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Pathogen-Related Spirochetes Identified within Gingival Tissue from Patients with Acute Necrotizing Ulcerative Gingivitis

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The purpose of this investigation was to determine whether monoclonal antibodies against pathogen-restricted antigens of *Treponema pallidum* subsp. *pallidum* could be used as probes for spirochetes in diseased gingival tissue from subjects with acute necrotizing ulcerative gingivitis. A biotin-streptavidin system was used to identify spirochetes bound by monoclonal antibodies in cryostat sections of tissue. Twelve of 16 tissue samples from diseased sites, but none of 8 tissue specimens from healthy sites, reacted with pathogen-restricted antibodies. Organisms were found in intact epithelium and connective tissues adjacent to ulcers. Staining intensity was often high in perivascular locations and around vesicular spaces. Monoclonal antibodies to *Bacteroides gingivalis* and *Treponema denticola* were each reactive with diseased gingival tissues, but staining was usually restricted to ulcerated areas. These studies extend recent observations that showed that subjects with acute necrotizing ulcerative gingivitis had both pathogen-related spirochetes in dental plaque and serum immunoglobulin G to pathogen-restricted antigens on *T. pallidum* subspecies, suggesting that pathogen-related spirochetes may be associated with the pathogenesis of certain periodontal diseases.

Spirochetes, including *Treponema denticola*, can be found in dental plaque above the gum line (supragingival plaque) of children and young adults with healthy periodontal tissues (2). Spirochetes are a common component of plaque associated with diseased periodontal tissues, particularly below the gum line in the crevice between the epithelium of the gingiva and the cementum of the tooth roots (subgingival plaque; the crevice is commonly referred to as a periodontal pocket when its depth exceeds 2 to 3 mm). Organisms in subgingival plaque are predominantly gram negative and motile, as opposed to the gram-positive, nonmotile bacteria which predominate in supragingival plaque. At present there is no direct evidence that any plaque microorganism is the etiologic agent of gingivitis or periodontitis. However, periodontal disease sometimes results in necrosis of the gingival epithelium, and spirochetes have been identified by transmission electron microscopy within unaffected connective tissue under necrotic gingival lesions from subjects with acute necrotizing ulcerative gingivitis (ANUG) (7–9) and have been observed up to 400 μm from the basement membrane of the crevicular epithelium, well in advance of necrotic lesions (3). A variety of spirochetes have been observed in subgingival plaque associated with ANUG (10), but it is not known which plaque spirochetes are able to penetrate periodontal tissues.

Recently, Riviere et al. (19) used *T. denticola* and *T. pallidum* monoclonal antibodies (MAbs) to identify spirochetes in supragingival plaque from subjects with clinically healthy gingivae and in subgingival plaque from normal subjects, from subjects with ANUG, and from subjects with chronic adult periodontitis. Whereas *T. denticola* was observed in supragingival plaque collected from clinically healthy sites, pathogen-related oral spirochetes (PROS) were not found under those conditions. PROS are unknown spirochetes that are similar in size and shape to other oral spirochetes and to *T. pallidum* subsp. *pallidum* (reference 19 and unpublished observations), and they react with MAbs previously thought to react only with pathogenic *T. pallidum* subspecies and *T. pertenue* (1, 12, 13). Subgingival plaque from apparently healthy sites rarely contained either *T. denticola* or PROS. In contrast, most subgingival plaque samples from patients with ANUG or periodontitis had both types of spirochetes. Furthermore, subjects with ANUG had serum immunoglobulin G (IgG) that bound pathogen-specific antigens on molecules from *T. pallidum* subsp. *pallidum*, while serum from healthy controls did not have these specificities. Although these observations suggest the presence of a heretofore unrecognized oral spirochete that shares antigens with pathogenic treponemes, it is not possible to determine from these data whether *T. denticola* and/or PROS have the potential to penetrate either necrotic or intact periodontal tissues. The purpose of this investigation was to determine whether MAbs to *T. denticola* and to *T. pallidum* could be used to identify spirochetes in and beyond necrotic gingival lesions.

