The surface protease of *Treponema denticola* mediates attachment of the bacteria to epithelial cells

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Abstract

Attachment of *Treponema denticola* ATCC 35405 was studied using a new epithelial model consisting of cultured periodontal ligament epithelial cells. *T. denticola* bound rapidly but selectively to the epithelial cells. Even at a high concentration of 1 x 10^{11} bacteria/ml, 31% of the epithelial cells did not bind any treponemes in confluent cultures. Some of the cells, however, bound large numbers of spirochetes. In sparse cultures with migrating epithelial cells higher amounts of *T. denticola* were bound than in confluent cultures. Electron microscopy showed direct contact of *T. denticola* and epithelial cell membranes without apparent preference in the adhesion sites in either of the cell types. Fibronectin and bovine serum albumin did not inhibit binding of *T. denticola* to the epithelial cells. In contrast, serum and fibrinogen markedly inhibited binding. Pretreatment of *T. denticola* with proteinase K, heating at 60°C or exposure to pH 3.2 inhibited the attachment by 30 %, 80% and 89 %, respectively, suggesting that *T. denticola* proteins were involved in the attachment. Protease inhibitors, phenylmethylsulfonyl fluoride and p-chloromercuribenzoic acid that inhibited the chymotrypsin-like protease of *T. denticola* also inhibited the attachment of the spirochetes to the epithelial cells. Purified chymotrypsin-like protease bound rapidly to the epithelial cells, and specific antibodies against the protease inhibited attachment of the treponemes to the epithelial cells. The results suggest that the binding to epithelial cells involves the *T. denticola* surface bound chymotrypsin-like protease.

Introduction

Oral spirochetes form a significant part of the microbial flora in advanced human periodontitis [1,2]. In the gingival pocket, the spirochetes have been shown to localize primarily on the loose surface layer of the plaque adjacent to the epithelium [3,4]. Species of the genus *Treponema* may have an important role in disease progress [5,6]. *Treponema denticola* the best known representative of this group of species possesses several potential virulence factors [7,8]. These include a variety of hydrolytic enzymes [9-15] cytotoxic peptidoglycan [16], factors interfering with fibroblast [17] and lymphocyte function [18]), hemagglutinating and hemolytic activity [19,20], and enhancement of neutrophil chemotaxis [21]. We have found that many of the cytopathic effects of *T. denticola* may be attributed to its surface-associated chymotrypsin-like protease [22]. Recent studies have shown that *T. denticola* binds to the extracellular matrix molecules laminin, fibronectin, types I and IV collagen and fibrinogen [23-25]. *T. denticola* has ability to attach to some cultured epithelial cells [26] and gingival fibroblasts [27,28]. Although the attachment to and invasion of the periodontal epithelial lining is of particular interest in the etiology of periodontal diseases, the mechanisms of these events are poorly understood. In this paper we examine binding of *T. denticola* to epithelium utilizing a novel periodontal epithelial culture model [29].

Materials and Methods

Bacterial cultures and suspensions.

T. denticola ATCC 35405 was grown and maintained in NOS broth containing (per 100 ml) heart infusion broth (1.25 g; Difco Laboratories, Detroit, Mich.), trypticase (1 g; BBL Microbiology Systems, Cockeysville, MD.), yeast extract (0.25 g; Difco), sodium thioglycolate (0.05 g; Difco), L-cysteine hydrochloride (0.1 g; Sigma Chemical Co., St. Louis, MO.), L-asparagine (0.025 g; Sigma), glucose (0.2 g; Difco), thiamine pyrophosphate (0.6 mg; Sigma), isobutyric acid, isovaleric acid and valeric acid (all fatty acids 0.001 % [vol/vol]; Sigma), sodium bicarbonate (0.2 % [wt/vol]; Fisher), heat inactivated horse serum (1 ml; Gibco, Burlington, Ontario, Canada), pH 7.4 [25]. Purity of the cultures was checked under phase contrast microscopy. A 3 d old *T. denticola* culture was transferred into 13 ml screw cap centrifugation tubes in a anaerobic chamber (Coy, Ann Arbor, MI.) and harvested by centrifugation (10 000 x g, 5 min, 4°C). The bacteria were resuspended and serially diluted with a known volume of phosphate buffered saline (PBS), pH 7.3 or minimum essential medium Eagle, alpha modification (αMEM; StemCell Technologies Inc., Vancouver, Canada) with 0, 1, or 15 % fetal bovine serum (FBS). The number of spirochetes was counted using a Petroff-Hausser bacterial counter (Hausser and Son, Philadelphia, PA.) under phase-contrast microscope.

