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The adsorption of endotoxin molecule in a microporous polyethylene hollow fibre membrane

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SUMMARY

The microporous polyethylene hollow fibre membrane is capable of adsorbing small-sized lipopolysaccharides (LPS) prepared by sonication dispersion, column chromatography on Sephadex G-75 and filtration through a filter membrane with a nominal pore size of 0·025 μm. Small-sized LPS had a one-thousandth of endotoxin activity as compared to intact LPS, when determined by the Synthetic Chromogenic Substrate method of LAL with a specific endotoxin activity of 73·7 ng/μg LPS. Fluorescent microscopy of fluorescein conjugated LPS on a microporous polyethylene hollow fibre showed that fluorescein-LPS was adsorbed through the entire depth of the membrane texture. Accordingly the adsorption capacity of the filter for small-sized LPS was determined as 1·65 mg LPS/3·68 m^2 surface/116 mg fibre/module.

INTRODUCTION

The major pyrogenic substance in water is endotoxin (lipopolysaccharide, LPS). Gram-negative bacteria produce individual endotoxin on their cell surfaces and release it into the surrounding medium following cell lsyis. The amphipathic LPS molecule makes various aggregation forms (Weiser & Rothfield, 1968; Hannecart-Pokorni, Dekegel & Dupuydt, 1973). LPS in big forms can, in turn, be converted into smaller ones under various environmental conditions (Ribi *et al.* 1966; Leive, Schvlin & Mergenhagen, 1968; Shands & Chun, 1980) and smaller ones exist as free

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forms or combine to form hybrids (Rudbach, 1970; Seid & Sadoff, 1981). Such aggregation and disaggregation of LPS causes problems, e.g. in quality control during pharmaceutical manufacturing processes and the consequent clinical application. Recently, a microporous polyethylene hollow fiber (EHF, a commercial product of Mitsubishi Rayon Co., Tokyo) has been developed as a water-purifying medium which removes bacterial cells, fine particles and endotoxin from water (Kamiki *et al.* 1982; Kamiki *et al.* 1985). In the preceding paper (Sawada *et al.* 1986), an adsorbent produced from EHF membrane is reported to have the capability of adsorbing various kinds of endotoxin. To confirm the binding of endotoxin molecules on the surface of the EHF membrane in filtration process, we designed a model experiment using authentic LPS isolated from *Escherichia coli* 0113.

The characterization of the solubility or the dispersion degree of LPS is crucial for the evaluation of endotoxins and assessment of their removal. Sonication dispersion has been recently studied and recommended to solubilize or disperse LPS (Ogawa & Kanoh, 1984). The LPS molecules prepared by filtration through a membrane having a nominal pore size of 0·025 μm was designated here as the small-sized LPS. This paper describes (1) the preparation of small sized LPS and its characterization; (2) the removal capacity of EHF membrane for small sized LPS; and (3) the adsorption mode of LPS-fluorescein isothiocyanate conjugate in the EHF membrane texture.

MATERIALS AND METHODS

The EHF membrane and module

Characteristics of the EHF membrane (Code: EHF 390-C, Mitsubishi Rayon Co., Tokyo) were as described by Kamiki *et al.* (1982). A module for experiment was made by using 128 filaments of the EHF membrane as shown in Fig. 1. The total surface area as determined by the nitrogen adsorption method (Bohra, 1984) is approximately 31·7 m^2/g fibre. To permeabilize the EHF membrane in the module it was treated with 30 ml of 99% ethanol and then 50 ml of endotoxin-free water (Otsuka Pharmaceutical Co., Naruto). Water flux through the membrane in the module was adjusted with a Mini-Pump (TMP 10H, Toyo Kagaku Sangyo Co., Tokyo) to 0·5 ml/min at 25 °C.

