Removal of Endotoxin from Culture Supernatant of *Bordetella pertussis* with Aminated Poly(γ-methyl L-glutamate) Spherical Beads

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Attempts were made to prepare adsorbents having a high affinity for endotoxin in the culture supernatant of *Bordetella pertussis*. When poly(γ-methyl L-glutamate) (PMLG) was used as a matrix and amino groups as the ligand, the highest affinity for endotoxin was attained even at a high ionic strength (μ=0.2—0.4). PMLG beads containing amino groups of about 3.2 meq/g selectively removed endotoxin from the culture supernatant of *B. pertussis* without affecting the protective antigens. It was demonstrated that 1 ml of the wet adsorbent adsorbed 4.5 mg of endotoxin. The beads of PMLG derivatives, therefore, are considered to be a useful adsorbent for the removal of endotoxin from the pertussis vaccine, affecting neither filamentous hemagglutinin nor pertussis toxin.

Keywords endotoxin; *Bordetella pertussis*; lipopolysaccharide; poly(γ-methyl L-glutamate); spherical bead; polyamino acid; amino group; filamentous hemagglutinin; pertussis toxin

Introduction

Endotoxin (lipopolysaccharide; LPS), a constituent of the cell wall of gram-negative bacteria, is ubiquitously distributed among plants, animals and environments. Because of its potent biological activities causing pyrogenic and shock reactions in man and animals,[1] it is necessary to remove even nanogram quantities of endotoxin from drugs and fluids before injection. Recently, some endotoxin adsorbents such as histidine-immobilized polysaccharides[2,3] and chitosan beads[4] have become commercially available. The endotoxin-adsorption capacity of these adsorbents, however, decreases with an increase in the ionic strength, and little endotoxin is adsorbed at an ionic strength as high as μ=0.2—0.4.

The antigenic proteins in the culture supernatant of *Bordetella pertussis* and tetanus and diphtheria toxoids, are vulnerable substances and sometimes aggregate themselves at a low ionic strengths of μ=0.01—0.1 because of their hydrophobicity.[5] We attempted, therefore, to develop novel endotoxin adsorbents which can retain the affinity for endotoxin even at a high ionic strength. We have already reported that some spherical beads prepared from poly(γ-methyl L-glutamate)(PMLG) without any other support were useful as endotoxin adsorbents.[6,7] When amino groups were incorporated as a ligand, the adsorbent exhibited the highest affinity for endotoxin in tetanus and diphtheria vaccine materials, even at a high ionic strength (μ=0.17), without affecting their substantial antigens.

This report describes novel adsorbents for the removal of endotoxin in the culture supernatant of *B. pertussis*, a gram-negative bacterium. It also describes the adsorption capacity of one of these adsorbents and its characteristics.

Experimental

Materials Endotoxin preparations from *Escherichia coli* UKT: B and O111: B4 were purchased from Wako Junyaku, Osaka, and Difco Laboratories, Detroit, MI, respectively. Limulus HS II-test Wako (Limulus amoebocyte lysate) was a product of Wako.

Pyro Sep was purchased from Daicel, Tokyo and diaminoalkanes were from Nacalai Tesque, Kyoto. PMLG was supplied by Ajinomoto, Tokyo. All other chemicals were of an analytical-reagent grade. Endotoxin-free water was obtained from Chemo-Sero Therapeutic Research Institute, Kumamoto.

Filamentous hemagglutinin (F-HA) and pertussis toxin (PT) antigen[8] prepared from a culture supernatant of *B. pertussis* Tohama were provided by Chemo-Sero Therapeutic Research Institute. The antigens were purified by the column method described previously.[9]

Preparation of PMLG Adsorbents PMLG adsorbent with diameters of 44 to 105 μm was prepared by the "suspension and evaporation" method reported by us.[10,11] Amino groups as the ligand were introduced into PMLG beads by aminolysis with hydrazine or various diaminoalkanes (diaminoethane and diaminohexane) as described previously.[7]

Determination of Amino-Group Contents of Adsorbents The amino groups were quantified by pH titration and elemental analysis as described previously.[7]

Adsorption of Endotoxin The affinity of the adsorbent for endotoxin was measured by a batchwise method as follows: A 2-ml portion of wet adsorbent was washed with 100 ml of 2.0 M sodium chloride on a glass filter and equilibrated with a phosphate buffer with different ionic strengths. A portion of 0.2 to 2.0 ml of wet adsorbent was suspended in 2—18 ml of an endotoxin solution. The suspension was shaken for 2 h at 25 °C and filtered through a Milipore filter (0.45 μm) to remove the adsorbent. The filtrate's endotoxin concentration was determined.