Purification of chymotrypsin-like protease from *T. denticola*.

The chymotrypsin-like protease of *T. denticola* ATCC 35405 was purified by preparative polyacrylamide gel electrophoresis as previously described [12]. The 95 kDa protease was identified by overlaying part of the gel with Whatman paper soaked in 1 mM succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide (SAAPNA, Sigma) in 0.05 M Tris hydrochloride-0.2 M NaCl buffer (pH 7.8). After 15 min, enzyme activity was detected as a yellow band on both the gel and the Whatman paper. The section corresponding to the active enzyme was cut out and the protease was electro-eluted using a Bio-Rad electro-eluting unit. The eluate was dialyzed against 10 mM Tris HCl pH 7.5, lyophilized, then reconstituted in the same buffer to give a concentration of 100 μg/ml.

Chymotrypsin-like protease assay.

The chymotrypsin-like activity of *T. denticola* was assayed as follows: quadruplicates of 50 μl bacteria samples in 50 mM Tris, pH 7.8 were loaded into wells of a 96-well plate. 50 mM SAAPNA substrate stock was prepared by dissolving in N,N,-dimethyl formamide. The reaction mixtures containing the bacteria, 1 mM SAAPNA, 50 mM Tris-0.4 M NaCl-10 mM dithiothreitol, pH 7.8 were incubated at 37°C for 30 min. Color formation of the chromogenic substrate was then monitored at 405 nm using a 2550 EIA Reader (Bio-Rad).

Antibodies.

Polyclonal anti-*T. denticola* 53 kDa and 95 kDa surface antigen antibodies and antibody against whole *T. denticola* ATCC 35405 cells were raised in New Zealand White rabbits as previously described [30,25] . Goat anti-rabbit IgG conjugated either with fluorescein isothiocyanate (FITC; ICN, Ontario, Canada) or alkaline phosphatase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) were used for immunofluorescence studies or enzyme-linked immunosorbent assay (ELISA) respectively. IgG was affinity purified using protein A-Sepharose CL-4B (Sigma) column.

Epithelial cell culture.

Porcine periodontal ligament epithelial cells (PLE, or epithelial cell rests of Malassez) were isolated as described previously [31]. Frozen stock of the PLE were thawed and cultured in αMEM containing 0.01 % penicillin G (w/v), 0.1 % gentamycin sulfate (v/v), 1.2 % fungizone (v/v) (Gibco BRL: 30 μg amphotericin B and 24.6 μg sodium deoxycholate per ml final concentration), and 15 % FBS (Gibco BRL, 15 % FBS-αMEMS) and maintained in a humidified, 5 % CO_2 atmosphere at 37°C. The pure epithelial cell explant expressed K 5, 6, 14, 16 and 19 as their main cytokeratins [29]. For the experiments, PLE between the fifth and tenth passage were harvested by trypsinization and resuspended in 15 % FBS αMEMS. Appropriate dilutions of the cell suspension were plated onto wells of 8-well chamber slides (Nunc, Naperville, Illinois.) or 96-well flat

bottom tissue culture plates (Falcon) and incubated for varying periods in 15% FBS-αMEMS prior to adding spirochete.

Bacterial binding assay.

