MICROBIAL PATHOGENICITY

Purification of immunosuppressive factor from *Capnocytophaga ochracea*

K. OCHIAI, H. SENPUKU* and T. KURITA-OUCHIAI

Department of Microbiology, Nihon University, School of Dentistry at Matsudo, Chiba 271 and *Department of Oral Science, National Institute of Infectious Disease, Shinjuku-ku, Tokyo 162, Japan

Capnocytophaga, one of the genera of oral bacteria, has been implicated in the pathogenesis of several diseases, including endocarditis, septicaemia and disorders of the oral cavity such as abscesses and periodontal disease. This study examined sonic extracts (SE) of *Capnocytophaga* strains for their ability to alter lymphocyte function. The SE of tested *Capnocytophaga* caused dose-dependent suppression of spleen cells in response to mitogen. This suppressive effect was heat-labile and sensitive to the proteolytic enzyme pronase E. The suppressive factor (SF) was purified from SE of *C. ochracea* by a combination of ultrogel-AcA34, high-pressure liquid DEAE ion-exchange chromatography and hydroxyapatite columns, which revealed a single band of 14 kDa by SDS-PAGE. Rabbit anti-serum against the purified SF inhibited the immunosuppression induced by SE of *C. ochracea* with the recovery of lymphocyte proliferation.

Introduction

The genus *Capnocytophaga* is part of the normal oral microflora found in the absence of oral disease [1]. *Capnocytophaga* spp. frequently have been isolated in large numbers from periodontal lesions of patients with localised juvenile periodontitis and other forms of periodontal disease [2–4]. An animal study with *Capnocytophaga*-mono-infected animals has clearly revealed its periodontopathic potential [5].

Dental plaque is extremely dense in bacterial cell content because of frequent interbacterial aggregations [6]. *C. ochracea* exhibits co-aggregation mediated by lectin–carbohydrate interaction. Furthermore, co-aggregated bacterial cells exhibit resistance to phagocytosis and the bactericidal action of polymorphonuclear leucocytes (PMNLs) [7]. As *Capnocytophaga* spp. can colonise periodontal pockets and possibly invade the surrounding tissue, investigation of the organism's ability to resist host defences is important for an understanding of the bacterial aetiology of periodontal disease and other infections. *Capnocytophaga* spp. also have the ability to inhibit the proliferation of human fibroblasts and promote the release of lysosomal enzyme from PMNLs, that contribute to the infection and the

destruction of gingival tissue [8]. Previous work has suggested that *Capnocytophaga* spp. cause neutrophil abnormalities in morphology and locomotion [9] and release of the products that inhibit neutrophil chemotaxis *in vitro* [10]. Moreover, *Capnocytophaga* spp. can degrade IgA and IgG [11].

Recently, several reports have described periodontopathic bacteria, such as *Actinobacillus* [12, 13] and *Capnocytophaga* spp. as the cause of bacteraemia and septicaemia in non-compromised and compromised hosts [14–17].

Several investigators have reported that some periodontopathic bacteria can suppress immune responses [18]. However, there is little information on the pathogenic potential of immunosuppressive substances in any periodontal disease.

Studies in this laboratory have investigated the immunoadjuvant effects of periodontopathic bacteria and the kinetics and the immunological role of immunosuppressive factor (SF) produced by these bacteria [19]. Previous reports showed that *A. actinomycetemcomitans* produces a low mol. wt SF and that it affects CD4/CD8 ratios and cytokine production [20–22]. The sonic extract (SE) from the cytoplasmic soluble fraction from *C. ochracea* also suppresses immunoglobulin production and causes a CD4/CD8 ratio disorder [23]. In the present study the SF from the cytoplasmic soluble fraction of

Received 12 May 1997; revised version accepted 28 March 1998.

Corresponding author: Dr K. Ochiai.

C. ochracea was purified by extensive column chromatography and examined for the suppression of proliferation of murine spleen cells.

Materials and methods

Bacterial strains and growth conditions

C. ochracea ATCC 33563 and *C. sputigena* ATCC 33123 were obtained from the American Type Culture Collection (Rockville, MD, USA). *Porphyromonas gingivalis* FDC 381 was also employed as a representative strain of periodontopathic bacteria in this study. The cells were grown in Brain Heart Infusion (BHI) Broth (Difco) supplemented with haemin 5 µg/ml and menadione 0.4 µg/ml in an anaerobic system (Model 1024, Forma Scientific, Marietta, OH, USA) for 2 days.