Determination of endotoxin activity

Determination of endotoxin in water samples was as described in the preceding paper (Sawada *et al.* 1986). A *Limulus* amoebocyte lysate (LAL) based kit, Pyrodick®, was purchased from Seikagaku Kogyo Co., Tokyo. An authentic LPS isolated from *E. coli* 0111:B4 (Difco Lab., Detroit, Michigan) was suspended in endotoxin-free water and treated with a sonicator (water-bath type, Nippon Rikagaku Kikai Co., Tokyo) at maximum power for 10 min at 70 °C. Endotoxin-free water was also used as a control sample. All glassware for pyrogen tests was heated at 250 °C for 2 h.

Carbocyanine dye reaction

A carbocyanine dye reaction was carried out by the method of Zey & Jackson (1973); briefly, 1·8 ml of acetate buffer (0·03 M, pH 4·0) and 0·9 ml of the dye

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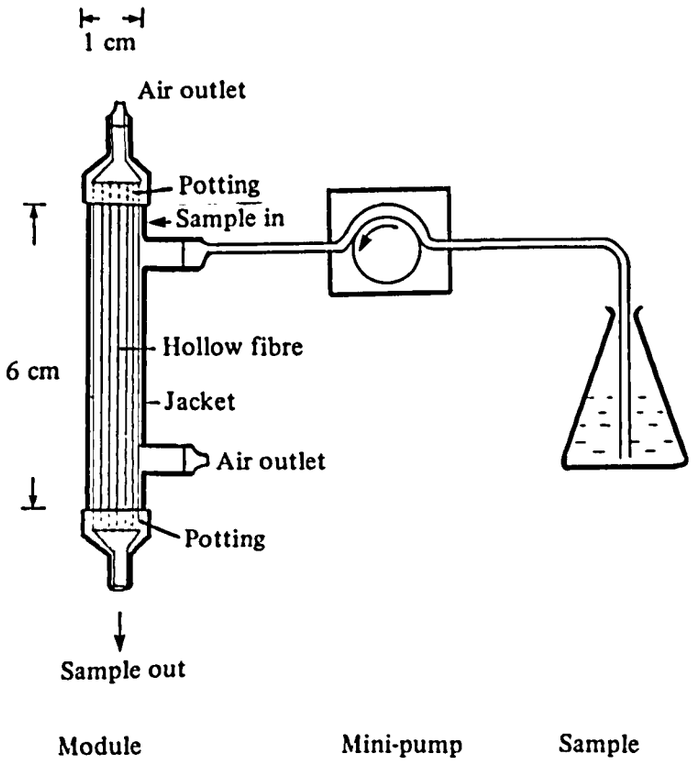

Fig. 1. A schematic diagram of filtration treatment.

reagent were added to a 0·3 ml sample, and the mixture was incubated at 30 °C for 15 min in the dark. Absorbance was determined by a spectrophotometer (Shimadzu UV-250 with attached recording unit OPI-2, Shimadzu Co., Kyoto).

Determination of the glucose concentration in LPS

The glucose concentration in LPS was estimated with anthrone (Spiro, 1966); briefly 2 ml of anthrone reagent was added to 1 ml of sample and the mixture heated at 95 °C for 15 min. The absorbance at 625 nm was measured. α-D-Glucose was used for the standard curve. LPS from *E. coli* 0113 contained 12 μg glucose/40 μg LPS.

LPS from E. coli 0113 *and sonication treatment*

LPS was extracted from a broth culture of *E. coli* 0113 by the hot-phenol method (Westphal & Lüderitz, 1954). In order to purify LPS, extraction was repeated three times and lyophilized to give a white powder. The LPS (2 mg) was suspended in 10 ml of buffer A (Tris-HCl, pH 7·1 containing 200 mM NaCl) and pulse-sonicated at 20 Kc, 20 W in a water-bath by a sonicator (Sonifier® Cell Disruptor 200, Branson Sonic Power Co., Dansbury, Conn.). At times indicated, 2 ml aliquots were removed and used for various assays.

