

Chemical Composition and Immunobiological Activities of Sodium Dodecyl Sulphate Extracts from the Cell Envelopes of *Actinobacillus actinomycetemcomitans*, *Bacteroides gingivalis* and *Fusobacterium nucleatum*

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The chemical composition and immunobiological activities *in vivo* and *in vitro* of sodium dodecyl sulphate extracts (SDS-SE) derived from periodontopathic bacteria (three strains of *Actinobacillus actinomycetemcomitans*, two strains of *Bacteroides gingivalis*, and one strain of *Fusobacterium nucleatum*) were investigated. The main components of SDS-SE were protein and lipid, with negligible amounts of peptidoglycan and lipopolysaccharide. Immunopotentiating activity was detected in both delayed-type hypersensitivity and antibody formation against the elicitation of a protein antigen with the SDS-SE preparations of *A. actinomycetemcomitans* ATCC 29524 and *B. gingivalis* 381 and 1021. On the other hand the SDS-SE of *A. actinomycetemcomitans* ATCC 29522 enhanced only the induction of a delayed-type hypersensitivity response. All the SDS-SE preparations had mitogenic activity to splenocytes from BALB/c *nu/nu*, C3H/HeN and C3H/HeJ mice. Migration-stimulating activity for human peripheral blood monocytes was detected especially in the SDS-SE preparations of *A. actinomycetemcomitans* ATCC 29524 and Y4. All of the SDS-SE samples inhibited [³H]thymidine uptake in human gingival fibroblasts and caused degradation of the cells. The results suggest that the cell membrane components extractable with sodium dodecyl sulphate from periodontopathic bacteria are involved in the pathogenesis of periodontal disease.

INTRODUCTION

In periodontal lesions, accumulation of supra- or subgingival plaque proceeds to inflammation of the gingiva, and other reactions, such as denaturation of collagen and alveolar bone loss (Macrina & Ranney, 1982). Gram-negative anaerobic rods in subgingival plaque play an important role in periodontal destruction by two different mechanisms. The causes of periodontal disease are supposed to be the direct toxicity of the bacterial cell components (Hausmann *et al.*, 1975; Baehni *et al.*, 1981; Iino & Hopps, 1984; Progulské *et al.*, 1984), toxic bacterial metabolites (Singer & Buckner, 1981) and the consequence of both humoral and cell-mediated immune reactions against bacterial-cellular components (Lehner, 1975; Page & Schroeder, 1981). Murayama *et al.* (1982) found that among the cellular fractions of a periodontopathic *Capnocytophaga* sp., the SDS-soluble fraction from the cell envelope showed

Abbreviations: FBS, foetal bovine serum; FMLP, *N*-formyl-L-methionyl-leucyl-phenylalanine; KDO, 2-keto-3-deoxyoctonate; MDP, muramyl dipeptide; PHA, phytohaemagglutinin; SDS-SE, SDS-soluble extract; SE, soluble extract.

the most immunobiological activity. The aim of the present study was to examine SDS extracts from the cell envelopes of *Actinobacillus actinomycetemcomitans*, *Bacteroides gingivalis* and *Fusobacterium nucleatum*, all bacteria associated with periodontopathic disease, for such immunobiological activities.

METHODS

Growth conditions. *A. actinomycetemcomitans* strains Y4 (originally isolated from the gingival sulcus of a patient with juvenile periodontitis by S. S. Socransky, Forsyth Dental Center, Boston, Mass., USA: Baehni *et al.*, 1979), ATCC 29522 (isolated from a mandibular abscess) and ATCC 29524 (isolated from a chest aspirate) were grown in brain-heart infusion broth (Difco) supplemented with 0.5% (w/v) yeast extract (Difco) and 0.05% (w/v) cysteine hydrochloride (Wako Pure Chemical Industries). *F. nucleatum* ATCC 25586 (isolated from a cervico-facial lesion) was grown in a similar medium except that 0.075% cysteine hydrochloride was used. ATCC strains were obtained from the American Type Culture Collection (Rockville, Md, USA). *B. gingivalis* strains 381 and 1021 (isolated from subgingival plaque) were generous gifts from S. S. Socransky; they were grown in brain-heart infusion broth supplemented with 0.0005% (w/v) haemin and 0.0001% (w/v) vitamin K (Wako).

All strains were incubated under anaerobic conditions in a chamber containing N₂/CO₂/H₂ (80:10:10, by vol.) (Yuyama Irika, Osaka) at 37 °C for 72 h. The cells were collected by centrifugation at 10000 g for 20 min, washed twice with 10 mM-phosphate-buffered saline pH 7.0 (PBS), once with distilled water and finally lyophilized.

Preparation of cell envelopes, soluble extract (SE) and SDS-SE. Glass beads (5.0 g; 0.18 mm diam., Takashima Shoten, Tokyo) were added to a suspension containing 1 g lyophilized bacterial cells in 40 ml 50 mM-phosphate buffer pH 7.0, and the mixture was sonicated in a machine (type UR-200P, Tomy Seiko, Tokyo) with an output of 185–200 W for 15 min at 4 °C. The sonicated cell suspensions were centrifuged at 1000 g for 20 min to remove undisturbed cells, and the supernates were pooled and centrifuged at 100000 g for 60 min. The supernate was dialysed repeatedly against distilled water at 4 °C (soluble extract, SE) and lyophilized. The pellet was washed three times with distilled water, suspended in 40 ml distilled water, mixed with an equal volume of 2% (w/v) SDS, stirred at 22 °C for 24 h and centrifuged at 100000 g for 60 min. The supernate was dialysed against distilled water containing 1.5% (w/v) Dowex-1 × 1 (Dow Chemical Co.) at 4 °C for 5 d. The ion-exchange resin suspension was changed twice a day. The dialysed extract was centrifuged at 20000 g for 30 min to remove small amounts of insoluble material. The final supernate was lyophilized (SDS-soluble extract, SDS-SE).

