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Adsorption of endotoxin on glass in the presence of rhIL-11

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Abstract

Poor recovery of spiked endotoxin in the *Limulus* amebocyte lysate assay (LAL assay) was observed in the presence of recombinant human interleukin-11 (rhIL-11), a cationic, hydrophobic protein. Detection of endotoxin activity remaining in the empty glass tubes in which endotoxin and rhIL-11 mixtures were incubated indicated adsorption of endotoxin on glass. At low concentrations of rhIL-11, a correlation between endotoxin adsorbed on glass and a decrease of endotoxin in solution was observed. Adsorption of rhIL-11 on glass correlated with adsorption of endotoxin, which indicates that rhIL-11 mediates adsorption of endotoxin on glass. Consequently, adsorption of endotoxin on glass may occur in the presence of other substances which bind to both of endotoxin and glass. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Endotoxin; LAL; Protein; Endotoxin-protein complexes; Adsorption on glass

1. Introduction

Endotoxins (lipopolysaccharides) are major components of the exterior membrane of gram-negative bacteria. They are responsible for inducing toxic-shock syndrome, nonspecific activation of the immune system, and activation of the complement cascade [1]. The *Limulus* amebocyte lysate (LAL) assay is widely used for detecting endotoxin contamination in many pharmaceutical, industrial and research samples [2]. While many substances are known to interfere with the LAL assay, most of these problems can be resolved by dilution since the interference usually depends on substance concentration [3]. However, inhibition caused by cationic proteins such as lysozyme, ribonuclease A, human immunoglobulin G and basic fibroblast growth factor cannot be resolved by dilution, dilution-heating or other treatments, which may be due to the inability of these treatments to dissociate protein-endotoxin complexes [4]. Other cationic proteins such as protamine [5], bacterial permeability-increasing protein [6] and macrophage cationic protein [7], are known to interact with endotoxin as well. Endotoxin also interacts with hydrophobic regions in addition to basic regions of peptides or proteins [8,9], suggesting that endotoxin may also interact with hydrophobic proteins in addition to cationic ones.

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Recombinant human interleukin-11 (rhIL-11) [10], developed as a drug for the prevention of thrombocytopenia, is a highly cationic (p$I > 11$) and hydrophobic protein because of its high content of basic residues, especially arginine (10%), and hydrophobic residues, especially leucine (23%). It is critical to monitor endotoxin contamination for quality control of pharmaceutical rhIL-11. As is the case with other cationic or hydrophobic proteins, poor recovery of spiked endotoxin was observed in the LAL assay of rhIL-11. In this paper, the LAL assay interference induced by rhIL-11 was examined and the cause of the interference was investigated.

2. Materials and methods

2.1. Materials and reagents

Pyrogen-free recombinant human IL-11 was obtained from the Genetics Institute (Cambridge, MA, USA). Lipopolysaccharide standard (*Escherichia coli* UKT-B), lyophilized *Limulus* amebocyte lysate and pyrogen-free glass tubes (12×75 mm) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Pyrogen-free water was from Otsuka Pharmaceuticals (Tokyo, Japan). All other chemicals were of analytical grade.

2.2. Endotoxin assay

Endotoxin concentrations were determined using the kinetic turbidmetric method following the instructions of the manufacturer (Wako). In a pyrogen-free glass tube, 100 μl of LAL reagent was mixed with an equal volume of a sample solution, and then incubated at 37°C for 60 min. Turbidity was measured with the Toxinometer ET-301 Analysis Module (Wako).

2.3. Time-course of endotoxin recovery in the presence of rhIL-11

Two hundred microliters of endotoxin (0.2 EU ml^{-1}), 180 μl of water and 20 μl of rhIL-11 (100 μg ml^{-1}) were added to a glass tube. The mixture was vortexed for 30 s, and then incubated at 4°C for 2, 4 and 6 h. At each timepoint, the solution was vortexed for 30 s again, and 100 μl of the mixture was transferred to another glass tube for the LAL assay. Subsequently, 100 μl of LAL reagent was added to the tube and endotoxin concentrations were determined. As a control, 400 μl of endotoxin (0.1 EU ml^{-1}) was added to a glass tube and was treated equivalently.

