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Phototargeting Oral Black-Pigmented Bacteria

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We have found that broadband light (380 to 520 nm) rapidly and selectively kills oral black-pigmented bacteria (BPB) in pure cultures and in dental plaque samples obtained from human subjects with chronic periodontitis. We hypothesize that this killing effect is a result of light excitation of their endogenous porphyrins. Cultures of *Prevotella intermedia* and *P. nigrescens* were killed by 4.2 J/cm^2, whereas *P. melaninogenica* required 21 J/cm^2. Exposure to light with a fluence of 42 J/cm^2 produced 99% killing of *P. gingivalis*. High-performance liquid chromatography demonstrated the presence of various amounts of different porphyrin molecules in BPB. The amounts of endogenous porphyrin in BPB were 267 (*P. intermedia*), 47 (*P. nigrescens*), 41 (*P. melaninogenica*), and 2.2 (*P. gingivalis*) ng/mg. Analysis of bacteria in dental plaque samples by DNA-DNA hybridization for 40 taxa before and after phototherapy showed that the growth of the four BPB was decreased by 2 and 3 times after irradiation at energy fluences of 4.2 and 21 J/cm^2, respectively, whereas the growth of the remaining 36 microorganisms was decreased by 1.5 times at both energy fluences. The present study suggests that intraoral light exposure may be used to control BPB growth and possibly benefit patients with periodontal disease.

Dental plaque is a biofilm that develops naturally on teeth. It consists of aggregates of 500 to 600 different bacterial taxa embedded in a matrix of polymers of bacterial and salivary origin (30). In healthy subjects, dental plaque remains stable for prolonged periods of time because of a dynamic balance among the resident members of its microbial community (12). Disease arises when the microbial homeostasis within the plaque breaks down because of disruption of the habitat's ecology (14). In periodontal disease, there is a shift in the composition of subgingival plaque's microflora that colonizes tooth surfaces and epithelial cells in the periodontal pocket to a more proteolytic gram-negative anaerobic community including the pigmented rods in the genera *Porphyromonas* and *Prevotella* (19, 29). Black-pigmented anaerobes such as *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Prevotella nigrescens* have been implicated as pathogens associated with the initiation and progression of periodontitis (3–5, 31, 32). These species depend largely on external heme as an iron source for their growth (20) and accumulate a cell surface black pigment that mainly consists of μ-oxobisheme of iron protoporphyrin IX (PpIX) in *P. gingivalis* (25) and monomeric iron PpIX (hematin) in *P. intermedia* and *P. nigrescens* (26). Although iron PpIX is the predominant pigment in black-pigmented bacteria (BPB), these species also accumulate various amounts of iron-free PpIX (23, 24), which is photosensitive (18). The UV-visible absorption spectrum of porphyrins exhibits an intense peak at around 405 nm, followed by several weaker peaks at 505, 540, 575, and 630 nm (27). As a result, excitation of PpIX by light causes energy transfer from the PpIX triplet state to molecular oxygen to produce the excited-state singlet

oxygen (type II photoprocess), which can then oxidize and destroy various biological molecules such as lipids, proteins, and nucleic acids (22). Inactivation of oral BPB by visible light has been reported previously with an argon laser (6, 7) and a helium-neon laser (9) at high-energy fluences ranging of up to 360 J/cm^2.

In the present study, we have investigated the effect of broadband light (380 to 520 nm) on BPB in pure cultures, as well as in dental plaque samples obtained from humans with chronic periodontitis. Our hypothesis was that blue light could achieve rapid and selective elimination of periodontopathogenic BPB by exciting their endogenous porphyrins.

MATERIALS AND METHODS

Microorganisms. The pure bacterial strains used in this study were *Porphyromonas gingivalis* ATCC 33277, *P. intermedia* ATCC 25611, *P. nigrescens* ATCC 33563, *Prevotella melaninogenica* ATCC 25845, and *Streptococcus constellatus* ATCC 27823. Cultures were maintained by weekly subculture in Trypticase soy agar with 5 μg of hemin per ml, 0.3 μg of vitamin K per ml, and 5% sheep blood (manufactured plates from Northeast Labs, Waterville, Maine). Cultures were grown in the presence of 80% N_2, 10% H_2, and 10% CO_2 at 35°C in an anaerobic chamber for 48 to 72 h. On the day of the experiment, the cells were harvested by centrifugation and resuspended in brain heart infusion broth (Becton Dickson & Company, Sparks, Md.). Cells were dispersed by sonication and repeated passage through Pasteur pipettes. For adjustment of inoculum density, cell numbers were estimated in a spectrophotometer (wavelength, 600 nm; 0.1 optical density [OD] unit equals approximately 10^8 cells/ml) in 1-ml cuvettes.

