Molecular Identification of an Invasive Gingival Bacterial Community

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A woman with neutropenia developed gingival hyperplasia. Biopsy showed invasion of gingival tissue with mats of filamentous organisms, and molecular analysis by polymerase chain reaction and fluorescence in situ hybridization revealed *Capnocytophaga sputigena, Leptotrichia* species, and *Fusobacterium nucleatum*. Oral bacterial flora may cause invasive gingival disease with hyperplasia in immunocompromised patients.

The mouth is a reservoir of bacteria with high species diversity [1]. Some species can cause invasive disease in patients with cancer who have compromised mucosal barriers because of radiation therapy or chemotherapy or who have concomitant neutropenia [2, 3]. Identification of bacterial pathogens in oral lesions is challenging because cultivation of specimens from this nonsterile site invariably yields a diversity of bacteria, making associations between cultivated bacteria and particular oral lesions tenuous. Furthermore, histological detection of bacteria in tissues by means of Gram staining can be difficult, because some gram-negative bacteria are not easily distinguished among the background of tissue cells. Molecular methods such as PCR and fluorescence in situ hybridization (FISH) can be used to detect, identify, and localize bacteria associated with occult bacterial lesions of the mouth.

Case report. A 45-year-old woman who had acute myelogenous leukemia diagnosed underwent chemotherapy and 2 consecutive transplantations (separated by 2 months) of peripheral blood stem cells from matched unrelated donors, with nonmyeloablative preparative regimens that included fludarabine therapy and total body irradiation. Both grafts failed, resulting in persistent neutropenia.

Thirty days after receiving the second allogeneic hematopoietic stem cell transplant, the patient reported mouth and jaw pain, fever, chills, anorexia, and malaise. On examination, gingival hypertrophy and right submandibular lymphadenopathy were noted. There were no other oral mucosal lesions. The patient had been taking cyclosporine and mycophenolate mofetil for graft-versus-host disease prophylaxis and amlodipine therapy for hypertension. These medications were discontinued. She experienced neutropenia for 4 weeks, with an absolute neutrophil count of 0 cells/μL. A blood culture grew *Staphylococcus epidermidis* and *Streptococcus salivarius*. The patient was given vancomycin therapy for gram-positive bacteremia and was given levofloxacin prophylaxis for neutropenia.

After 1 week of receiving antibiotic therapy, she was seen by the Oral Medicine service of the Seattle Cancer Care Alliance and was noted to have hypertrophy, primarily of the maxillary anterior attached gingiva, with minimal involvement of the interdental papilla. The tissue was firm to palpation. A gingival punch biopsy specimen was obtained from the anterior left maxillary gingiva above the lateral incisor and canine and was immediately placed in formalin for histological examination. Two days later, a follow-up blood sample was obtained, and the blood culture grew *Capnocytophaga sputigena,* a finding confirmed by 16S rDNA sequencing. The patient reported no fever, chills, or sweats but reported ongoing gingival pain, anorexia, and malaise. These symptoms and gingival hypertrophy resolved after treatment with imipenem, despite persistent neutropenia. Review of the gingival biopsy specimen showed hyperplastic stratified squamous epithelium. Deep in the tissue and at the margins of the resected specimen, there was a dense infiltrate of small filamentous organisms and minimal inflammatory response (figure 1). Organisms were visible with hematoxylin stain and methenamine silver stain but not with Brown-Brenn tissue Gram stain or Fite-Faraco stain. Culture of a gingival swab specimen grew normal oral flora. The identity of the filamentous microorganism(s) present in the deep gingival tissue was unknown.

Methods. For broad-range 16S rDNA PCR, sections of gingival tissue were cut from the paraffin block, dewaxed, digested to liberate DNA, and subjected to PCR, as described elsewhere [4], by use of primers Bact16S-338F (5′-ACTCCTRCGGGAGGCAGCAG-3′) and Bact16S-805R (5′-GACTACCAGGGTATCTAATCC-3′), which are complementary to highly

