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Membrane Components of *Treponema denticola* Trigger Proteinase Release from Human Polymorphonuclear Leukocytes

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Abstract. Tissue destruction during periodontitis is believed to be primarily brought about by leukocyte proteinases. We postulate that oral spirochetes cause discharge of polymorphonuclear leukocyte (PMN) lysosomal enzymes. Effects of *Treponema denticola* 53-kDa outer membrane protein, lipopolysaccharide (LPS), and peptidoglycan on degranulation of matrix metalloproteinases (MMP)-8 (collagenase) and -9 (gelatinase), cathepsin G, and elastase by human peripheral blood PMNs were studied by specific enzyme assays and Western blot analysis. *T. denticola* 53-kDa outer membrane protein was found to be a particularly efficient inducer of MMP-8 release. The induction was comparable with that of phorbol myristate acetate, a known inducer of PMN specific granule discharge. All of the treponemal substances, most notably the 53-kDa protein and LPS, induced release of MMP-9, a component of C-type granules. Both collagenase and gelatinase released from PMNs were mostly in active forms. Release of cathepsin G and elastase was also observed with the 53-kDa protein treatment. The other *T. denticola* substances did not induce release of these serine proteinases. Lactate dehydrogenase was not released from PMNs by the treatments, indicating that the degranulation was specific and not caused by toxic effects of the substances. This was confirmed by transmission electron microscopy of PMNs treated with the 53-kDa protein that showed rapid vacuole formation and cell shape changes but no disintegration of the cells. Thus, *T. denticola* may participate in the PMN-dependent extracellular matrix degradation during the course of periodontal inflammation by triggering the secretion and activation of matrix metalloproteinases.

Key words: *Treponema denticola*, neutrophils, degranulation, proteinases, matrix metalloproteinases, periodontitis.

Introduction

The number of spirochetes increases dramatically in subgingival plaque with periodontal disease severity. In advanced adult periodontitis, spirochetes constitute up to 50% of the total microscopic plaque flora (Listgarten, 1976; Armitage *et al.*, 1982; Moore *et al.*, 1991). *Treponema denticola* is one of the oral spirochetes associated with periodontitis (Moore *et al.*, 1991; Riviere *et al.*, 1992) and acute necrotizing gingivitis (Riviere *et al.*, 1991). Several potential virulence factors have been identified in the organism (Holt and Bramanti, 1991; Ellen *et al.*, 1994). A major antigen of *T. denticola* is a 53-kDa outer membrane protein (Haapasalo *et al.*, 1992). *T. denticola* also possesses a versatile outer-membrane-associated chymotrypsin-like proteinase with potential to degrade basement membrane proteins laminin-1 and type IV collagen (Uitto *et al.*, 1988a,b; Grenier *et al.*, 1990). It has become obvious, however, that collagenase and other matrix-degrading proteinases present in diseased periodontal tissues are primarily derived from polymorphonuclear leukocytes (PMNs) (Uitto *et al.*, 1981; Sorsa *et al.*, 1991; Mäkelä *et al.*, 1994). PMNs contain several proteinases that are stored in different subcellular PMN compartments. Collagenase (MMP-8) and PMN gelatinase-associated lipocalin (NGAL) are components of specific (secondary) PMN granules, while gelatinase (MMP-9) is stored in C-type granules (tertiary granules) (Janoff, 1972; Borregaard *et al.*, 1993). During or after release from PMNs, the latent forms of the matrix metalloproteinases are converted into active forms through proteolytic cleavage or by reactive oxygen species (Weiss, 1989; Saari *et al.*, 1990). Interestingly, procollagenase can also be activated by *T. denticola* proteases (Uitto *et al.*, 1986, 1992; Sorsa *et al.*, 1992). Serine proteinases elastase and cathepsin G are components of azurophilic (primary) PMN granules (Borregaard *et al.*, 1993). *T. denticola* has been shown to exert cytopathic effects on gingival fibroblasts (Weinberg and Holt, 1990; Baehni *et al.*, 1992), epithelial cells (Keulers *et al.*, 1993; Uitto *et al.*, 1995), lymphocytes (Shenker *et al.*, 1984), and red blood cells (Grenier, 1991). Very little information exists

about the action of treponemes on PMNs. We report here the potential of *T. denticola* membrane components, the 53-kDa outer membrane protein, lipopolysaccharide (LPS) and peptidoglycan, to induce release of matrix-degrading neutral proteinases of human PMNs.