HPLC analysis. For extraction of total porphyrins from *P. gingivalis*, *P. intermedia*, *P. nigrescens*, and *P. melaninogenica*, a two-phase method was used that included the use of acidified ethyl acetate (ethyl acetate-glacial acetic acid at 2:1), followed by 1 M HCl. Iron-containing porphyrins (heme) was extracted into the organic solvent but not extracted back into the acid phase. Thus, heme compounds were excluded. Porphyrins were quantified by scanning from 640 to 670 nm with an excitation wavelength of 400 nm with a Fluoromax-3 spectrofluorometer (Jobin Yvon, Edison, N.J.). The level of total porphyrins was calculated on the basis of a reference porphyrin mixture standard (see Fig. 3). Porphyrins were fractionated by a reversed-phase high-performance liquid chromatography (HPLC) method (11). The Waters HPLC system (Waters, Milford, Mass.) consisted of a 600E system controller, a 717 autosampler, a 470 fluorescence detec-

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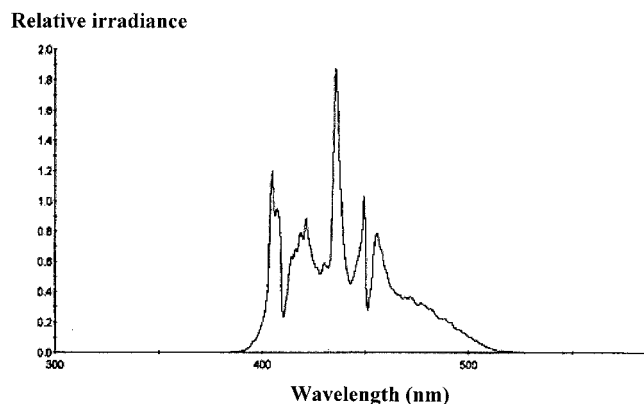
Relative irradiance

FIG. 1. Emission spectrum of the light source provided by Brite-Smile, Inc., Walnut Creek, Calif.

tor, and a 745B data module for peak integration. Separation of porphyrins was performed on a Phenomenex C_{18} Bondclone column (150 by 3.9 mm; Phenomenex, Torrance, Calif.).

Subjects and plaque samples. Samples of subgingival plaque were taken from 15 patients. Permission to collect dental plaque samples was authorized by Institutional Review Board-approved informant consent. All patients were diagnosed as having chronic periodontitis with pockets greater than 3 mm in depth. None of them used antibiotics or had undergone periodontal treatment during the 3 months prior to sampling. Dental plaque samples were taken from the supra- and subgingival mesiobuccal aspects of premolars or molars in each patient with individual sterile Gracey curettes. After their removal, the samples were placed immediately into an Eppendorf tube with 5 ml of prereduced anaerobically sterilized Ringer's solution. Cells were dispersed by sonication and repeated passage through Pasteur pipettes. Cell numbers were measured in a spectrophotometer with 1-ml tubes (1 OD unit equals approximately 10^9 cells/ml at 600 nm).

Light source. The irradiation source (BriteSmile model BS3000; BriteSmile, Inc., Walnut Creek, Calif.) consisted of two Mejiro metal halide gas plasma lamps with reflecting elements. The lamps are attached to two (one each) optical fiber bundles that lead to a "front end" that breaks each bundle up into three rectangular emitting output areas. The spectral range of the light source was 380 to 520 nm (Fig. 1). A strong peak occurred at 435 nm, and secondary peaks occurred at 405, 420, 450, and 455 nm (Fig. 1). The light source emitted 1.7% of the total energy in the UV A range of the spectrum (380 to 400 nm).