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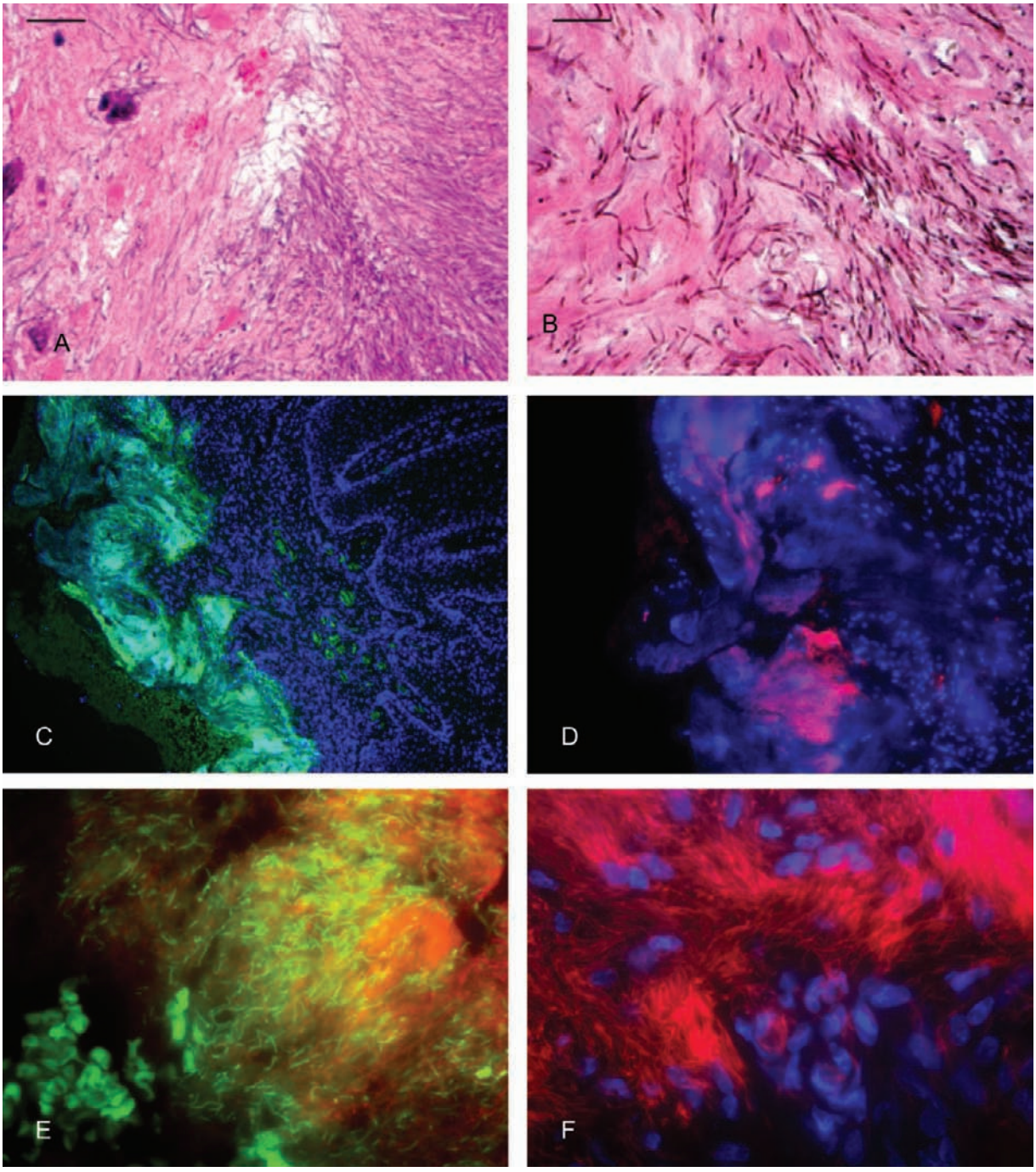
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conserved regions of the bacterial 16S rRNA gene and amplify an ~467-bp segment. Thirty-five cycles of PCR were performed with high-purity *Taq* polymerase (*Taq* LD; Applied Biosystems). Digestion controls lacking tissue were processed in the same manner and were subjected to PCR, as were the no-template PCR controls. Amplification products were cloned into *Escherichia coli* by means of a TOPO-TA vector (Invitrogen). Forty-one bacterial clones from the gingival tissue PCR were chosen. M13-amplified 16S rDNA inserts were screened for sequence diversity using restriction fragment–length polymorphism (RFLP) analysis with *Hin*P1 and *Hae*III restriction enzymes. Clone inserts with unique RFLP patterns were sequenced. Amplified 16S rDNA sequences were aligned with known 16S rDNA sequences in GenBank to identify bacteria with the use of the basic local alignment search tool (BLAST) of the National Center for Biotechnology Information.