Materials and methods

Purification of *T. denticola* components

Potential pathogenic factors were purified from cultured *T. denticola* ATCC 35405 cells. The 53-kDa outer membrane protein was purified by fast protein liquid chromatography anion-exchange chromatography of Triton X-100 extracts of the bacteria as described previously in detail (Haapasalo *et al.*, 1992). The preparation contained a small amount of chymotrypsin-like protease as a contaminant. Peptidoglycan was purified by means of a modification of the method of Heckels and Virji (1988) and by LPS according to the method of Darveau and Hancock (1983), as described previously (Grenier and Uitto, 1993). Preliminary experiments indicated that effects of the substances were concentration-dependent. For the sake of consistency, 15 µg/mL was chosen as the concentration for comparison of the effects of the three *T. denticola* components.

PMN cultures

Blood was obtained from healthy donors who consented to a protocol that was reviewed and approved by the Ethical Committee of Oulu University, Finland. Heparinized peripheral blood (PB) was centrifuged for 10 min at 200 x *g*. Supernatant was removed, and the cells were washed twice with Hanks' balanced salt solution (HBSS, Orion Diagnostica D-27, Helsinki, Finland). PMNs were prepared by dextran sedimentation followed by hypotonic lysis of contaminating red blood cells in 50 mM Tris, 6 mM NH_4Cl, pH 7.2, buffer before Lymphoprep (specific activity 1.078 g/mL, Nyegaard, Oslo) density gradient isolation. The proportion of contaminating mononuclear cells was less than 5%. Freshly isolated PMNs (3×10^7 cells in 3 mL of HBSS) were incubated with the test substances at 37°C in air for 5 to 120 min with a constant gently shaking. As positive control, the PMN degranulation was triggered by phorbol myristate acetate (PMA, 50 ng/mL).

Enzyme assays

After the triggering periods, the PMN suspensions were chilled on ice and centrifuged at 500 x *g* for 10 min. The cell-free medium was analyzed for collagenase (MMP-8), gelatinase (MMP-9), elastase, and cathepsin G activities. For collagenase assay, the samples were incubated with 1.5 µM soluble type I collagen for 2 to 6 hr at 22°C. Collagenase activity was measured by the conversion of the intact α-chains to αA-(3/4 α) degradation products by means of a SDS-7.5% polyacrylamide gel electrophoresis/laser-densitometric assay (Sorsa *et al.*, 1992). SDS-polyacrylamide gel electrophoresis involved in the various assays was done according to Laemmli (1970). MMP-9 activity was assayed by gelatin (heat-denatured type I collagen) zymography as described earlier (O'Grady *et al.*, 1984). Briefly,

gelatin, 1 mg/mL, was mixed with 10% polyacrylamide. Following electrophoresis, the slab gels were incubated for 2 hr with 2.5% Triton-X 100 (30 min) and then washed with distilled water. The gels were then incubated for 4 hr at 37°C in 50 mM Tris-HCl, 0.15 M NaCl, 5 mM $CaCl_2$, and 5 µM $ZnCl_2$, pH 7.8. The gels were stained with Coomassie brilliant blue and destained with 20% methanol/5% acetic acid (20°C), and the molecular weights of gelatinolytic zones were evaluated by comparison with pre-stained molecular weight standards. We assayed total gelatinase activity by incubating 10 µL of PMN medium with soluble ^{125}I-labeled gelatin (Sigma; 1.5 µM, 20,000 cpm) for 3 hr at 37°C (Mäkelä *et al.*, 1994). The degradation products were counted for radioactivity after the undegraded substrate was removed by precipitation with 20% trichloroacetic acid. The PMN-released material was assayed for elastase activity with the synthetic peptide succinyl-alanyl-alanyl-prolyl-valyl-p-nitroanilide (SAAVNA, 1 mM; Sigma, St. Louis, MO, USA) and for cathepsin G activity with succinyl-alanyl-alanyl-prolyl-phenylalanine-p-nitroanilide (SAAPNA, 1 mM, Sigma) as substrates, as described previously (Bieth *et al.*, 1974). Incubation time with 10 µL of medium and 1 mM chromogenic substrates was 2 hr at 37°C. One enzyme unit is defined as the amount of enzyme producing optical density change of 0.001/min at 405 nm. In all enzyme assays, *T. denticola* samples incubated without PMN's were included, and the activity values were subtracted from the corresponding values of PMN's incubated with the bacterial substance. Lactate dehydrogenase was assayed in medium to indicate the PMN viability. A 50-µL quantity of medium sample was incubated with 70 mM pyruvate, 6 mM EDTA, and 0.18 mM NADH in 60 mM Tris buffer, pH 7.4, at 37°C. The LDH activity was measured spectrophotometrically at 340 nm as previously described (Grenier and Uitto, 1993).

