRP37 method. Endotoxin is a very important causative agent of sepsis and is also important in the selection of antibiotics. The LRP37 method does not require special instruments and is expected to be widely used clinically. We expect that the standard for endotoxin measurement will change to this method in 5 years.

AUTHOR CONTRIBUTIONS

G Takahashi designed the study and wrote the initial draft of the manuscript. K Inada, K Sato and Y Inoue contributed to the analysis and interpretation of data and assisted in the preparation of the manuscript. All other authors have contributed to data collection and interpretation, and critically reviewed the manuscript. All authors approved the final version of the manuscript, and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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FINANCIAL & COMPETING INTERESTS DISCLOSURE

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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ETHICAL CONDUCT OF RESEARCH

Ethical approval for the study (Ethical Committee no. H29-174) was provided by the Ethical Committee of Iwate Medical University, Morioka, Japan (Chairperson: Y Sato) on 15 March 2018. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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