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Activation of the Interleukin-1β Precursor by *Treponema denticola*: a Potential Role in Chronic Inflammatory Periodontal Diseases

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There are several indications suggesting that interleukin-1β (IL-1β) may play an important role in inflammatory periodontal diseases. We hypothesized that periodontal sites would represent a unique combination of both cellular sources of IL-1β precursor (pro-IL-1β) and microbial proteases and proposed that *Treponema denticola*, a suspected periodontal pathogen, would play a critical role in the inflammatory nature of adult chronic periodontitis by activating pro-IL-1β. The aim of this study was thus to demonstrate the proteolytic cleavage and activation of the inactive precursor pro-IL-1β by *T. denticola*. After incubation of bacterial cells with recombinant pro-IL-1β, proteolytic cleavage was monitored by Western immunoblotting, and the biological activity of the digestion products was tested in a bioassay. We report here that *T. denticola* can cleave pro-IL-1β to yield two fragments with molecular masses of 18 and 19 kDa. Cleavage products showed a dose-dependent biological activity in the thymocyte proliferation bioassay, and this activity was inhibited by anti-IL-1β neutralizing antibodies. These results suggest that *T. denticola* may have a proinflammatory role in periodontal diseases.

Periodontitis, which affects the tissues surrounding and supporting the teeth, is the most common chronic inflammatory disorder in adults (38). The fact that the progression of periodontitis is episodic, with active and inactive phases, suggests opposing bacterial challenges and host defenses. Over the past years, many research groups have reported associations between specific bacterial species and the different forms of periodontitis (8). These studies suggested that periodontitis is a mixed infection involving several bacterial species acting in a cooperative or synergistic fashion (6). Spirochetes, including *Treponema denticola*, are found in high proportions in diseased periodontal sites (17, 25). *T. denticola* is known to produce a variety of proteolytic enzymes active against immunoglobulins, serum protease inhibitors, and basement membrane components (7, 18, 29, 31, 36). It is thus believed to participate in the perturbation of host defense mechanisms and in tissue destruction.

Although there is no doubt that certain bacterial factors play a direct role in the etiology of periodontitis, such factors may also indirectly lead to tissue destruction caused by the host's own defense mechanisms (2, 33). Inflammation is a localized protective response elicited by injury and infection. Whereas the absence of inflammatory response leads to a compromised host, excessive or sustained inflammation leads to chronic inflammatory diseases. This is because inflammation is an exceedingly potent amplification system involving the recruitment of humoral and cellular components of the immune system via a network of soluble mediators. Proinflammatory cytokines, such as interleukin-1 (IL-1), are key factors in the initiation and development of the inflammatory cascade. In addition, IL-1, which has pleiotropic effects on almost every cell type, can participate in attacking local tissues in periodontal disease by inducing collagenase production by fibroblasts and by promoting cartilage and bone resorption (4, 28, 34).

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IL-1β is a proinflammatory cytokine produced as an inactive precursor (pro-IL-1β) by various cell types, including monocytes, macrophages, and keratinocytes (4). This precursor, with a molecular mass of 31 kDa, must be cleaved enzymatically into a 17.5-kDa fragment to have biological activities (1, 3, 9, 20, 21, 26). Cells producing pro-IL-1β do not all possess the enzymatic activity required for the cleavage and activation of the inactive precursor. For example, keratinocytes produce but do not activate the pro-IL-1β, whereas monocytes use a highly selective IL-1β-converting enzyme (ICE) for cleaving between amino acids 116 (Asp) and 117 (Ala) to produce mature IL-1β (3, 14). Monocyte ICE is a member of the cysteine protease family. Proteolytic cleavage and activation of pro-IL-1β may also result from the action of mast cell chymase (24) or the pancreatic chymotrypsin (20, 23), whereas human cathepsin G and elastase (10) can yield fragments with lower biological activity.

