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Immunological Activities of *Capnocytophaga* Cellular Components

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Whole cells of a clinical isolate (strain S-3) of the genus *Capnocytophaga* were divided into cell envelope (CE) and cytoplasm (CP) fractions by mechanical disintegration followed by differential centrifugation, and a part of the CE fraction was further fractionated by sodium dodecyl sulfate (SDS) treatment into the peptidoglycan and SDS-supernatant fractions. The other part of the CE was extracted with butanol-water or hot phenol-water to isolate butanol-lipopolysaccharide and phenol-lipopolysaccharide, respectively. All of the test fractions except CP exhibited multifold immunomodulating activities, namely, the adjuvant activities to cellular as well as humoral immune responses against ovalbumin in guinea pigs, the mitogenicity on splenocytes of guinea pigs and BALB/c mice (but not on their thymocytes), the stimulation of guinea pig peritoneal macrophages (in terms of increased glucosamine uptake), and the activation of the human complement system through alternative as well as classical pathways. In addition, the test fractions other than the CP evoked dermatoxic reactions on rabbit skin with characteristic variations among them. The immunomodulating activities of SDS-supernatant were noteworthy in view of the fact that this fraction was essentially free of muramic acid and diaminopimelic acid and did not cause the gelation of horseshoe crab amoebocyte lysate except when it was used at the very high dose, suggesting that there was practically no contamination by peptidoglycans and lipopolysaccharides in the SDS-supernatant.

Members of the genus *Capnocytophaga* are implicated as possible etiological agents of periodontal diseases. Although morphological and biochemical traits of *Capnocytophaga* have been fairly well characterized (11, 16, 25, 35), only a few studies have dealt with possible mechanisms by which *Capnocytophaga* may be involved in pathogenesis of periodontal diseases. Whole cells of *Capnocytophaga* release lysosomal enzymes from human polymorphonuclear leukocytes (32). A sonic extract was found to inhibit the migration of human polymorphonuclear leukocytes (22) and to suppress the proliferation of human fibroblasts (27). However, little is known of biological activities of well-defined subcellular components of the *Capnocytophaga*, except the study by Stevens et al. (26), which demonstrated that an endotoxin from *C. sputigena* strain 4 exhibited significantly lower biological activities (pyogenicity, cytotoxicity, and mitogenicity) than endotoxin preparations from other gram-negative microorganisms.

As one of the approaches to evaluate the possible involvement of this organism in pathogenesis of periodontal diseases, the present study was undertaken to examine systematically a variety of the in vitro and in vivo immunomodulating activities of subcellular fractions obtained from a clinical isolate of *Capnocytophaga*.

MATERIALS AND METHODS

Microorganism. *Capnocytophaga* strain S-3 was isolated from a 17-year-old female with localized juvenile periodontitis at the Department of Oral Biology, State University of New York at Buffalo, and identified according to standard procedures described previously (10; Y. Murayama, P. A. Mashimo, and M. J. Levine, J. Dent. Res. **58** [special issue A]:965, 1978).

Cultural conditions. Strain S-3 which was grown on sheep blood agar plates for 3 days at 37°C in H_2 and CO_2 GasPak anaerobic jar (BBL Microbiology Systems, Cockeysville, Md.) was subcultured for 4 days under anaerobic conditions in 1 liter of brain heart infusion (Difco Laboratories, Detroit, Mich.) supplemented with polypeptone (BBL; 0.5%, wt/vol), yeast extract (Difco, 0.5%), glucose (1%), K_2HPO_4 (0.5%), and $MgSO_4 \cdot 7H_2O$ (0.002%). An entire growth of the subculture was then transferred to 10 liters of the above medium and incubated for 4 days at 37°C in a CO_2 incubator containing 10% CO_2 gas.

Cells were harvested by centrifugation at 1,500 × *g*

for 20 min and washed three times with 0.01 M sodium phosphate buffer (pH 7.2) containing 0.154 M NaCl and twice with deionized water.

