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Immunobiological Activities of a Porin Fraction Isolated from *Fusobacterium nucleatum* ATCC 10953

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From *Fusobacterium nucleatum* ATCC 10953 cell envelope fraction whose inner membranes had been removed by treatment with sodium *N*-lauroyl sarcosinate, an outer membrane protein (37,000 M_r in a native state) was prepared by extraction with lithium dodecyl sulfate. The protein thus obtained showed distinct porin activity, namely, the ability to form hydrophilic diffusion pores by incorporation into the artificial liposome membrane. The porin fraction exhibited strong immunobiological activities in the in vitro assays: B-cell mitogenicity and polyclonal B-cell activation on murine splenocytes, stimulatory effects on guinea pig peritoneal macrophages, and enhancement of the migration of human blood monocytes. The porin fraction also exhibited immunoadjuvant activity to increase the antibody production against sheep erythrocytes in the spleen of mice that were immunized by sheep erythrocytes with porin. Although chemical analyses revealed that the test porin fraction contained a considerable amount of lipopolysaccharide (LPS) (around 12% of the fraction), the studies with LPS-nonresponding C3H/HeJ mice and on the inhibitory effects of polymyxin B strongly suggest that most of the above bioactivities are due to porin protein itself, not to coexistent LPS in the porin fraction.

A variety of anaerobic gram-negative rods have been isolated at high rates from the periodontal pockets of patients with periodontal diseases. *Fusobacterium nucleatum* is predominant among these periodontitis-associated bacteria (26). The view that endotoxic lipopolysaccharides (LPS) and cell wall peptidoglycans of periodontitis-associated bacteria play an important role in the pathogenesis of periodontal diseases is generally accepted on the basis of experimental and clinical studies (6, 10, 30).

Evidence has accumulated that outer membrane proteins, which are cell surface components of various gram-negative bacteria, like LPS and peptidoglycans, exhibit powerful immunobiological activities, many of which are common to those of LPS and peptidoglycans (4, 20, 27, 32). This strongly suggests that the outer membrane proteins of periodontitis-associated gram-negative bacteria are involved in the pathological processes of periodontal diseases. Nevertheless, the bioactivities of the outer membrane of periodontitis-associated bacteria have not been studied, except by us (12, 21). Although we found that the sodium dodecyl sulfate (SDS) extract from the cell envelope of several oral gram-negative bacteria, including *F. nucleatum*, exhibited marked immunostimulatory activity, we did not determine the chemical entity responsible for the observed bioactivity. In the present study, we isolated porin, one of the major outer membrane proteins, in almost homogeneous (but not pure) form from *F. nucleatum* and examined the bioactivities of the protein in in vivo and in vitro assays, paying special attention to exclude the possible involvement of coexisting LPS in the observed bioactivities.

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MATERIALS AND METHODS

Bacterial strain and cultivating conditions. *F. nucleatum* ATCC 10953 maintained in a blood agar plate was inoculated in GAM broth (Nissui Seiyaku Co., Tokyo, Japan) and grown at 37°C for 3 days in an N_2-H_2-CO_2 (85:10:5) atmosphere in an anaerobic culture system (model 1024; Forma Scientific Inc., Marietta, Ohio). This culture was transferred to 10 times the volume of GAM broth and cultivated for an additional 3 days in the gaseous environment described above. Cells were harvested by centrifugation at 8,000 × *g* for 30 min and washed twice with 50 mM phosphate-buffered saline (PBS; pH 7.2) and once with distilled water to lyophilize them.

Preparation of cell envelope fraction. The lyophilized cells (5.6 g [dry weight unless otherwise specified]) were suspended in 80 ml of 50 mM PBS (pH 7.2) supplemented with 1 mg of DNase I (bovine pancreas; Sigma Chemical Co., St. Louis, Mo.) and 1 mg of RNase A (type I-AS from bovine pancreas; Sigma). The cell suspension was disrupted with an ultrasonicator (Insonator, model 200 M; Kubota, Tokyo) at 4°C with an output voltage of 180 to 200 W for 20 min. After undisrupted cells were removed by low-speed centrifugation (1,000 × *g*) for 10 min, the supernatant containing broken cells was centrifuged at 100,000 × *g* for 90 min to recover the cell envelope fraction as a sediment. The cell envelope fraction was washed by centrifugation five times with 50 mM PBS (pH 7.2) and twice with distilled water. Thus, 900 mg of the cell envelope was obtained at a yield of 16%.

Preparation of porin fraction. The cell envelope fraction (200 mg) was suspended in 20 ml of 0.5% sodium lauryl sarkosinate (SLS; Sigma) aqueous solution, and the suspension was gently stirred at 25°C for 30 min to solubilize the inner membrane by the method of Filip et al. (8). The SLS-treated envelope fraction was centrifuged at 100,000 ×

g for 90 min, and the sediment was suspended in 20 ml of 2% lithium dodecyl sulfate (LDS; BDH Chemicals Ltd., Poole, England) dissolved in 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Sigma)–lithium hydroxide (HEPES-LiOH) buffer. After gentle stirring at 4°C for 10 min, the suspension was centrifuged at 100,000 × *g* for 90 min. The supernatant, which contained outer membrane proteins solubilized by LDS extraction of the SLS-treated cell envelope, was applied on a Sephacryl S-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) column (1.6 by 100 cm) that had been equilibrated with 20 mM HEPES-LiOH buffer containing 1% LDS and 0.5 M LiCl. The same buffer was used for elution of the applied materials. The eluate was monitored by determination of the absorbance at 280 nm (OD_{280}). Three peak fractions (A, B, and C in Fig. 1) were separately pooled. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Lugtenberg et al. (18). The samples were heated with SDS at 100°C for 5 min unless otherwise specified. Fraction A, giving practically a single 41,000-M_r (41K) protein band, was thoroughly dialyzed against distilled water and concentrated with a rotary evaporator to 10 ml. The concentrate was mixed with 2 volumes of cold acetone, and the precipitated protein was suspended in 10 ml of distilled water. The acetone precipitation procedures were repeated (three times in total) to remove LDS as completely as possible. The final preparation was lyophilized. The yield was 29 mg, 14.5% of the cell envelope fraction.