Analytical methods. Hexose, pentose and methylpentose were detected by the anthrone (Ashwell, 1957), orcinol (Ashwell, 1957) and cysteine/sulphuric acid (Dische & Shettles, 1948) methods, respectively. Glycerol and total phosphate were estimated by the methods of Lambert & Neish (1950) and Lowry *et al.* (1954), respectively. Protein was determined by the Lowry method. 2-Keto-3-deoxyoctonate (KDO) was quantified by the method of Weissbach & Hurwitz (1959) after hydrolysis of the SDS-SE samples in 0.005 M-H₂SO₄ at 100 °C for 20 min. The quantity of SDS bound to protein was assessed by the partition detergent/dye salt method of Hayashi (1975); serum albumin and oleic acid were used as references for the correction.

Qualitative and quantitative analyses of amino acids and amino sugars were performed with an amino acid analyser (type 835, Hitachi). Specimens were hydrolysed with 6 M-HCl in a sealed tube under N₂ at 100 °C for 14 h. Hydrolysates were evaporated under reduced pressure and dried in a vacuum-desiccator containing solid NaOH to remove HCl. No corrections were made for the destruction of amino sugars and amino acids during the acid hydrolysis.

Gas-liquid chromatography (GLC) of fatty acids. Fatty acids were analysed, after methanolysis, in a gas-liquid chromatograph (type 163, Hitachi) with a column (200 cm) of 10% (w/v) DEGS on Chromosorb W as described by Ikemoto *et al.* (1978), with *cis*-11-eicosenoic acid (P. L. Biochemicals) as an internal standard. Hydroxylated fatty acids were also methylated and separated on Kieselgel 60 F₂₅₄ plates (20 × 20 cm, Merck) in light petroleum (b.p. 30–60 °C)/diethyl ether (1:1, v/v) at room temperature for 60 min. β -Hydroxymyristic acid (Wako) and β -hydroxypalmitic acid (isolated from *Azospirillum brasiliense* JCM 1225: Ohta & Hattori, 1983) were used as references. The separated hydroxylated fatty acids were detected by spraying the plate with 0.02% (w/v) rhodamine 6 G in ethanol, and examining it under ultraviolet irradiation. The zones of reaction were collected by scraping the Kieselgel off the plate and extracting with diethyl ether. The fatty acids in these samples were quantified by GLC.

Limulus assay. The endotoxin content of SDS-SE samples was determined by the *Limulus* Toxicolor test (Iwanaga *et al.*, 1978) by courtesy of M. Ooki and S. Tanaka, Tokyo Institute of Seikagaku Kogyo, Tokyo.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). This was done with 10% (w/v) acrylamide in 0.1% (w/v) SDS in a vertical slab gel apparatus according to Laemmli (1970), with slight modification. Slab gels were fixed and stained with 10% (v/v) acetic acid, 45% (v/v) methanol, 0.1% (w/v) Coomassie brilliant blue R at room temperature for 1 h, and destained overnight with 7.5% acetic acid, 10% methanol at room temperature. The following marker proteins (Oriental Yeast Co. and Seikagaku Kogyo Co.) were used for molecular mass

calibration: RNA polymerase (β , 140 kDa), horse heart muscle cytochrome *c* (hexamer, 74.4 kDa; tetramer, 49.6 kDa; trimer, 37.2 kDa; dimer, 24.8 kDa; and monomer, 12.4 kDa).

Assay of immunopotentiating activity. The immunoadjuvant activity *in vivo* was tested as described by Kotani *et al.* (1975). Groups of five female guinea-pigs were injected in the left hind food-pad with 0.2 ml of an emulsion prepared by mixing 1.0 mg ovalbumin (grade V, Sigma) as immunogen and 0.1 mg SDS-SE, both dissolved in PBS, with Freund's incomplete adjuvant (Difco). The delayed-type hypersensitivity reaction to ovalbumin was measured by the corneal reaction, and the anti-ovalbumin precipitin titre was assayed 3 and 4 weeks after immunization.

The mitogenic activity of SDS-SE. The effect of SDS-SE preparations (0.1, 1 and 10 μg) on mouse splenocytes was assayed by measuring the incorporation of [^3H]thymidine (Takada *et al.*, 1979). Splenocytes were isolated from BALB/c *nu/nu* (6-week-old Nihhon Clea, Tokyo), C3H/HeN (female, 10-week-old, Nihhon Charles-River, serum Atsuki, Saitama) and C3H/HeJ (female, 14-week-old, Jackson Laboratories, Miami, FL, USA) mice. Lipopolysaccharide (LPS) from *Escherichia coli* O127:B8 (Difco), *E. coli* O111:B4 and *Salmonella minnesota* Re595 (List Biological Laboratories), and phytohaemagglutinin (PHA: Wellcome HA16, Wellcome Reagents Ltd) were used as references.