Photptherapy studies. (i) Bacterial cultures. Suspensions of bacteria (10^8/ml) were placed in the wells of 24-well plates. All four BPB and *S. constellatus* (non-black-pigmented control species) were exposed to light from the halogen lamp at room temperature from above with fluences ranging from 0 to 42 J/cm^2 at an irradiance of 70 mW/cm^2. The measured temperature rise in the medium was less than 3°C during exposure to an irradiation fluence of 42 J/cm^2. All plates were kept covered during illumination in order to maintain the sterility of the culture. After illumination of the appropriate wells, serial dilutions of the contents of each well were prepared in brain heart infusion broth, and 100-μl aliquots were spread over the surfaces of blood agar plates enriched with vitamin K, *N*-acetylmuramic acid, and hemin. The plates were incubated anaerobically at 35°C for 7 days. Survival fractions in each well were calculated by counting the CFU on the plates and dividing by the number of colonies from control plates that were not exposed to light and kept at room temperature for periods equal to the irradiation times.

(ii) Pooled dental plaque. Dispersed dental plaque (10^8/ml) was placed in the wells of 24-well plates and exposed to light with fluences of 4.2 and 21 J/cm^2 at an irradiance of 70 mW/cm^2. After illumination, survival was estimated by two methods, i.e., by counting CFU as described above and then performing total DNA probe counts of 40 bacterial species (Table 1) by checkerboard DNA-DNA hybridization (28). For DNA probe analysis, Tris-EDTA buffer (1.5 ml) was added to the plates and the bacterial colonies were scraped off the surface with sterile L-shaped glass rods. The suspensions were placed into individual Eppendorf tubes and sonicated for 10 s to break up clumps. Each suspension was adjusted to a final OD of 1.0, which corresponded to approximately 10^9 cells. Ten microliters of the suspension (10^7 cells) was removed and placed in another Eppendorf tube with 140 μl of TE buffer and 150 μl of 0.5 M NaOH. The samples were lysed, and the DNA was placed in lanes on positively charged nylon membrane with a Minislot device (Immunetics, Cambridge, Mass.). After fixation of the DNA to the membrane, the membrane was placed in a Miniblotter 45 (Immunetics) with the lanes of DNA perpendicular to the lanes of the device. Digoxigenin-labeled whole genomic DNA probes for 40 bacterial taxa (Table 1) were hybridized in individual lanes of the Miniblotter. After hybridization, the membranes were washed at high stringency and the DNA probes were detected with antibody to digoxigenin conjugated with alkaline phosphatase for chemifluorescence detection. Signals were detected with AttoPhos substrate (Amersham Life Science, Arlington Heights, Ill.) and scanned with a Storm Fluorimager (Molecular Dynamics, Sunnyvale, Calif.). Computer-generated images were analyzed to determine the fluorescence intensity associated with each sample and probe. Two lanes in each membrane contained DNA standards with 1 ng (10^5 bacteria) and 10 ng (10^6 bacteria) of each species. The sensitivity of the assay was adjusted to permit detection of 10^4 cells of a given species by adjusting the concentration of each DNA probe. The measured fluorescence intensities were converted to absolute counts by comparison with the standards on the same membrane. Failure to detect a signal was recorded as zero. Inhibition of BPB

TABLE 1. Strains used for the development of DNA probes[a]

Bacterium	Strain(s)	Bacterium	Strain(s)
Actinobacillus actinomycetemcomitans	43718, 29523	*Gemella morbillorum*	27824
Actinomyces gerencseriae	23860	*Leptotrichia buccalis*	14201
Actinomyces israelii	12102	*Neisseria mucosa*	19696
Actinomyces naeslundii genospecies 1	12104	*Peptostreptococcus micros*	33270
Actinomyces naeslundii genospecies 2	43146	*Porphyromonas gingivalis*	33277
Actinomyces odontolyticus	17929	*Prevotella intermedia*	25611
Tannerella forsythensis	43037	*Prevotella melaninogenica*	25845
Campylobacter gracilis	33236	*Prevotella nigrescens*	33563
Campylobacter rectus	33238	*Propionibacterium acnes*	11827, 11828
Campylobacter showae	51146	*Selenomonas noxia*	43541
Capnocytophaga gingivalis	33624	*Streptococcus anginosus*	33397
Capnocytophaga ochracea	33596	*Streptococcus constellatus*	27823
Capnocytophaga sputigena	33612	*Streptococcus gordonii*	10558
Eikenella corrodens	23834	*Streptococcus intermedius*	27335
Eubacterium nodatum	33099	*Streptococcus mitis*	49456
Eubacterium saburreum	33271	*Streptococcus oratis*	35037
Fusobacterium nucleatum subsp. *nucleatum*	25586	*Streptococcus sanguis*	10556
Fusobacterium nucleatum subsp. *polymorphum*	10953	*Treponema denticola*	BI
Fusobacterium nucleatum subsp. *vincentii*	49256	*Treponema socranskii*	SI
Fusobacterium periodonticum	33693	*Veillonella parvula*	10790

