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Effect of Blood on Lethal Photosensitization of Bacteria in Subgingival Plaque from Patients with Chronic Periodontitis

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Abstract. The purpose of this study was to determine whether bacteria in subgingival plaque samples from patients with chronic periodontitis could be sensitized to killing by low-power laser light in the presence of blood. Toluidine blue O (45 $\mu g\,ml^{-1}$) was added to the plaque samples which were then exposed to light from a 7.3 mW HeNe laser for 30 or 120 s in the presence and absence of 10% horse blood. Viable counts of various groups and species of bacteria were determined before and after irradiation. A substantial bactericidal effect was obtained after irradiation for 30 s regardless of whether or not blood was present. However, in most cases irradiation in the absence of blood resulted in greater decreases in the viable counts of aerobes, anaerobes, streptococci and black-pigmented anaerobes. When the samples were irradiated for 120 s, 10% blood had little effect on the kills attained and elimination of periodontopathogenic species (black-pigmented anaerobes and *Fusobacterium nucleatum*) was achieved both in the presence and absence of blood.

INTRODUCTION

Chronic periodontitis is one of the most prevalent diseases of mankind and is a major cause of tooth loss in adults. The disease is a consequence of the accumulation of plaque on the tooth surface below the gingival margin. It is now recognized that a number of bacterial species are responsible for the tissue destruction that accompanies the disease and these include black-pigmented Gram-negative obligate anaerobes (e.g. *Porphyromonas gingivalis*, *Prevotella intermedia*) and *Fusobacterium nucleatum* (1). Current methods of treating chronic periodontitis are based on mechanical removal of subgingival plaque and this may be supplemented with topically-applied antibiotics and antiseptics (2). Long-term use of such agents, however, may lead to the development of resistance in the target organisms so that alternative ways of killing subgingival bacteria which could avoid this problem are clearly desirable. One possible means of doing this would be to employ a photochemotherapeutic approach. Recently, we have shown that oral bacteria can be killed by light from a low-power (HeNe) laser once they have been sensitized by low concentrations of a dye such as toluidine blue O (3). Organisms susceptible to such treatment include the periodontopathogenic species *Porphyromonas gingivalis* and *Fusobacterium nucleatum*. Furthermore, it has also been shown that these organisms may be killed in this way even when they are present in the highly mixed microfloras found in subgingival plaque samples from patients with chronic periodontitis (4). Photolysis of periodontopathogenic bacteria, therefore, may be a convenient means of eliminating these bacteria from the disease lesion—the periodontal pocket. The photosensitizer could be applied topically to the periodontal pocket and then laser light could be delivered via an optical fibre inserted into the pocket. However, introduction of such a fibre may cause bleeding within the lesion (as does insertion of a dental probe) and this may prevent lethal photosensitization of the bacteria present. The purpose of this study, therefore, was to determine whether bacteria in subgingival plaque samples could be killed by low-power laser light in the presence of blood in vitro.

MATERIALS AND METHODS

Subjects

Samples of subgingival plaque were taken from

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patients attending the School of Dental Hygiene, Eastman Dental Hospital. All patients were diagnosed as having chronic periodontitis with pockets greater than 5 mm in depth and radiological evidence of bone loss. None had used antibiotics or undergone periodontal treatment during the 6 months prior to sampling.

A total of 6 patients were investigated all of whom were females with ages ranging from 19 to 56 years with a mean of 37.8 years.

Plaque samples

After removal of supragingival plaque, a subgingival plaque sample was taken from a single site in each patient using a swab formed from calcium alginate wool wound on a barbed endodontic broach. The swab was immediately placed into 0.5 ml of pre-reduced Calgon-Ringers solution (Oxoid Ltd, Basingstoke, UK) and transported to the laboratory. Processing of the sample commenced within 5 min from the time it was taken.

Sample processing

Each sample was vortex-mixed until the calcium alginate had dissolved.