Human monocyte migration. The stimulation of migration of human monocytes by SDS-SE (0.1, 1 and 10 μg ml^{-1}) was assayed as described by Ogawa *et al.* (1982) using a multiwell chemotaxis assembly (Neuro Probe). Human peripheral blood monocytes were washed and resuspended to a cell density of 5×10^6 cells ml^{-1} in Gey's BSA solution (Gey's balanced salt solution containing 2%, w/v, bovine serum albumin and 20 mM-HEPES). *N*-Formyl-L-methionyl-leucyl-phenylalanine (FMLP) and LPS-stimulated serum were used as positive controls. The migration index was expressed as the ratio of cells which migrated in the presence of test or positive control materials to cells which migrated in medium alone.

Cytotoxic action of SDS-SE preparations on fibroblasts derived from human gingiva. Cultures of human gingival fibroblasts were obtained from the resected gingiva of a patient with marginal periodontal disease. Isolated gingiva was treated with crystalline trypsin, and dispersed cells were propagated and maintained in Eagle MEM medium (Nissui Seiyaku Co.) supplemented with 10% (w/v) foetal bovine serum (FBS; Gibco). In the assay system, cells were used after subculturing several times.

The inhibition of cell proliferation caused by SDS-SE samples was measured by the reduction in [^3H]thymidine incorporation, as follows. A confluent layer of human gingival fibroblasts grown in Eagle MEM medium supplemented with 5% FBS (Gibco) was trypsinized and dispersed in the medium. The cells collected by centrifugation were washed with Eagle MEM medium and resuspended in the same medium supplemented with 5% FBS to give 5×10^5 cells ml^{-1} . Portions of the cell suspension (200 μl , 1×10^5 cells) were distributed to each well of plastic microplates (96 flat-bottom wells, Corning), and incubated in an atmosphere of air/ CO_2 (95:5, v/v) at 37 °C for 48 h. The supernatant medium of the cell monolayer was replaced by MEM medium supplemented with 0.5% FBS and incubated for a further 20 h. SDS-SE samples were then added to the wells to give final concentrations of 0.5, 2, 8, 10, 20, 50, 100 and 200 μg ml^{-1} , and incubated for 20 h. [^3H]Thymidine (0.6 μCi , 22.2 kBq; Amersham Japan) was added to each of the wells, and the plate was incubated for 3.5 h and then washed with PBS. Trichloroacetic acid (200 μl of 5%, w/v, solution) was added to each well, followed by incubation at room temperature for 30 min and washing with ethanol/diethyl ether (3:1, v/v). The washed cells were digested with 0.3 M-NaOH at 37 °C for 20 min and neutralized with 6 M-HCl, then removed from the wells and dispersed into a suspension. The suspensions were transferred into scintillation vials and a cocktail of xylene base was added. Radioactivity was determined in a liquid scintillation counter (type LSC-700, Aloka, Tokyo). The assay was done with triplicate cultures and the mean value was determined.

The morphological change of fibroblast cells (5×10^5 cells per Petri dish, 35 \times 10 mm, Corning) caused by the addition of SDS-SE samples (0.5–200 μg) was observed after 24–48 h incubation, by Nikon inverted-type microscopy (Diaphoto-TMD, Nihhon Kogaku Industrial Co.).

RESULTS

Isolation of SE and SDS-SE. The recovery rates of SE and SDS-SE from whole cells of *A. actinomycetemcomitans* and *B. gingivalis* were 75–80% (SE, 27–31%; SDS-SE, 49–51%) and 66–72% (SE, 29–31%; SDS-SE, 38–39%), respectively. Contamination of the test specimens with endotoxin was checked by the *Limulus* Toxicolor test. Tests with equivalent weights of *E. coli* O111:B4 LPS were within the range 0.007–0.02%.

Chemical composition of SDS-SE. The major components of SDS-SE were protein (42–78%) and lipid (9.9–26.7% as fatty acids), with small amounts (<5%) of hexose, pentose, methylpentose, hexosamine, phosphorus and glycerol (the two *B. gingivalis* strains, however,

Table 1. *Amino acid and amino sugar composition (residues per 100 amino acid residues) of SDS-SE preparations*

Constituent	<i>A. actinomycetemcomitans</i>			<i>B. gingivalis</i>		<i>F. nucleatum</i>
	ATCC 29522	ATCC 29524	Y4	381	1021	ATCC 25586
Aspartic acid	10.2	10.2	9.2	7.2	7.8	10.2
Threonine	5.0	5.3	4.5	3.7	3.9	5.3
Serine	5.0	4.9	4.7	5.4	5.8	4.8
Glutamic acid	11.2	11.3	10.3	6.6	8.3	11.1
Proline	3.5	3.6	3.6	2.9	3.0	3.3
Glycine	8.1	7.9	7.8	7.2	7.6	7.6
Alanine	10.2	10.0	9.4	6.9	7.4	7.4
Cystine	ND	0.4	0.2	ND	ND	0.3
Valine	6.8	6.6	5.8	3.7	4.2	5.3
Diaminopimelic acid	0.5	0.4	0.2	0.9	0.2	ND
Methionine	0.5	0.2	0.2	0.6	0.7	1.8
Isoleucine	4.2	4.2	2.0	2.9	3.2	5.3
Leucine	7.4	7.4	7.2	5.4	6.0	7.2
Tyrosine	2.7	2.8	2.7	2.6	2.8	3.2
Phenylalanine	3.2	3.2	3.1	3.2	2.8	3.4
Ornithine	ND	ND	ND	ND	ND	0.1
Lysine	9.7	9.4	5.8	3.4	4.4	8.7
Ammonia	3.8	3.4	13.0	11.7	11.8	8.5
Histidine	1.6	1.7	1.8	1.1	1.4	1.3
Arginine	4.7	4.7	4.5	2.0	3.5	3.6
Glucosamine	0.9	1.5	2.9	18.3	14.4	1.2
Galactosamine	0.9	0.9	0.9	0.9	0.7	0.1