Attachment of *T. denticola* to cells was measured by immunofluorescence microscopy or ELISA. PLE cells were plated and maintained in wells of chamber slides to 30 or 95 % confluent as described above. For immunofluorescence studies the cells were washed with αMEM once and mixed with 0.2 ml of *T. denticola* suspended in αMEM. The mixture was incubated at 37°C in an atmosphere of air and 5% CO_2. In ELISA experiments *T. denticola* cells (1.4 x 10^{10} / ml) or 20 μg/ml chymotrypsin-like protease were added (20°C in air). If not otherwise stated the test medium was αMEM without serum. Controls consisted of PLE exposed to a corresponding amount of the spirochete transport medium.

Preparation of samples for fluorescence microscopy was as follows: After a 2 h challenge by *T. denticola*, PLE cultures were washed twice with αMEM, fixed with 2 % formaldehyde-0.05 % glutaraldehyde-PBS for 30 min and washed twice with PBS. The PLE were then subjected to the following treatment at 4°C: 1) permeablization by 0.2 % saponin (Sigma) in PBS for 20 min, 2) aldehyde group blocking by 0.5 mg/ml sodium borohydride (Fisher Scientific) in 0.2 % saponin-PBS for 20 min, 3) blocking by 3 % bovine serum albumin (BSA; Sigma) in 0.2 % saponin-PBS for 30 min, 4) anti-*T. denticola* 53 kDa surface antigen antibody (1 : 3 000) in 3% BSA-0.2 % saponin-PBS for 1 h, 5) PBS rinse, 6) goat anti-rabbit

IgG-FITC (1:200) mixed with 5 units/ml of rhodamine phalloidin (Molecular Probes, Eugene, Or.) in 3 % BSA-0.2 % saponin-PBS for 1 h, and 7) PBS rinse. The specimen was dehydrated in 30, 50, and 70 % ethanol, mounted by 10 % (w/v) 1,4 diazabicylco-[2,2]-octane (Sigma) and 0.02 % (w/v) sodium azide (Fisher Scientific) in 1:1 PBS-glycerol, and then examined under a fluorescent microscope (LSM10, Zeiss, Germany). The effect of fetal bovine serum, BSA (Sigma), human fibrinogen (Sigma), human fibronectin (Sigma), on the attachment of *T. denticola* to epithelial cells was studied as above. The effect of IgG antibodies on attachment was studied by pre-incubating *T. denticola* with antibodies and then washing the *T. denticola* with PBS before adding them to epithelial cells.

ELISA for *T. denticola* adhesion was performed as follows: The bacteria or the PLE were treated with the test compounds for 1 h in PBS at 20°C and then washed twice with PBS. After blocking the wells with 3 % BSA in PBS and washing twice with PBS, 200 µl of *T. denticola* cells (1.4 x 10^{10} / ml) in PBS was added to the wells. Following incubation for 1 h the wells were rinsed twice with 0.05 % Tween 20 in PBS and once with PBS. *T. denticola* whole cell antiserum (1 : 3 000 in 1 % BSA-PBS) was added to the wells incubated for 1 h, and rinsed as above, and then incubated for 1 h with goat-anti-rabbit antibody conjugated to alkaline phosphatase (1 : 3 000; Bethesda Research Laboratories). Color formation of the chromogenic substrate for alkaline phosphatase (p-nitrophenylphosphate; Sigma) was monitored at 405 nm using a 2550 EIA Reader. Attachment of PLE to the well after the treatment was assayed by Olympus CK2 inverted microscope.

Electron microscopy.

For scanning electron microscopy the cells were fixed with 2.5 % glutaraldehyde in PBS at 4°C for 16 h and processed by the tannic acid technique of Katsumoto et al. [32]. Specimens were mounted with silver dag, DC-sputtered with 15-20 nm gold in an Edwards coating unit (Gibco), and examined with a Cambridge Stereoscan microscope (Cambridge, U.K.).

For thin sections the cells were fixed at 4°C in 2.5 % glutaraldehyde for 2 hours. The specimens were post-fixed in 1 % osmium tetroxide for 20 min, stained in block in 1 % aqueous uranyl acetate, dehydrated, and embedded in Epon-Araldite. Ultrathin sections were placed on copper grids, stained sequentially with saturated uranyl acetate and lead citrate [33], and examined with a Phillips 300 transmission electron microscope.

