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Hemagglutination Activity of *Treponema denticola* Grown in Serum-Free Medium in Continuous Culture

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Hemagglutination by different *Treponema denticola* strains was observed for erythrocytes of human, horse, bovine, and rabbit origin. The growth of *T. denticola* ATCC 33520 in serum-free medium in continuous culture enabled us to study the hemagglutinating activity of freshly harvested spirochetes of a defined physiological status. The hemagglutinating activity was cell bound and not related to motility or appendages, such as fimbriae. The activity was destroyed by proteolytic enzymes, heat, and alkylation, indicating that the agglutinin is of a proteinaceous nature. In addition, periodate oxidation of the spirochetes indicated the involvement of carbohydrate groups. Microscopic inspection of the hemagglutination mixtures at the titration endpoints revealed that only a part of the spirochete population was involved in the hemagglutination process. The hemagglutinating activity was found to be growth phase related. The activity was blocked by serum, while of all tested amino acids and carbohydrates, only sialic acid blocked the activity at low concentrations. In conclusion, we found a hemagglutinating activity in *T. denticola* which was cell bound and growth phase related. The agglutinin may be a glycoprotein, like lectin, that recognizes sialic acid as a receptor.

Oral spirochetes are mainly found in the microbiota of the gingival crevice and periodontal pockets in association with periodontal disease (17, 18, 22, 30). Histological investigations have revealed spirochetes at the front of subgingival plaque in rapid progressive periodontitis, in the periodontal tissues of ulcerative gingivitis, and in the junctional epithelium of experimentally induced periodontal lesions (15, 21, 28). Oral *Treponema* species possess a variety of enzymatic activities which may play a role in soft tissue and bone destruction (7, 14, 20, 24). The proteolytic activities of *Treponema denticola* are unique among those of the cultivable *Treponema* species of oral and non-oral origin (19). The involvement of oral spirochetes in the progression of periodontal lesions and tissue invasion identifies these bacteria as putative pathogens in periodontal disease. Successful treatment of periodontal pockets leads to a reduction of spirochetes in the subgingival microbiota as well as to a reduction of specific proteolytic activities of *T. denticola* in the pockets (16, 18, 26). Little is known about the colonization of the subgingival area, although adherence is believed to play a role in the colonization process (10).

Recently, the adherence of *T. pallidum* and *T. hyodysenteriae* to different cell types and of *T. denticola* to saliva-coated hydroxyapatite, fibroblasts, and keratinocytes was described (2, 3, 8, 25, 27, 29). Glycosaminoglycans and probably fibronectin seem to play a role in the adherence of *T. pallidum*, while sialic acid (*N*-acetylneuraminic acid) may be involved with the receptor-binding ligands on *T. hyodysenteriae* and *T. pallidum* (2, 8, 29). Adhesins recognizing galactosyl and mannosyl as well as fibronectin receptors have been indicated to play a role in the adherence of *T. denticola* to fibroblasts and fibronectin-coated coverslips (5, 32).

The hemagglutinating activity of *T. denticola* ATCC 33520 is used in the present study as a model in the investigation of the adherence properties of spirochetes harvested from a continuous culture in a serum-free medium.

MATERIALS AND METHODS

Bacteria and cultures. *T. denticola* ATCC 33520, ATCC 35405, and ATCC 35404 were obtained from the American Type Culture Collection. Strain L11D and *T. vincentii* LA1 were obtained from N. S. Taichman, Dental School, University of Pennsylvania. *T. denticola* F1, NY 545, NY 535, B11, and B12 are our own isolates from human periodontitis (19). The strains were grown in GM1 broth supplemented with 0.3% heat-inactivated bovine serum (1) or in Proteose-Trypticase-yeast (PTY) medium (10 g of Proteose Peptone no. 2 [Difco, Detroit, Mich.], 5 g of Trypticase peptone [BBL, Cockeysville, Md.], 2.5 g of KCl, and 0.5 g of L-cysteine HCl [Merck, Darmstadt, Germany] per liter). The medium was titrated to pH 7.0 with 1 N KOH and heat sterilized. After the medium cooled, a filter-sterilized mixture of 5 ml of 10% (wt/vol) $NaHCO_3$, 0.025 g of thiamine PP_i (Sigma, Amsterdam, The Netherlands), and 5 ml of volatile fatty acid solution was added. The volatile fatty acid solution contained 100 ml of 0.1 N KOH and 0.5 ml of isobutyric acid, 0.5 ml of DL-2-methylbutyric acid, 0.5 ml of isovaleric acid, and 0.5 ml of valeric acid (all from Merck).