SE preparation

Micro-organisms were harvested by centrifugation (10 000 *g*), then washed three times with phosphate-buffered saline (PBS, pH 7.2). SE from the harvested bacterial cells was prepared by a method reported previously [19]. Briefly, the bacterial cells were resuspended in PBS and sonicated for 45 min on ice with a sonicator (Ohtake Works, Tokyo, Japan). Disruption of the bacteria was confirmed by microscopy. The samples were centrifuged at 8000 *g* for 30 min, and the cell-free supernate was stored at -20°C until used for the experiments. The protein concentrations in the SE were estimated with the BioRad protein assay (BioRad, Richmond, CA, USA). The following SE preparation was also employed for some experiments: SE in 50 mM potassium phosphate buffer (pH 7.2) was heated for 10 min at 100°C or treated with pronase E (Kaken Kogyo Ltd, Tokyo, Japan) for 24 h at 37°C. After treatment, the preparations were dialysed against distilled water. Polymixin B (Sigma)-treated SE (37°C, 30 min) was also prepared to study the effect of lipopolysaccharides (LPS) in SE.

Mice

C3H/HeN mice aged 7–8 weeks were obtained from Shizuoka Experimental (Hamamatsu, Japan) and LPS-low responder C3H/HeJ mice were obtained from Nihon-Clea (Tokyo, Japan). These mice were maintained in horizontal laminar flow cabinets and provided with sterile food and water *ad libitum* and were used at 8–9 weeks of age.

Proliferation assay

Spleen cells from C3H/HeN mice were washed and resuspended in RPMI 1640 supplemented with fetal calf serum 5%, 2.0 mM L-glutamine, kanamycin 0.2 mg/ml and 15 mM N-2-hydroxyethylpiperazine-N-2-ethansulphonic acid (HEPES) buffer (pH 7.2), and adjusted to 3.5×10^6 viable cells/ml before culture.

Lymphoid cells were cultured in triplicate in a 96-well microculture plate in a total volume of 0.2 ml of medium containing either Con A (Sigma) 0.5 µg or LPS (Sigma) 1.0 µg. Different concentrations of SE were added to these samples and cells were incubated for 72 h at 37°C in humidified air containing CO₂ 5%. Cultures were pulsed with 3.7 kBq of [³H]thymidine ([³H]TdR, Amersham, Buckinghamshire) during the final 6 h of the inoculation. After the labelling period, cells were harvested on to glass fibre filters with a multiple-cell harvester type MHC MO-YR 1162 (Biotech, Tokyo, Japan). The filters were dried, placed in vials with scintillation fluid and analysed with a scintillation counter (type LSC-673, Aloka, Tokyo, Japan). Each assay was repeated at least three times.

Extraction of LPS

Phenol-water-extracted LPS and butanol-water-extracted LPS were also prepared by the methods described previously [24, 25]. Briefly, lyophilised cells (10 g) were suspended in pyrogen-free water and phenol 90%. The mixture was stirred vigorously at 65°C for 20 min and then centrifuged at 7000 *g* for 20 min. The aqueous phase was removed, and the phenol phase and insoluble precipitate were re-extracted with water. The aqueous phase was dialysed extensively against distilled water, lyophilised and termed PW-LPS. The same weight of lyophilised cells was suspended in NaCl 0.85% in water. An equal volume of water-saturated butanol was added, and the aqueous phase was obtained after stirring the reaction mixture and centrifugation. The combined extracts of the aqueous phase were dialysed against distilled water and lyophilised. This was termed BW-LPS.