Preparation of LPS and peptidoglycan. LPS was prepared by extraction of lyophilized whole cells (2.0 g) with a hot phenol-water mixture, by the modified Westphal method described by Kim and Watson (14), at a yield of 20%. A peptidoglycan specimen, on the other hand, was prepared by boiling the LDS-insoluble fraction (200 mg), the residue after extraction of the SLS-treated cell envelope, with 2% LDS at 4°C as described in the preceding section, in 10 ml of HEPES-LiOH containing 4% LDS for 1 h. The insoluble residue was recovered by centrifugation at 100,000 × *g* for 90 min and washed five times with distilled water by centrifugation to obtain the peptidoglycan fraction, at a yield of 40% of the cell envelope (80 mg). These LPS and peptidoglycan preparations obtained from the same *F. nucleatum* whole cells for the outer membrane protein were used as references throughout the experiments.

Assay for porin activity. Porin activity was measured by the liposome-swelling method described by Luckey and Nikaido (17) with minor modifications (23). Briefly, egg phosphatidylcholine (2.6 μmol) and dicetylphosphate (0.1 μmol) were dried as a film at the bottom of a test tube, and an indicated amount of the test fraction was added. Liposomes were made by the standard precedure (23), i.e., by drying the sonicated test material-phospholipid mixture under reduced pressure and suspending the mixture in 0.6 ml of 15% dextran T-40 (Pharmacia) in 5 mM Tris hydrochloride buffer (pH 7.4). Portions of these liposomes (15 μl) were added to 0.7 ml of a 30 mM solution of arabinose in 5 mM Tris hydrochloride buffer (pH 7.4), in a semimicrocuvette, the contents were rapidly mixed, and the OD_{400} was recorded. The rate of penetration of arabinose was determined by the initial rate of swelling of the liposome membranes added with the isotonic solution of the sugar. In a control experiment, a porin fraction which had been denatured by heating in 1% SDS at 100°C for 10 min and precipitated with acetone to remove SDS was used for the liposome-swelling assay.

Detection of constituents characteristic of LPS and peptidoglycan. The content of amino acids and amino sugars was determined with an automatic amino acid analyzer (model 835 equipped with a no. 2617 cation-exchange resin; Hitachi, Tokyo) on specimens hydrolyzed in 6 N HCl at 100°C for 14 h in a sealed tube. The analytical conditions were set up to detect muramic acid and lanthionine, the characteristic constituents of *F. nucleatum* ATCC 10953 peptidoglycans, as sensitively as possible. Fatty acid determination was done by the method of Ikemoto et al. (11). Special attention was paid to detect 3-hydroxy fatty acids, in particular 3-hydroxymyristic acid, a characteristic fatty acid of the lipid A portion of LPS of the test organisms, and to estimate LPS in the test outer membrane protein fraction.

Immunobiological activities. (i) Mitogenicity. Splenocytes from athymic BALB/c nude (8 weeks old, male; Clea Japan, Osaka), C3H/HeN (12 weeks old, male; Charles River Japan, Osaka), and C3H/HeJ, an LPS nonresponder line (12 weeks old, male; Jackson Laboratories, Bar Harbor, Maine) mice were used for the assay. The splenocytes (5×10^5 cells per 200 μl per well) were cultured in triplicate with test materials (none in the control) for 48 h in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS; Flow Laboratories, North Ryde, Australia) under 5% CO_2 in air. During the final 24 h, 0.5 μCi of [^{3}H]thymidine (Amersham, Buckinghamshire, England) per well was added, and its incorporation by splenocytes was measured by the conventional liquid scintillation method. In some experiments, the test specimens pretreated with polymyxin B (Sigma) were also examined for their mitogenicity in murine splenocytes to exclude the influences of LPS coexisting in the test specimen.

(ii) PBA. Splenocytes (10^7 cells per 2 ml per well) from BALB/c (8 weeks old, male; Charles River Japan), C3H/HeN, or C3H/HeJ mice (described above) were cultured with or without test materials for 72 h in 10% FCS-RPMI 1640 medium under 5% CO_2 in air, in triplicate for each culture. The number of hemolytic plaque-forming cells (PFC) against 2,4,6-trinitrophenylated (TNP) sheep erythrocytes (SRBC) was determined in a Cunningham chamber (5). The polyclonal B-cell activation (PBA) ability of test materials was expressed as the ratio of PFC count in a test culture to that in the respective control culture, i.e., as a stimulation index. The influence of the addition of polymyxin B to test materials was also examined in this assay.