[a] All strains were obtained from the American Type Culture Collection except *T. denticola* B1 and *T. socranskii* S1, which were obtained from The Forsyth Institute.
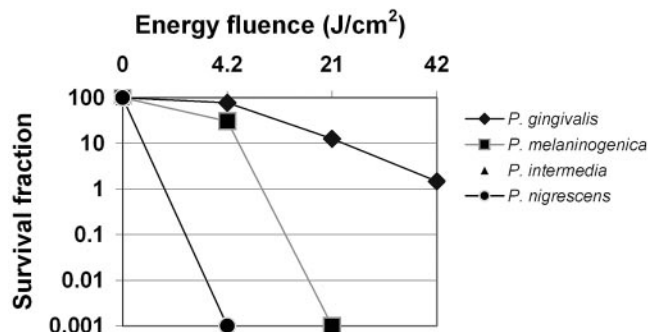
FIG. 2. Survival fraction of oral species following irradiation of planktonic cell suspensions. A survival fraction of 100% represents 10^8 microorganisms. Each plotted point is the mean of triplicate determinations.

growth was defined as the ratio of DNA probe counts before exposure to light to those after exposure to light. Differences between mean growth inhibition ratios or percentages were tested for statistical significance with Student's t test.

RESULTS

Photodestruction of bacterial cultures. The effects of increasing light doses from the light source on cultures of BPB are shown in Fig. 2. *P. intermedia* and *P. nigrescens* were completely killed by exposure to light with a fluence of 4.2 J/cm² (1 min of irradiation). *P. melaninogenica* was reduced by 70% by exposure to 4.2 J/cm² ($P < 0.008$) and completely killed by exposure to 21 J/cm² (5 min of irradiation). The *P. gingivalis* survival fractions were 77.25% ($P < 0.001$), 12.55% ($P < 0.00002$), and 1.48% ($P < 0.000001$) after exposure to light with fluences of 4.2, 21, and 42 J/cm², respectively. *S. constellatus*, a nonpigmented species, was unaffected by irradiation (data not shown).