MATERIALS AND METHODS

Subjects. Subjects with ANUG (n = 16) were diagnosed according to published criteria (6). The subjects were 15 to 30 years of age and presented with clinical evidence of spontaneous gingival bleeding, with acute necrosis and/or ulceration of more than one interdental papilla, and with pain of sudden onset and of gingival origin with a duration of at least 2 days. Informed consent was obtained from all participants. Normal tissue (n = 8) was saved from discarded healthy gingiva removed from extraction sites in patients without periodontal disease. These normal tissue samples included interdental papillae and adjacent gingiva and represented the same areas collected from patients with ANUG.

Tissue preparation. Interdental papillae at affected sites

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TABLE 1. MAbs used in this study

MAb	Investigator	Reference	Prototype strain or subspecies
Bg	L. Simonson	24	*B. gingivalis* ATCC 33277
TDII	L. Simonson	25	*T. denticola* ATCC 33521
TDIII	L. Simonson	23	*T. denticola* ATCC 33520 *T. denticola* ATCC 35404
TDXI	A. Nilius[a]	15	*T. denticola* ATCC 35405
TDXIII	A. Nilius	15	*T. denticola* ST10[b]
H9-1	S. Lukehart	9	*T. pallidum* subsp. *pallidum*, *T. pallidum* subsp. *pertenue*
H9-2	S. Lukehart	9	*T. pallidum* subsp. *pallidum*, *T. pallidum* subsp. *pertenue*
B1A1	S. Lukehart[c]	11	*T. pallidum* subsp. *pallidum*, *T. pallidum* subsp. *pertenue*

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[b] A generous gift from R. Smibert, Virginia Polytechnic Institute and State University, Blacksburg, Va.
[c] University of Washington, Seattle.

were excised, rinsed immediately in phosphate-buffered saline (pH 7.2), and snap frozen in liquid nitrogen. Tissue was stored at −70°C. Frozen serial sections, approximately 6 μm thick, were dried onto glass slides coated with chromalum gelatin (0.05 g of chromium potassium sulfatedodecahydrate and 0.05 g of gelatin in 100 ml of distilled water), immersed in cold 100% acetone for 15 to 30 s, air dried, and stored at 0°C. Negative control normal gingival tissue and positive control tissue from rabbit testes infected with *T. pallidum* subsp. *pallidum* (Nichols strain) were treated in the same manner.

MAbs. The monoclonal IgG antibodies used in this study are identified in Table 1. TDII,IAA11 (serovar B), will hereafter be referred to as TDII, TDIII,IIIBB2 (serovar C) will be referred to as TDIII, TDXI,R8B8R8E3 (serovar A) will be referred to as TDXI, and TDXIII,R9D9 (serovar D) will be referred to as TDXIII. BgII,VF9/2d will be referred to as Bg. Bg was used as a nonspirochetal MAb control. None of the MAbs described above reacts with *T. vincentii*, *T. scoliodontum*, *T. socranskii* subsp. *socranskii*, *T. socranskii* subsp. *buccale*, *T. socranskii* subsp. *paredis*, *T. pectinovorum* (15, 23, 24), *T. phagedenis* biotype Reiter, *T. refringens*, or *T. pallidum* subsp. *pallidum* (2). MAbs B1A1 and H9-1 react with determinants on the 47-kDa lipoprotein antigen, and H9-2 reacts with a 37-kDa endoflagellar sheath antigen of *T. pallidum* subspecies *pallidum* and *pertenue* (11, 13) but not with cultivable treponemes (2, 11, 13).