(iii) Adjuvant activity. Groups of BALB/c mice (five per group) or C3H/HeJ mice (seven per group) were immunized by intraperitoneal injection of 2×10^7 SRBC in 1.0 ml of PBS added with 50 μg of test materials (none in the control). One week later, the number of PFC against SRBC per 10^6 splenocytes and per spleen was determined by direct and indirect methods.

(iv) Stimulatory effects on guinea pig peritoneal macrophages. We measured inhibition of thymidine uptake, enhancement of glucosamine incorporation, and stimulation of superoxide anion (O_2^-) generation by peritoneal macrophages treated with test materials as described previously (16). In brief, oil-induced macrophages obtained from the peritoneal wash of a Hartley albino guinea pig (female, weighing ca. 500 g; Nihon Rabbit Kyoukai, Osaka, Japan) that had received an intraperitoneal injection of 20 ml of sterile liquid paraffin (Wako Pure Chemical Ltd., Osaka, Japan) 4 days before, were cultured with test specimens (none in the control) for 48 h in 10% FCS-RPMI 1640 medium at a density of 5×10^5 cells per 0.5 ml per well on a 24-well microculture plate (Falcon 3047). For the assay of thymidine and glucosamine incorporation, the cells were pulsed with 0.2 μCi of [^{3}H]thymidine and 0.1 μCi of [^{14}C]glucosamine hydrochloride during the final 24 h and 6 h

of the culture, respectively. After completion of the culture, the radioactivity in the trichloroacetic acid-insoluble fraction of each culture was measured by the liquid scintillation method. For the assay of superoxide anion generation, the peritoneal macrophages cultured with test specimens for 48 h, as described above, were incubated with 1 ml of 50 μM cytochrome *c* (from horse heart, type VI; Sigma) in HEPES buffer at 37°C for 10 min. Wheat germ agglutinin (4 mg/ml; Sigma) and cytochalasin E (500 μg/ml; Sigma) were added (10 μl of each), and the culture was incubated at 37°C for 15 min. The rate of reduction of cytochrome *c* in the reaction mixture was measured by recording the $OD_{550-540}$ with a double-beam spectrophotometer (model 200-20; Hitachi) and expressed as nanomoles per minute per 10^6 cells (molar absorption coefficient, 19.1×10^3). The possible inhibitory effect of polymyxin B on enhancement of superoxide anion generation by the porin fraction was examined to check the involvement of coexistent LPS in stimulation of macrophages.

(v) Migration of human peripheral blood monocytes. The assay for enhanced migration of monocytes by test materials was performed with a multiwell chemotaxis assembly (Neuro Probe, Cabin John, Md.) as described previously (24). The number of monocytes that migrated to the surface of the membrane sheet adjacent to the lower well containing an added test specimen was determined in triplicate for each experimental point. The migration-enhancing effect was expressed as the ratio of the number of monocytes in a test to that in the respective control. A fresh human serum sample (diluted 1:2) whose complement cascade was activated by incubation with LPS from *Salmonella enteritidis* (Difco Laboratories) and 10^{-8} M *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (Sigma) were used as positive controls.

Statistical analysis. The statistical significance of differences between each test and its respective control was examined by Student's *t* test.

RESULTS

Isolation of outer membrane protein. Figure 1 illustrates the elution profile obtained with an LDS extract of the SLS-treated cell envelope by gel filtration on a Sephacryl S-200 column. The SLS-insoluble but LDS-soluble fraction of the envelope gave one major peak fraction, which was followed by two minor peak fractions. Analysis by SDS-PAGE showed that the major peak fraction was practically homogeneous and consisted of a protein with an apparent molecular weight of 41,000, while the parent material gave additional minor bands (Fig. 2). In addition, the protein migrated at the position of 37,000 M_r without heat treatment prior to electrophoresis (Fig. 3, lane N).

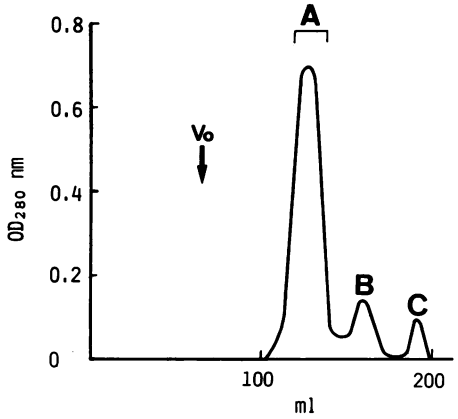

FIG. 1. Elution profile of *F. nucleatum* outer membrane proteins on Sephacryl S-200 column. An LDS extract of the SLS-treated cell envelope was applied to a column (1.6 by 100 cm) equilibrated with 20 mM HEPES-LiOH, pH 8.0, added with 0.5% LDS and 0.5 M LiCl. Elution was made with the same buffer. The OD_{280} was continuously recorded. Each fraction was subjected to analysis by SDS-PAGE. The main peak fraction (A) was pooled as indicated by the bracket. V_0, Void volume.

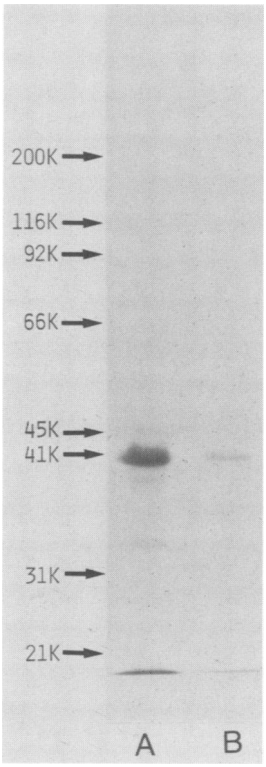

FIG. 2. SDS-PAGE patterns of the parent LDS extracts (lane A) and the major peak fraction (lane B). Samples were treated at 100°C for 10 min in sample buffer (1% SDS, 0.125 M Tris, 25% glycerol, and 3% 2-mercaptoethanol) before electrophoresis. Amounts applied were 60 μg (lane A) and 15 μg (lane B).