Preparation of subcellular components. (i) CE and CP. One volume of the washed cells (about 7.5 g, wet weight) was added to three volumes of 0.05 M phosphate buffer supplemented with 1 M NaCl, pH 7.0. The resulting cell suspension was transferred in a heavy-walled glass tube (8 by 12 cm; B. Braun, Melsungen, West Germany) containing 30 g of glass beads (0.17 to 0.18 mm in diameter; B. Braun), and cells were disrupted with a Braun cell homogenizer (model MSK) under cooling with liquid CO_2. The suspension of disrupted cells was centrifuged at 480 × *g* to remove intact cells which escaped disintegration, and the resulting supernatant fluid was separated into a supernatant mainly consisting of cytoplasm and a pellet of cell envelope by high-speed centrifugation at 100,000 × *g* for 60 min. The supernatant was dialyzed with cellophan seamless tubing (Union Carbide Co., Chicago, Ill.) against deionized water and lyophilized (designated as cytoplasm fraction [CP]). The pellet was washed by centrifugation (100,000 × *g*, 60 min) with 1 M NaCl–0.05 M phosphate buffer, pH 7.0, until the optical densities at 260 and 280 mm were reduced to the minimum and constant values (0.03 and 0.05, respectively). The final residue was further washed three times with deionized water and lyophilized (designated as cell envelope fraction [CE]). Totals of 4 g of the CE and 0.77 g of the CP were recovered from 6 g of whole cells (30.5 g, wet weight).

(ii) PG and SDS-supernatant fraction. A 1-g sample of CE was suspended in 200 ml of 0.05 M phosphate buffer, pH 7.0, containing 20 mg of pronase (Pronase P; 45,000 PUK/g, Kaken Chemical Co., Tokyo, Japan). Two milliliters of chloroform was added to the suspension, which was then incubated at 37°C for 36 h. After completion of digestion, a pellet was recovered by centrifugation (100,000 × *g*, 60 min) and then treated with 60 ml of 1% sodium dodecyl sulfate (SDS) in 0.05 M phosphate buffer, pH 7.0, with stirring at room temperature for 24 h. A remaining insoluble pellet collected by centrifugation at 100,000 × *g* for 60 min was treated again with 4% SDS at 100°C for 60 min. The pellet was washed six times each with 6 M urea and deionized water and then lyophilized (designated as peptidoglycan fraction [PG]).

The material extracted by cold SDS treatment was extensively dialyzed against several changes of deionized water containing 1.5% (wt/vol) Dowex 1-X8 (Dow Chemical Co., Midland, Mich.) at room temperature and then against deionized water at 4°C and lyophilized (SDS-supernatant fraction).

Totals of 15 mg of the PG and 180 mg of the SDS-sup were recovered from 1 g of the cell envelope fraction.

(iii) LPS. Lipopolysaccharide (LPS) was prepared by two extraction procedures. A specimen of LPS was prepared from the aqueous phase of a butanol extract (butanol-LPS) of the CE by the method of Morrison and Leive (19). Another specimen of LPS was extracted by treatment of the CE with 45% (vol/vol) aqueous phenol at 65°C (phenol-LPS) as described by Westphal et al. (34).

Recoveries from the CE on a weight basis were 3.6% with butanol-LPS and 2.5% with phenol-LPS.

Chemical analysis. Hexose (glucose as a standard), methylpentose (as rhamnose), and 2-keto-3-deoxy sugar (as 3-deoxyoctulosonic acid; KDO) were determined colorimetrically by the anthrone method (6), the cysteine-sulfuric acid method (1), and the method of Osborn (21), respectively. Total hexosamine was quantitated by the method described by Ghuysen et al. (7). Amino acid analysis was carried out by using an amino acid analyzer (model KLA-3B; Hitachi Ltd., Tokyo, Japan) as described before (14).

Assay for biological activities. (i) Immunopotentiating activities. Stimulation of serum antibody level and induction of delayed-type hypersensitivity, *i.e.*, corneal reaction and delayed skin reaction by test cellular components against ovalbumin (grade V; Sigma Chemical Co., St. Louis, Mo.) were assayed as previously described (15), using a group of five guinea pigs. Briefly, at weeks 2 and 3 after immunization, the guinea pigs were injected intracorneally with ovalbumin solution. The corneal reaction was examined after 48 h. At week 4 after immunization, the delayed skin responses were determined after intracutaneous injection of the ovalbumin. A few days after the skin test, the guinea pigs were bled by cardiac puncture. The levels of antiovalbumin in the serum were measured quantitatively. The induction of delayed-type hypersensitivity was also checked by the in vitro assay of antigen-dependent blastogenic response as described by Ivanyi and Lehner (13) with minor modifications. The heparinized blood specimen (0.1 ml) was incubated with 10 μg of ovalbumin dissolved in 1 ml of RPMI 1640 medium which was supplemented with 10% fetal calf serum (Flow Laboratories, Rockville, Md.), 100 U of penicillin G, and 100 μg of streptomycin sulfate. The incubation was carried out for 5 days at 37°C in a 5% CO_2 incubator. Quadruplicate cultures were set up with each of the test specimens (saline in a control). At 24 h before the culture was terminated, 1 μCi of [^{3}H]thymidine (TKA 305; Radiochemical Centre, Amersham, England) was added. The incubation was terminated by adding 1 ml of cold distilled water and stirring for 30 s. Lymphocytes were collected on glass microfiber paper (Whatman GF/C) by filtration and washed successively with 10 ml of 0.154 M NaCl, 10 ml of 5% trichloroacetic acid, and 5 ml of ethanol. Radioactivity of the lymphocytes collected on the paper was determined as described previously (31). The result was expressed as the stimulation index, i.e., the test culture/control culture mean count ratio.

