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NOTES

Detection of *Treponema denticola* in Atherosclerotic Lesions

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We examined 26 atherosclerotic lesions and 14 nondiseased aorta specimens to detect the periodontopathogenic part of the bacterial 16S rRNA locus by PCR. *Treponema denticola* sequence of the 16S rRNA locus was found in 6 out of 26 DNA samples (23.1%) from the formalin-fixed, paraffin-embeded atherosclerotic lesions obtained during surgery but not in any of the 14 nondiseased aorta samples from deceased persons. Utilizing immunofluorescence microscopy, we observed aggregated antigenic particles reacting with rabbit antiserum against *T. denticola* in thin sections of the PCR-positive samples, but we could not detect any reacting particles in the PCR-negative thin sections.

Recent epidemiological studies have established that rheological and hemostatic factors are related to vascular diseases. These factors are potential biological effectors which may interact with known risk factors such as hyperlipidemia, smoking, and infections to promote vascular events. A number of studies have suggested that infectious organisms may play a role in the etiology and epidemiology of atherosclerosis and related diseases (5, 6, 9, 29). For one such organism, *Chlamydia pneumoniae*, there is mounting evidence associating infections with a greater risk of atherosclerosis, myocardial infarction, and chronic coronary heart disease (3, 20, 26). Persistent infections by the obligate intracellular gram-negative bacteria are involved in a wide spectrum of respiratory diseases. It is now recognized that chronic oral infections, such as adult periodontitis, may have important long-term sequelae (2, 7, 10, 16, 19, 21–24, 28). An inflammatory response to endothelial cell injury and dysfunction caused by these infections may lead to atherosclerosis (26). Recently, Haraszthey et al. (11) reported that periodontal organisms such as *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Bacteroides forsythus*, and *Prevotella intermedia* were detected in atheromatous plaque by PCR. In the present study, we attempted to detect periodontopathic bacterial DNA in atherosclerotic vascular lesions.

To detect *A. actinomycetemcomitans, P. gingivalis, B. forsythus, Campylobacter rectus*, and *Treponema denticola*, we used the PCR and followed a double-blind protocol. The primers for detecting part of the bacterial 16S rRNA locus by PCR were synthesized in accordance with previously reported procedures (1, 30). Primer pairs for *T. denticola* (5′-TAATACCG AATGTGCTCATTTACAT-3′ and 5-TCAAAGAAGCATTC CCTCTTCTTCTTA-3′), *B. forsythus* (5′-GCGTATGTAACC TGCCCGCA-3′ and 5′-TGCTTCAGTGTCAGTTATACCT-3′), and *C. rectus* (5′-TTTCGGAGCGTAAAACTCCTTTTC-3′ and 5′-TTTCTGCAAGCAGACACTTTT-3′) were designed on the basis of 16S rRNA (1). Primer pairs for *P. gingivalis* (5′-ATAATGGAGAACAGCAGGAA-3′ and 5′-TCTTGCC AACCAGTTCCATTGC-3′) and *A. actinomycetemcomitans* (5′-CAGCAAGCTGCACAGTTTGCAAA-3′ and 5′-CATTA GTTAATGCCGGGCCGTCT-3′) were designed on the basis of fimbriae and leukotoxin (30). Primer pairs for *C. pneumoniae* (5′-TGACAACTGTAGAAATACAGC-3′ and 5′-GG TTGAGRTCAACGACTTAAGG-3′) were designed based on the 16S rRNA sequence by Jantos et al. (17). The preparation of the reaction mixture depended on the individual report. PCRs were performed according to these reports. Briefly, reactions for *T. denticola, B. forsythus*, and *C. rectus* included an initial denaturation step at 95°C for 2 min; 36 cycles of a denaturation step at 95°C for 30 s, an annealing step at 60°C for 1 min, and a final step at 72°C for 1 min, and a final step of 72°C for 2 min. Reactions for *P. gingivalis* and *A. actinomycetemcomitans* included 32 cycles of a denaturation step at 95°C for 30 s, an annealing step at 55°C for 30 s, and a final step at 72°C for 1 min. Reactions for *C. pneumoniae* included an initial denaturation step at 95°C for 2 min; 40 cycles of a denaturation step at 94°C for 15 s, an annealing step at 55°C for 15 s, and a final step at 72°C for 35 s; and a final step at 72°C for 10 min. In all experiments, negative and positive controls were used to confirm the results of the reactions. DNA samples were extracted from formalin-fixed, paraffin-embedded blocks of atherosclerotic vascular lesions from 26 autopsy samples from individuals with vascular diseases and 14 samples from nondiseased aorta specimens from 14 deceased persons without vascular disease, using a DNA extraction solution (Dexpat; Takara, Otsu, Japan) according to the manufacturer's protocol. A number of standard precautions were taken to prevent the occurrence of spurious results due to contamination; these included the use of dedicated laboratory space, Dexpat re-