Western blot analysis

The molecular forms of PMN collagenase, gelatinase, and PMN gelatinase-associated lipocalin (NGAL) released by the potential pathogenic components of *T. denticola* were analyzed by Western-blotting with specific antisera used as previously described (Sorsa *et al.*, 1994). The specific antiserum against human PMN collagenase (MMP-8) was kindly provided by Dr. Jürgen Michaelis, Department of Pathology, Christchurch School of Medicine, Christchurch, New Zealand. Studies on specificity and characterization of the antibody have been published earlier (Michaelis *et al.*, 1990). Specific rabbit polyclonal antisera against human PMN gelatinase (MMP-9) and NGAL were provided by Drs. Lars Kjeldsen and Nils Borregaard, Granulocyte Research Laboratory, Rigshospitalet, Copenhagen, Denmark (Kjeldsen *et al.*, 1993). After heat-denaturation (100°C for 5 min) in Laemmli's sample buffer, PMN-released material containing 10 µg protein was subjected to SDS-10% polyacrylamide gel electrophoresis (Laemmli, 1970). After electrophoresis, proteins in the gel were electrotransferred onto a nitrocellulose membrane. After the unoccupied sites were blocked with casein, the membrane was first reacted with the primary antibody (1:1000 dilution) and then with alkaline-phosphatase-conjugated secondary antibody. Immunoreactive proteins were visualized by means of 5-bromo-
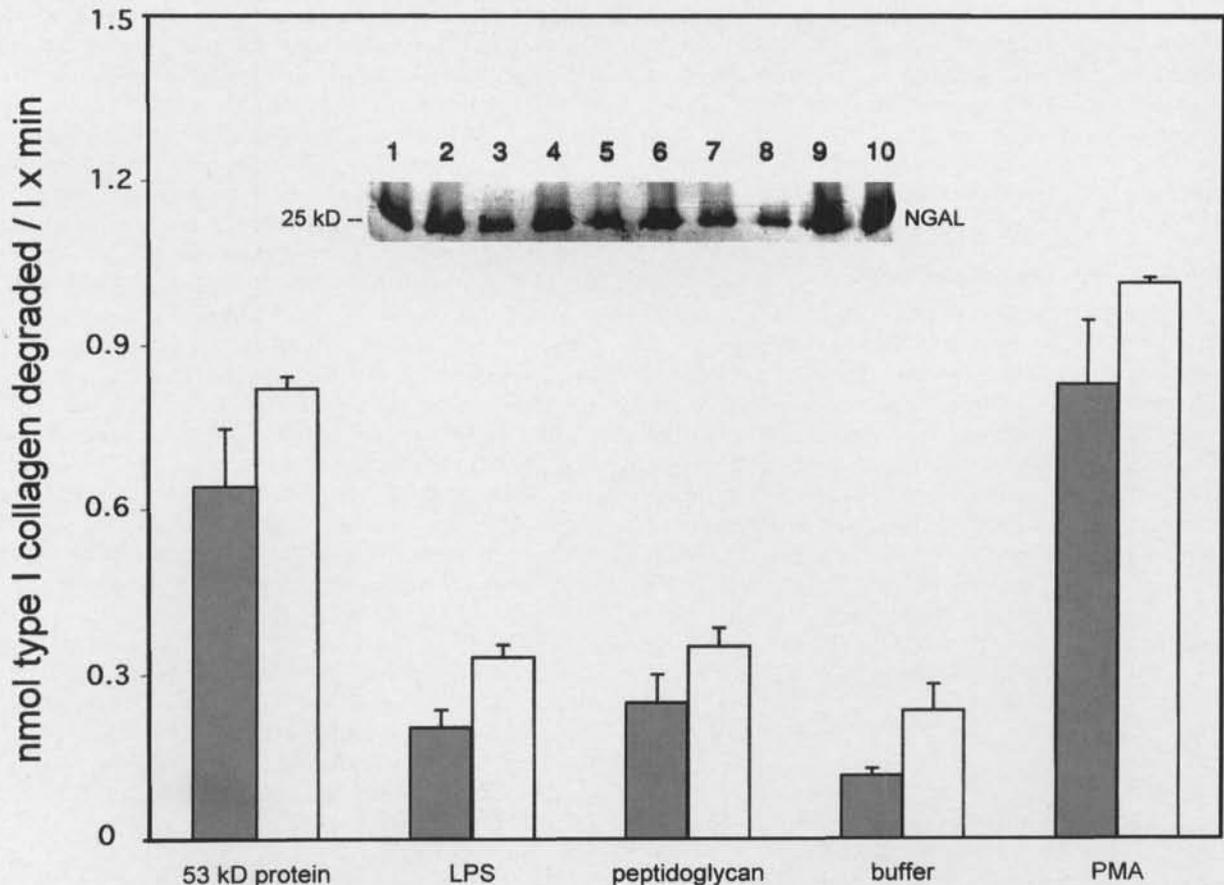
Figure 1. Effects of *T. denticola* membrane components on release of PMN collagenase and PMN gelatinase-associated lipocalin (NGAL). 53-kDa adhesin/porin, LPS, peptidoglycan, each 15 μg/mL, or phorbol myristate acetate (PMA), 50 ng/mL, was incubated with PMNs at 37°C. After 40 min of incubation, medium was assayed for collagenase activity with soluble type I collagen as substrate, without activation (shaded columns), or following activation with 1 mM aminophenylmercuric acetate (open columns). Values are means ± SD. Activity of all *T. denticola* substance samples and PMA was significantly different from that of the buffer control (p < 0.01, Student's *t* test). NGAL was detected, by Western blot analysis, in medium after 25 min (insert, lanes 1, 3, 5, 7, 9) or after 40 min (lanes 2, 4, 6, 8, 10) of incubation.