Batch-grown spirochetes were collected after 4 days of culturing at 37°C in an anaerobic glove box (Coy, Ann Arbor, Mich.) in a nitrogen atmosphere with 5% H_2 and 4% CO_2. Most tests were performed with *T. denticola* ATCC 33520 grown in serum-free PTY medium in continuous culture in a chemostat at pH 7.0, at a redux potential of −532 mV, at 37°C, at a dilution rate of 0.04 h^{-1}, and in a nitrogen atmosphere with 5% H_2 and 4% CO_2, resulting in a density of 5×10^8 spirochetes per ml and an optical density at 550 nm of 0.650. The spirochetes in the steady state of this culture did not show motility in phase-contrast microscopy.

Samples of spirochetes were obtained directly from the chemostat vessel or from the batch cultures. The samples were centrifuged for 20 min at 27,000 × *g* at 4°C. The pellets

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were resuspended by being vortexed in phosphate-buffered saline (PBS) (pH 7.2) with 25 mM $MgCl_2$ (MBS) to reach a concentration of at least 4×10^9 spirochetes per ml. The actual spirochete concentration in each test was estimated in a counting chamber (Hawksley, Lancing, United Kingdom) by phase-contrast microscopy.

Hemagglutination. Human, rabbit, horse, and bovine erythrocytes were harvested from EDTA-treated blood. Packed erythrocytes were washed two times in PBS (pH 7.2) and finally suspended in MBS to a concentration of 4×10^7 erythrocytes per ml, as microscopically estimated. The erythrocyte suspensions were stored at 4°C and used within 7 days after preparation. Serial twofold dilutions of spirochete suspensions in 0.1 ml of MBS were made in multiwell U-bottom microtiter plates (Hycult, Uden, The Netherlands). To each well was added 0.1 ml of erythrocyte suspension. In most tests, human O-type erythrocytes were used, unless otherwise indicated. The trays were kept overnight at 4°C, and hemagglutination was read the next morning. The endpoint was defined as the highest dilution showing complete hemagglutination. The titer is reported as the reciprocal of the endpoint dilution, based on an initial spirochete concentration of 4×10^9/ml. The titer as a percentage of the control titer was calculated by dividing the titer of the control by the titer of the test and multiplying by 100. All tests were performed on at least two different occasions in duplicate.

Statistical analyses were performed with the Kruskal-Wallis one-way analysis of variance (ANOVA).

Heat treatment. Five-milliliter portions of spirochete suspensions in MBS were incubated at 35, 40, 45, 50, and 55°C. Samples were taken from each portion at 5, 10, 20, 40, and 80 min, cooled, and titrated on ice.

Alkylation. Spirochetes were suspended in phosphate-buffered 2 and 4% (vol/vol) formaldehyde and 1.7% (vol/vol) glutaraldehyde (pH 7.2). At 15, 30, 60, 120, and 180 min, 0.2-ml samples were taken, added to 1.3 ml of MBS, centrifuged, resuspended in MBS, and titrated.

Periodate oxidation. Spirochetes were suspended in 0.025 M $NaIO_4$ in 0.1 M acetate buffer (pH 4.6) and in the same buffer as a control. After incubation for 2 h at 4°C, the spirochetes were harvested by centrifugation, washed once, suspended in MBS to the original concentration, and titrated.