In the case of the first three plaque samples, 30 μl aliquots were dispensed into each of six wells of a microtitre plate. Seven μl of defibrinated horse blood were then added to one of the wells followed by 30 μl of 100 $\mu g\,ml^{-1}$ toluidine blue O (TBO) and the suspension immediately exposed to light from a HeNe laser with a power output of 7.3 mW (NEC Corporation, Japan) for 30 s while being mixed on a magnetic stirrer. After exposure, the suspension was serially diluted in pre-reduced brain heart infusion (BHI) broth (Oxoid Ltd) and plated onto a range of media as described below. The procedure was then repeated in an identical manner on another 30 μl aliquot of the plaque suspension. Another two wells were processed in the same way except that 7 μl of water were added instead of blood.

In order to determine the effect of the dye itself on bacterial viability, 7 μl of water were added to two 30 μl aliquots of the plaque suspension followed by 30 μl of the dye solution. These were then mixed for 30 s (without exposure to laser light), diluted in BHI broth and plated onto the culture media.

Additional controls were prepared by adding 30 μl of water and 7 μl of blood or 37 μl of water to 30 μl aliquots of the plaque suspension which were then mixed for 30 s without exposure to the laser light.

The above was repeated on a further three plaque samples except that the period of exposure to laser light was 120 s.

Culture methods

Each series of bacterial suspensions obtained as described above was then plated, in duplicate, onto the following media: (i) Wilkins Chalgren (WC) agar (Oxoid Ltd) supplemented with 10% (v/v) horse blood. (ii) Mitis-salivarius (MS) medium (Difco Ltd, East Molesey, UK) for the cultivation of streptococci. (iii) A selective medium for *Fusobacterium nucleatum* (5).

All plates were then incubated anaerobically at 37°C for up to 10 days. A further set of WC plates was incubated for up to 5 days in an aerobic, CO_2-enriched, atmosphere. At the end of the incubation period, colonies on the aerobically-incubated and anaerobically-incubated WC plates were enumerated. These were designated the 'total aerobic' and 'total anaerobic' counts respectively. Black-pigmented colonies on the WC plates incubated anaerobically were also enumerated then sub-cultured onto fresh WC plates for incubation aerobically and anaerobically. Those black-pigmented colonies which did not grow aerobically and consisted of Gram-negative bacilli were termed black-pigmented anaerobes.

Colonies growing on the MS plates were enumerated, Gram-stained and tested for catalase production. Those colonies consisting of catalase-negative Gram-positive cocci were considered to be streptococci.

Colonies growing on the selective medium for *F. nucleatum* were Gram-stained and those consisting of Gram-negative fusiform bacilli were enumerated.

Statistical analysis

Data were analysed by comparing, for each sample, the proportion killed in the presence of blood with the proportion killed in its absence using a standard z-test for the comparison of two independent proportions. Ninety-five per cent confidence intervals were then calculated for the true population proportional differences and the results are shown in Tables 1 and 2.

Table 1. Kills achieved in plaque samples in the presence and absence of 10% (v/v) horse blood after irradiation with laser light for 30 s in the presence of 45 $\mu g\,ml^{-1}$ toluidine blue O

Category of organism	Sample	Percentage killed		95% C.I. for per cent proportional differences
		Blood present	Blood absent	
Aerobes	1	58.6	93.3	32.9–36.5
	2	42.2	84.9	41.8–43.8
	3	71.7	92.6	15.7–25.9
Anaerobes	1	71.9	86.6	14.0–15.5
	2	64.4	75.6	10.1–12.2
	3	56.8	70.8	10.9–17.2
Black-pigmented anaerobes	1	87.6	100.0	6.5–18.3
	2	68.4	100.0	26.3–36.8
	3	100.0	100.0	NS
Fusobacterium nucleatum	1	100.0	100.0	NS
	2	60.0	100.0	36.2–43.8
	3	100.0	100.0	NS
Streptococci	1	84.3	95.4	9.0–13.1
	2	38.0	90.2	49.7–54.6
	3	95.3	100.0	1.0–8.3

NS, Not significant.

