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A Major Antigen on the Outer Envelope of a Human Oral Spirochete, *Treponema denticola*

TOSHIHIKO UMEMOTO,[1]* ISAMU NAMIKAWA,[1] HIROHISA SUIDO,[2] AND SHOJIRO ASAI[1]

Department of Oral Microbiology, School of Dentistry, Asahi University, Hozumi, Gifu,[1] and Oral Care Laboratory, Sunstar Inc., Takatuki, Osaka,[2] Japan

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Human oral spirochetes are prominent inhabitants of subgingival plaque in patients with periodontal disease. By immunoelectron microscopy using protein A-gold complexes and either polyclonal mouse antiserum against the 53-kDa antigen or 53-kDa-antigen-specific monoclonal antibody, a major polypeptide antigen, with a molecular weight of 53,000 (molecular size, 53 kilodaltons [kDa]), of a human oral spirochete, *Treponema denticola* ATCC 33520, was found to localize on the surface of the outer envelope.

Human oral spirochetes increase in number in subgingival sulci of patients with periodontal disease (9). *Treponema denticola*, an easily isolated and cultured oral spirochete in humans, has an immunosuppressive substance which inhibits human fibroblast and lymphocyte proliferation (1), and this microorganism can also produce tissue-damaging enzymes (11, 15) and induce the production of antibody (5). In fact, periodontal patients with higher proportions of spirochetes in subgingival plaques have sera with higher levels of antibodies against oral spirochetes, including *T. denticola*, when compared with periodontally healthy individuals infected with a lower number of spirochetes (10).

We previously reported (16, 18) that rabbit antiserum against whole cells of *T. denticola* ATCC 33520 strongly react with a polypeptide antigen of this strain having a molecular weight of 53,000 (molecular size, 53 kilodaltons [kDa]) and that human sera from patients with necrotizing ulcerative gingivitis and localized juvenile periodontitis are also reactive with the same antigen. In this strain, therefore, the 53-kDa antigen seemed to be a potent in vivo immunogen for antibody production and to associate with periodontal disease.

In this study, the 53-kDa major antigen of *T. denticola* ATCC 33520 was localized by immunoelectron microscopy using protein A-gold complexes and the polyclonal or monoclonal antibody against the 53-kDa antigen.

MATERIALS AND METHODS

Bacteria. Eight treponemes, *Treponema denticola* ATCC 33520, ATCC 35404, and ATCC 35405, *T. socranskii* subsp. *socranskii* ATCC 35536, a human oral isolate strain G7201, *T. phagedenis* biotype Kazan 8 and biotype Reiter, and an avirulent variant *T. pallidum* ATCC 27087, were used in this study. All strains were anaerobically grown in TYGVS medium at 37°C for 4 days (11). The human oral isolate strain G7201 possesses three to four axial flagella at subterminal ends of an individual cell (17). Each strain was harvested by centrifugation and washed twice in 0.01 M phosphate-buffered saline (PBS; pH 7.2).

SDS-PAGE and immunoblotting. Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done by using 1-mm-thick slab gels with stacking and separating gels of 3% (wt/vol) and 10% polyacrylamide, respectively, by the method of Laemmli (7). Proteins on SDS-polyacrylamide gels were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Richmond, Calif.) by the method of Towbin et al. (14). Reactivities of the separated polypeptide antigens with a polyclonal mouse antiserum or a monoclonal antibody, both against the 53-kDa antigen as described above, were examined by use of horseradish peroxidase-conjugated affinity-purified anti-mouse immunoglobulins (immunoglobulin G [IgG], IgA, and IgM, heavy and light chain specific) at a dilution of 1:100 or 1:1,000 (Organon Teknika, Malvern, Pa.).