4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium.

Electron microscopy

The effect of the 53-kDa surface protein of *T. denticola* on PMN cell morphology was studied by transmission electron microscopy. PMN cells were incubated as above with 50 ng/mL, 1 μg/mL, or 15 μg/mL of the 53-kDa protein for 2, 10, and 40 min at 20°C. After the incubations, the samples were immediately fixed with 3% buffered glutaraldehyde for 2 hr at 20°C, and centrifuged at 1000 x g for 3 min. The samples were then washed twice with phosphate buffer (pH 7.3) and prepared for electron microscopy as previously described (Lounatmaa, 1985). The electron micrographs of the thin-sectioned specimens were taken with a JEM-1200EX transmission electron microscope at 60 kV.

Results

Small amounts of collagenase were released by unstimulated PMNs following a 40-minute incubation period. As expected, phorbol myristate acetate (PMA) increased release of active collagenase of PMNs by about seven-fold (Fig. 1). Pre-

treatment (40 min) of PMNs with *T. denticola* LPS and peptidoglycan resulted in moderate release of collagenase (Fig. 1). In contrast, *T. denticola* 53-kDa outer membrane protein promoted a collagenase release comparable with that of PMA treatment. The released collagenase was mostly in an active form (Fig. 1). Similar trends were observed in release of NGAL, another component of specific granules. About a three-fold increase in NGAL protein release was observed by 53-kDa protein treatment (insert in Fig. 1). A time-dependence experiment showed that the 53-kDa protein induced a steady release of active collagenase up to about 60 min, similar to the PMN's treated with PMA (Fig. 2). Pre-treatment with *T. denticola* LPS and peptidoglycan resulted in a much slower collagenase release. The 53-kDa protein and LPS were the most effective of tested substances to induce release of active gelatinase, a component of tertiary PMN granules (Fig. 3). In gelatin zymography, about 90% of gelatinase activity was found in the 92-kDa region (not shown). Peptidoglycan released active gelatinase significantly less than LPS or the 53-kDa protein, and as effectively as PMA (Fig. 3). In a separate experiment, none of

the test substances was found to contain proteolytic activity against collagen, gelatin, or the elastin substrate.

Molecular forms of collagenase and gelatinase released by PMNs were examined by Western blot analysis with use of specific MMP-8 (collagenase) and MMP-9 (gelatinase) antibodies. Medium of unstimulated cells showed weak immunoreactive bands with apparent molecular weights of 85 and 92 kDa, respectively (Fig. 4, lanes 1 and 2). The same immunoreactive bands were detected after treatments of PMNs with PMA, the 53-kDa protein, LPS, or peptidoglycan. The relative intensity of the bands roughly corresponded to the enzyme activity values measured in the different samples. Incubation of PMN's for 40 or 90 min with the 53-kDa protein resulted in formation of additional weaker protein bands reactive with the MMP-9 antibody at molecular weights both higher and lower than 92 kDa (Fig. 4B, lanes 5 and 6).

