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Adsorption of endotoxins on glass in the presence of cationic proteins

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Abstract

Endotoxin activity was detected in empty glass tubes where endotoxins were incubated with lysozyme, histone or RNaseA, indicating adsorption of endotoxins on glass in the presence of cationic proteins. In the case of lysozyme, the recovery of spiked endotoxins (90.0%) using polystyrene tubes for incubation was much greater than the recovery (28.5%) using glass tubes, suggesting that lysozyme-mediated adsorption of endotoxins on glass is a major cause of poor recovery of spiked endotoxins in the LAL assay using glass tubes. In contrast, the recovery of spiked endotoxins (64.7%) using polystyrene tubes in the presence of the non-cationic protein BSA was less than the recovery (103.9%) using glass tubes. The difference in endotoxin recovery using glass or polystyrene tubes in the presence of cationic proteins or BSA can be explained by differences in protein adsorption on the tubes. Consequently, care must be exercised in selecting containers used for the LAL assay of proteins which bind to endotoxins.

Keywords: Endotoxins; LAL assay; Cationic protein; Adsorption; Glass; Polystyrene

1. Introduction

Endotoxins (lipopolysaccharides) are present in the exterior membranes of gram-negative bacteria. They produce a variety of biological effects, including pathological phenomena and endotoxin-shock syndrome [1]. The *Limulus* amebocyte lysate (LAL) assay, which is a highly sensitive and simple method for detecting endotoxins, is frequently used in many pharmaceutical, industrial and research samples [2–6].

Endotoxins bind to proteins such as lysozyme [7], histone [8], and serum albumin [9]. Owing to the negatively charged phosphate groups on lipid A, endotoxins interact more strongly with cationic proteins [10–16], often causing poor recovery of spiked endotoxins in the LAL assay [16]. Petsch et al. reported that proteinase K digestion of the cationic proteins before the LAL assay improves endotoxin recovery [15]. Endotoxins are also adsorbed on containers such as polypropylene tubes, which also causes poor recovery [3,4]. To avoid adsorption of endotoxins to tube walls, glass tubes are generally used in the LAL assay [3–6].

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It was previously reported that endotoxins are adsorbed on glass in the presence of rhIL-11, a highly cationic ($pI > 11$) protein [16]. However, it is unclear whether this phenomenon is specific for rhIL-11 or occurs in the presence of other cationic proteins. In this paper, adsorption of endotoxins on glass was investigated in the presence of various cationic proteins. Polystyrene tubes were also assessed to determine a possible alternative to glass tubes for the LAL assay of the proteins.

2. Materials and methods

2.1. Materials and reagents

Lysozyme from chicken egg white, histone II-S from calf thymus, ribonuclease A (RNaseA) from bovine pancreas, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO); lipopolysaccharide standard (*Escherichia coli* UKT-B), lyophilized *Limulus* amebocyte lysate and pyrogen-free borosilicate glass tubes (12×75 mm) from Wako (Osaka, Japan); pyrogen-free polystyrene tubes from Beckton Dickinson and Company (Lincoln Park, NJ); and pyrogen-free water 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. Endotoxin adsorption on glass in the presence of proteins

Fifty microliters of endotoxins (2 EU ml^{-1}) and appropriate volumes of each protein solution 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 incubated at 4°C for 2 h. After incubation, the entire mixture in the glass tube was transferred to a fresh glass tube. 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 then endotoxin concentrations were determined. Endotoxin adsorption on glass is expressed as percent of the endotoxin concentration detected from the original glass tube divided by the sum of the concentrations from both glass tubes.

2.4. Endotoxin recovery using separate glass tubes, the same glass tube or a polystyrene tube

For the LAL assays performed using separate glass or polystyrene tubes for incubation, 50 μl of endotoxins (2 EU ml^{-1}), 10 μl of 0.1 mg ml^{-1} lysozyme solution and 40 μl of water were mixed into each glass or polystyrene tube. For control experiments, either the endotoxin or the lysozyme solution was replaced by water. The mixture was incubated at 4°C for 2 h. After incubation, the entire mixture in the tube was transferred to a fresh glass tube where 100 μl of LAL reagent was added, and then endotoxin concentrations were determined.