Preparation of fractions from bacterial cells

Cells of each strain (wet weight 17.5 g) were resuspended in PBS and homogenised with glass beads (0.10–0.11 mm, 40 g) in a Braun cell homogeniser (Model MSK, B Braun, Germany). Samples were kept standing at room temperature for 10 min; suspensions were then centrifuged at 8000 *g* for 20 min. Each supernate was obtained as a cytoplasmic fraction (CPF) after centrifugation at 100 000 *g* for 60 min and dialysis against distilled water followed by lyophilisation. The precipitate was resuspended in 0.05 M phosphate buffer (pH 7.0) and stirred for 30 min at room temperature with the addition of an equal amount of buffer containing n-lauroylsarcosine (N-dodecanol-N-methylglycine) 2%. The supernate was obtained by centrifugation as described above. This was termed the inner membrane fraction (IMF) after dialysis against distilled water and lyophilisation.

Purification of SF

To prepare the cytoplasmic fraction, the homogenised fraction was further centrifuged at 100 000 *g* for

60 min. The supernates containing cytoplasm were dialysed extensively against 20 mM Tris buffer (pH 8.2). The CPF from *C. ochracea* was dialysed against 10 mM Tris buffer (pH 7.4) and concentrated ($\times 10$) by Centricon 10 (Amicon, Lexington, MA, USA) and applied to an Ultrogel-AcA34 column, 6×40 cm, (IBF Biotechnics, France) pre-equilibrated with the same buffer. The column was then washed extensively. Ten-ml fractions were collected and monitored for UV absorbance and immunosuppressive activity (reduction of mitogen-induced [3 H] TdR incorporation by LPS). Active fractions were pooled, dialysed against 10 mM Tris buffer (pH 7.4) and then applied to an HPLC-DEAE 5PW (Toso, Tokyo, Japan) column pre-equilibrated in the same buffer. The column was then extensively washed and eluted with a linear NaCl gradient (0–0.5 M). Active fractions were pooled, concentrated by membrane filtration (Centricon 10; Amicon) and applied to an HPLC-hydroxyapatite-MP column, 8.8×10 cm, (Kanto Chemical, Tokyo, Japan). The column was washed extensively with the same buffer and eluted with a linear KH_2PO_4 – K_2HPO_4 gradient (0–0.5 M). Active immunosuppressive fractions were pooled and concentrated after dialysis.

SDS-PAGE

Samples at each step in the purification procedure were examined by SDS-PAGE. Samples were loaded at concentrations of 10–50 μg of protein/lane. Electrophoresis was performed at 15 mA for 60 min with resolving 15% and stacking 4% gels. Proteins were stained with Coomassie Brilliant Blue R-250. A low mol.-wt standard (BioRad) was used for the mol. wt estimate. The periodic acid-Schiff base method was also used for determination of the homogeneity of SF.

Enzyme and heat treatments of SF

The purified SF (4.0 μg) in 50 mM potassium phosphate buffer (pH 7.2) was heated for 10 min at 100°C. Pronase sensitivity was also tested by incubation of SF with pronase E (Kaken Kogyo) at 37°C for 2 h. The enzyme was inactivated by heating for 10 min at 100°C. After treatment, the preparation was dialysed against distilled water at 4°C. The samples were tested with a mitogen-induced proliferation assay with mouse spleen cells.

Preparation of anti-SF serum

Three New Zealand White rabbits (Shizuoka Experimental, Hamamatsu, Japan) were given four subcutaneous injections with 0.1 ml of a 1:1 emulsion mixture of purified SF from *C. ochracea* and complete Freund's adjuvant (Difco) on days 0 and 14. On day 28, rabbits were boosted with an inter-muscular injection of antigen. A total of 500 μg of purified SF was given to each rabbit. Serological analysis of various antigens with rabbit anti-SF serum was done by Western

blotting. Anti-SF serum was obtained from these rabbits and assayed for its inhibitory action on immunosuppression against SE.

Western blotting

Antigen preparations were transferred electrophoretically to a nitrocellulose filter after SDS-PAGE separation [26]. The filter was soaked in 50 mM Tris-HCl buffer (pH 7.3) containing 0.15 M NaCl and low-fat milk 5% and shaken gently for 30 min. The strips of nitrocellulose filter were incubated with antiserum for 4 h at room temperature. After washing and blocking, the membrane was incubated with a solution of horseradish peroxidase-conjugated anti-rabbit IgG mouse monoclonal antibody (Zymed Laboratories, San Francisco, CA, USA), followed by incubation in a solution containing 4-chloro-naphthol 0.05%.

