Factors influencing the susceptibility of Gram-negative bacteria to toluidine blue O-mediated lethal photosensitization

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N. KÖMERIK AND M. WILSON. 2002.

Aims: Bacteria can be killed by red light in the presence of a photosensitizer. The purpose of this study was to evaluate the effect of physiological and environmental factors on the susceptibility of some bacteria associated with oral infections in immunocompromised patients to killing by the photosensitizer toluidine blue O (TBO).

Methods and Results: Suspensions of *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae* in human saliva, horse serum or saline were exposed to light from a helium/ neon laser in the presence of TBO. Additional suspensions at various growth phases and pHs were treated in an identical manner. Survivors were enumerated by viable counting. All three species were susceptible to lethal photosensitization under all of the conditions tested. The presence of serum and, to a lesser extent, saliva decreased the level of kill attained. The bactericidal effect was reduced at acid pHs but was unaffected by the growth phase of the organism.

Conclusions: The composition and pH of the fluid in which bacteria are suspended influenced the effectiveness of TBO-mediated lethal photosensitization, whereas killing was unaffected by the growth phase of the organism.

Significance and Impact of the Study: Environmental factors operating in the mouths of patients with mucositis could reduce the effectiveness of TBO-mediated lethal photosensitization of bacteria associated with this condition.

INTRODUCTION

Infections caused by Gram-negative bacilli, such as *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae*, are common in immunocompromised patients and often result in a bacteraemia (Schimpff 1993). Colonization of these opportunistic organisms has been reported in the oral cavity of cancer patients undergoing radiotherapy for the head and neck (Makkonen *et al.* 1989; Spijkervet *et al.* 1990) and chemotherapy (Greenberg *et al.* 1982; Galili *et al.* 1992). The growth of such organisms in the mouth may constitute a source of serious systemic infection via the mucositis lesions caused by the cytotoxic effects of cancer

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treatment modalities. Furthermore, virulence factors, such as lipopolysaccharide produced by Gram-negative bacilli, are thought to accelerate tissue damage in mucositis through the induction of pro-inflammatory cytokine release (Sonis 1998). Elimination of these opportunistic organisms from the oral cavity of these patients may prevent the risk of local and systemic infections as well as improving mucositis; a variety of antimicrobial agents have been shown to be beneficial in reducing the degree of mucositis (Spijkervet *et al.* 1990; Symonds *et al.* 1996). However, resistance of Gram-negative bacilli to many antimicrobial agents is a growing cause for concern (Kern *et al.* 1994) and, therefore, alternative methods for the eradication of these pathogenic micro-organisms are required.

Photodynamic therapy is a local, repeatable and non-invasive technique which might be an effective alternative to antibiotics for the treatment of local infections. It can be

defined as the eradication of target cells by reactive oxygen species produced by the interaction between a photosensitizing agent and light of an appropriate wavelength (Dougherty *et al.* 1998). Photodynamic therapy has been exhaustively studied for the treatment of neoplasms; however, the antimicrobial application of this therapy has also been suggested, as a wide range of organisms have been reported to be very susceptible to this approach *in vitro* (Paardekooper *et al.* 1995; Wilson and Yianni 1995; Mohr *et al.* 1997). Gram-negative bacilli (*E. coli*, *Ps. aeruginosa* and *Kl. pneumoniae*) in saline suspensions have been shown to be very susceptible to killing by red light in the presence of toluidine blue O (TBO; Komerik *et al.* 1997). However, under *in vivo* conditions, there are several environmental factors that may determine the effectiveness of TBO-mediated lethal photosensitization against micro-organisms. In the mouth, organisms are mainly surrounded by saliva, which contains many organic and inorganic components. A decrease in the pH of the saliva of cancer patients undergoing cytotoxic therapies to around 5·0–7·0 has been reported (Ben-Aryeh *et al.* 1975). Serum exudate from the ulcerated mucositis lesions may also contribute to the fluid environment of the organisms. In addition, bacteria will be present in various growth phases and may respond differently to antimicrobial strategies under each condition. It is, therefore, essential to evaluate whether killing of target bacteria by this method is also effective under conditions that would be encountered in the oral cavity. Moreover, understanding the effect of biological factors on photosensitization may enable manipulation of the treatment procedure to achieve optimal results. The aim of the present study was to investigate the effect of serum, saliva, different pH values and bacterial growth phase on the level of kill achieved by photosensitization.