Column chromatography of Sephadex G-75

Sephadex G-75 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) was washed extensively with endotoxin-free water, and replaced with buffer A and a column (1·5 × 20 cm) was prepared. The LPS-containing sample was loaded onto the column and eluted with buffer A. The flow rate of chromatography was adjusted to 1 ml/min. The filtrate (2 ml) was collected in each tube.

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Preparation of small sized LPS

A membrane filter (diameter 22 mm) with a nominal pore of 0·025 μm (Millipore Co., Bedford, MA) was rinsed with endotoxin-free water and set in an adaptor for syringe injection. The filtrate (approximately 1 ml) was collected and absorbance of the filtrate was determined. LPS which passed through the EHF membrane was defined as small-sized LPS in this paper. To prepare a large volume of small-sized LPS, LPS sonicated for 1 min was filtered through a membrane (0·025 μm) in a filtration unit, Lab Cassett (Millipore Co.). The sonicated LPS and the small sized LPS were used on the day without freezing.

Removal capacity of EHF membrane for small sized LPS

Small-sized LPS at an optical density of 0·21 at 214 nm, which is equivalent to 100 μg/ml, was prepared by adjusting the concentration with buffer A. The LPS solution was continuously applied onto the EHF membrane in a module at a flow rate of 0·5 ml/min at 25 °C. The filtrate (1·5 ml) was collected in each fraction.

Observation of FITC-LPS adsorbed EHF membrane

Fluorescein isothiocyanate conjugate (LPS-FITC; Skelly, Munkenbeck & Morrison, 1979) was purchased from List Biological Lab. (Campbell, CA). An LPS isolated from *E. coli* 055:B5 was used for the preparation (23·3 moles of FITC per mole of LPS, List Biological Lab. Technological Data). The FITC-LPS (0·5 mg) dissolved in 50 ml of buffer A was divided into two parts. One part, 20 ml of the solution, was applied to one module, and the other part, 30 ml, to another module. Each module was broken and the EHF membrane was taken out and left in the dark for a few days. The FITC-LPS adsorbed EHF was fixed in paraffin and sliced by a microtome to 100 μm length. The cut end of EHF was observed under fluorescence microscopy (Olympus BH with a filter of 515 nm, Olympus Co., Tokyo).

Chemicals

Carbocyanine dye and anthrone were purchased from Eastman Kodak Co. (Rochester, New York) and Ishidzu Pharmaceutical Co. (Osaka), respectively. Other chemicals were of analytical reagent trade.

RESULTS

Sonication effect on the characteristics of LPS

The LPS isolated from *E. coli* 0113 was chosen because it is well-characterized (Ribi *et al.* 1966; Niwa *et al.* 1969) and is used as the US Standard (EC-2). The LPS suspended in buffer A was sonication-dispersed. At the indicated time sonicated samples were incubated with carbocyanine dye solution. The dominant moiety of LPS which reacts with the dye was lipid A (Ogawa & Kanoh, 1984). Accordingly, the absorption maximum of the LPS (0113) and dye conjugate was set at 470 nm. The absorbance at 470 nm increased during the first 30 sec of sonication and then levelled off to a slight increase. This indicates that the LPS molecules were fairly dispersed in the first 30 sec of sonication. Endotoxin activity was determined by the Synthetic Chromogenic Substrate method (Iwanaga *et al.*

Table 1. *Reactivities of LPS with carbocyanine and LAL following sonication*

Sample	Sonication period (min)	Carbocyanine dye reaction* (470 nm)	Endotoxin activity†		Absorbance at 214 nm	
			unfiltered (mg/ml)	filtered‡ (μg/ml)	unfiltered	filtered
Blank control	0	0·076	0·00	0·00	0·00	0·00
LPS	0	0·168	0·15	0·16	1·28	0·15
LPS	0·5	0·631	0·61	4·3	0·51	0·26
LPS	1	0·653	0·73	5·2	0·51	0·32
LPS	2	0·660	0·84	5·2	0·46	0·35
LPS	5	0·656	0·91	5·5	0·46	0·38

* A 100 μl aliquot (20 μg LPS) is used for the assay.

