Implantation of *Bacteroides gingivalis* in Nonhuman Primates Initiates Progression of Periodontitis

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Although periodontitis is a bacterial disease, its multidimensional nature and its bacterial complexity have made it difficult to definitively prove that specific microorganisms initiate the disease process. The successful implantation of a rifampin-resistant strain of the putative periodontal pathogen *Bacteroides gingivalis* into the periodontal microbiota of monkeys (*Macaca fascicularis*) resulted in an increase in the systemic levels of antibody to the microorganism and rapid and significant bone loss.

PERIODONTITIS IS A BACTERIALLY INduced inflammatory process that results in destruction of the connective tissue and bone that support the teeth. Evidence (*1–6*) suggests that different subgingival microbiota are associated with different clinical forms of the disease. Although it has been estimated that more than 200 different microbial species inhabit the subgingival area of periodontally diseased sites, only a limited number of these species have been implicated as putative periodontal pathogens (*1, 3–8*). At present, *Actinobacillus actinomycetemcomitans* is the only accepted etiologic agent for a specific periodontal disease, localized juvenile periodontitis (LJP) (*5, 8*). The association of specific pathogens with the most common form of periodontal disease, adult periodontitis, is more confounding than LJP because of the complexity of the microbiota, the apparent transient nature of disease progression, and the variability in microbiological data (*5, 9*). Several clinical studies have indicated that proportional increases or ecological "blooms" of a few specific microorganisms may be associated with progression of periodontitis (*10, 11*).

Eight adult female cynomolgus monkeys (*Macaca fascicularis*) with naturally occurring plaque, calculus, and gingivitis but with no detectable *Bacteroides gingivalis* were used. Animals were maintained in accordance with the guidelines of the University of Texas Health Science Center at San Antonio. Microbiological methods and sampling procedures were as previously described (*12–14*). Subgingival samplings at 0, 2, and 4 months during the preexperimental period revealed no *B. gingivalis* isolates from any site in these animals.

Baseline clinical, radiographic, microbiological, and immunological characteristics of the animals were determined. Four animals were then administered rifampin (10 mg/kg; three times per week intramuscularly) during the 8 weeks of period 1 (*15*). The other four animals served as controls and received placebo (phosphate-buffered saline) injections. Rifampin and placebo injections were continued for the entire experiment. Animals were monitored as above, at three separate times during period 1. After this initial period, four posterior teeth were ligated (*14*) to induce periodontitis, which initiated period 2. Period 3 commenced at 20 weeks after ligation and involved the implantation of rifampin-resistant *B. gingivalis* into two selected ligated sites in each animal in both groups of monkeys. Monitoring was continued at 4-week intervals for 12 weeks.

Clinical and radiographic assessments were conducted "blind," and plaque accumulation and inflammation of gingival tissue were assessed by standard procedures (*16*). Changes in alveolar bone support were determined by a standardized radiographic protocol, and the radiographic films were analyzed by computer-assisted densitometric image analysis (CADIA), which quantitates the area as well as the magnitude of density change as compared to the initial baseline films (*17*).

Bacteroides gingivalis strain 3079.03, isolated from a ligature-induced periodontitis site in a cynomolgus monkey, was used to derive the rifampin-resistant mutants. High-level rifampin-resistant mutants were selected in a multistep process that produced *B. gingivalis* strain 3079.03-R1 (Bg^R) which was resistant to rifampin (25 g/ml) and was used in the animal implantation experiments.

For implantation and subsequent monitoring of subgingival samples, Bg^R was grown on culture media (*12*) + rifampin (20 μg/ml) for 5 days. Just before implantation, the cultures were removed from the Brewer jars used for anaerobic transport, and a 10-μl sample was drawn into a Hamilton microsyringe which had a blunted needle tip. The syringe tip was carefully placed into the subgingival region of the periodontal pocket, and an approximately 8-μl sample was slowly injected into the pocket. This procedure was repeated three times per week for 2 weeks, followed by a 7-week rest period and then another week of implantation.