Porin activity of the major outer membrane protein. The porin activity of the isolated major outer membrane protein was examined in terms of its ability to form hydrophilic diffusion pores by incorporation into an artificial liposome membrane. Liposomes containing the unheated 37K protein (Fig. 3, lane N) exhibited a remarkable increase in the initial rate of swelling due to the penetration of arabinose into liposomes, while the liposomes containing the denatured 41K protein (Fig. 3, lane D) heated at 100°C for 10 min in a 1% SDS solution was scarcely active (Fig. 4). The purified 37K outer membrane protein having the porin activity will be referred to as the porin fraction hereafter.

Presence or absence of LPS and peptidoglycan in the porin fraction. Amino acid and amino sugar analyses revealed that the porin fraction did not contain any detectable amounts of lanthionine or muramic acid, characteristic components of the peptidoglycans of the test organism (13, 28) (data not shown).

Table 1 shows the fatty acid composition of the porin fraction and the reference LPS specimen. The finding to be noted is that the test porin fraction contained 2.95 μg of

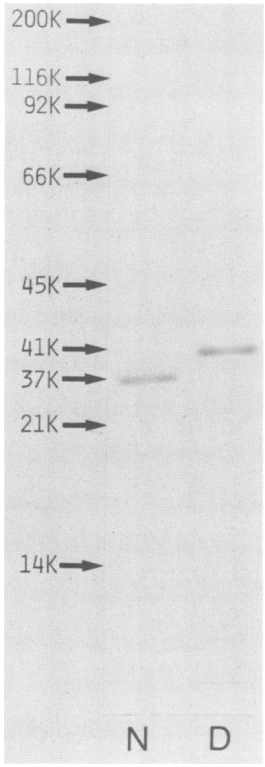

FIG. 3. Heat modifiability of the porin protein of *F. nucleatum*. The test fraction was heated at 100°C for 10 min in 1% SDS solution (lane D) or left unheated (lane N) prior to SDS-PAGE. The amount applied was 20 μg (dry weight) per lane.

3-hydroxymyristic acid (3-$OHC_{14:0}$) per mg, which corresponds to 12.4% of the amount of the reference LPS (23.8 μg/mg). This finding indicates that the porin fraction contained a chemically detectable amount (12.4%) of LPS. Three other 3-hydroxy fatty acids (3-$OHC_{8:0}$, 3-$OHC_{10:0}$, and 3-$OHC_{12:0}$) found in small amounts in the porin fraction,

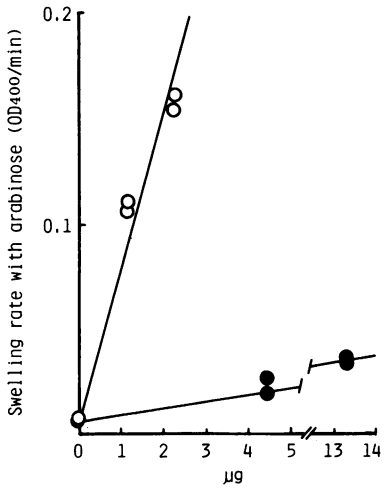

FIG. 4. Swelling rates of the artificial liposome membrane in which various amounts of outer membrane protein were incorporated. Symbols: ○, nontreated; ●, denatured by heating in 1% SDS aqueous solution at 100°C for 10 min, followed by acetone precipitation to remove SDS for the swelling assay.

but practically undetectable in the reference LPS, might be degradation products of LDS or SLS retained by the porin protein even after purification. Myristic ($C_{14:0}$), palmitic ($C_{16:0}$), and oleic ($C_{18:1}$) acids, the latter two of which are minor components of the reference LPS, were the major fatty acids found in the porin fraction.

Immunobiological activities of the porin fraction. (i) Mitogenicity. The porin fraction exhibited powerful mitogenic activity on the splenocytes of nude mice, which did not respond to phytohemagglutinin, and the activity was stronger than that of the reference LPS on a weight basis (Fig. 5). A remarkable finding was that the test porin fraction activated not only the splenocytes of LPS-responding mice (C3H/HeN mice) but also those of LPS-nonresponding mice (C3H/HeJ mice) under assay conditions in which the reference LPS was mitogenic on the splenocytes of the former but not on those of the latter strain and phytohemagglutinin powerfully stimulated the splenocytes of both strains. The possibility that the observed mitogenicity of the porin fraction on the splenocytes of BALB/c *nu/nu* and C3H/HeN mice was due to coexistent LPS was further excluded by examination of the inhibitory effects of polymyxin B (Fig. 6). Polymyxin B scarcely inhibited the mitogenicity of the porin fraction on the splenocytes of BALB/c *nu/nu*, C3H/HeN, and C3H/HeJ mice, whereas the mitogenicity of the reference LPS on the splenocytes of the first two mouse strains was completely inhibited by the addition of polymyxin B at a weight ratio higher than 1:1.