Polyclonal antiserum and monoclonal antibody. The cell lysate of *T. denticola* ATCC 33520 was prepared as described previously (18). Polypeptides of the cell lysate and protein standards were separated on SDS-10% polyacrylamide gels and stained with Coomassie brilliant blue. The acrylamide gel strips containing the 53-kDa antigen bands of *T. denticola* ATCC 33520 exclusively were pooled and homogenized for use as an immunogen by using a mortar and pestle with 2 ml of PBS (pH 7.0). The polyclonal mouse antiserum and monoclonal mouse antibody, both against the same immunogen, were prepared by intramuscularly immunizing three times at 2-week intervals 8-week-old female BALB/c mice with the 53-kDa antigen (protein content, approximately 8 μg) emulsified in complete Freund adjuvant (Difco Laboratories, Detroit, Mich.). Preimmune mouse sera (negative control) and the polyclonal mouse antiserum were collected prior to immunization and 4 days following the final immunization, respectively. Mouse hybridomas were generated by a modified method of Gefter et al. (4). Briefly, the spleen cells from the mice immunized with the 53-kDa antigen as described above were fused for 2 min with hypoxanthine-, aminopterin-, and thymidine-sensitive mouse myeloma cells P3-NS1-Ag4-1, at a 5/1 splenocyte-myeloma cell ratio in 50% polyethylene glycol 4000 (Merck & Co., Inc., Rahway, N.J.). The cells were washed in RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), suspended in hypoxanthine-aminopterin-thymidine medium, plated at 6×10^5 cells per well in flat-bottom microdilution plates, and incubated at 37°C with 6% carbon dioxide in a humid atmosphere. Of 211 wells, 128 showed growth of hybridomas in hypoxanthine-aminopterin-thymidine medium. Culture supernatants from growth-positive wells were tested for antibody against the 53-kDa antigen by an indirect enzyme-linked immunosorbent assay (ELISA) as described below. Antibody-positive cell lines were cloned by limiting dilution with feeder cells in 96-well tissue culture plates

* Corresponding author.

(Corning Glass Works, Corning, N.Y.). A high-titer monoclonal-antibody-producing clone, p1-11c, was used in this study. Immunodiffusion tests were performed to determine the monoclonal antibody subclass on microscope slides with 1% agar in PBS.

Agglutination reaction. Each cell suspension of the eight treponemes used was mixed for 15 min at room temperature with an equal volume of polyclonal mouse anti-53-kDa-antigen serum (1:128 dilution) or a 19-fold culture supernatant concentrate containing the monoclonal antibody to the 53-kDa antigen. The resultant clumps were gently washed twice in PBS (pH 7.2), stained negatively with 1% phosphotungstic acid, and observed in a transmission electron microscope (JEM 1200EX; JEOL, Tokyo, Japan) operated at 80 kV.

ELISA. An indirect ELISA was carried out to detect the cell lines producing 53-kDa-antigen-specific antibody. Antigens consisted of a cell lysate from *T. denticola* ATCC 33520 (18). The microdilution wells of flat-bottom plates (Corning) were coated with 100 μl of a diluted (1:128) cell lysate overnight at 4°C and filled with 100 μl of PBS containing 1% bovine serum albumin. After 1 h of incubation at room temperature, the wells were washed three times in PBS containing 0.05% Tween 20, dried, and stored at 4°C until use. The antigen-coated wells were filled with culture supernatants of hybridomas and left for 2 h at room temperature. The wells were then washed twice in PBS-Tween 20, and 100 μl of alkaline phosphatase-conjugated affinity-purified goat anti-mouse immunoglobulins (IgG, IgA, and IgM; Zymed Laboratories, Inc., San Francisco, Calif.) diluted 1:100 in PBS-Tween 20 was added to each well. After 2 h of incubation at room temperature, the wells were again washed with PBS-Tween 20, and the reaction was developed with 100 μl of *p*-nitrophenylphosphate (1 mg/ml) in 10% diethanolamine buffer (pH 9.8) and was stopped after 30 min at room temperature by the addition of 50 μl of 3 N NaOH solution. The A_{410} of each well was read with a microELISA minireader (MR-590; Dynatech Instruments, Inc., Santa Monica, Calif.).

Immunoelectron microscopy. Fresh cells of *T. denticola* ATCC 33520 were gently washed three times in PBS (pH 7.2) and resuspended (10 mg [fresh weight] per ml) in the same buffer. A 10-μl sample of the bacterial suspension was incubated for 60 min at room temperature with each 100 μl of preimmune mouse serum (negative control) at a dilution of 1:50, the polyclonal mouse 53-kDa antigen-specific antiserum at a dilution of 1:50, or a 19-fold-concentrated culture supernatant of the 53-kDa-antigen-specific monoclonal-antigen-producing hybridoma. The cells were washed twice in PBS and were reacted with protein A-gold complexes (particle size, 15 nm; E-Y Laboratories, Inc., San Mateo, Calif.) for 30 min at room temperature. The cells were washed again twice in distilled water, mounted on collodion membrane-coated nickel grids, negatively stained with 1% phosphotungstic acid, and observed in an electron microscope.

