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Filtration Removal of Endotoxin (Pyrogens) in Solution in Different States of Aggregation

KATHLEEN J. SWEADNER,† MARK FORTE,†† AND LITA L. NELSEN*
Millipore Corporation, Bedford, Massachusetts 01730

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Bacterial lipopolysaccharides are recognized as the major cause of pyrogenic reactions from parenteral solutions. Molecular filtration was used to remove these pyrogenic molecules (endotoxins) from contaminated parenteral solutions. Because bacterial lipopolysaccharides can exist in different states of aggregation, depending on the composition of the solution they are suspended in, the full range of possible states of aggregation was examined by using filters with a wide range of pore sizes. Filters of different pore sizes retained endotoxin lipopolysaccharide presumed to be in the vesicle form, the micelle form, or the detergent-solubilized form in aqueous solutions. Endotoxins (pyrogens) were successfully removed from artificially contaminated solutions of concentrated antibiotics by using filters of 10,000-nominal-molecular-weight limit.

The principle method for keeping drugs, vaccines, and parenterals of all kinds free of contaminating bacterial endotoxin is to keep the manufacturing process and all subsequent handling sterile. Because maintaining sterility is difficult in many processes, as in the production of vaccines and biological drugs such as antibiotics, a reliable method is needed for removing endotoxin from an accidentally contaminated product. Conventional heat or chemical sterilization, which only kills live bacteria, does not alter the toxic activity of the pyrogens; conventional sterilization by filtration removes whole bacteria but not the endotoxic fragments. The ion-exchange, charcoal, barium sulfate, and heat incubation procedures now being explored by others to remove endotoxin are two (or more)-step batch procedures that are often unreproducible and accompanied by high losses of the substance being purified (7, 10). Asbestos fiber beds can be used to remove pyrogens (3), but their use in the United States is prohibited by Food and Drug Administration regulation. Work in this laboratory has concentrated on using molecular filtration to remove endotoxin from contaminated solutions by separation according to size. It has the advantages of being straightforward, highly efficient, inert, and nondestructive to sensitive biological fluids.

The endotoxin from gram-negative bacteria resides in the lipopolysaccharide (LPS), which, with phospholipid and protein, makes up the bulk of the outer cell membrane. The LPS is an amphiphile with a large hydrophilic polysaccharide chain and a hydrophobic, fatty acid-containing tail. When isolated, the LPS aggregates in aqueous solution as one would expect of a major constituent of a biological membrane. Early attempts in this laboratory to remove endotoxin from contaminated solutions by using molecular filtration met with a bewildering variability in results. It became apparent that the solution properties of bacterial endotoxin as an amphiphile would have to be understood in detail before its behavior with a molecular filter of a given effective pore size could be predicted with any certainty. The work reported in this paper is the result of systematic manipulation of the state of aggregation of *Escherichia coli* LPS. This information can be used to predict the state of aggregation of endotoxin in a given solution, so that an appropriate molecular filter can be selected for removing it.

† Present address: Department of Neurobiology, Harvard Medical School, Boston, MA 02115.
†† Present address: Department of Chemistry, Boston College, Chesnut Hill, MA 02167.

MATERIALS AND METHODS

Purified *E. coli* LPS was obtained from Mallinckrodt. Mallinckrodt Pyrogent *Limulus* amoebocyte lysate was used routinely to measure endotoxin. Lysate was resuspended in 2.25 ml of pyrogen-free 100 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride in 0.9% NaCl, pH 7.1. *Limulus* lysate forms a firmer gel in up to 1.0 M Tris buffer than in distilled water, and the buffer ensures that the pH of the sample does not affect gel formation (unpublished data). The Mallinckrodt *Limulus* lysate results correlated well with results obtained with Worthington or Difco *Limulus* lysate and with rabbit tests on samples sent to outside laboratories. Pyrogen-free water was produced in the laboratory

by distillation or by distillation followed by a Millipore RO (reverse osmosis) system. Sodium cholate and deoxycholate were obtained from Sigma Chemical Co. All other compounds were reagent grade.