We measured antibody levels to *B. gingivalis*, *B. intermedius*, *A. actinomycetemcomitans*, and *Fusobacterium nucleatum* by using a modification of an enzyme-linked immunosorbent assay (ELISA) technique (*18, 19*). Briefly, cells were cultured and used as antigens as previously described (*18, 19*). The antigen-coated plates were incubated with monkey sera, and the system was developed with affinity-purified goat antibodies to human immunoglobulin G (IgG) or IgM (Behring Diagnostics) followed by affinity-purified rabbit antibodies to goat IgG conjugated to alkaline phosphatase (Behring Diagnostics). Antibodies to human immuoglobulins effectively detect the monkey antibody with an efficiency of 45 to 50% for IgG and 40 to 60% for IgM when compared to both human antibody preparations or to antibodies to monkey immunogloblins. Also, preliminary studies have shown that monoclonal antibodies to human IgG sub-

Table 1. Subgingival levels of *B. gingivalis* and bone loss after implantation of *B. gingivalis* (Bg^R).

Treatment	Animal	Maximum percentages of Bg^R after implantation*		Maximum change in CADIA after implantation†	
		Site 1	Site 2	Site 1	Site 2
Placebo	M93	ND	ND	0.0	0.0
Rifampin	M87	ND	ND	11.6	2.5
Placebo	M95	ND	6%	0.0	0.0
Rifampin	M91	3%	4%	0.0	0.0
Placebo	NO5	ND	10%	0.0	15.4
Placebo	NO3	7%	13%	9.3	5.8
Rifampin	M99	14%	18%	7.0	8.4
Rifampin	M79	18%	27%	8.9	11.3

*Maximum levels of Bg^R reached as the percentage of the total cultivable microbiota. Expressed as the nearest whole percentage. ND, nondetectable levels. †Decrease after implantation in bone density (in gray scale units, 0 to 255) (*17*) relative to values before implantation. Changes in CADIA values ±6.6 represent a significant loss of bone density (*17*).

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classes can detect similar epitopes on *M. fascicularis* immunoglobulins. Changes in antibody levels were analyzed by means of ^{2} analysis. Antibody activity is expressed as ELISA units on the basis of the inclusion of a reference serum on each plate. The reference serum was obtained as a pool from nonhuman primates actively immunized with formalinized *B. gingivalis* strain 3079.03.

In animals receiving placebo, 8 weeks of ligation induced periodontal bone loss in 10 of 16 experimental sites with three of four animals exhibiting disease. In animals receiving rifampin, all sites in one animal exhibited significant bone loss at 8 weeks of ligation, but the other 12 experimental sites showed no change. After implantation with *B. gingivalis*, significant radiographic bone loss was observed in five of eight sites in rifampin animals and two of eight sites in placebo animals (Table 1). Six of eight control sites in rifampin animals which were ligated, but not implanted, exhibited no bone loss at any time during the experimental period, and the two other nonimplanted sites in rifampin animals that lost bone after ligation showed no further progression during the implantation period.

All subgingival sites that were implanted with Bg^R exhibited no change in clinical levels of plaque. Clinical examination of the animals at the end of this time period, period 3, revealed that 80% of the implanted sites in the placebo animals and all implanted sites in the rifampin animals bled on gentle probing with a periodontal instrument.

Bacteria of strain Bg^R were isolated from 10 of 16 experimental sites with six of eight animals having at least one positive site (Table 1). Peak levels of 3 to 27% Bg^R were recovered. Of the two animals from which Bg^R could not be recovered, one was a placebo animal (M93) that did not develop periodontitis at any time during the experimental period in spite of having ligatures in place for 32 weeks, and the other animal (M87) was a rifampin animal which exhibited disease progression after implantation. Two other animals (M95 and M91) had peak recoverable levels of 4 to 6% Bg^R with no evidence of disease progression after implantation.

In the other four animals (NO5, NO3, M99, and M79 in Table 1) Bg^R was recovered from seven of eight experimental sites and achieved peak levels of between 7 and 27% of the total cultivable microbiota. These seven sites also exhibited progression of radiographic bone loss after implantation. Implanted sites with significant bone loss as assessed radiographically had significantly higher levels of Bg^R than sites without measurable bone loss [13.4 ± 8.7% (SD) versus 2.9 ± 4.4%, $t = -3.16$, $P < 0.01$].

Antibody changes after implantation indicated a substantial increase of IgM antibody levels to the homologous microorganism in five out of eight of the monkeys (Table 2). In addition, six out of eight of the monkeys showed increased serum IgG antibody to *B. gingivalis*; however, significantly fewer changes in levels of IgM or IgG were detected to *B. intermedius*, *F. nucleatum*, or *A. actinomycetemcomitans* (2 out of 48; $\chi^2 = 27.06$, $P < 0.001$).