The possibility that microbial enzymes display such convertase activity was investigated in *Streptococcus pyogenes*, and it was found that the exotoxin B, also a cysteine protease, can cleave and activate pro-IL-1β (13). We hypothesized that periodontal sites would represent a unique combination of both cellular sources of IL-1β precursor (epithelial cells, monocytes, neutrophils, etc.) and microbial proteases, and we proposed that *T. denticola* may play a critical role in the inflammatory nature of periodontitis by activating pro-IL-1β. The aim of the study was thus to demonstrate the proteolytic cleavage and activation of pro-IL-1β by *T. denticola*.

MATERIALS AND METHODS

Bacteria and growth conditions. The type strain *T. denticola* ATCC 35405 was grown in oral spirochete medium as previously described (16). Cultures were incubated in an anaerobic chamber (N_2-H_2-CO_2 [80:10:10]) at 37°C for 5 days (late exponential growth phase). Bacteria were harvested by centrifugation (10,000 × *g* for 15 min), washed twice in 0.1 M Tris hydrochloride buffer (Tris-HCl) (pH 8.0), and resuspended in the same buffer to an optical density of 2.0 at 660 nm.

Fluorometric assay for ICE-like activity. The fluorogenic substrate acetyl-Tyr-Val-Ala-Asp-7-amido-4-methylcoumarin was obtained from Peptides International (Louisville, Ky.). A stock solution was prepared at a concentration of 2

mM in dimethyl formamide and kept at −80°C. Before the assay was performed, the substrate was prepared at 50 μM in 0.1 M Tris-HCl (pH 8.0), and 50 μl was applied to a Whatman no. 1 paper strip (6 by 60 mm; Whatman Inc., Fairfield, N.J.). Bacteria obtained from the centrifugation of 100 μl of the bacterial suspension were applied to the strip. After an incubation of 2 h at 37°C in a humid atmosphere, hydrolysis of the substrate was monitored by observation under a UV lamp (wavelength of 366 nm). A positive reaction was indicated by the presence of a fluorescent spot on the paper strip.

Western immunoblotting assay for ICE-like activity. Recombinant human pro-IL-1β (12 μl; 1 μg/ml of Tris-HCl [pH 8.0]; Cistron Biotechnology, Pine Brook, N.J.) was incubated with the cell suspension of *T. denticola* (12 μl) for 3 h at 37°C. In one experiment, the degradation was evaluated by incubating the assay mixture for various periods of time (30 s; 5, 10, 15, 30, and 60 min; and 2, 3, 5, 8, and 25 h). After incubation, cells were removed by centrifugation (10,000 × *g* for 10 min). One part of denaturing buffer (0.125 M Tris-HCl [pH 6.8], 20% glycerol, 40% sodium dodecyl sulfate [SDS], 10% β-mercaptoethanol, 5% bromophenol blue) was then added to 3 parts of the sample, and the mixture was boiled for 5 min. Assays without bacteria or with bovine pancreatic chymotrypsin (12 μg; Sigma Chemical Company, St. Louis, Mo.) instead of bacteria were also performed. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) on a 12% resolving gel, using the buffer system of Laemmli (15) at a constant voltage of 100 V, and then electrophoretically transferred onto a nitrocellulose membrane. After blocking of the unreacted sites by a 90-min incubation in 20 mM Tris–500 mM NaCl buffer containing 1 M glucose, 10% glycerol, 0.6% Tween 20, and 2.5% bovine serum albumin, the membrane was incubated for 60 min with goat anti-IL-1β immunoglobulin G antibodies (1/500 dilution, 2 μg/ml; R&D Systems, Minneapolis, Minn.) and then with biotin-conjugated donkey anti-goat immunoglobulin G antibodies (1/1,000 dilution, 1.2 μg/ml; Jackson ImmunoResearch Laboratories Inc., West Grove, Pa.) for 30 min. Alkaline phosphatase-conjugated streptavidin (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) was used at a dilution of 1/500, and the enzymatic reaction was developed in a solution containing nitroblue tetrazolium chloride (1.65 mg) and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (0.8 mg) in 100 mM Tris-HCl, pH 9.5 (5 ml), containing 100 mM NaCl and 50 mM $MgCl_2$. SDS-PAGE markers from Bio-Rad Laboratories Ltd. (Mississauga, Ontario, Canada), recombinant pro-IL-1β, and mature IL-1β were included on each gel. The effect of heat treatment of *T. denticola* was evaluated by incubating the cell suspension at 56, 70, or 100°C for 30 min before the incubation with the IL-1β precursor. The effects of selected protease inhibitors on the cleavage of pro-IL-1β by *T. denticola* were also investigated. Iodoacetamide (for cysteine proteases), *p*-chloromercuriphenylsulfonic acid (for cysteine proteases), EDTA (for metalloproteases), 4-(2-aminoethyl)benzenesulfonyl fluoride (for serine proteases), or pepstatin A (for aspartic proteases) was included in the assay mixture at a concentration of 5 mM, 15 min prior to the addition of pro-IL-1β. Inhibitors were obtained from Sigma.