(ii) Dermatoxicities. Testing was done by the method of Schuster et al. (23) and of Narita (20) with some modifications. Briefly, white Japanese domestic rabbits weighing 2 to 2.5 kg, with the hair of the upper flank removed with barium sulfide solution, were injected with 0.2-ml aliquots of a saline solution or suspension (2 mg/ml) of test specimens into the depilated skin, after the disappearance of acute inflammation caused by barium sulfide (usually 7 days later). A maximum of 15 injections was made on each rabbit with two or three sites for one test specimen. Acute and chronic skin lesions produced within 30 days were evaluated for redness, swelling, nodulation and induration. The index of the lesions was the product of the major axis and minor axis of involvement. Another parameter was the total number of nodules of multinodular lesions.

(iii) Mitogenic activity. Assay was made on splenocytes and thymocytes of guinea pigs and BALB/c mice in terms of stimulation of [^{3}H]thymidine uptake (31).

TABLE 1. General chemical properties of subcellular fractions isolated from *Capnocytophaga*

Fraction	Hexose (%)	Hexosamine (%)	Methylpentose (%)	KDO (nmol/mg)	P (%)	Protein (%)
Whole cell	3.0	2.5	2.6	2.5	1.6	76.0
CE	2.2	2.3	0.8	9.7	0.9	62.0
CP	ND[a]	ND	0.1	9.3	ND	98.0
Butanol-LPS	14.4	15.3	4.2	20.4	1.8	7.4
Phenol-LPS	11.6	9.0	1.0	22.8	2.0	2.8
PG	5.7	7.2	0.7	0.7	1.6	10.0
SDS-supernatant	7.1	6.4	3.2	3.5	1.2	44.0

[a] ND, Not determined.

(iv) Macrophage-stimulating activity. Activation of peritoneal macrophages from guinea pigs was measured by increased incorporation of [^{14}C]glucosamine (Radiochemical Centre) (30).

(v) Activation of human complement system. Complement activation was determined as described previously (12).

(vi) Horseshoe crab amoebocyte lysate gelation activity. Activity to gelate the amoebocyte lysate of the horseshoe crab, *Tachypleus tridentatus*, was assayed with a Pre-Gel reagent (Teikoku-zooki Co., Tokyo, Japan). The activity was expressed as the lowest concentration in nanograms per 0.2 ml of Pre-Gel needed to form a solid gel.

RESULTS

Chemical characterization of subcellular fractions prepared for biological assays. As summarized in Table 1, CE and CP, particularly CP, were mainly composed of protein, whereas the PG, butanol-LPS, and phenol-LPS fractions, all of which were derived from the CE, were rich in sugars: hexose, hexosamine, and methylpentose. As expected, both LPS preparations showed high contents of a KDO. As shown in Table 2, amino acid and amino sugar analyses revealed that the major constituents of PG were glucosamine, muramic acid, alanine, glutamic acid, and diaminopimelic acid, suggesting that the cell wall PG of the test organism was a diaminopimelic acid type. Other amino acids and galactosamine (probably of non-PG origin) were also detected, but only in small amounts. Neither muramic acid nor diaminopimelic acid was detected in LPS preparations and the SDS-supernatant fraction. The absence of diaminopimelic acid was confirmed by paper chromatography (the descending technique, using a mixture of *n*-butanol–pyridine–water–acetic acid [60:40:30:3, vol/vol] as the developing solvent), which gave more clear-cut differentiation between diaminopimelic acid and methionine than the analysis with an amino acid autoanalyzer.