Treatment with proteolytic and hydrolytic enzymes. For attempted digestion of the agglutinin, the following enzymes were used: type I trypsin (10.00 BAEE units per mg), type VII chymotrypsin (40 to 60 U/mg), type VI protease (8 to 10 U/mg), type XI protease (2 to 10 U/mg), β-*N*-acetylglucosaminidase (74 U/mg), and chondroitinase ABC (0.2 to 1 U/mg) (all from Sigma); pronase E (70 PU/mg) and hyaluronidase (250 USP-E/mg) (both from Merck); and papain (30 U/mg) and neuraminidase (25 U/mg) (both from Boehringer [Mannheim, Germany]). Spirochetes (4×10^9 cells per ml of MBS) were incubated in a final enzyme concentration of 1 mg/ml for 2 h at 37°C as described by Bowden et al. (2). The controls were incubated in MBS only. After incubation, the spirochetes were harvested by centrifugation, washed once, suspended in MBS to the original concentration, and titrated.

Antisera. Native nonimmune and polyclonal antisera were kindly provided by S. Rijpkema, (The National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands), who immunized rabbits with sonicates of whole *T. denticola* ATCC 33520 or *T. vincentii* LA1 cells. The immune sera showed evident reactivity in immunoblots by numerous strongly reacting bands. They showed limited cross-reactivity with *T. pallidum* and *T. phagedenis* and no cross-reactivity with *Borrelia burgdorferi*, *Leptospira biflexa*, and *L. interrogans*. In the inhibition tests, spirochetes were incubated in serum dilutions of 1:50 and 1:800 in MBS. After incubation for 1 h at 20°C, the spirochetes were harvested by centrifugation, washed once, and titrated.

TABLE 1. Hemagglutination titers for *T. denticola* ATCC 33520 and erythrocytes of different origins

Erythrocytes	Median titer (range)	Mean rank
Human[a]		
A type	64 (2–64)	9
B type	32 (2–64)	7.5
O type	32 (2–64)	7.5
Animal[b]		
Rabbit	64 (16–512)	6.5
Horse	128 (32–512)	7.5
Bovine	16 (8–64)	3.3

[a] Kruskal-Wallis one-way ANOVA; chi-square = 0.37, not significant.
[b] Kruskal-Wallis one-way ANOVA; chi-square = 2.84, not significant.

Hemagglutination inhibition tests. Amino acids and carbohydrates were screened for inhibition of the hemagglutinating activity. D-(+)-Glucose, D-(−)-fructose, D-(+)-galactose, D-(+)-mannose, L-(−)-fucose, lactose, raffinose, L-alanine, L-arginine, L-lysine, L-proline, L-serine, L-phenylalanine, L-glutamine, glucuronic acid, galacturonic acid, *N*-acetylglucosamine, *N*-acetylgalactosamine, *N*-acetylneuraminic acid, gelatin, pectin, heparin, and chondroitin (all from Merck) were added to the spirochete suspensions at three concentrations, 50, 25, and 12.5 mM, unless otherwise indicated. Human mixed saliva and sublingual saliva were collected from two volunteers and centrifuged at 27,000 × *g* for 15 min. The saliva supernatant was used as an incubation fluid. After 1 h of incubation of the suspensions at 20°C, the spirochetes were harvested by centrifugation, suspended in MBS to the original concentration, and titrated.

RESULTS

Hemagglutination. Human A-, B-, and O-type erythrocytes were examined in five tests. In addition, rabbit, horse, and bovine erythrocytes were examined in four tests. All tests were performed in duplicate with *T. denticola* ATCC 33520 harvested from a continuous culture in the steady state. No significant difference in hemagglutinating activity among human A-, B-, and O-type or animal erythrocytes was observed (Table 1). Washing of the spirochetes in PBS instead of in MBS resulted in a 50% reduction in hemagglutinating activity. Hemagglutinating activity was only observed in the spirochetal fraction and not in the spent culture supernatant or washings. Microscopic inspection of the contents of the hemagglutination wells at the titration endpoints revealed many nonadhering spirochetes (Fig. 1).