None of the *T. denticola* substances triggered release of the PMN elastase component of primary granules, over the buffer control within 40 min. In 120-minute incubations, however, the 53-kDa protein caused significant PMN elastase secretion. The 53-kDa protein also prompted a rapid liberation of cathepsin G (Table). The other *T. denticola* factors did not have any effect on cathepsin G discharge. Lactate dehydrogenase was not released from the PMN's by any of the factors, even after the long incubation times (120 min), indicating that the granule release was specific and not due to cell lysis. However, PMN cells that were incubated with 15 µg/mL of the 53-kDa protein showed clear changes in cellular morphology when examined by transmission electron microscopy (Fig. 5). Numerous small intracytoplasmic vacuoles were present, and the cells had an irregular shape, unlike the spherical, untreated, PMNs. The vacuoles had a diameter of 0.2 to 0.6 µm, and most of them were lined by a membrane. They often contained small spherical granules and some amorphous material (Fig. 5C). Lysed PMN cells were not observed. Vacuole formation was already seen in some cells, even after a short incubation time (5 min) with 15 µg/mL of the 53-kDa protein. After a 40-minute incubation period, vacuolization clearly increased. During this time period, 1 µg/mL of the 53-kDa protein also caused some vacuole formation (not shown).

Discussion

PMNs play a critical role in the body's defense against a bacterial challenge. For that purpose, stimulated PMNs release microbicidal substances, inflammatory mediators, and hydrolytic enzymes (Welsh *et al.*, 1971; Janoff, 1972; Borregaard *et al.*, 1993). While aimed at destroying the microbes and removing the tissue degradation product, these compounds also damage the extracellular matrix of the host tissue (Weiss, 1989). *In vitro* studies have shown that PMN-derived metalloproteinases and serine proteinases can efficiently degrade extracellular matrix constituents and activate complement components, thus having the potential to mediate tissue destruction (Janoff, 1972; Borregaard *et al.*, 1993). Matrix metalloproteinase activity derived from PMNs is markedly increased in the gingival tissue or tissue exudate (Uitto *et al.*, 1981; Sorsa *et al.*, 1991) and saliva (Uitto *et al.*,
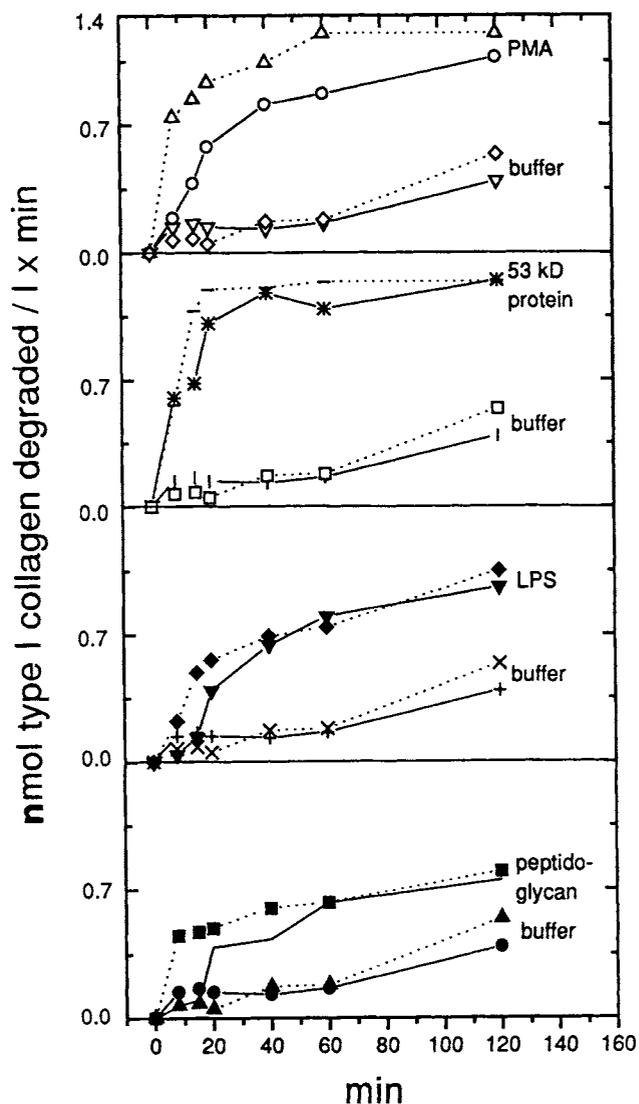
Figure 2. Time-dependent collagenase release from PMNs exposed to *T. denticola* membrane components. Concentrations of the *T. denticola* components and PMA were 15 µg/mL and 50 ng/mL, respectively. Incubation of medium samples and type I collagen was done at 22°C without collagenase activation (solid lines) or after activation with 1 mM aminophenyl mercuric acetate (broken lines).