2.4. Endotoxin recovery in the presence of various concentrations of rhIL-11

For the LAL assays performed in the same glass tube as the incubation, 50 μl of endotoxin (0.2 EU ml^{-1}), an appropriate volume of rhIL-11 (100 μg ml^{-1} or more) and water were mixed into a glass tube to yield desired protein concentrations. The total volume of the mixture was 100 μl. The mixture was vortexed for 30 s, and incubated at 4°C for 3 h. After incubation, 100 μl of LAL reagent was directly added to the glass tube and endotoxin concentrations were determined.

For the LAL assays performed in separate glass tube from the incubation, the mixture with endotoxin and rhIL-11 was prepared as describe above, and after incubation the mixture was transferred from the glass tube used for incubation to a fresh glass tube where the LAL assay was performed.

2.5. Endotoxin adsorption on glass

Fifty microliters of endotoxin (0.2 EU ml^{-1}), 45 μl of water and 5 μl of rhIL-11 (100 μg ml^{-1}) were added to a glass tube. The mixture was carefully vortexed for 30 s to minimize the attached mixture on the glass wall above the upper liquid level of the mixture. The mixture was incubated at 4°C for 2, 4 and 6 h. At each timepoint the entire mixture in the glass tube was transferred to another one. Subsequently, 100 μl of pyrogen-free water was added to the original tube. One hundred microliters of LAL reagent was added to both glass tubes and endotoxin concentrations were determined.

2.6. Protein adsorption on glass

Two hundred microliters of endotoxin (0.2 EU ml^{-1}), an appropriate volume of rhIL-11 and water were added to a glass tube to yield desired protein concentrations. The total volume of the mixture was 400 μl. The mixture was vortexed for 30 s, and incubated at 4°C for 3 h. After the incubation, the entire mixture in the glass tube was transferred to a polypropylene tube for HPLC determination (Waters) of protein concentration.

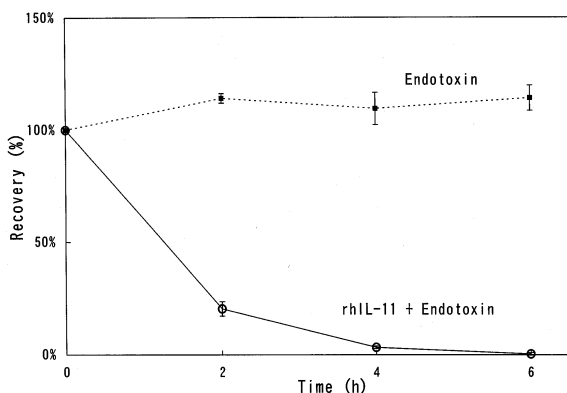

Fig. 1. Time-course of endotoxin recovery in the presence or absence of rhIL-11. Results are expressed as the mean ± SD from three assays. The final concentrations of endotoxin and rhIL-11 were 0.1 EU ml^{-1} and 5 μg ml^{-1}, respectively.

Table 1
Relationship between endotoxin recovery and rhIL-11 concentration when conducting incubation and the LAL assay in the same glass tube and in a separate one[a]

rhIL-11 Protein concentration (μg ml^{-1})	Endotoxin recovery (%)	
	The same glass tube	Separate glass tube
5	115.8 ± 2.9	19.2 ± 1.6
10	87.9 ± 19.4	19.3 ± 1.3
25	70.2 ± 21.5	70.9 ± 1.4
50	87.0 ± 5.8	77.9 ± 5.0
75	73.1 ± 1.5	78.6 ± 2.8
100	71.1 ± 3.4	73.1 ± 0.0
125	71.6 ± 1.0	68.2 ± 2.2
500	65.9 ± 5.8	42.7 ± 2.5
1000	44.3 ± 5.4	20.0 ± 0.3

[a] Results are expressed as the mean ± S.D. from three assays. The recoveries of endotoxin were expressed as percent of the obtained concentrations divided by the added endotoxin concentrations.