Statistical analysis

Statistical analysis was performed with Student's *t* test.

Results

Effect of SE on mitogen

SEs of tested bacteria were first examined for their ability to alter mitogen-induced spleen cell proliferation as shown in Fig. 1A and B. A low concentration (5 μg /well) of the SE from *P. gingivalis* enhanced cell proliferation induced by Con A or LPS. However, a high concentration (20–40 μg /well) caused a dose-dependent inhibition. Two strains of *Capnocytophaga* revealed a strong immunomodulative activity, even at low concentrations. In particular, *C. ochracea* demonstrated maximum inhibition on mitogen-induced proliferation at 5–10 μg /well. The addition of this SE (5 μg /well) inhibited 65.2% of mitogenic effect of Con A and 75.1% of LPS. Moreover, the addition of 10 μg /well and 20 μg /well inhibited 90.4% and 99.2% of the proliferation induced by Con A and LPS, respectively. The effect of incubation time with SE on the proliferation of spleen cells was also examined. Pre-incubation with SE (1–6 h) strongly inhibited the proliferation induced by LPS (data not shown). However, pre-incubation with SE did not affect the survival ratio of spleen cells which was examined by the trypan blue exclusion method (survival ratio of pre-exposed cells > 95.3%). Therefore, the maximum inhibition dose (20 μg) of SE and pre-incubation of SE and the cells for 1 h was selected for the following experiments.

As it was important to determine which components in SE induced suppression, two strains of *Capnocytophaga* that had demonstrated strong immunosuppression were selected to elucidate what the effective components in the SEs were. The heat- and pronase-treated SEs were employed in these series of

Fig. 1. Effects of SE on the mitogenicity induced by Con A and LPS. Mouse (C3H/HeN) spleen cells were incubated at 37°C for 60 min with various amounts of SE from *C. ochracea* (○), *C. sputigena* (●), *P. gingivalis* (Δ) followed by the addition of an optimal mitogenic dose of Con A (0.5 μg) and LPS (1 μg) as described in *Materials and methods*. [³H]TdR incorporation was measured after incubation for 3 days. Points represent the mean values from three different experiments (reproducibility 81.2%). Control values were 0.67 SD 0.08 kBq (Con A) and 0.75 SD 0.11 kBq (LPS). Vertical bars represent the SEM. Significantly different from the respective controls: ***p* < 0.01.

experiments (Fig. 2A and B). The mitogen-induced proliferation was restored to various degrees by these treatments; heat-treatment of SE from *C. ochracea* produced recovery of up to 78.2% (Con A) and 90.4% (LPS) of the control, and with pronase-treatment, 85.1% (Con A) and 80.3% (LPS) (Fig. 2A and B). Similar recoveries were obtained for SE from *C. sputigena*. Spleen cells from LPS-low responder mice, C3H/HeJ, were also used to examine the inhibitory effect of native SE from two strains of *Capnocytophaga* employing the same procedure as above. Similar results were obtained with C3H/HeN mice (Fig. 3). SEs from strains of *Capnocytophaga* produced a strong suppression of 95.1–99.0% of the control, respectively, for both mitogens. Polymixin B (an LPS inhibitor) was used to elucidate the effect of LPS in SE. Polymixin B did not affect the suppression induced by these strains under the conditions of the reaction mixture containing SE (20 μg) and different concentrations of polymixin B (0.2–2.0 μg) (data not shown).

Suppressive effect of prepared fractions

Four different fractions were prepared from *C. ochracea* which strongly suppressed Con A- and LPS-induced cell proliferation (Fig. 4A and B). PW-LPS and BW-LPS did not have a strong immunomodulatory activity with either mitogen. The suppressive effect was not observed at tested concentrations of IMF. However, CPF from *C. ochracea* significantly suppressed Con A- and LPS-induced cell proliferation, dose dependently. Addition of 1 μg of CPF caused 60.4% inhibition of cell proliferation induced by Con A and LPS, and 20 μg induced 90.2% inhibition. This suppression was

Fig. 2. Effects of heat or pronase treatment on SE from *Capnocytophaga*. Mouse spleen cells were incubated with heat- or pronase-treated SE (20 μg) followed by the addition of an optimal mitogenic dose of Con A and LPS. [³H]TdR incorporation was measured after incubation for 3 days. Bars represent the mean value and SEM. Control (■), SE (□), heat-treated SE (▨), pronase-treated SE (▩). Significantly different from the respective controls: **p* < 0.05, ***p* < 0.01.