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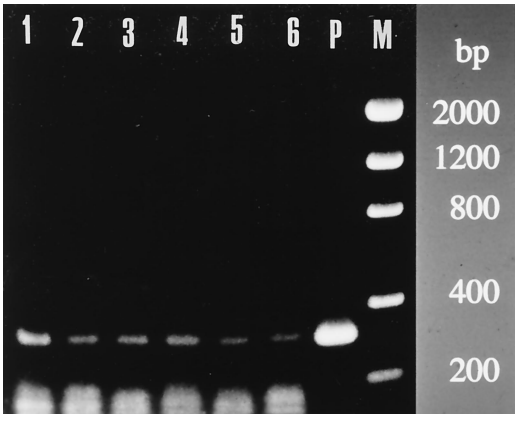

FIG. 1. Amplified bands of the *T. denticola* 16S rRNA. Lanes 1 to 6, DNA samples extracted from the atherosclerotic aortas obtained from surgically excised tissue of patients with vascular disease (Table 1); Lane P, positive control; lane M, positive base pair markers. Strong bands are present in lanes 1 to 4, and faint bands are present in lanes 5 and 6 at positions, corresponding to the 316-bp *T. denticola* 16S rRNA fragment.

agents, and pipetting with filters for pre- and post-PCR analysis and the testing of positive and negative control samples which included 10^3 *T. denticola* cells per μl and 5 μl of autoclaved water, respectively, in parallel with the test samples. In all examinations in this study, we checked for contamination of the target bacterial species and confirmed that there was no contamination in the negative samples.

We detected 316-bp bands of *T. denticola* DNA in the DNA samples extracted from 6 of 26 sites (23.1%) in diseased atherosclerotic aortas by 32 amplified cycles, but we could not detect such bands in samples from the 14 nondiseased aortas. Chi-square analysis showed that the relationship between atherosclerosis and detection of *T. denticola* is not statistically significant ($P = 0.0512$), but the P value is close to a statistically significant level. The amplified DNA bands are summarized in Fig. 1. All *T. denticola* DNA-positive samples were obtained from patients who had received surgical operations. The ages, sexes, and clinical diagnoses of the patients with the positive samples in which *T. denticola* sequence (fragment) of the 16S rRNA locus was detected are summarized in Table 1. All of the six samples in Table 1 were obtained from surgically excised tissues. We noted that patients 1, 2, 4, 5, and 6 had periodontitis lesions. To confirm that the amplified products were from *T. denticola*, the amplified DNA products from these samples were cloned into the pCR-100 vector using a cloning kit (Invitrogen, San Diego, Calif.) and sequenced by the dideoxy-chain termination method (27). We confirmed that the DNA sequences of the six amplified DNA samples were identical to those of *T. denticola* ATCC 33520 strain rRNA. Homology searches of the amplified bands were performed using the BLAST system. The degree of identity between the amplified fragment and the sequence deposited in the National Center for Biotechnology Information was 98%. The observed variation is minor, and in every case the sequence with the highest score in BLAST analysis was that from *T. denticola*. We examined the six samples in which the *T. denticola* sequence of the 16S rRNA locus was detected for the presence of a DNA band of *C. pneumoniae* using previously described primers (17), but we could not find any such band. Unexpectedly, no PCR-amplified DNA bands of *A. actinomycetemcomitans, P. gingivalis, B. forsythus*, or *C. rectus* were observed in 26 samples with atherosclerotic vascular lesions using the primers in this study.