† Concentration of LPS was 0·2 mg/ml. For assay, the sample was diluted with endotoxin-free water. Data are a mean of duplicate determinations.

‡ Sample was filtered by a membrane with a nominal pore size of 0·025 μm.

1978; Harada-Suzuki *et al.* 1982) of LAL. This method has an advantage of quantitative determination with a fairly good accuracy and reproducibility (Usami & Shimohira, 1981; Fujita & Nakahara, 1982). Endotoxin activity resulted in an increase after 30 sec of sonication and a gradual increase by further treatment (Table 1). The endotoxin activity of the sonicated LPS filtered through a membrane with a nominal pore size of 0·025 μm was 6–7 % of the unfiltered LPS. Absorption at 214 nm in the filtrate increased with prolonged periods of sonication, while absorbance at 214 nm, where almost all organic compounds have an absorbance in aqueous solution, decreased gradually following sonication in unfiltered material. This suggests that the LPS suspension was dispersed.

Gel-filtration of sonicated LPS and size estimation

In order to estimate the molecular size of the sonicated LPS, Kanoh, Kohlage & Siegert (1968) used a pyrogen-free column of Bio-Gel P-100. Similarly gel-filtration through a column of Sephadex G-75 was carried out in this paper. The elution profile was followed by reading the absorbance at 214 nm. Recovery of LPS from the column was over 85 in absorption base. Two ml of LPS dispersed for 1 min showed a major peak, Peak I, and a minor peak, Peak II (Fig. 2). A 1·5 ml aliquot of each fraction was next filtered by a filter membrane with a nominal pore size of 0·025 μm. Two peaks of absorbance in the filtrate were obtained as indicated by bars. Each of the peak fractions were combined and endotoxin activity was determined. As shown in Table 2, the Peak I filtrate had a poor activity in the LPS test, and Peak II had much less activity than the Peak I filtrate in absorption base. Endotoxin in Peak I had the highest activity. Since Peak II seemed to contain something like a by-product of LPS, e.g. an oxidized product (Ogawa & Kanoh, 1986), endotoxin in the Peak I filtrate was used in this study as a small-sized LPS.

Determination of the specific activity of small-sized LPS

The LPS concentration of the Peak I filtrate was determined from the glucose concentration by an anthrone reaction. Intact LPS contained 12 μg of glucose in 40 μg LPS (30 % glucose/LPS). The Peak I filtrate at A 214 nm: 0·1 contained 14·25 μg glucose/ml. Therefore, Peak I filtrate at A 214 nm:0·1 contains 47·5 μg LPS/ml. From the endotoxin activity in Table 2, the specific endotoxin activity of the Peak I filtrate was obtained, being 73·7 ng activity/μg LPS.

Removing capacity of EHF membrane for small-sized LPS

Small-sized LPS in the Peak I filtrate was passed through the EHF membrane in a module as shown in Fig. 1 and the LPS concentration in the filtrate was measured (Fig. 3). From an elution profile indicated by oblique lines, the amount of small-sized LPS adsorbed on the EHF membrane was estimated, resulting in 1·65 mg/116 mg fibre/3·68 m^2/module.

To confirm that molecular size distribution of LPS in the filtrate is the same size as the small sized LPS, a filtrate fraction number 10 in Fig. 3, 2 ml aliquot, was analysed on a column of Sephadex G-75. The retention fraction of LPS in the fraction number 10 appeared in fraction 13 (Fig. 4). Peak I filtrate was also analysed. Although small-sized LPS was prepared from Peak I fraction (a main

Table 2. *Correlation between LPS concentration and the endotoxin activity*

Peak fraction	Endotoxin concentration (μg/ml/A214 nm:0·1)
I	1150·0
II	0·4
I Filtrate	3·5

Calculation based on A214 nm:0·1.