TABLE 2. Amino acid and amino sugar composition of subcellular fractions isolated from *Capnocytophaga*

Amino acid and amino sugar	nmol/mg				
	CE	Butanol-LPS	Phenol-LPS	PG	SDS-supernatant
Aspartic acid	469.4	52.5	38.4	14.8	253.2
Threonine	295.7	20.1	16.1	21.6	127.7
Serine	371.6	57.7	88.3	57.6	214.1
Muramic acid	tr	0	0	162.8	0
Glutamic acid	569.2	71.0	71.0	283.0	178.8
Proline	167.2	0	0	0	55.6
Glycine	477.9	74.4	121.1	82.0	303.8
Alanine	520.5	233.8	163.1	436.0	233.8
Valine	367.8	27.8	14.9	21.6	148.2
Methionine	99.8	tr	15.7	0	46.1
Diaminopimelic acid	64.7	0	0	218.0	0
Isoleucine	318.3	16.3	9.6	16.8	120.2
Leucine	489.4	16.1	15.2	tr	199.8
Thyrocine	191.6	tr	tr	tr	83.7
Phenylalanine	245.8	tr	tr	tr	160.8
Glucosamine	41.3	117.0	341.8	161.8	42.9
Galactosamine	tr	120.0	257.9	28.0	29.5
Lysine	611.8	21.8	46.9	21.4	350.4
Histidine	65.1	0	0	tr	29.5
Arginine	252.7	65.0	37.7	11.8	128.6

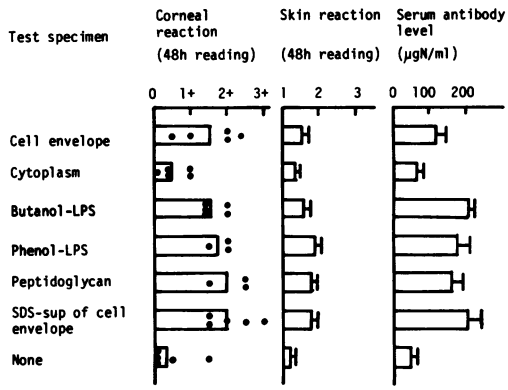

FIG. 1. Immunopotentiating activities of subcellular fractions from *Capnocytophaga* to induce delayed-type hypersensitivity (corneal and delayed-skin reactions) and to stimulate serum antibody levels in guinea pigs. Animals were injected in the left hind footpad with a water-in-mineral oil emulsion containing ovalbumin (1 mg) and a test specimen (0.2 mg). Results are expressed as a mean of five guinea pigs per group. ●, Individual reading in corneal reaction; ⊢⊣, standard error in skin reaction and serum antibody content.

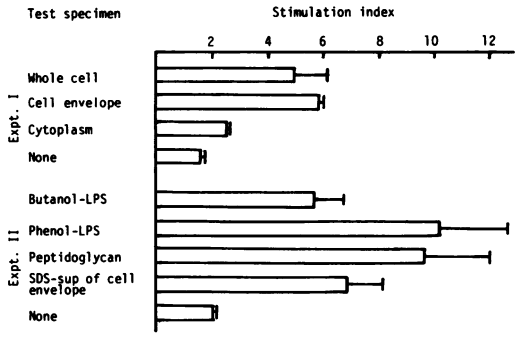

FIG. 2. Antigen-dependent blastogenic response of peripheral blood lymphocytes from guinea pigs sensitized with ovalbumin and test subcellular fractions. Lymphocytes were obtained from animals of the test and control groups (see Fig. 1) 1 month after the immunization and were assayed by ovalbumin-dependent blastogenic response. Results are expressed as the mean of the stimulation index; ⊢⊣, standard error.

High contents of amino sugars, particularly galactosamine, were noted with both LPS specimens.

Immunomodulating activities of *Capnocytophaga* subcellular fractions. (i) Immunopotentiating activities. Figure 1 shows that all of the test fractions except CP were active in induction of delayed-type hypersensitivity as well as in stimulation of circulating antibody level at a dosage of 200 μg per guinea pig. The development of delayed-type hypersensitivity in the animals sensitized with ovalbumin in combination with CE, both LPS preparations, PG, and SDS-supernatant as an adjuvant was ascertained by antigen-dependent blastogenic responses of peripheral lymphocytes, which were taken from the animals showing positive corneal and delayed skin reactions. The circulating lymphocytes of the animals mentioned above showed a significantly higher incorporation of [^{3}H]thymidine than those from the control animals as presented in Fig. 2.