The working dilution for each MAb was determined by titration against specific bacteria dried onto glass slides as previously described (2). Hybridoma culture supernatants containing B1A1, H9-1, and H9-2 were pooled (final concentrations of 24.9, 9.7, and 24.7 μg of IgG per ml, respectively) to detect spirochetes possessing antigens homologous with pathogenic treponemes (these pooled MAbs will hereafter be referred to as PT MAbs). Hybridoma culture supernatants containing TDII, TDIII, TDXI, and TDXIII were pooled (final concentrations of 4.2, 38.5, 19.5, and 2.7 μg of IgG per ml, respectively) to detect *T. denticola* (Td MAbs). Hybridoma culture supernatant containing Bg was used alone at a final concentration of 4.0 μg of IgG per ml.

Immunohistochemistry. Frozen tissue sections on glass slides were thawed, dried at room temperature to remove water, fixed in cold 100% acetone for 10 min, air dried to remove acetone, and rehydrated in Tris-buffered saline (TBS) (pH 7.2) for 5 min. Nonspecific binding sites in tissue sections were blocked for 10 min with TBS supplemented with 10% normal rabbit serum (catalog no. 01-6101; Zymed Laboratories, Inc., South San Francisco, Calif.), 0.05% gelatin, and 0.02% Tween 20. Excess blocking solution was removed, and each MAb preparation (Bg, PT, and Td) was incubated on separate sections of ANUG or control tissues at room temperature for 30 min. Control sections for each subject were incubated with TBS instead of MAbs (preliminary studies showed that inappropriate MAbs did not produce stain [data not shown]). The slides were then rinsed three times for 1 min each time with TBS containing 0.02% Tween 20. Biotinylated rabbit anti-mouse IgM plus IgG plus IgA (catalog no. P50010; Zymed) was added for 10 min, and then the slides were washed as before. Streptavidin-alkaline phosphatase conjugate (catalog no. P50091; Zymed) diluted 1:100 in TBS–0.02% Tween 20 was added for 10 min. The slides were washed again, and then *p*-nitrophenyl phosphate (substrate) and fast blue (chromogen) in alkaline buffer (catalog no. 00-2204; Zymed) supplemented with levamisole (catalog no. 00-2205; Zymed) to block endogenous alkaline phosphatase (16) were added for approximately 5 min. The slides were washed in distilled water and dried, and the coverslips were mounted with a glycerol-based aqueous medium (catalog no. 00-8001; Zymed). Some slides were counterstained with eosin before mounting.

Tissue evaluation. Specimens were examined at a magnification of 1,000×. The distribution of stain was evaluated in three regions: (i) within affected tissues, (ii) on or in the epithelium on either side of lesions, and (iii) connective tissue adjacent to lesions. A lesion was defined as that part of a specimen containing disrupted epithelium and connective tissue with interstitial spaces or vacuoles.

Whenever possible, two or more slides were examined with each MAb preparation for each subject, and the evaluator was unaware of the MAb used. A subject was considered positive for a given MAb if reactivity was observed in any region on any slide. Proportions of subjects reactive with each MAb preparation were compared by the χ^2 test for two independent samples. Significance was set at $P < 0.05$.

Each positive sample was also evaluated for the intensity of stain produced by each MAb preparation. Each of at least 10 high-power fields was given a score ranging from 0 to 4+, where 0 represents a lack of stain (as found with normal gingiva) and 4+ represents heavy staining (observed in infected rabbit testes treated with PT MAbs). Scores for each subject were averaged, and the mean and standard deviation were computed for each MAb. The significance of differences between means was determined by the two-tailed *t* test. Significance was set at $P < 0.05$.

RESULTS

Controls. Bg and Td MAbs did not stain *T. pallidum*-infected control rabbit tissue, while PT MAbs produced areas of heavily concentrated stain.

Reactivity of normal and ANUG gingiva with MAbs. Table 2 shows that no MAb preparation reacted with normal human gingiva. In contrast, 12 of 16 tissue samples of gingiva from ANUG sites were reactive with PT MAbs, 9 of 16 were reactive with Td MAbs, and 8 of 16 were reactive with Bg MAb. When reactivity was considered irrespective of distribution, there were no significant differences in proportions of ANUG samples stained with the three MAb preparations.