RESULTS

Specificities of polyclonal antiserum and monoclonal antibody against the 53-kDa antigen. The SDS-PAGE polypeptide pattern of the cell lysates from *T. denticola* ATCC 33520 was different from those of the seven other treponemes examined (Fig. 1). Of polypeptide bands ranging from approximately 15 to 140 kDa in molecular size, a 53-kDa band was a major peptide in *T. denticola* ATCC 33520. *T. denticola* ATCC 35404 and ATCC 35405 showed identical

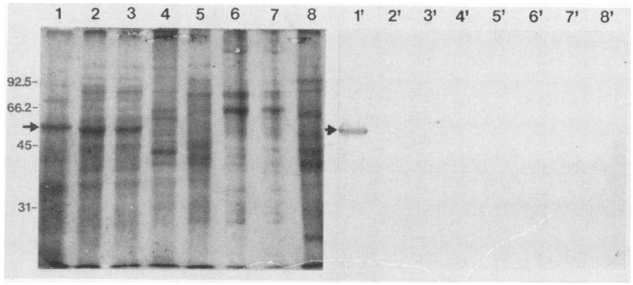

FIG. 1. Polypeptide patterns of eight *Treponema* strains and immunoblot analysis. The polypeptides of cell lysates from *T. denticola* ATCC 33520 (lane 1), *T. denticola* ATCC 35404 (lane 2), *T. denticola* ATCC 35405 (lane 3), *T. socranskii* subsp. *socranskii* ATCC 35536 (lane 5), human oral isolate strain G7201 (lane 5), *T. phagedenis* biotype Kazan 8 (lane 6), *T. phagedenis* biotype Reiter (lane 7), and *T. pallidum* ATCC 27087 (lane 8) were separated on an SDS-10% acrylamide gel and stained with Coomassie brilliant blue. Lanes 1' to 8' show the immunoblots of the polypeptides from eight strains incubated with the polyclonal mouse antiserum against the 53-kDa antigen of *T. denticola* ATCC 33520. The arrows indicate the 53-kDa antigen band of *T. denticola* ATCC 33520. The numbers on the left represent the molecular sizes (in kilodaltons) of the protein standards.

Coomassie brilliant blue-stained SDS-PAGE profiles, with main bands of approximately 50 kDa. *T. socranskii* subsp. *socranskii* ATCC 35536 had a main polypeptide band of 43 kDa, while the SDS-PAGE polypeptide pattern of human oral isolate strain G7201 resembled that of *T. pallidum* ATCC 27087 in that they both were without 50-kDa peptide bands. Two biotypes of *T. phagedenis* showed identical SDS-PAGE peptide patterns; this pattern was different from those of the six other treponemes examined. Immunoblot analysis revealed that the polyclonal mouse antiserum against the 53-kDa antigen of *T. denticola* ATCC 33520 reacted with the 53-kDa major peptide antigen of *T. denticola* ATCC 33520 but not with any other peptides from the seven other heterologous treponemes used (Fig. 1).

A representative cloned hybridoma, p1-11c, produced the specific monoclonal antibody against the 53-kDa antigen of *T. denticola* ATCC 33520. The immunoblot analysis demonstrated that the monoclonal-antibody-containing culture supernatant (19-fold concentrate) of the hybridoma p1-11c reacted specifically with the 53-kDa polypeptide of *T. denticola* ATCC 33520 (Fig. 2). The antibody class of this monoclonal antibody was determined to be IgG3 by double-diffusion analysis.

Agglutination reaction. *T. denticola* ATCC 33520 cells were agglutinated into clumps when mixed with the polyclonal mouse antiserum against the 53-kDa antigen or with the culture supernatant containing the 53-kDa-antigen-specific monoclonal antibody. However, the same antiserum or the monoclonal-antibody-containing culture concentrate could not agglutinate the seven other treponemes examined. When the resultant bacterial clumps were observed by electron microscopy, the surfaces of the outer envelopes of individual *T. denticola* ATCC 33520 cells were firmly attached to form the bacterial clumps (Fig. 3).