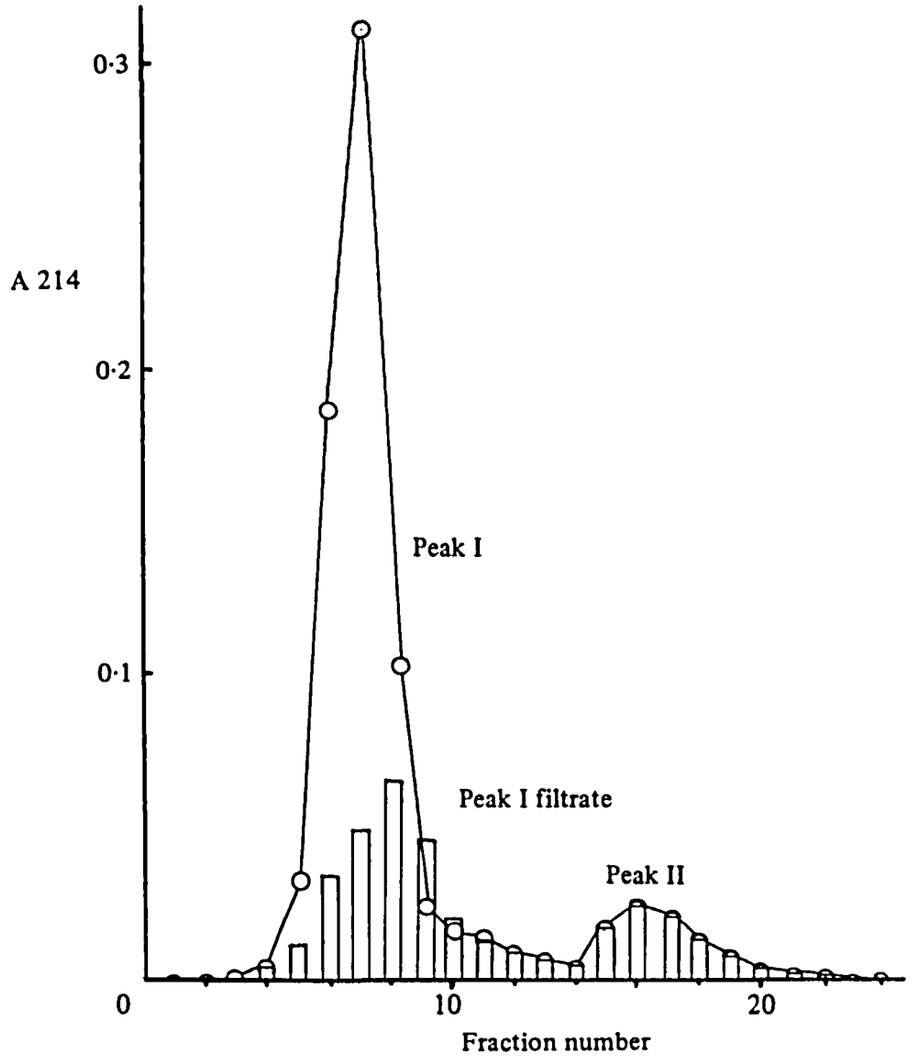

Fig. 2. Chromatographic profile of LPS sonicated on a column of Sephadex G-75 and filtration profile with a membrane. -O-, Sonicated for 1 min; ▭, filtered with a membrane.

peak fraction 8) in Fig. 2, it was eluted in fraction number 13. Recovery of the small-sized LPS through the gel filtration was approximately 85% in absorption base. Specific endotoxin activity of the fractions 12–14 (Fig. 4) obtained from the filtrate 10 (Fig. 3) was 3·0 μg/ A 214 nm:0·1/ml.