MATERIALS AND METHODS

Photosensitizer and light source

Toluidine blue O was obtained from Sigma Chemicals (Poole, UK) and kept in the dark. The light source used was a helium/neon gas laser (Spectra Physics, Tokyo, Japan) which produces light with a wavelength of 632·8 nm. The power output of the machine was 35 mW. The light was directed via adjusted mirrors to illuminate the wells of a 96-well microtitre plate with a spot area of 0·283 cm^2, which gives an irradiance of 0·12 W cm^{-2}.

Organisms and growth conditions

The organisms investigated were *Ps. aeruginosa* NCTC 10662, *E. coli* NCTC 10418 and *Kl. pneumonia* NCTC 9633. They were maintained by subculturing on Wilkins Chalgren (WC) blood agar (Oxoid, Basingstoke, UK) overnight aerobically at 37°C. Bacterial suspensions used for the experiments were grown in WC broth (Oxoid) under the same conditions and all contained approximately 10^{10}–10^{11} cfu ml^{-1}.

Effect of saliva and serum

Saliva was collected from five healthy volunteers, pooled and mixed vigorously to obtain a homogeneous suspension. Overnight cultures of bacteria were centrifuged at 5000 *g* for 15 min and the supernatant fluids removed. The bacteria were resuspended in 10 ml of the pooled saliva, horse serum (Oxoid) or 0·85% (w/v) NaCl. Aliquots (100 μl) of each of the resulting bacterial suspensions were transferred to wells of a 96-well microtitre plate (Sterilin, Stone, UK). Equal volumes of TBO (dissolved in either saliva, horse serum or 0·85% (w/v) NaCl) were added to give a final concentration of 25 μg ml^{-1}. After a 60-s incubation period, suspensions were exposed to various doses of laser light (22·3–74·4 J cm^{-2}). Control groups received 100 μl of the appropriate suspension fluid without TBO and were not irradiated. The number of viable bacteria surviving in each of the microwells was then determined as follows. Serial 1 in 10 dilutions of the suspensions in the microwell plates were prepared in nutrient broth and 50-μl aliquots of each were plated in duplicate onto MacConkey agar. Following overnight aerobic incubation at 37°C, the resulting colonies were counted and hence the number of viable bacteria in each suspension was calculated.

Effect of pH

Overnight cultures of bacteria were harvested by centrifugation at 5000 *g* for 15 min. The organisms were then resuspended in citrate–phosphate buffers at various pH values (pH 4·0–8·0). Toluidine blue O was dissolved in the appropriate buffers for each experimental condition. The bacterial suspensions (100 μl) at each pH value were then either exposed to light of 22·3 J cm^{-2} following the addition of 100 μl TBO (50 μg ml^{-1}) or received relevant buffer with no laser irradiation. The number of viable bacteria in each well was determined by viable counting on WC blood agar.

Effect of growth phase

Following the construction of a growth curve from measurements of the optical density of the bacterial suspensions at 540 nm (O.D.$_{540}$), cultures were harvested from the lag, mid-logarithmic and stationary phases by centrifugation at

5000 *g* for 15 min. They were then resuspended in saline to give suspensions with similar optical densities. Aliquots (100 μl) of each culture at each growth phase received equal volumes of 50 μg ml^{-1} TBO solution in 0·85% (w/v) NaCl. Some wells were exposed to 22·3 J cm^{-2} of laser light after 60 s incubation. Control groups received saline and were not exposed to light. The number of viable bacteria in each well was determined by viable counting on WC blood agar.