Statistics.

The controls were normalized and the data were presented as mean percentage to facilitate comparison. The corresponding standard deviation was deduced according to the method of standard deviation measurement in functional relationships [34]. When applicable, difference between means were evaluated by non-parametric multiple comparison (Bonferroni test).

Results

T. denticola bound rapidly to epithelial cells. At 15 minutes near maximum attachment was obtained, and after 2 h the number of attached bacteria declined. The attachment was more efficient at 4°C than at 20°C (Fig 1 and 2A). Electron microscopic examination showed direct binding of *T. denticola* to the epithelial cell membrane (Fig 3A and 3B). In some areas *T. denticola* appeared to attach through vesicles on the bacterial cell envelope. Our study did not reveal any specific sites of *T. denticola* or the epithelial cells favoring the adhesion. In some cells, though, the treponemes were attached by their ends to small spherical bodies on the epithelial cells (Fig 3A).

Larger numbers of epithelial cells bound *T. denticola* as the spirochete concentration in the incubation increased (Fig 4). The density of the epithelial cell culture affected the bacterial binding. In 30 % confluent cultures the maximum number of*T. denticola* -binding epithelial cells (90% of the cells) was obtained at a concentration of 4 x 9^9 bacteria/ml, while in near confluent cultures the maximum proportion of bacteria-binding cells was lower (70 % of the cells) and it was obtained at much higher *T. denticola* concentration (2 x 10^{10} bacteria/ml)(Fig 4).

The number of bacteria bound per epithelial cell was highly variable (Fig 1,Table 1). Even at high bacterial concentrations a considerable proportion of cells bound no or only a few bacteria. In other cells of the same cultures up to 80 spirochetes could be counted.

Serum inhibited the attachment of the treponemes. In the absence of serum or in the presence of 1 % serum *T. denticola* binding was taking place at much lower bacterial concentrations than in the presence of 15% serum (Fig 4B). Some major serum proteins were tested for their effect on binding. Fibrinogen strongly inhibited the binding, serum albumin and fibronectin had no effect at the studied concentrations (Table 2, experiment 1).

To further characterize the nature of *T. denticola* binding to epithelial cells, the spirochetes were treated with a variety of compounds prior to measuring the attachment by the ELISA method (Table 3). Proteinase K treatment of *T. denticola* inhibited its binding by 30%, mixed glycosidase had no effect. Pretreatment at pH 3.8 had no effect but exposure to pH 3.2 inhibited the binding by 89 %. An inhibition of 80% was obtained by heating the bacteria at 60°C. Phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor and p-chloromercuribenzoic acid (pCMBA), an inhibitor of thiol proteases inhibited the attachment by 62 % and 78 % respectively (Table 3).

All treatments that inhibited *T. denticola* binding, i.e. heating, pH 3.2, PMSF and pCMBA also inhibited the activity of the chymotrypsin-like enzyme of *T. denticola* (Table 3). We therefore studied if antibodies against the chymotrypsin-like protease have an effect on the epithelial cell binding. Anti-chymotrypsin-like protease IgG and anti-whole cell IgG significantly inhibited the binding (Table 2). Antibodies against the 53 kDa major surface protein of *T. denticola* did had no effect on the binding.

Finally, the adhesion of the chymotrypsin-like enzyme to the periodontal ligament epithelial cells was studied. Similar to whole*T. denticola*, the protease bound rapidly to the cells and. After 15 min, binding of the protease appeared to decline (Fig 2B). The binding was somewhat better at 4°C than at 20°C