Fig. 3. Effects of SE on the mitogenicity of spleen cells from C3H/HeJ mice. Spleen cells were incubated with SE (20 μ g) followed by the addition of Con A and LPS. Control (▨), *C. ochracea* (■) *C. sputigena* (□). Significantly different from the respective controls. ** $p < 0.01$.

Fig. 4. Effects of various components of *C. ochracea* on the mitogenicity induced by Con A and LPS. Mouse spleen cells were incubated with various amounts of CPF (○), IMF (●), PW-LPS (□) or BW-LPS (■) from *C. ochracea*, followed by the addition of an optimal dose of Con A and LPS. Points represent the mean value from three different experiments (reproducibility 85.4%). Vertical bars represent the SEM. Control values were 0.81 SD 0.07 kBq (Con A) and 0.89 SD 0.11 kBq (LPS). Significantly different from the respective controls: * $p < 0.05$, ** $p < 0.01$.

restored by either heat or pronase treatment of this CPF (data not shown). These data suggest that SF from CPF of *C. ochracea* was at least proteinaceous in part.

Purification of SF

SF from CPF was purified by a combination of gel-filtration and HPLC-DEAE. As shown in Fig. 5B, the immunosuppressive fraction was obtained as a sharp peak. However, as several other bands were detected in this sample by SDS-PAGE (Fig. 6, lane D), the concentrated active fraction was further applied to a hydroxyapatite-MP column and eluted with elution buffer. SF fractions were purified by hydroxyapatite-MP column and then subjected to SDS-PAGE analysis. This clearly revealed one band that corresponded to a mol. wt of *c.* 14 kDa by Coomassie Brilliant Blue R-25 staining (Fig. 6, lane E). SF did not display any bands, as tested for the PAS method (data not shown). When this SF was examined for activity, ID₅₀ was 16.7 ng for LPS stimulation and 25.0 ng for Con A.

Inhibition of immunosuppressive activity of SF by rabbit anti-SF serum

The results of Western blotting showed that the rabbit anti-SF serum revealed a single band which was identical with SF in the lane of the DEAE-5PW preparation (Fig. 6, lane F). A difference was found in the serum titre. Therefore, the serum that possessed the highest titre was employed in the assay for inhibition of immunosuppressive activity of SF by rabbit anti-SF serum. This was examined by the proliferation assay system, stimulated with LPS. The addition of diluted anti-serum (1 in 100, 1 in 500) inhibited SE-induced immunosuppression 96.7% and 89.2%, and SF-induced immunosuppression 98.2% and 96.4%, respectively.

Characterisation of SF

The heat- and pronase-treated SF was employed in this study. The mitogen-induced proliferation was restored to various degrees by these treatments; heat treatment of SF produced recovery of up to 92.3% (Con A) and 97.6% (LPS) of the control, with pronase treatment producing recovery to 94.2% (Con A) and 98.1% (LPS).

Discussion

The *Capnocytophaga* spp. tested suppressed murine lymphocyte proliferative responses to mitogens with low amounts of SE (Fig. 1). In particular, *C. ochracea* revealed a strong immunosuppressive effect on the cell proliferation of murine spleen cells. The SF was purified from CPF by a column chromatography technique. At the first stage of the experiment, the suppressive activity of SE from *C. ochracea* decreased or was destroyed by either heat or pronase treatment

Fig. 5. Purification procedure of SF from *C. ochracea*. (A) CPF was applied on the Ultrogel-AcA 34 gel filtration column and eluted with 10 mM Tris-HCl buffer (pH 7.2); 20 ml of each fraction were collected at a flow rate of 1.6 ml/min. Suppressive activity of each fraction was estimated by $[^3\text{H}]\text{TdR}$ incorporation (see *Materials and methods*). (B) The biologically active fraction was applied to a DEAE-5PW column, and eluted by a linear gradient of 10 mM Tris-HCl buffer (pH 7.2) containing 0–0.5 M NaCl; 1 ml of each fraction was collected at a flow rate of 2 ml/min. (C) The biologically active fraction was applied on a hydroxyapatite-MP column, and eluted with 10 mM Tris-HCl buffer (pH 7.2) containing 0–0.5 M KH_2PO_4 ; 1 ml of each fraction was collected at a flow rate of 2 ml/min.