2.7. Protein concentration

Protein concentrations were determined by reversed-phase HPLC using a Waters LC system consisting of a Model 626 non-metallic pump, a Model 700 autosampler and a Model 490 UV detector. The mobile phases used include solvent A (0.1% TFA) and solvent B (0.1% TFA in 90% acetonitrile). A Vydac C4 column (4.6 × 50 mm; Hesperia, CA) was initially equilibrated at 30% B at a flow rate of 1 ml min^{-1}. The separation was performed by a linear gradient of 30% B to 100% B for 5 min at a flow rate of 1 ml min^{-1} at 37°C. Detection was by UV absorbance at 214 nm.

3. Results and discussion

3.1. Decreased endotoxin recovery due to rhIL-11

Endotoxin recovery data after mixture of rhIL-11 with endotoxin in a glass tube and incubation at 4°C are shown in Fig. 1. As incubation time in a glass tube increased, endotoxin recovery decreased substantially in the presence of rhIL-11, whereas it was constant in the absence of the cytokine (Fig. 1). These results show that rhIL-11 mediates reduced endotoxin recovery in the LAL assay. This reaction proceeds over time and by 2 h, only about 20% of the endotoxin present was recovered as measured by the LAL assay.

3.2. Endotoxin recovery in the presence of various concentrations of rhIL-11

The relationship between endotoxin recovery and rhIL-11 concentration was examined. First, rhIL-11 was mixed with endotoxin in a glass tube, and incubated at 4°C for 3 h and the LAL assays were performed in the same glass tube without transfer. The results are shown in Table 1. Poor recovery of endotoxin in the LAL assay was

observed in the presence of rhIL-11. A largely constant fraction (25–30%) of the endotoxin was withdrawn from assay readouts at rhIL-11 concentrations ranging from 25 to 500 μg ml^{-1}. Presumably, saturation of binding sites for the protein molecules at the glass surface is attained at about 25 μg ml^{-1}. High protein concentrations are reported to interfere with the *Limulus* coagulation cascade [4], which is a likely rational for the markedly low value observed at 1000 μg ml^{-1} of the cytokine. In a second series of experiments, rhIL-11 was mixed with endotoxin and incubated at 4°C in a glass tube and transferred to another fresh tube, in which the LAL assay was performed (Table 1). Similar to results from the above experimental setting, about 70–80% of the endotoxin added was reported by assay over a wide concentration range of rhIL-11, and a further decline at the highest protein loads. In this case, however, a very notable interference with the LAL assay occurred also at low concentrations of rhIL-11 (Table 1). This is quite different from results of the first experimental series. Endotoxin may not be transferred to the fresh tube for the LAL assay from the glass tube used for incubation in which most of rhIL-11 should also remain at low concentrations due to adsorption on glass. This cannot be explained by the interference with the *Limulus* coagulation cascade by rhIL-11, but can be explained by the hypothesis that endotoxin adsorption on glass is mediated by rhIL-11.

3.3. Adsorption of endotoxin on glass in the presence of rhIL-11

To examine adsorption of endotoxin on glass, amounts of endotoxin adsorbed in a glass tube were measured directly using the LAL assay. After mixture of endotoxin with rhIL-11 in a glass tube and incubation at 4°C during 2, 4 or 6 h, the solution was transferred from the tube to a new equivalent. Water was then added to the tube used for incubation, and the amounts of endotoxin in the two glass tubes were measured using the LAL assay. As shown in Fig. 2, a gradually increasing amount of endotoxin, over a period of 6 h, was detected in the tube that was employed for incubation only. In an identical experimental setting, except that rhIL-11 was omitted, endotoxin activity was not recorded in the equivalent tube (not shown). Moreover, increasing values of endotoxin concentration in the added water (incubation tube) correlated well with decreasing estimates in the transferred mixture (Fig. 2). These findings show that endotoxin is adsorbed on glass in the presence of rhIL-11.