The relation between the primary experimental parameters is shown in Table 2. Five animals exhibited significant periodontal bone loss after implantation with *B. gingivalis*. The implanted microorganism could be recovered from the subgingival plaque of at least one site of four of these animals at peak levels of ≥10% of the cultivable microbiota, and IgM antibody levels increased to the homologous microorganism in four of the animals. The one animal (M99) that exhibited no increased antibody response had exceptionally high IgM levels before implantation.

Three animals exhibited no significant loss of periodontal bone after implantation. *Bacteroides gingivalis* was never recovered at the 10% level, and IgM levels increased in only one of the three animals.

Although many studies have associated various microorganisms with periodontal diseases, definitive proof of the role of specific microorganisms in the etiology of periodontitis remains unclear. Although there is considerable evidence of the essential role of bacteria in the initiation of periodontal disease, specific microbial changes associated with clinical signs of periodontitis may in fact be secondary ecological phenomena rather than causative associations. Thus, microbial changes identified after detection of clinical change may suggest causation but do not prove that specific microorganisms initiate disease. Such studies have been further complicated by the fact that the progression of periodontitis in humans can still only be detected by imprecise clinical measurements that must be made repeatedly over several months.

Although animal models have been useful in providing some information relevant to the relation between microorganisms and the periodontal disease process (*20*), these models are inherently complicated, making data interpretations in some instances very difficult. In addition, anaerobes, which predominate in the periodontal microbiota, cannot be predictably implanted in germ-free or gnotobiotic animals and therefore have been studied only minimally.

Of the large number of microbial species that may be isolated from periodontitis sites, *B. gingivalis* is repeatedly isolated from advanced periodontitis lesions in adults (*3, 5, 21*) and has been associated with the progression of periodontitis in humans and nonhuman primates (*7, 14, 21, 22*). Further, a majority of patients classified as having adult periodontitis exhibit elevated peripheral blood antibody levels to *B. gingivalis* (*23, 24*).

In spite of the strong associations between *B. gingivalis* and periodontitis, definitive proof of its periodontal pathogenicity has been elusive. Attempts to implant it into the oral cavity of germ-free rodents have for the most part been unsuccessful. For example, while Nagahata and co-workers (*25*) used a ligated hamster model inoculated with cotton threads that were soaked in a suspension of *B. gingivalis*, they were unable to demonstrate histological differences in bone resorption between control and inoculated animals. Although inflammation persisted in the inoculated animals at 8 weeks after ligation, there were signs of healing in control animals. Wyss and Guggenheim (*26*) inoculated conventional rats with either *Actinomyces viscosum*, *B. gingivalis*, or both.

Table 2. Relation of bone loss to recovery of Bg^R and change in antibody levels after implantation with *B. gingivalis*.

Animal	Significant bone loss	Bg^R ≥10%	IgM antibody levels		Increase	IgG antibody levels		Increase
			Before	After		Before	After	
NO5	+	+	51.3*	204†	+‡	20.5	33.7	+/−§
NO3	+	+	102.5	447	+	80.8	85.6	+
M99	+	+	220.9	140	−	57	37	−
M79	+	+	98.6	269	+	43.3	101	+
M87	+	−	81.1	106	+	78.1	127	+
M93	−	−	108.4	129	+	33.5	43	+
M95	−	−	121.2	110	−	34.4	31	−
M91	−	−	106.4	94	−	31.6	40.3	+/−

*Mean ± 2 SD of antibody levels in ELISA units before implantation. †Mean level of peak response in ELISA units after implantation. ‡At least two time points after implantation were significantly elevated compared to before implantation. §Only one time point after implantation was significantly elevated compared to before implantation.

Bacteroides gingivalis was successfully reisolated from the inoculated animals, but bone loss in *B. gingivalis*–associated animals was greater than in uninoculated controls only in the mandible [bone loss, 4.52 ± 0.33 mm (SD) in controls versus 5.10 ± 0.22 mm in experimental animals].