RESULTS

The effect of saliva and serum on the lethal photosensitization of the organisms can be seen in Fig. 1. In all cases, the bactericidal effect was light energy dose dependent. Neither irradiation of the organisms in the absence of TBO nor incubation with TBO in the dark had a significant effect on the viability of any of the organisms. The highest kills were
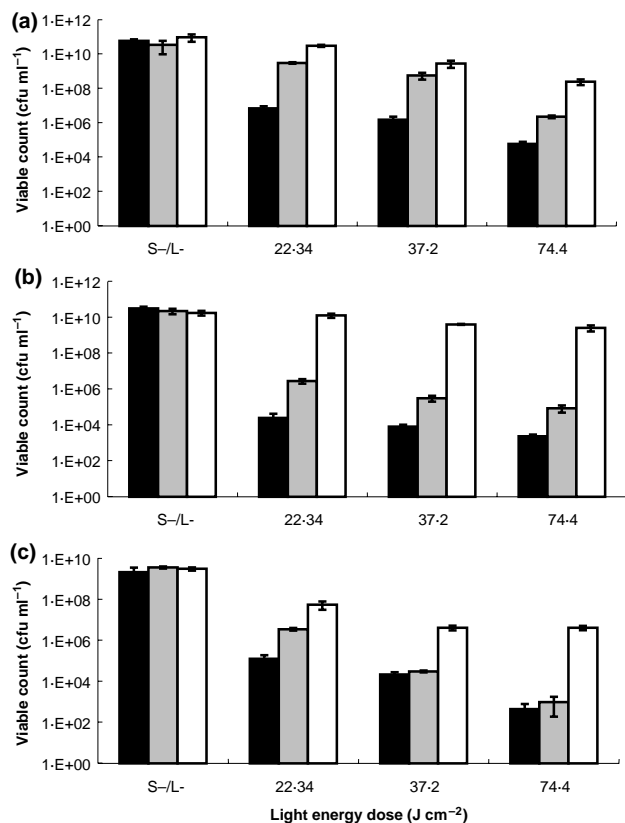
obtained when the organisms were suspended in saline, whereas photosensitization was least effective in the presence of 100% horse serum. Although the presence of saliva impaired the effectiveness of photosensitization, substantial levels of kill (> 5 log$_{10}$ reductions) could be achieved using the higher energy doses. In the presence of serum, > 2 log$_{10}$ reductions in the viable count were obtained at 74·4 J cm^{-2} for *Ps. aeruginosa* and *Kl. pneumoniae*, but this light dose had little effect on the viability of *E. coli*.

The pH of the suspending fluid had a marked effect on lethal photosensitization of the organisms (Fig. 2). In general, greater kills were obtained at the higher pHs with at least a 5 log$_{10}$ reduction in the viable count of all three organisms at a pH of 8·0. The viability of the *Ps. aeruginosa* controls was adversely affected by acidic conditions (pH 4·0 and 5·0). Furthermore, at these pH values. the smallest reductions in the viability of *E. coli* (1·5 log$_{10}$) and *Kl. pneumoniae* (2·7 log$_{10}$) were obtained. The lowest levels of kill for all of the organisms were obtained at pH 4·0. All three species displayed a similar susceptibility to lethal
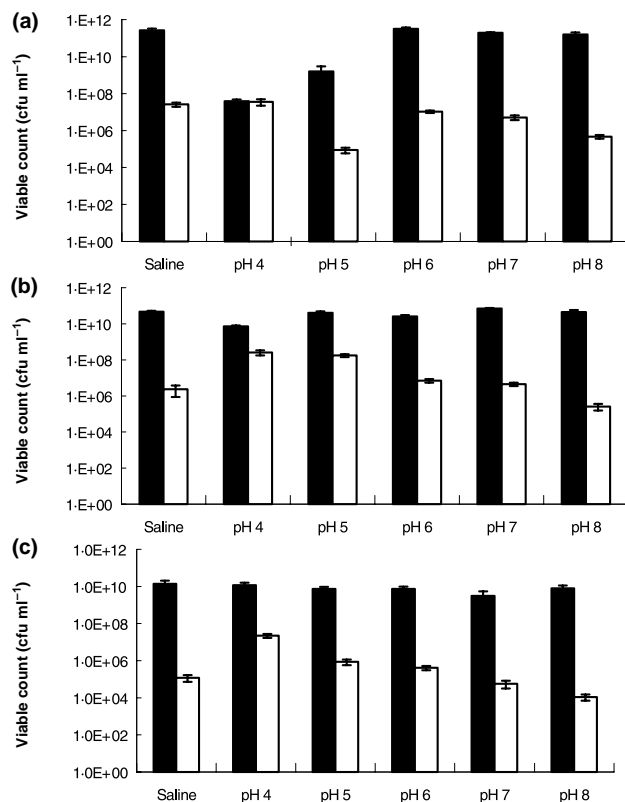
Fig. 1 Effect of saliva and serum on the photosensitization of (a) *Pseudomonas aeruginosa*, (b) *Escherichia coli* and (c) *Klebsiella pneumoniae*. Bacteria were either exposed to various light doses in the presence of toluidine blue O (S + L +) or were kept in the dark in the absence of the sensitizer (S − L −). Bars represent mean values (*n* = 4) of the viable counts and error bars represent S.D. ■, Bacteria suspended in saline; ▨, bacteria suspended in saliva; □, bacteria suspended in serum