(ii) PBA activity. The porin fraction markedly increased the number of hemolytic PFC against TNP-SRBC in the spleens from BALB/c, C3H/HeN, and C3H/HeJ mice, while the reference LPS was strongly active on the splenocytes from BALB/c and C3H/HeN mice, but scarcely active on those from C3H/HeJ mice (Fig. 7). The PBA activity of the porin fraction was not decreased by the addition of polymyxin B, unlike that of the reference LPS (Table 2).

(iii) Antigen-specific immunoadjuvant activity. The immunoadjuvant activity of the porin fraction was compared with that of the reference LPS by examination of the increase in direct and indirect hemolytic PFC in the spleens of BALB/c mice which were immunized with SRBC plus the test specimen. The porin fraction caused a significant increase in direct and indirect PFC counts against SRBC both per whole spleen and per 10^6 splenocytes, as did the reference LPS (Table 3). A significant increase in total spleen cells was

TABLE 1. Fatty acid composition of the porin and LPS fractions of *F. nucleatum*[a]

Fatty acid	Porin, μg/mg (%)	LPS, μg/mg (%)
$C_{12:0}$	ND[b]	0.10 (0.18)
$C_{14:0}$	4.79 (31.92)	30.33 (52.29)
$C_{15:0}$	0.06 (0.39)	0.26 (0.44)
$C_{16:0}$	2.33 (15.55)	1.38 (2.38)
$C_{16:1}$	0.57 (3.79)	0.26 (0.44)
$C_{18:0}$	0.31 (2.09)	0.43 (0.74)
$C_{18:1}$	2.08 (13.86)	0.02 (0.04)
3-OH $C_{8:0}$	0.27 (1.82)	0.04 (0.07)
3-OH $C_{10:0}$	0.87 (5.82)	ND
3-OH $C_{12:0}$	0.44 (2.96)	ND
3-OH $C_{14:0}$	2.95 (19.69)	23.82 (41.07)
Unidentified	0.32 (2.13)	0.61 (1.06)
Total	15.0 (100)	58.0 (100)

[a] Data are expressed per milligram of test specimen and as a percentage of total fatty acids (in parentheses).
[b] ND, Not detected.

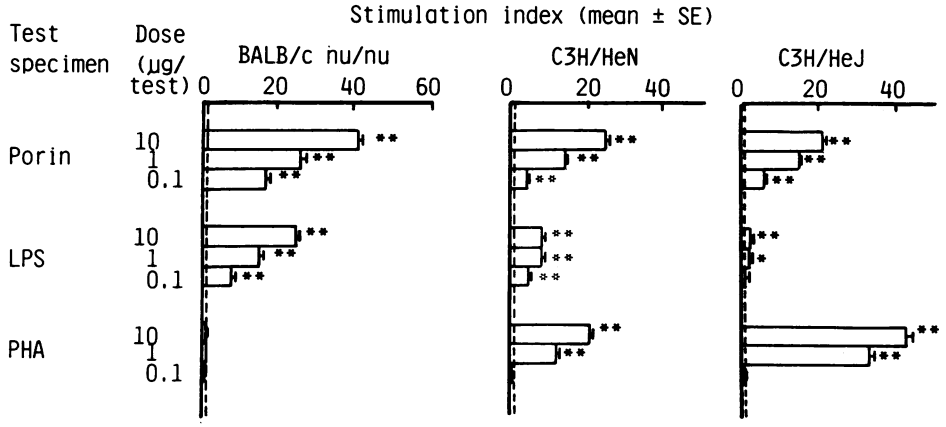

FIG. 5. Mitogenic effect of the porin and LPS fractions of *F. nucleatum* on splenocytes from athymic BALB/c *nu/nu*, C3H/HeN (LPS responder), and C3H/HeJ (LPS nonresponder) mice. The cells (5×10^5) were cultured with the indicated doses of test materials (none in the control cultures) for 2 days in quadruplicate. The counts in control cultures were as follows: BALB/c *nu/nu*, 3,156 ± 189 dpm; C3H/HeN, 3,671 ± 110 dpm; C3H/HeJ, 2,395 ± 96 dpm. The stimulating activity of a test specimen was expressed as the ratio of test to control values (a stimulation index) and examined for statistical significance by Student's *t* test (*, $P < 0.05$; **, $P < 0.01$ versus controls). PHA, Phytohemagglutinin (Wellcome Research Laboratories, Beckenham, England); SE, standard error.