Table 2. Kills achieved in plaque samples in the presence and absence of 10% (v/v) horse blood after irradiation with laser light for 120 s in the presence of 45 $\mu g\,ml^{-1}$ toluidine blue O

Category of organism	Sample	Percentage killed		95% C.I. for per cent proportional differences
		Blood present	Blood absent	
Aerobes	4	87.4	100.0	11.3–13.9
	5	99.6	100.0	0.3–0.6
	6	100.0	100.0	NS
Anaerobes	4	74.4	97.6	21.7–24.7
	5	94.3	98.9	4.3–4.9
	6	95.5	98.5	1.4–4.4
Black-pigmented anaerobes	4	100.0	100.0	NS
	5	100.0	100.0	NS
	6	100.0	100.0	NS
Fusobacterium nucleatum	4	100.0	100.0	NS
	5	100.0	100.0	NS
	6	100.0	100.0	NS
Streptococci	4	74.2	100.0	18.8–32.7
	5	100.0	100.0	NS
	6	100.0	100.0	NS

NS, Not significant.

RESULTS

The total viable anaerobic count (i.e. the viable count of facultative plus obligate anaerobes) of the plaque suspensions prior to laser irradiation ranged from 2.17×10^5 to 44.99×10^5 cfu ml^{-1} (Table 3). Black-pigmented anaerobes and *F. nucleatum* comprised between 1.2 and 3.1% and between 0.4 and 4.8%, respectively, of the total anaerobic count. The addition of 45 $\mu g\,ml^{-1}$ TBO had no statistically significant effect on the viability of any of the target organisms (Table 3). In general, the presence of 10% horse blood was similarly without effect on bacterial viability with the single exception of sample 3 where it caused a significant decrease in the number of *F. nucleatum* (Table 3).

Irradiation of plaque samples with laser light for 30 s in the presence of 45 $\mu g\,ml^{-1}$ TBO resulted in significant decreases (between 38 and 100%) in the viability of the target organisms in all samples regardless of whether or not blood was present (Fig. 1, Table 1). In 12 of 15 instances the kills achieved in the absence of blood were greater than those achieved in the presence of blood and the differences in kills were statistically significant. However, in

Table 3. The effect on the viable count of aerobes, anaerobes, black-pigmented anaerobes, *Fusobacterium nucleatum* and streptococci of adding blood or toluidine blue O to subgingival plaque samples from patients with chronic periodontitis. Figures represent the mean ($n = 4$) ± s.d.

Category of organism	Sample treatment	Viable count (cfu ml^{-1}) × 10^5 Sample					
		1	2	3	4	5	6
Aerobes	D−B−	4.95 ± 1.31	18.62 ± 1.53	0.49 ± 0.16	2.57 ± 0.62	15.71 ± 3.36	1.06 ± 0.34
	D−B+	4.79 ± 1.34	17.94 ± 2.48	0.49 ± 0.16	2.50 ± 0.77	13.44 ± 5.39	0.98 ± 0.34
	D+B−	5.18 ± 0.11	18.33 ± 2.59	0.38 ± 0.09	2.79 ± 0.80	14.89 ± 2.66	1.16 ± 0.39
Anaerobes	D−B−	25.67 ± 12.06	44.99 ± 16.39	2.17 ± 0.78	4.29 ± 1.37	43.14 ± 15.6	2.41 ± 0.79
	D−B+	22.58 ± 8.84	47.72 ± 14.29	1.89 ± 0.80	3.58 ± 0.68	49.13 ± 17.54	2.97 ± 0.73
	D+B−	22.07 ± 8.45	54.39 ± 1.50	2.09 ± 0.76	3.52 ± 0.96	4.17 ± 1.52	3.00 ± 1.17
Black-pigmented anaerobes	D−B−	0.46 ± 0.23	0.83 ± 0.17	0.06 ± 0.01	0.08 ± 0.02	0.52 ± 0.15	0.07 ± 0.03
	D−B+	0.53 ± 0.24	0.86 ± 0.19	0.05 ± 0.01	0.09 ± 0.02	0.59 ± 0.17	0.07 ± 0.03
	D+B−	0.59 ± 0.29	0.86 ± 0.12	0.05 ± 0.02	0.07 ± 0.01	0.49 ± 0.12	0.06 ± 0.01
Fusobacterium nucleatum	D−B−	0.09 ± 0.03	1.89 ± 0.23	0.04 ± 0.01	0.03 ± 0.01	0.29 ± 0.09	0.12 ± 0.03
	D−B+	0.09 ± 0.01	1.80 ± 0.31	0.03 ± 0.01*	0.03 ± 0.01	0.29 ± 0.05	0.09 ± 0.03
	D+B−	0.08 ± 0.01	2.05 ± 0.23	0.03 ± 0.01	0.02 ± 0.01	0.23 ± 0.06	0.09 ± 0.02
Streptococci	D−B−	1.89 ± 0.56	3.27 ± 0.72	0.30 ± 0.05	0.39 ± 0.03	12.11 ± 0.89	0.43 ± 0.03
	D−B+	2.04 ± 0.66	3.19 ± 0.48	0.29 ± 0.03	0.44 ± 0.10	11.94 ± 1.19	0.41 ± 0.08
	D+B−	1.79 ± 0.44	3.36 ± 0.93	0.27 ± 0.03	0.42 ± 0.07	12.81 ± 1.11	0.31 ± 0.09