Incubation with 10% bovine serum albumin in 2 M acetic acid (pH 4) resulted in no microscopic indications of glycosaminoglycans in the form of precipitates on the spirochetal surface, as found for *T. pallidum* by Fitzgerald et al. (8).

The hemagglutinating activity of nine *T. denticola* strains was examined in seven different tests. The strains were harvested from 5-day-old batch cultures in GM1 broth. Strains with a relatively high median titer above the mean of

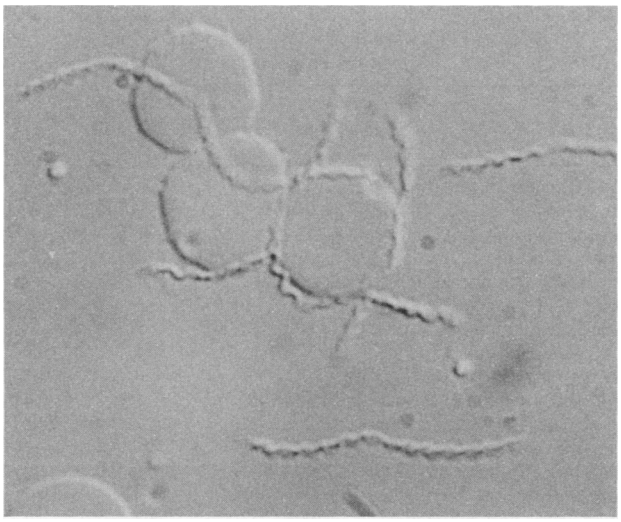

FIG. 1. Hemagglutination of O-type erythrocytes with *T. denticola* ATCC 33520 at the titration endpoint. Interference microscopy.

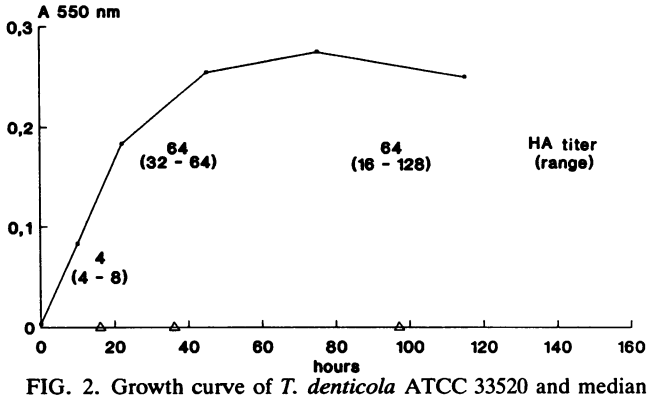

FIG. 2. Growth curve of *T. denticola* ATCC 33520 and median hemagglutination (HA) titers. Symbols: ■, optical density at 550 nm; △, test times.

ranks of 27.5 were F1, ATCC 35405, ATCC 33520, and L11D. A median titer below the mean of ranks of 27.5 was found for strains NY 545, NY 535, B12, ATCC 35404, and B11 (Table 2).

Growth phase. Culture vessels containing 100 ml of prereduced PTY broth were inoculated at 24-h intervals with 5-ml samples of the continuous culture of *T. denticola* ATCC 33520 in three different tests. Inoculation and incubation were done in the anaerobic glove box. At the end of the incubation period, the optical density of the culture and the hemagglutination titers were estimated. Figure 2 shows the growth curve and the median hemagglutination titers for three separate tests at various times. The median titer of the spirochetes harvested during the early growth phase was significantly lower than the median titers of the spirochetes harvested at the end of the exponential growth phase and the stationary phase (Wilcoxon rank test, $P = 0.02$). Motility was not observed in any sample.

Heat treatment and alkylation. The effect of heat on the hemagglutinating activity was tested by incubation of *T. denticola* ATCC 33520 in MBS at 35, 40, 45, 50, and 55°C (Fig. 3). The hemagglutinating activity was found to be sensitive to temperatures above 40°C.