1990; Mäkelä *et al.*, 1994; McCulloch, 1994) of periodontitis patients. Conversely, the total amount of matrix metalloproteinases in the periodontium as well as the ratio of active to latent enzyme are decreased following periodontal therapy (Hakkarainen *et al.*, 1988; McCulloch, 1994).

The goal of the present study was to determine whether membrane components of *T. denticola*—i.e., 53-kDa outer membrane protein, LPS, or peptidoglycan—can trigger PMNs to release matrix-degrading proteinases. Interestingly, while all of the components caused some release of the PMN proteinases, the different granules were selectively targeted by the substances. The 53-kDa outer membrane protein strongly stimulated release of collagenase and NGAL, a member of the lipocalin family binding to PMN gelatinase (Kjeldsen *et al.*, 1993). Both the rate and the extent of collagenase release from
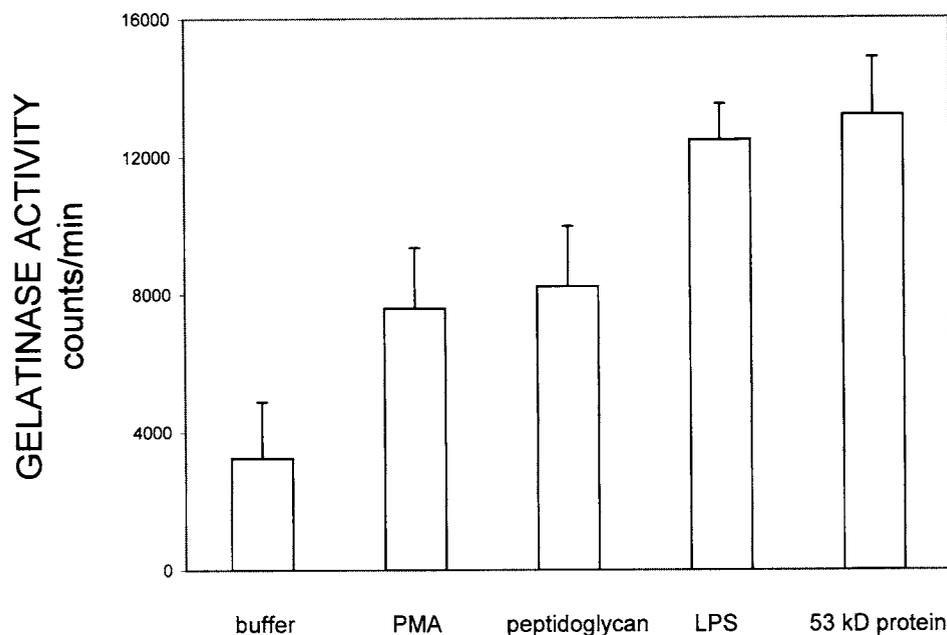
Figure 3. Release of gelatinase activity from PMNs exposed to *T. denticola* membrane components. PMNs were incubated with 15 µg/mL of the bacterial substances or 50 ng/mL of PMA for 40 min at 37°C. Medium samples were incubated with ^{125}I-labeled gelatin for 3 hr at 37°C. Values are means ± SD (n = 4) of radioactivity of the degradation products. Activity of all *T. denticola* substance samples and PMA was significantly different from that of the buffer control (p < 0.01, Student's *t* test).