Biological assay for IL-1β activity. (i) Preparation of test sample. Equal volumes (100 μl) of the bacterial suspension and pro-IL-1β (1 μg/ml), both in 0.1 M Tris-HCl, were mixed and incubated at 37°C for 3 h. The mixture was then centrifuged (10 min at 10,000 × *g*), and the supernatant fluid was filter (0.22-μm pore size) sterilized and serially diluted in RPMI 1640–F-12 (1:1) medium containing 5% Fetal Clone 1 (HyClone Laboratories Inc., Logan, Utah), 2 mM glutamine, gentamicin (50 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and 50 mM β-mercaptoethanol prior to being added in 50-μl aliquots to the thymocyte assay.

(ii) Thymocyte assay. The thymocyte assay was based on the procedure described by Gery and Waksman (5). Thymocytes from 6- to 8-week-old male DBA-2 mice (Charles River Breeding Farms, Saint-Constant, Québec, Canada) were washed three times and suspended at a concentration of 3×10^7 cells/ml in RPMI 1640–F-12 (1:1) medium containing the various supplements as described above. Thymocytes (7.5×10^6 cells/ml) were added to flat-bottom 96-wells tissue culture plates. Wells also received serial dilutions of the test samples along with suboptimal doses (0.5 to 0.75 μg/ml) of concanavalin A (ConA; Flow Laboratories, Mississauga, Ontario, Canada). The final volume of the assay was 200 μl. Plates were incubated for 72 h at 37°C in 5% CO_2. The specificity of the reaction was assessed by the addition of goat anti-human IL-1β (0.6 μg/ml; R&D Systems) neutralizing antibodies in duplicate wells. All assays were performed in triplicate, and the mean ± standard deviation was calculated. A standard curve of mature IL-1β (0 to 1,000 pg/ml) was included in each of three similar experiments from which the data presented are derived.

MTT reduction assay. An MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay (27) was used to measure cell proliferation in individual wells. Briefly, half (100 μl) of the supernatant fluid was withdrawn, and 20 μl of MTT (Sigma) at a concentration of 2.5 mg/ml in Hanks balanced salt solution was added to each well. Cultures were incubated for 4 h at 37°C in an atmosphere of 5% CO_2. Formazan was then dissolved by adding 120 μl of a mixture of dimethyl formamide and 15% SDS in 0.04 N HCl for an overnight incubation at room temperature. Absorbance was read at 550 nm (test wavelength) with a 690-nm reference wavelength.