Table 3 indicates the relative intensity of staining in positive tissues for each MAb preparation. When stain intensity was considered irrespective of frequency or distri-

TABLE 2. Gingival tissue reactivity with MAbs

Tissue samples (*n*)	No. of samples reactive[a] with indicated MAb(s)		
	PT[b]	Td[c]	Bg
Normal (8)	0	0	0
ANUG (16)	12	9[d]	8[d]

[a] Reactivity means one or more positive reactions anywhere in the tissue on any slide for a subject.
[b] B1A1, H9-1, and H9-2 MAbs, which react with pathogen-restricted antigenic determinants on *T. pallidum* subspecies *pallidum* and *pertenue*, were pooled.
[c] *T. denticola* MAbs TDII (serovar B), TDIII (serovar C), TDXI (serovar A), and TDXIII (serovar D) were pooled.
[d] Not different from PT MAbs by the χ^2 test.

bution of positive reactions, there were no differences between the three MAbs.

Distribution of bacteria within ANUG tissue. PT MAbs reacted with PROS with equal frequencies in all three zones of tissue from ANUG sites (Table 4). PT MAbs reacted with PROS in intact epithelium and within connective tissues adjacent to lesions (Fig. 1A), including perivascular locations (Fig. 2). High-power (1,000×) magnification revealed positively stained bacteria with spirochetal morphology (data not shown). In contrast, Td antibodies reacted primarily within lesions (Fig. 1B) and there was no significant difference between the proportion of samples positive for *T. denticola* and the proportion positive for PROS. *T. denticola* was found less frequently in intact epithelium or connective tissues than were PROS ($P < 0.01$ for each zone) and *T. denticola* was not detected unless PT MAb-reactive PROS were also present. Bg MAb reactivity was found less often in epithelium ($P < 0.001$), lesions ($P < 0.01$), and connective tissue ($P < 0.01$) than were PROS.

DISCUSSION

Spirochetes are a major component of dental plaque around lesions of necrotizing ulcerative gingivitis in humans,

TABLE 3. Intensity of MAb reactivity within ANUG tissue

MAb(s) and tissue region[a] (no. of positive samples)	Mean reaction intensity (SD)[b]	*P*[c]
PT (12)		
Epithelium	2.0 (1.0)	
Lesion	2.8 (0.9)	
Connective tissue	2.5 (0.7)	
Td (9)		
Epithelium	1.2 (0.5)	NS
Lesion	3.0 (1.2)	NS
Connective tissue	2.5 (1.4)	NS
Bg (8)		
Epithelium	0.9	
Lesion	2.1 (0.8)	NS
Connective tissue	1.5 (0.6)	<0.05

[a] Epithelium indicates epithelium on surface of specimen; Lesion indicates reaction within affected tissue of lesion; Connective tissue indicates intact connective tissues around lesion.
[b] Positive reactions were graded from 1+ to 4+. Three to five slides per subject were graded for each zone; the results were averaged for each subject. The mean and standard deviation were calculated from the average values for each positive subject.
[c] Compared with PT MAbs by the two-tailed *t* test. NS, not significant.

TABLE 4. Distribution[a] of MAb reactivity within ANUG tissue

MAb(s) and tissue region (no. of reactive samples/total no. of samples)	No. of samples positive in region (%)[b]	*P*[c]
PT (12/16)		
Epithelium	12 (100)	
Lesion	12 (100)	
Connective tissue	12 (100)	
Td (9/16)		
Epithelium	3 (33)	<0.01
Lesion	7 (78)	NS
Connective tissue	4 (44)	<0.01
Bg (8/16)		
Epithelium	2 (25)	<0.001
Lesion	4 (50)	<0.01
Connective tissue	4 (50)	<0.01

[a] Distribution of stain produced by each MAb preparation in indicated regions of tissue (see Table 3, footnote *a*).
[b] Values in parentheses are percentages of the total number of positive specimens for the indicated MAb preparation.
[c] Compared with PT MAbs by the χ^2 test. NS, not significant ($P > 0.05$).