(ii) Dermatoxicities for rabbits. The test fractions other than CP intradermally injected produced localized acute (primary) inflammation, i.e., redness and swelling which developed within 24 h, although there were differences in the extent of reaction from specimen to specimen. Characteristic variations among the test fractions were noticed with chronic lesions which appeared after the acute lesions had subsided. Since pilot studies showed that there was con-

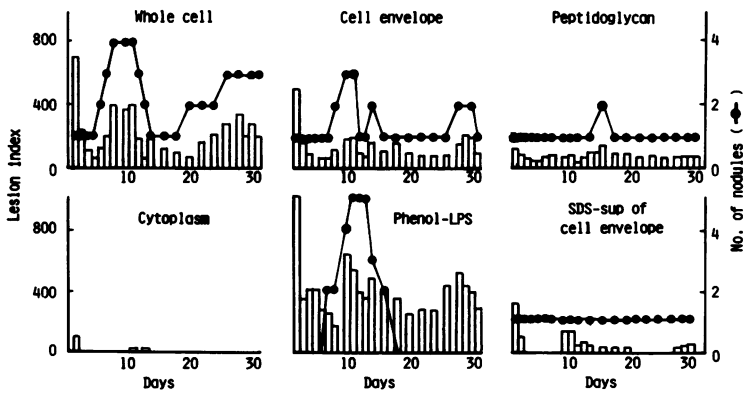

FIG. 3. Activities of subcellular fractions (0.4 mg each) from *Capnocytophaga* producing lesions (redness, swelling, nodulation, and induration) on the skin of a rabbit. Results are expressed by lesion index (major axis [mm] × minor axis [mm] of involvement) and the number of nodules involved in a lesion (●).

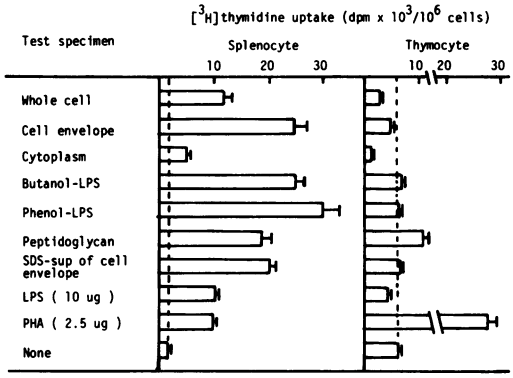

FIG. 4. Mitogenic effects of subcellular fractions (100 μg each) from *Capnocytophaga* on splenocytes and thymocytes from guinea pigs. Results are expressed as mean disintegrations of [^{3}H]thymidine incorporation per minute per 10^6 cells in quadruplicate cultures. LPS (LPS B from *Salmonella enteritidis* was from Difco); PHA, phytohemagglutinin (Difco); ⊢⊣, standard error.

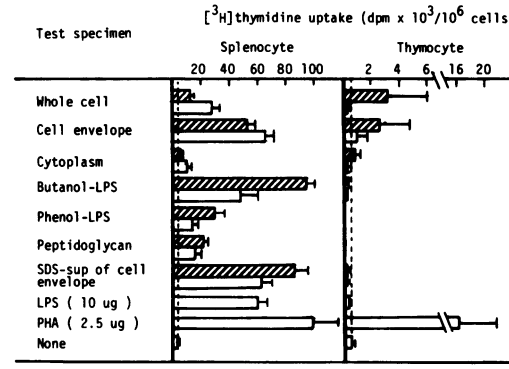

FIG. 5. Mitogenic effects of subcellular fractions from *Capnocytophaga* on splenocytes and thymocytes from BALB/c mice. Results are expressed as mean disintegrations of [^{3}H]thymidine incorporation per minute per 10^6 cells in quadruplicate cultures. Dose of test specimens: ▨, 100 μg; □, 10 μg. PHA, Phytohemagglutinin (Difco); ⊢⊣, standard error.

siderable variation among individual animals with regard to the time course of the chronic lesions produced by the same specimen, comparative studies between the test fractions were made on the same animal. Profiles of the reactions shown by one representative rabbit are shown in Fig. 3. Injection of the whole cell fraction and CE produced chronic lesions characterized by recurrent multinodular lesions and simultaneous recrudescence of the redness and swelling. The chronic lesions evoked by the phenol-LPS were also the recrudescence of both severe redness and diffuse swelling developing over the primary lesion and the multinodular lesions which appeared between day 5 and day 18. The primary lesions of PG, which consisted of weak induration and redness, persisted until the end of the observation period for 30 days, with some exacerbation on day 17. In the case of the SDS-supernatant, the primary lesion showed mild redness with a nodule, and after this subsided, an indurated incrustation appeared at and around the injection site on day 10. The CP never produced any lesions.