HPLC analysis. HPLC revealed that BPB expressed different porphyrin patterns (Fig. 3). The percent porphyrin content in BPB is shown in Table 2. The amounts of porphyrin were 267, 47, 41, and 2.2 ng/mg of protein in *P. intermedia*, *P. nigrescens*, *P. melaninogenica*, and *P. gingivalis*, respectively. The
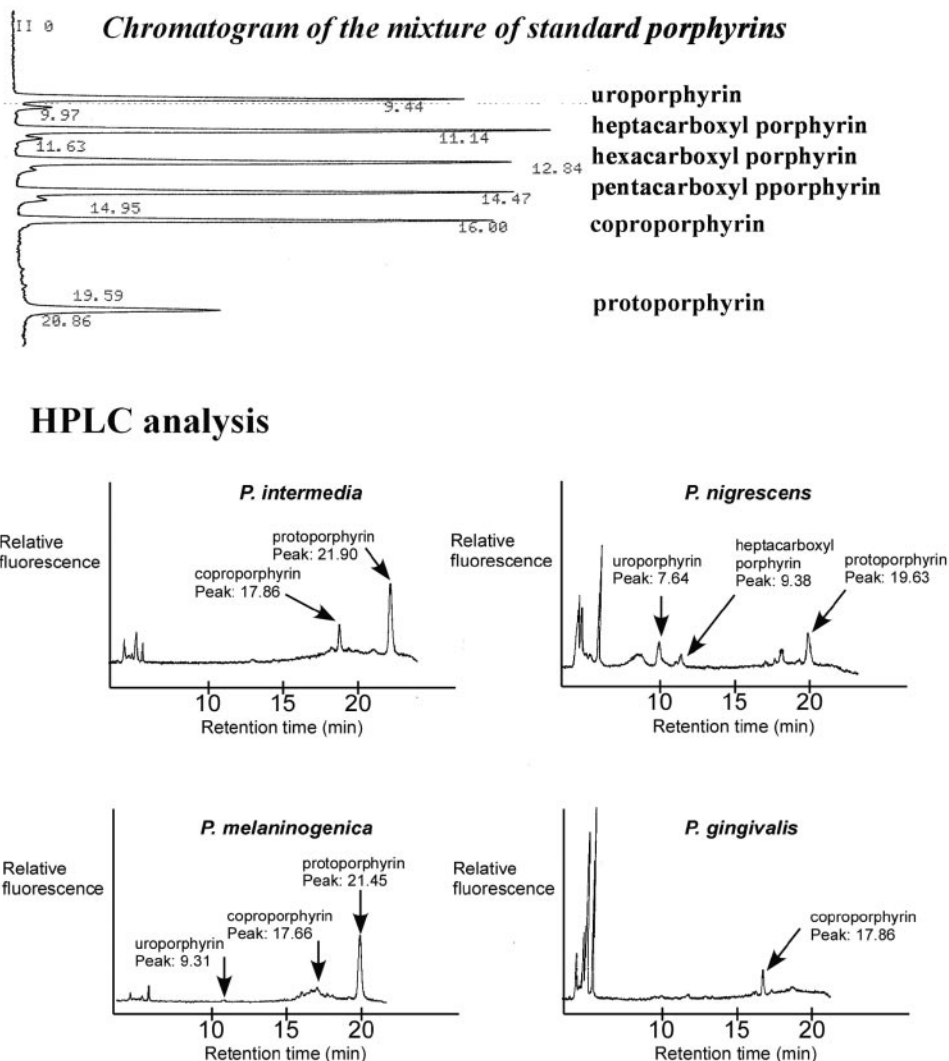

FIG. 3. Chromatogram of a mixture of standard porphyrins in the order of decreasing retention time and HPLC analysis of the porphyrin content of BPB.

TABLE 2. Porphyrin contents expressed in BPB

Porphyrin	% of porphyrin content of:			
	P. inter-media	P. nigres-cens	P. melani-nogenica	P. gingi-valis
Uroporphyrin		33	2	
Heptacarboxyl porphyrin		16		
Hexacarboxyl porphyrin				
Pentacarboxyl porphyrin				
Isocoproporphyrin				
Coproporphyrin	17		12	100
Protoporphyrin	83	51	85	

large signals appearing at the solvent front in the chromatograms of *P. nigrescens* and *P. gingivalis* represent low-molecular-weight fluorescent compounds of bacterial origin (Fig. 3).

Phototherapy of dental plaque microorganisms. (i) CFU. Figure 4 shows the reduction in the total number of CFU after exposure of dental plaque samples to light with energy fluences of 4.2 and 21 J/cm^2. The survival fractions were reduced by 17% ($P < 0.00002$) and 25% ($P < 0.0000007$), respectively.

(ii) Checkerboard DNA-DNA hybridization. Figure 5 shows the growth inhibition ratios of BPB after exposure of dental plaque samples to light with energy fluences of 4.2 and 21 J/cm^2. The order of growth inhibition was *P. melaninogenica* > *P. nigrescens* > *P. intermedia* > *P. gingivalis* for both energy fluences. The growth inhibition ratios of all BPB were statistically significantly different from those of controls at both energy fluences ($P < 0.05$). On the other hand, the growth inhibition ratios of BPB at 21 J/cm^2 were not statistically significantly different from those obtained at 4.2 J/cm^2 ($P > 0.05$), with the exception of *P. intermedia* ($P < 0.02$). The growth of all four BPB was suppressed 2 and 2.8 times at energy fluences of 4.2 and 21 J/cm^2, respectively ($P < 0.05$), whereas the remaining 36 microorganisms (Table 1) were inhibited 1.5 times at both energy fluences (Fig. 6).