Location of the major antigen by immunoelectron microscopy. The location of the 53-kDa major antigen of *T. denticola* ATCC 33520 was determined by immunoelectron microscopy; protein A-gold particles used as a probe were labeled after incubation with mouse sera or with the 53-kDa-antigen-specific monoclonal antibody. *T. denticola* ATCC 33520 cells treated with preimmune mouse control

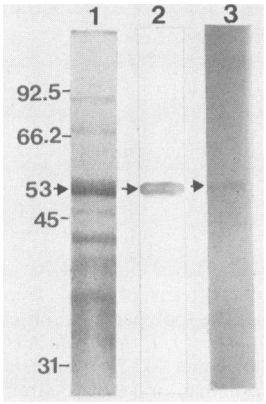

FIG. 2. Immunoblot analysis of the cell lysate from *T. denticola* ATCC 33520 with monoclonal antibody. The lysate proteins stained with Coomassie brilliant blue (lane 1) were reacted with the polyclonal mouse antiserum against the 53-kDa antigen (lane 2) or the 53-kDa-antigen-specific monoclonal antibody (lane 3). The 53-kDa bands (arrows) were lined up for comparison. Molecular size standards (in kilodaltons) are shown on the left.

serum were not immunolabeled with protein A-gold particles (Fig. 4A), while the ATCC 33520 cells, which had been preincubated with the polyclonal mouse antiserum against the 53-kDa antigen or with the monoclonal-antibody-containing culture supernatant, possessed the particles on the surfaces of the outer envelopes (Fig. 4B and 4C). Neither an axial flagellum nor a wall-membrane complex of the treponemal cell, which might have been exposed by partial disruption of the outer envelope during cell preparations, was labeled with the gold particles.

DISCUSSION

The present study adds to the finding by Simonson et al. (13) of a specific antigen on the outer envelope of *T. denticola*. *T. denticola* ATCC 33520 cells were agglutinated when mixed with the mouse anti-53-kDa-antigen serum which could not agglutinate seven other human oral treponemes examined, while the outer envelopes of treponeme ATCC 33520 cells were attached to form clumps in the presence of anti-53-kDa-antigen serum. The 53-kDa-antigen-specific monoclonal antibody also showed an ability to agglutinate the ATCC 33520 cells. In addition, the rabbit polyclonal antiserum to the intact ATCC 33520 cells showed an ability to agglutinate the fragmented membrane vesicles or outer envelopes (18). All these findings suggest that the 53-kDa major antigen of *T. denticola* ATCC 33520 may originate from the outer envelope. Immunoelectron microscopy using protein A-gold colloidal complexes with either the polyclonal or the monoclonal antibody against the 53-kDa antigen demonstrated that of the cellular components of *T. denticola* ATCC 33520, only the outer envelopes were specifically labeled with the gold particles. This observation clearly indicates that the 53-kDa peptide antigens are specifically located on the surface of the outer envelope of this microorganism. Simonson et al. (13) reported a surface antigen on the outer envelope of *T. denticola* ATCC 35521 by using monoclonal antibodies. Although the chemical nature of the surface antigen has not been defined, the antigen reported by Simonson et al. appears to be different