(iii) Mitogenic activity. The mitogenic effects of subcellular fractions on splenocytes and thymocytes of guinea pigs and BALB/c mice are presented in Fig. 4 and 5. All of the test fractions except the CP showed a remarkable definite mitogenicity on splenocytes of both guinea pigs and BALB/c mice. Slight mitogenicity was noticed with the PG for thymocytes of guinea pig and with the whole cells and CE for those of mouse. The other fractions tested were essentially inactive.

(iv) Macrophage-stimulating activity. All test fractions significantly stimulated glucosamine incorporation of guinea pig peritoneal macrophages (Fig. 6).

(v) Activation of human complement system. Figure 7 shows a dose-response curve of the complement activation of a pool of normal fresh human serum by subcellular fractions from *Capnocytophaga*. Less than 200 μg of CE and SDS-supernatant added to 1 ml of serum consumed 50% of the hemolytic activity of the complement system, and a maximal and almost complete consumption was achieved at a dose of 400 μg/ml of the serum. The PG and LPS preparations also activated the complement system, but the extent of complement consumption remained

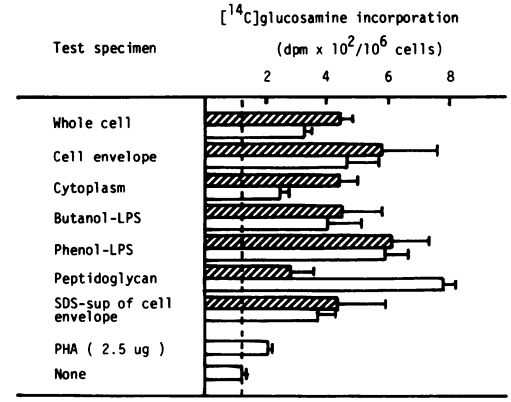

FIG. 6. Macrophage-stimulating activity of subcellular fractions from *Capnocytophaga*. Results are expressed as mean disintegrations of [^{14}C]glucosamine incorporation per minute per 10^6 cells in triplicate cultures. Dose of test specimens: ▨, 100 μg; □, 10 μg. PHA, Phytohemagglutinin (Difco); ⊢⊣, standard error.

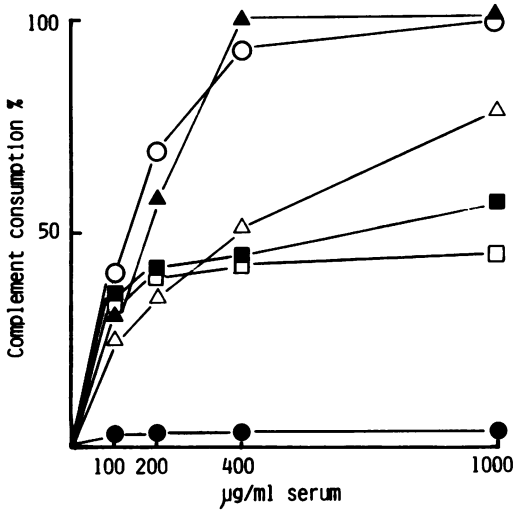

FIG. 7. Dose-response curve of subcellular fractions from *Capnocytophaga* activating the complement system in pooled human serum. Symbols: ○, CE; ●, CP; □, butanol-LPS fraction; ■, phenol-LPS fraction; △, PG; ▲, SDS-supernatant fraction of CE.

below 50% even at the highest dose of 400 μg. The CP was quite inactive.

Complement component (C1, C4, C2, C3, and C5) consumption profiles of the serum treated with the test fractions are illustrated in Fig. 8. The remarkable consumption of C1 and C4 was observed with the serum treated with each of test fractions other than CP, suggesting that these fractions activated the complement system through the classical pathway. However, the test for blocking effects of a chelating agent, ethylene glycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid (EGTA) on the activation by PG, phenol-LPS, and SDS-supernatant revealed that these subcellular components markedly activated the complement system even in the presence of EGTA. This finding strongly suggests that these subcellular fractions activated the complement system through the alternative as well as classical pathways.

(vi) Amoebocyte lysate gelation activity. The result shown in Table 3 indicates that a minimum effective concentration of the phenol-LPS was around 2×10^{-4} μg per 0.2 ml of the reaction system, whereas the SDS-supernatant and CP needed 20 and 10 μg, respectively, which are the 10^5-fold and 5×10^4-fold minimum effective doses of the phenol-LPS needed to form a solid gel, respectively. This finding indicates that possible contamination of the SDS-supernatant and CP by endotoxic LPS was negligible, if any.