Recovery of small-sized LPS adsorbed on the EHF membrane

To confirm whether adsorbed small-sized LPS can be released, LPS adsorbed EHF membrane was rinsed with 20 ml of 70 (v/v)% ethanol and 2 ml aliquots were analysed on a column of Sephadex G-75. From peak intensity, an approxi-

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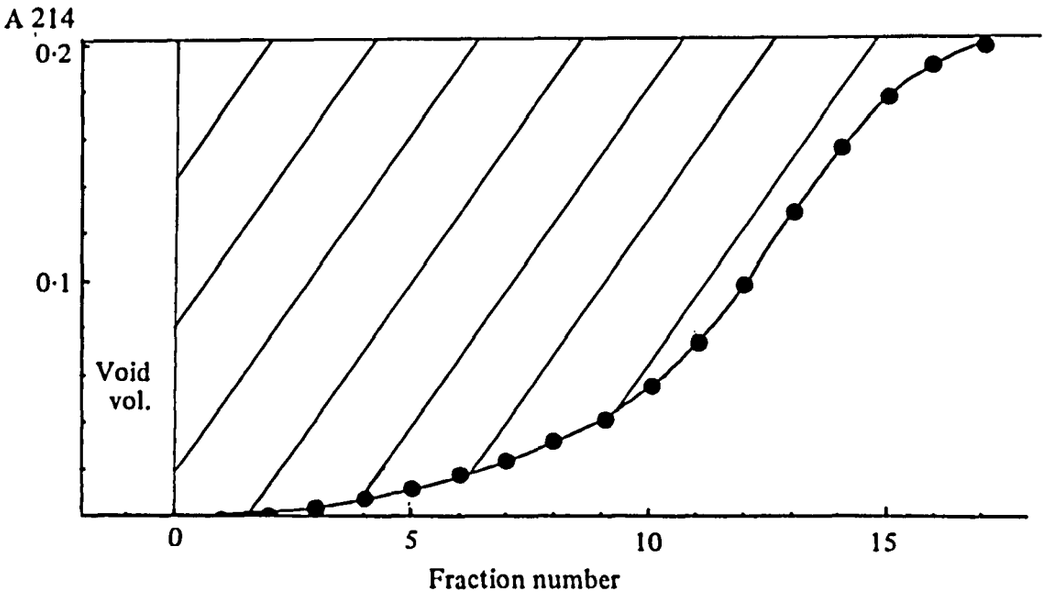

Fig. 3. Removing capacity of EHF membrane for small-sized LPS.

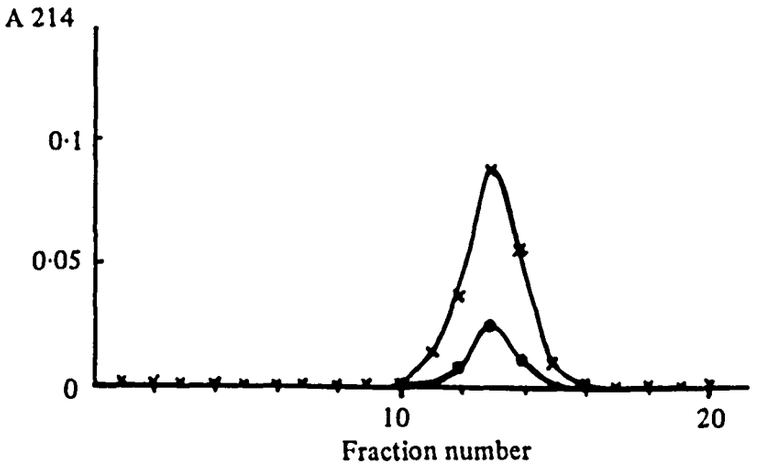

Fig. 4. Chromatographic pattern of the Peak I filtrate and the peak fraction 10 in Fig. 2. -×-, Filtrate with a membrane; -●-, Fraction 10 in Fig. 2.

mately 92 % recovery of LPS, identical with small-sized LPS, from EHF membrane was obtained (data not shown).