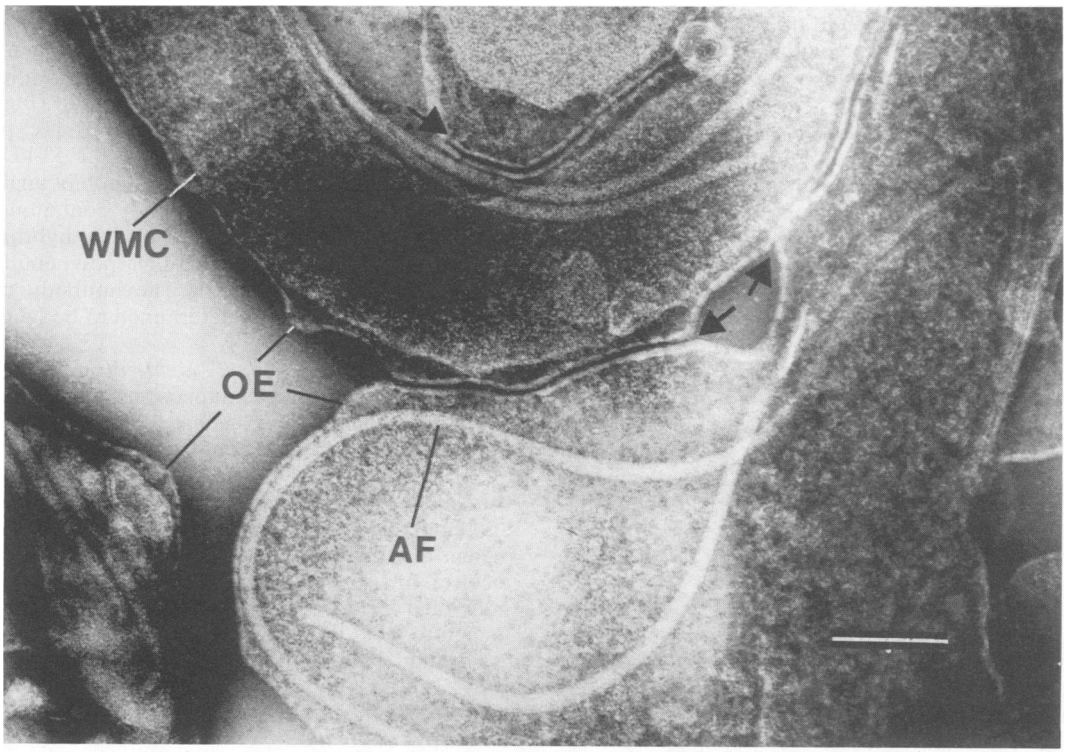

FIG. 3. Electron micrograph showing agglutinated outer envelopes of *T. denticola* ATCC 33520 cells in the presence of the polyclonal mouse antiserum against 53-kDa antigen. The negatively stained outer envelopes in clumps are seen to attach firmly (arrows). OE, Outer envelope; WMC, wall-membrane complex; AF, axial flagellum. Bar, 100 nm.

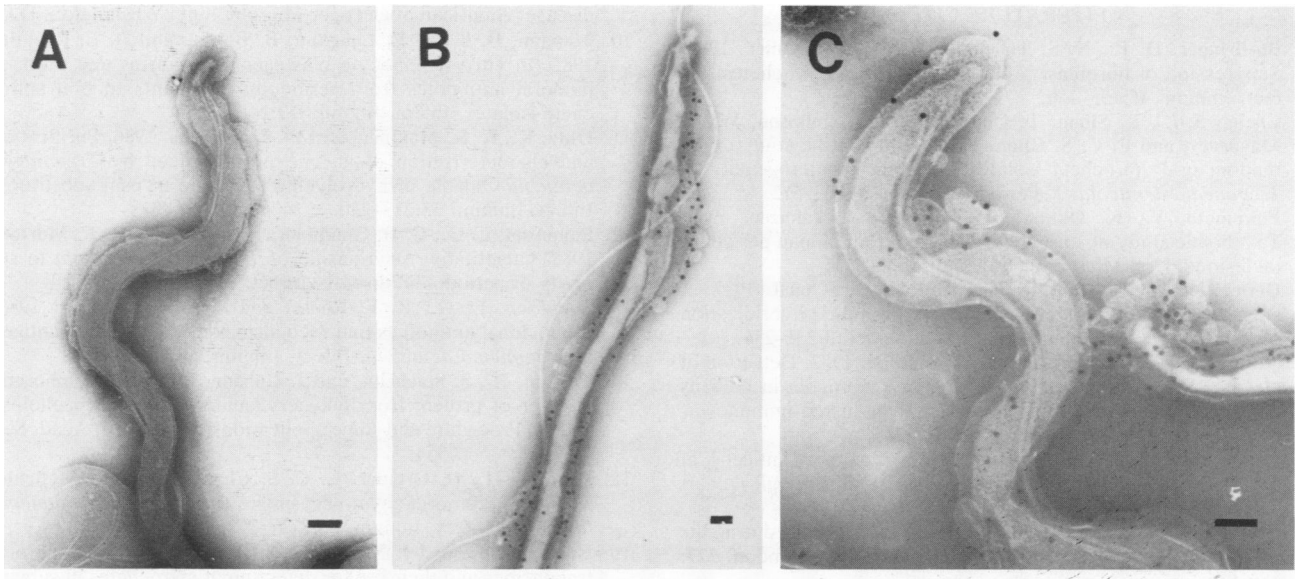

FIG. 4. Electron micrographs showing negatively stained *T. denticola* ATCC 33520 after incubation with preimmune mouse serum, polyclonal mouse serum, or monoclonal antibody, followed by incubation with protein A-gold particles. The cells incubated with the preimmune serum (A), the polyclonal antiserum against the 53-kDa antigen (B), or the monoclonal-antibody-containing culture supernatant (C) were reacted with protein A-gold complexes. Gold particles were specifically distributed on the outer envelopes of *T. denticola* ATCC 33520 cells incubated with the polyclonal (B) and monoclonal (C) antibodies. Bars, 150 nm.

from the 53-kDa surface antigen of *T. denticola* ATCC 33520 described here because a monoclonal antibody to the surface antigen of *T. denticola* ATCC 33521, which was prepared by Simonson et al., showed no reactivity with *T. denticola* ATCC 33520.