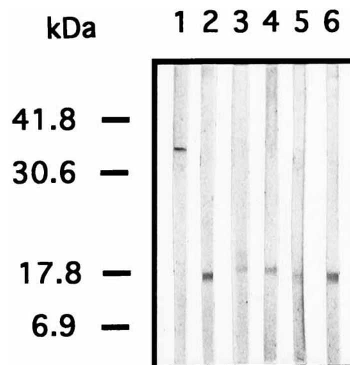

FIG. 1. Western immunoblot analysis of the cleavage of the IL-1β precursor by *T. denticola*. Lane 1, reference recombinant pro-IL-1β (12 ng); lane 2, reference recombinant mature IL-1β (12 ng); lane 3, pro-IL-1β (12 ng) incubated for 3 h with *T. denticola*; lane 4, pro-IL-1β (12 ng) incubated for 1 h with pancreatic chymotrypsin; lane 5, mature IL-1β (12 ng) incubated for 2 h with *T. denticola*; lane 6; mature IL-1β (12 ng) incubated for 2 h with pancreatic chymotrypsin. Molecular weight markers (indicated on the left) were, from top to bottom, carbonic anhydrase, soybean trypsin inhibitor, lysozyme, and aprotinin.

RESULTS

Enzymatic cleavage of pro-IL-1β by *T. denticola*. Using a simple qualitative assay, *T. denticola* cells were found to hydrolyze the fluorogenic substrate acetyl-Tyr-Val-Ala-Asp-7-amido-4-methylcoumarin. As this substrate closely matches the corresponding amino-terminal sequence (Tyr[113]-Val[114]-Cys[115]-Asp[116]) of the cleavage site of the ICE (35), this result suggests that *T. denticola* shares the unique specificity of monocyte ICE.

We then tested whether *T. denticola* was also active against the natural substrate pro-IL-1β. This was done by incubating *T. denticola* with recombinant pro-IL-1β and then analyzing the degradation products (in supernatant fluids) by SDS-PAGE and Western immunoblotting. After a 3-h incubation at 37°C, *T. denticola* had cleaved the pro-IL-1β to yield two fragments with molecular masses of 18 and 19 kDa (Fig. 1). As pancreatic chymotrypsin has been previously shown to produce an active fragment of about 18 kDa (20, 23), this enzyme was included to more easily localize low-molecular-weight fragments that are likely to possess mature IL-1β activity. Incubation (2 h) of mature IL-1β with *T. denticola* revealed a significant degradation of the molecule, yielding no fragments detectable in our assay.

Using this assay, it was found that the digestion of pro-IL-1β by *T. denticola* occurs very quickly (Fig. 2). A fragment of approximately 21 kDa is produced in a first step (within 30 s). This polypeptide is further degraded with the production of the 19-kDa fragment, followed by the 18-kDa fragment. After 3 h, the 18-kDa polypeptide is predominant. Longer incubation times were associated with a further degradation of this fragment by *T. denticola*. When *T. denticola* was recovered after a 3-h incubation period with the IL-1β precursor and submitted to the electrophoretic procedure, it appeared that no cleavage fragments attached to the *T. denticola* cell surface (data not shown). The cleavage of pro-IL-1β by *T. denticola* was found to be heat sensitive. Treating bacterial cells at 70°C for 30 min partially prevented the degradation of pro-IL-1β, whereas a complete loss of activity was observed after treatment at 100°C. Protease inhibitors were added to assay mixtures in order to characterize the class of proteolytic enzymes involved in the degradation of pro-IL-1β. Whereas none of the inhibitors tested could completely inhibit cleavage of the pro-IL-1β, *p*-