but it is not known which of the many types of plaque spirochetes are able to penetrate gingival epithelium (7, 9) into healthy connective tissues surrounding ulcerated gingiva (3, 7). The current study indicates that PROS are often found in tissues beyond lesions, whereas *T. denticola* and *Bacteroides gingivalis* are found significantly less frequently beyond lesions. Furthermore, *T. denticola* and *B. gingivalis* were usually found at diseased sites when PROS were also present, and they produced approximately the same intensity of stain as did PROS. If the intensity of stain in tissue is related to the number of bacteria present, then these observations indicate that when *T. denticola* can get into tissue it is there in about the same density as PROS; however, PROS seem to be able to get beyond necrotic lesions to a much greater extent than either *T. denticola* or *B. gingivalis*. We have shown previously (2) that PT MAb-reactive spirochetes are not present in plaque around healthy periodontal tissues but that both *T. denticola* and *B. gingivalis* are present in plaque from healthy sites, even in young children. Taken together, these observations suggest that PT MAb-reactive spirochetes may have a stronger association with disease than either *T. denticola* or *B. gingivalis*.

Among identified spirochetes, those that are able to penetrate intact integument and invade tissues are recognized pathogens. The pathogenic treponemes, including the etiologic agents of nonvenereal treponemal diseases (*T. pallidum* subsp. *endemicum*, endemic syphilis; *T. pallidum* subsp. *pertenue*, yaws; and *T. carateum*, pinta) as well as the syphilis treponeme (*T. pallidum* subsp. *pallidum*), all gain entry into the body through epithelium or mucosal surfaces (4, 21). Furthermore, *T. pallidum* subsp. *pallidum* is able to penetrate tight intercellular junctions of endothelial cell monolayers (26) and can migrate through epithelium and underlying connective tissues in vitro (5, 18). In vivo, this invasive ability leads to dissemination from inoculation sites to remote anatomical locations (14, 20). In contrast, cultivable, nonpathogenic *T. phagedenis* biotype Reiter and other cultivable oral treponemes, including *T. denticola* (17), cannot invade cell monolayers or tissue.

The current study used MAbs to pathogenic treponemes to demonstrate that PROS are found in epithelium and