Adsorption mode of the EHF membrane for FITC-LPS

The fluorescein conjugated LPS was passed through the EHF membrane and the adsorption mode of FITC-LPS was observed under a fluorescence microscopy. The membrane texture was stained all over with fluorescein. The intensity of fluorescein seemed to depend on the concentration of FITC-LPS loaded (Fig. 5).

Formation of large-sized LPS from the small-sized LPS

To see whether the small-sized LPS forms large-sized LPS under certain conditions, a solution of the small-sized LPS was left at 4 °C for 7 days. The solution was then analysed on a column of Sephadex G-75. Recovery of LPS from the column was 93 % in absorption base at 214 nm. Approximately 7 % of total absorption eluted was in fraction numbers 7–9. A specific endotoxin activity of the large-sized LPS was 1063 μg/ml/A214 nm:0·1.

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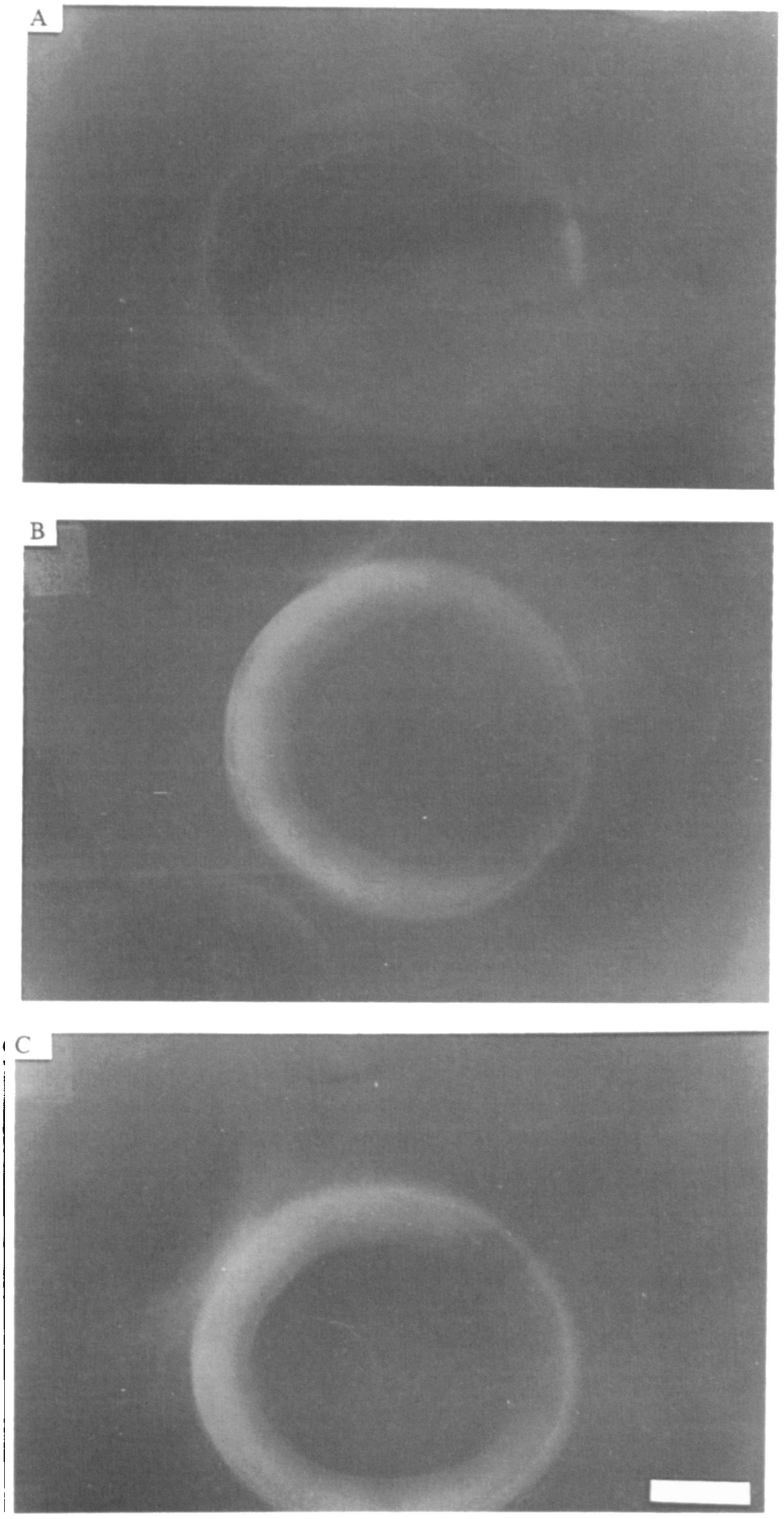