D−B−, Samples exposed to neither dye nor blood; D−B+, samples exposed to 10% (v/v) horse blood in the absence of dye; D−B+, samples exposed to 45 $\mu g\, ml^{-1}$ toluidine blue O in the absence of blood. *Significantly different from D−B− ($p < 0.05$).

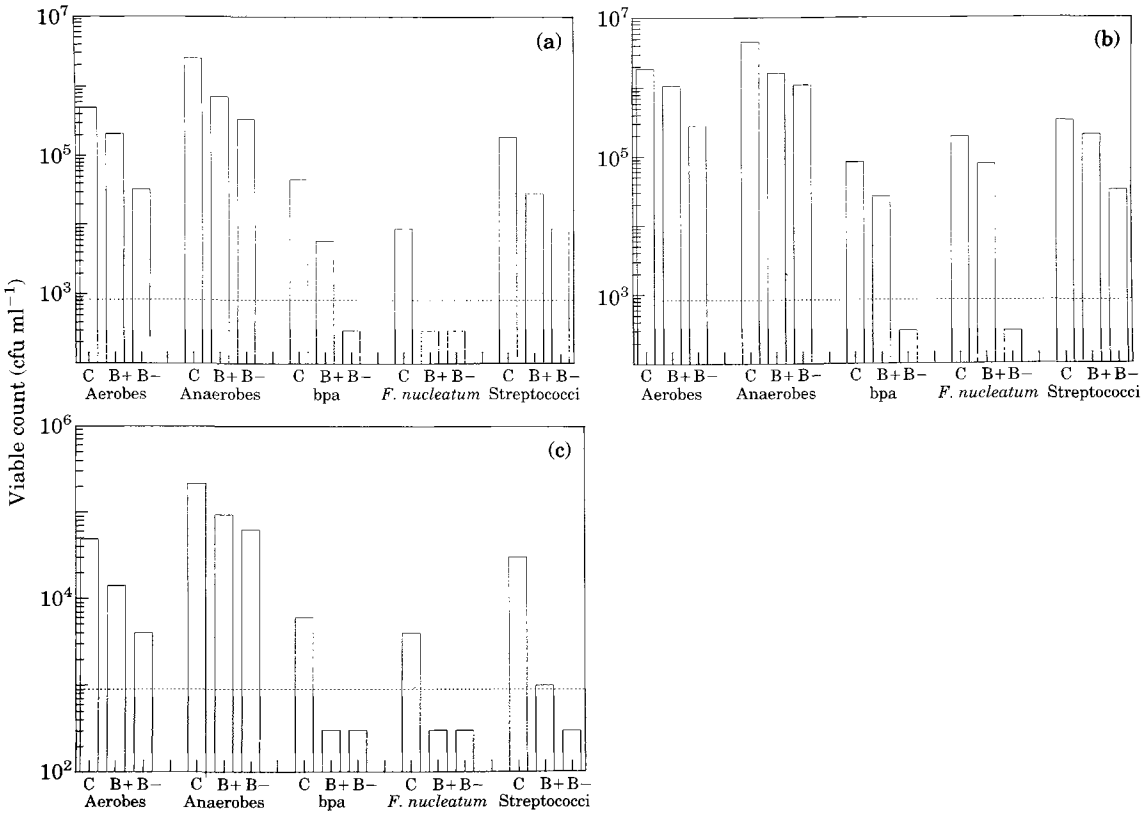

Fig. 1. Viable counts of bacteria in subgingival plaque samples after irradiation with light from a 7.3 mW HeNe laser for 30 s in the presence (B+) and absence (B−) of 10% horse blood. The samples also contained toluidine blue O (45 μg ml^{-1}) except for the controls (C) which were not irradiated with laser light and did not contain blood (bpa, black-pigmented anaerobes). The horizontal line represents the detection limit (900 cfu ml^{-1}) of the viable counting method used. (a) Sample 1, (b) sample 2 and (c) sample 3.

the case of *F. nucleatum*, in two of the samples (1 and 3) the organism was undetectable following irradiation in the presence and absence of blood. Similarly, black-pigmented anaerobes were undetectable in sample 3 after irradiation regardless of whether or not blood was present.

Differences in kills achieved in the presence and absence of blood were less marked when the samples were irradiated for 120 s (Fig. 2). However, where differences were apparent (on six of 15 occasions), greater kills were always achieved in the blood-free samples (Table 2). Black-pigmented anaerobes and *F. nucleatum* were not detectable in any of the three plaque samples after irradiation in the presence or absence of blood.

DISCUSSION

Previous studies of the effects of laser light on bacteria associated with chronic periodontitis have shown that these organisms may be killed when exposed to low doses of HeNe laser light in the presence of photosensitizers such as toluidine blue O and methylene blue (6). Furthermore, such organisms have been shown to be susceptible to killing under conditions more closely mirroring those which would be encountered in vivo. Hence, they could be killed when in the form of biofilms (7) and when they comprised part of the highly-mixed microfloras characteristic of the disease lesion (4). The results of the present investigation have also shown that substantial reductions in the viability of bacteria in subgingival plaques from patients with chronic periodontitis can be achieved by irradiation with low doses of HeNe laser light (energy dose = 219 mJ; energy density = 16.5 J cm^{-2}) in the presence of 45 μg ml^{-1} TBO. The results have also demonstrated that the presence of 10% horse blood can reduce the kills achieved although this could be overcome, in general, by increasing the light energy dose to 876 mJ (energy density = 66 J cm^{-2}). Of particular interest was the finding that the presence of blood had least effect on the killing of those organisms implicated in the pathogenesis of chronic periodontitis, i.e. black-pigmented anaerobes and *F.*