Discussion

Epithelial cells of different tissue origin and at varying stages of differentiation exhibit quite specific characteristics. These include organization of membrane bound and pericellular molecules that are responsible for the bacterial adhesion [35-37]. For these reasons it is important to use an appropriate cell type when studying binding of the periodontal microbes. We selected periodontal ligament epithelial cells (PLE) for our model cells in the attachment studies because they resemble junctional epithelial cells in many aspects. Both cells have the same developmental origin, the cells of the embryonic primary epithelial band. The cultured PLE cell lines do not keratinize and express a cytokeratin pattern very similar to junctional epithelial cells *in vivo* [29]. Cultured PLE cells express a number of matrix molecules and their receptors. Fibronectin, tenascin, laminin, kalinin and several β_1-integrins have been detected in PLE cultures [38, Uitto et al., unpublished data]. The PLE cells in culture also produce hyaluronic acid and various other glycosaminoglycans [39].

We have previously shown that *T. denticola* binds to a number of extracellular matrix and serum proteins, including type I and IV collagens, fibronectin, laminin, fibrinogen and serum albumin [24,25]. It was therefore surprising to find relatively limited spirochete attachment to epithelial cells carrying an array of different proteins on their surface. Scarce *T. denticola* attachment has also been found using other epithelial cells [26]. The periodontal ligament epithelial cells respond to the *T. denticola* challenge in different ways. Some cells bound none or only a few treponemes while at least 80 bacteria could be counted on other cells. Increasing the number of *T. denticola* did not cause a major shift in this binding pattern. Preferential attachment of *T. denticola* to a subpopulation of spherical cells of rat palatal epithelial cell cultures has been described [40]. The different amounts of *T. denticola* binding receptors appear to be associated with the different physiological status of the epithelial cells. The proportion of PLE cells binding *T. denticola* and the number of attached *T. denticola* per epithelial cell were higher in young sparse epithelial cell cultures than in older near confluent cultures. Migrating epithelial cells with free edges and active cytoplasmic protrusions are able to form effective attachments with other cells, substrate or particles. The cells of epithelial sheets with stabilized cell contacts lose their adhesiveness and ability to phagocytize [41,42]. This probably results by organization of the supramembraneous microfilament network [43] and loss or relocation of cell surface proteins, e.g. integrins [36,38]. We have recently found that migrating epithelial cells are more vulnerable to the cytopathic effects than cells of stationary epithelium [22].

Serum inhibited the spirochete attachment to the epithelial cells. Gingival crevicular fluid containing many serum components [44] may therefore protect epithelial cells from *T. denticola* binding. We asked if albumin, fibrinogen or fibronectin are responsible for the inhibitory effect. Fibrinogen strongly inhibited the attachment but the two other proteins did not. The finding suggests that *T. denticola* binding to epithelial cells is specific and possibly mediated by an adhesin recognizing also fibrinogen.

Heating and proteinase K treatment inhibited *T. denticola* binding to epithelial cells suggesting that bacterial proteins are involved in the process. The fact that treatment of *T. denticola* with protease inhibitors PMSF and pCMBA inhibited both cell binding and the chymotrypsin protease activity suggested that the outer membrane associated protease played a role in the *T. denticola* attachment. This idea was supported by the findings that antibodies against the chymotrypsin like protease inhibited the binding and that the purified protease attached to PLE cells. Interestingly, pretreatment of *T. denticola* with PMSF does not influence attachment to human gingival fibroblasts [45] and increases attachment to HEp-2 epithelial cells up to nine fold [46]. Probably, the contradiction between these findings are due to differences in the molecular texture of the different cell surfaces. This further emphasizes the importance of the proper selection of the cell model for bacterial attachment experiments.

The observation that the cell binding of whole *T. denticola* and purified chymotrypsin-like protease increased quickly and then declined indicates that the attachment is a dynamic process. Less avid binding at a

higher temperature may result from degradation of the receptor molecule by the protease and release of the attached bacteria. Degradation of fibronectin, concomitant with *T. denticola* binding, has been described in fibroblast cultures [45]. Possibly, the adhesin and the protease are located at a close proximity on *T. denticola* surface. A protease associated adhesin has been earlier described in another major periodontopathogen, *Porphyromonas gingivalis* [47]. We have observed that the surface located chymotrypsin-like protease is associated with a major hexagonal array surface adhesin/porin of *T. denticola* [25,48]. These two proteins are relatively difficult to dissociate by conventional purification procedures. We postulate that the chymotrypsin-like protease exists in a complex with the outer membrane protein and together they are responsible for adhesion and invasion capacity of the organism.