Filtration experiments were performed at room temperature in 25-mm, magnetically stirred molecular filtration cells (Millipore Corp.) under 25-lb/in^2 air pressure. The cells were rendered pyrogen-free by washing with soap and water followed by rinsing overnight in a continuous stream of pyrogen-free water. All glassware and forceps were sterilized by heating for 3 h at 200°C, and all fluid transfers and measurements were done with pyrogen-free Plastipak disposable syringes (Becton, Dickinson). A sterile Millipore Swinnex-25 filter was inserted between the cell and the compressed air supply to prevent contamination from dust or droplets. The cell was flushed with pyrogen-free water several times after the filter was wetted and mounted. A sample of the final rinse of each cell was assayed with the *Limulus* lysate test to check that the entire apparatus was pyrogen-free downstream from the filter before an experiment was begun.

E. coli LPS from a 500-μg/ml stock solution, stored at 4°C, was made up in the test solution to 1 μg/ml, mixed thoroughly, and used that same day. One milliliter of the test solution was set aside for endotoxin assay, and 9 ml was applied to the filter. The first 5 ml of the filtrate was collected and mixed thoroughly. Parallel 1:9 serial dilutions were performed on the original sample and the filtrate, and each dilution was tested with *Limulus* amoebocyte lysate. Dilutions were made with pyrogen-free portions of the same solution used for the filtration experiment so that all samples tested would be of identical composition. An exception to this protocol had to be made for the experiments using cholate and deoxycholate. In the presence of these detergents, even 100 μg of *E. coli* endotoxin per ml was not detected by the *Limulus* lysate test. The detergent had to be diluted by a factor of 100 with detergent-free water, presumably allowing some reaggregation of dissociated LPS subunits, before activity could be measured. Consequently, the protocol for experiments with cholate and deoxycholate became: challenge the filter with 10 μg of endotoxin solution per ml, dilute the sample and the filtrate 1:100 with water, and then perform serial dilutions with a pyrogen-free detergent solution similarly diluted.

Filters were selected that have pore sizes that decrease in steps of approximately a factor of 10. The filters used were Millipore EGWP (0.22-μm nominal pore size), VSWP (0.025-μm nominal pore size), PSVP (10^6 nominal-molecular-weight limit [nmwl]), PTHK (10^5 nmwl), and PTGC (10^4 nmwl). (The nmwl is defined by the ability to retain approximately 90% of globular proteins of a given molecular weight. The nmwl values are given only to indicate the range in pore sizes used; they cannot be used to assign an absolute size to an LPS particle because the asymmetry of a rod-shaped particle could lead to either more or less passage through a filter of a given pore size, depending on the flexibility of the rod. Samples of the filters were soaked overnight in 1 ml of pyrogen-free water, and both the water and the filters were tested for endotoxin by the *Limulus* lysate assay. All samples were negative.)

RESULTS

Filtration of LPS in different states of aggregation. In a representative filtration experiment, a PTGC (10,000 nmwl) filter was challenged with a solution of 1 μg of endotoxin per ml in saline, and serial dilutions of the original sample and of the filtrate were performed. With the lysate test calibrated with the sample solution, the concentration of endotoxin in the filtrate can be calculated from the number of 10-fold serial dilutions needed to reach the same end point, below which the endotoxin is too dilute to be detected by the *Limulus* lysate test. In the experiment reported in Table 1, then, the limit of sensitivity of the lysate test was of the order of 10^{-10} g/ml. Based on this, concentration of endotoxin in the filtrate must be less than 10^{-10} g/ml, indicating that less than 0.01% of the endotoxin passed the filter.