For the LAL assays performed using the same glass tube, 50 μl of endotoxins (2 EU ml^{-1}), 10 μl of 0.1 mg ml^{-1}), lysozyme solution and 40 μl of water were mixed into a glass tube. For control experiments, either the endotoxin or the lysozyme solution was replaced by water. The mixture was incubated at 4°C for 2 h. After incubation, 100 μl of LAL reagent was added directly to the glass tube and endotoxin concentrations were determined.

2.5. Protein adsorption on glass or polystyrene

Two hundred microliters of endotoxins (2 EU ml^{-1}), an appropriate volume of each protein solution and water were added to a glass or polystyrene tube to yield desired protein concentrations. The total volume of the mixture was 400 μl. The mixture was incubated at 4°C for 2 h. After incubation, the entire mixture in the glass

tube was transferred to an HPLC tube, and protein concentrations were determined by reversed-phase HPLC using an HP1090M system (Hewlett Packard). 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 0% B at a flow rate of 1 ml min^{-1}. The separation was performed by a linear gradient of 0%–100% B over 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. Endotoxin adsorption on glass in the presence of lysozyme

Endotoxins and the cationic protein lysozyme (p*I* 11), were mixed and incubated at 4°C for 2 h in a glass tube. The mixture in the glass tube was transferred to a fresh tube and endotoxin activity was measured in both glass tubes after water was added to the original tube for incubation, as described in Materials and methods. Table 1 shows endotoxin activity remained in the original glass tube even after the entire mixture was transferred. At a concentration of less than 50 μg ml^{-1} lysozyme, endotoxin activity detected from the original glass tube was higher than that from the fresh glass tube. These results show that endotoxins are readily adsorbed on glass in the presence of lysozyme.

To confirm endotoxin adsorption on glass, incubation and the LAL assay were performed in either the same glass tube or separate tubes. In the experiment using the same glass tube, endotoxins and lysozyme were incubated at 4°C for 2 h in a glass tube, and the LAL reagent was added directly to this solution without transfer after incubation, and then endotoxin concentration was measured. In the experiment using separate glass tubes, the entire mixture in each glass tube was transferred after incubation to a fresh glass tube where endotoxin concentration was measured after the LAL reagent was added. The results are shown in Table 2. The recovery (28.5%) of endotoxins using separate glass tubes was greatly reduced compared with the recovery (80.4%) using the same glass tube. Since the only difference between the two experiments lay in the transfer of the mixture from the original glass tube used for incubation to the fresh glass tube for the LAL assay, the reduced endotoxin recovery can be explained by adsorption of endotoxins in the original glass tube for incubation (Table 1). These results also indicate that endotoxins are readily adsorbed on glass in the presence of lysozyme.

The lysozyme reagent itself contained some endogenous endotoxins, which was also measured by assaying lysozyme solutions without spiked endotoxins in the same way. The endotoxin activity of the lysozyme sample obtained using separate glass tubes was also less than that using the same glass tube (Table 2), also suggesting adsorption of endotoxins on glass. These results indicate that endotoxins present in lysozyme preparations

Table 1
Adsorption of endotoxins on glass in the presence of lysozyme[a]

Lysozyme concentration	Endotoxins (EU ml^{-1})		Endotoxin adsorption on glass (%)
(μg ml^{-1})	Original glass tube	Fresh glass tube	
5	0.85 ± 0.19	0.59 ± 0.19	58.9
10	0.95 ± 0.04	0.36 ± 0.03	72.2
25	0.78 ± 0.22	0.56 ± 0.05	58.2
50	1.06 ± 0.43	1.25 ± 0.92	45.9
100	1.33 ± 0.15	1.67 ± 0.12	44.4

[a] Results are expressed as the mean ± S.D. from three assays. The apparent lysozyme concentration-dependence of the sum of the endotoxin concentrations was due to endotoxins present in the lysozyme reagent.

Table 2
Recovery of endotoxins using a glass tube in the presence of lysozyme[a]

Sample	Endotoxins (EU ml^{-1})	
	Separate glass tubes[b]	The same glass tube[c]
Endotoxins	0.44 ± 0.09	1.17 ± 0.14**
+ Lysozyme		
Endotoxins	1.49 ± 0.76	1.25 ± 0.06
Lysozyme	0.04 ± 0.01	0.20 ± 0.09*
Recovery (%)	28.5	80.4

[a] Results are expressed as the mean ± S.D. from three assays. Recovery of endotoxins is expressed as percent of the endotoxin concentration detected from the mixture with endotoxins and lysozyme divided by the sum of the concentrations of separate endotoxin and lysozyme tests. The concentration of lysozyme, when present, was 10 μg ml^{-1}.