Determination of Endotoxin-Adsorption Capacity The endotoxin-adsorption capacity of each adsorbent was determined by a column method as follows: A 0.8-ml portion of wet adsorbent was packed in a sterilized glass column (0.3 × 10 cm). The column was washed with 50 ml of 2.0 M sodium chloride and then equilibrated with 0.02 M of a phosphate buffer (pH 7.2, μ=0.17). An endotoxin solution (10 μg/ml, *E. coli* O111: B4 LPS) was passed through the column at a flow rate of 0.2 ml/min at room temperature.

Endotoxin Assay Endotoxin was assayed by turbidimetric time assay at 660 nm, a Limulus test method, with Toxinometer ET-201 (Wako).[12] *E. coli* UKT: B LPS was used as the standard endotoxin.

Antigen Assay F-HA and PT antigens were determined by enzyme-linked immunosorbent assay (F-HA ELISA and PT ELISA).[8] Enzymatic reactions were measured with a MR-580 Micro-ELISA Reader (Dinatech) at 405 nm. One ELISA unit was defined arbitrarily with purified F-HA as the reference.

Results and Discussion

Adsorption of endotoxin to PMLG adsorbents was examined by the batchwise method. The adsorption capacity was increased by the incorporation of amino groups into the PMLG beads as reported previously.[7] As summarized in Table I, the PMLG beads aminated with hydrazine or one of the various diaminoalkanes (diaminoethane and diaminohexane) satisfactorily removed endotoxin from endotoxin solutions (pH 7.2, μ=0.02—0.2). When diaminoethane was incorporated as the ligand, the adsorbent showed the highest affinity for endotoxin.

Electron micrographs of typical examples of adsorbents prepared from PMLG by our method[10] are shown in

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Table I. Effects of Ligands on Adsorption of Endotoxin

Adsorbent	Ligand	Quantity of amino groups (meq/g)	Conc. of endotoxin in filtrate (ng/ml) $\mu=0.02$	$\mu=0.20$
PMLG	$-COOCH_3$	<0.01	94	125
PMLG-HYD	$-CONHNH_2$	1.9	3	29
PMLG-ED-0.8	$-CONH(CH_2)_2NH_2$	0.8	<0.1	<0.1
PMLG-ED-3.2	$-CONH(CH_2)_2NH_2$	3.2	<0.1	<0.1
PMLG-HD	$-CONH(CH_2)_6NH_2$	1.0	0.8	20

The endotoxin-affinities of various PMLG adsorbents were determined by the batchwise method with 100 mg of adsorbent and 2 ml of an endotoxin solution (*E. coli* UKT: B, 250 ng/ml, pH 7.2). PMLG-HYD-ED, -HD: poly(γ-methyl L-glutamate)hydrazine, diaminoethane, diaminohexane.

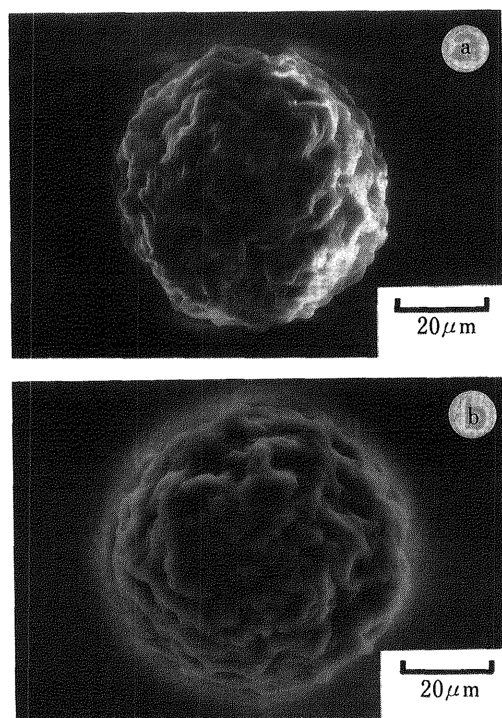

Fig. 1. Electron Micrographs of Adsorbents

(a) a PMLG sphere; (b) a PMLG-ED ($-NH_2$: 3.2 meq/g) sphere.

Fig. 1. Figure 1a shows a non-aminated bead and Fig. 1b the one aminated with diaminoethane ($-NH_2$: 3.2 meq/g). Each adsorbent is a porous spherical particle with a diameter of about 50 μm.

Effects of ionic strength on the adsorption of endotoxin to various adsorbents were examined in a phosphate buffer of varying ionic strengths (Fig. 2). The endotoxin-adsorption capacity of each adsorbent decreased with an increase in ionic strength. Pyro Sep adsorbed little endotoxin at a high ionic strength of $\mu=0.2$—0.8. This property is very disadvantageous for selective removal of endotoxin from protein solutions, because some antigenic substances lose their activities at a low ionic strength of $\mu=0.01$—0.1. By contrast, PMLG beads showed a high affinity for endotoxin at a high ionic strength of $\mu=0.2$—0.4.