Incubation of *T. denticola* ATCC 33520 in 2 and 4% formaldehyde resulted in a significant loss of activity which was proportional to the formaldehyde concentration (Fig. 4). Incubation with 1.7% glutaraldehyde resulted in an immediate inhibition of the hemagglutinating activity.

Enzyme and periodate treatments. The influence of proteolytic and hydrolytic enzymes on the hemagglutinating activity of *T. denticola* ATCC 33520 was examined in duplicate in two tests. Most of the proteolytic enzymes reduced the hemagglutinating activity to 12% of the control level. Pronase E and papain resulted in a reduction to 25% of the control level. Phenylmethylsulfonyl fluoride neutralized the effect of trypsin. Incubation of the spirochetes with a number of hydrolytic enzymes resulted in no reduction of the hemagglutinating activity (Table 3).

Treatment with sodium periodate in 0.1 M acetate buffer reduced the hemagglutinating activity to 12% of the control level; acetate buffer alone did not influence the activity (Table 3).

Hemagglutination inhibition tests. Different carbohydrates and amino acids were investigated in a number of tests for blocking of the hemagglutinating activity of *T. denticola* ATCC 33520. Of all the tested substances, only *N*-acetylneuraminic acid resulted in a significant decrease in the hemagglutinating activity (Table 4). Human mixed saliva and sublingual saliva resulted in a consistent 50% reduction in the hemagglutinating activity.

Native polyclonal antisera against *T. denticola* ATCC

TABLE 2. Hemagglutination titers for different *T. denticola* strains

Strain	Median titer (range)	Mean rank[a]
F1	32 (16–64)	47
ATCC 35405	32 (8–64)	43
ATCC 33520	32 (2–64)	42
L11D	16 (4–64)	36
NY 535	4 (2–16)	24
NY 545	4 (4–16)	23
B12	8 (1–16)	22
ATCC 35404	4 (1–32)	22
B11	1 (1–64)	15

[a] Kruskal-Wallis one-way ANOVA; chi-square = 3.93, $P = 0.02$. Mean of ranks, 27.5.

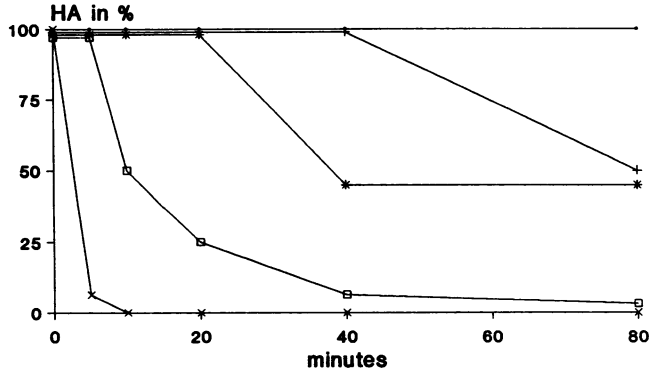

FIG. 3. Effect of heat on *T. denticola* ATCC 33520 hemagglutinating activity (HA). Symbols: ■, 35°C; +, 40°C; *, 45°C; □, 50°C; ×, 55°C.

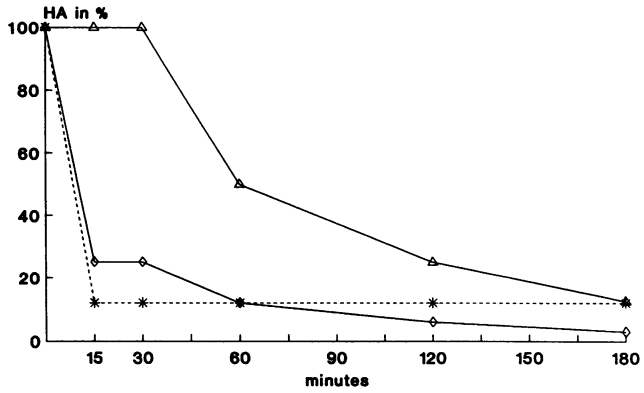

FIG. 4. Effect of formaldehyde and glutaraldehyde on *T. denticola* ATCC 33520 hemagglutinating activity (HA). Symbols: △, 2% formaldehyde; ◇, 4% formaldehyde; *, 1.7% glutaraldehyde.