Implantation of Bg^R in a large background of the normal microbiota demonstrated that there was a direct connection between the implanted Bg^R and clinical characteristics of periodontitis. The Bg^R emerged from this complex plaque ecological niche in such a way that it produced a "burst" of bone loss indicating that the emergence of *B. gingivalis* in the subgingival microbiota is capable of inducing progression of periodontitis. *Bacteroides gingivalis* therefore is capable of functioning as a primary pathogen in periodontal disease, and the potential of microbial changes to induce a burst of disease progression is thus confirmed. As a result, factors that allow or promote both the colonization and emergence of this microorganism become of great importance to the control of periodontitis.

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Dominant Role of N-Type Ca^{2+} Channels in Evoked Release of Norepinephrine from Sympathetic Neurons

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Multiple types of calcium channels have been found in neurons, but uncertainty remains about which ones are involved in stimulus-secretion coupling. Two types of calcium channels in rat sympathetic neurons were described, and their relative importance in controlling norepinephrine release was analyzed. N-type and L-type calcium channels differed in voltage dependence, unitary barium conductance, and pharmacology. Nitrendipine inhibited activity of L-type channels but not N-type channels. Potassium-evoked norepinephrine release was markedly reduced by cadmium and the conesnail peptide toxin ω-*Conus geographus* toxin VIA, agents that block both N- and L-type channels, but was little affected by nitrendipine at concentrations that strongly reduce calcium influx, as measured by fura-2. Thus N-type calcium channels play a dominant role in the depolarization-evoked release of norepinephrine.

CALCIUM ENTRY VIA VOLTAGE-gated Ca^{2+} channels is essential for neurotransmitter release (*1*). The existence of multiple types of neuronal Ca^{2+} channels (*2–4*) has raised questions about which channel (or channels) contributes to transmitter release. With notable exceptions [for example, see (*5–7*)], evoked release of neurotransmitters is largely resistant to dihydropyridine (DHP) antagonists in systems such as brain slices (*8*), cultured neurons (*9*), and synaptosomes (*10, 11*), which suggests that transmitter release may be dominated by DHP-resistant Ca^{2+} channels (*4, 9, 11, 12*). To test this hypothesis, we have studied the relation between Ca^{2+} entry and norepinephrine (NE) release in rat sympathetic neurons.

Whole-cell recordings (*13*) in rat sympathetic neurons (*14*) provided evidence for two types of Ca^{2+} channels that differed in their time and voltage dependence of inactivation. Calcium ion channel currents carried by 10 m*M* external Ba^{2+} were recorded with Na^+ and K^+ channel currents blocked (Fig. 1). Depolarizing pulses from different holding potentials (HPs) to a test potential of +10 mV evoked a decaying inward current and a late inward current which showed differing dependence on the HP. The late current amplitude was nearly maximal at HP = −60 mV, while the decaying current grew progressively larger as the HP approached −120 mV. These results are similar to those from chick dorsal root ganglion (DRG) neurons (*3, 4*) and have been attributed to the presence of two populations of Ca^{2+} channels—the L-type Ca^{2+} channel, which contributes maintained currents from depolarized HPs, and the N-type Ca^{2+} channel, which inactivates during maintained depolarizations but requires negative HPs to reprime. The rate of inactivation is much slower in sympathetic neurons than in DRG neurons.

Cell-attached patch recordings of single-channel activity provided evidence for distinct types of Ca^{2+} conductance (Fig. 1B). With 110 m*M* Ba^{2+} as the external charge carrier and isotonic K^+ outside the cell to bring its resting potential to zero, depolarizing pulses from an HP of −80 to −20 mV evoked unitary Ca^{2+} channel openings of roughly 1 and 2 pA in amplitude. The larger unitary currents occasionally appeared grouped in bursts of long-lasting openings (fifth sweep), a characteristic of L-type Ca^{2+} channels in other cells (*15*). The voltage dependence of the smaller and larger unitary currents corresponded to slope conductances of 11 and 27 pS (eight patches total). These slope conductances are close to average values for N- and L-type Ca^{2+} channels in chick DRG neurons, 13 and 25 pS, respectively (*4*). The Ca^{2+} channel with the smaller unitary conductance has the same dependence on holding potential as component N in whole-cell recordings (Fig. 1A). Unitary events seen with depolarizations from an HP of −80 mV (Fig. 1D) largely disappeared when the HP was shifted to −40 mV for 30 seconds or more (Fig. 1E) and reappeared after an HP of −80 mV was restored (Fig. 1F). In cell-attached patches,

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