For *Capnocytophaga*-specific 16S rDNA PCR, we designed primers that amplify an ~337-bp segment of 16S rDNA from bacteria in the *Capnocytophaga* genus: Capno16S-147F (5′-GGATAGCCCGAAGAAATTTGGAT-3′) and Capno16S-484R (5′-CGTCATCAAAGTACACGTACTCCTTAT-3′). After 40 cycles of PCR, amplification products were electrophoresed on 2% agarose gels and were visualized with ethidium bromide stain and ultraviolet transillumination. Amplification products were sequenced and aligned with known bacterial 16S rDNA sequences from GenBank to identify species.

We designed fluorescently labeled oligonucleotide probes that target genus-specific regions of bacterial 16S rRNA for in situ hybridization with bacteria in gingival tissue sections and for localization of bacteria by fluorescence microscopy. Probes were designed for the detection of *Leptotrichia* species (5′-TCCAGTGARCTATCTTCATCATC-fluorescein-3′) and *Capnocytophaga* species (5′-TTAATCCAAATTTCTTCGGGCTATC-Cyanine 3-3′). A probe for *Fusobacterium* species (5′-CTAATGGGACGCAAAGCTCTCTC-Cyanine 3-3′) was created on the basis of a template available at ProbeBase (http://www.microbial-ecology.de/probebase). Formalin-fixed paraffin-embedded gingival tissue was sectioned, placed on microscope slides, and dewaxed. FISH was performed as described elsewhere [5], with 15% formamide in the hybridization buffer and a hybridization temperature of 45°C. A broad-range 16S rRNA bacterial probe (Eub338–Cyanine 5) and the DNA-staining dye 4′,6-diamidino-2-phenylindole (DAPI) were also used to detect bacteria. Cultivated strains of *Fusobacterium nucleatum* (ATCC 25586), *Leptotrichia buccalis* (ATCC 14201), and *Capnocytophaga sputigena* (ATCC 33612) were used to test the specificity of the bacterial probes. Digital images were generated with a Nikon epifluorescence microscope and Metavue image analysis software (Universal Imaging).

Results. Broad-range 16S rRNA gene PCR of gingival tissue produced bands of the appropriate size on gel electrophoresis, whereas PCR results for the digest control and no-template controls were negative. Analysis for RFLP diversity among cloned PCR products revealed the presence of two 16S rDNA sequence types among the 41 clones evaluated. Sequencing showed that one 16S rDNA sequence was 99.8% similar to that of a *Leptotrichia* species isolate detected in the human mouth by PCR (Genbank accession number AF287816), and the second 16S rDNA sequence type was 100% similar to that of *Fusobacterium nucleatum* subspecies *canifelium*, a bacterium detected in the mouths of dogs and cats.

An amplification product was generated when *Capnocytophaga*-specific PCR was performed on the gingival tissue digest but not when it was performed on the digest control and no-template controls. Sequence alignment analysis of this amplicon yielded 100% similarity to 16S rDNA from *Capnocytophaga sputigena*, the bacterium that was also recovered from blood culture.

Testing of the bacterial probes by use of cultivated bacteria showed that the *Leptotrichia* and *Capnocytophaga* probes were highly specific, whereas the *Fusobacterium* probe showed some uptake in *Leptotrichia buccalis*. FISH demonstrated that *Fuso-*

Figure 1. Micrographs of gingival tissue specimens obtained from a patient with gingival hyperplasia. *A,* Thin bacterial filaments appear blue with the use of hematoxylin and eosin stain, and there are few human cells visible in this field. *B,* Bacteria appear as darkly stained chaining rods with tapered points with the use of methenamine silver stain. In *A* and *B,* bars are 10 μm in length (original magnification, ×1000). *C–F,* Micrographs of gingival tissue sections subjected to fluorescence in situ hybridization (FISH). *C,* Bacteria hybridizing with the broad-range bacterial probe Eub338-Cy5 (*pseudocolor light green*) form a dense band in the deep gingiva (original magnification, ×100). RBCs autofluoresce and appear dark green at the base of the biopsy (indicating hemorrhage) and in blood vessels. Human cell nuclei appear blue with 4′,6-diamidino-2-phenylindole (DAPI) stain, and the gingival surface with rete-like prominences is located to the right of the image. In *D* (image of the base of biopsy), bacteria hybridizing with the *Capnocytophaga* probe appear red and form clusters within the larger group of bacteria stained blue with DAPI (original magnification, ×200). Human nuclei also take up the DAPI stain and appear as discrete blue dots. *E,* Bacteria hybridizing with the *Leptotrichia* probe (*green*) and the *Fusobacterium* probe (*red*) are intertwined (original magnification, ×1000). RBCs autofluoresce and appear green in the lower left-hand corner of the image. *F,* At the edge of the bacterial mat, filamentous bacteria hybridize with the broad-range bacterial probe Eub338-Cy5 (*pseudocolor red*), and human cell nuclei are stained blue with DAPI (original magnification, ×1000). FISH confirmed that *Fusobacterium, Leptotrichia,* and *Capnocytophaga* species all formed filamentous structures in the gingival tissue. Human tissue was replaced with bacteria in some zones. By means of FISH, the thin filaments seen on the hematoxylin and eosin–stained section appear to correspond to *Fusobacterium* or *Capnocytophaga* species, and the wider rods seen on the methenamine silver–stained section appear to correspond to *Leptotrichia* species.