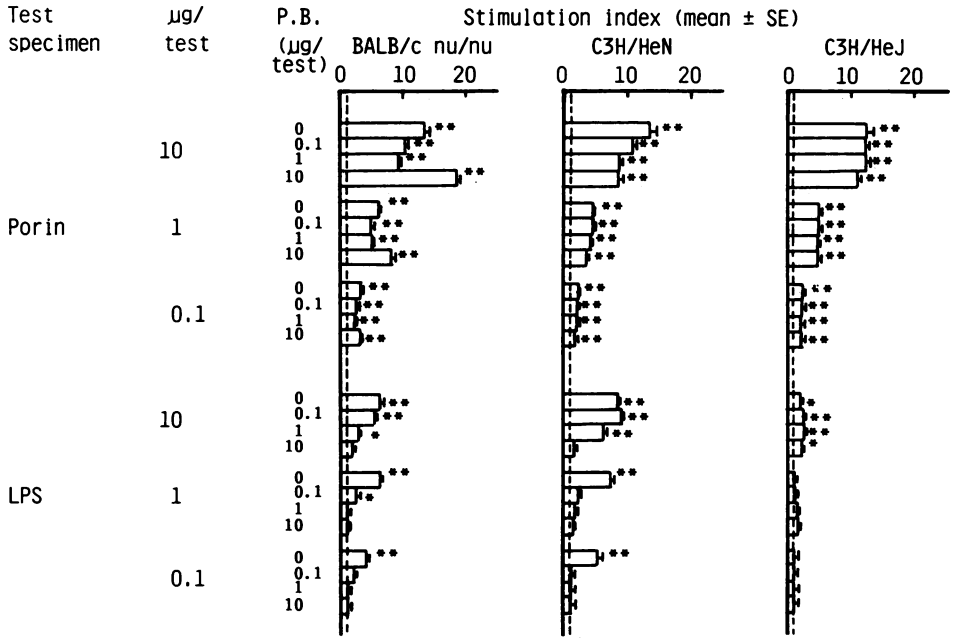

FIG. 6. Effect of polymyxin B (P. B.) on the mitogenic activities of the porin and LPS fractions of *F. nucleatum* on the splenocytes of athymic BALB/c *nu/nu*, C3H/HeN, and C3H/HeJ mice. The counts in control cultures were as follows. No polymyxin B control: BALB/c *nu/nu*, 3,190 ± 177 dpm; C3H/HeN, 3,741 ± 139 dpm; C3H/HeJ, 2,480 ± 212 dpm. Added polymyxin B (1 μg per test) control: BALB/c *nu/nu*, 2,153 ± 158 dpm; C3H/HeN, 3,080 ± 138 dpm; C3H/HeJ, 2,055 ± 112 dpm. Added polymyxin B (10 μg per test) control: BALB/c *nu/nu*, 1,655 ± 27 dpm; C3H/HeN, 2,844 ± 72 dpm; C3H/HeJ, 1,591 ± 101 dpm. *, $P < 0.05$; **, $P < 0.01$ versus untreated controls.

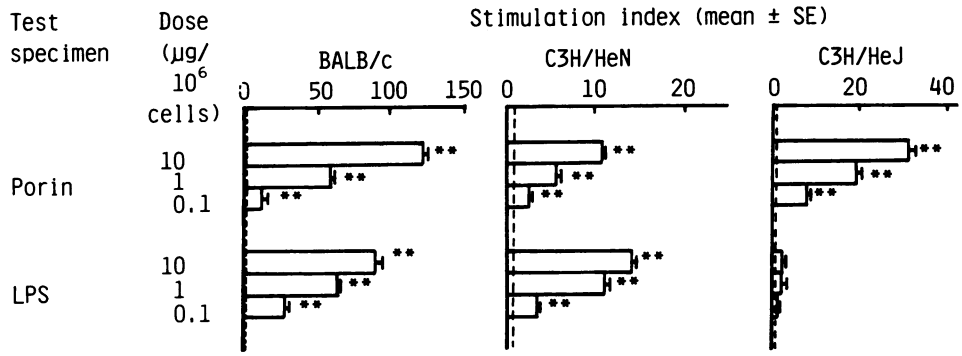

FIG. 7. PBA by the porin and LPS fractions of *F. nucleatum* on splenocytes from BALB/c, C3H/HeN, and C3H/HeJ mice. The cells (6×10^6) were cultured with the indicated doses of test specimens (none in the control) for 3 days in triplicate, and the number of direct PFC was determined. The PFC counts in control assays were: BALB/c, 3.9 ± 0.4; C3H/HeN, 8.5 ± 0.6; C3H/HeJ, 1.9 ± 0.4. *, $P < 0.05$; **, $P < 0.01$ versus controls.

TABLE 2. Effect of polymyxin B on PBA of BALB/c mouse splenocytes by the porin and LPS fractions of *F. nucleatum*[a]

Test specimen and dose (μg/test)	Hemolytic PFC count at polymyxin B sulfate concn (μg/test):		
	0	1	10
None (control)	4	6	6
Porin			
0.1	51[b]	84[b]	72[b]
1	241[b]	215[b]	197[b]
10	492[b]	502[b]	479[b]
LPS			
0.1	122[b]	23[c]	8
1	257[b]	86[b]	32[c]
10	359[b]	136[b]	33[c]

[a] Results are expressed as the mean of triplicates of anti-TNP-SRBC hemolytic PFC per 6×10^6 cells.
[b] $P < 0.01$ versus respective control.
[c] $P < 0.05$ versus respective control.

noted in the mice receiving the reference LPS as the adjuvant, but not in the mice receiving the porin fraction with SRBC.

A limited amount of the test porin fraction did not allow us to include the porin-alone control in the present assay to evaluate the possible effects of the PBA properties of the porin on the observed increase in hemolytic PFC counts. However, a highly significant increase in the indirect PFC count that was caused by the test porin fraction and is generally known to be scarcely affected by PBA may indicate the immunoadjuvant activity of the porin fraction. The possibility that the immunoadjuvant activity of the porin fraction is due to coexistent LPS was excluded by the experiment with C3H/HeJ mice presented in Table 4; the porin fraction caused a marked increase in direct anti-SRBC PFC per spleen and per 10^6 splenocytes, but adjuvant activity of the reference LPS was not observed.