[b] The incubation and the LAL assay were performed in separate glass tubes.

[c] The incubation and the LAL assay were performed in the same glass tube.

* $P<0.05$ versus separate glass tubes.

** $P<0.01$ versus separate glass tubes.

may not be accurately measured in the LAL assay if glass tubes are used in sample preparation.

3.2. Endotoxin adsorption on glass in the presence of various proteins

To examine how general this effect is, endotoxin adsorption on glass was assessed in the presence of the cationic proteins histone (p*I* 11) and RNase A (p*I* 9), and the non-cationic protein BSA (p*I* 5). As shown in Table 3 and Table 4, endotoxins were adsorbed on glass in the presence of histone and RNase A, indicating that endotoxins are adsorbed on glass in the presence of cationic proteins in general. In contrast, little adsorption of endotoxins was observed in the presence of BSA (Table 5). However, it has been reported that serum albumin binds to endotoxins [9], suggesting that the difference in endotoxin adsorption on glass between the cationic proteins and BSA is not due to differences in binding of endotoxins to proteins. To investigate the cause of this difference, adsorption of protein itself on glass was investigated. After the mixtures of endotoxins and proteins were incubated at 4°C for 2 h in a glass tube, protein concentrations were determined. The results are shown in Table 6. Lysozyme, histone and RNase A all were adsorbed on glass. However, little adsorption of BSA on glass was observed, which was significantly different from the results for cationic proteins. This is due to the attraction between the positively charged cationic proteins and the negatively charged glass surface, indicating that differences in endotoxin adsorption on glass in the presence of proteins is due primarily to differences in protein adsorption on glass.

Table 3
Adsorption of endotoxins on glass in the presence of histone[a]

Histone concentration (μg ml^{-1})	Endotoxins (EU ml^{-1})		Endotoxin adsorption on glass (%)
	Original glass tube	Fresh glass tube	
5	1.54 ± 0.09	3.26 ± 0.26	32.1
10	0.86 ± 0.07	6.70 ± 0.32	11.4
25	0.84 ± 0.11	10.08 ± 0.54	7.7
50	1.21 ± 0.47	–[b]	
100	2.12 ± 0.14	–[b]	

[a] Results are expressed as the mean ± S.D. from three assays. The apparent histone concentration-dependence of the sum of the endotoxin concentrations was due to endotoxins present in the histone reagent.

[b] The endotoxin concentrations were beyond the working ranges of the LAL assay due to endotoxins present in the histone reagent.

Table 4
Adsorption of endotoxins on glass in the presence of RNaseA

RNaseA concentration	Endotoxins (EU ml^{-1})		Endotoxin adsorption on glass (%)
(μg ml^{-1})	Original glass tube	Fresh glass tube	
5	0.36	1.21	22.9
10	0.22	0.94	18.9
25	0.27	1.02	21.3
50	0.23	1.08	17.9
100	0.11	1.14	9.4

Table 5
Adsorption of endotoxins on glass in the presence of BSA

BSA concentration	Endotoxins (EU ml^{-1})		Endotoxin adsorption on glass (%)
(μg ml^{-1})	Original glass tube	Fresh glass tube	
5	0.05	1.32	3.9
10	0.07	1.37	5.1
25	0.07	1.68	4.1
50	0.03	1.15	2.7
100	0.03	1.37	2.4

Table 6
Adsorption of proteins on glass and polystyrene

Protein	Recovery (%)					
Concentration	Glass				Polystyrene	
(μg ml^{-1})	Lysozyme	Histone	RNaseA	BSA	Lysozyme	BSA
5	39.3	32.3	59.7	88.6	94.0	69.7
10	67.7	59.5	80.0	98.8	98.6	76.8
25	83.1	85.1	89.2	98.2	98.3	91.5
50	85.5	95.9	98.8	99.4	98.4	96.5
100	93.7	95.4	96.4	100.3	96.4	97.3