TABLE 1. Age, sex, and clinical diagnosis of patients with atherosclerosis in whom *T. denticola* 16S rRNA was detected

Patient	Age (yr)	Sex	Clinical diagnosis
1	64	Male	Abdominal aortic aneurysm
2	64	Male	Abdominal aortic aneurysm
3	42	Female	Thoracal aortic aneurysm
4	62	Male	Thoracal aortic aneurysm
5	76	Male	Dissecting aneurysm
6	68	Male	Arcus aortic aneurysm

We examined the PCR-positive samples by an immunofluorescence technique for the presence of antigens of *T. denticola*. Rabbit antiserum against *T. denticola* was obtained by repeated inoculation of the cells into male New Zealand White rabbits as described in our previous papers (14, 15). Thin sections of the paraffin block samples were incubated with rabbit antiserum diluted 1:250 or 1:500 with phosphate-buffered saline (PBS) (pH 7.2) at room temperature for 60 min and then washed thoroughly with PBS. The samples were then incubated with fluorescence-conjugated goat anti-rabbit immunoglobulin G (Cappel, ICN Pharmaceuticals, Inc., Aurora, Ohio) diluted 1:1,000 with PBS. The samples were observed by immunofluorescence microscopy (Axiophot 2; Carl Zeiss, Jena, Germany). Panels 1A, 2A, 3A, and 4A in Fig. 2 are thin sections of atherosclerotic lesions which were stained with hematoxylin-eosin and in which we detected *T. denticola* DNA, as shown in Table 1 and Fig. 1. Panels 1B, 2B, 3B, and 4B in Fig. 2 are the immunofluorescence photographs corresponding to panels 1A, 2A, 3A, and 4A. The stained particles were observed in these sections. We detected some fluorescent particles in the foam cells within the fibrolipid lesions. The locations of the immunostained antigenic particles were similar to those reported for *C. pneumoniae* by Kuo et al. (20). However, we could not detect any spiral cells in the thin sections stained with the sera examined. It is possible that aggregated antigens of *T. denticola* were stained with the rabbit antiserum. No distinct fluorescent particle was observed when the sections were stained with rabbit serum absorbed with whole *T. denticola* cells. No fluorescent particle was observed in sections in which we could not detect the DNA band of 316 bp.

Oral gram-positive and gram-negative bacteria have frequently been identified in bacteremia and may play a role in vascular diseases (8, 12, 13). In addition, phagocytes in periodontal lesions may engulf various bacterial cells and their antigens (19). The bacterial cells and phagocytes may then penetrate the gingival tissues, be transported via the circulation to the heart, and adhere to the endothelium of an artery. These deposited bacteria can then stimulate the release of inflammatory cytokines and initiate the formation of the characteristic foam cells associated with atherosclerosis. Recently, Chiu (4) reported that oral bacteria, including *Streptococcus sanguis* and *P. gingivalis*, could be detected in vascular lesions. We demonstrated earlier that a protease, dentilisin, of *T. denticola* can degrade the intracellular matrix (14, 15). In addition