ND, Not detected.

had a hexose content of 7.9–8.6%). KDO contents were between 0.06 and 0.11%. The amino acid and amino sugar composition of SDS-SE is shown in Table 1. The major amino acids detected in SDS-SE of *A. actinomycetemcomitans* and *F. nucleatum* were glutamic acid, aspartic acid, alanine and lysine. In the SDS-SE of *B. gingivalis* large amounts of glucosamine were detected. Diaminopimelic acid (<0.9%) was found but muramic acid and lanthionine were not detected in any of the specimens tested.

SDS-PAGE analysis of SDS-SE preparations. The SDS-PAGE pattern showed similarities among the three strains of *A. actinomycetemcomitans* and between the two strains of *B. gingivalis* (Fig. 1). The molecular masses of the main bands were 46, 38, 34 and 28 kDa in the SDS-SE of *A. actinomycetemcomitans*, and 46 and 43 kDa in that of *F. nucleatum*; the SDS-SE of *B. gingivalis* showed diffused bands of lower molecular mass.

Measurement of bound SDS. The amount of SDS in the test specimens of *A. actinomycetemcomitans*, *B. gingivalis* and *F. nucleatum*, determined by the partition detergent/dye salt method, was 10.9–11.5%, 12.4–14.8% and 8.9%, respectively.

Fatty acid analysis. The fatty acid composition of SDS-SE preparations analysed by GLC is shown in Table 2. Large amounts of C_{14:0}, C_{16:0}, C_{16:1} and 3-OH C_{8:0} fatty acids and small amounts of 3-OH C_{14:0} and 3-OH C_{i-10:0} fatty acids were found in the specimens of *A. actinomycetemcomitans*. In the specimens of *B. gingivalis*, 3-OH C_{8:0} and 3-OH C_{i-10:0} fatty acids were found, but C_{16:0}, C_{16:1} and 3-OH C_{14:0} fatty acids were less abundant than in *A. actinomycetemcomitans*, and 3-OH C_{10:0}, C_{i-13:0}, C_{i-15:0}, 3-OH C_{i-15:0} and 3-OH C_{i-17:0} fatty acids, not found in *A. actinomycetemcomitans*, were detected.

Immunobiological activity in vivo and in vitro of SDS-SE preparations. The immunopotentiating activity on the cell-mediated and humoral immune responses of SDS-SE preparations was

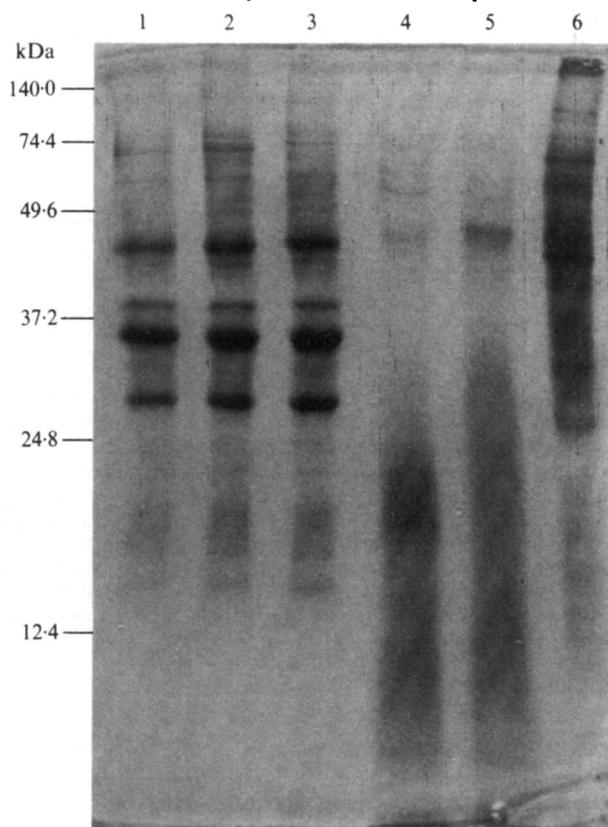


Fig. 1. SDS-PAGE of SDS-SE. Lanes: 1, *A. actinomycetemcomitans* ATCC 29522; 2, *A. actinomycetemcomitans* ATCC 29524; 3, *A. actinomycetemcomitans* Y4; 4, *B. gingivalis* 381; 5, *B. gingivalis* 1021; 6, *F. nucleatum* ATCC 25586.