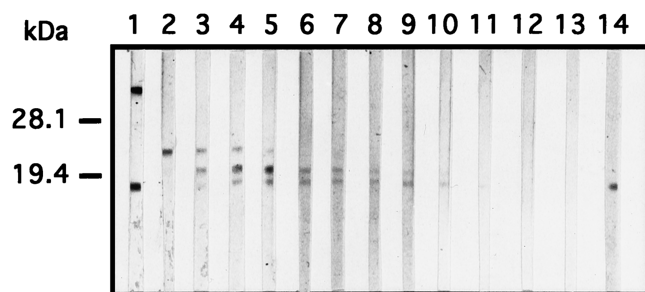

FIG. 2. Western immunoblot analysis of the degradation of the IL-1β precursor by *T. denticola* as a function of time. Lane 1, reference recombinant pro-IL-1β (12 ng) and mature IL-1β (12 ng). In lanes 2 to 12, pro-IL-1β (12 ng) was incubated for various periods of time with *T. denticola* before electrophoresis. Lane 2, 30-s incubation; lane 3, 5-min incubation; lane 4, 10-min incubation; lane 5, 15-min incubation; lane 6, 30-min incubation; lane 7, 60-min incubation; lane 8, 2-h incubation; lane 9, 3-h incubation; lane 10, 5-h incubation; lane 11, 8-h incubation; lane 12, 25-h incubation; lane 13, control of stability of mature IL-1β by a 25-h incubation with mature IL-1β instead of pro-IL-1β; lane 14, reference mature IL-1β (12 ng). Molecular weight markers were soybean trypsin inhibitor (28.1 kDa) and lysozyme (19.4 kDa).

chloromercuriphenylsulfonic acid, an inhibitor of cysteine proteases, prevented the generation of the 18- and 19-kDa fragments without affecting the production of the 21-kDa fragment (data not shown). However, the present experiments using a crude cell-derived mixture do not lead to a firm conclusion as to the nature of the enzyme(s) involved.

Cleavage of pro-IL-1β by *T. denticola* generates biologically active fragments. In the second part of the study, we evaluated whether the cleavage of pro-IL-1β by *T. denticola* yields biologically active fragments comparable to mature IL-1β. Thymocytes are sensitive to the simultaneous effects of ConA and IL-1β. When a suboptimal concentration of ConA was added to a thymocyte suspension in the presence of IL-1β, cell proliferation was observed in a dose-dependent manner (Fig. 3A). This effect was neutralized by incorporating a polyclonal antibody specific to IL-1β in the assay.

The supernatant fluid fraction containing the fragments (18 and 19 kDa) produced by *T. denticola* after incubation (3 h) with pro-IL-1β was found to possess potent IL-1β-like activity, as shown by an extended plateau between dilutions 1/12 and 1/40 in the dose-response curve (Fig. 3B). Indeed, a 1/400 dilution of the supernatant fluid caused cell proliferation comparable to that produced by the presence of approximately 3.3 pg of mature IL-1β/ml (Fig. 3A). The biological activity of the cleavage products was 82% inhibited by incorporating anti-IL-1β antibodies into the assay. No cell proliferation was observed when a control supernatant fluid (bacteria incubated in the absence of pro-IL-1β) was assayed, indicating that the proliferative effect is not due to stimulatory molecules released from the *T. denticola* cell surface.

DISCUSSION

IL-1β was detected in the gingival crevicular fluids of patients with periodontitis at higher concentrations than in subjects in a healthy control group (11, 30). Local production is suggested by the presence of IL-1β-positive cells in the lamina propria of gingival tissues and by a threefold increase of these cells in affected periodontal tissues (12). Unfortunately, the cellular sources of mature IL-1 were not identified in these studies.

Although inflammatory macrophages can synthesize and process the IL-1 precursor in its mature form, it is also possible that epithelial reservoirs of pro-IL-1 are taken up by microbial proteases, especially in trauma and tissue stress sites. In this study, we demonstrated the ability of *T. denticola* to cleave the inactive pro-IL-1β precursor with the production of biologically active fragments. The exact nature of the *T. denticola* enzyme(s) involved in the cleavage of pro-IL-1β is not clear. *T. denticola* is known to produce a 95-kDa chymotrypsin-like enzyme (36). As several of the proteases already known to activate pro-IL-1β have a chymotrypsin specificity, the 95-kDa protease of *T. denticola* may have a potential role to play in proteolytic activation. Preliminary assays using the purified enzyme did not produce the 18- or 19-kDa fragment but rather produced the 21-kDa polypeptide (data not shown). This 21-kDa fragment is likely to be an intermediary in the formation of the lower-molecular-weight polypeptides. This suggests that the chymotrypsin-like enzyme of *T. denticola* may be one of the enzymes involved in the activation of pro-IL-1β. The specific cleavage of the ICE substrate by *T. denticola* suggests the existence of a bacterial equivalent of the mammalian convertase whose role in *T. denticola* physiology is still unknown. The fact that none of the protease inhibitors tested were able to completely prevent cleavage of the inactive IL-1β precursor by