Fig. 6. SDS-PAGE analysis and Western blotting of SF from *C. ochracea*. Lane **A**, standard low mol. wt maker; **B**, CPF; **C**, Ultra-gel-AcA 34; **D**, DEAE-5PW; **E**, purified SF; **F**, Western blotting with DEAE-5PW and rabbit anti-SF serum.

(Fig. 2). After the purification procedure, SDS-PAGE analysis revealed that SF migrated as a single band corresponding to a molecular mass of 14 kDa. This molecule was protease and heat sensitive. The data clearly indicate that immunosuppression induced by SF from *C. ochracea* may be attributable to proteinaceous components. However, other components in SE, such as LPS, may contribute to the immunosuppression. Therefore, the following experiments were performed. LPS low responder C3H/HeJ mice were employed to clarify the participation of LPS in this suppression. Complete inhibition of cellular proliferation induced by either Con A or LPS was also found in C3H/HeJ mice (Fig. 3). Moreover, no immunomodulative activity was observed in experiments with two different preparations of LPS (PW-LPS and BW-LPS extracted from *Capnocytophaga* strains; Fig. 4A and B). Generally LPS demonstrates strong immunomodulative and biological activities [27]. Mixtures of different concentrations of polymixin B with SE did not inhibit suppressive activity (data not shown). The data clearly indicated that LPS of *Capnocytophaga* strains did not have any pathogenic potential in tests for various biological activities. A previous study also reported that LPS isolated from *Capnocytophaga* spp. did not have any pathogenic features [28].

It has been shown previously that the suppressive effect on the murine lymphocyte mitogenic response was caused by the exopolysaccharide from *C. ochracea* [29, 30]. Gormand *et al.* [17] reported

similar inhibition of lymphocyte response to these mitogens, and the inhibition was more potent with bacterial suspension than supernate. The SF described here was located in the CPF of *C. ochracea*, suggesting that the cytoplasmic SF remained intracellular and that the bacterial suspension contained a sufficient amount of SF to inhibit lymphocyte proliferation.

Purified T or B cells were not employed in this study. Therefore, the respective effect of SF on each subset of lymphocytes cannot be explained from these data. However, pre-incubation with SF and splenic cells revealed different cell proliferation induced by Con A and LPS (data not shown). A decrease of CD4/CD8 ratio and lymphocyte proliferation induced by Con A has been reported in an endocarditis patient with *C. ochracea* [17] and IL-2 receptor expression and Con A-induced proliferation of peripheral blood mononuclear cells were decreased during *C. ochracea* infection. Differences in response to mitogens and clinical aspects involving lymphocytes may suggest that disorder of a specific lymphocyte subset results in immunosuppression.

A. actinomycetemcomitans has been a suspected aetiological agent in localised juvenile and adult periodontitis, demonstrating a variety of virulence factors in in-vitro experiments. It can also cause disease such as bacterial endocarditis, meningitis, septicæmia and abscesses in extra-oral sites [12, 13].

A. actinomycetemcomitans produces a protease-labile, non-cytotoxic low mol. wt (14 kDa) suppressive factor [22]. Previous studies have shown that priming with a high concentration of SE from *A. actinomycetemcomitans* induced an immunosuppressive effect on immunoglobulin production, CD4/CD8 ratios and blastogenic activity to mitogen [20–22]. This inhibitory mechanism was, in part, clarified by the demonstration of suppression of IL-2 synthesis, IL-2 receptor expression and IL-6 secretion [20, 21]. Finally, *A. actinomycetemcomitans* produced the development of humoral or cell-mediated immune responses, or both, via the modulation of the T-cell subset and cytokine production. The evidence found in *A. actinomycetemcomitans* infection indicates the possibility that similar mechanisms may exist in capnocytophaga infection.