appear to be important in *T. denticola* interaction with PMNs. Vacuole formation was already observed after a five-minute treatment of PMNs with the 53-kDa outer membrane protein. It is not yet clear how the vacuole formation is related to the release of the lysosomal granules and whether the bacterial-PMN interaction involves a specific signal transduction pathway. The fact that the specificity and the time-dependency of the enzyme release were very similar to those produced by PMA suggests that the protein kinase C pathway is operational. Alternatively, the 53-kDa porin may perturb the membrane function by rapidly fusing to the PMN membranes and inducing lysosomal discharge. In any case, the release of granular contents was not due to PMN disintegration. None of the factors caused lactate dehydrogenase release indicative of cell integrity. *T. denticola* LPS and peptidoglycan also stimulated PMN matrix metalloproteinase release. These treponemal membrane components may trigger degranulation during intracellular PMN digestion. Phagocytosis of the *T. denticola* has been observed *in vitro* (Olsen *et al.*, 1984). *T. denticola* peptidoglycan has been shown to exert strong toxic effects on cultured epithelial cells (Grenier and Uitto, 1993). The effects of the substances were found to be concentration-dependent. At present, it is difficult to calculate the actual amounts of the *T. denticola* components that come into contact with the PMNs in the periodontal pocket. Because massive amounts of spirochetes can face the epithelium *in vivo* (Listgarten, 1976), and because they may also release their surface components as outer membrane vesicles (Egli *et al.*, 1993; Uitto *et al.*, 1995), it can be assumed that on some areas of

specific granules were comparable with those caused by PMA, an activator of protein kinase C and a potent inducer of PMN-specific granule release (Bell, 1986; Kjeldsen, 1995). The 53-kDa protein and LPS were the strongest stimulators of gelatinase (MMP-9) release from C-type granules. The primary granules appeared to be less affected by the *T. denticola* components, with the exception of the 53-kDa protein that caused both cathepsin G and elastase release from PMNs.

The 53-kDa protein is a major surface antigen of *T. denticola*, and it possesses a dual function as both porin and adhesin (Haapasalo *et al.*, 1992; Egli *et al.*, 1993). Using immunogold electron microscopy, we observed that the surface proteins shed by the organism penetrate epithelial cells rapidly and cause vacuole formation (Uitto *et al.*, 1995). These properties also

Table. Time-dependent release of elastase, cathepsin G, and lactate dehydrogenase (LDH) from human neutrophils incubated for 20, 40, or 120 min in the presence of 50 ng/mL phorbol myristate acetate (PMA), 15 µg/mL *T. denticola* 53-kDa protein, 15 µg/mL *T. denticola* LPS, or 15 µg/mL *T. denticola* peptidoglycan

| Sample | Enzyme Activity[a] in Neutrophil Supernatant | | | | | | | | |
| | Elastase | | | Cathepsin G | | | LDH | | |
	20	40	120	20	40	120	20	40	120
Buffer	0.12 ± 0.02	0.13 ± 0.02	0.14 ± 0.02	0.23 ± 0.01	0.21 ± 0.04	0.17 ± 0.06	0	0	0
PMA	0.96 ± 0.06[b]	1.30 ± 0.29[b]	1.33 ± 1.21[b]	1.45 ± 0.02[b]	2.58 ± 0.08[b]	2.52 ± 0.24[b]	0	0	0
53-kDa protein	0.18 ± 0.03	0.18 ± 0.05	1.61 ± 0.18[b]	3.38 ± 0.13[b]	3.82 ± 0.17[b]	3.98 ± 0.18[b]	0	0	0
LPS	0.16 ± 0.03	0.17 ± 0.05	0.18 ± 0.02	0.25 ± 0.04	0.41 ± 0.04[c]	0.31 ± 0.04	0	0	0
Peptidoglycan	0.12 ± 0.03	0.09 ± 0.01	0.29 ± 0.04	0.11 ± 0.01	0.15 ± 0.01	0.48 ± 0.16	0	0	0