33520 and *T. vincentii* LA1 were tested in three different tests with, as the test organism, *T. denticola* ATCC 33520 harvested from the continuous culture. The hemagglutinating activity of serum-treated spirochetes was significantly reduced. The reduction was related to the serum concentration and was of the same magnitude for spirochetes treated with the two different polyclonal sera or nonimmune serum (Table 5).

DISCUSSION

The agglutination of erythrocytes and attachment to other cells have been described for different oral and non-oral bacteria and have been related to specific adhesin-receptor interactions (6, 9, 23). The attachment of *Treponema* species to various cell types has been investigated (2, 8, 25, 27, 29). However, hemagglutination by *Treponema* species was, at least at the time of review of this paper, not described, possibly because of growth conditions, the difficulty of growing these organisms in large enough amounts, and the tested *Treponema* species and strains. For example, we found no hemagglutination by *T. phagedenis* and *T. hyodysenteriae* strains (data not shown). The present findings revealed a difference in hemagglutinating activity between the tested *T. denticola* strains, a result which could indicate a difference in the expression of the putative hemagglutinin.

The hemagglutinating activity was found to be growth phase related. A relationship between hemagglutinating activity and growth was also observed for cultures of *Porphyromonas gingivalis* (12), while in *Escherichia coli* the production of adhesins was found to be a function of growth rate (31). The low titer of spirochetes harvested during the early growth phase indicates that the amount of agglutinin or the fraction of agglutinating spirochetes is at least 15 times lower in these spirochetes than in spirochetes harvested at the stationary phase. Fluctuations in the adherent population have been observed in many lectin-producing bacteria (23) and can be of importance in the persistence of a species in a desquamating habitat, such as the gingival sulcus, from which adherent bacteria are readily removed with the shed epithelial cells and in which at least some of the progeny have to colonize new available surfaces (9).

Microscopic inspection of the hemagglutination mixtures at the titration endpoints revealed many unattached spirochetes, indicating that only a part of the spirochete population is involved in the hemagglutination process. This phenomenon was also noted in attachment studies with keratinocytes (13).

The growth of *T. denticola* ATCC 33520 in serum-free medium in continuous culture enabled us to study the hemagglutinating activity of freshly harvested spirochetes of

TABLE 3. Effect on hemagglutinating activity of enzyme and periodate treatments of *T. denticola* ATCC 33520

Treatment[a]	Hemagglutination (% of control level[b])
Trypsin	12
Trypsin + phenylmethylsulfonyl fluoride	100
Chymotrypsin	12
Protease type VI and type XI	12
Pronase E	25
Papain	25
Neuraminidase	100
β-*N*-Acetylglucosaminidase	100
Chondroitinase	100
Hyaluronidase	100
Periodate (0.025 M)	12
Acetate buffer (0.1 M, pH 4.6)	100

[a] Enzymes were tested at 1 mg/ml, with incubation for 2 h at 37°C, in duplicate in two tests. Periodate was tested in 0.1 M acetate buffer (pH 4.6), with incubation for 2 h at 4°C.
[b] Control treatment was MBS.

TABLE 4. Hemagglutinating activity of *T. denticola* ATCC 33520 treated with carbohydrates, amino acids, and other organic substrates

Substrate	Dose	Hemagglutination (% of control level[a])	No. of tests
D-(+)-Glucose, D-(−)-fructose, D-(+)-galactose, D-(+)-mannose, L-(−)-fucose, lactose, and raffinose	50 mM	100	4
Glucuronic acid and galacturonic acid	50 mM	100	2
N-Acetylneuraminic acid	10 mM	6[b]	3
N-Acetylglucosamine and *N*-acetylgalactosamine	50 mM	100	2
L-Alanine, L-arginine, L-lysine, L-proline, L-serine, L-phenylalanine, and L-glutamine	50 mM	100	4
Gelatin, pectin, heparin, and chondroitin	0.2%	100	2
Mixed saliva and sublingual saliva	100%	50[b]	2

[a] Control treatment was MBS.
[b] Results for the different tests were identical.