Table 2. Fatty acid composition (% of total fatty acids) of SDS-SE preparations

Fatty acid*	<i>A. actinomycetemcomitans</i>			<i>B. gingivalis</i>		<i>F. nucleatum</i>
	ATCC 29522	ATCC 29524	Y4	381	1021	ATCC 25586
12:0	tr.	0.1	tr.	ND	tr.	ND
14:0	11.1	12.1	12.4	1.8	4.3	4.1
15:0	0.1	tr.	tr.	ND	ND	ND
16:0	34.1	28.9	29.4	8.4	6.0	7.4
16:1	25.9	15.7	23.9	2.2	2.7	8.1
17:0	ND	ND	ND	ND	tr.	ND
18:0	1.6	1.6	1.7	0.5	ND	1.5
18:1	1.4	0.9	1.2	0.4	0.7	18.8
3-OH 8:0	12.7	28.9	20.8	15.3	11.7	29.0
3-OH 14:0	4.7	4.1	5.4	0.2	0.2	1.6
3-OH 16:0	ND	ND	ND	3.5	4.4	ND
<i>i</i> -13	ND	ND	ND	1.5	2.2	ND
<i>i</i> -15	ND	ND	ND	39.4	38.4	ND
3-OH <i>i</i> -10:0	3.8	4.6	3.9	6.5	6.0	7.4
3-OH <i>i</i> -15:0	ND	ND	ND	3.5	2.2	ND
3-OH <i>i</i> -17:0	ND	ND	ND	15.5	10.0	ND
Unknown	4.6	3.1	1.3	1.3	1.5	5.1

tr., trace; ND, not detected.

* *i*, *iso*-acid; 3-OH, 3-hydroxy acid; 16:0, a straight-chain saturated acid of 16 carbons; 16:1, a straight-chain unsaturated acid of 16 carbons with one double bond.

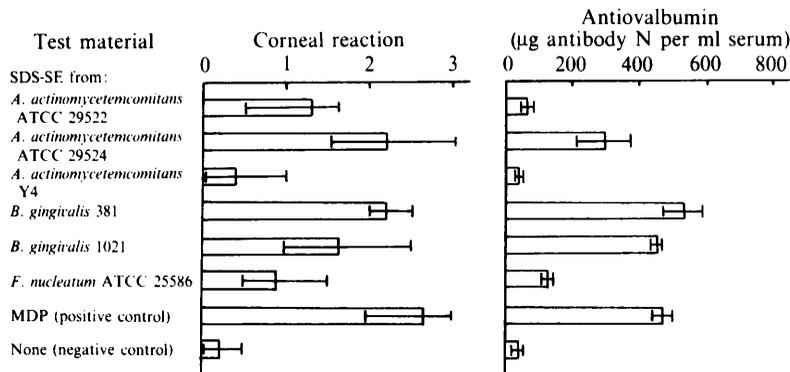


Fig. 2. Immunopotentiating activity of SDS-SE on cellular and humoral immunity to ovalbumin. Groups of five female albino guinea-pigs were immunized by footpad injection of 1 mg ovalbumin with 0.1 mg SDS-SE or MDP (muramyl dipeptide) as an emulsion in PBS/Freund's incomplete adjuvant with ovalbumin as a test antigen. The corneal reaction was measured 21 d after sensitization to evaluate delayed-type hypersensitivity, and the mean and range of the response are shown. The guinea pigs were given an intracorneal injection of 2% (w/v) ovalbumin in saline to make a transient white spot of opacity about 5 mm in diameter. The eyes were examined after 24 and 48 h and the corneal reaction was graded from 3 (whole cornea thickened, opaque and greyish-white) to 1 (slight opacity). When no visible difference from the uninjected eye was detected the reaction was graded as 0. The antiovalbumin precipitin level (μg antibody nitrogen per ml serum) was measured 30 d after sensitization, and the mean and standard error are shown.

determined (Fig. 2). SDS-SE of *A. actinomycetemcomitans* ATCC 29524 and *B. gingivalis* 381 and 1021 potentiated both induction of a delayed-type hypersensitivity and antibody formation against the antigen, ovalbumin. The activity was as potent as that of muramyl dipeptide (MDP). The mitogenic activity of SDS-SE preparations on splenocytes of athymic BALB/c *nu/nu* mice was tested using doses of 0.1, 1.0 and 10 μg (Fig. 3a). All the preparations showed mitogenic activity more potent than that of *E. coli* LPS. The mitogenic activity of the specimens on splenocytes of C3H/HeN (LPS high responder) and C3H/HeJ (LPS low responder) mice was also examined (Fig. 3b). Mitogenic activity of SDS-SE preparations was observed in both types of mice; this activity was more potent than that found with *E. coli* LPS or *S. minnesota* LPS. The human peripheral monocyte migration-stimulating activity of SDS-SE specimens was lower than that found with FMLP (10^{-8} M) or with LPS-stimulated serum (1:10), but the SDS-SE of *F. nucleatum* and *A. actinomycetemcomitans* ATCC 29522 and Y4 (dose of 1.0 $\mu\text{g ml}^{-1}$) showed a stimulation index of more than 3.0. The specimens of *B. gingivalis* 1021 (1.0 $\mu\text{g ml}^{-1}$), *B. gingivalis* 381 (10.0 $\mu\text{g ml}^{-1}$), *A. actinomycetemcomitans* ATCC 29524 (0.1 $\mu\text{g ml}^{-1}$) showed stimulation indices of 2-3 (Fig. 4).

The effect of SDS-SE preparations on DNA synthesis in human fibroblasts was examined. Incorporation of [^3H]thymidine by human gingival fibroblasts was inhibited completely by all the SDS-SE preparations at 20.0 $\mu\text{g ml}^{-1}$ (Fig. 5). However, the dose required to cause 50% inhibition of incorporation was lowest for *A. actinomycetemcomitans* SDS-SE. The effect of SDS alone (0.5, 1.0, 2.0 and 5.0 $\mu\text{g ml}^{-1}$, i.e. equivalent amounts of SDS estimated to be present in each of the preparations) on fibroblasts was also tested (Fig. 5): it had no inhibitory effect.