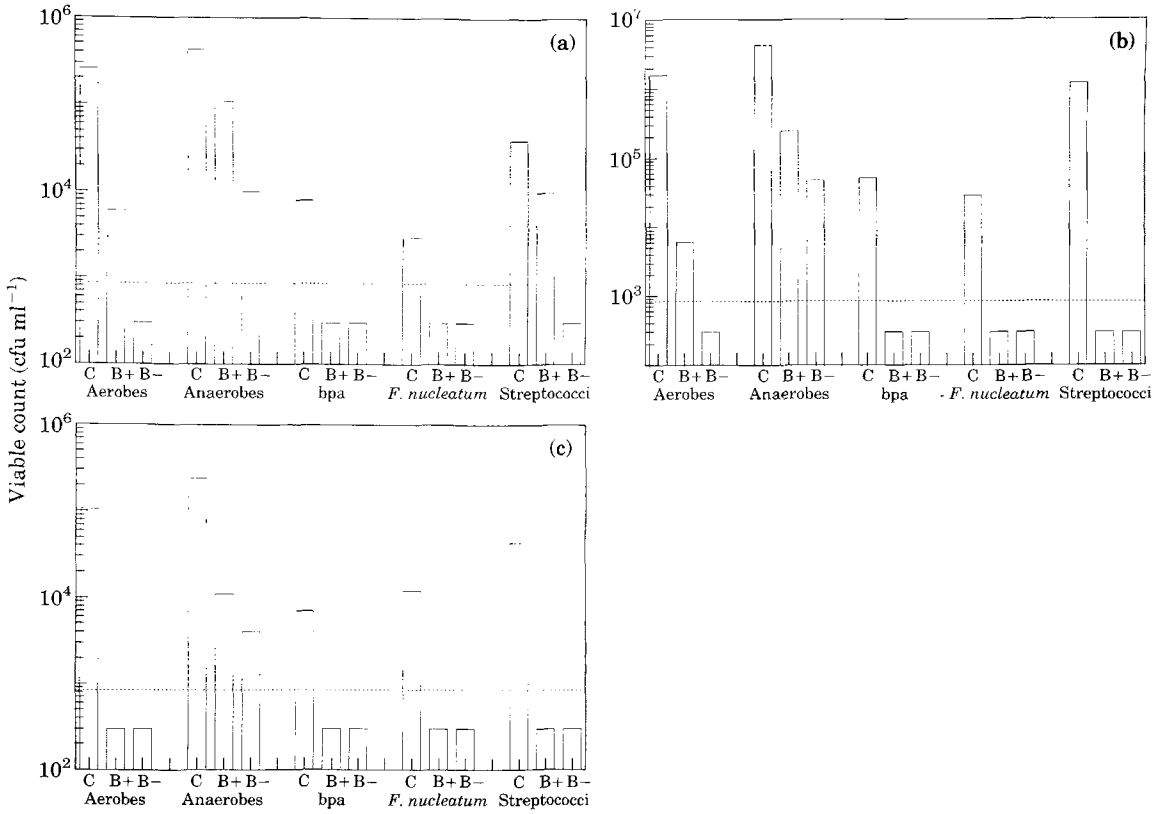

Fig. 2. Viable counts of bacteria in subgingival plaque samples after irradiation with light from a 7.3 mW HeNe laser for 120 s in the presence (B+) and absence (B−) of 10% horse blood. The samples also contained toluidine blue O (45 μg ml^{-1}) except for the controls (C) which were not irradiated with laser light and did not contain blood (bpa, black-pigmented anaerobes). The horizontal line represents the detection limit (900 cfu ml^{-1}) of the viable counting method used. (a) Sample 4, (b) sample 5 and (c) sample 6.

nucleatum. This is a significant finding when considering the potential application of photodynamic therapy for chronic periodontitis. A convenient way of delivering laser light to the sensitized bacteria would be via an optical fibre inserted into the periodontal pocket. However, it is well known that introduction of such a device into the pocket may result in bleeding. In fact insertion of a dental probe into a periodontal pocket is used as a diagnostic aid —bleeding is indicative of an active phase of the disease suggesting the likelihood of further tissue destruction in the lesion (8).

The decreased bactericidal effect observed in the presence of blood may be attributable to the reduced availability of the TBO for bacterial sensitization because of binding to cellular or soluble blood components, or to the free radical-scavenging effect of such a high concentration of organic matter. With regard to the latter, Nitzan et al (9) reported that lethal photosensitization of *Staphylococcus aureus* is less effective in culture medium than in saline. However, this may not be a major factor in the case of oral bacteria as studies of the lethal photosensitization of pure cultures of such organisms (as opposed to plaque samples) have shown that appreciable kills can be achieved in the presence of high concentrations of organic matter such as a brain/heart digest (6, 10). Elucidation of the reason for the reduced killing of plaque bacteria in the presence of blood clearly requires further investigation.

Although the complexity of the microflora of subgingival plaque limits the number of samples that can be processed in this type of investigation, the results of this pilot study have shown that the presence of 10% blood does interfere with the lethal photosensitization of periodontopathogenic bacteria in such samples resulting in decreased kills. However, this could be overcome by increasing the light dose. Hence, at an energy dose of 876 mJ (energy density = 66 J cm^{-2}) periodontopathogenic species were eliminated from the samples regardless of whether or not blood was present. If such kills can be achieved in vivo, this technique may be a useful means of eliminating periodontopathogenic bacteria from periodontal pockets.

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