TABLE 5. Hemagglutinating activity of *T. denticola* ATCC 33520 treated with polyclonal antisera and nonimmune serum

Serum	Median (range) hemagglutination (% of control level[a]) at the following serum dilution:	
	1:50	1:800
Anti-*T. denticola* ATCC 33520	6 (6–12)	50 (25–50)
Anti-*T. vincentii* LA1	6 (6–12)	50 (25–100)
Nonimmune	6 (6–12)	50 (12–100)

[a] Control treatment was MBS.

a defined physiological status. The hemagglutinating activity was cell bound and not related to appendages, such as fimbriae. (Electron microscopic examination failed to reveal the presence of such appendages [4].) The activity seemed not to depend on motility, because no motility was observed in samples taken in the different stages of the batch-grown cultures as well as in the steady state of the continuous culture. The hemagglutinating activity was destroyed by proteolytic enzymes, heat, and alkylation, indicating that the agglutinin is of a proteinaceous nature. In addition, the periodate sensitivity indicated the involvement of carbohydrate groups. Glycosaminoglycan has been found on the surface of *T. pallidum* strains and some *T. denticola* strains and probably originates from the host or serum in the culture medium (8, 25). In the present study, *T. denticola* ATCC 33520 was grown in serum-free medium. This strain did not show a glycosaminoglycan-positive reaction, and its hemagglutinating activity was not influenced by hyaluronidase or chondroitinase treatment, indicating that surface-bound glycosaminoglycan was probably not involved in hemagglutination by *T. denticola* ATCC 33520.

Incubation of *T. denticola* ATCC 33520 with native rabbit nonimmune serum or native rabbit antiserum resulted in a dose-response inhibition of the hemagglutinating activity. This result was obtained after washing of the serum-treated treponemes and suggests that a serum factor(s) binds to the spirochetes, causing an inhibition of hemagglutination. A dose-response inhibition of the adherence of *T. denticola* to human gingival fibroblasts was found by Weinberg and Holt (32) with heat-inactivated fetal bovine serum. In addition, Weinberg and Holt (32) postulated that there were lectin-like adhesins on the *T. denticola* surface with affinities for galactose and mannose. A different lectin seems to play a role in *T. denticola* ATCC 33520, since galactose and mannose had no effect on the hemagglutinating activity and of all the tested carbohydrates, only *N*-acetylneuraminic acid (sialic acid) blocked the activity at low concentrations. Comparable results were found in studies of the adherence of *T. denticola* to keratinocytes (13) and in a hemagglutination study (11) published during the review process for this manuscript.

Sialoglycoproteins and sialoglycolipids are present on different cell surfaces, including the erythrocyte membrane. Also, serum and salivary proteoglycans carry sialic acid molecules, possibly explaining the inhibition by nonimmune serum and the low but consistent inhibitory effect of saliva in the hemagglutination tests. Sialic acid has been implicated in the attachment process for *T. pallidum*, *T. hyodysenteriae*, and other microorganisms (2, 23, 29). It is postulated that, in the development of supragingival plaque, oral streptococci adhere to sialoglycoconjugates of the pellicle on the tooth surface and that *Actinomyces* species then adhere to galactose residues made available by their neuraminidase activity (10). These oral bacteria and the sialated salivary proteoglycans seem to make the supragingival environment unattractive for the attachment of *T. denticola*. However, sialic acid receptors may be available on the desquamating junctional epithelium in the subgingival environment, the habitat of the oral spirochetes. Whether the adhesins of *T. denticola* play a role in the ecology of the subgingival microbiota remains to be proven.

In conclusion, we found a hemagglutinating activity in *T. denticola* which was cell bound and growth phase related. The hemagglutinating activity seems to be of protein and carbohydrate nature and may be a glycoprotein, like lectin, that recognizes sialic acid as a receptor.

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