(iv) Macrophage-activating activity. Stimulation of guinea pig peritoneal macrophages by the porin fraction was tested by inhibition of thymidine uptake, increase in glucosamine incorporation (Fig. 8), and enhanced generation of superoxide anions (Fig. 9). The porin fraction significantly stimulated all of the above functions of macrophages, as did the reference LPS. The ability of the porin fraction to enhance the generation of superoxide anions, like the other bioactivities described above, was not inhibited by polymyxin B, in sharp contrast to the high responsiveness of LPS to the inhibitory effect of polymyxin B (Fig. 9).

(v) Monocyte migration enhancement. The porin fraction significantly enhanced the migration of human peripheral blood monocytes (Fig. 10) under assay conditions in which the reference LPS was found inactive, in accordance with our previous finding (15).

DISCUSSION

We isolated a porin fraction of *F. nucleatum*, one of the most predominant bacterial species in the periodontal pockets of adult patients with periodontitis, by the following procedures: preparation of an outer membrane-rich fraction, which mainly consisted of an outer membrane protein (41K

TABLE 3. Adjuvant activity of the porin and LPS fractions of *F. nucleatum* to increase anti-SRBC hemolytic PFC count in the spleen of BALB/c mice immunized with SRBC together with the test specimens[a]

Test specimen (50 μg)	Total spleen cells (10^6)	PFC count (10^4/spleen)		PFC count (no./10^6 cells)	
		Direct	Indirect	Direct	Indirect
Porin	226 ± 12	8.70 ± 0.32[b]	20.6 ± 1.22[b]	385 ± 14[c]	913 ± 54[b]
LPS	290 ± 13[b]	9.95 ± 2.55[c]	21.8 ± 3.77[b]	343 ± 88	752 ± 130[c]
None	183 ± 11	4.44 ± 0.58	6.0 ± 1.34	243 ± 32	327 ± 73

[a] BALB/c mice (five per group) received an intraperitoneal injection of 0.1 ml of PBS containing 50 μg of the test specimen and 2×10^7 SRBC. Seven days later, the splenocytes of each mouse were subjected to direct and indirect hemolytic PFC assays. Data are expressed as the mean hemolytic PFC count ± standard error of the mean.
[b] $P < 0.01$ versus untreated control.
[c] $P < 0.05$ versus untreated control.

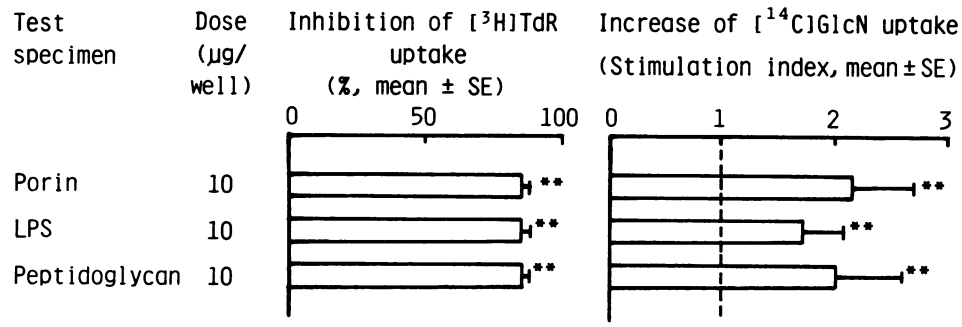

FIG. 8. Stimulatory effects of the porin and reference fractions of *F. nucleatum* on thymidine (TdR) and glucosamine (GlcN) uptake by guinea pig peritoneal macrophages. The cells (5×10^5) were cultured with 10 μg of test specimens per well (none in the control) for 2 days in duplicate. The counts in control cultures were: [^{3}H]thymidine incorporation, 16,520 dpm; [^{14}C]glucosamine incorporation, 543 ± 143 dpm. *, $P < 0.05$; **, $P < 0.01$ versus controls.

TABLE 4. Adjuvant activity of the porin and LPS fractions of *F. nucleatum* to increase the anti-SRBC hemolytic PFC count in the spleen of C3H/HeJ immunized with SRBC together with the test specimen[a]

Test specimen (50 μg)	Total spleen cells (10^6)	PFC count	
		Direct (10^4/spleen)	Direct (no./10^6 cells)
Porin	159 ± 12	10.39 ± 0.96[b]	654 ± 60[b]
LPS	154 ± 10	1.89 ± 0.33	123 ± 21
None	139 ± 13	1.54 ± 0.23	111 ± 17

[a] C3H/HeJ mice (seven per group) received an intraperitoneal injection of 0.1 ml of PBS containing 50 μg of test specimens and 2×10^7 SRBC. Seven days later, the splenocytes of each mouse were submitted to direct hemolytic PFC assays. Data are expressed as the mean hemolytic PFC count ± standard error of the mean.

[b] $P < 0.01$ versus untreated control.

protein after heating in SDS solution and 37K protein in the native state) and peptidoglycan, by exhaustive washing and SLS treatment of the insoluble residue of mechanically disrupted cells, and extraction of the porin from the outer membrane-rich fraction with 2% LDS at 4°C. DiRienzo and Rosan (7) reported isolation of a major cell envelope protein (designated HM-1) from a crude cell envelope fraction of *F. nucleatum*, but a strain (FDC 364) different from ours, by extraction with 2% SDS at 100°C. The HM-1 protein could be the same protein as the one purified in the present study, because they both appeared to be very abundant in the envelope fraction and to have similar heat modifiability, one of the characteristics of porin proteins (22). We found that the LDS extraction method at 4°C gave *Fusobacterium* porin in a native form.