bacterium, Leptotrichia, and *Capnocytophaga* were all present in gingival tissue as thin filamentous organisms (figure 1). Fusobacteria formed the thinnest filaments, whereas *Leptotrichia* species were slightly wider with tapered ends, and these 2 types of bacteria appeared to exist as an interdigitated community. *Capnocytophaga* species also formed thin filaments but were found more focally in discrete clusters within this bacterial community and were the minority genus. These bacteria formed a dense mat in the deep gingiva, with much of the tissue replaced by bacteria, as evidenced by the paucity of human cell nuclei visualized in DAPI-stained sections harboring bacteria.

Discussion. Our patient was severely immunosuppressed after 2 failed allogeneic hematopoietic cell transplantations and persistent neutropenia. She did not have frank mucositis but had gingival hyperplasia that initially was not suspected to be an infectious process. However, biopsy showed evidence of gingival infection with a community of at least 3 filamentous bacteria. It is possible that the gingival hyperplasia in this patient was a result of invasion by oral bacteria and their replication in gingival tissue without a normal inflammatory response. It is also possible that this patient developed gingival hyperplasia because of the use of drugs such as cyclosporine or amlodipine [6], and the oral bacteria invaded the already altered tissue. Bacterial invasion of the gingiva may be one cause of gingival hypertrophy in immunocompromised patients, and this possibility should be explored by using FISH with broad-range bacterial probes to determine whether bacteria exist in hyperplastic tissue from other patients. Molecular methods can help identify uncultivated oral pathogens associated with mucosal lesions in patients with neutropenia, which is an important attribute because many oral bacteria have not been successfully cultivated in the laboratory [1]. Identifying these pathogens can help focus treatment with antibiotics.

Fusobacterium nucleatum, Leptotrichia species, and *Capnocytophaga sputigena* are considered to be normal oral flora of humans [7]. *Fusobacterium* species are strict anaerobes, whereas *Leptotrichia* and *Capnocytophaga* species can grow under microaerophilic, hypercapneic conditions. The 16S rDNA sequence from the *Fusobacterium* species detected in the gingival tissue most closely matched that of the subspecies *canifelium,* and the patient may have acquired this strain from her cat. The 3 bacteria tend to be resistant to vancomycin and quinolone antibiotics, agents that the patient was receiving at the time of biopsy, but they are sensitive to imipenem, and this agent produced a clinical response. Detection of *Capnocytophaga sputigena* in the deep gingival tissue (by PCR and FISH) and in a blood culture specimen demonstrated that this was an invasive process. *Capnocytophaga* was not detected in the broad-range 16S rDNA PCR analysis, probably because it was a minority species and thus poorly represented in the clone library. Despite bacteremia, neutropenia, and a high burden of bacteria in gingival tissue, this patient tolerated the infection remarkably well, which suggests that these bacteria have relatively low virulence.

Cases of bacteremia due to *Capnocytophaga, Fusobacterium,* and *Leptotrichia* species have been described in patients with cancer and in recipients of hematopoietic cell transplants, particularly in the presence of mucositis [8–12]. In a study of bone marrow transplant recipients receiving quinolone prophylaxis, *Fusobacterium nucleatum* and *Leptotrichia buccalis* were the most common causes of anaerobic bloodstream infection [10]. This case illustrates that anaerobic bacteria may produce atypical oral pathology, as well as bacteremia, in immunocompromised patients.

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