DISCUSSION

Studies on physiological and morphological features of *Capnocytophaga* (11, 16, 25, 35) have revealed that this genus can be divided into three distinct biogroups, *C. ochracea*, *C. sputigena*, and *C. gingivalis*. The *Capnocytophaga* strain S-3 used in the present study was shown by Murayama et al. (J. Dent Res. **58**[special issue A]:965, 1978) to be physiologically similar to strain 25, which was one of the reference strains kindly supplied by S. S. Socransky (For-

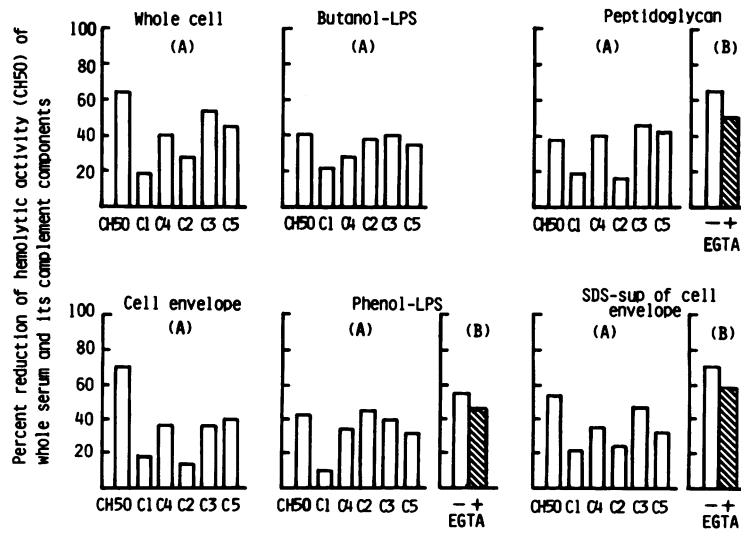

FIG. 8. Complement component consumption profiles in pooled human serum treated with subcellular fractions (0.2 mg/ml of serum) from *Capnocytophaga* (A) and comparison of complement consumption in the presence and absence of EGTA (100 mM) and $MgCl_2$ (50 mM) (B). CH_{50}, 50% of the hemolytic activity of the complement system.

TABLE 3. Gelation of amoebocyte lysate of horseshoe crab *(T. tridentatus)* by subcellular fractions isolated from *Capnocytophaga*

Test specimen	Gel formation at dose (ng/0.2 ml of assay mixture)[a]:										
	10^3	10^2	20	10	2	1	0.2	2×10^{-2}	2×10^{-3}	2×10^{-4}	2×10^{-5}
SDS-supernatant	++	+	±	−	−						
Cytoplasm	++	++	++	+	−						
Phenol-LPS					++		++	+	+	+	−

[a] ++, Solid gel that was not broken by slanting was formed; +, solid gel was formed, but was easily moved as a mass by slanting; ±, no solid gel was formed, but the viscosity of assay mixture was increased; −, no change.

syth Dental Center, Boston, Mass.) and classified as *C. ochracea* (25, 36).

Although possible involvement of *Capnocytophaga* in pathogenesis of periodontal diseases has been discussed in recent papers (22, 26, 27, 32), much remains to be elucidated about the mechanism of action. Test specimens used in previous studies to clarify possible etiological roles played by cellular components of *Capnocytophaga* were mostly crude soluble extracts which were obtained by sonication of whole cells and were vague in both chemical properties and their origin. With this in mind, we have made efforts to prepare subcellular fractions so as to avoid missing any of the cellular constituents.

The CE and CP were obtained from mechanically disrupted whole cells of the *Capnocytophaga*. The CE was then divided into a soluble (SDS-supernatant) and an insoluble (PG) fractions by hot SDS treatment. Chemical analyses proved that the PG specimen thus isolated was sufficiently pure, although it contained small amounts of non-PG amino acids. With regard to LPS (endotoxin), which characterizes the cell surface layer of gram-negative bacteria, we employed two different extraction procedures. The hot aqueous phenol extraction procedure is the most widely employed method for extraction of endotoxic LPS and is believed to be suitable for isolation of a purified specimen. However, Skidmore et al. (24) and Goodman et al. (9) demonstrated that the method of extraction played a critical role in determining the immunological activities. Therefore, the aqueous butanol method, which seemed to be milder in regard to extraction conditions, was used in addition to the hot phenol method.