3.3. Endotoxin recovery using polystyrene tubes in the presence of lysozyme or BSA

Since lysozyme itself is not adsorbed on polystyrene (Table 6), endotoxins and lysozyme were incubated in a polystyrene tube to prevent adsorption of endotoxins on glass in the presence of lysozyme, followed by transfer of the mixture into a fresh glass tube where the LAL assay was performed. The only difference from previous experiments using separate glass tubes (Table 2) was to use polystyrene tubes for incubation instead of glass tubes. The results are shown in Table 7. The recovery of endotoxins was 90.0% when the incubation was performed in the polystyrene tube, which was much greater than the recovery (28.5%) using a glass tube for incubation (Table 2). This result suggests that cationic protein-mediated endotoxin adsorption on glass causes poor recovery of spiked endotoxins in the LAL assay.

BSA-mediated endotoxin adsorption was also assessed in polystyrene and glass tubes. Adsorption of BSA on polystyrene was observed at low BSA concentrations, which was different from adsorption on glass (Table 6). The recovery of endotoxins (64.7%) using polystyrene tubes was less than the recovery (103.9%) using glass tubes (Table 7). Again, the only difference between the two experiments was whether the tubes for incubation were polystyrene tubes or glass tubes. These results indicate that adsorption of endotoxins on polystyrene occurs in the presence of BSA.

It has been reported that many proteins bind to endotoxins [7–16]. Adsorption of endotoxins on glass in the presence of cationic proteins can be explained by the hypothesis that endotoxins and cationic proteins form complexes, which are adsorbed on glass. It has also been reported that complexes between endotoxins and lysozyme reduce endotoxin activity in the LAL assay compared with assays conducted with endotoxins alone [15]. The slight decrease of endotoxin recovery (80.4%) using the same glass tube (Table 2) or endotoxin recovery (90.0%) using polystyrene tubes (Table 7) support this idea. However, the loss of endotoxin activity due to this effect was much less than that due to adsorption of endotoxins on glass. The results shown in Table 7 indicate that most of the loss of spiked endotoxins in the LAL assay in the presence of cationic proteins can be recovered using a polystyrene tube. In contrast, the results for BSA shown in Table 7 also suggest that endotoxin adsorption on polystyrene occurs in the presence of a non-cationic protein. Therefore, in the case of the LAL assay for a compound which binds to endotoxins, it is necessary to use a suitable container which adsorbs neither endotoxins nor the compound to avoid endotoxin adsorption on the container.

Table 7
Recovery of endotoxins using either glass or polystyrene tubes in the presence of lysozyme or BSA[a]

	Endotoxins (EU ml^{-1})		
Sample	Lysozyme Polystyrene[b]	BSA Glass[c]	BSA Polystyrene[b]
Endotoxins + Protein	1.30 ± 0.22*	1.19 ± 0.07	0.77 ± 0.06**
Endotoxins	1.30 ± 0.34	1.14 ± 0.04	1.19 ± 0.28
Protein	0.15 ± 0.02*	0.003>	0.003>
Recovery (%)	90.0	103.9	64.7

[a] Results are expressed as the mean ± S.D. from three assays. Recovery of endotoxins is expressed as percent of the endotoxin concentration detected from the mixture with endotoxins and protein divided by the sum of the concentrations of separate endotoxin and the protein tests. The concentration of protein, when present, was 10 μg ml^{-1}.

[b] The incubation was performed in a polystyrene tube, and the LAL assay in a glass tube.

[c] The incubation and the LAL assay were performed in separate glass tubes.

* $P<0.05$ versus separate glass tubes in Table 2.

** $P<0.05$ versus glass.

In conclusion, endotoxin activity was detected from the empty glass tubes where endotoxins were incubated with lysozyme, histone or RNase A, indicating that endotoxins are adsorbed on glass in the presence of cationic proteins. More than 80% of spiked endotoxins could be recovered using the same glass tube for the incubation and the LAL assay, or a polystyrene tube for incubation, indicating that lysozyme-mediated adsorption of endotoxins on glass is a major cause of poor recovery of spiked endotoxins in the LAL assay. Furthermore, in the case of BSA, poor recovery of spiked endotoxins was observed using a polystyrene tube, indicating that adsorption of endotoxins on containers may occur in the presence of proteins which bind to both of endotoxins and the containers. Consequently, care must be exercised in selecting containers used for the LAL assay of proteins which bind to endotoxins.

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