The effects of the amino-group content of the PMLG beads on the recovery of the two antigen proteins (F-HA and PT) were examined in 0.02 M of a phosphate buffer (pH 7.2, $\mu=0.2$) (Fig. 3). Considerable amounts of both antigens were adsorbed to non-aminated PMLG beads; 75% of F-HA and 25% of PT were adsorbed. These results show that the hydrophobicity of PT is lower than that of F-HA. Adsorption of these antigens decreased with an increase in the amino-group content of the PMLG beads. When the amino-group content was 3.2 meq/g, high recoveries of the two antigens were observed.

Effects of the amino-group contents of the PMLG beads and the ionic strength on the adsorption of endotoxin from an F-HA fraction of *B. pertussis* were examined

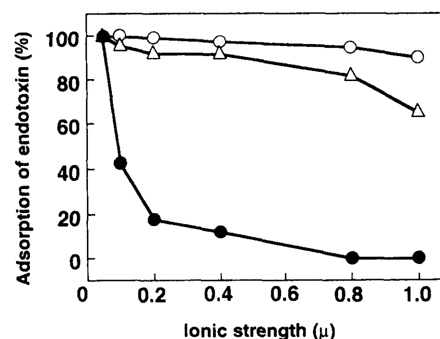

Fig. 2. Effect of the Ionic Strength on Adsorption of Endotoxin

Adsorption of endotoxin was carried out by the batchwise method with 0.5 g of wet adsorbent and 5 ml of an endotoxin solution (*E. coli* O111: B4 LPS 500 ng/ml, pH 7.0, $\mu=0.05$—1.0). Adsorbent, amino-group contents (meq/g): (○) PMLG-ED, 3.2; (△) PMLG-ED, 0.8; (●) Pyro Sep, 1.1.

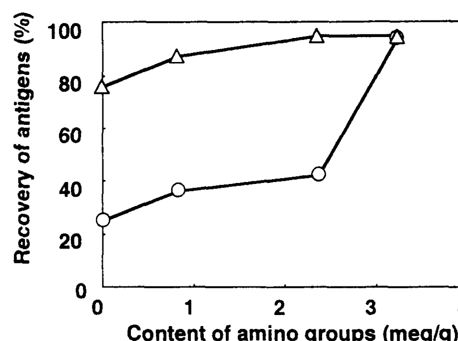

Fig. 3. Effects of Amino-Group Contents of PMLG-ED Adsorbents on the Recovery of Antigens

Recovery of the antigens was determined by the batchwise method with 2 ml of wet PMLG-ED adsorbent and 18 ml of antigen (pH 7.2, $\mu=0.2$) of *B. Pertussis*. ○, F-HA; △, PT.

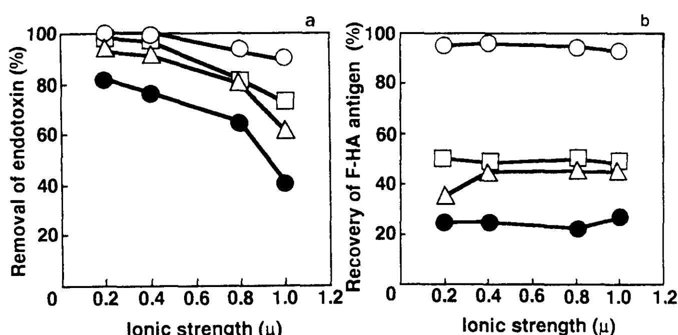

Fig. 4. Removal of Endotoxin (a) and Recovery of the Antigens of *B. pertussis* (b)

Affinity for endotoxin was determined by the batchwise method with 2.0 ml of PMLG-ED (amino-group contents (meq/g): ○, 3.2; □, 2.4; △, 0.8; ●, <0.1) and 18 ml of a sample solution (LPS 130 μg/ml, antigen 5000 ELISA-units/ml, pH 7.2, $\mu=0.2$—1.0).

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(Fig. 4). With each adsorbent, the endotoxin-adsorption capacity decreased with an increase in ionic strength. PMLG-ED (-NH_2: 3.2 meq/g) beads maintained the highest affinity for endotoxin at an ionic strength as high as $\mu=0.2$—0.4 (Fig. 4a). On the other hand, as shown in Fig. 4b, adsorption of F-HA decreased with an increase in the amino-group content of the PMLG beads. With PMLG-ED (–NH_2: 3.2 meq/g), a high recovery of F-HA was observed after the removal of endotoxin. Little F-HA was adsorbed at an ionic strength as high as $\mu=0.2$—1.0.