The native porin fraction we isolated exhibited powerful stimulatory effects on murine splenocytes and guinea pig peritoneal macrophages in a variety of in vitro assays. The possibility that these bioactivities of the porin fraction are due to coexistent LPS was excluded by use of LPS-nonresponding C3H/HeJ mice and by demonstration of lack of inhibitory effects of polymyxin B, which is well known to inhibit the bioactivities of LPS (2, 25).

There have been many reports that outer membrane proteins and lipoproteins which were prepared from various gram-negative bacteria exhibited powerful stimulatory effects on immunologically competent cells, especially B lymphocytes, in in vitro assays (1, 9, 19, 29, 31). Regarding periodontitis-associated bacteria, Murayama et al. (21) reported that SDS-extractable outer membrane proteins prepared from a *Capnocytophaga* sp. exhibited a variety of immunostimulatory activities in vivo as well as in in vitro assays. Kato et al. (12) also reported similar findings with three strains of *Actinobacillus actinomycetemcomitans*, two strains of *Bacteroides gingivalis*, and a strain of *F. nucleatum*. However, the test fraction was reported to reveal several major protein bands in SDS-PAGE, and the involvement of LPS in the manifestation of the described bioactivi-

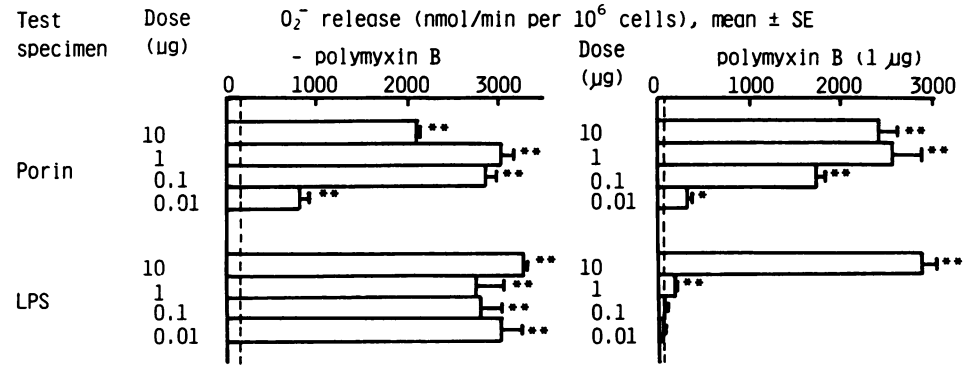

FIG. 9. Enhancement of superoxide anion (O_2^-) generation by guinea pig peritoneal macrophages treated with the porin and LPS fractions of *F. nucleatum* in the presence and absence of polymyxin B. The cells (10^6) were cultured with the indicated doses of test materials in the presence or absence of 1 μg of polymyxin B for 48 h. The values (mean ± SE) in the polymyxin B-free control and polymyxin B (1 μg per culture)-added control were 162 ± 36 and 63 ± 13 nmol/min per 10^6 cells, respectively. *, $P < 0.05$; **, $P < 0.01$ versus controls.

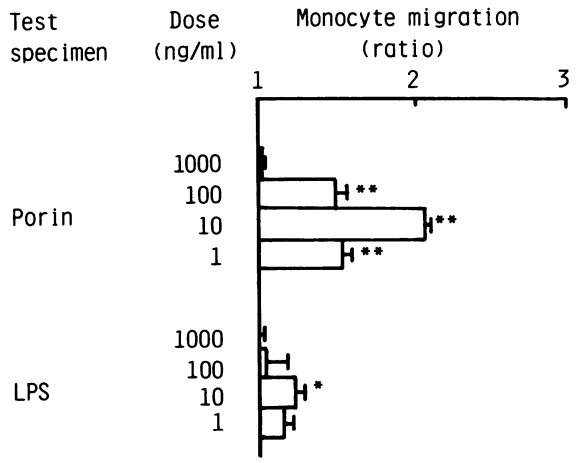

FIG. 10. Enhancement of monocyte migration in response to the porin protein and no or little enhancement by the reference LPS. Determinations were carried out in triplicate at each dose of test specimen. The number (ratio) of cells per oil immersion field in positive controls and those with no addition (controls) were as follows: 1:10 dilution of LPS-activated serum, 130.3 ± 8.3 (5.59 ± 0.35); 10^{-8} M FMLP, 106.3 ± 10.2 (4.56 ± 0.44); no addition, 23.3 ± 0.3 (1.00 ± 0.01). *, $P < 0.05$; **, $P < 0.01$ versus controls.

ties has not been adequately excluded. Similar criticism may be applied to other studies so far reported.

The findings reported here support the assumption that the porin protein of *F. nucleatum* may play a significant role in the pathogenesis of adult periodontitis, by consideration of the following facts. The bioactivities of the porin were comparable to those of LPS. The recovery rate of porin proteins from the cell envelope was 14.5%, but the real content of porin proteins is much higher (presumably around 30%), because the considerable part of porin proteins in the cell envelope was solubilized with the inner membranes by SLS treatment and so is significantly higher than that of LPS (2% of the whole cells, consequently 12% of the envelope by calculation). Furthermore, the porin protein and LPS of *F. nucleatum* may work synergistically in the manifestation of pathogenic actions which may lead to periodontal diseases. Further purification of the porin fraction to eliminate coexistent LPS is required to test this possibility.

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