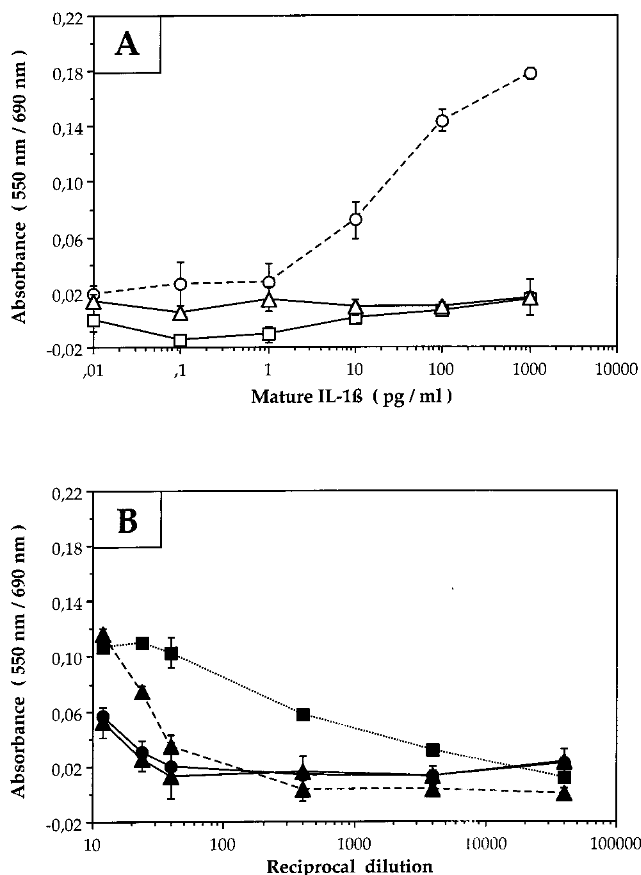

FIG. 3. Detection of biologically active fragments of pro-IL-1β generated by *T. denticola*. The thymocyte proliferation assay and the MTT colorimetric assay were used to generate the standard curve of recombinant mature IL-1β (A; ○) and the dose-response curve of biologically active fragments in supernatant fluids (various dilutions) of *T. denticola* incubated for 3 h with pro-IL-1β (12 ng) (B; ■). Also shown are the control responses of mature IL-1β alone, without ConA (A; □), and of *T. denticola* supernatant fluids incubated without pro-IL-1β (B; ▲—▲) and neutralization curves of mouse IL-1β (A; △) and of supernatant fluids of *T. denticola* incubated for 3 h with pro-IL-1β (12 ng) (B; ▲- - -▲) or in the absence of pro-IL-1β (B; ●) with 0.6 μg of specific anti-IL-1β antibodies per ml. Absorbance values of unstimulated control cells were substracted.

T. denticola cells is more evidence suggesting that the production of the 18- and 19-kDa fragments may require the participation of more that one proteolytic enzyme.

As *T. denticola* is present in high proportions in affected periodontal sites, it is possible that it can gain access to the epithelial and phagocyte IL-1β precursor and generate biologically active fragments. Indeed, *T. denticola* can attach to epithelial cells and increase permeability of the epithelium, thus favoring invasion of periodontal tissues (37). Interestingly, the presence of spirochetes has been previously demonstrated in histological tissue sections (22, 32). The cleavage of the IL-1β precursor could occur via secreted enzymes or via a reaction at the bacterial cell surface, as *T. denticola* has been shown to possess both surface and secreted forms of proteases (19). If this is so, *T. denticola* could exert a proinflammatory role in periodontal diseases and thus display a doubly deleterious effect on periodontal tissues by virtue of its own enzymes and by activating a dangerous inflammation cascade. The ability of other periodontopathogenic bacteria to activate the IL-1β precursor is now being investigated in our laboratory.

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