From these results, we assumed that the high affinity of aminated PMLG beads for endotoxin was induced by a cooperative interaction of the cationic and weak hydrophobic properties of the beads. The endotoxin-adsorption capacity of aminated PMLG beads, being dependent on the ionic strength, suggests cationic interaction. Adsorption of F-HA, being independent of the ionic strength, suggests the hydrophobic interaction of PMLG beads themselves, as shown in Fig. 3. Although the PMLG beads themselves show a hydrophobic tendency,[11,13] the incorporation of amino groups decreased the hydrophobic interaction with the F-HA antigen proteins. The high affinity of aminated PMLG beads for endotoxin is related to the anionic properties induced by phosphoric acid groups and to the hydrophobicity of the lipophilic groups of endotoxin.[14]

Selective removal of endotoxin from the two antigen preparations from the culture supernatant of *B. pertussis* was examined with PMLG-ED (–NH_2: 3.2 meq/g) beads at an ionic strength of $\mu=0.17$, which is equivalent to that of physiological saline. As shown in Table II, high recoveries of the two antigens of *B. pertussis* were obtained after the removal of endotoxin.

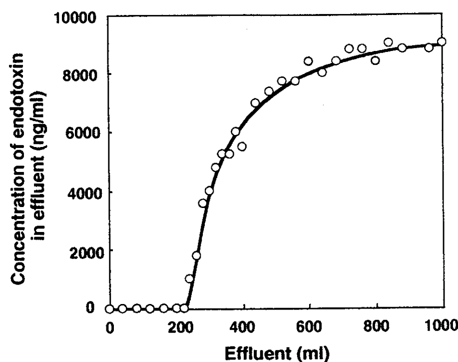

Fig. 5. The Endotoxin-Adsorption Capacity of PMLG-ED Adsorbent

Endotoxin (*E. coli* O111: B4 LPS) was dissolved in 0.02 M PBS (pH 7.2, $\mu=0.17$) at a concentration of 10 μg/ml. For determination of the affinity of PMLG-ED (amino-group content, 3.2 meq/g) adsorbent for endotoxin. 10-ml fractions were collected and the endotoxin concentration of each fraction was determined.

Table II. Removal of Endotoxin from Two Antigen Samples of *B. pertussis* with PMLG-ED Adsorbents

Sample solution		Conc. of endotoxin (ng/ml)		Recovery of antigen after treatment (%)
Antigen	Conc. (in ELISA units/ml)	Before treatment	After treatment	
F-HA	5000	130000	<0.1	94
PT	4500	45000	<0.1	99

The endotoxin-affinities of PMLG-ED adsorbents (–NH_2: 3.2 meq/g) were determined by the batchwise method with 2 ml of wet adsorbent and 18 ml of a sample solution (pH 7.2, $\mu=0.17$). Antigen assay: ELISA.

It is not so simple to remove endotoxin from F-HA and PT preparations, because they are from a gram-negative bacterium and therefore contain large quantities of endotoxin and their substantial antigens tend to lose their potencies at a low ionic strength of $\mu=0.01$—0.1. It is essential to eliminate endotoxin from an injection solution to a concentration of 0.1 ng/ml or lower[3] because of its potent biological activities which cause pyrogenic and shock reactions in mammals.[1] As shown in Table II, the endotoxin concentrations of the antigen fractions from *B. pertussis* were reduced to lower then 0.1 ng/ml upon treatment with PMLG-ED (–NH_2: 3.2 meq/g) beads at an ionic strength equivalent to physiological saline without affecting the protective antigens. These results show that the endotoxin-adsorption capacity of PMLG-ED beads is higher than that of Pyro Sep at a high ionic strength.

The endotoxin-adsorption capacity of PMLG-ED (–NH_2: 3.2 meq/g) beads was determined as follows: An endotoxin solution (10 μg/ml) was applied to a column (0.3 × 10 cm) until the endotoxin concentration of the effluent became equal to that of the charged endotoxin solution. As shown in Fig. 5, the adsorbent was slowly saturated with endotoxin. The adsorption capacity of 1 ml of the adsorbent was 4.5 mg endotoxin (*E. coli* O111: B4 LPS) at a high ionic strength of $\mu=0.17$. Minobe *et al.*[2] found also that the endotoxin-adsorption capacity of 1 ml of imidazole-immobilized polysaccharides such as Pyro Sep was 0.9 mg (*E. coli* O128: B12 LPS), but little endotoxin was adsorbed at a high ionic strength of $\mu \geqq 0.1$. These results show that the endotoxin-adsorption capacity of PMLG-ED beads is higher than that of Pyro Sep.

In addition, as previously reported,[11,16] the PMLG beads showed a remarkable resistance to high flow rate in liquid chromatography due to the rigid structure of the peptide chains and of the intermolecular hydrogen bonds.

The results of the present study demonstrated that affinity chromatography on aminated PMLG beads can remove endotoxin in the pertussis vaccine to a concentration lower than 0.1 ng/ml without affecting the potencies of the protective antigens.

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