Some of the recent cases of bacteraemia have originated from the oropharynx, although the gastrointestinal tract has been the most common source of bacteraemia [31–33]. The high frequency of systemic infection due to *C. ochracea* suggests that periodontal disease has to be considered as a possible precursor of systemic infection. It may be said that the classic source of bacteraemia in compromised or non-compromised patients, i.e., colonic microflora, appears to have been replaced by oral microflora in the anaerobic-capnophilic bacteraemias. Furthermore, another problem of concern in capnocytophaga infection is that strongly β -lactamase-positive *Capnocytophaga* strains have been isolated from the patients [34, 35].

This study has demonstrated that *C. ochracea* produces a proteinaceous inhibitory factor. The intracellular SF and exopolysaccharide may affect the host defence system by their combined actions both locally and systemically. Therefore, studies on the effect of SF as immunosuppressive treatment in capnocytophaga infection would be of great interest. The exact kinetics and immunological role of purified SF on the human immune system require further study.

This work was supported in part by grants-in-aid for the Frontier Science from the Ministry of Education, Science, Sports and Culture of Japan.

References

- Holt SC, Kinder SA. *Capnocytophaga*. In: Staley JT, Bryant MP, Pfennig N, Holt JG (eds) *Bergey's Manual of systematic bacteriology*, vol 3. Baltimore, Williams and Wilkins. 1989: 2050–2058.
- Slots J. The predominant cultivable organisms in juvenile periodontitis. *Scand J Dent Res* 1976; **84**: 1–10.
- Holdeman LV, Moore WEC, Cato EP, Burmeister JA, Palcanis KG, Ranney RR. Distribution of *Capnocytophaga* in periodontal microfloras. *J Periodont Res* 1985; **20**: 475–483.
- Haffajee AD, Socransky SS, Smith C, Dibart S. The use of DNA probes to examine the distribution of subgingival species in subjects with different levels of periodontal destruction. *J Clin Periodontol* 1992; **19**: 84–91.
- Irving JT, Socransky SS, Tanner ACR. Histological changes in experimental periodontal disease in rats monoinfected with gram-negative organisms. *J Periodont Res* 1978; **13**: 326–332.
- McIntire FC. Specific surface components and microbial coaggregation. In: Mergenhagen SE, Rosan B (eds) *Molecular bases of oral microbial adhesion*. Washington DC, American Society for Microbiology. 1985: 153–158.
- Ochiai K, Kurita-Ochiai T, Kamino K, Ikeda T. Effect of coaggregation on the pathogenicity of oral bacteria. *J Med Microbiol* 1993; **39**: 183–190.
- Stevens RH, Sela MN, Shapira J, Hammond BF. Detection of fibroblast proliferation inhibitory factor from *Capnocytophaga sputigena*. *Infect Immun* 1980; **27**: 271–275.
- Shurin SB, Socransky SS, Sweeney E, Stossel TP. A neutrophil disorder induced by *Capnocytophaga*, a dental micro-organism. *N Engl J Med* 1979; **301**: 849–854.
- Van Dyke TE, Bartholomew E, Genco RJ, Slots J, Levine MJ. Inhibition of neutrophil chemotaxis by soluble bacterial products. *J Periodontol* 1982; **53**: 502–508.
- Kilian M. Degradation of immunoglobulins A1, A2, and G by suspected principal periodontal pathogens. *Infect Immun* 1981; **34**: 757–765.
- Siegmán-Igra Y, Schwartz D, Ophir O, Konforti N. Endocarditis caused by *Actinobacillus actinomycetemcomitans*. *Eur J Clin Microbiol* 1984; **3**: 556–559.
- Verhaaren H, Claeys G, Verschraegen G, de Niel C, Leroy J, Clement D. Endocarditis from a dental focus. Importance of oral hygiene in valvar heart disease. *Int J Cardiol* 1989; **23**: 343–347.