The effect of SDS-SE preparations on the morphology of fibroblasts was tested. Morphological changes, which involved rounding of the cells and a decrease in the number of cells attached to the glass surface, were graded from A to D, with A representing the control culture of fibroblasts, B representing partially damaged cells and a small number of round cells, C representing many damaged cells and many round cells, and D representing a high proportion of damaged cells. Fig. 6 shows representative examples of morphological changes caused by the addition of SDS-SE of *B. gingivalis* 381 (grade B, 100 $\mu\text{g ml}^{-1}$, 24 h; C, 100 $\mu\text{g ml}^{-1}$, 48 h; D, 200 $\mu\text{g ml}^{-1}$, 48 h). The scores for morphological changes caused by the addition of SDS-SE

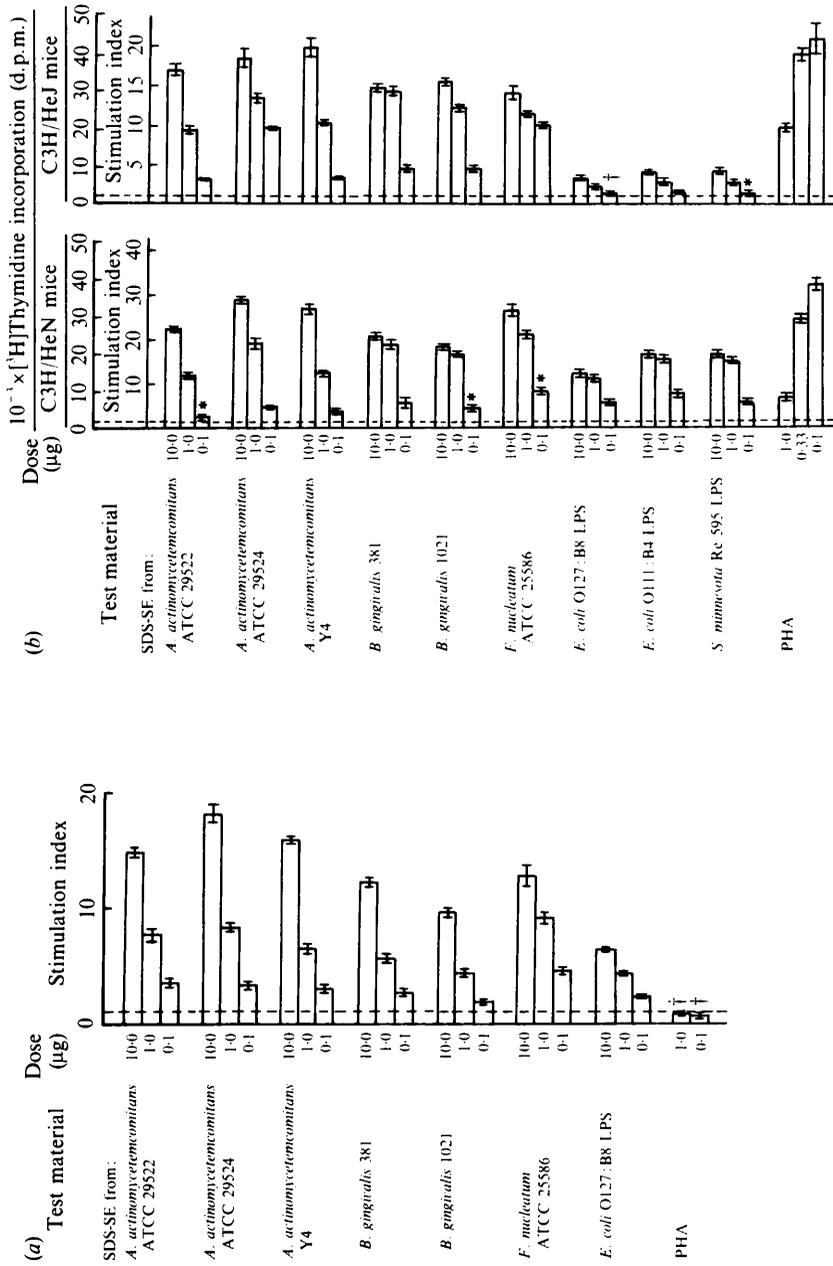


Fig. 3. (a) Mitogenic effects of SDS-SE on splenocytes from athymic BALB/c nu/nu mice (male, 6-week-old). The mean \pm standard error of the control culture (medium alone) was 2219 ± 292 d.p.m. per 5×10^5 cells. All results were significantly different from the control ($P < 0.01$), with the exception of that marked †. (b) Mitogenic effects of SDS-SE on splenocytes from C3H/HeN (female, 10-week-old) and C3H/HeJ (female, 14-week-old) mice. All results were significantly different ($P < 0.01$) from the respective control with the exception of those marked * ($P < 0.05$) and † (not significantly different).