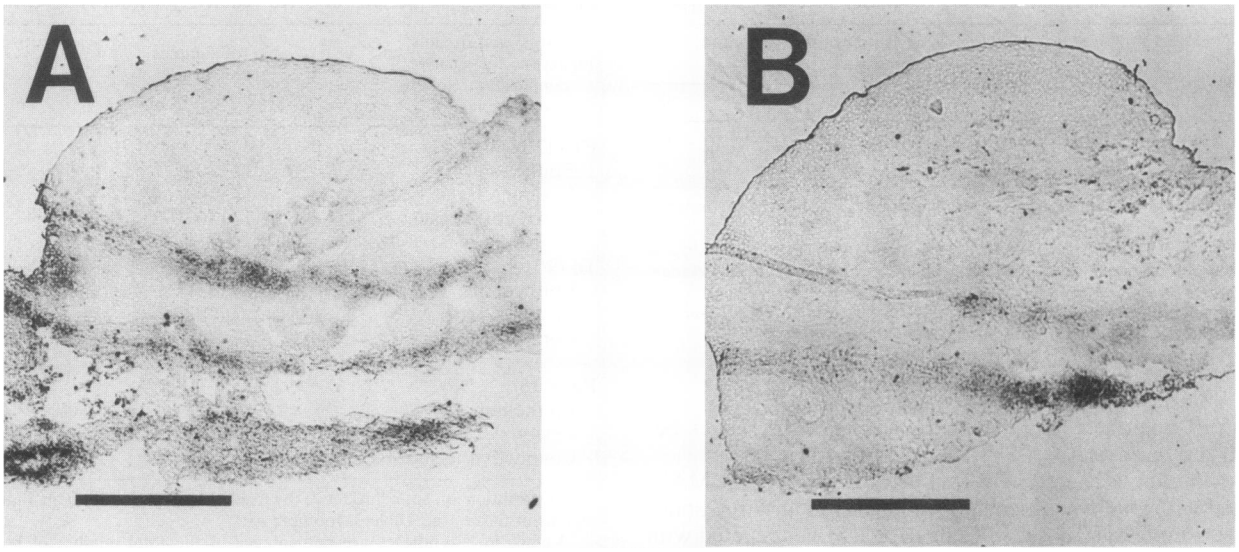

FIG. 1. Cross-sections through the buccal side of a necrotic interdental papilla obtained from subject A10. Cryostat tissue sections were treated with either PT MAbs (A) or Td MAbs (B). PT MAbs reacted with antigen in the necrotic area of the lesion (necrosis is seen as an area of ragged concavity on the surface at the left in panel A and as a vesicular area at the right in panel B; the necrotic surface is not in the plane of the section shown in panel B), in intact epithelium (the epithelium is the exterior covering of the tissue dome at the top of each panel), and in connective tissues surrounding the lesion (the connective tissue is the relatively clear area under the epithelium and contains PT MAb-dependent stain). Td MAbs primarily stained treponemes within the lesion. Bar, 0.1 mm.

connective tissues adjacent to ulcerative lesions in the majority of samples from patients with ANUG. PT MAb-reactive spirochetes were found around vesicular spaces in epithelium and connective tissues and were also observed in intact epithelium and connective tissues beyond damaged areas. However, although these new spirochetes had a stronger association with the disease process and were found in tissues beyond necrotic areas more often than was *T. denticola* or *B. gingivalis*, the evidence gathered in this investigation does not prove that PT MAb-reactive spirochetes are involved in the etiology of ANUG. This study does suggest, however, that a new *T. pallidum* subspecies or perhaps a new *Treponema* species has been identified and that, because of its relatedness to other pathogenic treponemes, it may be a good candidate for an invasive plaque bacterium. Further experiments, including isolation and characterization, are required before its pathogenic potential can be defined and its relatedness to known oral treponemes and pathogenic spirochetes can be established. Furthermore, although it is possible that spirochetes found in gingiva by other investigators (3, 7–9) are PROS, the lack of correlative data prevents extrapolation from those studies to the current work.

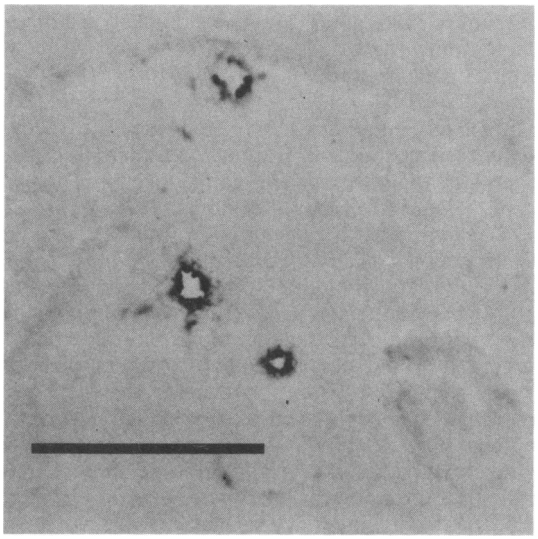

FIG. 2. Perivascular stain in gingival tissue from a patient with ANUG, developed after treatment with PT MAbs. Bar, 0.5 mm.

In summary, intact epithelium and connective tissues around necrotic gingiva, but not healthy gingiva, contain spirochetes that bear antigens heretofore thought to be restricted to pathogenic treponemes. These data suggest that a newly recognized spirochete, closely related to *T. pallidum*, is associated with necrotizing ulcerative gingivitis. Further experiments are needed to identify these spirochetes and to define their role in periodontal diseases.

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