Studies on the immunopotentiating activities of cellular components of bacteria in vivo have so far concentrated on PG in gram-positive bacteria and endotoxic LPS and PG in gram-negative bacteria. Thus, there is little information about immunomodulating activities of any other cellular components. Our finding that the SDS-supernatant fraction exerted a variety of remarkable immunomodulating activities comparable to those of endotoxic LPS and PG is noteworthy. At present, very little information is available on the chemical entity of the SDS-supernatant responsible for the multifold biological activities observed. The possibility that the biological activity shown by the SDS-supernatant is due to either contaminating PG or LPS can be excluded by the following facts. The amino acid and amino sugar analysis of the SDS-supernatant revealed a practical absence of muramic acid and diaminopimelic acid, characteristic components of bacterial PG. As far as LPS contamination is concerned, the KDO determination showed small amounts of the deoxy sugar in the SDS-supernatant. It should be pointed out, however, that the assay method used is not specific for KDO and that the CP which had negligible biological activities contained 2.5 times more KDO than was found in the SDS-supernatant. Furthermore, the dermotoxic reaction produced by the SDS-supernatant was quite different from that by the LPS, and although the amoebocyte lysate was gelated by the SDS-supernatant about 10^4 to 10^5 times the minimal dosage of the phenol-LPS was required to get a positive reaction. Taking together these facts, we can almost certainly exclude the possibility that the immunomodulating activities of the SDS-supernatant are associated with LPS.

There have been several studies demonstrating that bacterial cell surface components other than PG and LPS exert a mitogenic activity on lymphocytes. Namely, a fraction derived from the cytoplasmic membranes of nocardia (3–5), a lipid A-associated protein from gram-negative bacteria (2, 8), and substances prepared from the cell membranes of *Staphylococcus aureus* L forms (29, 30) have been studied. However, the chemical entities responsible for the observed mitogenic activities have not yet been clarified in all these substances.

The SDS-supernatant was shown to activate the complement system in human serum. It is well known that a lipid A portion of endotoxic LPS activates the classical pathway, and a polysaccharide moiety causes the activation of the alternative pathway (18). PG from gram-positive

bacteria (12, 37; S. Kotani, A. Kawasaki, S. Inai, K. Nagaki, M. Matsumoto, M. Inage, K. Yokogawa, S. Kawata, and A. Inoue, Int. J. Immunopharmacol. 2:213, 1980) and from *Treponema pallidum* (33) have been shown to activate the alternative as well as classical pathways of the human complement system. No bacterial components other than the polysaccharide moiety of endotoxic LPS and cell wall PG have been reported to activate the alternative pathway. So, the finding that the SDS-supernatant is active in this respect is interesting.

Regarding the mitogenic effect of *Capnocytophaga*, Diggens and Clagett (J. Dent. Res. 57[special issue A]:859, 1979) have reported that the *Capnocytophaga* butanol-LPS is a potent mitogen on splenocytes from all mouse strains tested, but the phenol-LPS stimulates the splenocytes from only one mouse strain. In contrast, Stevens et al. (27) reported that the *Capnocytophaga* LPS which was extracted by using hot phenol-water scarcely exhibits mitogenicity on mouse splenocytes. As for *Escherichia coli*, Skidmore et al. (24) have observed that the butanol-LPS contains potent mitogenic activity to spleen cells from C3H/HeJ mice, but the phenol-LPS does not. The biochemical basis of these observations was clarified by Morrison et al. (17) and Sultzer and Goodman (28), who showed that the butanol-LPS preparation contains a low-molecular-weight protein which is tightly bound to the lipid A moiety of LPS, whereas the phenol-extracted preparation lacks this protein. From these findings, it is suggested that the butanol-LPS from *Capnocytophaga* also contains some additional biologically active components which may be missed during the phenol extraction. In our study, however, the splenocytes from guinea pigs showed strong mitogenic responses to both butanol-LPS and phenol-LPS, but the splenocytes from mice only weakly reacted to both LPS preparations.

In conclusion, the present study shows that various components from the cell envelope of *Capnocytophaga* strain S-3 have multifold biological activities which should play important roles in the pathogenesis of periodontal diseases. It should be pointed out, however, that the observed immunomodulating activities of the PG and LPS are by no means unique properties of *Capnocytophaga*, but the common trait shared by almost all of cell wall PG of parasitic bacteria (18, 31; Kotani et al., Int. J. Immunopharmacol. 2:213, 1980). However, it would be interesting to see whether the number of immunomodulating activities of the SDS-supernatant fraction is unique to *Capnocytophaga* and to isolate the chemical entity responsible for these activities. A study along this line is currently in progress.

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