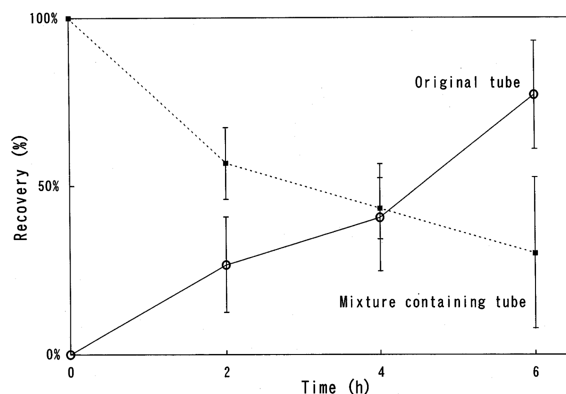

Fig. 2. Endotoxin recovery detected from a glass tube and from a mixture. Vortexing was performed when endotoxin and rhIL-11 were mixed. Results are expressed as the mean ± SD from three assays. The final concentrations of endotoxin and rhIL-11 were 0.1 EU ml^{-1} and 5 μg ml^{-1}, respectively.

3.4. Adsorption of rhIL-11 on glass

To confirm adsorption of rhIL-11 on glass, solutions containing various concentrations of rhIL-11 were transferred to glass tubes and incubated at 4°C. The concentrations of rhIL-11 in solution after the incubation step were measured by HPLC as described in Section 2. The results are shown in Table 2. At very low concentrations of rhIL-11, marked poor recoveries were recorded at the end of the incubation period. The extensive depletion observed at low cytokine loads and the steep increase of recovery seen at the higher concentrations are conceivably due to saturation of binding sites the glass surface. These figures correlate with the LAL assay readouts of endotoxin, which display a recovery plateau (at about 70% of the amount added) in the presence of 25 to 125–

Table 2
Adsorption of rhIL-11 on glass[a]

RhIL-11 Protein concentration ($\mu g\ ml^{-1}$)	rhIL-11 Recovery (%)
5	1.3 ± 2.3
10	7.7 ± 2.9
25	51.0 ± 3.9
50	74.3 ± 0.9
75	85.8 ± 1.1
100	92.6 ± 1.0
125	96.2 ± 0.7
500	99.4 ± 0.5
1000	99.8 ± 0.2

[a] Results are expressed as the mean ± S.D. from three assays. The recoveries of rhIL-11 were expressed as percent of the obtained values divided by the added rhIL-11 concentrations.

500 $\mu g\ ml^{-1}$ of rhIL-11 (Table 1). Accordingly, adsorption of endotoxin on glass occurred in parallel with that of rhIL-11, suggesting that the cytokine is a mediator in this process.

When poor recovery of endotoxin is observed in the LAL assay, dilution of a sample is recommended [3] since most inhibition problems in the LAL assay usually depend on the concentration of the sample. However, in the case of rhIL-11, excess dilution enhances adsorption of endotoxin on glass, which decreases apparent endotoxin recovery as a result. Adsorption of protein can usually be avoided by using plastic tubes. It has been reported, however, that such tubes can either inhibit or enhance readouts from the LAL assay [11], and plastic tubes which are suitable for endotoxin determination of rhIL-11 preparations, using the LAL system, have not been identified. Protease treatment before onset of the LAL assay procedure [6] or determination of endotoxin using a different assay system [12], are candidate remedies against endotoxin adsorption on glass in the presence of rhIL-11.

Indeed, Petsch et al. reported that the interference with the LAL assay caused by lysozyme, another highly cationic and hydrophobic protein, was observed when the lysozyme sample was strongly diluted before the LAL assay [4]. Therefore, adsorption of endotoxin on glass may occur in the presence of other cationic or hydrophobic proteins, or other substances which can bind to both of endotoxin and glass.

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