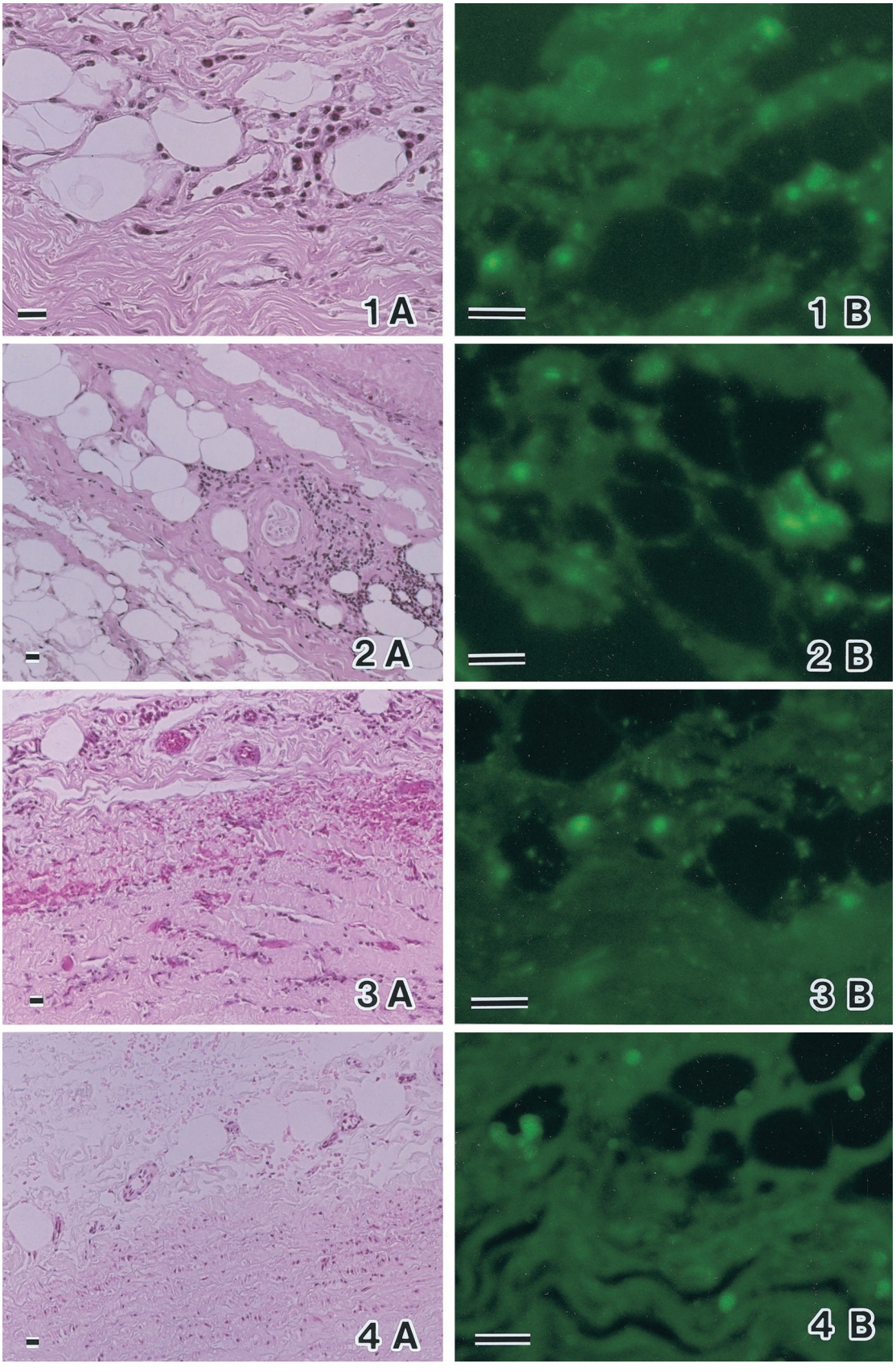

FIG. 2. (1A, 2A, 3A, and 4A) Thin sections of atherosclerotic lesions, stained with hematoxylin and eosin, in which strong *T. denticola* DNA bands were detected (Table 1 and Fig. 1). (1B, 2B, 3B, and 4B) Thin sections corresponding to panels 1A through 4A, respectively, stained with rabbit antiserum against *T. denticola*. Clear immunofluorescent particles can be observed in localized areas of foam cells or between small muscles in panels 1B through 4B. Bars, 20 μm.

to the motility of *T. denticola* cells, it is possible that the protease activity may also play a role in their penetration of periodontal tissues by infiltrating between the gingival cells and into the bloodstream. Recently, Peters et al. (25) showed that oral treponemes penetrated endothelial cells. The affinity of *Treponema pallidum* for vascular structures is well known (18), but the molecular basis for this property is unknown. We found that five of the six *T. denticola*-positive aorta samples were from patients with periodontitis. In order to confirm that *T. denticola* can penetrate into the gingival tissues and that the spirochetes carried by phagocytes can play a role in atherosclerosis, additional studies with humans and studies with animal models will be needed. Moreover, the presence of oral spirochetes in some aorta lesions does not necessarily imply an etiological role for the spirochetes in atherosclerosis.

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