Similar experiments were performed for the combinations of filters and solutions represented in Table 2. EGWP (0.22 μm) is a cellulosic filter suitable for retaining bacteria for filtration sterilization. It did not hold back endotoxin at all. VSWP, which can be used for the concentration of many viruses, retained endotoxin when it was in water or salt solutions, but when ethylenediaminetetraacetic acid (EDTA) was added to chelate divalent cations, the endotoxin passed the filter. PSVP (10^6 nmwl) and PTHK (10^5 nmwl) filters retained endotoxin when it was in solutions with chelators of divalent cations, but began to pass it when detergents were present to break down

TABLE 1. *Results of a representative experiment*[a]

Dilution	Limulus lysate assay	
	Sample	Filtrate
0	+	−
1:10	+	−
1:10^2	+	−
1:10^3	+	−
1:10^4	+	−
1:10^5	−	−
1:10^6	−	
1:10^7	−	
1:10^8	−	

[a] A 0.9% NaCl solution was contaminated with 1 μg of *E. coli* endotoxin per ml and filtered through a PTHK (100,000 nmwl) filter. Serial dilutions were tested for endotoxin with *Limulus* lysate test. Symbols: +, visible gel formation; −, no visible gel formation.

TABLE 2. *Molecular filtration removal of LPS endotoxin from solutions of E. coli endotoxin*

Sample		Approx concn (g/ml) of endotoxin in filtrate from[a]:				
Composition	Endotoxin concn (g/ml)	EGWP (0.22 μm)	VSWP (0.025 μm)	PSVP (10^6 nmwl)	PTHK (10^5 nmwl)	PTGC (10^4 nmwl)
Water	10^{-6}	10^{-6}	$<10^{-10}$	$<10^{-10}$	$<10^{-10}$	$<10^{-10}$
0.9% NaCl	10^{-6}	10^{-6}	$<10^{-10}$	$<10^{-10}$	$<10^{-10}$	$<10^{-10}$
5 mM $MgCl_2$	10^{-6}	10^{-6}	$\leq 10^{-10}$	$<10^{-10}$	$<10^{-10}$	
5 mM EDTA	10^{-6}		10^{-6}	$<10^{-10}$	$<10^{-10}$	
0.5% sodium cholate	10^{-5}			10^{-5}	10^{-7}	$<10^{-10}$
1% sodium cholate	10^{-5}			10^{-6}	10^{-7}	$<10^{-10}$
2% sodium cholate, 5 mM EDTA	10^{-5}			10^{-5}	10^{-5}	$<10^{-10}$
1% deoxycholate	10^{-5}			10^{-5}	10^{-5}	$<10^{-10}$

[a] 100 pg/ml (10^{-10} g/ml) was generally the lower limit of sensitivity of the *Limulus* assay in our laboratory.

the endotoxin into smaller particles. The PTGC filter (10^4 nmwl) did not pass measurable amounts of endotoxin in any solution.

It was possible to remove endotoxin from tap water (Bedford, Mass.) by passage through a PTGC filter. The treatment lowered the level of *Limulus* lysate-reacting material to below the detectable level. In equivalents of *E. coli* LPS, this was a reduction from about 1 to 10 ng/ml to less than 100 pg/ml.

Filtration of antibiotic solutions. Two types of antibiotics were artificially contaminated with endotoxin and purified by molecular filtration. A 10% solution of sodium cephalothin (Keflin; Eli Lilly & Co.) was made 1 μg/ml in *E. coli* endotoxin. The solution was filtered through a PTGC membrane (10,000 nmwl), using a 25-mm stirred cell in the protocol described above. Results indicated that PTGC membranes removed the endotoxin to levels lower than the sensitivity of the *Limulus* lysate assay. Similar results were obtained with samples of 33% disodium carbenicillin (Beecham Laboratories) in water artificially contaminated with 400 ng of *E. coli* endotoxin (Difco Laboratories) per ml and filtered through PTGC membranes. The filtrate was *Limulus* negative. (A larger-scale experiment on a similar solution of artificially contaminated [400 ng of endotoxin per ml] carbenicillin was run, using 0.05 m^2 of PTGC membrane. More than 3 liters of solution was filtered. The filtrate was negative to the *Limulus* lysate assay even at the end of the run, indicating that no "leaching" of pyrogen through the membrane occurred, even after significant quantities of sample had been exposed to the membrane.)