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Figure Legends

FIG 1. *T. denticola* binding to periodontal ligament epithelial cells. The interaction was observed under fluorescence microscopy using FITC-labeled antibody for *T. denticola* immunostaining and rhodamine/phalloidin for actin detection. A 30% confluent epithelial culture incubated with 2.5 x 10^8 spirochete/ml for 2 h at 37°C.

FIG 2. Time-dependent binding of *T. denticola* and its chymotrypsin-like protease to periodontal ligament epithelial cells. *T. denticola* (A; 1.4 x 10^{10} spirochete/ml) or purified protease (B; 20 μg/ml) was added to confluent epithelial cultures: open circles, 4°C; solid circles, 20°C. The binding was assayed with ELISA using anti-whole *T. denticola* antibodies.

FIG 3. Electron microscopy of *T. denticola* adhesion to periodontal ligament epithelial cells. **A:** Scanning electron micrograph. **B:** A thin section showing both direct cell to cell contact and attachment mediated by *T. denticola* vesicles (arrow).

FIG 4. Percentage of periodontal ligament epithelial cells binding at least one bacterium after challenged by *T. denticola*. High (A) or low (B) density epithelial cultures were incubated for 2 h at 37°C with *T. denticola* in αMEM containing different fetal bovine serum concentrations: solid circle, 15 % serum, open square, 1 % serum; open circle, no serum. Values are mean ± S.D. of 4 samples.

TABLE 1. The number of *T. denticola* binding to individual epithelial cells in sparse and dense cultures[a].

Number of bacteria bound	30% confluent PLE	95 % confluent PLE	
	3.5[b]	10[b]	100[b]
0	7	79	31
1 - 5	50	14	45
6 - 10	28	2	5
11 - 15	11	1	2
16 - 20	2	1	3
21 - 25	1	1	1
26 - 35	1	1	5
36 - 45	0	1	4
> 46	0	1	4

Periodontal ligament epithelial cells (PLE) were incubated with *T. denticola* in 15% FBS-αMEM for 2 h at 37°C.