- Forlenza SW, Newman MG, Lipsey AI, Siegel SE, Blachman U. *Capnocytophaga* species: a newly recognised clinical entity in granulocytopenic patients. *Lancet* 1980; **115**: 567–568.
- Hauk AA, Sugar AM, Rushing JL. *Capnocytophaga* septicemia. *N Engl J Med* 1980; **302**: 922.
- Parenti DM, Snyderman DR. *Capnocytophaga* species: infections in nonimmunocompromised and immunocompromised hosts. *J Infect Dis* 1985; **151**: 140–147.
- Gormand F, Etienne J, Rigal D, Bonnard M, Fleurette J, Beaune J. T lymphocyte disorder after *Capnocytophaga ochracea* endocarditis. *Infection* 1989; **17**: 382–384.
- Shenker BJ. Immunosuppression: an etiopathogenic mechanism. In: Guggenheim B (ed) *Periodontology today*. Basel, Kraeger. 1988: 178–186.
- Ochiai K, Kurita T, Nishimura K, Ikeda T. Immunoadjuvant effects of periodontitis-associated bacteria. *J Periodont Res* 1989; **24**: 322–328.
- Kurita-Ochiai T, Ochiai K, Ikeda T. Immunosuppressive effect induced by *Actinobacillus actinomycetemcomitans*: effect on immunoglobulin production and lymphokine synthesis. *Oral Microbiol Immunol* 1992; **7**: 338–343.
- Kurita-Ochiai T, Ochiai K, Saito N, Ikeda T. Adoptive transfer of suppressor T cells induced by *Actinobacillus actinomycetemcomitans* regulates immune response. *J Periodont Res* 1994; **29**: 1–8.
- Kurita-Ochiai T, Ochiai K. Immunosuppressive factor from *Actinobacillus actinomycetemcomitans* down regulates cytokine production. *Infect Immun* 1996; **64**: 50–54.
- Ochiai K, Kurita-Ochiai T, Ikeda T. Effect of immunosuppression induced by periodontopathic bacteria on secondary infection. *J Dent Res* 1991; **70** (Special issue): 791.
- Morrison DC, Leive L. Fractions of lipopolysaccharide from *Escherichia coli* O111: B4 prepared by tow extraction procedures. *J Biol Chem* 1975; **250**: 2911–2919.
- Westphal O, Jann K. Bacterial lipopolysaccharides. Extraction with phenol-water and further application of the procedure. *Methods Carbohydr Chem* 1965; **5**: 83–91.
- Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979; **76**: 4350–4354.
- Nishihara T, Fujiwara T, Koga T, Hamada S. Chemical composition and immunobiological properties of lipopolysaccharide and lipid-associated proteoglycan from *Actinobacillus actinomycetemcomitans*. *J Periodont Res* 1986; **21**: 521–530.
- Stevens RH, Sela MN, McArthur WP, Nowotny A, Hammond BF. Biological and chemical characterization of endotoxin from *Capnocytophaga sputigena*. *Infect Immun* 1980; **27**: 246–254.
- Bolton RW, Dyer JK. Suppression of murine lymphocyte mitogen responses by exopolysaccharide from *Capnocytophaga ochracea*. *Infect Immun* 1983; **39**: 476–479.
- Bolton RW, Dyer JK, Reinhardt RA, Okano DK. Modulation of *in vitro* human lymphocyte responses by an exopolysaccharide

- from *Capnocytophaga ochracea*. *J Dent Res* 1983; **62**: 1186–1189.
31. Baquero F, Fernandez J, Dronda F *et al*. Capnophilic and anaerobic bacteremia in neutropenic patients: an oral source. *Rev Infect Dis* 1990; **12** Suppl 2: S157–S160.
 32. Funada H, Machi T, Yoneyama H *et al*. *Capnocytophaga sputigena* bacteremia associated with acute leukemia. *Kansenshogaku Zasshi* 1993; **67**: 622–628.
 33. Fujita S, Ayukawa T, Tonohata A. [Identification of *Capnocytophaga* species by microplate hybridization method, and its restriction endonuclease digestion patterns.] *Rinsho Byori* 1995; **43**: 1066–1070.
 34. Rummens JL, Gordts B, Van Landuyt HW. *In vitro* susceptibility of *Capnocytophaga* species to 29 antimicrobial agents. *Antimicrob Agents Chemother* 1986; **30**: 739–742.
 35. Arlet G, Sanson-le Pors MJ, Castaigne S, Perol Y. Isolation of a strain of β -lactamase-producing *Capnocytophaga ochracea*. *J Infect Dis* 1987; **155**: 1346.