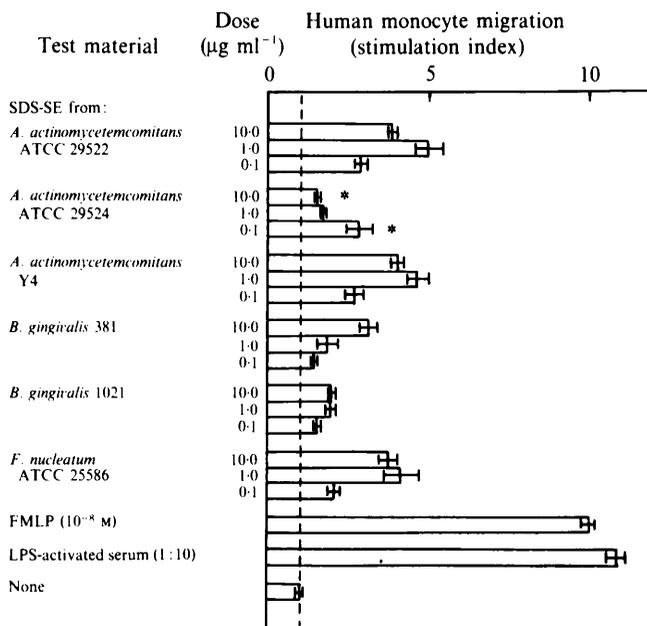


Fig. 4. Migration-stimulating activity of SDS-SE on human peripheral blood monocytes. The mean number \pm standard error of migrated monocytes of the positive controls was 73.3 ± 2.2 for FMLP (10^{-8} M) and 79.3 ± 3.3 for LPS-activated serum (1:10), and that of the negative control was 7.3 ± 0.3 . All results were significantly different from the control ($P < 0.01$) with the exception of that marked * ($P < 0.05$).

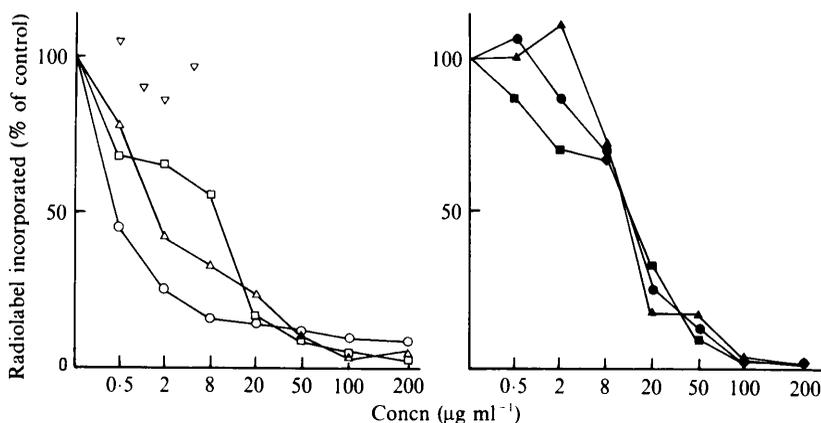


Fig. 5. Effects of SDS-SE and SDS on the synthesis of DNA by human gingival fibroblasts. \triangle , *A. actinomycetemcomitans* ATCC 29522; \square , *A. actinomycetemcomitans* ATCC 29524; \circ , *A. actinomycetemcomitans* Y4; ∇ , SDS; \bullet , *B. gingivalis* 381; \blacktriangle , *B. gingivalis* 1021; \blacksquare , *F. nucleatum* ATCC 25586.

specimens ($100 \mu\text{g ml}^{-1}$) after 24 or 48 h were: *B. gingivalis* 1021 (D, 24 h), *F. nucleatum* (D, 48 h), *B. gingivalis* 381 (C, 48 h), *A. actinomycetemcomitans* ATCC 29522 (B, 48 h), ATCC 29524 (B, 48 h). However, SDS-SE of *A. actinomycetemcomitans* Y4 caused definite morphological change of fibroblasts (grade C) after 48 h incubation only at a concentration of $200 \mu\text{g ml}^{-1}$.

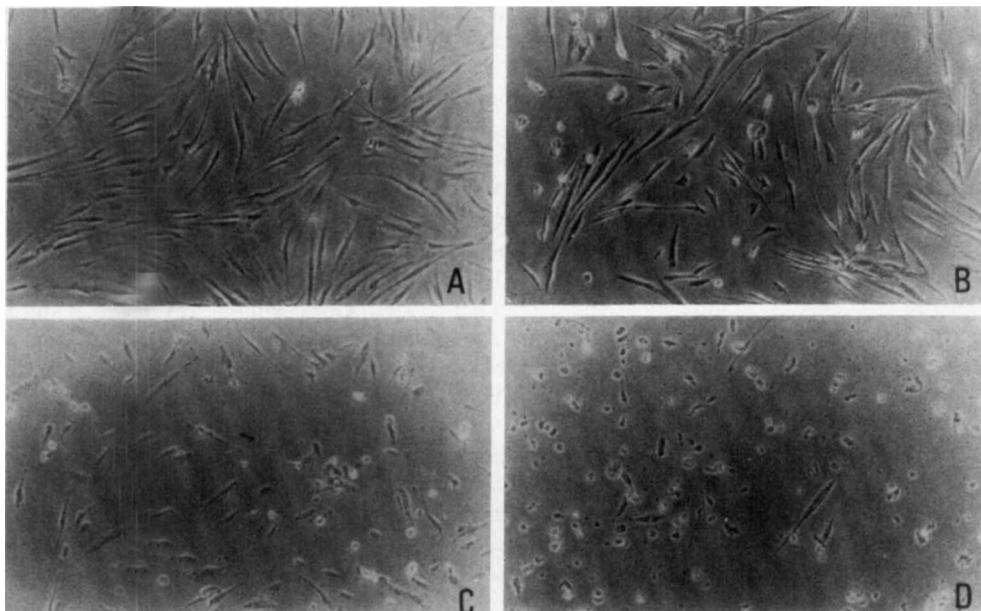


Fig. 6. Effect of *B. gingivalis* 381 SDS-SE on human gingival fibroblasts. Degree of morphological change: A, control cells (48 h); B, partially damaged cells (100 µg, 24 h); C, severely damaged cells (100 µg, 48 h); D, completely damaged cells (200 µg, 48 h). Magnification $\times 200$.