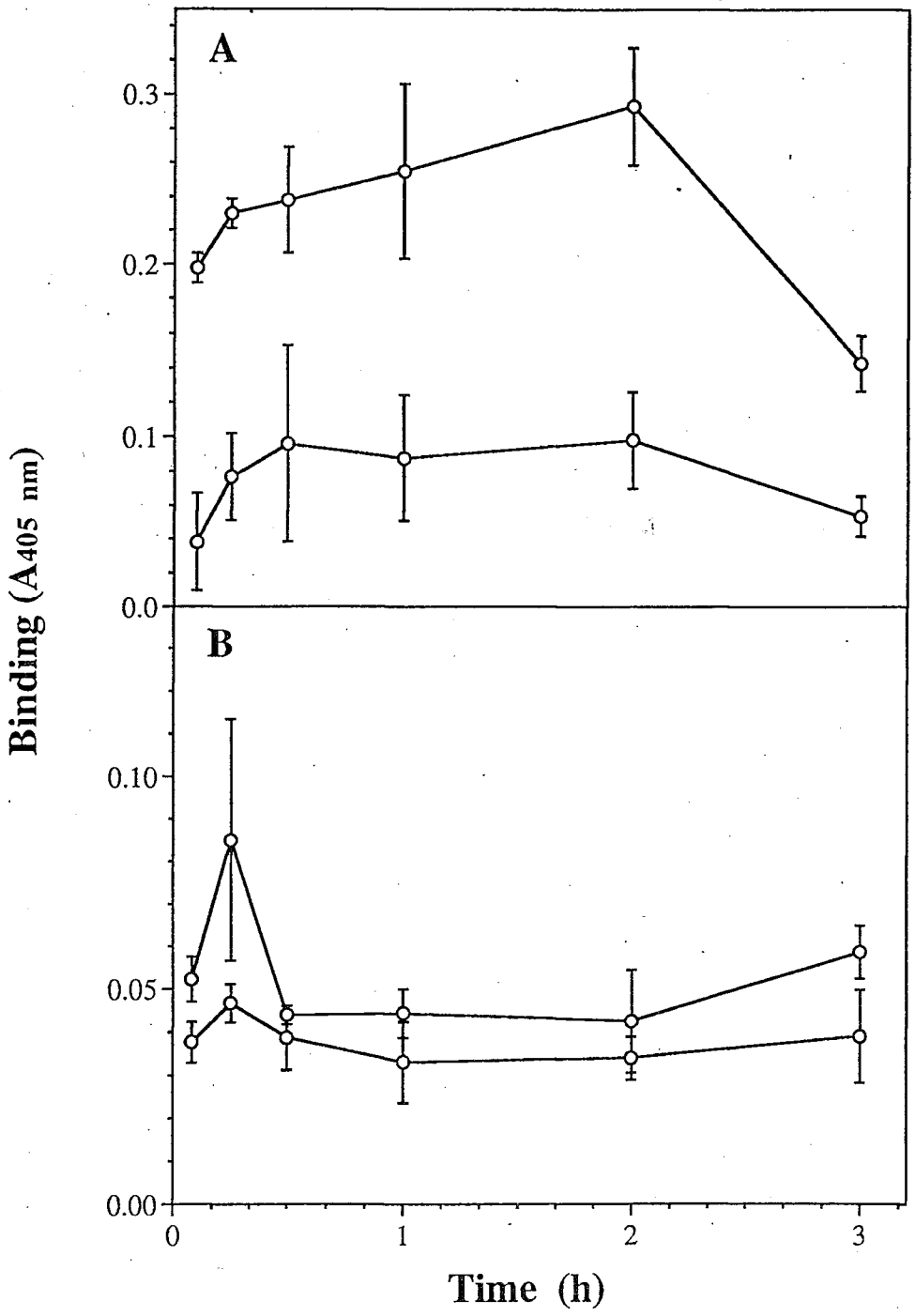
[a] The number of bacteria bound are given as percentage of total epithelial cells counted