Fig. 2 Effect of pH on the lethal photosensitization of (a) *Pseudomonas aeruginosa*, (b) *Escherichia coli* and (c) *Klebsiella pneumoniae*. Bars represent mean values (*n* = 4) of the viable counts before (■) and after (□) photosensitization (toluidine blue O 25 μg ml^{-1}; light 22·3 J cm^{-2}). Error bars represent S.D.
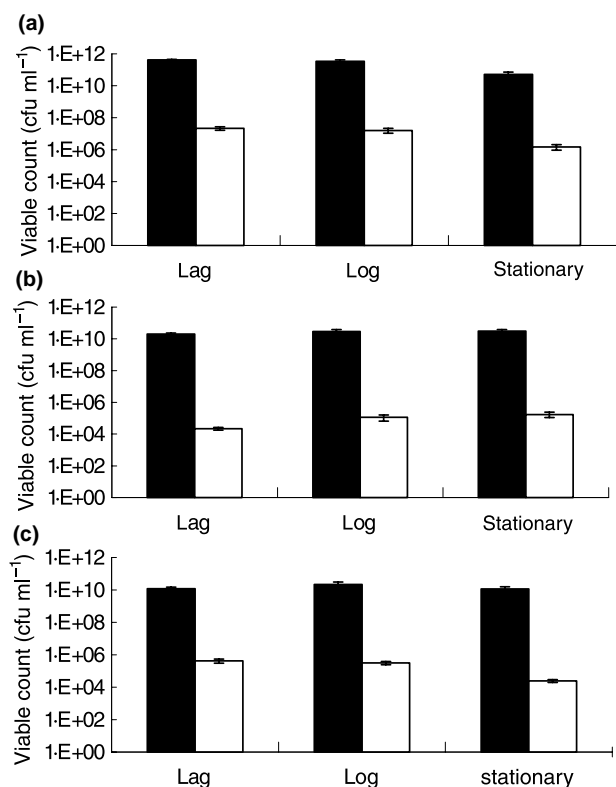
(a)

(b)

(c)

Fig. 3 The effect of growth phase on the susceptibility to photosensitization of (a) *Pseudomonas aeruginosa*, (b) *Escherichia coli* and (c) *Klebsiella pneumoniae*. Bars represent mean values ($n = 4$) of the viable counts before (■) and after (□) photosensitization (toluidine blue O 25 μg ml^{-1}; light 22·3 J cm^{-2}). Error bars represent S.D.

photosensitization in saline (pH 7·4) and in pH 7·0 buffer. Reductions of approximately 4·5 and 4 log$_{10}$ were obtained in *E. coli*, *Kl. pneumoniae* and *Ps. aeruginosa* suspensions, respectively.

Figure 3 shows the response of the three bacteria in different growth phases to light in the presence of TBO. As can be seen, the susceptibility to TBO-mediated photosensitization of all three organisms in all growth phases was very similar.

DISCUSSION

Certain molecules (known as photosensitizers) generate cytotoxic species (mainly singlet oxygen) when irradiated with light of an appropriate wavelength (Ochsner 1997). On absorbing a photon, the photosensitizer molecule is promoted to a high energy state (known as the triplet state) which then transfers its energy to an oxygen molecule resulting in the generation of singlet oxygen. This phenomenon can be used to kill bacteria in a process termed lethal photosensitization (Wilson 1993). The results of this study have shown