The human oral treponeme *T. denticola* has been classified on the basis of cultural, morphological, biochemical, and physiological characteristics and DNA homology (2). This species, however, seems to be composed of serologically various groups or serotypes (5, 6). *T. denticola* ATCC 35404 and ATCC 35405 showed identical SDS-PAGE patterns, with 50-kDa major peptide bands. The 53-kDa-antigen-specific monoclonal antibody, as well as the mouse polyclonal anti-53-kDa antigen, reacted with the 53-kDa antigen of strain ATCC 33520. However, the mouse polyclonal antiserum against the same antigen did not react with any other polypeptides from two strains of *T. denticola*. An SDS-PAGE pattern of the sonicate from *T. denticola* ATCC 33520 also appears to be different from that of ATCC 33521 (3). In addition, *T. denticola* ATCC 33520 differs from *T. denticola* ATCC 35404 and ATCC 35405 in SDS-PAGE pattern and antiserum reactivity. On the basis of reactivity of the surface antigen, therefore, the species *T. denticola* may include at least three different serogroups, represented by ATCC 33520, ATCC 33521, and ATCC 35404 (or ATCC 35405).

The polyclonal antiserum against the axial flagella of *T. phagedenis* biotype Kazan 5 showed cross-reactivity with *T. phagedenis* biotype Reiter, *T. denticola*, *T. pectinovorum*, *T. vincentii*, *T. pallidum* subsp. *pallidum* Nichols, *Spirochaeta aurantia*, and *Spirochaeta halophila* (8). This suggests that the axial flagellum protein may be relatively common among the spirochetes. In contrast, the surface antigens on outer envelopes seem to be rather specific to strains or species of spirochetes (13). The mouse antiserum against the 53-kDa antigen of *T. denticola* ATCC 33520 did not react with any other polypeptides from two strains of *T. denticola*, one strain of *T. socranskii*, a human oral isolate, two biotypes of *T. phagedenis*, and one strain of *T. pallidum*. We have not yet screened clinical isolates for the 53-kDa antigen. However, indirect immunofluorescence microscopy using rabbit antiserum to *T. denticola* ATCC 33520 indicated that the relative proportions of oral spirochetes with a serologically positive reaction vary up to approximately 20% of the total spirochetes in subgingival plaque samples from adult periodontitis patients (16). Since the rabbit antisera to *T. denticola* ATCC 33520 contained a high titer of antibody to the 53-kDa antigen, the 53-kDa antigen-carrying spirochetes may be present in the human oral cavity.

Four strains of human oral spirochetes, including *T. denticola* ATCC 33520, were shown to possess one to three major peptide antigens reactive with the corresponding rabbit antisera (18). In *T. denticola* ATCC 33520, the major peptide antigen was the 53-kDa surface antigen found on the outer envelope, as indicated above. The major peptide antigens detected in three other strains by the immunoblotting procedure may include their surface antigens, as in the case of *T. denticola* ATCC 33520. These surface antigens of human oral treponemes may be potent in vivo immunogens for spirochete antibody production in the infected host. The antibodies may participate in inflammatory reactions as immune complexes, resulting in periodontal tissue damage. Simonson et al. (12), using a monoclonal antibody specific for a surface antigen of *T. denticola*, also implicated *T. denticola* as one of the etiologic agents of periodontal disease by demonstrating that the *T. denticola* content in subgingival plaque samples of periodontitis patients is almost twice that of healthy subjects or of patients with moderate periodontitis. Therefore, the surface antigens of *T. denticola* and the monoclonal antibodies specific for these antigens seem to be useful tools for the study of serogrouping and periodontopathogenesis of this microorganism.

Chemical characteristics of the purified 53-kDa antigen as determined by affinity chromatography and the biological activities of this antigen in relation to macrophages and polymorphonuclear neutrophils will be reported elsewhere.

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