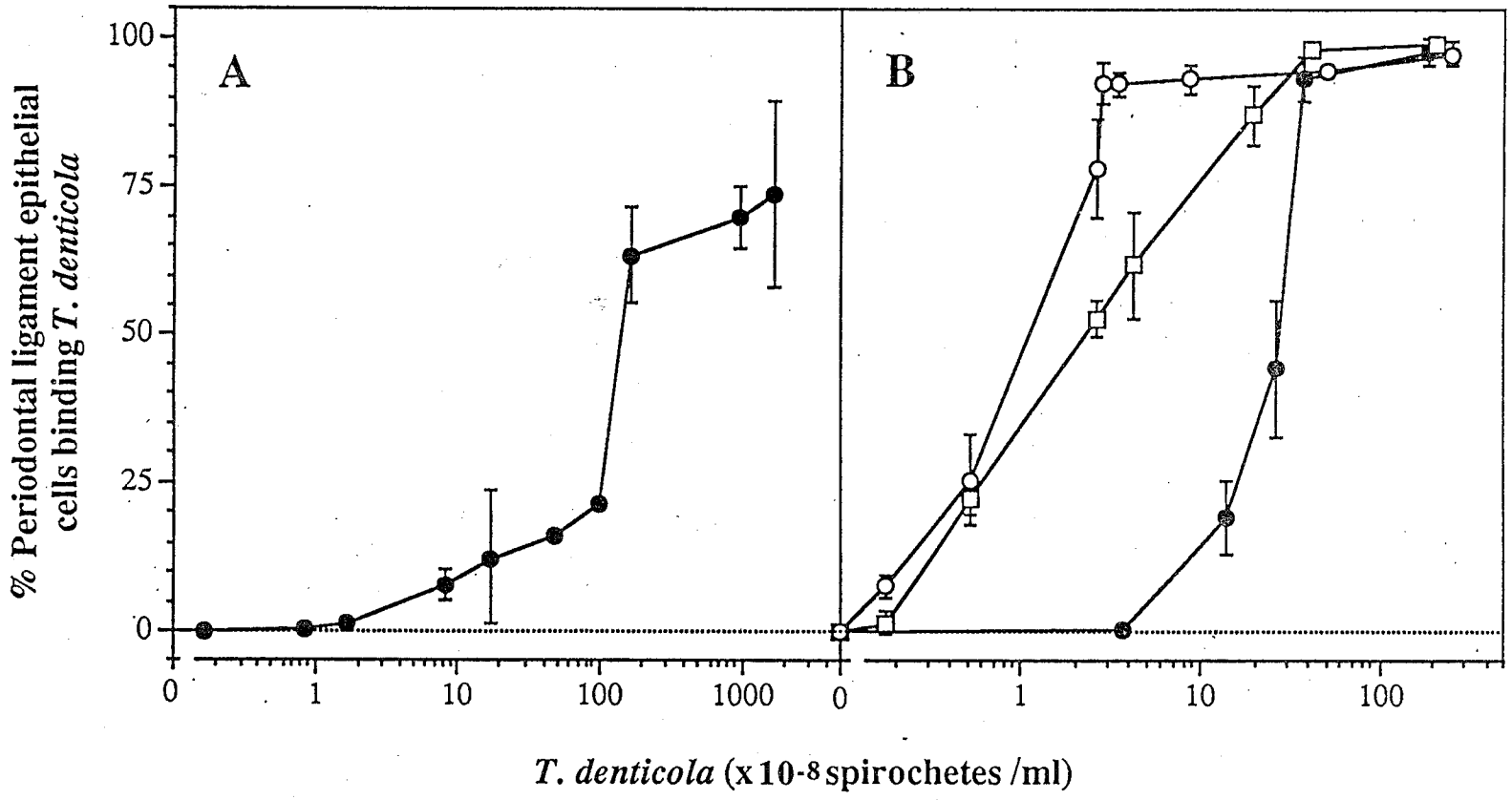
[b]Concentration of *T. denticola* in the incubation $x10^{-9}$ bacteria/ml.

TABLE 3. Effect of various treatments on binding and the chymotrypsin-like protease activity of *T. denticola*.

T. denticola treatment	Enzyme activity[a] (mean % ± S.D.)	*T. denticola* attachment to epithelial cells[b] (mean % ± S.D.)
None	100.0 ± 5.4	100.0 ± 8.9
PMSF, 1 mM	5.8 ± 1.1*	38.0 ± 3.3*
pCMBA, 2 mM	1.7 ± 0.4*	22.3 ± 2.1*
Cysteine, 50 mM	ND[c]	86.3 ± 5.6
Mixed glycosidase, 100 µg/ml	ND	92.7 ± 6.9
Proteinase K, 100 µg/ml	ND	69.7 ± 5.5*
pH		
3.2	5.6 ± 0.3*	11.0 ± 1.9*
3.8	76.7 ± 0.8*	87.0 ± 2.3
Heating at 70°C	2.5 ± 0.4*	22.2 ± 7.8*

[a]Following 1 h treatment, the spirochetes (1.4×10^{10}) were washed in PBS and assayed for SAAPNA degradation.
[b]*T. denticola* attachment measured by ELISA.
[c]ND = not determined.
*$p < 0.05$, Bonferroni test.


A
B
0.3
0.2
0.1
0.0
0.10
0.05
0.00
0
1
2
3
Binding (A405 nm)
Time (h)


A
B
100
75
50
25
0
0
1
10
100
1000
0
1
10
100
% Periodontal ligament epithelial cells binding T. denticola
T. denticola (x10-8 spirochetes /ml)