[a]   The enzyme activities are expressed as International Units, mean ± SE; n = 4.
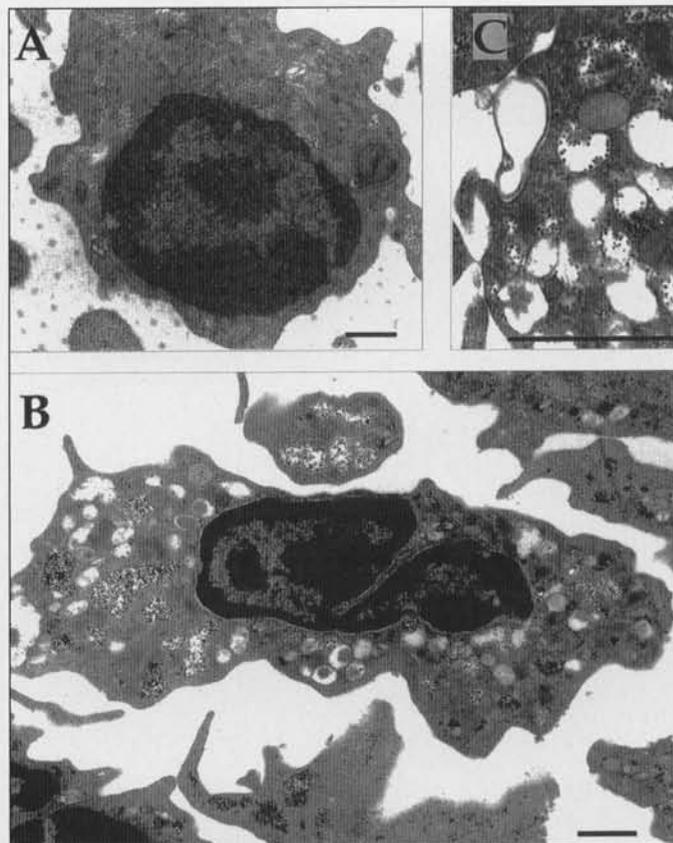[b]   p < 0.001.
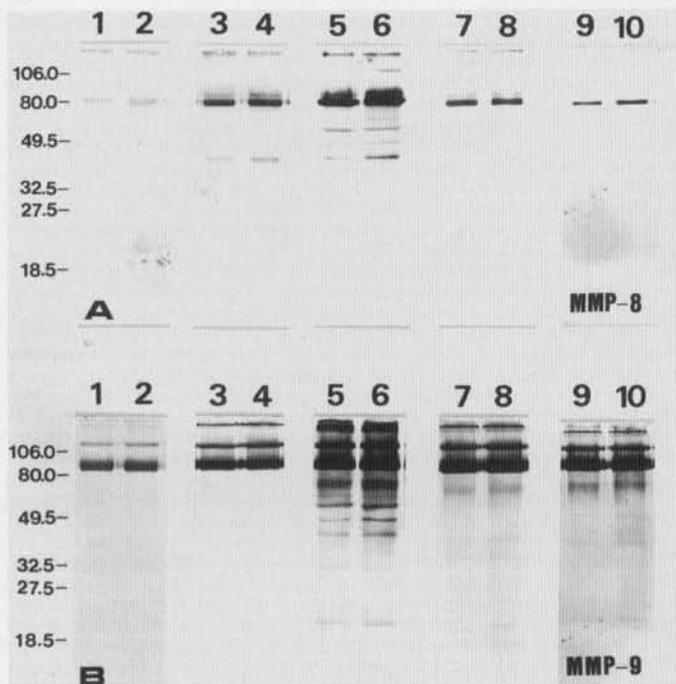
Figure 4. Western blot analysis of collagenase (A) and gelatinase (B) released from PMNs exposed to *T. denticola* membrane components. PMNs were incubated in buffer (1, 2), with 50 ng/mL of PMA (3, 4), 15 µg/mL of 53-kDa outer membrane protein (5, 6), 15 µg/mL of LPS (7, 8), or 15 µg/mL of peptidoglycan (9, 10), for 40 min (1, 3, 5, 7, 9) or 90 min (2, 4, 6, 8, 10).

Figure 5. Transmission electron microscopy of PMNs exposed to *T. denticola* 53-kDa outer membrane protein. (A) An untreated control PMN. (B) PMNs incubated in the presence of 15 µg/mL of the 53-kDa protein for 40 min. (C) A larger magnification of vacuoles observed in the cytoplasm of the treated PMNs. Bars = 1 µm.

the periodontal pocket these concentrations may be very high.

The 53-kDa protein also triggered release of PMN elastase and cathepsin G. Besides directly acting on extracellular matrix components, these proteinases may participate in activation of matrix metalloproteinases (Capodici and Berg, 1989; Saari *et al.*, 1990). The initial collagenase and gelatinase activation is probably due to autoactivation by reactive oxygen species generated in PMN's in response to the bacterial toxins (Weiss, 1989). Interestingly, porins from meningococci and gonococci increase induced intracellular hydrogen peroxide production in PMNs (Bjerknes *et al.*, 1995). The oxidative activation of matrix metalloproteinases can be subsequently potentiated by PMN cathepsin G, and the *T. denticola* chymotrypsin-like proteinase (Sorsa *et al.*, 1992). Thus, *T. denticola* has multiple mechanisms to promote tissue destruction during periodontal inflammation. First, its interaction with PMNs triggers release of serine proteinases and matrix metalloproteinases. Second, its potent chymotrypsin-like surface proteinase may directly participate in tissue degradation and activation of matrix metalloproteinases.

Acknowledgments

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