that lethal photosensitization of the three Gram-negative bacterial species investigated is adversely affected by the presence of serum and, to a lesser extent, saliva. A number of other studies have also reported that the presence of other organic materials (blood or nutrient broth), as well as saliva and serum, offers some protection to bacteria against lethal photosensitization (Nitzan *et al.* 1989; Wilson *et al.* 1993; Wilson and Pratten 1995; Bhatti *et al.* 1997). The decreased effectiveness of photosensitization in the presence of saliva and serum is most probably due to the protein content of these fluids, as proteins can affect light-mediated killing of bacteria in a number of ways. Firstly, proteins may absorb light, thereby reducing the number of photons available to interact with photosensitizer molecules and so reducing the yield of cytotoxic molecules produced (Wilson and Pratten 1995). Secondly, proteins may compete with the bacteria for photosensitizer molecules, thus decreasing the number of photosensitizer molecules available for binding to the target bacteria. Bhatti *et al.* (1997) reported that the uptake of TBO by bacteria was fivefold less in serum compared with that in saline. Thirdly, singlet oxygen has an extremely short lifetime (Moan and Berg 1991) and, unless generated in close proximity to cells, it would be unlikely to produce any cytotoxic effect. Accordingly, proteins may protect bacteria from cytotoxic species generated in the supernatant fluid. The culture medium has also been reported to quench singlet oxygen and free radicals produced by photosensitizers (Nitzan *et al.* 1989). In the present study, the bacterial levels of kill achieved in the presence of serum were not as high as in the presence of saliva. The reason for this could be related to the higher protein content of horse serum (5–9·5 g 100 ml^{-1}) compared with that of saliva (0·15–0·25 g 100 ml^{-1}) (Cole and Eastoe 1988). Other studies have shown that the efficacy of lethal photosensitization is inversely related to the protein content of the fluid in which the bacteria are suspended. For example, Nitzan *et al.* (1998) demonstrated that *Acinetobacter baumannii* could be photosensitized by a cationic porphyrin in a suspension medium with a low protein content (2 mg ml^{-1}) but that killing was impaired when a suspension fluid containing 15 mg protein ml^{-1} was used.

The pH of the medium was also found to have a marked effect on the extent of lethal photosensitization. The properties and behaviour of different photosensitizers and organisms are known to be affected by pH. Deuteroporphyrin has been shown to be most effective at killing *Staphylococcus aureus* at pH 6·5 and less so in more acidic, as well as basic, conditions (Nitzan *et al.* 1989). This has been attributed to the increased penetration by the porphyrin due to changes in membrane fluidity and the increased binding of the photosensitizer to the bacteria at pH 6·5. Cationic sensitizers, however, were reported to be more effective in neutral pH conditions (Nitzan *et al.* 1987).

Likewise, inactivation of yeast cells (Ito 1977) and *Porphyromonas gingivalis* (Bhatti *et al.* 1997) was found to be greater at neutral pHs. The results of this study have shown that an alkaline environment (pH 8·0) promoted the effect of photosensitization as opposed to an acidic environment (pH 4·0 and 5·0) in which decreased bacterial kills were observed. A high pH may increase the penetration of TBO into the cells. Hence, Wakayama *et al.* (1980) reported higher uptake of TBO molecules by *E. coli* cells at a slightly basic pH. Higher pH values may also promote the production and effectiveness of cytotoxic molecules. Pottier *et al.* (1975) reported an increase in levels of kill at higher pH values of TBO solutions and indicated that this was due to the increased cytotoxicity of singlet oxygen molecules. Tuite and Kelly (1993) found that the lifetime of the triplet state is increased at higher pH values due to the greater alkalinity of the excited state of the photosensitizer compared with its ground state. Likewise, higher concentrations of protons in a lower pH environment may interfere with the generation of singlet oxygen or other free radicals.

Gram-negative bacteria in the stationary phase of growth have a thickened outer membrane and are generally less affected by environmental changes and antibacterial agents than are log phase cells. They may, therefore, be expected to be less susceptible to lethal photosensitization. Bhatti *et al.* (1997) reported that *P. gingivalis* was less sensitive to photosensitization with TBO in the stationary phase than in the lag or log phases, although substantial kills were achieved in all phases. However, varying results have been reported with different photosensitizing agents. The susceptibility of *Staph. aureus* to photosensitization was found to be independent of growth phase when ALS$_2$Pc was used (Wilson and Pratten 1995), while Nitzan *et al.* (1989) reported that this organism was most sensitive in the mid-log phase when deuteroporphyrin was used. However, Venezio *et al.* (1985) demonstrated that lag phase cells sensitized by haematoporphyrin derivative were the most susceptible to photosensitization. The results of this study showed that all three organisms exhibited similar susceptibility to photosensitization in all stages of growth.

Photodynamic therapy is a non-cumulative, local treatment which may be an appropriate antimicrobial strategy for the treatment of local infections, e.g. infections in the mouth of immunocompromised patients. However, the saliva of cancer patients undergoing radiotherapy has been shown to have an increased viscosity and has a tendency to be acidic. The inhibitory effects of serum, saliva and low pH values on TBO-mediated photosensitization as demonstrated in this study may have an impact on the clinical effectiveness of this approach. However, it may be possible to overcome such detrimental effects by the simple expedient of asking the patient to rinse their mouth with a mild alkaline solution such as bicarbonate; this would dilute the protein content of the saliva and increase the pH.

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