DISCUSSION

In healthy subjects, dental plaque remains stable for prolonged periods of time because of a dynamic balance among the resident members of its microbial community (15). A major
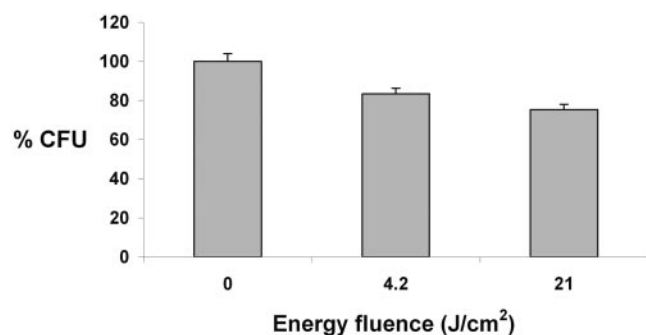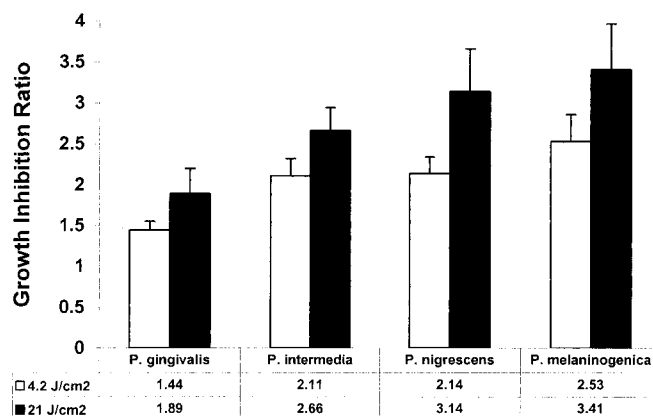

FIG. 5. Inhibition of BPB growth after exposure to light. The bars represent the ratio of DNA counts before exposure to light to those after exposure to light (mean ± standard error of the mean, $n = 15$ subjects).

	P. gingivalis	P. intermedia	P. nigrescens	P. melaninogenica
□ 4.2 J/cm2	1.44	2.11	2.14	2.53
■ 21 J/cm2	1.89	2.66	3.14	3.41

disturbance of the local habitat can cause a breakdown of this microbial homeostasis that may lead to enrichment of the microbial community by pathogens (13, 14). The primary goal of a strategy for disease prevention and control should be specific suppression of key pathogens, such as *P. gingivalis*, which may result in an increase in the microbial flora that is associated with health. The specific hypothesis of this study was that blue light could achieve a rapid and selective elimination of oral BPB by exciting their endogenous porphyrins.

Inactivation of oral BPB, such as *P. intermedia* and *P. gingivalis*, by visible light from an argon laser (wavelength range, 488 to 514 nm) (6, 7) and a helium-neon laser (wavelength, 633 nm) (9) at high-energy fluences ranging from 200 to 360 J/cm^2 has been reported previously. The 380- to 520-nm spectral range of light used in our studies matches the strongest porphyrin photoexcitation band at 405 to 415 nm and a small band at 505 nm (27). The green and red lights used in the above-mentioned studies (6, 7, 9) targeted only two small absorption peaks of endogenous porphyrins, and thus high-energy flu-

FIG. 4. Reduction in total CFU counts after exposure of pooled dental plaque samples to visible light at 4.2 and 21 J/cm^2. Each bar is the mean of values obtained from 15 patients (data from each patient were representative of three independent experiments). Error bars denote the standard error of the mean.
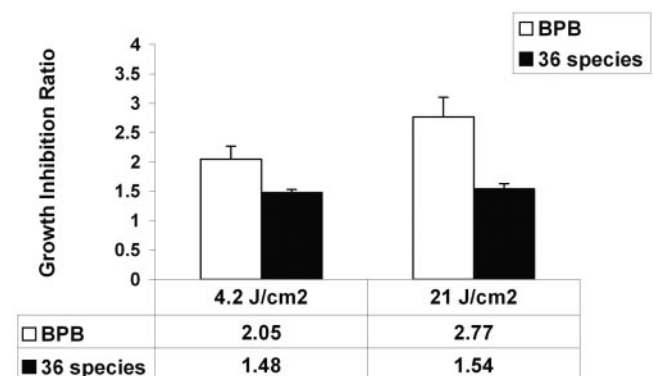

FIG. 6. Suppression of the BPB group (four species) and that of 36 other microorganisms after exposure of dental plaque to light. The bars represent the mean growth inhibition ratios (ratios of DNA counts before exposure to light to those after exposure to light) obtained from 15 patients (data from each patient were representative of three independent experiments), and the error bars represent the standard error of the mean.