DISCUSSION

LPS is believed to be arranged in a bilayer, analogous to a bilayer of phospholipid, in which the hydrophilic components are exposed to the aqueous environment while the hydrophobic fatty acid tails are sandwiched together in the center of the bilayer (1, 2, 4). In the case of bacterial LPS, this structure is apparently stabilized by divalent cations, because removal of Ca^{2+} and Mg^{2+} causes the bilayer to break down (5) into what appear to be micelles of 300,000 to 1,000,000 molecular weight. These appear as small rods or disks in the electron microscope, 20 to 70 nm long (rods) or in diameter (disks) and 3 to 7 nm thick (1, 12). Some strains of bacteria appear to give micelles of smaller size (8). The micelles can be further broken down in the presence of detergent or bile salts (6, 9, 13, 14). The particles can then no longer be seen in the electron microscope, but their size and shape, as calculated from sedimentation velocity, density, and viscosity measurements, are 0.8 to 1.2 nm in diameter and 20 to 70 nm long (2, 11), consistent with what is known of their chemical structure. The molecular weight is 10,000 to 20,000. The best evidence that these reductions in size are the result of breaking noncovalent bonds is that each step is freely reversible. When the detergent is dialyzed out and divalent cations are added back to the LPS, micelles and then membranous structures reassemble themselves (1, 2, 6, 8, 9, 11). The expected aggregation state for LPS from wild-type gram-negative bacteria in solutions of various compositions is summarized in Table 3, as gleaned from a number of published sources. The data presented in this paper corroborate the work of these other laboratories in describing the existence of bacterial LPS in states of aggregation of vastly different sizes in solution. All endotoxic activity passed a filter of 0.22-μm nominal pore size (EGWP) and was retained on a filter of 0.025-μm nominal pore size (VSWP), in the presence or absence of

TABLE 3. *Aggregation state for bacterial LPS resuspended in solutions of various compositions*

Solution	Aggregation state	Reference
Water	Vesicle	1, 4
5 mM $MgCl_2$, $CaCl_2$	Vesicle	1, 8
5 mM EDTA	Micelle	1, 5, 8, 13
1.0% sodium deoxycholate	Micelle or subunit	2, 12
2.0% sodium deoxycholate + 5 mM EDTA	Subunit	2, 12

salt. The combination of our data and published electron micrographs (1, 4, 13) suggests that the highest aggregate state in dilute solution is a bilayer sheet or vesicle with a diameter on the order of 0.1 μm. Smaller aggregates and possibly single subunits of LPS are seen when the compositions of the solutions are manipulated. The bilayer forms can be retained on VSWP, the micelles on PSVP, and all smaller forms on PTGC. The ground work is laid for the application of molecular filtration to the removal of endotoxin from contaminated solutions of small molecules. For example, all low-molecular-weight drugs, salts, and nutritional compounds should easily pass a PTGC membrane, leaving contaminating endotoxin behind. We have shown that solutions of sodium cephalothin and carbenicillin can be separated from 400 ng of *E. coli* endotoxin per ml in a single filtration through a PTGC membrane with no significant loss of antibiotic. Such a procedure, accomplishing a separation on the basis of size, avoids the problems of adsorption and product losses that are found when ion-exchange, charcoal, or barium sulfate methods are tried for removing endotoxin, and it does not have the problem of introducing a toxic element into the solution, as the asbestos filter method does. As another example, it might prove possible to remove endotoxins from viruses by retaining the viruses on a filter such as PSVP or VWSP and disaggregating and washing through the endotoxin with a solution of EDTA. In this way virus concentration and endotoxin removal could be accomplished in the same step.

Molecular filtration, then, may be of wide applicability for the removal of bacterial endotoxins from solutions. It is necessary to assess the state of aggregation of the endotoxin in the particular solution to be purified and to choose a filter of pore size suitable for getting a good separation of endotoxin from the solute being purified. If the endotoxin and solute are of similar size, it may be possible in many cases to manipulate the effective size of the endotoxin, as outlined here, and facilitate molecular filtration separation.

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