Fig. 5. Microscopic feature of FITC-LPS adsorbed EHF membrane. A, untreated control; B, FITC-LPS (20 ml) loaded; C, FITC-LPS (30 mL) loaded. A bar indicates 100 μm.

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DISCUSSION

The solubility or dispersion degree of LPS in an aqueous solution is directly responsible for the difference of endotoxicity. That is, LPS molecules with a small size have less reactivity to LAL test and less pyrogenicity in rabbits (Marx *et al.* 1968; Sweadner, Forte & Nelsen, 1977; Kanoh & Kawasaki, 1980). Likewise, small sized LPS prepared from *E. coli* 0113 by sonication dispersion and membrane filtration (0·025 μm in pore size) showed very poor reactivity to the LAL test (Table 2). Kanoh also observed that, following either sonication treatment or Triton X-100 treatment, LPS exists as small-sized LPS with a range of 0·01–0·02 μm as determined by electron microscopy by staining with uranyl acetate as well as sucrose gradient centrifugation (unpublished data). The small-sized LPS prepared seemed to have a similar size as observed above. The small-sized LPS may, therefore, be the short rodlets, not long string and spherical forms. A problem of small-sized LPS is that it forms large-sized LPS under various conditions which give high pyrogenicity. Indeed, we observed the formation of large-sized LPS from the small-sized LPS at a low temperature; this was determined by specific endotoxin activity of LAL test for LPS eluted from a column of Sephadex G-75. This observation is consistent with the report that deoxycholate-treated LPS could be reaggregated to the large LPS following dialysis against deoxycholate-free water (Ribi *et al.* 1966).

The EHF 390C membrane was found to have a maximum pore size of 0·04 μm as determined by several kinds of spherical, uniform particles and various viruses (Sawada, unpublished data). If endotoxin molecules are of larger size than this pore size, it would be removed by a filtration mechanism. The small-sized LPS was initially removed from the filtrates and was leaked out gradually in successive feedings (Fig. 3). This result clearly indicates that small-sized LPS was adsorbed and leaked out because of the reduction of binding sites on the EHF membrane. The adsorption mode of LPS was visualized by staining the EHF membrane with fluorescein. Not only the membrane surface, but also the entire depth of the membrane texture had been stained (Fig. 5).

It is well known that an ultrafiltration (below 0·02 μm, Porter, 1975) technique can provide endotoxin-free water (Sweadner, Forte, & Nelsen, 1977; Cradock *et al.* 1978). Because current ultrafiltration membranes have no specificity to bind endotoxin, contaminating particles are also removed from water. Therefore, the membrane soon faces fouling. EHF membrane has a relatively high water flux because of microfiltration in pore range (Porter, 1975). EHF membrane can simply be rinsed with organic solvents such as ethanol or acetone to simultaneously rejuvenate and sterilize the membrane. It was also found that removal efficiency of endotoxin from water at a high temperature, e.g. 70 °C, was greater than that of low temperature (data not shown). This result is in good agreement with our preceding observation (Sawada *et al.* 1986). Thus, EHF membrane is unique medium for water purification and is shown to have the capacity of adsorbing a kind of LPS in water by the filtration process.

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