	4.2 J/cm2	21 J/cm2
□ BPB	2.05	2.77
■ 36 species	1.48	1.54

ences were required to achieve bacterial inactivation. Blue light has also been used for eradication of *Propionibacterium acnes*, the gram-positive species that causes acne (1, 21), which produces endogenous porphyrins (mainly coproporphyrin and PpIX) that absorb energy in the near-UV and blue parts of the light spectrum (8, 10, 16, 17). A significant improvement in inflammatory lesions of patients with acne vulgaris after exposure to blue light with peaks at 405 and 420 nm has been demonstrated (21). However, the cumulative energy fluences used (320 J/cm^2) were much higher than those in our studies (21).

Our results showed the presence of different porphyrin patterns expressed in BPB (Table 2). The amount of endogenous porphyrin produced in *P. intermedia* was 120, 6.5, and 5.5 times higher than those in *P. gingivalis*, *P. melaninogenica*, and *P. nigrescens*, respectively. Although *P. gingivalis* and *P. melaninogenica* showed less susceptibility to blue light than *P. intermedia* did, as expected, both *P. nigrescens* and *P. intermedia* were completely killed after 1 min of irradiation. There are two ways to explain this discrepancy. It is possible that *P. intermedia* requires a lower-energy fluence for complete killing than *P. nigrescens* does and/or the porphyrin content of microorganisms may not be the sole determinant of photosensitivity.

The checkerboard DNA-DNA hybridization technique was used for identification and enumeration of bacterial species in dental plaque samples before and after exposure to light with whole genomic probes for 40 test taxa (28). This method offers advantages over the reverse-capture oligonucleotide method (2), which uses synthetic oligonucleotide probes. The latter, although highly specific, has proven difficult to use in applications requiring quantitative estimates of bacterial numbers because of difficulties in controlling the PCR step required for multiple samples and the lack of a suitable universal probe for oral bacteria. The whole-genomic method has permitted estimation of bacterial numbers by adjusting the average level of alkaline phosphatase labeling. With probit modeling, the response function of each of the probes was found to be linear in $\log_{10}$ N with a correlation coefficient of 0.97. The detection limit of this method was estimated by computing the number equivalent to 1.97 times the standard deviation of background samples, the lowest value that one can say is statistically significantly different from zero ($P < 0.05$).

When dental plaque samples from human subjects were irradiated, *P. melaninogenica* showed the highest susceptibility to light, followed by *P. nigrescens*, *P. intermedia*, and *P. gingivalis* (Fig. 5). All of the *Prevotella* species showed similar patterns of susceptibility to light, with growth inhibition ratios ranging between 2.1 (4.2 J/cm^2) and 3.4 (21 J/cm^2). The growth of *P. gingivalis* was inhibited 1.4 (4.2 J/cm^2) to 1.9 (21 J/cm^2) times. These data are in accordance with those obtained in a previous study in which exposure of human subgingival plaque samples to red light at 633 nm led to 60 and 40% elimination of *Prevotella* species and *P. gingivalis*, respectively (9). However, the energy fluence delivered to the species was 360 J/cm^2 since the red light corresponded to the long-wavelength absorption maximum of porphyrins. The same study demonstrated a reduction in the number of CFU of other anaerobic and aerobic dental plaque microorganisms by 50% as a result of their exposure to red light (9). In our study, the reduction of CFU in dental plaque samples was 25% (Fig. 4) after exposure

to light with an energy fluence of 21 J/cm^2. The microbial analysis showed that the growth of the remaining 36 taxa was suppressed 1.5 times at both energy fluences whereas the growth of all four BPB was inhibited 2 to 2.8 times (Fig. 6). Some of these non-black-pigmented species may also contain porphyrins and/or other cell pigments, which can explain their susceptibility to light.

These data suggest that visible light could be used prophylactically to stabilize the normal microbial composition of plaque by suppressing potentially pathogenic BPB. Compared with other forms of periodontal therapy (scaling, mouthwashes, surgery), this form of treatment would offer many advantages; it is painless, rapid, and devoid of drug toxicity; has no effect on taste; and is selective in its effect.

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