DISCUSSION

A. actinomycetemcomitans is a predominant bacterium isolated from patients with localized juvenile periodontitis, and a high antibody titre is also found in these patients (Slots & Genco, 1984). *B. gingivalis* has been isolated from the lesion site of rapidly progressing alveolar bone destruction and advancing periodontal lesion with severe gingivitis. Higher levels of immunoglobulin G were observed in patients with severe adult periodontitis and generalized juvenile periodontitis than in healthy controls (Slots & Genco, 1984). *F. nucleatum* is frequently isolated from human gingival crevice regions and is the predominant isolate from patients with chronic gingivitis, early and advanced periodontitis and juvenile periodontitis (Slots & Genco, 1984).

Several authors have reported the biological activity (direct toxicity) of SE from periodontopathic bacteria: leucotoxic activity of extracts of *A. actinomycetemcomitans*, and inhibitory activity of extracts of *A. actinomycetemcomitans*, *Campylobacter sputigena* and *Bacteroides asaccharolyticus* on the proliferation of human mucosal epithelial cells and fibroblasts (Stevens *et al.*, 1980, 1983; Kamen, 1983) have been described. Similar biological activities of cell envelope components of Gram-negative bacteria, especially LPS, lipid A and peptidoglycan have been reported (Sveen, 1977; Kiley & Holt, 1980; Iino & Hopps, 1984). Treatment of cell envelopes with SDS solubilizes both the inner and outer membranes (Filip *et al.*, 1973); the major constituents of the SDS-SE preparations reported here were protein and lipid. In the fatty acid analysis of SDS-SE of *A. actinomycetemcomitans*, 3-OH C_{8:0} and 3-OH C_{i-10:0} fatty acids were found in addition to C_{16:0}, C_{16:1} and 3-OH C_{i-14:0} fatty acids. The former two fatty acids were not found in the cell membrane of *A. actinomycetemcomitans* by Calhoun *et al.* (1981). In *B. gingivalis* SDS-SE, the concentration of C_{i-15:0} detected agreed with the report of Mayberry (1980), and 3-OH C_{16:0}, 3-OH C_{i-15:0} and 3-OH C_{i-17:0} fatty acids, not found in *A. actinomycetemcomitans* SDS-SE, were detected in addition to 3-OH C_{8:0}. Complete identification of the fatty acids will require analysis by GC-MS.

The KDO content of the SDS-SE preparations was $<0.01\%$, and the endotoxin content was

0.007–0.02%, suggesting that endotoxin contamination was negligible. The splenocytes of C3H/HeJ (LPS non-responder) mice responded to the SDS-SE as well as those of C3H/HeN mice, indicating that a B cell mitogen other than LPS was involved in SDS-SE (however, it should be stated that *B. gingivalis* LPS extracted by the phenol/water method, which had no detectable heptose and KDO, elicited a response with splenocytes of C3H/HeJ mice: Koga *et al.*, 1985). Neither muramic acid nor lanthionine was detected in the SDS-SE preparations, and diaminopimelic acid was present in trace amounts, suggesting minimal contamination with peptidoglycan. The results suggest that the major components solubilized by SDS from the cell envelopes of periodontopathic bacteria are essentially derived from the inner membrane.

Adjuvant activity of SDS-SE was tested using guinea-pigs with incomplete Freund's adjuvant and ovalbumin as the immunogen. SDS-SE from *A. actinomycetemcomitans* ATCC 29524 and *B. gingivalis* 381 and 1021 potentiated both cellular and humoral immunity, but the SDS-SE from *A. actinomycetemcomitans* ATCC 29522 potentiated only cellular immunity. The reason for this difference among the preparations of SDS-SE is unknown. Mitogenic activity on splenocytes from mice, exceeding that of authentic LPS, was detected in all the SDS-SE preparations. Migration-stimulating activity toward human peripheral monocytes was also detected but the SDS-SE preparations of *A. actinomycetemcomitans* ATCC 29524 and *B. gingivalis* 381 and 1021, which were adjuvant-active, were less active in this assay.

The precise component, whether protein and/or lipid, of SDS-SE responsible for the immunobiological activity is unknown. We tried to remove lipid from SDS-SE by chloroform extraction, but lipid removal was incomplete and partial inhibitory activity on the proliferation of gingival fibroblasts remained.

Nagai *et al.* (1984) used SE and SDS-SE from periodontopathic bacteria (*A. actinomycetemcomitans* and *B. gingivalis*) as test antigens for an ELISA to detect serum antibody in patients with various types of periodontal disease. They found that both antigens were reactive to the test sera, but some of the sera (31–59%) showed different antibody titres against each test antigen.

Soluble extracts obtained by SDS treatment from cell-surface components of periodontopathic bacteria may contain an active principle responsible for direct toxicity in collagenous-tissue destruction by affecting gingival fibroblasts, and immunopotentiating activity and activation of macrophage–lymphocyte interactions may contribute to sensitization of patients to the periodontopathic antigens via humoral and/or cell-mediated immune mechanisms.

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